MSC THESIS

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Investigation of mobile phase additives in HPLC-MS/MS method used for the determination of PFAS in drinking water

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1. Contents

2. Abbreviations

ESI	Electrospray ionization
(U)HPLC	UHPLC and/or HPLC
[M + H]+	Positive ion mode
[M- H]-	Negative ion mode
$C_8HF_{15}O_2$	Perflorooctane
CID	Collision-induced dissociation
DL	Detection Limit
ECHA	European Chemicals Agency
ELU	Eluent
EPA	United Environmental Protection Agency
FASA	Perfluoroalkane sulfonamide
FASAA	Perfluoroalkane sulfonamido acetic acid
FASE	Perfluoroalkane sulfonamido ethanol
FDA	Food and Drug Administration
FIA	Flow injection analyses
FOSA	Perfluorooctane sulphonamide
GenX	Hexafluoropropylene oxide-dimer acid
H+	Hydrogen ion
HFPO-DA	Hexafluoropropylene oxide-dimer acid / GenX
HPLC	High Performance Liquid Chromatography
HPLC/MS/MS	High-Performance Liquid Chromatography / Tandem Mass Spectrometry
LC	Liquid Chromatography
LC/MS/MS	Liquid Chromatography/Tandem Mass Spectrometry
LOD	Limit of detection
mM	Millimolar
MRL	Minimum Reporting Limit
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PAP	Polyfluoroalkyl phosphate ester
PFAA	Perfluoroalkyl acid
PFAS	Per- and polyfluoroalkyl substance
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonic acid
PFCA	Perfluoroalkyl carboxylic acid
PFDoDA	Perfluorododecanoic acid
PFDoDS	Perfluorododecane sulfonic acid
PFDS	Perfluorodecane sulfonic acid
PFHpS	Perfluoroheptane sulfonic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid

PFNS	Perfluorononane sulfonic acid
PFOA	Perfluorooctanoic acid
PFOPA	Perfluorooctyl phosphonic acid
PFOS	Perfluorooctane sulfonic acid
PFPA	Perfluoroalkyl phosphonic acid
PFPS	Perfluoropentane sulfonic acid
PFSA	Perfluoroalkane sulfonic acid
PFTrDS	Perfluorotridecane sulfonic acid
PFUnDS	Perfluoroundecane sulfonic acid
pH	Potential of Hydrogen
pKa	Negative base-10 logarithm of the acid dissociation constant
POP	Persistent organic pollutants
Q1	First quadrupole
R-COO–	Carboxylate
R-SO3–	Sulfonate group
SAICM	Strategic Approach to International Chemical Management
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
TOF-MS	Time-of-flight mass spectrometry
TWI	Tolerable Weekly Intake
UHPLC	Ultra High Performance Liquid Chromatography

3. Introduction

Per- and poly-fluoroalkyl substances (PFAS) are a large group of environmental pollutants that emerge from human activity, they did not occur naturally in the environment. They are widely used in industrial applications and consumer products which contribute to global contamination (Cousins et al., 2020). Nowadays, a lot of public and private bodies address this problem, for instance, Strategic Approach to International Chemical Management (SAICM) has listed PFAS as an issue of concern. Furthermore, the Stockholm Convention listed the first two PFAS groups as persistent organic pollutants (POP) namely perfluorooctane sulfonic acid (PFOS) and related compounds in 2009 and perfluorooctanoic acid (PFOA) and related compounds added in 2019 (UNEP, 2019). Recently the European Commission published its Chemicals Strategy including phasing out the use of PFAS in the EU unless their use is essential (Communication from the Commission to the European Parliament, 2020). According to the current state of knowledge, several PFAS substances are classified as carcinogenic, developmentally toxic, endocrine, immunotoxic and genotoxic as well as having an influence on the metabolism (Joensen et al., 2009; Schrenk et al., 2020). There are studies that highlighted a specific concern and spotlight about the long-chain PFAA because of their persistence, bioaccumulation, and toxicity and most of them have high water solubility. The persistent organic chemicals are characterized by resistance to environmental degradation, long half-lives, and the potential to bioaccumulate (UNEP, 2019). Per- and polyfluoroalkyl substances (PFAS) are characterize for one of the chemical classes with these properties. They are now widespread throughout the world's ecosystem, including in animals and humans. PFAS also have been found in drinking water due to contamination of surface water or groundwater and the unavailability of PFAS limits values in water resources. Additionally, several hundred million people globally are exposed to PFOS, PFOA and other PFAS from drinking water pollution due to contamination of ground and surface water (Andrews & Naidenko, 2020). Recently, a study in the US estimates that 200 million Americans could have PFAS in their drinking water at a concentration of 1 ng/L and therefore above levels considered safe (Andrews & Naidenko, 2020; Grandjean & Budtz-Jørgensen, 2013). The new group limit has been established in Europe by Drinking Water Directive in 2021 (2023 in Hungary) for a 'Total PFAS' of 0.5 μg/L or the limit for the 'Sum of 20 PFAS' of 0.1 μg/L in drinking water. The main responsibility for the contamination of groundwater in the US, Europe and Japan is the military activity in many areas of firefighting foam frequency. Therefore, the importance of the topic is raised even more by the war in Ukraine.

Based on the newly published regulations, assessing the exposure risk related to drinking water in all countries, including Hungary is very important. So far, no such survey has been carried out in Hungary. Due to the low limit values, it is extremely important to develop an analytical method that has the lowest possible measurement limit. According to the US EPA Method 537.1 Determination Of Selected Per- And Polyfluorinated Alkyl Substances In Drinking Water By Solid Phase Extraction And Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS), they have listed the limit of detection (LOD) between 0.7 and 3 ng/L, which must be fulfilled in the case of an analytical method suitable for the purpose.

4. Objectives

The aim of this thesis work was to choose the best mobile phase additive in the analysis of multi-compound PFAS in order to get a higher signal intensity and the most selective and sensitive method which can later be validated and thus applied to the analysis of PFAS in drinking water samples. In order to make the compounds detectable as little as possible, the main aim of this thesis was to examine the effect of different mobile phase additives on signal generation.

5. Literature Review

5.1. Terminology and properties of per- and polyfluoroalkyl substances (PFAS)

Fluoroalkyl compounds are divided into two sub-groups which are perfluoroalkyl substances (per-fluoroalkyl substances with a fully fluorinated alkyl tail) and so-called precursors (poly-fluoroalkyl substances which are partially fluorinated alkyl tail). Terminology of 'perfluorinated' contains compounds where F atoms have replaced all H atoms attached to C atoms in the non-fluorinated analogues, and 'polyfluorinated' means that all H atoms attached to one or several (but not all) C atoms have been replaced by F atoms. This structure provides many of these substances immiscible with aqueous or hydrocarbon solvents the fact that they are both hydrophobic and oleophobic. This kind of characteristic of PFAS contributed to the surface active behaviour. Surface active behaviour of PFAS due to the hydrophilic head (water-loving) and hydrophobic and lipophobic tail (water and oil repellent). The surface-active behaviour of the PFASs contains the fluorinated backbone of both hydrophobic (water repelling) and oleophobic/lipophobic (oil/fat repelling) while the terminal functional group of hydrophilic (water-loving) (Figure 1).

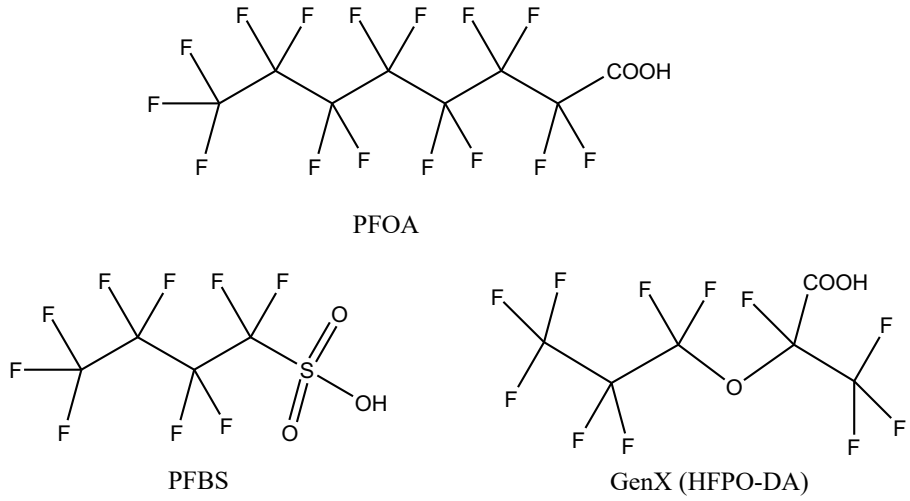

Figure 1. : Chemical structure of some PFA

This means those PFAS compounds tend to partition to interfaces, such as between air and water with the fluorinated backbone residing in air and the terminal functional group residing in water (or other hydrophilic materials). The PFAS partitioning behaviour also is affected by the alkyl chain length and the charge on the terminal functional group. In general, PFASs with shorter alkyl chain lengths are more water soluble than those with longer lengths. Adsorption

to soil surfaces has been shown to be greater for PFASs with longer alkyl chain lengths (Anderson et al., 2016).

Substances that consider more hydrophobic than hydrocarbons are perfluoroalkyl substances. For example, perflorooctane, $C_8HF_{15}O_2$ is not capable of being mixed with the more polar octane. In organic chemistry, the C-F bond is known as the most stable single bond, because F has the biggest electronegativity of all elements. This means that F has no urge to form any bonds (such as covalent) or interactions (such as H-bridge or Van der Walls) with the non-bonding electron pair. Because of this, many perfluorinated PFASs are inert against hydrolysis, photolysis, microbial degradation and metabolism, even though at relatively high temperatures. This extraordinary chemical and thermal stability is favourable in industrial applications, but it also makes some PFASs very persistent in the global environment. Because of the oleophobic and hydrophobic nature of the fluorinated carbon chain, PFASs provide highly useful and durable properties as monomer surfactants as well as incorporated into polymers (Kissa, 2001).

