NMR Metabolomics Analysis

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Adapted from slides previously prepared by Drs. Wimal Pathmasiri and Delisha Stewart

NIH Common Fund Eastern Regional Comprehensive Metabolomics Resource Core (ERCMRC)

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Outline of Today's Training

- Introduction:
- NMR Metabolomics:
  - Study Design
  - Sample Preparation
  - Data Acquisition
  - Data Pre-processing
  - Statistical Analysis
  - Library Matching
  - Pathway Analysis

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NMR Metabolomics

- Broad Spectrum
  - High throughput
  - NMR Binning
  - Multivariate analysis and other statistics
  - Identifying bins important for separating study groups
  - Library matching of bins to metabolites
- Targeted Metabolomics
  - Identifying a set of metabolites
  - Quantifying metabolites
  - Multivariate analysis and other statistics
- Pathway analysis
  - Use identified metabolites
  - Use other omics data for integrated analysis

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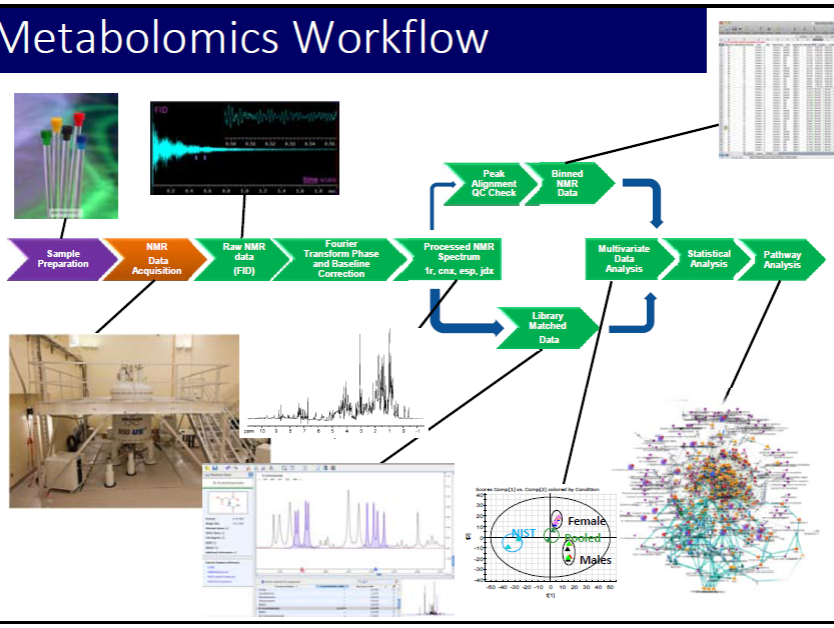
NMR Metabolomics Workflow



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Free Software available for NMR Metabolomics

- NMR Data Processing
  - ACD Software for Academics (ACD Labs, Toronto, Canada)
- Multivariate data analysis
  - MetaboAnalyst 3.0 (http://www.metaboanalyst.ca)
  - MetATT (http://metatt.metabolomics.ca/MetATT/)
  - MUMA (http://www.biomolnmr.org/software.html)
  - Other R-packages
- Library matching and Identification
  - BATMAN (Imperial College), Bayesil (David Wishart lab)
  - Use of databases
    - Birmingham Metabolite library, HMDB, BMRB
- Pathway analysis
  - Metaboanalyst, metaP Server, Met-PA, Cytoscape, KEGG, IMPALA

Also available through www.metabolomicsworkbench.org

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Other Software available for NMR Metabolomics

COMMERCIAL

- NMR Data-preprocessing
  - ACD Software (ACD Labs, Toronto, Canada)
  - Chenomx NMR Suite 8.1 Professional
- Multivariate data analysis
  - SIMCA 14
- Other statistical analysis
  - SAS, SPSS
- Library matching and quantification
  - Chenomx NMR Suite 8.1 Professional
- Pathway analysis
  - GeneGo (MetaCore Module)
  - Ingenuity Pathway Analysis (IPA)

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Sample Preparation, Data Acquisition, and Pre-processing

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Important Steps in Metabolomics Analysis

- Study design Considerations
  - Factors such as gender, ethnicity, age, BMI (human studies)
  - Species, strains, feed, housing (animal studies)
- Sample collection
  - Collection vials, anticoagulant use (heparin, citrate, EDTA)
- Sample storage
  - -80 °C is optimal, minimize freeze-thaw cycles
  - -20 °C is sometimes more practical (i.e. field studies)
- Sample preparation
  - Optimize the methods and use them consistently throughout study
  - Daily balance and pipette checks
- Use Quality Check (QC) samples
  - Pooled QC samples (Phenotypic and combined pooled samples)
  - Use matching external pooled QC samples where pool samples cannot be prepared from study samples
- **Optimize all procedures and use them consistently throughout the study**

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Check the samples and the Metadata

- **Why are these serum samples straw colored?**
  - Are these samples actually plasma or urine?
- **Why are there more samples in the box than listed on the inventory emailed?**
  - The wrong box was pulled from their biorepository and shipped.
- **There is only 3 pieces of dry ice in this box!**
  - Did they really pack these "precious samples" in a way to risk them thawing?
- **Check every label on the samples shipped to verify they match the inventory.**
  - Most sample labels will match, but the wrong tubes can get pulled meaning the right samples were not shipped.
  - Sometimes hand-written labels are illegible and will require further communication to verify the sample ID.
- **Check the metadata.**
  - Did they really send us female controls to compare with male cases?
- **Communicate sample and metadata discrepancies/issues immediately.**
  - Use of pictures here can be very helpful.

