Protein Estimation Methods

Stephen Harvey

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UNIVERSITY OF MINNESOTA

Center for Mass Spectrometry and Proteomics | Phone | (612)625-2280 | (612)625-2279

Protein in Biological Fluids

Plasma

60 to 80mg/mL

CSF

0.15 to 0.6mg/mL

Urine

0 to 0.8mg/mL healthy

2.O to 20mg/mL mild disease

>100mg/mL chronic kidney disease

BALF

<100µg/mL

Mass Spectrometry

- MALDI and Q-TOF

  60,000 Da protein requires 1.0mg/mL

- Triple Quadrople

  <10 pg on column (less than ng/mL)

- ITRAQ – Peptides labeled

  Requires 40 µg for each aliquot

Dynamic range and Variability of proteins

Plasma

Serum albumin (55% of all)

↓

Interleukin 6, 0 to 5 pg/mL

Urine

Uromodulin (30% n-glycosylated)

Sample preparation

- Concentrate/dilute sample
- Purification
  - Buffers and high salt
  - Detergents for integral protein
- Immunoprecipation (IP)
  - Detergents throughout procedure
- Gel electrophoresis

IP methods - points of concern

Lysis buffers - Tris, SDS, Triton x-100, EDTA

Dilution buffer - Tris, SDS, Triton x-100, EDTA

Wash buffers - Tris, SDS, Triton x-100, EDTA

Elution buffer - Tris, SDS, DTT

Total protein concentration

- Ultraviolet absorption

- Colorimetric assays

  Bradford

  Bicinchoninic Acid assay (BCA)

Ultraviolet Absorption

- Proteins
  - Absorbance at 280nm:
    - Tryptophan, Tyrosine
  - Absorbance at 205nm:
    - Peptide bond, sensitive, more interference
- Peptides
  - Absorbance between 200-230nm:
    - Aromatic A.A., histidine, cysteine, methionine

Considerations

Over 10-fold range in UV absorption at 280nm

Extinction coeff: e = ABS/concentration (L)

Varies with pH and ionic strength

- Strongly interfering compounds.

  Nucleic acids - measure at 260 and 280nm

  If $OD_{260}/OD_{280} < 1.0$, ~95% protein

  [protein] = $1.55A_{280} - 0.76A_{260}$ (ref. below)

  Buffers - especially at 205nm

  Detergents - especially at 205nm

  Unsaturated compounds - 205nm

Considerations con't

- Loss to cuvette walls

  Dilute solutions measured at 205nm

Stoscheck, C, *Methods in Enzymology*, Vol 182, pg 54-56

Colorimetric Assays

Bicinchoninic Acid assay (BCA)

- 20 to 2000µg/mL
- Cu^{2+} complexes with C, W, Y and peptide bonds
- Reduced to Cu^{+}
- Cu^{+} reacts with BCA reagent:
- Absorbance at 562nm

Coomassie Blue Protein Assay (Bradford)

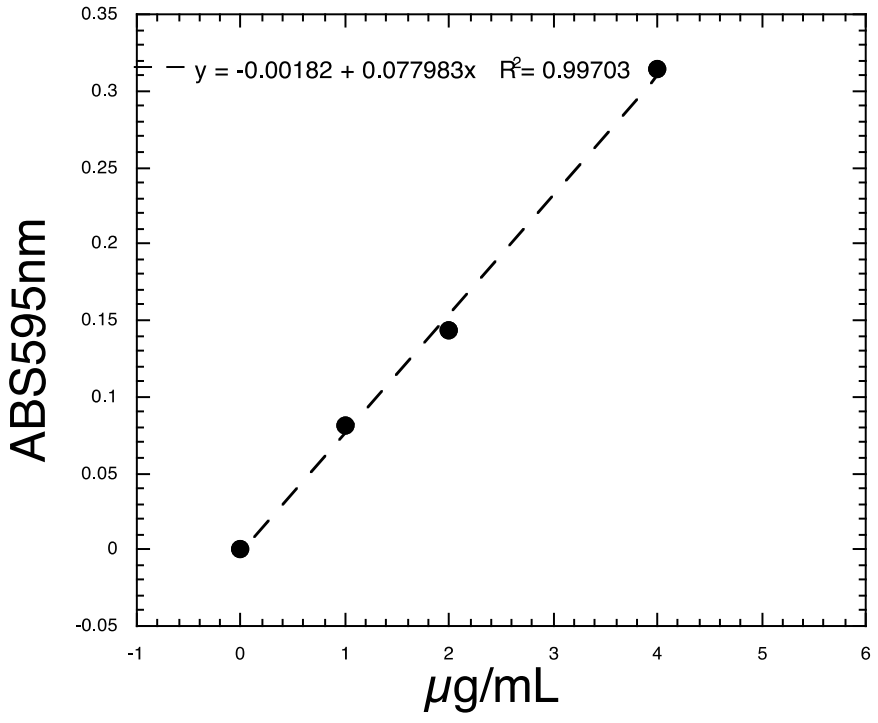
- 1.25 to 1400µg/mL
- Basic and aromatic residues stabilizes anionic form of dye.
- Peptides of over 10 residues
- Absorbance at 595nm

Considerations

- Determine linear range.
- Use similar standard to sample
- Determine concentration from standard curve
- Bradford - High protein to protein variability
  Sensitive to interfering compounds
- BCA is less sensitive to interfering compound.
- Microassays using these reagents are particularly sensitive to interfering agent (up 40-fold more).

