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## ► To cite this version:

Clémence Gély, Yoan Monneau, Maggy Hologne, Karine Faure. Impact of conditioning runs on hydrophilic interaction chromatography repeatability and its application as a second dimension in online comprehensive two-dimensional liquid chromatography. *Journal of Separation Science*, 2024, 47, 10.1002/jssc.202300935 . hal-04590635

**HAL Id: hal-04590635**

**<https://hal.science/hal-04590635v1>**

Submitted on 28 May 2024

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

4           Clémence Gély<sup>a</sup>, Yoan R. Monneau<sup>a</sup>, Maggy Hologne<sup>a</sup>, Karine Faure<sup>a\*</sup>

5 <sup>a</sup>Universite Claude Bernard Lyon 1, ISA UMR 5280, CNRS, 5 rue de la Doua, 69100 Villeurbanne, France

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7 \*Correspondence to:

8 Karine Faure, Institut des Sciences Analytiques, UMR 5280, 5 Rue de la Doua, 69100  
9 Villeurbanne, France

10 Email: karine.faure@isa-lyon.fr

## 11 **Abstract**

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

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

## 31 **Abbreviations**<sup>1</sup>

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### <sup>1</sup> **Abbreviations**

1D-LC: One-dimensional liquid chromatography

<sup>1</sup>D: First dimension

<sup>2</sup>D: Second dimension

LCxLC: Online comprehensive two-dimensional liquid chromatography

RP: Reverse phase

## 32 1. Introduction

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

44 The HILIC technique relies on the adsorption of a water layer on the stationary phase,  
45 necessitating extended equilibration times, especially when used in gradient mode. For  
46 example, the literature suggests a minimum of 40 column volumes for a complete  
47 equilibration, depending on the stationary phase and solutes [4,5]. Nevertheless, shorter  
48 partial equilibration periods are also feasible [4,6–8]. For example, McCalley showed that an  
49 equilibration step of 12.2 column volumes yields satisfactory retention time repeatability [6],  
50 while even as few as 5 column volumes were sufficient for consistent separation of antibiotic  
51 and antiretroviral compounds [4]. Such long equilibration times have encouraged the use of  
52 HILIC as the first dimension in online LCxLC setups. As so, the utilization of HILICxRPLC  
53 separation has yielded promising outcomes in the analysis of proteins and their glycoforms  
54 [9,10], industrial polymers [11], and food products such as phenolic compounds [12–15], as  
55 well as lipids analysis [16].

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

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

73 The modulation system used in online LCxLC requires a fast second dimension. In gradient  
74 mode, the <sup>1</sup>D modulation time (<sup>1</sup>t<sub>mod</sub>) is directly related to the <sup>2</sup>D gradient time (<sup>2</sup>t<sub>g</sub>) and  
75 equilibration time (<sup>2</sup>t<sub>eq</sub>), the time to equilibrate the column to the initial gradient conditions, as  
76 shown in equation (1).

$$77 \quad {}^1t_{\text{mod}} = {}^2t_{\text{g}} + {}^2t_{\text{eq}} \quad (1)$$

78 To increase the peak capacity of the second dimension, the <sup>2</sup>D slope should be shallow to  
79 ensure <sup>2</sup>t<sub>g</sub> is as long as practically feasible. To avoid an increase in the modulation time,  
80 which may lead to under-sampling of the first dimension [3,29], it is crucial to minimize <sup>2</sup>t<sub>eq</sub>.  
81 The equilibration time depends on both the equilibration volume required to achieve stable  
82 retention times and the flow rate. The maximal flow rate is always used in the second  
83 dimension. Consequently, the only parameter that can be reduced is the equilibration  
84 volume.

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

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

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

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

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

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

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

148

149      **2. Material and methods**

150      2.1. Chemicals

151      LC-MS grade acetonitrile was purchased from Riedel-de-Haën (Seelze, Germany). Uridine  
152      (purity  $\geq$  99%), nortriptyline hydrochloride (purity  $\geq$  98%), amoxicillin (purity between 95.0-  
153      100.0%), saccharose (purity  $\geq$  99.5%), 4-hydroxybenzoic acid (purity  $\geq$  99%), and sodium  
154      chloride were obtained from Aldrich (Steinheim, Germany). Analytical reagent-grade  
155      ammonium acetate, formic acid (98-100%), and calcium chloride were purchased from  
156      Fisher Scientific (Illkirch, France) and Carl Roth (Karlsruhe, Germany). Deionized water was  
157      produced using an Elga Purelab Classic UV purification system from Veolia water STI  
158      (Décine-Charpieu, France). HYase (Hyaluronidase from *Streptomyces hyalurolyticus*) and  
159      chABC (Chondroitinase ABC from *Proteus vulgaris*) were acquired from Sigma Aldrich (Saint  
160      Quentin Fallavier, France).

