Target Monitoring and Non-target Discovery of Halogenated Contaminants in Water, Wildlife and Humans

by

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Abstract

Poly- and per-fluoroalkyl substances (PFASs) are a vast group of environmental contaminants that have been used for almost 70 years and are now ubiquitously distributed around the world. The perfluoroalkyl chain imparts environmental persistence, and long-chain perfluorinated acids are of special concerns due to their high bioaccumulation potentials and associated toxicities. Since the year 2000, there has been a global trend towards restricting long-chain perfluorinated acids and their precursors while shifting to alternative fluorinated chemicals, thus humans and wildlife are now exposed to traditional PFASs and a growing list of alternative PFASs. This thesis addresses questions of current and future PFAS exposure by investigating: (1) how is human exposure to perfluoroctanesulfonate (PFOS) changing over time, and what is the relative contribution from PFOS precursors (PreFOS), and (2) what unknown legacy or alternative PFASs are also emitted to the environment, and can any of these accumulate in organisms?

PFOS is among the most prominent PFAS in the environment and is the dominant PFAS in human serum. **Chapter 2** explored the temporal trends of PFOS concentrations in human serum (U.S. and Sweden), and included a close examination of its isomer and enantiomer profiles which were hypothesized to serve as biomarkers of exposure to PreFOS. Despite decreasing PFOS concentrations, isomer and enantiomer profiles suggest that the relative importance of PreFOS exposure is increasing for the Swedish population. In the U.S., the profiles further suggest that males and older people have relatively more PreFOS exposure than females and younger people. This study highlights many unknowns about the pathways leading to PFOS exposure, and suggests that unknown PreFOS molecules are important contributors to human body burdens.

In **Chapter 3**, I developed and applied a powerful method for discovery of unknown PFASs in water. Using a wastewater sample collected from a fluorochemical industrial park in China, a sensitive and fast non-target PFAS discovery method featuring "in-source fragmentation flagging" was developed on an HPLC-Orbitrap MS system. The non-target method revealed 5 new homologous classes of PFASs in the wastewater (**Chapter 3**), and 10 new classes in pooled fish samples from two locations in China (**Chapter 4**). Most discovered PFASs were long chain (i.e. with 8-17 carbon atoms) poly/perfluoroalkyl sulfonates with hydrogen substitution, or with ether, double bond or carbonyl structures, probably reflecting the major alternative structures that are being produced today in China; now one of the major PFAS manufacturing centers in the world. Additionally, 3 classes of chlorine-substituted poly/perfluoroalkyl acids and 2 classes of poly/perfluoroalkyl amines were discovered (**Chapter 3** and **Chapter 4**). Chlorine substitution of the perfluoroalkyl chain likely increases the bioaccumulation potential, and amines are a general concern due to their genotoxicity. It is unclear whether these new substances are historically emitted PFASs, or contemporary alternatives still in production, but larger-scale monitoring studies and relevant toxicological tests should be followed for these newly discovered PFASs to better understand their risks.

In **Chapter 5**, I extended the non-target methodology to analysis of polar bear serum for fluorinated and chlorinated contaminants by introduction of a sample preparation step based on stir-bar sorptive extraction. The purpose was to increase sensitivity for organohalogen discovery while minimizing endogenous interferences, and the method performed well for a range of polar and ionic analytes. In pooled polar bear serum (Beaufort Sea and Western Hudson Bay), I discovered a wide variety of polar chlorinated contaminants and four homologous classes of poly/perfluorinated sulfonates. Among the discoveries were 7 classes of polychlorinated

biphenyl (PCB metabolites), 2 of which are entirely novel, and 5 of which are reported for the first time in polar bears. These discoveries may explain missing "mass balances" in previous effects-directed analyses of polar bear serum contamination, and demonstrate the power of non-target analytical chemistry for improving characterization of exposomes and of the environmental fate of chemicals.

Preface

Chapter 2 research involved human specimens and received research ethics approval from the University of Alberta Research Ethics Board: "Temporal trends of human exposure pathways to perfluorooctanesulfonate (PFOS)", No. Pro00030128, May 24, 2012. **Chapter 2** was an international research collaboration initiated by Dr. R. Vestergren (Norwegian Institute for Air Research) and led by my supervisor, Professor J.W. Martin (University of Alberta) with Professor U. Berger (Stockholm University), Dr. A. Glynn (Swedish National Food Agency) and Dr. G.W. Olsen (3M Company). I was responsible for the study design, sample preparation, instrumental analysis, data collection and processing as well as manuscript composition. Dr. Martin and Dr. A.S. Pereira assisted with study design and instrumental analysis, respectively. Dr. S. Beesoon helped with my sample preparation skill training. Drs. Berger, Glynn and Olsen provided the Swedish and U.S. human serum samples, respectively, and they, together with Dr. Vestergren, all contributed to manuscript editing and revision. Dr. Martin was the senior/corresponding author and was involved in concept formation, study design, international collaboration establishment and manuscript composition. **Chapter 2** has been published as:

> Yanna Liu, Alberto S. Pereira, Sanjay Beesoon, Robin Vestergren, Urs Berger, Geary W. Olsen, Anders Glynn, Jonathan W. Martin. Temporal Trends of Perfluorooctanesulfonate Isomer and Enantiomer Patterns in Archived Swedish and American Serum Samples. *Environ Int*, 2015, 75, 215-22.

For **Chapter 3**, I conducted sample preparation, instrumental analysis, data collection and processing, and manuscript composition. Dr. Pereira contributed to the establishment of the instrumental method. Dr. Martin was the senior/corresponding author and was involved in conceptualizing of the approach and manuscript composition. **Chapter 3** was published as:

> Yanna Liu, Alberto S. Pereira, Jonathan W. Martin. Discovery of C_5–C_{17} Poly- and Perfluoroalkyl Substances in Water by In-Line SPE-HPLC-Orbitrap with In-Source Fragmentation Flagging. *Anal Chem*, 2015, 87, (8), 4260-8.

Chapter 4 of this thesis has been submitted for publication. I conceptualized and designed the study, conducted sample preparation, instrumental analysis, data collection and analysis, and manuscript composition. Dr. Martin was the senior/corresponding author and was involved in manuscript composition.

Chapter 5 was an international collaboration led by my supervisor, Professor J.W. Martin (University of Alberta) and me, with Dr. E. Richardson (Environment and Climate Change Canada) and Professor H.J. Lehmler (University of Iowa) as lead collaborators. I designed the study, conducted method development, instrumental analysis, data collection and analysis and manuscript composition. Drs. E. Richardson, A.E. Derocher (University of Alberta) and N.J. Lunn (Environment and Climate Change Canada) provided polar bear serum samples from their archives. Drs. Lehmler, L.W. Robertson (University of Iowa) and X. Li (University of Iowa) provided some native PCB metabolite standards used in the study. **Chapter 5** is now under peer-review. All co-authors participated in manuscript editing and revision. Dr. Martin was the senior/corresponding author and was involved with conceptualization of method development for stir-bar sorptive extraction, international collaboration establishment, data interpretation and manuscript composition.

To my parents

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List of Abbreviations

CID	collision induced dissociation
ESI	electrospray ionization
FTICR	Fourier-transform ion cyclotron resonance
HCD	higher collisional dissociation
HPLC-MS	high performance liquid chromatography coupled with mass spectrometry
MDL	method detection limit
MQL	method quantification limit
PFAAs	poly/perfluoroalkyl acids and their salts
PFASs	poly/perfluoroalkyl substances
PCBs	polychlorinated biphenyls
PFCAs	poly/perfluoroalkyl carboxylic acids and their salts
PreFOS	precursors of perfluorooctane sulfonic acid and sulfonates
PFOA	perfluorooctanoic acids and its salts
POPs	persistent organic pollutants
PFOS	perfluorooctane sulfonic acid and sulfonates
PFSAs	poly/perfluoroalkyl sulfonic acids and their salts
qTOF	quadrupole time-of-flight
RP	resolving power
SBSE	stir bar sportive extraction
SID	source induced dissociation
SPE	solid phase extraction
(u)HRMS	(ultra)high resolving power mass spectrometry

Chapter 1. Introduction

1.1 Poly- and Per-Fluoroalkyl Substances (PFASs)

1.1.1 PFASs and their Applications

PFASs are a wide range of manufactured organic chemicals whereby several (i.e. polyfluoro-) or all (i.e. perfluoro-) hydrogens that would normally be attached to carbon atoms have been replaced by fluorine atoms.[1] Highly fluorinated alkyl chains have unique physicochemical properties such as excellent oil, water and stain repellency, high thermal and chemical stability, and can reduce surface tension of aqueous solutions at low concentrations. PFASs have been extensively used in an array of industrial, commercial and consumer products since the 1950s,[2, 3] including for hydraulic fluid, carpet and fabric stain repellents, non-stick cookware manufacturing, polishes, aqueous film-forming foams (AFFFs), in semiconductor devices, paints and in electronics.

PFASs include a vast array of different structural compound classes (**Figure 1**). They may be used in products as small molecules (e.g. perfluorooctanesulfonate in Fluorad^{TM} branded carpet spot cleaner), incorporated into polymers (e.g. fluoropolymers or perfluoropolyethers) where the polymeric backbone itself is fluorinated (e.g. Teflon PTFE), or into side chain polymers, which have a non-fluorinated polymeric backbone with repeating fluorinated side chains (e.g. Scotchgard^{TM} carpet protector).[2] The most common historic classes of PFASs that were used directly were the perfluoroalkyl acids (PFAAs), including but not limited to perfluoroalkyl carboxylates (PFCAs, COO^-) and sulfonates (PFSAs, SO_3^-). A wide variety of PFAA precursors also exist, including many poly/perfluoroalkyl alcohols and/or sulfonamides which were used directly or incorporated to side-chain polymers.[1] Such precursors can degrade to PFAAs, making it difficult to know the ultimate source of PFAAs measured in people or the environment.

1.1.2 Ubiquitous Global Distribution of PFASs

Organic forms of fluorine were first reported in human serum in 1968.[4] In 1976, Taves *et al*.,[5] by using nuclear magnetic resonance (NMR), tentatively identified perfluorooctanoic acid (PFOA, C_8-PFCA, $C_7F_{15}COO^-$) in pooled U.S. human plasma. In the next 30 years, the organic

fluorine monitoring has mostly been performed in occupationally exposed workers,[6] because the analytical methods available then (e.g. ashing) had very high detection limits (μg/mL). It was not until the late 1990s, when liquid chromatography coupled with mass spectrometry was commonly available, that detection of background PFASs in environmental or biological samples first became possible. In a seminal report by the 3M Company (3M Co.) in 2001, PFOA, perfluorooctanesulfonate (PFOS, C_8-PFSA, $C_8F_{17}SO_3^-$), perfluorooctanesulfonamide (PFOSA, a PFOS precursor, $C_8F_{17}SO_2NH_2$) and perfluorohexanesulfonate (PFHxS, C_6-PFSA, $C_6F_{13}SO_3^-$) were identified in human serum from the general population of the United States.[7] Since then, a series of PFAAs ranging from C_4-C_{14} have been detected in human,[8, 9] wildlife[8, 10-12] and environmental samples.[8, 13]

The strong C-F bond contributes to PFAS environmental persistence.[14, 15] PFOS has not been shown to degrade under environmental conditions,[16] and it resists biological[17] and microbial biotransformation.[17-19] No degradation of perfluorobutanesulfonate (PFBS, C_4-PFSA), PFOS or 6:2 fluorotelomer sulfonate ($C_6F_{13}C_2H_4SO_3^-$) was detected after 2 - 3.4 years of anaerobic incubation with three different sludge.[18] The high stability and moderate water solubility of PFAAs allows them to travel long-distances via ocean currents.[20-23] Moreover, wide distribution of semi-volatile PFAA precursors in air (e.g. 6:2 fluorotelomer alcohol, 6:2 FTOH, $C_6F_{13}C_2H_4OH$), and their atmospheric oxidation, also contributes to the global dissemination of PFAAs, including in the Arctic.[12, 17]

1.1.3 The Hazardous Long-Chain PFAAs

Among all PFASs in the environment, PFSAs with ≥ 6 carbons and PFCAs with ≥7 carbons are of particular concern for wildlife and humans due to their combination of hazardous properties.[2] Here we refer to this group of compounds as long-chain PFAAs. In addition to their environmental persistence as discussed above, long-chain PFAAs show a tendency to bioaccumulate in wildlife and are very difficult for humans to excrete. This group of substances binds to serum protein, including albumin (>99% of PFOS and >95% of PFOA is bound to albumin),[24-26] β-lipoprotein,[27, 28] and liver fatty acid-binding protein[29-31] which contributes to their bioaccumulation and the bioaccumulation potential generally increases with increasing perfluoroalkyl chain length.[32-35] The human serum elimination half-life for a short-chain PFAA such as PFBS in only 1 month,[36] but for PFOS is 5 years.[37] Similarly, the bioaccumulation

factor (log BAF) in lake trout was 4.1 for PFOS and 3.2 for PFOA,[38] whereas PFBS[32, 39] and PFCAs with ≤ C_6 are considered "not bio-accumulative".[34, 40]

The long-chain PFAAs are also associated with multiple toxicities,[41-44] and can bind to various hormone receptors or carrier proteins, including estrogen receptor,[45, 46] androgen receptor,[45, 47] and transthyretin.[28, 45, 46] Both PFOS and PFOA are developmental toxicants,[48-50] peroxisome proliferators[51] and endocrine disruptors[52-58] in animals and humans. Long-chain PFAAs also have been associated with alteration of cell membrane properties,[59, 60] causing adverse effects on immune systems, and can induce reproductive toxicity,[61] neurotoxicity,[62, 63] hepatotoxicity[64, 65] and renal toxicity.[66] Developmental toxicity is one of the most sensitive endpoints,[54, 67] as fetuses/neonates are vulnerable, and long-chain PFASs pass placental barriers[68, 69] and can be excreted into milk. [68-70]

1.1.4 Regulatory Actions and Interventions for Long-Chain PFAAs

With concern about the health risks of long-chain PFAA exposure, regional, national and international regulatory agencies have enacted some rules and regulations,[71] while PFAS manufacturers also implemented certain voluntary actions with the aim to reduce emissions and minimize future environmental and human exposure. Given that exposure of animals and humans to long-chain PFAAs may come from a combination of direct exposure, originating from industrial use and release as well as consumer product application and disposal, and indirect exposure from various precursors, which degrade in the environment or are metabolized in organisms,[17] most regulations and industrial phase-outs have included both PFAAs and their many precursors.

PFOS and PFOA were the first PFASs to be addressed, shortly after their discovery in humans and wildlife. Between 2000 and 2002, the 3M Co., which was the world's largest PFOS producer covering 80% of the global market at that time,[72] phased out its PFOS and PFOS precursor (PreFOS) as well as its PFOA and PFOA precursor production in the U.S., Western Europe and Japan.[73] In 2002, the U.S. Environmental Protection Agency (U.S. EPA) published a *Significant New Use Rule (SNUR)*, requiring manufacturers or importers to notify EPA before commencing the manufacture or import of PFOS and PreFOS.[74] In 2006, a *Stewardship Agreement*[75] between eight leading companies, including 3M Co., DuPont and Arkema, and the U.S. EPA was enacted which aimed for a 95% PFOA and PFOA precursor emission reduction in

each company (measured from the year 2000 baseline) by 2010, and virtual elimination by year 2015. According to the annual progress reports,[76] all participating companies claimed to have been fulfilling their goals in each year. The European Union (EU) began to restrict PFOS and PFOA related production and use in 2006 under the *European Union Marketing and Use* Directive,[77] and under the *Registration, Evaluation, Authorization and Restriction of Chemicals* (REACH) program since 2013.[78] In 2009, PFOS and PreFOS were categorized as Persistent Organic Pollutants (POPs) under *Annex B* of the international *Stockholm Convention*.[79] As of June 1, 2014, Norway became the first country which bans PFOA use in solid and liquid consumer products as well as in textiles and carpets;[80] the ban was extended to semiconductors and photographic coatings on January 1, 2016.[80]

Emission controls on a broader range of long-chain PFCAs have also been enacted gradually. For example, 5 PFAS companies in Canada signed the *5-Year Voluntary Environmental Performance Agreement*[81] in 2010, and met the goal of eliminating C_8-C_{20} PFCAs and their precursors in Canadian commerce by 2015. In 2016, the Canadian environmental and health authorities published *Regulations Amending the Prohibition of Certain Toxic Substances Regulations 2012 (RA-PTSR)*,[82] prohibiting the use of long-chain PFCAs (and PFOS) in commercial products. The U.S. EPA issued additional *SNURs* in 2015,[83] requiring manufacturers, importers and processors of long-chain PFCAs to submit notifications before commercializing their products. Similarly, the EU listed C_8-C_{14} PFCAs as substances of very high concern under REACH in 2013, requiring "registration, notification, and duty to communicate" on products containing these substances.[84]

1.1.5 PFASs Production and Use Today

In this section, current trends in worldwide PFAS production and use are discussed, following the series of regulatory and industrial control actions discussed above.

1.1.5.1 Transitioning to Alternative Chemistries

Regulations on existing long-chain PFAAs have driven PFAS manufacturers to develop alternatives. According to very limited publicly available information, four categories of replacements are likely being produced.[2, 85] Two of these categories are alternative PFASs and are discussed below, while the other two categories are non-fluorinated chemicals, and non-

chemical alternatives. However, examples of these last two categories are scarce due to the unique nature of PFASs. Some tested candidates do not meet the requirements, particularly that of imparting extremely low surface tension.[2, 86] Hydrocarbon surfactants have been proposed as a good substitute in some photographic industries[2] and in firefighting foams,[87] but no commercialization has yet been reported to our knowledge.

Short-chain PFASs, including PFSAs with $<C_6$ and PFCAs with $<C_7$ and their precursors,[2] are likely the major alternatives being used today. One major transition has been from $\geq C_6$-based PFSAs to C_4-based PFSAs. The 3M Co. commercialized PFBS based surface treatment products in 2003,[88] and tetraethylammonium PFBS was registered under REACH in 2013 for metal plating.[89] Other manufacturers have transitioned from $\geq C_8$-PFCAs to C_6-PFCAs,[86] and from 8:2 based fluorotelomers (e.g. 8:2 FTOHs, $C_8F_{17}C_2H_4OH$) to 6:2 FTOHs. For example, DuPont marketed its 6:2 FTOH AFFFs (e.g. $C_6F_{13}C_2H_4SO_2NHC_3H_6N^+(CH_3)_2CH_2COO^-$) beginning in 2008.[90] Some manufacturers in China and Italy were reported to use C_6-PFSA based-substitutes (e.g. PFHxS) for historical C_8-PFSA based-applications, instead the shorter C_4-based chemistries;[16,17] PFHxS is actually defined as long-chain PFSA, and is more persistent in humans than PFOS (half-life 7.5 years vs 5 years).[2, 37] Nevertheless, this general transition to short-chain PFASs is due to their structural, physical and chemical similarities to long-chain PFASs (e.g. amphiphobic, stable and surface-active), while generally being easier to excrete,[91, 92] less bioaccumulative,[34] and as claimed by manufacturers "less toxic" compared to longer-chain PFASs.[93]

The second type of replacement is structurally modified PFASs. By inserting functionalities or atoms that are expected to aid degradation or metabolism[1] (e.g. ethers, hydrocarbons and ketones) into perfluoroalkyl chains, the resulting new PFASs are assumed to be less persistent and less accumulative. In fluoropolymer manufacture, perfluoropolyethers (PFEs) were reported to have replaced traditional PFOA salts as new processing aids,[85] such as ADONA ($CF_3OCF_2CF_2CF_2OCHFCOO^-NH_4^+$) from the 3M Co.,[94] GenX ($CF_3CF_2CF_2OCF(CF_3)COO^-NH_4^+$) from Dupont,[95] cyclic (e.g. dioxane based structure)[96] or polymeric functionalized PFEs (e.g. ClC_3F_6O-$[CF_2CF(CF_3)O]_m$-$[CF(CF_3)O]_nCF_2COOH$, m = 1-4, n = 0-2) from Solvay,[97] and 6:2 fluorotelomer carboxylic acid (6:2 FTCA, $C_6F_{13}CH_2COO^-$) from a Chinese company.[98] In surface treatment of food contact materials, Solvay used polyfluoropolyether phosphate (e.g. $(HO)_2P(O)O(OCH_2CH_2)_nCF_2CH_2(CF_2O)_p$-

$(OCF_2CF_2)_qOCF_2CH_2$-$(OCH_2CH_2)_n$-$OP(O)(HO)_2$, n=1-2, p/q=1-3) to replace PFOS/PreFOS-phosphates (e.g. $[C_8F_{17}SO_2N(C_2H_5)C_2H_4O]_2PO_2H$).[99] In AFFFs, the 3M Co. developed a perfluoroalkyl ketone based fire suppression agent (i.e. $CF_3CF_2C(O)CF(CF_3)_2$)[100] after its PFOS-related production was ceased.

1.1.5.2 Continued Long-Chain PFAS Production and Use

Given the difficulty to find appropriate chemical substitutes having high performance similar to traditional long-chain PFASs, continued use of long-chain PFASs is known. In fact, most or all of the regulations discussed above were issued with affiliated "exemption" files which specify acceptable purposes for continued use. For example, the *Stockholm Convention* allows continued PFOS and PreFOS use in aviation applications, in AFFFs, in surface treatments, in paper packaging and in semiconductors applications.[79] The Canadian *RA-PTSR* does not prohibit long-chain PFCAs when they are present in manufactured items,[82] and Norway still allows continued PFOA use in food packaging, food contact materials and medical devices[80] despite being the first country to ban PFOA in consumer products.

1.1.5.3 PFAS Manufacturing in Developing Countries

Since around the year 2000, and as domestic regulatory scrutiny and public concern over PFAS-related health risks grew, most of the major historical PFAS manufacturers have started up new manufacturing plants in developing countries, mainly in continental Asia (e.g. China and India)[101] where economic growth is preferable and few regulations have been enacted to control PFASs. For example, in 2001, a High-Tech Fluorine Chemical Industrial Park was built in Changshu, Jiangsu, and more than 40 fluorochemical manufacturing companies have built plants in it, including 3M and DuPont from the U.S., Arkema from France, Solvay from Belgium and Daikin from Japan.[102, 103] With time, more plants have been built in other cities in China, including but not limited to Shanghai (3M,[104] DuPont,[105] Daikin[106]), Zhangqiu (3M[104]), Ulanqab (3M[104]), Shenzhen (DuPont[105]), Suzhou (Daikin[106]) and Quzhou (Solvay[107]). At the same time, as major historical manufacturers reduce or discontinue their long-chain PFAS production, new manufacturers in developing countries have stepped forward to begin large scale PFAS production, including of traditional long-chain PFASs to fill the growing market gap for these substances.[108] Built in 2001, the Dongyue fluoro-silicon industrial park has mostly Chinese companies in it. It is now the world's largest PTFE manufacturing center, and is the primary

supplier of big companies including DuPont and Daikin.[109] More local companies have emerged with the continued build-up of fluorochemical industrial parks in more Chinese cities, such as in Zhangqiu in Shandong Province, Taicang and Taizhou in Jiangsu Province, Quzhou in Zhejiang Province, Ulanqab in Inner Mongolia Province, Fuxin in Liaoning Province and in Sanming in Fujian Province.[110]

1.1.6 Concerns Over the Status Quo of PFAS Production and Use

Likely due to efforts from both industry and regulatory bodies over the last 17 years, PFOS, PFOA and some of their precursors have shown worldwide decreasing trends in various samples since around 2000.[111] Meanwhile, short-chain PFASs (e.g. PFBS)[112, 113] and some structurally modified alternative PFASs have started to increase. Human PFAS temporal trends are summarized in **Table 1**. Canada, benefiting from its domestic controls for long-chain PFCAs,[81, 82] has shown background levels of C_8-C_{11} PFCAs in its large-scale 2009-2011 Canadian Health Measures Surveys (mean 2.3, 0.8, 0.2 and 0.1 ng/mL, respectively).[114] While these are indeed encouraging results, current PFAS production and use globally remains concerning, and future human PFAS exposure problems are far from resolved. Members of the scientific community have, in fact, called for an outright PFAS phase-out by encouraging significant innovations to develop fluorine-free alternative chemicals.[115, 116]

1.1.6.1 Continued Long-Chain PFAS Production and Unknown Precursors

Contrary to the decreasing trends discussed above at many global locations, PFOS and PFOA in some Chinese people has surged by 13× and 6× times, respectively, from 1999 to 2002.[117] Moreover, long-chain PFCAs (mostly C_9-C_{12} PFCAs) have shown consistent upward trends (or sometimes levelling-off) in people from almost all countries (**Table 1**, no human data from China, but increasing C_7 and C9 PFCAs were seen in dolphins[112]). Despite the decreasing PFOS and PFOA trends in almost all developed countries, rates of decrease are usually much slower than the intrinsic biological elimination rates known from monitoring of retired PFAS industry employees.[37] Reported rates of decrease are summarized in **Table 1**. Taking PFOS as an example, which has an intrinsic half-life of 5 years,[37] PFOS should therefore decline at a rate of -14%/yr, and 50%, 75%, 88% and 94% of PFOS should be eliminated after 5, 10, 15 and 20 years, respectively. This slower apparent elimination likely implies continuing PFOS exposure in the

real world.

PFAS manufacturing is now mainly conducted in developing countries where few or no regulations have yet been enacted. No PFAS regulations are noted in India.[71] China has begun to restrict new installations of PFOS and PFOA production facilities since 2011,[118] and to ban PFOS uses in some applications since 2014.[119] Little data is available on the production volumes of PFASs in these countries, but according to some estimations China's annual PFOS and PreFOS production surged from <50 tonnes (t) to >200 t between 2004-2006,[120] followed by a decrease to 100 t in 2008.[121] According to a survey conducted by the China Association of Fluorine and Silicone Industry in 2013, the annual PFOS and PreFOS production in China was 100 - 160 t between 2009-2012, with one-quarter to one-third being exported.[108] By 2015, at least 12 local companies, 9 in Hubei province and 3 in Fujian province, were known to still be producing PFOS and PreFOS.[108] PFOA is widely used as processing aid in the production polymers (e.g. PTFE).[2] The Donyue fluoro-silicon industrial park in China ranks first in the world's PTFE production[102, 123, 124] (e.g. 34,000 t in 2015[122]), which covers only 30% of domestic PTFE production. With a cumulative emission of 250 t PFOA between 2007 and 2012,[125, 126] China has rapidly become the world's largest PFOA emitter. India's PFOA production has likely increased dramatically since 2010 as its PTFE production increased more than 3× between 2010 and 2011. The total C_4-C_{14} PFCA (but mainly long-chain PFCAs) emission from fluoropolymer production sites in China, India, Russia and Poland combined were estimated to have increased from ~50 to ~200 t/year from 2000 to 2010,[127] in contrast to the trend in the U.S., Western Europe and Japan where combined emissions decreased from ~150 t/year to almost zero over the same time period. Therefore, large scale long-chain PFAS production has been occurring in these emerging economies, and local emissions will likely affect the rest of the world due to intensified global trading activities (e.g. half of China's PFOS/PreFOS production was designated for export in 2006[120]) and the long-range transport potential of PFASs.

Historical long-chain PFAS applications have been continuing lawfully in certain countries, allowed by the exemptions listed in almost all regulations. As discussed in ***Section 1.1.5.2***, PFOS and hundreds of PreFOS molecules were exempted under the *Stockholm Convention* for continued use in AFFFs, surface treatments, paper packaging and metal plating applications, despite that these are their major historical applications. For long-chain PFCAs, the U.S.[83] and the EU[84] only began an "importing notification" requirement 2-5 years ago, and no associated

regulations exist in developing countries where large-scale production, import, sale and use of these chemicals is continuing today.

Another concern over long-chain PFASs is related to their precursors, a vast array of polyfluoroalkyl substances which can degrade or be metabolized to persistent PFAAs. These pose human and environmental health risks due to their decomposition to PFAAs, but also pose (mostly) unknown toxicological risks when living organisms are exposed to these various substances. Despite their long history of use, there are still large unknowns in their chemical identities, including residual impurities in their formulations, their environmental distribution, human exposure, transformation rates/efficiencies and toxicities. For example, it was not until the year 2010 that diester polyfluoroalkyl phosphates[128] and N-methyl-/N-ethyl-perfluorooctanamides[129] were discovered as precursors to PFCAs, in addition to the FTOHs which were shown to be metabolized to PFCAs in the 1980s.[130] More than 100 PreFOS molecules were listed in the *Stockholm Convention*,[79] but fewer than 10 of these (e.g. PFOSA, perfluorooctane-sulfonamidoacetates, perfluorooctanesulfonamidoethanols, and their N-methyl/N-ethyl derivatives) have ever been directly monitored in the environment.[17] Hindered by unknown identities or lack of authentic standards, the downstream studies (e.g. human exposure and toxicities) remain scarce, and continuing uncertainty remains for predicting human long-chain PFCA and PFSA exposure.

1.1.6.2 Problematic Alternative PFASs and Large Unknowns in Their Identities

Corresponding to the industrial transition to alternative PFASs, increasing C_4- and C_6-PFSA (i.e. PFBS and PFHxS) concentrations have recently been observed[112, 113, 131] (**Table 1**). Moreover, perfluorobutanesulfonamide (PFBSA), which is a short-chain precursor to PFBS was recently reported in fish from the Netherlands, Canada and the U.S.,[132] and pioneering studies have revealed structurally modified alternative PFASs (e.g. PFECAs) in water samples downstream of a fluorochemical facility.[133, 134] Although more studies are certainly required to evaluate the safety of these new substances as alternatives, some of the few existing studies have already suggested that these poly-/per- fluorinated alternatives may not be as safe as expected.

First, the alternative PFASs are often still persistent, and maybe as persistent as traditional PFAAs. Trifluoroacetic acid, PFBS, and 6:2 fluorotelomer sulfonic acid (6:2 FTSA, $C_6F_{13}CH_2CH_2SO_3^-$) were similarly resistant to microbial degradation as PFOS, showing no

degradation after 32 weeks of incubation under aerobic conditions;[18, 135] and perfluoroether chains were shown to be as resistant to abiotic and biotic degradation as perfluoroalkyl chains.[136] Even if the structurally modified PFASs do break at the modified bonds (e.g. ether bonds), the resulting degradation products may still be persistent.[115, 116]

Second, the alternative PFASs do not necessarily have lower bioaccumulation potentials than traditional long-chain PFAAs. PFHxS has a slower urinary excretion[137] and a longer elimination half-life (7.5 years vs 5.0 years) than PFOS in humans,[42, 137, 138] and is therefore more bioaccumulative. C_4-PFCA (perfluorobutyric acid, PFBA) also had a slower renal clearance rate than C_6-PFHxA (perfluorohexanoic acid, PFHxA).[92] Only one elimination study with structurally modified PFASs has been reported,[139] whereby $CF_3OC_3F_6OCHFCF_2COO^-$ (a PFECA molecule) had a faster serum elimination than PFOA in humans, but a much slower elimination in rats. Of further considerations is that despite lower nominal bioaccumulation potentials (measured or assumed), this may be negated by higher exposure due to a greater amount being required in products to achieve equal performance to legacy PFAAs.[116, 140]

Third, these alternative PFASs are not necessarily less toxic. PFHxS was shown to be more hepato-toxic than PFOS in rats,[141] and PFHxA was more acutely toxic than PFOA to aquatic life.[142] Without any traditional long-chain PFAS being tested as a reference, two PFECAs were found to be associated with hepatotoxicity, genotoxicity and developmental toxicity.[94] Moreover, the alternatives may exert very different toxicities than traditional long-chain PFASs due to different distributions in the body.[43] For example, PFBA is predominately distributed to human lungs, kidneys and brain, whereas PFOS accumulates preferentially in the livers and bones.[143]

A final concern over alternative PFASs is the large unknown in their chemical identities. The currently available information on alternatives is only "a small selection of alternatives",[101] while many still remain unknown. In some countries (e.g. the U.S[144]), manufacturers and importers are required to submit compound information for regulatory review before any alternative can be introduced. These data, however, remain publicly inaccessible due to confidentiality or trade secret concerns. In developing countries, few regulatory requirements have been made for manufacturing new chemicals for industrial or commercial purposes.[101] It is therefore difficult to know what alternatives we are and will be exposed to, what chemicals to monitor for in the ambient environment or in goods exported around the globe, and what the associated risks may be. This causes concerns as overall PFAS production in developing

countries has been increasing.

1.1.7 Future Human PFAS Exposure and Key Question.

Collectively, continued long-chain PFAS production in certain exempted applications, and substitutions by problematic known and unknown alternative PFASs will not likely mitigate future human or environmental health risks. For better actions to be taken in the future, the following questions should be answered:

The core question for long-chain PFAS exposure is **how are we exposed today, and how will we be exposed in future?** Even if all long-chain PFAS production was to stop immediately, human long-chain PFAS exposure would continue into the future due to the time-lag of products' life-cycles (from chemical application to emission from current-use products and from disposed waste), and, maybe more importantly, due to the likely slow environmental degradation of known and unknown precursors which enter landfills. For example, the incorporation of PFAA precursors into side-chain polymers, and the time frame for hydrolysis of these side-chains remains highly uncertain.[145-147] Based on current-use and discarded products containing 8:2 FTOH side-chain polymers, a modelling study suggested that global annual FTOH emission will likely continue to increase until after 2040.[148] Both the long-chain PFAAs and their precursors pose health risks and contribute to human and environmental exposure of PFAAs, thus it is important to figure out how the relative extent of direct and indirect exposure will change in the long term. Knowing this will help to elucidate the major human exposure sources, thus facilitating sound emission control actions to minimize future exposures.

The ultimate question for alternative PFASs is their safety. There are several historical examples of structurally similar chemicals being used as substitutes for the banned or restricted chemical substances, and these all turned out to be less than successful. For example, chlorofluorocarbons were replaced by hydrochlorofluorocarbons,[149] and polybrominated diphenyl ethers were replaced by various halogenated flame retardants.[150] To address the concern that we may be making the same mistakes again requires immediate and systematic risk characterization for these alternatives. However, before any evaluation can be performed, the first question is **what are the chemical identities of current alternative PFASs?** Except for the short-chain PFASs, there are large uncertainties in the chemical identities of short-chain precursors and of structurally modified PFASs. Early discovery of these chemicals would enable

safety evaluation studies and regulatory actions if deemed necessary.

1.2 How Will Future Human PFOS and PreFOS Exposure Change?

PFOS remains today as the dominant PFAS in human serum. Its exposure sources are not well known but thought to include direct PFOS exposure, direct exposure to PreFOS, as well as direct exposure to PFOS which was emitted to the environment as PreFOS.[17] Due to large unknowns in PreFOS identities, it is not feasible to perform top-down monitoring studies where all PreFOS molecules are quantified in human biofluids or environmental media. It is preferable to employ a bottom-up method in which direct PreFOS monitoring could be avoided.

PFOS isomer profiles[151] and enantiomer profiles[152] were proposed as biomarkers of PreFOS exposure in people, specifically as a means for evaluating the relative extent that PreFOS has contributed to the body-burden of PFOS. This theory is based on the fact that commercially relevant PFOS and PreFOS have branched isomer contents of 30% (i.e. 70% linear)[153, 154] and for chiral branched isomers of PFOS the starting enantiomer fraction should be 0.5 (i.e. equal amount of both enantiomers)[155] and an assumption that only biological biotransformation (i.e. of PreFOS to PFOS) would change these profiles appreciably. Proof-of-principle studies have demonstrated these assumptions for branched PreFOS isomers[156, 157] and for enantiomers of 1*m*-PFOS.[152] Therefore, measuring PFOS isomer and/or enantiomer profiles in humans may be an indirect bottom-up method to estimate the relative contribution from indirect PreFOS exposure. A temporal trend study of these biomarkers, as described in **Chapter 2**, would allow prediction of future human exposure scenarios.

1.3 Unknown PFAS Identification

As discussed in Section ***1.1.6.1*** and ***1.1.7***, there are large unknowns in the chemical identities of historically emitted PFAA precursors and alternative PFASs. In mass balance studies of total organic fluorine, significant proportions of unidentifiable organic fluorine are frequently reported in environmental (50 to ≥99%[158, 159]) and biological samples (15 to ≥99%).[39, 160-162] Among the PFAAs (e.g. C_4-C_{14} PFCAs and C_4-C_8 PFSAs) that increase in effluent after municipal wastewater treatment,[163-165] aerobic biotransformation[166] and/or chemical oxidization (e.g. by persulfate) processes,[167-172] only about half of the mass balance can be attributed to known precursors, confirming the existence of unidentified precursors in relevant environmental

media. Recently, a perfluorooctanesulfonamido ethanol-based phosphate diester was discovered in marine sediments[173] and human sera,[174] thus exposing a fraction of the unknown organic fluorine content. Similarly, recent discoveries of PFECAs and PFESAs in water demonstrates that unknown alternative PFASs are currently present in our environment.[133, 134]

Unknown PFASs contribute to a substantial proportion of unknown organic fluorine in the environment, thereby representing a great source of uncertainty for environmental and human health risks. Developing analytical methods capable of discovering and characterizing identities of these unknown PFASs will facilitate this knowledge through enabling important studies on the persistence, bioaccumulation and toxicity of new substances.

1.3.1 Target *vs* Non-Target Analysis

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS) is what first enabled the discovery of PFASs in humans and in the environment,[7, 9] and such instruments have gained popularity for trace environmental contaminant analysis for a broad range of polar and ionic chemicals. Quadrupole, triple quadrupole, and quadrupole ion traps are the workhorse mass analyzers for highly sensitive detection of targeted analytes today. For a comprehensive target analysis, the detection of any specific compound in a sample with these instruments has traditionally depended on the availability of authentic chemical standards, which provides characteristic information such as chromatographic retention time and MS/MS spectral information. The standards also allow exquisitely sensitive and quantitative sample extraction methods to be developed, by enhancing recovery for the targeted molecule while simultaneously removing interferences. Such targeted approaches are usually of high selectivity and high sensitivity (e.g. femto- to picogram in absolute mass[175]). They, however, are only able to study a small number of compounds, and are highly dependent on the availability of commercially available authentic standards or *de novo* synthesis. With increasing numbers of chemicals being produced each year,[176] it is getting more expensive and impossible to analyze a long list of chemicals of potential environmental relevance. Besides, these mass spectrometers only provide unit resolution (i.e. ±0.5 amu), even when operating in multiple reaction monitoring (MRM) mode, the possibility of generating false positive results increases as the sample matrix becomes increasingly complex.

A paradigm shift in the way that environmental contaminants are discovered and

monitored is called "non-target" analysis, enabled by modern advances in full-scan high resolution mass spectrometry (HRMS), and in fact ultra-HRMS (uHRMS). Non-target analysis makes it possible to discover and detect a broad range of compounds in a single run, without any *a priori* information or authentic standard available. This is based on the combination of the high resolving power (RP) or resolution, which reduces interferences, high mass accuracy, which allows for assignment of plausible molecular formulae for unknown spectral peaks, high scan rates, which allow for coupling with various types of chromatography, and high sensitivity in full-scan acquisition, which makes the detection of subtle contaminants possible in complex sample matrices. Mass spectral RP, or resolution, is the ability of an instrument to resolve two peaks of very close mass-to-charge ratios [*m/z*], defined at a specified *m/z* (usually at *m/z* 200-400) by the full width at half maximum peak height ($m/m_{1/2}$). Mass accuracy is the difference between the measured and the true *m/z* of an ion, defined as the ratio of mass difference ($m_{1/2}$) to the true *m/z* (typically expressed as parts-per-million, ppm). Commercially available HRMS instruments include various quadrupole time-of-flight instruments (qTOF, RP≥30,000, mass accuracy ≤5 ppm, scan rate 1-10 Hz, sensitivity picogram absolute mass in full-scan),[133, 177-179] and uHRMS instruments include Orbitrap (Orbitrap-MS, RP≥120,000, mass accuracy ≤3 ppm, scan rate 1-2 Hz, sensitivity femto- to picogram absolute mass in full-scan)[175, 180] and Fourier-transform ion cyclotron resonance (FTICR-MS, RP≥1,000,000, mass accuracy ≤2 ppm, scan rate 0.3-1 Hz, sensitivity picogram absolute mass in full-scan).[179] Furthermore, unlike previous generations of HRMS, such as magnetic sectors, todays' HRMS and uHRMS instruments are capable of full scan without appreciable loss of sensitivity, RP, or mass accuracy.

Non-target analysis has commonly been applied in molecular life sciences[181, 182] and is gaining popularity in environmental analytical chemistry. As a general guideline, RP ≥60,000 is recommended for reliable empirical formula assignment of compounds in mixtures composed of C, H, O, N, F and P.[175] In the most complex samples such as petroleum, uHRMS (i.e. RP ≥100,000) is required to resolve isobars containing S[183] (e.g. to differentiate C_3 vs H_4S, Δm =3.37 mDa[184]). MS/MS or MS^n may help to elucidate the structure of an unknown molecule after molecular formula assignment, but in practice authentic standards are still required to conclusively confirm the presence of a molecule after it is detected. Moreover, although non-target mass spectrometry does not require any *a priori* information, specific compounds or long-lists of compounds may be screened using "suspect-screening" approaches,[185, 186] or classes of

substances could be targeted based on common mass spectral behavior.[187, 188]

1.3.2 Non-Target Analysis Workflow

A general workflow for unknown environmental contaminant discovery by HPLC-HRMS is: (1) producing highly resolved full-scan spectra to reveal all ions in the sample; (2) "picking out" potential environmental contaminant ions from complex background signals; (3) assigning plausible molecular formulae for candidate ions; (4) MS^n fragmenting candidate ions for structural characterization, and (5) structure confirmation based on spectral databases or authentic standards. Among these, step 2 is perhaps most difficult and critical, as the likely challenge is to distinguish trace signals of environmental contaminants from a complex mixture of major and minor natural/endogenous compounds; akin to finding the proverbial needle(s) in the haystack.

1.3.3 Non-Target Techniques for Differentiating PFAS Signals from Matrix Background

When no *a priori* information is available, techniques are required to distinguish analyte ions of interest the complex matrix. Ion distinguishing techniques will vary for different compound classes (e.g. isotopic patterns for brominated compound discovery[189]), and some have previously been developed or proposed for filtering PFAS ions from non-fluorinated background signals, including both post- and pre-HRMS-analysis filtering techniques. The four post-HRMS-analysis data filtering techniques are:

1) *Negative mass defects*. Mass defects ($\Delta m/z$) are defined as the difference between the nominal and exact mass of an atom or molecule. Fluorine atoms have a negative mass defect ($\Delta m/z$ -0.0016), hydrogen atoms have a positive mass defect (m/z +0.0079), while carbon has zero mass defect. Therefore, compared to hydrocarbons which have positive mass defects, PFASs will tend to have low or negative mass defects. However, this can be affected by other negative mass defect elements, including oxygen ($\Delta m/z$ -0.0051), sulfur ($\Delta m/z$ -0.0279) and phosphorus ($\Delta m/z$ -0.0262), and therefore should be used together with other techniques.[99, 177, 178]
2) *Homologous series*. PFASs are often manufactured as a series of chain-length homologues, and the m/z spacing is either ±49.997 (i.e. -CF_2-) or ±99.994 (i.e. -CF_2CF_2-),

depending on whether electrochemical fluorination or telomerization was used in their manufacturing. This technique can be used with either low-resolution MS (e.g. fast atom bombardment MS, FAB-MS[177]) or HRMS (e.g. qTOF[134, 190] and FT-ICR[179]) but has greater diagnostic potential in the case of HRMS.

3) *Kendrick Mass Defect Plot (KMDP).* The KMDP is a quick data screening technique for visualizing all analytes in a sample, and for homologous series discovery.[191] Homologous compounds share the same general formula and only differ by chain lengths. For homologous hydrocarbons with repeating CH_2 units, the absolute molecular or ion masses among homologues differ by n × 14.01565 Da, where n is an integer, representing the difference in numbers of total CH_2 units. Therefore, by multiplying by 14/14.01565 (i.e. normalizing the mass of CH_2 from 14.01565 to exactly 14.00000), all homologues' masses will be re-scaled to the Kendrick mass,[192] and produce identical Kendrick mass defects (i.e. difference between nominal Kendrick mass and exact Kendrick mass). Further, by plotting the resultant Kendrick mass defects *vs* nominal masses (i.e. KMDP), all homologues will display in horizontal lines, making the entire homologous class stand out from other chemicals and background signals. Thus, by applying different parameters in mass re-scaling, the KMDP can be used to discover different classes of homologues, such as -O_2- series (multiply by 32/31.989830), -SO_2- series (multiply by 64/63.961902),[193, 194] and -CF_2- series (i.e. PFASs, multiply by 50/49.996806).[133, 177-179, 195]

4) *Case-control study design*. As frequently used in metabolomics studies,[196] the comparison of spectra belonging to a control group can be statistically compared to spectra of experimental or treatment groups to highlight those ions significantly affected by the treatment.[133, 195, 197, 198] Analogies to this approach have found application for environmental contaminant discovery, for example the comparison of samples from upstream and downstream of a fluorochemical industry have revealed certain PFASs discharged into natural water.[133] However, "control" samples are not always feasible, especially when considering the ubiquitous distribution of PFASs in the environment. Furthermore, this technique usually requires repeated sample injections to accommodate the statistical treatment of the data.

The above post-HRMS-analysis techniques are often used together to narrow down

candidate PFAS ion lists, for example by focusing on homologous ions with negative mass defects.[177, 198] To further accelerate or simplify prospective PFAS ion filtering, some researchers also explored pre-HRMS-analysis techniques. Two examples of this are noted for PFASs. Before the HRMS analysis, D'Agostino *et al.*[179] fractionated the AFFF sample into four fractions with two different types of solid phase extraction cartridges, and subjected each fraction to combustion ion chromatography. Then, only fraction(s) with high fluorine signals were proceeded for HRMS analysis. Qin *et al.*,[199] as a proof-of-principle, analyzed PFAS-spiked samples by HPLC coupled to continuum source molecular absorption spectrometry for flagging retention times of the spiked PFAS. Subsequent HRMS analysis could then be focused on the portion eluting during that time period. The success of these two approaches for analysis of environmental samples may be limited by the high detection limits of combustion ion chromatography (2-10 ng F/mL,[161, 179] 3-15 ng/mL PFOS equivalent) and continuum source molecular absorption spectrometry (0.1-300 ng F/mL,[199] 0.15-154 ng/mL PFOS equivalent).

1.3.4 More Efficient PFAS Signal Discrimination Techniques Needed

Despite availability of the above multiple signal filtering techniques, non-target PFAS discovery by mass spectrometry only began in 2010, and to date has predominately been focused on identification of active PFAS ingredients or impurities in industrial formulations[99] or commercial products, including food contact materials.[177, 179, 200, 201] In these cases, PFASs are present at relatively high levels and there is little background interference, compared to environmental samples. The only attempts at non-target PFAS discovery in environmental or human samples also used samples with high PFASs: water from downstream of a fluorochemical industry,[133] and serum samples from AFFF-exposed firefighters.[198] The slow progression to background environmental and biological samples, where PFASs may be present at trace levels may be attributable to insufficient capability of the available filtering techniques to distinguish subtle PFAS signals in super-complex mass spectra.

We are therefore interested to explore, in addition to the above pre- and post-HRMS-analysis mass filtering techniques, whether an "in-HRMS-analysis" technique could be developed to further facilitate PFAS signal discrimination from massive background signals. Although reported after completion of work in this thesis, a data-independent precursor isolation and characteristic fragment method for brominated compound discovery is noted,[202, 203] where

fragmentation spectra of all detectable ions were produced in a Q-Exactive Obitrap MS, and only ions with characteristic ^{78}Br and ^{81}Br fragments were targeted for further elucidation. Such methods have the advantage of incorporating signal filtering, empirical formula confirmation, and MS/MS structural characterization into a single step, and can potentially be run with the commonly available qTOF-MS. However, the method requires many injections (>10 injections in the study) and produces high numbers of spectra for analysis.[202, 203] It is therefore worthwhile exploring a fast and simple in-HRMS-analysis technique that can be used for large-scale PFAS discovery in the future.

1.4 Research Questions and Proposed Studies

Due to the changing PFAS environmental and human exposure and associated uncertain human health risks, it is important now to **better understand the extent of human PFAA precursor exposure**, and to identify **what unknown alternative PFASs the environment may be exposed to in future**. To this end, I undertook the following four projects:

1. **To explore the temporal trends of PFOS isomer and enantiomer profiles in human serum, biomarkers of PreFOS exposure:** Human serum samples were analyzed from the U.S., where large-scale and long-term PFOS/PreFOS production once took place, and from Sweden, which had no known historical PFOS/PreFOS production. This study was designed to provide information on how the PreFOS exposure may have changed over 2-3 decades in the two countries and is expected to give hints to predict exposure sources in the future.
2. **To develop a fast and simple non-target PFAS discovery method for complex sample matrices** with a new in-HRMS-analysis PFAS signal filtering technique. A wastewater sample discharged from a Chinese fluorochemical manufacturing park was used for method development and validation.
3. **To apply the developed non-target PFAS discovery method to fish.** Fish livers from two natural water systems in China were tested, and this is expected to provide information on what PFASs that have been or are currently being released in China may bioaccumulate in fish bound for market.
4. **To extend the developed non-target method for organohalogen discovery in polar bears.** Polar bears have long been recognized as highly exposed to halogenated

environmental contaminants.[11, 204-206] Serum samples from polar bears in two Canadian Arctic regions were examined with the possibility of identifying new contaminants which might explain a toxicologically relevant unknown fraction in previous effects-directed analysis work.[207]

The results of the thesis are expected to provide information on how future human PFAS exposure may change, what new PFASs may be emitted today in new source regions that could be regulated before they are global contaminants, and what new PFASs can bioaccumulate in animals. The results may furthermore enable others to conduct risk characterization or risk assessment on these halogenated contaminants, with the goal of minimizing future environmental and human health risks.

1.5 Figures and Table

1.5.1 Figures

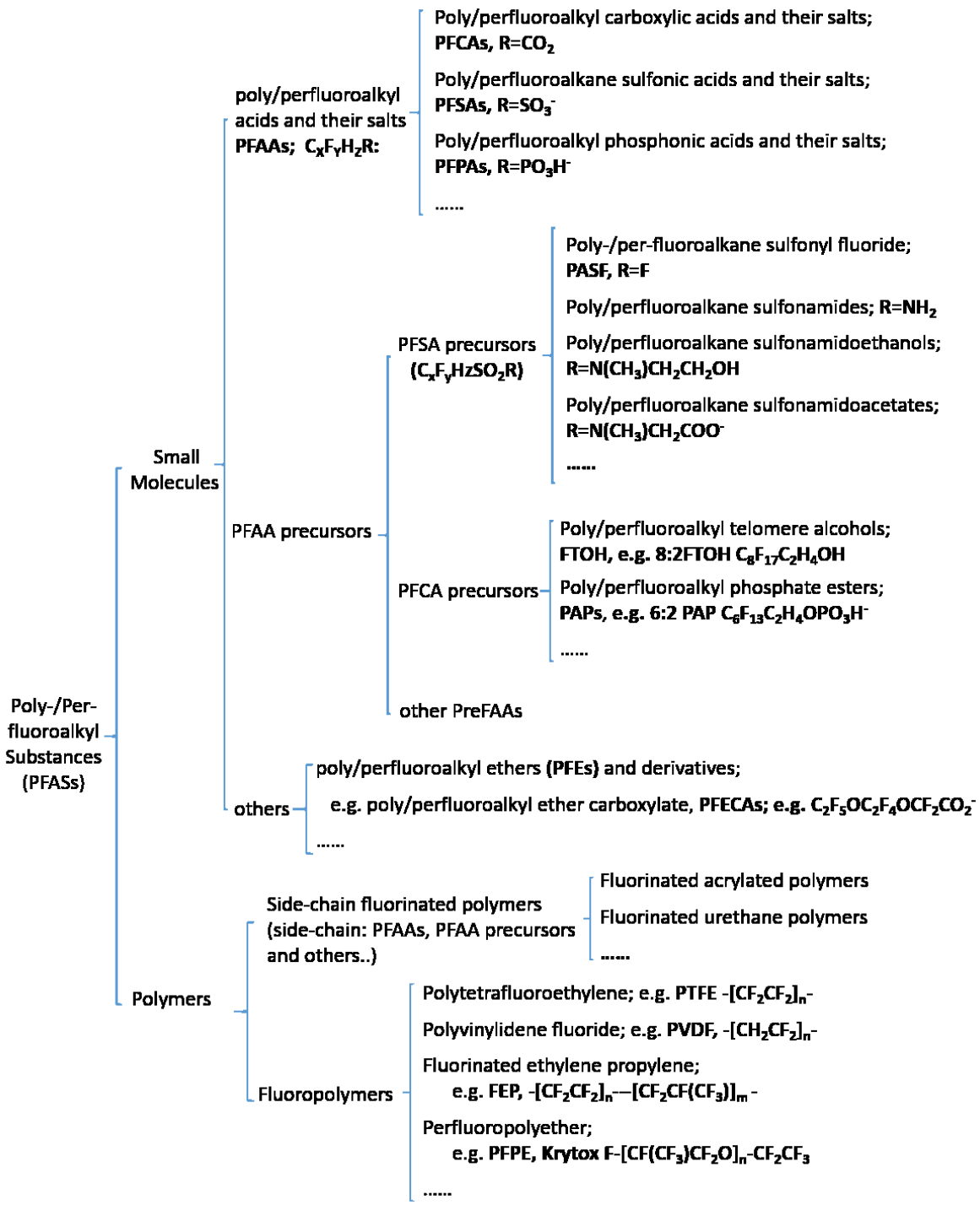

Figure 1. General classification of PFASs.

1.5.2 Tables

Table 1. Temporal trends of PFASs in humans since time of the 3M Co. phase-out in year 2000. Rates of change are expressed as positive (+) or negative (-) percentage changes per year, or over the period shown.

	the U.S.	Canada	Sweden	Norway	Germany	Australia	P.R. China
PFOS	-9%/yr 03-13[208] -88% 00-15[209] -55% 99-08[210] -83% 99-14[211]	-22% from 07-09 to 09-11;[114, 212] decrease 92-04 in Nunavik mothers[c,213]	-8%/yr 96-10[113] -8%/yr 97-12[214] -6%/yr 08-15[215]	decrease 98-06[216]	decrease 00-10[174]	-66% 02-11[217] -17%/yr 02-13[218]	+1300% 99-02[117] stable 08-12[219]
PFOSA[a]	decrease 99-08[211]		-22%/yr 96-10[113] -21%/yr 97-12[214]				
FOSAA[a]			-20%/yr 97-12[214]		decrease 00-10[174]		
MeFOSAA[a]	increase00-06[209] decrease 06-15[209] -89% 99-12[211]		-20%/yr 97-12[214]		decrease 00-10[174]		
EtFOSAA[a]			-39%/yr 97-12[214]		decrease 00-10[174]		
Di-SAmPAP[a]					stable 95-10[174]		
PFOA	-5%/yr 03-13[208] -77% 00-15[209] stable 03-08[210] -51% 99-14[211]	stable from 07-09 to 09-11[114, 212]	-3%/yr 96-10[113] -7%/yr 08-15[215]	decrease 98-06[216]	-5%~-9%/yr 95-10[220]	-66% 02-11[217] -13%/yr 02-13[218]	+600% 99-02[117] stable 08-12[219]
8:2 FTSA[b]						-13%/yr 02-13[218]	
6:2/6:2DiPAP[b]					stable 95-10[220]		
6:2/8:2DiPAP[b]			increase 97-12[214]				
8:2/8:2DiPAP[b]			increase 97-12[214]				

Table 1. Continued:

	the U.S.	Canada	Sweden	Norway	Germany	Australia	P.R. China
C_6-PFSA	-6%/yr 03-13[208] -61% 00-15[209] stable 99-08[210, 211] -17% 09-14[211]	-26% from 07-09 to 09-11[114, 212]	+8%/yr 96-10[113] +8%/yr 08-15[215]		stable 95-10[174]	-15%/yr 02-11[218]	
C_7-PFSA						-13%/yr 02-11[218]	
C_{10}-PFSA			-10%/yr 96-10[113]			decrease 02-11[218]	
C_7-PFCA			-8%/yr 08-15[215]	stable 98-06[216]		stable 02-04[218] decrease 04-11[218]	
C_9-PFCA	-2%/yr 03-13[208] increase00-06[209] decrease 06-15[209] +250% 99-08[210] +210% 99-10[211] stable 11-14[211]		+4%/yr 96-10[113] stable 08-15[215]	increase 76-06[216]	increase 95-10[220]	increase 02-06[4] decrease 06-11[218]	
C_{10}-PFCA	-1%/yr 03-13[208] increase00-06[209] decrease 06-15[209] increase 99-04[211] stable 04-10[211] increase 11-14[211]		+4%/yr 96-10[113] -4%/yr 08-15[215]	increase 76-06[216]	increase 95-10[220]	increase 02-04[218] stable 04-13[218]	
C_{11}-PFCA	-3%/yr 03-13[208] +600% 09-12[211]		+3%/yr 08-15[215]		increase 95-10[220]	increase 02-04[218] stable 04-13[218]	
C_{12}-PFCA				stable 98-06[216]		+130% 02-13[218]	
C_4-PFSA			+11%/yr 96-10[113]	stable 98-06[216]			
Total PFASs		+2.6%/yr 06-10 Nunavik preschoolers[c,221]					

[a] PFOSA (perfluorooctanesulfonamide), PFOSAA (perfluorooctanesulfonamidoacetate), MeFOSAA (N-methyl perfluorooctanesulfonamidoacetate), EtFOSAA (N-ethyl perfluorooctanesulfonamidoacetate) and Di-SAmPAP (diethyl perfluorooctanesulfonamidoethanol-based polyfluoroalkyl phosphate ester) are PFOS precursors

[b] 8:2FTSA (8:2 fluorotelomer sulfonic acid), 6:2/6:2 DiPAP, 6:2/8:2 DiPAP and 8:2 DiAP (polyfluoroalkyl phosphoric acid diesters) are PFCA precursors

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Chapter 2. Temporal Trends of PFOS Isomer and Enantiomer Profiles in Two Populations*

2.1 Introduction

PFOS has been widely used in various products since 1946 for its oil and water repellency and high stability.[1] Its environmental[2] and biological persistence,[3] and its bioaccumulation potential[4] have made PFOS ubiquitously distributed in the environment, in wildlife, and in human populations.[5-7] PFOS is eliminated slowly in humans, with a reported serum elimination $t_{0.5}$ of approximately 4.8 years (95% confidence interval (CI), 4.0-5.8).[3] Among PFOS rodent toxicity studies, many adverse effects have been noted at high concentrations, but developmental toxicity[8-11] is one of the most sensitive endpoints.

PFOS precursors include a variety of compounds that can potentially degrade to PFOS in the environment, or be metabolized to PFOS in organisms.[5] PreFOS was used directly in some applications but was also covalently linked into large fluorinated side-chain polymers,[5] and total historic emissions of PreFOS likely exceeded PFOS.[12, 13] The long-term hydrolytic stability of covalently bound PreFOS in side chain polymers is an unresolved question,[14-16] thus there are uncertainties in future emissions of PreFOS. Furthermore, PFOS and ~100 PreFOS compounds were recognized as POPs under the *Stockholm Convention* in 2009,[17] but the agreement allowed their continued production in countries where there are no domestic restrictions (e.g. China), and little information is available on the specific uses of PreFOS today.

Except for a few PreFOS molecules (e.g. PFOSA, FOSAAs and FOSEs), many historically manufactured PreFOS molecules have never been analyzed in any samples. Total extractable organic fluorine analysis has shown a significant proportion of unknown organic fluorine in various samples (e.g. 60-90% in seawater,[18] ~70% in dolphin livers[19] and 15-67% in Chinese whole blood[20]), raising the possibility that unidentified PreFOS may contribute to the unknown organic fluorine signal. Furthermore, new PreFOS molecules continue to be discovered, for

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* A version of this chapter has been published. Y. Liu, A.S. Pereira, S. Beesoon, R. Vestergren, U. Berger, G.W. Olsen, A. Glynn, J.W. Martin. *Environ Int*, 2015, 75, 215-22. Reprint with permission.

example a PreFOS-based phosphate diester was recently identified in marine sediment[21] and in human serum[22] for the first time.

In humans, PFOS body burdens are predicted to result from a combination of direct exposure to PFOS (**Figure 2-1**, pathway 1 and 3), and indirect exposure to PreFOS, which can be transformed to PFOS internally (**Figure 2-1**, pathway 2).[5, 23] Using occurrence data for PFOS and PreFOS in various human exposure media, modeling studies estimated that direct PFOS exposure usually dominated general population exposure (e.g. 90%[24, 25]). Even so, the relative contribution of PreFOS may be up to 60-80% in high exposure sub-populations (i.e. those individuals with high PFOS in serum) due to the large variability in PreFOS concentrations in the indoor environment.[25] Nevertheless, these modeled predictions may underestimate the true importance of PreFOS, because (i) they cannot account for PreFOS compounds that have never been monitored, and (ii) they do not take into account that some fraction of PFOS in exposure media (e.g. dietary fish) may originate from environmental biotransformation of PreFOS (**Figure 2-1**, pathway 3; e.g. metabolism of PreFOS to PFOS in fish that people later ingest[26]).

Following the phase-out of PFOS and PreFOS by the 3M in the U.S, Western Europe and Japan between 2000 and 2002,[27] decreasing PFOS temporal trends were detected in different populations from North America[28-31] and Europe.[22, 32-34] However, how each of these trends were influenced by variations in PFOS and PreFOS exposure is unknown. Understanding the relative contribution of PreFOS to PFOS exposure may help to explain the observed trends, to elucidate major exposure sources, and also to predict future exposure.

Benskin *et al*.[35] proposed that PFOS branched isomer content >30% in human serum was possibly a biomarker of significant PreFOS exposure. This principle is supported by knowledge that historical commercially relevant batches of PFOS contained a consistent branched isomer composition of ~30% branched and ~70% linear,[36, 37] and that all major branched PFOS isomers are excreted more efficiently than linear PFOS (*n*-PFOS) via human urine.[38] Therefore, if an individual's exposure is dominated by direct PFOS exposure, the branched isomer content in the body should be <30%, not >30% as is most commonly observed. Commercially relevant batches of PreFOS were also ~30% branched,[36, 37] and studies have shown the preferential excretion of linear PreFOS (i.e. PFOSA) compared to branched PreFOS through urine in rodents[39] and in humans,[38] resulting in preferential retention of branched FOSA isomers that can then be

biotransformed to branched PFOS. Moreover, branched PreFOS isomers are metabolized faster than linear PreFOS *in vitro* by human liver microsomes[40] and *in vivo* in rats.[41] Thus, if an individual's exposure to PFOS is dominated by biotransformation of PreFOS, then it is at least feasible for the branched PFOS isomer content in the body to be >30%, as previously hypothesized by Martin *et al.*[5]

PFOS enantiomer profiles in serum were also proposed as a biomarker of human exposure to PreFOSs.[42] This is because: (i) many branched PFOS and PreFOS isomers are chiral[43] and were manufactured as racemic mixtures (i.e. 50:50) of two non-superimposable enantiomers (i.e. mirror image molecules); (ii) *in vitro* biotransformation rates of 1*m*-PreFOSs (i.e. a α-perfluoromethyl branched PreFOS isomer, see "Nomenclature" in 2.2.1) to 1*m*-PFOS differ for the two enantiomers,[42] and (iii) from rat studies there is currently no evidence for differential absorption, distribution or excretion of 1*m*-PFOS enantiomers.[44] Thus, the detection of significantly non-racemic 1*m*-PFOS enantiomer fractions in humans (i.e. when the ratio of the two enantiomers is significantly different from 1:1) may point to the contribution of PreFOS (**Figure 2-1**, pathways 2 and 3), because direct PFOS exposure alone should always result in a racemic 1*m*-PFOS enantiomer signal (based on (i) and (iii)). Significantly non-racemic signatures of 1*m*-PFOS have already been reported in Canadians[44] and in aquatic organisms from Lake Ontario.[45]

Martin *et al*.[5] hypothesized that PFOS isomer profiles and 1*m*-PFOS enantiomer fractions could both be used as biomarkers of human exposure to PreFOS. Therefore, in the current study both biomarkers were analyzed in two sets of archived human samples to achieve two objectives: first, as a cross-validation exercise (i.e. if both biomarkers measure the same phenomenon, they should be correlated), and second, to evaluate how relative exposure to PFOS and PreFOS might have changed over time in two geographically distinct populations.

2.2. Experimental Section[†]

[†] Drs. Urs Berger and Anders Glynn, and Geary W. Olsen provided the Swedish archived human serum extracts, and the U.S. human serum/plasma samples, respectively. Dr. Alberto S. Pereira assisted in instrumental analysis.

2.2.1 Nomenclature

PFOS isomer nomenclature was adopted from Benskin *et al*..[46] Briefly, linear PFOS is termed *n*-PFOS, perfluoroisopropyl-PFOS is termed *iso*-PFOS, while the remaining mono-perfluoromethyl isomers are abbreviated as #*m*-PFOS, where # indicates the carbon position of perfluoromethyl branching. ∑PFOS refers to the sum of all detected PFOS isomers.

2.2.2 Chemical and Reagents.

Standard br-PFOSK (>98%; a technical mixture of 78.8% *n*-PFOS, 10% *iso*-PFOS, 1.2% 1*m*-PFOS, 1.9% 3*m*-PFOS, 2.2% 4*m*-PFOS, 4.5% 5*m*-PFOS, 0.71% of m_2-PFOS), MPFAC-MXA (>98%; containing mass labeled *n*-$^{13}C_4$-PFOS) and racemic 1*m*-PFOS (4.6mg/mL in methanol) were obtained from Wellington Laboratories (Guelph, ON, Canada). Sterile filtered calf serum was purchased from Lampire Biological Laboratories (Pilersville, PA, USA). Optima-grade tetrahydrofuran, formic acid (98%), glacial acetic acid (>99%), ammonium acetate and triethylamine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Optima grade methanol and water were obtained from Fisher Scientific (Ottawa, ON, Canada).

2.2.3 Human Serum Sample Sources

Ethics approval was obtained from the Health Research Ethics Board of the University of Alberta to analyze archived human serum samples from Sweden and the U.S. Archived serum samples from Swedish primiparous women in Uppsala (**Table 2-1**) were originally collected and analyzed for the "Persistent Organic Pollutants (POPs) in Uppsala Primiparas" study.[32, 47] Three extracts from pooled primiparous women's serum samples were obtained each year during 1996-2010 (except 2003 and 2005). Sample details and pooling scheme were can be found elsewhere[47] and were also detailed in **Table 2-1**. In total, 36 extracts of pooled serum, and 4 procedural blanks were shipped to the University of Alberta and stored at -20°C prior to analysis.

Archived American serum or plasma samples from 1974,[48] 1989,[48] 2000/2001,[48] 2006[49] and 2010[28] were previously collected from individuals residing in the Hagerstown, Maryland, U.S.A. (**Table 2-1**). The 1974 serum and 1989 plasma samples were originally obtained from an archived database of two large community-based cohorts,[48] and were analyzed for PFAS trends. Unlike the original analyses performed on these samples, the present study did not include paired

samples from the same individuals. The 2000/2001serum,[48] 2006 plasma[49] and 2010 plasma[28] samples were originally collected for a series of cross-sectional studies that were designed to measure PFASs in American Red Cross adult blood. For the current study, 12 samples from each year were randomly selected: 3 from young males (YM, 25-35yr); 3 from young females (YF, 25-35yr); 3 from older males (OM, >50yr) and 3 from older females (OF, >50yr) (**Table 2-1**). In total, 60 samples were shipped to the University of Alberta and stored at -20°C.

2.2.3 Extraction of American Human Samples.

The American samples along with procedural blanks were extracted with a modified SPE method developed by Kuklenyik *et al.*[50] Only polypropylene labware was used, and all of them were rinsed with optima-grade 50%methanol/50%water three times prior to use. Oasis HLB cartridges (Waters, 60 mg/3 mL) were pre-conditioned with 2 mL methanol followed by 2 mL 0.1 M formic acid. 0.5 mL serum/plasma sample, spiked with 10 µL of 100 ng/mL MPFAC-MXA, was acidified with 2 mL of 0.1 M formic acid. The sample mixture was thoroughly mixed on a vortex mixer followed by a 15-min sonication. Together with the solution obtained by rinsing the tube used to contain the sample with 0.5 mL 0.1 M formic acid three times, each sample was loaded and allowed to slowly pass the cartridge by gravity. After being washed with 2 mL of 0.1 M formic acid, 4 mL of 0.1 M formic acid/methanol (50%:50%) and 1 mL of 1% ammonium hydroxide in water successively, the cartridges were vented by air for 20 seconds. Analytes were eluted with 1mL of 1% ammonium hydroxide in methanol into 15 mL centrifuge tubes and evaporated to near-dryness under a gentle steam of high-purity nitrogen at 55 °C. The obtained extracts, after being reconstituted with the solution obtained by rinsing the 15 mL centrifuge tubes with mobile phase of initial composition, was transferred to 750 µL polypropylene vials (Supelco) with polyethylene caps (National Scientific).

Results for serum and plasma are known to be comparable,[51] and thus no adjustments were made to the data. Biomonitoring of contaminants in individual samples usually results in log-normal distribution, whereas using pooled samples results in bell-shaped normal distributions.[52] Pooled samples are better for detecting small annual changes in a temporal trend study[53] because of the smaller intra-year variation (e.g. arithmetic means and smaller variances,[54] whereas individual samples are better for identifying important determinants of exposure.

2.2.4 Instrumental Analysis

2.2.4.1 PFOS isomer profile analysis

PFOS isomer profile analysis was performed on a Schimadzu LC-20AD (Shimadzu Corporation, Tokyo, Japan) HPLC system coupled to an Applied Biosystems hybrid triple-quadrupole linear ion trap API4000 mass spectrometer (MDS Sciex, Concord, ON, Canada). The isomer separation method used here was adopted from a previously published method.[46] Briefly, 20 μL of each extract were injected onto a FluoroSep RP Octyl HPLC column (100 Å pore size, 3 μm particle size, 2.1 mm i.d., 150 mm length, ES Industries, West Berlin, NJ, USA) heated to 40°C with a flow rate of 0.15 mL/min. An Eclipse XDB-C18 column (5 μm particle size, 4.6 mm i.d., 150 mm length, Agilent, Santa Clara, CA, USA) was connected upstream of the injector to trap the instrumental-sourced analytes,[55] thereby reducing the background signal. The mobile phase gradient started from 60%A (5 mM formic acid adjusted to pH=4 with ammonium hydroxide) and 40%B (methanol). The initial condition was held for 0.3 min before ramping to 64% B by 1.9 min, and then successively increased to 66% by 5.9 min, 70% by 7.9 min, 78% by 40 min, and finally reaching 100% by 42 min. After holding for 13 min, the percent of B rapidly returned to the initial condition by 66 min, and then equilibrated for 25 min prior to the next injection. The API4000 mass spectrometer was equipped with an electrospray interface operating at 450 °C in negative ion mode. MRM mode was used for data collection, and the specific tandem mass spectrometry transitions for each isomer can be found in **Table 2-2**. Typical isomer separations are presented in **Figure 2-2**, obtained from a pooled Swedish sample.

Analyst™ (v.1.5.1, ABSciex, ON, CA) was used to collect and process mass spectral data. As recommended by Riddell *et al*.,[56] each PFOS isomer was quantified against its own methanol-based calibration curve, except 3*m*- and 5*m*-PFOS, which were quantified together due to incomplete chromatographic resolution (**Figure 2-2**). Linear $^{13}C_4$-PFOS was used as the internal standard for all isomer quantification.

2.2.4.2 1*m*-PFOS Enantiomer Profile Analysis

1*m*-PFOS enantiomer profile analysis was achieved with a separate HPLC-MS/MS analysis of the same extracts used in the isomer analysis. Enantiomer separation was performed on an Agilent 1100 (Agilent Technologies, Palo Alto, CA) HPLC system, and the separation method developed by Wang *et al*.[44] was adopted. Briefly, two Chiralpak QN-AX HPLC columns

(5 μm silica-gel, 2.1 mm i.d., 150 mm length, Chiral technologies Inc. PA) in tandem, following a C18 guard column (5 μm particle size, 3.0 mm i.d., 4 mm length, Phenomenex, Torrance, CA) were used. Isocratic mobile phase of tetrahydrofuran (THF) : 0.2 M formic acid : water : tri-ethylamine (70% : 20% : 10% : 0.05 by volume) was flowing through the system at 0.12 mL/min. 20 μL of each extract (30-40 μL for few samples with low 1*m*-PFOS concentration quantified in isomer profile analysis) were injected. An Applied Biosystems API5000 triple-quadrupole mass spectrometer was used for detection. It was equipped with a Turbo V ion spray interface (MDS Sciex, Concord, ON, Canada), which was operating at 500 °C in negative ion mode.

Analyst 1.5.1 was used for collecting and processing mass spectral data. Enantiomer fractions (EFs) were used to describe quantitatively the relative proportions of 1*m*-PFOS enantiomers, and were calculated by dividing the peak area of the 1st eluting enantiomer by the combined peak area of the two enantiomers[57] after deconvolution using Peakfit software (v4.6, Systat Software, San Jose, CA). A typical enantiomer separation for a pooled Swedish serum sample, and the resulting deconvoluted chromatograms are presented in **Figure 2-3**.

2.2.5 Quality Control (QC)

Instrumental methanol blanks were injected every 10 samples to monitor carry-over in both analyses. Standards of br-PFOS and 1*m*-PFOS were analyzed after every 10 samples in the isomer and enantiomer analyses, respectively, to monitor any instrumental drift. Procedural blanks were analyzed with human samples to monitor background PFOS contamination. Method detection limit (MDL) and method quantification limit (MQL) were defined as the concentration in the sample corresponding to a chromatographic peak with a signal-to-noise ratio of 3 and 10, respectively, when no response was detected for the analyte in procedural blanks. When the analyte was present in procedural blanks (i.e. *n*-PFOS), MDL and MQL were defined as the mean blank concentration plus 3 or 10 times the standard deviation, respectively. MDLs and MQLs for the isomer analysis are reported in **Table 2-2** . For the chiral 1*m*-PFOS EF analysis, MDL and MQL of racemic 1*m*-PFOS were 0.46 pg and 1.5 pg on-column, respectively.

Recoveries of both sample extraction methods (i.e. SPE and protein precipitation), and matrix effects of both analytical methods (i.e. isomer and enantiomer separations) were assessed by the method introduced by Marchi *et al.*[58] In isomer separation, triplicate pre-spiked and post-

spiked samples were prepared by spiking 20 ng/mL of br-PFOSK standard into calf serum before and after the extraction process, while the solvent-based one were prepared by dissolving br-PFOSK standard into mobile phase to produce a 20 ng/mL sample. Recovery and matrix effects were then calculated by dividing the concentration of post-spiked sample by that of pre-spiked one, and by dividing the concentration of solvent-based sample by post-spike one, respectively. Measured PFOS isomer recoveries for SPE and protein precipitation methods were comparable, as were any matrix effects (**Table 2-2**), and %br-PFOS calculations were not significantly affected by extraction technique or matrix effects.

2.2.6 Racemic range of 1*m*-PFOS Enantiomer Fractions (EFs)

A total of 15 racemic 1*m*-PFOS standard injections were made under the following solvent conditions: 5 in methanol, 5 in 50% acetonitrile/50% 4 mM ammonium acetate (to match the Swedish extracts), and 5 in 40% methanol/60% 5 mM ammonium acetate pH=4 (to match the American extracts). The determined EFs ranged from 0.482 to 0.496 (**Figure 2-3**), and no statistical difference (p>0.05) was observed among the arithmetic means of the three different solvent conditions. The effect of both SPE and protein precipitation extraction methods were tested by spiking 46 pg of racemic standard into calf serum before and after the extraction, respectively, and EFs were also in the range of 0.482-0.496, showing no measurable influence. Matrix effects were also examined by spiking 46 pg of racemic standard into 100 μL of two human extracts (one with a high native concentration, and the other with a low native concentration of 1*m*-PFOS) from each country. The determined EFs after background correction ranged from 0.481 to 0.500, showing only a subtle matrix effect for human serum. Thus, for purposes of this study, the racemic EF range was conservatively defined as 0.480-0.500, similar to what was used by Wang *et al*.[44] and Asher *et al.*[45]

2.2.7 Statistical analysis

Temporal trends of ∑PFOS, %br-PFOS and 1*m*-PFOS EF were examined by piecewise regression in both Swedish and American populations, using the beginning of the phase-out (year 2000) as the breakpoint. Piecewise simple linear regression (PSLR, **Model 1**) was used for Swedish primiparous women's data, while piecewise multiple linear regression (PMLR, **Model 2**) was employed for American adult data to examine possible age and gender differences. ∑PFOS

data in Swedish and American samples were log-transformed prior to analysis because they did not meet the criterion for normality, as determined by the Shapiro-Wilk test. All %br-PFOS and 1*m*-PFOS EF data were normally distributed and did not require transformation.

Multiple linear regression (MLR) was used to determine the correlation between the two potential biomarkers, %br-PFOS and 1*m*-PFOS EF, to account for potential covariates (i.e. "year" in Swedish samples; and "year", "age" and "gender" in American samples). All statistical tests were performed with Stata (v8.0, StataCorp LP, Texas, USA), and $p<0.05$ was considered to be statistically significant.

Model 1 PSLR and **Model 2** PMLR are shown below, where $\boldsymbol{y}$ is the relevant PFOS measurement, ***year*** is the time vector, ***d*** is a dummy variable (0, if ***year***<2000 and 1, if ***year***≥2000), and ***age*** and ***gender*** are vectors for the age and gender categories, respectively.

$$\boldsymbol{y} = \beta_0 \cdot \boldsymbol{year} + \beta_1 \cdot (\boldsymbol{year}-2000) \cdot \boldsymbol{d} + \varepsilon \quad \textbf{(Model 1)}$$

$$\boldsymbol{y} = \beta_0 \cdot \boldsymbol{year} + \beta_1 \cdot (\boldsymbol{year}-2000) \cdot \boldsymbol{d} + \beta_a \cdot \boldsymbol{age} + \beta_g \cdot \boldsymbol{gender} + \beta_{ag} \cdot \boldsymbol{age} \cdot \boldsymbol{gender} + \varepsilon \quad \textbf{(Model 2)}$$

2.3 Results and Discussion

2.3.1 Temporal trends of ∑PFOS

All analyzed PFOS isomers were >MQL (**Table 2-2**) in all samples from both countries. For Swedish samples,[32, 47] the high homogeneity of the donors (all participants were primiparous women of nearly the same age from the same county, and all samples were collected three weeks after delivery), the large number of sampling years, and the pooling,[32, 47, 59] together likely contributed to high statistical power for temporal trend detection.[53] PSLR on log-∑PFOS showed no significant temporal trend up to 2000, but between 2000 and 2010 the downward trend was statistically significant (**Figure 2-4A**). This is consistent with the report by Glynn *et al.*,[47] who analyzed the same samples and stated that the decreasing trend only occurred after 2001, but did not analyze trends for the two separate periods. The trend detected here is broadly consistent with trends observed in Norwegians[33] and Germans,[22, 60] whereby the PFOS concentration in serum was stable from the mid-1990s to 2000/2001, but declined afterwards.

The population halving time,[22, 28] that is the amount of time required for a compound to fall to 50% of the amount at the beginning of a period in a population, was calculated by simple

linear regression (SLR) using only the data from 2000 onwards in both populations. The population halving time is appropriate when describing trends from cross-sectional data, and is not equivalent to the apparent human elimination half-life, nor the intrinsic half-life.[61] For Swedish samples, population halving time was estimated to be 4.8 (95% CI, 3.8-6.6) years by SLR on log-∑PFOS in the 2000-2010 period (p<0.001, R^2=0.74). Glynn *et al*.[32] formerly reported a longer population halving time [8.2 years (95% CI 6.3-12)] in the same Swedish samples, but this is because the whole dataset from 1996 to 2010 was used rather than only the 2000-2010 period. In fact, by treating the whole dataset together here, a longer population halving time of 7.1 (95%CI 5.9-9.0) years was obtained, demonstrating good agreement with Glynn *et al*.,[32] despite different isomer quantification methods used.

In American samples, temporal trend analysis by PMLR on log-∑PFOS showed no significant temporal trend from 1974 to 2000/2001, but in the 2000/2001-2010 period the downward trend was statistically significant (**Figure 2-4B**). Both of these observations were consistent with the study on the larger dataset from which the current samples were drawn.[28, 48] Furthermore, the 2000/2001-2010 decline is broadly in line with two American NHANES studies.[29, 62] It was confirmed in the analysis that neither age nor gender confounded the temporal trend analysis. The population halving time, based on the 2000/2001-2010 data, was 5.4 years (95% CI, 3.8-9.7), close to the 4.3 year estimate in the larger population from which this subset was drawn,[28] thus the subset of samples examined here was representative.

Taken together, the decline of PFOS between 2000 and 2010 in Swedes and Americans in this study mirrored each other, and corresponded with the time of the phase-out, suggesting significant exposure mitigation by the phase-out. Nevertheless, we understand very little about what pathways of exposure resulted in these declines, thus it is of interest to know the relative contribution that PFOS and PreFOS have played over time.

2.3.2 Temporal trends of %br-PFOS

Triplicate analyses of a relevant historic 3M electrochemical fluorination (ECF) PFOS standard in the current study revealed 30±2.7% (mean ± standard deviation (S.D.)) branched PFOS content (%br-PFOS). Taking this as the reference, only 3 Swedish pooled samples (2 from 1996, and 1 from 1999), and 1 American individual sample (from 1989), could be characterized

as having an "ECF-like" PFOS isomer signature. For all other samples, %br-PFOS was greater than 33% (**Figure 2-5**), except for one sample from an American young female which had an exceptionally low %br-PFOS of 27%. Such enrichment of branched isomers, relative to historic ECF PFOS, is consistent with other human monitoring studies (**Table 2-3A**).

Direct PSLR on %br-PFOS in pooled Swedish samples from primiparous women showed no significant trend in %br-PFOS between 1996 and 2000, but a significant upward trend between 2000 and 2010 (**Figure 2-5A**). The increase of %br-PFOS is consistent with the general trend noted by Glynn *et al*.[32] for the same samples, and was confirmed here statistically. The slightly lower %br-PFOS values determined by Glynn *et al.* (mean values from 31% in 1996 to 39% in 2010) than in the current study (means from 32% in 1996 to 45% in 2010) is mostly likely attributable to the different calibration method used by Glynn *et al*.

As discussed, enriched branched PFOS isomer profiles (i.e. %br-PFOS >30%) might be explained by PreFOS exposure. If so, the increasing %br-PFOS temporal trend suggests that the relative importance of PreFOS exposure has been increasing compared to direct PFOS exposure. There are no convincing Swedish data suggesting that exposure to PreFOS has increased over time in an absolute sense, but the trend observed here could be explained if pathway 1 has diminished more rapidly than pathways 2 and 3 (**Figure 2-1**).

An alternative explanation that must be considered for the increasing %br-PFOS temporal trend is the potential for environmental or biological processes to affect PFOS isomer profiles in human exposure media. For instance, soils preferentially adsorb the more hydrophobic linear PFOS,[63, 64] causing enriched branched PFOS isomer profiles in water (**Table 2-3B**). Conversely, it is known that fish excrete branched PFOS isomers more efficiently,[65] resulting in enriched linear PFOS isomer profiles in fish meat (**Table 2-3C**). For the general Swedish population, fish is an important dietary component.[66] However, almost all fish samples previously examined were enriched in *n*-PFOS (**Table 2-3C**). Furthermore, %br-PFOS in archived herring, an important commercial fish species landed in Swedish harbors, actually declined between 1991 (7% branched) and 2011 (<4% branched).[67] An estimation of different food categories in the general Swedish population suggested that the contribution of fish product to dietary PFOS has increased (i.e. ~40% in 1999, ~60% in 2005 and ~85% in 2010).[66] Thus, it is difficult to reconcile %br-PFOS isomer patterns and temporal trends in fish, with isomer patterns and trends in the current

Swedish serum samples.

