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A study of column equilibration time in hydrophilic interaction chromatography.

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Keywords: hydrophilic interaction chromatography; HILIC; equilibration.

Highlights

- Full column equilibration for isocratic analysis in HILIC takes up to 1 hour.
- Equilibration best measured by retention time constancy, not baseline disturbance.
- Equilibration depends on column, flow and pre-equilibration (“storage”) solvent.

- Repeatable partial equilibration possible in ~ 5min. for gradient elution.
- Selectivity changes dependent on equilibration time, so it should be held constant.

Abstract

The time taken to achieve full column equilibration for isocratic analysis of acidic, basic, and neutral solutes in hydrophilic interaction liquid chromatography (HILIC) was compared using the mobile phase disturbance method, column efficiency measurements and retention time stability. Full equilibration, which could take up to an hour, was best measured by the last procedure and was found to depend on the nature of the stationary phase, the pre-equilibrium (e.g. storage) solvent and the flow rate. While longer equilibration times are a relatively minor inconvenience in isocratic analysis, they are surprisingly not a barrier to the use of gradient elution in HILIC. A repeatable partial equilibration giving retention time stability equivalent to that in isocratic analysis was demonstrated for an equilibration time of only ~5 min., using as few as 2 preliminary conditioning runs on a column that had taken the longest time to achieve full equilibration. Due to selectivity changes that occur dependent on the equilibration time, it is necessary to use identical gradient conditions in a series of analyses, which however appears to be facile on a modern HPLC instrument.

1. Introduction.

Hydrophilic interaction chromatography (HILIC) is particularly suited to the analysis of polar and ionised solutes that can be difficult to retain using reversed-phase (RP) techniques [1, 2]. It has been used for important applications particularly in pharmaceutical analysis [3], for determination of amino acids, peptides and proteins [4] and in metabolomics [5, 6]. A number of reviews on the technique and its applications have been published recently [3, 7-9]. For those solutes that can be analysed by either technique, HILIC offers a useful alternative selectivity to RP as shown by low correlation of retention factors, a feature that may also be useful for 2 dimensional separations [10]. HILIC has a number of advantages over RP, which include the low backpressure that results from the organic-rich eluents typically employed, leading to the possibilities for use of long columns and/or high flow rates [11]; increased sensitivity in linkage with mass spectrometry [12, 13], and good peak shapes with basic compounds that can give asymmetric peaks in RP separations [11].

A perceived disadvantage of HILIC is the apparently long equilibration time of the column with the mobile phase. However, there are relatively few rigorous studies that have investigated this phenomenon in detail. Shollenberger and Bell [14] in some innovative studies investigated re-equilibration in HILIC using an aqueous gradient of 5-50 % 5 mM ammonium acetate (AA) in ACN. Further experiments were performed where the buffer concentration was increased from 2-10 mM AA in a constant concentration of 90 % ACN. The test solutes included polar neutral and basic compounds. A bare silica and a pentahydroxy silica phase were investigated. For the aqueous gradient studies, it was found that repeatable results were obtained after a re-equilibration time of as little as 2 min., whereas full equilibration of the column took considerably longer, although these times were not stated. Results were broadly similar on both columns studied, and seemed mostly independent of solute type. These findings have some similarity to those found by Carr and co-workers in RP chromatography, who showed repeatable partial re-equilibration was possible with only 2 column volumes for fast cycling of the second dimension in 2D analysis [15, 16]. Again, full equilibration took considerably longer. In the buffer gradient study [14], little effect was found of equilibration time on retention of polar neutral compounds. This result is explicable considering the generally small effect of buffer

