

# COMPARISON OF IMAC, SCX AND WAX FOR MDLC MS/MS ANALYSIS OF PHOSPHOPEPTIDES IN PROTEOME DIGESTS

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# INTRODUCTION

Although protein phosphorylation is an important post translational modification (PTM), researchers who study it are hampered by the fact that it usually occurs on proteins expressed in low abundance in cells, and then only to a small percentage of the protein's total population.

Immobilized Metal Affinity Chromatography (IMAC) is one of the most common methods for trace enrichment of phosphorylated proteins and peptides prior to LC/MS analysis, but IMAC suffers from nonselective binding and variable recovery.

This study looks at Strong Cation Exchange (SCX) and Weak Anion Exchange (WAX) as alternate modes of chromatography and compares them to IMAC for trace enrichment of phosphopeptides in complex proteome digests prior to LC/MS analysis.

# EXPERIMENTAL

**MDLC**

**Michrom Paradigm MS4B**

**100% Metal Ion Free Fluid Path**

**Columns**

**IMAC**

**1x10mm Poros 20 MC**

**WAX**

**1x10mm 5 $\mu$  300A Poly WAX LP**

**SCX**

**1x10mm 5 $\mu$  300A Poly SEA**

**Nanotrap**

**150 $\mu$  x50mm Peptide Nanotrap**

**NanoRP**

**75 $\mu$  x100mm 5 $\mu$  200A Magic C18**

**MS**

**Michrom Nanotrap Platform**

**Thermo LCQ ITMS**

# NEED FOR A METAL ION FREE MDLC-MS

One of the main concerns with the use of IMAC is the fact that phosphopeptides (PP) bind to the metal ions on the IMAC column and the proper conditions must be used to insure complete dissociation of the PP-metal bonds (the strength of the PP-metal bond is a function of the PP and the metal, so it is difficult to find universal IMAC conditions).

A variation of IMAC can also occur in RPLC, if the HPLC system has any metal parts (ie SS) that can leach trace metal ions onto the RPLC column. Since RPLC columns are known to tightly bind metal ions and RPLC elution solvents are totally different than IMAC elution solvents (organic vs. high salt), PP may irreversibly bind to an RPLC column used in a HPLC system with any SS parts.

The Paradigm MS4B MDLC used in this study was designed to be 100% metal ion free, with the fluid path exposed to only teflon, titanium, PEEK, sapphire and ceramic to insure that no losses of PP occurred in the D2 RPLC separations.

# METHODOLOGY

This study investigates a variety of IMAC, SCX and WAX HPLC columns, using a range of different mobile phases to determine the best conditions for trace enrichment.

A mixed casein tryptic digest was used to assess the selectivity and recovery of the various stationary and mobile phases. Three peptides with a single phosphorylation site were identified in our digest as follows:

$\alpha$ -S1 Casein PP-A	VPQLEIVPNpSAEER	MW - 1659
$\alpha$ -S2 Casein PP-B	TVDMESpTEVFTKK	MW - 1594
$\beta$ Casein PP-C	KFQpSEEQQQTEDELQDK	MW - 2189

The optimum phosphopeptide trace enrichment conditions were then coupled to nanoscale RP LC MS/MS to yield an MDLC MS/MS method for direct analysis of phosphopeptides in complex proteome digests.

# RESULTS OF IMAC STUDIES WITH CASEIN

IMAC is a popular technique used by researchers for trace enrichment of PP from complex protein digests, but it has limitations which make it unsuitable for high throughput proteomics. Great care must be taken to choose and load the correct metal ion and optimize the elution conditions to obtain reliable results.

We chose a commercial IMAC column and followed the manufacturers protocol for preparing the column ( $\text{Ga}^{+2}$ ), loading the sample (casein digest), flushing out the unbound peptides and eluting the bound peptides. LC/MS analysis of the bound fraction showed that one of the 3 PP gave good recovery, one gave poor recovery and the third was not recovered. The bound fraction contained several nonphosphorylated peptides, as shown in the next slide.



