# Metabolomics and metabolite identification – where are we now and the route ahead?


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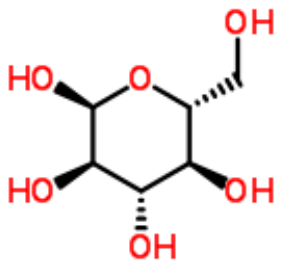
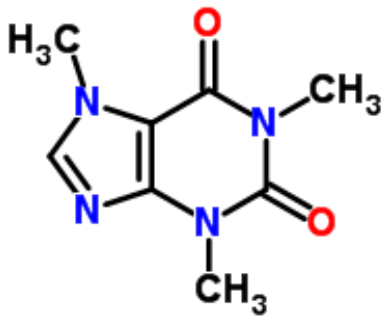
# A tutorial – my views

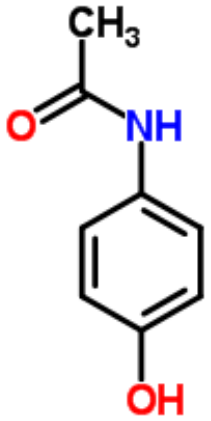



- Introduce our abilities and common difficulties/ limitations of metabolite identification in complex metabolomic samples
  - describe common workflows for GC-MS and UPLC-MS




- Describe the reporting standards for metabolite identification

- Discuss what innovative tools are required or are being developed



# Component characterisation of simple solutions

- Relatively easy for analytical chemists to characterise a single component solution!

- Many tools available for characterisation of unknowns
  - mass spectrometry (MS)
  - nuclear magnetic resonance (NMR) spectroscopy
  - ultraviolet spectroscopy (UV)
  - infrared spectroscopy (IR)
  - elemental analysis


- Many of these are not appropriate for complex multi-component solutions
  - metabolomic samples are very complex (contain 100-1000s of metabolites)
  - mass spectrometry and NMR are two tools commonly applied in the analysis of complex metabolomic samples

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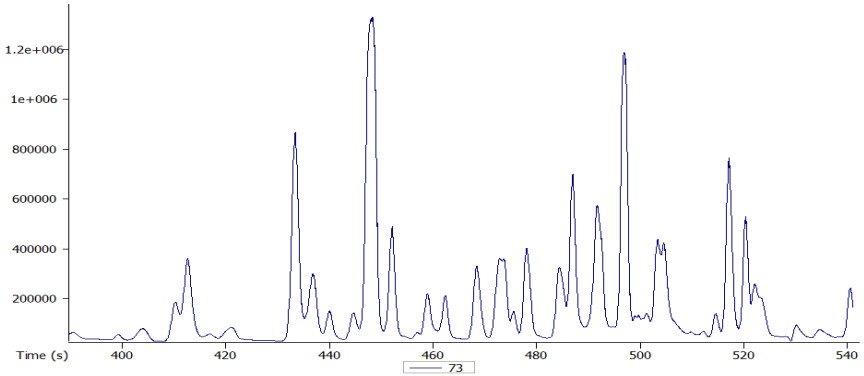
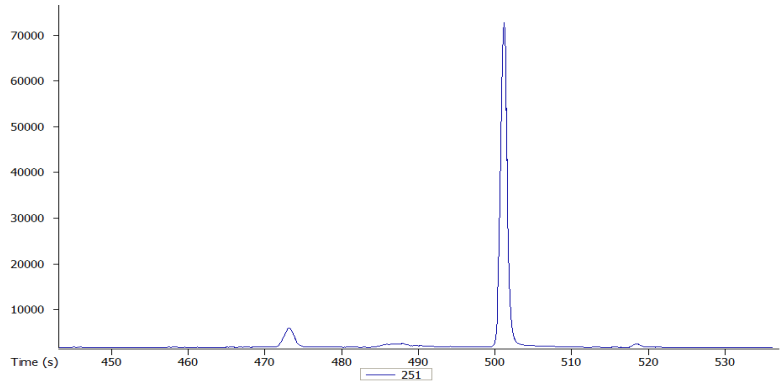
# Untargeted *vs.* (semi-)targeted metabolomic studies

## METABOLIC PROFILING or UNTARGETED ANALYSIS




- (semi)-quantitative (global) detection of a wide range of metabolites
- Orbitrap, TOF, Q-TOF, IT, Q, FTICR
- data acquisition without *a priori* knowledge of biologically interesting metabolites
- metabolite identification required post data acquisition

## TARGETED OR SEMI-TARGETED ANALYSIS




- quantification of a smaller number of (related) metabolites for
  - targeted = generally less than 20
  - semi-targeted = low 100s
- QQQ
- metabolite identity already known
  - no further metabolite identification required

**This seminar discusses metabolite identification in data acquired applying metabolic profiling strategies (i.e. complex samples containing 100-1000s of metabolites)**

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# Metabolite identification – A BOTTLENECK IN METABOLOMICS

For metabolomics to be successful it is essential to derive biological knowledge from analytical data - a view emphasised by a recent Metabolomics ASMS Workshop Survey 2009 which found that the biggest bottlenecks in metabolomics were thought to be identification of metabolites (35%) and assignment of biological interest (22%).

*http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics-Survey-2009*

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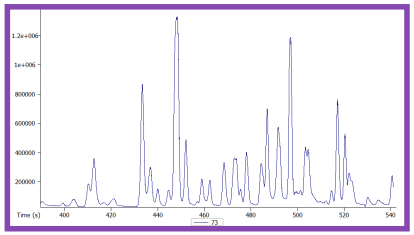
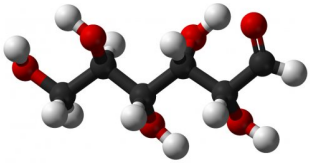
# Why is metabolite identification a bottleneck?

