Application Note

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HPLC-DAD Analysis of Nucleotides Using a Fully Inert Flowpath

Agilent 1260 Infinity II Bio-Inert LC System and a PEEK-lined Agilent InfinityLab Poroshell 120 HILIC-Z Column

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Abstract

Phosphorylated compounds such as nucleotides are known to interact with stainless steel components leading to recovery issues in HPLC and LC/MS. This Application Note describes the analysis of nucleotides using the Agilent 1260 Infinity II Bio-Inert LC System and a PEEK-lined Agilent InfinityLab Poroshell 120 HILIC-Z column. The fully inert flowpath, governed by the setup, leads to substantially improved recoveries of these challenging phosphorylated solutes.

Introduction

The chromatographic analysis of phosphorylated species is highly challenging. Interaction between phosphate groups and stainless steel components in the flowpath leads to peak broadening and peak loss. This phenomenon has been described for phosphorylated peptides, phosphosugars, and nucleotides, among others[1-6]. We recently demonstrated that phosphorylated glycans can successfully be analyzed when the entire sample flowpath is metal-free[7]. To achieve this, an Agilent 1260 Infinity II Bio-Inert LC System and a PEEK-lined HILIC column were used. Here, we extend this work to other challenging solutes, for example, nucleotides. In the analysis of nucleotides, precautions are generally taken to prevent the formation of metal-phosphate complexes. These include operating the instrument at high pH values, using ion pairing reagents, by treatment of stainless steel components with phosphoric acid or carbonate, or by adding metal chelators to the mobile phase[8]. We demonstrate the analysis of nucleotides on a fully inert flowpath using a 1260 Infinity II Bio-Inert LC and a PEEK-lined Agilent InfinityLab Poroshell 120 HILIC-Z column.

Experimental

Materials

Acetonitrile and water were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, ammonium hydroxide, nucleosides, and nucleotides were purchased from Merck (Darmstadt, Germany) (Figure 1).

Sample preparation

Nucleotides and nucleosides were dissolved in water at a concentration of 100 ppm.

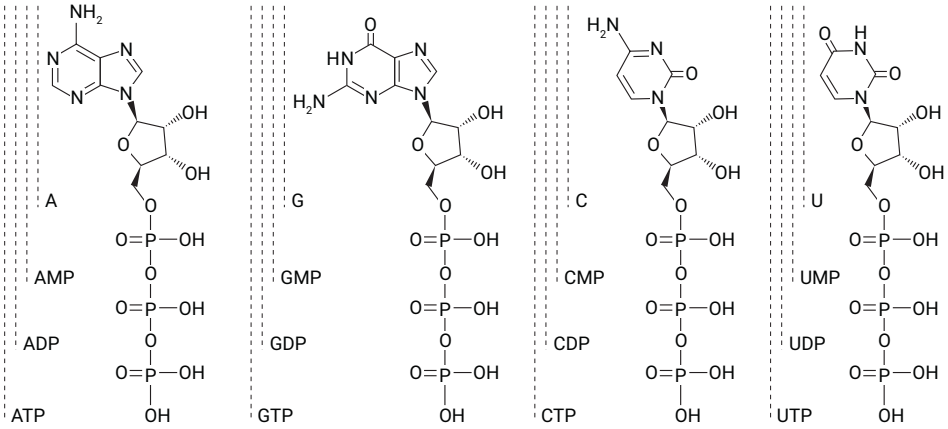

Figure 1. Nucleosides and nucleotides used in this study. A: adenosine; G: guanosine; C: cytidine; U: uridine.

Instrumentation

HILIC-DAD measurements were performed with the following setup:

Agilent 1260 Infinity II Bio-Inert LC System equipped with:

- Agilent 1260 Infinity II Bio-Inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-Inert Multisampler (G5668A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with Bio-Inert Heat Exchanger (option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert standard flow cell (10 mm, option #028)

Agilent 1260 Infinity II LC System equipped with:

- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Multiple Wavelength Detector (G7165A) with standard flow cell (10 mm, option #018)

Agilent OpenLab CDS version 2.1 software was used.

