Advances in Superficially Porous Particles for Increased Method Development Flexibility and Scalability

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One of the most significant recent advancements in LC column technology is the new generation of superficially porous silica particles. These particles provide similar efficiency to smaller diameter totally porous particles but with significantly lower backpressure. While chromatographers enjoy the ultra-high efficiency of these particles, they also desire more selectivity options to facilitate method development. Early introductions of SPP columns focused on expanding selectivity options with new phase chemistries, but recent innovations have enabled the use of pH as a selectivity tool through new approaches to particle development. Additionally, expansion outside the small molecule arena into biopharmaceutical applications with multiple particle and pore size options is further fuelling growth in usage of these Fast LC columns.

Over the past few years, superficially porous particle (SPP) columns have seen tremendous growth as users have recognised their advantages over traditional totally porous columns. For new methods, the popularity of SPP columns has exceeded that of sub-2 µm columns. Major benefits include robustness, high efficiency, and low backpressure. Modern superficially porous particle columns were introduced in 2006, when Kirkland and co-workers commercialised the first sub-3 µm SPP column (DeStefano, Langlois, & Kirkland, 2008). Since that time, multiple manufacturers have introduced their own families of SPP columns, including AMT HALO, Phenomenex Kinetex, Agilent Technologies Poroshell 120, and Waters CORTECS, a recent introduction to the SPP market. Additionally, many smaller companies have started to offer their own SPP columns as well, leading to wide adoption of these technologies in the HPLC chromatographic laboratory.

These columns have been shown to improve separation efficiency significantly (Long & Wang, 2012), enabling the ability to utilise faster methods to achieve higher throughput. With more and more laboratories focusing on increasing sample throughput, migrating to these SPP technologies can provide significant advantages over totally porous columns. Furthermore, adopting larger particle size SPP columns versus sub-2 µm columns translates to lower backpressures, enabling chromatographers to get the most out of their LC systems.

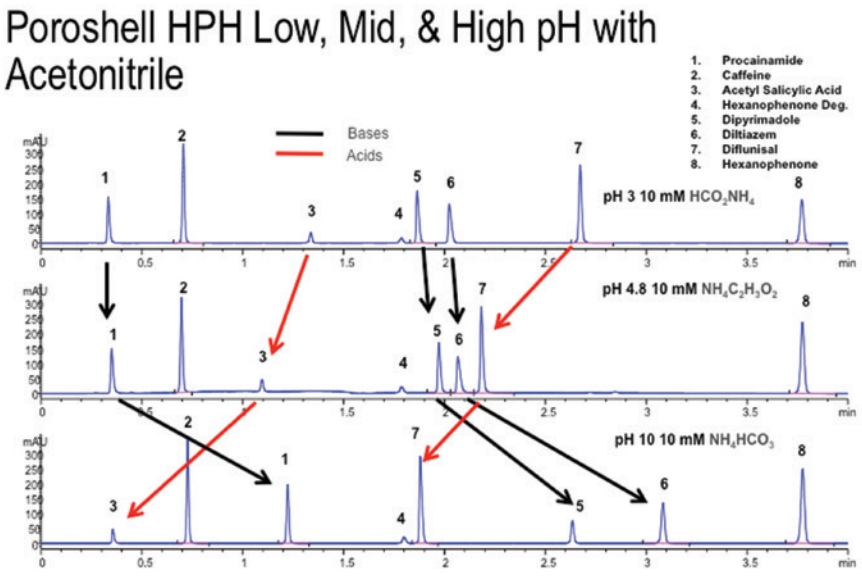

Figure 1

While migrating to SPP columns provides significant advantages over totally porous columns, there have been some challenges associated when the technology was initially launched. Method development, for example, typically takes advantage of multiple chemistries to exploit various aspects of analyte interactions for achieving desired resolutions. Early in the release of this column technology, chemistry choices were limited, reducing the options for method development. However, as the column families expanded, so have the number of chemistries, increasing the flexibility for chromatographers. Additionally, recent improvements to the particles themselves enable robust operation under high pH conditions (Use of Agilent Poroshell HPH-C18 Columns at Elevated pH as a Tool for Method Development, 2014), enabling the utilisation of pH as a method development tool without the fear of reduced column lifetime under elevated pH conditions.

