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Demonstrating Improved Sensitivity and Dynamic Range with MaxPeak High Performance Surfaces (HPS) Technology: A Case Study on the Detection of Nucleotides

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Abstract

Acidic metal-sensitive analytes are challenging to robustly and sensitively assay by LC-MS. Numerous attempts have been made to mitigate analyte losses by modulating the chemical attraction to metal surfaces, but none have produced a universal solution that is compatible with highly sensitive UPLC-MS analyses. Using adenosine nucleotides as model analytes, we have demonstrated that the Waters ACQUITY Premier LC System with HPS Technology offers superior protection against metal-analyte interactions without compromising the benefits of high-efficiency UPLC separations with sensitive MS detection.

Benefits

- This case study shows when and how chromatographic surfaces can be of significant influence for analyte detection.
- Waters ACQUITY Premier LC System and Columns with MaxPeak High Performance Surfaces can

dramatically improve the recovery and peak shape of metal sensitive analytes, like nucleotides and their analogs.

Introduction

Nucleotides are the building blocks of DNA and RNA and they play a central role in metabolism. Analyzing and quantifying them is thus crucial to understanding biological functions and activities.[1,2] Over the years, the study of nucleotides and their analogs has received growing attention. In one very important instance, nucleotide type molecules are being studied and applied as RNA polymerase inhibitors to treat viral infectious diseases[3] such as Hepatitis C,[4] certain viral cancers,[5] Ebola,[6] and even more recently, SARS-CoV-2 (COVID-19).[7]

One of the challenges in LC-MS analyses of nucleotides is their affinity for metal surfaces, especially stainless-steel and corroded stainless-steel, which can cause low recoveries.[8,9] Such losses can be severe at low analyte concentrations and thus negatively impact assay sensitivity. To avoid these detrimental losses, analysts have employed many different workarounds. Some have tested metal-free LC-MS instruments equipped with polymeric or silica-based components, which turned out to be incompatible with modern sub-2-µm particle UPLC methods.[9] Others have found limited success by adding metal-chelating additives to mobile phases to suppress metal-analyte interactions. Ultimately though, this has narrowed their method development options.[10]

To this end, Waters has developed MaxPeak High Performance Surfaces (HPS) Technology that offers new inert surfaces for both LC instruments and columns.[11] In this application note, we will demonstrate the benefit of MaxPeak HPS Technology using adenosine and its phosphorylated derivatives as test analytes and will thereby show a case study on when and how chromatographic surfaces can be of significant influence to analyte detection.

Experimental

Sample Description:

Mixture of ATP, ADP, AMP, and adenosine in water

LC Conditions

System:	ACQUITY Premier UPLC, ACQUITY UPLC I-Class PLUS
Detection:	Triple Quad MS
Vials:	TruView Vials
Column(s):	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 50 mm in standard stainless-steel hardware (p/n: 186003538) ACQUITY Premier HSS T3, 1.8 µm, 2.1 x 50 mm (p/n: 186009467)
Column temp.:	35 °C
Sample temp.:	20 °C
Injection volume:	1 µL
Flow rate:	0.5 mL/min
Mobile phase A:	10 mM ammonium acetate at pH 6.8
Mobile phase B:	Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	98.8	1.2	–
0.2	0.5	98.8	1.2	6
0.8	0.5	81.0	19.0	6
0.9	0.5	81.0	19.0	6
1.0	0.5	98.8	1.2	6
3.0	0.5	98.8	1.2	11

MS Conditions

System:	Xevo TQ-XS
Ionization mode:	Negative electrospray (ES-)
Acquisition range:	SRM: see table below
Capillary voltage:	-0.3 kV
Collision energy:	See table below
Cone voltage:	See table below

	MW	Transition	Cone voltage (V)	Collision energy (eV)
ATP	507.18	505.96>158.84	30	30
ADP	427.20	425.98>134.00	48	22
AMP	347.22	346.00>96.85	50	20
Adenosine	267.24	266.02>134.00	32	18

Data Management

Chromatography software:	MassLynx, v4.2
MS software:	MassLynx, v4.2
Informatics:	N/A

