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# Low Adsorption HPLC Columns Based on MaxPeak High Performance Surfaces

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## CHALLENGES IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SEPARATIONS OF METAL-SENSITIVE ANALYTES

Because of its manufacturability and pressure capabilities, stainless steel has long been the preferred material for constructing HPLC columns. However, stainless steel hardware is susceptible to corrosion[1] and can negatively impact the peak shape and recovery of some analytes.[2-4] Titanium and nickel-cobalt alloys have thus been used as alternative materials for particular applications.[5] While these alternatives exhibit improved corrosion resistance, they still present major challenges for some analyses.[6]

The interaction between certain classes of analytes and metal surfaces results from electron sharing. Most metals are covered with a thin layer of metal oxide.[1] Transition metal ions in this layer are electron deficient, which leads them to act as Lewis acids. Meanwhile, many analyte molecules contain moieties that are electron rich, such as phosphate and carboxylate groups. Being Lewis bases, these molecules can adsorb to transition metal ions on the surface of HPLC columns. This interaction becomes very significant if a molecule contains multiple electron rich moieties arranged such that it can adsorb by multi-dentate chelation. In practice, this may result in poor chromatographic peak shape, severe analyte losses, and quantitative inaccuracies.

In a workaround, chromatographers often address issues with metallic column hardware by adding chelators like EDTA to the mobile phase or sample diluent.[4,7] Volatile chelators such as citric acid and acetylacetone have been used in mobile phases for LC-MS analyses.[8, 9] However, the use of chelators can negatively impact chromatographic selectivity and MS performance.

Because of these effects, HPLC analyses of metal-sensitive compounds have required the use of metal-free columns. Polyether ether ketone (PEEK) has historically been the material of choice to use in place of stainless steel. While PEEK is a reasonably good fit for HPLC (<5000 psi), it does not have the mechanical strength needed to be viable on its own under ultrahigh pressure conditions (≥5000 psi). Steel-clad PEEK tubing has recently been developed to allow higher pressure operation. It has been shown that the internal diameters of PEEK tubing can exhibit more variability than those of stainless steel and titanium tubing.[10] Therefore, comparatively large retention shifts might be encountered upon switching between PEEK-lined columns. In addition, PEEK is not compatible with some solvents, notably tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and chlorinated solvents such as chloroform and methylene chloride.[11] Moreover, PEEK is a relatively hydrophobic polymer. Because of hydrophobic secondary interactions, it can sometimes be necessary to passivate PEEK surfaces via multiple sample injections.[12, 13]

### COMPARING MaxPeak HPS FOR PREMIER COLUMNS AND QuanRecovery VIALS AND PLATES

MaxPeak High Performance Surfaces are a collection of novel surfaces designed to address the shortcomings of materials traditionally used in chromatographic analyses. Reversed-phase and HILIC PREMIER columns are constructed with a novel LC surface composed of a chemically resistant hybrid organic/inorganic material that minimizes the interaction of analytes with metallic flow paths. In contrast, QuanRecovery Plates and Vials are based on an oxygen-enriched surface that serves to block and mitigate hydrophobic adsorption between analytes and polypropylene.

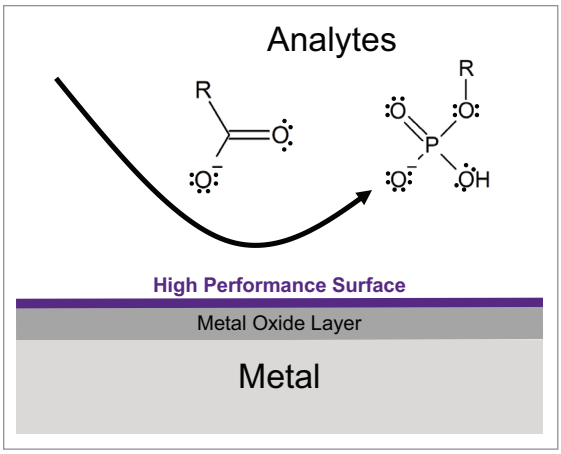


*Figure 1. A MaxPeak High Performance Surface as utilized in the PREMIER columns to impede electron rich analytes from interacting with metallic hardware.*

