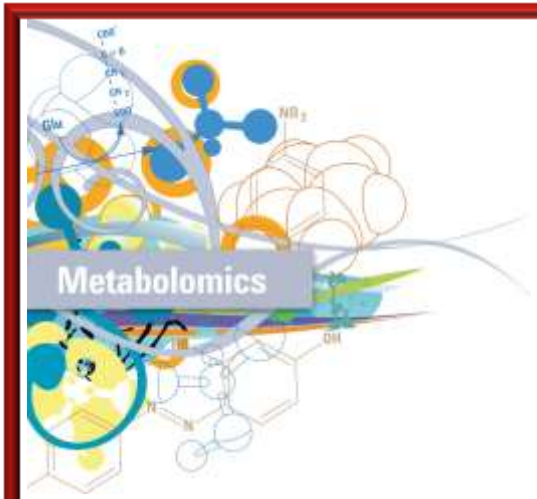


High Throughput Workflow for Global Metabolomic Profiling of Biological Samples with RapidFire online SPE/MS system

Michelle Romm, Ph.D.

Applications Scientist,

Agilent Technologies, Inc.

What is High Throughput?

# of Samples	RapidFire/MS	LC/MS
N=1	14 secs	30 mins
N=100	23 mins	50 hrs
N=1,000	3.8 hrs	20.8 days
N=10,000	38.8 hrs (1.6 days)	208.3 days

120X Faster

Why High Throughput Profiling?

Is Faster better?

When

Method Development

pH
Solvents/Additives
Chemistry
Timings/Flows

Large Population Studies

N= 5
50
500
5000

Better Experiments!

RapidFire/MS System

RapidFire 365 Online SPE/MS System

- **BenchBot plate handling robot**
  - 63 plate capacity (384 well plates = 24,192 samples)
  - Off-deck reach capability
- **Quaternary pumps**
  - Solvent switching capability
    - Capable of running multiple disparate methods in one batch
- **12 position automated cartridge changer**
- **Automated method development**
  - Perform multiple injections of a single sample with different SPE cartridges and mobile phases
    - C18, C8, C4, CN, Phenyl, HILIC, Graphitic Carbon
    - Simply run multiple samples overnight and select your ideal conditions the next morning

Aspirate	Load/Wash	Elute	Re-equilibrate
600ms	3000ms	8000ms	1000ms

For Research Use Only. Not for Use in Diagnostic Procedures.

Method Development: Refining the Experiments

- Cartridge chemistries
- Ionization mode
- Loading solvents/times
- Elution times/solvents

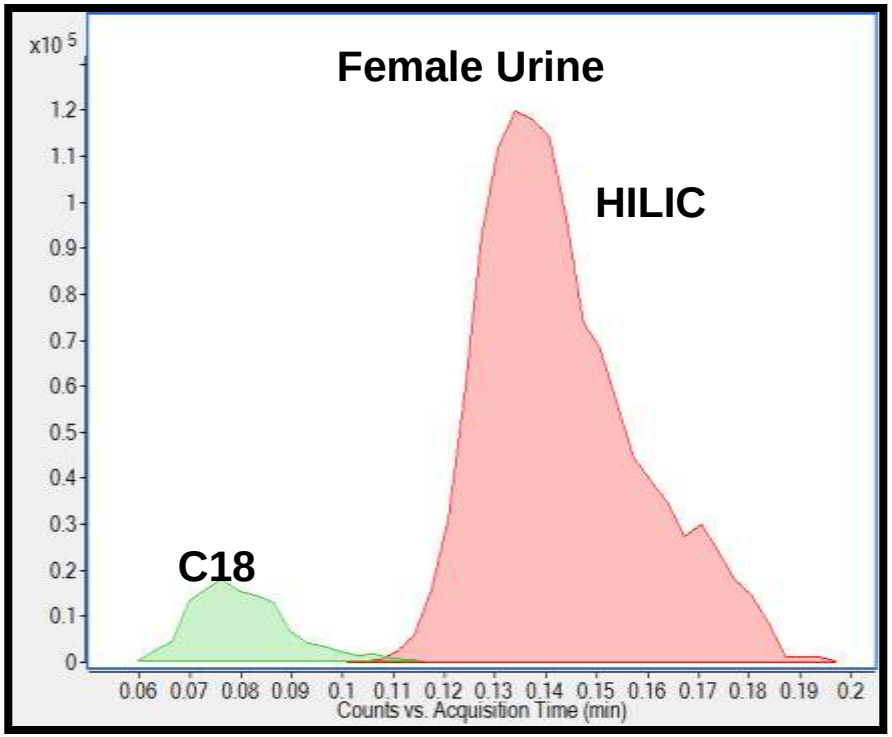
One solution doesn't fit everyone, this system allows FLEXIBILITY!

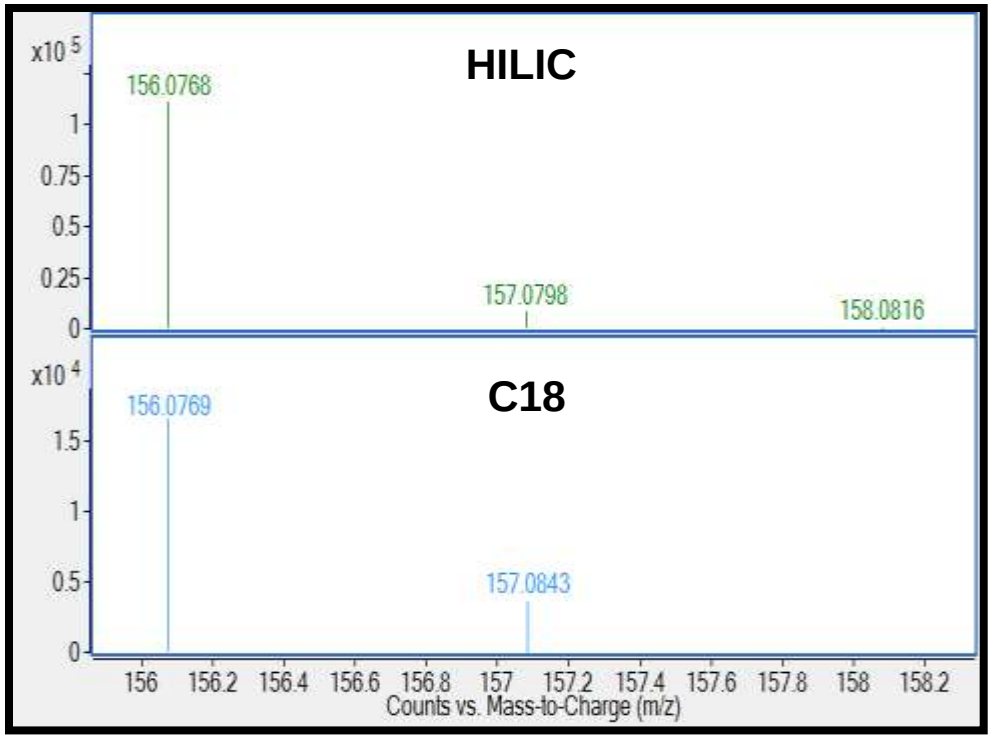
Comparison of SPE Chemistries: HILIC vs. C18 SPE/MS for a Single Feature in Urine Sample

Compound List

Automatically Show Columns

Show/Hide	File	Mass	m/z	RT	Quality Score	Height	Ions	Polarity	Algorithm
☑	Inj00012-MetStab C18-A1.d	155.0696	156.0769	0.08	70.2	16551	2	Positive	Find by Molecular Feature
☑	Inj00011-MetStab C18-A1.d	155.0695	156.0768	0.14	100	111020	3	Positive	Find by Molecular Feature

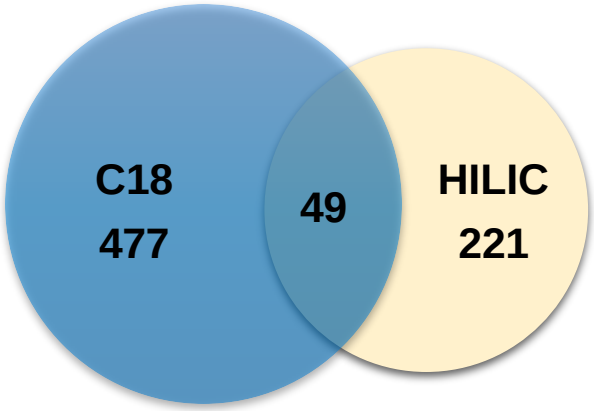




Comparison of Chemistries: C18 vs. HILIC SPE/MS (+ESI) in Urine

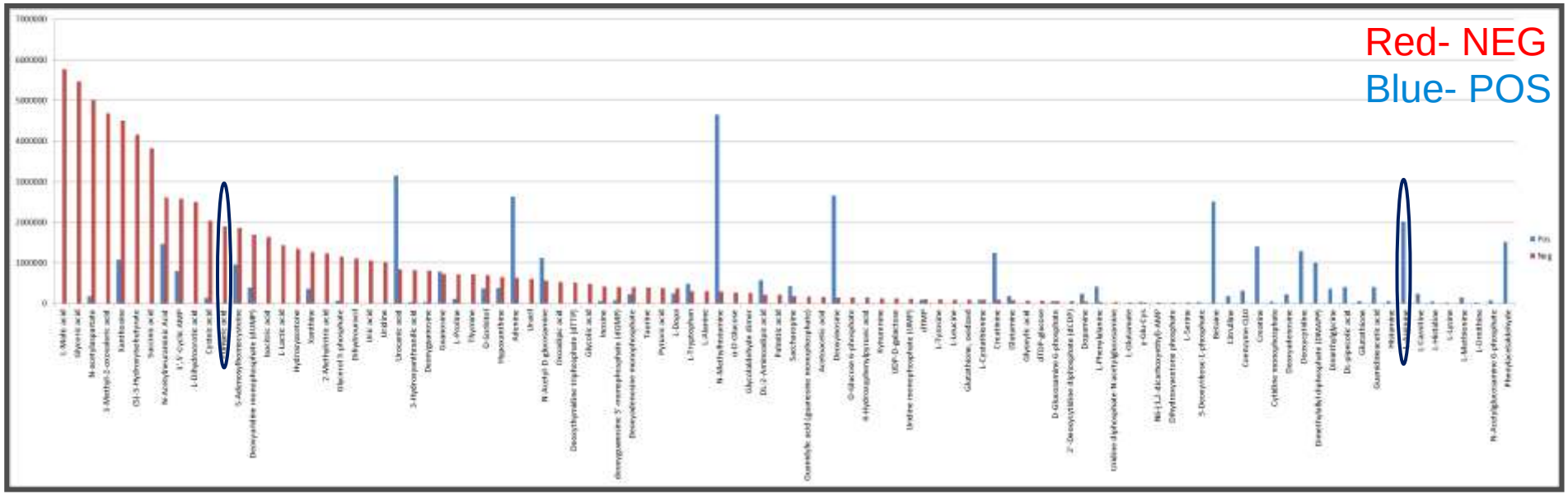
- ✓ 526 metabolites annotated by C18 SPE/MS
- ✓ 270 metabolites annotated by HILIC SPE/MS
- ✓ 49 annotated metabolites common between HILIC and C18 SPE/MS

747 unique metabolites (100% reproducibility; CV<15%)



