Technical Note

Serial RPLC-HILIC coupling hyphenated with Orbitrap mass spectrometric detection - Next generation in Non-Target Screening

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Abstract

For almost ten years the serial coupling of RPLC and HILIC columns (i.e., reversed phase liquid chromatography and hydrophilic interaction liquid chromatography) is well known in its combination with high accuracy and high-resolution mass spectrometric detection (HRMS). In that time, several studies have been published using this hyphenation in various disciplines like environmental analysis (e.g., aqueous samples), food analysis (e.g., drinking water and beverages), (plant) metabolism analysis, process monitoring (e.g., various oxidation techniques), and others.
The polarity-extended chromatographic separation allows the simultaneous separation of non-polar, polar and very polar molecules in one run and remains the same in coupling with HRMS, since the early days. Now, an updated serial setup using novel HPLC equipment in combination with an Orbitrap Exploris 120 mass spectrometer was developed and is ready to be used for future challenge of mass spectrometry and in special in non-target screening.

This technical note presents a state-of-the-art system and the performance within the system. The method development is included reflecting the systematic observation of quality parameter within the instrumental analysis strategy.

Motivation

The increasing interest in holistic views on complex samples forces chromatographic separation techniques to become more broaden and directly compatible with sensitive and accurate and high-resolution spectrometric detection (HRMS). In an early overview in 2012 [1] it has been shown that polarity-extended chromatographic separation techniques (like the serial coupling of RPLC with HILIC) can separate simultaneously non-polar, polar and very polar molecules in one run. Two white paper manuscripts in this 'AFIN-TS Forum' format reflected the wide-spread application of serial RPLC-HILIC in combination with HRMS in water [2] and various other disciplines [3]. This separation technique was published in far more than 35 (peer-reviewed) articles (applied in various disciplines and measured as aqueous solutions or other solvent extracts). Therewith molecules could be separated in a very wide polarity range (up to log(D) values – 7 to + 7) [3,4]. Consequently, the system was used more or less unchanged -with only slight modifications- now for more than 10 years.

On this anniversary and due to new separation equipment available on the HPLC market (like ultra-high-pressure liquid phase pumps, sub-2 µm particles, etc.) the original instrumental setup was totally rebuilt and established with up-to-date instruments and equipment for the next decade. Thereby the sample injection stability (especially for HILIC separation performance), the retention time robustness (< 2%) and other quality criteria shall remain the same as earlier [4]. On the other hand, the separation should speed up (from 58 minutes down to about 35 minutes), the formerly applied salt gradient in the mobile phase should be prevented-in HILIC and the starting content of water should be at 5%. Last but not least due to new mass spectrometric ion source concepts the former implemented make-up flow could (sometimes) become superfluous. The arrangement of the instrumental components (Figure 1) remains the same like in the formerly described hyphenation.

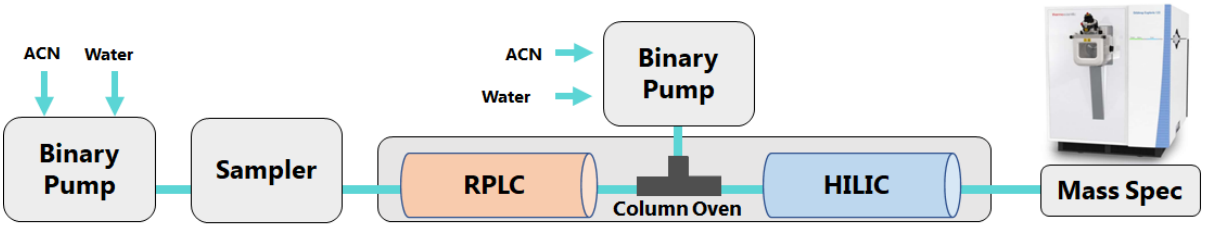


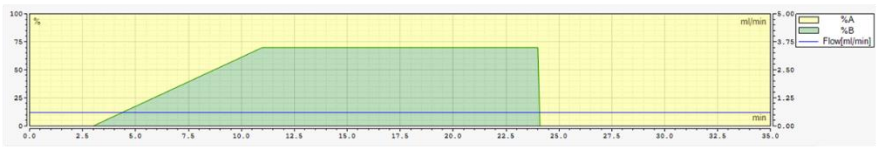
Figure 1: Scheme of the serial column coupling hyphenated with an Orbitrap mass spectrometer. The details of the new experimental setup can be found in the Methods part of this article.