5.2. PFAS in Water

PFAS (per- and polyfluoroalkyl substances) are a group of man-made compounds that have been detected in drinking water supplies across the globe. PFAS exposure has been associated with several health effects such as liver damage, thyroid hormone disruption, developmental and reproductive effects, and immune system dysfunction (Hu et al., 2019). PFAS are extremely persistent in the environment and have long half-lives in humans, leading to concerns about their potential bioaccumulation and biomagnification (UNEP, 2019). The source of PFAS contamination in drinking water is based on manufacturing and industrial facilities mainly(Pamela Lein & Peter Andrew, 2023). Besides that, the source of pollution also came from military installations, airports, and landfills (Pamela Lein & Peter Andrew, 2023). Incineration facilities have been connected to PFAS contamination in vegetables grown on agricultural fields where it was applied, and wastewater treatment facilities have been associated with PFAS contamination in biosolids produced (Brown et al., 2020). Due to the high persistence of PFASs and the fact that their head part is water-soluble, they are important pollutants of the water base. Due to their environmental cycle, they appear in both underground and surface water. Concerns have been raised regarding environmental exposure because PFASs are ubiquitously distributed in the environment through long-range transport via the water. In addition, humans worldwide are exposed to PFASs through contaminated drinking

water. In response to this, the European Commission has set a maximum permissible limit in drinking water on 12th January 2021 (DIRECTIVE (EU) 2020/2184, 2020). By 12 January 2026, Member States shall take the measures necessary to ensure that water intended for human consumption complies with the parametric values set out in Annex I for "PFAS total" of 0.5 μg/L or "Sum of PFAS" of 0.1 μg/L. "PFAS Total" means the totality of per- and polyfluoroalkyl substances. This parametric value shall only apply once technical guidelines for monitoring this parameter are developed. "Sum of PFAS" means the sum of per- and polyfluoroalkyl substances considered a concern as regards water intended for human consumption. This list contains the substances listed in Table 1. Member States may then decide to use either one or both of the parameters 'PFAS Total' or 'Sum of PFAS. Hungary follows the EU legislation and regulates the Drinking Water for human consumption in the Governmental Regulation number 5/2023 (I. 12.) which was released on the 12th of January, 2023 (5/2023. (I. 12.) Government decree, 2023).

Table 1. : List of "Sum of PFAS" substances

Name of "Sum of PFAS" substances
PFBA (perfluorobutanoic acid)
PFPA (perfluoroalkyl phosphonic acid
PFHxA (perfluorohexanoic acid)
PFHpA (perfluoroheptanoic acid)
PFOA (perfluorooctanoic acid)
PFNA (perfluorononanoic acid)
PFDA (perfluorodecanoic acid)
PFUnDA (perfluoroundecanoic acid)
PFDoDA (perfluorododecanoic acid)
PFTrDA (perfluorotridecanoic acid)
PFPS (perfluoropentane sulfonic acid)
PFHxS (perfluorohexanesulfonic acid)
PFHpS (perfluoroheptanesulfonic acid)
PFOS (perfluorooctanesulfonic acid)
PFNS (perfluorononanesulfonic acid)
PFDS (perfluorodecanesulfonic acid)
PFUnDS (Perfluoroundecane sulfonic acid)
PFDoDS (Perfluorododecane sulfonic acid)
PFTrDS (Perfluorotridecane sulfonic acid)

PFAS are not easily degraded and are challenging to destroy because they are made for long-term stability. Several treatment technologies have been developed to remove PFAS from drinking water, including activated carbon adsorption, reverse osmosis, and ion exchange (US EPA, 2018). The efficacy of these treatment technologies can vary depending on the specific PFAS compound and the water quality parameters (e.g., pH, temperature) of the source water. A recent study by the Environmental Working Group found that PFAS contamination of drinking water is much more widespread than previously believed, affecting more than 200 million Americans across all 50 states (Pamela Lein & Peter Andrew, 2023). In conclusion, PFAS contamination of drinking water is a major public health concern that requires continued research and regulatory action to protect human health and the environment.

5.3. Human exposure and toxicology

PFASs are known for high persistence and for that perfluorinated parts of PFAS molecules are not degraded in soils or groundwater hence these PFASs have become “forever chemicals” (Kwiatkowski et al., 2020). Therefore, it's important to look into and maybe rehabilitate contaminated areas and polluted groundwater that have an influence on communities. Industrial landfills with high PFAS concentrations are susceptible to long-term leakage and may eventually need to be excavated to safeguard human health. (Oliaei et al., 2013). Since the first discovery of PFASs in human blood (Olsen et al., 1999), internal human exposure to these substances has been researched in numerous studies conducted all over the world (Haug et al., 2011; Hölzer et al., 2011). Temporal trend analysis revealed a recent drop in the levels of PFOS and PFOA in human serum (Glynn et al., 2012; Olsen et al., 2012; Yeung et al., 2013), which is probably related to the phase-out of these chemicals' production by 3M between 2000 and 2002. However, on the other hand, long-chain longer chain PFCAs showed a recent increase in humans in several studies (Glynn et al., 2012; Yeung et al., 2013)

Multiple pathways exist for human exposure to PFASs, including ingestion of food, water, breast milk, non-food items, skin contact, inhalation of household dust, and air inhalation. All of these have been listed as key human exposure pathways to PFASs for the general population, however, (Jogsten et al., 2009) recognized dietary intake as the main one. Moreover, it appears that the exposure pattern varies according to the kind of PFAS, geographic region, food varieties, and eating habits. For instance, eating fish is thought to be a significant method to be exposure to PFOS (Vestergren et al., 2012), while drinking water was identified as a significant

route of exposure for short-chain PFCA and PFSA homologues (Vestergren et al., 2012). Drinking water's contribution to blood PFAS concentrations can vary between individuals acc ording to consumption rates and toxicogenetics (Hu et al., 2019). Public drinking water systems serving at least six million people per year went over the PFAS lifetime health advisory level between 2013 and 2015, according to the US Environmental Protection Agency (Hu et al., 2016)

5.4. GenX

PFAS has been used widely in consumer and industrial products since 1940's because of their resistance to grease, oil, water and also heat. This chemical has been used in water-resistant fabric and carpeting, cleaning products, paints, cookware, food packaging, food processing equipment and fire-fighting foams (Food and Drug Administration (FDA), 2022). Nowadays instead of PFA acids for example PFOA researchers have explored a safer replacement which is hexafluoropropylene oxide dimer acid (HFPO-DA) also known as GenX (Figure 1). A representative potential of GenX has a comparatively short carbon chain length and a lower tendency to accumulate in humans hence GenX has been used as a substitute for other PFASs (Yoo et al., 2021). GenX provides lower bioaccumulation when compared to PFOA (Gannon et al., 2016) (Tian & Sun, 2019). The production of GenX has been increased gradually based on the consideration of GenX as a recognizable difference compound compared to other short-chain members of PFAS, due to the ether functional group in the chemical structure of GenX being a more environmentally friendly compound. However, GenX is considered more hydrophilic than other PFASs and it has the characteristic of reduced molecular size, providing more difficulty in separation in the water treatment facility (Sun et al., 2016). Gen X generally has been widely scattered in rivers worldwide involving South Korea, China, the United Kingdom, the Netherlands, Sweden, and the United States (Pan et al., 2018). GenX considers a local pollutant due to the exposure route in the drinking water near fluorochemical processing sites in the Netherlands with concentrations ranging from 1.4 to 8.0 ng/L (Gebbink & van Leeuwen, 2020). Furthermore, a study based on a computer model that analysed data from rat laboratories in Sweden concluded that GenX is more toxic than PFOA (Gomis et al., 2018). Due to its widespread use and potential health effects, GenX has received significant attention in the scientific literature. The molecular formula of GenX is $C_6HF_{11}O_3$, the image of the chemical structure of GenX was shown in Figure 1.

5.5. Analytical methods of PFAS investigation

With the aid of a newly created analytical technique based on tandem mass spectrometry and high-performance liquid chromatography (HPLC/MS/MS), the global environmental presence of PFASs in wildlife was first identified in 2001 (Giesy & Kannan, 2001). The most widespread method used to detect PFAS is a target method with LC-MS/MS that is mostly used in UHPLC MS/MS because of the high sensitivity and extremely low detection limit. For aqueous samples like drinking water, liquid chromatography works best and enables the separation of the various PFAS of interest. A tandem mass spectrometry apparatus, or triple quadrupole, consisting of two quadrupoles in series with a collision cell in the middle, was used specifically for this investigation. This detector configuration enables the approach to be more selective by filtering out particular mass transitions for each analyte (precursor/product ion). Separation of the analytes happened during HPLC by the interaction of the compound with the stationary phase and mobile phase. During the separation process, the precursor ions are selected based on their mass-to-charge ratio (m/z) and their behaviour in chromatographic separation. In the MS/MS part where precursor and product ions will be chosen based on the optimization process. The selection of product ions depends on the optimization process, which involves testing different collision energies and isolation windows. The most abundant and specific product ions are chosen for quantification and confirmation of the compound of interest. In the case of perfluoroalkyl carboxylic acids and sulfonic acids, the precursor ions are well known and mostly the deprotonated molecule, however, differences can be read in the literature for the ether-type compounds, such as the GenX. The mass spectrum of GenX has been studied using high-resolution mass spectrometry, and several possible ion formations have been proposed. The multiple reaction monitoring (MRM) transitions for the HFPO-DA (GenX) analysis in the first quadrupole (Q1) were separated followed by collision-induced dissociation (CID) with optimal collision energy in the collision cell, most investigations use the 329 m/z precursor, which is the deprotonated molecule (L. Mullin et al., 2019). The loss of -CO_2 (285 m/z), the breakdown of the ether linkage that produced C_3F_7 (169 m/z), and further fragmentation of the C-chain that produced C_2F_5 (119 m/z) was represented by the product ions (L. Mullin et al., 2019). The mass spectrum of GenX using time-of-flight mass spectrometry (TOF-MS) can be seen in Figure 2 (Strynar et al., 2015).

