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Low Adsorption UPLC Systems and Columns Based on MaxPeak High Performance Surfaces: The ACQUITY Premier Solution

T. H. Walter, M. Trudeau, J. Simeone, P. Rainville, A. V. Patel, M. A. Lauber, J. Kellett, M. DeLano, K. Brennan, C. Boissel, R. Birdsall, K. Berthelette
Waters Corporation, Milford, MA, USA

CHALLENGES IN HPLC SEPARATIONS OF METAL-SENSITIVE ANALYTES

Since the earliest development of high-performance liquid chromatography (HPLC), stainless steel has been the preferred material of construction for chromatographs and columns. However, it has long been observed that stainless-steel hardware can cause poor peak shapes and low recoveries for certain analytes.[1–4] Compounds that show this behavior typically contain phosphate and/or carboxylate groups, although some analytes with other electron-rich functional groups have been reported to show similar issues.[1] The severity of adsorption has been found to increase with the number of these functional groups in the analyte.[3,4] These issues become highly problematic when the stainless steel is corroded,[5] which can occur due to exposure to mobile phases that are highly acidic and/or contain chloride salts.[6] Iron ions released from stainless-steel components have also been reported to adsorb on stationary phases, where they may interact with analytes, causing reactions including complexation,[1] epimerization,[7] and oxidation.[8] Another issue associated with HPLC systems and columns constructed from stainless steel is the presence of iron adducts in mass spectra.[9,10]

One approach to mitigate these issues is to use alternative metals such as titanium and nickel-cobalt alloys (e.g., MP35N) for components in the flow path of HPLC systems.[11] 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.[12,13] Polyether ether ketone (PEEK) is another material that has been used in place of stainless steel in HPLC systems and columns. While this engineering plastic has been used for HPLC (<5000 psi), it lacks the mechanical strength to tolerate ultrahigh pressure conditions (≥5000 psi) unless cladded with steel. In addition, PEEK swells when in contact with some solvents, notably tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), chloroform, and methylene chloride.[14] PEEK is also relatively hydrophobic, making it sometimes necessary to passivate PEEK surfaces via multiple sample injections to mitigate hydrophobic secondary interactions.[15,16]

An alternative approach to mitigate analyte interactions with metallic HPLC hardware is to add chelators like ethylenediaminetetraacetic acid (EDTA) to the mobile phase or sample diluent.[4,17] Other chelators such as citric acid,[18] acetylacetone,[19] and medronic acid[20] have been used in mobile phases for LC-MS analyses. However, chelators can cause ion suppression and may be difficult to remove from HPLC systems. Chelators are also known to cause corrosion of stainless steel.[6]

MAXPEAK HIGH PERFORMANCE SURFACES FOR UPLC INSTRUMENTS AND COLUMNS

To address these challenges, Waters has developed a family of new technologies, named MaxPeak™ High Performance Surfaces (HPS). When utilized for UPLC™ System and Column hardware, this technology provides a highly effective barrier that mitigates undesired interactions with metal surfaces (Figure 1). The surface chemistry may be tailored to give properties that are optimal 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 particles.[21] This MaxPeak HPS LC surface is composed of a highly crosslinked layer containing ethylene-bridged siloxane groups. The water contact angle for this layer has been measured to be 30°, indicating a more hydrophilic surface than that of PEEK, which has been reported to have a water contact angle of 70–90°.[22] The use of MaxPeak HPS Technology for column hardware has previously been described.[23] While the column accounts for the largest proportion of the metal surface area, the system contribution is also significant. Considering an ACQUITY™ UPLC H-Class System with a 2.1 x 50 mm ACQUITY UPLC Column, calculations indicate that the system contributes approximately 30% of the total metal surface area accessible to the analytes. Thus, to minimize adsorption, MaxPeak HPS Technology must be used for both the column and the system.

