High Pressure Direct Protein Extraction from Tissue – Trypsin Digest with Pressure Cycling Technology (PCT)

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Applications of Ultra-high Pressure in Biotechnology

Harvard Medical School

Joseph B. Martin Conference Center

Boston, MA 02115

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Mizzou
University of Missouri

PCT Application a

- Direct tissue extraction
  - Protein
  - Oligonucleotides
  - Small molecules

Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. *J Biomol Tech* 2006; **17**: 173.

Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA. *Electrophoresis* 2007; **28**: 1022.

Pevsner P, Vecchione D, Stall B, Remsen T, Anand S, Stern, A. British Mass Spectrometry Society Annual Meeting, Edinburgh, Scotland, September 9-12, 2007

Pevsner P, Vecchione D, Remsen T, Kessler P, Levers N, Yang P, Stern A, Samuels H. British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK, December 8-12, 2007.

Pevsner P, Vecchione D, 1 Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK, December 8-12, 2007

PCT Application b

- Trypsin Digest

Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG,2nd, Smith RD. *J Proteome Res* 2008; **7**: 3276. DOI: 10.1021/pr7008077.

Pevsner PH, Melamed J, Remsen T, Kogus A, Francois F, Kessler P, Stern A, Anand S.

Biomarkers Med 2009; **3**: 55.

Introduction a

- PCT reduces trypsin digest time from hours to minutes.
- Current methods often use heat and prolonged digest intervals.
- Samples digested in minutes with PCT give virtually the same results as those digested for one hour at 55°C.

Introduction b

- Samples for this study
  - Trypsin digest of protein calibrant mixture for matrix-assisted laser desorption ionization mass spectrometry (MALDI)
  - Trypsin digest of tissue extract for liquid chromatography mass spectrometry (LCMS)
    - normal mouse tongue and heart
    - irradiated mouse tongue and heart

Why pressure? a

- Denaturation takes place when the protein-protein and protein-solvent interactions are interrupted. Applying pressure causes an increase in the number of water-water and protein-water hydrogen bonds, while the number of protein-protein hydrogen bonds decrease, resulting in denaturation of the protein.

Silva JL, Weber G. *Annu Rev Phys Chem* 1993.

- High pressure exposes a protein's hydrophobic groups to water which allows molecules of water to penetrate the protein and increase the internal pressure, destabilizing the hydrogen bonds that are responsible for the secondary and tertiary structure of the protein. As a result, the protein is destabilized and unfolds.

Ruan K, Lange R, Bec N, Balny C. *Biochem Biophys Res Commun* 1997; **239**: 150.

Why pressure? b

- Pressures between 29,000- 145,000 PSI (2-10 kbar) are adequate to denature protein, however, pressures >35,000 PSI will denature trypsin.

Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG, Smith RD. *J Proteome Res* 2008; **7**: 3276.

- Under high pressure, non-native salt bridges, hydrophobic, and electrostatic interactions are disrupted within the protein. The application of pressure has also been shown to increase protein charge which increases electrostatic repulsions. These repulsions favor a conformational change within the protein (i.e. unfolding).

Balny C. *Journal of Physics: Condensed Matter* 2004; **16**: S1245.

The role of temperature

- Temperature plays a significant role in a protein's susceptibility to proteolytic digestion.
- Depending on the stability of a given protein, temperatures up to 60°C can be used.

Sim TS, Kim EM, Joo HS, Kim BG, Kim YK. *Lab Chip* 2006; **6**: 1056.

The role of time

Long digest intervals have been cited as a major disadvantage in protein studies and prompted a search for viable time-saving alternatives, such as PCT.

Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG, Smith RD. *J Proteome Res* 2008; **7**: 3276.

Trypsin Auto-digestion

- Trypsin auto-digestion often occurs in longer digest intervals and at higher temperatures, resulting in inaccurate MALDI analysis.
- To minimize excessive auto-digestion, the trypsin-substrate ratio is kept very low (~1:50), and the temperature below 55 ºC.
  - Ratio determines the extended length of time needed for digestion to occur.
  - A low ratio diminishes the noise present in MALDI spectra by reducing trypsin autodigestion peptides.

Sim TS, Kim EM, Joo HS, Kim BG, Kim YK. *Lab Chip* 2006; **6**: 1056.

Methods a

- Matrix-assisted laser desorption (MALDI) mass spectrometry
- Trypsin digest with PCT
  - 16 samples of BSA
  - 16 samples of Cytochrome-C
- Trypsin Digest Control (55°C for one hour)

Methods b

- Materials
  - HPLC-grade water
  - Powdered BSA and Cytochrome-C
  - Trypsin- mass spectrometry grade treated with TCPK, a protease inhibitor
  - Peptide Calibrants
    - Angiotensin I-1296.68 (M+H)+ monoisotopic,
    - Neurotensin-1672.92 (M+H) + monoisotopic,
    - ACTH (clip 1-17)-2093.09 (M+H)+ monoisotopic
  - Protein Calibrants
    - Insulin 5733.49 Da
    - Cytochrome c 12,361.96 Da
    - Apomyoglobin 16,952.27 Da
    - Aldolase 39,211.28 Da
    - Bovine serum albumin 66,429.09

Methods c

- **Trypsin Preparation**
  - Trypsin (1µg/µL) dissolved in 100 mM ammonium bicarbonate.
- **Calibrant Preparation**
  - Calibrants dissolved in 0.1% TFA/Acetonitrile (ACN), 30/70.

Methods d

- BSA
  - 1158 µg (10 µg/ µL ammonium bicarb 100 mM) /trypsin 2 µg (1 µg/ µL ammonium bicarb 100 mM), total160 µL
- Cytochrome-C
  - 1158 µg (10 µg/ µL ammonium bicarb 100 mM) /trypsin 2 µg (1 µg/ µL ammonium bicarb 100 mM), total160 µL
- Sixteen samples of BSA and trypsin (158 µL /1158 µg BSA and 2 µL/2 µg trypsin), and sixteen samples of Cytochrome-C and trypsin (158 µL/ 1158 µg Cytochrome-C and 2 µL/2µg trypsin) were placed in individual microtubes.

PCT Protocol

- Experimental Protocol
  - Four samples of each protein barocycled at ambient temperature and 35 K psi for
    - 1 cycle (20 seconds)
    - 5 cycles
    - 10 cycles
    - 20 cycles
- Control Protocol
  - Four samples of each protein were digested with trypsin for 1 hour at 55° C.

MALDI preparation

- MALDI Matrix Preparation
  - 70% ACN/ 30% CHCA (cyano-4-hydroxycinnamic acid) matrix solution, 5 mg/mL, prepared for MALDI TOF-TOF.
- MALDI Calibration Wells
  - Calibration wells created by micropipetting 1 µL of each of the 2 peptide mixes in between sample wells and covering them with 2 µL of matrix.
- MALDI Plate Preparation
  - Two µL of each BSA and Cytochrome-C samples micropipetted onto separate wells of a stainless steel MALDI conduction plate for mass spectrometric analysis.
  - The samples were covered with 2 µL of matrix and then dried in an oven at 55 °C.

MALDI Spectrometric Analysis

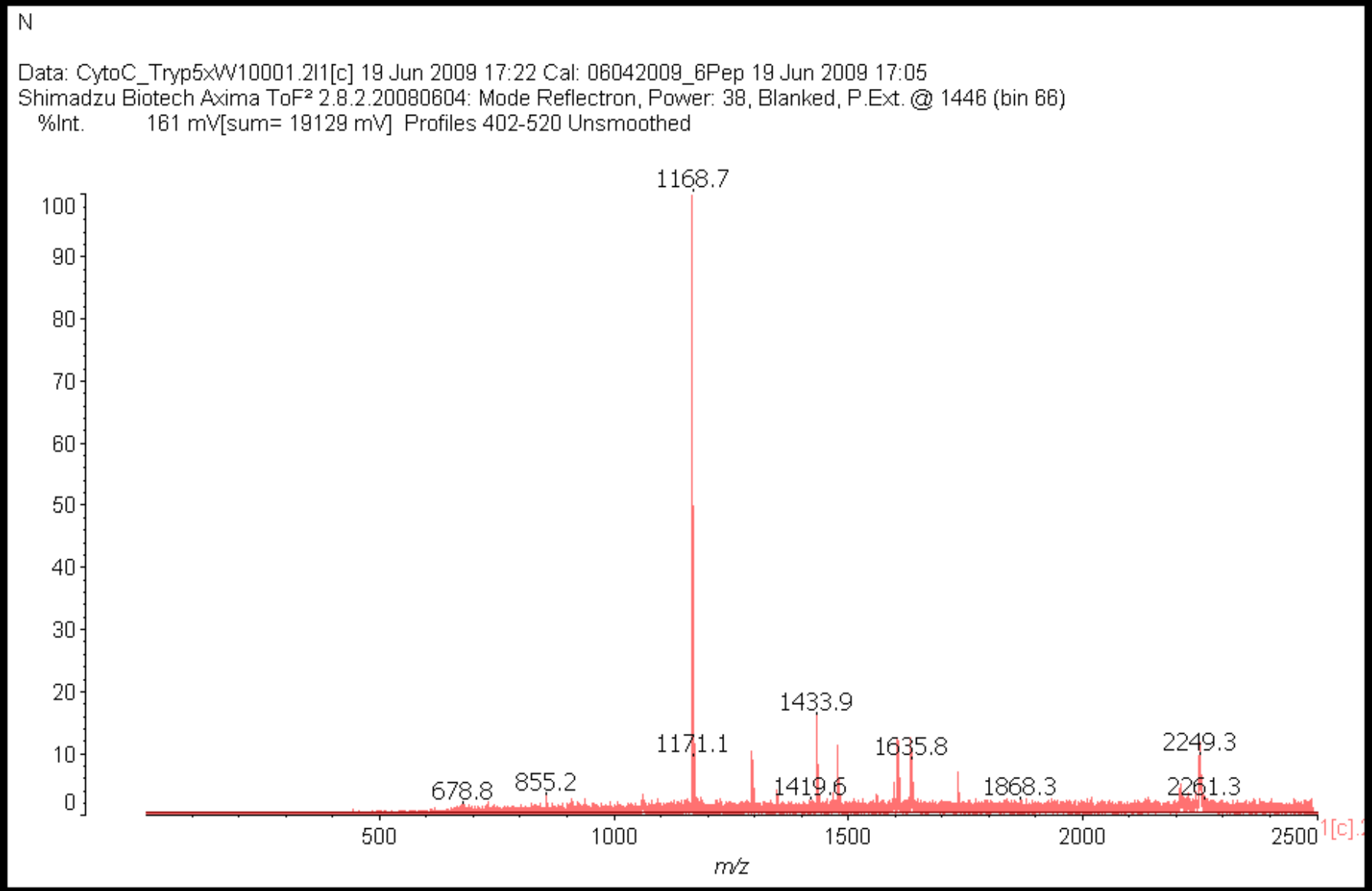
- Results were obtained using a Shimadzu Axima MALDI TOF-TOF.
- Analysis was conducted in reflectron mode.