Method development with SPP columns using pH as a selectivity tool

Method development often involves the separation of mixtures of analytes, both simple and complex, and selectivity plays an important role in resolution of these

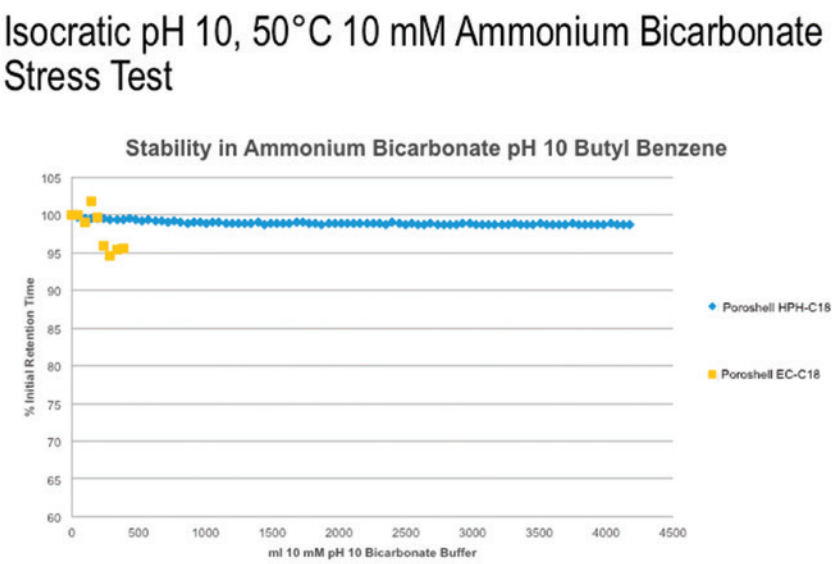

Figure 2

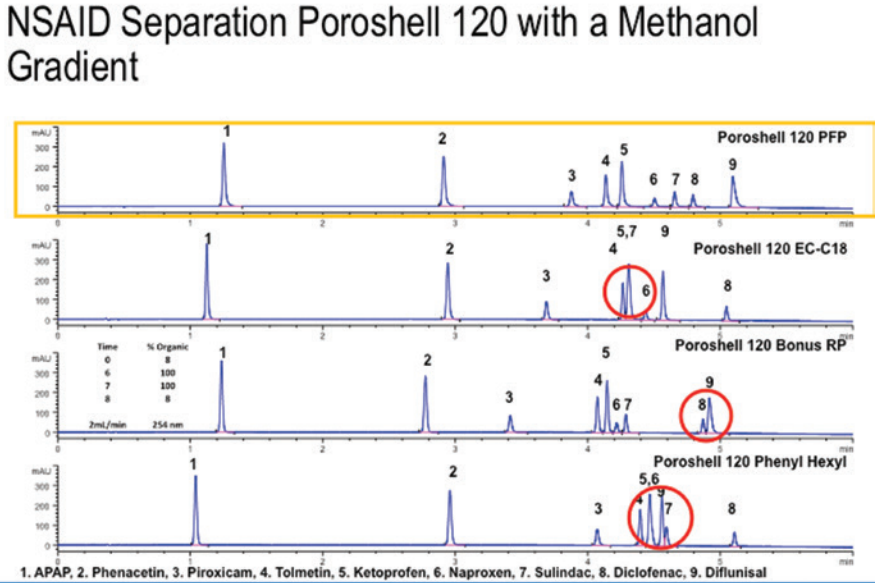

Figure 3

analytes. Selectivity can be controlled though several factors, including the choice of stationary phase, the type of organic modifier, gradient slope, flow rate, and temperature. For ionisable compounds, pH of the buffer is also a powerful parameter. Optimising the separation of ionisable compounds to find robust conditions has become an important part of method development in liquid chromatography. Most pharmaceutical and biological compounds contain ionisable moieties such as carboxylic or amino groups. Because retention in reversed-phase liquid chromatography (RPLC) using traditional alkyl phases is strongly dependent upon the analyte charge, pH can be used to make large changes in selectivity. At a pH below their pKa, acids have their maximum retention because they are neutral, but bases have their minimum retention because they are fully charged. At basic pH (above the pKa of the compound), bases have their maximum retention because they are neutral, and acids are fully ionised and have their minimum retention. For the best peak shape, retention and sample loading of basic analytes in RPLC, the mobile phase pH should be two units higher than the pKa of the compound of interest. For many pharmaceutically derived compounds this in itself can be challenging since these compounds will typically have multiple pKa's, but in general performing a separation at a higher pH will have a beneficial effect on the peak shape. The retention of neutral compounds is unaffected by pH.

