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Inventarisatie toegevoegde waarde van SFC

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KWR

Bridging Science to Practice

Report

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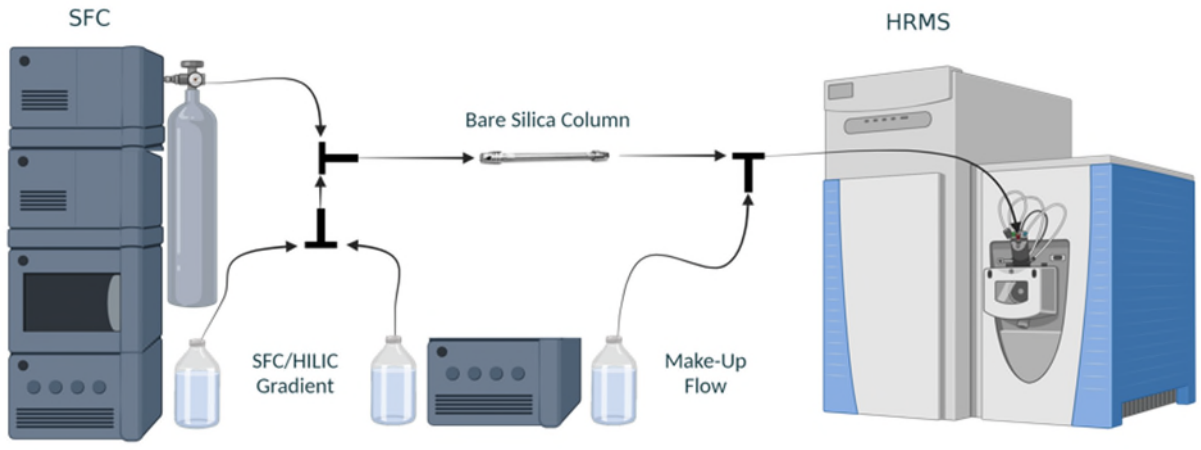
Managementsamevatting

Superkritische vloeistofchromatografie (SFC) heeft toegevoegde waarde bij analyse van polaire stoffen, experimenten met aangepaste apparatuur nodig voor optimalisatie

Authors: Elvio Amato, Erik Emke, Nienke Meekel

Zeer polaire organische verbindingen vormen een uitdaging voor de afvalwaterzuivering door hun sterk hydrofiele gedrag. Met conventionele reversed-phase vloeistofchromatografie (RPLC) worden deze verbindingen vaak onvoldoende vastgehouden. Daarom zijn nieuwe methoden ontwikkeld voor scheiding en detectie van sterk polaire organische verbindingen, zoals superkritische vloeistofchromatografie (SFC). Deze techniek kan opgeloste stoffen scheiden over een breder polariteitsbereik dan afzonderlijke LC-methoden. Wel zorgt gebruik van superkritische vloeistoffen voor extra complicerende factoren. Experimenten met een SFC-apparaat bij de Universiteit van Amsterdam (UvA) laten zien dat SFC potentieel heeft voor scheiding van een breed scala chemische stoffen met verschillende polariteiten. De ontwikkelde SFC-MS methode detecteerde en scheidde met succes 14 van de 22 geselecteerde stoffen over een breed polariteitsbereik, 8 stoffen werden niet gedetecteerd. Een speciale opstelling is nodig voor verder onderzoek naar bijvoorbeeld alternatieve monsterbehandeling voor voldoende gevoeligheid. Een ternaire gradiëntopstelling (in wezen dubbele chromatografie met SFC en hydrofiele interactiechromatografie (HILIC)) is een interessante optie voor optimale retentie en scheiding.



SFC-HILIC-MS setup.

Belang: potentieel groenere en goedkopere methode voor analyse polaire stoffen

Zeer polaire organische verbindingen vormen een uitdaging voor afvalwaterzuiveringsprocessen vanwege hun sterke hydrofiele gedrag en worden vaak onvoldoende vastgehouden door conventionele scheidingstechnieken op basis van reversed-phase vloeistofchromatografie (RPLC). Om dit probleem aan te pakken, zijn inmiddels nieuwe methoden ontwikkeld voor scheiding en detectie van sterk polaire organische verbindingen, waaronder superkritische vloeistofchromatografie (SFC). Deze techniek gebruikt van superkritische vloeistoffen als mobiele fase en kan opgeloste stoffen scheiden over een breder polariteitsbereik dan afzonderlijke LC-methoden, in verschillende milieus (zoals afvalwater, oppervlaktewater en grondwater), biedt snellere analyses dan traditionele LC-technieken én gebruikt

aanzienlijk minder organische oplosmiddelen, waardoor de kosten en de afvalproductie dalen. Het is een groener alternatief voor conventionele methoden met vergelijkbare resultaten als de combinatie van hydrofiele interactiechromatografie (HILIC) met reversed-phase LC (RPLC). SFC gebruikt dezelfde kolommen als LC en de integratie van een SFC-systeem in standaard analytische laboratoria lijkt qua infrastructuur en veiligheidseisen geen al te grote uitdaging. Het gebruik van superkritische vloeistoffen brengt echter extra factoren mee om rekening mee te houden, zoals tegendruk en samendrukbaarheid van de mobiele fase. Nader onderzoek was nodig om te achterhalen of voor SFC een ingewikkeldere methode moet worden ontwikkeld en of en wanneer SFC HILIC kan aanvullen dan wel overtreffen, waardoor verbindingen kunnen worden gemeten die met HILIC niet detecteerbaar zijn.

Aanpak: experimenten met SCF-apparatuur bij UvA

Na literatuuronderzoek zijn met partners van drinkwaterbedrijven en laboratoria samen (i) geschikte verbindingen geselecteerd voor het experimentele werk, dat plaatsvond met een SFC-UV-apparaat bij de Universiteit van Amsterdam (UvA). Het experimentele werk omvatte verder: (ii) kiezen van de chromatografische kolom en uitvoeren van initiële scheidingsbeoordelingen, (iii) optimaliseren van het scheidingsproces en (iv) integreren van massaspectrometrie (MS) voor verdere analyse.

Resultaten: scheiding over een breed polariteits-bereik werkt, invulling optimale opstelling

De ontwikkelde SFC-MS methode detecteerde en scheidde met succes 14 van de 22 geselecteerde stoffen over een breed polariteitsbereik. 8 stoffen konden niet worden gedetecteerd om verschillende redenen, waaronder lage gevoeligheid, het gebruikte type apparatuur en initiële instelcondities. Met de geselecteerde verbindingen werden verschillende belangrijke stappen onderzocht, wat leidde tot de volgende resultaten:

1. Een make-up flowsamenstelling van pure methanol (MeOH) leverde de beste resultaten en gevoeligheid na de kolom voor ionisatie, terwijl toevoegingen zoals mierenzuur (FA), ammoniumfluoride (AmF) en ammoniumacetaat (AmAc) leidden tot verminderde signaalintensiteit en ionenonderdrukkingseffecten;
2. Het gebruik van acetonitril (ACN) als organisch verdunningsmiddel maakte injectie van water (10-50%) op de kolom mogelijk zonder afbreuk te doen aan piekvorm, intensiteit, gebied en MS-gevoeligheid. Dit biedt een voordeel ten opzichte van chromatografiemethoden zoals hydrofiele interactie vloeistofchromatografie (HILIC).
3. Het gebruik van ACN als modificator verbeterde de algehele scheiding in vergelijking met methanol. Deze keuze biedt voordelen bij monstervoorbereiding en -behandeling.

Concluderend: de optimale opstelling voor SFC-MS bestaat uit het gebruik van ACN als modificator en 100% MeOH voor make-up om de ionisatie te verbeteren. Injectie met 10-50% water is haalbaar, maar kan de piekvorm beïnvloeden, afhankelijk van de verbinding. ACN heeft de voorkeur boven MeOH als injectievloeistof. De combinatie van SFC en HILIC lijkt veelbelovend voor het benutten van de sterke punten van beide technieken.

Toepassing: monsterbehandeling en concentratie-bereik verder onderzoeken met aangepaste SFC

Voor een grondiger beoordeling van de SFC-prestaties moet aangepaste SFC-apparatuur worden gebruikt. Om voldoende gevoeligheid te bereiken moeten de monsterbehandeling en het concentratiebereik moeten verder worden onderzocht en geoptimaliseerd. Het gebruik van een ternaire gradiëntopstelling, waarbij in wezen een dubbele chromatografiescheiding plaatsvindt (SFC en hydrofiele interactiechromatografie (HILIC)), is een interessante optie om de beste retentie en scheiding te bereiken.

Rapport

Dit onderzoek is beschreven in het rapport *Inventarisatie toegevoegde waarde van SFC* (BTO-2024.040).

Contents

1 Literature review

1.1 Introduction

Supercritical fluid chromatography (SFC) is a separation technique that relies on the use of supercritical fluids as a mobile phase. Supercritical fluids are characterised by reduced viscosity and increased diffusivity compared to liquids, which result in enhanced separation efficiencies. Early applications of SFC focused on the separation of enantiomers (Bieber and Letzel, 2021; Calcaterra and D'Acquarica, 2018; Pérez-Fernández et al., 2011; Rice et al., 2020) and analysis and extraction of organic compounds (Berset and Holzer, 1999; Hawthorne et al., 2000; Luo and Schrader, 2022), however, the benefits of SFC for the determination of polar and very polar compounds have been recently reviewed (Bieber et al., 2017; Losacco et al., 2021; Sen et al., 2016). SFC operates over a relatively large polarity range, including also very polar substances that are typically difficult to measure using conventional reversed-phase liquid chromatography (RPLC). Very polar substances can be measured with Hydrophilic Interaction Liquid Chromatography (HILIC) but permanently negatively charged compounds cannot be measured only with Mixed-mode chromatography. Precautions have to be taken to prevent adsorption to metal oxides on the other hand slight changes with these precautions (eq. using acids) can have a dramatic effect on the retention times. Applications of SFC in water quality monitoring have thus far included wastewater, groundwater and surface water samples. SFC gained popularity also as a more green and sustainable analytical technique compared to conventional liquid chromatography (LC), i.e., CO_2 is generally recycled from industrial processes and significantly reduces consumption of organic solvents and production of waste (Płotka-Wasylka et al., 2017).

1.2 Theory

In supercritical fluid chromatography (SFC), the mobile phase is maintained above its critical point, where a difference between gaseous and liquid states can no longer be observed (Figure 1). At these conditions, the material is defined as a supercritical fluid; however, this is not considered a state of matter, i.e., during phase changes (e.g., condensation and evaporation) the physical properties (e.g., density, viscosity and diffusivity) of the material change abruptly, while in the supercritical region the physical properties of a pure compound show continuous rather than abrupt variations.

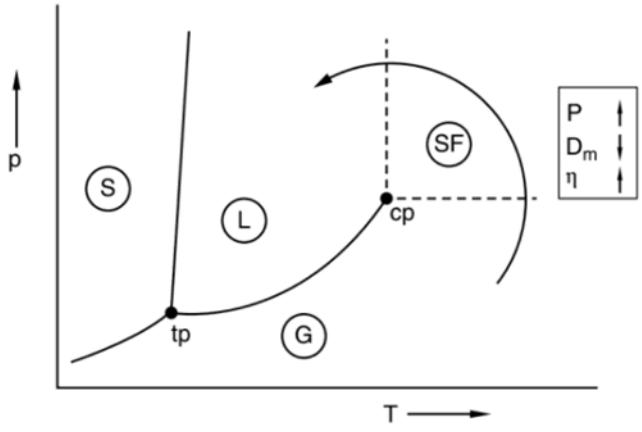


Figure 1. Phase diagram for a single pure component, illustrating areas in which solid (S), liquid (L), gaseous (G), and supercritical (SF) conditions occur. tp is the triple point and cp is the critical point. A gas can be transferred into a liquid by following the arrow. In doing so, the density, the viscosity, and the diffusion coefficient change continuously from gas-like to liquid-like values, but no phase change is observed (Schoenmakers, P.J. and Uunk, L.G.M., "Mobile and stationary phases for supercritical fluid chromatography").

A supercritical fluid is characterised by a lower viscosity and a higher diffusion coefficient than a liquid, which provide the fluid with properties that are between those of LC and GC (Chester et al., 1996; Taylor, 2008). SFC generally uses instrumentation that is almost identical to that used in high performance liquid chromatography (HPLC). CO_2 is typically used as mobile phase, however, applications of water in its supercritical state have also been reported (Dembek and Bocian, 2020). The intermolecular interactions between CO_2 molecules are relatively weak, however, when molecules are compressed, the density of the resulting fluid approaches that of a fluid. Despite the higher density, the intermolecular forces are still weak and solutes dissolved in the CO_2 can still diffuse rapidly through it.

Since CO_2 is a highly nonpolar solvent, an organic cosolvent, generally referred to as 'modifier' (e.g., methanol, ethanol or isopropanol), and/or additives (e.g., water, ammonium hydroxide, ammonium acetate, formate, formic and trifluoroacetic acids) are typically added to the mobile phase to enable the separation of polar solutes (Konya et al., 2020; Lesellier, 2020; Lesellier and West, 2015; Ovchinnikov et al., 2022; Si-Hung et al., 2022). However, the addition of a polar modifier decreases diffusion coefficients significantly.