Matrix Science MASCOT Search Parameters

- Peptide mass fingerprint (PMF) mode.
- Search enzyme set to trypsin.
- Variable modifications set to Oxidation (M) (methionine)
- Mass values set to monoisotopic
- Protein mass unrestricted
- Peptide mass tolerance set at ±1.5Da.
- Peptide charge state set at 1+.
- Search set to permit a maximum of 2 missed cleavages.

Results (MALDI)

- Analysis resulted in the positive identification of Cytochrome-C and BSA.
  - All MASCOT scores were significant matches.
  - Numbered scores represent exact matches.



Cytochrome-C – 5 Cycles

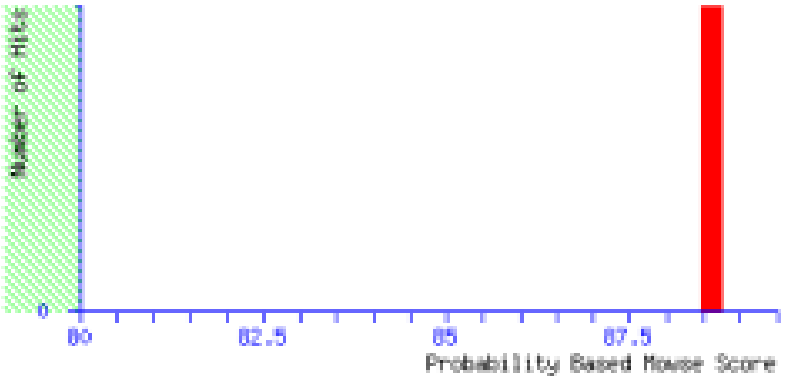
Mascot Score : 89

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 5 cycles W1
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 21:20:25 GMT
Top Score : 89 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).

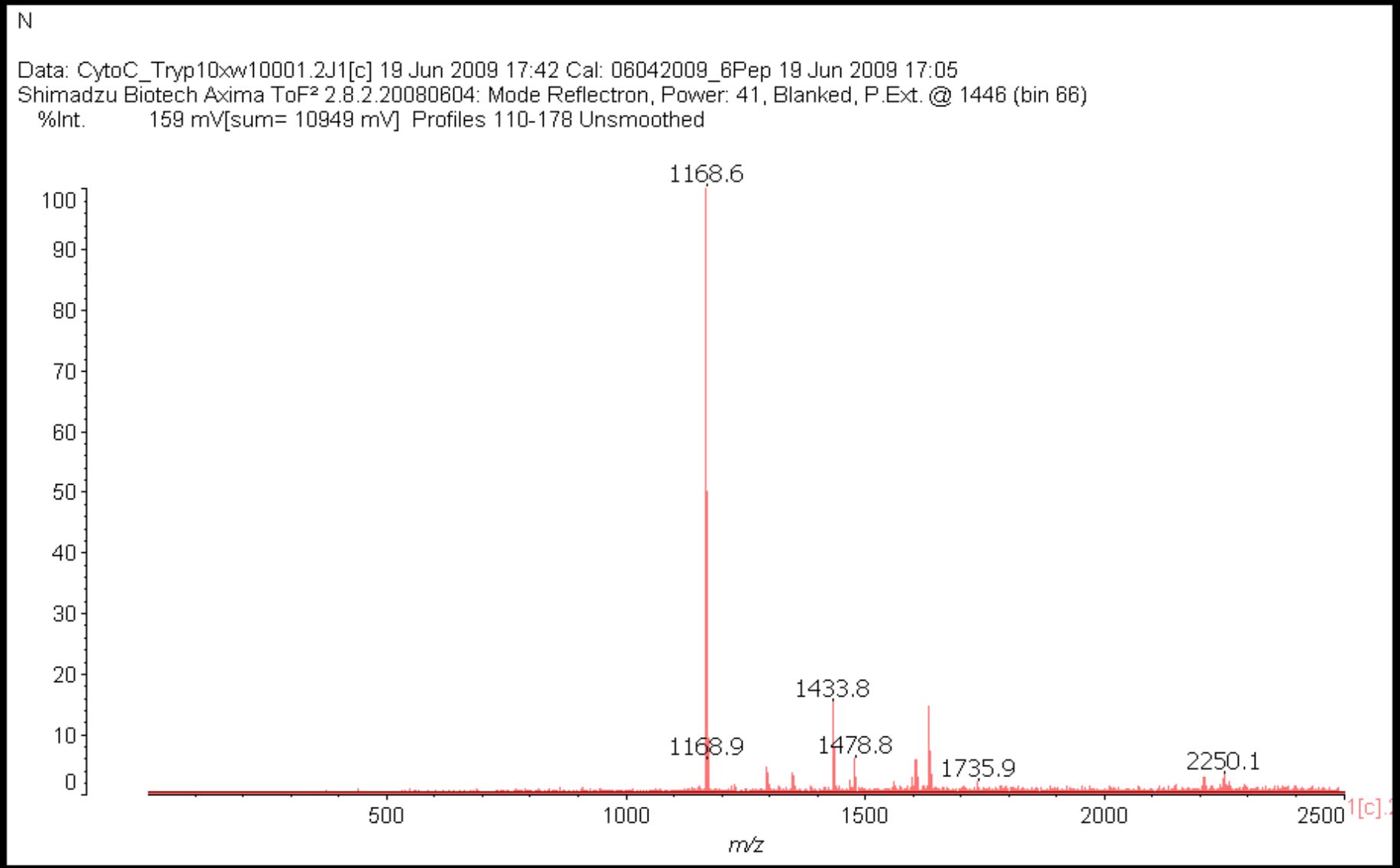


Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

1. CYC_HORSE Mass: 11825 Score: 89 Expect: 0.00065 Queries matched: 9
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



Cytochrome-C – 10 Cycles

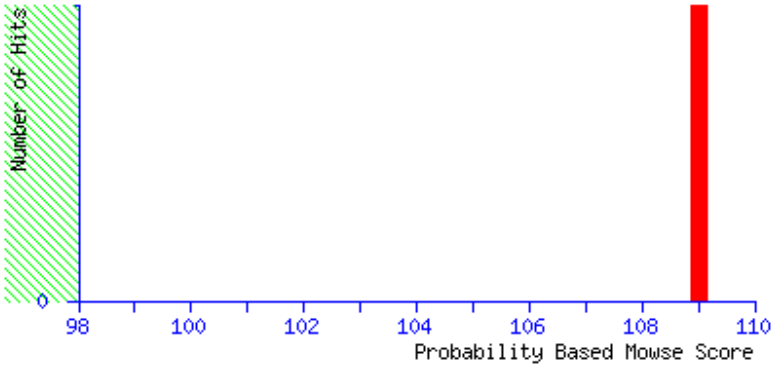
Mascot Score : 109

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 10x w1
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 21:34:58 GMT
Top Score : 109 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



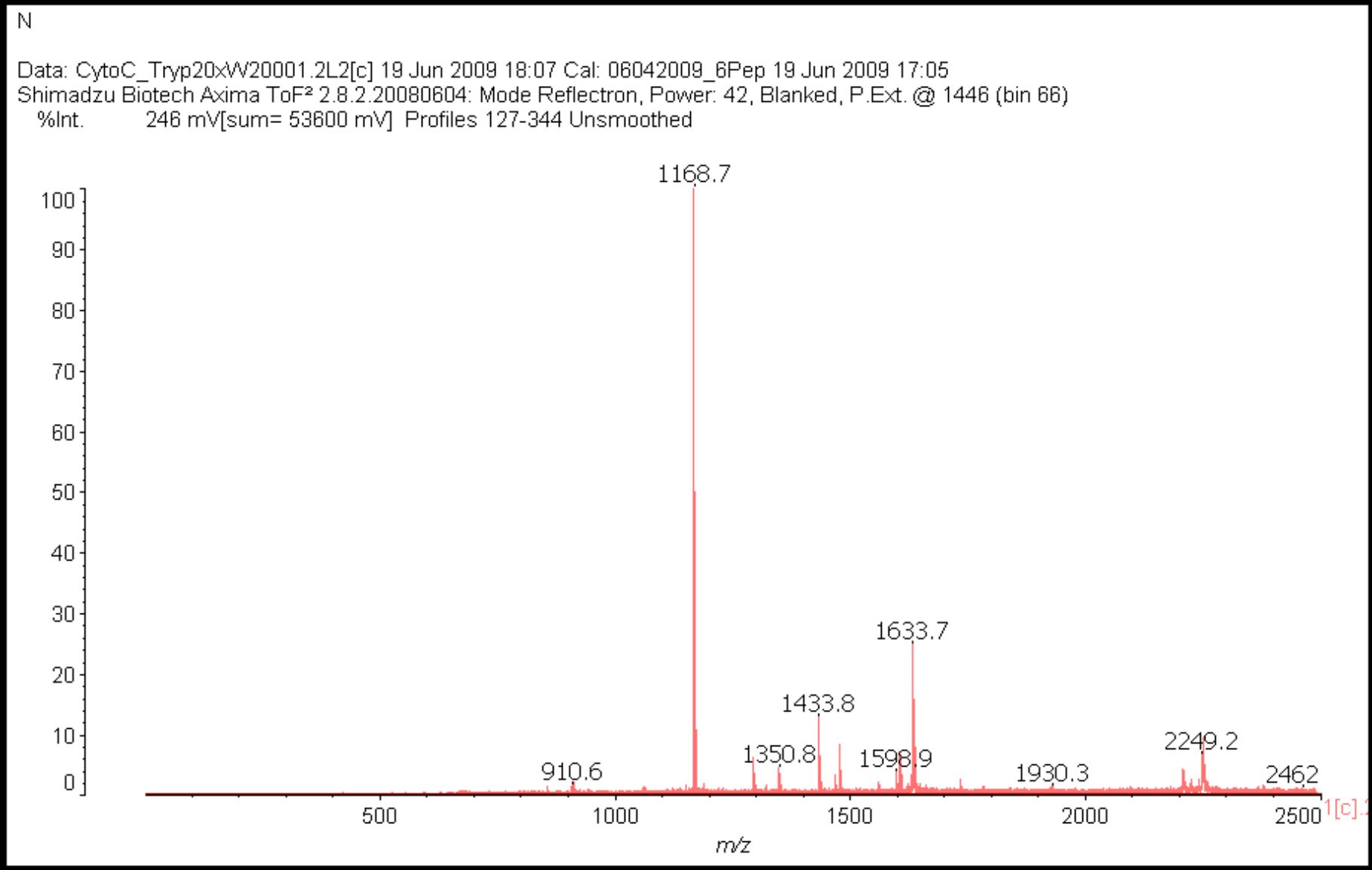
Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

