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HPLC Method Development

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

BUILD BETTER HPLC METHODS WITH CRAWFORD SCIENTIFIC

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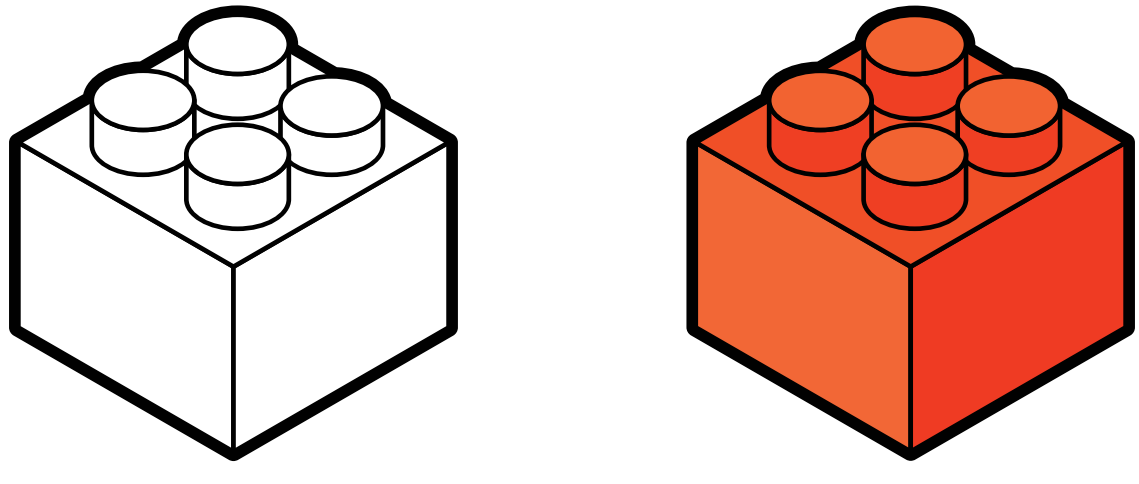

GENERIC METHODS:
THE POTLUCK SUPPER OF ANALYTICAL CHEMISTRY?

As we enter the generalization phase of the industrialization of analytical science, we find ourselves striving for the generic in as many areas as possible.

Pareto is omnipotent, his law guides our efforts in method development for HPLC, GC, and sample preparation. We drive for 80% of every analysis being covered by a single method, so we can devote our "thinking time" to the 20% of "difficult" separations, which don't work with our generic protocols. In theory this should mean that we can spend 20% of our time doing high throughput analysis and 80% of our time solving the issues with our more difficult separations.

It's expected. It's how industrialization works. It's going to be OK—isn't it?
But read on and I'll try to point out why the generic method may be taking us down a road that few of us want to go.

I read recently of efforts (1) to produce a generic HPLC method for pharmaceutical analysis of new chemical entities (NCE's) using 0.05% (v/v) formic acid (circa. pH 3.0) and acetonitrile eluent, a –C18 core shell stationary phase (dp 2.7 µm, 50 mm x 2.1 mm) and relatively steep gradient (30–100% B in 1 min), using an UHPLC system. The method, using a number of NCE test probes, produced a good separation with a peak capacity (Pc) around 100.

The article is by a respected author and was published on a highly respected website. It's a good read—but that's not where the story ends.

Beware of the Pitfalls in this Approach

What if, in final use, the laboratory has not yet moved to UHPLC and is still using an HPLC system with limited pressure capability?

Well then we may need to move to a different column and flow rate combination, the selection was a 50 mm x 3.0 mm column using 2.7µm core-shell particles with the same stationary phase (naturally).
Problem solved, and using one of the ubiquitous "method translators" which are available, we needn't have to think about getting out the calculator—the software will work out the new flow rate and perhaps injection volume that we require.

Of course, if the dwell volumes of the two systems are different, we may need to compensate for this. A quick measurement of the dwell volumes of each system entered into the translation software will compensate and avoid any changes in retention or selectivity. If we don't know the dwell volumes, or don't know how to measure them—well, it's a generic method and we can probably change the gradient a little to make sure the separation is satisfactory.

As the method has a very short run time, this shouldn't take too long, and starting the gradient before we make the injection should compensate for the larger dwell volume in the HPLC vs the UHPLC system. Most data systems will allow this these days.

It's a nice approach. At pH 3 we can assume that we are far enough away from analyte pKa values to avoid retention time/resolution changes due to small changes in pH. Therefore, we should have a reasonably robust method. Acetonitrile generally produces high peak capacities, has low UV cut-off, and lower viscosity to help keep back-pressures low. The formic acid at pH 3 will hopefully improve analyte ionization efficiency in electrospray mass spectrometer sources, if MS detection is preferred.

If we need to perform stability indicating analysis, where we are separating structural analogues of the NCEs, we may be able to move to a longer segmented gradient with a shallow initial stage to help with retention of hydrophilic analytes; a middle stage with a slope to allow the separation of the analogues which may have similar LogP (LogD) values; and then a ballistic gradient to elute any highly retained matrix components. These gradients can often be sub 10-minutes and computer modelling can be used to help predict optimum gradient conditions.

To improve peak shape or provide better pH control — in case we stray closer to the pKa of any of the analytes — the author suggests an aqueous eluent component of 20 mM ammonium formate at pH 3.7. We may expect retention and/or selectivity changes at this stage due to the higher pH and the change in ionic strength of the mobile phase.
If we are using UV detection at lower wavelengths, we may need to use 0.03% formic acid (v/v) in the acetonitrile and 0.05% formic acid (v/v) (aq) to balance the absorbance of the eluents and avoid baseline drift during the gradient methods.

For more complex separations, or NCEs with structurally similar impurities or degradants, we might explore different column chemistry or analysis at high pH. This will result in different ionization states for ionogenic compounds and usually improved peak shape for basic analytes.

So, is this a good approach?

Yes—it's commendable in the sense that various approaches are suggested for overcoming different challenges in pharmaceutical development and one has a good underlying sense of when and why changes may be required. Generic? It's highly debatable.

I would ask a simple question. If you don't get a suitable separation — or worse, if you don't realize the separation is suitable (i.e. due to analyte co-elution) — without a good underlying sense of the principles of chromatography, where do you go next?

This is the crux of my argument — we need to be able to realize something has gone wrong and act upon it. The more generic we make things, the less we think about what non-optimal might look like, and the harder it is to correct.

GENERIC METHODS:
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Recognize when peak shapes are affecting resolution or reproducible integration. Recognize when retention time variability, due to small changes in eluent pH when analyzing ionogenic compounds, may be leading to poor resolution in a chromatogram or poor quantitative results (and how to act accordingly).

Understand the links between sample diluent, injection volume, and pH control (for example, buffering) to avoid peak shape and retention time issues. Understand what to do in order to optimize gradient conditions in order to separate analytes whose structure and/or physico-chemical properties are similar, without knowing something of the nature of gradient HPLC and ways of predicting gradient range and slope. Otherwise, we often revert to, what I call, "wing of bat and eye of newt" development. That is—change something in the eluent or method (potion), give it a stir, and see what happens, without any idea of what is producing the change or what to do if it doesn't work.

Importantly, we also need to develop a sense of when trying to optimize a method is futile and we require a radically different approach—such as a change in stationary phase chemistry, or a change to the mode of chromatography. One needs to be able to assess when shorter columns are not producing enough plates to derive a separation and when a longer chromatographic bed is needed so that efficiency (N) can be used to aid the separation when selectivity is not optimal.

I absolutely applaud the move toward the generic (and let's face it, it's going to continue whether I like it or not). And, I really like the article cited above, including the author's treatment of the approaches taken to meet the demands of the various types of analysis. Nonetheless, what is of paramount importance is that we don't lose sight of the underlying scientific principles.

We must learn to understand the risks in generic method approaches and work hard to improve our skills in order to recognize when problems occur. Moreover, we can use our knowledge of chromatography to devise solutions to the problems and have enough strategies in our toolbox to do this effectively and with insight. These are the 20% of the separations that the Pareto principle tells us we will now have 80% of our time to fix. The problem is—given the throughput demands of the modern laboratory and the "black box" nature of instruments and chromatographic conditions, that 80% of time we are supposed to dedicate to recognizing problems and solving them is actually no time at all!

I've often found that looking at any set of chromatographic conditions and the attributes of our sample and analytes (if we are lucky enough to know them) and asking "why are those conditions used and what could go wrong" can be a great help in increasing my knowledge of chromatography and the requirements for particular applications. Most important of all—if you don't know—find out!

Being of the "generic generation" is not shameful—it's a really exciting time in analytical chemistry—as long as you remain informed enough to make the decision if what you are looking at falls into Pareto's 80% category or 20% category…

References;

- *1. M.W. Dong, A Universal Reversed-Phase HPLC Method for Pharmaceutical Analysis, LCGC North Am., 34(6), 408–419 (2016).*

IF AT FIRST YOU DON'T SUCCEED:
WHERE NEXT IN HPLC METHOD DEVELOPMENT!

Do you ever look with envy at other labs and think 'I wish we were equipped with such a sophisticated and effective HPLC method development system'?
Systems that are equipped with several columns, eluent reservoirs, and columns switching valves that are connected to automated software — these labs are the labs that can test a wide number of column and eluent combinations, in order to find the best conditions during the initial phase of method development.
While these advanced systems are flashy and efficient, they don't present a universal panacea in HPLC method development — a simple, pared-down approach can still achieve a great deal. In my experience, a well-designed single screening experiment can reveal a lot about the sample at hand and inform future method choices. However, I also find that interpretations of initial screen results are regularly where less experienced method developers get stuck.

Never fear — this article aims to help. In it, you'll find some simple scenarios to guide you through the next steps. Over the years, I've designed some robust decision trees that can aid future experimentation as, together, we progress through the method development paradigm. These trees are designed to maximise return on effort — so that simple experiments can be tried and tested before more complex experiments or time-consuming steps are undertaken.

Step 1 – Arrange your screening experiment to shoot for k* (let's call that an Average Gradient Retention Factor) of around 5.

The formula we use here is;

$$k^* = \frac{t_g F}{\Delta \emptyset \, V_m \, S}$$

t_g is the gradient time in minutes, F is the eluent flow rate in mL/min, ΔΦ is the change in eluent composition (i.e. 0.4 for a 20 to 60% B gradient), V_m is the interstitial volume of the column, which is estimated by;

$$V_m = \pi \left(\frac{d_c}{2}\right)^2 L \, 0.68$$

S is a shape selectivity factor which can be estimated by, 0.25 $M_{w0.5}$ (for analytes < 1000Da a value of 5 is typically used for S)
Some of this is predicated on the column dimensions used which determines V_m, and some of the more common column dimensions give the following interstitial column volumes;

Column Dimensions (mm)	Interstitial Volume (V_m) (mL)
150 x 4.6	1.7
100 x 4.6	1.1
100 x 2.1	0.24
50 x 2.1	0.12

Table 1: Interstitial Column Volumes (V_m) for various popular HPLC column dimensions.

For screening experiments – we like a nice wide gradient range to take care of the various polarities of analyte that we might encounter, so let's say a range of 0.8 which will mean setting the gradient from 10%B to 90%B (not forgetting that the we hold the gradient at 90% for a considerable time to assess if there are any really strongly retained analytes).

I'm going to assume an eluent flow rate of 1.0 mL/min for the 4.6mm internal diameter columns and 0.5 mL/min for the 2.1 mm internal diameter columns.

Armed with all this information we can re-arrange Equation 1 to give us a gradient time figure which will result in a k* value of 5 for each of the columns shown in Table 1;

$$\frac{\Delta\emptyset\, V_m\, Sk^*}{F} = t_g$$

$$\frac{0.8 \times 1.7 \times 5 \times 5}{1} = 34\, mins.$$

Calculating for each of the column volume and flow rate combinations gives gradient times shown in Table 2.

Column Dimensions (mm)	Interstitial Volume (V_m) (mL)	Flow rate (mL)	Gradient Time (for k*=5) (mins)
150 x 4.6	1.7	1.0	34
100 x 4.6	1.1	1.0	22
100 x 2.1	0.24	0.5	9.6
50 x 2.1	0.12	0.5	4.8

Each value calculated using a shape factor (S) of 5.

