Journal of Chromatography A 1715 (2024) 464608

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Practical examination of flow rate effects and influence of the stationary phase water layer on peak shape and retention in hydrophilic interaction liquid chromatography

David V. McCalley

Centre for Research in Biosciences, University of the West of England, Frenchay, Bristol, United Kingdom of Great Britain and Northern Ireland BS16 1QY, UK

ARTICLE INFO

Keywords:
HPLC
HILIC
Mobile phase
Flow rate

ABSTRACT

The effect of flow velocity on retention and peak shape of neutral, acidic and basic probe compounds was studied using seven different UHPLC hydrophilic interaction chromatography (HILIC) columns. Surprisingly on some columns, the retention factor k was found to vary somewhat with flow velocity, due to the combined effects of pressure and of frictional heating on retention. The selectivity of different HILIC columns was much greater than typically found with RP columns. The volume of the water layer on the HILIC columns was measured using the toluene exclusion procedure. For the neutral solute uridine, a good correlation was found between the volume of the water layer and retention, indicating the likely domination of a partition mechanism. For the ionogenic solutes, the correlation was generally poor, due to the presence of strong additional mechanisms such as ionic retention and repulsion. Reduced Van Deemter plots for uridine showed a negative correlation between the reduced b coefficient and the volume of the water layer, which can be attributed to reduced surface diffusion in this viscous layer. Once again, the behaviour of ionic solutes was complex on some columns making detailed interpretation difficult.

1. Introduction

Hydrophilic interaction chromatography (HILIC) has become a useful complementary technique to RP-LC especially for the analysis of polar/ionised solutes that are difficult to retain by RP [1,2]. For samples amenable to both techniques, HILIC has some advantages over RP. Furthermore, recent studies have been successful in overcoming some of the limitations of HILIC such as long equilibration times [3,4], injection solvent incompatibility difficulties [5] and deleterious interactions with metals [6]. Its range of applications extends even to large biopharmaceuticals such as monoclonal antibodies [7,8].

It is generally accepted that partition of solutes between a layer of water held on the surface of a polar column and the bulk mobile phase is a major contributor to retention of neutral solutes, whereas ionic retention and repulsion effects are also important for ionized solutes. McCalley and Neue measured the pore volume occupied by water by monitoring the exclusion of toluene from the water layer [9]. Guo adapted this method and applied it to a number of different HILIC columns [10]. Furthermore, he used it to estimate the phase ratio of columns and hence the % of the retention of neutral molecules contributed by adsorption and partition [11]. The basic method of measuring the water layer volume was later validated by Gritti and co-workers using a theoretical approach [12].

Recent research has included studies of the kinetics of HILIC separation, comparing the results with those from RP separations [13–17]. The finding that the Van Deemter B term is generally smaller in HILIC than RP is significant in that it suggests that slow diffusion takes place in the (viscous) water layer adsorbed on the column surface. This slow diffusion may limit the performance of HILIC at elevated flow rates. More recently, very low intra-particle diffusion rates have been measured in polymeric zwitterionic HILIC columns (both polymeric and monolithic), indicating that diffusion inside the pores is at least 100 times smaller than in the bulk mobile phase [18]. Gritti [12] compared various ethylene bridged hybrid silica columns (bare silica, amide, zwitterionic), showing the intra-particle diffusivity normalised to the bulk diffusivity decreased from 0.33 on the BEH silica to 0.10 for amide to 0.03 for the zwitterionic column. The retention of neutrals, which increased markedly from the silica to the amide to the zwitterionic column, was correlated to the volume of water as measured on the column surface by the toluene exclusion procedure.

The aims of the present study can be summarised as:

E-mail address: David.Mccalley@uwe.ac.uk.

https://doi.org/10.1016/j.chroma.2023.464608
Received 26 October 2023; Received in revised form 18 December 2023; Accepted 24 December 2023

- To compare the flow rate performance of a number of representative HILIC columns from different selectivity groups including bare silica, zwitterionic, neutral amide, and columns with anionic or cationic groups. Most previous data pertains only to bare silica HILIC or to a few types of stationary phase.
- To compare the flow performance of the phases with acidic and basic as well as the usual neutral solutes. Retention data for these solutes should also allow some rationalisation of selectivity on the different column types.
- To measure the volume of the water layer on columns of the same geometry packed with different very small particle stationary phases (sps).
- To investigate the possible correlation between solute retention and water layer volume.
- To attempt correlation between the extent of the water layer on these columns and the reduced van Deemter coefficients b and c.

The aim was to present a practical view of flow dependence of retention and efficiency, using a variety of HILIC columns/solutes. These experiments should be of use to practitioners hoping to optimise analysis conditions in HILIC.

2. Experimental

Experiments were performed using a 1290 ultra-high performance liquid chromatograph (UHPLC, Agilent, Waldbronn, Germany) comprising a binary pump, autosampler and photodiode array UV detector (0.6 µL low dispersion flow cell of 10 mm path length) set at 210/254 nm (bandwidth 4 nm) with data collection rate 160 Hz. The instrument was fitted with the ultra-low dispersion needle seat and flow-through needle options. The columns used are specified in Table 1. The temperature of the oven was set at 30 °C. The mobile phase (mp) was 5 mM ammonium formate (AF) buffer pH 4.4 in 90 % ACN-water, at a flow of 0.05 to 0.70 mL/ min. 5 mM AF maintains good buffering properties with minimal suppression of MS sensitivity, if using this method of detection. ${}^{w}_{w}$pH was measured before addition of the organic solvent. Columns were equilibrated in the mp for at least 1 h at 0.4 mL/min. 1 µL injections of 20 mg/L solutes uridine(URD, log D $_{\mathrm{pH4.4}} = -2.1$); 3-hydroxybenzoic acid (3OH-BA, log D $_{\mathrm{pH\ 4.4}} = 1.10$), nortriptyline (NOR log D $_{\mathrm{pH4.4}} = 0.94$) and trimethylphenylammonium chloride (TMPAC, log D $_{\mathrm{pH4.4}} = -2.15$) were purchased from Sigma-Aldrich (Poole, U.K.). Log D values were obtained from the programs Marvin (ChemAxon, Budapest, Hungary) and I-Lab (ACD, Toronto, Canada). Each solute was prepared at a concentration of 20 mg/ L in the mobile phase; samples (1 µL) were injected in duplicate. Solvents and additives ACN, (gradient UV grade), AF, formic acid (FA) and ammonia solution (all MS grade) were from Fisher (Loughborough, U.K.). Column efficiency was measured using the half-height procedure. This method gives a reasonable quantitative and relative indication of peak shape, when peaks are mostly symmetrical, as in the present study. The method for calculation of the column efficiency of such peaks also gives good precision. Curve fitting was performed using Origin 6.1 software (OriginLab, Northampton USA).