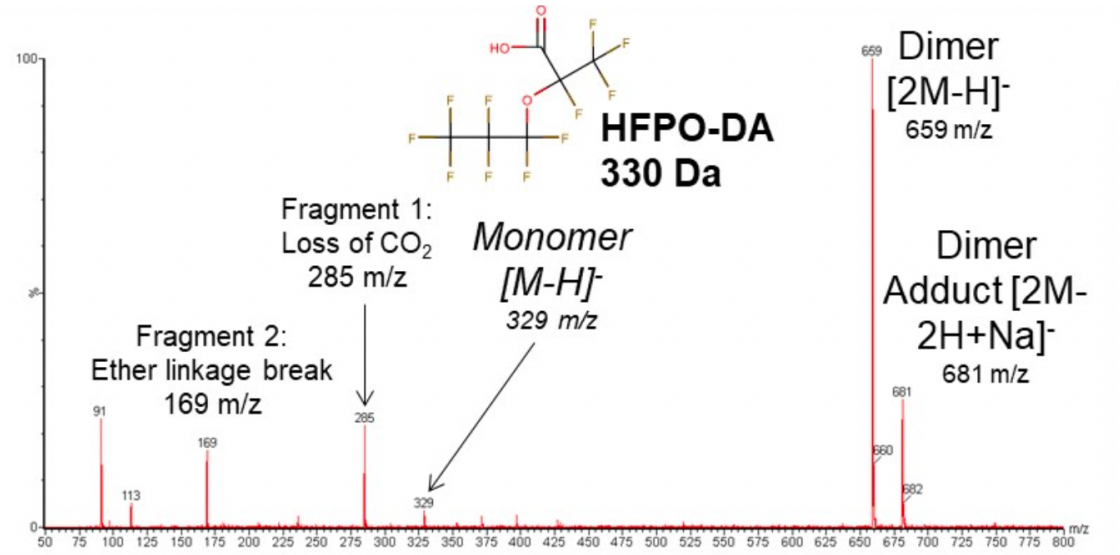

Figure 2. : Observations in spectra: dimer formation, fragmentation and adduct formation of GenX (Strynar et al., 2015)

Important to mention, that based on Figure 2. several ion formations are occurring in the mass spectrometer, which is not preferable from a sensitivity point of view. The deprotonated dimer formation resulted in the most intensive signal in the case of GenX. The selection of the most intense precursor and product ions is very important since the EU limit value is very low, therefore the method requires high sensitivity, adequate accuracy in the magnitude of ng/L, and the expected limit of detection (LOD) value is between 0.3 and 3 ng/L for drinking water samples (Shoemaker & Dan Tettenhorst, 2020).

5.6. Mobile Phase Additives

Regarding the instrumental analytical method of such a multi compound measurement, the greatest variability is with regard to the mobile phase. It is known that different mobile phase additives have a great influence on ESI ionization. The choice of mobile phase additives can have a significant impact on the efficiency and sensitivity of ESI (electrospray ionization) in the detection of PFASs. ESI is a widely used technique in mass spectrometry that relies on generating ions by applying an electric field to a liquid sample. In ESI, the mobile phase acts as a carrier for the analyte molecules and helps to nebulize the sample into small droplets. The droplets then enter the mass spectrometer where they are ionized and analysed. The type of mobile phase and additives used can affect the ionization efficiency and sensitivity of ESI. For example, acidic or basic additives such as formic acid or ammonia can help to improve ionization efficiency by protonating or deprotonating the analyte molecules, leading to

increased sensitivity (Garcia, 2005). Other additives like acetonitrile or methanol can help to improve the ionization efficiency and reduce signal suppression by reducing surface tension and increasing droplet size. Different mobile phase additives can also affect the overall quality of the ESI spectrum, including the signal intensity, peak shape, and background noise. Thus, careful optimization of the mobile phase conditions is essential for achieving the best possible ESI results.

The choice of mobile phase additive depends on several factors such as the polarity of the PFASs, the functional groups of the molecule, the sample matrix, and the analytical instrument. Regarding the mobile phase additives, there is conflicting information in the literature and often the choice is based on routine analytical procedures rather than on scientific theories and tests. Researchers have reported that using formic acid as a mobile phase additive can improve the limit of detection (LOD) and signal intensity for PFASs compared to other additives (Poothong et al., 2017). However, in another publication, even the acetic acid proved to reach higher signal intensities (Wu et al., 2004). Ammonium acetate has been found to be an effective mobile phase additive for the analysis of highly polar PFASs, while trifluoroacetic acid has been found to enhance the detection of less polar PFASs (Garcia, 2005). Some studies have also reported that the use of mixtures of mobile phase additives can further improve the ESI ionization efficiency and selectivity for PFASs (Lauren Mullin et al., 2018). It is important to note that the choice of mobile phase additive must be carefully optimized to avoid potential interference or suppression of the ESI signal by co-eluting compounds or matrix effects. In almost all publications, C18 column with an aqueous and methanol/acetonitrile phase containing 5–50 mM ammonium acetate is typically used to form ionic PFAS in ESI source (Nakayama et al., 2019).

5.7. The "wrong-way-around" phenomenon

The "wrong-way-around" phenomenon is well-known that occurs during electrospray ionization (ESI) of strong base or strong acidic solutions. The appearance of intense [M + H]+ ions during electrospray ionization of strongly basic solutions and intense [MH]- ions during electrospray ionization of strongly acidic solutions is known as the "wrong-way-round" phenomenon (Wu et al., 2004). In a solution phase, PFAS are in deprotonated form, for example, R-SO−3 and R-COO− either due to high pH or very low pH, because the pKa of these compounds is very low (Table 2). In gas phase chemistry especially in MS systems that include ESI, when there will be a lot of droplets of eluents and analytes, the situation is slightly different compared to the solution phase. There is a tip of ESI that consist of metal wire with a

strong electric field connected to it. In the case of PFAS, the measurement takes place in negative ion mode whereby in the state of electrophoretic charge separation. The tip in this state has a strong electric field hence it's related to the phenomenon of reduction. In the system during measurement there is an eluent consisting of water, however, the literature stated that acid is also needed. This kind of idea is contrary to the solution-phase concept when higher pH is needed in order to have deprotonated acid form. Meanwhile, based on this "wrong way around" phenomenon a lower pH is needed instead (Mansoori et al., 1997). Acetic acid is usually used and the reductions happened when electron acceptation happened (acid accept electron) and the product will be an acetate ion and the formation of hydrogen gas. In the phase of solvent evaporation, there will be droplets with acetate ions forming on the surface hence when the solvent is evaporated, the acetate ion needs a proton to form acid. In this stage, the analytes will be deprotonated. The phenomenon is characterized by the appearance of an intense [M-H] ion during ESI of acidic solutions (Wu et al., 2004). The main theory behind this phenomenon is that the strong basicity or acidity of the solution leads to proton transfer in the opposite direction than expected. The study found out that some weak acids significantly increased the negative-ion ESI response (Wu et al., 2004).

Table 2. : pKa values of some PFAS

List of PFASs		Molecular Formula	pK_a value
Perfluorodecanoic acid	PFDA	$C_{10}HF_{19}O_2$	2.58
Perfluorododecanoic acid	PFDoA	$C_{12}HF_{23}O_2$	0.52 ± 0.10
Perfluoroheptanoic acid	PFHpA	$C_7HF_{13}O_2$	-2.29
Perfluorohexanoic acid	PFHxA	$C_6\ HF_{11}O_2$	-0.16
Perfluorononanoic acid	PFNA	$C_9\ HF_{17}O_2$	-0.21
Perfluorooctanoic acid	PFOA	$C_8HF_{15}O_2$	2.84
Perfluorotetradecanoic acid	PFTA	$C_{14}HF_{27}O_2$	0.52 ± 0.10
Perfluorobutanesulfonic acid	PFBS	$C_4HF_99O_3S$	-3.31
Perfluorohexanesulfonic acid	PFHxS	$C_6HF_{13}O_3S$	0.14
Perfluorooctanesulfonic acid	PFOS	$C_8HF_{17}O_3S$	<1.0

6. Materials and Methods

6.1 Materials

6.1.1. Chemicals

Ammonium acetate, ammonium hydrogen carbonate, methanol, distilled water and acetic acid were purchased from the same company as was written in the diploma thesis of Majercsik, 2020. Multi-PFAS Analyte Primary Dilution Standard mix solution (18 analytes in 1.2ml methanol containing 4% water, 2mg/L for each) was purchased from Kromat Kft. (all of the analytes shown in Table 3).

Table 3. : List of analytes measured in this work

Compound name	
Perfluorotetradecanoic acid	PFTeDA
Perfluorotridecanoic acid	PFTrDA
11-Chloroeicosafluoro-3-oxaundecane-1-sulfonic acid	11Cl-PF3OUDS
perfluorododecanoic acid	PFDoA
N-ethylperfluorooctane sulfonamidoacetic acid	N-EtFOSAA
N-Methylperfluorooctanesulfonamidoacetic acid	N-MeFOSAA
Perfluoroundecanoic acid	PFUnA /PFUnDA
9-Chlorohexadecafluoro-3-Oxanone-1-Sulfonic Acid	9Cl-PF3ONS
Perfluorodecanoic acid	PFDA
Perfluorooctane sulfonic acid	PFOS
Perfluorononanoic acid	PFNA
Perfluorooctanoic acid	PFOA
Perfluorohexane sulfonic acid	PFHxS
4,8-dioxa-3H-perfluorononanoic acid	ADONA
Perfluoroheptanoic acid	PFHpA
Perfluorohexanoic acid	PFHxA
Perfluorobutane sulfonic acid	PFBS
Hexafluoropropylene oxide-dimer acid	HFPO-DA / GenX

6.1.2. Apparatus

Cellulose-acetate syringe filter, 100 ml volumetric flask, reagent bottles, HPLC-MS/MS instrument, Zorbax EclipsePlus C18 RRHD chromatographic column with the particle size of 1.8μm and with a dimension of 2.1 x 50mm, glass equipment and pipettes were purchased from the same company as was written in the diploma thesis of Majercsik, 2020.

