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EVALUATION AND OPTIMIZATION OF REVERSE PHASE COLUMN MATERIAL FOR THE ANALYSES OF COMPLEX TRYPTIC PEPTIDE MIXTURES BY LC/MS AND LC/MS/MS

Iain Campuzano, Michael Desor, Therese McKenna, James Langridge
Waters Corporation, Manchester, UK

INTRODUCTION

Electrospray mass spectrometry coupled to nanobore reverse phase chromatography has rapidly become the chosen method for the separation and characterization of enzymatically digested protein samples. A key component in the MS analysis is the complexity of the sample that elutes into the mass spectrometer, from the HPLC column, at any given moment in time. This can have a profound influence on the number of peptides and hence proteins identified, and also the dynamic range of the identified components. Therefore, the peak capacity of the chromatographic system is often an important attribute. Additional chromatographic attributes such as retention time reproducibility and injection methods can have profound effect on the results, especially if relative quantification is desired.

In this poster we investigate different stationary phases for peptide mapping and in particular we will focus on how to improve chromatographic separations over those methods currently in use. Data will be presented showing the improved peptide recovery, particularly of small, hydrophilic peptides from a protein digest, resulting in an increased level of protein coverage.

EXPERIMENTAL

All data was acquired on a Waters® Micromass® Q-Tof™ Global and Waters modular CapLC® system. The following column configuration was used to separate tryptic digests.

The amount of 100 fmol of Yeast Enolase digest was first loaded onto a trapping/guard column, at a flow rate of 15 µL/min for 3 minutes in 0.1% formic acid solvent. The guard column consisted of Symmetry® C_{18} OPTI-PAK, 300 µm x 5 mm, followed by a Graphitized Carbon column 180 µm x 30 mm (Figure 1). Following the loading phase, the 10-port Stream Select Module back-flushes (Figure 2) the trapped peptides onto the analytical column. The analytical column used was a Waters NanoEase™ 75 µm x 15 cm Atlantis™ dC_{18} capillary column. The pumping system delivers a flow of 4.5 µL/min, which is split to give a resultant flow through the analytical column of 300 nL/min. Peptides were resolved using an acetonitrile gradient. Details of gradient are listed below.

The above loading/trapping procedure and gradient were also used to separate the tryptically digested peptides of Yeast Enolase in the absence of the Graphitized Carbon column.

Chromatographic columns

Waters NanoEase dC_{18} 75 µm x 150 mm 3 µm

Symmetry C_{18} OPTI-PAK 300 µm x 5 mm

Graphitized Carbon 180 µm x 30 mm

Gradient

Time	% Solvent B
0	5
3	5
60	40
65	85
70	85
71	5
90	5

Solvent A, 5% ACN (v/v) 0.1% HCOOH (v/v)

Solvent B, 95% ACN (v/v) 0.1% HCOOH (v/v)

Mass Spectrometry

Mass spectroscopic analysis was carried out on a Waters Micromass Q-Tof Global mass spectrometer fitted with a nano ZSpray™ source. The instrument was calibrated over the mass range m/z 50-1600, using the fragment ions of Glu-fibrinopeptide B. Data Directed Acquisition (DDA™) was performed on the tryptically digested samples. An initial TOF-MS survey scan was acquired over the mass range m/z 300-1600, with the switching criteria for MS to MS/MS switching including ion intensity and charge state. The Q-Tof

Global was programmed to ignore singly charged ions and perform MS/MS on, and up to three co-eluting species, with 1-second integration times. Switch back into MS survey mode was triggered after a time period of 4 seconds. The collision energy used to perform MS/MS was varied according to the mass and the charge state of the eluting peptide. Data for MS/MS was acquired over the mass range m/z 50-1600.

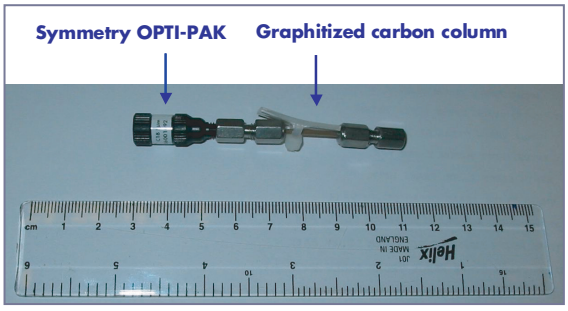

Figure 1. The configuration of Symmetry C_{18} OPTI-PAK trapping column and graphitized carbon column. The Symmetry OPTI-PAK trapping cartridge screws directly into the connector of the graphitized carbon column, thus minimizing any dead-volume.

