Pharma

Polarity-extended chromatography – setup, method transfer, and optimization for serial RPLC-HILIC coupling with mass spectrometric detection

A powerful hyphenation for non-targeted screening

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Keywords

Vanquish Flex UHPLC, Orbitrap Exploris 120 mass spectrometer, non-targeted screening, NTS, polarity-extended chromatography, serial coupling, HILIC, reversed phase, method transfer, method optimization

Application benefits

- Serial coupling of RPLC and HILIC enables separation of non-polar to very polar organic compounds in a single injection.
- Efficient and time-saving separation and detection of molecules are achieved across a wide range of polarities by using state-of-the art LC and MS systems.

Goal

Method transfer from an HPLC to a UHPLC-MS setup for compounds with a polarity range from logD -7 to logD +7.

Introduction

The increasing interest in a holistic understanding of complex samples requires chromatographic separation techniques suitable for the analysis of various compound classes with different physicochemical properties that are compatible with mass spectrometry (i.e., electrospray ionization). In an early publication,[1] it was demonstrated that polarity-extended chromatographic separation techniques (like the serial coupling of reversed phase liquid chromatography (RPLC) with hydrophilic interaction liquid chromatography (HILIC)) can separate non-polar, polar, and very polar molecules in one run.

Two white papers reflect the wide-spread application of serial RPLC-HILIC in combination with high-resolution accurate-mass (HRAM) mass spectrometry (MS) in water analysis[2] and various other fields of application[3]. Using RPLC-HILIC, molecules with a wide polarity range covering logD values from -7 to +7 are successfully separated.[3,4] This separation technique is widely accepted and has been in use for over 10 years.

Due to the commercial availability of improved (U)HPLC products, the HPLC instrumental setup was completely rebuilt and transferred to modern UHPLC instrumentation and consumables. The initial HPLC hardware could tolerate pressure up to 600 bar, whereas the columns and T-piece only supported pressures up to 400 and 350 bar, respectively. With the new instrumentation and equipment, pressures up to 1,034 bar become possible, which can significantly improve separation speed and efficiency.

The goal for the new setup was to maintain the sequential elution of HILIC and RP retained compounds, retention time (RT) stability, and peak area precision while decreasing the run time significantly compared to the original setup.[5] Improvements in the MS ion source design allowed for a simplification of the setup by eliminating the isocratic pump previously used for make-up flow prior to the MS inlet.

In addition, the Thermo Scientific™ Orbitrap Exploris™ series mass spectrometers with the heated electrospray ionization (HESI) source allow efficient polarity switching within an analytical run. This helps to further increase analytical efficiency and sample throughput. Thus, a combination of modern UHPLC instrumentation and state-of-the-art MS instruments is a perfect match for polarity-extended non-targeted analyses.

Figure 1 shows the HPLC setup (A) and the UHPLC setup (B).

Experimental

Chemicals

- Water, LC-MS grade, Merck
- Acetonitrile, LC-MS grade, Honeywell
- Methanol, LC-MS grade, Honeywell
- Ammonium acetate (>98% purity), Sigma-Aldrich
- Analytical standards from various vendors

Sample handling

- Thermo Scientific™ SureSTART™ 2 mL Glass Snap Top Vials, Level 3 High Performance Applications (P/N 6PRV11-1P)
- Thermo Scientific™ SureSTART™ 11 mm Snap Caps, Level 3 High Performance Applications (P/N 6PRC11STS1)

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC-HRAM MS system consisting of:

- Vanquish System Base Horizon/Flex (P/N VF-S01-A)
- Vanquish Binary Pump F (P/N VF-P10-A), with 10 µL mixer
- Vanquish Binary Pump F (P/N VF-P10-A), with 150 µL mixer
- Vanquish Split Sampler FT (P/N VF-A10-A)
- Vanquish Column Compartment H (P/N VH-C10-A-02)
- Tee piece, Thermo Scientific (P/N U-428)
- Orbitrap Exploris 120 mass spectrometer with HESI source (P/N BRE725531)

