Quantification of small molecules, peptides and proteins

ABSOLUTE

• Focus - Selected Reaction Monitoring (SRM)/Multiple Reaction Monitoring (MRM) scan Mode as method of quantitation

• External or internal standards

RELATIVE

• mTRAQ reagents
HPLC and Mass Spectrometry (MS) offer SENSITIVE and SPECIFIC methods to study biological processes

• HPLC: Capillary LC (e.g., 75 um ID column) provides sensitivity at the femtomole level for many compounds; also:
  – very small amounts of material are consumed

• MS: Provides specificity due to mass measurement and provides sensitivity for many molecules

⇒ 2D LC MSMS performs 4 dimensional separation.
Q-Trap Multiple Reaction Monitor

Capillary LC column

Q0

Skimmer

Q1

Q2
(Collision cell)

Q3/LIT

EM Detector

Peptide Selection

Fragmentation

Fragment Selection

MRM Signal

Intensity

Time

University of Minnesota
Center for Mass Spectrometry and Proteomics | Phone | (612)625-2280 | (612)625-2279
Experimental Methods

Example 1: Quantification – MRM mode
Small Molecules

Example 2: Quantification – MRM mode
Proteins and Peptides

Example 3: Phosphorylation
Global vs targeted
**Calibration - External Standard Method**

Standards = analyte \( n_{A,i} \) \( i = 1 \ldots 5 \) in \( V_A \)

\[ c_1 = \frac{n_1}{V_A} \quad c_2 = \frac{n_2}{V_A} \ldots \]

Unknown Sample = analyte \( n_U \) in \( V_A \)

Analysis

\( R_1, R_2, R_3 \ldots R_6 \)

Plot

\[ R_A = k \cdot n_A + S_{bl} \]

\[ y = mx + b \]

Analysis

Measure \( R_U \)

Response, \( R_{A,i} \)

Amount, \( n_{A,i} \)

5/11/2008
FIGURE 1. The secondary structure of *E. coli* tRNALys(UUU) (a) and the chemical structure of t6A (b).
Selected Reaction Monitoring in a Triple Quadrupole

- **Q1 (413)**
- **Q2 collision cell**
- **Q3 (280.9)**

Set on mass of precursor ion (T6A)

Fragment precursor ion

Transmit only diagnostic product ion

All fixed ➔ Very Selective Scan

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Experimental Design

Bulk purification of tRNA
Enzymatically hydrolysed to nucleotides
Purified by running zip-tip c18
LC on C18 RP in presence of ammonium acetate
MRM analysis

T6A  413> 280.9 and 136
Adenosine 268>136

Ratio of T6A to Adenosine was determined
XIC of +MRM (4 pairs): 267.000/136.000 Da ID: adenosine_136 from Sample 22 (jixxx002_leixx039_11855_20120620_100000fm) of jix...
Max. 3090.0 cps.

XIC of +MRM (4 pairs): 413.000/281.000 Da ID: T6A_281 from Sample 22 (jixxx002_leixx039_11855_20120620_100000fm) of jixxx002...
Max. 1.3e4 cps.

XIC of +MRM (4 pairs): 268.000/136.000 Da ID: adenosine_136 from Sample 22 (jixxx002_leixx039_11855_20120620_100000fm) of jix...
Max. 130.0 cps.
XIC of +MRM (4 pairs): 267.000/136.000 Da ID: adenosine_136 from Sample 17 (jixxx002_leixx039_11855_20120620_10000fm) of jixx...

Max. 100.0 cps.

XIC of +MRM (4 pairs): 413.000/136.000 Da ID: T6A_136 from Sample 17 (jixxx002_leixx039_11855_20120620_10000fm) of jixx002...

Max. 1060.0 cps.

XIC of +MRM (4 pairs): 268.000/136.000 Da ID: adenosine_136 from Sample 17 (jixxx002_leixx039_11855_20120620_10000fm) of jix...

Max. 2810.0 cps.

XIC of +MRM (4 pairs): 413.000/281.000 Da ID: T6A_281 from Sample 17 (jixxx002_leixx039_11855_20120620_10000fm) of jixxx002...

Max. 1350.0 cps.
Calibration for T6A_281: $y = 0.51544x + 186.90287$ ($r = 0.99996$) (weighting: None)
Wild-type

XIC of +MRM (4 pairs): 267.000/136.000 Da ID: adenosine_136 from Sample 3 (jixxx002_leixx039_11855_20120620_W29) of jixxx002...
Max. 1690.0 cps.