- Tools available for identification of a limited number of metabolites in a semi-automated process (traditional analytical chemistry)
  - these are being applied for identification of 100-1000s of metabolites
  - limited number of these tools which have been developed and experimentally validated for high-throughput metabolite identification of all metabolites
- Metabolomes and raw data are complex
- 7800+ metabolites in human body (not including gut microflora –derived, drug-derived and many lipids)
- Qualitative description of all metabolomes is not complete (and not electronically available)
- Different physicochemical properties (diversity is greater than proteome for example)





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# Mass spectrometers provide many advantages for metabolite identification in metabolomics

- **Sensitive detection** (sub-micromoles.$L^{-1}$ to millimoles.$L^{-1}$)
  - detection of 100-1000s of metabolic features/metabolites
- **High mass resolution** (5000 to >200 000+ FWHM)
  - ability to separate features of similar but not identical monoisotopic mass
- **High mass accuracy**(< 5ppm)
  - ability to accurately determine the mass of detected metabolic features
  - molecular formula determination
- **Gas phase ion fragmentation**
  - GC-MS using EI sources (or CI or QQQ)
  - LC-MS using QQQ, Q-TOF or LIT for MS/MS
  - structural determination
- **Isotope patterns and relative isotope abundance (RIA)**
- **New developments in instruments and computational tools**

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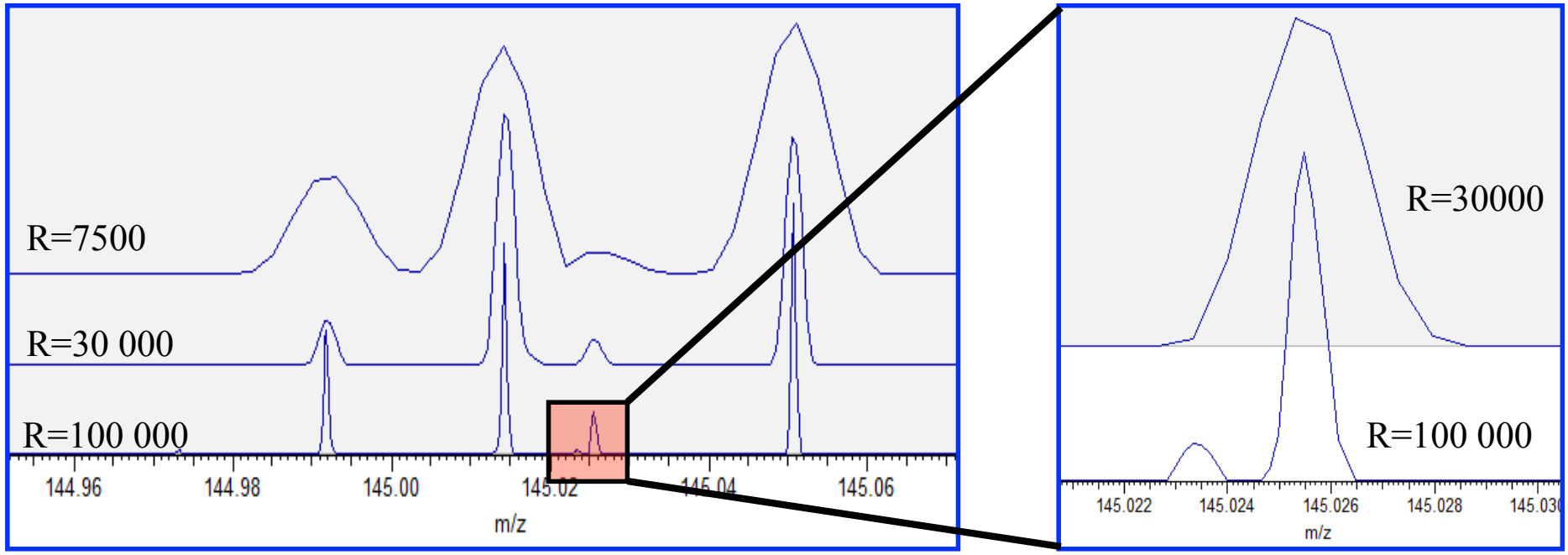
# Technological advances during the last decade!




***Typically we detect 1.5 to 3 times more mass peaks in direct infusion experiments when applying a mass resolution of 100 000 compared to 7500***

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# Levels of metabolite identification

- Sumner et al. Proposed minimum reporting standards for chemical analysis, Metabolomics, 2007, 3(3), 211-221
- Currently, four levels of metabolite identifications can be reported
- Not defining how to perform metabolite identification but defining how to report it

| Level | Confidence of Identity | Level of Evidence |
|---|---|---|
| 1 | Confidently identified compounds. | Comparison of two or more orthogonal properties with an authentic chemical standard analysed under identical analytical conditions. |
| 2 | Putatively annotated compounds | Based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries, without reference to authentic chemical standards. |
| 3 | Putatively annotated compound classes | Based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class. |
| 4 | Unknown compounds | Although unidentified and unclassified, these metabolites can still be differentiated and quantified based upon spectral data. |

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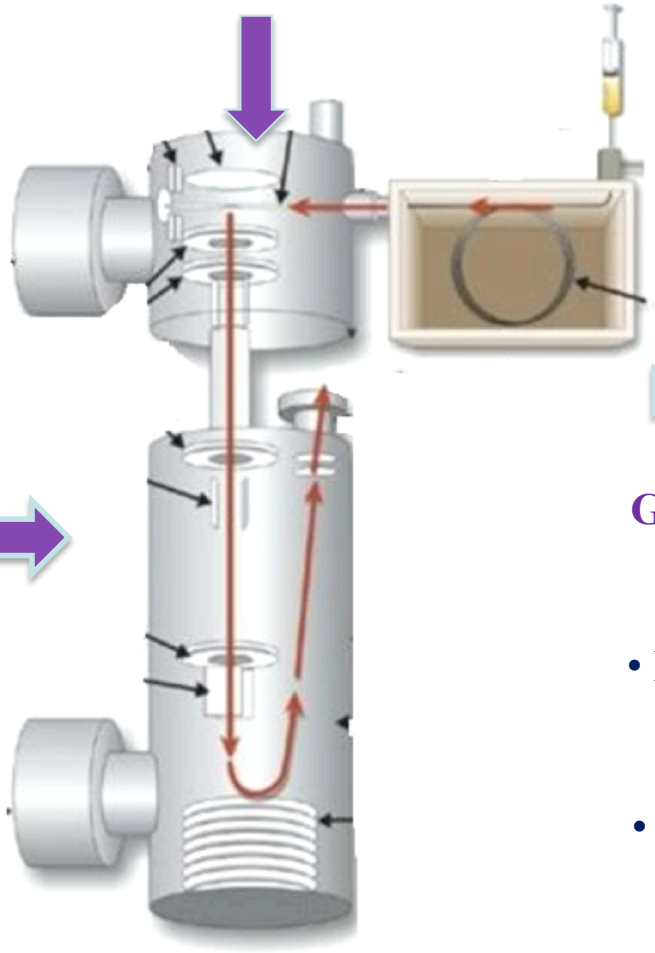
# Gas Chromatography-Mass Spectrometry (GC-MS)

