Untargeted Investigation of Non-Alcoholic Fatty Liver Disease Using Effective Mutiplatform GC-MS Instrumentation

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Introduction

Background

- Non-alcoholic fatty liver disease (NAFLD) is a major medical issue in developed countries.
- NAFLD is the most common liver disease and parallels the obesity epidemic in the US.
- The purpose of this investigation is to utilize high performance Gas Chromatography Time-of-Flight Mass Spectrometry (GC-TOFMS) for increased analysis throughput of complex samples, followed by comprehensive, multidimensional chromatography and high resolution Time-of-Flight mass spectrometry (GCxGC-HRT) for discovery and confirmation of NAFLD biomarkers.

Methods

Samples

Rodents were fed different levels of copper with either distilled water or 30% fructose (w/v) for a period of several weeks. The animals were then euthanized and liver/plasma samples collected for TOFMS analysis. Metabolites were extracted with a water/methanol solvent mixture, dried extensively, and derivatized via a standard two-step procedure (MEOX, MTBSTFA). This methodology minimized sugar interferences (e.g., monosaccharides and disaccharides), and produced stable derivatives of alcohols, lactones, acids, amino acids, diacids, fatty acids, and sterols that could be easily analyzed by GC-TOFMS.

Instrumentation

Samples were analyzed using a combination of nominal and high resolution GC-TOFMS and GCxGC-HRT. Complementary electron and chemical ionization methods were also employed to assist in identification of metabolites. Acquired data were processed using untargeted methods for peak determination, spectral deconvolution, accurate mass formula determination, and spectral similarity searches for confident metabolite identifications.

Table 1. GC-TOFMS Parameters

Gas Chromatograph	**Agilent 7890 with LECO L-Pal3 Autosampler**
Injection	1 µL, 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

Figure 1. Pegasus® BT GC-TOFMS.

Table 2. GC and GCxGC-HRT Parameters

Gas Chromatograph	**Agilent 7890 with LECO L-Pal3 Autosampler**
Injection	1µL, Split 20:1; 280 °C (1 µL, Splitless for CI)
Carrier Gas	He @ 1.0 mL/min, Constant Flow
Column 1	Rxi-5 MS, 30 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 i.d. x 0.25 µm (Restek, Bellefonte, PA, USA)
Temperature Program	60 °C (0.50 min), ramped 5 °C/min to 270 °C (6 min) Secondary oven maintained +10 °C relative to primary oven
Thermal Modulation (GCxGC)	4s with temperature maintained +15 °C relative to secondary oven
Mass Spectrometer	**LECO Pegasus HRT**
Ion Source Temperature	250 °C (EI); 200 °C (CI)
Ionization Mode	EI and 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)

Figure 2. Pegasus GC-HRT 4D.

Throughput: GC-TOFMS

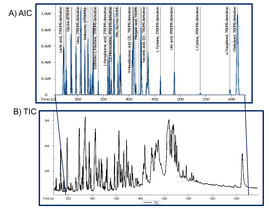


Figure 3: A) eXtracted Ion Chromatogram (XIC) and B) TIC of a liver sample.

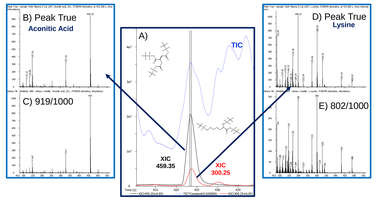


Figure 4. A) ChromaTOF® brand software deconvolution results - liver sample. Peak True (deconvoluted) and library spectra for aconitic acid (B,C) and lysine (D,E).

Table 3. Representative analytes in liver sample (Similarity Ave. = 886)

Metabolite	*Formula*	*R.T.(Sec)*	*Similarity*
Lactic acid, 2TBDMS derivative	$C_{15}H_{34}O_3Si_2$	273	866
Glycolic acid, 2TBDMS derivative	$C_{14}H_{32}O_3Si_2$	276	865
Alanine 6TBDMS	$C_{21}H_{53}NO_2Si_4$	283	894
Glycine, 2TBDMS derivative	$C_{14}H_{33}NO_2Si_2$	287	909
3-Hydroxybutyric acid, (R)-, 2TBDMS derivative	$C_{16}H_{34}O_3Si_2$	294	911
L-2-Aminobutyric acid, 2TBDMS derivative	$C_{16}H_{35}NO_2Si_2$	297	889
ß-Alanine, 2TBDMS derivative	$C_{15}H_{35}NO_2Si_2$	303	856
Dimethylpentobarbital	$C_{13}H_{24}N_2O_3$	306	900
Valine dTBDMS	$C_{17}H_{39}NO_2Si_2$	307	864
L-Leucine, 2TBDMS derivative	$C_{18}H_{41}NO_2Si_2$	314	917
Niacinamide, TBDMS derivative	$C_{13}H_{24}N_2OSi$	318	922
Isoleucine, 2TBDMS derivative	$C_{18}H_{41}NO_2Si_2$	320	943
4-Aminobutanoic acid, 2TBDMS derivative	$C_{16}H_{39}NO_2Si_2$	325	831
Uracil, 2TBDMS derivative	$C_{16}H_{30}N_2O_2Si_2$	327	929
L-Proline, 2TBDMS derivative	$C_{17}H_{37}NO_2Si_2$	328	918
Itaconic acid, 2TBDMS derivative	$C_{17}H_{32}O_4Si_2$	331	864
D-Pyroglutamic acid, 2TBDMS derivative	$C_{17}H_{35}NO_3Si_2$	360	854
Met, (2TBDMS)	$C_{17}H_{39}NO_2SSi_2$	363	868