SFC can be defined as a normal phase technique, i.e., peaks elute from lower to higher polarity. However, SFC provides advantages compared to normal phase HPLC, i.e., equilibration is faster, and even aqueous-based samples can be injected (Bieber and Letzel, 2021; Ovchinnikov et al., 2022). Overall, the same stationary phases (and in some cases the same column) used in HPLC can be used in SFC. An overview of the performance of different stationary phases (i.e., pentafluorophenyl, non-polar alkyl, polar alkyl, aromatic, and polar phases) useful for method development in SFC is available in the literature (West et al., 2016). The addition of a modifier is a crucial point to achieve separations over a wide polarity range as simply using pure liquid CO_2 offers a polarity similar to hexane and significantly limits the scope of the separation. However, the addition of a modifier forces the supercritical fluid into the subcritical region. In addition to modifiers, additives are also often included to further help with the selectivity, efficiency and elution strength of the separation (van de Velde et al., 2020). This unique combination of supercritical fluid, modifiers and additives offers flexibility in the analytes that can be analyzed and eluted, and, in the case of water contaminants, widens the possible polarity range for analysis (van de Velde et al., 2020). Such flexibility is not offered nor possible with any other separation technique at the moment (Figure 2) (Tisler et al., 2023).

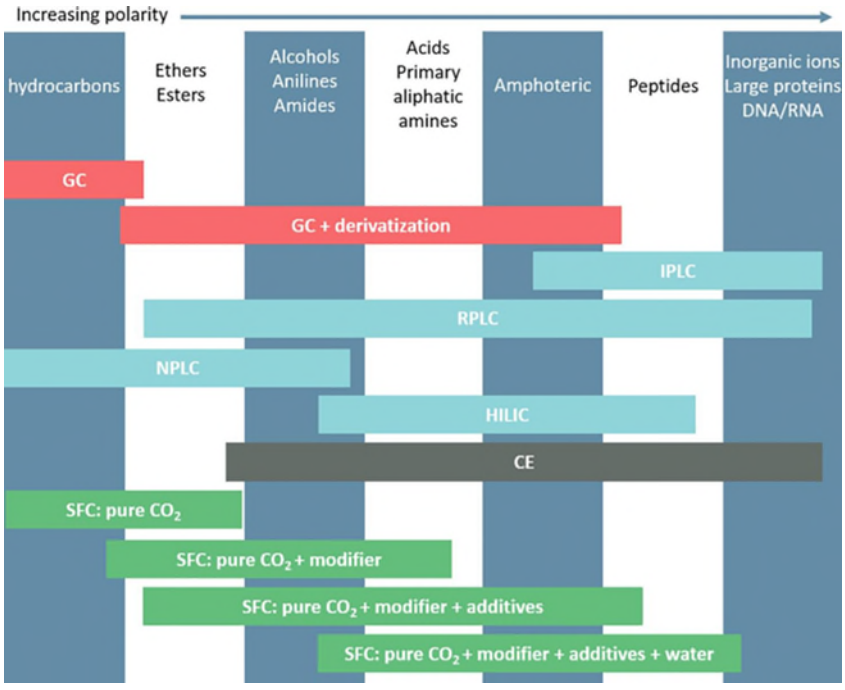


Figure 2: SFC polarity/chemical composition coverage range in comparison to other common chromatographic techniques (Tarafder, 2016; van de Velde et al., 2020). Abbreviations: ion pair (IPLC), normal phase (NPLC), hydrophilic interaction (HILIC) liquid chromatography, capillary electrophoresis (CE)

1.3 Comparison with conventional LC

1.3.1 Advantages

SFC offers some advantages over commonly used separation techniques, including a greater polarity range of separable compounds (e.g., log*D* from -8 to 10 at pH 7) (Bieber et al., 2017; Losacco et al., 2021), increased mobile phase flow rates (e.g., 1~4 mL/min) (West et al., 2016), and lower costs per analysis. The polarity range of SFC is comparable to that obtained by combining HILIC and RPLC (Figure 3), and can be achieved using only one stationary phase and in a smaller elution window (Bieber et al., 2017; Losacco et al., 2021). This allows the separation and simultaneous determination of nonpolar, polar, and very polar compounds in one experimental run. In addition, due to the low viscosity and high diffusion of the mobile phase, the re-equilibration time, flow rates, and mass transfer of SFC are faster than those of LC (Bieber and Letzel, 2021; Grand-Guillaume Perrenoud et al., 2012; Lesellier and West, 2015; Losacco et al., 2021).

SFC is less affected from negative impacts by matrix compounds which impact retention, such as salts. However, matrix effects appear to be inconsistent between SFC and LC, making these techniques complementary to each other (Bieber et al., 2017). Finally, detection is traditionally based on Diode array detection (DAD) which is useful when less complicated samples are analysed in high concentrations. Or during method development which allows a relative low operating cost before transferring the method to a more sensitive detection technique. Disadvantage is that when optimizing a large group of compounds chromophores are needed to be able to detect. But when there is a need for increased sensitivity and a broader range, mass spectrometry is used. Depending on the available equipment this can be a triple quadruple (QqQ) or for accurate massspectromters (Q-TOF or Orbitrap type instruments. Also increased sensitivity has been observed for UHPSFC–MS over UHPLC–MS, which was attributed to a more efficient desolvation due to the MeOH and decompressed CO_2 mixture (Grand-Guillaume Perrenoud et al., 2014).

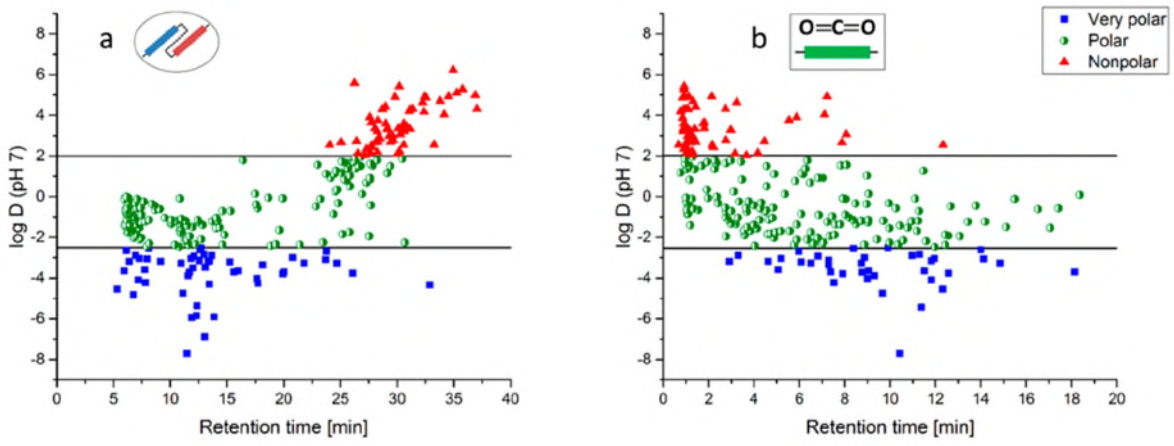


Figure 3. Relationships between logD and retention time of reference compounds for (a) the HILIC-RPLC and (b) HILIC-SFC 2D systems (Bieber et al. 2017).

The CO_2 used for SFC separations is a relatively cheap industrial by-product. Combined with the low consumption of organic solvents, this results in low costs for solvents purchase and disposal. This is an economic but also ecologic benefit in high sample throughput, because the solvents make SFC separations greener and ultimately more sustainable than LC separations (Dembek and Bocian, 2020; Taylor, 2009).

1.3.2 Disadvantages

It appears that method development in SFC is more laborious than in LC. SFC separations are influenced by several factors, which are not present or adjustable in LC, such as the backpressure, or the compressibility of the mobile phase, which impacts mobile phase density (Bieber and Letzel, 2021). Also, there is currently limited knowledge about SFC retention mechanisms. In LC, the retention time of molecules can be used to estimate their polarity, whereas for SFC the retention time is not linked to polarity (Bieber et al., 2017). However, some authors suggest that method development is faster for SFC compared to HILIC or RPLC (Lesellier and West, 2015).

Some studies have shown that the use of additives may result in chemical modification of either the solid phase, mobile phase or solutes (Ovchinnikov et al., 2022). In addition, SFC is more difficult to investigate than LC since supercritical fluids exist only under pressure and cannot be simply kept in a glass flask to perform measurements of solubility, pH, density, viscosity etc. However, indirect methods of measuring physical and chemical properties of supercritical fluids and investigating their effect on retention mechanisms in SFC are often applied (Ovchinnikov et al., 2022). Furthermore, injection volumes used for SFC (<10µL) are usually smaller than those used for HILIC and RP-LC (25-100 µl), and analysis of water samples typically require preconcentration steps (e.g., SPE). Also, the coupling with MS is still at an early stage of development.

Direct injection of water samples is not possible due to the low polarity of supercritical CO_2, (sCO_2) and generally extensive sample preparation is required for solvent exchange. However, applications of methanol-modified CO_2 that incorporated 5% (w/w) of water have been reported (Ashraf-Khorassani et al., 2012; Liu et al., 2019; Sen et al., 2016). Sen et al. (2016) used a 1:3 urine:methanol ratio to investigate metabolites in urine samples, and 1:1 methanol:water mixtures for chromatographic method development. Similarly, Bieber et al. (2017) used a mixture of reference standards in acetonitrile:water 50:50 to asses polar organic compounds using SFC.

1.4 Potential added value for the water sector

Due to their strong hydrophilic behaviour, very polar organic compounds are typically difficult to remove by wastewater purification processes (Reemtsma et al., 2016). In addition, these compounds are poorly retained by most commonly used separation techniques which rely on RPLC, and new approaches have been developed for the separation and detection of very polar organic compounds, including normal phase liquid chromatography (NPLC), ion chromatography (IC), hydrophilic interaction liquid chromatography (HILIC), and, more recently, also SFC.

Bieber et al. (2017) found that 80% of the compounds measured in wastewater treatment plant (WWTP) effluent samples showed a negative log*D* value (at pH 7). The authors compared the performance of reverse-phase liquid chromatography coupled with a HILIC column (RPLC-HILIC) and SFC-HILIC using (i) a mixture of reference standard compounds (very polar, polar, and nonpolar compounds combined into working standard mixtures with a final concentration of 10 µM per compound in acetonitrile:water 50:50, v/v) and (ii) samples of wastewater effluent. In the reference standard test, LC and SFC separated and detected a similar number of very polar compounds (log*D* < -2.5) using a HILIC column. While these compounds were mainly retained and detected using HILIC, RPLC measured a small fraction of very polar compounds that were not detected using HILIC. Fifteen very polar (log*D* < -2.5), 10 polar (-2.5 < log*D* < 2.0), and 4 nonpolar compounds (log*D* > 2) were detected by RPLC-HILIC/TOF-MS only, while 1 very polar, 10 polar, and 1 nonpolar compound were detected by SFC/TOF-MS only. However, authors suggested that the detection of individual compounds may be improved by altering ionization parameters of the electrospray ionization (ESI) source or adjusting the separation method.

In wastewater samples, RPLC-HILIC/TOF-MS detected a total of 58 compounds, containing 13 very polar, 42 polar, and 3 nonpolar substances. All very polar compounds were retained by HILIC, as well as 35 of the 42 polar

compounds. The remaining 7 polar and 3 nonpolar compounds eluted from the RP column. By SFC/TOF-MS 42 of the standard compounds could be detected in the water sample, including 3 very polar, 35 polar, and 4 nonpolar compounds. While the polarity range of these compounds was comparable to the range of RPLC-HILIC/TOF-MS, it appears that LC-HILIC may retain more very polar compounds than SFC-HILIC. However, the use of additives and modifiers considerably changes the performance of SFC (Konya et al., 2020; Lesellier, 2020; Ovchinnikov et al., 2022; Si-Hung et al., 2022), and methods may be adapted to specifically target very polar organic substances.

Ozonation is a commonly used treatment for the removal of micropollutants in WWTP, however, this approach may generate transformation products (TP) that are typically more polar than their precursors due to oxidation processes. Seiwert et al. (2021) compared the performance of RPLC-HRMS and SFC-HRMS (equipped with a BEH column) for the detection of TPs in WWTP effluent samples following ozonation treatments. Authors performed both suspect and NTS analyses, and found that (i) the two techniques detected a similar number of TPs in suspect screening mode, (ii) SFC-HRMS detected more TPs than RPLC-HRMS in the 50 to 200 m/z range, and (iii) RPLC-HRMS measured more TPs than SFC-HRMS in the 200 to 500 m/z range. Authors suggested that features showing lower molecular weight may be associated with more polar compounds.

A SFC-HRMS approach was developed for the analysis of persistent and highly polar substances in surface, ground-, and drinking water samples (Schulze et al., 2020). Results showed that SFC (coupled with a BEH column) is a suitable technique for the monitoring of highly polar compounds in water, while conventional RPLC-MS/MS analyses were characterized by poor retention and peak shape for these compounds. In a later study, a similar SFC-HRMS method was compared to HILIC-HRMS on the basis of suspect screening of >1000 of potential persistent and mobile chemicals in surface water samples (Neuwald et al., 2021). A total of 64 candidate compounds were identified, of which only 31 were detected by both techniques (Figure 4). This suggests that HILIC and SFC may be complementary to each other. For instance, some flame retardants (e.g., tris(1-chloro-2-propyl) phosphate (TCPP) and tris(2-chloroethyl)phosphate (TCEP)), contrast agents (diatrizoic acid (DAA)), organic sulfonates (naphthalene-1-sulfonic acid (NAPSA) and p-toluenesulfonate (PTSS)) pharmaceuticals and their transformation products (e.g., losartan and valsartan acid) were measured only by SFC, while chemicals such as 2-(2-(dimethylamino)-ethoxy)ethanol (DMAEE), tetrabutylammonium (TetraBuAm), tributylmethylammonium (TriBuMeAm), 2-phenyl-1H-benzimidazole-5-sulfonate (PhBenzImiSA), ethyltrimethylammonium (EtTriMeAm), and dimethyldidecylammonium (DiMeDiDecAm) were found only using HILIC. However, the differences between substances that were detected by only one of the two techniques could not be clearly explained based on their physicochemical properties. Overall candidate compounds were characterised by high polarity, low molecular weight, and relatively high number of heteroatoms.