concentration on retention of neutrals in HILIC [17]. In contrast, much greater changes in retention with equilibration time occurred for basic compounds, particularly on the silica column, whose retention is known to be dominated by ionic interactions. Nevertheless, it appeared that a similar short repeatable equilibrium in gradients was obtainable in all cases. A further interesting study by Bell [18] using silica, pentafluorphenyl, penta hydroxyl and zwitterionic columns with acid, basic and neutral probes, concentrated on isocratic full equilibration studies with pre-flushing exclusively in 60% ACN-water followed by equilibration in 5 mM (pH unadjusted) ammonium formate (AF) in 98 % ACN. It was suggested that full equilibration coincided with a shift in the UV background response of the detector, and that shift took longest with 98 % ACN-AF, which was the constant mobile phase composition used for all further studies. The correspondence of the shift with full equilibration was apparently demonstrated for all stationary phases studied. It appeared that stationary phases with high affinity for water equilibrated faster than phases such as silica, which has a low affinity for water [19]. Other phases that do not absorb an appreciable amount of water also equilibrated more rapidly. Limited quantitative data analysis was presented with these preliminary findings.

The aim of the present work was to investigate further the full and partial equilibration of HILIC stationary phases. Some questions addressed included the effect of the phase type i.e. fully porous (FPP) or superficially porous (SPP)) on equilibration time; the effect of stationary phase ligand; the effect of buffer in the pre-equilibration or “storage” solvent (organic-water or organic- buffer); whether baseline shifts in UV detector response can indeed provide a reliable indicator of full column equilibration; and the effect of gradient slope on the repeatability of retention in partial equilibration. Clearly, to study the effects of all these parameters in detail would necessitate a very large number of experiments, but we hoped to obtain at least some useful pointers for further studies.

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 flow cell). The columns (all 10 cm x 0.21 cm ID) were Cortecs silica (SPP, particle size 1.6 μm, pore diameter 90

Å, surface area 100 m^2/g), BEH silica (FPP, particle size 1.7 μm, pore diameter 137 Å, surface area 186 m^2/g) and BEH amide (FPP, particle size 1.7 μm, pore diameter 130 Å, surface area 185 m^2/g) all from Waters (Milford, USA); Advance Bioglycan (amide) (SPP, particle size 2.7 μm, pore diameter and surface area unavailable (Agilent); ZIC HILIC (FPP, particle size 3.5 μm, pore diameter 100 Å, surface area unavailable) and ZIC-cHILIC (FPP, particle size 3.0 μm, pore diameter 100 Å, surface area unavailable) both from Merck (Darmstadt, Germany). Acetonitrile (gradient UV grade), formic acid and ammonium formate were purchased from Fisher (Loughborough, U.K.). All test solutes were obtained from Sigma-Aldrich (Poole, U.K.). Standards were prepared at concentrations of 20-50 mg/L and injected dissolved in the buffered 95% ACN mobile phase. Buffered mobile phases were prepared by adjusting the pH of the aqueous portion before addition of ACN ($_w^w$ pH); both solvents were metered by weight and the premixed solvent delivered from a single pump [20]. Flow rate for equilibration and analysis was 0.5 mL/min. except where stated. For the isocratic experiments, columns were pre-equilibrated in the appropriate mobile phase (90 % ACN-water, 60% ACN-water or 5 mM AF pH 4.4 in 60 % ACN) for at least 2 hours prior to equilibration with the analysis solvent (exclusively 5 mM AF pH 4.4 in 95% ACN). The pump was carefully primed with the analysis solvent up to the end of the capillary connecting the injector to column, such that the analysis solvent immediately entered the column after time t = 0 min. The equilibration times mentioned represent the time between this solvent switch and the beginning of the chromatographic run indicated. For gradient elution, columns were equilibrated with a number of conditioning gradient runs (detailed below) where the data was not used other than to establish whether partial equilibration had occurred. Estimates of pK_a were taken as the average of values predicted by the programs Marvin (Chem Axon, Budapest, Hungary) and ACD I Lab (ACD, Toronto, Canada).