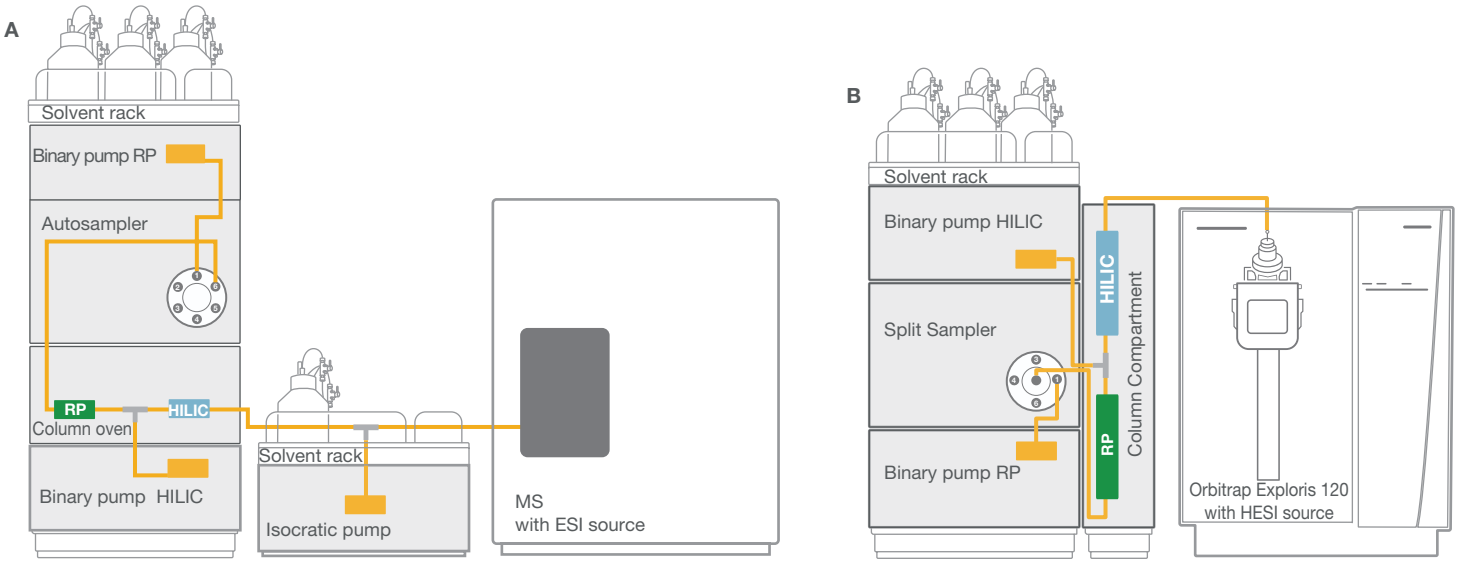

Figure 1. Schemes of serial RPLC-HILIC coupling. (A) HPLC setup[5] and (B) UHPLC setup

Sample preparation

Information on applied standard compounds (in the logD range from -7 to +7 at pH 7) is given in a former publication.[4] Standards were prepared in individual stock solutions of 1 mM, dissolved in acetonitrile, acetonitrile/water (50/50, v/v), or methanol and stored at 4 °C before use. For analyses, the compounds were combined in a mixture with a concentration of 2 µmol/L for each compound.

Mobile phase consideration

A concentration of 5 mmol/L ammonium acetate (NH_4Ac) in RPLC solvents (e.g., mobile phase B in Table 1) should not be exceeded due to limited solubility in acetonitrile (ACN).

Chromatographic conditions

Table 1. Chromatographic conditions for the UHPLC setup

Parameter	Value					
Columns	RPLC: Thermo Scientific™ Accucore™ C18, 50 × 2.1 mm, 2.6 µm (P/N 17126-052130) HILIC: Thermo Scientific™ Syncronis™ HILIC 100 × 2.1 mm, 1.7 µm (P/N 97502-102130)					
Mobile phase	RPLC: A: H_2O/ACN 95/5 (v/v) with 5 mM NH_4Ac B: ACN/H_2O 95/5 (v/v) with 5 mM NH_4Ac HILIC: A: ACN B: H_2O/ACN 95/5 (v/v)					
Gradient	RPLC			HILIC		
	Time (min)	%B	Flow rate (mL/min)	Time (min)	%B	Flow rate (mL/min)
	0.0	0	0.035	0.0	0	0.600
	8.0	0	0.035	3.0	0	0.600
	9.1	0	0.200	11.0	70	0.600
	21.0	100	0.200	24.0	70	0.600
	23.9	100	0.200	24.1	0	0.600
	24.0	0	0.200	35.0	0	0.600
	24.5	0	0.035			
	35.0	0	0.035			
Column temperature	40 °C					
Autosampler temperature	8 °C					
Autosampler wash solvent	Water/Methanol 90/10 (v/v)					
Injection volume	10 µL					

Figure 2 presents the RPLC-HILIC method overview.

