The Discovery of Potential Cancer Biomarkers in Human Plasma Using GC- and GCxGC-TOFMS

David E. Alonso[1], Habtom Ressom[2], Cristina Di Poto[2], and Joseph E. Binkley[1]; [1]LECO Corporation, St. Joseph, MI; [2]Georgetown University Medical School, Washington D.C.

Introduction

- Hepatocellular carcinoma (HCC) is the leading cause of cancer-related deaths worldwide (9th in the US)
- *Risk Factors:* Hepatitis (B &C), alcoholism, diabetes, obesity, and nonalcoholic fatty liver disease
- *Critical Need:* Early stage intervention and effective medical treatment for HCC

Objectives

- To complement a much larger study investigating metabolite levels in HCC versus liver cirrhosis (CIRR) patients
- To identify potential HCC biomarkers using a non-targeted, multiplatform approach

Samples

Plasma

Metabolite Extraction (ACN/*i*-PrOH/H_2O)

64 Patient Samples HCC & CIRR)

Derivatization

Methoxymation: 30 µL of methoxylamine (20 mg/mL in Pyridine), heat/agitate at 60 °C for 30 minutes

Silylation: 70 µL of MSTFA, heat/agitate at 60 °C for 30 minutes

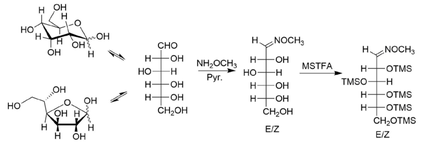


Figure 1: Two-step derivatization of glucose

Instrument Parameters

Gas Chromatograph Autosampler	**LECO GCxGC (Dual Stage Quad Jet Thermal Modulator) LECO L-PAL 3**
Injection	1µL, Split 20:1; 250 °C
Carrier Gas	He @ 1.4 mL/min, Constant Flow
Column Set	Rxi-5ms, 30 m x 0.25 mm i.d. x 0.25 µm (Restek) Rxi-17sil ms, 0.6 m x 0.25 mm i.d. x 0.25 µm (Restek)
Temperature Program	50 °C (1 min), ramped 10 °C/min to 300 °C (12 min) Secondary oven offset was 5 °C relative to primary oven
Modulation	3s modulation; temperature offset 15 °C relative to secondary oven
Mass Spectrometer	**LECO Pegasus® BT**
Ion Source Temperature	250 °C
Ionization Mode	EI
Mass Range (m/z)	45-750
Acquisition Rate	10 spectra/s (200 spectra/s GCxGC-TOFMS)

Pegasus BT 4D

GC-TOFMS Results

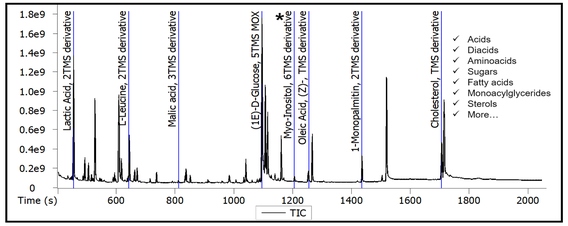


Figure 2: TIC showing the chemical diversity of plasma sample metabolites identified by GC-TOFMS.

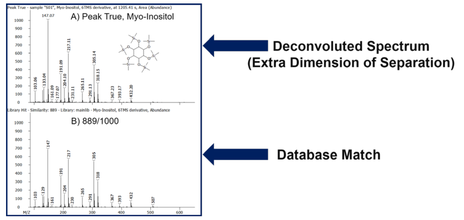


Figure 3: A) Peak True (Deconvoluted), and B) library spectra for myo-inositol.

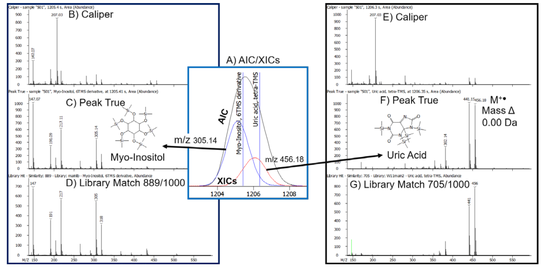


Figure 4: A) Analytical Ion Chromatogram (AIC) showing extracted ions (XICs) for coeluting myo-inositol & uric acid metabolites. Caliper, Peak True and Library spectra for myo-inositol (B-D) and uric acid (E-G). Identification of uric acid is supported by mass Δ calculations (MΔ = $m/z_{obs} - m/z_{calc}$).

