Application Note

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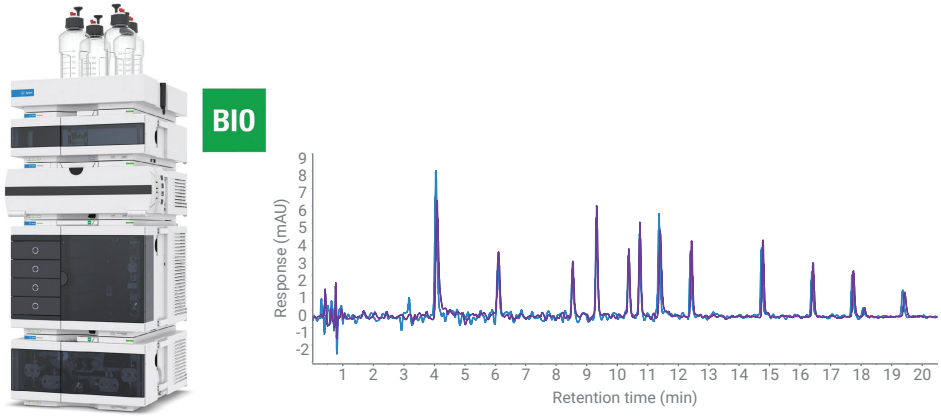
Enhanced Sensitivity for Phosphopeptide Analysis

Phosphopeptide analysis with the Agilent 1290 Infinity II Bio LC System



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Abstract

Performance for the Agilent 1290 Infinity II Bio LC System for phosphopeptide analysis is compared to the standard stainless steel (SST)-based Agilent 1290 Infinity II LC System. A complex peptide sample consisting of 12 peptides, two of which are phosphorylated, was separated. Differences in recovery of the phosphorylated peptides relative to the nonphosphorylated compounds were evaluated. To prevent the sample from interacting with the column housing, a column with PEEK-lined hardware was used.

Both LC systems demonstrated consistent injection precision, indicating reliable hardware — a must for system-to-system comparison. Subsequent measurements with lower sample amounts showed reduced recovery of the phosphorylated peptides with the SST-based LC system. The results clearly demonstrate the benefits of a biocompatible LC flow path when analyzing biomolecules and samples that may interact with stainless steel surfaces.

Introduction

The introduction of biocompatible and bio-inert materials in LC systems was a great improvement in terms of hardware robustness and data quality and validity. Biomolecules and derivatives such as proteins, peptides, nucleic acids, hybrid, and heavily charged molecules are challenging to analyze in terms of recovery and reproducibility due to their tendency to interact nonspecifically with the ferrous surface in the flow path. In the low pressure range, polymer materials are widely used to suppress nonspecific interactions of analytes, whereas in the high pressure range, biocompatible materials such as titanium, corrosion-resistant metal alloys, and ceramics come into play.

In this application note, the performance of the Agilent 1290 Infinity II LC System and the Agilent 1290 Infinity II Bio LC System is evaluated for a phosphorylated peptide separation.

Experimental

Equipment

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

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A) with Agilent Bio Jet Weaver (G7132-60135)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent Quick Connect Bio Heat Exchanger Std. (G7116-60071) and Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with Agilent Bio Standard Flow Cell for VWD, 10 mm (G1314-60188)

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

- Agilent 1290 Infinity II High-Speed Pump (G7120A) with Agilent Jet Weaver (G4220-60006)
- Agilent 1290 Infinity II Multisampler (G7167B) with Agilent InfinityLab Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Heat Exchanger Std. (G7116-60015) and Agilent Thermal Equilibration Devices (G711-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with Agilent Standard Flow Cell for VWD, 10 mm (G1314-60186)

Software

Agilent OpenLab software suite version 2.5 or later with Agilent OpenLab LC driver 3.5.32

Column

Agilent AdvanceBio EC-C18, 2.1 × 100 mm, 2.7 µm, PEEK-lined (part number 675775-902)

Chemicals

All solvents were LC grade. 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). Acetonitrile, Lichrosolv was obtained from VWR International, Darmstadt, Germany. Trifluoroacetic acid (TFA) was purchased from Honeywell, Fluka, Seelze, Germany.

