Metabolomics Analysis of *Phaseolus vulgaris L.* Affect on a Breast Cancer Rat Model

Compound Identification Using an Accurate Mass Retention Time Library

Steven Fischer
Senior Application Scientist
LC/MS Marketing
Agilent Technologies,
Santa Clara, CA

Topics

Compound identification challenges

Compound identification using AMRT

- Accurate mass matching
- Retention time matching
- Isotope matching

LC/MS metabolomics example

- *Phaseolus vulgaris L.* Affect on a Breast Cancer Rat Model

Metabolomics Compound Identification Challenges

Compounds can be classified as:

1. Known metabolite
   - Make it easy to identify
2. Known chemical but not a known metabolite
   - Make it possible to identify using MS/MS
3. Unknown chemical and unknown metabolite
   - Major project to identify

Metabolite Identification Schemes

1. Search a mass spectral library
   - **Agilent Fiehn GC/MS Metabolomics Library**
   - Metabolomics specific LC/MS/MS Library
2. Search a database, buy the standard and re-chromatograph
   - **METLIN AMRT Database**
3. Interpret an MS/MS spectra
   - MS/MS pattern matching software
4. Purify the compound and analyze by NMR with mass spectral data support
   - Mass directed purification systems

Compound Identification Using an Accurate Mass Retention Time Library

Analysis Of Metabolites Using METLIN With Retention Time And Isotope Pattern Scoring

Increase specificity of identification

•Search on mono-isotopic accurate mass

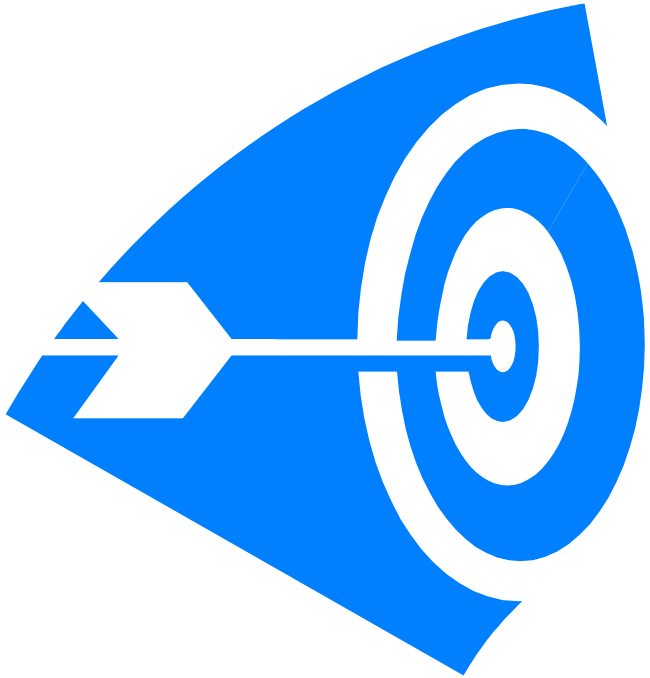
•Require retention time

•Calculate empirical formula using full isotope pattern

Generate empirical formula for un-matched data

•Add found but unknown compound to database for future tracking

  – retention time and proposed formula



Molecular Formula and METLIN Personal Metabolite Database Matching Applied to the Identification of Compounds Generated by LC/TOF-MS, T. Sana et al, *Journal of Biomolecular Techniques* 1 9:258–266 2008

METLIN Personal Database Overview

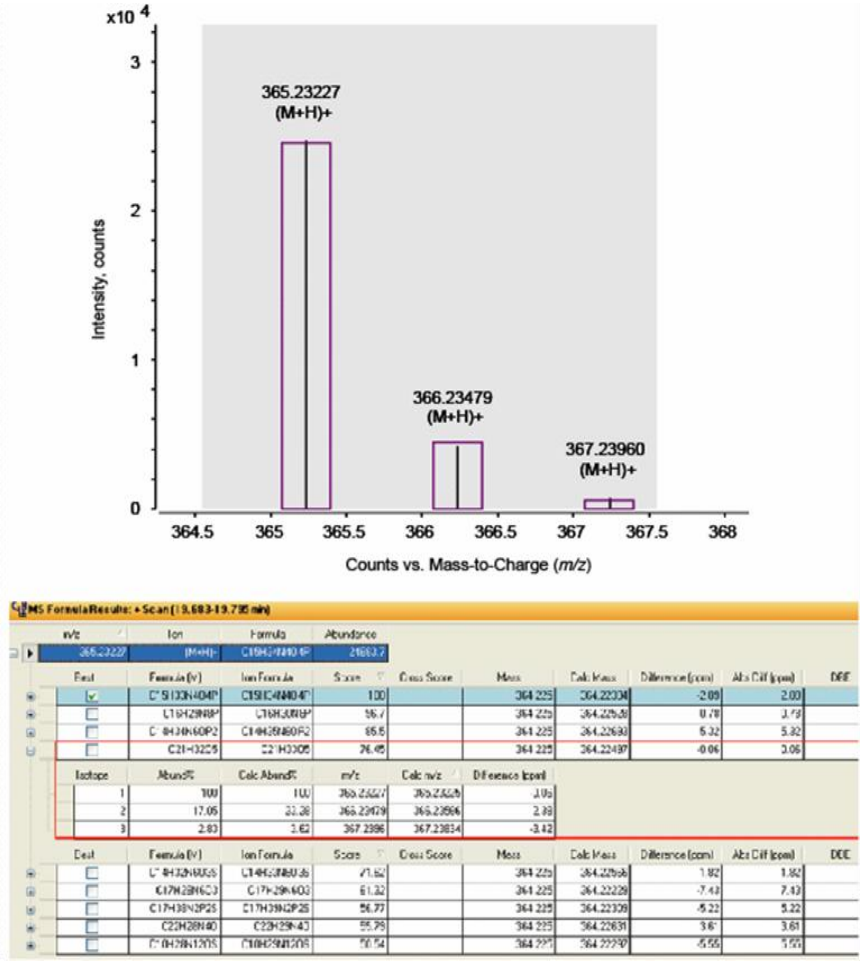
Metabolite-specific database for metabolomics research

