A Novel Monomeric C_{18} Bonded Phase for Improved Resolution & Sensitivity in LC-MS/MS Analysis of Peptides

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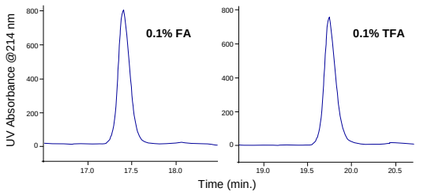
ABSTRACT- The separation of protein enzymatic digests by RP-HPLC is a standard method in protein research and in the development of well-characterized biotechnology pharmaceuticals. Resolution on silica-based RP columns is determined by the nature of the silica, as well as, the silanes and the chemistry that is used to bond them to the stationary phase. Typically, 300 Å C18 chemistries have carbon coverage ranging from 2.8 to 3.6 µmol/m^2. The performance of a novel, monomeric bonded 300 Å C18 adsorbent (VYDAC© EVEREST™) with carbon coverage >4 µmol/m^2 is described. The resolution and sensitivity of this stationary phase was assessed using an LC-MS/MS analysis of several tryptic digests including: digests of individual proteins (serum albumin and green fluorescent protein); a complex sample comprised of a dozen proteins of different abundances; ribosomes; and whole bacterial proteomes. The tryptic peptides were run on nano/capillary or microbore C_{18} columns. LC-MS/MS analyses were performed on an ABI Q TRAP, an ABI Q-STAR, and a Thermo Finnigan LCQ Deca using a solvent system comprised of acetonitrile/water with 0.1 to 0.5% formic acid, or with 0.05% TFA, with subsequent database searching using Mascot and/or SEQUEST. With the complex sample of a dozen proteins, the score from Mascot was higher for a number of protein identifications on the novel phase compared to a small pore (100 Å) C_{18} material. This corresponds to higher individual peptide scores, which is usually indicative of better MS/MS. The novel phase also performed well with ribosomes and complex proteome samples, based on SEQUEST results.

Technical Specifications
5 µm Silica
300 Å Pore Diameter
C_{18} Monomeric Bonding
Carbon Coverage > 4 µmol/m^2

Applications
Peptide Mapping
Purification of Synthetic Peptides
High Loading Applications
Proteome Studies

Fig. 1. WNPESLNT Peptide Response with Formic Acid or TFA: 1 µg Load on 4.6 mm i.d.

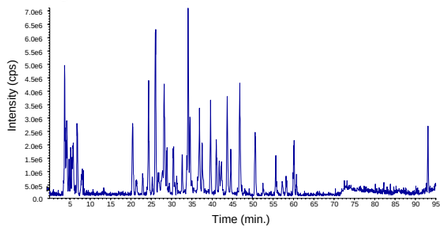


The novel C_{18} media (300 Å pore size, 5 µm particle size) was packed in a 4.6 mm x 250 mm column (VYDAC 238EV54). Mobile phase: A: 0.1% v/v formic acid or TFA in water; mobile phase B: 0.1% v/v formic acid or TFA in 70:30 acetonitrile: water. Gradient: 2%B per minute. Flow Rate: 1 mL/min. Peptide load: 100 µL injection of a 10 µM solution (1 µg).

➔ Peak width and shape does not change by switching from TFA to formic acid. This indicates excellent carbon coverage of the silica.

Data courtesy of Ali Kettani, Memorial Sloan-Kettering Cancer Center; currently at Bruker Daltonics, Inc.

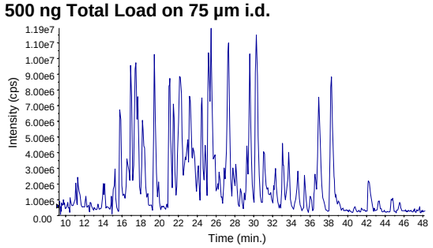
Fig. 2. BPC of Tryptic Digest of BSA: 580 ng Load on 300 µm i.d.