In contrast to the Swedish samples, a temporal trend for %br-PFOS was not detected in American samples by PMLR (**Figure 2-5B**). This may be due to lower statistical power (e.g. the small sample size and the relatively large intra-year variability due to the use of individual samples), compared to the Swedish pooled serum data set, or may reflect the absence of a true temporal trend. Interestingly though, both gender and age were significant variables in the PMLR analysis. Older Americans had significantly higher (Δ=3.6) %br-PFOS than younger Americans, after adjusting for gender, and American males were significantly higher (Δ=4.7) in %br-PFOS than females, after adjusting for age. The latter is consistent with a report of significantly higher %br-PFOS in males (33%) than females (30%) in Norway[68] and a recent study in 50 Chinese young couples.[69] According to our hypothesis, the significantly lower %br-PFOS in American females could be a result of relatively lower PreFOS exposure compared to males. However, it might also be explained by isomer-selective placental transfer during pregnancy, which favors branched PFOS isomers and results in reduced %br-PFOS in maternal serum.[70, 71] Scarce isomer specific studies exist on menstrual and lactation elimination of PFOS, however, the significantly higher %br-PFOS in the husband than their wives, none of which have had a baby,[69] may point to a preferential branched PFOS elimination in females. In another study a statistically unchanged PFOS isomer profile was determined in mothers across one year's lactation,[72] suggesting that no isomer selection happened in lactation.

Interestingly, American males[28, 48, 49, 73] and older adults[48] had higher ∑PFOS than females and younger adults, respectively, in the larger American study population from which this small subset of samples was drawn. This, together with the same age and gender differences detected here for %br-PFOS, shows a tendency that individuals with higher ∑PFOS concentrations may have higher %br-PFOS. This is in agreement with Zhang *et al.*,[74] who observed a significant positive correlation between ∑PFOS and %br-PFOS in a Chinese population.

To determine if all branched PFOS isomers were behaving similarly, we also examined correlations among the isomers, in terms of absolute concentration and percentage contribution to ∑PFOS in both populations. Spearman rank correlation was used here, because most data sets did not meet criteria for normality (**Table 2-4** and **Table 2-5**). In both populations, except for 1*m*-PFOS in Swedish samples, the individual branched PFOS isomer concentrations were always

positively correlated, indicating consistent temporal trends of the major PFOS isomers in both populations. With respect to percentage of each PFOS isomer, 1*m*-, (3+5)*m*- and 4*m*-PFOS, together comprised 63±0.69% and 68±0.80% of total branched PFOS in Swedes and Americans, respectively, and were always positively correlated with each other. This pattern, to some extent, suggests the similarity in environmental or biological disposition among the majority of branched PFOS isomers, or the similarity in the metabolism of the corresponding PreFOS isomers. *Iso*-PFOS, the single most prominent individual branched PFOS isomer in both populations (36±4.1% in Swedes and 31±6.2%in Americans), did not correlate with any other PFOS isomer, suggesting a distinct behavior in either *iso*-PFOS disposition or *iso*-PreFOS metabolism. An analogous situation was reported by Zhang *et al.*,[69] who found that 1*m*-, (3+5)*m*- and 4*m*-PFOS were enriched to a similar extent in humans when compared to the ECF PFOS, while *iso*-PFOS was enriched to a much lesser extent.

2.3.3 Temporal Trends of 1*m*-PFOS EFs

All samples from both countries showed non-racemic EFs for 1*m*-PFOS (i.e. outside the range of 0.480-0.500). Additionally, except for one American sample (EF=0.512, from 1989), all samples had EFs below 0.480. This general finding is in line with the directionality of non-racemic EFs previously measured in 8 Canadian pregnant women, and 7 members of a highly ScotchGard™-exposed Canadian family,[44] suggesting a common 1*m*-PreFOS enantioselective metabolic preference in most people examined to date. PSLR gave a significant decreasing temporal trend in 1*m*-PFOS EFs between 1996 and 2000, but no significant trend between 2000 and 2010 in Swedish primiparous women (**Figure 2-6A**). No significant 1m-PFOS EF temporal trend was observed in Americans (**Figure 2-6B**), however, both age and gender were found to be significant predictors. Males had significantly lower (Δ=0.02) 1m-PFOS EFs than females after adjusting for age, and the elderly had significantly lower (Δ=0.03) 1*m*-PFOS EFs than the young after adjusting for gender.

As hypothesized by Martin *et al.*,[5] non-racemic 1*m*-PFOS EFs may point to PreFOS exposure by pathways 2 and 3 (**Figure 2-1**). Based on the limited available evidence, the only known enantioselective process that can lead to non-racemic 1*m*-PFOS is the biotransformation of 1*m*-PreFOS.[42] Therefore, the observed 1*m*-PFOS EF trend in Swedish population, and its age/gender difference in Americans, suggested an increased relative contribution of PreFOS

exposure in Swedes over time, and relatively higher PreFOS exposure in male or old Americans. However, there are other non-PreFOS-relevant processes that could cause non-racemic signatures of 1*m*-PFOS EFs. Certain environmental or biological processes may favor one specific 1*m*-PFOS enantiomer. For example, it is currently uncertain if human elimination of 1*m*-PFOS (e.g. urine[38] or feces[75]) is enantioselective. In rats, there was no evidence for enantioselective urinary elimination or tissue distribution,[44] but species differences could exist. The observed age and sex differences could possibly explained by enantiospecific menses,[76] placental transfer[70, 71] and lactation[32, 70, 77] in young women, but enantioselective studies of these processes have not been done for PFOS, thus it is acknowledged that there remains some uncertainty in interpreting 1*m*-PFOS EF data.

2.3.4 Comparing %br-PFOS and 1*m*-PFOS EF

A close examination of individual data revealed that the American with the exceptionally high 1*m*-PFOS EF (0.512) was the same individual with the lowest %br-PFOS (27%). Furthermore, the 3 pooled Swedish samples having the highest 1*m*-PFOS EF (0.475, 0.462 and 0.446, respectively) also had the lowest %br-PFOS (30%, 32% and 33%, respectively). MLR analysis between %br-PFOS and 1*m*-PFOS EFs, after correcting for potential covariates (i.e. "year" in Swedish samples, and "year", "age" and "gender" in American samples), revealed significant correlations between the two biomarkers in American samples (**Figure 2-7B**), but not when using all the Swedish samples (**Figure 2-7A**). However, when categorizing the Swedish samples into two time periods (before and after the phase-out), a significant correlation between %br-PFOS and 1*m*-PFOS EF was observed in the 1996-2000 period (p=0.01) but not in the 2000-2010 period (p=0.36) (**Figure 2-7A**). The *a priori* hypothesis, proposed by Martin *et al*.,[5] was that both measurements could be biomarkers of PreFOS exposure, and the current results offer some support to this theory. However, 1*m*-PFOS EFs only explain 40% of the variation in %br-PFOS (41% in Swedish samples for the 1996-2000 period, and 42% in American samples), indicating that the two biomarkers are not exactly equivalent. Further studies of PFOS isomer content in important human exposure media (e.g. in diet, to rule out a high %br-PFOS dietary intake), and comparison of the 1*m*-PFOS EF in paired serum and urine samples (to rule out enantioselective human excretion) are priorities for future research.

Both isomer and enantiomer biomarkers suggest an increase in the relative importance of

PreFOS exposure in this Swedish population since the late 1990s. If true, the apparent contradictions between (i) the low %br-PFOS in Swedish herring (and most other fish) and the high %br-PFOS in the current Swedish sera, and (ii) between the decreasing %br-PFOS in Swedish herring (from 7% in 1991 to <4% in 2011[67]) and the increasing %br-PFOS in the current Swedish population over the same period, might suggest increasing relative significance of non-dietary PreFOS exposure pathways (e.g. consumer products[78]). The American samples, however, showed no clear trend in PFOS isomer or enantiomer profiles, making the relative PFOS and PreFOS exposure trends unknown. Future studies on larger sample sets, with smaller gaps between sampling times, and in populations that are representative of the underlying general population would be of great help. Furthermore, longitudinal samples from the same people, rather than the cross-sectional samples as used in the current study, would be better for temporal trend studies, since factors such as age and birth-period[79] may have confounded the results here.

2.4 Figures and Tables

2.4.1 Figures

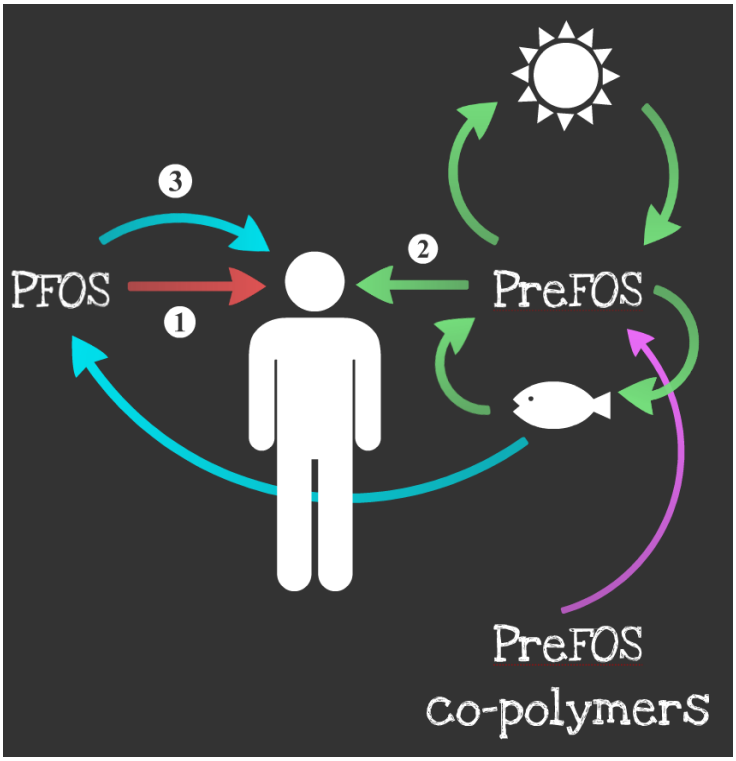

Figure 2-1. Summary schematic for three primary PFOS exposure pathways. As discussed by Martin *et al*.,[5] (1) PFOS may be emitted directly to the environment and absorbed directly in humans (red arrow); (2) PreFOS may be emitted to the environment (directly, or degraded from copolymers (pink line)). The emitted PreFOS might first be transformed to other forms of PreFOS (biotic or abiotic), but some form of PreFOS is directly absorbed into the human body (green arrows), a fraction of which can then be metabolized to PFOS; and (3) PreFOS may be emitted to the environment and transformed to PFOS abiotically or biotically (e.g. fish, or microbes), and the resultant PFOS could then be absorbed directly in humans (blue arrows).

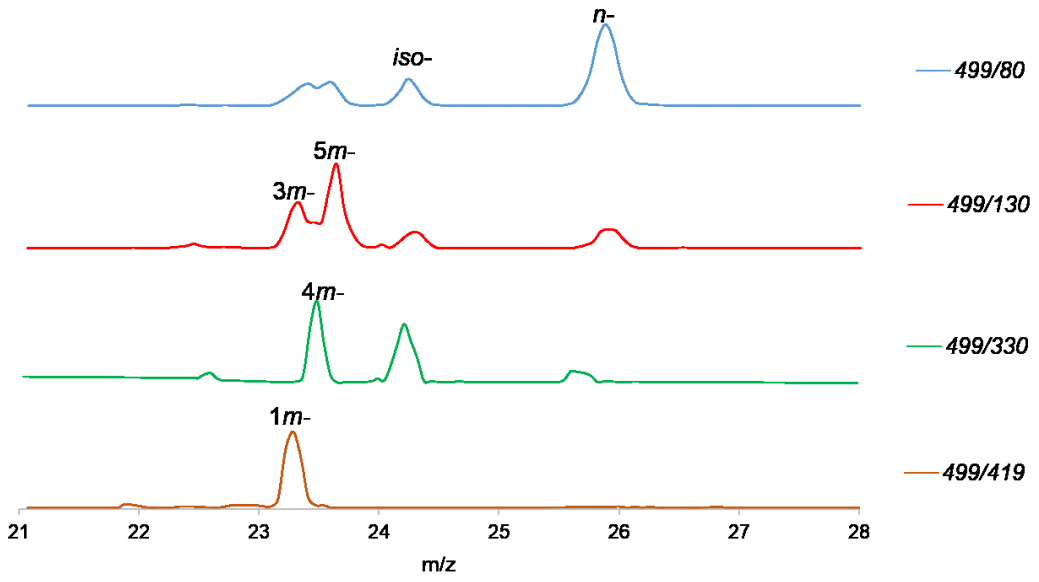

Figure 2-2. Typical PFOS isomer chromatograms. Numbers beside each chromatogram (i.e. "499/#") refer to tandem mass spectrometry transitions used to monitor and quantify different isomers.

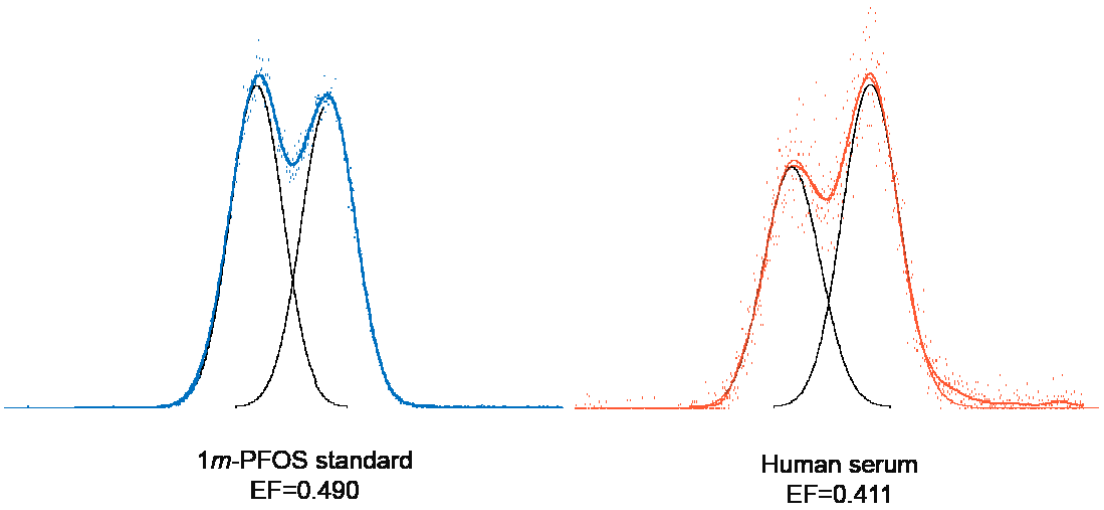

Figure 2-3. Typical 1*m*-PFOS enantiomer chromatograms for 1*m*-PFOS standard (blue) and for a pooled Swedish serum sample (red). The black lines are deconvoluted traces processed by Peakfit.

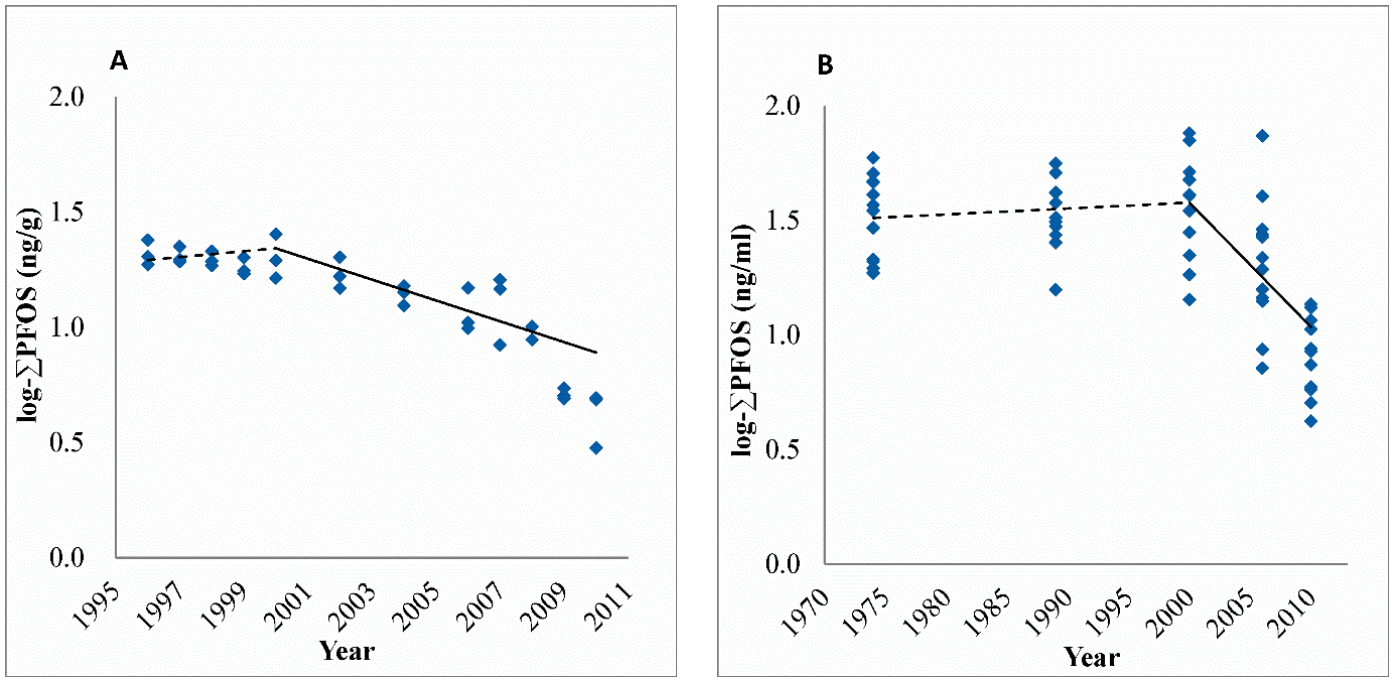

Figure 2-4. Temporal trends of log-transformed total PFOS (log-∑PFOS) in pooled Swedish serum samples from primiparous women (A, $p_{1996\text{-}2000}$=0.47, $p_{2000\text{-}2010}$<0.001) and in individual American adult serum/plasma samples (B, $p_{1974\text{-}2000}$=0.50, $p_{2000\text{-}2010}$<0.001). Black lines indicate piecewise linear regression (solid and dashed lines indicate statistically significant and insignificant regressions, respectively) over different time periods.

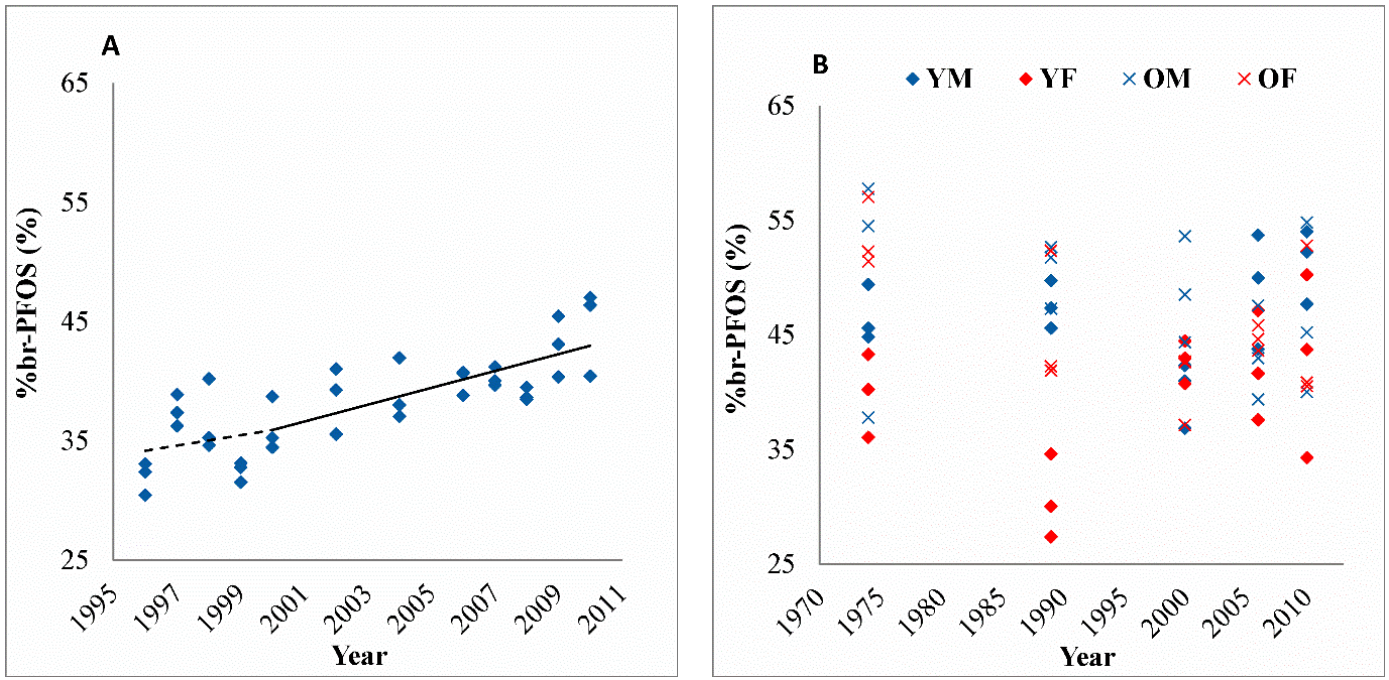

Figure 2-5. Temporal trends of percent branched PFOS isomers (%br-PFOS) in pooled Swedish serum samples from primiparous women (A, $p_{1996\text{-}2000}$=0.30, $p_{2000\text{-}2010}$<0.001) and in individual American adult serum/plasma samples (B, p_{gender}=0.003, p_{age}=0.02). The black lines indicate piecewise linear regression (solid and dashed lines indicate statistically significant and insignificant regressions, respectively, over different time periods). The four groups in American samples are young males ("YM"), young females ("YF"), old males ("OM") and old females ("OF").

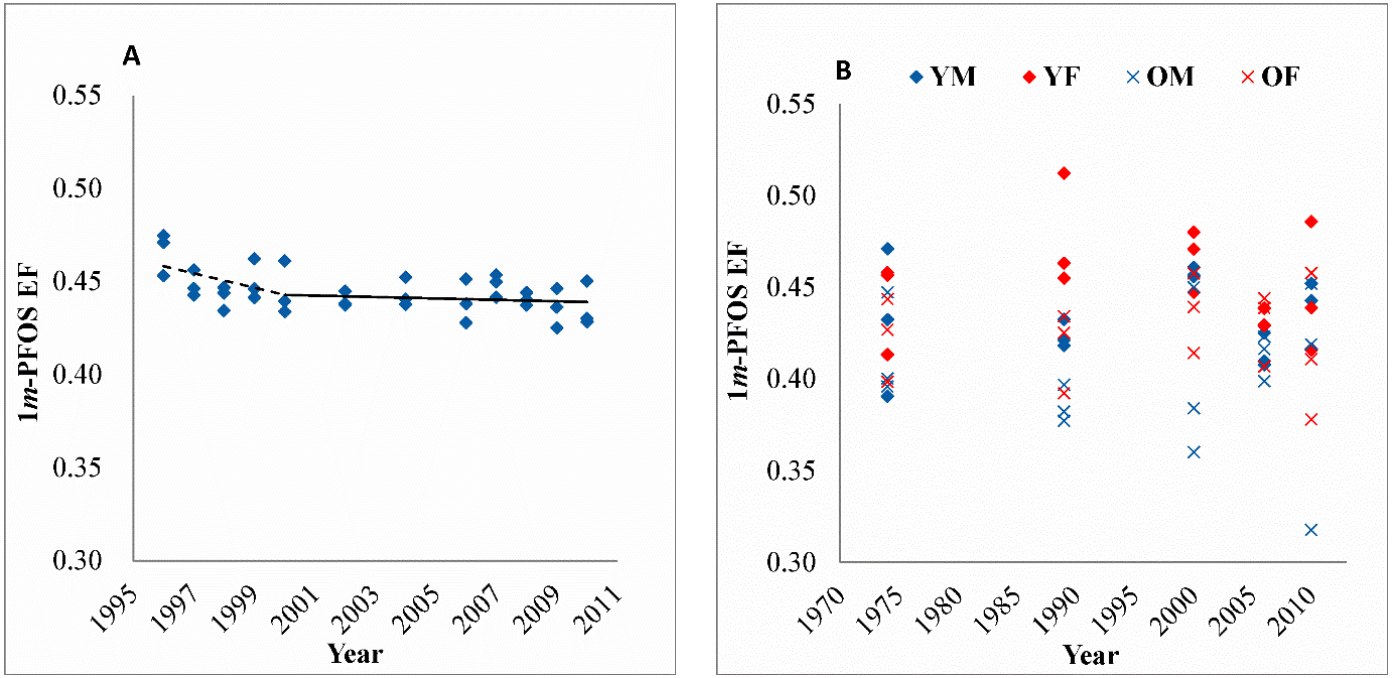

Figure 2-6. Temporal trends of enantiomer fractions of 1*m*-PFOS (1*m*-PFOS EFs) in pooled Swedish serum samples from primiparous women (A, $p_{1996\text{-}2000}$=0.016, $p_{2000\text{-}2010}$=0.49) and in individual American adult serum/plasma samples (B, p_{gender}=0.004, p_{age}<0.001). The black lines indicate piecewise linear regression (solid and dashed lines indicate statistically significant and insignificant regressions, respectively, over different time periods). The four groups in American samples are young males ("YM"), young females ("YF"), old males ("OM") and old females ("OF").

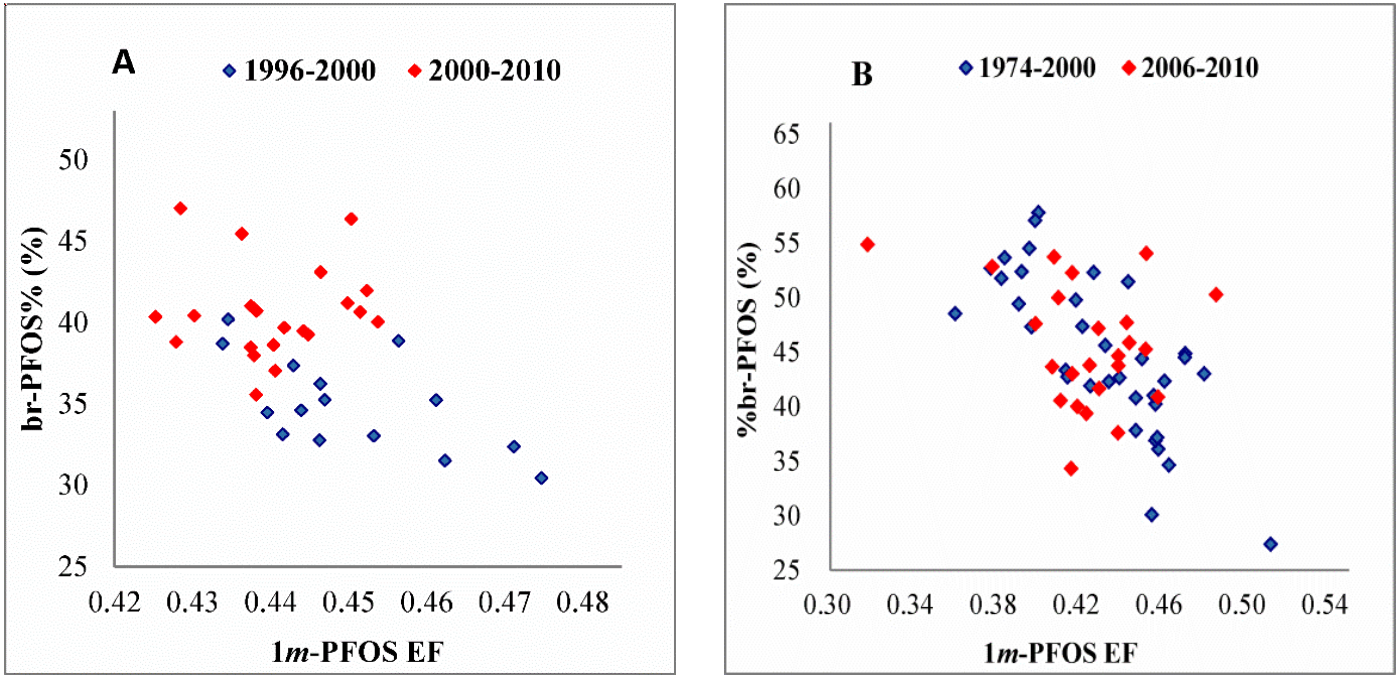

Figure 2-7. Plots of percent branched PFOS isomers (%br-PFOS) *vs* enantiomer fractions of 1*m*-PFOS (1*m*-PFOS EF) in pooled Swedish serum samples from primiparous women (A, $p_{1996\text{-}2000}$=0.01) and in individual American adult serum/plasma samples (B, p<0.001).

2.4.2 Tables

Table 2-1. Comparison of Swedish and the U.S. human serum samples

	Swedish Samples[32, 47]	**the U.S. Samples**[28, 48, 49, 73]
Location	Uppsala, Sweden	Hagerstown, Maryland, USA
Participants	19-41year-old primiparous women 3 weeks after delivery	young males (25-35yrs); young females (25-35yrs) old males (>50yrs); old females (>50yrs)
Sampling year	1996, 1997, 1998, 1999, 2000/2001, 2002, 2004, 2006, 2007, 2008, 2009, 2010	1974, 1989, 2000/2001, 2006, 2010
Sample type	extracts of pooled serum (by protein precipitation method[1])	individual serum (1974[48] and 2000/2001[73]) individual plasma (1989[48], 2006[49], 2010[28])
Sample number/year	3 extracts of pooled serum/year (6-7, 20-21, 24-25, 5-6, 9-10, 10-11, 10-11, 10, 9-10, 10, 10, 10 individual samples, respectively, in each pool from 1996-2010)	12 individual samples/year (3 individual samples for each subgroup)
Total sample number	36 extracts of pooled serum sample and 4 procedural blanks	60 individual samples

Table 2-2. MRM transitions, MDLs, MQLs, recoveries and matrix effects of individual PFOS isomer in PFOS isomer analysis.

PFOS isomer	MRM transition	linear regression r^2	MDL ng/mL	MQL ng/mL	SPE for the U.S. samples		Protein Precipitation for Swedish samples	
					Recovery (%)[α]	Matrix Effects (%)[α]	Recovery (%)[α]	Matrix Effects (%)[α]
***n*-**	499→80	0.9982	0.080	0.20	107±1	116±5	97±6	114±5
1*m*-	499→419	0.9942	0.0030	0.0090	98±8	108±2	100±4	87±3
(3*m*+5*m*)-	499→130	0.9978	0.010	0.035	108±4	98±6	94±7	71±2
4*m*-	499→330	0.9952	0.015	0.045	98±2	111±2	108±3	72±3
***iso*-**	499→80	0.9970	0.015	0.050	105±1	119±4	95±8	95±6

[α] Recovery and matrix effects were assessed by analyzing 20ng/mL br-PFOSK spiked calf serum samples (expressed here as Mean ± S.E.)

Table 2-3. Review of previous PFOS isomer studies.

(A) In human samples

Author, Year	Samples description (determined %br-PFOS in samples)	%br-PFOS in ECF PFOS
Zhang *et al*.[69] 2014	Adult serum (mean value 40.8%) from 50 Chinese couples in North China, 2012	~28%
Zhang *et al*.,[74] 2013	adult serum (51.9%, range 22.7-82.7%) from two cities in North China, 2010	29.7%
Beesoon *et al*.,[70] 2011	Paired maternal serum (36%, range 27-44%), and cord serum (46%, range 36-54%) from Vancouver, Canada, 2007-2008	30±2.7%
Haug *et al.,*[33] 2009	Pooled serums samples from different county hospitals in Norway (32, 36, 40, 43% for 1976, 1987, 1998 and 2007, respectively)	/
Riddell *et al*.,[56] 2009	Pooled serum samples from donors consumed fish caught around the Great Lakes, 2006; pooled serum from humans across U.S., 2006 (30-50% for both)	/
Karrman *et al*.,[80] 2007	Plasma samples from Swedish adults 1997-2002 (34%, range 30-50%); plasma samples from young adults in United Kingdom, 2003 (40%); human serum from Australia from 2002-2003 (41%)	21-34%
Rylander *et al*.,[68] 2009	Adult males (33%, range 0-51%) and females (30%, 0-44%) from Andenes, Norway, 2005. The population is known to have a high consumption of various kinds of seafood (on average 31%, range 0-51%)	/
Rylander e*t al*.,[81] 2009	Plasma samples from pregnant women in a coastal city and an inland district in the same province in south central Vietnam, 2005. Samples were taken within 3 hours after delivery (on average 19%, range 7-83%)	/

(B) In the environmental samples

Author, Year	Samples description (determined %br-PFOS in samples)	%br-PFOS in ECF PFOS
Karrman *et al*.,[63] 2011	Soils around a AFFF-used training site in Flesland airport (average 21%, increased with the distance from the site); Water (39-42%), sediment (average: 21.5%) and fish liver (10-13%) from the lake the seepage water	37
Houde *et al*.,[64] 2008	Bulk water (44-57%), sediment (11-19%) and invertebrates and fish (<=22%) from Lake Ontario, Canada, sampled in 2002	23
Asher *et al*.,[45] 2012	Bulk water (31.2%), sediment (3.7%) and aquatic species (<=10%) from Lake Ontario, Canada, sampled in 2007-2008	/
Benskin *et al*.,[82] 2010	Coastal Asian seawater: Tomakomai Bay, Japan, 2003 (31.4%-38.7%); Tokyo Bay, Japan, 2008(35.4%); Sea of Japan, 2008 (38.8-39.6%); Hangzhou, China, 2008 (30.7%); Shanghai, China 2008(29.2%) Mississippi river, 2009: water (51.9%); sediment (11-19%); North Sea Canal Water from Netherland, 2005 (~34%)	27.2%
Yu *et al*.,[83] 2013	Water samples from Huai River Basin (32.4%, 29.0-35.0%) and Taihu Lake (45.1, 48.1-62.5%), China, 2011	29.1% (21.8-34.7%)
Ullah *et al*.,[84] 2011	Tap water from Stockholm University, Sweden (32%), Institute for Environment and Sustainability, Ispra, Italy (26%), University of Antwerp, Belgium (38%), University of Amsterdam, The Netherlands (38%), VU University, Amsterdam, The Netherlands (42%), Norwegian Institute for Air Research, Tromso Norway (30%) and Fraunhofer Institute, Schmallenberg, Germany (29%)	21.2%

(C) In wildlife samples

Author, Year	Samples description (determined %br-PFOS in samples)	%br-PFOS in ECF PFOS
Powley *et al*.,[85] 2008	Cod (~50%), ringed and bearded seal blood (4%) from Western Canadian Arctic, 2004	26%
Gebbink *et al*.,[86] 2010	Herring gull eggs (1.7-5%) from Great Lakes, North America (NA), 2007	21-35%
O'Brien *et al*.,[87] 2011	Direct inject technical PFOS into the air cell of chicken eggs prior to incubation, and detected ~16% in liver of the embryonic chickens	37.3
Sharpe *et al.,*[65] 2010	Enriched *n*-PFOS in laboratory exposed zebra fish and rainbow trout compared to the technical PFOS in food	/
Chu *et al*.,[88] 2009	Herring gull eggs from Great Lakes, NA, 1989 (5.5%); Double crested cormorant eggs from Great Lakes, North America, 2003 (4.1%); Polar bear plasma from Norwegian Arctic, 2007 (17.6%); Polar bear livers from Nunavut, Canadian Arctic, 2007 (7.6%)	35
Kumar *et al*.,[89] 2009	Aquatic wildlife, including sharks, from Georgia coast, USA, 2006-2007 (11-23%)	/
Greaves *et al*.,[90] 2013	Blood (15%) and liver (7%) samples of polar bear from Greenland, 2006	21-35%
Rotander *et al.,*[91] 2012	Whale livers (5-7%) from Faroe Islands 1984-1989; ringed seal liver (9% in 1984 to 17% in 2006) from north West Greenland	/
Huber *et al*.,[92] 2012	Harbor porpoise liver samples from Baltic (~10%) and North Sea (15%) 1991-2008	/
Lloyd *et al*.,[93] 2009	Whitebait (39%), Roe deer (42%), Cromer crab (14%) and Carp (11%)	~29%
Ullah *et al.,*[94] 2012	10 duplicate food samples from individuals in southern part of Germany during Apr. to Oct.2005 (15-36%); Carp from Netherland (22%), pig livers from supermarkets in Norway, Belgium and Czech Republic (23%)	21.2%
Ullah *et al.,*[67] 2014	Archived Herring muscle and liver samples during 1991-2011 (≤10% in all); fishes from Baltic Sea, Sweden, Lake Ijssel, Netherland and Amsterdam etc. (3%-18%)	/

Table 2-4. Spearman correlation coefficients (r value) and significant levels (p value) among #-PFOS (*#=total*, *total branched*, *1m*, *(3+5)m*, *4m*, *iso*, m_2, *n*-) and *%#*-PFOS in Swedish pooled serum samples. *p* values in red indicate statistically significant.

(A). Spearman correlation among PFOS isomer concentration in pooled Swedish serum samples (ng/g)

		total	**%br-**	**%1*m*-**	**%(3+5)*m*-**	**%4*m*-**	**%*iso*-**	**%*n*-**
total	r value	1.000						
	p value							
%br-	r value	.951*	1.000					
	p value	.000						
%1*m*-	r value	.463*	.558*	1.000				
	p value	.005	.000					
%(3+5)*m*-	r value	.943*	.989*	.554*	1.000			
	p value	.000	.000	.000				
%4*m*-	r value	.854*	.942*	.541*	.927*	1.000		
	p value	.000	.000	.001	.000			
%*iso*-	r value	.983*	.980*	.481*	.964*	.898*	1.000	
	p value	.000	.000	.003	.000	.000		
%*n*-	r value	.986*	.915*	.397*	.909*	.796*	.959*	1.000
	p value	.000	.000	.016	.000	.000	.000	

* $p<0.05$

(B) Spearman correlation among Percent PFOS isomer in pooled Swedish serum samples (%#-PFOS)

		%br-	**%1*m*-**	**%(3+5)*m*-**	**%4*m*-**	**%iso-**	**%*n*-**
%br-	r value	1.000					
	p value						
%1*m*-	r value	.771*	1.000				
	p value	.000					
%(3+5)*m*-	r value	.939*	.753*	1.000			
	p value	.000	.000				
%4*m*-	r value	.800*	.512*	.660*	1.000		
	p value	.000	.001	.000			
%*iso*-	r value	.194	-.278	.141	.155	1.000	
	p value	.258	.100	.413	.366		
%*n*-	r value	-1.000*	-.771*	-.939*	-.800*	-.194	1.000
	p value		.000	.000	.000	.258	

* $p<0.05$

Table 2-5. Spearman correlation coefficients and significant levels (*p* value) among #-PFOS (*#=1m, (3+5)m, 4m, iso, m_2, n-*) and %#-PFOS in American serum/plasma samples. *p* values in red indicate statistically significant.

(A) Spearman correlation among PFOS isomer concentration in American serum/plasma samples (ng/mL)

		total	**%br-**	**%1*m*-**	**%(3+5)*m*-**	**%4*m*-**	**%*iso*-**	**%*n*-**
total	r value	1.000						
	p value							
%br-	r value	.971*	1.000					
	p value	.000						
%1*m*-	r value	.683*	.739*	1.000				
	p value	.000	.000					
%(3+5)*m*-	r value	.968*	.995*	.706*	1.000			
	p value	.000	.000	.000				
%4*m*-	r value	.960*	.989*	.731*	.991*	1.000		
	p value	.000	.000	.000	.000			
%*iso*-	r value	.947*	.948*	.567*	.950*	.924*	1.000	
	p value	.000	.000	.000	.000	.000		
%*n*-	r value	.988*	.937*	.625*	.937*	.923*	.929*	1.000
	p value	.000	.000	.000	.000	.000	.000	

*$p<0.05$

(B) Spearman correlation among Percent PFOS isomer in American serum/plasma samples (%#-PFOS)

		%br-	**%1*m*-**	**%(3+5)*m*-**	**%4*m*-**	**%*iso*-**	**%*n*-**
%br-	r value	1.000					
	p value						
%1*m*-	r value	.483*	1.000				
	p value	.000					
%(3+5)*m*-	r value	.864*	.270*	1.000			
	p value	.000	.037				
%4*m*-	r value	.864*	.327*	.852*	1.000		
	p value	.000	.011	.000			
%*iso*-	r value	.221	-.525*	.227	.096	1.000	
	p value	.090	.000	.081	.464		
%*n*-	r value	-1.000*	-.483*	-.864*	-.864*	-.221	1.000
	p value		.000	.000	.000	.090	

* $p<0.05$

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Chapter 3. Discovery of C5-C17 PFASs in Water by In-Line SPE-HPLC-Orbitrap with In-Source Fragmentation Flagging‡

3.1 Introduction

Due to their unique water and oil repellency, high surface activity, and thermostability, PFASs have been used since the 1950s as surfactants and surface protectors for various industrial, commercial and consumer product applications. However, since the widespread discovery of PFOS and PFOA in the environment in 2001,[1, 2] the highly persistent[3] and sometimes bioaccumulative[4] nature of long-chain PFASs has come to be recognized. There has since been a global trend towards restricting the use of some long-chain PFASs (e.g. the *Stockholm Convention* on PFOS and its precursors[5]) and transitioning to alternative PFASs that are believed to be less bioaccumulative or less persistent.[6]

A recent review[6] of alternative PFASs showed that traditional long-chain PFASs, short-chain PFASs, and structurally modified long-chain PFASs (e.g. per- or polyfluoropolyethers) are currently in production. However, experts agree that there is little publicly available information on all alternative PFASs now being manufactured and marketed, including their chemical structures. For this reason, among several others, the recent Helsingor Statement[7] and Madrid Statement[8] called on the international community to limit the production and use of PFASs altogether.

Quantitative mass balance studies comparing total extractable organic fluorine (EOF) to the sum concentrations of known PFASs have consistently demonstrated a significant proportion of unknown organic fluorine compounds in various sample types: 50 to ≥99% unknown in water[9] or sediment,[10] and 15 to ≥99% unknown in biological samples including worms and shrimp,[11] wild rat/mouse blood[12] and even human blood.[13] These unknown PFASs represent a great source of uncertainty for environmental and human health risk assessment, thus their discovery and

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characterization is of high importance. Total organic fluorine measurements, including by CIC, provide a quantitative indication of how much organic fluorine is present in a sample, but this method is not selective enough to identify unknown fluorinated chemicals.

HRMS makes the characterization of unknown compounds possible based on the inherent combination of high spectral RP (i.e. ≥ 10,000) and high mass accuracy (i.e. ≤5ppm).[14-17] This enables the assignment of molecular formulae to observed *m/z* peaks, for example by using QTOF-MS. In highly complex matrices, uHRMS (i.e. RP≥100,000) can provide additional discovery power by further base-line resolving the features in mass spectra, allowing minor analytes to be uncovered and thus their chemical formulae to also be predicted. Technologies that are capable of uHRMS include Orbitrap-MS and FTICR-MS.

A critical step in unknown PFAS identification is to distinguish the few fluorinated compounds, usually present at low levels, from non-fluorinated compounds, many at high levels, in complex environmental or biological samples. A few approaches have already been described for non-targeted PFAS screening. Qin *et al*.[18] used HPLC-continuum source high resolution molecular absorption to flag the presence of model fluorinated compounds, followed by off-line analysis of HPLC fractions by low resolution MS. This proof-of-principle method has the potential advantage of quantifying unknown fluorine (i.e. ng F), but so far has not been reported to have identified any unknown fluorinated compounds in real samples.

MS-based approaches have taken advantage of negative mass-defects (e.g. in the range of -0.100 to 0.150[14)] and chain-length homologues (*m/z* spacing equals to ±50 or ±100). Trier *et al*.,[17] scanned industrial product blends using qTOF-MS and found >100 PFASs by only focusing on homologous ions with negative mass defect. Place *et al*.[14] examined several AFFF products by fast atom bombardment MS to screen for such ion series, followed by a secondary qTOF-MS analysis to examine the mass defects for the suspected ions series. D'Agostino *et al*.[15] used TOF-CIC to prescreen AFFF fractions, and then used the above approaches to flag specific PFASs in high fluorine AFFF fractions by HRMS (qTOF) and uHRMS (FTICR-MS). To date, the only applications of the above methods to non-targeted discovery of PFASs in environmental samples were by Strynar *et al*.[16] who, by focusing on ions present in water downstream of a fluorochemical plant, but not upstream, reported unique homologous PFAS compounds using TOF-MS; and by Crimmins *et al*.[19] who, by screening ions with negative mass defects in lake

trout extract tentatively proposed a new short-chain chlorine substituted PFAS.

In our experience with the analysis of complex environmental samples by uHRMS, mass defect and homologous ion-series profiling are indeed useful approaches, but on their own leave too much ambiguity in detection and characterization of unknowns. This is because known PFASs often contain other heteroatoms (e.g. S, P and O) which also decrease the molecular MD. Furthermore, even given an instrumental mass accuracy of ≤3 ppm there is often more than a single theoretical empirical formula that can be assigned to an unknown *m/z* peak when allowing unrestricted formula predictions from only C, H, O, N, S, P, F and Cl atoms.

Here we build on previous HRMS and uHRMS applications for unknown PFAS detection by attempting to overcome some of the above limitations by (i) boosting sensitivity with large volume injection to in-line solid phase extraction (in-line SPE), and (ii) increasing overall specificity to PFASs discovery by (a) flagging unknown PFAS retention times by in-source fragmentation scanning and (b) subsequent Orbitrap MS/MS experiments to confirm the assigned molecular formulae and elucidate structure. With only two injections of a single wastewater sample, the method was shown to be sensitive and fast by discovery of 36 new PFAS compounds that have not previously been reported in the environment.

3.2 Experimental Section[§]

3.2.1 Chemical and Reagents

Optima water and methanol were from Fisher Scientific (Ottawa, ON, Canada). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Model PFAS standards (**Table 3-1**) were purchased from Wellington Laboratories (Guelph, ON, Canada).

3.2.2 Sample Collection and Preparation

A combined aqueous wastewater released from a fluorochemical manufacturing park in China was sampled in Jan. 2014. Optima water in the same polypropylene sampling container was also taken to the field and was analyzed as a field blank. Both samples were shipped to the

[§] Ms. Manli Qian helped in wastewater collection and shipped the sample to the University of Alberta.

University of Alberta, Edmonton, Canada and stored at -20°C prior to analysis. The only sample preparation was centrifugation, whereby 30 mL of the sample, field blank, and procedural blank (i.e. Optima water) were transferred to 50 mL polypropylene centrifuge tubes (Fisher Scientific, USA) and spun at 4500 rpm for 30 min at room temperature. After spinning, supernatant was transferred to 10 mL autosampler vials for analysis.

3.2.3 HPLC-LTQ-Orbitrap-MS

Centrifuged samples were directly injected to an in-line C18 SPE column (Hypersil Gold, 2 cm length, 2.1 mm i.d., 12 µm particle) by large volume injection (2-5 mL). The SPE eluent was directed to a reversed-phase C18 HPLC column (Hypersil Gold, 5 cm length, 2.1 mm i.d., 1.9 µm particles) whose chromatographic eluent was connected to a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite^{TM} , Thermo Fisher Scientific, CA, USA). An Ascentis Express F5 HPLC column (10 cm length, 2.1 mm i.d., 2.7µm particles) was used to confirm certain analytes. Total run time was 43 min and the mobile phase program for SPE and the analytical column gradient elution is described in **Table 3-2**.

The Orbitrap Elite^{TM} was operated in negative electrospray ionization (ESI) mode in full scan (nominal RP=120,000 at *m/z*=400) and in in-source fragmentation flagging scan (nominal RP=15,000 at *m/z*=400). Capillary voltage was 5kV and capillary and vaporizer temperatures were 300 and 350 °C, respectively. Sheath gas flow, auxiliary gas flow and sweep gas flow were set to 40, 5 and 2 (arbitrary units), respectively. Data acquisition and analysis was performed by *Xcalibur* software (v2.2, Thermo Fisher).

3.2.4 Unknown PFAS Discovery and Characterization Workflow

The workflow included two injections. The first injection simultaneously accomplished untargted HPLC retention time flagging, and empirical formula assignment. The second injection was used for targeted MS/MS in HRMS mode for empirical formula confirmation and structural characterization.

3.2.4.1 First Injection

The Orbitrap-MS was programmed to cycle back and forth between 3 parallel MS events: two distinct HRMS in-source fragmentation flagging scans (each at ~8 Hz), and uHRMS full

scanning (at ~2 Hz). Full-scan orbitrap-MS was performed between *m/z*=100-2000, and nominal RP was set to 120,000 at *m/z*=400. The fragmentation flagging scans were primarily designed to flag the retention time of compounds containing a perfluorinated moiety by producing diagnostic C_xF_y fragments (e.g. $C_nF_{2n+1}^-$ and $C_nF_{2n-1}^-$) in the S-Lens region of the instrument, after elution from HPLC. To optimize fragment production, model native PFASs (**Table 3-1**) were tested under different source-induced dissociation (SID) energies in the S-lens region, and 50 eV was optimal in producing diagnostic C_xF_y fragments (e.g. $C_2F_5^-$ *m/z*=118.993 and $C_3F_5^-$ *m/z*=130.992). The diagnostic fragments were detected by full scanning in the *m/z* range of 50-300, with nominal RP set to 15,000 (RP calculated by dividing nominal *m/z* by spectral peak width at half-maximum). These in-source fragmentation conditions also produced other diagnostic fragments, such as SO_3F^- *m/z*=79.956, PO_3^- *m/z*=78.958 and SO_4H^- *m/z*=96.959, which further aided elemental composition assignment and structural elucidation. Technically, any theorized diagnostic fragments with $m/z \geq 50$ can be monitored by this technique.

Chlorine atoms are present in some PFAS alternatives,[6, 20] and these have previously been shown to produce chloride (Cl^-) in MS/MS spectra.[20] It was therefore hypothesized that Cl^- could also be monitored during in-source fragmentation conditions to flag the presence of Cl-substituted PFASs. Thus another in-source fragmentation flagging scanning event was added to the method with scanning in the *m/z* range of 30-100, also using SID=50eV, but here the ion trap mass analyzer (unit resolution) of the Orbitrap Elite™ was used, instead of the Orbitrap detector, due to the low mass cut-off (*m/z*=50) of the latter.

Once a suspect PFAS ion was identified through full-scan and in-source fragmentation flagging scan, we confirmed that the ion was not present in blank samples, then performed a manual PFAS homologue search of the chromatograms by searching for ±50 and/or ±100 ions (more precisely, 49.996 and 99.993, respectively). Manual searching of the extracted ion chromatograms provided retention time information to confirm that the larger homologues corresponded to longer retention times, as would be expected by physical properties.

CF_2 normalized Kendrick mass defect (KMD, **Equation 3-1, 3-2 and 3-3**) plot (KMDP), by plotting KMDs against nominal Kendrick masses (NKMs, **Equation 3-2**), was used as an additional preliminary screening method for the existence of PFASs and their homologous series, which distribute as horizontal lines in KMDPs. KMDP is capable of quickly filtering out

homologous *m/z*'s in full-scan mass spectra of tested samples.[21-23] Typical PFASs have mass defects of -0.1- 0.15.[14] This, in the *m/z* range of 100-1000, will produce KMD values ≤0.10 or ≥0.80, which will fall within two separate regions in the KMDP. To combine the two regions into one, 0.10 was added to all KMDs that were <0.1, thereby resulting in "adjusted KMDs" (AKMD) from 0.80 to 1.10, the typical PFAS range (**Equation 3-4**). Some model PFASs were measured, and the resultant AKMDs were within the range (i.e. 0.95-1.05, **Figure 3-1**). In the current study, all ions in the sample with intensity $\geq 5\times10^3$ were plotted in AKMDP, and the focus was on ions with AKMD of 0.8-1.1.

Kendrick mass (KM) = measured mass × 50/49.99681 (**Equation 3-1**)

NKM = KM (round up, 0 digit) (**Equation 3-2**)

KMD = (NKM – KM) (round, 3 digits) (**Equation 3-3**)

AKMD = KMD+1, if KMD<0.1(**Equation 3-4**)

For each suspect PFAS homologue series, molecular formula prediction (*Xcalibur* software) began with the smallest ion in the series. A general elemental composition limit was set (i.e. carbon: 1-60, hydrogen: 0-60, fluorine: 1-100, oxygen 0-8, nitrogen: 0-2, sulfur: 0-1 and phosphorous: 0-2), but was refined when justified by isotopic patterns, or by in source fragmentation flagging data. For example, one Cl atom would be allowed in the formula when a 3:1 of $[M]^-$ and $[M+2]^-$ ions was evident, or when a Cl^- peak was evident in in-source fragmentation flagging scan. Mass accuracy always had to be ≤ 5ppm for the empirical formula to be tentatively accepted. Proposed molecular formula were further filtered according to the "Seven Golden Rules" (element number restrictions, LEWIS and senior check, isotopic pattern filter, H/C ratio check, heteroatom ratio check, element probability check and TMS check) for organic chemical formulae.[24] Molecular formula filtering for larger ions was informed by the proposed molecular formula for the smallest ion in the respective series, but other requirements (e.g. mass accuracy error) had to be fulfilled. Ultimately, molecular formulae were confirmed by MS/MS characterization, described below.

3.2.4.2 Second Injection

MS/MS experiments were conducted on at least one member of each homologous series of suspected PFASs to confirm the proposed molecular formula, and further to characterize the structure. Like the first injection, this was also done in HPLC mode to avoid interferences, and various collision induced dissociation (CID, e.g. 10, 20, 35eV) and higher collisional dissociation (HCD, e.g. 50, 60, 80, 100eV) energy functions were used for the same injection. The product ions of CID and HCD were detected in the Orbitrap mass analyzer (RP=30,000), thereby allowing molecular formulae of each major product ion to be determined accurately. By this strategy, a proposed molecular formula would be discarded if product ions contained atoms (different atoms, or atoms in the wrong proportion) that could not be explained by the suspected molecular formula of the parent ion.

3.2.5 Quality Control

Field and procedural blanks were analyzed along with each sample injection to check for analyte carryover or background response. To evaluate the approximate sensitivity range of the method (which in reality depends on the individual analyte), method detection limit (MDL) was determined for model mass-labelled PFASs by injecting triplicate 5 mL samples spiked at various concentrations in the wastewater (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 ng/mL). The lowest concentration that could be reliably differentiated from background noise (e.g. produce a discernible peak when no background noise was evident, or signal/noise ≥10 if background noise was present) was defined as the MDL. Procedural recovery for individual native PFASs injected to the in-line SPE was evaluated by calculating the peak area produced from injection of 5 mL of 1 ng/mL stock aqueous solution (i.e. 5 ng on-column) compared to the peak area produced when injecting 100 μL of 50 ng/mL standard (i.e. 5 ng in methanol/water=10/90) directly to HPLC.

3.3 Results and Discussion

3.3.1 Method Performance

Determined MDLs (**Table 3-3**) for model mass-labelled PFASs ranged from 0.005 to 0.2 ng/mL, corresponding to 0.003-0.2 ng F/mL (i.e. 0.02-0.7 ng F on-column). This is comparable to MDLs reported in targeted PFAS analysis by sensitive multiple reaction monitoring analysis using triple quadrupoles,[14] and up to 3 orders of magnitude more sensitive than untargeted EOF

or total fluorine (TF) analysis by combustion ion chromatography (2-10 ng F/mL for EOF, and 2 ng F/mL for TF).[13]

Procedural recoveries by in-line SPE for most native PFASs were in the range of 50-120% (**Figure 3-2**), but lower for short-chain PFAS analytes (e.g. PFBS ~17%, PFBA and PFPeA <5%), and very long-chain PFAS analytes (e.g. bis(1H, 1H, 2H, 2H-perfluorodecyl) phosphate 30%). Other SPE column chemistries could be tested to extend the range of analytes in future, but this was not a focus in the current work.

For the industrial wastewater sample, a highly complex chromatogram (**Figure 3-3**A) and mass spectrum was revealed (**Figure 3-3**B and C). For the ~2000 ions observed in full scan mode (over the ~40min chromatogram) with intensity $\geq 5\times10^3$ (**Figure 3-4**A), approximately half fell within the characteristic PFAS AKMD range of 0.8~1.1 (**Figure 3-1** and **Figure 3-4**). Common PFAS standards fall within the range of 0.9-1.1 (**Figure 3-1**), therefore the zoomed in view of this region is shown, encompassing ~300 unknown ions (**Figure 3-4**B). Measured RPs were always ≥ 120,000 for ions below *m/z* 500 in full-scan mode (**Figure 3-5**A), and were > 15,000 for fragments with *m/z* ≤ 300 by in-source fragmentation flagging scanning in the Orbitrap (**Figure 3-5**B), which was sufficient to resolve ions with a theoretical mass split of 0.004 Da and 0.01 Da, respectively.

As a final validation of the method performance and sensitivity, we examined the wastewater chromatogram for presence of PFCAs, which are routinely observed in water.[1] The C6-C11 PFCAs were detected (mass accuracy -0.47 to -0.07 ppm, blue diamonds in **Figure 3-4**B, C11 PFCA not shown due to intensity $\leq 5\times10^3$). Confirmation of PFCAs was accomplished by matching retention times to authentic standards, observation of the heavy natural isotope (^{13}C), and $[M\text{-}CO_2]^-$ ions at the same retention time in full-scan (blue triangles and dots, respectively, in **Figure 3-4**B). Characteristic $C_nF_{2n+1}^-$ fragment ions (i.e. $C_2F_5^-$ and $C_3F_7^-$) were also observed for these PFCAs in the in-source fragmentation flagging scan (peak "a - c" in **Figure 3-6**A). The concentrations were ~90, 0.5, 20, 0.1, 0.01 and 0.005ng/mL in the wastewater sample for C6-C11 PFCAs, respectively, demonstrating the method sensitivity. Perfluorinated sulfonates (e.g. PFOS) were all below MDLs in this wastewater sample, suggesting that companies in the fluorochemical manufacturing park were not using these PFASs in their processes.

3.3.2 Unknown PFAS Discovery

Class 1 Discovery: Hydro-Substituted Perfluorocarboxylates (H-PFCAs)

In-source fragmentation flagging revealed several $C_2F_5^-$ and $C_3F_7^-$ chromatographic peaks (1B-1J in **Figure 3-6**A). In full-scan at the corresponding retention times, a series of ions differing by *m/z* 50 were observed (1A-1J in **Table 3-4**; "**Class 1**" in **Figure 3-6**B, 1A, 1K and 1L not visible due to lower intensity). Further, these ions were distributed in the same horizontal line in the PFAS region of the CF_2 normalized AKMDP (red diamonds in **Figure 3-4**B), and with increasing *m/z* these had increased retention times on the C18 column ("**Class 1**" in **Table 3-4** and **Figure 3-6**B).

For all ions in this class, a $[M-64]^-$ fragment ion was observed at the same retention time with the same peak shape in full-scan mode (**Figure 3-4**B and **Figure 3-7**), corresponding to neutral loss of HF and CO_2. For the most intense species (i.e. 1B-1E in **Table 3-4**), the ^{13}C stable isotope was observed ($[M]^- : [M+1]^- \approx 100:1$) for both the parent ion and the $[M-64]^-$ ion (red diamonds, red triangles, red crosses and red multiplication signs, respectively, in **Figure 3-4**B and **Figure 3-7**). Molecular formula generation for peak 1B revealed a formula of $C_7F_{12}HO_2^-$, with only 0.013 ppm error. For the other ions in this series (1A and 1C-1L), the general formula $C_nF_{2n-2}HO_2^-$ was a good match, with ppm error ranging from -1.95 to -0.95.

MS/MS experiments further confirmed the assigned empirical formula. For example, for the parent ion with formula $[C_{10}F_{18}HO_2]^-$, each product ion was assigned a formula within 5 ppm error using only C (0-10), F (0-18), H (0-1), and O (0-2) (**Figure 3-7**). With regards to structure, in MS/MS spectra the combined neutral loss of CO_2 and HF was confirmed, strongly indicating a mono-H-substituted PFCA (e.g. **Figure 3-7** for peak 2E, $C_{10}F_{18}HO_2^-$). The exact location of the H substitution cannot be confirmed, but the presence of $C_2F_5^-$ and $C_3F_7^-$ in the MS/MS spectrum (**Figure 3-7**) indicates that the H atom is not near to the terminal perfluoromethyl group. Interestingly, a 2-H substituted PFOA was reported as a possible PFOA degradation product in experimentally incubated soil microsomes.[25] The only other literature report of mono-H substituted PFCA is for ω-H substituted PFCAs, which were reported to facilitate the emulsion polymerization of vinyl fluoride,[26] but the above evidence indicates that the **Class 1** ions observed here are not ω-H substituted PFCAs (i.e. H is not on the terminal perfluoromethyl group).

It is important to mention that all of the ions (except for 1A) in this series were also detected during procedural and field blank sample analysis, however response in the wastewater sample was between ~5 (peak 1J and 1L) and ~300 (peak 1B and 1C) times higher than for the blank samples (**Figure 3-8**). The source of this blank response was later determined to be the mobile phase, or the HPLC system itself since there were measurable **Class 1** PFAS responses when running the method program without injections. Furthermore, when the column equilibration time was extended to 60 min, the response of these ions increased up to 12-fold in the following run without injection (**Figure 3-9**). The consistently low response in blanks did not hinder identification of these ions in the current sample, but such background could hinder further environmental monitoring and should therefore be eliminated with a trap column, as is routinely done for PFOA and PFOS analysis today.[27, 28]

Class 2 Discovery: Chlorine-Substituted Perfluorocarboxylates (Cl-PFCAs)

In-source fragmentation flagging revealed compelling evidence for several chlorinated organic species based on simultaneous appearance of $^{35}Cl^-$ and $^{37}Cl^-$ at several HPLC retention times (**Figure 3-10**A). Several of these chloride ion peaks corresponded to the retention times of 8 higher *m/z* ions (2A-2H in **Table 3-4**, "**Class 2**" in **Figure 3-10**B; 2A and 2H not visible due to low intensity). These ions were not detected in either procedural blank or field blank, and fell into a horizontal line in the PFAS region of the CF_2 normalized AKMDP (orange diamonds in **Figure 3-4**B). This combined evidence suggested that these compounds were a homologous series of chlorine-substituted polyfluorinated molecules differing by a CF_2 (i.e. *m/z* 50). Moreover, this ion series displayed increasing retention times with increasing *m/z* (**Figure 3-10**B).

For all ions in this class, an isotopic pattern of $[M]^-$: $[M+2]^-$ = 3:1 was observed in full-scan (**Figure 3-11**A), indicating the presence of only one chlorine atom per molecule. For the smallest ion in this series, *m/z*=278.946, only 2 molecular formulae were possible: $C_5O_2ClF_8^-$ (-0.050 ppm mass error) and $C_3H_5O_6ClF_4P^-$ (1.76 ppm). The latter was excluded as it is impossible by the Seven Golden Rules. Thus, the former empirical formula assignment was tentatively accepted, and the general formula $ClC_nF_{2n-2}O_2^-$ was also a very good fit for all larger ions in the homologous series with reasonable mass accuracy error (i.e. -1.19 to 0.25 ppm). Moreover, the corresponding ^{13}C, ^{37}Cl and even (^{13}C+^{37}Cl) isotopic ion series were observed at the same

retention time in full-scan spectrum with reasonable mass accuracy (orange triangles -1.68 to -0.42 ppm, squares -1.53 to -0.16 ppm, and short lines -2.63 to -0.94 ppm in **Figure 3-4**B, and **Figure 3-11**A). The observation of a corresponding decarboxylated ion $[M-44]^-$ for each compound in this series at the same retention time in full-scan (orange crosses in **Figure 3-4**B and **Figure 3-11**A) is consistent with the identification of this series as carboxylic acids.

In CID MS/MS mode, the parent ions were successfully isolated with zero or low energy (e.g. CID 0, 10, 25eV) and are visible in the MS/MS spectra. However, no stable meaningful product ions were observed except for $[M-44]^-$ (mass accuracy 1.3ppm, **Figure 3-11**B), confirming the carboxylic moiety. Neither parent ion, nor the $[M-44]^-$ product ion was observed when MS/MS energy was increased (e.g. CID≥35eV). The reason for this is likely that the Cl atom is lost in MS/MS as chloride, as we readily observed through in-source fragmentation flagging of $^{35}Cl^-$ and $^{37}Cl^-$ (**Figure 3-10**A), leaving only a neutral fluorinated carbon chain which cannot be detected. Thus, the structure of the Cl-substituted perfluorinated carbon chain remains uncertain for this new class of PFASs.

It is only germane to note that for other widely used PFASs containing a single Cl atom, it is present at the terminal carbon of a linear perfluorinated chain,[6, 20, 29] including the commercially available sulfonic acid F-53B (molecular structure $ClCF_2C_5F_{10}OCF_2CF_2SO_3^-$).[25] A *Scifinder* database search showed that both $ClC_5F_{10}CO_2^-$ and $ClC_8F_{16}CO_2^-$ (the same formulae with 1B and 1E, **Table 3-4**) have been assigned CAS registry numbers (127843-88-3 and 45296-50-2, respectively). The former is a branched isomer, and the latter is shown as a linear isomer, but both have their Cl atom on the terminal carbon. Meissner *et al*.[29] synthesized branched Cl substituted PFCAs for fire-extinguishing agents, but none of the chemicals in this class have been reported in any product or environmental sample.

Class 3 Discovery: Polyfluorinated Sulfates (PFSfs)

This class of substances was initially flagged by two fragment peaks of SO_3F^- and SO_4H^-, at retention times of 12.7 and 16.5 min (3B and 3C in **Figure 3-6**A), and suspected parent ions from full-scan were *m/z* 374.975 and 438.988, respectively (**Figure 3-6**B). The *m/z* difference between these two ions is 64, corresponding to a $(CHF)_2$ unit. Because this ion series is not built on repeating CF_2 or C_2F_4 units, they do not display on a horizontal line in the CF_2 normalized AKMDP, and therefore would be difficult to reveal using only the AKMDP strategy. A manual

check of the full-scan data for homologous ions of 374.975 and 438.988 with *m/z* difference of 64 was therefore performed, revealing a series of 6 homologous ions which were not present in procedural blank or field blank sample ("**Class 3**" in **Table 3-4**, 3A-3F in **Figure 3-6**B, yellow diamonds in **Figure 3-4**B). This homologous series of ions have different KMD values, but with the same difference between each two adjacent homologous ions, and therefore display as a sloped line in the AKMDP (yellow diamonds in **Figure 3-4**B). The unique ratio of $[M]^-$: $[M+1]^-$ was approximately 100:4 for the whole series. This isotopic pattern, together with the two in-source fragments SO_3F^- and SO_4H^-, suggests a single sulfur atom.

Molecular formula generation for the smallest ion in the series was proposed as $C_5F_8H_3SO_4^-$, with -1.73 ppm error. For the homologous series, the general formula of $C_nF_{n+3}H_{n-2}SO_4^-$ was also a good fit (ppm error ranged from -2.15 to -0.87). Very detailed MS/MS spectra for this PFAS class (**Figure 3-12**) further confirmed the assigned empirical formula (mass accuracy error ≤5ppm for all product ions). At higher collision energy, the appearance of sulfate ion (*m/z* 96.961, SO_4H^-), as well as the corresponding SO_3H^- peaks at the same retention times during in-source fragmentation flagging scans, suggests that the molecules are polyfluorinated sulfates ("**Class 3**" in **Table 3-4**), rather than sulfonates. PFOS and F-53B are per-/poly-fluorinated sulfonates and we confirmed that these do not produce the SO_4H^- fragment ion under any condition (**Figure 3-13**).

For peak 3C ($C_9F_{12}H_7SO_4^-$), the parent ion was shown to sequentially lose HF at least four times. This does not allow the exact structure of the polyfluoroalkyl chain to be known, but it must therefore contain repeating $-CF_2CH_2-$ or -CHF- units. Vinylidene difluoride polymer ($-CF_2CH_2-$) and co-polymers (e.g. with trifluoroethylene, chlorotrifluorothylene and hexafluoroisobutylene) were reported for some commercial applications such as in exterior metal siding, in electronics, and in chemical processing equipment.[30]

Class 4 Discovery: Hydro-Substituted Unsaturated Perfluorinated Ethers/Alcohols (H-uPFE/As)

This series of PFAS homologues was exclusively found in the wastewater sample. They were initially flagged by simultaneous $C_3F_5^-$ and $C_4F_7^-$ peaks at a retention time of 22.5 min (4D in **Figure 3-6**A), and the suspected parent ion was 428.977 from full scanning. A check in the AKMDP region revealed a series of 5 ions differing by *m/z* 50 (4A-4E in **Figure 3-6**B, "**Class 4**"

in **Table 3-4**, green triangles in **Figure 3-4**B). The proposed molecular formula for peak 4A (*m/z* 328.984) was $C_7F_{12}HO^-$ (ppm error -1.2), and the general formula $C_nF_{2n-2}HO^-$ was a good match for the whole series (ppm error ranged from -2.3 to -1.5). The deficiency of two fluorine or hydrogen atoms indicates a double bond equivalent (ring or double bond).

MS/MS experiments further confirmed the assigned empirical formula with mass accuracy error within 2 ppm for the product ions. The MS/MS spectra in this class (e.g. compound 4C, **Figure 3-14**) showed a sequential loss of HF and CF_2O, suggesting that this series of ions are unsaturated mono-H-perfluorinated ethers or acidic alcohols (**Table 3-4**). Ether functionalities do not normally ionize in electrospray negative mode, thus these ions could be fragments of larger molecules or polymers (e.g. hydrocarbon polymers with side-chain polyfluorinated groups).[31] Alternatively, this series of PFASs could be polyfluorinated alcohols, which would be acidic due to the electron withdrawing fluorine atoms,[32] and only for this reason are detected in negative ion mode here. The structure for this group of substances is therefore much more uncertain than for **Class 1-3**, but the above evidence is strong that their molecular formulae are indeed $C_nF_{2n-2}HO^-$.

Class 5 Discovery: Chlorine-Substituted Unsaturated Perfluorinated Ethers/Alcohols (Cl-PFE/As)

Two other small Cl^- peaks in the in-source fragmentation flagging scan were noticed (5C and 5D in **Figure 3-10**A). Examination of the full-scan data at these retention times revealed two suspect PFAS ions with a mass difference of *m/z*=50, and AKMDP plotting revealed that these were part of a homologous series of four-ions (cyan diamonds in **Figure 3-4**B) which were only observed in the wastewater sample. One more potential ion in this series was noticed in a manual check of extracted ion chromatograms. Collectively, this ion group showed increasing retention time with increasing *m/z* (5A-5E in **Figure 3-10**B and "**Class 5**" in **Table 3-4**).

In addition to evidence for chlorine atoms from in-source fragmentation flagging, the full scan isotopic pattern of $[M]^-$: $[M+2]^-$ was 3:1 for all ions in this class, diagnostic of monochlorinated compounds. Molecular formula generation was performed on peak 4A (**Figure 3-10B**, **Table 3-4**) by allowing 1 chlorine atom, and the formula $ClC_6F_{10}O^-$ was the best match (mass error -0.45ppm) out of only two possibilities. Moreover, the general formula $ClC_nF_{2n-2}O^-$ was an excellent match to the other 4 ions in this homologous series, with mass accuracy ranging between -1.31 to 0.050 ppm.

Structural characterization by MS/MS fragmentation of peak 2C ($ClC_8F_{14}O^-$), the most intense ion in the series, revealed that neutral loss of CF_2O was the major product ion (*m/z* 346.95) in both CID and higher energy HCD (**Figure 3-15**), indicative of an ether or alcohol functionality. An increase of the HCD fragmentation energy produced 7 more intense fragments (**Figure 3-15**) which together provided high confidence that the assigned molecular formula was correct, including two monochlorinated fragments; and the mass accuracy error for each fragment ion ranged from -2.1 to 2.5 ppm. The additional observation of $C_2F_5^-$ and $C_3F_7^-$ ions suggest a terminal C_3F_7 moiety. The general formula, as well as the $C_3F_5^-$, $C_4F_7^-$ and $C_5F_9^-$ product ion series, furthermore indicate a double bond equivalent in the perfluoroalkyl structure. Collectively, these data were insufficient to allow structures to be confidently proposed, and it is also possible that each peak is composed of more than 1 isomer. Overall, we can propose that the compounds are similar to **Class 4** compounds, either unsaturated ethers that break down from larger molecules or polymers under ionization conditions, or that these are acidic alcohols. In a review of currently used alternatives to long-chain PFASs, Wang *et al*.[20] reported a Cl substituted sulfonate F-53B and a Cl substituted fluoropolymer poly-ether (CAS No. 329238-24-6) from Solvay, used for polytetrafluoroethylene (PTFE) and polyvinylidene fluoride (PVDF) production. The current **Class 5** compounds may be related to these, but are chemically distinct.

3.4 Significance

In untargeted environmental sample analysis, achieving detection and characterization of trace compounds in complex matrices is a challenge akin to finding a needle in the proverbial haystack. Place *et al*.[14] and D'Agostino *et al.*[15] used AFFF commercial material to identify new PFASs (diluted samples were used in the analysis), thus sensitivity and interferences were not primary challenges in previous work. Here a complex industrial wastewater was analyzed, where even the most prominent PFAS, perfluorohexanoic acid (PFHxA), was only present at parts-per-billion (ppb) concentration. Fragmentation flagging scans were a very useful method to allow finding several needles (i.e. 36 previously unknown PFASs) in the haystack (i.e. a complex environmental sample with >2000 total species and >300 in the mass defect region of PFASs). The sensitivity challenge was overcome by in-line SPE for sample pre-concentration in the current study. Assuming similar response factors as the model PFASs, most new PFASs identified here were present at mid to low ppb levels (i.e. <20 ng/mL). For the targeted PFCAs,

the overall method and workflow was shown to be capable of detecting concentrations as low as 0.005 ng/mL. In-line SPE is not only faster and more reproducible than off-line methods, because evaporation and reconstitution procedures are avoided, but it is also a very clean method with little chance of spurious contamination.

Methods with improved recoveries for short-chain PFASs[33] (e.g. with a different SPE column) would be beneficial in future non-targeted PFAS analysis, especially when considering the general trend of switching from long-chain PFASs to short-chain alternatives. Only negative ESI mode was used in the current study. Place *et al.*[14] and D'Agostino *et al.*[15] identified a large proportion of PFASs in positive ion mode, thus the same workflow developed here could be run in positive ESI mode, as well as with atmospheric pressure chemical ionization (APCI) or photoionization sources (APPI).

Complete structural characterization and confirmation of chemical structures cannot be achieved with the current workflow – not without authentic standards to match retention time and MS/MS spectra. Nevertheless, as demonstrated here, uHRMS instrumentation is capable of confirming molecular formulae with very high confidence (accurate mass, stable isotopes, fragments, and MS/MS experiments in high-resolution mode), leaving very little ambiguity to the novel PFAS classes identified here. Even without authentic standards, the current wastewater sample can be used as a reference material to develop targeted methods for environmental monitoring (e.g. HPLC-MS/MS), to determine the wider environmental relevance of the 36 novel PFAS species described here. To confirm the structure(s) would require expensive and time consuming *de novo* organic synthesis, because none of the current species are commercially available today as authentic standards. To aid in future structural elucidation, one option is for the HPLC eluent from the current method to be fractionated and analyzed off-line by other techniques (e.g. NMR).

Historically, sensitive and specific trace analytical methods have been a limitation that has delayed the identification of global contaminants, including most POPs (including PFOS) which were used for many decades prior to their discovery in people and the global environment. Today's advanced analytical technologies raise new possibilities for early discovery of environmental contaminants by sensitive non-targeted methods. Such discoveries could allow

emission controls or regulatory action to be implemented earlier, thereby minimizing widespread contamination.

3.5 Figures and Tables

3.5.1 Figures

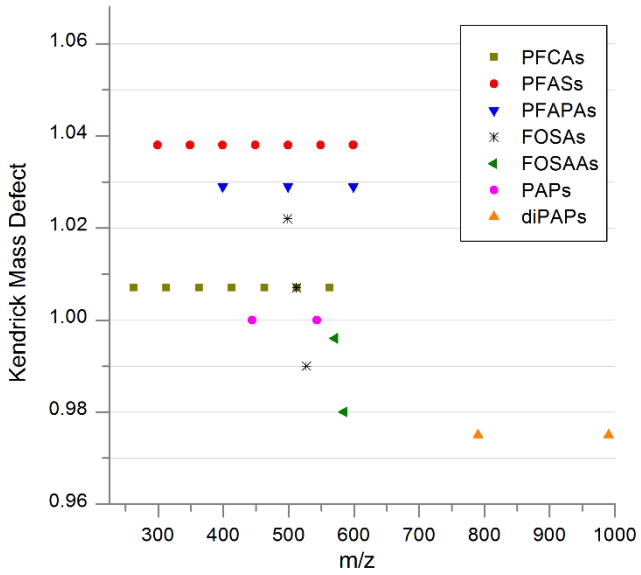

Figure 3-1. Adjusted Kendrick mass defect plot (AKMDP) of model PFASs.

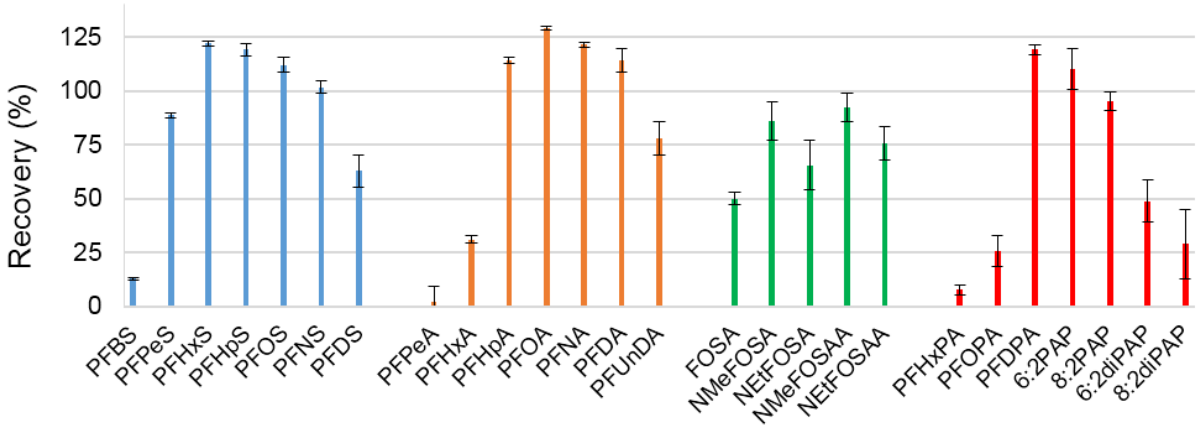

Figure 3-2. Procedural recoveries of model native PFASs for the in-line SPE method. Error bars represent standard error of the mean.

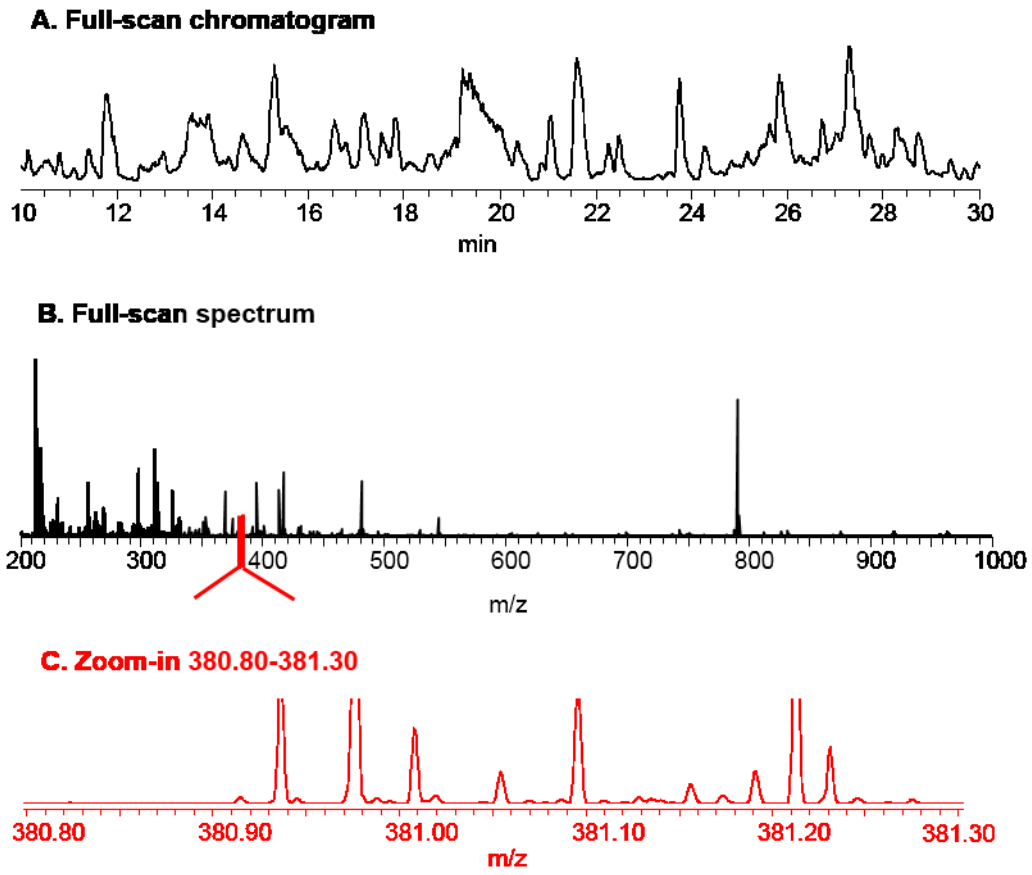

Figure 3-3. Full-scan chromatogram of the industrial wastewater on C_{18} column (**A**), and its mass spectrum (**B**) and zoomed-in mass spectrum over a range of 0.5 *m/z* units (**C**).

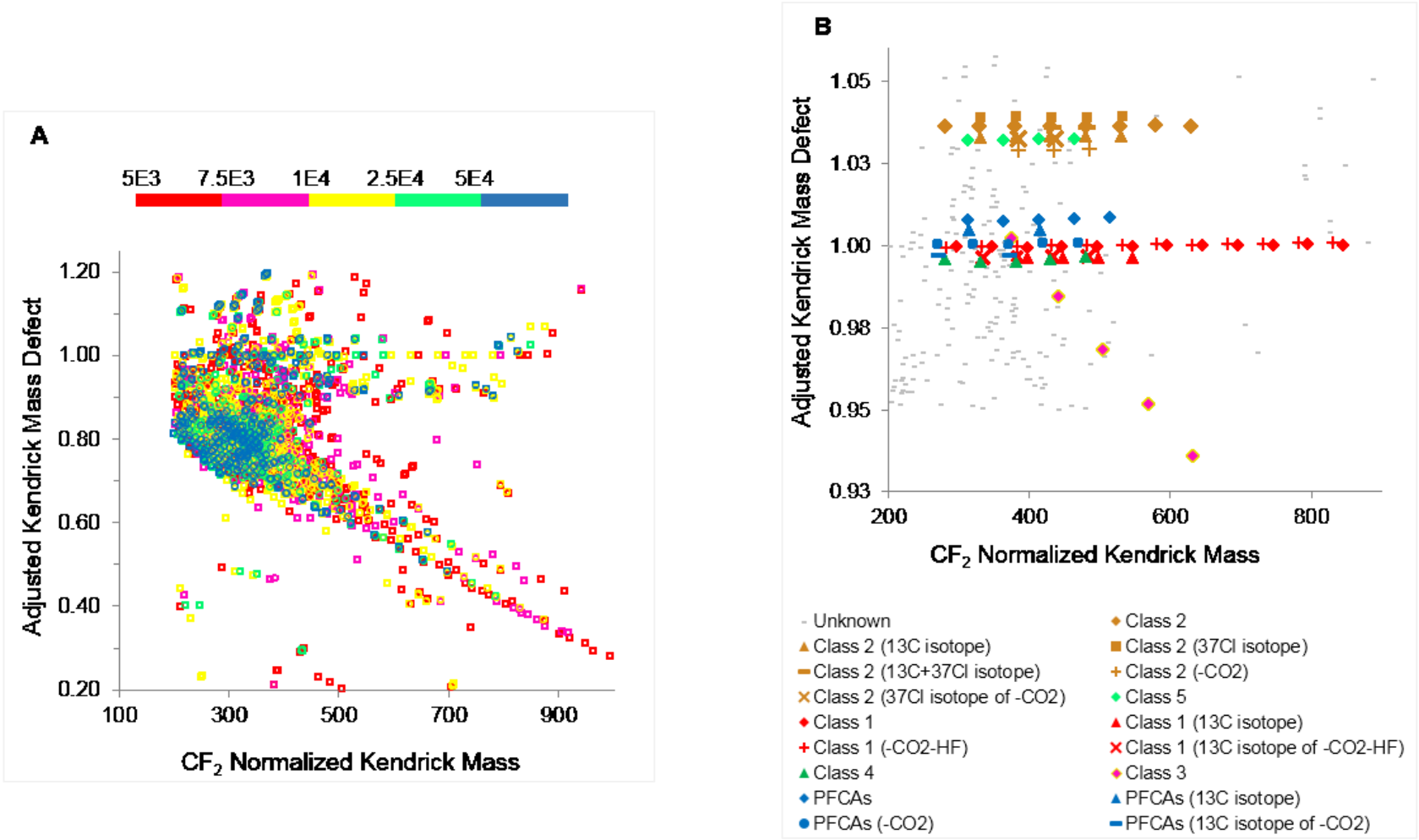

Figure 3-4. CF_2 normalized adjusted Kendrick mass defect plots of all ions detected in the wastewater sample that were $\geq 5\times10^3$ in intensity (**A**, ~2000 ions), and of ions within the characteristic PFAS adjusted Kendrick mass defect range of 0.90-1.10 (**B**, ~300 ions).

