

HIGH THROUGHPUT LC/MSD ANALYSIS OF SIGNATURE PEPTIDES FROM BIOLOGICALLY SIGNIFICANT MARKER PROTEINS

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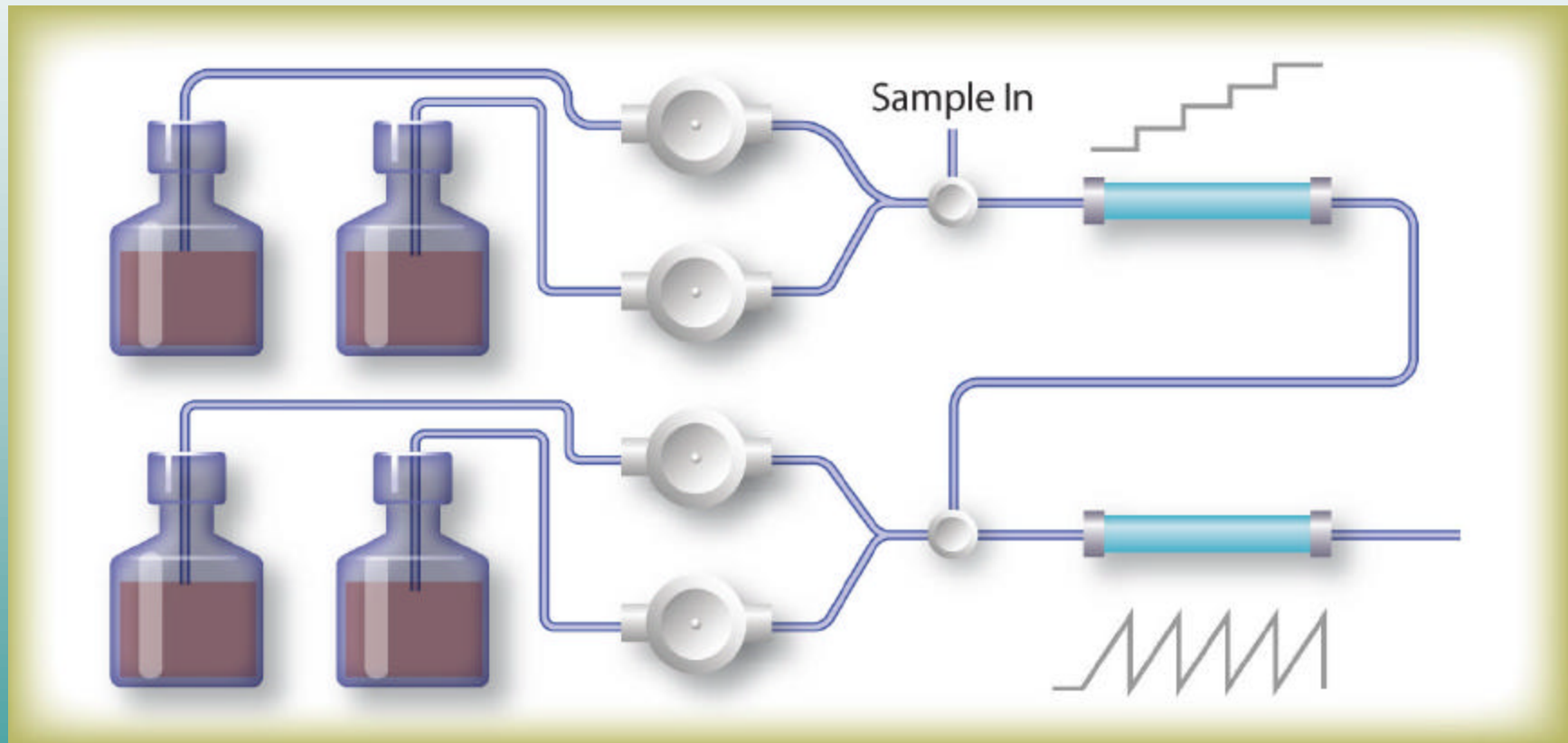
INTRODUCTION

Proteomics and the role that proteins play in the structure and function of biological systems is currently a subject of great interest. Shotgun proteomics is a term that's been used to describe the analytical task of identifying thousands of proteins in whole cell extracts, and many basic researchers are actively working in this area. Functional proteomics is a term that's been used to describe the analytical task of characterizing those proteins which play a biologically significant role in various cell functions, and many applied researchers are looking at specific areas of interest. Once a protein that represent a particular disease state has been identified and characterized, high throughput quantitation of this marker protein becomes an even greater analytical challenge. Marker proteins can be used diagnostically for early disease detection, or therapeutically to monitor the effect of drugs on the treatment of diseases. Using a model protein in urine, this study describes a strategy for high throughput LC/MS analysis of patient samples for marker protein quantitation. Using an automated 2D protein isolation step, in situ digestion, ballistic peptide mapping and ESI-TOF/MSD, 96-384 samples can be analyzed in one day.