3. Results and Discussion.

3.1 Choice of column, solutes and selectivity; choice of mobile phase.

A column set for the equilibration experiments should contain examples that give different selectivity and are also commonly used for HILIC separations. A previous study indicated that silica, amide and zwitterionic phases provide a range of different selectivity and retention for acidic, basic and neutral solutes [17]. The test set

included examples of these phases, with some in fully porous particle (FPP) format and others as superficially porous (SPP) “shell” phases. Uridine, nortriptyline and 4-OH benzoic acid were chosen as examples of neutral, basic and acidic solutes respectively. Nortriptyline is a strong base (pK_a 10.2) and should be completed protonated under the experimental conditions. 4-OH benzoic acid is a weak acid (pK_a 4.5) but shows good retention on zwitterionic HILIC phases [17]. Under the analysis conditions used, it should be partially ionised [21]. In contrast, stronger acids like sulfonic acids may suffer poor retention due to the dominant effect of repulsion from negatively charged silanol groups on silica-based phases.

Fig. 1 shows the remarkably different selectivity of these 3 solutes on 6 different HILIC phases when fully equilibrated with 5 mM AF pH 4.4 in 95 % ACN. These chromatograms illustrate an advantage of HILIC in manipulating a separation by change of stationary phase. The selectivity of the two bare silica phases Cortecs (SPP) and BEH (FPP) silica was similar. Both gave highest retention of nortriptyline (peak 3) due to attractive interactions with negatively charged residual silanols [22], which may in contrast be at least partially shielded on the bonded phases. The BEH phases are based on a hybrid inorganic-organic substrate known to possess a low concentration of acidic silanols [17]. The low pore occupancy of water in silica columns (7-9% in 80% ACN [19]) and consequent poor hydrophilic selectivity [17] explains the low retention of uridine (peak 1). Both zwitterionic phases gave high retention of 4-OH benzoic acid (peak 2). These phases have thick polymeric layers that may shield solutes from silanol interactions. Some attractive ionic interactions of acidic solutes may also take place with the positively charged quaternary ammonium group in the column’s bonded ligand. This is in the distal position in ZIC-cHILIC (phosphorylcholine) possibly explaining greater retention of the acid than occurs with the ZIC-HILIC (sulfobetaine) phase which has the quaternary group in the proximal position. The polymeric layers also encourage substantial occupancy of the pore volume with water (20-25% in 80% ACN-buffer [19]) . The thick water layer and resulting hydrophilic selectivity of these phases [17] also result in high retention of the neutral solute (peak 1). The amide columns appear to show moderate silanol effects, with greatest retention of the neutral solute. Pore occupancy data is not available for these particular amide column brands.

Throughout this study, the final equilibration mobile phase used for analysis was 5 mM aqueous AF in 95% ACN, chosen to give appropriate solute retention. In

our experience, smaller concentrations of the aqueous phase can give rise to baseline and retention instability, whereas higher concentrations lead to low retention of the solutes used in this study. Flow rates were typically 0.5 mls/min., which was somewhat above the optimum flow rate for columns of the physical dimensions and particle size used (see experimental). However, we presumed that higher flows would encourage equilibration, and could be used prior to a subsequent analytical run at lower flow if desired. (This presumption was indeed confirmed by the results shown below- see 3.4). Pre-equilibration solvents included 5 mM aqueous AF in 60 % ACN, which is likely to be the strongest solvent used at the end of a gradient. 90 % ACN was also chosen, as this is a typical column storage solvent, allowing estimation of the time required to equilibrate a column "out of the box". Columns were washed with these pre-equilibration solvents for at least 2 hours before each equilibration experiment was carried out.

3.2 Changes in column selectivity with equilibration time.

Column equilibration might be expected to affect solutes of different structure in different ways [18]. Fig. 2a shows (top) the analysis of the solute mix with a ZIC-cHILIC column fully pre- equilibrated (2 hours) and analysed with 5 mM AF in 60 % ACN. As expected, the retention of all solutes was poor using this strong solvent. As equilibration time with buffer in 95 % ACN was increased, the selectivity of the separation clearly changed with peaks eluting in the order 3,2,1; 2,3,1;3,2,1 and finally 3,1,2. It is interesting that peaks 2 and 1 inevitably increase in retention with equilibration time, as might be expected as the mobile phase becomes increasingly hydrophobic, whereas peak 3 (nortriptyline) shows an initial increase in retention followed by a decrease in retention. It is difficult to explain this observation, but we speculate that change in the mobile phase may cause disruption in the water layer exposing the basic solute to ionic interactions with exposed ionised silanols. It could be that buffer components partition initially into the bulk mobile as the concentration of water in the mobile phase is increased, leaving the silanols more exposed to interact with basic solutes. Fig. 2b shows a somewhat similar change in selectivity for the BEH amide column under the same conditions. However, the movement of the nortriptyline peak is more complex, with decreases followed by small increases in retention.

