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Evaluating Relative Retention of Polar Stationary Phases in Hydrophilic Interaction Chromatography

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Abstract: A large number of polar stationary phases with diverse chemistry have been developed for various applications in hydrophilic interaction chromatography (HILIC). However, column manufacturers employ different testing procedures to evaluate retention of the polar stationary phases. This renders the retention data impossible for comparison and makes it difficult for the users to select the right stationary phase based on retention. We have evaluated 25 polar stationary phases using cytosine and uracil as the model compounds in various mobile phase conditions. These stationary phases show a wide range of retention characteristics for the model compounds. The ranking of the stationary phases does not change drastically with the acetonitrile level in the mobile phase.

Keywords: stationary phase; retention; cytosine

1. Introduction

When selecting a stationary phase for chromatographic application, column retention is one of the important parameters for the users to consider. Column manufacturers typically provide retention information obtained with an internal testing procedure. To compare relative retention of different stationary phases, it is critical to employ a common testing procedure including the test compound and conditions. In reversed-phase liquid chromatography (RPLC), the relative retention of the stationary phase is typically evaluated by measuring the retention of a hydrophobic model compound (e.g., toluene). Goldberg compared the retention of various RPLC columns using the retention data of anthracene [1]. Mac Mod published a more comprehensive evaluation of 59 C18 columns from various brands and manufacturers by using toluene as the test compound [2].

As hydrophilic interaction chromatography (HILIC) continues to grow in popularity, more and more stationary phases are being developed for the separation of polar compounds [3–5]. Most of the commercially available stationary phases have been evaluated for HILIC separation, but the published studies typically focus on the selectivity of various stationary phases [5–12]. Different probe pairs have been used to evaluate specific interactions between the probe compounds and the stationary phases [9,10]. Chemometric methods are employed to identify selectivity patterns and classify the stationary phases [11,12]. It is well known that chromatographic resolution is dependent on separation efficiency, selectivity, and retention as shown in the equation below:

$$R_S = \frac{\sqrt{N}}{4}\frac{\alpha - 1}{\alpha}\frac{k_2}{k_2 - 1} \quad (1)$$

where N is the separation efficiency, α the selectivity factor and k_2 the retention factor. The equation above indicates that the retention factor is equally important to achieve desired separation. Although selectivity evaluation is based on the retention data of the probe compounds, it is difficult to find the retention data directly in the published studies. From a practical point of view, it is important

to understand both the retention and selectivity in order to select the right stationary phase for specific applications.

Table 1 shows a survey of the testing procedures employed by the manufacturers of the polar stationary phases. The survey results clearly demonstrate that the testing protocols vary significantly among the manufacturers of HILIC columns. Even when some manufacturers use cytosine as the test compound, the mobile phase conditions are not always the same. Another possible reason for the lack of a common testing procedure is related to the complex retention mechanisms of HILIC. It is widely acknowledged that hydrophilic partitioning, polar interactions including surface adsorption, and electrostatic interactions can all potentially contribute to the retention in HILIC [13–15]. The electrostatic interactions (either repulsive or attractive) take place between the charged analytes and the functional groups on the stationary phase surface, and can be modulated by the mobile phase pH and salt concentration. Ideally, the test compound should not be ionized in the testing condition to avoid the electrostatic interactions. All non-ionized compounds experience both hydrophilic partitioning and polar interactions. Previous studies indicate that the retention based on hydrophilic partitioning and polar interactions is influenced by the mobile phase conditions [14,16,17]. Therefore, selecting the appropriate mobile phase is very important for evaluating the retention of the polar stationary phases.

Table 1. Survey of testing procedures of hydrophilic interaction chromatography (HILIC) column manufacturers.