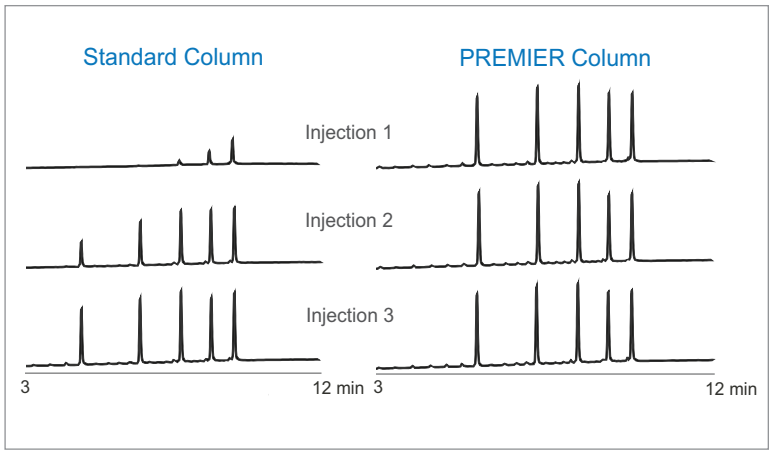


*Figure 2. Comparison of results for a standard versus a PREMIER column for the separation of the MassPREP Oligonucleotide Standard. Acetonitrile gradient separations were carried out using ACQUITY UPLC Oligonucleotide BEH $C_{18}$, 130 Å, 1.7 µm, 2.1 x 50 mm Columns. The aqueous mobile phase was 25 mM hexylammonium acetate (pH 6.0), the column temperature was 60 °C, and the flow rate was 0.4 mL/min. The peaks were detected by absorbance at 260 nm. Three consecutive injections were made of a mixture containing 10 pmol of each oligonucleotide.*

## MaxPeak HIGH PERFORMANCE SURFACES FOR HPLC COLUMNS

To address these challenges, Waters™ developed a family of technologies named MaxPeak High Performance Surfaces (HPS). When utilized for HPLC column hardware, this technology provides a highly effective barrier that mitigates undesired interactions with metal surfaces (Figure 1). This has been implemented using in-house, specialized manufacturing. This new manufacturing process involves a rigorous level of quality control. The surface chemistry can be tailored to give properties that are most appropriate for one or more intended chromatographic modes. We have developed a surface chemistry that is particularly well suited to reversed-phase (RP) and hydrophilic interaction chromatography (HILIC) and is based on a hybrid organic/inorganic surface that is similar to that of BEH (ethylene-bridged hybrid) particles.[14] This MaxPeak HPS LC surface is comprised of a highly crosslinked layer containing ethylene-bridged siloxane groups. This is different than the MaxPeak HPS technology that has been used on QuanRecovery™ Autosampler Vials and Plates to mitigate hydrophobic non-specific binding (NSB). In summary, MaxPeak HPS technologies have been created based on an understanding of the properties of chromatographic surfaces required to mitigate undesired interactions with analytes in demanding LC applications.

## BENEFITS TO CHROMATOGRAPHY AND LC-MS

MaxPeak HPS offers improvements in the separation and detection of metal-sensitive analytes, ranging from organic acids and organophosphates to oligonucleotides, peptides, glycans, and phospholipids. These improvements include a reduced requirement for conditioning, greater recovery, improved peak symmetry, lower detection limits, and higher quality mass spectra.

Some of these benefits are illustrated in Figures 2 and 3, wherein chromatographic results and mass spectra are displayed. Figure 2 shows chromatograms from gradient separations of a mixture of oligonucleotides obtained using two columns. The sample was the MassPREP™ Oligonucleotide Standard (a mixture of deoxythymidines with 15, 20, 25, 30, and 35 nucleotides). ACQUITY™ UPLC™ Oligonucleotide BEH $C_{18}$, 130 Å 1.7 µm Columns using standard stainless steel versus MaxPeak HPS hardware (designated by the name PREMIER ) were compared. Three consecutive injections were made on each column. The results show that the standard column gave very low peak heights in the first injection, with the heights gradually increasing in the subsequent injections. In contrast, the PREMIER column gave more consistent peak heights for all three injections. This demonstrates that PREMIER columns have a reduced need for conditioning to achieve consistent peak heights for metal-sensitive analytes.

