

# Sample preparations in metabolomics

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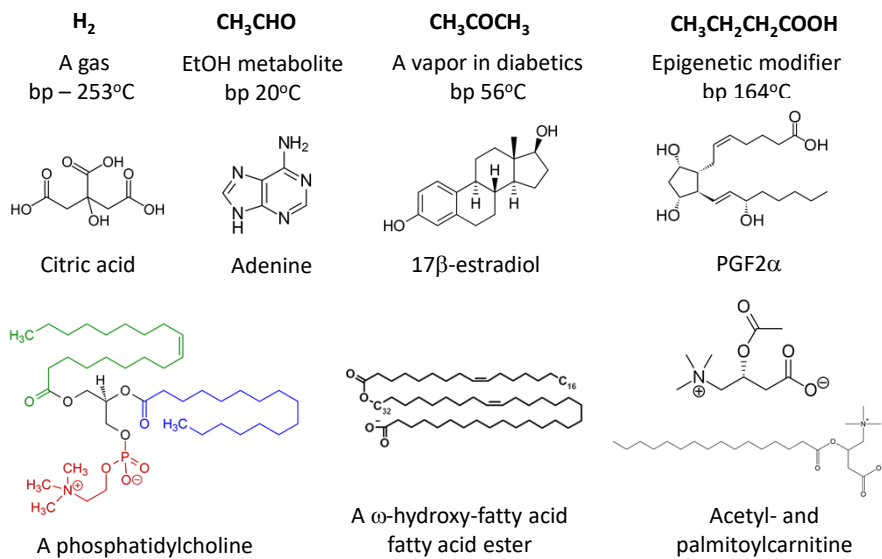
## Backgrounds

- Metabolite coverage (>8000 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

Vuckonic et al. Anal Bioanal Chem 2012

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## Metabolites/metabolome are structurally diverse



Source: Dr. Barnes' slides

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## A typical metabolomics workflow

1. Sample collection
2. Extraction
3. Analysis of the extracted compounds
4. Data Processing
5. Metabolite annotation
6. Biological interpretation

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## 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**

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## Sample preparation

- **Collection and quenching**
- **Storage**
- **Homogenization**
- **Extraction**

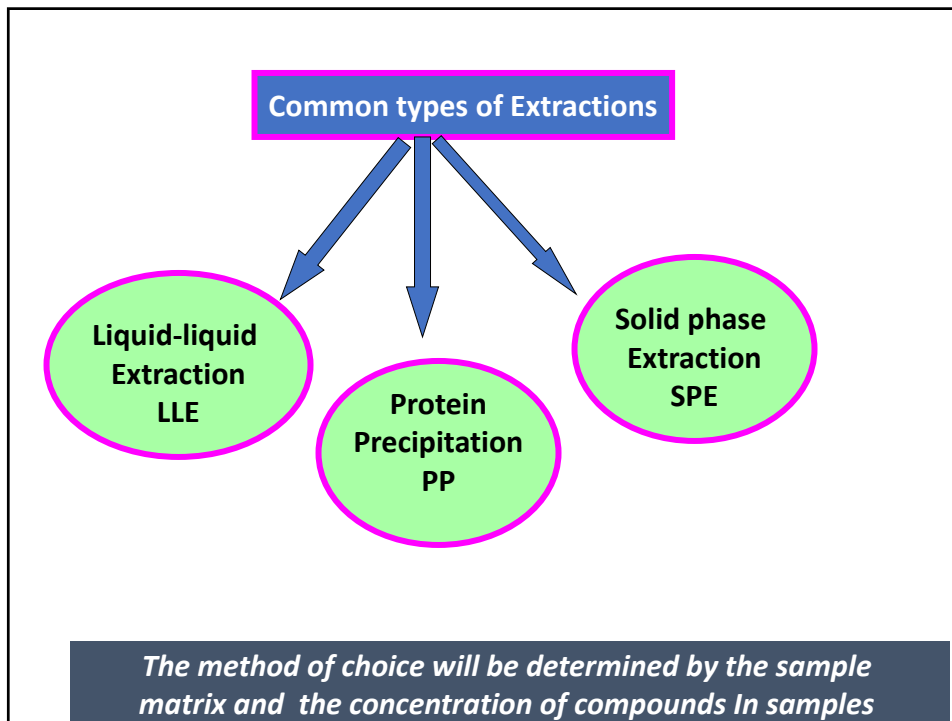
Mushtaq et al. Phytochem. Anal. 2014

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## Problems facing with extraction and analysis

