



A Comparison of Fully Porous C18 Reversed Phase HPLC Columns

Introduction

The most commonly used LC reversed-phase alkyl bonded stationary phase is octadecyl carbon chain (C18)-bonded silica, which is denoted as USP classification L1. C18 columns have a broad applicability from pharmaceuticals to food and environmental analyses. However, not all C18 columns are alike. Simply swapping a C18 column from one manufacturer to another can result in differences in retention time, resolution and even selectivity. Differences can arise due to variations in hydrophobicity, silanol activity, packing quality, particle size distribution, and silica purity, to name a few.¹

This technical note provides details of a comparative study between twenty-four silica-based C18 phases, from a number of manufacturers covering the following areas:

- Relative Hydrophobicity Study
- Efficiencies for a Neutral Compound
- Evaluation of Silanol Activity

Table 1 shows the C18 (150 x 4.6 mm, 5 µm) silica-based columns included in this comparative study. A combination of Type A and Type B phases are included, all of which are classed as 'small-pore'. High purity silicas (e.g. Type B) have lower and more tightly controlled levels of metal ions than older generation silicas (Type A). LC columns always have several parameters quoted including pore size, carbon load, and surface area.

Carbon load refers to the % (by weight) carbon content of the stationary phase bonded to the support material. In general, stationary phases with higher carbon loads result in more hydrophobic surfaces and tend to retain hydrophobic analytes more strongly than stationary phases with lower carbon loads. It is important to note that carbon loading does not account for the degree or type of endcapping used, in which the % carbon will differ between various endcapping groups.^{1,2}

The surface area refers to the total area of the solid surface in an adsorbent (e.g. porous silica particles). Columns with high surface areas may exhibit greater retention, resolution, and capacity. However, columns with lower surface areas will typically equilibrate faster. Most interactions which occur between analytes and stationary phase occur within the internal pore structure of the silica particles, in which 99 % of the available surface area resides.²

Small-pore particle columns typically have pore sizes ranging from about 60-150 Å and are used for the analysis of 'small molecules'. Pore diameter is not usually a primary consideration when selecting a column for small-molecule analysis.³

It is important to note that, without chromatographic evaluation, these parameters will not provide enough information alone to accurately predict selectivity and retention.

Table 1. LC column stationary phase specifications for twenty-four C18 150 x 4.6 mm, 5 µm columns from various manufacturers investigated in these studies.

Column	Carbon Load (%)	Pore size (Å)	Surface Area (m ² /g)	End capping	Silica Type (A/B)
ACE 5 C18	15.5	100	300	Yes	B
Atlantis C18	12.0	100	330	Yes	B
Brownlee Analytical C18	13.0	110	180	Yes	A
Brownlee Validated C18	20.0	100	300	Yes	B
Gemini C18	14.0	110	375	Yes	B
Hypersil BDS C18	11.0	130	170	Yes	A
Hypersil Gold C18	10.0	175	220	Yes	B
Hypersil ODS	10.0	120	170	Yes	A
Inertsil ODS-2	18.5	150	320	Yes	B
Inertsil ODS-3	15.0	100	450	Yes	B
InertSustain C18	14.0	100	350	Yes	B
Kromasil C18	20.0	100	320	Yes	B
Luna C18 (2)	17.5	100	400	Yes	B
Prodigy ODS-3	15.5	100	450	Yes	B
Quasar C18	17.0	100	380	Yes	B
Symmetry C18	19.1	100	335	Yes	B
XBridge C18	18.0	130	185	Yes	B
XSelect CSH C18	15.0	130	185	Yes	B
XSelect HSS C18	15.0	100	230	Yes	B
XTerra MS C18	15.5	125	175	Yes	B
YMC-Pack ODS-A	17.0	120	330	Yes	B
Zorbax Eclipse XDB-C18	10.0	80	180	Yes	B
Zorbax Extend-C18	12.5	80	180	Yes	B
Zorbax SB-C18	10.0	80	180	No	B

Relative Hydrophobicity Study

Comparison of Hydrophobicity

Hydrophobicity is a measure of the bonded stationary phase's ability to hydrophobically interact with analyte carbon groups. In this study, only C18 alkyl stationary phases have been considered. The retention factor (k) is a method of evaluating how much interaction the analyte has had with the alkyl chain.

k is calculated using the retention time of the analyte and the time at which an unretained compound elutes (e.g. uracil in reversed-phase HPLC) (Equation 1). It is considered best practice to have a k value between 2-10 for good separations. The k value for a hydrophobic/neutral compound (toluene is commonly used and was investigated in this study) is a good marker of hydrophobicity.

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

Equation 1. Retention factor (k) equation, where t_r is the retention time of the analyte, and t_0 is the time at which the unretained compound elutes.⁴

Low k values (< 1) indicate little to no retention has taken place, which can be problematic. In cases such as these, potential impurities can easily co-elute, resulting in poor selectivity. Higher k values indicate that the analyte is more highly retained. A stationary phase with greater hydrophobicity will experience greater analyte retention (thus greater k) for a

hydrophobic compound.^{4,5} Excessive retention reduces sample throughput, with increased analysis times, and the peak height will also decrease as peak bandwidth increases.⁵ However, there is scope to reduce longer run times by switching to a less hydrophobic phase (e.g. C18 to a C8).

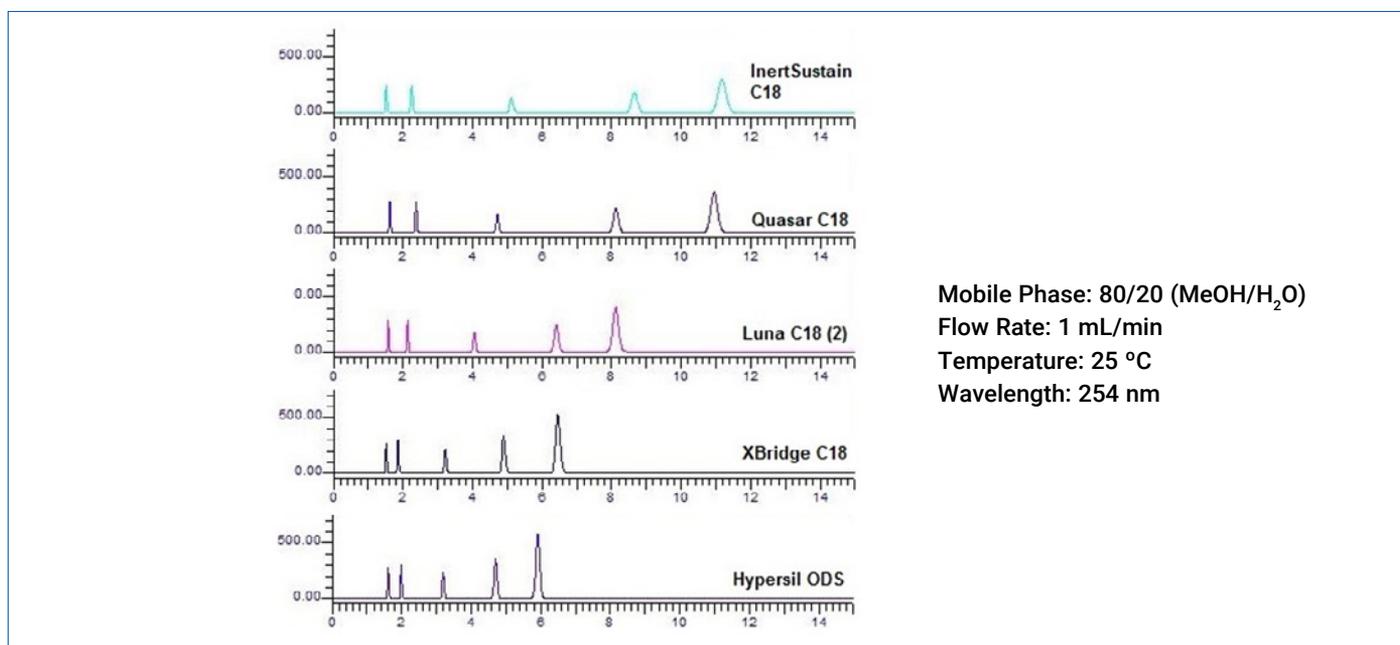
Some benefits of increased retention include:

- Greater opportunity to resolve analytes without increasing column length.
- Water content of the mobile phase does not need to be increased to improve retention, yielding greater MS sensitivity.