## IONISATION SOURCES PROVIDE:

• **ELECTRON IMPACT** - PROVIDES REPRODUCIBLE GAS-PHASE FRAGMENTATION FOR STRUCTURE ELUCIDATION

• **CHEMICAL IONISATION** - NO FRAGMENTATION, ACCURATE MASS MEASUREMENT OF MOLECULAR ION



## MASS SPECTROMETRY PROVIDES:

• HIGH MASS RESOLUTION

• HIGH MASS ACCURACY (SOME NOT ALL INSTRUMENTS),

• HIGH SCAN SPEEDS / ACQUISITION RATES TO ACCURATELY DEFINE NARROW CHROMATOGRAPHIC PEAKS

• HIGH SENSITIVITY

## GAS CHROMATOGRAPHY PROVIDES:

• REPRODUCIBLE

• HIGH RESOLUTION (PEAK WIDTHS OF A FEW SECONDS),

• RETENTION INDICES FOR METHOD AND LIBRARY TRANSFERABILITY

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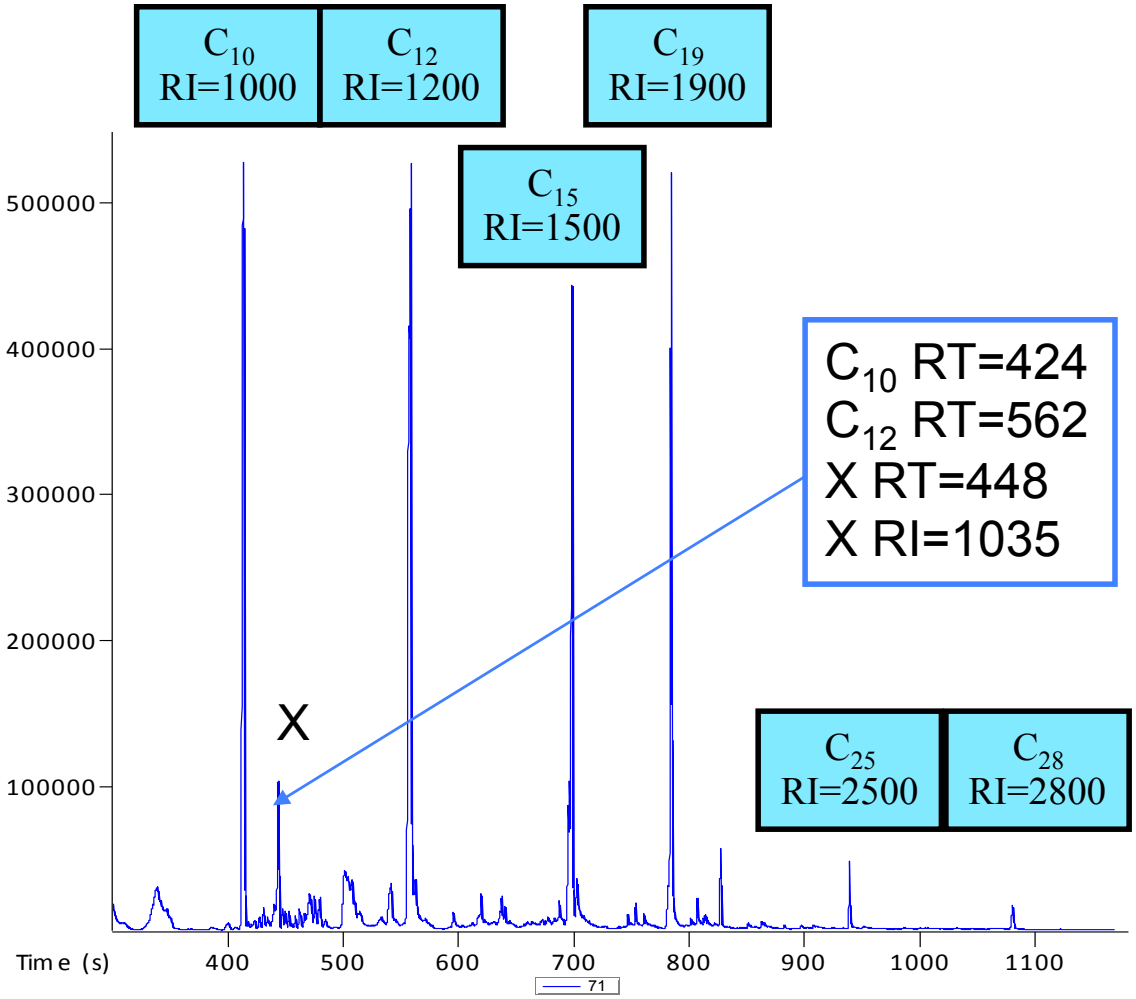
# Retention indices




Applies a series of homologous compounds
e.g. n-alkanes
e.g. fatty acid methyl esters

- Normalisation of retention time range

- Minimises errors associated with drift in retention time

- Can be applied across different GC columns and instruments (method and mass spectral library transferability)

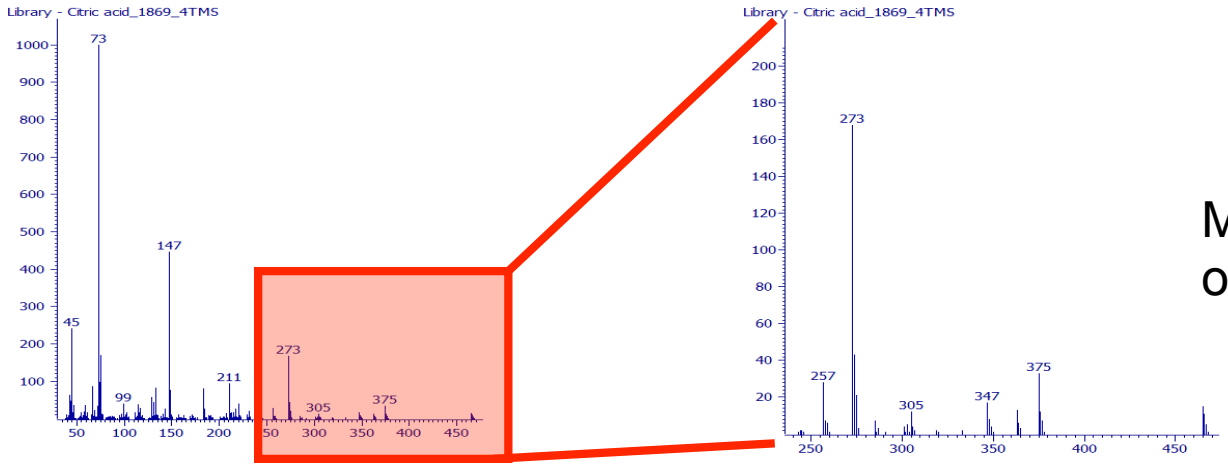
# Mass spectral libraries and library matching

