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**Impact of conditioning runs on hydrophilic interaction chromatography (HILIC)
repeatability and its application as a second dimension in online comprehensive two-
dimensional liquid chromatography.**

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Abstract

A common separation approach for polar compounds involves coupling reversed-phase liquid chromatography (RPLC) with hydrophilic interaction chromatography (HILIC) in two-dimensional chromatography. The higher proportion of acetonitrile used in the HILIC mobile phase, which enhances mass spectrometry detection, encourages its use in the second dimension. Previous studies demonstrated that the HILIC column can be partially equilibrated within very short timeframes without compromising retention time stability, rendering it suitable in online LCxLC setups. In addition, a specific number of conditioning cycles seems necessary to establish stable retention times. Here, the repeatability of HILIC when employed as second dimension in LCxLC was investigated, with a focus on determining the required number of conditioning cycles to achieve repeatable retention times. Various parameters influenced by the LCxLC online modulation system were studied, such as steep gradient slopes up to 8%, and very short equilibration times, less than or equal to dead time, as well as injection volume and solvent, which depend on the first dimension. Finally, the use of HILIC as a second dimension with tailored conditioning runs was applied to the analysis of hyaluronic acid hydrogel digests. The application of an RPLCxHILIC method using five conditioning runs, yielded exceptional stability in second-dimension retention times.

Keywords: RPLCxHILIC, conditioning runs, two-dimensional liquid chromatography, repeatability, hyaluronic hydrogel

Abbreviations¹

¹ Abbreviations

1D-LC: One-dimensional liquid chromatography

¹D: First dimension

²D: Second dimension

LCxLC: Online comprehensive two-dimensional liquid chromatography

RP: Reverse phase

1. Introduction

Online comprehensive two-dimensional liquid chromatography (LCxLC) is widely employed for the analysis of complex samples due to its high peak capacity. To achieve optimal results in these methods, it is essential for the two chromatographic systems involved to be as orthogonal as possible. The most commonly utilized combination is RPLCxRPLC, which involves two reversed-phase modes with potential adjustments to the mobile phase pH [1]. This combination is preferred due to the compatibility of the mobile phases and the rapid equilibration of the RP stationary phases. However, this separation mode has limited orthogonality and may not be suitable for highly polar compounds. In such cases, the HILIC mode can be employed as one of the two dimensions, although this necessitates more complex method development due to the mobile phase incompatibilities and potential injection effects [2,3].

The HILIC technique relies on the adsorption of a water layer on the stationary phase, necessitating extended equilibration times, especially when used in gradient mode. For example, the literature suggests a minimum of 40 column volumes for a complete equilibration, depending on the stationary phase and solutes [4,5]. Nevertheless, shorter partial equilibration periods are also feasible [4,6–8]. For example, McCalley showed that an equilibration step of 12.2 column volumes yields satisfactory retention time repeatability [6], while even as few as 5 column volumes were sufficient for consistent separation of antibiotic and antiretroviral compounds [4]. Such long equilibration times have encouraged the use of HILIC as the first dimension in online LCxLC setups. As so, the utilization of HILICxRPLC separation has yielded promising outcomes in the analysis of proteins and their glycoforms [9,10], industrial polymers [11], and food products such as phenolic compounds [12–15], as well as lipids analysis [16].

Nonetheless, HILIC as a second dimension is also of genuine interest. Its mobile phase is primarily composed of an organic solvent, typically acetonitrile (ACN), which has a lower viscosity than water, enabling faster separation in the second dimension. In addition, several studies have shown that HILIC can improve the sensitivity in electrospray ionization (ESI)-MS detection [17–20]. This improvement is mainly due to the desolvation process promoted by the high proportion of ACN in HILIC, as well as to the ionization mechanisms, such as proton transfer, pKa of analytes in the mobile phase, and mobile phase pH [18]. A comparative study of HILICxRPLC and RPLCxHILIC for the analysis of phenolic compounds demonstrated the detection of a higher number of compounds when HILIC was used as the second dimension before MS [21]. A similar result was observed in the comparison of RPLCxHILIC and HILICxRPLC dimension orders for lipid profiling in a biological sample [22].

Due to the considerable advantages of utilizing HILIC as the second dimension for the analysis of polar compounds, the RPLCxHILIC setup has garnered increasing interest over the last three years, although its implementation remains relatively limited [23]. Most of the reported setups used HILIC in an isocratic mode to avoid equilibration issues since the solvent proportions are maintained constant throughout the analysis [24–28]. However, this approach may restrict the elution range of analytes.

The modulation system used in online LCxLC requires a fast second dimension. In gradient mode, the ¹D modulation time (¹t_{mod}) is directly related to the ²D gradient time (²t_g) and equilibration time (²t_{eq}), the time to equilibrate the column to the initial gradient conditions, as shown in equation (1).

$${}^1t_{\text{mod}} = {}^2t_{\text{g}} + {}^2t_{\text{eq}} \quad (1)$$

To increase the peak capacity of the second dimension, the ²D slope should be shallow to ensure ²t_g is as long as practically feasible. To avoid an increase in the modulation time, which may lead to under-sampling of the first dimension [3,29], it is crucial to minimize ²t_{eq}. The equilibration time depends on both the equilibration volume required to achieve stable retention times and the flow rate. The maximal flow rate is always used in the second dimension. Consequently, the only parameter that can be reduced is the equilibration volume.

Few attempts explored the use of HILIC in gradient mode as a second dimension. In the development of RPLCxHILIC methods for peptides [30] and lipids analysis [22,31], equilibration volumes of three to six volumes (V_c) of HILIC column, as it is typically used for one-dimensional RPLC, were employed. For the analysis of phenolic compounds [21], samples from traditional Chinese medicine [32], and a chiral LCxHILIC method used for amino acid separation [33], equilibration volumes of one- to two-column volumes, typical for second-dimensional RPLC, were utilized. In a recent study, the ²D HILIC column was equilibrated with less than one-column volume (0.6 V_c) [34], without any distortion of the spots, which indicates repeatability between modulations. Nevertheless, evaluation of the repeatability of the second dimension, such as monitoring ²t_r between modulations or between repeated injections was not conducted. Despite the common recommendations for full equilibration in HILIC, these results highlight that HILIC can be used with very small equilibration volumes.

