NCBS – MS FACILITY:

PROTOCOL FOR THE DETERMINATION OF INTACT MASS OF PEPTIDE/PROTEIN SAMPLE

AIM: Determine the intact mass of the Peptide/protein sample.

The process is described in three parts.
First part describes the instrument set up and chemicals required for the analysis.
The second part describes the calibration of the instrument using horse heart Myoglobin.
The third exemplifies the determination of intact mass of a protein sample.

PROCEDURE:

I. Instrument and Chemicals

Waters Ultima QTOF (former Micromass)
Software - MassLynx 4.1

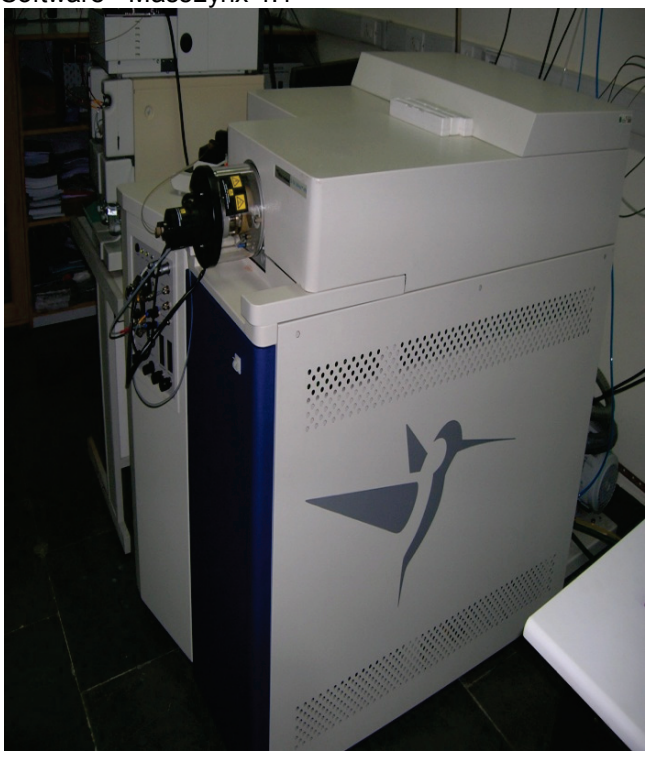

Figure 1) Waters Q-TOF Ultima mass spectrometer (former Micromass)

Chemicals:
Myoglobin from Equine Heart (Sigma Aldrich Cat#M1182
Water (LC/MS grade-Fluka)
Acetonitrile (LC/MS grade –Fluka)
Methanol (LC/MS grade –Fluka)
Formic Acid (LC/MS grade - Fluka)

Instrument requirements:

1. MS Tune file – C:\qtof_users\karthik.pro\acqudb\protein_hhm_pos100720.ipr
2. Tune parameters are summarized in the following Excel file (QTOF computer -C:\Documents and Settings\labusers\My Documents\karthik results\MS parameters)
   – MS Parameters for reference
3. Put on the API gas(Ultra High pure Nitrogen) and Collision gas(Argon)
4. Change the capillary voltage from 0 to 3.3 and Desolvation temperature from 20 to 200(these parameters are kept at 0 and 20 when the instrument is idle.
5. 250µl syringe and Infusion pump

II. Calibration of the Instrument with Horse Heart Myoglobin

A. Prepare the following solutions:

1. Spray mix – 0.1% Formic acid in 50:50 acetonitrile: water
2. Myoglobin stock solution – 100µM in water(stored in -20C)
3. 5µl of the above stock diluted to 1ml using spray mix - to give 250fmoles/µl
4. 50:50 methanol: water (for cleaning syringe)

B. Perform a Blank run, this serves as a primary check:

1. Clean the syringe 3-4 times with 50:50 methanol: water.
2. Fill the syringe with spray mix and allow it to run for a few minutes.
3. Press Acquire (on the tune page) – give a filename and add details in the text box. Set the mass range from 100 to 4000 m/z. Acquire around 200 scans
4. Allow the spray to stabilize. You will see some small masses (156.6676 and 213.2656- corresponding to the spray mix solvent) in the spectrum. Check for response, ideally a response of about 150 to 200 ions/scan is good. Record the spectrum – for reference (Figure 2)