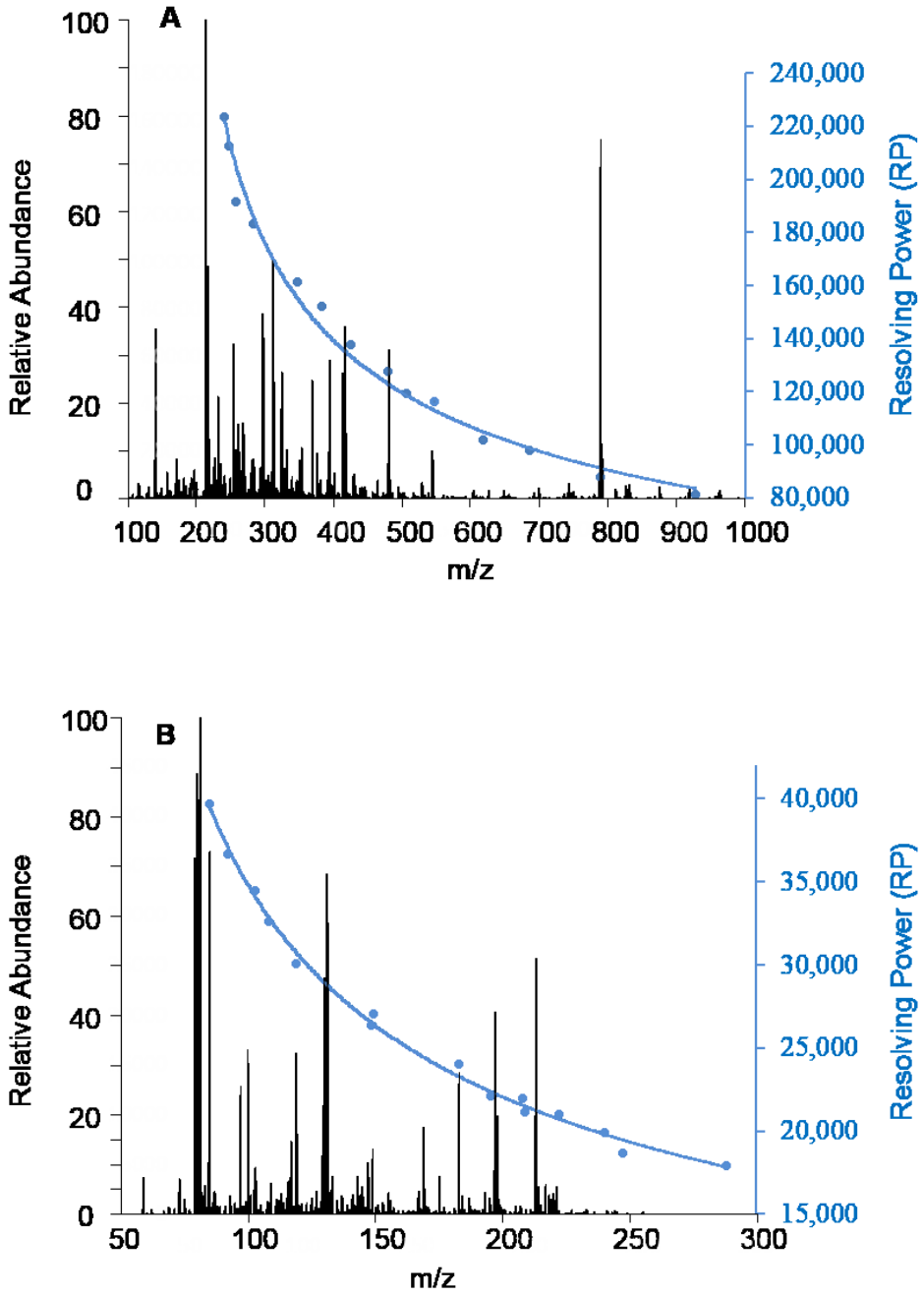

Figure 3-5. Mass spectra (black) and measured resolving power values (blue) in both full-scan (**A**) and in-source fragmentation flagging scan (**B**) of the wastewater sample from a fluorochemical manufacturing park.

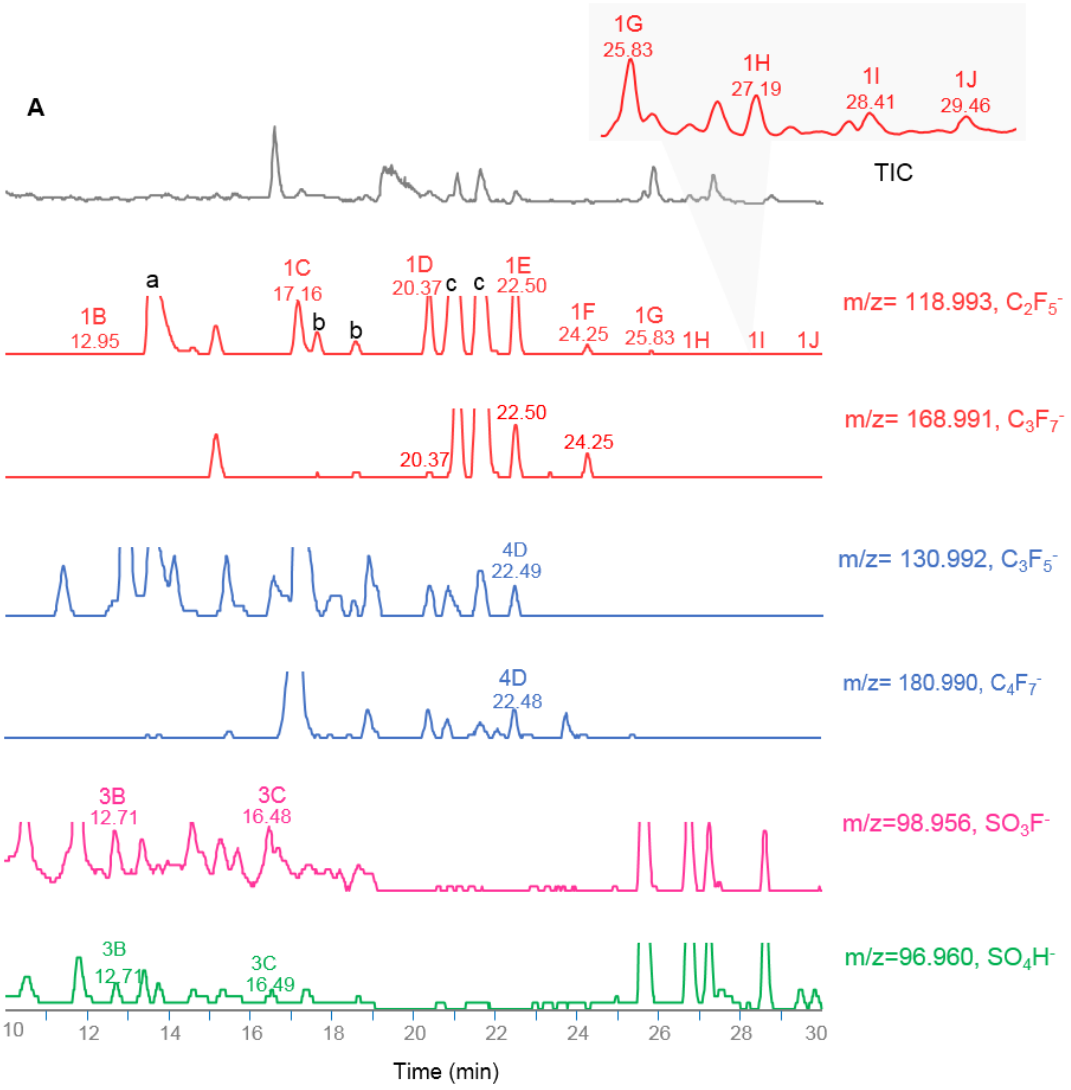

Figure 3-6. Extracted ion chromatograms $[C_2F_5]^-$, $[C_3F_7]^-$, $[C_3F_5]^-$, $[C_4F_7]^-$, $[SO_3F]^-$ and $[SO_4H]^-$ in-source fragments (SID=50 eV, **A**), and all **Class 1**, **3** and **4** homologous PFASs detected in the industrial wastewater sample (**B**). Shaded insets in **Figure 3-6A** and **Figure 3-6B** are zoomed-in chromatograms of in-source $[C_2F_5]^-$ fragments during 25.5-30 min (A), of **Class 1** "1G-1L" homologues during 25-32 min, respectively. Labeled peaks correspond to the identified novel PFASs, and are consistent with labels in **Table 3-4**. Peaks labeled "a" through "c" in **Figure 3-6A** correspond to C6-C8 PFCAs.

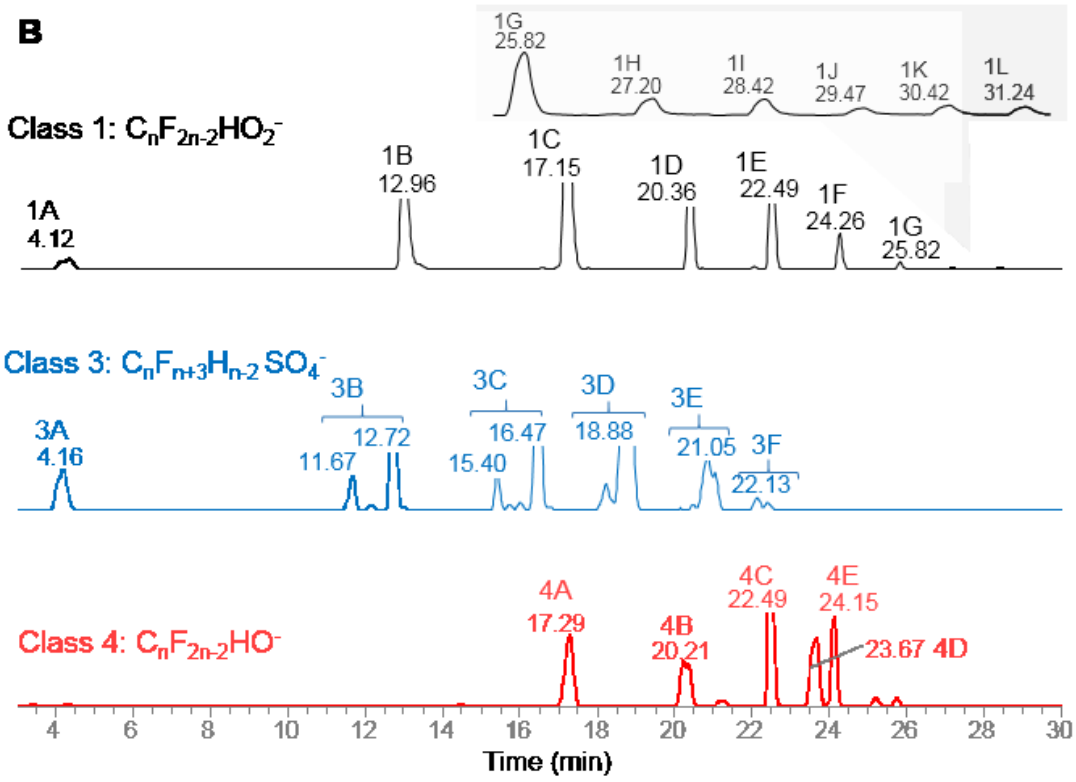

Figure 3-6. Extracted ion chromatograms $[C_2F_5]^-$, $[C_3F_7]^-$, $[C_3F_5]^-$, $[C_4F_7]^-$, $[SO_3F]^-$ and $[SO_4H]^-$ in-source fragments (SID=50 eV, **A**), and all **Class 1**, **3** and **4** homologous PFASs detected in the industrial wastewater sample (**B**). Shaded insets in **Figure 3-6A** and **Figure 3-6B** are zoomed-in chromatograms of in-source $[C_2F_5]^-$ fragments during 25.5-30 min (A), of **Class 1** "1G-1L" homologues during 25-32 min, respectively. Labeled peaks correspond to the identified novel PFASs, and are consistent with labels in **Table 3-4**. Peaks labeled "a" through "c" in **Figure 3-6A** correspond to C6-C8 PFCAs.

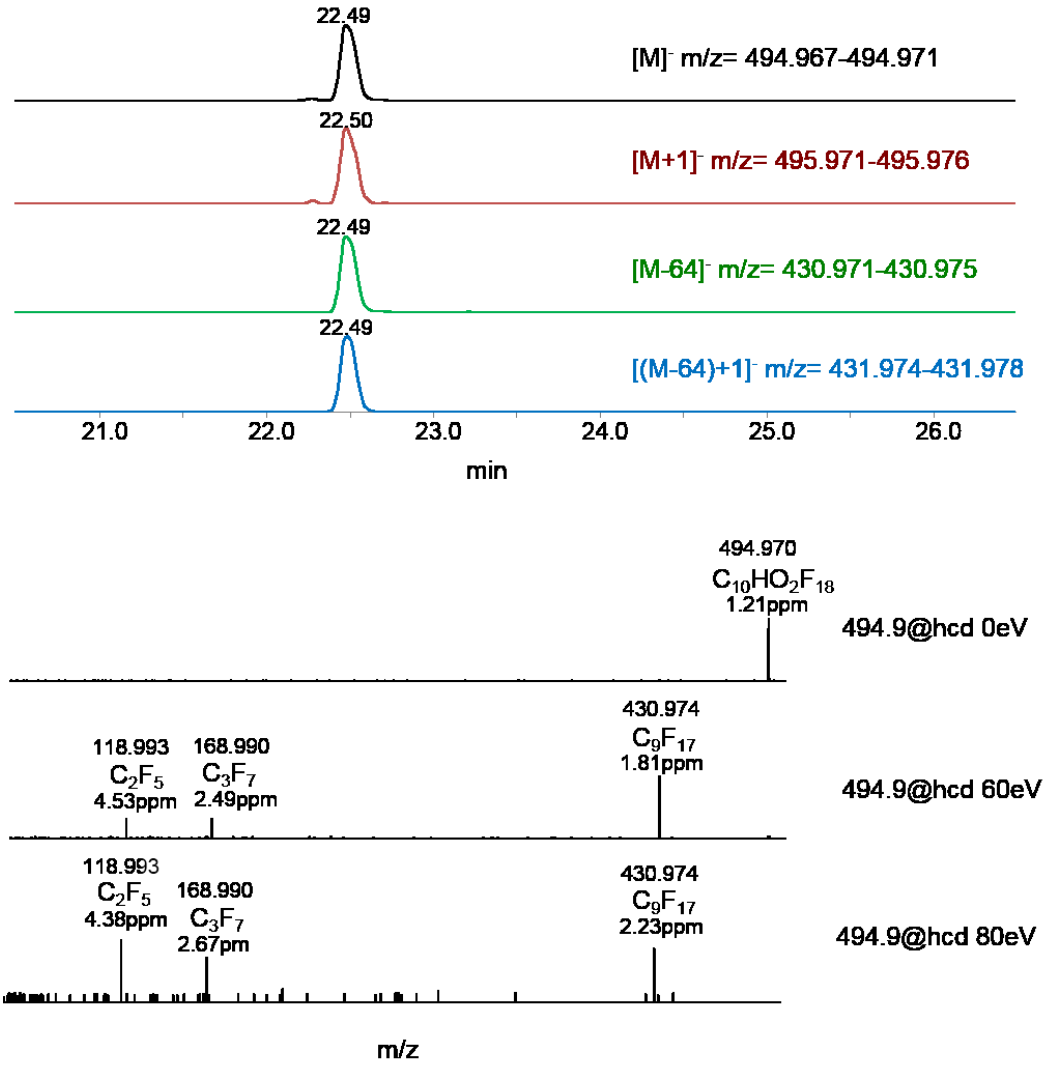

Figure 3-7. Extracted ion chromatograms of a **Class 1** homologue with *m/z* = 494.969 ($C_{10}F_{18}HO_2^-$) and its in-source fragment with *m/z* = 430.974 ($C_9F_{17}^-$) (**A**), and MS/MS spectra (**B**).

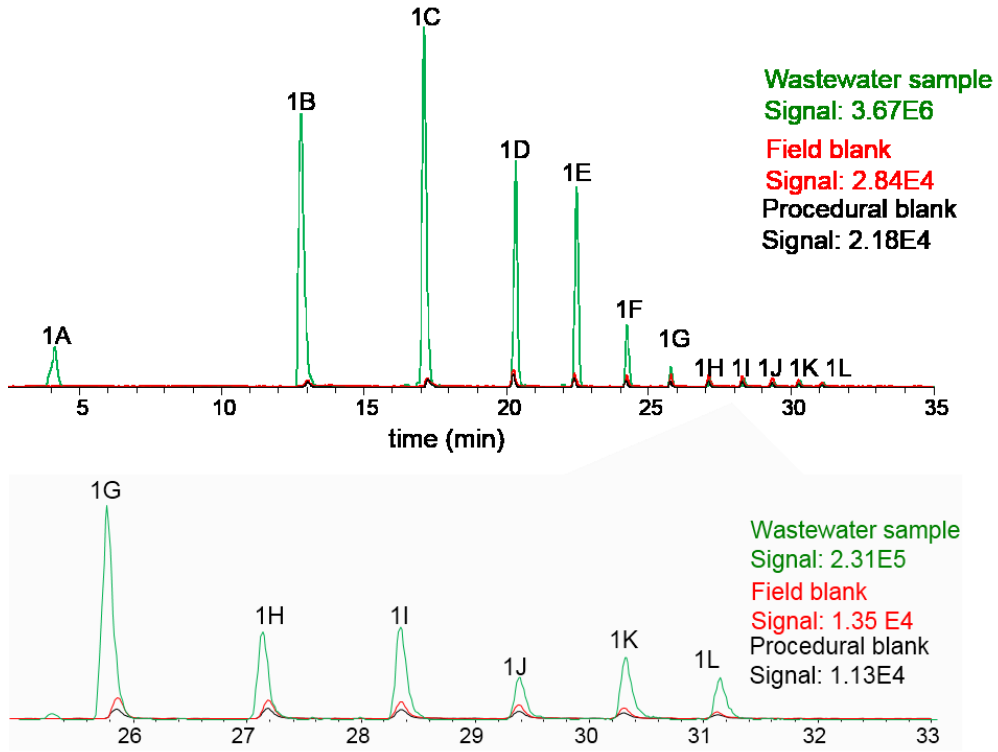

Figure 3-8. Extracted ion chromatograms of all **Class 1** homologous PFASs in procedural blank (black line), field blank (red line) and the industrial wastewater (green line).

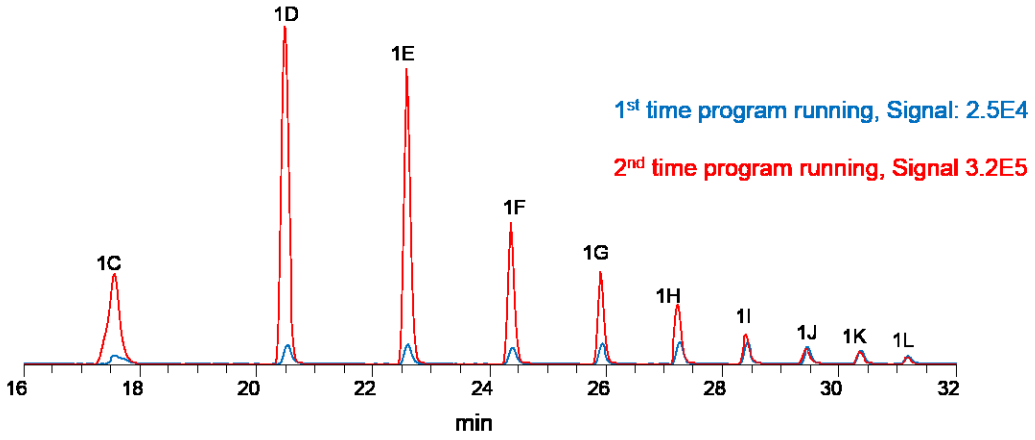

Figure 3-9. Extracted ion chromatograms of selected **Class 1** PFASs when running the method program twice without any injection. The second analysis (red line) was performed after 60 min of equilibration after the first analysis (blue line) was made.

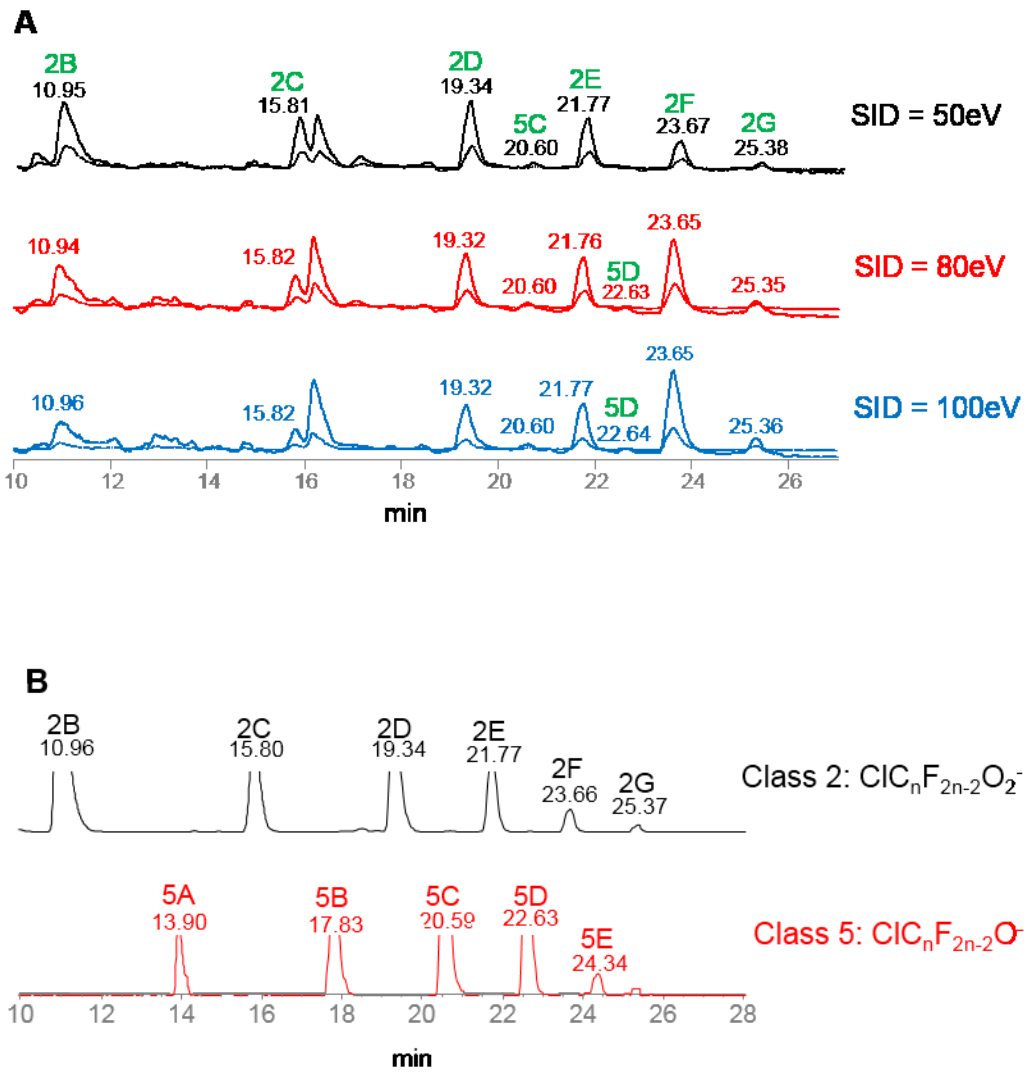

Figure 3-10. Extracted ion chromatograms of in-source $[^{35}Cl]^-$ and $[^{37}Cl]^-$ fragments (**A**) and all **Class 2** and **Class 5** homologous PFASs (**B**) detected in the industrial wastewater sample. The separation was performed on the F5 column. Labeled peaks in **Figure 3-10B** correspond to the identified novel PFASs, are consistent with labels in **Table 3-4** and with discussion in the main text.

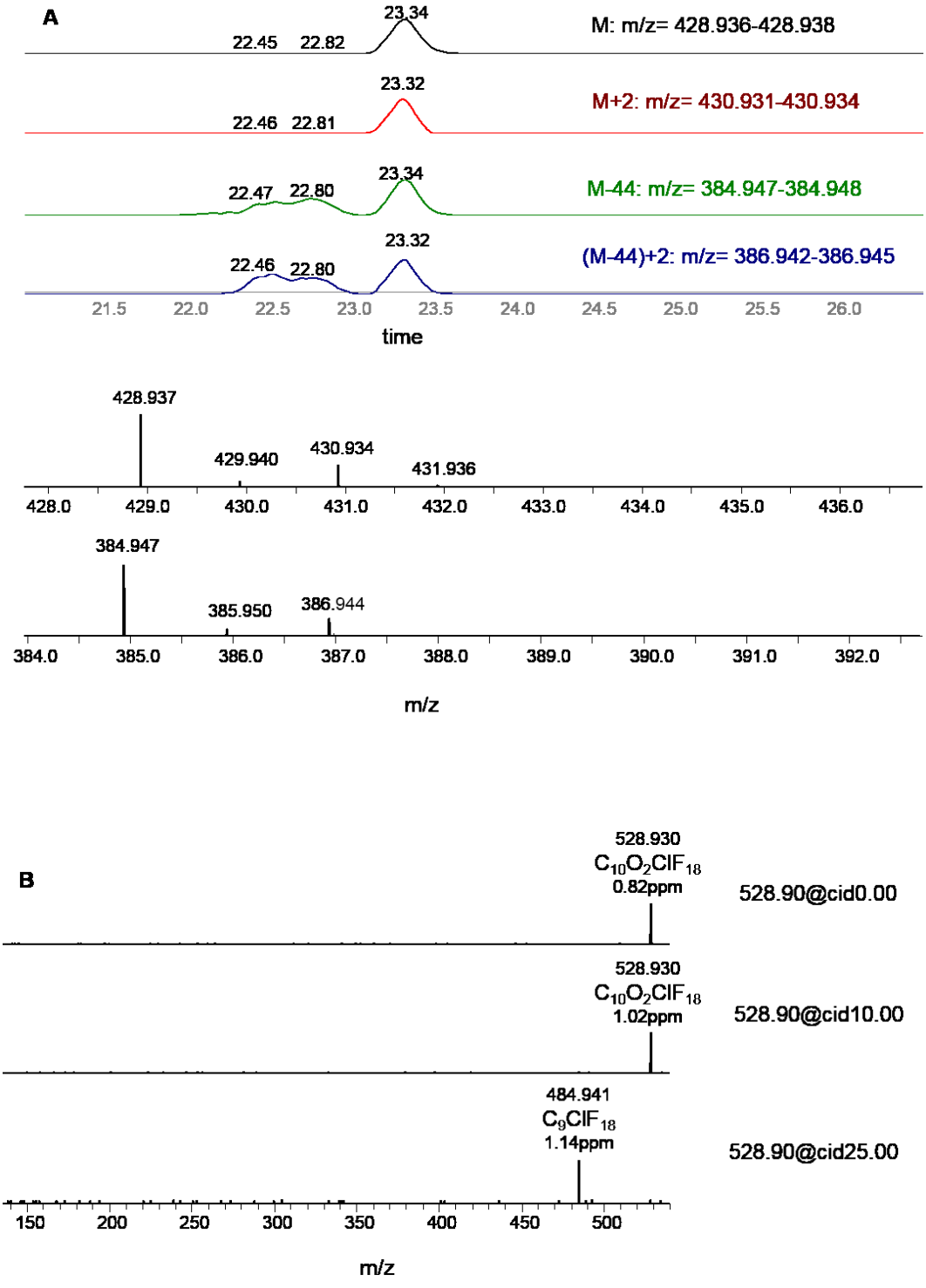

Figure 3-11. Extracted ion chromatograms and mass spectra of a **Class 2** homologue with *m/z*=428.937 ($ClC_8F_{16}O_2^-$) and its in-source fragment with *m/z*=384.947 ($ClC_7F_{16}^-$) (**A**), and MS/MS spectra of another **Class 2** homologue with *m/z*=528.931 (**B**).

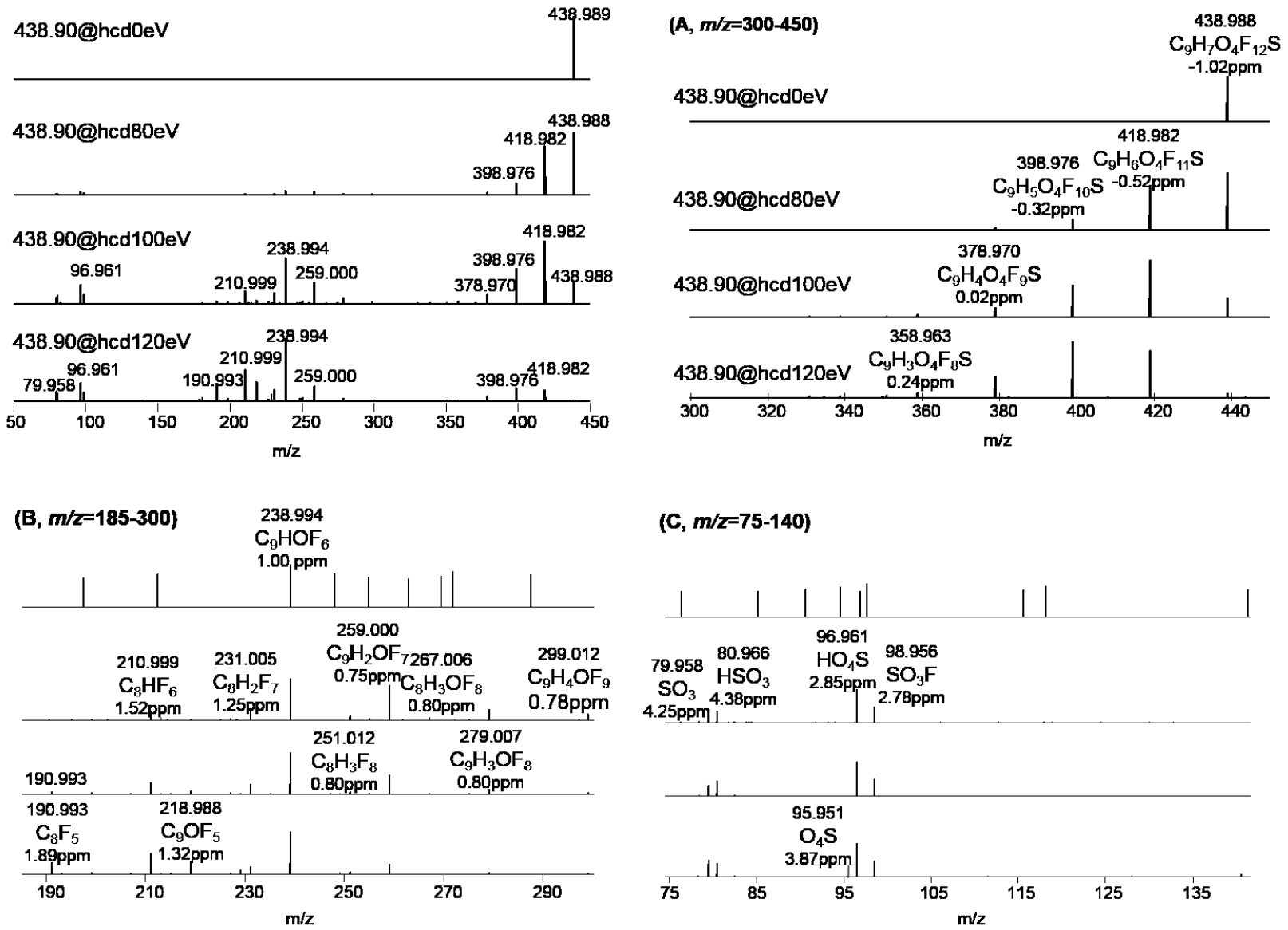

Figure 3-12. MS/MS spectra of a **Class 3** homologue with *m/z*=483.988 ($C_9F_{12}H_7SO_4^-$). Figure **A**, **B** and **C** are zoomed-in spectra.

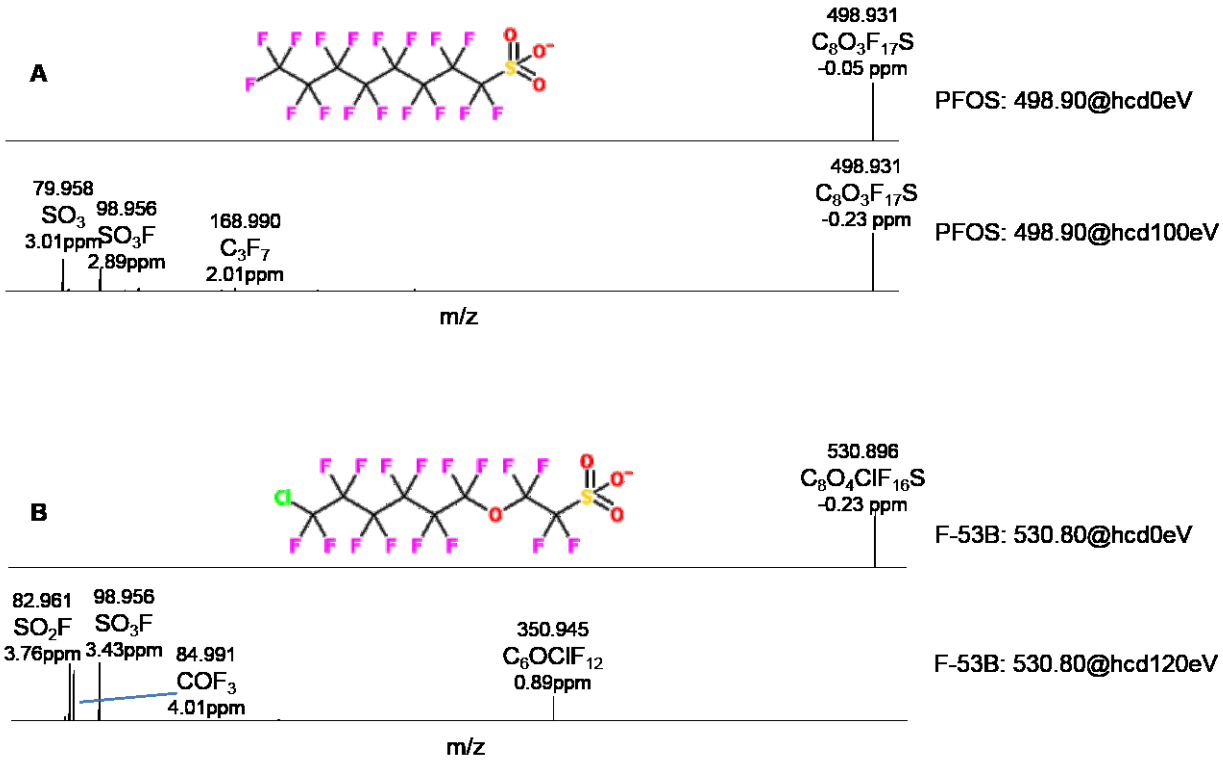

Figure 3-13. MS/MS spectra of PFOS ($C_8F_{17}SO_3^-$) and F-53B standards ($ClC_8F_{16}SO_3^-$).

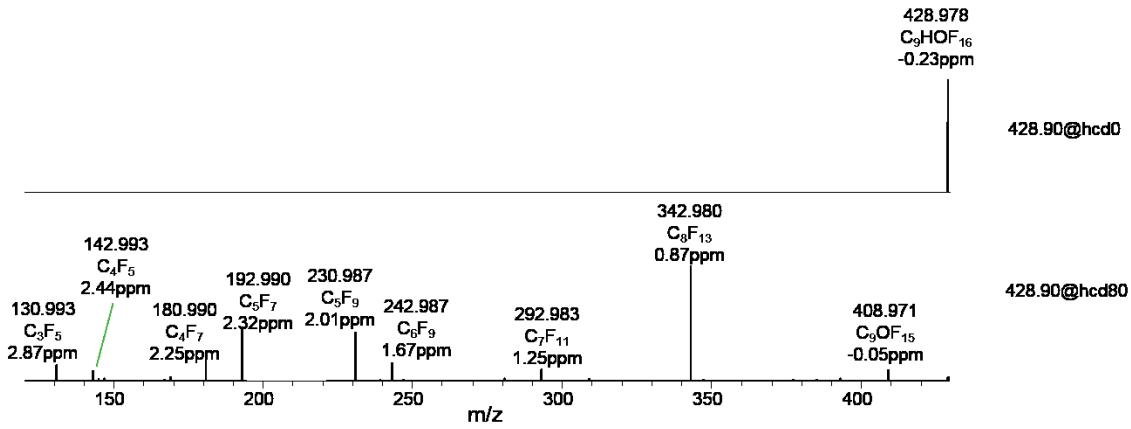

Figure 3-14. MS/MS spectra of a **Class 4** homologue with *m/z* 428.978 ($C_9F_{16}HO^-$).

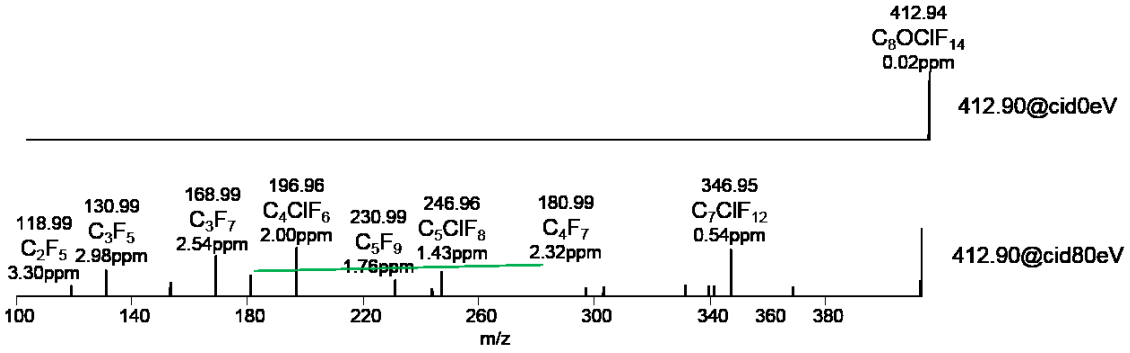

Figure 3-15. MS/MS spectra of a **Class 5** homologue with *m/z* 412.942 ($ClC_8F_{14}O^-$).

3.5.2 Tables

Table 3-1. Model PFASs used in the current study.

PFASs	Full Name	Abbreviation	Structure
PFCAs	perfluoro-n-butanoic acid	PFBA	$CF_3(CF_2)_2COO^-$
	perfluoro-n-pentanoic acid	PFPeA	$CF_3(CF_2)_3COO^-$
	perfluoro-n-hexanoic acid	PFHxA	$CF_3(CF_2)_4COO^-$
	perfluoro-n-heptanoic acid	PFHpA	$CF_3(CF_2)_5COO^-$
	perfluoro-n-octanoic acid	PFOA	$CF_3(CF_2)_6COO^-$
	perfluoro-n-nonanoic acid	PFNA	$CF_3(CF_2)_7COO^-$
	perfluoro-n-decanoic acid	PFDA	$CF_3(CF_2)_8COO^-$
	perfluoro-n-undecanoic acid	PFUnDA	$CF_3(CF_2)_9COO^-$
PFSAs	perfluoro-1-butanesulfonate	PFBS	$CF_3(CF_2)_3SO_3^-$
	perfluoro-1-pentanesulfonate	PFPeS	$CF_3(CF_2)_4SO_3^-$
	perfluoro-1-hexanesulfonate	PFHxS	$CF_3(CF_2)_5SO_3^-$
	perfluoro-1-heptanesulfonate	PFHpS	$CF_3(CF_2)_6SO_3^-$
	perfluoro-1-octanesulfonate	PFOS	$CF_3(CF_2)_7SO_3^-$
	perfluoro-1-nonanesulfonate	PFNS	$CF_3(CF_2)_8SO_3^-$
	perfluoro-1-decanesulfonate	PFDS	$CF_3(CF_2)_9SO_3^-$
PFOSAs	perfluoro-1-octanesulfonamide	FOSA	$CF_3(CF_2)_7SO_2NH^-$
	N-methylperfluoro-1-octanesulfonamide	Me-FOSA	$CF_3(CF_2)_7SO_2N^-(CH_3)$
	N-ethylperfluoro-1-octanesulfonamide	Et-FOSA	$CF_3(CF_2)_7SO_2N^-(C_2H_5)$
FOSAAs	N-methylperfluoro-1-octanesulfonamidoacetic acid	Me-FOSAA	$CF_3(CF_2)_7SO_2N(CH_3)CH_2CO_2^-$
	N-ethylperfluoro-1-octanesulfonamidoacetic acid	Et-FOSAA	$CF_3(CF_2)_7SO_2N(C_2H_5)CH_2CO_2^-$
PFPs	perfluorohexylphosphonic acid	PFHxPA	$CF_3(CF_2)_5P(O)(OH)O^-$
	perfluorooctylphosphonic acid	PFOPA	$CF_3(CF_2)_7P(O)(OH)O^-$
	perfluorodecylphosphonic acid	PFDPA	$CF_3(CF_2)_9P(O)(OH)O^-$
PAPs	1H, 1H, 2H, 2H-perfluorooctylphosphate	6:2PAP	$CF_3(CF_2)_5CH_2CH_2OP(O)(OH)O^-$
	1H, 1H, 2H, 2H-perfluorodecylphosphate	8:2PAP	$CF_3(CF_2)_7CH_2CH_2OP(O)(OH)O^-$
	Bis(1H, 1H, 2H, 2H-perfluorooctyl)phosphate	6:2 diPAP	$[CF_3(CF_2)_5CH_2CH_2O]_2P(O)O^-$
	Bis(1H, 1H, 2H, 2H-perfluorodecyl)phosphate	8:2 diPAP	$[CF_3(CF_2)_7CH_2CH_2O]_2P(O)O^-$

Table 3-2. Mobile phase program of the in-line SPE-HPLC-Orbitrap MS method.

Time min	Flow mL/min	Grad[α]	A%[2]	B%[3]	Valve	Flow mL/min	Grad[α]	A%[β]	B%[β]
0.00	2.00	Step	99	1	-	0.50	Step	99	1
3.67	0.25	Step	0	100	switch[γ]	0.75	Ramp	99	1
7.67	0.25	Step	0	100	-	0.50	Ramp	70	30
12.67	0.25	Step	0	100	-	0.50	Ramp	50	50
21.67	0.50	Step	0	100	-	0.50	Ramp	1	99
41.67	0.50	Step	99	1	-	0.50	Step	1	99
42.67	0.50	Step	99	1	-	0.50	Step	99	1

[α] "Grad" means gradient mode (i.e. step function or linear ramp);

[β] mobile phase "A" and "B" are water and methanol, respectively, with 2 mM ammonium acetate in both

[γ] "switch" refers to the switch of the valve from in-line SPE loading position, to elution position.

Table 3-3. Method detection limits (MDLs) of model mass-labelled PFAS standards in the in-line SPE method, measured by spiking different concentrations of mass-labelled PFAS standards into the wastewater samples. MDLs of PFASs in ng/mL unit are measured data, and other MDLs are back-calculated data from them.

PFASs	MDLs			
	PFAS, ng/mL	PFAS, ng	Fluorine, ng/mL	Fluorine, ng
$^{13}C_2$-PFHxA	0.2	1.00	0.13	0.66
$^{13}C_4$-PFOA	0.02	0.10	0.01	0.07
^{13}C5-PFNA	0.01	0.05	0.01	0.03
$^{13}C_4$-PFDA	0.01	0.05	0.01	0.04
$^{13}C_2$-PFUdA	0.01	0.05	0.01	0.04
$^{13}C_2$-PFDodA	0.005	0.03	0.004	0.02
$^{18}O_2$-PFHxS	0.01	0.05	0.01	0.03
$^{13}C_4$-PFOS	0.005	0.025	0.003	0.02
^{3}d-NMeFOSA	0.2	1.0	0.13	0.63
^{5}d-NEtFOSA	0.2	1.0	0.12	0.61
$^{13}C_2$-6:2PAP	0.05	0.25	0.03	0.14
$^{13}C_2$-8:2PAP	0.1	0.5	0.06	0.30
$^{13}C_4$-8:2 diPAP	0.2	1.0	0.13	0.65

Table 3-4. Summary of the identified PFASs in the wastewater sample from a fluorochemical manufacturing park in China.

Class No.	Name and Proposed Structure	Labels	Theoretical *m/z*	Molecular Ion
1	hydro-substituted perfluorocarboxylates H-PFCAs $H(C_nF_{2n})COO^-$ n=5-16	1A	294.98114	$C_6F_{10}HO_2^-$
		1B	344.97904	$C_7F_{12}HO_2^-$
		1C	394.97585	$C_8F_{14}HO_2^-$
		1D	444.97265	$C_9F_{16}HO_2^-$
		1E	494.96946	$C_{10}F_{18}HO_2^-$
		1F	544.96627	$C_{11}F_{20}HO_2^-$
		1G	594.96307	$C_{12}F_{22}HO_2^-$
		1H	644.95988	$C_{13}F_{24}HO_2^-$
		1I	694.95669	$C_{14}F_{26}HO_2^-$
		1J	744.95205	$C_{15}F_{28}HO_2^-$
		1K	794.95030	$C_{16}F_{30}HO_2^-$
		1L	844.94601	$C_{17}F_{32}HO_2^-$
2	chlorine-substituted perfluorocarboxylates Cl-PFCAs $ClC_nF_{2n}COO^-$ n=4-11	2A	278.94646	$ClC_5F_8O_2^-$
		2B	328.94313	$ClC_6F_{10}O_2^-$
		2C	378.94007	$ClC_7F_{12}O_2^-$
		2D	428.93704	$ClC_8F_{14}O_2^-$
		2E	478.93368	$ClC_9F_{16}O_2^-$
		2F	528.93049	$ClC_{10}F_{18}O_2^-$
		2G	578.92729	$ClC_{11}F_{20}O_2^-$
		2H	628.92410	$ClC_{12}F_{22}O_2^-$
3	polyfluorinated sulfates PFSfs $C_2F_5(C_2H_2F_2)_nCHFOSO_3^-$ n=1-6	3A	310.96298	$C_5F_8H_3SO_4^-$
		3B	374.97543	$C_7F_{10}H_5SO_4^-$
		3C	438.98789	$C_9F_{12}H_7SO_4^-$
		3D	502.99982	$C_{11}F_{14}H_9SO_4^-$
		3E	567.01280	$C_{13}F_{16}H_{11}SO_4^-$
		3F	631.02526	$C_{15}F_{18}H_{13}SO_4^-$
4	hydro-substituted unsaturated perfluorinated ethers/alcohols H-uPFE/As	4A	328.98413	$C_7F_{12}HO^-$
		4B	378.98093	$C_8F_{14}HO^-$
		4C	428.97674	$C_9F_{16}HO^-$
		4D	478.97455	$C_{10}F_{18}HO^-$
		4E	528.97135	$C_{11}F_{20}HO^-$
5	chlorine-substituted unsaturated perfluorinated ethers/alcohols Cl-uPFE/As	5A	312.94835	$ClC_6F_{10}O^-$
		5B	362.94515	$ClC_7F_{12}O^-$
		5C	412.94196	$ClC_8F_{14}O^-$
		5D	462.93877	$ClC_9F_{16}O^-$
		5E	512.93557	$ClC_{10}F_{18}O^-$

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Chapter 4. Non-Target Mass Spectrometry Reveals New Classes of Perfluoroalkyl Substances in Fish from the Yangtze River and Tangxun Lake, China

4.1 Introduction

Many long-chain poly- and perfluoroalkyl substances (PFASs) are now globally distributed as contaminants.[1, 2] Long-term human and environmental health concerns have arisen due to their known environmental persistence,[3] bioaccumulation potential[4] and toxicological effects.[5-7] Since their first discovery in the environment since 2001, industrial phase-outs[8-10] and domestic and international restrictions and regulations[9-16] have been enacted in an effort to minimize future exposure to known PFASs. Some consequences of these actions include a global geographic shift of PFAS manufacturing to countries with fewer restrictions, and a shift in chemistry towards alternative PFASs[17-19] that have uncertain environmental fate and toxicological effects.

After the 3M Co. phased out its C_8 production in North America, Europe and Japan between 2000 and 2002, developing countries, mainly in continental Asia (e.g. China), increased their PFAS production,[20] and contamination of local environments[21, 22] and of human serum[23] has followed. The link between a contaminated environment and local people is well described for fisheries around Tangxun Lake in China.[22] Here, wastewater from an industrial region is known to have contaminated the lake water, such that it now contains very high concentrations of long chain PFASs, including perfluorooctanesulfonate (PFOS, 73-1650 ng/L) and perfluorooctanoate (PFOA, 71-1390 ng/L) as well as known alternative-PFASs such as perfluorobutanesulfonate (PFBS, 2240-4520 ng/L) and perfluorobutanoate (PFBA, 1820-6280 ng/L). In blood of fishery employees who live around this lake, and who also likely consume the fish, serum concentrations of PFOS were extremely high, up to 31,400 ng/mL (median 10,400 ng/mL).[24] Moreover, use of PFASs in China may have global consequences, as shown for F-53B ($ClC_6F_{12}OC_2F_4SO_3K$, CAS No. 73606-19-6), a metal plating mist suppressant chemical used only in China since the 1970s.[25-27] F-53B is detectable in Chinese environmental[26-28] and biological samples,[28-31] but was

also recently detected in Greenland ringed seals and polar bears.[32]

Shorter-chain PFASs and structurally modified long-chain perfluorinated acids, such as by incorporation of ether bonds or of non-fluorinated carbons, are two broad categories of known alternative-PFASs.[18] Manufacturers claim such alternatives are less bioaccumulative and "safe for their intended use",[33, 34] but there are few publicly available toxicity studies. In many cases the alternative-PFASs, or their predicted breakdown products, do not avoid the problem of environmental persistence. For example, trifluoroacetic acid, PFBS and 6:2 fluorotelomer sulfonic acid were similarly resistant to microbial degradation as PFOS,[35, 36] and perfluoroether chains were as resistant as perfluoroalkyl chains to abiotic and biotic degradation.[37] Moreover, shorter-chain alternatives do not necessarily avoid unwanted bioaccumulative properties. For example, the C_6 perfluoroalkyl sulfonate (PFSA) is more biopersistent in humans than the C_8-PFSA (i.e. PFOS),[38-40] and perfluorobutanesulfonamide (a precursor of PFBS) was recently detected in fish[41] (mean 0.04-5.35 ng/g wet weight in fish in Canada, and 80.12 ng/g in fish in the Netherlands), suggestive of its bioaccumulation potential.

Another concern is that unknown PFASs are now being intentionally manufactured, or produced as byproducts, and that these may already be adding to environmental contamination. At a fluorochemical manufacturing site on the Yangtze River in China, our previous work to flag unknown PFASs by non-target high-resolution mass spectrometry (Nt-HRMS) in wastewater revealed 36 new PFASs (**Chapter 3**).[42] These included 3 PFAS classes that had not previously been reported in the environment: polyfluorinated sulfates ($C_nF_{n+3}H_{n-2}SO_4^-$), chlorine-substituted perfluorocarboxylates ($ClC_nF_{2n}CO_2^-$), and hydro-substituted perfluorocarboxylates ($HC_nF_{2n}CO_2^-$). Moreover, it is generally reported that a high percentage (i.e. >90%) of the total mass balance of organic fluoride is from unknown compounds, including in environmental samples[43, 44] and biological samples.[31, 45, 46] These unknowns may be attributable to unidentified legacy PFASs, or to contemporary use of alternative-PFASs, and it is important to identify these unknowns to accurately evaluate or mitigate risks of PFAS exposure today and in the future.

Nt-HRMS techniques are valuable tools for chemical discovery owing to their high sensitivity for detection, high spectral resolving power to avoid isobaric interferences, and high mass accuracy which enables accurate prediction of empirical formulae for unknown molecules. Their successful applications to PFAS discovery in water samples[42, 47-49] and in human serum[50]

are already demonstrated.

In the current study we adapted our Nt-HRMS method for PFAS discovery in water (**Chapter 3**)[42] to discovery of potentially bioaccumulative PFASs in fish tissue collected at two strategic locations in China. The first location was on the Yangtze River, approximately 10 km downstream of the fluorochemical manufacturing park where we previously identified new PFASs in wastewater[42] which is known to be released to the river.[51] The second location was at Tanxun Lake, which is now well recognized as a highly contaminated system with an active fishery.[22-24] The fish from these two water systems have long been sold in markets for consumption by local people, or potentially transported for sale to other regions of China. The current study will help to reveal potentially bioaccumulative PFASs which may be a risk to aquatic wildlife and surrounding human populations.

4.2 Material and Methods**

4.2.1 Chemical and Reagents

Optima water and methanol were from Fisher Scientific (Ottawa, ON, Canada), and ammonium acetate and 1-methyl piperidine (1-MP) were from Sigma-Aldrich (St. Louis, MO, USA). A standard of decafluoro-4-(pentafluoroethyl)-cyclohexanesulfonate (4-PFCHES, CAS 335-24-0) was from Wako Chemicals (Richmond, VA, USA), and a chlorinated perfluorinated ether sulfonate (F-53B, CAS 73606-19-6) industrial standard was from Shanghai Maikun Chemical Co., Ltd. (Shanghai, China). A historical industrial PFOS standard was donated by the 3M Co. (St. Paul, MN, USA).

4.2.2 Sample Collection

In October 2015, local Chinese fishermen were recruited to collect fish from two freshwater locations in China (**Figure 4-1**). One location was in the Yangtze River, in a region

** Ms. Manli Qian helped in fishermen recruiting and fish liver collection. Ms. Xinxin Ma (Nankai University) freeze-dried individual fish liver samples, and Mr. Liang Wang shipped the dried samples to the University of Alberta. Dr. Amila O De Silva (Environment and Climate Change Canada) provided the 4-PFECHS standard.

approximately 10 km downstream of a known wastewater treatment plant (WWTP, same as in **Chapter 3**) that services a major fluorochemical manufacturing park in Changshu, Jiangsu province.[42] The other location was in Tangxun Lake in Wuhan, Hubei province. This lake receives discharge from a WWTP that processes both domestic and industrial wastewater and has been previously identified as a contaminated water body.[22] Fish from both locations are routinely caught by commercial fisheries and are sold in local markets.

Three fish species were collected from the Yangtze River, including common carp (*Cyprinus carpio*), silver carp (*Hypophthaimichthys molitrix*) and bighead carp (*Aristichthys nobilis*). The same three species were collected from Tangxun Lake, as well as a fourth species, white Amur bream (*Parabramis pekinensis*). Between 3 and 7 individual fish were sampled for each species at each location. Whole liver was collected from each individual fish, weighed (wet weight, **Table 4-1**), bagged separately in polypropylene plastic bags, and shipped overnight in an ice box to Nankai University (Tianjin, China) where all liver samples and ~10 g potassium chloride granules (as quality control for possible laboratory contamination) were freeze dried, weighed (dry weight, **Table 4-1**) and ultimately shipped to the University of Alberta for Nt-HRMS analysis.

4.2.3 Sample Preparation

Freeze-dried fish livers and the procedural blank salts were wrapped in aluminum foil and pulverized using a small hammer. Between 0.20 - 0.31 g of individual livers from the same species at each location were pooled, resulting in 3 pooled samples for the Yangtze River and 4 pooled samples for Tangxun Lake (**Table 4-1**). The procedural blank salts and pooled dry liver powder (0.5 g) was extracted 3 times with 1.5 mL acidified acetonitrile (1% formic acid) in a 15 mL centrifuge tube, and centrifuged at 3000 rpm at 15 ℃ for 20 min. An aliquot (1000 μL) of the supernatant was taken, mixed with 4 mL water and loaded to pre-conditioned weak anion exchange cartridges (500 mg WAX/6 mL, Waters). After washing successively with 1 mL 2% formic acid, 2 mL water and 2 mL methanol, the cartridges were eluted with 2 mL 1% NH_4OH in methanol. The eluents were concentrated under a gentle stream of ultrapure nitrogen and reconstituted in 200 μL of 50% water/50% methanol. Injections (10-50 μL) of the final extract were made to HPLC-Orbitrap. Procedural blanks (0.5 mL Optima water) were extracted along with fish livers to monitor possible laboratory contamination during the extraction. An

instrumental blank (50% water/50% methanol) was also injected to monitor for any instrumental background.

4.2.4 Instrumental Analysis

The non-target PFAS discovery method developed in **Chapter 3**[42] was applied with some modifications. An Accela HPLC coupled with an Orbitrap Elite™ hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for analysis. Chromatographic separations were performed on an Xselect CSH C18 XP column (130 Å pore size, 2.5 µm particle size, 3 mm i.d., 150 mm length, Waters, Milford, MA) at 0.5 mL/min, and operating pressure was between 400-900 bar. Both mobile phase A (water) and B (methanol) contained 2 mM ammonium acetate and 2 mM 1-MP ($pH_{mobile\ phase\ A}$=9.8). The HPLC gradient program was as follows: starting with 38% B, increased linearly to 50% B between 0.17 and 2.67 min, then to 99% B at 41 min, held for 6 min, and returning to 38% B with a hold for 6 min before the next injection. Sample injection volume was between 20 - 50 µL.

The Orbitrap was operated in negative electrospray ionization (ESI) mode. Capillary voltage, capillary temperature, vaporizer temperature, sheath gas, auxiliary gas and sweep gas were 5 kV, 275 °C, 325 °C, 40, 5 and 2 (arbitrary units), respectively. Two injections were made for the Nt-HRMS method. Analyte ions of unknown fluorinated contaminants are discovered in the first injection by performing the following parallel scans: (1) full scan (*m/z*=150-1500, Orbitrap mass analyzer set to RP 120,000); (2) in-source fragmentation flagging scan I (*m/z*=50-500, SID energy set to 80 or 100 eV, Orbitrap mass analyzer set to RP 30,000); and (3) in-source fragmentation scan II (*m/z*=30-100, SID energy set to 80 or 100 eV, ion-trap mass analyzer). Plausible molecular formulae were then assigned based on collective information including mass accuracy (i.e. within ±5ppm for all discovered ions and their isotopic ions), isotopic patterns (e.g. isotopic peaks $[M]^-/[M+2]^-$=3:1 indicates one chlorine atom), and compatibility of the predicted formula with in-source fragment ions (e.g. in-source $[SO_3]^-$ indicates at least one sulfur and three oxygen atoms must be present in the intact ion). In the second injection, collision induced dissociation (CID) (0-80eV, He gas) and/or higher-energy collisional dissociation (HCD, 0-155eV, N_2 gas) MS^n (where n=2 - 4 stages of ion isolation and fragmentation) analyses were performed to further confirm the proposed molecular formula while also attempting to characterize structure of the unknown ions.

4.2.5. Data Presentation

Legacy PFASs (e.g. PFCAs and PFSAs) were monitored using authentic standards, and non-target PFASs were discovered by the Nt-HRMS method. For each class of non-target PFAS homologues discovered, plausible structures were proposed and confidence levels (CL=1-5) were assigned according to established criteria.[52] Briefly, CL=1 represents confirmed structures by match to reference standards, level 2 represents probable structures by comparing to library spectra or by diagnostic evidence, and level 3 represents tentative candidates whose possible structure can be proposed but lack sufficient information to assign an exact structure (e.g. position of a chlorine atom on an aromatic ring). CL=4 is used when the unknown analyte ion can be assigned an unambiguous formula, but no structural information is available. Level 5 is assigned when analyte ions cannot be assigned a single empirical formula with confidence.

Quantification and semi-quantification were performed for legacy PFASs and non-target PFASs detected in fish livers, respectively. Each legacy PFAS was quantified in pooled fish liver extract (ng/mL) against its own methanol-based calibration curve, and back-calculated to the concentration in wet liver (ng/g wet weight, **Table 4-2**) using the average wet/dry weight liver ratio among all individual fish in the pool (**Table 4-1**). Authentic standards were not available for most non-target analytes. In full recognition of analytical uncertainties, semi-quantification was nevertheless performed with legacy PFAS standards (**Table 4-3**). For characterized non-target PFCAs and PFSAs, the semi-quantification was performed assuming the same molar unit response as the same or the closest chain-length legacy PFCAs and PFSAs, respectively. For non-target PFASs of other structures (i.e. non-perfluoroalkyl acids), the same molar unit response as PFOS was arbitrarily assumed for all analytes, regardless of chain-lengths. For the purpose of comparison with legacy PFASs in fish liver, the semi-quantified molar concentrations in extracts were further transformed to ng/g wet weight liver.

4.3 Results and Discussion

4.3.1 Legacy PFASs

Legacy PFCAs (C_4-C_{14}) and PFSAs (C_4-C_{10}) were detected in all fish samples from both locations. PFOS-precursors (e.g. N-methyl perfluorooctanesulfonamidoacetate) and PFCA

precursors (e.g. 8:2 polyfluoroalkyl phosphoric acid monoester) were occasionally detected in some samples, while perfluorophosphonic acids were not detected in any sample (**Table 4-2**, **Figure 4-2**). PFCAs were generally present at very low levels in all fish, whereas PFSAs were the major legacy PFASs detected. The Tangxun lake fish generally had higher levels of PFSAs than the Yangtze River fish. Highest concentrations were in bream fish from Tangxun Lake, but the same species was not available for comparison in the Yangtze River. PFOS was the dominant PFAS in all fish samples, contributing 58-72% of all legacy PFASs in the Yangtze River fish and 93-97% in Tangxun Lake fish. The exceptionally high PFOS in bream fish in Tangxun Lake (750 ng/g wet weight) is consistent with a previous report where PFOS was detected at 540-1900 ng/g levels in crucian carp liver collected in the same lake two years earlier.[29]

4.3.2 Newly Discovered Non-Target PFASs

Using the Nt-HRMS discovery methods, in total 10 homologous classes of PFASs were discovered in the 7 pooled fish liver samples. None of these non-target analytes were detected in any quality control blanks, allowing us to rule out laboratory and instrumental background as their sources. Each PFAS class detected had at least 5 chain-length homologues, and isomers of a given homologue were commonly, but not always, observed. At each location, the homologue and isomer profiles were very similar between fish species, although minor analytes in one fish species were sometimes being absent in another due to differences in concentrations between species.

For structural elucidation of the unknown PFASs, 1-2 abundant homologues in each class were analyzed by MS^n experiments in one of the fish species that had relatively high responses (e.g. bream fish in Tangxun Lake). Because of insufficient signal to conduct MS^n experiments with less abundant homologues, proposed structures are generalized for the whole class. Each discovered class of PFAS is presented with detailed discussion of the mass spectral evidence and novelty for each class is always discussed with respect to previous reports.

Class 1: Terminal Chlorine Substituted Perfluoroalkylcarboxylates, terminal Cl-PFCAs, $[ClC_{n-1}F_{2n-2}COO]^-$, n=8-15

A homologous class of chlorinated PFASs was discovered in all 3 fish samples from the Yangtze River by in-source fragmentation scanning for $[^{35}Cl]^-$ and $[^{37}Cl]^-$ peaks. The associated

ion formulae for these non-target analytes, also considering the $[M]^-/[M+2]^-$=3:1 isotopic pattern, was consistent with Cl-PFCAs having 8-15 carbon atoms (**Figure 4-3.1**, **Table 4-4**). Only one isomer was observed for each homologue, and 8 total analytes were detected in this class.

Structural elucidation by MS^2 was performed for the prospective C_9 homologue in pooled common carp liver (**Figure 4-3.2**). Observation of the $[M\text{-}CO_2]^-$ fragment indicated a carboxylic acid functional group. $[M\text{-}CO_2]^-$ was the only fragment observed under low fragmentation energy, and no fragment could be seen when fragmentation energy increased (scanning range *m/z* 50-500). Thus, the dominant fragmentation pathway was yield of $[Cl]^-$ ion, leaving a neutral charge on the perfluoroalkyl chain. Taking the evidence together with the high abundance of $[^{35}Cl]^-$ and $[^{37}Cl]^-$ from in-source fragmentation, we proposed a terminal chlorine substituted PFCA structure, CL=3, for this homologous class (**Table 4-4**).

We previously reported this same class of analytes (C_5-C_{12}) in influent water[42] to a WWTP (**Chapter 3**) located ~10 km upstream of where these fish were collected in the Yangtze River. This is the first ambient environmental measurement of Cl-PFCAs, and we are not of previous reports. Compared to our previous analysis of WWTP influent, shorter C_5-C_7 homologues were absent in fish while longer C_{13}-C_{15} homologues were present, likely indicating a higher bioaccumulation potential for long-chain homologues, a well-known phenomenon among legacy PFASs.[4, 53-55] Moreover, the C_8 homologue which dominated the Cl-PFCA class in the wastewater[42] from **Chapter 3**, had the lowest response among all homologues in fish livers here (**Figure 4-3.1**) and was estimated to be present at the lowest concentrations (**Table 4-3**). The C_{10} and C_{11} homologues dominated the profile in fish, with estimated concentrations of 17 and 13 ng/g wet weight, respectively, in pooled common carp liver.

Class 2: x:2 Chlorine Substituted Perfluoroalkyl Ether Sulfonates, x:2 Cl-PFAESs, $[ClC_{n-2}F_{2n-4}OC_2F_4SO_3]^-$, n=7-10, 12, 14

Another homologous class of non-target analytes was detected by in-source fragmentation flagging of $[Cl]^-$ which aligned with various fluorinated ion fragments: $[SO_2F]^-$, $[COF_3]^-$ and $[SO_3F]^-$ (**Figure 4-4.1**). These were observed in all pooled samples from both locations, and the corresponding molecular ions in full-scan corresponded to $ClC_nF_{2n}SO_4^-$ where n=7-10, 12 and 14 carbons (**Table 4-4**). Only one isomer was detected for each chain-length homologue.

MS^2 analyses were performed for both C_8 and C_{10} homologues in pooled common carp

liver from the Yangtze River (**Figure 4-4.2**). We also analyzed an authentic industrial standard of F-53B ($CClF_2C_5F_{10}OC_2F_4SO_3^-$) which shares a common empirical formula with the unknown C_8 homologue. The retention time (**Figure 4-4.1**) and MS^2 spectrum (**Figure 4-4.2**) of the unknown C_8 homologue in carp matched the C_8 homologue in F-53B**.** Moreover, the unknown C_{10} homologue in carp matched retention time and MS^2 spectrum of residual C_{10} homologue in F-53B (**Figure 4-4.1** and **Figure 4-4.2**). Thus, for these two homologues the structures were confirmed (CL=1) as 6:2 terminal chlorine substituted perfluoroalkyl ether sulfonate (6:2 Cl-PFAES, $CClF_2C_5F_{10}OC_2F_4SO_3^-$, also known as F-53B) and 8:2 Cl-PFAES ($CClF_2C_3F_6OC_2F_4SO_3^-$). Although we did not explore structure of other residual carbon chain-length homologues ($ClC_nF_{2n}SO_4^-$, where n=6, 7 and 9) by MS^n, these were also detected in the F-53B standard, and matched retention times of the C_7 and C_9 analytes in fish (**Figure 4-4.1**). Thus, it is highly likely that these unknown analytes in fish are 5:2 and 7:2 Cl-PFAESs. Remaining homologues were therefore assigned as x:2 Cl-PFAES, CL=2.

In previous reports, 6:2, 8:2 and 10:2 Cl-PFAESs were detected in samples from China, including 6:2 (i.e. F-53B) in chrome plating discharge and downstream surface water,[27] 6:2, 8:2 and 10:2 in municipal sludge,[26] 6:2 in air,[28] 6:2 in crucian carps (including from Tanxun Lake),[29] 6:2, 8:2[30, 31] and 10:2[31] in human biofluids, and 6:2 in Greenland ringed seals and polar bears.[32] In the current study, 5:2, 7:2, and 12:2 Cl-PFAESs, are reported for the first time in any sample type (**Figure 4-4.1**, **Table 4-4**).

Between the two locations, the Yangtze River fish (5:2-8:2, 10:2 and 12:2) had more homologues than the Tangxun Lake fish (6:2-8:2). The (semi-)quantification was performed against a calibration curve of 6:2 Cl-PFAES, and concentrations of x:2 Cl-PFAESs in the Yangtze River fish were generally higher than for Tangxun Lake (**Table 4-3**). This probably suggests a unique local F-53B exposure source for the Yangtze River, especially when considering the fact that the Yangtze River (a lotic aquatic system) has a higher dilution rate than Tangxun Lake (a lentic system). 6:2 Cl-PFAES is usually detected at comparable or higher levels than PFOS.[27-31] In the current study, 6:2 Cl-PFAES was present at similar concentrations as PFOS (67%-276% of PFOS, **Figure 4-2**) in the Yangtze River fish, and comparable (127%) to the concentration measured in crucian carp liver in other Chinese waters.[29] In Tangxun Lake, due to the exceptionally high PFOS concentrations, 6:2 Cl-PFAES was present at lower relative concentrations than PFOS (1.1-6.2% of PFOS) (**Table 4-3**, **Figure 4-2**), consistent with

measurements in crucian carp liver two.[29]

F-53B is a mist suppressant used in the chrome plating industry in China since the late 1970s.[25, 26] Five Cl-PFAES homologues (4:2-8:2) were detected in the industrial F-53B standard (**Figure 4-4.1**). Widespread x:2 Cl-PFAES detection in different areas of China indicates ubiquitous F-53B exposure in this country, and detection of 6:2 Cl-PFAES in Greenland ringed seals and polar bear[32] further suggests its long-range transport potential. Airborne F-53B in a Chinese city has increased 5-fold between 2007 and 2014,[28] possibly indicating an increasing problem. 6:2 Cl-PFAES has a log-bioaccumulation factor of 4.3 in crucian carp, compared to 3.3 for PFOS.[29] It has been termed "the most bio-persistent PFAS in humans reported to date" with a median half-life of 18.5 years, compared to 7.7 years for PFOS.[31] The acute toxicity,[27] embryo-toxicity and cardiac developmental effects[56] of 6:2 Cl-PFAES in zebrafish has been described. Large scale targeted monitoring and risk assessment of x:2 Cl-PFAESs, as a class, may be warranted in the future.

Class 3: Cyclic or Unsaturated PFSAs, $[C_nF_{2n-1}SO_3]^-$, n=7-14

Here, a homologous class of non-target analytes was discovered by in-source fragmentation flagging of $[SO_3]^-$ and $[SO_3F]^-$ that appeared in all 4 pooled fish liver samples from Tangxun Lake. In full scan, these fragments corresponded to molecular ions similar to perfluoroalkyl sulfonates with 7-14 carbon atoms, but with 2 fewer F atoms (**Figure 4-5.1**, **Table 4-4**); owing to a double bond or ring in their structures (i.e. $C_nF_{2n-1}SO_3^-$). More than 50 partially resolved isomer peaks were observed among all chain-length homologues in this class.

MS^2 analyses were performed for all C_8 isomers ($C_8F_{15}SO_3^-$) in pooled bream fish (**Figure 4-5.2**). The presence of $[SO_3]^-$ and $[SO_3F]^-$ for all isomers confirmed the in-source fragments and indicated the presence of a sulfonate group next to a fluorinated chain; most isomers also yielded the intact $C_8F_{15}^-$ ion after neutral loss of SO_3. Short perfluoroalkenyl fragments (i.e. $C_4F_7^-$) confirmed that some of the isomers had structures with a double-bond, whereas $C_5F_9^-$ and $C_6F_{11}^-$ ion were more ambiguous either representing longer perfluoroalkenyl moieties, or 5-6 membered rings.[57] Unlike all isomers in the fish, MS^2 of authentic 4-PFECHS standard ($C_8F_{15}SO_3^-$) did not yield the $[SO_3]^-$ ion, but exclusively the $[SO_3F]^-$ ion (**Figure 4-5.3**a); the structure for this standard is a sulfonate group attached directly to a 6-membered perfluoroalkyl ring. The absence of $[SO_3]^-$ fragment was also observed for 1-perfluoromethyl-PFOS standard (**Figure 4-5.3**b),

where SO_3 is directly connected to a tertiary carbon, similar to 4-PFECHS. Thus, any perfluoroalkyl ring-containing structures in fish (e.g. possibly isomers 2 or 5) likely have a -CF_2- or -C_2F_4- between the sulfonate and the ring, and any branched unsaturated perfluoroalkyl sulfonate structures likely to have the branch located at non-α carbons (**Figure 4-5.2**). The general structure for this class was therefore assigned as cyclic or unsaturated PFSAs, CL=3 (**Table 4-4**).

We also detected residual C_8 (≥9 isomers) and C_9 (≥4 isomers) cyclic or unsaturated PFSA homologues in a standard of historical commercial PFOS (denoted as "3M-PFOS" hereafter), and using retention time and MS^2 spectral matches we confirmed that unknown isomer 2 and 9 of the C_8 homologue in fish (**Figure 4-5.2**) are the same unknown isomers in 3M-PFOS. Thus, these substances may be present in the environment as residual byproduct of historical PFOS. In the four Tangxun Lake fish samples, this class of analytes present at <10% of legacy PFSA concentrations (**Figure 4-2**), but the C_9, C_{11} and C_{12} homologues were more prominent in some samples (**Table 4-3**). The bream fish showed higher levels than other species (**Figure 4-2**).

We are unaware of any previous reports of unsaturated PFSAs in any biological sample, but in total 5 cyclic PFSA have been previously reported in waters,[58, 59] sediment,[57] amphipods[59] and in fish from the Great Lakes[58] and St. Lawrence River.[60] Among them, 4-PFECHS[57-61] and perfluoro-4-methylcyclohexanesulfonate (4-PFMeCHS)[58] were previously confirmed. Unsaturated or cyclic PFSA isomers with 4-13 carbons were reported in 3M commercial products, including a 3M PFOS standard, in aqueous film-forming foams (AFFFs),[62] and in concrete impacted by AFFF.[63]

Class 4: Monoether-PFSAs, $[C_nF_{2n+1}SO_4]^-$, n=4-12

Another homologous group of analytes was discovered through fragmentation flagging by $[C_4F_9O]^-$ which coeluted with $[SO_3]^-$, $[SO_2F]^-$, $[SO_3F]^-$ and $[C_3F_7]^-$ in all pooled fish liver samples from Tangxun Lake. These fragments corresponded to molecular ions with the general formula $[C_nF_{2n+1}SO_4]^-$ with 4-12 carbons (**Figure 4-6.1**, **Table 4-4**). More than 30 isomers (some only partially resolved) were observed among all chain-length homologue in this class.

MS^2 was performed for all prospective C_8 isomers in Tangxun Lake bighead carp (**Figure 4-6.2**). While observations of $[SO_3]^-$, $[SO_3F]^-$ and perfluoroalkyl ions (e.g. $[C_3F_7]^-$) are similar to classic perfluorinated sulfonates, the high abundance of oxy-perfluoroalkyl fragments (e.g.

$[C_4F_9O]^-$) suggested the presence of an ether moiety.[64] The possibility that these unknowns were perfluoroalkyl sulfates (i.e. R_f-OSO_3^-) was ruled out due to absence of $[C_8F_{17}O]^-$, formed via neutral loss of SO_3,[42, 65] as well as absence of $[SO_4]^-$ ion; [42, 47] both were also observed for polyfluorinated sulfates in **Chapter 3**. This class was therefore proposed as monoether-PFSAs CL=3 (**Table 4-4**), and possible structures are shown in **Figure 4-6.2**. The $[SO_3]^-$ ion was absent for isomers 3 and 6, likely indicating a tertiary α-carbon (as discussed in **Class 3**, among all mono-CF_3 branched PFOS isomers only α-branched PFOS produced no $[SO_3]^-$ fragment,[66] **Figure 4-5.3**).

The only previous report of monoether-PFSAs was a C_6-analyte detected in AFFF-exposed firefighter serum,[50] which was proposed as $CF_3OC_5F_{10}SO_3^-$ based on its MS^2 spectrum. We detected residual C_8 (≥6 isomers) and C_9 ether-PFSAs (≥6 isomers) in 3M-PFOS, but none of these matched with those in fish. C_8 and C_9 homologues were the dominant homologues in all four Tangxun Lake fish species, and bream fish showed the highest estimated concentrations (~50 ng/g for both homologues, **Table 4-3**). Compared to legacy PFSAs of the same chain-length, this class was generally present at 10-fold lower concentrations (**Figure 4-2**), however, some long-chain homologues were comparable (e.g. C_9 homologues were present at 60-190% of PFNS concentrations).). It is unclear if the presence of monoether-PFSAs in these fish is a result of historical PFOS production, or due to contemporary production as replacements to legacy PFSAs.[19]

Class 5: Diether-PFSA, $[C_nF_{2n+1}SO_5]^-$, n=6-12

Here another homologous group of analytes was discovered through in-source fragmentation flagging by $[C_2F_5O]^-$ in all Tangxun Lake pooled fish livers. In full-scan, these flags corresponded to the general molecular ion formula $C_nF_{2n+1}SO_5^-$ with 6-12 carbons (**Figure 4-7.1**, **Table 4-4**). This empirical formula is similar to classic perfluoroalkyl sulfonates (e.g. PFOS) but with two additional oxygen atoms, and the only possible way these oxygens can be incorporated to the structure is through ether bonds. Multiple partially resolved isomers were observed for homologues with 8 or more carbons (smaller homologues had only weak intensity and no isomers were observed), and the total number of discovered analytes was estimated at over 25.

MS^2 analyses were performed for all three C_8 isomers in pooled liver of bream fish from

Tangxun Lake (**Figure 4-7.2**). Fragments $[SO_3]^-$, $[CO_3F]^-$ and $[SO_3F]^-$ are indicative of a sulfonate group, and the high response of perfluoroalkoxy $[C_nF_{2n+1}O]^-$ and dioxy $[C_nF_{2n+1}O_2]^-$ fragment ions provided empirical evidence to support the assignment of this class as diether-PFASs, with CL=3 (**Table 4-4**).

These analytes have not previously been reported, but two C_7 analytes detected in water downstream of a fluorochemical facility were tentatively proposed as something similar: a diether-polyfluoroalkyl sulfonate ($CF_3CHFOCF_2C(CF_3)OC_2F_4SO_3^-$, and a diether-unsaturated perfluoroalkyl sulfonate ($CF_2=CFOCF_2C(CF_3)OC_2F_4SO_3^-$).[48] Diether perfluoroalkyl carboxylates (e.g. $C_2F_5OC_2F_4OCF_2COO^-$, CAS No. 908020-52-0) have been reported to be manufactured by companies as alternatives to PFOA,[19] thus it is possible that the novel diether-PFSAs detected here were manufactured intentionally as PFOS alternatives. The semi-quantified concentrations for this class were relatively low in all fish samples, with the dominant C_9 homologue at <3 ng/g (**Table 4-3**). Bream fish had the highest levels of all homologues in this class (**Figure 4-2**).

Class 6: Enol-Ether-, Cyclic-Ether- or Carbonyl-PFSAs, $[C_nF_{2n-1}SO_4]^-$, n=6-13

Here a homologous group of analytes was discovered by in-source fragmentation flagging of $[C_3F_5]^-$ and $[C_4F_7]^-$ which coeluted with $[SO_3]^-$ and $[SO_3F]^-$ in all pooled fish liver samples from Tangxun Lake. In full-scan, these fragments corresponded to molecular ions with the general formula of $C_nF_{2n-1}SO_4^-$ with 6-13 carbon atoms (**Figure 4-8.1**, **Table 4-4**). More than 40 partially resolved isomers were observed among all homologues in this class.

MS^2 was performed for all C_8 isomers in bream fish from Tangxun Lake (**Figure 4-8.2**). $[SO_3]^-$, $[SO_2F]^-$ and $[SO_3F]^-$ indicated an underlying PFSA structure. Neutral $C_nF_{2n}O$ loss (e.g. production of $C_7F_{13}SO_3^-$ and $C_6F_{11}SO_3^-$) and perfluoroalkenyl fragments were commonly observed. This, along with DBE=1, allows three feasible structures with CL=3 (**Figure 4-8.2**, **Table 4-4**):

(1) Cyclic ether-PFSAs. Neutral CH_2O loss is characteristic of cyclic ethers in electron ionization[67] and collision-activated dissociation[68] in GC-MS. (2) Unsaturated ether-PFSAs. Perfluoroalkoxy fragments are usually expected for perfluoroalkyl ethers.[48, 64] However, no such fragments were observed for any C_8- isomer, possibly indicating an enol ether structure where the existence of a nearby double bond may have reduced production of the perfluoroalkoxy fragment. Neutral CH_2O loss was commonly observed for anisoles and methoxypyridines in

electron ionization.[69] (3) Carbonyl-PFSAs. Fragments $C_6F_{11}SO_3^-$ for isomer 7 and 9 can be formed via cleavage of a $CF_3C(O)$- moiety, while $C_7F_{13}SO_3^-$ for isomer 2-6 and 8 may be due to a terminal carbonyl fluoride (i.e. an acid fluoride). However, we believe it unlikely that such a compound would be hydrolytically stable under our alkali mobile phase condition, not to mention persistent enough to be present in fish livers (**Table 4-4**).

Residual $C_8F_{15}SO_4^-$ (6 isomers) and $C_9F_{17}SO_4^-$ (3 isomers) ions were also detected in 3M-PFOS. By retention and MS^2 matches, isomers 2, 5 and 8 of the C_8 homologue in fish samples are likey of the same chemical identity as two major and one minor C_8 isomers in 3M-PFOS. This class of analytes could therefore be byproducts in historical perfluoroalkyl sulfonate production, or perhaps are manufactured intentionally as new alternatives to PFOS. The total semi-quantified concentration for this class was between 0.02-18 ng/g in bream fish, and <1 ng/g in the other species (**Table 4-3**, **Figure 4-2**).

Cyclic ether PFSAs have not previously been reported. A C_7-analyte detected in water downstream of a fluorochemical production facility was proposed as an enol ether-perfluoroalkyl sulfonate (CF_2=$CFOCF_2C(CF_3)OC_2F_4SO_3^-$, as discussed in **Class 5**, but only based on its exact mass (i.e. no spectral information).[48] Based on MS^2 spectra, a homologous (C_4-C_{13}) class of compounds detected in AFFF-impacted concrete[63] and a C_8 analyte detected in AFFF-exposed firefighters' were proposed as ketone-PFSAs.

Class 7: Diether-Unsaturated-, Cyclic-Ether- or Carbonyl-Ether-PFSAs, $[C_nF_{2n-1}SO_5]^-$, n=6-13

Here a homologous group of analytes was flagged by the in-source fragment ions $[C_2F_5O]^-$ and $[C_3F_5O_2]^-$, which coeluted with $[SO_3]^-$, $[SO_3F]^-$, $[C_2F_5]^-$ and $[C_3F_7]^-$ in all pooled fish from Tangxun Lake. In full-scan, these ions corresponded to molecular ions having the general molecular ion formula of $[C_nF_{2n-1}SO_5]^-$ with between 6-13 carbons (**Figure 4-9.1**, **Table 4-4**). This empirical formula is similar to **Class 6** analytes, but with one additional oxygen atom. More than 30 partially resolved isomers were observed among all homologues.