The equilibration of HILIC columns has been the subject of several studies in both isocratic and gradient modes. McCalley investigated the effect of stationary phase and pre-equilibration solvent on the complete equilibration time [5,6]. Notably, the choice of the stationary phase was found to have a significant effect, with zwitterionic phases requiring

longer equilibration compared to other phases. Furthermore, pre-equilibration conditions resembling the equilibration conditions imply faster equilibration, so the final gradient composition may influence the equilibration time. Increasing the column temperature from 20 to 45 °C, as well as decreasing the surface area of the stationary phase, were found to decrease the equilibration time. The author also observed that the duration of a partial equilibration in gradient mode significantly influences the selectivity [6]. Despite these observations, retention times remained reproducible for all partial equilibration times tested, with a relative standard deviation of less than 0.2%, similar to repeatability after a full column equilibration. Thus, full equilibration is not required to achieve stable HILIC retention. Another important aspect is the need for several "conditioning runs" or "blank gradients" to stabilize compound retention. In McCalley's studies [5,6], 1-6 injections were performed without data collection to achieve retention repeatability. Similarly, in a study focusing on the repeatability of HILIC analysis of antibiotic and antiretroviral compounds, six conditioning runs (successive elution gradient programs performed before injection) were carried out: five with full equilibration time and one with the studied partial equilibration time [4]. Finally, Mc Calley suggested that "*higher precision could be obtained by increasing the number of conditioning runs*" [6].

Seidl et al. studied the influence of mobile phase (pH, buffer concentration, preparation method), flow rate (0.3 to 1.2 mL/min), analytes, stationary phase properties (chemistry and column length), and equilibration time, on the retention time stability. They showed that increasing the flow rate reduced the equilibration time and enhanced repeatability, while buffer concentration and mobile phase pH did not affect repeatability, except for the ZIC-HILIC column. Remarkably, after a fast equilibration of 3 s, corresponding to two-column volume, retention time repeatability showed a standard deviation lower than 0.001 min, enabling for a HILICxHILIC analysis. Nonetheless, the ²D normalized gradient slope, which remained below 2%, was slow for a second dimension. Unfortunately, the authors did not mention any conditioning runs.

In one-dimensional HILIC separations, conditioning runs are often performed before the sample sequence. For instance, metabolomic studies recommended 5 to 20 conditioning runs [35–39], while glycoproteins analysis suggested two conditioning runs [40]. These conditioning runs are crucial for achieving repeatable HILIC results. However, to the best of our knowledge, their specific impact has not been investigated in the context of HILIC as the second dimension of LCxLC.

In this study, we aimed to assess the repeatability of HILIC when used as the second dimension in a gradient-mode LCxLC. Our primary focus is determining the number of

conditioning runs required to achieve stable retention times. Given the constraints of the LCxLC online modulation system, the second dimension must be as short as possible, which requires a steep gradient slope and a minimal equilibration time. Therefore, we evaluated the effect of both the gradient slope (with normalized values over 2%) and the equilibration volume (less than one column volume) on retention time repeatability. The immobilized water layer in the HILIC may be disturbed by the injection of a large amount of water from the ¹D RPLC sampling, potentially influencing the equilibration process and the retention time repeatability. Therefore, the effect of solvent and injection volume on the required number of conditioning runs was also investigated. Finally, we applied the obtained results to a repeatability assessment of an RPLCxHILIC method developed for the analysis of an industrial mixture of cross-linked polysaccharides derived from a hyaluronic hydrogel.

2. Material and methods

2.1. Chemicals

LC-MS grade acetonitrile was purchased from Riedel-de-Haën (Seelze, Germany). Uridine (purity $\geq 99\%$), nortriptyline hydrochloride (purity $\geq 98\%$), amoxicillin (purity between 95.0-100.0%), saccharose (purity $\geq 99.5\%$), 4-hydroxybenzoic acid (purity $\geq 99\%$), and sodium chloride were obtained from Aldrich (Steinheim, Germany). Analytical reagent-grade ammonium acetate, formic acid (98-100%), and calcium chloride were purchased from Fisher Scientific (Illkirch, France) and Carl Roth (Karlsruhe, Germany). Deionized water was produced using an Elga Purelab Classic UV purification system from Veolia water STI (Décine-Charpieu, France). HYase (Hyaluronidase from *Streptomyces hyalurolyticus*) and chABC (Chondroitinase ABC from *Proteus vulgaris*) were acquired from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2. Sample preparation

Individual stock solutions were prepared in water at a concentration of 1 mg/mL for amoxicillin, saccharose, and 4-hydroxybenzoic acid, and prepared in acetonitrile at a concentration of 1 mg/mL for uridine and nortriptyline. Working solutions were then derived from these stock solutions, with concentrations of 10 $\mu\text{g/mL}$ for saccharose, 20 $\mu\text{g/mL}$ for nortriptyline and uridine, 30 $\mu\text{g/mL}$ for 4-hydroxybenzoic acid, and 40 $\mu\text{g/mL}$ for amoxicillin. The volumes of the diluting solvents, acetonitrile and water, were adjusted to achieve a final composition of 95/5, 50/50, or 5/95 $\text{H}_2\text{O}/\text{ACN}$ (v/v) as required by the experiment.

The enzymatic digestion of crosslinked polysaccharides was performed as follows: two syringes of hyaluronic acid hydrogel supplied by Allergan were mixed with 200 mL of a 150 mM NaCl saline solution and then filtered through 0.22 μm nylon filters (GE Water & Process Technologies, Feasterville-Trevose, USA). Subsequently, the hydrogel was washed with 200 mL of ultrapure water and then filtered once more before being solubilized into 20 mL of water and freeze-dried. The resulting lyophilisate was dissolved in 1 mM ammonium acetate and 10 mM calcium chloride solution to achieve a concentration of 2 mg/mL. The enzymatic digestion was performed by adding 100 μL of HYase (300 U/mL) and incubating overnight at 37°C with continuous mixing at 150 RPM. Then, 100 μL of chABC at 5 U/mL was added every 3 hours, with continuous incubation and mixing, and a final addition was made approximately 12 hours later. The progression of digestion was monitored by measuring the optical density at 232 nm using a NanoDrop One^c (ThermoScientific, Waltham, USA). Following digestion, the sample was freeze-dried, and a 30 mg/mL suspension of the digested powder in water was prepared for further analysis.