- Database installed on a PC
- Contains ~22000 compounds
- Manual and batch searches
  - Query based on monoisotopic mass
- Customizable
  - Add compounds
  - Assign chromatographic retention times to metabolites
  - Create subset databases
- Works with other Agilent software

Calculating a Molecular Formula (MFG) With Database Searching

Dihydrocortisol example

- Database search
  - Uses only mono-isotopic mass; loses isotope information
  - Only compounds in the database can be matched; database selection creates specificity based on biology
- Molecular formula calculation (MFG)
  - Uses mass values of all isotopes, including adducts
  - Support database match; if no retention time information present
  - Provides useful information; even if no database match

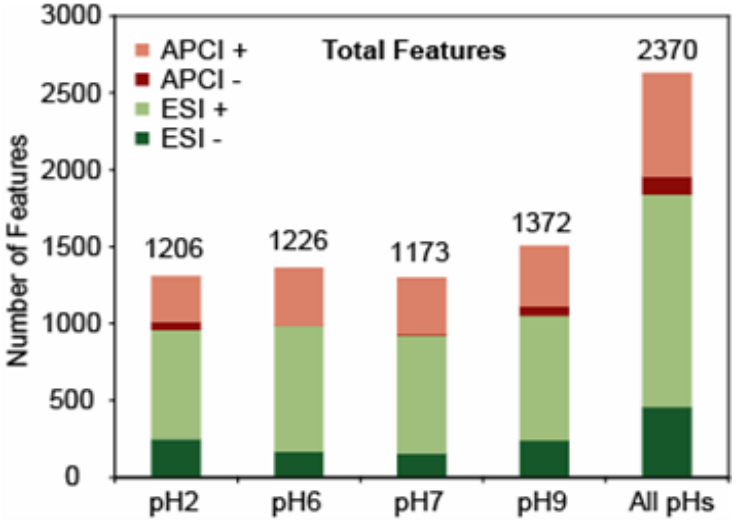


Erythrocyte Metabolites Extracted At Different pH And Ionization At Different Modes

No single ionization mode detects all analytes

1. ESI 67% vs. APCI 25%
   - Common to both 7.3%
2. Pos mode 74% vs. Neg mode 22%
   - Common to both 3.9%

Need method for ESI (+/-) and APCI (+/-)



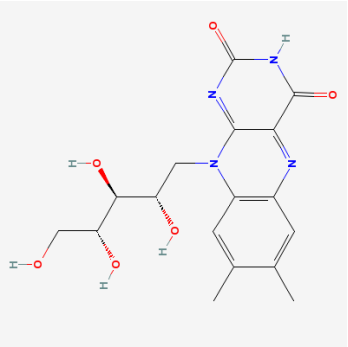
A sample extraction and chromatographic strategy for increasing LC/MS detection coverage of the erythrocyte metabolome, T.R. Sana et al., *J. Chromatography* B871 (2008) 314-321

Chromatography method must be compatible with ESI & APCI and positive & negative ionization

