Biomarker Identification in Nonalcoholic Fatty Liver Disease via GC/MS

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Introduction

- *Non-alcoholic fatty liver disease (NAFLD) is a major medical problem in developed countries*
- *NAFLD is associated with:*
  - *Increase obesity and insulin resistance*
  - *Metabolic syndrome*
- *NAFLD is influenced by lifestyle:*
  - *Exercise*
  - *Diet*
- *Research Samples & Study*
  - *168 Rodent samples (original sample set, Pegasus 4D Study)*
  - *Investigate two critical factors: 1) Increased fructose consumption and 2) Inadequate Cu intake*
  - *Complementary investigation: Utilize a more sensitive instrument (20 fg) with extended linear dynamic range (10^5) to further characterize the samples*
- *Analysis Objective: Identify potential NAFLD biomarkers in pooled rodent samples using high performance, benchtop GC-TOFMS (Figure 1)*

Figure 1. Pegasus® BT GC-TOFMS.

Workflow & Methods

- *Sample preparation*
  - *Dry: 1) Speed Vac & 2) Lyophilizer*
  - *Derivatize: 1) MEOX (30 µL, 20mg/mL) & 2) MTBSTFA (20 µL)*
  - *(For each step, heat at 60 °C for 1 hr)*
- *Analyze derivatized samples using high performance GC-TOFMS, ChromaTOF® brand software, and statistical processing software*
- *Instrument Parameters*

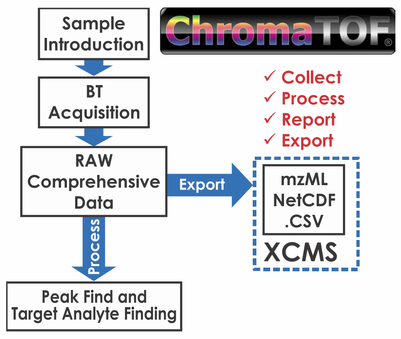


Figure 2. Analysis Workflow.

Table 1. GC-TOFMS Parameters

Gas Chromatograph	**Agilent 7890 with LECO L-Pal3 Autosampler**
Injection	*1µL, Split 20:1; 270 °C*
Carrier Gas	*He @ 0.8 mL/min, Constant Flow*
Column	*Rxi-5 Sil MS, 20 m x 0.18 mm i.d. x 0.18 µm (Restek, Bellefonte, PA, USA)*
Temperature Program	*60 °C (0.50 min), ramped 36 °C/min to 320 °C (3 min)*
Mass Spectrometer	**LECO Pegasus BT**
Ion Source Temperature	*250 °C*
Ionization Mode	*EI*
Mass Range (m/z)	*45-600*
Acquisition Rate	*10 spectra/s*

GC-TOFMS Results

Figure 3. AICs for 6 pooled liver samples.

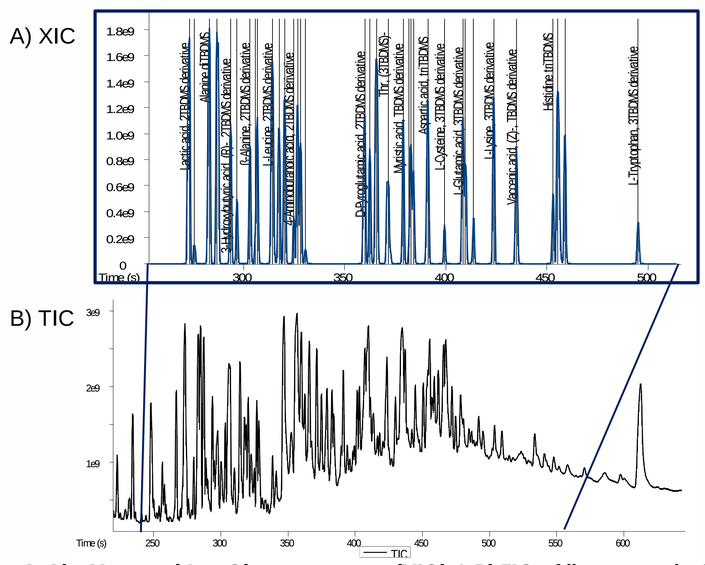


Figure 4. A) eXtracted Ion Chromatogram (XIC) & B) TIC of liver sample 6*.

