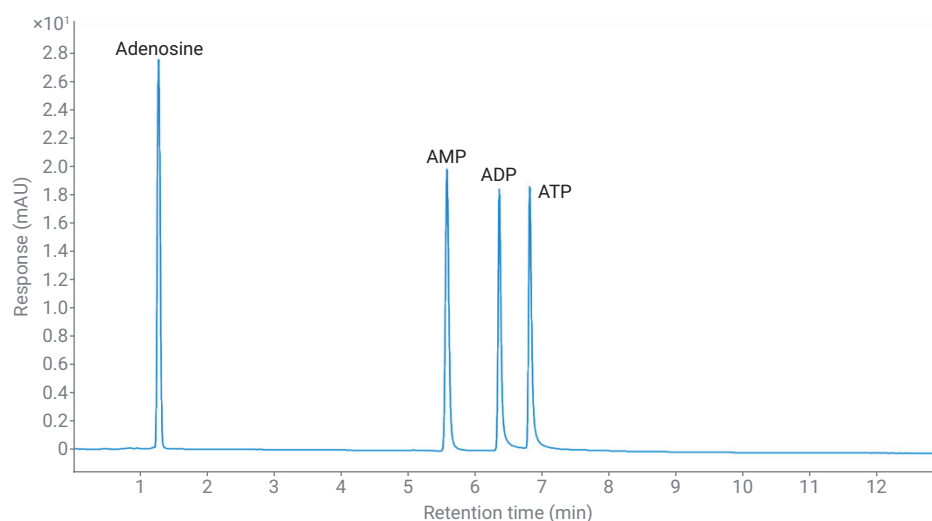


# Comparability Studies for the Analysis of Nucleotides on Four Different LC Systems

## Bio versus stainless steel systems



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### Abstract

Phosphorylated compounds are challenging molecules for stainless steel-based liquid chromatographic (LC) systems. Poor peak shape and sample loss can impact the data quality, and hence decrease confidence in the generated data. In this application note, adenosine and three corresponding nucleotides were analyzed on four different Agilent LC systems: two low-adsorbent LC systems, and two stainless steel-based systems. The performance of the four systems with respect to peak shape and sample loss was compared and evaluated. Both low-adsorbent Bio LC systems showed clearly superior performance over the stainless steel LC systems, with highly reproducible data.

## Introduction

Chromatographers aim for baseline separated peaks with symmetrical Gaussian peak shapes. Peak symmetry and high recovery rates are indicative of a well-behaving LC system. A well-behaving LC system is considered to be a system with no unwanted or unpredictable interactions between samples and chromatographic surfaces. Adsorptive interactions often lead to altered peak shapes like asymmetric peaks or peak tailing, and the recovery of the analyzed samples can be significantly reduced.<sup>1,2</sup>

Phosphorylated species are particularly challenging for stainless steel systems, as the interaction between the phosphate groups and the iron-containing stainless steel components in the flow path can lead to peak tailing and sample loss. Nucleotides are especially sensitive to this kind of interaction<sup>3</sup>. However, it was also described for other phosphorylated compounds like peptides, sugars, lipids, and glycans.<sup>4</sup>

One possibility for preventing the interaction of the phosphorylated samples with the stainless steel components is to execute conditioning procedures, to inactivate polar surface groups and reduce the amount of metals eluting from the LC system. However, these procedures mostly include time-consuming and cumbersome overnight flushing with strong metal chelators or acidic solutions, potentially harming the LC system and reducing productivity and instrument lifetime.<sup>2,5</sup>

The use of low-adsorption LC systems such as the Agilent 1260 Infinity II Bio-inert LC, the Agilent 1260 Infinity II Prime Bio LC, and the Agilent 1290 Infinity II Bio LC improve analyte recovery and limit undesired interaction, without the need for tedious conditioning procedures.<sup>2</sup> This application note

compares the analysis of adenosine and its phosphorylated derivatives with respect to sample loss, peak shape, and reproducibility using a PEEK-lined HILIC column on four different Agilent LC systems: two low adsorption LC systems, and two stainless steel-based systems.