SFC is mainly known for its applications in enantiomeric separation. Some studies have reported applications of SFC for the separation of pharmaceutical and drugs that may be of interest for the drinking water sector. For instance, illicit drugs and pharmaceuticals may undergo enantiomer-specific enrichment or depletion in WWTP and in the environment (Kasprzyk-Hordern and Baker, 2012a), which may lead to varying ecotoxicological impact on water quality based on the different biological activity and effects exerted by different enantiomeric forms. However, enantiomeric separation of illicit drugs has been achieved also using LC-based methods (Wang et al., 2021). Enantiomeric separation is also useful for sewage epidemiological studies concerning the estimation of the use of illicit drugs (Kasprzyk-Hordern and Baker, 2012b). Applications of SFC for the separation of chiral enantiomers such as beta-blockers, benzodiazepines, antidepressants, and insecticides in water samples have been also reported (Chen et al., 2015; Rice et al., 2020).

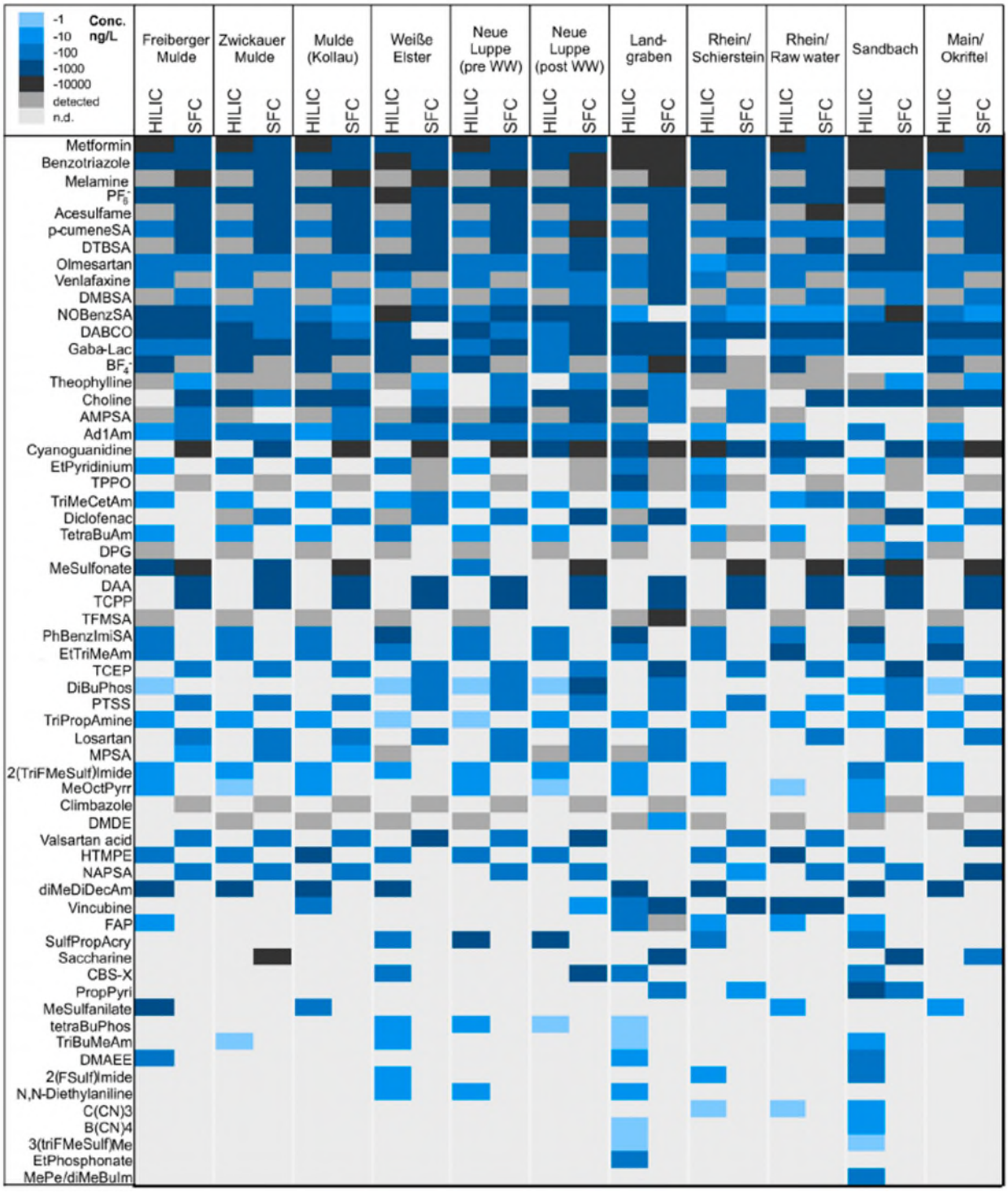


Figure 4. Heatmap of estimated concentrations of persistent and mobile substances measured in water samples confirmed by reference standards (Neuwald et al., 2021).

Table 1: Compounds measured by SFC and HILIC (from Neuwald et al., 2021

Compound	Chromatography	logKow	logD
Tris(1-chloro-2-propyl) phosphate	SFC	2.59	-
Tris(2-chloroethyl) phosphate	SFC	1.78	-
Diatrizoic acid	SFC	1.37	-
diatrizoic acid	SFC	-	-
p-toluenesulfonate	SFC	-0.62	-
Losartan	SFC	4.01	-
Valsartan acid	SFC	-	-
2-(2-(dimethylamino)-ethoxy)ethanol	HILIC	-	-
tetrabutylammonium	HILIC	-	-
tributylmethylammonium	HILIC	-	-
2-phenyl-1H-benzimidazole-5-sulfonate	HILIC	-	-
ethyltrimethylammonium	HILIC	-	-
dimethyldidecylammonium	HILIC	-	-
Bis(fluorosulfonyl)imide	HILIC	-	-
Tricyanomethanide	HILIC	-	-
Tetracyanoborate	HILIC	-	-
Tris(trifluoromethylsulfonyl)methanide	HILIC	-	-
Ethylphosphonate	HILIC	-	-
2,3-dimethylbutyl imidazolium	HILIC	-	-

1.5 Implementation of SFC at KWR and drinking water laboratories

1.5.1 Back-pressure regulator

Since in SFC the mobile phase is a compressed gas, a backpressure regulator is required on the system outlet to ensure the mobile phase remains in the super critical fluid phase (Figure 1) throughout the separation. Any deviation will result in different chromatographic properties but can also be used as an advantage to separate a different kind of class of compounds and hence combine essentially two different techniques (SFC/HILIC).

1.5.2 MS hyphenation

Early applications of SFC relied on capillary columns, thus, coupling with MS required ionization sources typically used for gas chromatography (GC) (i.e., electron ionization (EI) and chemical ionization (CI)). In modern SFC separation is performed using packed columns, and ionization sources such as ESI and atmospheric pressure chemical ionization (APCI) are commonly used (Pilařová et al., 2019; Toribio et al., 2021). An example of a SFC setup is shown in Figure 5. Supercritical fluid needs to be depressurized before introduction in the ionization source; this requires a dedicated interface to avoid issues related to decompression of the fluid, such as potential precipitation of analyte due to change in density and decreasing temperatures (Tarafder, 2018; van de Velde et al., 2020). The interfaces used for SFC-MS hyphenation can be classified in two main groups, full and split flow introduction (Toribio et al., 2021) (Figure 5); however, various options are available (Figure 6).

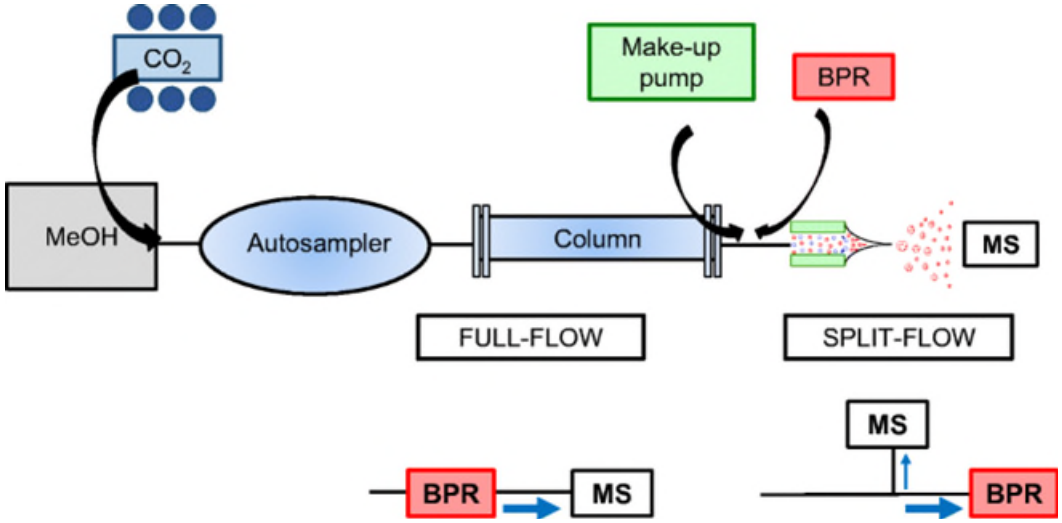


Figure 5. Schematic of a supercritical fluid chromatography system (Akbal and Hopfgartner, 2020).

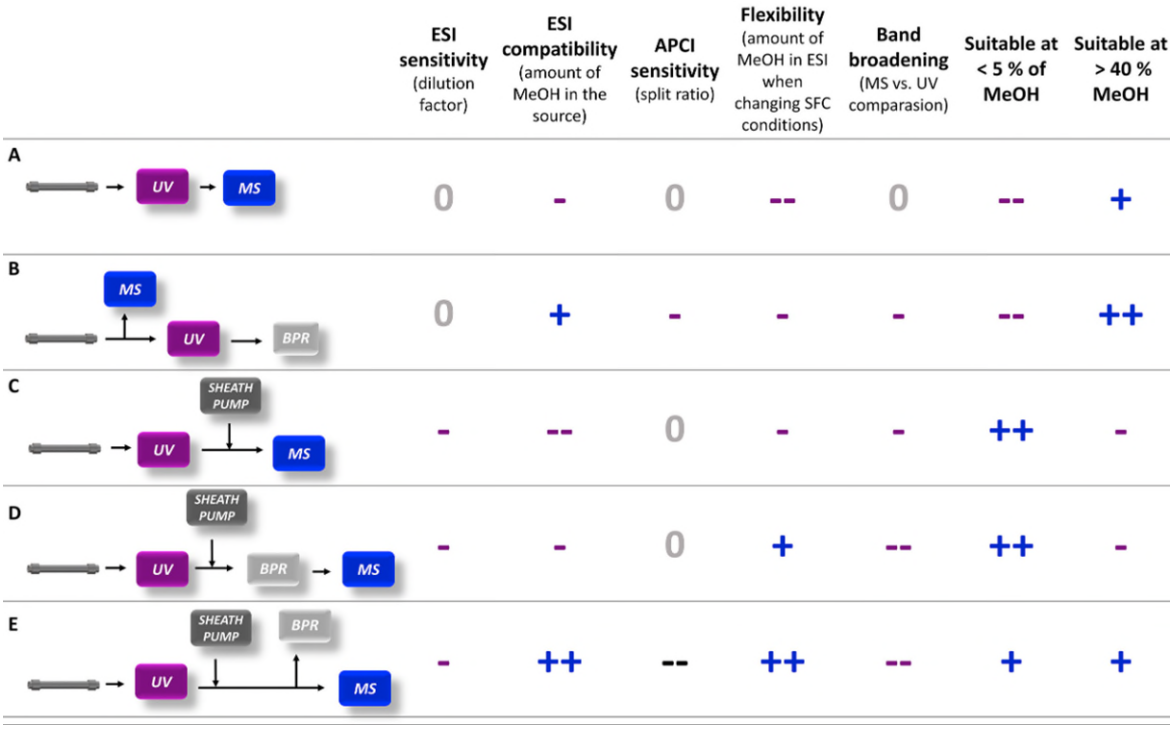


Figure 6. Schematic of SFC-MS interfaces (Pilařová et al., 2019).

In full flow introduction the full flow from the SFC system enters the MS detector. In split flow introduction only a small portion enters the MS. With modern SFC systems pressures up to 600 or 1000 bar can be achieved and allow ultra-high-performance supercritical fluid chromatography (UHPSFC) to be performed (Bieber and Letzel, 2021). Both interfaces use a make-up fluid introduced before the MS detector, which prevents solute precipitation when CO_2 volatilizes, especially when a small percentage of modifier is used. The make-up fluid enhances the ionization in ESI, however, this results in sample dilution and loss of sensitivity. Under most conventional operating conditions, the dilution factor varies between 1.1 and 1.5 (Grand-Guillaume Perrenoud et al., 2014).

1.5.3 CO_2 purity

Bulk CO_2 is typically of high purity (many grades are often > 99.99 % purity). Beverage or food grade may be a suitable choice. Some analytical SFC systems have a powerful chiller that liquefies the vapor phase from the cylinder, making eductor or dip tube extending from the valve to the bottom of the cylinder unnecessary. By using the vapor phase and not the liquid phase, the fluid is distilled just before use, leaving any non-volatile contaminants behind in the cylinder.

