EFFICIENT USE OF PEPTIDE MAPPING FOR CHARACTERIZING NATIVE PROTEIN STRUCTURE AND STRUCTURAL VARIANTS

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INTRODUCTION

High resolution chromatography, accurate mass LC-MS, and software tools have been combined to more efficiently correlate peptide maps with protein structure. HPLC is the established first step in deducing structure from these fragments because differences in sequence yield chromatographically separate peaks. The resolution can be enhanced by applying UPLC® separation principles to the peptide mixtures. HPLC and UPLC peptide maps will be compared. The optimized chromatography still requires confirmation of peak identity and purity so it is useful to couple the separation to the exact mass measurements possible with an oa-ToF mass spectrometer. Peptides can be identified based on molecular weight, and coelutions can be detected. This additional information links the chromatographic pattern to the structure of the protein. At the same time the comparison of the HPLC and UPLC separations shows an improvement in the utility of the MS because the peptide spectra are simpler and easier to interpret when more completely resolved samples are introduced into the MS. Complete interpretation of complex LC/MS chromatograms with accurate mass measurement is time-consuming and labor intensive. New specialized software has been developed for these large data sets. The peptide peaks are detected by the Apex3D and Peptide3D algorithms to deconvolute multiply-charged ions and combine isotopes. These algorithms are embedded in Biopharmalynx. Detected MS peaks are matched to the structural features of the proteins with rigorous comparison and search algorithms. The combination of UPLC, oa-Tof MS, and advanced software act synergistically to improve the interpretation of peptide maps.

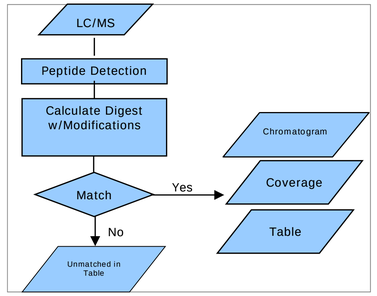


Figure 1. High resolution LC/MS data from protein digests are processed to automatically determine the molecular weights of peptides, match them to sequence and identify modifiers.

METHODS

MATERIALS

Peptides:
MassPREP™ Phosphorylase b Digestion Standard; Waters
[Glu[1]]-Fibrinopeptide B; Sigma

Columns:

HPLC
XBridge™ BEH 300 C_{18} 3.5µm 2.1x250mm Column

UPLC
ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x150mm Column

ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x50mm Column
ACQUITY UPLC™BEH 130 C_{18} 1.7µm 2.1x100mm Column
ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x100mm Column
ACQUITY UPLC™HSS C_{18} 1.8µm 2.1x100mm Column
ACQUITY UPLC™HSS T3 C_{18} 1.8µm 2.1x100mm Column
ACQUITY UPLC™HSS C_{18}SB 1.8µm 2.1x100mm Column

Chemicals:
Hydrogen peroxide; Sigma
Formic Acid; Pierce
Trifluoroacetic Acid; Pierce
Milli Q® water
Acetonitrile; Fisher Optima
Methanol; Fisher HPLC Grade

Instruments
Waters® ACQUITY UPLC® System including:
ACQUITY Binary Solvent Manager
ACQUITY Sample Manager
ACQUITY Column Heater
ACQUITY TUV Detector
Waters® Column Heater Module
Waters® Micromass LCT Premier Mass Spectrometer

Samples

Control:
The native digest is the MassPREP™ Phosphorylase b Digestion Standard. Add 250µL water to 1 vial. Vortex. Final concentration of the digest is 4pmol/µL

Analyte:
Oxidized digest: Add 250µL 0.01% hydrogen peroxide to 1 vial MassPREP™ Phosphorylase b Digestion Standard. Vortex. Incubate at room temperature for 2 hours. Final concentration of the digest is 4pmol/µL.