## Experimental

### Equipment

The Agilent 1260 Infinity II Bio-Inert LC comprised the following modules:

- Agilent 1260 Infinity II Bio-Inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-Inert Multisampler (G5668A) equipped with Agilent InfinityLab sample thermostat (G5668A#101)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) equipped with a bio-inert standard flow heat exchanger
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert standard flow cell, 10 mm (G4212-60007)

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger (G7116-60071)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L, RFID tag (G1314-60189)

The Agilent 1290 Infinity II LC System comprised the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler (G7167B) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a micro flow cell, 3 mm, 2  $\mu$ L (G1314-60187)

The Agilent 1260 Infinity II LC System comprised the following modules:

- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1290 Infinity II Multisampler (G7167A) with Sample Thermostat (option #101)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with standard flow heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a micro flow cell, 3 mm, 2  $\mu$ L (G1314-60187)

### Software

Agilent OpenLab CDS Version 2.5 or later versions

### Column

InfinityLab Poroshell 120 HILIC-Z, 2.1  $\times$  150, 2.7  $\mu$ m, PEEK-lined (part number 673775-924)

## Chemicals

All solvents were LC grade. Acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Ammonium acetate, ammonium hydroxide, adenosine, adenosine 5'-monophosphate, adenosine 5'-diphosphate sodium salt, and adenosine 5'-triphosphate disodium salt hydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

## Samples

Adenosine, adenosine 5'-monophosphate, adenosine 5'-diphosphate sodium salt, and adenosine 5'-triphosphate disodium salt hydrate were dissolved in warm water and mixed in 80, 133, 200, and 400 µM to generate equimolar concentrations.

**Note:** Adenosine is dissolved best in warm water as the solubility in cold water is less efficient.

## Buffer preparation

100 mM ammonium acetate stock solution was prepared, and the pH was adjusted to pH 9. The prepared stock solution was filtered using a 0.2 µm membrane filter. For 1 L of eluent A, 100 mL of stock solution was mixed with 900 mL water. For eluent B, 100 mL of stock solution was mixed with 900 mL of acetonitrile.

## Method

**Table 1.** Chromatographic conditions.

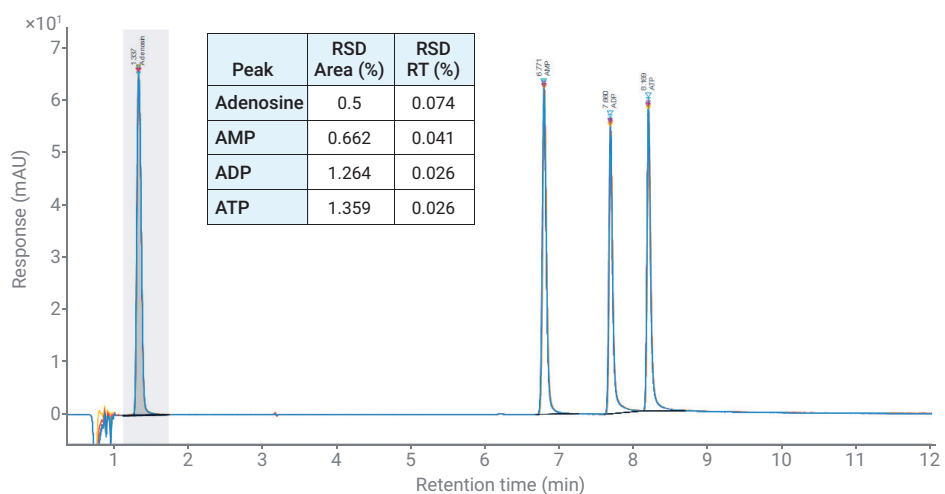
Parameter	Value
Solvent	A) 10 mM ammonium acetate, pH 9 B) ACN:100 mM ammonium acetate, pH 9 (9:1 (v:v))
Gradient	0 minutes: 90 %B 12 minutes: 50 %B 13 minutes: 50 %B Post time 10 minutes
Flow Rate	0.400 mL/min
Temperature	35 °C
Detection	260 nm 20 Hz
Injection	Injection volume: 1 µL Sample temperature: 10 °C Needle wash: 3 seconds in water