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Sample Preparation for Metabolomics Analysis

Current sample preparation practices (in brief)

- **Biofluids**
  - Dilute with D_2O/ buffer/ 0.9% Saline
  - Add internal standard (ISTD, eg. Chenomx) solution or formate (for serum).
  - Centrifuge and transfer an aliquot into NMR tube
- **Tissue and Cells**
  - Homogenization performed in ice cold 50/50 acetonitrile/water
  - Supernatant dried down (lyophilized)
  - Reconstituted in D_2O and ISTD (eg. Chenomx) solution
- **Pooled QC Samples (Sample Unlimited)**
  - Mix equal volume of study samples to get pooled QC samples
  - 10% QC samples
- **Pooled QC Samples (Sample Limited)**
  - Use independent pool of similar samples
  - 10% QC samples
- **Daily balance and pipette check**

Samples are randomized for preparation and data acquisition

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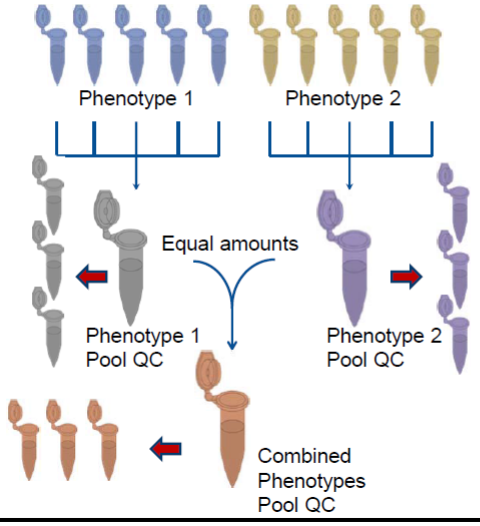
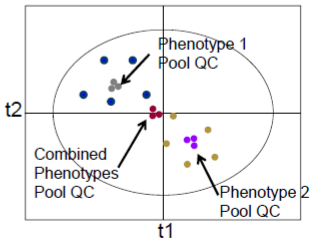
Preparing Pooled QC Samples



- Aliquots from each sample in the study phenotype are pooled (phenotypic pool)
- Equal amount of each phenotypic pools are pooled (Combined phenotypic pool)
- Replicates of pools are prepared
- Pool samples are prepared along with the study samples



Pooled samples should cluster tightly

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NMR Data Acquisition

- 1D NMR
  - 1st increment of NOESY
    - noesyprid (Bruker)
  - CPMG (serum or plasma)
    - cpmgpr1d (Bruker)
    - To remove broadening of signals due to macromolecules (eg. Proteins and lipids)
- 2D NMR (for structure elucidation)
  - 2D J-Resolved
  - COSY
  - TOCSY
  - HSQC
  - HMBC

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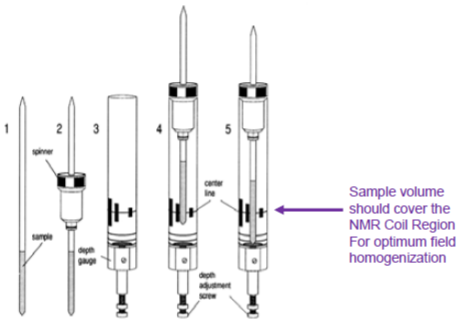
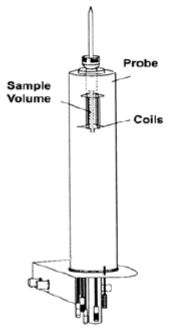
Sample Amount in NMR tube



Sample volume should cover the NMR Coil Region For optimum field homogenization



- At least 10% D_2O in the sample
- Optimum volume
  - 550 – 600 uL (5mm tube)
  - 200 uL (3 mm tube)
- Sample gauge is used

For very small sample amounts, a NMR with a microcoil probe is an option.

AVANCE Beginners User Guide 004 (Bruker, Germany)

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NMR Data

- A typical 1H NMR Spectrum consists of thousands of sharp lines or signals.
- The intensity of the peak is directly related to the number of protons underlying the peak.
- The position of a particular peak in the X-axis of the NMR spectrum is called the "Chemical Shift" and it is measured in ppm scale
- The NMR spectrum obtained for the biological sample is referenced using a reference compound such as DSS, TSP, or Formate added to the sample in sample preparation step.
- pH indicator may also be used (for example, Imidazole)

DSS=4,4-dimethyl-4-silapentane-1-sulfonic acid, TSP=Trimethylsilyl propionate

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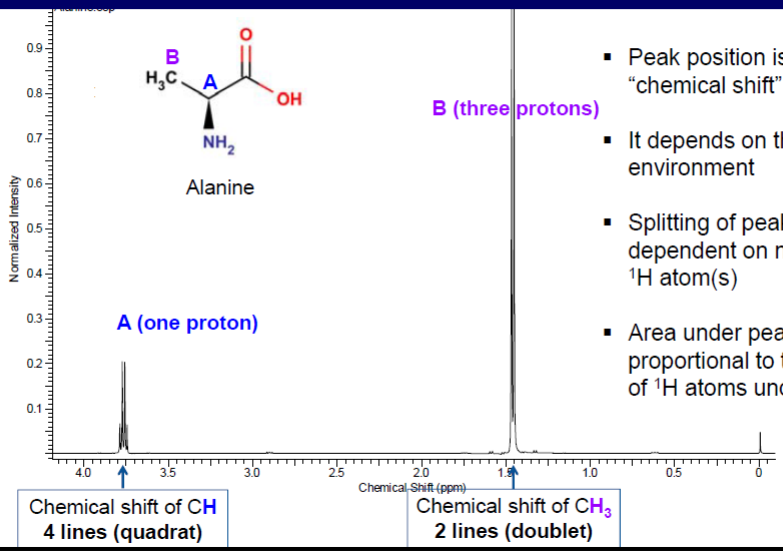
^{1}H NMR Spectrum for Alanine



- Peak position is called "chemical shift"
- It depends on the chemical environment
- Splitting of peak is dependent on neighboring ^{1}H atom(s)
- Area under peak proportional to the number of ^{1}H atoms underlying it

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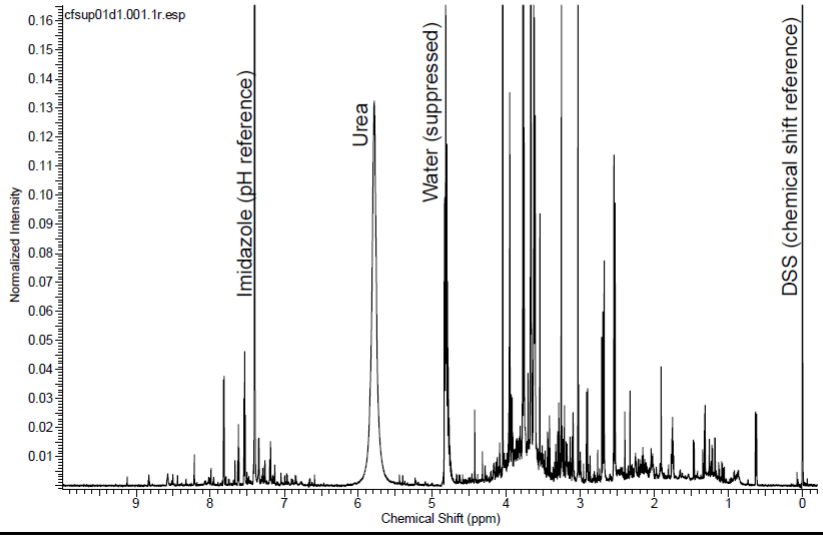
Typical ^{1}H NMR Spectrum of Urine