Table 2: Gradient times required to achieve a k* of 5 for various combinations of HPLC column volume, eluent flow rate and gradient range.

IF AT FIRST YOU DON'T SUCCEED:
WHERE NEXT IN HPLC METHOD DEVELOPMENT!

So now we have gathered some useful information and we can consider undertaking our initial experiments – I've proposed below a set of conditions based on a good quality C18 column, however if you know something of the physical chemical properties of your analyte, you may want to choose a more appropriate column chemistry (see Ref 1 and 2 for more details) or eluent pH value;

Column: C18 (150 x 4.6mm)

Eluent: [A] MeCN [B] Water [C] 2% TFA v/v 200mM Ammonium Acetate (aq) using the following gradient table

Gradient: 10% to 80% in 34 minutes (see Table 2 for Gradient Time details)
(use an initial hold time (5% of the gradient time) if the method is expected to be transferred to different equipment or laboratories for dwell volume matching)

Time (mins)	%A	%B	%C
0	85	10	5
1.5	85	10	5
34	5	90	5

Here we have opted for an acidic mobile phase at constant ionic strength (0.1% v/v TFA, 10mM ammonium acetate) which will very much help in the interpretation of where to go next when we obtain the results from the initial screening experiment.

Flow Rate: 1 mL/min (for this column volume – see Table 2 for relevant details for other column dimensions)

Diluent: 10% MeCN (aq) (or as close as possible to this concentration given solubility constraints, to avoid peak shape deformation)

Detection: Electrospray API -MS in pos/neg switching mode if possible or UV detection at an appropriate wavelength for the sample type (choose 254 nm if the analyte chemistry is unknown)

Ok – so let's assume we have undertaken our initial screening experiments, so what do we do with the results, as unless you are extremely lucky, you are unlikely to get a perfect separation from the screening experiment – and if you do, you can write to me and let me know!

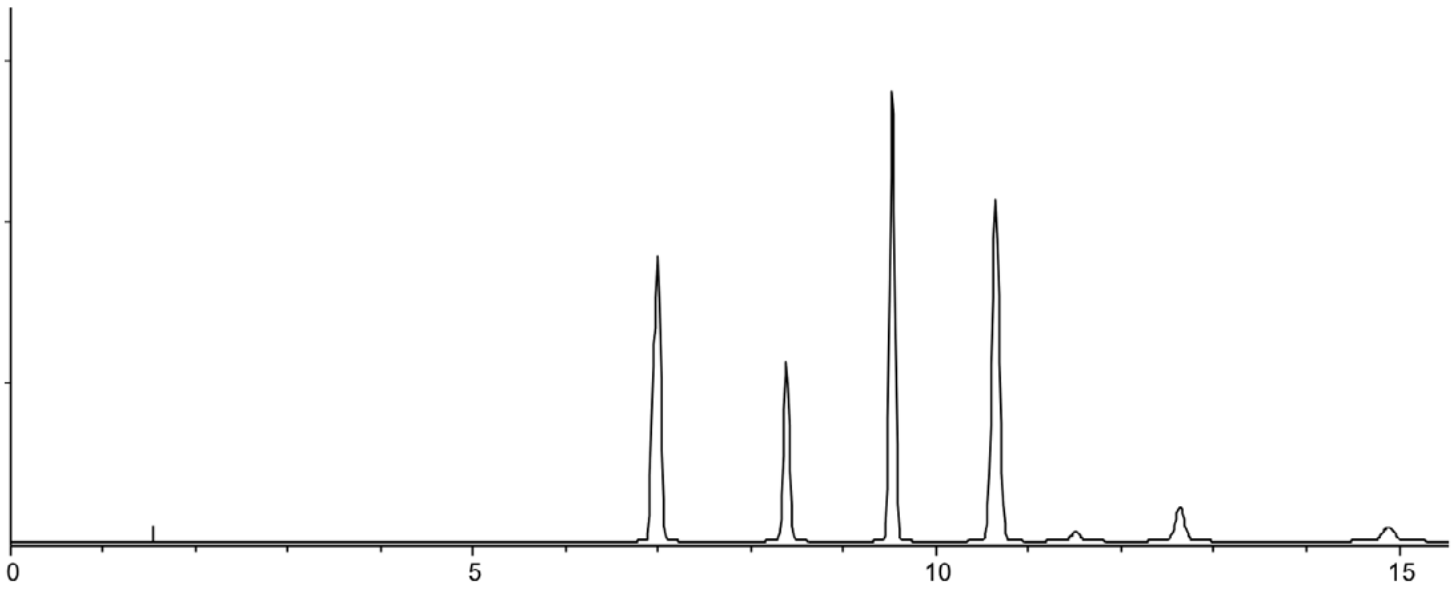
IF AT FIRST YOU DON'T SUCCEED:
WHERE NEXT IN HPLC METHOD DEVELOPMENT!

I'm going to outline a few typical scenarios here and then show you some very simple logic flows and decision trees to follow in order to make the most efficient use of your method development time. The principle here is that we are adopting a 'kill quickly' policy and performing a limited number of experiments post the initial screen before stopping optimisation and changing to a new column. This avoids the funnel of diminishing returns, where one is tempted to perform an (often long) series of 'tweaks' in order to achieve a satisfactory separation. This latter approach is both time consuming a rarely leads to robust HPLC methods and the kill quickly philosophy is much more time efficient in the long run.

In this simulated experiment we are separating a mixture of 5 pharmaceutical test compounds and three impurity peaks. We can expect the test compounds to be at a higher concentration than any of the impurities.

Scenario 1: Some of my peaks are missing

Only 7 of the 8 expected peaks can be seen in the chromatogram



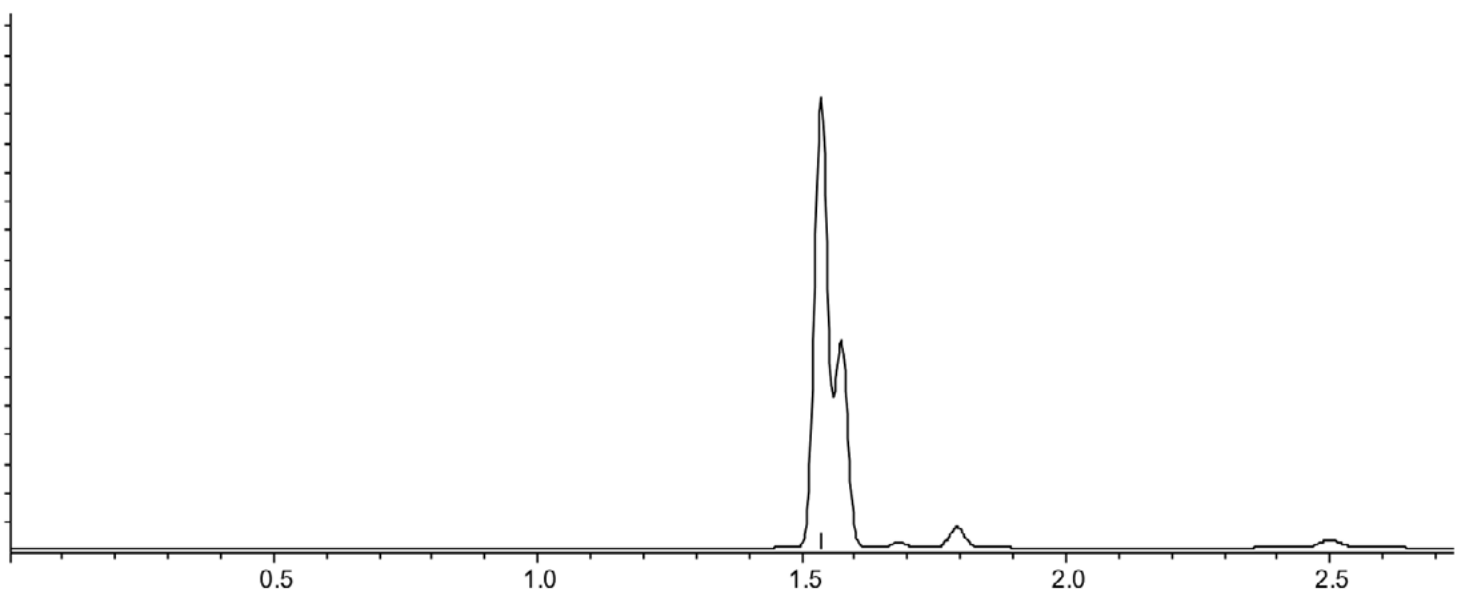
Reasons:

- Irreversible or very long retention (see Scenario 3)
- Co-elution (see scenario 5)
- No or poor analyte retention (see Scenario 2)

Actions:

- Investigate elongated retention by repeating the screening with a hold at the end of the chromatogram the same length as the gradient time used – examine the isocratic hold section for the missing peak
- Proceed to Reasons 2 & 3

Scenario 2: All peaks elute very quickly



Reasons:

- Poor interaction between analyte and stationary phase (possibly due to polarity mismatch between analyte and stationary phase)

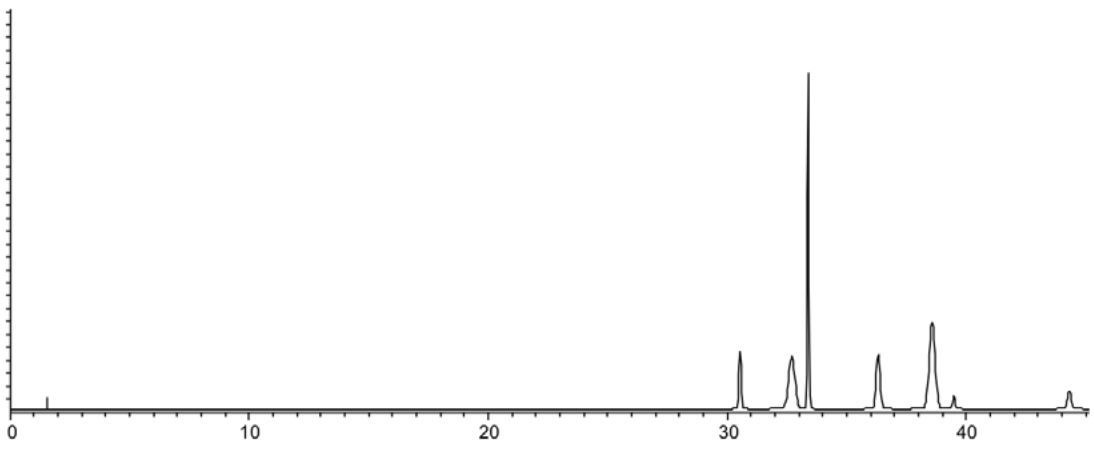
Actions:

- Run two further experiments at pH 5.0 and 7.5 and assess retention behaviour
- Increased retention indicates one or more strongly basic analytes – consider further optimising pH if k values (calculated using Equation 4) are between 1 and 15 (ideally between 2 and 10).

$$k = \frac{t_r - t_0}{t_0}$$

  Where t_r is the analyte retention time and t_0 is the system hold-up time (dead volume)
  Note that the use of retention factor (k) values within gradient analysis is not strictly indicative of retention behaviour but these figures can be used as a rough guide to the applicability of the method
- If any analytes remain at k = 2 or below, consider switching to a different Stationary Phase

Scenario 3: All peals elute very slowly

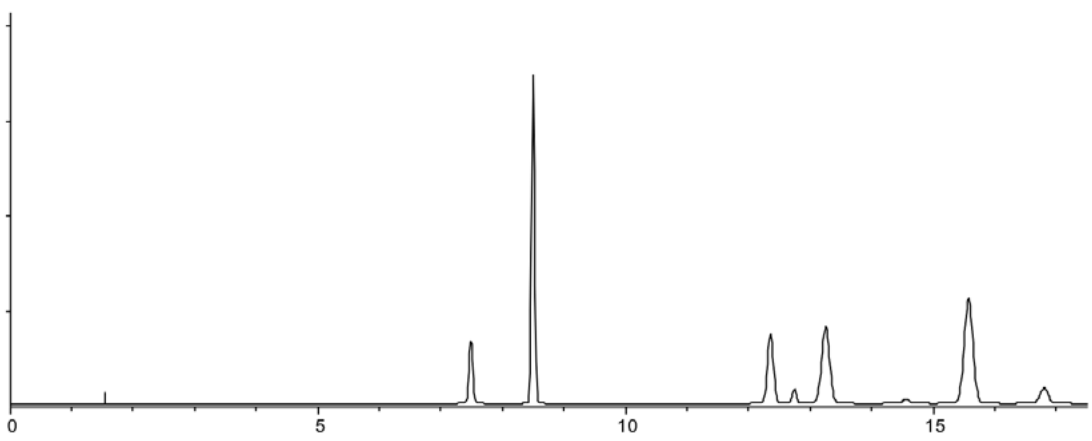


Reasons:

- Interaction between analytes and stationary phase is too strong, due to the analytes being extremely hydrophobic (high LogP values)

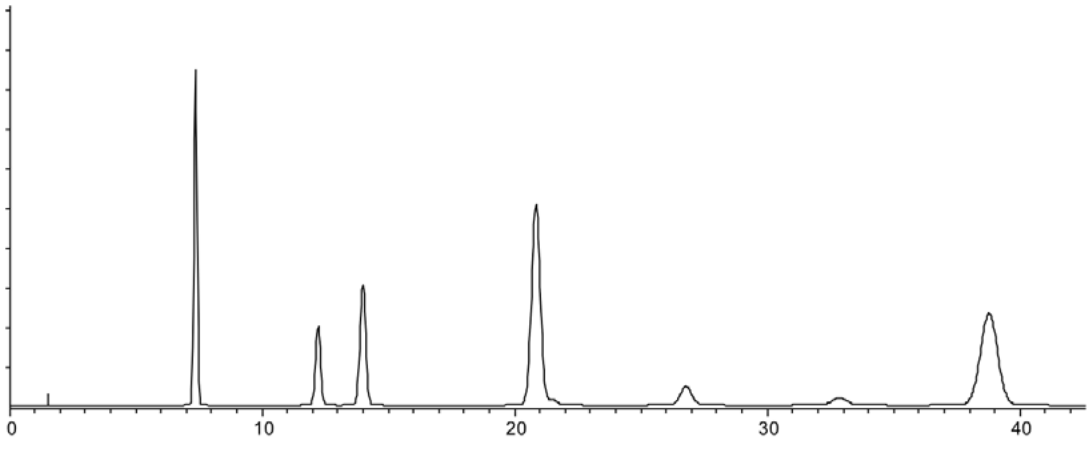
Actions:

- If all peaks elute within the isocratic portion at the end of the gradient, consider changing to a less retentive column (C8 / C4, Phenyl etc.) or altering the eluent pH
- Repeat the experiment at pH 5.5 and 7.5 and assess the retention behaviour – go to step 3
- If most of the analytes elute within the gradient time, either after the initial screen or the pH adjustment experiments, calculate the elution composition at which the first peak elutes (remember to account for any system gradient dwell volume) and subtract 20%B.
- Carry out a further experiment starting at the gradient composition calculated in step 3 but with the same gradient rate as the starting experiment (%B change / minute) – in this case the first peak elutes at approximately 75% B therefore the gradient will start at 50% and the rate of change from the original screen is 2.5%B per minute. Therefore, the new gradient will be 50%B to 90% in 16 minutes.
- It this produces more reasonable retention times, consider optimising gradient slope by having and doubling the gradient rate (1.25%B per minute, 50%B to 90%B in 32 minutes and 5% per minute, 50%B to 90%B in 8 minutes) and estimate the best gradient slope (rate) using the three chromatograms obtained at 1.25, 2.5 and 5%B/min.



Same sample separated using 50 – 85% B in 20 mins pH 5.5

Scenario 4: Peaks show wide range of retention behaviours

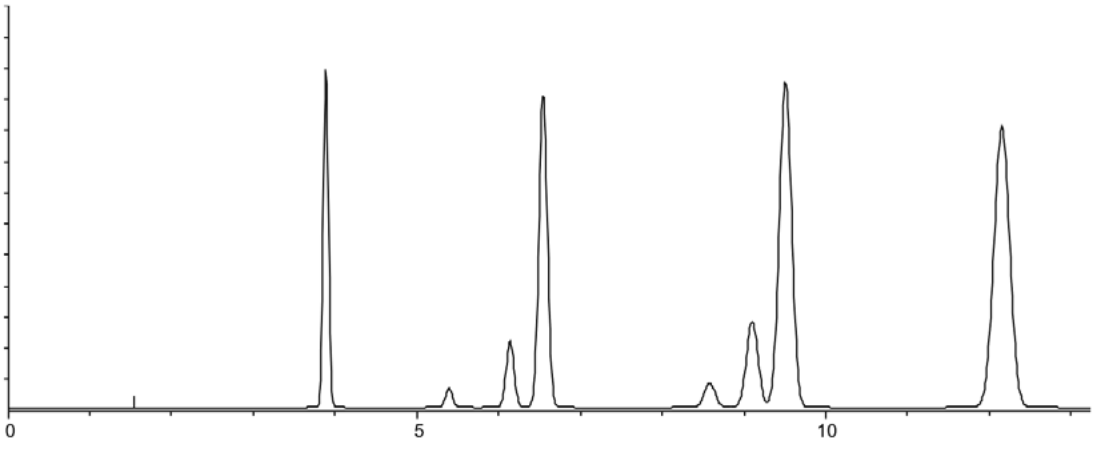


Reasons:

- Analytes have a wide range of LogP or LogD values and therefore show widely different interaction with the stationary phase. This can be due to the native hydrophobicity of the analytes or that hydrophobicity is modified by the presence of ionisable groups and the extent of ionisation, which is controlled by eluent pH

Actions:

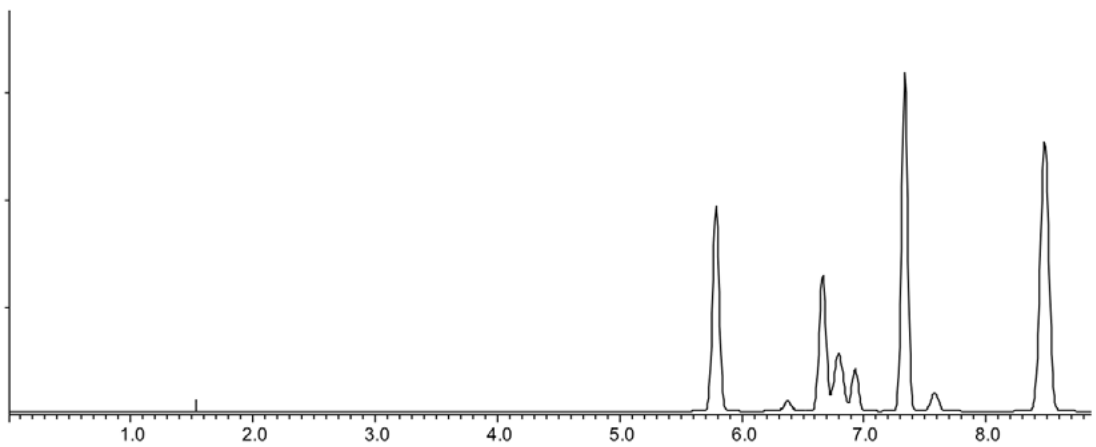
- Double the gradient rate to 5%B per minute (i.e. 10%B to 90%B in 16 minutes) and estimate the best gradient slope between 2.5%B and 5.0%B per minute. Exercise extreme caution when evaluating co-elution and ensure that peaks are tracked either by peak area or UV spectral identification or using MS detection.
- Ideal retention factor range (see Equation 4) is between 2 and 10
- If no suitable gradient slope can be estimated, repeat the initial screening experiment at pH 5.0 and 7.5 to assess the possibility for pH optimisation
- If no combination of gradient slope and pH can be identified for a suitable separation, consider changing to a different stationary phase and repeat the initial screening exercise.



Chromatogram resulting from gradient slope of 5% per minute at pH 5.5. Whilst further development is required to improve resolution (and robustness), all peaks are separated within a reasonable retention range.

IF AT FIRST YOU DON'T SUCCEED:
WHERE NEXT IN HPLC METHOD DEVELOPMENT!

Scenario 5: Co-elution in the middle of the chromatogram / compressed retention range

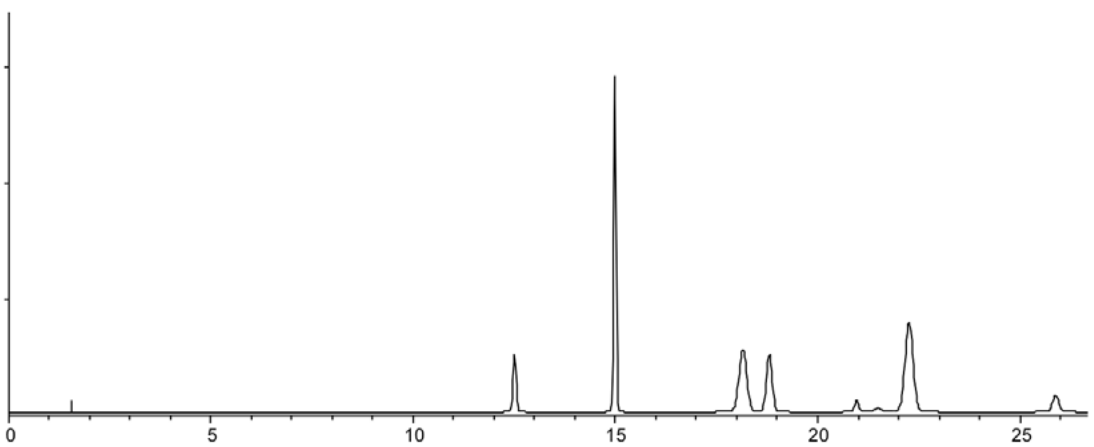


Reasons:

- Analytes have similar LogP or LogD values and are interacting with the stationary phase in a similar fashion

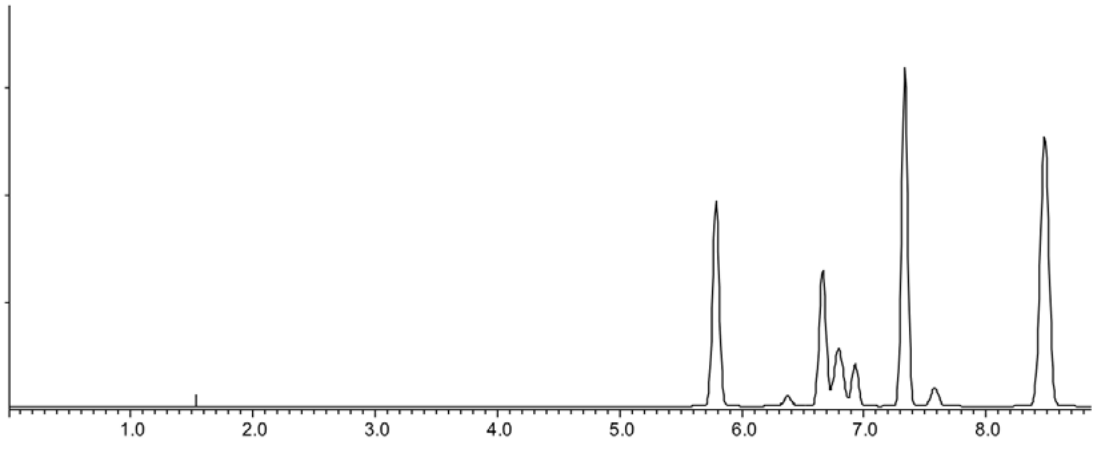
Actions:

- Calculate the eluent composition at the elution time of the first peak at subtract 10%B (in this case the first peak elutes at around 20% B therefore the gradient would start at 5% B)
- Halve the gradient rate from the original screening separation (from 2.5%B per minute to 1.25%B per minute) so the gradient would new be 5%B to 90%B in 60 minutes (approximately)
- Assess the new chromatogram and estimate a suitable gradient slope (%B/min) and upper %B limit for the method. Exercise extreme caution when evaluating co-elution and ensure that peaks are tracked either by peak area or UV spectral identification or using MS detection.
- If a suitable gradient range cannot be identified – consider changing to Methanol as an alternative modifier using the initial screening conditions for the first experiment and re-start at Number 1 in this list.
- Whilst it may be tempting to use pH to optimise the separation prior to the above steps, when peaks are compressed as shown here, it will be very difficult to find an optimum pH and the method is likely to be non-robust. Once a suitable gradient slope and modifier have been achieved, the peaks should elute over a wider retention range and pH optimisation should be possible. Further experiments at pH 5.5 and 7.5 will enable an estimation of the optimal pH from the 3 chromatograms obtained (pH 2.1, 5.5 and 7.5).
- If a suitable separation cannot be achieved, select a more suitable HPLC column and repeat the initial screening exercise.



