Sample preparation in metabolomics

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Backgrounds

• Metabolite coverage (~8500 endogenous and 40,000 exogenous metabolites human metabolomes) with wide dynamic concentration range
• Retaining of analytes and removal of undesirable matrix components - pre-concentration step
• It affects qualitative and quantitative analysis of metabolites and hence biological interpretation
• Avoiding loss/degradation (quenching and rapid extraction)
• Non-selective (global or untargeted) and selective (targeted) extraction of metabolites
• Simple, rapid, reproducible and quantitative recovery of metabolites
Metabolites/metabolome are structurally diverse

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Description</th>
<th>Boiling Point</th>
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</thead>
<tbody>
<tr>
<td>H₂</td>
<td>A gas</td>
<td></td>
<td>bp – 253°C</td>
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<tr>
<td>CH₃CHO</td>
<td>EtOH metabolite</td>
<td>A vapor in diabetics</td>
<td>bp 56°C</td>
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<tr>
<td>CH₃COCH₃</td>
<td></td>
<td></td>
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<tr>
<td>CH₃CH₂CH₂COOH</td>
<td>Epigenetic modifier</td>
<td></td>
<td>bp 164°C</td>
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<tr>
<td>Citric acid</td>
<td></td>
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<tr>
<td>Adenine</td>
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<tr>
<td>17β-estradiol</td>
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<td></td>
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<tr>
<td>PGF₂α</td>
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<td></td>
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<tr>
<td>A phosphatidylcholine</td>
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<td></td>
<td></td>
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<tr>
<td>A ω-hydroxy-fatty acid fatty acid ester</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Acetyl- and palmitoylcarnitine</td>
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</tbody>
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Source: Dr. Barnes’ slides

Biological samples

- Bio-fluids- urine, plasma, bile, saliva etc.
- Fecal samples
- Muscles/epithelial tissues
- Plant- roots, leaves
- *In vitro* microscopic cell culture- culture medium, cell lysates
Sample preparation

• Collection and quenching

• Homogenization

• Extraction


The method of choice will be determined by the sample matrix and the concentration of compounds in samples.
Extraction of Metabolites from Cells-
intra-cellular metabolites

- **Adherent cells in petri dish/flask**
  - Prepare ice-cold physiologic saline
  - Tilt plate/flask and remove cell culture medium with vacuum pipet from cellular monolayer
  - Immediately add 10 ml ice-cold physiologic saline, swirl and remove medium with vacuum pipet (less than 10 sec)-**quenching metabolism starts**
  - Spike with IS and add MeOH cooled in dry ice (-43°C)-**quenching/extraction**
  - Incubate at 0-4°C for 30 min, centrifuge and transfer the supernatant into a new tube, concentrate (evaporation under nitrogen, lyophilization etc) if necessary and store -20 °C until analysis

- **Suspended or non-adherent cells**
  - Remove cell medium from the culture flask/dish and transfer to tubes, centrifuge at low speed and pellet the cells
  - Discard the medium and add 1 ml of MeOH cooled in dry ice (-43°C)/2 x 10⁶ cells
  - Incubate at 0-4°C for 30 min, centrifuge to remove the supernatant into a new tube, concentrate if necessary and store -20 °C until analysis

Adopted from Dr. Barnes slides

Tissue – metabolite extraction

- **Tissue MUST BE snap-frozen (liq N₂) to prevent further metabolism**
- **Grind the tissue in a pestle and mortar**
  - Pre-cool in liq N₂
  - Pour powder as a slurry into extraction tube
  - Allow N₂ to evaporate
- **Add 4 volumes of pre-cooled (-20°C) MeOH**
  - Extract at 0–4°C for 30 min
  - Centrifuge – collect supernatant
  - Re-extract and centrifuge
  - Combine supernatants
Urine

- Urines can be spot (collected at the time) or 24-hour collections
  - The 24-hour collection is an integral of urinary output
  - For rat studies, best collected using a metabolic cage where the urine drips into a beaker set in a container filled with dry ice
  - For mice, roll them on their back – they will pee for you
- It’s worth noting that urine resides in the bladder at ~37°C for several hours before it is collected
  - Once it’s out of the bladder, it will be exposed to microbes that may alter its composition
  - For clinical studies, the urine can be collected and then placed in a refrigerator – some add ascorbic acid (1%) or 10% sodium azide

Urine storage and extraction

- Once collected, urine is mixed and its total volume noted
  - Best if (say) five to ten 1 ml aliquots are taken and stored at -80°C
  - These can be thawed one time to begin extraction
- Urines must be centrifuged to remove particulate matter
  - Cleared human urine could be used directly (need to divert the initial eluate since it is predominantly electrolytes and very hydrophilic metabolites such as urea, glucose, etc.)
  - Rodent urines contain MUP proteins – these must be precipitated by adding 4 volumes of ice-cold MeOH
    - Precipitated protein removed by centrifugation
    - Supernatant is evaporated to dryness under N₂ and re-dissolved in water
Blood, plasma and serum

- Blood consists of cells (reticulocytes, white cells/monocytes and plasma or serum)
- Plasma requires the use of heparin or EDTA
  - Heparin is preferred for NMR analysis
  - EDTA is preferred for LC-MS analysis
- Serum has no required additions, but be careful not to lyse the reticulocytes since the released heme is highly oxidative
  - add 50 mM nitriloacetic acid to complex Fe^{2+/3+}
- Store in 1 ml aliquots at -80°C
- Small animals – mice, zebrafish – yield only µl volumes

