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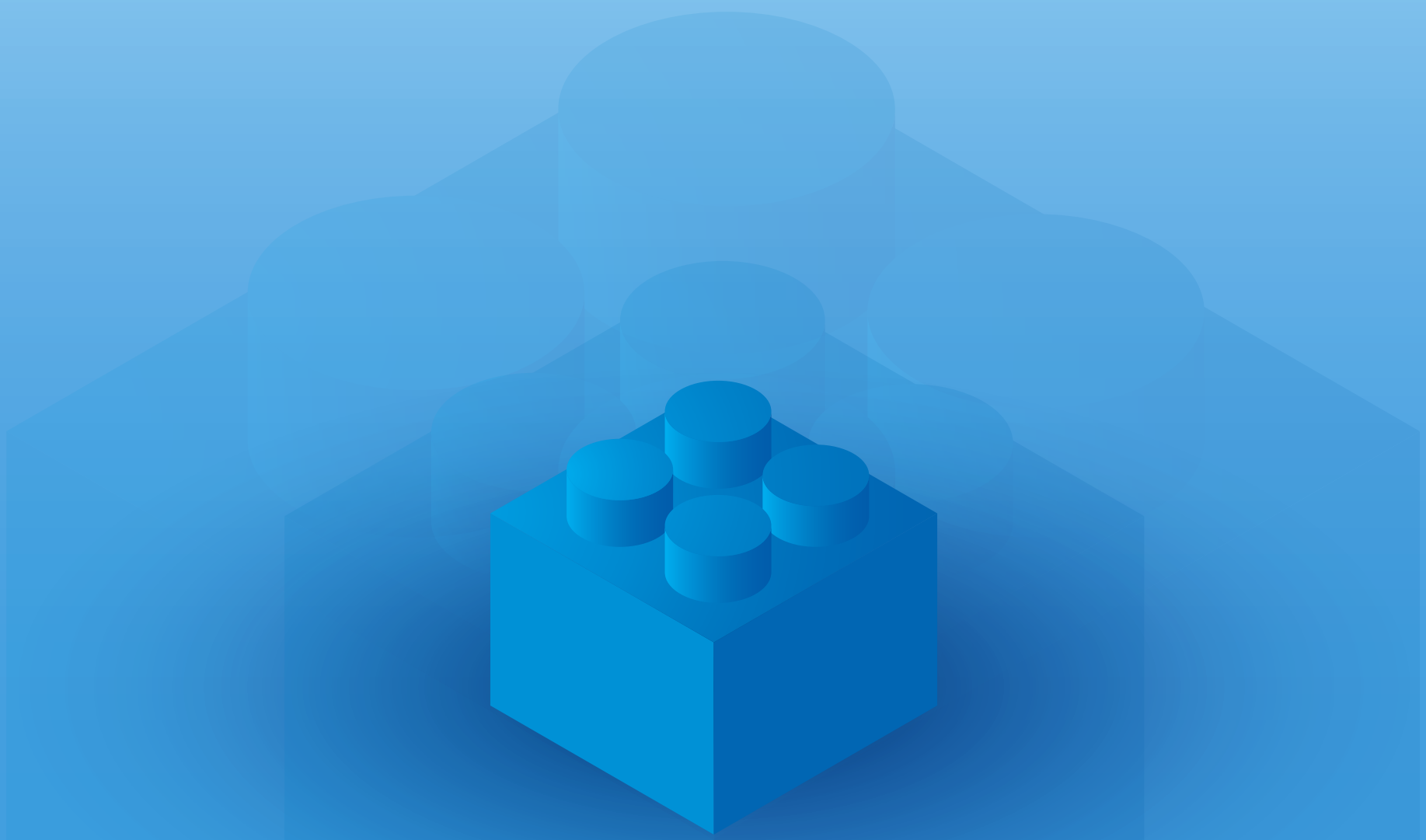

HPLC Method Development

A collection of articles designed to help improve your HPLC Method Development knowledge and skills.

VOLUME III

BUILD BETTER HPLC METHODS WITH CRAWFORD SCIENTIFIC

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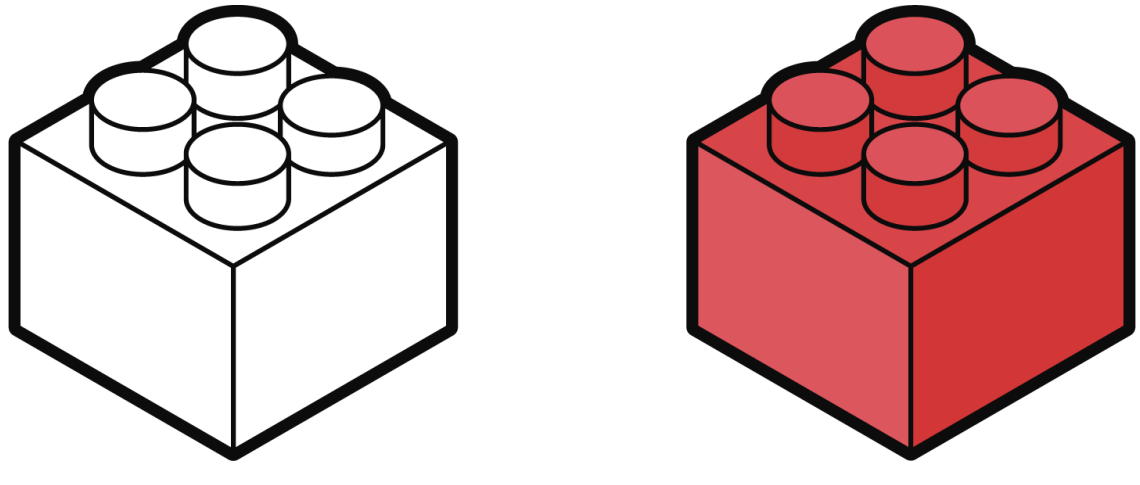

Mobile phase selection is key for gaining insight into the selectivity that each stationary phase has to offer. Remember that this is a combination of the bonded phase ligand, the silica substrate, and any post-bonding treatments (such as end-capping). Ideally, we want to screen the sample with the widest of mobile phase chemistries as possible in order to fully map the selectivity possibilities. This involves changing modifier, pH, as well as the strength and type of buffer.

The selectivity obtained using methanol and acetonitrile are often very different. Especially, when acidic, basic and/or aromatic analytes are involved. This is due to the different physicochemical properties of these common HPLC solvents.

Figure 1 highlights the major differences between the physicochemical properties of common solvents, showing how this might affect the various types of analyte that we deal with.

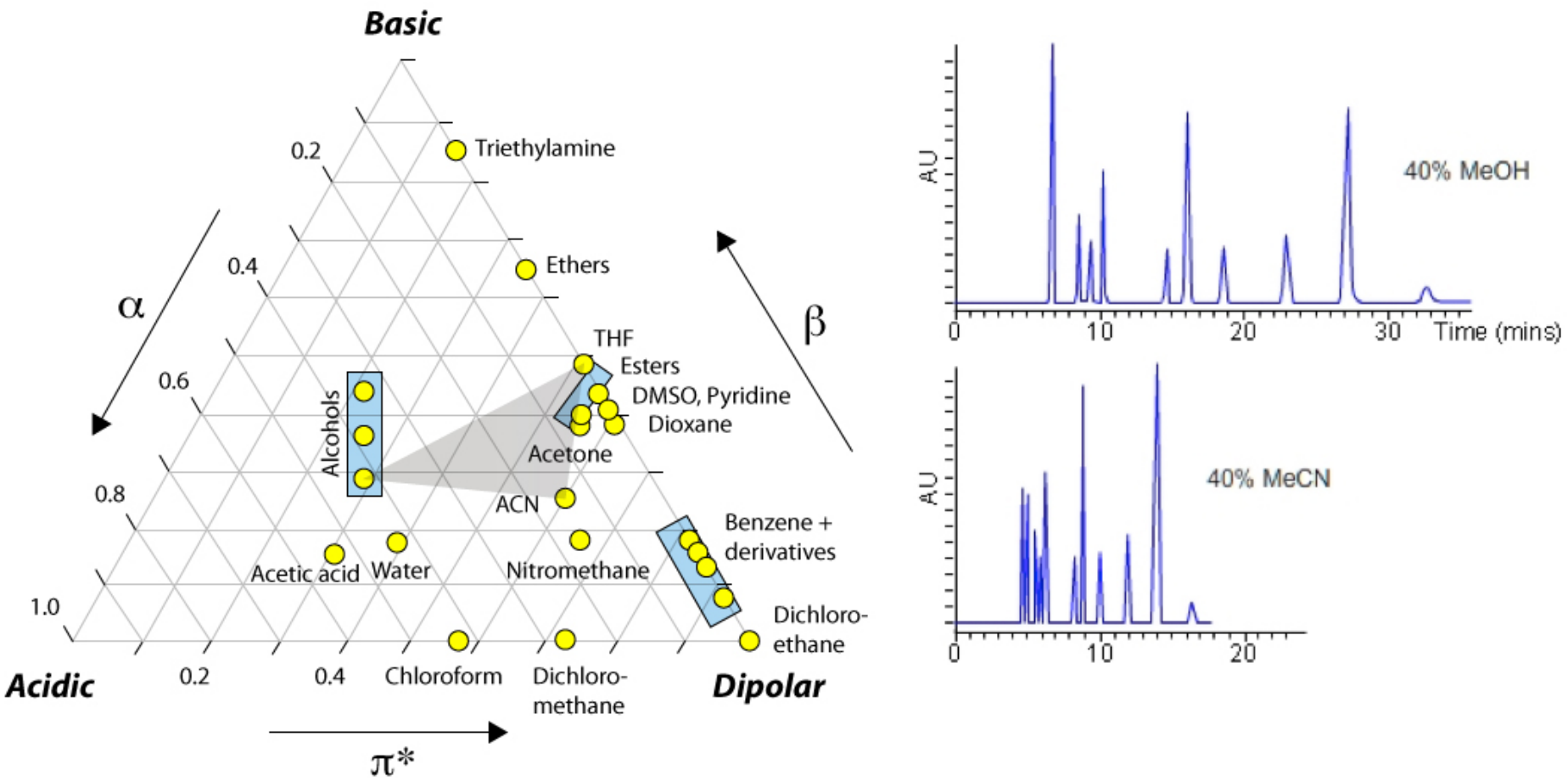

Figure 1: Selectivity (and retention) changes due to the differences in physicochemical properties of the organic modifier.

Solvent	Dipole π*	Acidity α	Basicity β
MeOH	0.28	0.43	0.29
MeCN	0.60	0.15	0.25

Table 1: Relative properties of Methanol and Acetonitrile which influence retention and selectivity in reversed phase HPLC.

pH & Buffer Choice

The relative acidity and basicity of each modifier have different effects on the solubility of the analyte in the mobile phase in relation to the stationary phase. As a result, this effects selectivity between analytes. For this reason, a screen to investigate the selectivity 'map' for any separation should include chromatograms generated using both acetonitrile and methanol.

For ionisable molecules, the pH of the eluent will affect the degree of analyte dissociation. Hence, retention is impacted, due to the relative decrease or increase in polarity as the molecule becomes more or less ionised. As all analytes have a different extent of dissociation at various pH values, altering the mobile phase pH will change the relative retention of each ionisable analyte. This impacts selectivity.

Depending upon the nature of the analyte, one might want to choose acidic, neutral, or basic pH to investigate a wide-range of pH values. However, if using modelling or optimisation software to assist method development, the range of pH values used may need to be reduced in order to create a valid model.

