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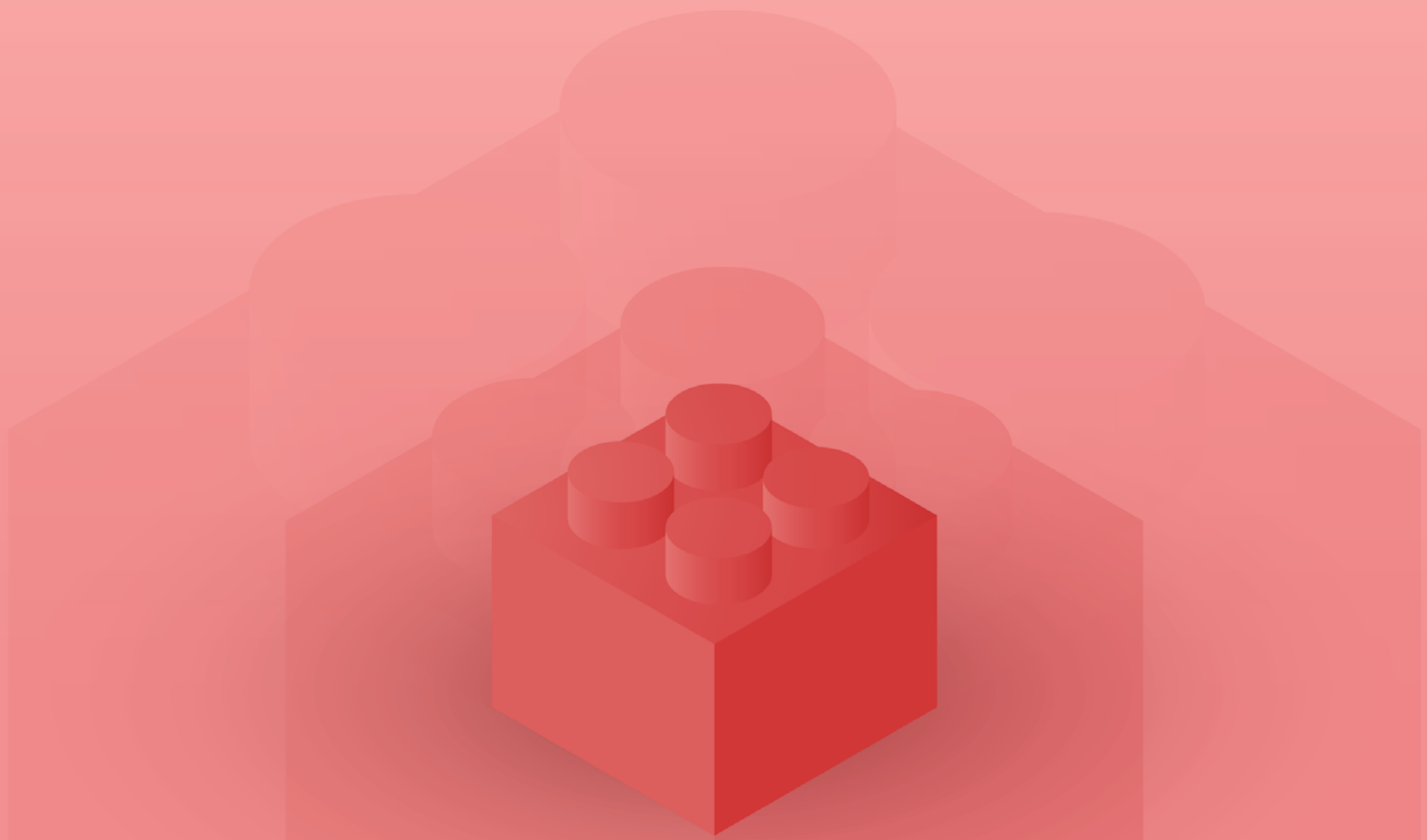

HPLC Method Development

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

VOLUME II

BUILD BETTER HPLC METHODS WITH CRAWFORD SCIENTIFIC

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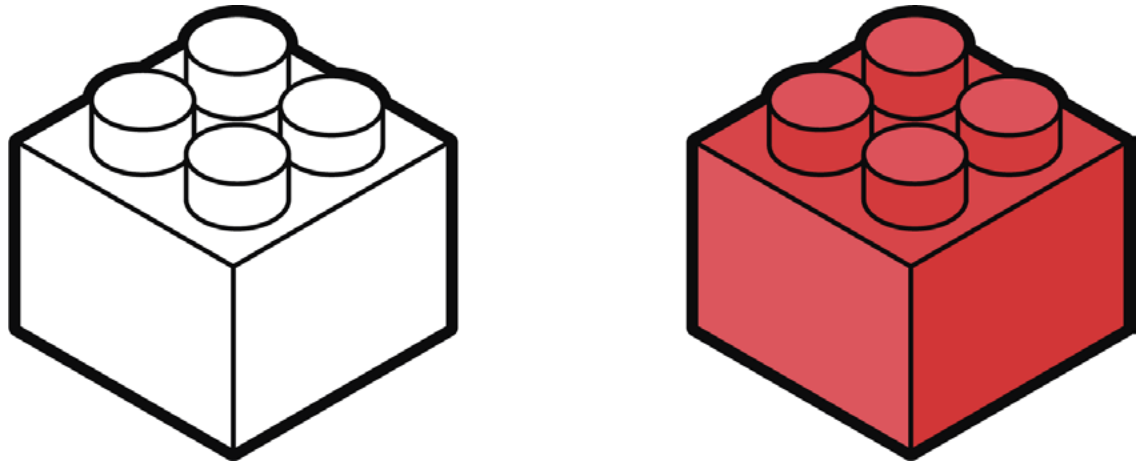


COLUMN SELECTION FOR HPLC METHOD DEVELOPMENT

The approaches to method development vary widely, especially column selection in HPLC. When I ask about column selection criteria I get a range of responses from:

- We use our favourite columns
- Our column screen has been developed for our applications types over a number of years
- We look for applications from literature and vendors using similar analytes
- We use what worked last time for a similar analyte / matrix
- We use whatever is on the instrument or the first column we put our hand on in the drawer

These responses range from laissez-faire to well researched but, when pushed, even the most diligent of analysts fail to grasp the key considerations in column screening. The toolkit approach should provide a highly efficient and wide-ranging exploration of the separation possibilities for the application under investigation — in particular the selectivity which will drive the separation under development. Whilst it's true that a wide range of modifiers, pHs, additives, and gradient profiles are an important part of the selectivity 'space' it is also true that the nature of the stationary phase is a large contributor to selectivity. Yet analysts are often a lot more cautious when discussing the factors that drive stationary phase choice.