161      2.2. Sample preparation

162      Individual stock solutions were prepared in water at a concentration of 1 mg/mL for  
163      amoxicillin, saccharose, and 4-hydroxybenzoic acid, and prepared in acetonitrile at a  
164      concentration of 1 mg/mL for uridine and nortriptyline. Working solutions were then derived  
165      from these stock solutions, with concentrations of 10  $\mu$ g/mL for saccharose, 20  $\mu$ g/mL for  
166      nortriptyline and uridine, 30  $\mu$ g/mL for 4-hydroxybenzoic acid, and 40  $\mu$ g/mL for amoxicillin.  
167      The volumes of the diluting solvents, acetonitrile and water, were adjusted to achieve a final  
168      composition of 95/5, 50/50, or 5/95 H<sub>2</sub>O/ACN (v/v) as required by the experiment.

169      The enzymatic digestion of crosslinked polysaccharides was performed as follows: two  
170      syringes of hyaluronic acid hydrogel supplied by Allergan were mixed with 200 mL of a 150  
171      mM NaCl saline solution and then filtered through 0.22  $\mu$ m nylon filters (GE Water & Process  
172      Technologies, Feasterville-Trevose, USA). Subsequently, the hydrogel was washed with 200  
173      mL of ultrapure water and then filtered once more before being solubilized into 20 mL of  
174      water and freeze-dried. The resulting lyophilisate was dissolved in 1 mM ammonium acetate  
175      and 10 mM calcium chloride solution to achieve a concentration of 2 mg/mL. The enzymatic  
176      digestion was performed by adding 100  $\mu$ L of HYase (300 U/mL) and incubating overnight at  
177      37°C with continuous mixing at 150 RPM. Then, 100  $\mu$ L of chABC at 5 U/mL was added  
178      every 3 hours, with continuous incubation and mixing, and a final addition was made  
179      approximately 12 hours later. The progression of digestion was monitored by measuring the  
180      optical density at 232 nm using a NanoDrop One<sup>c</sup> (ThermoScientific, Waltham, USA).  
181      Following digestion, the sample was freeze-dried, and a 30 mg/mL suspension of the  
182      digested powder in water was prepared for further analysis.



183 2.3. Instrumentations

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

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

195 The data were processed using the Agilent MassHunter workstation Qualitative Analysis  
196 10.0, and 2D plots were realized with Matlab (R2020a, 9.8.01323502).

197 2.4. One-dimensional HILIC

198 The 1D HILIC separations were performed using three different columns: an Acquity BEH  
199 Amide column (150x2.1 mm; 1.7  $\mu\text{m}$ , 130  $\text{\AA}$ ), an Acquity BEH HILIC (100x2.1 mm; 1.7 $\mu\text{m}$ ,  
200 130  $\text{\AA}$ ) from Waters (Milford, MA, USA), and a SeQuant ZIC-HILIC column (100x2.1 mm;  
201 3.5 $\mu\text{m}$ , 200  $\text{\AA}$ ). The column temperature was maintained at 45  $^{\circ}\text{C}$ . The mobile phase  
202 consisted of two components: (A) ACN and (B)  $\text{H}_2\text{O}$ +20 mM ammonium acetate, at pH=6.8.  
203 Prior to each set of experiments, the column was flushed with ten column volumes of mobile  
204 phase composed of 40% B. The gradient was programmed to obtain a normalized slope of 2,  
205 5, and 8 %, depending on the specific experiment. The flow rate and the isocratic step were  
206 optimized to achieve an appropriate equilibration volume, taking into account the instrument  
207 injection time (30s). Injection volumes were set at either 1 % or 5 % of the column volume,  
208 respectively 3  $\mu\text{L}$  and 15  $\mu\text{L}$  for BEH Amide and 2.4  $\mu\text{L}$  for both BEH HILIC and ZIC-HILIC  
209 corresponding to 1% for column volume. Each set of experiments involved ten consecutive  
210 injections.