6.2 Methods

The experimental design of the investigation of mobile phase additives was presented in Table 4. The tests were run in two steps. First, a preliminary investigation was done using only two compounds, namely PFOA and GenX. Second, after the multi-standards arrived at the department, the same experiments were done with all 18 compounds. The main aim of the studies was to investigate the best mobile phase additive, resulting in the highest signal of the PFA compounds.

Table 4. : The experiment design of mobile phase additive

Preliminary study (PFOA and GenX)	Multi-PFAS study
Experiment 1. Test of buffer salts a) FIA	**Experiment 3.**Test of buffer salts a) FIA b) Chromatography
Experiment 2. Test of pH a) FIA b) Chromatography	**Experiment 4.**Test of pH a) FIA b) Chromatography

6.2.1. Flow Injection Analyses (FIA)

For flow injection analyses (FIA) the UHPLC-MS/MS system without column, with high injected concentration together with slow flow rate was used in order to have time for data collection. The analyser was applied in selected ion monitoring (SIM) mode measuring the precursor ions. All experiments were done in three replicates. The measurement parameters are shown in table Table 5. The FIA measurement is fast and simple, however uses isocratic elution, which means defined water/organic phase composition during ionization. However, the organic-water ratio of the mobile phase plays an important role in the formation of the MS

signal through its modifying effect of surface tension of drop, hence the intensity of the signal under chromatographic conditions should be also determined (Table 4).

6.2.2. Chromatographic analyses

In the case of the chromatographic experiment, the column was installed and the separation and detection were done in MRM mode using 5μl injection of 100ng/ml standard solutions. The chromatographic separation as well as the parameters of detection (such as selection of precursors/products, ion source parameters, gradient program etc.) were optimized before, not shown in this work. All experiments were done in three replicates. The measurement parameters of chromatographic analyses are shown in Table 5.

Table 5. : Measurement parameters of FIA and Chromatography

	FIA	**Chromatography**
Composition of mobile phase	different (Table 6 and 7)	
Ratio of mobile phases	isocratic (50:50 = A:B)	gradient elution
Flow rate	0.200 mL/min	0.400mL/min
Column and termostate	No column 40°C	Zorbax EclipsePlus C18 RRHD 1.8µl (2.1 x 50mm), 40°C
Detection	SIM mode	MRM mode
Injected concentration	1000 ng/ml	100 ng/ml
Injection volume	5.0 µl	
Evaluation	peak area under the curve	

6.2.3. Test of buffer salts (Experiments 1 & 3)

In order to check the effect of buffer salt type on the signal intensities of PFAS, six different mobile phases were prepared, in which two different salts in three different concentration have been tested (ELU_1-6, Table 6). In advance, a stock solution of 100mM ammonium-acetate as well as ammonium hydrogen carbonate was prepared by solving 770.8mg and 791mg salts in about 60ml water, respectively. The solutions were filtered through a 0.45 µm cellulose acetate syringe filter into a 100.0ml of volumetric flask and were filled to the line. The eluents were prepared by appropriately diluting the stock solutions (Table 6). All of the eluents were used for flow injection as well as for chromatographic analyses in experiment 1 and 3, according to the table 4.

Table 6. : Different compositions of mobile phases used for testing the effect of buffer salts on the signal intensity of PFAS

Notation of mobile phase	Composition of the water-phase eluent (A)	Composition of the organic solvent-phase eluent (B)
ELU_1	20 mM ammonium acetate	MeOH
ELU_2	10 mM ammonium acetate	MeOH
ELU_3	4 mM ammonium acetate	MeOH
ELU_4	20 mM ammonium hydrogen carbonate	MeOH
ELU_5	10 mM ammonium hydrogen carbonate	MeOH
ELU_6	4 mM ammonium hydrogen carbonate	MeOH

6.2.4. Test of pH (Experiments 2 & 4)

Eluents with different pH were prepared in order to check the effect of acidity on the signal generation of PFAS in the mass spectrometer. In advance, the pH of the solutions was measured, in order to check the suitability with the silica-gel column pH limit between 2 and 9. The list of the eluent for this experiment was tabulated in Table 7. All of the eluents were used for flow injection as well as for chromatographic analyses in experiments 2 and 4, according to Table 4.

Table 7. : Different mobile phases used for testing the effect of pH on the signal intensity of PFAS (AA: acetic acid)

Notation of mobile phase	Composition of eluent A	Composition of eluent B
ELU_A	4mM ammonium hydrogen carbonate	MeOH
ELU_B	4mM ammonium hydrogen carbonate + 0.01% AA	MeOH
ELU_C	4mM ammonium hydrogen carbonate + 0.05% AA	MeOH
ELU_D	4mM ammonium hydrogen carbonate + 0.1% AA	MeOH
ELU_E	4mM ammonium hydrogen carbonate + 1% AA	MeOH
ELU_F	4mM ammonium hydrogen carbonate + NH_4OH, pH 8.0	MeOH
ELU_G	water	MeOH

7. Results

7.1. Preliminary study (PFOA and GenX)

7.1.1. Investigation of mobile phase salts on the signal of PFOA and GenX (Experiment 1.)

In the preliminary study, the FIA analysis was used to check the signal intensity of mix GenX and PFOA with different salt compositions and concentrations of the mobile phases. The flow-injection-analysis (FIA) of standard mix solution was a very fast way to investigate the effectiveness of ionization of compounds. The measuring lasts only 1 minute with a slow flow rate and the instrument was working in single ion monitoring (SIM) mode to check the molecule ion formation. Without a column, the compounds are reaching the detector at the same time forming a relatively wide peak. Similarly, to the chromatography, the area under the curve is evaluated (Figure 3).

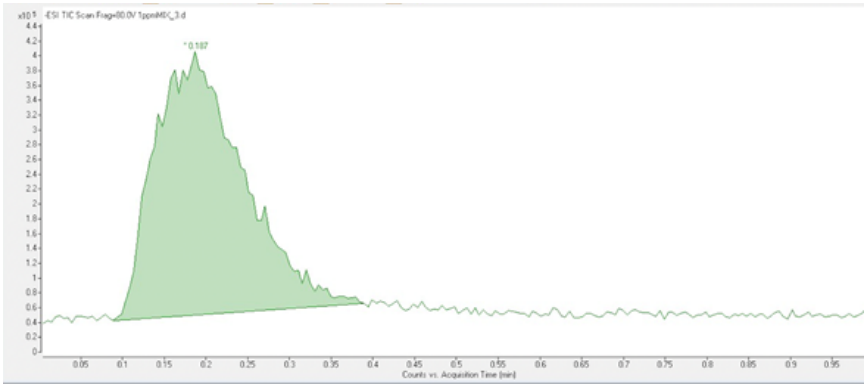

Figure 3. : Chromatogram of the Flow Injection Analyses of mix Gen X and PFOA using ELU_3 in a preliminary study.

In this preliminary experiment, we searched for the right buffer type and concentration which result in the highest signal of the two compounds. It can be seen from the results presented in Figure 4, that one precursor mass of PFOA (namely 413.0) and four precursor masses of GenX (namely 391.0, 329.0, 285.0 and 658.9) have been monitored for signal intensity, the selection was based on literature data (Figure 2). The aim was to choose one dominant precursor mass for GenX, together with minimizing the concurrent signals. Based on the result, we can conclude that in the case of GenX, neither the deprotonated nor the carbonated (in the case of ELU 4-5-6) molecule ions can be the dominant precursor (Figure 4). Hence, the decarboxylated molecule ion (285) should be chosen as the precursor. Therefore, the dimer formation (658.9) was also not preferred, it should be avoided, as possible. Considering these aspects, the carbonated eluent resulted in the best signals. All carbonated eluent considered, the lower

concentration (4mM) favours ion formation the most. Eluent with 4mM ammonium hydrogen carbonate became the optimal composition, in which the efficiency of dimer formation was lower, but the dominant precursors were present with the highest intensity for both compounds.

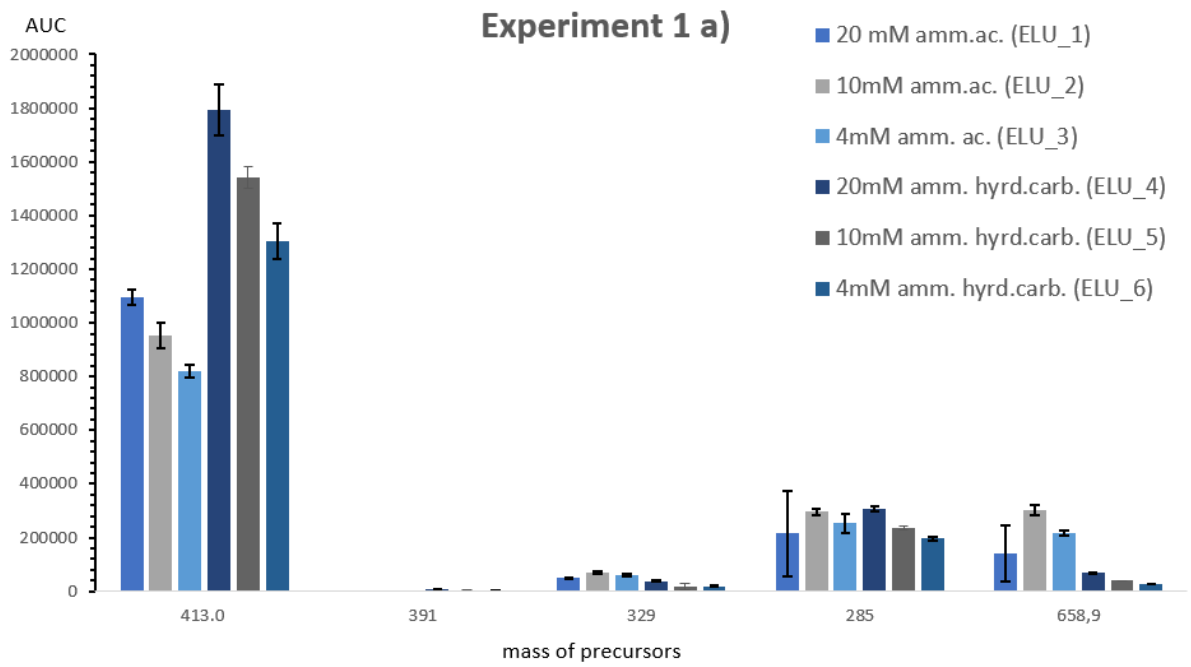

Figure 4. : Experiment 1 a): Flow Injection Analyses of 1 mg/L PFOA and GenX standard mix solution using mobile phases with different salt contents

7.1.2. Investigation of mobile phase pH on the signal of PFOA and GenX (Experiment 2.)

In the second FIA experiment, using the optimal 4mM carbonated buffer, the effect of mobile phase pH was investigated (Table 8, Figure 5). We used acetic acid for acidification because the gas phase Gibbs Free Energies of acetic acid dissociation was the highest, compared to formic acid or other acids (Wu et al., 2004). This was also supported by Hua & Jenke, 2012, who mentioned buffers containing acetic acid were the preferred choice for negative ESI. In negative ion mode, an acidic mobile phase containing acetate anion improved ESI responses for acidic compounds, primarily due to gas phase effects (Hua & Jenke, 2012).