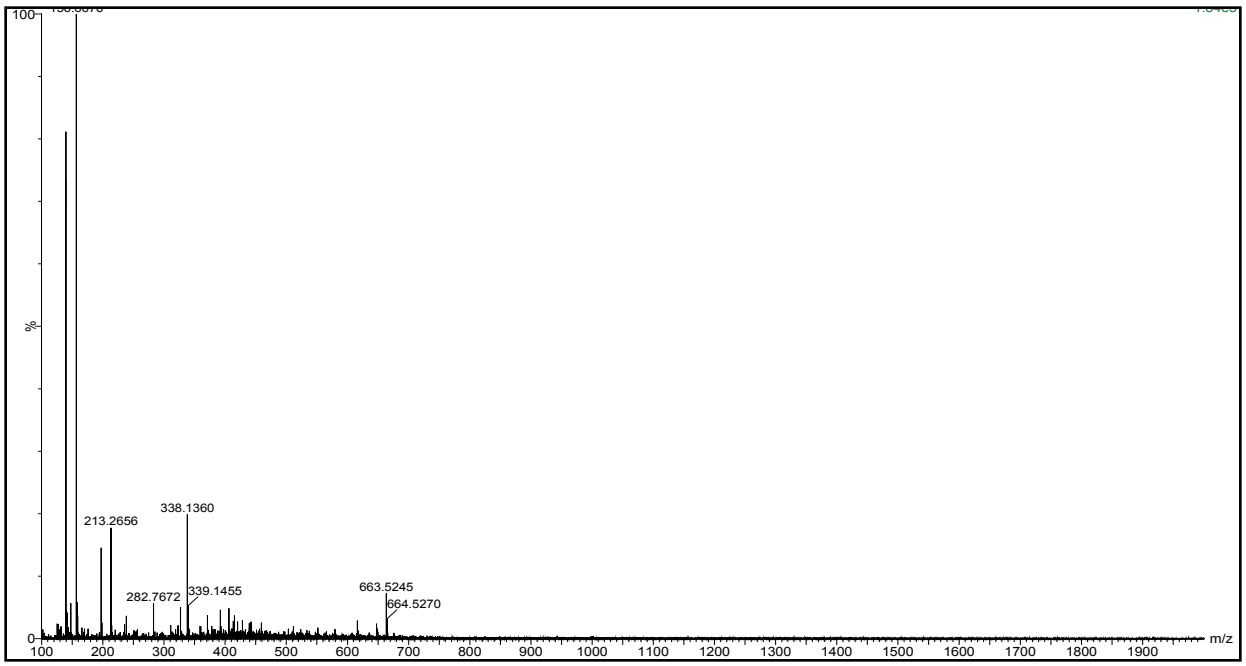

Figure 2) Representative spectrum of a blank solvent (acetonitrile: water 50:50 (v:v) with 0.1% formic acid)

C. **Set the instrument to uncalibrated state:**
Go to **Calibration** on the main menu and open **Uncal.cal**

D. Calibration with Horse Heart Myoglobin:

1. Fill the syringe with 250fmoles/µl of myoglobulin solution, and run the sample for a few minutes.
2. Press Acquire (on the tune page) – give a filename for ex.120705_MYO_1 and add details in the text box. Set the mass range from 500 to 2000 m/z.
3. Allow the spray to stabilize. You will see a charge series (typical of a protein-Figure2) in the spectrum. Check for response, ideally a response of about 150 to 200 ions/scan is good(excluding m/z 616.5276- corresponding to Heam)

