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The Application of Mass Spectrometry to Metabonomics

Chris Stumpf, Waters Corporation

What is Metabonomics?

- Jeremy Nicholson first used the term ‘metabonomics’ and defined it as:
  - The quantitative measurement of time-related multiparametric metabolic responses for multi-cellular systems
- Metabonomics is the study of endogenous metabolites and differences in the complement of these metabolites caused by external stimuli
  - exposure to a toxin
  - administration of a therapeutic agent
  - disease

Why Metabonomics?

- Metabonomics is an extension of genomics and proteomics
- "Metabolic changes are real-world end points, whereas gene expression changes are not; they merely indicate the potential for an end-point change."
  - Jeremy K. Nicholson, John Connelly, John C. Lindon and Elaine Holmes. Metabonomics: a platform for studying drug toxicity and gene function. Nat. Rev. Drug Disc. 1, 153-161 (2002).
- Studying changes in metabolite concentrations can give valuable information on gene function and mechanisms of disease and toxicity.
- Potential biomarkers of disease and toxicity

MetaboNomics vs. MetaboLomics

- Metabonomics
  - used primarily by the pharmaceutical and related industries
  - concentrates on mammalian systems
  - many practitioners started using NMR
  - typical application:
    - discovery of markers of toxicity for drug candidate screening
- Metabolomics
  - used primarily by plant science and related sciences
  - study of plants and lower organisms
  - extension of metabolic profiling
  - typical application:
    - investigation of genetic modification

What are the Challenges?

- Highly complex samples
  - plants may contain 1000's of primary and secondary metabolites
- Wide variety of compound classes
- Wide range of metabolite concentrations
- Large number of samples
- Data analysis is challenging and requires statistical approach
  - principal component analysis
  - partial least squares - discriminant analysis

NMR and LC-MS are Complementary

- Advantages of NMR
  - Information-rich spectra
  - Detects wide range of compounds
  - Large dynamic range
  - Quantitative response
  - Direct analysis of biofluids and intact tissue samples
- Disadvantages of NMR
  - No chromatographic separation possible within time frame
  - Some compounds masked by other metabolite signals
  - Areas of the spectrum hidden by xenobiotic signals
  - Relatively insensitive
  - Some functional groups are NMR invisible

NMR and LC-MS are Complementary

- Using LC-MS on a quadrupole-time of flight mass spectrometer
- Advantages of LC-MS
  - Sensitivity - detection of lower level metabolites
  - Information rich - exact mass measurement
  - On-line chromatography reduces masking of low level metabolites
  - Can use MS/MS to aid identification
  - Easier to remove xenobiotic contributions
- Disadvantages of LC-MS
  - Lower dynamic range than NMR
  - Need multiple chromatographic methods - retention and separation of polar metabolites
  - Isomeric metabolites
  - Response compound dependent

Metabonomics : Study of Known Toxins

- 20 rat urine samples supplied by GSK
- Two time points: 0-8 hr and 8-24hr
- 3 x 2 controls
- 3 compounds, 3 samples per time point, 2 time points
- Simple Sample Prep: Samples centrifuged then diluted 1:4 with distilled water prior to analysis by LC/MS

LC/MS methodology

- Chromatography:
  - Column: Waters XTerra® C18 100 x 2mm 3.5µm
  - Eluent: Reverse Phase
  - Flow Rate: 600 uL/min (slower for LC/MS/MS)
- Mass spectrometry
  - Quattro *micro*™ or Q-Tof micro™
  - Scan range 100-1000m/z
  - Data collected from 0-10mins (30 minutes for LC/MS/MS)

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LC/MS TIC data: Qualitative Differences