The Hydrophobic Subtraction Model of Stationary Phase Selectivity

It is particularly important to be aware of the characteristics of stationary phase and it's support which drives selectivity. A good set of descriptors can be found in the hydrophobic subtraction model [1] of column selectivity which is used by the Product Quality Research Institute (PQRI) to build their database of column characteristics:
http://apps.usp.org/app/USPNF/columnsDB.html

This model can be very simply described using the graphic shown in Figure 1

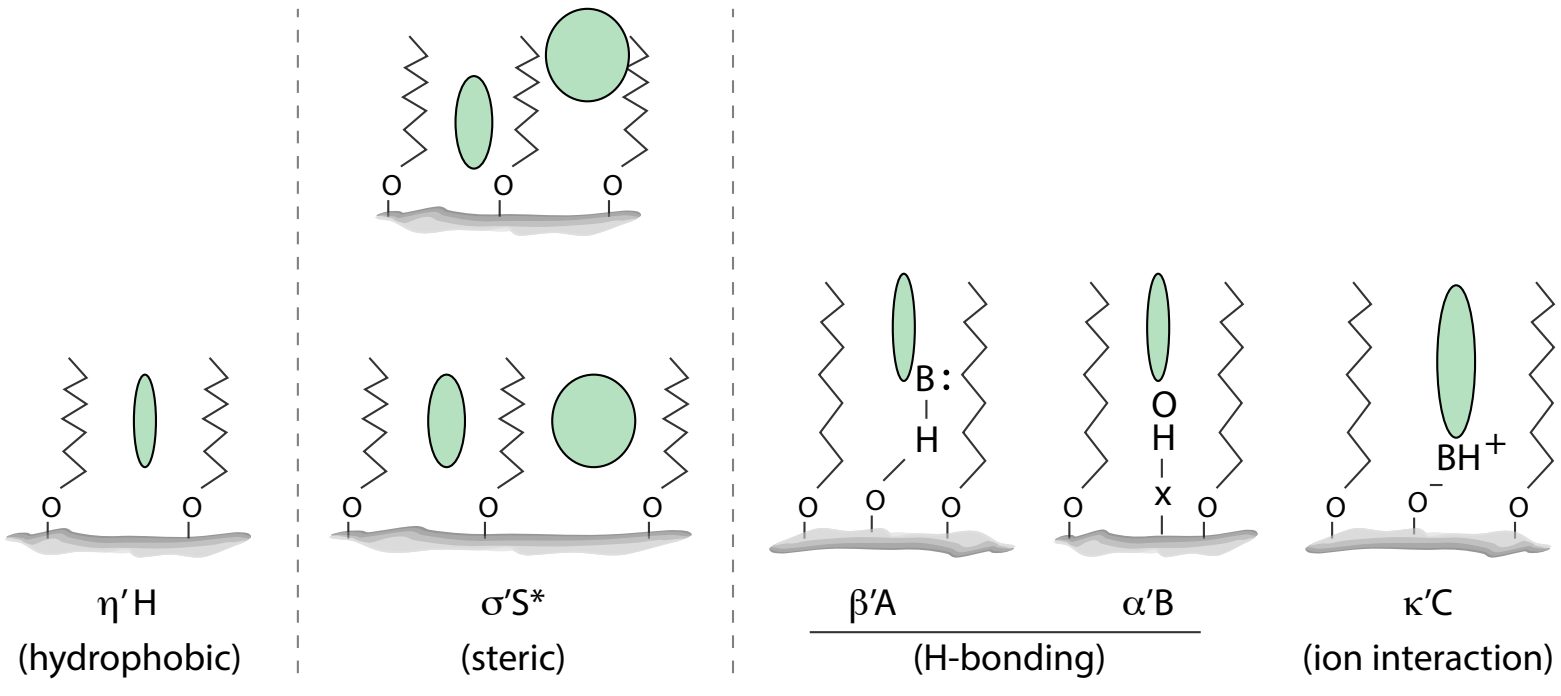


Figure 1: Representation of five solute–column interactions of the Hydrophobic Subtraction Model.

The terms beneath each picture in Figure 1 represent the terms which the hydrophobic subtraction model defines as being of primary importance in determining separation selectivity behaviour of the analyte and column. These are determined using retention and selectivity measurements on each column using a defined set of chemical test probes.
The primary characteristics can be very simply described as:

H – is the hydrophobicity (retentivity) of the phase and is dictated by the type and bonding density of the ligand

S* - Steric selectivity, the ability to separate molecules whose shape, size, and polar functional groups differ. It's dictated by the type and bonding density of the ligand

A – the stationary phase acidity, represents the ability of the phase to hydrogen bond with basic functional groups of the analyte and a contributor is analyte interactions with acidic silanol groups on the silica substrate

B – the stationary phase basicity, represents the ability of the phase to hydrogen bond with analyte acidic functional groups which can be via surface metal ions or a layer of sorbed water at the silica surface

C – represents the ion exchange capacity of the column, which changes with increasing pH as the acidic silanol groups on the silica surface increase in charge between pH 2.8 and pH 7.0 (the two values at which the ion exchange capacity is measured in the model), thus affecting the degree of secondary interaction between polar and ionisable analyte functional groups and the silica surface

It's important to highlight that each of the interactions above can contribute in a positive way to the selectivity of the separation.

Contribution of HSM Model terms to Column Selectivity

The key to improving column selection, lies in understanding which characteristics dominate with each phase and what affects the characteristics have on the separation of certain (your!!) analytes. Before I go on to consider how this might be achieved, consider the number of times in the above list that the word silica appears versus the number of times I mention 'ligand'.

Whilst all of the key characteristics are affected by the bonded phase ligand, at least three of them are highly influenced by the nature and surface treatment of the underlying silica onto which the stationary phase ligands are bonded. Given that, in order to properly explore the selectivity space available, one needs to be varying the support material as well as the bonded phase ligand in order to get the 'full range' of selectivity that might be available for a separation.

So, if your screen contains a set of columns from a single manufacturer where the only difference is in the bonded phase ligand, you need to consider if you are fully exploring the column selectivity space available. Any nervousness about variability in the nature of the underlying silica can be almost wholly discounted in modern times as well established manufacturers have good control over any batch to batch variability in either the bonded phase or the silica substrate.

Visualisation of Column Comparisons

One way to visualise the differences between columns is to compare them using radar plots — where the key differences and dominant characteristics can be displayed. Some rudimentary empirical experiments can then be made to assess which of the characteristics is best at driving the separations which you perform. This may be a set of columns which show very subtle selectivity differences if you work on similar molecules and have identified the key characteristics for a successful separation. Alternatively, if you are running a general screen, or if the analyte nature or structure is unknown, it may be a column set with widely differing properties. Some typical radar plots are shown in Figures 2 to 4.

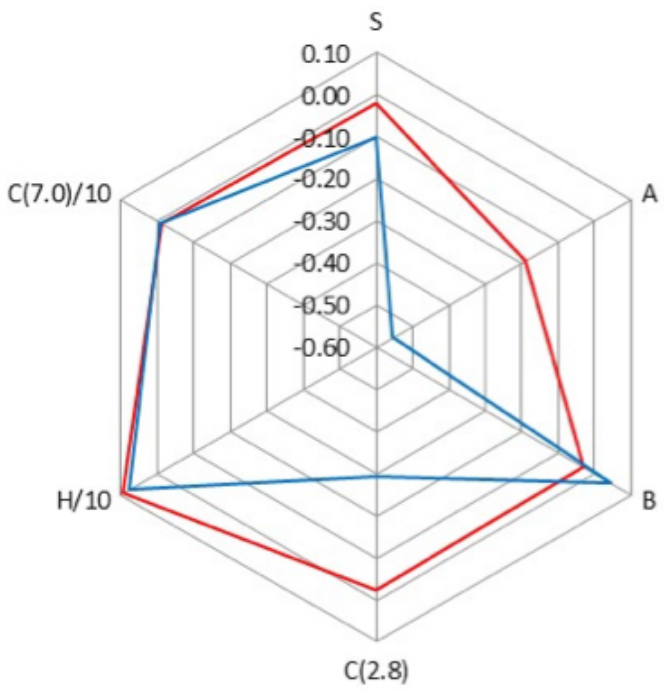


Figure 2: Comparison of properties of two different C18 (L1) HPLC column stationary phases using a radar plot of the PQRI column classification properties which are in turn based on the terms of the hydrophobic subtraction model.