Samples

- Agilent Ten-Peptide Standard (part number 5190-0583)
- pp60 c-src (521-533) peptide (singly phosphorylated) trifluoroacetate salt (part number 4031184.0001, Bachem, Bubendorf, Switzerland)
- YEEI peptide (singly phosphorylated) trifluoroacetate salt (part number Y4377, Sigma, Saint Louis, Missouri, USA)

Singly phosphorylated peptides have been shown to exhibit lower nonspecific interaction with metal surfaces than multiphosphorylated peptides.[1] Since the current study focuses on UV-based detection, and the availability of larger quantities of multiphosphorylated peptides is limited, easier-to-access singly phosphorylated peptides were used.

Sample preparation (sample stock) for phosphopeptide mix:

1. Dissolve both phosphorylated peptides to 1 mg/mL in water/acetonitrile 80/20.
2. Mix 40 µL of pp60 with 10 µL of YEEI and 50 µL of water/acetonitrile 80/20.
3. Use the mix from the previous step to dissolve the Agilent Ten-Peptide Standard.

For injection precision measurements:
Dilute sample stock 1:2 (v/v) with water/acetonitrile 80/20.

For recovery measurements:
Dilute sample stock 1:10 (v/v) with water/acetonitrile 80/20.

Separation method

Table 1. Phosphopeptide mix separation method conditions.

Parameter	Value
Solvent	A) Water B) Acetonitrile, both with 0.1% TFA
Gradient	Time (min) B (%) 0.00 10 2.00 10 20.00 47 20.01 95 20.50 95 20.51 10 Stop time: 20.50 min Post time: 10 min
Pump Settings	Minimum stroke: automatic, synchronized Compressibility: use solvent types
Flow Rate	0.4 mL/min
Temperature	55 °C, using Agilent Thermal Equilibration Devices
Detection	220 nm 20 Hz
Injection	Injection volume: 1 or 5 µL (as indicated) Sample temperature: 10 °C Needle wash: 10 s in acetonitrile:water 1:1

Results and discussion

Combining phosphopeptides together with nonphosphorylated peptides allows the latter to act as an internal standard to quantitate nonspecific interactions of phosphopeptides within the sample flow path. Two commercially available phosphopeptides (pp60 and YEEI) were mixed with the Agilent Ten-Peptide Standard. A separation method was developed that permits the direct comparison of the complex sample run on a 1290 Infinity II LC System and a 1290 Infinity II Bio LC System. The use of an LC column with biocompatible hardware allows the detection of system-only-related effects.

Table 2 shows the general injection precision of both LC systems. A higher concentration sample was used to avoid integrator-related artifacts due to low peak height. Both systems were validated for precise injection performance, which is mandatory for addressing the subject of this study. Using this high concentration sample, the 1290 Infinity II LC System was slightly less precise (higher % RSD) due to a single outlier (peak 11).

Table 2. Injection precision results for the Agilent 1290 Infinity II Bio LC System and Agilent 1290 Infinity II LC System. Ten 5 µL injections of the phosphopeptide mix of were separated on an Agilent AdvanceBio EC-C18 PEEK-lined column. The average peak area (n = 10) was used to calculate standard deviation and injection precision (% RSD).

