Sample Prep for Metabolomics experiments -PLANT TISSUES-

Ute Roessner
Metabolomics Australia
School of Botany, The University of Melbourne
Workshop Agenda

Ute Roessner – Sample prep for plant tissues

Rick Dunn – Sample prep for mammalian tissues

David deSouza – Sample prep for microbiological samples

Dan Jones – Sample prep for specialised tissues and analyses
What is metabolism?

The word metabolism comes from the Greek metabolē and means change or transformation.

What is a metabolite?

A substance that takes part in the process of metabolism, which involves the breakdown of complex organic constituents of the body with the liberation of energy for use in bodily functioning. The various compounds that take part in or are formed by these reactions are called metabolites.

What is a metabolic pathway?

A metabolic pathway is a series of chemical reactions occurring within a cell.
What is Metabolomics about?

This is the analytical approach of large-scale, non-targeted and high-throughput analysis of metabolites in a biological system.

The real challenge of metabolomics is to comprehensively cover the analysis of very many compounds having very many different chemical structures.
Metabolomics Approaches

1. **Target Analysis**
   - Detection and quantification of a small set of target compounds (identified) → HPLC

2. **Metabolite Profiling**
   - Detection and quantification of a large set of metabolites (identified and unidentified) → GC-MS

Metabolomics Approaches

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3. **Metabolomics**
   - Detection and quantification of as many metabolites as possible → LC-MS/MS and GC-MS, NMR

4. **Metabolic Fingerprinting**
   - Generation of a metabolic “signature” of a sample → FT-IR, flow injection MS/MS, FT-MS
   → high-throughput screening

Typical metabolomics approach:

1. Tissue harvest
2. Shock freeze
3. Homogenization
4. Extraction procedure
5. Derivatization
6. Data analysis
7. Data interpretation
Challenges of Metabolomics

1. Chemical differences of metabolites
2. Quenching of Metabolism
3. High variability of abundance
4. Different approaches have to be established for
   a) Extraction
   b) Separation
   c) Detection
   d) Quantitation
5. Identification of novel compounds
6. Automation of data evaluation
7. Data-handling, -mining and visualisation
8. Data interpretation
Chemical diversity of metabolites

Chemical structures
Size, weight, polarity, stability etc.

Formular: \( \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{glucose} \rightarrow \text{glycogen} \)

Molecular weight:
- \( \text{CO}_2 \): 44
- \( \text{H}_2\text{O} \): 18
- \( \text{glucose} \): 180
- \( \text{glycogen} \): \( n \times 180 \)

highly apolar: lipids, fatty acids, waxes, terpenes
highly polar: carotenoids, chlorophylls, steroids, flavonoids, amino acids, organic acids, organic amines, alkaloids, nucleosides, sugars, nucleotides, phosphates, metals, salts
Isomers

Glucose  Mannose  Galactose

Modifications

D-glucose  D-glucose-6-phosphate

hydroxyproline  2-amino-2-deoxy-D-glucose  N-acetyl-D-glucosamine

There is no “ultimate” analytical technology to analyse all the structural different compounds simultaneously.
Abundance of Metabolites

<table>
<thead>
<tr>
<th>Metabolite / Cell</th>
<th>Amount (Moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6</td>
<td>10^{-12} (pico)</td>
</tr>
<tr>
<td>10^5</td>
<td>10^{-13}</td>
</tr>
<tr>
<td>10^4</td>
<td>10^{-14}</td>
</tr>
<tr>
<td>10^3</td>
<td>10^{-15} (femto)</td>
</tr>
<tr>
<td>10^2</td>
<td>10^{-16}</td>
</tr>
<tr>
<td>10^1</td>
<td>10^{-17}</td>
</tr>
<tr>
<td>10^0</td>
<td>10^{-18} (atto)</td>
</tr>
</tbody>
</table>

Dynamic Ranges
- NMR
- LC-UV
- GC- or LC-MS
- CE-MS
- CE-LIF
Workflow for Metabolomics

Plants of interest ➔ Tissue harvest ➔ Homogenisation

Aliquots

Extractions, phase separations, enrichments, purifications

GC-MS, LC-MS/MS, CE-MS, FT-MS, Spectroscopy, NMR etc.