The Analytical Solution

The application of smaller particles in the stationary phases in combination with ultra-high pressure liquid phase pumps could speed up the separation to a total of 35 minutes. This is due to higher flow rates in a) the HILIC column (1.7 µm particles size) with 600 µL/min flow rate and b) the RPLC column (2.6 µm particles size) with maximum 200 µL/min flow rate (Figure 2), resulting in faster mobile phase gradients and re-equilibration.

HILIC

t [min]	%B	Flow rate [mL/min]
0	0	0.6
3	0	0.6
11	70	0.6
24	70	0.6
24.1	0	0.6
35	0	0.6



RPLC

t [min]	%B	Flow rate [mL/min]
0	0	0.035
9	0	0.035
9.1	0	0.2
21	100	0.2
23.9	100	0.2
24	0	0.2
24.1	0	0.035
35	0	0.035

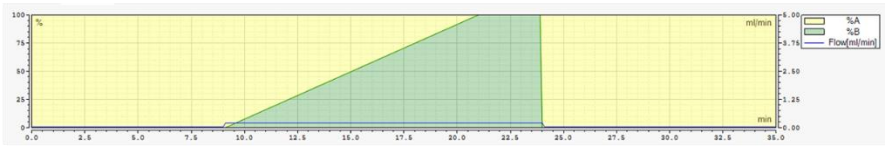


Figure 2: Overview of the setup conditions of the flow system.

