

Analysis of Highly Polar Compounds by SFC/Q-TOF MS with Identification using Database and Library Searches

Enhanced fluidity liquid chromatography (EFLC) using high modifier concentration at elevated system pressure

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Abstract

This Application Note demonstrates the ability of the Agilent 1260 Infinity II Analytical SFC System to separate highly polar compounds on different columns using an increased amount of modifier, a technique known as enhanced fluidity liquid chromatography (EFLC). The development of a supercritical fluid chromatography (SFC) separation method for 46 highly polar compounds on different columns is shown. The compounds were detected by accurate-mass Q-TOF MS measurement, and identified by database search on accurate mass. Compounds with known MS/MS spectra were identified by confirmation with fragment ions. For proof concept, a river water sample was spiked with the compounds and analyzed.

Introduction

Targeted and untargeted screening of river water by high-resolution liquid chromatography/mass spectrometry (HR-LC/MS) to get an overview of organic contaminants released to the environment has become increasingly popular. Most of these methods cover the semipolar compounds typically analyzed by LC/MS.¹ To gain a complete picture, the highly polar compounds must also be taken into consideration. Often, these substances are persistent, similar to artificial sweeteners or X-ray contrast agents, others are toxic or biologically active such as haloacetic acids or metformin. Although well ionized by electrospray ionization (ESI), analysis by LC/MS is not easy because these compounds do not show good retention on reversed phase columns, and often coelute with inorganic salts, which leads to ion suppression and insufficient limits of detection.

In contrast to HPLC, SFC, which makes use of CO₂ in the supercritical state, offers performance advantages in terms of higher separation speed at lower backpressure. This is due to the CO₂, which has a lower viscosity, increased diffusion, and better mass-transfer capabilities compared to classical HPLC mobile phases.

However, due to the expansion of the application of SFC to more polar compounds, modifiers, such as methanol, must be used to elute and separate such compounds by the chromatographic columns. This changes the physical behavior of CO₂ to such an extent that it is no longer in the supercritical state for higher modifier concentrations, for example above 20% methanol. Fortunately, this liquefied CO₂ still gives the same advantages to the elution mixture.

The requirement to separate highly polar or even ionic compounds makes it necessary to apply modifier concentrations above 90% containing even ionic buffers. This has been demonstrated by means of the application of SFC for metabolomic studies.² In recent literature, this chromatographic condition is referred to EF-LC and used in multiple applications from analytical to preparative scale.³ As a drawback, the pressure in the system is increasing from typical SFC conditions to typical HPLC conditions with the increasing part of the modifier. However, the Agilent 1260 Infinity II SFC pump with its pressure capability up to 600 bar can easily cope with these circumstances.

This Application Note demonstrates the separation of highly polar compounds on different columns by means of modifier concentrations up to 95% including additional additives. The compounds were directly transferred to an accurate-mass Q-TOF LC/MS system and ionized in an Agilent Jet Steam source. The identification was done by database and library search based on accurate mass. Finally, a spiked river water sample was analyzed directly after dilution with modifier.

Experimental

Instrumentation

Agilent 1260 Infinity II SFC System comprised:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1290 Infinity Valve Drive (G1170A) with 12-position/13-port solvent selection valves (G4235A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II Diode Array Detector (G7115A) with high-pressure SFC flow cell (G4301-60200)

- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) (G7116B) with Agilent InfinityLab Quick Change eight-column selection valve (G4239C)
- Agilent 6545 accurate-mass Q-TOF LC/MS

Instrumental setup

The SFC system was plumbed in a standard configuration. The SFC pump was connected to a 12-solvent selection valve, which was clustered through software with the pump. The MCT was equipped with an eight-column selection valve and two column tag reader elements. After the backpressure regulator (BPR), the expanding SFC flow was directly connected to the Jet Steam Source. Due to the high content of methanol and additives, an additional make up was not necessary to support the ionization in the Jet Stream ion source.

Columns

- Agilent ZORBAX SB-CN, 150 × 4.6 mm, 5 μm (p/n 883975-905)
- ZORBAX RxSil, 150 × 4.6 mm, 5 μm (p/n 883975-901)
- ZORBAX HILIC Plus RRHD, 100 × 3.0 mm, 1.8 μm (p/n 959758-301)
- Agilent InfinityLab Poroshell 120 HILIC-Z, 100 × 3.0 mm, 2.7 μm (p/n 685975-324)

Software

- Agilent MassHunter Q-TOF Acquisition software, Version B.09.00
- Agilent MassHunter Qualitative software, Version 10
- Agilent MassHunter Water Screening PCDL, Version B.07.00 (p/n G6882AA)