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

218 2.5. RPLCxHILIC conditions for the analysis of a digested hyaluronic acid hydrogel  
 219 The hyaluronic acid hydrogel was analyzed with a RPLCxHILIC-QToF method. The <sup>1</sup>D RPLC  
 220 was performed using a Luna Omega Polar C18 (50x1 mm, 1.7 μm) column purchased from  
 221 Phenomenex (Torrance, California, USA), and the <sup>2</sup>D HILIC separation was carried out using  
 222 the BEH Amide column (50x2.1 mm, 1.7 μm). Details of the RPLCxHILIC conditions can be  
 223 found in Table 1. The instrument settings were drying gas temperature, 200°C; drying gas  
 224 flow rate, 12 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 350°C; sheath gas  
 225 flow rate, 11 L/min; fragmentor, 185 V; capillary voltage, 5000 V; electrospray positive mode.  
 226 Mass spectra data were collected using the full scan mode with a mass range of 100–3200  
 227 m/z and an acquisition rate of 5 Hz. To perform the necessary <sup>2</sup>D conditioning runs before  
 228 compound elution, the sample was injected four minutes after the beginning of the gradient.

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

	First dimension ( <sup>1</sup> D)	Second dimension ( <sup>2</sup> 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 <sub>2</sub> O+10 mM ammonium acetate / B: ACN	A: ACN / B: H <sub>2</sub> 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 <sub>0</sub> )	0.12 min (1.1t <sub>0</sub> )
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	

231  
 232 A 2D-chromatogram is constituted of a succession of 2D fraction separations. The <sup>1</sup>D  
 233 retention time, <sup>1</sup>tr of a given solute was assigned to the peak apex of its most intense peak  
 234 between the fractions containing this solute, while the <sup>2</sup>D retention time, <sup>2</sup>tr representing the  
 235 time spent in the second dimension, was calculated as the remaining between the <sup>1</sup>D

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

$$238 \quad t_r = n t_s + t_r \quad (2)$$

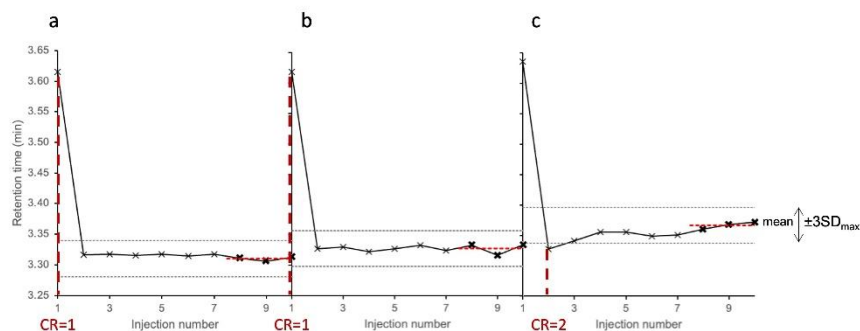
### 239 **3. Results and discussion**

240 Comprehensive two-dimensional liquid chromatography (LCxLC) requires a rapid  
241 equilibration of the second-dimensional column while maintaining repeatable retention times.  
242 Previous studies recommending long equilibration times in HILIC implied that using this  
243 separation mode as a second dimension in LCxLC might be impractical. However, several  
244 promising studies using HILIC as a second dimension indicated that HILIC separations can  
245 be reproducible even with a very short equilibration time of less than one column volume  
246 [8,21,34]. Furthermore, studies on repeatability in HILIC suggested that it can be improved by  
247 increasing the number of conditioning runs [5,6].

248

#### 249 **3.1. Methodology**

250 This study aims to determine the necessary number of conditioning runs for achieving  
251 consistent retention times in conditions compatible with the use of HILIC as a second  
252 dimension. To this end, ten consecutive sample injections were performed and repeated on  
253 three different days, as illustrated in Figure 1. The standard deviation of the last three  
254 injections was calculated for each day. It was assumed that the variability between injections  
255 8, 9, and 10 solely resulted from method variability rather than a lack of conditioning runs.  
256 Then, the maximum standard deviation among the three days (D1, D2, and D3), denoted as  
257  $SD_{max}$ , was used to establish the retention window. This allowed us to consider the maximum  
258 method-induced variability that could be observed over the course of three days. If the  
259 calculated standard deviation was less than the acquisition rate (one point every 0.0002 min  
260 in UV and 0.005 min in MS),  $SD_{max}$  was set equal to 0.0002 min or 0.005 min as appropriate.  
261 When the retention time of injection  $n$  (denoted as  $t_{r_n}$ ) fell within the retention window defined  
262 as  $mean(tr) \pm 3 SD_{max}$ , with  $mean(tr)$  representing the average retention time of the three last  
263 injections in the experiment, the injections were deemed repeatable starting from injection  $n$ .  
264 In such cases, the required number of conditioning runs (CR) was  $n-1$ .