Table 2. Representative analytes in liver sample 6

Name	*Formula*	*R.T. (s)*	*Area*	*Similarity*
Lactic acid, 2TBDMS derivative	$C_{15}H_{34}O_3Si_2$	273	3889358872	866
Glycolic acid, 2TBDMS derivative	$C_{14}H_{32}O_3Si_2$	276	392513491	865
Alanine diTBDMS	$C_{15}H_{35}NO_2Si_2$	283	2627670348	894
Glycine, 2TBDMS derivative	$C_{14}H_{33}NO_2Si_2$	287	2093903802	909
3-Hydroxybutyric acid, (R)-, 2TBDMS derivative	$C_{16}H_{36}O_3Si_2$	294	1338064236	911
L-2-Aminobutyric acid, 2TBDMS derivative	$C_{16}H_{37}NO_2Si_2$	297	980488165	889
ß-Alanine, 2TBDMS derivative	$C_{15}H_{35}NO_2Si_2$	303	811778683	856
Dimethylpentobarbital	$C_{13}H_{22}N_2O_3$	306	445555160	900
Valine diTBDMS	$C_{17}H_{39}NO_2Si_2$	307	2085644362	864
L-Leucine, 2TBDMS derivative	$C_{18}H_{41}NO_2Si_2$	314	3168854906	937
Niacinamide, TBDMS derivative	$C_{12}H_{20}N_2OSi$	318	2512779336	922
Isoleucine, 2TBDMS derivative	$C_{18}H_{41}NO_2Si_2$	320	2056141263	943
4-Aminobutanoic acid, 2TBDMS derivative	$C_{16}H_{37}NO_2Si_2$	325	460048677	831
Uracil, 2TBDMS derivative	$C_{16}H_{32}N_2O_2Si_2$	327	3063305152	929
L-Proline, 2TBDMS derivative	$C_{17}H_{37}NO_2Si_2$	328	2171908625	918
Itaconic acid, 2TBDMS derivative	$C_{17}H_{34}O_4Si_2$	331	211731605	864
D-Pyroglutamic acid, 2TBDMS derivative	$C_{17}H_{35}NO_3Si_2$	360	2010302971	854
Met, (2TBDMS)	$C_{17}H_{39}NO_2SSi_2$	363	795167581	868

Name	*Formula*	*R.T. (s)*	*Area*	*Similarity*
L-Serine, 3TBDMS derivative	$C_{21}H_{49}NO_3Si_3$	366	1804043501	910
Thr, (3TBDMS)-	$C_{22}H_{51}NO_3Si_3$	372	1907273568	870
Myristic acid, TBDMS derivative	$C_{20}H_{42}O_2Si$	379	2626194161	906
Alanylglycine, 2TBDMS derivative	$C_{17}H_{38}N_2O_3Si_2$	382	246632471	802
L-Phenylalanine, 2TBDMS derivative	$C_{21}H_{39}NO_2Si_2$	383	861540012	913
Malic acid, 3TBDMS derivative	$C_{22}H_{48}O_5Si_3$	384	1330224256	852
Aspartic acid, triTBDMS	$C_{22}H_{49}NO_4Si_3$	391	1444900099	897
L-Cysteine, 3TBDMS derivative	$C_{21}H_{49}NO_2SSi_3$	399	533411147	943
L-Glutamic acid, 3TBDMS derivative	$C_{23}H_{51}NO_4Si_3$	408	1539429832	919
Palmitic Acid, TBDMS derivative	$C_{22}H_{46}O_2Si$	410	1491608293	833
L-Asparagine, 3TBDMS derivative	$C_{22}H_{50}N_2O_3Si_3$	414	1044773406	873
L-Lysine, 3TBDMS derivative	$C_{24}H_{56}N_2O_2Si_3$	424	1125874542	917
Vaccenic acid, (Z)-, TBDMS derivative	$C_{24}H_{46}O_2Si$	435	4203899325	837
Histidine triTBDMS	$C_{24}H_{51}N_3O_2Si_3$	453	1428415311	895
Arachidonic Acid, TBDMS derivative	$C_{26}H_{46}O_2Si$	455	1921897209	899
L-Tyrosine, 3TBDMS derivative	$C_{27}H_{53}NO_3Si_3$	459	1990728852	922
Uric acid, 4TBDMS derivative	$C_{29}H_{68}N_4O_3Si_4$	488	239470552	898
L-Tryptophan, 3TBDMS derivative	$C_{29}H_{54}N_2O_2Si_3$	495	1189857045	866
Cholesterol, TBDMS derivative	$C_{33}H_{66}OSi$	630	17933415	823