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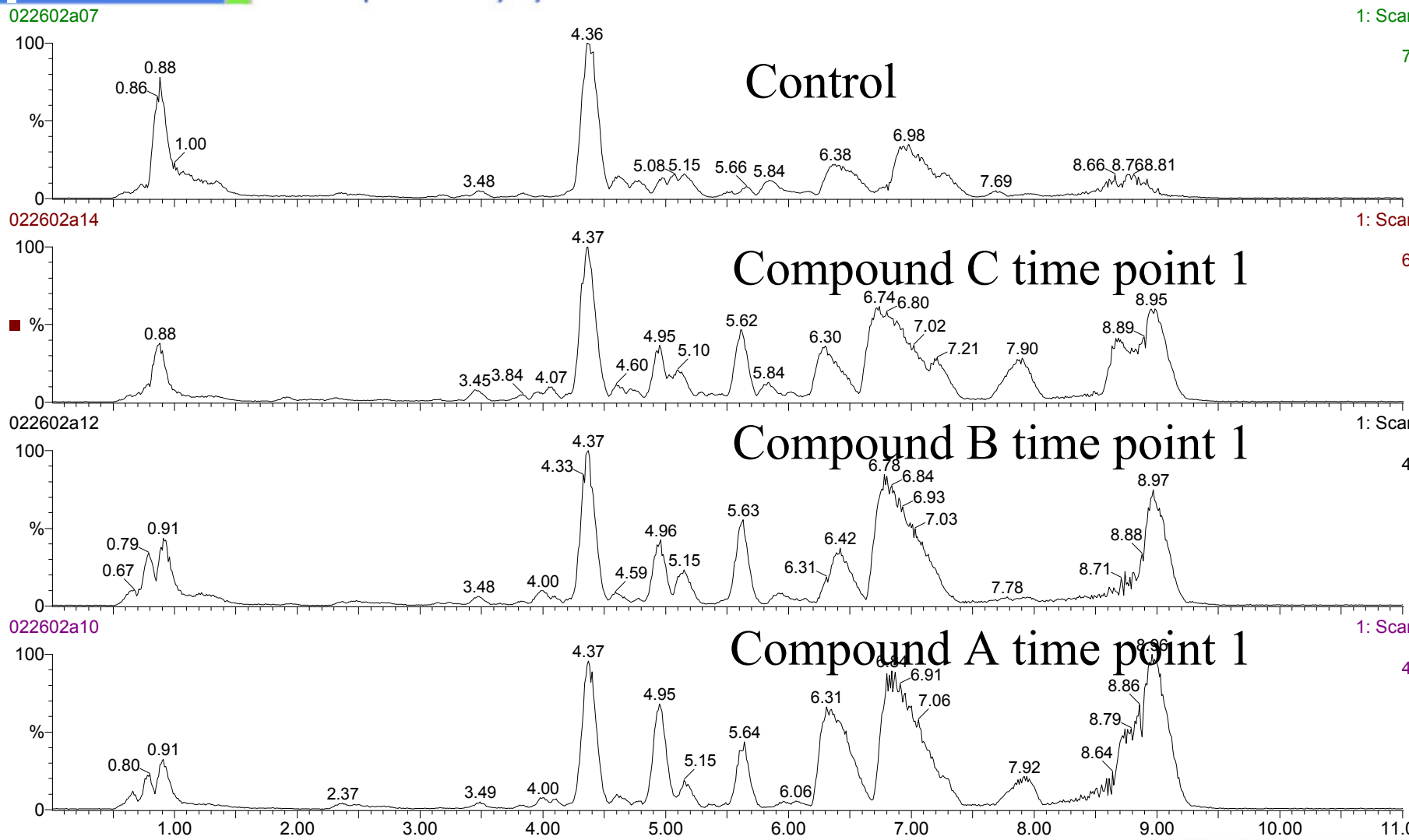

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Statistical Analysis of the Data

- Biomarkers can be found by analyzing the mass spectra one-by-one. However this is cumbersome.
- Principal Component Analysis (PCA) is a multi-variate analysis technique that provides a global view of the data
- PCA shows clusters in data sets and allows the constituents causing the differences between the clusters to be highlighted
- More subtle differences may be highlighted by supervised techniques such as partial least squares - discriminant analysis (PLS-DA)
- These statistical techniques can highlight features in a data set that are not easily found by eye.