MaxPeak HPS technology has also been found to improve peptide separations. Pronounced benefits have been found not only for phosphorylated peptides but also for peptides containing multiple acidic residues, such as the so-called PENNYK peptide from humanized monoclonal antibodies.[15] Figure 3A shows zoomed MS chromatograms

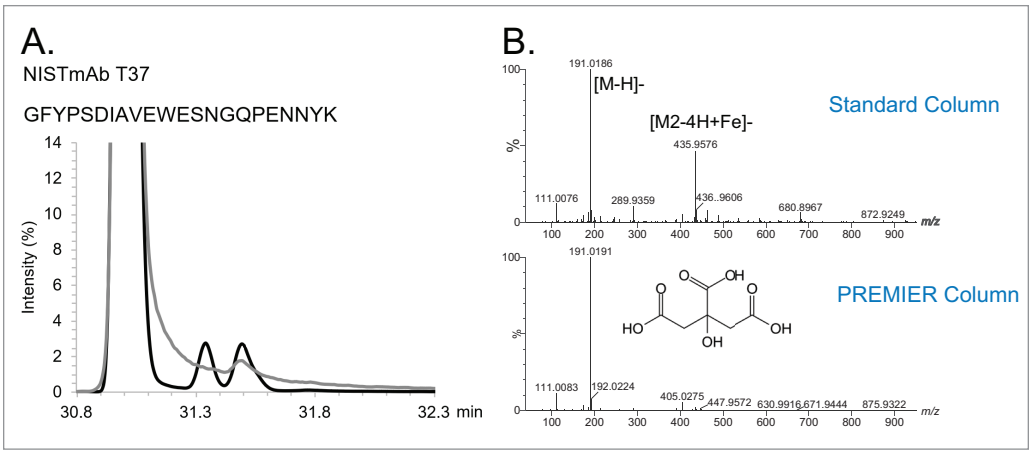


*Figure 3. Benefits of MaxPeak HPS technology for LC-MS analyses. (A) MS chromatogram of tryptic peptide 37 from NISTmAb using a gradient separation with mobile phases containing 0.1% (v/v) formic acid with ACQUITY UPLC CSH $C_{18}$, 130 Å, 1.7 µm Columns, based on standard column hardware (gray trace) versus MaxPeak HPS hardware (black trace). (B) Negative ion mode mass spectra for citric acid obtained with a 0.1% formic acid mobile phase and ACQUITY UPLC CSH Phenyl Hexyl, 1.7 µm Columns packed into either standard hardware (top) or MaxPeak HPS hardware (bottom).*

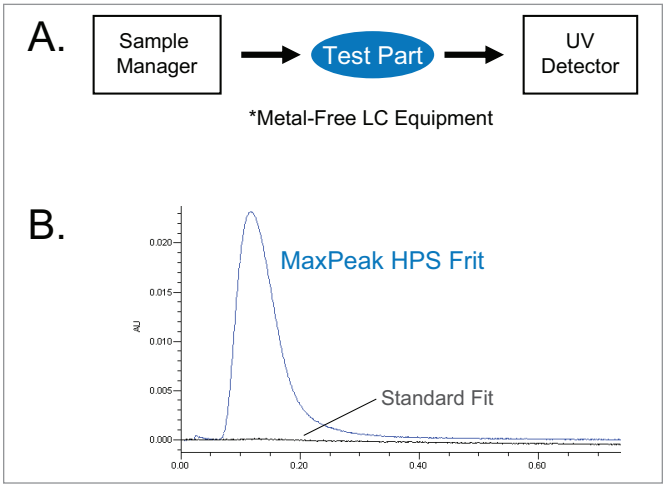


*Figure 4. Quality control test for in-house manufacturing of MaxPeak HPS hardware. (A) Schematic of instrument setup. (B) Test results for a MaxPeak HPS frit (blue) and a standard frit (black) for an injection of 10 ng of ATP.*

of this peptide as separated using a standard ACQUITY UPLC CSH $C_{18}$, 130 Å, 1.7 µm Column versus the PREMIER version. Upon analyzing the bottom 10% of the peak, dramatically reduced tailing can be seen to be afforded by the PREMIER column, allowing two minor peaks to be resolved and accurately quantified. These minor peaks arise from deamidated variants of the PENNYK peptide, and their relative areas need to be monitored for quality control of monoclonal antibody therapeutics.[15]

MaxPeak HPS technology has been observed to be of benefit for the chromatography of many challenging analytes. Complementing these effects is the fact that this technology can enhance the quality of mass spectra. Figure 3B provides data from one such example, in which citric acid cycle metabolites were analyzed using LC-MS with either a standard or an ACQUITY PREMIER CSH Phenyl Hexyl, 1.7 µm Column. When using the standard column, a significant number of the collected negative ion mode spectra were found to be contaminated with peaks from iron ion adducts. In contrast, spectra obtained when using a PREMIER column are cleanly populated with deprotonated molecular ions. This improves the detection sensitivity and the quality of MS library matches. With its combination of benefits, MaxPeak HPS technology presents a new means to obtain improved MS data quality and more accurate results.