1. CYC_HORSE Mass: 11825 Score: 109 Expect: 5.9e-06 Queries matched: 12
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



Cytochrome-C – 20 Cycles

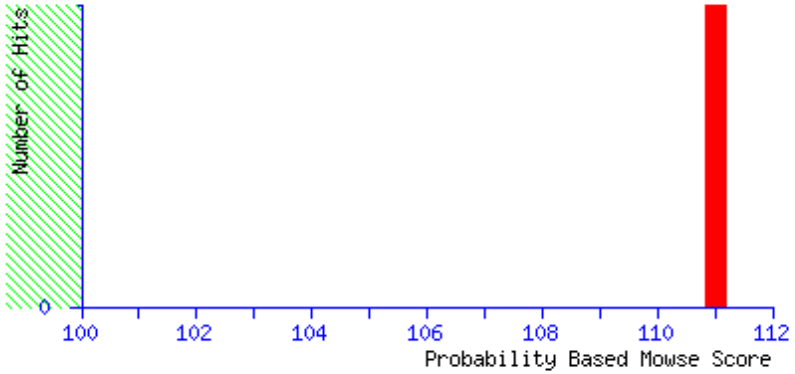
Mascot Score : 111

{MATRIX SCIENCE} Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 20xW2
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 22:01:13 GMT
Top Score : 111 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



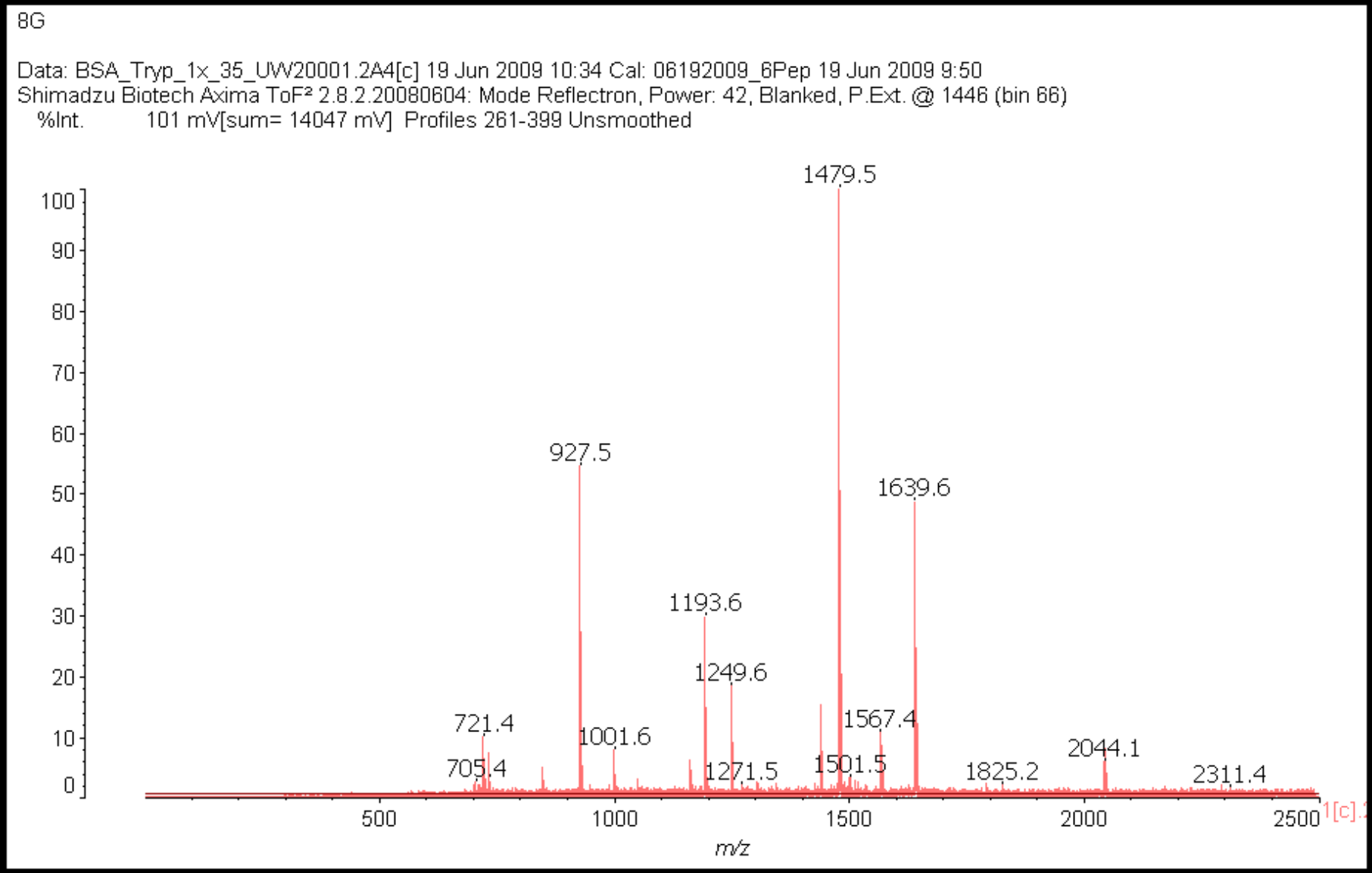
Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

1. CYC_HORSE Mass: 11825 Score: 111 Expect: 3.7e-06 Queries matched: 11
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



BSA – 1 Cycle

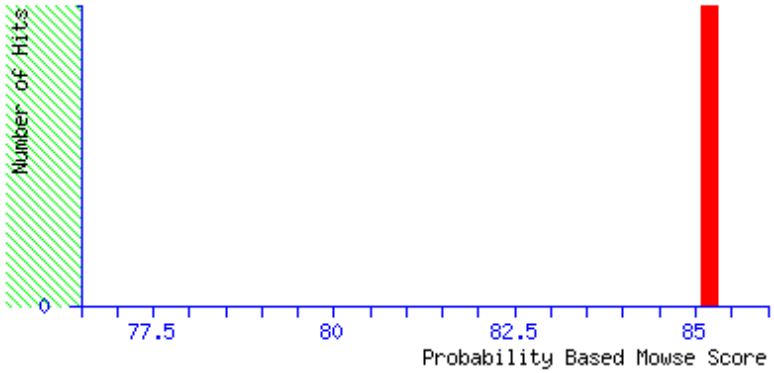
Mascot Score : 85

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA Trypsin 10 cycles2MSMS
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 14:28:34 GMT
Top Score : 85 for ALBU_BOVIN, Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



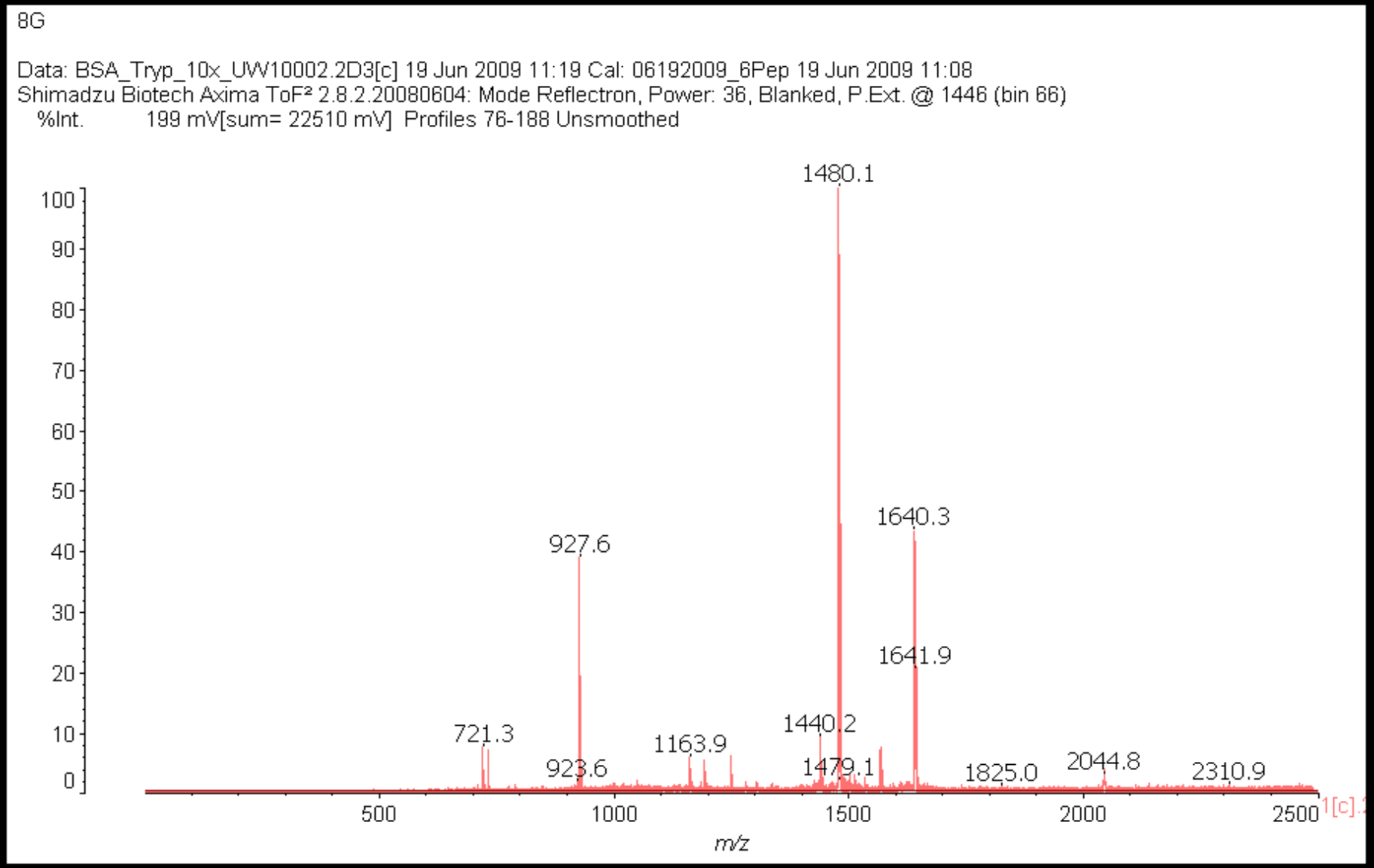
Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

1. ALBU_BOVIN Mass: 69248 Score: 85 Expect: 0.0014 Queries matched: 14
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4