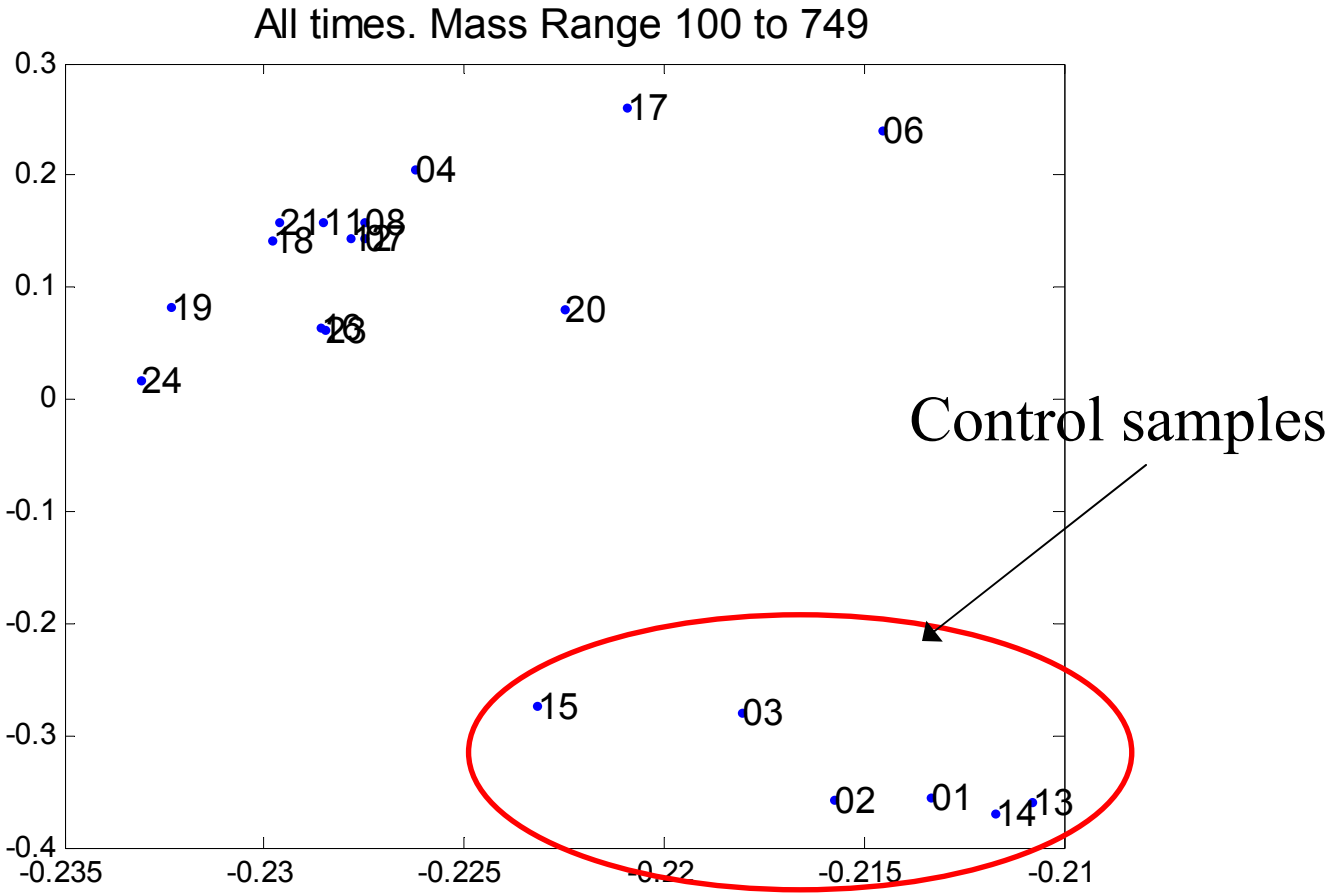
Raw Data Processing

- Need to reduce data set for statistical analysis
- Spectra combined into 1 minute time slices (10 spectra/sample)
- Spectra in 1 amu ‘bins’
- Mass-intensity pair lists exported to Excel and then to MatLab
- PCA performed in MatLab

- Note: this is a simplistic treatment of the data. Future work will concentrate on deconvolution of the data to retain retention time information.