- Mass spectral libraries
  - constructed with authentic chemical standards
  - multiple libraries available, none are comprehensive
  - we apply The Manchester Metabolomics Database (MMD) library

- Comparing the mass spectrum of an authentic chemical standard against the mass spectrum of an unknown metabolite
  - Compares and scores depending on number of matched ions and relative intensity of those ions

- Provides a confidence score on match (out of 1000 or as a %)

- Difficulty in trimethylsilyl spectra as m/z 73 and 147 are common in most TMS-metabolites at high intensity and so matching can be compromised
  - differences can be based on a limited number of high m/z ions with a low response




Mass spectrum of citric acid

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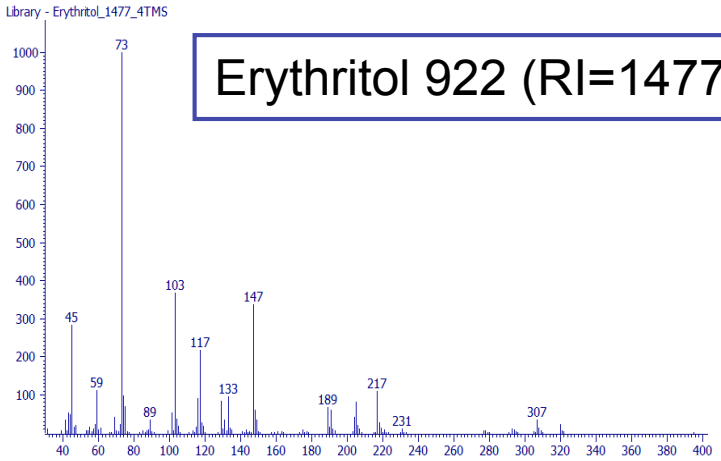
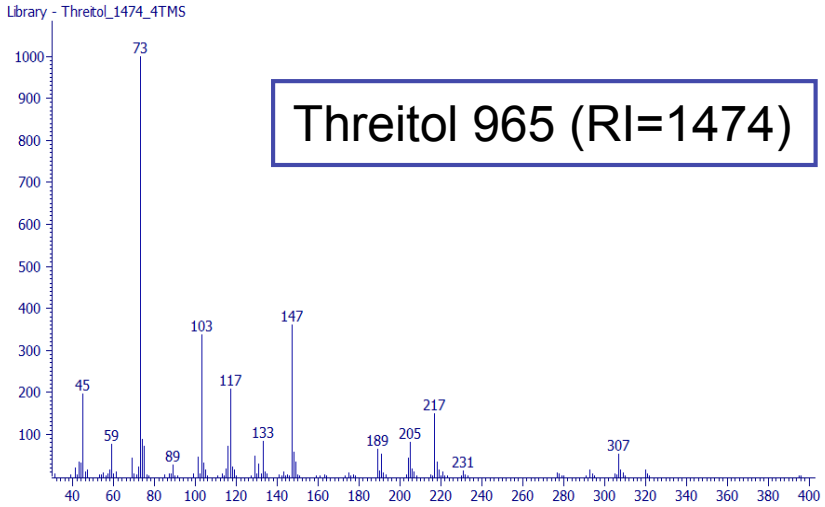
# Problems to consider

- Metabolites of similar chemical structures have a similar chemical structure and may have similar retention index and mass spectrum
- Targeted separation methods required
- Report as X and/or Y