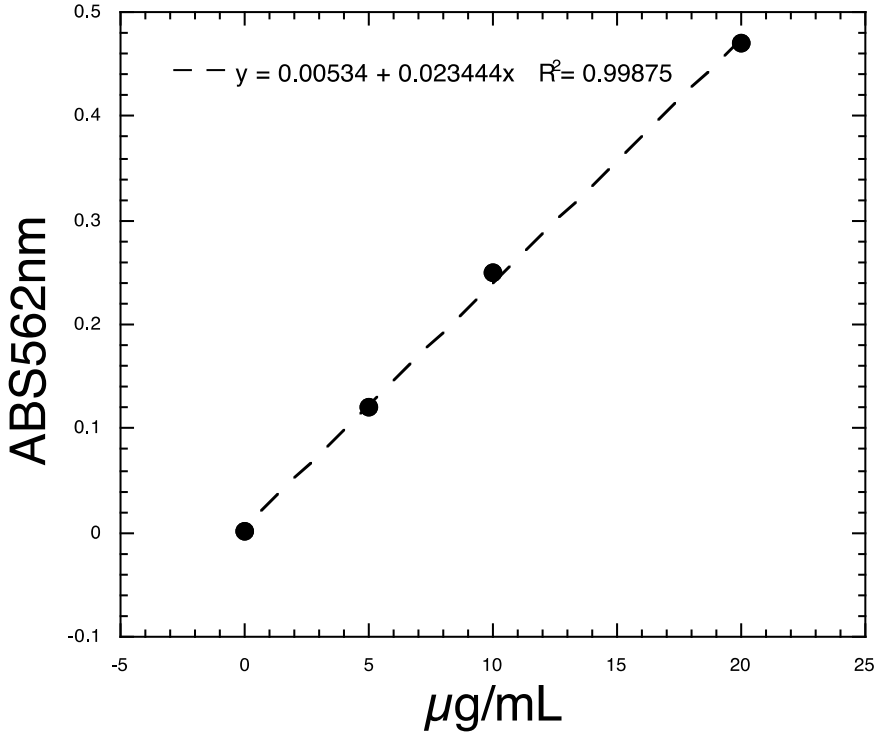
Bradford (BioRad) and BCA (Pierce) standard curves
1.0mg/mL bovine serum albumin used as standard

Bradford assay



1 to 4µg/mL

BCA assay



5 to 20µg/mL

Uromodulin concentration measure

1.0mg/mL BSA used as standard

Assay	Conc .(point)	Conc. (slope)
Bradford(low)	not detected	ND
(high)	0.05mg/mL	ND
BCA (low)	0.17mg/mL	0.18mg/mL
(high)	0.64mg/mL	NA
UV_{280nm}(high)	0.75mg/mL	NA
UV_{280nm}(Standard)	1.02mg/mL	NA

Compatible with protein assay

Compound	Bradford	BCA	280nm/205nm
Triton X-100	0.1%	1.0%	0.02%/<0.01%
Tween 20	0.01%	1.0%	0.3%/0.1%
CHAPS	10%	1.0%	1.0%/1.0%
SDS	0.1%	1.0%	0.1%/0.1%
Octyl-B-glucoside	0.5%	5.0%	10%/ND
Tris	2.0 M	0.1 M	0.5 M/40mM
Phosphate	1.0 M	2 M	1.0 M/50mM
Sodium chloride	5.0 M	1.0 M	>1.0 M/0.6 M

Compatible with MS analysis

Compound	Bradford	BCA	LC-MS
Triton X-100	0.1%	1.0%	not
Tween 20	0.01%	1.0%	not
CHAPS	10%	1.0%	SDS-PAGE
SDS	0.1%	1.0%	0.1%SDS-PAGE
Octyl-B-glucoside	0.5%	5.0%	yes
Tris	2.0 M	0.1 M	not
Phosphate	1.0 M	2 M	not
Sodium chloride	5.0 M	1.0 M	not