1.5.4 Accessibility of the critical point

The critical point of pure CO_2 is readily accessible at just over 31 °C and 70 bar. This simply means that CO_2 can be compressed to a dense fluid at relatively low temperatures and pressures. A dense solvent at relatively low temperature is unlikely to damage temperature sensitive, labile compounds. The need for only modest pressures to achieve a dense solvent is convenient and does not impose a significant technical or energy penalty. However, The density of CO_2 changes over a wide range with changes in temperature and pressure. At 40 °C, most of the change in density occurs over only a narrow range of pressure between about 70 and 110 bar. Operation in this region means that small changes in pressure produce large changes in density and retention. As the temperature is increased, the curves tend to flatten out, creating a shallower gradient of density against pressure. Most pressure programming has been done at elevated temperatures since it is easier to make small changes in density on a shallower slope.

1.5.5 Safety

CO_2 is not toxic at low concentrations, however, at high concentrations it can be lethal. Cylinders of high-pressure CO_2 are commonly used in restaurants and cafeterias for carbonated drinks. Many fire extinguishers also contain high-pressure CO_2 and are widely distributed throughout factories and office buildings. The concentration of CO_2 in properly designed SFC laboratories is much lower than is typical in occupied conference rooms or theatres. Large quantities of CO_2 are seldom stored directly in the laboratory, so large-scale escapes are unlikely. CO_2 is denser than air and can tend to accumulate near the floor of poorly ventilated spaces. Sensors and alarms should be mounted near waist level. Oxygen sensors are not necessary since in any potentially dangerous situation the oxygen level is likely to be near normal, even when there is a dangerous level of CO_2. When using CO_2 as the primary component in the mobile phase, it is almost impossible to get the mobile phase to burn.

1.6 Conclusions

SFC appears to be a suitable technique for the efficient and reproducible separation of chemicals in environmental samples, including wastewater, surface water, and groundwater. Compared to conventional LC methods, the use of supercritical fluids allows for faster analysis to be achieved, and largely reduces the use of organic solvents, resulting in lower costs and waste production. SFC separates solutes within a polarity range that considerably exceeds that of individual LC techniques, i.e., it is comparable to that obtained by coupling HILIC with RPLC. However, additional parameters that come into play when supercritical fluids are used (e.g., backpressure and compressibility of the mobile phase) may result in more laborious method development. Nevertheless, SFC uses the same columns that are commonly used in LC, and the integration of a SFC system in typical instrumental laboratories does not seem particularly challenging in terms of infostructure and safety requirements (i.e., SFC operational characteristics are between those of GC and LC, and typical pressures do not exceed those of UPLC). Applications of SFC for the detection of very polar compounds are attractive, however, for these compounds results suggest that SFC may be complementary rather than more performing than HILIC, i.e., SFC can measure compounds that are not detectable by HILIC and vice versa.

2 SFC in practice

2.1 Experimental approach

The experimental work was carried out at the University of Amsterdam (UvA), in collaboration with the group of Dr Andrea Gargano, where a SFC-UV apparatus was available for testing. The approach involved the following steps: (i) the selection of suitable compounds, performed in consultation with partners from the drinking water companies and laboratories, (ii) the selection of the chromatographic column and preliminary assessment of the separation, (iii) optimization of the separation, and (iv) hyphenation with MS (Figure 7).

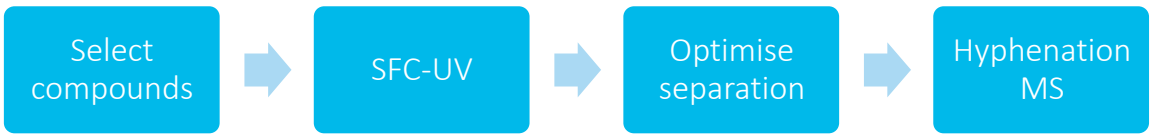


Figure 7. Schematic of the experimental approach.

2.2 Chemicals and reagents

Methanol absolute (MeOH) ULC/MS – CC/SFC grade and acetonitrile (ACN) LC-MS grade were purchased from Biosolve (Valkenswaard, The Netherlands), and ultrapure water (18.2 MΩ cm) was obtained using a Sartorius Arium 611UV system (Göttingen, Germany). Carbon dioxide (CO_2, purity 4.6) was purchased from Nippon gases (Vlaardingen, The Netherlands). Ammonium formate (AmF, ≥ 99%), ammonium acetate (AmAc, ≥ 99%) and formic acid (FA, ≥ 98%) were all purchased from Sigma Aldrich (Darmstadt, Germany). Oxypurinol (93.7%) and maleic hydrazide (99.87%) were obtained from LGC Dr. Ehrenstorfer (Augsburg, Germany). 1,5-naphthalenedisulfonic acid tetrahydrate (96.5%), pyrazole (97.5%), poly(melamine-co-formaldehyde) methylated solution, maleimide (≥ 98.5%) and melamine (98.5%), bisphenol-A (≥ 99.0%), cotinine (analytical standard, ≥ 98.5%) and paraquat dichloride hydrate (analytical standard, ≥ 98.0) were obtained from Merk Life Science (Darmstradt, Germany). 5-fluorouracil (≥ 99.0%) was obtained from TCI Europe (Zwijndrecht, Belgium). Cyanuric acid (≥ 97.5%) was obtained from Acros Organics (Geel, Belgium). Glycerol was obtained from Boom (Meppel, The Netherlands). 1,4-dioxane, (aminomethyl)phosphonic acid, desphenyl chloridizon, diglyme, tetraglyme, glyphosate and sucralose were all obtained in-house from the KWR water research institute (Nieuwegein, The Netherlands).

2.3 Compounds selection

The performance of SFC was investigated using compounds that are typically challenging to measure by conventional RPLC. The polarity range of these compounds varied from -4.70 to 3.32 (Table 2). These analytes were grouped into three categories based on their respective logP values according to Kah and Brown (2008): very polar logP < -2.5, polar -2.5 < logP < 2.0, non-polar logP > 2.0. These compounds were selected to cover a suitable range of polarities and included substances indicated by the drinking water companies and laboratories as relevant for the drinking water sector and particularly challenging to determine with conventional analytical approaches. Care was taken to ensure that at least 10 compounds were UV active.

Table 2: Compounds selected for SFC-MS testing.

Compound	pKa	logP	Exact Mass (Da)	Ion Observed	Category
(Aminomethyl)phosphonic acid	2.35, 5.90, 10.80	-4.70	111.0085	N.D	Very polar
Paraquat	11.00	-4.22	186.1157	N.D	Very polar
Glyphosate	2.34, 5.73, 10.20	-3.40	169.0140	N.D	Very polar
Maleic hydrazide	5.62	-1.96	112.0273	$[M+H]^+$	Polar
Cyanuric acid	6.88, 11.40, 13.50	-1.95	129.0174	$[M-H]^-$	Polar
Glycerol	14.40	-1.76	92.0473	N.D	Polar
Oxypurinol	6.25	-1.72	152.0334	$[M+H]^+$	Polar
Melamine	5.00	-1.37	126.0654	$[M+H]^+$	Polar
Sucralose	12.52	-1.00	396.0146	$[M+NH_4]^+$ / [M-H]-	Polar
1,5-napthalenedisulfonic acid	2.40	-0.94	287.9762	$[M-H]^-$	Polar
5-fluorouracil	8.02	-0.89	130.0179	$[M-H]^-$	Polar
Maleimide	8.52	-0.76	97.0164	N.D	Polar
Tetraglyme	-3.50	-0.70	222.1467	$[M+Na]^+$	Polar
Diglyme	-3.70	-0.36	134.0943	$[M+H]^+$	Polar
Desphenyl Chloradizon	3.38	-0.30	145.0043	$[M+H]^+$	Polar
1,4-dioxane	-3.90	-0.27	88.0524	N.D	Polar
Cotinine	4.80	0.07	176.0950	$[M+H]^+$	Polar
Poly(melamine-co-formaledhyde)	7.01	0.18	432.8400	N.D	Polar
Pyrazole	2.48	0.26	68.0374	N.D	Polar
Valsartan	4.73	1.50	435.2270	$[M+H]^+$/ $[M-H]^-$	Polar
Valsartan acid	4.73, 3.9	2.3	266.0804	$[M+H]^+$/ $[M-H]^-$	Non-polar
Bisphenol-A	9.60	3.32	228.1150	$[M-H]^-$	Non-polar

pKa, logP, and exact mass were taken from PubChem. N.D = not detected.

2.4 Sample preparation

Standard solutions were prepared in MeOH to a working concentration of 1 mg/mL. When necessary, sonication (30 minutes at 30 °C) was applied to fully dissolve the chemicals. Substances were injected individually and as a mixture. The effect of the addition of water to the chromatographic separation and peak shape of the compounds was investigated by preparing a series of mixtures of organic solvent and water (i.e., 90/10, 80/20, 70/30, 60/40 and 50/50 organic:water) obtained by adding MeOH or ACN to a water solution containing the desired chemical mixture at a concentration of 0.5 mg/mL.

2.5 Supercritical fluid chromatography and mass spectrometry setup

2.5.1 SFC-UV

The SFC setup comprised a Waters Acquity UPC^2 (Waters, Milford, United States) equipped with a diode array detector. This system was initially used to investigate chromatographic separation, this allowed for a fast orientation method on the chromophoric compounds included in the selection. In a later stage. Traditional DAD flowcell are rated up to 70 bar and cannot be used for SFC since it is below the supercritical point of CO_2. That is why a special a flowcell which can withstand higher pressures is needed. In a later stage, the detector was replaced with a mass spectrometer to improve the detection of all compounds. The wavelengths selected for measurements were 215 and 254 nm as well as scan mode from 200-300 nm. The column used for separations was the Acquity Virdis® BEH (3.0 mm I.D x 100 mm, 3.5 µm). For all analyses a binary linear gradient was used consisting of CO_2 and 95/5 MeOH/H_2O + 50 mM AmF (v/v, modifier) (Table 3). The column temperature was kept constant at 40°C, the auto backpressure regulator (ABPR) held at 120 bars, and a flow rate of 0.500 mL min^{-1} was used for all separations. The injection volume was 5 µL. These initial parameters and conditions were adapted from Desfontaine et al. (2017).

Table 3: SFC-MS linear gradient.

Time (min)	CO_2 (A, %)	AmF modifier (B, %)
0.00	98	2
1.00	98	2
16.00	0	100
17.00	0	100
17.01	98	2
18.50	98	2

2.5.2 SFC-MS

The flow from SFC separation and make-up flow were first mixed in a T-piece and then supplied to a quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Compact QTOF, Bruker Daltonics, Bremen, Germany, introduced in 2008), through another T-piece, in which the majority of the flow was directed to the MS and the other split to the ABPR (Figure 8). This required a flow gradient for the make-up flow to ensure that the effluent supplied to the MS consisted of 100% organic modifier (Table 4). The composition of the make-up flow was also tested using pure MeOH and 90/10 MeOH/H_2O (v/v) + (i) 0.1% FA, (ii) 50 mM AmF and (iii) 50 mM AmAc.

All samples were analyzed both in positive and negative mode using electrospray ionization (ESI) source. A mass range of 50-1500 m/z, end plate offset of -500 V, nebulizer gas of 3.9 bar, dry gas flow of 8.9 L/min, dry temperature of 220°C was used for both polarity modes. A capillary voltage of +3500 V and -3400 V was used for positive and negative ion mode respectively. For calibration of the TOF system a calibration mixture of 90/10 IPA/H_2O + 10 mM sodium formate for which a calibration score exceeding 98.0% was accepted and used.

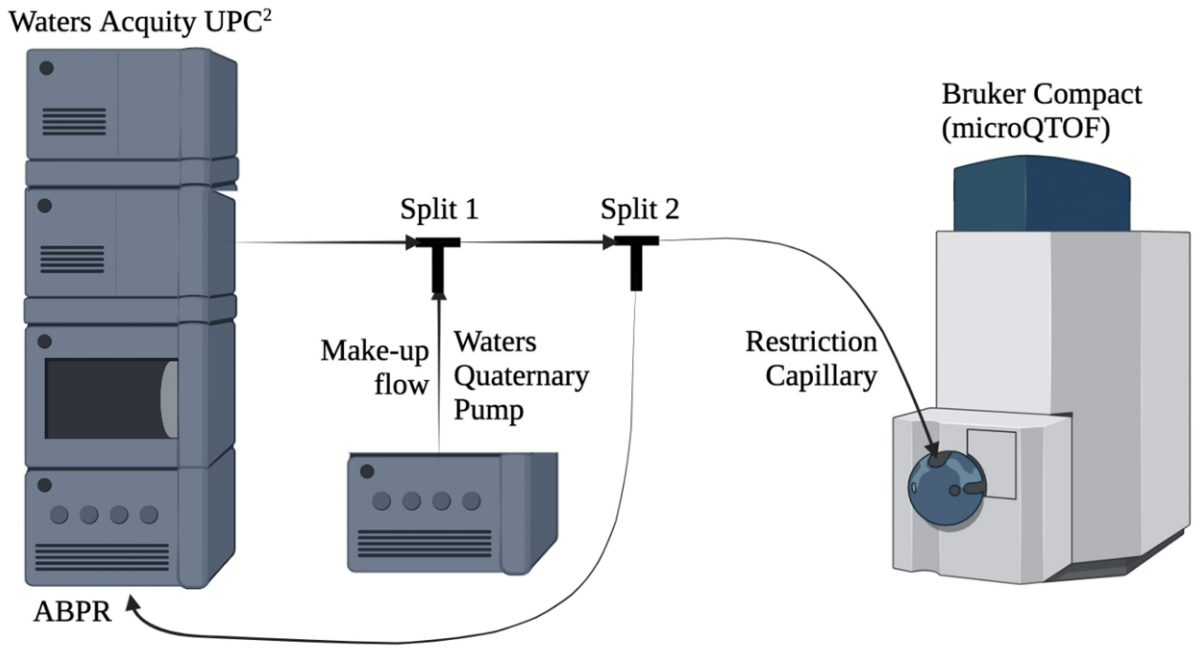


Figure 8. Schematic representation of the SFC-MS setup.