Figure 1 shows the chromatograms of a neutral compound test mix obtained on a selection of columns within the study with differing levels of hydrophobicity. Figure 2 details the k values obtained for toluene on the C18 phases, from the most retentive to the least.

Columns such as the XSelect HSS C18 and Luna C18 (2) provide moderate retention. However, PerkinElmer's Quasar C18 phase is among the more retentive phases due to the high surface area, combined with its optimised C18 ligand bonding process. This gives Quasar, and other similarly retentive phases, greater opportunity to resolve analytes without increasing the column length.

The InertSustain C18 also demonstrated a high k value for toluene. However, peaks are broader, which can translate to lower efficiencies, and this is indeed observed when calculating efficiencies. Conversely, the Hypersil Gold C18 demonstrated the least retention, with a k value for toluene below one. Low k values can cause issues with reproducibility and can cause a column to fail method validation.



Mobile Phase: 80/20 (MeOH/H₂O)
Flow Rate: 1 mL/min
Temperature: 25 °C
Wavelength: 254 nm

Figure 1. Chromatograms of a neutral compound test mix on C18 columns of varying hydrophobicity. Elution order: uracil, dimethyl phthalate, toluene, biphenyl, phenanthrene.

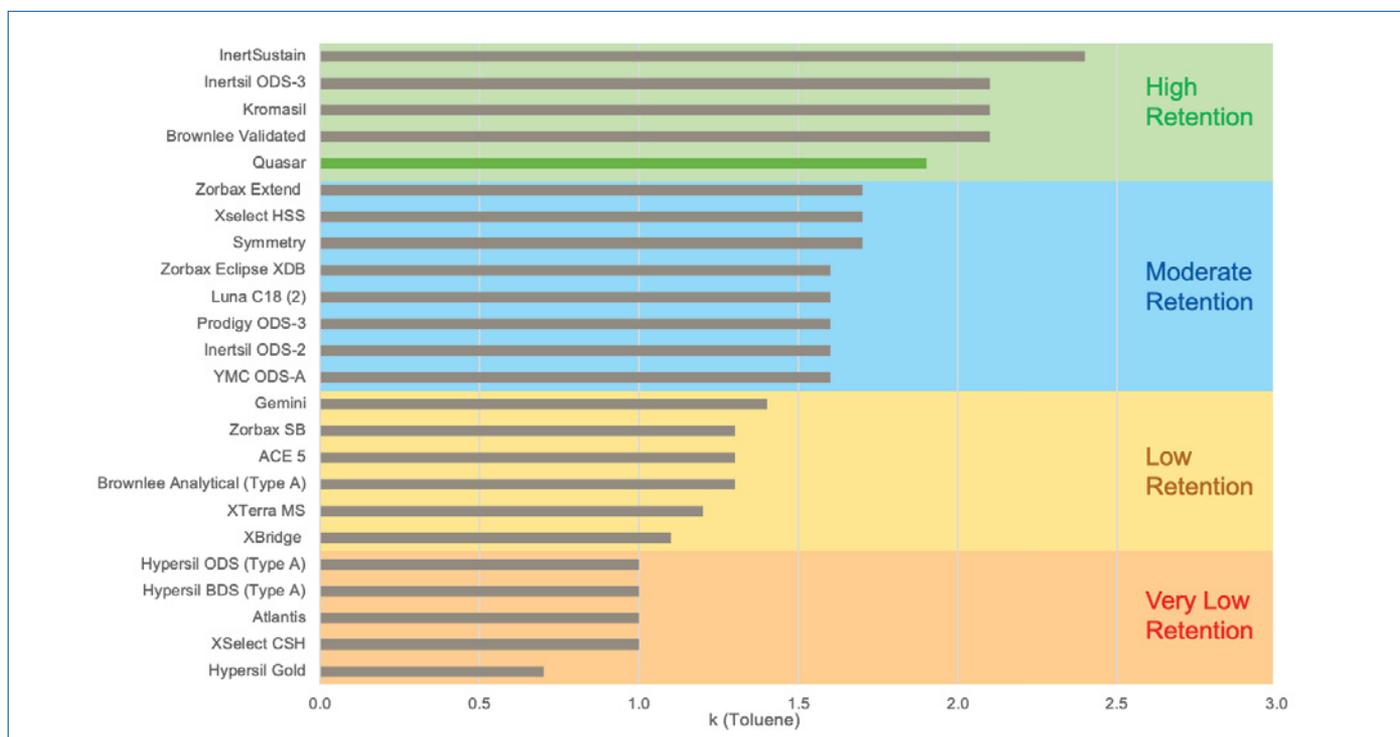


Figure 2. C18 phases ranked according to k' value of toluene.

Comparison of Column Efficiency

High retention needs to be accompanied by good peak shape for the most effective separations. Therefore, it is important to calculate column efficiencies (of toluene in this instance) to gain insight into overall column performance.

Column efficiency is a measure of the peak dispersion on the column, which reflects the column performance. It is typically reported as plates (N) or plates per meter (N/m). For a given column length, a greater number of plates implies less dispersion of chromatographic bands, narrower peaks, and a more efficient separation.⁶ Column packing quality, column dimensions, particle size, and formation of voids within the column all contribute to the column efficiency. It is important to note that later eluting peaks, which look broad in comparison with early eluting peaks, may have a higher column efficiency.⁵

There are various methods of calculating column efficiency, including the Foley-Dorsey method (Figure 3a) and Tangential methods (Figure 3b). In this study, the USP compliant tangential method has been used as it is widely adopted by the industry.

Using a neutral compound (such as toluene) for efficiency measurements allows retention from unwanted secondary interactions to be minimized. This ensures the primary interaction is the hydrophobic interaction between the alkyl chain and the neutral analyte; that in turn enables the data generated to provide a greater indication on factors such as particle size, packing efficiency, and particle size distribution. Smaller average particle sizes, higher quality packing and narrow particle size distributions all tend to result in higher efficiencies.⁶

Figure 4 shows the column efficiencies (N/m) obtained for toluene using the C18 columns included in this study. The data was obtained using the same neutral test mix as in the hydrophobicity study. Figure 5 illustrates the efficiency of toluene obtained on a selection of columns. High efficiencies were observed on several columns including Brownlee Validated C18, Luna C18 (2), and ACE 5 C18. However, much higher efficiencies were obtained on various columns, including Quasar C18. Higher efficiencies generally indicate narrower peaks, and thus allow greater scope to resolve critical pairs.

Figure 6 demonstrates a comparison between the efficiencies and retention of toluene for each of the columns. In the hydrophobicity study, the InertSustain C18 showed to be slightly more retentive than Quasar C18. However, high retention needs to be accompanied by good peak shape, and the InertSustain C18 did not show good peak shape when compared with Quasar C18 (Figure 7); it demonstrated 30 % less efficiency for toluene.