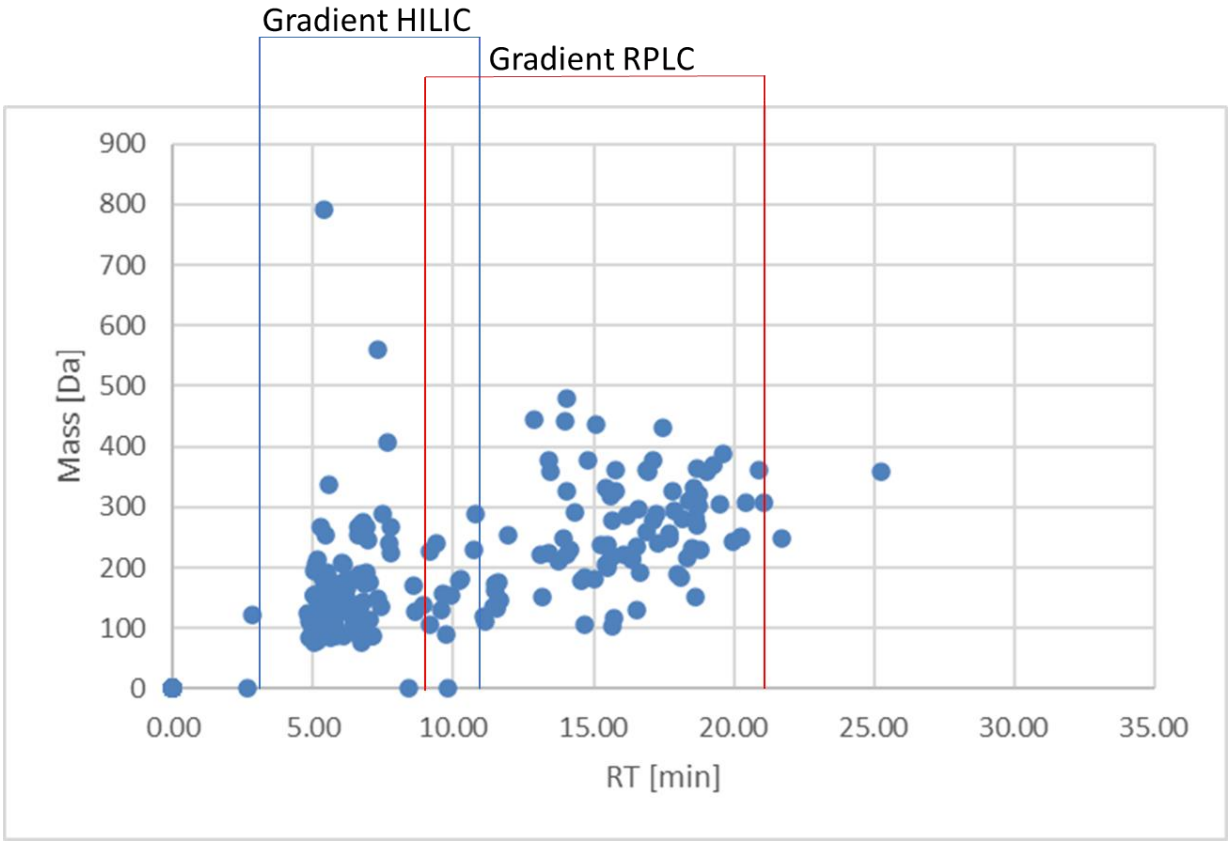


Figure 3: Retention time – mass plot in the new serial coupling for 296 organic molecules.

Figure 3 schematically presents the reference substances which were separated with the final flow gradient in the HILIC column (see left hand side the blue box) and the subsequent flow gradient in the RPLC column (see right hand side the red box)

Material and Methods

Chemicals and Solutions

Ultrapure water (H_2O) and acetonitrile (ACN) were obtained at LC–MS grade from Merck (Darmstadt, Germany) and Honeywell (Morristown, NJ, USA), respectively. Ammonium acetate (NH_4Ac) was purchased from Sigma-Aldrich (Seelze, Germany). Information on applied standard compounds (in the

log D (at pH 7) range of -7 to +7) is given in a former publication [4] These standards were obtained from ACROS organics (Thermo Fisher Scientific, Geel, Belgium), Alfa Aesar (Thermo Fisher Scientific, Karlsruhe, Germany), Cayman Chemical Company (Ann Arbor, Michigan, U.S.A.), CHEMOS GmbH (Regenstauf, Germany), Dr. Ehrenstorfer (Augsburg, Germany), Merck KGaA (Darmstadt, Germany), Fluka (Buchs, Switzerland), Sigma-Aldrich (Seelze, Germany), Supleco (Bellefonte, Pennsylvania, U.S.A.), and TCI (Eschborn, Germany).They were prepared in individual stock solutions of 1000 µmol/L, 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.

Instrumental Setup

The chromatographic setup consisted of a Vanquish Flex LC system (Thermo Fisher Scientific, Germering, Germany) with two binary pumps, an autosampler and one column oven, which contained a HILIC and a RPLC column coupled in series via a T-piece with a mixing frit (Upchurch, IDEX Europe GmbH, Erlangen, Germany). The RP separation was carried out on a ThermoFisher Accucore C18, (50 x 2.1 mm, 2.6 µm, P/N 17126-052130). The mobile phase of the RP separation pump consisted of A: H_2O/ACN 95/5 (v/v) with 5 mM NH_4Ac and for B: ACN/H_2O 95/5 (v/v) with 5 mM NH_4Ac. The HILIC separation was carried out on a ThermoFisher Synchronis HILIC (100 x 2.1 mm, 1.7µm, P/N 97502-102130). The mobile phase of the HILIC separation pumps consisted for solution A: ACN and for solution B: H_2O/ACN 95/5 (v/v).

Information on the gradients can be found in Figure 2 (left side). The injection volume was 10 µL.

The chromatographic system was connected to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific GmbH; Dreieich, Germany) equipped with an electrospray ionization (H-ESI) source. The source was operated at spray voltages of 3500 and -2500 V in the positive and negative modes, respectively. Sheath gas, auxiliary gas and sweep gas were set to 50, 8 and 0 (arbitrary units), respectively. The capillary temperature and the vaporizer temperature were set to 320 and 550 °C, respectively. In order to obtain NTS data, a mass range of 70–1000 Da was scanned at a resolution of 60,000 (full width at half maximum at m/z 200). MS2 spectra were acquired in the data-dependent acquisition mode at a resolution of 30,000 by employing a collision energy steps of 15 and 45 V. The

four most abundant precursor ions were selected to trigger after one scan cycle and afterwards excluded for 7 s.