Table 4: Make-up flow gradient. The CO2 and modifier B flow rate are obtained from Table 3.

Time (min)	CO_2 Flow Rate (mL min^{-1})	Modifier Flow Rate (mL min^{-1})	Make Up Flow Rate (mL min^{-1})
0.00	0.490	0.010	0.490
1.00	0.490	0.010	0.490
16.00	0.000	0.500	0.000
17.00	0.000	0.500	0.000
17.01	0.490	0.010	0.490
18.50	0.490	0.010	0.490

2.6 Data Processing

Chromatographic data from the SFC was obtained and processed using Empower 3 Software (version 7.30). Mass spectrometric data was obtained and processed using Bruker Compass DataAnalysis 1.3 (version 4.0 SP4). From Bruker Compass DataAnalysis, extracted ion chromatograms (XIC) were used in order to obtain retention times, peak area/intensity, S/N ratios, and to visualize mass spectra to calculate mass accuracy. The tailing factor (T_f), which provides a measure of the tailing effect of a peak, was calculated using

$$T_f = \frac{a+b}{2a} \qquad \text{Eq. 1}$$

where a and b are the front and back width of the peak at 5% of the peak height, respectively, and 2a assumes a perfect Gaussian and symmetrical peak.

3 Results and discussion

3.1 Chromatographic separation

A total of 14 compounds were detected by SFC-MS, including 12 polar compounds and 2 non-polar compounds (Figure 9; Table 2). None of the very polar compounds were detected. The retention times ranged between 1 and 11 min over a 18.5 min gradient run. In SFC it is expected that non-polar analytes elute at the beginning of the gradient when the CO_2 composition is high and more polar elute later when modifier composition increases. However, this trend was not observed as the least polar compounds, valsartan acid and bisphenol-A, did not elute before the other more polar compounds (Figure 10). In addition, the range of polarities found within the polar category also deviated from the expected retention mechanisms in SFC. It should be noted that for some of the analytes it may be difficult to accurately produce XIC due to the relative low abundance of the ions. However, overlap between retention times of compounds with different polarity was also observed by Bieber et al. (2017), where the elution order expected for SFC was confirmed following the analysis of a much greater number of compounds than here (Figure 4).

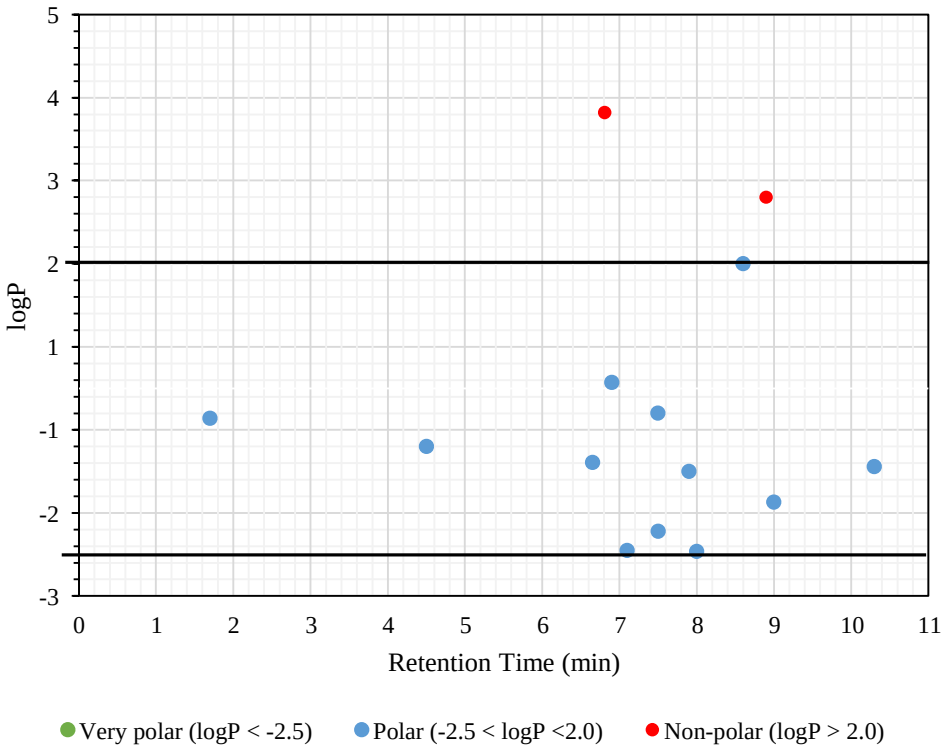


Figure 9. Retention time vs logP for detectable analytes in both positive and negative ion mode (Table 2). This data was obtained using MeOH as a make-up flow. Separation was done on a Acquity Virdis® BEH (3.0 mm I.D x 100 mm, 3.5 µm) Black lines indicate the cut-off points for each logP category (Table 2).

For (Aminomethyl)phosphonic acid (AMPA) and glyphosate, two of the three very polar compounds investigated in this study, the lack of detectability could be a result of the sample preparation used. These two compounds are highly soluble in pure water, however, when organic solvent are added immediate precipitation is observed. This was tested by dissolving each compound in water and progressively adding MeOH or ACN in 10% increments. Results showed that even at an organic solvent percentage as low as 30% the analytes precipitated. The amount of water injected in

SFC is typically < 50% (Section 1.3.2), and this may contribute to poor solvation of AMPA and glyphosate in the injection solvent which results in lack of detection.

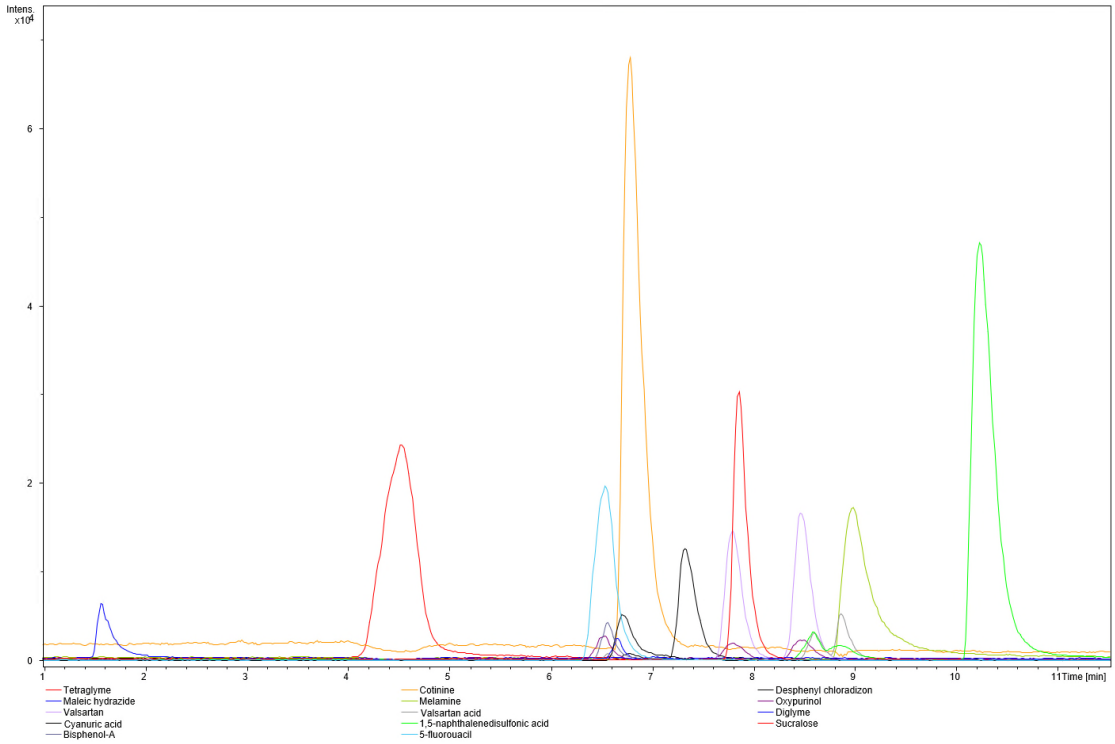


Figure 10. XIC of all 14 detectable analytes using MeOH as the make-up flow in positive and negative ion mode. Legend: burgundy is tetraglyme, light blue is desphenyl chloridizon, light green is melamine, magenta is valsartan, purple is oxypurinol, black is cyanuric acid, turquoise is 5-fluorouacil, grey is cotinine, blue is maleic hydrazide, red is sucralose, orange is valsartan acid, lime is 1,5-naphthalenedisulfonic acid and pink is bisphenol-A.

The remaining six analytes that could not be detected were pyrazole, 1,4-dioxane, glycerol, maleimide, paraquat and poly(melamine-co-formaledhyde. The age of the QTOF instrument (2008) and the fact that it was not optimized for the really small molecules was one of the reasons. Pyrazole, 1,4-dioxane, glycerol and maleimide are relatively small molecules (68.0374, 88.0524, 92.0473 and 97.0164 Da respectively), for which poor ionization efficiency and sensitivity. Both positive and negative ionization modes resulted in no detection, even when tautomerization reactions were considered (Sarkar et al., 2019). For pyrazole, the use of atmospheric pressure chemical ionization as an ion source may be a suitable alternative to ESI as it significantly increases the ionization efficiency, especially for small molecules containing a pyrazole group (Jiang et al., 2022). However, it has been reported that ESI can also successfully ionize pyrazole, but in conditions that are not suitable for SFC (Abdighahroudi et al., 2020). For 1,4-dioxane, GCMS is typically the analytical approach of choice.

Paraquat has a molecular mass of 186.1157 Da when singly charged, however considering the permanent 2^+ charge it should be detected at a mass of 93.0578 Da, which is a difficult mass range to detect ions. This is a similar issue that was experienced for pyrazole with low mass detectability. The singly charged ion was expected, but not observed (Tsao et al., 2016). Another possible factor contributing to loss of detection of paraquat is poor elution from the column, despite including 100% organic solvent in the elution gradient. This would indicate that the elution strength of the mobile phase is insufficient to elute this very polar compound during the separation.

For poly(melamine-co-formaledhyde), the lack of detection can be attributed to both low sensitivity as well as poor separation. For this polymer, the separation nor MS were optimized. In addition, the mass spectra deviated from what is expected when analyzing a polymer, i.e., no polymer distribution was observed in MS. For this reason, as well as the broad and coeluting peaks, poly(melamine-co-formaledhyde) was not further investigated.

Valsartan and valsartan acid showed the most intense signal in the XIC and underwent complete in-source fragmentation (Figure 11), resulting in the complete loss of the protonated monoisotopic ion. This was most likely caused by excessively high source temperature for these compounds. This also hinders the quantifying on MSMS signal if the in-source fragmentation is not always constant. Overall, satisfactory separation was achieved for the remaining compounds.

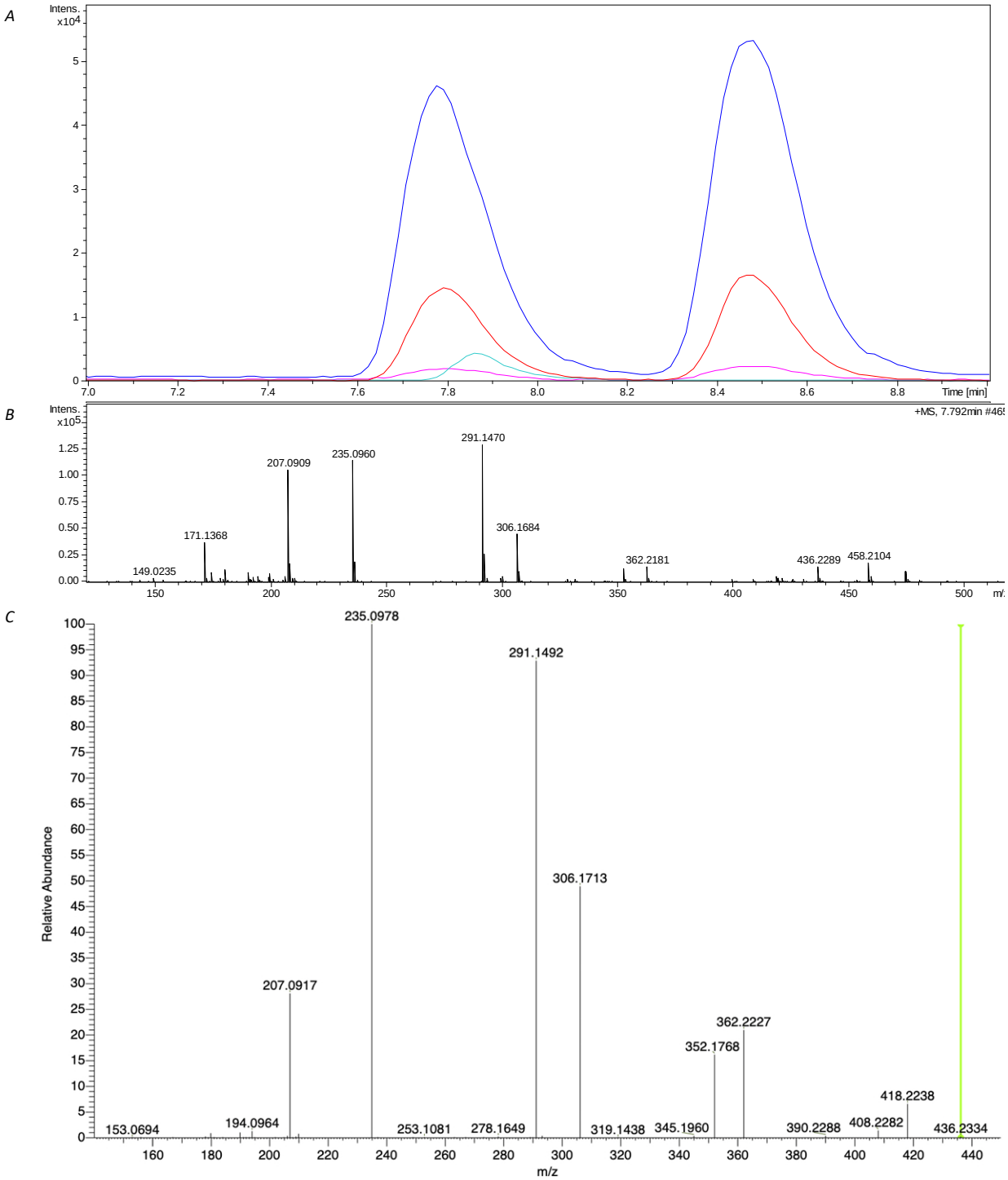


Figure 11: (A) XIC from positive ion mode data using MeOH as make-up flow. (B) Mass spectra of valsartan showing in-source fragmentation, [M+H]+ peak at 346.2289 m/z. (C) MS/MS spectra of valsartan taken from mzCloud, green line is the monoisotopic peak. Note: XIC found in (B) was recorded with a mass error of 13.7 ppm at m/z 436.2289. Legend: red, blue, pink and turquoise are all valsartan but are found in the XIC of 1,5-naphthalenedisulfonic acid, oxypurinol and sucralose respectively

3.2 Make up flow composition

The make-up flow solutions were prepared in 90/10 MeOH/H_2O (v/v) using 0.1% FA, 50 mM AmF and 50 mM AmAc (Figure 12). Compared to pure MeOH (Figure 10), there was a 5-fold decrease in intensity across the MS signal for most analytes. This was unexpected as the post-column infusion of additives typically improves ionization efficiency (Grand-Guillaume Perrenoud et al., 2014). In positive ion mode, the addition of FA and AmAc to the make-up flow had similar effects in terms of sensitivity. This change in intensity may be explained by the addition of salts that could suppress the signal and was particularly noticeable for cotinine in AmAc. For the remaining 13 analytes, the intensity reduction observed was the same for both compositions.

