Lipidomics workshop 11/12.9.2019

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Slides 1-20 are modified based on *www.msg.ucsf.edu/agard/maldi/IntrotoMS.ppt*,

downloaded 17.05.2017

Aims:

If you/we do lipidomics, understand

- what you do
- what we do
- what you get
- what you can do with it

Schedule:

Wed.:

- Basics of MS
- Lipidomics MS in details
- Sample preparation
- Principles of data recording and processing

Thu.:

- Lab: Sample preparation
- Lab: Data recording
- Comp: Data processing

What does a mass spectrometer do?

A mass spectrometer measures masses

(Andrej Shevchenko)

→ in addition, modern MS generates molecular fragments and measures their masses

→ intensity information can be used for quantification

→ structural information

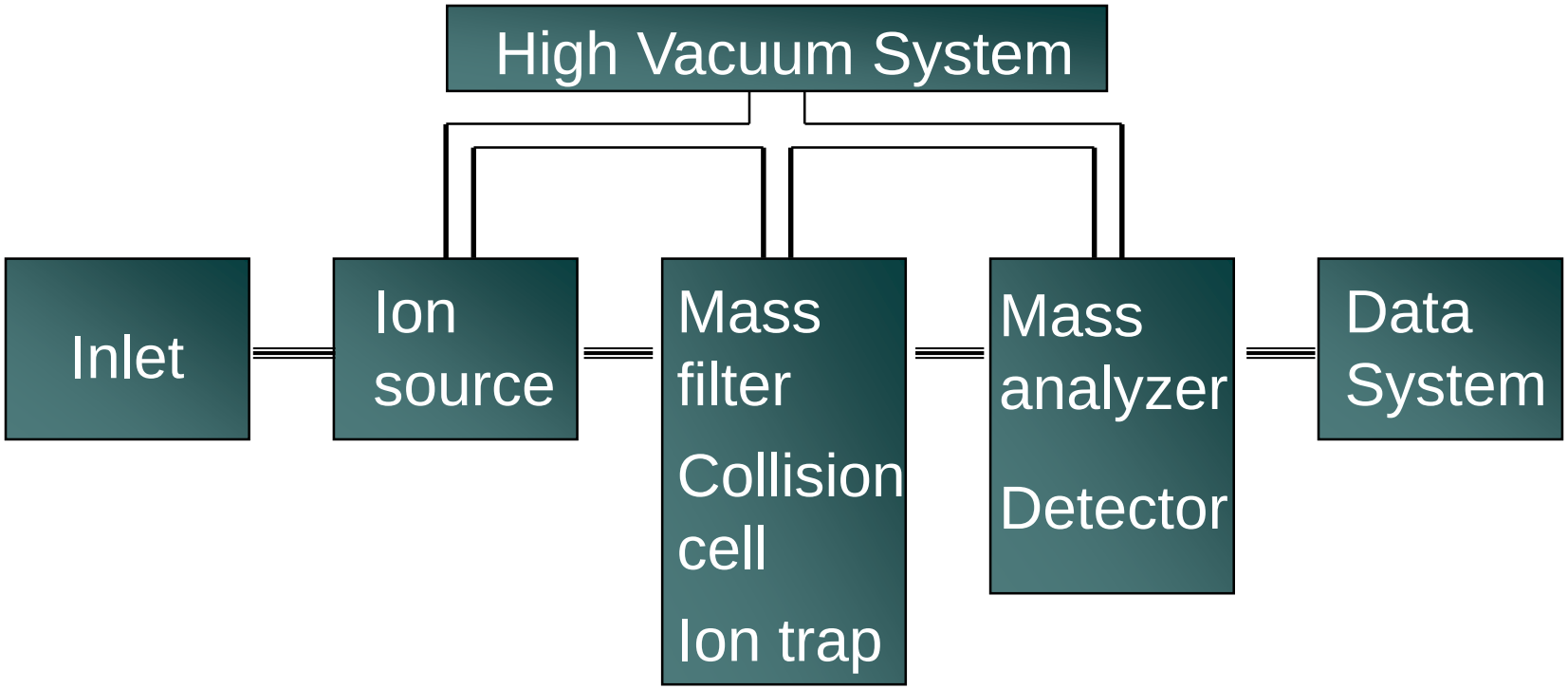
What are mass measurements good for?

To identify, verify, and quantitate:

→ any kind of biomolecule

→ any kind of synthetic molecule

Mass Spectrometer Block Diagram



Ion Sources make ions from sample molecules

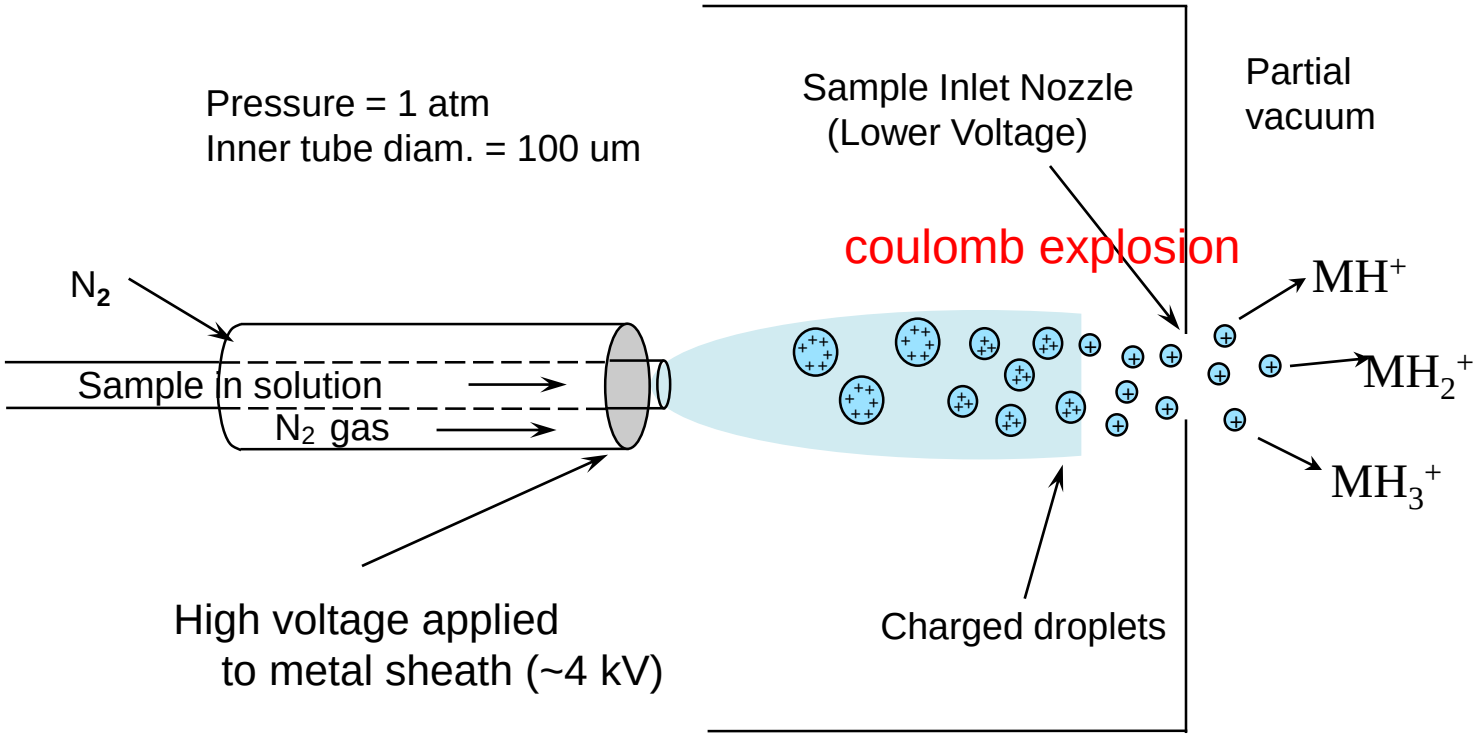
MALDI (matrix assisted laser desorption ionization)

- sample solution mixed with aromatic organic acids, then completely dried, then activated by UV-Laser
- Discontinous stream of ions
- peptides, proteins, rarely used for lipids

ESI (electrospray ionization)

- sample solution sprayed directly
- continuous stream of ions
- Peptides , lipids, small molecules

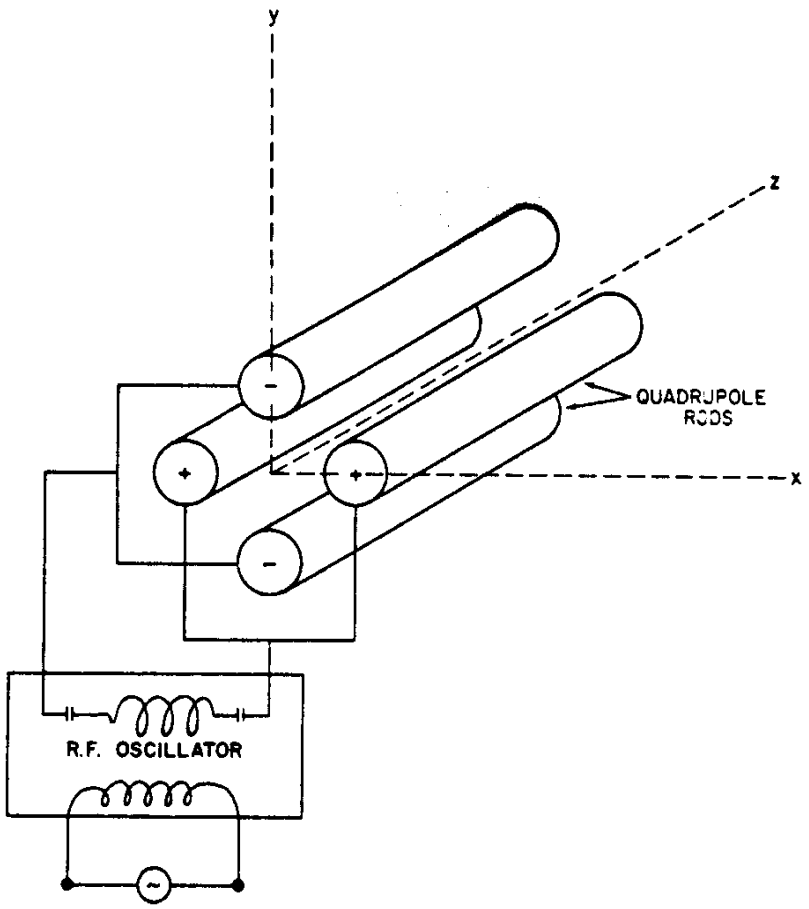
ESI Electrospray ionization:



Mass analyzers separate ions based on their mass-to-charge ratio (m/z)

- Operate under high vacuum (keeps ions from bumping into gas molecules)
- Actually measure mass-to-charge ratio of ions (m/z)
- Key specifications are resolution, mass measurement accuracy, and sensitivity.
- Several kinds exist: for bioanalysis, quadrupole, time-of-flight, ion trap, orbitrap are most used.

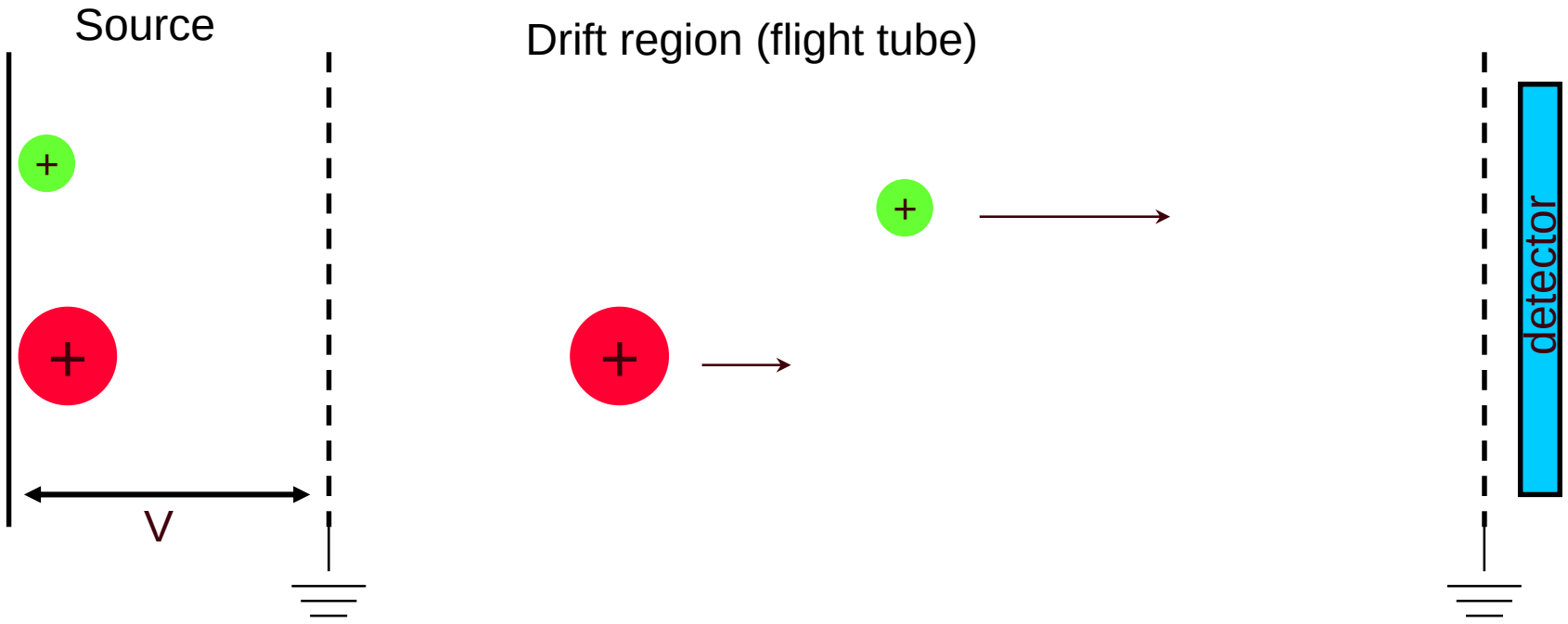
Quadrupole Mass Analyzer



Uses a combination of RF and DC voltages to operate as a mass filter.

- Has four parallel metal rods.
- Lets one mass range pass through at a time.
- Can scan through all masses or sit at one fixed mass.

Time-of-flight (TOF) Mass Analyzer



- Ions are fed into the source and accelerated in pulses (t=0).
- smaller m/z gives higher speed v (1/2 $mv^2 = zV$)
- The drift region is field free.
- Measures the time for ions to reach the detector.
- Small ions reach the detector before large ones.

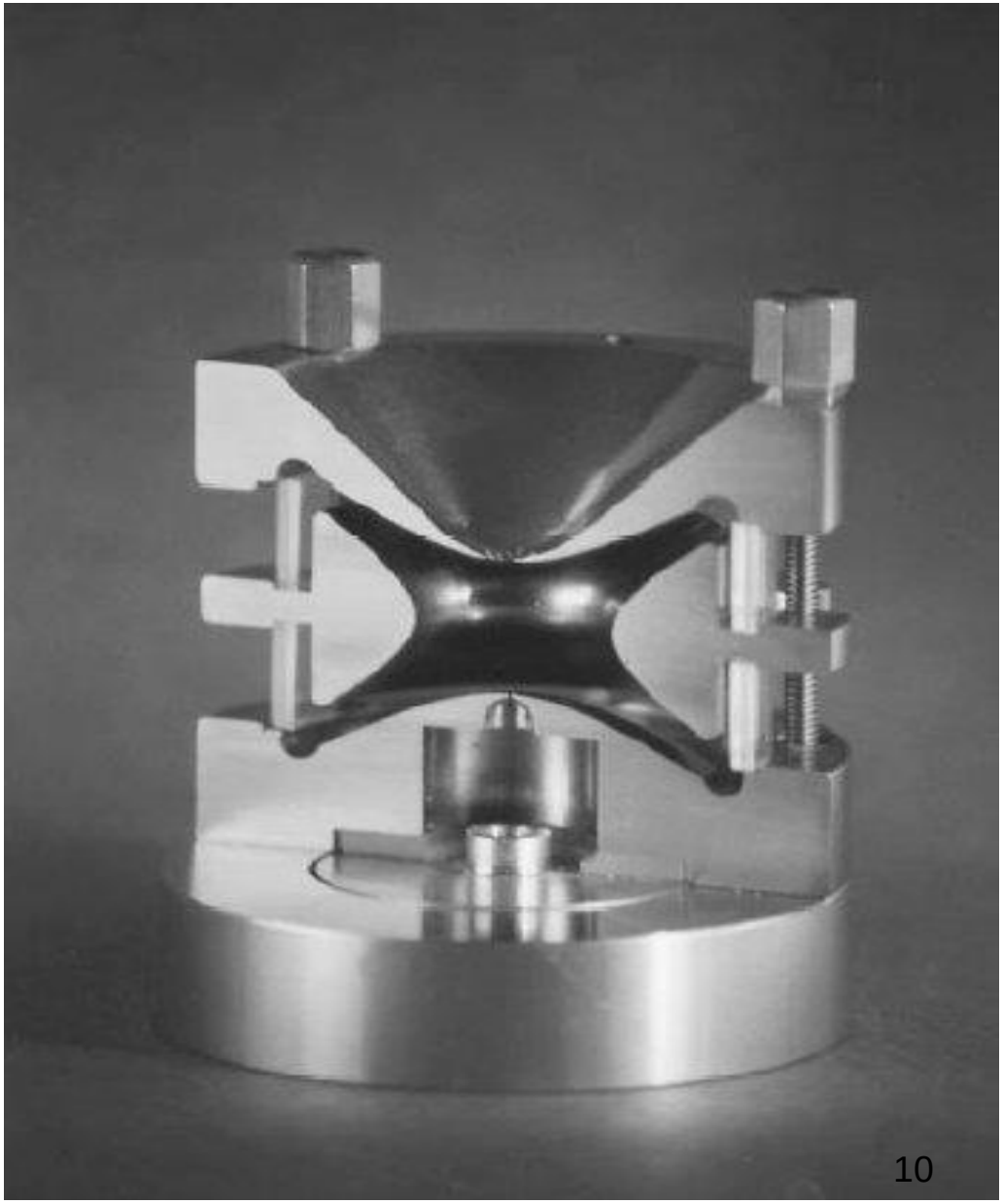
Ion Trap Mass Analyzer (Wolfgang Paul, NP Physics 1989)

can store ions

analyze the mass

select masses

useful for low abundant ions



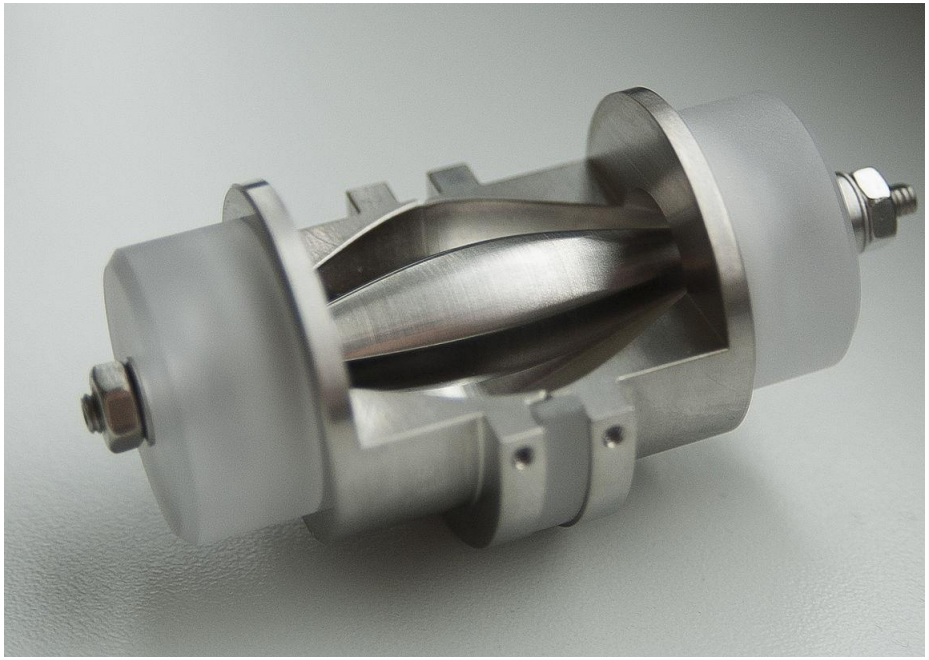
Cut away side view

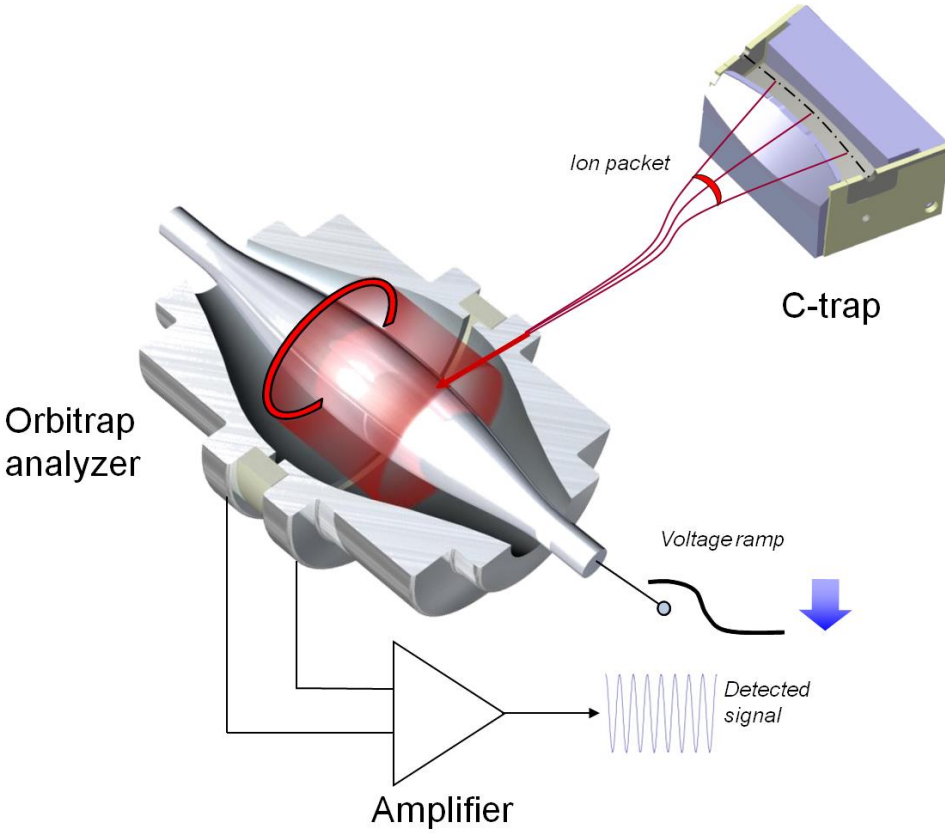
Orbitrap (Makarov, 1999)

analyzes masses

with incredible precision and

resolution





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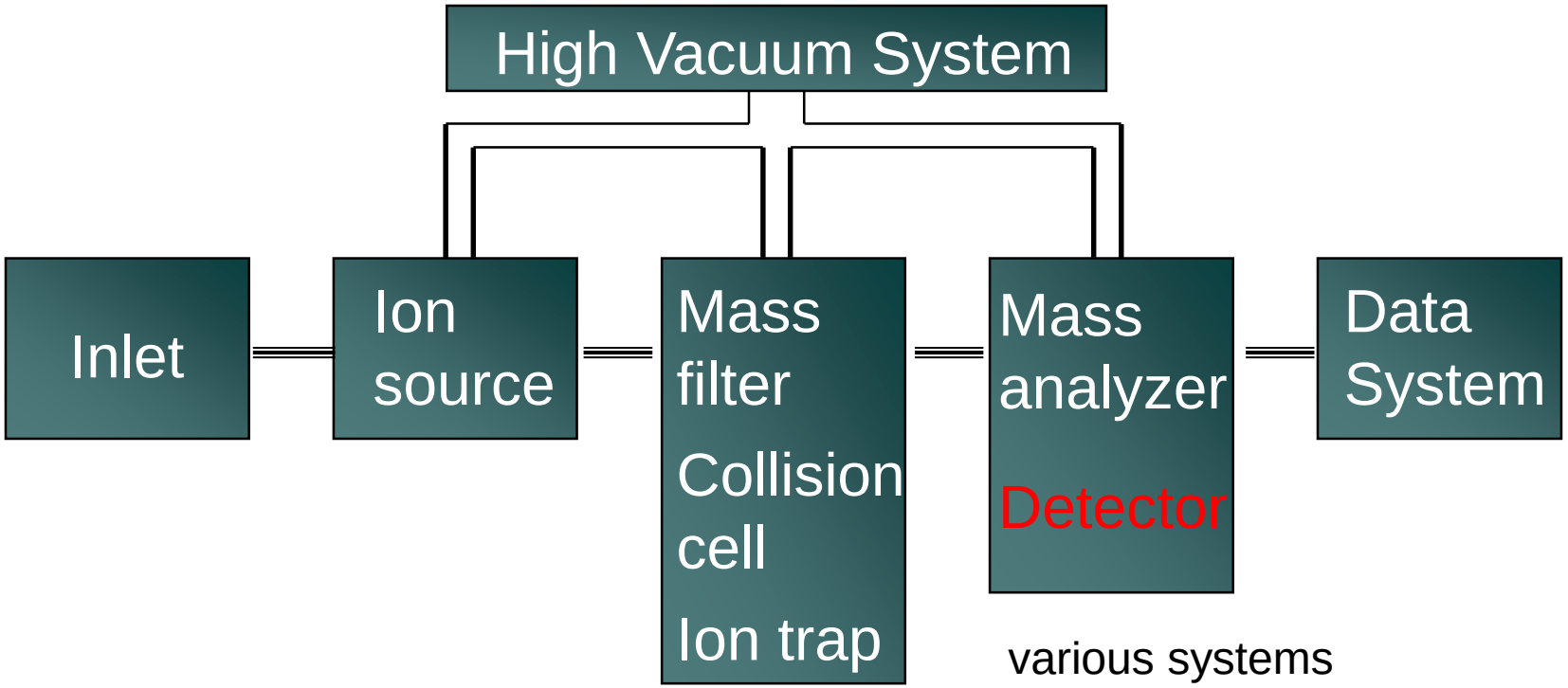
Mass Spectrometer Block Diagram



various systems
amplify ion counts like a PMT

orbitrap is both an analyzer and detector

How do mass spectrometers get their names?