Poor selection of the buffer type and concentration is where many come unstuck. Find the pKa of some common buffers in Table 2. For maximum buffering capacity, get eluent pH as close to the buffer pKa value as possible. Ideally, within +/- 1 pH of the buffer pKa. By following this rule, a buffer strength of 10mM may be enough to effectively control the eluent pH.

Buffer (10mM)	pKa	Buffer Range (pH adjusting species)
Ammonium Acetate	4.8 9.2	3.8 – 5.8 (acetic acid) 8.2 – 10.2 (ammonia)
Ammonium Formate	3.8 9.2	2.8 – 4.8 (formic acid) 8.2 – 10.2 (ammonia)
Ammonium Hydroxide	9.2	8.2 – 10.2 (ammonia)
Trifluoroacetic acid (0.1%)	<2	2.06 (Not a buffer)
Di Sodium Monohydrogen Orthophosphate	2.1	1.1 – 3.1 (phosphoric acid)
Mono Sodium Dihydrogen Orthophosphate	7.2	6.2 – 8.2 (phosphoric acid)

Table 2: Useful buffers and their buffering ranges for HPLC method development.

There are some 'tricky' pH ranges in MS detection while avoiding solid (non-volatile) buffers (phosphates). The range between pH 6 and pH 8 is foremost. However, a 2-4-6 approach is popular, using 0.1% TFA (pH~2), ammonium formate or formic acid adjusted to pH 4, and ammonium acetate or acetic acid adjusted between pH 5.8 – 6.0. Otherwise, determine if an alkaline pH is more suitable to your sample types and adjust the pH values used accordingly.

Additionally, the use of TFA will change the nature of the stationary phase surface as it pairs with any basic species on the silica surface. It is also vital that the column is re-equilibrated properly between each run, meaning the re-equilibration of around 10 column volumes. This degree of rigour when switching solvent systems is necessary even when TFA is not being used; however, the number of column volumes required may be lower.

Realise The Full Selectivity Range For Each Column

Now consider the optimum flow rate (linear velocity), gradient range, and gradient slope for the screening experiment. This is necessary to realise the full selectivity range for each column.

First the most straightforward — gradient range. Run from 5 to 95% B to explore the range of eluotropic strength necessary for each separation. Hold at the top eluent composition (2 – 3 column volumes) to ensure all sample components have been eluted.

For eluent flow (linear velocity) the highest peak capacity in gradient mode is typically found at the highest flow rates. However, to ensure operation within the pressure limits of the system, and for practical purposes, follow the rule of thumb guidelines shown in Table 3.

Column Internal Diameter (mm)	Flow Rate (mL/min)
4.6	1.0
3.0	0.4
2.1	0.2

Table 3: Typical mobile phase flow rates for HPLC column of various dimension.

The flow rates above are adjusted using the square ratio of the column diameters based on an optimum flow of the 4.6mm column of 1.0 mL/min (i.e. (32 / 4.62) x 1 = 0.425).

The maximum peak capacity in gradient HPLC is reached at the highest flow rates. In practice, the 3.0 and 2.1 columns are often run at 0.5 mL/min. even with sub 2mm packing materials. However, for the remainder of this exercise, assume the flow rates in Table 3.

Finally, consider the gradient slope employed for the screen. Use the re-arranged gradient equation in terms of k* (average gradient retention factor) and aim for an average gradient retention factor between 2 and 5;

$$\mathbf{t_g = 5 \times k^* \times \Delta\Phi \times Vm / F}$$

Where k* is the average gradient retention, ΔΦ is the change in %B (5% - 95% B = 0.9 for this equation), Vm is the column interstitial volume (π x radius2 x length x 0.6) and F is the flow rate (mL/min.).
For a 50 x 2.1mm column, at 0.2 mL/min and a gradient of 5 – 95% B, to obtain a k* value of 3 the gradient time would need to be;

$$\mathbf{t_g = 5 \times 3 \times 0.9 \times 0.104 / 0.2 = 7 \text{ minutes}}$$

Note that a value of 3 has been used for k*; however, the gradient slope (gradient time if we assume a fixed starting and ending composition) can be adjusted for k* values between 2 and 5. Some common column dimension/gradient slope combinations are shown in Table 4;

Column Dimensions (mm)	Flow Rate (mL/min.)	Gradient Time (mins.)	k*2 – k*5 (mins.)
150 x 4.6	1.0	20	15.3 – 38.3
100 x 3.0	0.4	14	10.8 – 27.1
50 x 2.1	0.2	7	4.7 – 11.7

Table 4: Common conditions for HPLC screening with columns of various dimensions.

In practice, a range of stationary phase chemistries can be screened in columns with dimensions 50 x 2.1, 1.9mm (for example), using eluents with pH as close to 2, 4 and 6 as possible. See the results in Figure 2.

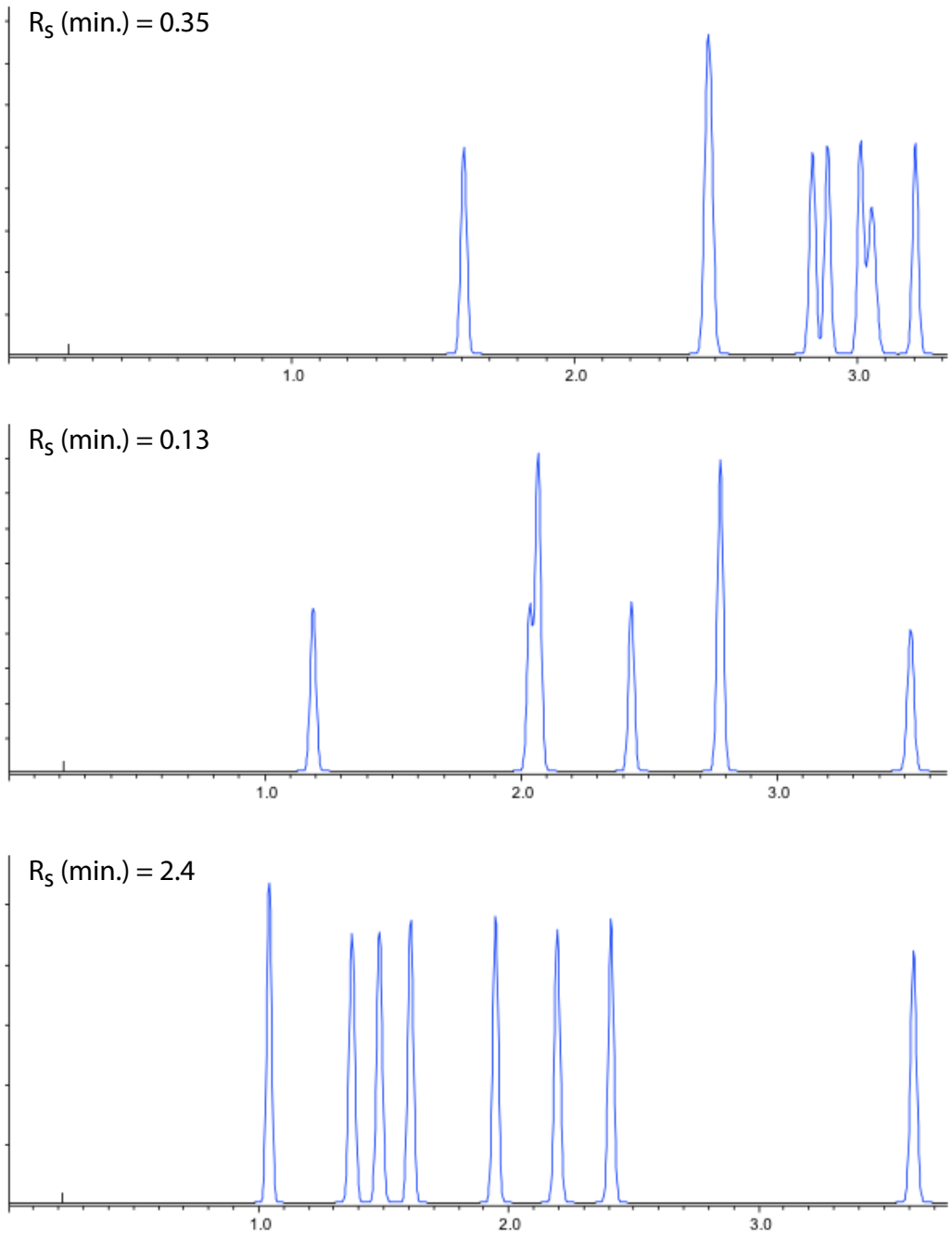

Figure 2: Stationary phase screen (C18 50 x 2.1 mm, 1.9 mm) of a test mix of 8 acidic, basic and neutral compounds using mobile phases of 0.1% v/v TFA (top), 10 mM ammonium formate (pH 4) and 10 mM ammonium acetate (pH 5.8).

Gradient conditions 5 – 95% B Acetonitrile in 5 minutes.

Using figure 2, screen the same three pH values with methanol as the organic modifier, making 6 experiments with each stationary phase. Then repeat all six experiments using the different stationary phases under investigation.

These chromatograms can be assessed by eye — in combination with the efficiency, selectivity, peak shape, and resolution values — to assess which combination will be taken forward for optimisation. This also ascertains if a different set of stationary phases or pH ranges needs to be employed.

If using computer optimisation software, the system will be able to predict (within limits) the optimum combination of variables. Then users can assess if a usable set of conditions is obtainable with this set of stationary phases and pH range.

One final word on platform approaches of this type. It is easy to experience poor efficiency due to poor peak shape of the analytes. This is created by a mismatch between the sample diluent and the mobile phase under investigation. It is good practice to keep the eluotropic strength of the sample diluent as low as possible (low %B). If possible (check the solubility of the analytes carefully), have six samples prepared in each of the 6 starting eluent compositions.

HAVE WE FORGOTTEN THE ADVANTAGES OF CORE-SHELL PARTICLES?

In the late noughties we couldn't avoid webinars, seminars, and online calculators extolling the virtues of the 'new' core-shell particle morphology. Used by HPLC column manufacturers, they promised higher performance at lower backpressures. Many wondered if this was a reaction to the introduction of sub-2µm HPLC particles and UHPLC instrumentation (around 2004). Particularly, the speed and efficiency which this approach brought to our industry.

The hype around core-shell particles has died down. In fact, lately it has proved difficult to find an online method translator — most manufacturers have core-shell versions of their popular phases from their fully porous particle (FPP) ranges. So, are we in danger of forgetting the advantages which core-shell particles can bring to methods which still use FPPs?

Put differently, why do 5µm or 3µm FPPs still exist? And why haven't more migrated their methods to this superior technology? After all, 2.7µm (or similar) and 5µm core-shell particles are widely available. Why not update our legacy methods, or seek to develop new methods using core-shell particles?

Arguably, as there are many legacy methods which work perfectly well on FPPs, there is no real driver to change. That being said, many scientists struggle with methods where an improvement in efficiency (and, resultantly, resolution) would deliver significant benefits — solving issues with troublesome methods or identifying how shortened analytical run times may help with throughput problems. This article is for those who seek to improve or replace existing methods on a limited budget for new instruments.

As well as a refresher on the maths required to undertake method transfer from FPPs to superficially porous particles (SPPs), this methodology imposes a further constraint by working within the new USP General Chapter proposition <621> which, as of July-22-2019, is in draught form [1]. For a more detailed explanation of the proposed changes to Chapter <621> see reference [2].

