Effects of Increased Fructose Consumption and Inadequate Copper Intake on the Pathogenesis of Nonalcoholic Fatty Liver Disease (NAFLD): A Feces, Plasma and Liver Metabolomics Study

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Introduction

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Inflammation, oxidative stress, dyslipidemia, insulin resistance, and obesity are key clinical risk factors for the progression of nonalcoholic fatty liver disease (NAFLD).[1] Interactions between diet, liver, and immune system play an important role in this disease.[2] Increased fructose consumption and inadequate copper intake are two critical factors that may contribute to metabolic syndrome and lead to the development of diseases such as NAFLD.[3] For example, increased consumption of high fructose corn syrup has been associated with liver fibrosis severity in subjects with NAFLD.[4]

Metabolomics is a viable method for identifying compounds associated with NAFLD. In this investigation, high quality data facilitate the identification of key metabolites differentiating normal versus diseased species.

Objectives

- To further investigate the relationship between copper deficiency and fructose-induced fatty liver disease.
- Implement the use of enhanced, comprehensive chromatography and high resolution time-of-flight mass spectrometry for separation and confident identification of metabolites in samples

Technology

GERSTEL

Sample Introduction

Data Acquisition & Processing

LECO Pegasus® GC-HRT & HRT 4D with ChromaTOF-HRT® Software

Instrument Attribute	Value
Mass Accuracy	<1 ppm
Mass Range	10-1500 m/z
Resolving Power	up to 50,000
Data Acquisition Speed	Up to 200 sps
Ionization	EI, PCI

Experimental

Samples

84 Samples, six groups:

Group	Copper Level	High Fructose Diet
1	Adequate	Yes
2	Low	Yes
3	Supplemental	Yes
4	Adequate	no
5	Low	no
6	Supplemental	no

Samples were extracted with methanol and water and derivatized using a two step process:

- Methoximation (30 µL of MEOX, 60°C, 1 hr)
- Silylation (20 µL MTBSTFA, 60°C, 1 hr)

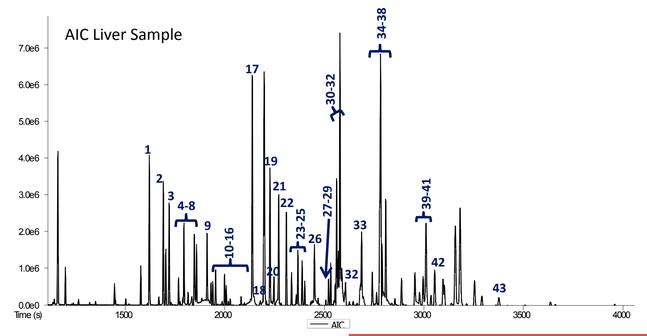
Instrument Parameters

Gas Chromatograph	Agilent 7890 with Dual Stage Quad Jet Modulator and MPS2 Autosampler
Injection	1 µL, Split 20:1, 280°C (1µL Splitless for CI)
Carrier Gas	He @ 1.0 ml/min, Constant Flow
Column 1	Rxi-5 ms, 60 m x 0.25 mm i.d. x 0.25 µm (Restek, Bellefonte, PA, USA)
Column 2	Rxi-17 sil ms, 0.60 m x 0.25 mm x 0.25 µm coating (Restek, Bellefonte, PA, USA)
Temperature Program	0.5 min at 60°C, ramped 5°C/min to 270°C, held 6 min Secondary oven maintained +10°C relative to primary oven
Thermal Modulation (GCxGC)	4 s with temperature maintained +15°C relative to 2nd oven
Mass Spectrometer	**LECO Pegasus® HRT 4D**
Ion Source Temperature	250 °C (EI); 200 °C (CI)
Acquisition Mode	High Resolution, R = 25,000 (FWHM)
Ionization Mode	EI and or CI (Reagent Gas: 5% NH_3 in CH_4)
Mass Range (m/z)	30-510 (EI); 60-1500 (CI)
Acquisition Rate	10 spectra/s (200 spectra/s GCxGC)

Processing & Statistical Methods

- Experimental data was processed using ChromaTOF brand software (Peak Selection, Compound Identification). Compounds were rapidly and confidently identified using high quality data and effective software tools.
- Statistical processing was performed using MetPP Software (Alignment, Metabolite Quantification).
- 38 compounds were identified as significant in determining the relationship between copper and fructose intake. These included acids (lactic acid, 3-hydroxybutyric acid, 2-aminobutyric acid), amino acids (aspartic acid, cysteine, proline, glutamic acid, ornithine), diacids (succinic acid, malic acid, fumaric acid), and fatty acids (myristic, pentanedecanoic acid, palmitic acid).

