Faster Time to Results for Ultra-Performance Liquid Chromatographic Separations of Metal-Sensitive Analytes

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Abstract

Many practitioners of HPLC think of analytical speed only in terms of the time required to carry out the separation. However, when separating analytes that interact with the metal surfaces in HPLC instruments and columns, analytical speed may be negatively impacted by the time required for conditioning. This is the case for important classes of analytes such as oligonucleotides, acidic peptides and anionic polar metabolites. In a recent advance, an innovative technology has been developed to mitigate interactions with the metal components of HPLC systems and columns, reducing the time needed to obtain accurate, reproducible results for metal-sensitive analytes.

Introduction

Analytical scientists are constantly under pressure to produce results quickly. In seeking out more efficient ways to accomplish their tasks, separation scientists look at all aspects of their instrumentation and methods. In 2004, the introduction of Ultra-Performance Liquid Chromatography (UPLC™) improved the speed with which analytical scientists could carry out LC separations [1 - 3]. The combination of columns packed with < 2 µm particles and low-dispersion high-pressure tolerant instrumentation greatly reduced separation times.

However, for some applications, the analysis time is negatively impacted by the need for extensive conditioning of the LC system and column before accurate and reproducible results can be obtained. One of the root causes of the need for conditioning is the interaction between certain analytes and the metal surfaces of HPLC systems and columns. It has long been known that stainless steel hardware can cause poor peak shapes and low recoveries for some analytes [4 - 7]. Compounds that show this behaviour typically contain phosphate and/or carboxylate groups, although some analytes with other electron-rich functional groups have been reported to show similar issues [4]. The severity of adsorption has been found to increase with the number of these functional groups in the analyte [6, 7]. Adsorption becomes particularly problematic when the stainless steel is corroded [8], which can occur due to exposure to mobile phases that are highly acidic and/or contain chloride salts, among other conditions [9].

One approach to mitigate these issues is to use alternative metals such as titanium or nickel-cobalt alloys (e.g. MP35N) for components in the flow path of the HPLC system [10]. While these alternatives exhibit improved corrosion resistance, making them useful for applications that require mobile phases with high salt concentrations, they still may adsorb certain analytes [11]. To avoid metal surfaces altogether, organic polymers such as polyether ether ketone (PEEK) have been used in HPLC systems and columns. However, this engineering plastic lacks the mechanical strength necessary to

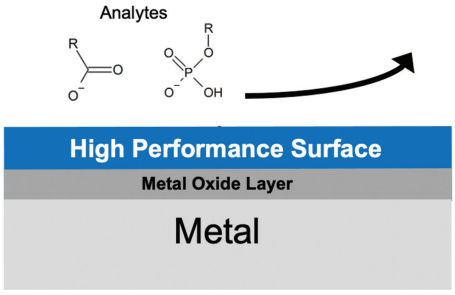

Figure 1. A MaxPeak High Performance Surface impedes interactions of electron-rich analytes with metallic hardware.

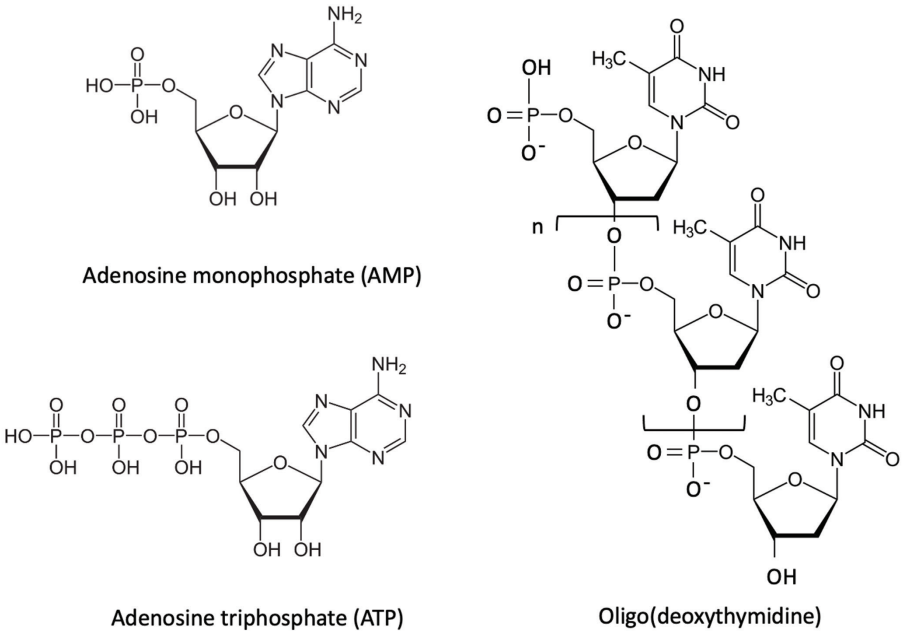

Figure 2. Chemical structures of the test analytes.