Results and Discussion

Adenosine and its phosphorylated derivatives, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), and adenosine-5′-monophosphate (AMP) (Table 1), were analyzed using a typical reversed-phase LC-MS condition and a triple quadrupole mass spectrometer. Figure 1 shows example separations of the four mentioned compounds. When the sample was injected onto a standard LC-MS system consisting of stainless-steel components and column hardware, the highly phosphorylated ATP and ADP were completely lost during the analysis. Meanwhile, the singly phosphorylated AMP was observed as a barely detectable peak with significant tailing (Figure 1a). When the same sample was injected onto an ACQUITY Premier Column using an ACQUITY Premier System (Figure 1c), all four adenosine-containing compounds were readily detected, with appreciable peak areas and significantly less tailing. This remarkable improvement in peak area and shape is attributed to the effective suppression of metal-analyte interactions, enabled by the proprietary MaxPeak High Performance Surfaces. It is interesting to compare these two chromatograms with an intermediate case, where the sample

was injected onto an ACQUITY Premier Column using a stainless-steel LC system (Figure 1b). While detectable peaks were observed for ATP and ADP, the peaks were significantly smaller compared to those obtained with the ACQUITY Premier LC and Column (Figure 1c). While AMP suffered a less severe loss than ATP or ADP, its peak shape was significantly distorted, making integration difficult. This result clearly demonstrates that metal-analyte interactions can be very disruptive for certain analytes and that it is important in such situations to use inert chromatographic surfaces.

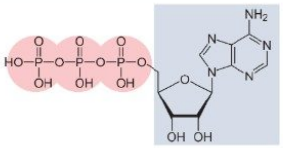
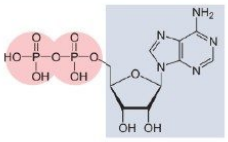
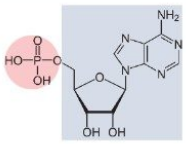
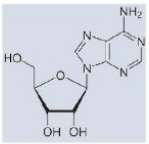
	ATP Adenosine-5'-triphosphate	ADP Adenosine-5'-diphosphate	AMP Adenosine-5'-monophosphate	Adenosine
				
	$C_{10}H_{15}N_5O_{13}P_3$	$C_{10}H_{15}N_5O_{10}P_2$	$C_{10}H_{15}N_5O_7P$	$C_{10}H_{15}N_5O_4$
MW	507.18	427.20	340.22	267.24
Log P*	-5.8	-4.7	-4.7	-2.1
pK_a* (strongest acid)	0.9	1.77	1.22	12.45

Table 1. Test compounds. The log P and pKa values are from the Human Metabolome Database, calculated using the ChemAxon algorithm (http://hmdb.ca/).