2.3. Instrumentations

The 1D-LC was carried out with an LC1290 Infinity I system hyphenated with an Infinity Lab MSD (Mass Selective Detector, model 6125B) and diode-array UV absorbance detector equipped with a 0.6 μL flow-cell, set at 80 Hz, all from Agilent Technologies (Waldbronn, Germany). The system is equipped with two high-pressure binary pumps, and an auto-sampler featuring a flow-through needle injector with an injection time of 30 s. The LC-UV-MS system was controlled by Agilent OpenLab software.

The analysis of the digested hyaluronic acid hydrogel was performed using a 2D-LC1290 Infinity II hyphenated with an Agilent QTOF mass spectrometer (model G6545B). The dwell volumes for the first and the second dimension were 170 μL and 80 μL (loop volume excluded), respectively. The 2D-LC-UV was operated by Agilent OpenLab software, while the QTOF mass spectrometer was controlled by Agilent MassHunter software.

The data were processed using the Agilent MassHunter workstation Qualitative Analysis 10.0, and 2D plots were realized with Matlab (R2020a, 9.8.01323502).

2.4. One-dimensional HILIC

The 1D HILIC separations were performed using three different columns: an Acquity BEH Amide column (150x2.1 mm; 1.7 μm , 130 Å), an Acquity BEH HILIC (100x2.1 mm; 1.7 μm , 130 Å) from Waters (Milford, MA, USA), and a SeQuant ZIC-HILIC column (100x2.1 mm; 3.5 μm , 200 Å). The column temperature was maintained at 45 °C. The mobile phase consisted of two components: (A) ACN and (B) H₂O+20 mM ammonium acetate, at pH=6.8. Prior to each set of experiments, the column was flushed with ten column volumes of mobile phase composed of 40% B. The gradient was programmed to obtain a normalized slope of 2, 5, and 8 %, depending on the specific experiment. The flow rate and the isocratic step were optimized to achieve an appropriate equilibration volume, taking into account the instrument injection time (30s). Injection volumes were set at either 1 % or 5 % of the column volume, respectively 3 μL and 15 μL for BEH Amide and 2.4 μL for both BEH HILIC and ZIC-HILIC corresponding to 1% for column volume. Each set of experiments involved ten consecutive injections.

Amoxicillin, uridine, and 4-hydroxybenzoic acid were detected using UV absorbance at 275 nm, while nortriptyline was detected at 215 nm. Saccharose was detected using MS with electrospray ionization in negative mode, specifically following the ion m/z 341 in single ion monitoring mode. For the MS detection of saccharose, the following parameters were utilized: the drying gas temperature, 350°C, the drying gas flow, 12 L/min, the nebulizer pressure, 35 psi, the capillary voltage, 6000 V, the fragmentor voltage, 100 V, and the acquisition rate, 3.3 Hz.

2.5. RPLCxHILIC conditions for the analysis of a digested hyaluronic acid hydrogel

The hyaluronic acid hydrogel was analyzed with a RPLCxHILIC-QToF method. The ¹D RPLC was performed using a Luna Omega Polar C18 (50x1 mm, 1.7 μm) column purchased from Phenomenex (Torrance, California, USA), and the ²D HILIC separation was carried out using the BEH Amide column (50x2.1 mm, 1.7 μm). Details of the RPLCxHILIC conditions can be found in Table 1. The instrument settings were drying gas temperature, 200°C; drying gas flow rate, 12 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 350°C; sheath gas flow rate, 11 L/min; fragmentor, 185 V; capillary voltage, 5000 V; electrospray positive mode. Mass spectra data were collected using the full scan mode with a mass range of 100–3200 m/z and an acquisition rate of 5 Hz. To perform the necessary ²D conditioning runs before compound elution, the sample was injected four minutes after the beginning of the gradient.

Table 1: Experimental conditions of the RPLCxHILIC setup for the digest hydrogel separation in figure 6.

	First dimension (¹ D)	Second dimension (² D)
Stationary phase	Luna Omega Polar C18 (RP)	BEH amide (HILIC)
Column geometry	50x1 mm, 1.7 μm	50x2.1 mm; 1.7 μm
Mobile phase	A: H ₂ O+10 mM ammonium acetate / B: ACN	A: ACN / B: H ₂ O+20 mM ammonium acetate
Normalized slope	1.15	6.54
Gradient step	0% B for 4 min 0-25% B in 60 min 25-0% B in 2.75 min	5-70% B in 1.1 min 70-5% B in 0.11 min
Equilibration time	8.25 min (3t ₀)	0.12 min (1.1t ₀)
Temperature	30°C	50°C
Flow rate	10 μL/min	1.1 mL/min
Split	4:1, waste: loop	2:1, UV: MS
Injection	Delayed injection at 4.0 min 1 μL	3 μL
Sampling time (ts)	1.33 min	

A 2D-chromatogram is constituted of a succession of 2D fraction separations. The ¹D retention time, ¹tr of a given solute was assigned to the peak apex of its most intense peak between the fractions containing this solute, while the ²D retention time, ²tr representing the time spent in the second dimension, was calculated as the remaining between the ¹D

retention time 1t_r and the sampling time (also called modulation time), t_s , with n the number of modulations prior to the peak apex (Eq.2).

$$^1t_r = n t_s + ^2t_r \quad (2)$$

3. Results and discussion

Comprehensive two-dimensional liquid chromatography (LCxLC) requires a rapid equilibration of the second-dimensional column while maintaining repeatable retention times. Previous studies recommending long equilibration times in HILIC implied that using this separation mode as a second dimension in LCxLC might be impractical. However, several promising studies using HILIC as a second dimension indicated that HILIC separations can be reproducible even with a very short equilibration time of less than one column volume [8,21,34]. Furthermore, studies on repeatability in HILIC suggested that it can be improved by increasing the number of conditioning runs [5,6].