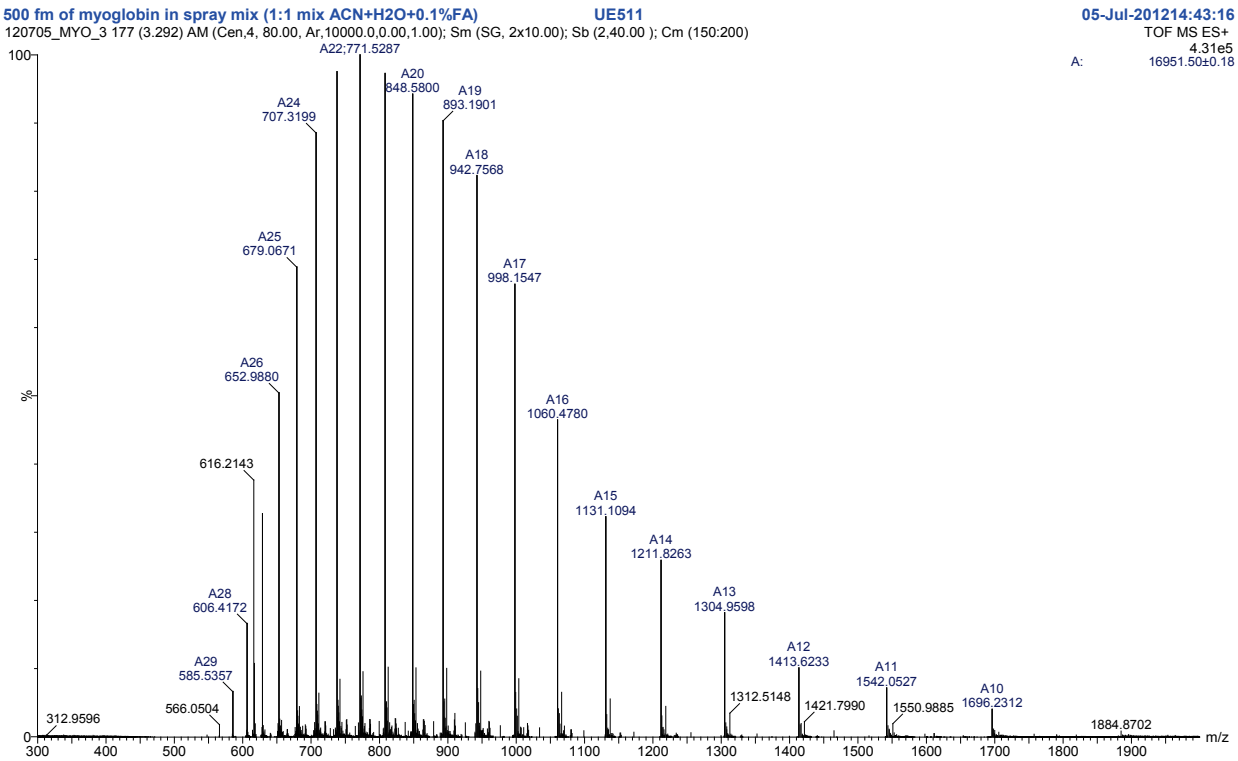

Figure 3) Representative spectrum showing the charge series of Horse Heart Myoglobin at a concentration of 250 fmol/µl.

4. Stop the acquisition.
5. Re- Acquire – give a calibration filename for ex.120705_MYO_Cal_1.and add details in the text box. Set the mass range from 500 to 2000 m/z.
6. Combine necessary amounts of scans to get a better spectra (Figure 4),

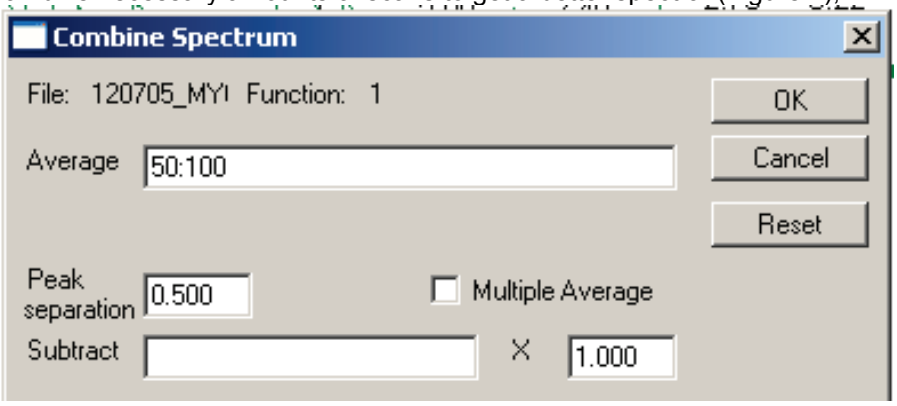

Figure 4): Screen shot of the Combine/Integrate spectra process menu of MassLynx software

7. Process the combined spectra, i.e.do a background subtraction, smooth and centroid using Mass measure (Figure 5) from the process menu. Record the spectrum – for reference.
8. Save the spectrum – saves as AccMass**xxx**
9. Go to **Calibration** on the main menu
10. Here go to **Calibrate** ⟶ **from File** ⟶ **Display Calibration Graphs –** browse to the file for ex.**120705_MYO_Cal_1** ⟶ go to history, you will find the saved AccMass file , click on it and say OK
11. A window opens with a report of number of matches and RMS residual value. To improve the calibration, matches which are totally off can be removed by zooming and right clicking on the

reference and data file uncalibrated (m/z match which was off will be removed).Go to display default to see the improvement.

12. Then once satisfied with the number of matches and RMS residual value (at least 15-16 good matches out of 21 and RMS residual value of less than $4e^{-1}$) - go to **finished** and accept calibration, and do file save as- Save file with date-format YYMMDD_MYO_Cal.cal.
(Important never save over the Uncal.cal file , always use **save as**)
13. After calibration the mass of Myoglobin can be determined (deconvolution) by using the spectrum after mass measure should be used. Go to **Process** → **Components** → **Find auto** (Figure 6). Here give the mass range, for ex. 6000 to 20000 for Myoglobin and say find First. It returns the mass of the protein.