Types of ion sources:

- Electrospray (ESI)
- Matrix Assisted Laser Desorption Ionization (MALDI)

Types of mass analyzers:

- Quadrupole (Quad, Q)
- Ion Trap
- Time-of-Flight (TOF)

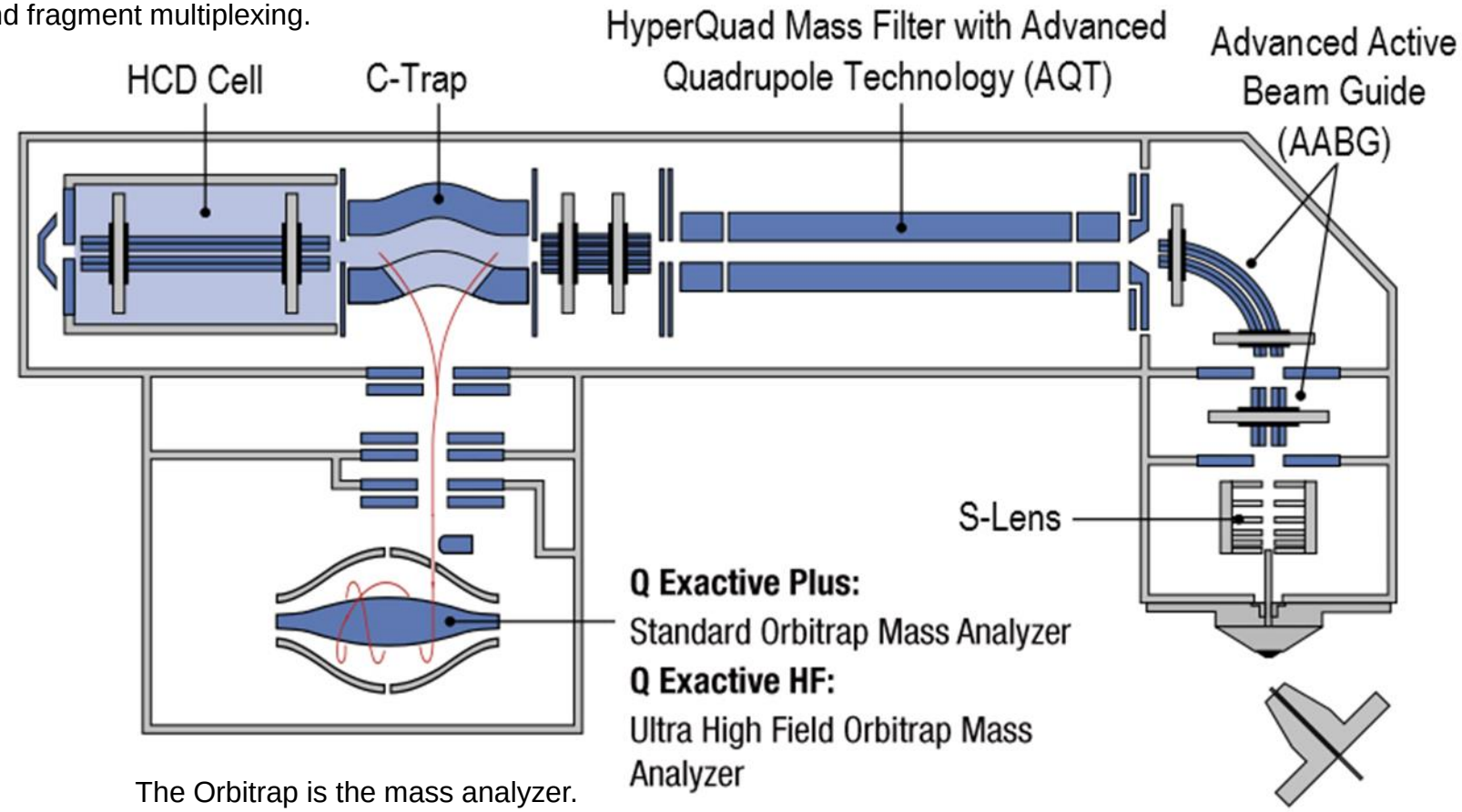
- Either source type can work with either analyzer type: "MALDI-TOF," "ESI-Quad."

- Analyzers can be combined to create "hybrid" instruments. ESI-QQQ, MALDI QQ TOF, Q Trap

- Our machine is a ESI-Q-ion trap-Orbitrap called Q-Exactive Plus

The HCD can collide **and store** ions, allowing stepped collision energies and fragment multiplexing.

The quadrupole selects ions for further analysis with min window of 0.4 Da, max 2000 Da



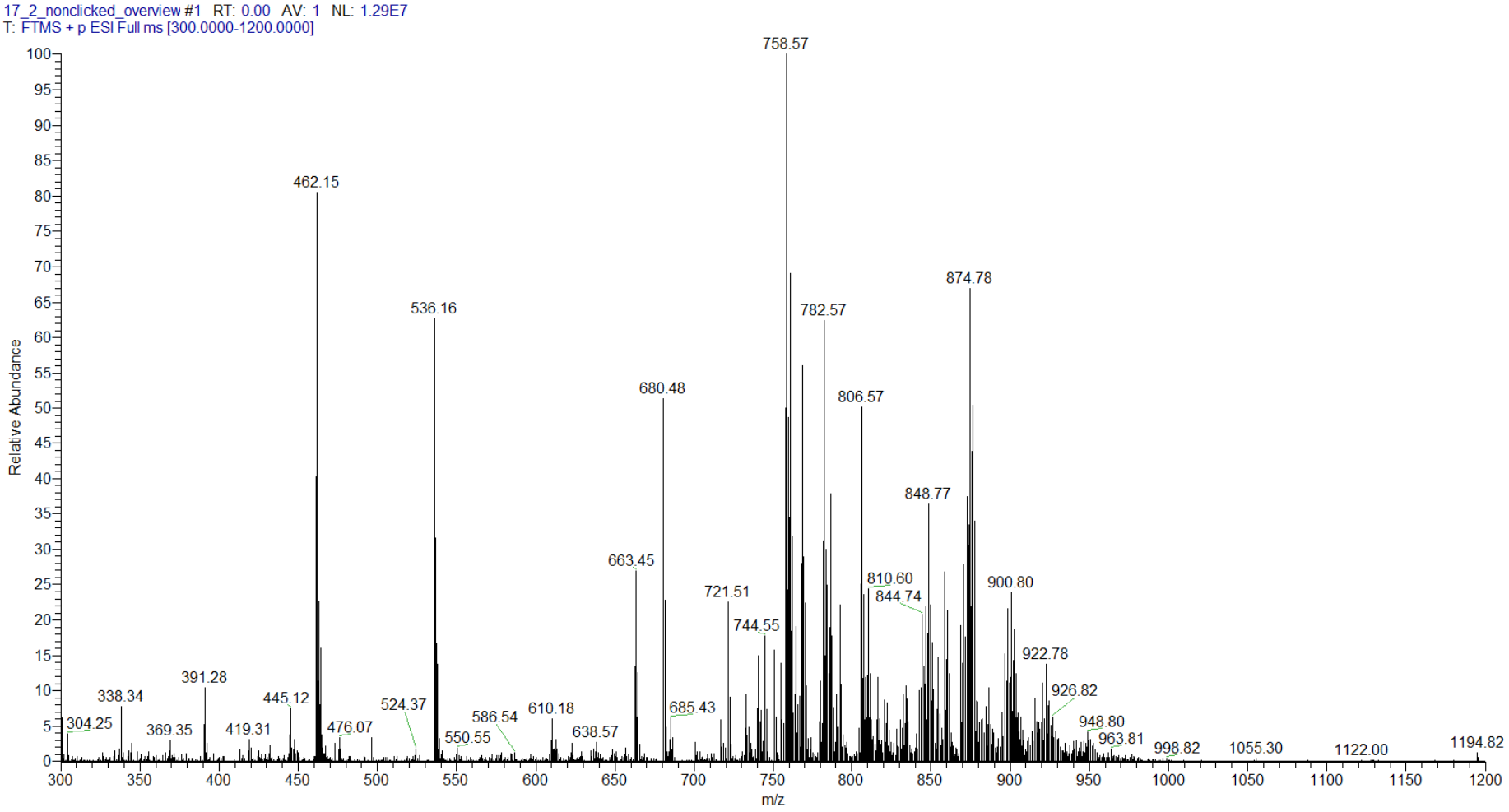
The Orbitrap is the mass analyzer. All measurements are done here

The ESI ion source

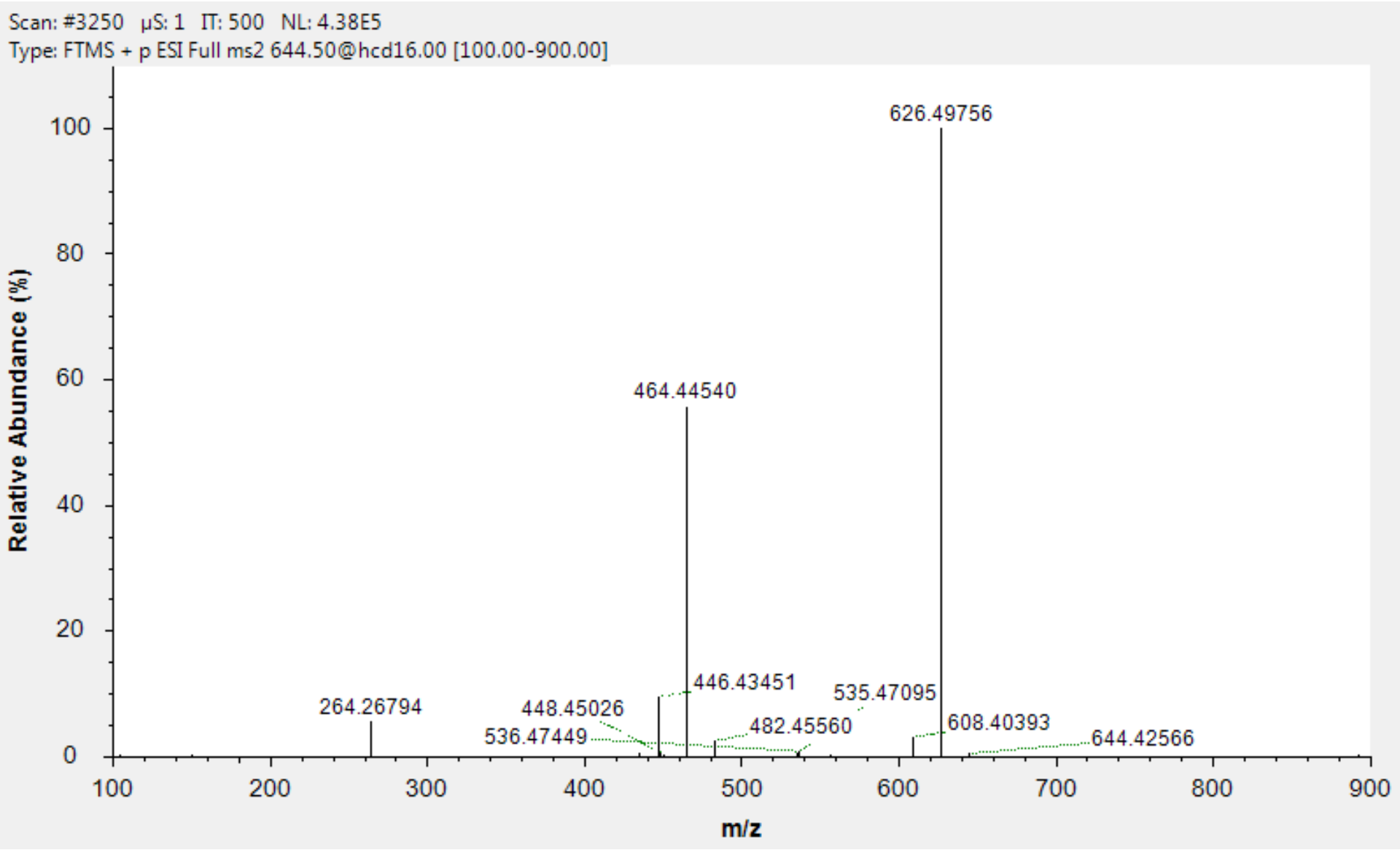
Precision: < 1 ppm PC 34:2 = 758.5694

Resolution: 280,000 → baseline separation between 758.565 and 758.570

Spectra 1: MS1



Spectra 2: MS2



Spectra 3: Stable isotopes of most relevant elements

Isotope	Nominal Mass	Exact Mass	Abundance
^{1}H	1	1.0078	99.985%
^{2}H	2	2.0141	0.015
^{12}C	12	12.0000	98.89
^{13}C	13	13.0034	1.11
^{14}N	14	14.0031	99.64
^{15}N	15	15.0001	0.36
^{16}O	16	15.9949	99.76
^{17}O	17	16.9991	0.04
^{18}O	18	17.9992	0.20

each C in the sum formula will contribute about 1% of the heavy isotope → isotope peaks

each H in the sum formula contributes 0.008 to the mass after the decimal point → “mass defect” characteristic for molecule classes

Spectra 4: Isobaric species

- compounds with the same nominal but different exact mass
- in practice: compounds that your spectrometer cannot resolve
- In that case, they cannot be distinguished by mass alone
- need additional information:
- retention time in HPLC or similar
- mass of characteristic fragments

isobars: PC[34:2] / PS[34:3] →758.5694 / 758.4966 as $[M+H]^+$

In contrast **isomers**: citrate/isocitrate, PC [16:0/18:2]/PC [17:1/17:1], peptides or oligos with same AAs or nucleotides but different sequence, all hexose phosphates, all HETEs, ...

Spectra 5: mass defects

Name	mass of MH^+	sum formula
PS(38:7)	806.4966	C44H73O10N1P1
PI-O(32:4) x NH_4	806.5178	C41H77O12P1N1
PC(38:6)	806.5694	C46H81O8N1P1
TG(48:9) x NH_4	806.6293	C51H84O6N1
GlcCer(24:3)	806.6504	C48H88O8N1
TG(47:2) x NH_4	806.7232	C50H96O6N1
CE(28:2) x NH_4	806.7749	C55H100O2N1

Spectra 6: the N-Rule

- **N-rule**: compounds with odd number of N have odd-numbered nominal masses

  CH_4: 16 NH_3: 17

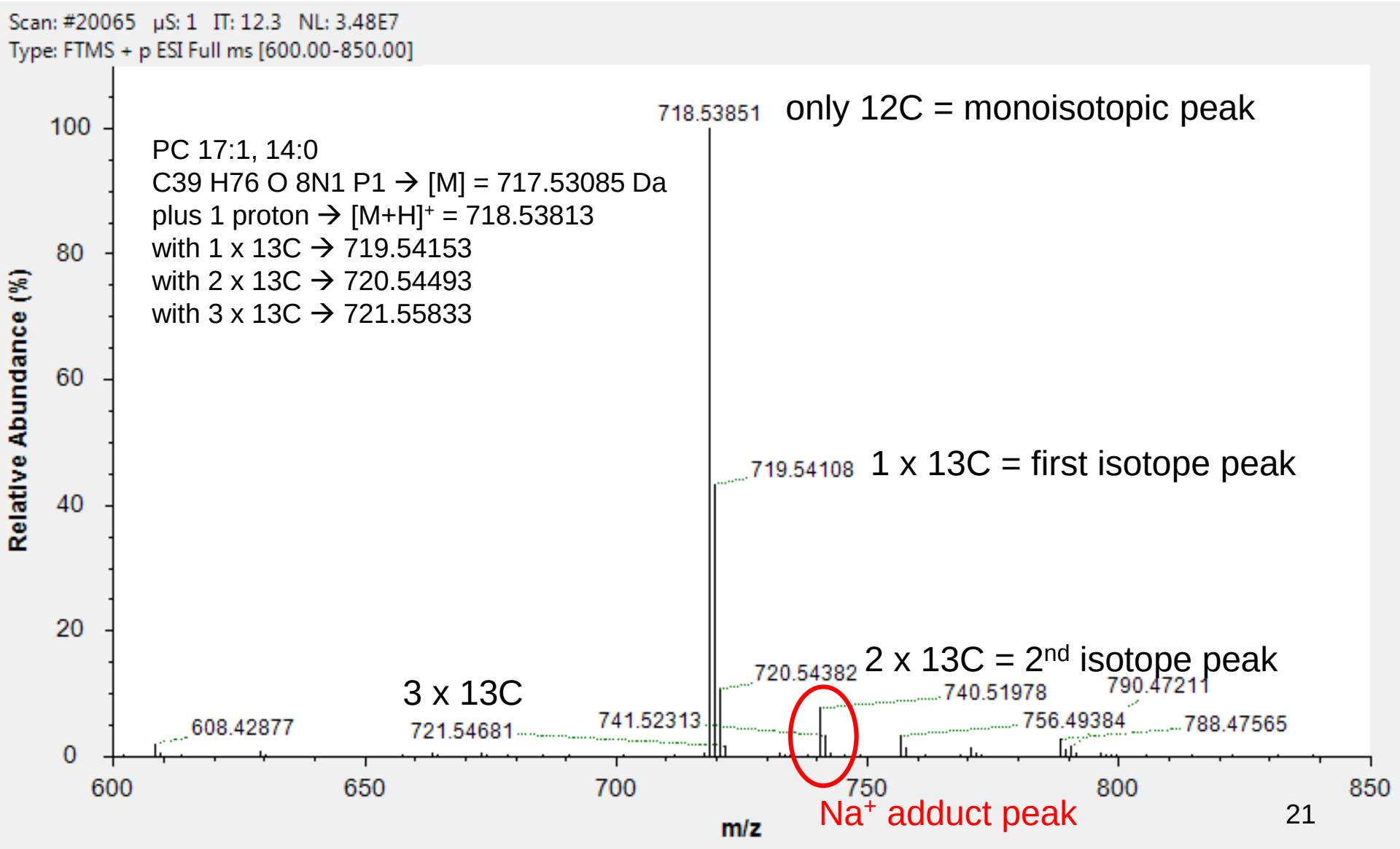
  t-Butane: 58 Trimethylamine: 59

→ as $[M + H]^+$ or $[M - H]^-$, these have **even** numbers

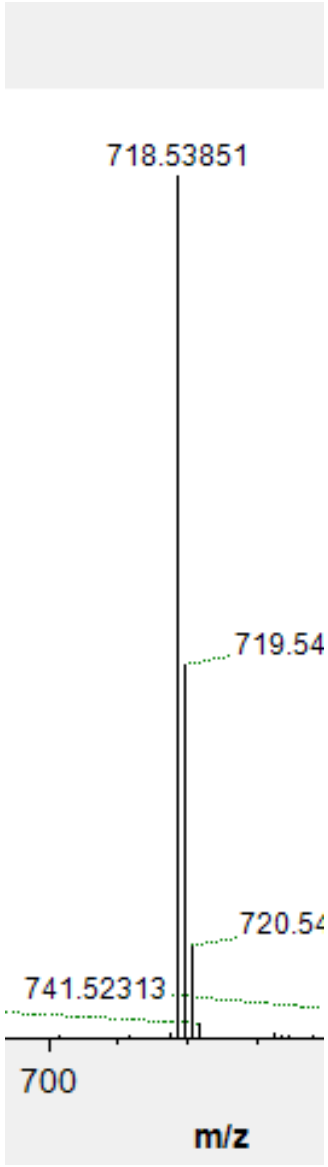
→ **even nominal mass in lipid MS means 1, 3, 5... N**

→ **attention with ammonium adducts: the adduct ion contains one N**

Spectra 7: Isotope peaks



Spectra 8: Mass accuracy



Mass accuracy = (theoretical mass – measured mass)/theoretical mass

Mass accuracy depends on the instrument design and quality

Quadrupole: 2 – 5 ppm
TOF: 2 – 5 ppm
Orbitrap: 0.1 – 1 ppm
FTICR: 0.1 – 1 ppm

Example:

Mass accuracy = (theoretical mass – measured mass)/theoretical mass

= - 0.00038 / 718.54 = -0.00000053 = -0.53 ppm

Spectra 9: Resolution

Same definition and rules as in HPLC (see there)

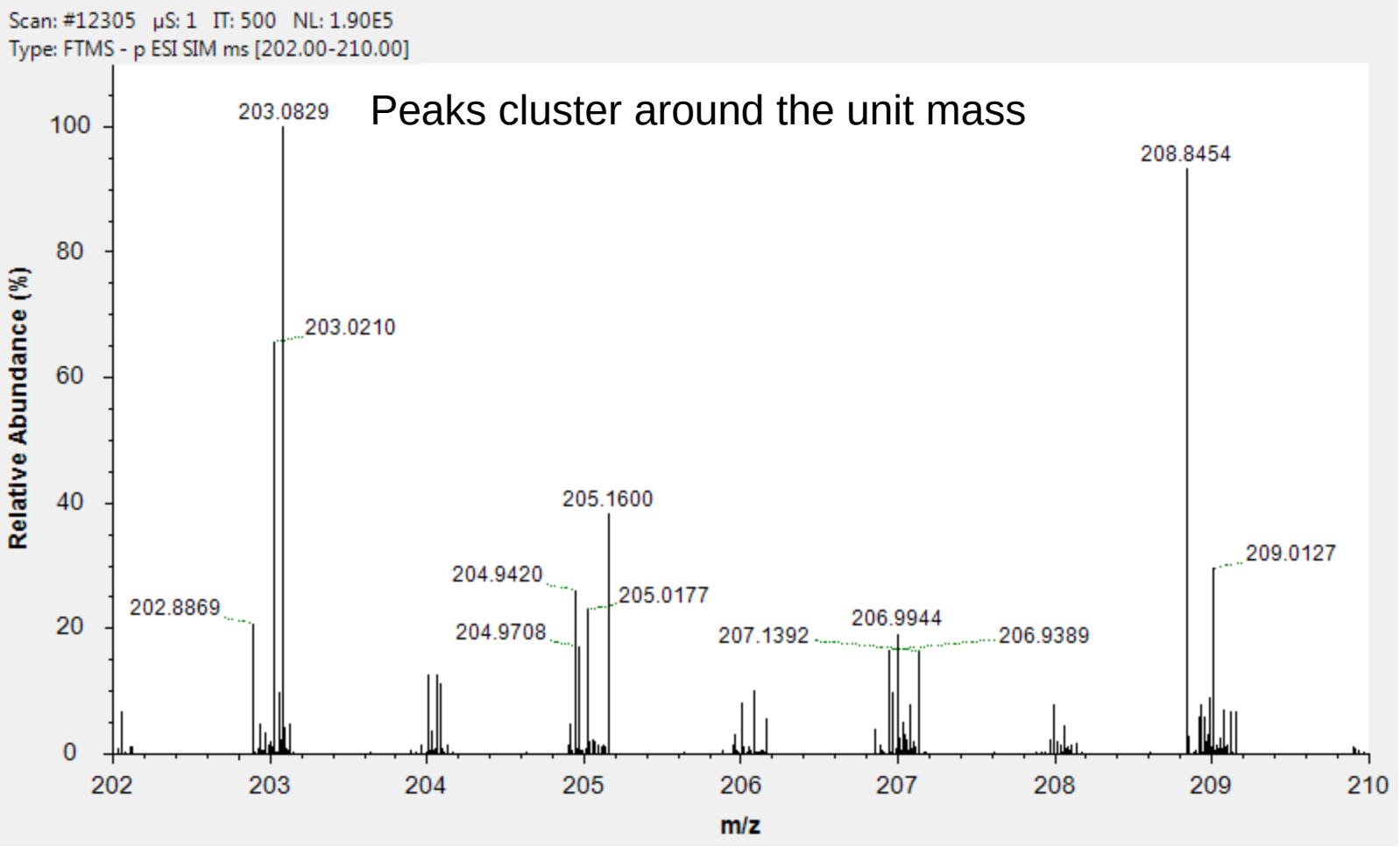
To resolve two peaks, their distance should be larger than the Half Maximum Peak width (FWHM)

$R = M/\Delta M$

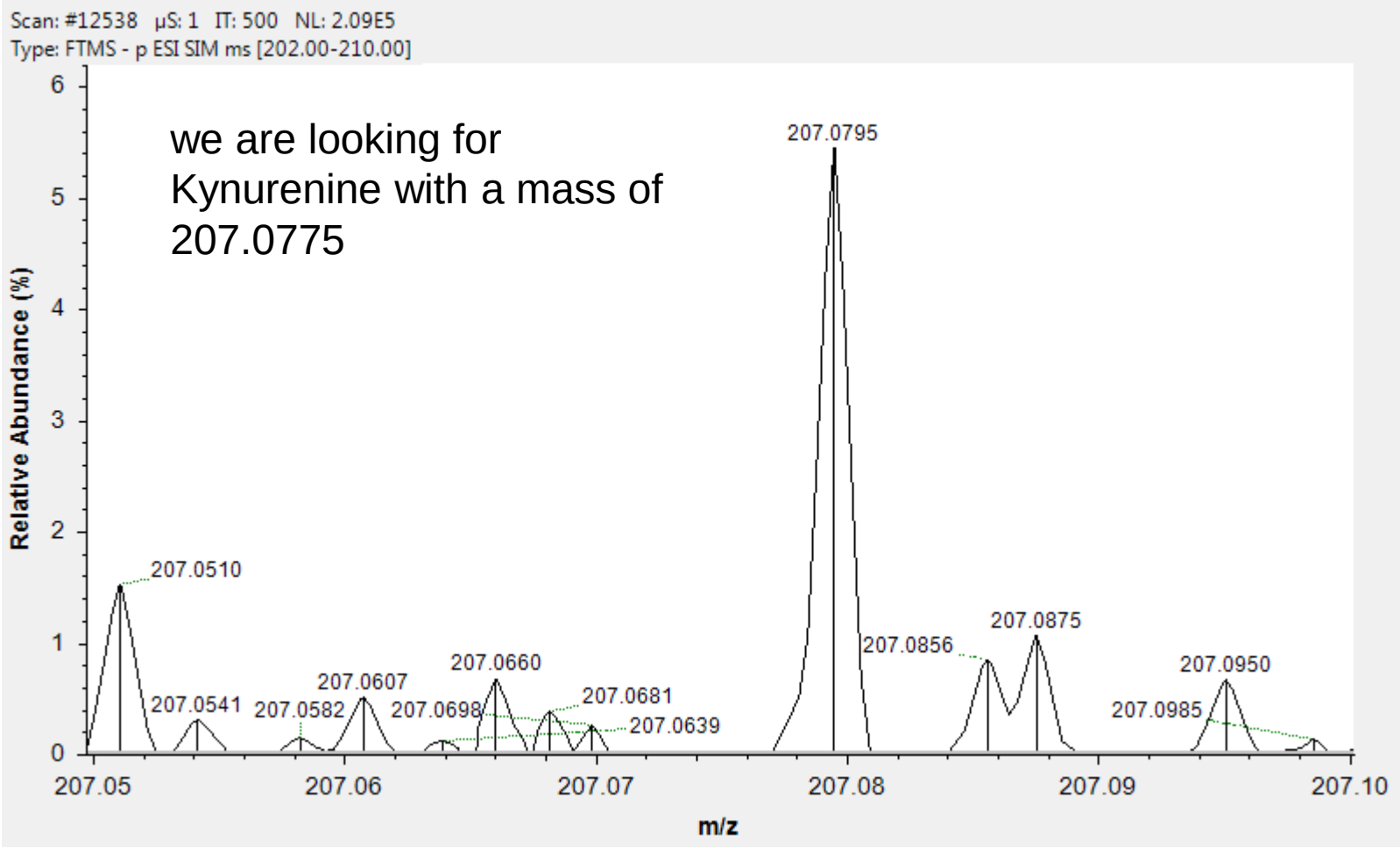
strongly depends on the mass analyzer

Ion trap	1000	resolves 13C isotope peaks
quadrupole	1000	
TOF	10000 – 50000	resolves many class isobars
Orbitrap	100000 – 500000	resolves isotope-isobars ($2x^{2}H$ vs $4 \times {}^{1}H$)
FT-ICR	1000000	resolves nearly everything