To translate the various method parameters from the FPP to the SPP column, use the following equations;

Flow Rate (Equation 1)

$$F_2 = F1 \times \left[\left(d_{c2}^2 \times d_{p1} \right) / \left(d_{c1}^2 \times d_{p2} \right) \right]$$

Gradient Segment Times (Equation 2)

$$t_{G2} = t_{G1} \times (F_1 / F_2) [(L_2 \times d_{c2}^2) / (L_1 \times d_{c1}^2)]$$

HAVE WE FORGOTTEN THE ADVANTAGES OF CORE-SHELL PARTICLES?

When changing column or particle size dimensions for USP methods, comply with the requirements that;

L/d_p = -25% to +50%

or

N = -25% to +50%

That is, the column length (L) to particle size (dp) ratio must not fall outside these limits.

In this case, we wish to translate the USP method for lanzoprazole and its impurities, using a 150 x 4.6mm column with 5µm FPP, to an SPP column which uses 2.7µm particles.

Typically, we can choose columns of 100, 75, or 50mm in length. We need to decide which of these columns would comply with the requirements of Chapter <621>

L	dp	L/dp ratio	% change
150	4.6	32,600	-
100	2.7	37,100	+14
75	2.7	27,800	-15
50	2.7	18,500	-44

We can see that the 100mm or 75mm columns are available as options when switching to 2.7µm SPP formats. Assume that we wish to take full advantage of the speed gains offered by SPP particles and that we will opt for the 75mm column.

Furthermore, reduce the internal diameter of the HPLC column, providing that the linear velocity of the eluent is maintained. To ensure that this is the case, use equation 2 to calculate the eluent flow rate.

There's usually a choice between 3mm or 2.1mm internal diameter columns; however, be careful when reducing internal diameter, as making large changes to the instrument dwell volume (VD) to column volume (VM) ratio has been shown to induce changes in selectivity when using gradient methods [3]. Whilst a detailed treatment of this topic is outside the scope of this discussion, it's recommended to seek further information from reference 3, which also details which changes to the gradient program might be made in order to avoid selectivity issues when large changes in VD/VM are required.

The interstitial volumes of both the SPPs and FPPs can be estimated if some facts about the materials in use ar known. Measuring this directly by measuring the dead time (volume) of the system with and without the column installed might be the best approach.

The porosity ep of the particles (ask the manufacturer for this information) might be required. Or, the porosity of the corresponding SPP might need to be estimated, using the relationship;

ε_p (FPP) = 0.68 *(information obtained from the manufacturer or estimated from dead time marker)*

Equation 3

$$\varepsilon_p\ (\mathrm{SPP}) = (x_{inter} + x_{intra})\varepsilon_{p\ (FPP)}$$

Equation 4

$$\varepsilon_p\ (\mathrm{SPP}) = \left(\left(1 - \frac{\pi}{6}\right) + \left(\frac{\pi\left(d_p^3 - d_{core}^3\right)}{6d_p^3}\right)\right)\varepsilon_{p\ (FPP)}$$

(SPP particle diameter = 2.7µm and core diameter = 1.7µm)

$$\varepsilon_p\ (\mathrm{SPP}) = 0.59$$

Estimating the extra column volume for our three columns can then be achieved using **Equation 5**;

$$V_M = \pi\left(\frac{d}{2}\right)^2 L\varepsilon_p$$

Assuming the system dwell volume is around 300mL the VD/VM ratio changes here would be;

150 x 4.6 mm column using FPP VM = 1700µL, VD/VM = 0.18
75 x 3.0 mm column using SPP VM = 313µL, VD/VM = 0.96
75 x 2.1 mm column using SPP VM = 153µL, VD/VM = 1.96

Then the required gradient adjustment required for the two reduced dimension columns can be estimated using the following equation;

$$z = \left(\frac{V_{D1}}{V_{M1}} - \frac{V_{D2}}{V_{M2}}\right)\left(\frac{V_{M2}}{F_2}\right)$$

$$z = (0.18 - 0.96)\left(\frac{313}{800}\right)$$

z = -0.31

If the value for Z is negative, then the gradient needs to be started Z minutes prior to sample injection (possible with newer HPLC systems). If positive, an isocratic hold of Z minutes needs to be inserted at the start of the gradient.

Working through the various calculations, we arrive at the following sets of conditions;

Original Method;
Column – 150 x 4.6 mm 5µm (FPP)
Flow rate – 1 mL/min
Gradient –

Time (mins)	Solution A (%)	Solution B (%)
0	90	10
40	20	80
50	20	80
51	90	10
60	90	90

(A is 100% water, B is acetontrile:water:triethylamine (160:40:1) adjusted to pH 7.0 with phosphoric acid)

Translated method 1;
Column – 75 x 3.0 mm 2.7µm (SPP)
Flow rate – 0.79 mL/min (rounded to 0.8 mL/min) - *Calculated from Equation 1*
Gradient –

Time (mins)	Solution A (%)	Solution B (%)
-0.3	90	10
10.7	20	80
13.4	20	80
14.4	90	10
18.3	90	90

Calculated using Equation 6

Subsequent lines calculated using Equation 2

Equilibration time calculated using (10x column volume) / flow rate)

Note that the gradient here must be started 0.3 minutes prior to sample injection to retain the selectivity. If this is not possible with existing equipment, consider using the same internal diameter column as the original, but with reduced column length (i.e. 75 x 4.6mm in this case). Furthermore, the re-equilibration time has been calculated using a 10x column volume. This may be shortened, which can be assessed empirically.

Translated method 2;

Column – 75 x 2.1 mm 2.7µm (SPP)

Flow rate – 0.39 mL/min (rounded to 0.4 mL/min) - *Calculated from Equation 1*

Gradient –

Time (mins)	Solution A (%)	Solution B (%)
-0.7	90	10
10.4	20	80
13.0	20	80
14.0	90	10
17.8	90	90

Calculated using Equation 6

Subsequent lines calculated using Equation 2

Equilibration time calculated using (10x column volume) / flow rate)

Here the gradient needs to be started 0.7 minutes prior to sample injection.

The chromtaograms obtained using these three methods are shown in Figure 1;

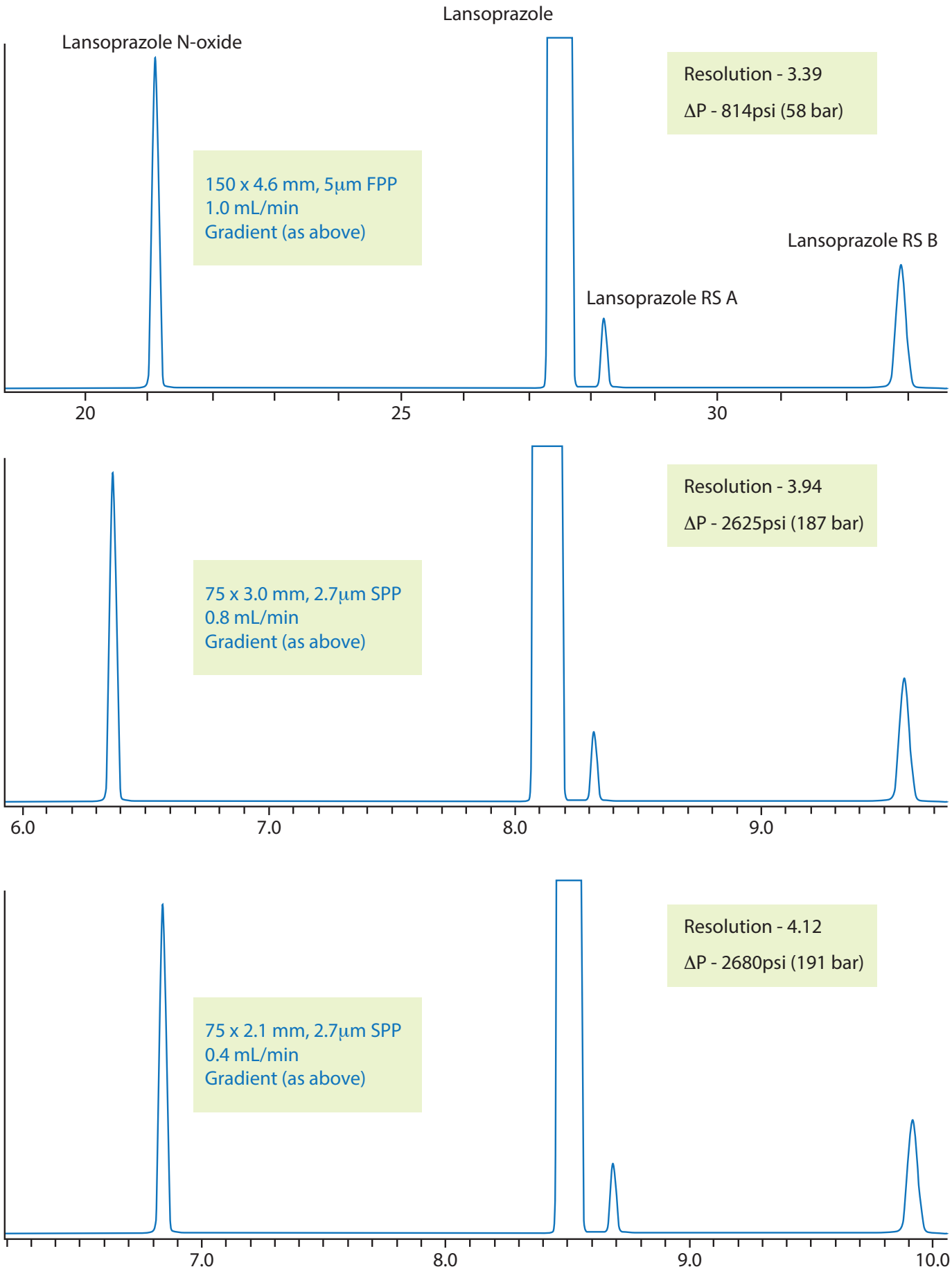

Figure 1: Resulting chromatogram with figures of merit for the original USP method for Lansoprazole impurities and the same method employed using Chapter <621> allowable changes and 2.7µm Superficially Porous Particles with 75mm x 3.0mm (middle) and 75 x 2.1mm (bottom)

Another important adjustment is the volume of sample injected. As column dimensions are reduced, and the particle morphology is changed, the amount of stationary phase available is reduced. This risks overloading the column, fronting peaks, thereby reducing the accuracy and reproducibility of quantitation. The following formula is very useful, which is based on a well-established mathematical relationship. However, it further reduces the injected volume by 30% to account for the marginally lower loadability of the superficially porous particles. Reducing by a further 30% is generally more than is required to account for the reduction in available surface area, and the injection volume may be increased empirically if extra sensitivity is required.

Equation 7

$$V_{i1} = V_{i2} \times \left(\frac{d_2}{d_1}\right)^2 \times \left(\frac{L_2}{L_1}\right) \times 0.7$$

$$V_{i1} = 40 \times \left(\frac{3}{4.6}\right)^2 \times \left(\frac{75}{150}\right) \times 0.7$$

$V_{i1} \cong 6uL$ (5µL used in practice for the 3mm i.d. column and 3µL used for the 2.1mm i.d. column)

Note that this formula is not recognised by USP <621> the current version of which (USP 40 – NF 35) says: 'The injection volume can be adjusted as far as it is consistent with accepted precision, linearity, and detection limits. Note that excessive injection volume can lead to unacceptable band broadening, causing a reduction in N and resolution, which applies to both gradient and isocratic separations. Therefore, the suitability of the injection volume using the method in equation 7 should be assessed empirically.

The chromatograms in figure 1show that either of the superficially porous core-shell column methods are more than three times faster than the USP method using the original conditions. This improvement in speed is due to the increased efficiency of core-shell columns, which allow appreciably smaller columns to be used without compromising the efficiency and resolution of the separation. This increase in the speed of analysis can be very important in high throughput laboratories.