GC-HRT



Peak	Name	R.T. (s)	Formula	Area	Similarity
1	Lactic acid (2TBDMS)	1620.9	$C_{15}H_{34}O_3Si_2$	26499926	907
2	Alanine (2TBDMS)	1691	$C_{15}H_{35}NO_2Si_2$	17955434	954
3	Glycine (2TBDMS)	1720.3	$C_{14}H_{33}NO_2Si_2$	15469625	943
4	3-Hydroxybutyric acid, (R)- (2TBDMS)	1767.7	$C_{16}H_{36}O_3Si_2$	3058664	902
5	2-Aminobutyric acid (2TBDMS)	1789.5	$C_{16}H_{37}NO_2Si_2$	1105272	850
6	β-Alanine (2TBDMS)	1834.3	$C_{15}H_{35}NO_2Si_2$	1585626	845
7	Urea (2TBDMS)	1846.9	$C_{13}H_{32}N_2OSi_2$	10424563	852
8	Valine (2TBDMS)	1857.9	$C_{17}H_{39}NO_2Si_2$	9208842	928
9	Leucine (2TBDMS)	1911	$C_{18}H_{41}NO_2Si_2$	12991642	942
10	Niacinamide (2TBDMS)	1930.8	$C_{17}H_{26}N_2OSi$	5050307	886
11	Isoleucine (2TBDMS)	1953.87	$C_{18}H_{41}NO_2Si_2$	5083582	939
12	γ-Aminobutyric acid (2TBDMS)	1986.73	$C_{16}H_{37}NO_2Si_2$	618481	787
13	Uracil (2TBDMS)	1998.7	$C_{16}H_{32}N_2O_2Si_2$	6226121	906
14	Proline (2TBDMS)	2006.9	$C_{17}H_{37}NO_2Si_2$	4513020	923
15	Itaconic acid (2TBDMS)	2074.5	$C_{15}H_{30}O_4Si_2$	233343	823
16	Caffeine	2128.6	$C_8H_{10}N_4O_2$	550298	894
17	Glycerol (3TBDMS)	2138.2	$C_{15}H_{posO_3Si_3$	42397566	942
18	Methyl palmitate	2187.47	$C_{17}H_{34}O_2$	252777	808
19	Pyroglutamic acid (2TBDMS)	2227	$C_{15}H_{29}NO_3Si_2$	23335396	943
20	Methionine (2TBDMS)	2246.9	$C_{17}H_{39}NO_2SiS_2$	4318712	908
21	Serine (3TBDMS)	2270.8	$C_{21}H_{49}NO_3Si_3$	19457429	906

