APPLICATIONS OF MASS SPECTROMETRY IN A BIOLOGICS REGULATORY ORGANIZATION

Terry Cyr, Daryl Smith, Marybeth Creskey, Lisa Walrond, Yves Aubin, Genevieve Gingras, Michael Johnston, Michel Girard, Aaron Farnsworth, Sean Li

Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Products and Foods Branch, Health Canada, Ottawa, Ontario

terry.cyr@hc-sc.gc.ca  613 957-1068
Overview:

non-prescriptive approach - potential advantages

- Why mass spectrometry
- Why electrospray
- Why are you so picky
- Why, Why, Why?
Biological products

- Primary sequence
- Tertiary structure
- Post translational modifications
- Degradation products
- Host cell proteins
- Bioavailability
- Potency
Vaccine components

Influenza proteins -
three or four strains 15 μg hemagglutinin/0.5mL ea
A(H1N1)   A(H3N2)   B for 2013/14
  ● A/California/7/2009 (H1N1)
  ● A/Texas/50/2012 (H3N2)
  ● B/Massachusetts/2/2012 (Yamagata lineage)
  ● B/Brisbane/60/2008 (Victoria lineage)

Clinically relevant non-medicinal ingredients:
  - egg proteins (for most products)
  - trace: antibiotic, detergent
Current Methods

● Comparison of vaccine to reference antigen by SRID.

  Dependent on:
  – Quality of reference antigen
  – Reliability of reference antigen quantification
  – Reliability of SRID quantification

Limitations:
  – No identification or quantification of NA
  – Timeframe for generation of standards and antisera

● Method for host cell proteins - ovalbumin
Desired method attributes

- Analysis with low potential for altering the product – differential losses, oxidation, hydrolysis...
- Rapid with high reproducibility
- Discriminate between products and strains
- Able to analyze formulated product – 4 strains
Influenza strain identification

Hemagglutinin (HA)

Egg proteins
Increased instrument resolution and sensitivity.

Increased peptide IDs, ~50% sequence coverage

Hundreds of ambiguous IDs
### Similar Proteins

<table>
<thead>
<tr>
<th>Sequence Coverage</th>
<th>Protein</th>
<th>Accession</th>
<th>Prob</th>
<th>%Spec</th>
<th>#Pep</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Dunedin/2/20...</td>
<td>gi</td>
<td>109675818</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Wellington/18/...</td>
<td>gi</td>
<td>110733667</td>
<td>100%</td>
<td>3.3%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Wellington/10/...</td>
<td>gi</td>
<td>112788642</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Vaikato/13/2...</td>
<td>gi</td>
<td>112796999</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Vaikato/14/2...</td>
<td>gi</td>
<td>112791691</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Wellington/14/...</td>
<td>gi</td>
<td>115344566</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Michigan/120/...</td>
<td>gi</td>
<td>115371365</td>
<td>100%</td>
<td>3.3%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Michigan/5/20...</td>
<td>gi</td>
<td>115371375</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Vaikato/11/2...</td>
<td>gi</td>
<td>115521484</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/South Austral...</td>
<td>gi</td>
<td>115607765</td>
<td>100%</td>
<td>3.3%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/South Austral...</td>
<td>gi</td>
<td>115607784</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New South W...</td>
<td>gi</td>
<td>117571148</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Vaikato/4/20...</td>
<td>gi</td>
<td>117572393</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Vaikato/17/2...</td>
<td>gi</td>
<td>131052778</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Western Aust...</td>
<td>gi</td>
<td>133752844</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Wellington/6/2...</td>
<td>gi</td>
<td>133981721</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Florida/08/20...</td>
<td>gi</td>
<td>158123374</td>
<td>100%</td>
<td>3.3%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Minnesota/17/...</td>
<td>gi</td>
<td>158123382</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New Mexico/...</td>
<td>gi</td>
<td>156123384</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/North Carolin...</td>
<td>gi</td>
<td>156123386</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Auckland/584...</td>
<td>gi</td>
<td>156536299</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/Auckland/597...</td>
<td>gi</td>
<td>156536318</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>haemagglutinin [Influenza A virus (A/New Caledo...</td>
<td>gi</td>
<td>19049784</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New Caledoni...</td>
<td>gi</td>
<td>33622382</td>
<td>100%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New York/22/...</td>
<td>gi</td>
<td>73763195</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New York/23/...</td>
<td>gi</td>
<td>74477208</td>
<td>100%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>hemagglutinin [Influenza A virus (A/New York/22/...</td>
<td>gi</td>
<td>75168283</td>
<td>100%</td>
<td>3.4%</td>
</tr>
</tbody>
</table>
1 dose vaccine (15 µg HA/500 µl)

