Chromatography Focus

THE GATEWAY TO DIFFICULT SEPARATIONS, SELECTIVITY OR EFFICIENCY?

Separations scientists are facing an increasing number of HPLC Columns that are offering either orthogonal selectivity or extremely high plate numbers (efficiency) compared to those available say, 5 years ago. When faced with new separations that need to be performed, and in many cases these are getting more complex, which route do they opt to pursue at the method development stage?

Much recent commercial activity has seen the promotion of ultra high efficiency columns, either by virtue of small particle sizes or use of elevated temperature (or both) as an option but we also have some unique novel chemistries, which are designed to offer alternate selectivity to the 'traditional' end-capped C18 (L1 designation in the US Pharmacopoeia). In this two-part article we shall examine the advantages of each route along with the shortfalls and show examples of successful separations.

At the recent HPLC 2008 Symposium Professor Klaus Unger [1] gave a talk which was a 'Where are we now' review of packed columns and monolith technologies. He concluded that although advances in efficiency and different technology indeed gave some advantages, perhaps the time had come to revisit the increasing range of selectivities of the stationary phase as a way of achieving the desired separation.

RESOLUTION, SELECTIVITY AND PLATE COUNT

All of the above parameters are related in the Resolution equations [2] defined as

Resolution *(Rs)*: Ability of a column to separate chromatographic peaks;

$Rs = (t_{R2} - t_{R1})/[(w_{b1} + w_{b2})/2]$, where t_{R2} and t_{R1} are the retention times of the two peaks and w_b is the baseline width of the peaks. It is usually expressed in terms of the separation of two peaks. A value of 1 is considered to be the minimum for a measurable separation to occur and to allow good quantitation. A value of 0.6 Is required to discern a valley between two equal height peaks. A value of 1.5 is considered sufficient for baseline resolution for two peaks of equal height. Values of 1.7 or greater are generally desirable for rugged methods.

Resolution equation: Also called the general resolution equation and the Purnell equation;

$R = 4\sqrt{N}[(\alpha-1)/\alpha][k/(1+k)]$, where N is the efficiency, α is the separation factor and k is the retention factor.

The resolution equation therefore contains a Selectivity term $(\alpha-1)/\alpha$, Retardation term $k/(1+k)$ and a Dispersion term $\sqrt{N}$ [3] – any of which can affect the resolution and hence the potential usefulness of the separation of the components (often more than 2!).

In order to obtain optimum selectivity then the selectivity coefficient that characterises the distribution equilibrium of two solutes a and b between the stationary and mobile phases must be grater than 1, ideally up to a value of 1.2. There are various ways in which the coefficient may be optimised to give orthogonal selectivity and preparation of the stationery phase is that most commonly utilised (mobile phase composition, temperature, mixed (or multidimensional modes)) amongst the others possible, as it encompasses several steps from support choice to conditioning of the bonded phase with which to impart the 'magic ingredient' to expand the selectivity possibilities.

When trying to separate with low alpha values it is tempting to think that just a few extra plates are what are required. How many columns are thrown out after failing an SOP test that has been set for pre-determined Rs when the maximum attainable with the method is only just greater. If you have the opportunity to try a more selective phase you should explore the possibilities.

From a manufacturers point of view it is more than just showing how innovative they can be in producing a phase with alternate selectivity but it must have demonstrable advantages over existing products, be stable under likely mobile phase conditions and have a controllable manufacturing process to give reproducible performance. At the support stage they can control the physical characteristics such as surface area, porosity, pH and in the case of silica, the different types of silanols on the surface. At the modification stage the chemical characteristics can be controlled such as % carbon (retention) and orientation on the surface – all of which will affect the selectivity and give it uniqueness.

As with all stationary phases the choice of support can have significant impact on the bonded phases even more so with the availability of different types of supports as well as the seemingly ubiquitous Type A (first generation typically made from sodium silicate sols) and Type B silica (aka 'high purity' silica with lower residual metal content), we also have the more recent silica hybrid particles, Porous Graphitic Carbon and polystyrene/divinyl benzene to consider.