Peak	Name	R.T. (s)	Formula	Area	Similarity
22	Threonine (3TBDMS)	2309.3	$C_{22}H_{51}NO_3Si_3$	15473469	901
23	Myristic acid (TBDMS)	2367.2	$C_{20}H_{42}O_2Si$	9439940	942
24	Phenylalanine (2TBDMS)	2388.4	$C_{21}H_{39}NO_2Si_2$	6978920	923
25	Malic acid (3TBDMS)	2401.8	$C_{22}H_{54}O_5Si_3$	5064551	868
26	Aspartic acid (3TBDMS)	2450.1	$C_{22}H_{51}NO_4Si_3$	11161151	906
27	Pentadecanoic acid (TBDMS)	2474.4	$C_{21}H_{44}O_2Si$	443342	889
28	Cysteine (3TBDMS)	2507.3	$C_{21}H_{49}NO_2SSi_3$	1225312	905
29	Hypoxanthine (2TBDMS)	2532.14	$C_{17}H_{32}N_4OSi_2$	8955364	934
30	Glutamic acid (3TBDMS)	2570.5	$C_{23}H_{51}NO_4Si_3$	7372099	886
31	Ornithine (3TBDMS)	2577	$C_{23}H_{53}N_2O_2Si_3$	3862368	869
32	Palmitic acid (TBDMS)	2578.8	$C_{22}H_{46}O_2Si$	67425484	948
33	Asparagine (3TBDMS)	2607	$C_{22}H_{52}N_2O_3Si_3$	3432828	885
34	Lysine (3TBDMS)	2687.32	$C_{24}H_{56}N_2O_2Si_3$	10165440	919
35	Glutamine (3TBDMS)	2741	$C_{23}H_{52}N_2O_4Si_3$	5838457	866
36	Linoelaidic acid (TBDMS)	2778.27	$C_{24}H_{46}O_2Si$	14548967	901
37	13-Octadecenoic acid, (E)- (TBDMS)	2782.6	$C_{24}H_{48}O_2Si$	65579108	950
38	Vaccenic acid, (E)-, (TBDMS)	2790.2	$C_{24}H_{48}O_2Si$	21296722	913
39	Stearic acid (TBDMS)	2808.9	$C_{24}H_{50}O_2Si$	24751093	947
40	Histidine (3TBDMS)	2978.5	$C_{24}H_{51}N_3O_2Si_3$	3631498	923
41	Arachidonic acid (TBDMS)	3011	$C_{26}H_{46}O_2Si$	13123283	922
42	Tyrosine (3TBDMS)	3054.6	$C_{27}H_{53}NO_3Si_3$	11693508	929
43	Docosenoxic (TBDMS)	3376.9	$C_{28}H_{56}O_2Si$	2179290	812

Figure 1. AIC and Table of representative compounds in liver tissue. Metabolites were automatically identified through ChromaTOF-HRT processing.

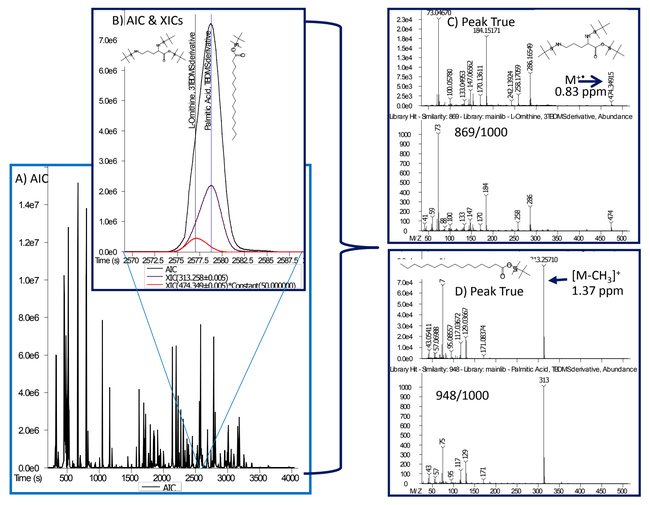


Figure 2. AIC and XIC expansion of liver tissue demonstrating benefits of High Resolution Deconvolution. Peak true and library mass spectral data for ornithine & palmitic acid.

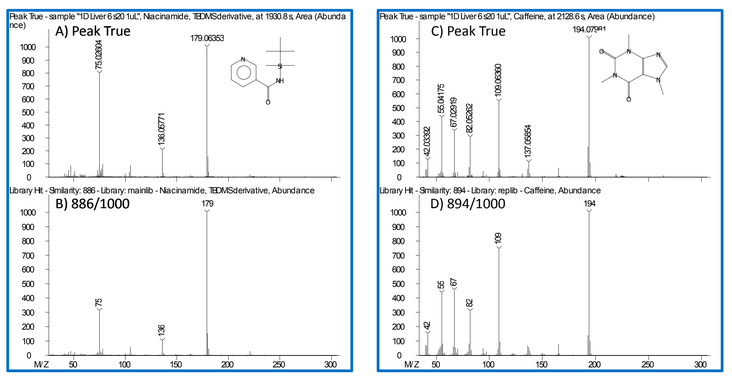


Figure 3. Peak true and library mass spectral data for niacinamide and caffeine in liver tissue.