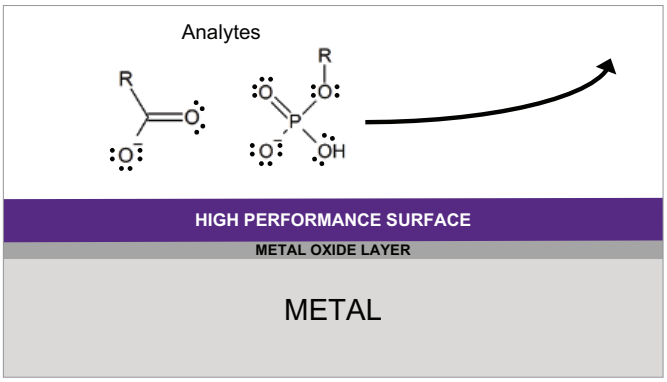

Figure 1. A MaxPeak High Performance Surface as utilized in the ACQUITY Premier Solution to impede electron rich analytes from interacting with metallic hardware.

THE ACQUITY PREMIER SYSTEM

To achieve optimal UPLC performance for metal-sensitive analytes, MaxPeak HPS Technology has been applied to the components of a new UPLC system, named ACQUITY Premier System (Figure 2). The combination of corrosion-resistant materials and ethylene-bridged siloxane surfaces used in this system avoids contamination with metal ions and mitigates the interaction of analytes with metal surfaces. To support a wide range of applications, ACQUITY Premier Systems are available with either binary or quaternary solvent managers and one-, two-, four-, or six-column managers. The sample manager uses a flow-through needle design. The system is compatible with Waters optical detectors (ACQUITY TUV, PDA eλ, and FLR), mass spectrometers, and associated system software (Empower,™ MassLynx,™ or UNIFI™). This system is intended to be used with columns in which MaxPeak HPS Technology has been applied to the metal hardware. These are named ACQUITY Premier Columns. The combination of the ACQUITY Premier System and ACQUITY Premier Columns is called the ACQUITY Premier Solution.

Figure 2. An ACQUITY Premier System and Column with MaxPeak HPS Technology. A) An LC instrument designed with a combination of corrosion-resistant components and hybrid organic/inorganic surfaces for sensitive, high efficiency RP and HILIC separations of metal-sensitive compounds. B) A complementary ACQUITY Premier Column constructed with hybrid organic/inorganic hardware surfaces.

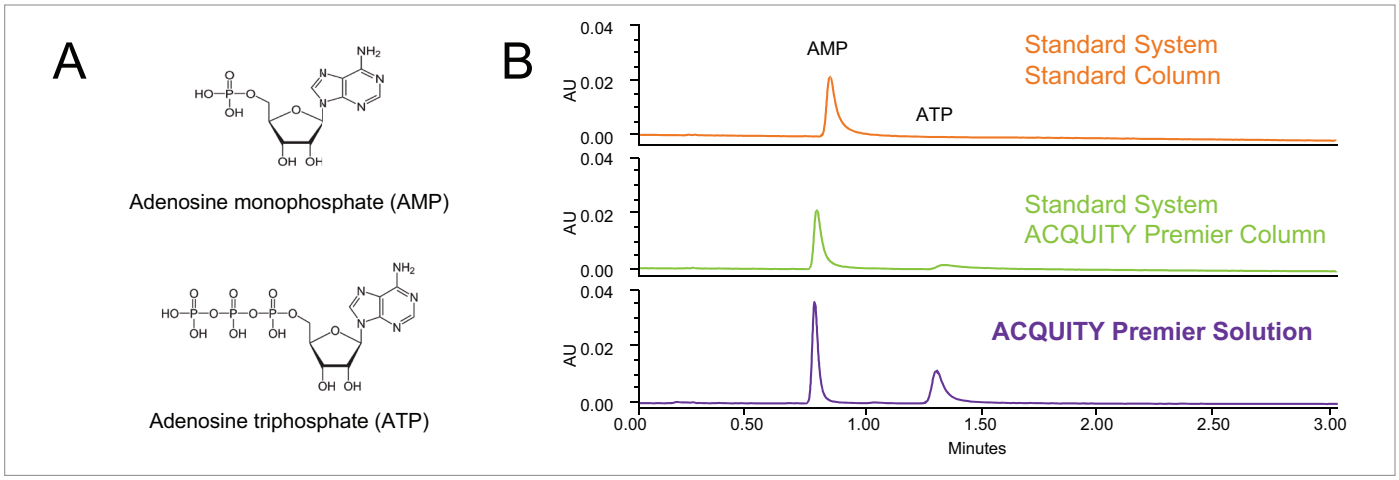

Figure 3. A) Chemical structures of AMP and ATP. B) Chromatograms obtained using a standard UPLC system and column vs the same system with an ACQUITY Premier Column and the ACQUITY Premier Solution. A mixture of ATP and AMP was separated at 20 ng mass loads on ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 50 mm Columns using a 65/35 (v/v) acetonitrile/aqueous 60 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.