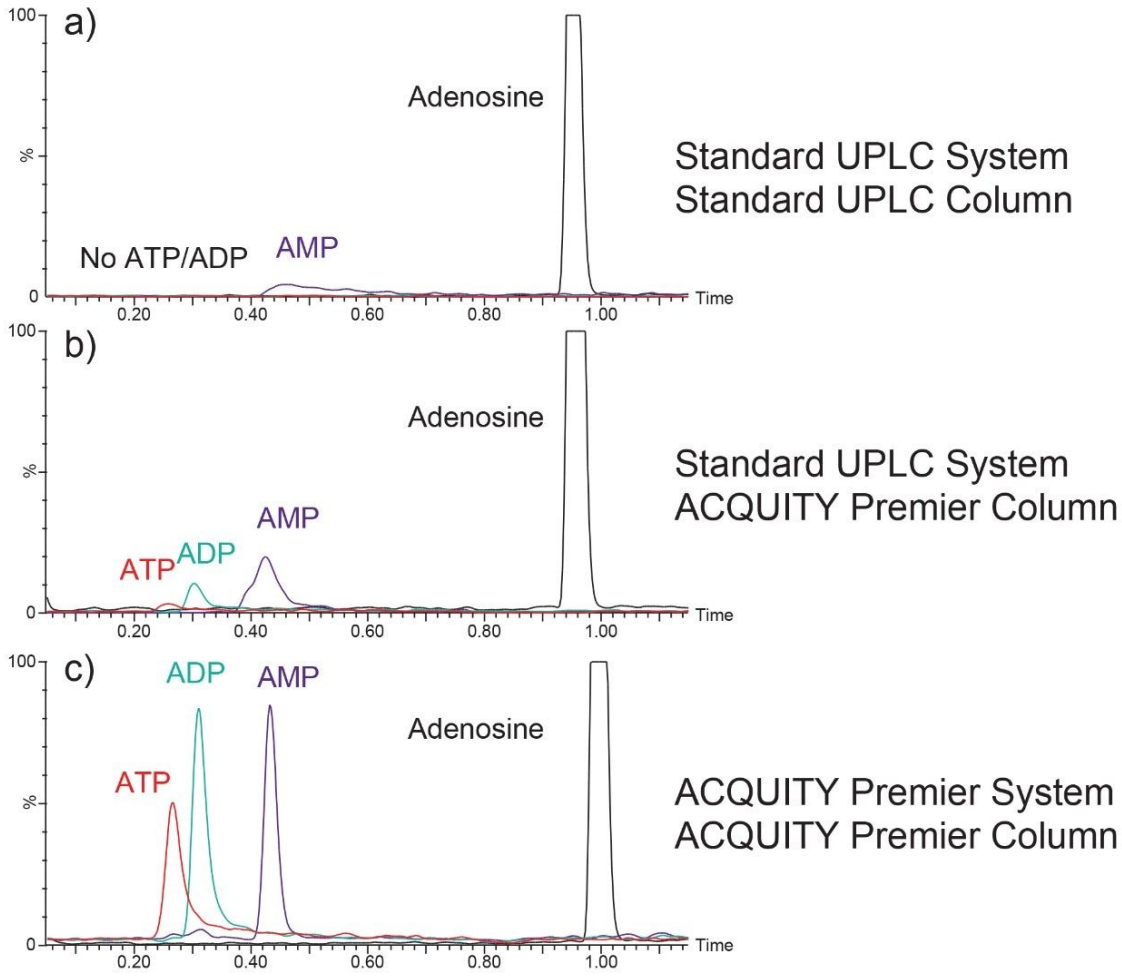


Figure 1. Example chromatograms from 1 µL injections of mixture samples containing ATP, ADP, AMP, and adenosine (20 pg/µL each).

To further demonstrate the importance of these inert LC surfaces, calibration curves for the four compounds were plotted using log-log scaling (Figure 2a), while the corresponding numerical results have been summarized in Table 2. ATP and ADP posed the greatest challenge for achieving a linear calibration curve. When these compounds were analyzed with the standard LC system and standard column, no peak was observed even at a relatively high concentration of 2000 pg/µL (2 ng column load). Replacing the standard stainless-steel column with an ACQUITY Premier Column enabled the detection of ATP and ADP, but the calibration ranges were significantly narrower compared to those acquired using the fully inert ACQUITY Premier System with an ACQUITY Premier Column. The analyte losses directly impacted the lower limit of detection. An example can be

found with AMP, a less acidic compound than ATP or ADP. While AMP did not suffer from as much adsorptive loss as ATP or ADP in all the three combinations of systems and columns that we tested, the sensitivity with which it was detected still varied significantly. The lower limit of detection for AMP was an order of magnitude higher (Table 2) and the slope of the calibration curve was lower (Figure 2b) when a standard UPLC system and column were used. Employing the ACQUITY Premier LC together with an ACQUITY Premier Column offered the best sensitivity for all four analytes: quantitation of sub-pg/µL concentrations was possible and calibration ranges of over three orders of magnitude could be established.