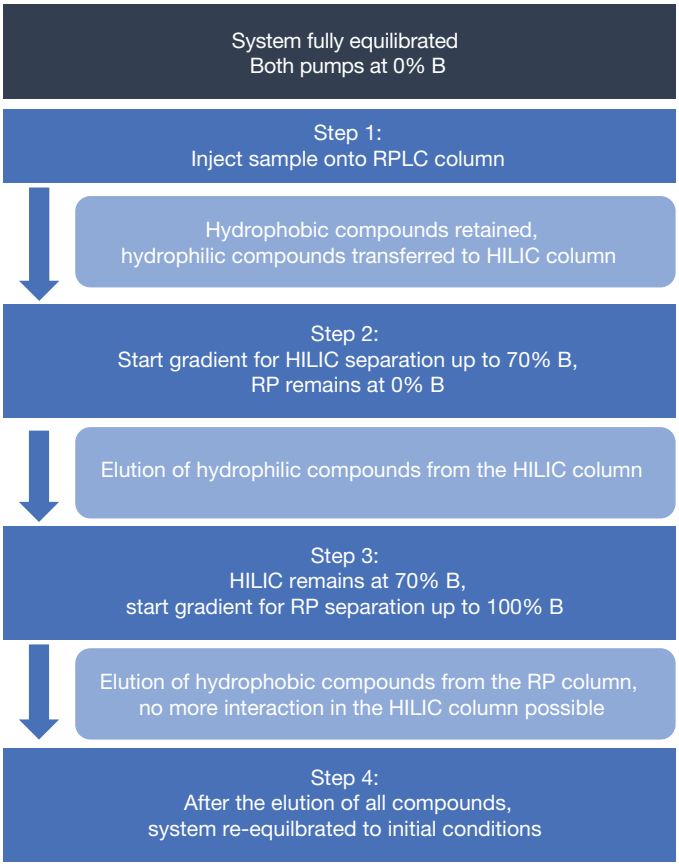

Figure 2. RPLC-HILIC method overview

MS settings

Table 2. Instrument and scan settings for the mass spectrometer

Parameter	Value
HESI source settings	
Vaporizer temperature	400 °C
Ion transfer tube temperature	320 °C
Source voltage (positive-negative switching)	+3,500 V, -2,500 V
Sheath gas flow	50
Aux gas flow	8
Sweep gas flow	0
MS scan settings	
Mass range	*m/z* 70–1000
FullMS resolution	60,000 @ *m/z* 200
MS^2 settings	DDA, Top 4
MS^2 resolution	30,000
Collision energy	Stepped 15 to 45 eV

Chromatography Data System

Thermo Scientific™ Chromeleon™ 7.3.1 Chromatography Data System (CDS) was used for data acquisition and processing.

Results and discussion

Method transfer from HPLC to UHPLC

General considerations

The instrumental setup, namely the serial combination of RP and HILIC columns in one separation method, requires some general considerations:

- This setup allows the injection of complex samples, containing very polar to non-polar compounds, and the separation and sequential elution of these compounds in one run. In contrast to a typical 2D LC setup, compounds are separated in one or the other column rather than in both. Since compounds are eluted from the RP column (hydrophobic compounds) or the HILIC column (hydrophilic compounds), retention time can be used to assess a compound polarity.

- HILIC separations require a stable water layer on the particle surface of the stationary phase. For stable retention conditions a minimum of 5% water in the organic solvent at starting conditions is required.

- HILIC retention mechanisms (especially when using zwitterionic stationary phases) also include electrostatic interactions, so the concentration of salts and buffers in the mobile phase can strongly affect the retention behavior of compounds. Therefore, the concentration of additives must be kept as constant and reproducible as possible during elution of compounds from the HILIC column (first elution step). Changing additive concentrations after the elution of compounds from HILIC (e.g., when elution from RP is started) does not affect retention anymore.

- The serial LC method will be coupled to MS detection, so the use of volatile salts and buffers (e.g., ammonium acetate) is mandatory. To ensure sufficient solubility of ammonium acetate in the mobile phase solvents, it is suitable to use a mixture of 95% acetonitrile and 5% water (and vice versa) containing up to about 6 to 7 mmol/L of ammonium acetate. It is preferred to add the salts to the RP solvents because in the HILIC separation, pure acetonitrile is required as solvent and there the solubility of ammonium acetate is highly limited. In addition, since the consumption of HILIC solvents is higher than solvents in RP, it is more practical to use pure solvents here. This reduces the necessity of mixing solvents and eliminates a source of potential deviation.

- For the HILIC separation, acetonitrile and water are required as solvents. However, the use of pure water should be avoided, due to biological contamination risks.

- The combination of RP and HILIC separations in one method requires a thorough adjustment of mobile phase composition and flow rates between modes. In addition, column void volumes and the gradient delay time need to be considered. Column void volume can be estimated based on column length and inner diameter. The gradient delay time is strongly dependent on the flow rate of the mobile phase and the volume of the static mixer in the pump. The goal is to find a combination of mobile phase solvents and flow rates that ensures an initial water content in the HILIC column of approximately 5% and a total flow rate of less than 800 µL/min for sufficient evaporation and ionization conditions in the subsequent electrospray ionization.

- The sequential elution of compounds in this coupling of RP and HILIC provides the benefit that retention time information can be used to assess a compound's hydrophobicity. Non-polar to medium polar compounds are retained in the RP column, while polar to very polar compounds, which are not retained by RP, are transferred to the HILIC column and eluted from there. The sequential elution results in two elution windows of compounds with HILIC-retained compounds in the first section of the chromatogram, followed by RP-retained compounds. Especially for applications in which unknown compounds are separated (e.g., non-target screening), the information about compound hydrophobicity can be an important aspect for identification. Thus, elution windows of RP and HILIC separations should clearly be separated in the chromatographic method. To achieve this, the mobile phase gradients of both separations need to be approximated very carefully.

The original method was developed on an HPLC instrument using a 50 × 3 mm core-shell RPLC column with 2.7 µm particle size in combination with a 150 × 2.1 mm zwitterionic HILIC column with 5 µm particle size. The method run time including equilibration is 58 minutes. The method was transferred to a UHPLC instrument with a 50 × 2.1 mm core-shell RP column with 2.6 µm particle size and a 100 × 2.1 mm zwitterionic HILIC column with 1.7 µm particle size. Due to increased column pressure stability, the backpressure could be increased up to 1,034 bar, allowing higher flow rates and water content in the mobile phase compositions. These parameters substantially reduced the run time to 35 minutes and lowered the solvent consumption (for further details please refer to the later section).

