Millennial Scientific

WHITE PAPER

Capillary-HPLC Comparison of NanoPak-C All-Carbon, Porous Graphitic Carbon and C18 for NanoLC–MS of a Bovine Serum Albumin (BSA) Tryptic Digest

Balaji Sitharaman

Contact us at: https://www.millennialscientific.com/contact.

Abstract

Silica-based C18 reversed-phase columns are the standard for LC–MS-based peptide mapping but offer limited retention for very hydrophilic peptides, glycans, glycopeptides, PTM-rich peptides, and polar metabolites. NanoPak-C All-Carbon microbeads are a new class of porous graphitic media synthesized via a microfluidic route, designed to provide stronger, orthogonal retention for such analytes. In this study, All-Carbon capillary columns (5 µm and 3.5 µm) were compared with a commercial PGC column (5 µm) and silica C18 columns (3 µm and 5 µm) under identical nanoLC–MS conditions using an 80 fmol BSA tryptic digest. All 75 µm × 250 mm columns were independently packed and evaluated on a Bruker/Michrom NanoAdvance UHPLC–Thermo LTQ XL system. C18 delivered the highest peak density and remains preferred for general peptide mapping. All-Carbon showed stronger retention, slightly higher efficiency than the PGC comparator, faster and more reproducible conditioning, and a cleaner MS baseline, with further efficiency gains for the 3.5 µm format. These data position NanoPak-C All-Carbon microbeads as complementary tools for polar analytes within proteomics.

1. Introduction

Silica-based C18 reversed-phase columns remain the standard choice for LC–MS-based peptide mapping [1]. However, they offer limited retention and selectivity for very hydrophilic peptides, glycopeptides, glycans, PTM-rich peptides, and polar small molecules [1]. Porous graphite carbon (PGC) phases have been reported to provide mixed hydrophobic, polar, and planar interactions, leading to stronger retention and orthogonal selectivity relative to C18 [2]. However, PGC behavior in proteomics applications is known to differ substantially from C18 [3] and requires careful evaluation.

NanoPak-C porous graphitic carbon microbeads (All-Carbon microbeads) represent a new class of porous graphitic media synthesized by a microfluidic based method [4]. Their composition and synthesis method are key differentiators compared to other PGCs. In particular, All-Carbon microbeads packed capillary HPLC columns could act as a niche tool for polar analytes including glycans, glycopeptides, PTM-rich peptides, and metabolites often under retained by conventional C18. At the same time, the All-Carbon microbead synthesis platform provides a toolbox to customize composition, structure, and surface chemistry [4], creating opportunities to tailor all-carbon media for different separation problems within proteomics.

This technical note compares All Carbon Microbeads (5 µm and 3.5 µm diameters) with commercially-available PGC n (5 µm diameter) and silica C18 capillary columns (3 µm and 5 µm diameters) under identical nanoLC–MS conditions using a low-femtomole bovine serum albumin tryptic digest (BSATD). The goal is to define the performance envelope of All Carbon microbeads in a proteomics-relevant setting and clarify where all-carbon media provides

advantages relative to conventional C18. To ensure an unbiased evaluation, all column packing and performance studies described here were carried out by an independent collaborator experienced in capillary nanoLC–MS column preparation.

---

2. Experimental

2.1 LC system and chromatographic conditions

All separations were performed on a Bruker/Michrom NanoAdvance UHPLC operated in capillary nanoLC mode. The following LC conditions were used for all runs:

- Solvent A: 0.1% formic acid in water
- Solvent B: 100% acetonitrile
- Gradient: 5–55% B in 30 min (linear)
- Flow rate: 300 nL/min
- Column format: 75 µm ID × 250 mm fused-silica capillaries

2.2 Columns evaluated

- **All-carbon (NanoPak-C / Millennial)**
  - All-Carbon 5 µm, 75 µm × 250 mm
  - All-Carbon 3.5 µm, 75 µm × 250 mm

- **PGC (Commercial comparator not manufactured by us)**
  - PGC 5 µm, 75 µm × 250 mm

- **Silica-based C18 reference (not manufactured by us; used as benchmark only)**
  - C18 5 µm, 75 µm × 250 mm
  - C18 3 µm , 75 µm × 250 mm

All columns were operated under the same gradient and flow conditions to enable direct comparison of retention profiles and chromatographic performance.