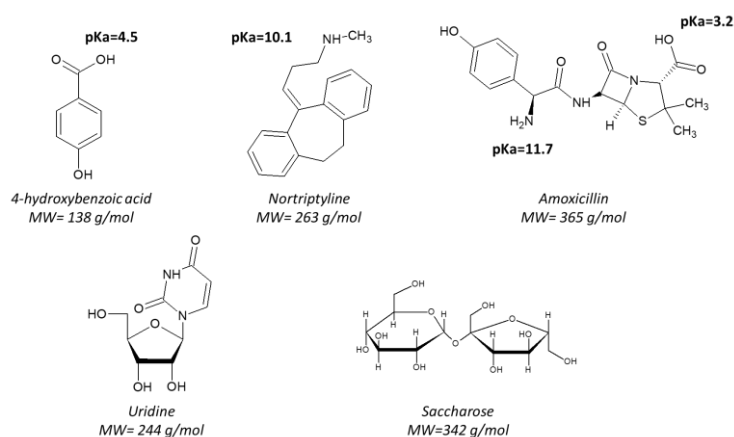


265

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

270

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



275

276 Figure 2: Structure and physicochemical properties of studied compounds

277

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

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

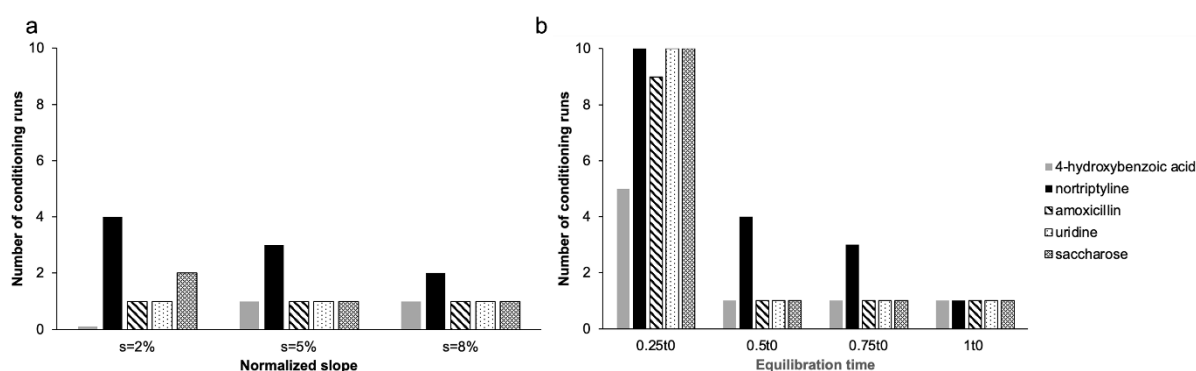
284 carried out using a BEH Amide column with a standardized slope of 5%, a mobile phase  
285 composition of 5% to 90% water, and an equilibration time equivalent to 0.5 column's dead  
286 time ( $t_0$ ). Compounds were injected over the course of three days, each injection being 1% of  
287 column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

293

### 294 3.2. Effect of gradient slope and equilibration time

295 In the second dimension of the LCxLC, the compounds must elute during the modulation  
296 period of the first dimension. This typically leads to a steep <sup>2</sup>D gradient and a final gradient  
297 composition enriched with the stronger solvent. However, increasing the aqueous content  
298 over 40% in the mobile phase disrupts the water layer characteristic of the HILIC mode  
299 [43,44]. Moreover, McCalley suggested that the number of conditioning runs should be  
300 adapted to the slope [5].

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



307

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

311 Injection volume, 3  $\mu\text{L}$  in 50/50  $\text{H}_2\text{O}/\text{ACN}$ . (a) Equilibration time  $0.5 t_0$ , (b) normalized slope 5  
312 %.