EXPERIMENTAL

- **2D Protein HPLC** Michrom Paradigm MS4 or MX4
D1 Column - Protein CF Magic Bullet
D2 Column - Protein SEC Column
- **In-Situ Digestion** Michrom Paradigm MX4
R/A/Trypsin Digest in A/S
- **Peptide LC/MSD** Michrom Paradigm MX4 LC/MSD
Column - 3u 200A Magic C18 Bullet
Detection - UV₂₀₅ and ESI-TOF/MS

2D PROTEIN LC ON PARADIGM MS4 OR MX4



2D LC PROTEIN PURIFICATION

The new Michrom Paradigm MX4 LC/MSD is an integrated LC/MSD system that combines a High Throughput Autosampler (CTC Mini Pal), a Ballistic Multi-Dimensional Separations Module (Michrom Paradigm MS4) and an ESI-TOF Mass Selective Detector (Michrom) with applications kits and software developed for a variety of pharmaceutical and biochemical applications.

The MX4 is well suited for the high throughput needs of marker protein quantitation in diagnostic and therapeutic applications. The first step in this assay is a rapid 2D LC protein purification which isolates the marker protein of interest from the majority of other proteins present in the sample. The modes of HPLC can be varied according to the sample and specific protein of interest, as shown on the slide that follows.

In this study, urine was spiked with a known protein and then purified by chromatofocusing (CF) and size exclusion (SEC) which removed over 95% of the interfering proteins in the sample.

2D PROTEIN LC PURIFICATION MODES

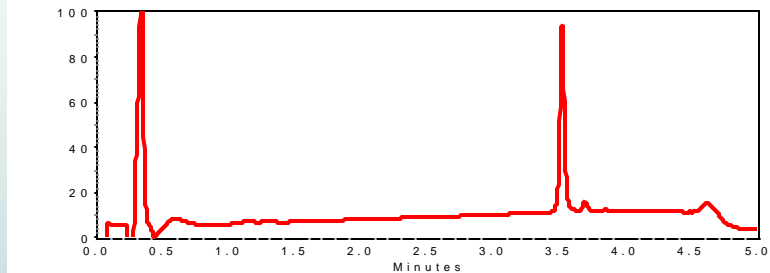
First Dimension (D1)

- Affinity
- Anion Exchange
- Cation Exchange
- **Chromatofocusing**
- HIC
- HILIC
- Reversed Phase
- Size Exclusion

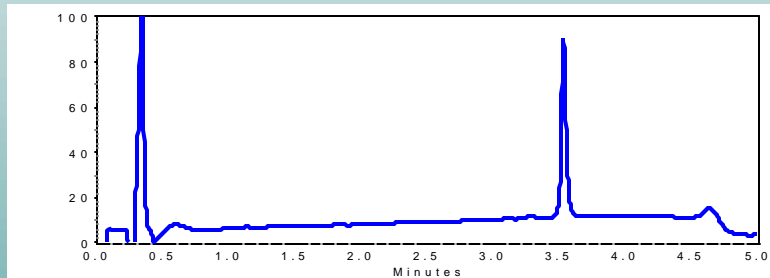
Second Dimension (D2)

- Affinity
- Anion Exchange
- Cation Exchange
- **Chromatofocusing**
- HIC
- HILIC
- Reversed Phase
- **Size Exclusion**

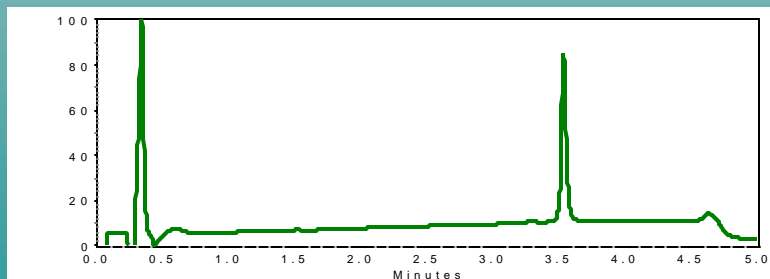
2D LC MARKER PROTEIN PURIFICATION



- **Top Chromatogram (UV) Shows RPLC of Marker Protein Standard Before 2D LC Purification**