Manufacturer	Stationary Phase	Test Compound	Mobile Phase
Advanced Chromatography Technology (ACE)	HILIC-A HILIC-B HILIC-N	Caffeine Uracil Uridine	90% ACN + 10% 100 mM ammonium formate (pH 4.7)
EMD Millipore	ZIC-HILIC ZIC-cHILIC	Uracil, cytosine	80% ACN + 20% 25 mM ammonium acetate
ES Industry	Epic-HILIC	Uracil, cytosine	90% ACN + 10% water
HilliCon	iHILIC Fusion iHILIC Fusion (+)	Uracil, cytosine	80% ACN + 20% 25 mM ammonium acetate (pH 6.8)
Nacalai Tesque	Cosmosil HILIC	Uracil, uridine	90% ACN + 10% water
Phenomenex	Luna-HILIC	Uracil, cytosine	90% ACN, 10mM ammonium formate
PolyLC	Hydroxyethyl A	Toluene	Methanol
SiliCycle	SiliChrom HILIC	Tested under normal phase conditions	
Thermo Scientific	Accucore Amide	Uridine	75% ACN + 25% 10 mM ammonium acetate (pH 5.4)
	Accurcore Urea	Acetylsalicylic acid	90% ACN, 10 mM ammonium acetate (pH 5)
Tosoh Bioscience	TSKgel Amide-80	Uracil	85% ACN + 15% water
Waters	Cortecs HILIC Atlantis HILIC	Adenine, cytosine, thymine	90% ACN, 10 mM Ammonium formate
	XBridge Amide	Cytosine, thymidine	80% ACN + 20% 20 mM ammonium formate (pH 3)
YMC	YMC-pack Amino	Sugars	75% ACN + 25% water
	YMC-Diol YMC-PVA Sil	Tested under normal phase conditions	

This study aims to investigate the retention of various polar stationary phases in HILIC under different mobile phase conditions. It is not the intention of this study to compare the selectivity of the selected stationary phases. Hence, only one pair of the test compounds is selected for this study. Ideally, the retention of the test compounds should be based on the predominant retention mechanism,

for example, toluene retention in RPLC. However, the complexity of the retention mechanisms makes it more difficult to select an ideal test compound for retention evaluation in HILIC. This study evaluates the retention of a large number of polar stationary phases using two selected test compounds (cytosine and uracil) under different mobile phase conditions. We hope that the column manufactures can adopt a common test protocol based on the results of this study.

2. Materials and Methods

All the polar stationary phases selected for this study were either purchased from or kindly donated by the column manufacturers. Table 2 presents the details of all the stationary phases including stationary phase chemistry, particle size, pore size, and column dimension. HPLC grade acetonitrile (ACN) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained from an in-house Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium acetate (ultrapure grade) was obtained from Amresco (Solon, OH, USA). Stock solutions of ammonium acetate (100 and 200 mM) were prepared by dissolving the appropriate amount of ammonium acetate in purified water. The pH of the stock ammonium acetate solutions was in the range of 6.8 to 7.0 without any adjustment. The mobile phase was mixed online by quaternary gradient pumps with acetonitrile, water, and ammonium acetate stock solutions at various proportions to achieve the desired acetonitrile content and ammonium acetate concentration. Uracil, cytosine and toluene were purchased from Sigma-Aldrich (St. Louis, MO, USA). The test solution of uracil and cytosine was prepared at approximately 0.1 mg/mL in a mixture of ACN and water (90/10, *v*/*v*). Toluene was spiked into the sample solution as the void marker.

Table 2. Detailed Information of the selected polar stationary phases.

Column Name	Stationary Phase Type	Column Information		
		Particle Size (μm)	Pore Size (Å)	Dimension (mm)
ACE HILIC-A	Acidic	3	100	4.6 × 150
ACE HILIC-B	Basic	3	100	4.6 × 150
ACE HILIC-N	Neutral	3	100	4.6 × 150
Cortecs HILIC	Silica	2.7	83	3.0 × 150
Atlantis HILIC 1	Silica	3	98	4.6 × 150
Atlantis HILIC 2		5	96	4.6 × 250
XBridge Amide	Amide	3.5	142	2.1 × 150
Accucore Amide	Amide	2.6	150	2.1 × 150
Accucore Urea	Urea	2.6	80	2.1 × 150
TSkgel-Amide 80	Amide	3	150	4.6 × 150
SiliChrom HILIC	Urea	5	100	4.6 × 250
Cosmosil HILIC	Triazole	5	120	4.6 × 250
LUNA HILIC 1	Cross-linked diol	5.8	204	4.6 × 250
LUNA HILIC 2		2.9	187	4.6 × 150
YMC-Pack NH2	Amino	5	120	4.6 × 250
YMC Diol-NP	Diol	5	120	4.6 × 250
YMC PVA-Sil	PVA	5	120	4.6 × 250
Epic HILIC-HC	Polyhydroxyl	5	120	4.6 × 250
Hydroxyethyl A	2-Hydroxyethyl aspartamide	5	100	4.6 × 200

