White Paper

Pushing the Boundaries of Chromatographic Separation with Inert HPLC Column Hardware

A comprehensive evaluation of inertness, stability, and analytical sensitivity

Author

Jordy Hsiao,
Mohammadali Masigol,
Richard Closser,
Lucas Serge, Li-Chih Hu,
Norwin von-Doehren,
Andrea Angelo P. Tripodi, and
Anne Blackwell
Agilent Technologies, Inc.

Introduction

Achieving reliable chromatographic separation of biologically relevant molecules remains a persistent challenge in analytical science. Our early work using hydrophilic interaction chromatography (HILIC) showed promise for separating underivatized amino acids[1], but exposed limitations in detecting and resolving acidic metabolites such as those containing multiple phosphate or carboxylate groups.[2] These compounds play essential roles in cellular pathways like the tricarboxylic acid (TCA) cycle, making their accurate analysis critical.

This challenge led us to investigate the broader issue of analyte interactions with metal surfaces along the sample flow path.[3] Our survey of the literature and internal studies revealed widespread signal suppression and poor peak shapes across various stationary phases, including reversed-phase, ion exchange, and size exclusion when analyzing phosphopeptides[4-5], phosphorylated glycans[6], monoclonal antibodies[7], and pharmaceutical compounds.[8] These effects were often linked to interactions with metal surfaces in column and system hardware.

In response, we developed the Agilent Altura HPLC column platform with Ultra Inert technology, engineered for inertness and optimized for high-sensitivity separations. These are coated stainless-steel (SS) columns, where the inert coating blocks analyte-metal interactions, while keeping the strength, consistency, and high-pressure benefits of SS column hardware.

This white paper outlines the performance of Altura columns across a range of analytes and conditions, demonstrating improvements in chromatographic resolution and analytical sensitivity for metal-sensitive compounds, while ensuring chemical stability and batch reproducibility. Our goal is to share insights that can help analytical scientists overcome common hardware-related limitations and accelerate method development for challenging biomolecules.

Experimental

Samples

- Polar metabolites including organic acids (that is, glutamine, glutamate, and malate) and phosphorylated nucleotides (for example, AMP, ADP, and ATP) were purchased from Sigma-Aldrich.
- Phosphopeptide samples were acquired from AnaSpec (AS-61145), with the sequence and phosphorylated residues annotated in Table 1.

Table 1. Phosphopeptide sequences used in this study. (pS) indicates phosphorylated serine and (pY) indicates phosphorylated tyrosine.

Identifier	Sequences
a	SFVLNPTNIGM(pS)KSSQGHVTK
b	FQ(pS)EEQQQTEDELQDK
c	TRDIYETD(pY)YRK
d	TRDI(pY)ETD(pY)(pY)RK

Analytical columns

- Agilent HILIC-Z column with SS column hardware, 100 Å, 2.1 × 150 mm, 2.7 µm
- Agilent Altura HILIC-Z column with Ultra Inert technology, 100 Å, 2.1 × 150 mm, 2.7 µm
- Agilent C18 column with SS column hardware, 120 Å, 2.1 × 150 mm, 2.7 µm
- Agilent Altura C18 column with Ultra Inert technology, 120 Å, 2.1 × 150 mm, 2.7 µm
- Competitor 1 C18 column with inert column hardware, 130 Å, 2.1 × 150 mm, 2.5 µm
- Competitor 2 C18 column with inert column hardware, 300 Å, 2.1 × 150 mm, 1.9 µm

Instruments

LC/MS analysis was conducted using the following instrument configuration:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler with sample thermostat (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Table 2. LC parameters for Figure 4 competitive comparisons.

Agilent 1290 Infinity II Bio LC System	
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Column Temperature	40 °C
Flow Rate	0.4 mL/min
Sampler Temperature	8 °C
Gradient	Time (min) %B 0 2 1.5 2 11.5 32 12 2 18 2

Table 3. MS parameters for Figure 4 competitive comparisons.