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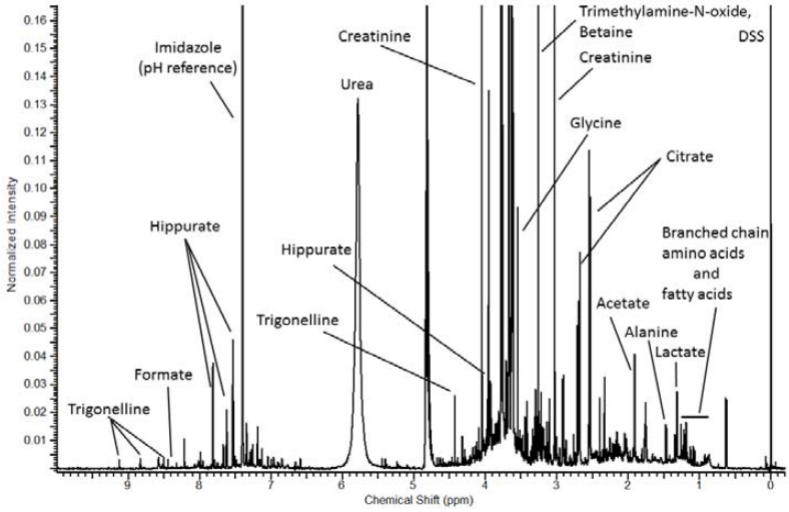
Typical ^{1}H NMR Spectrum of Urine



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Collecting NMR Data at UAB

- Metabolomics samples can be submitted to the Central Alabama High-Field NMR Facility for spectral acquisition
- Cost is $14/sample for standard 1D collection
- Turnaround time varies, but if coordinated in advance is usually less than 48 hours
- Contact Will Placzek (placzek@uab.edu)

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Moving from Raw data to sample analysis

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Data Pre-processing

- After NMR data acquisition, the result is a set of spectra for all samples.
- For each spectrum, quality of the spectra should be assessed.
  - Line shape, Phase, Baseline
- Spectra should be referenced
  - Compounds commonly used: DSS, TSP, Formate
- Variations of pH, ionic strength of samples has effects on chemical shift
  - Peak alignment
  - Binning or Bucket integration

High quality data are needed

- Remove unwanted regions
- Normalize data (remove variation in concentration of samples)

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Quality Control Steps

- Quality of metabolomics analysis depends on data quality
- Typical problems
  - Water peak (suppression issues)
  - Baseline (not set at zero and not a flat line)
  - Alignment of peaks (chemical shift, due to pH variation)
  - Variation in concentration (eg. Urine)
- High quality of data is needed for best results

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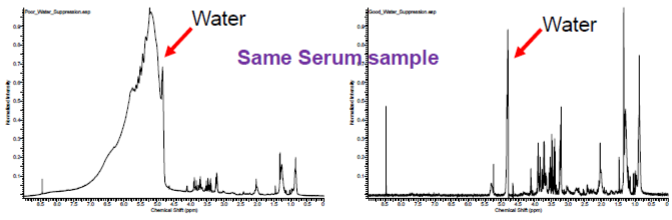
Water Suppression Effects and Other Artifacts

- If water is not correctly suppressed or removed there will be effects on normalization
- Need to remove other artifacts
- Remove drug or drug metabolites



Poor water suppression Good water suppression

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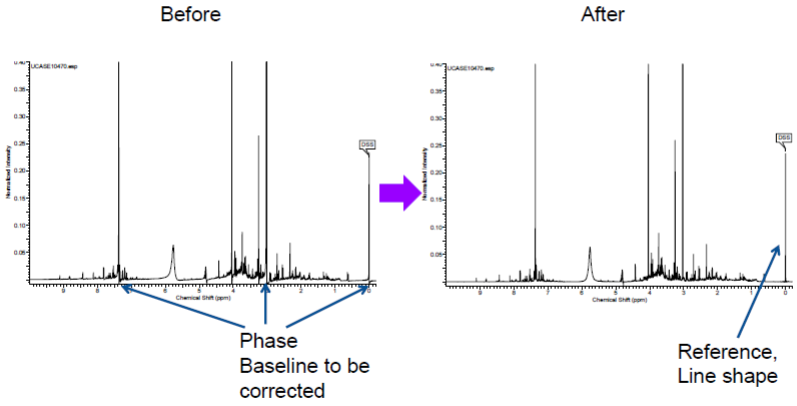
NMR Pre-processing



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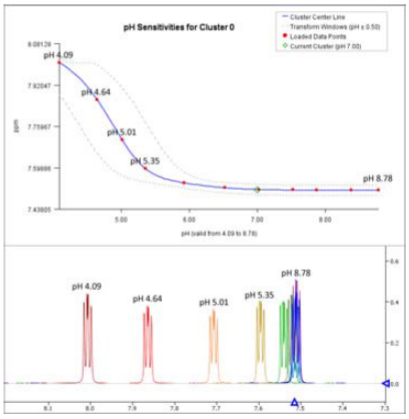
pH Dependence of Chemical Shift

Chemical shift variability
- pH
- ionic strength
- metal concentration

Methods to overcome this problem
- Use a buffer when preparing samples
- Binning (Bucketing)
  - Fixed binning
  - Intelligent binning
  - Optimized binning
- Available data alignment tools
  - Recursive Segment-wise Peak Alignment (RSPA)
  - Icoshift
  - speaq



http://www.chenomx.com/software/software.php
Savorani , F. et al, Journal of Magnetic Resonance, Volume 202, Issue 2, 2010, 190 – 202
Vu, T. N. et al., *BMC Bioinformatics* 2011, 12:405

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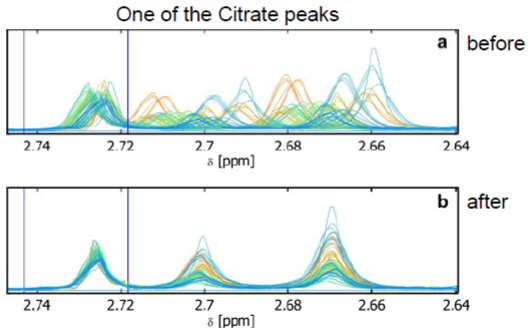
Peak Alignment