Transfer to filter (10K MWCO)

Reduction, alkylation, quench reaction

Centrifugation wash steps
Deglycosylation (+3)

Protein digestion - centrifuge enzyme solution through filter

Dry down flowthrough (=peptides)
Resuspend in injection buffer

Synapt : LC-MSMS
Triplicate injections
Autocat exclusion lists

Peak list processing
Merge 6 LC-MSMS runs search
In-house influenza database
MS/MS of $2^+ (K)VDDGFLDIWTYNAELLVLENER(T)$
2736.33 AMU
On filter digestion, replicate analyses with exclusion lists

Hundreds of peptide IDs, >90% sequence coverage

Routinely Achieve Unambiguous ID

Strain ID – Method Development

http://rosemarywashington.files.wordpress.com/

Routinely Achieve Unambiguous ID
Hemagglutinin and neuraminidase sequences in the 2007–2008 trivalent vaccine showing identified sequence in bold red. N-Glycosylation sites are highlighted in green, as determined by incorporation of 18O during enzymatic deglycosylation with PNGase F. Blue = partially glycosylated, as evidenced by the identification of both the modified and unmodified peptides. Note that the low observation of partially glycosylated peptides by this approach suggests that the glycosylation of the identified peptides is near 100%.
MS Protein Quantification

- intensity distributions tend to be similar between proteins

![Signal Intensity of Peptides from Three Equimolar Proteins](chart.png)

Average signal intensity from the three most intense peptides ~ protein amount

(15% for proteins of similar mass)
Quantification of antigens can be made by comparing to a spiked reference standard.

**Average Top 3 Peptide Intensity**

- **Reference**: 30 μg/mL
- **Protein A**: 25 μg/mL
- **Protein B**: 104 μg/mL
- **Protein C**: 7.4 μg/mL
trivalent vaccine (15 µg HA/500 µl)

heat 100 °C 5 min
cool to room temperature

add: Rapigest
ADH as internal standard

Digest: high enzyme/short time

Halt reaction and cleave Rapigest

Dilute with 1.5x injection solvent

Synapt: MSE
Glu-Fib lockspray

PLGS peak list generation and database search
Results - Quantification

NA Calculated Concentrations

 ug/mL

<table>
<thead>
<tr>
<th>N1</th>
<th>N2</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. Antigen 1</td>
<td>Ref. Antigen 2</td>
<td>Manufacturer 1</td>
</tr>
<tr>
<td>Manufacturer 2</td>
<td>Manufacturer 3</td>
<td></td>
</tr>
</tbody>
</table>
Comparison of NA by MS versus Western blot.
Hemagglutinin quantification in influenza vaccines, monovalent bulks, and reference antigens as determined with triplicate MSE analysis. Expected amount (shown as black solid line) is 15 g/dose for all samples except pan-1, which is 7.5 g/dose. (a) HA content of three seasonal (different years) trivalent vaccines from Manufacturer 1 (tri-1 through tri-3) and of 2010–11 trivalent vaccines from two manufacturers (tri-3 and tri-4) and the corresponding reference antigens. (b) HA content of H1N1 monovalent vaccines, bulk vaccine, and reference antigen. SRID data from the manufacturer and regulatory agency are shown for pan-2 through pan-9 in orange and yellow points.
Overview – issues to consider

- Reducing, capping, deglycosylation increased RSD – minimize sample handling
- Pipetting very crucial – coat each tip
- Minimize missed cleavages and in-source fragments (split peptide signal intensity)
- High enzyme/short incubation produced lowest RSD
- Primary standard quantitation – used ADH, NIST BSA
- Databases:
  - Specific for each vaccine
  - Use strain identification results to augment quant database
QCONCAT = quantification contatamer(1) (Method 2)

- Determine optimal tryptic peptide for quant
  - In this case use top 3
- Design a gene to encode the polypeptide
- Vector is expressed, isolated, digested
- Vector is expressed in labelled medium
- Labelled polypeptide is isolated and purified
- Labelled polypeptide is added to samples prior to analysis to provide equimolar internal standards.