Agilent 6545 XT AdvanceBio LC/Q-TOF	
Ion Source	Agilent Dual Jet Stream ESI source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	8 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Fragmentor	175 V
Skimmer	65 V
Reference Mass	322.0481, 922.0098
MS1 Range	*m/z* 300 to 1,250
Acquisition Rate	1 spectra/second

Results and discussion

Demonstrating the impact of inert hardware on chromatographic performance

To evaluate the practical benefits of our new inert column platform, we compared its performance against conventional SS hardware using a range of biologically relevant analytes. As shown in Figure 1, the Altura columns with Ultra Inert technology consistently outperformed the SS column across three representative compound classes.

In the first comparison, peptides containing multiple acidic residues or phosphates (peptide “b” and peptide “d”) were undetectable when using the SS hardware. Meanwhile, peptide “c”, which contains a phosphorylated tyrosine residue, had 50% less signal in peak height when using the SS hardware. Notably, peptide “a” signals were comparable between the SS and inert hardware. These results highlight the sensitivity differences towards metal adsorption amongst the varied peptide sequences and acidic modifications.

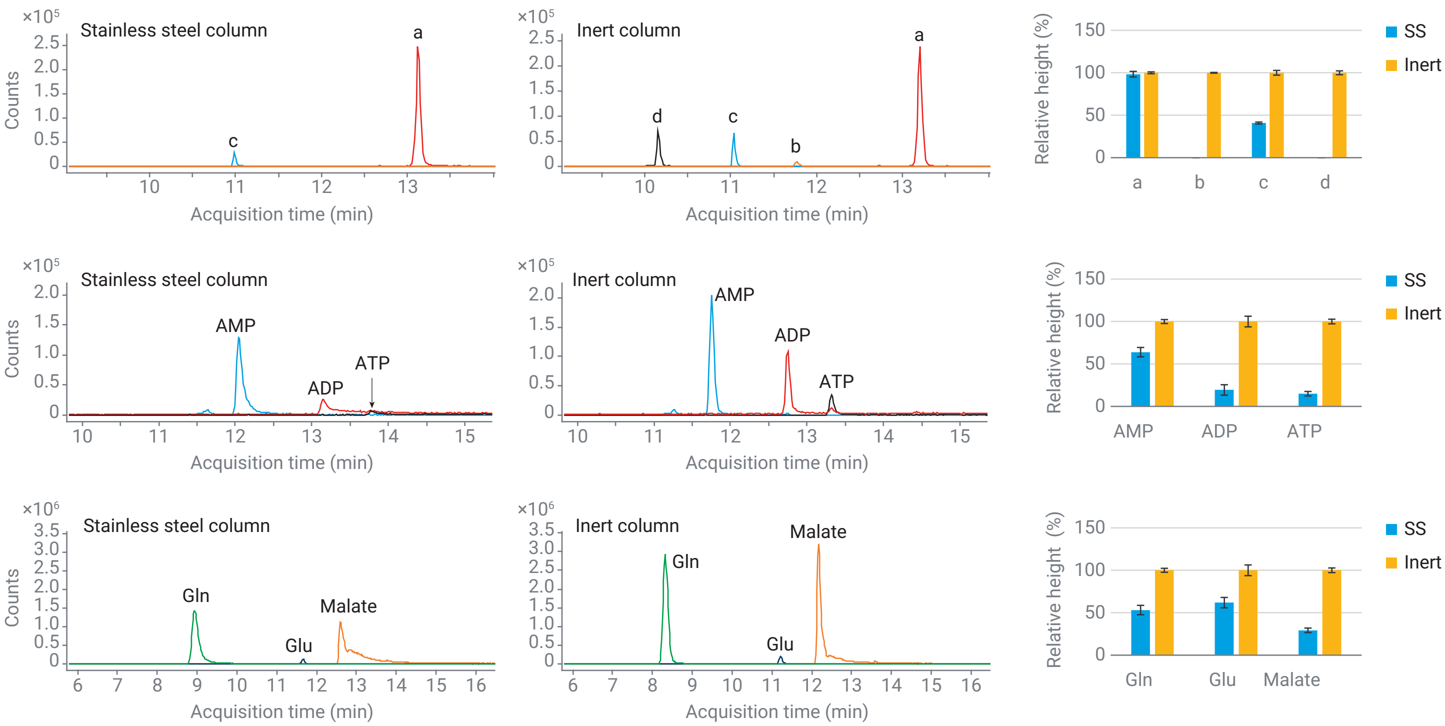

Figure 1. Comparison of chromatographic performance between stainless steel (SS) and Agilent Altura Ultra Inert HPLC column hardware across three compound classes: synthetic peptides, phosphorylated nucleotides (AMP, ADP, and ATP), and acidic metabolites (glutamine, glutamate, and malate). The synthetic peptides were resolved by reversed-phase (RP) separation, while the phosphorylated nucleotides and acidic metabolites were separated by hydrophilic interaction chromatography (HILIC). Chromatograms and corresponding bar graphs showed significantly higher relative signal intensities with the Altura columns, highlighting their inertness and enhanced analyte recovery.

Moreover, the results highlight the new Altura column's inertness, which reduces analyte loss. Similarly, in the second set, phosphorylated nucleotides (AMP, ADP, and ATP) exhibited significantly higher signal intensities and improved peak shapes with the inert column, indicating reduced interaction with metal surfaces. Lastly, acidic metabolites such as glutamine, glutamate, and malate showed enhanced recovery and sharper peaks, further demonstrating the new inert column's ability to mitigate adsorption effects.