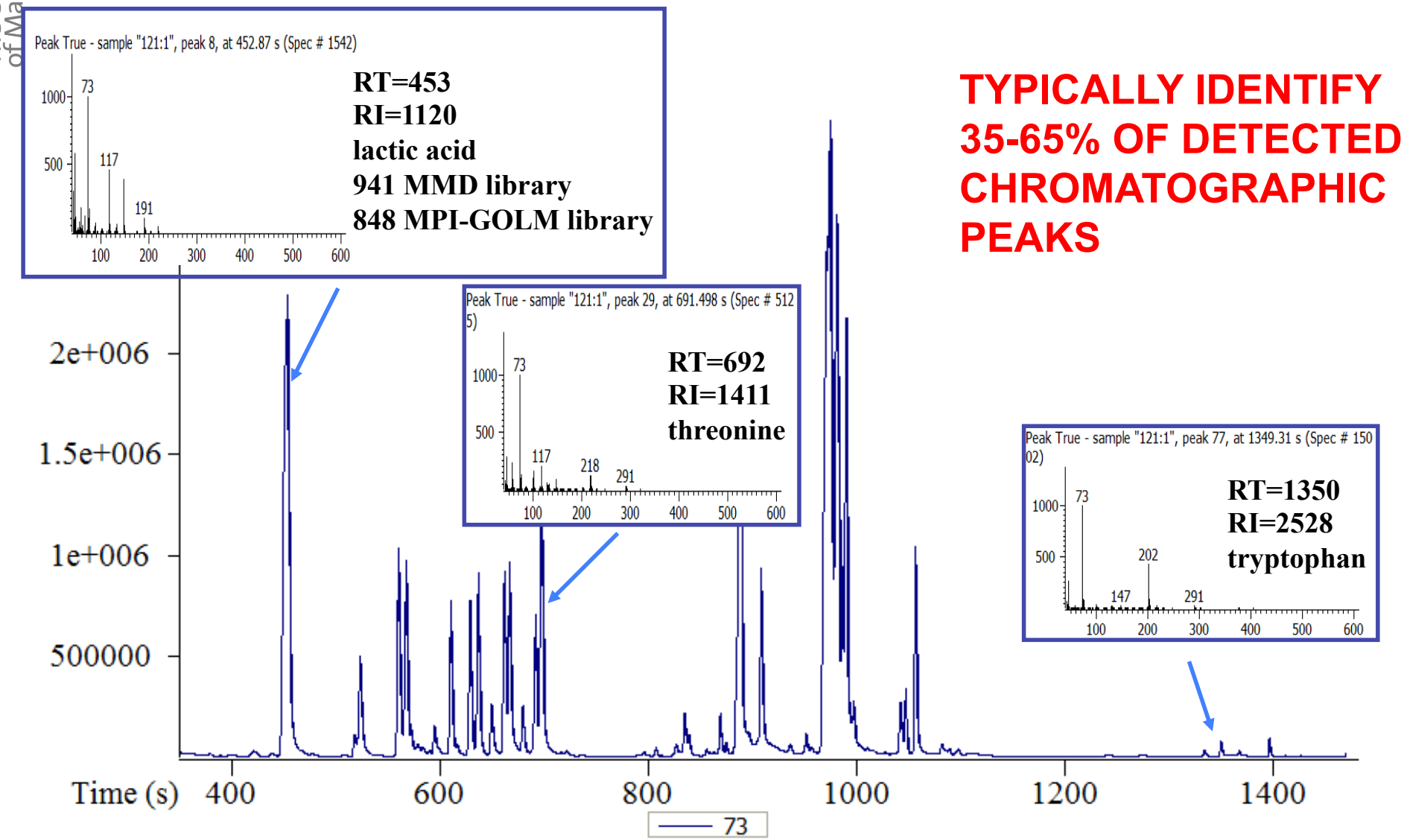

Erythritol 922 (RI=1477)




Threitol 965 (RI=1474)

Erythritol 922 (RI=1477) AND/OR
Threitol 965 (RI=1474)

# Mammalian cell footprint sample



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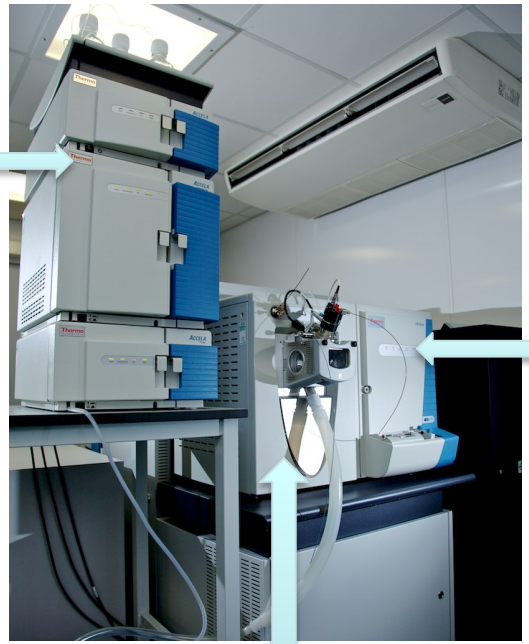
# Electrospray Ionisation (ESI): DIMS, LC-MS, UPLC-MS and CE-MS

## UPLC/UHPLC PROVIDES:

- REPRODUCIBLILITY
- HIGH RESOLUTION (PEAK WIDTHS OF A FEW SECONDS),
- NO RETENTION INDICES FOR METHOD AND LIBRARY TRANSFERS



## MASS SPECTROMETRY PROVIDES:

- HIGH MASS RESOLUTION
- HIGH MASS ACCURACY (SOME NOT ALL INSTRUMENTS),
- HIGH SCAN SPEEDS / ACQUISITION RATES FOR TO ACCURATELY DEFINE NARROW CHROMATOGRAPHIC PEAKS
- HIGH SENSITIVITY
- MS/MS OR $MS^n$ CAPABILITIES FOR MOLECULAR ION FRAGMENTATION (ALL ION OR SELECTED ION)

## IONISATION SOURCES PROVIDE:

- ELECTROSPRAY OR APCI
- REPRODUCIBLE
- MINIMAL/NO ION FRAGMENTATION
- COMPLEX ADDUCT FORMATION (LIQUID AND GAS-PHASE

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# Routine workflow applied

- Accurate *m/z* data acquisition

1. Apply data related to ion type

- Molecular formula determination

1. Applications of rules to filter (e.g. relative isotope abundances)
2. Comparison of MF to metabolite databases

- Putative metabolite identification

- MS/MS or $MS^n$ fragmentation
  - experimental and authentic standard / *in-silico*