Method

Parameter	Value
Column	InfinityLab Poroshell 120 HILIC-Z, stainless steel, 2.1 × 100 mm, 2.7 µm (p/n 685775-924) InfinityLab Poroshell 120 HILIC-Z, PEEK-lined, 2.1 × 100 mm, 2.7 µm (p/n 675775-924)
Mobile Phase A	10 mM NH_4-acetate, pH 9
Mobile Phase B	Acetonitrile/100 mM NH_4-acetate, pH 9 (90:10) (v/v)
Gradient	0 to 12 minutes: 90 to 48 %B 12 to 13 minutes: 48 %B 13 to 13.1 minutes: 48 to 90 %B 13.1 to 22 minutes: 90 %B
Flow Rate	0.4 mL/min
Column Temperature	35 °C
Injection Volume	1 µL
Detection	260 nm, bandwidth 4 nm, no reference, 10 Hz

Results and Discussion

A recent Application Note demonstrated significant peak tailing and area reduction when analyzing adenosine triphosphate (ATP) on a stainless steel instrument. It was hypothesized that the formation of phosphate-iron complexes caused these problems. By contrast, Gaussian peak shapes and full recovery were obtained on an instrument with a metal-free flowpath, that is, an Agilent 1260 Infinity Bio-Inert Quaternary LC[6]. The effect of the column was not described in that study. The recent introduction of PEEK-lined columns with stainless steel housing has opened opportunities for the LC analysis of challenging solutes on fully inert flowpaths.

Figures 2 to 5 show the analysis of various nucleotides and nucleosides on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC. An enormous discrepancy was noticed when analyzing the nucleotides on a stainless steel or PEEK-lined column. The nonphosphorylated nucleosides adenosine, cytidine, guanosine, and uridine behave equally well on both columns, illustrating equal column quality. The phosphorylated species, however, show tailing peaks and poor recovery on the stainless steel column. The higher the number of phosphate groups, the lower the recovery. As evidenced from the data, the instrument itself also has a nonnegligible impact on peak shape and recovery.