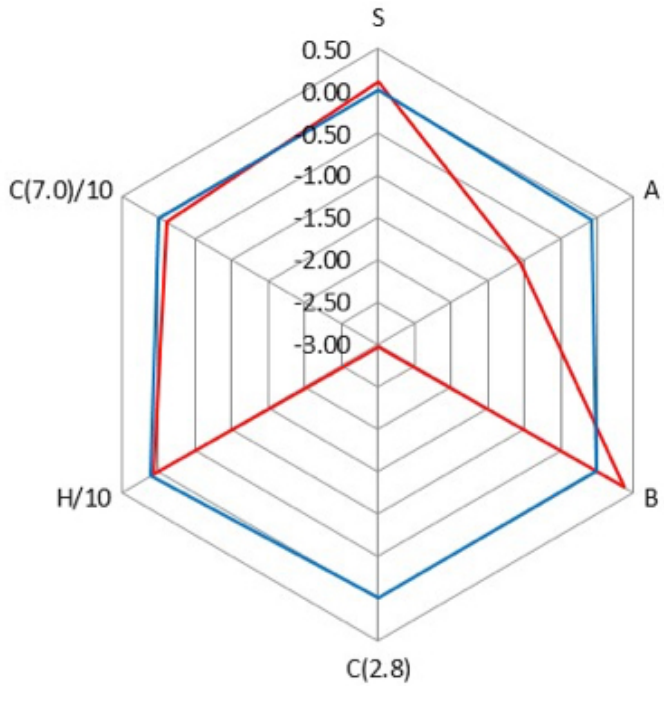


Figure 3: Comparison of properties of a Polar Embedded C18 column (L60) and a C18 column (L1) stationary phases using the radar plot of the PQRI column classification.

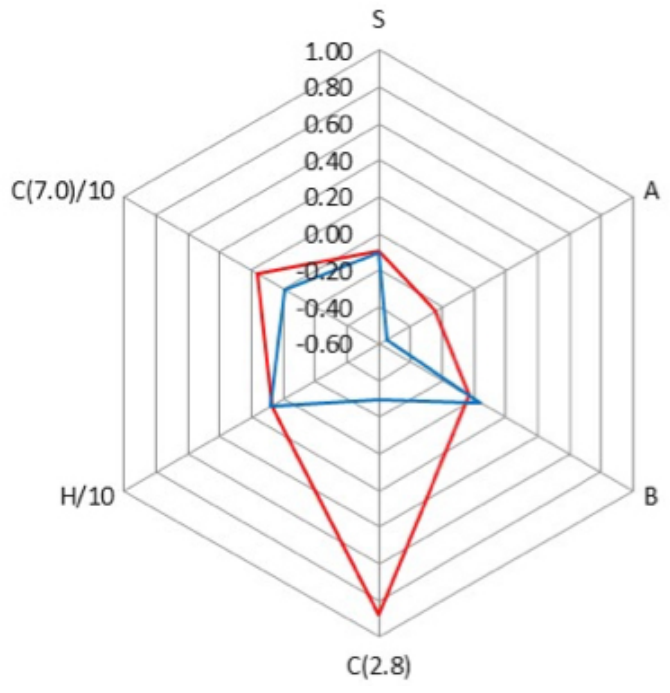


Figure 4: Comparison of properties of a PentaFluoroPropyl (PFP) column (L43) and a Phenyl column (L11) stationary phases using the radar plot of the PQRI column classification.

One should note the scales on Figures 2, 3 and 4.

Figure 2 shows subtle differences (full scale of radar plot is 0.35 units) in Steric (S), Ion Exchange (silanol acidity) (C2.8) and Surface Acidity (A) between the two C18 phases. However very large differences (full scale of radar plot is 3.5 units) between Ion Exchange capacity, Surface Acidity and Surface Basicity (B) are highlighted between the C18 and Polar Embedded columns compared in Figure 3.

Figure 4 compares two columns whose selectivity differs only in the bonded phase as the underlying silica used is identical. One column contains an aromatic system which is highly electron rich (Phenyl) and the other an aromatic system which is highly electron deficient (PFP) and as such the characteristics are markedly different (full scale of radar plot is 1.6 units).

The differences between the phases in Figure 2 are driven primarily by the underlying silica and bonding density changes. In Figure 3, they're driven by a combination of differences in the bonded phase as well as the differences in the underlying silica. The differences in selectivity between the columns in Figure 4 are predominantly due to differences in the bonded phase.

Conclusions

The key to good column selection is knowing the dominant characteristics required from the chosen column set which drive the separation of your analyte types or, alternatively, by covering the widest selectivity range possible if screening unknown or unfamiliar analytes. Column selection for one-off method development, and as part of screening databases, can be improved through the use of in-silico models such as the PQRI database and a better understanding of selectivity models (such as the hydrophobic subtraction model). An empirical appreciation of the correlation between column properties and various analyte structural features can also be highly beneficial in this regard.

If you'd like to know more about your column sets, or help with choosing orthogonal phases for your screen or a particular method development then please contact me at tony@crawfordscientific.com

References

[1] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, and P.W. Carr, J. Chromatogr. A 961, 195–215 (2002).

SCREENING PLATFORMS FOR HPLC METHOD DEVELOPMENT

I'm often asked to help with the development of column 'screening' platforms and automated development systems. Whilst this covers a large amount of analytical science there are some common elements to this type of approach, perhaps the most important of which is column selection. Unsurprising given that 'selectivity' is the most powerful tool we have in chromatography and we all know that the best way to optimise selectivity is to choose the most appropriate stationary phase.

I want to expand upon this important topic with some more pragmatic reflections and advice which I hope will be helpful to those who seek to develop a more rapid and effective approach to column selection and HPLC method development.

We might seek a 'single' solution approach which will lead to a satisfactory method for every analyte encountered but, in my experience, this does not exist. Think of the type and variety of analyte molecular structure, relative hydrophobicity (solubility), functional group chemistry, physico-chemistry, stereo chemistry, etc. which you might encounter and this becomes obvious. Unless you work with a particularly narrow, and very stable, range of compounds. Further, the intended outcomes and attributes of the methods under development may also differ vastly and the requirements of a method for identification and/or quantification of impurities and degradants, will differ widely from a potency assay. These methods may also differ in the type of detector which is available or required, in order to obtain fit for purpose data. This may also impact on the HPLC method being designed.

That being said, there are approaches which can improve the effectiveness of method development for 'broad' categories of method types or analytes and it is worthwhile highlighting some considerations which will dictate approaches to the implementation of screening and automated development protocols.

Method Attributes

It is necessary to define the scope and attributes of the methods being developed in order to select appropriate screening methods and, crucially, to know when to stop optimisation. Most screening approaches follow the broad workflow;

- Screen a number of stationary phases based on the analyte types or application
- During screening – investigate the column selectivity with different organic modifiers, buffers (and perhaps pH values)
- Choose the most likely one or two combinations and further optimise the separation conditions (often with the assistance of optimisation software or calculated estimates of optimum gradient conditions / pH etc.)

Stage 3 of this process is often the most time consuming and suffers from the greatest degree of 'overwork'. In properly defining method attributes and having discipline in the approaches used for optimisation, a great deal of time can be saved in chasing lost causes before reverting to an alternative stationary phase. It's that age old problem - if you don't know where you are going, how do you know when you have arrived?'