Peak Number/ Name	Peak Area Average	Peak Area Standard Deviation	Injection Precision (% RSD)
Agilent 1290 Infinity II Bio LC System			
1/pp60	1,181.27	5.67	0.48
2	436.01	4.65	1.07
3	291.76	2.61	0.89
4	581.00	1.56	0.27
5	374.86	2.78	0.74
6	501.27	3.83	0.76
7/YEEI	766.66	2.80	0.36
8	403.19	3.51	0.87
9	411.21	1.48	0.36
10	294.78	1.16	0.39
11	284.98	4.75	1.67
12	219.83	0.65	0.30
Agilent 1290 Infinity II LC System			
1/pp60	1,240.45	3.70	0.30
2	482.24	5.39	1.12
3	329.35	3.93	1.19
4	624.90	1.46	0.23
5	393.12	1.64	0.42
6	533.82	1.62	0.30
7/YEEI	800.24	8.60	1.07
8	433.62	1.36	0.31
9	441.85	1.22	0.28
10	322.10	2.30	0.72
11	299.46	10.77	3.60
12	217.38	3.00	1.38

To test for nonspecific interactions between the phosphopeptides and stainless steel surfaces, seven injections were made with a lower concentration sample. The direct comparison of peptide peak areas between the two systems reveals a significantly higher value for the phosphopeptide pp60 on the biocompatible LC system (Figure 1). Peak areas of the nonphosphorylated peptides in the sample are not similarly affected. The higher peak area for pp60 is possibly an indication for a phosphopeptide-specific effect. However, it is not conclusive since the two LC systems use different detectors, lamps, and UV cells that can also cause diverse results. The higher peak area for pp60 on the biocompatible LC is interesting, though, because the peak areas of the nonphosphorylated compounds, on average, are all slightly lower on the same biocompatible LC system.

The lack of nonspecific interaction for the second phosphopeptide YEEI over the course of seven measurements demonstrates the inherent differences in affinity to stainless steel surfaces within this class of molecules.

Also note that the phosphopeptides used in this study are both singly phosphorylated, and the expected effects are smaller than for multiphosphorylated molecules.[2]

Two UV traces of single injections from both LC systems are overlaid in Figure 2 (after blank subtraction), demonstrating differences in peak height for the eluting constituents. The two peptides with significantly higher peak height on the biocompatible LC represent the two

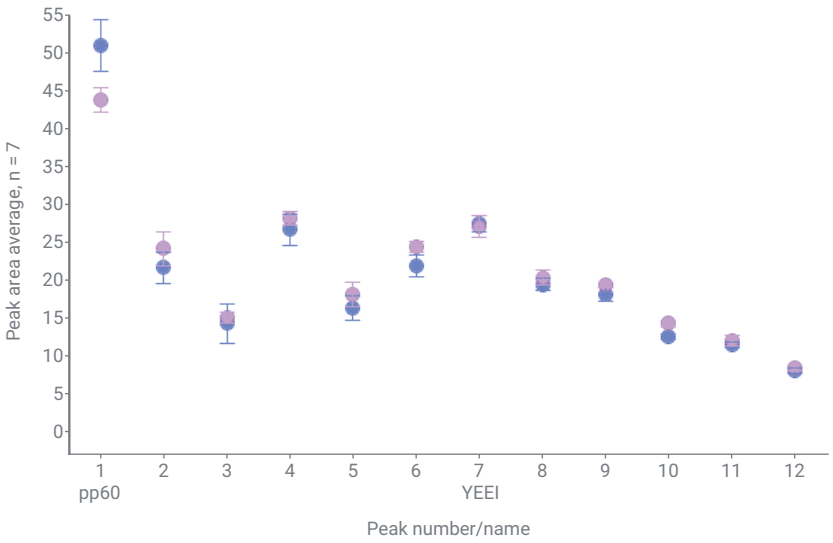
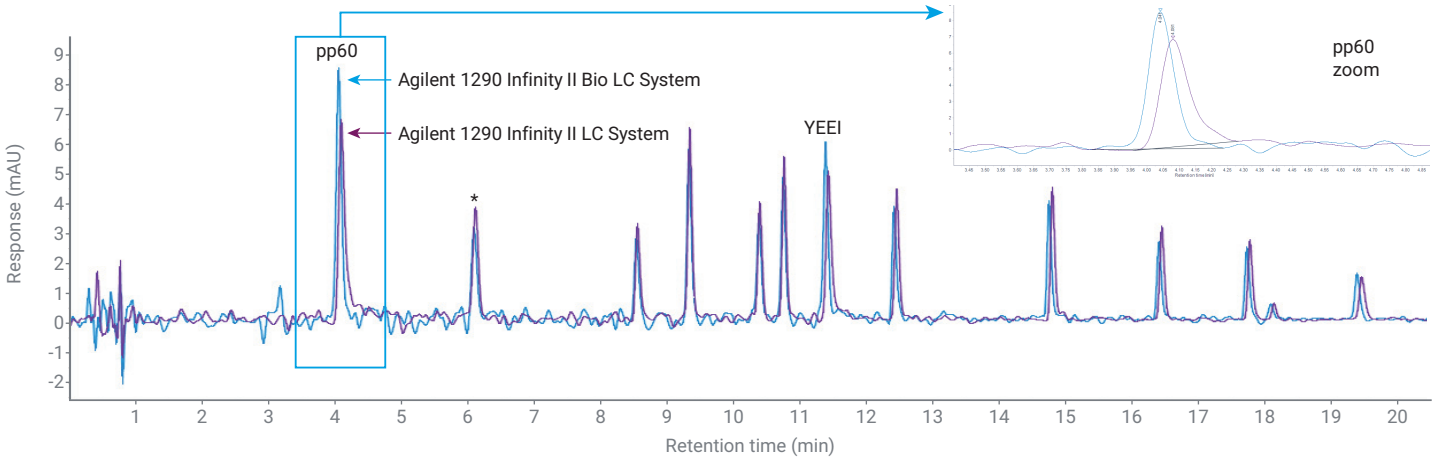