Example

icoshift



Savorani , F. et al, Journal of Magnetic Resonance, Volume 202, Issue 2, 2010, 190 - 202

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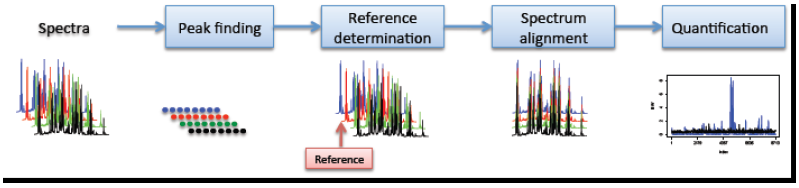
Peak Alignment

Example

speaq



Vu, T. N. et al., *BMC Bioinformatics* 2011, **12**:405

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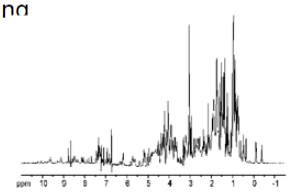
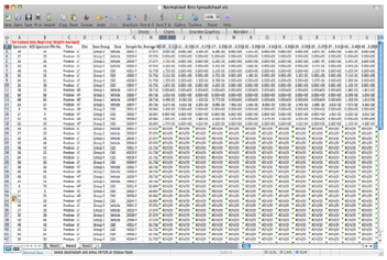
NMR Binning

- A form of quantification that consists of segmenting a spectrum into small areas (bins/buckets) and attaining an integral value for that segment
- Binning attempts to minimize effects from variations in peak positions caused by pH, ionic strength, and other factors.
- Two main types of binning
  - Fixed binning
  - Flexible binning





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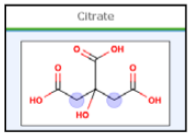
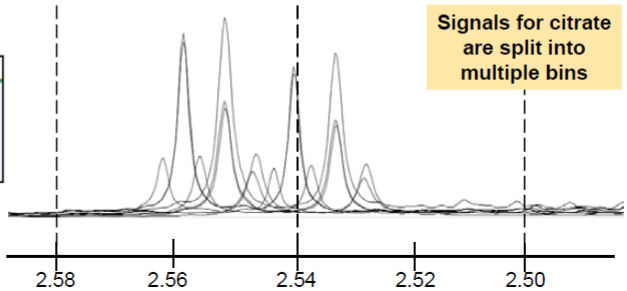
NMR Binning

Peak shift can cause the same peak across multiple samples to fall into different bins

- The entire NMR spectrum is split into evenly spaced integral regions with a spectral window of typically 0.04 ppm.
- The major drawback of fixed binning is the non-flexibility of the boundaries.
- If a peak crosses the border between two bins it can significantly influence your data analysis



Fixed Binning



Signals for citrate are split into multiple bins

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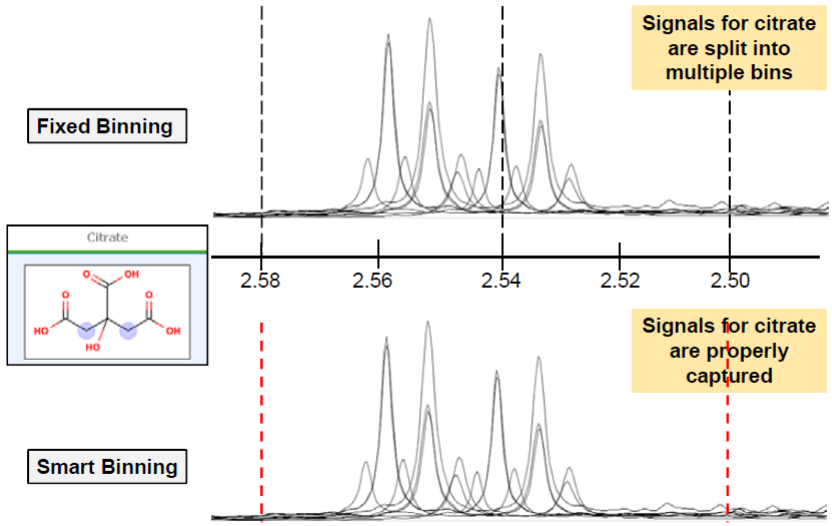
NMR Binning



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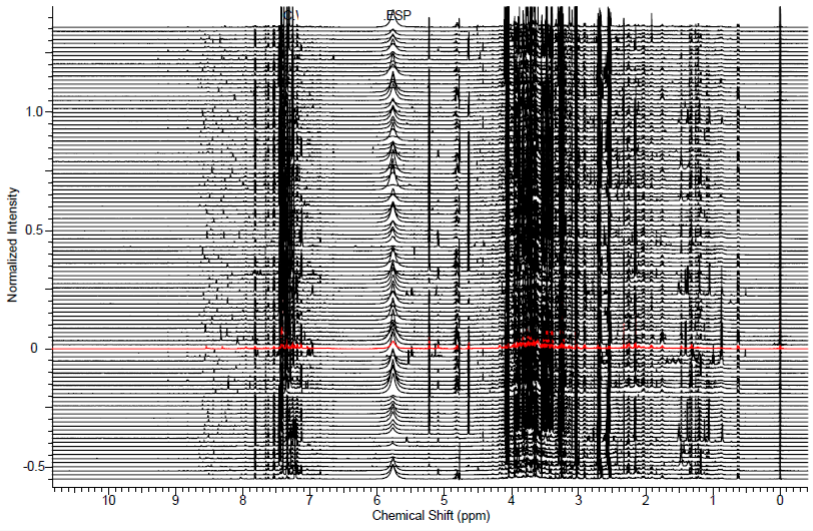
NMR Binning