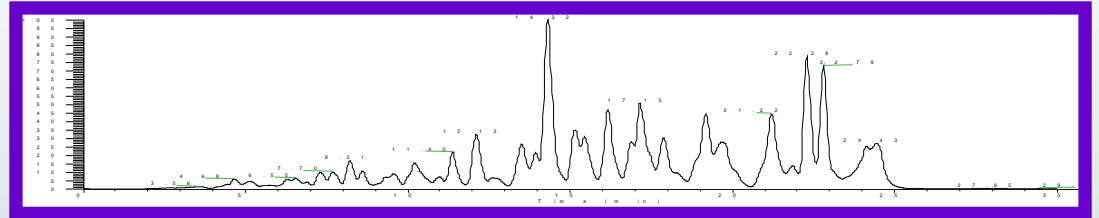
# RESULTS OF SCX STUDIES WITH CASEIN

After analyzing many proteome digests by 2DLC MS/MS, it was noted that any PP present in these samples were usually found in the flow through from the first dimension SCX separation, but not in subsequent SCX steps. This suggested that SCX might be useful for trace enrichment of PP, since most nonphosphorylated peptides (positively charged at low pH) are retained by the SCX column.

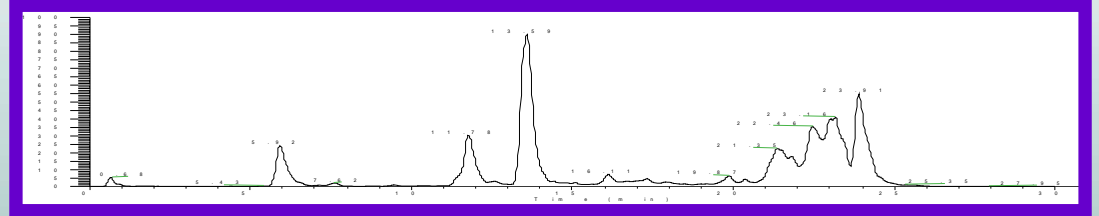
Using Michrom's standard 2D peptide LC MS/MS protocol, a  $\beta$ -casein digest was loaded onto a SCX microtrap column and the unbound peptides were flushed onto a peptide nanotrap and then analyzed by nano RPLC MS/MS. As shown on the slide that follows, only 2 of the three  $\beta$ -casein PP were recovered in the SCX flow through and several non-phosphorylated peptides were also detected.

# RESULTS OF SCX STUDIES WITH CASEIN

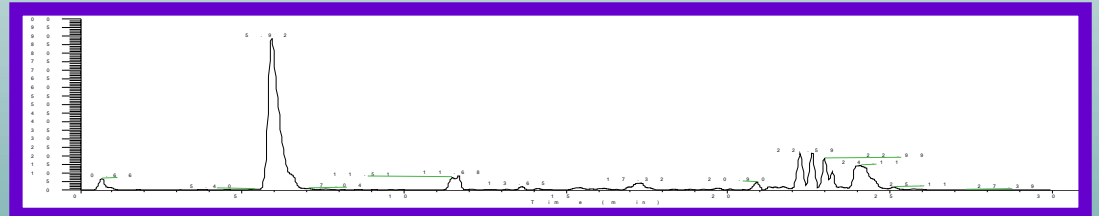
LCMS Base Peak Trace  
Entire Casein Digest



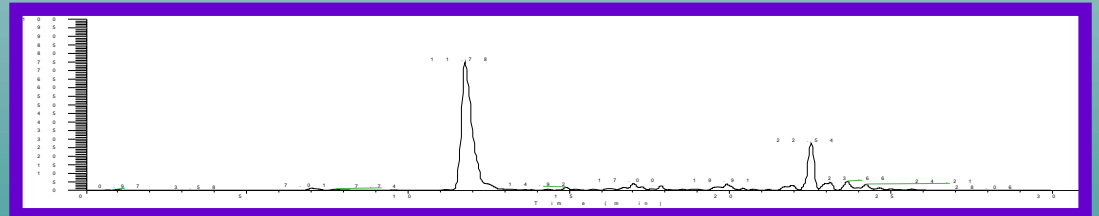
LCMS Base Peak Trace  
SCX Unbound Peptides