MS^2 analysis was performed for all C_{10} isomers in silver carp from Tangxun Lake (**Figure 4-9.2**). The $[SO_3]^-$ and $[SO_3F]^-$ fragment ions observed for all isomers are characteristic of PFSAs. Similar to Class 6, neutral loss of $[C_nF_{2n}O]$ and perfluoroalkenyl fragments ions (e.g. $[C_3F_5]^-$ or $[C_4F_7]^-$) were observed for all isomers, indicating the same three feasible

functionalities as above: enol ether-, cyclic ether- or carbonyl-PFSAs. The high $[C_2F_5O]^-$ fragment for isomer 1-3, as observed for **Class 4** monoether-PFSAs and **Class 5** diether-PFSAs, indicates an additional terminal ethylether (example structures for isomer 2 in **Figure 4-9.2**), while the absence of an obvious perfluoroalkoxy fragment for isomer 4 and 5 may indicate an internal ether structure (i.e. see proposed dioxy-cyclic PFASs or a mixed carbonyl-ether-PFSA for isomer 4 in **Figure 4-9.2**). Thus, diether-unsaturated-, cyclic-ether- or carbonyl-ether-PFASs are all possible for this class, with CL=3 (**Table 4-4**).

This class of ions was not detected in the historical 3M-PFOS standard. In fish livers, most analytes were estimated to be <1 ng/g, except in bream fish, where the dominant C_{10} homologue was present at ~3.0 ng/g (**Table 4-3**, **Figure 4-2**). The only previous report of a chemical belonging to this class was a study by Strynar *et al*.[48] who, proposed a C_7 analyte detected in water as diether-unsaturated PFSA (i.e. $CF_2{=}CFOCF_2CF(CF_3)OC_2F_4SO_3^-$) as discussed in **Class 5** and **6**, and based only on its exact mass..

Class 8: Perfluoroalkyl Amine Carboxyl Esters, $[C_nF_{2n-6}H_6O_6N]^-$, n=13-16

For this class of analytes, discovery was initially for a series of small nitrogen containing ions that we later realized were fragments of larger precursor molecules, which dissociated even under the soft ionization conditions of our full scan mode. Here, the discovery process is described sequentially, from smallest to largest ions, as it is instructive for characterization of the analyte structure(s) in this class.

A homologous group of nitrogen containing ions, with the general formula of $[C_nF_{2n+2}N]^-$ (n=5-12), was initially discovered by in-source $[C_3F_7]^-$ fragments in all Tangxun Lake pooled fish liver (**Figure 4-10.1**, **Table 4-4**) with many isomers observed for each homologue. Structural characterization of the C_5 (**Figure 4-10.2**a) and C_8 homologues (**Figure 4-10.2**b) suggested a perfluoroalkyl amine structure for these ions. Although branching of the perfluoroalkyl chain could possibly have given rise to the high number of isomers, the identical MS^2 profiles, especially for those of low carbon-chain homologues (e.g. $C_5F_{12}N^-$ in **Figure 4-10.2**), raised the possibility that these analytes may actually be identical or very similar fragments coming from larger precursor molecules.

Manual searching for higher molecular weight precursors was performed at the same retention times, and the first class of plausible precursor ions had the general molecular formula

of $[C_nF_{2n}O_2N]^-$ with 9-16 carbons (**Figure 4-10.3**, **Table 4-4**). Each $[C_nF_{2n}O_2N]^-$ homologue was composed of multiple isomeric peaks, and each peak had at least one $[C_nF_{2n+2}N]^-$ ion peak at the same retention time (shaded peaks in **Figure 4-10.3**), consistent with these being precursor ions. This was confirmed by MS^2 fragmentation of all $C_{12}F_{24}O_2N^-$ isomers (**Figure 4-10.4**a), as well as the major isomer of the C_{10}-C_{13} homologues (**Figure 4-10.4**b), whereby $[C_8F_{18}N]^-$ and/or $[C_5F_{12}N]^-$ ions were produced under various dissociation conditions. Neutral loss of $C_nF_{2n-2}O_2$ (n=4-6) was commonly observed for the C_{10}-C_{13} homologues, corresponding to either a perfluorocarboxylate or carboxyl ester moiety (see proposed structures for the major C_8 isomer in **Figure 4-10.4**).

Continued manual search for even higher molecular weight precursors, at the same retention times, revealed two classes of homologues with the general formulae: $[C_nF_{2n-2}H_2O_5N]^-$ (n=10-17, **Figure 4-10.5**) and $[C_nF_{2n-6}H_6O_6N]^-$ (n=13-16, **Figure 4-10.6**). Almost all $[C_nF_{2n-2}H_2O_5N]^-$ ions had a corresponding $[C_nF_{2n}O_2N]^-$ peak at the same retention time (shaded peaks in **Figure 4-10.3**), and all $[C_nF_{2n-6}H_6O_6N]^-$ ions had a corresponding $[C_nF_{2n-2}H_2O_5N]^-$ peak at the same retention time (shaded peaks in **Figure 4-10.5**), indicating these as precursors.

Collectively, these data suggest that the analytes actually present in fish livers are homologues and isomers of $[C_nF_{2n-6}H_6O_6N]^-$, while the $[C_nF_{2n-2}H_2O_5N]^-$, $[C_nF_{2n}O_2N]^-$ and $[C_nF_{2n+1}N]^-$ fragments are formed spontaneously in the source of the instrument. Neither $[C_nF_{2n-2}H_2O_5N]^-$ nor $[C_nF_{2n-6}H_6O_6N]^-$ homologues could be successfully selected in HCD or CID MS^2 analysis, making the structure of $[C_nF_{2n-6}H_6O_6N]^-$ uncertain (CL=4, **Table 4-4**). Nevertheless, based on characterization of the $[C_nF_{2n}O_2N]^-$ fragments, a general core structure of perfluoroalkyl amine carboxyl esters is likely for the $[C_nF_{2n-6}H_6O_6N]^-$ homologues.

The number of analytes detected for this $[C_nF_{2n-6}H_6O_6N]^-$ homologous class was estimated by counting the number of different retention times for the $[C_nF_{2n+2}N]^-$ fragments, and at least 50 $[C_nF_{2n-6}H_6O_6N]^-$ analytes could be confirmed in fish livers. Semi-quantification for this class was performed using PFOS calibration curves, and the total concentration was calculated by adding up $[C_nF_{2n-6}H_6O_6N]^-$ and all 3 classes of fragments. Thus, total **Class 8** analytes were present at >10 ng/g in all 4 fish species, and reached a level of ~630 ng/g in bream fish, comparable to the level of PFOS (**Figure 4-2**). The C_{15} homologue eluting at 24.37 min was taken as an example, and its sequential fragmentation pathway to $[C_{13}F_{24}H_2O_5N]^-$, to $[C_{12}F_{24}O_2N]^-$ and eventually to

$[C_8F_{18}N]^-$ was proposed (**Figure 4-10.7**).

Simple perfluoroalkyl amines are marketed for use in electronics testing, heat transfer[70, 71] and as artificial oxygen carriers for medical purposes.[72-74] Perfluoroalkyl amines are also among the starting materials for manufacturing fluoropolymers (e.g. polyurethanes and polyamides[71]). The only previous report of a perfluoroamine in the environment was perfluorotributylamine, which is among the 3M *Fluorinert*™ products,[70, 75, 76] detected in urban air in Toronto.[77] It is noteable that perfluorotributylamine has an atmospheric lifetime of 500 years, and has been concluded to have "the highest radiative efficiency of any compound detected".[77]

Class 9: Unsaturated Perfluoroalkyl Amine Carboxylates, or Unsaturated Perfluoroalkyl Amine Carboxyl Esters, $[C_nF_{2n}NO_2]^-$, n=10-15.

Another homologous group of ions with the general formula $[C_nF_{2n}N]^-$ (n=6-15) was discovered through in-source $[C_3F_5]^-$ fragments in all Tangxun Lake pooled fish livers (**Figure 4-11.1**). This empirical formula is similar to the above perfluoroalkyl amine fragments of **Class 8** analytes, but with two fewer F atoms, owing to a double bond or ring. The unsaturated perfluoroalkyl amine structure was confirmed by MS^2 experiments with all C_8 isomers (CL=3, **Figure 4-11.2**). Manual searching for possible higher molecular weight precursors revealed one homologous class of ions having the general molecular formula $[C_nF_{2n-2}NO_2]^-$ (n=10-15, **Class 9**, **Figure 4-11.3**), similar to the homologous fragments $[C_nF_{2n}NO_2]^-$ in **Class 8**. MS^2 fragmentation of all $C_{12}F_{22}NO_2^-$ isomers confirmed the general structure for this class as unsaturated perfluoroalkyl amine carboxylates, or carboxyl esters (CL=3, **Figure 4-11.4**).

The total number of analytes detected for **Class 9** $[C_nF_{2n-2}NO_2]^-$ was estimated by first counting the total different retention times of the $[C_nF_{2n}N]^-$ fragments (**Figure 4-11.1**) and then subtracting those where **Class 8** $[C_nF_{2n+2}N]^-$ fragments were also coeluting (**Figure 4-10.1**) due to the possibility that an unsaturated $C_nF_{2n-2}NO_2^-$ can fragment to both saturated $[C_nF_{2n+2}N]^-$ and unsaturated $[C_nF_{2n}N]^-$ fragments in the source (e.g. $C_{12}F_{22}NO_2^-$ at retention time 22.80 min produced $C_5F_{12}N^-$ and $C_7F_{14}N^-$ in MS^2 CID analysis in **Figure 4-11.4**, and a $C_5F_{12}N^-$ and a $C_7F_{14}N^-$ fragments also present in full-scan in **Figure 4-10.1** and **Figure 4-11.1**, respectively). The semi-quantified concentration for **Class 9** was ~10× lower than for **Class 8** (**Table 4-3**, **Figure 4-2**).

Class 10: Perfluorinated Pyrroles or Imidazoles, $[C_nF_{2n-3}N_2O]^-$, n=9-18

Finally, a homologous group of analytes was discovered by in-source fragmentation flagging for $[C_3F_5O]^-$ and $[C_3F_7]^-$. In all Tangxun Lake pooled fish liver samples, these flagging ions corresponded to a series of compounds belonging to the general molecular formula $C_nF_{2n-3}N_2O^-$ with 9-17 carbons (**Figure 4-12.1**, **Table 4-4**). Multiple isomers were observed for most homologues, and more than 40 analytes were detected in this untargeted class.

Deep structural elucidation was performed by MS^2, MS^3, and MS^4 for the early-eluting isomer of the smallest homologue (i.e. C_9, **Figure 4-12.2**a). Unlike any of the other classes characterized above, here radical loss, or radical formation, was commonly observed, indicating an aromatic moiety.[78-81] The DBE value of 3 does not allow a phenyl in the molecule, thereby indicating a 5-member heteroaromatic moiety (i.e. furan, pyrrole, pyrazole, imidazole, oxazole and isoxazole). The observed loss of $\bullet OCF_3$ confirmed that the oxygen was not in the ring, therefore excluding furans, oxazoles and isoxazoles. Further, azo-compounds (-N=N-) can lose N_2 in negative mode,[82] an and the absence of such fragmentation narrowed down the possible core structures to either pyrroles or imidazoles.

The loss of $\bullet C_4F_9$ (i.e. *m/z*=436.98 → 217.99) and of C_4F_{10} (*m/z*=436.98 → 198.99) suggested a C_4F_9 substituent; based on the analogy that $\bullet CH_3$ and CH_4 losses are observed for isopropyl-substituted phenyls.[81] Losses of $\bullet CF_3$ (*m/z*=436.98 → 367.97 and 217.99 →149.00), of $\bullet OCF_3$ (*m/z*=217.99 → 133.00) and of $\bullet CF_3$+CO (*m/z*=217.99 → 121.00) indicated a CF_3O substituent; based on analogy that CH_3O substituted aromatic compounds lose $\bullet CH_3$, $\bullet OCH_3$[82-84] and ($\bullet CH_3$+CO).[81, 82, 84, 85] Given these two likely substituents, the possible structures would either be $(CF_3O)(C_4F_9)(NF_2)$-pyrrole or $(CF_3O)(C_4F_9)(CF_3)$-imidazole, and the fragmentation pathways for both structures are proposed in **Figure 4-12.2**b. The structure for this class was therefore proposed as perfluorinated pyrroles or imidazoles, CL=3 (**Table 4-4**).

It is noticeable that isomers for each homologue eluted as two clusters, with one cluster eluting much later (e.g. 1st C_{12} isomer cluster eluting 16.98-19.85 min, 2nd cluster eluting 22.87-24.43 min, **Figure 4-17.1**). To examine structural differences between the two clusters, MS^2 - MS^5 analyses were performed for all C_{12} isomers, which were the most abundant homologue, and similar fragmentation patterns were observed (**Figure 4-17.3**). The very different retention times for the two clusters may be due to different core structures (e.g. pyrrole vs imidazole), or that one cluster or both clusters were in-source fragments from larger precursors. However, no precursor

homologues were discovered.

The estimated concentrations for analytes in this class, against a PFOS calibration curve, were usually lower than 1 ng/g in most fish (**Table 4-3**). However, the dominant C_{12} homologue was present at concentrations in the range of 3.8-208 ng/g (8-28% of the PFOS concentration in the same sample). Among all fish species, highest concentrations were found in bream fish.

We are not aware of any previous reports of perfluorinated pyrroles or imidazoles. Fluoropyrrole derivatives have been used in pharmaceutical applications (e.g. anti-inflammatory agents)[86-88] and as insecticides (e.g. chlorofenapyr[89]), and fluorinated imidazoles are useful reagents in organic chemistry,[90] but all these examples are not highly fluorinated. Perfluoroalkyl imidazoles are potent angiotensin II antagonists,[91] but no such commercialized drug is known.

4.4 Significance

The in-source fragmentation flagging method,[42] first developed for water analysis, was shown here as a powerful tool for unknown PFAS discovery in a complex biological matrix. The method has the potential to be used for discovering any class of compound that fragments to produce characteristic ions in the source. Here, in addition to perfluoroalkyl (e.g. $[C_2F_5]^-$) and perfluoroalkene fragments (e.g. $[C_3F_5]^-$), perfluoroether (e.g. $[C_2F_5O]^-$, $[C_3F_5O]^-$ and $[C_3F_5O_2]^-$), perfluoroamine (e.g. $[C_5F_{12}N]^-$ and $[C_8F_{16}N]^-$), chlorine ($[^{35}Cl]^-$ and $[^{37}Cl]^-$) and S-containing fragments ($[SO_3]^-$, $[SO_3F]^-$ and $[SO_3F]^-$) were used in the current study to flag and characterize unknown PFASs. As shown for most classes of substances discovered here, the method benefits tremendously from liquid chromatography to separate any isomeric homologues prior to structural elucidation. In this way, a much greater number of compounds were discovered relative to what would have been observed by direct infusion of the same extracts. Moreover, structural elucidation would have proven more difficult, and likely inaccurate, without chromatographic separation.

In total, more than 330 non-target PFAS analytes were detected in fish livers from the two locations. These belonged to 10 unique classes of PFASs. Except for 6:2 Cl-PFASE[29] (**Class 2**) and 4-PFECHS[58, 60] (**Class 3**), all non-target analytes are reported here in fish for the first time.

Considering all previous studies of PFASs in human, environmental or industrial and commercial standards, more than 165 non-target analytes belonging to 4 PFAS classes are

reported here for the first time, including **Class 5** diether-PFSAs, **Class 8** perfluoroalkyl amine carboxyl esters, **Class 9** unsaturated perfluoroalkyl amine carboxylates/ carboxyl esters, and **Class 10** perfluoroalkyl pyrroles or imidazoles. For the 6 PFAS classes that were previously reported in other samples or materials, new homologues and isomers were detected in the current study, including C_{13} - C_{15} Cl-PFCAs (**Class 1**, 3 analytes), 5:2, 7:2 and 12:2 Cl-PFAESs (**Class 2**, 3 analytes), C_{14} cyclic or unsaturated PFSAs (**Class 3**, >2 isomers), C_4 - C_5 and C_{10} - C_{12} monoether-PFSAs (**Class 4**, >15 analytes), C_{10} - C_{13} enol-ether-, cyclic-ether- or carbonyl-PFSAs (**Class 6**, ~30 analytes) and C_6 and C_8 - C_{13} diether-unsaturated-, cyclic-ether- or carbonyl-ether-PFSAs (**Class 7**, ~30 analytes).

The number of carbon atoms (e.g. chain-length) detected in the PFASs in the current study ranged from C_4 to C_{18}. Among the 73 homologues, 61 homologues had at least 8 carbons, showing the dominance of long-chain PFASs in these fish. This is not likely biased by the analytical method which was based on extraction using weak anion exchange, and the HPLC method was capable of retaining PFASs as short as 4 carbons (e.g. PFBA and PFBS was detected). The few number of shorter-chain PFSAs detected is most likely attributable to their lower bioaccumulation potentials, or that very few short-chain PFASs were, or are, emitted in these two regions of China.

The semi-quantified concentration for most of the 73 homologues were low (<1 ng/g, **Table 4-3**). However, some long-chain homologues showed very high concentrations (e.g. **Class 8** in Tangxun Lake bream fish). The total concentration for each PFAS class was calculated by summing up all homologues within the same class (**Figure 4-2**). In the Yangtze River fish, **Class 1** Cl-PFCAs and **Class 2** x:2 Cl-PFAESs were generally present at comparable levels in each fish species. In fish from Tangxun Lake, **Class 4** monoether-PFSAs, **Class 8** perfluoroalkyl amine carboxyl esters and **Class 10** perfluoroalkyl N-heterocycles were the three dominant PFAS classes, present at >10 ng/g in most samples. It is also noteworthy that, among the four fish species in Tangxun Lake, bream fish showed much higher concentrations for all detected legacy and most non-target PFASs than other species. This is possibly attributable to different feeding habits (omnivorous for bream *vs* filter feeder for silver carp and bighead carp).

Compared to the legacy PFASs, the newly identified PFASs have mono-chlorine substitution, double-bonds or rings, ether bonds, carbonyl or amine functional groups. Among

these, only ketone (i.e. carbonyl) and ether PFASs are known to have been produced intentionally in recent years as alternative PFASs.[19] However, ether PFSAs (and maybe carbonyl-PFSA) were also detected here as byproducts in historical 3M-PFOS. It is therefore difficult to know if any of the non-target PFASs detected here are from historical or contemporary production and emission. Particularly for Tanxun Lake, it would seem prudent to examine current wastewater releases for evidence of the many PFASs identified here. This information may help to minimize future exposure around this contaminated water body. Similarly, the WWTP plant upstream of where the Yangtze River fish were collected is also a point source to be monitored, as the **Class 2** Cl-PFCAs were previously detected in wastewater there.

Between the two sites examined, there was a clear difference in the types of PFASs detected in fish livers. With the exception of **Class 2** x:2 Cl-PFAESs, which were detected in both locations, 8 PFAS classes were exclusively discovered at Tangxun Lake, and 1 class was only detected in the Yangtze River (**Figure 4-2**). This most likely indicates that the sources of contamination were from unique point sources (i.e. wastewater from different fluorochemical industries), but it should also be kept in mind that we are comparing fish from a lake to fish from a river, where residence times, dilution and primary productivity are different. Future monitoring of water from more regions of China are warranted to understand the true spatial distribution and sources of all chemicals identified here.

The discovery of more than 330 non-target PFASs, predominantly long-chain PFASs, in fish demonstrates bioavailability of all these compounds, and is suggestive of a measurable bioaccumulation potential for these compounds whose environmental fate has not been previously studied. Given that all fish livers were sampled from fish destined for fish markets and likely widespread human consumption, the current findings raise immediate questions about health risks to exposed people.

4.5 Figures and Tables

4.5.1 Figures

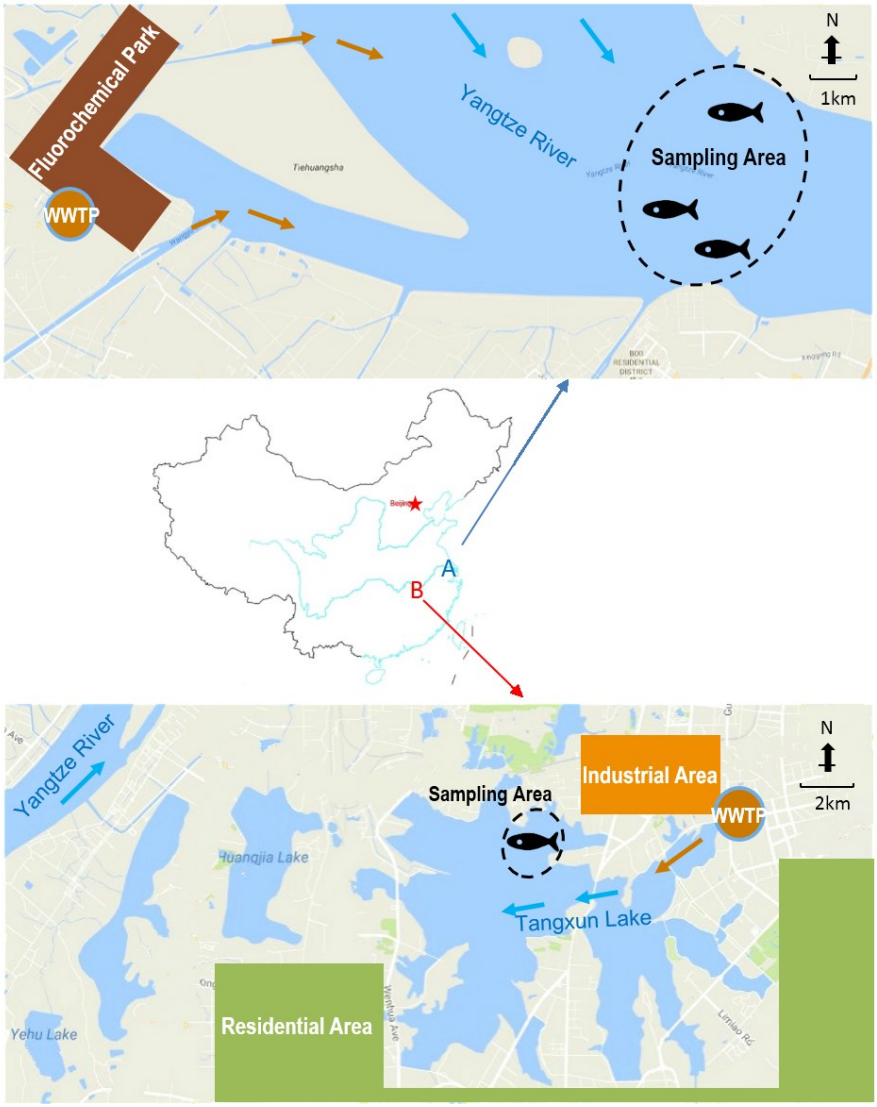


Figure 4-1. Fish collection sites at two locations in China: (**A**) Yangtze River in Changshu, Jiangsu. Here fishermen collected fish over a 30 km^2 region (black dashed lines) approximately 10 km downstream of a wastewater treatment plant (WWTP) discharge located in a fluorochemical manufacturing park (brown area); and (**B**) Tangxun Lake in Wuhan, Hubei, which receives wastewater discharge from a WWTP that processes both domestic (light green areas) and industrial wastewater (orange areas). Fishermen collected fish over an approximate 5 km^2 region (blacked dashed circle) in the lake.

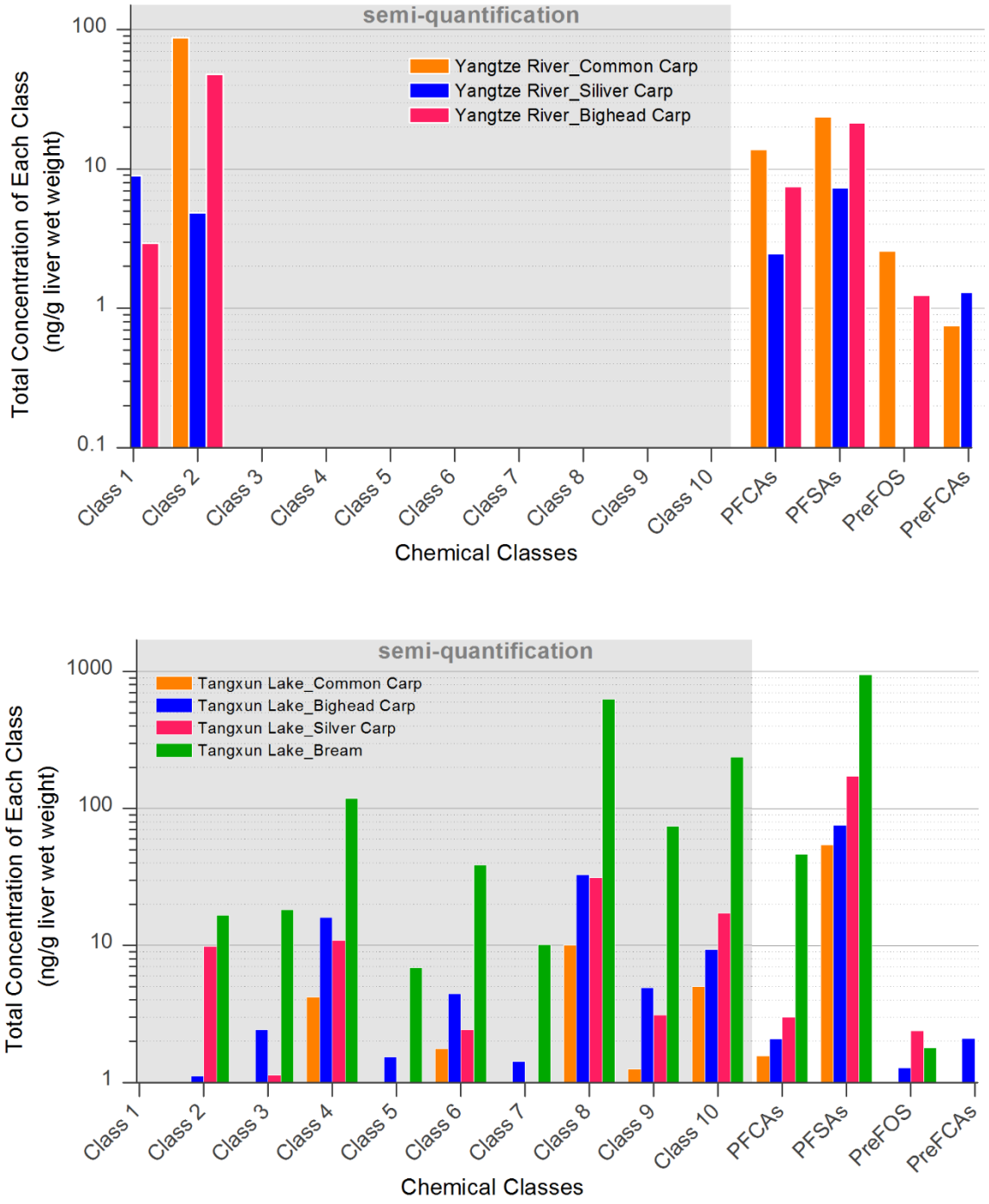


Figure 4-2. Total concentration of each PFAS class detected in pooled fish livers from the Yangtze River and Tangxun Lake (standard quantified PFAS: white background; semi-quantified PFAS: grey background). "PreFOS" are PFOS precursors, composed of PFOSA, MeFOSA and MeFOSAA, and "PreFCAs" are PFCA precursors, composed of 6:2 PAP and 8:2 PAP.

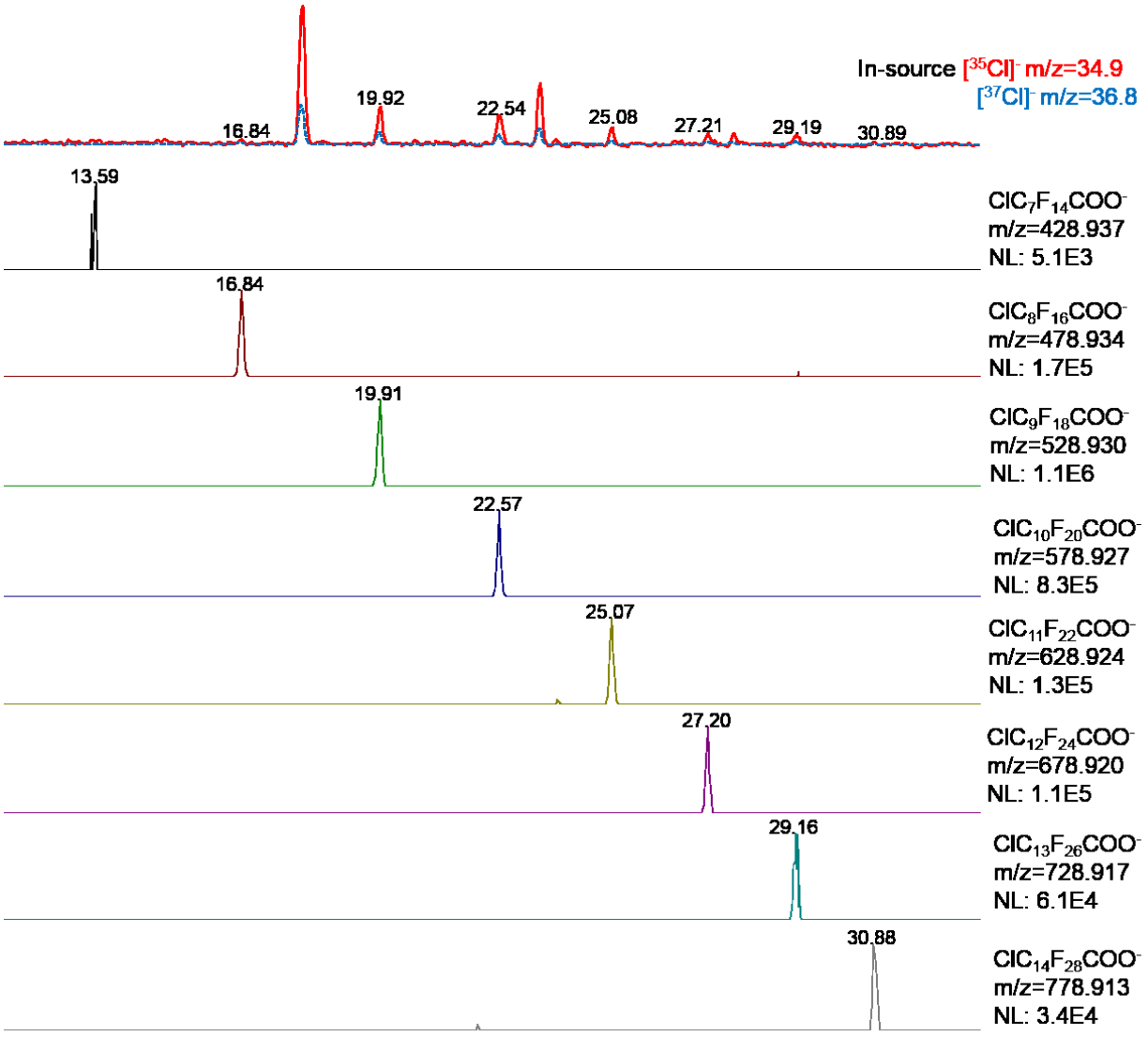


Figure 4-3.1. Extracted ion chromatograms of $[Cl]^-$ ($[^{35}Cl]^-$ in red and $[^{37}Cl]^-$ in blue) in-source fragments and **Class 1** Cl-PFCA ($ClC_{n-1}F_{2n-2}CO_2^-$) molecular ions detected in pooled common carp liver from the Yangtze River.

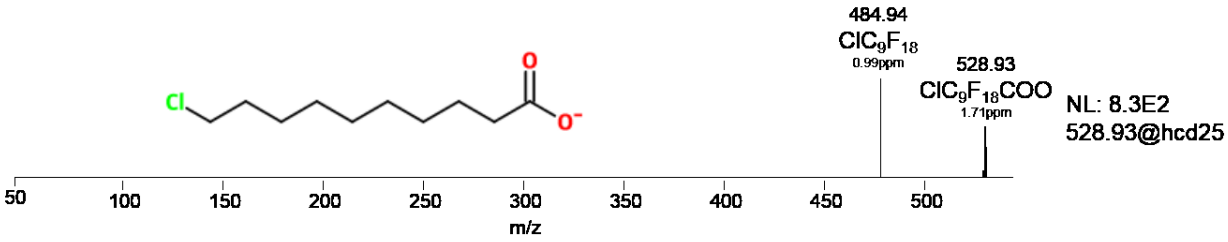


Figure 4-3.2. MS2 spectrum (HCD 25 eV) of C9-Cl-PFCA (**Class 1**, $ClC_9F_{18}CO_2^-$) in pooled common carp liver from the Yangtze River. A proposed structure is presented, F atoms not shown.

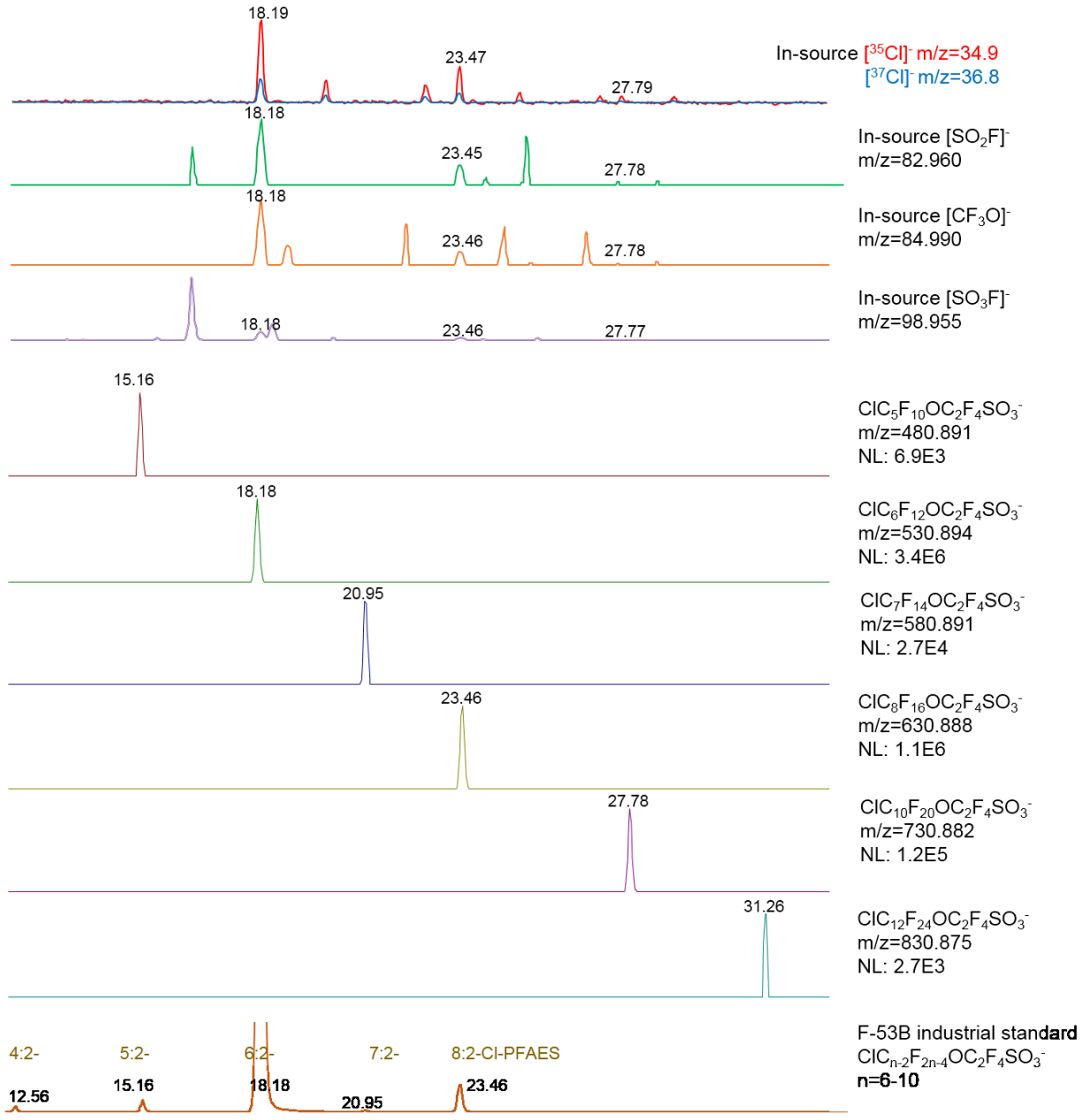


Figure 4-4.1. Extracted ion chromatograms of $[Cl]^-$, $[SO_2F]^-$, $[COF_3]^-$ and $[SO_3F]^-$ in-source fragments, and **Class 2** x:2 Cl-PFAES ($ClC_{n-2}F_{2n-4}OC_2F_4SO_3^-$) molecular ions detected in pooled common carp liver from the Yangtze River, and of x:2 Cl-PFAESs in F-53B industrial standard.

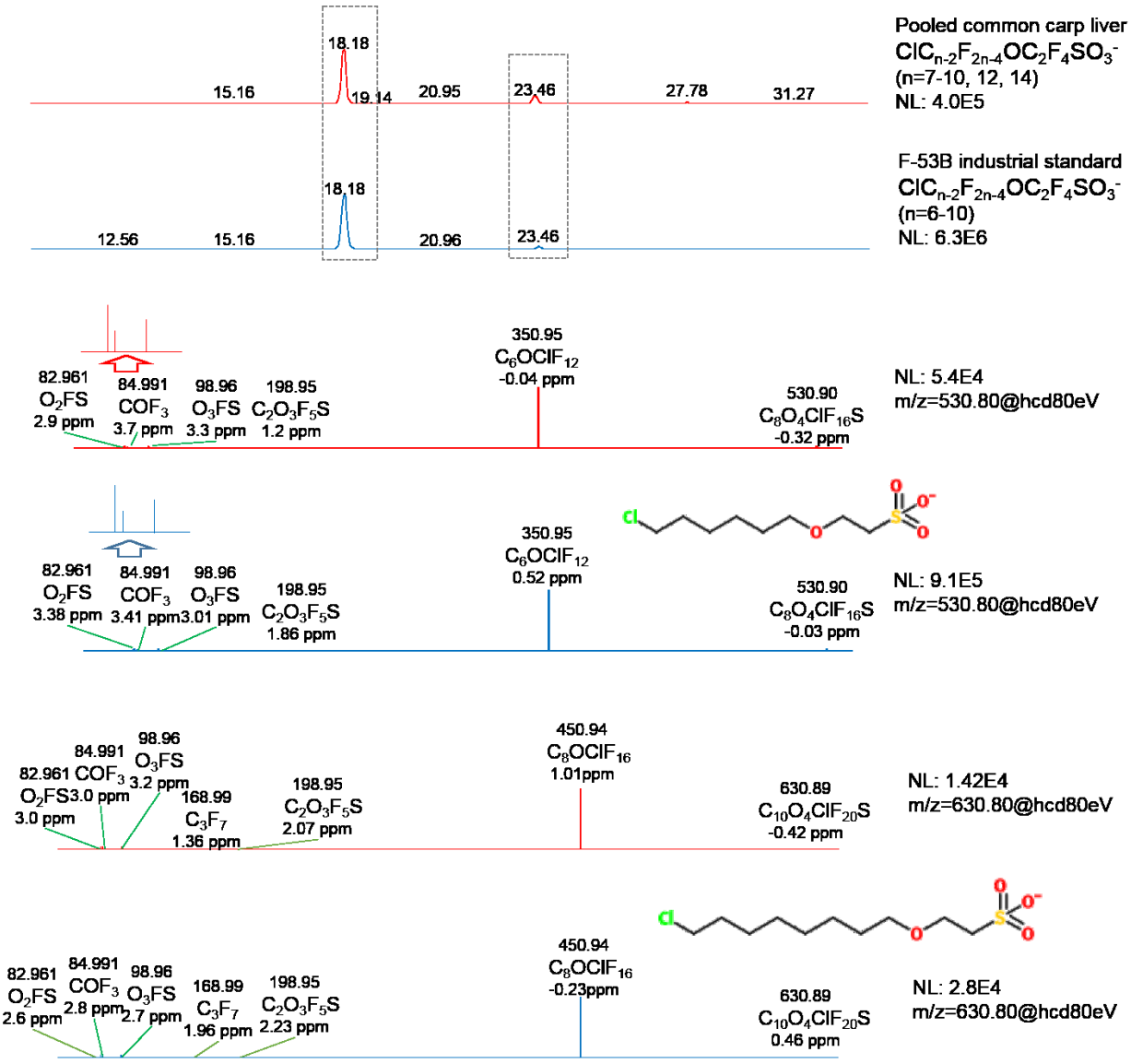


Figure 4-4.2. MS^2 spectra (HCD 80 eV) of C_8 ($ClC_8F_{16}SO_4^-$) and C_{10} x:2 Cl-PFAESs ($ClC_{10}F_{20}SO_4^-$, **Class 2**) in pooled common carp liver from Yangtze River, and in F-53B industrial standard.

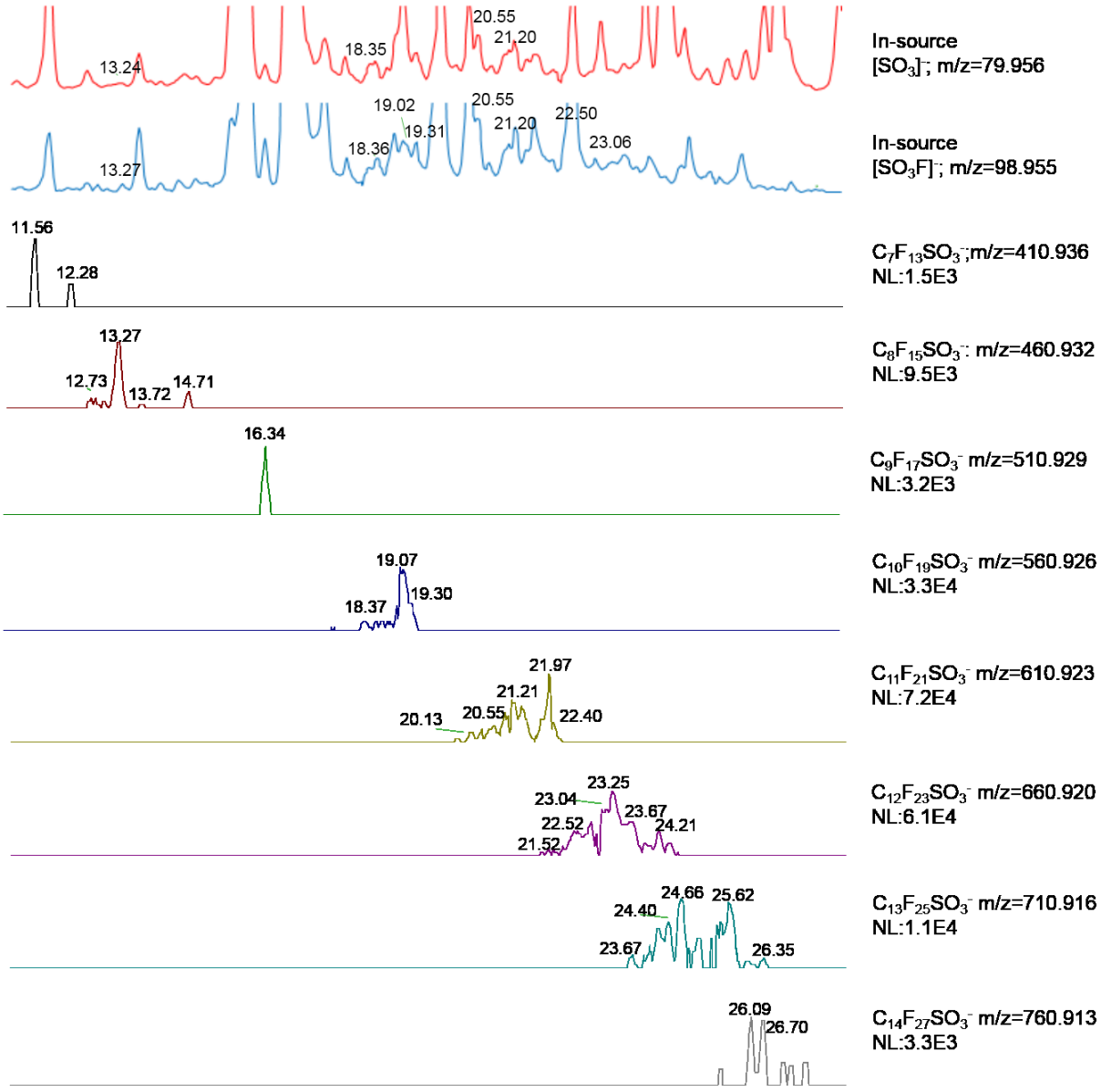


Figure 4-5.1. Extracted ion chromatograms of $[SO_3]^-$ and $[SO_3F]^-$ in-source fragments and **Class 3** cyclic or unsaturated PFSA ($C_nF_{2n-1}SO_3^-$) molecular ions detected in pooled bream liver from Tangxun Lake.

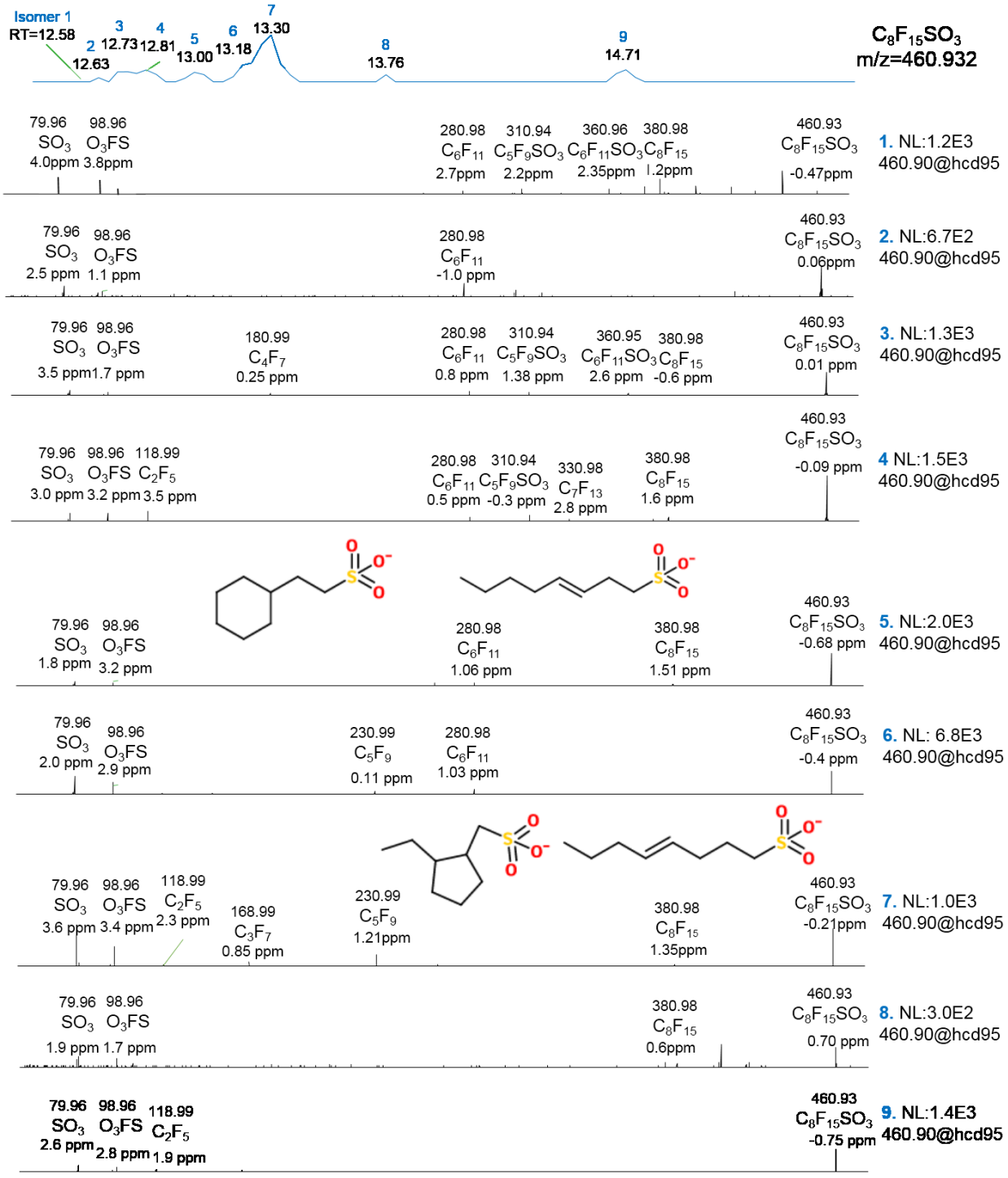


Figure 4-5.2. MS^2 spectra (HCD 95 eV) of C_8- cyclic or unsaturated PFSAs ($C_8F_{15}SO_3^-$, **Class 3**) in pooled bream fish liver from Tangxun Lake. Example structures are drawn for some isomers, F atoms not shown.

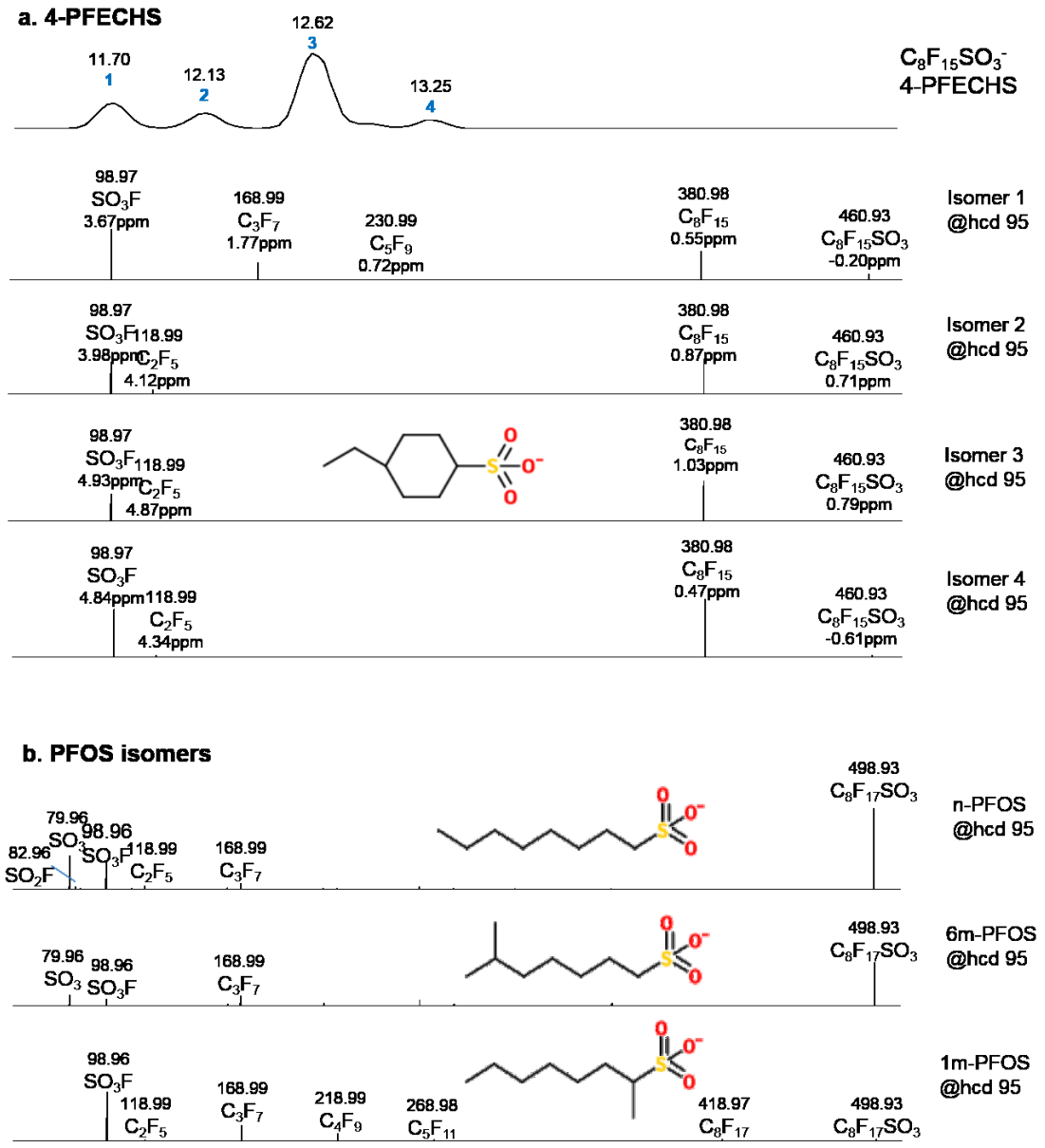


Figure 4-5.3. Extracted ion chromatogram and MS^2 spectra (HCD 95 eV) of isomers of 4-PFECHS standard (**a**), and MS^2 spectra of different PFOS isomer standards (**b**). 4-PFECHS isomer 3, being the most abundant peak, is probably 4-PFECHS.

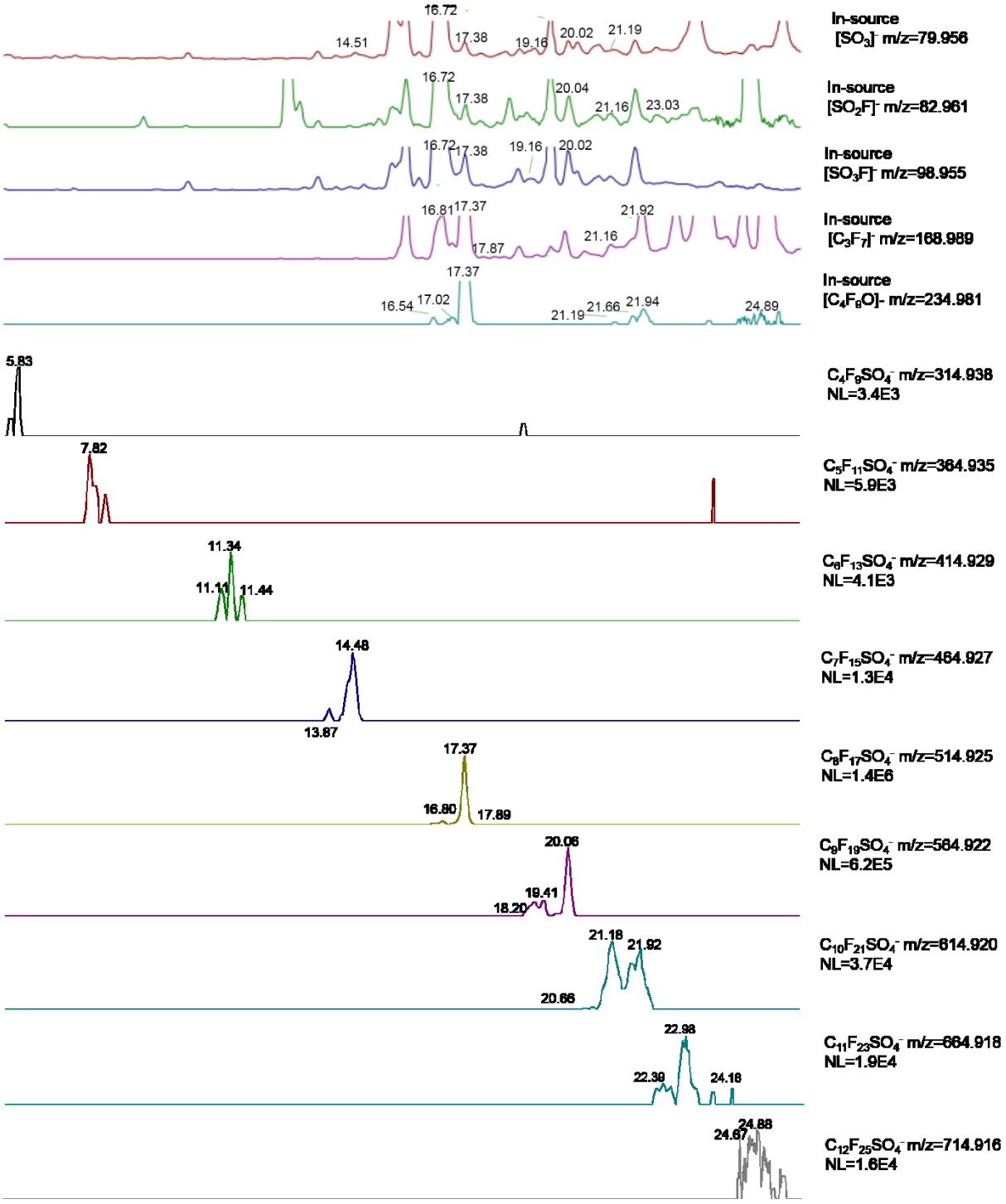


Figure 4-6.1. Extracted ion chromatograms of $[SO_3]^-$, $[SO_2F]^-$, $[SO_3F]^-$, $[C_3F_7]^-$ and $[C_4F_9O]^-$ in-source fragments, and **Class 4** monoether-PFSA ($C_nF_{2n+1}SO_4^-$) molecular ions detected in pooled bighead carp liver from Tangxun Lake.

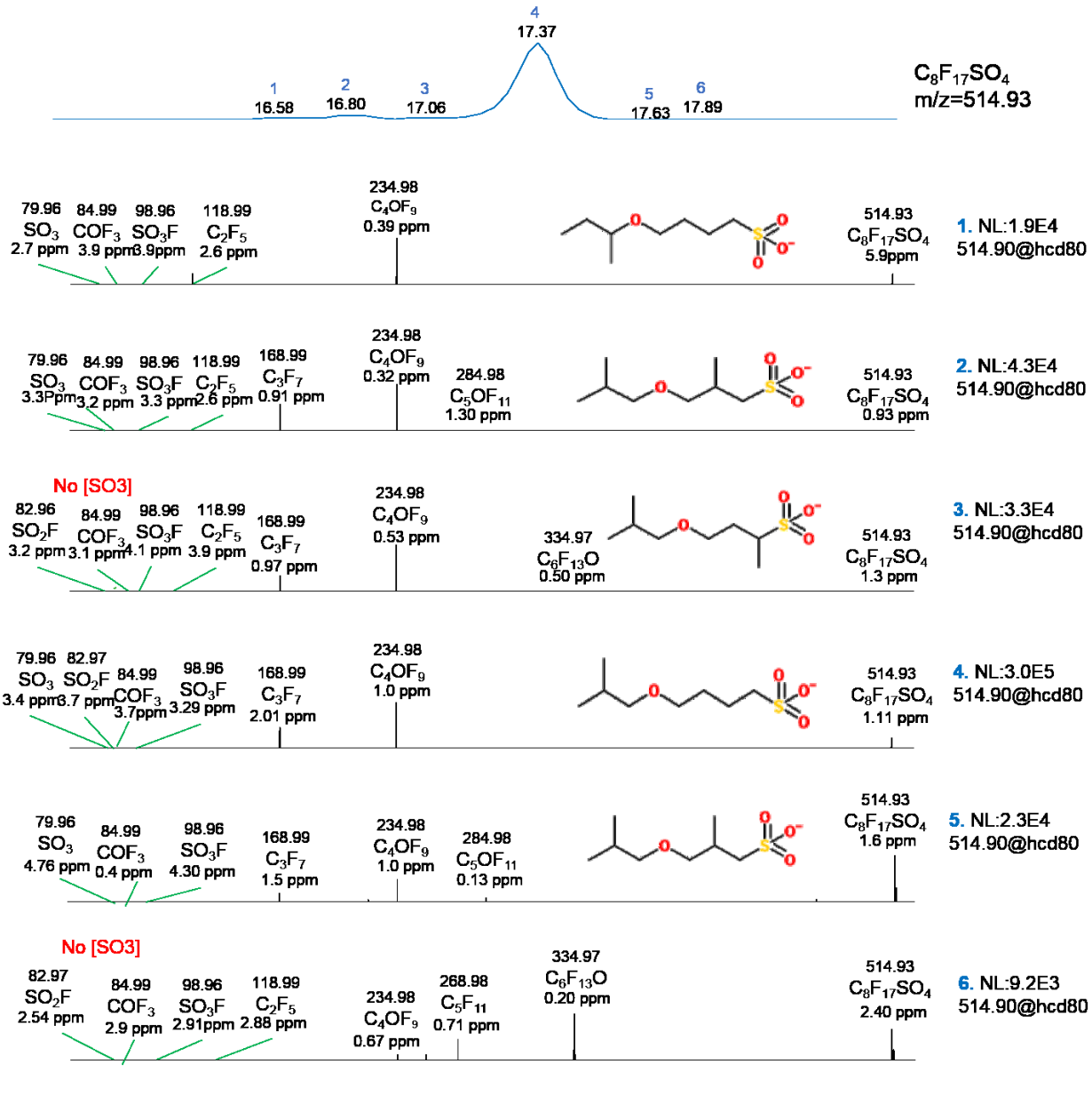


Figure 4-6.2. MS^2 spectra (HCD 80 eV) of all C_8-monoether-PFSA isomers ($C_8F_{17}SO_4^-$, **Class 4**) in pooled bighead fish liver from Tangxun Lake. Example structures are proposed, F atoms not shown.

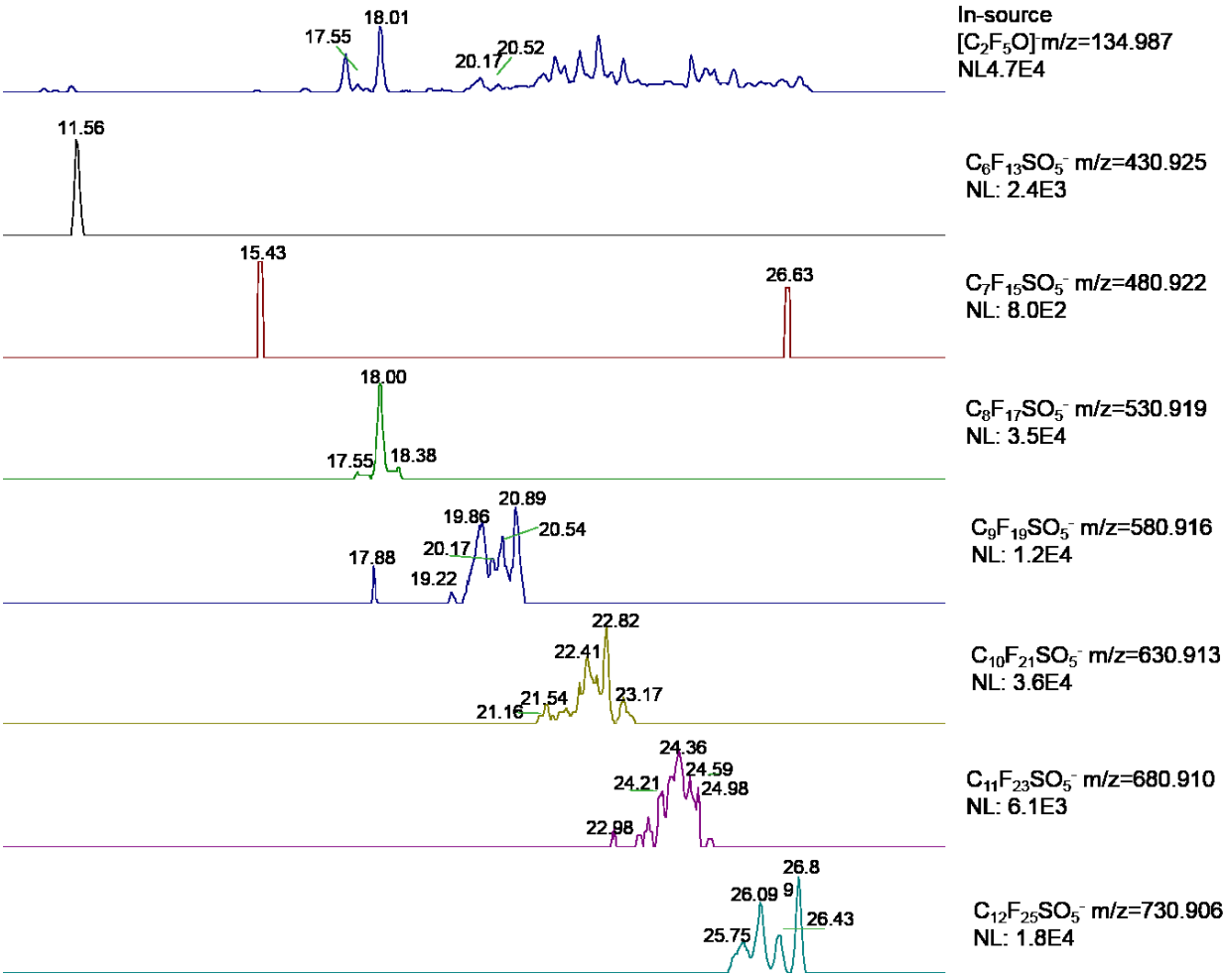


Figure 4-7.1. Extracted ion chromatograms of $[C_2F_5O]^-$ in-source fragments and **Class 5** diether-PFSA ($C_nF_{2n+1}SO_5^-$) molecular ions detected in pooled bream fish liver from Tangxun Lake.

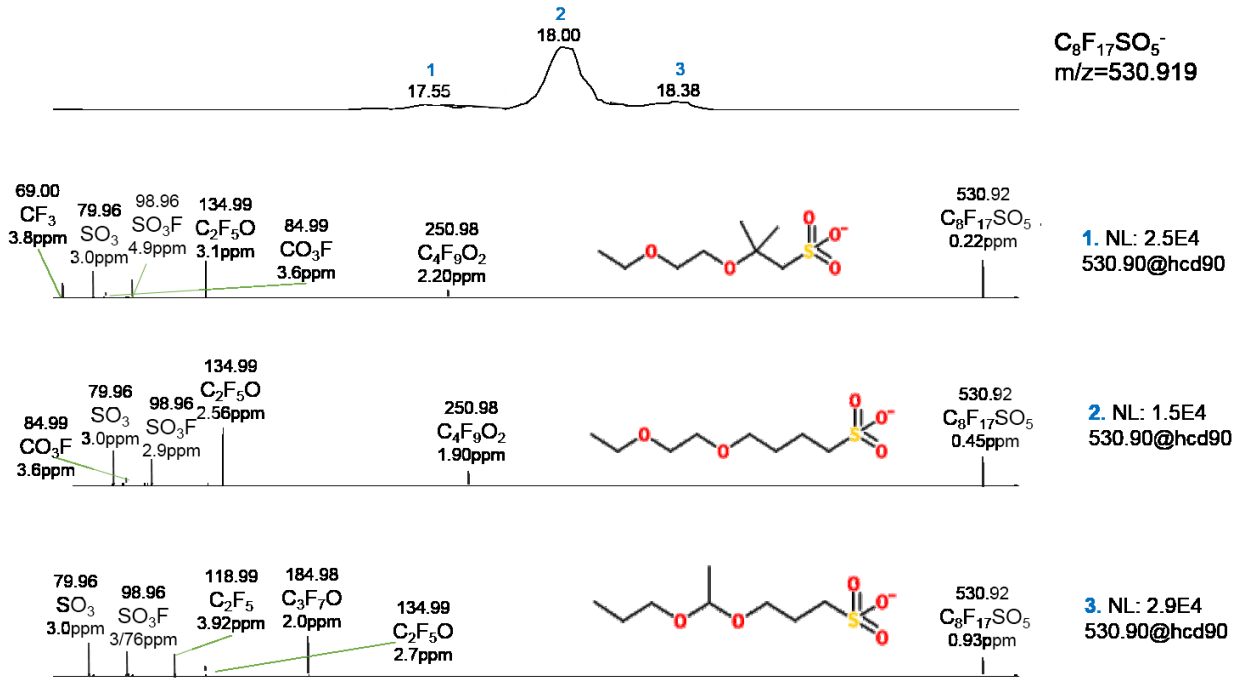


Figure 4-7.2. MS^2 spectra (HCD 90 eV) of selected C_8-diether-PFSA isomers ($C_8F_{17}SO_5^-$, **Class 5**) in pooled bream fish liver from Tangxun Lake. Example structures are drawn, F atoms not shown.

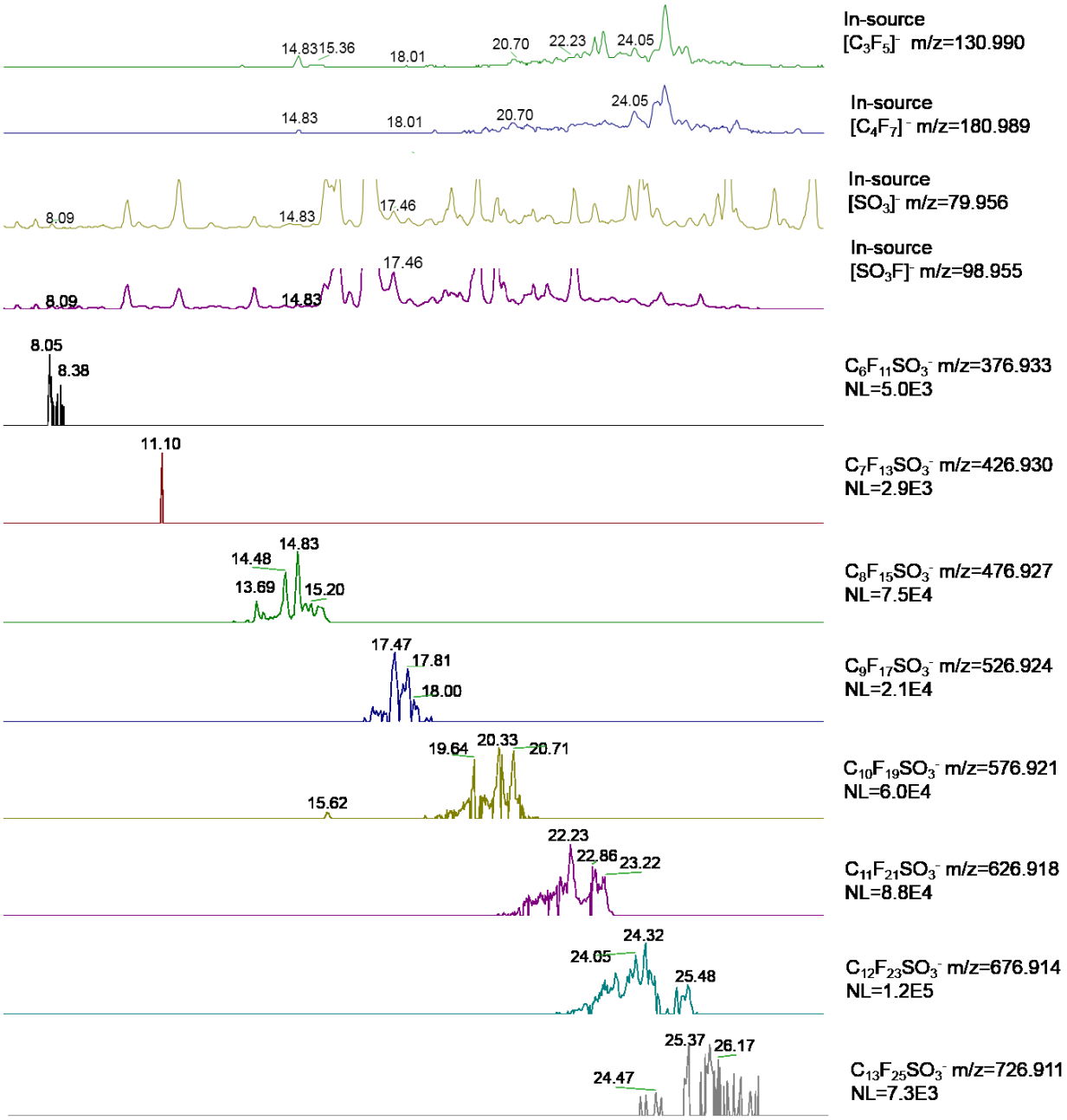


Figure 4-8.1. Extracted ion chromatograms of $[SO_3]^-$, $[SO_3F]^-$, $[C_3F_5]^-$ and $[C_4F_7]^-$ in-source fragments, and **Class 6** enol ether-, cyclic ether- or carbonyl-PFSA ($C_nF_{2n-1}SO_4^-$) molecular ions detected in pooled bream liver from Tangxun Lake.

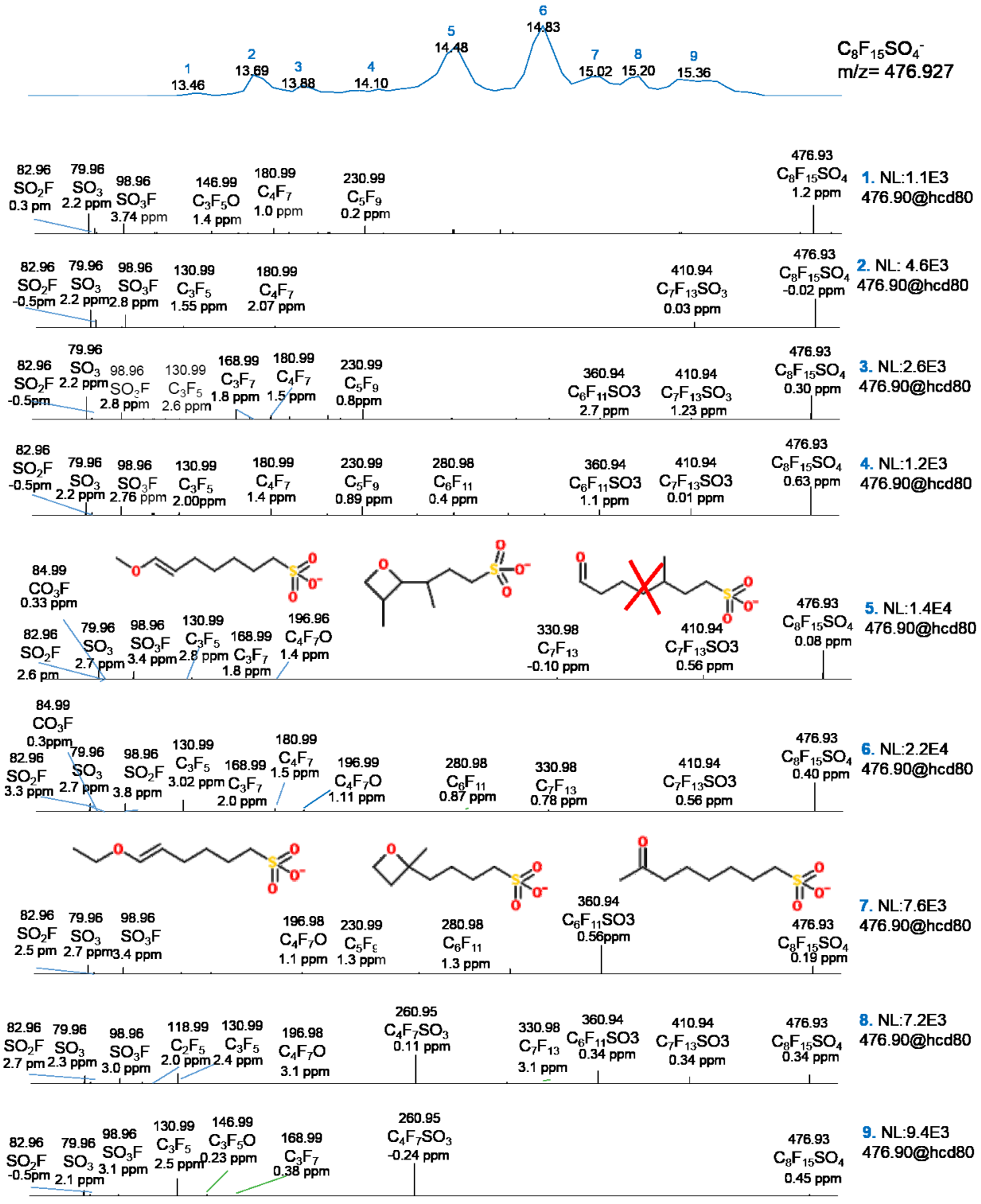


Figure 4-8.2. MS^2 spectra (HCD 80 eV) of all C_8- enol ether-, cyclic ether- or carbonyl-PFSA ($C_8F_{15}SO_4^-$, **Class 6**) isomers in pooled bream fish liver from Tangxun Lake. Example structures are proposed, F atoms not shown.

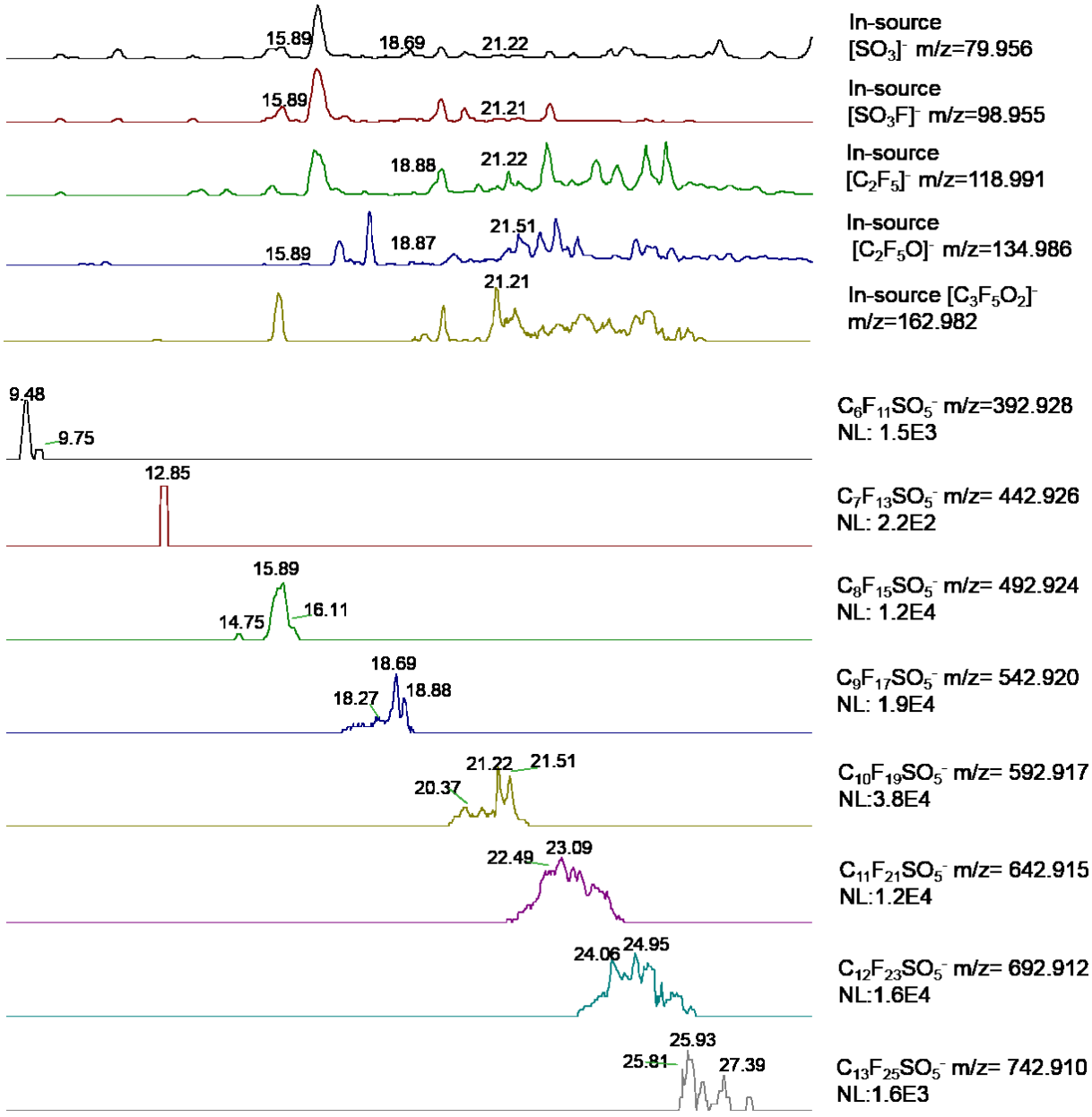


Figure 4-9.1. Extracted ion chromatograms of $[C_2F_5]^-$, $[C_2F_5O]^-$, $[C_3F_5O_2]^-$, $[SO_3]^-$ and $[SO_3F]^-$ in-source fragments, and **Class 7** diether-unsaturated-, cyclic-ether- or carbonyl-ether-PFSA ($C_nF_{2n-1}SO_5^-$) molecular ions detected in pooled bream fish liver from Tangxun Lake.

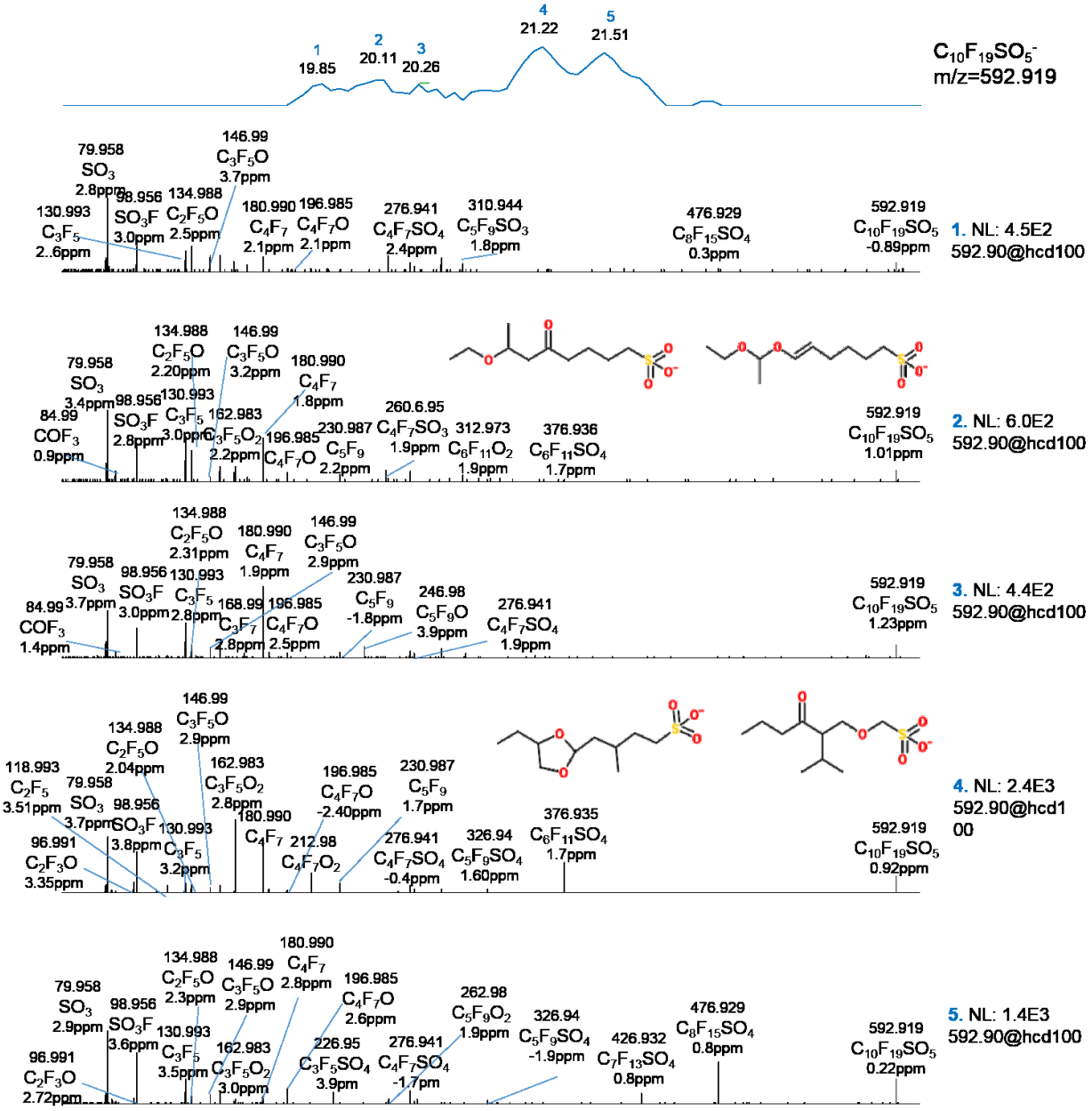


Figure 4-9.2. MS^2 spectra (HCD 100 eV) of all C_{10} diether-unsaturated-, cyclic-ether- or carbonyl-ether-PFSA isomers ($C_{10}F_{19}SO_5^-$, **Class 7**) in pooled silver carp liver from Tangxun Lake. Example structures are proposed for some isomers, F atoms not shown.