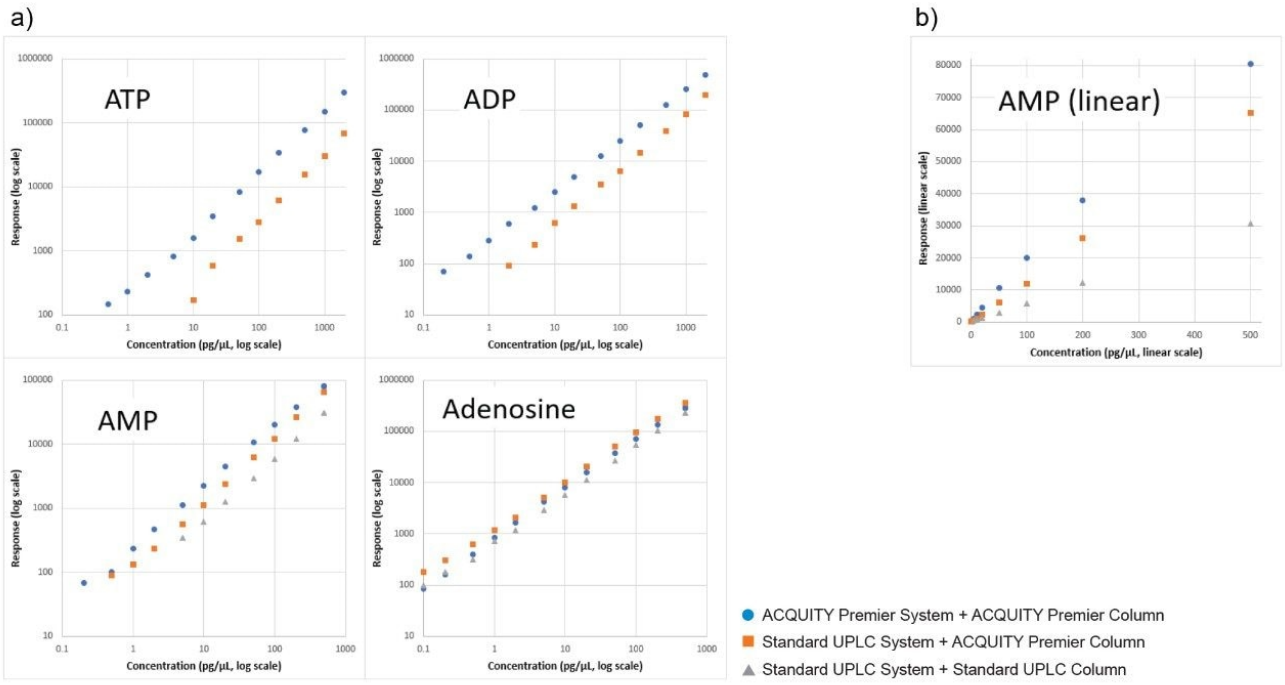


Figure 2. a) Log-log calibration curves for 4 compounds, and b) linear calibration curve for AMP.

	ACQUITY Premier System ACQUITY Premier Column		Standard UPLC System ACQUITY Premier Column		Standard UPLC System Standard UPLC Column	
	Range (pg/µL)	r^2	Range (pg/µL)	r^2	Range (pg/µL)	r^2
ATP	0.5–2000	0.9984	10–2000	0.9968	Not detected	–
ADP	0.2–2000	0.9996	2–2000	0.9873	Not detected	–
AMP	0.2–500	0.9877	0.5–500	0.9987	5 -500	0.9995
Adenosine	0.1–500	0.9877	0.1–500	0.9822	0.1 - 500	0.9947

Table 2. The calibration ranges for four compounds and their linearity when the samples were injected onto different columns and systems. All calibration curves were determined with 1/x weighting.

Adenosine, which does not contain a metal-sensitive phosphate group, yielded equivalent calibration curves and a calibration range of over three orders of magnitude regardless of the choice of LC system and column. This suggests that the observed sensitivity differences with phosphate-containing compounds was indeed due to problematic analyte-metal interactions and that the ACQUITY Premier technology made it possible to quantify ATP, ADP, and AMP without any bias or historical problems related to phosphorylated molecules.

Being that nucleotides are very polar analytes, extra care should be given to develop fit-for-purpose chromatographic methods, especially where very robust quantitation might be needed. Herein, an RPLC method with HSS T3 and ammonium acetate mobile phase was employed to complete a case study on the effects of inert chromatographic surfaces. The employed separation technique does not provide retention for each of the adenosine nucleotides. ATP, in fact, is seen to elute near the void. As such, this exact method may not be sufficiently robust to deal with complex sample matrices. Analysts might find it worthwhile to also investigate the addition of some mobile phase ion pairing and the use of MaxPeak High Performance Surfaces in the form of hydrophilic interaction chromatography (HILIC) and anion-exchange reversed-phase column technologies.

Conclusion

Analyzing adenosine nucleotides, such as ATP, ADP, and ADP, was found to be difficult using standard LC systems and columns made of stainless-steel components. The challenge can be attributed to the strong affinity

of these compounds towards exposed metal surfaces. The analytes with two or three phosphate groups showed the worst effects. With the standard LC and column, ATP and ADP were completely lost at and below 2 ng/µL sample concentrations. AMP, with a single phosphate group, showed comparatively minor losses. Nonetheless, it suffered from compromised assay sensitivity.

As shown with this case study, the ACQUITY Premier System can be effectively used with an ACQUITY Premier Column to overcome some metal adsorption challenges that can interfere with achieving robust quantitation. With this technology, new levels of performance will now become possible for the quantitation of nucleotides, whether the employed technique be based on a fit-for-purpose RPLC method, HILIC, or mixed mode chromatography.

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720007053, November 2020