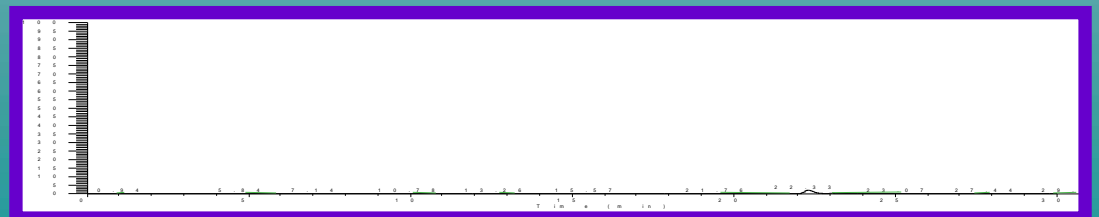
LCMS XIC  $M + 2 = 831$   
SCX Casein PP-A



LCMS XIC  $M + 2 = 798$   
SCX Casein PP-B



LCMS XIC  $M + 2 = 1095$   
SCX Casein PP-C



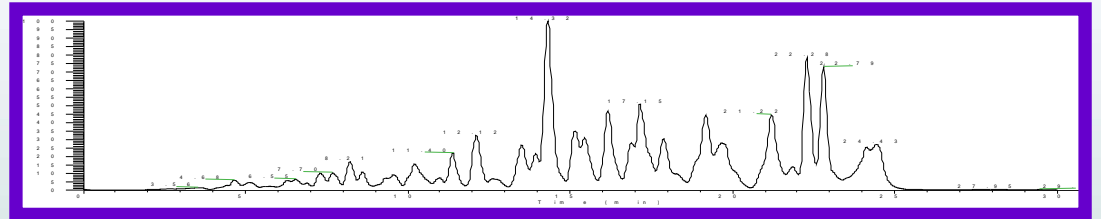
# RESULTS OF WAX STUDIES WITH CASEIN

Since phosphopeptides generally have higher pI's than non-phosphorylated peptides and they are negatively charged at high pH, we decided to also investigate the use of a WAX column to see if it would selectively retain PP in complex proteome samples.

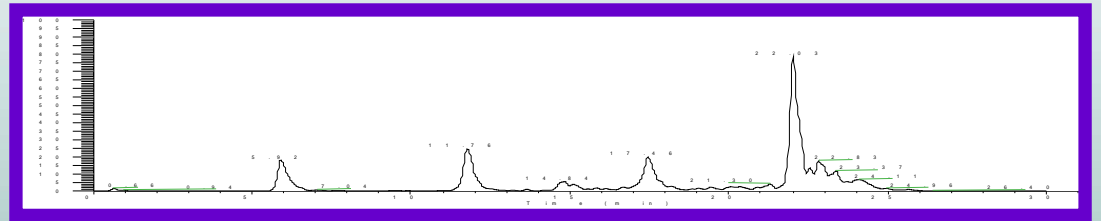
We looked at a variety of WAX materials under a various load/wash/elute conditions (pH, salt, organic, etc) to determine the best possible WAX protocol. Optimum results were obtained with a 5u 300A Poly WAX LP column, loading the sample (casein digest) at pH 6.2, flushing out the unbound peptides and then eluting the bound peptides with 1 M KCl. LC/MS analysis of the bound fraction showed that all 3 PP gave good recovery, and the bound fraction contained only a few nonphosphorylated peptides, as shown in the next slide.

# RESULTS OF WAX STUDIES WITH CASEIN

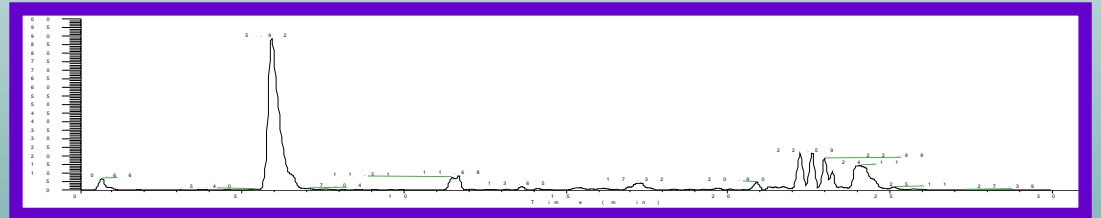
LCMS Base Peak Trace  
Entire Casein Digest