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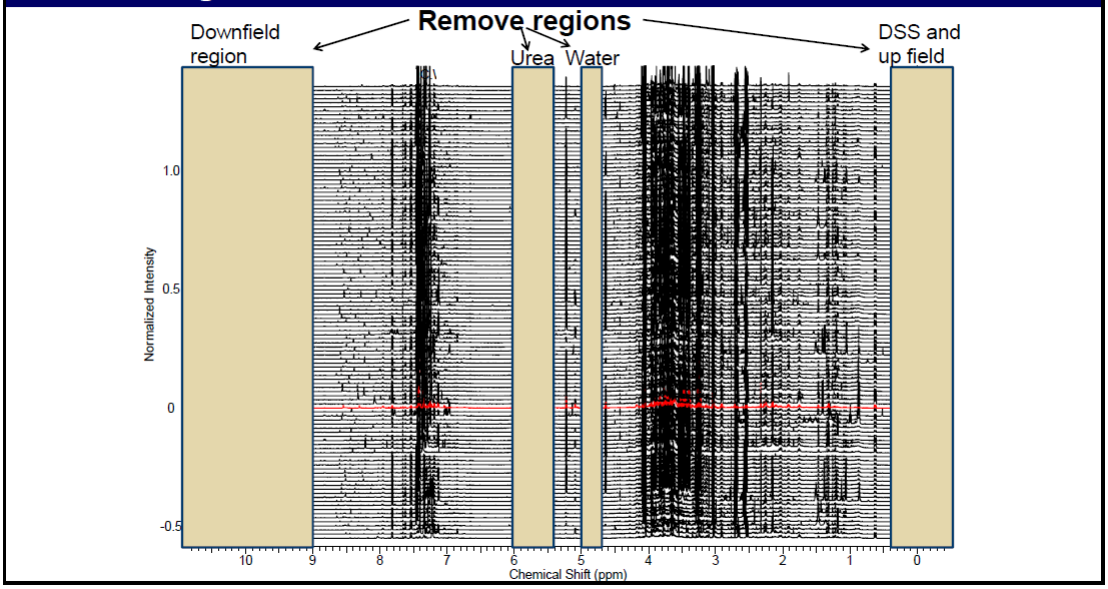
NMR Binning



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NMR Binning

- Integrate bins (0.04 ppm bin size)
- Normalize integral of each bin to the total integral of each spectrum
- Merge metadata
- Result is a spreadsheet ready for further multivariate data analysis and other statistical analysis

Sample ID	Disease Group	[0.40 .. 0.46]	[0.46 .. 0.52]	[0.52 .. 0.54]	[0.54 .. 0.57]	[0.57 .. 0.60]	[0.60 .. 0.66]	[0.66 .. 0.68]	[0.68 .. 0.71]	[0.71 .. 0.75]
C0559	Cases	7.60E-05	0.00E+00	7.32E-02	8.48E-02	3.20E-02	1.84E+00	1.31E-01	3.60E-01	3.67E-01
C0629	Cases	0.00E+00	1.78E-02	0.00E+00	2.18E-02	0.00E+00	1.08E+01	0.00E+00	0.00E+00	3.02E-02
C0640	Cases	3.44E-04	0.00E+00	1.83E-03	1.86E-04	0.00E+00	4.51E+00	0.00E+00	0.00E+00	0.00E+00
C0835	Cases	6.41E-04	0.00E+00	6.44E-03	0.00E+00	3.96E-03	3.28E+00	0.00E+00	5.12E-03	1.75E-02
D0613	Cases	6.63E-03	0.00E+00	0.00E+00	1.06E-02	0.00E+00	5.79E+00	0.00E+00	6.36E-02	3.02E-01
D0762	Cases	0.00E+00	0.00E+00	1.79E-02	1.98E-02	0.00E+00	9.37E+00	0.00E+00	0.00E+00	1.74E-02
D1113	Cases	3.14E-03	2.42E-03	8.02E-02	1.04E-01	5.32E-03	3.74E+00	0.00E+00	2.02E-02	1.84E-01
D1158	Cases	0.00E+00	3.71E-03	2.35E-02	4.83E-02	0.00E+00	5.02E+00	0.00E+00	1.91E-02	0.00E+00
D2090	Cases	0.00E+00	0.00E+00	2.45E-03	9.98E-04	0.00E+00	5.76E+00	0.00E+00	1.24E-02	1.04E-02
E0004	Cases	1.72E-03	0.00E+00	6.85E-02	3.05E-02	0.00E+00	1.47E+00	6.90E-02	3.61E-01	4.08E-01
E0195	Cases	0.00E+00	1.69E-03	5.57E-02	6.29E-02	0.00E+00	2.77E+00	1.34E-01	2.04E-01	4.56E-01
E0363	Cases	1.25E-03	0.00E+00	1.00E-02	1.00E-02	0.00E+00	9.17E+00	0.00E+00	1.08E-02	2.30E-02
E0369	Cases	4.11E-03	0.00E+00	2.23E-02	7.44E-02	3.08E-03	3.41E+00	0.00E+00	3.28E-02	9.09E-01
E0487	Cases	1.72E-03	0.00E+00	0.00E+00	1.00E-02	0.00E+00	4.00E+00	0.00E+00	1.36E-02	0.00E+00
F0036	Cases	1.66E-02	0.00E+00	0.00E+00	2.06E-02	0.00E+00	1.22E+01	1.04E-02	0.00E+00	5.97E-01
F0108	Cases	0.00E+00	2.31E-03	6.30E-03	1.11E-02	0.00E+00	7.17E+00	0.00E+00	1.65E-02	2.21E-01
A0233	Control	0.00E+00	1.86E-02	0.00E+00	1.82E-02	0.00E+00	1.61E+01	0.00E+00	2.91E-03	0.00E+00
A0490	Control	0.00E+00	0.00E+00	2.99E-03	3.60E-02	0.00E+00	2.97E+00	0.00E+00	4.00E-02	5.46E-01
A2003	Control	0.00E+00	0.00E+00	3.45E-02	2.20E-02	0.00E+00	1.80E+00	0.00E+00	0.00E+00	0.00E+00
C0586	Control	0.00E+00	1.69E-02	0.00E+00	6.64E-03	0.00E+00	1.92E+01	0.00E+00	6.51E-02	0.00E+00
C2177	Control	0.00E+00	0.00E+00	3.02E-02	3.59E-02	0.00E+00	2.35E+00	0.00E+00	3.19E-02	1.49E-01
D0177	Control	9.21E-03	0.00E+00	1.69E-02	1.47E-02	0.00E+00	2.43E+00	0.00E+00	4.46E-02	0.00E+00
D0729	Control	0.00E+00	1.88E-03	5.58E-02	7.87E-02	2.92E-02	3.16E+00	6.59E-02	2.80E-01	4.30E-01
D0909	Control	0.00E+00	1.08E-03	0.00E+00	5.69E-03	0.00E+00	2.49E+00	0.00E+00	1.01E-02	1.87E-01
D0945	Control	0.00E+00	4.79E-04	7.00E-03	0.00E+00	4.19E-03	3.99E+00	0.00E+00	1.11E-03	3.98E-02
D1174	Control	0.00E+00	9.33E-04	0.00E+00	3.43E-03	1.30E-02	7.21E+00	6.53E-03	0.00E+00	1.66E-02
D2054	Control	1.55E-03	0.00E+00	0.00E+00	1.22E-02	0.00E+00	2.07E+00	0.00E+00	1.28E-02	3.90E-01
D2062	Control	2.39E-05	0.00E+00	6.04E-02	2.99E-02	0.00E+00	4.94E+00	0.00E+00	9.95E-03	0.00E+00
D2079	Control	2.73E-02	0.00E+00	1.81E-03	1.17E-02	0.00E+00	3.38E+01	7.87E-02	0.00E+00	5.91E+00