SFC method

Parameter	Value
Solvent A	CO ₂
Modifier B	Methanol (30 mmol NH ₄ Ac or 3 mmol NH ₄ Ac)
SFC Flow Rate	1.5 mL/min (columns with 3.0 mm id), 2.0 mL/min (columns with 4.6 mm id)
Gradient	0 minutes – 5% B, 10 minutes – 95% B, 12 minutes – 95% B
Stop Time	12 minutes
Post Time	2 minutes
BPR Temperature	60 °C
BPR Pressure	140 bar
Column Temperature	40 °C
Injection Volume	1.0 µL (standard), 10 µL (diluted river water)
Feed Solvent	Methanol
Over Feed Volume	4 µL
Feed Speed	400 µL/min
Needle Wash	3 seconds in methanol

Q-TOF data acquisition

- All ions mode negative, m/z 50 to 1,000, scan rate 6 Hz, CEs: 0, 10, 30
- All ions mode positive, m/z 50 to 1,000, scan rate, 6 Hz, CEs: 0, 10, 30

Q-TOF data analysis

- Find by formula (with and without fragment confirmation)

The Q-TOF was operated in All Ions mode. In this mode, the collision cell operates with different collision energies while the quadrupole is set to transmission of all masses. This leads to mixed fragment spectra of all ions entering the collision cell. With the help of MS/MS spectra libraries, target fragment ions can be extracted out of these spectra, and assigned to the precursor ion using a coelution score. (Figure 1).

MS source conditions for SFC coupling

- Source parameters for SFC:
See Table 1

Table 1. Jet Stream ion source conditions for SFC.

MS Source Conditions	Positive Polarity	Negative Polarity
Gas Temperature	220 °C	220 °C
Gas Flow	9 L/min	9 L/min
Sheath Gas Temperature	350 °C	350 °C
Sheath Gas Flow	11 L/min	11 L/min
Nebulizer	50 psi	50 psi
Capillary	4,000 V	4,000 V
Nozzle	0 V	1,000 V

Samples

- Test mix of 46 highly polar compounds (Table 2), 10 ppm each in MeOH, was diluted to 1 ppm with methanol.
- A river water sample was spiked to 10 µg/L of the mixture and diluted with methanol (1:10).

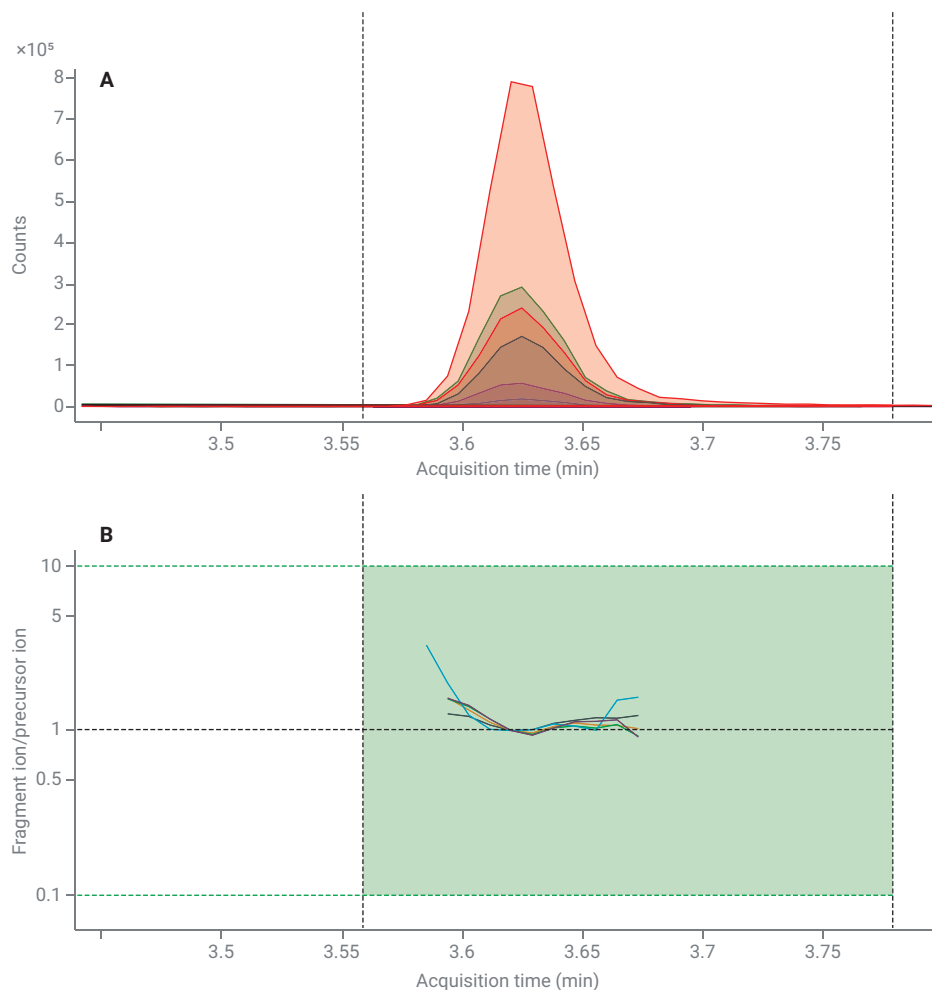


Figure 1. Overlay of precursor and fragment ions from an All Ions experiment for dapson (A) and coelution plot (B).