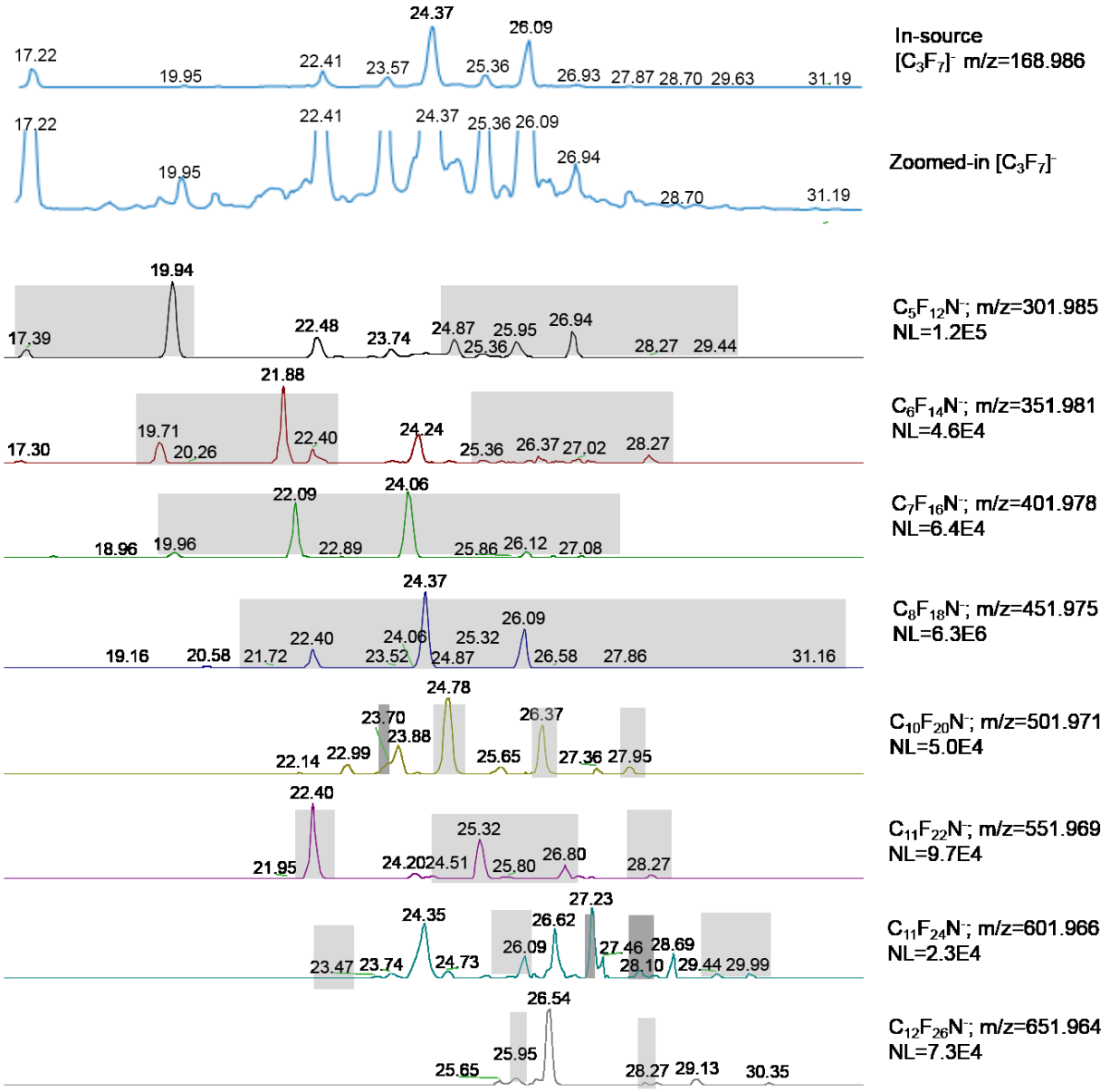


Figure 4-10.1. Extracted ion chromatograms of $[C_3F_7]^-$ in-source fragments, and perfluoroalkyl amine fragments ($C_nF_{2n+1}N^-$) detected in pooled bream fish liver from Tangxun Lake. All shaded peaks have a corresponding $C_nF_{2n}NO_2^-$ (**Figure 4-10.3**) peak at the same retention times.

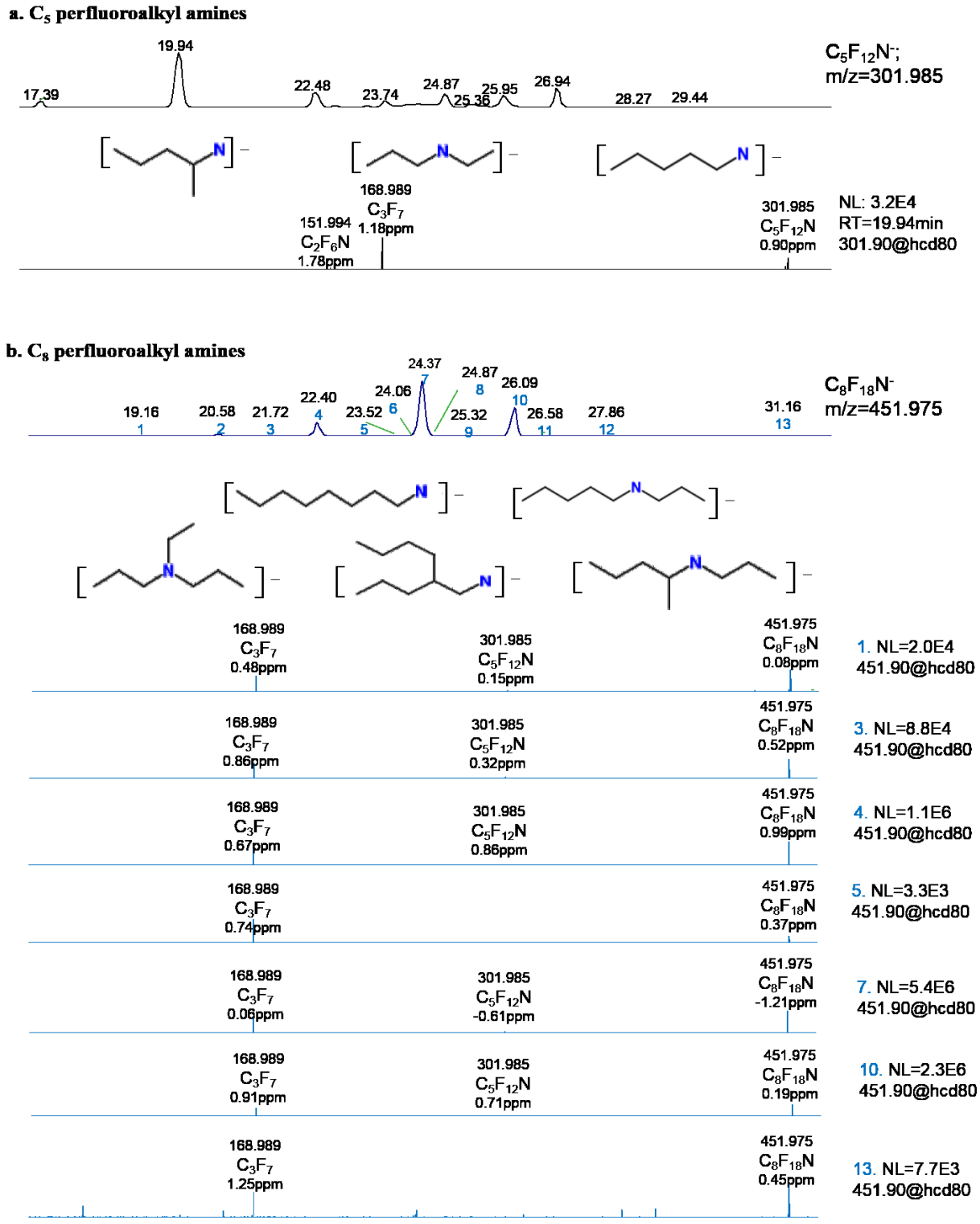


Figure 4-10.2. MS^2 spectra (HCD 80 eV) of a representative C_5 isomer ($C_5F_{12}N^-$, at retention time 19.94 min) and all C_8 isomers of perfluoroalkyl amine fragments ($C_8F_{18}N^-$) in bream liver from Tangxun Lake. Example structures are proposed, F atoms not shown.

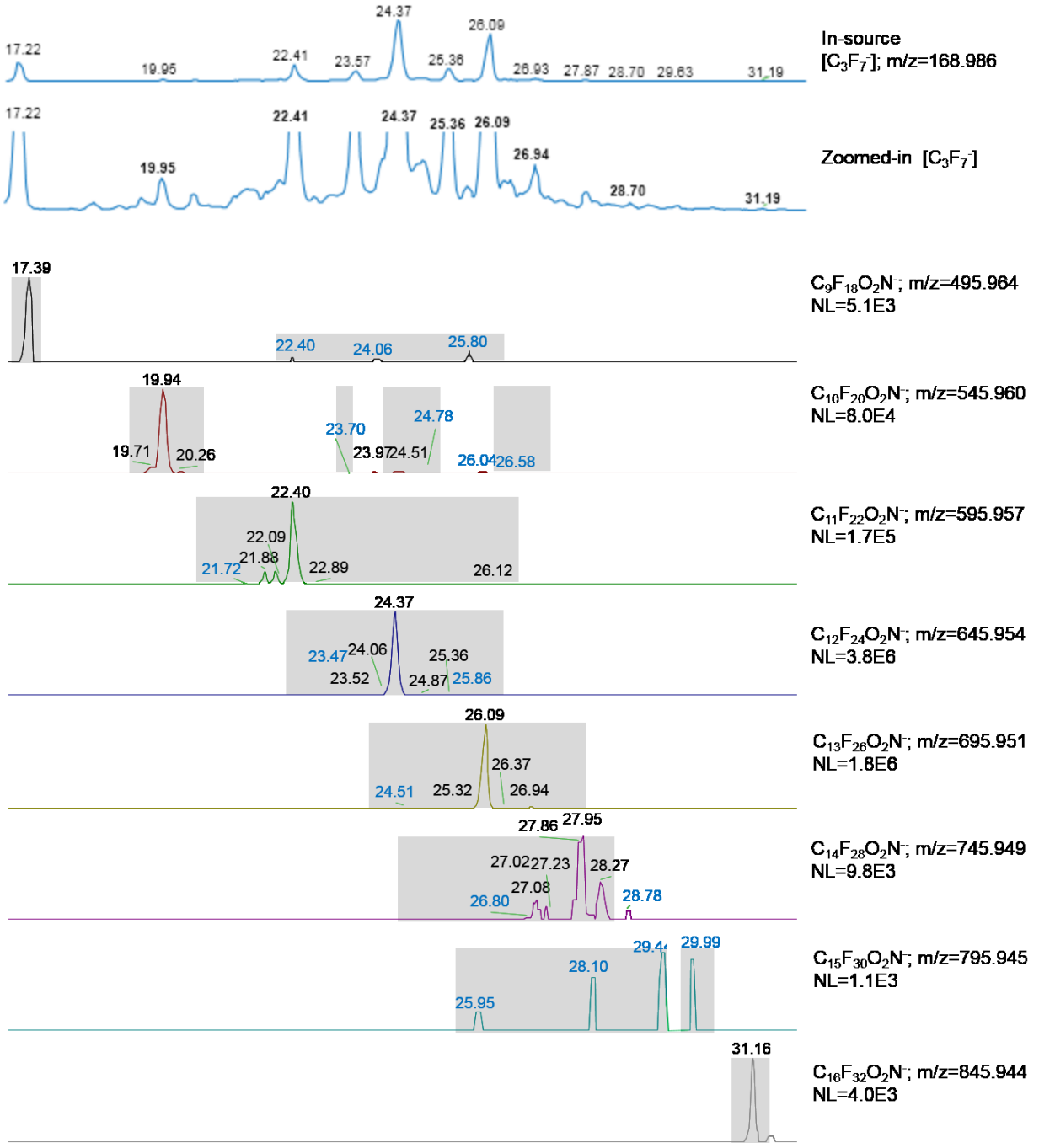


Figure 4-10.3. Extracted ion chromatograms of $[C_3F_7]^-$ in-source fragments, and perfluoroalkyl amine carboxylate or carboxyl ester fragments $[C_nF_{2n}O_2N]^-$ detected in pooled bream fish liver from Tangxun Lake. All shaded peaks have at least one corresponding $[C_nF_{2n+2}N]^-$ fragment peak (**Figure 4-10.1**) at the same retention times. Peaks with retention time labeled in blue represent minor analytes with peak height below 1000 counts.

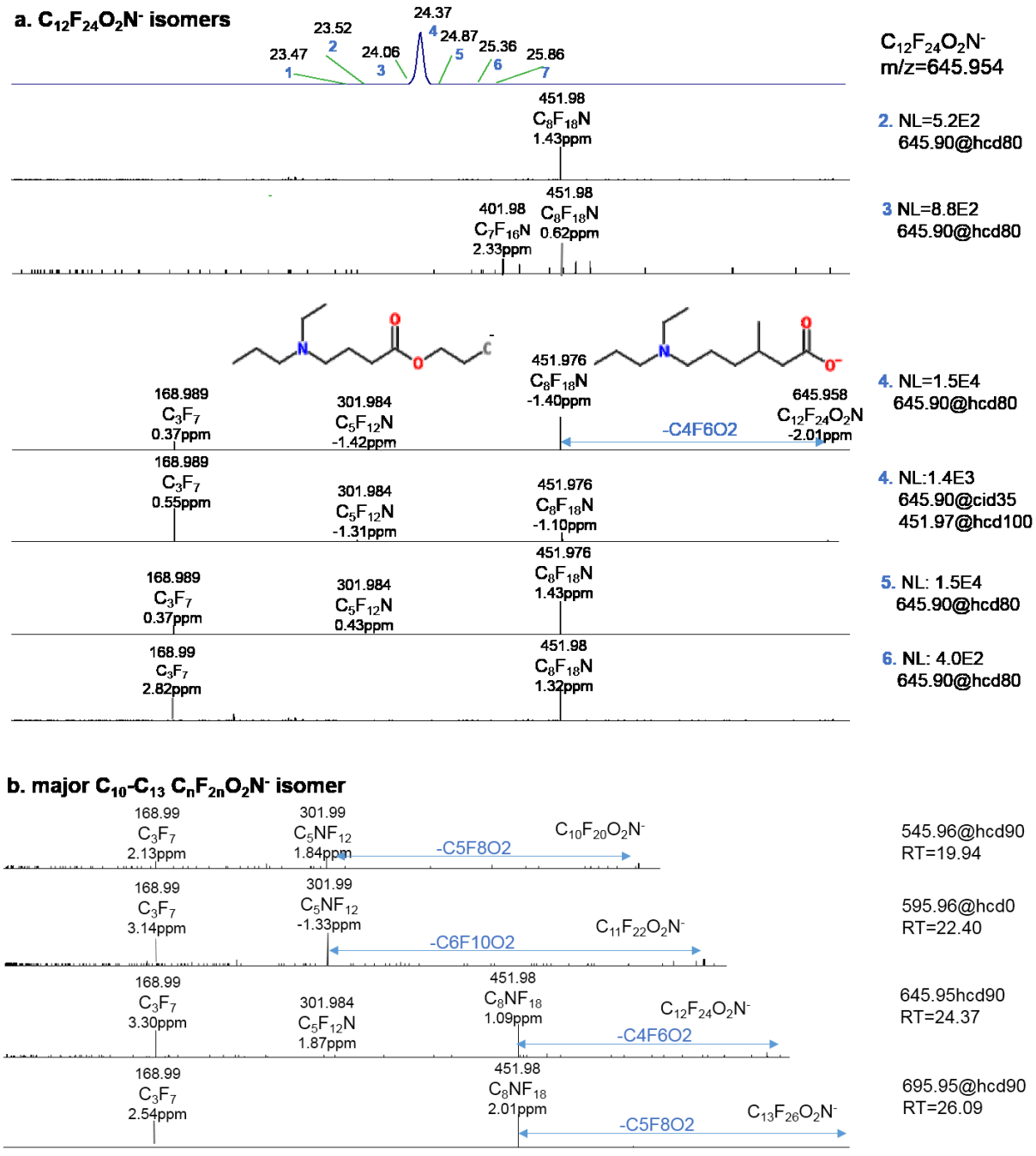


Figure 4-10.4. MS^2 spectra of all C_{12} isomers ($C_{12}F_{24}O_2N^-$, HCD 80e eV, **a**), and the major C_{10}- ($C_{10}F_{20}O_2N^-$, 19.94 min), C_{11}- ($C_{11}F_{22}O_2N^-$, 22.40 min) and C_{13}- ($C_{13}F_{26}O_2N^-$, 26.09 min) isomers ($C_nF_{2n}O_2N^-$, HCD 90eV, **b**) of perfluoroalkyl amine carboxylate or carboxyl ester fragments ($C_nF_{2n}O_2N^-$) in bream fish liver from Tangxun Lake. Example structures for the major $C_{12}F_{24}O_2N^-$ isomer are presented, F atoms not shown.

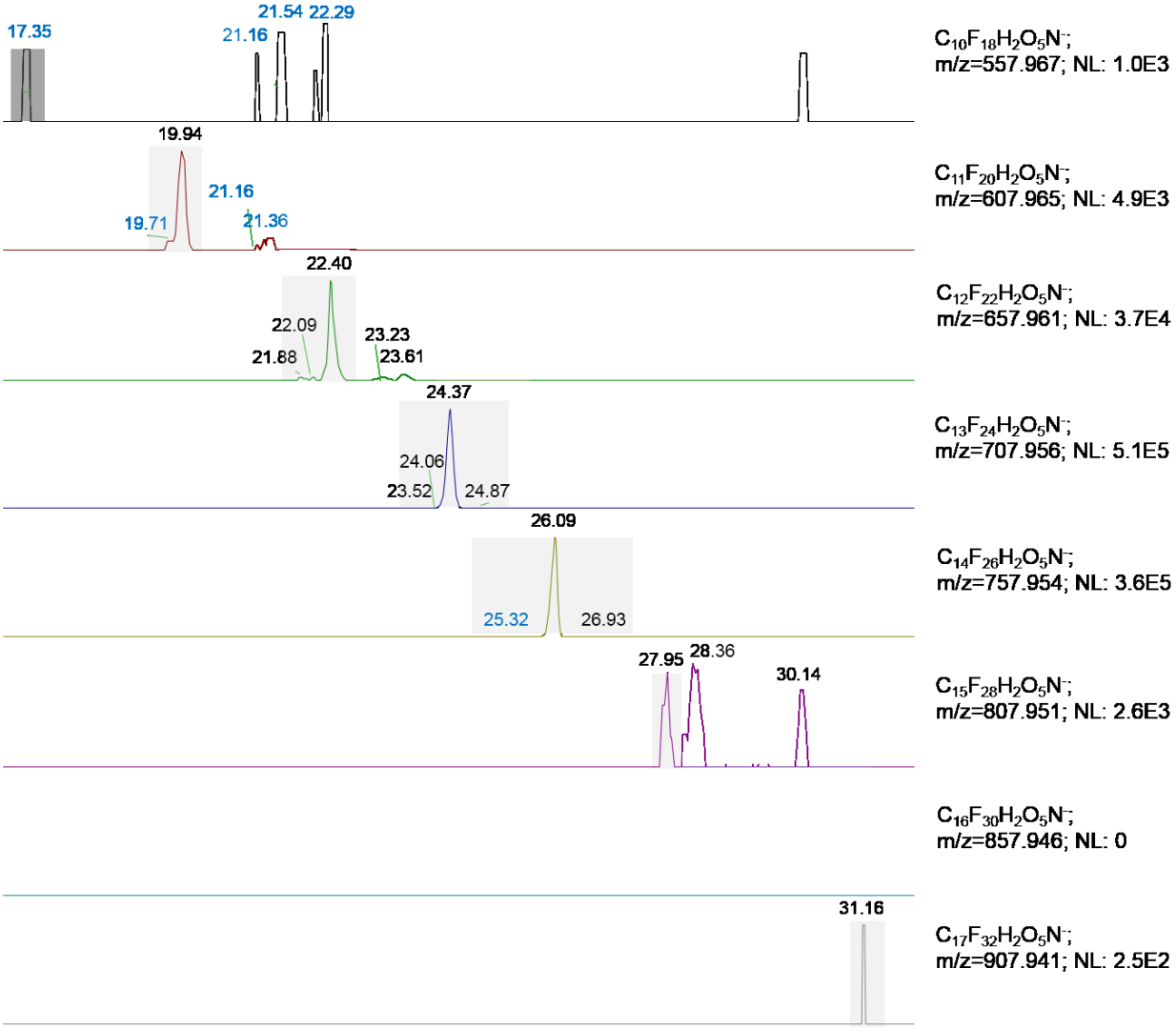


Figure 4-10.5. Extracted ion chromatograms of perfluoroalkyl amine carboxyl ester fragment ions ($C_nF_{2n-2}H_2O_5N^-$) detected in pooled bream fish liver from Tangxun Lake. All shaded peaks have at least one corresponding $C_nF_{2n}O_2N^-$ fragment peak (**Figure 4-10.3**) at the same retention times. Peaks with retention time labeled in blue represent minor analytes with peak height <1000 counts.

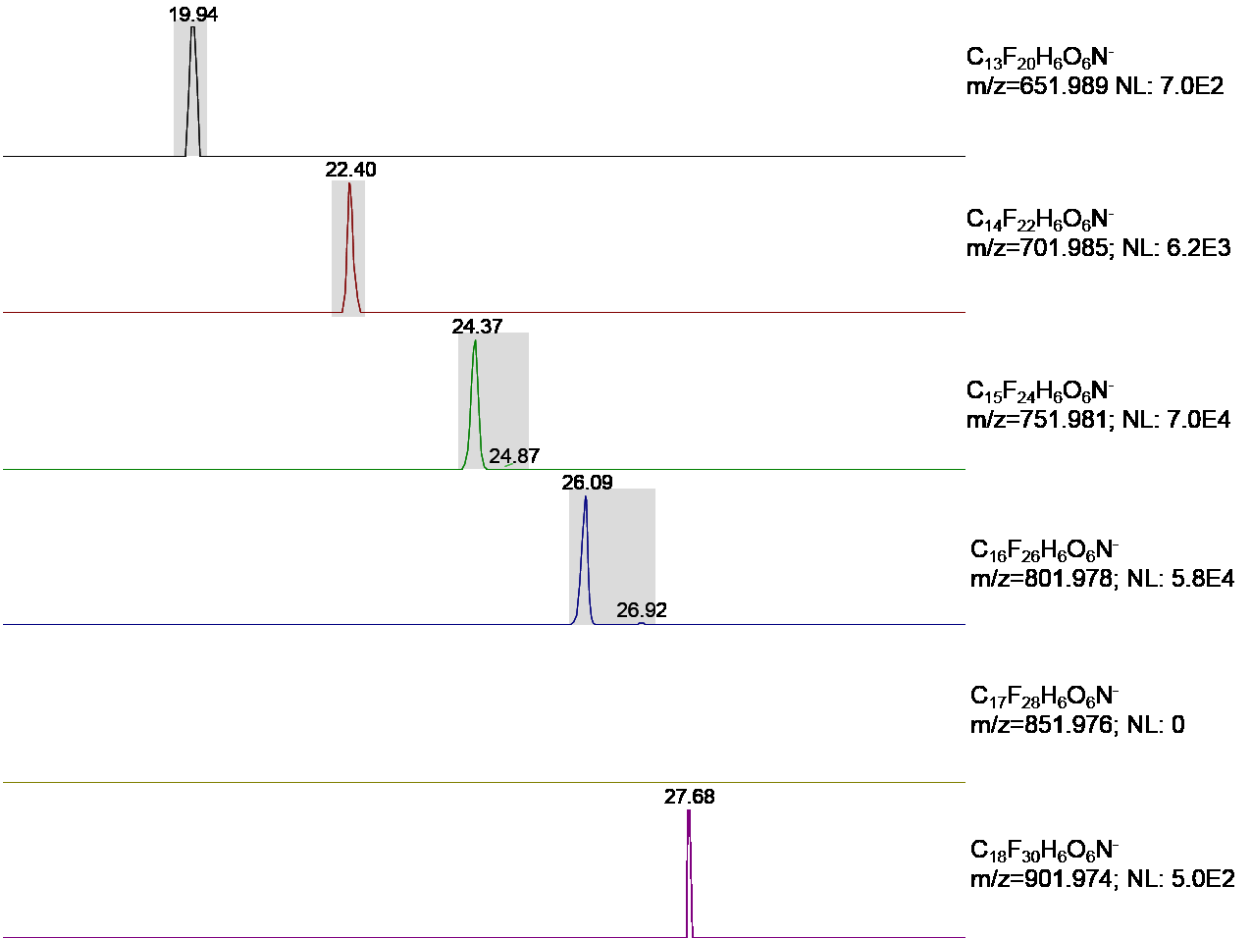


Figure 4-10.6. Extracted ion chromatograms of perfluoroalkyl amine carboxyl ester molecular ions ($C_nF_{2n-6}H_6O_6N^-$, **Class 8**) detected in pooled bream fish liver from Tangxun Lake. All shaded peaks have at least one corresponding $C_nF_{2n-2}H_2O_5N^-$ fragment peak (**Figure 4-10.5**) at the same retention times.

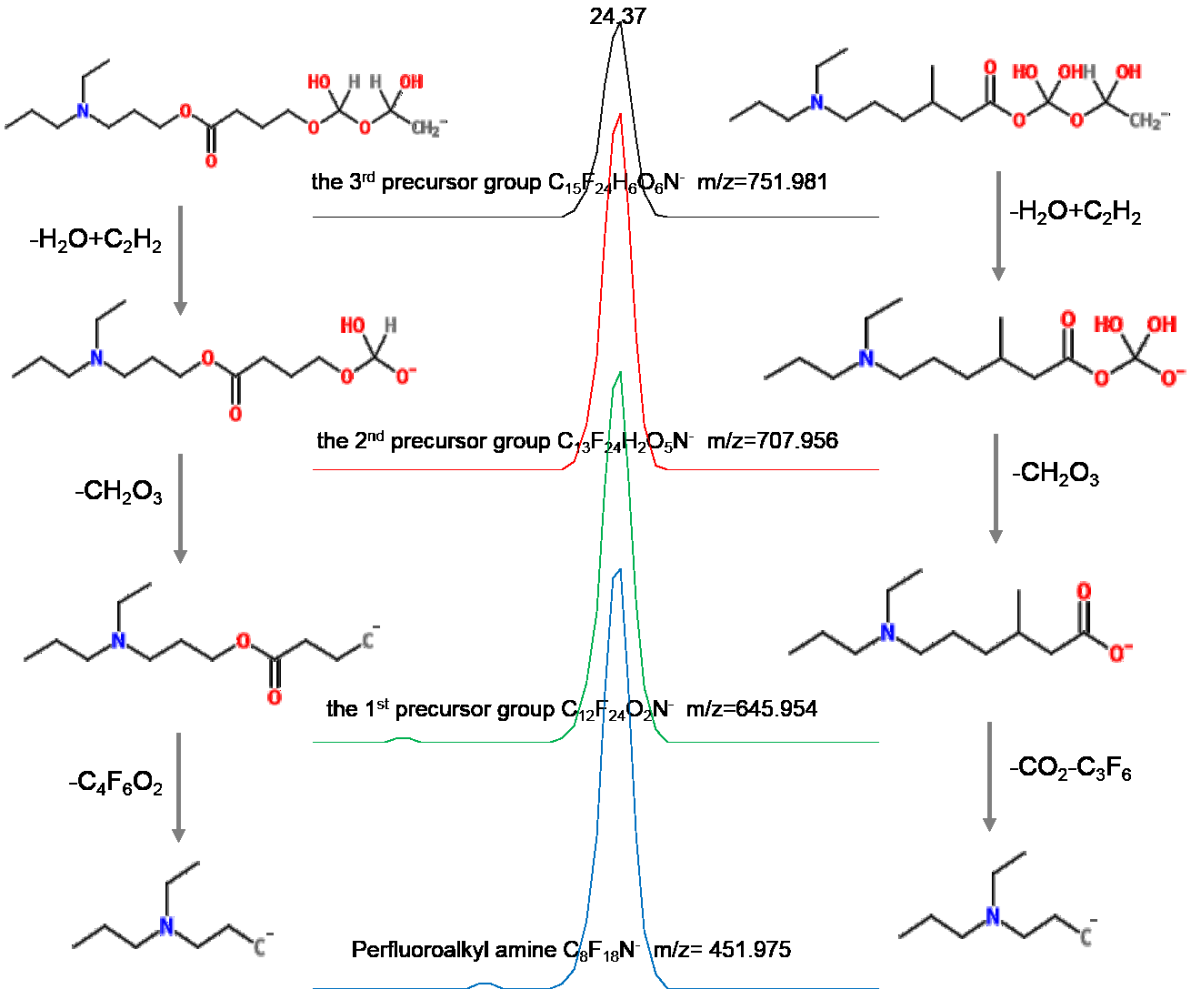


Figure 4-10.7. Extracted ion chromatograms of a C_{15} perfluoroalkyl amine carboxyl ester ($C_{15}F_{24}H_6O_6N^-$, **Class 8**) and its sequential fragments $[C_{13}F_{24}H_2O_5N]^-$, $[C_{12}F_{24}O_2N]^-$ and $[C_8F_{18}N]^-$, eluting at 24.37 min in pooled bream liver from Tangxun Lake. Example structures for each analyte are presented, and possible fragmentation pathways among them are proposed. Depending on two possible structures for $C_{12}F_{24}O_2N^-$, two pathways are presented.

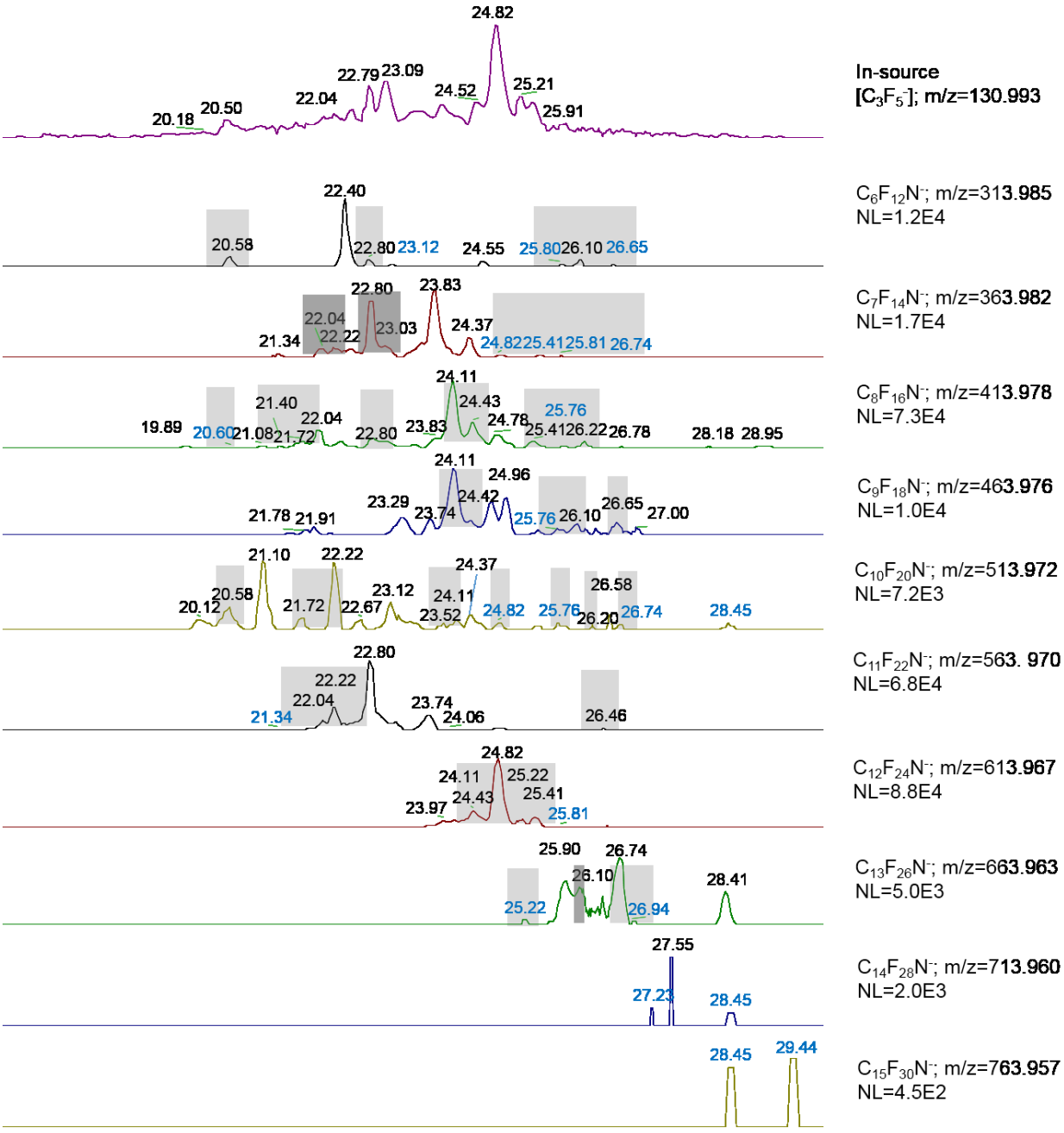


Figure 4-11.1 Extracted ion chromatograms of $[C_3F_5]^-$ in-source fragments and unsaturated perfluoroalkyl amine fragments $C_nF_{2n}N^-$ detected in pooled bream liver from Tangxun Lake. All shaded peaks have a corresponding **Class 19** (**Figure 4-11.3**) peak at the same retention times. Peaks with retention time labeled in blue represent minor analytes with peak height below 1000 counts.

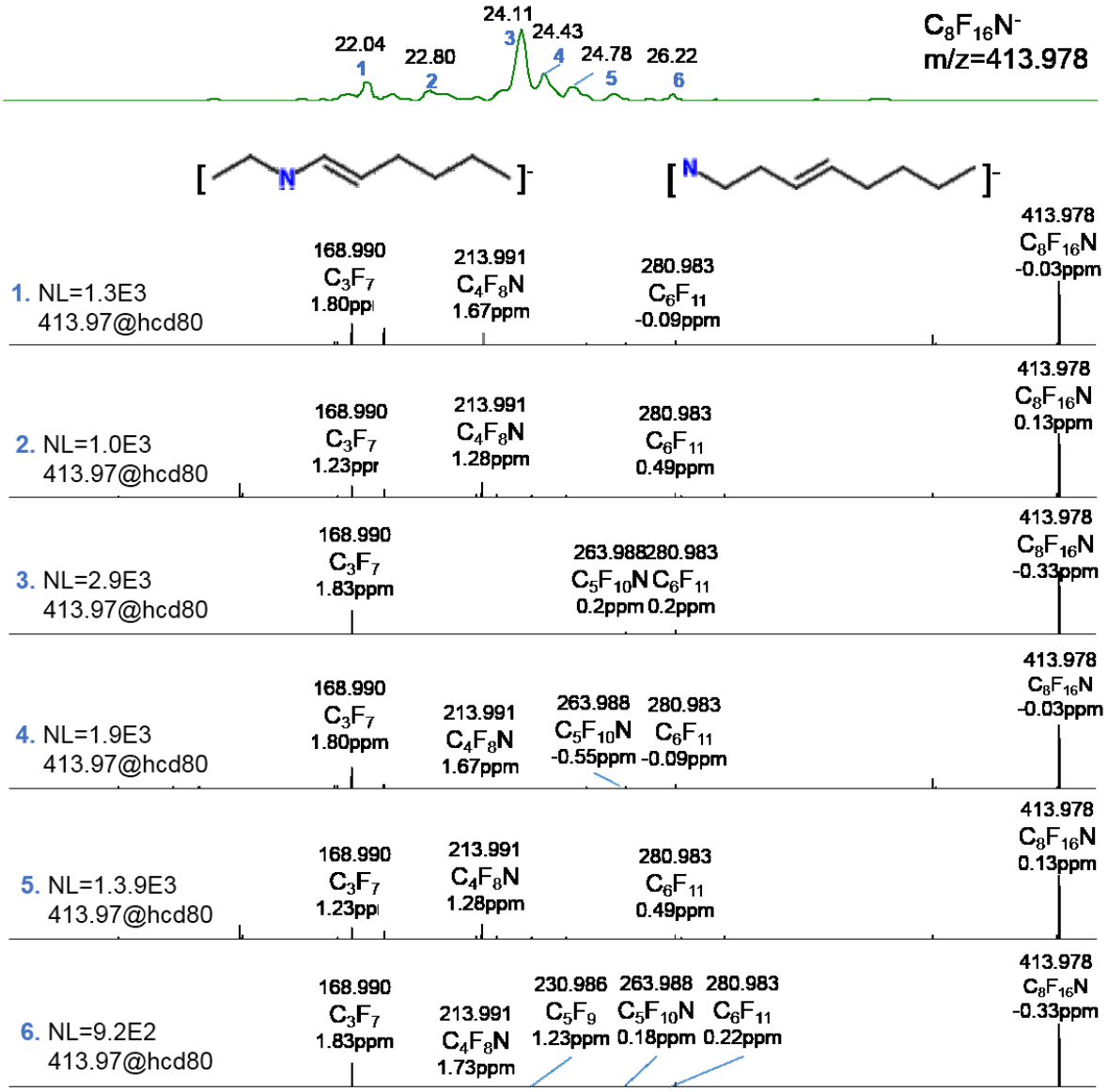


Figure 4-11.2 MS^2 spectra (HCD 80 eV) of selected isomers of C_8 unsaturated perfluoroalkyl amine fragments ($C_8F_{16}N^-$) in bream fish liver from Tangxun Lake. Example structures are proposed for isomer 1, F atoms not shown.

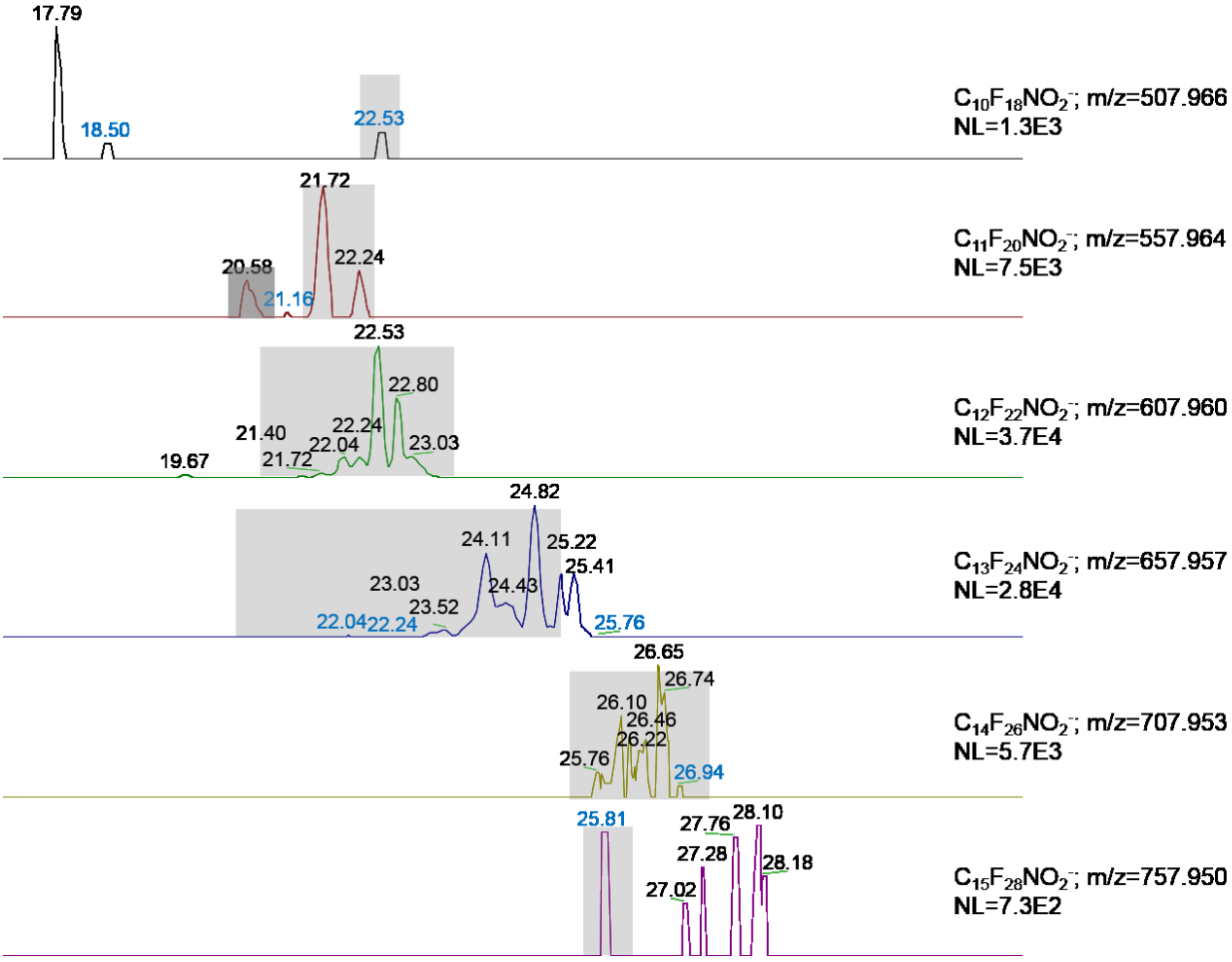


Figure 4-11.3. Extracted ion chromatograms of unsaturated perfluoroalkyl amine carboxylate or carboxyl ester ($C_nF_{2n-2}NO_2^-$, **Class 9**) molecular ions detected in pooled bream fish liver from Tangxun Lake. All shaded peaks have at least one corresponding $C_nF_{2n}N^-$ fragment peak (**Figure 4-11.1**) at the same retention times. Peaks with retention time labeled in blue represent minor analytes with peak height below 1000 counts.

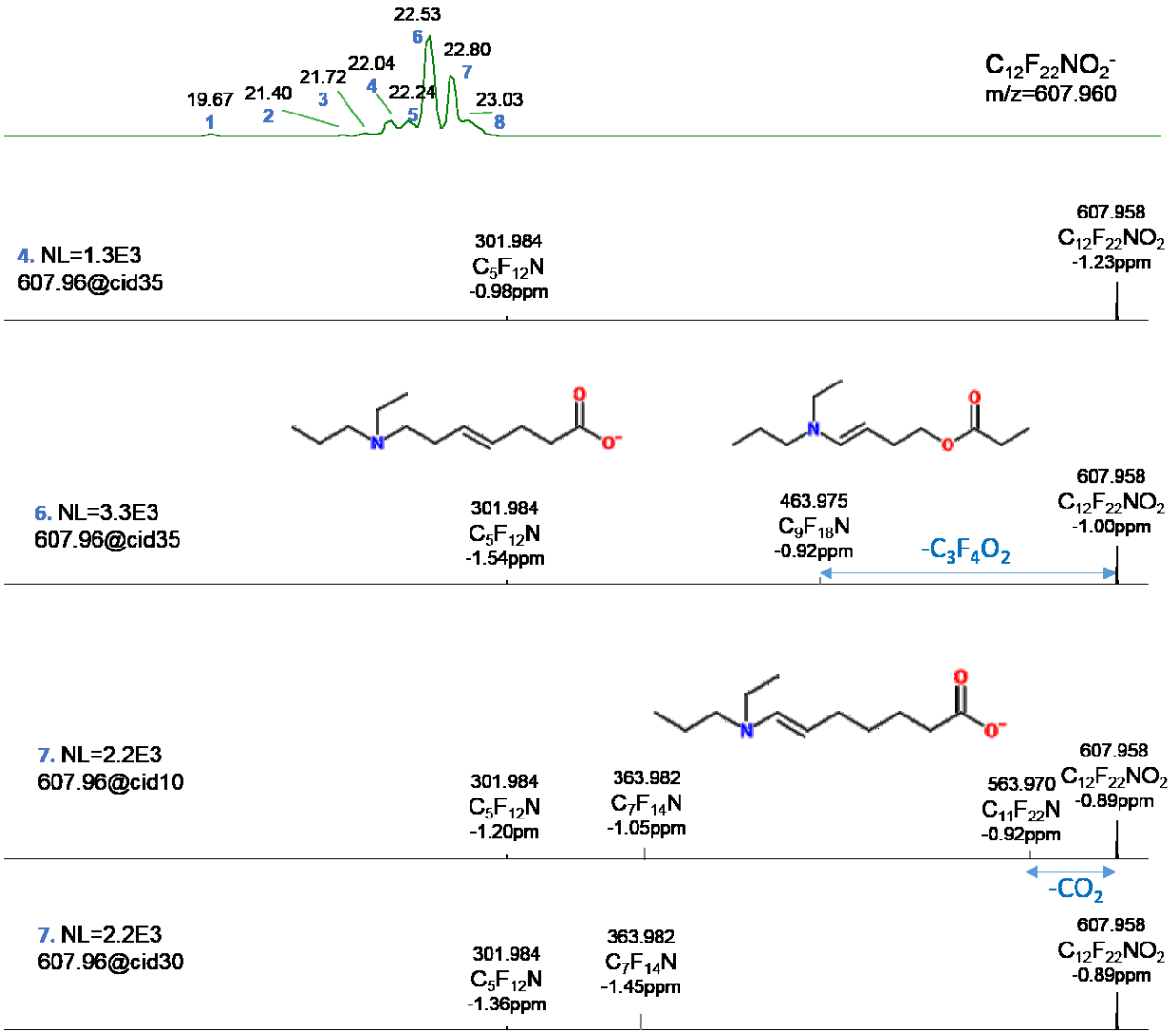


Figure 4-11.4. MS^2 spectra (CID 10-35 eV) of selected isomers of the C_{12} unsaturated perfluoroalkyl amine carboxylates or carboxyl esters ($C_{12}F_{22}O_2N^-$, **Class 9**). Example structures are proposed, F atoms not shown.

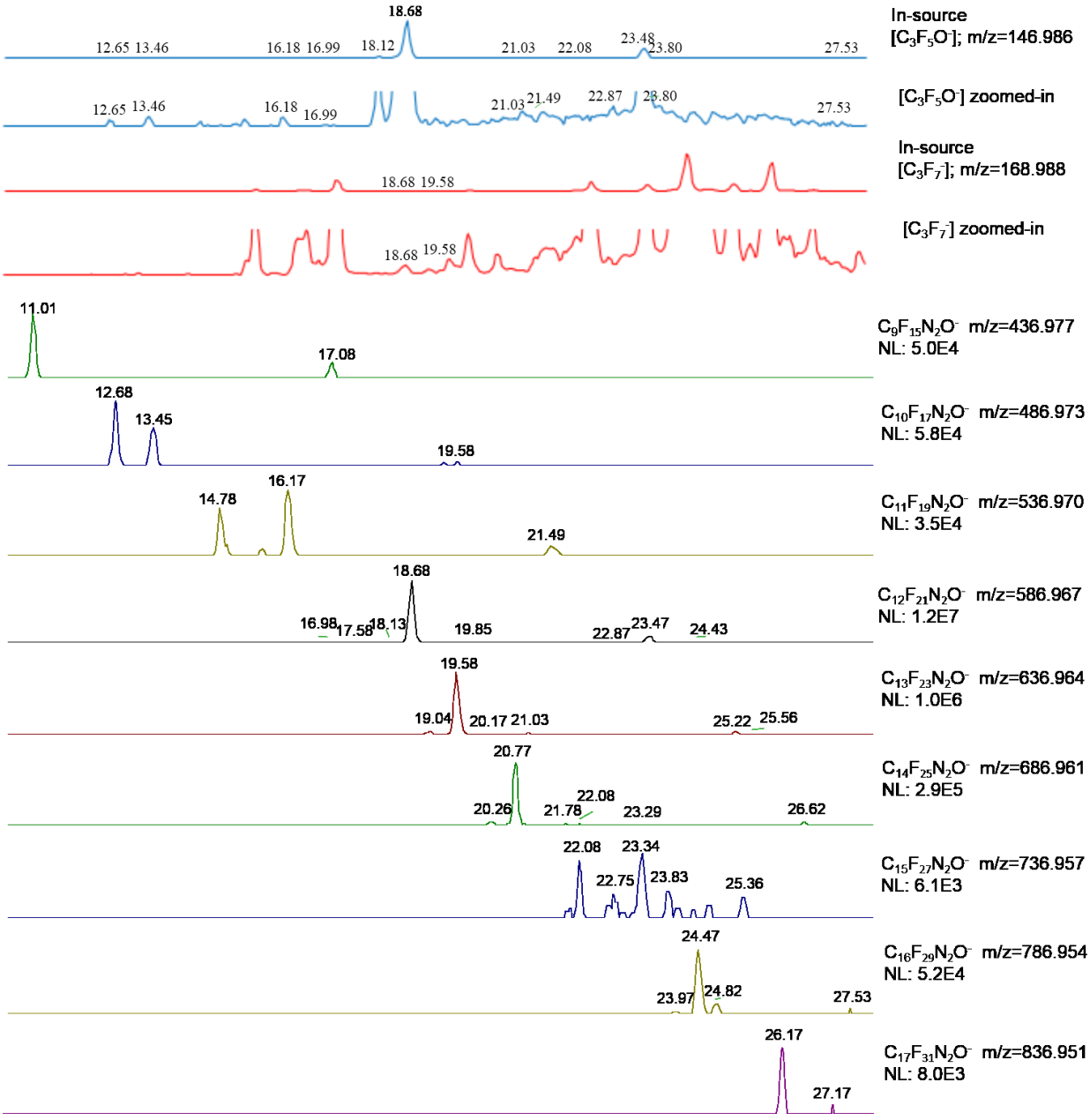


Figure 4-12.1 Extracted ion chromatograms of $[C_3F_5O]^-$ in-source fragments and **Class 10** perfluorinated pyrrole or imidazole ($C_nF_{2n-3}N_2O^-$) molecular ions detected in pooled bream fish liver from Tangxun Lake.

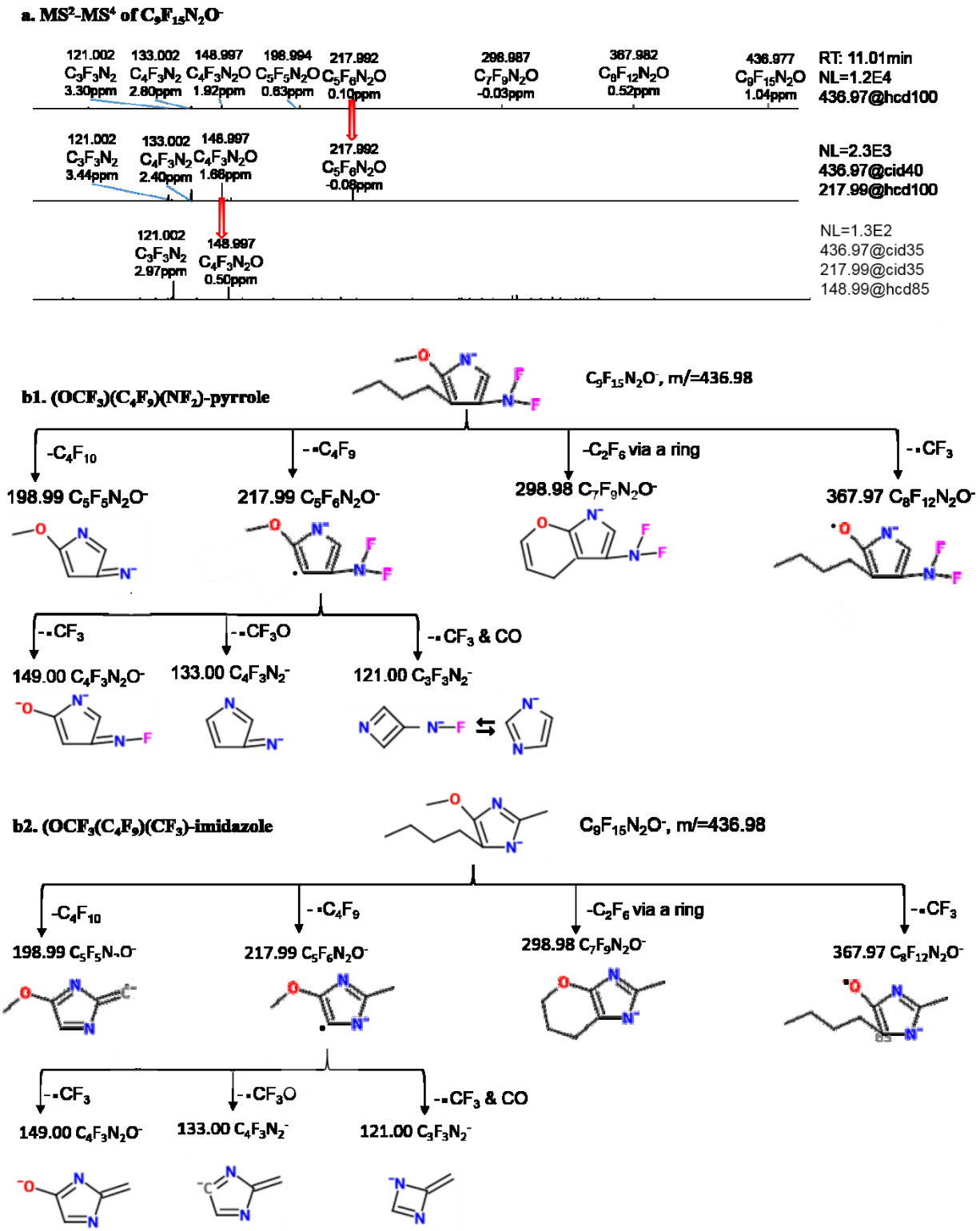


Figure 4-12.2. MS^2, MS^3 and MS^4 spectra of the major C_9 perfluorinated pyrrole or imidazole isomer eluting at 11.01 min ($C_9F_{15}N_2O^-$, **Class 10**, **a**) and proposed fragmentation pathways (**b**). Structures are presented, F atoms not shown.

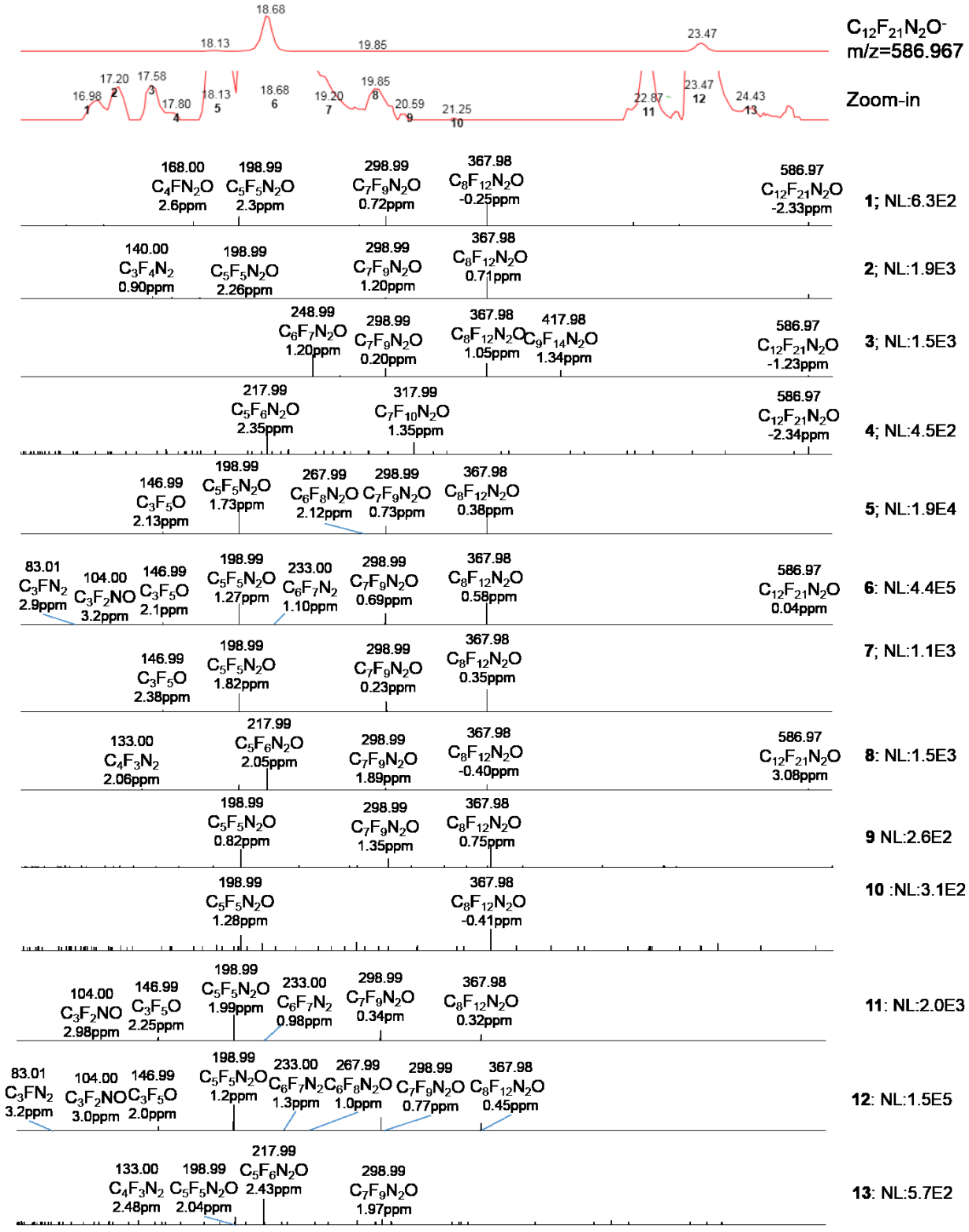


Figure 4-12.3 Chromatograms and MS^2 spectra (HCD 100 eV) of all C_{12} perfluorinated pyrrole or imidazole isomers ($C_{12}F_{21}N_2O^-$, **Class 10**) in bream fish liver from Tangxun Lake.

4.5.2 Tables

Table 4-1. Sample weight data (wet liver weight (g)/dry liver weight (g)) and ratio of liver dry/wet weight for each individual fish. Numbers in red represent the average wet/dry liver ratio of all individual fish in the same pool.

Yangtze River						Tangxun Lake							
Common Carp		Bighead Carp		Silver Carp		Common Carp		Bighead Carp		Silver Carp		Bream	
weights	ratio	weights	ratio	weights	ratio	weights	ratio	weights	ratio	weights	ratio	weights	ratio
4.9/0.31	16.1	9.9/1.48	6.8	21.5/1.34	14.9	79.8/2.69	29.5	80.5/5.40	14.9	98.6/3.40	29.0	41.1/3.67	11.2
5.7/0.71	7.0	20.1/1.12	15.2	20.4/2.12	9.6	47.6/3.20	14.9	80.0/4.39	18.2	100.2/6.30	15.8	88.7/3.71	23.9
5.3/0.45	11.1	20.1/1.91	10.5	39.8/3.77	10.6	80.1/4.45	18.0	98.9/4.34	22.8	97.5/5.98	16.3	41.4/9.20	4.4
15.0/1.58	9.5	21.7/1.30	16.7			79.9/4.43	18.0	82.3/4.84	17.0	94.8/6.32	15.1	85.9/4.45	19.3
10.8/1.13	8.8	23.4/1.65	14.1			83.9/4.22	19.9			83.4/4.53	18.4	88.3/5.54	16.0
						99.4/6.33	15.7			92.3/5.88	14.7	88.5/13.62	6.5
												104/11.0	9.5
	10.5		12.6		11.7		19.3		18.2		18.2		13.0
0.31 g dried liver from each individual for each species						0.20 g dried liver from each individual for each species							

Table 4-2. Legacy PFASs concentrations (ng/g w.w.) in pooled fish liver samples. Blank cells indicate not detected, while trace concentrations below the limit of quantification are shown as <LOQ. The LOQ value for each pooled fish sample is calculated back from instrumental LOQ (i.e. 0.01 ng/mL for each legacy standard).

	Yangtze River			**Tangxun Lake**			
	Common	Bighead	Silver	Common	Bighead	Silver	Bream
LOQ (ng/g w.w.)	0.0017	0.0015	0.0014	0.0009	0.0010	0.0010	0.0014
PFBA	<LOQ	0.07		0.01	0.01		2.6
PFPeA							<LOQ
PFHxA	0.21	0.09	0.06	0.01	0.03	0.06	0.05
PFHpA	<LOQ		<LOQ		<LOQ	<LOQ	<LOQ
PFOA	0.47	0.55	0.24	0.06	0.14	0.27	0.70
PFNA	0.19	0.12	0.06	0.03	0.05	0.16	0.58
PFDA	2.5	3.3	1.4	0.40	1.00	0.93	6.8
PFUnDA	6.2	2.9	0.19	0.30	0.41	1.4	22
PFDODA	1.9		0.22	0.34	0.25	0.17	8.6
PFTrDA	1.3	0.5	0.25	0.11	0.17		3.4
PFTeDA	1.1			0.28			2.0
PFHxDA							
PFODA							
Total	14	7.5	2.5	1.6	2.1	3.0	47
PFBS	<LOQ	<LOQ	0.04	1.2	0.61	11	178
PFPeS	<LOQ		<LOQ	0.09	<LOQ	0.60	1.0
PFHxS	0.41	0.02	0.02	1.2	0.18	2.4	7.5
PFHpS	0.13		0.18	0.62	0.30	1.2	5.3
PFOS	22	21	5.3	47	68	140	750
PFNS	0.80		1.8	2.3	3.7	7.2	75
PFDS	<LOQ		<LOQ	2.3	2.9	5.0	92
Total	24	22	7.4	54	76	170	950
PFOSA				0.16	0.42	0.18	0.59
MeFOSA	2.6					1.8	
EtFOSA							
MeFOSAA		1.3		0.33	0.86	0.43	1.2
EtFOSAA							
Total	2.6	1.3		0.49	1.3	2.4	1.8
6:2 PAP	0.24		0.61	0.40	1.1		
8:2 PAP	0.50		0.69	0.36	0.99		
6:2 DiPAP							
8:2 DiPAP							
Total	0.75		1.3	0.76	2.1		
PFHxPA							
PFOPA							
PFDPA							

Table 4-3. Semi-quantification of newly discovered PFASs (ng/g w.w.) in pooled fish liver samples. The "reference standard" (R.S.) for each newly discovered PFAS is a legacy PFAS whose calibration curves were used to semi-quantify the new PFAS by assuming the same molar response. Blank cells indicate not detected, while trace concentrations below the limit of quantification are shown as <LOQ. The LOQ value for each pooled fish sample is calculated back from instrumental LOQ (0.01 ng/mL for each legacy standard). Percent numbers in red are concentration ratios of the new PFAS to its reference standard in the same sample.

		Yangtze River						**Tangxun Lake**							
		Common		Bighead		Silver		Common		Bighead		Silver		Bream	
LOQ (ng/g w.w.)		0.0017		0.0015		0.0014		0.0009		0.0010		0.0010		0.0014	
	R.S.	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%
Class 1 Cl-PFCA															
C_8	C_8-PFCA	0.14	29%												
C_9	C_9-PFCA	2.1	110%	0.08	64%	0.63	1100								
C_{10}	C_{10}-PFCA	17	680%	1.5	45%	4.7	330%								
C_{11}	C_{11}-PFCA	13	220%	0.15	5%	3.0	1600%								
C_{12}	C_{12}-PFCA	1.1	58%	0.18		0.29	130%								
C_{13}	C_{13}-PFCA	1.3	110%			0.29	120%								
C_{14}	C_{14}-PFCA	2.2	190%	1.1											
C_{15}	C_{14}-PFCA	1.2	110%												
Total		38		2.9		8.9									
Class 2 x:2 Cl-PFAES															
C_7	6:2 Cl-PFAES	0.12	<1%												
C_8		62	100%	47	100%	3.6	100%	0.54	100%	0.99	100%	9.0	100%	16	100%
C_9		0.44	1%	0.28	1%	0.22	6%	0.06	10%		<1%	0.06	1%	0.25	2%
C_{10}		23	37%	0.94	2%	0.74	20%			0.16	17%	0.83	9%	0.48	3%
C_{12}		2.7	4%			0.28	8%								
C_{14}		0.15	<1%												
Total		88		48		4.8		0.60		1.1		9.9		17	

Table 4-3. Continued:

	R.S.	Yangtze River	Tangxun Lake							
			Common		Bighead		Silver		Bream	
			Conc.	%	Conc.	%	Conc.	%	Conc.	%
Class 3 cyclic/ unsaturated PFSA C_7	C_7-PFSA		0.04	6%					0.15	3%
C_8	C_8-PFSA		0.07	<1%	0.16	<1%	0.12	<1%	0.38	<1%
C_9	C_9-PFSA		0.38	17%	1.0	28%	0.42	6%	1.5	2%
C_{10}	C_{10}-PFSA		<LOQ		0.19	7%	0.07	1%	1.4	2%
C_{11}			0.18	8%	0.47	16%	0.23	5%	5.6	6%
C_{12}			0.30	13%	0.57	20%	0.29	6%	8.5	9%
C_{13}			0.02	1%	<LOQ		<LOQ		0.60	1%
C_{14}			<LOQ				<LOQ		0.05	<1%
Total			1.0		2.4		1.1		18	
Class 4 monoether-PFSA C_4	C_4-PFSA				<LOQ		<LOQ		<LOQ	
C_5	C_5-PFSA		<LOQ		<LOQ		<LOQ		0.14	14%
C_6	C_6-PFSA		0.04	3%	<LOQ		0.07	3%	0.24	3%
C_7	C_7-PFSA		0.08	13%	0.17	58%	0.14	12%	0.55	10%
C_8	C_8-PFSA		1.9	4%	7.7	11%	6.1	4%	44	6%
C_9	C_9-PFSA		1.8	76%	7.2	190%	4.3	60%	54	72%
C_{10}	C_{10}-PFSA		0.26	11%	0.80	27%	0.26	5%	11	12%
C_{11}			0.11	5%	0.13	5%	<LOQ		4.4	5%
C_{12}			0.06	3%	<LOQ		<LOQ		3.5	4%
Total			4.2		16		11		120	
Class 5 diether-PFSA C_6	C_6-PFSA		<LOQ		<LOQ		<LOQ		<LOQ	
C_7	C_7-PFSA		0.04	7%	0.11	36%			0.16	3%
C_8	C_8-PFSA		0.08	<1%	0.21	<1%	0.11	<1%	0.84	<1%
C_9	C_9-PFSA		0.45	19%	1.2	33%	0.47	7%	2.6	3%
C_{10}	C_{10}-PFSA		0.02	1%	0.03	1%	<LOQ		2.4	3%
C_{11}			<LOQ						0.24	<1%
C_{12}			<LOQ		<LOQ		<LOQ		0.71	1%
Total			0.59		1.5		0.58		6.9	

Table 4-3. Continued:

	R.S.	Yangtze River	Tangxun Lake							
			Common		Bighead		Silver		Bream	
			Conc.	%	Conc.	%	Conc.	%	Conc.	%
Class 6 enol ether-/cyclic ether-/carbonyl-PFSA										
C_6	C_6-PFSA		<LOQ				0.02	1%	0.02	<1%
C_7	C_7-PFSA		0.04	6%	0.10	32%	0.04	4%	0.15	3%
C_8	C_8-PFSA		0.35	1%	0.87	1%	0.80	1%	3.5	<1%
C_9	C_9-PFSA		0.43	18%	1.2	33%	0.48	7%	2.5	3%
C_{10}	C_{10}-PFSA		0.14	6%	0.45	15%	0.25	5%	5.3	6%
C_{11}			0.27	12%	0.74	25%	0.40	8%	9.4	10%
C_{12}			0.54	24%	1.1	38%	0.44	9%	18	19%
C_{13}			<LOQ		<LOQ				0.2	<1%
Total			1.8		4.5		2.4		38	
Class 7 diether-unsaturated-/cyclic-ether-/carbonyl-ether-PFSA										
C_6	C_6-PFSA		<LOQ				<LOQ		<LOQ	
C_7	C_7-PFSA		0.04	6%					0.14	3%
C_8	C_8-PFSA		0.08	<1%	0.19	<1%	0.11	<1%	0.70	<1%
C_9	C_9-PFSA		0.43	19%	1.2	33%	0.56	8%	2.5	3%
C_{10}	C_{10}-PFSA		0.05	2%	0.02	1%	0.06	1%	3.0	3%
C_{11}			<LOQ		<LOQ		<LOQ		1.9	2%
C_{12}			0.02	1%	<LOQ		<LOQ		1.8	2%
C_{13}									<LOQ	
Total			0.62		1.4		0.72		10	
Class 8 fragment $C_nF_{2n+2}N^-$										
C_5	C_8-PFSA		0.10	<1%	1.0	1%	0.73	1%	8.2	1%
C_6			0.05	<1%	0.34	<1%	0.22	<1%	2.8	<1%
C_7			0.07	<1%	0.37	1%	0.20	<1%	3.9	1%
C_8			4.2	9%	19	27%	18	12%	310	41%
C_9			0.07	<1%	0.33	<1%	0.13	<1%	5.6	1%
C_{10}			0.09	<1%	0.33	<1%	0.20	<1%	7.1	1%
C_{11}			0.07	<1%	0.22	<1%	0.07	<1%	3.7	<1%
C_{12}			0.10	<1%	0.63	1%	0.39	<1%	4.0	1%
Total			4.7		22		20		350	

Table 4-3. Continued:

	R.S.	Yangtze River	Tangxun Lake							
			Common		Bighead		Silver		Bream	
			Conc.	%	Conc.	%	Conc.	%	Conc.	%
Class 8 fragment $C_nF_{2n}NO_2^-$ C_9	C_8-PFSA		0.05	<1%	0.14	<1%	0.08	<1%	0.43	<1%
C_{10}			0.11	<1%	0.65	1%	0.54	<1%	4.5	1%
C_{11}			0.19	<1%	0.40	1%	0.18	<1%	9.3	1%
C_{12}			2.4	5%	0.91	1%	3.8	3%	140	19%
C_{13}			0.92	2%	5.5	8%	5.0	3%	65	9%
C_{14}			0.08	<1%	0.22	<1%	0.11	<1%	1.9	<1%
C_{15}									0.28	<1%
C_{16}									0.42	<1%
Total			3.8		7.8		9.8		220	
Class 8 fragment $C_nF_{2n-2}H_2O_5N^-$ C_{10}	C_8-PFSA		<LOQ				<LOQ		<LOQ	
C_{11}			0.04	6%					0.14	3%
C_{12}			0.08	<1%	0.19	<1%	0.11	<1%	0.70	<1%
C_{13}			0.43	19%	1.2	33%	0.56	8%	2.5	3%
C_{14}			0.05	2%	0.02	1%	0.06	1%	3.0	3%
C_{15}			<LOQ		<LOQ		<LOQ		1.9	2%
C_{16}			0.02	1%	<LOQ		<LOQ		1.8	2%
C_{17}									<LOQ	
Total			1.5		2.0		1.3		52	
Class 8 $C_nF_{2n-6}H_6O_6N^-$ C_{13}	C_8-PFSA				0.16	<1%			0.23	<1%
C_{14}			<LOQ		0.19	<1%	0.08	0%	0.32	<1%
C_{15}					0.40	1%			1.2	<1%
C_{16}					0.22	<1%			1.1	<1%
C_{17}									<LOQ	
C_{18}									<LOQ	
Total					0.97		0.08		2.9	
Total Class 8			10		33		31		620	

Table 4-3. Continued:

	R.S.	Yangtze River	Tangxun Lake							
			Common		Bighead		Silver		Bream	
			Conc.	%	Conc.	%	Conc.	%	Conc.	%
Class 9 Fragment $C_nF_{2n}N^-$ C_6	C_8-PFSA		0.03	<1%	0.08	<1%			0.46	<1%
C_7			0.03	<1%	0.15	<1%	0.05	<1%	1.8	<1%
C_8			0.14	<1%	0.72	1%	0.42	<1%	9.4	1%
C_9			0.05	<1%	0.18	<1%	0.07	<1%	1.9	<1%
C_{10}			0.06	<1%	0.19	<1%	0.10	<1%	1.5	<1%
C_{11}			0.12	<1%	0.63	1%	0.38	<1%	7.4	1%
C_{12}			0.13	<1%	0.56	1%	0.28	<1%	8.8	1%
C_{13}			0.06	<1%			0.09	<1%	1.2	<1%
C_{14}									<LOQ	
C_{15}									<LOQ	
Total			0.62		2.5		1.39		32	
Class 9 $C_nF_{2n-2}NO_2^-$ C_{10}	C_8-PFSA								<LOQ	
C_{11}			0.06	<1%	0.18	<1%	0.08	<1%	0.86	<1%
C_{12}			0.20	<1%	0.93	1%	0.09	<1%	12	2%
C_{13}			0.30	1%	0.86	1%	0.73	1%	16	2%
C_{14}			0.07	<1%	0.18	<1%	0.74	1%	9.3	1%
C_{15}					0.22	<1%	0.08	<1%	3.8	1%
Total			0.63		2.4		1.7		42	
Total Class 9			1.4		4.9		3.1		74	
Class 10 perfluorinated imidazoles/ pyrroles C_9	C_8-PFSA		0.04	<1%	0.21	<1%	0.19	<1%	0.72	<1%
C_{10}			0.06	<1%	0.36	1%	0.33	<1%	1.3	<1%
C_{11}			0.07	<1%	0.23	<1%	0.17	0%	0.94	<1%
C_{12}			3.8	8%	7.3	11%	16	11%	210	28%
C_{13}			0.83	2%	1.1	2%	0.13	<1%	19	3%
C_{14}			0.16	<1%	0.16	<1%			4.6	1%
C_{15}									0.38	<1%
C_{16}			0.06	<1%					1.2	<1%
C_{17}									0.4	0%
Total			5.0		9.3		17		240	

Table 4-4. Non-target PFASs detected in pooled fish liver (C=common carp, B=bighead carp; S=silver carp; Br=bream) from the Yangtze River and Tangxun Lake and associated confidence levels (CL=1-4). The range of carbon length homologues (n), total number of analytes detected in each class, and relevant previous reports are shown. Representative structure for one of the homologue (mostly C_8) in each class is shown, F atoms not shown.

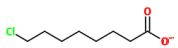
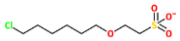
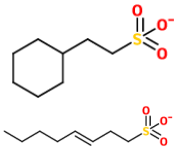
Class No.	PFAS Category	Molecular Ion Formula & Structure	Homologues Discovered in Yangtze River (n)			Homologues Discovered in Tangxun Lake (n)				Total Analytes	CL	Previous Reports
			C	B	S	C	B	S	Br			
1	terminal chlorine substituted perfluoroalkyl carboxylates (Cl-PFCAs)	$ClC_{n-1}F_{2n-2}CO_2^-$ 	8 9 10 11 12 13 14 15	9 10 11 12 13 14 15	9 10 11 12 13 14					8	3	n=5-12 in wastewater from a WWTP located ~10km upstream of location A[42]
2	x:2 chlorine substituted perfluoroalkyl ether sulfonates (x:2 Cl-PFASEs)	$ClC_nF_{2n}SO_4^-$ 	7 8 9 10 12 14	8 9 10	8 9 10 12	8 9	8 10	8 9 10	8 9 10	6	1 for n=6, 8; 2 for others	n=8 in chrome plating company discharge and downstream water;[27] in crucian carps[29] and in air[28] in China, as well as in Greenland ringed seal and polar bears[32] n=8, 10, 12 in municipal sewage sludge[26] and human serum in China[30, 31] n=6-10 in F-53B industrial standard in this study
3	cyclic or unsaturated perfluoroalkyl sulfonates	$C_nF_{2n-1}SO_3^-$ 				7 8 9 10 11 12 13 14	8 9 10 11 12 13	8 9 10 11 12 13 14	7 8 9 10 11 12 13 14	>50	3	In total four n=8 cyclic PFSAs, mainly 4-PFECHS, in standards,[57, 58] waters,[58, 59] sediment,[57] amphipods[59] and fish[58, 60] n=4-13 homologous in 3M commercial products and AFFFs,[62] in concrete from AFFF impacted fire training ground[63] n=8-9 in industrial 3M-PFOS standard in this study

Table 4-4. Continued:

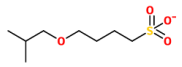
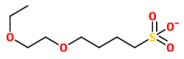
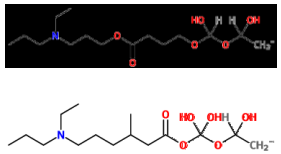
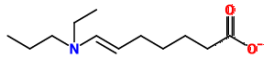
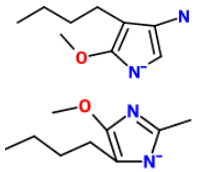
Class No.	PFAS Category	Molecular Ion Formula & Structure	Homologues Discovered in Yangtze River (n)			Homologues Discovered in Tangxun Lake (n)				Total Analytes	CL	Previous Reports
			C	B	S	C	B	C	B			
4	monoether-perfluoroalkyl sulfonates (monoether-PFSAs)	$C_nF_{2n+1}SO_4^-$ 				5 6 7 8 9 10 11 12	4 5 6 7 8 9 10 11 12	4 5 6 7 8 9 10 11	4 5 6 7 8 9 10 11 12	>30	3	n=6 in AFFF exposed firefighters' sera[50] n=8-9 in industrial 3M-PFOS standard in this study
5	diether-perfluoroalkyl sulfonates (dither-PFSAs)	$C_nF_{2n+1}SO_5^-$ 				6 7 8 9 10 11 12	6 7 8 9 10 12	6 8 9 10	6 7 8 9 10 11 12	>25	3	no report
6	enol ether-/cyclic ether-/carbonyl-perfluoroalkyl sulfonates	$C_nF_{2n-1}SO_4^-$				6 7 8 9 10 11 12 13	7 8 9 10 11 12 13	6 7 8 9 10 11 12	6 7 8 9 10 11 12 13	>40	3	n=8 ketone-PFAS proposed in AFFF exposed firefighters' sera;[50] n=7 enol-ether-PFSA proposed in water downstream of a fluorochemical production facility[48] n=4-13 in concrete from AFFF impacted fire training ground;[63] n=8-9 in industrial 3M-PFOS standard in the current study
7	diether-unsaturated-/cyclic-ether-/carbonyl-ether-perfluoroalkyl sulfonates	$C_nF_{2n-1}SO_5^-$				6 7 8 9 10 11 12	8 9 10 11 12	6 8 9 10 11 12	6 7 8 9 10 11 12 13	>30	3	n=7 diether unsaturated PFSA proposed in water downstream of a fluorochemical production facility[48]

Table 4-4. Continued:

Class No.	PFAS Category	Molecular Ion Formula & Structure	Homologues Discovered in Yangtze River (n)			Homologues Discovered in Tangxun Lake (n)				Total Analytes	CL	Previous Reports
			C	B	S	C	B	C	B			
8	perfluoroalkyl amine carboxyl esters	$C_nF_{2n-6}H_6O_6N^-$ 				13 14 15 16		14	13 14 15 16 18	>50	4	no report
9	unsaturated perfluoroalkyl amine carboxylate or carboxyl esters	$C_nF_{2n-2}O_2N^-$ 				11 12 13 14	10 11 12 13 14 15	10 11 12 13 14	10 11 12 13 14 15	≥50	3	no report
10	perfluorinated pyrroles or imidazoles	$C_nF_{2n-3}N_2O^-$ 				9 10 11 12 13 14 16	9 10 11 12 13 14	9 10 11 12 13	9 10 11 12 13 14 15 16 17	>40	3	no report

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Chapter 5. Non-target Mass Spectrometry Reveals Hundreds of Previously Unrecognized Organohalogen Compounds in Polar Bear Serum

5.1 Introduction

Polar bears (*Ursus maritimus*) are apex predators of the Arctic marine foodweb and have a diet composed largely of seal blubber. Their high trophic positon makes them vulnerable to high organohalogen contaminant exposure.[1, 2] In the mid-1970s, many classes of organochlorine pesticides and industrial chemicals were first identified in polar bear tissues, including dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs),[3] dieldrin, hexachlorobenzene, and hexachlorocyclohexane.[4] More recent discoveries found that polar bears are also exposed to persistent fluorinated and brominated organic contaminants, including perfluorinated acids[5, 6] and polybrominated diphenyl ethers (PBDEs).[7]

This contamination represents a considerable health concern, not only to polar bears but also to indigenous people who have long depended on marine mammals for subsistence.[8, 9] Among possible health effects in the polar bear, a recent review highlighted a weight of evidence for immune-suppression and endocrine disruption.[10] Based on this review, persistent metabolites of widespread environmental contaminants, such as the OH-PCBs, OH-PBDEs, methylsulfone PCBs ($MeSO_2$-PCBs) and *p,p′*-DDE are associated with such health effects in polar bear. As reviewed by Grimm *et al.*,[11] experimental evidence shows that various classes of PCB metabolites exhibit carcinogenic, neurologic and endocrine effects.

The detection and identification of unknown organohalogen chemicals has received previous attention because of their potential hazardous properties (e.g. environmental persistence, bioaccumulation and biomagnification), and it is known that a high proportion (i.e. 50%-99%) of the total organohalogen signal present in environmental and biological samples remains uncharacterized.[12-19] Moreover, effects-directed analysis of the transthyretin (TTR)-binding activity in polar bear cub blood revealed that only half the TTR-activity could be explained with

known contaminants including PCBs, OH-PCBs, and perfluorinated acids.[20] In a follow-up study, 3 new octachloro-OH-PCBs (Cl_8-OH-PCBs) were discovered, covering ~55% of the previously unidentified TTR-binding activities, but leaving ~25% of the biologically-active fraction unidentified.[21] To our knowledge, this unknown biologically active fraction of chemicals has still not been identified.

High resolution mass spectrometry (HRMS) has emerged as a powerful tool in non-target contaminant discovery. The combination of high spectral resolving power and high mass accuracy allows prediction of the empirical formula for unknown chemicals, while MS^n experiments can further help to elucidate unknown structures. Recent instrumental approaches to non-target HRMS (Nt-HRMS) screening of organohalogen compounds in environmental samples includes Orbitrap mass spectrometry with "fragmentation-flagging" for halogen ions.[22] We reported >35 new fluorinated and chlorinated compounds in a Chinese wastewater using HPLC-Orbitrap Elite™ by inducing fragmentation at the source, thereby using chloride ions ($^{35}Cl^-$ and $^{37}Cl^-$) or organofluorine ions (e.g. $CF_3CF_2^-$) as flags for unknown compounds which could then be identified by accurate mass and MS/MS experiments.[22] In a similar approach, Peng *et al*. detected thousands of organo-bromine[23] and organo-iodine[24] chromatographic peaks in lake sediments using Orbitrap Q Exactive™ and by inducing fragmentation in the collision cell to yield Br^- and I^-.

Existing applications for organohalogen discovery have mostly been focused on identification of active ingredients or impurities in commercial or industrial products,[25-28] with fewer successful applications to environmental samples[22, 23, 29, 30] and even fewer to complex biological samples (e.g. firefighters' serum[31]), partially owing to lower concentrations and more complex matrices for the latter sample types. Strategic sample preparation steps to minimize matrix signal, while simultaneously concentrating contaminant classes of interest are therefore important advances for new contaminant discoveries of biological relevance. Solid phase extraction (SPE)[25, 29] and organic solvent extraction[23, 31, 32] are the most commonly used sample preparation methods. However, to concentrate large volumes of biofluid sample (i.e. 5-10 mL) and to isolate a polar class of contaminants that we hypothesized to be present in serum, here we considered stir-bar sorptive extraction (SBSE) for sample preparation. This is a low cost and environmentally friendly sample preparation method that has advantages for large sample volumes, however only two commercially available adsorbent-coated stir bars are available for

SBSE: coated with polydimethylsiloxane (PDMS)[33, 34] or PDMS/ethylene glycol copolymer.[35] A home-made SBSE method using small segments of polyethersulfone (PES) capillary tubing was recently reported as an alternative, low cost approach to commercially available products.[36]

Here we analyzed two pooled polar bear serum samples from subpopulations in the Canadian Arctic (one from Western Hudson Bay, and the other from Beaufort Sea) with the aim of detecting unknown polar yet potentially biologically active chlorinated or fluorinated compounds. We stirred PES capillaries in pooled polar bear serum, and analyzed the extracts by Nt-HRMS with in-source fragmentation flagging,[22] and MS^n experiments were used for structural elucidation. A large number of previously unknown organohalogen compounds were revealed, and the relevant significance was discussed.