BSA – 10 Cycles

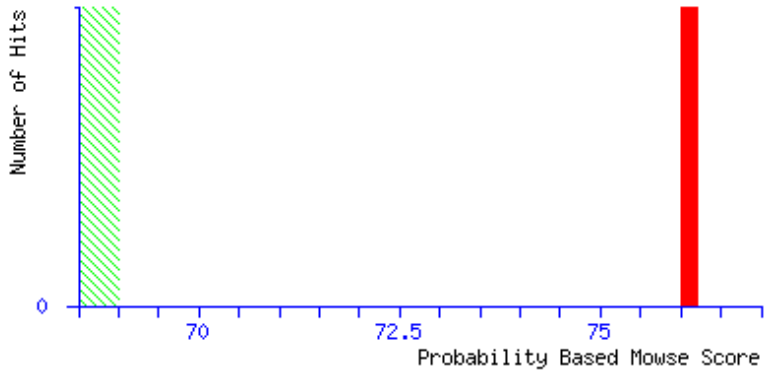
Mascot Score : 76

{MATRIX SCIENCE} Mascot Search Results

```
User            : Administrator
Email           :  jennifer.oprihory@nyumc.org
Search title    : BSA Trypsin 10 cycles2MSMS
Database        : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp       : 19 Jun 2009 at 15:12:38 GMT
Top Score       : 76 for ALBU_BOVIN, Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



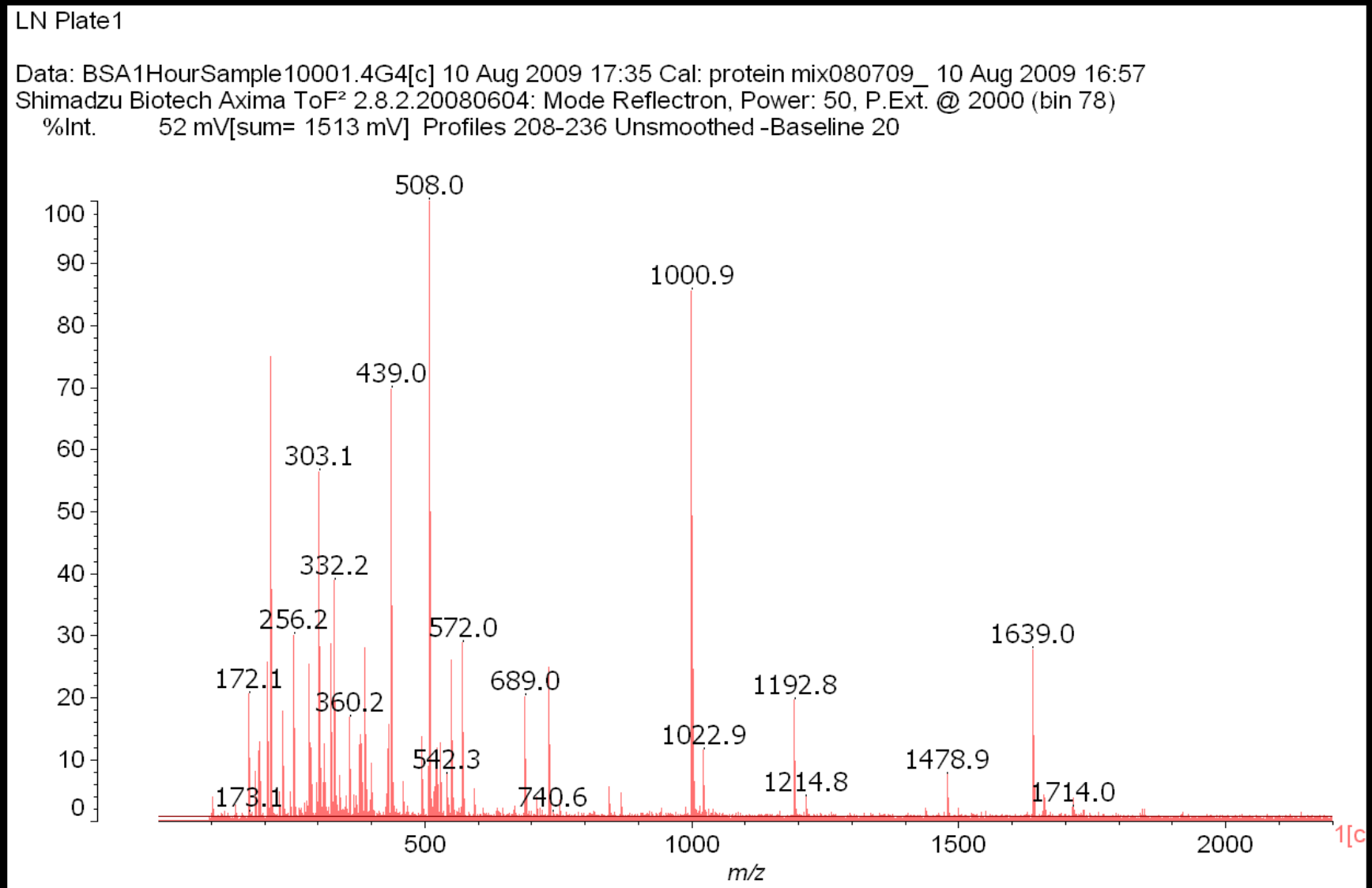
Concise Protein Summary Report

Format As: Concise Protein Summary — Help

Significance threshold p< 0.05 — Max. number of hits 5

Re-Search All | Search Unmatched

1. ALBU_BOVIN **Mass:** 69248 **Score:** 76 **Expect:** 0.012 **Queries matched:** 13
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4



BSA 1 Hour Digest

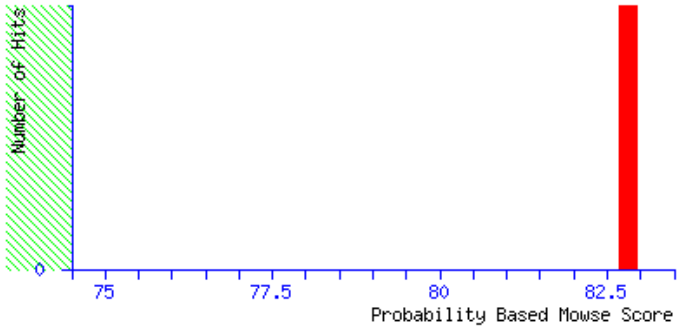
MASCOT Score: 83

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA2Jenn
Database : NCBInr 20090806 (9363125 sequences; 3207829265 residues)
Taxonomy : Mammalia (mammals) (728013 sequences)
Timestamp : 10 Aug 2009 at 21:43:39 GMT
Top Score : 83 for gi|76445989, serum albumin [Bos indicus]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 71 are significant (p<0.05).

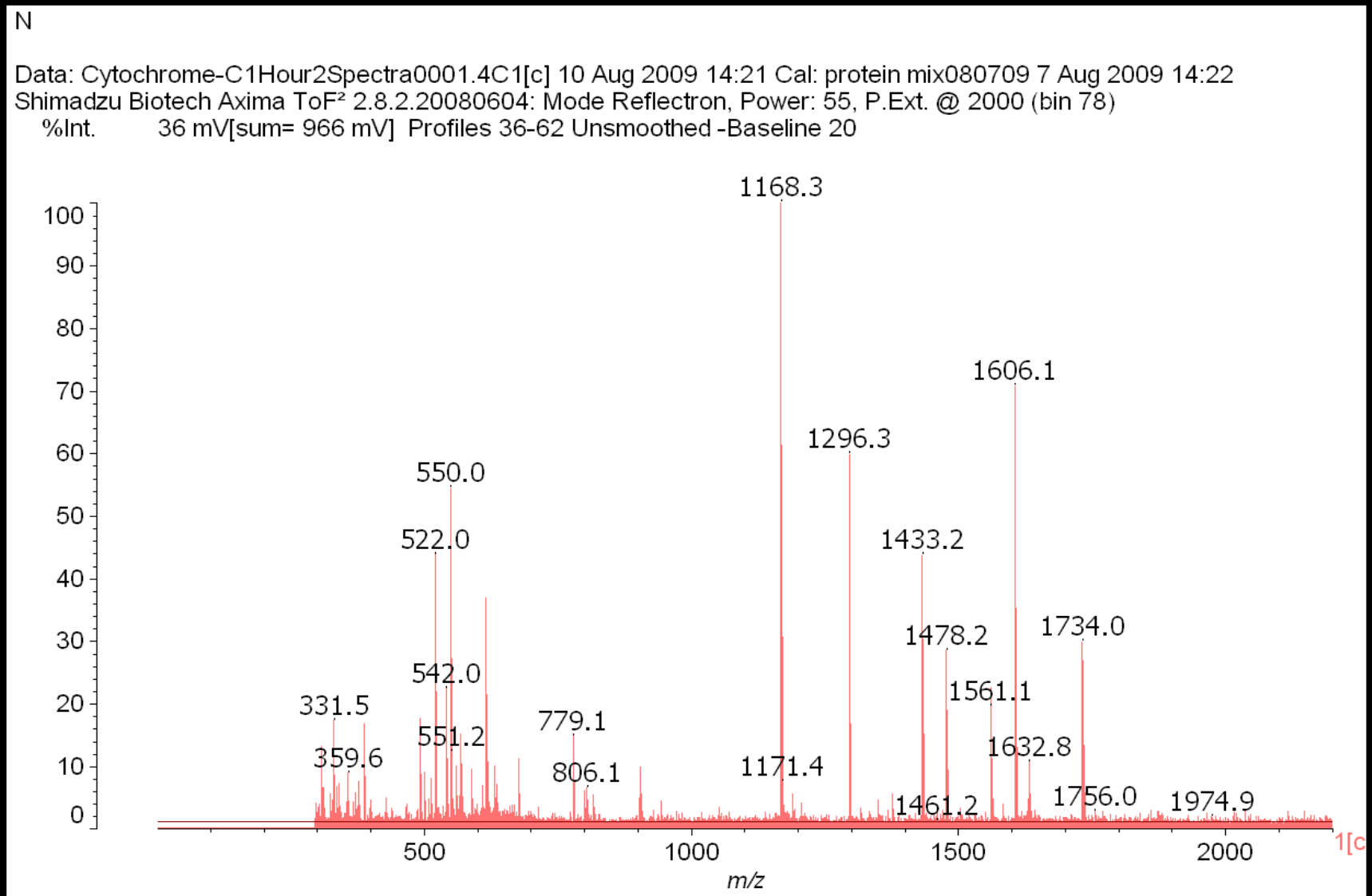


Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|76445989 **Mass:** 53890 **Score:** 83 **Expect:** 0.0038 **Queries matched:** 10
serum albumin [Bos indicus]