Figure 1. Peak area differences between the Agilent 1290 Infinity II Bio LC System (blue) and Agilent 1290 Infinity II LC System (magenta) using the phosphopeptide mix. Seven repeat 1 µL injections were separated on an Agilent AdvanceBio EC-C18 PEEK-lined column. Phosphopeptide numbers and names are as specified on the X-axis. Error bars indicate standard deviation values.



Instrument	*Peak Area Peak 2	Peak Area pp60	Peak Height pp60	Peak Symmetry pp60	*Peak Area Loss (%) pp60 – Peak 2	Peak Area YEEI	Peak Height YEEI	Peak Symmetry YEEI	*Peak Area Loss (%) YEEI – Peak 2
Agilent 1290 Infinity II Bio LC System	21.11	49.82	8.40	0.77	–	30.57	5.79	0.88	–
Agilent 1290 Infinity II LC System	20.53	43.53	6.59	0.67	11.23	28.19	4.91	0.75	5.47

Figure 2. Overlay of two signal traces from the Agilent 1290 Infinity II Bio LC System and Agilent 1290 Infinity II LC System (blank subtracted). A 1 µL injection of the phosphopeptide mix was separated on an Agilent AdvanceBio EC-C18 PEEK-lined column. The inset chromatogram and table show the phosphopeptides' (pp60 and YEEI) UV signals and results, respectively. An asterisk marks peak 2, which is used as a reference to calculate peak area loss values for phosphorylated peptides pp60 and YEEI on the 1290 Infinity II LC System.

phosphopeptides in the sample. All other nonphosphorylated peptides in the sample mixture exhibit no substantial difference between the LC systems. Zooming in on the pp60 peak validates the improvement in peak height and peak symmetry for the biocompatible LC system, which is summarized in the inset chromatogram and table in Figure 2. Also observed is a striking peak area loss of 11.23 and 5.47%, relative to peak 2, for pp60 and YEEI, respectively.

Peak area loss values were calculated according to Equation 1.