Table 2. Compounds identified by accurate-mass database search and Q-TOF measurement in ESI positive and negative ionization mode for each tested column. Green = identified; xxx = qualified, spectra in PCDL; xx = high score, no library spectrum; x = low score, no library spectrum; Red = not identified.

Name	Formula	Sil		CN		HILIC-Z		HILIC-Plus	
		neg	pos	neg	pos	neg	pos	neg	pos
1-Naphthalene Sulfonic Acid	C ₁₁ H ₇ SO ₃ H	xx		xx		x		xx	
2,4-Dinitrophenol	C ₆ H ₄ N ₂ O ₅	xxx		xxx		xxx		xxx	
3,5-Ditertbutyl Salicylic Acid	C ₁₅ H ₂₂ O ₃	xx		xx		xx	xx	xx	xx
4-(2-Hydroxyethyl)-morpholine	C ₆ H ₁₃ NO ₂		xx		xx				xx
6-Methyl-1,3,5-triazine-2,4-diamine	C ₄ H ₇ N ₅		xx		xx		xx		xx
Acesulfame (Acesulfame-K)	C ₄ H ₅ NO ₂ S	xxx		xxx		xxx		xxx	
Amantadine	C ₁₇ H ₁₇ N		xxx		xxx		xxx		xxx
Ametryne (Ametrex)	C ₉ H ₁₇ N ₃ S		xxx		xxx		xxx		xxx
Benzoguanamine	C ₉ H ₉ N ₅		xx		xx		xx		xx
Bromoacetic Acid	C ₂ H ₃ BrO ₂			xx				xx	
Bromodichloroacetic Acid	C ₂ HBrCl ₂ O ₂	xx		xx		x		xx	
BTA/Benzotriazole	C ₆ H ₅ N ₃			x	x			xxx	
Carbendazim (Azole)	C ₉ H ₉ N ₃ O ₂		xxx	xx	xxx	xx	xxx	xx	xxx
Chloroacetic Acid	C ₂ H ₃ ClO ₂			xx		x		xx	
Chloro-bromoacetic Acid	C ₂ H ₂ BrClO ₂	xx		xx		xx		xx	
Cyanuric Acid	C ₃ H ₃ N ₃ O ₃	xx		xx		x		xx	
Cyclamic Acid (Cyclamate)	C ₆ H ₁₃ NO ₃ S	xx	xx	xx	xx			xxx	
Dapson	C ₁₂ H ₁₂ N ₂ O ₂ S		xxx	xx	xxx	xx	xxx	xx	xxx
Desphenyl-chloridazon	C ₄ H ₄ ClN ₃ O	xx	xxx	xx	xxx	xx	xxx	xx	xxx
Diatrizoate (Amidotrizoic Acid)	C ₁₁ H ₉ N ₃ O ₄		xxx		xxx				xxx
Dibromoacetic Acid	C ₂ H ₂ Br ₂ O ₂	xx		xx		xx		xx	
Dichloroacetic Acid	C ₂ H ₂ Cl ₂ O ₂	xx		xx		xx		xx	
Dicyandiamide	C ₂ H ₄ N ₄	x			xx			xx	
Dicyandiamidine (Guanylurea)	C ₂ H ₆ N ₄ O		xxx		xxx		xxx	xx	xxx
Gabapentin	C ₉ H ₁₇ NO ₂		xxx		xxx		xxx	xxx	xxx
Gabapentinlactam	C ₉ H ₁₅ NO		xx		xx		xx		xx
Iopamidol	C ₁₇ H ₂₂ I ₃ N ₃ O ₈		xxx	xxx	xxx			xxx	xxx
Melamine	C ₃ H ₆ N ₆		xxx		xxx		xxx		xxx
Melamine, Hexakis(methoxymethyl)-	C ₁₅ H ₃ N ₆ O ₆		xx		xx		xx		xx
Metformin	C ₄ H ₁₁ N ₅		xxx		xxx		xxx		xxx
N-[3-(Dimethylamino)Propyl]-2-methylacrylamide	C ₉ H ₁₈ N ₂ O		xx		xx		xx		xx
Neo Heliopan Hydro/Phenylbenzimidazole Sulfonic Acid	C ₁₃ H ₁₁ N ₂ O ₃ S		xxx	xxx	xxx			xxx	xxx
Oxipurinol	C ₈ H ₈ N ₄ O ₂	xx		xx	xx	xx	x	xx	
p-Toluenesulfonic Acid	C ₇ H ₆ O ₃ S	xx		xx		xx		xx	
p-Toluenesulfonamid	C ₇ H ₉ NSO ₂		xxx	xxx	xxx	xxx	xxx	xxx	
Saccharin	C ₇ H ₅ NO ₃ S	xx		xx		xx		xxx	
Sodium Methyl Sulfate	CH ₃ SO ₄	x		x	x				
Sucralose	C ₁₂ H ₁₉ Cl ₃ O ₈	xxx	xxx	xxx	xxx	xx	xx	xxx	
Sulfamic Acid	H ₂ NSO ₃ H	xx		xx				xx	
Sulfanilic Acid	C ₆ H ₇ NO ₃ S	xx		xx		x		xx	
Tbz/Thiabendazole	C ₁₁ H ₉ N ₃ S		xxx	xx	xxx	xx	xxx	xx	xxx
Tcep/Tris(2-chloroethyl)phosphate	C ₆ H ₁₂ Cl ₃ O ₄ P		xxx		xxx		xxx		xxx
Tcpp/Tri-(2-chloroisopropyl)phosphate	C ₉ H ₁₈ Cl ₃ O ₄ P		xxx		xxx		xxx		xxx
Trichloroacetic Acid	C ₂ HCl ₃ O ₂	xxx		xxx		xxx		xxx	
Trifluoroacetic Acid	C ₂ HF ₃ O ₂	xx		xx		xx		xx	
Trifluoromethanesulfonic Acid	CHF ₃ O ₃ S	xx		xx		xx		xx	