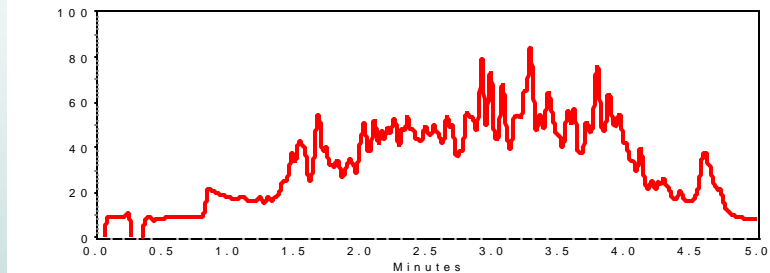


- **Middle Chromatogram (UV) Shows RPLC of Marker Protein After D1 CF LC Purification**

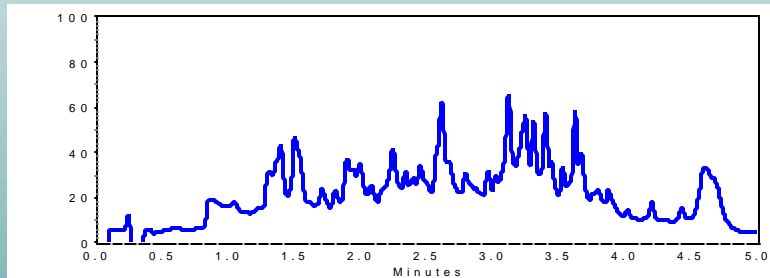


- **Bottom Chromatogram (UV) Shows RPLC of Marker Protein After D2 SEC LC Purification**

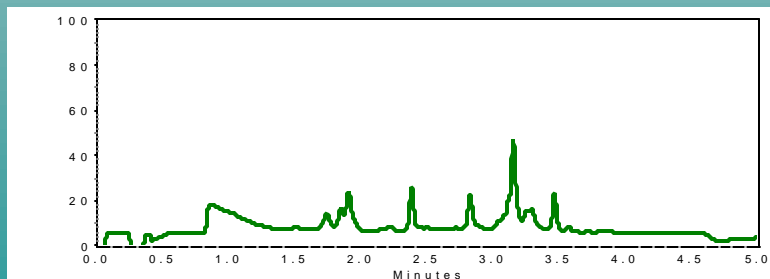
2D LC MARKER PROTEIN PURIFICATION



- **Top Chromatogram (UV) Shows RPLC of Proteins in Urine Before 2D LC Purification**



- **Middle Chromatogram (UV) Shows RPLC of Proteins in Urine After D1 CF LC Purification**



- **Bottom Chromatogram (UV) Shows RPLC of Proteins in Urine After D2 SEC LC Purification**

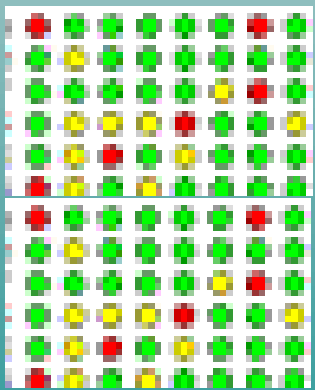
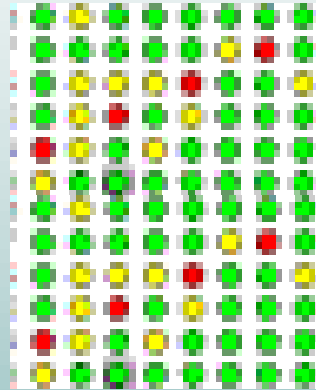
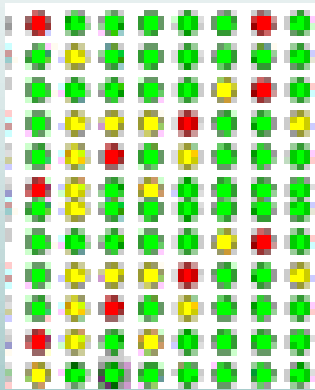
IN-SITU TRYPSIN DIGESTION

Once the samples were purified by rapid 2D LC, the fractions containing the marker protein of interest (collected into a second 96 well microtiter plate on the MX4 autosampler) were reduced, alkylated and digested with trypsin in the autosampler (8 hours). The digested samples were then analyzed by rapid gradient LC/UV/MSD to detect and quantify the signature peptide from the marker protein of interest.

Although this entire assay can be run on a single Paradigm MX4 system, the flexibility and throughput are limited. Since the 2D protein purification, in-situ digestion and RPLC peptide analysis are done serially, the throughput is limited to 96 samples per day.

