A Simple, Generally Applicable HILIC Method Development Platform Based Upon Selectivity

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Hydrophilic interaction chromatography (HILIC) separations are gaining popularity across many industry sectors and application areas. Despite this, method development experience for many analysts is still low compared to reversed-phase liquid chromatography (RPLC). In this work, a broadly applicable HILIC method development screening platform is proposed. Depending upon the analytes to be screened, a maximum of three stationary phases and three eluent pH values are selected providing nine chromatograms for evaluation prior to next steps to achieve the method objective. An example HILIC method development activity for caffeine and related substances (as example polar neutral analytes) is shown.

Introduction

Hydrophilic interaction chromatography (HILIC) has been developing and evolving as a separation mode for retaining neutral and polar analytes poorly retained in reversed-phase liquid chromatography (RPLC) since the early 1990s. Described as a technique employing a polar stationary phase (e.g. unbonded silica) with reversed-phase type eluents (e.g. aqueous-organic mixtures) [1], HILIC is different practically and mechanistically from both normal and reversed-phase liquid chromatography. Others have investigated and explored the different stationary phase and eluent selectivity for this separation mode [2,3]. Detailed studies into HILIC separation mechanisms have been described elsewhere and are not the purpose of this discussion [4-10]. However, it is clear that mechanistically HILIC is complex and provides multiple modes of interaction: between the analyte, stationary phase, eluent and water enriched layer at the stationary phase particle-eluent interface [9,11].

HILIC is highly suited to the analysis of polar to very polar analytes; an area that RPLC has typically achieved limited success without the use of ion-pair reagents or additives (both of which bring their own challenges). For this discussion, polar to very polar analytes are defined as those compounds with a log P value (i.e. octanol – water partition coefficient) of approximately zero or less. A log P value is a reasonable rough guide to the lipophilicity of an analyte. A log P value of zero or less indicates the analyte is highly water soluble or has significant hydrophilic properties and so more suited to a HILIC separation mode. The more lipophilic an analyte, the more suited it is for RPLC. Log D data (partitioning of an analyte as a function of ionisation at a specific eluent pH) can also help. The lower or more negative a log D value, the more ionised and polar the analyte indicating its suitability for HILIC separations. Of course, when dealing with unknown analytes, such data is not available and should method development analysts have analyte solubility / sample diluent concerns there are recommendations and guidance available [12].

As a general rule of thumb, if an analyte elutes before caffeine in RPLC (log P ~zero), it may be better suited to a HILIC separation mode. Figure 1 shows a schematic continuum from HILIC to RPLC separation modes with illustrative log P values and analytes. The area of overlap between the separation modes around caffeine typically causes the most discussion. In this area either mode may offer advantages – the choice is usually application driven.

HILIC offers a number of attractive characteristics and complementary benefits to RPLC. Despite this (and whilst the awareness and popularity of HILIC separations from peer reviewed literature and symposia is more widespread and growing [13]), the practical success and method development knowledge of HILIC remains some way behind RPLC. As with RPLC, there are a variety of approaches to HILIC method development that include: applying prior knowledge of separation conditions from related analytes, peer-reviewed literature work, iterative experiments, systematic screening experiments or design of experiments protocols. All approaches have pros and cons. Unless prior knowledge and experience of a compound or class is known, many analysts will apply a broad general scouting approach at one set of conditions (e.g. low pH, one stationary
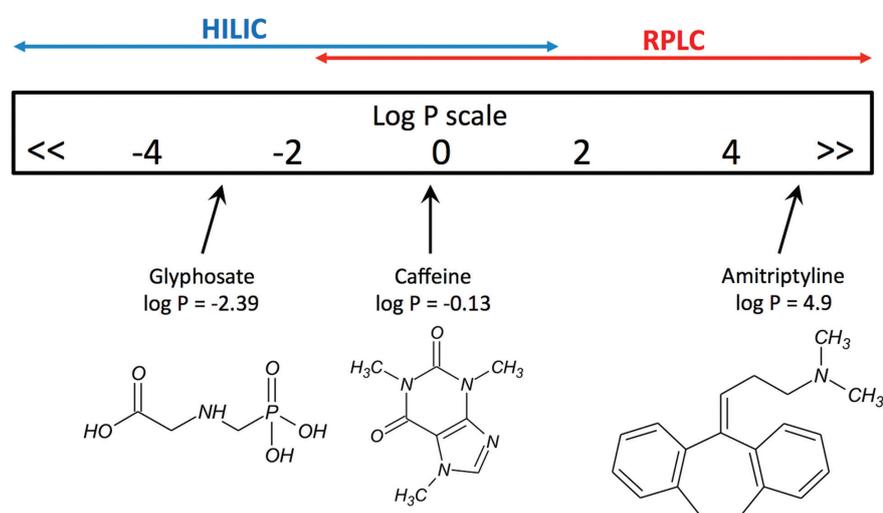