## Results and discussion

The order of analyses is an important factor in this application, as metal ions leaching from the HPLC equipment can impact the performance of the PEEK-lined separation column. First, the process was started in a completely PEEK-based low-adsorption system, the 1260 Infinity II Bio-Inert LC. Second, the analysis was transferred to the low-adsorption MP35N-based system, the 1260 Infinity II Prime Bio LC (MP35N is a nickel-cobalt-based alloy). Third, the analysis was transferred to the first stainless steel-based system,

the 1290 Infinity II LC, followed by the fourth system, the 1260 Infinity II LC. It is important to note that the exact same solvents, samples, and most importantly, the same column were used in all experiments. To prevent the phosphorylated samples from degradation, all samples were freshly prepared and cooled to 10 °C. All analyses were conducted consecutively within three days.

The first analysis on the Agilent 1260 Infinity II Bio-Inert LC is shown in Figure 1. All four peaks were baseline separated, with good peak shapes and excellent relative standard deviations (RSDs) for retention time (RT) and area. It has to be kept in mind that HILIC can be challenging in terms of peak shape, as unwanted, secondary interactions are more common compared to reverse phase chromatography.<sup>6</sup> Considering the demanding HILIC chromatography, the peak shapes appeared excellent, with only minimal tailing.



**Figure 1.** Separation of nucleotides on the Agilent 1260 Infinity II Bio-Inert LC, seven consecutive runs.

The second analysis on the Agilent 1260 Infinity II Prime Bio LC is shown in Figure 2. As already seen with the 1260 Infinity II Bio-Inert LC, all four peaks were baseline separated with good peak shapes and excellent relative standard deviations (RSDs) for retention time (RT) and area. The adenosine peak is higher in intensity compared to the three phosphorylated nucleotide peaks, due to the sharper peak shape of adenosine caused by the low-dispersion system. However, the area percentage of the four nucleotide peaks is comparable to the area percentage of the analysis in the 1260 Infinity II Bio-Inert LC, revealing equivalent performance of the two systems for the analysis of nucleotides. Due to the use of the DAD in this 1260 Infinity II Bio-Inert LC system, the peak intensities were overall higher, compared to the other systems where a VWD was used.

The third analysis on the stainless steel-based 1290 Infinity II LC looks completely different (Figure 3). While the adenosine peak is comparable to the previous two systems with respect to peak height/area and shape, the three phosphorylated nucleotides show quite different peak appearance. Adenosine can be considered here to be an internal standard, which should behave exactly the same in all LC systems due to the fact that it does not contain any iron-reactive phosphate groups. In contrast, AMP, and especially ADP and ATP, are extensively tailing. In addition, the amount of sample loss increases with increasing phosphate groups attached to the molecule (see also Figure 5). The interaction between the iron from the stainless steel capillaries and further components becomes visible.