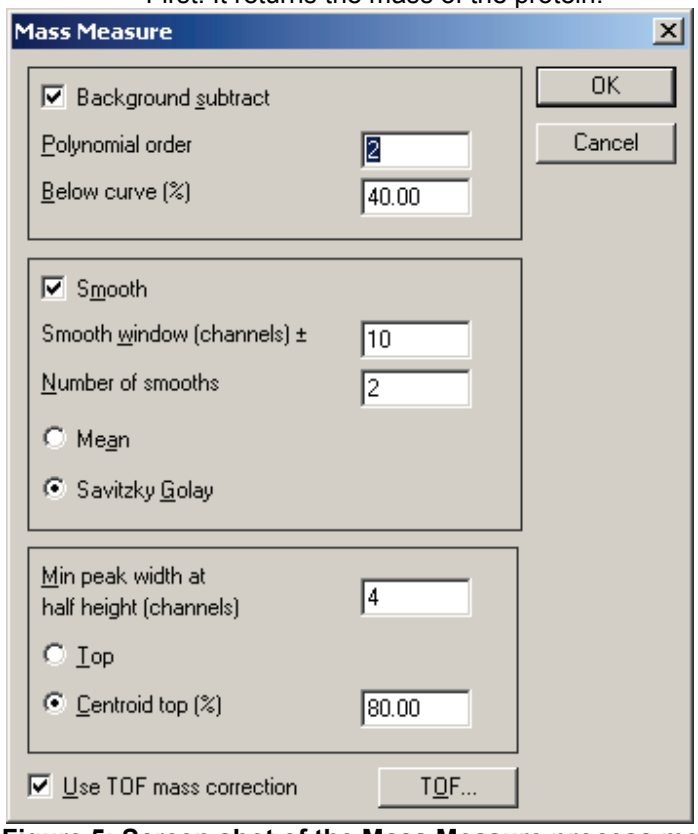

Figure 5: Screen shot of the Mass Measure process menu of MassLynx software

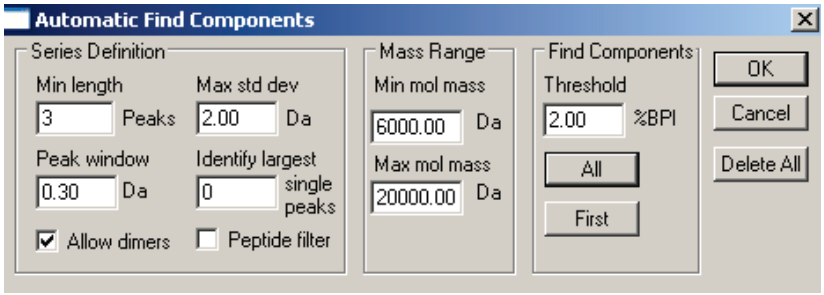

Figure 6: The Deconvolution menu of MassLynx

14. Mass of the protein can also be determined by using **maxEnt** from the process menu**,** after combining spectra.
15. Find the mass error in ppm (less than 10ppm is acceptable)
Expected mass of Horse heart Myoglobin is 16951.49 Da

III. Determination of Intact mass of unknown sample

1. Requirements:
   Obtain the following information from the user:
   Amount of protein/compound in each sample, additive/contaminants/buffer composition of sample, source of the sample, processing steps applied to purify the sample,
   Protein sequence, Monoisotopic mass of the protein (can be calculated from the sequence) or molecular formula of the compound, any other information such as SDS PAGE gel picture or structure if available.
2. Vortex the sample for 30mins at 1500rpm, centrifuge at 5000rpm for 5 min
3. Supernatant to be taken for further dilutions using Spray mix.
4. Run a blank infusion with spray mix
5. Press Acquire (on the tune page) – give a filename and add details in the text box. Set the mass range from 100 to 4000 m/z. Acquire around 200 scans.
6. Allow the spray to stabilize. You will see some small masses (156.6676 and 213.2656- corresponding to the spray mix solvent) in the spectrum. Check for response, ideally a response of about 150 to 200 ions/scan is good. Record the spectrum – for reference (Figure 1)
7. Sample analysis -Start with higher dilution 1:1000 ratio with Spray mix
8. Perform an initial survey scan with broader mass range to check the response of the compound being injected. Press Acquire (on the tune page) – give a filename and add details in the text box. Set the mass range from 100 to 4000 m/z. The mass range can be reduced if required after seeing the response.
9. Allow the spray to stabilize. Check for response, ideally a response of about 150 to 200 ions/scan is good.
10. If the response is low, dilutions like 1:200 or 1:50 ratio can be prepared accordingly.
11. Acquire with the dilution which gives good response – give a sample filename with user name and sample ID (for example120705_NITYA_Sample_1) and add details in the text box.
12. Combine necessary amounts of scans to get a better spectra (Figure 4),
13. Process the combined spectra if necessary, i.e.do a background subtraction, smooth and centroid using Mass measure (Figure 5) from the process menu. Record the spectrum – for reference.
14. The mass of the sample if it is a protein, can be determined (deconvolution) by using the spectrum after mass measure should be used. Go to **Process** ⟶ **Components** → **Find auto** (Figure 6). Here give the mass range depending on the expected mass of the sample, and say find First. It returns the mass of the protein.
15. Mass of the protein can also be determined by using **maxEnt** from the process menu**,** after combining spectra.

Best representative spectra with significant masses are provided to the user along with the results.