Figure 1: Schematic continuum between HILIC and RPLC separation modes with overlaid log P values and example analytes.
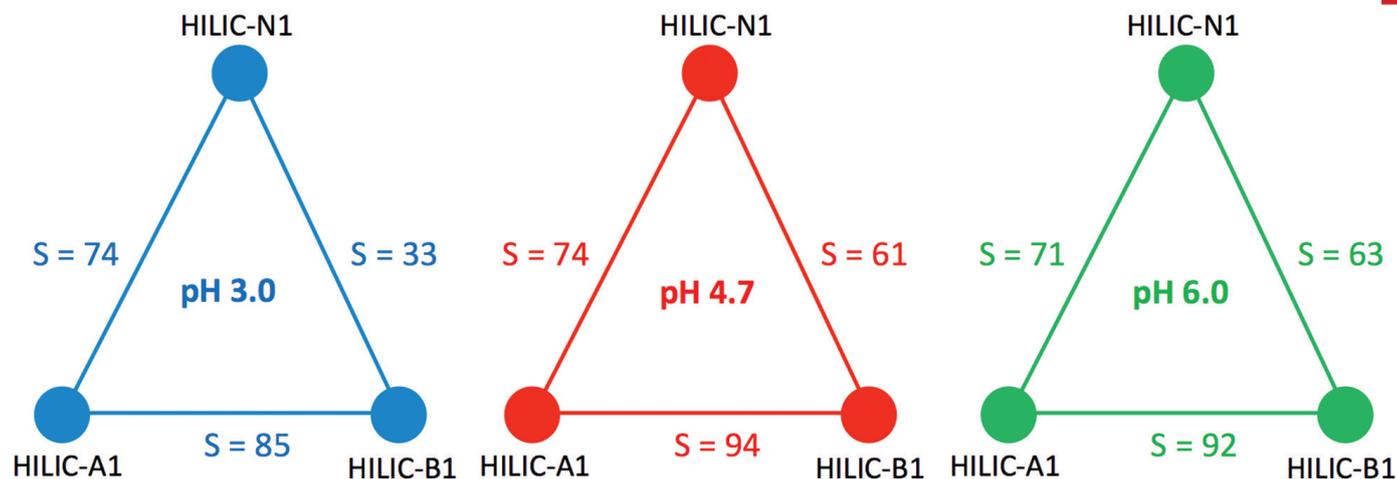
Figure 2: Selectivity diagrams showing data for each ACE HILIC phase at three different eluent pH values.

phase) followed by iterative steps to attempt separation. This path is popular, as it does not rely on access to literature databases or commercial software to define experiments. It can however take time. It is possible to generate improved and more extensive separations knowledge of analytes in a mixture by using a systematic screening method development approach where the defined experiments are based upon known differences in selectivity.

In this brief discussion, a selectivity-based systematic screening platform focussed on stationary phase and eluent pH for HILIC method development is described. This simple platform will be used to develop a HILIC method for caffeine (as model active ingredient) and four related substances present at 0.5 %w/w.

Experimental

Reagents and instrumentation

Chemicals, reagents, analytes, solvents and water were purchased from Sigma-Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK). Analyses used an Agilent 1100 LC quaternary instrument. The three stationary phases ACE HILIC-A1 (acidic character), ACE HILIC-N1 (neutral character) and ACE HILIC-B1 (basic character), 150 x 4.6 mm, 5 µm, are beta test columns from Advanced Chromatography Technologies Ltd (Aberdeen, UK).

