THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Knowledge that will change your world

July 23, 2018

6th Annual UAB Metabolomics Workshop

Recovering the metabolome

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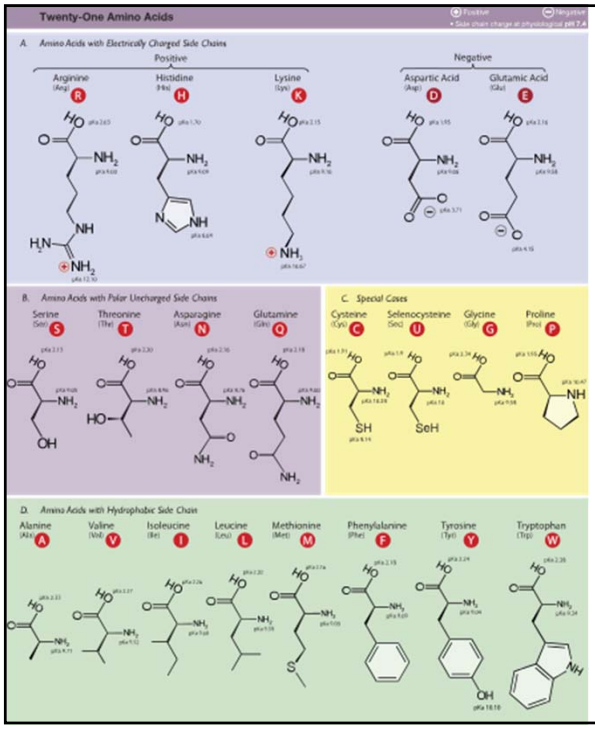
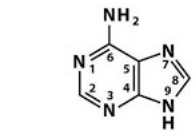
Synopsis

- **Comparison with the chemistry of proteins and DNA**
- **Samples**
  - Fluids, cells and tissues and "other" samples
- **Collection/storage**
  - Importance of timing/SOP, avoid plasticware
- **Extraction**
  - Keep cool (!), partitioning, pH, microwave, supercritical fluid
- **Standards**
  - Isotopes, related compounds, matrix effects
- **Sample clean up**
  - Solid phase, supported liquid phase

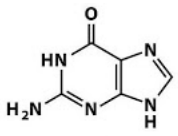


- Amino acids have similar backbones and side chains ranging from the hydrophobic aliphatic and aromatic groups to polar and charged groups.
- When assembled into proteins, many of the differences at the side chain level are largely averaged out.
- Proteins are separated by their mobility in SDS-PAGE gels (differences in MW) and their isoelectric points.
- In general, proteins can be extracted and analyzed using standard procedures.
  - MeOH, EtOH, MeCN
  - Sulfosalicylic acid
  - Trichloroacetic acid

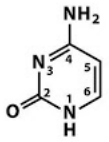
Chemistry of DNA bases



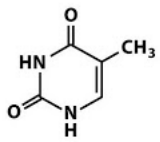
Adenine
(6-Aminopurine)



Guanine
(2-Amino-6-oxopurine)



Cytosine
(2-Oxo-4-aminopyrimidine)



Thymine
(2,4-Dioxo-5-methylpyrimidine)

- **Very little difference between the bases**
  - Just ± $-NH_2$ or –C=O and their positions
  - The sugar phosphate backbone is the same in DNA (deoxyribose), but different from RNA (ribose)
- DNA/RNA are recovered either with ice-cold EtOH, or selectively in the case of mRNA with oligoT