Table 2. *Cont.*

Column Name	Stationary Phase Type	Column Information		
		Particle Size (μm)	Pore Size (Å)	Dimension (mm)
ZIC-HILIC 1	Zwitterionic	5	200	4.6 × 150
ZIC-HILIC 2		3.5	200	4.6 × 150
ZIC-HILIC 3		3.5	100	4.6 × 150
ZIC-cHILIC	Zwitterionic	3	100	4.6 × 150
iHILIC-Fusion	Zwitterionic	3.5	100	3.0 × 150
iHILIC-Fusion (+)	Zwitterionic	3.5	100	3.0 × 150

An Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an online vacuum degasser, a quaternary gradient pump, an autosampler, a variable UV detector, and a thermostated column compartment was used for all the experiments. The flow rates (1.0, 0.5, and 0.2 mL/min) were matched to the column inner diameters (4.6, 3.0, and 2.1 mm ID). The injection volume was 2 μL and detection was made at 254 nm. Chromatograms were recorded by ChemStation for LC and LC/MS (Rev. C. 01. 06.).

3. Results

Accurate determination of the retention factor (k) depends on the void or hold-up time (t_0) measurement. The void time may be measured by minor disturbance of baseline, homologous series, or unretained marker compounds [18]. The complexity of the retention mechanism in HILIC makes it especially challenging to select an unretained marker. Neue and McCalley first proposed the use of toluene as an unretained marker in HILIC [19]. Dinh et al. further refined the method by taking the water uptake into consideration [20]. Toluene has been used as the marker compound to determine the void time in HILIC in several published studies [21–23]. In this study, we employed toluene to measure the void time of the selected stationary phases. The survey of the testing procedures of various column manufacturers (Table 1) reveals that cytosine and uracil are more commonly employed in the testing protocol. Cytosine is a pyrimidine nucleobase with pKa_1 ~ 4.63 [24], so it is not ionized in the mobile phase around neutral pH. Cytosine has been used to evaluate the performance of various polar stationary phases in HILIC [25]. Recently, Alpert demonstrated by comparing the retention of cytosine and cytidine that cytosine might have specific interactions with the Hydroxyethyl A phase [26]; however, the unusual elution pattern was not observed on Altantis HILIC silica, YMC Pack amino, TSKgel Amide-80, and ZIC-HILIC phases [25]. Uracil has two p*Ka* values: pKa_1 ~ 9.36 and pKa_2 ~ 13.49. It is negatively charged above pH 10 and not ionized around neutral pH [15]. In this study, cytosine and uracil were selected as the model compounds. Both compounds remain neutral and provide reasonable retention in the selected mobile phase conditions. Table 1 also indicates that the column manufactures use very different mobile phase conditions that have various levels of acetonitrile. In this study, we measured the retention factors of cytosine and uracil on 25 polar stationary phases (Table 2) at three levels of acetonitrile (75%, 85%, and 90%). All the mobile phases contain 5 mM ammonium acetate. Figure 1 shows the ranking chart of the selected polar stationary phases based on the retention factor of cytosine measure in the mobile phase containing 75% acetonitrile. The retention factors of uracil are also included in the chart, but the ranking order may be slightly different based on the uracil retention factor for some stationary phases.