*de novo* structural elucidation

comparison to mass spectral libraries

- Reduction of number of putative hits from accurate *m/z* data

- Authentic standard purchase or synthesis and comparative analysis

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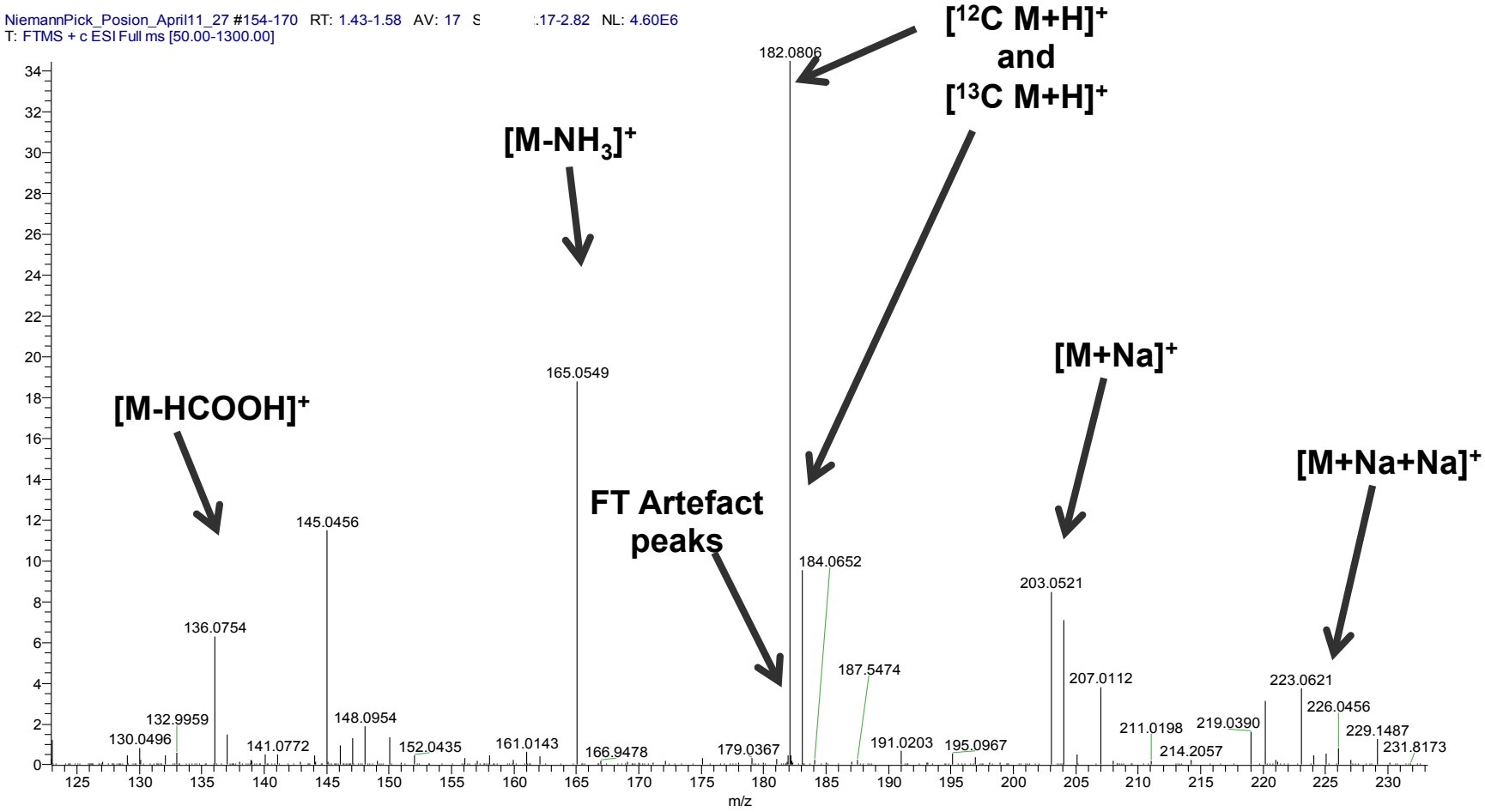
# The complexity of ESI data - tyrosine

## 17 IONS OF DIFFERENT MASS AND SAME RETENTION TIME CREATED FROM A SINGLE METABOLITE – NEED TO DEFINE THE ION TYPE FOR IDENTIFICATION OR HIGH PROBABILITY OF FALSE POSITIVE IDENTIFICATION



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# Accurate measurement of *m/z* as the first process applied

- However, high chance of false positives if type of ion is not determined before conversion to molecular formula
  - see Brown M, Dunn W.B., et al. Mass spectrometry tools and metabolite-specific databases for molecular identification in metabolomics. *The Analyst* 2009, 134, 1322-1332.
  - determine ion type first using accurate mass differences, RT and correlation analysis

BIOINFORMATICS ORIGINAL PAPER

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*Systems biology*

Advance Access publication February 16, 2011

**Automated workflows for accurate mass-based putative metabolite identification in LC/MS-derived metabolomic datasets**

Marie Brown[1], David C. Wedge[2], Royston Goodacre[2,3], Douglas B. Kell[2], Philip N. Baker[4], Louise C. Kenny[5], Mamas A. Mamas[1,6], Ludwig Neyses[1,6] and Warwick B. Dunn[1,2,3,7,*]

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Associate Editor: John Quackenbush

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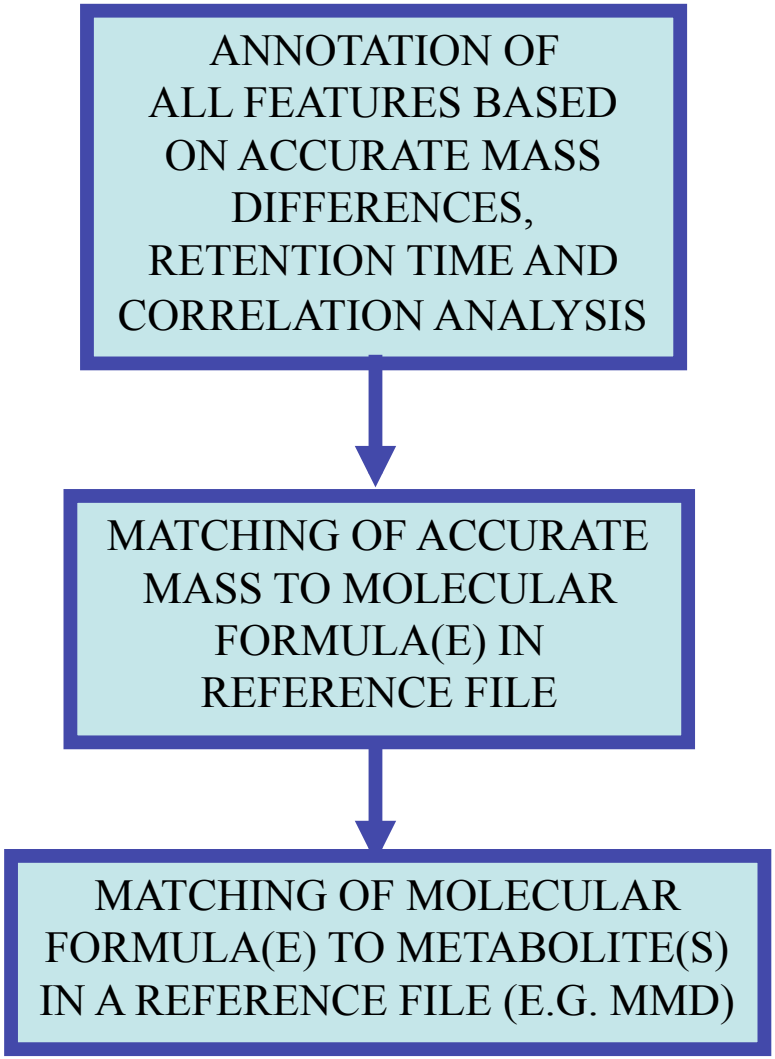
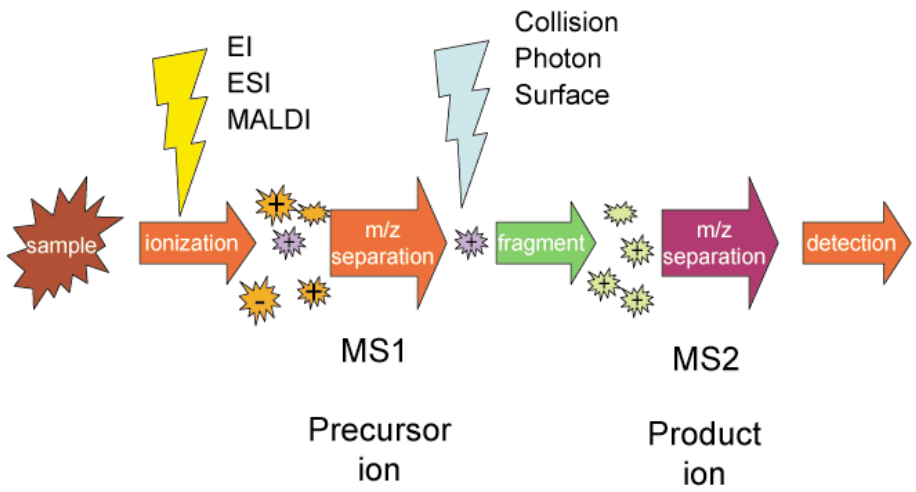
# PUTMEDID-LCMS