3.1. Methodology

This study aims to determine the necessary number of conditioning runs for achieving consistent retention times in conditions compatible with the use of HILIC as a second dimension. To this end, ten consecutive sample injections were performed and repeated on three different days, as illustrated in Figure 1. The standard deviation of the last three injections was calculated for each day. It was assumed that the variability between injections 8, 9, and 10 solely resulted from method variability rather than a lack of conditioning runs. Then, the maximum standard deviation among the three days (D1, D2, and D3), denoted as SD_{max} , was used to establish the retention window. This allowed us to consider the maximum method-induced variability that could be observed over the course of three days. If the calculated standard deviation was less than the acquisition rate (one point every 0.0002 min in UV and 0.005 min in MS), SD_{max} was set equal to 0.0002 min or 0.005 min as appropriate. When the retention time of injection n (denoted as t_{r_n}) fell within the retention window defined as $mean(tr) \pm 3 SD_{max}$, with $mean(tr)$ representing the average retention time of the three last injections in the experiment, the injections were deemed repeatable starting from injection n . In such cases, the required number of conditioning runs (CR) was $n-1$.

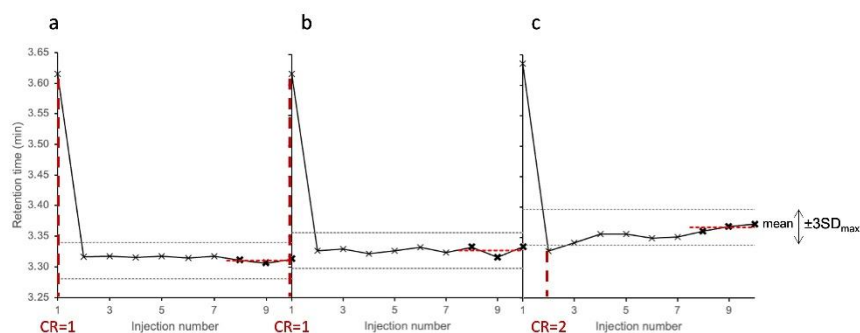


Figure 1: Evolution of amoxicillin retention time as a function of the number of injections on three different days. The black dotted lines represent the upper and lower limits between which retention time was considered repeatable and the red dotted lines represent the CR number.

A set of five compounds were selected (Figure 2): 4-hydroxybenzoic acid (acidic), nortriptyline (basic), amoxicillin (zwitterionic), uridine (neutral), and saccharose (neutral sugar). Three of these compounds, uridine, 4-hydroxybenzoic acid, and nortriptyline were the same as those studied by McCalley [5,6].

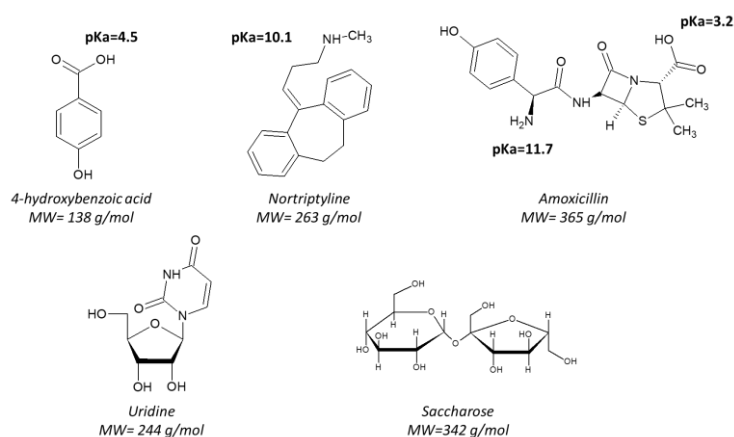


Figure 2: Structure and physicochemical properties of studied compounds

The effects of gradient and injection parameters were investigated for a BEH amide column. This column was chosen because of its pH and temperature stability [41] as well as its widespread use in saccharide separation [42]. To assess the effect of the stationary phase, two additional columns were also examined: the BEH HILIC and the ZIC HILIC.

To enable easier comparison of results, the initial experiment aimed to evaluate the inter-day repeatability of CR numbers determined under identical conditions. The experiments were

carried out using a BEH Amide column with a standardized slope of 5%, a mobile phase composition of 5% to 90% water, and an equilibration time equivalent to 0.5 column's dead time (t_0). Compounds were injected over the course of three days, each injection being 1% of column volume, with the diluting solvent consisting of 95% H₂O and 5% ACN (Figure S1 in the supplementary information). The variability between days was assessed by calculating the SDs over three days for the CR and this variability was compound dependent. As a result, a retention time window of ± 1 CR was observed for 4-hydroxybenzoic acid, amoxicillin, and uridine, while saccharose and nortriptyline exhibited a wider window of ± 2 CRs.

3.2. Effect of gradient slope and equilibration time

In the second dimension of the LCxLC, the compounds must elute during the modulation period of the first dimension. This typically leads to a steep ²D gradient and a final gradient composition enriched with the stronger solvent. However, increasing the aqueous content over 40% in the mobile phase disrupts the water layer characteristic of the HILIC mode [43,44]. Moreover, McCalley suggested that the number of conditioning runs should be adapted to the slope [5].

The effect of steep gradient slopes on the repeatability of HILIC retention times was examined in combination with a brief equilibration time (Figure 3a). Additionally, the experiments were performed with a final mobile phase composition of 90% water (except for the 2% gradient slope condition where the final composition was 70%). This approach was chosen to ensure a thorough disruption of the water layer, exposing the system to the worst-case scenario [45].