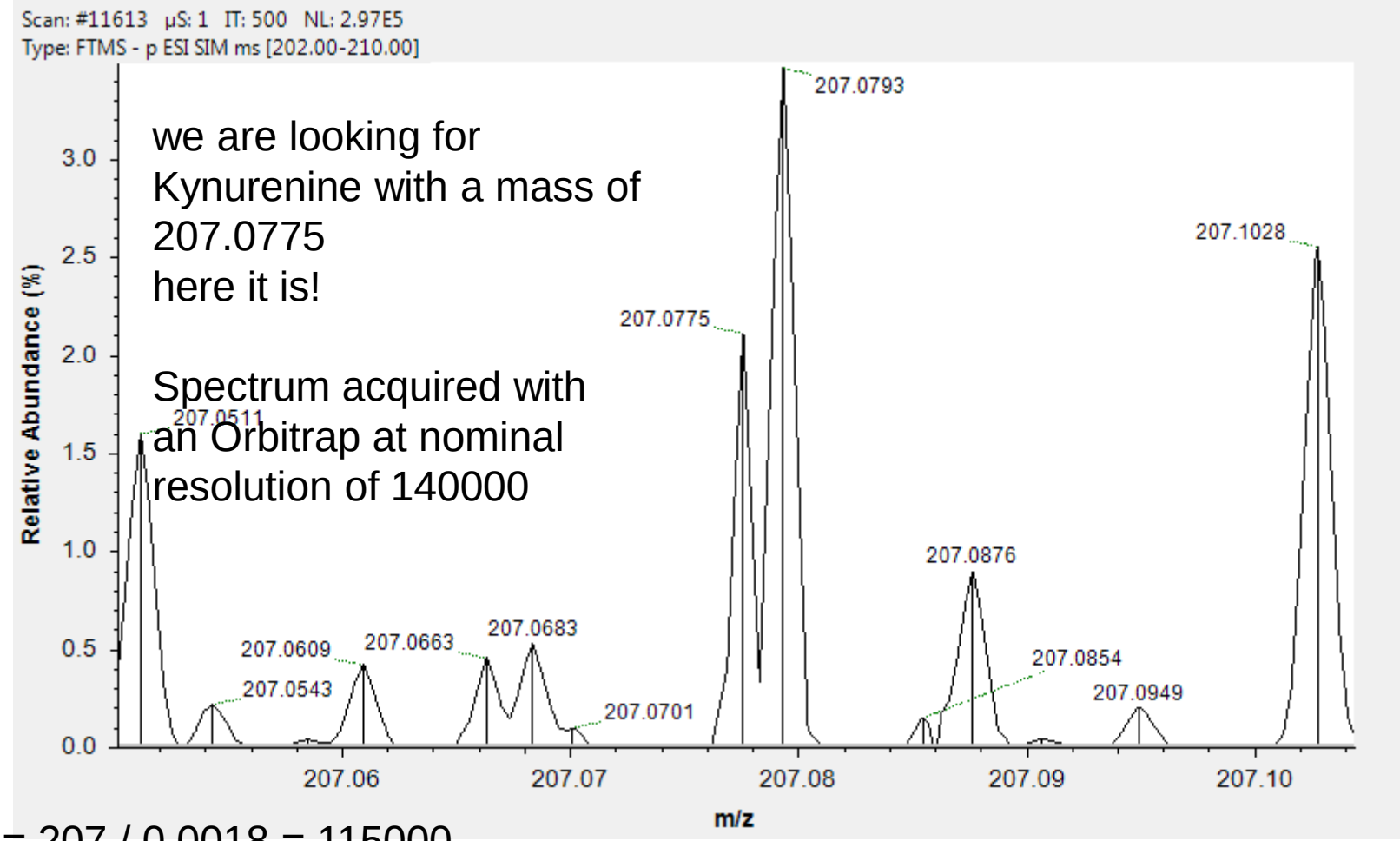
Spectra 10: Why is resolution important?



Spectra 10: Why is resolution important?



Spectra 10: Why is resolution important?



R = 207 / 0.0018 = 115000

quadrupole → you would miss the peak completely
TOF-analyzer → you would mix it up with the 207.0793 peak

Liquid or gas chromatography combined with Mass Spectrometry: LC-MS / GC-MS

- MS is directly coupled to the outflow of a chromatography
- spectra are taken at close time intervals
- chromatograms of each mass can be reconstructed from the spectra
- fragmentation can be included into the scan scheme
- LC-MS is the method for forensic, environmental and clinical quantitative MS of trace compounds

Tandem Mass Spectrometry

- Tandem MS is the combination of at least two mass analyzers with a collision cell
- the first analyzer selects the ions to be fragmented
- In the collision cell the ions are collided with a gas (nitrogen, helium)
- upon collision, the ions form fragments
- the second mass analyzer analyzes the fragments

Fragmentation 1

- In the collision cell the ions are collided with a gas (nitrogen, helium)
- upon collision, the ions form fragments
- the kinetic energy is given by the acceleration voltage
- unit is electronvolt eV
- 1 eV = 96.5 kJ/mol = 23.1 kcal/mol
- biomolecules are typ. fragmented at 10-60 eV
- fragmentation occurs at the most labile bonds first
- at higher collision energy, more stable bonds break
- fragmentation pattern are characteristic for substance classes and substance species

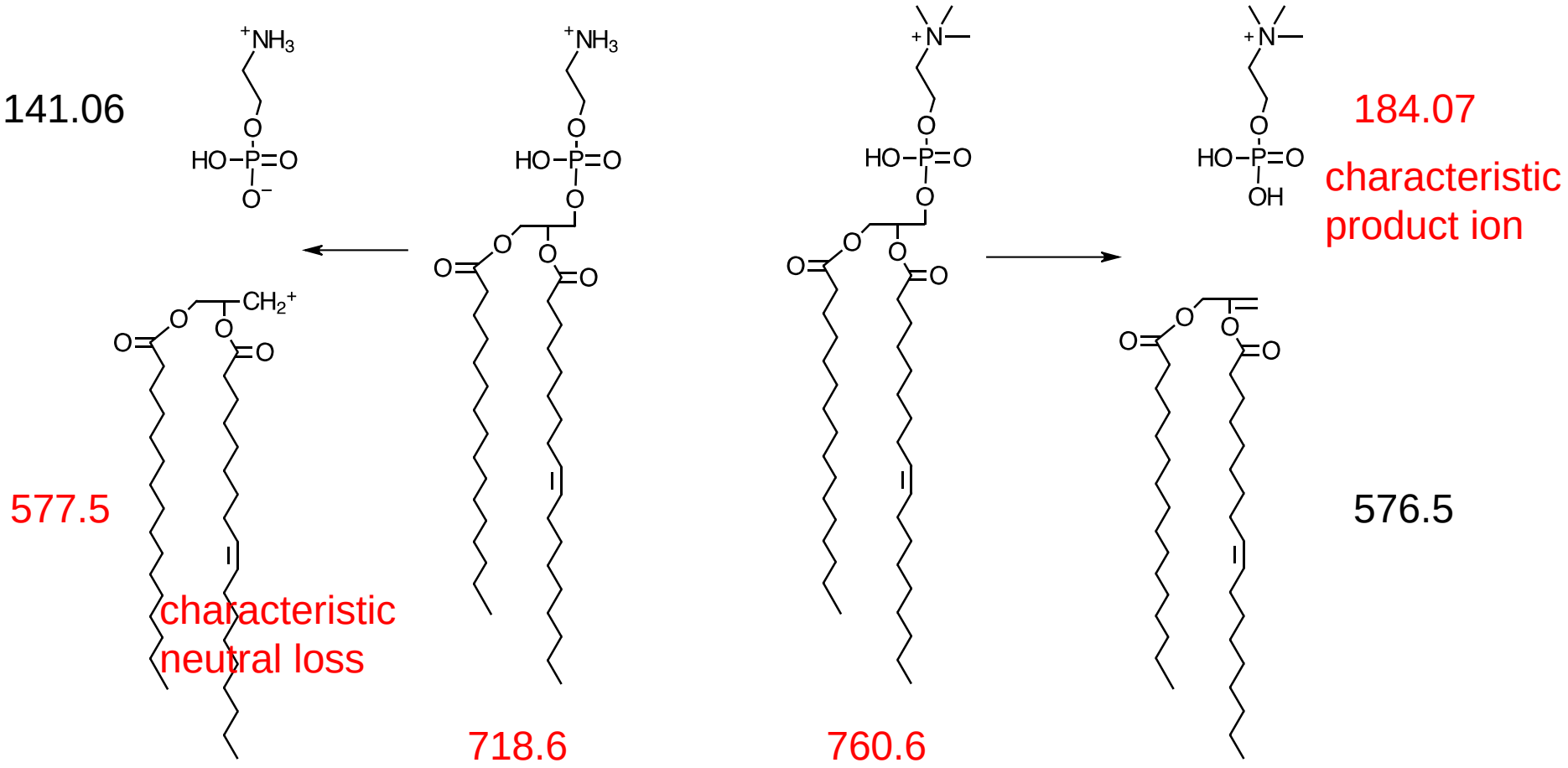
Fragmentation 2

- basics:
  - a single charged ion will fragment into an ion and a neutral remainder
  - only the charged fragment is detected after the collision; the neutral part is lost

Characteristic product ions or neutral losses

PE: neutral loss 141.06 in positive mode

PC: product ion 184.07 in positive mode

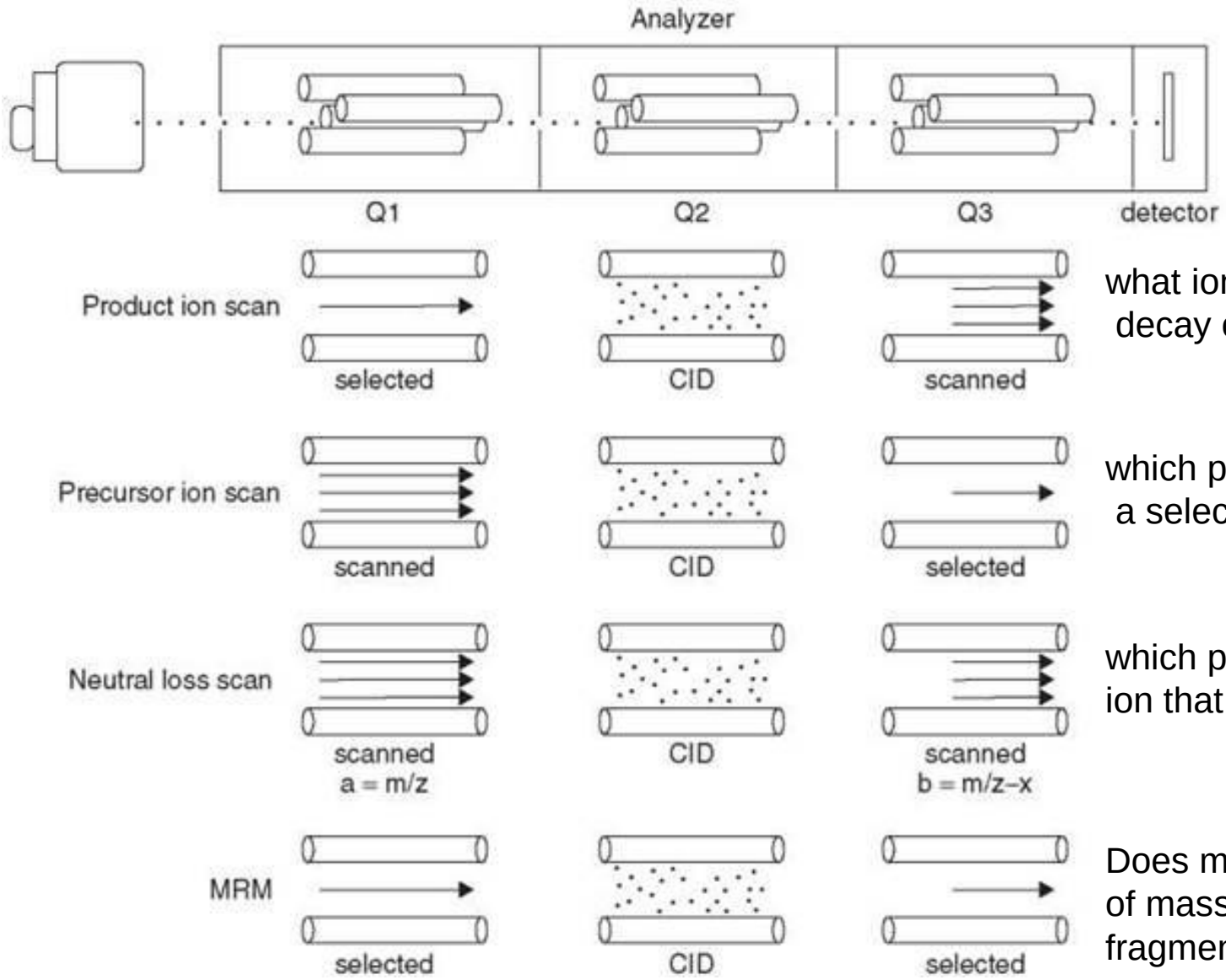


Fragmentation 3

- basics:
  - a single charged ion will fragment into an ion and a neutral remainder
  - only the charged fragment is detected after the collision; the neutral part is lost
  - some molecules give just one characteristic fragment
  - others produce several in parallel
  - biomolecules easily loose water (-18 Da), phosphoric acid (-98 or 80 Da) or CO_2 (44 Da)

- larger biomolecules often fragment along the biosynthetic units
  - lipids → fatty acids, phosphor-headgroups
  - peptides → amino acids

Fragmentation in a triple quad MS



what ions are produced by decay of selected precursor?

which precursors produce a selected product ion?

which precursors produce an ion that is smaller by XX Da?

Does my analyte contain an io of mass xx that produces a fragment of mass yy?

from Farwanah&Kolter: Lipidomics DOI: 10.1002/9780470048672.wecb291

We want to measure thousands of substances in one sample

How to deal with the complexity?

Shotgun MS

- samples are not pre-separated
- the entire mixture is directly infused into the ESI source
  - ➢ Constant composition over the entire analysis time
  - ➢ No variations in matrix effects
  - ➢ Analytes and int. stds. face identical conditions
  - ➢ No enrichment of anything
- Isobaric species are not separated
  - Needs high-resolution MS
  - ➢ Isomeric species are not separated
    - ➢ Needs diagnostic MS2
  - ➢ Rare species are suppressed or lost

LC-MS

- pre-separation by LC
- the column outflow is infused into the ESI source
  - ➢ Quickly changing composition over the analysis time
  - ➢ Strong variations in matrix effects
  - ➢ Analytes and int. stds. face different conditions
  - ➢ strong enrichment of substances
- Isobaric species are often separated
  - Works with low-resolution MS
  - ➢ Isomeric species may be separated
    - ➢ often needs diagnostic MS2
  - ➢ Rare species are detected

Strategy of data recording

For unequivocal identification: MS1 and MS2 data are needed

Shotgun MS

- Record MS1 data first (takes 30 s)
- Record MS2 data in 1 Da windows
  - Of the entire mass range
    - ➢ untargeted for discovery
      10 min for 1400 Da
  - With a target list
    - ➢ faster but limited
- The MS2 data contain the entire fragment spectrum
- Complete representation of sample
- Can be searched for anything

LC-MS

- Key problem: composition changes every second
  - ➢ No large scale MS2 raster data
- Record MS1 and MS2 alternatingly
- Substances elute as peaks with about 10-20 s peak time
  - ➢ needs same scan every 2 s
  - ➢ Max 10 diff. Scans at low res.
  - ➢ Or 4 scans at high res.
- ➢ At 20 min run time → 40 subst. + dirt at any moment
- ➢ Practically: either trace a couple of predefined MS2 scans over the entire run (classical LC-MS in trace analysis)
- ➢ Or change the MS2 scans according to expected substances at a given time (targeted LC-MS lipidomics)
- ➢ Or do MS2 for peaks measured in MS1 (DDA, i.e. in proteomics)

We do shotgun lipidomics

- Complete data coverage in MS1 and MS2
- Complete freedom in data analysis
- Very stable internal standardization
- Very reliable data
- Complete OMICS of all „abundant“ (500-2000) species

- No rare mediator lipids (Eicosanoids, S1P, PIPs)

- and the notorious cholesterol problem

Standards and quantification

1.) mass calibration

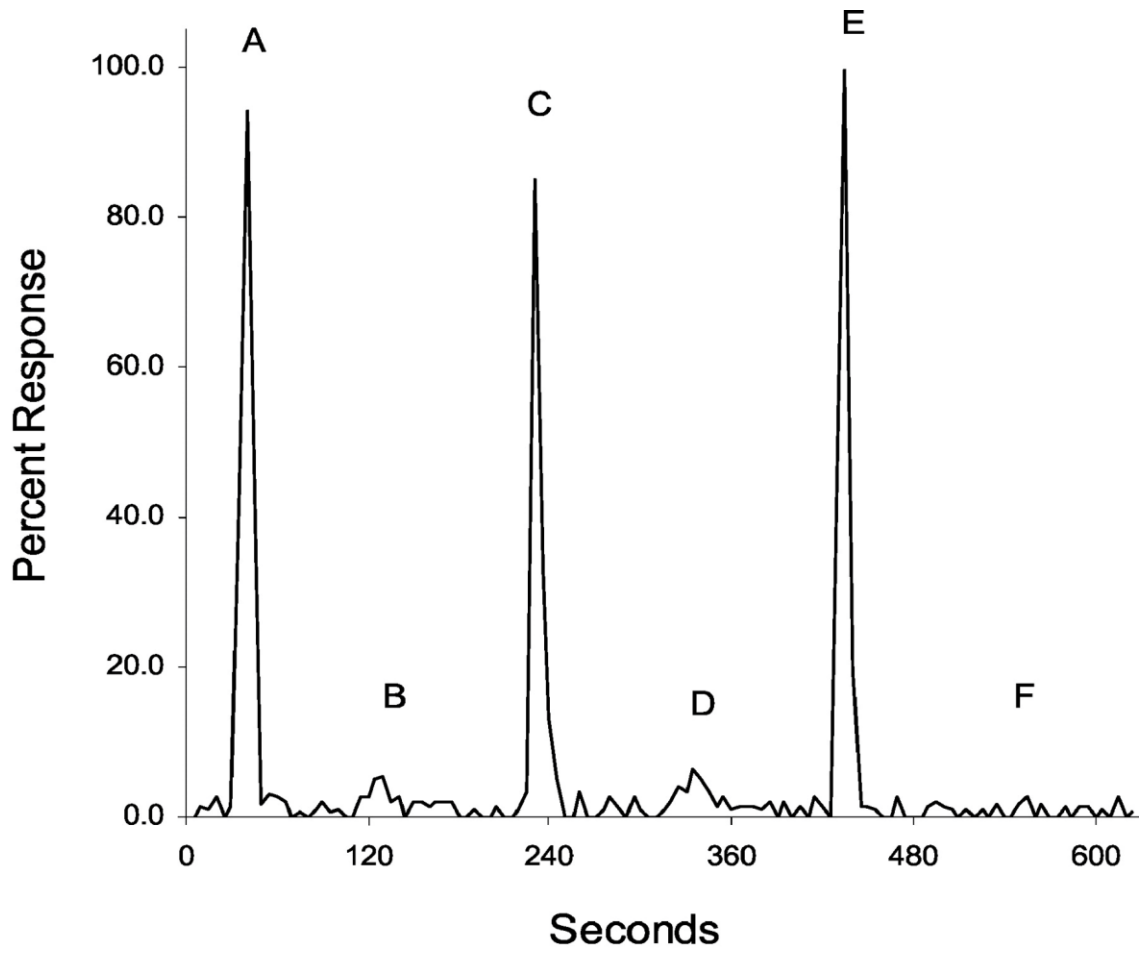
- with a mix of qualitative standards covering the relevant mass range, sprayed alone, separate for positive and negative mode

2.) absolute quantification:

- in principle, MS gives a direct quantification by counting the ions per second that reach the detection system,

but

Ion suppression



A, C, E: caffeine solution directly infused into MS

B, D, F: same amount of caffeine, but now mixed into serum and extracted with three different methods

Ion suppression and internal standardization

- any component of the analyte (called “matrix”) can suppress or enhance the detection efficiency of any peak
- → you can never compare absolute intensities measured in different preparations
- → the only way for quantitative MS is **internal standardization**
- **internal standard: the substance of interest with an isotope label or similar to distinguish the standard from the analyte**
- optimally added before the extraction procedure
- Lipid standards: for each class one lipid with unnatural fatty acid combination e.g. PC[17:1;14:0] or deuterium labeled FA
- Added during lipid extraction
- Quantification of all species of one class relative to the internal standard
- The set of standards added defines the scope of quantitative analysis
  - No ex-post quantification by later addition of standards and re-measuring

The IS list

Class	Species	Sum	Mass	Monoiso Mass	µl of 200 µg/ml in stock mix	µl per Mix vial	pmol per mix vial	pmol per 10 µl Mix	µg per 10 µl mix
PE	PE [31:1]	C36H70O8N1P1	675,91	675,4839048	2000	35,55555556	10520,796	210,416	0,142
PC	PC [31:1]	C39H76O8N1P1	717,99	717,530855	4000	71,11111111	19808,385	396,168	0,284
PS	PS [31:1]	C37H70O10N1P1	719,92	719,4737341	1000	17,77777778	4938,820	98,776	0,071
PI	PI [34:0]	C43H83O13P1	839,08	838,5571293	1000	17,77777778	4237,445	84,749	0,071
PA	PA [31:1]	C34H65O8P1	632,85	632,4417057	500	8,888888889	2809,161	56,183	0,036
PG	PG (28:0)	C34H66O10P1Na1	688,85	666,447185	500	8,888888889	2580,791	51,616	0,036
CL	CL [56:0]	C65H126O17P2	1241,63	1240,847026	500	8,888888889	1431,810	28,636	0,036
LPA	LPA (17:0)	C20H40O7P1Na1	446,491	424,25899	250	4,444444444	1990,833	39,817	0,018
LPC	LPC [17:1]	C25H50O7N1P1	507,64	507,3324896	250	4,444444444	1751,022	35,020	0,018
LPE	LPE (17:0)	C22H44NO7P	465,561	465,28553	250	4,444444444	1909,286	38,186	0,018
Cer	Cer [17:0]	C35H69O3N1	551,92	551,5277451	250	4,444444444	1610,539	32,211	0,018
SM	SM [17:0]	C40H81O6N2P1	717,05	716,583225	1000	17,77777778	4958,588	99,172	0,071
GlcCer	GlcCer [12:0]	C36H69O8N1	643,93	643,5023182	500	8,888888889	2760,825	55,216	0,036
GM3	GM3 (18:0-D3)	C59H105D3N2O21	1201,54	1182,755	250	4,444444444	739,791	14,796	0,018
TAG	TG [47:1]	C50H94O6	791,27	790,7050407	4000	71,11111111	17973,918	359,478	0,284
CE	CE [17:1]	C44H76O2	637,07	636,5845317	1000	17,77777778	5581,107	111,622	0,071
DAG	DG [31:1]	C34H64O5	552,87	552,4753752	500	8,888888889	3215,544	64,311	0,036
MAG	MG [17:1]	C20H38O4	342,51	342,2770097	500	8,888888889	5190,441	103,809	0,036
Chol	Chol [d6]	C27H40D6O	392,69	392,3925266	4000	71,11111111	36217,429	724,349	0,284
Carn	Car(15:0)	C22H43O4N1	386,56		250	4,444444444	2299,485	45,990	0,018
total					22500	400		2650,520	1,600
						= 80 µg/vial			50 samples/vial

Sample preparation: $CHCl_3$/MeOH/H_2O 2-Phase

Optimal:	reality:
• Isolate all lipids	• most
• Concentrate lipids	• yes
• Remove other ions	• most
• Remove proteins	• yes

Tissue culture cells:

Preferred procedure: direct extraction of cells from the culture dish.

Scale, cell number:

Normal cell lines (HeLa, COS7, HEK, HuH7, MEFs, MEB4, Macrophages and all other flat cells with similar content in material: **50000 - 200000 cells**, typically in a 12-well plate or equivalent dish.

High material content cells: primary hepatocytes, differentiated 3T3-L1 or similar: **5000 - 10000 cells**, in a 24-well plate or equivalent

Material needed: Chloroform, Methanol, water, isopropanol (all MS Grade), Acetic acid, ammonium acetate, internal standard mix
Original Eppendorf 1.5 ml plastic tubes. For optimal results, briefly wash the tubes once with methanol

Prepare:

Extraction Mix (for 10 Samples: 4.9 ml methanol, 1 ml chloroform, 100 µl internal standard mix)

1% Acetic acid in water

Spray buffer: 16 ml isopropanol, 10 ml methanol, 1720 µl water, 280 µl 1 M NH_4Ac in water

Procedure:

1) wash cells at least twice with PBS or similar salt, protein-free, no detergent

2) aspirate last wash solution as complete as possible. Too much residual water will ruin the experiment in the next step.