3. Results and discussion

3.1. *Is the retention factor in HILIC independent of flow?*

It was desirable first of all to establish a set of HILIC columns for the study which contained at least one example of columns that had different retention properties. Such a set should contain at least a bare silica column (cation exchange contribution to retention) an amino column (anion exchange) a neutral column (e.g. amide, hydrophilic retention) and a zwitterionic column [19] where the charges on closely spaced anionic and cationic ligands supposedly cancel each other out (quasi neutral with some ionic retention properties). A fundamental assumption in LC is that the retention factor k is independent of flow and of column length. However, careful examination of Fig. 1 shows that in practice and under certain conditions, k may not be exactly independent of flow. k was calculated from the formula

$$k = (t_R - t_0)/t_0$$

where t_R is the solute retention time and t_0 is the void time of the column measured by the retention of toluene. Changing the flow rate from 0.05 to 0.7 mL/min. (linear velocity 0.046 to 0.61 cm/s) resulted in a decrease in k on the ZWIT column of 15 %,13 %, 8 % and 8 % for TMPAC, URD, 3OH-BA and NOR respectively. These results may be attributed to the effects of frictional heating and to pressure. For the flow rate range above, pressure increased from 26 to 398 bar on the ZWIT column but only up to a max. of 138 bar on the 3.5 µm ZIC column. Increasing temperature generally causes a reduction in retention (although for some bases may cause an increase in retention), and increasing pressure alone also generally gives reduced retention in HILIC [1]. Thus, temperature and pressure effects usually act in the same direction. In RP, temperature and pressure effects generally act in opposite direction, and may (partially) cancel each other [1,15]. Clearly, these effects are dependent on parameters such as column particle size and external porosity. For instance, the Titan column (Fig. 1) shows little variation in k with flow rate which may be partially due to its somewhat larger particle diameter (1.9 µm, 14 to 221 bar) compared with the ZWIT column . For the ZIC column variations were very small in line with the lower pressures of this larger particle column. These effects on k were previously noted for a single HILIC silica column [15], but are shown here to be common for a range of HILIC columns.

3.2. *Measurement of the excess water held on the surface of the stationary phase*

The principle of simple measurement of the amount of excess water immobilised on the stationary phase was first established by McCalley and Neue [9]. The volume of water is obtained by subtracting the retention volume of the unretained solute toluene in the working mobile phase (e.g. 5 mM AF pH 4.4 in 90 % ACN at 0.4 mL/min.) from the retention volume of toluene using pure ACN as the mobile phase at the same flow. Other methods have verified the presence of the water layer such as the molecular simulation dynamics procedure of Tallarek [20] and the Karl Fischer titration procedure of Irgum [21].

The assumption in [9] is that toluene is (mostly) excluded from the

Table. 1
Column properties.

Column	Dimensions L x i.d.(cm)	Manufacturer	Type	Particle Diam. (µm)	Pore Diam. (Å)	Surface area m^2/g
BEH prem. amide	10 × 0.21	Waters (Milford, USA)	Amide	1.7	130	185
BEH ZWIT	10 × 0.21	Waters	Sulfobetaine	1.7	95	270
Torus DEA	10 × 0.21	Waters	Diethylaminoethyl (amino)	1.7	130	185
Cortecs silica	10 × 0.21	Waters	Shell silica	1.6	90	n/a
ZIC—HILIC	10 × 0.21	Merck-Sequant Darmstadt, D	Sulfobetaine	3.5	200	n/a
Titan	10 × 0.21	Merck-Supelco	Silica	1.9	n/a	n/a
Penta-HILIC	10 × 0.21	AMT Wilmington, USA	Pentahydroxy shell	2.0	n/a	n/a