Example: Determination of intact mass of β- casein(C6905- Sigma Aldrich, from bovine milk)

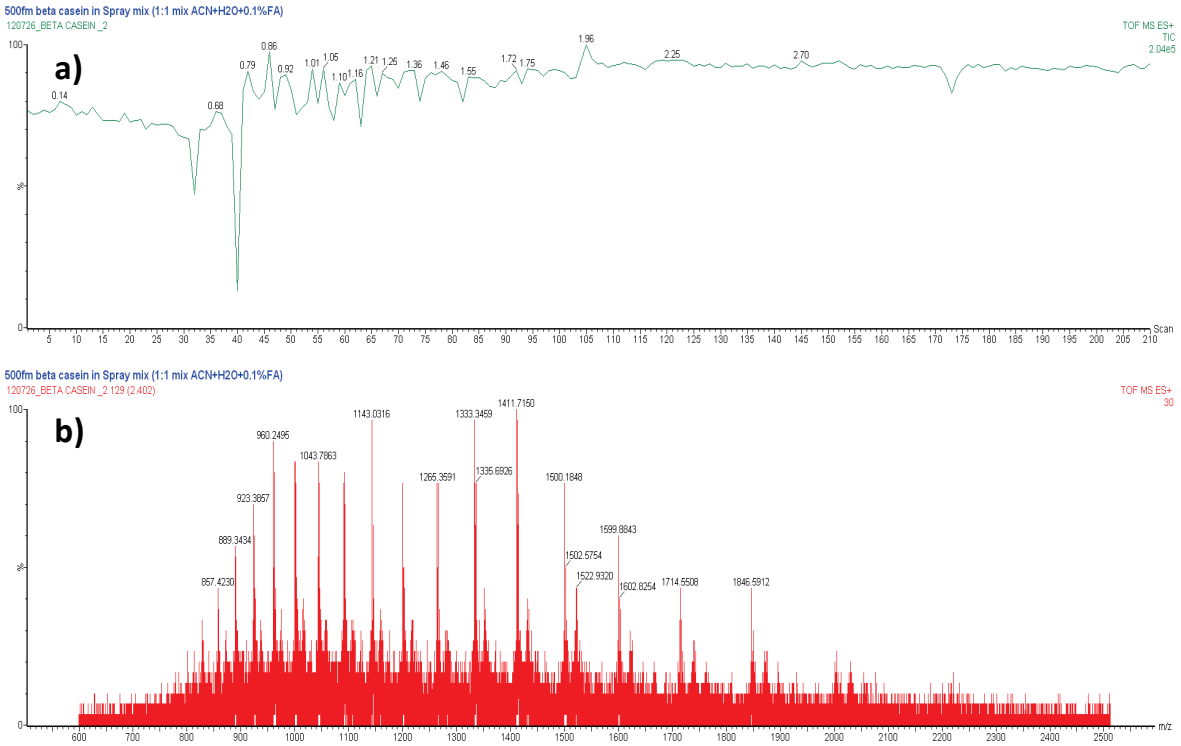

Figure 7) Direct infusion experiment of β-casein (C6905- Sigma Aldrich) with a concentration of 500fmol/µl in acetonitrile / water (1:1) with 0.1% formic acid. a) Total Ion chromatogram b) Single scan spectra