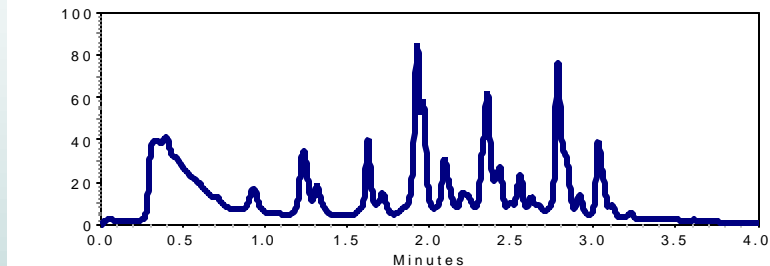
In order to increase the sample throughput and improve the flexibility of this assay, samples were prepared off line using a Paradigm MS4 Multi-Dimensional Separations Module. This allowed 384 (4 x 96) digested samples to be analyzed on the MX4 in a single day (5 minutes per sample).

DISPLAY OF 384 MARKER PROTEIN ASSAYS

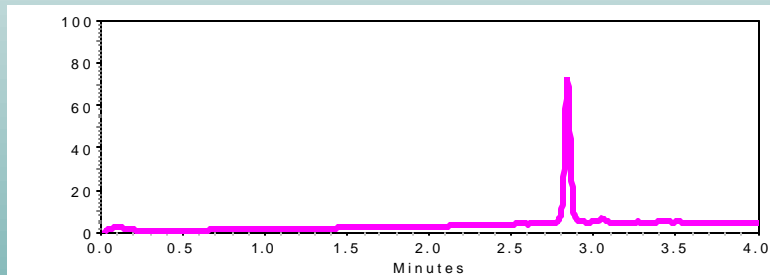


- Green wells represent patient samples that showed normal levels of marker protein
- Yellow wells represent patient samples that showed problems with marker protein determination
- Red wells represent patient samples that showed higher levels of marker protein