gi|30794280 **Mass:** 69278 **Score:** 68 **Expect:** 0.11 **Queries matched:** 10
albumin [Bos taurus]



Cyto C 1 Hour Digest

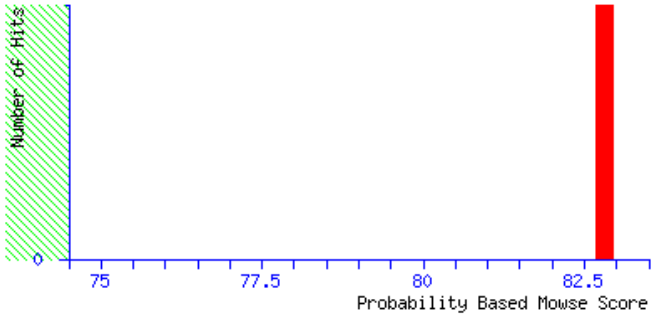
MASCOT Score: 83

MATRIX SCIENCE Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA2Jenn
Database : NCBInr 20090806 (9363125 sequences; 3207829265 residues)
Taxonomy : Mammalia (mammals) (728013 sequences)
Timestamp : 10 Aug 2009 at 21:43:39 GMT
Top Score : 83 for gi|76445989, serum albumin [Bos indicus]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 71 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

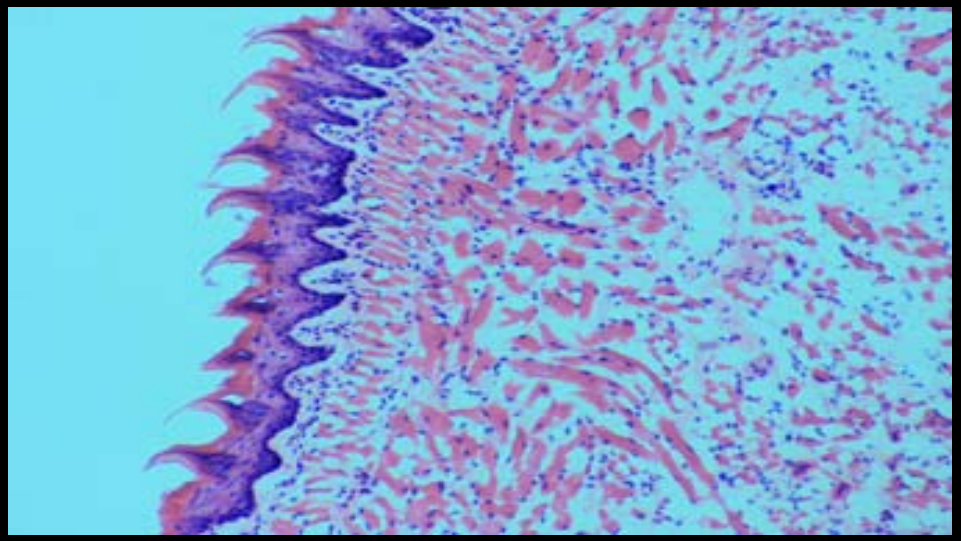
Re-Search All | Search Unmatched

1. gi|76445989 **Mass:** 53890 **Score:** 83 **Expect:** 0.0038 **Queries matched:** 10
serum albumin [Bos indicus]
gi|30794280 **Mass:** 69278 **Score:** 68 **Expect:** 0.11 **Queries matched:** 10
albumin [Bos taurus]

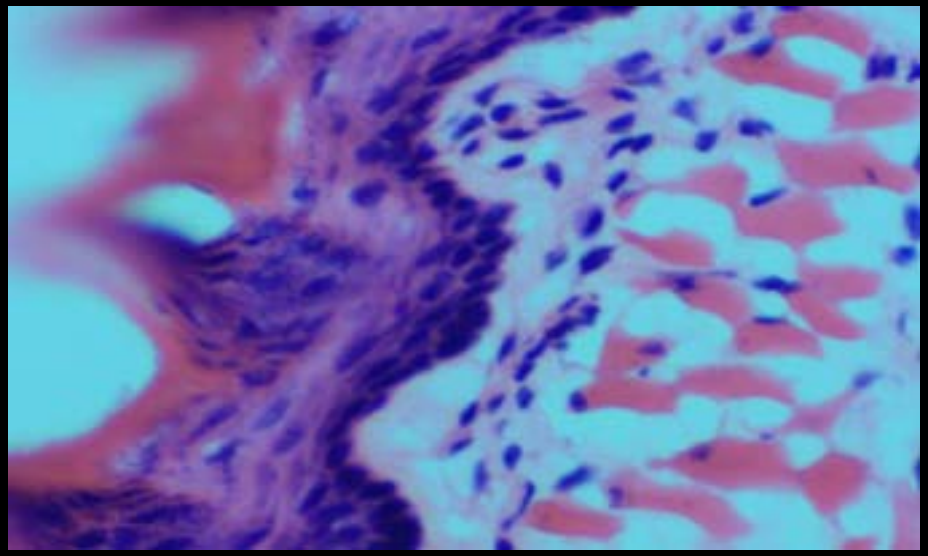
Results

BSA and Cytochrome-C were used to demonstrate proof of principle that trypsin digest could be achieved in minutes at ambient temperature.

H&E OF NORMAL MURINE TONGUE

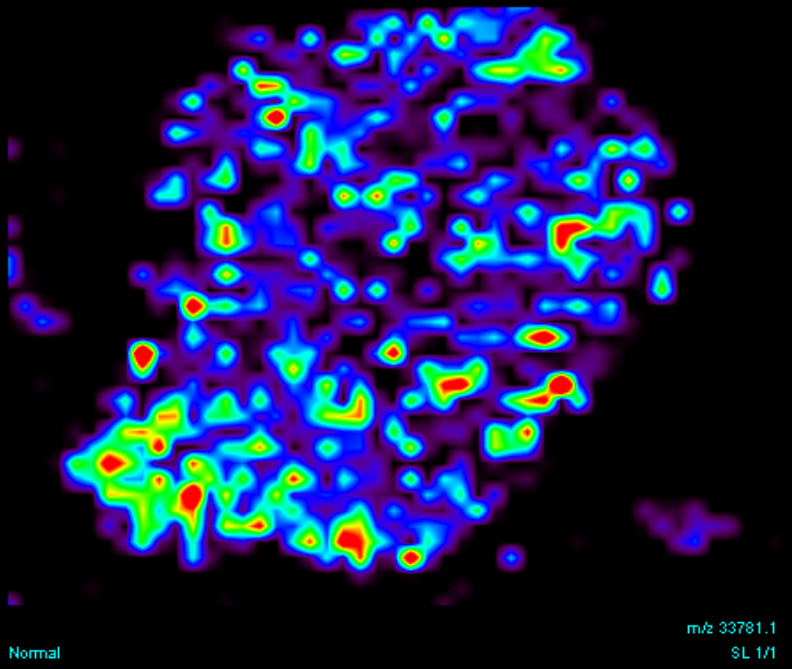


Histopathology of normal murine tongue 10X, longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.



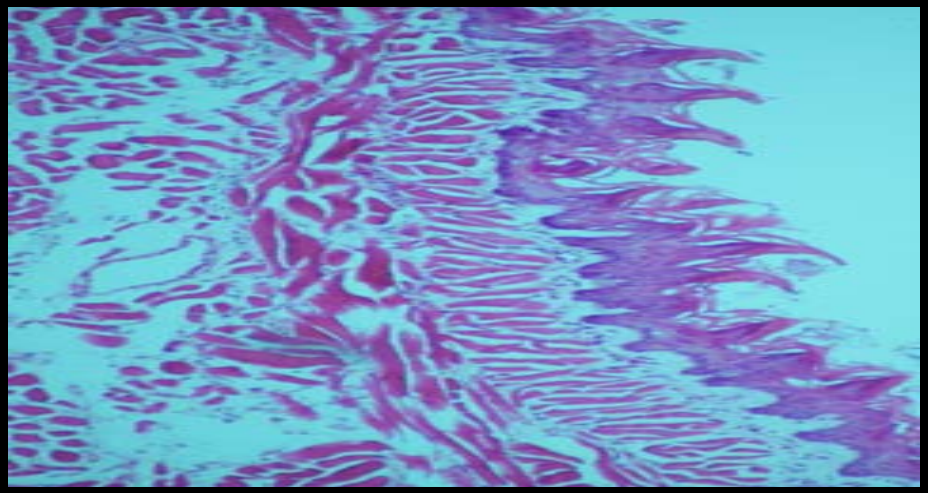
Histopathology of normal murine tongue, 63X longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.

Normal 33781.1 Da Double Charge Albumin

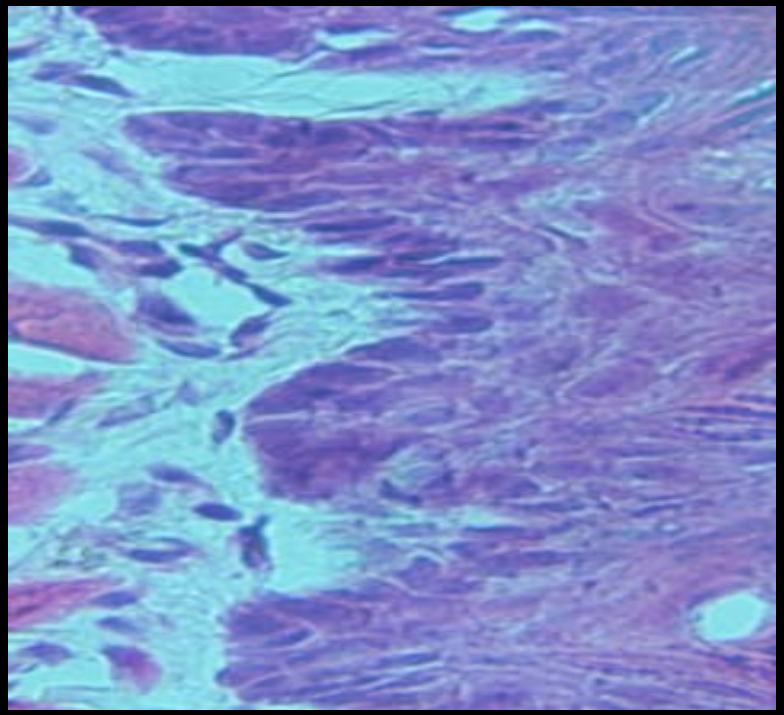