The addition of AmF significantly suppressed all analytes except for tetraglyme (Figure 12). It appears that a small peak could be ascribed to melamine, however, when looking at the MS spectrum the expected m/z is not distinguishable from the noise. In addition, when the m/z value is selected for which melamine is expected an error of 89.5 ppm is obtained, further confirming that melamine is not detectable. However, the large suppression is not observed for tetraglyme and the intensity is even 5-fold higher compared to pure MeOH (Figure 10). Generally, for the SFC-MS analysis of metal ion clusters such as $[M+Na]^+$, signal suppression is expected (Haglind et al., 2018). However, it appears that when AmF is added post-column and the overall AmF concentration directed to the MS increases this effect is not observed and the sensitivity rather increases.

Similar to positive ion mode, also for negative ion mode the make-up flow compositions of FA and AmF there is a 5-fold reduction in the intensity (Figure 12; Figure 10). When comparing between the two compositions, there seems to be a flip in the intensities between 5-fluorouracil and bisphenol-A. In FA 5-fluorouracil has a higher intensity than bisphenol A, whereas in AmAc bisphenol-A has a higher intensity (Figure 12). Therefore, it can be concluded that FA promotes and increases the ionization efficiency of 5-fluorouracil, whereas AmAc increases that of bisphenol-A. However, intensities are consistently lower than those obtained using pure MeOH as a make-up flow solvent (Figure 10).

For cyanuric acid (black) there is no signal detection for the analyte, which is to be expected as for all other make-up flow compositions the signal was very low. In addition, for the remaining three analytes, 1,5-napthalenedisulfonic acid, 5-fluorouracil and bisphenol-A, the signal intensity is also reduced, especially for 1,5-napthalenedisulfonic acid (green). Here the intense peak at 10.8 minutes is reduced to a peak that falls within the noise. All this is in line with what was previously described, as there is a higher salt concentration going to the MS, which in turn suppresses the majority of the signal.

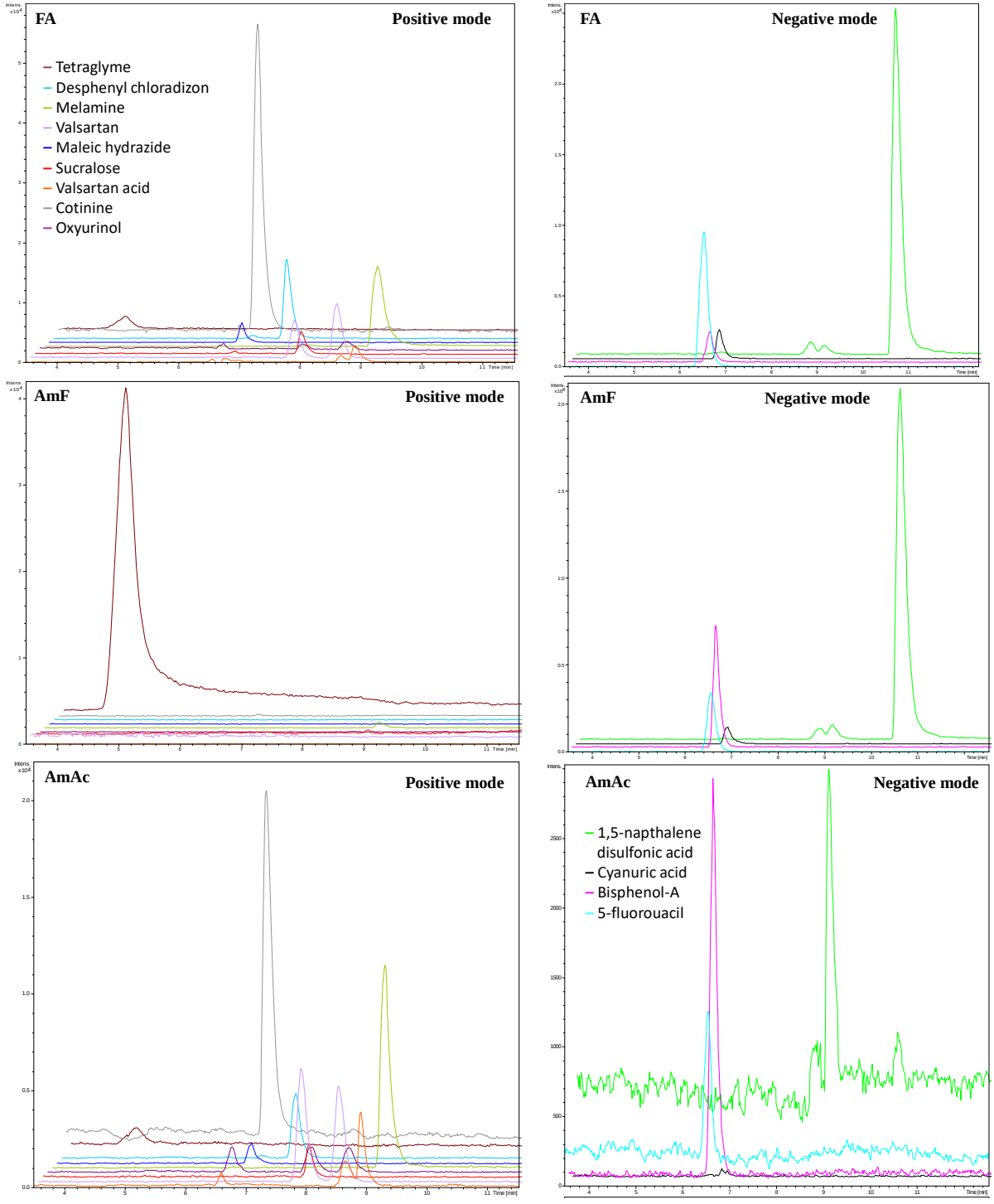


Figure 12: Summary of stacked XIC of different make-up flow additive compositions. Three make-up flow compositions were tested, 0.1% formic acid, 50 mM ammonium formate and 50 mM ammonium acetate.

3.3 Injection of water samples

For the analysis of water samples, the injection of more water is a critical aspect in terms of reducing the tedious sample preparation of real water samples. Therefore, to evaluate how much water content can be injected for the developed SFC-MS, different compositions were investigated both with MeOH and ACN. Injecting high amounts of water, such as 50:50 (v/v, organic/water), causes significant band broadening due to miscibility issues and solubilizing with SFC mobile phases (Desfontaine et al., 2017). These effects come with higher injection volumes as more water is then needed to mix with the mobile phase. To highlight this, examples for valsartan and cotinine are shown (Figure 13).

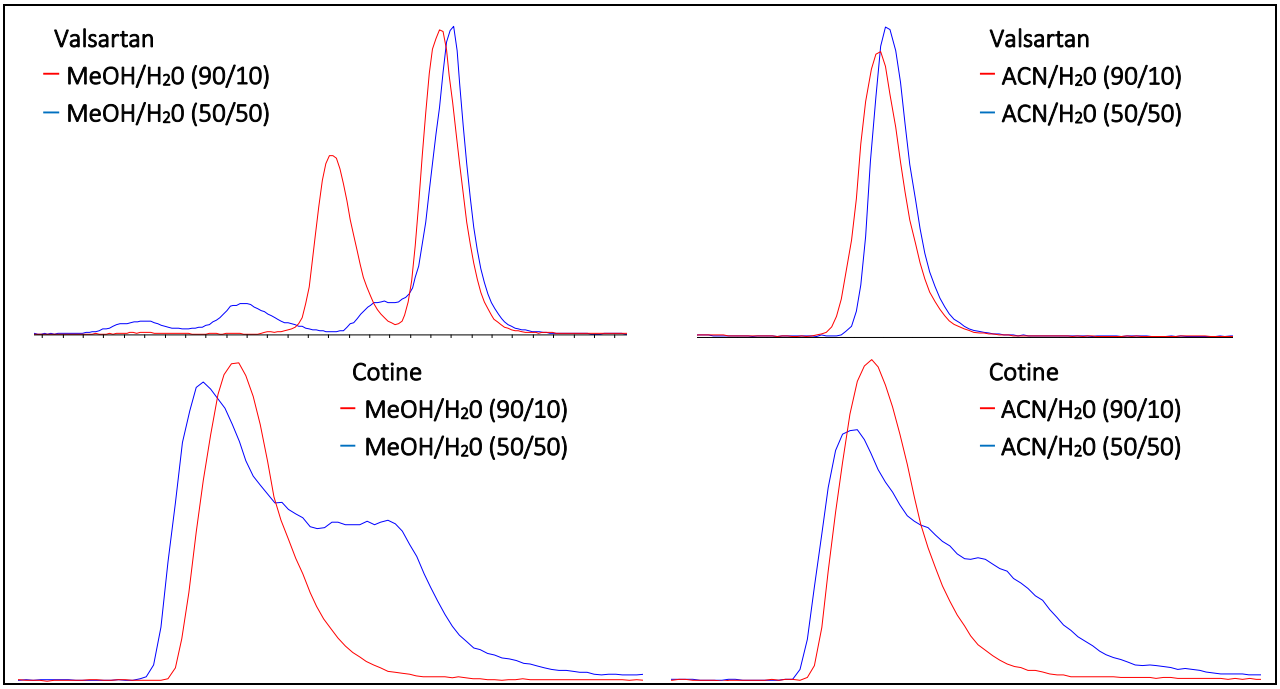


Figure 13: XIC of valsartan in MeOH (A) and ACN (B), and cotinine in MeOH (C) and ACN (D). Red and blue curves refer to injections at 90/10 (v/v) organic/H_2O and 50/50 (v/v) organic/H_2O, respectively.

For valsartan, an improvement in peak shape from MeOH to ACN is observed. Peak splitting occurring for 10% water was not observed when injecting 50% water, however, the peak shape was distorted and chromatographic separations impaired. When switching to ACN as injection solvent, no peak splitting was observed and in both cases the peak shape is significantly improved compared to MeOH. When injecting 50% water, a moderate increase in the signal intensity relative to 10% water was observed. For cotinine, both MeOH and ACN with 10% water content produced a good peak, although some tailing was observed. In contrast, when switching to 50% water injection the chromatographic separation was severely hampered and caused significant tailing for both solvents. Overall, for both compounds, the use of ACN resulted in good peak shapes. Tailing factors relative to the use of different organic solvents (i.e., MeOH and ACN) and water (10 and 50%) content are shown in Figure 14. It should be noted that the percentage of water was recorded from 10% to 50% in steps of 10% (10, 20, 30, 40, 50% H_2O), however, only the two extremes are reported here for comparisons.