Selectivity study

Columns were equilibrated for 100 column volumes using 10 mM ammonium formate at pH 3.0, 4.7 and 6.0, in MeCN/H_2O (90:10 v/v) with a flow rate of 1.5 mL/min. A concentrated stock solution of ammonium formate at each pH was prepared and diluted accordingly to ensure a constant buffer concentration. Exploring broader pH ranges for HILIC is possible with appropriate phases but suitable selectivity

and separation for a broad range of analytes has been found to be possible with eluent pH 3.0, 4.7 and 6.0. Temperature was 25°C with 230 and 254 nm detection. 48 pharmaceutically relevant compounds (0.5 mg/mL MeCN/H_2O 90:10 v/v) representing small molecule analytes with a broad range of physico-chemical properties (acids (pKa range from -0.43 to 4.6 for p-toluene sulphonic acid and 4-hydroxybenzoic acid respectively), bases (pKa range from 5.7 to 10.5 for melamine and tyramine respectively), neutrals and zwitterionics) were injected onto each column. The HILIC-A1 and HILIC-B1 retention data have been used previously to calculate alternative selectivity data on prototype ACE HILIC phases using 54 analytes [14]. In this current work commercially representative phases and a new HILIC-N1 phase are used. New selectivity data are recalculated for all three ACE HILIC phase combinations and presented in a new format as selectivity diagrams. The recalculated selectivity values are used to define a new generally applicable HILIC method development platform not previously described.

Gradient screening for the caffeine method development example

Columns were equilibrated for 50 column volumes at the starting gradient conditions prior to use. Mobile phases were A = 10 mM ammonium formate, pH 3.0 or pH 4.7 in MeCN/H_2O (94:6 v/v) and B = 10 mM ammonium formate, pH 3.0 or pH 4.7 in MeCN/H_2O (50:50 v/v). The gradient was 0-100 %B in 15 minutes at 1.5 mL/min. A constant total 10mM buffer concentration was kept across the gradient ranges to ensure no additional selectivity effects from varying buffer concentrations would be seen. Temperature = 25°C with detection at 275 nm. 2 µL injections of a 25 mg/mL caffeine mixture (with the related substances at 0.5 %w/w) in MeCN/H_2O (90:10 v/v).

Results and Discussion

Comparing the selectivity differences between the three HILIC stationary phases at each pH

The retention times were plotted on each stationary phase against each other for all 48 analytes to examine the degree of scatter i.e. selectivity differences. As an example, the retention time graph for HILIC-A1 versus HILIC-N1 at pH 4.7 gave a correlation coefficient (r^2) of 0.4476 (data not shown) indicating good scatter. It is clear there is a high degree of difference between these two phases under these conditions. This is to be expected due to the acidic and neutral nature of the phases respectively and the mixed properties of the analytes. With charged surfaces, acidic and basic phases will be dominated by electrostatic interactions with cationic and anionic analytes respectively. Neutral phases will have a high degree of hydrogen bonding with analytes as a principle mode of interaction. Nevertheless, a variety of ion-exchange, partitioning into the water enriched layer on the particles and other mechanisms affect the differences in retention and separation. Inserting the correlation coefficient into Equation 1 yields the selectivity descriptor value [15] which is determined as S = 74.

Equation 1
Selectivity Descriptor = $100 \times \sqrt{(1 - r^2)}$

A selectivity descriptor value < ~8 signifies the conditions do not provide significant differences in selectivity, whilst values > ~8 indicate increasingly different selectivity which is helpful for method development. This comparison approach is simple but powerful for comparing selectivity values for a broad range of analytes between phases, conditions or combinations thereof.