Salts
Polymers
Detergents

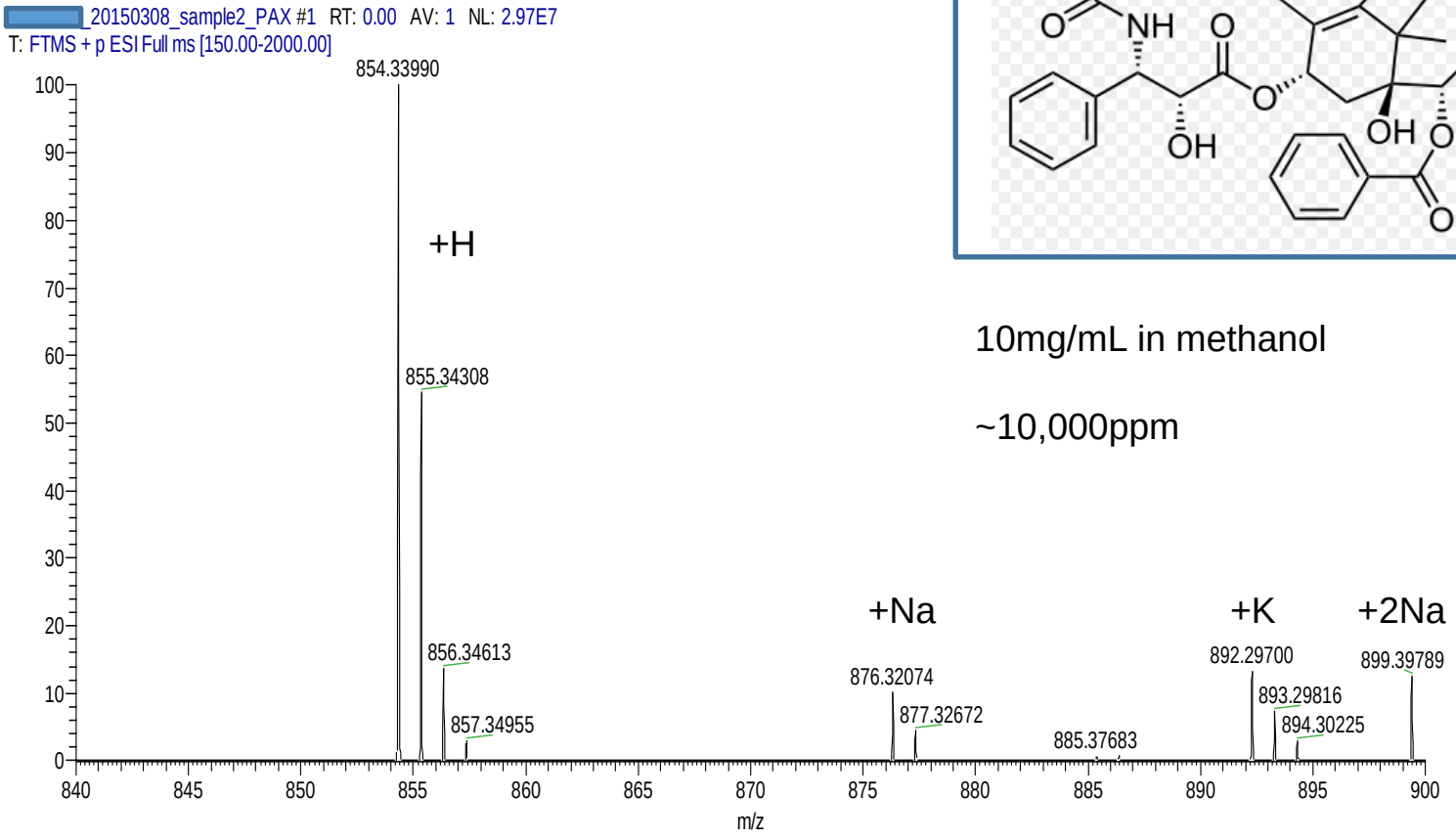
- Complicate interpretation
- Compete with charged ions
- Interfere with MALDI matrix
- Signal suppression
- Contaminate the instrument

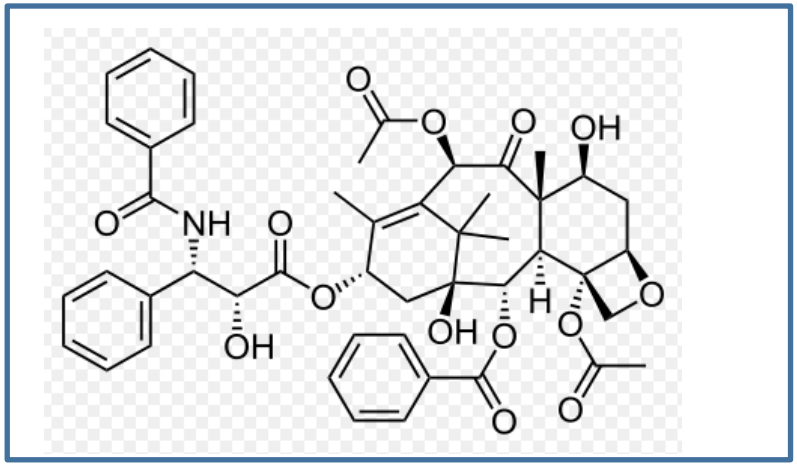
Example contaminant affects on mass spectrometry analysis