From Beynon – Nature Methods

(1) A **nucleic acid molecule** consisting of two or more identical copies of the same **sequences** covalently linked in **tandem**. Biology Online.
QCONCAT

- Two sequences have been generated and tested
- QconCat 1: 36 - Top 4 peptides from HA (3 strains), NA (3 strains), BSA, ADH and Ovalbumin directly to each other:
  Histidine Tag Leader- HA1a-HA1b-H1c-HA1d-HA2a-HA2bHA2c-HA2d-HA3a...
- QconCat2: 27 - Top 3 peptides only, with “sacrificial” linkers (3 amino acids preceding and following peptide of interest – as in protein) separated with an arginine:
  His tag Leader- S1S2S3-HA1a-S4S5S6-R-S7S8S9-HA1b-S10S11S12-R-S13S14S15-HA1c...
Vaccine – annual trivalent
Corrected value – based on the ratio to spiked BSA

Influenza vaccine - Quant Result

µg/mL

H1 | N1 | H3 | N2 | HB | NB

2h | 4h | O/N | Corr
HA quantitation (METHOD 3) by IDMS-MRM

- Method based on Williams et al / Vaccine 30 (2012) 2475 -2482
- HA glycoprotein quantification based on IDMS with pseudo-MRM data acquisition
- Protein quantitation based on peptide quantitation result from 2-3 peptides per protein
Peptide selection

- Avoid readily oxidizable amino acids (methionine)
- Avoid peptides containing cysteine
- Avoid peptides with a glycosylation motif
- Avoid overly hydrophilic/hydrophobic peptides (want peptides 7-12 a.a.)
- Ideally use peptides from different regions of the protein (complete digestion)
- Use conserved peptides
Peptide sequences of HA subtypes

A/California/07/2009

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVEKNVTTHSVNLLDEDKHNGKLCKLRGVAPHLGLGKCNIAGWLGNPECESLSTASSWSYIVETPSDDNGTCRYPTFIDYEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKSFYKNLWVLKKGNPSYPKLKSYSINDKGKEVLVLWGIHHPSTSADQQSLYQNADAYVFVGSRYSKKFKPEIAIRPKVRDQTEGRMNNYYYWTLEPGDKITFEATGNLVPRYPYAFAMERNAGSIIISDTPVHDCNNTCTQTPKGAINTSPLFQNIHPITGKCPKYVKSTDLRLATGLRNIPSIQRSGLFGAIAGFIIEGGWTGMDGWYGHQQQGSGYAADLKSTQNAIDEITNKVNSVIEKMTNQFTAVGKEFENLEKRIENLNNKVDGDGFDTYNTAELLVLLENERTLDYHDSNVKNLYEKVRSQLKNNAKEIGNGCCEFYYHCKDNTCMESVKNTYDYPKYSEEAKLNRRIIDGVKLESTRIYQILAIYSTVASSLVLVVLGSGLAISFWMCNSNGLSQCRIC

B/Massachusetts/2/2012-like (Yamagata lineage)

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATQTGEVNTGVIPTTPTKSYFANLGTGTKTRGKLCPDCLNCTDLDVALGRPMVCVTTPSAKASILHEVRVPVTSGCFPMHDRTKIRQLANLLRGYENIRLSTQNVDAEKAAPGGPYRLGTSGSCPNTSFKSFFMATMAWPDKNNKNATNPVTEVPICAEGEDQITVWGFHSSDDKQMKNLYGDSNPQKFTSSANGVTTTHYVSQIGGFDQTEDGGLPQGRIVVDMMQQKPGKTGTIVYQRGVLLPQKVCASGRSKVTKGSLPLIGEADCLHEGKGNKSKPYTYGHEHAKIANCIPWVKTPLKLNGNTYRPPAKLKLKERGFFGIAAGFLEGGWEGMIAGWHGDISPLAYGAVADLKTSTQAINEKTKNLNSLSELEVKNLQLRSGAMDELHNEIELEDEKVDLRLADTISSQIELAVLLSNEGIINSEDEHLLALERKLKMLGPASVIDIGNGCETKHHCNTLDRIAAGTFNAGEFSLPTFDLSNATAALNDGGLDNHTILLYSTAASSLAVTLMLAIYIFVMVSRDNVSCISCL
Peptide Standards