Same separation carried using a shallow gradient 30 – 65 %B in 30 minutes.

Scenario 6: Co-elution at the start and end (or throughout) the chromatogram

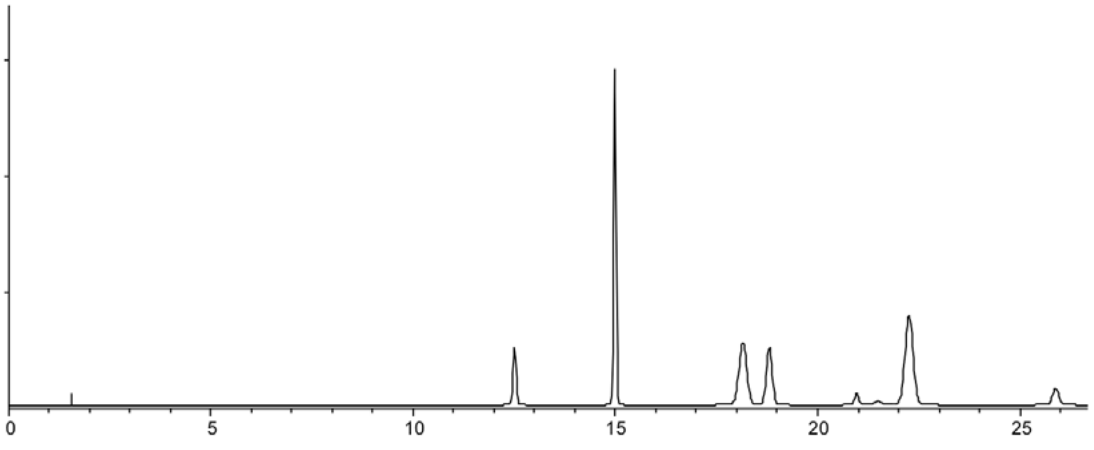


Reasons:

- Analytes have similar LogP or LogD values and are interacting with the stationary phase in a similar fashion
- Co-elution over the whole chromatogram indicates many similar species or sub-optimal selectivity, indicating a change is required to the stationary phase or eluent

Actions:

- Calculate the eluent composition at the elution time of the first peak at subtract 10%B (in this case the first peak elutes at around 20% B therefore the gradient would start at 5% B)
- Switch to Methanol as the modifier and repeat the screening exercise but starting the gradient at the composition calculated in step 1 – assess if the degree or number of co-elutions has improved. Move to step 3 with either acetonitrile or methanol.
- Repeat the screening separation at pH5.5 – does the selectivity change significantly? If so – conduct a further experiment at pH 7.5, if not consider a change to a different stationary phase
- From the three pH experiments, pH 2.1, 5.5 and 7.5 you will be able to estimate a suitable pH for the separation. If no suitable pH can be identified, consider using a stationary phase capable of withstanding high eluent pH and repeat the experiment at pH10. Re-evaluate the chromatography to assess if a pH between 7.5 and 10 might be suitable for the separation. If suitable separation cannot be achieved, consider using a stationary phase with an alternative selectivity (see references 1 and 2).



Same separation carried out using Methanol as the modifier at pH 6.5.

Whilst in almost all cases, further optimisation work will be required at the end of each process, the order in which changes are undertaken and the types of change will lead to a significant reduction in effort for those developing separations without the aid of optimisation software!

IF AT FIRST YOU DON'T SUCCEED:
WHERE NEXT IN HPLC METHOD DEVELOPMENT!

References;

- *Do you really know your stationary phase chemistry?*
- *Relating Analyte Properties to HPLC Column Selectivity the road to Nirvana*

DITCH YOUR MUNDANE HPLC METHODS: SUPERCHARGE YOUR CHROMATOGRAPHY

It's hard to know how much more effective your HPLC method can be if you don't know what an efficient method looks like in full-flow.

I'm not talking about investing hugely in your equipment but setting realistic, achievable goals. Because why spend all your money on a Ferrari when a Skoda Superb more than does the trick?

Get your HPLC methodology firing on all cylinders

I've outlined below the chromatographic conditions for the Chromatographic Purity USP monograph method for Lansoprazole;

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

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

The remainder of the conditions can be found in USP Monograph: Lansoprazole, USP 40-NF 25.

I've modelled the separation obtained for Lansoprazole and 3 known impurities obtained on a 4 year old HPLC system with reasonably low extra column volumes and dwell volume (Figure 1).

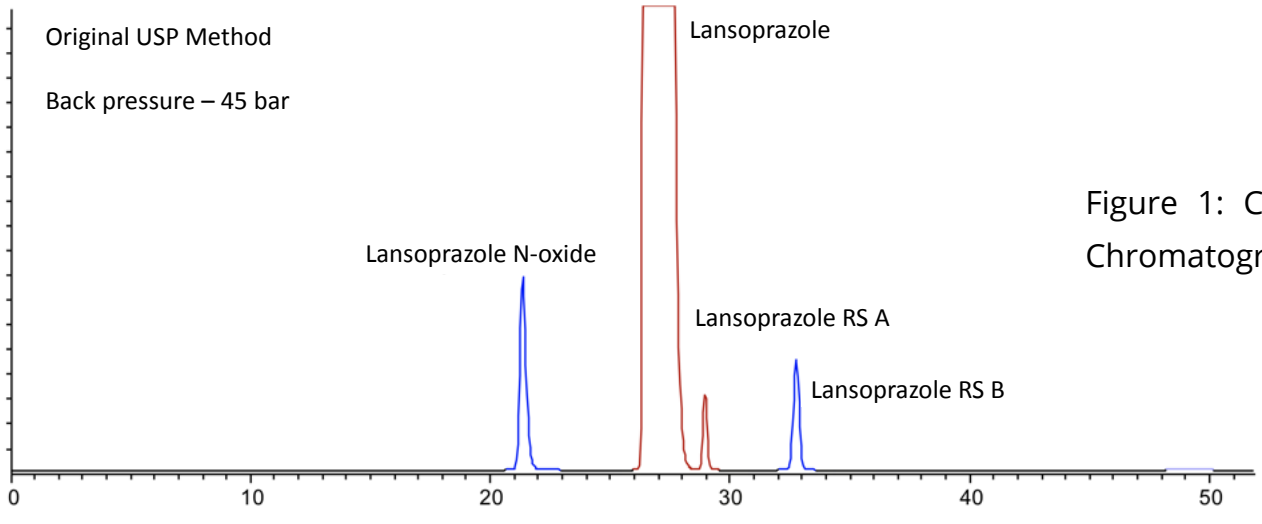


Figure 1: Computer model of Lansoprazole USP Chromatographic Purity separation.

One should note here that the relative responses should not be taken as accurate and that the tailing factor for the Lanzoprazole peak exceeded the required limits (TUSP = 1.5). We have increased the relative concentrations of each of the impurities for the purposes of this demonstration.

So – here we have our 'current vehicle' and we may be perfectly happy with its performance. But what might the separation look like if we 'tuned' it a little?

I would like to point out very clearly here that none of the following changes are acceptable under the current guidance of USP General Chapter <621>. But those who would like to see what might be theoretically possible please read on. For those who are not operating under legislative guidelines and who have the opportunity to improve methods, please also note that no changes should be made to methods without first validating the procedure according to your company guidelines.

The idea here is to keep within the operating pressure limits of a 400 bar to represent an 'achievable aspiration' in terms of the separation – i.e. one wouldn't need a 1000bar 'SuperSystem' in order to achieve the proposed improvements.

To be honest, I'm also not really trying to improve the separation in any other respect than the time taken. How quickly could an acceptable separation be achieved?

In the initial set of experiments I'm going to follow a rule recommended by the USP (General Chapter <621>) regarding the limits over which one should alter the ratio of column length to particle diameter;

L/dp (-25% to +50%)

Although once again I should state that as this is a gradient separation, these changes are not allowable under current USP guidance. However, these adjustments would be allowable if the separation was performed under isocratic conditions.

Following the USP recommendations I've outlined in Table 1 the changes to column length and column dimensions that we may be able to consider. I've also targeted the use of superficially porous particles which will deliver higher efficiency than their fully porous counterparts and hopefully preserve the quality of our separations throughout the changes.

Column Length (mm)	Particle Size (mm)	L/d_p ratio
150	5	30
100	4	25 (-17%)
100	2.7	37 (+23%)

Table 1: Changes to the column length to particle size ratio allowable for isocratic methods under USE General Chapter.

There is a further allowable change within the USP guidelines for isocratic methods of +/- 50% of the original flow rate, which might also be applied to our aspirational method. Note here again that some exceptions apply and that linear velocity should be maintained.

So let's look first at the separation obtained with scaled column dimensions and eluent low rate;

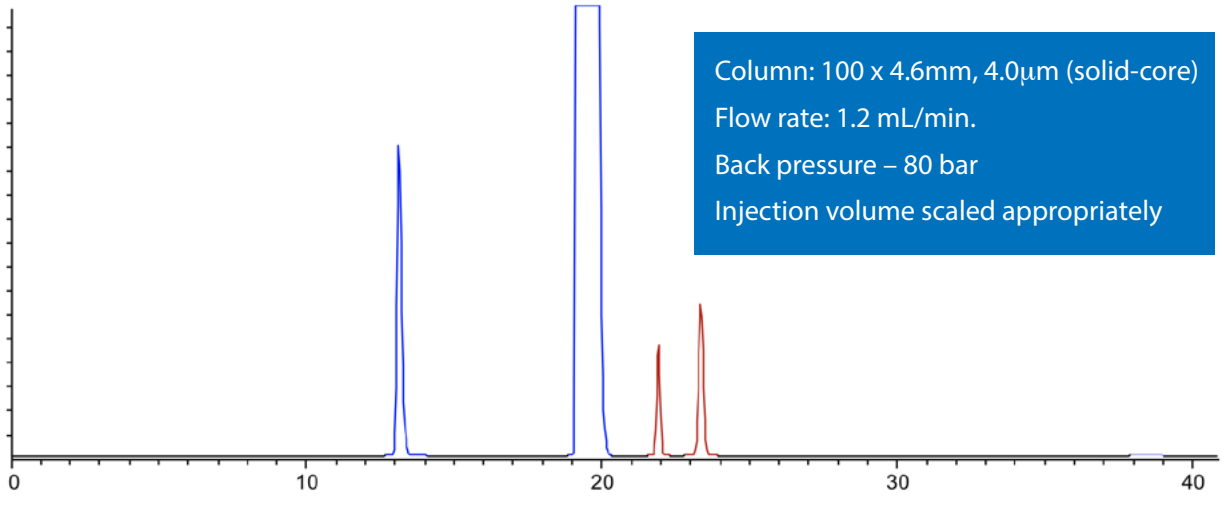


Figure 2: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the figure. (All other conditions are maintained from the USP monograph method.)