3) quickly add to each well 500 µl of Extraction Mix.

This is a critical step. If too much water was left, phase separation will occur with formation of a chloroform phase that will start to dissolve the plastic of the dish, to be recognized as a turbid, soft layer of "molten" plastic on the surface of the dish. If this happens, this sample is lost. Do not attempt to further process it. Quick addition of the Extraction Mix ensures that phase separation will not occur.

4) sonicate the entire plate for 10 sec in the bath sonicator at RT. Cells will either stay attached on the dish or suspend in the solvent mix

5) collect the entire material into an Eppi, spin at 20000 g for 2 min at RT

6) collect the supernatant into new Eppi, add 200 µl chloroform and 800 µl 1% AcOH in water. Shake manually for 5 sec, spin for 2 min at 20000 g at RT.

7) Remove by careful aspiration the upper phase

8) Transfer the entire lower phase (using a chloroform-rinsed yellow pipet tip) into a fresh Eppi.

9) Evaporate in the speed vac (45 ° C, 10 min, **do not overdry**)

10) Add 500 - 1000 µl spray buffer. Sonicate in the bath for 5 min.

11) spray positive and negative with the OMICS method files at 10 µl/min. Intensity of the 760.58 PC peak should preferably be between 2E6 and 3E7.

Animal tissues (except adipose tissue):

Preferred procedure: extraction of a tissue homogenate.
All normal tissues: Obtain a piece of tissue of **about 10 mg weight**.

Procedure:

1) collect fresh or frozen tissue into a pre-weighed Eppi. Note final weight.

2) add 500 µl water and quickly homogenize

3) Transfer 50 µl of the homogenate into fresh Eppi and add 500 µl Extraction Mix. Freeze the remaining homogenate for eventual future use, in particular for protein determination

4) sonicate for 2 min in the bath sonicator.

5) spin at 20000 g for 2 min **at RT**

6) collect the supernatant into new Eppi, add 200 µl chloroform and 750 µl 1% AcOH in water. Shake manually for 5 sec, spin for 2 min at 20000 g.

7) Remove by careful aspiration the upper phase….

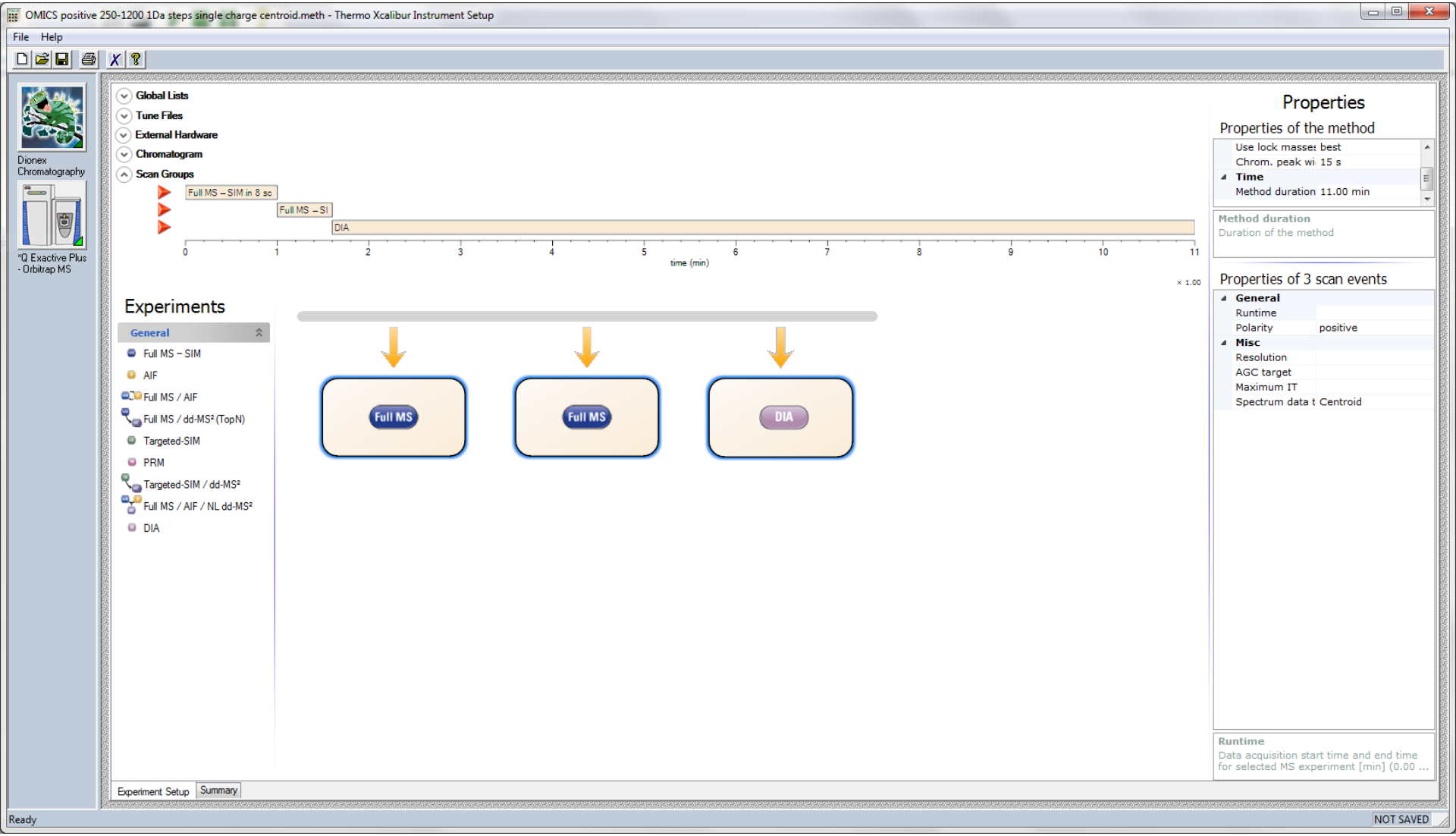
Adipose tissue and Serum/Plasma:

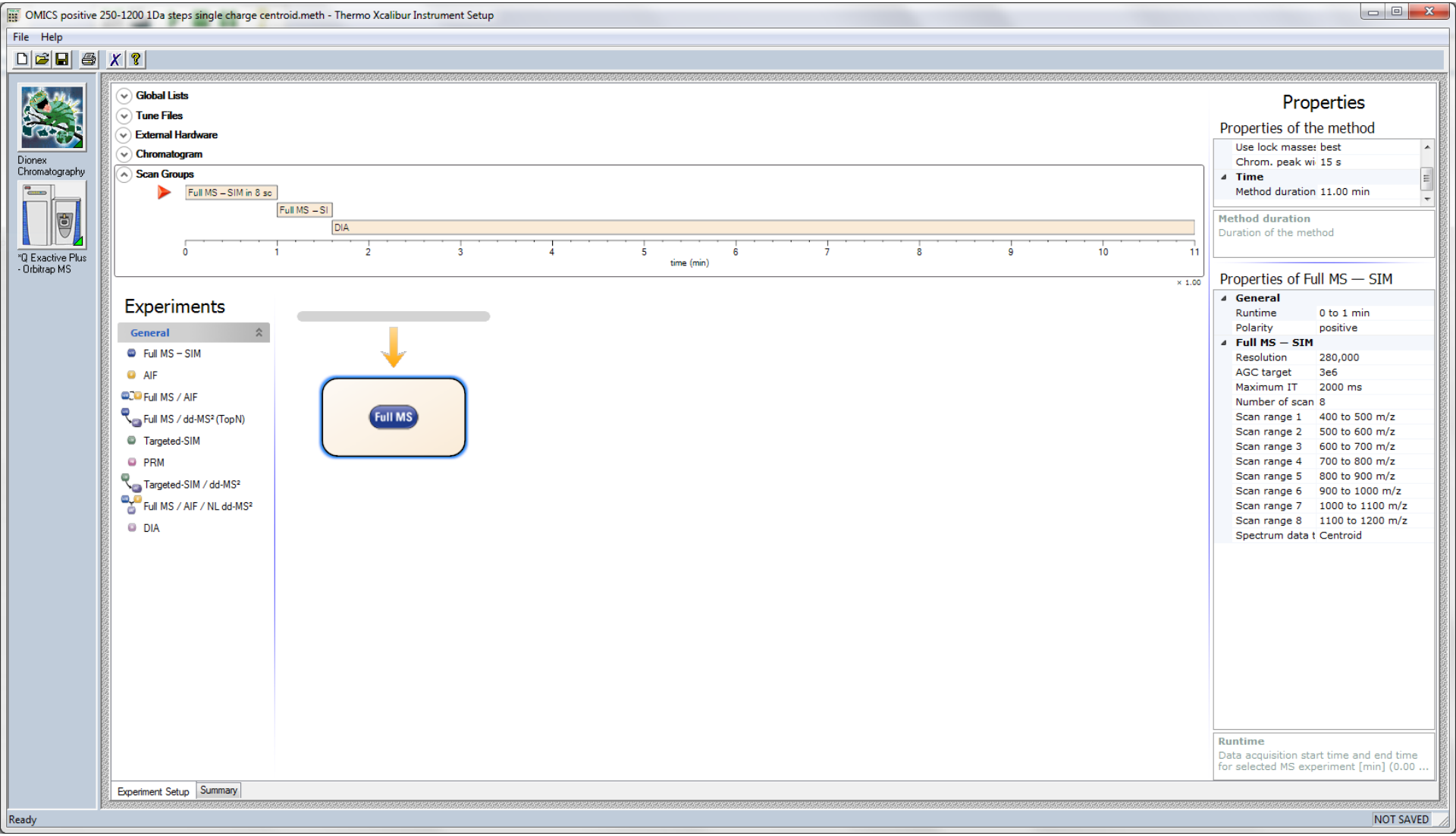
Adipose tissue contains very much TAG that suppresses other signals

Needs:

- more and different TAG-IS: for 2 mg tissue 30 µl extra IS-TAG 50:1-d4 (1000 µg/ml)
- Separation of TAG and other neutral lipids from phospholipids by phase distribution between hexane and 70% aq. ethanol
- Adipose tissue samples are three times the work of other samples

Plasma samples contain large amounts of sterol ester and need additional CE-IS. Extraction etc. is the normal procedure. 2µl plasma are optimal.


OMICS positive 250-1200 1Da steps single charge centroid.meth - Thermo Xcalibur Instrument Setup
File Help
Dionex Chromatography
*Q Exactive Plus - Orbitrap MS
Global Lists
Tune Files
External Hardware
Chromatogram
Scan Groups
Full MS – SIM in 8 sc
Full MS – SI
DIA
0 1 2 3 4 5 6 7 8 9 10 11
time (min)
× 1.00
Experiments
General
Full MS – SIM
AIF
Full MS / AIF
Full MS / dd-MS² (TopN)
Targeted-SIM
PRM
Targeted-SIM / dd-MS²
Full MS / AIF / NL dd-MS²
DIA
Full MS
Full MS
DIA
Properties
Properties of the method
Use lock masses best
Chrom. peak wi 15 s
Time
Method duration 11.00 min
Method duration
Duration of the method
Properties of 3 scan events
General
Runtime
Polarity positive
Misc
Resolution
AGC target
Maximum IT
Spectrum data t Centroid
Runtime
Data acquisition start time and end time for selected MS experiment [min] (0.00 ...
Experiment Setup
Summary
Ready
NOT SAVED


OMICS positive 250-1200 1Da steps single charge centroid.meth - Thermo Xcalibur Instrument Setup
File
Help
Dionex Chromatography
Q Exactive Plus - Orbitrap MS
Global Lists
Tune Files
External Hardware
Chromatogram
Scan Groups
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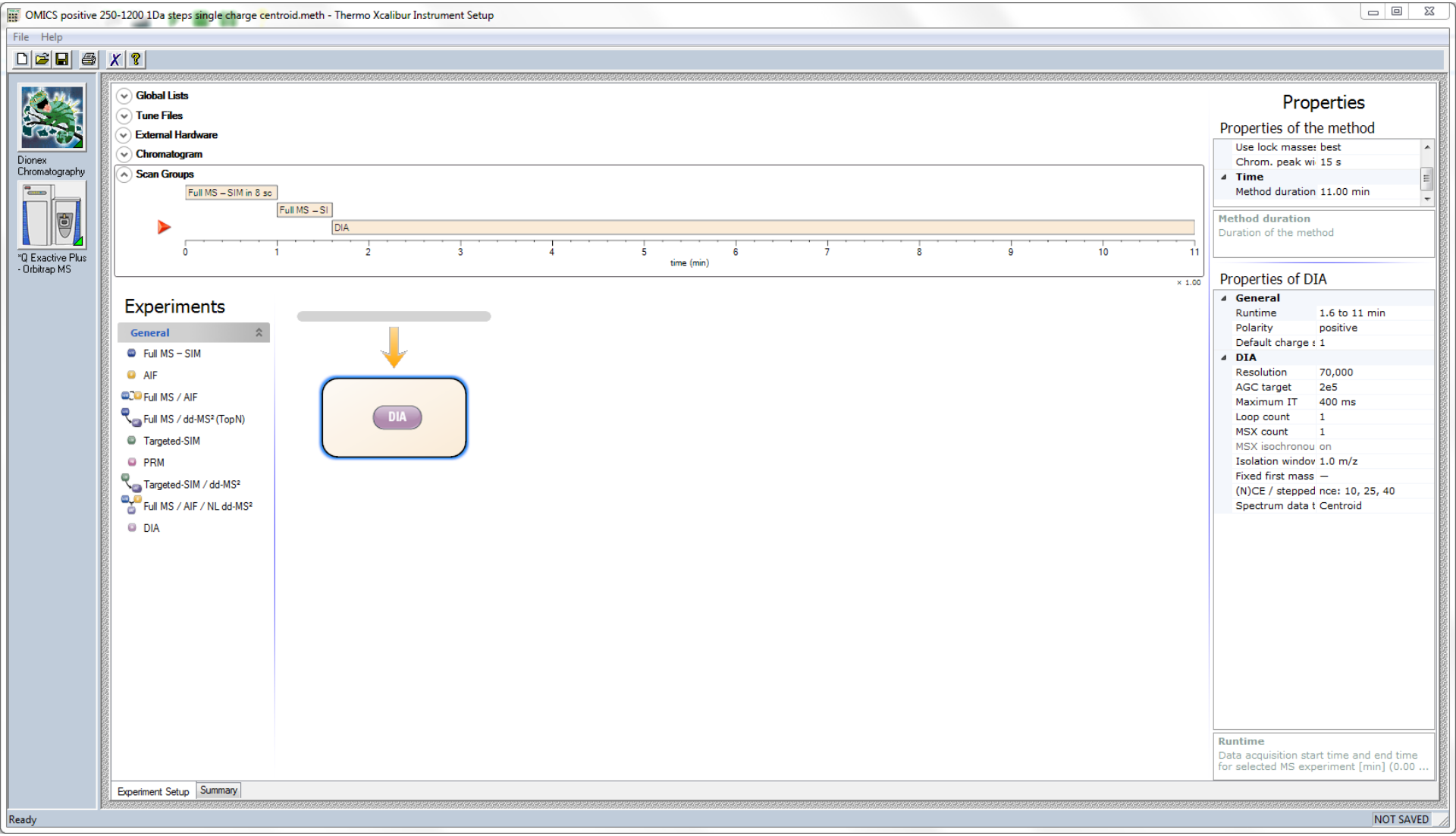
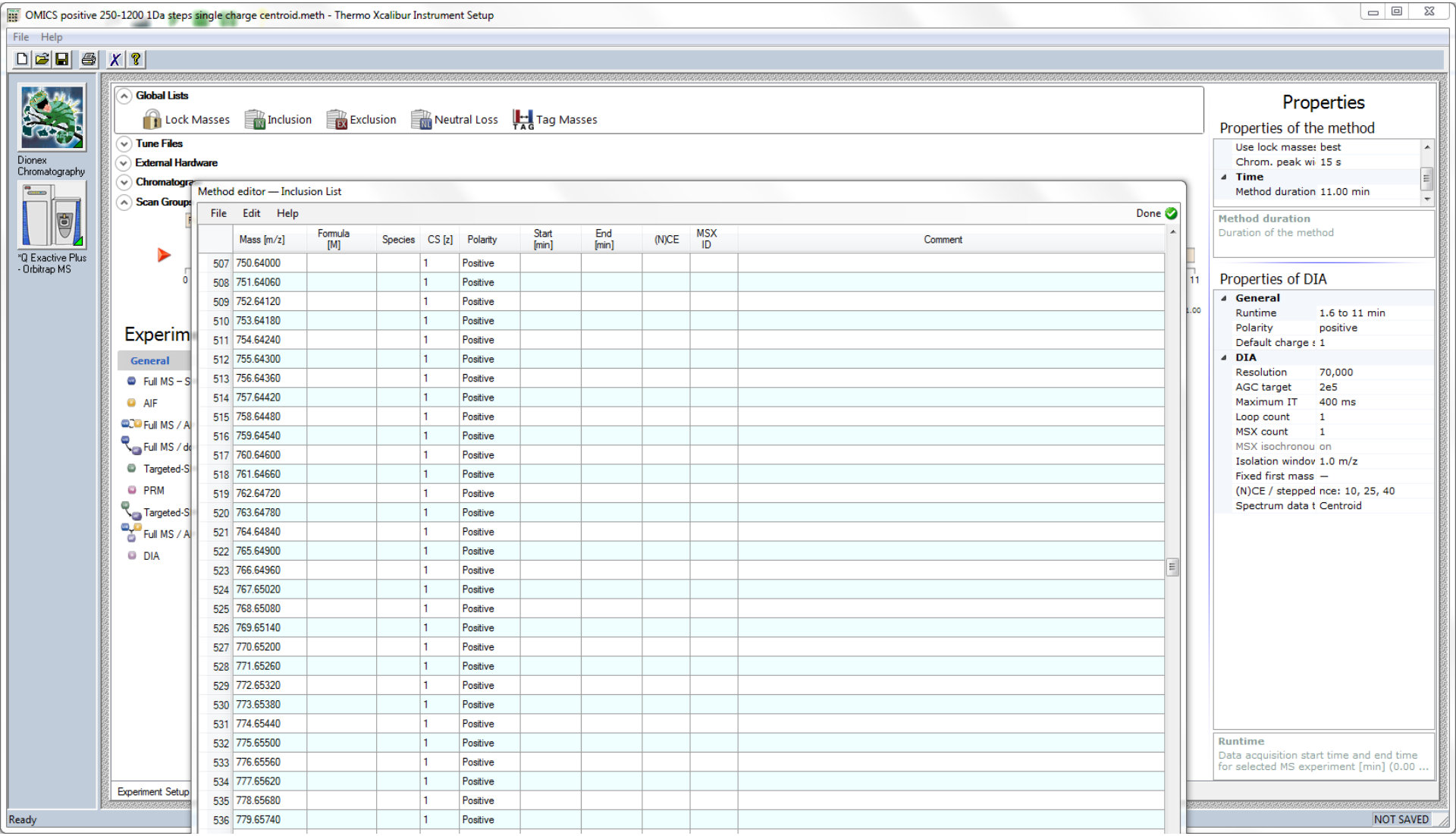
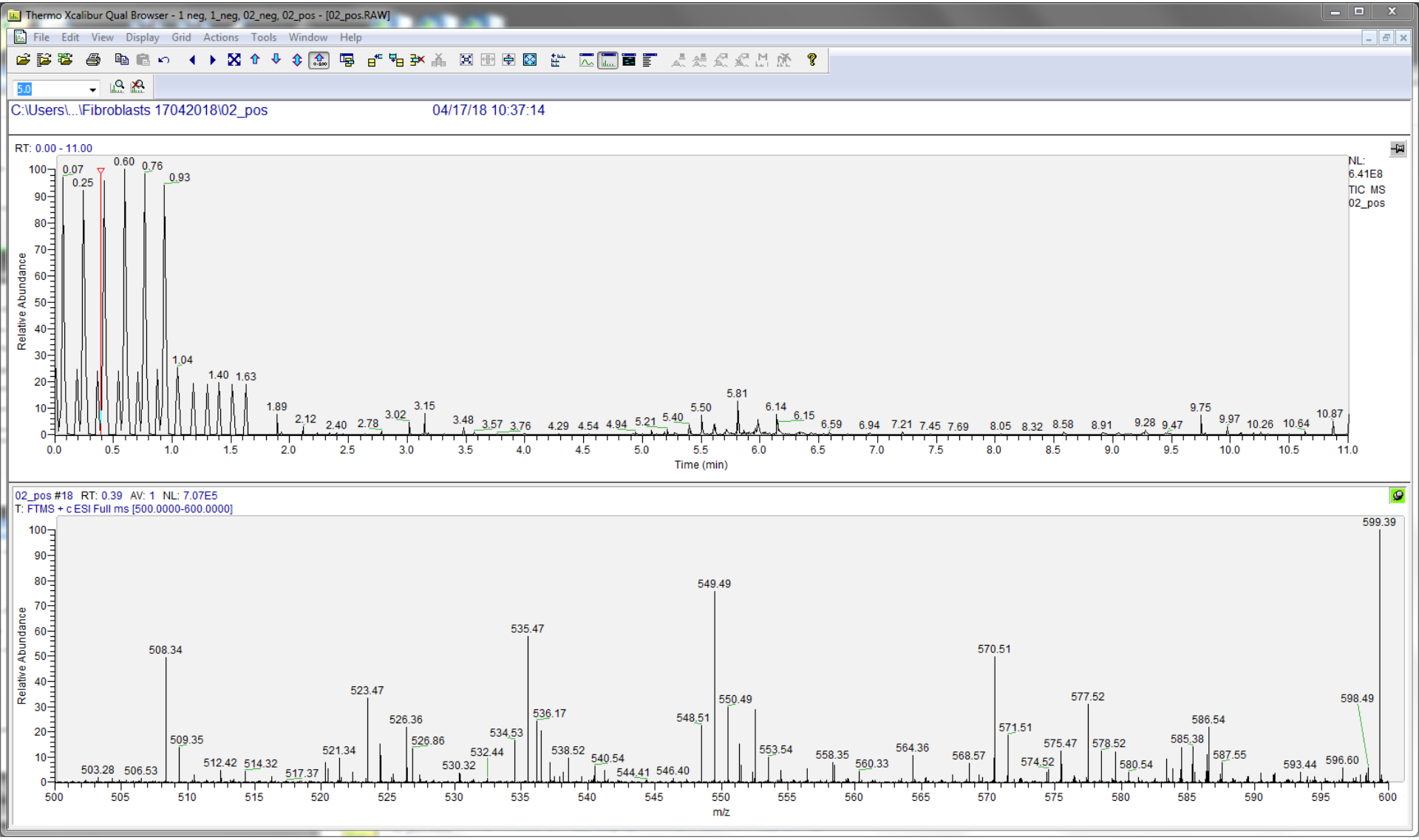
Full MS – SI
DIA
0
1
2
3
4
5
6
7
8
9
10
11
time (min)
× 1.00
Experiments
General
Full MS – SIM
AIF
Full MS / AIF
Full MS / dd-MS² (TopN)
Targeted-SIM
PRM
Targeted-SIM / dd-MS²
Full MS / AIF / NL dd-MS²
DIA
Full MS
Properties
Properties of the method
Use lock masses best
Chrom. peak wi 15 s
Time
Method duration 11.00 min
Method duration
Duration of the method
Properties of Full MS — SIM
General
Runtime 0 to 1 min
Polarity positive
Full MS — SIM
Resolution 280,000
AGC target 3e6
Maximum IT 2000 ms
Number of scan 8
Scan range 1 400 to 500 m/z
Scan range 2 500 to 600 m/z
Scan range 3 600 to 700 m/z
Scan range 4 700 to 800 m/z
Scan range 5 800 to 900 m/z
Scan range 6 900 to 1000 m/z
Scan range 7 1000 to 1100 m/z
Scan range 8 1100 to 1200 m/z
Spectrum data t Centroid
Runtime
Data acquisition start time and end time for selected MS experiment [min] (0.00 ...
Experiment Setup
Summary
Ready
NOT SAVED