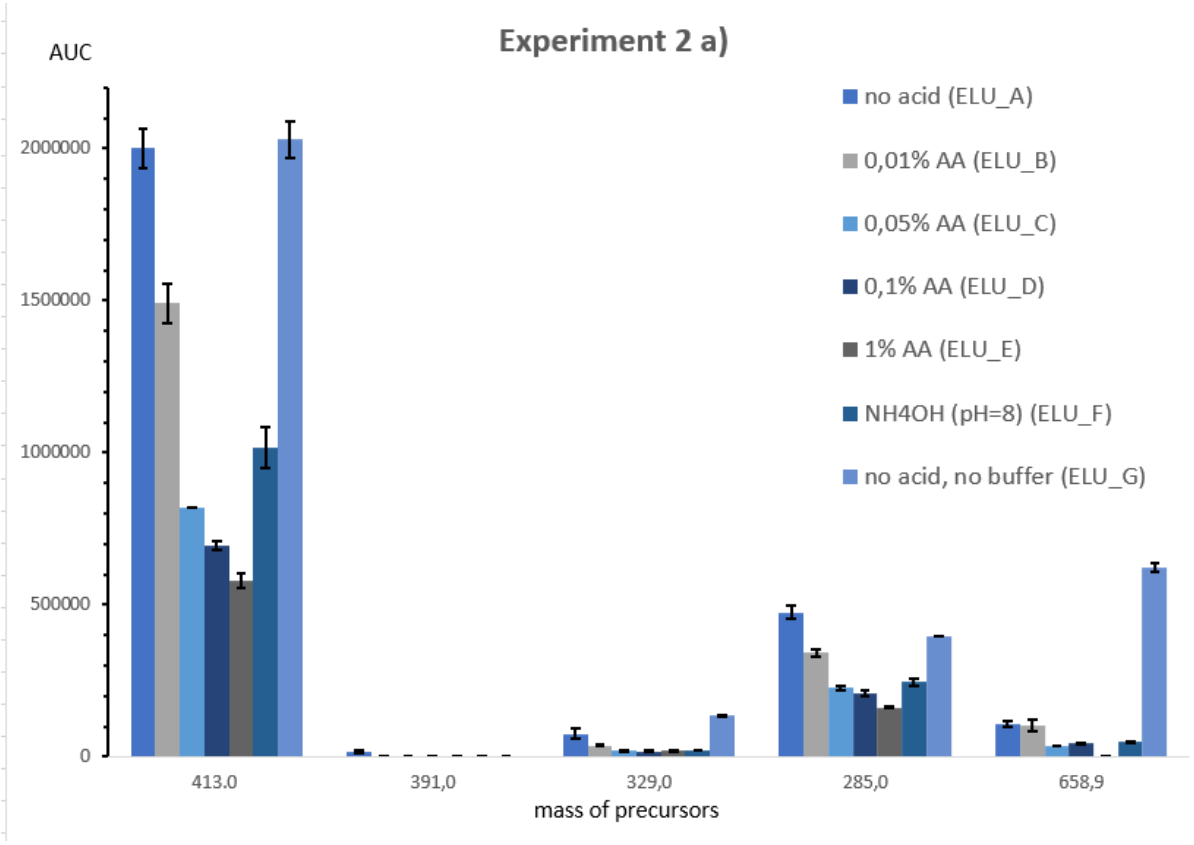

Figure 5. : Experiment 2 a): Flow Injection Analyses of 1 mg/L PFOA and GenX standard mix solution using mobile phases with different acid contents

It was interesting that the most intense signal was obtained for the eluents with non-buffered composition (without acid, (ELU_A) or without acid and salt (ELU_G). However, the literature data proved, that buffering of the mobile phase is important from the point of view of signal stability because the pH-changing effect of the sample matrix reduces the accuracy of the measurement (Hua & Jenke, 2012). Therefore, non-buffered alternatives were discarded. In the case of buffered mobile phases, the less the acidity, the higher the signal intensity that was experienced. The pH of the mobile phases was also determined, as supplemented data to our work (Table 8).

Table 8. : pH of different mobile phases used in Experiment 2 and 4 (AA: acetic acid)

	Composition of eluent A	pH
ELU_A	4mM ammonium hydrogen carbonate	6.3
ELU_B	4mM ammonium hydrogen carbonate + 0.01% AA	5.6
ELU_C	4mM ammonium hydrogen carbonate + 0.05% AA	4.3
ELU_D	4mM ammonium hydrogen carbonate + 0.1% AA	3.9
ELU_E	4mM ammonium hydrogen carbonate + 1% AA	3.0
ELU_F	4mM ammonium hydrogen carbonate + NH_4OH	8.0
ELU_G	water	7.3

Based on this preliminary FIA experiment, the ideal mobile phase composition is (A) 4mM ammonium hydrogen carbonate with 0.01% acetic acid in water, and (B) methanol. Important to mention, that during FIA measuring, the organic solvent/water ratio of the mobile phase was constant, and so was the surface tension of the droplets as well. However, during a multi-compound chromatographic run using gradient elution, the organic/inorganic composition of the mobile phase was changing, which contributed to the effect on the surface tension of the droplet in the ESI, hence on the ionisation too. Due to this reason, a chromatographic investigation of the signal intensity was also done for the final conclusion of the preliminary study. In order to use the highest sensitivity of the method, MRM mode was applied for chromatographic analyses, which have earlier been optimized.

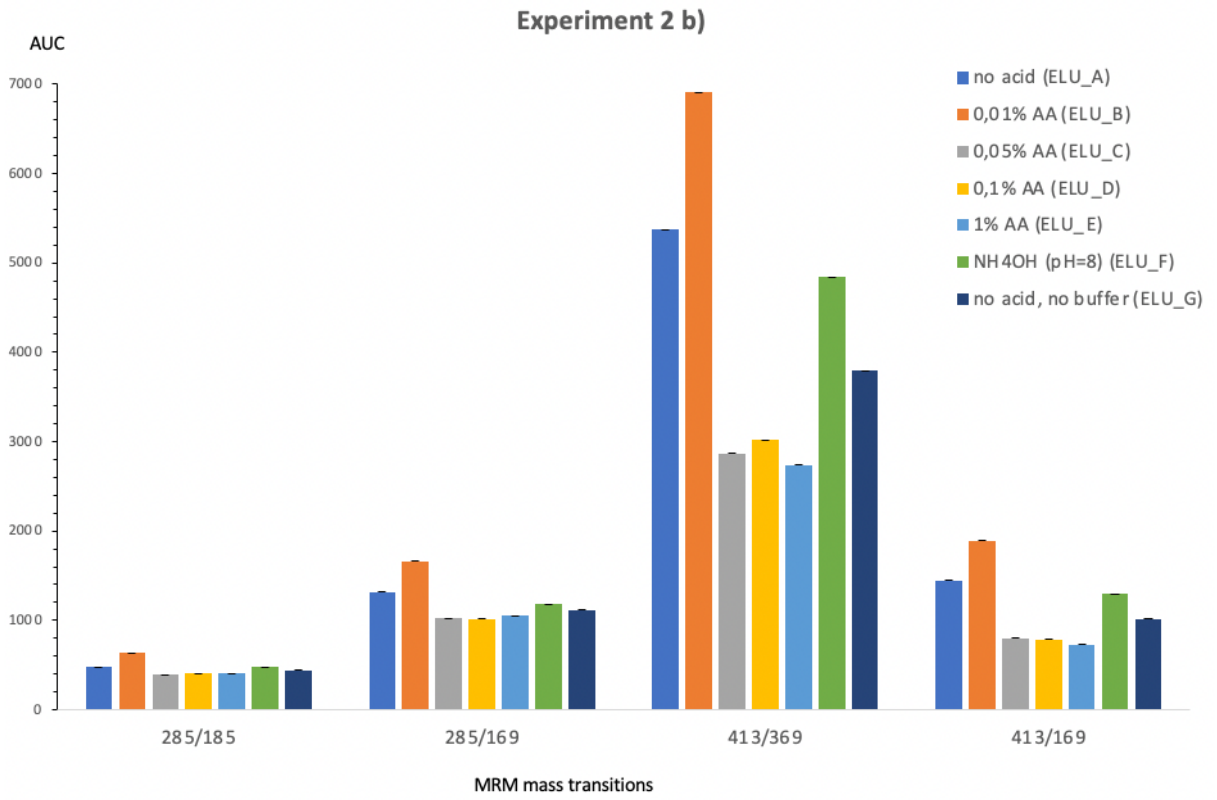

Figure 6. : Experiment 2 b): Chromatographic Analyses of 100 ng/ml PFOA and GenX standard mix solution using mobile phases with different acid contents

Figure 6 shows that buffering was important in the gradient elution method because eluent A and G resulted in lower signal intensities. In the case of all four MRM transitions, the lowest acid content (0.01%) help the ionization of the compounds to the most extent. Higher pH (eluent F) gave the second intensive signal, however long-term work at the limit value of the pH tolerance of the column (pH=9) was not preferred. Our results support the statement of Wu et al., 2004, that suggested a low amount of acidity in the mobile phase to ionize acidic compounds with good efficiency.

