

Separation of phospho and glyco peptides using porous graphitic carbon for the proteomic study of oncology patients

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Overview

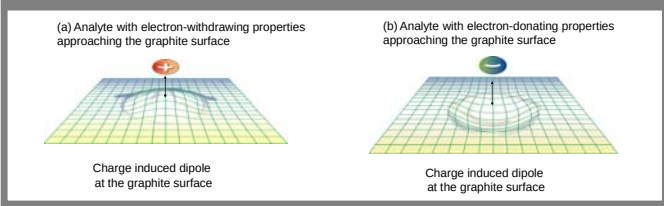
Aim: To develop an LC-MS method of analysis using Porous Graphitic Carbon (PGC) columns to retain small, polar peptides for the accurate detection and quantification of post-translational modifications (PTMs) such as phosphorylation

Introduction

The accurate detection and quantification of post-translational modifications (PTMs) of proteins such as phosphorylation remains a challenge in proteomics. Fractionation of proteins for their analysis and sequencing leads to the formation of a series of peptides, some of which are phosphorylated or glycosylated. These compounds are polar in nature and as a consequence their retention using conventional C18 reverse-phase packings is a challenge. 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 graphite (Figure 1). PGC is ideal to retain and resolve very polar, hydrophilic molecules, which are normally not retained under reverse-phase LC using typical MS compatible mobile phases. Furthermore, the stability of the graphite packing allows the use of mobile phases in the full pH range (0-14) and elevated temperatures, unlike standard silica-based reverse-phase packings.

The work presented herein demonstrates the advantages of using PGC for the analysis of polar peptides and phosphopeptides by LC/MS. By accurate consideration of pH, analyte isoelectric point (pI) and column temperature, retention profiles were generated for a set of standards.

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



Methods

Method 1:

Instrumentation: Thermo Scientific Surveyor and Thermo Scientific LCQ Deca
Columns: Thermo Scientific Hypercarb 5 µm, 50 x 2.1 mm; Thermo Scientific Hypersil GOLD 5 µm, 100 x 2.1 mm
LC Conditions: A: H₂O+0.1% Formic Acid B: MeCN+0.1% Formic acid; Gradient 1: 5-100% B in 10 min.
Flow rate: 0.2 mL/min; Temperature: 30° C; Detection: ESI+

Method 2:

Instrumentation: Thermo Scientific TLX, used in HPLC-mode, Thermo Scientific TSQ Vantage Triple Quadrupole MS-detector; Thermo Scientific HOT POCKET.
Columns: Hypercarb™ 5 µm, 100 x 2.1 mm.

LC Conditions:

Gradient separation			Mobile Phases			pH	A	B	Flow rate: 0.2 mL/min Temperatures: 25°, 30°, 40°, 50°, 60° C Detection: ESI+
Time/ mins	% A	% B							
0	100	0	H ₂ O + 0.1% Formic acid		2	MeCN + 0.1% Formic acid			
3	100	0	Ammonium acetate, 10 mM, pH 6		6	MeCN			
13	0	100	Ammonium acetate, 10 mM, pH 8		8	MeCN			
16	0	100	H ₂ O + 0.1% 1-methylpyrrolidine		10	MeCN + 0.1% 1-methylpyrrolidine			
17	100	0							
20	100	0							

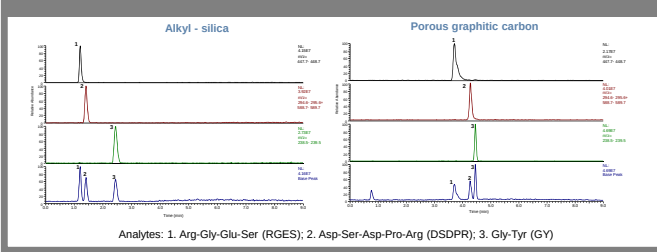
Peptides	One-letter abbreviation sequence	Isoelectric point
Gly-Tyr	GY	5.52
Asp-Ser-Asp-Pro-Arg	DSDPR	4.21
Arg-Gly-Glu-Ser	RGES	6.0
Gly-His-Lys	GHK	8.76
Tyr-Ser-Lys	TSK	8.41
Val-Tyr-Val	VVV	5.49
Asp-Ala-Asp-Gly-pTyr-Leu-Ile-Pro-Gln-Gln-Gly	DADEpYLIPQQG	2.68

Results

Analysis of small hydrophilic peptides

To demonstrate the retentivity of PGC against standard bonded silica packings, a mixture of three hydrophilic peptides (GY, RGES and DSDPR) was analysed according to Method 1. 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.



Analysis of a peptide mixture

To demonstrate the ability of PGC to allow for the one-step analysis of polar and non-polar peptide fragments, a mixture of 5 peptides was analysed. This was designed to contain 3 polar and 1 non-polar peptide as well as 1 polar phosphopeptide. The mixture was analysed using the full array of conditions described in method 2. The retention times were plotted as a function of temperature and pH to obtain a 3D surface plot.