3.3 Equilibration as measured by baseline disturbance, column efficiency or retention time stability?

Deciding equilibration time by visual inspection of a baseline disturbance as recommended by Bell and co-workers [18] seems initially a simple, intuitive and logical procedure and is used by many workers in all branches of HPLC analysis. A column must be fully equilibrated when the emerging effluent has a constant composition, which no longer changes with time. However, this method is not without potential difficulty. An immediate question that comes to mind is whether the sensitivity of the detection procedure which determines the consistency of the effluent is sufficient-will increased equilibration time be indicated at high detection sensitivity where changes in composition will be more apparent?

Fig. 3 (top) shows a pronounced upward baseline shift at about 0.5 min. into a chromatographic run started 11.9 min. after the eluent was changed from 5 mM AF pH 4.4 in 60 % ACN to the analysis solvent buffer in 95% ACN for the Cortecs silica column. The bottom chromatogram shows the same experiment using the BEH amide column where the shift occurred 12.5 min. after the eluent was changed. Both chromatograms were run at the same detection sensitivity (550 mAUFS). Whereas the top chromatogram appears to indicate a sudden change in the baseline, after which the detector signal appears to stabilise immediately, the bottom one indicates a much more gradual rise in the baseline after the initial increase at about 0.5 min into the run, and the baseline appears stable only after about 4 min. into the run. There is clearly some uncertainty as to the equilibration time using this method even though it may provide an approximate indicator of equilibration. Instead we calculated the time taken for the retention time of each peak to fall in the range of 99-101% of its retention time at full equilibration. These values are shown in Table 1 alongside the time period taken for the baseline shift to occur. Clearly, actual measure of the retention time stability in this way is a much stricter measure of full equilibration than the baseline shift procedure. For example, for the experiments depicted in Fig. 3 the baseline shift method gave values of 12.4 and 12.5 min. while the retention time procedure gave average values for the 3 solutes of 49 and 36 min. for the Cortecs silica and BEH amide columns respectively. In every case (Table 1) the baseline disturbance method gave an optimistic estimation of full retention time equilibration.

Column efficiency might be another method of measuring column equilibration with the mobile phase. For example, Fig. 2b clearly shows an improvement in the efficiency of the nortriptyline peak as a function of equilibration time on the BEH amide column. Fig. 4 shows a plot of column efficiency (measured by the half height procedure) against equilibration time for the BEH amide column pre-equilibrated with 5 mM AF pH 4.4 in 60 % ACN. The baseline shift time is indicated by a vertical red line. Clearly, there is an initial rapid improvement in efficiency, which gradually settles as equilibration time increases. The improvement may be due to stabilisation of the column water layer after the initial disrupting effect of the new mobile phase. The times of the runs enabling 99-100 % of N to be obtained were 79.0, 28.8 and 28.8 min. for uridine, 4-OH benzoic acid and nortriptyline respectively, whereas in terms of retention time they were 39.9, 39.9 and 28.8 min. respectively. Clearly, the stabilisation values from column efficiency are closer to those from retention time than the baseline shift measurement. However, as column efficiency can be affected also by the changing retention times (due in part to extra-column effects) and can be affected by solute mass injected, there seems no compelling reason to use it in preference to retention time measurements.

3.4 Effect of stationary phase, pre-equilibration conditions, flow rate and solute on equilibration time.

i) Effect of the pre-equilibration solvent and flow rate.