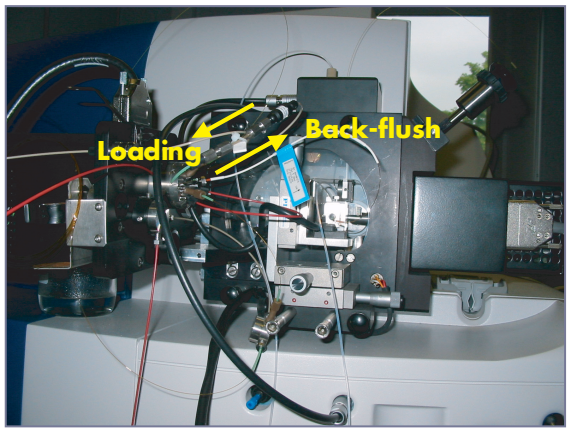

Figure 2. The loading and back-flushing configuration of the Symmetry C_{18} OPTI-PAK trapping column and graphitized carbon column when connected to the Stream Select Module.

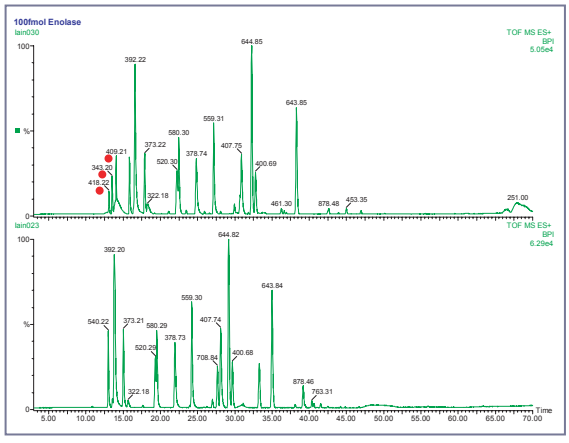

Figure 3. Chromatographic separation of 100 fmol of Yeast Enolase tryptic digest. The top chromatogram represents separation in the presence of the Symmetry C_{18} trapping column and Graphic carbon column, followed by Atlantis dC_{18} analytical separation. The three additional chromatographic peaks have been highlighted. The lower chromatogram represents separation in the presence of the Symmetry C_{18} trapping only, followed by Atlantis dC_{18} analytical separation.

AVSKVYARSVYDSRGNPTVEVELTTEKGVFRSIVPSGASTGVHEALEMRDGDKSKWM
GKGVLHAVKNVNDVIAPAFVKANIDVKDQKAVDDFLISLDGTANKSKLGANAILGVSLA
ASRAAAAEKNVPLYKHLADLSKSKTSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPT
GAKTFAEALRIGSEVYHNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIK
AAGHDGKVKIGLDBASSEFFKDGKYDLDFKNPNSDKSKWLTGPQLADLYHSLMKRYP
IVSIEDPFAEDDWEAWSHFFKTAGIQIVADDLTVTNPKRIATAIEKKAADALLLKVNQIGT
LSESIKAAQDSFAAGWGVMVSHRSGETEDTFIADLVVGLRTGQIKTGAPARSERLAKL
NQLLRIEEELGDNAVFAGENFHHGDKL

Figure 4. The amino acid sequence coverage map of Yeast Enolase. The red amino acid residues represent those obtained by trapping peptides in the presence of the Symmetry C_{18} only (66% sequence coverage). The underlined residues are the additional peptides obtained by trapping in the presence of a Symmetry C_{18} trapping column and a Graphic carbon column (6.6% sequence coverage).

RESULTS

The data would indicate that when trapping a tryptic peptide digest onto a Symmetry C_{18} trapping column, followed by an extremely hydrophobic stationary phase is advantageous. Any small hydrophilic peptides which are not normally retained by standard C_{18}, are trapped by the additional hydrophobic trapping column plumbed in series to the standard C_{18} trapping column.

The upper chromatogram in Figure 3 shows the presence of 3 additional chromatographic peaks at the beginning of the gradient, corresponding to the peptides of sequence GVFR, VYAR, WMGK, TGQIK, AAAEK and TGAPAR. Figure 4 shows the coverage map of Yeast Enolase when analyzed using the two differing trapping protocols. The additional 6 peptides noted above represents an additional 6.6% sequence coverage, therefore increasing sequence coverage from 68% to 74.6%. Note also that the overall chromatographic retention times of all the peptides change in the presence of the additional hydrophobic trapping column.

CONCLUSION

Upon analysis of 100 fmol of Yeast Enolase tryptic digest in the absence of the Graphitized Carbon trapping column, the percentage of amino acid sequence coverage obtained was 68%. In the presence of the Graphitized Carbon, the sequence coverage increased to 74.6%. The additional 6.6% sequence coverage represents 6 small hydrophilic peptides, which were not retained by the Symmetry C_{18} trapping column alone. Additionally, this trapping column configuration may be very useful in the analysis of small hydrophilic phosphopeptides, which are known to be problematic when analyzed by standard reverse phase chromatography.

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