7.2. Multi PFAS study

7.2.1. Investigation of mobile phase salts on the signal of multi-PFAS (Experiment 3.)

The second part of this work, we used the same mobile phases that were used in the preliminary study, for the investigation of 18 FPAS compounds (Table 3). The precursor ions as well as the MRM transitions and the chromatographic gradient program were collected from the literature data and have been optimized by us before the experiments (not shown in this work).

The FIA (Experiment 3 a) of 100ng/ml multi-PFAS standard solution was shown in Figure 7. Based on the results it can be seen, that for most analytes the carbonated buffers eventuated higher signal compared to the acetated buffers. However, 20mM hydrogen-carbonate concentration is too much for the good ionization of all the compounds. We assume, that maybe the intensive gas formation (CO_2) during ionization is responsible for signal suppression, in that case. A low amount of carbonate present (4mM) however well supported the ion formation in the ion source.

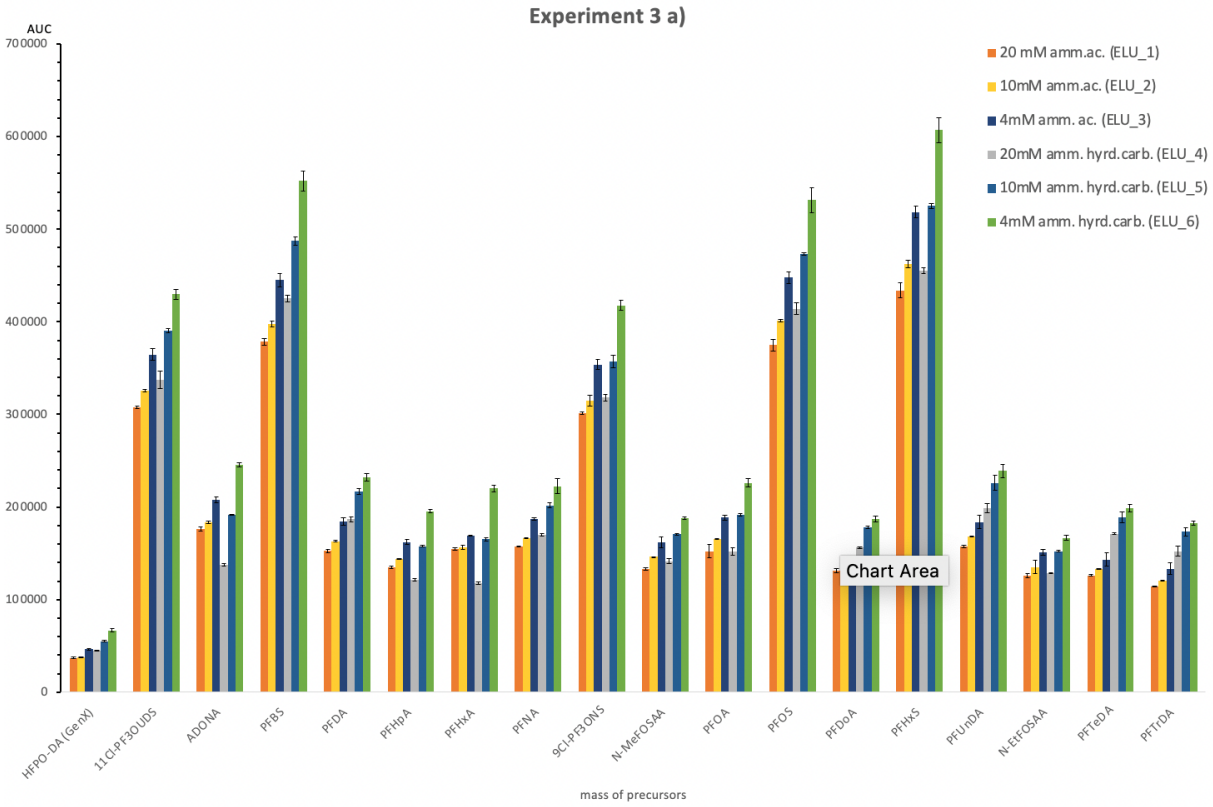

Figure 7. : Experiment 3 a): Flow Injection Analyses of 1 mg/L of multi-standard PFAS solution using mobile phases with different salt contents.

The chromatographic analyses of the same experiment showed similar results in the case of long-chain PFAS compounds, that the carbonated buffer is the preferred choice. While for the short carbon-chained species, and the acid-amides, the ionization was similar in both cases, only depending on the concentration of the buffer. It was clearly seen, that the lower buffer was preferable in all cases. Considering all compounds, the 4mM hydrogen-carbonate buffer was chosen for further experiments.

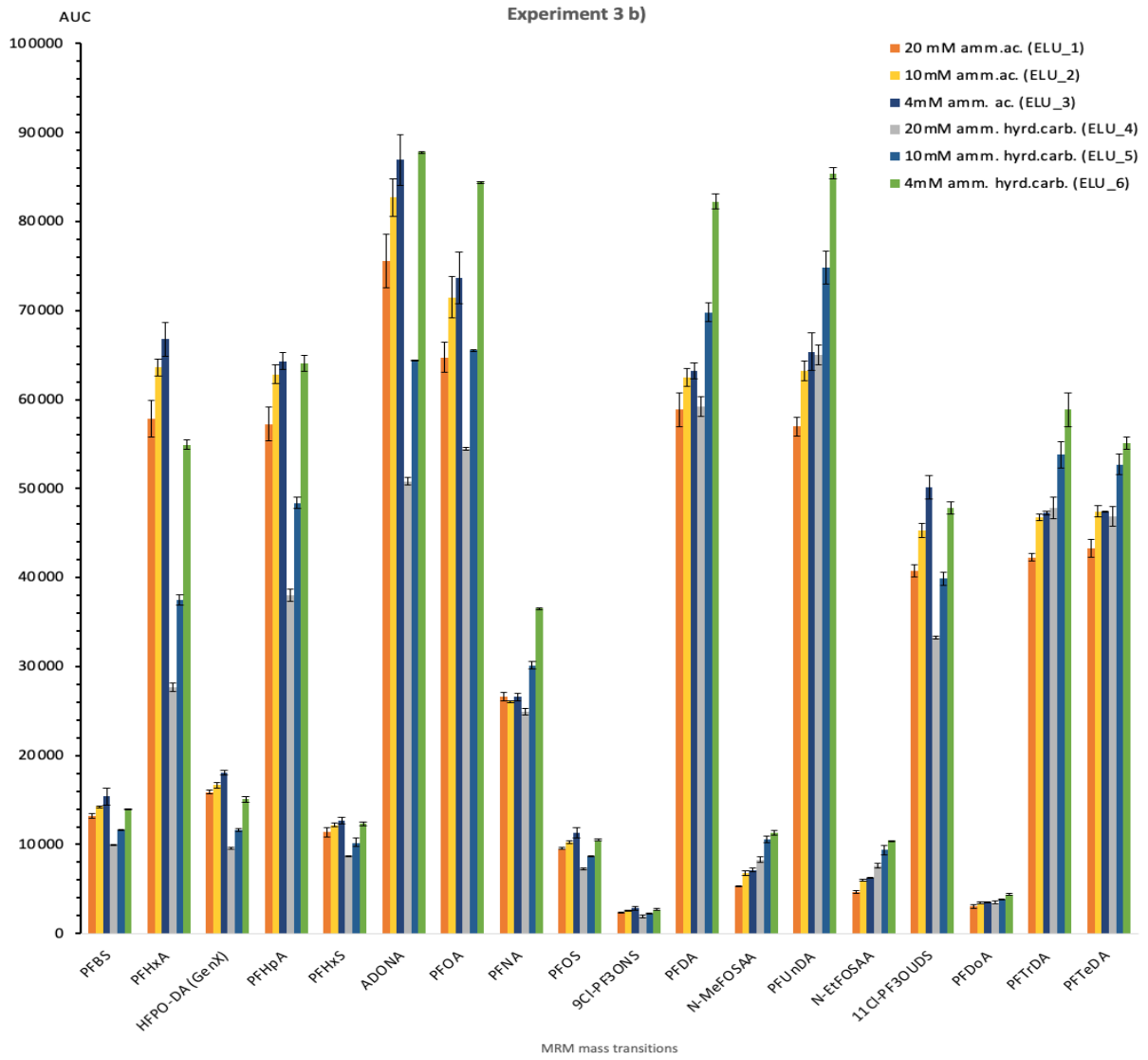

Figure 8. : Experiment 3 b): Chromatographic analyses of 100 ng/mL multi-standard PFAS using mobile phases with different acid contents.

7.2.2. Investigation of mobile phase pH on the signal of multi-PFAS (Experiment 4.)

In the second experiment regarding multi-PFAS investigation, the effect of mobile phase pH was tested (Figure 9). Both FIA and chromatographic analyses showed the same results regarding all of the compounds demonstrated in the preliminary trial. The non-buffered ELU G gave the highest signal intensity for all compounds in both trials, however with enormous high error. Examining the peak shapes of a chromatogram using a non-buffered mobile phase we can see the peak broadening effect of such circumstances (Figure 10). Considering the pH tolerance range of the column, the highest signal intensities were gained with the lowest acid

content, namely 0.01% acetic acid in the eluent. Based on the experiments of my thesis, it can be said that the ideal mobile phase of multi-PFAS analytical method was ElU_B with 4mM ammonium hydrogen carbonate 0.01% acetic acid (A) and MeOH (B). Using this method, a high-intensity chromatographic signal with a nice peak shape is obtained for each target component (Figure 10).