XIC of +MRM (4 pairs): 413.000/281.000 Da ID: T6A_281 from Sample 3 (jixxx002_leixx039_11855_20120620_W29) of jixxx002_leixx0...
Max. 1.8e5 cps.

XIC of +MRM (4 pairs): 413.000/136.000 Da ID: T6A_136 from Sample 3 (jixxx002_leixx039_11855_20120620_W29) of jixxx002_leixx0...
Max. 1.2e5 cps.

XIC of +MRM (4 pairs): 268.000/136.000 Da ID: adenosine_136 from Sample 3 (jixxx002_leixx039_11855_20120620_W29) of jixxx002_...
Max. 8.3e6 cps.
Y - uninduced

XIC of +MRM (4 pairs): 267.000/136.000 Da ID: adenosine_136 from Sample 27 (jixxx002_leixx039_11855_20120620_Y) of jixxx002_leixx039...
Max. 1010.0 cps.

XIC of +MRM (4 pairs): 413.000/136.000 Da ID: T6A_136 from Sample 27 (jixxx002_leixx039_11855_20120620_Y) of jixxx002_leixx039...
Max. 1.2e4 cps.

XIC of +MRM (4 pairs): 413.000/281.000 Da ID: T6A_281 from Sample 27 (jixxx002_leixx039_11855_20120620_Y) of jixxx002_leixx039...
Max. 1.8e4 cps.

XIC of +MRM (4 pairs): 268.000/136.000 Da ID: adenosine_136 from Sample 27 (jixxx002_leixx039_11855_20120620_Y) of jixxx002_leixx039...
Max. 6.5e6 cps.
Y+IPTG

XIC of +MRM (4 pairs): 267.000/136.000 Da ID: adenosine_136 from Sample 31 (jixxx002_leixx039_11855_20120620_Yiptg) of jixxx0...
Max. 870.0 cps.

XIC of +MRM (4 pairs): 413.000/281.000 Da ID: T6A_281 from Sample 31 (jixxx002_leixx039_11855_20120620_Yiptg) of jixxx002_leix...
Max. 1.0e5 cps.

XIC of +MRM (4 pairs): 413.000/136.000 Da ID: T6A_136 from Sample 31 (jixxx002_leixx039_11855_20120620_Yiptg) of jixxx002_leix...
Max. 6.9e4 cps.

XIC of +MRM (4 pairs): 268.000/136.000 Da ID: adenosine_136 from Sample 31 (jixxx002_leixx039_11855_20120620_Yiptg) of jixxx0...
Max. 6.6e6 cps.

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Determined that the T6A had increased when a target gene was induced with an IPTG inducible promoter.

Used ratio of an unlabeled internal standard housekeeping small molecule with similar characteristics as the target molecule.

Separated by LC
Internal Standard (IS) Method

Standards = analyte \( n_{A,i} \) in \( V_A \)

Unknown Sample = analyte \( n_U \) in matrix \( V_A \)

\[
\begin{align*}
C_1 &= \frac{n_1}{V_A} \\
C_2 &= \frac{n_2}{V_A} \\
C_{1,f} &= \frac{n_1}{V_A + v_{IS}} \\
C_{IS,f} &= \frac{n_{IS}}{V_A + v_{IS}} = \text{constant}
\end{align*}
\]

Internal Standard = IS \( n_{IS} \) in solvent \( V_{IS} \)

\[
\begin{align*}
C_{IS} &= \frac{n_{IS}}{V_{IS}} \\
C_{IS,f} &= \frac{n_{IS}}{V_A + v_{IS}} = \text{constant}
\end{align*}
\]

Analysis

Sample Work-up

\[
\begin{align*}
C_U &= \frac{n_U}{V_A} \\
C_{U,f} &= \frac{n_U}{V_A + v_{IS}}
\end{align*}
\]

5/11/2008
Multiple Reaction Monitoring in QTRAP

Q1 Precursor mass
Q2 collision cell
Q3 product ions

Capillary LC column
Transmit only precursor m/z Selected to Q2
CE optimized to produce diagnostic ions
only diagnostic product ions of acrylamide

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Glucose + Asparagine → H₂O

Acrylamide
Acrylamide Experimental design

Extract acrylamide from french fries

Spike with stable isotope

Process samples with SPE column

LCMSMS on product ions
72>55 and 75>58
Acrylamide
Standard Curve
ratio of H/L

Calibration for acryl72/55: \( y = 1.04935x - 0.00474 \) (weighting: None)
### Acrylamide Spread Sheet

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Conc</th>
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<th>Area Ratio</th>
<th>Calc Conc</th>
<th>Accuracy</th>
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ProteinPilot interpretation of MS/MS data