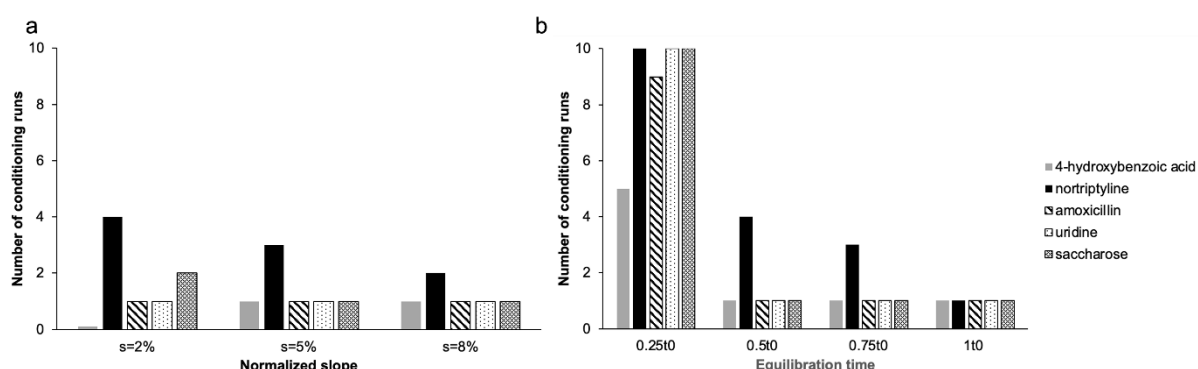


Figure 3: Effect of (a) the gradient normalized slope, and (b) the equilibration time on the number of conditioning runs. Column, BEH Amide, flow rate, 0.4 mL/min, (except for $t_{eq}=0.25 t_0$, flow rate 0.18 mL/min), gradient, from 5% to 90% B (except for $s=2\%$, from 5% to 70% B).

Injection volume, 3 μ L in 50/50 H₂O/ACN. (a) Equilibration time 0.5 t_0 , (b) normalized slope 5 %.

In contrast to the finding in McCalley's study [5], which stated that more CR are required for a 1%/min slope compared to a 5%/min slope, the results shown in Figure 3a indicate that the slope had no significant effect on the number of CRs required to reach repeatability, compared to inter-day variability (± 1 CR for 4-hydroxybenzoic acid, amoxicillin, and uridine, and ± 2 CRs for saccharose and nortriptyline). Both amoxicillin (zwitterionic) and uridine (neutral) required only one CR across all studied slopes. The variation of one conditioning run between the various gradient slopes, observed for 4-hydroxybenzoic acid (acidic) and saccharose (neutral), was not considered significant. A potential trend was noted for the basic solute nortriptyline, which required fewer CRs with steeper slopes. Consequently, slopes exceeding 2% (normalized with the column dead time) can be applied when using HILIC as a LCxLC second dimension. No slope greater than 8% was tested thus, as compounds may no longer elute beyond this value.

Subsequently, the use of very short equilibration times ranging from 0.25 t_0 to 1 t_0 have been investigated. The variation of equilibration times was achieved by adjusting an isocratic step before the gradient, taking into consideration the injection time (30 s), during which the column was flushed by the initial mobile phase. For the shortest equilibration time (0.25 t_0), the flow rate had to be reduced to 180 μ L/min due to the instrumental injection time.

Figure 3b illustrates that, for all compounds, retention times exhibited repeatability after a higher number of conditioning runs (between 5 and 10) when equilibration was set at 0.25 t_0 compared to longer equilibration time. In contrast, when the equilibration time was greater than or equal to 0.5 t_0 , it had no discernible effect on the required number of CRs. Since the retention of neutral compounds primarily depends on the water layer volume [43], we can reasonably conclude that a short equilibration volume of 0.5 V_0 is sufficient to facilitate the stabilization of the water layer on BEH amide column. For nortriptyline, the required CRs appeared to decrease with longer equilibration times. Notably, even with a short equilibration time of 0.5 t_0 , the relative standard deviations in retention times of the last three injections were very low, measuring below 0.12% for all the studied compounds.

This observation indicated that HILIC retention times remained repeatable even with an extremely brief equilibration time of less than one t_0 , provided that a certain number of conditioning runs were performed beforehand. This finding holds significant promise for the use of HILIC in two-dimensional liquid chromatography.

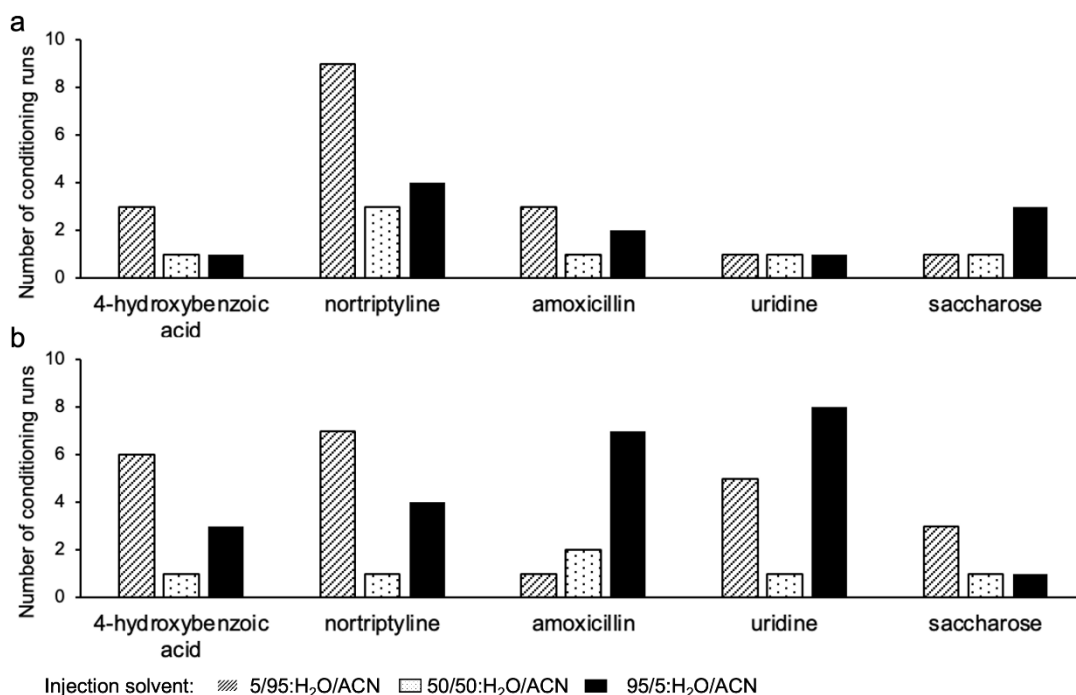
345

346 3.3. Effect of injection solvent type and volume

347 In online LCxLC, the composition of the injection solvent depends on the ¹D gradient
 348 composition if no specific instrumentation is used (e.g. Active Solvent Modulation). In
 349 addition, sometimes large volumes are injected. The effects of the incompatibility of mobile
 350 phases between HILIC and RPLC on peak shape have already been studied [46,47]. Here,
 351 we focused on the impact of the injection process on the repeatability of retention times, a
 352 relationship that has never been previously studied to our knowledge.