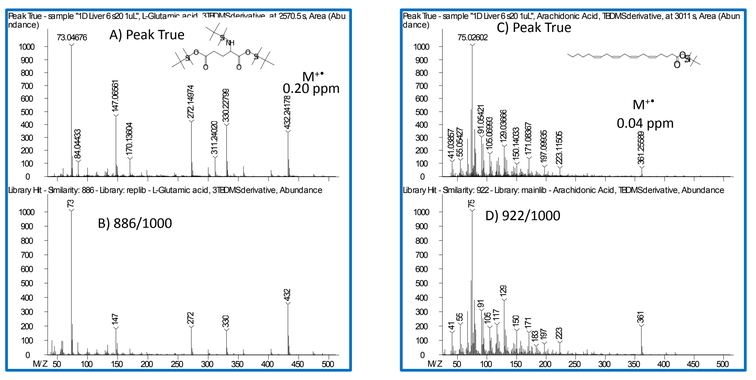
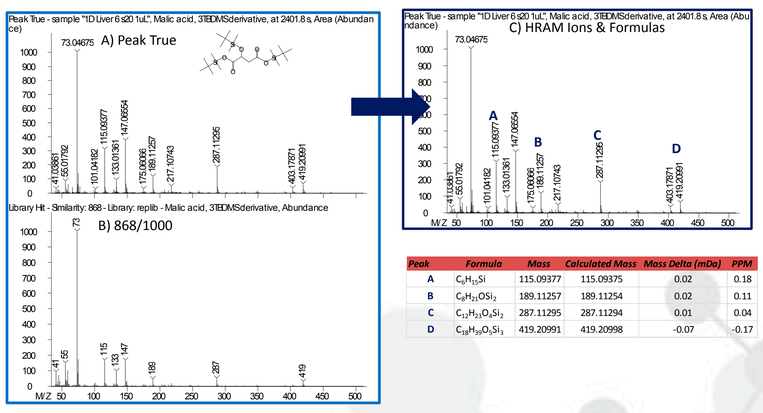


Figure 4. Peak true and library mass spectral data for glutamic acid and arachidonic acid in liver tissue. High resolution accurate mass ions (HRAM) and mass accuracy values for the molecular ions.



Peak	Formula	Mass	Calculated Mass	Mass Delta (mDa)	PPM
A	$C_5H_{15}Si$	115.09377	115.09375	0.02	0.18
B	$C_6H_{21}OSi_2$	189.11257	189.11254	0.02	0.11
C	$C_{12}H_{23}O_4Si_2$	287.11295	287.11294	0.01	0.04
D	$C_{18}H_{39}O_5Si_3$	419.20991	419.20998	-0.07	-0.17

Figure 5. Peak true and library mass spectral data for malic acid in liver tissue. HRAM fragment ions, formulas, and mass accuracy values.

Complementary EI and CI-HRT Data

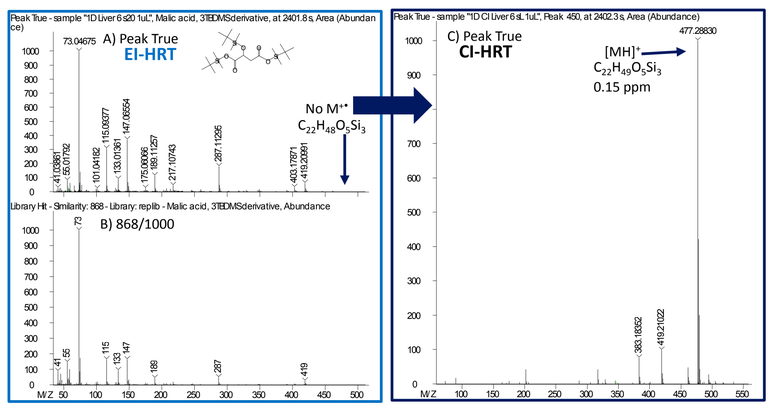
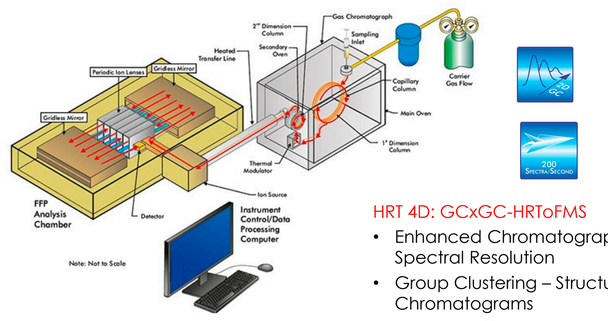


Figure 7. Peak True EI-HRT, Library, and CI-HRT Data for Malic Acid.