Additionally, by using Figure 6 as a guide, if a user switches from an XTerra MS C18 or Symmetry C18 to a Quasar C18, they could expect to observe a more efficient separation, with sharper peaks and greater retention for neutral compounds. Greater efficiency enhances the resolving power of the column and improves critical peak pair separation. If a method measures relative retention, then switching phases poses less of an issue. If, however, the method measures absolute retention and there is a fixed retention window, adjustments would need to be made to the mobile phase composition to alter the analyte retention profile.

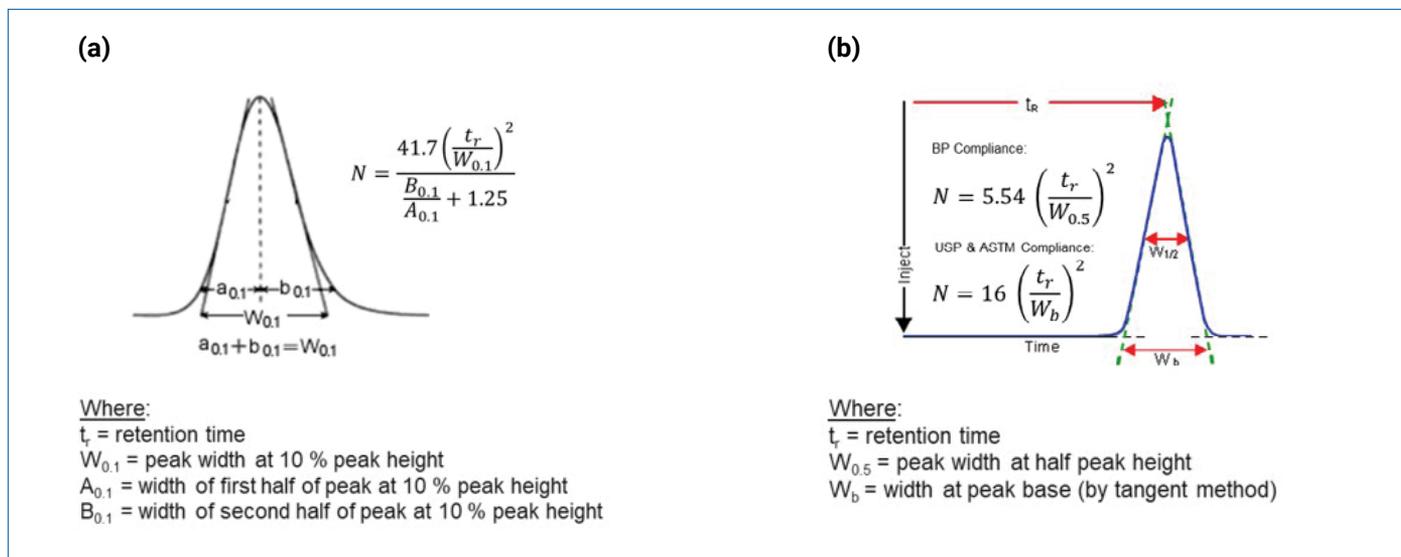


Figure 3. Foley-Dorsey approximation method (a) and Tangential method (b) for calculating column efficiency.^{7,8}

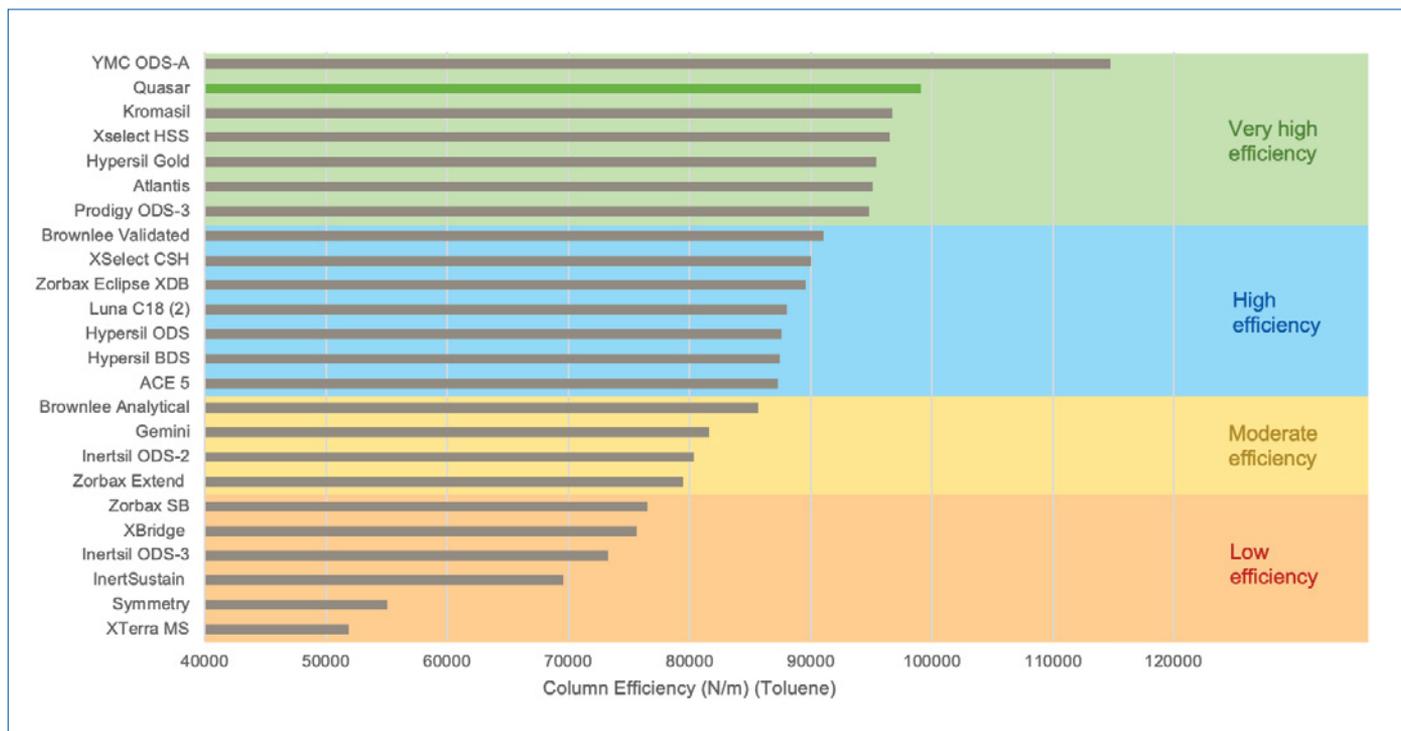


Figure 4. Column efficiencies (N/m) obtained for toluene for C18 150 x 4.6 mm, 5 μm columns.