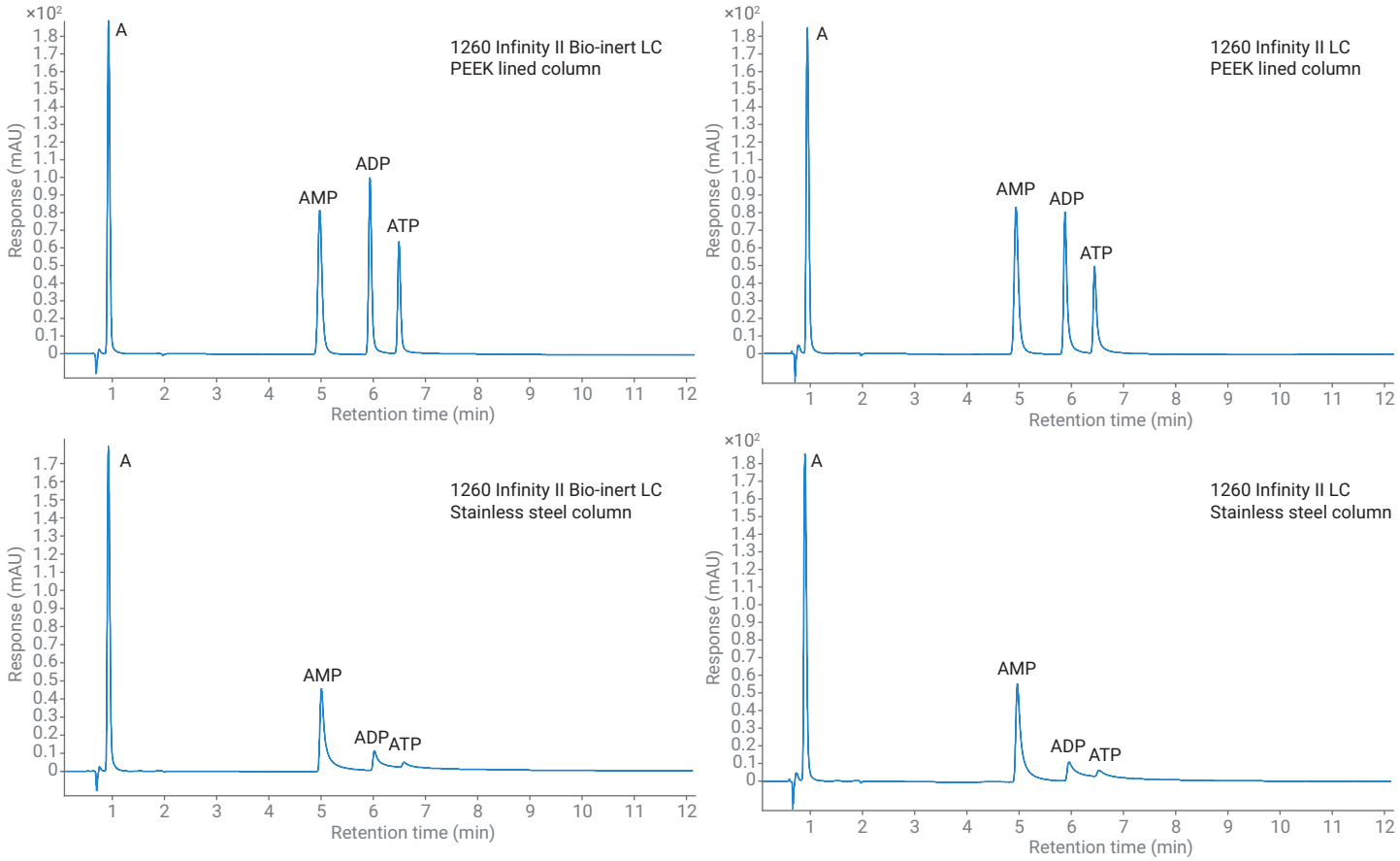

Figure 2. HILIC-DAD chromatograms of adenosine (A), AMP, ADP, and ATP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