The following sections describe the method transfer and optimization in more detail.

Method transfer and optimization for serial RPLC-HILIC coupling

As the first step in the method transfer, the HPLC method with the original columns, later called HPLC columns, was transferred to the UHPLC instrument. Due to different void volumes in the two systems, changes in retention times were observed. In the next step, the LC columns were exchanged, and the more pressure stable columns, later called UHPLC columns, were installed. This resulted in significantly changed retention times due to the different column dimensions and differences in adsorption behavior. In the next step, the solvents of the mobile phase compositions were adjusted based on the general considerations mentioned above.

Unlike the separation mechanism in RPLC using a polar-endcapped stationary phase, which is nearly independent of salt concentration, HILIC is very sensitive to changes in salt concentration. In the original HPLC method, a salt gradient influenced the water layer thickness in the HILIC separation.[4] This was caused by the mismatch between the RP and HILIC mobile phases, the RP consisting of a mixture of acetonitrile and 10 mM ammonium acetate in water. In the UHPLC method, however, both RP solvents were changed to mixtures of acetonitrile and water with a final content of 5 mM ammonium acetate. Consequently, the water layer is not influenced by the gradient and the salt content remains constant when compounds are eluted from the HILIC column. After this elution, flow rates are changed and so also the content of ammonium acetate in the composition. This does not affect HILIC elution anymore, which is already accomplished at that point.

The solvents in HILIC originally consisted of acetonitrile (solvent A) and water (solvent B). To decrease the risk of biological contamination, here solvent B was changed to water with 5% acetonitrile.

a) Method optimization

To maintain the separation quality after changing mobile phases and column dimensions, the gradients and flow rates were optimized on the new setup. Method optimization was first performed on the HILIC column followed by the RP column. This was necessary because the conditions in the HILIC column (e.g., flow rate and mobile phase composition) are influenced by the conditions in the RP column and the requirements for a stable and reproducible separation in HILIC are more complex.

Initially, the HILIC separation consisted of an isocratic hold at 100% acetonitrile for six minutes, followed by a ramp to 60% A (organic solvent) and 40% B (water) within seven minutes (5.7%/min). In method optimization, different combinations of isocratic initial hold times and gradient settings were tested (not all data shown). The hold time was of special importance because the gradient in the HILIC column should only start when compounds have passed through the RP column and arrived at the HILIC column. So, there is an important connection between the HILIC method and the RP method and column geometry (resulting void time). It was determined that a ramp to 70% B provided the best separation. Therefore, the gradient steepness was increased to 8.8%/min, resulting in a gradient from 0 to 70% B in eight minutes. Along with gradient optimization, different flow rates were tested. Here, the flow rate combination was an important aspect because the combined flow when entering the HILIC column defines the mobile phase composition during the HILIC separation and must be highly reproducible. The optimal flow rate of the HILIC pump was found to be 0.6 mL/min. The HILIC pump method started at 100% A (acetonitrile) and the required water content in the mobile phase was provided by the RP pump, which delivered 0.035 mL/min of 100% solvent A (water/acetonitrile 95/5 v/v with 5 mM ammonium acetate). The resulting mobile phase consisted of 5.2% water and 0.28 mM ammonium acetate.

The RP pump method started with an isocratic hold at 100% solvent A for seven minutes (that is when the HILIC-retained compounds are starting to elute), followed by a gradient to 50% solvent B within five minutes, an increase of the flow rate from 0.05 mL/min to 0.1 mL/min within one minute and a consecutive gradient to 100% within nine minutes.[1] During the optimization, this was simplified by separately optimizing the mobile phase gradient and the flow rate increase. The initial isocratic hold was necessary to guarantee the transfer of all polar to very polar compounds through the RP column to the HILIC column and to ensure a suitable mobile phase composition for the HILIC separation. After the HILIC elution was finished, the gradient in the RP pump could be started. In the optimized UHPLC method, the initial isocratic hold was performed for nine minutes with a flow rate of 0.035 mL/min followed by a flow rate increase to 0.2 mL/min within 0.1 minutes. This was mainly due to the void volume of the column and the resulting mobile phase composition in the HILIC column. The following mobile phase gradient increased the content of solvent B from 0 to 100% within twelve minutes (8.3%/min). The flow rate of 0.035 mL/min in the initial phase was determined by the previously optimized HILIC separation. The initial isocratic phase was necessary to allow all compounds that are not retained in the RP column to be transferred into the HILIC column for subsequent separation. For the elution of RP retained compounds, the flow rate was increased to speed up the separation and to improve peak shapes. Due to the significantly higher flow rate in HILIC with 70% water content at that time point, further interactions of RP separated compounds in the HILIC column were ruled out.[6]

To combine the two separations and to maintain the separate elution windows, the isocratic phases and starting times of the gradients were carefully adjusted.

Table 3 presents the gradient settings for the HPLC and the UHPLC methods. Figure 3 shows overlaid extracted ion chromatograms (XICs) for two example compounds in each optimization step of the HILIC separation, and Figure 4 shows the RP separation. The orange trace represents the HPLC method with HPLC columns. With the UHPLC column and the HPLC method (red trace), improved peak shape was obtained after transfer to the UHPLC instrument. Peak shape and retention were further improved by optimizing the chromatographic method conditions (blue trace).