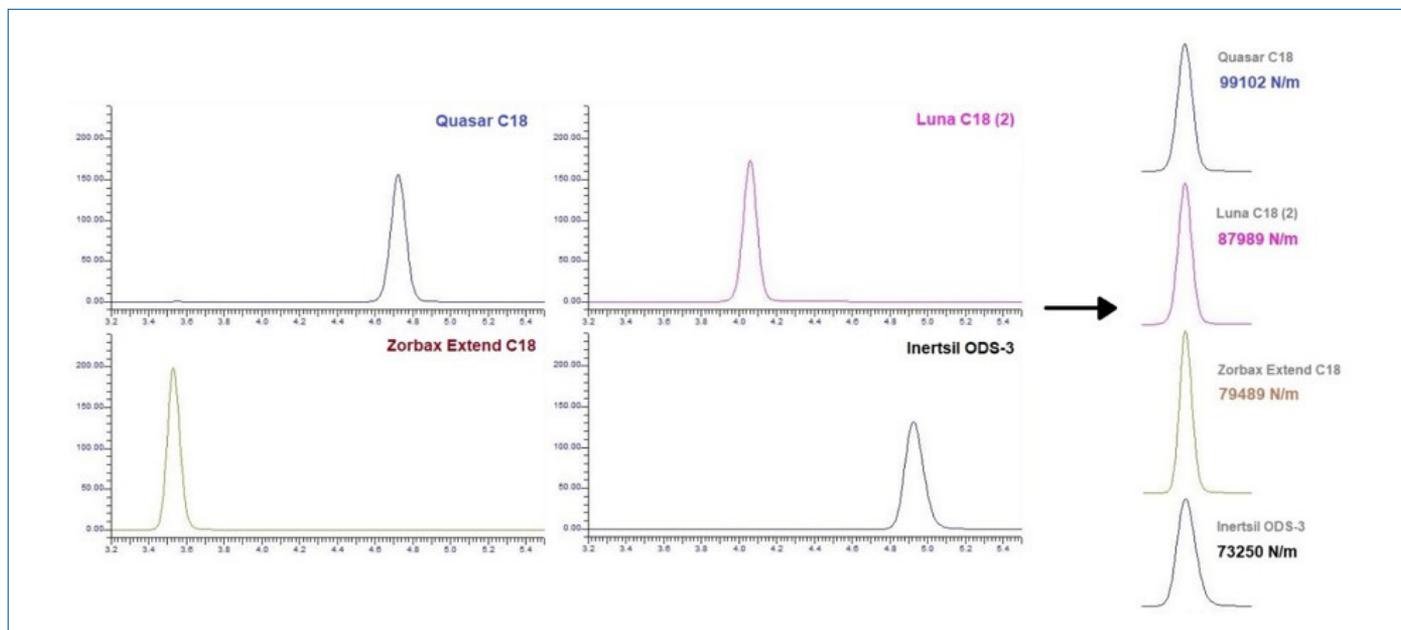


Figure 5. Comparison of various C18 columns for toluene peak shape. LC conditions: 80/20 (MeOH/H₂O) at 1 mL/min, 254 nm PDA detection, 25 °C oven temperature.

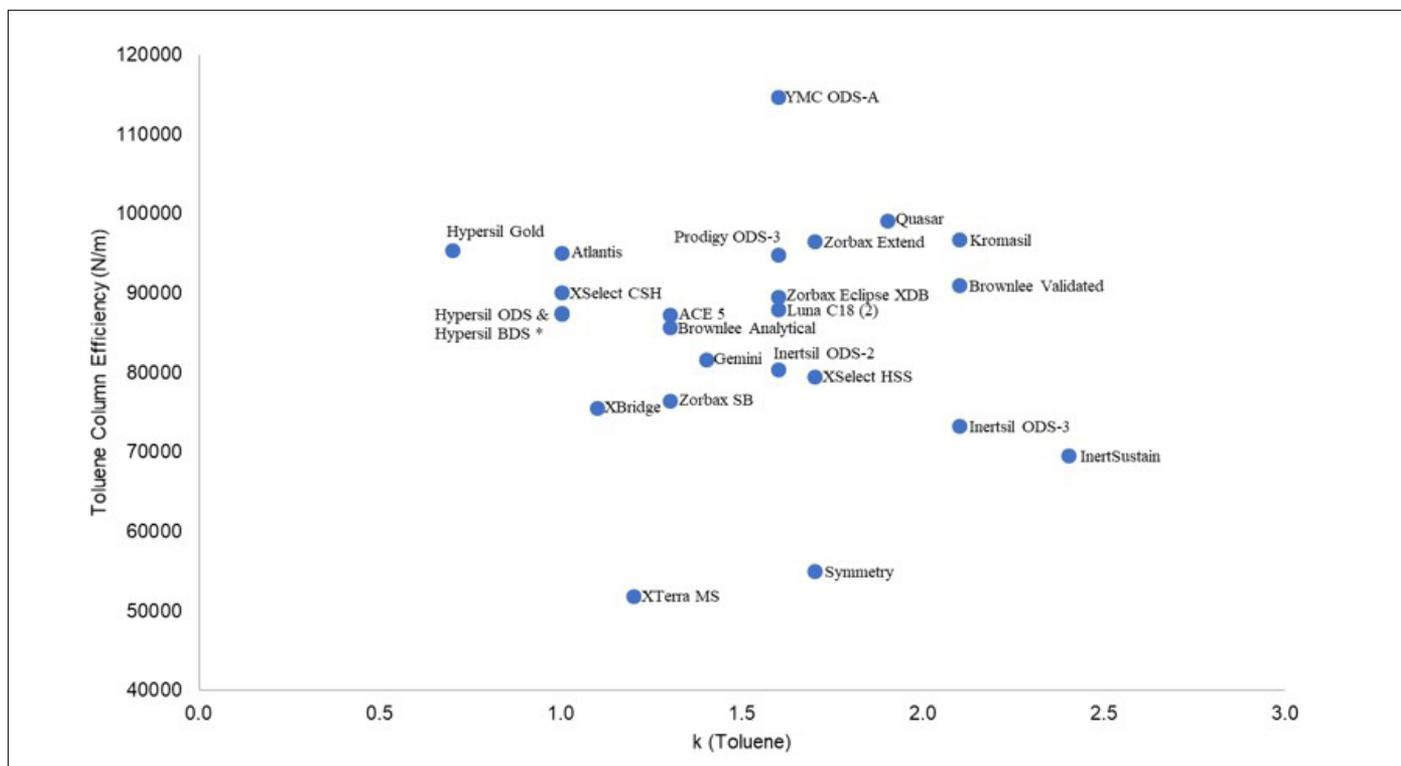


Figure 6. C18 columns ranked by retention and efficiency of toluene. (*Hypersil ODS and Hypersil BDS experienced similar k and column efficiency).