Conclusion – the recovery of proteins and DNA/RNA is straightforward

Chemistry of metabolites/metabolome

H_2

A gas
bp – 253°C

CH_3CHO

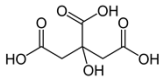
EtOH metabolite
bp 20°C

CH_3COCH_3

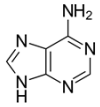
A vapor in diabetics
bp 56°C

$CH_3CH_2CH_2COOH$

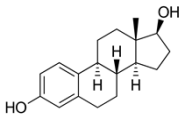
Epigenetic modifier
bp 164°C



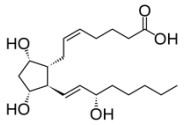
Citric acid



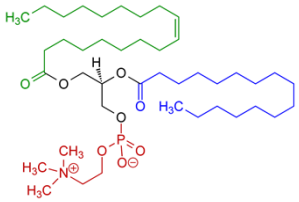
Adenine



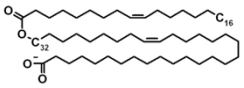
17β-estradiol



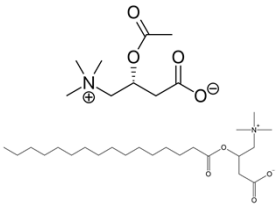
PGF2α



A phosphatidylcholine



A ω-hydroxy-fatty acid
fatty acid ester



Acetyl- and
palmitoylcarnitine

Conclusion

the metabolome is extremely diverse

Sampling the -omes

- **Germ-line DNA remains the same over a lifetime**
- **Somatic DNA may have modifications (limited), but they are stable**
- **mRNA is more dynamic**
- **Most proteins have long lifetimes**
  - PTMs can exhibit quick changes (30-60 sec) during signaling (phosphorylation/dephosphorylation)
- **Metabolites in bioenergetics have very short half-lives (seconds or sub-second for ATP)**
  - Need to freeze clamp
  - Chemical stability during extraction

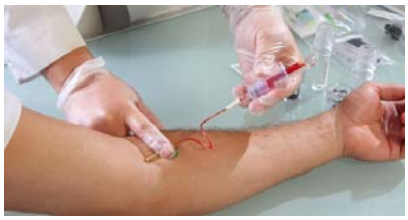
Metabolites from cells

- **Adherent cells in petri dish**
  - Prepare ice-cold physiologic saline
  - Tilt plate and remove medium with vacuum pipet
  - Immediately add 10 ml ice-cold physiologic saline, swirl and remove medium with vacuum pipet (less than 10 sec)
  - Add MeOH cooled in dry ice (-43^{o}C)
  - Incubate at 0-4^{o}C for 30 min
- **Suspended cells**
  - Rapidly filter through nylon membrane
  - Add MeOH cooled in dry ice (-43^{o}C) to the filter
  - Incubate at 0-4^{o}C for 30 min

Adapted from Kathleen Stringer
http://www.uab.edu/proteomics/metabolomics/workshop/2014/videos/stringer.html

Sample Collection

- **The first step in sample processing**
  - depends on the type of sample
  - depends on the source of the sample
    - clinical vs. experimental
- **Consistency is key**
  - uniformity of supplies
  - standard operating procedures (SOP)
  - prospective collection vs. samples of convenience

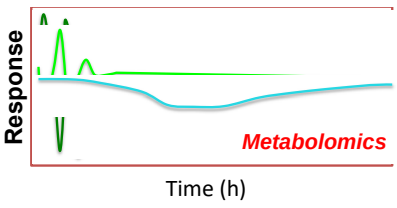


- **Universal "standards" do not yet exist but will be driven by the advancement of metabolomics technology**

image from www.usada.org

Adapted from Kathleen Stringer
http://www.uab.edu/proteomics/metabolomics/workshop/2014/videos/stringer.html

Sample Collection



- **Variables to consider**:
  - time of day and circadian variation
  - gender and age of subjects (mammalian)
  - diet, hydration, fasting state, exercise/activity

- **Collection vessel**
  - glass vs. plastic
  - laboratory vs. clinic
  - presently there are no "metabolomics tubes"

image adapted in part from D. Wishart, Bioinformatics.ca; June 13, 2011 under a creative commons license

Slupsky, CM., et al. Anal Chem 2007;79:6995-7004
Park, Y., et al. Am J Physiol Regul Integr Comp Physiol 2009;297:R202-9