It is possible to construct selectivity diagrams to visualise the data using the selectivity descriptor values determined
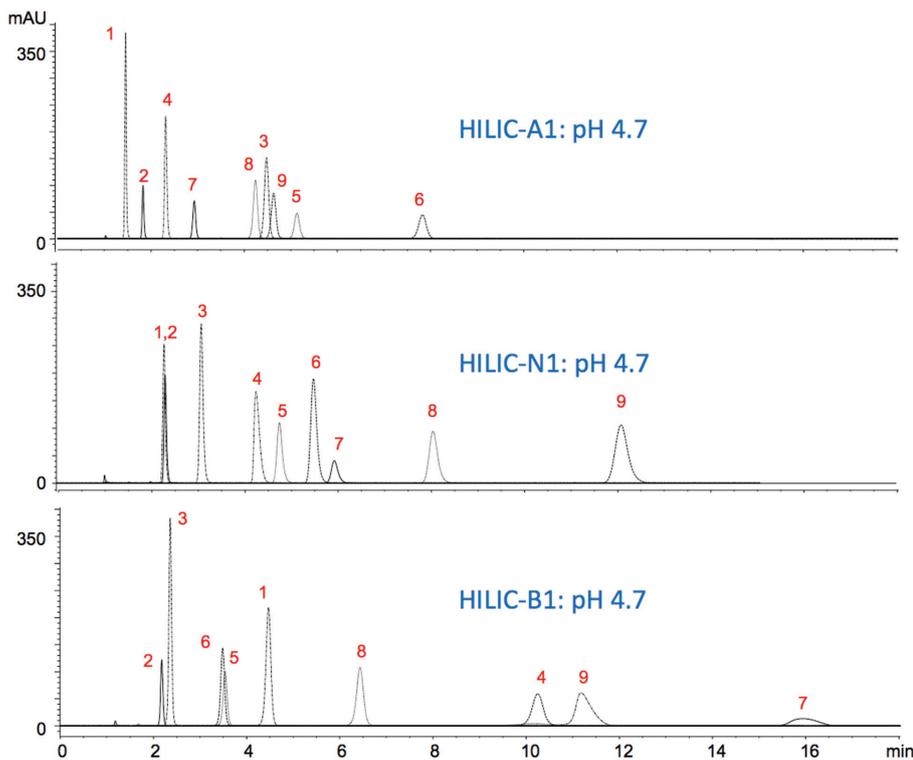
Figure 3: Chromatograms of ACE HILIC-A1, HILIC-N1 and HILIC-B1 phases at pH 4.7 with 3 acidic, 3 basic and 3 neutral analytes overlayed to show differences in retention and elution order. Conditions as in experimental. Peaks: 1= p-Toluene sulphonic acid, 2= Uracil, 3= Sotalol 4= 4-hydroxybenzoic acid, 5= Salbutamol, 6= Atenolol, 7= Mandelic acid, 8= Deoxycytidine 9= Cytidine

between stationary phase / eluent pH combinations. Figure 2 shows the selectivity diagrams for the three HILIC stationary phases at the 3 eluent pH values examined.

It is clear that there is a reasonable degree of difference (i.e. larger selectivity differences) between each of the three HILIC stationary phases at each individual eluent pH for the analytes. This is helpful and indicates that screening each of these three phases at any of the single eluent pH values would give differences in analyte retention and elution order. Figure 3 shows stacked overlays of single injections (taken from the 48 analyte mixture) containing example 3 acidic analytes, 3 basic analytes and 3 neutral analytes on HILIC-A1, HILIC-N1 and HILIC-B1 phases at pH 4.7.

It can be seen that there are significant differences in analyte retention times and elution orders when comparing the three different stationary phases. This supports the application of the selectivity diagrams to explain selectivity differences.

Comparing the selectivity differences between each eluent pH for the three HILIC phases

Using the selectivity descriptor data already acquired, it is possible to examine the impact of eluent pH on analyte retention / selectivity with each individual stationary phase. The selectivity diagrams are shown in Figure 4.

For HILIC-A1, HILIC-N1 and HILIC-B1, there are reasonable selectivity differences when comparing pH 3.0 / pH 4.7 and pH 3.0 /

pH 6.0 combinations on each phase. This indicates that for each individual stationary phase it is helpful to explore these eluent pH value combinations for differences in selectivity when developing methods. The HILIC-A and HILIC-B phases show larger selectivity values (i.e. great changes in analyte retention and elution order) as there is a dual effect of changes to the analyte (potentially ionisation) and stationary phase character (potentially ionisation plus silanol activity) as eluent pH is explored.

The HILIC-N1 phase displays the lowest selectivity values of 18 and 20 when comparing pH 3.0 / pH 4.7 and pH 3.0 / pH 6.0 combinations respectively. The neutral character of the HILIC-N1 phase would indicate no change in bonded phase ionisation. It is likely that changes in the acidic and basic analyte ionisation states and increasing silanol activity across the pH range explored are responsible. These changes are also likely to alter the weighting of the hydrogen bonding (and other) mechanisms with the neutral phase and are the most likely cause of differences in retention / elution observed.