tolerate UPLC pressures (≥5,000 psi) unless cladded with steel. In addition, PEEK is not compatible with some solvents, notably tetrahydrofuran, dimethyl sulphoxide, chloroform and methylene chloride [12]. PEEK is also relatively hydrophobic, and it may be necessary to condition PEEK surfaces via multiple sample injections to mitigate hydrophobic adsorption [13, 14].

To address the challenges posed by undesired surface adsorption, a family of new technologies named MaxPeak™ High Performance Surfaces (HPS) was recently developed. A hydrophilic surface that mitigates hydrophobic adsorption on plastic vials and 96-well plates was the first in the MaxPeak HPS family [15]. Here, we describe the second MaxPeak HPS chemistry, which was designed to provide a barrier to mitigate undesired interactions of analytes with metal surfaces in LC systems and columns (see Figure 1) [16]. The surface chemistry used in the work described here is based on a hybrid organic/inorganic composition that is similar to that of ethylene-bridged hybrid (BEH™) particles [17], and is well suited for reversed-phase and hydrophilic interaction chromatography. Here, we demonstrate the reduced need for conditioning afforded by this technology, which allows separation scientists to more quickly obtain accurate and reproducible results for metal-sensitive analytes.

Experimental

All UPLC and MS instruments, columns, and data systems were from Waters Corp. (Milford, MA, USA). Columns and instrumentation incorporating MaxPeak HPS technology are commercially available from Waters Corp. and carry the PREMIER™ brand name.

UPLC H-Class instruments equipped with a Quaternary Solvent Manager, a Flow-Through Needle Sample Manager, a CH-A column heater and a photodiode array (PDA) detector were used for the separations of adenosine monophosphate (AMP) and adenosine triphosphate (ATP). Both a standard instrument and one incorporating MaxPeak HPS technology were used. AMP and ATP were obtained as disodium salts from Millipore-Sigma (Burlington, MA, USA). The samples, freshly prepared daily in 100% water, contained 50 µg/mL each of AMP and ATP and 0.4 µL was injected onto UPLC BEH C18 1.7 µm, 2.1 x 50 mm columns. Separations were also performed using columns of the same dimensions and containing the same packing material but incorporating MaxPeak HPS technology. All tests were carried out using new columns. Isocratic separations were performed at 30°C using an aqueous 10 mM ammonium acetate (pH 6.8) mobile phase at a flow rate of 0.5 mL/min. The UV response at 260 nm was recorded using an Empower™ 3 Chromatography Data System.

The oligonucleotide separations were carried out using an H-Class Bio system with a PDA detector equipped with a 5 µL titanium flow cell. The UPLC system was conditioned with 500 pmol of a 39-mer oligodeoxythymidine. The sample was the MassPREP™ Oligonucleotide standard (Waters Corp., Milford, MA, USA) reconstituted in 200 µL of deionised water (giving a concentration of 5 pmol/µL of each oligonucleotide), with 2 µL injected onto UPLC Oligonucleotide BEH C18 1.7 µm, 2.1 x 50 mm columns. For comparison, separations were also performed using columns of the same dimensions and containing the same packing material, but incorporating MaxPeak HPS technology. Gradient separations were carried out at 60°C, with mobile phase A containing 25 mM hexylammonium acetate (pH 6.0) in water and mobile phase B comprising 50/50 (v/v) mobile phase A / acetonitrile. A linear gradient from 50 - 86% B was carried out in 12 min at a flow rate of 0.4 mL/min. The UV response at 260 nm was recorded using an Empower 3 Chromatography Data System.