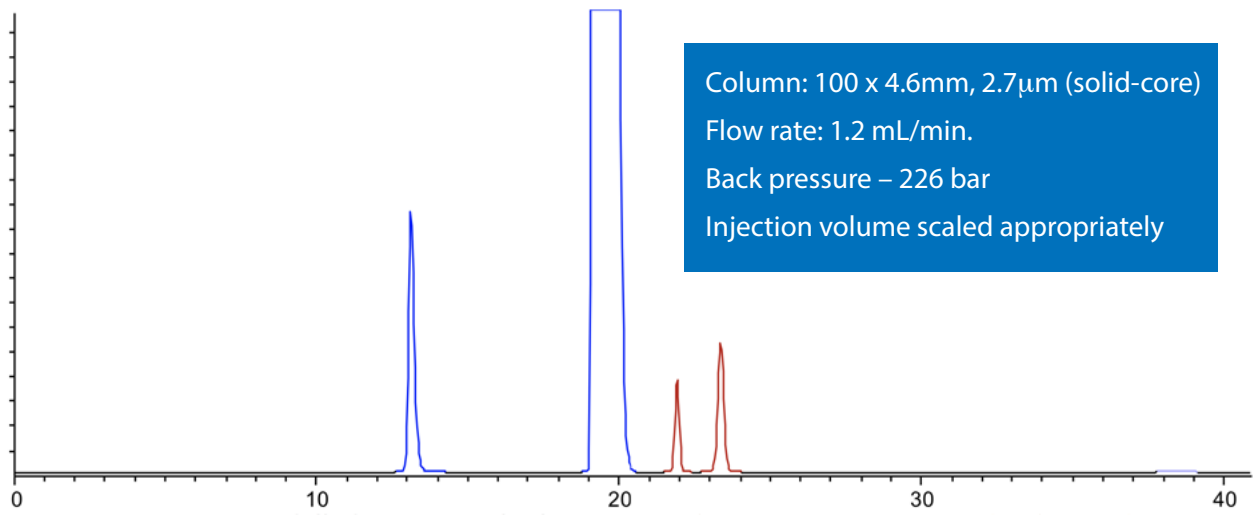


Figure 3: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the figure. (All other conditions are maintained from the USP monograph method.)

The resolution between any critical pair satisfies the method requirements and the retention time of the last peak is around 75% of the original method. In fact the resolution is so good, one might consider further reductions in analysis time.

So, far I've followed the USP General Chapter <621> requirements for the method adjustments – but now I'm going to break free of these constraints and begin to look at optimizing the gradient conditions.

There is an excellent relationship that we might use to maintain gradient slope (and hopefully the resolution) whilst making method adjustments (Equation 1);

$$G_s = V_m \Delta\Phi / F t_G$$

Where G_s is gradient slope, V_m the interstitial volume of eluent in the column, $\Delta\Phi$ the gradient range (changing % organic), F is the flow rate and t_G the gradient time.

Suppose I wanted to adjust the gradient time to 20 minutes but use the column specified in the original method.

Interstitial volume $(V_m) = \pi \times r^2 \times L \times W$

Where r is column radius (take care – not column diameter!), L is column length and W a porosity factor (0.68 can be used for fully porous particles and 0.55 for core-shell particles).

Interstitial volume (V_m) of USP method monograph (150 x 4.6mm) column is

$\pi \times 2.3^2 \times 150 \times 0.68$ @ 1695(mL) @ 1.7mL

So from equation 1 and the monograph method we can calculate the gradient slope value.

$V_m = 1.7$
$\Delta\Phi = 70$ (gradient is 10 to 80% B)
$F = 0.8$
$t_G = 40$
Gradient slope G_s (monograph method) = 1.7 x 70 / 0.8 x 40 = 3.72

So if we wanted to halve the gradient time we would need to retain a value of 3.72, and as we want to keep the column the same as the original method, we could either alter the gradient range (convenient but more likely to affect separation selectivity) or the flow rate, again convenient.

In order to retain a gradient slope value of 3.72 we would need to adjust the flow rate to 1.6 mL / min.
Gradient slope G_s (20 minute gradient, 150 x 4.6mm column) = 1.7 x 70 / 1.6 x 20 = 3.72

So let's see what the original monograph method would look like with a 20 minute gradient and a flow rate of 1.6 mL/min.

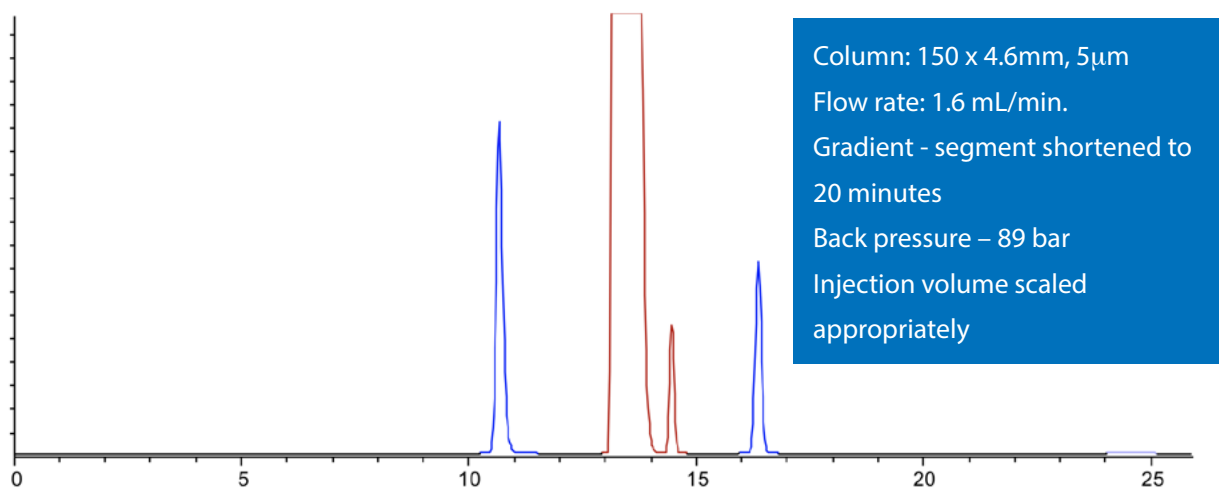


Figure 4: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the figure. (All other conditions are maintained from the USP monograph method.)

So we've pretty much halved the run time and not suffered any ill effects from lost resolution.

Ok – so we might say that we've tuned up the current car a little. How aspirational might we be in terms of increasing method throughput? Could we combine the shortened gradient time and adjustments to column dimensions and flow rate to produce something a little more exciting in terms of run time but maintaining the separation quality and critical method attributes?

Using Equation 1, I've modelled several separations using various column dimensions, in each case altering the method flow rate to maintain, as closely as possible, the gradient slope value for the 20 minute gradient time. In each case I'm trying to maintain the usability of the method on a 400bar HPLC system.

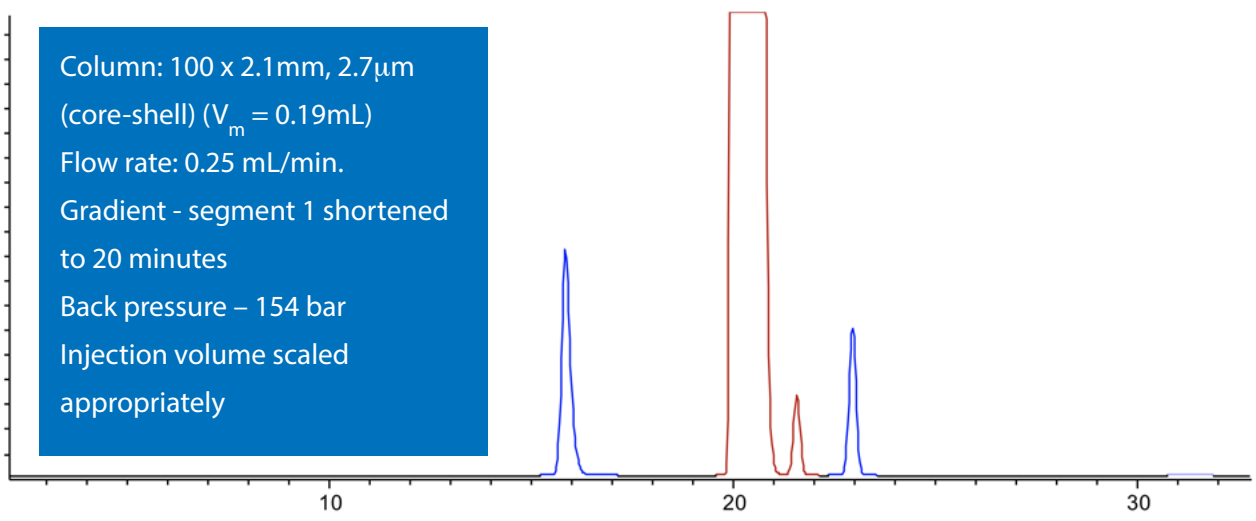


Figure 5: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the figure. (All other conditions are maintained from the USP monograph method.)

From Figure 5 we can see we've gone backwards a little! Reducing the column volume (especially the column internal diameter) has dictated that we need to significantly drop the flow rate in order to retain a constant gradient slope and the method is slower than the previous iteration.

So – after a few more in-silico iterations – I threw all of my tuning tools at the method and came up with the following method by simply adjusting the various parameters in Equation 1 to maintain the gradient slope.

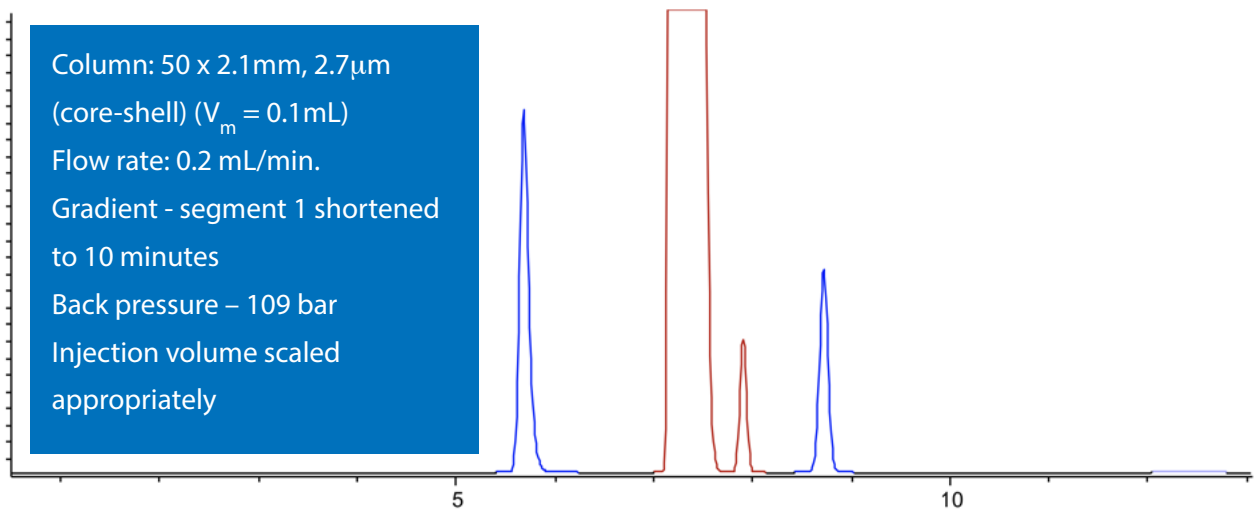


Figure 6: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the figure. (All other conditions are maintained from the USP monograph method.)

A pretty successful 'tune up' I'd say with an impressive 75% reduction in analysis time.

But let's not get too carried away with our Audi A8 equivalent separation without first assessing our driving skills! What if we had a much older system with much larger dwell and extra column volumes.

Development System (used to this point) – Dwell Volume 600µL / Extra Column Volume <10µL

Older System (more realistic) - Dwell Volume 1100µL / Extra Column Volume 25µL

Our supertuned method above may look something like this;

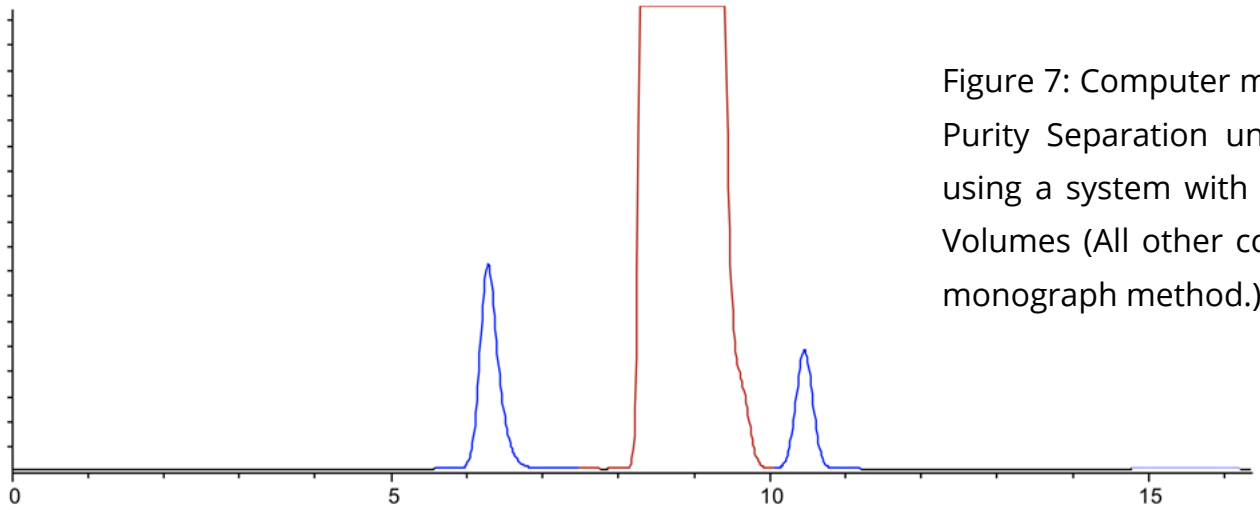


Figure 7: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in Figure 6 using a system with more realistic Dwell and Extra Column Volumes (All other conditions are maintained from the USP monograph method.)