Table 1: Representative Compounds in plasma (Untargeted Peak Find Processing)

Name	Formula	R.T. (s)	Similarity
Lactic Acid, 2TMS	$C_9H_{24}O_3Si_2$	454	941
Glycolic acid, 2TMS	$C_8H_{22}O_3Si_2$	467	882
ß-Alanine, 2TMS	$C_9H_{23}NO_2Si_2$	492	927
2-Hydroxybutyric acid, 2TMS	$C_{10}H_{24}O_3Si_2$	515	909
Oxalic acid, 2TMS	$C_8H_{20}O_4Si_2$	518	897
2-Hydroxyisovaleric acid, 2TMS	$C_{11}H_{26}O_3Si_2$	549	952
2-Aminobutyric acid, 2TMS	$C_{10}H_{25}NO_2Si_2$	555	883
3-Hydroxyisovaleric acid, 2TMS	$C_{11}H_{26}O_3Si_2$	587	787
2-Keto-N-Caproic acid, 2TMS MOX	$C_{13}H_{29}NO_3Si_2$	591	867
L-Valine, 2TMS	$C_{11}H_{27}NO_2Si_2$	594	901
Urea, N,N'-bis(trimethylsilyl)-	$C_7H_{20}N_2OSi_2$	608	787
L-Leucine, 2TMS	$C_{12}H_{29}NO_2Si_2$	641	919
Glycine, 3TMS	$C_{11}H_{29}NO_2Si_3$	644	931
Isoleucine, 2TMS	$C_{12}H_{29}NO_2Si_2$	659	876
L-Proline, 2TMS	$C_{11}H_{27}NO_2Si_2$	662	874
Fumaric acid, 2TMS	$C_{10}H_{20}O_4Si_2$	670	915
Succinic acid, 2TMS	$C_{10}H_{22}O_4Si_2$	672	753
Glyceric acid, 3TMS	$C_{12}H_{30}O_4Si_3$	691	869
Fumaric acid, 2TMS	$C_{10}H_{20}O_4Si_2$	698	829
2,3-Dihydroxybutanoic acid, 3TMS	$C_{13}H_{30}O_4Si_3$	703	784
Nonanoic acid, TMS	$C_{12}H_{26}O_2Si$	707	880
Serine, 3TMS	$C_{12}H_{29}NO_3Si_3$	715	896
L-Threonine, 3TMS	$C_{13}H_{31}NO_3Si_3$	737	946
5-Methyloxytesine, 3TMS	$C_{14}H_{31}NO_3Si_3$	762	833
ß-Alanine, 3TMS	$C_{12}H_{29}NO_2Si_3$	765	774
Aminomalonic acid, 3TMS	$C_{12}H_{29}NO_4Si_3$	800	832
Malic acid, 3TMS	$C_{13}H_{30}O_5Si_3$	812	849
Erythritol, 4TMS	$C_{16}H_{38}O_4Si_4$	831	879
L-Methionine, 2TMS	$C_{11}H_{27}NO_2SSi_2$	834	927
L-Proline, 5-oxo-, 3TMS	$C_{11}H_{25}NO_3Si_3$	837	931
Hydroxylamine, 2TMS	$C_6H_{19}NOSi_2$	841	821
L-Cysteine, 3TMS	$C_{12}H_{29}NO_2SSi_3$	861	870
2-Ketoglutaric acid, TMS MOX	$C_{10}H_{19}NO_5Si$	874	705
L-Ornithine, 3TMS	$C_{14}H_{34}N_2O_2Si_3$	901	775
Glutamic acid, 3TMS	$C_{14}H_{31}NO_4Si_3$	905	717
Phenylalanine, 2TMS	$C_{15}H_{25}NO_2Si_2$	911	922
Lauric acid, TMS	$C_{15}H_{32}O_2Si$	920	699
Asparagine, 3TMS	$C_{13}H_{30}N_2O_3Si_3$	941	855
5-Hydroxytryptophan, 4TMS	$C_{23}H_{44}N_2O_5Si_4$	943	722