SCREENING PLATFORMS FOR HPLC METHOD DEVELOPMENT

Typical method attributes, defined at the outset, may include;

- Favoured modifiers, eluent additives, or pH windows
- Favoured column manufacturers*
- Column dimensions
- Stationary phase particle size, pore size, and morphology
- Maximum runtime
- Maximum or minimum gradient time and limits of gradient slope (%B/min)
- Minimum resolution (including resolution of peaks on API compound tails
- Minimum peak efficiency (or maximum dispersion with UHPLC methods)
- Peak asymmetry
- Peak capacity (usually estimated using simple peak width measurements)
- Back pressure
- Repeatability & reproducibility

Many factors need to be considered in order to set a 'first intent' method specification as defined by some of the attributes above and might include;

- Equipment available within the developing lab and in the labs where the method will be implemented
- Availability of UHPLC equipment
- Requirement for mass spectrometric detection in-use
- Required throughput
- Likelihood for the method to require extremes of pH
- Complexity of the method – assay versus stability indicating versus impurity identification for example
- Equipment performance (gradient repeatability, extra column volume, pressure limits, detector characteristics etc.)
- Complexity of the resulting chromatogram and the requirement for good quantitative performance
- The need to validate the method and the legislative recommendations for validation criteria

* The preference for particular column manufacturers deserves a special mention. If the method is to be used in a wide range of locations or at geographically remote sites, choose columns from manufacturers with a wide ranging distribution network, who have a proven reputation for quality of production and perhaps a commitment to developing new and improved phases.

These are very important considerations, but should be tempered when considering the selectivity space being explored by the screening method, more of which later.

SCREENING PLATFORMS FOR HPLC METHOD DEVELOPMENT

Analyte and Sample Properties

Several general approaches to column & method screening platforms have emerged in the past 5 years or so, and many of these use the analyte and sample properties to influence the decision on which type of platform to implement. The properties typically considered include;

- Range of polarity (LogP/LogD)
- Structural similarity of the analytes
- Structural motifs which indicate a particular column type – highly aromatic / fluorinated / halogenated etc.
- Size / molecular weight range of analytes
- Ionogenicity of the analytes and range of pKa values involved
- Presence of conformers / isomers
- Chirality

The subject of chiral phase screening is not considered here as the topic is at least one blog entry on its own!

Popular screening approaches

As a result of the analyte survey, one might choose from one of several popular approaches including;

Maximised selectivity space screen (where log P/D and analyte chemistry are widely varying or the analyte characteristics are not known in advance)

4 – 6 columns which may typically include;

- C18
- C8
- PFP
- Phenyl (Phenyl Hexyl)
- Polar Embedded Phase
- Aq*
- Cyano

* Phase capable of use in 100% aqueous mobile phase and typically containing a polar endcapping or polar surface treatment)

Column characterisation databases might be employed. These allow the user to compare and contrast the various characteristics of each phase employed in order to maximise the selectivity space covered (although the list of 'preferred' column suppliers may somewhat limit the size of the available selectivity space).

There are many factors which influence the selectivity of a stationary phase and some of these are explored in various phase characterisation databases which I've previously written about:
Relating Analyte Properties to HPLC Column Selectivity - On the road to Nirvana

Another important factor is that not all columns are created equal, and it is important (perhaps even essential) to remember when choosing columns for screening that there can be huge variation in the selectivity of phases which are nominally of the same phase type.

Figure 1 shows the example of a 4 column wide selectivity screen where, for exactly the same nominal phases, the selectivity space (as indicated by the magnitude on the axes of the spider diagram as well as the general shape of the plots) is much broader when phases from different manufacturers are explored rather than choosing several phases from the same manufacturer. There are numerous reasons for these differences which arise from the different silica types, bonding chemistries, surface, and deactivation treatments etc. These variations should be kept in mind when selecting columns for screening. This is often taken into account during secondary column screening (see below) where variants of the same nominal phase chemistry are explored in order to further investigate the selectivity options of a column that has shown promise in a wide selectivity screen. In this instance, column selection for the secondary screen can be greatly influenced and helped by using the screening databases in order to select columns which show marked variation in the important selectivity characteristics.

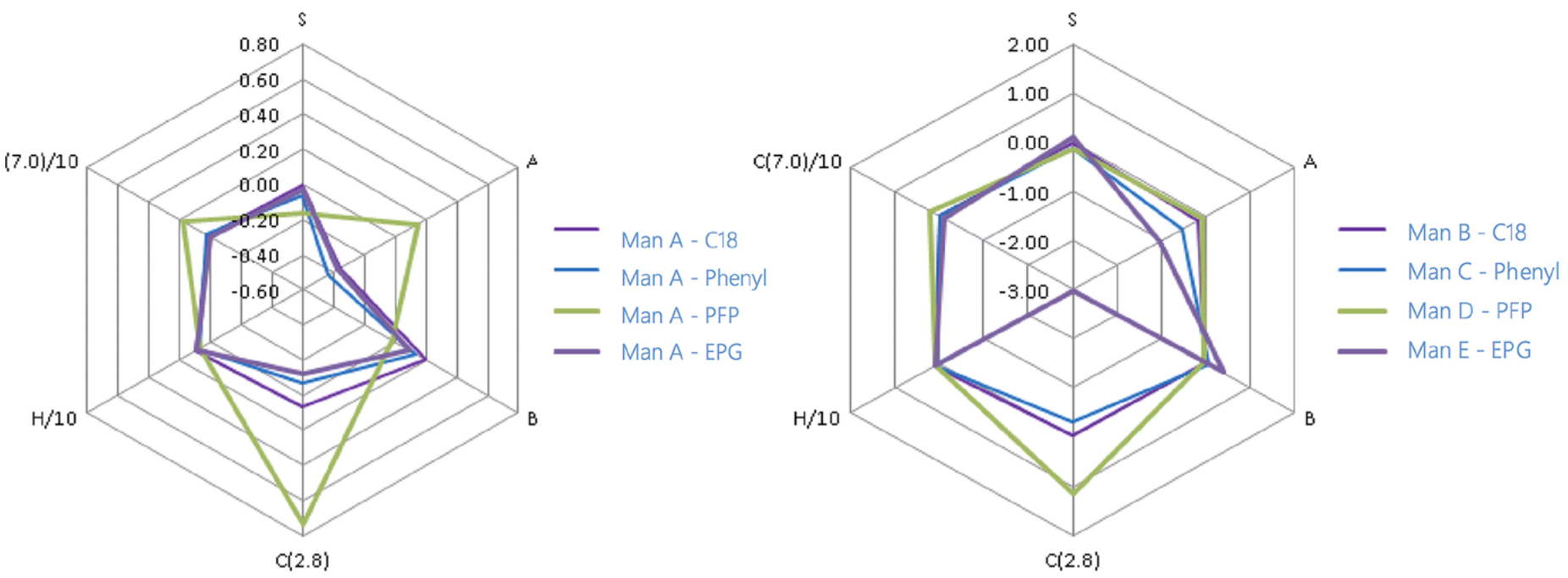


Figure 1: Spider diagram plot to indicate the relative selectivity space achieved when screening columns of nominally the same chemistry from the same manufacturer (Left) and different manufacturers (Right).