Fragmentation Evidence

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Precursor m/z 724.3

MS/MS data from captured peptide
BSA MRM/SRM spectrum

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<th>Dwell Time</th>
<th>ID</th>
<th>CE</th>
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<th>Dwell Time</th>
<th>ID</th>
<th>CE</th>
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<td>AEFVEVTK</td>
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</table>
RELATIVE QUANTITATION
mTRAQ Reagents
Duplex or triplex mass difference tags (Non-Isobaric Amine Tags)

Figure 1: Chemical Structure of mTRAQ® Reagents

Applied Biosystems product description
Obtain robust performance with Global Internal Standard (GIS) methodology. The GIS is prepared by labeling pooled samples with mTRAQ® Reagents \( \Delta 8 \). This GIS is then spiked into individual sample labeled with either mTRAQ® Reagents \( \Delta 0 \) or mTRAQ® Reagents \( \Delta 4 \) before MRM analysis.
Protein Characterization by Mass Spectrometry

Post Translational Modifications

• Protein Identification

Attributes that define protein states:
• Covalent modifications
• Cellular localizations
• Presence of ligands
• Alternate splicing
• Proteolytic cleavage
• Oligomeric state
• Protein conformation
## Select Post Translational Modifications

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Phosphorylated Protein Detection by Mass Spectrometry

Challenges
• Low abundance of modified protein (relative to unmodified or other proteins)
• MS signal suppression
• Ionization efficiency
• Data analysis inefficiency (software limitations)
• Phosphate group is labile under mass spectrometric conditions

Methods for success
• **Enrichment**
• Variation of proteolytic enzymes
• Both ESI and MALDI MS
• Multiple software packages and manual interpretation
ENRICHMENT for PHOSPHOPEPTIDES May be Crucial to Success

**Dual enrichment method**
1. SCX separation: at pH 2.7, phosphopeptides elute early
2. Immobilized Metal Affinity Chromatography (IMAC)

**IDENTIFICATION of 1000’s of phosphopeptides/proteins**

Villén J & Gygi SP 2008, Nature Protocols 3(10), 1630

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Crude Peptides

Phosphopeptide
Peptides Zip Tipped C18

Phosphopeptide

m/z

a.i.

5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
1300
1800
2300

1046.44
1296.49
1108.25
1174.24
1506.43
2061.58
2464.94

Sudha Marimanikkuppam (University of Minnesota)
Peptides Titania Tip

Phosphopeptide

Sudha Marimanikkuppam (University of Minnesota)
Phosphopeptide ID and Confidence in Site Localization

- Identification of phosphopeptides and localization of the phosphorylation sites is challenging
- Phosphate often dissociates from peptide and amino acid localization information is gone (neutral loss of phosphoric acid)
- Measures of confidence in phosphosite localization are not currently well-defined
### Predictable Mass Shifts for Phosphorylated Fragments

MQIFVK<$\text{Tp}$>LTK ($+80 = \text{HPO}_3$); $M_{\text{W, mono}} = 1288.67$

<table>
<thead>
<tr>
<th>$m/z$</th>
<th>b series</th>
<th>y series</th>
<th>$m/z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1142.6</td>
<td>MQIFVK&lt;$\text{Tp}$&gt;LT</td>
<td>K</td>
<td>147.1</td>
</tr>
<tr>
<td>1041.5</td>
<td>MQIFVK&lt;$\text{Tp}$&gt;L</td>
<td>TK</td>
<td>248.1</td>
</tr>
<tr>
<td>928.4</td>
<td>MQIFVK&lt;$\text{Tp}$&gt;</td>
<td>LTK</td>
<td>361.3</td>
</tr>
<tr>
<td>747.4</td>
<td>MQIFVK</td>
<td>$\text{Tp}$&lt;sup&gt;L&lt;/sup&gt;TK</td>
<td>542.3</td>
</tr>
<tr>
<td>619.3</td>
<td>MQIFV</td>
<td>$\text{K}$&lt;sup&gt;Tp&lt;/sup&gt;LTK</td>
<td>670.4</td>
</tr>
<tr>
<td>520.3</td>
<td>MQIF</td>
<td>$\text{V}$&lt;sup&gt;K&lt;/sup&gt;T&lt;sup&gt;Tp&lt;/sup&gt;LTK</td>
<td>769.4</td>
</tr>
<tr>
<td>373.2</td>
<td>MQI</td>
<td>$\text{F}$&lt;sup&gt;V&lt;/sup&gt;K&lt;sup&gt;Tp&lt;/sup&gt;LTK</td>
<td>919.5</td>
</tr>
<tr>
<td>260.1</td>
<td>MQ</td>
<td>$\text{I}$&lt;sup&gt;F&lt;/sup&gt;V&lt;sup&gt;K&lt;/sup&gt;T&lt;sup&gt;Tp&lt;/sup&gt;LTK</td>
<td>1029.6</td>
</tr>
<tr>
<td>132.0</td>
<td>M</td>
<td>$\text{Q}$&lt;sup&gt;I&lt;/sup&gt;IFV&lt;sup&gt;K&lt;/sup&gt;T&lt;sup&gt;Tp&lt;/sup&gt;LTK</td>
<td>1157.6</td>
</tr>
</tbody>
</table>