353 Three injection solvents were tested: the ideal case containing 5% water, an intermediate
 354 case with 50% water, and the worst case with 95% water in the sample diluent. The worst
 355 case can frequently occur in RPLCxHILIC, especially at the beginning of the ¹D gradient
 356 when highly polar compounds are eluted. Additionally, two injection volumes were evaluated,
 357 1% and 5% of the column volume. The study was performed with the minimum equilibration
 358 time necessary to achieve repeatable retention times: 0.5 t_0 .

359 The results are summarized in Figure 4, where the required number of conditioning runs to
 360 achieve repeatable retention times (CR) is plotted as a function of solvent and injection
 361 volume.



362

363 Figure 4: The number of conditioning runs for selected solutes determined for different
 364 injection solvent compositions (5%, 50%, and 95 % water) and two injection volumes: (a) 1%
 365 and (b) 5% of the column volume, and using the following settings: normalized slope 5%,

flow rate 0.4 mL/min, on BEH Amide column, equilibration time 0.5 t_0 . Injected volumes were (a) 3 μ L and (b) 15 μ L.

At low injection volume (1% of the column volume), the number of conditioning runs necessary for neutral and zwitterionic solutes remained unaffected by the water content in the sample diluent, as the CR variation was lower than inter-day variability (± 1 CR for 4-hydroxybenzoic acid, amoxicillin, and uridine, and ± 2 CRs for saccharose and nortriptyline). In contrast, nortriptyline and 4-hydroxybenzoic acid were notably affected: the higher the ACN content in the injection solvent, the greater the number of CR required. This finding was surprising because the proportions of the strong solvent injected were similar to the initial composition of the mobile phase. This discrepancy may be attributed to variations in the pKa values of the analytes in response to the proportion of organic solvent in the medium. Specifically, the pKa of acids increases with the proportion of acetonitrile, ranging from a pKa value 4 to 6.10 for 4-hydroxybenzoic acid at 0% ACN to 60% ACN [48]. Conversely, the pKa of bases decreases slightly [48,49]. Consequently, depending on the injection solvent, the environment at the head of the column could lead to modifications of ionic interactions.

No effect of solvent and injection volume on the number of CRs was observed for the most retained compound, saccharose (± 2 CRs inter-day variability). This remained true even when the water-enriched layer was disrupted by water injection (condition 95/5: H₂O/ACN). This lack of effect was likely due to its long retention, resulting in an on-column focusing of saccharose. A similar observation was made regarding the effect of equilibration time on compounds retention, as highly retained compounds remained unaffected by equilibration time [4].

On the other hand, when the injection volume was increased to 5% of the column volume (15 μ L), the trend was further confirmed for nortriptyline and 4-hydroxybenzoic acid. Moreover, for the acidic compound, the number of CRs increased with the injected volume, except for the intermediate composition of the diluent.

For uridine, the number of CRs increased with the injected volume, except for the 50/50: H₂O/ACN solvent proportion. The maximum number of required CRs was observed when the proportion of injected H₂O was maximal. The same result was observed for amoxicillin, with an increase in the number of CRs along with the increase in the injected volume and the injected proportion of water.

The increase in the number of CRs as the quantity of injected water increased was anticipated. Indeed, the aqueous phase serves as the strong solvent in HILIC, and the water

layer should be most affected under such conditions. However, the low number of CRs when the injection solvent closely resembled the intermediate composition of the gradient was more surprising. Although unexplained to date, the positive observations that injecting analytes into an intermediate solvent required fewer conditioning runs were consistent over the days and effective for all tested compounds. Unfortunately, this intermediate composition represents only a few sampling fractions when dealing with online RPLCxHILIC setups. Trends in the evolution of CRs with injection volume and solvent seemed to depend on the compounds and their nature, whether neutral or ionized, as well as on compound retention on the column. This may explain the varying recommendations found in the literature regarding the number of CRs to be performed in one-dimensional HILIC.

3.4. Effect of the stationary phase

The previous parameters were studied on a BEH Amide column. However, the stationary phase can influence the number of CRs. Specifically, the thickness of the water layer is linked to the nature of the stationary phase [50–53]. For example, bare silica stationary phases generate a thinner water layer than zwitterionic phases. Moreover, it was shown that the time required to achieve full equilibrium depends on the stationary phase; it is longer for the zwitterionic column and shorter for the bare silica column [6]. Therefore, the effect of the stationary phase on the number of CRs was investigated, comparing unbonded, amide-based, and zwitterionic stationary phases.

The number of CRs required for the BEH HILIC and ZIC-HILIC columns was compared to that obtained using the BEH Amide column (Figure 5a). The number of CRs was higher for both BEH HILIC and ZIC-HILIC columns compared to the BEH Amide column, except for 4-hydroxybenzoic acid and amoxicillin, which required the same number of CRs for BEH Amide and ZIC-HILIC columns. This effect can be explained by differences in solute retention between the two columns. For example, saccharose, which is strongly retained in the BEH Amide column (elution with 30% H₂O), which is well-suited for this compound, exhibited intermediate retention in ZIC-HILIC (elution between 20 and 27% H₂O) and very poor retention in the BEH HILIC column (elution at 5.4% H₂O) (Figure 5b). Consequently, it required a higher number of CRs with the BEH HILIC column than with the ZIC-HILIC and BEH Amide columns. On the other hand, 4-hydroxybenzoic acid was poorly retained by all three columns and required the same number of CRs. This result supports the hypothesis that the number of CRs is indeed related to compound retention.