Utilize multiple unique chemistries to increase the metabolite coverage

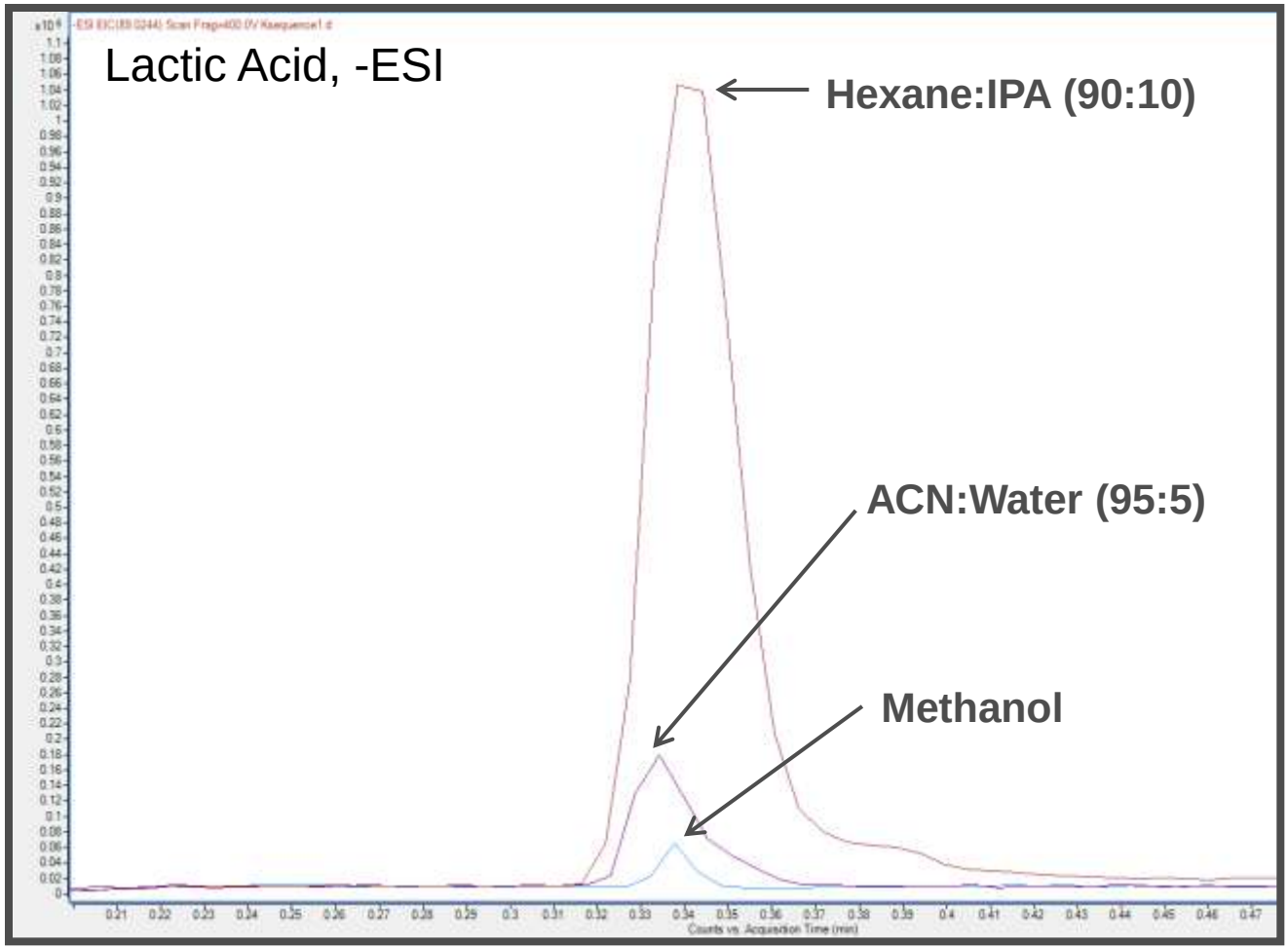
Comparison of ESI Mode: a Small Polar Library



- ~100 pure endogeneous human metabolite standards
  - small organic acids, biogenic amines, nucleotides, amino acids
- Custom packed HILIC material

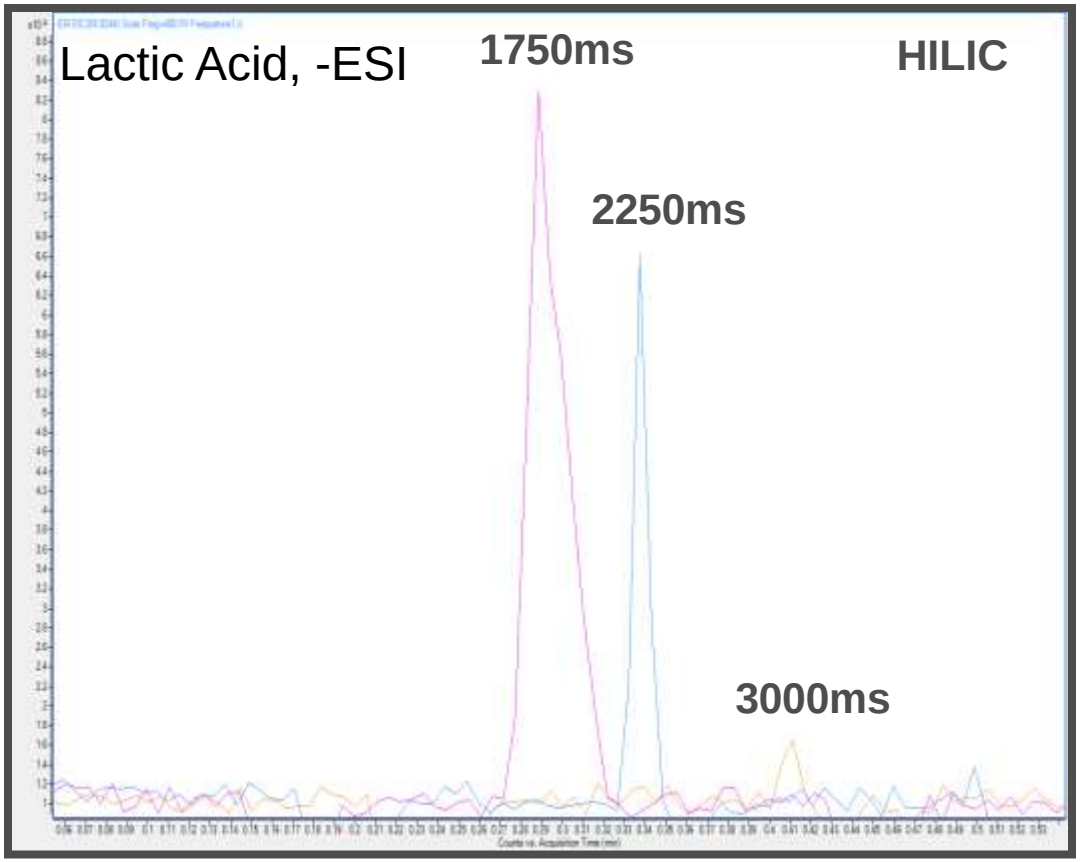
Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego

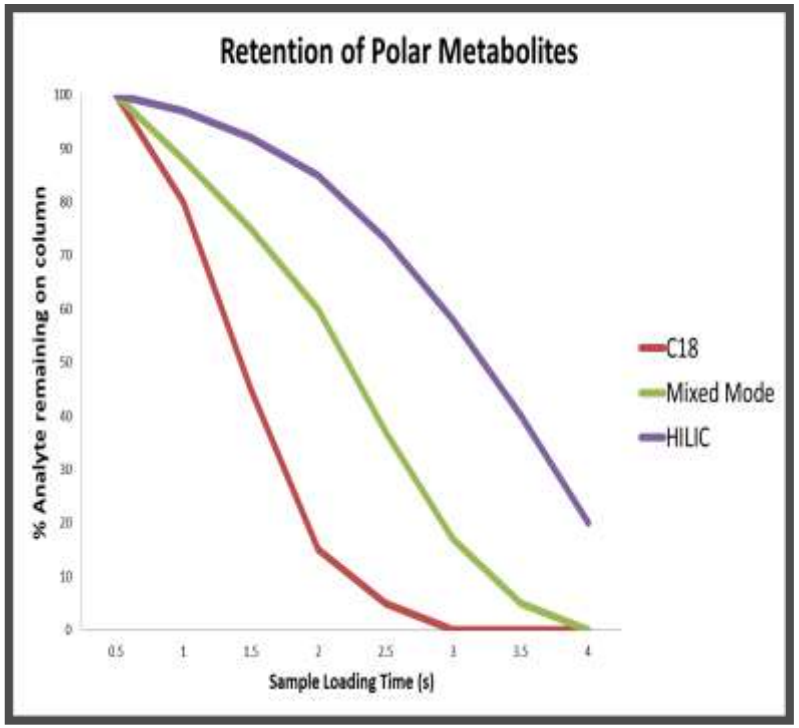
Comparison of Loading Solvents: HILIC Normal Phase



Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego

Comparison of Loading Times: Polar Analytes

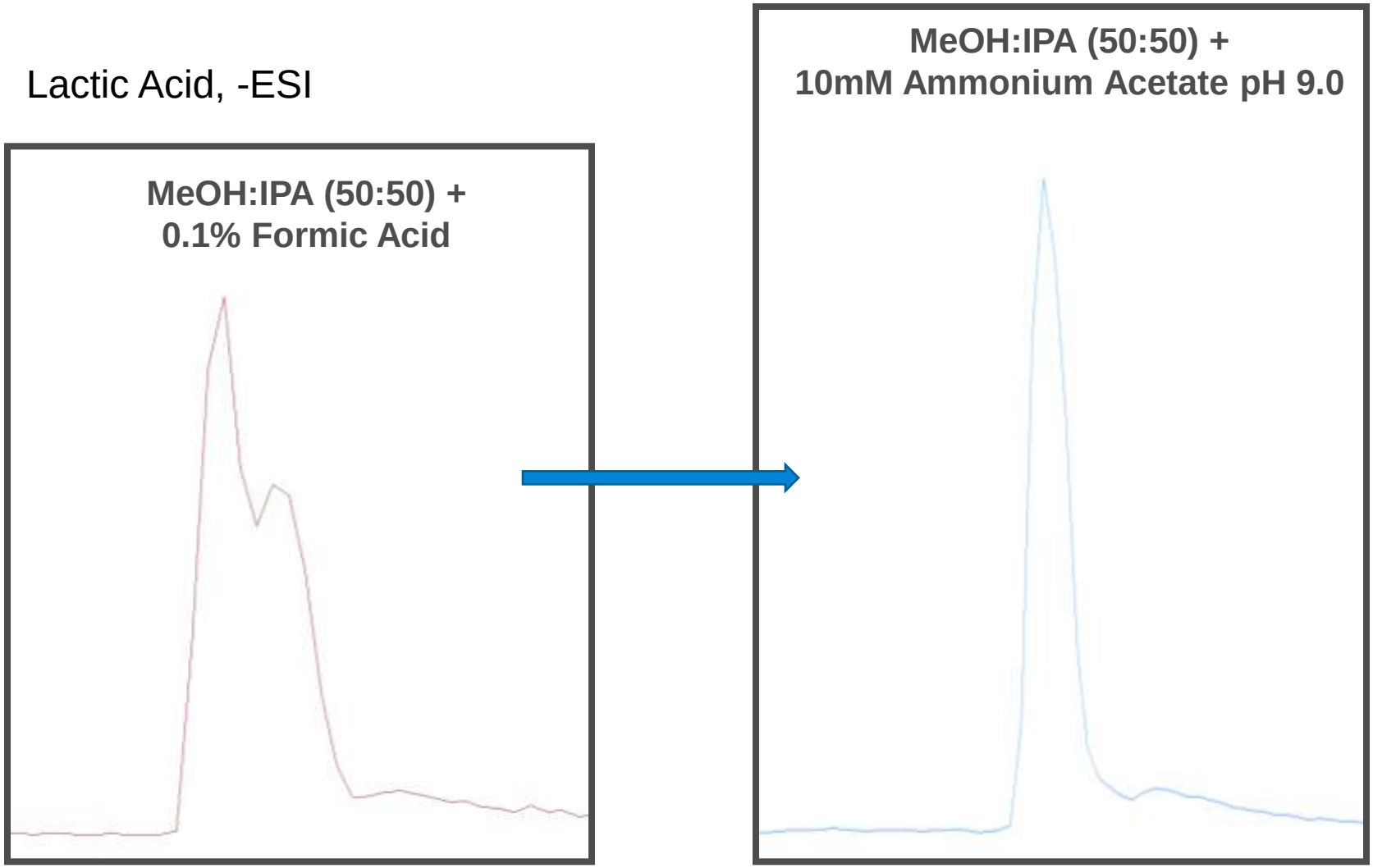




Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego

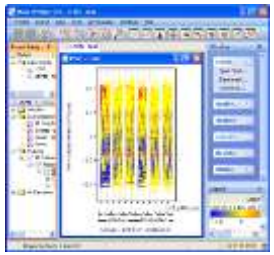
Comparison of Elution pH

Lactic Acid, -ESI



Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego

High Throughput Metabolomics Profiling Workflow

High Throughput Acquisition	Batch Data Processing	Data Analysis	Verification/ Confirmation/ Validation	High Throughput Quantitation
 RapidFire 365  iFunnel 6550 Q-TOF	**MassHunter Profinder (NEW)**  .d data input ↓ Recursive MFE ↓ Features .cef output	**Mass Profiler Professional (MPP)**  **Statistics** **Database Search** **Pathway Analysis** **Potential Targets**	**Pooled Sample/ Random Samples** **LC/MS Acquisition** FindbyFormula Verify Targets **LC/MS/MS Acquisition** Metlin Library/ SimLipid/ MSC **Confirm Targets** ↓ **Validate with Reference Standard**	 RapidFire 365  iFunnel 6490 QQQ

High-throughput Profiling: What is the Workflow?

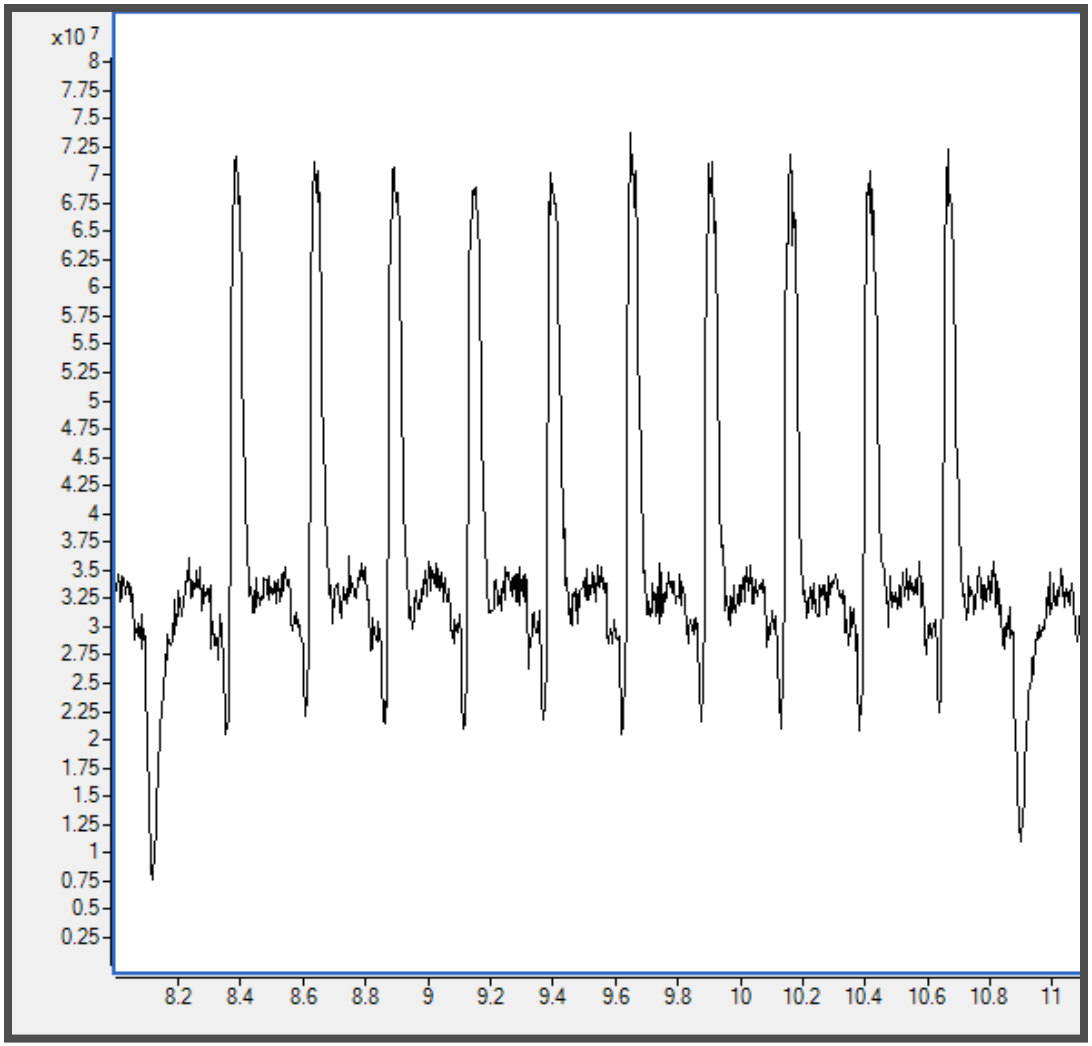
- Human urine
- Human serum lipidome
- Milk lipidome

Human Urine Metabolomics Profiling: Sample Prep

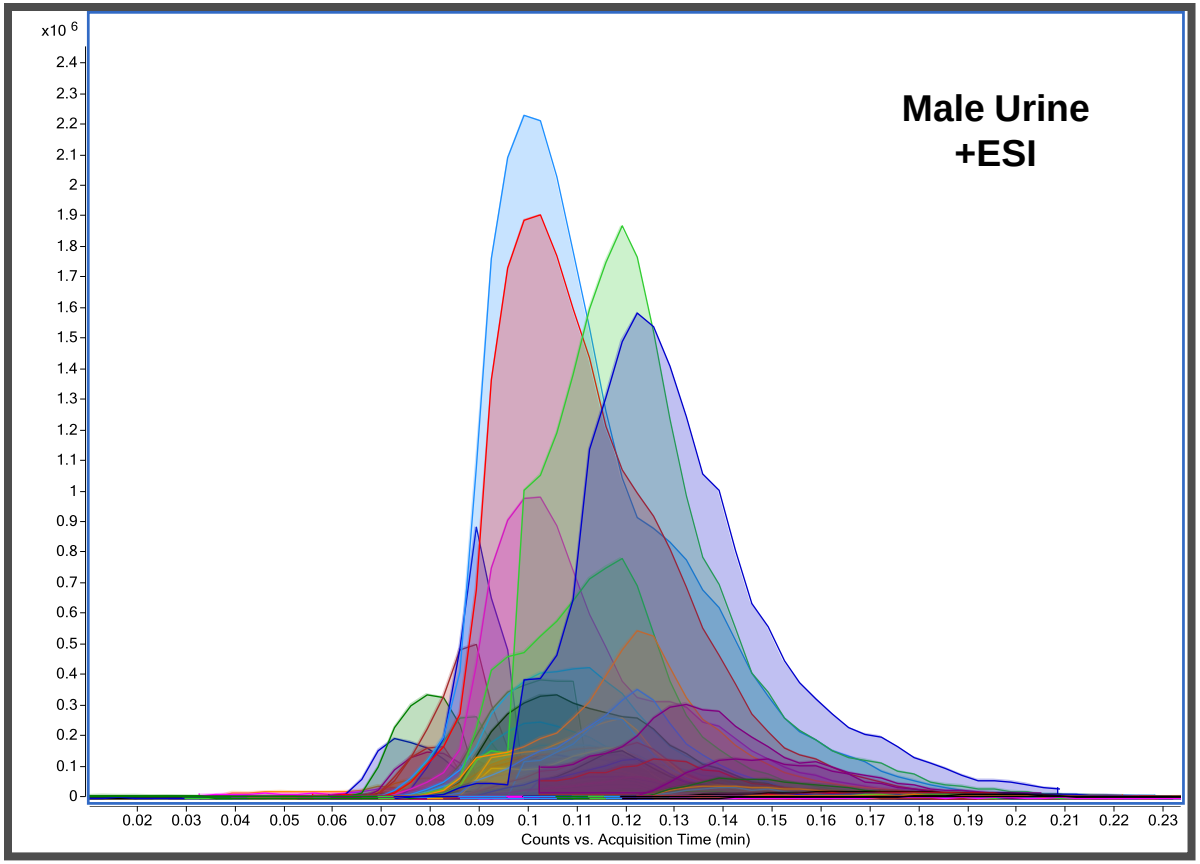
- Human urine, collected and pooled from healthy subjects (males and females), was purchased from Innovative Research Inc. (Novi, MI)
- Filter 400µL urine through a 0.22µm Ultrafree-MC® centrifugal filter units (Millipore) by centrifugation @ 12,000xg for 20min @ 4°C
- Pipette 50µL of filtered urine (10 replicates) into the Amicon Ultra-0.5mL Centrifugal Filter (3kDa Cut-off Ultracel-3 membrane) from Millipore
- Dilute with 350µL of Milli-Q quality water containing 0.2% Acetic acid, 1mM Ammonium acetate, and centrifuge @ 12000xg, 4°C for 30min to concentrate
- The flow-through represents the metabolomics sample
- Inject **10µL** of flow-through for SPE/MS as well as LC/MS/MS analysis

Human urine equivalent to 1.25µL was used for the metabolomics profiling studies

TIC for 10 Human Urine Samples (C18 SPE/MS)