The accompanying bar graphs quantify these improvements, showcasing the Altura columns with Ultra Inert technology delivering up to 2 to 3 times higher relative signal intensities across all compound classes. Moreover, peak resolution was significantly enhanced for most target analytes, as evidenced by the reduced tailing factor observed when using inert column hardware (Table 4). These results underscore the critical role of hardware surface chemistry in achieving reliable, high-sensitivity separations, particularly for analytes prone to metal interactions.

Table 4. Improved peak shape with inert column hardware. These results highlight the improvement in the tailing factor for the metal-sensitive analytes. Some analytes were not listed due to complete adsorption leading to unquantifiable results. Refer to Table 1 for peptide "a" and peptide "c" sequences.

	Tailing Factor (TF)					
	Peptide		Metabolites			
	a	c	Glutamine	Glutamate	AMP	ADP
SS	1.2	1.9	1.8	1.4	2.6	4.8
Inert	1.0	1.4	1.2	1.3	1.3	1.7
ΔTF (Inert - SS)	−0.2	−0.5	−0.6	−0.1	−1.3	−3.1

Evaluating chemical stability under harsh conditions

In addition to chromatographic performance, long-term chemical stability is a critical requirement for column hardware, especially when exposed to aggressive mobile phases or elevated temperatures. To assess the durability of the Altura column hardware with Ultra Inert technology, we conducted a series of stress tests simulating real-world analytical conditions.

Figure 2 outlines the experimental setup and results, where panel A shows the lifetime testing configuration. Empty inert columns were exposed and circulated with defined chemical solvents using an Agilent 1290 Infinity Flexible Cube system over a six-week period. Panel B details the LC/MS analysis workflow, employing an Agilent 1290 Infinity II bio LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer with MP35N or PEEK connection capillaries to confidently monitor analyte recovery and signal integrity without metal-mediated interferences along the sample flow path.

The results revealed the inert column's exceptional chemical resilience (Figures 2C and 2D) across a range of chemical treatments, including strong acid, strong base, concentrated phosphate-buffered saline (PBS), and ion-pairing reagents at elevated temperatures. The inert column showed minimal signal loss, even under the most aggressive conditions.

These findings confirm that the Ultra Inert column technology not only improves analyte recovery but also withstands prolonged exposure to chemically demanding workflows, making it a robust solution for high-sensitivity biomolecular analyses.

