Improving the Detection of Hydrophilic Peptides for Increased Protein Sequence Coverage and Enhanced Proteomic Analyses

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Introduction

Reversed-phase pre-columns, commonly referred to as trap columns, are widely used for capturing peptides from enzymatic digests of proteins to facilitate removal of interfering substances, or as a concentration step prior to their introduction (in-line) to a high resolution separation column and subsequent analysis by liquid chromatography tandem mass spectrometry (LC MS/MS). Binding of peptides to the trap column is therefore pivotal for a successful analysis. The parameters of contact time (flow rate) and loading solvent composition are examined to determine their effects on the ability of peptides to bind to a water wettable C18 stationary phase trap column.

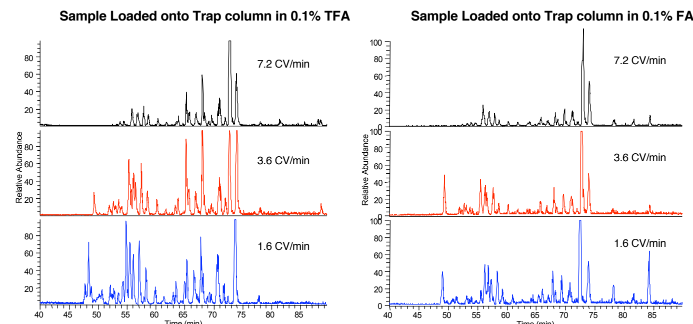
Methods

Fifty picomoles of human low density lipoprotein receptor-related protein-associated protein-1(RAP) (kindly provided by D.R. Strickland laboratory) was digested with TPCK treated bovine trypsin (Worthington) at a final concentration of 0.5μM in 50μl of 25mM ammonium bicarbonate, 20% acetonitrile, 5mM $CaCl_2$ for 4 hrs at 37°C. The digested protein was dried by rotary evaporation and resuspended in 0.1% formic (FA), or trifluoroacetic acid (TFA) and was diluted to 50 femtomoles/μl prior to analysis by LC MS/MS (LCQ Advantage, Thermo Fisher). The stationary phase for both the trap and separation column was a 5μm, TARGA® C18 (Higgins Analytical). The HPLC consisted of a single Waters Alliance® 2695 that was modified with a standard Rheodyne® 2-position 6-port valve inserted in the solvent flow path between the pump and the autosampler to allow flow to be directed through the autosampler for loading samples onto the trap column, and subsequently switched to bypass the autosampler during gradient separation. This configuration eliminated the relatively large system volume contained in the autosampler of the HPLC during the gradient separation portion of the chromatographic run. In addition the flow from the HPLC pump was split to achieve the low flow rates used in this study.

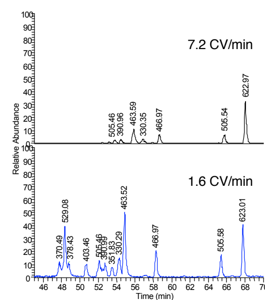
Samples consisting of 200fm of digested protein in a volume of 4μl of either 0.1% FA or 0.1% TFA were loaded onto a 250μ x 23mm trap column for 30 minutes at linear velocities (contact time) ranging from 1.6 to 7.2 column void-volumes/min (CV/min). To calculate linear velocity in CV/min, the void volume was estimated to be ~50% of the internal volume of the trap column. Bound peptides were eluted onto a 75μ x 200mm column equilibrated in 0.1% FA and eluted with a gradient of acetonitrile containing 0.1% FA at a flow rate of approximately 250nl/min. The gradient went from 0 to 5% acetonitrile in 5 min and then from 5 to 25% acetonitrile in 60 min. The trap and separation columns were re-equilibrated for 20 min prior to injection of the next sample.

LC MS/MS analysis was carried out using a Thermo-Finnigan LCQ Advantage® ion trap mass spectrometer utilizing nanospray ionization. MS analysis consisted of repetitive cycles of full MS scans over the range of 250 - 1800 m/z followed by MS/MS analysis of the three most abundant ions from the full MS scan. These ions were subsequently excluded from further MS analysis

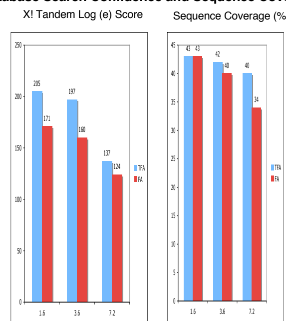
Increasing Contact Time Markedly Improves Trapping of Hydrophilic Peptides



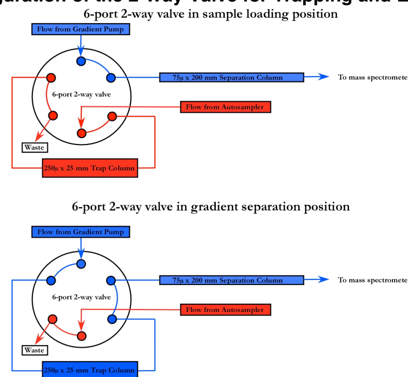
Partial Reconstructed Ion Chromatogram Showing Enhanced Retention of Specific Hydrophilic Peptides



Increased Retention of Hydrophilic Peptides Improves Database Search Confidence and Sequence Coverage



Configuration of the 2-Way Valve for Trapping and Elution of Peptides



A standard configuration of the 2-way valve allowing bound peptides to be back-flushed from the top of the trap column to the separation column where they are "refocused" providing minimal loss of resolution.

Results

Preliminary experiments where peptides were loaded onto a trap column at flow rates typically published in the literature (≥60 CV/min or equivalent to 15μl/min through a 0.5μl trap column) demonstrated a lower than expected yield of peptides in the early portion of the chromatogram.

Adding TFA to the sample solution for trap loading modestly improved the overall retention of peptides but was unable to effect retention of the hydrophilic peptides at a flow rate ≥ 7.2 CV/min.

Decreasing the flow rate at which peptides were loaded onto the trap had a dramatic effect on retention of hydrophilic peptides. These peptides were retained when the flow rate was reduced to 3.6 CV/min with maximal binding occurring at 1.6 CV/min.

The presence of TFA in the sample solution during trap loading showed increased retention of hydrophilic peptides compared to FA. However a subset of these peptides were not retained when the flow rate was increased from 1.6 to 3.6 CV/min.

The greater number of peptides retained when the sample was applied to the trap at lower flow rates and in the presence of 0.1% TFA improved sequence coverage and the X! Tandem Log (e) score.

Conclusions

Increasing the contact time of the peptides with the stationary phase allows retention of hydrophilic peptides that otherwise would not bind to the trap column at higher flow rates.

Contact time can be increased in either of two ways:

- **lowering the flow rate during trap column loading**
- **increasing the trap column volume by making it longer (not wider which would have adverse effects)**

TFA in the sample solution improves retention of hydrophilic peptides at a low flow rate but not at a high flow rate compared to FA.

Equilibration of the TARGA® C18 columns, a polar embedded stationary phase, in the complete absence of organic solvents provided more favorable conditions to retain hydrophilic peptides compared to other C18 stationary phases that require the presence of some organic solvent to allow access to the porous structure of the reversed-phase support.

Providing adequate contact time increases the number of peptides bound and available for analysis during the chromatographic run. This improves