It seemed that the number of CRs depended not only on the retention of the compound but also on the retention mechanism involved. When we examine the shape of the saccharose

peak eluted with the ZIC-HILIC column (Figure 5b), its deformation may be due to the thickness of the water layer [50], which is more pronounced with this column, or the different interactions that occur depending on the stationary phase. For example, the BEH Amide stationary phase mainly involved hydrogen bonding with the analytes, whereas the BEH HILIC and ZIC-HILIC columns also involved electrostatic interactions [42,43]. The effect of the stationary phase on the peak shape of saccharose was also observed on the BEH HILIC column, with a broadening of the peak. The peak shape of 4-hydroxybenzoic acid was less affected by the stationary phase than that of saccharose. In fact, only one peak tail was observed with the BEH Amide column. This could be due to the presence of a secondary interaction that is not significant enough to affect the number of CRs.

The detrimental impact of ionic interactions on the number of required CRs appeared to be confirmed by the behavior of the basic compound. Across all the columns tested, nortriptyline required more conditioning runs than the other solutes. In these tests, the aqueous phase has a pH value of 6.8, enabling positively charged nortriptyline to interact with negatively charged residual silanols. However, when the mobile phase was acidified to pH 3.15, thereby protonating the residual silanols, the number of required CRs decreased from 4 to only 1 on the BEH Amide column (data not shown). These results suggested a link between electrostatic interactions and the requirement of conditioning runs.

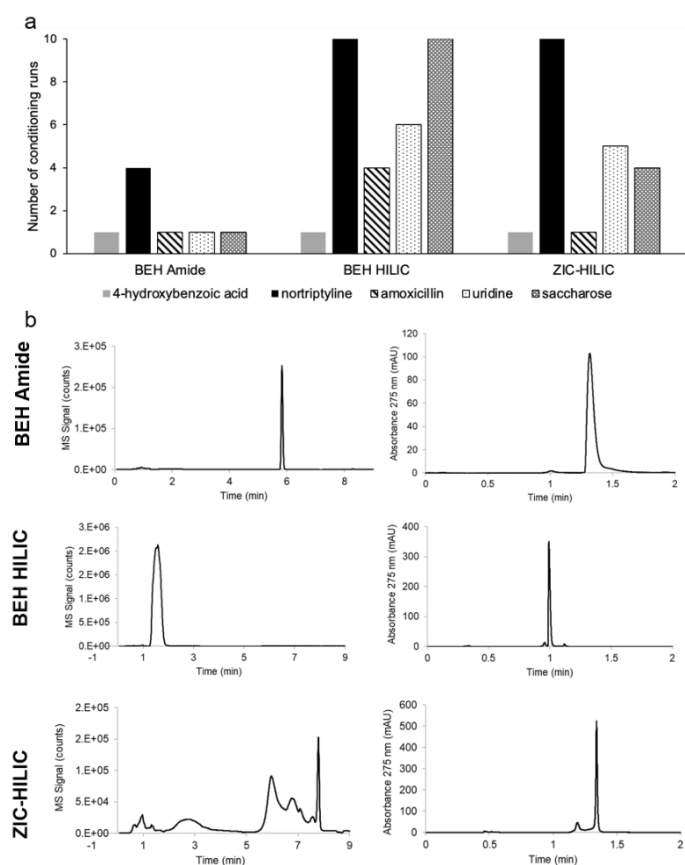


Figure 5: a) Number of conditioning runs according to the stationary phase of BEH Amide, BEH HILIC, and ZIC-HILIC. b) Chromatograms of saccharose and 4-hydroxybenzoic acid injected on BEH Amide, BEH HILIC, and ZIC-HILIC columns. Normalized slope 5%, equilibration time $0.5 t_0$ for the three columns, flow rate 0.4, 0.27, and 0.2 mL/min for the BEH Amide, BEH HILIC, and ZIC-HILIC, respectively. Studied solutes injected in 50/50 H₂O/ACN with a volume of 3 μ L for BEH Amide and 2.4 μ L for both BEH HILIC and ZIC-HILIC, corresponding to 1% of the column volume.

In this article, only three stationary phases were compared. A more comprehensive study of the number of required CRs as a function of the stationary phase would be necessary. Such a study would involve a broader panel of columns and compounds, to provide a deeper understanding of the phenomenon at the molecular level. The impact of water within the pores is acknowledged not only in terms of compound retention but also in relation to equilibration duration [54] and column efficiency. This is attributed to differences in compound mass transfer based on the water layer thickness [52]. A comparison of pore size was performed using a BEH Amide with 300Å pore size. At first sight, no significant impact was found on the number of conditioning runs required (Fig. S2). Nonetheless, the influence of these factors on the number of conditioning runs has not yet been completely investigated. Exploring this aspect may contribute significantly to our understanding of the underlying mechanisms.

4. Application to an industrial mixture of cross-linked polysaccharides from a hyaluronic hydrogel

Hyaluronic acid (HA) is a glycosaminoglycan consisted of a repetition of disaccharide units composed of a glucuronic acid and an N-acetyl-glucosamine. This naturally occurring polysaccharide find wide application in various medical and aesthetic procedures [55,56]. To enhance its resistance to biological degradation and its mechanical properties, a proprietary cross-linking agent is added to interconnect the polysaccharide chains, forming a viscous and biocompatible hydrogel. Accurate characterization of the location and the nature of the bonds between the polysaccharide chains and the cross-linker is essential to ensure product quality.

Upon enzymatic depolymerization of the hydrogel, a complex mixture of highly polar compounds is formed, including anionic oligomers from hyaluronic acid chains and polysaccharides-cross-linker structures containing both positively and negatively charged

moieties. Given the polarity of the analytes and the limited solubility of the sample in organic solvents, a RPLC was preferred for the first dimension. Moreover, consistent with the findings of Muller et al. [21], an enhanced sensitivity was obtained when coupling HILIC with MS compared to RPLC. When working at the same dilution factor, we observed that the MS signal for cross-linked polysaccharides was, on average, ten times higher when using HILIC-MS compared to RPLC-MS (for industrial confidentiality, spectra are not shown). Consequently, a RPLCxHILIC method was developed for the analysis of digested hyaluronic acid hydrogel. A Luna Omega Polar C18 column was chosen for the reverse phase separation due to its ability to operate at 100% aqueous mobile phase. For the second dimension, a BEH amide column was used due to its ability to separate polysaccharides based on chain length [57,58].