Until recently, all SPP materials possessed limited lifetime in higher pH buffers due to the silica based particle morphology. To achieve longer lifetimes it is necessary to protect the base particle by either surface modification or special bonding modification. Agilent addressed the issue of silica dissolution under elevated pH conditions with the introduction of Poroshell HPH-C18 and HPH-C8 technologies. This was achieved with a proprietary process that organically modifies the silica surface, enabling the chromatographic support to remain stable even under high pH. Furthermore, by adding this technology to the Agilent Poroshell 120 family, Poroshell 120 SPP columns can be used for all Fast LC method development needs, regardless of the mobile phase pH.

Figure 1 has shown how this chemistry offers additional flexibility in your method development. Here, a method with low, medium, and high pH was used to separate a mix of acids, bases, and neutral compounds. As the best resolution for all compounds was obtained under the higher pH conditions, this would be the best choice for the method in the future. This presents a problem with traditional silica based substrates. However, with the organically modified silica support in Poroshell HPH-C18, the column can be employed at an elevated pH with far less impact on column lifetime. Figure 2 demonstrates the effects of 1,600 injections over 10,000 column volumes, with another separation mixture of acids, bases, and neutrals, at pH 10. There is little impact on retention under elevated pH conditions. A recent addition in this space, the Phenomenex Kinetex EVO, also utilised an organically modified silica substrate; though this material is currently only available in the larger, 5µm, particle size columns.

Bonded phase expansions for flexible method development

While the advancements in pH stability improve the ease of method development, the availability of multiple phase chemistries also aids with respect to choices for method development. With the addition of the bonded chemistries available for high pH stability along with other chemistries that take advantage of other analyte interactions, the Poroshell 120 family has been expanded to include 12 bonded chemistries, offering multiple options for chromatographers.

Chromatographers are often seeking chemistries for the analysis of polar analytes, and one of the most recent bonded chemistry developments to address this