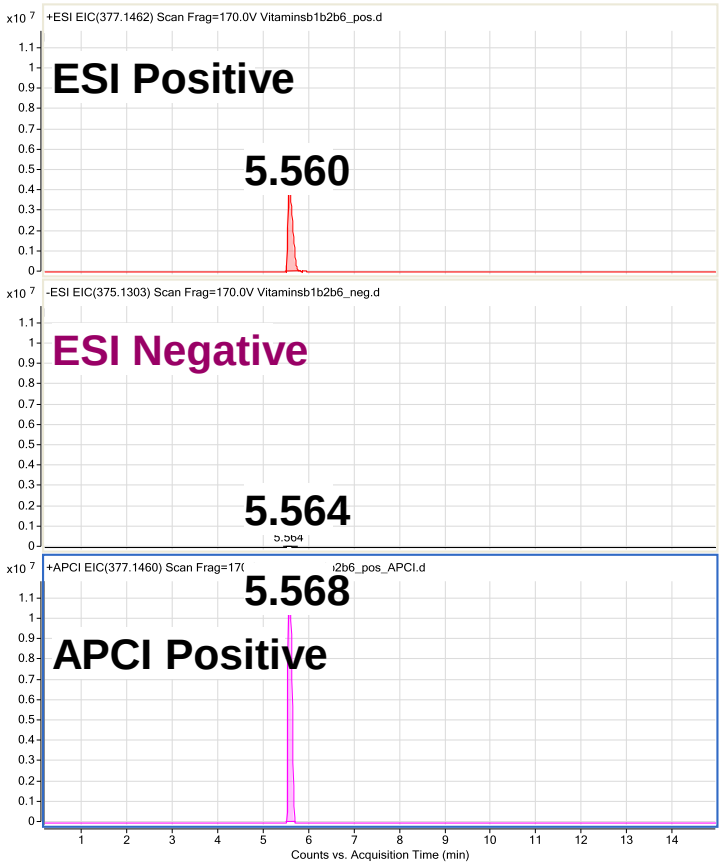
AMRT Analytical Methodolgy

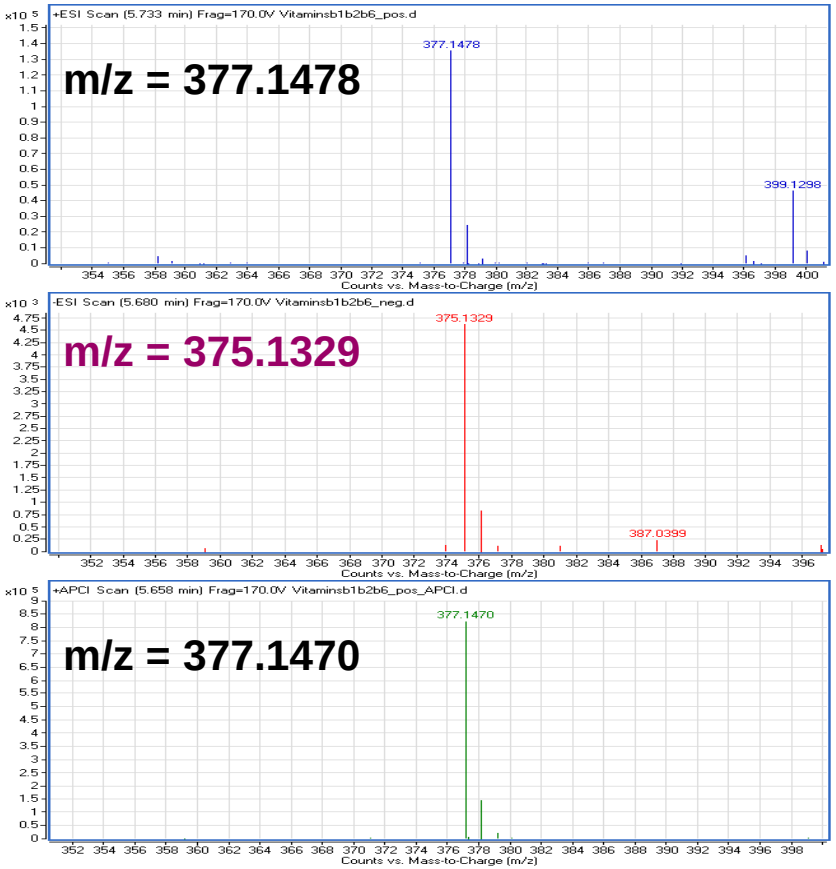
- Single reverse phase separation method
  - Water, methanol and acetic acid
  - Reverse phase column
    - 2.1 x 50 mm, 1.8 µm Zorbax SB-Aq
    - 2.1 x 30 mm, 3.5 µm Zorbax SB-C8
  - Flow rate 0.6 ml/min
  - Simple linear gradient
    - 2% methanol to 98% methanol in 13 minutes
    - 6 minute hold at 98% methanol
  - Cycle time 24 minutes
- Compatible with ESI / APCI and positive / negative ionization modes

Vitamin B2 Standard: $C_{17}H_{20}N_4O_6$ [M+H] = 377.1456, [M-H] =375.1310

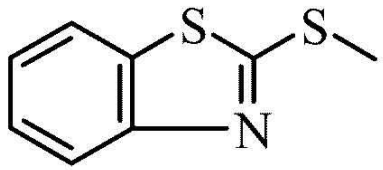


RT_{ave}=5.564

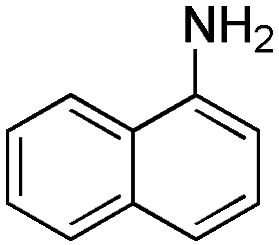




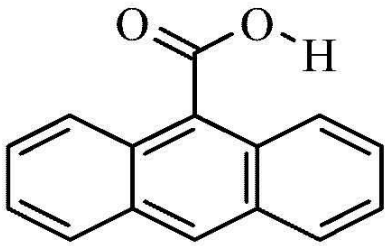
LC/MS Internal Standards



2-(methylthio)benzothiazole



1-naphthylamine



9-anthracene carboxylic acid

2-(methylthio)benzothiazole

- APCI only – positive ion only
- Recovery has significant dependence on pH and salt