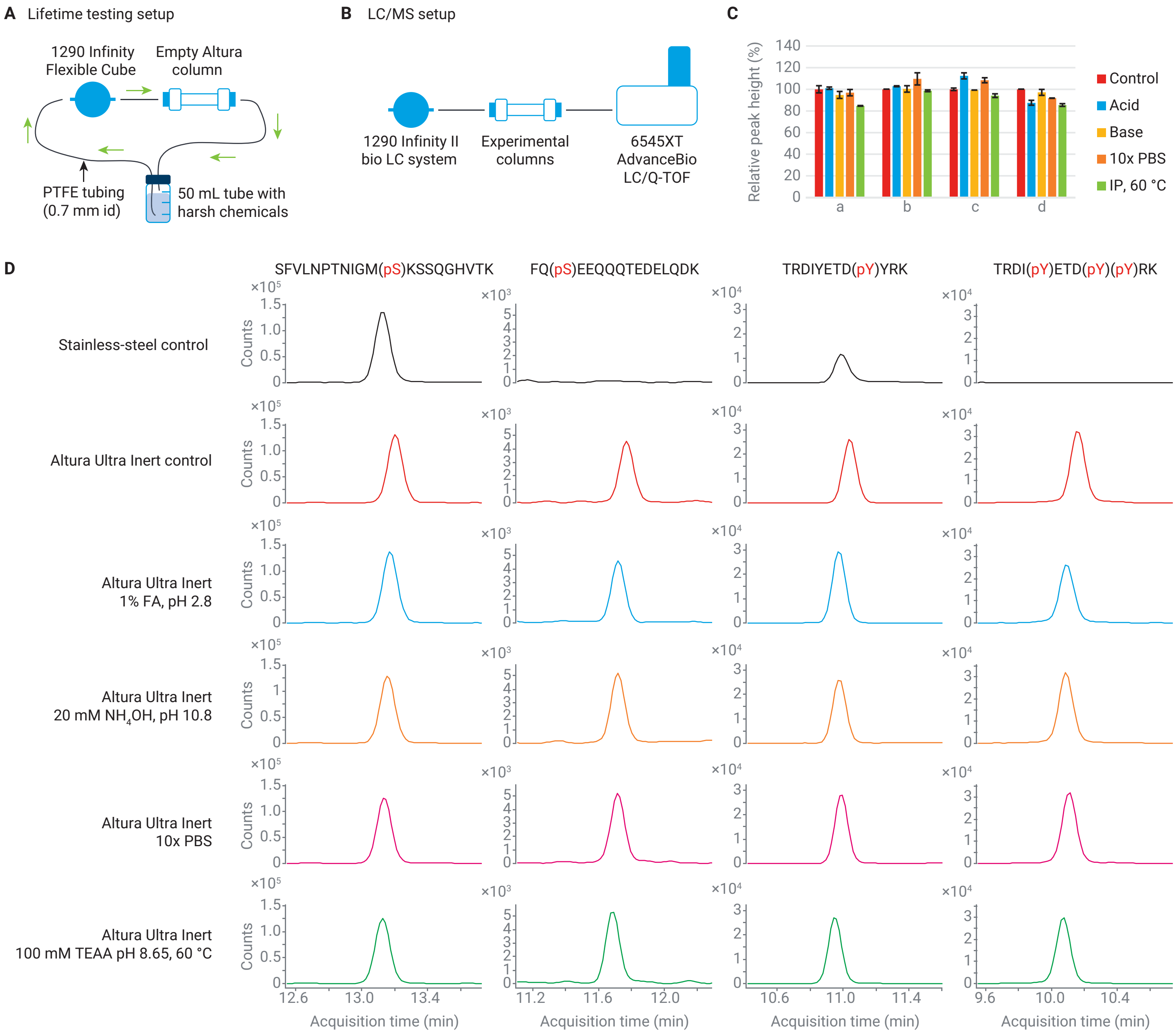

Figure 2. Evaluation of the Agilent Altura Ultra Inert columns' durability under aggressive chemical conditions. (A) Lifetime testing setup using an Agilent 1290 Infinity Flexible Cube. (B) LC/MS analysis workflow with an Agilent 1290 Infinity II bio LC system and Agilent 6545XT AdvanceBio LC/Q-TOF. (C) Bar graph showing relative signal intensities after exposure to acid, base, concentrated PBS, or ion-pairing reagents at elevated temperature, with the untreated column serving as the control. (D) Chromatograms illustrating qualitative differences amongst the chemically treated column hardware.

