

Sample preparations in metabolomics

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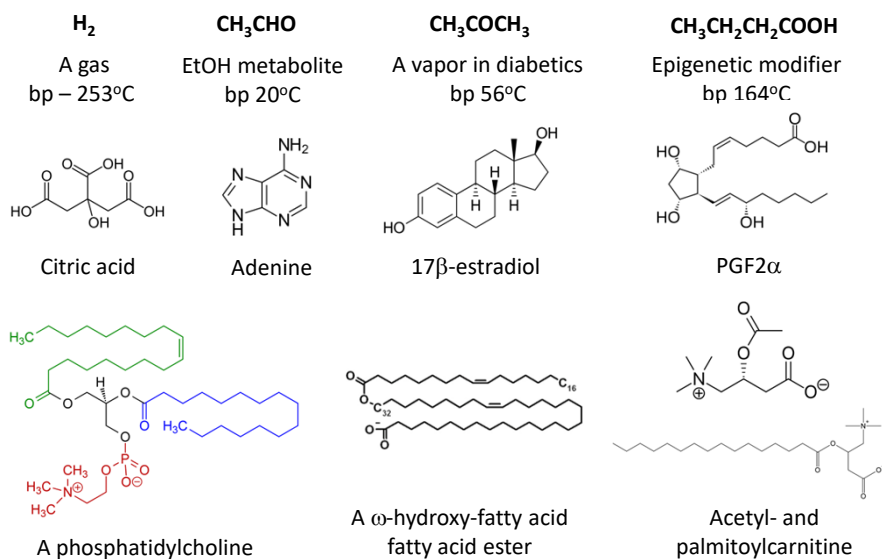
Backgrounds

- 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

Vuckovic 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 preparations

- **Collection** (filtration, centrifugation/addition of bacteriostatics) and quenching
- **Storage**
- **Homogenization**
- **Extraction**

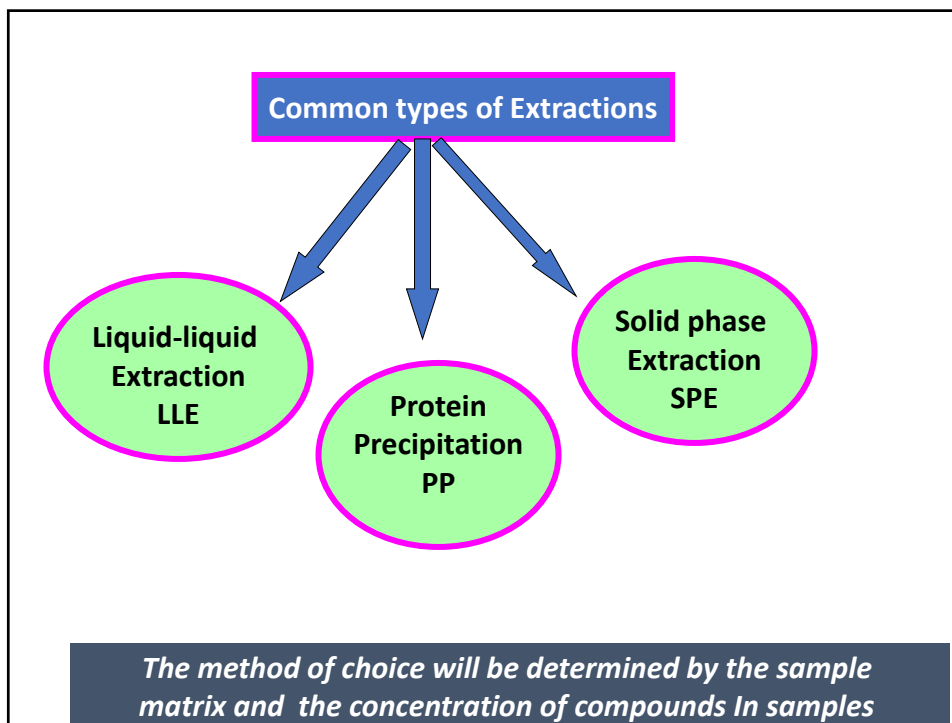
Mushtaq et al. Phytochem. Anal. 2014
Smith et al. Metabolites, 2020

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Prevalent challenges 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

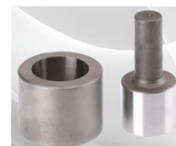
- Adherent cells in petri dish/flask
 - Prepare ice-cold physiologic saline (0.9% NaCl w/v)
 - Tilt plate/flask and remove cell culture medium with vacuum pipet from cellular monolayer
 - Immediately add 10 ml ice-cold physiologic saline, swirl and remove medium with vacuum pipet
 - Spike with IS and add cold MeOH (-20°C) and ice cold H₂O (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 CHCl₃ (-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 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



Source: Dr. Barnes' slides

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Urine

- **Urine 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 1% sodium azide (NaN₃).