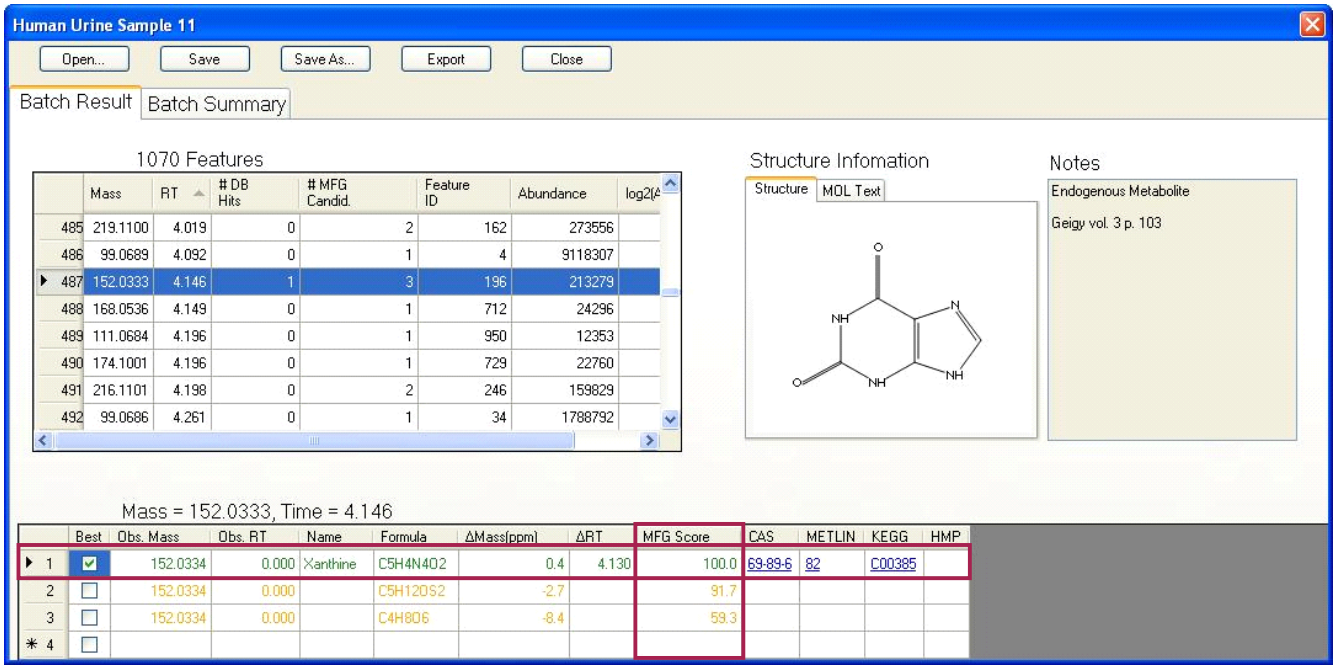
1-naphthylamine

- APCI and ESI – positive ion only
- Good solubility, especially at low pH

9-anthracene carboxylic acid

- APCI and ESI – positive and negative ion mode
- Good solubility, especially at high pH

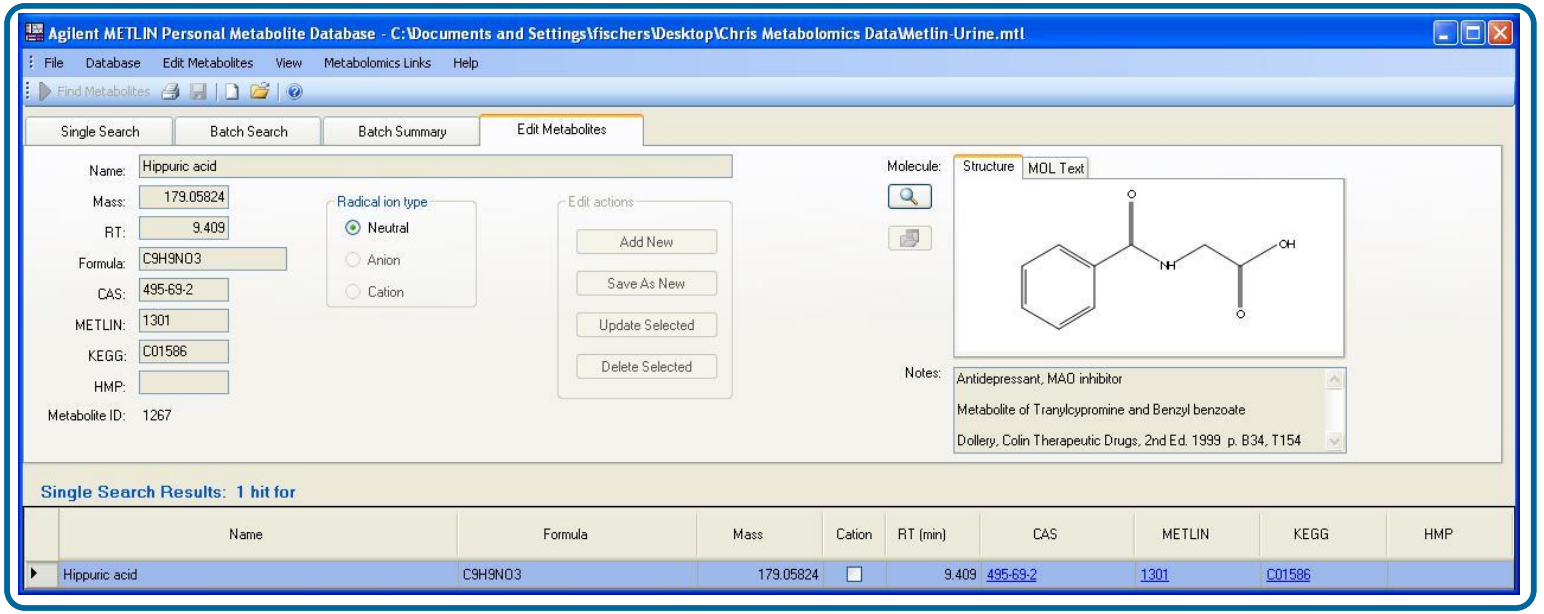
Using Accurate Mass, Retention Time Database Searching With Molecular Formula (MFG)



Results are returned prioritized by;