For the peptide separations, a UPLC H-Class Binary Bio PLUS system with a QDa™ mass detector and an Empower 3 Chromatography Data System was used. Experiments were carried out using both a standard instrument and one incorporating MaxPeak HPS technology. The sample was the mAb Tryptic Digestion standard (Waters Corp, Milford, MA) reconstituted in MS-grade water containing 0.1% formic acid at a concentration of 0.5 mg/mL, with 10 µL injected onto UPLC Peptide CSH™ C18 1.7 µm, 2.1 x 100 mm columns. For comparison, separations were also performed using columns of the same dimensions and containing the same packing material but incorporating MaxPeak HPS technology. Gradient separations were carried out at 60°C, with mobile phase A containing 0.1% formic acid in water and mobile phase B containing 0.1% formic acid in acetonitrile. A linear gradient from 1-35% B was carried out in 52 min at a flow rate of 0.2 mL/min. The QDa mass detector was operated in positive ion electrospray mode, using an acquisition range of 250 - 1250 m/z, a capillary voltage of 1.5 kV, cone voltage of 10 V and probe temperature of 600°C. Selected ion monitoring was implemented to monitor the T37 peptide using an m/z value of 849.2 representing the $[M+3H]^{+3}$ charge state.

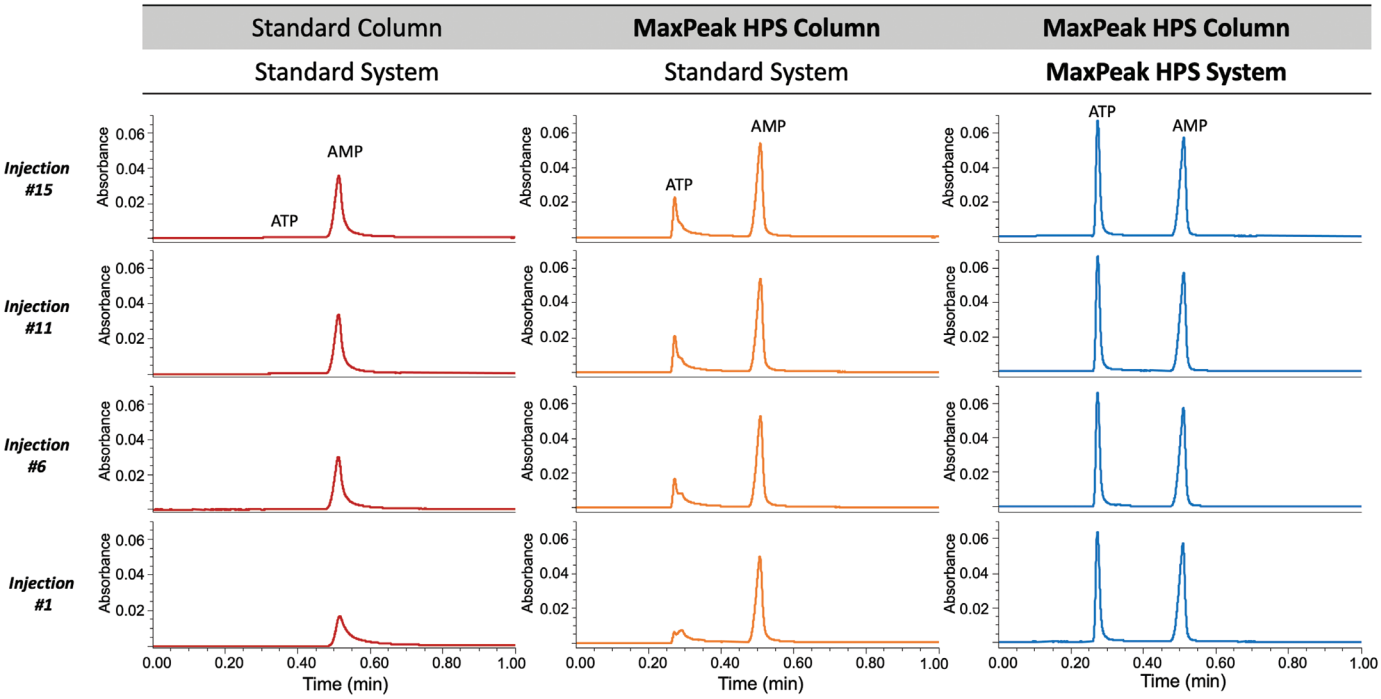

Figure 3. Comparison of chromatograms for the separation of a mixture of AMP and ATP using a standard UPLC system and column, a standard system with a MaxPeak HPS column and a MaxPeak HPS system and column. ACQUITY UPLC™ BEH C18 1.7 µm 2.1 x 50 mm columns were used with an aqueous 10 mM ammonium acetate (pH 6.8) mobile phase, 30°C column temperature and a 0.5 mL/min flow rate. The peaks were detected by absorbance at 260 nm. A series of fifteen injections was made (20 ng of each nucleotide) , and the results for injections 1, 6, 11 and 15 are shown