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**

Methanol:Chloroform Extraction

Whole Blood Extraction SOP

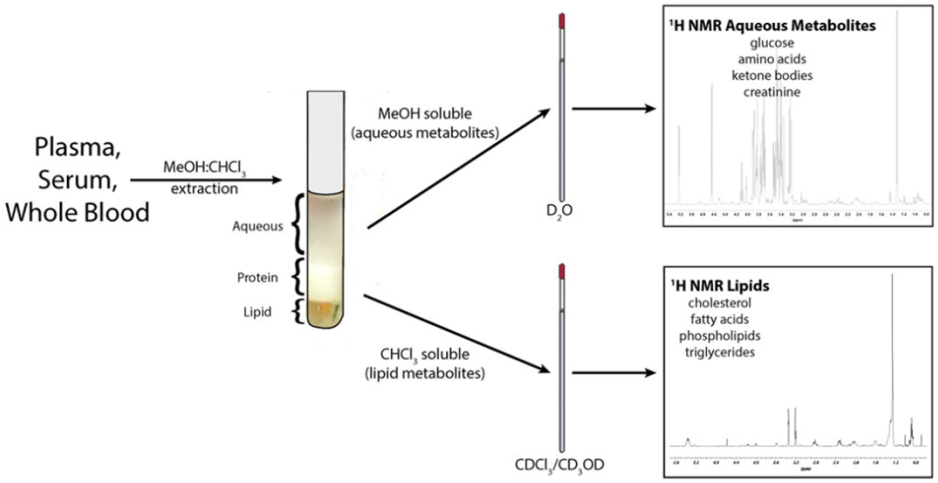
- **<u>Biomaterials Required</u>**:
  - ~0.5 to 1.0 mL plasma/serum or whole blood (per sample) collected with heparin*

Preservative will vary depending on planned analytical platform

- **<u>Other reagents and solutions</u>**:
  - Methanol and chloroform (reagent or HPLC grade)
    - mix 1:1 (vol/vol) fresh in a tightly sealed (Corning screw top) bottle that has been pre-cooled (-20°C)
    - store mixture (-20°C) so it is ice-cold when ready for use
  - Ice-cold DI water

Adapted from Kathleen Stringer
http://www.uab.edu/proteomics/metabolomics/workshop/2014/videos/stringer.html

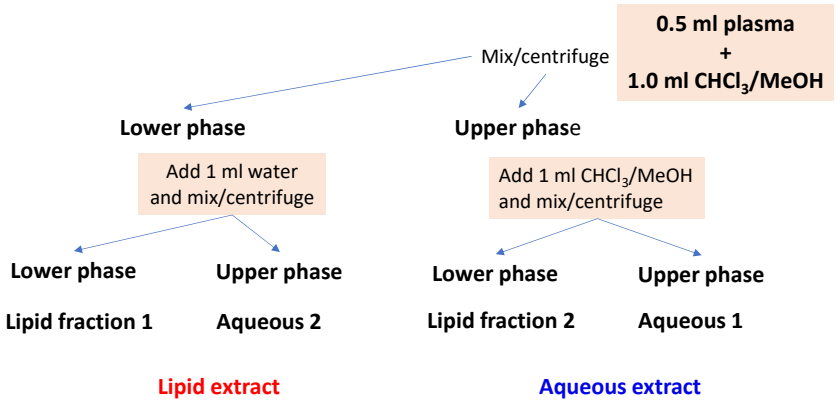
Chloroform-methanol extraction



Further extraction conditions

- **A fuller account of this method is given by Kathleen Stringer at the 2nd UAB Metabolomics Workshop**

http://www.uab.edu/proteomics/metabolomics/workshop/2014/videos/stringer.html



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 N_2 and re-dissolved in water