2.3 Mass spectrometry

LC effluent was analyzed using a Thermo LTQ XL ion trap mass spectrometer equipped with a Bruker/Michrom CaptiveSpray source configured for Thermo instruments.

Acquisition parameters were:

- MS1 scan range: 400–2000 m/z
- Heated capillary temperature: 200 °C
- Electrospray voltage: 1.4 kV

Chromatograms shown are **base peak chromatograms (BPC)** unless otherwise noted.

2.4 Sample

A commercial **bovine serum albumin tryptic digest (BSATD)** was used as a proteomics-relevant test mixture.

- Stock concentration: 400 fmol/µL
- Injection volume: 200 nL

- Load on column: 80 fmol BSATD per run

This low-femtomole loading regime is representative of high-sensitivity nanoLC–MS applications and is appropriate for probing stationary-phase efficiency and baseline quality.

2.5 Independent evaluation

Column packing, method execution, and chromatographic data acquisition were performed by an independent collaborator with extensive experience in capillary HPLC column preparation and nanoLC–MS for proteomics. This third-party evaluation was used to minimize bias in the comparison between NanoPak-C All Carbon, commercial PGC, and C18 reference columns.

---

3. Results and Discussion

3.1 C18 5 µm and C18 3 µm reference columns: peptide mapping benchmark

The **C18 5 µm** (**Figure 1a**) **and C18 3 µm** (**Figure 1d**) columns served as performance benchmarks for conventional peptide mapping. Under the 5–55% B gradient, both C18 columns generated **dense chromatograms with numerous resolved peaks** across the 6–25 min window, indicative of high peak capacity for a single-protein digest.

- The **C18 3 µm** chromatogram displays **narrower peaks and higher apparent peak density** compared with 5 µm C18, consistent with the increased efficiency expected from smaller particles.

- Baseline stability on C18 was **excellent**, with a low and flat baseline outside major elution regions, providing high signal-to-noise for the 80 fmol load.

These data confirm that, under the chosen conditions, the C18 columns provide a robust **reference for conventional tryptic peptide separations**, against which PGC performance can be interpreted.

3.2 PGC 5 µm: reference commercial comparator

The **PGC 5 µm** chromatogram (**Figure 1b**) shows several hallmarks of PGC behavior:

- Relative to C18, the main peptide population elutes **later in the gradient**, with many major peaks eluting between ~6 to 24 min, reflecting **stronger retention** on PGC's surface.

- Peak shapes are generally acceptable, but the **overall peak density is lower** than on C18 at the same sample load and gradient, yielding fewer discrete peaks over the full chromatographic window.

- The baseline outside the main elution region shows **elevated background and broader features** relative to C18, consistent with **stationary-phase bleed and/or release of strongly retained contaminants**.

These observations are in line with common experience: PGC offers **orthogonal selectivity and stronger retention,** but does not match optimized C18 for global peptide mapping in terms of peak capacity, and may contribute more to MS baseline at high sensitivity.

3.3 All Carbon 5 µm

All Carbon 5 µm (**Figure 1c**) chromatogram can be directly compared with the 5 µm PGC trace because all chromatographic and MS conditions, including gradient slope and sample load, were identical.

Key observations:

- **Retention behavior**: The overall elution window (~6-24 minutes) on All-Carbon 5 µm is similar to PGC, confirming that both materials provide strong PGC-type retention relative to C18. Major peptide peaks elute in the same approximate time window, indicating comparable selectivity.

- **Peak shape and efficiency**: Visual inspection of the base peak chromatograms shows that All-Carbon 5 µm exhibits **slightly sharper and more symmetric peaks** than PGC for many prominent peptide features, corresponding to a modest but consistent improvement in chromatographic efficiency at the same particle size.