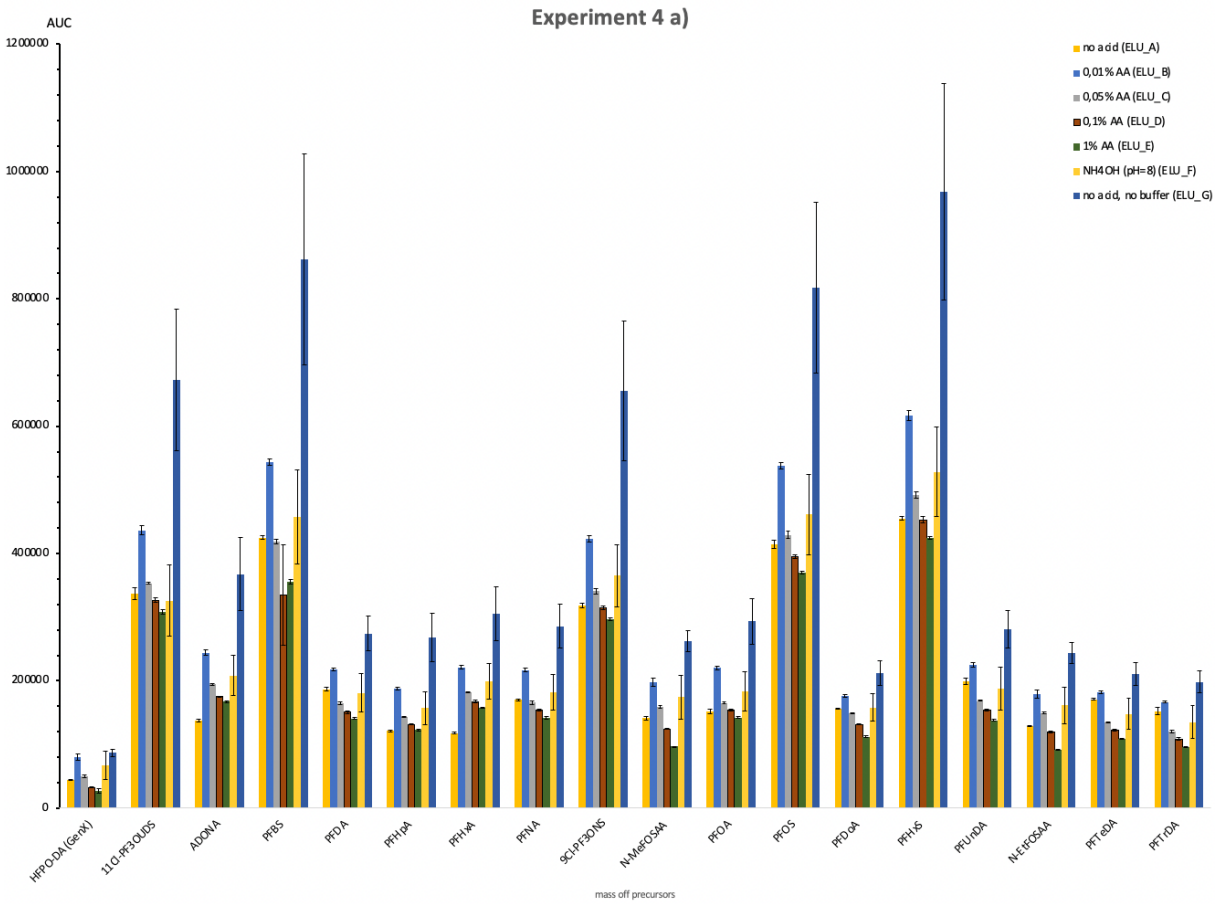

Figure 9. : Experiment 4 a): Flow injection analyses of 1 mg/L multi-PFAS standard mix using mobile phases with different salt contents.

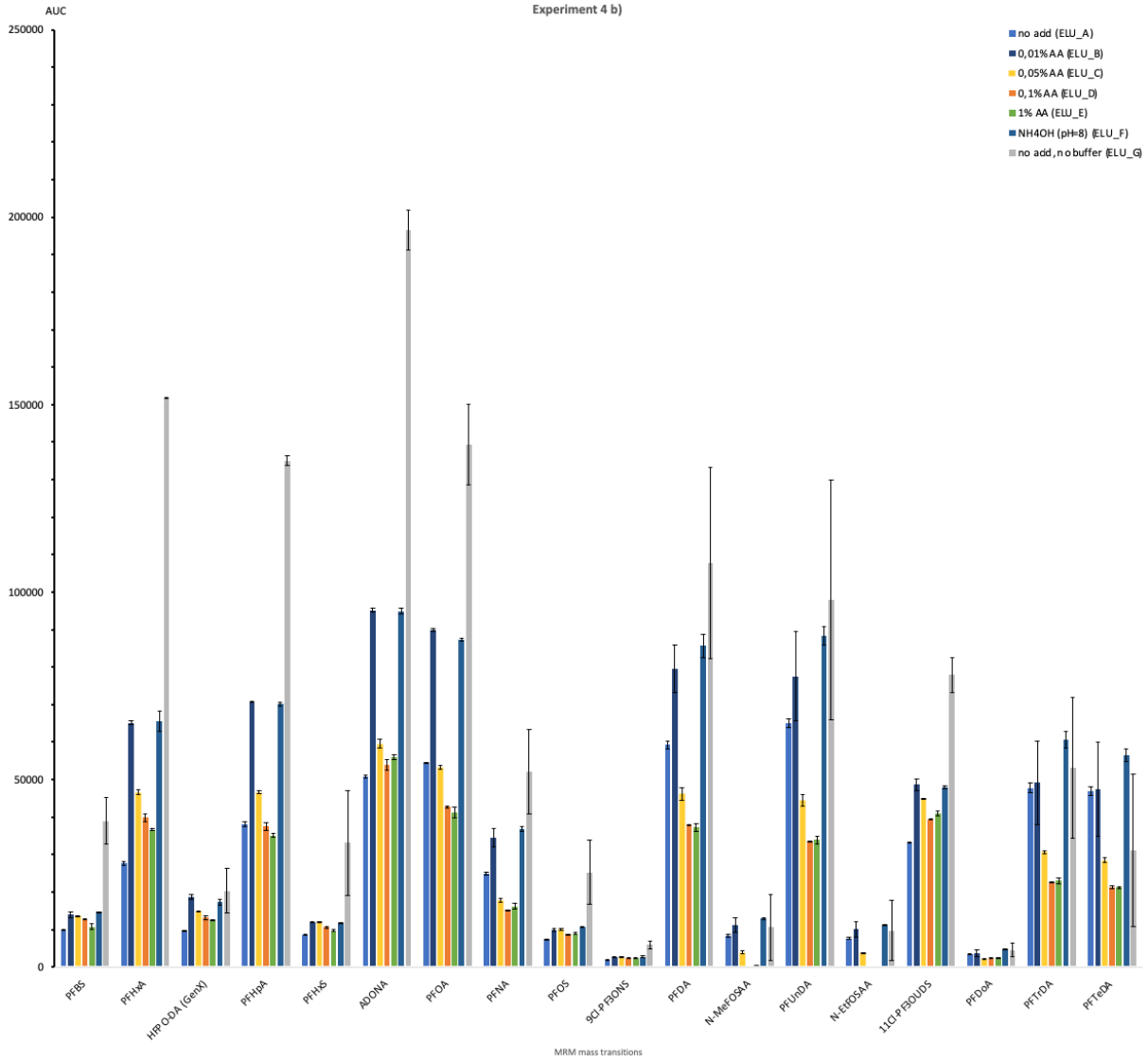

Figure 10. : Experiment 4 b): Chromatographic analysis of 100 ng/mL multi-PFAS standard mix using mobile phases with different acid contents.

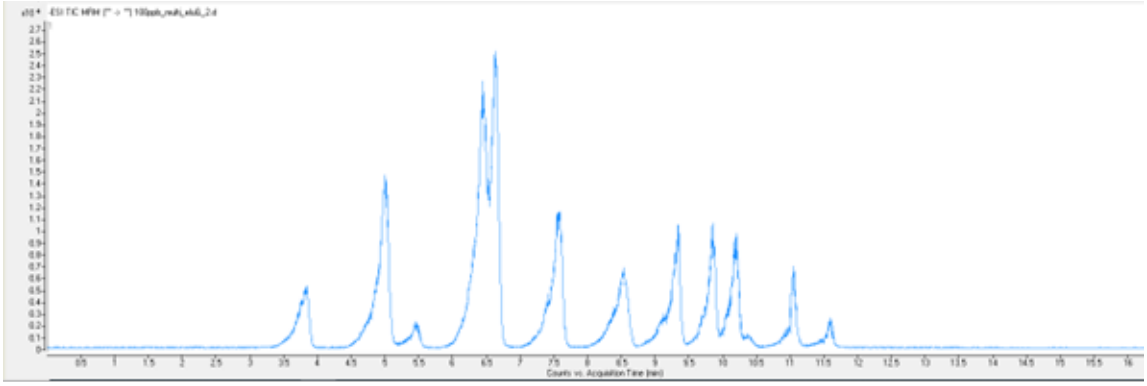

Figure 11. : Peak broadening in the case of multi-PFAS analysis using Eluent G (water, no additive, no buffer) (Experiment 4 b))