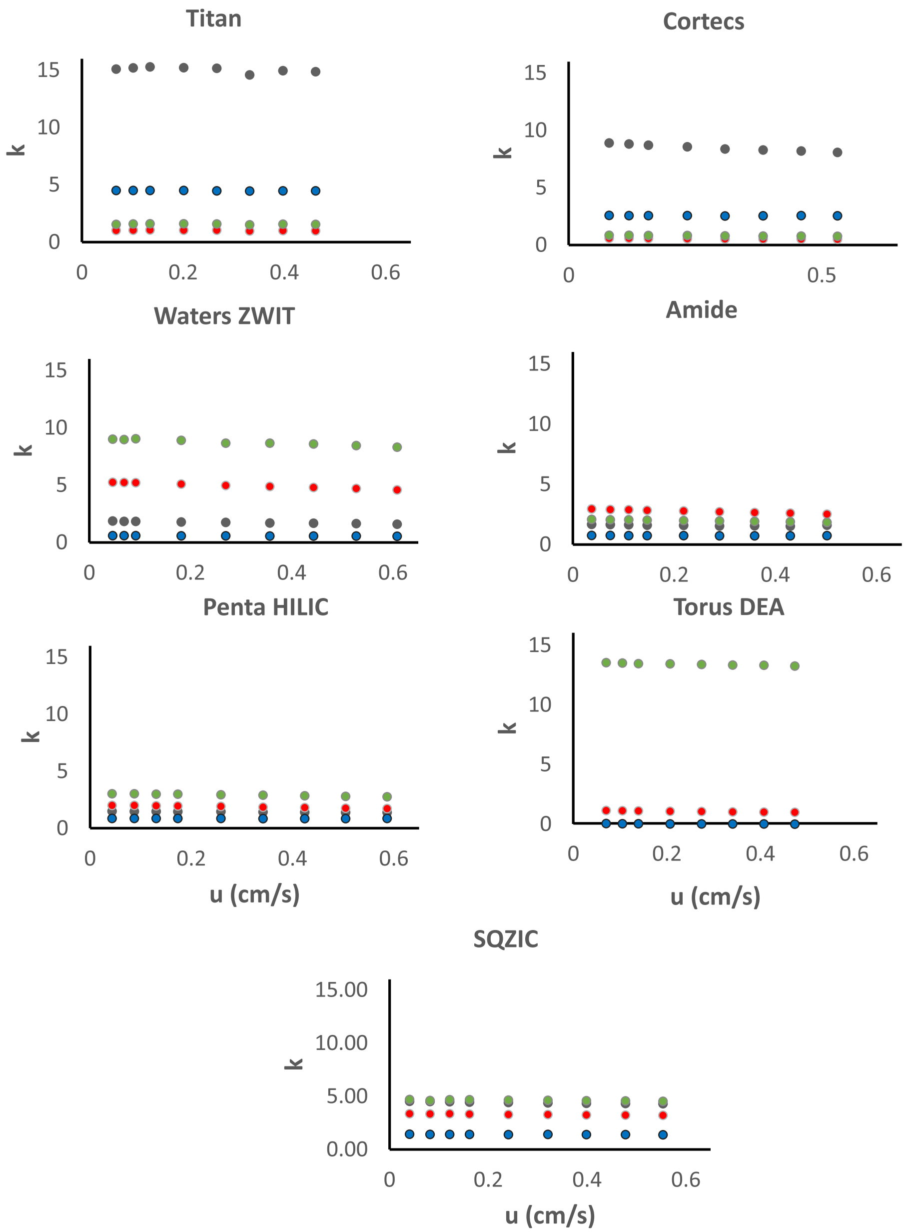

Fig. 1. Plots of retention factor against flow velocity for seven different HILIC columns. Mobile phase 5 mM AF pH 4.4 in 90 % ACN. Detection UV at 210 nm. Temperature 30 °C. Solutes: URD (red); 3OH-BA (green); NOR (blue); TMPAC (black).

water layer and thus has a smaller retention volume in the working mobile phase than in pure ACN (assuming no water layer exists in pure organic solvent). However, NMR studies [22] have shown that some toluene molecules can penetrate the water layer leading to very slight retention of this solute, but the effect seems to be very small. Furthermore, washing the column even with pure HPLC grade ACN may not remove all of the tightly bound water layer to polar column ligands or silanols [9], especially with underivatized silica sps. In order to minimise this latter effect, ACN dried over molecular sieve was used instead of normal HPLC grade ACN. However, this measure was demonstrated to be inconclusive, as the retention of toluene was similar to when using normal purified solvent.

The basic procedure [9] was usefully expanded and explained in a paper by Guo [10] who confirmed the basic relationship

$$V_{water} = V_{ACN} - V_M$$

where V_{water} is the volume of the water layer, V_{ACN} is the column holdup volume measured using toluene and pure ACN as the mobile phase, and V_M the same except using the working mobile phase. This equation was extensively validated in a theoretical paper by Gritti and co-workers [12]. Overall, the toluene exclusion method appears to be simple and accurate, and was thus selected for the present study.

Table 2 shows that the water layer in the columns ranged from 0.0088 mL for Cortex silica (a shell column) to 0.041 mL for the ZWIC phase. It is difficult to compare the results in Table 2 with those obtained by others using different methodologies because nominally similar columns may give different results according to factors such as the properties of the base material, the structure/coverage of any bonded ligands and of course, differences in the methodology used. Table 2 indicates that increasing water uptake occurs in the order:

Bare silica (low)∼ pentahydroxyl< BEH amide < amino <zwitterionic (high)

Jandera (as reported in [1]) gives the equivalent number of monomeric water layers inside the pores of the adsorbent surface at full saturation capacity as:

Table. 2
Reduced van Deemter coefficients and water layer volume for test solutes on seven different columns.

	dp(µm)		a	b	c	Water (mL)
BEH amide	1.7					
	tpp	Uridine	0.78	1.08	0.50	
		3-OH bz	1.03	0.89	0.59	0.0196
		Nortrip	2.22	0.77	0.34	
BEH zwitt.	1.7					
	tpp	Uridine	1.53	0.97	0.84	
		3-OH bz	2.21	0.78	0.49	0.0412
		Nortrip	16	−6.0	8.0	
Cortecs	1.6					
	shell	Uridine	0.27	2.18	0.41	
		3-OH bz	0.12	1.23	0.48	0.0088
		Nortrip	0.88	1.98	0.14	
Torus DEA	1.7					
	tpp	Uridine	0.69	1.17	0.32	
		3-OH bz	1.32	1.35	0.25	0.0216
		Nortrip	0.71	0.73	0.31	
Titan Si	1.9					
	tpp	Uridine	1.09	1.15	0.30	
		3-OH bz	0.90	0.78	0.50	0.0212
		Nortrip	0.56	2.37	0.25	
Penta HILIC	2					
	shell	Uridine	0.81	2.22	0.20	
		3-OH bz	4.23	0.65	0.21	0.0184
		Nortrip	1.16	0.94	0.15	
SQ-ZILIC	3.5					
	tpp	Uridine	1.23	0.69	0.33	
		3-OH bz	1.16	0.61	0.39	0.0292
		Nortrip	1.35	0.86	0.17	

tpp = totally porous particles.

Hydride< bare silica < pentahydroxyl< zwitterionic. Irgum's water uptake isotherms [21] indicate Bare silica< amino< amide < zwitterion

The difficulty in comparing different columns is indicated by the marked difference in the water uptake isotherms for amino columns from two different manufacturers even when measured using the same methodology in the same study [21]. Nevertheless, the methods show broad agreement.