Metabolite	*Formula*	*R.T.(Sec)*	*Similarity*
L-Serine, 3TBDMS derivative	$C_{21}H_{53}NO_3Si_3$	366	910
Thr, (3TBDMS)	$C_{22}H_{55}NO_3Si_3$	372	870
Myristic acid, TBDMS derivative	$C_{20}H_{42}O_2Si$	379	906
Alanylglycine, 2TBDMS derivative	$C_{17}H_{38}N_2O_3Si_2$	382	802
L-Phenylalanine, 2TBDMS derivative	$C_{24}H_{39}NO_2Si_2$	383	913
Malic acid, 3TBDMS derivative	$C_{22}H_{52}O_5Si_3$	384	852
Aspartic acid, triTBDMS	$C_{26}H_{59}NO_4Si_3$	391	897
L-Cysteine, 3TBDMS derivative	$C_{21}H_{53}NO_2SSi_3$	399	943
L-Glutamic acid, 3TBDMS derivative	$C_{26}H_{59}NO_4Si_3$	408	919
Palmitic Acid, TBDMS derivative	$C_{22}H_{46}O_2Si$	410	833
L-Asparagine, 3TBDMS derivative	$C_{25}H_{58}N_2O_3Si_3$	414	873
L-Lysine, 3TBDMS derivative	$C_{27}H_{64}N_2O_2Si_3$	424	917
Vaccenic acid, (Z)-, TBDMS derivative	$C_{24}H_{48}O_2Si$	435	837
Histidine triTBDMS	$C_{27}H_{57}N_3O_2Si_3$	453	895
Arachidonic Acid, TBDMS derivative	$C_{26}H_{46}O_2Si$	455	899
L-Tyrosine, 3TBDMS derivative	$C_{27}H_{53}NO_3Si_3$	459	922
Uric acid, 4TBDMS derivative	$C_{29}H_{64}N_4O_3Si_4$	488	898
L-Tryptophan, 3TBDMS derivative	$C_{29}H_{54}N_2O_2Si_3$	495	866
Cholesterol, TBDMS derivative	$C_{33}H_{58}OSi$	630	823

Statistical Analysis: Features → Metabolites

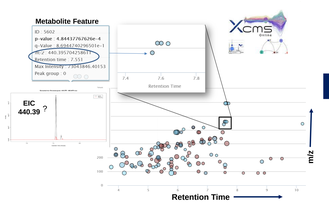
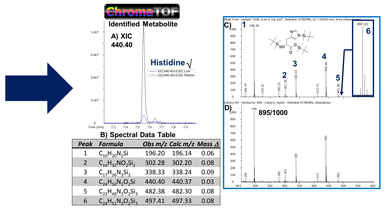


Figure 5. XCMS online Cloud Plot (m/z vs retention time) showing a metabolite feature with a retention time of 7.55 minutes and EIC m/z = 440.396.



Peak	Formula	Obs m/z	Calc m/z	Mass Δ
1	$C_{11}H_{24}N_2Si$	196.20	196.14	0.06
2	$C_{14}H_{28}NO_2Si$	302.28	302.20	0.08
3	$C_{14}H_{28}N_2Si$	338.33	338.24	0.09
4	$C_{18}H_{34}N_2O_2Si$	440.40	440.37	0.03
5	$C_{21}H_{54}N_2O_2Si_3$	482.38	482.30	0.08
6	$C_{24}H_{55}N_2O_2Si_4$	497.41	497.33	0.08

Figure 6. ChromaTOF - A) XIC 440.40; B) generated formulas for fragment and molecular ions; C,D) Peak True and library match data for histidine.