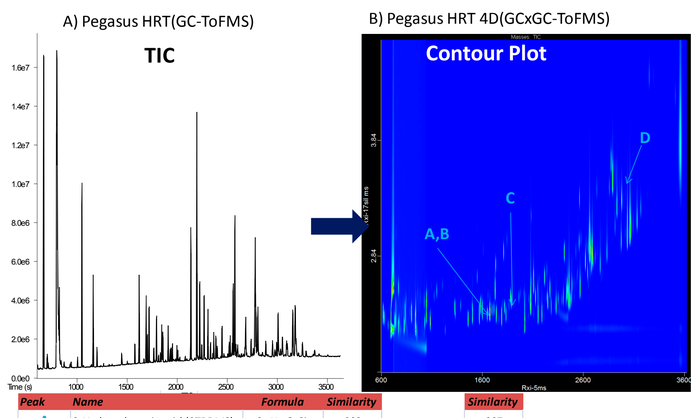
GCxGC-HRT



HRT 4D: GCxGC-HRToFMS

- Enhanced Chromatographic and Mass Spectral Resolution
- Group Clustering – Structured Chromatograms
- Improved Characterization of Compounds

Figure 8. GCxGC-HRT 4D Instrument



Peak	Name	Formula	Similarity	Similarity
A	3-Hydroxybutyric acid (2TBDMS)	$C_{16}H_{36}O_3Si_2$	902	907
B	L-2-Aminobutyric acid (2TBDMS)	$C_{16}H_{37}NO_2Si_2$	850	915
C	Fumaric acid (2TBDMS)	$C_{16}H_{32}O_4Si_2$	676	899
D	Pantothenic acid (2TBDMS)	$C_{27}H_{59}NO_5Si_3$	612	833

Figure 9. A) GC-HRT and B) GCxGC-HRT Data. Table illustrating the benefits of combining enhanced chromatographic and mass spectral resolution for confident identification of metabolites differentiating groups (e.g., fumaric acid).

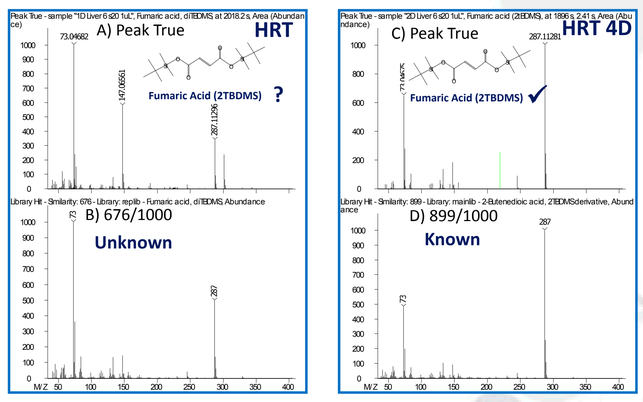


Figure 10. HRT and HRT 4D Mass Spectral Data for Fumaric Acid (Unknown → Known)

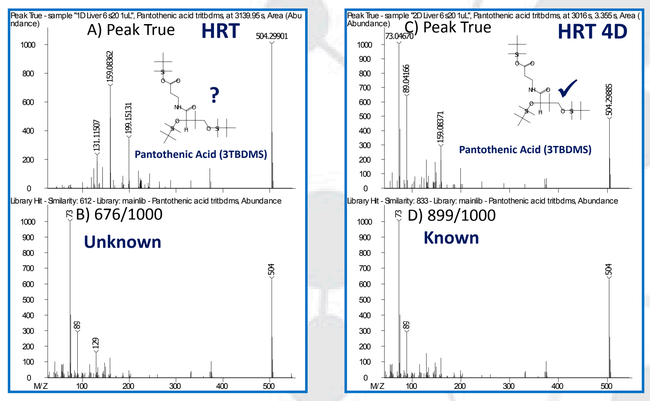


Figure 11. HRT and HRT 4D Mass Spectral Data for Pantothenic Acid (Unknown → Known)

Conclusion

- 38 metabolites detected with significant abundance alteration between the study groups were easily and automatically identified using high quality data and superior processing tools
- Enhanced two-dimensional chromatographic resolution, spectral similarity searches of large, well-established databases, and formula determinations using high resolution accurate mass ions increased confidence in metabolite characterization.

References
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