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
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)
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
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)
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**
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.
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

<table>
<thead>
<tr>
<th>Level</th>
<th>Confidence of Identity</th>
<th>Level of Evidence</th>
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<tbody>
<tr>
<td>1</td>
<td>Confidently identified compounds</td>
<td>Comparison of two or more orthogonal properties with an authentic chemical standard <strong>analysed under identical analytical conditions.</strong></td>
</tr>
<tr>
<td>2</td>
<td>Putatively annotated compounds</td>
<td>Based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries, without reference to authentic chemical standards.</td>
</tr>
<tr>
<td>3</td>
<td>Putatively annotated compound classes</td>
<td>Based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class.</td>
</tr>
<tr>
<td>4</td>
<td>Unknown compounds</td>
<td>Although unidentified and unclassified, these metabolites can still be differentiated and quantified based upon spectral data.</td>
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Gas Chromatography-Mass Spectrometry (GC-MS)
GAS CHROMATOGRAPHY PROVIDES:
- **REPRODUCIBLE**
- **HIGH RESOLUTION (PEAK WIDTHS OF A FEW SECONDS)**
- **RETENTION INDICES FOR METHOD AND LIBRARY TRANSFERABILITY**

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**
Retention indices

Applies a series of homologous compounds e.g. n-alkanes 
and 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
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) AND/OR Threitol 965 (RI=1474)
Mammalian cell footprint sample

RT=1350
RI=2528

tryptophan
RT=692
RI=1411

threonine
RT=453
RI=1120

lactic acid

941 MMD library
848 MPI-GOLM library

Typically identify 35-65% of detected chromatographic peaks
Electrospray Ionisation (ESI):
DIMS, LC-MS, UPLC-MS
and CE-MS
**UPLC/UHPLC PROVIDES:**
- Reproducibility
- High resolution (peak widths of a few seconds),
- No retention indices for method and library transfers

**IONISATION SOURCES PROVIDE:**
- Electro spray or APCI
- Reproducible
- Minimal/no ion fragmentation
- Complex adduct formation (liquid and gas-phase)

**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)
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
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
However, high chance of false positives if type of ion is not determined before conversion to molecular formula

- determine ion type first using accurate mass differences, RT and correlation analysis
• 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\textsuperscript{n}

- Gas-phase fragmentation through ion activation in a vacuum
  - 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\textsuperscript{n} where n can be greater than 2)
    - HCD in Orbitrap instruments (MS/MS)
  - Advantages and limitations (e.g. IT/LIT (1/3\textsuperscript{rd} rule))
  - Provide structural information
  - Apply to reduce number of potential molecular formula
    - like putting a jigsaw puzzle together

http://en.wikipedia.org/wiki/Tandem_mass_spectrometry
Complementary ion activation mechanisms are advisable – CID vs HCD

CID in a linear ion trap

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

HCD

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
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!!!!*
In-silico/computational tools in development

• Use data from ‘knowns’ or computational algorithms to predict ‘unknowns’
  • *In-silico* fragmentation for LC-MS
  • Substructure prediction for GC-MS
  • Retention time/index prediction
• Ionisation behaviour rules for ESI-MS
• Application of prior biological knowledge (biological samples are not random collections of chemicals but chemicals are linked by enzymatic reactions)
• Synthesis of novel metabolites
• In-vivo stable isotope labelling

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

• Metabolite identification is a highly complex process in metabolomics

• Mass spectrometry offers many tools for metabolite identification
  – accurate mass
  – MS/MS and MS
  – 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