3.3. Column retention and selectivity. Relationship of retention to water layer volume

Fig. 2 shows the exceptional differences in selectivity of these different sp types-these differences are much greater than those typically seen in RP-LC and can be considered as an advantage of HILIC. 7 columns were selected to include at least one of each of the selectivity groups [19]. All columns were of the same length and i.d. All contained particles of diameter ~ 2 µm to facilitate comparison of performance except ZIC, which was of the smallest particle size available (3.5 µm). The test solutes were 3OH- BA (acid, negatively charged), TMPAC and NOR (quaternary/basic, positively charged) and URD (neutral) under the experimental conditions (mp pH =4.4).

The two silica sps (Titan and Cortecs) showed similar selectivity with strong retention of TMPAC and NOR (peaks 4 and 5, Fig. 2). The (basic) DEA sp shows strong retention of the acid 3OH-BA (peak 3) whereas the positively charged solutes 4 and 5 are excluded, eluting before the void volume marker (toluene, peak 1). The neutral and quasi neutral sps (penta HILIC, zwitterionic, amide) show rather similar order of elution of the solutes. It is interesting that the Penta HILIC sp showed markedly different selectivity to the silica columns. Presumably the -OH groups on this sp are much less acidic that those on bare silica. The ZWIT and ZIC sps have rather similar selectivity; both are reported by the manufacturer to contain sulfobetaine ligands. The highest retention of (neutral) URD (peak 2) is shown by the two Zwitterionic sps, which are recommended to give the best separation of neutrals. This is expected due to the high water layer volume on the ZWIC and ZIC sps (0.041 and 0.029 mL respectively-see Table 2). Indeed, there is a good correlation between the water layer volume and the retention factor of URD (*r* = 0.89, Fig. 3). High levels of adsorbed water on zwitterionic sps have been attributed to the formation of a hydrogel with the polymeric ligands [21]. Guo [11] has suggested that the relation between the water layer volume and retention is likely to include contributions from adsorption and partition for neutral solutes. These contributions will vary from solute to solute. The correlation of the retention of 3OH-BA is much poorer (*r* = 0.50) can be attributed to additional strong attractive or repulsive interactions between solute and stationary phase other than neutral hydrophilic interactions. For instance, the DEA sp shows strong retention of 3OH-BA due to ionic retention which undoubtedly affects the correlation (see Fig. 3). The correlation of the retention of NOR with the water layer volume is poorer still. There are clearly strong additional interactions of NOR with the Titan silica sp, while DEA excludes this cationic probe due to repulsion. Despite the relatively poor correlation of *k* for NOR vs the water layer volume (*r*= −0.32), it is interesting that the graph shows a negative slope, which signifies that as the volume of the water layer increases, the retention of NOR tends to decrease. The retention of (protonated) NOR is likely to be dominated by strong ionic interaction with negatively charged column silanols. Increase in the volume of water may help to deactivate silanols and decrease this ionic retention.

3.4. Reduced van Deemter plots for the 3 solutes

The experimental plate heights as a function of flow rate were first corrected for instrumental band broadening by replacing the column with a short piece of narrow i.d. capillary tubing used to generate some back pressure in the instrument. The total bandspreading measured experimentally $\sigma^2_{\mathrm{tot,v}}$ equals the column bandspreading plus the instrumental bandspreading where

$$\sigma^2_{tot,v} = \sigma^2_{col,v} + \sigma^2_{ext,v}$$

the subscript col refers to the band spreading of the column itself and subscript ext refers to the bandspreading produced by the instrument [23,24]. σ_v in volume units can be obtained from σ_t measured in time units using the relationship

$$\sigma_v = \sigma_t F$$

where F is the volumetric flow rate typically measured in mL/min. The peaks obtained without the column in place were symmetrical in shape and showed no evidence of strong adsorption sites (e.g. metal-solute interactions) in the instrument [6].

Fig. 4 shows plots of the (dimensionless) reduced plate height vs the reduced velocity for 3 test solutes, corresponding to flow rates of 0.05 to 0.7 mL/min. TMPAC was omitted due to asymmetric peaks on some of the stationary phases and its low molar absorptivity which affected the reproducibility of the data. The data were fitted to the reduced form of the van Deemter equation.

$$h = a + b/\nu \ + c\nu$$

where *h* is the reduced plate height, *v* is the reduced velocity

$$\nu = ud_p / D_m$$

u is the linear velocity typically measured in cm/s, d_p the particle diameter in cm, D_m the diffusion coefficient of the solute in the mobile phase in cm^2/s; clearly v is also dimensionless. D_m values for a wide range of compounds in different solutes calculated using the Taylor–Aris method can be obtained from [25]. Optimum reduced velocity was generally in the range of 1.5–2.0, (corresponding to 0.2–0.3 mL/min.), generating (corrected) reduced plate height (h) typically of 2 (sometimes less for some solute/column combinations). Clearly, the behaviour of some solutes was anomalous e.g. NOR on the ZWIT column gave high values of h even at the lowest flow. This behaviour was similar using