Ensuring reproducibility across coated column batches

Reproducibility is a cornerstone of reliable analytical workflows, particularly in biopharmaceutical environments where consistency across manufacturing lots is essential. To assess batch-to-batch performance, we evaluated multiple production lots of the Altura Ultra Inert columns and compared them to an SS column.

As shown in Figure 3, all four inert column batches (labeled #1 through #4) delivered consistently high relative signal intensities across replicate analyses. In contrast, the SS control column exhibited lower signal recovery. The consistency of relative signal intensities across the batches further highlights the reproducibility of the hardware's inert surface chemistry and manufacturing process. These results also confirm that the Altura column platform delivers dependable performance across production lots, making it a robust and scalable solution for high-throughput and quality critical applications.

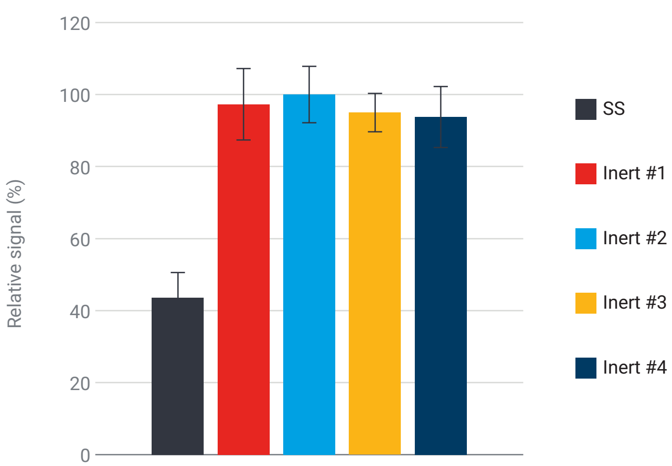

Figure 3. Assessment of batch-to-batch consistency for four Altura Ultra Inert column hardware production lots compared to a stainless-steel (SS) column. Bar graph shows relative signal intensities (peak height) for phosphopeptides with error bars (n = 8).

Superior detection of phosphorylated peptides

Phosphorylated peptides are notoriously difficult to analyze due to their strong affinity for metal surfaces, which often leads to poor analyte recovery and low analytical signal. To benchmark the performance of the Altura inert column against industry-standard alternatives, we conducted a comparative study using four representative phosphorylated peptide sequences.

The inert column hardware packed with end-capped C18 consistently delivered higher relative peak areas across a range of peptide concentrations when compared to the same C18-phase-packed SS, as well as two competitor C18 columns with inert hardware (Figure 4A). Each scatter plot illustrates the relationship between peptide amount (pmol) and signal response for each specific phosphopeptide sequence. In all cases, the Altura inert column (red) outperformed the other columns. These quantitative results are further illustrated in Figure 4B, which shows the chromatogram for each peptide at 0.5 pmol injection. The Altura inert column produced sharper, more intense peaks across all peptides, including multiply phosphorylated sequences such as TRDI(pY)ETD(pY)(pY)RK, highlighting its superior inertness and sensitivity.

Together, these figures demonstrate the Altura column's ability to preserve signal integrity and enhance sensitivity for labile, metal-sensitive analytes. This performance is especially valuable in phosphoproteomics workflows and other applications where detection sensitivity and quantitative accuracy are paramount.