Results and Discussion

The benefits of MaxPeak HPS technology may be demonstrated for the separation of nucleotides, which have been shown to adsorb onto stainless steel surfaces [5 - 8]. Adenosine triphosphate (ATP) (see Figure 2 for chemical structure) is a nucleotide that, in addition to being a precursor of DNA and RNA, is an important metabolite, providing energy to drive many cellular processes [18]. Consequently, the quantification of ATP is important in a number of different application areas. Shown in Figure 3 are a series of chromatograms demonstrating the separation of adenosine monophosphate (AMP) and ATP using standard vs MaxPeak HPS systems and columns. In each case, a series of fifteen injections was made. In the chromatograms obtained using the standard system and column, the peak for AMP shows significant tailing, which gradually decreases as more injections are made. ATP, which adsorbs more strongly due to its three phosphate groups, shows severe tailing and very low intensity, and is barely detectable in the fifteenth injection. When using the same standard system with a column that incorporates MaxPeak HPS technology, the AMP peak shows improved symmetry. However, the ATP peak is severely tailed, improving over the series of injections, but still showing severe tailing and low intensity in the fifteenth injection. In contrast, when using both a system and column incorporating MaxPeak HPS technology, the chromatograms show symmetrical peaks from the first to the last injection.

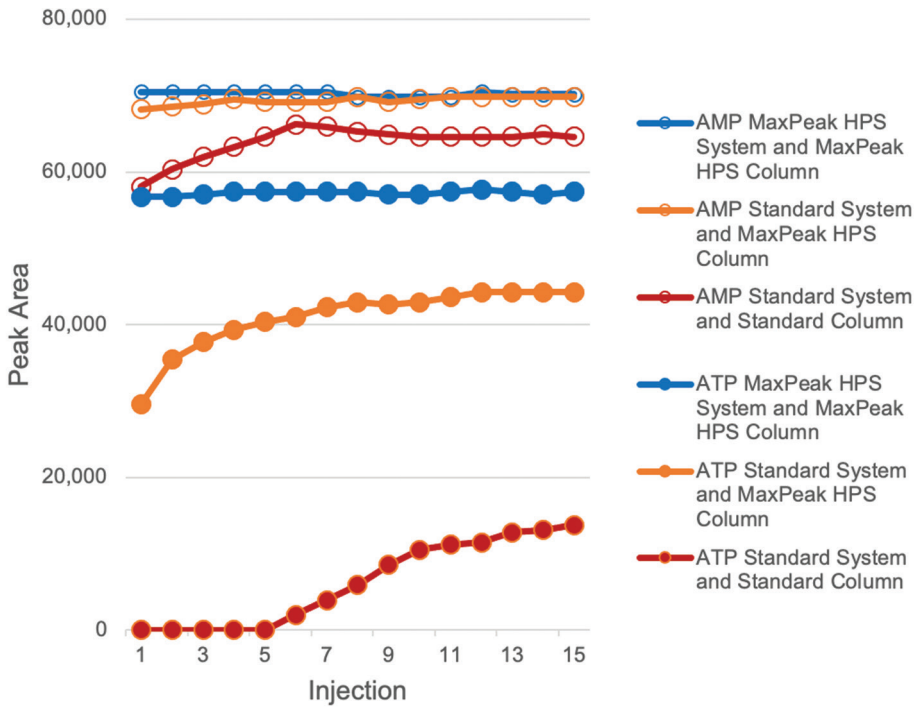

Figure 4. Comparison of peak areas of AMP and ATP vs injection number using a standard UPLC system and column, a standard system with a MaxPeak HPS column and a MaxPeak HPS system and column. The test conditions are described in the caption for Figure 3.

In Figure 4 we show the areas of the AMP and ATP peaks vs injection number for the three combinations of system and column. It is evident that besides the peak tailing observed when using a standard system and/or column, the peak areas are also lower relative to those observed when using both a system and column that incorporate MaxPeak HPS technology. This is particularly true for ATP, due to its strong adsorption on stainless steel surfaces. Thus, without substantially more conditioning than used in this experiment, the quantitative results