However, there are also situations in which the inherent efficiency increase achieved when using core-shell particles can be a great advantage. In the following example we used a system with a dwell volume of 300µL and extra column volume of 60µL, initially following the USP method. Figure 2 (top) shows that the resolution between the lansoprazole and lansoprazole RS A peaks would be unacceptable in a practical situation. Using the principles above, a method translation was produced which used a 2.7µm superficially porous particle with the same 150 x 4.6mm column dimension, which produced a significant improvement in resolution from 1.78 to 2.38, which may be usable in practice.

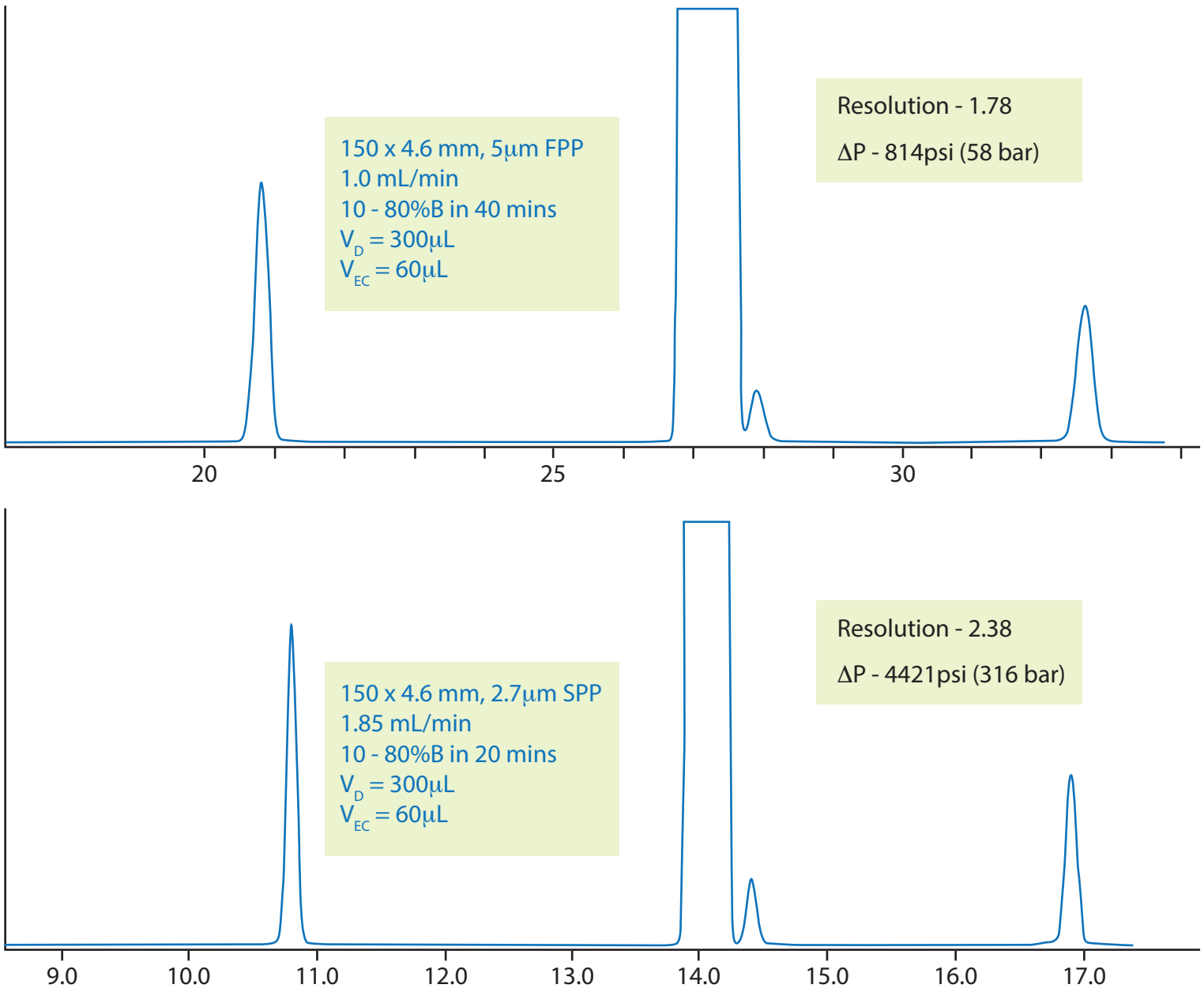

Figure 2: Resulting chromatogram with figures of merit, using a standard HPLC system, for the original USP method for Lansoprazole impurities (top) and the same method employed using Chapter <621> allowable changes and 2.7µm Superficially Porous Particles with the same column dimensions (bottom).

Using simple translation methods, we have demonstrated that chromatographic run times and quality of chromatography can be significantly improved by using superficially porous silica particles. Perhaps we should then ask our original question again — 'why would a fully porous particle be used when the use of superficially porous particles brings so many benefits'? Surely it couldn't be that the method translation effort is too much to bear?

Finally, consider the question of selectivity equivalence between the fully porous and superficially porous variants of manufacturers stationary phases. There is some variation in selectivity between FPP and SPP variants of nominally the same bonded phase from the same manufacturer. Whilst adjustments to the gradient profile, to account for VD/VM ratio differences, can help to address these issues, the fact remains that selectivity differences have been noted. Could this be a factor which makes the preferential use of SPPs a step too far for method translation? Of course, this would not be a problem if the SPP was used during the method development phase and we should also note the fidelity of selectivity between FPP and SPP variants are very good for some manufacturers.

HAVE WE FORGOTTEN THE ADVANTAGES OF CORE-SHELL PARTICLES?

References:

[1] C188676 (43(5) Harmonization Stage 4 General Chapter 621)

[2] https://www.crawfordscientific.com/technical/chromatography-blog/hplc-chromatography-tips/usp-chapter-621-changes

[3] http://www.chromatographyonline.com/translations-between-differing-liquid-chromatography-formats-advantages-principles-and-possible-pitfalls

UNDERSTANDING STATIONARY PHASES FOR HILIC SEPARATIONS

Mixed-mode chromatography can solve a number of analytical problems. However, due to the sheer number and type of variables that need to be considered, and optimised, many are wary of the complexity. How could these methods be robust? And, given the existing number of variables, is more selectivity really required?

HILIC chromatography involves hydrophilic partitioning. It can potentially require a combination of hydrogen bonding, ion exchange (analyte/stationary phase), more ion exchange (salt additives/stationary phase), ionic (charge) repulsion and ion exclusion (displacement) mechanisms. What's more, depending on the stationary phase used, partitioning may not be the dominant retention mechanism.

The point? HILIC chromatography is not straightforward and there may be a number of mechanisms in play which need to be considered. While mixed-mode chromatography is complex, HILIC is mixed-mode chromatography. At least some of the time.

This article will assist in understanding the nature of stationary phases and their interactions with analytes and mobile phase constituents. Without a basic understanding of HILIC stationary phases and the retention mechanisms in play, it is very difficult to understand this type of separation, what to do when things go wrong, or how to develop and optimise methods.

Take a simple amino stationary phase as an example. Examine some of the interactions which might be in play during a HILIC separation involving aminobenzoic acid.

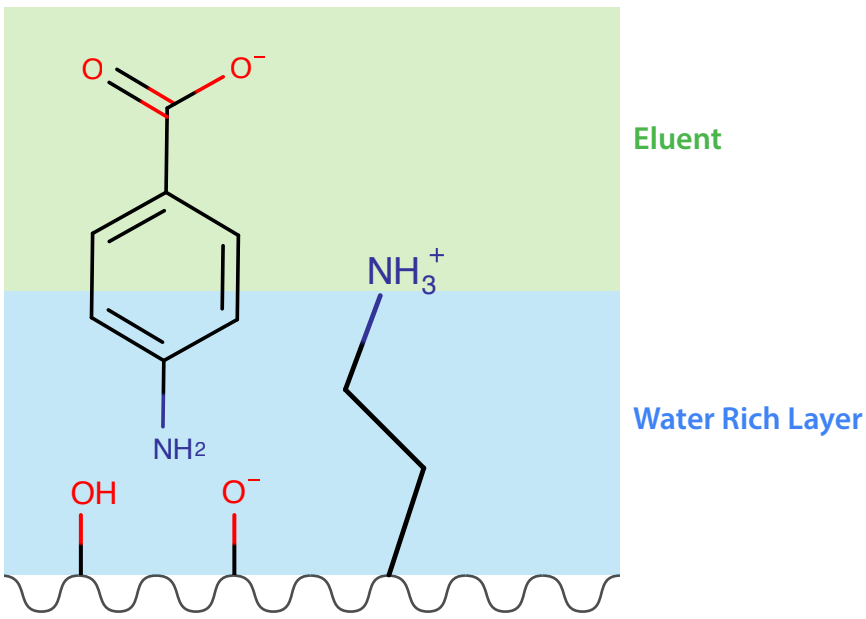

Figure 1: Schematic Representation of the HILIC Retention of 4-aminobenzoic acid on an amino stationary phase.

There are many possible contributions to retention from this very simple stationary phase and analyte combinations;

1) The hydrophilic nature of the stationary phase will dictate (amongst other factors) the thickness of the adsorbed water layer at the silica surface. In turn, this will dictate the retention by partitioning from the eluent into the water enriched layer for more polar (hydrophilic) analytes and analyte moieties (functional groups).

2) The thickness of this water layer may be affected by altering the % organic within the eluent.

3) Depending on the eluent pH the residual silanol groups on the silica surface may be charged (typically < pH4). Therefore, ion exchange may occur between analyte cationic groups.

4) If the analyte contains anionic groups, then ion repulsion may occur between the analyte and charged silanol groups on the silica surface (again, dependent upon eluent pH). Similarly, if the analyte is cationic there may be electrostatic repulsion between the bonded phase ligand and the analyte.

5) If either the silanol group or the analyte is not charged, then the presence of polar analyte functional groups may allow hydrogen bonding between the analyte and silica surface. Bonded phases containing polar neutral groups may be able to hydrogen bond with neutral polar analyte functional groups. This type of interaction can play a major role in HILIC separations.

6) Under the pH conditions shown, the stationary phase is positively charged and the analyte is negatively charged – resulting in electrostatic interactions.

7) If the eluent contains salts (ammonium acetate for example), the analyte electrostatic interactions may be overcome by increasing the concentration or counter ion strength (position within the Hofmeister series). This depends on the type and concentration of ions.

8) Van der Waals interactions will also be in play between the hydrophobic portions of the stationary phase and the analyte.

In summary, with this simple analyte/stationary phase combination, the following interactions & mechanisms of interaction will be possible:

- Hydrophilic partitioning
- Ion exchange
- Ion repulsion
- Hydrogen Bonding
- Ion Exclusion (depending upon the nature and concentration of eluent buffers)
- Van der Waals (hydrophobic) interactions between the analyte and stationary phase bonded ligand

A mixed-mode separation is in play. If all of the possible interactions are not understood and accounted for, then retention and selectivity in HILIC mode will be difficult to predict or influence using experimental variables.

The possibility and extent of these interactions will depend upon several experimental variables and will change with the nature of the stationary phase selected. Some stationary phases are only capable of some of the potential interactions. This will be explained in more detail.

Experimental Variables in HILIC

First, a brief summary of the experimental variables in HILIC chromatography and how they might be altered;

Hydrophilic Partitioning – The strength of hydrophilic partitioning into the water enriched layer is influenced by the stationary phase chosen and the amount of organic within the eluent. Higher organic will result in stronger partitioning of polar (hydrophilic) analytes into the water enriched layer; therefore, retention will increase. The eluent salt (buffer) concentration may also affect the thickness of the water layer.