As mentioned above, biocompatible LC systems that are required to sustain pressures of over ~9000 psi are commonly constructed using titanium and MP35N for components that contact the mobile phase. Currently, this includes the Thermo Scientific Vanquish line of LCs (and their Dionex UltiMate 3000 biocompatible predecessors), as well as the Agilent 1290 Infinity II Bio LC system. While these systems are resistant to corrosion when using high salt mobile phases common to biomolecule analyses, the use of titanium and MP35N does not prevent adsorption of metal-sensitive compounds. In addition, Agilent and Shimadzu both offer mid-pressure range instruments that make use of stainless-steel clad PEEK tubing. These systems suffer from the solvent incompatibilities and hydrophobicity of PEEK. In addition, the larger dispersion associated with these systems results in the loss of some or all the benefits of employing <2 µm particle columns. Only the ACQUITY Premier System combines low dispersion and corrosion-resistant metal components with inert surfaces to deliver a new level of performance for UPLC separations of metal-sensitive analytes.

EXAMPLES OF IMPROVED SEPARATIONS USING THE ACQUITY PREMIER SOLUTION

The benefit of using an ACQUITY Premier System in combination with an ACQUITY Premier Column (the ACQUITY Premier Solution) is demonstrated by the results shown in Figure 3. Here, a mixture of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) was separated at 20 ng individual mass loads under HILIC conditions using ACQUITY BEH Amide Columns. Chromatograms obtained using a standard UPLC System and a standard column show significant tailing for the AMP peak, while no peak was observed for ATP. Upon switching to an ACQUITY Premier Column, a peak could be obtained for ATP, although it exhibited severe tailing and a low peak area. It was not until the full ACQUITY Premier Solution was used that a sharp peak was achieved for ATP with the expected peak area.

The ACQUITY Premier Solution also provides benefits for certain pharmaceutical analytes. We previously demonstrated improved peak shape and greater peak areas for hydrocortisone sodium phosphate when an ACQUITY Premier Column was used in place of a standard column on a standard UPLC System.[23] Further improvement was observed for this analyte when using the ACQUITY Premier Solution. As shown in Figure 4, the largest slope of the calibration curve was obtained when using the ACQUITY Premier Solution. This combination also gave the smallest relative standard deviations (RSD) for peak area at the lowest loadings. The peak area RSD values were calculated from three sequential injections of 2, 20, and 50 ng on-column loads. While RSD values of 5–10% were observed using a standard UPLC System with either a standard or an ACQUITY Premier Column, the ACQUITY Premier Solution gave RSD values less than 0.5%. This demonstrates that the best reproducibility and sensitivity for metal-sensitive analytes are obtained using the ACQUITY Premier Solution.

The ACQUITY Premier Solution has been found to be particularly beneficial for separations of oligonucleotides. We previously demonstrated that a standard UPLC system and column initially showed very low peak heights for the MassPREP Oligonucleotide Standard, a mixture of