The novel C_{18} media (300 Å pore size, 5 µm particle size) was packed in a 300 µm x 150 mm column (VYDAC 238EV5.315). Mobile phase A: 0.1% v/v formic acid in water; mobile phase B: 0.1% v/v formic acid in acetonitrile. Gradient: 4% B for 5 min., 4-40% B in 70 min, 40-90% B in 10 min., 90% for 10 min., equilibrate at 4% B for 20 min. Flow Rate: 5 µL/min. Temperature: 22ºC. Peptide load: 0.1 µL injection of 5.8 µg/µL (580 ng or 9 pmol). HPLC System: LC Packings Ultimate Inert with Famos Autosampler. MS Detector: ABI Q-Trap.

➔ Theoretical sample capacity for optimal resolution on 300 µm i.d. = 1000 ng. This amount represents 58% of that capacity.

Fig. 3. BPC of "Mock" Pulldown Complex Std.: 10 Protein Mixture, 55% Excess of BSA 500 ng Total Load on 75 µm i.d.

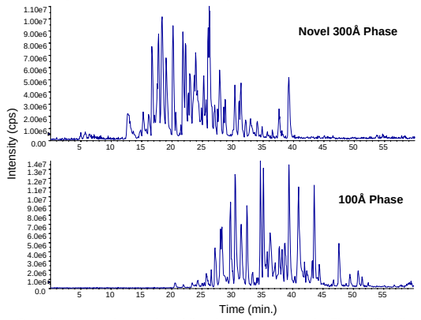


A mixture was formulated from 10 proteins of varying relative abundance with bovine serum albumin constituting the majority of the sample (55% by weight). This mixture was then digested with trypsin. Samples were loaded onto 1-mm trap cartridge using Switchos and washed for 2.5 minutes. The flow was then reversed onto a 75 µm x 150 mm column (VYDAC 238EV5.07515) and peptides eluted directly into MS. Mobile phase A: 0.1 % formic acid in water; mobile phase B: 0.1 % formic acid in acetonitrile. Gradient: 2% B for 2.5 min., 2-25 % B in 50 min. MS Detector: ABI Q-TRAP using IDA in LIT scan mode. Scan range m/z 300-1200. 1 EMS; 1 ER scan; 2 EPI scans. The spray tip was directly coupled to column end.

➔ Theoretical sample capacity for optimal resolution on a 75 µm i.d. = 50 ng. Loading was 10x higher, and the media still provides excellent performance.

Figs. 3 to 8: Data courtesy of Cindy Lou Chepanoske, Prolexys Pharmaceuticals.

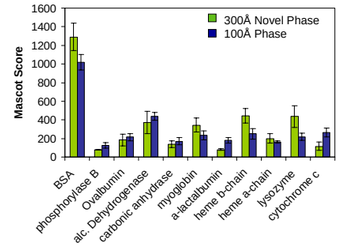
Fig. 4. BPC of "Mock" Pulldown Complex Std.: 10 Protein Mixture, 55% Excess of BSA 250 ng Total Load on 75 µm i.d.



Sample preparation and mobile phases same as in Fig. 3. Gradient for novel 300Å phase: 2% B for 2.5 min., 2-25 % B in 50 min. Gradient for 100 Å phase: 2% B for 2.5 min., 2-35 % B in 50 min. MS Detector: ABI Q-TRAP.

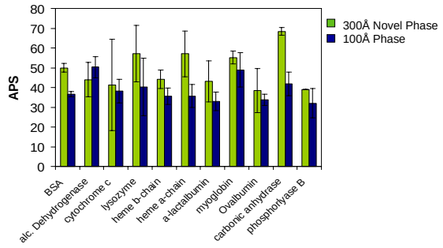
➔ The 300Å phase has lower surface area; thus, it exhibits a faster elution.

Fig. 5. Database Search Results for 250 ng "Mock" Mix (N = 3)



➔ The score returned from Mascot is higher for a number of protein identifications using the novel 300Å phase, even if more peptides were found using the small pore phase. This corresponds to higher individual peptide ion scores, which is usually indicative of higher quality MS/MS data.