IMS of normal murine tongue, longitudinal section. Note scattered foci of residual Albumin (red) within tongue vessels.

MURINE TONGUE H&E SECTION OF 1Gy TBI

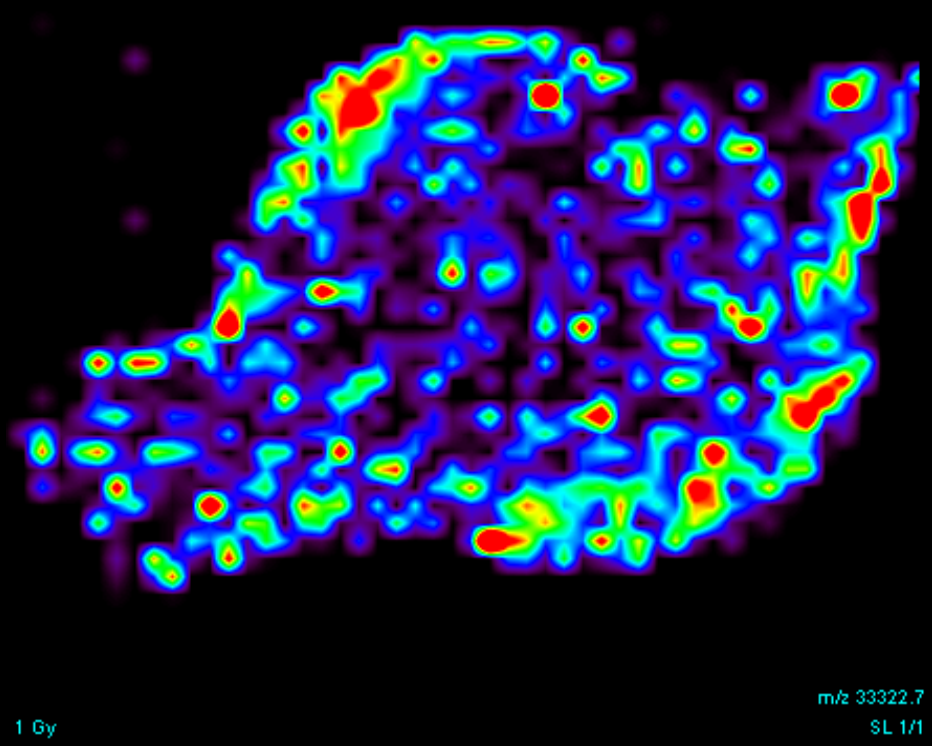


H&E section 10X one hour post 1 Gy TBI. Note destructive changes in the cornified spicules and edema in the basal layers



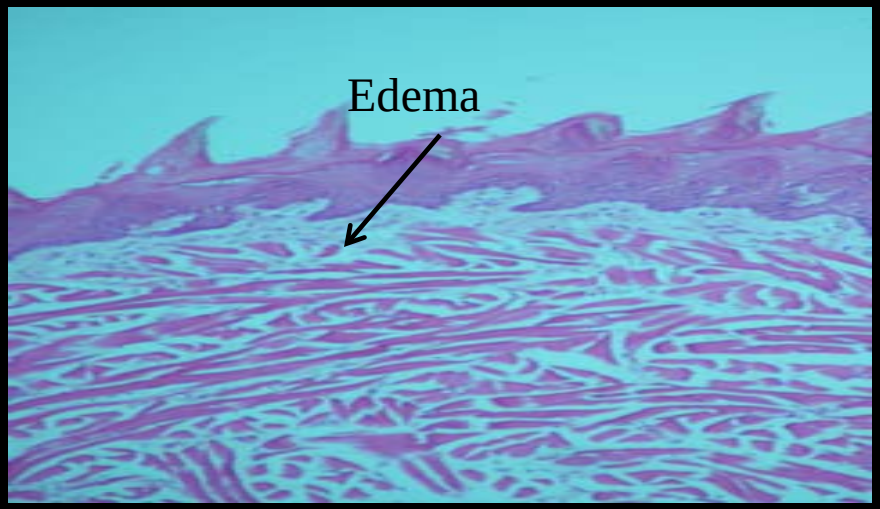
H&E section 63X one hour Post 1 Gy. Note swelling of basal cells and edema of sub-basal layer

1Gy 33,322.7 Da Double Charge Albumin

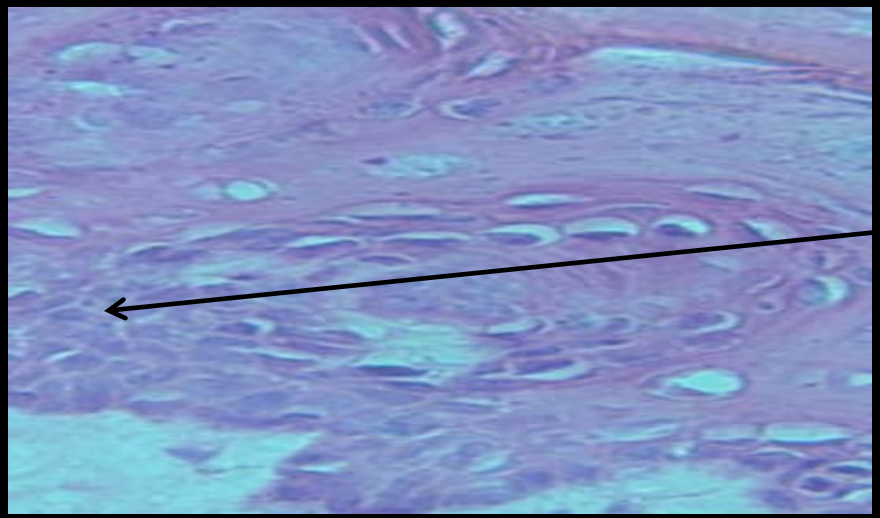


MALDI image of murine tongue one hour post 1 Gy TBI, longitudinal section. Note peripheral foci of Albumin

MURINE TONGUE H&E SECTION OF 2Gy TBI

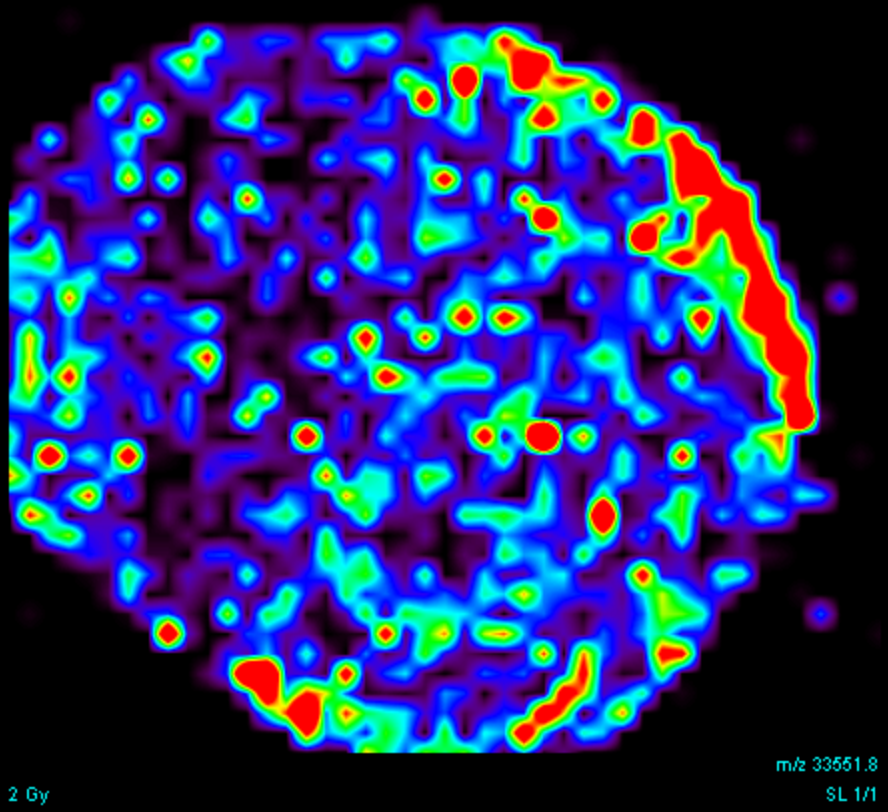


H&E section 10X of murine tongue one hour post 2 Gy TBI. Note progressive disruption of the cornified spicule layer, and increased edema of the sub-basement membrane layer



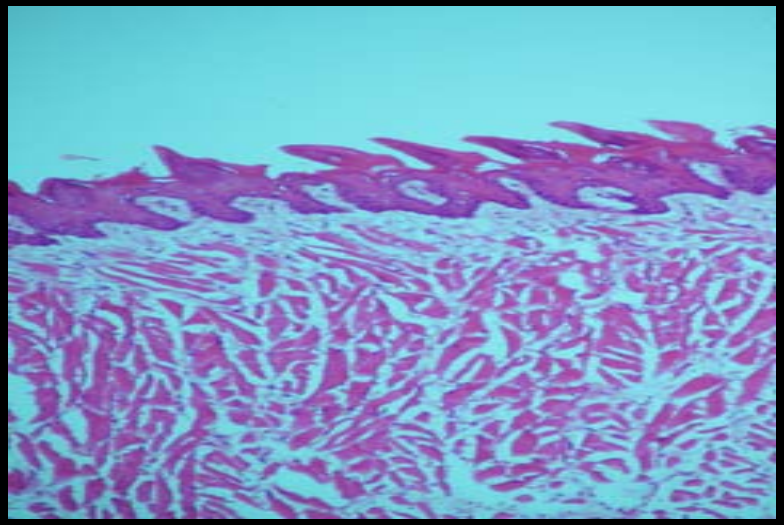
Post 2 Gy H&E section 63X. Note marked “smudging” and edema of the basal cells

2 Gy 33,551.1 Da Double Charge Albumin

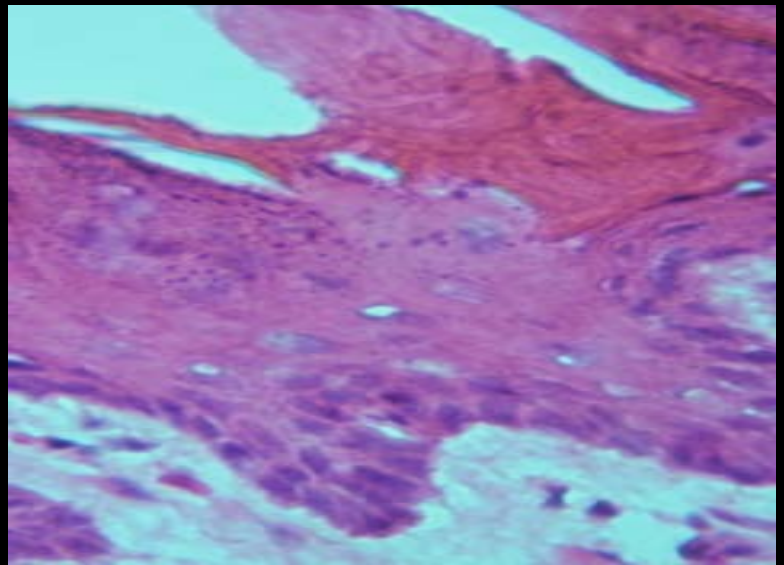


IMS, note marked increase in peripheral foci of Albumin compared to the post 1 Gy IMS (Red).

MURINE TONGUE H&E SECTION OF 3GY TBI

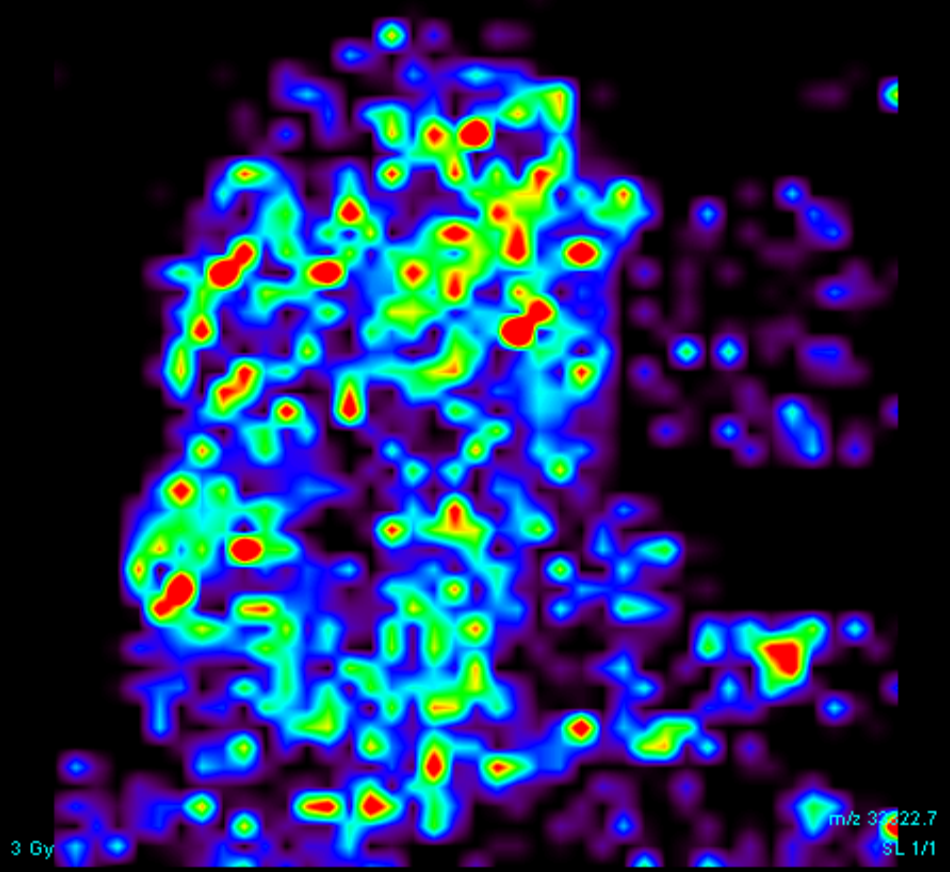


H&E section 10X of murine tongue one hour post 3 Gy TBI. Note progressive destructive changes and virtual complete loss of the cornified spicule layer corresponding to the loss of peripheral Albumin foci in the MALDI image; increased edema and damaged architecture of the center of the tongue, compared to the 2 Gy H&E section.