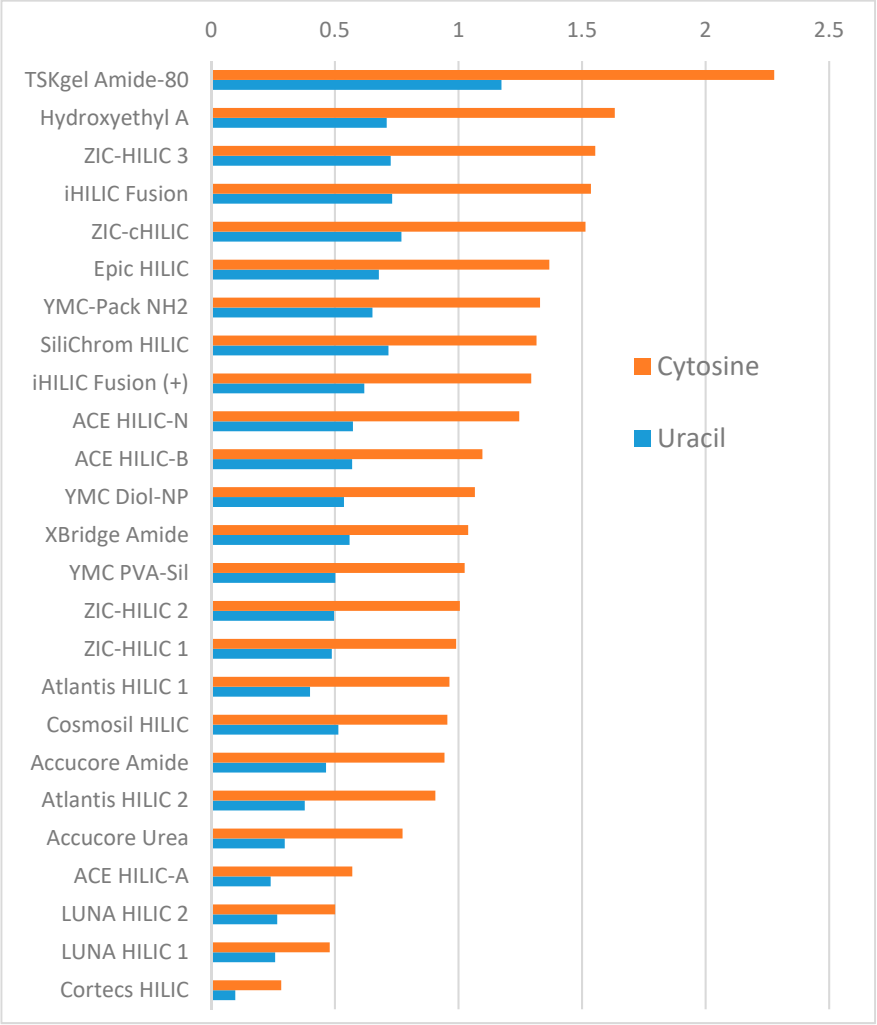

Figure 1. The retention factors of cytosine and uracil on 25 polar stationary phases in the mobile phase containing 75% acetonitrile and 5 mM ammonium acetate.

As shown in Figure 1, the retention of the polar stationary phases varies significantly in the same mobile phase conditions. TSKgel Amide-80 and Hydroxyethyl A columns take the top two spots based on the cytosine retention factor; and TSKgel Amide-80 seems to have much stronger retention than Hydroxyethyl A. The zwitter-ionic phases (e.g., ZIC-HILIC 3, ZIC-cHILIC, and iHILIC-Fusion) also show stronger retention for cytosine. In contrast, LUNA-HILIC phases with cross-linked diol groups display relatively low retention for cytosine. The Cortecs HILIC phase based on superficially porous silica has the weakest retention for cytosine. The retention data in Figure 2 indicates that the particle size has relatively insignificant effect on the retention (e.g., Altantis HILIC, LUNA HILIC and ZIC-HILIC phases); however, the pore size has a very significant effect on the retention. Two ZIC-HILIC phases with a larger pore size (200 Å) have significantly lower retention than their counterpart with a smaller pore size (100 Å). In comparison to cytosine, uracil has much smaller retention factors on the selected columns partially because cytosine (Log P ~ −1.73) is more polar than uracil (Log P ~ −1.07). This makes the selected stationary phases less differentiated based on the uracil retention factor. In some cases, the ranking of the stationary phase based on uracil changes noticeably (e.g., Hydroxyethyl A, ZIC-cHILIC, SiliChrom HILIC, and Cosmosil HILIC phases).