313

314 In contrast to the finding in McCalley's study [5], which stated that more CR are required for a  
315 1%/min slope compared to a 5%/min slope, the results shown in Figure 3a indicate that the  
316 slope had no significant effect on the number of CRs required to reach repeatability,  
317 compared to inter-day variability ( $\pm 1$  CR for 4-hydroxybenzoic acid, amoxicillin, and uridine,  
318 and  $\pm 2$  CRs for saccharose and nortriptyline). Both amoxicillin (zwitterionic) and uridine  
319 (neutral) required only one CR across all studied slopes. The variation of one conditioning  
320 run between the various gradient slopes, observed for 4-hydroxybenzoic acid (acidic) and  
321 saccharose (neutral), was not considered significant. A potential trend was noted for the  
322 basic solute nortriptyline, which required fewer CRs with steeper slopes. Consequently,  
323 slopes exceeding 2% (normalized with the column dead time) can be applied when using  
324 HILIC as a LCxLC second dimension. No slope greater than 8% was tested thus, as  
325 compounds may no longer elute beyond this value.

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

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

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

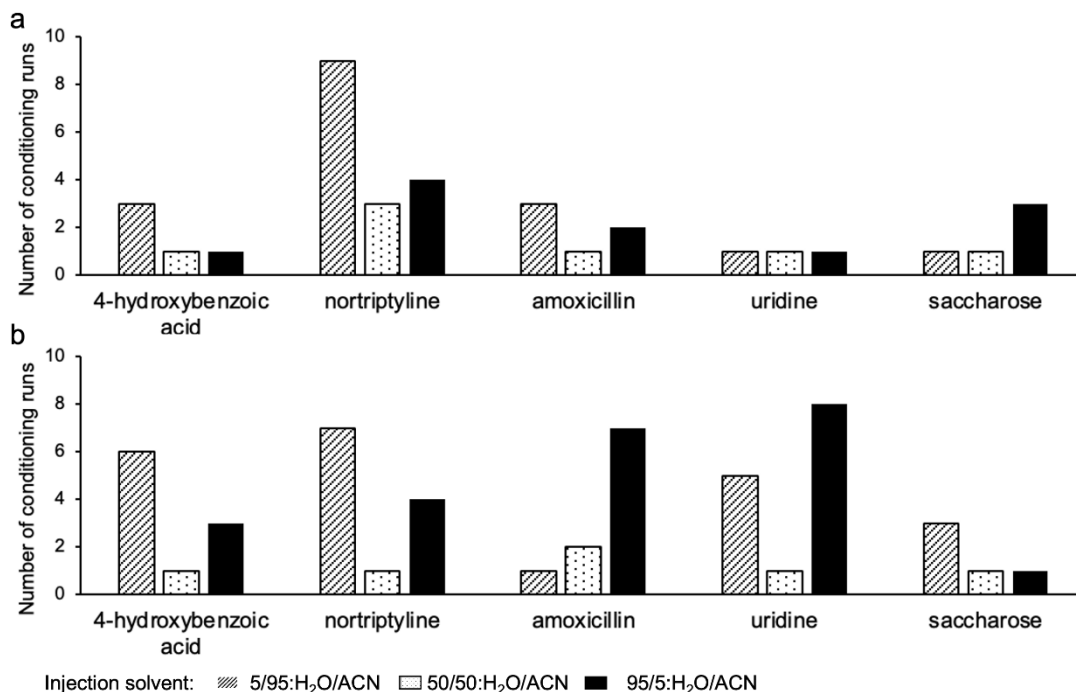
345

### 346 3.3. Effect of injection solvent type and volume

347 In online LCxLC, the composition of the injection solvent depends on the <sup>1</sup>D 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 <sup>1</sup>D 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%,

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

368

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

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

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

393 For uridine, the number of CRs increased with the injected volume, except for the 50/50:  
394 H<sub>2</sub>O/ACN solvent proportion. The maximum number of required CRs was observed when the  
395 proportion of injected H<sub>2</sub>O was maximal. The same result was observed for amoxicillin, with  
396 an increase in the number of CRs along with the increase in the injected volume and the  
397 injected proportion of water.