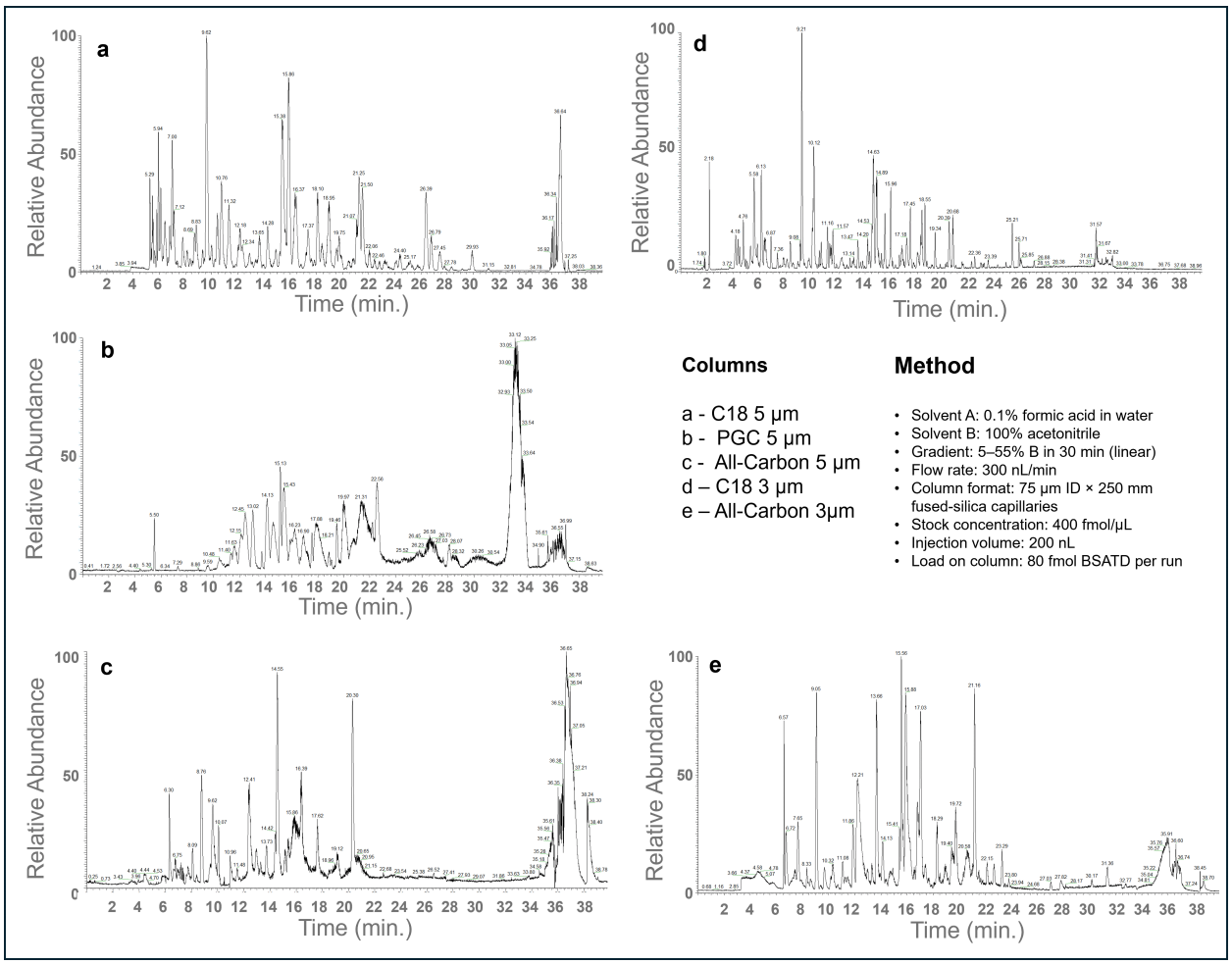

Figure 1. Representative chromatograms of bovine serum albumin tryptic digest (BSATD) separated using NanoLC on the following capillary HPLC columns: (a) C18 5 µm, (b) PGC 5 µm, (c) All-Carbon 5 µm, (d) C18 3 µm, (e) All-Carbon 3µm. Column format: 75 µm ID × 250 mm fused-silica capillaries