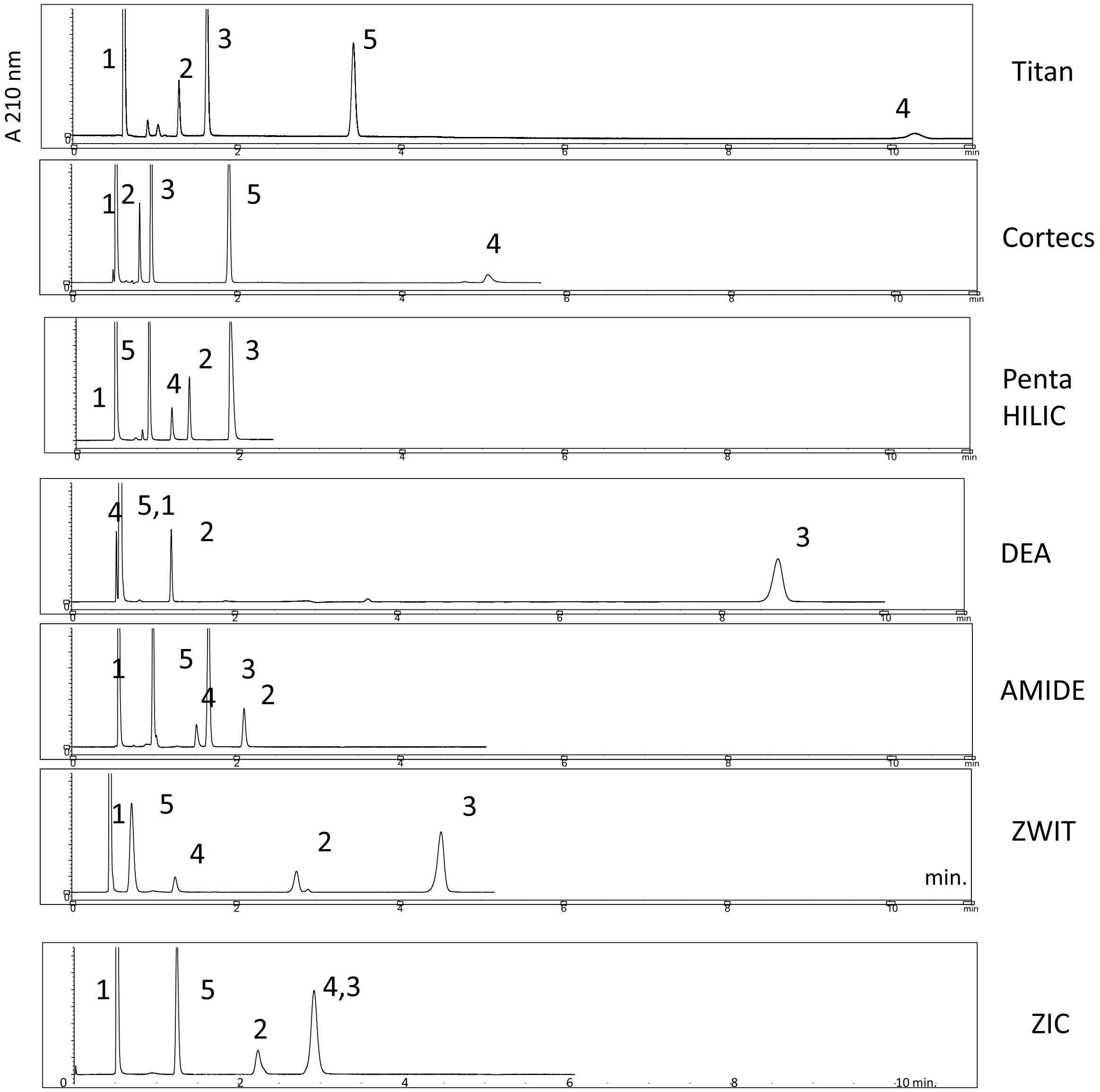

Fig. 2. Chromatograms of toluene (peak 1); URD (peak 2); 3OH- BA (peak 3); NOR (peak 4); TMPAC (peak 5) on seven different HILIC columns. Flow rate 0.4 mL/ min. Mobile phase and other conditions as Fig. 1.

TMPAC (both probes are positively charged) and can be attributed to strong adsorptive column sites. This effect is apparently absent from the ZIC column. Another seemingly anomalous result is the high value of h for 3OH-BA at all velocities on the penta HILIC column. Further study showed that this result was attributable to mass overloading effects (even using the standard 20 ng injections) resulting in fronting peaks at all velocities. Solute–solute interactions may occur for compounds with more hydrophobic character resulting in anti-Langmuir behaviour. Solubility in the thin layer of water on the penta HILIC phase may be a contributing issue to the overloading process [24].

Cabooter and co-workers found low b term coefficients (0.89–1.91) on three types of polymeric zwitterionic columns, representing low longitudinal dispersion in these columns [23]. In earlier work by Heaton et al., larger b coefficients were obtained on a Zorbax C18 silica RP column (4.74 to 6.31) compared with a similar silica HILIC column (2.10–3.08). Cabooter and co-workers used a Zorbax C18 column in the RP mode (b coefficients 3.29–6.29) followed by stripping the C18 with a hot solution of TFA in ACN, thus ensuring the similarity of the columns. The resultant bare silica column gave $b = 0.73$–1.27. It was noted that b coefficients in RP increased with increasing retention factor, as the solute spends more time in the sp, allowing increased surface diffusion, which is pronounced with this retention mechanism. The differences in b coefficient can be explained by slow stationary phase diffusion within the high viscosity low mobility water layer in HILIC compared with more rapid diffusion in the organic rich layer formed on a C18 surface [14–17].

Table 2 shows the reduced van Deemter a, b and c coefficients for the 3 test solutes on the 7 different columns used in the present study. The data for NOR on the Waters ZWIT column ($a = 16$, $b = -6.0$, $c = 8.0$) are clearly anomalous as shown in the reduced van Deemter plots (Fig. 4) and are excluded from the immediate discussion and correlations below. We intend to present in the future a more in-depth analysis of the results, using also peak parking experiments to allow a more accurate estimation of the b coefficients. This treatment should enable a clearer picture of kinetic performance to be obtained. Nevertheless, the current presentation shows what the experimentalist will see in practice. The data for the Merck ZIC column which also has sulfobetaine ligands contrasts strongly with that of the ZWIT column. This result might be due to the presence of some active sites for bases on the ZWIT column. The range of b coefficients for URD was 0.69 to 2.22 with the highest value for the