Specialist / Secondary screen (either after promising columns have been identified using the maximised selectivity screen or where analyte properties indicate a preference for a particular phase type)

3 – 6 columns which may include;

- Variants of C18 columns (different silica types / mono or bidentate / surface treatments / ligand density etc.)
- Variants of Phenyl Phases (diphenyl / biphenyl / phenyl hexyl / activated phenyl (containing oxygen atom in the alkyl spacer etc.)
- Phases for conformer / isomer separations (high density C18 phases, PFP phases and C30 phases)

And a host of other phase variant combinations

Polar screen (reversed phase) (where poor retention is seen in the maximised selectivity screen or where analyte structure and physical-chemical data indicate the requirement for more polar interaction)

3 – 6 columns which may include;

- Aq type phases
- Polar Embedded phases
- Mixed mode phases (acidic, basic or zwitterionic functionality)
- Diol phases
- Amide phases

Polar Screen (HILIC or Aqueous Normal Phase Mode) (where poor retention is seen in the maximised selectivity screen or where analyte structure and physical-chemical data indicate the requirement for more polar interaction)

3 – 6 columns from the following broad classifications;

- Diol
- Polyol
- Amido
- Amino
- Ionic / zwitterionic
- Cyano
- Carbamate

Each of these screens may use different mobile phase combinations to fully explore the selectivity space with each stationary phase. There are obviously a wide range of mobile phase variants which can be used to investigate the selectivity variations.

These are a few of the more popular combinations;

- Screen using both methanol and acetonitrile – they selectivity differences can be very large
- Screen over a wide range of modifier concentrations to ensure all analytes are eluted
- 0.1% TFA v/v is a popular choice for low pH work (pH ~ 2.1)
- 0.1% Formic Acid (FA) v/v gives a pH of around 2.7
- Note that both of the above have virtually no buffering capacity
- Ammonium Formate (10mM) pH 3.8 and Ammonium Acetate (10mM) pH 4.8 are useful buffers for exploring lower pH range is a useful mobile phase depending upon the analyte chemistry (if known) especially where mass spectral detection is used
- To explore the mid pH range – 10mM Ammoniuim Bicarbonate works well
- Ammonia (Ammonium Hydroxide) at pH 9 is useful for exploring the separation at high pH (note that the column should be suitable for high pH work or column dissolution will quickly occur!)

It's impractical to screen using all of the stationary phases with all of the eluent combinations above; therefore, if the analyte cheistry is unknown, the choice should be based on your typical applications or a screen across a wide pH range.

Computer aided optimisation software can be used to identify the most promising combinations of stationary phase with mobile phase composition or, if necessary, one might eyeball the separations to pick out the candidate combinations for further optimisation. From this point, the method will be further optimised and the best way to do this is to again use computer aided optimisation software or to use simple calculation to estimate the optimum gradient time, starting and ending composition.

For pH optimisation, if you have no access to computer optimisation software, then a series of experiments at varying mobile phase pH can be undertaken to select the optimum value and obtain the best separation selectivity. In practical terms, the pH 'window' explored can be as little as 3 pH units, depending on the results from the initial screen.

As mentioned above, the key to automated or rapid screening and development approaches is knowing when to stop exploring a particular mobile phase or stationary phase combination. Your protocol should be clear — if no satisfactory separation has been obtained, switch to the next most promising stationary phase and mobile phase combination or switch to a different screen altogether.

Conclusions

Screening experiments in HPLC should be designed to explore the maximum selectivity space possible — at least for the initial screen. Columns should be screened using various combinations of organic modifier and pH in order to identify the best combination. If a suitable combination cannot be identified from the initial screen, or to 'fine tune' a separation, a secondary screen can be employed to investigate subtle differences between the separation performance of columns with, nominally, the same chemistry. A secondary screen can also be utilised to investigate alternative approaches, such as the use of columns designed for the retention of more polar analytes.

THE SHAPE OF THINGS TO COME – POSSIBLE CHANGES TO USP CHAPTER <621>

One of the major limitations to the 'allowable' changes to USP HPLC methods was the restriction on alterations to the gradient profile of a method in order to meet system suitability requirements (USP 40 - NF35 (Supplement 2) General Chapter <621>). So how are they addressing this?

Proposed Changes to USP <Chapter 621> on 'allowable Changes'

The *C188676 (43(5) Harmonisation Stage 4 General Chapter 621)* was recently sent out for discussion and comment. This document contains proposed updates to the 'allowable change' regulations — some of these are very interesting! For reference, I've tabulated the current regulations and those which are being proposed;

Current Guidelines

Variable	Allowable changes	Isocratic	Gradient
Column Chemistry	Stationary phase may be changed within the same 'L' classification	Yes	Yes
pH	±0.2	Yes	Yes
Buffer Concentration	±10%	Yes	Yes
Mobile Phase	The lesser of ±30% relative or ±10% absolute for minor components	Yes	Yes (but not recommended)
UV Wavelength	0, but error ±3 nm OK	Yes	N
Column length and particle size	L/d_p = -25% to +50% or N = -25% to +50%	Yes	N
Column diameter	OK, if linear velocity is constant	Yes	No
Flow rate	OK if linear velocity is constant, plus an additional ±50%, with exceptions	Yes	No
Gradient profile	Changes to initial isocratic time of the gradient only	-	Yes
Injection volume	OK, if performance is OK	Yes	Yes
Column temperature	±10°C	Yes	Yes

Recommended method for adjusting flow rate to maintain constant linear velocity;

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

THE SHAPE OF THINGS TO COME –
POSSIBLE CHANGES TO USP CHAPTER <621>

Guidelines under discussion

Variable	Allowable changes	Isocratic	Gradient
Column Chemistry	No change of the physicochemical characteristics of the stationary phase permitted, i.e., chromatographic support, surface modification, and extent of chemical modification must be the same*	Yes	Yes
pH	±0.2	Yes	Yes
Buffer Concentration	±10%	Yes	Yes
Mobile Phase	The lesser of ±30% relative or ±2% absolute for minor components	Yes	Yes (but not recommended)
UV Wavelength	0, but error ±3 nm OK	Yes	No
Column length and particle size	L/dp = -25% to +50%	Yes	N
or	OK, if linear velocity is constant	Yes	No
N = -25% to +50%	Yes	No	No
Column diameter	OK, if linear velocity is constant	Yes	No
Flow rate	OK if linear velocity is constant, plus an additional ±50%, with exceptions	Yes	No
Gradient profile	Adjust each gradient segment according to Equation (1) below **	-	Yes
Injection volume	OK, if performance is OK	Yes	Yes
Column temperature	±10°C	Yes	Yes

* A change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided the other column chemistry requirements are met.

Recommended method for adjusting each gradient segment;

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

** Composition/gradient: Adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- System suitability requirements are fulfilled
- Principal peak(s) elute(s) within ±15% of the indicated retention time(s); this requirement does not apply when the column dimensions are changed
- Final composition of the mobile phase is not weaker in elution power than the prescribed composition

As you can see, there are proposed changes to the eluent composition, further qualification of changes to the stationary phase, and guidance on allowable changes to the gradient profile. This last one is critical.
It's worth re-iterating at this point that, at present, these are merely proposals — they may never be implemented. However, it's worth understanding what they could mean in practice in the likelihood that they are eventually enacted.

Lanzoprazole Impurity Determination – optimised using original and proposed allowable changes

Starting with the proposed adjustments to the gradient profile, we can use the USP method for Lanzoprazole (and its impurities) alongside an allowable change in column dimensions and flow rate to explore what an 'adjusted' method may look like.
The USP method conditions for the analysis are;

Column:	L1 150 x 4.6mm, 5µm
Injection:	40 µL
Detection:	UV 285 nm
Flow Rate:	0.8 mL/min
Temperature:	Ambient (controlled 35°C)
Mobile Phase (v/v)	
Solution A:	100% Water
Solution B:	Acetonitrile, water, triethylamine (160:40:1) adjusted to pH 7.0 with phosphoric acid.