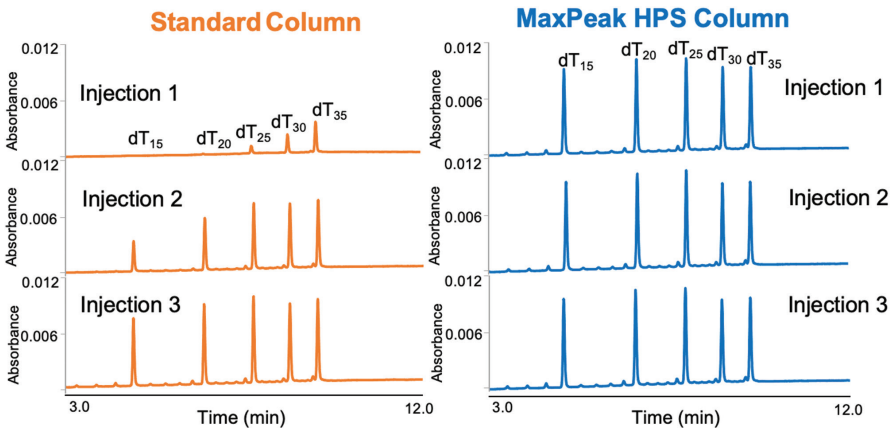

Figure 5: Comparison of chromatograms for a standard versus a MaxPeak HPS column for the separation of the MassPREP Oligonucleotide Standard, a mixture of five oligodeoxythymidines (dT) with 15 - 35 nucleotides. On each column, three consecutive injections were made of a mixture containing 10 pmol of each oligonucleotide. Acetonitrile gradient separations were carried out using ACQUITY UPLC Oligonucleotide BEH C18, 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.

obtained using the standard system and column would not reflect the true amount of ATP in the sample. In addition, the precision would suffer because of the changing peak area with injection number. In contrast, when using both a system and column that incorporate MaxPeak HPS technology, consistent peak areas were observed from the first injection.

MaxPeak HPS technology has been found to be particularly valuable for separations of oligonucleotides [19], which are important for several high profile uses, including as vaccines [20], therapeutics [21], and diagnostics, e.g. as primers for the polymerase chain reaction (PCR) [22]. LC and LC/MS methods are important for quality control of these modalities [23]. An example of the effect of metal surfaces in a UPLC system and column on the separation of a mixture of oligonucleotides is shown in Figure 5. The sample contained a mixture of deoxythymidines (see Figure 2 for the chemical structure) with 15, 20, 25, 30 and 35 nucleotides, which were separated using either a standard UPLC column or a UPLC column that incorporated MaxPeak HPS technology. Three consecutive injections were made on both columns. With the standard column, low peak heights were observed in the first injection, and the heights gradually increased in subsequent injections. Thus, with a standard column, a number of conditioning injections are necessary before reproducible results may be obtained. However, even after extensive conditioning, accurate quantification may be challenging because the effects of conditioning are often transient [19]. In contrast, the column incorporating MaxPeak HPS technology gave reproducible peak heights for all three injections. This demonstrates the reduced need for conditioning when using MaxPeak HPS technology.

Another important class of analytes that benefits from MaxPeak HPS technology is acidic peptides, that is, peptides that exhibit a net negative charge at neutral pH [24]. Such peptides may occur, for example, in the enzymatic treatment of proteins. The separation of proteolytic digests is a fundamental tool used to characterise therapeutic proteins, such as monoclonal antibodies (mAb). When coupled to mass spectrometry, these peptide maps provide information on the amino acid sequence, as well as the presence of related impurities such as deamidated species. An example of an important acidic peptide present in tryptic digests of humanised monoclonal antibodies is the T37 peptide located in the Fc constant region, which has been linked to the therapeutic efficacy of mAb-based drug products [25]. This peptide contains four acidic amino acid residues and only one basic amino acid (see Figure 6A), and hence is negatively charged over a wide pH range. When using a standard system and column with the acidic low ionic strength mobile phases which are optimal for LC/MS (water and acetonitrile containing 0.1% formic

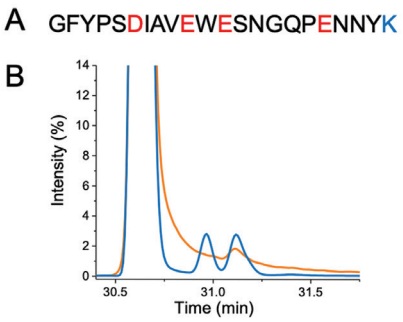

Figure 6. A) Amino acid sequence of tryptic peptide 37 from NIST mAb, with the four acidic residues shown in red and the basic residue in blue; B) Overlaid chromatograms showing the peak for this peptide using conventional (orange trace) versus MaxPeak HPS (blue trace) system and column hardware. Acetonitrile gradient separations were carried out employing mobile phases containing 0.1% (v/v) formic acid with ACQUITY UPLC CSH 130 Å C18 1.7 µm 2.1 x 100 mm columns. The column temperature was 60°C and the flow rate was 0.2 mL/min The peaks were detected using ESI+ MS (SIR mode). The minor peaks are from deamidated variants. The data were time-aligned to facilitate comparison.