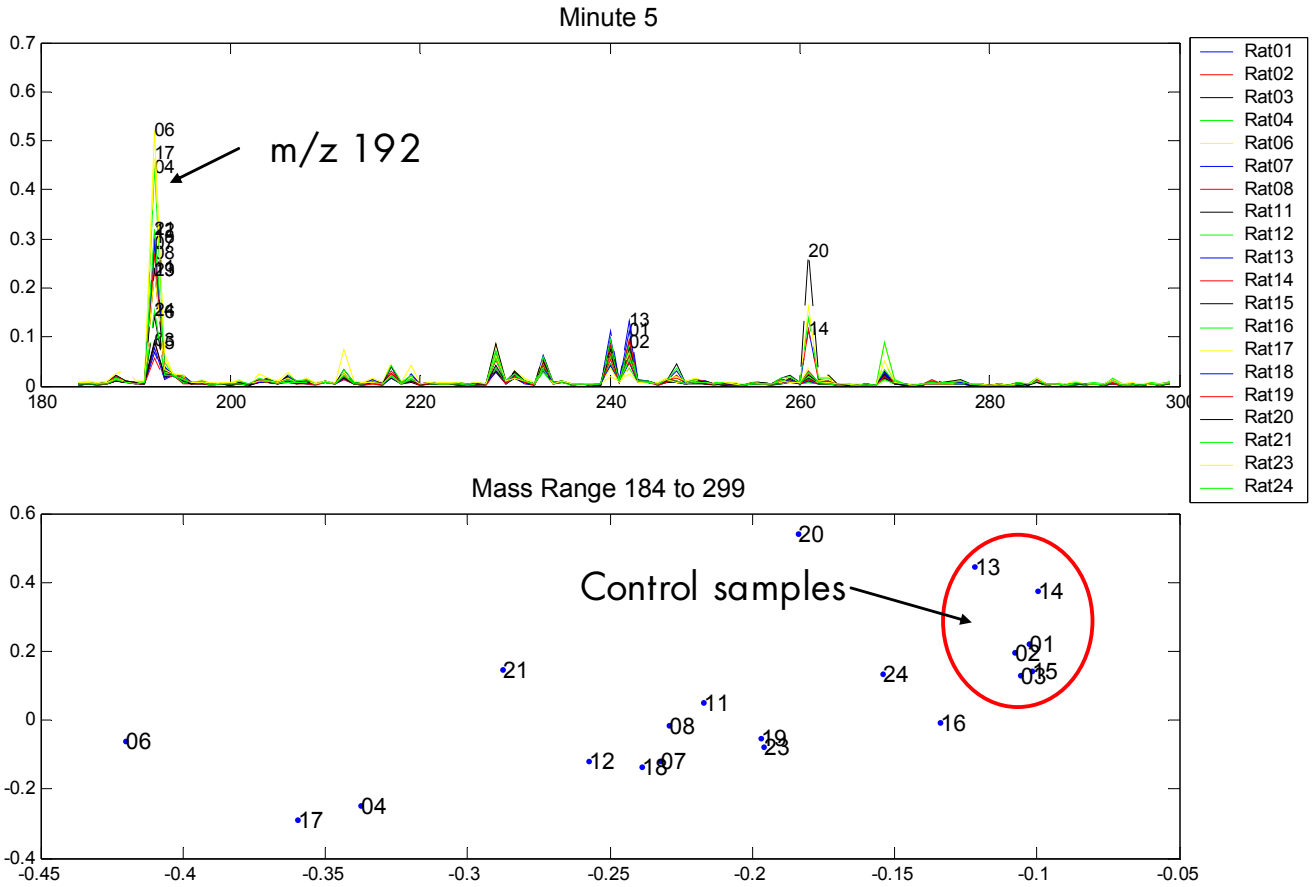
PCA for the whole data set

MassLynx -> Excel -> MatLab (PCA)



Data from March 2002

PCA investigation from Minute 5 of Chromatogram



Note: LC Retention Information is Utilized!

Ions identified as responsible for PCA separation

Compound dosed	Analyte m/z value	Change
A	283	10 fold increase
A	461	5 fold increase
A	187	10 fold increase
B	338	2 fold reduction
B	283	10 fold increase
B	461	10 fold increase
B	187	10 fold increase
C	283	20 fold increase
C	187	30 fold increase
A,B,C	192	3 fold increase

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LC/MS of 192 ion using a Q-Tof

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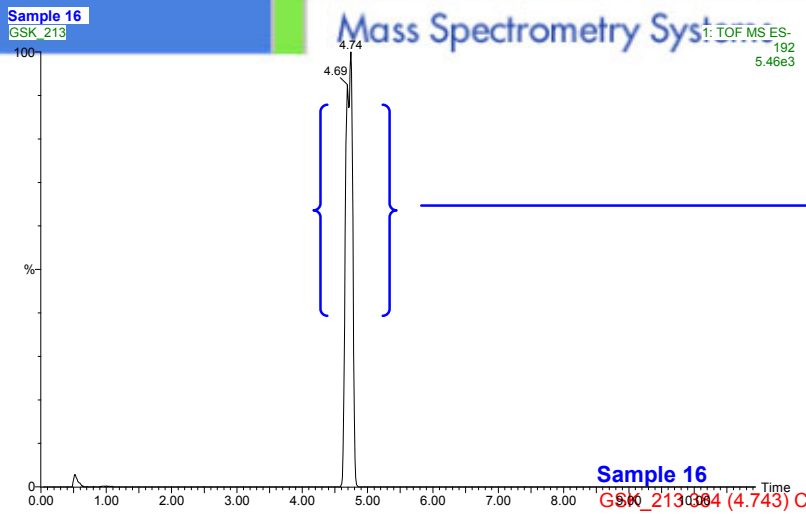
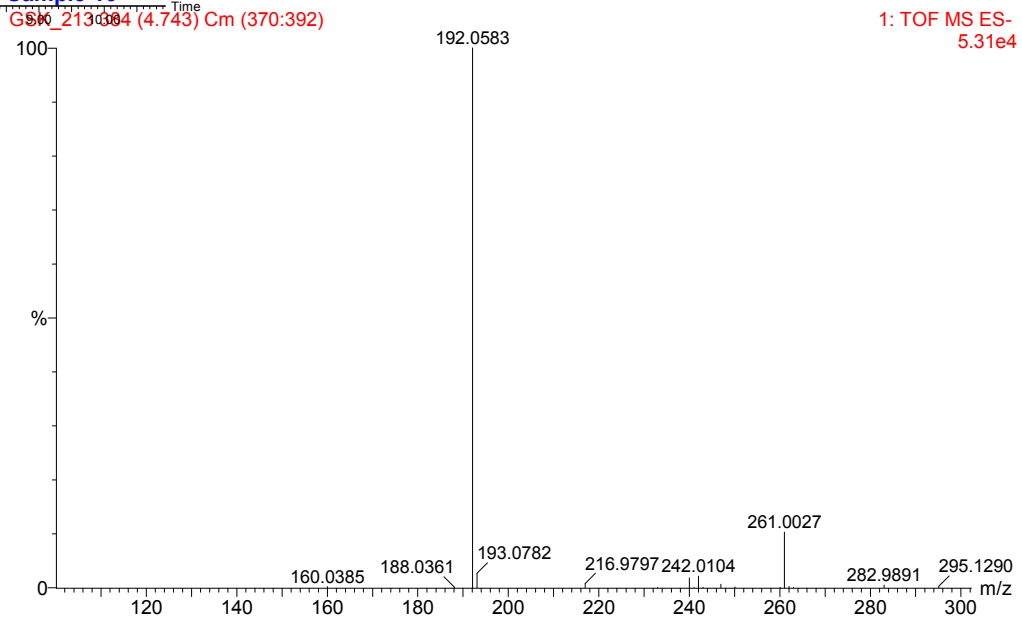


Accurate mass information obtained for the 192 ion was used to determine the elemental composition $C_{10}H_{10}NO_3$.