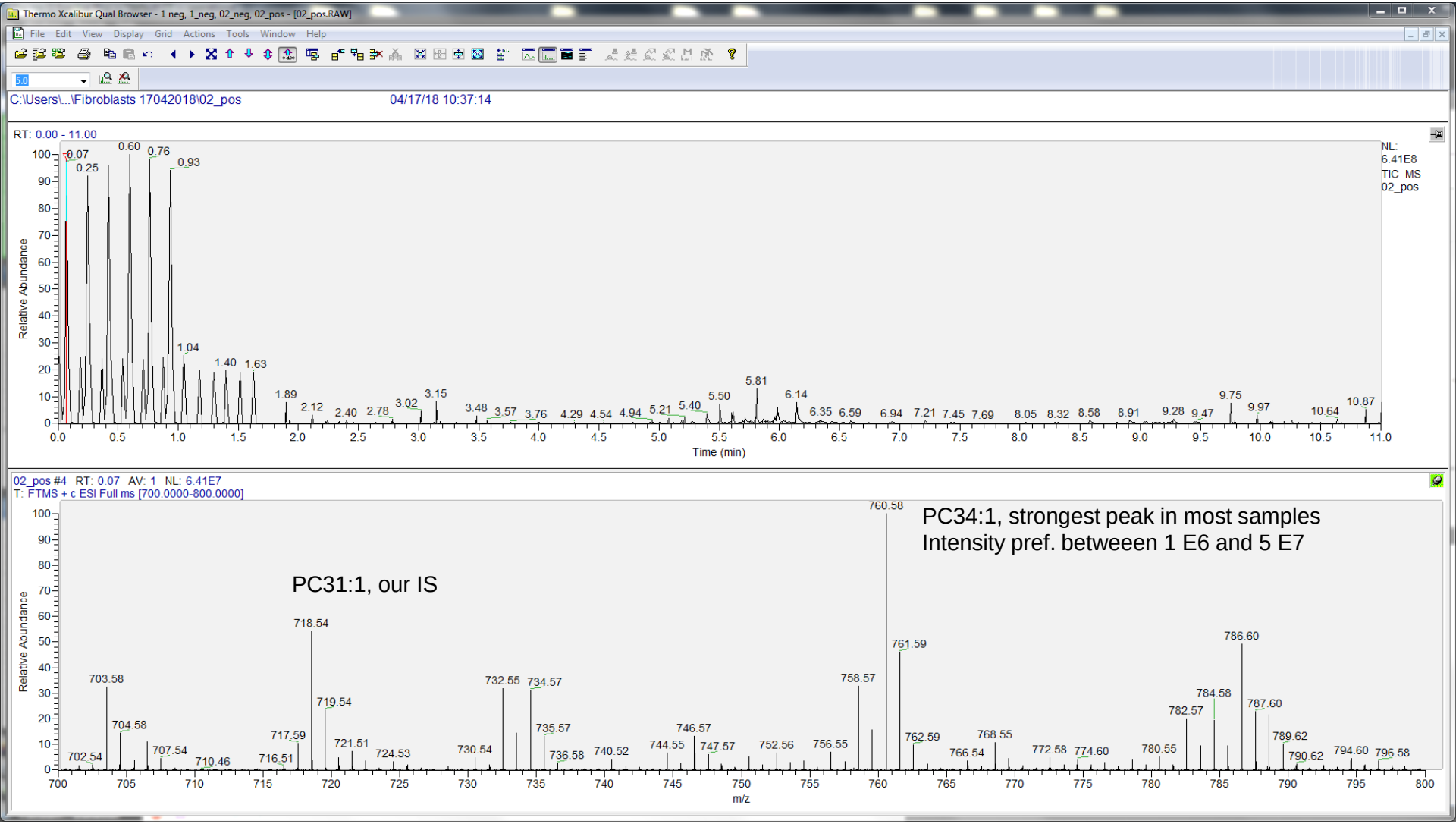

OMICS positive 250-1200 1Da steps single charge centroid.meth - Thermo Xcalibur Instrument Setup
File Help
Dionex Chromatography
Q Exactive Plus - Orbitrap MS
Global Lists
Tune Files
External Hardware
Chromatogram
Scan Groups
Full MS – SIM in 8 sc
Full MS – SI
DIA
0 1 2 3 4 5 6 7 8 9 10 11
time (min)
× 1.00
Experiments
General
Full MS – SIM
AIF
Full MS / AIF
Full MS / dd-MS² (TopN)
Targeted-SIM
PRM
Targeted-SIM / dd-MS²
Full MS / AIF / NL dd-MS²
DIA
DIA
Properties
Properties of the method
Use lock masses best
Chrom. peak wi 15 s
Time
Method duration 11.00 min
Method duration
Duration of the method
Properties of DIA
General
Runtime 1.6 to 11 min
Polarity positive
Default charge s 1
DIA
Resolution 70,000
AGC target 2e5
Maximum IT 400 ms
Loop count 1
MSX count 1
MSX isochronou on
Isolation windov 1.0 m/z
Fixed first mass —
(N)CE / stepped nce: 10, 25, 40
Spectrum data t Centroid
Runtime
Data acquisition start time and end time for selected MS experiment [min] (0.00 ...
Experiment Setup
Summary
Ready
NOT SAVED


OMICS positive 250-1200 1Da steps single charge centroid.meth - Thermo Xcalibur Instrument Setup
File Help
Dionex Chromatography
Q Exactive Plus - Orbitrap MS
Global Lists
Lock Masses
Inclusion
Exclusion
Neutral Loss
Tag Masses
Tune Files
External Hardware
Chromatogra
Scan Groups
Experim
General
Full MS – S
AIF
Full MS / A
Full MS / d
Targeted-S
PRM
Targeted-S
Full MS / A
DIA
Experiment Setup
Method editor — Inclusion List
File Edit Help
Done
Mass [m/z] Formula [M] Species CS [z] Polarity Start [min] End [min] (N)CE MSX ID Comment
507 750.64000 1 Positive
508 751.64060 1 Positive
509 752.64120 1 Positive
510 753.64180 1 Positive
511 754.64240 1 Positive
512 755.64300 1 Positive
513 756.64360 1 Positive
514 757.64420 1 Positive
515 758.64480 1 Positive
516 759.64540 1 Positive
517 760.64600 1 Positive
518 761.64660 1 Positive
519 762.64720 1 Positive
520 763.64780 1 Positive
521 764.64840 1 Positive
522 765.64900 1 Positive
523 766.64960 1 Positive
524 767.65020 1 Positive
525 768.65080 1 Positive
526 769.65140 1 Positive
527 770.65200 1 Positive
528 771.65260 1 Positive
529 772.65320 1 Positive
530 773.65380 1 Positive
531 774.65440 1 Positive
532 775.65500 1 Positive
533 776.65560 1 Positive
534 777.65620 1 Positive
535 778.65680 1 Positive
536 779.65740 1 Positive
Properties
Properties of the method
Use lock masses best
Chrom. peak wi 15 s
Time
Method duration 11.00 min
Method duration
Duration of the method
Properties of DIA
General
Runtime 1.6 to 11 min
Polarity positive
Default charge s 1
DIA
Resolution 70,000
AGC target 2e5
Maximum IT 400 ms
Loop count 1
MSX count 1
MSX isochronou on
Isolation windov 1.0 m/z
Fixed first mass —
(N)CE / stepped nce: 10, 25, 40
Spectrum data t Centroid
Runtime
Data acquisition start time and end time for selected MS experiment [min] (0.00 ...
Ready
NOT SAVED

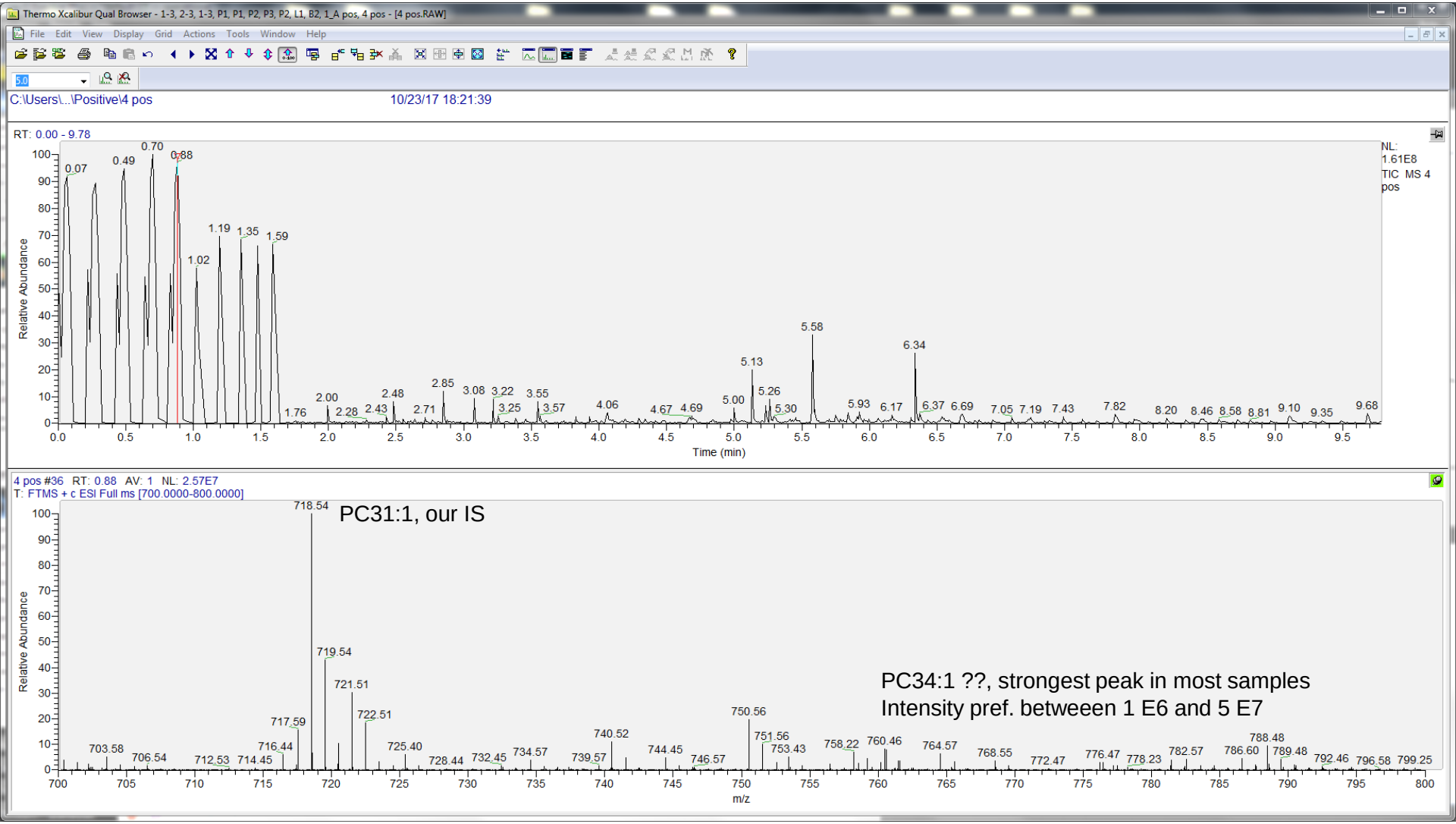
Quick quality check: this is a nice sample



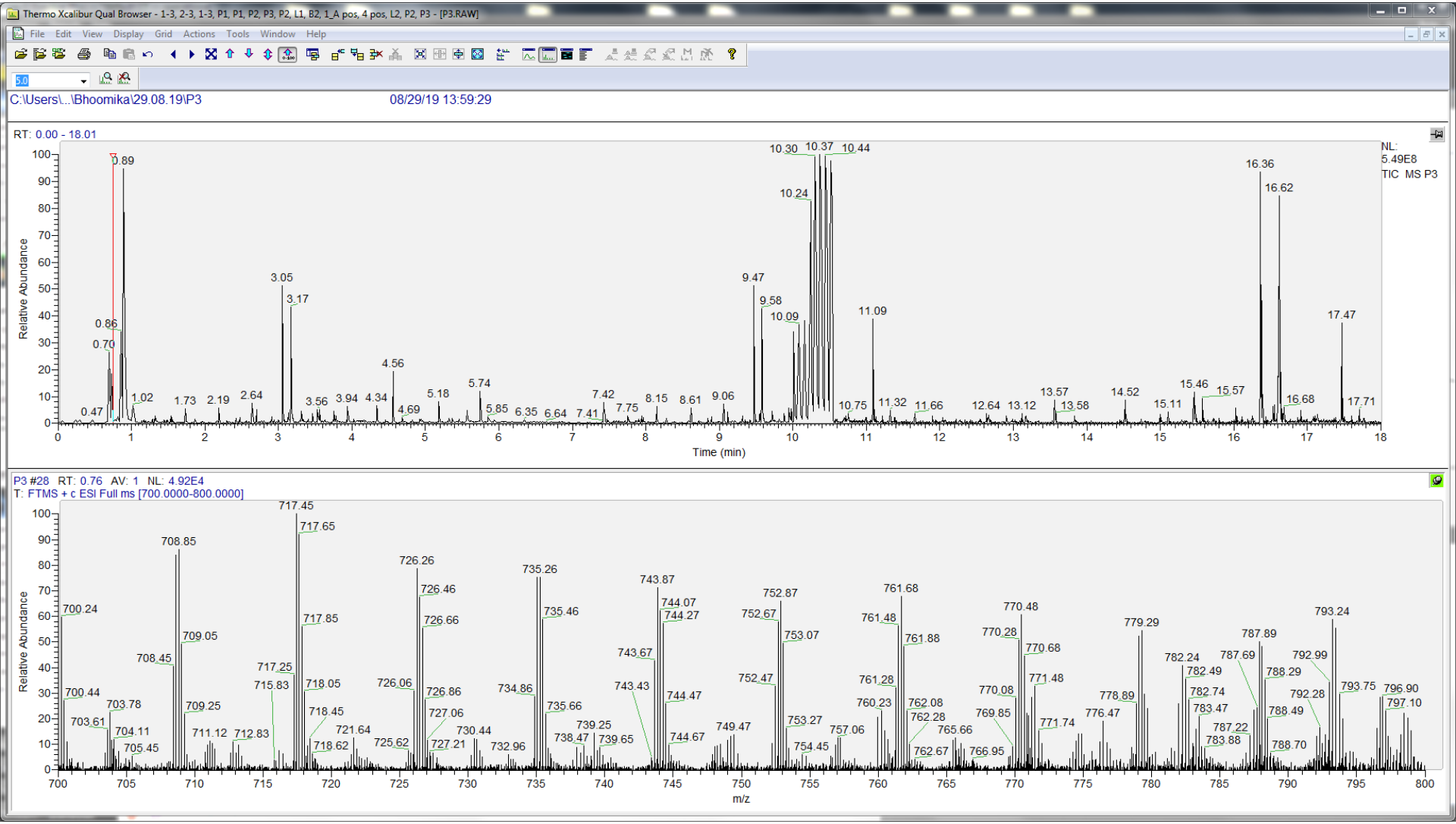
Quick quality check: this is a nice sample



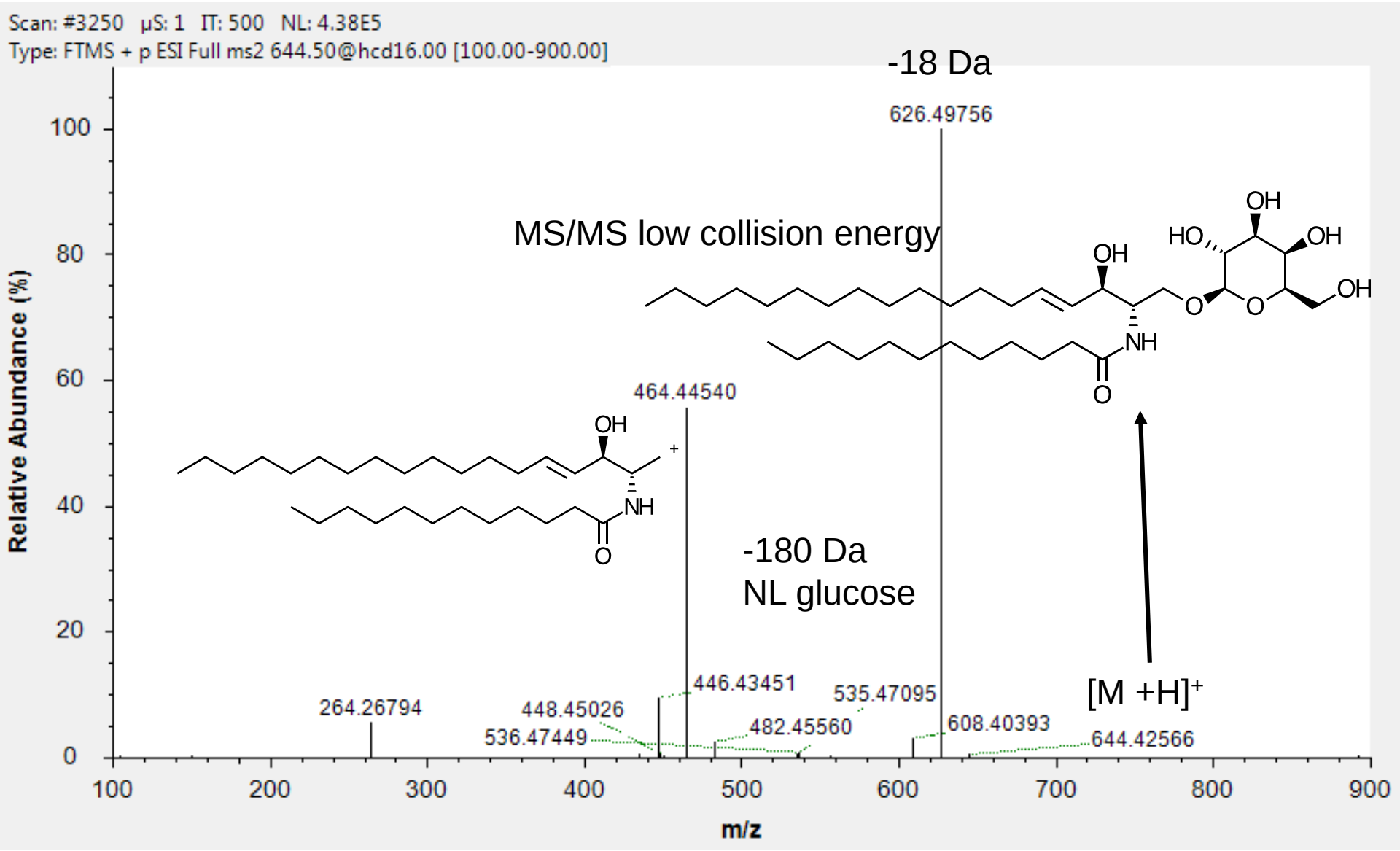
Quick quality check: this is a good quality, low intensity sample



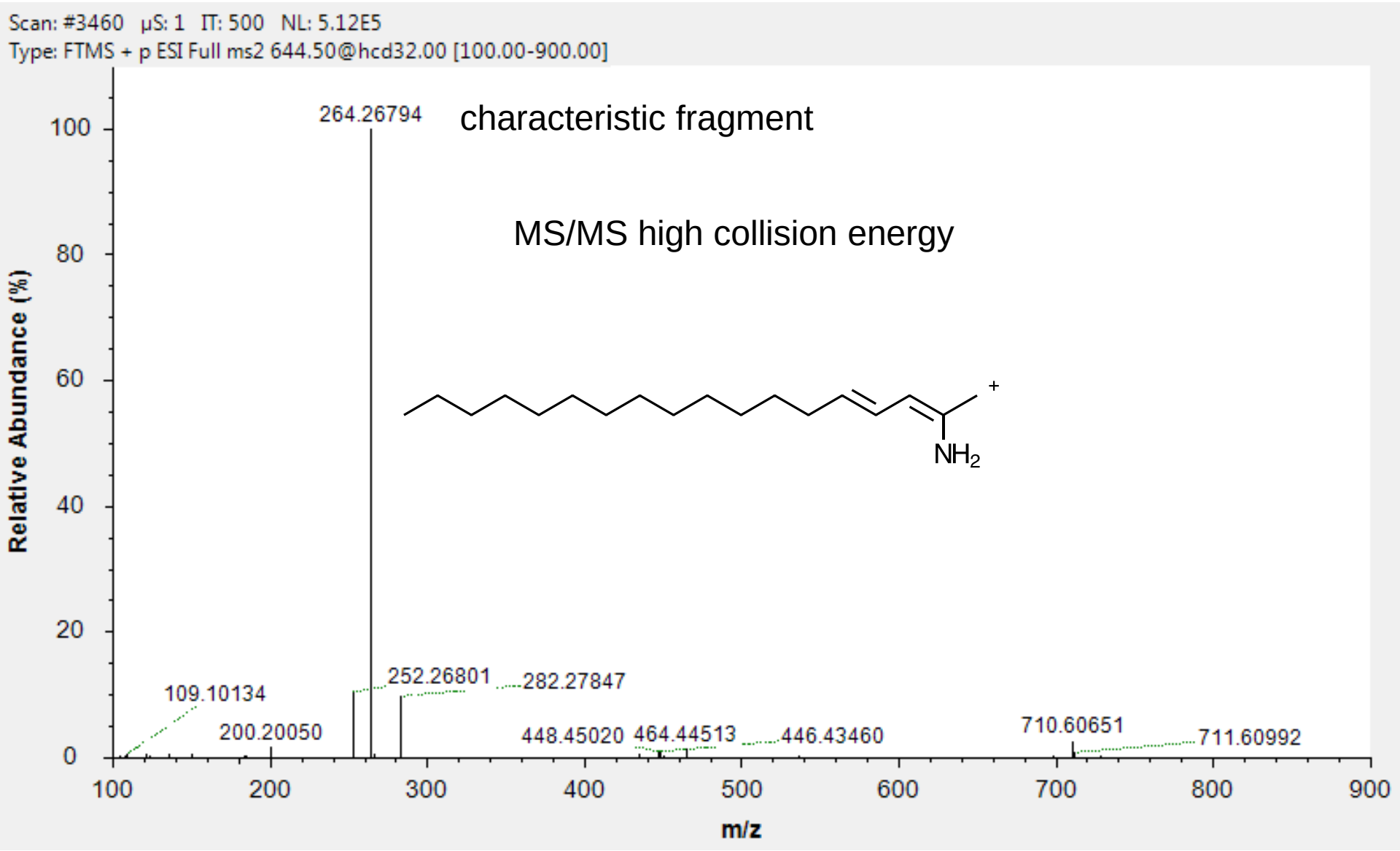
Quick quality check: this is a bad sample



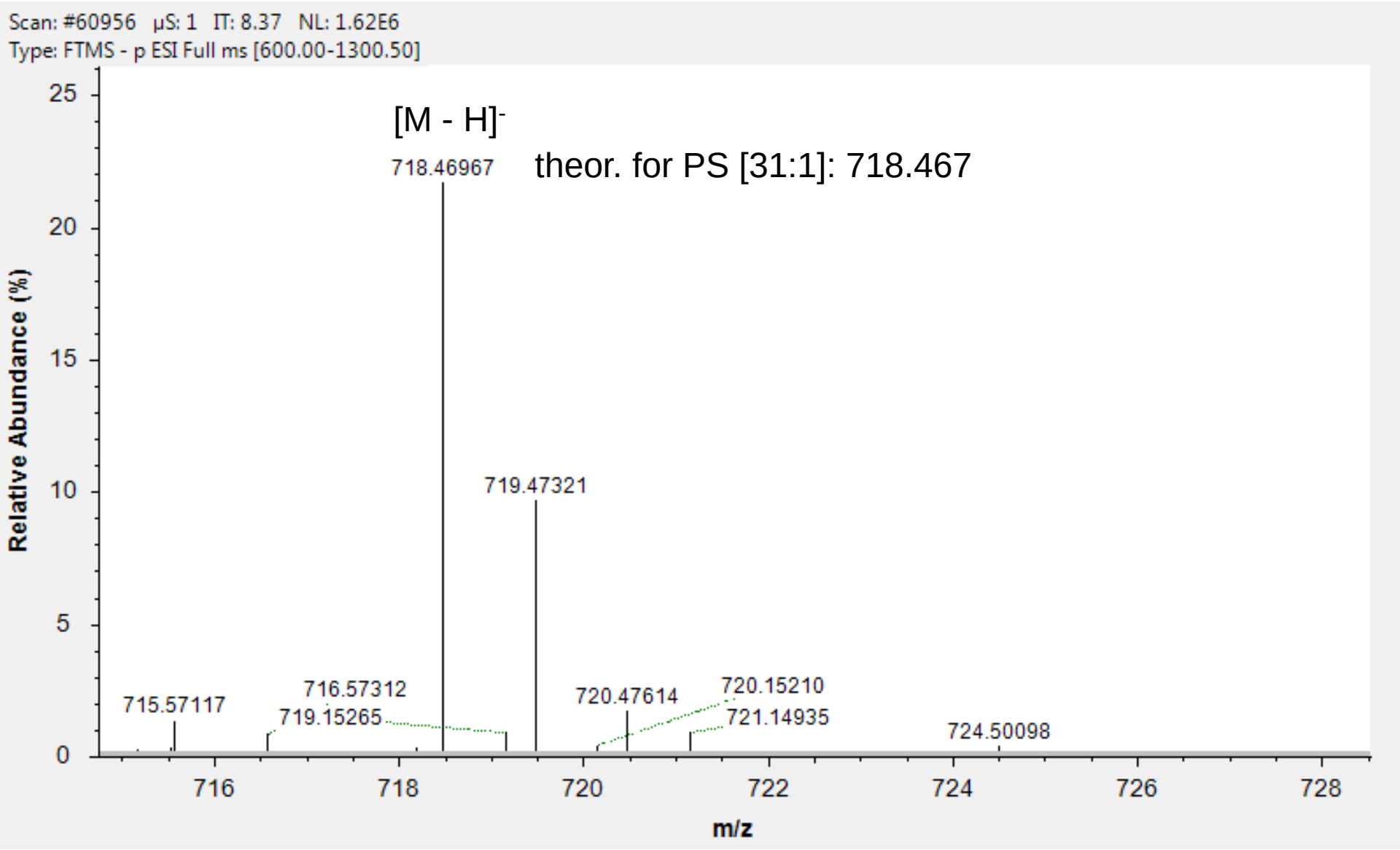
Lipid identification by characteristic fragmentation: GlcCer