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Urine storage and extraction

- **Urine 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

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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 μl 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 (NaCl , Na thioglycollate, Na_2HPO_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 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

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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 CHCl₃
Agitation/sonication (10 sec)

↓
1.0 mL H₂O + 1.25 mL CHCl₃
Vigorous shaking, centrifugation

↓

	Aqueous phase
	Lipid soluble CHCl ₃ layer

↓
Concentration, reconstitution and analysis

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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

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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
- **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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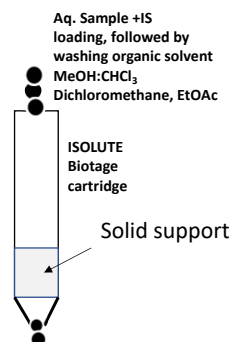
Quantification

- **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 C₁₇ or stable isotope labeling through metabolism-AA-d₄).
- **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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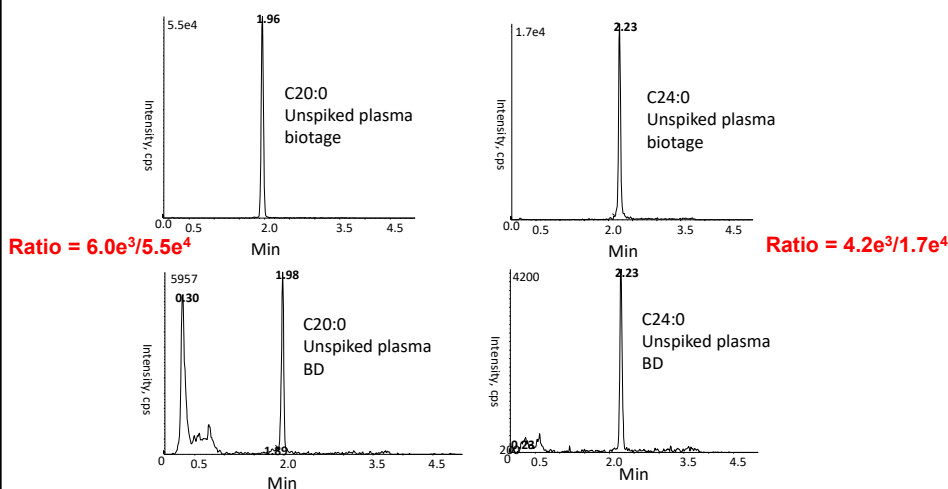
Supported Liquid Extraction (SLE)

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

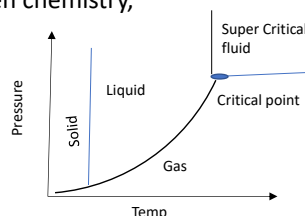


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Supercritical Fluid Extraction (SFE)

Extraction of bioactive natural products

- Extraction method involving the use of supercritical solvent CO₂ 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 CO₂ can extract a wide range of compounds
- Inexpensive, faster and environmental-friendly - Green chemistry, renewable solvent
- Extraction of thermally-labile compounds



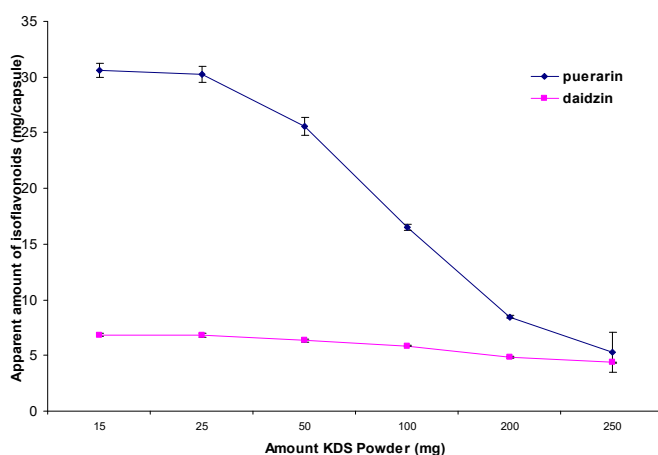
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Microwave-assisted solvent extraction (MAE)

- 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.