Electrostatic Interactions – depend upon the degree of ionisation of;

- The analyte (ionogenic or non-ionogenic)
- The bonded phase (if ionogenic)
- The silica surface (residual silanol groups)

To manipulate a HILIC separation using pH, the solution pH and analyte pKa value must be known in order to estimate the degree of charge of the analyte. It is especially important to note that under the highly organic conditions that are typical of HILIC separations, both the analyte pKa and solution pH will differ from literature values; or from the measured pH of the aqueous phase only. Some very approximate rules of thumb can be employed to help us assess the solution pH and degree of analyte ionisation;

% Acetonitrile in Eluent	Target pH	Shift from aqueous pH
90	2	+3.5
90	4	+2.5
90	6	+2.0
75	2	+2.5
75	4	+2.0
75	6	+1.5
50	2	+1.5
50	4	+1.0
50	6	+0.5

% Acetonitrile in Eluent	Shift from Apparent Analyte pKa (Basic Analytes)
90	-1.0
75	-0.6
50	-0.1

Table 1 & 2: Adjustments to eluent pH and analyte pKa in highly organic eluents.

Take care when planning a HILIC separation involving ionogenic analytes or interpreting chromatography. Adjust thinking on the degree of analyte ionisation according to the amount of acetonitrile within the eluent, the literature value of the analyte pKa, and the target eluent pH.

Some stationary phases are designed to carry a full charge in solution (permanently ionised) and others, such as the zwitterionic phases, carry both positive and negative charges to reduce the strength of electrostatic interactions. These phases carry smaller net negative or positive charges depending upon the terminal group of the bonded phase. We will discuss the various stationary phases and their ability to interact electrostatically below. However, we should note that with some ligands such as the pentafluoropropyl (PFP) stationary phase, retention is almost totally dominated by the strength of electrostatic interactions with analytes. Very little hydrophilic partitioning is involved.

Of course, when considering the electrostatic interactions between analyte and bonded phase, we should not forget that there may also be repulsion between them, where both are either anionic or cationic. These repulsive forces will act to reduce retention and alter selectivity between ionogenic and non-ionogenic analytes.

The silica surface will have a pKa of around 4, so increasing eluent pH above 4 will make the silica surface increasingly anionic. Therefore, electrostatic attraction or repulsion also need to be taken into account when interpreting HILIC separations.

Hydrogen Bonding – Polar analytes and stationary phase moieties may be able to hydrogen bond in order to increase retention and alter the relative selectivity of the separation between polar and less-polar analytes depending upon the degree of hydrogen bonding.

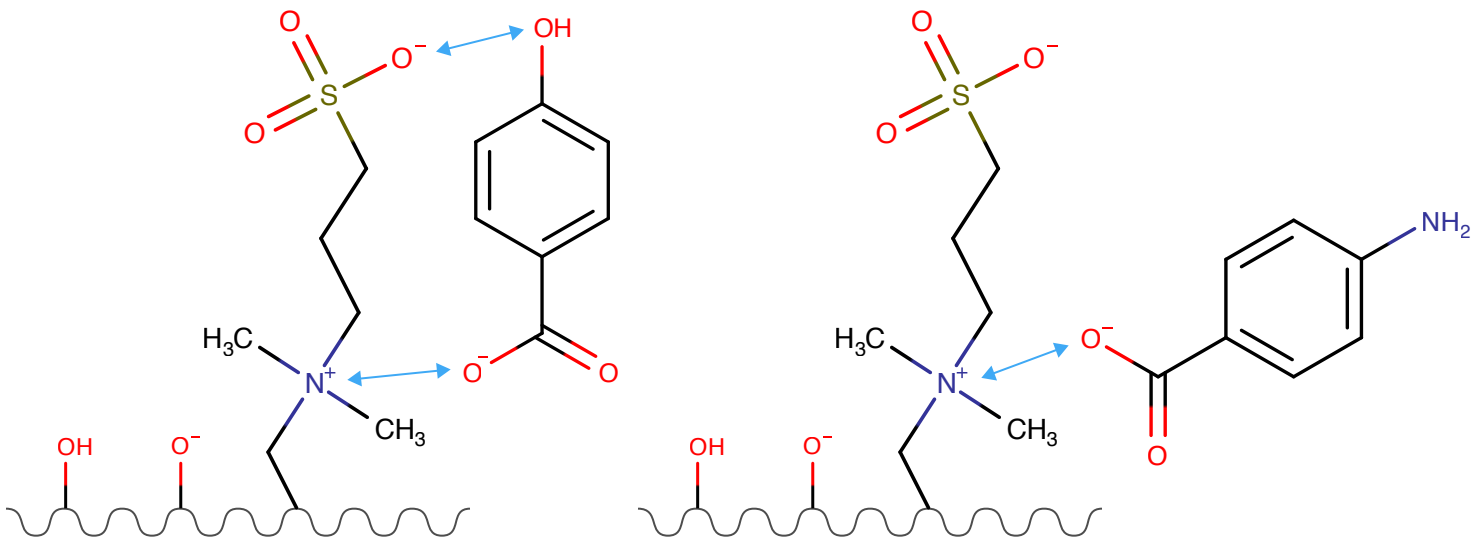

Figure 2: Interactions of 4 hydroxy benzoic acid and 4 amiono benzoic acid (right) with a zwitterionic stationary phase.

Figure 2 shows 4-hydroxy aminobenzoic acid (left) undergoing both electrostatic and hydrogen-bonding interactions with the zwitterionic stationary phase. Hydrogen-bonding of 4-aminobenzoic acid is not possible; therefore, a shorter retention time of this analyte may be expected. Both analytes will undergo hydrophilic partitioning and electrostatic interactions. The effects of increased or reduced hydrogen-bonding may be less significant.

The effects of hydrogen bonding in HILIC separations can be critical when separating polar neutral analytes.

Ion Exclusion (use of buffers) – Typically, the type and concentration of salt used in the eluent system will affect the degree of electrostatic interactions between the analyte and the stationary phase. For some separations, altering the buffer between a formate and acetate system can have a profound effect on the selectivity of the separation. Similarly, the concentration of the buffer will attenuate the degree of electrostatic interactions. Salt concentrations between 5mM and 100mM are typical. Higher buffer salt concentrations tend to lead to shorter retention times when electrostatic retention mechanisms are in play. A good starting point for investigations into the effects of buffer concentration is around 20mM. If using eluents containing 95% organic, the buffer concentration should be kept below 15mM to avoid buffer precipitation.

It's also important to note that the amount of salt can also affect the thickness of the water layer. This can alter retention when dealing with neutral analytes. Higher buffer concentrations can lead to thicker water enriched layers and longer retention times for neutral analytes.

Popular Stationary Phases | Interaction & Retention Mechanisms

What are the most popular stationary phase types? What types of interaction & retention mechanisms we can expect;

Zwitterionic Stationary Phases

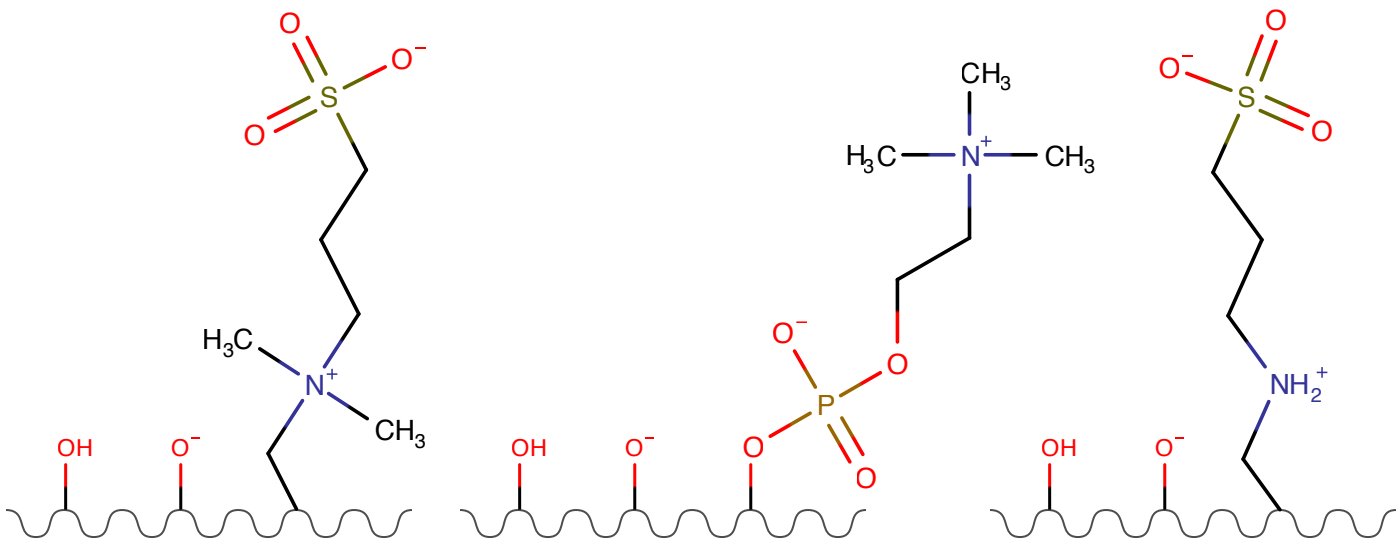

Figure 3: Zwitterionic Stationary Phases.

Partitioning	Medium
Ion Exchange	Medium
Hydrogen Bonding	High

These phases tend to have thick water layers and will undergo electrostatic interactions. The charge tends to be relatively low as the 1:1 ratio of positive and negative groups on each ligand is somewhat balanced. The overall net charge is dictated by the terminal group on each phase (so net negative when the sulphonic acid group is terminal on the ligand, for example). The overall extent of charge will not be affected by the solution pH and this can often simplify the planning and interpretation of separations, where the pH affects only the degree of ionisation of any ionogenic analytes.

Amino Phases

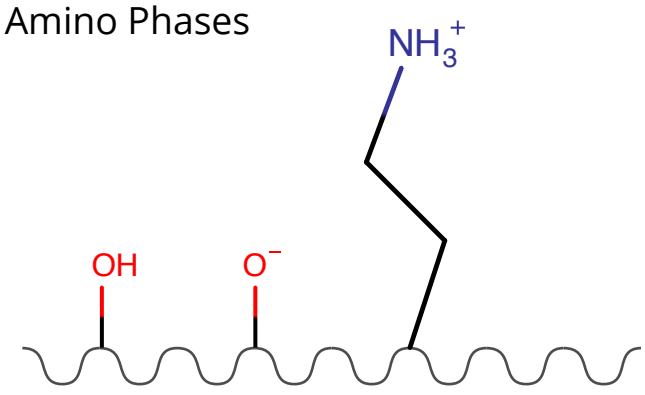

Figure 4: Amino Phases.

Partitioning	High
Ion Exchange	High
Hydrogen Bonding	Medium

The degree of amino phase charge can be influenced by eluent pH. They are particularly retentive for acidic compounds but often suffer from increased equilibration times. Care should be taken when dealing with aldehydes as there is the possibility of the formation of Schiff bases with the stationary phase.