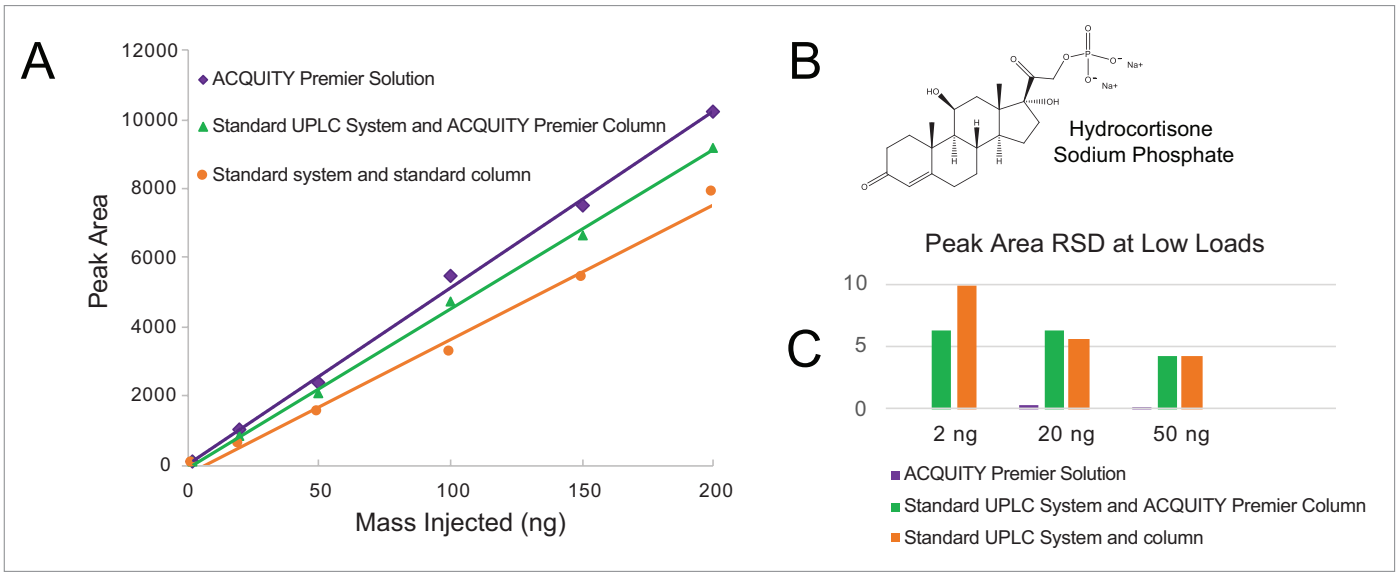

Figure 4. A) Calibration curves for hydrocortisone sodium phosphate obtained using a standard UPLC system and column vs the same system with an ACQUITY Premier Column and the ACQUITY Premier Solution. Detection was by UV absorbance at 246 nm. The columns were ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 50 mm and acetonitrile gradient separations were carried out using a 10 mM ammonium formate (pH 3.0) aqueous mobile phase. B) Chemical structure of hydrocortisone sodium phosphate. C) Comparison of the relative standard deviations of the peak areas for the three lowest mass loads, based on three consecutive injections.