- AMRT match and MFG
- AMRT only
- Database match and MFG
- Database match
- MFG

Reverse Phase, Accurate Mass, Retention Time (AMRT) Metabolite Database



Retention times were added to the database by running standards

- Database is separation specific; retention times change if separation is changed
- Compounds need to be retained (k'>2) in order for retention time to add specificity
- Compounds of same mass ranked differently if retention time information present
- RP-AMRT currently contains 363 database entries with retention time

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Steven Fischer
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Henry J. Thompson
Meghan M. Caulum
Cancer Prevention Laboratory
Colorado State University

Dry Bean / Rat Tumor Study

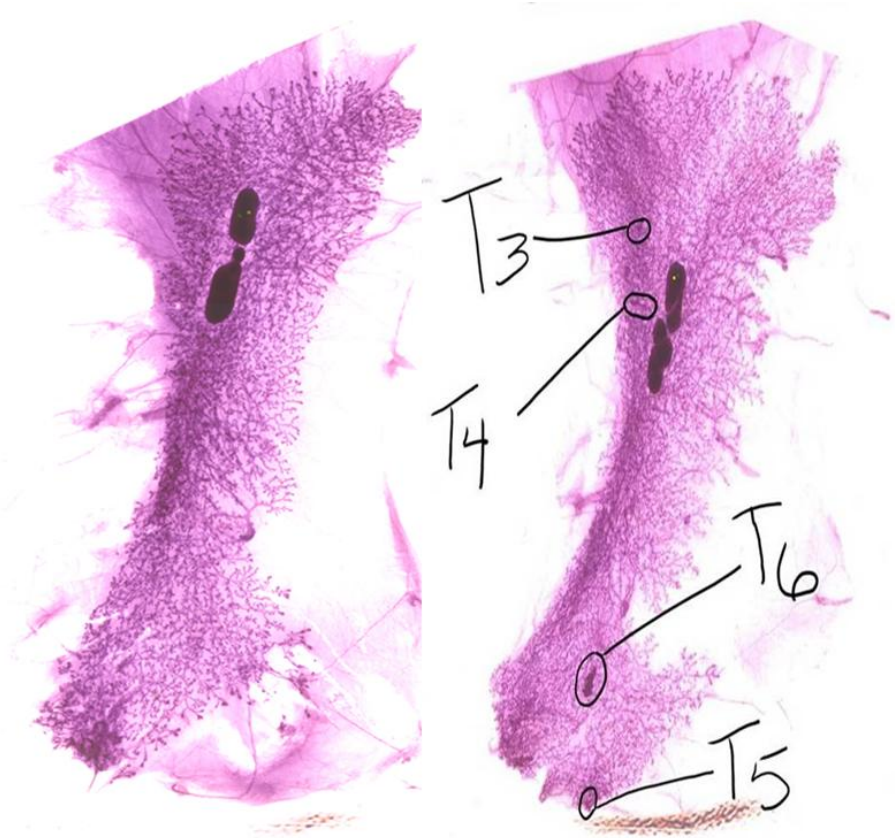
Breast cancer rat model responds to different dry beans

- White kidney bean is most effective
- Navy bean is intermediately effective
- Red bean is partially effective
- Rat chow has no effect

Are there observable chemical differences in beans?

Can the differences be seen in plasma, gland or tumor?

Treatment	Sample Number	incidence (%)	average tumors per	Tumor burden g/rat
Control	30	93.3	3.2	0.48
Navy Bean	30	70.0	1.9	0.34
White Kidney Bean	30	66.7	1.0	0.05



Study Design

Diet test

- 10 lots produced of each
  - House blend rat chow AIN-93G
  - 60 % white kidney to chow blend
  - 60 % navy to chow blend
  - 60 % red to chow blend

Animal - Diet test

- 10 animals feed diet of
  - House blend rat chow AIN-93G
  - 60 % white kidney added to chow
  - 60 % navy added to chow
  - 60 % red added to chow

Sample Processing Protocol

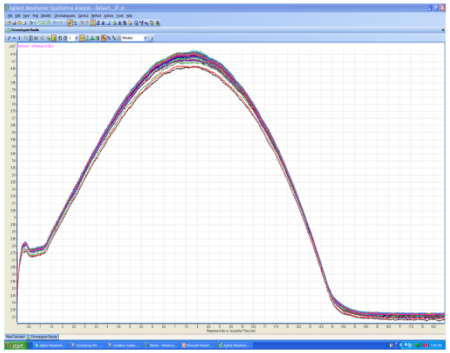
Sample extraction

- Diet 50 mg, Plasma 100 ul, tissue 100 mg
- water / methanol / chloroform
  - pH 2 and pH 9

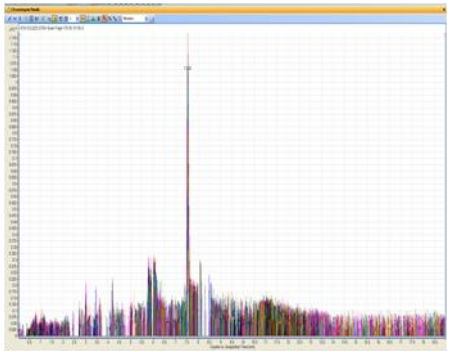
Extract preparation

- Speed-Vac to dryness
- Protein precipitate with 75% ACN
- Speed-Vac to dryness
- Reconstitute with 50 ul methanol, vortex then 50 ul water, vortex
- Blend pH 2 and pH 9 extracts
- Inject 5 ul into LC/MS system