Chemicals

All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22 μm membrane point-of-use cartridge (Millipak). Chemicals were purchased from Sigma-Aldrich (Germany).

Results and discussion

Development of an SFC separation method

The first step in the development of the SFC separation method for the complete set of 46 highly polar compounds was to choose an organic modifier solvent. The solvent with highest SFC elution strength, methanol, was chosen for the initial scouting experiments. As an initial gradient, a generic increase of modifier from 5 to 95% in 10 minutes was applied. As a set of stationary phases, columns with silica, cyano, HILIC, and zwitterionic HILIC, which are typically applied for polar compounds, were used. As an additive for the organic modifier, ammonium acetate was chosen. This MS-friendly additive helps to ionize the compounds in the source, and has an effect on the compound/stationary-phase interaction, especially with ionic compounds and HILIC-Z phases. To identify the compounds that were eluted from the tested columns, the generated Q-TOF data were screened against an accurate mass database and library. The experiments were done in positive and negative ionization mode.

Figure 2A shows that the compounds that were detected in positive ionization mode eluted over the complete gradient window using the Rx-Sil column. A higher additive concentration was applied to improve peak shapes. Three compounds (early eluting *tris*(2-chlorethyl)phosphate (TCPP), medium eluting 6-methyl-1,3,5-triazin-2,4-diamin, and late eluting metformin)

were used as trace compounds to compare the selectivity of the tested columns. In comparison, Figures 2B to 2D show the results achieved with the other tested columns. It was found that another good stationary phase for the separation was the cyano

phase (Figure 2B). Good peak shapes were achieved through a low additive concentration, which is favorable for the MS source. The eluting compounds were equally distributed within an elution time window up to six minutes. The HILIC-Z column showed elution up to 7.2 minutes