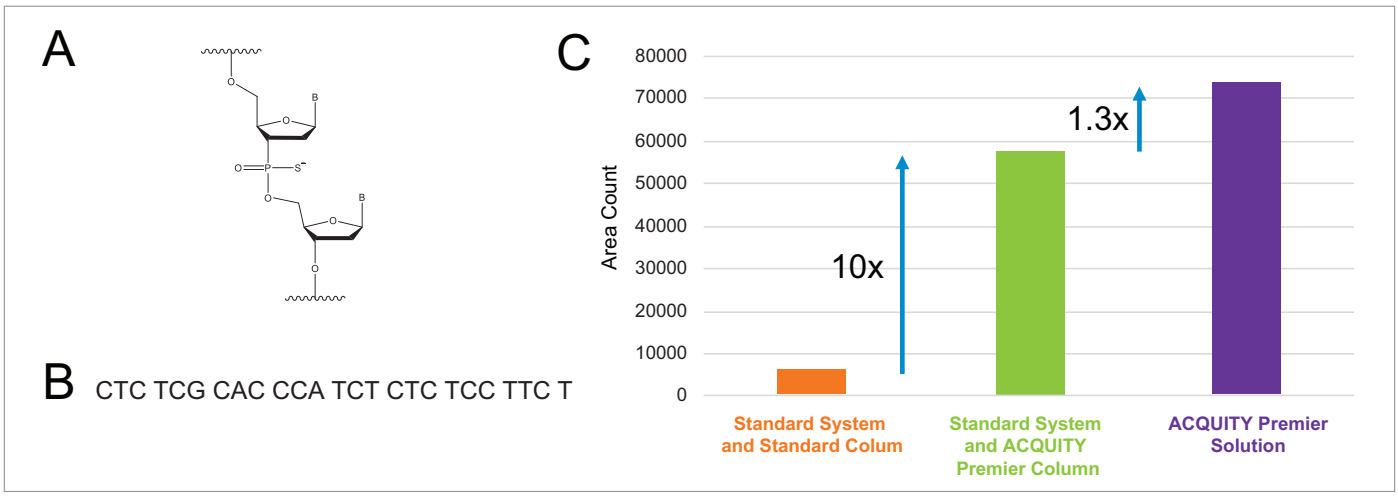

Figure 5. A) Generalized structure of phosphorothioate modified DNA. B) Nucleotide sequence of GEM91. C) Comparison of peak areas obtained for GEM91 by LC-MS/MS using a standard UPLC system and column vs the same system with an ACQUITY Premier Column and the ACQUITY Premier Solution. The samples contained 10 µg/mL of GEM91, and 10 µL was injected. ACQUITY Oligonucleotide BEH C_{18}, 1.7 µm, 2.1 x 50 mm Columns were used with mobile phases containing 150 mM hexafluoroisopropropanol and 5 mM hexylamine, with a methanol gradient. A Xevo TQ-XS Tandem Mass Spectrometer was used.

deoxythymidines with 15, 20, 25, 30, and 35 nucleotides.[23] The peak areas gradually increased as more injections were made, as the system and column became conditioned. In contrast, when an ACQUITY Premier Column was used, consistent peak areas were observed from the first injection. Here, the sensitivity obtained using an ACQUITY Premier System and Column was compared to that observed using their standard counterparts for a bioanalysis application. The analyte used for this study was GEM91, an antisense oligonucleotide phosphorothioate that has been investigated as a treatment for HIV-1.[24] The analyte was detected using tandem mass spectrometry, with a Xevo™ TQ-XS System. The analysis conditions and sample preparation have previously been described.[25] The results shown in Figure 5 demonstrate a 13-fold increase in peak area for a 100 ng on-column load of GEM91 when using an ACQUITY Premier System and Column compared to their standard counterparts. The use of a standard UPLC System with an ACQUITY Premier Column showed a smaller improvement, with a 10-fold increase in peak area vs the same system with a standard column.