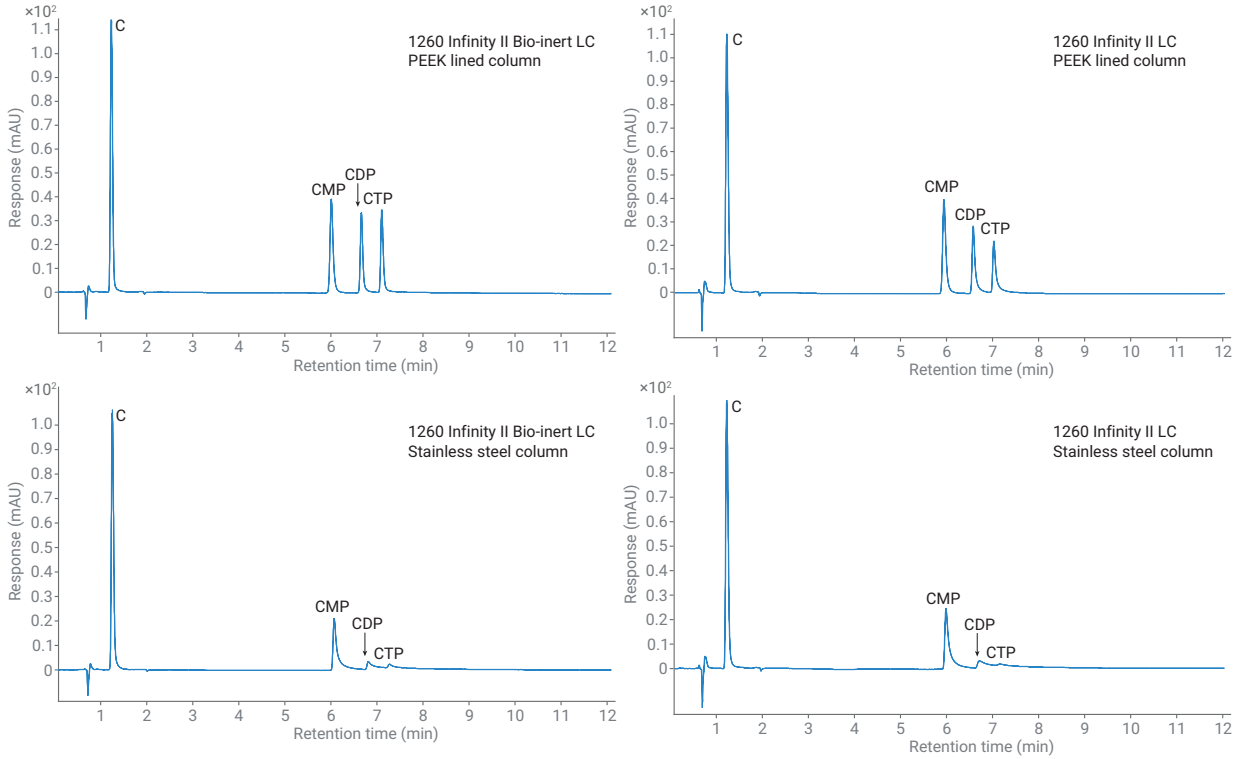

Figure 3. HILIC-DAD chromatograms of cytidine (C), CMP, CDP, and CTP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

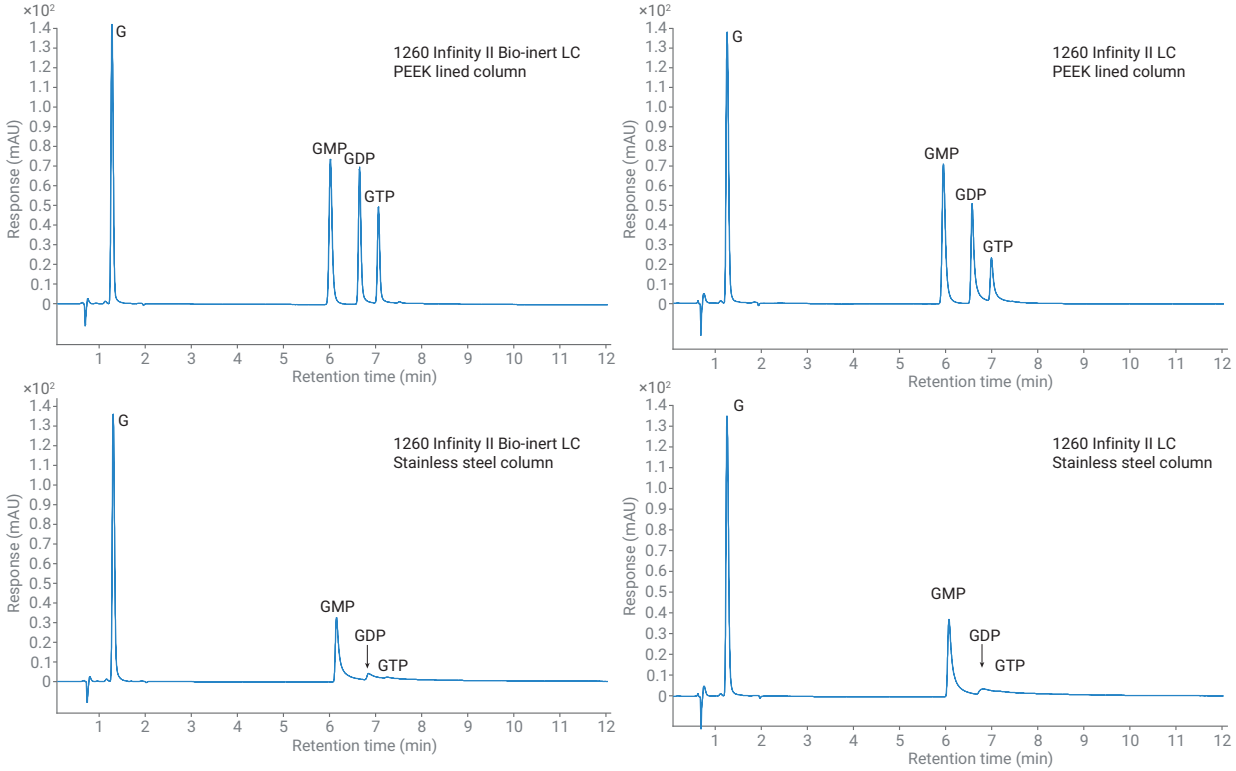

Figure 4. HILIC-DAD chromatograms of guanosine (G), GMP, GDP, and GTP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