EIC of a Single Injection of Urine Sample on C18 RapidFire SPE/MS

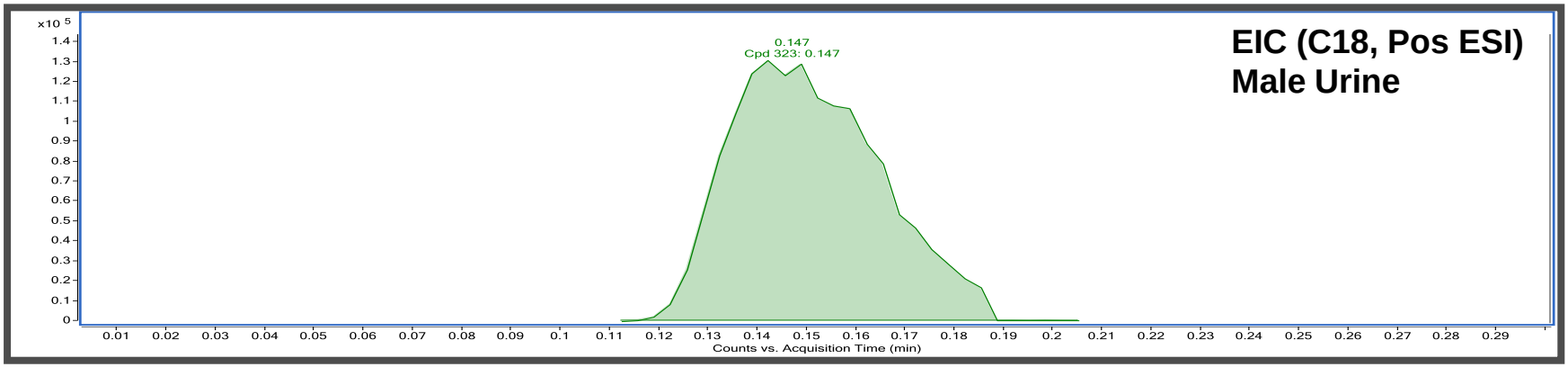


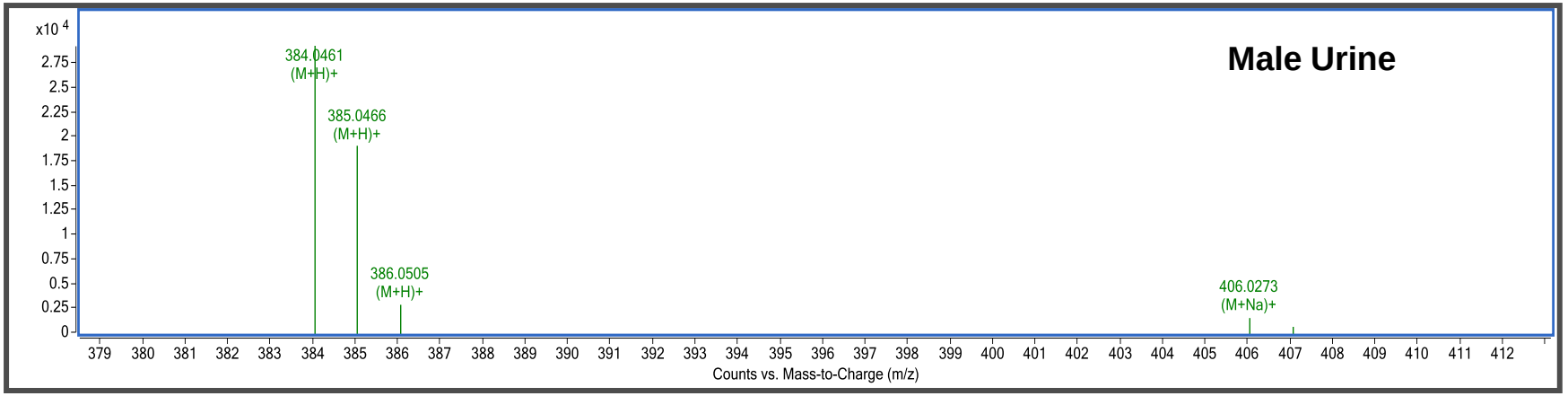
EIC Overlay of a Single Feature in Urine on C18 RapidFire SPE/MS

Compound List

Automatically Show Columns

Show/Hide	Cpd	File	Mass	m/z	RT	Quality Score	Height	Ions	Polarity	Algorithm
☑	318	Inj00037-MetStab C18-B1.d	267.0691	268.0764	0.145	100	8518	3	Positive	Find by Molecular Feature
☑	320	Inj00037-MetStab C18-B1.d	517.0538	518.061	0.146	100	4607	3	Positive	Find by Molecular Feature
☑	323	Inj00037-MetStab C18-B1.d	383.0388	384.0461	0.147	100	108704	6	Positive	Find by Molecular Feature
☑	324	Inj00037-MetStab C18-B1.d	373.018	374.0253	0.15	100	3354	3	Positive	Find by Molecular Feature





ProFinder for High Throughput Batch Analysis of Samples

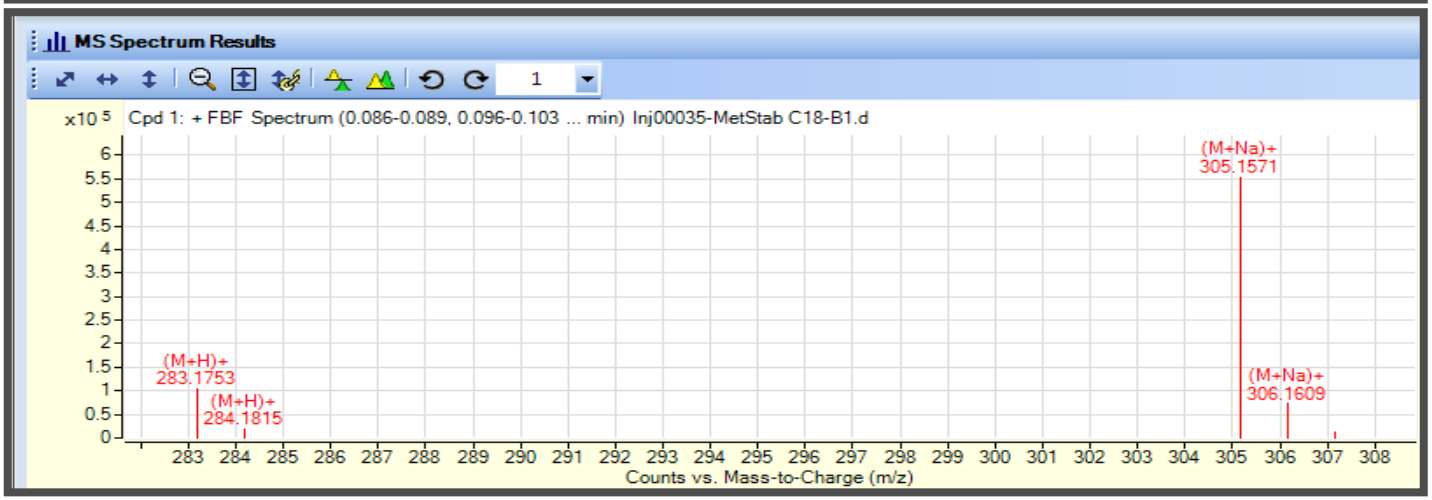
Automatically Show Columns

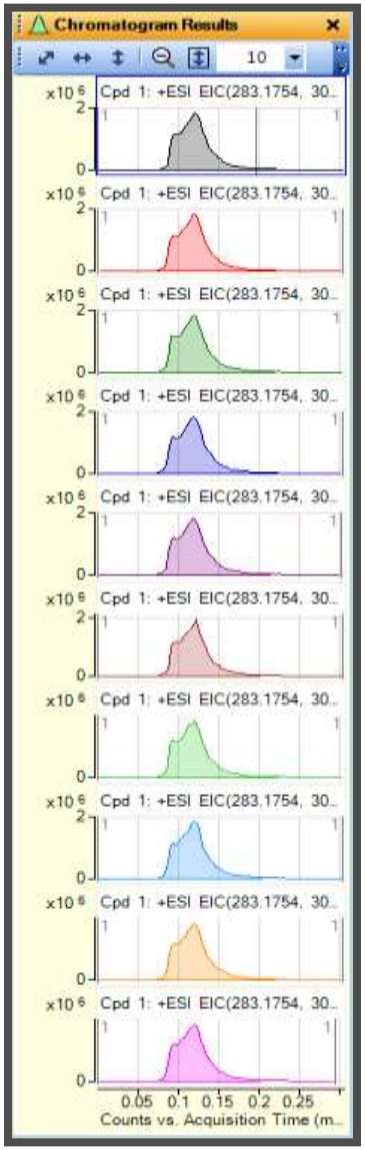
Group	RT (Tgt)	RT (med)	Found	Missed	%RSD (Tgt)	Height (med)	Mass (Tgt)	RSD (Mass, ppm)	RT (span)	RT (m
1	0.115	0.119	10	0	0.3	1817658	282.1683	0.41	0.004	0.151

Compound Details

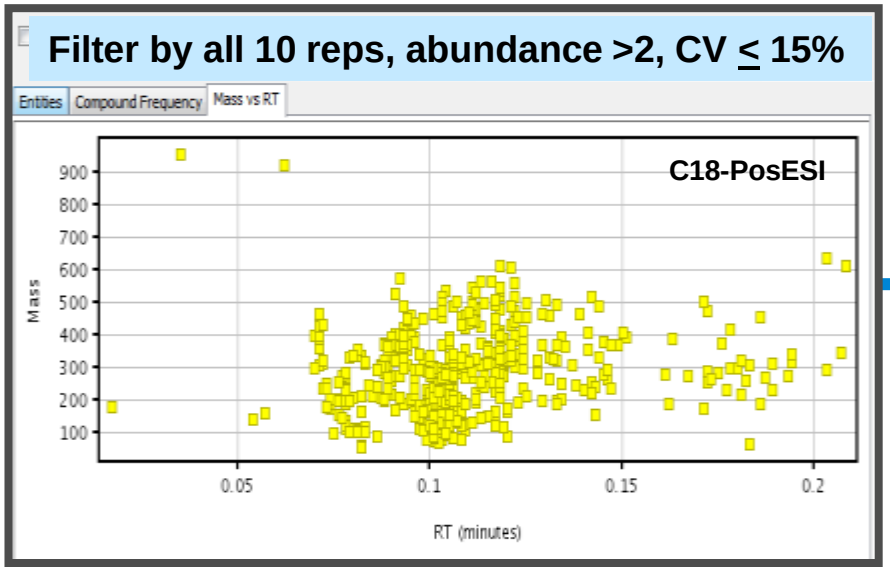
Automatically Show Columns

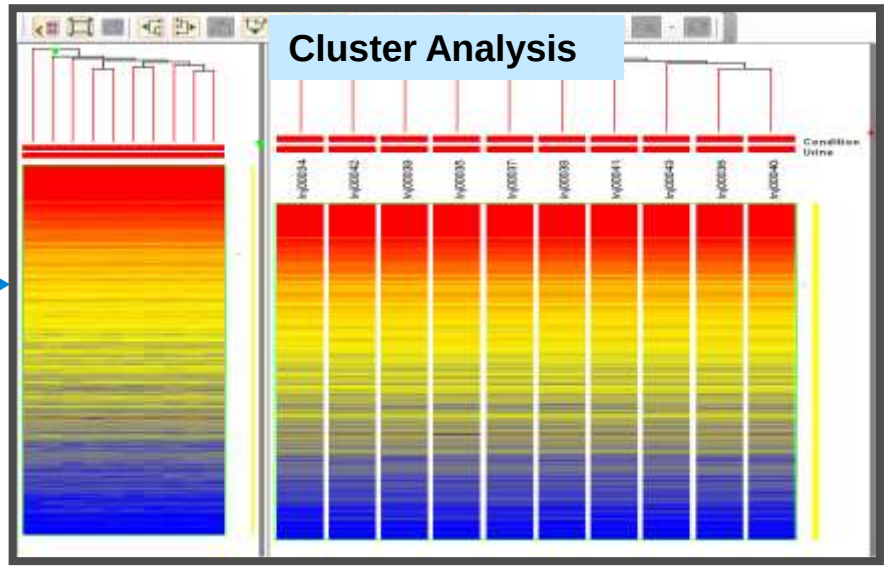
File	Score (Tgt)	Score (MFE)	Area	Height	Mass	Diff (Tgt, ppm)	RT	Ions	Z Count
Inj00034-MetStab C18-B1.d	99.62	100	4744036	1821442	282.1681	-0.71	0.12	5	1
Inj00035-MetStab C18-B1.d	99.01	100	4769787	1824081	282.1679	-1.46	0.119	5	1
Inj00036-MetStab C18-B1.d	99.88	100	4786381	1821291	282.1683	-0.06	0.118	5	1
Inj00037-MetStab C18-B1.d	99.81	100	4748902	1814025	282.1682	-0.3	0.119	5	1
Inj00038-MetStab C18-B1.d	99.43	80	4768715	1812210	282.168	-0.96	0.118	5	1
Inj00039-MetStab C18-B1.d	99.76	100	4724047	1935793	282.1681	-0.45	0.121	5	1
Inj00040-MetStab C18-B1.d	99.69	100	4763608	1798235	282.1681	-0.65	0.119	5	1
Inj00041-MetStab C18-B1.d	99.74	100	4744920	1825080	282.1681	-0.62	0.12	5	1
Inj00042-MetStab C18-B1.d	99.8	100	4749936	1798975	282.1682	-0.39	0.119	5	1
Inj00043-MetStab C18-B1.d	99.87	100	4649658	1756119	282.1682	-0.2	0.121	5	1



