Sample preparation in metabolomics

Jeevan Prasain, PhD
Department of Pharmacology and Toxicology, UAB
jprasain@uab.edu

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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**

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Metabolites/metabolome are structurally diverse

H_2

A gas
bp – 253°C

CH_3CHO

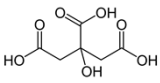
EtOH metabolite
bp 20°C

CH_3COCH_3

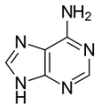
A vapor in diabetics
bp 56°C

$CH_3CH_2CH_2COOH$

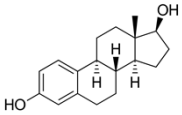
Epigenetic modifier
bp 164°C



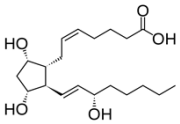
Citric acid



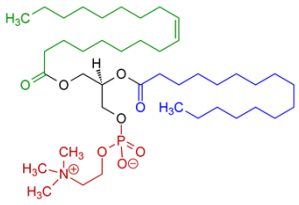
Adenine



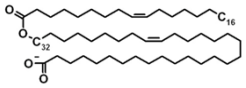
17β-estradiol



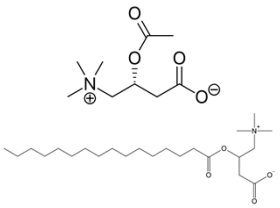
PGF2α



A phosphatidylcholine



A ω-hydroxy-fatty acid
fatty acid ester



Acetyl- and
palmitoylcarnitine

Source: Dr. Barnes' slides

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Sample preparation is important

- **Metabolites content**
- **Data quality**
- **Biological interpretation of results obtained**

Wawrzyniak et al., Scientific Rep. 2018

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Sample preparation

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

Mushtaq et al. Phytochem. Anal. 2014

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Biological samples

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

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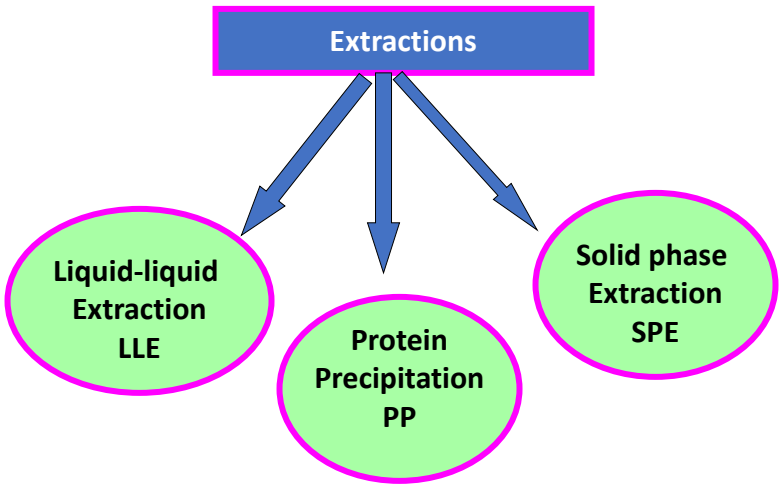

The method of choice will be determined by the sample matrix and the concentration of compounds In samples

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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
  - Spike with IS and add cold MeOH (-20^{o}C) and ice cold H20 (400 ul each 1:1 v/v)
  - Scrape the well with a cell scraper, and transfer the extract into an eppendorf tube containing 400 uL of CHCl3 (-20^{o}C) quenching/extraction
  - 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 ^{0}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 follow the similar procedure as described above for adherent cells. (quenching, extraction and separation of phases)

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

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Tissue – metabolite extraction

- **Tissue MUST BE snap-frozen (liq N_2) to prevent further metabolism**
- **Grind the tissue in a pestle and mortar**
  - Pre-cool in liq N_2
  - Pour powder as a slurry into extraction tube
  - Allow N_2 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

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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_2 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 $Fe^{2+/3+}$
- **Store in 1 ml aliquots at -80°C**
- **Small animals – mice, zebrafish – yield only µl volumes**

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Fecal collection and extraction

- **Considered as a functional readout of gut microbial activities**
- **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 N_2
  - 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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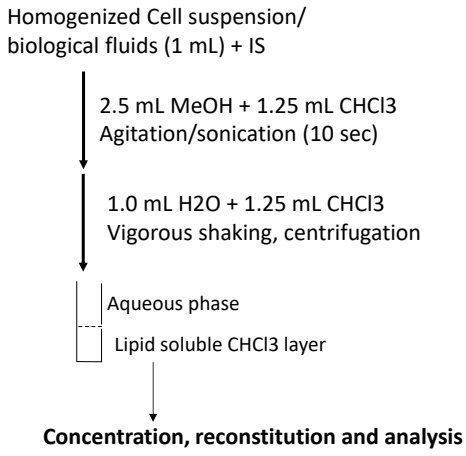
Extraction of lipids

Auto-oxidation and pH are two important issues

Bligh/Dyer extraction



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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

- Defined as the operational techniques and activities used to measure and report these quality requirements after data acquisition (*Broadhurst et al., Metabolomics 2018*).
- 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, make sure experiment method is working properly
- Negative controls- where no change is expected
- Sham controls- incidental effects induced by the procedure or operation as a control
- Vehicle controls

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_{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.