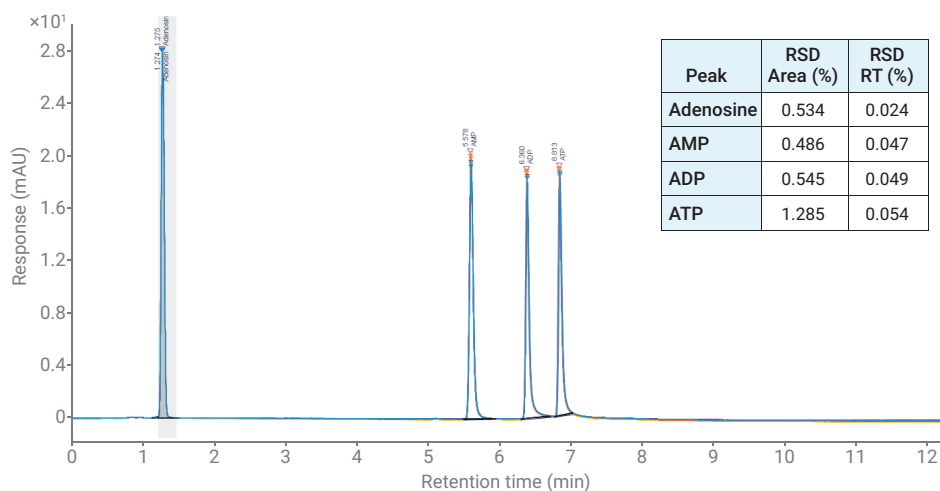


Figure 2. Separation of nucleotides on the Agilent 1260 Infinity II Prime Bio LC, seven consecutive runs.

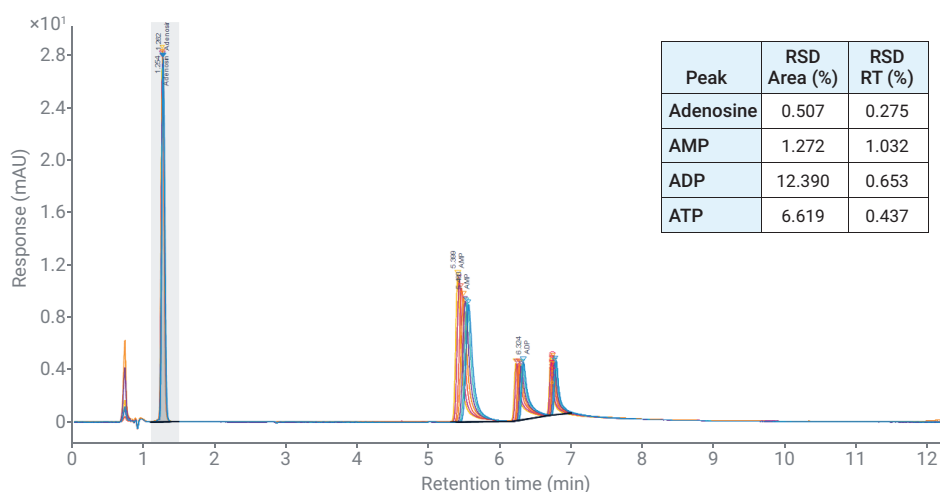


Figure 3. Separation of nucleotides on the Agilent 1290 Infinity II LC, seven consecutive runs.

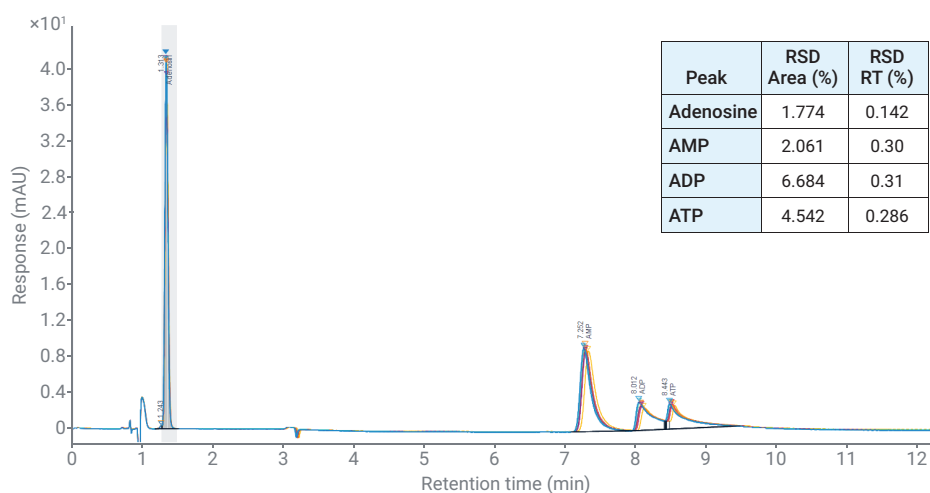


Figure 4. Separation of nucleotides on the Agilent 1260 Infinity II LC, seven consecutive runs.