In total, 150 compounds were analyzed with logD values (at pH 7) ranging from -8.63 to 6.22. Figure 5 shows a RT/logD plot where the light-yellow shaded area represents the HILIC elution window, with the majority of compounds having logD values <0, and the light-green shaded area represents the RP elution window, with the majority of compounds having logD values >0.

Table 3. Optimization of RP and HILIC gradient conditions from HPLC setup to UHPLC setup.
HPLC column in HILIC: 150 × 2.1 mm and 5 µm particle size; UHPLC column: 100 × 2.1 mm and 1.7 µm particle size. HPLC column in RP: 50 × 3 mm with 2.7 µm particle size; UHPLC column: 50 × 2.1 mm with 2.6 µm particle size.

HPLC method					UHPLC method				
	RP		HILIC			RP		HILIC	
Time (min)	B (%)	Flow rate (mL/min)	B (%)	Flow rate (mL/min)	Time (min)	B (%)	Flow rate (mL/min)	B (%)	Flow rate (mL/min)
0.0	-	-	0	0.400	0.0	0	0.035	0	0.600
6.0	-	-	0	0.400	3.0	-	-	0	0.600
7.0	0	0.050	-	-	8.0	0	0.035	-	-
12.0	50	0.050	-	-	9.1	0	0.200	-	-
13.0	50	0.100	40	0.400	11.0	-	-	70	0.600
22.0	100	0.100	-	-	21.0	100	0.200	-	-
32.0	100	0.100	40	0.400	23.9	100	0.200	-	-
33	0	0.100	0	0.800	24.0	0	0.200	70	0.600
53	0	0.100	0	0.800	24.5	0	0.035	0	0.600
54	0	0.050	0	0.400	35.0	0	0.035	0	0.600
58	0	0.050	0	0.400	-	-	-	-	-

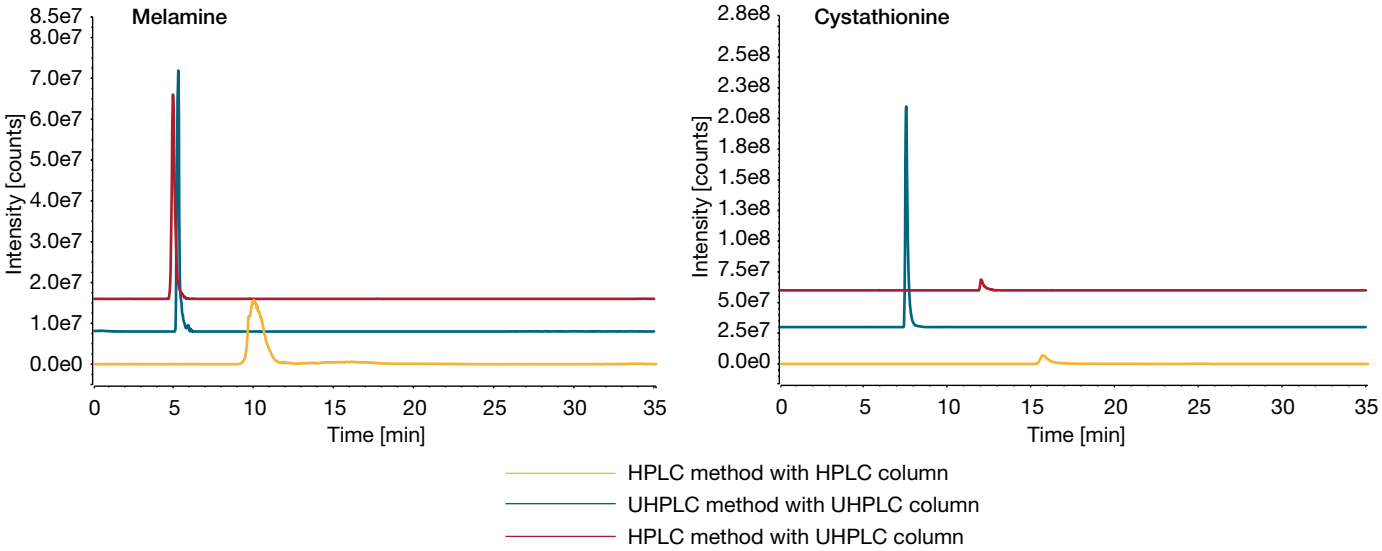

Figure 3. HILIC separation: Overlay XICs for melamine (logD at pH 7 = -2.02) and cystathionine (logD at pH 7 = -3.57). Orange trace = HPLC method with HPLC column; red trace = HPLC method with UHPLC column, and blue trace = UHPLC method and UHPLC column. The HPLC column was 150 × 2.1 mm (5 µm particle size) and the UHPLC column was 100 × 2.1 mm (1.7 µm particle size).