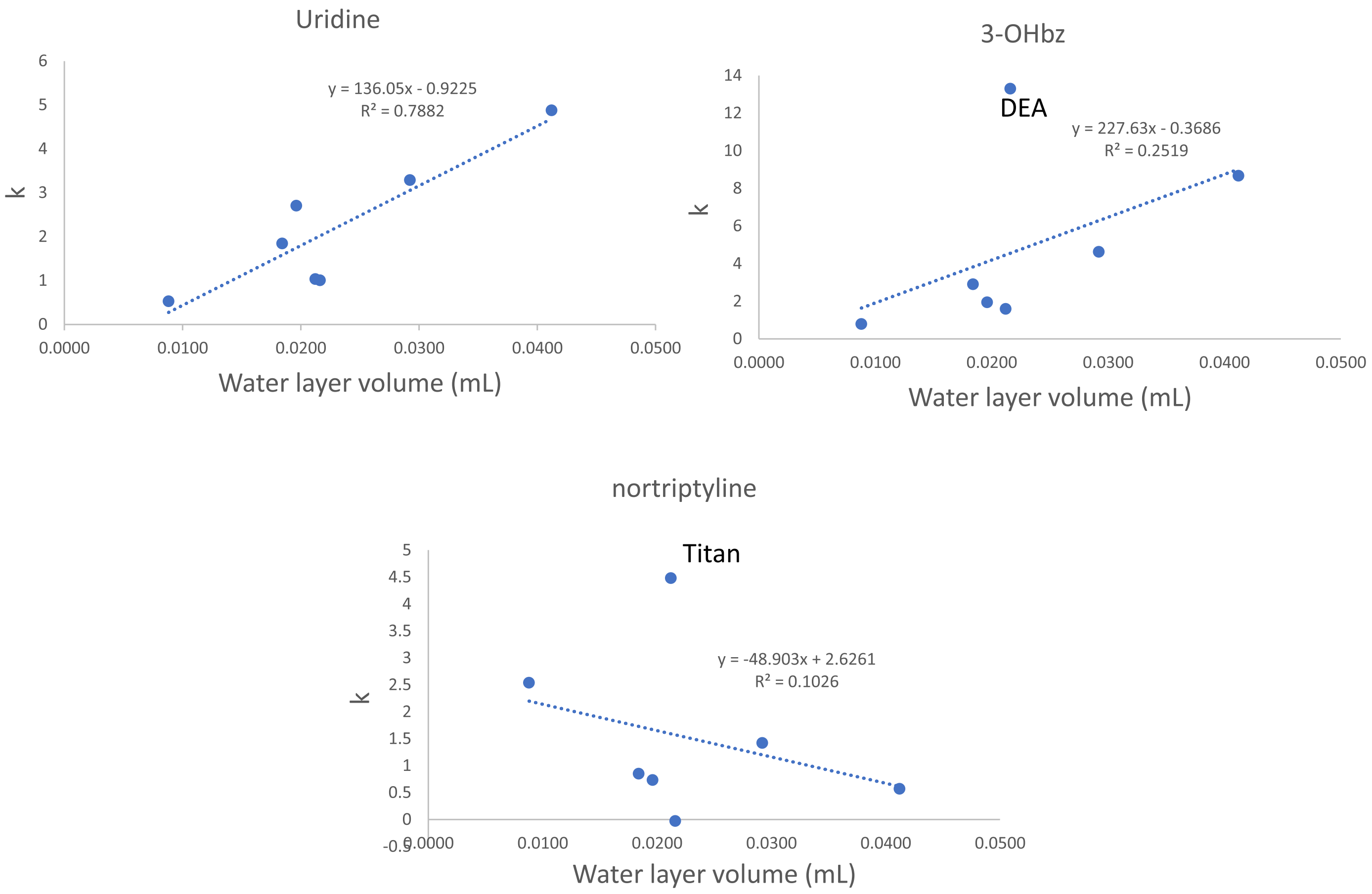

Fig. 3. Correlation plots of retention factor vs adsorbed water layer volume for seven HILIC columns using URD, 3OH-BA and NOR as test solutes. For other conditions, see Fig. 2.

Pentahilic column (water layer 0.0184 mL) and the lowest for the ZIC column (water layer 0.0292 mL). These low values agree with the reports of Cabooter and Heaton. Note that the present study uses a much greater range of HILIC columns and solutes than previous investigations.

In our study, the correlation coefficient r between the reduced b coefficient for URD and the water layer volume was −0.82, in line with the arguments above which suggest that hindered diffusion takes place as the water layer volume increases. A moderate correlation of −0.53 was obtained with 3OH-BA, and $r = -0.45$ for NOR. These poorer correlations can be explained by the complexity of the retention mechanism for ionised solutes. Nevertheless, the reduced b coefficients for 3OH-BA and NOR on these HILIC columns remain low.

The reduced c coefficients are also shown in Table 2. Previously these were found to be 2 to 3 times smaller in RP with a C18 column than with a single bare silica column [15]; the values for c in HILIC in the present study are similarly high (range 0.20–0.84; 0.21–0.59; 0.14–0.34 for URD, 3OH-BA and NOR respectively. Cabooter [17] also found larger c coefficients in a silica HILIC column than for the equivalent RP column. In the present study The c coefficients gave correlation with the water layer volume $r = 0.66$, −0.18 and 0.24 for URD, 3OH-BA and NOR respectively. In theory, positive correlations would be expected as increasingly hindered mass transfer would be expected as the volume of the water layer increased. The low correlations of c with the water layer volume might also be due to experimental error resulting from the limited range of the size of the coefficients.

The high c coefficients for HILIC suggest that the VD curves at high velocity will be steeper than in RP and thus HILIC is less suitable for fast analysis than RPLC. Nevertheless, the practical analyst may still find higher efficiency for the same solute at high flow in HILIC compared with RP analysis. This difference is due to the consideration of non-reduced parameters where the higher D_m values in typical HILIC mobile phases (high ACN concentrations) are not factored out as is the case in reduced plots.

Gritti also found elevated c coefficients for the single neutral solute cytosine on the Waters ZWIT phase, attributing this finding to the enhanced water layer on this column [12].

4. Conclusions

The measured retention factor in HILIC is not always exactly constant as shown for a range of different columns. In HILIC, retention usually decreases with the increasing temperature that results from frictional heating produced as the flow rate is increased. Pressure alone tends to decrease retention in HILIC while it usually increases in RP-LC. These effects act in the same direction in HILIC and thus can produce important decreases in retention as the flow rate is increased. Different HILIC columns have widely different selectivity, which can be advantageous in method development.