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



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

410

#### 411 3.4. Effect of the stationary phase

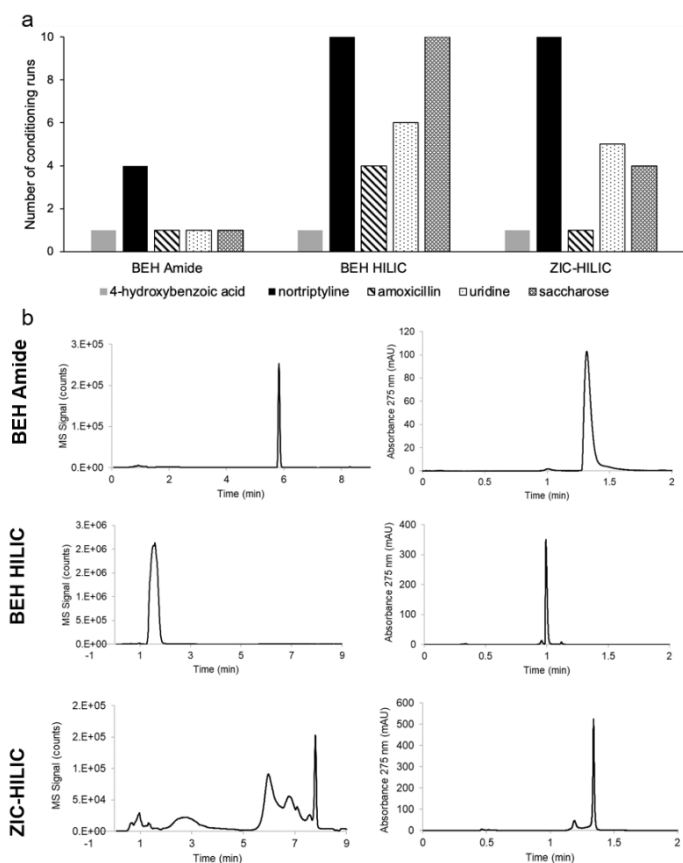
412 The previous parameters were studied on a BEH Amide column. However, the stationary  
413 phase can influence the number of CRs. Specifically, the thickness of the water layer is  
414 linked to the nature of the stationary phase [50–53]. For example, bare silica stationary  
415 phases generate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

420 The number of CRs required for the BEH HILIC and ZIC-HILIC columns was compared to  
421 that obtained using the BEH Amide column (Figure 5a). The number of CRs was higher for  
422 both BEH HILIC and ZIC-HILIC columns compared to the BEH Amide column, except for of  
423 4-hydroxybenzoic acid and amoxicillin, which required the same number of CRs for BEH  
424 Amide and ZIC-HILIC columns. This effect can be explained by differences in solute  
425 retention between the two columns. For example, saccharose, which is strongly retained in  
426 the BEH Amide column (elution with 30% H<sub>2</sub>O), which is well-suited for this compound,  
427 exhibited intermediate retention in ZIC-HILIC (elution between 20 and 27% H<sub>2</sub>O) and very  
428 poor retention in the BEH HILIC column (elution at 5.4% H<sub>2</sub>O) (Figure 5b). Consequently, it  
429 required a higher number of CRs with the BEH HILIC column than with the ZIC-HILIC and  
430 BEH Amide columns. On the other hand, 4-hydroxybenzoic acid was poorly retained by all  
431 three columns and required the same number of CRs. This result supports the hypothesis  
432 that the number of CRs is indeed related to compound retention.

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

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

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



454 Figure 5: a) Number of conditioning runs according to the stationary phase of BEH Amide,  
455 BEH HILIC, and ZIC-HILIC. b) Chromatograms of saccharose and 4-hydroxybenzoic acid  
456 injected on BEH Amide, BEH HILIC, and ZIC-HILIC columns. Normalized slope 5%,  
457 equilibration time  $0.5 t_0$  for the three columns, flow rate 0.4, 0.27, and 0.2 mL/min for the BEH  
458 Amide, BEH HILIC, and ZIC-HILIC, respectively. Studied solutes injected in 50/50 H<sub>2</sub>O/ACN  
459 with a volume of 3  $\mu$ L for BEH Amide and 2.4  $\mu$ L for both BEH HILIC and ZIC-HILIC,  
460 corresponding to 1% of the column volume.

