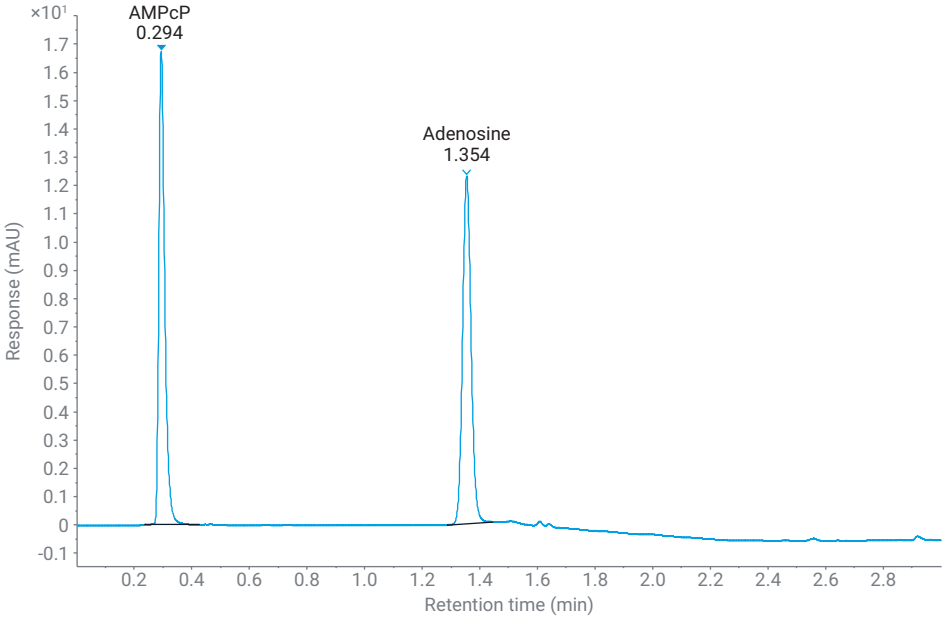
Technical Overview

# Demonstrating Inertness for the Analysis of Nucleotides on the Agilent 1290 Infinity II Bio LC





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## Abstract

Low-adsorption ultra-high performance liquid chromatography (UHPLC) systems are the preferred choice to analyze metal-chelating components. This application note shows the analysis of a test sample using equimolar amounts of adenosine and one corresponding nucleotide. The sample reveals interactions between the phosphate groups of the nucleotide and LC metal surfaces after calculation of the area ratios between the two molecules. With a ratio of 0.95 ±0.01, the Agilent 1290 Infinity II Bio LC showed excellent performance as a functioning low-adsorption system. The system delivered data with the highest confidence for the analysis of metal-sensitive compounds.

## Introduction

Inertness of an LC system is a favorable goal in the chromatographic world. However, how can inertness be defined? An inert LC system can be considered as a system with no unwanted or unpredictable interactions between samples and surfaces within the chromatographic system. 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.[1,2]

The interaction between chelating analytes and metals, especially phosphate groups and iron-containing stainless steel components, is particularly challenging. To demonstrate the inertness of LC systems, metal-sensitive compounds such as nucleotides can be used as indicators of system inertness. The immense effect of the iron-containing LC flow path on peak tailing and sample loss of nucleotides was previously shown in a comparison of Bio LCs versus stainless steel LC equivalents.[3]

In this application note, an established test sample[4] using an equimolar mixture of adenosine and a nonhydrolyzable analog of adenosine diphosphate (adenosine 5'-(α, β-methylene) diphosphate (AMPcP)) was used. The sample results demonstrated the inertness of the Agilent Infinity II 1290 Bio LC. The 1290 Infinity II Bio LC system is composed of MP35N as the main material with no iron-containing stainless steel parts in the flow path. This adsorption system is specially designed for conditions used in biochromatography: even high salt concentrations such as 2 M NaCl, up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl are tolerated even for long-term application without the risk of wearing, maintaining excellent reproducibility and stability.

## Experimental

### Equipment

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

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- 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 µL, RFID tag (G1314-60189)

### Software

Agilent OpenLab CDS version 2.6 or later versions

### Column

ACQUITY PREMIER HSS T3 column, 1.8 µm, 2.1 mm x 50 mm (part number 186009467)

**Note:** The column, sample, solvents, and other boundary conditions were the same according to reference 4.

### 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 and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Steinheim, Germany).

### Samples

Waters PREMIER AMPcp and adenosine standard (part number 186009755). The standard was reconstituted in 200 µL of water. It contains 3.400 µg (7.6 nmol) AMPcP and 2.140 µg (8 nmol) adenosine per vial, an almost equimolar amount of both molecules.