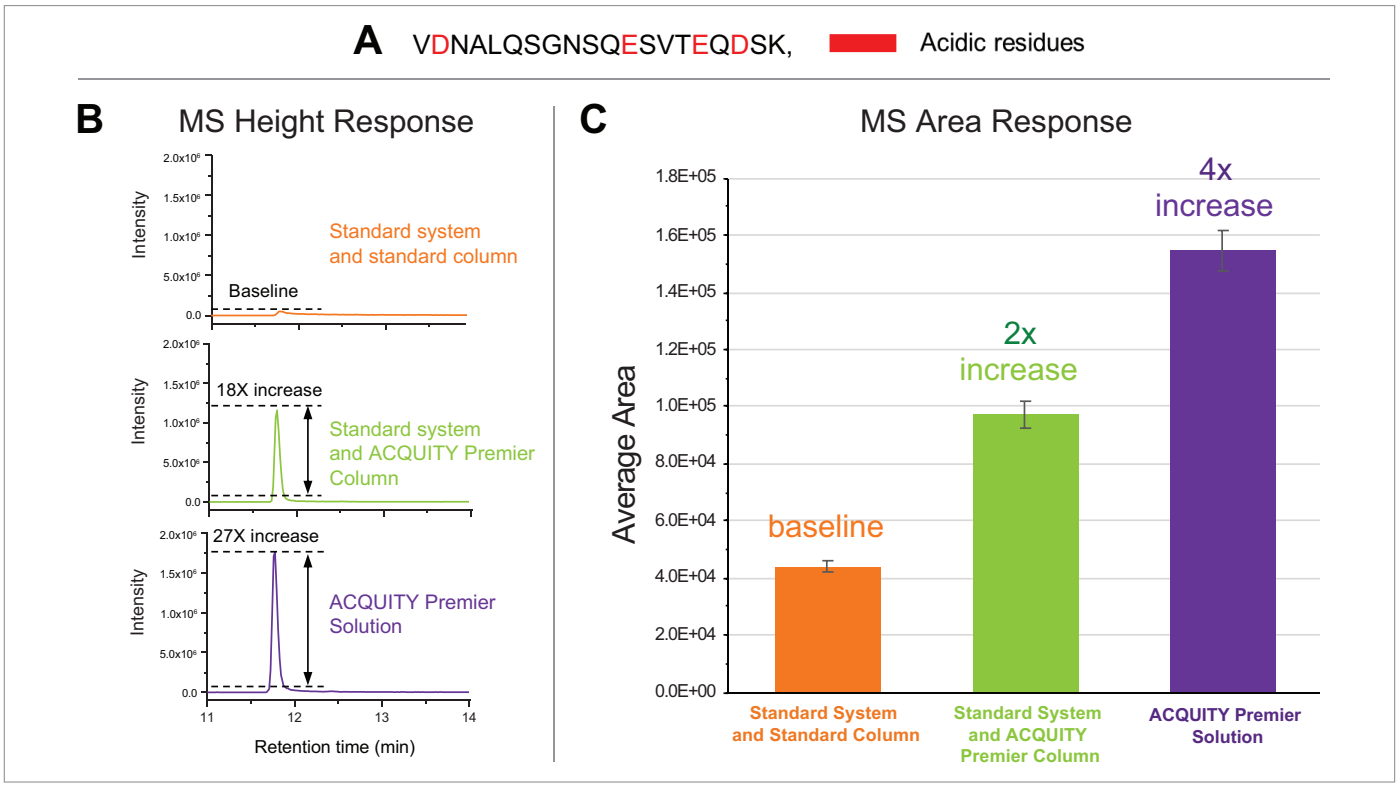

Figure 6. A) Amino acid sequence of the LC:T14 peptide from trypsin-digested NIST mAb. B) Comparison of peak heights obtained for this peptide (0.2 µg mass load) using LC-MS with a conventional UPLC system and column vs the same system with an ACQUITY Premier Column and the ACQUITY Premier Solution. C) Comparison of peak areas. Acetontrile gradient separations were carried out using 0.1% formic acid and ACQUITY UPLC CSH™ C_{18}, 1.7 µm, 2.1 x 100 mm columns. A SYNAPT™ XS High Resolution Mass Spectrometer was used to detect the peak.

The ACQUITY Premier Solution has also been found to improve peptide separations. Pronounced benefits have been observed not only for phosphorylated peptides but also for peptides containing multiple acidic amino acid residues. We previously demonstrated reduced peak tailing for the so-called PENNYK peptide[26] from trypsin-digested NISTmAb.[23] The reduced tailing allowed 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.[26] Another example of MaxPeak HPS Technology giving improved peak shape for an acidic peptide is shown in Figure 6. The LC:T14 peptide from trypsin-digested NIST mAb contains four acidic amino acid residues, and shows severe peak tailing when separated using a standard UPLC system and column. When the same sample was separated using a standard UPLC system and an ACQUITY Premier Column, we observed a dramatic improvement in peak shape for this peptide, leading to an 18-fold increase in peak height and a 2-fold increase in peak area, detected using MS.
The experimental conditions were previously described.[27]
When using the ACQUITY Premier Solution, an additional 50% increase in peak height and a 2-fold increase in peak area was obtained. Relative to the results observed using a standard UPLC System and Column, the ACQUITY Premier Solution gave a 27-fold increase in peak height and a 4-fold increase in peak area for the LC:T14 peptide.

SYSTEM QUALIFICATION TESTING

When using any analytical instrument, it is important to ensure proper system performance, often done in the form of instrument qualification and/or inclusion of system suitability measurements prior to analyses. To this end, two Quality Control Reference Materials (QCRMs) were developed to assess system inertness, each including the metal-sensitive probe compound adenosine 5′-(α,β-methylene)diphosphate (AMPcP). One QCRM includes only AMPcP and is intended for use without a column to evaluate/troubleshoot the LC flow path. Injecting a sample containing only AMPcP onto an ACQUITY Premier System with a union in place of a column, AMPcP peak area was nearly doubled, and the peak area RSD was reduced from 6.4% to 0.4% over six replicate injections compared to a traditional metal alloy containing biocompatible UPLC (see Figure 7B).