Post 3 Gy H&E section 63X. Note severe damage at the base of the spicules, chromatin debris, and edema compared to the 2 Gy H&E.

3Gy 33,322.7 Da Double Charge Albumin

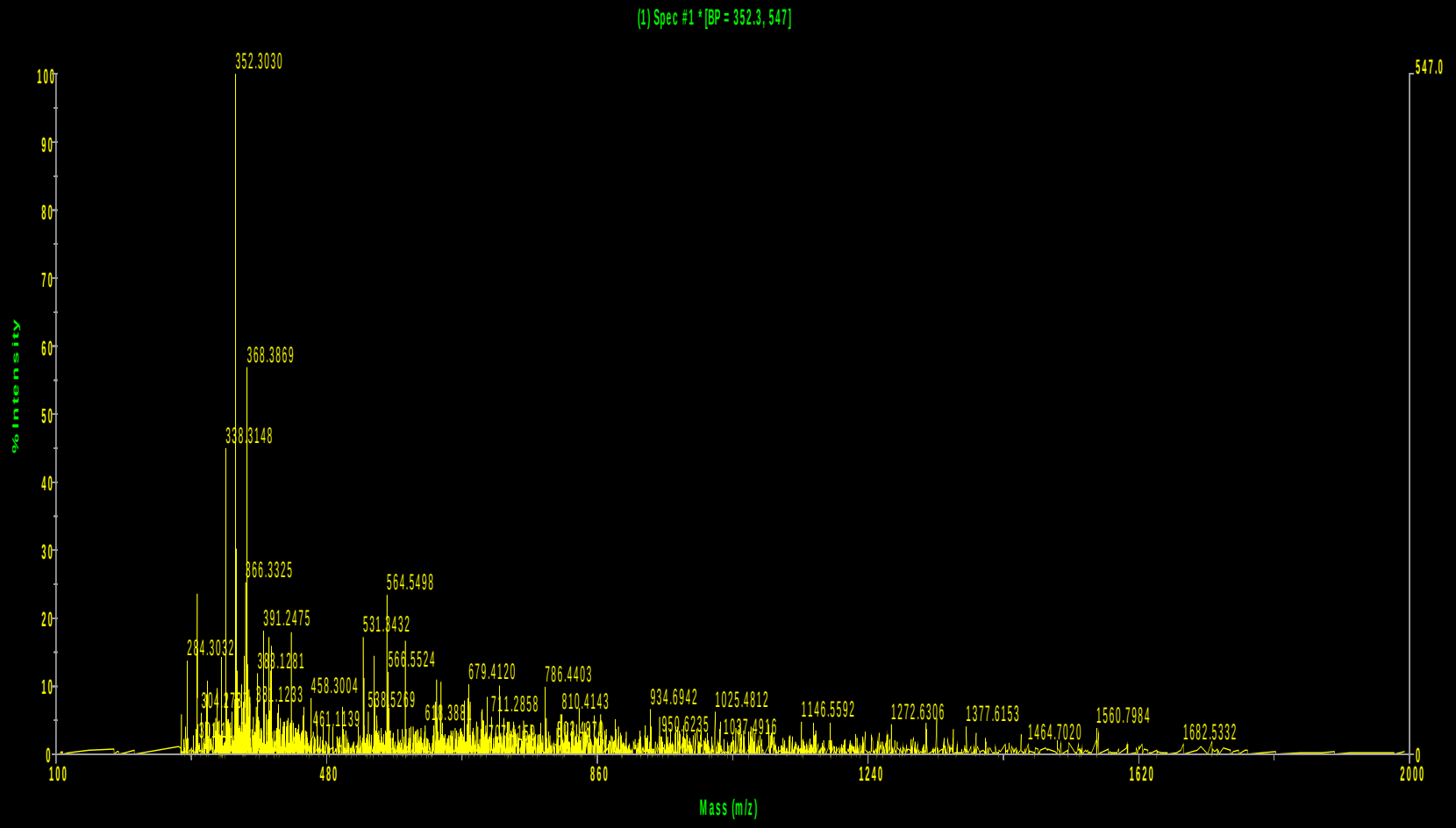


IMS of murine tongue one hour post 3 Gy TBI. Note loss of peripheral albumin and increase in central foci of Albumin. This is due to tissue loss at the periphery and increased destruction of the center of the tongue. Albumin (Red) compare with the post 2 Gy image

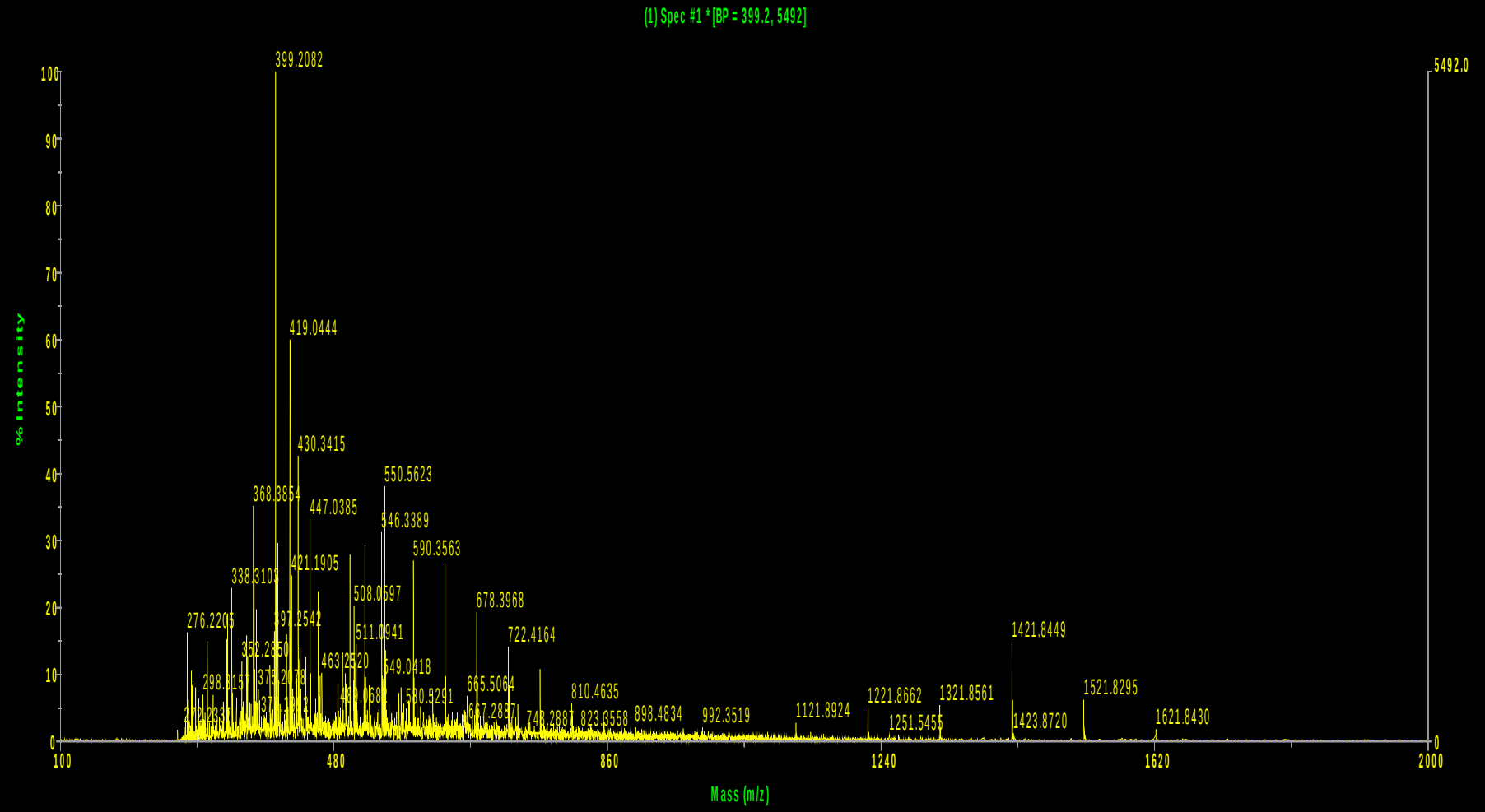
Results (LCMS)

- Successful identification of proteins from mouse tongue and heart
  - Tongue
    - Post 1 Gy, hemoglobin subunit α
    - post 2 Gy, fatty acid-binding protein adipocyte
    - post 3 Gy, hemoglobin α chains was identified. Albumin was identified post 1, 2 and 3 Gy.
  - Heart
    - Actin, alpha cardiac muscle 1 OS=Mus musculus

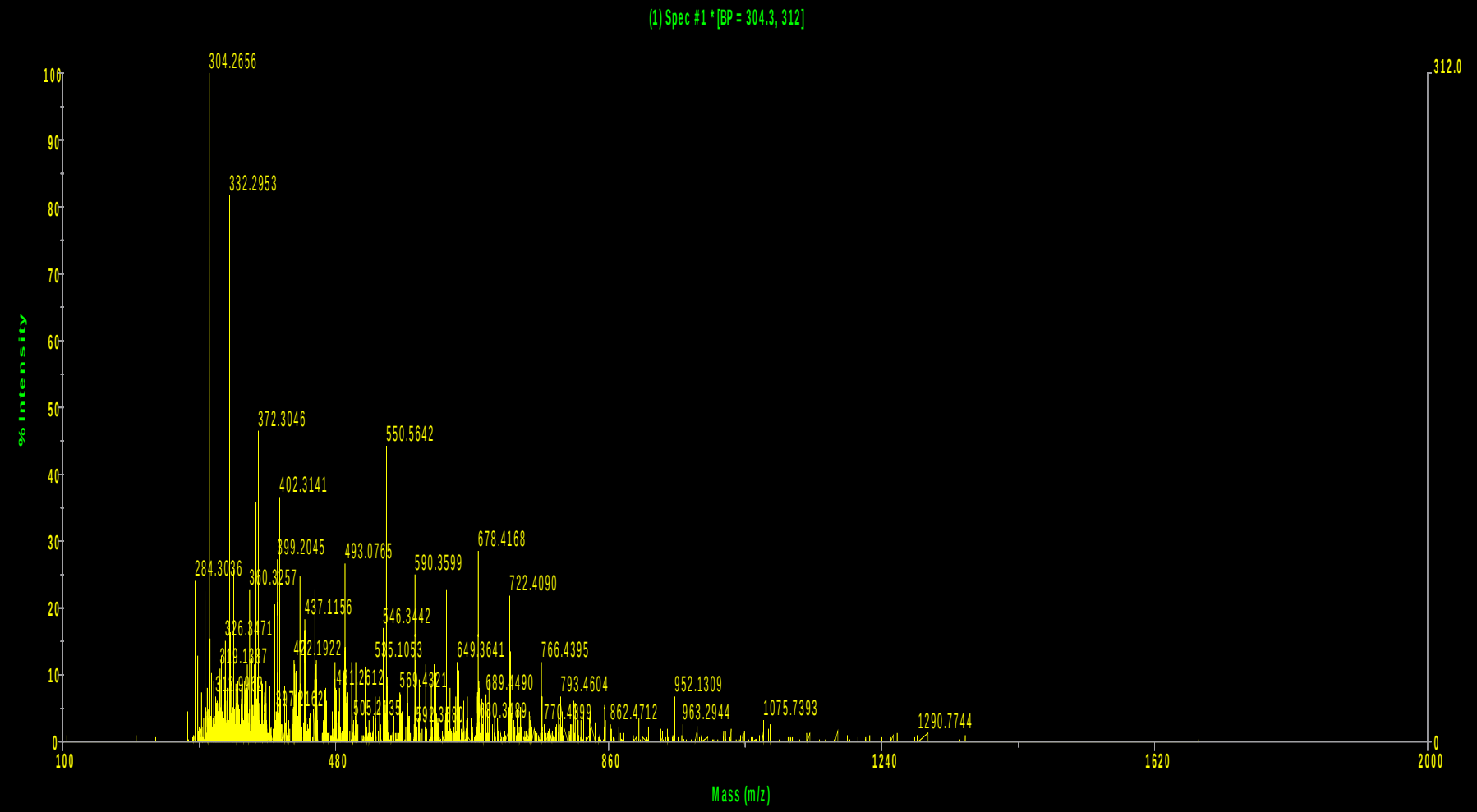
LCMS spectrum tongue normal



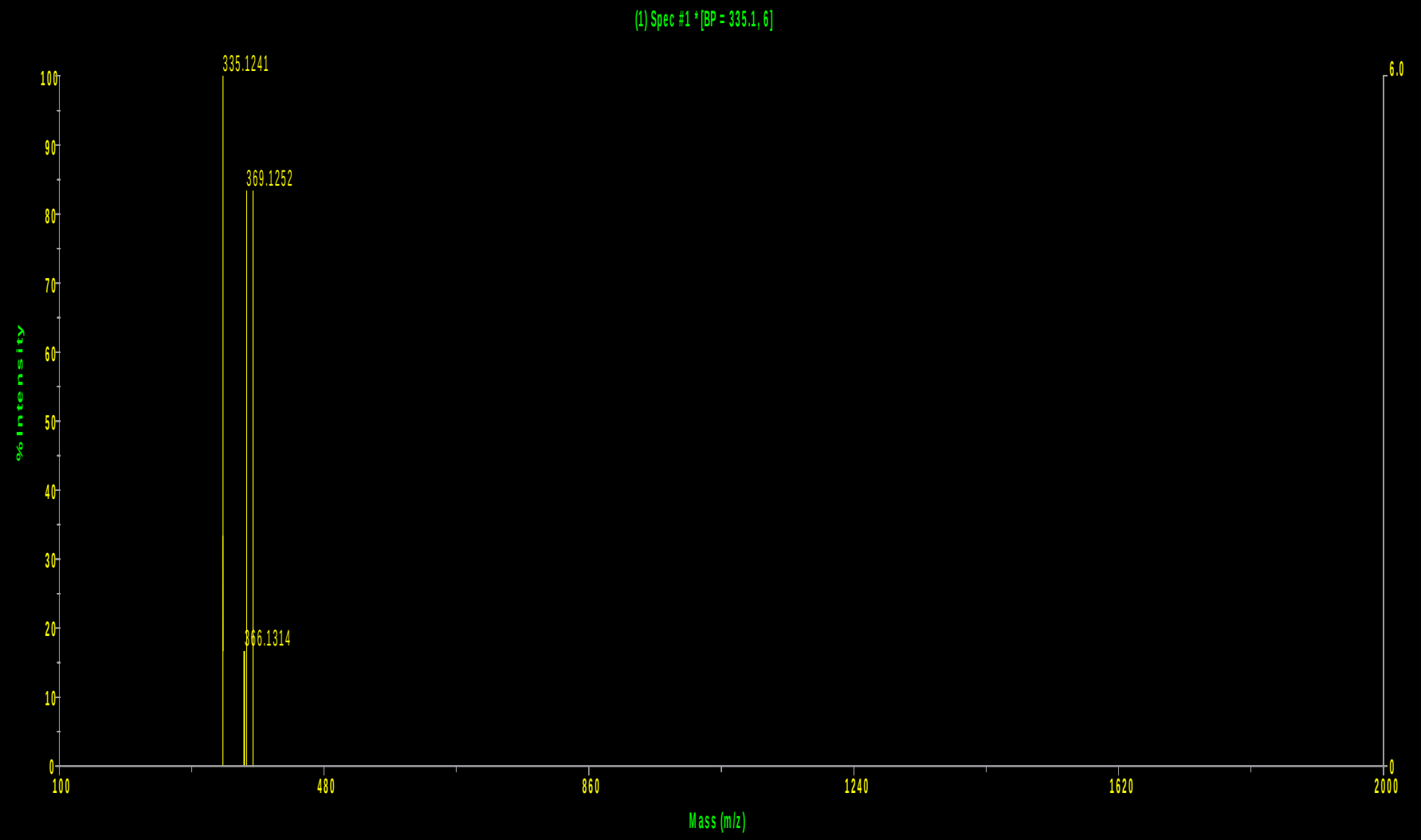
LCMS spectrum tongue post 1 Gy



LCMS spectrum tongue post 2 Gy



LCMS spectrum tongue 3 Gy



Normal Mouse Tongue

Q9CXH5 – Mus :hemoglobin, beta adult major chain

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9JJ20 – Mus : 14-3-3 protein sigma; Sfn MGI 1891831 Synonyms: Mme1 EMBL AAF36093.1

Q64475 Mus: histone H2B type 1-B (h2B-143)H2B1B_.

Q9Z1R9 Mus: - trypsinogen 16