Fig. 5 shows plots of retention as a % of retention at full equilibrium against equilibration time for BEH silica and BEH amide columns with pre-equilibration in either 60 % ACN-buffer or 90% ACN-water. In all cases, 99-101% equilibration clearly takes longer than the baseline disturbance, indicated by a vertical red line. This is particularly the case for pre-equilibration in the buffered mobile phase. Fig. 6 summarises the data for all six columns that indicate equilibration is faster when the column has been pre-equilibrated with 90 % ACN-water than 60 % ACN-buffer.

Fig. 7 shows similar equilibration plots for the BEH amide column with different pre-equilibration mobile phases and different flow rates. Clearly, equilibration takes longer when the equilibration mobile phase (95 % ACN- buffer) flow is at 0.25 mL/min. compared with 0.5 mL/min., but the relationship with flow is evidently not simply a proportional one. For instance, when the pre-equilibration

solvent is 60 % ACN buffer, full equilibration takes more than twice as long at the lower flow rate. However, with 90 % ACN-water as the pre-equilibration solvent, full equilibration takes longer, but certainly not twice as long at the lower flow. It is possible that several factors may influence the effect of flow. For instance, static equilibration might occur even in the absence of flow due to diffusion of the mobile phase into the pores of the column, causing faster equilibration than expected at low flow. Conversely, the higher pressures that result from the use of higher flow may encourage penetration of the mobile phase into the column pores, resulting in faster equilibration at high flow. Nevertheless, equilibration of the column at higher flow prior to analysis can be recommended. Fig. 7 also indicates that pre-equilibration in 60 % ACN-water results in less favourable equilibration times than when carried out in 90 % ACN-water. This result (which is also observed for the Cortecs silica column, Table 1) suggests that stabilisation is more rapid when the water content of the pre-equilibration solvent closely matches that of the equilibration solvent, as less disruption of the water layer occurs. The presence or absence of buffer salt in the pre-equilibration solvent appears to be a less important factor, although somewhat faster equilibration was achieved for the BEH column in buffered 60 % ACN than in 60 % ACN-water (Fig. 7).

ii) Nature of the solute

The nature of the solute (neutral, basic or acidic) does not seem to have a major effect on equilibration time (see Fig. 6). However, equilibration of the basic solute nortriptyline does appear to be somewhat more rapid than the neutral or acidic solutes in many cases. Surprisingly, 4-OH benzoic acid, whose retention is very susceptible to changes in pH under the conditions of analysis [21], does not appear on average to take significantly longer to reach equilibrium than the neutral solute uridine. The initial elevation in the retention of nortriptyline, followed by a slow decrease is indicated for the amide column under both pre-equilbration conditions in Fig. 5a (see also discussion in Section 3.2). However, this is hardly shown for the BEH silica phase, which demonstrates a slow increase in retention of all solutes on equilibration (Fig. 5b).

iii) Effect of the stationary phase.

Fig. 8 shows equilibration times of the 6 stationary phases deduced from the baseline shift, with pre-equilibration in either 90 % ACN-water or 60 % ACN-buffer. As noted above, these times are more optimistic than those shown in Fig. 6, based on 99-101 % of retention at full equilibrium. It is notable that the disturbance method indicates relatively little difference in equilibration time for the two pre-equilibration solvents (90 % ACN-water or 60 % ACN-buffer), unlike the results in Fig. 6 and Table 1, which indicate considerably faster equilibration in the former solvent. Fig. 8 suggests the most rapid equilibration for the bare silica columns, and the least rapid for the polymeric bonded zwitterionic columns having thicker water layers. The amide columns gave intermediate results, although no data on the water layer thickness has been published for these particular column brands. These results broadly agree with those reported by Bell [18]. The full equilibration times as indicated instead by retention time measurements (Fig. 6 and Table 1) give a less clear picture of the relationship between stationary phase and equilibration time. The polymeric zwitterionic columns are again indicated as the slowest to equilibrate, but the differences between these and the other columns are not so pronounced. There is no indication from Fig. 6 that the shell columns (Agilent glycan and Cortecs silica) equilibrate faster than their totally porous equivalents (BEH amide and BEH silica) although no firm conclusions can be drawn as these stationary phases differ in so many other ways (e.g. particle size, nature of the base silica). Indeed it is difficult to find equivalent commercially available totally porous and shell HILIC columns even from the same manufacturer to carry out such a study.