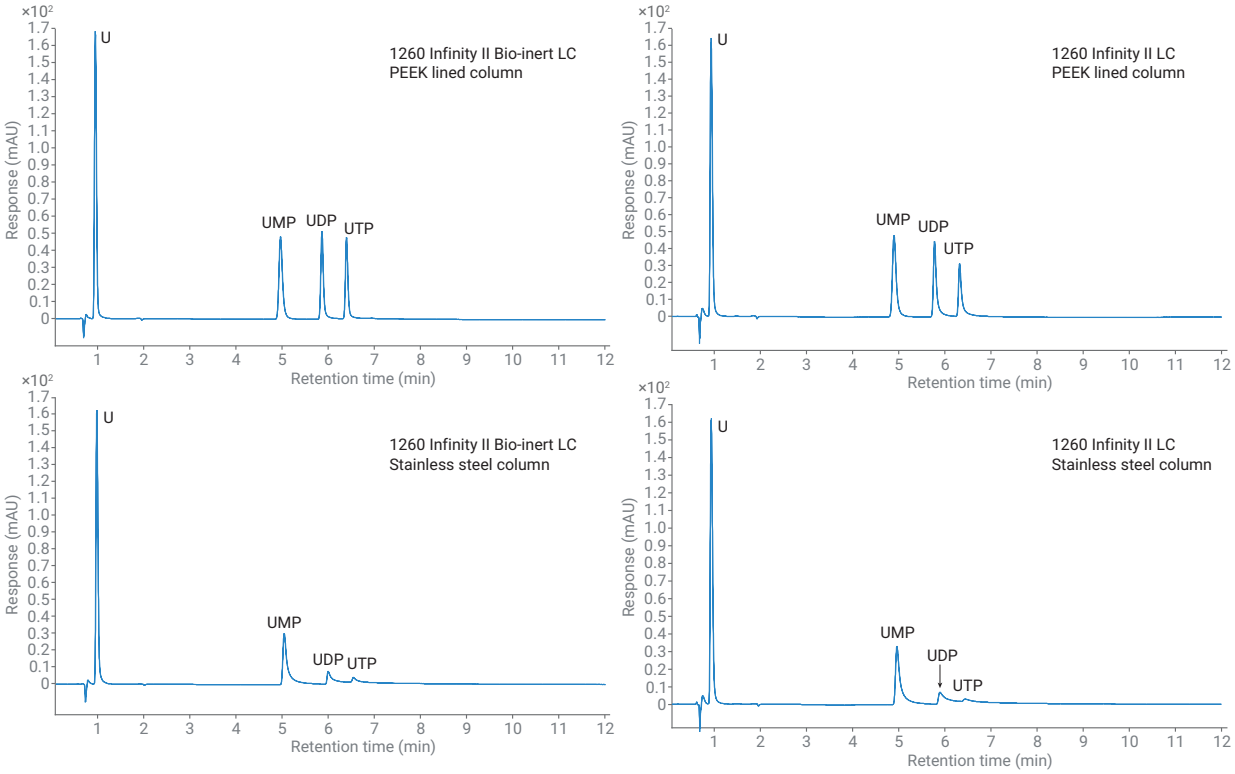

Figure 5. HILIC-DAD chromatograms of uridine (U), UMP, UDP, and UTP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

On the PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on the 1260 Infinity II Bio-Inert LC, improved peak shapes and improved recoveries were observed for all nucleotides. This shows the importance of removing all metal parts from the flowpath (instrument and column inertness). The data shown in Table 1 further support these findings. Upon evolving from an inert to a stainless steel flowpath, a decrease in area recovery is observed. This decrease accompanies an even more substantial lowering of peak height recovery, illustrating the worsening of the peak shape.