The volume of the water layer, determined by the toluene exclusion method, is greatest for columns with extensive polymerised hydrophilic layers such as the zwitterionic columns investigated here. The retention of the neutral solute URD correlates reasonably well with the water layer volume on various columns. However, care must be exercised in the interpretation of such results even for neutral solutes due to the solute dependent contributions of adsorption and partition to retention. The adsorption and partition mechanism may be influenced differently by the extent of the water layer. The correlation of the retention of an acidic probe with the water layer volume was much less, indicating the effect on retention of mechanisms other than hydrophilic processes such as ionic repulsion or attraction. The retention of the basic probe NOR

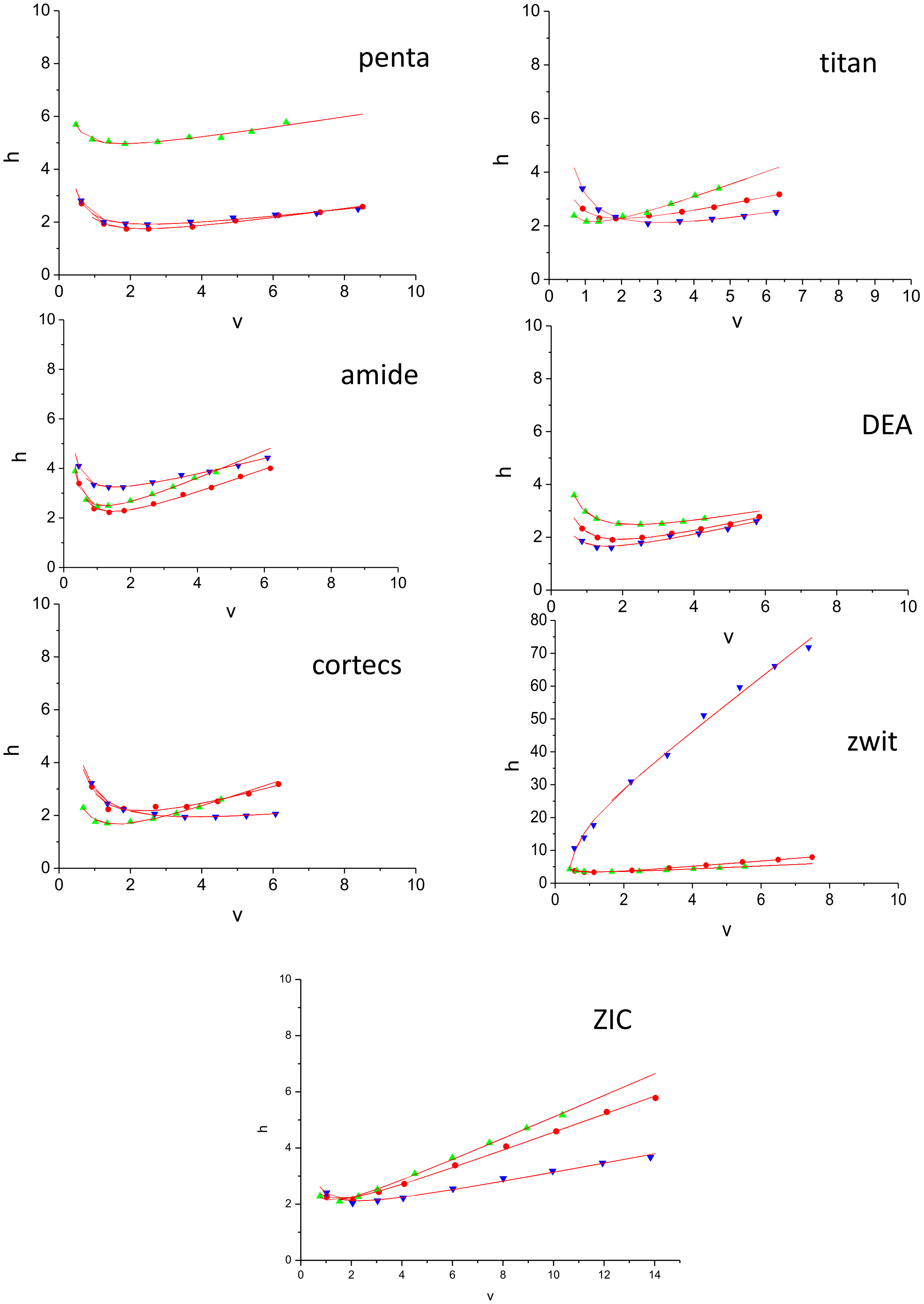

Fig. 4. plots of reduced plate height vs the reduced velocity for seven HILIC columns with data fitted to the reduced van Deemter equation. Other conditions as Figs 1 and 2.

showed a negative correlation with the volume of the water layer. This result may be indicative of shielding of ionised strong retention sites (silanols) as the volume of water increases.

A reasonable negative correlation was observed between the reduced b coefficient of URD on the seven columns and the volume of the water layer. This result is attributable to reduced surface diffusion in the viscous water layer on the surface of the stationary phase-diffusion becomes more restricted as the water layer volume increases. Moderate negative correlation was also observed for NOR and 3OH-BA. As before, the complexity of the retention mechanism may be responsible.

In theory, c coefficients should increase with the volume of the water layer, reflecting the adverse effect on mass transfer. However, our results indicated rather poor correlation except for URD. This result may be indicative of errors resulting from the rather small range of c coefficients in the columns used. Throughout, it is much more difficult to interpret flow rate effects with ionised rather than neutral solutes. Some unusually strong retention or repulsion effects can occur with such solutes.

CRediT authorship contribution statement

David V. McCalley: was the sole contributor to this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors thank Agilent Technologies (Waldbronn, Germany) for the gift of the HPLC instrument, and Waters (Milford, USA), AMT (Wilmington, USA) and Merck (Darmstadt, D) for the gift of some of the columns used in this work.

References

[1] D.V. McCalley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1523 (2017) 49–71, https://doi.org/10.1016/j.chroma.2017.06.026.

[2] A. Cavazzini, M. Catani, A. Felinger, Hydrophilic interaction liquid chromatography, in: Liquid Chromatography, Fundamentals and Instrumentation, 3rd ed, 1, 2023, pp. 227–249, https://doi.org/10.1016/B978-0-323-99868-7.00030-8.

[3] D.V. McCalley, Managing the column equilibration time in hydrophilic interaction chromatography, J. Chromatogr. A 1612 (2020) 460655, https://doi.org/10.1016/j.chroma.2019.460655.