Since the data evaluated in Figure 2 are just a snapshot of two single injections, Table 3 reveals peak area loss results for pp60 and YEEI from the average of all 10 repetitions (not blank subtracted). Furthermore, these values were calculated relative to all nonphosphorylated peptides in the sample. The range of peak area loss, depending on the reference peptide applied, indicates an individual binding affinity of the peptides to metal surfaces. For pp60, peak area loss rises up significantly to 28%, whereas for YEEI the effect is much weaker (up to 17%), and therefore the significance of nonspecific interaction is not clear.

$$\text{Area loss} = 100 - \left(\frac{A_{\text{peak 2,SST}}}{A_{\text{phosphopeptide,SST}}} \times \frac{A_{\text{phosphopeptide,bio}}}{A_{\text{peak 2,bio}}} \times 100 \right)$$

Equation 1. Determining peak area loss of phosphorylated peptides pp60 and YEEI relative to nonphosphorylated peptides in a stainless-steel-based (SST) LC system. Where: $A_{\text{peak 2,SST}}$ is the peak area of peak 2 on the SST LC system, $A_{\text{phosphopeptide,SST}}$ is the peak area of phosphopeptide on the SST LC system, $A_{\text{phosphopeptide,bio}}$ is the peak area of phosphopeptide on the bio LC system, and $A_{\text{peak 2,bio}}$ is the peak area of peak 2 on the bio LC system.

Table 3. Peak area loss of phosphopeptides pp60 and YEEI for the Agilent 1290 Infinity II System. Ten repeat 1 µL injections of the phosphopeptide mix were separated on an Agilent AdvanceBio EC-C18 PEEK-lined column. Peak area average (n = 10) was used to calculate peak area loss for pp60 and YEEI relative to the other sample components.

Peak Number/ Name	Retention Time (min)	Peak Area Average (n = 10)	pp60 Peak Area Loss (%)	YEEI Peak Area Loss (%)
Agilent 1290 Infinity II Bio LC System				
1/pp60	4.04	50.01	–	–
2	6.08	21.43	–	–
3	8.53	14.08	–	–
4	9.32	26.61	–	–
5	10.36	16.28	–	–
6	10.73	22.14	–	–
7/YEEI	11.36	27.91	–	–
8	12.39	19.05	–	–
9	14.72	18.10	–	–
10	16.38	12.53	–	–
11	17.71	11.46	–	–
12	19.36	8.14	–	–
Agilent 1290 Infinity II LC System				
1/pp60	4.08	44.14	n/a	n/a
2	6.10	23.70	25.30	14.56
3	8.54	15.17	21.99	11.53
4	9.33	28.39	20.90	10.54
5	10.38	18.13	26.10	15.29
6	10.75	24.44	25.01	14.30
7/YEEI	11.41	26.95	n/a	n/a
8	12.43	20.00	18.96	8.76
9	14.77	19.33	20.98	10.61
10	16.42	14.17	28.09	17.11
11	17.75	11.87	17.29	7.23
12	19.43	8.40	17.02	6.99

Conclusion

In this application note, the tendency and extent of nonspecific binding of phosphorylated peptides to the Agilent 1290 Infinity II Bio LC System was compared to its stainless-steel-based counterpart. After proving accurate injection performance, the evaluation revealed a significant nonspecific interaction for the phosphopeptide pp60 with a recovery loss of up to 28% on a stainless-steel-based LC system. For the second phosphorylated peptide in this test, YEEI, a recovery loss of up to 17% was determined, and its significance is uncertain under the given test conditions. Overall, the results demonstrate the benefit of using LC systems with biocompatible surface materials over stainless steel due to its minimized susceptibility to bind low abundant sample compounds such as phosphorylated peptides.

References

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2. Schneider, S. Comparability Studies for the Analysis of Nucleotides on Four Different LC Systems. *Agilent Technologies application note*, publication number 5994-4392EN, **2021**.

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