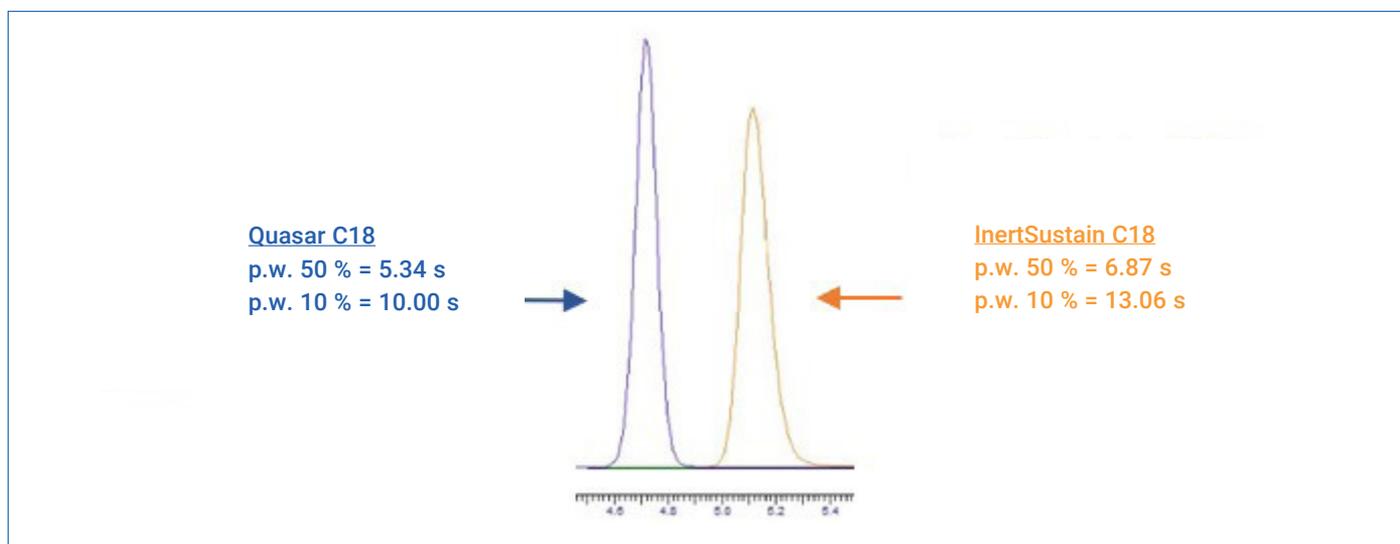


Figure 7. Toluene peak obtained using Quasar C18 (blue) and InertSustain C18 (orange) with peak widths (p.w.) shown at 50 % and 10 % peak height.

The YMC ODS-A phase showed the highest column efficiency for toluene, with the second ranking being Quasar C18. However, when observing the backpressures of these two columns, YMC ODS-A generated much higher backpressure than Quasar C18, 2020 psi as against 1660 psi respectively, a 22 % increase. Packing particle size is one of the largest factors which influences backpressure. Whilst smaller particles yield higher efficiencies, they also result in higher backpressures. The backpressure observed for the YMC ODS-A phase is more indicative of a 4 μm phase, as opposed to a 5 μm phase. Continuous use of LC columns under higher backpressures leads to deterioration in overall column efficiency, which can be detrimental to column lifetime. It may also limit the method development scope, specifically in terms of being unable to use faster flow rates necessary for rapid analysis.

Figure 8 shows a comparison of the column efficiencies obtained for toluene and the backpressures observed for a selection of columns. A reasonable mid-range backpressure was observed using Quasar. This allows Quasar to operate easily at higher flow rates and stay within pressure limitations of the pump and column. Conversely, the backpressure observed using the Zorbax Extend C18 was relatively low, but this phase also showed a much lower efficiency. Lower efficiencies indicate broader peaks, which can reduce the resolving power of the column. The XTerra MS, however, combined two unfavorable features, higher backpressure and very low efficiency.

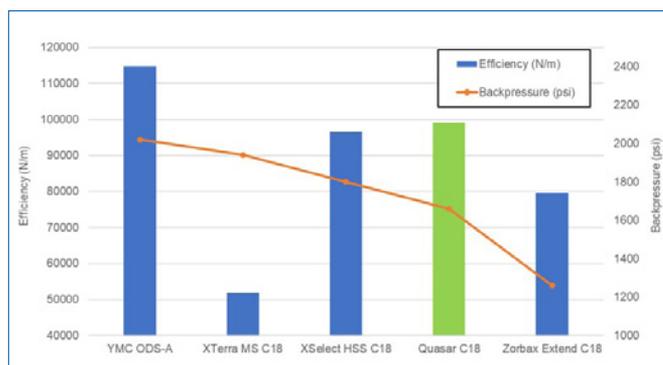


Figure 8. Comparison of toluene column efficiency and backpressure for a selection of C18 columns.

Evaluation of Silanol Activity

Using neutral compounds are useful for determining packing efficiency and efficiency of a column. However, it does not indicate the activity of the silica surface or how the column might perform when separating basic/cationic compounds. Basic compounds can frequently interact with weakly acidic underivatized residual silanols (SiOH) on the surface of the silica particles. This unwanted secondary retention causes the analytes to be retained longer than the 'bulk' of the analyte molecules, which results in peak tailing and impacts on the separation efficiency of the column.⁹ Ion-exchange and hydrogen bonding are thought to be the two most significant interactions which govern the process.¹⁰

The secondary retention is most problematic when the column is used between pH 4 – 8, in which ion-exchange governs the interaction process. Within this range, ionized silanols (SiO⁻) (Figure 9) can form ion-exchange sites which interact with protonated bases and cause secondary retention (Figure 10).¹¹

Below pH 3, hydrogen bonding dominates and residual silanol groups tend to be in the less active un-ionized form.^{1,10}

Silica purity is an important factor for LC columns in order to obtain peaks with little tailing and maintain high efficiency separations. Surface metals (M⁺) can further activate free silanol groups, making them even more acidic, and become even more likely to interact with basic analytes. High purity silicas (e.g. Type B) have lower and more tightly controlled levels of metal ions than older generation silicas (Type A). These properties result in lower silanol activity and better peak shape for basic compounds.¹²

Efficiencies and peak shapes of basic compounds are affected by silanol interactions, thus comparison of efficiency and tailing factors for a basic compound, such as pyridine (used in this

study), can be used as an indicator of silanol activity. Phases with 'very low' levels of silanol activity tend to also have higher column efficiencies.¹⁰

The tailing factor is a measure of peak tailing, which is calculated using the equation shown in Figure 11 at 5 % peak height. A tailing factor of one indicates a perfectly symmetrical peak. Peak tailing occurs when the tailing factor is >1, and peak fronting occurs when the tailing factor is <1. Calculation at 5 % peak height, rather than at 10 %, provides a more rigorous test and better discrimination between phases.

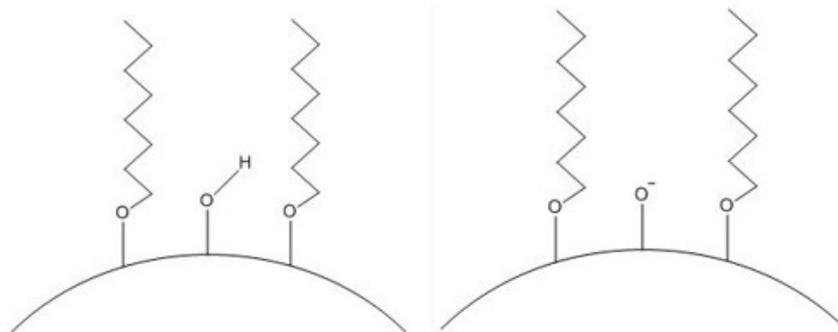


Figure 9. Unionized silanol (Si-OH) (left) and ionized silanol (SiO⁻) (right).

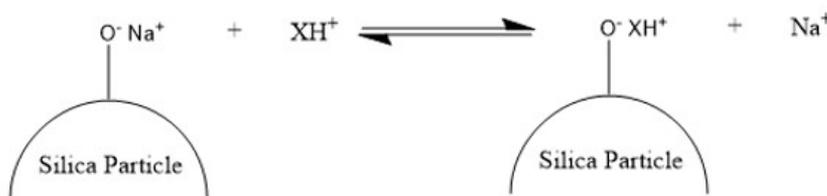
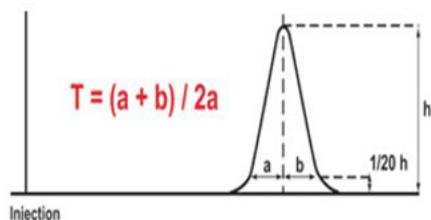


Figure 10. Ion-exchange interaction between a protonated basic compound (XH⁺) and an ionized silanol group, which often results in peak tailing.¹¹



Where:

T = Tailing factor

a = peak width between the peak maximum and the front edge of the peak at 5 % of the peak height

b = peak width between the peak maximum and the back edge of the peak at 5 % of the peak height

Figure 11. Tailing factor equation and method.