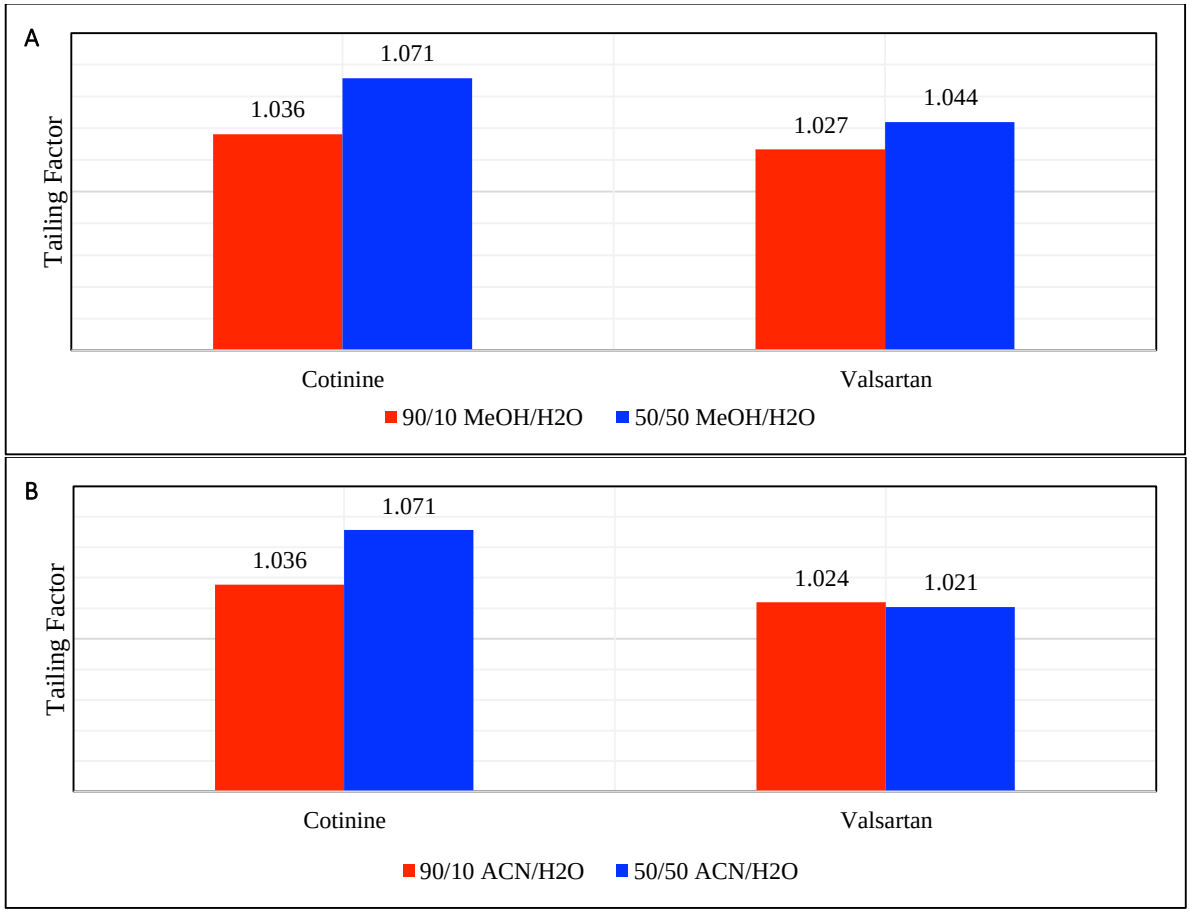


Figure 14: Tailing factors calculated from XIC found in Figure 9 for valsartan and cotinine in MeOH (A) and ACN (B). Legend: red is injections at 90/10 (v/v) organic/H2O and blue is injections at 50/50 (v/v) organic/H2O

3.4 Detection

The relatively old microQTOF used for most of the analyses offered a lower ion transmission, which may not have sufficed for some of the analytes to be detected. In a later stage, the more recent instrument Orbitrap Q Exactive plus became available and was used for hyphenation with the SFC. Direct infusion of the standards showed a sufficient ionization for the majority of compounds. Hence the decision was made to transfer the SFC equipment to the Q Exactive.

3.5 Effect of the mobile phase

The Q Exactive was used to test different columns and modifiers (MeOH and ACN). For this experiments the instrumental conditions as described in chapter 2 were used except that for HILIC (HILIC ATLNTIS) and RPLC a different column was used matching the dimensions of the SFC column. Tests were performed using the setup shown in Figure 15. The columns used included:

1 HILIC (Figure 16)
2 Traditional RPLC (Figure 17)
3 SFC with Methanol (Figure 18
4 SFC with Acetonitrile (Figure 19)

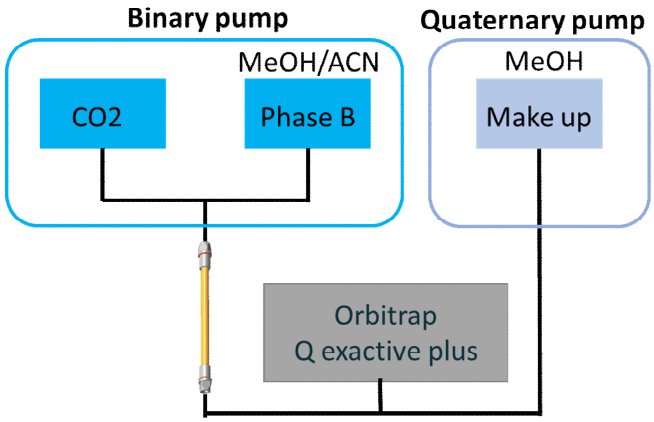


Figure 15: Setup with the Orbitrap Q-exactive

The HILIC approach (Figure 16) showed early eluting compounds with sub optimized peak shapes.

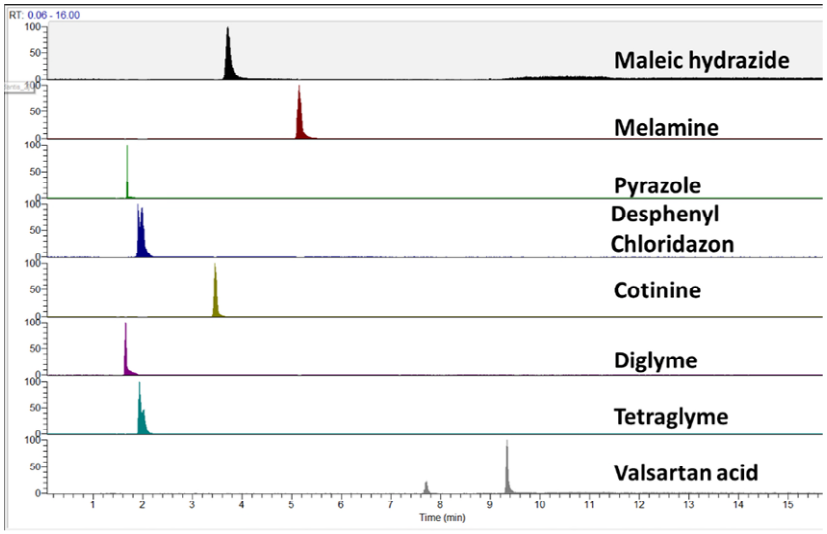


Figure 16: Test mix HILIC method.

When the setup was switched to a RPLC method this resulted in even shorter retention times or almost no retention at all.

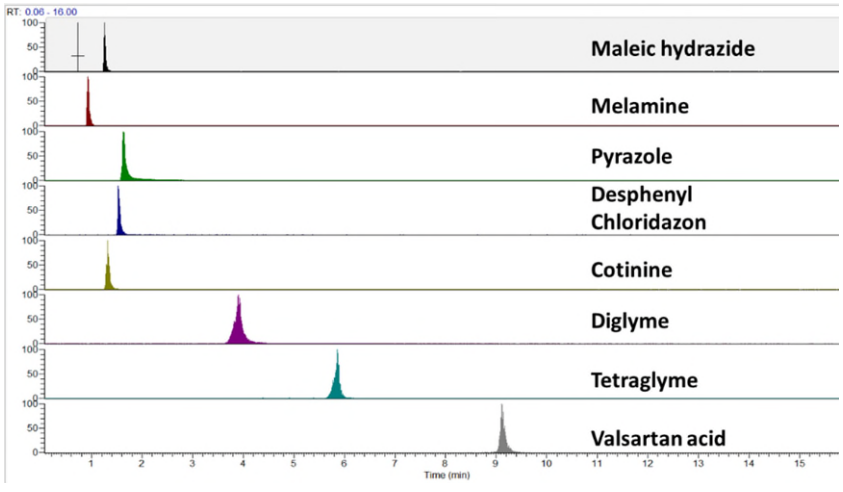


Figure 17: Test mix RPLC method.

When the setup was switched to a SFC method with methanol as a modifier retention improved, but for some compounds the peak shape worsened.

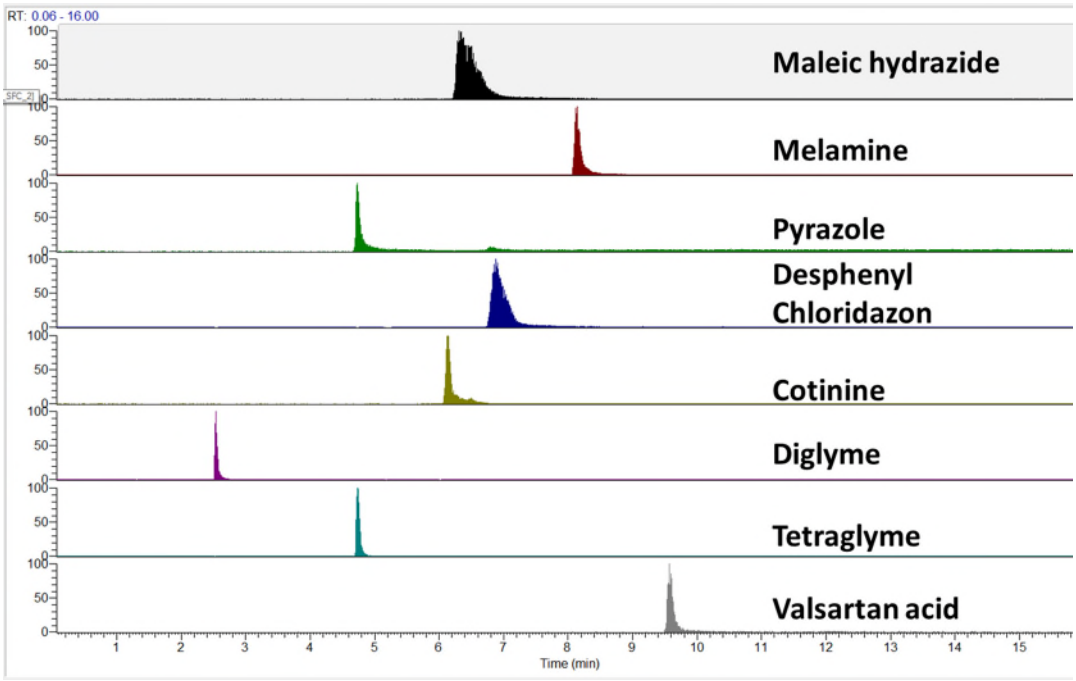


Figure 18: Test mix with SFC (MeOH) method.

When using SFC with ACN as a modifier both the shape of the peaks and separation improved.

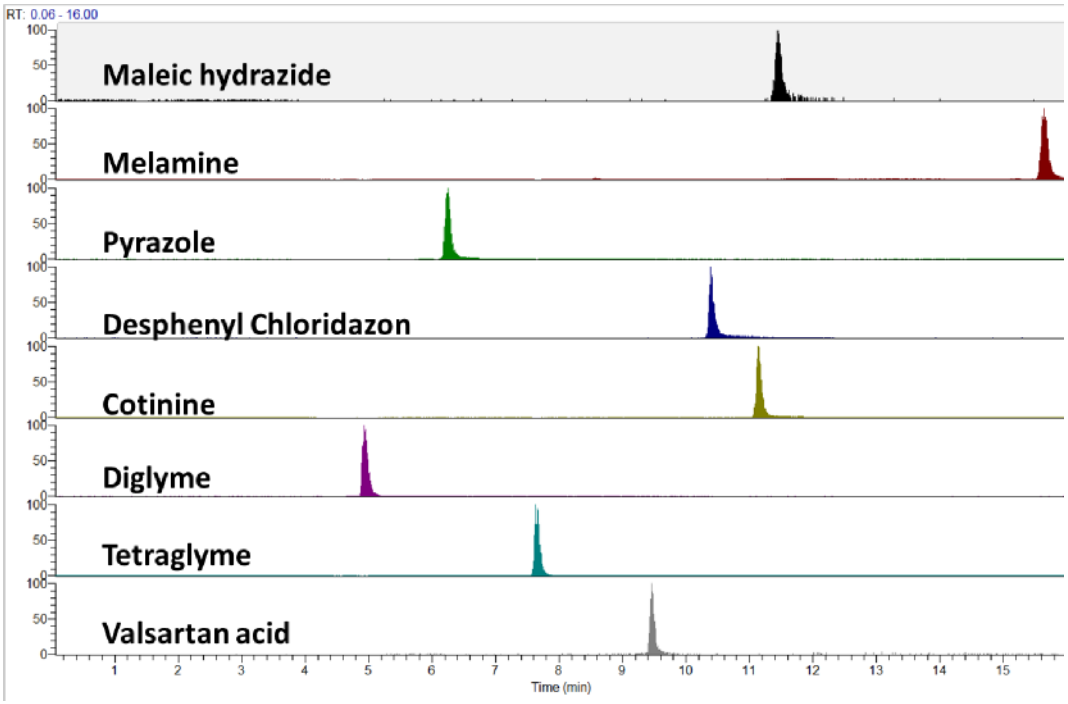


Figure 19: Test mix with SFC (ACN) method.

The hyphenation with a more recent accurate mass spectrometer showed to be much more promising. Moreover the switch to ACN as a modifier overall increased retention time but showed a much better general separation and peak shape. The separation achieved can also help to reduce matrix suppression. This was not further studied to what extend it can influence ionisation but should be explored in further detail.

3.6 The combination of supercritical and HILIC

With SFC the really high polar compounds such as Glyphosate and AMPA are not eluting. This can be due to several reasons, including the effect of the water content. Using a light amount of water in the SFC separation will have a negative affect on the peak shape or prevent a proper elution from a compound. For some compounds it will not be a problem but even injecting a few microliters of water will deteriorate peak shape (see chapter 3.3). The switch to acetonitrile as an injection solvent (3.3) and as a modifier (3.5) has shown to be effective. An interesting solution to tackle all these together is to use a ternary gradient which essentially combines SFC with HILIC (Baker et al., 2017)(Figure 20).

