

Porous Graphitic Carbon for Sample Preparation and LC/MS Analysis of Hydrophilic Biomolecules

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Abstract

Purpose: To demonstrate the advantages of using porous graphitic carbon (PGC) in micro-scale SPE and LC/MS analysis of polar molecules of biological interest such as small hydrophilic peptides and phosphopeptides.

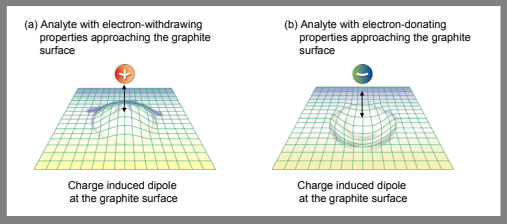
Introduction

The sensitivity of the analysis of small hydrophilic peptides by mass spectrometric detection is often compromised by the presence of salts and non-volatile buffers. These peptides are not retained and, therefore, are often found in the flow-through fraction from a C18 LC column, the type of stationary phase most commonly used for the separation of proteolytic digests of proteins. The analysis of the flow-through fraction requires either a stationary phase that can retain the peptides away from the solvent front, where the biological salts and buffers elute¹, or a sample clean-up step to remove the salts. Porous Graphitic Carbon (PGC) is a material that provides strong retention of very polar compounds; the retention mechanism involves a charge-induced interaction of the polar analyte with the polarizable surface of the graphite² (Figure 1). PGC is ideal to retain and resolve very polar, hydrophilic molecules, which are normally not retained under reversed-phase LC using typical MS compatible mobile phases.

Micro-scale solid phase extraction (SPE) can be used as a sample purification process to remove contaminants; this technique has the advantage of effectively handling limited sample volumes (low microlitre) to maximise sensitivity. When the analytes are very hydrophilic it is necessary to select a sorbent that provides good retention and minimises sample loss through breakthrough in the aqueous matrix.

The work presented in this paper demonstrates the advantages of using PGC in micro-scale SPE and LC/MS analysis of a phosphopeptide and di-, tri- and penta-peptides containing polar and basic terminal amino acid residues. Analytical parameters investigated are chromatographic retention and resolution, spectral cleanliness and sample recovery.

FIGURE 1. Schematic representation of a point charge approaching the graphite surface.



Materials & Methods

• Columns - Hypercarb™ 5 μm, 50 x 2.1 mm; Hypersil GOLD™ 5 μm, 100 x 2.1 mm (Thermo Scientific, Bellefonte, PA). Instrumentation: Finnigan™ Surveyor™ and Finnigan LCQ™ Deca (Thermo Scientific, San Jose, CA). Micro-scale SPE: HyperSep™ Hypercarb Tips 10 - 200 μL volume (Thermo Scientific, Bellefonte, PA).

• LC/MS conditions:

Mobile phase: A - H₂O + 0.1% formic acid; B - ACN + 0.1% formic acid; Gradient : 5 to 100% B in 10 min; Flow rate: 0.2 mL/min; Temperature: 30 °C; Detection: +ESI

• Micro-scale SPE protocol:

Solvents: A - H₂O + 0.1% formic acid; B - H₂O / ACN (30:70) + 0.1% formic acid.

Tip conditioning: Aspirate and expel 5 times 20 μL of solvent B. Aspirate and expel 5 times 20 μL of solvent A.

Sample loading (binding): Aspirate and expel 20 times 20 μL of sample.

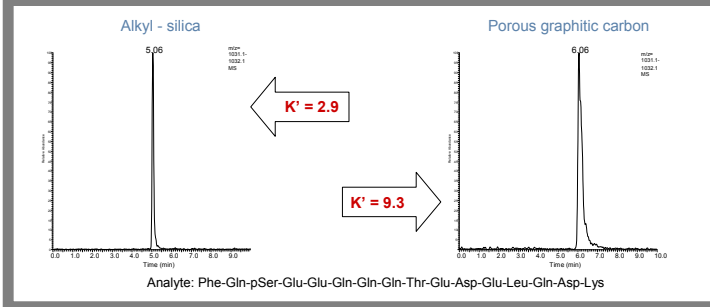
Sample washing: Aspirate and expel 5 times 20 μL of solvent A, discarding the expelled solvent each time.