Figure 12 shows the tailing factors for pyridine using the C18 columns included in the study. The mobile phase conditions were at near neutral pH to promote interaction between pyridine and silanols. No buffer was used in this study as its presence in the mobile phase can mask potential interactions of the silica surface and the analyte.

The Quasar C18 phase demonstrated excellent peak shape with very low tailing ($T = 1.29$) when compared with other phases (e.g. XSelect HSS, $T = 1.93$), due to its ultra-high purity silica base and low residual silanol activity.

The YMC ODS-A and Kromasil C18 columns both show high efficiencies in neutral separation and ranked in the top group; conversely, they demonstrated lower rankings in the silanol activity study. Much higher peak tailing is observed when they are compared with Quasar C18, with tailing factors of 2.26 and 3.04 respectively. These results indicate that their silica surfaces cause more unwanted secondary interactions with basic compounds (Figure 13).

As expected, the highly acidic, traditional, type A silicas (Hypersil BDS, Brownlee Analytical C18 and Hypersil ODS) showed extremely strong retention of pyridine, with very broad to no elution over a 30-minute period.

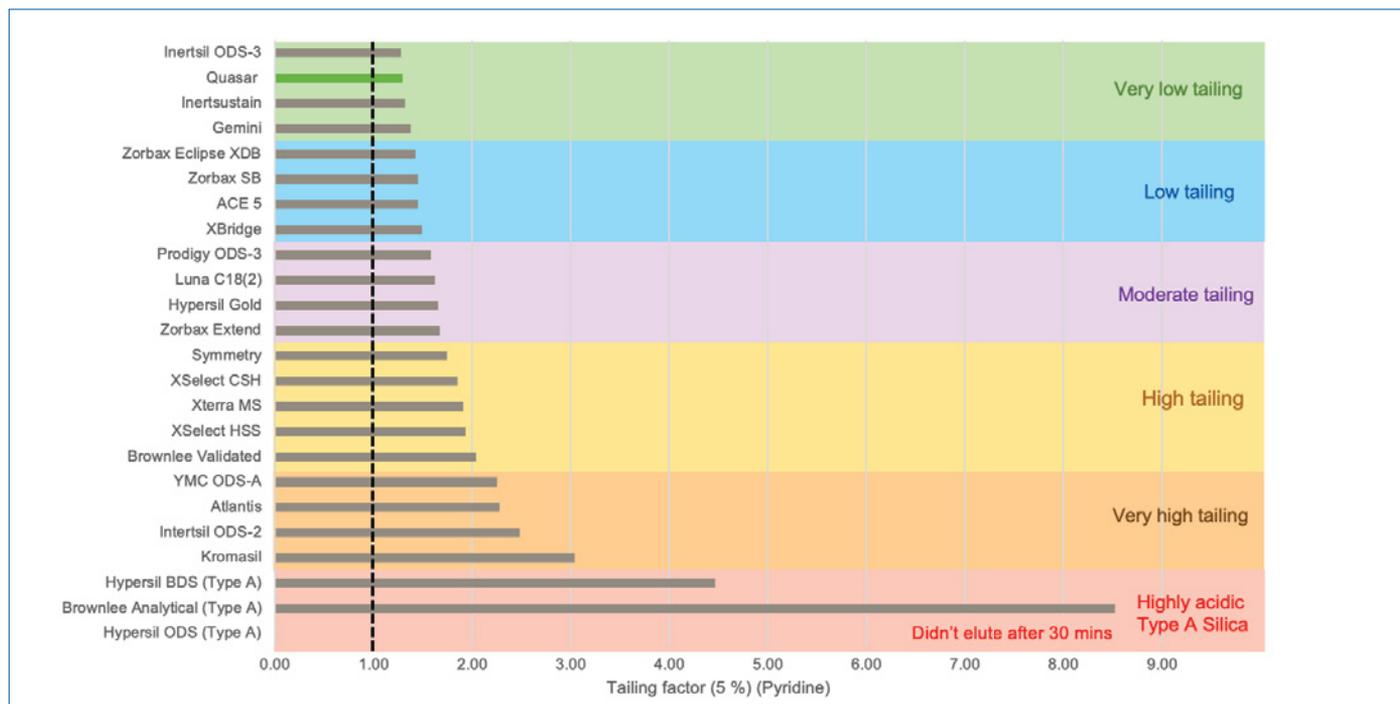


Figure 12. Tailing factors (5%) obtained for pyridine for the C18 150 x 4.6 mm, 5 μm columns.

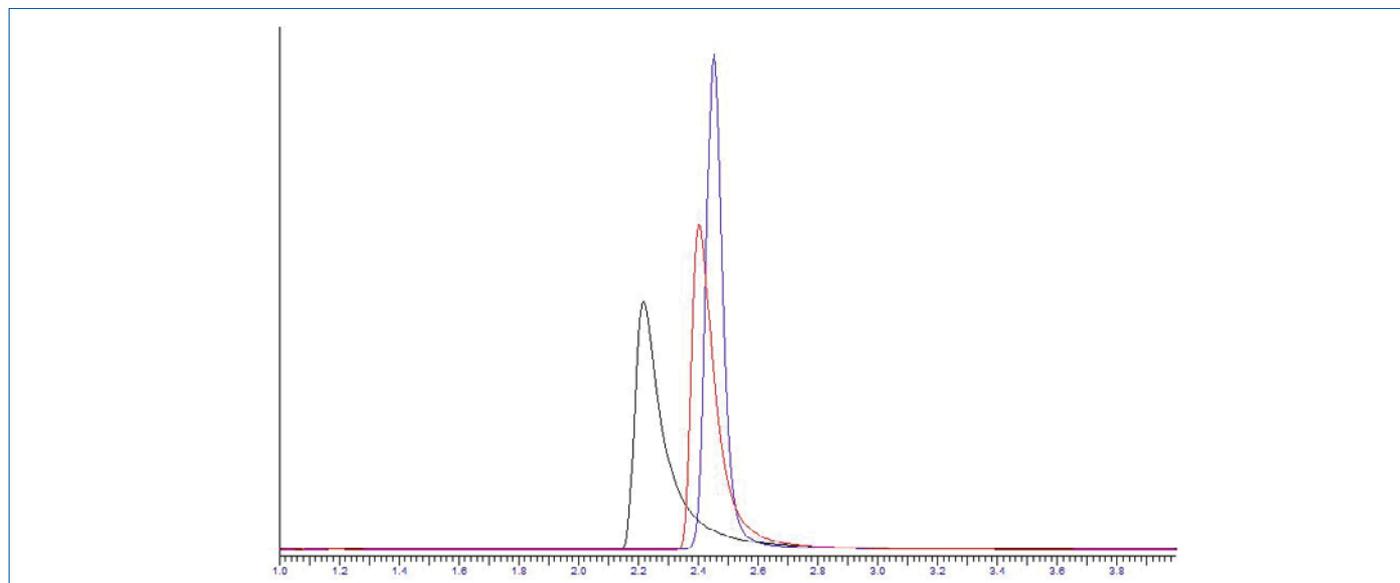


Figure 13. Pyridine peak obtained using Quasar C18 at 2.45 mins (blue), YMC ODS-A at 2.40 mins (red), and Kromasil C18 at 2.22 mins (black).