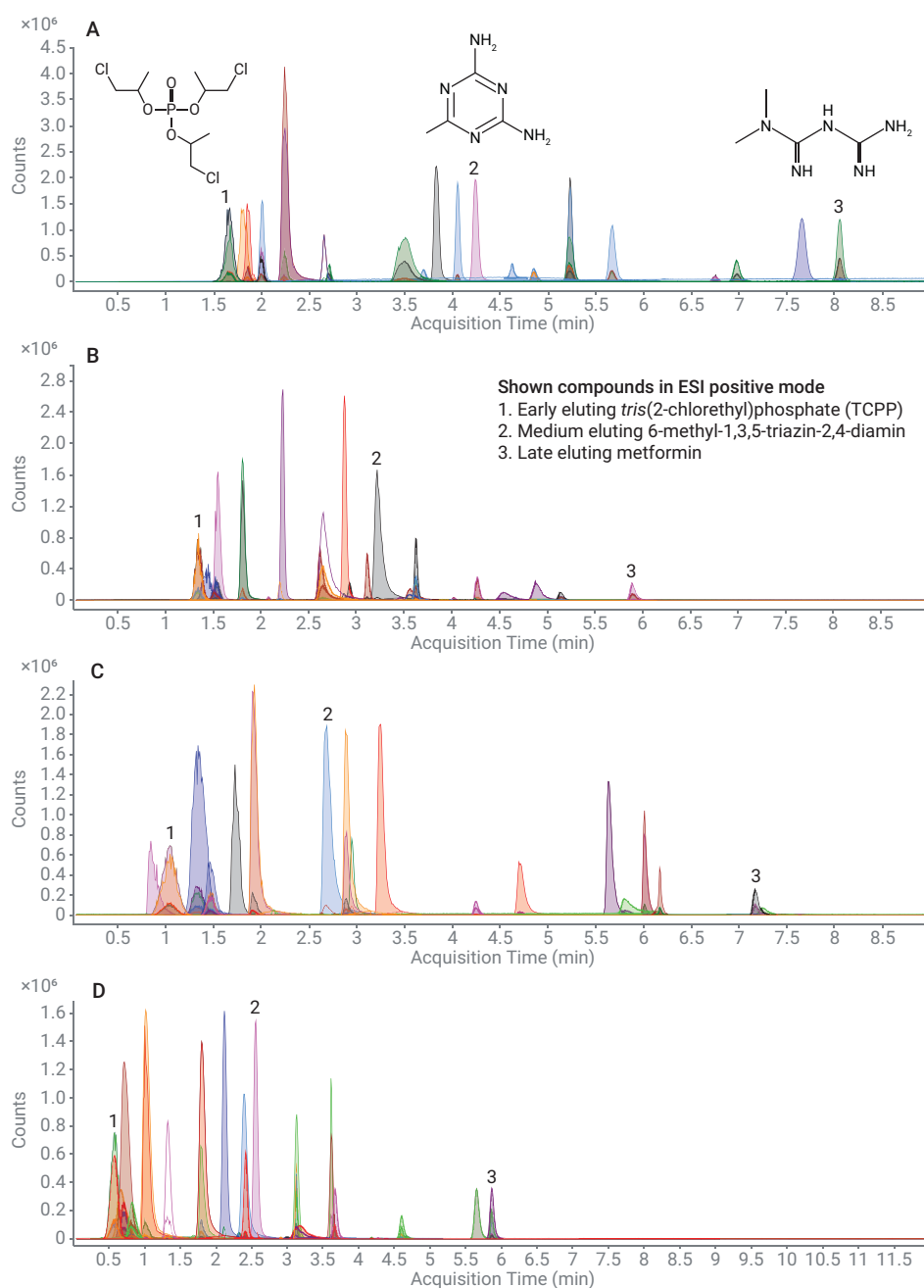


Figure 2. Column screening of the SFC separation of highly polar compounds with accurate-mass Q-TOF measurement in positive ESI mode and database search. A: Agilent ZORBAX Rx-Sil column (methanol + 30 mM NH_4Ac), B: ZORBAX SB-CN column (methanol + 3 mM NH_4Ac), C: Poroshell 120 HILIC-Z column (methanol + 3 mM NH_4Ac), D: ZORBAX HILIC Plus column (methanol + 3 mM NH_4Ac).

with larger groups of compounds eluting early and at the end of this time frame (Figure 2C). The HILIC Plus column showed a stronger shift to earlier elution times (Figure 2D).

The negative ionization mode was tested respectively (Figure 3A to 3D). The silica columns showed most of the compounds eluting in the middle of the run time (Figure 3A). The other columns showed a broader elution time frame (Figures 3B to 3D). With HILIC phases, the compounds eluted more towards the beginning or the end of the elution window (Figures 3C and 3D). Table 2 outlines the final results of compounds identified per column and their data quality. The HILIC Plus column showed the highest number of noneluted and unidentified compounds.