Sample elution: Aspirate and expel 20 times 20 μL of solvent B, collecting the expelled solution in a clean micro-centrifuge tube. Transfer solution to micro-vial for injection.

Results

i) Phosphopeptide retention on PGC

FIGURE 1. Comparison of the capacity factor of a monophosphorylated peptide on an alkyl-silica phase and PGC.



When analyzed under identical conditions the capacity factor for the monophosphopeptide on a porous graphitic carbon column is 3 times greater than when analyzed using an alkyl-silica stationary phase (Figure 1). On the PGC column the retention mechanism is a combination of hydrophobic or dispersive interaction with the hydrophobic amino acid chain, and a polar interaction between the phosphate group and the polarizable surface of the graphite.

ii) Di- to penta- hydrophilic peptides

In Figure 2 the retention of a di-, tetra- and a penta-peptide is compared on the alkyl-silica phase and on PGC. On the alkyl-silica phase, typically used in the separation of proteolytic digests, RGES elutes at the solvent front, closely followed by DSDPR. The basic (Arg) and alcohol (Ser) terminal residues make these short peptides hydrophilic and difficult to retain under conventional reversed-phase LC/MS conditions. On the PGC column these short peptides are well retained away from the solvent front.

FIGURE 2. Comparison of the retention of 3 hydrophilic peptides on alkyl-silica and porous graphitic carbon. PGC provides higher retention and different selectivity.

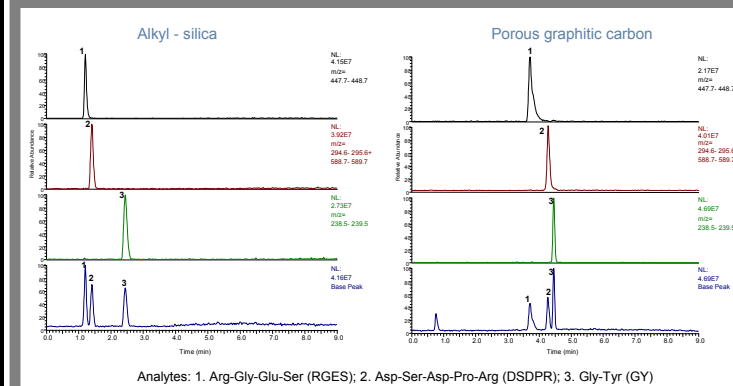
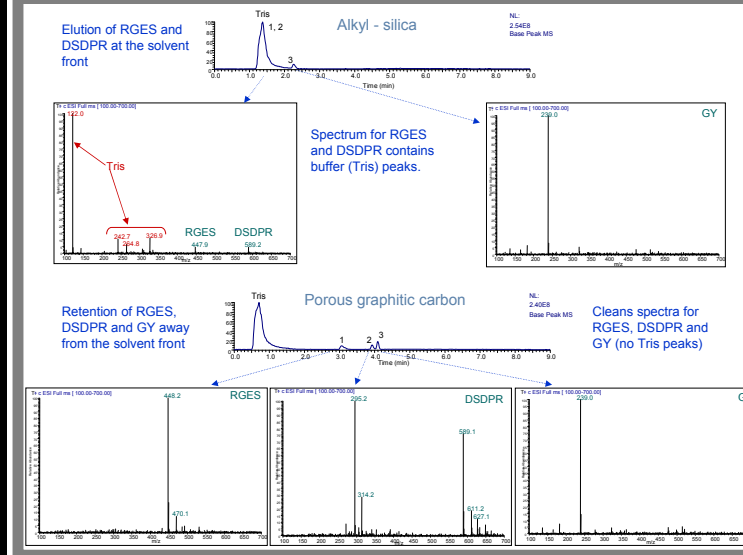
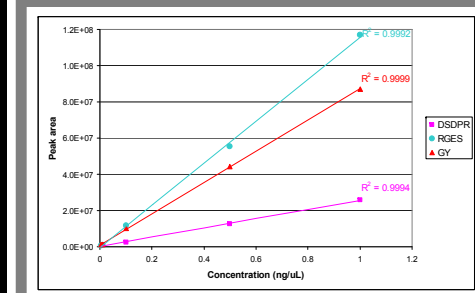