Time and space constraints prevent too much discussion but we can consider the Type A and Type B silicas, and as shown in *Figure 1*, there are different types of silanols present in differing concentrations

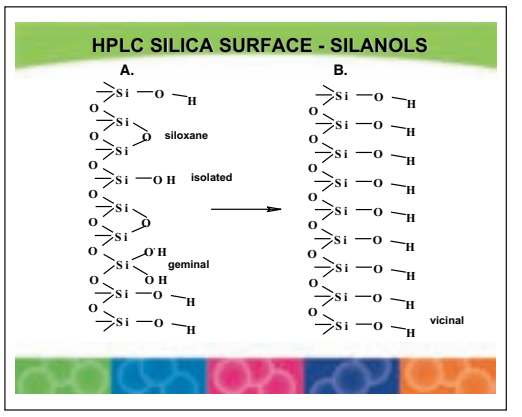

Figure 1. Surface Silanols

Each class of silanol has its own effect on the separation and even after undergoing extensive reactions with C18 chains to cover a large area of the surface, the presence of 'unreacted' silanols was always viewed as a negative aspect of Columns with that particular characteristic

Table 1 overleaf summarises some of the variables that can affect selectivity, some more than others, to give an overview of how manufacturers have a range of choices when developing new `alternate' selectivity columns. For the sake of brevity I have limited this to silica since this is the most commonly used support.

In the last 10 years or so attention had been paid by manufacturers to develop mainly C18 phases where the surfaces of the silica underwent some degree of 'deactivation' prior to bonding to limit the tendency of residual silanols, particularly isolated acidic ones, causing tailing peaks with basic compounds.

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Table 1. Impact of typical variables on stationary phases.

Variable	Range/Options	Impact
Surface Area	100 - 550 m^2g^{-1}	Determines retention
Mean pore size	60 - 300Å	Limits the size of bonded phase chemistries which can be chemically bound onto the surface
Particle size	1 - 10µm	Limits efficiency of column.
Metal content	Type A: upwards of 5,000 ppm Type B; usually <10ppm total	Lower metal concentrations favour purer separations but specific ions can be beneficial to certain separations.
Silanol types	Geminal, Vicinal, Isolated, Acidic, Siloxane	Silanols remaining after bonding can affect peak shape of acids or bases
Surface pH	3 -7.5	Indicates nature of silica surface and type/nature of silanols present
1st Bonding	C18, C8, C4, C1, Amino, Cyano, Diol, Phenyl, Di-Phenyl, Fluorine containing compounds, Polar embedded groupings, Amide linked groups.	Should determine primary mechanism of the separation and minimising effect of silica surface on certain analytes
Bonding Density	1 – 6 µmols/m^2	Low values indicate 'thin'coverage of silica surface and increased chances of unreacted silanols partaking in the separation – unless this is of benefit.
End Capping	TMS, Polar Bondings, Ion exchange, Normal phase bondings. Controlled 'naked'silanols.	Ability to bring second mechanism into the separation. Useful to tailor make selectivity in mixed polarity samples

The drive was usually more focussed on the stability of the bonded phase under mobile phase conditions where first generation silica which usually had some residual silanol activity 'peering through' the C18 coating and causing tailing issues with basic, acidic and chelating compounds. Not that this was always a bad trait to have since some non, or poorly end capped first generation silica exhibited mixed mode properties due to the unreacted silanols which made them suitable for certain compound types. With the mixed interactions it was possible to achieve additional retention of polar groups and obtain separations that were impossible to achieve with end-capped phases and certainly not achievable with base deactivated high surface loading materials such as Inertsil and Kromasil at the time. Unfortunately reproducibility could be difficult to maintain.

Catecholamines are a good example of where the underlying activity of the silica can have a great effect on the selectivity. Clinical assays are still carried out using Type A silica phases, such as Spherisorb ODS2, Techsphere and Hypersil ODS simply because the early eluting Adrenaline and Noradrenaline cannot resolve on a deactivated phase and the highly aqueous mobile phases are not compatible with the high hydrophobicity. Not withstanding the fact that there are serious problems with using such active silicas but it is often a case of when not broke, don't try to fix it.