As mentioned previously, column efficiency can be calculated using tangential methods or the Foley-Dorsey method. The latter method, used in the silanol activity study, provides a more accurate calculation of theoretical plates by introducing equation parameters which accommodate the peak asymmetry.¹³

Figure 14 shows the column efficiencies (N/m) obtained for pyridine for the C18 columns included in this study. Figure 15 illustrates a few examples of the pyridine peak shape obtained on different columns. High efficiencies were obtained on the Inertsil

ODS-3 and Zorbax Eclipse XDB-C18. However, Quasar C18 demonstrated the highest efficiency for pyridine, with a value of 44,185 N/m.

The YMC ODS-A phase demonstrated excellent efficiency for neutral compounds but produced very low column efficiency for pyridine. When compared with the Quasar C18, the YMC ODS-A showed a 74 % drop in efficiency. Additionally, Kromasil C18 demonstrated very high efficiency for neutral compounds, but showed extremely low efficiency for pyridine. In comparison with Quasar C18, a 90 % drop in efficiency for pyridine was observed.

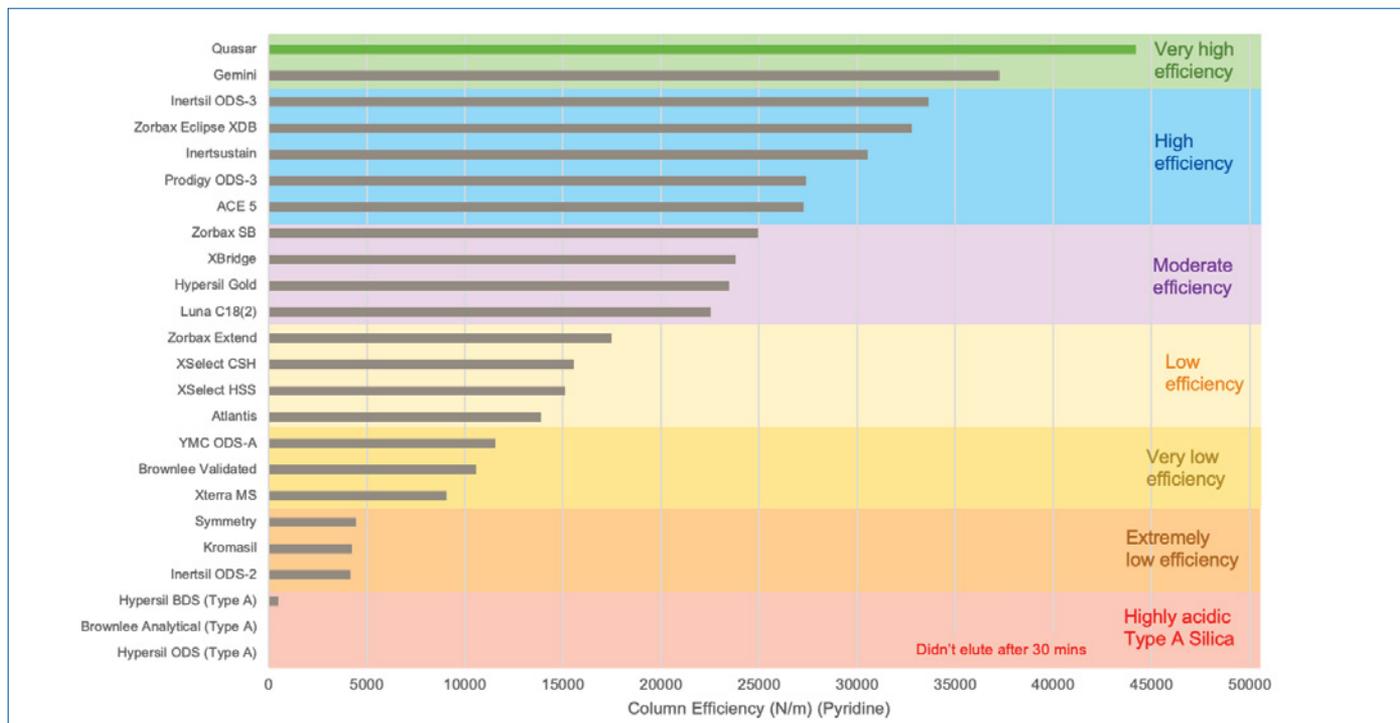


Figure 14. Column efficiencies (Foley-Dorsey) obtained for pyridine for all C18 150 x 4.6 mm, 5 μm columns.

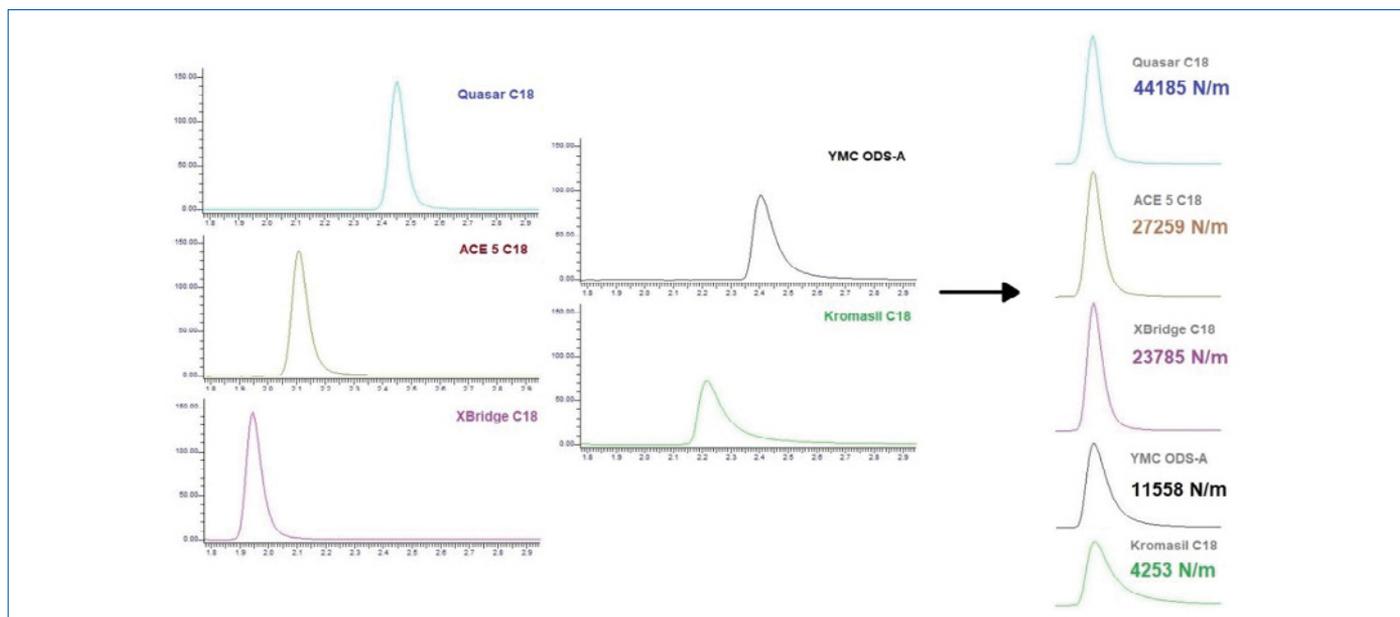


Figure 15. Comparison of various C18 columns for column efficiency of pyridine and peak shape. LC conditions: 60/40 (MeOH/H₂O) at 1 mL/min, 254 nm PDA detection, 25 °C oven temperature.