5.2 Experimental Section[††]

5.2.1 Nomenclature

Chlorinated compound congeners are abbreviated as "$Cl_{\#}$-A-B", where # indicates the number of chlorine atoms in the molecule, "A" indicates important functional groups (e.g. hydroxyl group, OH), and "B" represents the core structure (e.g. polychlorinated biphenyl, PCB). Poly- and perfluoroalkyl compound homologues are termed as "$C_{\#}$-A-B", where # indicates the total carbon chain length, "A" indicates important functional groups (e.g. ether), and "B" is abbreviation of the core structure (e.g. perfluoroalkyl sulfonates, PFSAs). Whenever the structure is confirmed, the commonly recognized names will be used, for example, 3, 3'-dichlorpbiphenyl will be termed as PCB 11 according to the Ballschmiter-Zell nomenclature[37], and perfluorooctanesulfonate will be acronymed PFOS.[38]

5.2.2 Chemicals and Reagents

Optima grade water, methanol and acetonitrile were from Fisher Scientific (Ottawa, ON,

[††] Drs. Evan Richardson, Andrew E. Derocher and Nicholas J. Lunn provided polar bear serum samples. Drs. Hans-Joachim Lehmler, Larry W. Robertson and Xueshu Li provided native SO_4-PCB standards, and Dr. Amila O De Silva (Environment and Climate Change Canada) provided the 4-PFECHS standard.

Canada). Ammonium acetate, 1-methyl piperidine (1-MP), tetrabutylammonium bromide (TBABr), sodium hydroxide and commercial calf serum were from Sigma-Aldrich (St. Louis, MO, USA). Four hexachloro hydroxylated PCB (Cl_6-OH-PCB) and three Cl_7-OH-PCB standards were from Wellington Laboratories (Guelph, ON, Canada, **Table 5-1**). 14 monochloro to tetrachloro PCB sulfate standards (Cl-Cl_4 SO_4-PCBs) and 2 monochloro dihydroxylated standards (Cl-DiOH-PCBs, **Table 5-1**) were previously synthesized at the University of Iowa.[39-45] Triclosan (i.e. 2, 4, 4,'-trichloro-2'-hydroxydiphenyl ether, a hydroxylated pentachlorodiphenyl ether, OH-Cl_5-PCDE) was from Sigma-Aldrich. Perfluoroalkyl carboxylate (PFCA), perfluoroalkyl sulfonate (PFSAs), and perfluorooctanesulfonamide standards were from Wellington Laboratories (**Table 5-2**). A standard of decafluoro-4-(pentafluoroethyl)-cyclohexanesulfonate (4-PFCHES, CAS 335-24-0) was from Wako Chemicals (Richmond, VA, USA). A chlorinated perfluorinated ether sulfonate (F-53B, CAS 73606-19-6) industrial standard was from Shanghai Maikun Chemical Co., Ltd. (Shanghai, China). A historical industrial PFOS standard was donated by the 3M Co. (St. Paul, MN, USA).

PDMS (1 mm thickness,10 mm length) coated Twister bars were from Gerstel (Mulheim an der Ruhr, Germany), PDMS tubing (2.2 mm o.d., 0.5 mm i.d., cut into 10 mm length segments) and PES sheets (48 mm length, 48 mm width, 2 mm height, cut into 8 mm length, 2 mm width, 2 mm height segments) were from Good Fellow (Edmonton, AB, Canada), and PES capillary tubing (UltraPES0.7, 0.7 mm o.d., 150 μm wall thickness, cut into 10 mm length segments) was from 3M Deutschland GmbH (Membranes Business Unit, Wuppertal, Germany). HybridSPE® (500mg/6mL) cartridges were from Sigma-Aldrich, and Cleanascite™ was from BioTech Support Group (NJ, USA). Nalgene® surface-free cellulose acetate filters (0.2 μm pore size) and Nalgene® combined glass fiber and cellulose acetate filters (0.2 μm pore size) were from Sigma-Aldrich, polyvinylidene fluoride filters (0.2 μm pore size) were from Millipore Ltd. (Etobicoke, ON, Canada) and combined glass fiber and polytetrafluorethylene (PTFE, 0.2 μm pore size) filters were from Phenomenex (Torrance, CA, USA).

5.2.3 Polar Bear Serum Collection and Pooling

Two polar bear monitoring programs began in Western Hudson Bay[46, 47] and in the Beaufort Sea [48] in the 1980s. Each year, serum samples from individual polar bears of both sex and of different ages (1-25 yrs) were collected and archived at -80 ℃. Capture and handling

protocols were in accordance with the Canadian Council on Animal Care guidelines and approved by the Environment and Climate Change Canada, Prairie and Northern Region Animal Care Committee and the University of Alberta Biological Sciences Animal Policy and Welfare Committee. For the current work, 14 and 22 individual serum samples from Western Hudson Bay and Beaufort Sea, respectively, were withdrawn from the archive, stored at -80 ℃ for a month (along with the procedural blanks discussed in section **5.2.4**) and pooled by location (**Table 5-3**), resulting in two pooled polar bear serum samples.

5.2.4 Sample Extraction by SBSE

Based on an established SBSE method for extracting PFASs from large volume water samples,[36] a similar SBSE method for extracting PFASs and polychloroalkyl compounds from 5 mL serum matrix was developed using authentic standards of PFASs (**Table 5-2**), OH-PCBs and SO_4-PCBs (**Table 5-1**). A mixed standard stock solution containing 200 ng/mL of each individual PFAS, OH-PCB and SO4-PCB standards was prepared. 5 mL of calf serum was spiked at 1 ng/mL of the mixed standard, and four different extraction materials (1 PDMS twister, surface area=144 mm^2 Volume=94 mm^3; 2 PDMS tube segments, S=169 mm^2 V=72 mm^3; 3 PES sheet segments, S=144 m^2 V=64 mm^3; and 7 PES capillary segments, S=154 mm^2 V=27 mm^3 were compared by recovery and matrix effects), three different protein precipitation methods (saturated aqueous ammonium sulfate, aqueous 10% trichloroacetic acid, and acetonitrile), phospholipid removal methods (HybridSPE® and Cleanascite™), and different extraction parameters (pH 7.0, 9.5 and 10.5; number of PES capillary segments 5, 10 and 20; concentration of ion-pairing reagent TBABr 28 μM and 56 μM; extraction time 24, 36 and 48 h; and desorption time 30 min and 60 min) were tested to obtain optimal extraction condition. In addition, four different filters (Nalgene® surface-free cellulose acetate, Nalgene® combined glass fiber and cellulose acetate, polyvinylidene fluoride, and combined glass fiber and PTFE) were tested for their absorption capacity to native standards by passing through 15 mL of 0.5 ng/mL mixed standard (in 50% H_2O/50%MeOH). The PTFE-coated magnetic stir bar was also examined for its standard absorption during extraction (< 2% standard was absorbed). The developed SBSE method is presented in the following.

For extraction of samples, an aliquot of 5 mL of pooled serum sample from each location was mixed with 15 mL acetonitrile, vortexed and centrifuged at 4415 g and at 15 ℃ for 30 min

to remove serum proteins. The ~20 mL supernatants were loaded to two methanol-preconditioned HybridSPE cartridges (~10 mL for each) and eluted under gravity for phospholipid removal. Combined eluents (free from phospholipids) were concentrated with high purity nitrogen gas to approximately 3 mL. The pH was then adjusted to 10.5 with 0.05 M Na_2CO_3 (~1.8 mL), and the volume was made up to 10 mL with water. Then 0.09 g TBABr (i.e. 28 μM), a polytetrafluoro-ethylene (PTFE) coated magnetic stir bar, and ten 10 mm PES capillary segments (preconditioned with methanol for 48 h and washed with water before use) were added to the pH adjusted sample. Samples were sealed and placed on a magnetic stirrer (700 rpm) for 36 h at room temperature. All PES segments were then removed with tweezers, rinsed with water, dried with dust-free tissues (Kimtech Science), and immersed in 1 mL methanol inside 1.5 mL glass vials for analyte desorption. After 30 min, PES segments were removed from the vial and the extracts were dried under high purity nitrogen gas, reconstituted in 200 μL of 50% water/50% methanol and filtered with Nalgene filters when necessary. Extracts (10 - 50 μL; 0.25 - 1.25 mL of polar bear serum equivalent) were injected to the HPLC-Orbitrap MS system for analysis.

Triplicate procedural blanks, prepared with calf serum, were extracted along with pooled polar bear samples to monitor for possible laboratory contamination. Triplicate instrumental procedural blanks, prepared with 50% methanol/50% water, were also injected during sample analysis to check for any instrumental background. To consider possible chemical migration from contaminated cryovials during long-term sample archiving, two sets of triplicate quality control samples were also prepared: (1) calf serum aliquoted to the same containers (×10) used for polar bear serum storage (2.0 mL/container), sealed and stored at -80℃ (along with the individual polar bear serum samples) for 1 month before extraction, and (2) calf serum aliquoted and sealed in the same containers used for polar bear serum storage, but allowed to sit at room temperature for 1 month before extraction. The individual samples in each set were pooled, extracted and analyzed along with the polar bear serum samples.

5.2.5 HPLC-LTQ-Orbitrap-MS

As described for analysis of fish liver extracts in **Chapter 4**, HPLC-Orbitrap was employed for non-target discovery of perfluoroalkyl and/or chlorinated compounds. Briefly, a combination of in-source fragmentation flagging, full scan and MS^n experiments were used to

detect and characterize unknown halogenated substances. Fluorinated and chlorinated fragment ions (e.g. $[C_2F_5]^-$ and $[Cl]^-$) and chlorinated fragments detected by in-source fragmentation flagging were used to flag retention times of halogenated molecular ions in full-scan mode. Exact masses (within ±5ppm) of the molecular ions, isotopic patterns and in-source fragments were used to assign empirical formulae.

5.2.6 Data Presentation

For each PFAS chain-length homologous class (i.e. a series of compounds share the same general formula but with different chain-lengths) or chlorinated congener series class (i.e. a series of compounds share the same general formula but with different chlorine substitutions), structure elucidation was based on MS^n (where n=2-3 stages of ion isolation and fragmentation) for 1 - 2 of the major homologues or congeners in the class. For each non-target class of PFAS homologues discovered, plausible structures were proposed and confidence levels (CL=1-5) were assigned according to established criteria for non-target mass spectrometry; where CL=1 is the highest level of confidence, confirmed by authentic standard.[49]

5.2.7 Quantitative Method Performance

Although the focus of the study was discovery, and not quantification, a mixed standard stock solution containing 200 ng/mL of each of individual PFAS, OH-PCB and SO_4-PCB standards (**Table 5-1** and **Table 5-2**) was used to evaluate quantitative method performance. The standard was diluted to 0, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5.0, 10, 12.5, 25ng/mL in 50% water/50% methanol, and 20 µL of each was injected to evaluate instrumental detection limits (IDLs, **Table 5-4**). Similarly, 5 mL aliquots of calf serum were spiked at 0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 ng/mL, and 20 µL of the final SBSE extracts were injected to measure method detection limits (MDLs, **Table 5-4**) of the overall SBSE-HPLC-Orbitrap method. Recovery, matrix effect and the total extraction efficiency of the SBSE method was also evaluated by using two aliquots of 5 mL calf serum. The 200 ng/mL stock solution was spiked (12.5 µL) in one aliquot before the SBSE extraction (termed "pre-spiked"), and in the other during the reconstitution step after extraction (termed "post-spiked"). Same volume (20 µL) of the final extracts of both pre-spiked and post-spiked samples, and 12.5 ng/mL mixed standard in 50% water/50% methanol (termed "standard") were injected. Recovery,

matrix effect and the total extraction efficiency was calculated by peak area ratio of pre-spiked/post-spiked, post-spiked/standard, and pre-spiked/standard, respectively.[50]

5.2.8 Quantitative Structure Activity Relationships (QSARs)

QSARs were processed with the Toxicity Estimation Software Tool[51] (TEST, EPA, version 4.2.1). A 96 h fathead minnow 50% lethal concentration (LC_{50}, lethal concentration required to kill 50% of the tested population) and the developmental toxicity were predicted as endpoints. Five different methods[51] (detailed in **Table 5-5**) were used in each toxicity prediction, and the average value was presented.

5.3 Results and Discussion

5.3.1 Method Performance

The main objective of the method development was to maximize method sensitivity for contaminant discovery for a wide range of analytes, whereas quantitation (recovery or linearity) was not a priority. Sensitivities of the SBSE-HPLC-Orbitrap method for PFAS detection ranged from 0.005 ng/mL to 0.25 ng/mL (**Table 5-4**), which is better than those reported for some targeted HPLC-MS/MS analyses of serum. For example, the MDL of PFOS in the current study was 0.005 ng/mL, which is lower than the MDL of 0.2 ng/g in an ENVI-Carb-HPLC-MS/MS analysis[52] or the MDL of 0.08 ng/mL in an SPE-HPLC-MS/MS method for serum.[53] Back *et al*.[54] obtained increased sensitivities for PFASs in aqueous samples using large volume injection (900μL), with MDLs ranging from 0.00071-0.067 ng/mL. Our MDLs are comparable for a few known PFASs (e.g. PFHxS: 0.005 ng/mL *vs* 0.0017ng/mL[54]). MDLs for authentic OH-PCBs were within the range of 0.005-0.05 ng/mL, which is comparable to MDLs reported for targeted analysis by GC-MS/MS (e.g. 0.01 ng/mL[55]) or HPLC-MS/MS (e.g. Cl_5-, Cl_6-, Cl_7-OH-PCB: 0.001ng/g[56]). The SBSE method generally had >50% recoveries for long chain PFASs (>C_{12} PFCAs and >C_6 PFSAs) as well as for most OH-PCBs and SO_4-PCBs (**Figure 5-1**). Matrix effects for C_6-C_{11} PFASs and a few OH-PCBs and SO_4-PCBs were measurable but acceptable for this large volume of serum. However, short- and very long chain PFASs, as well as most SO_4-PCBs, had major matrix effects. Nevertheless, the MDLs descried above take recoveries and matrix effects into account, and the overall method performance was good for a wide range of

polar and ionic analytes.

5.3.2 Organochlorine and Organofluorine Contaminant Discovery

The fragmentation flagging scans of pooled serum extracts from Hudson Bay and Beaufort Sea showed hundreds of peaks (e.g. $[^{35}Cl]^-$ and $[^{37}Cl]^-$ peaks, and $[C_2F_5]^-$, $[COF_3]^-$, $[SO_2F]^-$ peaks, **Figure 5-2**) corresponding to unknown organochlorine and organofluorine analytes. In total, 16 contaminant classes (317 peaks), including 12 organochlorine classes, 3 organofluorine classes and 1 mixed organo-chlorine/fluorine class were discovered in the two pooled serum samples, with none of these detected in any quality control blank. The 16 classes were broadly categorized into 3 major groups for discussion below: Group 1, PCB metabolites (**Class 1.1** through **Class 1.7**, 272 peaks total), Group 2, other polychlorinated compounds (**Class 2.1** through **Class 2.5**, 11 peaks total), and Group 3, perfluoroalkyl sulfonates (**Class 3.1** through **Class 3.4**, 34 peaks total). All detected congeners or isomers within each class will be presented, including previously known and new discoveries, but newly discovered compounds will be highlighted.

Some general notes and assumptions are also important to state at the outset. First, the detected analyte profiles (known and unknown chemicals) in each class were very similar between the two pooled polar bear serum samples. This is demonstrated by comparison of in-source organochlorine and organofluorine fragment chromatograms in **Figure 5-2**. For simplicity, only results for the Hudson Bay sample are shown because peak intensities were generally higher in this pooled sample, except for **Class 3.4** (x:2 Cl-PFAES), where signals were higher in the Beaufort Sea sample. Second, multiple peaks (or isomers, peaks of ≤1000 counts in intensity were not considered) were observed for nearly all discovered ions, and the core structures that were elucidated by MS^n experimentation with the major isomers (within a class) were assumed to be true for minor peaks within the same class. Third, the core structure elucidated for one homologue, or congener, was assumed to be representative for the whole class of contaminants identified based on their analyte ions.

5.3.3 Group 1. PCB Metabolites

Class 1.1: Mono-hydroxylated PCBs, OH-PCBs, $[C_{12}OCl_nH_{9-n}]^-$, n=3-9

Reports have noted the presence of approximately 40 OH-PCBs in polar bear serum, with

approximately 25 Cl_5- to Cl_9-OH-PCBs (i.e. n=5-9 Cl) confirmed by authentic standards already.[21, 56-63] Here by Nt-HRMS 54 analytes were detected in both pooled polar bear serum samples that fit the empirical formula of OH-PCBs, but in this case ranged from Cl_3- to Cl_9-OH-PCB congeners (**Figure 5-3.1**, **Table 5-6**).

MS^2 experiments were performed for all prospective OH-PCB congeners detected in the Hudson Bay sample (e.g. Cl_6- and Cl_9-OH-PCB results in **Figure 5-3.2**), as well as for injections of seven authentic OH-PCB standards (**Figure 5-3.3**). Same fragmentation patterns were observed in the sample compared to authentic standards, with consecutive neutral losses of HCl, followed by a neutral loss of CO (for peaks of high intensity) and/or neutral loss of Cl_2 (for highly chlorinated Cl_7-Cl_9 congeners, e.g. Cl_9 congener in **Figure 5-3.2**b). By matching retention times and MS^2 spectra to authentic standards, the most abundant Cl_6- and Cl_7-OH-PCB congeners in both pooled polar bear sera were confirmed as 4-OH-PCB146 and 4-OH-187, respectively (CL=1, **Table 5-6**). This discovery is not novel as 4-OH-PCB146 and 4-OH-187 are known major OH-PCB congeners in many biotic organisms,[58, 64] including polar bears.[21, 57-60, 62]

For the rest of the unknown OH-PCB congeners, $[C_2OCl]^-$ fragment were absent for all congeners, but it presented for all authentic *meta* OH-PCB standards (i.e. 3- or 3'-OH-PCBs, **Figure 5-3.3**), suggests a dominance of *para* OH-PCBs (i.e. 4- or 4'-OH-PCBs), and is consistent with previous studies.[21, 57-60, 62] Further lending evidence to the core structures of these non-target analytes, breaking of the biphenyl bond was observed for a Cl_7-OH-PCB standard (i.e. $[C_6HOCl_4]^-$ for 4-OH-PCB 187, **Figure 5-3.3**b) and for some Cl_6-Cl_9 congeners in polar bears, including $[C_6HOCl_4]^-$ for one Cl_6- (congener 8 in **Figure 5-3.2**a), two Cl_7- and one Cl_8-congeners, and $[C_6OCl_5]^-$ for one Cl_8- and one Cl_9- (**Figure 5-3.2**b) congeners. The combined evidence allowed high confidence for assigning this whole class of non-targeted analytes as OH-PCBs, CL=3 was assigned for all OH-PCBs that could not be matched to an authentic standard (**Table 5-6**). Thus, for most of the OH-PCBs, the exact position of the OH and Cl substitution is uncertain using the current methodology.

More than 100 structurally confirmed OH-PCB congeners, varying from Cl- to Cl_9-OH-PCBs have been reported in various samples (e.g. water,[65-67] sediment,[65, 67-69] air,[67, 70] fish,[58, 65, 71] birds,[58, 72] seals,[58, 72, 73] polar bears,[21, 57-60, 62] humans[11, 61] and commercial Aroclors[68]). In polar bears, among the ~40 congeners previously detected,[21, 57-62] the Cl_5- to Cl_7- congeners are most

common, while Cl_4- (3 congeners of unknown structure[56]), Cl_8- (4'-OH-PCB 201 and 3-PCB 203[21]) and Cl_9 (4'-OH-PCB 208[60-62]) are rarely reported. Here then, the novel discoveries in polar bear are the Cl_3-OH-PCBs (4 congeners) and a greater number of Cl_4-OH-PCBs (approximately 10 congeners) (**Table 5-6**). Failure to detect any Cl- or Cl_2-OH-PCB congeners in the current study and in previous reports is most likely attributable to their low bioaccumulation potentials in polar bears,[11, 57] or in the marine foodweb.[74]

Class 1.2: Di-hydroxylated PCBs, DiOH-PCBs, $[C_{12}O_2Cl_nH_{9-n}]^-$, n=4-8

Only one DiOH-PCB (i.e. 4,4'-diOH PCB 202) has been reported in polar bear.[21, 57, 60-62] Nt-HRMS analysis in the current study detected 37 peaks present in both pooled serum samples that fit the empirical formula of Cl_4- to Cl_8-DiOH-PCBs (**Figure 5-4.1**, **Table 5-6**).

MS^2 experiments were performed with putative Cl_5- (**Figure 5-4.2**a) and Cl_8-DiOH-PCB congeners (**Figure 5-4.2**b) in the Hudson Bay sample). Characteristic **Class 1.1** OH-PCB fragmentation patterns were observed, such as consecutive loss of HCl and CO. Moreover, the diagnostic neutral loss of two CO was observed for DiOH-PCB congeners present at high intensity in serum (e.g. Cl_5-DiOH-PCB congener 10 and 12, **Figure 5-4.3**a, and Cl_8-DiOH-PCB, **Figure 5-4.3**b), which is consistent with a DiOH-PCB structure and was observed for two authentic Cl-DiOH-PCB standards (**Figure 5-4.3**).

Because DiOH-PCBs have the same molecular formula as hydroxylated polychlorinated diphenyl ethers (OH-PCDEs), we considered if the detected peaks may be diphenyl ethers. A Cl_3-OH-PCDE standard (2, 4, 4,'-trichloro-2'-OH-PCDE, also known as triclosan) was therefore analyzed. Unlike the two authentic DiOH-PCB standards or any of the non-target analytes we believed to be DiOH-PCBs, no stable MS^2 fragment was obtained for Triclosan. By in-source fragmentation, strong $[C_6H_3OCl_2]^-$ and $[C_6H_3O_2Cl]^{\bullet-}$ ions were produced (**Figure 5-4.4**), indicating preferential breaking of the ether-bond for OH-PCDEs. Moreover, the retention time of Triclosan (24.7 min, **Figure 5-4.4**) was much longer than the prospective DiOH-PCBs with the same numbers of chlorine substitution (12-17 min, **Figure 5-4.3**).

Taken together, our data confirm that the non-target analytes in polar bear serum are DiOH-PCBs (CL=3) (**Table 5-6**), but with the position of chlorine and hydroxyl substitution being uncertain. Both authentic catechol DiOH-PCB standards produced $[C_2H_3O_2]^-$ ions in MS^2 (**Figure 5-4.3**), which was not observed for any of the Cl_5- and Cl_8-DiOH-PCBs examined by

MS^2 in polar bears here (**Figure 5-4.2**), suggesting a dominance of congeners with non-adjacent hydroxyl groups. This is consistent with previous reports in surface water[66] and sediments,[68] lake trout,[71] birds,[60] polar bears[21, 57, 60-62] and humans,[11, 61] where all DiOH-PCBs detected have one OH group on each phenyl ring.[11, 21, 57, 60-62, 66, 68, 71] In total, six DiOH-PCB congeners with 5-8 chlorine atoms were previously reported in water,[66] fish,[71] humans,[11] polar bears[21, 57, 60-62] and commercial Aroclors.[68] Thus, the novelty of the current Nt-HRMS work in polar bear serum is the detection of 37 DiOH-PCB congeners, ranging from Cl_4- to Cl_8-, including 7 Cl_4-DiOH-PCBs that have not been reported in any sample type previously (**Table 5-6**).

Class 1.3: PCB Sulfates, SO_4-PCBs, $[C_{12}SO_4Cl_nH_{9-n}]^-$, n=2-8

SO_4-PCBs have never been reported in polar bears. Here 51 peaks were detected in two pooled samples that fit the empirical formula of Cl_2- through to Cl_8-SO_4-PCBs (**Figure 5-5.1**, **Table 5-6**).

MS^2 analyses were performed for all prospective SO_4-PCB congeners detected in the Hudson Bay sample (e.g. **Figure 5-5.2** for Cl_4- and Cl_7-SO_4-PCBs), and for SO_4-PCB authentic standards with 1-4 chlorine atoms (**Figure 5-5.3**). The MS^2 of authentic standards and putative SO_4-PCBs were comparable and showed neutral SO_3 loss from the molecular ion and subsequent losses of HCl and CO. The loss of Cl_2 was observed only for highly chlorinated congeners (e.g. Cl_7-SO_4-PCB congener 4, **Figure 5-5.2**b). In some cases, the intact $[SO_3]^-$ ion was observed, both for standards and for analytes extracted from polar bear serum. Overall, the experimental evidence confirms that the non-target analytes in polar bear serum belong to SO_4-PCB structure. Moreover, based on matching of retention times and MS^2 spectra (**Figure 5-5.2** and **Figure 5-5.3**), we identified a Cl_2-SO_4-PCB (**Figure 5-5.1**) and a Cl_3-SO_4-PCB congener (**Figure 5-5.1**) in polar bears serum as 4-SO_4-PCB 8 and 4-SO_4-PCB 33, respectively (**Table 5-1**, **Figure 5-5.3**). Thus, CL=3 was assigned for the SO_4-PCB class, and CL=1 was assigned for the two structurally confirmed congeners.

Only four previous studies report the detection of SO_4-PCBs, including in lab studies of PCB 3 exposed rats (three SO_4-PCB 3 congeners detected)[75, 76] and PCB 11 exposed plants[77] (three SO_4-PCB 11congeners detected), and in humans with known PCB 11 exposure (one SO_4-PCB 11 detected).[78] In an *in vitro* study, Sacco *et al.*[79] incubated Cl_4- and a Cl_6-OH-PCB congener in liver cytosol or microsomes derived from adult polar bears and detected the

formation of SO_4-PCB. The discovery of a large number (i.e. 51) of SO_4-PCBs, including many highly chlorinated SO_4-PCB congeners (i.e. Cl_5 through Cl_8), in polar bear serum is an intriguing observation, especially considering the emerging evidence implicating adverse health outcomes of SO_4-PCBs in mammalian models (e.g. high binding affinity to transthyretin[80]).

Class 1.4: Hydroxylated PCB Sulfates: OH-SO4-PCBs, $[C_{12}SO_5Cl_nH_{9-n}]^-$, n=3-7

OH-SO_4-PCBs have not been detected previously in environmental samples. Here 42 peaks were detected by Nt-HRMS in both pooled polar bear serum samples that fit the empirical formula for Cl_3- through to Cl_8-OH-SO_4-PCBs (**Figure 5-6.1**, **Table 5-6**).

MS^2 experiments were performed for all prospective Cl_5-OH-SO_4-PCB congener peaks in polar bear (**Figure 5-6.2**), but no authentic standards were available. Consistent with a **Class 1.3** SO_4-PCB core structure, all congeners showed neutral loss of SO_3 from the molecular ion, and several showed continued loss of HCl or CO. We also observed loss of the fifth oxygen at CO for the most abundant congener (congener 6, **Figure 5-6.2**), which is consistent with the presence of an OH group as we observed for **Class 1.1** OH-PCBs. Therefore, this class of non-target analytes was identified as OH-SO_4-PCBs. The possibility of this class being isobaric SO_4-PCDEs was considered but ruled out because the biphenyl C-C bond was not cleaved in MS^2, even when all oxygen atoms had been lost (i.e. Congener 6, **Figure 5-6.2**). A confidence level "CL=3" was therefore assigned for this class (**Table 5-6**).

We are only aware of two reports of OH-SO_4-PCBs in the literature,[76, 81] and both were for laboratory studies where rats were exposed to PCB 3[81] or PCB 11:[76] two PCB 3 metabolites in urine[81] and one PCB 11 metabolite in bile[76] were tentatively identified as OH-SO_4-PCBs. Thus, the novelty of this Nt-HRMS discovery lies not only in the large number (i.e.42) of OH-SO_4-PCBs detected, but also the detection of a large number of highly chlorinated congeners (i.e. Cl_5- through Cl_8-) that have never been observed in samples, nor in any experimental work that we are aware of.

Class 1.5: Hydroxylated Methylsulfone PCBs, OH-$MeSO_2$-PCBs, $[C_{13}SO_3Cl_nH_{11-n}]^-$, n=3-8

OH-$MeSO_2$-PCBs have not been detected previously in environmental samples, but here 45 peaks were detected in both pooled samples that fit the empirical formula for Cl_3- through to Cl_8-OH-SO_2CH_3-PCBs (**Figure 5-7.1**, **Table 5-6**).

MS^2 analyses were performed for all prospective Cl_6-OH-SO_2CH_3-PCB congeners, but no authentic standards were available. MS^2 spectra (**Figure 5-7.2**) were more complex than for other PCB metabolites, but most unknown congeners showed an initial diagnostic loss of SO_2CH_2, demonstrating the methylsulfone functionality, and the third oxygen atom was typically lost as CO, consistent with an OH-PCB core structure. We generally characterized the MS^2 fragmentation of all unknown congeners as $[M\text{-}SR\text{-}mHCl\text{-}nCO]^-$, where SR=$SO_2CH_2$, •$SO_2CH_3$, $SOCH_2$, •$SOCH_3$, SCH_2, or •SCH_3, and m=0-2 and n=0-2. The •$SOCH_3$ or $SOCH_2$ loss is produced by -SO_2CH_3 rearranging to -OS(O)CH_3 in the mass spectrometer, a commonly observed rearrangement for $MeSO_2$-PCBs.[82-86] The SCH_2, or •SCH_3 loss may be due to a different rearrangement. In previous GC-MS work, the electron ionization spectra of two Cl_5-OH-SO_2CH_3-PCB authentic standards[87] showed fragmentation pattern that is consistent with the current analytes, specifically the loss of 114 amu (i.e. SO_2CH_3Cl) and 177 amu (i.e. $SO_2CH_3COCl_2$).

Taking the evidence together, this class of non-target analytes was identified as OH-SO_2CH_3-PCBs (CL=3, **Table 5-6**). Positions of $MeSO_2$- and OH substitutions are uncertain, but the observation of *m/z*=94.98 $[SO_3CH_3]^-$ for congeners 2-5 probably indicates proximity between the neutral SO_2CH_3 group and the hydroxyl oxygen, which could align to form a 6-membered ring if they were in adjacent positions of the same phenyl (**Figure 5-7.2**). The absence of this particular fragment for congener 1, on the other hand, may indicate a larger distance between the $MeSO_2$- and OH for this congener (e.g. in different rings), which might also explain the greater polarity and shorter retention time of congener 1.

The only report of OH-$MeSO_2$-PCBs was in human lung and adipose tissues collected from Yusho patients,[87] where a few peaks were proposed as Cl_4-Cl_6 OH-$MeSO_2$-PCBs using low resolution GC-MS, but no confirmation of the formula by HRMS was made, and none of these matched authentic standards that were available.

Class 1.6: Di-hydroxylated Methylsulfone PCBs, DiOH-$MeSO_2$-PCBs, $[C_{13}SO_4Cl_nH_{11-n}]^-$, n=2-7

To our knowledge, DiOH-SO_2CH_3-PCBs have never been reported in any sample or experimental situation. Here 22 peaks were detected in both pooled serum samples that fit the empirical formula of Cl_2- through to Cl_7-DiOH-SO_2CH_3-PCBs (**Figure 5-8.1**, **Table 5-6**).

MS^2 analyses were run for all prospective Cl_4- (**Figure 5-8.2**a) and Cl_6- (**Figure 5-8.2**b) congeners in the Hudson Bay sample, but no authentic standards were available. Similar to **Class 1.5** OH-$MeSO_2$-PCBs discussed above, the general fragmentation for both Cl_4 and Cl_6 unknowns was $[M\text{-}SR\text{-}mHCl\text{-}nCO]^-$, where SR=$SO_2CH_2$, •$SO_2CH_3$, $SOCH_2$, •$SOCH_3$, or •SCH_3, m=0-3 and n=0-2. The conclusion that this class has one more hydroxyl than **1.5** OH-$MeSO_2$-PCBs was drawn not only from exact mass and molecular formula predictions, but from observation of an additional neutral loss of the fourth oxygen atom as CO. This is shown in **Figure 5-8.2**a for Cl_4 congeners (i.e. *m/z*=155.95 $[C_7Cl_2H_3]^{\bullet-}$ formed from *m/z*=183.95 $[C_8Cl_2H_3O]^{\bullet-}$ after sequentially loss of SO_3CH_2 and OH), and in **Figure 5-8.2**b for Cl_6 congeners (i.e. *m/z*=331.9 $[M\text{-}SO_2CH_3\text{-}2CO]^-$ and *m/z*=260.9 $[M\text{-}SO_2CH_2\text{-}2HCl\text{-}2CO]^-$). The alternate possibility that these non-target analytes were isobaric congeners of OH-SO_2CH_3-PCDEs was ruled out by the MS^2 spectra, whereby all oxygen atoms could be lost without breaking the biphenyl structure. Thus, this class was confirmed at DiOH-SO_2CH_3-PCBs (CL=3, **Table 5-6**).

Class 1.7: PCB Sulfonates, SO_3-PCBs $[C_{12}SO_3Cl_nH_{9-n}]^-$, n=3-6

To the best of our knowledge, PCBs with sulfonate or sulfonic acid substitution have not been reported previously in environmental samples, nor in experimental studies. Here, 21 peaks were detected in both pooled samples that fit the empirical formula of Cl_3- through to Cl_6-SO_3-PCBs (**Figure 5-9.1**, **Table 5-6**).

MS^2 experiments were performed for all prospective Cl_4 congeners in the Hudson Bay serum sample (**Figure 5-9.2**). Characteristic neutral loss of SO_2 (i.e. *m/z*=304.91) was observed for each congener, which is analogous to fragmentation of alkylbenzenesulfonates.[88] Moreover, for the most abundant congeners (congeners 1, 2 and 6, **Figure 5-9.2**), diagnostic intact $[SO_3]^-$ and $[SO_3Cl]^-$ ions were observed, confirming the presence of sulfonate functionality. These two fragments are also analogous to $[SO_3]^-$ and $[SO_3F]^-$, two diagnostic fragments for PFASs.[89] While also considering neutral loss of HCl from the abundant congeners (**Figure 5-9.2**) the SO_3-PCB structure was therefore assigned for this class of non-target analytes (CL=3, **Table 5-6**).

5.3.4 Group 2. Other Polychlorinated Compounds

Class 2.1: Chlorinated Aromatic Compounds, $[C_8Cl_nH_7\text{-}nO]^-$, n=6 and 7

A Cl_6- and a Cl_7- analytes with the general molecular formula $C_8Cl_nH_{7-n}O^-$ were detected in

both serum samples (**Figure 5-10**). The high double bond equivalent (i.e. DBE=5) of the predicted formulae, and the high stability of the molecular structure (i.e. no C-C cleavage, even under very high HCD energy of 145 eV, **Figure 5-10**) suggested an aromatic structure. Both analytes showed neutral loss of Cl_2, and the Cl_6- analyte also showed neutral loss of HCl in MS^2 experiments. Overall, this suggests hydroxyl-chlorostyrene as the most probably structure (CL=3, **Figure 5-10**, **Table 5-6**).

It is noteworthy that 4-hydroxylated heptachlorostyrene (4-OH-HpCS) was previously detected in the plasma of fish,[90] humans,[91] ringed seald[92] and of polar bears,[92] and is believed to be a metabolite of the industrial byproduct octachlorostyrene.[90-92] The molecular formula of the Cl_7- non-target analyte detected here is consistent with 4-OH-HpCS. In polar bears, 4-OH-HpCS presents at 5-216 times higher levels than octachlorostyrene,[92] and to our knowledge, no Cl_6-hydroxylchlorostyrene has been reported in any sample yet.

Class 2.2: Chlorinated (Iso)Quinoline Sulfonates, $[C_9Cl_nH_{6-n}NSO_3]^-$, n=5 and 6

Two Cl_5- and one Cl_6- non-target analytes were detected in both pooled polar bear serum samples that fit the molecular formula of $C_9Cl_nH_{6-n}NSO_3^-$ (**Figure 5-11**). The high DBE value (i.e. 7) and the high stability of the nitrogen-containing C_8 core molecular structure under high fragmentation energy (e.g. HCD145) suggested an N-heterocyclic aromatic structure (i.e. quinoline or isoquinoline). In MS^2 analyses of the Cl_5- and Cl_6- major congeners, the sulfur atom was lost as $[M\text{-}SO_2]^-$ or $[M\text{-}SO_3]^-$ for both, and weak $[SO_3Cl]^-$ and $[SO_3]^-$ fragments were observed for the major Cl_5- congener, indicating a sulfonate functional group. The structure for this class was therefore proposed as chlorinated (iso)quinoline sulfonates with a confidence level of "3" (**Table 5-6**). We are not aware of such analytes being reported previously in any sample.

Fluorinated quinolines have been used in agriculture, in liquid crystals, cyanine dyes and pharmaceutics.[93-95] One of the most common methods to synthesize fluorinated quinolones is the displacement of chlorine substituents in highly chlorinated quinolines with fluorine.[93, 96, 97] The three non-target analytes discovered in both serum samples may originated from this industrial process.

Class 2.3: Tetrachlorinated Aromatic Sulfate, $[C_{14}Cl_4H_7SO_4]^-$

The molecular formula of a single non-target analyte was confirmed as $[C_{14}Cl_4H_7SO_4]^-$, and MS^n experiments were performed to explore molecular structure (**Figure 5-12**). The

fragment $[M\text{-}SO_3]^-$ and subsequent sequential losses of HCl and CO, as was observed for SO_4-PCBs (**Class 1.3**), indicated an aromatic sulfate structure. Multiple structures, including SO_4-DDE, are possible (CL=3, **Figure 5-12**, **Table 5-6**). It is noteworthy that DDE is among the most abundant environmental POPs,[98-100] and the molecular formula of the non-target analyte detected here is consistent with a DDE-sulfate (SO_4-DDE). SO_4-DDE has not been reported previously, but methyl sulfone DDE ($MeSO_2$-DDEs[99]) was detected in polar bears,[101, 102] and hydroxylated DDE isomers (OH-DDEs) were reported in sea birds and seals.[72] Further studies using an authentic standard are needed to further identify this metabolite.

Class 2.4: Heptachlorinated Hydroxylated Nitroaromatic Compounds $[C_{14}Cl_7H_5NO_6]^-$ and $[C_{14}Cl_7H_7NO_6]^-$

Two non-target Cl_7- analytes with the molecular formula $C_{14}Cl_7H_5NO_6^-$ (**Figure 5-13**a) and $C_{14}Cl_7H_7NO_6^-$ (**Figure 5-13**b) were detected in both serum samples. Losses of $\bullet NO_2$, $\bullet NO$ and/or HNO_2 from both analytes in MS^2 experiments are highly characteristic of nitroaromatic structures.[103-105] The DBE values of 9 and 8 indicated that at most two and one phenyl rings are allowed in the $C_{14}Cl_7H_5NO_6^-$ and $C_{14}Cl_7H_7NO_6^-$ molecular structures, respectively. The common fragmentation patterns for both compounds include consecutive neutral HCl losses, followed by up to two CO losses, suggesting the presence of at least two phenolic hydroxyl groups in both analytes. Taken together, the structure for these two analytes was assigned as hydroxylated nitroaromatics with a confidence level of "3" (**Table 5-6**). Despite that the exact structures remain unknown, nitroaromatics are characterized as persistent, acutely toxic, mutagenic and carcinogenic; and their oxidized and reduced products can also cause DNA damage.[106] We note that a hydroxylated nitro-PCDE is also a theoretically possible structure for $C_{14}Cl_7H_5NO_6^-$. Some legacy herbicides are nitro-PCDEs (e.g. nitrofen),[107] which can be metabolized to hydroxylated nitro-PCDEs.[108]

Class 2.5: Hexachlorinated compounds: $[C_{15}Cl_6H_{13}O_5]^-$ and $[C_{15}Cl_6H_{15}O_3]^-$

Two non-target ions detected in both serum samples fit to the molecular formula $C_{15}Cl_6H_{13}O_5^-$ (2 peaks, **Figure 5-14**a) and $C_{15}Cl_6H_{15}O_3^-$ (1 peak, **Figure 5-14**b), which were the only possible formula predictions when allowing reasonable atom numbers (i.e. 6 Cl, 0-100 C, 0-100 H, 0-1 N, 0-10 O and 0-1 P). Sequential HCl loss was observed for all 3 analytes in MS^2 spectra at low collision energy, with loss of CO_2 being observed for the major $C_{15}Cl_6H_{13}O_5^-$ peak,

suggestive of a carboxylate moiety. No stable fragments could be obtained at higher fragmentation energies, hindering further structural characterization. No structure therefore could be proposed for these three analytes, and thus a confidence level of "4" was assigned (**Table 5-6**).

5.3.5 Group 3. Perfluoroalkyl Sulfonates (PFSAs)

Class 3.1: Cyclic or Unsaturated PFSAs, $[C_nF_{2n-1}SO_3]^-$, n=8-10

A homologous class of analytes was discovered by in-source fragmentation flagging based on $[SO_3]^-$ and $[SO_3F]^-$ peaks in both samples which corresponded to molecular ions of PFSAs with 8-10 carbon atoms, but with 2 fewer F atoms (**Figure 5-15.1**). Multiple partially resolved isomers were observed for each carbon chain length-homologues, and at least 8 non-target analytes were discovered in this class based on the combined chromatographic and fragmentation behavior. In MS^2 experiments, $[SO_3]^-$ and $[SO_3F]^-$ were produced (**Figure 5-15.2**), consistent with in-source fragmentation behavior. Diagnostic perfluoroalkenyl ion fragments (e.g. $C_5F_9^-$, $C_8F_{15}^-$) were observed for all isomers, indicating a ring or double-bond in the core structure.

No authentic unsaturated PFSA standard is currently available, but we examined the only cyclic PFSA standard available, 4-PFECHS. In MS^2 experiments, 4-PFECHS produced perfluoroalkenyl fragment ions as well as $[SO_3F]^-$ (**Figure 5-15.3**a). However, it did not yield a $[SO_3]^-$ fragment which is characteristic of sulfonates, possibly because the sulfonate group is connected to a tertiary carbon. For example, the $[SO_3]^-$ fragment is also absent in the MS^2 spectrum of 1-perfluoromethyl-PFOS (**Figure 5-15.3**b). Thus, C_8-homologue isomer 2 (and possibly also isomer 4) in the polar bear serum could either be a cyclic PFSA, where SO_3 is directly connected to the ring, or an α-branched unsaturated PFSA (example structures are shown in **Figure 5-15.2**). In contrast, isomer 1 and 3 are probably cyclic or unsaturated PFSAs with a secondary α-carbon. None of the non-target analytes matched 4-PFECHS, but isomer 2 is possibly 2- or 3-PFECHS, since its retention time and MS^2 behavior was similar to an unknown isomer present in the 4-PFECHS standard. This class of analytes were proposed as unsaturated or cyclic PFSAs, thus with a confidence level of "3" (**Table 5-6**). We also detected residual C_8- (8 isomers) and C_9- (3 isomers) homologues in a historical commercial PFOS standard from 3M Co. (denoted as "3M-PFOS' hereafter, **Figure 5-15.1**), but none of the C_8- isomers matched those in polar bear serum.

We are unaware of any previous reports of unsaturated PFSAs in any biological sample, but in total 5 cyclic PFSAs have been previously reported in waters,[109, 110] sediment,[111] amphipods[110] and in fish from the Great Lakes[109] or St. Lawrence River.[112] Among them, 4-PFECHS[109-113] and perfluoro-4-methylcyclohexanesulfonate (4-PFMeCHS)[109] were structurally confirmed. Multiple unsaturated or cyclic PFSA isomers with 4-13 carbons were reported in 3M commercial products,[114] and in concrete impacted by AFFF.[115] We also reported C_7-C_{14} unsaturated or cyclic PFASs in fish from Chinese waters in **Chapter 4**.

Class 3.2: Ether-PFSAs, $[C_nF_{2n+1}SO_4]^-$, n=6-9

Here a homologous group of analytes was discovered by in-source fragmentation flagging based on $[SO_3]^-$, $[SO_3F]^-$ and $[C_2F_5O]^-$ peaks in both samples that corresponded to molecular ions of PFSAs with 6-9 carbons, but with an extra oxygen atom (**Figure 5-16.1**). Multiple isomers were evident for each carbon chain-length homologue, with at least 11 isomers resolved by retention time and fragmentation behavior. Further MS^2 structure elucidation was performed with two C_7 isomers (**Figure 5-16.2**), showing characteristic $[SO_3]^-$, $[SO_3F]^-$ and perfluoroalkyl (e.g. $[C_2F_5]^-$) fragments, as well as diagnostic perfluoroalkoxy fragments (e.g. $[C_2F_5O]^-$) indicating the presence of an ether linkage.[116] Based on the available evidence, these non-target analytes were identified as ether-PFSAs with a confidence level of "3" (**Table 5-6**). We also detected residual C_8- (3 isomers) and C_9-homologues (5 isomers) in the 3M-PFOS. Only the major C_8 isomer in polar bear sample had enough sensitivity for MS^2 analysis, and which is probably of the same chemical identity as the major C_8 isomer in the 3M-PFOS standard, judged by retention time and MS^2 fragmentation spectra. Industrial PFOS and other PFSA products may therefore be unrecognized sources of ether-PFASs in the environment and wildlife.

With the knowledge that a fluorochemical production facility was producing Nafion, Strynar *et al.*[117] tentatively proposed a C_6 analyte detected in water sample downstream of the facility as a dietherfluoroalkyl sulfonate ($CF_3CHFOCF_2C(CF_3)OC_2F_4SO_3^-$) only by matching the exact *m/z*. Another report of ether-PFSA was a C_6 analyte detected in serum samples from AFFF-exposed firefighters,[31] which was proposed as $CF_3OC_5F_{10}SO_3^-$ based on MS^2 spectrum. C_7-C_{14} ether PFSAs were also detected in fish livers in **Chapter 4**.

Class 3.3: Enol-Ether- or Cyclic-Ether- or Carbonyl-PFSAs, $[C_nF_{2n-1}SO_4]^-$, n=7-9

Similar to the **Class 3.2** ether-PFSAs, another homologous group of analytes was

discovered through in-source $[SO_3F]^-$, $[C_3F_5]^-$ and $[C_4F_7]^-$ peaks (**Figure 5-17.1**). These fragments corresponded to molecular ions of PFSAs with 7-9 carbons, but with 2 fewer F atoms and an extra oxygen atom. Multiple isomers were observed for each homologue, and at least 12 isomers were discovered for this class.

MS^2 analyses were performed with all C_8- isomers (**Figure 5-17.2**), and $[SO_3]^-$, $[SO_3F]^-$ and perfluoroalkenyl fragments (e.g. $[C_4F_7]^-$) for all isomers indicated a core-structure consistent with PFSAs. Neutral $C_nF_{2n}O$ loss (i.e. leaving $C_7F_{13}SO_3^-$ and $C_6F_{11}SO_3^-$) and perfluoroalkenyl fragments were commonly observed. This, along with DBE=1, makes three structures possible: (1) Cyclic ether-PFSAs; neutral CH_2O loss is characteristic of cyclic ethers in electron ionization[118] and collision-activated dissociation[119] in GC-MS. It is possible that similar fragmentation may occur for a perfluorinated cyclic ether-PFSA. (2) Unsaturated ether-PFSAs, in other words a structural combination of the above **Classes 5.3.5.1** and **5.3.5.2**. Perfluoroalkoxy fragments are usually expected for perfluoroalkyl ethers,[116, 117] but no such fragments were observed for any C_8- isomer here, possibly indicating an enol ether structure whereby the proximal double bond may weaken the production of a perfluoroalkoxy fragment. (3) Carbonyl-PFSAs. The $C_6F_{11}SO_3^-$ fragment for isomer 6 could be formed via neutral cleavage of a $CF_3C(O)$- terminal group. The ion $C_7F_{13}SO_3^-$ produced for most of the other isomers in this class indicates loss of carbonyl fluoride (COF_2) which could be due to a terminal acid fluoride moiety. However, we believe it unlikely that such a compound would be hydrolytically stable under our alkali mobile phase condition, not to mention persistent enough to be present in polar bears. Multiple structures are possible, and a confidence level "3" was assigned for this homologous class (**Table 5-6**). Residual $C_8F_{15}SO_4^-$ and $C_9F_{17}SO_4^-$ were also detected in the 3M-PFOS industrial standard. Three C_8- isomers (i.e. isomer 1, 4 and 6, **Figure 5-17.2**) detected in polar bear serum samples matched the retention times and MS^2 spectra of three C_8 isomers detected in the 3M-PFOS. The detection of this class of non-target analytes in polar bears may therefore be from industrial PFSA products, including PFOS.

There are no previous reports of cyclic-ether-PFSAs. A C_6-analyte detected in water downstream of a fluorochemical production facility was proposed as an enol-ether-PFSA (CF_2=$CFOCF_2C(CF_3)OC_2F_4SO_3^-$), but the only evidence presented was the accurate mass.[117] Based on MS^2 spectra, homologous compounds (C_4-C_{13}) detected in concrete that had been impacted by AFFF[115] and a C_8 analyte (i.e. $CF_3C(O)C_6F_{12}SO_3^-$) detected in AFFF-exposed

firefighters' serum samples were tentatively proposed as ketone-PFSAs (i.e. carbonyl-PFSAs). This homologous of compounds (C_6-C_{13}) were also detected in fish livers in **Chapter 4**.

Class 3.4: x:2 Chlorine Substituted Perfluoroalkyl Ether Sulfonates, x:2 Cl-PFAESs, $[ClC_nF_{2n}SO_4]^-$, n=6-8

Here, a homologous class of non-target analytes was discovered by in-source fragmentation flagging of $[Cl]^-$ peaks in the Beaufort Sea pooled serum sample (**Figure 5-18.1**), and the general formula was proposed as $ClC_nF_{2n}SO_4^-$ (where n=6-8). Only one isomer was observed for each chain-length homologue, and more homologues were detected in the Beaufort Sea sample (C_6-C_8) than in the Hudson Bay sample (C_8).

MS^2 analyses were performed for the C_8 homologue in the Beaufort Sea serum sample (**Figure 5-18.2**) and for an authentic industrial standard of F-53B ($CClF_2C_5F_{10}OC_2F_4SO_3^-$), which shares the same empirical formula with the non-target C_8 homologue. Retention time (**Figure 5-18.1**) and MS^2 spectrum (**Figure 5-18.2**) of the unknown C_8 homologue in the Beaufort Sea sample matched with the C_8 homologue of the F-53B standard. Residual C_6-C_{10} chain-length homologues were also detected in the F-53B standard (**Figure 5-18.1**). Retention times of the C_6 and C_7 homologues in the Beaufort Sea serum sample also matched with the C_6 and C_7 analytes in the F-53B standard. Thus, the structure for the non-target C_8 homologue in the Beaufort Sea serum sample was identified as 6:2 terminal chlorine substituted perfluoroalkyl ether sulfonate (6:2 Cl-PFAES) with a confidence level of "1", and the non-target C_6 and C_7 analytes in the Beaufort Sea sample were assigned as 4:2 and 5:2 Cl-PFAESs with a confidence level of "2" (**Figure 5-18.1**).

F-53B is a mist suppressant used in the chrome plating industry in China since the late 1970s,[121, 122] and 6:2, 8:2 and 10:2 Cl-PFAESs have been detected in different areas of China.[122-128] We also detected 5:2-10:2 and 12:2 homologues in fish from China in **Chapter 4**. A recent study reported 6:2 Cl-PFAES in Greenland ringed seals and polar bear.[128] Here, we detected 6:2 Cl-PFAES in both polar bear serum samples, and, for the first time, report 4:2 and 5:2 Cl-PFAESs in polar bears (Beaufort Sea).

6:2 Cl-PFAES is usually present at comparable or higher levels than PFOS.[123-127] It has a stronger bioaccumulation potential than PFOS (e.g. log-transformed bioaccumulation factor was 4.3 in crucian carps, in contrast to 3.3 for PFOS[125]), and has been described as "the most bio-

persistent PFAS in humans reported to date" (median half-life 18.5 yrs[127]). In addition, 6:2 l-PFAES has been shown to be associated with acute toxicity,[123] embryo-toxicity and cardiac development disrupting effects[129] in zebrafish.

5.4 Summary and Significance

5.4.1 Summary of Discoveries

By combining a sensitive SBSE extraction step to non-target HPLC-Orbitrap MS^n, we detected 16 classes of organohalogen compounds comprising at least 317 non-target analytes in two pooled polar bear serum samples (**Table 5-6**). Although many of these analytes may represent more than one co-eluting isomer, and most will require confirmation of exact molecular structure with authentic standards (which are not currently available), 15 of these classes (314 peaks) could be confidently assigned a core structure based on various mass spectral evidence (i.e. with a confidence level of 1-3).

Considering all historic contaminant biomonitoring studies in polar bears, at least 268 analytes are reported for the first time here. This includes 215 analytes belonging to 9 newly discovered classes in polar bear (51 SO_4-PCBs, 42 OH-SO_4-PCBs, 45 OH-$MeSO_2$-PCBs, 22 DiOH-$MeSO_2$-PCBs, 21 SO_3-PCBs, 3 chlorinated quinoline/isoquinoline sulfate, 8 cyclic or unsaturated PFSAs, 11 ether-PFSAs and 12 enol-ether-,cyclic-ether- or carbonyl-PFSAs) as well as 52 novel analytes that can be categorized into 3 previously detected classes in polar bear (14 OH-PCBs, 36 DiOH-PCBs and two x:2 Cl-PFAESs).

Considering all historic environmental monitoring and biomonitoring, as well as analysis of industrial or commercial materials, 209 analytes are reported here for the first time, including 46 analytes in 3 new contaminant classes (22 DiOH-$MeSO_2$-PCBs, 21 SO_3-PCBs and 3 chlorinated quinoline/isoquinoline sulfate) and 162 novel contaminants in 7 previously reported contaminant classes (31 DiOH-PCBs, 48 SO_4-PCBs, 42 OH-SO_4-PCBs, 30 OH-$MeSO_2$-PCBs, 10 ether-PFSAs and one x:2 Cl-PFAES).

5.4.2 Concentration Estimation

For each non-target homologue or congener class discovered in the current study,

combined isomer concentration was estimated by assuming the same molar unit response as structurally similar authentic standard (**Table 5-7**), and the molar concentrations (i.e. molar/mL) were further transformed back into mass concentration (i.e. ng/mL). Specifically, all poly/perchlorinated non-targets were semi-quantified using averaged responses of all OH-PCB and SO_4-PCB standards available in the current study, because they produced similar unit response, regardless of different degree of chlorination. Each non-target PFSA homologue was semi-quantified against the calibration curve of the same carbon chain-length legacy PFSA (e.g. C_8-ether PFSA quantified by PFOS), except x:2 Cl-PFAES, where the whole class was quantified by 6:2 Cl-PFAES standard.

Among the 7 classes of PCB metabolites, the estimated total concentration follows OH-$MeSO_2$-PCB (90.1 ng/mL) > OH-PCB (26.3 ng/mL) > SO_4-PCB (13.5 ng/mL) > OH-SO_4-PCB (4.2 ng/mL) > DiOH-PCB (3.1 ng/mL) > DiOH-$MeSO_2$-PCB (2.6 ng/mL) > SO_3-PCB (1.3 ng/mL) in the Hudson Bay sample, and OH-$MeSO_2$-PCB (55.0 ng/mL) > OH-PCB (6.7 ng/mL) > SO_4-PCB (4.5 ng/mL) > DiOH-$MeSO_2$-PCB (1.2 ng/mL) > OH-SO_4-PCB (0.8 ng/mL) > DiOH-PCB (0.6 ng/mL) > SO_3-PCB (0.5 ng/mL) for the Beaufort Sea sample (**Table 5-7**, **Figure 5-19**). $MeSO_2$-PCB, OH-PCB and SO_4-PCB are the three major PCB metabolite classes in both samples, account for 92% and 96% of total PCB metabolites in the Hudson Bay and Beaufort Sea sample. Only OH-PCBs and DiOH-PCBs were measured in polar bears, and OH-PCBs were thought to be the dominant metabolite class in serum.[60-62, 92, 130] Here, the new OH-$MeSO_2$-PCB class dominates in both samples, presenting at 3.4 and 8.2 times higher levels than OH-PCBs and accounting for 64% and 80% of all measured PCB metabolites in the Hudson Bay and Beaufort Sea sample, respectively (**Figure 5-20**).

Among the 5 classes of other polychlorinated compounds, **Class 2** and **Class 2.3** presented at relatively high levels (**Table 5-7**, **Figure 5-19**), with hydroxyl-chlorostyrene and SO_4-DDE being among the possible structures, respectively. Compared to legacy PFSAs, the discovered non-target PFSAs presented at relatively low levels (<0.2 ng/mL, **Figure 5-19**).

5.4.3 Toxicological Implications

There is little or no toxicological information for the majority of analytes detected here, but it is important to consider that all were found circulating in blood serum of polar bears. The

presence of contaminants in serum opens the possibility that they exert adverse physiological effects. For example, OH-PCBs, SO_4-PCBs and 4-OH-HpCS bind to serum proteins such as albumin[131] or transthyretin, which in turn can affect thyroid homeostasis.[80, 92, 131-134] Classic PFSAs (e.g. PFOS) also have a high affinity for serum protein, including albumin.[135] It is likely that the PCB metabolites and PFSAs discovered in the current study also bind to serum proteins. Additionally, almost all of the chlorinated chemicals discovered here contain an aromatic structure (**Class 2.5** structures are unclear) which are either formed via or can undergo bioactivation to reactive intermediates, such as epoxide, (semi)quinone or radical intermediates, that can covalently bind to DNA, RNA or proteins.[11] Moreover, nitroaromatics and both of their reductive and oxidative products are associated with mutagenic or carcinogenic toxicities.[106]

An initial QSAR assessment was therefore performed for each non-target chlorinated class to estimate their toxicities (no relevant data available for estimating fluorinated contaminant toxicities), and plausible representative structures were used as input for each class. Among the multiple possible structures for each of the **Group 2** chlorinated chemical class, only the structure relating to legacy chlorinated contaminants were tested (e.g. SO_4-DDE for **Class 2.3**). For PCB metabolites, representative structures for each class were proposed based on historical reports. Among historically reported mono-substituted PCB metabolites,[11, 21, 57-62, 65-73, 75, 77, 78, 81, 87] *para-* (i.e. 4 or 4') and *meta-* (i.e. 3 or 3'/5 or 5') substituted metabolites usually dominate, while *ortho-* (i.e. 2 or 2'/6 or 6') substituted ones are minor metabolites. Therefore, *para-* and *meta-*, *para-/para-, para-/meta- and meta-/meta-,* and *para-/para-/meta-*, *para-/meta-/meta-* and *meta-/meta-/meta-* substitution was taken as representative for PCB metabolites with 1, 2 and 3 functional groups, respectively. Among the few DiOH-PCBs previously reported in surface water[66] and sediments,[68] lake trout,[71] birds,[60] polar bears[21, 57, 60-62] and humans,[11, 61] all have one OH group on each phenyl ring.[11, 21, 57, 60-62, 66, 68, 71] However, in OH-PCB exposed rats studies[136, 137] or *in vitro* CYP isoenzyme incubation study,[138] catechol-PCBs (e.g. *para-/meta-*) were usually detected. Therefore, both situation will be considered for representative PCB metabolites with 2-3 functional groups. The extent of chlorination for PCB metabolites was set at 6 because the Cl_6-congeners were almost always among the major congeners in each of the 7 PCB metabolite classes (**Table 5-7**).

For all mono-substituted PCB metabolites and most DiOH-PCBs, the 96 h fathead minnow LC_{50} values were lower than the LC_{50} of the respective parent compound (**Table 5-5**), indicating

higher toxicity for the polar metabolite. When compared to OH-PCBs, most of metabolites, including the dominant OH-$MeSO_2$-PCBs, showed a higher toxicity. For other chlorinated aromatics, the more polar metabolites usually have a higher LC_{50} value than their neutral parent compounds. As expected, the tetrachlorinated hydroxylated nitroaromatic compound (**Class 2.4**) was estimated to have a much higher toxicity than other chlorinated contaminants. All metabolites, except for 4-OH-HpCS, were estimated to possess some developmental toxicity, whereas their parent chemicals were usually classified as non-developmental toxicant.

5.4.4 PCB Biotransformation Pathways

Grimm *et al.*,[11] Dhakal *et al.*[75] and Letcher *et al.*[139] previously summarized PCB biotransformation pathways, including OH-PCBs, DiOH-PCBs, SO_4-PCBs and $MeSO_2$-PCBs, OH-SO_4-PCBs, PCB-glucuronide conjugates (OGA-PCBs), OH-OGA-PCBs, SO_4-OGA-PCBs, mercapturic acids and OH-mercapturic acids. Here we adapt and expand the schemes by considering the new classes of PCB metabolites discovered here using a representative Cl_6-PCB congener (**Figure 5-20**).[75]

Only OH-PCBs and DiOH-PCBs were previously reported in polar bears, and the new mixed class of OH-SO_4-PCBs, as detected in PCB 3 exposed rats,[75] can be formed via sulfonation of a DiOH-PCB or by hydroxylation of a SO_4-PCB.[76] The other mixed metabolite classes, OH-$MeSO_2$-PCBs and the DiOH-$MeSO_2$-PCBs are likely formed via (successive) hydroxylation of $MeSO_2$-PCBs. Up to 50 congeners of $MeSO_2$-PCBs have been reported in various samples including polar bears,[11] making this suggestion feasible. The pathway leading to formation of the novel SO_3-PCB class is not clear, and requires further studies. However, sulfonic acid metabolites were previously reported for some compounds in environmental samples (e.g. chloroacetanilide herbicides in water[140] and soil[141-145]) or in rats (e.g. menthofuran,[146] andrographolide[147] and N-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-3,4,5,6-tetrahydrophalimide[148]), and the SO_3 is either added to a double bond[146-148] or to replace a chlorine atom.[140-145] With PCBs having unsaturated bonds and chlorine atoms, based on the above studies we can propose the following two possible pathways (red arrows in **Figure 5-20**): (1) Pathway 1 involves glutathione (GSH), which reacts with PCBs[141, 145, 149, 150] or PCB epoxides,[11, 75, 150] and the resultant GSH-PCBs are successively transformed to cysteine PCB conjugates and PCB thiols.[141, 145, 148, 149] While some SH-PCBs can further be

oxidized to $MeSO_2$-PCBs,[11, 75] some may have undergone oxidation to form SO_3-PCBs (2) Pathway 2 would be mostly mediated by gut flora, which reduces SO_4^{2-} in polar bears from direct environmental exposure, or indirectly from organosulfur compound oxidization (e.g. cysteine and GSH can be metabolized to SO_4^{2-} in mammals[151]) to SO_3H^-, and adds to PCBs. In the above three rat studies, the sulfonic acid metabolites were detected urine,[146, 147] faeces[148] and/or small intestine contents,[147] but not in bile,[146-148] suggesting that SO_3H-addition probably happens in the small intestine.[146, 148]

No glucuronide-conjugates (compound **I-III** in **Figure 5-20**) or mercapturic acids (compound **IV** and **V**) were detected in the current study. OGA-PCBs (compound **I**) was previously reported as minor metabolites in urine samples from PCB 3 exposed rats (not detected in serum),[75, 76] and were detected in *in vitro* incubation studies with microsomes derived from rats,[152] catfish[153] and polar bears.[154] Minor OH-OGA-PCBs (compound **II**) and SO_4-OGA-PCBs (compound **III**) were reported in urine, but not serum, from PCB 3 exposed rats.[75] Non-detect of any glucuronide metabolites either indicates their low abundance in polar bear serum, or because the SBSE method has poor recovery for metabolites due to the high water solubility of glucuronic acid. Similar to glucuronide-conjugates, mercapturic acids (compound **IV**)[75, 155] and OH-mercapturic acids (compound **V**) were only detected in urine samples from PCB 3 exposed rats (not detected in serum),[75] and the absence of such metabolites in the current study might also be attributed to their low concentrations in serum. $MeSO_2$-PCBs (compound **IV**, previously detected in polar bears[139]) do not ionize in ESI and therefore would not be detected with the current methodology.

5.4.5 Future Studies

A recent review of temporal trend studies in Arctic biota reported an overall decreasing trend for polychlorinated contaminants (e.g. ∑PCBs, *p,'p*-DDE), and increasing trends for perfluorinated (e.g. classic PFSAs) and polybrominated contaminants.[156] However, it is unclear if metabolites follow the same general trends as the parent polychlorinated contaminants, and today the situation is further complicated by climate change. In Greenland, recent climate change is believed to have caused a "levelling-off/moderate increase" in ∑PCBs in polar bears during 2000-2010.[157] Future studies on temporal trends of the discovered chloro- and fluoro-compounds are therefore warranted while also considering spatial differences across the Arctic.

The discovery of a series of new PCB metabolites and other non-target poly/perchlorinated aromatic compounds in polar bears indicates that historical exposure to chlorinated contaminants has been underestimated. The presence of new non-target PFASs also raise questions about whether these PFASs are from historical PFAS exposure or contemporary alternative manufacturing. Toxicological consideration should be given to these novel classes of chemicals, either in future effects-directed analyses or basic toxicology tests. While challenging, understanding the individual- and population-level effects of these new pollutants raises additional concerns for the conservation and management of polar bears.

5.5 Figures and Tables

5.5.1 Figures

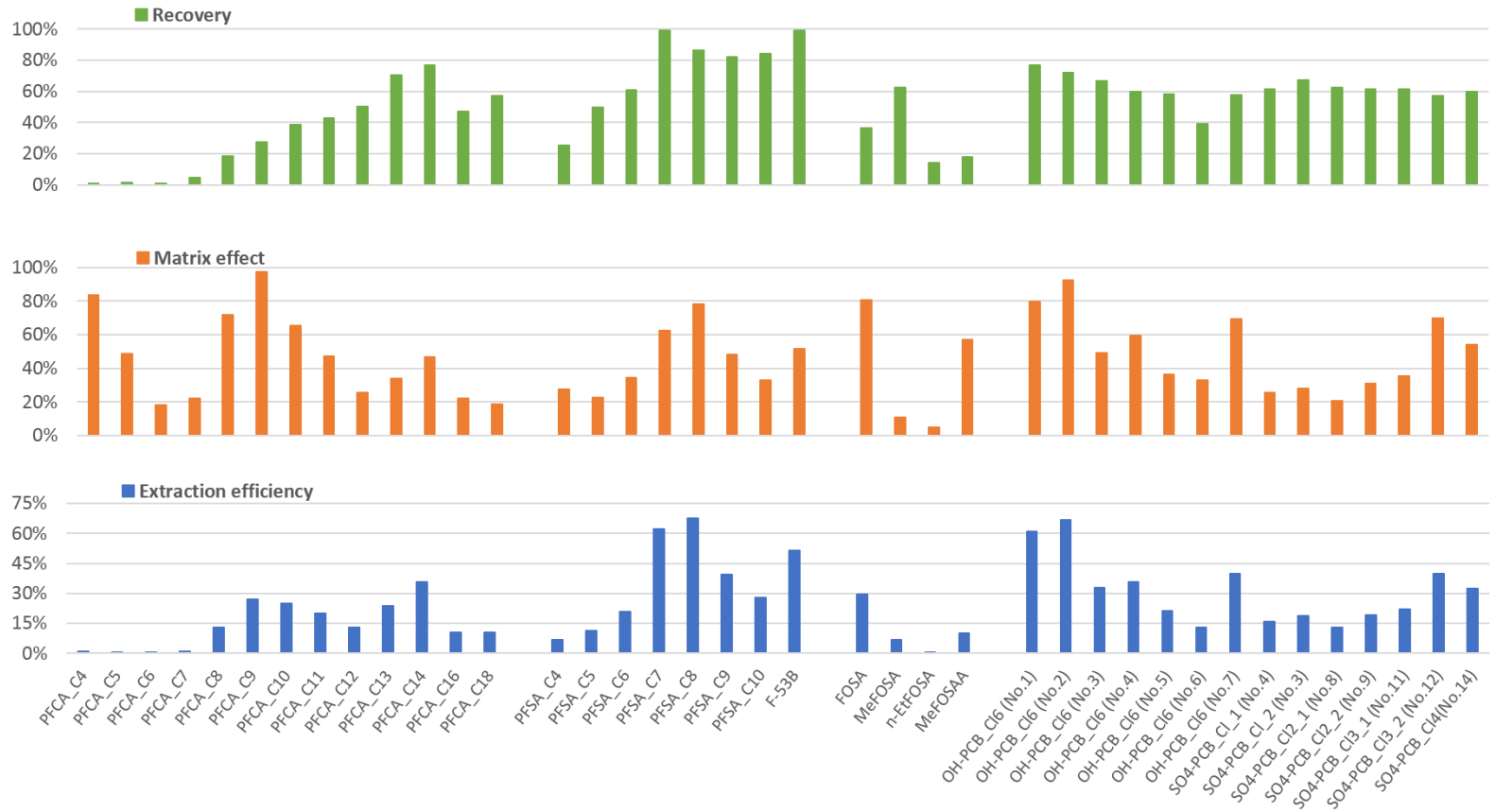


Figure 5-1. Recovery, matrix effect and total extraction efficiency of the SBSE-HPLC-Orbitrap method.

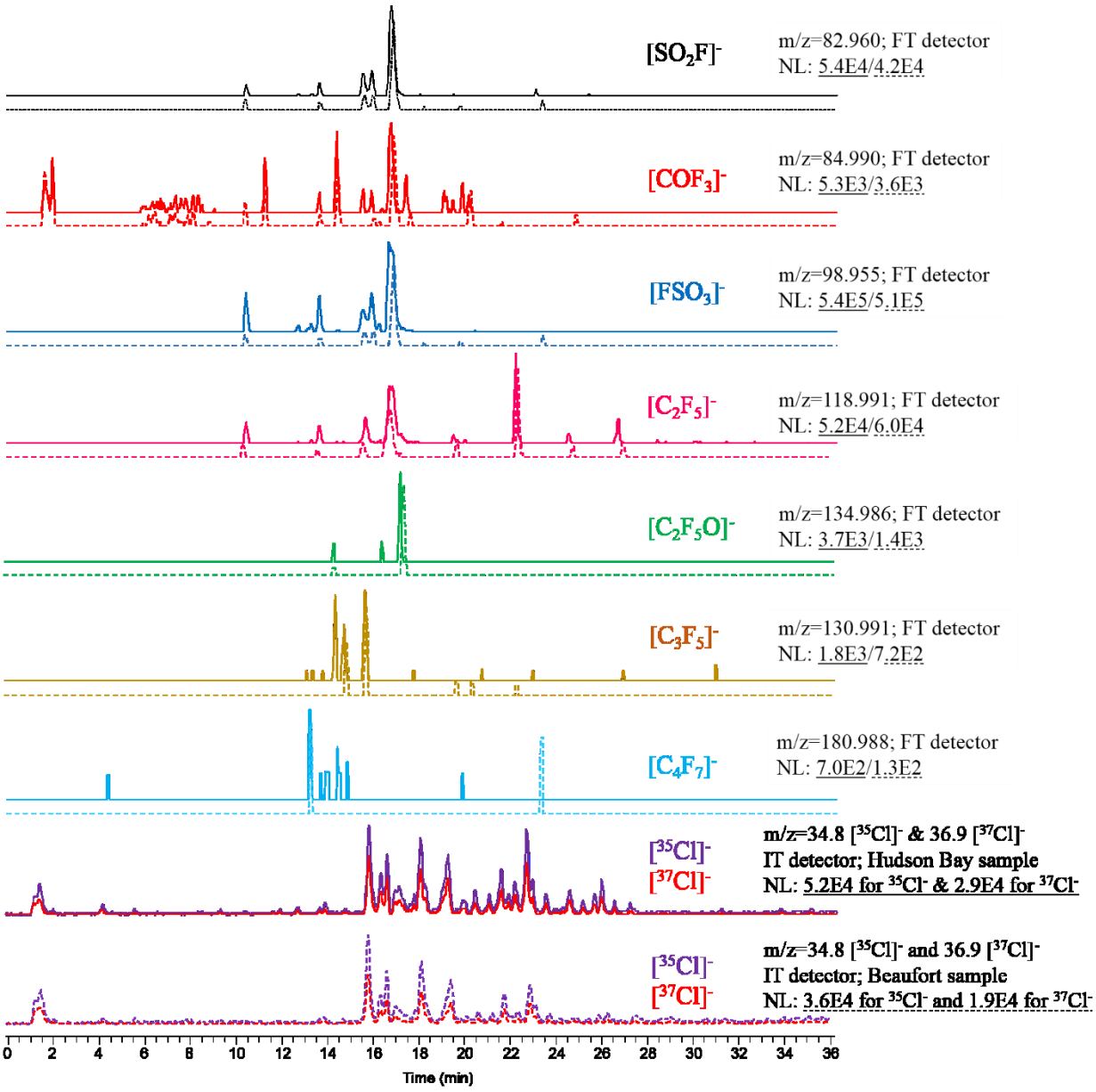


Figure 5-2. Fluorine-containing fragments detected by the Orbitrap mass analyzer (each chromatogram is for the theoretical mass of the extracted ion shown ±5 ppm), and $[Cl]^-$ fragments detected by the ion trap detector (±0.5 amu) in in-source fragmentation flagging scans in pooled polar bear serum samples from Western Hudson Bay (solid lines) and Beaufort Sea (dotted lines). The "NL" numbers are the peak height when injecting 20 µL of the SBSE extract (500 µL of polar bear serum equivalent).

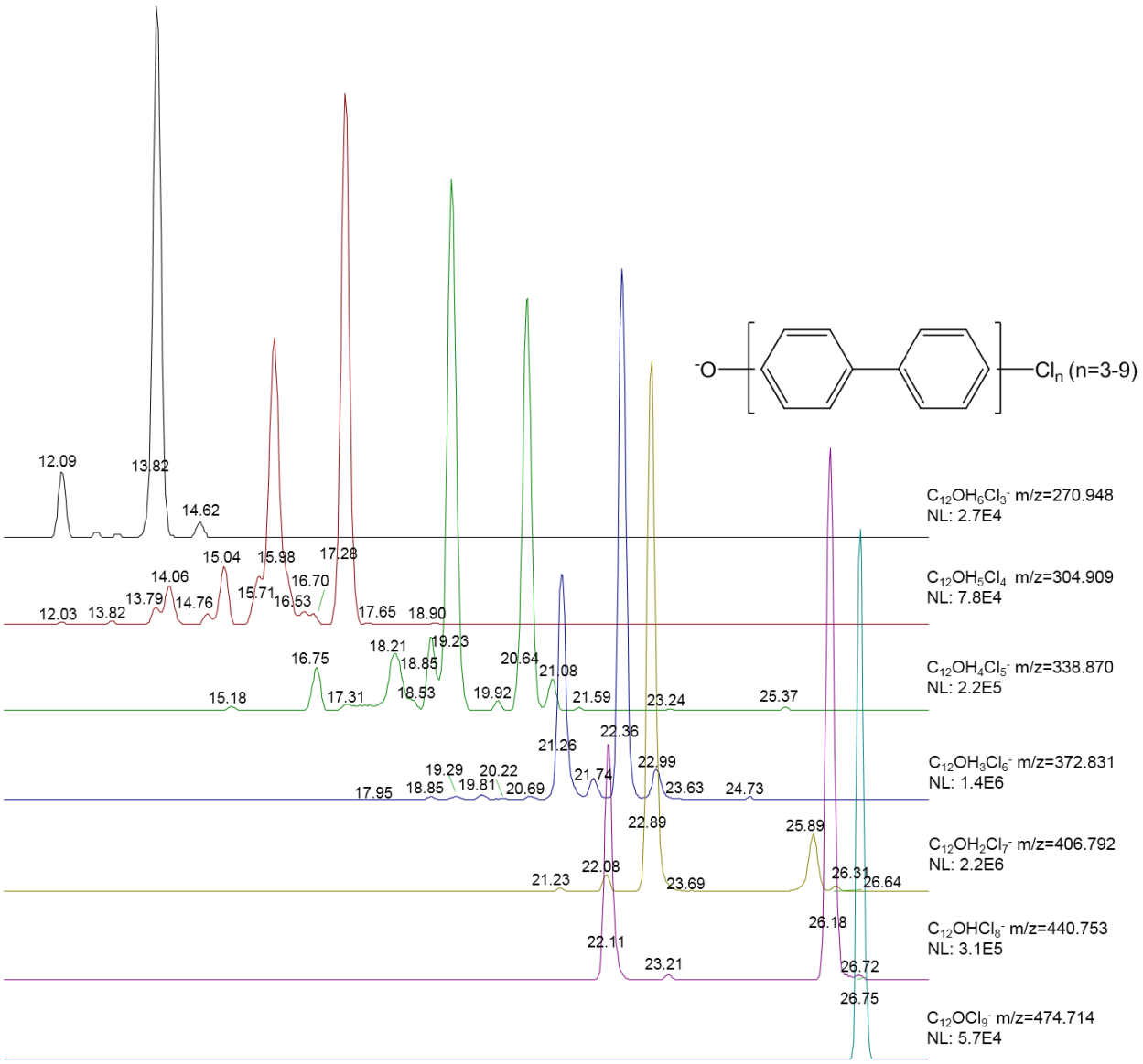


Figure 5-3.1. Extracted ion chromatograms of **Class 1.1** OH-PCB congeners ($C_{12}OCl_nH_{9-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms correspond to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass (i.e. ions where all chlorine are ^{35}Cl isotopes).

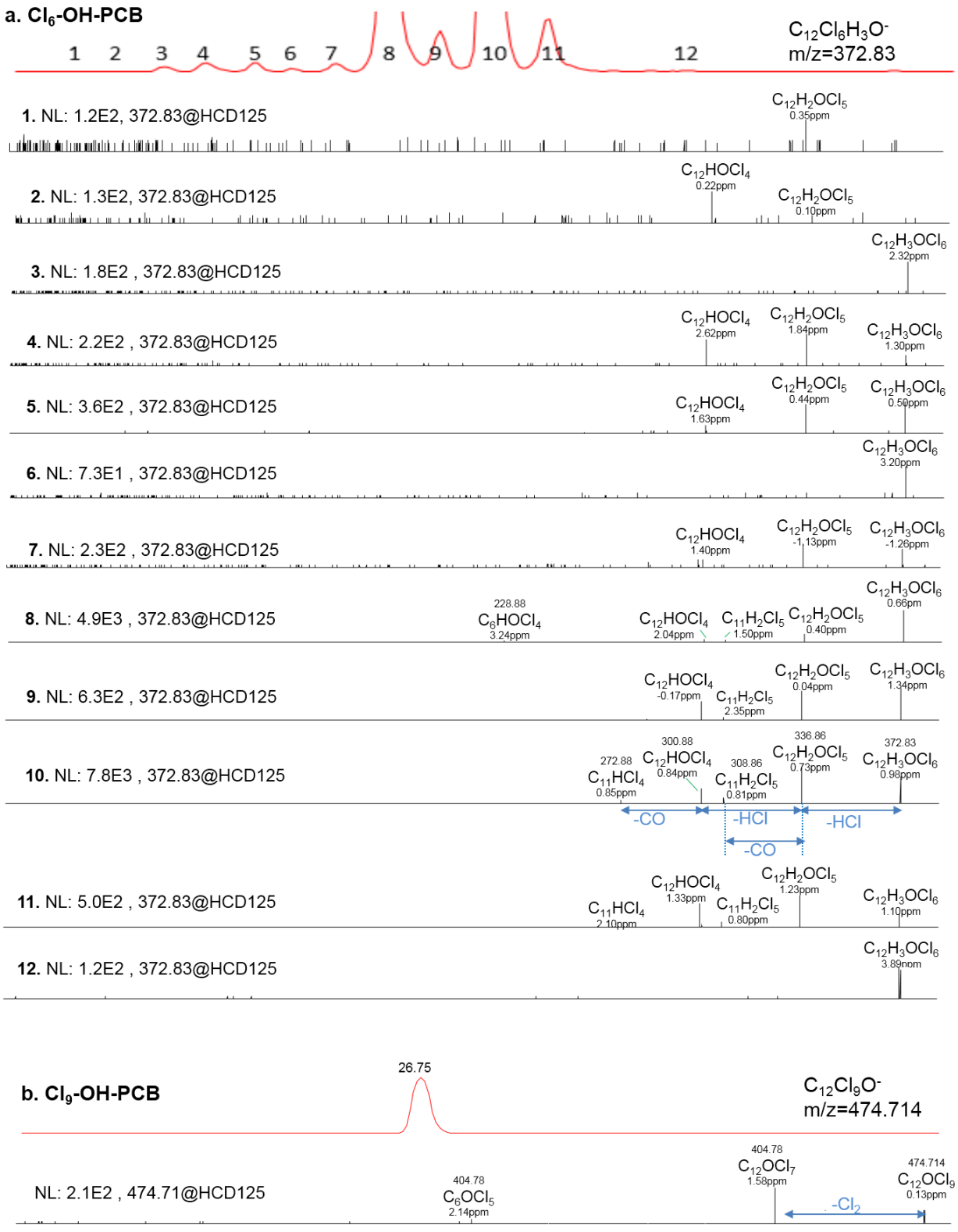


Figure 5-3.2. MS^2 spectra (HCD 125 eV) of all Cl_6-OH-PCB ($C_{12}OH_3Cl_6^-$, **a**) and Cl_9-OH-PCB congeners ($C_{12}OCl_9^-$, **Class 1.1**, **b**) in the Hudson Bay pooled polar bear serum.

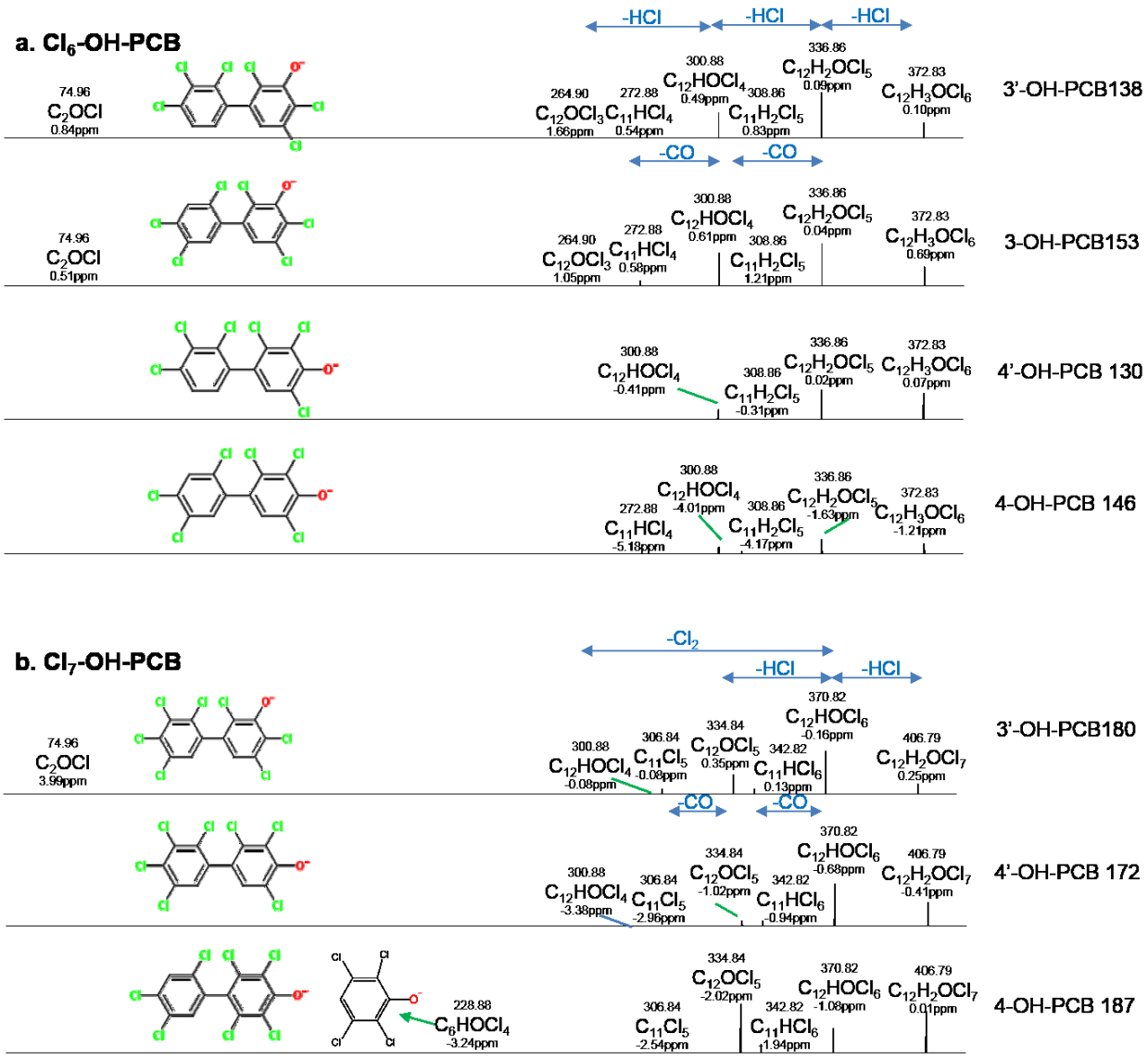


Figure 5-3.3. MS^2 spectra (HCD 125 eV) of four authentic Cl_6-OH-PCB standards ($C_{12}OH_4Cl_6$, **a**) and of three Cl_7-OH-PCB standards ($C_{12}OH_3Cl_7$, **b**).