Gradient Table;

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

We wish to translate the method to the following column - L1 50 x 2.1mm, 1.9mm. In this case the stationary phase will remain unchanged. This change is allowed under the current guidance as the L/d_p ratio change is ~12%.
The flow rate translation in order to maintain constant linear velocity is;

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

$$F_2 = 0.8 \times \left[\left(2.1_{c2}^2 \times 5_{p1} \right) / \left(d4.6_{c1}^2 \times d1.9_{p2} \right) \right]$$

$$F_2 = 0.44\ mL/min$$

THE SHAPE OF THINGS TO COME –
POSSIBLE CHANGES TO USP CHAPTER <621>

To illustrate the changes to the gradient table – we have translated the first section of the original gradient;

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

$$t_{G2} = 40 \times (0.8/0.44)[(50 \times 2.1_{c2}^2)/(150 \times 4.6_{c1}^2)]$$

$$t_{G2} = 5.06\ (rounded\ to\ 5.1\ mins)$$

So, the gradient table for the new method would be;

Time (mins)	Solution A (%)	Solution B (%)
0	90	10
5.1	20	80
6.3	20	80
6.4	90	10
7.6	90	90

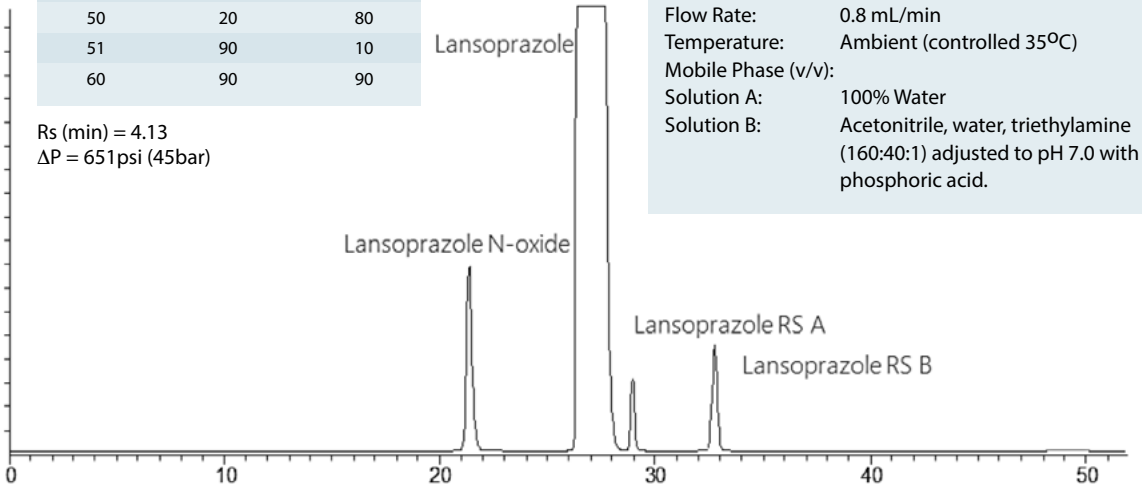
The resulting chromatograms for the two methods would look as follows;

Original Method

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

Rs (min) = 4.13
ΔP = 651psi (45bar)

Column: L1 150 x 4.6mm, 5µm
Injection: 40 µL
Detection: UV 285 nm
Flow Rate: 0.8 mL/min
Temperature: Ambient (controlled 35°C)
Mobile Phase (v/v):
Solution A: 100% Water
Solution B: Acetonitrile, water, triethylamine (160:40:1) adjusted to pH 7.0 with phosphoric acid.

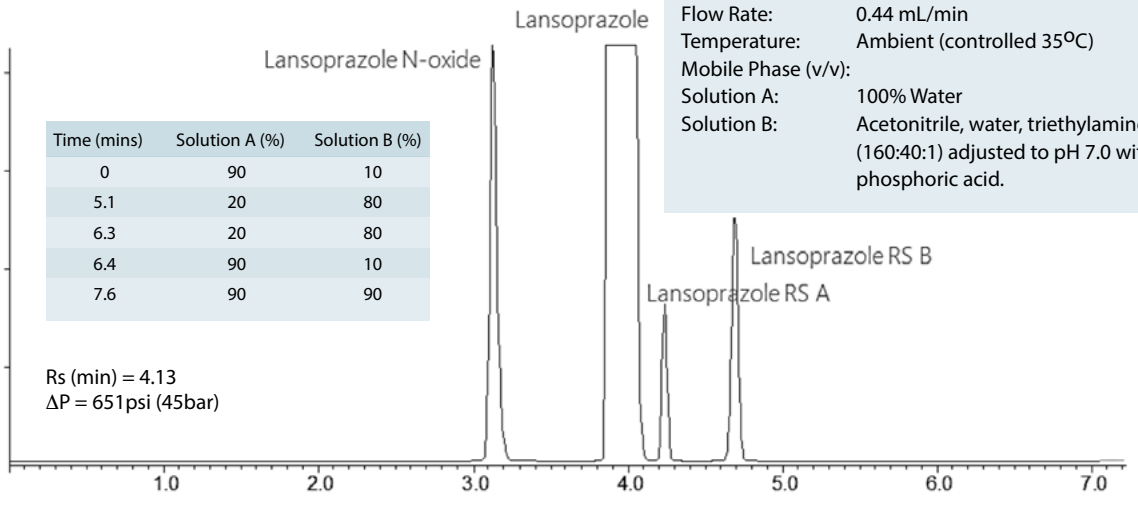


New Method

Column: L1 50 x 2.1mm, 1.9µm
Injection: 2 µL
Detection: UV 285 nm
Flow Rate: 0.44 mL/min
Temperature: Ambient (controlled 35°C)
Mobile Phase (v/v):
Solution A: 100% Water
Solution B: Acetonitrile, water, triethylamine (160:40:1) adjusted to pH 7.0 with phosphoric acid.

Time (mins)	Solution A (%)	Solution B (%)
0	90	10
5.1	20	80
6.3	20	80
6.4	90	10
7.6	90	90

Rs (min) = 4.13
ΔP = 651psi (45bar)



For those with 600Bar HPLC systems or better, the second chromatogram offers a much higher throughput option, while still complying with the proposed guidelines.

Stumbling blocks on new allowable changes to column stationary phase

The other 'major' change which concerns some further clarification on allowable changes to stationary phases requires attention.

The guidance under consultation states;

No change of the physicochemical characteristics of the stationary phase permitted, i.e., chromatographic support, surface modification, and extent of chemical modification must be the same; a change from totally porous particle (TPP) columns to superficially porous particle (SPP) columns is allowed provided these requirements are met.

As it stands, this is a little ambiguous to be useful.

What would constitute a change in the chromatographic support if swapping from a TPP to an SPP is allowed?

What now constitutes a change in surface modification? Does this refer to the bonded phase ligand, any endcapping reagents, or surface area? If so, there will be little scope for changing from one manufacturer's phase to another – even if they are considered to be the equivalent. If the 'extent of chemical modification' refers to the carbon load – this is a very crude measure of retentivity and, in some cases, selectivity.

The wish to tighten the guidelines ought to be applauded as, of the almost 200 commercially available columns in the L1 category, the chromatographic behaviour within this group can change drastically.

A better approach to stationary phase allowable changes?

Perhaps this is the ideal time to place more focus on the selectivity databases which exist, which can help to demonstrate the 'equivalence' of selectivity of two different phases.