Ave. = 886/1000

GC-TOFMS Results

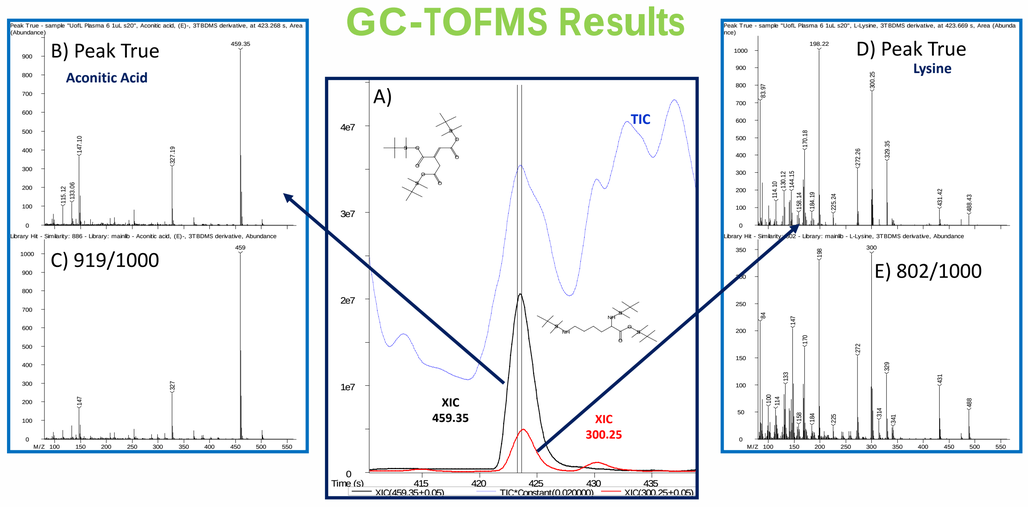


Figure 5. A) ChromaTOF deconvolution results - liver sample 6. Peak True (deconvoluted) and library spectra for aconitic acid (B,C) and lysine (D,E).