Metadata

Normalized binned data

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Data Normalization, Transformation, and Scaling

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Normalization

- Normalization reduces the sample to sample variability due to differences in sample concentrations—particularly important when the matrix is urine
  - Normalization to total intensity is the most common method
    - For each sample, divide the individual bin integral by the total integrated intensity
  - Other Methods
    - Normalize to a peak that is always present in the same concentration, for example normalizing to creatinine
    - Probabilistic quotient normalization
    - Quantile and cubic spline normalization

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Centering, Scaling, and Transformations

I Centering $\tilde{x}_{ij} = x_{ij} - \bar{x}_i$

II Autoscaling $\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{s_i}$

Range scaling $\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\left(x_{i_{max}} - x_{i_{min}}\right)}$

Pareto scaling $\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{s_i}}$

Vast scaling $\tilde{x}_{ij} = \frac{\left(x_{ij} - \bar{x}_i\right)}{s_i} \cdot \frac{\bar{x}_i}{s_i}$

Level scaling $\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\bar{x}_i}$

III Log transformation $\tilde{x}_{ij} = {}^{10}\log\left(x_{ij}\right)$ $\tilde{\tilde{x}}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$

Power transformation $\tilde{x}_{ij} = \sqrt{\left(x_{ij}\right)}$ $\tilde{\tilde{x}}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$

Analysis results vary depending on the scaling/ transformation methods used.

Van den Berg et al 1006, BMC Genomics, 7, 142

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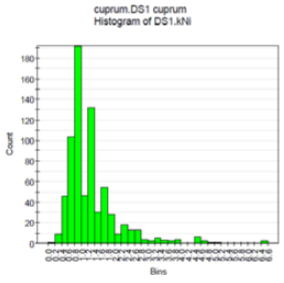
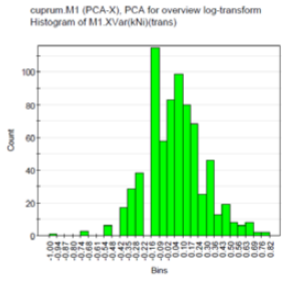
Data Transformation

- Before transformation
  - skew distribution



- After log-transformation
  - More close to normal distribution



Susan Wicklund, Multivariate data analysis for omics, Sept 2-3 2008, Umetrics training

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Scaling

- Unit variance (autoscaling) divides the bin intensity by the standard deviation
  - May increase your baseline noise
  - Dimensionless value after scaling
- Pareto scaling divides the bin intensity by the square root of the standard deviation
  - Not dimensionless after scaling
- For NMR data, centering with pareto scaling is commonly used

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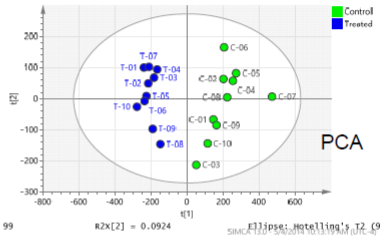
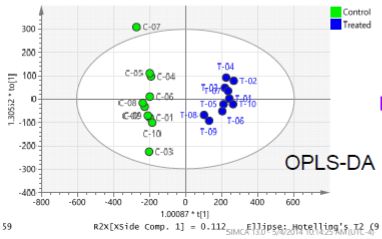
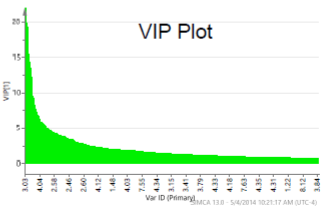
Multivariate Data Analysis and Other Statistical Analyses

- Mean centered and scaled data
- Non-supervised analysis
  - Principal component analysis (PCA)
- Supervised analysis
  - PLS-DA and OPLS-DA
- Loadings plots and VIP Plots to identify discriminatory bins
- p-Value, fold change







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Library Matching
Pathway Analysis

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Chenomx Library

- Over 320 metabolites
- pH sensitive library of ^{1}H NMR Spectra
- Customizable