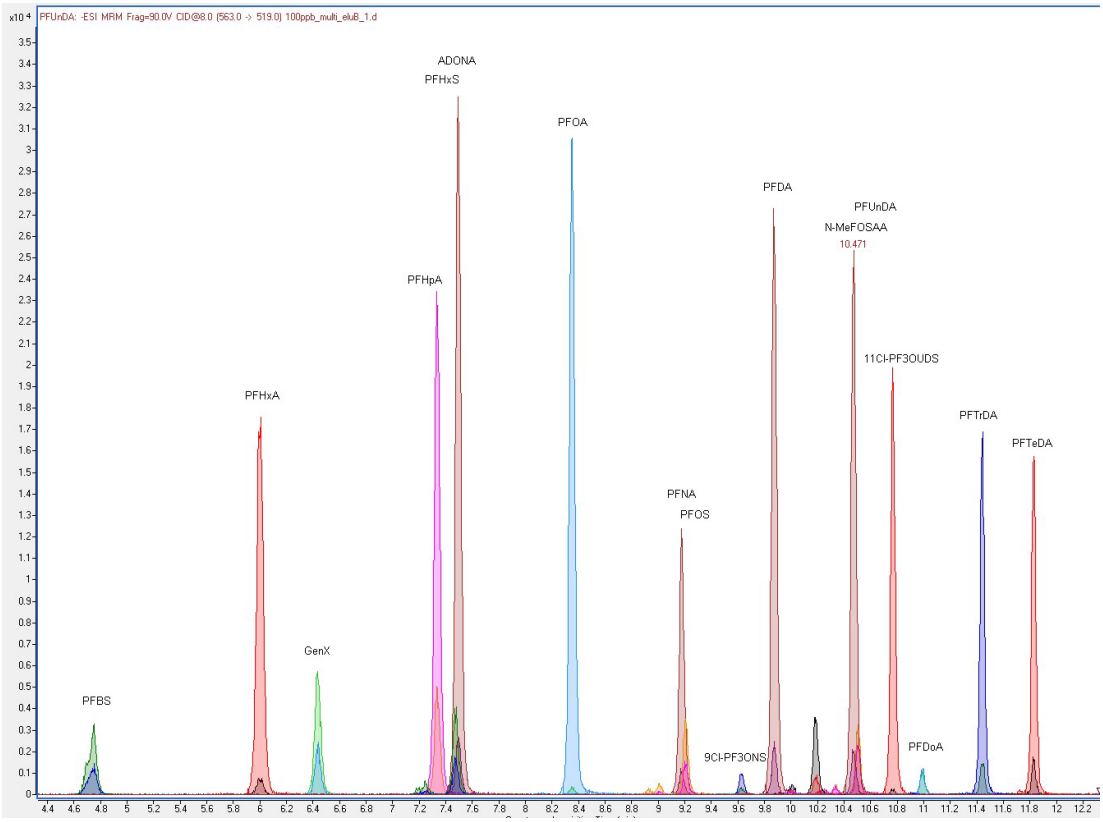

Figure 12. : Total ion chromatogram of 100ng/mL multi-PFAS standard solution using (A) 4mM ammonium hydrogen carbonate (ELU_B) with 0.01% AA and (B) MeOH

8. Conclusions

As a result of my work, I managed to select the mobile phase additive, which mostly helps the signal generation of PFAS components during multicomponent measurement. Despite the fact that in the literature there is only one case Lauren Mullin et al., 2018 that use of ammonium hydrogen carbonate in PFAS analyses, we consider the performed tests to be an important and special result. My work confirms the idea that instead of the routine adoption of literary data, it is worth focusing on the details of the methods and investigating all the parameters for individual devices.

In the thesis work, the best mobile phase additive was 4mM ammonium hydrogen carbonate with 0.01% acetic acid as eluent A and MeOH as eluent B. This study proves that the mobile phase additive in negative ion mode was helping in the detection of PFAS with high sensitivity by increasing the overall signal and decreasing unintended fragmentation.

9. Summary

This thesis work discusses mobile phase additives in order to investigate the signal of PFAS compounds. Per- and poly-fluoroalkyl substances (PFAS) are a large group of environmental pollutants. They are widespread throughout the world's ecosystem, including in animals and humans and also can be found in drinking water. The new limit has been established by Drinking Water Directive in 2021 in the EU for a 'Sum of PFAS' of 0.1 μg/L, which limit value must be measured by all member states from 2026. In order to make the compounds measurable with HPLC- MS/MS method with as low a concentration as possible, the aim of this work was to examine the effect of different mobile phase additives on the signal generation of multi-PFAS standard solution. The methodology was based on tandem mass spectrometry (MS/MS) and ultrahigh-pressure liquid chromatography (UHPLC) methods.

In a preliminary experiment, two substances—PFAO and hexafluoropropylene oxide-dimer acid, also referred to as GenX—were tested first before the multi-standard PFAS solution was used. The selection of multi-standard PFAS (18 compounds) was based on the United Environmental Protection Agency's (EPA) Method 537.1: Determination of Selected PFAS in Drinking Water by Solid Phase Extraction (SPE) and LC/MS/MS (2018/2020). The investigation involved Flow Injection Analyses (FIA) with no column and a slow flow rate. The analyser was applied in selected ion monitoring (SIM) mode measuring the precursor ions. Besides that, chromatographic analyses with column installed was also used, and the separation and detection were done in optimized with multiple reaction monitoring (MRM) mode.

This study involved a series of eluents with buffer salts including the different concentrations of ammonium acetate and ammonium hydrogen carbonate. Additionally, the effect of mobile phase pH on signal generation was also investigated.

The results show that the addition of ammonium hydrogen carbonate and acetic acid to the mobile phase significantly improves the detection of PFASs in negative ion mode. The best mobile phase additive for detecting PFASs using UHPLC/MS/MS in negative ion mode was a combination of (eluent A) 4mM ammonium hydrogen carbonate and 0.01% acetic acid and (eluent B) MeOH.

As a continuation of the work, by transforming the developed method into a dynamic MRM method, we can measure a multi-PFAS solution with a concentration of 1ng/ml with the appropriate intensity (Figure 13)

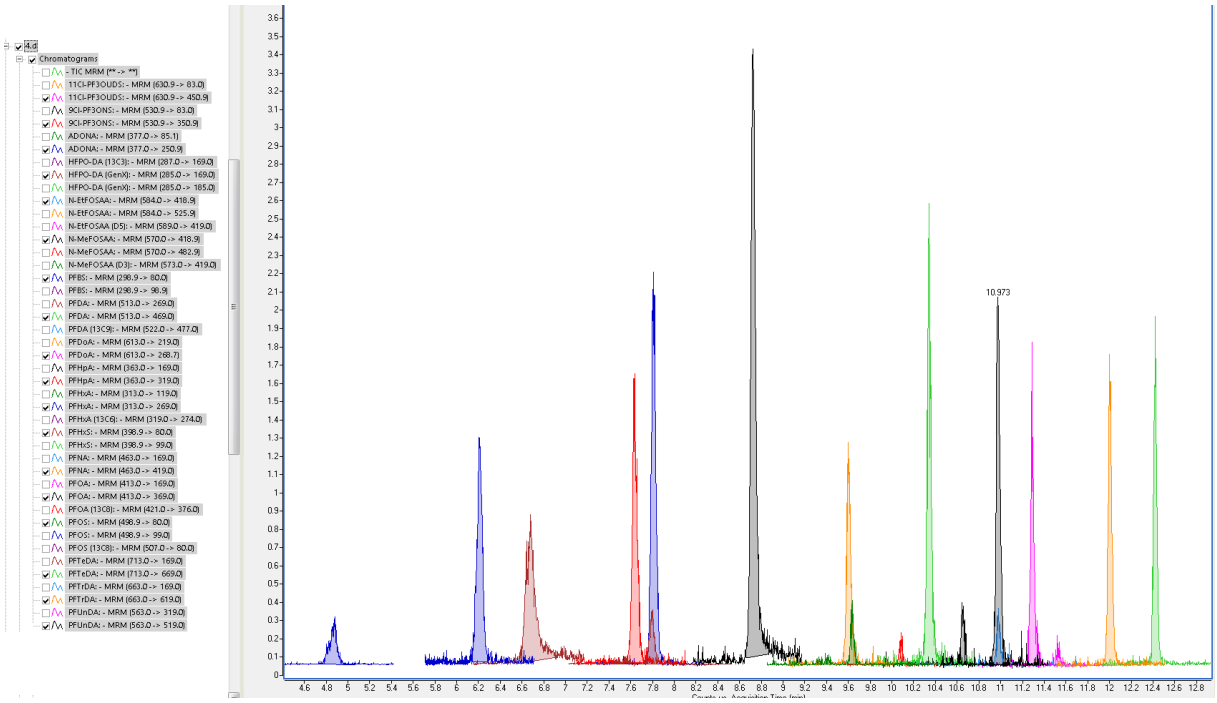

Figure 13. : Overlayed total ion chromatogram of 1 ng/mL multi-PFAS standard solution using (A) 4mM ammonium hydrogen carbonate (ELU_B) with 0.01% AA and (B) MeOH mobile phase in dMRM measuring mode (only the quantitative transitions are illustrated)

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“I am humbled and grateful for the blessings and guidance I have received from God, which gave me the strength and perseverance to overcome obstacles and achieve my goals."

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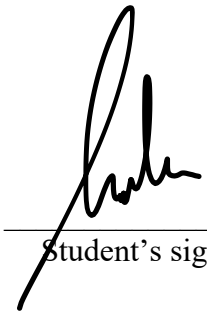

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I, the undersigned Siti Amirah Binti Hasan (Neptun code: ET5ULK) student at MSc Food Safety and Quality Engineering programme request that my diploma thesis titled Investigation of mobile phase additives in HPLC-MS/MS method used for the investigation of PFAS in drinking water (name of supervisor(s): Dr Sörös Csilla Marczika Andrásné) be encrypted by applying point c) of Section 95 (5) of the Study and Examination Regulations of the Hungarian University of Agriculture and Life Sciences (hereinafter referred to as 'SER'). I understand that if my request is approved, the encryption of the diploma thesis will cover 5 years following the successful defense, in accordance with point c) of Section 95 (5) of SER.

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I, the undersigned[1] Dr Sörös Csilla Marczika Andrásné Professor (name and position of company representative), as the representative of the Hungarian University of Agriculture and Life Sciences, 1118 Budapest, Villányi út 29-43. (name and address of the company), request that the diploma thesis titled Investigation of mobile phase additives in HPLC-MS/MS method used for the investigation of PFAS in drinking water made by Siti Amirah Binti Hasan (Neptun code: ET5ULK) by using the data provided by Hungarian University of Agriculture and Life Sciences be encrypted.

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