- **Metabolite concentration range (pM-mM), high turnover rate**
- **Structural diversity ( $10^5$ ), chemical stability and ionizability**
- **Quantitation of endogenous metabolites- lack of analyte free matrix**
- **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

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## Extraction of Metabolites from Cells- intra-cellular metabolites

- Adherent cells in petri dish/flask
  - Prepare ice-cold physiologic saline (0.9% NaCl w/v)
  - 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
  - Spike with IS and add cold MeOH (-20°C) and ice cold H<sub>2</sub>O (400 ul each 1:1 v/v)-  
**quenching**
  - Scrape the well with a cell scraper, and transfer the extract into an eppendorf tube containing 400 uL of CHCl<sub>3</sub> (-20°C)
  - Agitate the cell extract for 20min at 1400 rpm, followed by 5min of centrifugation at a minimum of 16,100 x g and transfer the phases into a new tube, concentrate (evaporation under nitrogen, lyophilization etc) if necessary and store -20 °C until analysis
- Suspended or non-adherent cells
  - Centrifuge cells in medium at 250 x g for 5 min to separate the medium and cell pellets.
  - Wash cells with ice cold saline and follow the similar procedure as above.  
**(quenching, extraction and separation of phases)**

Adopted from Dr. Barnes slides and Sapcariu et al. MethodsX 2014

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## Tissue – metabolite extraction

- Tissue **MUST BE** snap-frozen (liq N<sub>2</sub>) to prevent further metabolism
- Grind the tissue in a pestle and mortar
  - Pre-cool in liq N<sub>2</sub>
  - Pour powder as a slurry into extraction tube
  - Allow N<sub>2</sub> 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



Source: Dr. Barnes' slides

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## 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

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## Urine storage and 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 N<sub>2</sub> and re-dissolved in water

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## 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  $\text{Fe}^{2+/3+}$
- **Store in 1 ml aliquots at -80°C**
- **Small animals – mice, zebrafish – yield only  $\mu\text{l}$  volumes**

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## 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 ( $\text{NaCl}$ , Na thioglycollate,  $\text{Na}_2\text{HPO}_4$ , pH 8.4) minimal medium
  - Glycerol added to prevent freezing when stored at -20°C

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## Fecal extraction

- **Treat frozen feces like tissue**
  - Powder in liq N<sub>2</sub>
  - 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

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## 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 CHCl<sub>3</sub>  
Agitation/sonication (10 sec)

↓  
1.0 mL H<sub>2</sub>O + 1.25 mL CHCl<sub>3</sub>  
Vigorous shaking, centrifugation

↓  
Aqueous phase  
Lipid soluble CHCl<sub>3</sub> layer

↓  
Concentration, reconstitution and analysis

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## Using isotopes to monitor recovery

- **Isotopically labeled compounds, particularly  $^{13}\text{C}$  (a stable isotope), behave the same as their unlabeled counterparts**
  - They have different masses – 1.003 Da for every  $^{13}\text{C}$
  - 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  $^{13}\text{C}$ -labeled yeast product

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## Choice of Good Internal Standards

- **A stable isotopically labeled IS is preferable**
  - If  $^{13}\text{C}$ , then there must be at least three  $^{13}\text{C}$  atoms to avoid contributions of natural abundance  $^{13}\text{C}$
- **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**

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## Quality control

- A pool of all or a batch of study samples- average metabolites (matrix and analytes) of all samples
- Assess the analytical variable of data- drift in Rt and ion signals
- Analyzed in a fixed interval of sample run

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## Controls

- Positive controls- where changes are expected
- Negative controls- where no change is expected
- Sham controls- incidental effects induced by the procedure or operation as a control

Vanisevic J and Want EJ., Metabolites 2019

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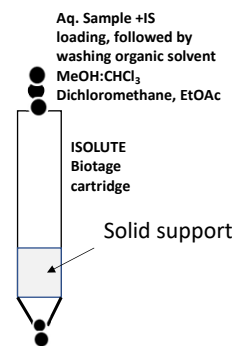
## Quantification

- **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<sub>17</sub> or stable isotope labeling through metabolism- AA-d<sub>4</sub>).
- **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.