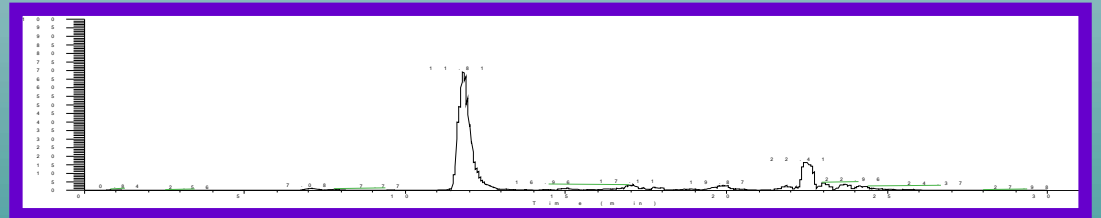
LCMS Base Peak Trace  
WAX Unbound Peptides



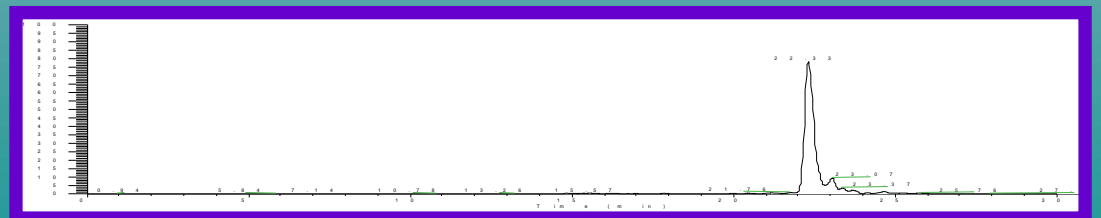
LCMS XIC  $M + 2 = 831$   
WAX Casein PP-A



LCMS XIC  $M + 2 = 798$   
WAX Casein PP-B



LCMS XIC  $M + 2 = 1095$   
WAX Casein PP-C



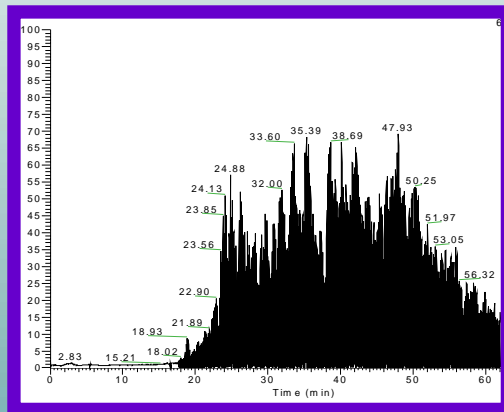
# MDLC MS/MS OF PP IN YEAST PROTEOME

From the data generated for casein in this study, we decided to use WAX as the first dimension of a MDLC system for phosphopeptide analysis in complex proteome samples. Using the same basic configuration as our “Industrial Mudpit” setup, but with a WAX trap in place of the SCX trap, we were able to automate this MDLC process from sample loading through nano RP LC/MS/MS analysis.

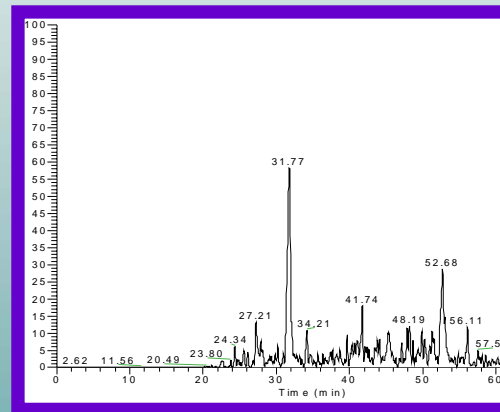
A whole yeast protein digest was used to test this MDLC system, and the results are seen on the slide that follows. A Sequest search of these samples identified a PP (MW 2060) from a phosphorylated yeast protein (Acetyl-CoA Synthetase) in the WAX bound fraction, but this protein was not found in the Sequest search of the whole yeast proteome sample.