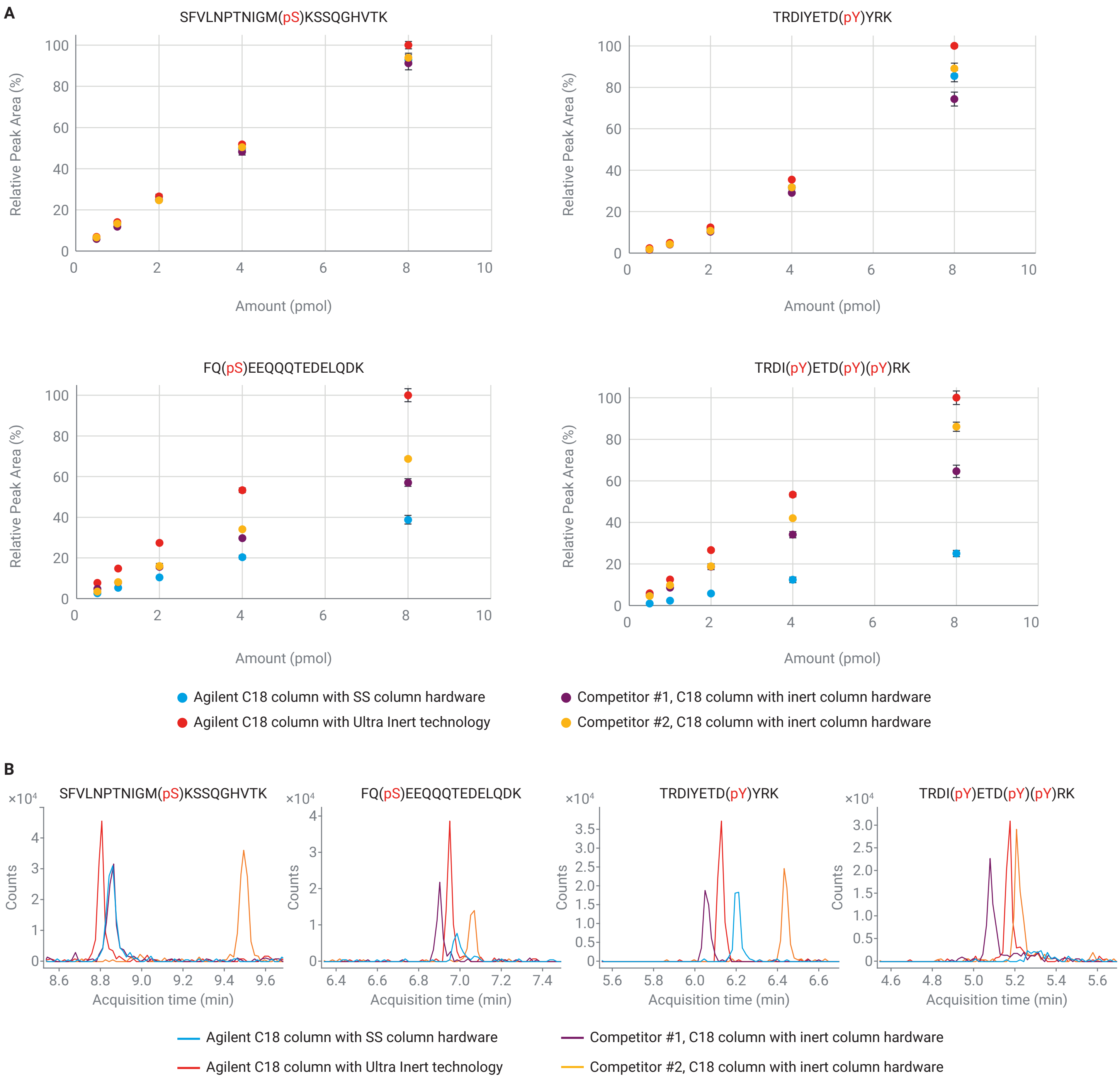

Figure 4. Competitive comparison for detecting phosphorylated peptides. (A) Scatter plots showing relative peak area versus peptide amount for four phosphorylated peptide sequences across four C18 columns: Agilent C18 in SS column hardware, Agilent C18 in inert column hardware, Competitor #1 C18 in inert column hardware, and Competitor #2 C18 in inert column hardware. (B) Corresponding chromatograms for each peptide confirm the Agilent Altura column's superior sensitivity and peak quality at 0.5 pmol injections of the indicated peptide.

System-wide inertness: column and LC platform synergy

While column hardware plays a critical role in minimizing analyte loss, the broader LC system can also contribute to optimal chromatographic performance. To evaluate the combined effects of column and system inertness, we tested four configurations: inert and SS columns paired with either a biocompatible LC system (bio LC) or a standard SS-based LC system.