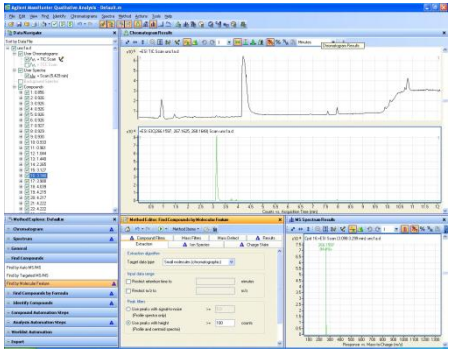
Data Analysis Workflow:
Bean, Mammary Gland, Plasma and Tumor



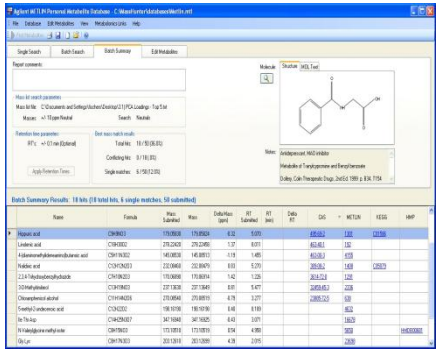
Verify System Pressure Reproducibility



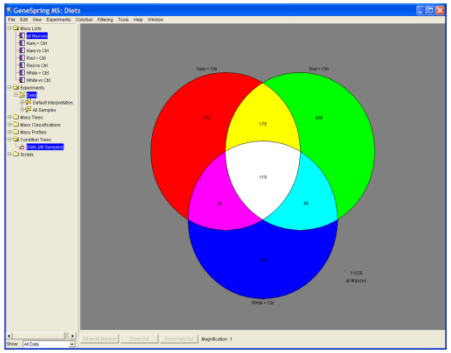
Verify Internal Standard Reproducibility



Find Features using Mass Hunter MFE



AMRT METLIN Search Of Sample Differences



Find Metabolite Differences

Align and Normalize Features

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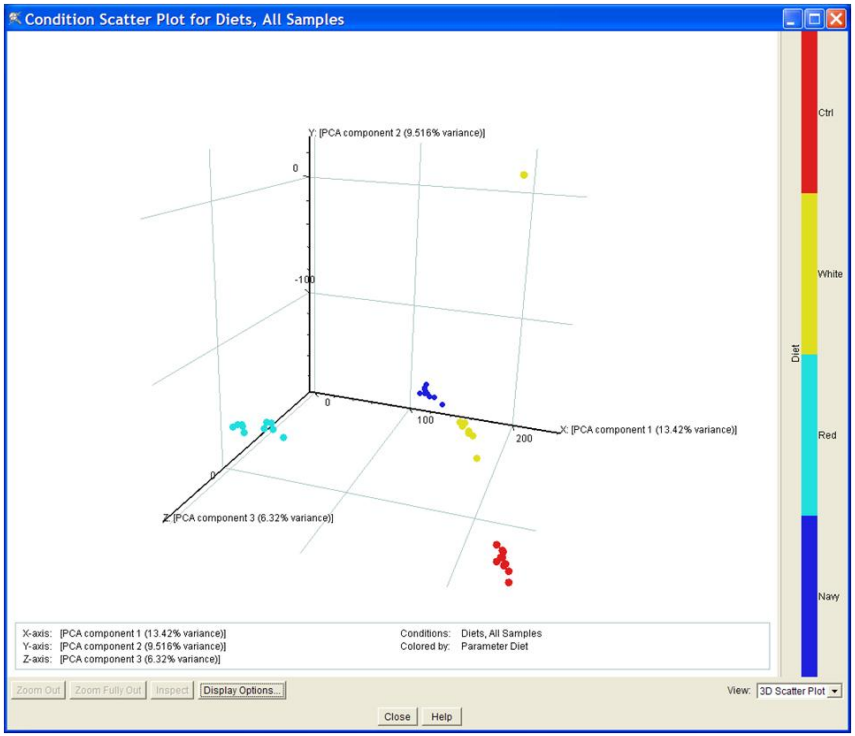
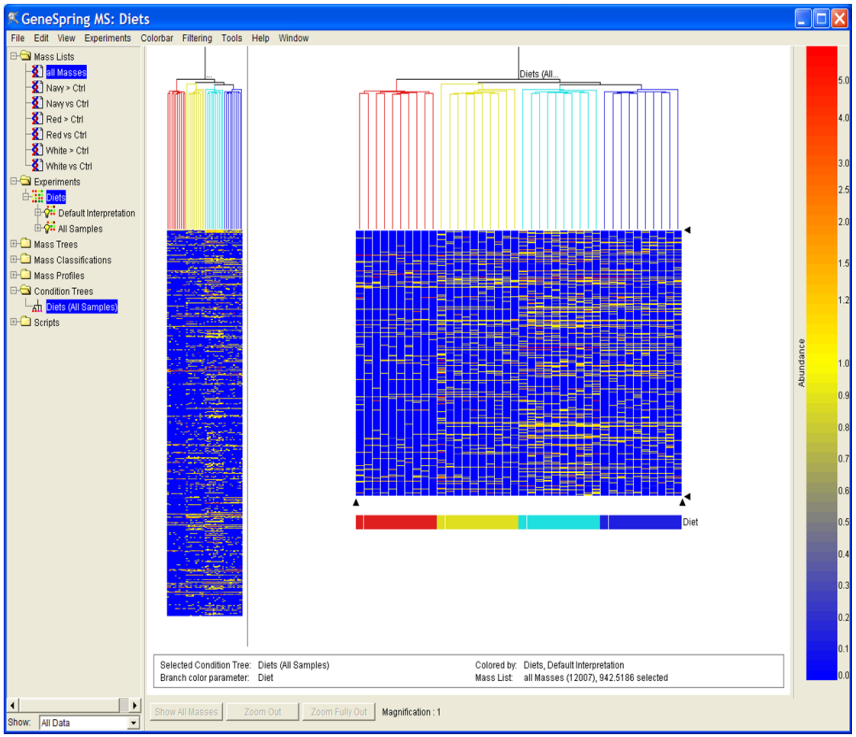
Initial Analysis Of Bean Supplemented Diets Data