## QUALITY ASSURANCE THROUGH DESIGN AND PROCESS CONTROL

Because MaxPeak HPS technology is produced by an in-house manufacturing process, the PREMIER columns come with a high degree of quality assurance. As mentioned earlier, a high-performance surface can be tuned to improve chromatographic performance and mitigate adsorption issues in several different applications. Consequently, it is imperative that it be tightly controlled. One key aspect of a surface is its hydrophobicity, which may be evaluated by measuring the contact angle between it and a small droplet of water.[16] A higher water contact angle corresponds to a more hydrophobic surface, whereas a smaller contact angle indicates one that is more hydrophilic. For reference, PEEK surfaces tend to exhibit water contact angles between 70 and 90 degrees,[17] whereas the ethylene-bridged hybrid MaxPeak HPS surface yields contact angles that are less than 60 degrees. To control the MaxPeak HPS manufacturing process, this and other physicochemical properties are measured to ensure consistency.

In the Waters quality control strategy, MaxPeak HPS manufacturing is also monitored to ensure that processed parts show high recoveries when used to analyze metal sensitive analytes. In this testing, a low mass load of adenosine triphosphate (ATP) is injected and passed through a frit that has been brought through the manufacturing process and tested on a column-free and metal-surface-free chromatographic test system. A simplified schematic of this apparatus is displayed in Figure 4, along with a test result that is typical of a MaxPeak HPS frit versus a standard, unprocessed metal frit. High recovery of ATP is confirmed to be achieved with the MaxPeak HPS frit. Using this test, high quality and consistent performance can be assured.

## CHEMICAL STABILITY TESTING

As important as it is to ensure that MaxPeak HPS hardware is reproducibly manufactured, it is also important that it stands up to demanding use scenarios. To this end, various forms of robustness testing have been carried out. For an initial round of testing, we explored the stability of the MaxPeak HPS surface to several high- and low-pH mobile phase compositions over a 16-hour period. Each test consisted of a repeated cycle of one hour of exposure to a stress condition, a flush to achieve equilibration, and a test injection of 10 ng of ATP. As above, testing was performed on a MaxPeak HPS frit. ATP recoveries obtained in these stress tests are shown in Figure 5. Against a pH 12 stress condition, no significant change in ATP recovery was observed at 60 °C, and only a small decrease was seen at 90 °C. A pH 1 stress condition at 60 °C resulted in little-to-no change in recovery, while a 20% decrease was observed at 90 °C.

Testing such as this has facilitated the recommendation of certain use conditions. At pH values from 2–11, 90 °C can be used without concern. Between pH 1–2 and 11–12, temperatures up to 60 °C may be used. Using an aqueous/organic mixture instead of a 100% aqueous mobile phase is expected to extend the lifetime.

These chemical stability results confirm that there are no special precautions needed for MaxPeak HPS columns under standard use conditions. The ethylene-bridged hybrid composition of the MaxPeak HPS surface requires no extra consideration versus those already made for the chemical robustness of the chromatographic stationary phase.