Interestingly when comparing pH 4.7 / pH 6.0 combination for each individual HILIC phase, the selectivity values are quite low (maximum value across all three phases for selectivity = 8). Analyte retention and elution order would not be significantly different between these conditions.

Conclusions of selectivity comparisons with three stationary phases and 3 eluent pH values

The selectivity descriptor values and selectivity diagrams help provide recommended stationary phase / eluent pH combinations for systematic method development. It is clear that the three different stationary phases (i.e. acidic, basic and neutral character) provide suitably different selectivity for a range of acidic, basic and neutral analytes. All three
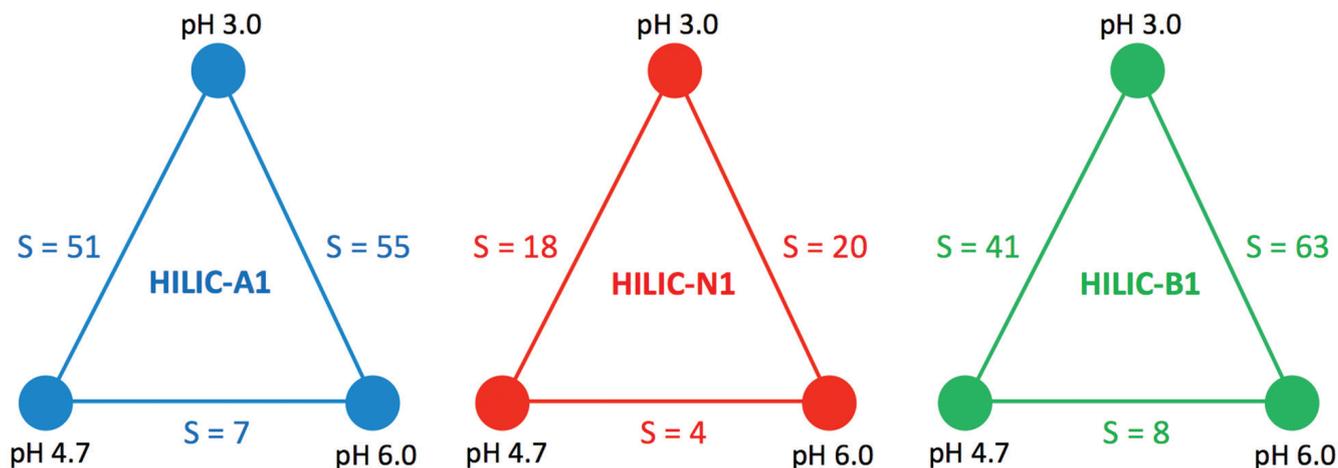

Figure 4: Selectivity diagrams examining the impact of eluent pH on each individual ACE HILIC stationary phase
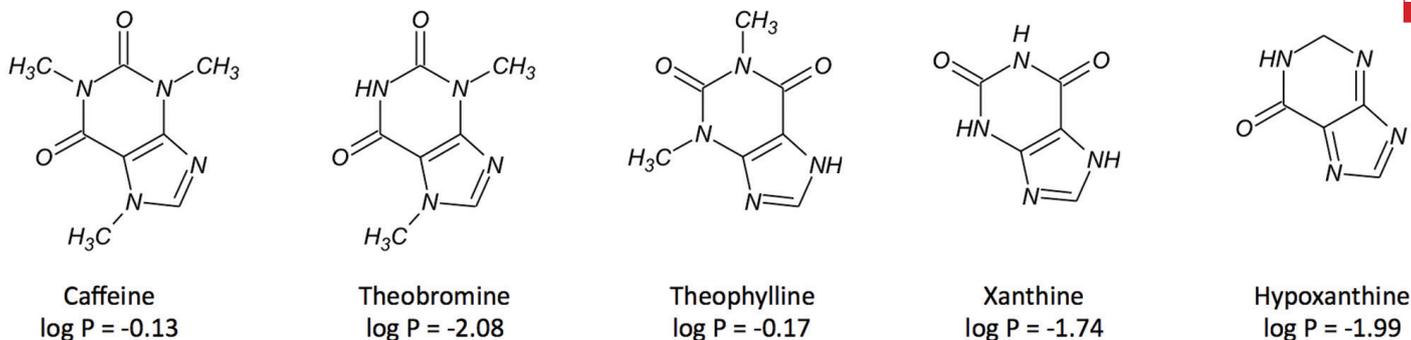
Caffeine
log P = -0.13