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

473

#### 474 **4. Application to an industrial mixture of cross-linked polysaccharides from a** 475 **hyaluronic hydrogel**

476

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

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

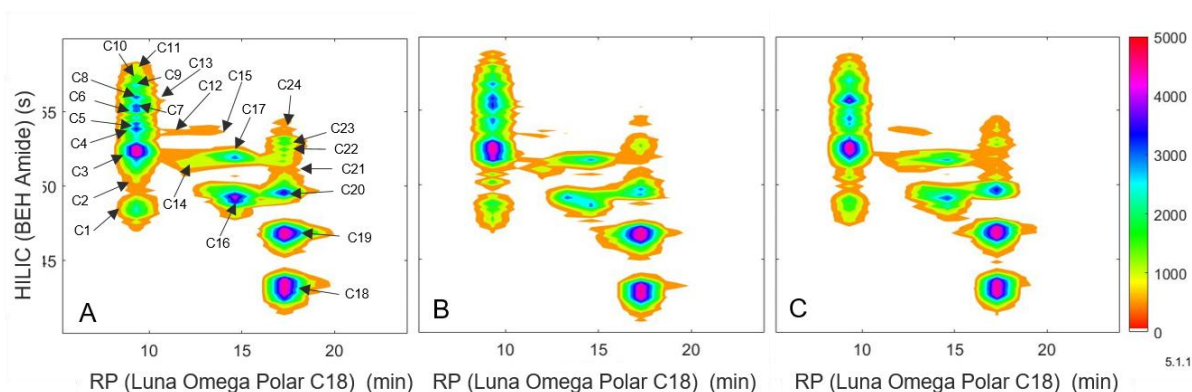
488 moieties. Given the polarity of the analytes and the limited solubility of the sample in organic  
489 solvents, a RPLC was preferred for the first dimension. Moreover, consistent with the  
490 findings of Muller et al. [21], an enhanced sensitivity was obtained when coupling HILIC with  
491 MS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

499 The number of injections required to achieve reproducible retention of the digested hydrogel  
500 compounds in HILIC was determined on a 1D setup by monitoring 5 specific ions. The study  
501 was performed for an injection volume of 5  $\mu\text{L}$  as 100%  $\text{H}_2\text{O}$  as the injection solvent,  
502 corresponding to the initial RPLC gradient conditions, while other conditions are listed in  
503 Table 1. The evolution of the retention time for the extracted ion peaks was used to evaluate  
504 the number of conditioning runs, following the procedure described in section 3.1. It was  
505 observed that the retention times became repeatable starting from the 6<sup>th</sup> injection (see  
506 Figure S3 in the supplementary information). Accordingly, five conditioning runs were  
507 required in second dimension.

508 The RPLCxHILIC method was optimized, taking into account several factors: the <sup>2</sup>D maximal  
509 injection volume, 5  $\mu\text{L}$ ; the lowest <sup>2</sup> $t_{\text{eq}}$  (limited by the <sup>2</sup>D delay time, set at 0.12 min), and the  
510 number of <sup>2</sup>D conditioning runs. In typical LCxLC instrument, the pumping systems, the  
511 sample injection, the modulation program and the successive <sup>2</sup>D gradients start  
512 simultaneously with the <sup>1</sup>D gradient program. The less retained compounds elute from <sup>1</sup>D  
513 column just after the <sup>1</sup>D dead time, meanwhile the <sup>2</sup>D column experiences a certain number  
514 of conditioning runs. In LCxLC analysis using HILIC as second dimension, the slow first  
515 dimension and the fast second dimension typically result in 6 and 12 CRs during the <sup>1</sup>D  $t_0$   
516 [8,21,22,30]. Unfortunately, due to the very wide elution range of the hyaluronic hydrogel  
517 solutes in HILIC, combined with the high viscosity of the large amount of water required at  
518 the end of the gradient, the <sup>2</sup>D separation extended to 1.1 min. Despite optimized geometry  
519 and flow rates only 2 conditioning runs could be achieved during the <sup>1</sup>D  $t_0$ . This led to  
520 significant variability of <sup>2</sup>D retention times. Subsequently, a delayed injection was  
521 programmed at 4.0 min, allowing for five modulations before the first eluting peak. The  
522 hydrogel digest mixture was then analyzed three times using the RPLCxHILIC method as  
523 described in Table 1.

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

530



531

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

535

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

## 546 5. Conclusion

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

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

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

#### 564 **Acknowledgement**

565 Allergan Aesthetics, an AbbVie company, funded this study. The authors would like to thank  
566 Alizee Dufour, Baptiste Schindler, Stephane Ogier et Aude Bernardin for their contributions  
567 to the manuscript. No honoraria or payments were made for this publication or for authorship.

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