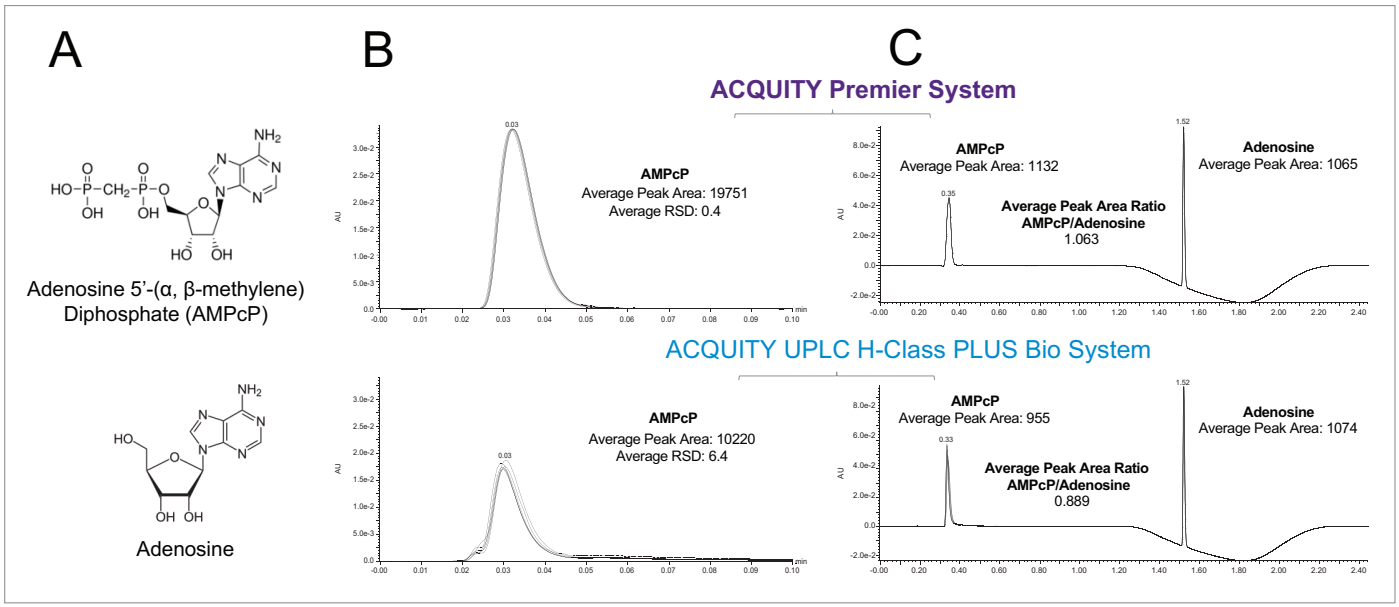

Figure 7. A) Structures of the test analytes used to evaluate system performance. B) Overlays of five replicate injections of the AMPcP standard on an ACQUITY Premier System (top) versus on an ACQUITY UPLC H-Class PLUS Bio System (bottom). The mobile phase was 50/50 water:acetonitrile. C) Overlays of five replicate injections of the AMPcP and adenosine standard on an ACQUITY Premier System (top) and five replicate injections on an ACQUITY UPLC H-Class PLUS Bio System (bottom). An ACQUITY Premier HSS T3, 1.8 µm, 2.1 x 50 mm Column was used, the temperature was 35 °C, and the peaks were detected by absorbance at 260 nm. A linear gradient from 5–95% B was carried out, where mobile phase A was 10 mM ammonium acetate in 99.8/0.2 (v/v) water/acetonitrile and mobile phase B contained 8 mM ammonium acetate and 0.1% trifluoroacetic acid in 79.8/20.2 (v/v) water/acetonitrile.