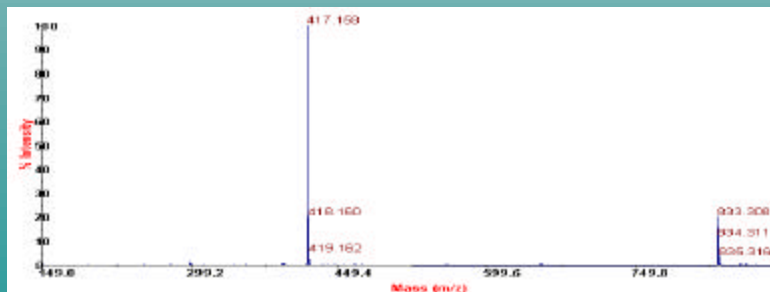
LC-MSD OF MARKER PROTEIN DIGEST



- Upper Trace Shows TIC For Trypsin Digestion of Marker Protein

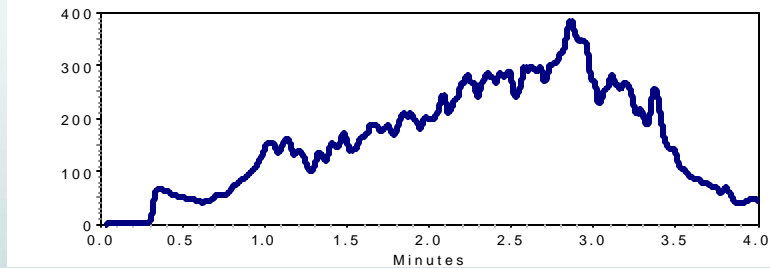


- Lower Trace Shows XIC (m/z 417.2) of Signature Peptide From Marker Protein

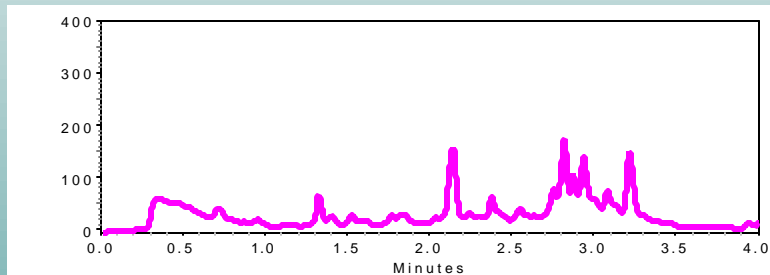


- ESI-TOF Mass Spectra of MW 834.3 Signature Peptide From Marker Protein

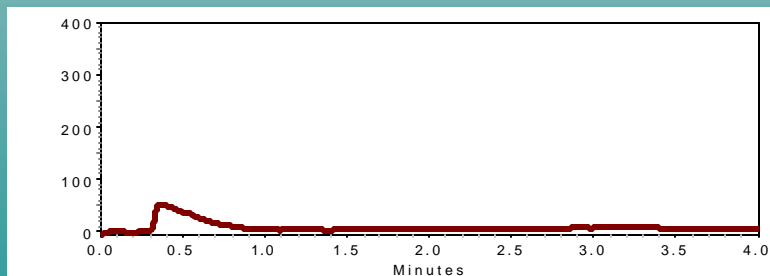
LC-MSD OF URINE PROTEINS DIGEST



- Top Trace Shows TIC For Trypsin Digest of Urine Proteins Before 2D LC

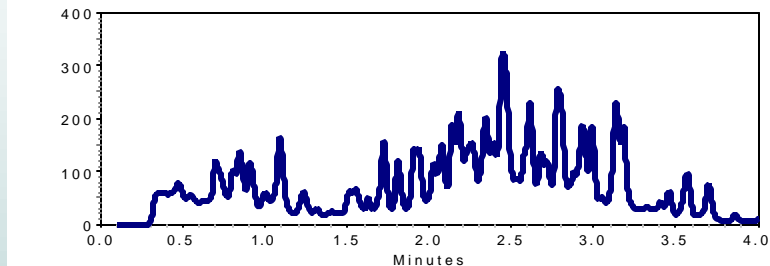


- Middle Trace Shows XIC (m/z 417.2) For Trypsin Digest of Urine Proteins Before 2D LC

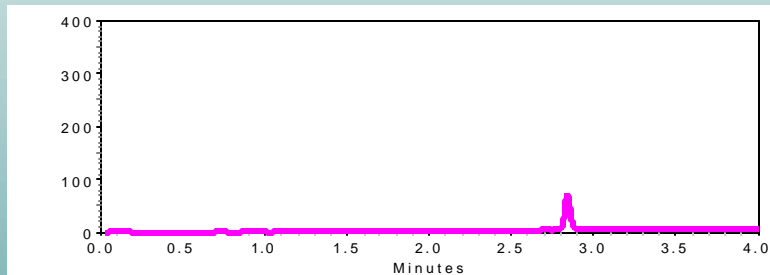


- Bottom Trace Shows TIC For Trypsin Digest of Reagent Blank Before 2D LC

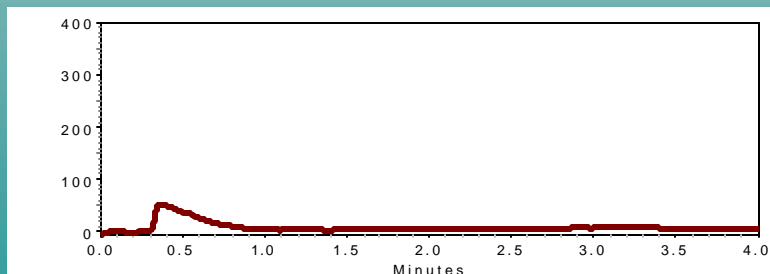
LC-MSD OF MARKER PROTEIN IN URINE



- Top Trace Shows TIC For Trypsin Digest of Urine Proteins After 2D LC



- Middle Trace Shows XIC (m/z 417.2) For Trypsin Digest of Urine Proteins After 2D LC



- Bottom Trace Shows TIC For Trypsin Digest of Reagent Blank After 2D LC

FUTURE PLANS

Some future work in this area will involve looking at other modes of protein HPLC that may be better suited to specific classes of proteins being analyzed as biomarkers. Affinity chromatography can be very selective for known proteins of interest, but protein specific ligands must be prepared for each assay and care must be taken to insure quantitative results. Reversed phase (RP) HPLC offers very high resolution for proteins that behave well, but is not well suited for very large, hydrophobic proteins.

Other protein purification and quantitation techniques such as ICAT will also be investigated to see if they could also be adapted to the MX4 platform for signature peptide quantitation of marker proteins in biological samples.

Once marker proteins are well established as being useful in clinical applications to help monitor the efficacy of new drug candidates, more work will also need to be done to insure that this signature peptide protocol follows the strict criteria for clinical studies.

CONCLUSIONS

- **The Paradigm MX4 is an Ideal Tool For Biomarker Screening**
- **Rapid MDLC With ESI-TOF/MSD Provides High Throughput**
- **2D Protein LC Modes Can be Varied For Specific Samples**
- **Rapid High Resolution XIC-MSD Gives Quantitative Results**
- **Hardware and Software Provides Flexibility For User Needs**