Theobromine
log P = -2.08

Theophylline
log P = -0.17

Xanthine
log P = -1.74

Hypoxanthine
log P = -1.99

Figure 5: Structures for caffeine and some related substances with log P data included

phases should be included in a method development approach. The eluent pH selectivity data indicates that combinations of pH 3.0 / pH 4.7 or pH 3.0 / pH 6.0 would provide the largest selectivity differences. Depending upon the analytes, it may not be necessary to explore all three pH values on all three phases for method development.

Assessment / optimisation of other main chromatography parameters for HILIC methods

If required, once initial analyte screening is complete, it is possible to explore other parameters to improve the separation further or optimise the method. Ionic strength can be influential in HILIC [11]. The effects of ionic strength depend upon the phase character and analyte properties. For chargeable phases (e.g. HILIC-A1 and HILIC-B1), any ion exchange mechanisms may be modified altering retention of ionic analytes. The adsorbed water layer (central to partitioning activities) may also change in character. As an example, HILIC-A1 has a negative charge on the surface, which attracts cationic buffer components. Increasing ionic strength may reduce the surface net negative charge reducing the retention of ionised basic analytes due to decreasing ion exchange. For ionised acidic analytes any reduction in the net negative surface charge may lead to increased analyte retention.

Other organic modifiers (apart from the popular aprotic acetonitrile) have been used in HILIC and have been reported with varying success [16] including benefits for MS detection [17]. For the eluent, hydrogen-bonding protic solvents such as the alcohols are typically avoided due to the potential to disrupt the aqueous layer (and therefore partitioning mechanism) surrounding the stationary phase. However, a wider range of solvents may be used for the sample diluent to overcome solubility concerns [12].

HILIC method development for caffeine plus related substances

Figure 5 shows the structure and log P

data [18] for caffeine and some related substances used for this HILIC method development exercise.

All compounds are polar neutral species. The negative log P data indicate a reasonable degree of hydrophilicity (particulary for theobromine, xanthine and hypoxanthine) making this mixture suitable for HILIC mode. As noted earlier, if the analyst did not know the structures of the analytes a good rule of thumb is that if an analyte elutes before caffeine in RPLC (log P ~zero), it may be better suited to a HILIC separation mode. As all analytes in this mixture are polar neutral, a rational choice of

method development screening conditions may be made. All three stationary phases will be screened but only pH 3.0 and pH 4.7 will be used. There seems little advantage to using pH 6.0 (which is helpful for acidic analytes to ensure full ionisation to improve electrostatic and other interactions). The pH 6.0 eluent would also increase the acidic character of the HILIC-A1 phase which may be useful for basic analytes, but is again, not necessary here.