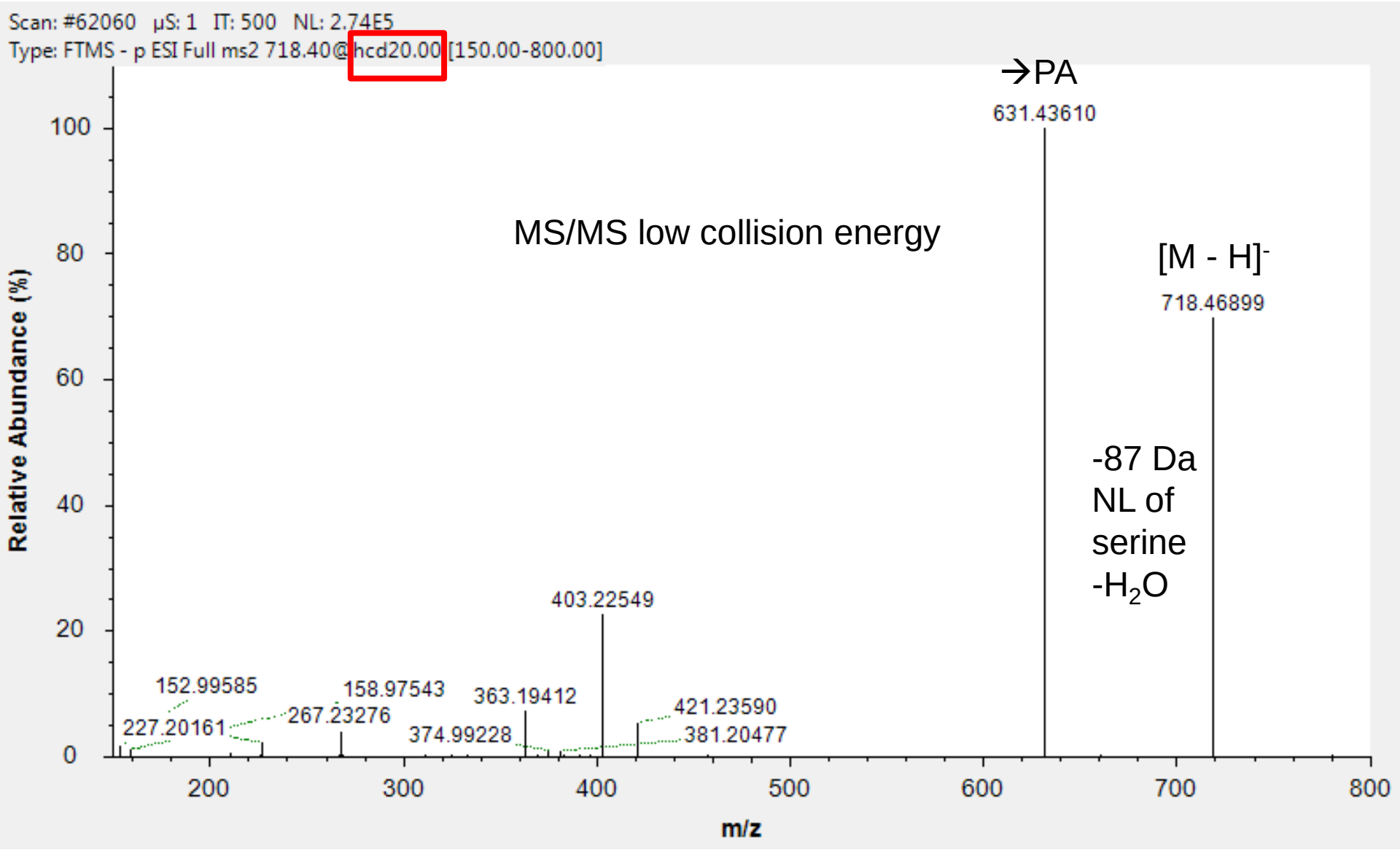
Lipid identification by characteristic fragmentation: GlcCer



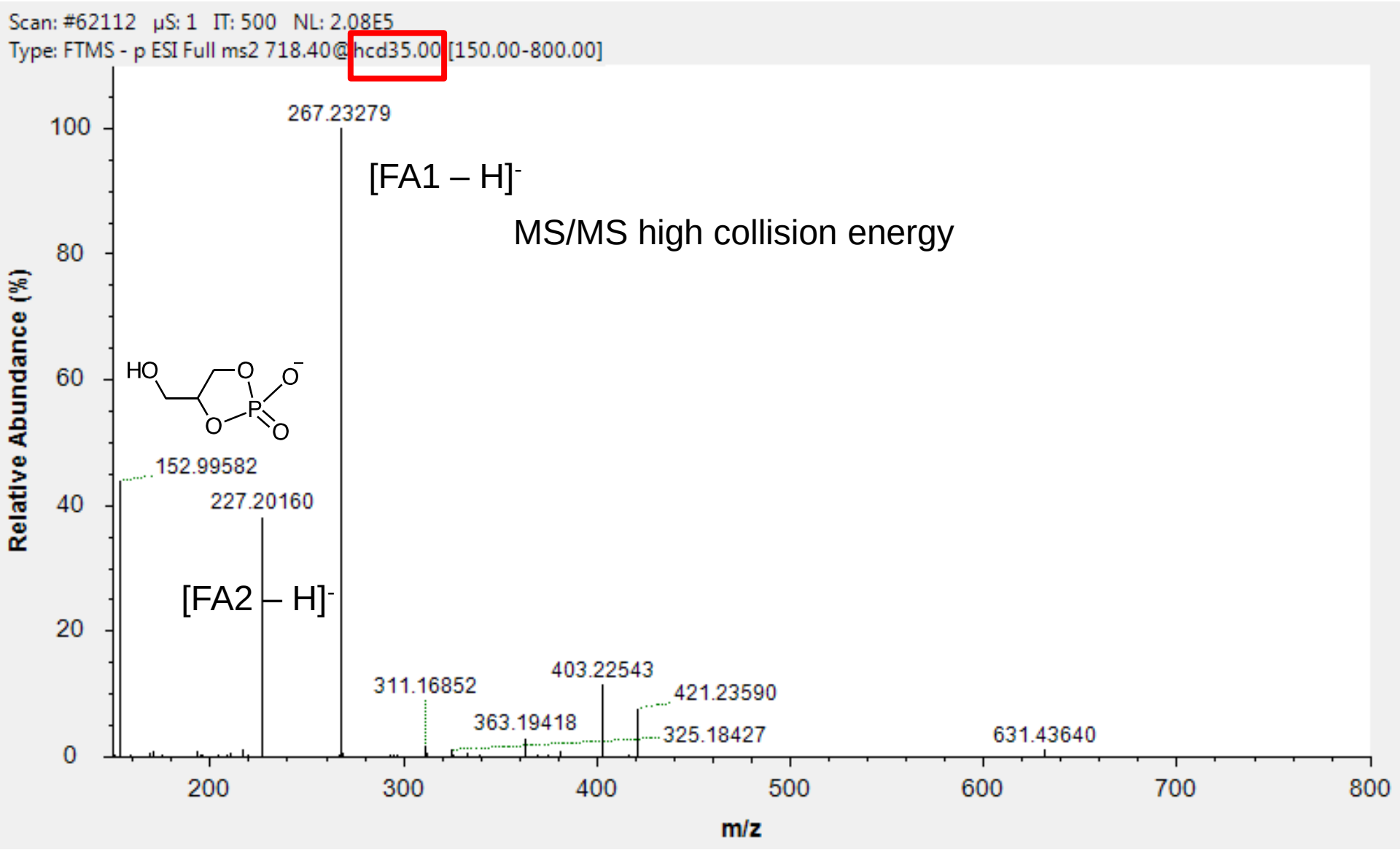
Lipid identification by characteristic fragmentation: PS neg.



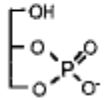
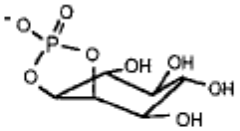
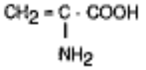
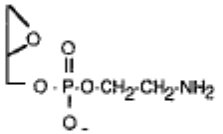
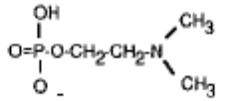
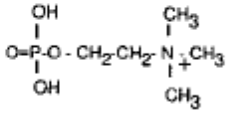
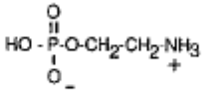
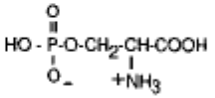
Lipid identification by characteristic fragmentation: PS



Lipid identification by characteristic fragmentation: PS

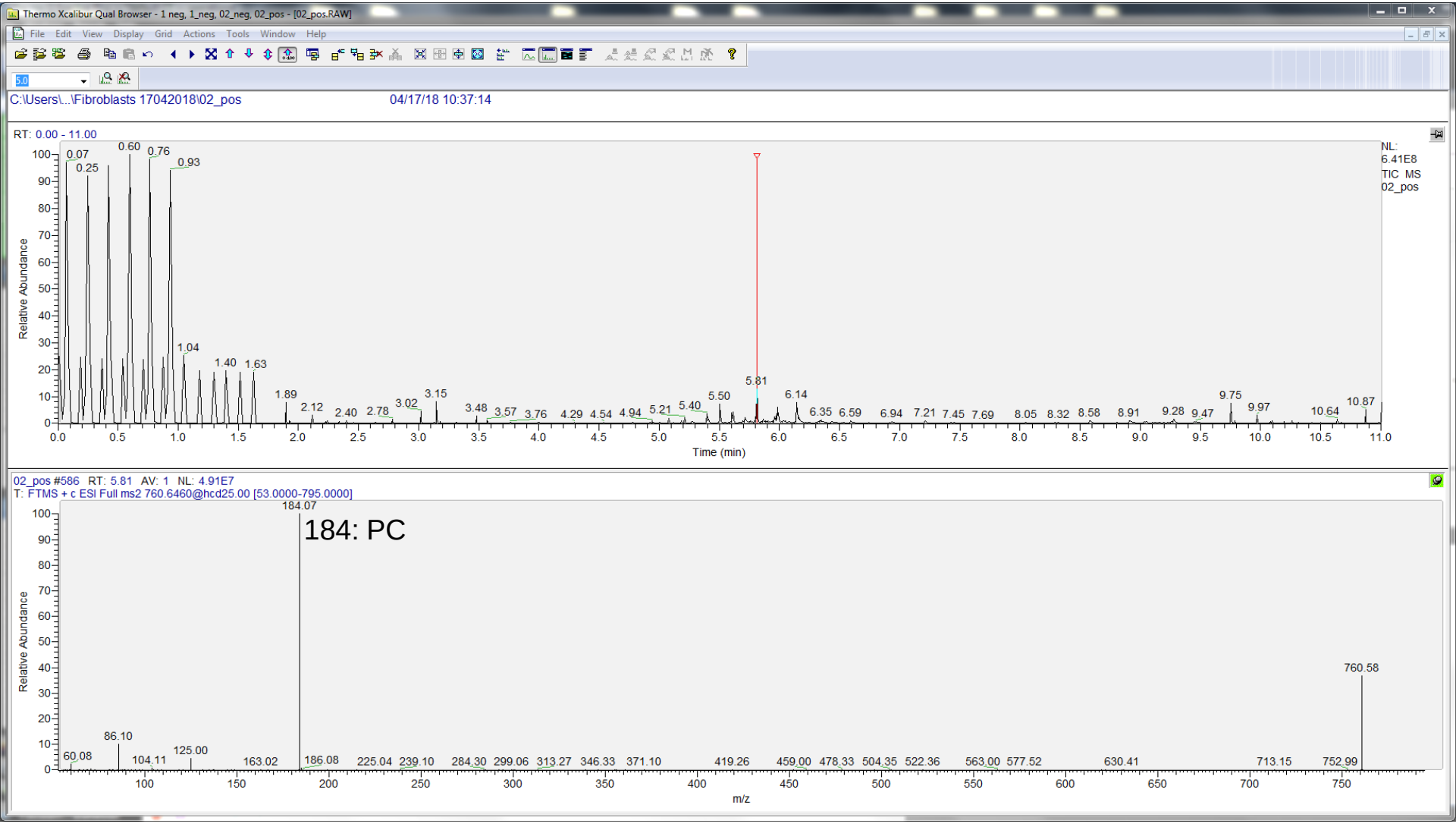


Lipid identification by characteristic fragmentation: overview

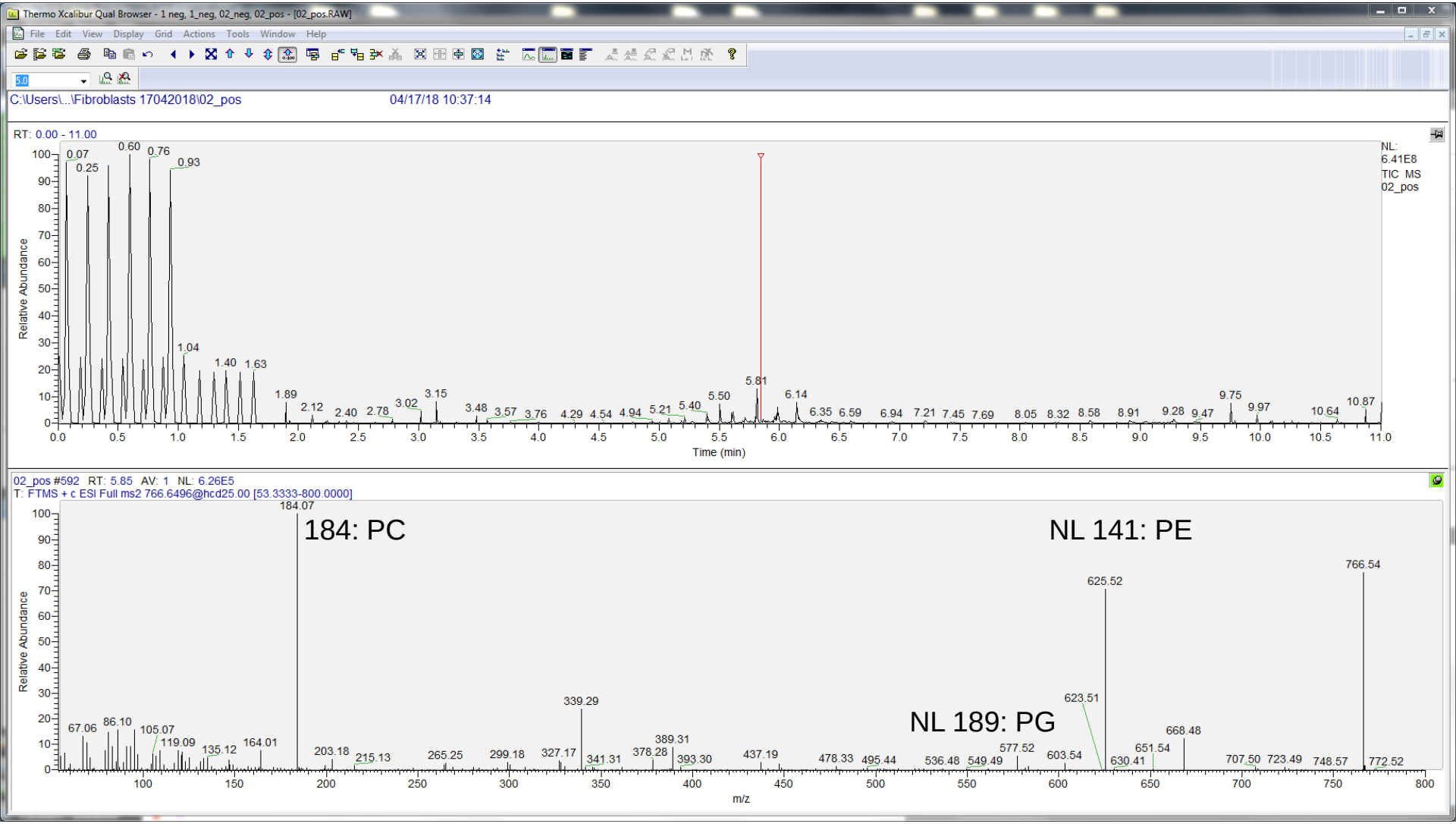
specificity	polarity	fragment structure	scan mode	fragment type	optimal collision offset
all $[M-H]^-$ ions of glycerophospho-lipids	neg.		precursor of 153	glycerol-phosphate $-H_2O$	+50 V
phosphatidyl-inositol	neg.		precursor of 241	head group $-H_2O$	+45 V
phosphatidyl-serine	neg.		neutral loss of 87	head group $-H_3PO_4$	+28 V
phosphatidyl-ethanolamine	neg.		precursor of 196	dilyso $-H_2O$	+50 V
sphingomyelin	neg.		sCID + precursor of 168	head group (demethyl-ated)	sCID +65 & +40 V
phosphatidyl-choline and sphingomyelin	pos.		precursor of 184	head group	-35 V
phosphatidyl-ethanolamine	pos.		neutral loss of 141	head group	-25 V
phosphatidyl-serine	pos.		neutral loss of 185	head group	-22 V

[1] Brugger B, Erben G, Sandhoff R, Wieland FT & Lehmann WD (1997) Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 94, 2339–2344.

MS2 of m/z 760.6



MS2 of m/z 766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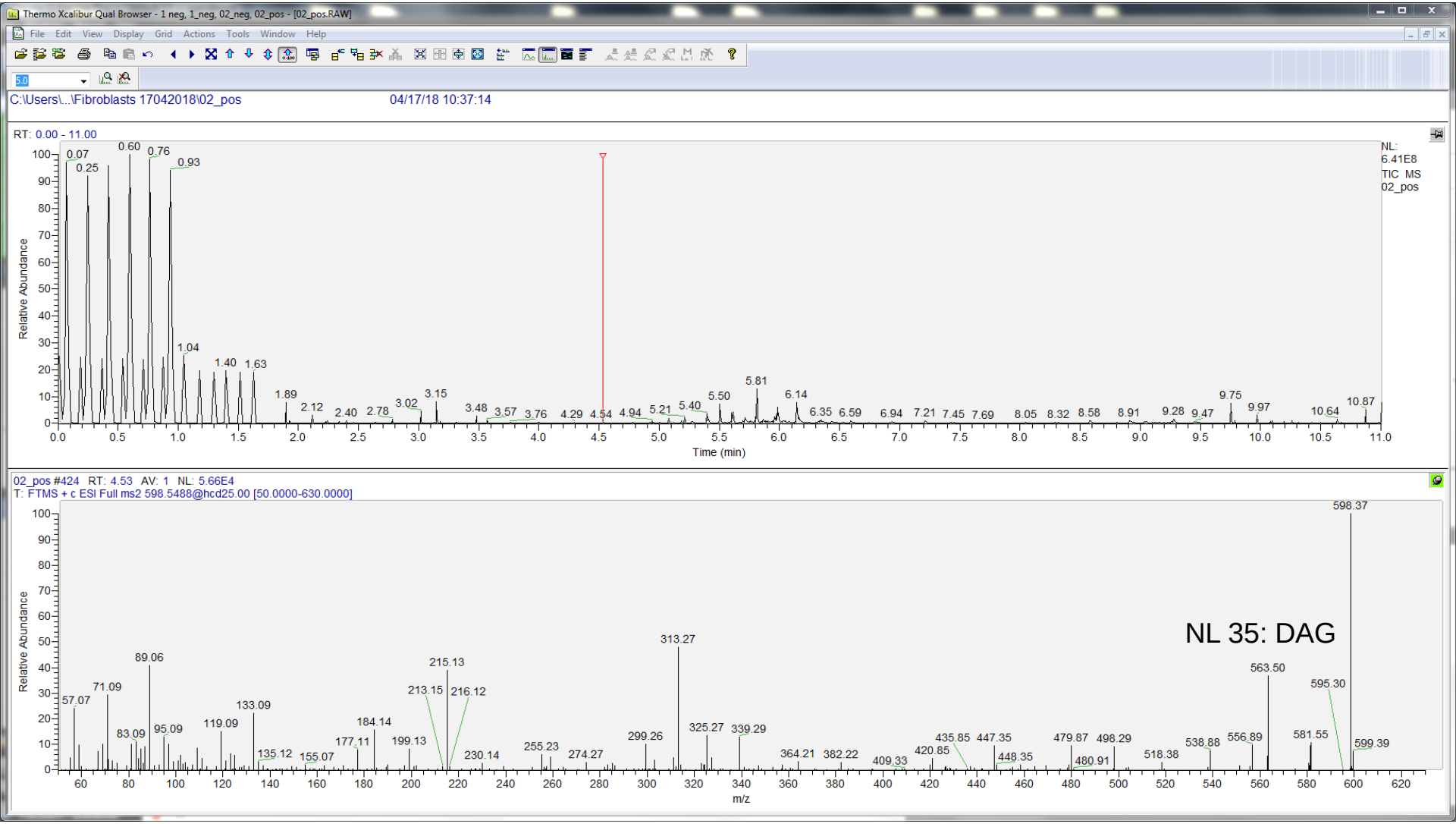
File Edit View Display Grid Actions Tools Window Help
C:\Users\...\Fibroblasts 17042018\02_pos 04/17/18 10:37:14
RT: 0.00 - 11.00
NL: 6.41E8 TIC MS 02_pos
Relative Abundance
Time (min)
02_pos #592 RT: 5.85 AV: 1 NL: 6.26E5
T: FTMS + c ESI Full ms2 766.6496@hcd25.00 [53.3333-800.0000]
184: PC
NL 141: PE
NL 189: PG
m/z

MS2 of m/z 766, zoom into precursor mass

MS2 of m/z 598


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
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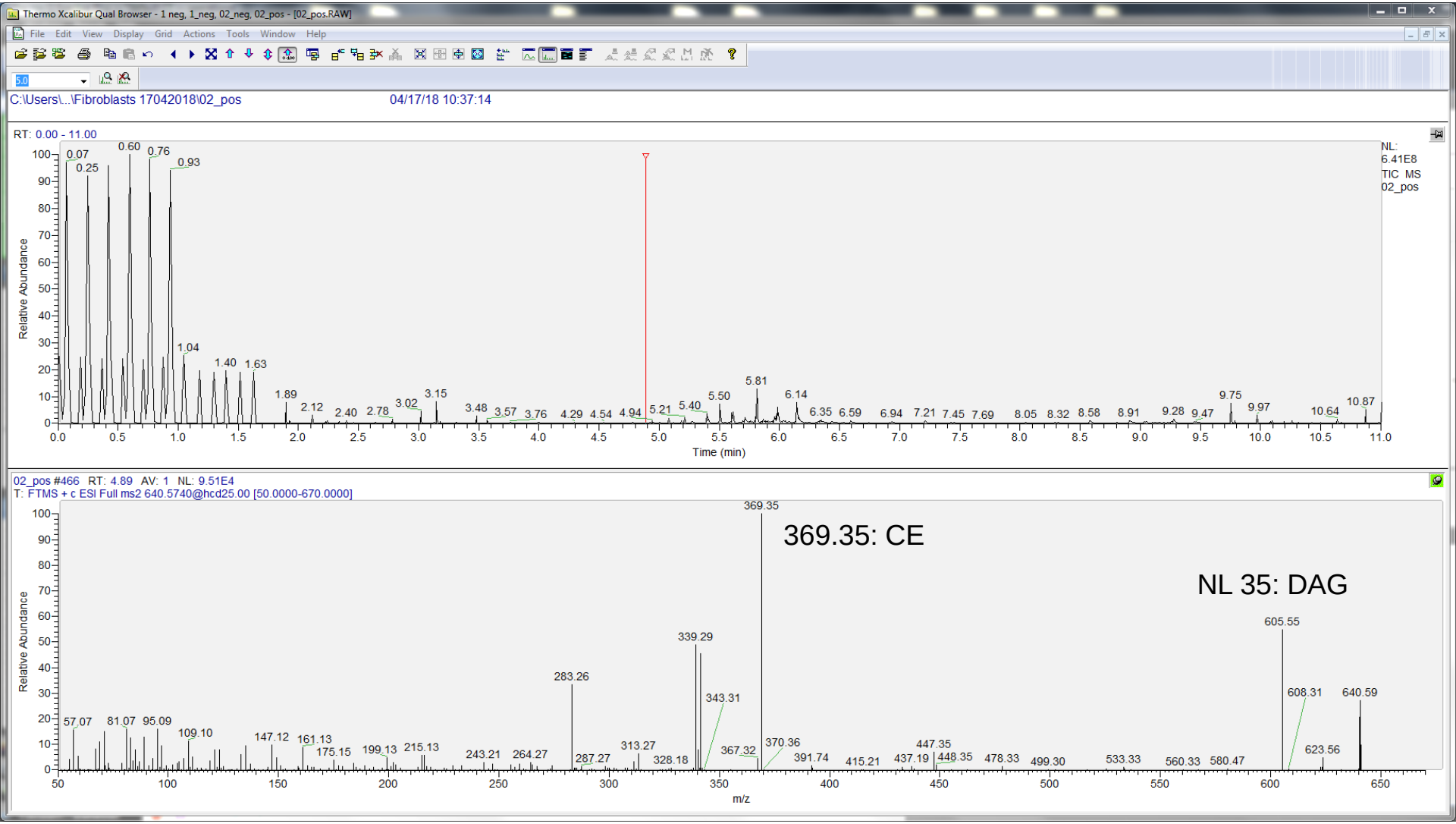
RT: 0.00 - 11.00
NL: 6.41E8 TIC MS 02_pos
Relative Abundance
Time (min)
02_pos #424 RT: 4.53 AV: 1 NL: 5.66E4
T: FTMS + c ESI Full ms2 598.5488@hcd25.00 [50.0000-630.0000]
NL 35: DAG
m/z