FIGURE 3. Comparison of the spectra obtained for 3 hydrophilic peptides in Tris buffer on alkyl-silica and porous graphitic carbon stationary phases.



A flow-through fraction from a proteolytic digest was simulated by diluting a solution containing three hydrophilic peptides in Tris buffer (100 mM, pH 8.0) to concentrations in the range of 15 to 30 pmol/μL. This fraction was injected and separated on an alkyl-silica phase and on PGC (Figure 3). On the alkyl-silica phase the two more polar peptides (RGES and DSDPR) elute at the solvent front, co-eluting with the chromatographic peak for Tris. The spectrum for these peptides is dominated by the Tris peaks at m/z 122, 243, 265 and 327, showing very weak signals for the [M+H]⁺ at m/z 448 and 589. On the other hand, the PGC stationary phase effectively separates these peptides away from the Tris chromatographic peak, producing clean and intense spectra, that allow for good identification.

FIGURE 4. Linearity data for concentrations of 5 μg/μL to 1 ng/μL of each peptide injected on the Hypercarb column.



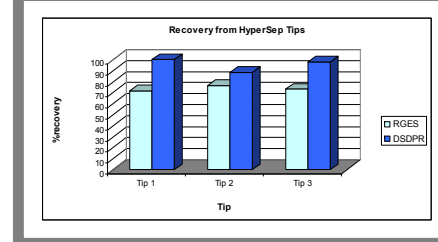
The chromatographic retention and resolution of the hydrophilic peptides on the PGC column enables quantitative data to be obtained. On Figure 4 the linearity of the response is demonstrated for concentrations in the range of 5 μg/μL to 1 ng/μL of each peptide.

iii) Microscale SPE

Desalting of hydrophilic peptides can also be accomplished by using microscale tips packed with porous graphitic carbon. This enables the off-line preparation and clean-up of biological samples for further analysis and identification by MS. In this approach it is important that the sorbent in the tip is capable of retaining the hydrophilic analytes with no breakthrough in the aqueous matrix, and that good recovery of the retained analytes from the tip is achieved.

A flow-through fraction from a proteolytic digest was simulated by diluting a solution containing RGES and DSDPR in Tris buffer (100 mM, pH 8.0) to concentrations of 0.1 and 0.5 ng/μL respectively. Following the procedure detailed in the methodology section, the recoveries were measured by comparison of the ESI-MS signal for the tip eluate with the ESI-MS signal for the solution of the same concentration. Recoveries are between 72 and 101%, as shown in Figure 6.

FIGURE 6. Recovery from HyperSep Hypercarb Tips for 2 peptides, RGES and DSDPR.



Conclusions

- Porous graphitic carbon columns show increased capacity factors over alkyl-silica columns for phosphorylated peptides.
- In contrast to alkyl-silica stationary phases, porous graphitic carbon retains small hydrophilic peptides away from the solvent front under typical reversed-phase LC/MS conditions; as a result the spectra are free from biological buffer and salts allowing for good peptide identification even at low levels.
- Quantitative analysis of small hydrophilic peptides on porous graphitic carbon columns can be achieved with excellent linearity.
- Micro-scale SPE with porous graphitic carbon packed tips gives good recovery of small hydrophilic peptides from buffer matrices.

References

- (1) E. T. Chin, D. I. Papac, *Anal. Biochem.*, 273 (1999) 179-185.
- (2) P. Ross, *LCGC Europe*, May 2000

Additional Information

For additional information, please browse our website: www.thermo.com/columns

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