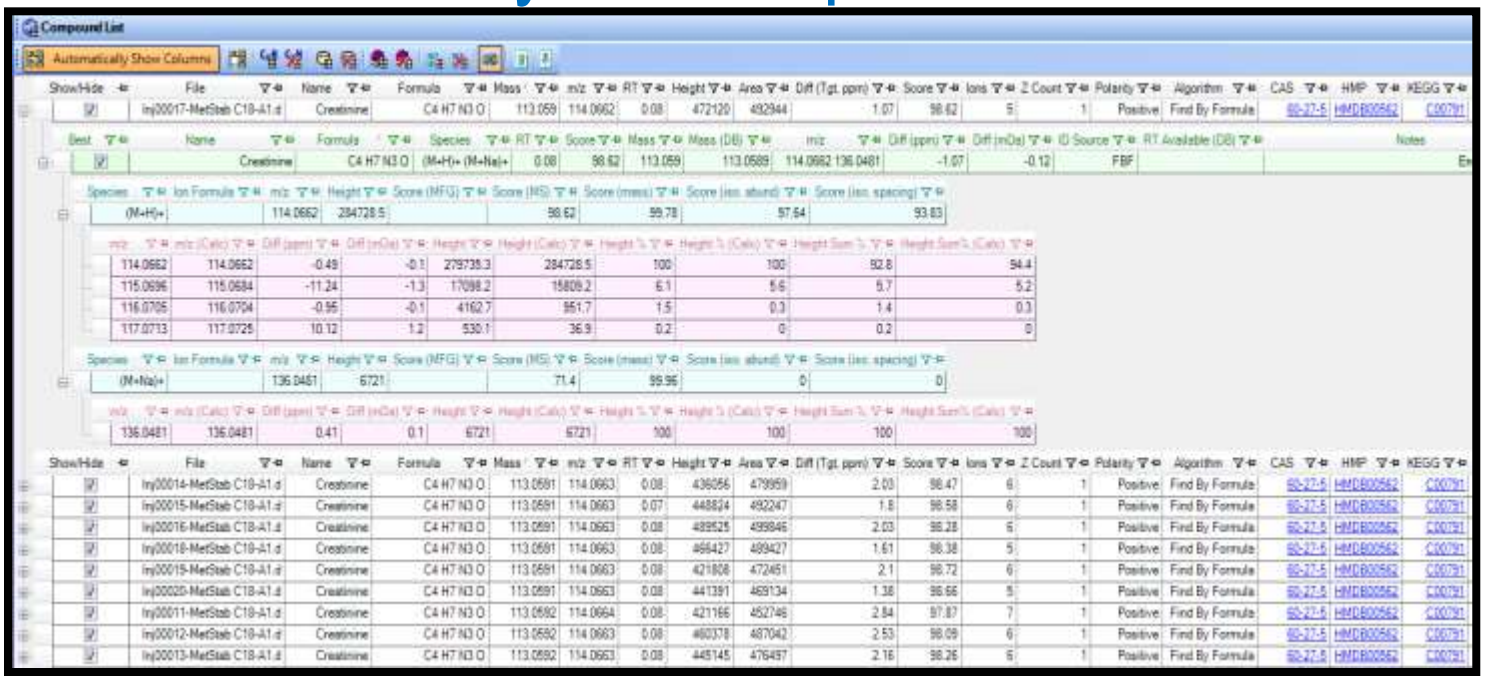


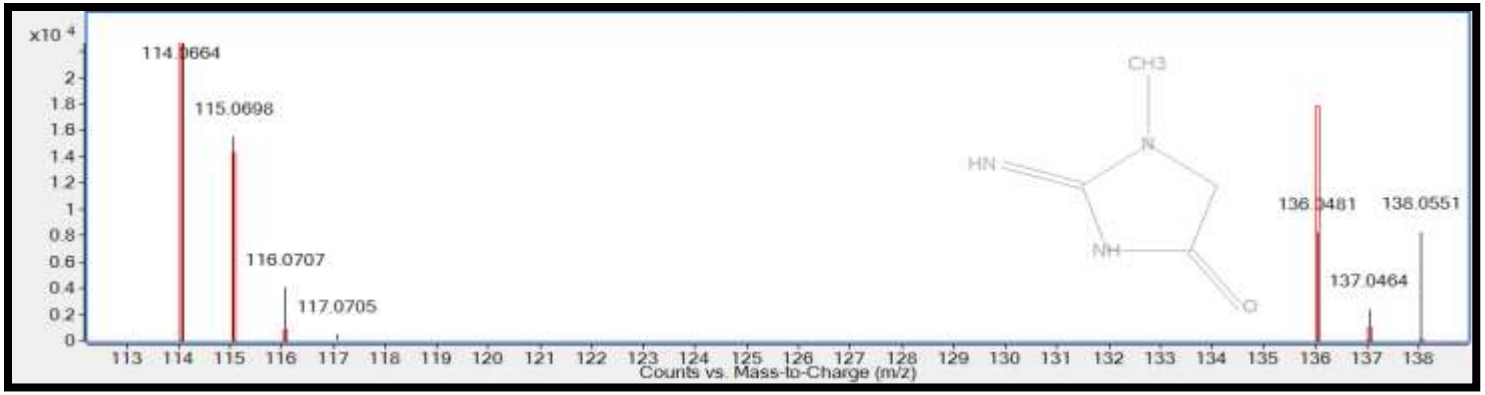
Mass Profiler Professional (MPP)