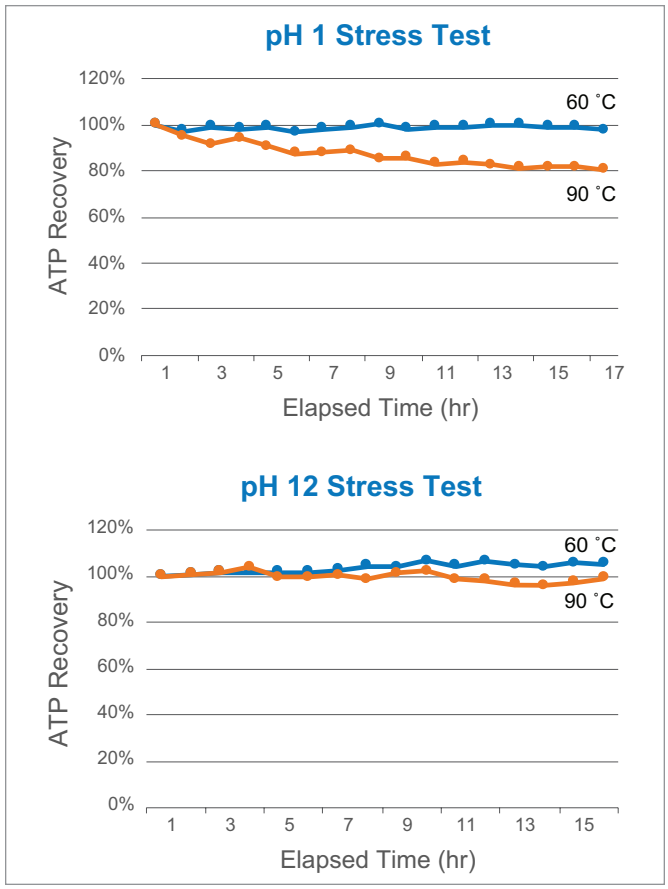


*Figure 5. Accelerated stability test results for a MaxPeak HPS frit. Low pH testing was performed with 1% TFA (pH 1) mobile phase at 60 and 90 °C. High-pH testing was performed with 10 mM sodium hydroxide (pH 12) mobile phase at 60 and 90 °C. The y-axes show the recoveries for ATP measured as shown in Figure 4.*

## EFFECTS OF MASS LOAD

The problem of the adsorption of sample components onto column hardware is well known, yet some analysts may have never experienced it or may be unaware of its impact on the quality of an analysis. This could be explained by several factors:

1. The analytes of interest do not exhibit affinity towards metallic surfaces.
2. The analysis is performed at concentration levels exceeding the adsorptive capacity of the column hardware surface and there is only a comparatively minor loss of analyte.
3. The LC column is partially conditioned during prior use with repetitive injections of the sample or sample-like components.
4. Analyte adsorption to metallic components is suppressed due to the selected mobile phase composition, such as one containing a phosphate buffer.