Mass shifts will occur only in fragments containing the **phos-**Thr (bold), therefore location of MODIFICATION can be pinpointed.
Three Fragmentation Methods Available
1. CID = collision induced dissociation (linear ion trap)
2. HCD = higher energy collision induced dissociation (multipole)
3. ETD = electron transfer dissociation

**Figure 1-2.** Schematic view of the LTQ Orbitrap Velos

CID in LIT (linear ion trap)

HCD in an octapole
#1 AEFAEVsK

CID

HCD
#2 LVNEVtEFAK

CID

HCD
#6 DIslSDyK

CID

HCD
Phosphorylation of Serine, Threonine and Tyrosine

- Amino acid delta mass = 79.9663 monoisotopic
- Neutral loss (S) = 97.9769 monoisotopic

PhosphoSerine: $\text{C}_3\text{H}_6\text{NPO}_3$

PhosphoThreonine: $\text{C}_4\text{H}_8\text{NPO}_3$

PhosphoTyrosine: $\text{C}_9\text{H}_{10}\text{NPO}_3$

DehydroAlanine: $\text{C}_3\text{H}_2\text{NO}$
Special Survey Scans

1. Neutral Loss Scan (Positive mode)
   utilize loss of phosphoric acid (49 m/z for +2 ions)

2. Precursor Ion Scan (Negative mode)
   utilize loss of $PO_3^-$
Targeted - Dephosphorylation

Phosphoserine
\[ \text{C}_3\text{H}_6\text{NPO}_5 (166.998) \]

Serine
\[ \text{C}_3\text{H}_5\text{NO}_2 (87.032) \]

Dehydroalanine
\[ \text{C}_3\text{H}_3\text{NO} (69.013) \]

- \( \text{HPO}_3 (80.0) \)
- \( \text{H}_3\text{PO}_4 (98.0) \) (\( \beta \)-elimination)
Neutral loss scan in QTRAP

Q1 Mass selection

Q2 collision cell

Q3 Scans & Isolates

Capillary LC column

Ion A+ H₃PO₄
Ion B
Ion C
Ion D
Ion E

A+ 49

A+ MS³

Scans across mass

Fragment ions one at a time

Mass difference is fixed

LDIFpSDFGGLK
Spiked phosphopeptide identified @ 19 min by neutral loss scan, AND subsequently sequenced by product ion scan.

Chu et al., ASMS 2002
Precursor ion Scan with Polarity Switching

-Prec(79) scan

Enhanced Resolution Scan

FQpSEEQQQTEDELQDK
Precursor Ion Scan in a QTrap

Q1 Mass selection: Capillary LC column
- Ion A + H₃PO₄
- Ion B
- Ion C
- Ion D
- Ion E
- Ion F

Q2 collision cell: Scans across mass range
- Fragment ions one at a time

Q3 pass only 79: Transmits only 79 triggers acquisition of all product ions from precursor that lost 79

Negative mode scan with positive mode for MS/MS!
Precursor ion Scan with Polarity Switching

-Prec(79) scan

Enhanced Resolution Scan

FQpSEEQQQTEDELQDK
MS/MS on ion 1031.3 for sequence determination
Lysine PTM’s: Methylation and Acetylation

• 2 most common PTM’s of lysine in histones and transcription-related proteins
• Me and Acetyl status are critical for studies of transcriptional regulation, gene control and cancer therapy
• Peptide hydrophobicity and steric hindrance increases
• Stable modification
Lysine PTM’s

Zhang K et al, 2004
Proteomics, 4, 1 – 10
# Lysine Modifications

<table>
<thead>
<tr>
<th>Δ *</th>
<th>PTM</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.01565</td>
<td>methyl</td>
<td>H(2) C</td>
</tr>
<tr>
<td>28.0313</td>
<td>dimethyl</td>
<td>H(4) C(2)</td>
</tr>
<tr>
<td>42.04695</td>
<td>trimethyl</td>
<td>H(6) C(3)</td>
</tr>
<tr>
<td>42.010565</td>
<td>acetyl</td>
<td>H(2) C(2) O</td>
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</tbody>
</table>