m/z (H+) 854.3382

SALTS

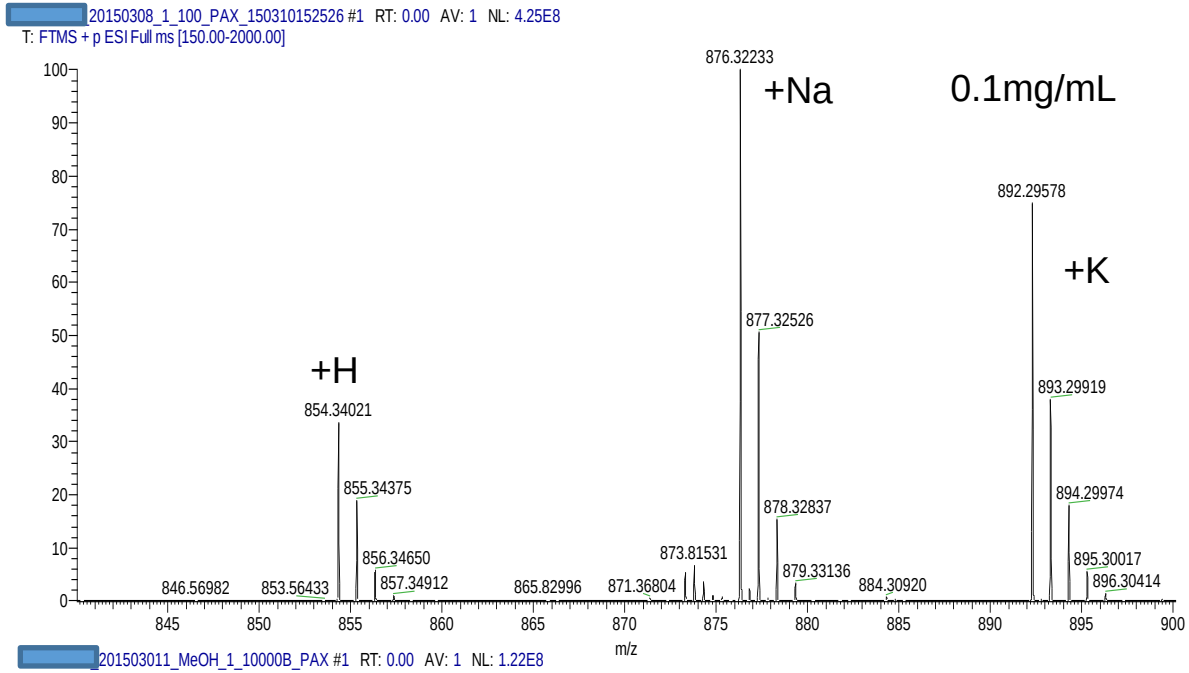
- Complicate interpretation

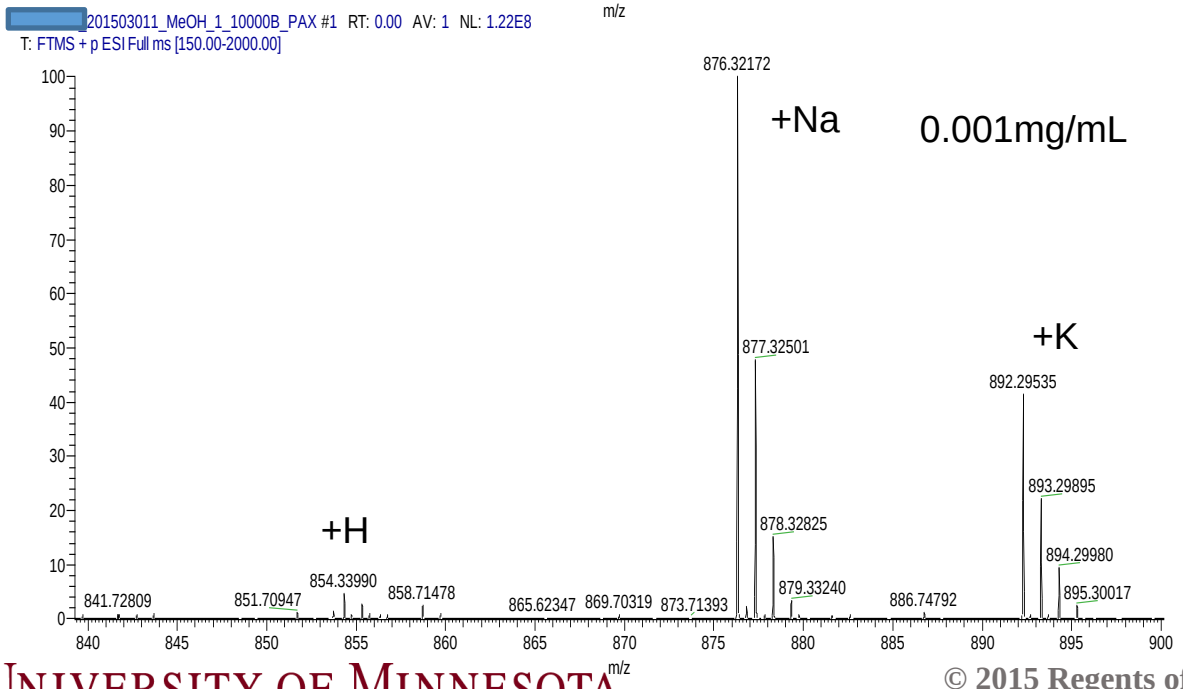




10mg/mL in methanol

~10,000ppm



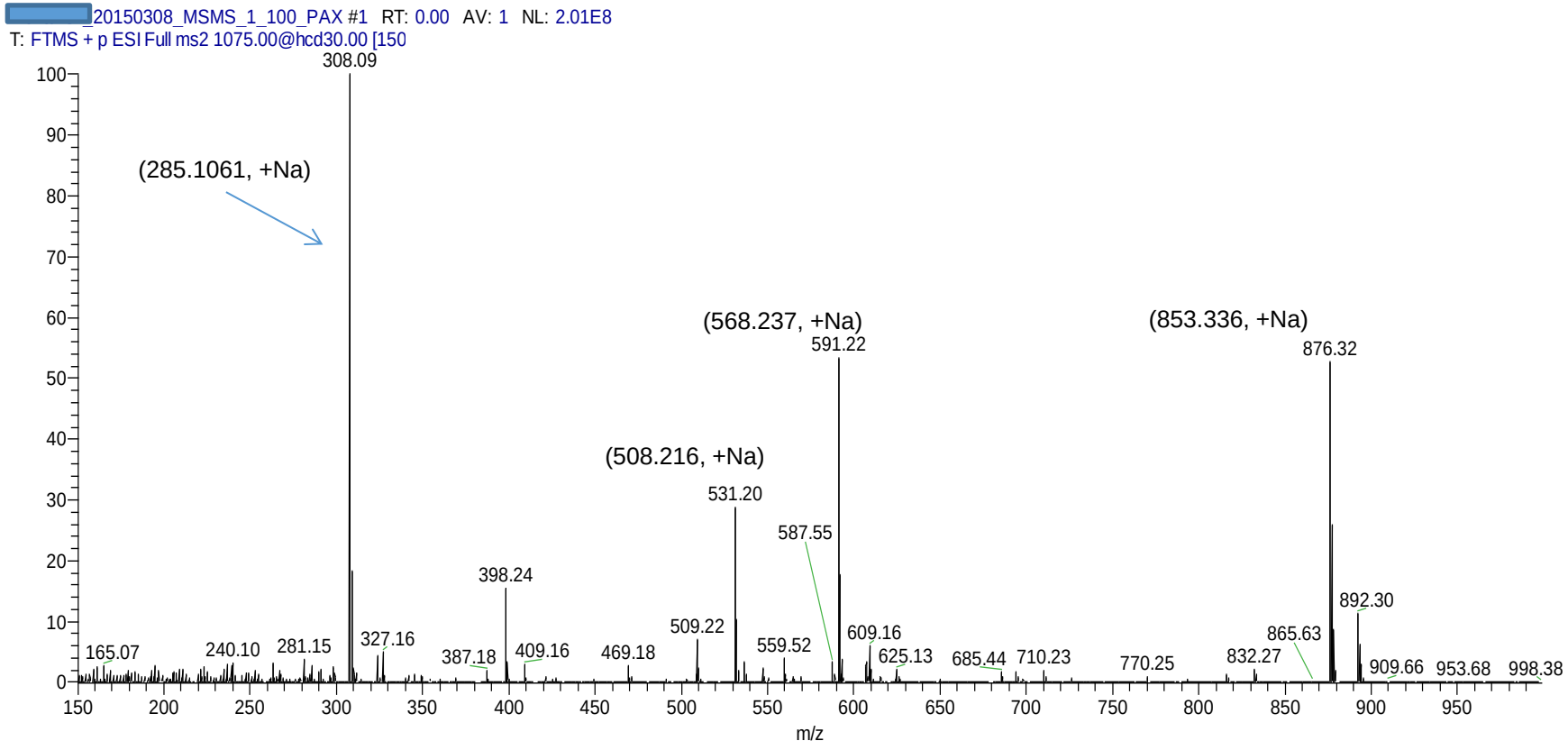