PCA of Bean Diet Data



Condition Tree of Bean Diet Data

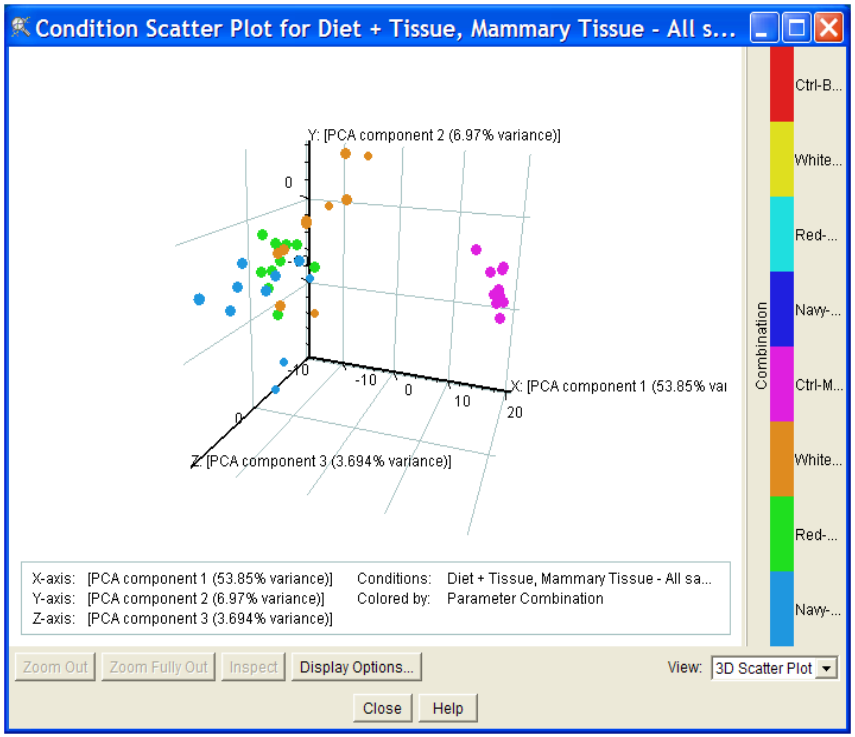


Identification Results Of Bean Diet Samples LC/MS; ESI-Positive Ion

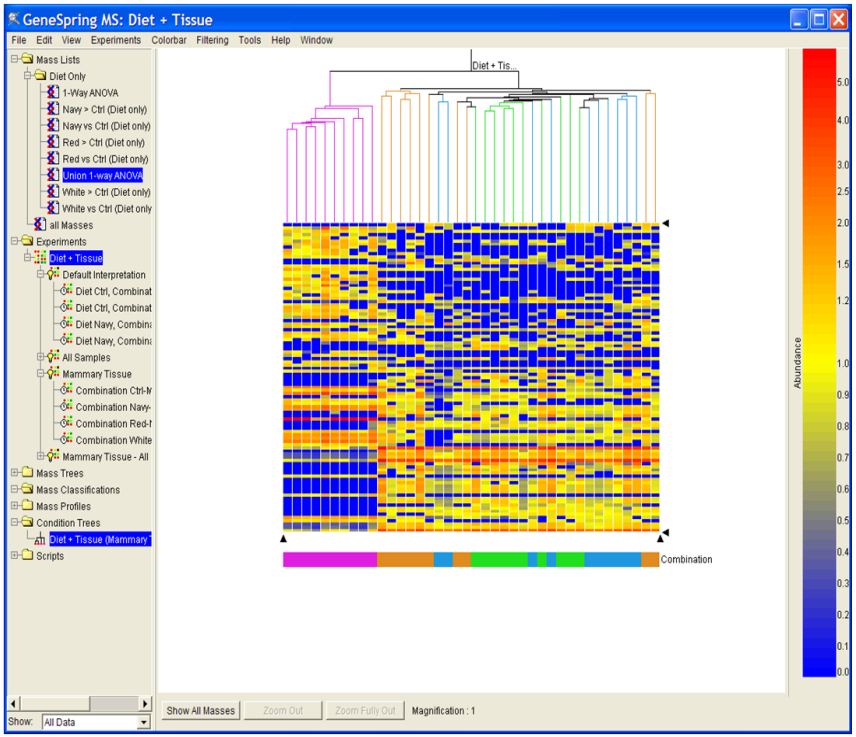
	Control		White		Navy		Red	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total Features	692	100.0%	1386	100.0%	1639	100.0%	2290	100.0%
DB+RT+MFG	7	1.0%	10	0.7%	10	0.6%	14	0.6%
DB+RT	0	0.0%	2	0.1%	1	0.1%	5	0.2%
DB+MFG	89	12.9%	322	23.2%	340	20.7%	481	21.0%
DB	31	4.5%	69	5.0%	90	5.5%	144	6.3%
MFG	444	64.2%	655	47.3%	716	43.7%	1025	44.8%
No match	121	17.5%	328	23.7%	482	29.4%	621	27.1%
	692		1386		1639		2290	