Figure 7 shows us that this separation is clearly not acceptable! Our aspirations have overleapt our abilities....

So let's back off a little and drive within the limitation may be our ability. This required some more modelling using the older system, however were still able to obtain a separation which reduced the overall run time by over 60%. The main changes are in the gradient time and crucially in the internal diameter of the column, which at 4.6mm is much more 'forgiving' in terms of the effects of larger extra column volume.

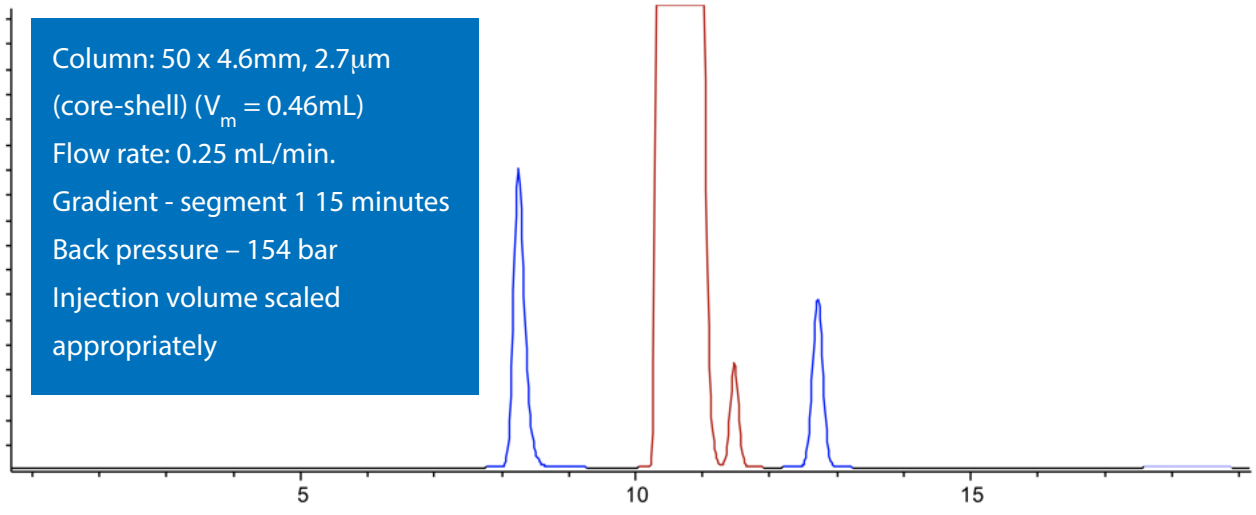


Figure 8: Computer modelled Lansoprazole chromatographic Purity Separation under the conditions stated in the Figure using a system with more realistic Dwell and Extra Column Volumes (All other conditions are maintained from the USP monograph method.)

Figure 8 shows us that the separation is satisfactory in terms of resolution and has a 60% run time compared to the original USP monograph method.

So what has been the point of this exercise?

- To highlight a bad monograph method? Not at all. Although I know this method is currently under review as there are other issues associated with it, from a chromatographic perspective it is fine, but dated and therefore longer than it needs to be.
- To annoy all of those who would like to improve their methods but can't due to legislative restrictions? No, I merely want to highlight what is possible and to give a more aspirational perspective on the use of more modern column and instrument technology.
- To encourage anyone to break the rules laid out by the USP? Absolutely not, you should never do this, however once in a while it's good to look up from our work and get a sense of what may be possible in the future, as the monograph methods catch up with newer technology.

For anyone using USP and other pharmacopeia methods, be aware that some changes to methods are possible and you should become familiar with the types of change that may be possible (using the appropriate Pharmacopeia) in order to improve a method or meet system suitability criteria.
For anyone laboring with methods using older column technology and who are fortunate enough to be able to make method changes, take the opportunity once in a while to 'tune' your methods or perhaps even to see what life might be like in the 'aspirational supercar'.

USEFUL FREE TOOLS FOR:
HPLC METHOD DEVELOPMENT

I've done that thing where I've stated a very interesting title — I hope I can deliver something which lives up to it. I dislike it when people 'overstate' their talk or poster titles at conferences to draw me in and then don't deliver against the promise – I'll let you judge how we go here. First, let me explain.

I was investigating a separation that I was adapting from a manufacturer's application note [1] and wanted to check the observable effects from changing column selectivity.

Here are the analytes and the separation on an Agilent Poroshell C18-EC column (end-capped) and the associated separation from a variety of steroids that we have been working on.

Conditions

Columns:	Agilent poroshell 120, 2.1 x 100 mm
Flow Rate:	0.4 mL/min.
Gradient:	25-80% MeCN/10 min. (0.1 formic acid in water and MeCN)
Temperature:	25^{O}C
Detection:	DAD 260,80 ref = off

Peaks

Hydrocortisone
β Estradiol
Androstadiene 3,17 dione
Testosterone
Ethinylestradiol
Estrone
Norethindrone acetate
Progesterone

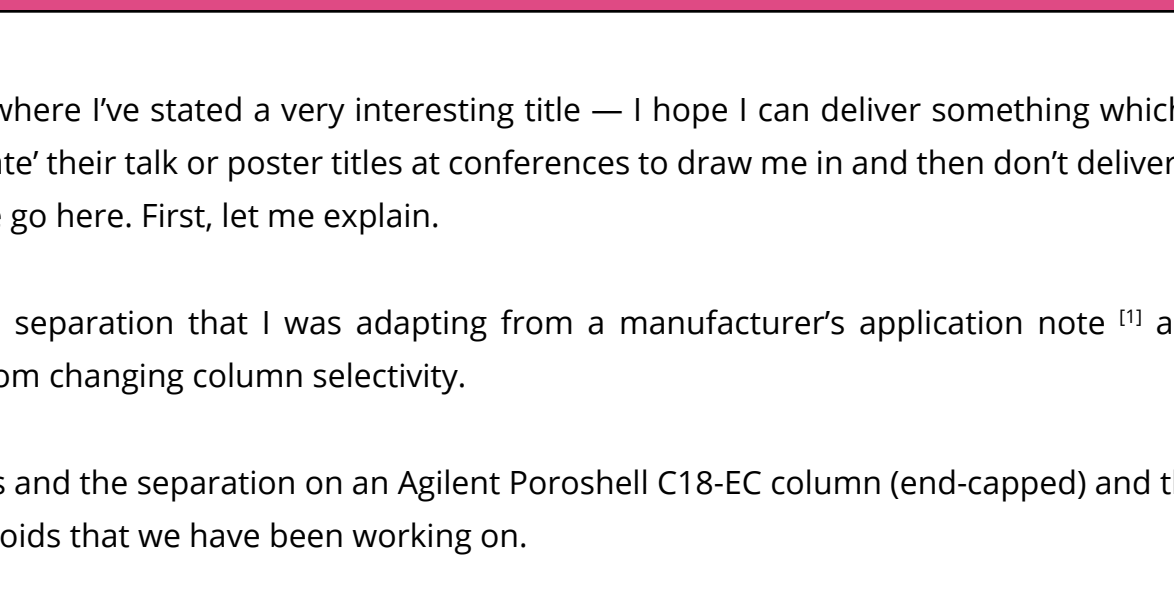


Figure 1: Initial separation of a range of steroid analytes using an end-capped C18 core-shell column.

I wanted to check the elution order and how this correlated with the hydrophobicity of the analytes and if any other factors were dictating retention and selectivity on this 'standard' end capped octadecylsilyl column.

When I started my career, some 30 years ago, this would have meant a good chunk of time spent with the Handbook of Chemistry and Physics [2] in the hope that I could find all of the relevant analytes. These days, it's possible to find most physicochemical information online. One website that is worth your attention is Chemicalize — https://chemaxon.com/products/chemicalize — developed and maintained by CHEMAXON. This contains most of the useful measurements that we need to help us understand the analyte behaviour of a wealth of common (and some not so common) species. To demonstrate the capability, figure 2 is a screenshot of a search on estrone.

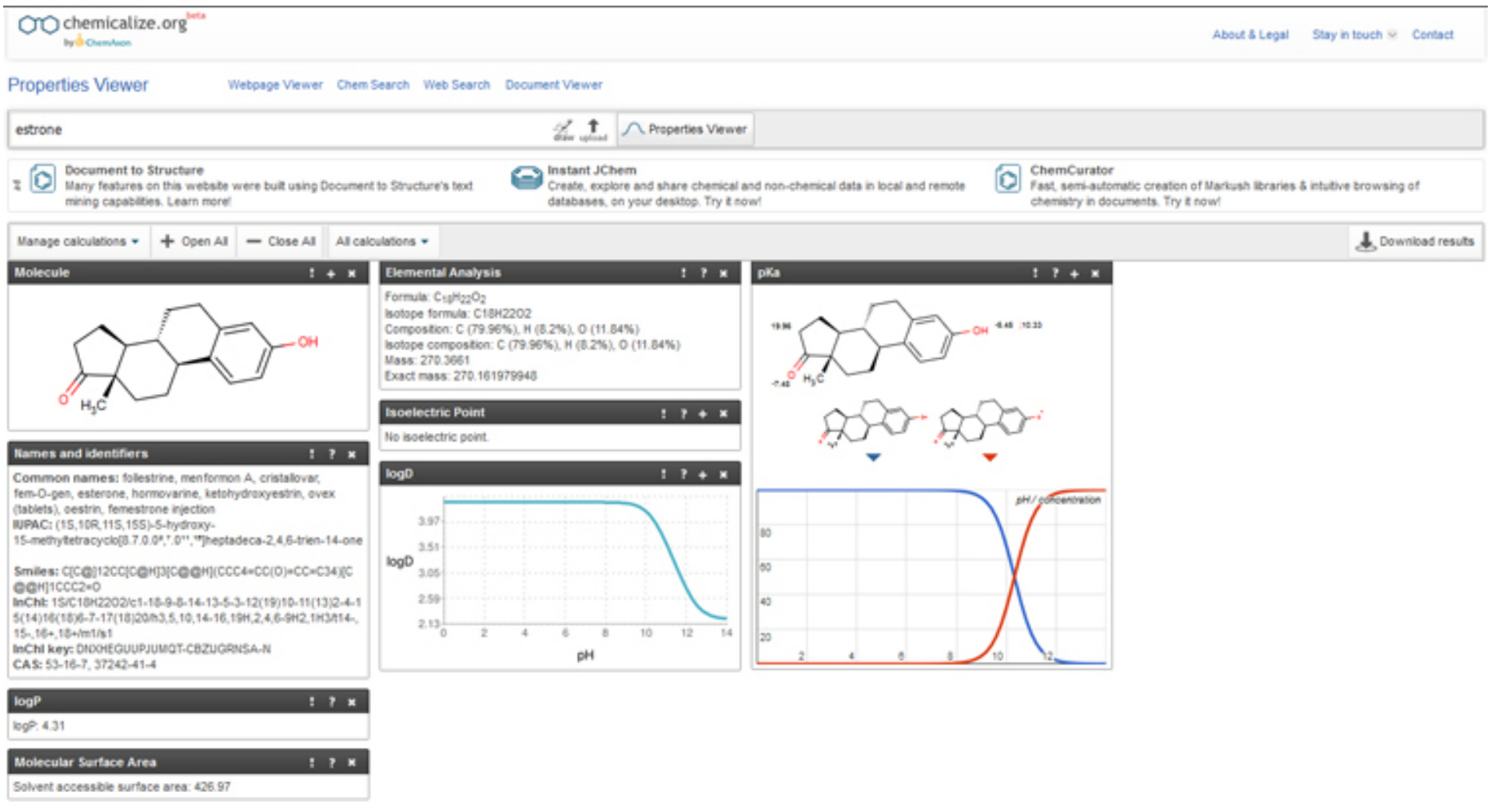


Figure 2: Screen shot of some of the information available from Chemicalize. (www.chemicalize.com)

I've only shown a limited amount of information available from the site in Figure 2 as this is what was required for my particular application but I'd encourage you to explore all of the other useful information for yourself. It is really straightforward to obtain information on hydrophobicity (LogP & LogD) as well as pKa, molecular weight, molecular geometry, polar nature, surface area, and other very useful data.