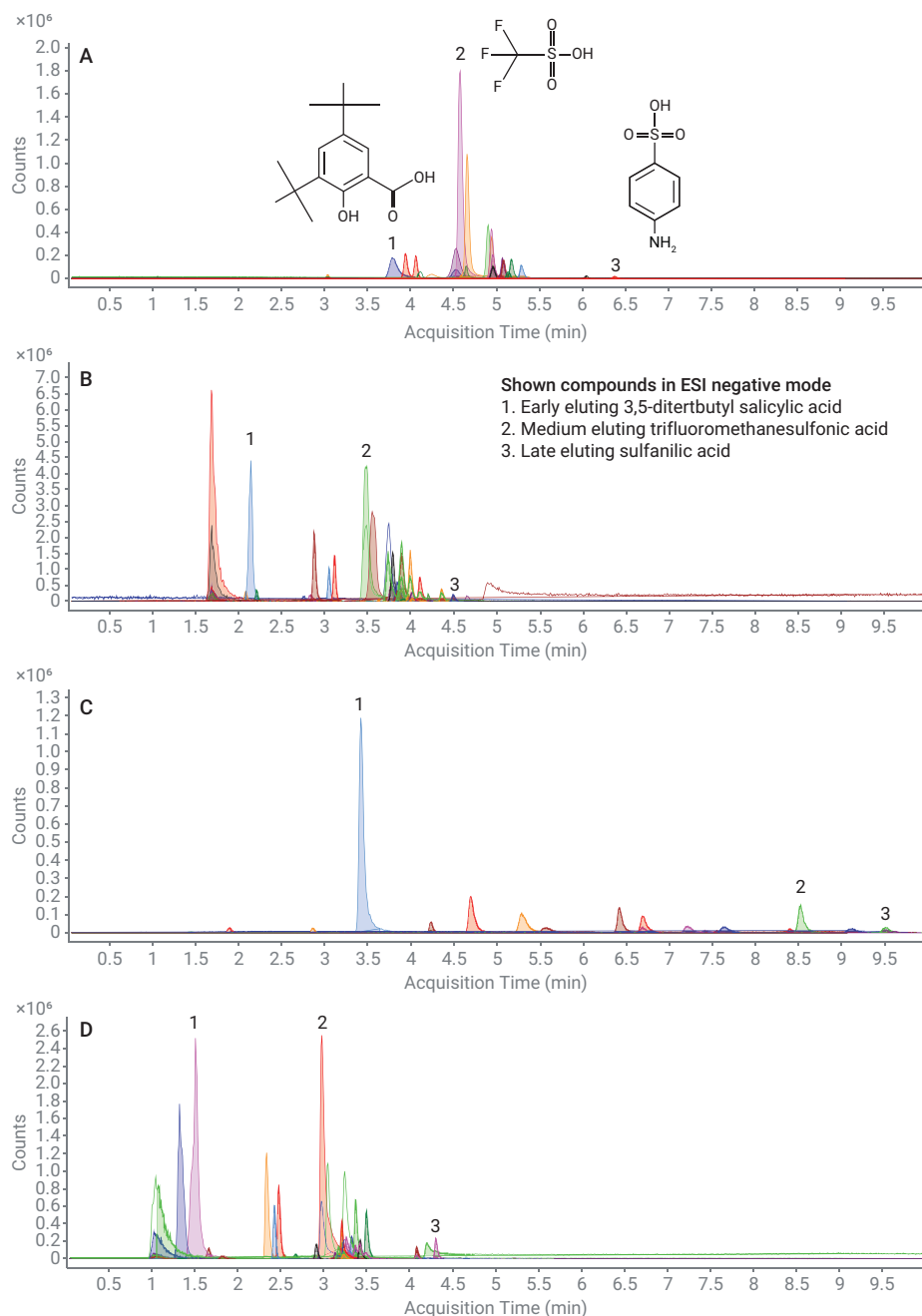


Figure 3. Column screening of the SFC separation of highly polar compounds with accurate-mass Q-TOF measurement in negative ESI mode and database search. A: Agilent ZORBAX Rx-Sil column (methanol + 30 mM NH_4Ac), B: ZORBAX SB-CN column (methanol + 3 mM NH_4Ac), C: Poroshell 120 HILIC-Z column (methanol + 3 mM NH_4Ac), D: ZORBAX HILIC Plus column (methanol + 3 mM NH_4Ac).

Analysis of a river water sample

The SB-CN column and the developed SFC conditions were applied for additional experiments to analyze a clean river water sample spiked with 10 µg/L (10 ppb) of the compounds. One part of the sample was diluted with one-part methanol, and used directly for injection. Different injection volumes from 1 to 10 µL were tested. For many compounds, there was a linear increase

in signal without peak broadening. This is shown in Figure 4 for one early eluting (*tris*-(2-chlorisopropyl)phosphat) and one late eluting (metformin) compound. Both show a linear increase in signal for an increase of the injection volume of the highly aqueous sample. The complete set of compounds identified at the 10 ppb level in river water, together with signal-to-noise ratios (S/N), is shown in Table 3. For example, four

compounds could be identified with a limit of detection (LOD) at 0.03 ppb, 37 compounds have a LOD below 3.0 ppb, and only one LOD was above 10 ppb (Figure 5). Only one compound (*p*-toluenesulfonamide) could not be found in the spiked river water sample. There were 22 compounds identified at the 10 ppb level in positive ESI mode and 23 compounds in negative ESI mode (Table 3).