- We have developed an *in-silico* suite of workflows for metabolite identification
  - automated and high-throughput
  - for holistic identification of all features
  - easy to use and idiot-proof (i.e. I can use it!!)
  - fill a gap in currently available tools
  - apply information on ion type to reduce number of false positives
- Three separate Taverna workflows have been developed
  - flexibility built in
  - converts accurate mass data to molecular formula(e) and potential metabolite
  - applies reference files which can be developed by the user to be instrument/organism specific
  - developed for Windows not Macs



# MS/MS and MS$^n$

- Gas-phase fragmentation through ion activation in a vacuum




http://en.wikipedia.org/wiki/Tandem_mass_spectrometry

- Specific to a single mass/metabolite OR all-ion fragmentation
- Different ion activation mechanisms available
  - Collision Induced Dissociation (CID) in a Q-TOF or QQQ (MS/MS)
  - CID in an ion trap/linear ion trap (MS$^n$ whewre n can be greater than 2)
  - HCD in Orbitrap instruments (MS/MS)
- Advantages and limitations (e.g. IT/LIT (1/3$^{rd}$ rule))
- Provide structural information
- Apply to reduce number of potential molecular formula
  - like putting a jigsaw puzzle together

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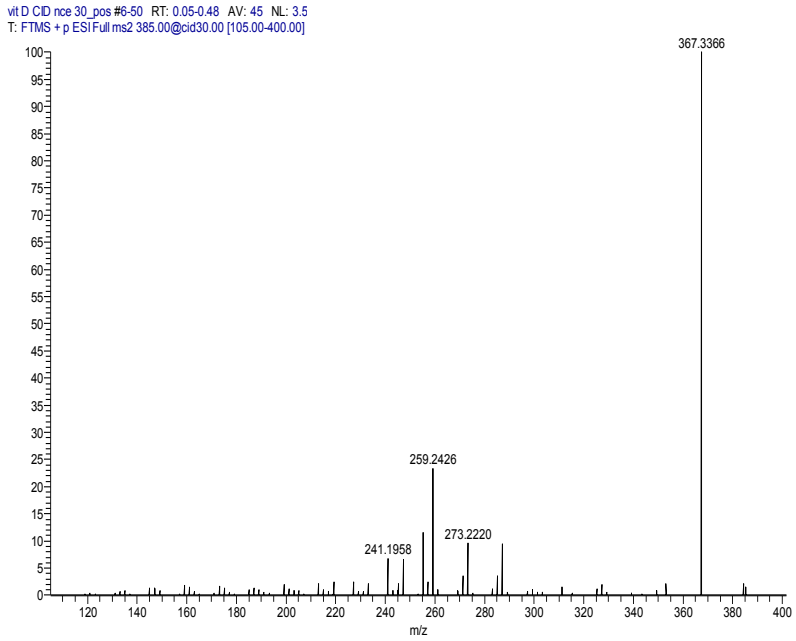
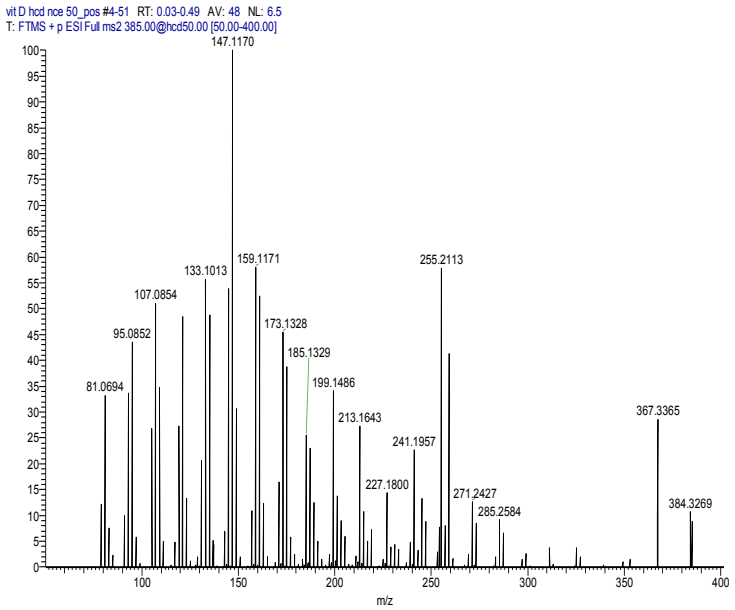
# Complementary ion activation mechanisms are advisable – CID *vs* HCD