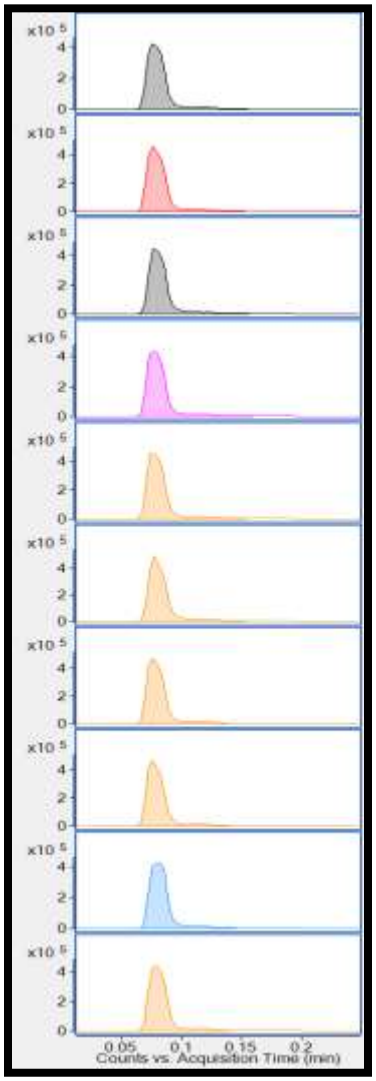




Annotation of Creatinine in Urine Samples by C18 RapidFire SPE/MS



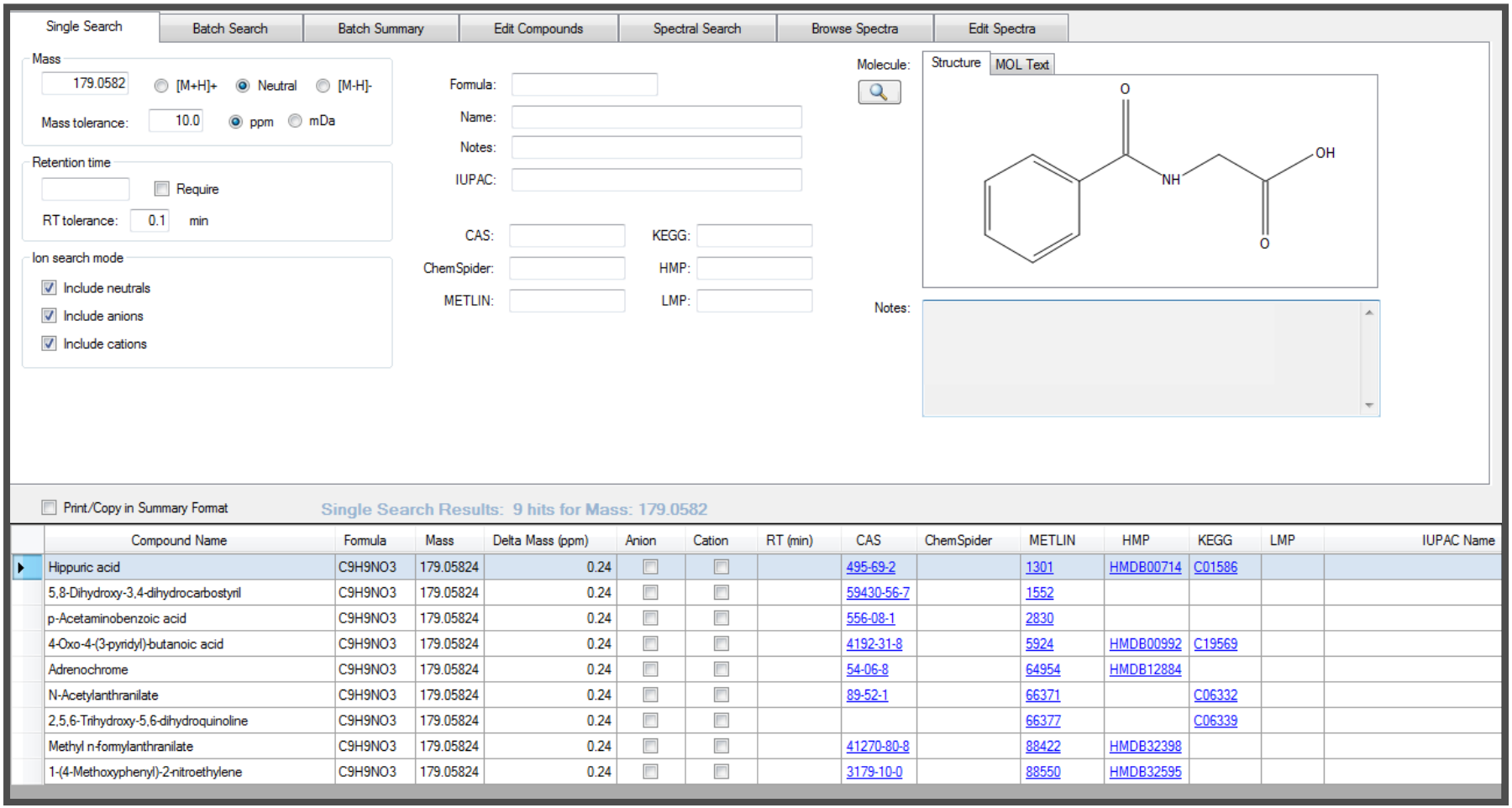




Select High Abundant Metabolites in Human Urine Annotated by SPE/MS

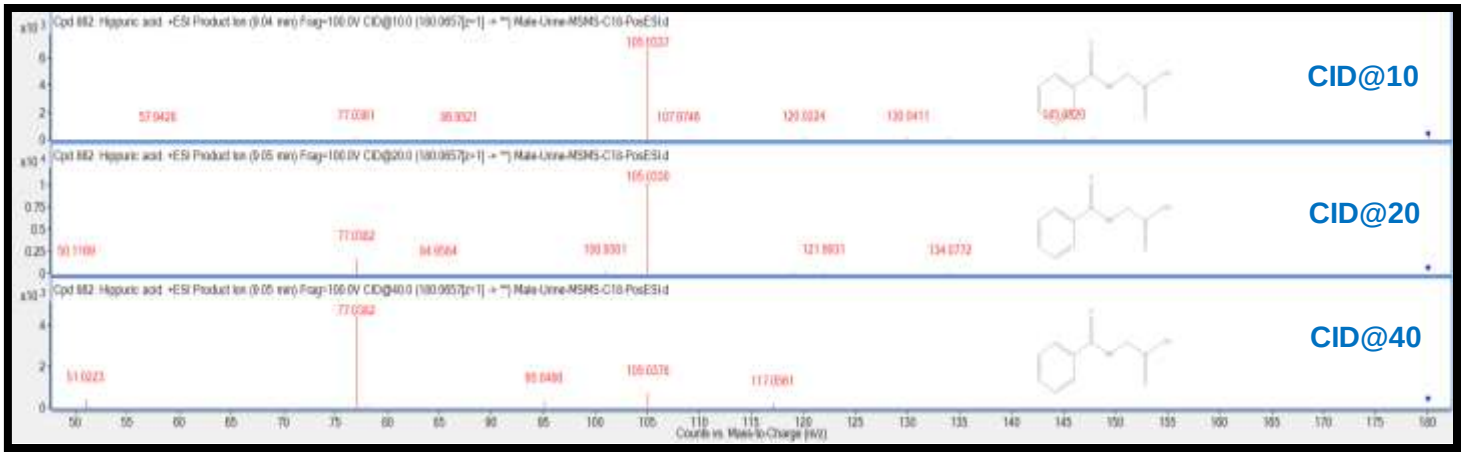
Compound	RF-C18 Male
Hippuric acid	5158031
Pirimicarb	3255466
Enprofylline	496924
Lys Asn Asp	420101
3-Hydroxyhippuric acid	410564
Pinacidil	359491
Nadolol	357228
4-(1-Hydroxy-2-(methylamino)ethyl)phenol	299538
Leu Gln Lys	287646
Triciribine	285932
3-Amino-2-naphthoic acid	277964
L-4-Hydroxy-3-methoxy-a-methylphenylalanine	266033
1-Methylxanthine	262927
AFMK	229545
Creatinine	225274
4-Chloroacetophenone	217597
Jasmolone glucoside	209979
Istamycin C1	192524
N-Acryloylglycine	183487
N2,N2-Dimethylguanosine	175089
N(alpha)-t-Butoxycarbonyl-L-leucine	171812
beta-Snyderol	135177
2-Methylguanosine	126988

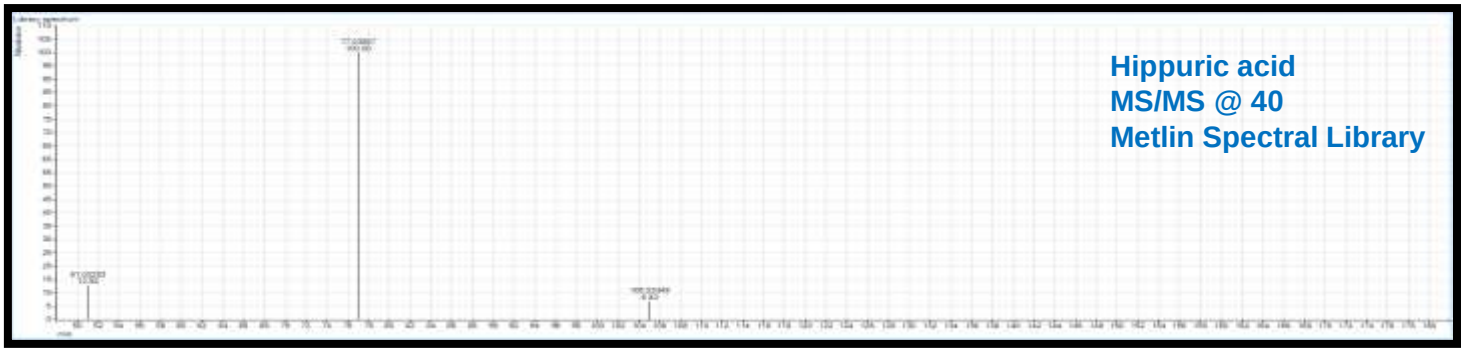
Metlin Library Check for Isomers/Isobars



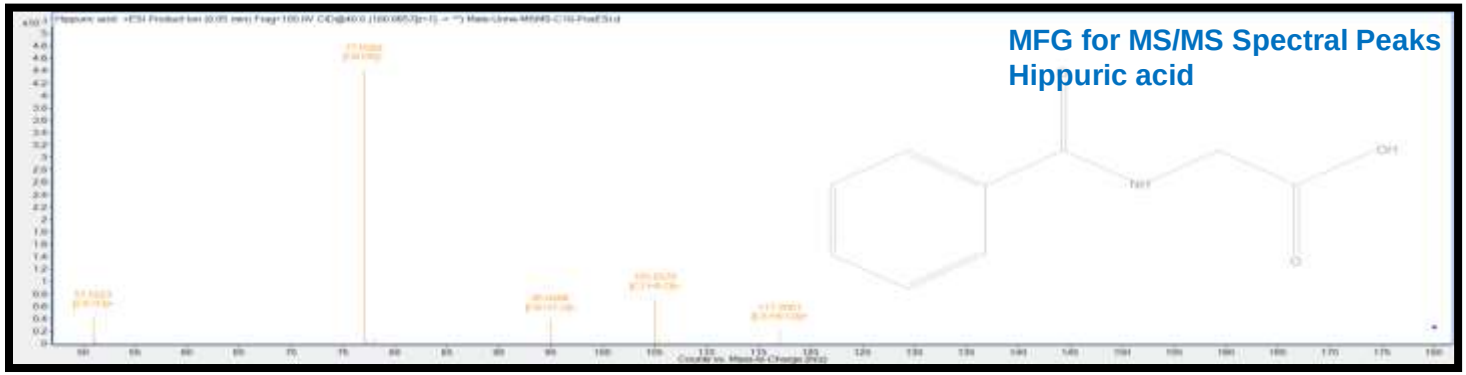
Compound Name	Formula	Mass	Delta Mass (ppm)	Anion	Cation	RT (min)	CAS	ChemSpider	METLIN	HMP	KEGG	LMP	IUPAC Name
Hippuric acid	C9H9NO3	179.05824	0.24				495-69-2		1301	HMDB00714	C01586		
5,8-Dihydroxy-3,4-dihydrocarbostyril	C9H9NO3	179.05824	0.24				59430-56-7		1552				
p-Acetaminobenzoic acid	C9H9NO3	179.05824	0.24				556-08-1		2830				
4-Oxo-4-(3-pyridyl)-butanoic acid	C9H9NO3	179.05824	0.24				4192-31-8		5924	HMDB00992	C19569		
Adrenochrome	C9H9NO3	179.05824	0.24				54-06-8		64954	HMDB12884			
N-Acetylanthranilate	C9H9NO3	179.05824	0.24				89-52-1		66371		C06332		
2,5,6-Trihydroxy-5,6-dihydroquinoline	C9H9NO3	179.05824	0.24						66377		C06339		
Methyl n-formylanthranilate	C9H9NO3	179.05824	0.24				41270-80-8		88422	HMDB32398			
1-(4-Methoxyphenyl)-2-nitroethylene	C9H9NO3	179.05824	0.24				3179-10-0		88550	HMDB32595			