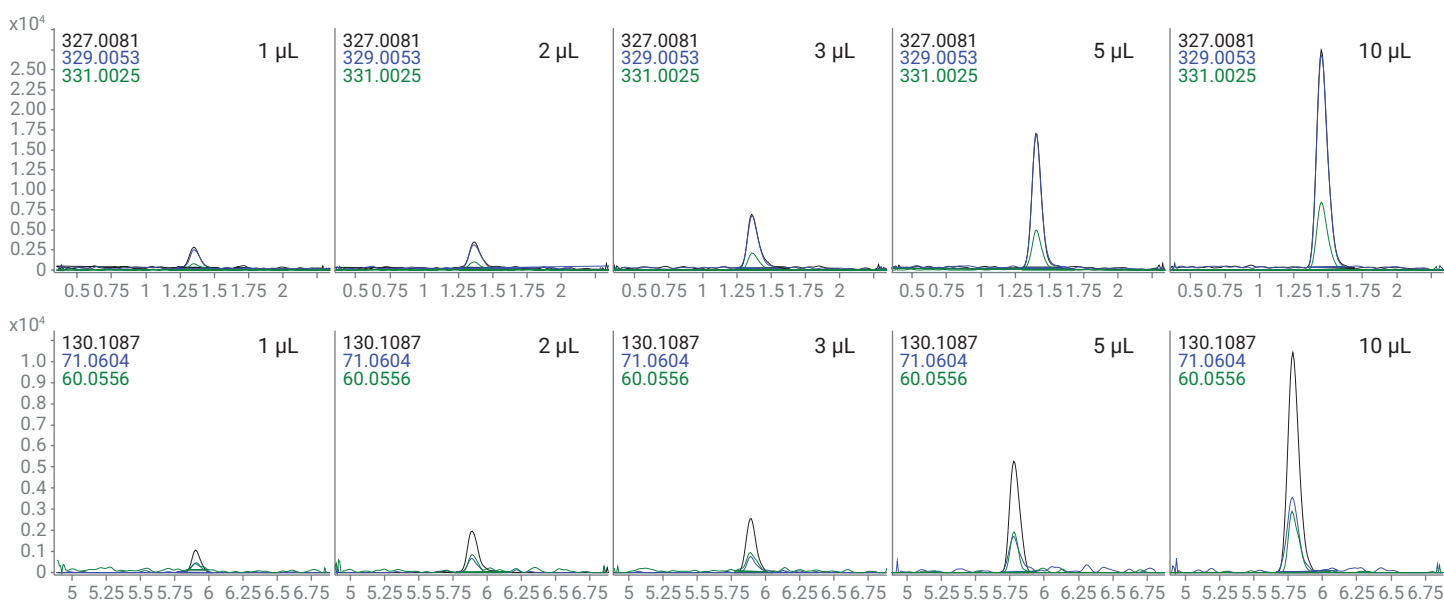


Figure 4. Linear increase of response for two compounds in spiked river water sample (10 µg/L and 1:1 dilution with methanol) from 1 to 10 µL injection volume on the cyano phase column. Top: early eluting TCP. Bottom: late eluting metformin.

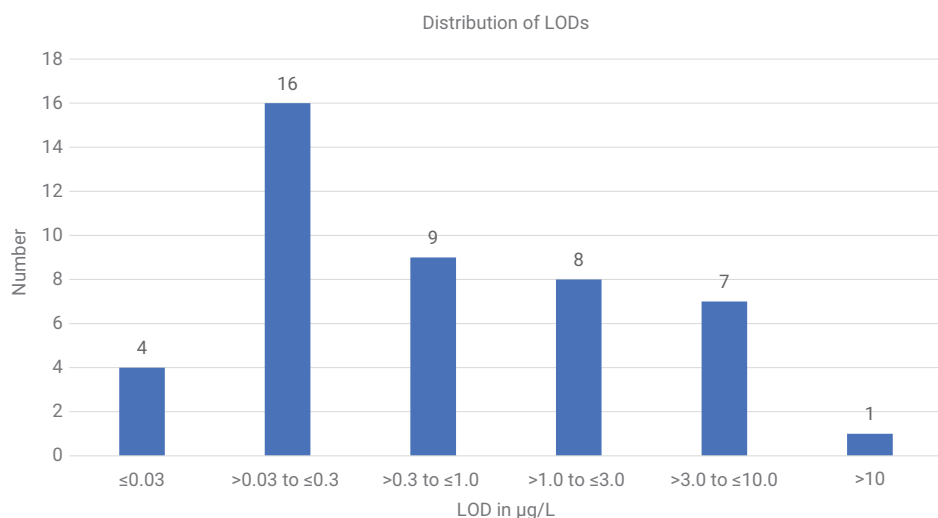


Figure 5. Results of the river water analysis on the cyano phase column, showing compounds detected after a 10 µL injection.

Table 3. All 45 compounds were found in the spiked river water sample after a 10 µL injection. A few were detected in negative and positive ESI mode. A signal-to-noise ratio of 3 was used to estimate an LOD.