Fecal collection

- Note: feces have been in the presence of a trillion bacteria at 37°C for several days during colonic passage
  - Some metabolism can occur after collection
  - Slowed by cooling – can be frozen as for tissue
- Sometimes feces are collected for microbiome analysis
  - Placed in Cary Blair (NaCl, Na thioglycollate, Na₂HPO₄, pH 8.4) minimal medium
  - Glycerol added to prevent freezing when stored at -20°C
Fecal extraction

- Treat frozen feces like tissue
  - Powder in liq N₂
  - Extract with 4 volumes of cooled (-20°C) MeOH
- Fresh feces
  - Extract with 4 volumes of cooled (-20°C) MeOH
- Feces in Cary-Blair medium
  - Extract with 4 volumes of cooled (-20°C) MeOH
- Feces in Cary-Blair medium plus glycerol
  - Disperse in aqueous medium and extract with ethyl acetate

Extraction of lipids

Auto-oxidation and pH are two important issues

Bligh/Dyer extraction

Homogenized Cell suspension/biological fluids (1 mL) + IS

- 2.5 mL MeOH + 1.25 mL CHCl₃
  - Agitation/sonication (10 sec)
- 1.0 mL H₂O + 1.25 mL CHCl₃
  - Vigorous shaking, centrifugation

- Aqueous phase
- Lipid soluble CHCl₃ layer

Concentration, reconstitution and analysis
Using isotopes to monitor recovery

- Isotopically labeled compounds, particularly $^{13}$C (a stable isotope), behave the same as their unlabeled counterparts
  - They have different masses – 1.003 Da for every $^{13}$C
  - Can be measured independently from the real metabolite
  - Not available for every metabolite
  - “All” metabolites would be very expensive
  - Alternative is to use the IROA Technologies reagent
    - An exhaustively $^{13}$C-labeled yeast product

Choice of Good Internal Standards

- A stable isotopically labeled IS is preferable
  - If $^{13}$C, then there must be at least three $^{13}$C atoms to avoid contributions of natural abundance $^{13}$C
- Or, a compound not found in the samples
  - In the absence of stable isotopically labeled internal standard, the unlabeled internal standard needs to be structurally similar to the analyte
- Should not react chemically with the analyte
Quantification

- **Relative quantification**
  - normalizes the metabolite signal that of an internal standard signal intensity in large scale un-targeted profiling (e.g., non-naturally occurring lipid standards - Cer C$_{17}$ or stable isotope labeling through metabolism- AA-d$_4$.

- **Absolute quantification**
  - based on external standards or internal isotopically labeled standards - targeted metabolomics.

- **Matrix effects**
  - Affect selectivity, accuracy and reproducibility.
  - Signal suppression or enhancement are major issues. Stable isotope labeled standards are needed.

Problems facing with extraction and analysis

- **Metabolite concentration range**- pM-mM
- **Structural diversity, chemical stability and ionizability**
- **Endogenous substances**
  - From matrix, i.e., organic or inorganic molecules present in the sample and that are retained in the final extract.
  - Examples: EDTA, phospholipids, drugs administered to the patient and proteins/peptides
- **Exogenous substances**,
  - molecules not present in the sample, but coming from various external sources during the sample preparation.
  - Detergents, plasticizers, solvent residues, column siloxanes
Supported Liquid Extraction (SLE)

- Aq. sample is adsorbed on a porous highly polar solid support - Diatomaceous earth
- Sufficiently adsorbs the entire volume of sample
- Non-polar compounds at the surface of solid support
- Target analytes should be in non-ionized form
- Eluted by non-polar solvent
- Simple, high throughput and extraction efficiency

Targeted analysis of ceramides-MRM chromatograms showing simultaneous determination of ceramides (C₄-C₂₄)
Quantitative analysis of ceramides; Poor recoveries of non-polar ceramides in Bligh-Dyer (BD) liquid-liquid extraction compared to Biotage (supported liquid extraction)

Supercritical Fluid Extraction (SFE)
Extraction of bioactive natural products

- Extraction method involving the use of supercritical solvent in extracting non-polar to moderately polar analytes from solid matrices
- Use of solvents above the critical conditions for temperature and pressure - super critical carbon dioxide
- Able to penetrate solid matrix (botanical products) and solubilize compounds
- Inexpensive, faster and environmental friendly - Green chemistry, renewable solvent
- Extraction of thermally-labile compounds
Microwave-assisted solvent extraction (MAE)

- Use of microwave energy to heat liquid organic solvent in contact with sample
  - Watch out for thermal degradation
- Non-ionizing, fast and effective extraction with limited volume of solvent
- Moisture or water serves as target for microwave heating
- Special approved microwave equipment should be used, not domestic microwave oven

The ratio of botanical material to extracting solvent plays important role in efficient extraction of phytochemicals

Extractability of isoflavones from various amounts kudzu dietary supplement powder in 5 mL of 80% aq. MeOH

Conclusions

• Development of optimal extraction method for a biological sample remains a significant challenge.
• Although conventional extraction methods SPE, PPT, and LLE are widely used, newer methods such as supported liquid extraction may be used for extracting many non-polar compounds in biological samples efficiently.