[4] D.V. McCalley, A study of column equilibration time in hydrophilic interaction chromatography, J. Chromatogr. A 1554 (2018) 61–70, https://doi.org/10.1016/j.chroma.2018.04.016.

[5] M.R. Taylor, J.K. D.V.McCalley, Managing sample introduction problems in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1700 (2023) 464006, https://doi.org/10.1016/j.chroma.2023.464006.

[6] D.V. McCalley, Influence of metals in the column or instrument on performance in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1663 (2022) 462751, https://doi.org/10.1016/j.chroma.2021.462751.

[7] V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Hydrophilic interaction chromatography hyphenated with mass spectrometry: a powerful analytical tool for the comparison of originator and biosimilar therapeutic monoclonal antibodies at the middle-up level of analysis, Anal. Chem. 89 (2017) 2086–2092, https://doi.org/10.1021/acs.analchem.6b04726.

[8] A. Periat, S. Fekete, A. Cusumano, J.L. Veuthey, A. Beck, M. Lauber, D. Guillarme, Potential of hydrophilic interaction chromatography for analytical characterisation of protein biopharmaceuticals, J. Chromatogr. A 1448 (2016) 81–92, https://doi.org/10.10106/j.chroma.2016.04.056.

[9] D.V. McCalley, U.D. Neue, Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography, J. Chromatogr. A 1192 (2008) 225–229, https://doi.org/10.1016/j.chroma.2008.03.049.

[10] Y. Guo, N. Bhalodia, B. Fattal, Y. Serris, Evaluating the adsorbed water layer on polar stationary phases for hydrophilic interaction chromatography (hilic), Separations 6 (2019) 19–28, https://doi.org/10.3390/separations6020019.

[11] Y. Guo, D. Baran, Hydrophilic partitioning or surface adsorption? A quantitative assessment of retention mechanisms for hydrophilic interaction chromatography (HILIC), Molecules 28 (2023) 6459, https://doi.org/10.3390/molecules28186459.

[12] B.A. Alden, F.G.J. McLaughlin, T.H. Walter, Retention and mass transfer properties of the series of unbonded, amine bonded and alkylsulfobetaine-bonded ethylene bridged hybrid hydrophilic interaction liquid chromatography columns, J. Chromatogr. A 1692 (2023) 463828, https://doi.org/10.1016/j.chroma.2023.463828.

[13] G. Desmet, Broeckhoven K, S. Deridder, D. Cabooter, Review of recent insights in the measurement and modelling of the B term dispersion and related mass transfer properties in liquid chromatography, Anal. Chim. Acta 1214 (2022) 339955, https://doi.org/10.1016/j.aca.2022.339955.

[14] H. Song, G. Desmet, D. Cabooter, Assessment of intra particle diffusion in hydrophilic interaction chromatography and reversed-phase liquid chromatography under conditions of identical packing structure, J. Chromatogr. A 1523 (2017) 204–214, https://doi.org/10.1016/j.chroma.2017.06.068.

[15] J.C. Heaton, X. Wang, W.E. Barber, S.M.C. Buckenmaier, Practical observations on the performance of bare silica in hydrophilic interaction chromatography compared with reversed-phase liquid chromatography, J. Chromatogr. A 1328 (2014) 7–15, https://doi.org/10.1016/j.chroma.2013.12.058.

[16] J.C. Heaton, D.V. McCalley, Comparison of the kinetic performance and retentivity of sub- 2 μm core shell, hybrid and conventional bare silica phases ihydrophilic interaction chromatography, J. Chromatogr. A 1371 (2014) 106–116, https://doi.org/10.1016/j.chroma.2014.10.013.

[17] H. Song, G. Desmet, D. Cabooter, Evaluation of the kinetic performance differences between hydrophilic-interaction liquid chromatography and reversed-phase liquid chromatography under conditions of indentical packing structure, Anal. Chem. 87 (2015) 12331–12339, https://doi.org/10.1021/acs.analchem.5b03697.

[18] H. Li, G. Desmet, Z. Jiang, D. Cabooter, On the occurrence of very low intra particle diffusion rates in zwitterionic hydrophilic interaction liquid chromatography polymer columns, J. Chromatogr. A 1683 (2022) 463531, https://doi.org/10.1016/j.chroma.2022.463531.

[19] T. Jonsson, N.P.D.K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 5880–5891, https://doi.org/10.1016/j.chroma.2011.06.037.

[20] S.M. Melnikov, A. Holtzel, A. Seidel-Morgenstern, U. Tallarek, A molecular dynamics study of the partition mechanism in hydrophilic interaction chromatography, Angew. Chem. Int. Edit. 51 (2012) 6251–6254, https://doi.org/10.1002/anie.201201096.

[21] N.P. Dinh, T. Jonsson, K. Irgum, Water uptake on polar stationary phases under conditions for hydrophilic interaction chromatography and its relation to solute retention, J. Chromatogr. A 1320 (2013) 33–47, https://doi.org/10.1016/j.chroma.2013.09.06122.

[22] A. Shamshir, N.P. DInh, T. Sparmann, M.J. Ashiq, K. Irgum, Interaction of toluene with polar stationary phases under conditions typical of hydrophilic interaction chromatography probed by saturation transfer difference nuclear magnetic resonance spectroscopy, J. Chromatogr. A 1588 (2019) 58–67. 10.1016/j.chroma.2018.11.02.

[23] H. Li, Z. Jiang, G. Desmet. D. Cabooter, In depth performance analysis of monolithic and particulate zwitterionic hydrophilic interaction liquid chromatography polymer columns, Molecules 28 (2023) 2902, https://doi.org/10.3390/molecules28072902.

[24] D.V. McCalley, Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds? J. Chromatogr. A 1171 (2007) 46–55, https://doi.org/10.1016/j.chroma.2007.09.047.

[25] H. Song, Y. Vanderheyden, E. Adams, Desmet G, D. Cabooter, Extensive database of liquid phase diffusion coefficients of some frequently used test molecules in reversed-phase liquid chromatography and hydrophilic interaction liquid chromatography, J. Chromatogr. A 1455 (2016) 102–112, https://doi.org/10.1016/j.chroma.2016.05.054.