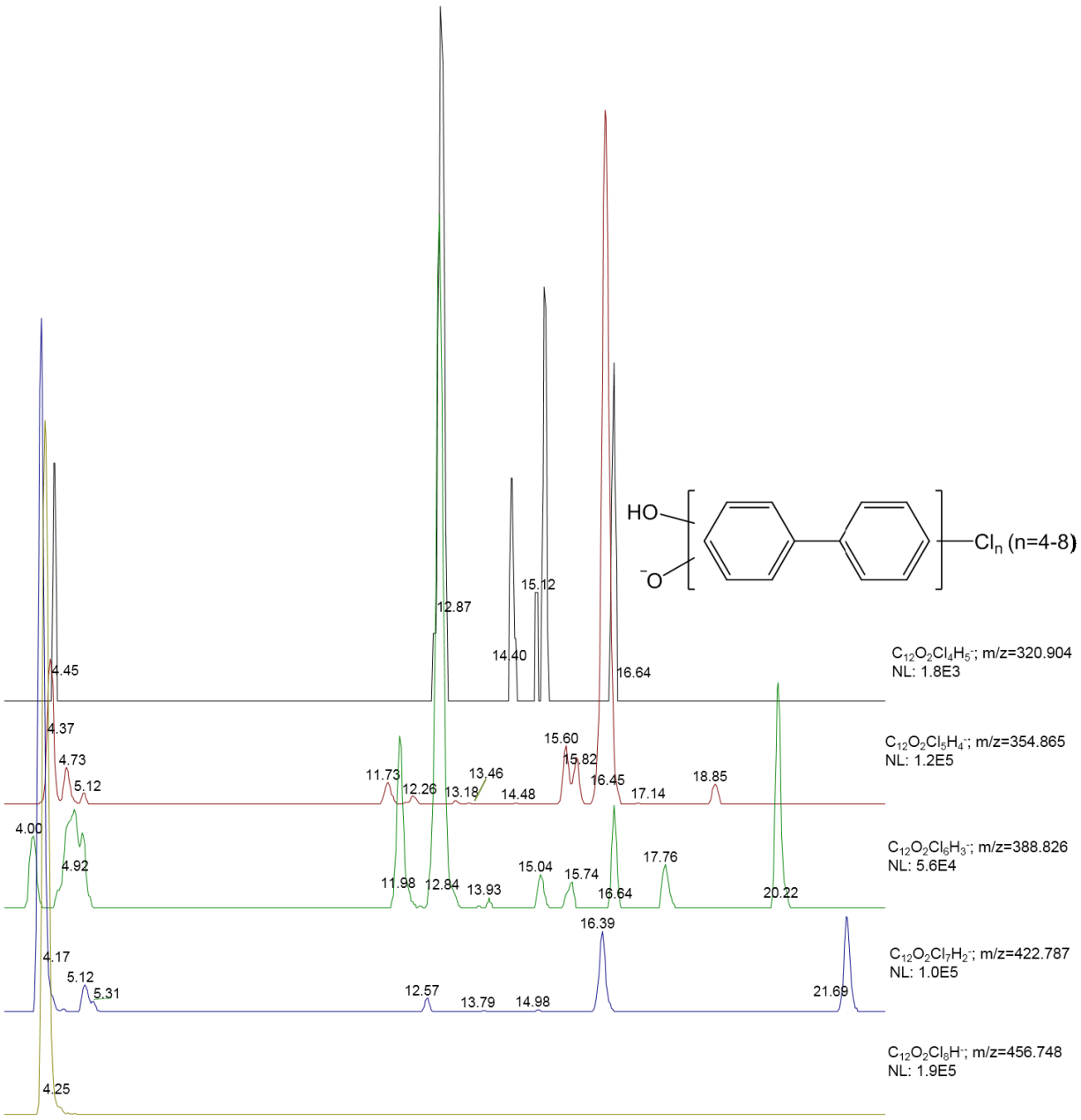


Figure 5-4.1 Extracted ion chromatograms of **Class 1.2** DiOH-PCB congeners ($C_{12}O_2Cl_nH_{9-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.

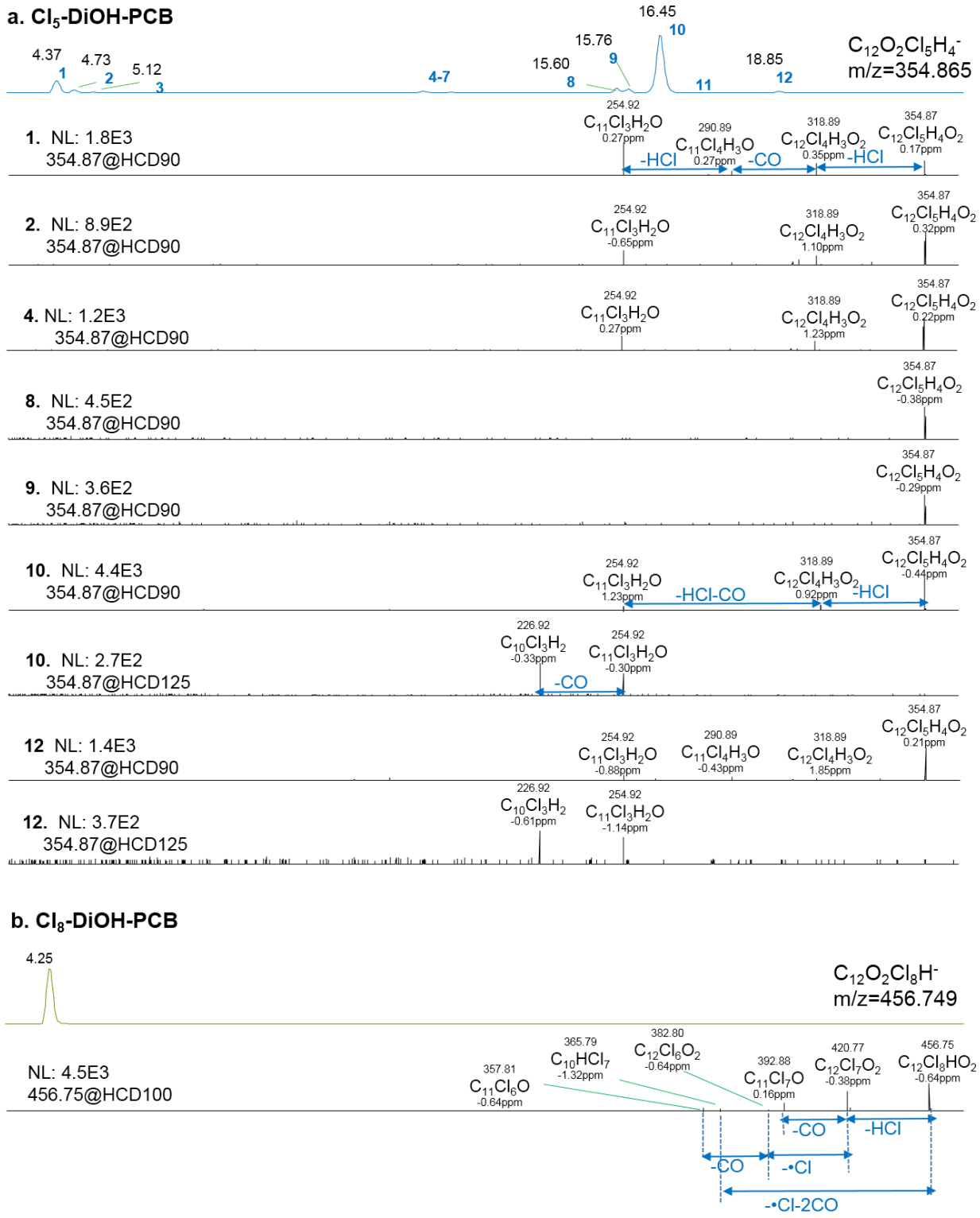


Figure 5-4.2. MS^2 spectra (HCD 90-125 eV) of selected Cl_5-DiOH-PCB ($C_{12}H_4Cl_5O_2^-$, **a**) and Cl_8-DiOH-PCB congeners ($C_{12}HCl_8O_2^-$, **Class 1.2**, **b**) in the Hudson Bay pooled polar bear serum.

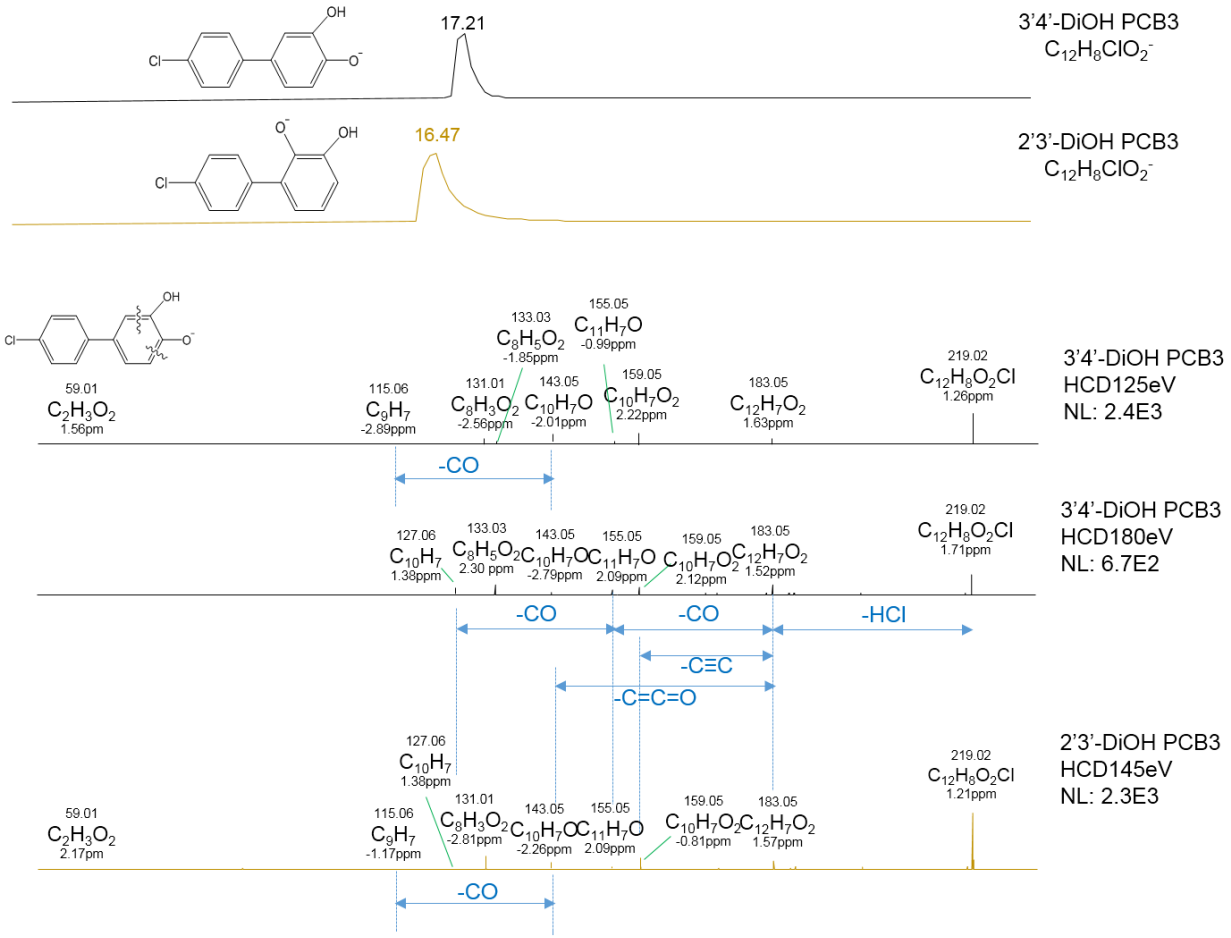


Figure 5-4.3. Extracted ion chromatograms and MS^2 spectra (HCD 125-180 eV) of 3', 4'-DiOH PCB 3 and of 2', 3'-DiOH PCB 3 standards ($C_{12}H_8O_2Cl^-$).

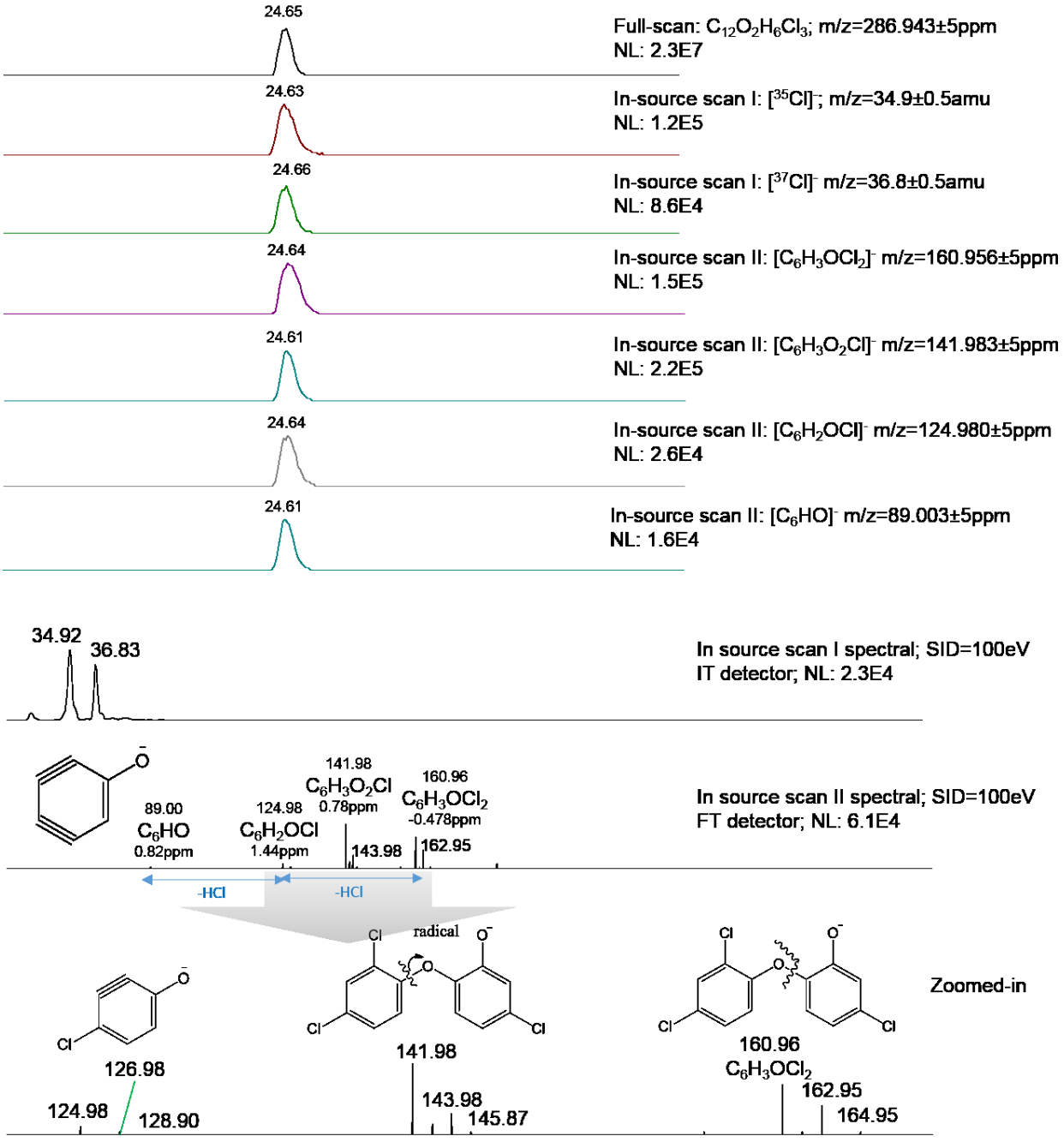


Figure 5-4.4. Extracted ion chromatograms of triclosan ($C_{12}O_2H_6Cl_3^-$) in full scan and its fragments in in-source fragmentation scan.

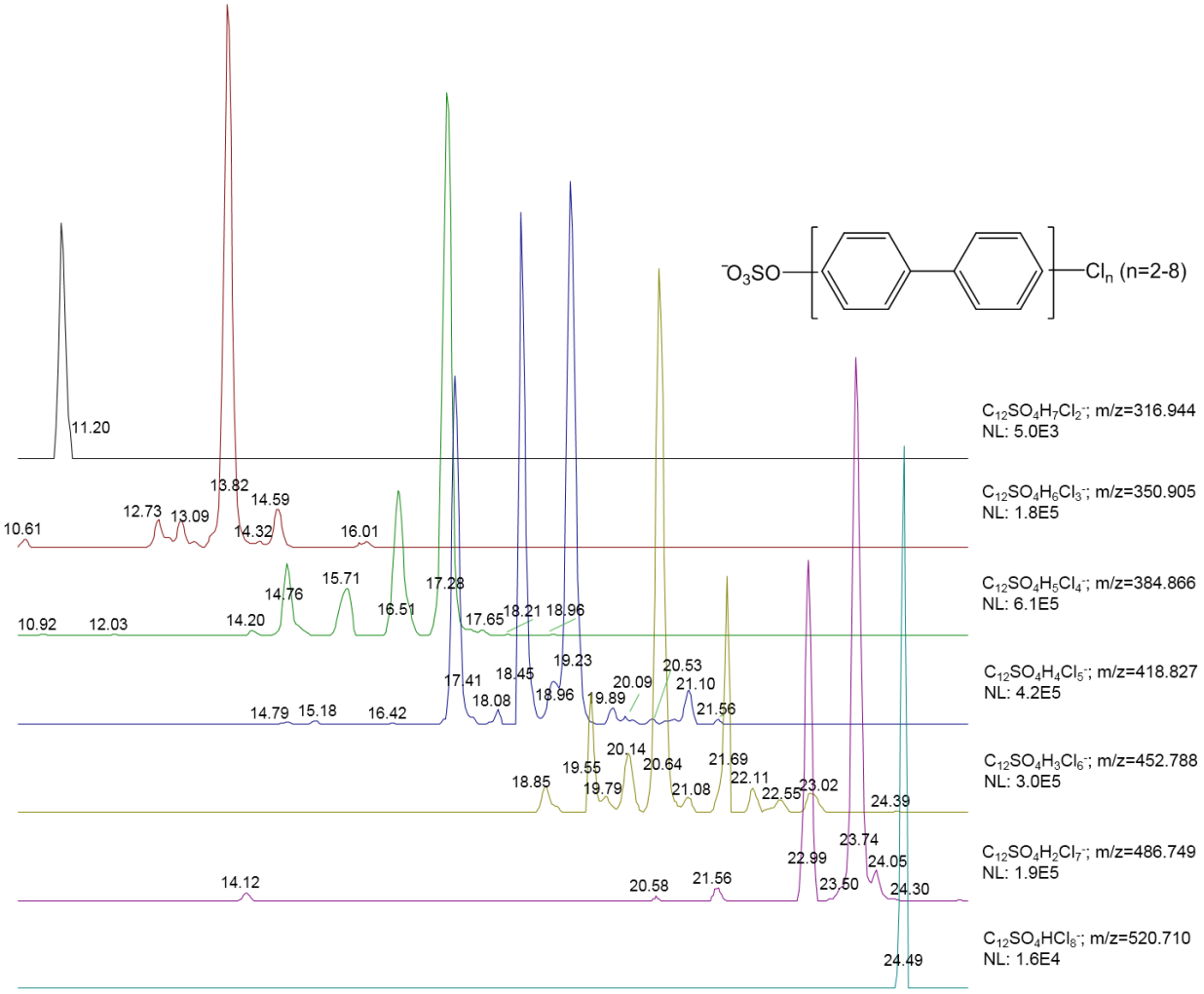


Figure 5-5.1. Extracted ion chromatograms of **Class 1.3** SO_4-PCB congeners ($C_{12}SO_4Cl_nH_{9-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.

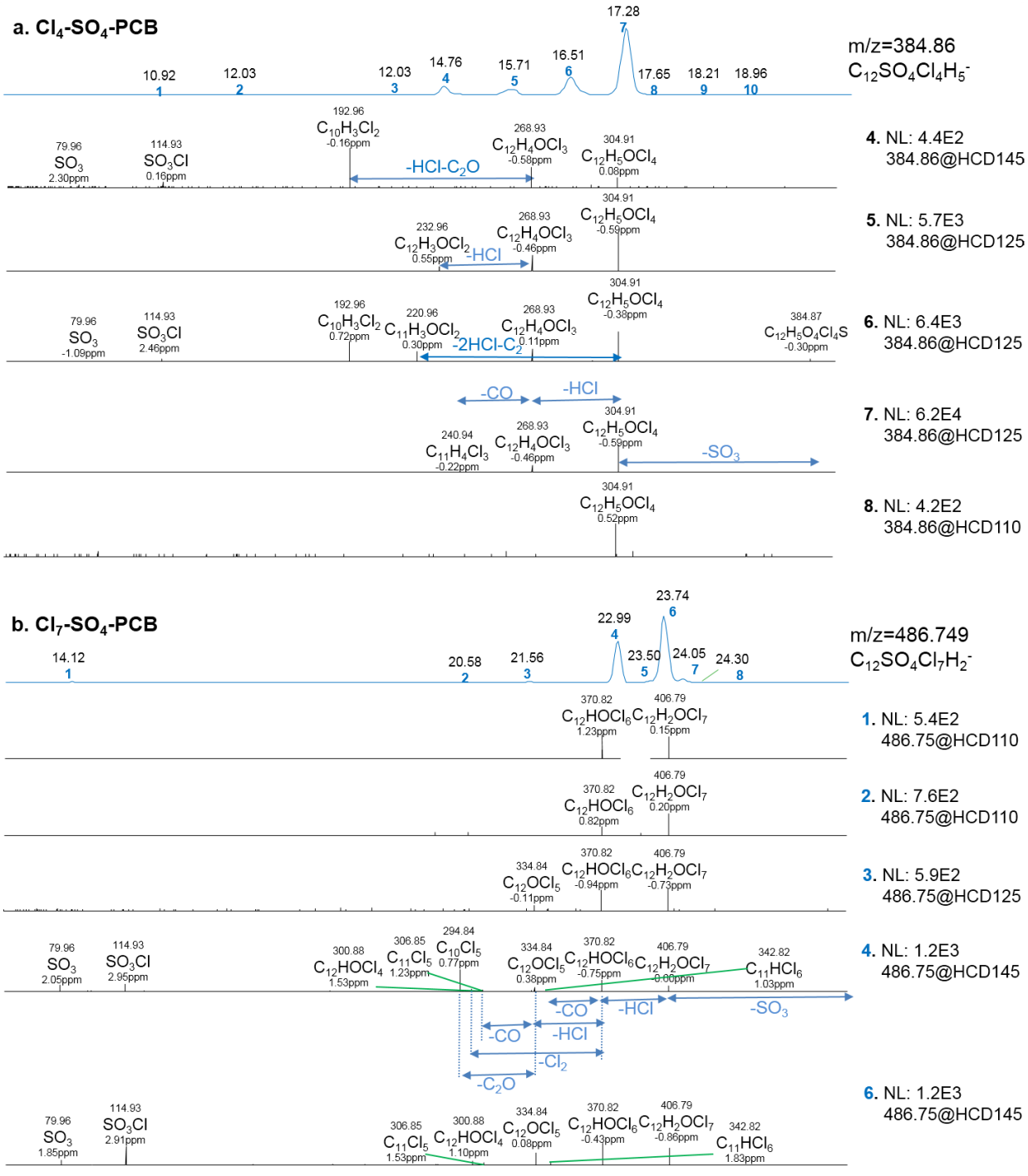


Figure 5-5.2. MS^2 spectra (HCD 110-145 eV) of selected Cl_4-SO_4-PCB ($C_{12}H_5Cl_4SO_4^-$, **a**) and Cl_7-SO_4-PCB congeners ($C_{12}H_2Cl_7SO_4^-$, **Class 1.3**, **b**) in the Hudson Bay pooled polar bear serum.

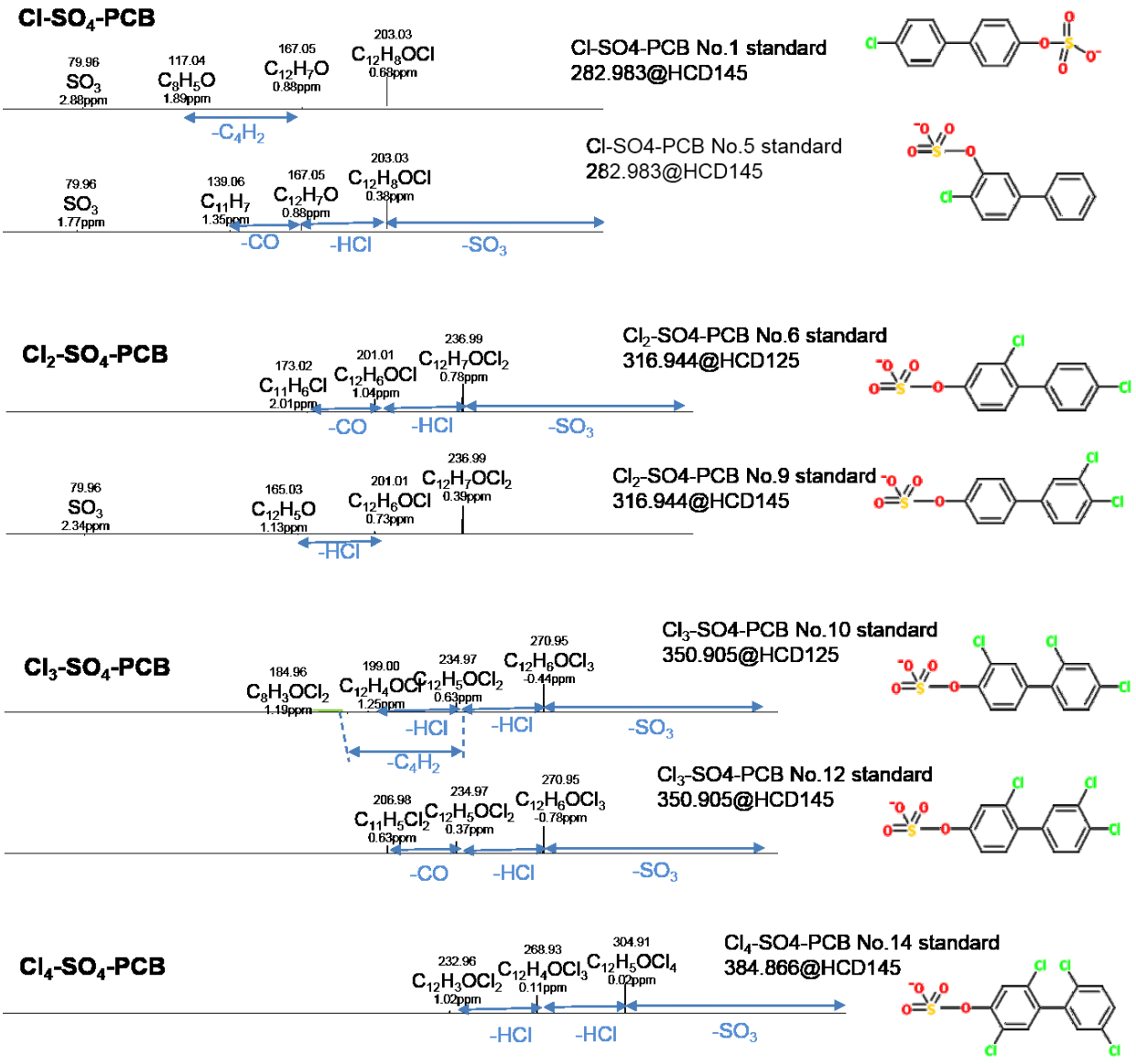


Figure 5-5.3. MS^2 spectra (HCD 145 eV) of selected Cl-SO_4-PCB through Cl_4-SO_4-PCB standards ($C_{12}SO_4ClH_8^-$, $C_{12}SO_4Cl_2H_7^-$, $C_{12}SO_4Cl_3H_6^-$ and $C_{12}SO_4Cl_4H_5^-$).

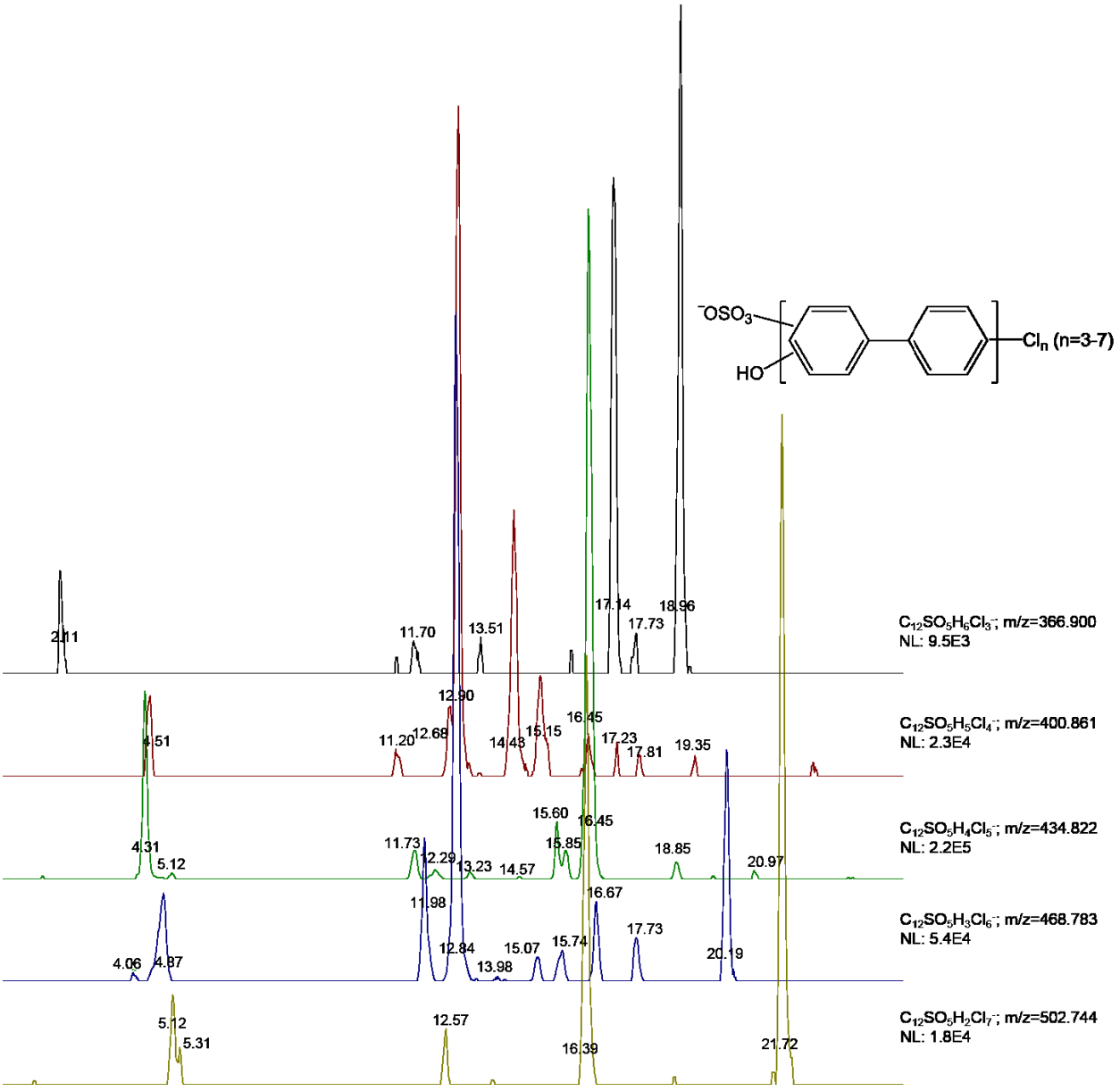


Figure 5-6.1.Extracted ion chromatograms of **Class 1.4** OH-SO_4-PCB congeners ($C_{12}SO_5Cl_nH_{9-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.

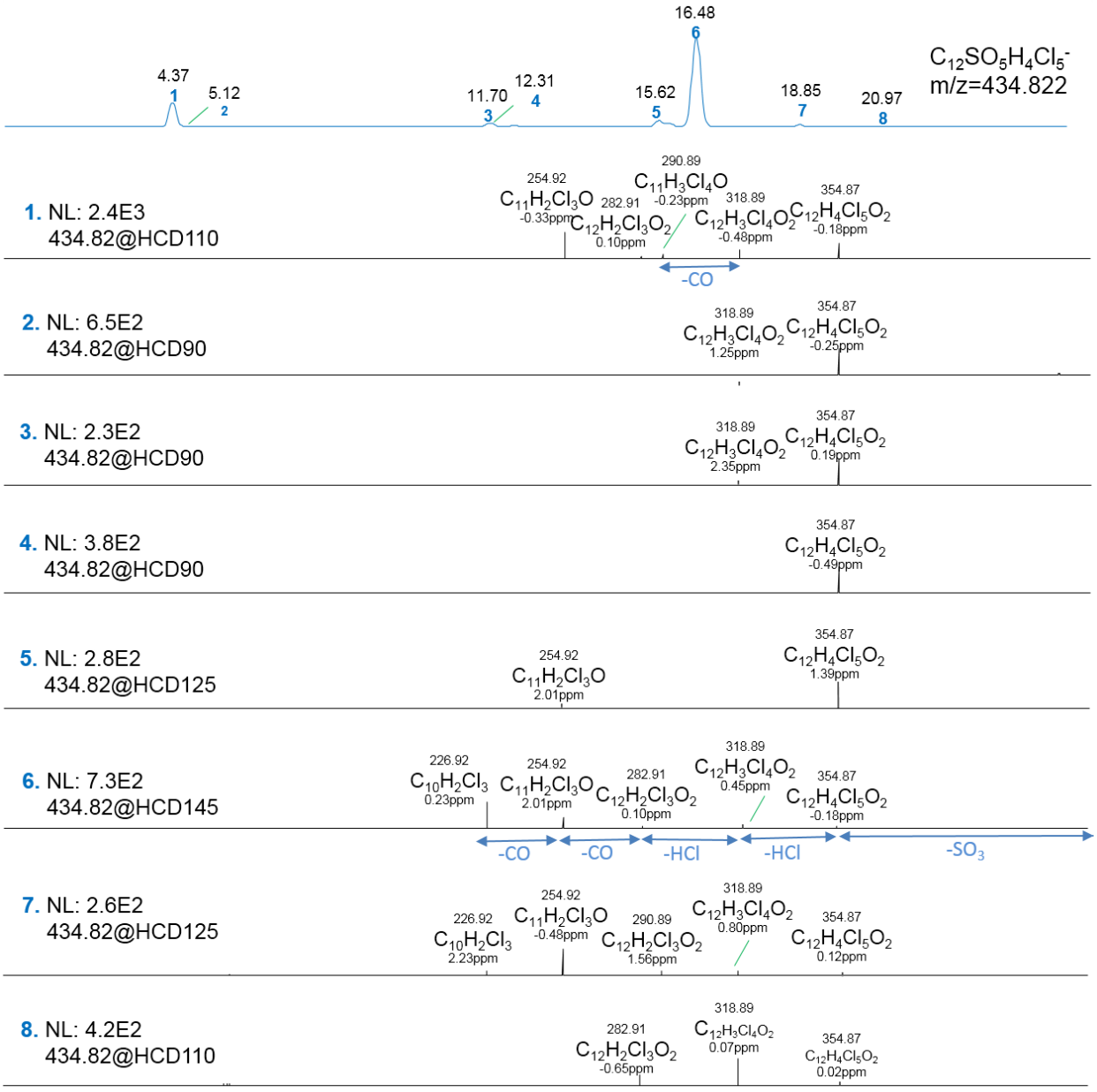


Figure 5-6.2.MS^2 spectra (HCD 90-145 eV) of selected Cl_5-OH-SO_4-PCB ($C_{12}H_4Cl_5SO_5^-$, **Class 1.4**) congeners in the Hudson Bay pooled polar bear serum.

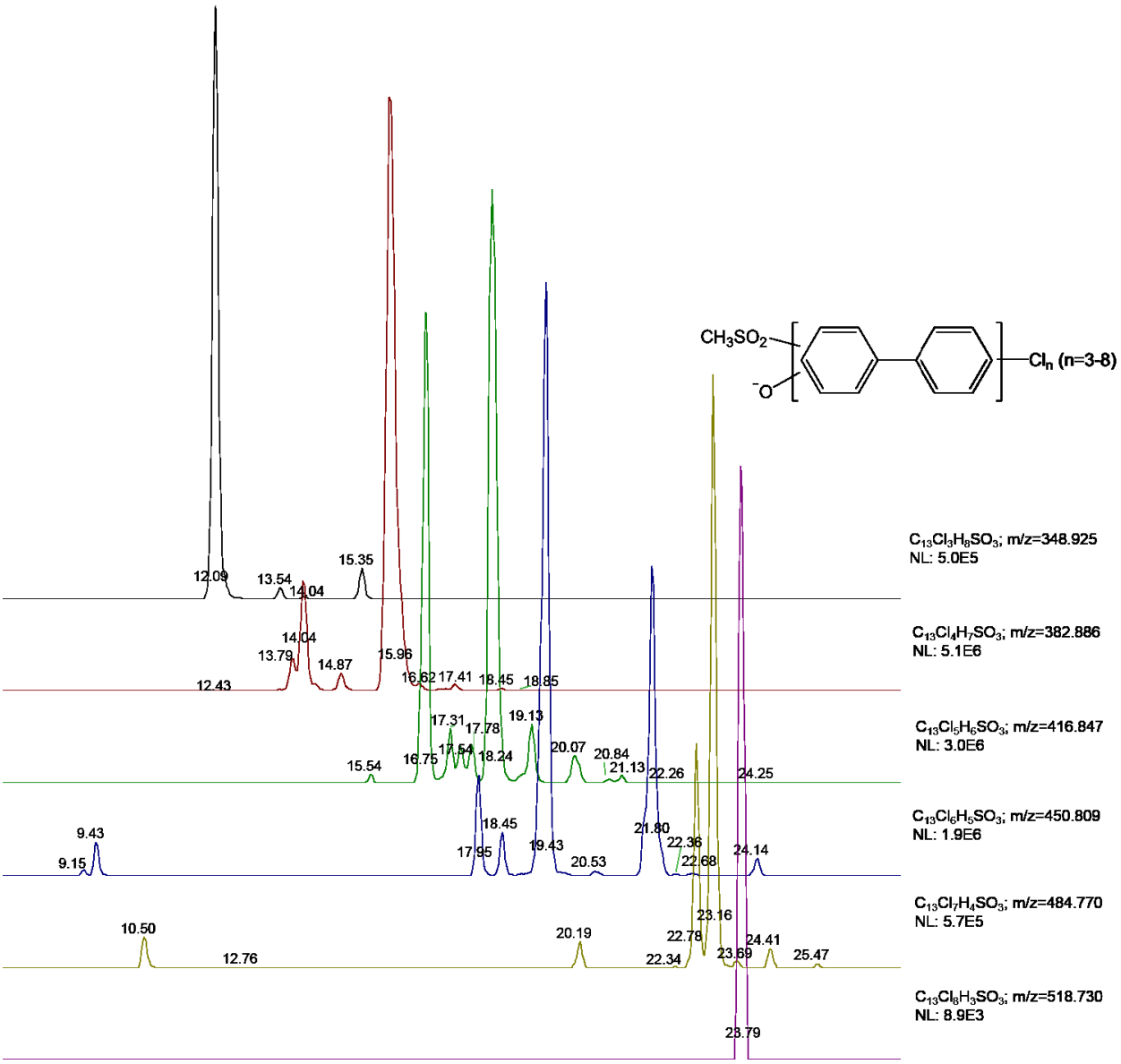


Figure 5-7.1. Extracted ion chromatograms of **Class 1.5** OH-$MeSO_2CH_3$-PCB congeners ($C_{13}SO_3Cl_nH_{11-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.

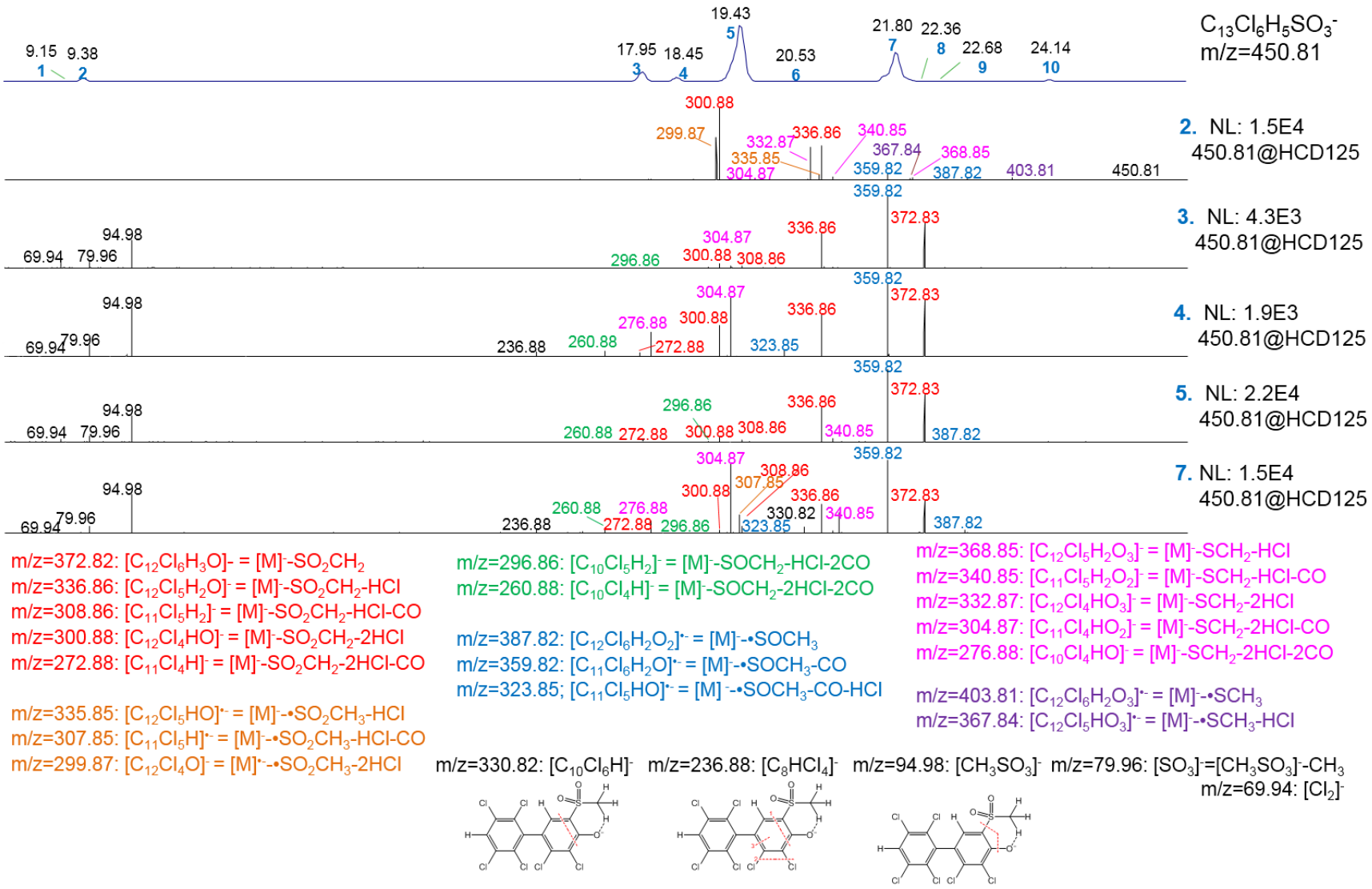


Figure 5-7.2. MS^2 spectra (HCD 125 eV) of selected Cl_6-OH-$MeSO_2$-PCB congeners ($C_{13}Cl_6H_5SO_3^-$, **Class 1.5**) in the Hudson Bay pooled polar bear serum. Proposed fragmentation sites and mechanisms of formation are shown.

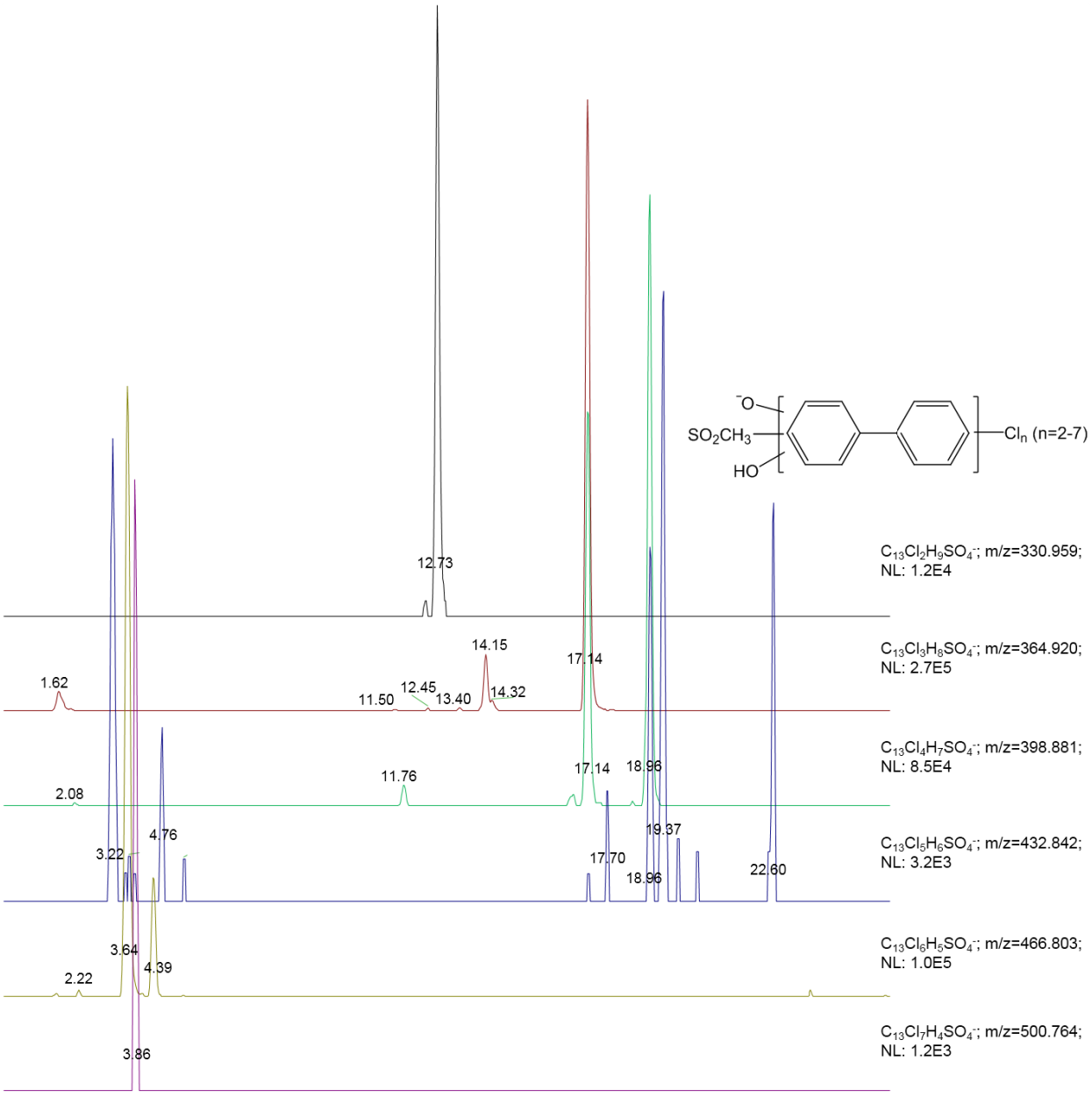
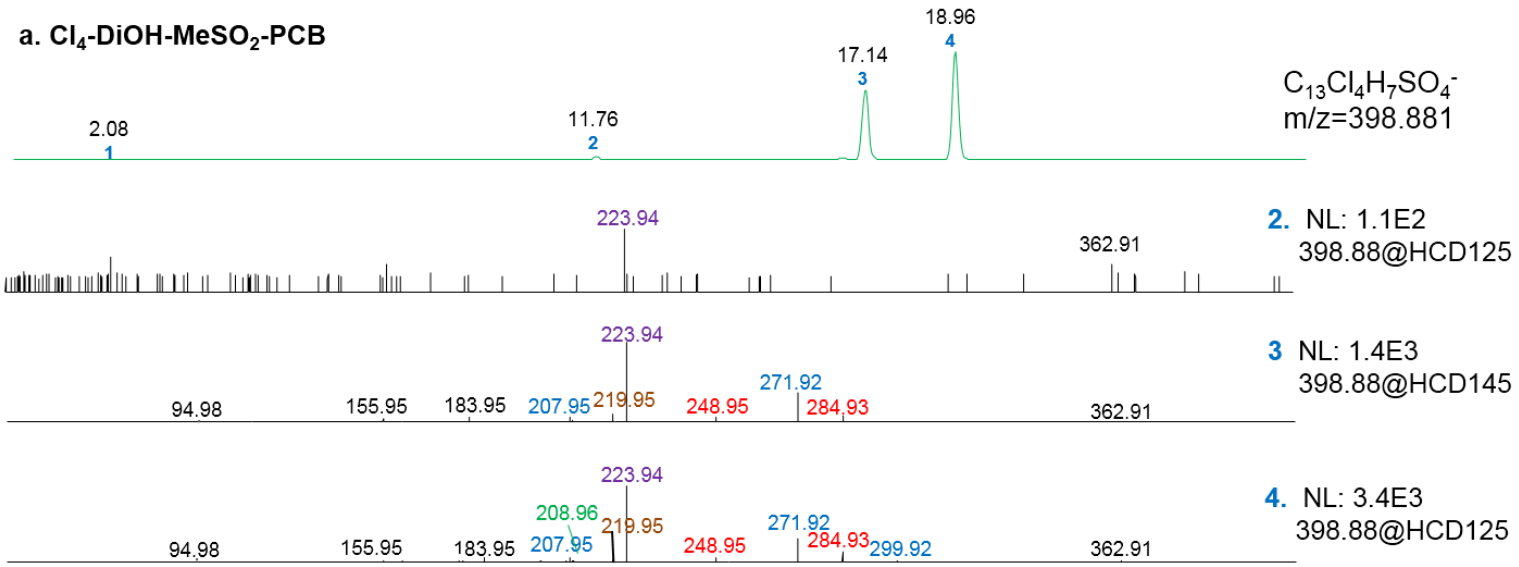


Figure 5-8.1. Extracted ion chromatograms of **Class 1.6** DiOH-MeSO_2-PCB congeners ($C_{13}SO_4Cl_nH_{11-n}^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.



m/z=284.93: $[C_{12}Cl_3H_4O_2]^-$ = $[M]^-$-SO_2CH_2-HCl

m/z=248.95: $[C_{12}Cl_2H_3O_2]^-$ = $[M]^-$-SO_2CH_2-2HCl

m/z=219.95: $[C_{11}Cl_2H_2O]^{\bullet-}$ = $[M]^-$-•SO_2CH_3-2HCl-CO

m/z=208.96: $[C_{10}Cl_2H_3O]^-$ = $[M]^-$-$SOCH_2$-2HCl-2CO

m/z=299.92: $[C_{12}Cl_3H_3O_3]^{\bullet-}$ = $[M]^-$-•$SOCH_3$-HCl

m/z=271.92: $[C_{11}Cl_3H_3O_2]^{\bullet-}$ = $[M]^-$-•$SOCH_3$-HCl-CO

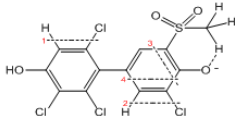
m/z=207.95: $[C_{10}Cl_2H_2O]^{\bullet-}$ = $[M]^-$-•$SOCH_3$-2HCl-2CO

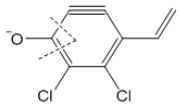
m/z=223.94: $[C_{10}Cl_2H_2O_2]^{\bullet-}$ = $[M]^-$-•SCH_3-2HCl-2CO

m/z=362.91: $[C_{13}Cl_3H_6SO_4]^-$ = [M]—HCl

m/z=94.98: $[CH_3SO_3]^-$

m/z=183.95: $[C_8Cl_2H_2O]^{\bullet-}$ m/z=155.95: $[C_7Cl_2H_2]^{\bullet-}$ = [m/z=183.95]$^{\bullet-}$-CO





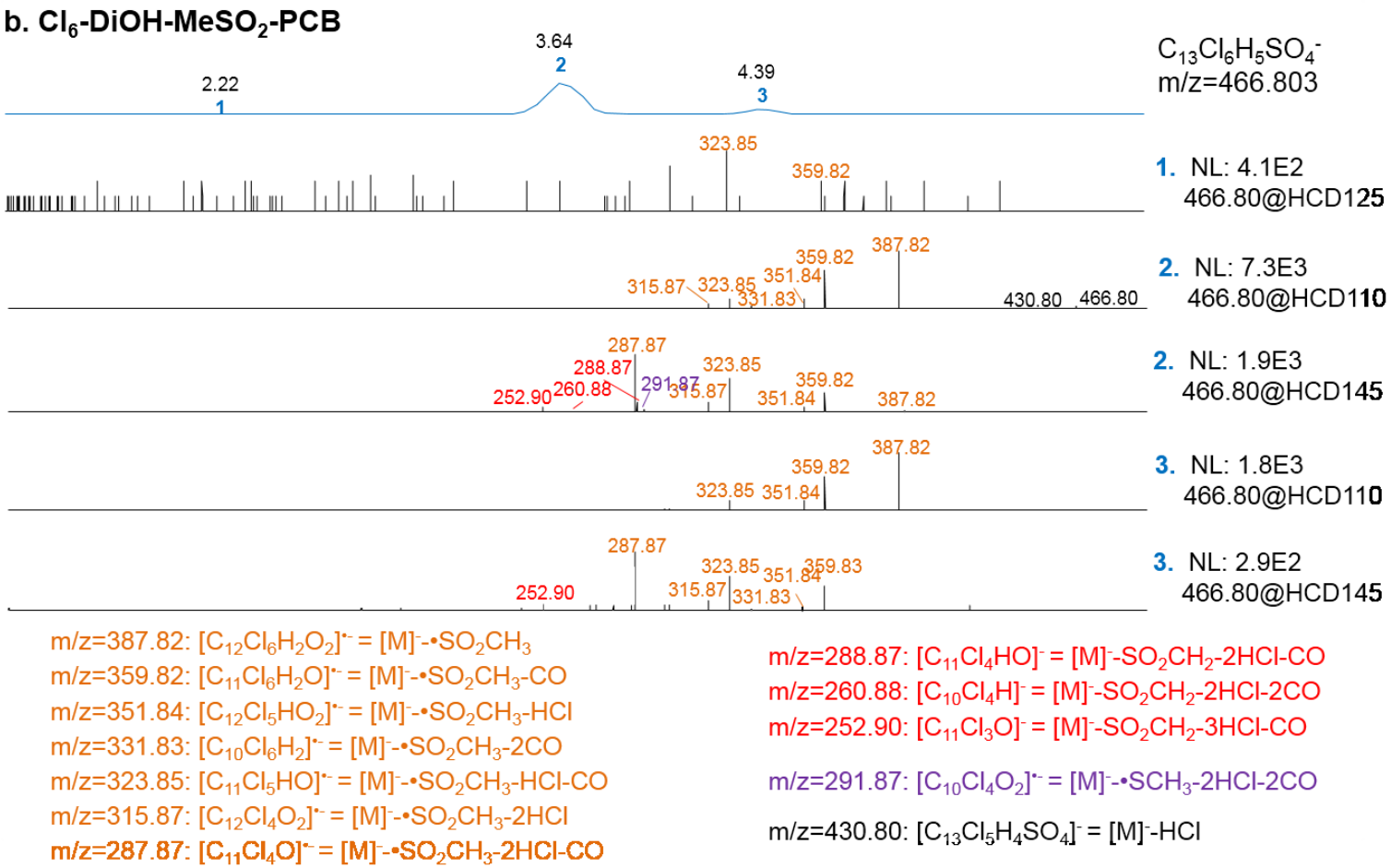


Figure 5-8.2. MS^2 spectra (110-145 eV) of selected Cl_4- ($C_{13}Cl_4H_7SO_4^-$, **a**) and all Cl_6-DiOH-$MeSO_2$-PCB congeners ($C_{13}Cl_6H_5SO_4^-$, **Class 1.6**, **b**) in the Hudson Bay pooled polar bear serum, and the formation mechanism for each fragment is proposed.

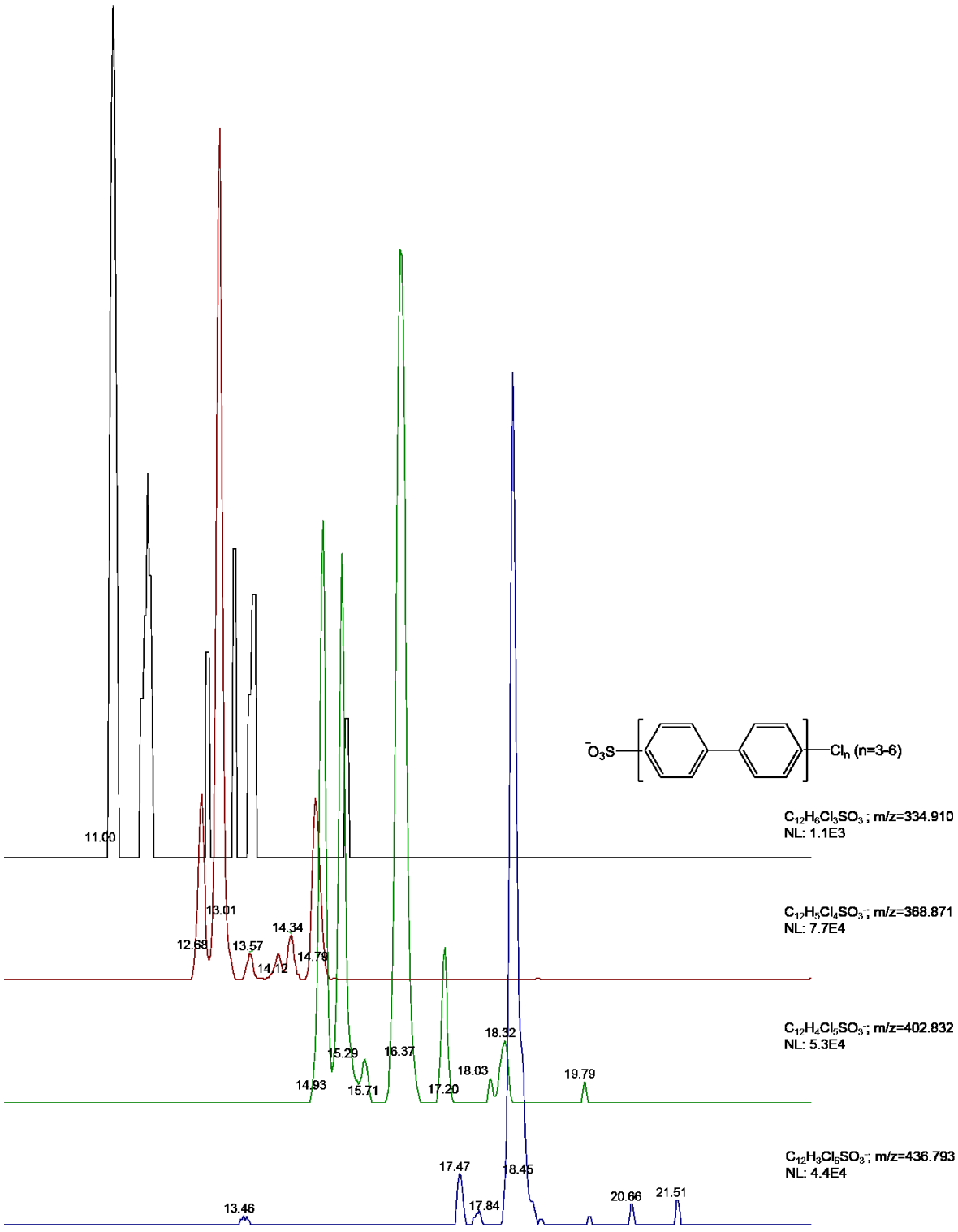


Figure 5-9.1. Extracted ion chromatograms of **Class 1.7** SO_3-PCB congeners ($C_{12}Cl_nH_{9-n}SO_3^-$) in the Hudson Bay pooled polar bear serum. The extracted ion chromatograms corresponds to theoretical molecular ion (*m/z* ± 5 ppm) with the lowest isotopic mass.

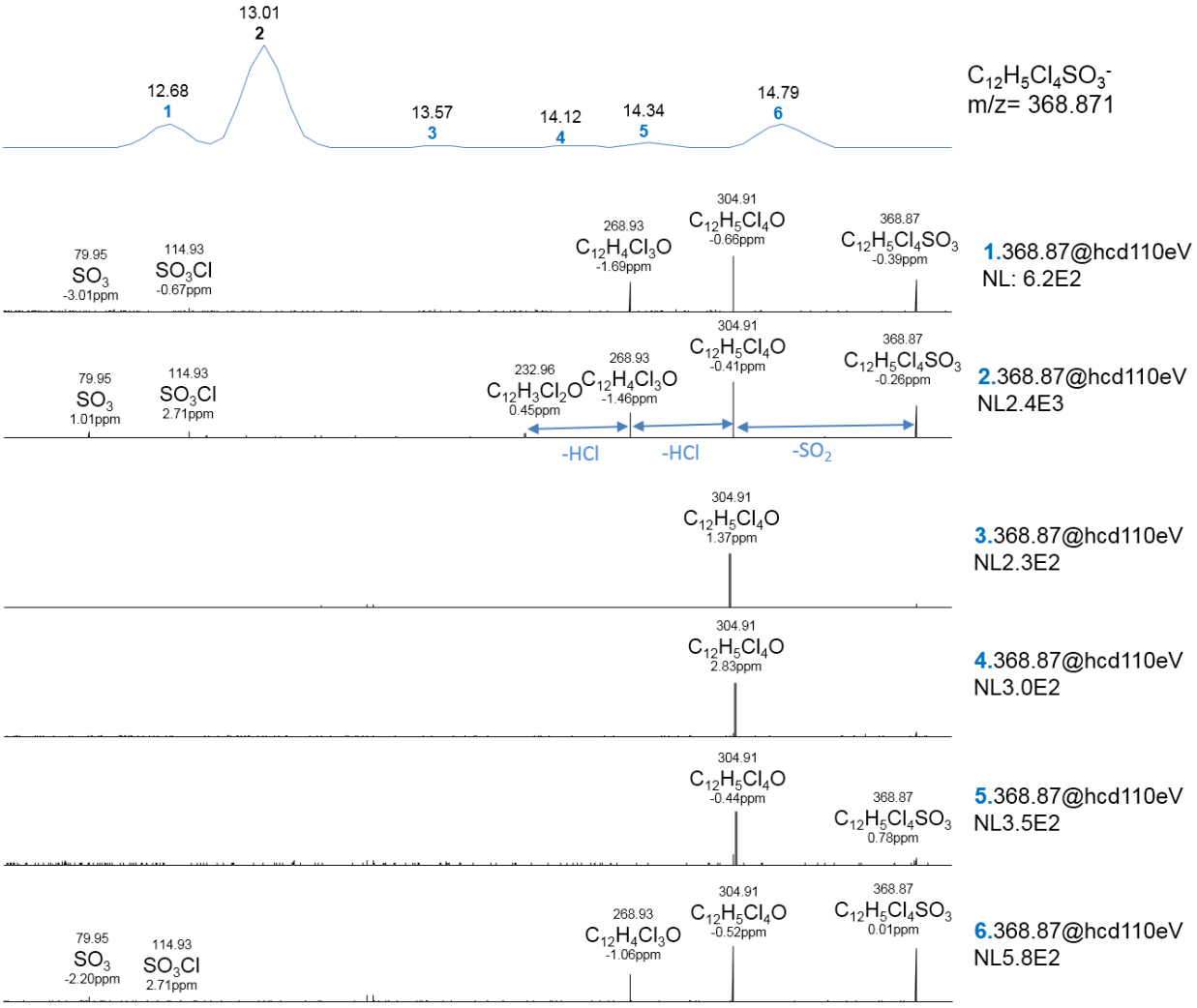


Figure 5-9.2. MS^2 spectra (HCD 110 eV) of all Cl_4-SO_3-PCB congeners ($C_{12}H_5Cl_4SO_3^-$, **Class 1.7**) in the Hudson Bay pooled polar bear serum.

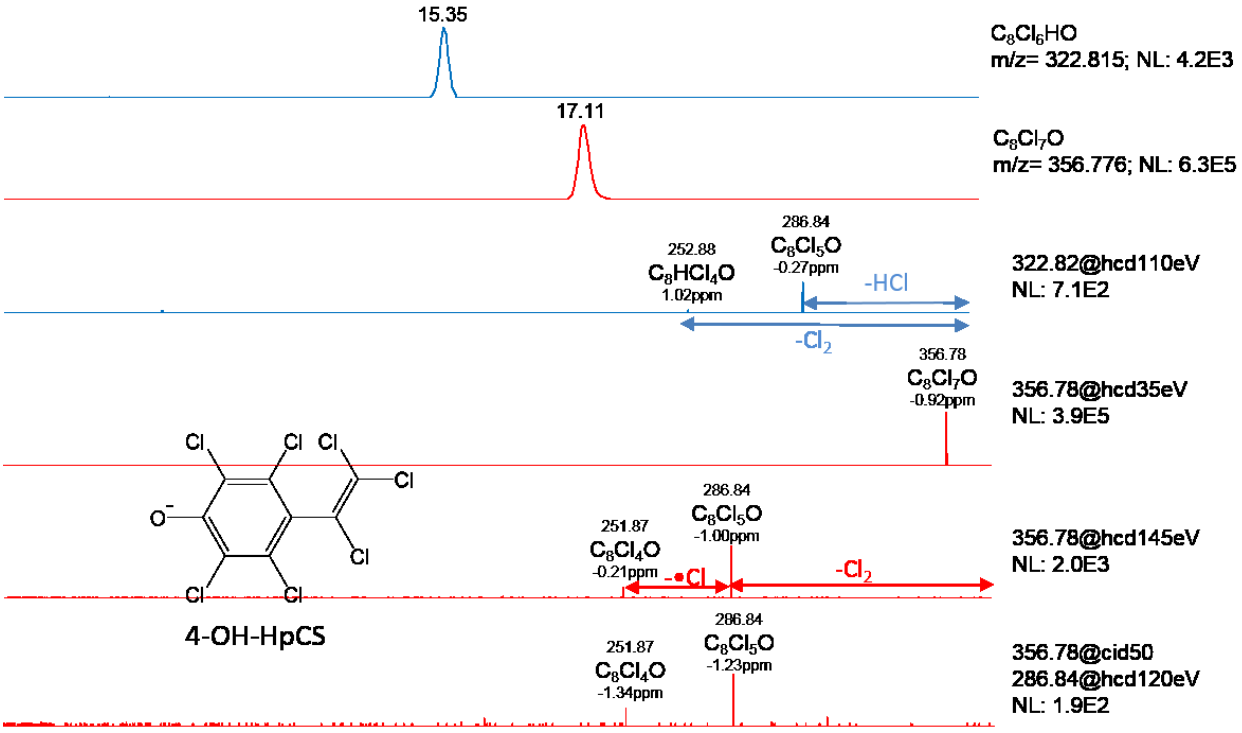


Figure 5-10. Extracted ion chromatograms and MS^2 spectra (HCD 35-145 eV) of **Class 2.1** chlorinated aromatic compounds ($C_8Cl_nH_{7-n}O^-$) in the Hudson Bay pooled polar bear serum. The structure of 4-hydroxylated heptachlorostyrene (4-OH-HpCS) is shown.

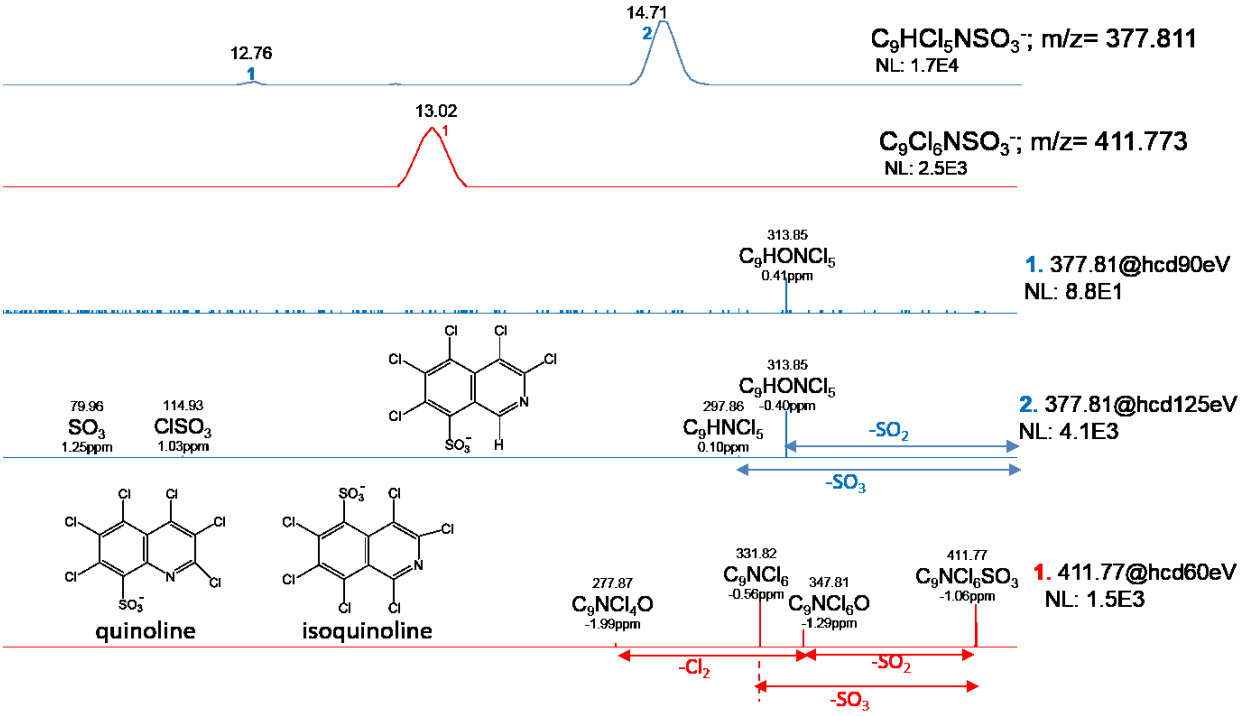


Figure 5-11. Extracted ion chromatograms and MS^2 (HCD 60-125 eV) spectra of **Class 2.2** chlorinated (iso)quinoline sulfonates ($C_9Cl_nH_{6-n}NSO_3^-$) in the Hudson Bay pooled polar bear serum. Proposed structures are shown.

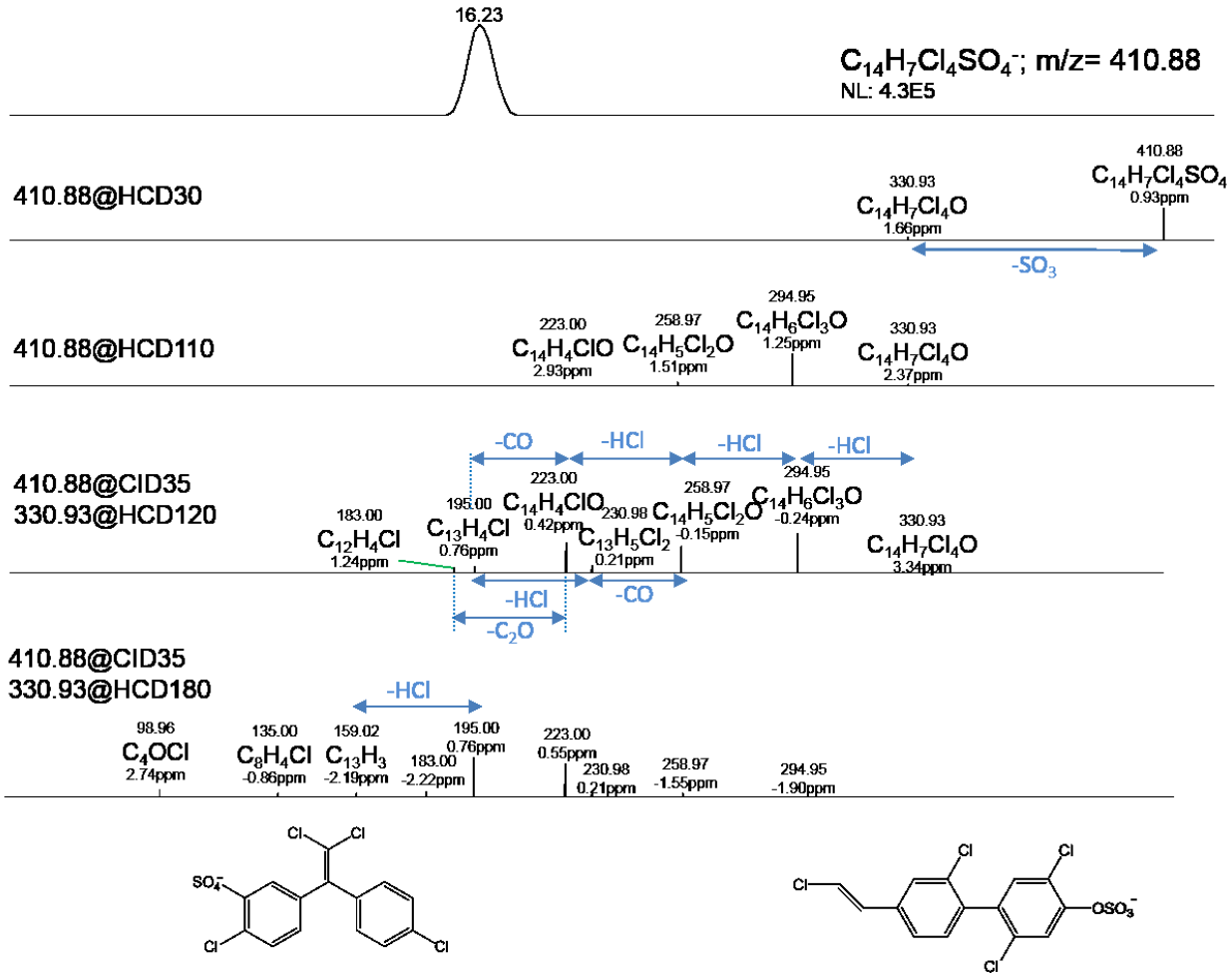


Figure 5-12. Extracted ion chromatogram, and MS^2 (HCD 30-110 eV) and MS^3 spectra (CID 35 eV➔HCD 120-180 eV) of **Class 2.3** tetrachlorinated aromatic sulfate ($C_{14}Cl_4H_7SO_4^-$) in the Hudson Bay pooled polar bear serum. Examples of possible structures, including SO_4-DDE, are shown.

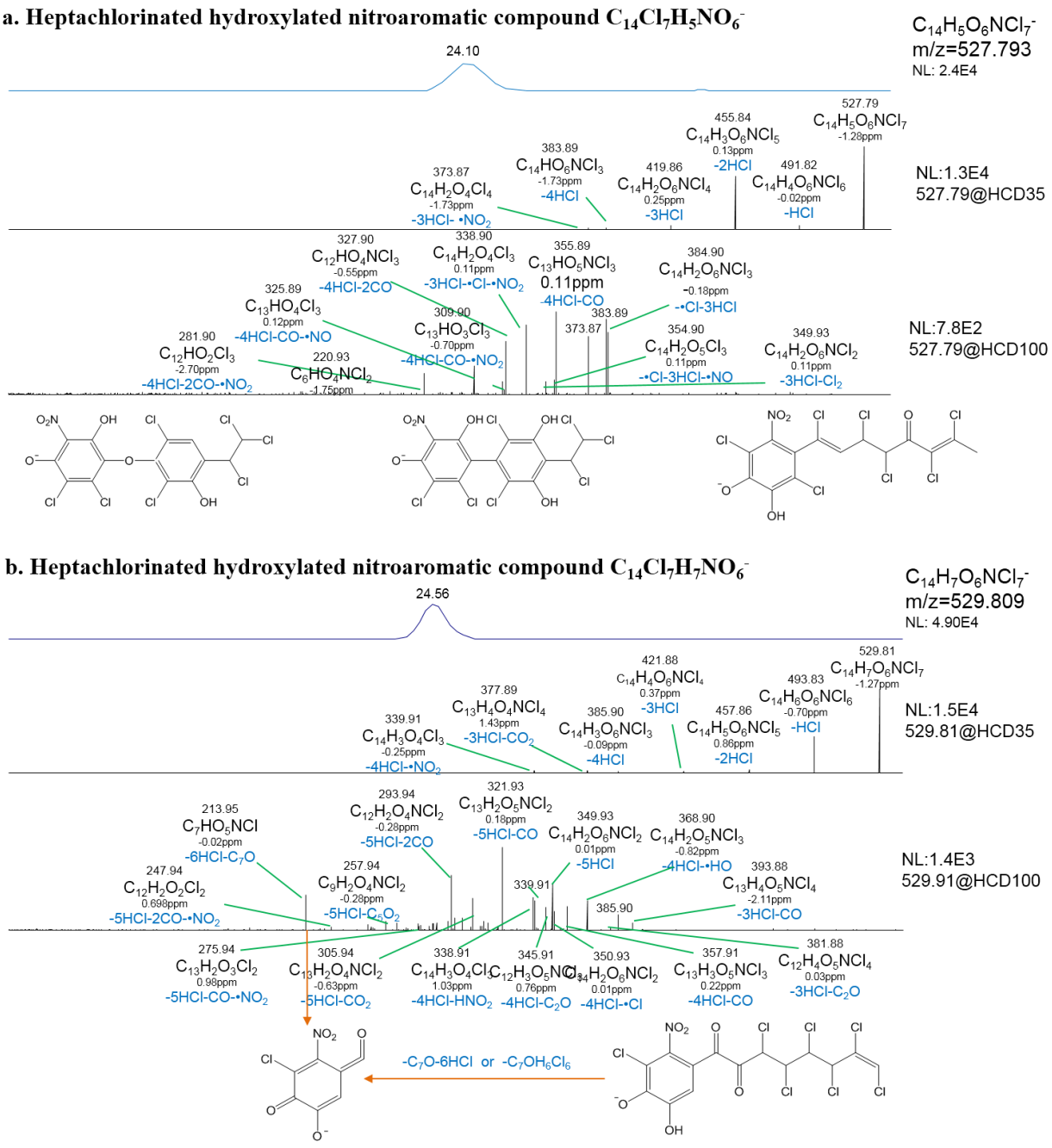


Figure 5-13. Extracted ion chromatograms and MS^2 spectra (HCD 35-100 eV) of **Class 2.4** heptachlorinated hydroxylated nitroaromatic compounds ($C_{14}Cl_7H_5NO_6^-$, **a**; and $C_{14}Cl_7H_7NO_6^-$, **b**) in the Hudson Bay pooled polar bear serum. Examples of possible structures are shown.

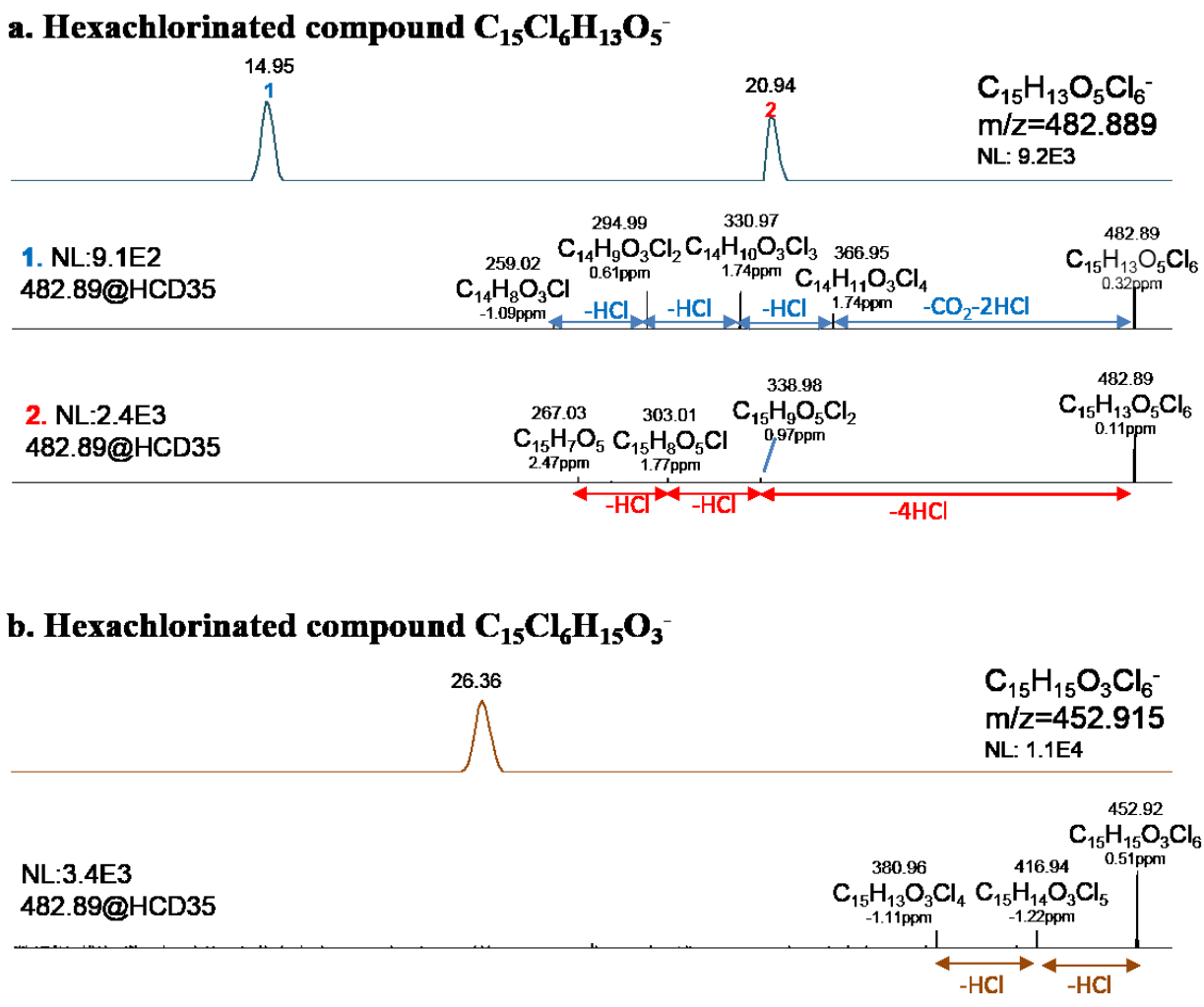


Figure 5-14. Extracted ion chromatograms and MS^2 spectra (HCD 35 eV) of **Class 2.5** hexachlorinated compounds ($C_{15}Cl_6H_{13}O_5^-$, **a**; and $C_{15}Cl_6H_{15}O_3^-$; **b**) in the Hudson Bay pooled polar bear serum.