Similar to the analysis on the 1290 Infinity II LC, the analysis on the 1260 Infinity II LC shows also immense peak tailing in combination with high sample loss for the phosphorylated nucleotides, see Figure 4 and 5.

Figure 5 summarizes the results with respect to sample loss in all four analyzed systems. The area loss was calculated using adenosine as internal standard. As no phosphate groups are attached to adenosine, no interaction between the iron-containing components of the LCs is expected. Therefore, a mathematical formula was used to calculate area loss (Equation 1):

The assumption is that the 1260 Infinity II Bio-inert system with only PEEK-cladded capillaries and other bio-inert materials is the gold standard. To exclude other factors like columns and supplies, the area loss values were normalized to the 1260 Infinity II Bio-inert system.

First, the area loss increases with increasing attached phosphate groups, as the higher the phosphate amount, the higher the interaction with iron-based materials.

Second, it becomes obvious that the area loss is only seen in the SST systems, and no difference in area loss is observed in the two Bio Systems. Hence, it can be reasoned that the MP35N-based system – the 1260 Infinity II Prime Bio LC – is equivalently suited for nucleotide analysis compared to the Agilent 1260 Infinity II Bio-Inert LC.

Different concentrations of nucleotides were analyzed to evaluate the influence of the concentration factor with respect to sample loss on the SST-based 1290 Infinity II LC. Figure 6 shows a diagram for the amount of sample loss in percent, versus different concentrations of the phosphorylated nucleotides. Four different concentrations were analyzed: 80, 133, 200, and 400 µM. As

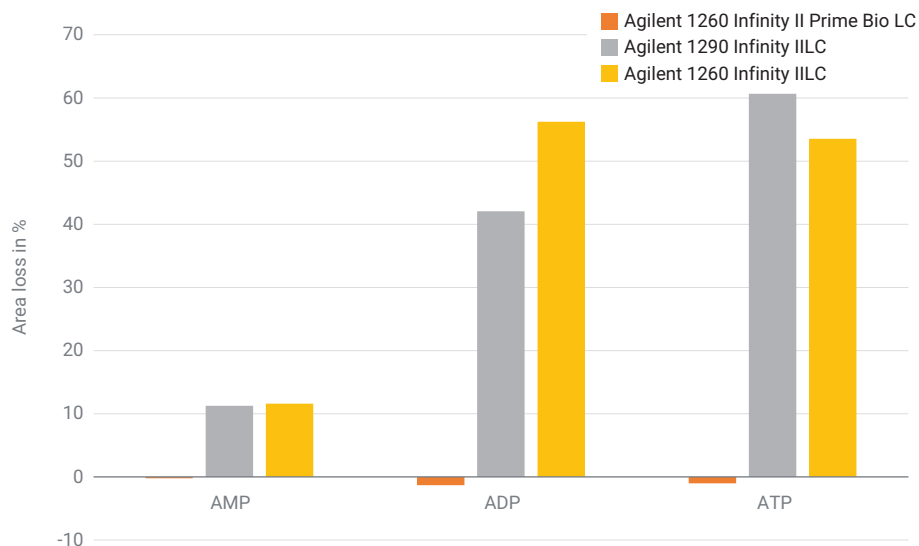
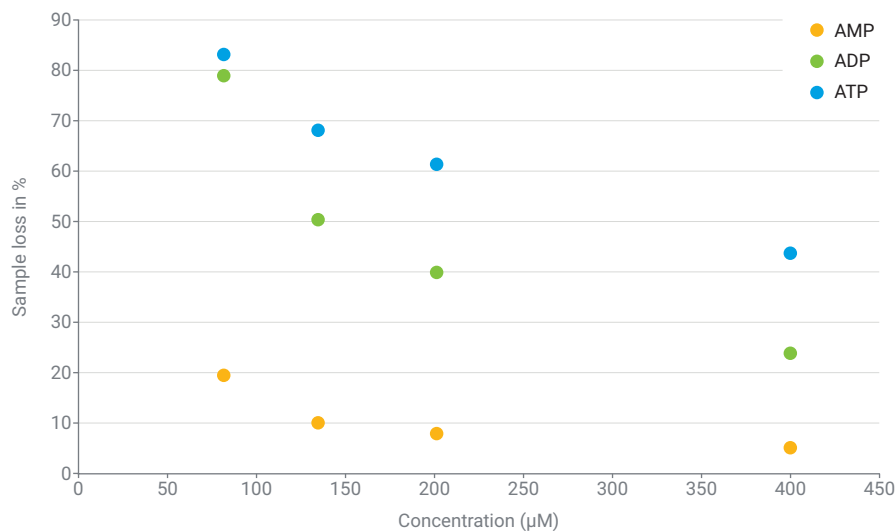