As shown in Figure 5, the bio LC system paired with the Altura column (panel i) delivered the highest signal intensities for phosphopeptides, confirming the importance of minimizing metal interactions throughout the entire flow path.

In contrast, the standard LC system with an SS column (panel iv) showed the lowest signal recovery, underscoring the cumulative impact of non-inert surfaces. Transitioning from the standard LC system with SS column (panel iv) to a fully inert configuration (panel i) resulted in a 25x increase in the peak area for peptide b.

Interestingly, when comparing panel iv to panel ii, the relative signal intensity significantly improved when switching from an SS column to an inert column. The peak area for peptide b increased 14x with this simple column substitution. The results suggest that having an inert column is key for maximizing the analytical signal when using an SS LC, enabling users to gain better sensitivity from their existing SS LC systems.

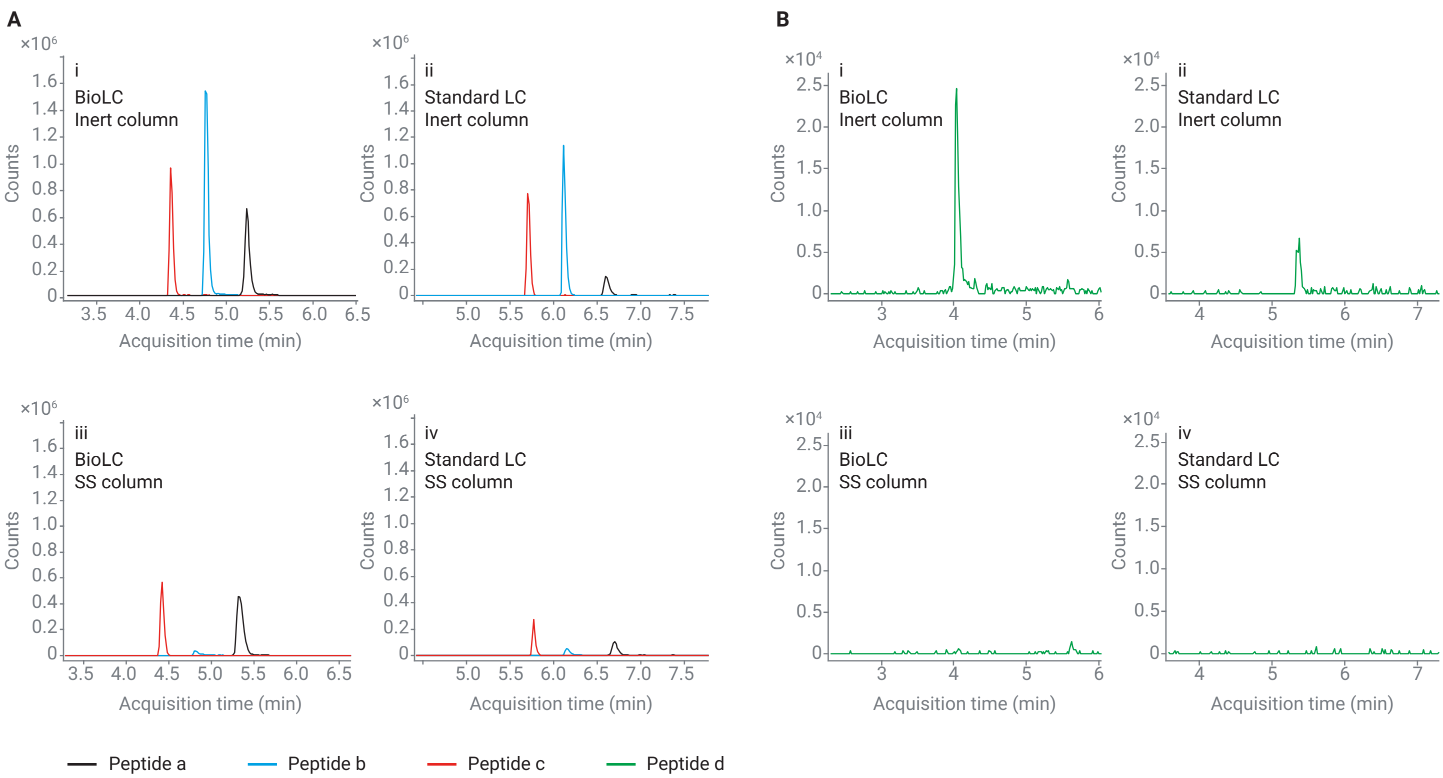

Figure 5. Evaluating inert column and LC systems with phosphopeptides. Comparison of phosphopeptide (0.5 pmol) signal recovery across four system configurations: (i) inert column with bio LC, (ii) inert column with standard SS-based LC, (iii) SS column with bio LC, and (iv) SS column with standard SS-based LC. The fully inert flow path yielded the highest signal intensities, while the standard LC + SS column setup showed the lowest. Results highlight the importance of full-system inertness for optimal phosphopeptide detection, as well as the value of an inert column with an existing SS system. Peptide d is shown separately for clarity due to lower signal response than peptides a–c. Refer to Table 1 for peptide sequences.

Conclusion

This white paper presents a comprehensive evaluation of Ultra Inert HPLC column technology, designed to address longstanding challenges in the chromatographic analysis of biologically relevant, metal-sensitive compounds. Through a series of targeted experiments, we demonstrate that the Agilent Altura columns offer significant improvements in analyte recovery, signal intensity, and peak shape particularly for acidic metabolites and phosphorylated peptides.

Key findings include the following:

- Enhanced chromatographic performance across diverse compound classes, with sharper peaks and higher signal intensities (Figure 1)