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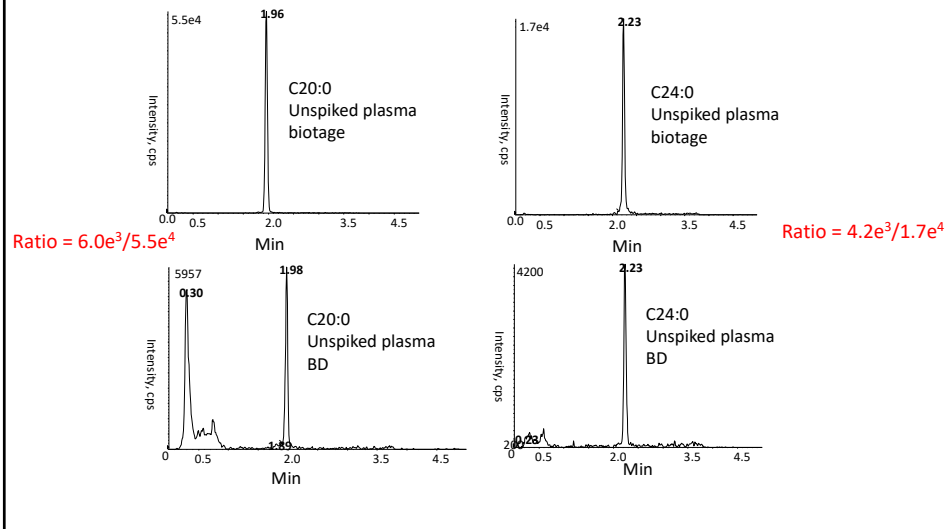
## Supported Liquid Extraction (SLE)

- 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



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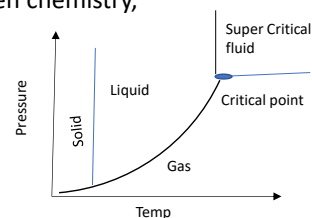
**Quantitative analysis of ceramides;  
Poor recoveries of non-polar ceramides in Bligh-Dyer (BD) liquid-liquid  
extraction compared to Biotage (supported liquid extraction)**



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## Supercritical Fluid Extraction (SFE) Extraction of bioactive natural products

- Extraction method involving the use of supercritical solvent CO<sub>2</sub> 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
- By controlling the levels of pressure/temperature, supercritical CO<sub>2</sub> can extract a wide range of compounds
- Inexpensive, faster and environmental-friendly - Green chemistry, renewable solvent
- Extraction of thermally-labile compounds



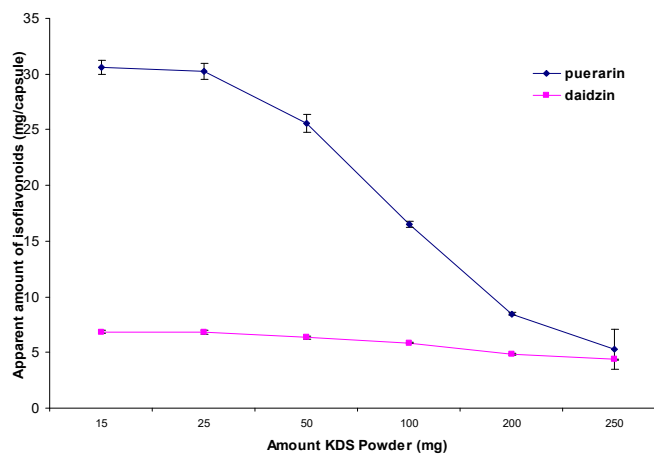
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## Microwave-assisted solvent extraction (MAE)

- 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

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## 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

*Prasain et al. J. Agric. Food Chem., 2003*

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## 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.