* Monoisotopic delta mass

**Use of High Resolution Mass Spectrometry for PTM Discrimination, example:**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MH+1 (mono)</th>
<th>Δ mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>KQLATK(Acetyl)AAR</td>
<td>1028.6211</td>
<td>0.0364 Da, 35 ppm</td>
</tr>
<tr>
<td>KQLATK(Trimethyl)AAR</td>
<td>1028.6575</td>
<td></td>
</tr>
</tbody>
</table>
Strategies for PTM ID/localization

• Signature low mass fragment ions
• Test search program with std peptides
• Precursor Ion Scan (Me-R)
• Neutral Loss Scan (Me-R)
• Heavy methyl SILAC
Amino Acid

Immonium Ion

\[ R + \text{NH}_2=\text{CH} \]

‘Low Mass Marker’ Ions

Zhang K et al, 2004 Proteomics, 4, 1 – 10
Diagnostic Low Mass Marker Ions in MS/MS Spectra Used for Peptide Sequence Interpretation

<table>
<thead>
<tr>
<th>Residue</th>
<th>3-letter code</th>
<th>1-letter code</th>
<th>Immonium ion*</th>
<th>Related ions*</th>
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</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>129</td>
<td>59,70,73,87,100,112</td>
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<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
<td>87</td>
<td>70</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
<td>88</td>
<td>70</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
<td>102</td>
<td></td>
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<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
<td>101</td>
<td>56,84,129</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
<td>30</td>
<td></td>
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<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
<td>110</td>
<td>82,121,123,138,166</td>
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<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
<td>86</td>
<td>44,72</td>
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<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
<td>86</td>
<td>44,72</td>
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<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
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<td>70,84,112,129</td>
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<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
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<td>Phenylalanine</td>
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<td>Proline</td>
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<td>P</td>
<td>70</td>
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<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
<td>60</td>
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<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
<td>74</td>
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<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>159</td>
<td>77,117,130,132,170,171</td>
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<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>136</td>
<td>91,107</td>
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<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>72</td>
<td>41,55,69</td>
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</tbody>
</table>

Immonium and related ion masses after Falick, 1993 #690 and Papayannopoulos, 1995 #681. * Bold face indicates strong signals, italic indicates weak.
Low Mass Marker Ions from Side Change Rearrangements or Elimination Reactions of Mono- Di- and Tri-Methyl Lysines and Acetyl Lysine

- LYSINE
- Me-LYSINE
- 2Me-LYSINE
- 3Me-LYSINE
- acetyl-LYSINE

- $m/z$ 84
- $m/z$ 84 and 98
- $m/z$ 84
- $m/z$ 84
- $m/z$ 84 and 126
sPRG Std PTM Modified Peptide Methylation and Acetylation Detection by CMSP Velos Orbitrap

Database searches
ProteinPilot
Sequest/Scaffold (limited var mods)
<table>
<thead>
<tr>
<th>Residue</th>
<th>b</th>
<th>b+2</th>
<th>y</th>
<th>y+2</th>
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<tbody>
<tr>
<td>F</td>
<td>148.0757</td>
<td>74.5415</td>
<td>1294.7042</td>
<td>647.8557</td>
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<tr>
<td>P</td>
<td>245.1285</td>
<td>123.0679</td>
<td>1147.6358</td>
<td>574.3216</td>
</tr>
<tr>
<td>K[3Me]</td>
<td>415.2704</td>
<td>208.1388</td>
<td>1050.5830</td>
<td>525.7951</td>
</tr>
<tr>
<td>A</td>
<td>486.3075</td>
<td>243.6574</td>
<td>880.4411</td>
<td>440.7242</td>
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<tr>
<td>E</td>
<td>615.3501</td>
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<tr>
<td>V</td>
<td>1061.5666</td>
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<td>167.1103</td>
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<tr>
<td>S</td>
<td>1148.5986</td>
<td>574.8030</td>
<td>234.1448</td>
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<td>K</td>
<td>1276.6936</td>
<td>638.8504</td>
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<td>74.0600</td>
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Correct
higgi022_091711_sPRG_purepepHCD_40_01 #3354  RT: 33.82  AV: 1  NL: 3.46E3
T: FTMS + c NSI d Full ms2 432.24@hcd40.00 [111.00-1310.00]

FPK$_{3Me}$AEFAEVSK

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