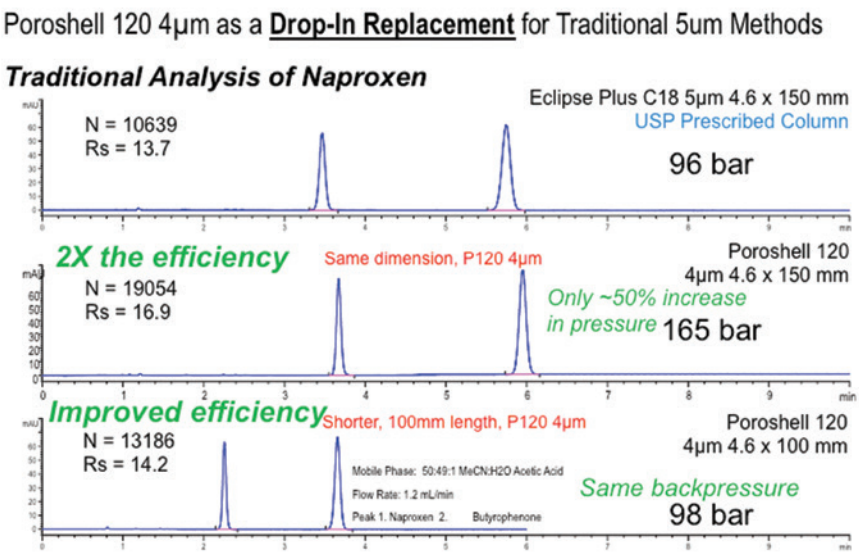

Figure 4

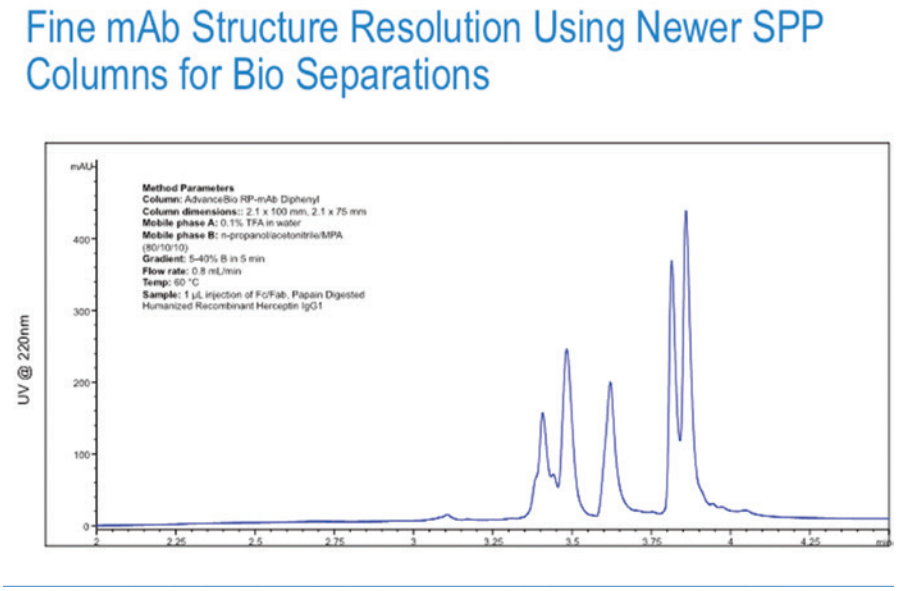

Figure 5

need has been the addition of the PFP chemistry. The PFP chemistry has steadily gained popularity over the past few years as chromatographers seek to enhance separations of polar compounds containing hydroxyl, carboxyl, or other polar groups. Furthermore, because of the fluorinated phenyl ring structure, PFP chemistries can enhance the separation of positional isomers and halogenated compounds (Przybyciel, 2006).

In Figure 3, one can see the improved performance PFP offers in the separation and resolution of non-steroidal anti-inflammatory drugs (NSAIDs). Bonus RP and Phenyl-Hexyl – both chemistries which are typically recognised for their orthogonality when compared to C18 chemistries – do not resolve all compounds, while the Poroshell 120 PFP completely resolved all analytes of interest in this case. While not indicative that all compounds of this class can be separated using a PFP phase, it illustrated the value in having multiple chemistries at ones disposal, and why manufacturers continue to release additional chemistries with unique polar character, demonstrated most recently in the release of the Phenomenex Kinetex F5 chemistry.

Hurdles with sub-3 and sub-2 µm SPP columns

While method development has been made easier through the availability of more phase chemistries, there are still many cases where the transition to sub-3 µm SPP columns cannot be performed. In order to properly adopt modern Fast LC columns such as these, LC systems must be configured for low dead volume to take advantage of the improved performance.

Also, since many users are transitioning from traditional 5 µm columns, the increased back pressure, while often still maintained below 400 bar, can cause some issues. Systems configured with PEEK tubing and PEEK fittings offer the flexibility in system configuration and setup, but can be limiting in column options due to their inherent pressure limits, limiting users to 200 bar operation.