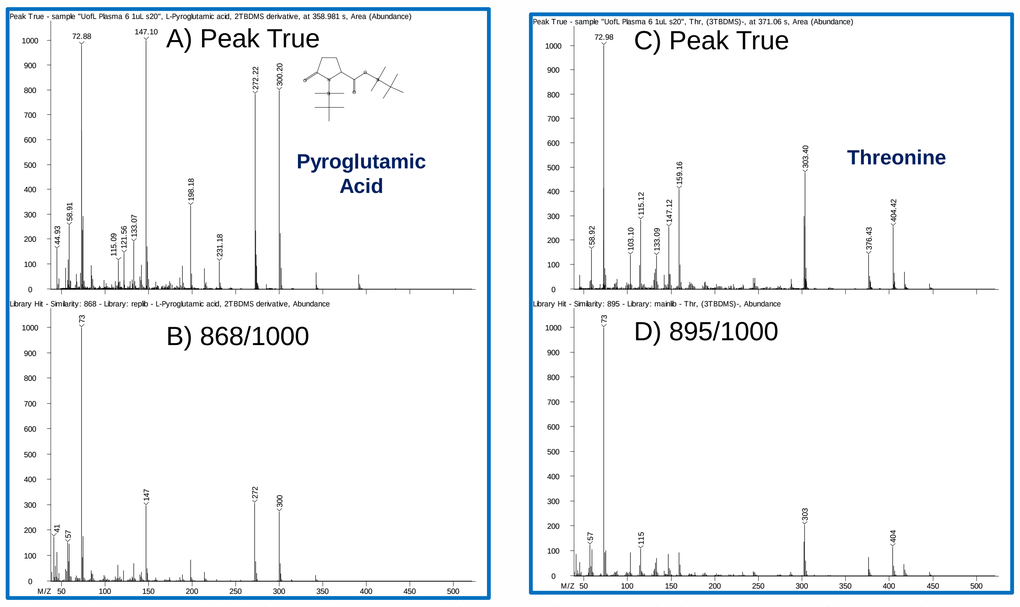


Figure 6. Pegasus BT high quality spectral data: Peak True and library spectra for pyroglutamic acid (A,B) and threonine (C,D).

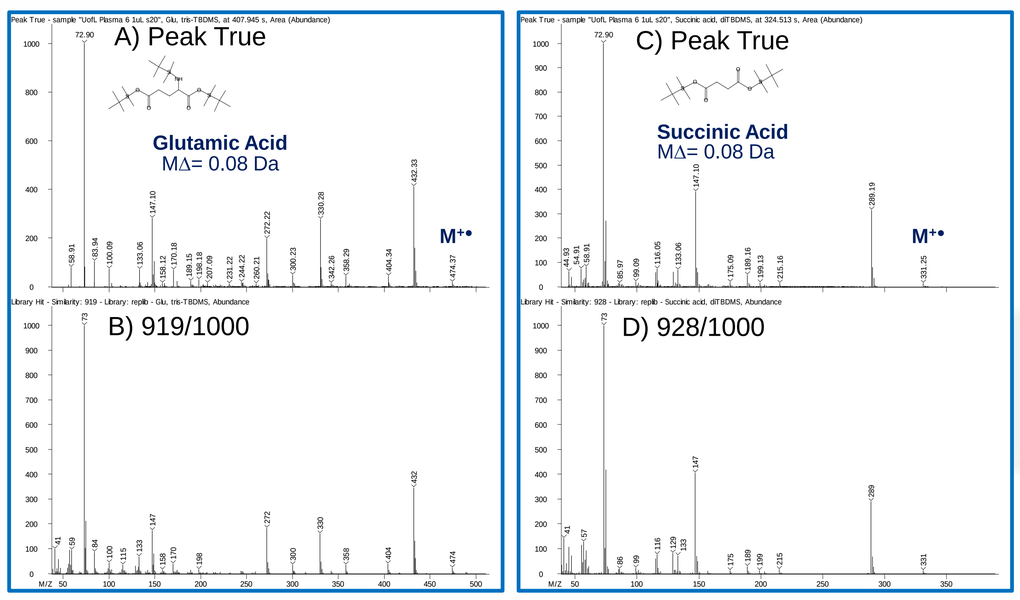


Figure 7. Pegasus BT high performance spectral data with mass delta values <0.10 Da): Peak True and library spectra for glutamic acid (A,B) and succinic acid (C,D).

Pegasus BT Data Export and Statistical Analysis

- *Raw data was exported (netCDF) using ChromaTOF (Figure 8)*
- *Data was uploaded and processed using XCMS online (xcmsonline.scripps.edu)*
- *PCA and Cloud Plot data (Figure 9) were used to find features that differentiated samples*
- *Potential biomarker metabolites for NAFLD were confidently identified using ChromaTOF (Figure 10)*
- *Potential biomarkers characterized included: Amino acids (e.g., histidine, alanine. valine, etc.), fatty acids (e.g., arachidonic acid) and misc. compounds (e.g., uridine, 7-methylxanthine)*

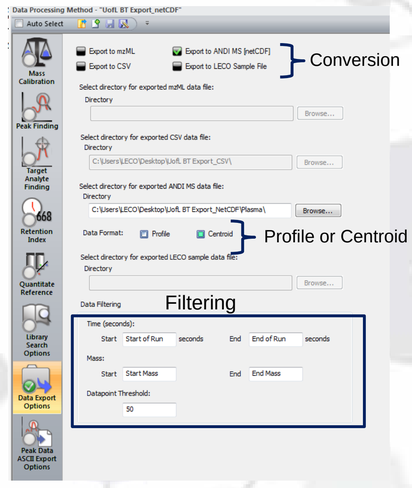


Figure 8. ChromaTOF raw data export feature.