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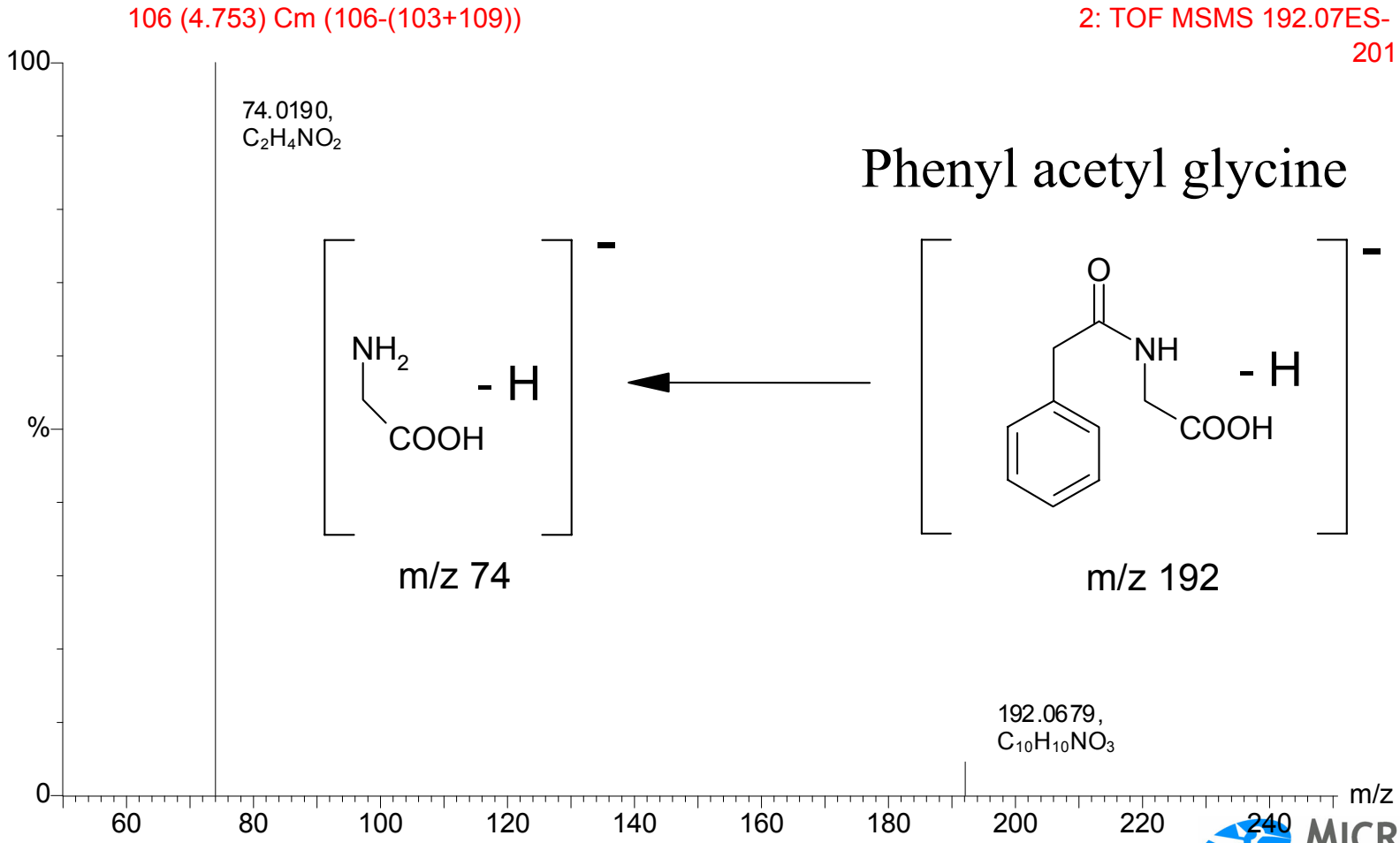
LC/MS/MS of 192 ion



Identified by MS but missed by NMR!

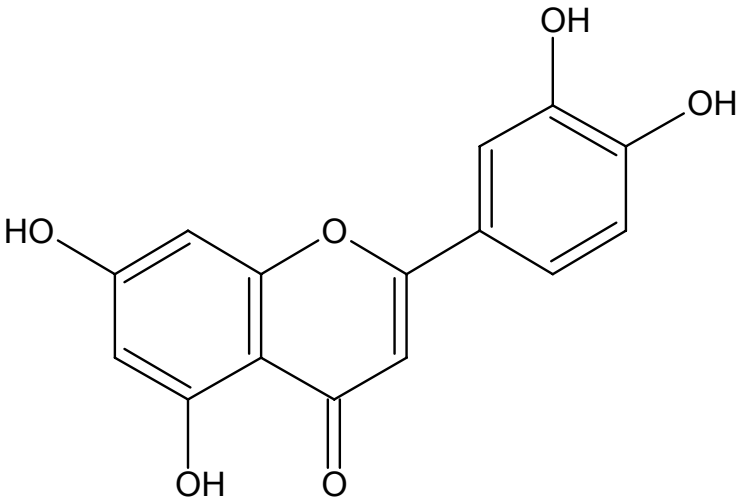
Other compounds identified, but due to confidentiality agreement they cannot be shown.