- Synthetic HA peptides from MidWest Bio-tech
- Incorporate one amino acid with $^{13}$C and $^{15}$N

<table>
<thead>
<tr>
<th>HA Subtype</th>
<th>Peptide Sequence</th>
<th>$\Delta$ Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3N2</td>
<td>STQAAIDQINGK</td>
<td>7</td>
</tr>
<tr>
<td>H3N2</td>
<td>DEALNNR</td>
<td>7</td>
</tr>
<tr>
<td>H3N2</td>
<td>EFSEVEGR</td>
<td>6</td>
</tr>
<tr>
<td>H1N1</td>
<td>EQLSSVSSFHER</td>
<td>10</td>
</tr>
<tr>
<td>H1N1</td>
<td>TLDYHDSNVKK</td>
<td>6</td>
</tr>
<tr>
<td>H1N1</td>
<td>VNSVIEK</td>
<td>6</td>
</tr>
<tr>
<td>HB</td>
<td>NLNSLELEVK</td>
<td>6</td>
</tr>
<tr>
<td>HB</td>
<td>SYFANLK</td>
<td>7</td>
</tr>
<tr>
<td>HB (Yamagata)</td>
<td>GVLLPQK</td>
<td>8</td>
</tr>
</tbody>
</table>
Vaccine 1 2013-2014, µg HA / mL vaccine

- H1N1: 37.1, 41.4, 45.0
- H3N2: 42.2, 40.9, 45.5
- HB: 70.0, 81.6, 77.6

Legend:
- Blue: Vaccine 1, prep-1
- Red: Vaccine 1, prep-2
- Green: Vaccine 1, prep-3
Mean triplicate prep. Quant based on analysis of 1 peptide (STQSAIDQITGK)
Advantages of Analysis by MS

**Strain confirmation and quantification** for both HA and NA without reference antigen or antibodies.

Identification and quantification of host cell proteins: *chicken egg proteins*.

Characterization of **post-translational** modifications.

Analytical timeline of **days from weeks**.
Counterfeit Drugs

Figure 2. Base peak ion chromatogram
“Designer” Counterfeit Drugs

Figure 1: Chemical structures of nortadalafil (1, R = H), tadalafil (2, R = CH3), sildenafil (3, R = O), sildenafil thione (4 R = S), and dimethylsildenafil thione (5).
“Designer” Counterfeit Biological
2157 Proteins at 50.0% Minimum 2 Min # Peptides 4.8% Decoy FDR 61709 Spectra at 50.0% Minimum 0.84% Decoy FDR

**STEM Cell protein analysis**

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Molecular Weight</th>
<th>Probability</th>
<th>Hsp1b1_1</th>
<th>Hsp1b2_1</th>
<th>Hsp2_1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>over 95%</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80% to 94%</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50% to 79%</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20% to 46%</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0% to 16%</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Bio View: 2306 Proteins in 2010 Clusters With 101 Decays

### 43 proteins that differ by 3
Plant-derived biologic drugs – use existing guidelines

- proof-of-concept *in vitro* and/or *in vivo*
- pharmacological (PK/PD) effects
- toxicological effects, including short-term and long-term toxicology studies
- allergens → need for allergenicity testing
- immunogenicity → PTMs unique to plant expression systems
- toxicants (hemolytic agents, neurotoxins)
- pathogens, pesticides, fertilizers and heavy metals
Pattern of albumin glycation

Determination of Supplier-To-Supplier and Lot-To-Lot Variability in Glycation of Recombinant Human Serum Albumin Expressed in Oryza sativa - accepted PLOS ONE
Centre for Vaccine Evaluation Research Team

**MS**  Marybeth Creskey, Dr. Daryl Smith, Lisa Walrond

**HPLC & CE**  Dr. Michel Girard, Barry Lorbetskie

**NMR**  Dr. Yves Aubin and Genvieve Gingras and protein expression

**Virology**  Dr. Sean Li, Dr. Aaron Farnsworth

**Protein Folding**  Dr. Michael Johnston, Grant Frahm

**Stem cells**  Dr. Michael Rosu-Myles, Dr. Jessie Lavoie
Thank you for the invitation and your attention!

Questions are welcome.