3.5 Equilibration in gradient elution.

While somewhat longer equilibration times in isocratic analysis can be relatively easily overcome by use of a single (longer) conditioning period prior to analysis of a batch of samples, the same is potentially not true for gradient analysis. If a full equilibration time of an hour is required on some columns for stable retention to be attained after pre-equilibration in a strong solvent like 60 % ACN-buffer, and this time had to be left between each analysis, then the throughput of the procedure would be severely limited. However, the concept of repeatable partial equilibration as proposed by Carr and co-workers [15, 16] may also be applicable in HILIC [14]. For

this study we chose the ZIC-cHILIC column, as it appeared to take the longest time to equilibrate in isocratic analysis (Table 1) and thus presented a possible worst case scenario.

Table 2 shows the relative standard deviation (rsd) of the retention times of 6 consecutive gradient analyses for the three test solutes using a fast gradient of 95-60% ACN (both containing 5 mM AF pH 4.4) in 7 min. (i.e. an increase in water concentration of 5 % / min.). The 6 runs were performed after 2 conditioning runs of the same gradient from which no data were utilised in the repeatability calculations. Runs were performed after equilibration times of 5.6, 10.6, 15.6 and 30.6 min. The equilibration time included the cycle time of the autosampler (0.60 min.) during which time the initial buffered solvent (95 % ACN) continued to pass through the column. In all cases the % rsd of the runs was < 0.2 %, and the results were comparable or better than those shown for the isocratic runs on the same column (after equilibration for 61.5 min.). No dependence of repeatability on the equilibration time for the gradient runs was apparent, and the runs after 5.6 min equilibration were as repeatable as those after 30.6 min. equilibration (see Table 2) . Fig. 9a however shows that the selectivity of the separation varies according to the equilibration time, as expected from the results in the previous isocratic experiments. Thus the retention of nortriptyline (peak 3) decreases, whereas the retention of uridine (peak 1) increases slightly, and that of 4-OH benzoic acid (peak 2) more substantially, with equilibration time. Clearly, it is necessary to fix the equilibration time at the same value to obtain consistent results. Fig. 9b and Table 2 show results of a similar experiment, using a much slower gradient (5-12 % water in 7 min., i.e. 1% /min.). Repeatability was very similar, although up to 6 conditioning runs were necessary in some cases to stabilise retention values before highly repeatable data could be collected under some conditions.

The interpretation of results under gradient elution conditions is complex, in that the exact composition of the starting (weak) mobile phase in the gradient differs according to the time of equilibration, as higher concentrations of the strong (aqueous) solvent remain unpurged from the column at short equilibration times. Although the absolute concentrations of residual aqueous solvent are likely to be very low, their relative amounts may still be influential, possibly leading in part to the different retention and selectivities shown. Nevertheless, the results of the present

and previous studies [14] indicate the practicality of repeatable gradient elution in HILC, despite longer full equilibration times applicable for isocratic work.

4. Conclusions

Column equilibration in HILIC was studied under both isocratic and gradient elution conditions using acidic, basic and neutral test solutes. 6 different stationary phases were chosen and shown to have markedly different selectivity for these solutes in the same mobile phase (5 mM AF pH 4.4 in 95 % ACN). This mobile phase was used for as the final equilibration and analysis solvent in all experiments. In isocratic analysis, full equilibration took up to 1 hour dependent on conditions. The selectivity of the separation changed with equilibration time, suggesting that full equilibration is always necessary. It has been suggested that full equilibration can be monitored through the baseline disturbance produced. While this can give a rough estimate of equilibration, it underestimates the time necessary to produce retention times to within 1% of those at full equilibration. The pre-equilibration state of the column (e.g. the storage solvent) has a profound effect on time taken for subsequent equilibration. Close matching of the aqueous concentration in pre-equilibration and equilibration solvents seems to be more effective in reducing the equilibration time than the presence or absence of buffer. This effect may be due to more rapid stabilisation of the water layer. The nature of the solute (acid, base or neutral) did not seem to have a major effect on equilibration time. In broad agreement with previously published work, polymeric zwitterionic columns with thick water layers took longer to equilibrate (up to an hour with some conditions) than silica columns, which have thinner water layers. No indication was found that superficially porous stationary phases equilibrated more rapidly than their totally porous counterparts.