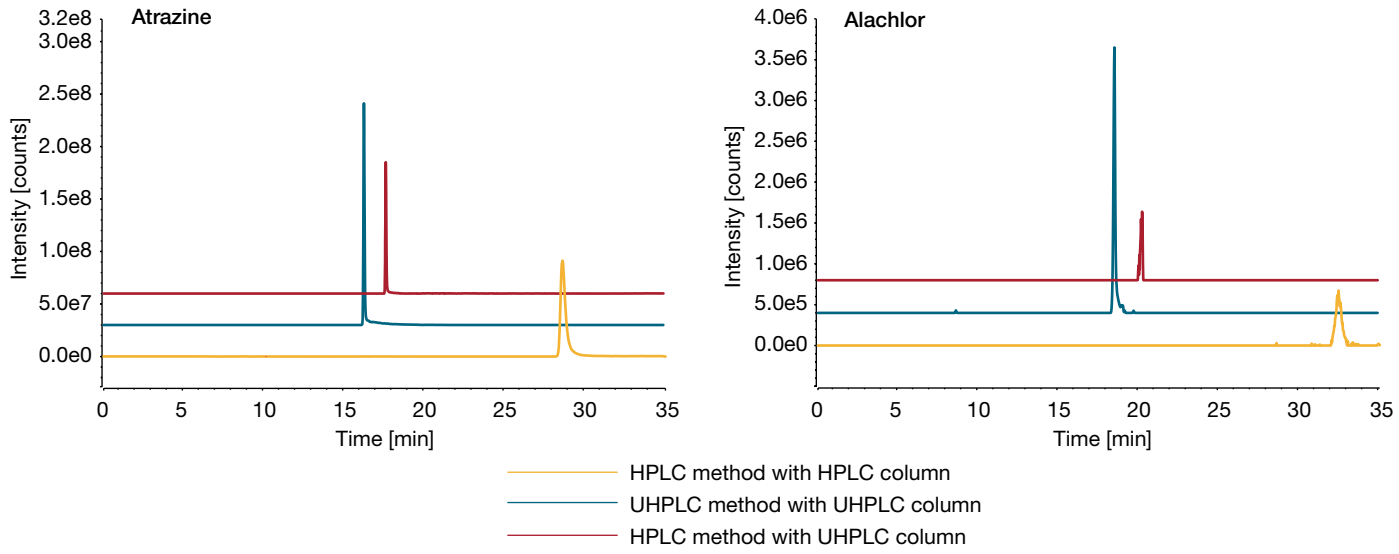

Figure 4. RP separation: Overlay XICs for atrazine (logD at pH 7 = 2.20), and alachlor (logD at pH 7 = 3.59). Orange trace = HPLC method with HPLC column; red trace = HPLC method with UHPLC column, and blue trace = UHPLC method and UHPLC column. The HPLC column was 50 × 3.0 mm (2.7 µm particle size) and the UHPLC column was 50 × 2.1 mm (2.6 µm particle size).