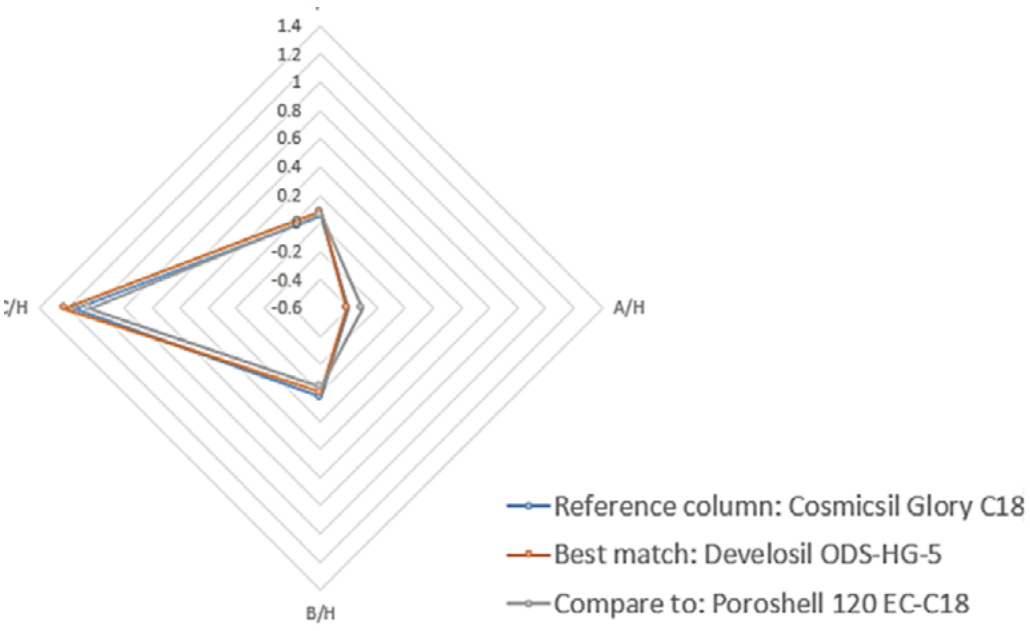
At Crawford Scientific, we use a model based on the PQRI selectivity database (Hydrophobic Subtraction Model, HSM [1]), alongside some other key metrics to help clients understand the equivalence of reversed phase HPLC stationary phases.

Alongside the HSM model, we use a linear regression model for measuring the equivalence of each selectivity characteristic [2,3] and other checks to demonstrate the theoretical equivalence of columns. We have found success with this model and some examples of reports and graphical comparisons are shown below for reference.

The Fs comparison number is the PQRI / HSM similarity factor, the total equivalence metric is based on a regression analysis of the differences in each HSM factor between the two columns — each reduced by the hydrophobicity. The Q comparison predicts the likelihood of critical resolution being maintained from the original separation. The model is built to account for the presence and strength of acids and bases within the analyte mix, as well as the eluent pH.

THE SHAPE OF THINGS TO COME –
POSSIBLE CHANGES TO USP CHAPTER <621>

Column Matching ra	Column name	Fs	Q comparison	Equivalence	Total Equivalenc	USP	Phase Chemistry
Reference column	Cosmicsil Glory C18	0	resolution maintained	Equivalent	0.000	L1	C18
1	Develosil ODS-HG-5	0.112	resolution maintained	Equivalent	0.105	L1	C18
2	Poroshell 120 EC-C18	0.265	resolution maintained	Equivalent	0.121	L1	C18
4	Discovery C18	0.314	resolution maintained	Similar	0.165	L1	C18
13	Hypurity C18	0.492	resolution maintained	Similar	0.168	L1	C18
3	Develosil ODS-UG-5	0.294	resolution maintained	Equivalent	0.198	L1	C18
18	Xtimate C18	0.534	resolution maintained	Similar	0.204	L1	C18
12	XBridge C18	0.459	resolution maintained	Similar	0.209	L1	C18
6	ProntoSIL 200 C18 H	0.361	resolution maintained	Similar	0.225	L1	C18
5	ProntoSIL 200 C18 H	0.361	resolution maintained	Similar	0.225	L1	C18
7	Venusil XBP C18(L)	0.379	resolution maintained	Similar	0.267	L1	C18
8	Zodiac C18(1)	0.441	resolution maintained	Similar	0.287	L1	C18
38	Alltima HP C18	0.766	resolution maintained	Similar	0.299	L1	C18
10	XTerra MS C18	0.451	resolution maintained	Similar	0.300	L1	C18
15	Hypersil Beta Basic-18	0.509	resolution maintained	Similar	0.304	L1	C18
98	Acquity UPLC BEH C18	1.129	resolution maintained	Similar	0.318	L1	C18
23	ProntoSIL 120 C18 H	0.615	resolution maintained	Similar	0.324	L1	C18



This approach would be far more applicable in defining the selectivity equivalence of columns and would be much less ambiguous (some might say more scientific) than the current proposed wording.

However, maybe the intention is that the stationary phase cannot be changed? I would find this very restrictive in my own work, and I'm sure many of other chromatographers would agree.

Tighter restrictions on changes to isocratic eluent composition

We should also briefly mention the narrowing of the allowable adjustment to the absolute percentage of minor mobile phase constituents. In some ways the new proposed maximum variation of 2% absolute is more sensible and is welcomed, as the previous allowable range could be too wide when the minor component is present at larger percentages in the isocratic mobile phase.

As an example;

50:50 Aq : Organic eluent - 30% change to either component would be 15% alteration of either aqueous or organic (35 - 65%A or B) so the 10% absolute limit applies and the allowable range is 40:60 or 60:40.

This would result in a large change in retention (by a factor of around x3!)

The new guidelines would limit this change to 48:52 or 52:48, as a worst-case scenario, which I feel is much more appropriate.

Conclusions

It's excellent to see that compendial authorities are considering updating 'allowable change' regulations, and the updates to allow changes to gradient profiles certainly open up a whole new world. Perhaps it wasn't the initial intention of the allowable changes, these new recommendations would certainly allow many outdated USP methods to be brought up to date somewhat.

However, there are some reservations around the new restrictions on allowable changes to stationary phase chemistry. We will need to wait for further clarification before fully endorsing the new recommendations.

References

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THERE'S MORE TO HPLC METHOD TRANSFER PROBLEMS THAN DIFFERENCES IN SYSTEM DWELL VOLUME!

The overriding majority of articles on problems with the Technical Transfer of HPLC methods ultimately focus on differences between HPLC dwell volumes.

I acknowledge that this issue needs to be considered, especially given the number of different HPLC and UHPLC systems, the high or low pressure mixing variants of each, and the likelihood of needing to transfer methods between them. Indeed, this issue has also been widely recognised by instrument vendors, some of whom now offer the ability to adjust the 'apparent dwell volume' of the system within the software / firmware in order to match that of the originator lab instrumentation.

However, as the title suggests, there are many more issues which can cause problems in the transfer of HPLC methods. I wanted to highlight some common issues that come across my desk, in the hope that it will help you avoid these problems in your own practice.

What follows is general guidance, and whilst I haven't illustrated each item with a specific example, I've referenced further resources where applicable.

Check the grade and make of solvents used. There are, for example, many different grades of acetonitrile and methanol, stabilised or unstabilised THF, and matching the solvent quality used for eluent preparation and sample diluent between originator and receiving lab is very important. Try to include the manufacturer name and part number in transfer protocols where necessary.

Ensure that the sample preparation section of the protocol is fully explicit. Dissolving a solid in 50:50 acetonitrile:water or dissolving the solid in acetonitrile, followed by the addition of the same volume of water, may produce very different dissolution results with samples of varying solubility.

Be very explicit with the nature of the eluent preparation. 0.1% v/v and 0.1% w/v solutions of TFA produce markedly different solution pH. Where the eluent pH is a critical variable, be sure to say so and indicate that care must be taken to achieve the desired value and, where possible, an acceptable range. When preparing eluents gravimetrically is critical, make this explicit.