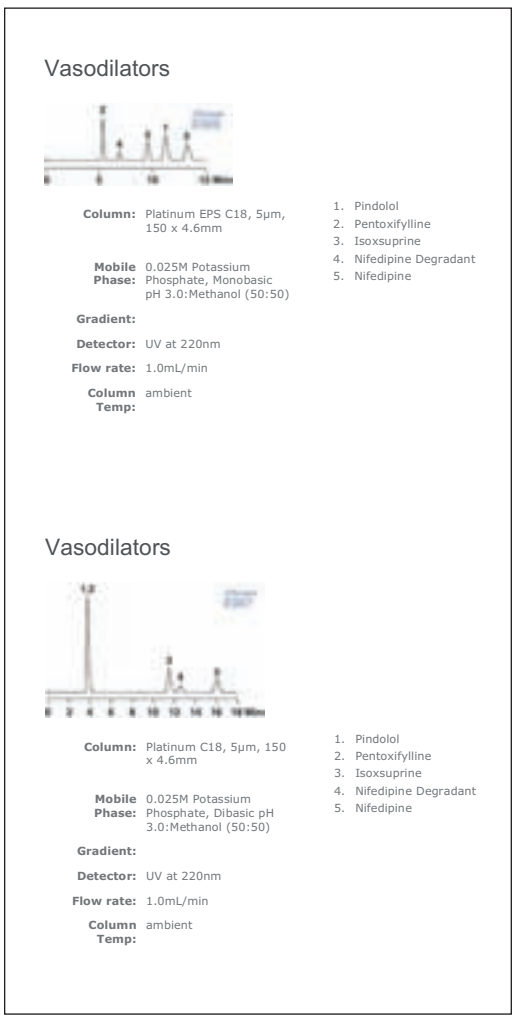

Figure 2. (Platinum Vasodilators) - Selectivity changes due to Polar end capping

An example showing orthogonal selectivity to standard C18's by utilising these mixed mode interactions is shown in *Figure 2*. Fundamentally it is base deactivated silica with low metal content and a uniform layer of vicinal saloons with a low coverage bonding. In the EPS format (Extra Polar Selectivity) it is non-endcapped but because there are no acidic silanols (or very low level) peak shape with bases is fine. However due to the mixed mode mechanism, RP and NP, polar molecules (Pinedolol and Isoxsuprine) are retained longer and can shift position relative to a standard C18.

In addition, pH effects can be used to move peaks around so the chromatographer has more scope to improve the chromatography. Another benefit is that with a low C18 (or C8) coverage, retention of non-polars in mixed polarity samples is much less than normal so often isocratic elution can be used instead of gradient.[4,5]

Sticking with the C18 phases since they are by far the largest used phase, more attention has recently been paid to controlling the surface of the silica prior to bonding and then changing the end capping chemistries to impart another, different, mechanism to the phase. This results in different selectivity compared to older C18`s coated with Tri-methyl silane (TMS) or similar hydrophobic groups.

In *Figure 3 (a) and (b)*, the end capping moiety is changed (both contain polar groups which are designed to affect retention and hence selectivity) and so the retention of the very polar compounds, DOPAC and 5-HIAA has been dramatically affected.