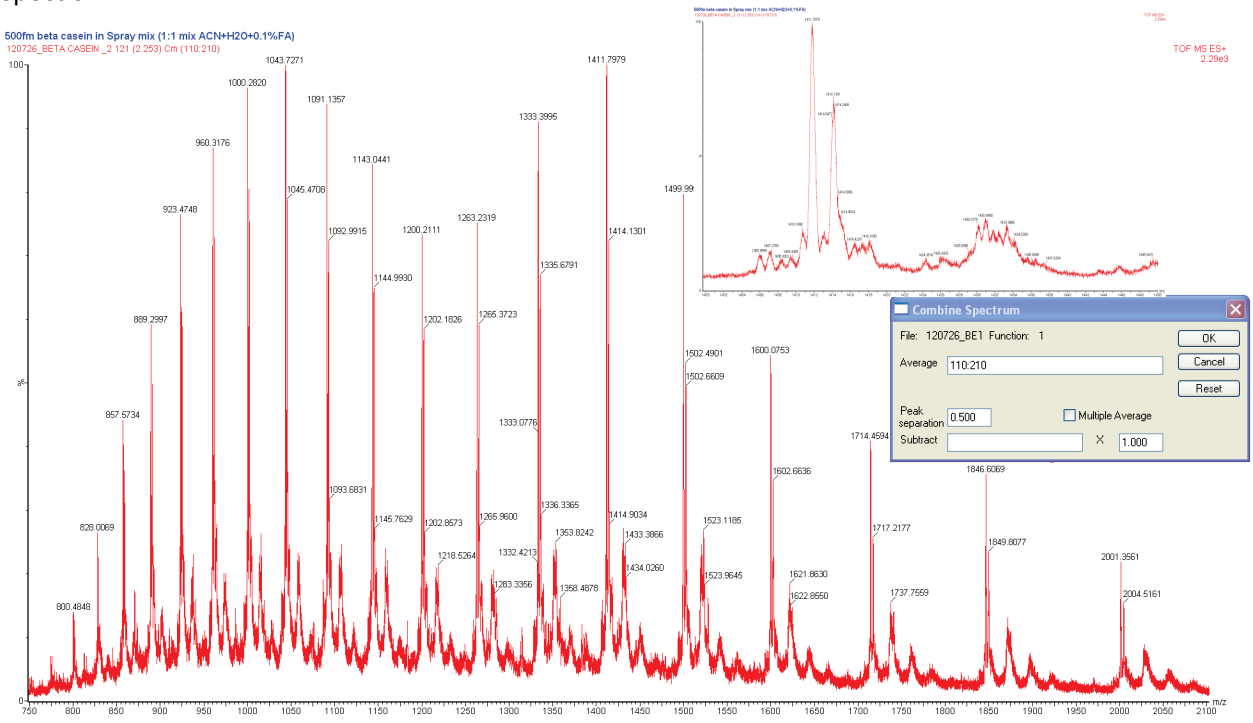

Figure 8) Original spectrum obtained by combining 100 scans. Inlet for the m/z region 1400 – 1450 indicates two main components. Screenshot of used parameters

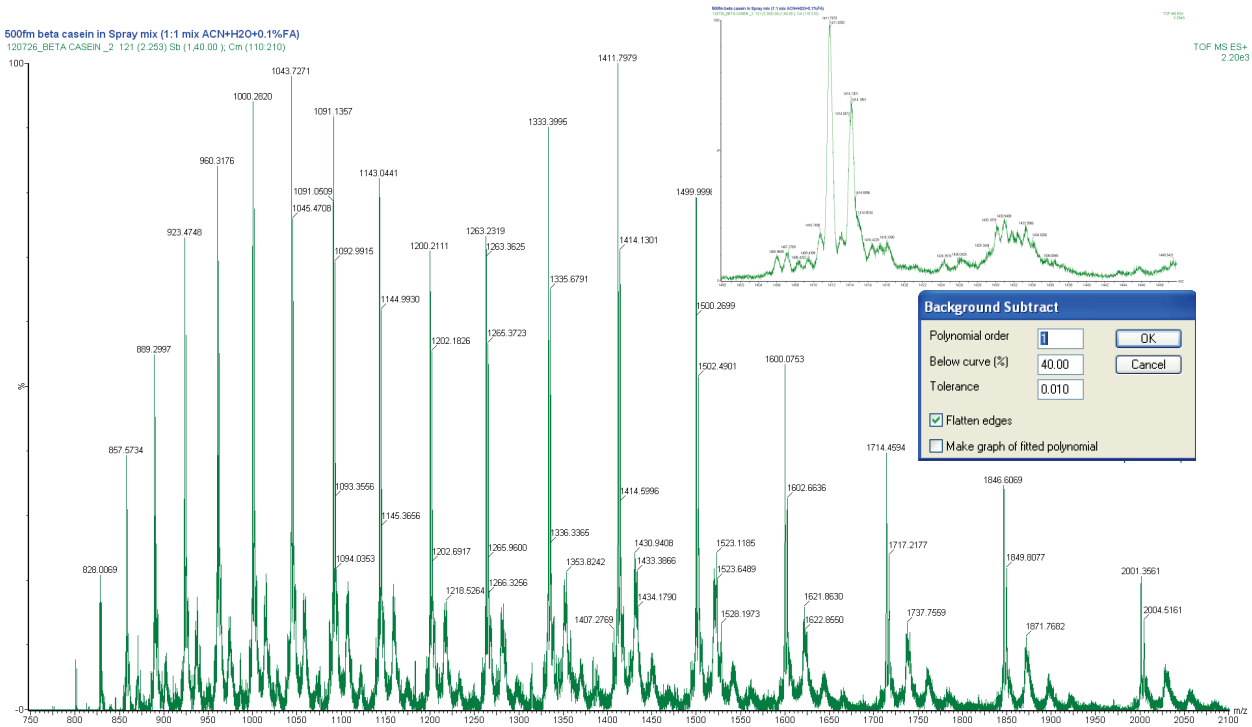

Figure 9) Processed spectrum obtained after background subtraction. Inlet for the m/z region 1400 – 1450. Screenshot of used parameters.