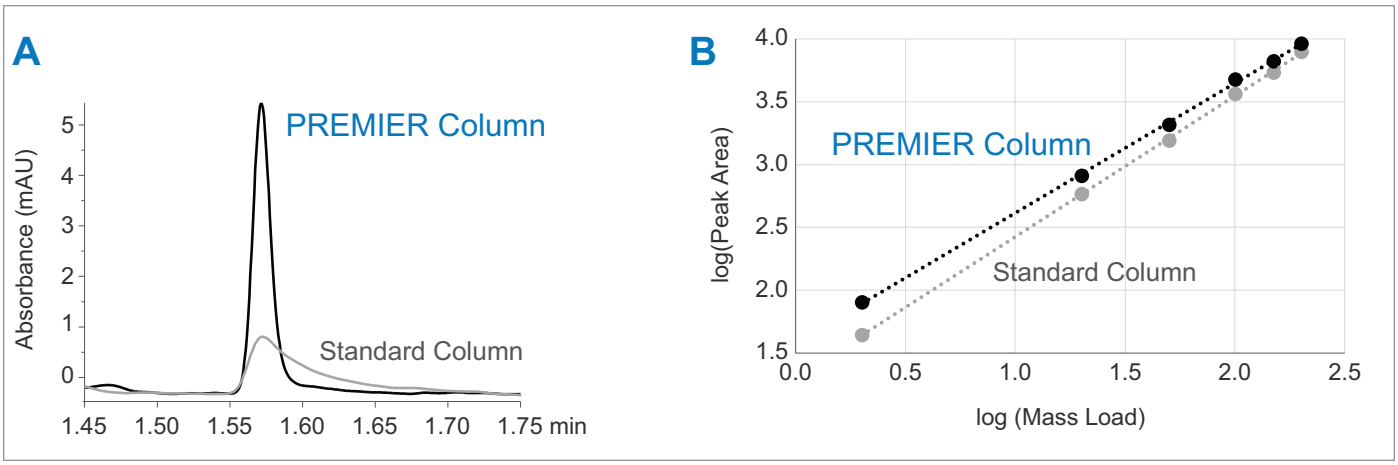


*Figure 6. (A) Comparison of the chromatograms obtained for hydrocortisone phosphate using PREMIER (black trace) and standard (gray trace) ACQUITY UPLC BEH $C_{18}$, 130 Å, 1.7 µm, 2.1 x 50 mm Columns. The mass load was 2 ng. Acetonitrile gradient separations were carried out with a 10 mM ammonium formate (pH 3.0) aqueous mobile phase, a flow rate of 0.5 mL/min and a temperature of 30 °C. The peaks were detected using a PDA detector, with the 246 nm channel displayed. (B) Log-log plot of peak area vs mass load for hydrocortisone phosphate separated using PREMIER (black symbols) and standard (gray symbols) columns, with mass loads from 2–200 ng.*

We investigated the dependency on mass load using example separations of hydrocortisone phosphate detected by absorbance at 246 nm. Acetonitrile gradient separations were carried out using 10 mM ammonium formate (pH 3.0) as the aqueous mobile phase and a temperature of 30 °C. Standard and ACQUITY PREMIER BEH $C_{18}$, 130 Å, 1.7 µm, 2.1 x 50 mm Columns were used. The mass load of hydrocortisone phosphate ranged from 2–200 ng on-column. Figure 6A shows a comparison of the chromatograms obtained using the two columns for the lowest mass load, demonstrating the narrower, more symmetric peak observed using the PREMIER column. The area of the peak obtained using the PREMIER column is also 83% greater than that obtained with the standard column. Figure 6B shows a plot of the log of the peak area vs the log of the mass load. The results demonstrate that higher peak areas are observed over the entire range when using the PREMIER column, with the relative differences becoming larger as the mass load decreases. This has been observed for other analytes as well and appears to be a general trend.

## ADDITIONAL CONSIDERATIONS

While the column is generally the greatest contributor to undesirable interactions with metal-sensitive analytes, the HPLC system and the mobile phase may also have an impact. The HPLC system should be conditioned following the manufacturer's guidance. When preparing the mobile phase, it is advised to use LC-MS quality reagents that are certified to contain no more than ppb levels of metal impurities. Mobile phase containers should also be chosen to avoid metal ion contamination (plastic containers are recommended), and metal sinker filters should not be used. In some challenging LC-MS applications, MaxPeak HPS column hardware may not resolve all issues caused by analyte adsorption. In these cases, a low concentration (sub-millimolar) of a chelator, such as citric acid, may be added to the mobile phase to mitigate any residual adsorption.[18]

Finally, some samples might contain a significant concentration of metal ions. Accordingly, it is foreseeable that some assays may benefit from adding chelators and/or suitable internal standards to samples.[19] In which case, residual adsorptive sites will be transiently conditioned with each sample injection. Similarly, it might even be advantageous in some instances to include a chelating additive in a sample preparation process, especially where complicated adsorption and Lewis acid-base interactions might be at play.

## CONCLUSION

PREMIER columns, constructed with MaxPeak High Performance Surfaces, provide a means to improve the LC analysis of analytes that are sensitive to adsorption on metal surfaces. Without trading UPLC performance and without doping mobile phases with chelators, it is now possible to achieve high recoveries and sharp peaks for many historically challenging analytes. With MaxPeak HPS technology, analytes are impeded from Lewis acid-base interactions and adsorptive losses. This technology has proven to be effective in improving the analysis of electron-rich compounds, such as those containing phosphate and carboxylate groups. The benefits of the technology are most pronounced for low analyte loads. Dramatic improvements are seen in RP and HILIC applications for organic acids, organophosphates, oligonucleotides, peptides, glycans, and phospholipids.

To ensure the quality and reliability of this technology, Waters has established an in-house capability for manufacturing and quality assurance testing. Most importantly, key aspects of the MaxPeak HPS manufacturing process are monitored, including the hydrophobicity and inertness of MaxPeak HPS parts. Moreover, parts made using MaxPeak HPS technology have been subjected to stress testing to confirm robustness. It has been demonstrated that MaxPeak HPS hardware is stable from pH 1–12. No special considerations are needed for the use of PREMIER columns. In sum, PREMIER columns provide chromatographers with a new option for analyzing metal-sensitive compounds and establishing more accurate, reproducible, and robust analytical methods.

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