1,3-Dihydroxyacetone, 1,3-Dimethylurate, 1,6-Anhydro-β-D-glucose, 1,7-Dimethylxanthine, 1-Methylnicotinamide, 2'-Deoxyadenosine, 2'-Deoxyguanosine, 2'-Deoxyinosine, 2-Aminoadipate, 2-Aminobutyrate, 2-Ethylacrylate, 2-Furoate, 2-Hydroxy-3-methylvalerate, 2-Hydroxybutyrate, 2-Hydroxyglutarate, 2-Hydroxyisobutyrate, 2-Hydroxyisocaproate, 2-Hydroxyisovalerate, 2-Hydroxyphenylacetate, 2-Hydroxyvalerate, 2-Methylglutarate, 2-Octenoate, 2-Oxobutyrate, 2-Oxocaproate, 2-[illegible]ate, 2-Phosphoglycerate, 3,4-Dihydroxymandelate, 3,5-Dibromotyrosine, 3-Aminoisobutyrate, 3-Chlorotyrosine, 3-Hydroxy-3-methylglutarate, 3-Hydroxybutyrate, 3-Hydroxyisovalerate, 3-Hydroxymandelate, 3-Hydroxyphenylacetate, 3-Indoxylsulfate, 3-Methyl-2-oxovalerate, 3-Methyladipate, 3-Methylxanthine, 3-Phenyllactate, 3-Phenylpropionate, 4-Aminobutyrate, 4-Aminohippurate, 4-Hydroxy-3-methoxymandelate, [illegible], 4-Hydroxyphenyllactate, 4-Pyridoxate, 5,6-Dihydrothymine, 5,6-Dihydrouracil, 5-Aminolevulinate, 5-Hydroxyindole-3-acetate, 5-Hydroxylysine, 5-Methoxysalicylate, Acetaldehyde, Acetamide, Acetaminophen, Acetate, Acetoacetate, Acetone, Acetylsalicylate, Adenine, Adenosine, Adipate, Alanine, Allantoin, Alloisoleucine, Anserine, Arginine, Argininosuccinate, Asparagine, Aspartate, Benzoate, Betaine, [illegible], Caffeine, Caprate, Caprylate, Carnitine, Carnosine, Choline, Cinnamate, Citrate, Citrulline, Creatine, Creatinine, Cysteine, Cystine, Cytidine, Cytosine, DSS (Chemical Shift Indicator), Dimethylamine, Epicatechin, Ethanol, Ethanolamine, Ethylene glycol, Ethylmalonate, Ferulate, Formate, Fructose, Fucose, Fumarate, Galactarate, Galactitol, Galactonate, Galactose, Gentisate, Glucarate, Glucose, Glutamate, Glutamine, Glutarate, Glutaric acid monomethyl ester, Glutathione, Glycerate, Glycerol, Glycine, Glycolate, Glycylproline, Guanidoacetate, Guanine, Hippurate, Histidine, Homocitrulline, Homocystine, Homogentisate, Homoserine, Homovanillate, Hypoxanthine, Ibuprofen, Imidazole, Indole-3-acetate, Inosine, Isobutyrate, Isocaproate, Isocitrate, Isoleucine, Isopropanol, Isovalerate, Kynurenate, Kynurenine, Lactate, Lactose, Leucine, Levulinate, Lysine, Malate, Maleate, Malonate, Mannitol, Mannose, Methanol, Methionine, Methylamine, Methylguanidine, Methylmalonate, Methylsuccinate, N,N-Dimethylformamide, N,N-Dimethylglycine, N-Acetylaspartate, N-Acetylglutamate, N-Acetylglycine, N-Carbamoyl-β-alanine, N-Carbamoylaspartate, N-Isovaleroylglycine, NAD+, Niacinamide, Nicotinate, O-Acetylcarnitine, O-Phosphocholine, O-Phosphoethanolamine, O-Phosphoserine, Ornithine, Oxalacetate, Oxypurinol, Pantothenate, Phenol, Phenylacetate, Phenylacetylglycine, Phenylalanine, Pimelate, Proline, Propionate, Propylene glycol, Protocatechuate, Pyridoxine, Pyroglutamate, Pyruvate, Quinolinate, Riboflavin, Ribose, S-Adenosylhomocysteine, S-Sulfocysteine, Salicylate, Salicylurate, Sarcosine, Serine, Suberate, Succinate, Succinylacetone, Sucrose, Tartrate, Taurine, Theophylline, Threonate, Threonine, Thymine, Thymol, Tiglylglycine, Trigonelline, Trimethylamine, Trimethylamine N-oxide, Tryptophan, Tyramine, Tyrosine, Uracil, Urea, Uridine, Urocanate, Valerate, Valine, Valproate, Vanillate, Xanthine, Xanthosine, Xylose, cis-Aconitate, myo-Inositol, o-Cresol, p-Cresol, trans-4-Hydroxy-L-proline, trans-Aconitate, β-Alanine, π-Methylhistidine, τ-Methylhistidine

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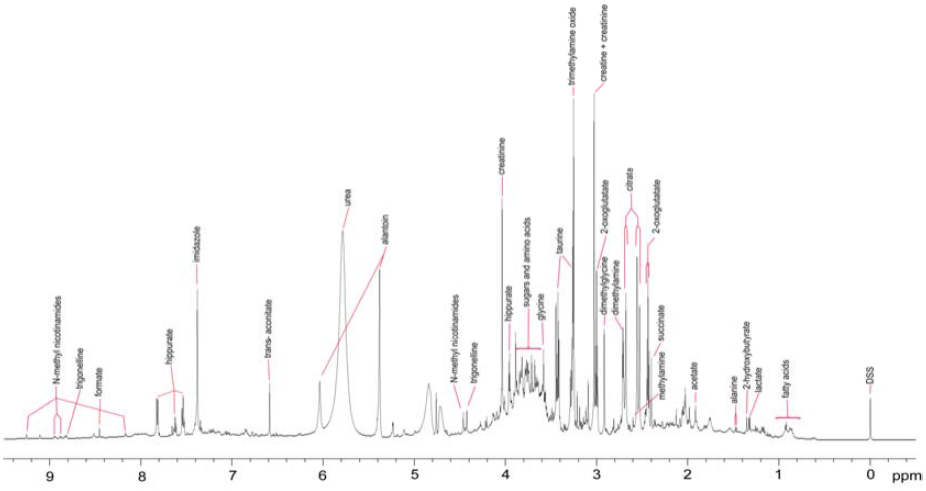
NMR Spectrum of Urine with Chenomx Library Fit of Metabolites



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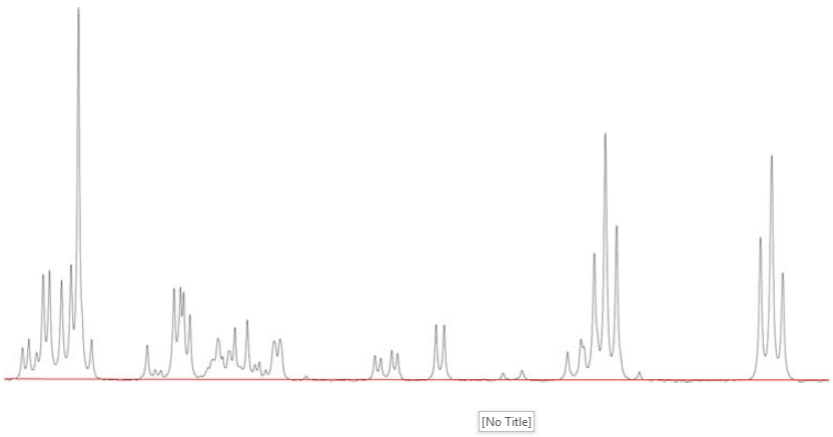
Fitting of Metabolites