1. Effect of pH

Polar peptides:

Retention of Polar fragments GHK and TSK is greatly affected by the pH of the mobile phase. Both these peptides have a basic terminal lysinyl residue and feature no acidic residues other than the carboxy terminus. At the isoelectric point (pI), the peptides are in an overall zero charge state. When the pH of the mobile phase is increased, reaching near-pI values, the retention of the peptides is greatly increased (Figures 3 and 4).

FIGURE 3. Left: Retention profile of the peptide GHK as a function of pH and temperature. Right: Retention time shift with varying pH of the peptide GHK at 30° C.

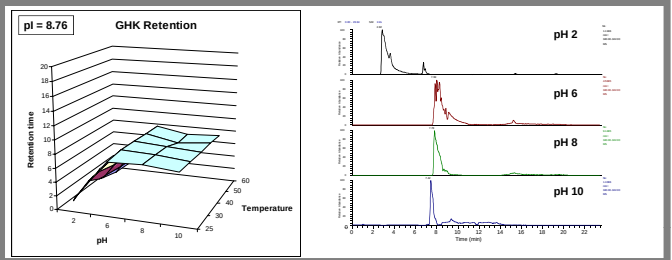


FIGURE 4. Retention profile of the peptide TSK as a function of pH and temperature.

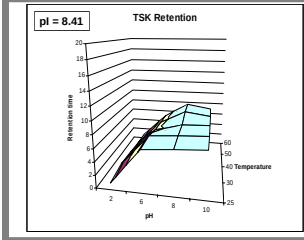
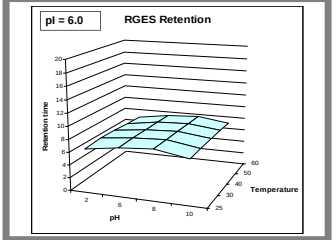
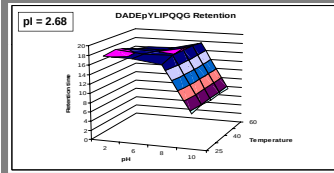


FIGURE 5. Retention profile of the peptide RGES as a function of pH and temperature.



The peptide RGES, which contains both acidic (Glu) and basic (Arg) residues, showed to be less affected by the pH of the mobile phase (Figure 4). Across the studied pH range, the retention time was found to vary by less than 1 minute, even at pH 10, 4 units above the pI.

FIGURE 6. Retention profile of the phospho-peptide DADEpYLIPQQG as a function of pH and temperature.

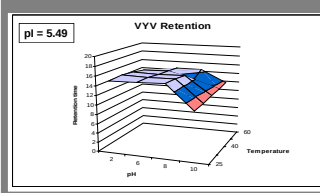


Phosphorylated peptides:

Figure 6 shows the retention surface of phospho-peptide DADEpYLIPQQG. The trend follows that observed for GHK and TSK, where retention is increased when the pH of the mobile phase is closer to the pI of the fragment.

At pH values greatly above the pI, the fragment is predominantly ionised. Consequently, retention on the PGC surface is dictated primarily by induced dipole-interactions, resulting in decreased retention times.

FIGURE 7. Retention profile of the hydrophobic peptide VVV as a function of pH and temperature.



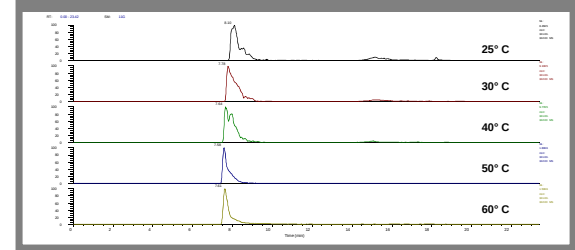
Non-polar peptides:

Retention of the peptide VVV showed to be affected only above pH 8, 2.5 units greater than the pI (Figure 7). The overall increase in charged states at higher pH may result in decreased hydrophobic interactions, explaining the drop in retention. This trend mirrors that observed with phospho-peptide DADEpYLIPQQG.

2. Effect of Temperature

The effect of temperature on retention was found to be minimal across the range of peptides under consideration. For polar fragments, the increase in temperature was found to improve peak shape. Figure 8 shows the effect of Temperature on the peak shape of the fragment GHK; with increasing temperature, little effect is observed on the retention time, however peak symmetry is much improved.

FIGURE 8. Effect of temperature on peak shape of peptide GHK analysed at pH 8.



Conclusions and Future Work

- Porous Graphitic Carbon has shown to give greatly improved retention of polar peptides compared to standard silica based reverse-phase packings. The pH of the mobile phase was found to greatly influence the retention of each species; the highest retention was found when the pH draws nearer to the pI of the fragment.
- The mono-phosphopeptide DADEpYLIPQQG was shown to be well retained on the PGC surface; however the retention varies significantly with the mobile phase pH.
- The analysis of peptide mixtures containing both polar and non-polar fragments has been achieved in one step, using a singular stationary phase.
- In future studies the possibility of using pH gradients will be investigated in order to develop a "one-pot" universal method of analysis. Capillary and nano-bore PGC columns will be tested in order to achieve improved sensitivity for complex mixtures from protein digests.

References

1. Hypercarb™ Applications Notebook, <http://www.separatedbyexperience.com/pages/pages.php?page=hypercarbresources>

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