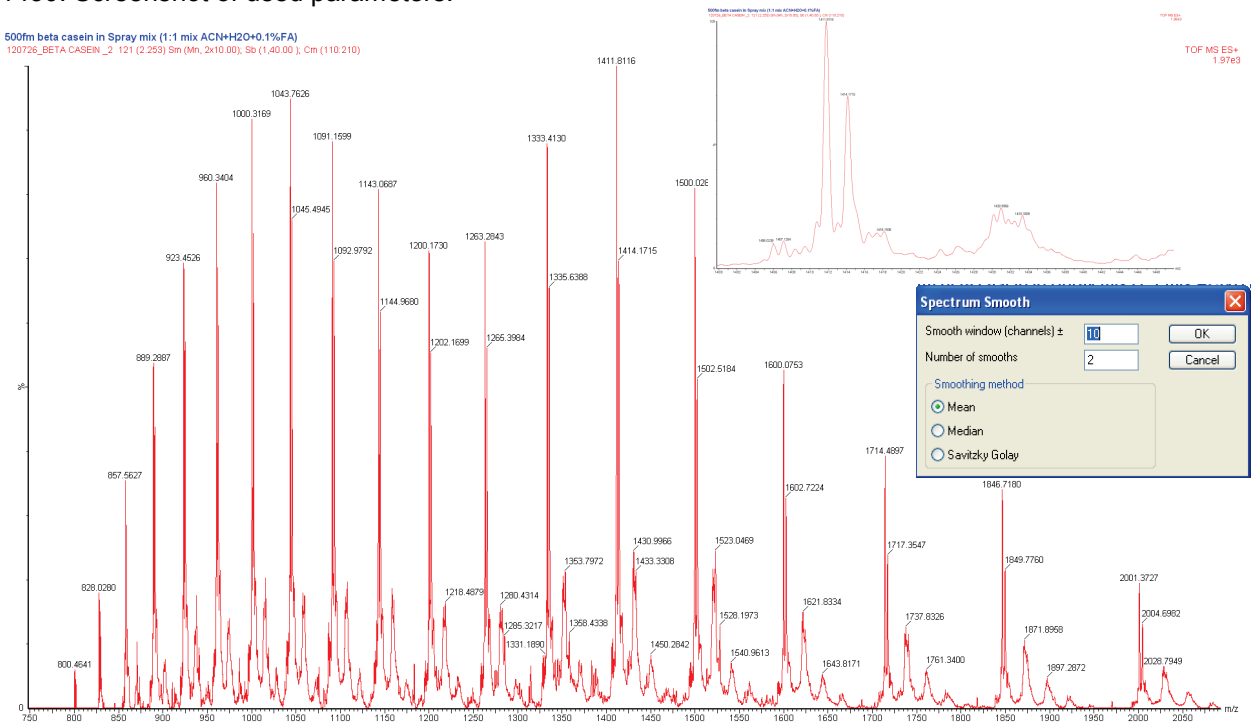

Figure 10) Processed spectrum obtained after smoothing. Inlet for the m/z region 1400 – 1450. Screenshot of used parameters.