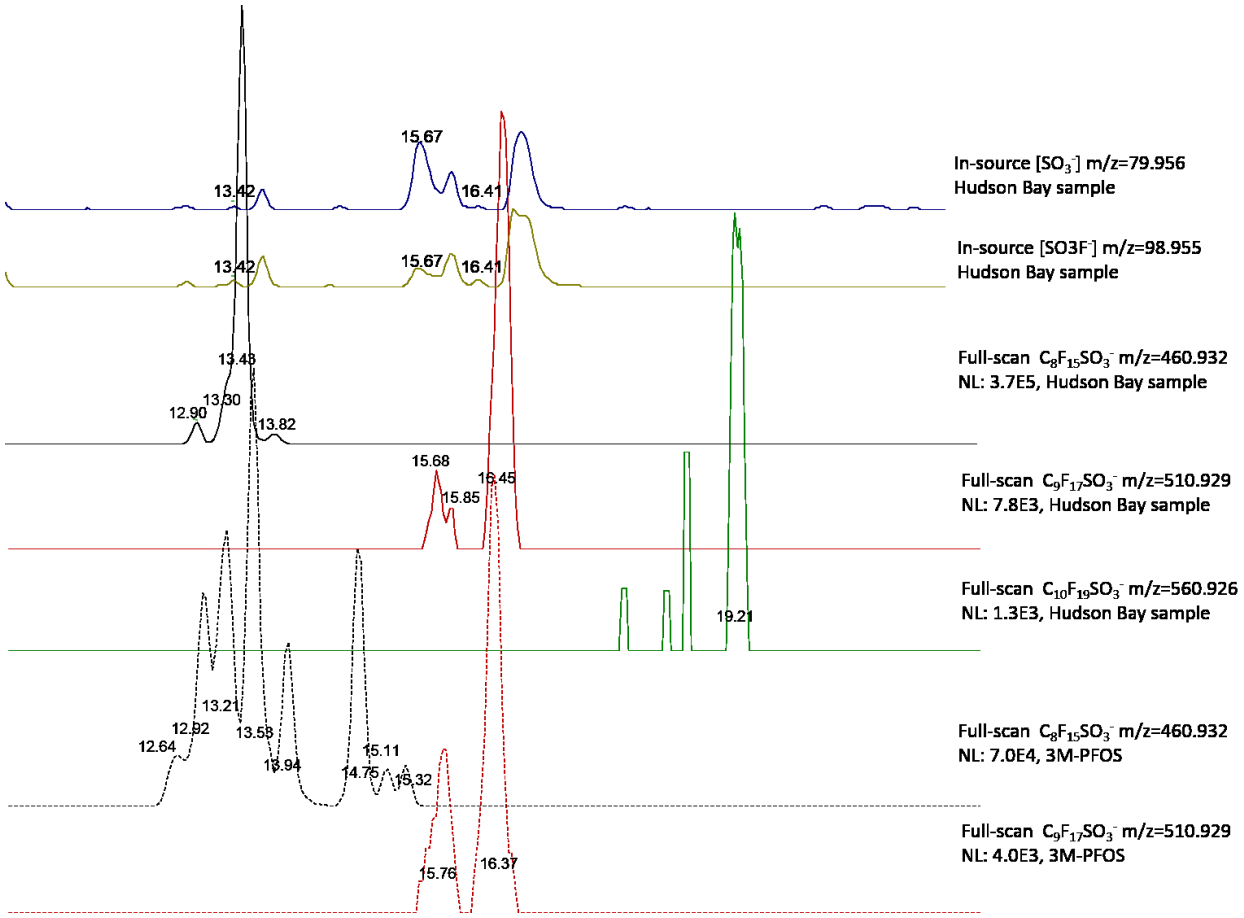


Figure 5-15.1. Extracted ion chromatograms of $[SO_3]^-$ and $[SO_3F]^-$ in-source fragments, and **Class 3.1** cyclic and/or unsaturated PFSA homologues ($C_nF_{2n-1}SO_3^-$) detected in the Hudson Bay pooled polar bear serum (solid line), and in the 3M-PFOS industrial standard (dashed line). All chromatograms are for the theoretical mass shown ±5 ppm.

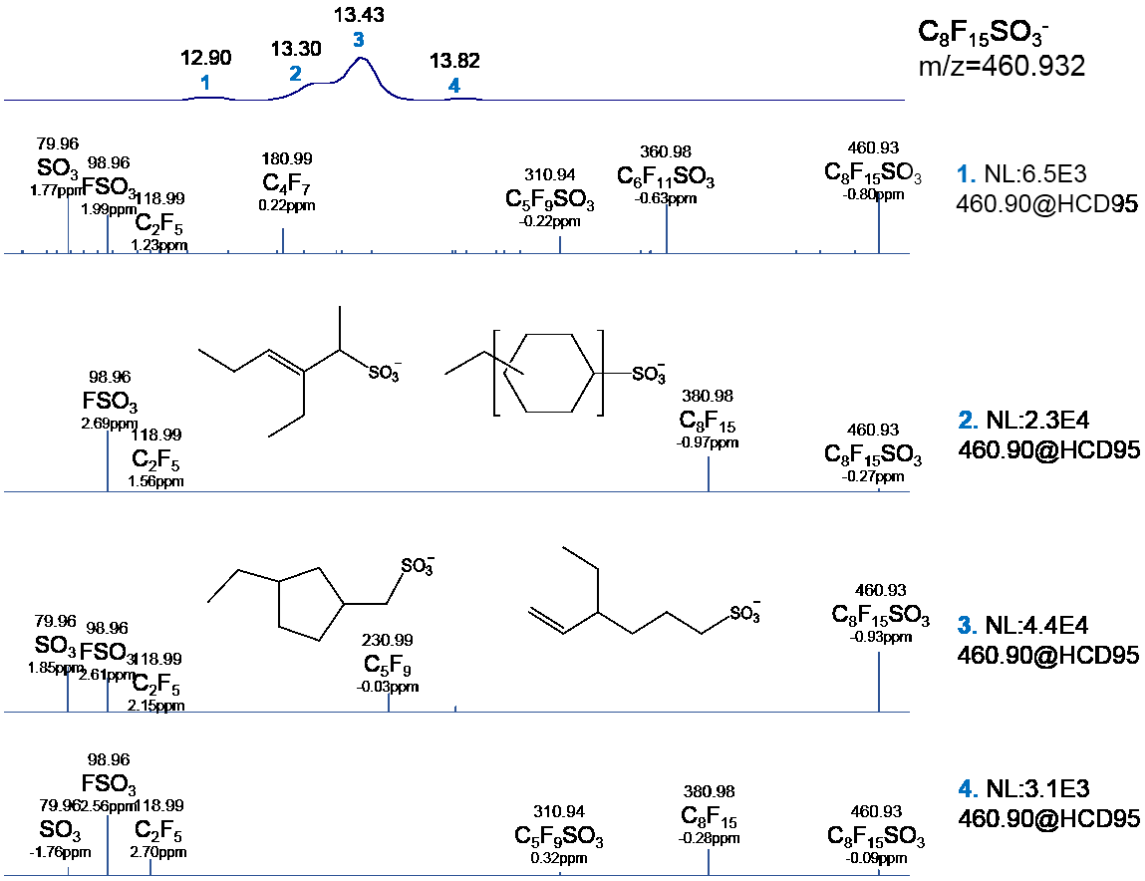


Figure 5-15.2. MS^2 spectra (HCD 95 eV) of all C_8- cyclic or unsaturated PFSA isomers ($C_8F_{15}SO_3^-$, **Class 3.1**) in the Hudson Bay pooled polar bear serum. Examples of possible structures for some isomers are proposed, F atoms not shown.

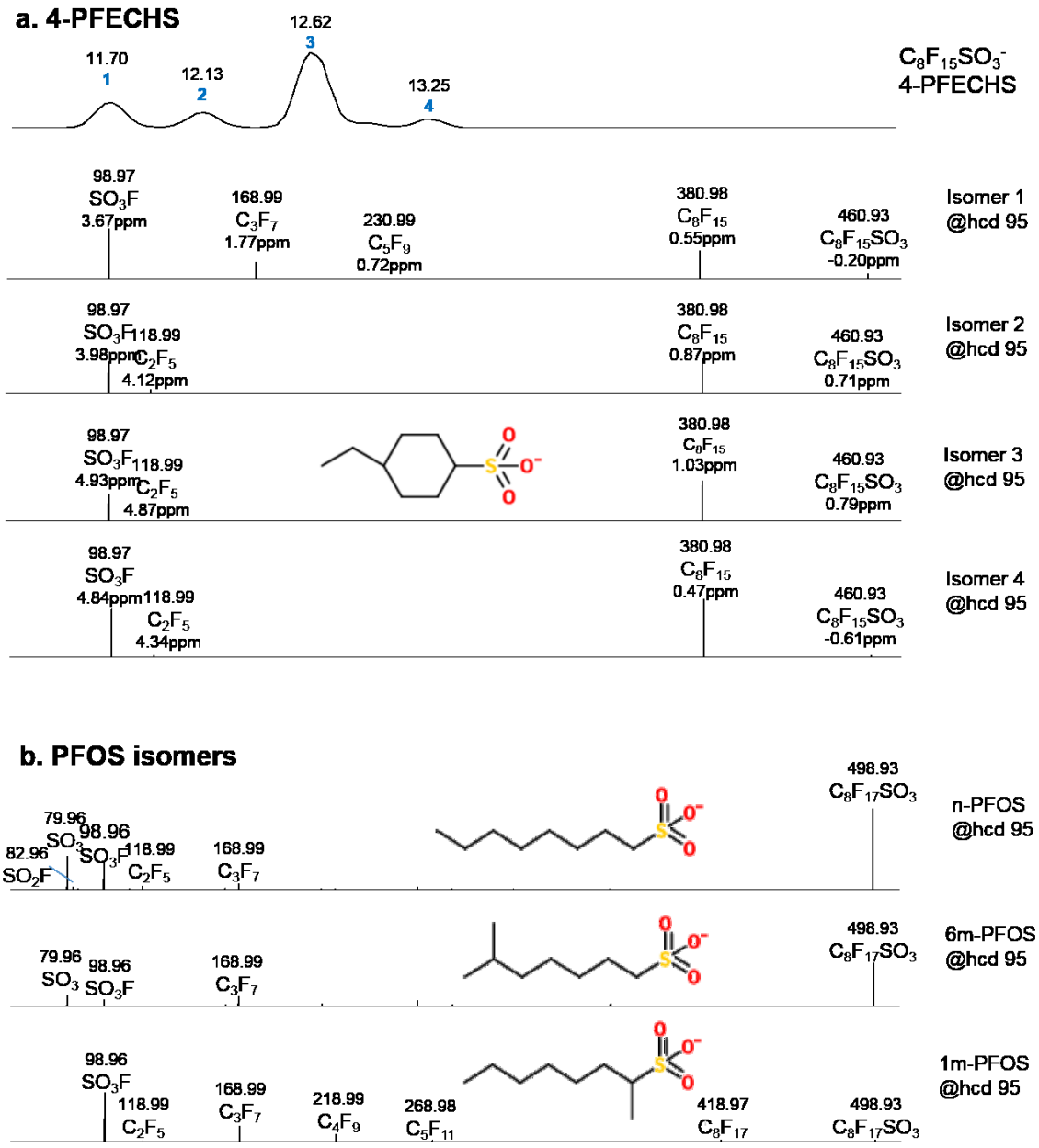


Figure 5-15.3. Extracted ion chromatogram and MS^2 spectra (HCD 95 eV) of 4-PFECHS and byproduct isomers in 4-PFECHS standard (**a**), and MS^2 spectra (HCD 95 eV) of different PFOS isomer standards (**b**). The isomer 3 in 4-PFECHS standard, being the most abundant peak, is probably 4-PFECHS. Structures of 4-PFECHS and PFOS isomers are shown, F atoms not shown.

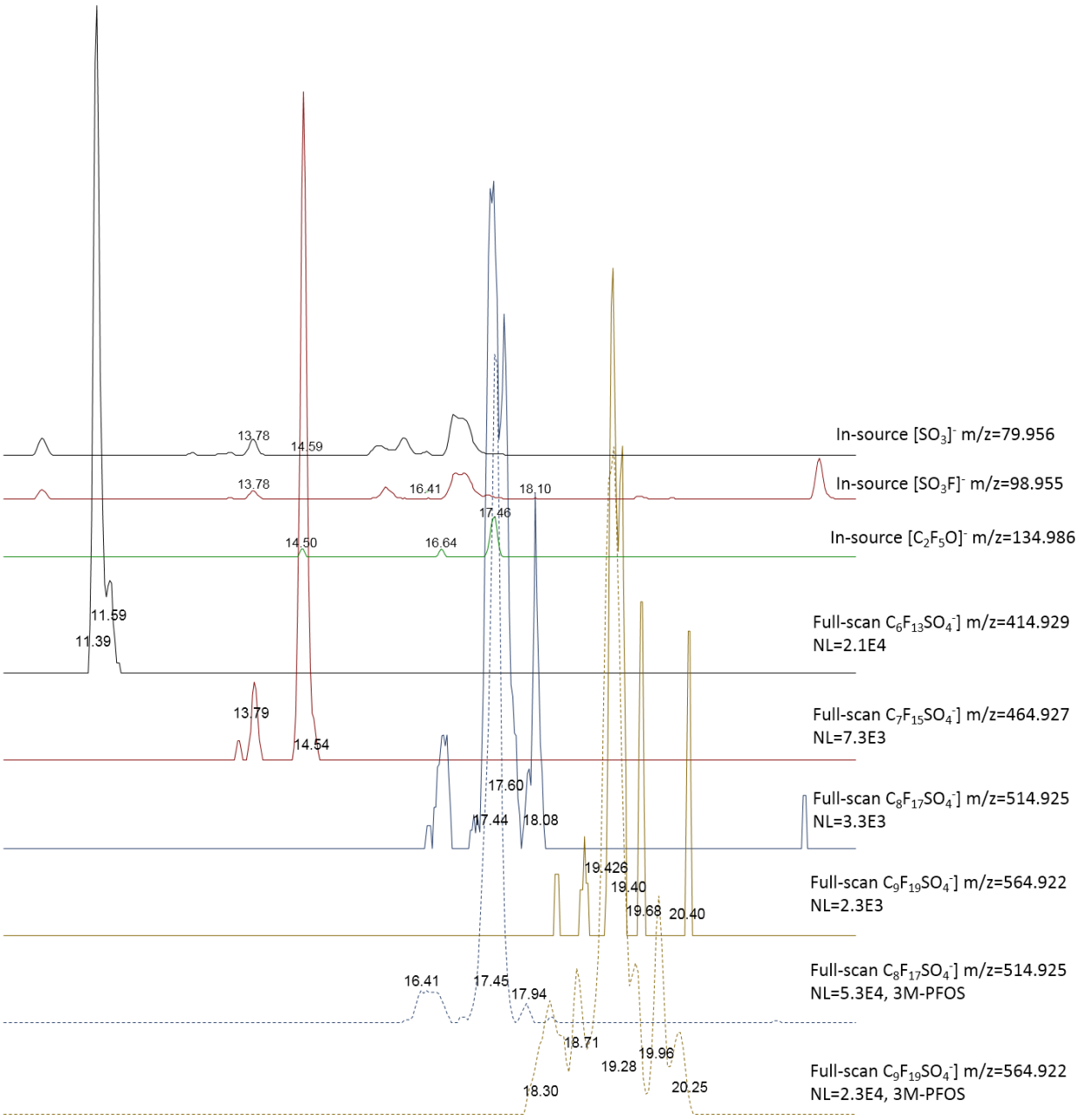


Figure 5-16.1. Extracted ion chromatograms of $[SO_3]^-$, $[SO_3F]^-$ and $[C_2F_5O]^-$ in-source fragments, and **Class 3.2** ether-PFSA homologues ($C_nF_{2n+1}SO_4^-$) detected in the Hudson Bay pooled polar bear serum (solid line), and in the 3M-PFOS industrial standard (dashed line). All chromatograms are for the theoretical mass shown ±5 ppm.

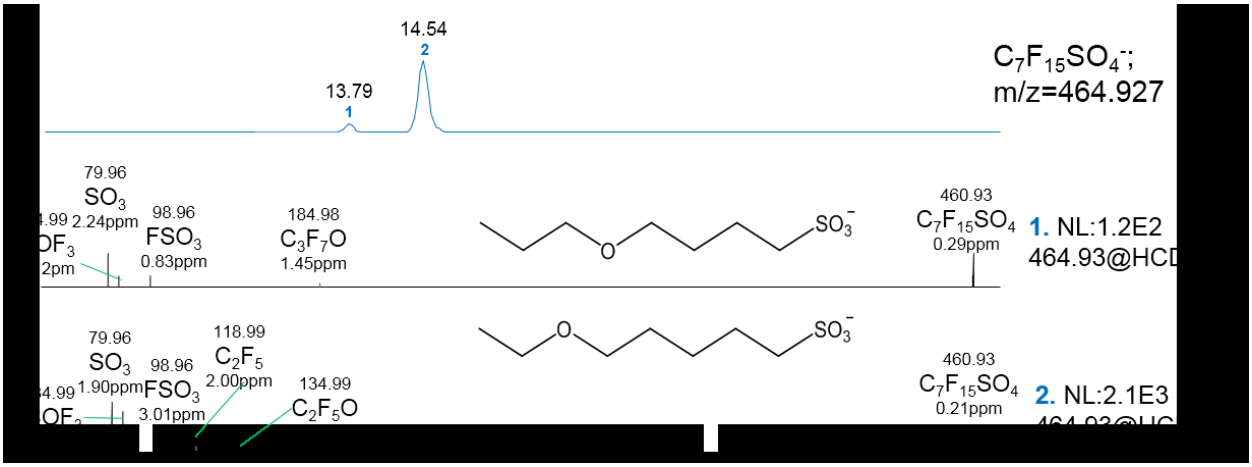


Figure 5-16.2. MS^2 spectra (HCD 110 eV) of all C_7-ether-PFSA isomers ($C_7F_{15}SO_4^-$, **Class 3.2**) in the Hudson Bay pooled polar bear serum. Proposed structure for each isomer is presented, F atoms not shown.

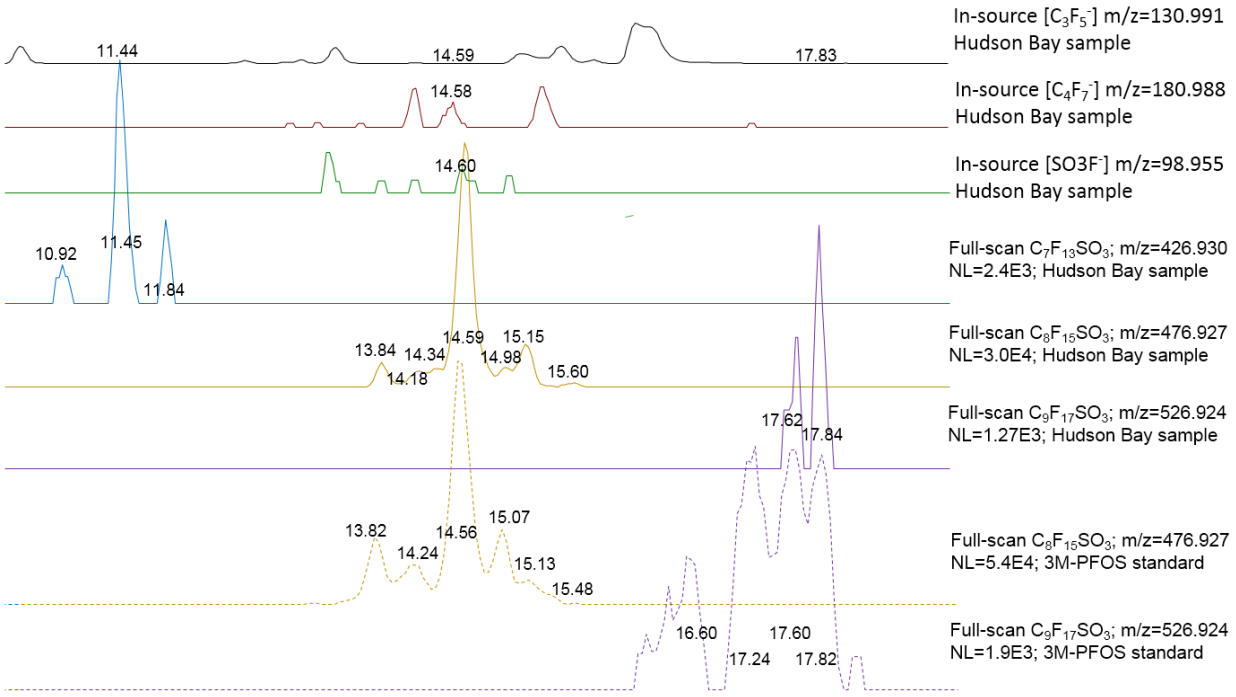


Figure 5-17.1. Extracted ion chromatograms of $[SO_3F]^-$, $[C_3F_5]^-$ and $[C_4F_7]^-$ in-source fragments, and **Class 3.3** enol-ether-, cyclic-ether- or carbonyl-PFSA homologues ($C_nF_{2n-1}SO_4^-$) detected in the Hudson Bay pooled polar bear serum (solid line), and in the 3M-PFOS industrial standard (dashed line). All chromatograms are for the theoretical mass shown ±5 ppm.

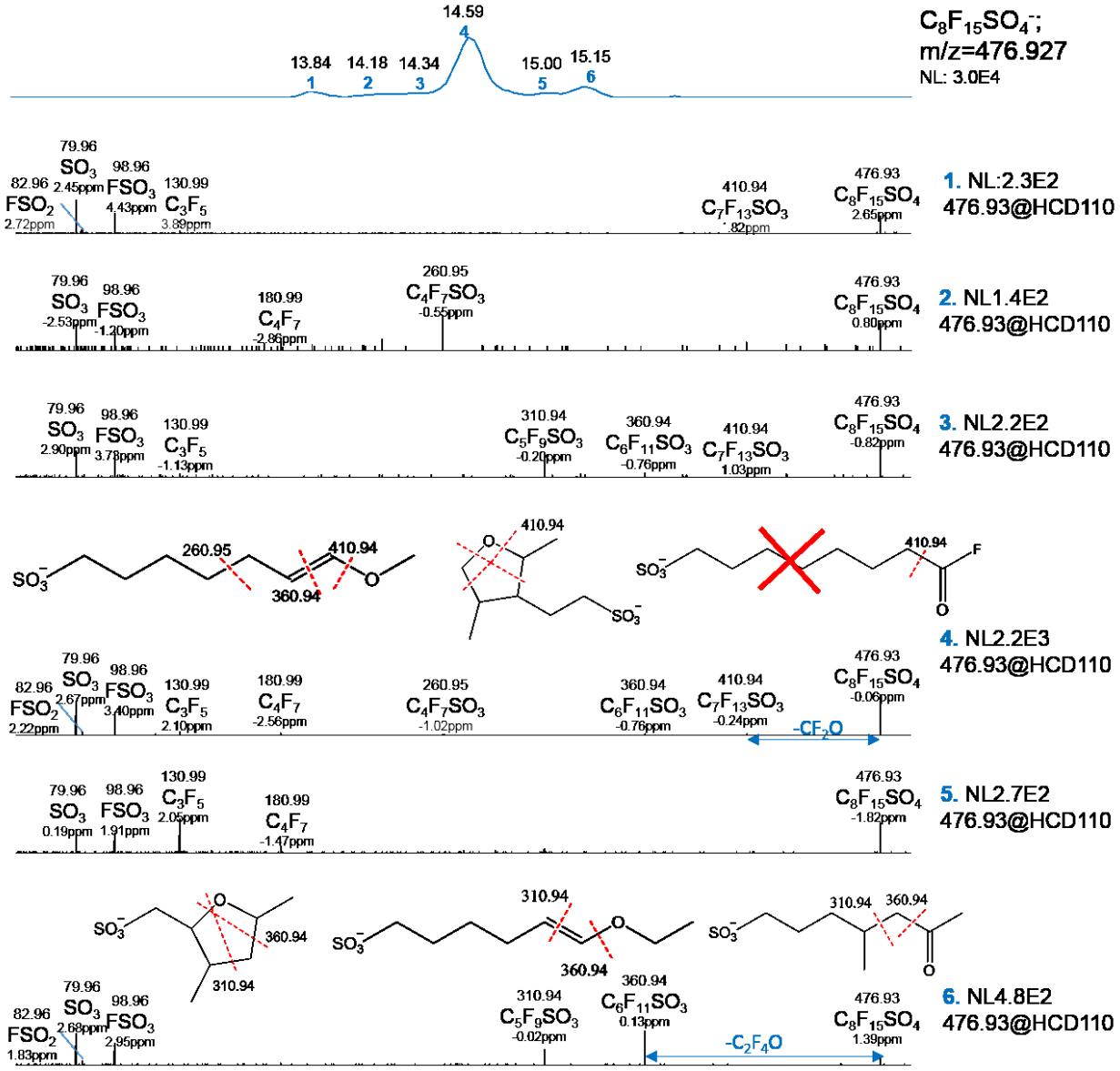


Figure 5-17.2. MS^2 spectra (HCD 110 eV) of all C_8- enol-ether-, cyclic-ether- or carbonyl-PFSA isomers ($C_8F_{15}SO_4^-$, **Class 3.3**) in the Hudson Bay pooled polar bear serum. Examples of possible structures for isomers 4 and 6 are presented, F atoms not shown.

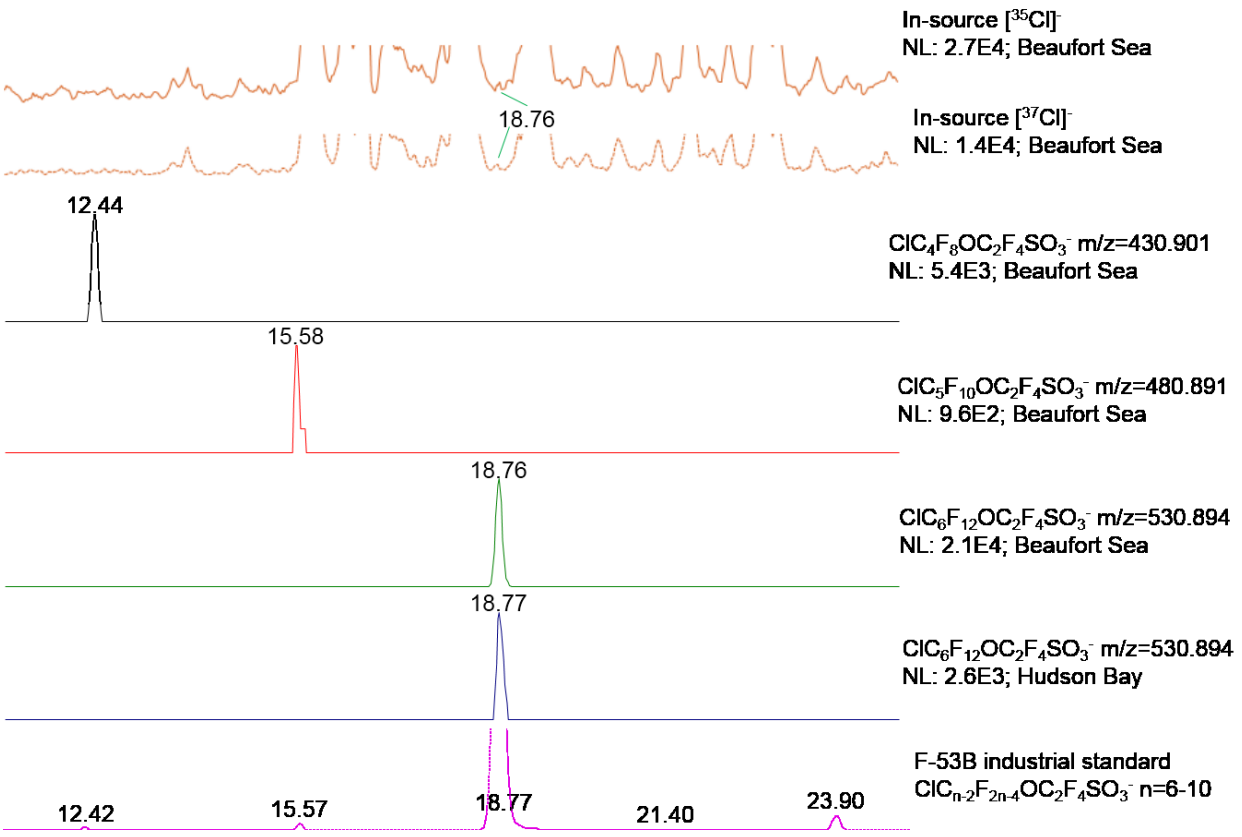


Figure 5-18.1 Extracted ion chromatograms of $[^{35}Cl]^-$ and $[^{37}Cl]^-$ in-source fragments, and **Class 3.4** x:2 Cl-PFAES homologues ($ClC_nF_{2n}SO_4^-$) detected in the Hudson Bay pooled polar bear serum (n=8), in Beaufort Sea pooled polar bear serum (n=6-8), and in F-53B industrial standard (n=6-10). All chromatograms are for the theoretical mass shown ±5 ppm.

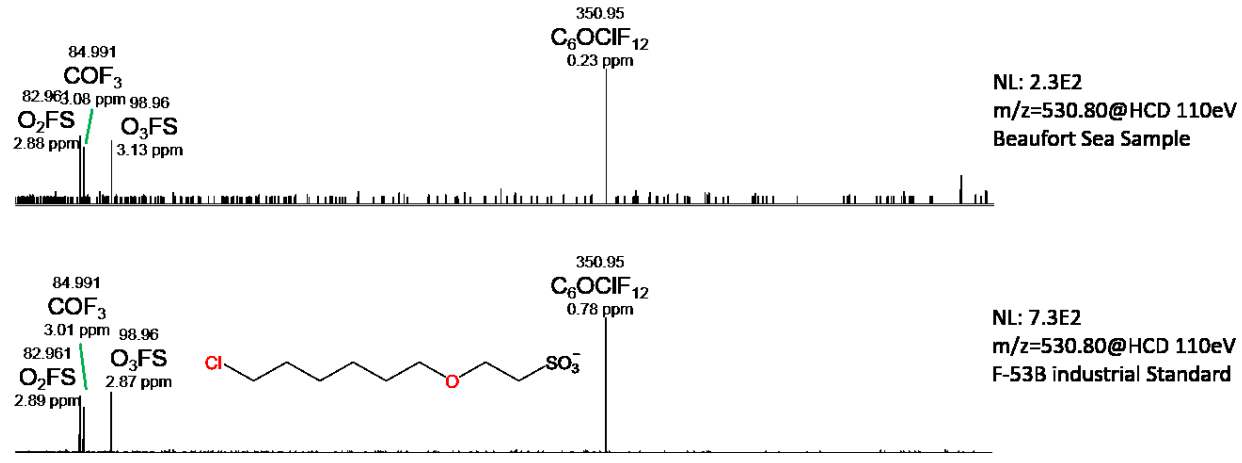


Figure 5-18.2 MS^2 spectra (HCD 110 eV) of C_8- x:2 Cl-PFAES ($ClC_8F_{16}SO_4^-$, **Class 3.4**) in Beaufort Sea pooled polar bear serum, and in F-53B industrial standard.

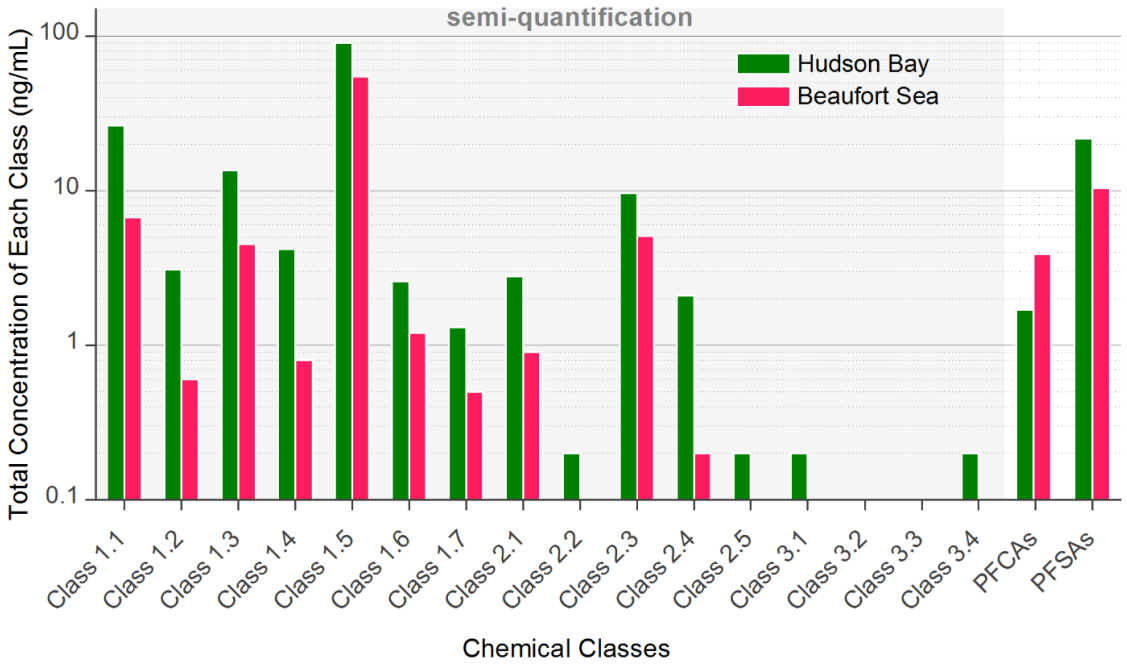


Figure 5-19. Total concentration of each class of halogenated compounds detected in pooled polar bear serum samples from Hudson Bay and Beaufort Sea (standard quantified compound classes: white background; semi-quantified compound classes: grey background).

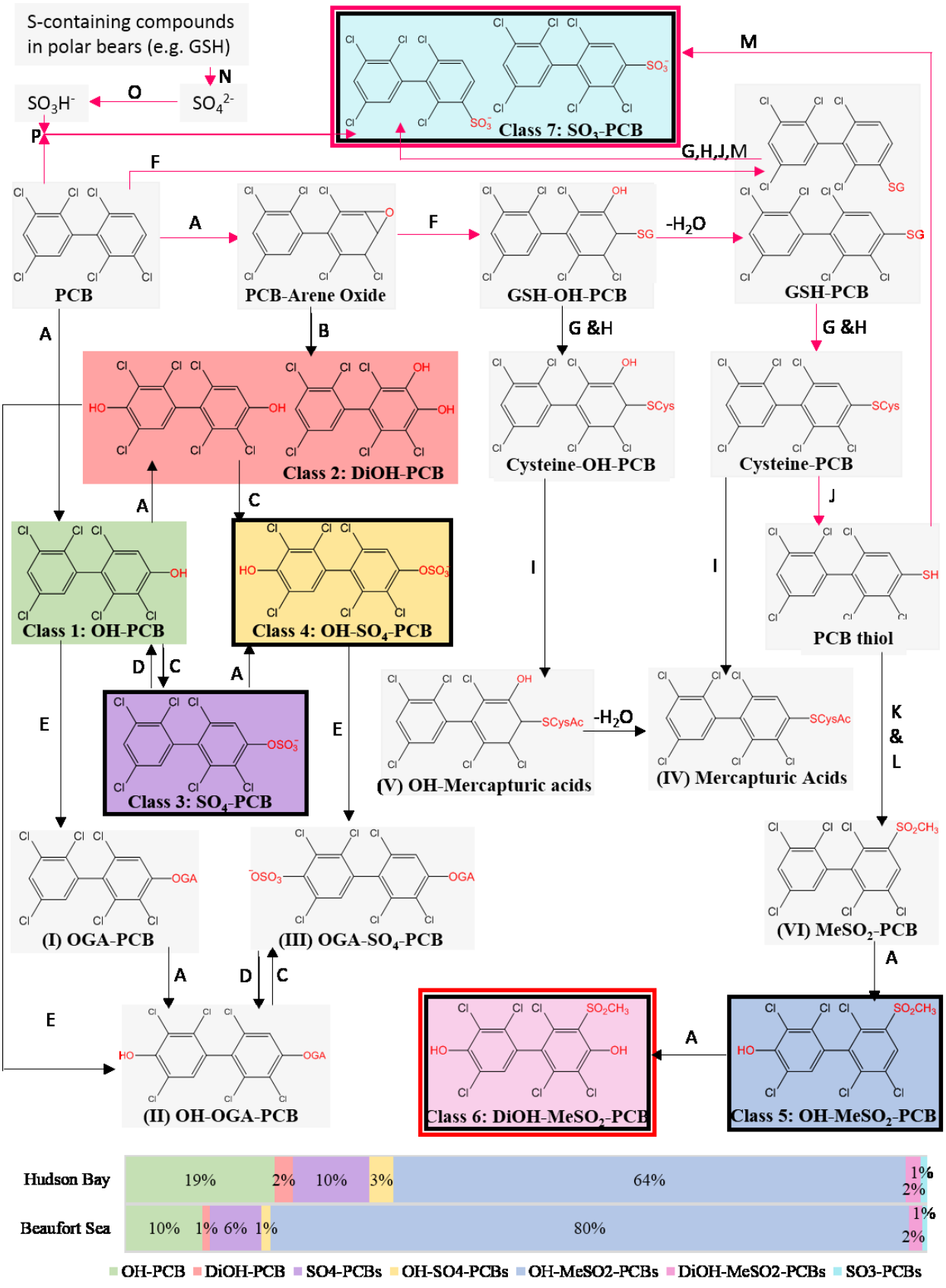

S-containing compounds in polar bears (e.g. GSH)
N
O
P
M
F
A
B
C
D
E
G &H
I
J
K & L
G,H,J,M
-H2O
PCB
PCB-Arene Oxide
GSH-OH-PCB
GSH-PCB
Cysteine-OH-PCB
Cysteine-PCB
PCB thiol
Class 1: OH-PCB
Class 2: DiOH-PCB
Class 3: SO4-PCB
Class 4: OH-SO4-PCB
Class 5: OH-MeSO2-PCBs
Class 6: DiOH-MeSO2-PCB
Class 7: SO3-PCB
(I) OGA-PCB
(II) OH-OGA-PCB
(III) OGA-SO4-PCB
(IV) Mercapturic Acids
(V) OH-Mercapturic acids
(VI) MeSO2-PCB
Hudson Bay
19%
2%
10%
3%
64%
1%
2%
Beaufort Sea
10%
1%
6%
1%
80%
1%
2%
OH-PCB
DiOH-PCB
SO4-PCBs
OH-SO4-PCBs
OH-MeSO2-PCBs
DiOH-MeSO2-PCBs
SO3-PCBs

Figure 5-20. Proposed metabolic scheme for a representative Cl_6-PCB congener (enzymes involved are listed in the following), and percentage bar graphs for all the 7 classes of PCB metabolite (**Class 1.1** - **1.7**) detected in pooled polar bear serum samples from both the Hudson Bay and Beaufort Sea. Metabolites with black and red outlines represent metabolites, for the first time, reported in polar bears and in all environmental and biological samples, respectively. Metabolites I-VI were previously reported but not detected in the current study. Metabolites IV and VI do not ionize in the current HPLC-ESI-Orbitrap system.

Enzymes: A. CYP450; B. Epoxide hydrolase; C. Sulfotransferase; D. Sulfatases; E. UDP-glucuronosyltransferase; F. Glutathione S-transferase; G. γ–Glutamyl transpeptidase; H. cysteinylglycine dipeptidase; I. Cysteine S-conjugate N-acetyltransferase; J. Cysteine S-conjugate β-lyase; K. Thiol S-methyltransferase; L. CYP or FMO; M. Oxidation; N. Oxidation; O. Reduced by gut floral; P. Directly added by gut flora

5.5.2 Tables

Table 5-1. OH-PCB, DiOH-PCB, SO_4-PCB and triclosan standards used in the current study.

Category	Source	Name or Abbreviation
Hydroxylated PCBs (OH-PCBs)	Wellington Lab	2,2',3',4,4',5-hexachloro-3'-hydroxybiphenyl (3'-OH-PCB 138, OH-PCB No.1)
		2,2',4,4',5, 5'-hexachloro-3-hydroxybiphenyl (3-OH-PCB 153, OH-PCB No.2)
		2,2',3,3',4', 5-hexachloro-4'-hydroxybiphenyl (4'-OH-PCB 130, OH-PCB No.3)
		2,2',3,4',5, 5'-hexachloro-4-hydroxybiphenyl (4-OH-PCB 146, OH-PCB No.4)
		2,2',3',4, 4',5,5'-heptachloro-3'-hydroxybiphenyl (3'-OH-PCB 180, OH-PCB No.5)
		2,2',3,3',4',5, 5'-heptachloro-4'-hydroxybiphenyl (4'-OH-PCB 172, OH-PCB No.6)
		2,2',3,4',5, 5',6-heptachloro-4-hydroxybiphenyl (4-OH-PCB 187, OH-PCB No.7)
Dihydroxylated PCBs (DiOH-PCBs)	Synthesized[39]	4-chloro-2',3'-dihydroxybiohenyl (2',3'-diOH-PCB 3)
		4-chloro-3',4'-dihydroxybiohenyl (3',4'-diOH-PCB 3)
PCB sulfates (SO_4-PCBs)	Synthesized[3, 9, 41-43, 45]	4-chlorobiphenyl-4'-sulfate (4'-SO_4-PCB 3, SO_4-PCB No.1)
		4-chlorobiphenyl-2'-sulfate (2'-SO_4-PCB 3, SO_4-PCB No.2)
		4-chlorobiphenyl-3'-sulfate (3'- SO_4-PCB 3, SO_4-PCB No.3)
		4-chlorobiphenyl-2-sulfate (2-SO_4-PCB 3, SO_4-PCB No.4)
		4-chlorobiphenyl-3-sulfate (3-SO_4-PCB 3, SO_4-PCB No.5)
		2,4'-dichlorobiphenyl-4-sulfate (4-SO_4-PCB 8, SO_4-PCB No.6)
		2,5-dichlorobiphenyl-4'-sulfate (4'-SO_4-PCB 9, SO_4-PCB No.7)
		3,3'-dichlorobiphenyl-4-sulfate (4-SO_4-PCB 11, SO_4-PCB No.8)
		3,4-dichlorobiphenyl-4'-sulfate (4'-SO_4-PCB 12, SO_4-PCB No.9)
		2,3',4-trichlorobiphenyl-4'-sulfate (4'-SO_4-PCB 25, SO_4-PCB No.10)
		2,3',5-trichlorobiphenyl-4'-sulfate (4'-SO_4-PCB 26, SO_4-PCB No.11)
		2,3',4'-trichlorobiphenyl-4-sulfate (4-SO_4-PCB 33, SO_4-PCB No.12)
		3,3',4-trichlorobiphenyl-4'-sulfate (4'-SO_4-PCB 35, SO_4-PCB No.13)
		2,2',5,5'-tetrachlorobiphenyl-4-sulfate (4-SO_4-PCB 52, SO_4-PCB No.14)
Hydroxylated PCDE	Sigma Aldrich	2, 4, 4,'-trichloro-2'-hydroxydiphenyl ether

Table 5-2. PFAS standards used in the current study

Native PFASs	Full name	Abbreviation	Molecular Formula
Perfluorinated Carboxylic Acids (PFCAs)	Perfluoro-n-butanoic acid	PFBA	$CF_3(CF_2)_2COO^-$
	Perfluoro-n-pentanoic acid	PFPeA	$CF_3(CF_2)_3COO^-$
	Perfluoro-n-hexanoic acid	PFHxA	$CF_3(CF_2)_4COO^-$
	Perfluoro-n-heptanoic acid	PFHpA	$CF_3(CF_2)_5COO^-$
	Perfluoro-n-octanoic acid	PFOA	$CF_3(CF_2)_6COO^-$
	Perfluoro-n-nonanoic acid	PFNA	$CF_3(CF_2)_7COO^-$
	Perfluoro-n-decanoic acid	PFDA	$CF_3(CF_2)_8COO^-$
	Perfluoro-n-undecanoic acid	PFUnDA	$CF_3(CF_2)_9COO^-$
	Perfluoro-n-dodecanoic acid	PFDoDA	$CF_3(CF_2)_{10}COO^-$
	Perfluoro-n-tridecanoic acid	PFTriDA	$CF_3(CF_2)_{11}COO^-$
	Perfluoro-n-tetradecanoic acid	PFTeDA	$CF_3(CF_2)_{12}COO^-$
	Perfluoro-n-hexanedecanoic acid	PFHxDA	$CF_3(CF_2)_{14}COO^-$
	Perfluoro-n-octadecanoic acid	PFODA	$CF_3(CF_2)_{16}COO^-$
Perfluorinated sulfonates (PFSAs)	Perfluoro-1-butanesulfonate	PFBS	$CF_3(CF_2)_3SO_3^-$
	Perfluoro-1-pentanesulfonate	PFPeS	$CF_3(CF_2)_4SO_3^-$
	Perfluoro-1-hexanesulfonate	PFHxS	$CF_3(CF_2)_5SO_3^-$
	Perfluoro-1-heptanesulfonate	PFHpS	$CF_3(CF_2)_6SO_3^-$
	Perfluoro-1-octanesulfonate	PFOS	$CF_3(CF_2)_7SO_3^-$
	Perfluoro-1-nonanesulfonate	PFNS	$CF_3(CF_2)_8SO_3^-$
	Perfluoro-1-decanesulfonate	PFDS	$CF_3(CF_2)_9SO_3^-$
	decafluoro-4-(pentafluoroethyl)-cyclohexanesulfonate	4-PFECHS	$C_2F_5(C_6F_4)SO_3^-$
Perfluorooctane sulfonamides (FOSAs)	Perfluoro-1-octanesulfonamide	FOSA	$CF_3(CF_2)_7SO_2NH^-$
	N-methylperfluoro-1-octanesulfonamide	MeFOSA	$CF_3(CF_2)_7SO_2N^-CH_3$
	N-ethylperfluoro-1-octane sulfonamide	EtFOSA	$CF_3(CF_2)_7SO_2N^-C_2H_5$
	N-ethylperfluoro-1-octane sulfonamidoacetic acid	MeFOSAA	$CF_3(CF_2)_7SO_2N(C_2H_5)$-$CH_2COO^-$

Table 5-3. Demographic information of pooled polar bear serum samples

Location	Collection Date	Age (yrs)	Sex	Volume (mL)
Western Hudson Bay	1985/04/17	9	M	3.0
	1985/04/19	7	M	3.3
	1986/09/17	18	M	3.8
	1987 spring	23	M	3.2
	1988/09/11	12	M	4.5
	1992 spring	10	F	3.3
	1993/07/22	18	M	7.5
	1993/11/09	9	M	7.5
	1994/04/08	14	F	2.0
	2000/03/02	18	F	1.5
	2003 spring	19	M	3.0
	2008/09/08	18	M	1.8
	2009/03/02	18	F	1.5
	2010 fall	4	F	1.5
Beaufort Sea	1987 spring	23	M	3.0
	2003 spring	19	M	3.5
	2007/04/28	1	M	1.7
	2007/04/28	11	F	2.1
	2007/04/28	8	F	1.8
	2007/05/06	14	F	1.8
	2008/04/19	13	F	1.8
	2009/04/17	7	M	1.8
	2009/04/18	16	F	1.8
	2009/04/19	15	F	1.7
	2009/04/20	5	F	1.8
	2009/04/20	7	F	2.0
	2009/04/30	7	F	1.8
	2010/04/20	14	F	1.8
	2010/04/21	8	F	1.8
	2010/04/22	22	F	1.8
	2010/04/23	20	F	1.5
	2010/04/25	5	F	1.7
	2010/04/25	13	F	1.7
	2010/04/27	5	F	1.8
	2010/04/27	3	F	1.7
	2010/04/27	12	F	1.8

Table 5-4. Instrumental detection limits (IDL, ng/mL) of SBSE-HPLC-Orbitrap method detection limits (MDLs, ng/mL) of legacy PFASs and some OH-PCB and SO_4-PCB standards. The former was measured by injecting 20 μL of different concentrations of mixed standards in water/methanol = 50/50, and the latter was measured by injecting 20 μL of SBSE extracts of 5 mL calf serum spiked at different concentrations.

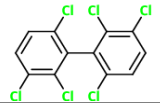
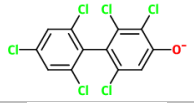
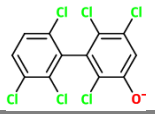
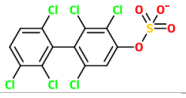
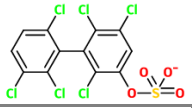
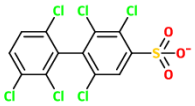
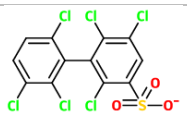
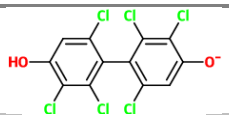
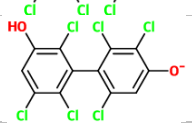
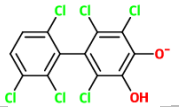
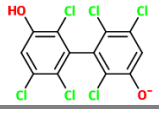
Standards		**IDLs (ng/mL)**	**MDLs (ng/mL) (in the original 5mL serum)**
PFCAs	C_4	0.01	0.025
	C_5	0.025	0.1
	C_6	0.005	0.10
	C_7	0.01	0.1
	C_8	0.025	0.005
	C_9	0.01	0.005
	C_{10}	0.025	0.005
	C_{11}	0.01	0.005
	C_{12}	0.025	0.05
	C_{13}	0.05	0.05
	C_{14}	0.5	0.1
	C_{16}	0.5	0.1
	C_{18}	1.0	0.25
PFSAs	C_4	0.0025	0.05
	C_5	0.0025	0.05
	C_6	0.0025	0.005
	C_7	0.0025	0.005
	C_8	0.0025	0.005
	C_9	0.01	0.005
	C_{10}	0.01	0.005
F-53B	C_8	0.0025	0.005
PFOS Precursors	FOSA	0.025	0.005
	MeFOSA	0.1	0.005
	MeFOSAA	0.01	0.005

Table 5-4. Continued:

	Standards	IDLs ng/mL	MDLs (ng/mL) (in the original 5mL serum)
OH-PCBs	Cl_6 (No. 1 standard*)	0.001	0.025
	Cl_6 (No. 2 standard*)	0.005	0.005
	Cl_6 (No. 3 standard*)	0.025	0.01
	Cl_6 (No. 4 standard*)	0.01	0.005
	Cl_7 (No. 5 standard*)	0.001	0.005
	Cl_7 (No. 6 standard*)	0.005	0.005
	Cl_7 (No. 7 standard*)	0.005	0.01
SO_4-PCBs	Cl (No. 4 standard*)	0.025	0.01
	Cl (No. 3 standard*)	0.01	0.05
	Cl_2 (No. 8 standard*)	0.005	0.005
	Cl_2 (No. 9 standard*)	0.0025	0.005
	Cl_3 (No. 11 standard*)	0.0025	0.005
	Cl_3 (No. 12 standard*)	0.005	0.01
	Cl_4 (No. 14 standard*)	0.01	0.01

* "No. # standard", where # corresponds to the numbering in **Table 5-1**.

Table 5-5. Predicated 96-hour fathead minnow LC_{50} values and developmental toxicities for representative PCB metabolites and other polychlorinated aromatic compounds by TEST*,[51]

	Structure	96 h fathead minnow LC_{50} (mg/L)	Development Toxicity
PCBs		0.0108	0.33; non-toxicant
Class 1.1 OH-PCB		0.0671	0.78; toxicant
		0.0383	0.79; toxicant
Class 1.3 SO_4-PCB		0.00295	1.31; toxicant
		0.00288	1.01; toxicant
Class 1.7 SO_3-PCB		0.0591	0.95; toxicant
		0.00362	1.00, toxicant
Class 1.2 DiOH-PCB		0.0247	0.93; toxicant
		0.0279	0.94; toxicant
		0.0154	0.87; toxicant
		0.0181	0.86; toxicant

Both toxicity prediction is the average results from 5 different methods: (1) hierarchical clustering: estimated using the weighted average of the predictions from several different models which are obtained by using Ward's method to divide the training set into a series of structurally similar clusters. A genetic algorithm-based technique is used to generate models for each cluster; (2) single model: estimated using a multilinear regression model that is fit to the training set (using molecular descriptors as independent variables) using a genetic algorithm-based approach; (3) group contribution: estimated using a multilinear regression model that is fit to the training set (using molecular fragment counts as independent variables); (4) FDA: estimated using a new model that is fit to the chemicals that are most similar to the test compound; and (5) nearest neighbor: estimated by taking an average of the three chemicals in the training set that are most similar to the test chemical

Table 5-5. Continued:

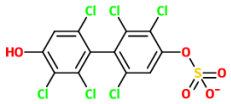
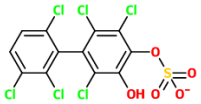
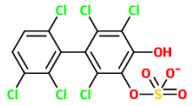
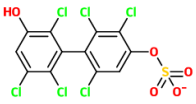
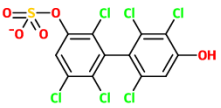
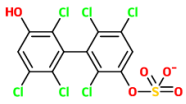
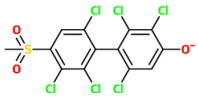
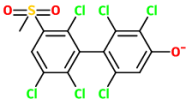
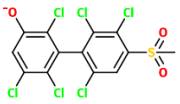
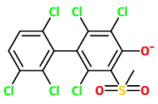
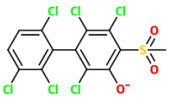
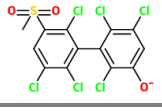
	Structure	96 h fathead minnow LC_{50} (mg/L)	Development Toxicity
Class 1.4 OH-SO_4-PCB		0.0789	1.21; toxicant
		0.0171	1.18; toxicant
		0.0390	1.20; toxicant
		0.0124	1.27; toxicant
		0.0253	1.25; toxicant
		0.0230	1.21; toxicant
Class 1.5 OH-$MeSO_2$-PCB		0.0131	0.92; toxicant
		0.24	0.98; toxicant
		0.0783	0.93; toxicant
		0.0406	0.94; toxicant
		0.0490	0.86; toxicant
		0.0531	0.85; toxicant

Table 5-5. Continued:

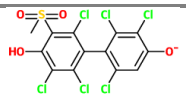
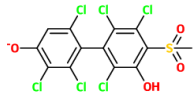
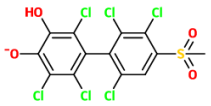
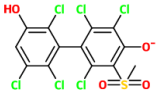
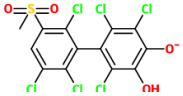
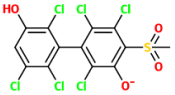
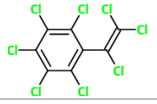
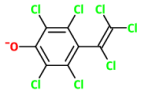
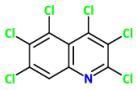
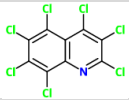
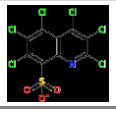
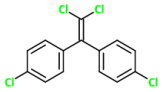
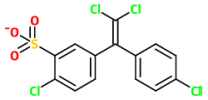
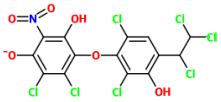
	Structure	96 h fathead minnow LC_{50} (mg/L)	Development Toxicity
Class 1.6 DiOH-$MeSO_2$-PCB		0.0208	1.04; toxicant
		0.0342	1.07; toxicant
		0.0210	1.04; toxicant
		0.15	1.07; toxicant
		0.0134	1.06; toxicant
		0.0301	099; toxicant
Class 2.1 chlorinated aromatics e.g. hydroxyl-chlorostyrene		0.00579	-0.24; non-toxicant
		0.0141	0.27; non-toxicant
Class 2.2 chlorinated (iso)quinoline sulfonates		0.0604	0.42; non-toxicant
		0.0210	0.10; non-toxicant
		0.56	0.68; toxicant
Class 2.3 tetrachlorinated aromatic sulfate e.g. SO_4-DDE		0.15	0.46; non-toxicant
		0.19	1.03; toxicant
Class 2.4 heptachlorinated hydroxylated nitroaromatics		0.000878	0.90; toxicant

Table 5-6. Non-target PFSAs and poly/perchlorinated compounds detected in polar bear serum samples.

Category	Class No.	Name & Abbreviation	Ion Formula	Total Analytes	Confidence Level	Previous Reports in Polar Bears & in Other Samples
PCB metabolites	1.1	hydroxylated PCBs OH-PCB	$C_{12}Cl_nH_{9-n}O^-$ n=3-9	54	3; 1 for two congeners	~40 congeners (Cl_4-Cl_9) detected in polar bears, and 25 of them structurally confirmed (Cl_5-Cl_9)[21, 57-60, 62] in total >100 congeners (Cl-Cl_9) reported in other samples[11, 21, 57-62, 65-73]
	1.2	di-hydroxylated PCB DiOH-PCBs	$C_{12}Cl_nH_{9-n}O_2^-$ n=4-8	37	3	One Cl_8 congener confirmed in polar bears[21, 57, 60-62] in total 6 congeners confirmed (Cl_5-Cl_8) in other samples[11, 21, 57, 60-62, 66, 68, 71]
	1.3	PCB sulfate SO_4-PCBs	$C_{12}Cl_nH_{9-n}SO_4^-$ n=2-8	51	3; 1 for two congeners	in total 6 congeners (3 for Cl- and 3 for Cl_2-) proposed in PCB 3 and PCB 11 exposed rats[75, 76] and plants,[77] and in human with known PCB 11 exposure[78]
	1.4	hydroxylated PCB sulfates OH-SO_4-PCBs	$C_{12}Cl_nH_{9-n}SO_5^-$ n=3-7	42	3	in total 3 OH-SO_4-PCB congeners (2 for Cl- and 1 for Cl_2-) proposed in PCB 3[81] and PCB 11 exposed rats[76]
	1.5	hydroxylated methylsulfone PCBs OH-$MeSO_2$-PCBs	$C_{13}Cl_nH_{11-n}SO_3^-$ n=3-8	45	3	approximately 15 peaks (Cl_4-Cl_6) proposed as OH-$MeSO_2$-PCBs in Yusho patients[87]
	1.6	di-hydroxylated methylsulfone PCBs DiOH-$MeSO_2$-PCBs	$C_{13}Cl_nH_{11-n}SO_4^-$ n=2-7	22	3	no report
	1.7	PCB sulfonate SO_3-PCBs	$C_{12}Cl_nH_{11-n}SO_3^-$ n=3-6	21	3	no report

Table 5-6. Continued:

Category	Class No.	Name & Abbreviation	Ion Formula	Total Analytes	Confidence Level	Previous Reports in Polar Bears & in Other Samples
Other poly-chlorinated compounds	2.1	chlorinated aromatic compounds (e.g. hydroxyl-chlorostyrene)	$C_8Cl_nH_{7-n}O^-$ n=6-7	2	3	4-OH-HpCS in polar bears[92] 4-OH-HpCS in fish,[90] humans,[91] ringed seal[92]
	2.2	chlorinated (iso)quinoline sulfonates	$C_9Cl_nH_{6-n}NSO_3^-$ n=5-6	3	3	no report
	2.3	tetrachlorinated aromatic sulfate (e.g. DDE sulfate)	$C_{14}Cl_4H_7SO_4^-$	1	3	/
	2.4	heptachlorinated hydroxylated nitroaromatic compounds	$C_{14}Cl_7H_5NO_6^-$	1	3	/
			$C_{14}Cl_7H_7NO_6^-$	1		
	2.5	hexachlorinated compounds	$C_{15}Cl_6H_{13}O_5^-$	2	4	/
			$C_{15}Cl_6H_{15}O_3^-$	1		

Table 5-6. Continued:

Category	Class No.	Name & Abbreviation	Ion Formula	Total Analytes	Confidence level	Previous Reports in Polar Bears & in Other Samples
Per-fluoroalkyl sulfonates (PFSAs)	3.1	cyclic or unsaturated PFSAs	$C_nF_{2n-1}SO_3^-$ n=8-10	8	3	n=4-13 in 3M commercial products, industrial PFOS and AFFFs;[114] n=7-14 in fish liver (**Chapter 4**); n=8-9 in industrial 3M-PFOS standard in total 5 C_8-cyclic PFSAs in industrial 4-PFECHS,[109, 111] waters,[109, 110] sediments,[111] fish;[109, 110, 112] only 4-PFECHS[109-113] and 4-PFMeCHS[109] structurally confirmed
	3.2	ether PFSAs	$C_nF_{2n+1}SO_4^-$ n=6-9	11	3	A C_6-ether-PFSA proposed in firefighter' sera[31] n=4-12 in fish liver (**Chapter 4**); n=8-9 in industrial 3M-PFOS standard
	3.3	enol-ether-, cyclic-ether- or carbonyl-PFSAs	$C_nF_{2n-1}SO_4^-$ n=7-9	12	3	n=4-13 in concrete from AFFF impacted fire training ground;[115] n=6-13 in fish liver (**Chapter 4**); n=8-9 in industrial 3M-PFOS standard n=8 ketone-PFSA proposed in firefighters' sera[31]
	3.4	x:2 chlorine substituted perfluoroalkyl ether sulfonates x:2 Cl-PFAESs	$ClC_nF_{2n}SO_4^-$ n=6-8	3	1 for one homologue, 2 for others	n=8 in Greenland ringed seal and polar bears[128] in total 3 homologues proposed (n=8, 10, 12) in chrome plating company discharge and downstream surface water,[123] municipal sewage sludge;[122] air sample[124] in China, fish[125] and in Chinese human serum[127] and urine samples[127] n=7-12 and 14 in fish liver (**Chapter 4**) n=6-10 in F-53B industrial standard

Table 5-7. Quantified legacy PFASs and semi-quantified non-target PFSAs and chlorinated compounds discovered in both pooled polar bear serum samples from Western Hudson Bay and Beaufort Sea. The LOQ value (0.004 ng/mL) for each pooled sample is calculated back from instrumental LOQ (i.e. 0.01 ng/mL for each legacy standard). The "reference standard" for each non-target analyte is an authentic standard whose calibration curve was used to semi-quantify the non-target assuming the same molar response. Percent numbers in red are concentration ratios of each analyte in its homologous/congener class.

	Reference Standard	**Western Hudson Bay Pooled Polar bear Serum**		**Beaufort Sea Pooled Polar bear**	
		Conc. (ng/mL) in pooled serum	% in each class	Conc. (ng/mL) in pooled serum	% in each class
Class 1.1 OH-PCBs					
Cl_3	averaged OH-PCB and SO_4-PCB standards	0.1	0.3%	<0.1	0.5%
Cl_4		0.6	2.2%	0.2	3.3%
Cl_5		2.1	7.9%	0.7	9.8%
Cl_6		8.9	34%	2.4	35%
Cl_7		12	47%	3.0	45%
Cl_8		2.2	8.3%	0.4	5.6%
Cl_9		0.2	0.8%	<0.1	0.4%
Total		26		6.7	
Class 1.2 DiOH-PCBs					
Cl_4	averaged OH-PCB and SO_4-PCB standards	<0.1	0.5%	<0.1	0.7%
Cl_5		0.8	26%	0.2	32%
Cl_6		0.7	21%	<0.1	10%
Cl_7		0.6	20%	0.1	23%
Cl_8		1.0	33%	0.2	34%
Total		3.1		0.6	
Class 1.3 SO_4-PCBs					
Cl_2	averaged OH-PCB and SO_4-PCB standards	<0.1	0.1%	<0.1	<0.1%
Cl_3		0.7	4.9%	0.4	8.3%
Cl_4		4.4	32%	1.0	22%
Cl_5		5.4	40%	2.0	44%
Cl_6		1.8	13%	0.8	18%
Cl_7		1.3	9.8%	0.3	7.6%
Cl_8		<0.1	0.2%	<0.1	0.1%
Total		14		4.5	

Table 5-7. Continued:

	Reference Standard	Western Hudson Bay Pooled Polar bear Serum		Beaufort Sea Pooled Polar bear	
		Conc. (ng/mL) in pooled serum	% in each class	Conc. (ng/mL) in pooled serum	% in each class
Class 1.4 OH-SO_4-PCBs	averaged OH-PCB and SO_4-PCB standards				
Cl_3		0.1	2.7%	<0.1	3.0%
Cl_4		0.3	7.6%	0.1	12%
Cl_5		2.5	60%	0.6	70%
Cl_6		0.9	21%	<0.1	8.9%
Cl_7		0.4	9.6%	<0.1	5.8%
Cl_8		<0.1	<1%		
Total		4.2		0.8	
Class 1.5 OH-$MeSO_2$-PCBs	averaged OH-PCB and SO_4-PCB standards				
Cl_3		2.0	2.2%	0.5	0.9%
Cl_4		39	43%	28	53%
Cl_5		32	35%	18	33%
Cl_6		15	16%	7.0	13%
Cl_7		3.2	3.6%	1.2	2.2%
Cl_8		<0.1	0.04%	<0.1	<0.1%
Total		90		55	
Class 1.6 DiOH-$MeSO_2$-PCBs	averaged OH-PCB and SO_4-PCB standards				
Cl_2		<0.1	1.6%	<0.1	1.2%
Cl_3		1.1	43%	0.8	68%
Cl_4		0.6	25%	0.2	14%
Cl_5		<0.1	1.7%	<0.1	0.7%
Cl_6		0.8	29%	0.2	16%
Cl_7		<0.1	0.2%		
Total		2.6		1.2	
Class 1.7 SO_3-PCBs	averaged OH-PCB and SO_4-PCB standards				
Cl_3		<0.1	0.2%		
Cl_4		0.4	33%	0.1	21%
Cl_5		0.6	49%	0.2	49%
Cl_6		0.2	18%	0.1	30%
Total		1.3		0.4	

Table 5-7. Continued:

	Reference Standard	**Western Hudson Bay Polar Bear Serum**		**Beaufort Sea Polar Bear Serum**	
		Conc. (ng/mL) in pooled serum	% in each class	Conc. (ng/mL) in pooled serum	% in each class
Class 2.1 chlorinated aromatic compounds_Cl_6	averaged OH-PCB and SO_4-PCB standards	<0.1	/	<0.1	/
Cl_7		2.8	/	0.9	/
Total		2.8		0.9	
Class 2.2 chlorinated (iso)quinoline sulfonates_Cl_5		0.2	/	0.1	/
Cl_6		<0.1	/	<0.1	/
Total		0.2		0.2	
Class 2.3 tetrachlorinated aromatic sulfate_Cl_4		9.6	/	5.2	/
Class 2.4 heptachlorinated hydroxylated nitro-aromatic compounds_Cl_7H_5		0.1	/	0.1	/
Cl_7H_7		2.0	/	0.1	/
Total		2.1		0.2	
Class 2.5 hexachlorinated compounds_Cl_6H_{13}		0.1	/	<0.1	/
Cl_6H_{15}		0.1	/	<0.1	/
Total		0.2		<0.1	

Table 5-7. Continued:

	Reference Standard	**Western Hudson Bay Polar Bear Serum**		**Beaufort Sea Polar Bear Serum**	
		Conc. (ng/mL) in pooled serum	% in each class	Conc. (ng/mL) in pooled serum	% in each class
Class 3.1 cyclic or unsaturated PFSAs_C_8	PFOS	0.1	73%	<0.1	49%
C_9	PFNS	<0.1	5%	<0.1	7%
C_{10}	PFDS	<0.1	22%	<0.1	44%
Total		0.2		0.1	
Class 3.2 ether PFSAs_C_6	PFHxS	<0.1	6%	<0.1	5%
C_7	PFHpS	<0.1	13%	<0.1	11%
C_8	PFOS	<0.1	19%	<0.1	13%
C_9	PFNS	<0.1	62%	<0.1	71%
Total		<0.1		0.1	
Class 3.3 enol-ether-, cyclic-ether- or carbonyl-PFSAs_C_7	PFHpS	<0.1	5%	<0.1	6%
C_8	PFOS	0.1	53%	<0.1	31%
C_9	PFNS	<0.1	42%	<0.1	62%
Total		0.1		0.1	
Class 3.4 x:2 Cl-PFAESs_C_6	6:2 Cl-PFAES	/		<0.1	21%
C_7		/		<0.1	13%
C_8		0.2	100%	<0.1	66%
Total		0.2		<0.1	

Table 5-7. Continued:

	Reference Standard	Western Hudson Bay Polar Bear Serum		Beaufort Sea Polar Bear Serum	
		Conc. (ng/mL) in pooled serum	% in each class	Conc. (ng/mL) in pooled serum	% in each class
PFCAs_C4	PFCAs_C4	/	/	/	/
C_5	C_5	/	/	/	/
C_6	C_6	/	/	/	/
C_7	C_7	/	/	/	/
C_8	C_8	<0.1	2%	<0.1	2%
C_9	C_9	0.4	26%	1.4	37%
C_{10}	C_{10}	0.1	8%	0.6	15%
C_{11}	C_{11}	1.0	58%	1.7	44%
C_{12}	C_{12}	<0.1	0.2%	<0.1	0.2%
C_{13}	C_{13}	<0.1	4%	<0.1	2%
C_{14}	C_{14}	<0.1	2%	/	/
C_{16}	C_{16}	/	/	/	/
C_{18}	C_{18}	/	/	/	
Total		1.7		3.9	
PFSAs_C4	PFSAs_C4	/	/	/	/
C_5	C_5	<LOQ		<LOQ	
C_6	C_6	0.7	3%	0.7	7%
C_7	C_7	0.9	4%	0.4	4%
C_8	C_8	20	93%	9.2	89%
C_9	C_9	<0.1	0.4%	<0.1	0.5%
C_{10}	C_{10}	<LOQ	<LOQ	<LOQ	
Total		22		10	
PFOSA	PFOSA	<0.1	12%	/	/
MeFOSA	MeFOSA	0.1	88%	/	/
MeFOSAA	MeFOSAA	/	/	/	/
EtFOSA	EtFOSA	/	/	/	/
EtFOSAA	EtFOSAA	/	/	/	/
Total		0.1			

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Chapter 6. Summary, Conclusions and Future Work

6.1 Summary and Conclusions

6.1.1 Background and Problems

The global distribution,[1, 2] extreme persistence,[3] high bioaccumulation potential[4] and toxicological effects of long-chain PFASs[5-7] have led to increasing human and environmental health concern. Since the year 2000, industrial phase-outs[8-10] as well as domestic and international restrictions and regulations[9-16] have been enacted to minimize future human and environmental exposure. These actions have led to a general transitioning towards manufacturing of alternative-PFASs, as well as a geographic shift of PFAS manufacturing into countries with fewer restrictions.

The 3M Company (3M Co.), historically the world's largest PFAS manufacturer, phased out its PFOS and PreFOS production between years 2000 and 2002.[17] Since then, there has been a general decreasing trend in PFOS concentrations in human serum samples collected in North America[18-21] and Europe.[22-25] However, how these trends have been, or will continue to be, influenced by relative changes in direct PFOS or indirect PreFOS exposure was not known. As unknown amounts of PFOS and PreFOS continue to be produced for exempted applications, and as unknown quantities of historically emitted PreFOS continue to degrade in the environment, humans are expected to be exposed to PFOS long into the future. Understanding the relative PFOS and PreFOS contribution will help to understand major exposure sources and may allow prediction of future exposure.

According to very limited publically available information, short-chain PFASs and structurally modified long-chain PFASs are the two major alternatives that are currently being produced.[26] However, the types of modified structures used in alternative-PFASs largely remain unknown, and the fact that these alternatives are still polyfluoro-based raises concerns about their persistence and safety. Historically, structurally similar hydrochlorofluorocarbons were used to replace chlorofluorocarbons,[27] but turned out still to be hazardous. Discovery of such unknown

alternative-PFASs near their sources will reveal what PFASs are now being used, and which may continue to represent an environmental hazard in the future, thereby enabling early safety evaluation studies before they become global contaminants.

6.1.2 Thesis Scope

With the aim of understanding overall current and future human PFAS exposure, my thesis has been focused on two research questions: (1) what is the relative amount of human PFOS exposure that comes from PreFOS, and how has this been changing since the widespread discovery of PFOS and its precursors in human biofluids, and (2) to what alternative PFASs are we likely to be exposed to in the future. Question (1) was addressed in **Chapter 2**, by exploring the temporal trends of PFOS isomers and enantiomers as biomarkers of relative PreFOS exposure in the U.S and Swedish populations. Question (2) was addressed in **Chapters 3** and **4** through method development of a non-target analytical approach and workflow that effectively enabled several organofluorine discoveries in fluorochemical industry wastewater, as well as in downstream fish in China, which is now the major PFAS production center in the world. In **Chapter 5** an effective sample preparation step, based on stir-bar sorptive extraction (SBSE), was paired with the untargeted analytical workflow to discover other unique PFASs in polar bears, and serendipitously led to discovery of hundreds of previously unrecognized organochlorine contaminants and associated metabolites in serum of polar bears from two populations. Thus, the thesis research succeeded in answering the general questions that were set out, but also served to demonstrate the power of non-target analysis to discover, not only new contaminants, but also old ones which have eluded detection for many decades.

6.1.3 How We Are and Will Be Exposed to PFOS

Archived human serum samples from an American and Swedish population were examined for PFOS isomer and enantiomer profiles in **Chapter 2**, both of which were previously proposed as biomarkers of relative PreFOS exposure in humans.[1] Both biomarkers indicated a relative increasing importance of PreFOS exposure in the Swedish population from 1996 to 2010. In the U.S. population, no temporal trend was observed but clear age and sex differences were observed for both biomarkers. Specifically, males and older people had significantly higher PreFOS exposure than females and younger people for all the 5 years between 1975 and 2010.

The study of both populations suggested that despite overall decreasing PFOS in all humans since the 3M phase-out, the fraction of exposure from PreFOS has declined more slowly than direct PFOS exposure. Based on conservative assumptions and the PFOS isomer and enantiomer profiles, the amount of PFOS in human serum from PreFOS has increased from 3 - 7% in 1995 to 13 - 21% in 2010 in the Swedish population, and may continue to trend upwards in the future. This raises some concern over PreFOS, a vast group of PFASs that has the same long history as PFOS, and that has probably been emitted in even greater amounts than PFOS,[28, 29] even though only a few molecules have historically been monitored. Future studies on PreFOS are suggested to reveal their chemical identities, to elucidate their exposure sources and potential for adverse biological effects. A focus on exposure to older males may be most relevant.

PFOS has almost always been the most prominent legacy long-chain PFAS detected in various samples,[1] and was the first PFAS to be phased out[8] or regulated.[30] This PFOS study therefore shed light on overall legacy long-chain PFAS exposure, probably suggesting an overall increasingly important precursor exposure and highlighting future research needs for identifying historically unknown PFASs, which led us to **Chapter 3**.

6.1.4 What Other PFASs May Be Entering the Environment?

6.1.4.1 Non-target PFAS Discovery Method Development

The large unknowns in alternative-PFAS that are currently being produced, and of unknown precursors to legacy PFAAs that have historically been emitted represent an important source of uncertainty for environmental and human health risk assessment. In **Chapter 3**, by using a wastewater sample discharged from a fluorochemical industrial park in China, I developed a fast and sensitive non-target PFAS discovery method on an HPLC-Orbitrap MS.

The method has the following characteristics: (1) the in-source fragmentation spectra, collected parallel to full-scan spectra, allow minute PFAS signals to be revealed from massive background signals, thereby simplifying and accelerating PFAS signal filtering; (2) the in-source fragmentation scans, which are performed by applying a voltage in the source (e.g. in the S-lens areas for Orbitrap Elite^{TM}), can be achieved in all commercialized HRMS, making its application in other HRMS systems quite straightforward; (3) the in-line SPE technique allows up to 5 mL of sample to be injected and analyzed, greatly boosting method sensitivity to a level (0.005 - 0.2

ng/mL) comparable to target MRM-analysis on triple quadrupole or quadrupole ion trap MS systems;[31] and (4) the method has the potential to be used for discovering any class of compound which can fragment to produce characteristic ions in the source (e.g. chlorinated compounds can produce Cl^-, brominated compounds can produce Br^-).

Historically, identification of environmental contaminants could only be performed with targeted methods and authentic standards, and with a hypothesis that an analyte should be present. Recent advances in HRMS raise new possibilities for early discovery of environmental contaminants without such *a priori* information. The establishment of this non-target method will greatly facilitate the discovery of unknown PFASs, and potentially other contaminants, thereby contributing to minimizing widespread contamination of our environment in the future.

6.1.4.2 Non-target PFAS Discovery in Environmental and Biological Samples

Since around the year 2000, there has been a global shift in PFAS manufacturing from developed countries towards emerging economies in countries like China and India. In contrast to the decreasing PFOS concentrations in Westerners,[22-25] PFOS in residents in Shenyang, China, has surged from 1.8 ng/mL in 1999[32] to 79 ng/mL in 2004,[33] and up to 31400 ng/mL of PFOS was detected in serum of Chinese fishermen in 2013.[34] This, along with limited industrial data,[35, 36] suggests that China has probably become one of the most important and active PFAS production centers in the world. Unknown PFAS identification in samples from China is therefore of particular importance, and will likely help to reveal the major alternative classes that are being manufactured and marketed in China, and perhaps exported around the world.

In the industrial wastewater sample from China (**Chapter 3**), 5 new homologous classes (36 analytes) of PFASs with carbon chains ranging from 5 to 17 were discovered, composed mostly of hydro- or chlorine-substituted legacy PFCAs and PFSAs. While the hydro-substituted PFASs could be a type of alternatives, where hydrogen was inserted to make the compound easier to degrade, the chlorinated compounds are likely to have bioaccumulation potentials that rival or exceed their perfluorinated counterparts.

Further in non-target PFAS discovery, and specifically to understand the environmental relevance and possibility for bioaccumulation of the discovered PFASs, we analyzed fish samples (**Chapter 4)** collected from two water systems in China, one of which was downstream of the WWTP sampled in **Chapter 3**. Pooled fish livers were extracted with an off-line SPE

method and subjected to HPLC-Orbitrap for analysis. In total, 10 classes of new PFASs (>330 analytes) were revealed, with 4 classes not previously reported in any samples. The discovered PFASs are composed of unsaturated/cyclic-, ether-, carbonyl- or chlorine-substituted PFASs, two classes of poly/perfluoroamines and a perfluorinated N-heterocyclics. Only ketone- and ether-PFASs are known to have been produced intentionally in recent years by some companies as alternative PFASs,[37] and it is unclear whether the other detected structures were from historical or contemporary PFAS production and emission.

Chapter 3 and **Chapter 4** suggested that vast numbers of unknown PFASs have been, or are, currently manufactured and emitted in China. Most of the discovered PFASs are indeed structurally modified. While the persistence of structurally modified PFASs is still a question requiring extensive study, most of them (61 out of the total 73 homologues) have v≥8 carbons and thus have high potential to bioaccumulate. For example, in **Chapter 4**, both the C_8- and C_9 monoether-PFASs, against a PFOS calibration curve, were semi-quantified to be present at ~50 ng/g w.w. in fish livers. As expected, the chlorinated PFCAs discovered in the upstream wastewater sample in **Chapter 3**, were also detected in the downstream fish samples (**Chapter 4**), confirming their bioavailability and suggesting a bioaccumulation potential. In addition, the discovery of per/polyfluoroamines and perfluorinated N-heterocycles is intriguing and worth special attention as their structures are very different from legacy PFASs, and are estimated to be present at extreme concentrations (e.g. **Class 8** perfluoroamine 630 ng/g w.w. and C_{12}-perfluoro-N-heterocycle 210 ng/g w.w. in bream fish).

6.1.4.3 Non-target Halogenated Organic Discoveries in Polar Bears

To explore the potential of the developed non-target method in **Chapter 3**, I further applied it to polar bear serum samples in **Chapter 5**. Polar bears have long been known to be exposed to high levels of hazardous organohalogen contaminants,[38, 39] and previous work has suggested an unknown fraction of thyroid disruptors in polar bear serum.[40, 41]

To increase signal intensities for non-target PFASs and chlorinated contaminants in polar bear serum, a SBSE method was developed using polyethersulfone segments as the sorptive material to concentrate analytes in 5 mL serum samples. Compared to the commonly used SPE sample preparation method in environmental field, SBSE can work with much higher volumes of samples (e.g. 100 mL wastewater[42]) without significantly increasing the cost. However, on the

other hand, only two types of commercialized coated stir bars, PDMS[43, 44] and PDMS/ethylene glycol,[45] are currently available. The development of more sorptive materials for SBSE, as the various packing materials used in SPE cartridges, is therefore highly desired.

Polar bear serum from individual bears, collected in various years, from two Canadian Arctic regions were pooled by location, extracted by a SBSE method, and subjected to the HPLC-Orbitrap method for analysis. In total, 4 PFAS classes (>30 analytes) and 12 chlorinated classes were discovered (>280 analytes, including 7 PCB metabolite classes, 4 other polychloro-aromatic classes, and 1 uncharacterized polychlorinated classes). Among them, 9 classes, including 5 PCB metabolite classes, were reported for the first time in polar bears.

The successful discovery of an array of previously unrecognized PFASs and chlorinated substances in polar bears, demonstrates the capability of the non-target PFAS method (**Chapter 3**) to discover other classes of contaminants, and indicates that historical exposure to halogenated contaminants in polar bears has been substantially underestimated and not fully understood. The wider relevance of these discoveries to the Arctic marine foodweb, to Inuit, and to background populations around the world has yet to be established.

6.2 Future Work

Overall, this thesis contributes to the understanding of past and future human PFAS exposure: legacy long-chain PFAS exposure will continue, and the relative importance of precursors is likely to increase. In today's source regions, a vast array of previously unknown PFASs are being emitted to our environment and could result in persist long term effects over a much broader area. As suggested in **Chapter 5**, wildlife and humans have likely been long-exposed to a greater number of legacy contaminants, or their transformation products, than previously understood. While two major research questions were answered in this thesis, some new research questions arose at the same time.

In **Chapter 2**, while the U.S. samples showed clear age and gender differences, the study failed to show any temporal trend for PreFOS exposure. Future studies on better temporal samples (e.g. larger sample sizes, smaller sampling gaps, and higher homogeneity) could help to clarify future human PFOS exposure in that country. From a broad perspective, similar studies exploring the relative direct exposure and indirect precursor exposure should be performed for

other long-chain PFASs, especially those are currently showing upward trends (e.g. C_9-C_{11} PFCAs and PFHxS, **Table 1**), and in more countries for a better understanding of overall human exposure trends around the world.

As indicated by the study in **Chapter 2**, precursor exposures are likely gaining in relative importance. However, in the non-target discovery studies in **Chapters 3-5**, PreFOS molecules were not discovered. While this could be due to the low abundance of such precursors in the tested samples, the single use of negative electrospray mode is likely another possible reason. Barzen-Hanson[46] have recently reported some previously unknown PreFOS molecules using positive electrospray mode. In the future, positive mode, as well as different ionization modes (e.g. APCI, APPI) should also be run to better recognize the spectrum of unknown PFASs that are currently present in our environment. New non-target discovery techniques should also be explored. Peng *et al*.[47] recently developed a data-independent precursor isolation and characteristic fragment method for brominated compound discovery on Orbitrap Q Exactive, and ABSciex has also released a Triple TOF® 6600 system featuring *SWATH* (i.e., MS/MS^{ALL}) function.[48] Also, with GC-HRMS (e.g. Q Exactive™ GC Orbitrap[49]) becoming available, non-target discovery of less- or even non-polar PFASs, if there are any, becomes feasible.

The non-target PFAS discovery work in **Chapters 3-5** revealed previously unknown PFASs and chlorinated substances, and downstream studies of these contaminants should be conducted to understand their distribution, toxicological profiles and their overall risks. For example, a temporal trend study of these new compounds will help to determine whether they are increasing in samples, and thus worth of special attention in the future.

As demonstrated in **Chapter 5**, the developed non-target PFAS discovery method has great potential to be applied for identifying other types of environmental contaminants than PFASs alone, and such trials in the future should be encouraged. Further, for a specific contaminant class, the non-target method could be applied to different types of environmental samples or different wildlife tissues. This is expected to reveal all the possible forms of the contaminant, and will help gain a better understanding of total exposure, and of environmental fate and (bio)transformation pathways. For example, legacy PreFOS compounds have been detected at low levels in serum samples, a matrix in which PFOS monitoring studies have most commonly been performed. However, it has been shown that in blood, PFOSA (a PreFOS

molecule) is predominately distributed in the red blood cell fraction, rather than in serum.[50] To also include the red blood cell fractions in future studies will probably increase the probability of discovering historically unknown PreFOS in biological samples.

Sample preparation is crucial in non-target discovery, especially when discovering subtle contaminants in complex sample matrices. In-line SPE and SBSE methods were developed for non-target PFAS discovery in a wastewater sample (**Chapter 3**) and in polar bear sera (**Chapter 5**), respectively. However, both methods only showed high extraction efficiencies for certain PFASs (e.g. longer chain PFSAs), and the SBSE method unfortunately removes poly-/per-fluoroalkyl phosphonic acids in the phospholipid-removal step. Development of new sample preparation techniques with higher extraction efficiencies for a wider spectrum of PFASs is therefore desirable. The currently available SiliaPrep™ Fluorochrom SPE cartridges[51] packed with fluorinated sorbent are probably a good candidate for future method development.

Finally, the current uHRMS-based non-target chemical discovery and characterization method is capable of confirming molecular formulae and for proposing core molecular structures with high confidence. However, for complete structural characterization of unknowns, standard synthesis or coupling with techniques such as nuclear magnetic resonance would be needed.

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