Amide Phases

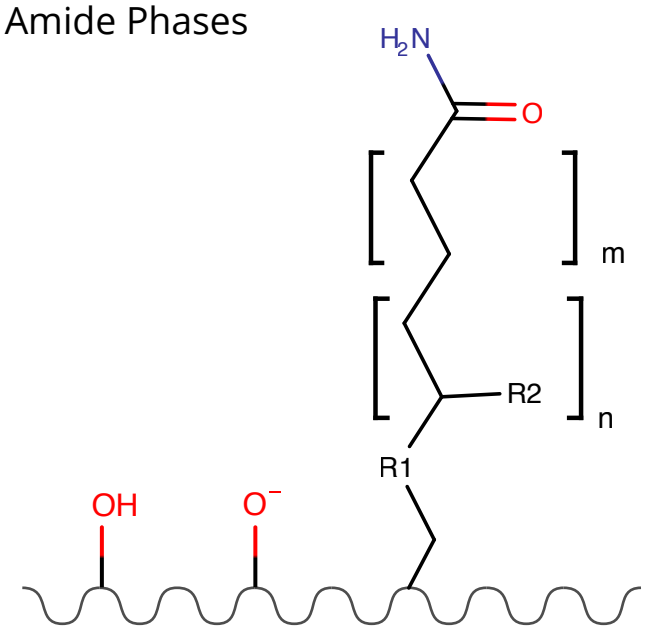

Figure 5: Amide Phases.

Partitioning	High
Ion Exchange	None
Hydrogen Bonding	Strong

In most commercial phases, the alkyl spacer length is short. These phases are not ionisable, meaning that the retention mechanism is easier to understand. Retention and selectivity can be controlled by lower salt concentrations.

Diol and Polyol Phases

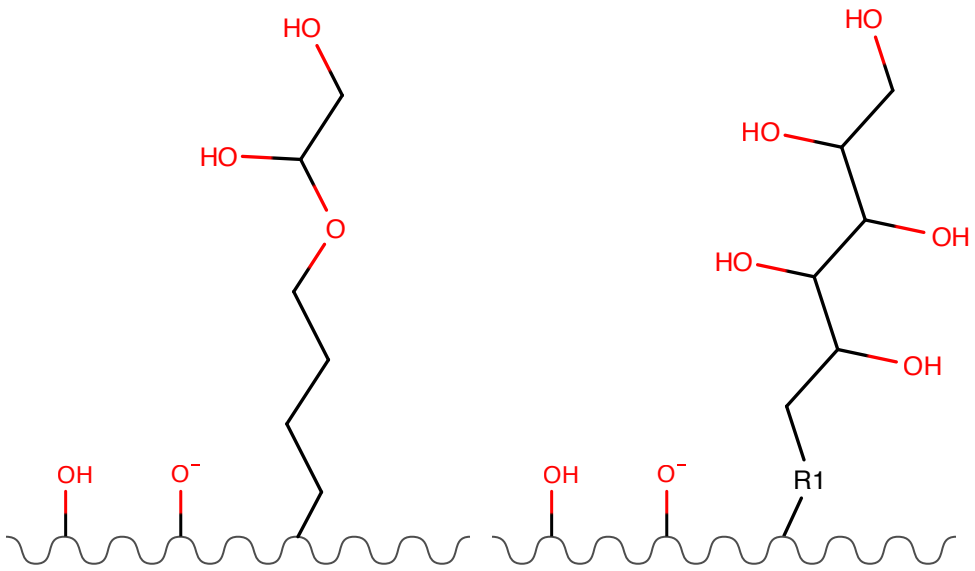

Figure 6: Diol and Polyol Phases.

Partitioning	High
Ion Exchange	None
Hydrogen Bonding	High

These phases are neutral and tend to have lower hydrophilic partitioning (more shallow water layers). The degree of hydrogen bonding is critical.

Bare Silica Phases

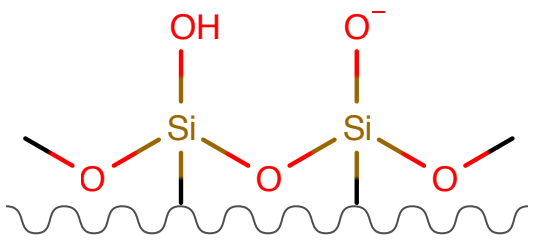

Figure 7: Bare Silica Phases.

Partitioning	Medium
Ion Exchange	High (>pH4-5)
Hydrogen Bonding	Medium

Whilst bare silica was popular in the early days of HILIC separations, silica surfaces are less homogenous. The pKa of the silanol groups can vary depending on the metal ion content and surface treatments. For these reasons, bare silica is less popular now that dedicated bonded phases are available.

PentaFluoro Propyl (PFP) Phases

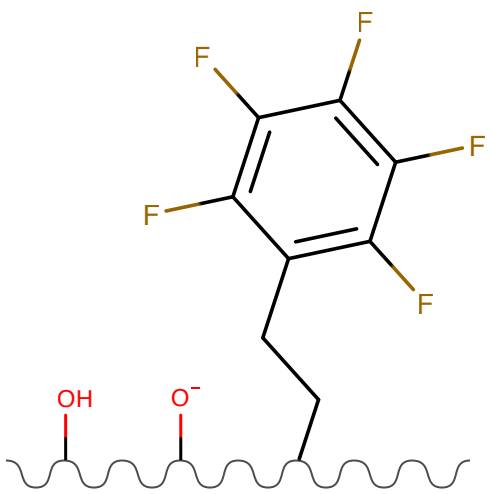

Figure 8: PentaFluoro Propyl (PFP) Phases.

Partitioning	Low
Ion Exchange	High
Hydrogen Bonding	High

These phases separate almost solely on the strength of electrostatic interactions between the analyte and stationary phase, with very little hydrophobic partitioning.

Interpreting HILIC Separations

There are a number of more specialist phases available. However, the above treatment covers the most popular phase types and gives a flavour of the multiple retention mechanisms possible within the HILIC separation mode. Without a good understanding of the stationary phase and its possible interactions, it is very difficult to interpret or plan effective HILIC separations.

The more eagle-eyed reader will note that the Cyano phase has been omitted from the list above. Their lack of hydrogen bonding capability and poor retention of polar analytes — even at high organic concentrations — makes them less favourable for HILIC separations.

GETTING THE MOST FROM PHENYL STATIONARY PHASES FOR HPLC

There has been a trend towards 'lazy' chromatography in the industry of late. This can be seen when 0.1% (w/w or w/v!) TFA or formic acid is used to 'buffer' the eluent system, well away from the pKa of analyte molecules. This leaves most acidic analytes in the ion suppressed form and most basic analytes in the ionised form. The approach avoids issues with retention time drift and effectively eliminates pH as a variable used to control retention or selectivity for separations involving ionogenic analytes.

This creates more reliance on other variables to control the selectivity. The prime suspects are the stationary phase and organic modifier (type and eluotropic ratio). Exploring the selectivity of other stationary phase types is important, in case the usual column of first intent (typically a C18 or C8 phase) does not produce a satisfactory separation.

Phenyl Phases

Phenyl type phases can be useful when separating a variety of analytes. This includes aromatic, polycyclic, and unsaturated species, due to the π-π interactions between the electron-rich double bonds within the analyte and stationary phase phenyl moieties. Relative retention compared to more hydrophobic phases is reported to increase in the order:

aliphatics < sunstituted benzenes < polyaromatic hydrocarbons ≅ nitroaromatics

which correlates to the degree of π-electrons within the solute [1].

It should be noted here that these phases also undergo hydrophobic interactions with analytes, which often dominate the overall strength of the interaction. Further, phenyl phases undergo weak electrostatic, hydrogen bonding, and dipole-dipole interactions with analytes. Under some classification systems, they strongly differentiate on what are sometimes called analyte steric factors.

Understanding the chemical nature of the various types of phenyl phase can help to rationalise variation in retention and selectivity compared to separation on other phases.

Figure 1 shows just a snapshot of the various chemistries available which are generally described as phenyl or phenyl hexyl phases. These fall under the USP classification L11.

There are many more variants available. Pentafluorophenyl (PFP, USP L43) phases have markedly different properties and will not be discussed.

The chemistry of each of the phenyl phases is explained in the Figure 1 legend, however the main differences include; the number of aromatic groups (mono versus biphenyl), the length of the alkyl spacer between the silica surface and the phenyl group, the nature of the substituent groups on the bonded ligands (typically methyl or more sterically bulky isobutyl groups), the inclusion of an oxygen atom in the linker to activate the π electron system in the aromatic ring, and finally whether the silica stationary surface is endcapped or not.

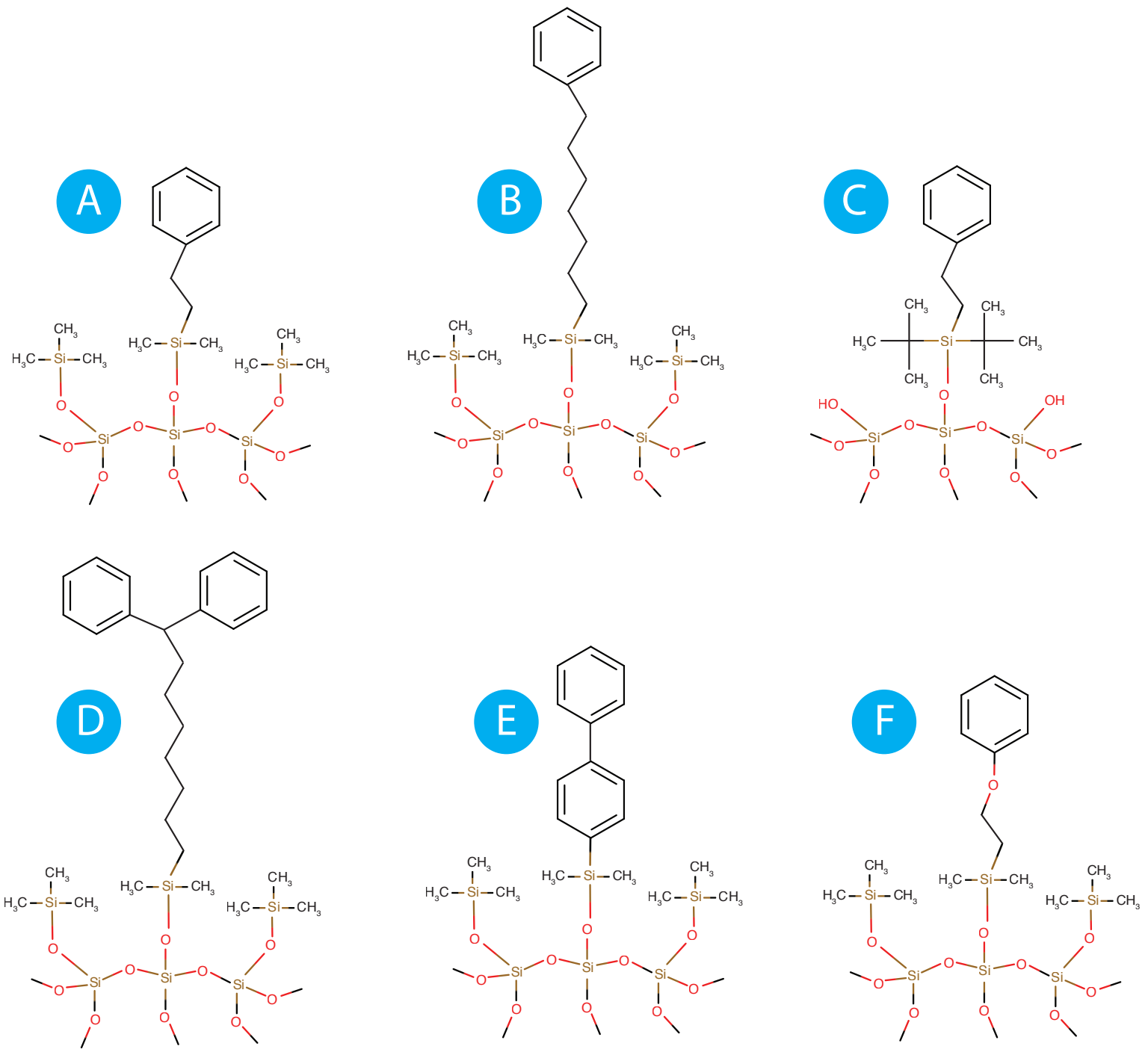

Figure 1: Various forms of phenyl HPLC bonded phase:

A) Ethyl phenyl with methyl side groups and an endcapped silica surface
B) Phenyl hexyl phase with extended (hexyl) ligand spacer methyl side groups and an endcapped silica surface
C) Ethyl phenyl ligand with steric protection (isobutyl) side groups and a non-endcapped surface
D) Hexyl biphenyl with methyl side groups and endcapped silica surface
E) Biphenyl phase with methyl side groups and endcapped silica surface
F) Oxygen activated phenyl ethyl phenyl phase with methyl side groups and endcapped silica surface

Figures 2 and 3 show an investigation [1] which can be used to highlight some of the key properties of phenyl phases and how they might best be used to explore alternative retention and selectivity when C18 phases fail to produce a satisfactory separation.