Compound Name	Formula	Ionization Mode	RT	S/N Ratio [Peak to Peak]	Estimated LOD [µg/L] (Calculated From S/N Ratio)
1-Naphthalene Sulfonic Acid	C ₁₁ H ₇ SO ₃ H	negative	4.013	172	0.17
2,4-Dinitrophenol	C ₆ H ₄ N ₂ O ₅	negative	1.841	59	0.51
3,5-Ditertbutyl Salicylic Acid	C ₁₅ H ₂₂ O ₃	negative	2.038	328	0.09
4-(2-Hydroxyethyl)-morpholine	C ₆ H ₁₃ NO ₂	positive	3.792	30	1.00
6-Methyl-1,3,5-triazine-2,4-diamine	C ₄ H ₇ N ₅	positive	3.169	273	0.11
Acesulfame (Acesulfame-K)	C ₄ H ₅ NO ₄ S	negative	2.038	122	0.25
Amantadine	C ₁₁ H ₁₇ N	positive	4.182	188	0.16
Ametryne (Ametrex)	C ₉ H ₁₇ N ₅ S	positive	3.792	202	0.15
Benzoguanamine	C ₉ H ₉ N ₅	positive	2.904	381	0.08
Bromoacetic Acid	C ₂ H ₃ BrO ₂	negative	2.770	2	15.00
Bromodichloroacetic Acid	C ₂ HBrCl ₂ O ₂	negative	3.792	3	10.00
BTA/Benzotriazole	C ₆ H ₅ N ₃	positive	2.367	23	1.30
Carbendazim (Azole)	C ₉ H ₉ N ₃ O ₂	positive	1.870	38,000	0.0008
Chloroacetic Acid	C ₂ H ₃ ClO ₂	negative	2.840	170	0.18
Chloro-Bromoacetic Acid	C ₂ H ₂ BrClO ₂	negative	3.678	9	3.33
Cyanuric Acid	C ₃ H ₃ N ₃ O ₃	negative	2.408	101	0.30
Cyclamic Acid (Cyclamate)	C ₆ H ₁₃ NO ₂ S	negative	3.792	94	0.32
Dapson	C ₁₂ H ₁₂ N ₂ O ₂ S	negative	3.607	10	3.00
Dapson	C ₁₂ H ₁₂ N ₂ O ₂ S	positive	3.607	16	1.88
Desphenyl-chloridazon	C ₄ H ₄ ClN ₃ O	negative	3.095	111	0.27
Desphenyl-chloridazon	C ₄ H ₄ ClN ₃ O	positive	3.095	26	1.15
Diatrizoate (Amidotrizoic Acid)	C ₁₁ H ₉ N ₃ O ₄	positive	4.585	4	7.50
Dibromoacetic Acid	C ₂ H ₂ Br ₂ O ₂	negative	3.695	20	1.50
Dichloroacetic Acid	C ₂ H ₂ Cl ₂ O ₂	negative	3.634	32	0.94
Dicyandiamide	C ₂ H ₄ N ₄	negative	2.913	107	0.28
Dicyandiamide	C ₂ H ₄ N ₄	positive	2.913	150	0.20
Dicyandiamidine (Guanylurea)	C ₂ H ₆ N ₄ O	negative	3.495	5	6.00
Dicyandiamidine (Guanylurea)	C ₂ H ₆ N ₄ O	positive	3.495	29	1.03
Gabapentin	C ₉ H ₁₇ NO ₂	positive	5.035	90	0.33
Gabapentinlactam	C ₉ H ₁₅ NO	positive	2.111	114	0.26
Iopamidol	C ₁₇ H ₂₂ I ₃ N ₉ O ₈	negative	3.942	3	10.00
Melamine	C ₃ H ₆ N ₆	positive	4.470	24	1.25
Melamine, Hexakis(methoxymethyl)-	C ₁₅ H ₃ N ₆ O ₆	positive	1.547	113	0.27
Metformin	C ₄ H ₁₁ N ₅	positive	5.784	10,000	0.003
N-[3-(Dimethylamino)Propyl]-2-Methylacrylamide	C ₉ H ₁₈ N ₂ O	positive	4.738	97	0.31
Neo Heliopan Hydro/Phenylbenzimidazole Sulfonic Acid	C ₁₃ H ₁₁ N ₂ O ₃ S	negative	4.420	14	2.14
Neo Heliopan Hydro/Phenylbenzimidazole Sulfonic Acid	C ₁₃ H ₁₁ N ₂ O ₃ S	positive	4.420	1,000	0.03
Oxipurinol	C ₅ H ₄ N ₄ O ₂	negative	2.884	108	0.28
p-Toluenesulfonic Acid	C ₇ H ₈ O ₃ S	negative	3.872	87	0.34
Saccharin	C ₇ H ₅ NO ₃ S	negative	3.748	22	1.36
Sodium Methyl Sulfate	CH ₃ SO ₄	negative	3.651	55	0.55
Sucralose	C ₁₂ H ₁₉ Cl ₃ O ₈	negative	3.043	26	1.15
Sulfamic Acid	H ₂ NSO ₃ H	negative	4.101	17	1.76
Sulfanilic Acid	C ₆ H ₇ NO ₃ S	negative	4.286	49	0.61
TBZ/Thiabendazole	C ₁₁ H ₇ N ₃ S	positive	2.690	9	3.33
TCEP/Tris(2-Chloroethyl)phosphate	C ₆ H ₁₂ Cl ₃ O ₄ P	positive	1.617	13,400	0.002
TCPP/Tri-(2-Chloroisopropyl)phosphate	C ₉ H ₁₈ Cl ₃ O ₄ P	positive	1.450	171	0.18
Trichloroacetic Acid	C ₂ HCl ₃ O ₂	negative	3.739	4	7.50
Trifluoroacetic Acid	C ₂ HF ₃ O ₂	negative	3.431	3	10.00
Trifluoromethanesulfonic Acid	CHF ₃ O ₃ S	negative	3.404	188	0.16

Conclusion

This Application Note demonstrates the use of high organic solvent modifier concentration for the separation of very polar compounds by SFC (EFLC) in combination with Q-TOF mass spectrometry detection and accurate-mass database and library identification in a screening method for water samples. The Agilent 1260 Infinity II Analytical SFC System is able to cope with the increased pressure (up to 600 bar) which could be caused by the high content of organic modifier in liquified CO₂. In the examined set of highly polar compounds, LODs to <0.03 ppb were achieved, even in a highly aqueous spiked river water sample (diluted with organic solvent). This method can be converted to a targeted method on a tandem quadrupole MS to achieve even lower LODs.

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