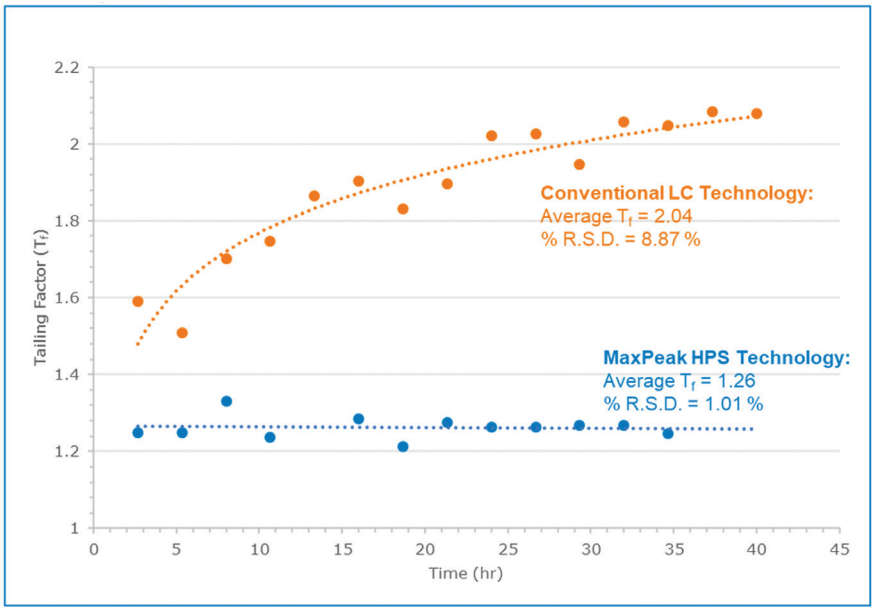

Figure 7. Plot of tailing factors for tryptic peptide 37 from NIST mAb vs time using conventional (orange trace) versus MaxPeak HPS (blue trace) system and column hardware. The systems were washed with 30% phosphoric acid, then water, before carrying out the peptide separations. The test conditions are given in the caption for Figure 6.

acid), significant tailing is observed for the T37 peak, obscuring the presence of small trailing peaks (see Figure 6B). In contrast, using both a system and column that incorporate MaxPeak HPS technology, the T37 peak is sharp and symmetric, allowing accurate quantification of the trailing peaks. These minor peaks arise from deamidated variants, and their areas relative to the main T37 peak are monitored for quality control of therapeutic monoclonal antibodies [25].

In an attempt to reduce the tailing of the T37 peptide peak when using conventional metal surface technology, the UPLC system was conditioned by washing with a 30% phosphoric acid solution for 20 min followed by flushing with water until the pH was neutral. This was found to temporarily reduce the tailing of the T37 peak, but the tailing factor increased over 48 h of exposure to the 0.1% formic acid mobile phases, indicating an increase in surface adsorption activity. This is shown in Figure 7. In contrast, with MaxPeak HPS technology similar results were obtained with and without the phosphoric acid wash, with consistent assay performance observed from beginning to end when this separation was performed over the same time period. Equally important is that the tailing factor was lower when using both a system and column that incorporate MaxPeak HPS technology, resulting in excellent resolution of the deamidation variants, allowing them to be accurately and reproducibly quantified.

Conclusions

These results demonstrate the challenges posed by interactions of analytes with the metal surfaces in conventional UPLC systems and columns. Mitigation of these interactions using time-consuming conditioning approaches can add hours to the analysis time, and don't guarantee that accurate and reproducible results will be obtained. Using the new capabilities afforded by systems and columns that incorporate MaxPeak HPS technology, separations of metal-sensitive analytes may now be carried out without extensive conditioning protocols, providing accurate and reproducible results in a much shorter time. As demonstrated here, this technology is important for a range of analyte classes, including acidic polar metabolites, oligonucleotides and acidic peptides. Other analytes containing phosphate and/or carboxylate groups show similar benefits [16], as do some compounds containing additional electron-rich functional groups. Consequently, this technology is likely to be beneficial for a range of applications.

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