MS2 of m/z 640


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
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RT: 0.00 - 11.00
NL: 6.41E8 TIC MS 02_pos
Relative Abundance
Time (min)
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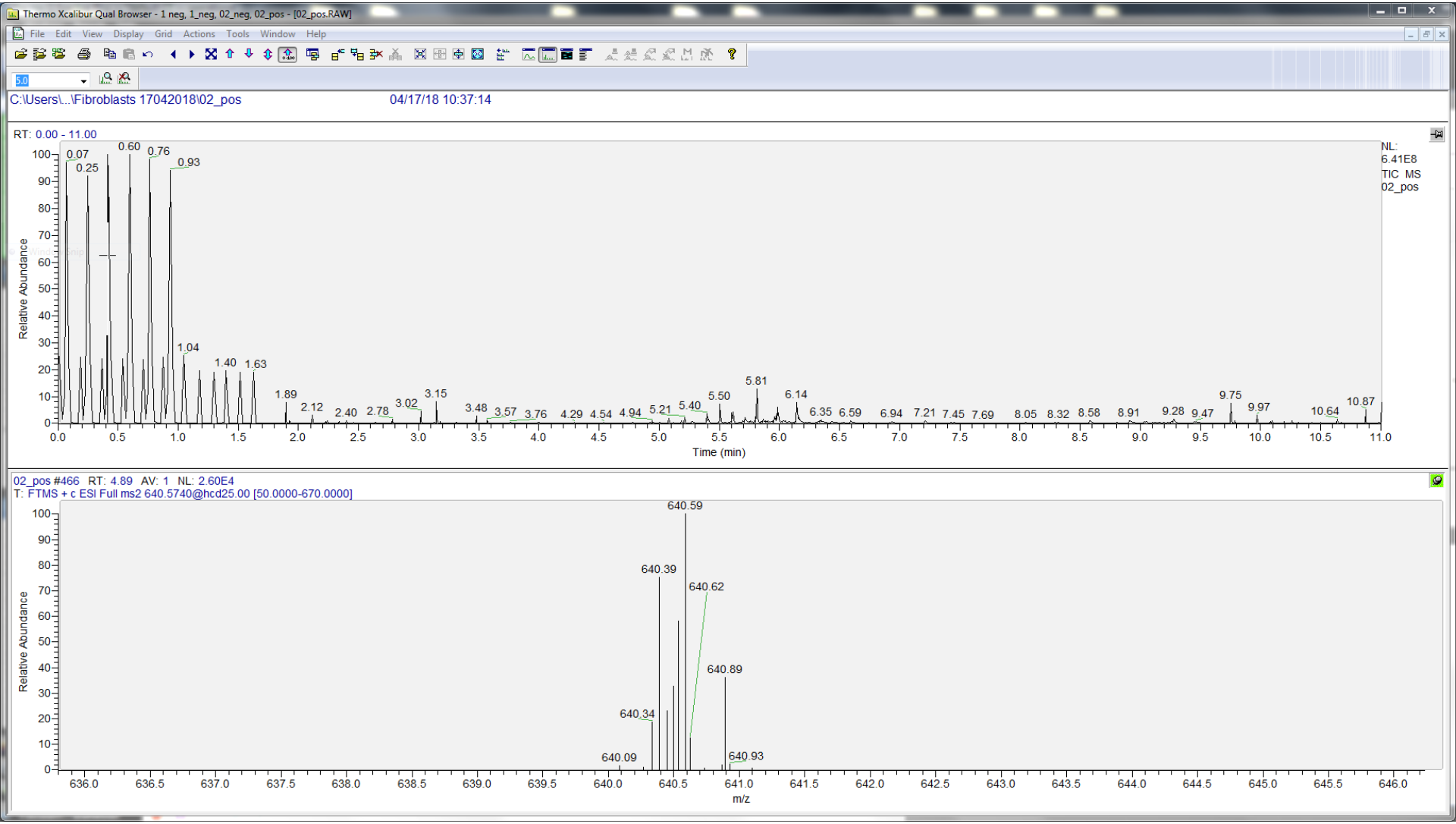
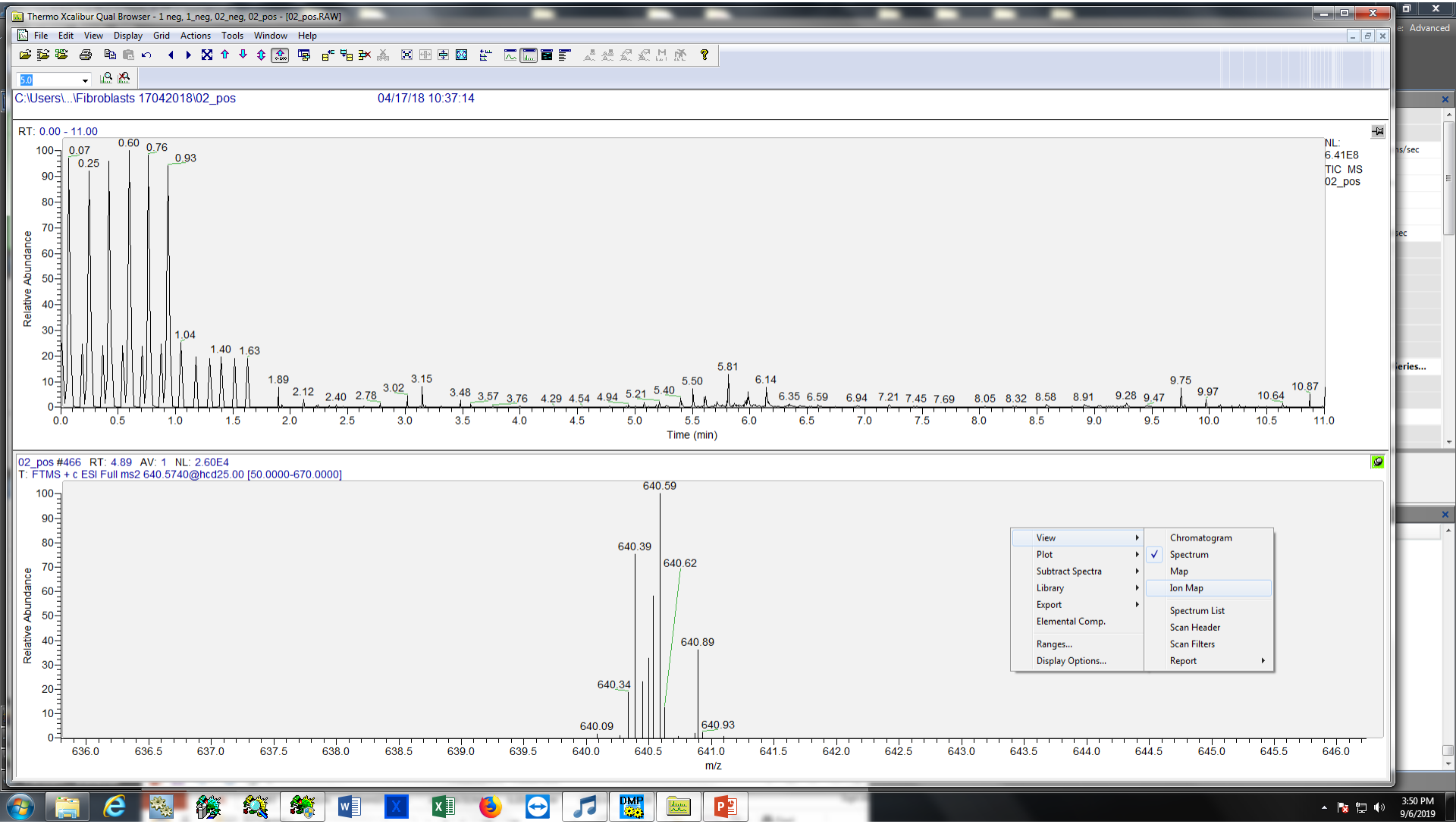
T: FTMS + c ESI Full ms2 640.5740@hcd25.00 [50.0000-670.0000]
369.35: CE
NL 35: DAG
m/z

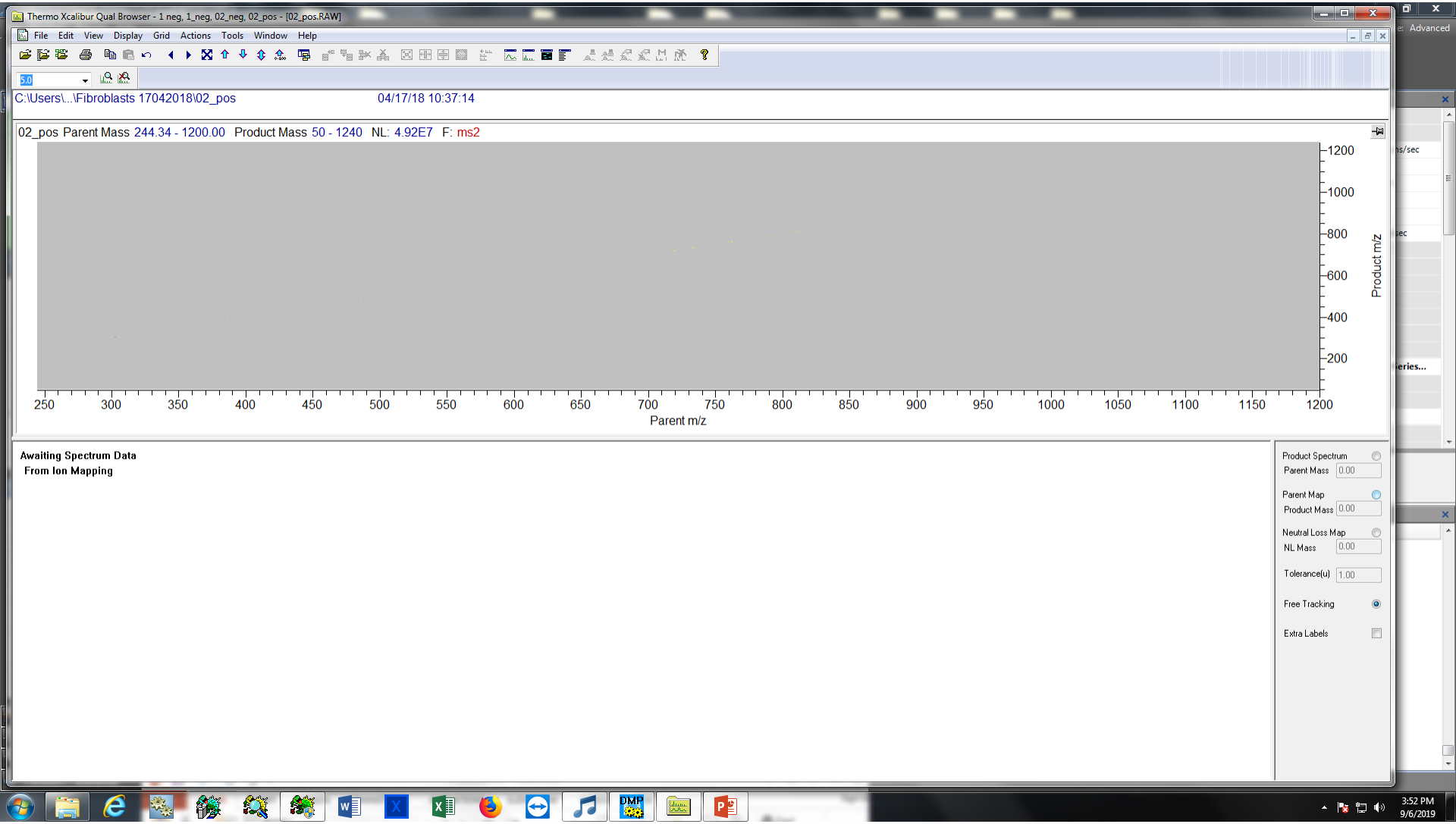
MS2 of m/z 640, zoom into precursor



Class	IS Species	polarity	Identification by			
PE	PE [31:1]	pos	NL 141	loss of phosphoethanolamine		
PC	PC [31:1]	pos	FR 184.07	release of phosphocholine		
PS	PS [31:1]	pos	NL185.01	loss of phosphoserine		
PI	PI [34:0]	pos (NH4+)	NL 277.06	loss of phosphoinositol and NH3		
PA	PA [31:1]	pos (NH4+)	NL 115.00	loss of phosphate and NH3		
PG	PG (28:0)	pos (NH4+)	NL 189.04	loss of phosphoglycerol and NH3		
CL	CL [56:0]	neg	FR 152.99	release of cyclic glycerolphosphate		
LPA	LPA (17:0)	pos (NH4+)	NL 115.00	loss of phosphate and NH3		
LPC	LPC [17:1]	pos	FR 184.07	release of phosphocholine		
LPE	LPE (17:0)	pos	NL 141	loss of phosphoethanolamine		
Cer	Cer [17:0]	pos	FR 264.27	release of the sphingoid base backbone		
SM	SM [17:0]	pos	pos	FR 184.07	release of phosphocholine	
GlcCer	GlcCer [12:0]	pos	FR 264.27	release of the sphingoid base backbone		
GM3	GM3 (18:0-D3	neg	FR 290.09	release of the sialic acid anion		
TAG	TG [47:1]	pos (NH4+)	MS1 of NH4 adduct			
CE	CE [17:1]	pos (NH4+)	FR 369.35	release of cholesten cation		
DAG	DG [31:1]	pos (NH4+)	NL 35.04	loss of water and ammonia		
MAG	MG [17:1]	pos (NH4+)	NL 35.04	loss of water and ammonia		
Chol-d6	Chol [d6]	pos (NH4+)	FR 369.35	release of cholesten cation		
Carn	Car(15:0)	pos	FR 85.03	release of butenoic acid cation from the carnitin		

Emulated fragment ion or neutral loss scan: the ion map function of Xcalibur

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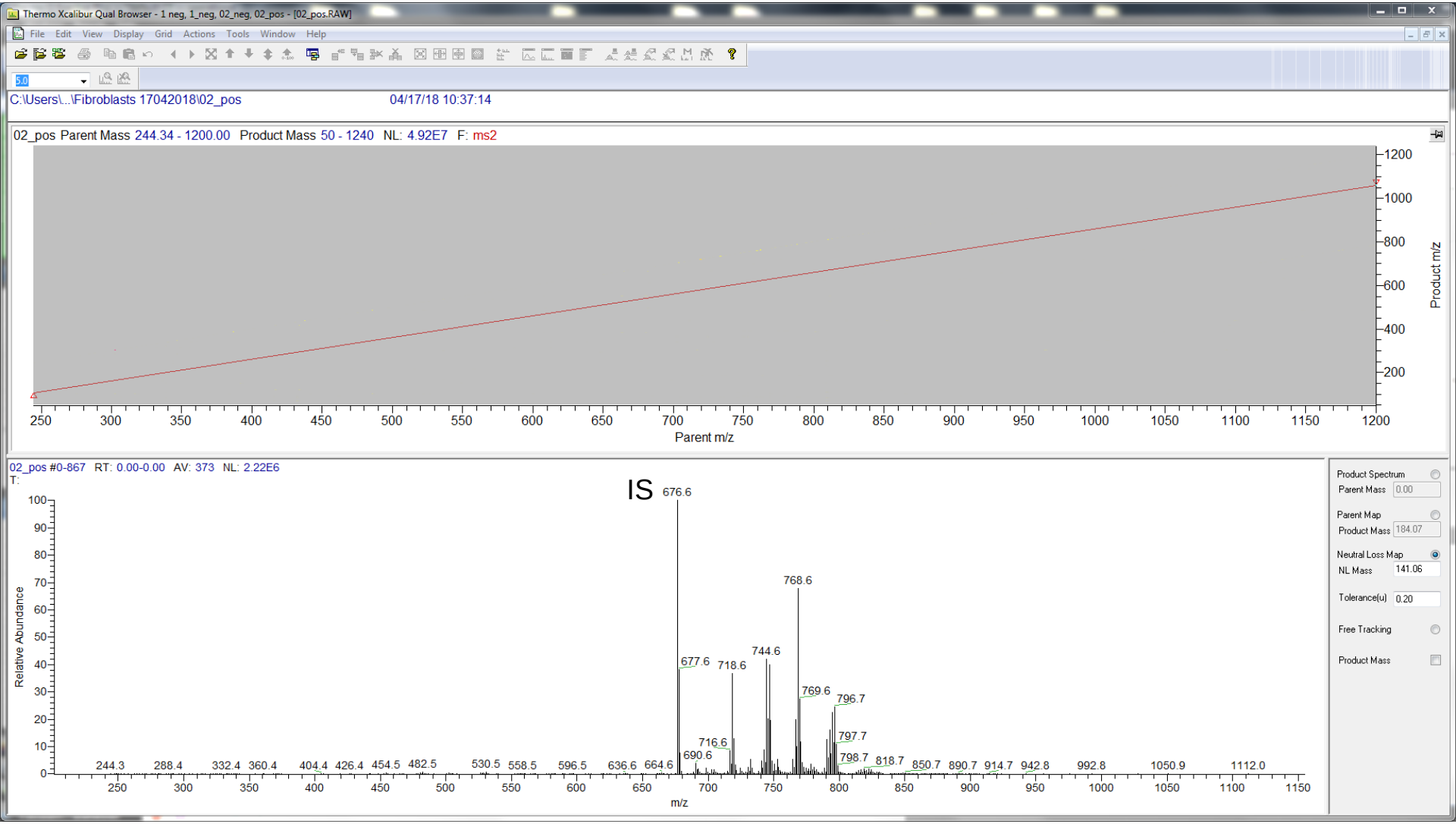
C:\Users\...\Fibroblasts 17042018\02_pos 04/17/18 10:37:14
02_pos Parent Mass 244.34 - 1200.00 Product Mass 50 - 1240 NL: 4.92E7 F: ms2
Product m/z
Parent m/z
Awaiting Spectrum Data
From Ion Mapping
Product Spectrum
Parent Mass
Parent Map
Product Mass
Neutral Loss Map
NL Mass
Tolerance(u)
Free Tracking
Extra Labels

NL 141: PE


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
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Parent m/z
Product m/z
02_pos #0-867 RT: 0.00-0.00 AV: 373 NL: 2.22E6
T:
IS 676.6
768.6
677.6
718.6
744.6
769.6
796.7
797.7
716.6
690.6
798.7
Relative Abundance
m/z
Product Spectrum
Parent Mass 0.00
Parent Map
Product Mass 184.07
Neutral Loss Map
NL Mass 141.06
Tolerance(u) 0.20
Free Tracking
Product Mass

How does it work?

Neutral loss map function:

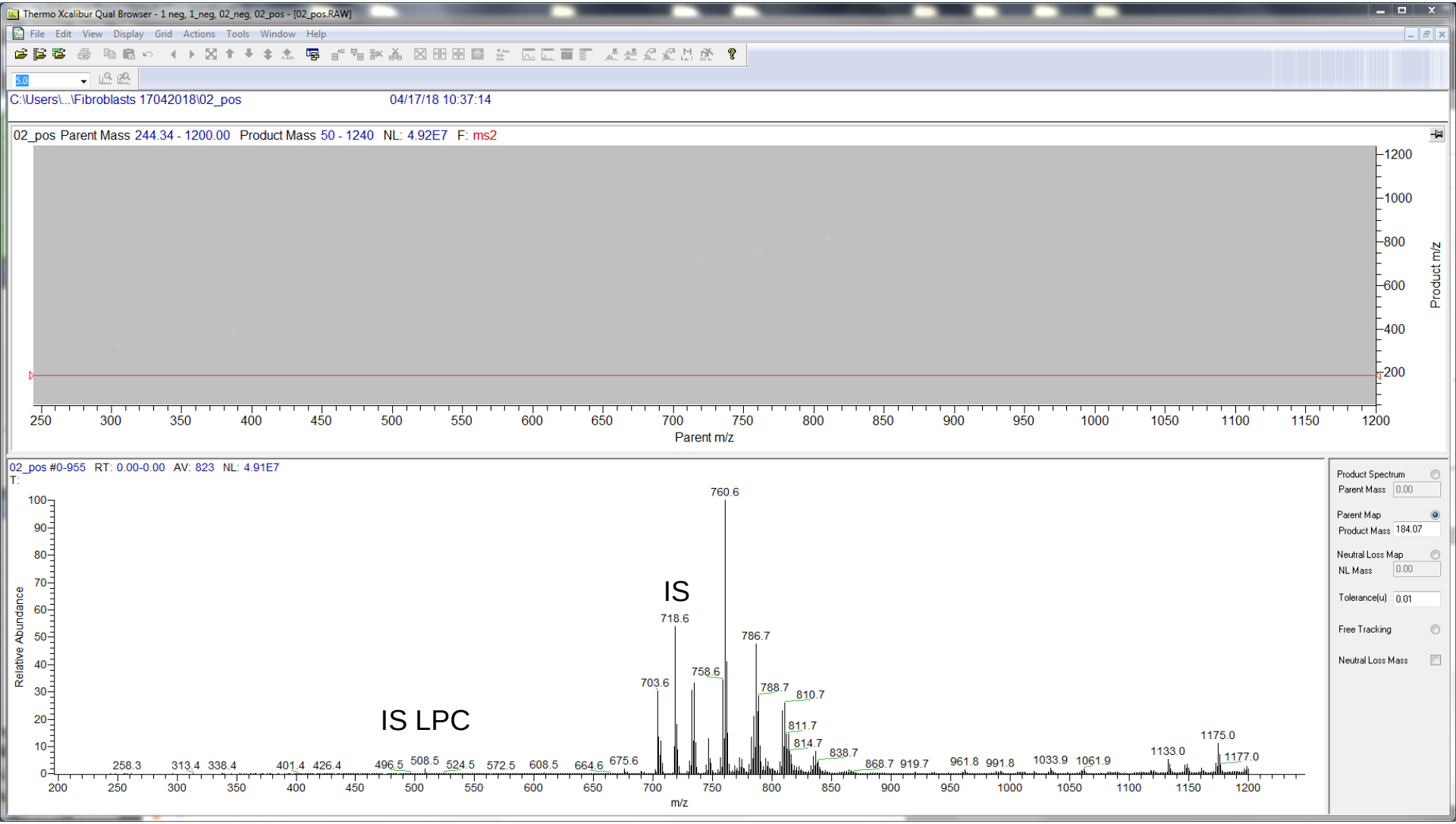
The software takes

- Each single MS2 scan of the raw file
- Finds the according center of the MS2 window from the inclusion list and uses this as a precursor mass (attn.: this is usually not the real precursor mass)
- Subtracts from that mass the given NL mass (141.06) to give a target mass window with the width that is given by the tolerance that was typed in
- Looks for peaks that fall into this target window and sums up their intensities
- Takes that intensity sum and ascribes it to the „precursor mass“
- Shows the resulting spectrum

Problem: since the real precursor mass is unknown, you need relatively large tolerances not to miss peaks → **can give false positive peaks**

and you do not get a name of your lipid

Prec of 184.07: PC + SM



How does it work?

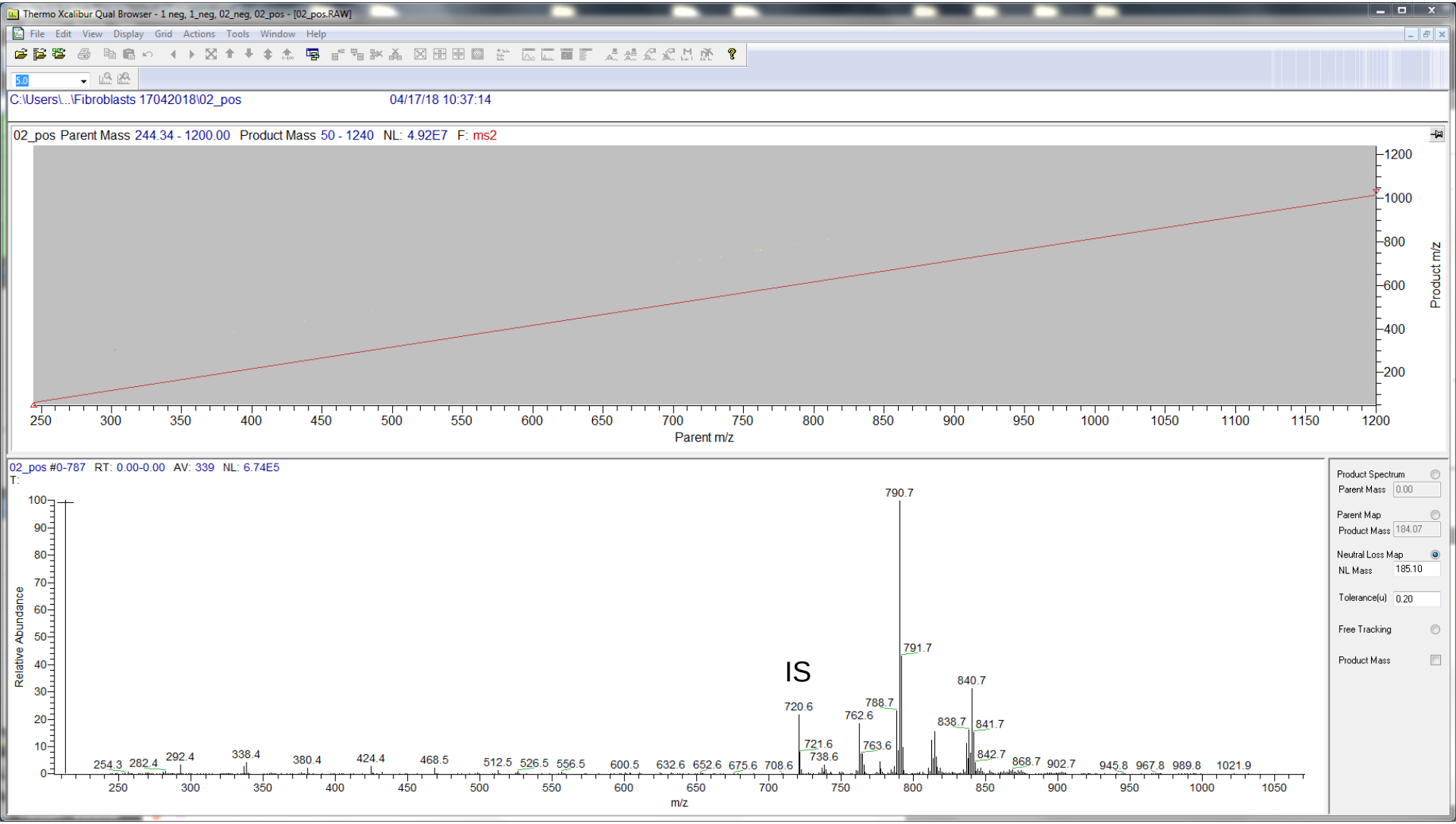
Parent map function:

The software takes

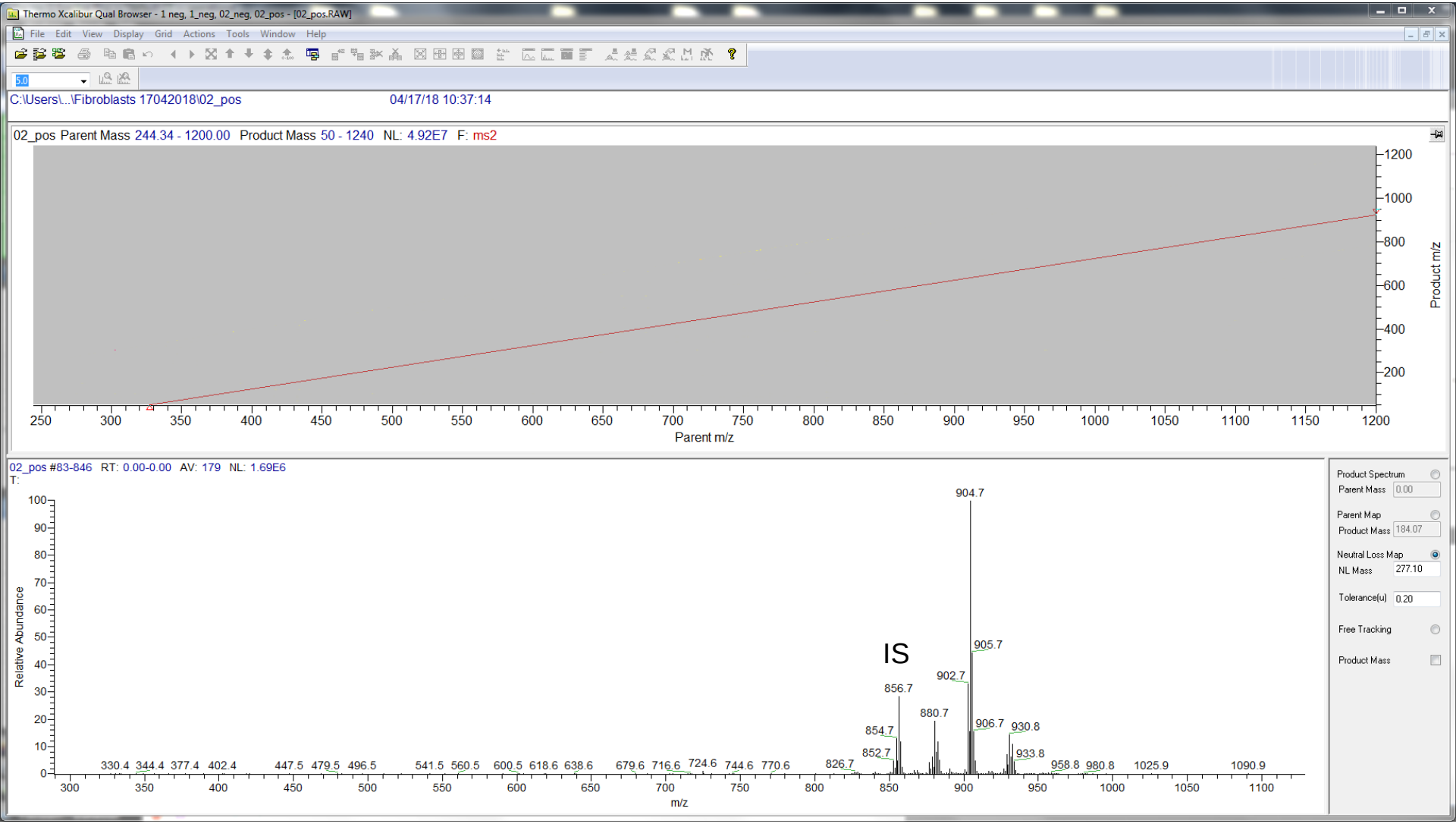
- Each single MS2 scan of the raw file
- Finds the according center of the MS2 window from the inclusion list and uses this as a precursor mass
- Looks for peaks of the product mass +/- tolerance defined by the user and sums up their intensities
- Takes that intensity sum and ascribes it to the precursor mass
- Shows the resulting spectrum