Although the time taken for full equilibration might be seen as a serious drawback in gradient elution, repeatable partial equilibrium could be achieved after as little as ~ 5 min. even using the column requiring the longest time for full equilibration. Results were broadly in agreement with those from a previous study [14]. Retention time reproducibility was equivalent to that obtained in isocratic analysis, and data collection could be commenced in many cases after as little as 2 conditioning runs when using either steep of shallow gradient programs. These results may also have some significance for the potential use of HILIC in the second dimension of 2-dimensional LC, where rapid equilibration of the column is required.

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6. Legend to Figures

Fig. 1 Selectivity of different columns at full equilibrium. Mobile phase 5 mM AF pH 4.4 in 95 % ACN, 0.5 mL/min. Injection volume 1 μL. Column temperature 30 °C. UV detection at 215 nm. Peak identities 1 = uridine; 2 = 4-OH benzoic acid; 3 = nortriptyline. Flow rate 0.5 mL/min.

Fig.2 Selectivity as a function of equilibration time. Times in italics represent the period elapsed since change from the pre-equilibration solvent (60 % ACN 5mM AF pH4.4) to the analysis eluent (95% ACN 5mM AF pH 4.4) at the beginning of each chromatogram for a) ZIC-cHILIC and b) BEH amide columns. Other conditions as Fig. 1.

Fig. 3 Baseline shifts for (top) Cortecs silica column. Start of run 12.4 min. after solvent change from 60 % ACN-buffer to 95 % ACN-buffer; (bottom) BEH amide column, start of run 12.5 min. after same solvent change.

Fig. 4 Column efficiency as a % of fully equilibrated efficiency plotted as a function of equilibration time. BEH amide column pre-equilibrated in 60 % ACN-5mM buffer. Other conditions as Fig. 1.

Fig. 5 a) Equilibration of BEH amide column using (top) pre-equilibration in 90 % ACN-water, (bottom) 60 % ACN containing 5 mM AF pH 4.4. b) Same but with BEH silica column. Analysis (equilibration eluent) as Fig. 1. Other conditions as Fig. 1.

Fig. 6 Time to achieve retention to within +/- 1% of that at full equilibration for different columns. (top) pre-equilibration in 90 % ACN-water (bottom) pre-equilibration in 60 % ACN-buffer.

Fig. 7 Time to achieve retention to within +/- 1% of that at full equilibration. BEH amide column with various pre-equilibration eluents and flow rates.

Fig. 8 Baseline disturbance time (min,) for different columns in 2 different pre-equilibration solvents.

Fig. 9 Retention and selectivity changes with equilibration time in gradient elution. Column: ZIC-cHILIC. a) gradient 5mM AF pH 4.4 in 95% ACN to 5 mM AF pH 4.4 in 60 % ACN in 7 min. (increase in water concentration of 5 %/min. b) gradient 5mM AF

pH 4.4 in 95% ACN to 5 mM AF pH 4.4 in 88 % ACN in 7 min. (increase in water concentration of 1 %/min). Peak 4 = toluene (void volume marker).