CID in a linear ion trap

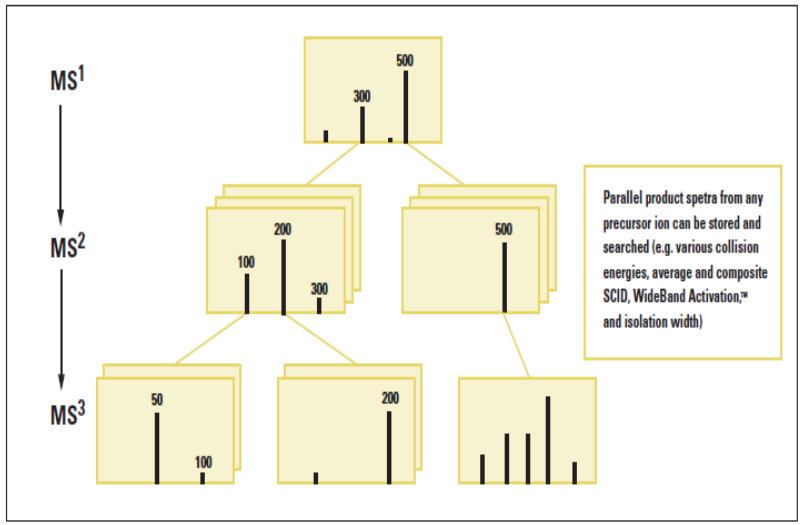



HCD

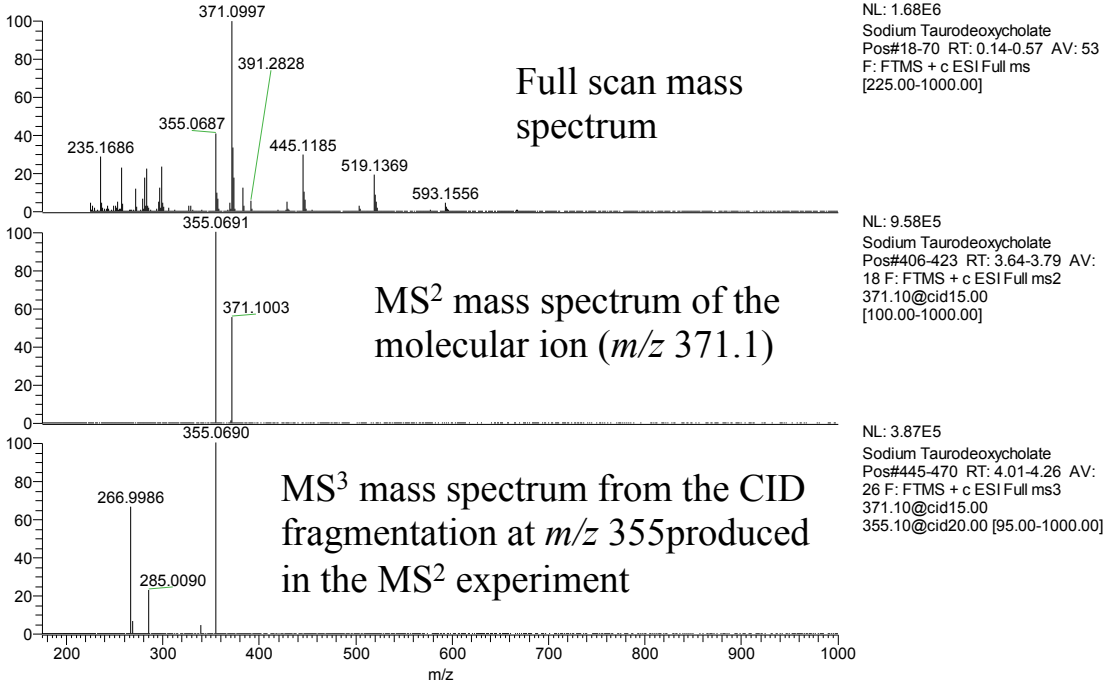
- CID and HCD can (but not always) provide complementary mass spectral data
- Comparable with CID and ECD in proteomics

Thanks to Dr Graham Mullard in CADET/School of Biomedicine for providing the data

# $MS^n$ – mass spectral trees and increased specificity




Parent to daughter to grand-daughter to great grand-daughter




**Courtesy of ThermoFisher Scientific**

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# Problems to consider

- Not all metabolites present in a diverse range of metabolomes are known and electronically tagged
  - *can only apply comparative data if metabolites are present in these databases/ libraries*

- Mass spectral libraries
  - *not all known metabolites are commercially available and so are present in mass spectral libraries*
  - *LC-MS/MS libraries are significantly less developed than for GC-MS*
  - *LC-MS/MS libraries – are they transferable? RT and mass spectra*

- Data are complex
  - *one metabolite = multiple features*
  - *false positives*

- No automated workflows employing multiple strategies
  - *alot of manual work still involved!!!!*

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# In-silico/computational tools in development

- Use data from ‘knowns’ or computational algorithms to predict ‘unknowns’
- *In-silico* fragmentation for LC-MS
  - MassFrontier (http://www.highchem.com/massfrontier/mass-frontier.html)
- Substructure prediction for GC-MS
  - Hummel J, et al., Decision tree supported substructure prediction of metabolites from GC-MS profiles. Metabolomics. 2010, 6(2), 322-333.
- Retention time/index prediction
  - Kumari S, et al., Applying in-silico retention index and mass spectra matching for identification of unknown metabolites in accurate mass GC-TOF mass spectrometry. Anal Chem. 2011, 83(15):5895-902
- Ionisation behaviour rules for ESI-MS
  - Draper J et al. Metabolite signal identification in accurate mass metabolomics data with MZedDB, an interactive m/z annotation tool utilising predicted **ionisation** behaviour 'rules’. BMC Bioinformatics. 2009, 10:227.
- Application of prior biological knowledge (biological samples are not random collections of chemicals but chemicals are linked by enzymatic reactions)
  - Weber RJM et al. MI-Pack: Increased confidence of metabolite identification in mass spectra by integrating accurate masses and metabolic pathways. Chemometrics and Intelligent Laboratory Systems, 2010, 104, 75-82.
- Synthesis of novel metabolites
- In-vivo stable isotope labelling

- **If all else fails……isolation of metabolite and *de novo* structural elucidation**

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# Summary

- Metabolite identification is a highly complex process in metabolomics
- Mass spectrometry offers many tools for metabolite identification
  - accurate mass
  - MS/MS and $MS^n$
  - retention time and retention index
  - mass spectral libraries
  - computational tools
- Limited automated and high-throughput INTEGRATED workflows available as of yet (especially for ESI-MS)
- Unable to identify all metabolites in a sample currently and we are a long way off
- Require a slow cataloguing of metabolites present in a diverse range of metabolomes across many research groups and their database integration
- We are currently on an important developmental journey which is essential for metabolomics to be successful