The second QCRM utilizes an equimolar combination of AMPcP and adenosine. Adenosine has the same base molecular structure as AMPcP but lacks the phosphate moiety that adsorbs to metal surfaces and is used as a negative control. Adenosine can be used to evaluate the overall health and performance of the system, while the AMPcP can be used to assess the potential for binding to metal surfaces. This sample requires the use of a chromatographic column. Comparing the ratio of AMPcP to adenosine can help to indicate the presence of metal binding sites in the flow path (including the chromatographic column), with this ratio decreasing as the number of binding sites increases. As shown in Figure 7C, injecting a sample containing AMPcP and adenosine with an ACQUITY Premier Column installed yielded an area ratio closer to the expected value of one when the ACQUITY Premier System was used versus a conventional metal alloy based biocompatible UPLC system. Considering that the adenosine peak areas differed by less than 1% on the two systems, this suggests that there was some interaction between AMPcP and the conventional metal alloy based biocompatible UPLC system, resulting in a reduced peak area for AMPcP. The peak height variability for AMPcP was also greater for this system compared to the ACQUITY Premier System, as shown by the RSD values of 9.0% for the metal alloy-based biocompatible system vs 0.8% for the ACQUITY Premier System over five replicate injections.

MOBILE PHASES AND SAMPLE PREPARATION

The interaction of analytes with metal surfaces is highly dependent on the composition of the mobile phase. One key aspect is the pH. Studies of nucleotides and oligonucleotides have demonstrated that the most severe adsorption on stainless steel occurs with acidic mobile phases.[4,5,28] When using basic mobile phases, adsorption is greatly reduced. A likely explanation for this is that the oxide layer on stainless steel has an isoelectric point close to 7.[29] When the pH is lower than 7, the oxide layer is positively charged, and may interact electrostatically with analytes that are negatively charged.

Another important consideration when separating metal-sensitive analytes is mobile phase purity. When preparing the mobile phase, LC-MS quality reagents should be used. Mobile phase containers should be chosen to avoid ionic contaminants, which may leach from glass bottles. Plastic containers are recommended, preferably certified clean polyethylene bottles. Metal sinker filters should not be used. In some challenging LC-MS applications, MaxPeak HPS hardware may not resolve all issues caused by analyte adsorption. In these cases, a low concentration (sub-millimolar) of a chelating additive, such as citric acid, may be added to the mobile phase to mitigate any residual adsorption.[30]

Ionic contamination caused by glass sample containers is another issue to avoid. Plastic sample containers are recommended. For analytes that may adsorb to the hydrophobic surface of plastic containers, low-bind containers such as QuanRecovery™ Vials and Plates should be used.[31] Some samples might contain a significant concentration of metal ions and may benefit from the addition of chelators and/or suitable internal standards to the sample solvent to bind the metal ions.[32] Similarly, it might be advantageous in some instances to include a chelating additive in the sample preparation process.

CONCLUSIONS

The ACQUITY Premier System, constructed with corrosion-resistant components and MaxPeak High Performance Surfaces, provides improved separations of analytes that adsorb on metal surfaces. The best results are obtained when this system is used together with ACQUITY Premier Columns, which incorporate the same ethylene-bridged hybrid surface. With the ACQUITY Premier Solution, the need for conditioning with samples or standards is greatly reduced, leading to significant time savings as well as improved precision and accuracy. This solution has proven to be effective in RP and HILIC separations of a range of analytes, including organophosphates, organic acids, oligonucleotides, acidic peptides, sialylated glycans and certain phospholipids. Improved performance has also been observed for some analytes containing other electron-rich functional groups, such as amine, amide, hydroxyl, and ether groups.

Because adsorptive losses become more severe as the mass injected decreases, the ACQUITY Premier Solution is particularly valuable for applications where low levels of metal-sensitive compounds need to be quantified. This makes the ACQUITY Premier Solution ideal for use with mass spectrometry detection. Applications such as bioanalysis of oligonucleotides and other metal-sensitive analytes, metabolomics studies of acidic polar metabolites, and proteomics investigations of phosphopeptides all show benefits from the use of the ACQUITY Premier Solution.

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