LC/MS/MS Confirmation of Hippuric acid

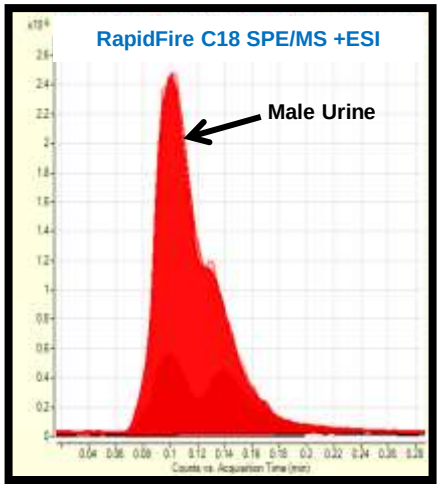




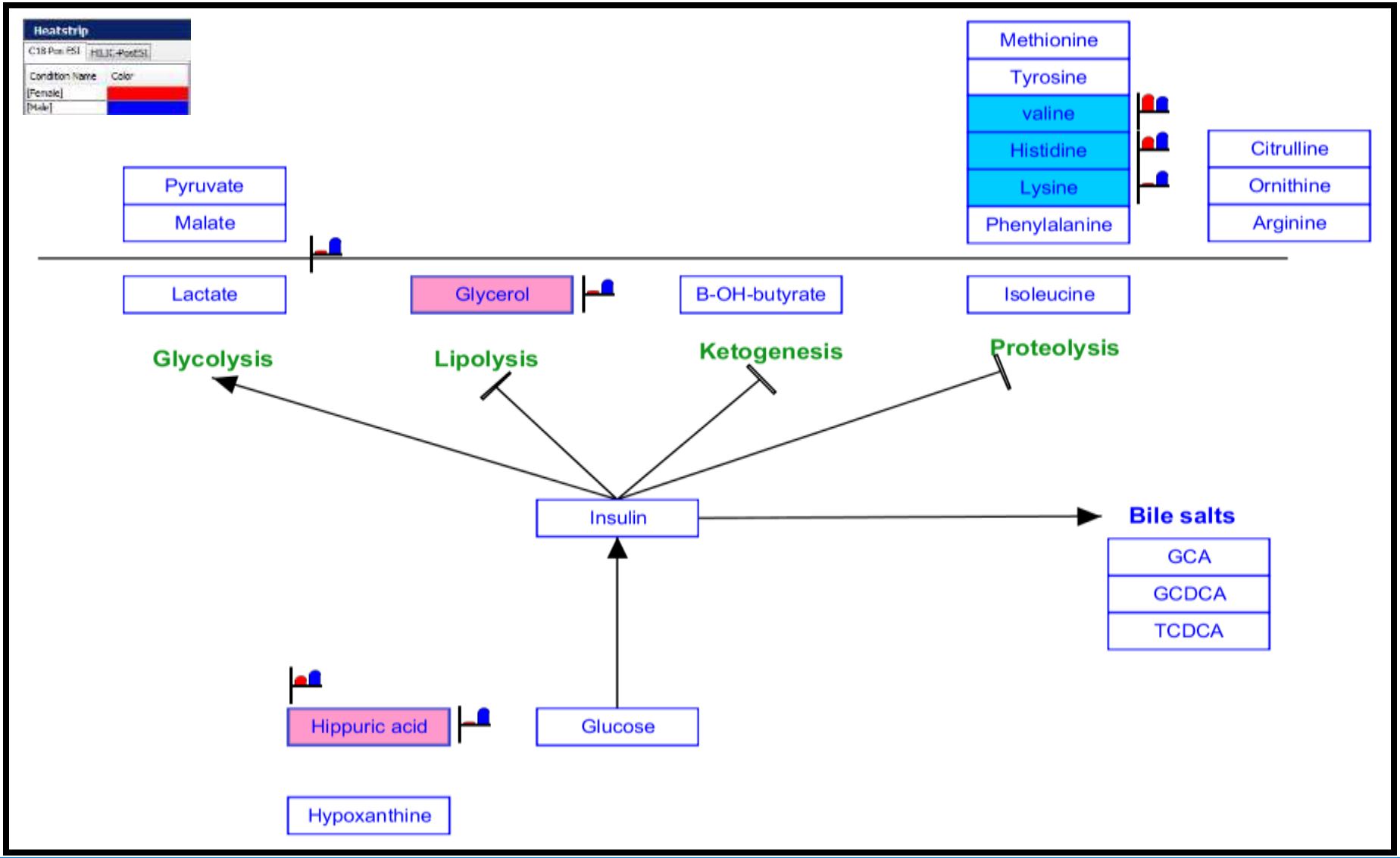
Hippuric acid
MS/MS @ 40
Metlin Spectral Library



MFG for MS/MS Spectral Peaks
Hippuric acid



Glucose Homeostasis Pathway (WP661_45308)

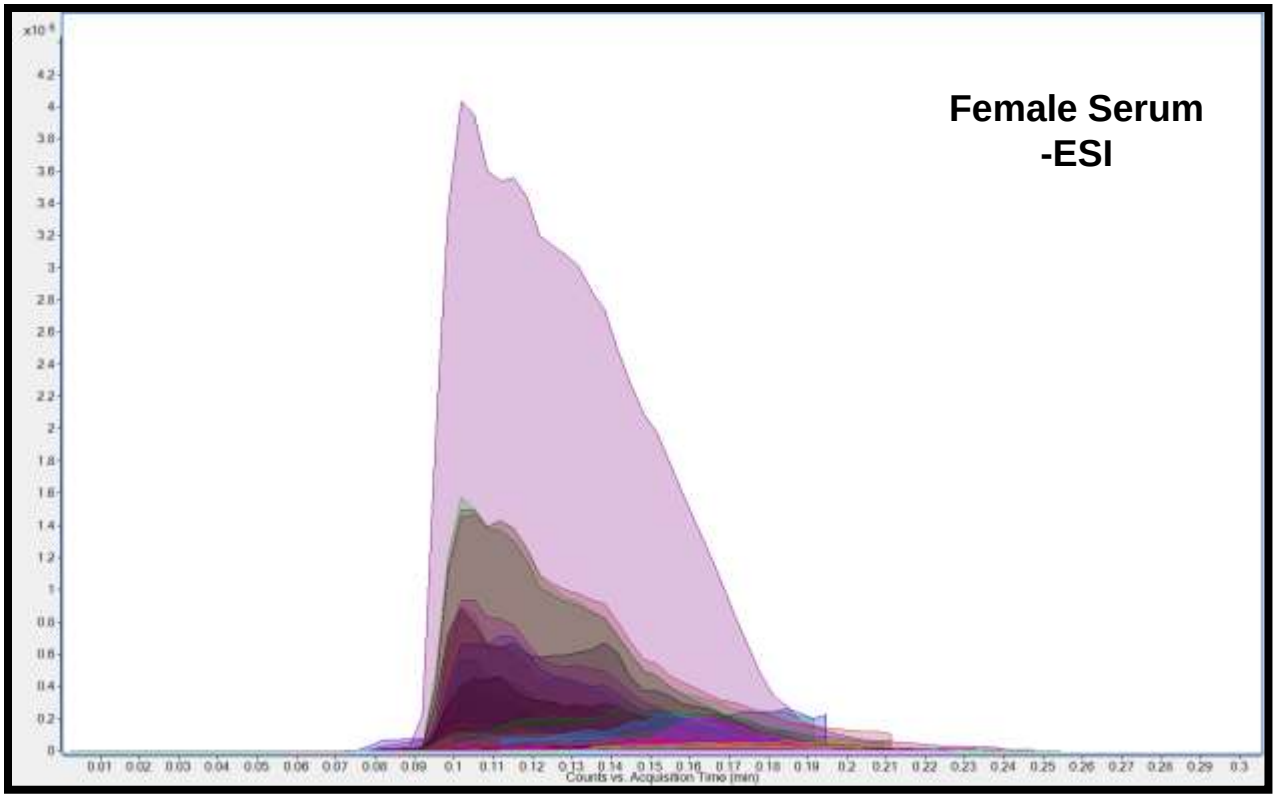


Human Serum Lipidomics Profiling: Sample Prep

- Human serum, collected and pooled from healthy subjects (males and females), was purchased from Innovative Research Inc. (Novi, MI)
- Filter 400µL serum through a 0.22µm Ultrafree-MC® centrifugal filter units (Millipore) by centrifugation @ 12,000xg for 20min @ 4°C
- Pipette 100µL filtered serum into a Protein LoBind® Eppendorf tube
- Add 700µL ice-cold Methanol:Isopropanol, 1%Acetic Acid, vortex 30sec
- Equilibrate for 10min at room temp and Centrifuge at ~12000xg, 20min, 4°C
- The organic supernatant represents the lipidomics sample
- Inject **10µL** for SPE/MS as well as LC/MS/MS analysis

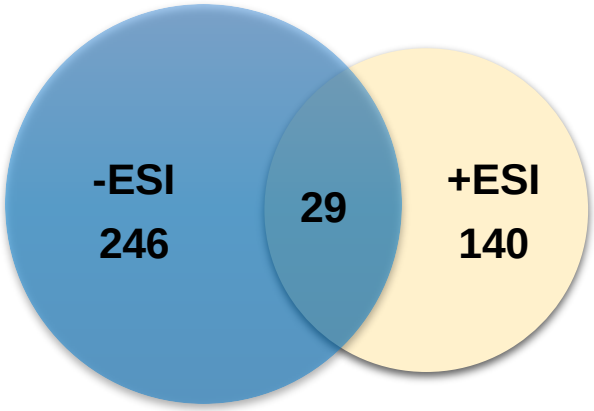
Human serum equivalent to 1.25µL was used for the lipidomics studies

EIC of a Single Injection of Serum Sample on C18 RapidFire SPE/MS



Lipidomics Profiling of Human Serum by C18 SPE/MS

- ✓ 169 lipids annotated in +ESI mode
- ✓ 275 lipids annotated in –ESI mode
- ✓ 29 annotated lipids common between +ESI and –ESI modes



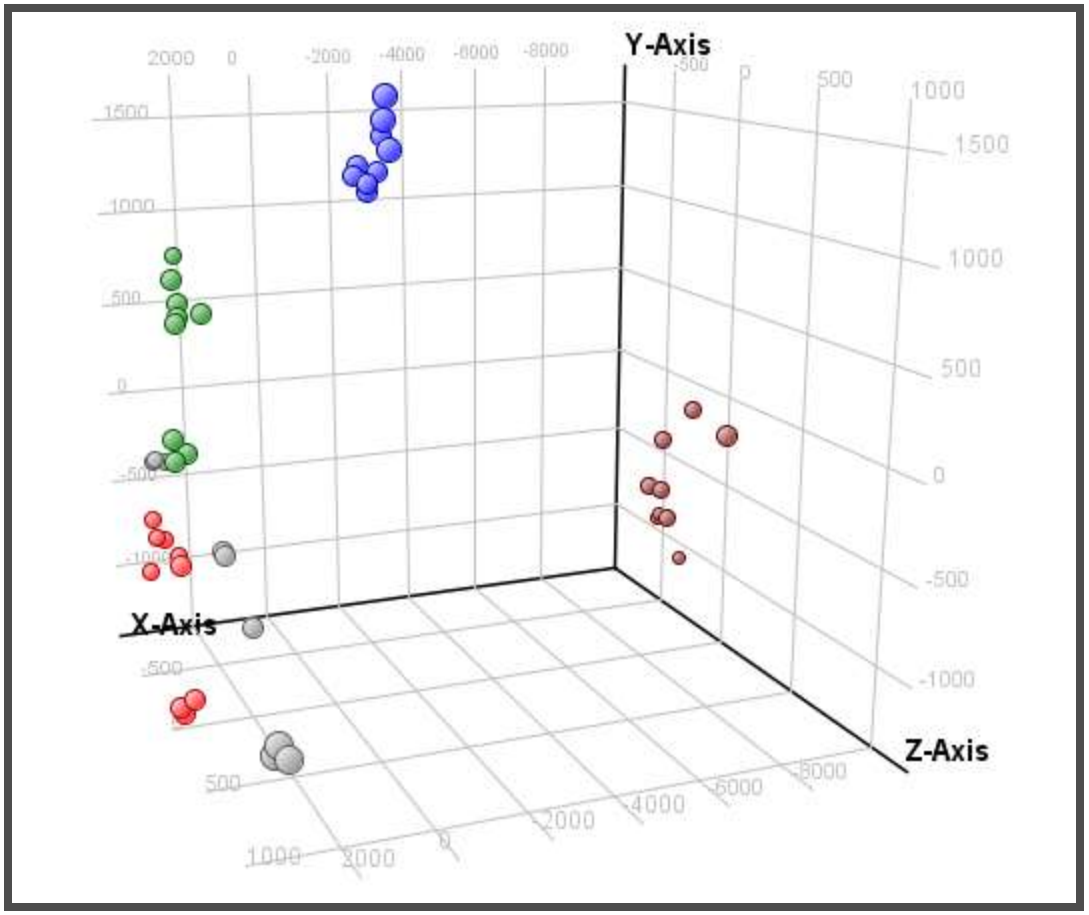
415 unique lipids

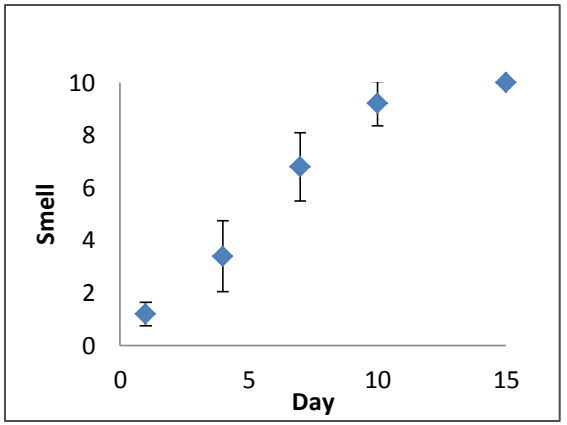
Milk Lipidomics Profiling: Sample Prep

- Pipette 150µL of milk into a Protein LoBind® Eppendorf tube
- Add 1200µL ice-cold Methanol:Isopropanol 1%Acetic Acid, vortex 30sec
- Equilibrate for 10min at room temp and Centrifuge at ~12000xg, 10min, 4°C
- Transfer the organic supernatant into a separate Protein LoBind® Eppendorf tube.
- Store at -80°C
- Inject 10µL of sample for SPE/MS