Diluted in Sigma Optima grade Methanol >2ppm sodium
>1ppm potassium

~100ppm

~1.0ppm

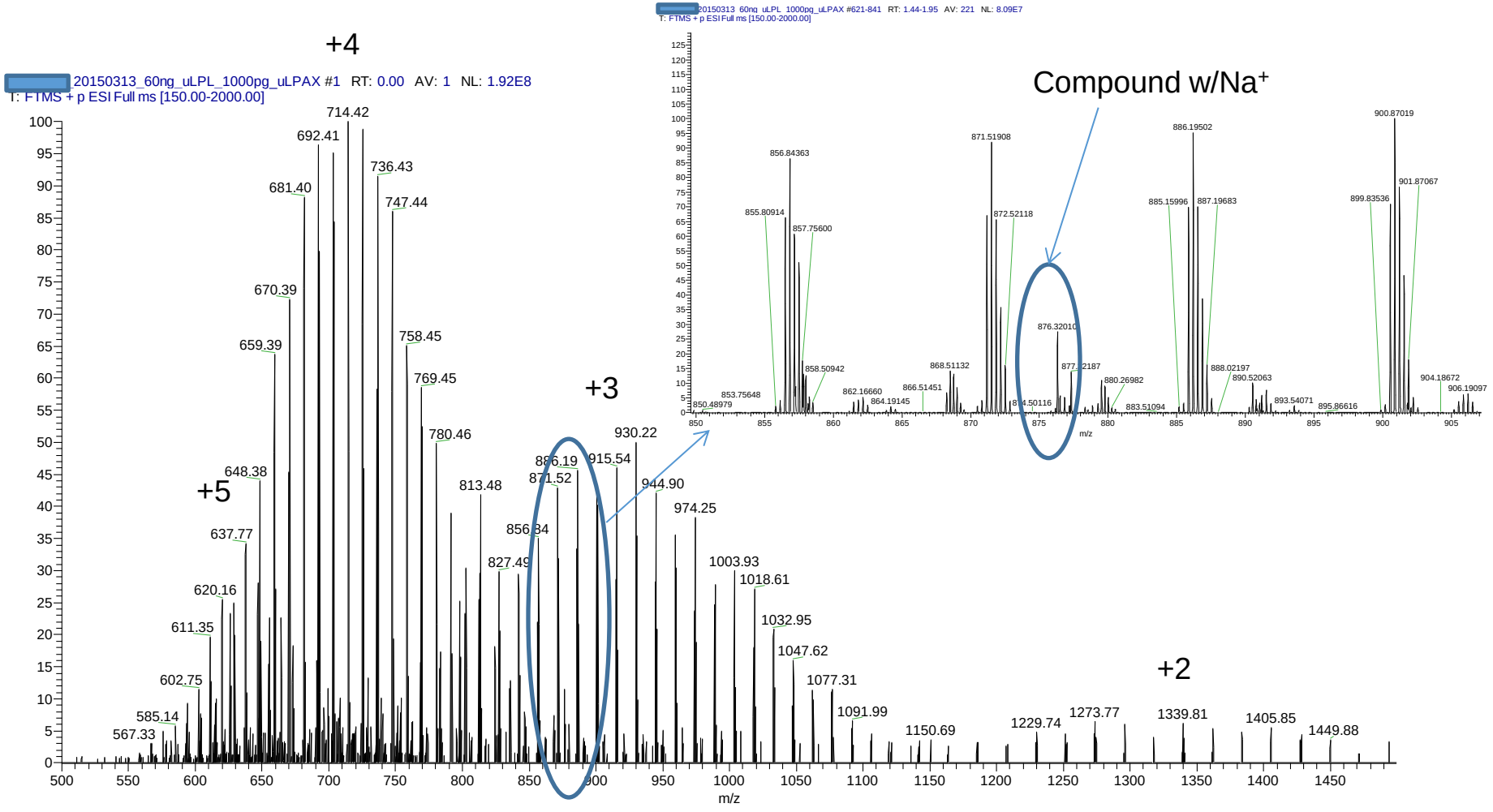
Fragmentation of compound.



- Expected fragment peaks are present complexed with sodium (+23,).

- Salts present in sample alter observed m/z.
- Salt adducts may produce different ion signal response.
- Salt adduct are present with fragmentation. Complicating identification.