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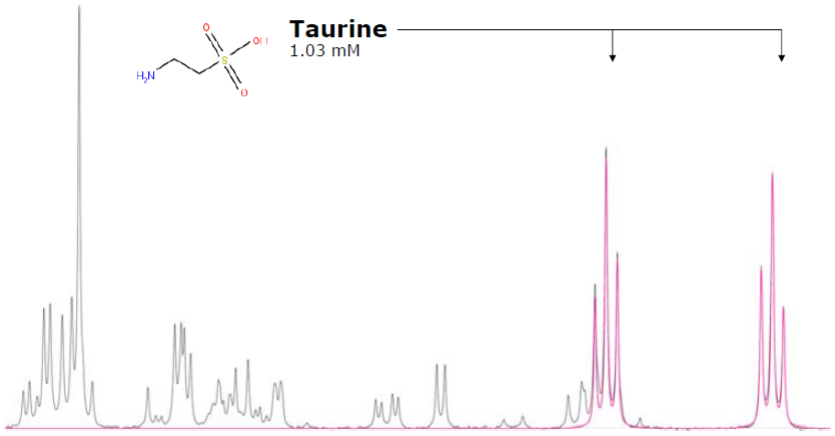
Fitting Taurine



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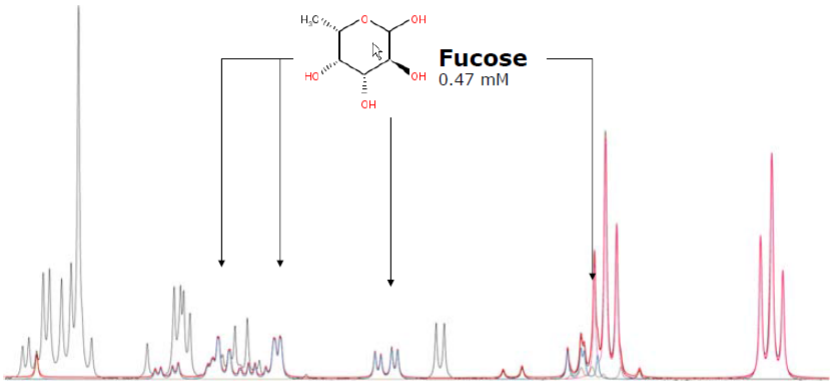
Fitting Fucose



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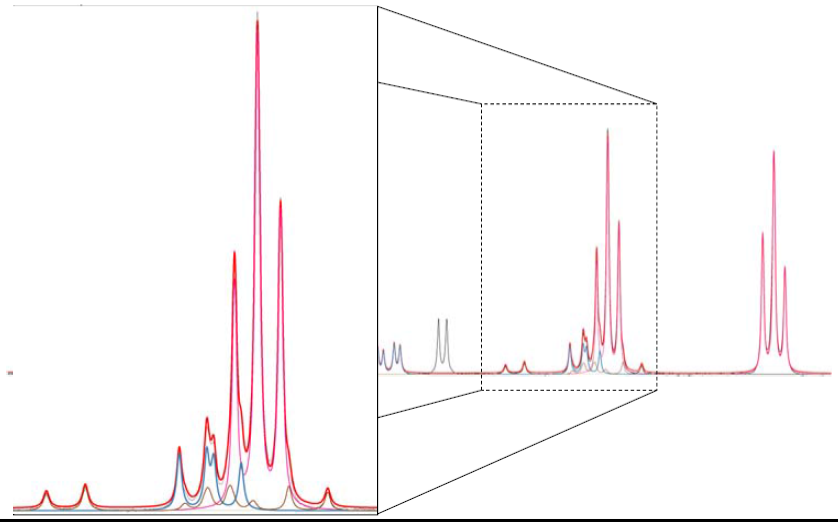
Additive Fit



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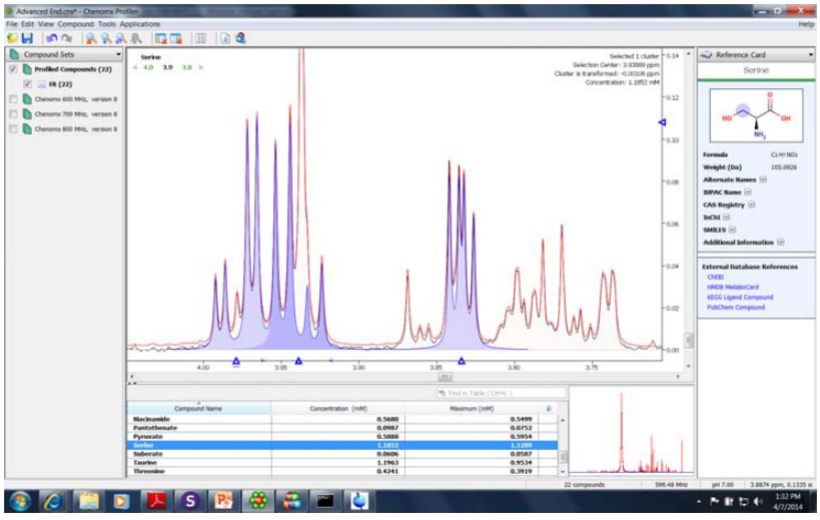
Additive Fit



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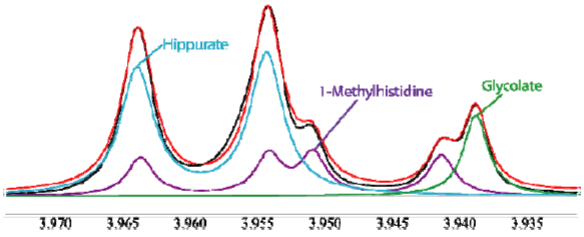
Chenomx Helps Resolving Ambiguity in Highly Overlapped Regions



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Interpreting Results and Pathway Analysis

Once we have performed a metabolomics analysis:

- We find some important metabolites that are responsible for the separation of study groups.
- The next questions are
  - What does it means?
  - How do you correlate these finding to your study questions?
  - Does it explain any findings that are meaningful for your study hypotheses?
  - Does it generate a new hypothesis?

- How do you answer these questions?
  - Next step is to interpret results and perform metabolic pathway analysis

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Interpreting Results and Pathway Analysis

- There are a number of freely available software
  - meta-P Server, Metaboanalyst, Met-PA, web based KEGG Pathways, Cytoscape.
  - GeneGo, Ingenuity Pathway Analysis (Commercial)
- Another way of interpreting metabolomics results is to use traditional biochemistry text books.
- The input for pathway analysis is typically a list of metabolites (with any fold change or p-value information)
- Genomics, transcriptomics, and/or proteomics data can be integrated
- Once these pathways are identified, you may perform a targeted metabolomics analysis to validate the findings from global analysis.

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