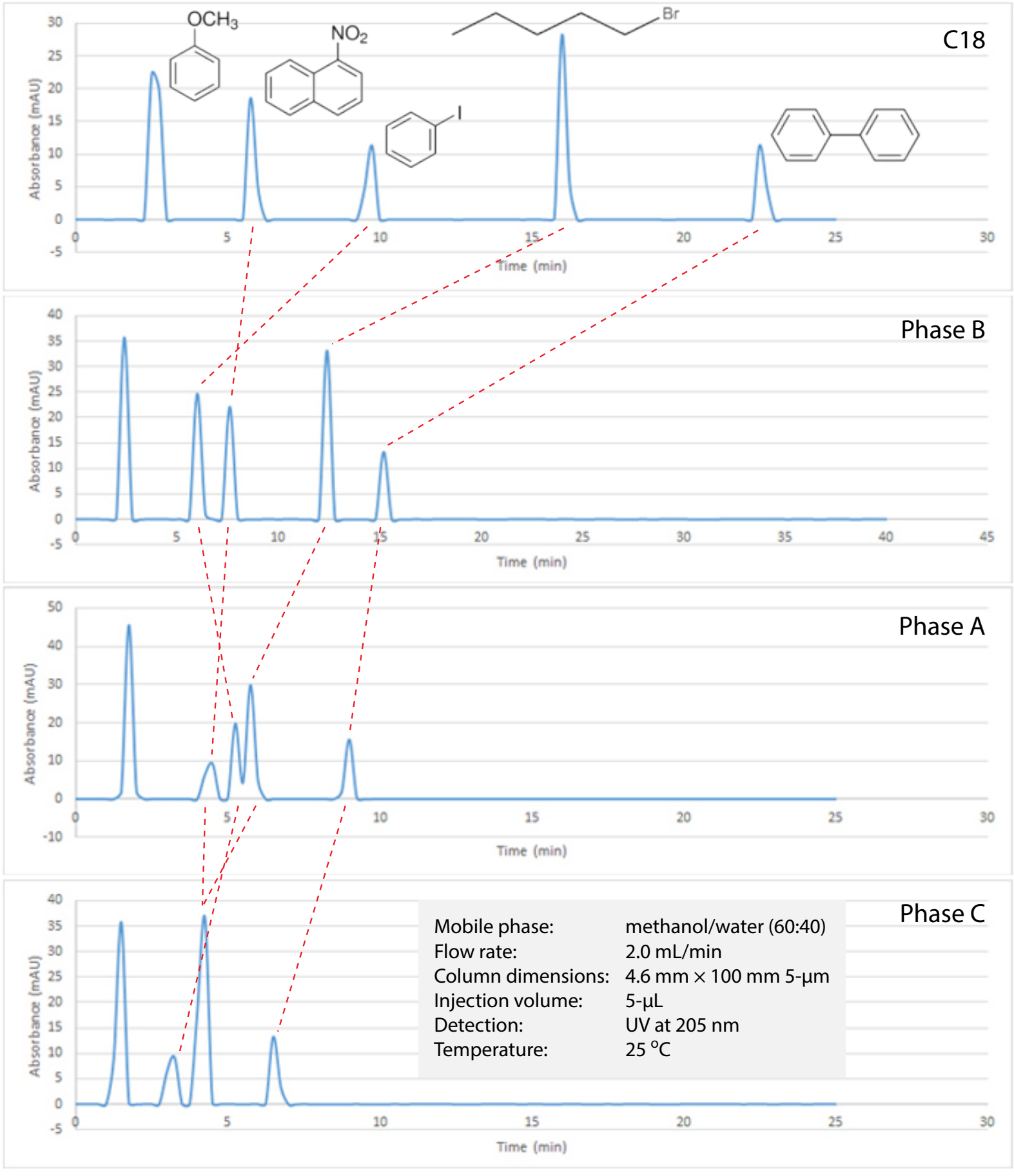

Figure 2: Separation of a range of analytes using C18 and various types of phenyl stationary phase using methanol as organic modifier.

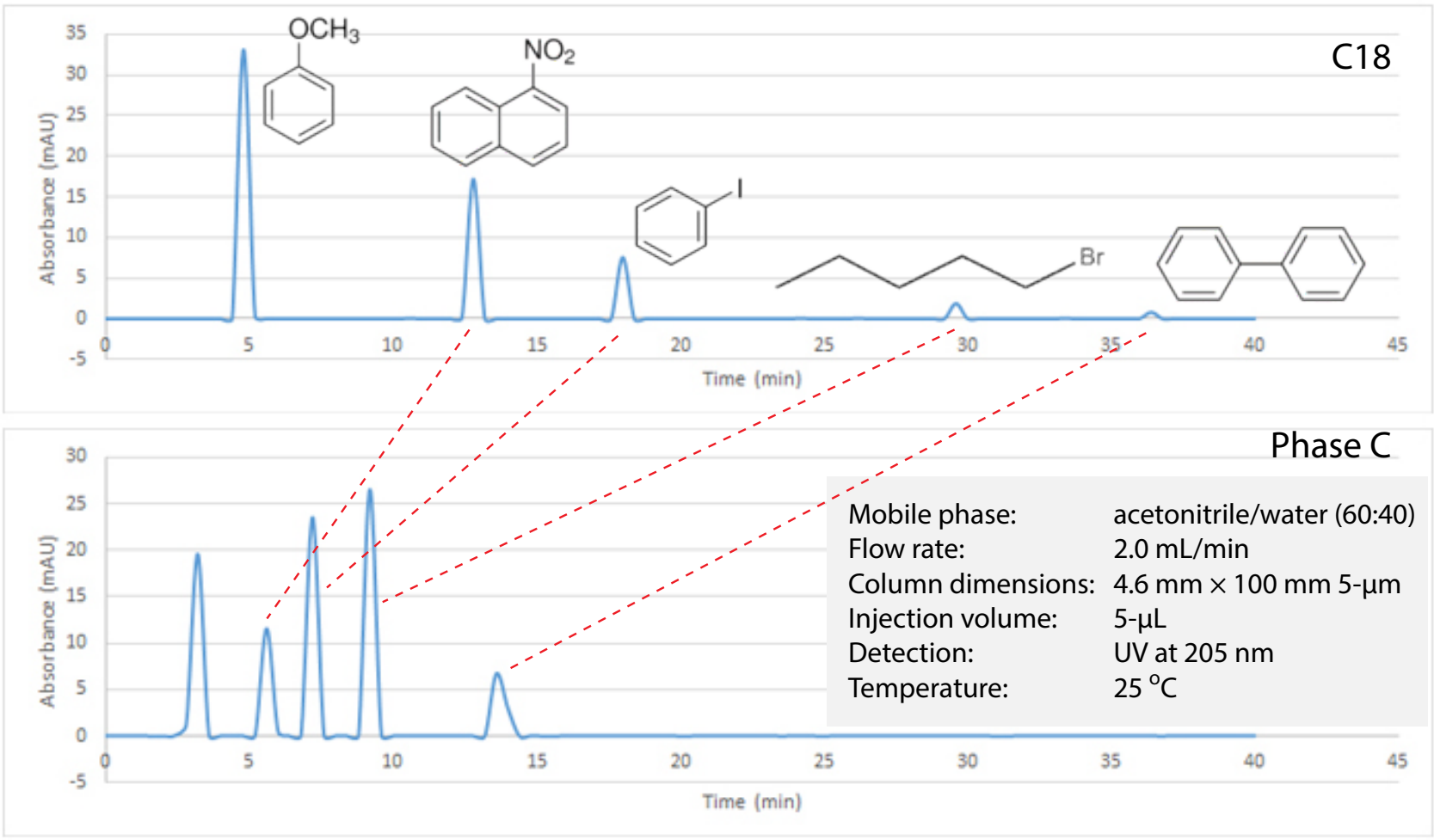

Figure 3: Separation of a range of analytes using C18 and a non-endcapped ethyl phenyl stationary phase using acetonitrile as organic modifier.

The compounds used in the investigation from left to right in the uppermost chromatogram of Figure 2 are;

- Anisole – a substituted benzene with an electron donating group
- 1-Nitronapthalene – a nitro-substituted aromatic with an electron withdrawing group
- Iodobenzene – a substituted benzene with an electron withdrawing group
- 1-Bromopentane – an aliphatic compound with electron withdrawing group
- Biphenyl – a polyaromatic hydrocarbon (PAH)

When comparing the behaviour of analytes with different stationary phases, it is also useful to analyse a number of analytes of the same class and compare the retention factor or retention time on both phases via a scatter plot with linear regression.

The slope of the regression plot indicates the relative strengths of analyte/stationary interaction compared to the base column (a C18 in this case) — where a slope of 1 indicates equal strengths of interaction for the two columns. The slope values may be compared to assess relative strengths of interaction on each of the columns and between classes of compound.

The regression value gives an indication of the nature of the analyte/stationary phase interaction. Low regression values indicate that the mechanisms of interaction differ and one might expect an orthogonal selectivity compared to the base column. Data of this type is represented in Table 1 and Figure 4.