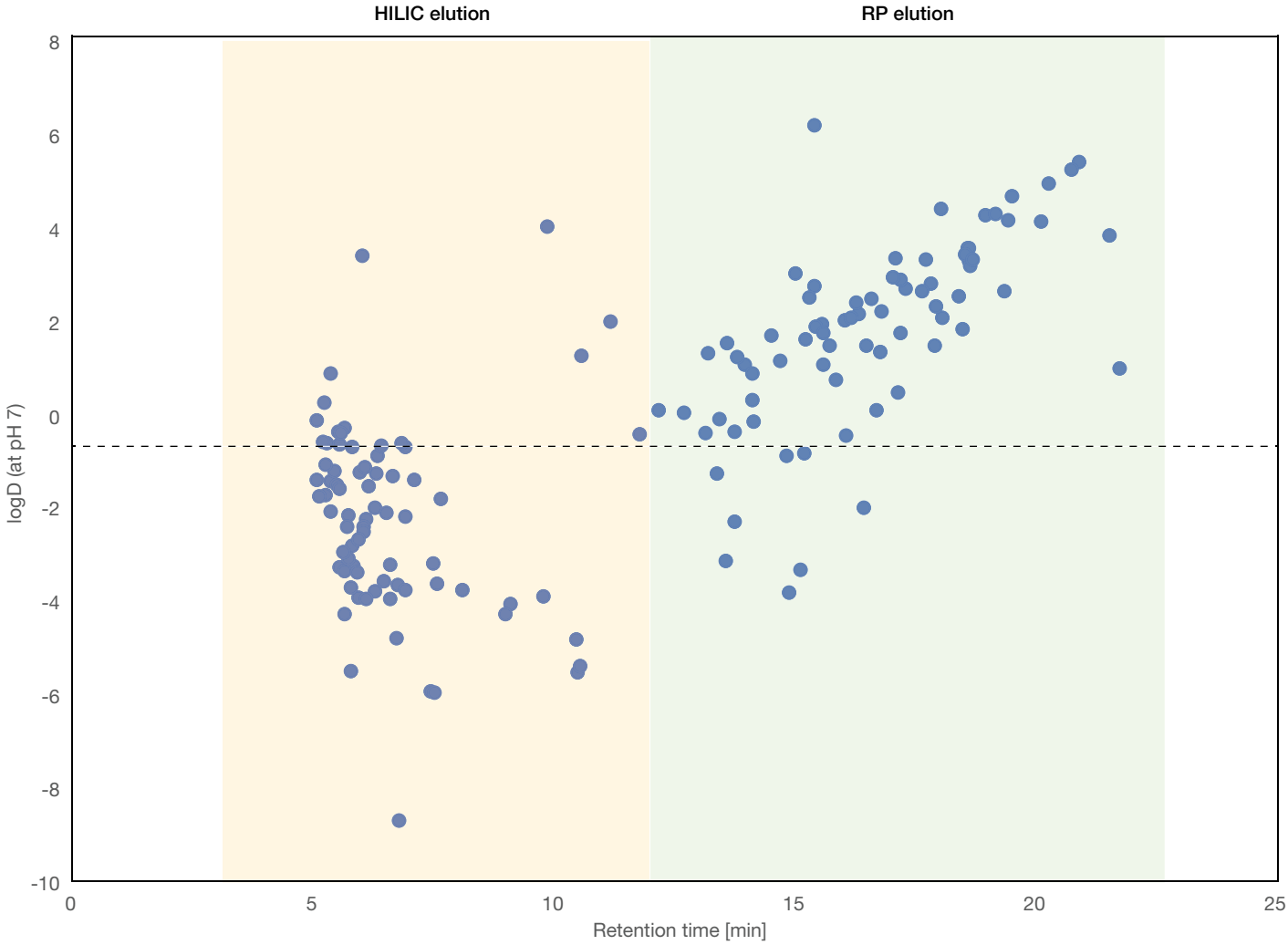

Figure 5. RT/logD plot of a mixture containing 150 compounds obtained with the optimized UHPLC method

b) Reconditioning conditions

Reconditioning of the chromatographic system is important to ensure reproducible separations. Equilibrium in the column is not dependent on re-equilibration time but on the volume of solvent passing through the column. RPLC requires about 10 column volumes, whereas HILIC requires up to 20–30 column volumes for full re-equilibration. With a column dimension of 100 × 2.1 mm for the HILIC column, the column volume is approximately 250 µL. With the pre-defined flow rates of 0.035 mL/min from the RP pump and 0.6 mL/min from the HILIC pump, the re-equilibration should be sufficient within eight to twelve minutes. The re-equilibration state was tested with several injections after defined re-equilibration times. It was determined that a re-equilibration phase of eleven minutes resulted in sufficient RT reproducibility (Figure 6) and peak shape (Figure 7). Insufficient equilibration can cause significantly changed retention behavior, due to altered retention effects.

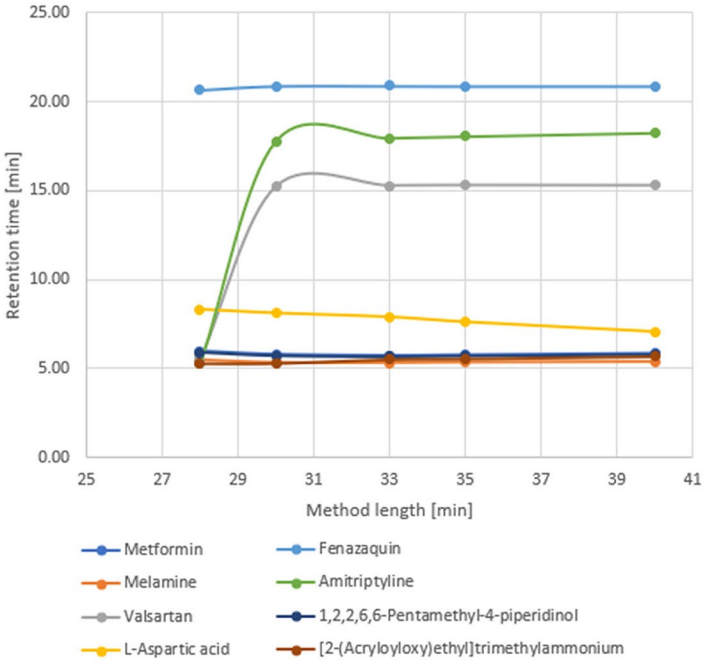

Figure 6. Retention time plots of experiments with different re-equilibration times resulting in different method length. Metformin logD at pH 7 = -2.06; Melamin logD at pH 7 = -2.02; Valsartan logD at pH 7 = 2.54; L-Aspartic acid logD at pH 7 = -5.37; Fenazaquin logD at pH 7 = 5.42; Amitriptyline logD at pH 7 = 2.12; 1,2,2,6,6-pentamethyl-4-piperidinol logD at pH 7 = -2.11; [2-(Acryloyloxy)ethyl]trimethylammonium logD at pH 7 = -3.23.