Lockspray:
Glu-fibrinopeptide 1pmole/µL in 0.1% Formic Acid in 50% water, 50% methanol

Chromatography
Mobile phase A1: 0.02% TFA in Water
Mobile phase A2: 0.1% Formic Acid in Water
Mobile phase B1: 0.018% TFA in Acetonitrile
Mobile phase B2: 0.1% Formic Acid in Acetonitrile
Weak needle wash: 0.2%TFA with 5% Acetonitrile
Strong needle wash: 20% Mobile phase A:80% Mobile phase B
Flow rate 0.2mL/min
Injection: 10µL

RESULTS

Comparing HPLC and UPLC Peptide Maps

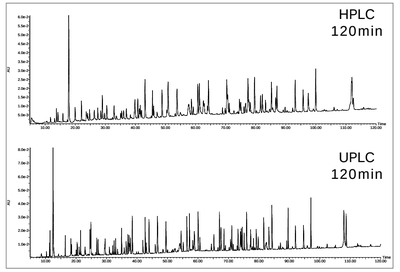


Figure 2. HPLC and UPLC Separations at Constant Gradient Time. Both columns are run from 0-50%B over 180min. The increased number of peaks with UPLC is readily apparent.

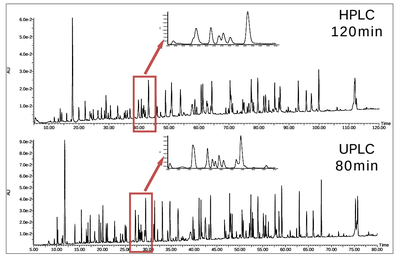


Figure 3. HPLC and UPLC at Constant Gradient Slope. The gradient duration is scaled to column volume (250mm HPLC to 150mm UPLC) so gradient slope is constant. The UPLC run time is 60% that of the HPLC. Constant selectivity is confirmed (inset) with extracted mass chromatograms for the selected region.

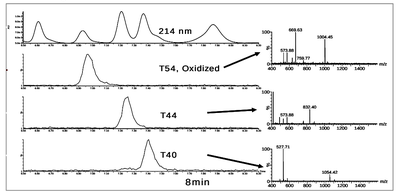


Figure 4. Separation on 2.1x50mm UPLC Column (ACQUITY UPLC™BEH 300 C_{18} 1.7µm); 12.5-16%B in 7.67min at 34°. Scaling to a 50mm column can reduce run time. In the resulting separation, the oxidized T54 peptide is well resolved from the adjacent peaks. The chromatographic peak is sufficiently well-separated for identification and quantitative analysis using UV detection.

Automated Identification and Quantitation of Peaks in UPLC/MS Peptide Maps

A

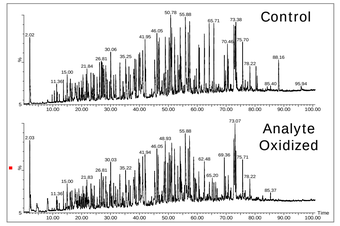
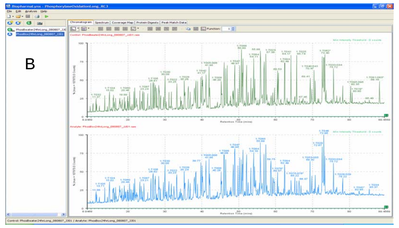


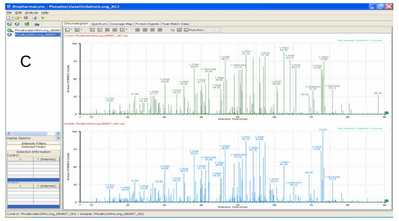
Figure 5. Forty picomoles of the trypsin digest of a 97 kDa protein, phosphorylase b, were analyzed by LC/MS. The analyte sample was treated to forced degradation with peroxide with the goal to determine the oxidation state of the protein before and after treatment. Bioparmalynx software was used to interpret and quantitate the LC/MS data. Panel A shows the Total Ion Chromatograms for the samples. Panel B shows the same TICs in Biopharmalynx annotated with the peptide identifiers. Panel C shows a graphical view of the tabular results. Panel D shows a difference plot in the region where an oxidized peptide elutes. Panel E shows a few columns of the data table including control retention time, mass, and intensity. Panel F shows the region of the peak match table where the versions of T41 are located. In panel G the example peptide, T41, shows oxidation of the control sample was 1.88%. Oxidation of the Analyte sample was 66.26%