Fig. 6. Comparison of Average Peptide Score (APS, N=3) for Each Component of "Mock" Mix



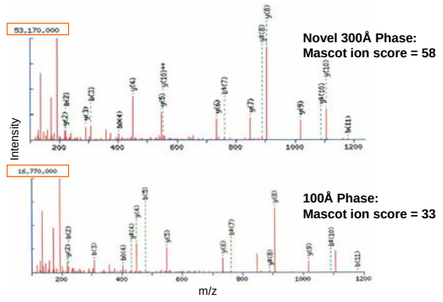
To explain why some of the MS/MS data from the novel 300Å phase is better, the average peptide score (APS) in each component for the 2 phases was determined. This APS number, a parameter used often by Prolexys Pharmaceuticals to assess data quality, is the score/non-redundant peptides. For instance:

Score 200 for 3 peptides: APS = 67
Score 200 for 4 peptides: APS = 50

In the experience of researchers at Prolexys Pharmaceuticals a higher APS score correlates well with higher quality MS/MS data. This correlation between APS and spectral quality is a function of a number of factors, of which the quality and nature of chromatography is a significant contributor.

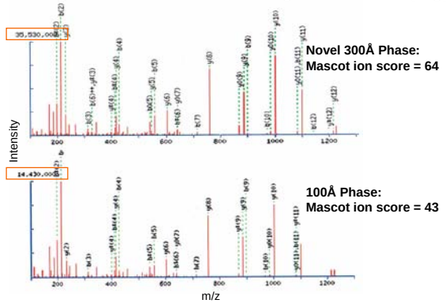
➔ The APS score is higher for the 300Å phase in a number of instances.

Fig. 7. MS/MS Fragmentation of GYSLGNWVCAAK from Lysozyme (Chicken)



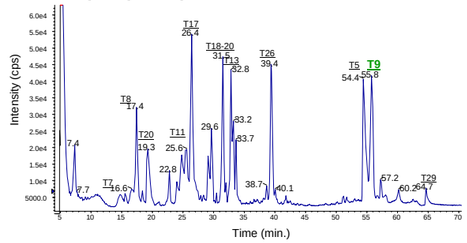
➔ Higher fragment ion intensities are observed for the 300Å phase. Compare number on top of y-axis of each mass spectrum. These good quality spectra play a part in the higher Mascot ion scores.

Fig. 8. MS/MS Fragmentation of VNVDEVGGEALGR from Hemoglobin ß-chain (Pig)



➔ Higher fragment ion intensities are observed for the 300Å phase. Compare number on top of y-axis of each mass spectrum. These good quality spectra play a part in the higher Mascot ion scores.

Fig. 9. TIC of Tryptic Digest of Green Fluorescent Protein (GFP): 150 pmol Load on 1 mm i.d.

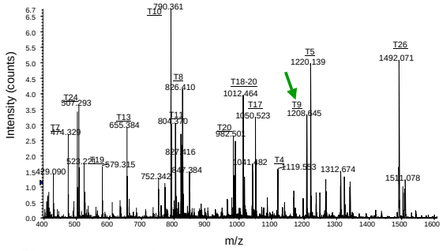


The ratio of protein to trypsin was 50:1. The GFP was heat denatured, reduced with triscarboxyethylphosphine, alkylated with iodoacetamide, and then digested 20 hours at 37°C. Chromatography was performed on a 1 mm x 250 mm column (VYDAC 238EV51). Mobile phase: A: 2% acetonitrile, 0.05% TFA; mobile phase B: 90% acetonitrile, 0.05% TFA. Gradient: 5 to 65% B in 60 min. Flow Rate: 50 µL/min. Injection volume: 25 µL GFP digest. MS Detector: ABI Q-STAR.

➔ Good resolution of the hydrophobic peptides is observed.

Figs. 9 & 10: Data courtesy of Larry Gross, Research Specialist, Howard Hughes Medical Institute.

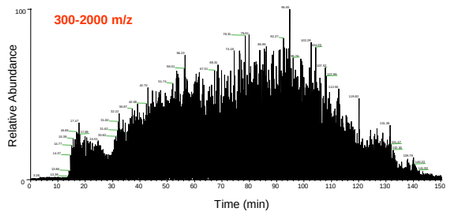
Fig. 10. Mass Spectrum of Tryptic Digest of GFP



T9 is chromophore peptide.
// LPVPWPTLVTTLTYGVQC*FSR //
*carboxyamidomethyl

T9 monoisotopic mass expected: 2414.2
Observed: 2414.3

Figure 11: LC-MS/MS TIC of Tryptic Digest of *R. palustrus* Ribosomes: 300 µm i.d.