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Problems facing with extraction and analysis

- **Metabolite concentration range-** (Prostaglandins, estradiol, pM), (glucose, total cholesterol, 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 samples, but coming from various external sources during the sample preparation.**
  - **Detergents, plasticizers, solvent residues, column siloxanes**

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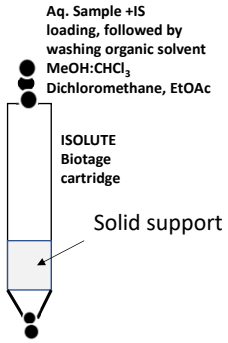
Supported Liquid Extraction (SLE)

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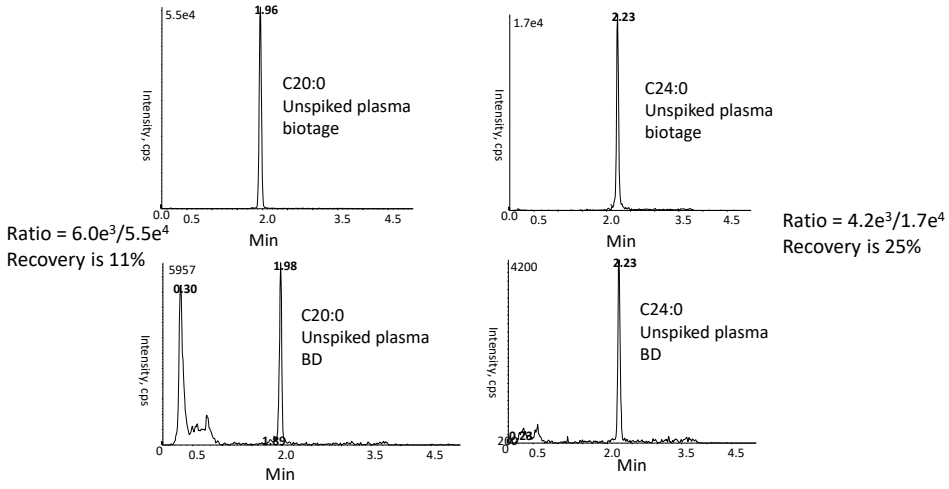
Quantitative analysis of ceramides;
Poor recoveries of non-polar ceramides in Bligh-Dyer (BD) liquid-liquid extraction compared to Biotage (supported liquid extraction)



Ratio = $6.0e^3/5.5e^4$
Recovery is 11%

Ratio = $4.2e^3/1.7e^4$
Recovery is 25%

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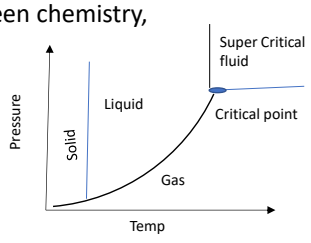
Supercritical Fluid Extraction (SFE)

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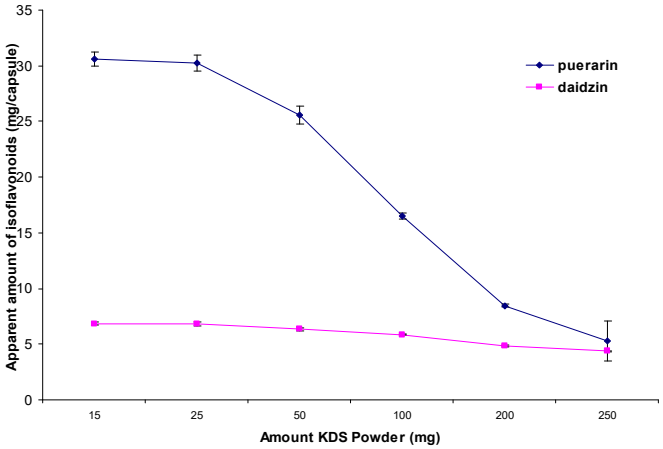
The ratio of botanical material to extracting solvent plays important role in efficient extraction of phytochemicals



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.**

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