### Method

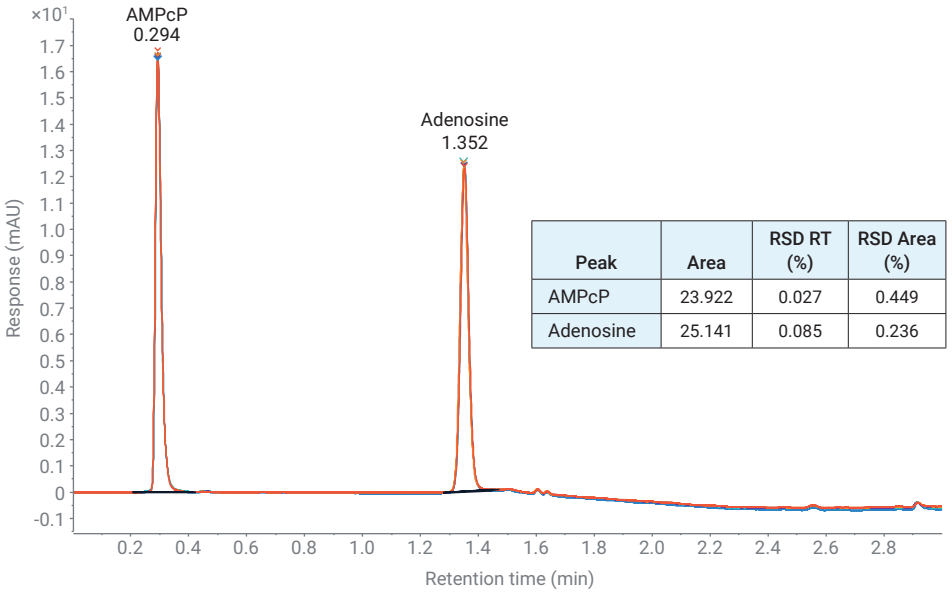
**Table 1.** Chromatographic conditions.

| Parameter | Value |
|---|---|
| Solvent | A) 10 mM Ammonium acetate in water<br>B) 90% ACN + 10% water, 10 mM ammonium acetate, 0.1% TFA |
| Gradient | 0 min: 5% B<br>3 min: 95% B<br><br>Stop time: 3 min<br>Post time: 5 min |
| Flow Rate | 0.400 mL/min |
| Temperature | 35 °C |
| Detection | 260 nm<br>20 Hz |
| Injection | Injection volume: 1 µL<br>Sample temperature: 10 °C<br>Needle wash: 3 s in water |

## Results and discussion

An established test sample containing adenosine and AMPcP was used to analyze the 1290 Infinity II Bio LC for inertness. Due to the electron-rich phosphate groups in nucleotides, there is a high risk of adsorption to exposed iron-containing metal sites. The sample contains adenosine as neutral/negative control probe and AMPcP as an example of a nucleotide. Adenosine has the same molecular structural base as AMPcP but without any attached phosphate moieties that are prone to adsorb to metal surfaces. With this equimolar formulation, the peak areas can be compared, and the ratio can be determined. In an optimal inert system, this ratio is considered approximately 1 as both molecules should not show any adsorption to the flow path surfaces, assuming the UV detectors used show comparable performance. In a stainless steel system, the ratio of AMPcP/adenosine is typically well below 1.

Figure 1 shows the analysis of five subsequent runs of the AMPcP/adenosine on the 1290 Infinity II Bio LC using an inert column housing. The results can therefore be used to evaluate the influence of the LC system only. Besides excellent reproducibility results for retention time (RT) and area (Figure 1), the area ratio of AMPcP/adenosine was determined to be 0.95. This value indicates that the 1290 Infinity II Bio LC system is perfectly suited to analyze nucleotides and similar phosphorylated compounds with high confidence.




| Peak | Area | RSD RT (%) | RSD Area (%) |
|---|---|---|---|
| AMPcP | 23.922 | 0.027 | 0.449 |
| Adenosine | 25.141 | 0.085 | 0.236 |

**Figure 1.** Overlay of five subsequent runs of the testing sample on the Agilent 1290 Infinity II Bio LC. The area ratio of AMPcP/Adenosine was 0.95.

## Conclusion

The Agilent 1290 Infinity II Bio LC as a low-adsorption LC system using MP35N as the main material shows excellent performance for the analysis of iron-sensitive phosphorylated nucleotides. The sample using equivalent amounts of adenosine as a neutral standard, plus AMPcP as an iron-reactive component, was used to determine the amount of adsorption to LC metal surfaces. With a resulting ratio of 0.95, the 1290 Infinity II Bio LC delivered excellent performance for low-adsorptive interactions as well as high reproducibility of RT and area. The 1290 Infinity II Bio LC systems can be highly recommended for the reproducible analysis of nucleotides and comparable compounds that have high affinity to metal surfaces. The system minimizes the risk of losing sample to the LC components for more trust and confidence in the generated data.

## References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2. Fekete, S. Defining Material Used in Biopharmaceutical Analysis. *LCGC Europe*, LCGC Europe-06-01-**2021**, *34(6)*, 245–248.
3. Schneider, S. Comparability Studies for the Analysis of Nucleotides on Four Different LC Systems. *Agilent Technologies application note*, 5994–4392EN, **2021**.
4. Patel, A. *et al.* PREMIER Standards to Investigate the Inertness of Chromatographic Surfaces. *Waters Corporation application note*, 720007105EN, **2021**.


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