Table 2 shows the RSD values obtained on retention time, peak area, area %, and peak height for the quadruplicate analysis of adenosine, AMP, ADP, and ATP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC. It can be concluded that full inertness results in improved figures-of-merit for the analysis of these challenging solutes. Figure 6 shows the corresponding chromatograms.

Table 1. Retention time, peak area, area %, and peak height for the different solutes analyzed on a PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and recoveries obtained for the different solutes analyzed on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-inert LC and 1260 Infinity II LC.

	PEEK Lined Column and Bio-inert HPLC System (Full Inert)				% Recovery SS Column/SS HPLC Versus Fully Inert (Area)	% Recovery SS Column/SS HPLC Versus Fully Inert (Height)	% Recovery SS Column/Inert HPLC Versus Fully Inert (Area)	% Recovery SS Column/Inert HPLC Versus Fully Inert (Height)	% Recovery PEEK-Lined Column/SS HPLC Versus Fully Inert (Area)	% Recovery PEEK-Lined Column/SS HPLC Versus Fully Inert (Height)
Compound	RT (min)	Area	Area%	Height						
Adenosine	0.86	633.8	34.6	189.8	106.2	95.8	100.1	95.5	109.1	98.3
AMP	4.91	477.9	26.1	82.1	96.2	66.5	82.3	56.4	108.1	102.6
ADP	5.88	431.2	23.6	100.8	41.3	11.0	35.3	11.7	96.5	80.7
ATP	6.43	288.0	15.7	64.5	49.2	9.9	40.8	8.2	102.5	78.3
Cytidine	1.18	379.7	44.2	113.1	110.0	94.7	100.3	94.6	110.8	96.9
CMP	5.98	192.4	22.4	39.1	95.2	61.9	80.9	55.1	109.0	101.9
CDP	6.64	138.7	16.1	33.7	41.5	9.9	35.8	10.9	105.5	85.1
CTP	7.09	148.4	17.3	34.7	30.7	5.7	31.7	7.4	86.1	64.2
Guanosine	1.21	496.0	36.7	139.2	109.1	96.3	100.2	95.4	110.2	98.6
GMP	6.01	360.3	26.7	72.5	90.0	51.5	71.9	44.6	110.0	98.0
GDP	6.65	288.7	21.4	68.6	27.0	5.6	24.2	6.3	101.6	74.2
GTP	7.06	205.6	15.2	48.7	25.8	5.7	27.0	5.0	82.0	48.3
Uridine	0.88	523.1	42.6	168.5	110.3	96.1	101.1	96.2	111.1	98.0
UMP	4.96	275.5	22.4	48.3	100.1	68.7	87.4	63.1	108.8	100.5
UDP	5.87	222.7	18.1	51.8	50.2	14.0	41.8	15.5	105.3	87.0
UTP	6.41	206.4	16.8	48.1	55.8	7.0	50.6	9.3	89.4	66.4

Table 2. RSD on retention time, peak area, area %, and peak height for adenosine, AMP, ADP, and ATP analyzed in quadruplicate on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

	SS Column and SS HPLC System				SS Column and Bio-Inert HPLC System				PEEK-Lined Column and SS HPLC System				PEEK-Lined Column and Bio-inert HPLC System (Full Inert)			
Compound	RT (min)	Area	Area%	Height	RT (min)	Area	Area%	Height	RT (min)	Area	Area%	Height	RT (min)	Area	Area%	Height
Adenosine	0.10	1.69	2.24	0.80	0.11	0.11	1.98	0.11	0.10	0.44	0.27	0.34	0.06	0.14	0.14	0.23
AMP	0.11	1.62	2.26	2.24	0.18	2.04	0.15	4.07	0.03	0.63	0.11	0.50	0.04	0.14	0.04	0.53
ADP	0.21	10.52	7.00	21.04	0.12	6.65	4.83	14.32	0.04	0.94	0.32	1.21	0.02	0.46	0.32	0.20
ATP	0.28	10.35	6.82	16.91	0.14	5.20	3.34	13.07	0.04	0.96	0.34	1.79	0.02	0.19	0.17	0.32