- Exceptional chemical stability under harsh conditions, ensuring long-term durability and consistent performance (Figure 2)
- Reliable batch-to-batch reproducibility, supporting deployment in high-throughput environments (Figure 3)
- Superior detection of phosphorylated peptides, outperforming conventional column technologies in both sensitivity and peak shape (Figures 4)
- The importance of system-wide inertness, with optimal results achieved when pairing the Altura column with a bio LC system (Figure 5)

Together, these results highlight the Altura columns as a robust, high-performance solution for challenging separations. By minimizing unwanted interactions and maximizing signal fidelity, this technology enables more confident quantitation, deeper analytical coverage, and greater efficiency in method development.

References

1. Wei, T.; *et al.* The use of HILIC Zwitterionic Phase Superficially Porous Particles for Metabolomics Analysis. *LCGC Suppl.* **2018**, *36(6)*, 30–35.
2. Hsiao, 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.
3. Hsiao, J.; *et al.* Troubleshooting LC Separations of Biomolecules, Part 1: Background, and the Meaning of Inertness. *LCGC Eur.* **2020**, *33(3)*, 122–126.
4. Fleitz, A.; *et al.* Enhanced Detection of Multiply Phosphorylated Peptides and Identification of Their Sites of Modification. *Anal. Chem.* **2013**, *85(18)*, 8566–8576.
5. Winter, D.; *et al.* Citrate Boosts the Performance of Phosphopeptide Analysis by UPLC-ESI-MS/MS. *J. Proteome Res.* **2009**, *8(1)*, 418–424.
6. Sandra, P.; *et al.* Analyzing Phosphorylated N-Glycans with Recovery on Bio-Inert LC Systems and PEEK-Lined HILIC Columns. *LCGC Eur.* **2018**, *31(10)*, 566–571.
7. Rivera, B.; *et al.* Bioinert Versus Biocompatible: The Benefits of Different Column Materials in Liquid Chromatography Separations. *LCGC Suppl.* **2018**, *36(6)*, 24–29.
8. De Pra, M.; *et al.* Effects of Titanium Contamination Caused by Iron-Free High-Performance Liquid Chromatography Systems on Peak Shape and Retention of Drugs with Chelating Properties. *J. Chromatogr A* **2020**, *1611*, 460619.

www.agilent.com

DE-009624

This information is subject to change without notice.

Printed in the USA, September 11, 2025
5994-8618EN

Agilent
Trusted Answers