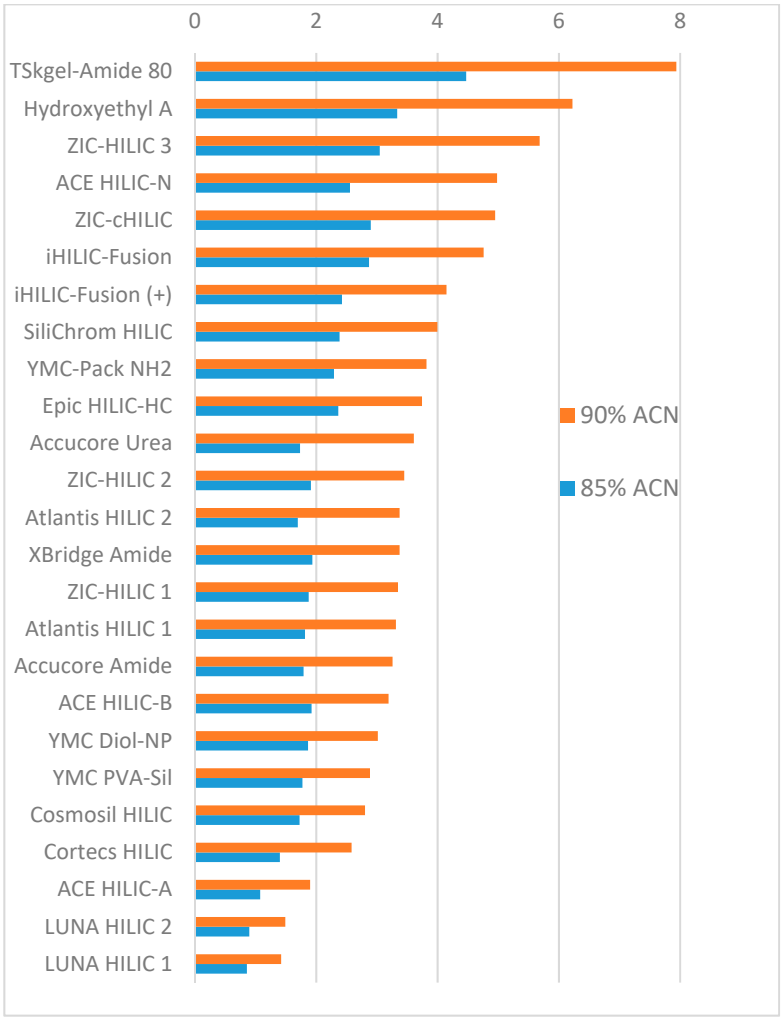

Figure 2. Cytosine retention factors on 25 polar stationary phases measured in the mobile phase containing 85% and 90% acetonitrile. Ammonium acetate concentration remains at 5 mM.

Figure 2 shows the ranking chart of the selected stationary phases based on cytosine retention factor in the mobile phase containing 85% and 90% acetonitrile. As expected, the retention of cytosine became much stronger at higher acetonitrile levels.

The overall ranking of the selected stationary phases does not change significantly in the mobile phase containing different levels of acetonitrile (Figures 1 and 2). TSKgel Amide-80, Hydroxyethyl A and ZIC-HILIC 3 phases remain the top three and ACE HILIC A and LUNA HILIC phases are at the bottom of the ranking at all the acetonitrile levels. However, the ranking of some stationary phases changes noticeably when the acetonitrile content increases. For example, the ACE HILIC-N phase ranks much higher in 90% acetonitrile than in 75% acetonitrile. The bare silica phases (Atlantis HILIC and Cortecs HILIC) jump up in the ranking in the mobile phase containing 90% acetonitrile. It is interesting to note that the superficially porous silica-based Accucore Urea phase moves up significantly in the ranking at 90% acetonitrile surpassing Accucore Amide. On the other hand, the stationary phases with hydroxyl groups on the packing surface (Epic HILIC-HC, YMC Diol-NP, and YMC PVA Sil) all drop in the ranking when the acetonitrile level increases.