Figure 5. Area loss in percent, compared to the Agilent 1260 Infinity II Bio-Inert LC.

Equation 1.

$$\text{Area loss} = 100 - \left( \frac{A_{\text{AXP,SS}}}{A_{\text{Adenosine,SS}}} \times \frac{A_{\text{Adenosine,bio}}}{A_{\text{AXP,bio}}} \times 100 \right)$$



Sample Loss in %	80 µM	133 µM	200 µM	400 µM
Adenosine	0	0	0	0
AMP	19.71	10.23	8.13	5.19
ADP	79.12	50.49	39.99	24.11
ATP	83.31	68.15	61.55	43.86

Figure 6. Sample loss for the phosphorylated nucleotides analyzed in the SST-based Agilent 1290 Infinity II LC.

described above, the effect of sample loss is more drastic with ADP and ATP. For example, at small concentrations of 80  $\mu\text{M}$ , the relative sample loss for ATP is over 80%. For five times higher concentrations of 400  $\mu\text{M}$ , the relative sample loss is reduced to approximately 44%. Again, for higher concentrations, the effect of the LC system becomes insignificant – whereas the effect of the used column (PEEK-lined versus SST) is more substantial<sup>3</sup>.

## Conclusion

Both low-adsorption Bio LC versions (the complete bio-inert Agilent 1260 Infinity II Bio-Inert LC and the Agilent 1260 Infinity II Prime Bio LC as a biocompatible LC system using MP35N as main material) show excellent and very comparable performance for the analysis of iron-sensitive phosphorylated nucleotides without the need for time-consuming passivation procedures. In contrast, without passivation, the stainless steel-based LC systems (the Agilent 1290 Infinity II LC and Agilent 1260 Infinity II LC) showed immense tailing and area loss for the phosphorylated compounds, especially ADP and ATP. Both SST LC systems showed lower precision of RT and area for the phosphorylated compounds compared to the Bio LC systems. The sample loss was shown to be highly dependent on the concentration – the lower the concentration, the more pronounced the area loss. Both Bio LC systems can therefore be highly recommended for the reproducible analysis of nucleotides, minimizing the risk of losing sample to the system components, for more trust and confidence in the generated data.

## References

1. Wakamatsu A.; Morimoto, K.; Kudoh, S. A Severe Peak Tailing of Phosphate Compounds Caused by Interaction with Stainless Steel Used for Liquid Chromatography and Electrospray Mass Spectrometry. *J. Sep. Sci.* **2005**, *28*, 1823–1830.
2. Fekete, S. Defining Material Used in Biopharmaceutical Analysis. *LCGC Europe* **2021**, *34*, 245–248.
3. HPLC-DAD Analysis of Nucleotides Using a Fully Inert Flowpath. *Agilent Technologies application note*, publication number 5994-0680EN, **2019**.
4. Tuytten, R. *et al.* Stainless Steel Electrospray Probe: a Dead End for Phosphorylated Organic Compounds? *J. Chromatogr. A* **2006**, *1104*, 209–221.
5. Hsiao, J. J. *et al.* Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive. *Anal. Chem.* **2018**, *90*(15), 9457–9464.
6. Heaton, J. C.; McCalley, D. V. Some Factors that Can Lead to Poor Peak Shape in Hydrophilic Interaction Chromatography, and Possibilities for Their Remediation. *J. Chromatogr. A* **2016**, *1427*, 37–44.

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