- **Conditioning behavior**: Based on discussions with the experimentalist, **5 µm All-Carbon conditioned more quickly and more reproducibly than 5 µm PGC**, requiring fewer gradient runs to achieve stable retention and peak shapes at low femtomole loadings. This is a practical advantage for nanoLC–MS users who need to deploy PGC phases efficiently.

- **Baseline and bleed**: At matched sensitivity, **All-Carbon exhibited a cleaner MS baseline than PGC**, indicating **lower stationary-phase bleed and reduced contribution to background signal** under the conditions tested. In non-peak regions of the chromatograms, All-Carbon shows a flatter baseline than PGC at comparable retention times.

Taken together, these observations show that All-Carbon 5 µm not only matches PGC in retention and selectivity, but also offers **faster, more reproducible conditioning and lower bleed**, which are important differentiators in high-sensitivity nanoLC–MS workflows.

3.4 All-Carbon 3.5 µm: higher-efficiency all-carbon option

Reducing All-Carbon microbead diameter size from **5 µm to 3.5 µm** yields a clear improvement in chromatography. The **All-Carbon 3.5 µm** chromatogram (**Figure 1e**) shows:

- **Narrower peak widths** for many major peptide peaks, reflecting higher column efficiency at smaller particle size.

- **Improved resolution** between partially overlapping features compared with All-Carbon 5 µm, particularly in mid-gradient regions where peptide density is highest.

- A similar overall retention profile and elution window to All-Carbon 5 µm, indicating that selectivity is preserved while efficiency is increased.

All-Carbon 3.5 µm provides both **the expected PGC retention** and a **performance step-up in efficiency**, analogous to the improvement observed when moving from 5 µm to 3 µm C18.

3.5 All-Carbon vs PGC vs C18: Retention and selectivity trade-offs

Comparing All-Carbon, PGC and C18 chromatograms highlights several important trade-offs:

- **Retention strength**: For the same gradient (5–55% B) and sample, All-Carbon and PGC retain peptides more strongly than C18. In practice, equivalent retention on C18 is often achievable with **shallower gradients (e.g., 5–35% B)**, reinforcing that all-carbon media may require **~20% higher final organic content** to elute comparable peptide populations.

- **Peak capacity and mapping**: C18 chromatograms (especially 3 µm) exhibit **higher peak density and apparent peak capacity** for a global digest, making them more suitable for routine bottom-up proteomics workflows where maximal identifications are desired. All-Carbon and PGC shows fewer total resolved peaks for the same digest, although individual peaks can be well shaped.

- **Selectivity**: All-Carbon mixed-mode retention makes it attractive for **very hydrophilic or isomeric analytes** (e.g., glycans, glycopeptides, PTM-rich peptides, polar metabolites), but the same selectivity can be less optimal for broad peptide mapping, where hydrophobicity-driven separation on C18 is often more predictable.

Taken together, these observations support a complementary positioning: C18 for **general peptide mapping**, and **All-Carbon as a niche tool for polar analytes such as glycans, glycopeptides, PTM-rich peptides, and metabolites**, where stronger retention and orthogonal all-carbon selectivity are advantageous. At the same time, the **All-Carbon microbead synthesis route provides a tunable toolbox** to adjust composition, structure, and surface function [4]. Building on the current products, efforts are underway to develop additional all-carbon compositions that more closely match **C18-like performance for general peptide mapping**, while preserving the benefits of an all-carbon support.

3.6 Practical considerations: conditioning and baseline behavior

Both All-Carbon and PGC columns required more extensive conditioning than C18 to achieve stable retention and optimal sensitivity at low femtomole loadings, but **clear differences emerged between All-Carbon and PGC**.