I've seen a rise in the popularity of buffering sample diluent solutions in order to improve the robustness of methods where analyte pKa values fall at the lower or upper end of the pH range and where 'generic' eluents such as 0.1% TFA, 0.1% Formic acid, or Ammonia are used to achieve the desired eluent pH. This won't really solve the the lack of buffering as the sample diluent mixes with the eluent, but it can help. However, it is more unusual, and the originating lab should make an effort to explain any unusual aspects of their methods in the transfer protocol or supporting documents.

THERE'S MORE TO HPLC METHOD TRANSFER PROBLEMS THAN DIFFERENCES IN SYSTEM DWELL VOLUME!

So many method transfer problems are associated with sample vials. Ensure that you include the part numbers for the vial, caps and inserts that are used for the method. Vials of lower quality may contain residues which could interfere with chromatography, may have adsorptive sites which retain certain analytes, and different septa materials may contain different extractable compounds — again which can interfere with chromatography. The vial closure — whether snap cap, screw cap or crimp cap — can make a difference to the loss of volatile components and also the ingress of air into the vial headspace once the septum has been pierced for the first time.

I've known instances where results between sites have varied due to the fill level of the vials, typically due to the amount of air (oxygen) available for analyte oxidation, change of solution pH through ingress of CO_2 from the vial headspace or loss of a volatile into the vial headspace. So state the typical vial fill volume if you know this to be an issue.

If you filter the mobile phase or the sample solution, you need to be very explicit about the filter type used (including part numbers where appropriate). Different filter materials will have different absorptivity characteristics and should not be varied. Further, the surface area of the filter may make a difference to the filtering efficiency or absorptivity and should also be matched. This usually means using a filter of the same nominal 'volume'. Even maintaining the same filter material and size may not be good enough as manufacturing differences in the plastic housing materials may again mean different extractables or contaminants are released into the sample solution — again, potentially interfering with chromatography.

There are often measures that we take inadvertently to prevent issues from occurring and one should give careful consideration to each step of the process to consider where variability might occur. This could be something as simple as the type of cap used on the eluent reservoir. When I say cap, I mean lab film (parafilm), scrunched up blue roll, old solvent Winchester caps, the original caps supplied by the manufacturer, or some retro-fit caps which include a charcoal filter to protect workers from organic solvent vapours. Any differences in the eluent reservoir cap or closure can potentially influence the ingress of CO2 or the loss of volatile components. These issues can potentially change the eluotropic strength of the eluent and the selectivity of the separation through changes in eluent pH. Consider including a photograph of the system on which the method was validated to provide visual cues for the receiving laboratory.

Consider using constant ionic strength eluent programs. This can be a life saver in terms of baseline drift, variability, and/or noise during gradient analysis. Ultimately, this produces a much more transferable method. Note that the simplest way to achieve this it to use a quaternary pumping system, where three channels are typically used as shown in the Table below. A typical (simple) table for a ternary gradient system which produces a constant 20mM ionic strength is shown below to highlight the principle;

Time (min)	Pump Channel A (%)	Pump Channel B (%)	Pump Channel C (%)
0	75	5	20
20	15	65	20

A = water / B = MeOH / C = 100 mM buffer solution

THERE'S MORE TO HPLC METHOD TRANSFER PROBLEMS THAN DIFFERENCES IN SYSTEM DWELL VOLUME!

Pump compressibility settings can affect the reproducibility of the gradient. Ensure that the method contains the correct compressibility settings (especially where isocratic or binary pumps are used) and that the receiving lab is encouraged to set these correctly, as they are mostly overlooked.

There are various autosampler parameters which may vary unconsciously between laboratories and again these details should be explained as fully as possible in the transfer protocol. Depending upon the manufacturer and operating principle of the autosampler these variables may include;

- The composition of the needle wash solvent
- Whether the needle wash bottle / vial is capped or uncapped
- If a flush is used to wash the outside of the needle
- The number of flushes or sample 'pumps' / 'aspirations' of the injection syringe during sample aspiration as well as pre and post-injection washes
- The insertion depth of the needle into the sample vial (especially where a biphasic sample solution or supernatant above solid centrifugate is involved)

The wash program of the autosampler can be particularly important when considering the quantitative reproducibility of the method

The column re-equilibration program is typically mentioned in the method gradient timetable; however, I wonder how many of us consider the impact of the system dwell volume (apologies – I promised not to mention it!) on the system equilibration time (volume)? Much is written on the ability of modern HPLC systems to reproducibly re-equilibrate, even if this is not a 'fully equilibrated' state. All I can state is that I've seen issues with resolution between critical peak pairs in a separation which was directly attributable to differences in initial eluent composition created by not adjusting a gradient table between systems with different dwell volume.

Column and eluent temperature can also be the cause of variation, especially where the fundamental heating or cooling principle differs between instruments (for example when transferring from an instrument with Peltier heating to an instrument with forced air heating). The position of the column within the column compartment may produce local differences in the temperature gradient across the column (there will always be one!). Further, the volume of the heating unit through which the eluent passes prior to entering the column for pre-heating may differ between instruments (or, indeed, within the same instrument) and this should be established and matched as closely as possible. I've seen many issues associated with transfer between instruments in which one has an eluent pre-heating facility and the other doesn't. Again, these seemingly innocuous details have the potential to be make or break differences for difficult / susceptible separations.

There are a host of detector settings which can cause issues with method transfer, especially when transferring between diode array detectors from different manufacturers.

THERE'S MORE TO HPLC METHOD TRANSFER PROBLEMS THAN DIFFERENCES IN SYSTEM DWELL VOLUME!

As a minimum, consider;

- Matching the collection rate as well as any post-acquisition data filtering rates
- Maintaining the spectral resolution between detectors (actually as a means of controlling the inherent signal to noise ratio between instruments). This is typically controlled by altering the slitwidth settings of the instrument (depending upon manufacturer) and some manufacturers provide a 'resolution' setting which controls the amount of data which is retained from the photodiode array (post-acquisition filtering)
- The bandwidth setting for each discrete wavelength being acquired should be explicitly stated otherwise major differences in signal intensity may occur between the originating and receiving laboratory. Similarly, if a reference wavelength is used, both the wavelength and bandwidth of the reference signal should be explicitly stated.
- It is helpful to state the flow cell volume of the instrument used in the originator lab, as any significant variation between instruments can have a huge impact on efficiency and sensitivity.

Finally, the integration algorithm used for data processing can have an impact on the success of the method transfer. Where possible, the integration algorithm table (integration events table) should be included with the method, especially where the separation is complex and contains any of the following features;

- Drifting Baselines
- Split / shouldered peaks
- Poorly resolved / bunched peaks
- Badly fronting or tailing peaks

Differences between the integration processes from different manufacturers can be the cause of much frustration. Often, compliance with system suitability specification limits does not guarantee trouble free operation when 'real' samples are being analysed. Testing rejected or retained batches is always encouraged so that the integration parameters from the receiving instrument / computer data system can be tuned to match the performance of the originator lab.

Conclusion

Differences in dwell volume between pumping systems can give rise to method transfer issues; however, there are a host of other issues that need to be considered when troubleshooting method transfer problems. These can be related to both the chemistry of the separation, and the instrument settings used by the originator and receiving laboratories. These should all be considered during the investigation.

With more esoteric factors, problems often arise from the assumption that 'our practice is the same as their practice' and factors such as the quality of solvents used, the method for measuring and adjusting pH, and more minor instrument settings can often be insidious contributory factors.

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He has helped thousands of budding chromatography method developers using his own experiences and insights, working with students to improve knowledge and understanding of chromatographic processes and their application.