Data evaluation was conducted in a targeted approach using TraceFinder 5.1 (Thermo Fisher Scientific GmbH; Dreieich, Germany) based on an in-house compound database, containing the sum formula of all analyzed reference compounds.

Background – How to perform such a serial chromatographic coupling?

The systematic development strategy for a state-of-the art 'serial RPLC-HILIC coupling'

The new polarity-extended chromatographic separation setup with the serial RPLC-HILIC coupling totally satisfies the requirements as described in the motivation section above, thus it is worth to try it also in further laboratories for many other applications.

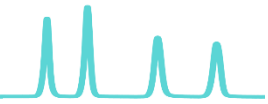
On the other hand, we realized that in the last decade many laboratories are struggling with a final decision of implementing a serial coupling of RPLC-HILIC instead of the routinely applied RP-setup. To clarify the easy but robust performance of this serial coupling the next sections present some key aspects in developing the new setup. Simultaneously, we hope that this highlights the robustness and applicability of such serial couplings (simply combining to gradient pumps via T-peace).

The column-separated method optimization for a state-of-the art 'serial RPLC-HILIC coupling'

At the beginning of the method optimization the RPLC and HILIC separations were evaluated separately in order to assess the contributions of the individual columns to the overall separations. Therefore, only single columns were tested or the mobile phase composition of the column which was not investigated in an experiment was held at high elution strength (high acetonitrile content in RPLC or high water content in HILIC).

a) HILIC separation

The initial method for the HILIC separation consisted of an isocratic initial phase of six minutes at 100% acetonitrile, followed by a mobile phase gradient to 60% acetonitrile and 40% water within seven



minutes (5.7%/min). In a first test, this method was used with the new setup and the new stationary phase. The mobile phase gradient was followed by an isocratic hold at 40% water in order to evaluate the elution of highly retained compounds. In a next step, the gradient was increased step by step, to promote the elution of these compounds. Finally, a gradient up to 70% water showed the most optimal performance. The gradient steepness was increased to 8.8%/min resulting in a gradient from 0 to 70% water in 8 minutes. The final flow rate was set to 0.6 mL/min

b, RPLC separation

The initial method consisted of an isocratic hold at beginning a 100% solvent A for seven minutes, followed by gradient to 50% solvent B within 5 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 9 minutes. This was simplified by decoupling the mobile phase gradients and the flow rate increase. In the final 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. The following mobile phase gradient increased the content of solvent B from 0 to 100% within 12 minutes (8.3%/min).

The column-coupled method optimization for a state-of-the art 'serial RPLC-HILIC coupling'

a) Salt Conditions

In the original method there was a salt gradient influencing the water layer thickness. This was due to the fact that the mobile phases consisted of mixtures of acetonitrile and 10 mM ammonium acetate in water. In the new method, the solvents were changed to mixtures of acetonitrile and water with a final content of 5 mM ammonium acetate. As a consequence, the water layer should not be influenced by changing salt contents during mobile phase gradients (thus hopefully a further increased robustness in HILIC). Whereas the separation mechanism in RPLC, with non-polar endcapped stationary phases is almost independent from the salt content, HILIC is very sensitive influenced by salt changing.

b) Reconditioning Conditions

Equilibrium is not dependent on re-equilibration time but on solvent volumes passing through the column through the column. RPLC requires about 10 column volumes whereas HILIC requires usually up to 20-30 column volumes for full re-equilibration. With a dimension 100 x 2.1 mm for the HILIC column, the column volume was approximately 250 µL. With the pre-defined mobile flow-rates of 0.035 mL/min from pump 1 and 0.6 mL/min from pump 2, the re-equilibration should be sufficient within 8 to 12 minutes. The re-equilibration state was tested with several injections after defined re-equilibration times. Finally, a re-equilibration phase of eleven minutes showed suitable results in RT reproducibility (Figure 4).