Separation
Detection
Identification
Quantification

Combined data
What is different when handling plant tissues?

- Photosynthesis - light dependency
- Anatomy (many different cell types)
- Complexity
- Dynamic range of metabolite levels
Light dependency – time of harvest

Urbanczyck-Wochniak et al. 2005
Tissue sampling

→ fast quenching of metabolism

→ statistics (replication)

→ controlled environment

→ comparing references (i.e. WT)

→ recoveries
Biological replication

*Individual vs pooling*

→ The more sensitive your method the better your biological replication!

→ *Aim: don’t pool samples!*
Sample prep for plant metabolite analyses

• Quenching of metabolism

• Extraction

• Separation from large molecules / other cell products

• if required further clean up / enrichment / purification
Quenching metabolism

- Stop any metabolic reaction in the sample
  - Cold (shock freezing)
  - Denaturing enzymes
  - Acids
  - Solvents (MeOH, Chloroform etc)
Metabolite release from the tissue/cell

- **Cell Disruption**

  - **Mechanical**
    - Liquid shear
      - Ultrasonics
      - Microwave
      - French press
      - Pressurized liquid extraction
      - Supercritical fluid extraction
    - Solid shear
      - Manual grinding
      - Ball mill
      - Others
  - **Non-Mechanical**
    - Enzymatic
      - Lysozyme
    - Chemical
      - Organic solvents alone
      - Methanol, chloroform and buffer
      - Boiling ethanol
      - Boiling water
      - Acid/alkali treatment
    - Physical
      - Osmotic shock
      - Freeze/thawing
      - Heating
Extraction

- Metabolite locations
  - Extra cellular/intracellular?
  - Select tissue of interest
  - Select organelle of interest?

- Select fraction of interest
  - Hydrophillic
  - Hydrophobic
Separation from “unwanted”

- Proteins
- Lipids
- Starch / cell wall / other carbohydrates
- DNA/RNA
- Chlorophyll and other pigments
- High abundant primary metabolites
- Salts
Crude (‘omics) vs Clean (targeted)

- Crude
- Phase separated
- SPE
- “pure”

Sensitivity

Selectivity
Special Targets may require special extractions

- Volatiles
- Very instable metabolites
- Extremely low abundant
- Lipids
- Fatty acids
Solvents

Decision which is best is influenced by chemical properties of metabolites of interest and method of separation and detection available

- Water
- Methanol / Ethanol
- Isopropanol
- Chloroform
- Acids (e.g. perchloric acid)
- Acetonitrile
- mixtures of either
Influence of analysis method to extraction

- **GC-MS**
  - Crude extraction possible
  - Selectivity comes through derivatisation

- **LC-MS**
  - Crude extraction possible
  - Need to remove salts, high molecular weights, pigments

- **LC-MS – MRM based quantitation**
  - Crude extraction possible, but further clean up recommended for increase of selectivity
Summary

- Before start “learn” about your metabolites of interest
- Any method from Literature needs to be validated for your tissue of interest
- Decision of ‘omics vs targeted and/or semiquantitative vs quantitative
- Biological replication – don’t pool!

*The better your sample prep the better the data!!*
Metabolomics
Hands-on Workshop

Overview: Hands-on experience (theory and practice) in state-of-the-art metabolomics technologies

Program:
- GC-MS based metabolite profiling
- LC-MS based metabolite profiling
- LC-MS based metabolite quantification
- NMR based metabolite profiling
- How to set up a metabolomics experiment

When: 20th to 22nd of October 2009

Where: Metabolomics Australia node, The University of Melbourne, Parkville Campus, Victoria

Cost: $300 (includes lecture notes, experimental requirements, catering)

More information and Registration:
Please contact Dr Ute Roessner
u.roessner@unimelb.edu.au
Phone: 03-8344 4099
www.metabolomics.net.au

Hurry as numbers are limited!
Thanks!

Recommended Reading:

Villas-Boas, Roessner, Smedsgaard and Nielsen 2007
Metabolome Analyses – An Introduction

Wiley & Sons