Initial HILIC gradient scouting runs (at each phase / eluent pH combination generating six chromatograms) were performed as described in the experimental section. The related
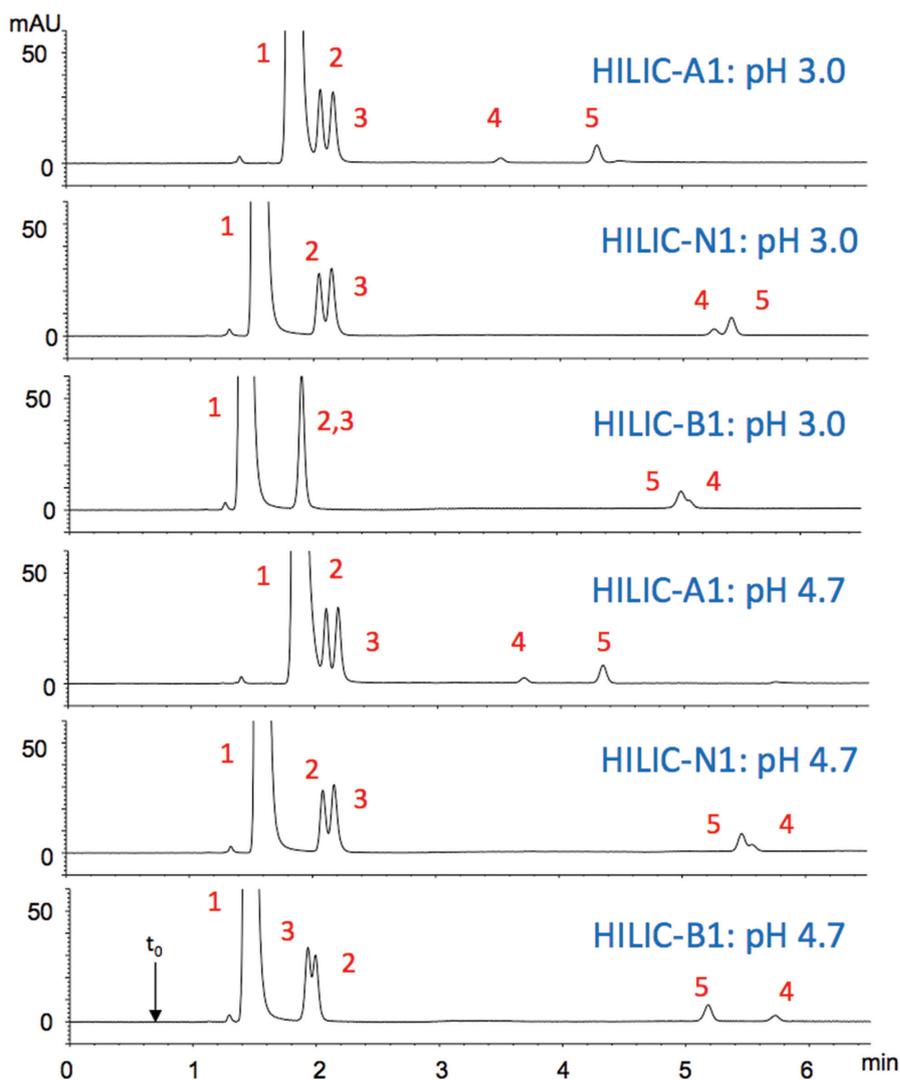

Figure 6: Initial stationary phase / eluent pH screening chromatograms for caffeine plus related substances (at 0.5% w/w). Conditions as in experimental. Peaks: 1 = Caffeine, 2 = Theophylline, 3 = Theobromine, 4 = Xanthine, 5 = Hypoxanthine.
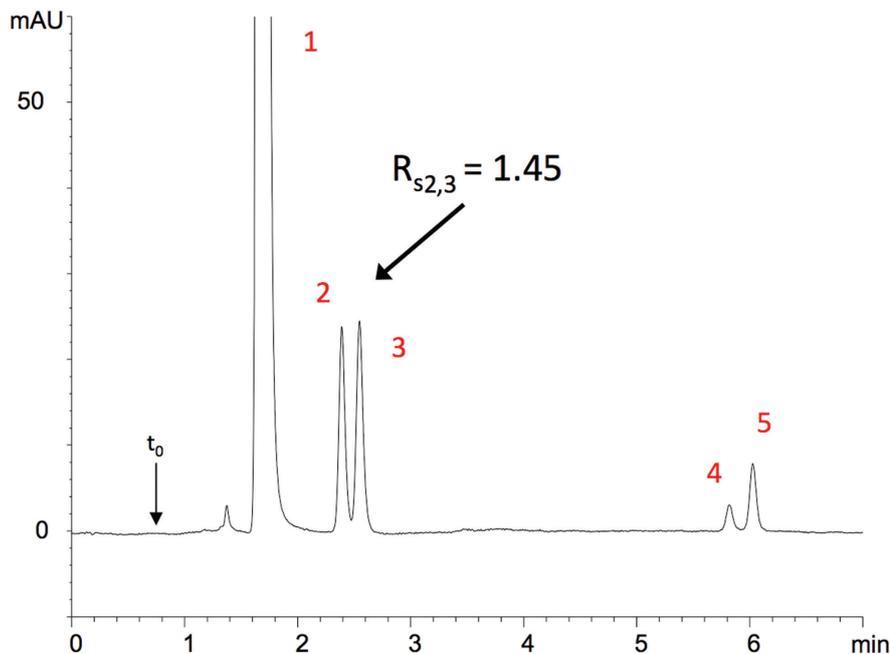
Figure 7: Final HILIC separation of caffeine plus related substances (at 0.5% w/w) on the ACE HILIC-N1 phase. Conditions: A= 10 mM ammonium formate, pH 3.0 in MeCN:water (96:4 v/v). B= 10 mM ammonium formate pH 3.0 in MeCN:water (50:50 v/v). 0-100% B gradient in 15 minutes, 1.5 mL/min, 2 µL, 15°C, 275 nm. Peak identities: 1 = Caffeine, 2 = Theophylline, 3 = Theobromine, 4 = Xanthine, 5 = Hypoxanthine.