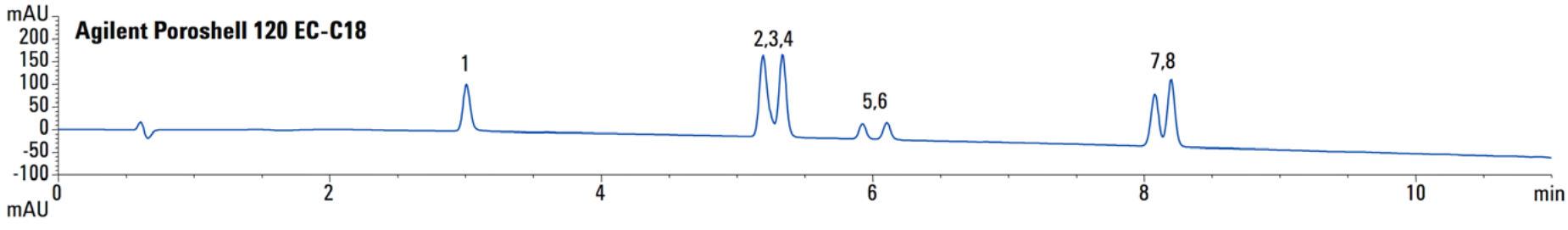
Figure 3 shows the C18 separation — again tabulated in elution order against the physicochemical parameters that are thought to affect this separation.

Conditions

Columns:	Agilent poroshell 120, 2.1 x 100 mm
Flow Rate:	0.4 mL/min.
Gradient:	25-80% MeCN/10 min. (0.1 formic acid in water and MeCN)
Temperature:	25°C
Detection:	DAD 260,80 ref = off

Peaks

Hydrocortisone
β Estradiol
Androstadiene 3,17 dione
Testosterone
Ethinylestradiol
Estrone
Norethindrone acetate
Progesterone



Compound	M.Wt.	pKa1	Log P	Solvent Accessible Surface Area (Acetonitrile)
Hydrocortisone	362.5		1.28	556.4
β Estradiol	272.4	10.33	3.75	436.4
Androstadienedione	284.4		3.93	438.2
Testosterone	284.4		3.37	477.7
Ethinylestradiol	296.2	10.33	3.9	459.6
Estrone	270.3	10.33	4.31	427
Norethindrone acetate	340.5		3.66	528.2
Progesterone	314.5		4.15	524.8

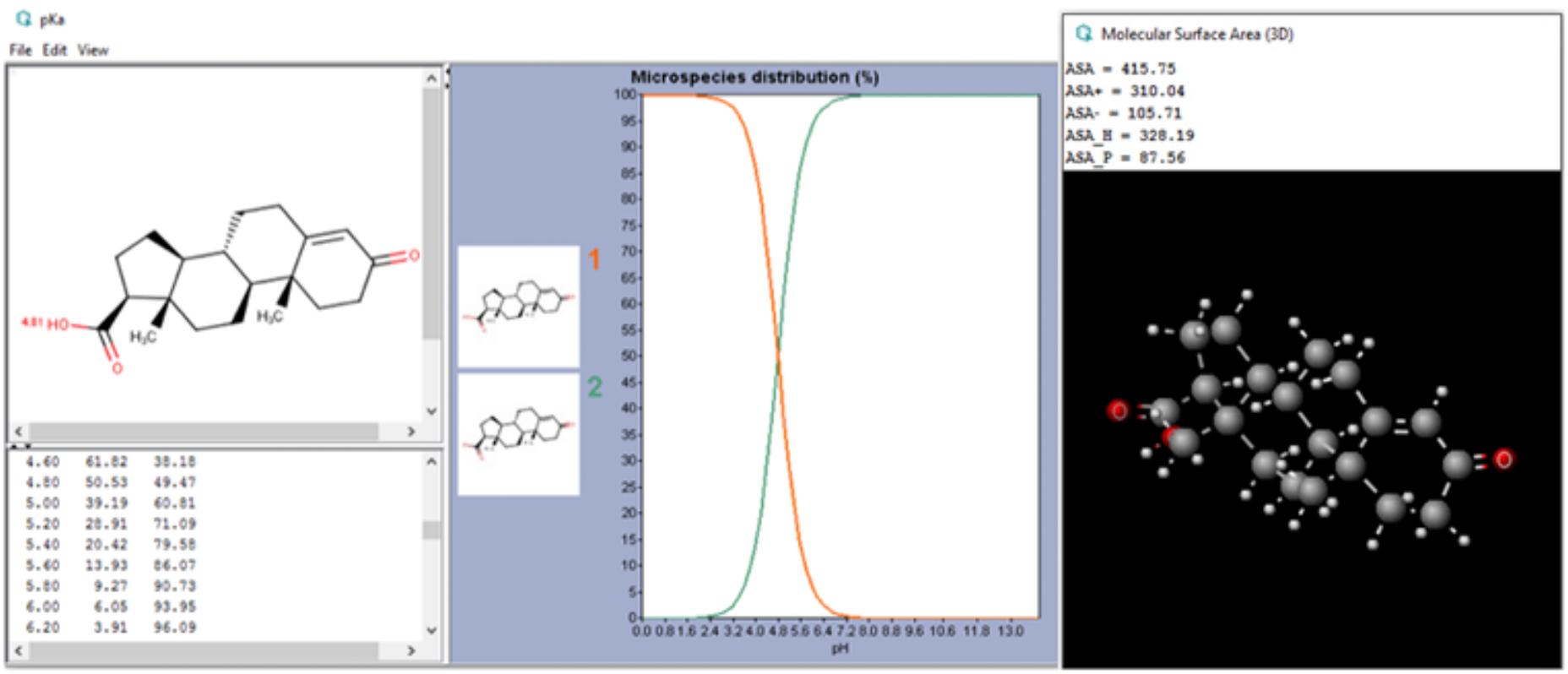
Figure 3: Initial separation of a range of steroid molecules using an end-capped C18 core-shell column and some relevant physicochemical chemical parameters of the analytes.

As you will see, for the most part, the elution order increases as hydrophobicity increases (increasing values of LogP, the octanol/water partition coefficient) as one might expect in reversed phase chromatography. However, the later-than-expected elution where this trend is not followed (by testosterone for example) can be explained by an increase in accessible acetonitrile solute surface area. Again, this is a very useful predictor of retention in reversed phase HPLC and represents the relative area available for the solvent or the stationary phase to interact with.

Before moving on to investigate the selectivity options from other phases, I wanted to predict the retention time of an analyte — an acid analogue of the androstadienedione molecule.

Unfortunately, this analyte is not contained within the Chemicalize database. But that's okay because ChemAxon offer another free resource called MarvinSketch (https://chemaxon.com/products/marvin) — a chemical structures drawing program containing some free plug-ins that can estimate/calculate the same physicochemical parameters which we've used to analyse the separation so far.

After drawing the acid analogue, the plug-ins were used to predict the physicochemical data which is shown in Figure 4.



pKa = 4.81

Solvent Accessible Surface area 554.6

LogP = 3.82

Figure 4: Some selected outputs from the calculation performed in MarvinSketch on the acid analogue of the Androstadienedione analyte.

We didn't have the analogue synthesised at the time of doing this predictive work, however, we could estimate that the analyte may elute in the fairly 'crowded' area around peaks two and three. So, how do we select columns in order to try and improve the separation between these closely eluting analytes while improving the separation between already co-eluting peak pairs?

Are there any tools available which will allow us to predict the type of phase that we might want to use?

There are several models for describing stationary phase selectivity which I've discussed previously (http://www.chromatographyonline.com/column-selection-hplc-method-development) so I won't cover the basics here – although some selectivity characteristics knowledge of the columns you wish to consider for the analysis is immensely helpful.

The next really useful tool then, is the database of column selectivity which is maintained by Dwight Stolls' group at Gustavus Adolphus College, which can be found at www.HPLCcolumns.org.

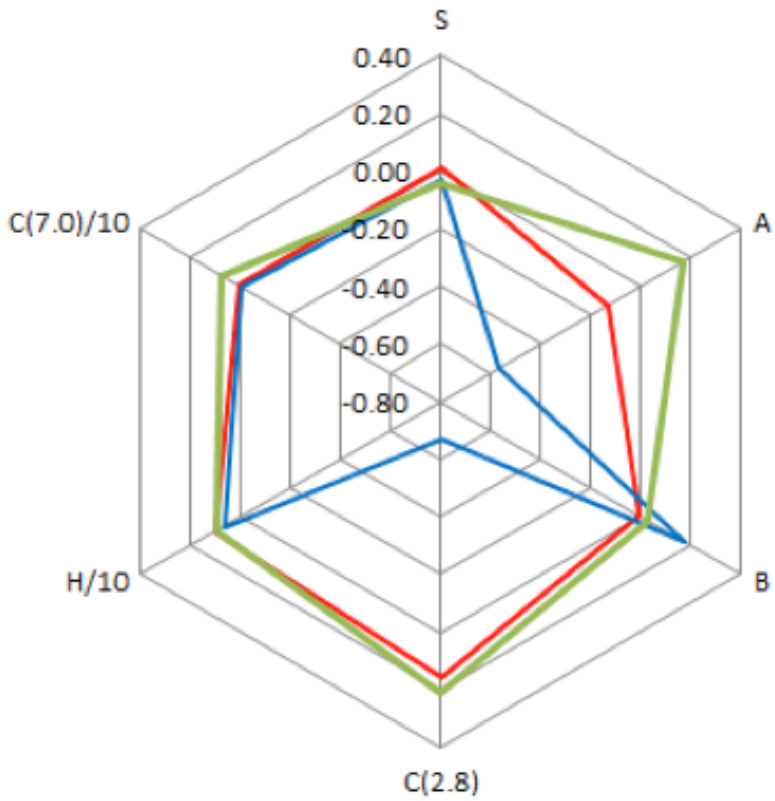
F_S	Name	Manufacturer	Silica type	H	S*	A	B	C (pH 2.8)	C (pH 7.0)
0.00	Poroshell 120 EC-C18	Agilent Technologies	B	1.02	0.00800	-0.130	-0.00400	0.161	0.123
5.96	Poroshell 120 EC-C8	Agilent Technologies	B	0.877	0.0110	-0.232	0.0230	0.127	0.0900
7.46	Poroshell 120 HPH C18	Agilent Technologies	B	1.03	0.00500	-0.140	-0.0140	0.0730	-0.00400
10.24	Zorbax Eclipse XDB-C18	Agilent Technologies	B	1.07	0.0200	-0.0600	-0.0300	0.0500	0.0800
11.76	Poroshell 120 SB-C18	Agilent Technologies	B	0.956	-0.0410	0.168	0.0250	0.210	0.763
12.30	Zorbax StableBond 300A C8	Agilent Technologies	B	0.700	-0.0800	0	0.0400	0.140	0.820
12.49	Zorbax StableBond 300A C18	Agilent Technologies	B	0.900	-0.0500	0.0400	0.0400	0.250	0.700
12.59	Zorbax StableBond 80A C18	Agilent Technologies	B	0.990	-0.0300	0.260	0	0.130	1.04
13.08	Poroshell 120 Phenyl-Hexyl	Agilent Technologies	phenyl	0.752	-0.0830	-0.394	0.0180	0.136	0.140
13.45	Zorbax Extend C18	Agilent Technologies	B	1.09	0.0500	0.0100	-0.0400	0.0300	0.0100

Figure 5: Selective screen shot from the www.HPLCcolumns.org

You will see that the database characterises the columns as per the parameters described in the hydrophobic subtraction model (HSM) [3].