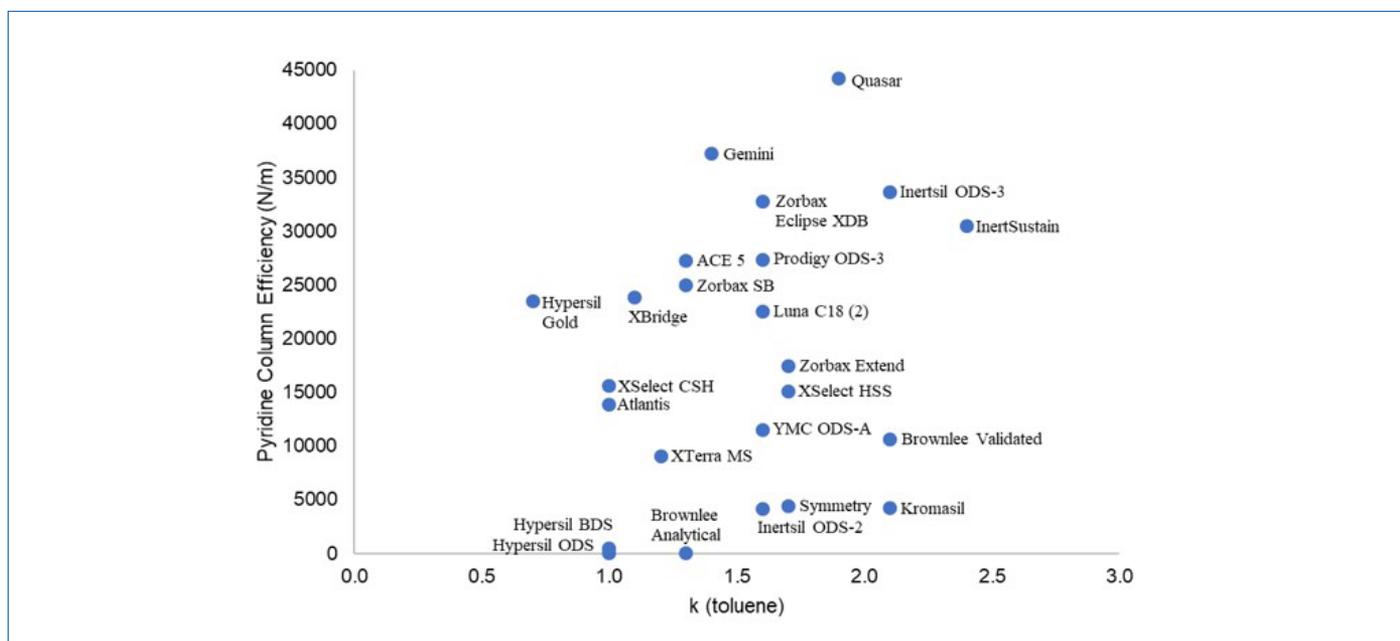


Figure 16. Column efficiency of pyridine vs. retention (k) of toluene for all C18 columns.

A plot of pyridine column efficiency against retention (k) of toluene (Figure 16) shows how the columns compare for the two tests. The Inertsil ODS-3 and Quasar C18 phases both show high retention for toluene and high efficiency for pyridine, with Quasar being more efficient than the Inertsil ODS-3. As such phases yield efficient separations, with good resolution, there is scope to optimize methods and reduce overall run times which in turn improves productivity. Conversely, columns such as Atlantis C18 and XTerra MS C18 show much reduced retention for toluene and much lower efficiencies for pyridine. For less retentive columns, such as these, the water content of the mobile phase would need to be increased to improve retention, which would result in lower MS sensitivity. Additionally, Kromasil C18 is an example of a column demonstrating high retention for toluene, a desirable attribute, but extremely low efficiency for the basic compound pyridine. This enhanced retention allows greater opportunity to resolve analytes without increasing column length. However, poor efficiencies are the result of poor peak shape which can also degrade the resolving power and cause difficulty in resolving critical peak pairs.

Conclusion

In this in-depth study, evaluation of relative hydrophobicity, efficiencies for a neutral compound, and silanol activity have been investigated for twenty-four silica-based C18 phases. It has been shown that, despite being the same bonded phase, there are differences between these columns. This study provides a guide to help with alternative column selection.

PerkinElmer's Quasar C18 phase has shown to have a highly retentive stationary phase due to the high surface area of the silica, combined with the optimized ligand bonding process applied. Highly retentive phases provide greater opportunity to resolve analytes without increasing the column length or increasing the mobile phase water content. MS sensitivity is enhanced with mobile phase containing low water content. In addition, the backpressures observed using Quasar allows the column to operate easily at higher flow rates and stay within pressure limitations of the pump and column.

High retention needs to be accompanied by good peak shape, which Quasar has shown. Quasar demonstrated very high column efficiencies for neutral compounds, with data for toluene presented. Additionally, Quasar has demonstrated very high efficiency and very low peak tailing for a basic compound, pyridine, due to its ultra-high purity silica base and low residual silanol activity.

It is important to note that a column which provides desirable separation and characteristics for one application, may not be the most suitable for another application. However, the comparative data obtained provides a good indicator as to how the columns evaluated might perform for neutral and basic analytes.

References

1. LCGC's CHROMacademy, All HPLC C18s are the same aren't they? Retrieved from <https://www.chromacademy.com/chromatography-HPLC-C18-all-the-same.html>. Date Accessed: March 2020.
2. LCGC's CHROMacademy, Selecting Reversed Phase Columns. Retrieved from <https://www.chromacademy.com/lms/sco5/10-Selecting-Reversed-Phase-Columns.html>. Date Accessed: March 2020.
3. The LCGC Blog, Things you should know about your HPLC column, Part I – Pore Sizes and Particle Diameters. Retrieved from <http://www.chromatographyonline.com/lcgc-blog-things-you-should-know-about-your-hplc-column-part-i-pore-sizes-and-particle-diameters>. Date accessed: March 2020.
4. M.W. Dong, Modern HPLC for Practicing Scientists, John Wiley & Sons, Inc., New Jersey, 2006.
5. LCGC's CHROMacademy, The Theory of HPLC Chromatographic Parameters. Retrieved from https://www.chromacademy.com/lms/sco2/Theory_Of_HPLC_Chromatographic_Parameters.pdf. Date accessed: March 2020.
6. B.A. Bidingmeyer, Practical HPLC Methodology and Applications, John Wiley & Sons, Inc., New York, 1992.
7. J.P. Foley & J.G. Dorsey, Equations for Calculation of Chromatographic Figures of Merit for Ideal and Skewed Peaks, Anal. Chem., 1983, 55, 730-737.
8. N.A. Parris, Instrumental Liquid Chromatography: A Practical Manual on High Performance Liquid Chromatographic Methods, Journal of Chromatography Library – Volume 5, Elsevier, The Netherlands, 1979.
9. LCGC's CHROMacademy, The Theory of HPLC Column Chemistry. Retrieved from https://www.chromacademy.com/lms/sco4/Theory_Of_HPLC_Column_Chemistry.pdf. Date accessed: March 2020.
10. M. Kele & P.C. Iraneta, CHROMATOGRAPHY: LIQUID | Column Testing and Evaluation, Chemistry, Encyclopedia of Separation Science, 2007, 1-7.
11. T. Tuzimski & J. Sherma, High Performance Liquid Chromatography in Pesticide Residue Analysis, Taylor & Francis Group, Florida, 2015.
12. LCGC's CHROMacademy, HPLC Troubleshooting Guide – Peak Tailing. Retrieved from <https://www.chromacademy.com/chromatography-HPLC-Troubleshooting-Peak-Tailing.html>. Date accessed: March 2020.
13. L. Ohannesian & A. Streeter, Handbook of Pharmaceutical Analysis, Marcel Dekker, Inc., New York, 2005.