Table 4. Partial list of potential NAFLD biomarkers identified using XCMS online and ChromaTOF. Metabolites A-D were upregulated in liver samples, while citric acid was found in greater quantities in plasma. Stearic Acid was tentatively identified with the marginally acceptable similarity score of 703/1000

Feature	*m/z*	*BT Similarity*	*Metabolite*
A	341.28	703	Stearic Acid?
B	418.36	897	Aspartic Acid
C	431.43	917	Lysine
D	440.39	895	Histidine
E	459.33	726	Citric Acid

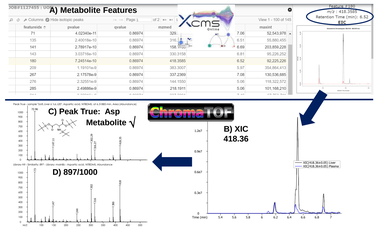
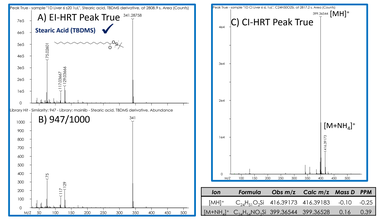


Figure 7. XCMS online Results Table showing a feature with a retention time of 6.52 minutes and EIC m/z = 418.36. ChromaTOF processing resulted in the identification of the metabolite aspartic acid.

Robust Characterization and Confirmation: GC-HRT

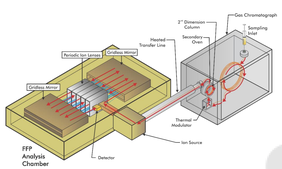


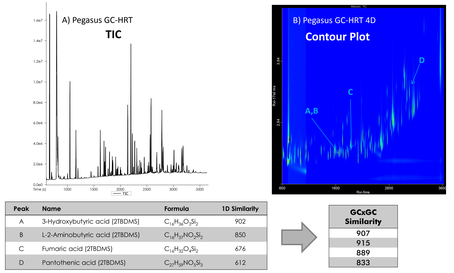
Ion	Formula	Obs m/z	Calc m/z	Mass D	PPM
[MH]+	$C_{24}H_{53}O_3Si$	416.39173	416.39183	-0.10	-0.25
[M+NH4]+	$C_{24}H_{57}NO_3Si$	399.36544	399.36528	0.16	0.39

Figure 8. Peak True EI-HRT, library match, and CI-HRT data for stearic acid.

Discovery: GCxGC-HRT

- Enhanced Chromatographic and Mass Spectral Resolution
- Group Clustering – Structured Chromatograms
- Removal of background interferences (e.g., column bleed, solvents, etc.)
- Improved Characterization of Compounds in Complex Matrices





Peak	Name	Formula	1D Similarity
A	3-Hydroxybutyric acid (2TBDMS)	$C_{16}H_{34}O_3Si_2$	902
B	L-2-Aminobutyric acid (2TBDMS)	$C_{16}H_{35}NO_2Si_2$	850
C	Fumaric acid (2TBDMS)	$C_{16}H_{30}O_4Si_2$	676
D	Pantothenic acid (2TBDMS)	$C_{29}H_{63}NO_5Si_3$	612

GCxGC Similarity
907
915
889
833

Figure 9. A) GC-HRT and B) GC-HRT 4D data. A table illustrating the benefits of combining enhanced chromatographic and mass spectral resolution for confident identification of metabolites differentiating the different groups (e.g., fumaric acid).

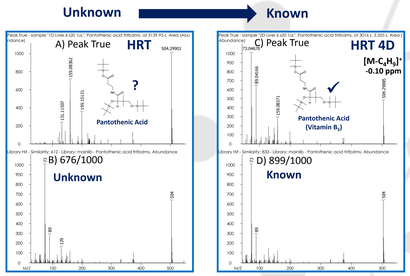


Figure 10. A) GC-HRT and B) GC-HRT 4D data for pantothenic acid (Vitamin B_5).

Summary

- High performance GC-TOFMS was used for increased analysis throughput of complex samples.
- GC-HRT facilitated confident identification of metabolites using complementary high resolution mass spectrometry ionization methods for formula determinations for fragment, molecular, and adduct ions.
- The combination of enhanced chromatographic and mass spectral resolution (Figure 11) led to superior analysis of complex matrices by transforming unknowns to known compounds in metabolomics data. Compounds were confidently identified as potential biomarkers for NAFLD as a direct result of this methodology.

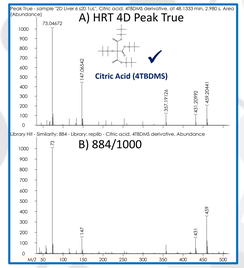


Figure 11. GC-HRT 4D data for citric acid.

Delivering the Right Results