Analysis Of Rat Mammary Gland After Four Different Bean Diets

PCA of Mammary Gland Data



Condition Tree of Mammary Gland Data



Identification Results Of Mammary-Bean Diet Samples, LC/MS; ESI-Positive Ion

	Control		White		Navy		Red	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total Features	1218	100.0%	1186	100.0%	1182	100.0%	1141	100.0%
DB+RT+MFG	10	0.8%	10	0.8%	10	0.8%	8	0.7%
DB+RT	0	0.0%	1	0.1%	1	0.1%	1	0.1%
DB+MFG	287	23.6%	284	23.9%	319	27.0%	272	23.8%
DB	18	1.5%	8	0.7%	8	0.7%	27	2.4%
MFG	743	61.0%	707	59.6%	688	58.2%	693	60.7%
No match	160	13.1%	176	14.8%	156	13.2%	140	12.3%
	1218		1186		1182		1141	

Bean Metabolites Found In Mammary Gland Tissue

White Bean

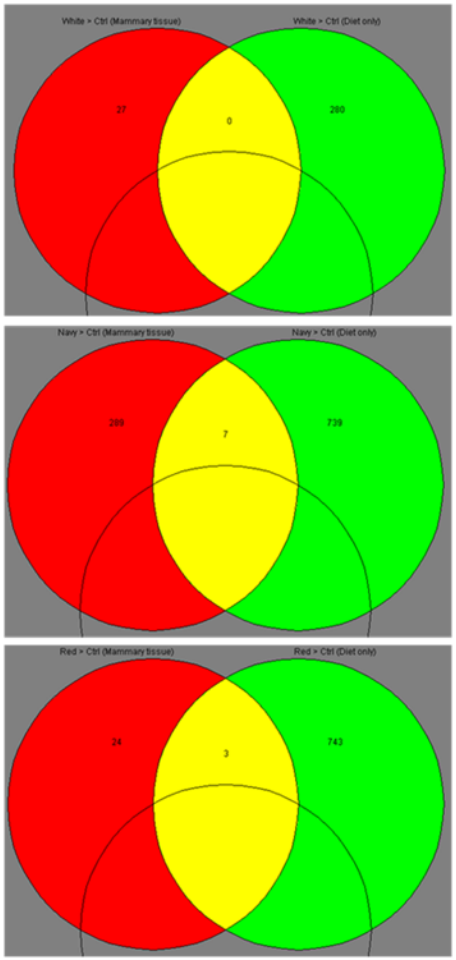
- Present in both – **0**
- Unique to tissue – **27**
- Unique to bean – **280**

Navy Bean

- Present in both – **7**
- Unique to tissue – **289**
- Unique to bean – **739**

Red Bean

- Present in both – **3**
- Unique to tissue – **24**
- Unique to bean – **743**



AMRT Search Results For Common Metabolites

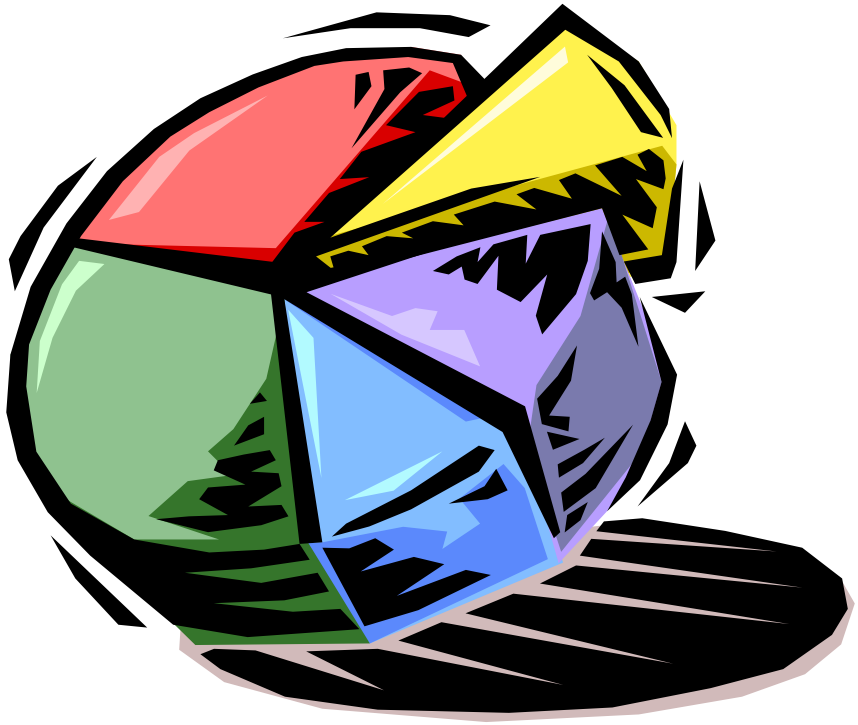
White Bean – 0

Navy Bean – 7

- DB + RT + MFG – 1 (niacinamide)
- DB + MFG – 2
- MFG – 4

Red Bean – 3

- DB + RT + MFG – 0
- DB + MFG – 2
- MFG - 1



Bean-Rat Model Observations With Regard to Identification

1 in 4 features were found in the METLIN database

- A RT database of 363 compounds is too small (~4% of DB matches)

4 in 7 features had only an empirical formula calculated

1 in 7 features were complete unknowns