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

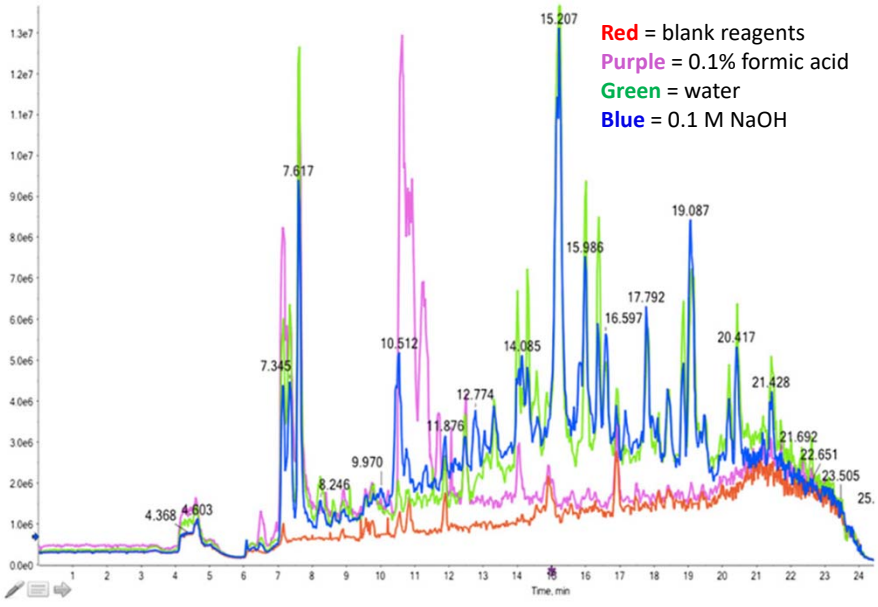
Fecal collection

- **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

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

Importance of pH



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.

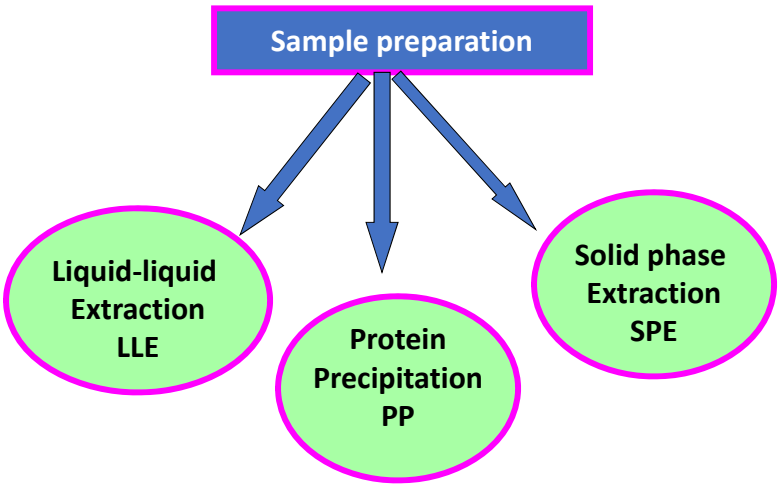
Problems facing with extraction and analysis

- **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

Objective of sample preparation for metabolomics

- Non-selective/selective- high metabolite coverage of a biological sample (~8500 endogenous and 40,000 exogenous metabolites human metabolomes)
- Retaining of analytes and removal of undesirable matrix components
- Pre-concentration step
- Simple, rapid, reproducible and quantitative recovery of metabolites

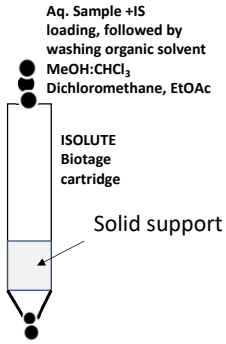
Sample preparation is a crucial step in removing the interfering compounds from biological matrix



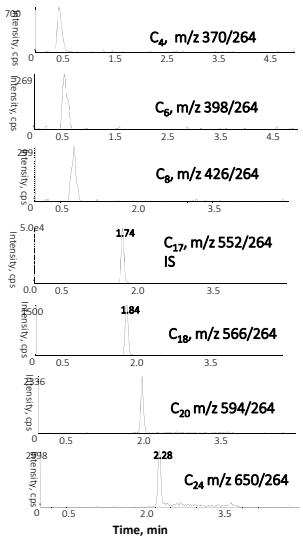
The method of choice will be determined by the sample matrix and the concentration of compounds In samples