In addition to the acetonitrile level, previous studies have demonstrated that the salt concentration can have significant effect on the retention of the neutral compounds in HILIC [25–27]. We selected three stationary phases, namely, ZIC-HILIC 3, XBridge Amide, and LUNA HILIC 2 to represent different levels of retentivity based on the previous results (Figures 1 and 2). The retention factors of the model compounds were measured with the ammonium acetate concentration in the range of

4–30 mM in the mobile phase containing 85% acetonitrile. Figure 3 shows the plots of the cytosine retention factor against the ammonium acetate concentration on the three stationary phases.

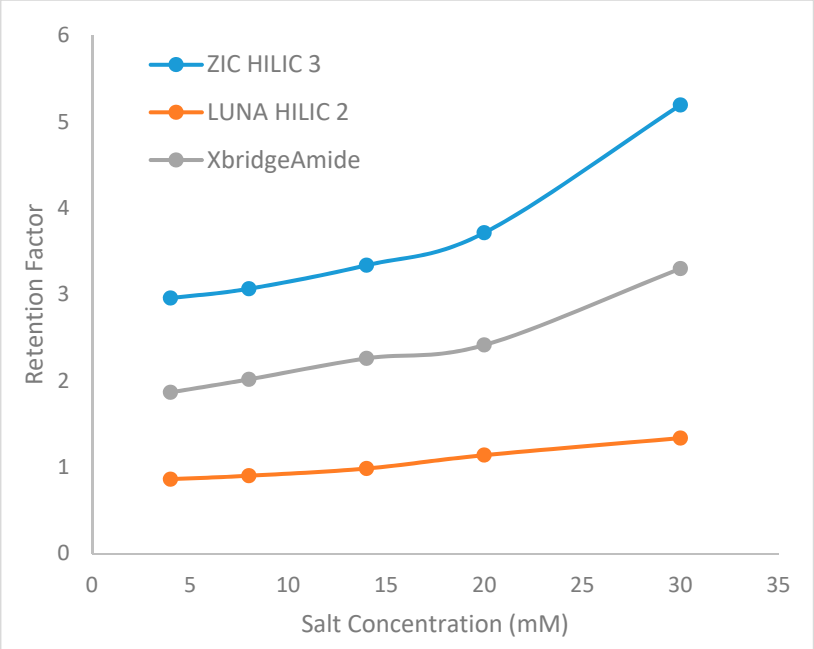

Figure 3. Cytosine retention factors on three polar stationary phases measured in the mobile phase containing 85% acetonitrile and ammonium acetate concentration 4–30 mM.

As shown in Figure 3, the cytosine retention factors increase with the ammonium acetate concentration in a non-linear fashion; however, the impact of the salt concentration seems to vary with the stationary phases. The least retentive LUNA HILIC phase experiences relatively small impact, but the salt concentration has a much more significant effect on the more retentive phase (e.g., ZIC-HILIC 3). Therefore, the salt concentration must be carefully selected for the testing procedure. More studies are ongoing to evaluate the effect of the salt concentration on other stationary phases. In this study, we selected a low salt concentration (5 mM) to minimize the impact of the salt concentration on the retention.

4. Conclusions

The results of this study demonstrate that the ability to retain polar compounds in HILIC varies significantly among the polar stationary phases currently available on the market. The current data provides general information on the ranking of the polar stationary phases based on the retention for the model compounds (e.g., cytosine). The ranking does not change drastically when the level of acetonitrile in the mobile phase changes, but the ranking for some stationary phases shifts noticeably. It should be emphasized that the ranking does not imply by any means the quality of the polar stationary phases, and should be considered together with the selectivity of the stationary phase for specific applications. The overall applicability of the stationary phase also depends on the physical chemical properties of the compounds and the chromatographic conditions. The study results also demonstrate that it is critical to use a common testing procedure to evaluate the retention of the polar stationary phases. The relative ranking depends on the test compounds and the mobile phase conditions. It is our hope that the data of this study can be useful to the column manufacturers towards adopting a common testing procedure.

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