Milk equivalent to 1 µL was used for the lipidomics studies

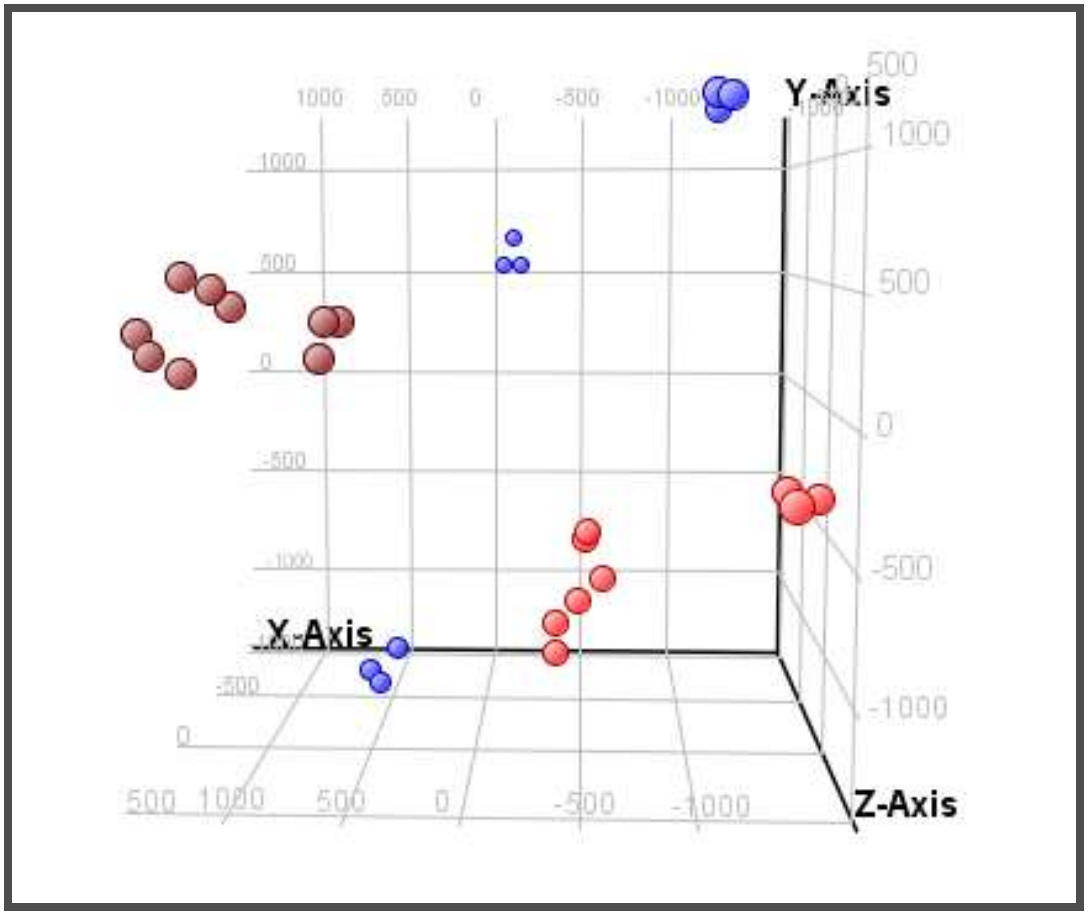
Whole Milk C4 SPE/MS (+ESI) – Time Course



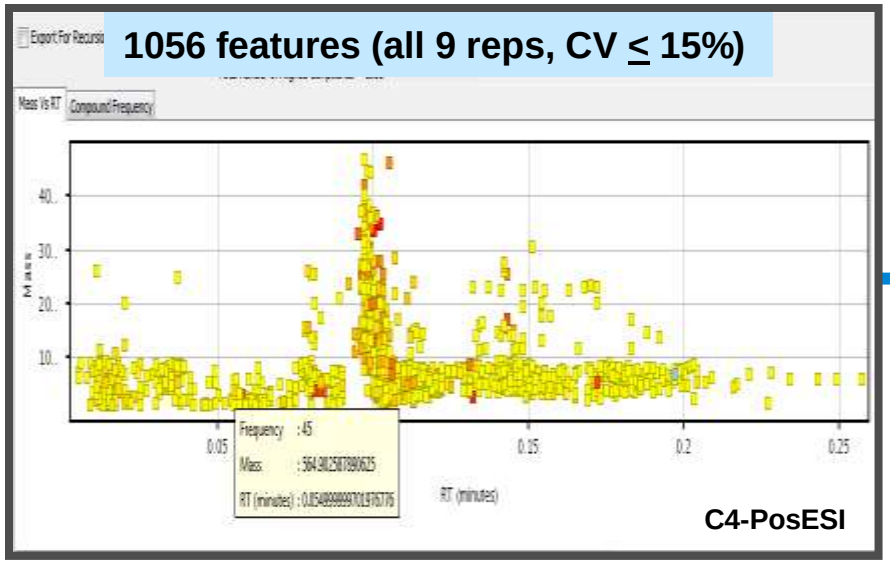


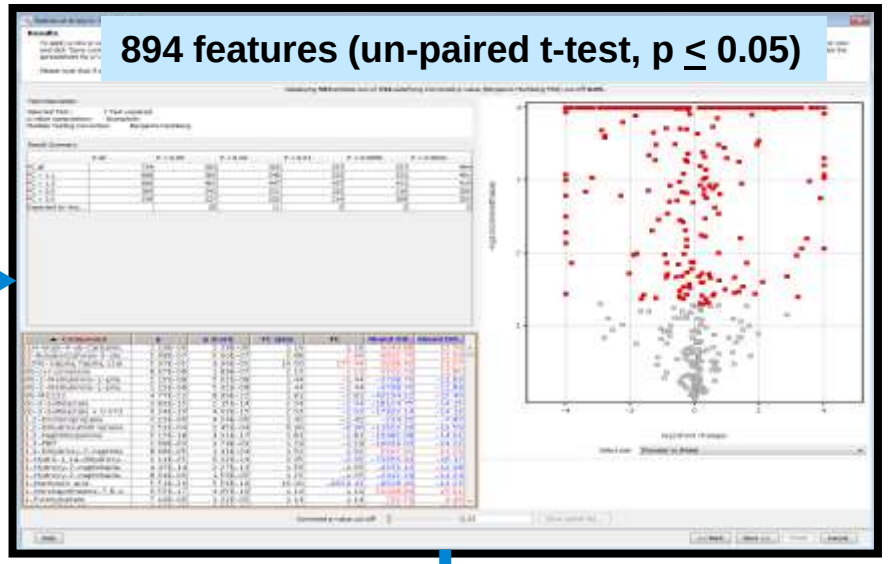
- Day1
- Day10
- Day15
- Day4
- Day7

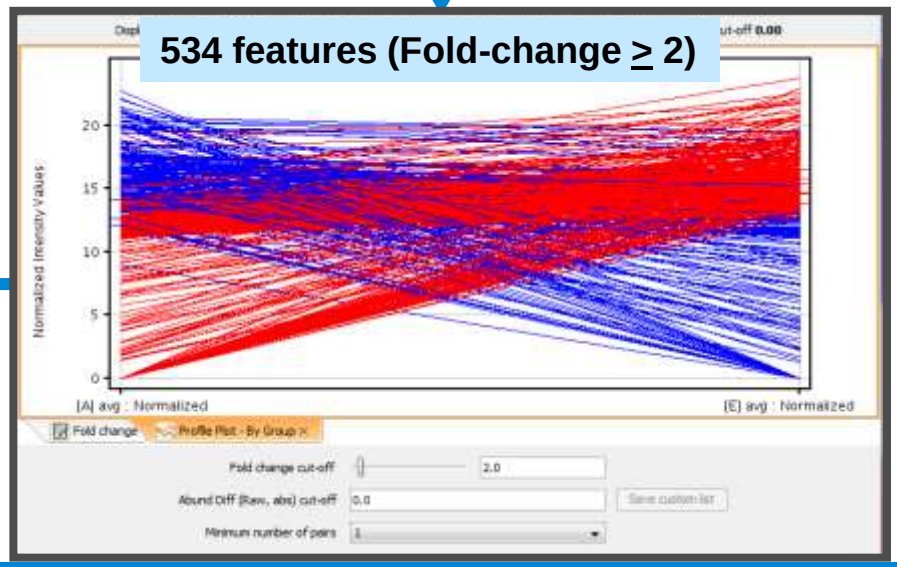
Whole Milk C4 SPE/MS (+ESI) – Days 1 to 7

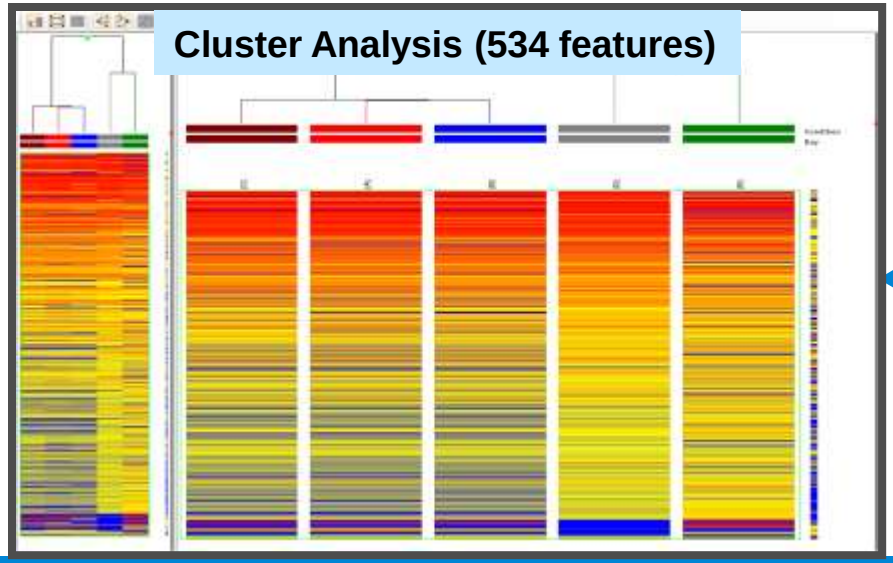


Mass Profiler Professional (MPP)









Summary

- Performed “high throughput” profiling of biological samples with RapidFire SPE/MS
- RapidFire data were fully integrated and compatible with Agilent software solutions (MassHunter, ProFinder, MPP, Metlin)
- Demonstrated reproducibility and fidelity of the current high throughput workflow for data processing and analysis
  - *Confirmed select metabolites/lipids by MS/MS analysis*

Take Home Message…

- Fast
  - Less than 15 secs per sample
  - Facilitates quick hypothesis verification
- Flexible
  - Ability to change method parameters quickly
  - Multiple binding chemistries available
- Reproducible
  - Replicates had less than a CV <15%
  - Extracted peaks were superimposable
- High fidelity of data
  - Annotations were confirmed by LC/MS/MS

Acknowledgements

I would like to thank Jeramie Watrous and Mohit Jain from University of California, San Diego.