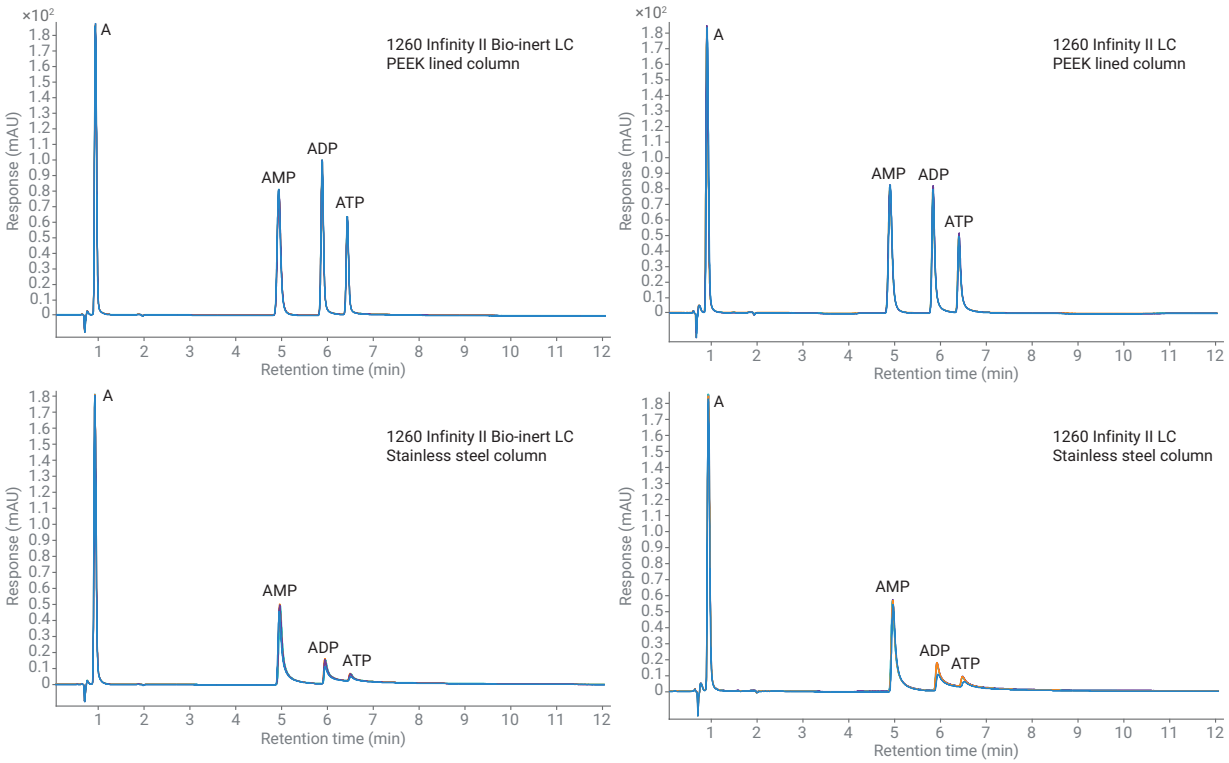

Figure 6. Quadruplicate injection of adenosine (A), AMP, ADP, and ATP on a stainless steel and PEEK-lined InfinityLab Poroshell 120 HILIC-Z column installed on a 1260 Infinity II Bio-Inert LC and 1260 Infinity II LC.

Conclusion

This Application Note describes the analysis of nucleotides using the 1260 Infinity II Bio-Inert LC and a PEEK-lined InfinityLab Poroshell 120 HILIC-Z column. It demonstrates that these challenging phosphorylated structures can successfully be analyzed when the entire flowpath is devoid of metal parts, that is, with instrument and column inertness.

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Printed in the USA, April 1, 2019
5994-0680EN

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