Annotated Total Ion Chromatograms

B

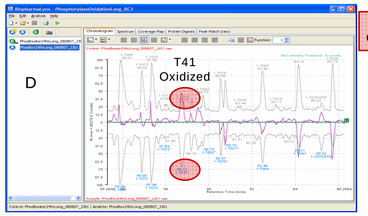


C



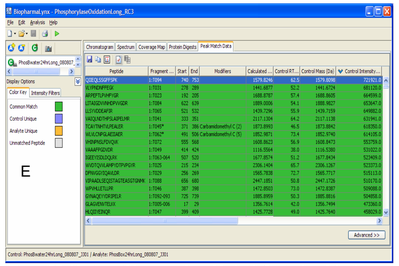
Annotated Difference Plot

D



T41 Oxidized

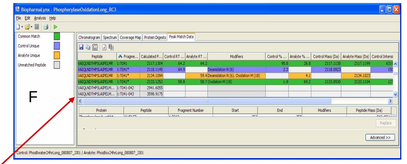
Peak Match Results



E

Figure 5E. Most peptides were found in both Control and Analyte samples (green).

Peak Match Table

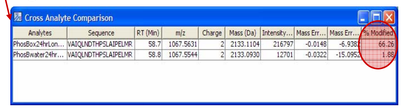


F

Figure 5F. Peptide T41 was found in the oxidized, native, deamidated (control only, blue) and deamidated/oxidized forms (Analyte only, orange) depending on the sample.

Cross Analyte Comparison

G



Cross Analyte Comparison

Analytes	Sequence	RT (Min)	m/z	Charge	Mass (Da)	Intensity ...	Mass Err...	Mass Err...	% Modified
PhosBox2Hr(on...	VAIQLNDTHPSLAIPELMR	58.7	1067.5631	2	2133.1104	216797	-0.0148	-6.936	66.26
PhosBreaker24hr...	VAIQLNDTHPSLAIPELMR	58.8	1067.5544	2	2133.0930	12701	-0.0322	-15.095	1.88

ALTERNATIVE COLUMN CHEMISTRIES FOR UPLC PEPTIDE MAPS

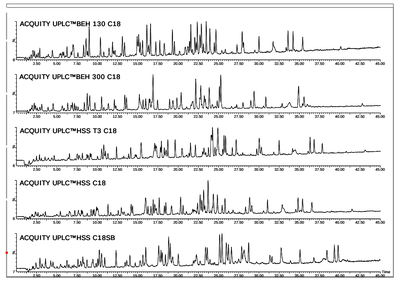


Figure 9. MassPrep Phosphorylase Digestion Standard Separated on 5 UPLC columns. The same sample was separated on 5 different UPLC columns. These packings represent two different base particle materials and four different bonding chemistries for the attachment of the C_{18} bonded phase. In all examples, elution was effected by a gradient from 5% to 50% acetonitrile in the presence of constant 0.1% Formic Acid at a flow rate of 0.2mL/min. The columns all give useful separations of this mixture. The selectivity differences among the materials are really quite obvious. No one column gives the best separation across the whole sample. On the other hand every column gives better resolution than others for some specific peptides. The HSS C18SB column does show more peak tailing than the other columns so it is not a likely first choice in an optimization experiment. The other four columns show good retention and peak shape with formic acid as a mobile phase modifier. They can be used, therefore, to maximize MS sensitivity while optimizing chromatographic quality.

CONCLUSION

- **UPLC® Peptide Mapping can be used to improve resolution, to reduce runtime without sacrificing resolution, and for quantitation of specific structural features.**
- **Developing UPLC maps exploits the usual mechanisms for reversed-phase chromatographic separations.**
- **Exact Mass LC/MS detection with UPLC Peptide Mapping maximizes information.**
- **Software tools provide efficient matching of chromatographic peaks with known structural features of the protein as well as modifications to that structure.**
- **New UPLC reversed-phase columns provide alternate selectivities for optimizing peptide maps**

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