# MDLC MS/MS OF PP IN YEAST PROTEOME

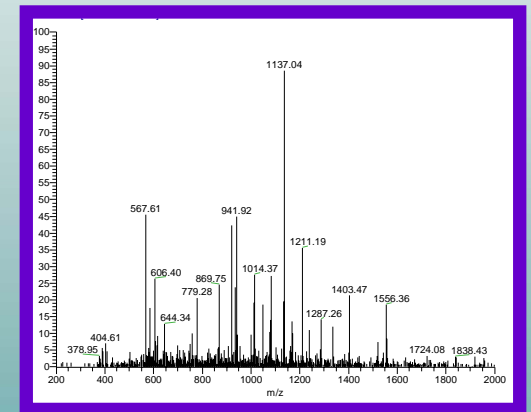
TIC Traces  
60 min Runs



XIC Traces  
M/Z 1031

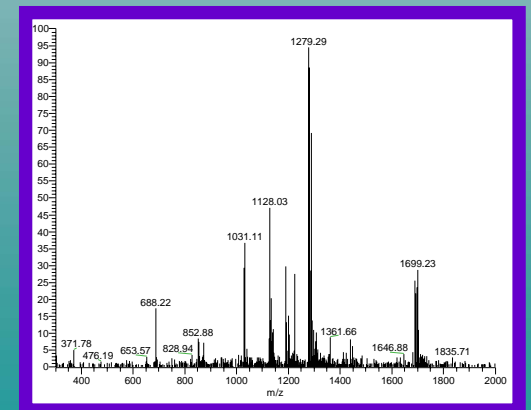
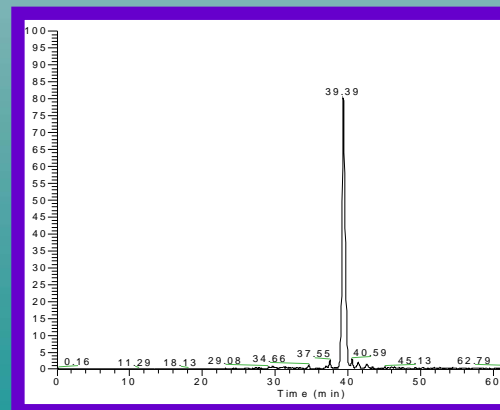
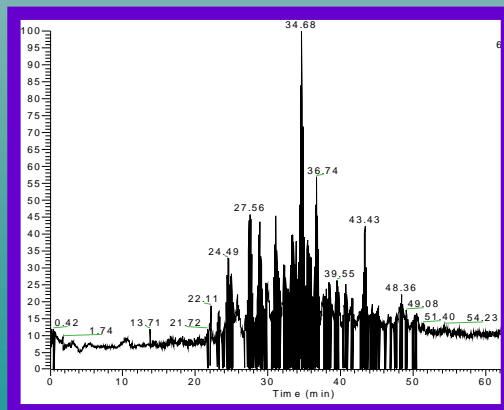


Full Spectra  
At RT 39.4 min



Whole  
Yeast  
Proteome

WAX  
Bound  
Fraction



# SUMMARY

This study has shown that although IMAC, SCX and WAX can be used to selectively retain PP, IMAC and SCX can lead to variable recovery and nonselective binding which may limit their usefulness. WAX appears to be a good alternative to IMAC and SCX for trace enrichment of phosphopeptides in complex proteome samples.

By substituting WAX for SCX in our “Industrial Mudpit” protocol and modifying the load/wash/elute chemistries, an automated robust MDLC system for enrichment of PP in proteomics samples is easily setup.

Future work will include looking at a wide range of PP standards to validate this MDLC method for capacity, recovery, selectivity, reproducibility and sensitivity.

# CONCLUSIONS

- **IMAC Has Several Limitations For Routine PP Analysis**
- **SCX Offers an Alternate to IMAC But is Not PP Selective**
- **WAX Appears to Give Better Results for PP vs IMAC or SCX**
- **WAX/RPLC is a Good MDLC Method for PP Enrichment**
- **Automation of PP MDLC on the Michrom MS4B is Simple**
- **PP Analysis Validates the Need For a Metal Ion Free MDLC**
- **Future Work Will Test Universal Applicability of WAX for PP**

# ACKNOWLEDGEMENTS

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