Problem: the real precursor mass is still unknown, but you can use small tolerances since you exactly know the product mass → no false positive peaks, but precursor masses may be slightly off

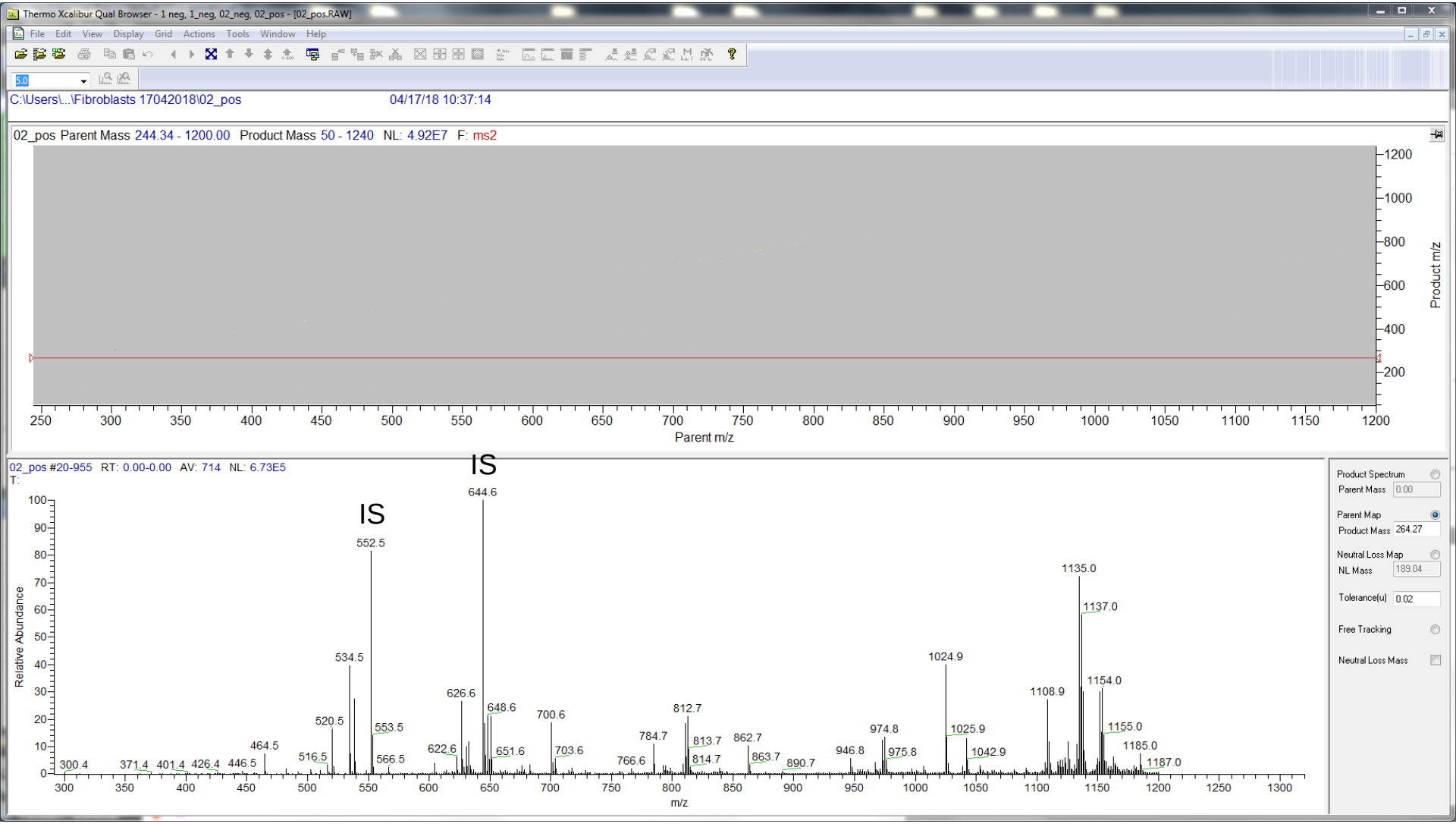
NL 185: PS


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
File Edit View Display Grid Actions Tools Window Help
C:\Users\...\Fibroblasts 17042018\02_pos 04/17/18 10:37:14
02_pos Parent Mass 244.34 - 1200.00 Product Mass 50 - 1240 NL: 4.92E7 F: ms2
Parent m/z
Product m/z
02_pos #0-787 RT: 0.00-0.00 AV: 339 NL: 6.74E5
T:
Relative Abundance
m/z
IS
790.7
791.7
840.7
720.6
788.7
762.6
838.7
841.7
721.6
738.6
763.6
842.7
868.7
902.7
945.8
967.8
989.8
1021.9
254.3
282.4
292.4
338.4
380.4
424.4
468.5
512.5
526.5
556.5
600.5
632.6
652.6
675.6
708.6
Product Spectrum
Parent Mass 0.00
Parent Map
Product Mass 184.07
Neutral Loss Map
NL Mass 185.10
Tolerance(u) 0.20
Free Tracking
Product Mass

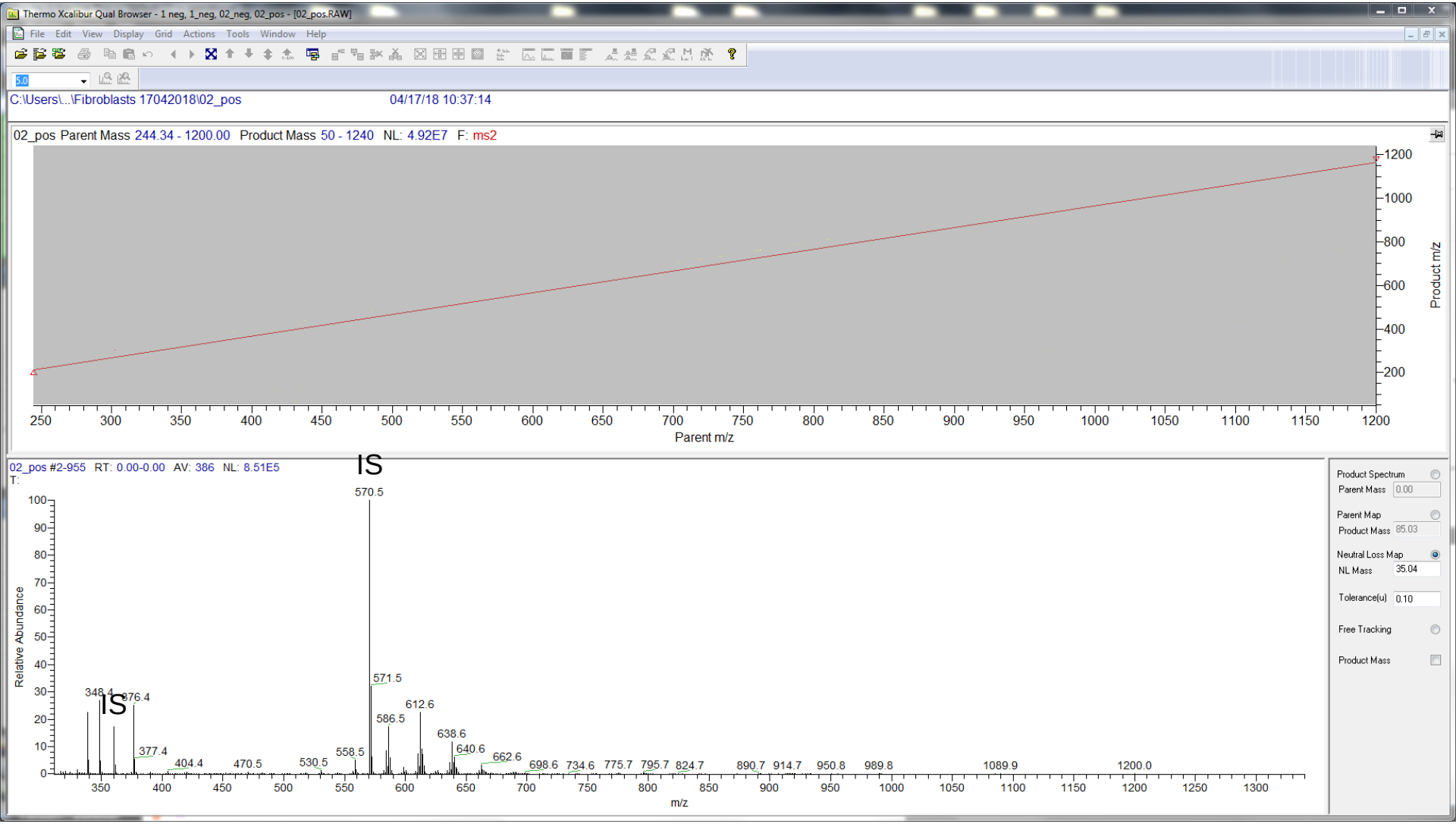
NL 277: PI


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
File Edit View Display Grid Actions Tools Window Help
C:\Users\...\Fibroblasts 17042018\02_pos 04/17/18 10:37:14
02_pos Parent Mass 244.34 - 1200.00 Product Mass 50 - 1240 NL: 4.92E7 F: ms2
Parent m/z
Product m/z
02_pos #83-846 RT: 0.00-0.00 AV: 179 NL: 1.69E6
T:
Relative Abundance
m/z
IS
904.7
905.7
902.7
856.7
880.7
906.7
930.8
854.7
852.7
933.8
Product Spectrum
Parent Mass 0.00
Parent Map
Product Mass 184.07
Neutral Loss Map
NL Mass 277.10
Tolerance(u) 0.20
Free Tracking
Product Mass

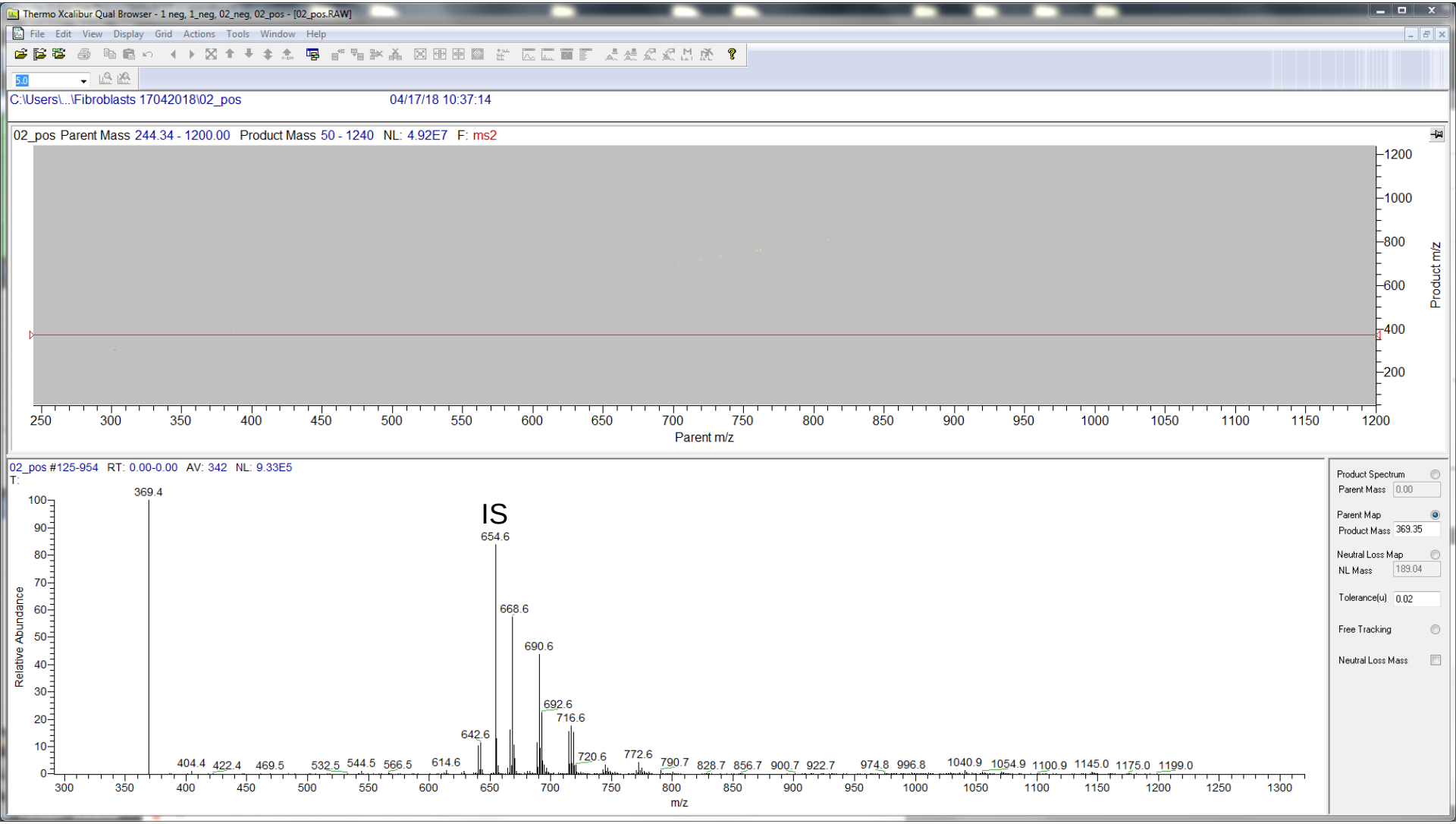
Prec of 264.27: Cer, GlcCer, LacCer



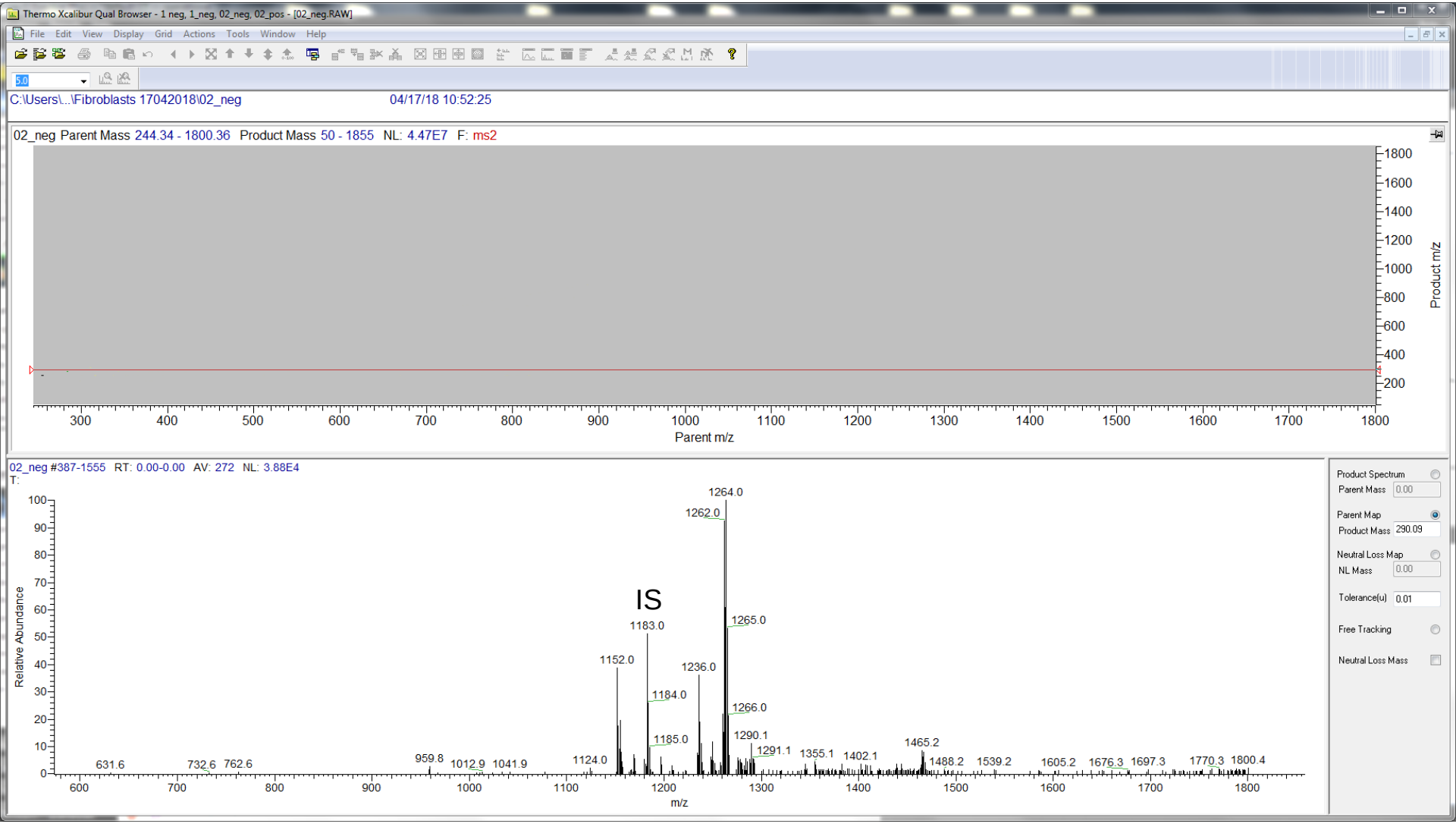
NL 35: DAG + MAG


Thermo Xcalibur Qual Browser - 1 neg, 1_neg, 02_neg, 02_pos - [02_pos.RAW]
File Edit View Display Grid Actions Tools Window Help
C:\Users\...\Fibroblasts 17042018\02_pos 04/17/18 10:37:14
02_pos Parent Mass 244.34 - 1200.00 Product Mass 50 - 1240 NL: 4.92E7 F: ms2
Parent m/z
Product m/z
02_pos #2-955 RT: 0.00-0.00 AV: 386 NL: 8.51E5
T:
IS
IS
570.5
571.5
612.6
586.5
638.6
640.6
662.6
348.4
376.4
377.4
404.4
470.5
530.5
558.5
698.6
734.6
775.7
795.7
824.7
890.7
914.7
950.8
989.8
1089.9
1200.0
Relative Abundance
m/z
Product Spectrum
Parent Mass 0.00
Parent Map
Product Mass 85.03
Neutral Loss Map
NL Mass 35.04
Tolerance(u) 0.10
Free Tracking
Product Mass

Prec of 369.35: CE



Prec of 290.09 negative: Gangliosides



Analyzing data with LipidXplorer

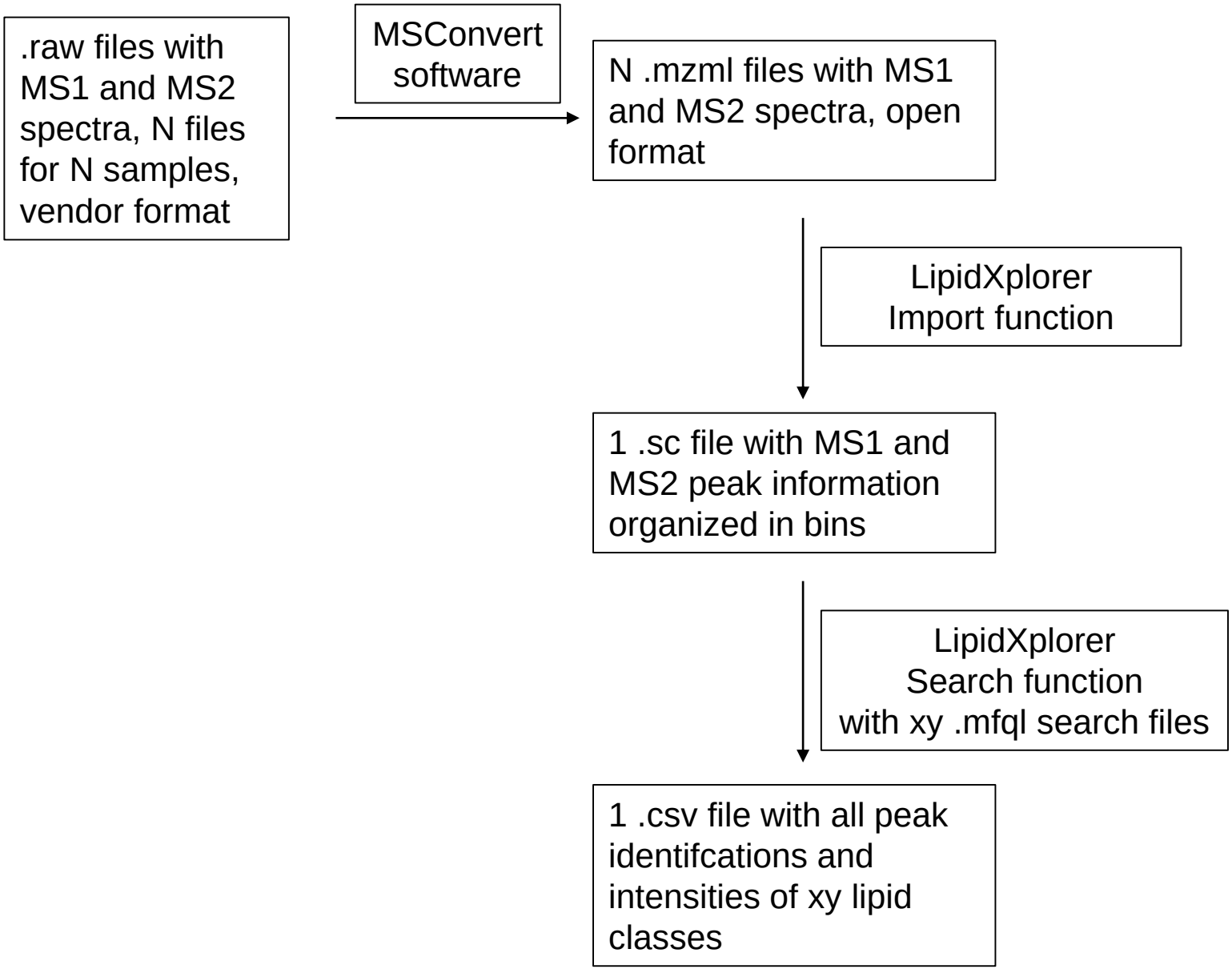
Herzog, R., Schwudke, D., Schuhmann, K., Sampaio, J.L., Bornstein, S.R., Schroeder, M., and Shevchenko, A. 2011. A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12(1): R8.

LipidXplorer is free:

https://wiki.mpi-cbg.de/lipidx/Main_Page

It is a python application and needs a windows PC

Install it on a computer with fast (not many) processor cores and at least 16 GB RAM


.raw files with MS1 and MS2 spectra, N files for N samples, vendor format
MSConvert software
N .mzml files with MS1 and MS2 spectra, open format
LipidXplorer Import function
1 .sc file with MS1 and MS2 peak information organized in bins
LipidXplorer Search function with xy .mfql search files
1 .csv file with all peak identifcations and intensities of xy lipid classes

The data import function

1. Find the first MS1 spectrum in the mzml file
2. generate an open mass list that contains bins that have the size of the mass resolution of the scan
3. find all peaks in the first MS1 spectrum and fill them into the bins of the list
4. Take the next MS1 scan and fill the peaks into the bins of the list
5. continue until all MS1 scans are binned into the list
6. Take the first MS2 scan
7. Identify its precursor center mass and mass window size
8. Go to the MS1 bin list and find all MS1 peaks within that MS2 precursor window
9. Generate another binned MS2 list with all peaks of the first MS2 scan
10. Make a logical assignment of the MS2 bin list to the MS1 peaks in the precursor window.
11. Do that with all (thousands!!) MS2 scans

12. Result: a list of MS1 peaks with their assigned MS2 peaks in a compacted form
    → this is the .sc (master scan) file

13. In the .sc files, replica measurements are already sorted into the bins
    → their individual masses are replaced by the common bin mass, but the individual intensities still exist as individual entries (but will be averaged in the final output)

The search function

1. opens the .sc file of the sample set
2. makes a representation of the file in the RAM that is more easy to search
   → this is usually expanding the size of the .sc file by a factor of 10
   → if you computer does not have enough RAM for it, it will do the following calculations on the hard drive instead of the RAM
   → this will take a long time
   → be sure you have enough RAM
3. opens the mfql search file
4. takes the precursor definition from the define section
5. Identify peaks in MS1 that fulfil these criteria and sets a flag there
6. takes the NL or fragment definition for MS2 from the define section
7. seaches the MS2 data connected to the flagged MS1 peaks to identify MS2 peaks that fit to the MS2 definitions
8. if applying, logical constrains by suchthat conditions are checked
9. peaks that fit all criteria are collected
10. and reported according to the parameters set in the report section of the .mfql file
11. Output is a .csv list that contains all the identified species in the lines in blocks as defined by the .mfql. The columns contain the reported parameters for each single species.

End