This database is very useful for finding the HSM data on just about every reversed phase column in existence, as well as looking for similar, orthogonal column chemistries, and highlighting columns which have dominant characteristics for the retention and separation of target analytes.

Putting the database above to use, I looked for two columns that I thought might give different characteristics to those of the C18 we started with. The results of my findings are shown in Figure 6. The excel spreadsheet radar plot output is from a tool that we created here at Crawford Scientific; however, making something similar is straightforward.



Poroshell 120 EC-C18
Poroshell 120 SB-C18
Poroshell 120 Bonus-RP

Column	Poroshell 120 EC-C18	Poroshell 120 SB-C18	Poroshell 120 Bonus-RP
H	1.023	0.956	0.686
S	0.008	-0.041	-0.030
A	-0.130	0.168	-0.573
B	-0.004	0.025	0.180
C(2.8)	0.161	0.210	-0.670
C(7.0)	0.123	0.763	-0.017

Figure 6: Column comparison data on the original column and two further candidates for the separation.

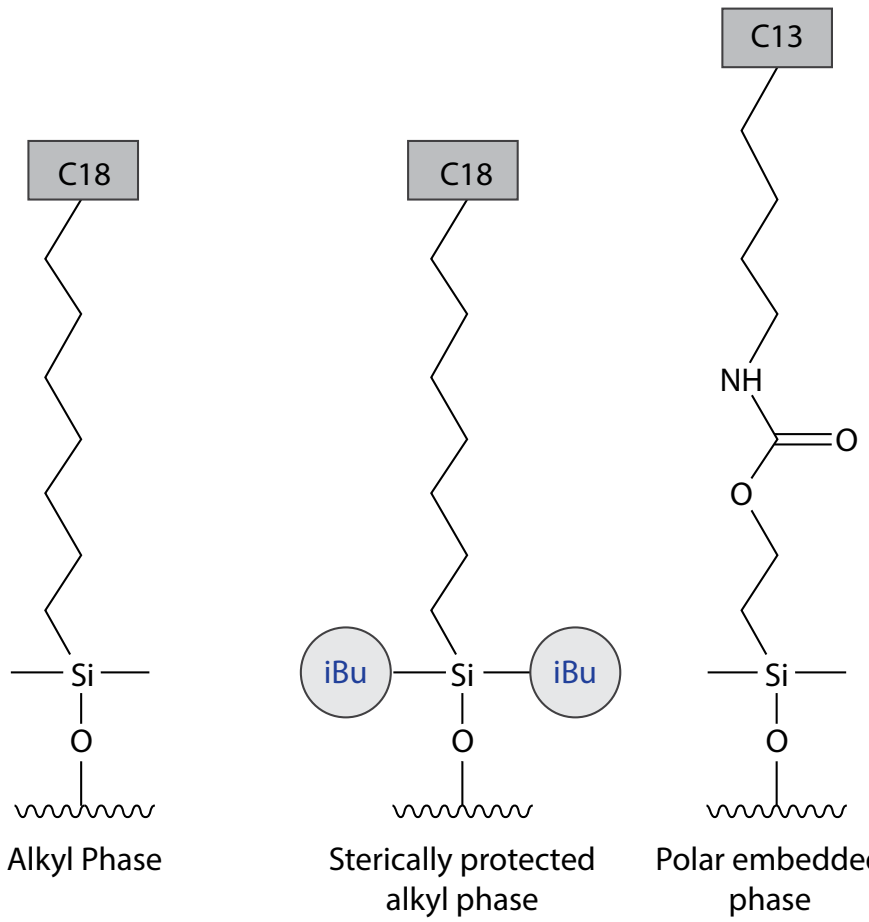
Before I go further, I should point out that this is unweighted data. Data should be weighted for the various analyte characteristics. The database at www.hplccolumns.org is really useful for this.

Secondly, I'm in no way claiming that we can do any sort of quantitative structure/activity correlation with the tools that I'm discussing. For that type of development, you should be following the work of Paul Haddad's group at the University of Tasmania and their various collaborators. I've cited one paper in Reference 4 for you to start from.

However, it is useful to begin to relate the selectivity properties of the columns to what 'might' and what 'does' happen in practice. Or even to select columns based on what interactions you THINK might be dominant in the analyte/stationary phase interaction. In this way, our column selection and method optimization can be much better informed.

Again, without going into too much detail – we can relate the results from the databases with the stationary phase characteristics to postulate what might happen when various phases are used. Here we picked a non-endcapped phase, which is sterically protected with bulky iso-butyl substituent groups on the bonded ligand, as well as a polar embedded column which typically contain a carbamate or imide functional group within the alkyl chain.

Note that the structures shown in Figure 7 are representative of the 'type' of ligand used — they do not represent the actual chemistry of these phases. Further, we've represented the chain lengths on each ligand in a rather unconventional way for convenience and space!



Column	Poroshell 120 EC-C18	Poroshell 120 SB-C18	Poroshell 120 Bonus-RP
H	1.023	0.956	0.686
S	0.008	-0.041	-0.030
A	-0.130	0.168	-0.573
B	-0.004	0.025	0.180
C(2.8)	0.161	0.210	-0.670
C(7.0)	0.123	0.763	-0.017

Figure 7: Stationary phases of the 'type' used in our experiments.

So, what differences in the separation can we expect from these alternative phases, given the data from the column selectivity database?

The StableBond (SB C18) column will give approximately the same hydrophobic retention but will show increased shape selectivity. This will result in increased retention for sterically more bulky and less polar analytes. It can be expected that more polar or ionogenic analytes will not show the same extended retention.

It's important to note that the StableBond column is non-endcapped, and had the separation been carried out at pH values above 6, the selectivity might have looked very different. This is due to the acidic surface silanol groups becoming ionized (pka is around 4) and the polarity of the surface changing markedly — becoming increasingly anionic — which is reflected in the high, pH 7.0, value displayed by the ion exchange capacity. However, the eluent for this experiment was pH 2.8 (approx.) after adjustment with 0.1% formic acid and as such we expect any acidic silanol groups to be non-ionised; therefore, they have less effect on the separation.

Figure 6 indicates that the polar embedded bonus RP column will show much greater retention of acidic species due to interaction with the embedded basic group in the ligand, and much lower retention of basic species, as any exposed surface silanol groups are 'shielded' by the embedded polar ligand. In general the hydrophobic retention of this column is lower, so any less polar compounds should show shortened retention times.

The actual chromatograms obtained are shown in Figure 8, alongside the original chromatogram from the end-capped C18.

Conditions

Columns:	Agilent poroshell 120, 2.1 x 100 mm
Flow Rate:	0.4 mL/min.
Gradient:	25-80% MeCN/10 min. (0.1 formic acid in water and MeCN)
Temperature:	25^{O}C
Detection:	DAD 260,80 ref = off

Peaks

Hydrocortisone
β Estradiol
Androstadiene 3,17 dione
Testosterone
Ethinylestradiol
Estrone
Norethindrone acetate
Progesterone

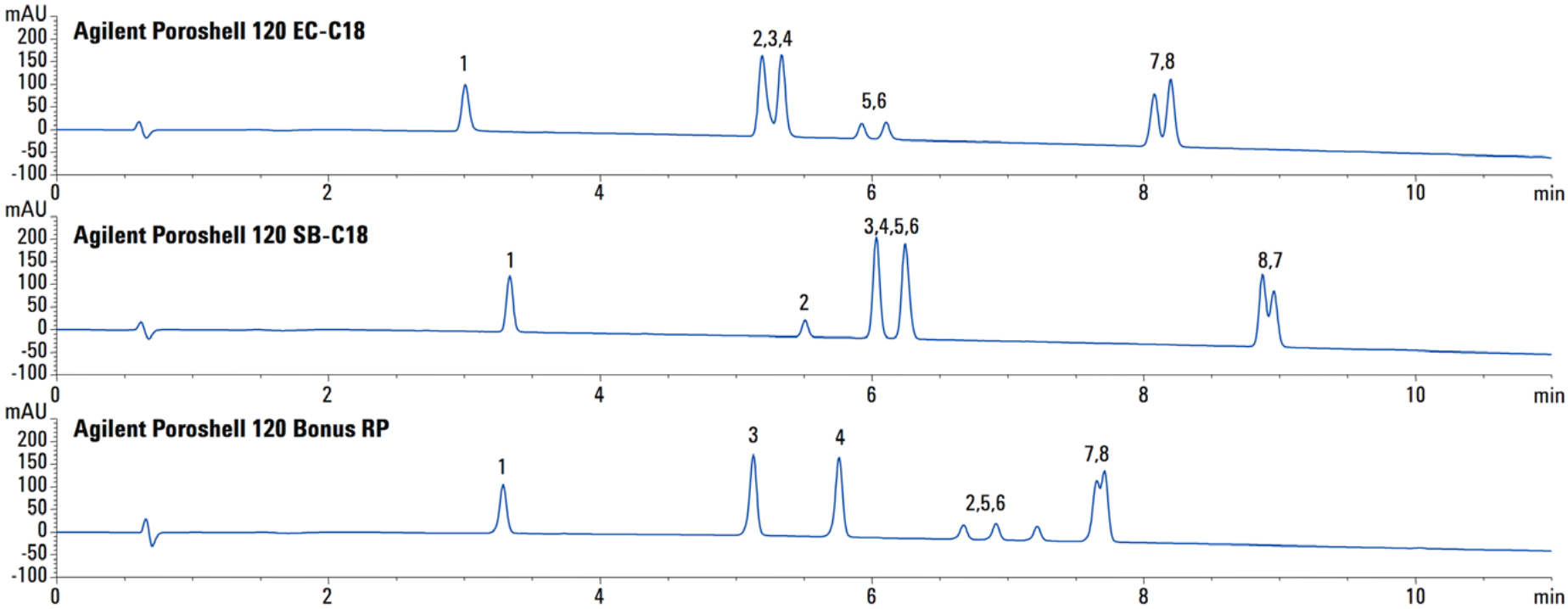


Figure 8: Separation of various steroid analytes using three different ligands to explore the selectivity obtained in comparison with changes predicted using a column comparison database.

Without undergoing an in-depth debrief on the results, it can be seen that the SB-C18 does indeed show extended retention for the less polar analytes and those without a polar or ionisable functional group are effectively retained to a lower degree which is entirely in keeping with an increase in the shape selectivity of the phase. Compounds 2, 5 and 6, which are very weakly acidic, are effected less markedly.

The bonus RP column shows reduced retention of the non-polar compounds and a markedly higher retention of the acidic compounds (2, 5 and 6) as predicted. This separation certainly gives a much better opportunity to 'insert' the acidic androstadienedione analogue which will hopefully belute just before or after compounds 7 and 8.

Whilst the separation was still not optimised, a successful separation was eventually affected using a phenyl hexyl functional column with methanol as the eluent modifier. My point here is — there are a host of tools available to help guide our HPLC method development and optimisation choices. We should all be taking full advantage of them!

I also appreciate that I've highlighted only a limited set of tools here and readers and vendors alike will be keen to let me know of their versions of these tools or other tools that can be used.

References;

1. *Fast Screening Methods for Steroids by HPLC with Agilent Poroshell 120 Columns, Agilent Application note 5991-0451*

2. *CRC Handbook of Chemistry and Physics, 96th Edition (15 Jun 2015), Editor-in-Chief W. M. Haynes*

3. *Wilson NS, Nelson MD, Dolan JW, Snyder LR, Wolcott RG, Carr PW Column selectivity in reversed-phase liquid chromatography: II. Effect of a change in conditions. Journal of Chromatography A 961, 195–215 (2002).*

4. *Talebi M, Schuster G, Shellie RA, Szucs R, Haddad PR. Performance comparison of partial least squares-related variable selection methods for quantitative structure retention relationships modelling of retention times in reversed-phase liquid chromatography. Journal of Chromatography A 1424, 69-76 (2015).*

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Tony Taylor has over 30 years of experience in developing chromatographic methods and is still learning. As the Technical Director of three varied contract and application development laboratories, he understands what frustrates analytical chemists and how to help them overcome problems.

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.