Compound	k' C18	k' Phenyl Ethyl	
Dimethoxybenzene	1.16	1.2	**Substituted Benzenes**
Ethylbenzene	11.55	3.11	
Anisole	2.62	1.43	
Benzonitrile	0.79	1.05	
Ethyl benzoate	4.39	2.71	
Toluene	6.18	1.97	
p-Dinitrobenzene	1.17	1.57	**Nitro-aromatics**
m-Dinitrobenzene	1.31	1.69	
1,3,5-Trinitrobenzene	1.04	2.01	
1-Nitronaphthalene	5.82	4.12	
1,3-Dinitronaphthalene	6.22	6.83	
1,5-Dinitronaphthalene	4.63	5.62	
Naphthalene	1069	3.44	**PAH**
2-Methylnaphthalene	20.98	5.63	
1-Methoxynaphthalene	13.48	4.76	
Biphenyl	22.59	6.57	
Phenanthrene	37	9.61	
Anthracene	43.53	10.81	
n-Pentyl acetate	4.8	2.64	**Aliphatics**
1-Nitropropane	0.43	0.55	
1-Nitrobutane	1.02	0.95	
1-Bromopropane	3.61	1.45	
1-Bromobutane	7.74	2.46	
1-Bromopentane	16.06	4.18	

Table 1: Retention data for various classes of compounds on C18 and phenyl ethyl stationary phases (analysis conditions as per Figure 2). [1]

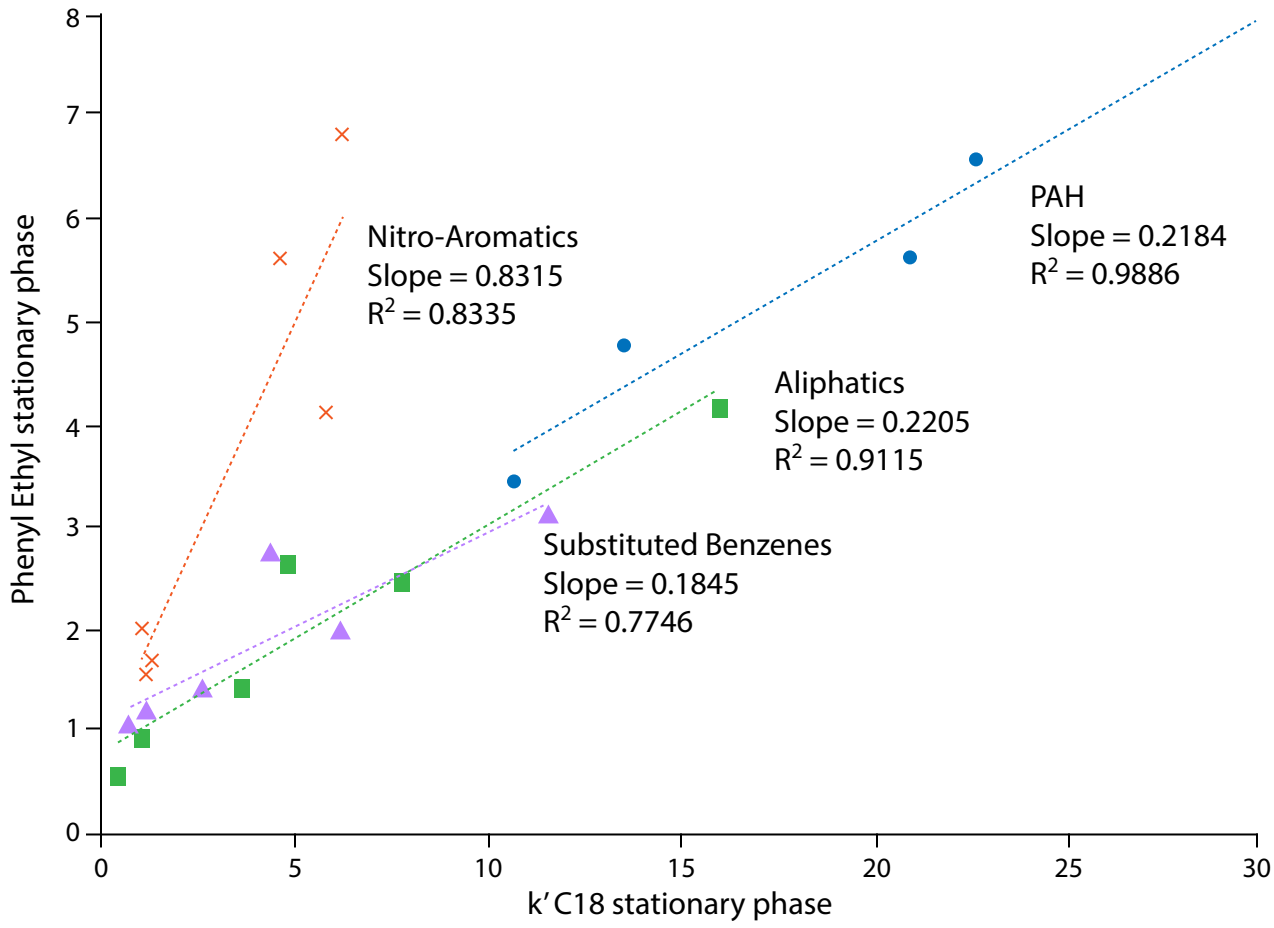

Figure 4: Correlation of retention factor data for various compound classes on C18 and phenyl ethyl stationary phases.

Examining the data collected, some opinions about phenyl based stationary phases begin to form.

Figure 1 shows that, when using methanol as the eluent modifier, whilst general retention for analytes decreases compared to the C18 phase, there is peak swapping. This indicates a change in selectivity, especially for nitro-aromatics and substituted benzene compounds, which is confirmed via the low linear regression values (R2) in Figure 4.

Figure 1 also shows that the phenyl hexyl phase shows the longest retention of the phenyl phases, due to the dominant nature of the hydrophobic interaction between the analyte and hexyl spacer of the bonded ligand. The difference in the strength of π-π interactions. It's most noticeable when using phenyl phases with shorter alkyl spacers as the hydrophobic interaction becomes less dominant.

The variation in slope for the regression lines for each class of compound shows that the strength of interaction between analyte and stationary phase varies for each of the compound classes investigated. The large differences in regression coefficient (R2) for each compound class indicates that the type of interaction between analyte and stationary phase changes for each compound class. This is widely attributed to the varying degree of π-π interaction which is possible between the analyte and the stationary phase. [2]

Nitro-aromatics which have electron-withdrawing groups (activating the π electron system within the ring) tend to be relatively more retained than aliphatic compounds on phenyl columns than C18 columns (slope is greater than that for the aliphatic compounds in Figure 4).

It is postulated that this is due to the π electron system accepting electrons from the phenyl stationary phase. It effectively acts as a Lewis base. The ability of the stationary phase to act as a Lewis base is amplified if an oxygen or electron-donating atom is included just below the aromatic group of the stationary phase (phase F in Figure 1); therefore, giving rise to stronger interactions with unsaturated systems with substituent electron-withdrawing groups. An enhanced change in selectivity relative to aliphatic or fully saturated analytes is also expected.

Substituted benzenes with electron-donating groups are retained less strongly relative to aliphatic compounds on phenyl versus C18 columns. This is due to lower π-π interactions between the analyte and stationary phase which are both electron-rich (lower slope for the linear correlation co-efficient in Figure 4).

The ability to differentiate more certainly between analytes with different degrees of π electron activity make phenyl phases highly suitable for the separation of position isomers of aromatic compounds and for aromatic analytes in which the nature of substituent groups changes (Figure 5).

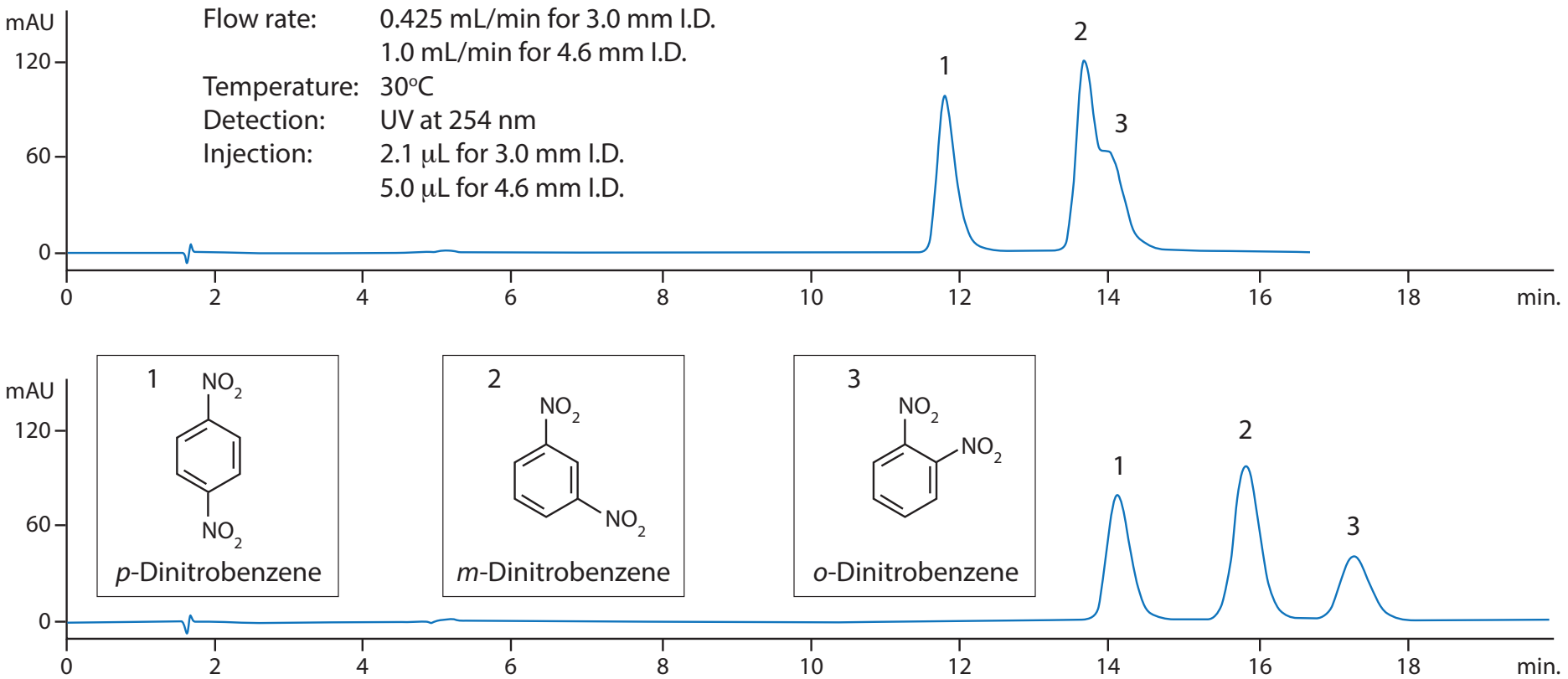

Figure 5: Enhanced separation of positional isomers of dinitrobenzene using a phenyl stationary phase (bottom) versus a C18 phase (top). [4]

When using acetonitrile as the organic modifier, the selectivity of the separation does not change when switching from a C18 to the ethyl phenyl column, indicating that the relative influence of the π-π interaction compared to the hydrophobic interaction is lessened than when using methanol as the modifier. This is often attributed to π-π interactions between the acetonitrile molecules and the π electron systems of the analyte and/or stationary phase. They act to compete with analyte/stationary phase interactions. [3]

GETTING THE MOST FROM PHENYL STATIONARY PHASES FOR HPLC

Knowing something of the stationary phase chemistry of phenyl based stationary phases will help you to select and rationalise your observations during HPLC method development. The same goes for the relative strengths of interaction with analyte molecules, as well as the surprising differences in retention and selectivity when using methanol as opposed to acetonitrile as the organic modifier. With less willingness to finesse our separations through eluent optimisation, knowledge of stationary phase chemistry and analyte interactions becomes increasingly important.

References:

[1] Comparison of Selectivity Differences Among Different Agilent ZORBAX Phenyl Columns using Acetonitrile or Methanol, Agilent Technologies Publication, 5990-4711EN

[2] J. León E. Reubsaet, Rune Vieskar, “Characterization of π-π Interactions Which Determine Retention of Aromatic Compounds in Reversed-Phase Liquid Chromatography,” Journal of Chromatography A, 841 (1999) 147-154.

[3] Min Yang, Steven Fazio, David Munch, Patrick Drumm, “Impact of Methanol and Acetonitrile on Separations Based on π-π Interactions with a Reversed-Phase Phenyl Column,” Journal of Chromatography A, 1097 (2005) 124-129.

[4] Comparison of separation selectivity among YMC-Triart series, YMC Application Note F130201AE

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