'Focussed' Metabolomics Application

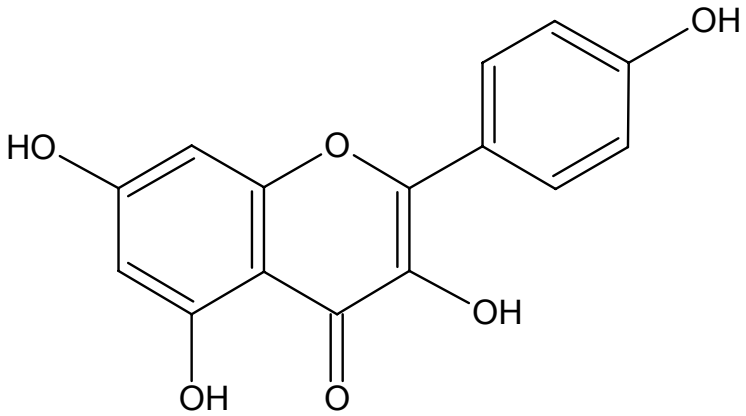
- Flavonoids play essential role in plant physiology
- Beneficial to human health
- Tomatoes contain only small amounts
- Aim to upregulate the flavonoid biosynthesis by genetic engineering
- Apart from the aglycone many variations exist as additions of various sugar moieties to the free hydroxyl groups
- Exact neutral loss acquisitions used to detect formation of new flavonoid-glycosides on a Q-Tof Ultima

  - Collaboration with Plant Research International, The Netherlands

Examples of Flavonoid Structures



Luteolin



Kaempferol

Potential Glycosylations

- Pentoses $C_5H_{10}O_5$ monoisotopic mass 132.0422
- Deoxyhexose $C_6H_{12}O_5$ monoisotopic mass 146.0579
- Hexosamine $C_6H_{13}NO_5$ monoisotopic mass 161.0688
- Hexose $C_6H_{12}O_6$ monoisotopic mass 162.0528
- Hexuronic acid $C_6H_{10}O_7$ monoisotopic mass 176.0321

*Different combinations of all of these were also monitored for neutral losses. 19 individual masses and/or combined were analysed

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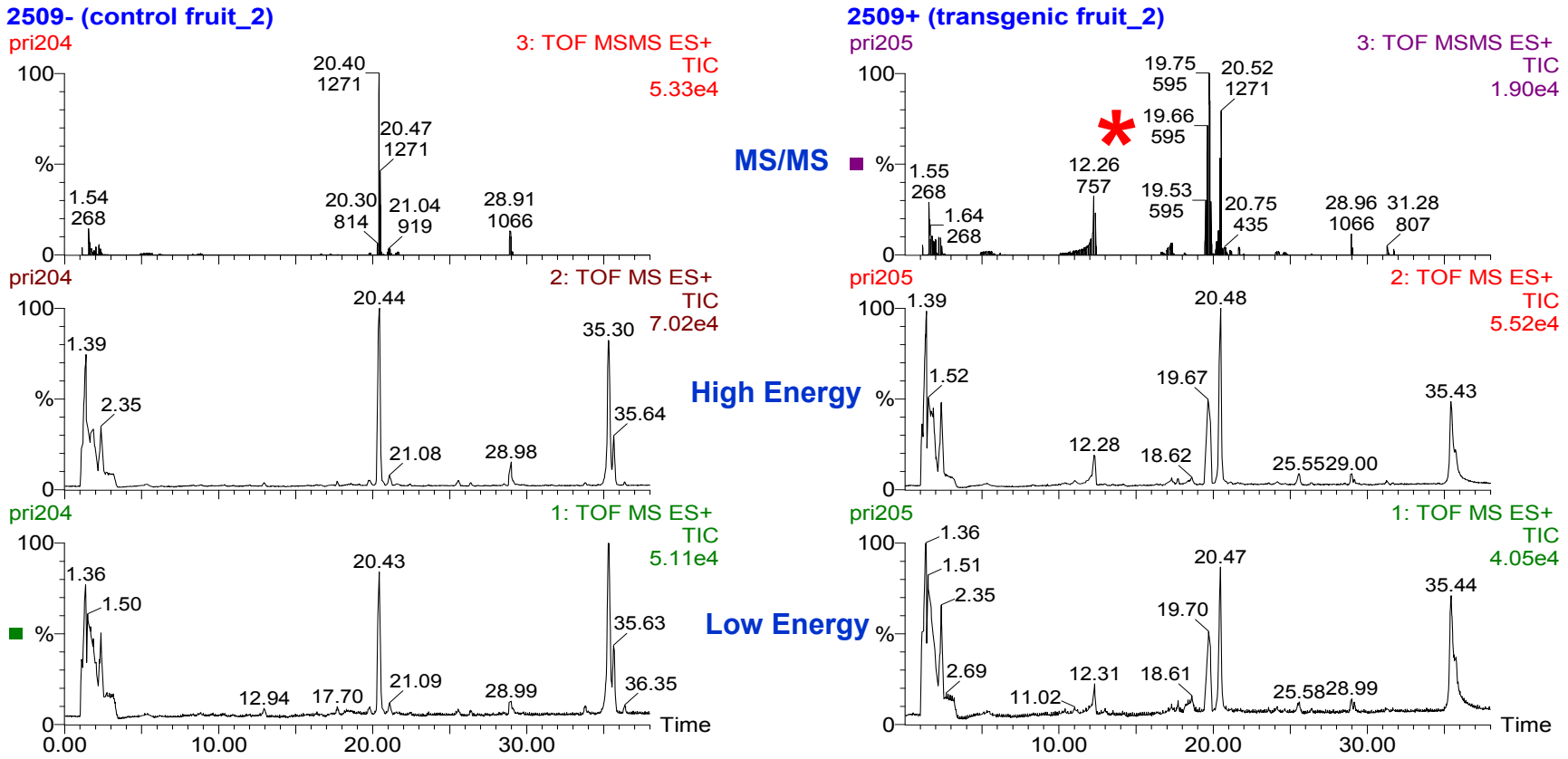
Neutral loss chromatograms from control and transgenic tomatoes