Conclusions

HILIC method development can appear complex to many analysts. In this discussion, a generally applicable, simple, HILIC method development screening platform based upon the power of selectivity using stationary phase and eluent pH has been described. Depending upon the properties of the analytes, it is possible to select a maximum of three stationary phases and three eluent pH values for the method development platform. This would provide nine chromatograms for the analyst to evaluate before deciding upon next steps to achieve the method objective. Using the HILIC method development workflow with subsequent method optimisation, a suitable HILIC separation of caffeine and related substances was achieved as an example polar analyte mixture.

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substances were spiked at 0.5% w/w to caffeine as the principle component in the sample. Figure 6 shows the zoomed in chromatogram stack plot for the six initial runs.

The elution order for xanthine and hypoxanthine (peaks 4 and 5 respectively) are seen to reverse on HILIC-B1 at pH 3.0, HILIC-N1 at pH 4.7 and HILIC B1 at pH 4.7. Caffeine is more retained on the HILIC A1 phase at pH 4.7. There is little change in analyte retention and no change in elution order when comparing the HILIC-A1 phase at pH 3.0 and pH 4.7. The acidic character of the phase and increased silanol activity at pH 4.7 over pH 3.0 do not appear to provide any advantage for this polar neutral mixture. There is a reversal in the elution order of xanthine and hypoxanthine on the HILIC-N1 phase between pH 3.0 and pH 4.7. The HILIC-N1 phase will undergo an increase in silanol activity between pH 3.0 and pH 4.7, but as the phase is neutral there will be no phase ionisation effects. Both analytes are polar neutrals so no ionisation state changes are likely either. It is possible that the partitioning into the adsorbed water layer mechanism has been affected by the pH changes leading to the elution order changes observed. Additionally, the degree of hydrogen bonding may be affected which could affect neutral or charged analyte interactions. The HILIC-B1 phase shows some selectivity changes between pH 3.0 and pH 4.7 with an increase in resolution between peak 4 and peak 5 and a reversal in elution order (the only phase and eluent pH combination this is observed) for peak 2 and peak 3 (although still coeluting). So despite these analytes being polar neutral there are significant selectivity effects observed across the phases and conditions explored.

Optimisation of other chromatography parameters to give the final HILIC method

Based upon these initial screening data (Figure 6), the HILIC-N1 conditions at pH 3.0 were selected for method optimisation with a goal to achieve separation of all components. The gradient starting conditions were modified to 10 mM ammonium formate, pH 3.0, in MeCN:water (96:4 v/v). The small increase in acetonitrile starting conditions should provide additional retention of the early eluting caffeine and coeluting theophylline and theobromine analytes. Subsequent injections gave a moderately improved separation between theophylline and theobromine. Further increases in the starting volume fraction of acetonitrile are not possible due to disruption of the adsorbed water layer around the stationary phase particles. Thus, the temperature was reduced to 15°C to achieve the final separation in Figure 7. The final chromatographic resolution value between the critical pair (i.e. closest eluting analytes in the chromatogram, which is peak 2 and peak 3) is 1.45 compared to a resolution value of 1.09 for this critical pair achieved from the initial screening work.

The separation of caffeine (as the principle component) and related substances (at 0.5%w/w) is considered acceptable and fit for its intended purpose after following the defined workflow with subsequent method optimisation.