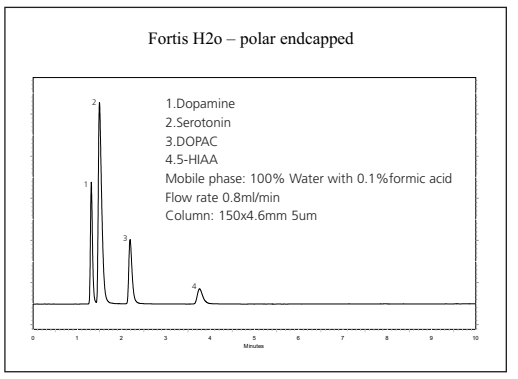

Figure 3a. H2o Cats vs dEV) - Selectivity with different polar capping chemistries

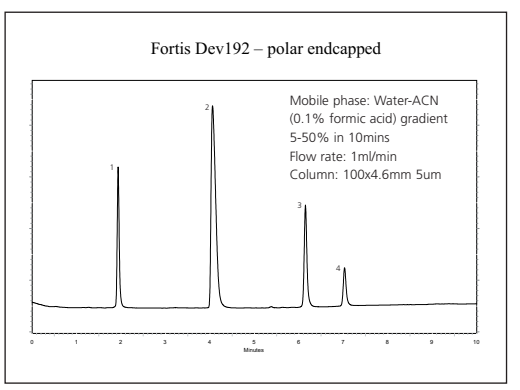

FIgure 3b. (H2o Cats vs dEV) - Selectivity with different polar capping chemistries

An interesting comparison using a common base silica (Type B) but different bonding chemistries and capping reagents is shown in *Figure 4*. In this case the base silica is the Hypersil GOLD support. A generic mobile phase was used (A; water + 0.1% formic acid, B; Methanol + 0.1% Formic Acid, 20-50% B gradient over 15 mins). All columns were based on 5µm particles in 150x4.6mm, flow was 1 ml/min and UV@280 nm was used. Analytes were from the Catechin group of flavenoids, and Chemistries looked at were a long alkyl chain material (>C8), C8, polar endcapped C18 (aQ), Cyano, Pentafluorophenyl (PFP) and Phenyl.

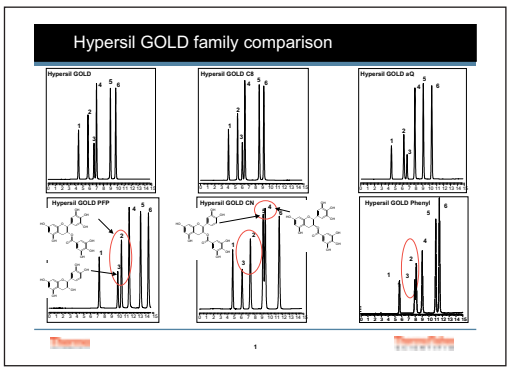

Figure 4. Selectivity changes due to Bonded phase/analyte interactions

Selectivity on the long alkyl chain chemistry and C8 is very similar, as expected, with only slightly less retention on C8 (gradient conditions account for the difference).

Resolution between 3 and 4 and 5 and 6 increases on aQ (polar endcapped C18) when compared with the C8 phase. The PFP phase produces a change in elution order of analytes 2 and 3, compared with the alkyl chain phases.

The extra retention of epigallocatechin gallate over epicatechin is caused by the additional substituted ring that interacts with the phenyl ring on the stationary phase. The Cyano phase shows another reversal of elution order between gallocatechin gallate and epicatechin gallate. This is likely to be caused by the additional hydroxy group in gallocatechin gallate

Most selective chromatography of all is to be found in Affinity and Chiral Chromatography. Where it is impossible to separate by any other chromatographic process the surface of these phases is the ultimate in selectivity. The chemistry is still based on relatively large particles with low efficiencies but gives high alpha values, where there is the correct match of analyte, mobile phase and 3D surface.

Clearly the options for the manufacture of a phase with unique selectivity properties appear to be endless with silica, primary and secondary variables to change almost to a 'pick and mix' scenario. The question remains as to whether it is more beneficial to maximize the plates available or to look for a stable, reproducible column with selectivity, which gives, required resolution using isocratic conditions.

Ideally I suppose the end game is to have a range of complimentary selective columns available in very high efficiency columns. This also poses questions for method development software developers as to how they tackle that issue in the face of increasing complexity of samples, particularly those emanating from the life science laboratories.

Part 2 of this article looking at the case for using the maximum plate strategy will appear in October issue of International Labmate.

Acknowledgements

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References

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