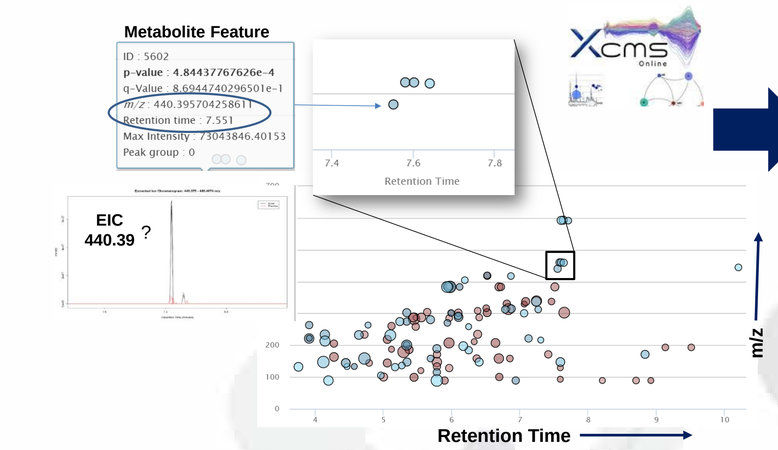
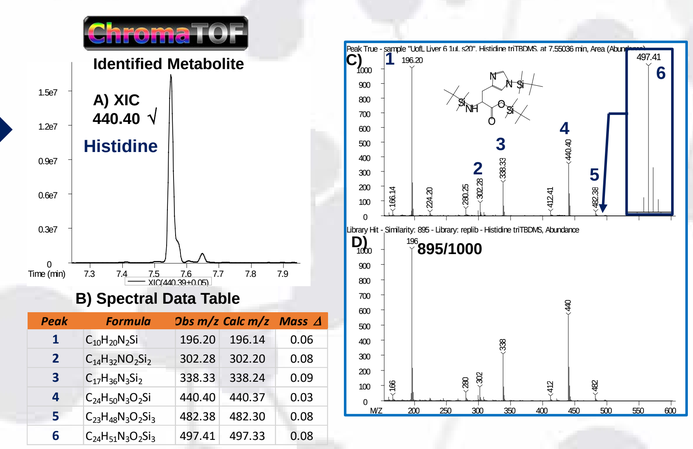


Figure 9. XCMS online Cloud Plot (m/z vs retention time) showing a metabolite feature with a p-value of 4.84e-4, retention time of 7.55 min. and EIC m/z = 440.396.



Peak	*Formula*	*Obs m/z*	*Calc m/z*	*Mass Δ*
1	$C_{10}H_{20}N_2Si$	196.20	196.14	0.06
2	$C_{14}H_{32}NO_2Si_2$	302.28	302.20	0.08
3	$C_{17}H_{36}N_3Si_2$	338.33	338.24	0.09
4	$C_{24}H_{50}N_3O_2Si$	440.40	440.37	0.03
5	$C_{23}H_{48}N_3O_2Si_3$	482.38	482.30	0.08
6	$C_{24}H_{51}N_3O_2Si_3$	497.41	497.33	0.08

Figure 10. ChromaTOF - A) XIC 440.40, B) Generated formulas for fragment and molecular ions, C,D) Peak True and library match data for histidine.

Delivering the Right Results

Summary

- *The Pegasus BT instrument produced high quality data*
- *This data was exported into statistical processing software for sample differentiation and feature selection*
- *ChromaTOF brand software was used to fully characterize metabolites using spectral similarity searches against large, well-established databases and formula determinations using data with mass deltas<0.10 Da*