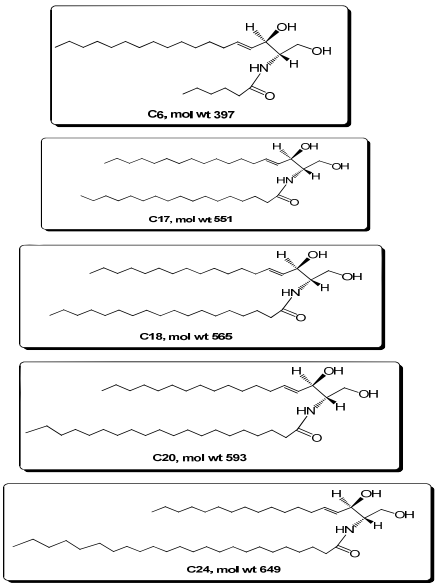
Supported Liquid Extraction (SLE)

- 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

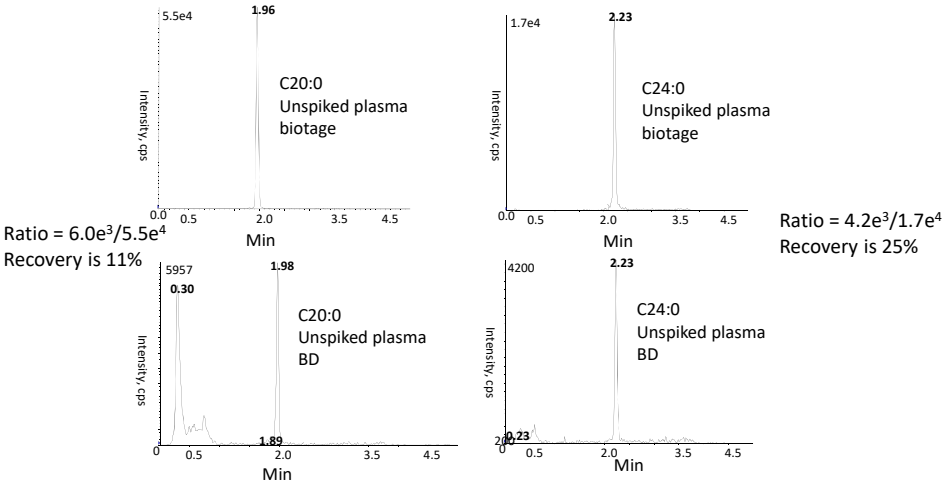


Targeted analysis of ceramides-MRM chromatograms showing simultaneous determination of ceramides (C_4-C_{24})





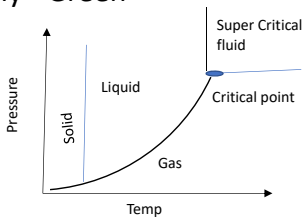
Sample preparation is a crucial step in quantitative analysis of ceramides; Poor recoveries of non-polar ceramides in Bligh-Dyer (BD) liquid-liquid extraction compared to Biotage (supported liquid extraction)



Supercritical Fluid Extraction (SFE)

Extraction of bioactive natural products

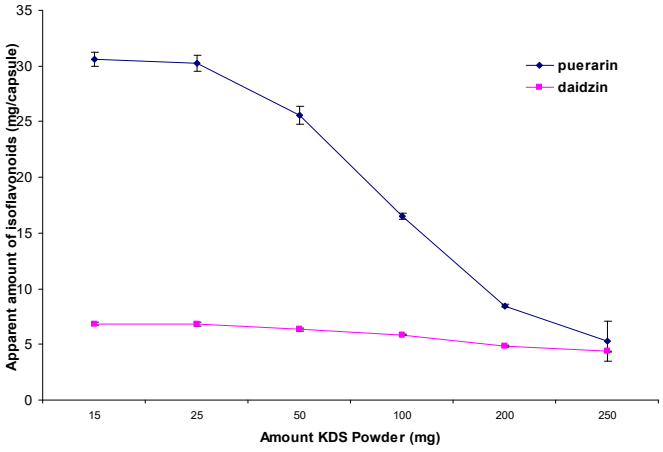
- Extraction method involving the use of supercritical solvent in extracting non-polar to moderately polar analytes from solid matrices
- Use of solvents above the critical conditions for temperature and pressure - super critical carbon dioxide
- Able to penetrate solid matrix (botanical products) and 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 ovens**

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

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.**

Questions?