- **Conditioning**: Both All-Carbon 5 µm and 3.5 µm columns reached stable performance in **fewer conditioning runs and with better run-to-run reproducibility** than PGC 5 µm. All-Carbon approached steady-state behavior more rapidly, whereas, PGC required more injections before retention times and peak shapes stabilized.

- **Baseline and bleed**: Under identical MS conditions, **All-Carbon consistently produced a lower and smoother baseline than PGC**, consistent with **reduced stationary-phase bleed and fewer strongly retained background components**. PGC chromatograms showed more pronounced baseline elevation and broad background features, especially at higher organic content.

Once conditioned, All-Carbon performance was stable and reproducible, while requiring **less conditioning overhead and contributing less to background noise** than PGC. For routine use, we still recommend multiple blank gradients and high-organic washes for any PGC phase, but All-Carbon's faster conditioning and cleaner baseline reduce the practical cost of adopting an all-carbon column in sensitive proteomics workflows.

---

4. Conclusions

Under matched nanoLC–MS conditions using an **80 fmol BSA tryptic digest**, we find that:

1. **NanoPak-C All-Carbon behaves as a robust graphitic carbon phase** with substantially stronger peptide retention than C18 and characteristic PGC selectivity, confirming its suitability for applications where conventional reversed-phase media under-retains critical analytes.

2. At **5 µm particle diameter**, **All-Carbon shows improved efficiency and peak shape relative to 5 µm PGC**, while maintaining a similar overall retention profile and elution window.

3. **All-Carbon conditions more quickly and more reproducibly than PGC**, reaching stable retention and peak performance in fewer runs, which reduces startup time and improves robustness in nanoLC–MS workflows.

4. **All-Carbon exhibits lower stationary-phase bleed than PGC under identical conditions**, leading to a **cleaner MS baseline and reduced background signal**—an important advantage at low femtomole sensitivity.

5. **All-Carbon 3.5 µm** provides a higher-efficiency all-carbon option, with narrower peaks and improved resolution compared with All-Carbon 5 µm, analogous to the gain observed when moving from 5 µm to 3 µm C18.

6. Compared with C18, All-Carbon and PGC materials show lower peak density for this global digest but offer stronger retention and distinct selectivity.

Taken together, All-Carbon microbeads are best suited as a **complementary tool for hydrophilic peptides, glycans, glycopeptides, PTM-rich peptides, and polar metabolites**, rather than a replacement for C18 in routine peptide mapping workflows.

These preliminary findings define the current suite of NanoPak-C All-Carbon microbead reverse phase media for demanding nanoLC–MS applications. The data presented here form the basis for further application notes in glycoproteomics, glycomics, and metabolomics, where all-carbon selectivity is expected to provide significant analytical benefit. More broadly, they illustrate how the All-Carbon microbead synthesis toolbox can be used to design additional all-carbon compositions—ranging from highly polar-selective phases to candidates targeting C18-like performance for general peptide mapping—ultimately enabling a suite of all-carbon supports tailored to diverse proteomics separations.

References

[1] K.L. Sanders, J.L. Edwards, Nano-Liquid Chromatography-Mass Spectrometry And Recent Applications In Omics Investigations, Analytical Methods 12(36) (2020) 4404-4417.

[2] M. Russo, M.R.T. Camillo, R. La Tella, F. Rigano, P. Donato, L. Mondello, P. Dugo, Principles And Applications Of Porous Graphitic Carbon Stationary Phase In Liquid Chromatography: An Update, Journal Of Chromatography A 1719 (2024) 464728.

[3] D.G. Delafield, H.N. Miles, W.A. Ricke, L. Li, Inclusion Of Porous Graphitic Carbon Chromatography Yields Greater Protein Identification And Compartment And Process Coverage And Enables More Reflective Protein-Level Label-Free Quantitation, Journal Of Proteome Research 22(11) (2023) 3508-3518.

[4] M.J. Parente, B. Sitharaman, Synthesis And Characterization Of Carbon Microbeads, ACS Omega 8(37) (2023) 34034-34043.