Control Fruit

Transgenic Fruit



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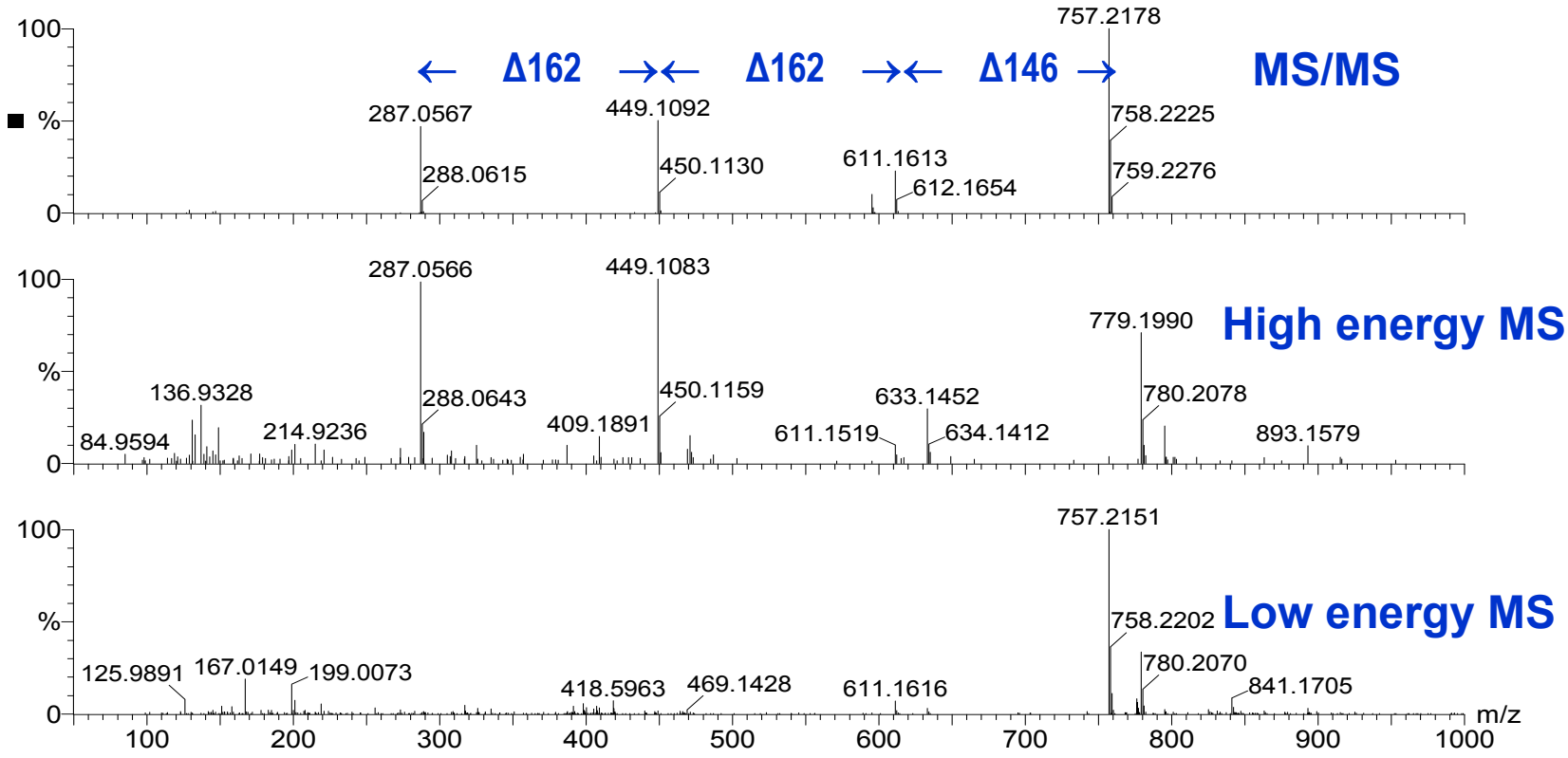
Spectra from peak at 12.3 min showing loss of 3 sugars (deoxyhexose + 2 hexose)