BAC33789 –Mus: Sgol1 shugoshin-like 1 [(S. pombe) (AK049517.1)]

Mouse Tongue 1hr post 1 GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

Q9CXH5 – Mus :hemoglobin, beta adult major chain

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9Z1R9 - Mus: - trypsinogen 16

Mouse Tongue 1hr post 2GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9CY54 - Mus: hemoglobin, beta adult major chain Symbol: Hbb-b1

Q9CY06 - MUS:hemoglobin, beta adult major chain

P02088 – Mus: HBMS Hemoglobin subunit beta

Q3UJH8 –Mus: glutamate oxaloacetate transaminase 1 EC=2.6.1.1

P11404 Mus: fatty acid-binding protein, heart FABPH.

P04117 – Mus: FABPA Fatty acid-binding protein, adipocyte (AFABP) also (Adipocyte lipid-binding protein) (ALBP)

S02654; **S00195** Mus: malate dehydrogenase DEMSMC - (EC 1.1.1.37), cytosolic – mouse, L-lactate dehydrogenase

Q3UBW7 – Mus: transferrin

Mouse Tongue 1hr post 3GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9CY12 – Mus: hemoglobin, beta adult major chain

Q3USS4 – Mus: glial fibrillary acidic protein

Q3TWV0 – Mus: vimentin
JC4030 – Mus: DnaJ-like protein MTJ1

Q9QZ05 (E2AK4) Mus: eukaryotic translation initiation factor 2-alpha kinase 4 EC=2.7.11.1

Conclusion

Trypsin digestion with PCT is rapid with higher yield than conventional methods.

High pressure (45K psi) PCT increases tissue protein extraction yields.

The application of extraction solvents will further increase both yield and specificity of extracted species.