As a result of these potential limitations, manufacturers of SPP columns expanded their range of particle options to include 4 and 5 µm SPP columns, with introductions from AMT, Phenomenex, Thermo Scientific, and Agilent. These columns can offer double the efficiency of a conventional 5 µm column, with minimal increases in backpressure, thus enabling the continued use of PEEK tubing and fittings and the ease of use that brings. Moreover, since the particle sizes are similar, these columns can often be upgraded as drop-in replacements, improving the separation with little to no method changes required. With half the column back pressures of the 2.7 µm SPP columns and efficiencies nearly double that of traditional totally porous 5 µm columns, users can achieve improved performance with the ease of dropping in a replacement column. An example of this is shown in the analysis of naproxen (Figure 4). Here, the USP standard analysis of naproxen was transitioned from an Eclipse Plus 5 µm C18 column to a Poroshell 120 4 µm EC-C18 column with no changes in column length or method parameters. The efficiency was improved nearly 50%, while the backpressure was maintained below 200 bar. Further improvements were made to shorten the column, reducing the analysis time by 50%, improving overall sample throughput. Additional improvements could be made using smaller SPP particle columns, albeit at the expense of slightly higher back pressures. The larger particle SPP columns offer a good compromise for increased performance while maintaining current methods and low back pressures.

Improvements in biopharmaceutical applications with new SPP column options

With many of the innovations in SPP columns focus on small molecule analysis, developments have recently turned toward biopharma applications for improvements. Key areas are in the analysis and characterisation of glycans. N-linked glycosylation is a critically important and very complex post-translational modification.

It therefore needs to be controlled and monitored throughout the development, processing, and manufacture of glycoproteins drugs. Therapeutic protein characteristics, including safety, efficacy, and serum half-life, can be affected by differences in their glycosylation pattern and so the analysis of these patterns is an important part of the characterisation of therapeutic glycoproteins, particularly mAbs. The Agilent AdvanceBio Glycan Mapping Columns, available in both totally porous sub-2 µm totally porous and 2.7 µm SPP columns take advantage of amide bonded chemistry to enable fast HILIC mode analysis of glycans. Additionally, AMT HALO released a similarly product, the HALO Penta-HILIC, targeted for N-linked Glycan and glycopeptide analysis.

Additionally, with the continued importance of biotherapeutic proteins, comprehensive characterisation is a prerequisite. It is of paramount importance for the production process to be highly consistent and robust, and that any change in process related impurities that could impact clinical outcomes and immunogenicity are avoided. Characterisation of monoclonal antibody (mAb) primary structure using reversed-phase liquid chromatography is a key activity in bio-pharma discovery, development and QA/QC. Comprehensive characterisation requires the analysis of intact, heavy and light chains, and Fc and Fab regions of the antibody using liquid chromatography often coupled with high resolution mass spectrometry. Increases in the speed and resolution offered by the HPLC columns used in these separations facilitate improvements in the quality of characterisation data. Product releases in this space include the Agilent AdvanceBio RP-mAb column - based on Poroshell technology, Phenomenex Aeris, and the AMT HALO Protein column. Each of these takes advantage of the improved performance with SPP columns, and is ideal for separation and analysis of mAbs and proteins for biotherapeutic monitoring. Each of these is made up of a particle in the 3 to 4 µm range with pore sizes optimised for larger biomolecules. In Figure 5 a fast separation of the fine structure detail is demonstrated on an Advancebio RP-mAb column, indicating the improved speed and resolution that can be obtained with these materials, performance typically reserved for smaller particle based bio-LC columns.

Conclusion

Through the continued improvements and innovations in particle structure and expansion in chemistries and applications, SPP columns have shown that they will continue to be a focal point in chromatographic analyses. With the performance improvements and method development flexibility they offer, they are growing in adoption with laboratories. And, as more and more methods are developed using SPP columns, they are beginning to be used extensively in QA and QC laboratories and across multiple application areas in addition to pharma and biopharma analyses, including food, environmental, and clinical research areas.

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