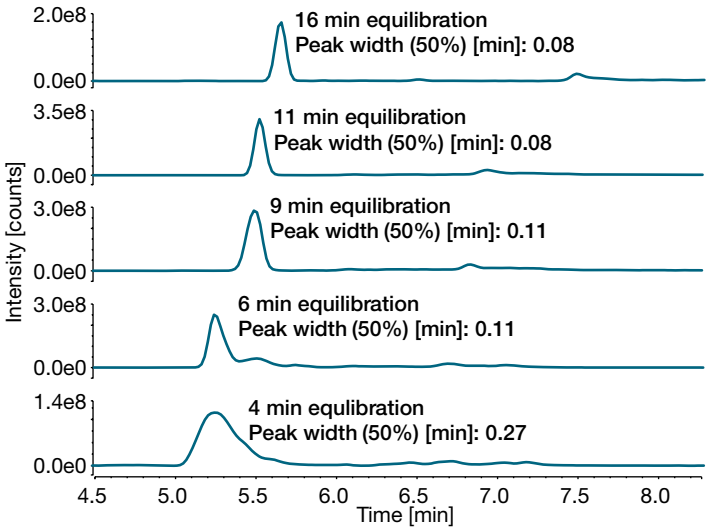

Figure 7. Chromatograms of [2-Acryloyloxy)ethyl]trimethylammonium (logD at pH 7 = -3.23) for different re-equilibration times. Peaks are labeled with equilibration time and peak width at half height.

c) Total analysis time

The total run time of the HPLC method was almost 60 minutes. Since modern LC instrumentation is highly robust under UHPLC conditions (UHPLC pump and sub 2 µm particles and/or core shell particles) and current MS technology operates at higher scan rates, the run time was significantly decreased from the original 58 minutes to 35 minutes. This was due to two independent factors, the separation speed/gradient steepness and the adjusted re-equilibration. This is a significant improvement in comparison to the original method and further helps to apply this method in high-throughput routine analysis.

Since the Orbitrap Exploris 120 mass spectrometer can switch between positive and negative ionization mode in one run, the total analysis time can also be significantly reduced. Thus, from initially 8 hours for a typical non-target screening experiment (one flushing blank, three replicate analyses of the sample in positive ionization mode, one flushing blank, three replicate analyses of the sample in negative ionization mode, each 58 minutes long), the time to analyze one sample can be reduced to approximately 2.5 hours (one flushing blank, three replicate analyses of the sample in positive and negative ionization mode in parallel, each 35 minutes). This is 40% of the initial analysis time (Figure 8). In addition, the solvent consumption was reduced substantially. The HPLC method consumed approximately 36.6 mL of solvent per run, which were 6.8 mL of water and 29.8 mL of acetonitrile in LC solvents of both pumps. This was reduced by the UHPLC method to 24.8 mL per run (8.9 mL of water and 15.9 mL of acetonitrile), which is a reduction by 32%. For one sequence, as shown in Figure 8, the HPLC method requires 292.4 mL of solvent, while the UHPLC method only consumes 99.2 mL. This is a savings of 18.7 mL of water and 174.3 mL of acetonitrile, a reduction of 66%.

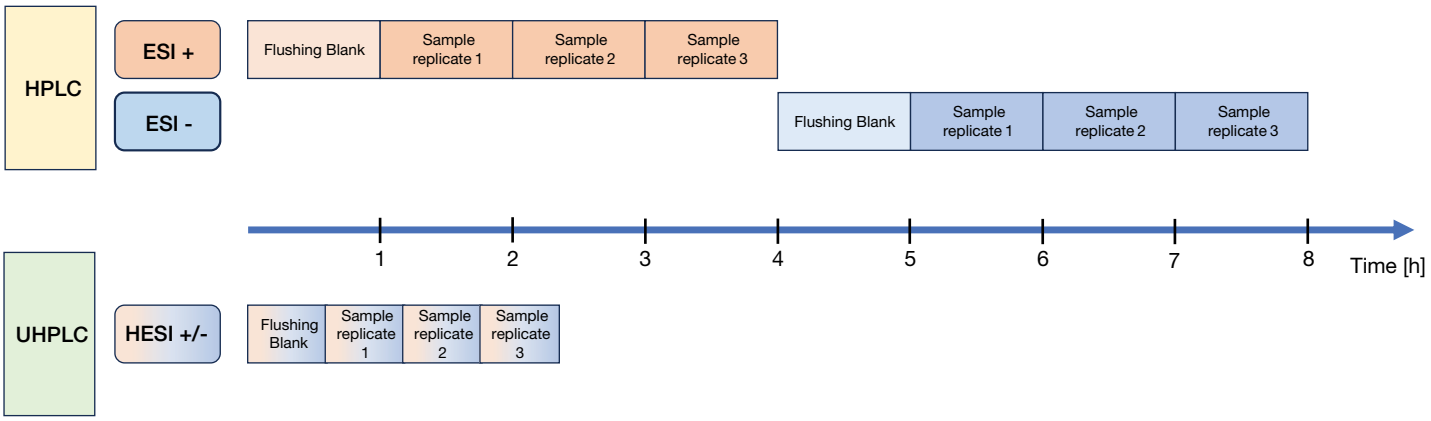

Figure 8. Total analysis time comparison of HPLC-MS und UHPLC-MS setup. The UHPLC-MS setup saves 70% of analysis time compared to the HPLC-MS setup.

Conclusion

A systematic transfer of the serial RPLC-HILIC coupling from an HPLC to an UHPLC setup with modern instrumentation and consumables was successfully performed. Transfer and optimization of the HPLC method allowed for:

- Reduction in separation time (including equilibration) by 40%
- Reduction in total analysis time of one sample using an Orbitrap Exploris 120 mass spectrometer with HESI positive/negative switching by 70%
- Reduction of solvent consumption by 32% per injection and 66% per sample

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