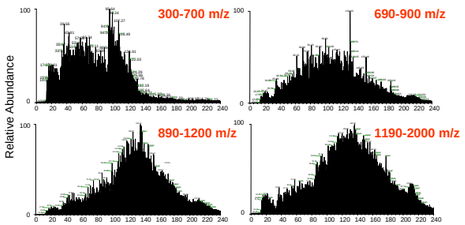
The ribosome prep was performed using 11 g of aerobically grown *R. palustrus*. Ribosomes were purified by sucrose cushion. Ribosomes were extracted with acetic acid, quantitated with BCA assay, and a total of 2 mg of extracted ribosomal proteins was digested with trypsin. The sample was desalted with a C_{18} SepPak and concentrated to ~500 µL. Column: 300 µm x 250 mm (VYDAC 238EV5.325). Mobile phase A: 95% H_2O, 5% acetonitrile, 0.5% formic acid; mobile phase B: 30% H_2O, 70% acetonitrile, 0.5% formic acid. Flow rate: 4 µL/min. HPLC System: LC Packings Ultimate Inert with Famos Autosampler. MS detector: Thermo Finnigan LCQ DECA ion trap equipped with an ESI source.

➔ 94 proteins were identified in a short time using a 1-D LC-MS/MS method.

The LCQ was operated in the data dependent mode, where the top four peaks in every full MS scan were subjected to MS/MS analysis. The dynamic exclusion feature of the Xcalibur software was enabled, with the following settings used: exclusion mass width: +/- 2.5 m/z, repeat count: 1, repeat duration: 0.5 min, exclusion duration: 1.00 min. All data files were searched with the TurboSEQUEST search engine and identifications were based on two or more high-scoring peptides per protein. The output data from the search was first stored in MS Excel for later use and then filtered further and sorted by gene locus number with DTASelect software obtained from John R. Yates III and co-workers at the Scripps Institution. The default settings of DTASelect were used for all searches, which include a minimum cross-correlation (X_{corr}) of 1.8 for +1 peptides, 2.5 for +2 peptides and 3.5 for +3 peptides.

Fig. 11: Data courtesy of Michael B. Strader, Chemical Sciences Division, ORNL.

Figure 12: LC-MS/MS TIC's of Tryptic Digest of Whole Bacterial Proteomes: 300 µm i.d.



The bacterial sample concentrations were as follows:

Organism	Conc. within Mixture
A. thaliana	1.8 mg/mL
S. cerivisae	3.0 mg/mL
R. palustrus	2.5 mg/mL
S. oneidensis	2.4 mg/mL
E. coli	8.0 µg/mL

Overall, each background component of the mixture is ~2.5 mg/mL in concentration, with the target *E. coli* at a dilution strength of 1:250 of the other components. The mixture was added to a glass centrifuge tube, and an appropriate amount of cold (-20°C) acetone was added to the mixture. The mixture was then incubated at -20°C for 30 minutes. After precipitation, the solution was centrifuged (10,000xg, 20 min.) at 4°C. Supernatant acetone was decanted, leaving behind only the proteins in a white, flaky pellet. Two mL guanidine/DTT was added and the sample was heated at 60°C for 1 h. The solution was diluted to 12 mL with Tris buffer. Sequencing grade trypsin was added at 1:50 (200 µg of trypsin). The sample was digested overnight at 37°C. The next day, trypsin was added at 1:100 (100 µg of trypsin). Digestion was run another 5 h at 37°C. DTT was added at 10mM and the solution was shaken at 37°C for 1 h. The sample was centrifuged to remove particulates and desalted with three C_{18} SepPak Plus cartridges. The sample was concentrated to 1 mL in mobile phase A, and then filtered.

➔ A large number (590) of proteins was identified using a 1-D LC-MS/MS method. For each injection, a separate mass range was scanned in order to increase dynamic range.

Fig. 12: Data courtesy of Nathan VerBerkmoes, Chemical Sciences Division, ORNL.

Conclusions

Novel and innovative bonding technology has been described on a 300Å silica-based, C_{18} reversed-phase adsorbent with high carbon coverage. Benefits for analyses of peptides include increase in signal intensity, due to sharper and more symmetric peaks, and improved resolution. The innovative bonding allows for higher recoveries and, hence, the ability to detect more trace level peptides of hydrophilic and hydrophobic nature. This phase performs well with complex proteome samples and can outperform smaller pore phases.

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