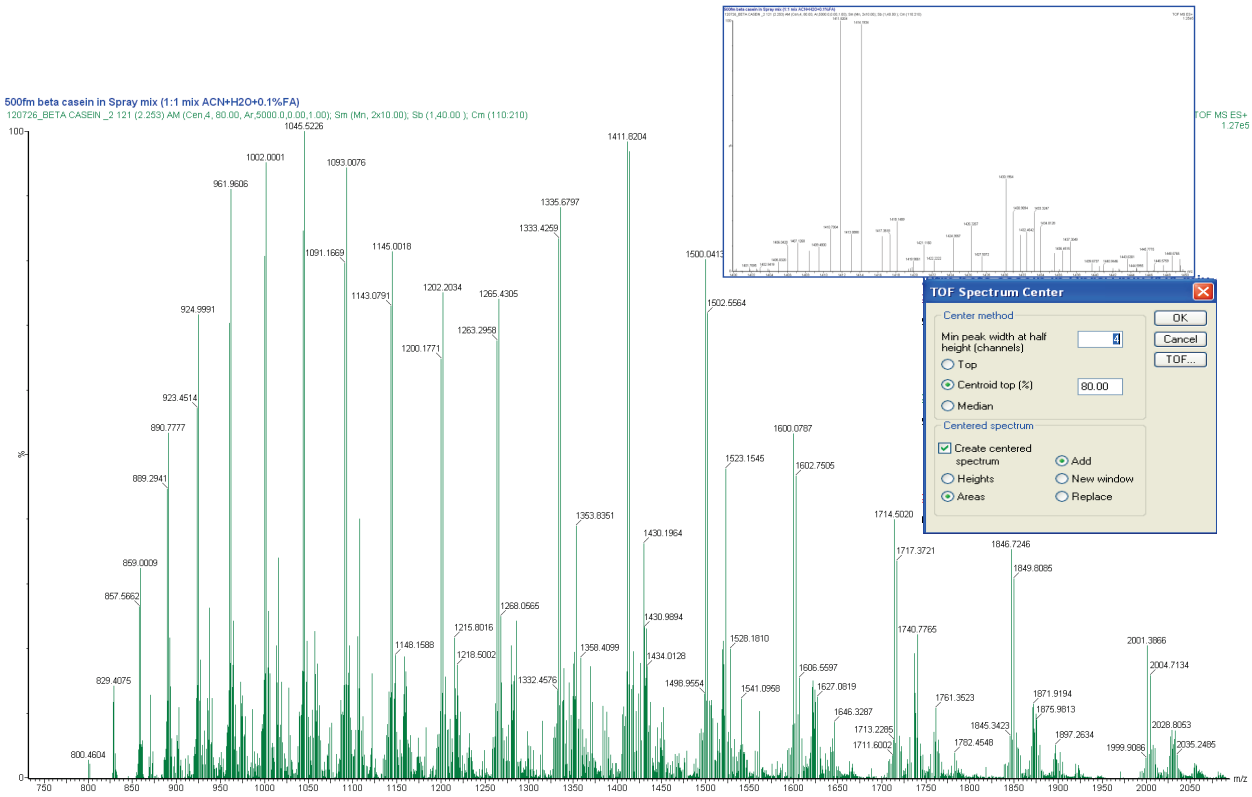

Figure 11) Processed spectrum obtained after performing centroidization processing. Inlet for the m/z region 1400 – 1450. Screenshot of used parameters.

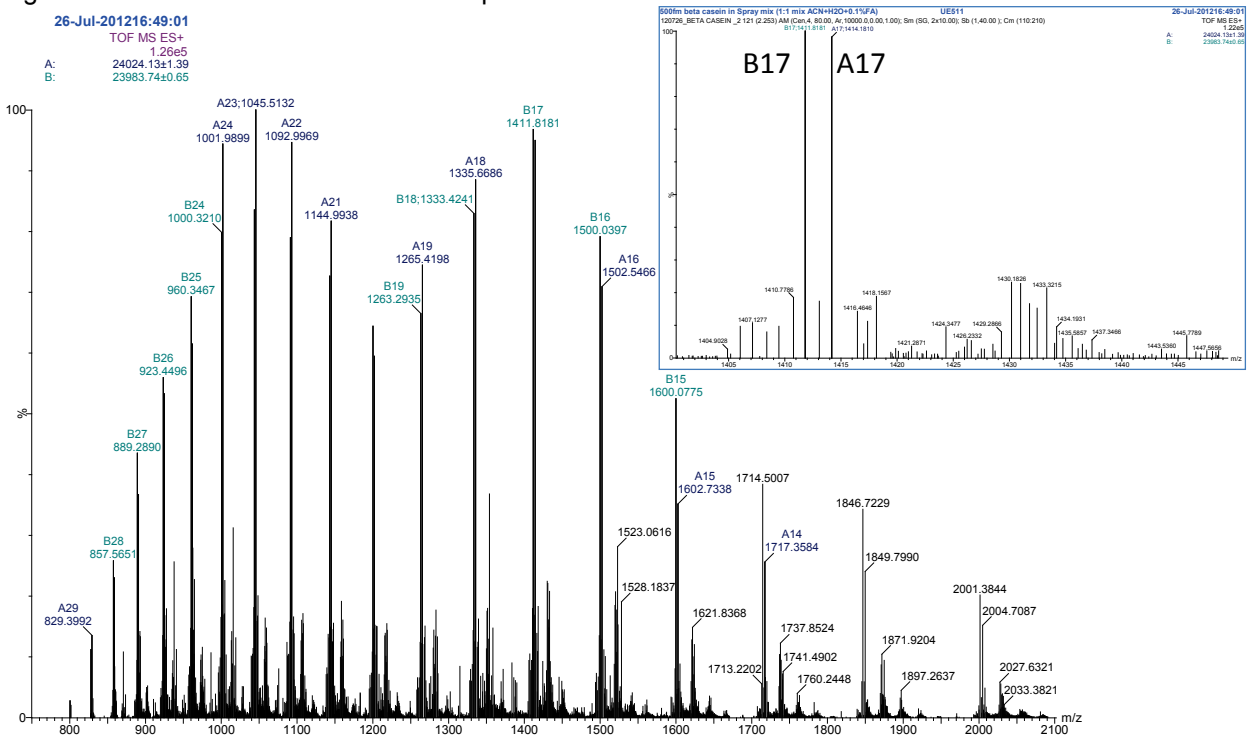

Figure 12) Spectrum of β-Casein after assigning charge series using MassLynx deconvolution process menu (Fig 5).

In our experimental approach we could identify two major genetic variants as described in [1]. Genetic variant A1 was detected with experimental molecular mass 24092.1±1.4 and A2 was detected with molecular mass of 2398.7±0.7.

References:

[1] J Proteome Res. 2009 March ; 8(3): 1347–1357. doi:10.1021/pr800720d

please refer to table 2 on page 26 :β-casein genetic variants,
Mass 24024.13 corresponds to variant A1
Mass 23983.74 corresponds to variant A2
Genetic variant B with a molecular mass of 24092.34 could not be identified in our experiment.