2509+ (transgenic fruit_2)



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Elemental composition report and structure for peak at 12.3 min

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Elemental Composition

File Edit View Process Help

Multiple Mass Analysis: 5 mass(es) processed

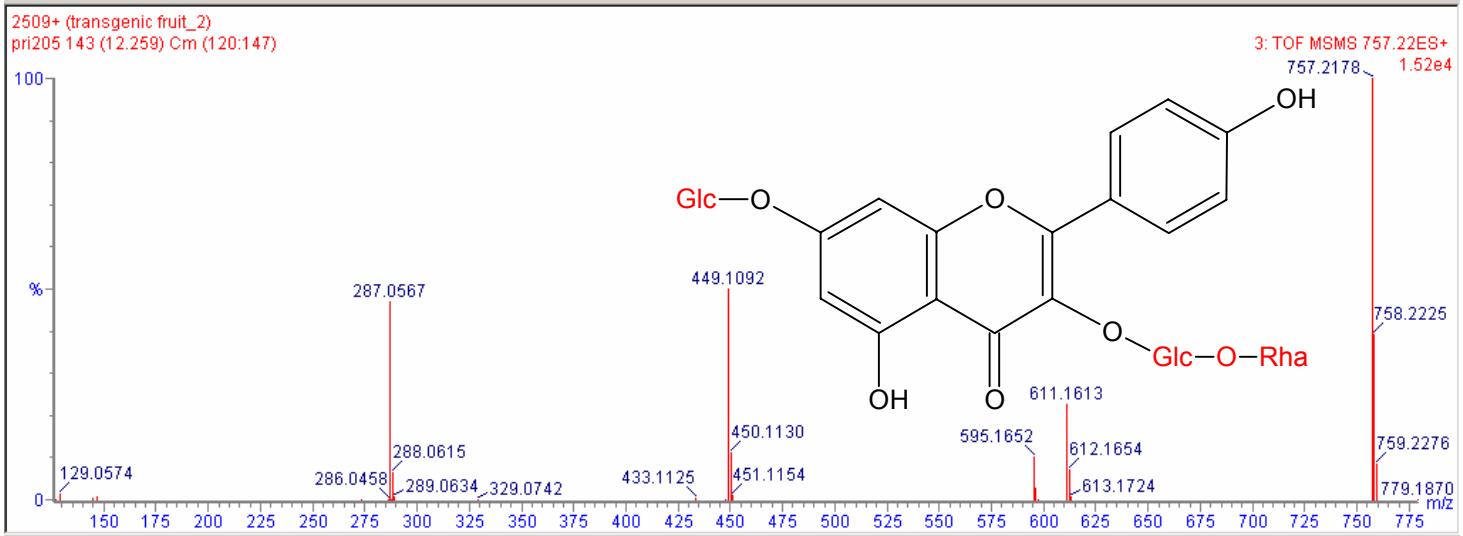
Tolerance = 6.0 PPM / DBE: min = -1.5, max = 15.0

Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions

845 formula(e) evaluated with 5 results within limits (up to 50 closest results for each mass)

Mass	RA	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	O
287.0567	46.80	287.0556	1.1	4.0	10.5	C15 H11 O6	1	15	11	6
449.1092	50.07	449.1084	0.8	1.8	11.5	C21 H21 O11	1	21	21	11
611.1613	22.67	611.1612	0.1	0.1	12.5	C27 H31 O16	1	27	31	16
757.2178	100.00	757.2191	-1.3	-1.7	13.5	C33 H41 O20	1	33	41	20
758.2225	39.19	758.2269	-4.4	-5.9	13.0	C33 H42 O20	1	33	42	20



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Conclusions

- Metabonomics
  - Simple reversed-phase LC/MS system allowed separation of the control from dosed samples using multi-variant analysis.
  - m/z of analytes responsible for the PCA separation were identified.
  - Analytes responsible for PCA separation were identified by LC/MS/MS and Q-TOF
- 'Focussed' Metabolomics
  - Selective and sensitive (+/- 20mDa window) tool for screening for glycosylated metabolites in complex extracts
  - Fast screening method for differentially produced glycosides in control and transgenic plants, containing MS and MS/MS information from one single chromatographic run
  - Exact mass MS and MS/MS (<5ppm RMS) provided information on the putative metabolites and their aglycone fragments