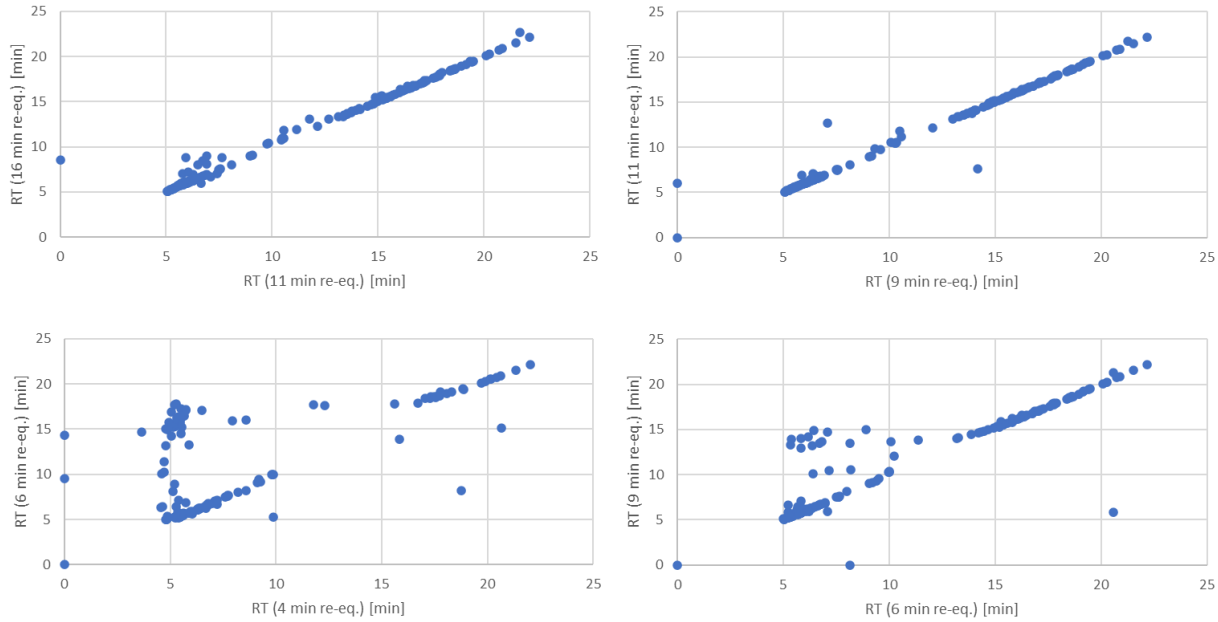


Figure 4: Retention time plots of injections with different re-equilibration phases.

c) Total run time

The original method was almost 60 minutes. Since liquid chromatography is highly stabile in ultra-high-pressure conditions (using UHPLC pumps and sub-2 µm-particles and core-shell particles) and since mass spectrometer work today in higher detection frequencies, the run time should and could significantly be reduced. This was due to two independent factors, the separation speed and the

adjusted re-equilibration phase. As a consequence, the final method could be speeded up to a total run time of 35 minutes. This is a significant improvement in comparison the original method and further helps to apply this method in high-throughput routine analysis.

RPLC-HILIC-ESI-Orbitrap-MS and NTS – a perfect match

The new RPLC-HILIC LC setup allows now to screen complex samples for very polar to non-polar compounds within 35 minutes, in contrast to nearly 60 minutes before. With the power of the Exploris MS instrument, this offers the chance to comprehensively screen samples in high-throughput. Since the Exploris 120 MS is also capable of analyzing positive and negative ionization mode in parallel, the analysis time can significantly be reduced. From initially 8 hours (1 flushing blank, three replicate analyses of the sample in positive ionization mode, 1 flushing blank, three replicate analyses of the sample in negative ionization mode, each 60 minutes long) the time to analyze one sample could be reduced to approximately 2.5 hours (1 flushing blank, three replicate analyses of the sample in positive and negative ionization mode in parallel, each 35 minutes). This is less than 1/3 of the initial analysis duration. All in all, a perfect setup for routine analysis.

The future and 'Applying serial RPLC-HILIC coupling in the next decade'

One decade after introducing the RPLC-HILIC-MS(/MS) into the analytical community (and in focus on non-target screening) the technique has come of age from pure discovering applications to a more broaden use in research, commercial and public laboratories. In recent years, several laboratories have installed the serial coupling of RPLC and HILIC. Some of them were supported by AFIN-TS in implementing their adapted analytical concept for receiving new insights in complex samples. The next step is a further distribution of polarity-extended chromatography in NTS on a global stage. The time has come, the systems are ready.

Acknowledgements

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