7. References

[1] D.V. McCalley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, J. Chromatogr. A, 1523 (2017) 49-71.
[2] H. Vlckova, K. Jezkova, K. Stetkova, H. Tomsikova, P. Solich, L. Novakova, Study of the retention behavior of small polar molecules on different types of stationary phases used in hydrophilic interaction liquid chromatography, J. Sep. Sci., 37 (2014) 1297-1307.
[3] Q. Zhang, F.Q. Yang, L. Ge, Y.J. Hu, Z.N. Xia, Recent applications of hydrophilic interaction liquid chromatography in pharmaceutical analysis, J. Sep. Sci., 40 (2017) 49-80.
[4] A. Periat, I.S. Krull, D. Guillarme, Applications of hydrophilic interaction chromatography to amino acids, peptides, and proteins, J. Sep. Sci., 38 (2015) 357-367.
[5] K. Spagou, I.D. Wilson, P. Masson, G. Theodoridis, N. Raikos, M. Coen, E. Holmes, J.C. Lindon, R.S. Plumb, J.K. Nicholson, E.J. Want, HILIC-UPLC-MS for exploratory urinary metabolic profiling in toxicological studies, Anal. Chem., 83 (2011) 382-390.
[6] K. Spagou, H. Tsoukali, N. Raikos, H. Gika, I.D. Wilson, G. Theodoridis, Hydrophilic interaction chromatography coupled to MS for metabonomic/metabolomic studies, J. Sep. Sci., 33 (2010) 716-727.
[7] Y. Guo, Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC), Analyst, 140 (2015) 6452-6466.
[8] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, J. Chromatogr. A, 1218 (2011) 5920-5938.
[9] P. Jandera, P. Janas, Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography. A review, Anal. Chim. Acta, 967 (2017) 12-32.
[10] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography, J. Chromatogr. A, 1217 (2010) 8230-8240.
[11] D.V. McCalley, Evaluation of the properties of a superficially porous silica stationary phase in hydrophilic interaction chromatography, J. Chromatogr. A, 1193 (2008) 85-91.
[12] A. Periat, J. Boccard, J.L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, J. Chromatogr. A, 1312 (2013) 49-57.
[13] A. Periat, I. Kohler, A. Bugey, S. Bieri, F. Versace, C. Staub, D. Guillarme, Hydrophilic interaction chromatography versus reversed phase liquid chromatography coupled to mass spectrometry: effect of electrospray ionization source geometry on sensitivity, J. Chromatogr. A, 1356 (2014) 211-220.
[14] D.L. Shollenberger, D.S. Bell, Investigation of reequilibration in hydrophilic interaction chromatography, LC-GC (Europe), 29 (2016) 687-692.
[15] A.P. Schellinger, D.R. Stoll, P.W. Carr, High speed gradient elution reversed phase liquid chromatography of bases in buffered eluents. Part II. Full equilibrium, J. Chromatogr. A, 1192 (2008) 54-61.
[16] A.P. Schellinger, D.R. Stoll, P.W. Carr, High-speed gradient elution reversed-phase liquid chromatography of bases in buffered eluents. Part I. Retention repeatability and column re-equilibration, J. Chromatogr. A, 1192 (2008) 41-53.

[17] A. Kumar, J.C. Heaton, D.V. McCalley, Practical investigation of the factors that affect the selectivity in hydrophilic interaction chromatography, J. Chromatogr. A, 1276 (2013) 33-46.
[18] D.S. Bell, Investigations of equilibration dynamics in hydrophilic interaction liquid chromatography (HILIC), Lecture given at HPLC 2017, Prague June 2017.
[19] 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.
[20] C.A. Lucy, C.B. Craven, C. Seidl, D.R. Stoll, Eluent preparation for hydrophilic interaction liquid chromatography, Part 1: Solvent volumes and buffer counterions, LC-GC Europe, 31 (2018) 22-27.
[21] D.V. McCalley, A study of the analysis of acidic solutes by hydrophilic interaction chromatography, J. Chromatogr. A, 1534 (2018) 64-74.
[22] C.D. Iverson, X. Gu, C.A. Lucy, The hydrophilicity vs. ion interaction selectivity plot revisited: The effect of mobile phase pH and buffer concentration on hydrophilic interaction liquid chromatography selectivity behavior, J. Chromatogr. A, 1458 (2016) 82-89.