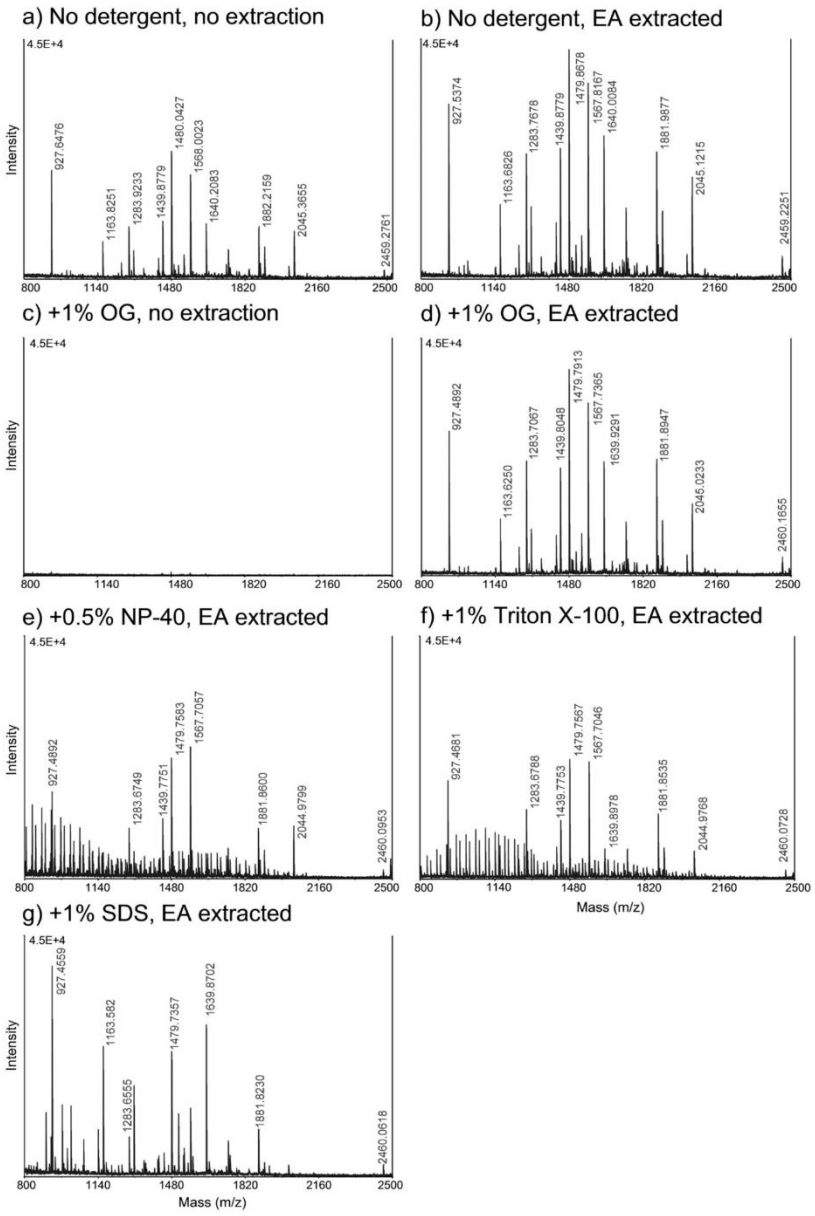
Polymers – Polyethylene glycol



Detergents

- Signal suppression
- Contaminate the instrument

octylglucoside
Before cleanup



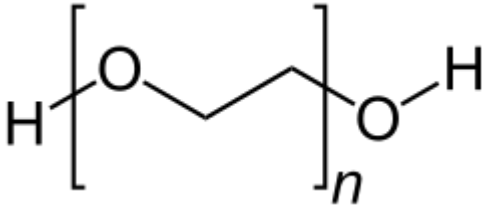
octylglucoside
After cleanup

Triton X-100
After cleanup

Yeung,Y, Nieves,E, Angeletti, R, Stanley, E Anal Biochem. 2008 Nov. 15; 382(2): 135-137

Polyethylene Glycol and detergents

- Complicate interpretation
- Compete with charged ions
- Interfere with MALDI matrix
- Signal suppression
- Contaminate the instrument



Ethylene oxide polymer

~44 m/z

Conclusions

Develop purification/enrichment techniques with both protein level assay and mass spectrometry compatibility in mind.

- Ultraviolet spectroscopy
  - 280nm proteins: 200 - 230nm proteins or peptides
    - Differential absorption at 280nm, determine extinction coefficient
    - Sensitive measure at 205nm, many interfering compounds

Conclusions con't

- Colorimetric assays
  - Dye-based, Bradford
  - Cu+2 based, BCA
    - Variable protein-to-protein sensitivity
    - Interfering compounds
- Mass spectrometry
  - picogram to microgram quantities required
  - Signal suppression
    - Detergents
    - Non volatile salts
    - Etc.

Compatible concentrations for protein assays

Compound	Bradford	BCA	UV280nm/205nm
Acetate	0.6 M	0.2M	0.1/0.01
Acetone	10%	10%	
Ammonium sulfate	1.0 M	1.5M	50%/9%
Ampholytes pH 3–10	0.5%		
BES	2.5 M		
Brij 35		5.0%	1%/1%
CHAPS	10%	1.0%	1%/1%
Citrate,	50 mM		<1.0mM 5%/<10mM
Deoxycholate	0.1%	5%	0.3%/0.1%
DNA/RNA	10.25mg		0.1mg/1.0µg
DMSO	5%	5%	20%/<10%
DTT	1.0 M	<1mM	3mM/0.1mM

Compound	Bradford	BCA	UV280nm/205nm
EDTA	0.2 M	10mM	30mM/0.2mM
EGTA	50 mM		
Glycerol	99%	10%	40%/5%
Glycine	0.1 M	1.0M	1.0M/5.0mM
Guanidine-HCl	2.0 M	4.0M	
HEPES	0.1 M	0.1M	ND/<20mM
β-Mercaptoethanol,	1.0 M	0.01%	10mM/<10mM
MES	0.7 M	0.1 M	
Methanol	10%	10%	
MOPS	0.2 M	0.1mM	
NP-40	0.25%	5.0%	
Octyl-B-glucoside	0.5%	5.0%	10%/ND
OTG	1.0%	5.0%	

Compound	Bradford	BCA	UV280nm/205nm
Phosphate	1.0 M	2 M	1.0 M/50mM
PIPES	0.5 M	0.5 M	
Potassium chloride	1.0 M	<10mM	100mM/50mM
SDS	0.1%	1.0%	0.1%/0.1%
Sodium chloride	5.0 M	1.0 M	>1.0 M/0.6 M
Sucrose	1.0 M	40%	2.0 M//0.5 M
Tris	2.0 M	0.1 M	0.5 M/40mM
Triton X-100	0.1%	1.0%	0.02%/<0.01%
Tween 20	0.01%	1.0%	0.3%/0.1%
Urea	6.0 M	3.0 M	>1.0 M/<0.1 M

Stoscheck, C, *Methods in Enzymology*, Vol 182, pg 52-53
Pierce, ref 1296.2
BioRad Laboratories Inc, technical note 1069

LC-MS compatible detergents

PPS Silent Surfactant
http://shop.expedeon.com/products/18-Protein-Solubility/
129-PPS-Silent-Surfactant/

Big CHAPS deoxy
http://www.emdmillipore.com/life-science-research/big-chap-deoxy/
EMD_BIO-256455/p_Ltab.s1L_.8AAAEWhmEfVhTm?PortalCatalogID=
merck4biosciences&CountryName=United+States+of+America

ASB series
http://www.emdmillipore.com/life-science-research/asb-16
/EMD_BIO-182755/p_hmmb.s1L_.4AAAEWhmEfVhTm