The number of injections required to achieve reproducible retention of the digested hydrogel compounds in HILIC was determined on a 1D setup by monitoring 5 specific ions. The study was performed for an injection volume of 5 μ L as 100% H₂O as the injection solvent, corresponding to the initial RPLC gradient conditions, while other conditions are listed in Table 1. The evolution of the retention time for the extracted ion peaks was used to evaluate the number of conditioning runs, following the procedure described in section 3.1. It was observed that the retention times became repeatable starting from the 6th injection (see Figure S3 in the supplementary information). Accordingly, five conditioning runs were required in second dimension.

The RPLCxHILIC method was optimized, taking into account several factors: the ²D maximal injection volume, 5 μ L; the lowest ²t_{eq} (limited by the ²D delay time, set at 0.12 min), and the number of ²D conditioning runs. In typical LCxLC instrument, the pumping systems, the sample injection, the modulation program and the successive ²D gradients start simultaneously with the ¹D gradient program. The less retained compounds elute from ¹D column just after the ¹D dead time, meanwhile the ²D column experiences a certain number of conditioning runs. In LCxLC analysis using HILIC as second dimension, the slow first dimension and the fast second dimension typically result in 6 and 12 CRs during the ¹D t₀ [8,21,22,30]. Unfortunately, due to the very wide elution range of the hyaluronic hydrogel solutes in HILIC, combined with the high viscosity of the large amount of water required at the end of the gradient, the ²D separation extended to 1.1 min. Despite optimized geometry and flow rates only 2 conditioning runs could be achieved during the ¹D t₀. This led to significant variability of ²D retention times. Subsequently, a delayed injection was programmed at 4.0 min, allowing for five modulations before the first eluting peak. The hydrogel digest mixture was then analyzed three times using the RPLCxHILIC method as described in Table 1.

The 2D plots obtained from the sum of the extracted ion chromatograms (EICs) of the crosslinker-bound polysaccharide units' specific ions over the three injections are shown in Figure 6. The standard deviation of the t_r values was lower than 0.5 s for the 24 spots and the relative standard deviation (RSD) was lower than 1%. These results demonstrate that HILIC can be established as the second dimension if enough conditioning runs are performed.

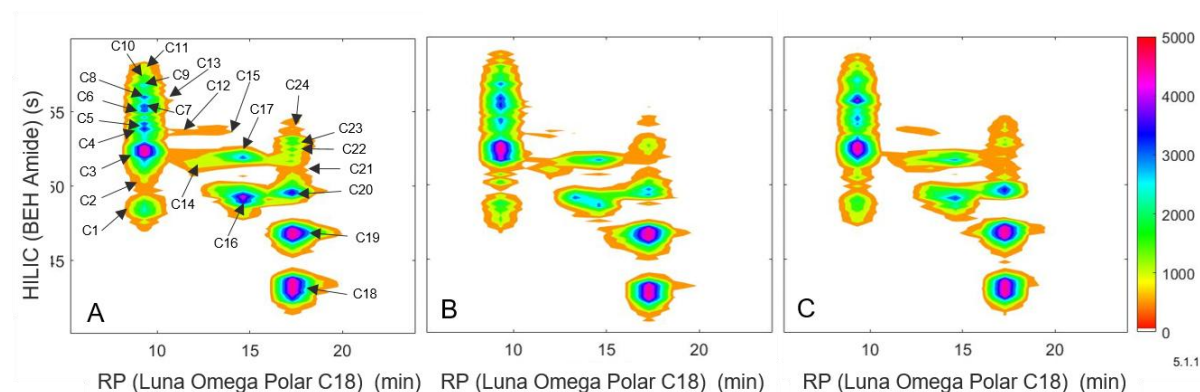


Figure 6: RPLCxHILIC chromatogram of a crosslinked hydrogel digest. Sum of EICs with 24 peaks of interest (C1-C24). Conditions as described in Table 1. Three analyses (A, B and C) performed the same day.

In Figure 6, the compounds were segregated into five rows in the 1^{D} RP dimension. The first row contained compounds C1 to C11, corresponding to HA polysaccharides with one pendant crosslinker (incomplete crosslinking reaction). The upper rows, encompassing compounds C12 to C24, represented compounds formed by two HA chains cross-linked by one or more cross-linkers. Their retention in RPLC depended on the number and position of cross-linkers. The second dimension separated the cross-linked polysaccharides according to the length of the carbohydrate chain. The spatial distribution on the 2D plot of these enzymatically digested compounds can provide valuable insights into the structure of the initial hydrogel. These results underscore the significance of employing an RPLCxHILIC method for the analysis of hyaluronic acid-based hydrogel.

5. Conclusion

In this paper, the repeatability of retention times in HILIC mode when used as the second dimension in on-line LCxLC has been investigated. Our focus was on 2DLC-dependent parameters, such as equilibration volume, slope, solvent, and injection volume. The impact of these parameters on the number of pre-injection conditioning runs required to achieve

repeatable retention times was investigated. For this purpose, a mixture of polar compounds was used. The results indicate that slope and equilibration volume greater than 0.5 dead volume do not significantly affect the number of required CRs. However, injected volume, solvent, and stationary phase had an effect, which varied depending on the compounds, their retention characteristics, and the interaction mechanisms involved. Further studies are necessary to gain a deeper understanding of the differing behavior observed in compounds with similar retention on the HILIC column. Notably, this study represents the first instance, to the best of our knowledge, of investigating the repeatability of HILIC retention times with an equilibration step lower than one column volume.

An RPLCxHILIC method for analyzing a digested HA hydrogel sample illustrated the feasibility of using HILIC as the second dimension, providing that an adequate number of conditioning runs were executed in the ²D. The results showed a good repeatability of the ²D retention times, with an RSD below 1%.

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