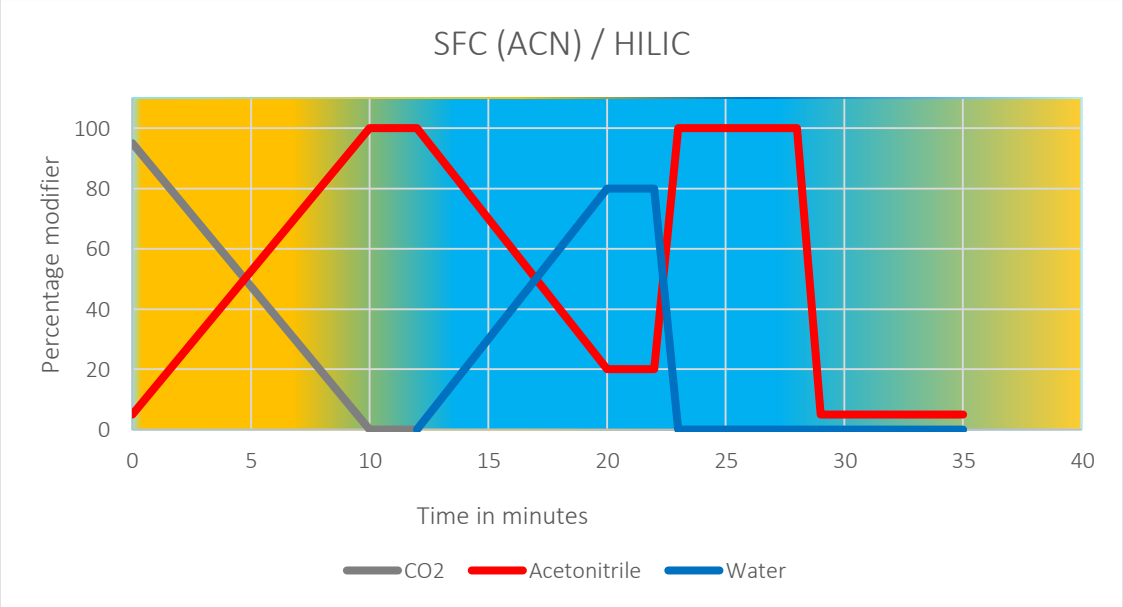


Figure 20:Ternary gradient used. In Yellow the SFC part and in Blue the HILIC part of the separation.

The first 12 minutes are true SFC and no water is used in the gradient process. At the next step a gradient is started when water is added and the CO_2 is finished. Essentially a HILIC gradient is started and the best of both worlds are combined. The first exploratory experiments using SFC ternary gradient and Orbitrap Q-Exactive are shown below. Using this approach, even a very polar compound such as paraquat was eluted (log Kow -4.22) (Figure 21).

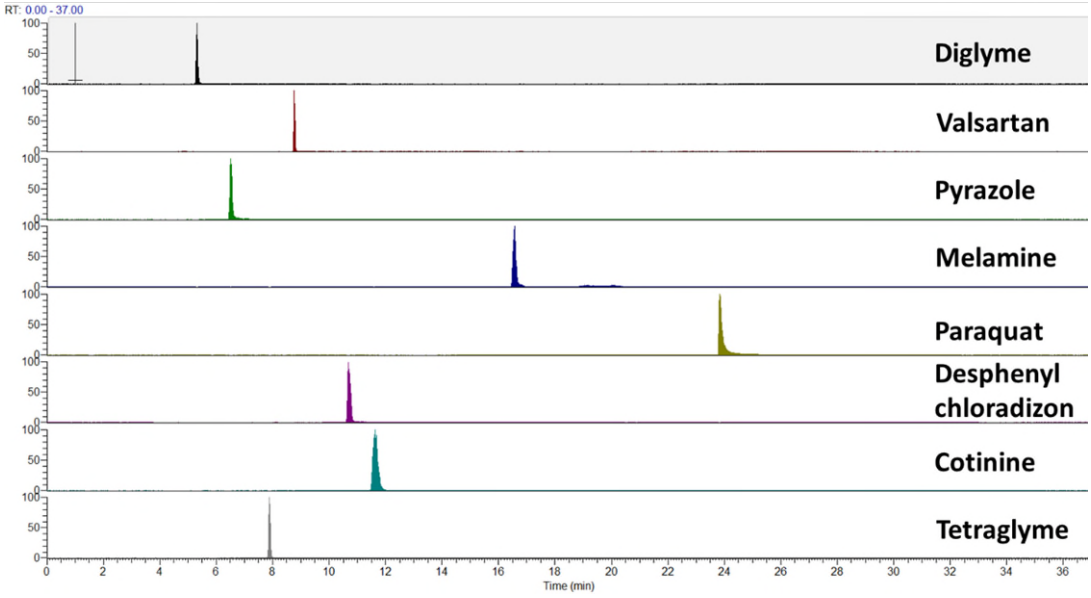


Figure 21: SFC (ACN)-HILIC ternary gradient preliminary experiments (positive mode).

In the negative mode even glyphosate and AMPA are eluted but peak shape is far from ideal.

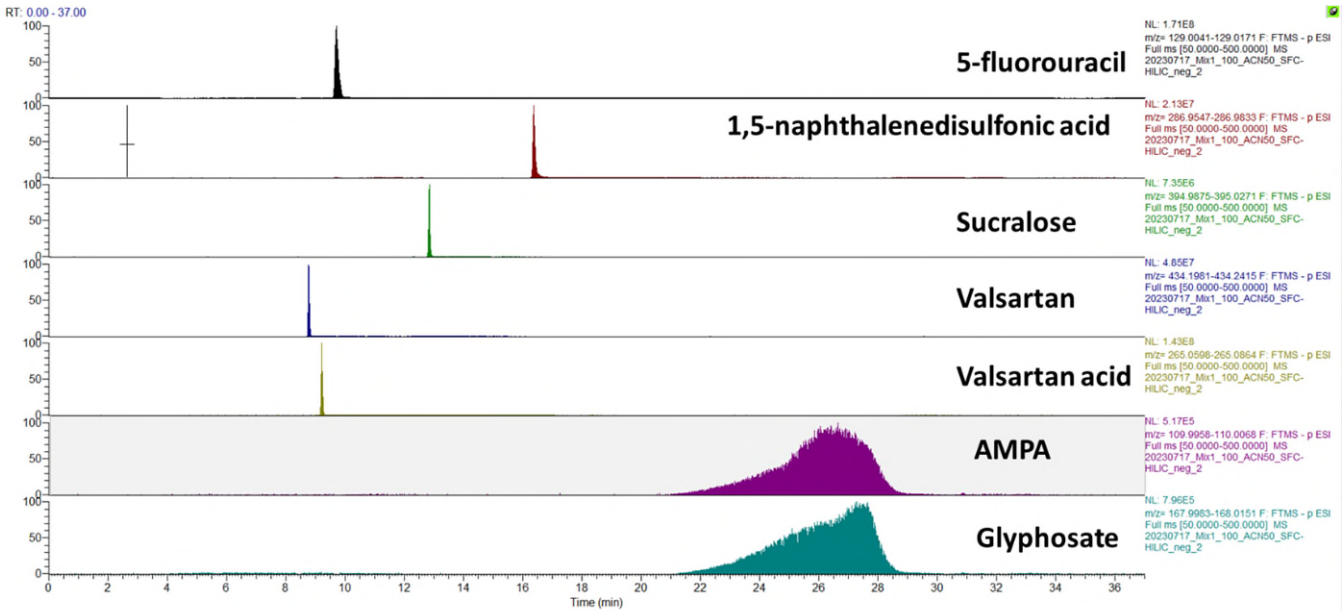


Figure 22: SFC-HILIC ternary gradient preliminary experiments (negative mode).

These promising results show opportunities for further exploration for a combination of SFC(ACN)/HILIC-MS setup. Especially since a large volume direct water injection is not possible with SFC and to achieve lower detection limits it is necessary to explore different sample handling techniques to increase the concentration factor.

4 Conclusions

The aim of this research was to gain insight into how and if SFC can be of an added for the analysis of water samples to determine polar contaminants. The majority of analytes, 14 out of 22, found in Table 1 were successfully detected and separated using the developed SFC-MS method encompassing a broad polarity range from apolar to polar. The 8 analytes that could not be detected due to several reasons:

- Sensitivity
- Equipment used
- Initial setup conditions

Several crucial steps were investigated with the set of selected compounds.

Makeup-flow

The impact of make-up flow composition was tested and it was found that the use of pure MeOH provides the best results and sensitivity post-column for ionisation of the set of analytes. Additions to the methanol such as FA, AmF and AmAc, which are typically used in SFC-MS setups, yielded mixed results but generally drastically decreased the intensity of the signal and ion suppression effects were observed.

Injection of water

The percentage of water that could be used as a diluent and injected onto the column was investigated in order to reduce sample preparation and handling when working with water samples. It was found that using ACN as organic diluent allows for injections of water (10-50%) without suffering in peak shape, intensity, area and MS sensitivity. This is a striking finding as traditional chromatography used for polar analytes, hydrophilic interaction liquid chromatography (HILIC), typically requires 95/5 ACN/H_2O (v/v). With the developed method the injection of up to 50% water is possible illustrating a clear advantage over using HILIC, although the total injection amount with this current setup was not exceeding 5 µL. This indicates that the setup is very sensitive to changes in water percentage.

Modifier use

Exploratory experiments with a slightly different setup and MS detector showed that using ACN (aprotic solvent) as a modifier enhanced the overall separation in contrast to MeOH (protic solvent). This will give a clear advantage when a suitable sample preparation and handling technique is chosen, and the final extract can be directed to 100% ACN content.

In conclusion, the most efficient setup for SFC-MS shows that the modifier ACN gave the best chromatographic separation, and the makeup flow should be 100% MeOH to improve ionization. Injection can be performed using 10-50% water, but effects on peak shape are compound dependent. ACN is preferred over MeOH as an injection fluid. The combination of SFC and HILIC seems very promising to combine the best of both worlds together.

5 Future Perspectives

The current approach for analyzing very polar compounds in water samples is to use HILIC or mixed-mode chromatography, depending on the compounds investigated. HILIC is essentially normal phase chromatography but with water miscible solvents. Mixed-mode chromatography is a reversed phase column with both positive and negative charged groups. With HILIC, permanently negatively charged compounds cannot be analyzed, and that is why mixed-mode chromatography is used. Both methods present limitations: HILIC injection solvent needs to be 95% acetonitrile and with mixed-mode robustness of the method is a problem, i.e., slight changes in ionic composition of a method result in effects on the analysis. In both methods, sample concentration will result in more matrix interference.

In this study, SFC-MS successfully covered a wide polarity range, however, very polar compounds such as glyphosate, paraquat and (Aminomethyl)phosphonic acid could not be detected. Yet, SFC in combination with HILIC proved to be an attractive approach that combines the strengths of both methods: SFC enables efficient separation over a broad range of polarity while HILIC allows retention of very polar analytes in a single run. In addition, with a ternary gradient (CO_2/ACN/H_2O), matrix interference could be reduced.

Some compounds were likely not detected due to sensitivity issues. The microQTOF used for all analyses offered a lower ion transmission, which may not have sufficed for some of the analytes to be detected. For this reason, using the Q Exactive plus Orbitrap could result in the analytes with lower signal to be detected. In addition, the use of the Velos LTQ ion trap could also yield better results due to improved ion transmission and ionization of the analytes, despite the decrease in resolution of the instrument. Furthermore, the Bruker source used in the microQTOF is generally not heated, whereas the ones on the two Thermo Scientific MS are heated. Preliminary studies on both the Orbitrap and LTQ have shown that the majority of the compounds that were not detected using the microQTOF are detectable. Therefore, switching to a newer type of MS could result in the successful analysis of more compounds. Not only using a Thermo Scientific instrument, but even using another Bruker Daltonics QTOF MS like the Maxis or Impact II would hopefully yield better results as the sensitivity and resolution of the instrument is immensely improved (longer flight tube).

We recommend to further explore the SFC-HILIC including (i) matrix effects, estimation of limits of quantitation and linearity range, and (ii) optimization of sample concentration using, for instance, a combined (pH optimized) SPE cleanup and freeze drying. Finally, a comparison with traditional methods using field samples should be included.

5.1 Practical implications for using SFC

It is not so straightforward to use any HPLC setup for SFC. The best option is to have a dedicated setup for SFC. Costs for a complete setup including DAD is approximately 100-125 kEuro. For the use of CO_2, cylinders with a dip tube can be used which is easier than a sperate chiller. They are relatively cheap and available in smaller cylinders. Points to keep mind when acquiring a SFC setup:

1. HPLC Pumpheads compress essentially the CO_2 and generates heat. To avoid abnormal behavior, a cooling system such as backwash of the seals is required.

2. sCO_2 is used in polymerization process and as a solvent, thus, materials present in a HPLC setup such as seals check valves and washers are prone to adsorption and may result in leakages.

3. A pump needs to be fitted with a manifold mixing valve which can accommodated the high pressure when sCO_2 is entering the manifold (> 55 bar). For example a Gradient Proportioning Valve (GPV) is on the low pressure side and does not meet the demands.

4. A Column heater is required to prevent temperature changes that can have detrimental effects on the peak shape.

5. When algorithms are used for pre-compressing problems may occur due to sCO_2 being more prone to compression than standard HPLC solvents.

6. Injection can be performed using a loop, but the best results were obtained by combining it with modifier stream injection. Essentially the injection is done in the modifier part of the setup (e.g., ACN or MeOH).

7. For method development a DAD is recommended but care must be taken since traditional flow cells cannot withstand the high backpressure (~250 Bar) associated with SFC.

8. A separate pump which is able to deliver a highly reproducible flow of makeup solvent to enhance ionization is required.

9. And finally a well-designed backpressure valve to keep the post column pressure constant is needed.

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