RapiGest SF
http://wwwp1.waters.com/webassets/cms/support/docs/715000122.pdf

ProteaseMax
http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20
Bulletins/101/ProteaseMAX%20Surfactant%20Trypsin%20Enhancer.pdf

N-octyl-B-glucopyranoside

Sodium Deoxycholate

LC-MS incompatible detergents

Trition X-100

NP-40*

Igepal

Bri-35

Tween-20

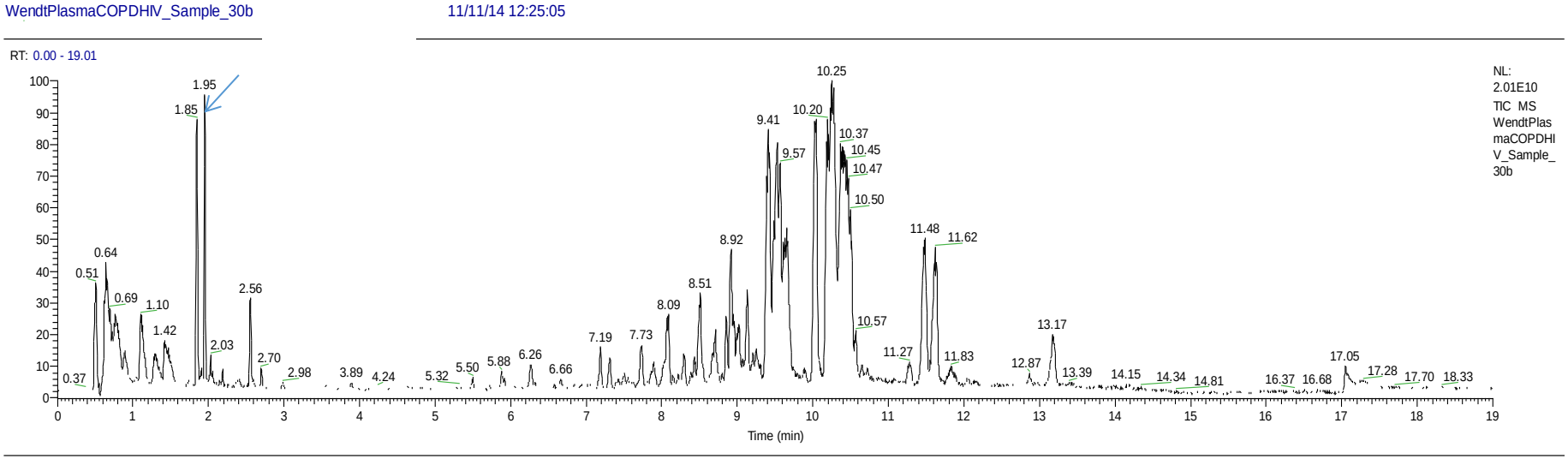
Octyl-B-thioglucopyranoside

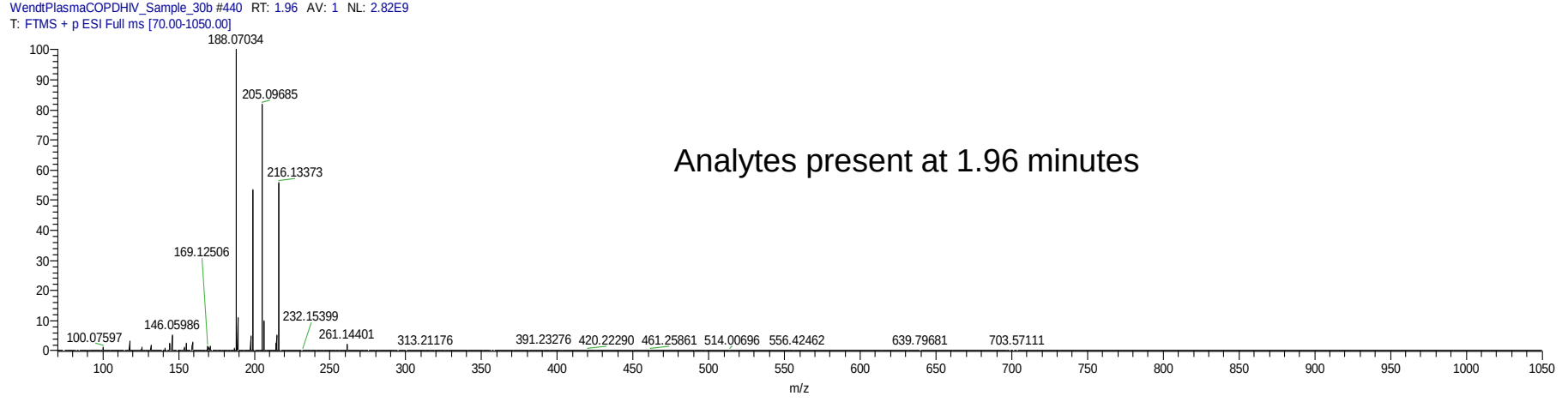
SDS*

CHAPS*

* May be removed by SDS-PAGE

Reverse-Phase C18 analysis of plasma





Analytes present at 1.96 minutes