Ribitol, 5TMS	$C_{17}H_{44}O_5Si_5$	973	882
Glutamine, 4TMS	$C_{19}H_{46}N_2O_4Si_4$	984	833
l-Glutamine, 3TMS	$C_{14}H_{34}N_2O_4Si_3$	1007	877
L-Ornithine, 4TMS	$C_{17}H_{42}N_2O_2Si_4$	1037	894
Citric acid, 4TMS	$C_{18}H_{44}O_7Si_4$	1040	871
Myristic acid, TMS	$C_{17}H_{36}O_2Si$	1046	861
Caffeine	$C_8H_{10}N_4O_2$	1056	919
1,5-Anhydroglucitol, 4TMS	$C_{18}H_{46}O_5Si_4$	1062	871
D-Fructose, 5TMS MOX	$C_{21}H_{51}NO_6Si_5$	1079	922
1E)-D-Galactose, 5TMS MOX	$C_{21}H_{51}NO_6Si_5$	1088	924
1E)-D-Glucose, 5TMS MOX	$C_{21}H_{51}NO_6Si_5$	1095	821
L-Lysine, 4TMS	$C_{18}H_{44}N_2O_2Si_4$	1099	820
L-Histidine, 3TMS	$C_{15}H_{29}N_3O_2Si_3$	1100	752
L-Tyrosine, 3TMS	$C_{18}H_{29}NO_3Si_3$	1110	855
Mannitol, 6TMS	$C_{24}H_{62}O_6Si_6$	1114	947
1(2)-D-Galactose, 5TMS MOX	$C_{21}H_{51}NO_6Si_5$	1119	817
Palmitoleic acid, TMS	$C_{19}H_{38}O_2Si$	1150	921
Palmitic Acid, TMS	$C_{19}H_{40}O_2Si$	1161	950
Myo-Inositol, 6TMS	$C_{24}H_{60}O_6Si_6$	1205	889
Uric acid, 4TMS	$C_{17}H_{36}N_4O_3Si_4$	1206	705
Margarate, TMS	$C_{20}H_{42}O_2Si$	1214	837
Linoleic acid, TMS	$C_{20}H_{40}O_2Si$	1251	929
Oleic Acid, (Z)-, TMS	$C_{21}H_{42}O_2Si$	1254	941
Stearic acid, TMS	$C_{21}H_{44}O_2Si$	1266	946
Lanthionine, 4TMS	$C_{21}H_{48}N_2O_4SSi_4$	1287	745
L-Cystine, 4TMS	$C_{24}H_{56}N_2O_4S_2Si_4$	1305	825
Pseudo uridine, 5TMS	$C_{24}H_{52}N_2O_6Si_5$	1329	756
Arachidonic acid, TMS	$C_{23}H_{40}O_2Si$	1332	928
1-Monomyristin, 2TMS	$C_{21}H_{44}O_4Si_2$	1346	728
Arachidic acid, TMS	$C_{23}H_{48}O_2Si$	1363	835
Embramine	$C_{28}H_{38}NO$	1374	916
Uridine, 3TMS	$C_{18}H_{36}N_2O_6Si_3$	1380	781
2-Palmitoylglycerol, 2TMS	$C_{25}H_{52}O_4Si_2$	1420	849
1-Monopalmitin, 2TMS	$C_{25}H_{52}O_4Si_2$	1435	908
2-Monostearin, 2TMS	$C_{25}H_{52}O_4Si_2$	1504	864
α-Glyceryl arachidate, 2TMS	$C_{29}H_{60}O_4Si_2$	1599	804
γ-Tocopherol, TMS	$C_{32}H_{58}O_2Si$	1616	806
ß-Tocopherol, TMS	$C_{32}H_{58}O_2Si$	1692	839
Cholesterol, TMS	$C_{30}H_{54}OSi$	1707	923
Campesterol, 1TMS	$C_{31}H_{54}OSi$	1774	723

Ave. Similarity = 855/1000

GCxGC-TOFMS Results (Untargeted)

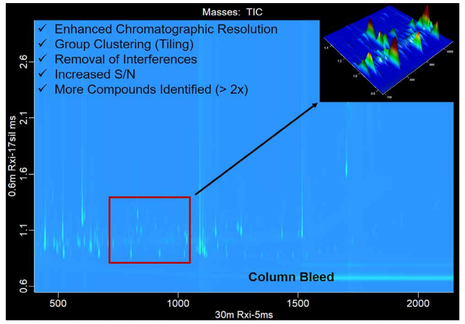
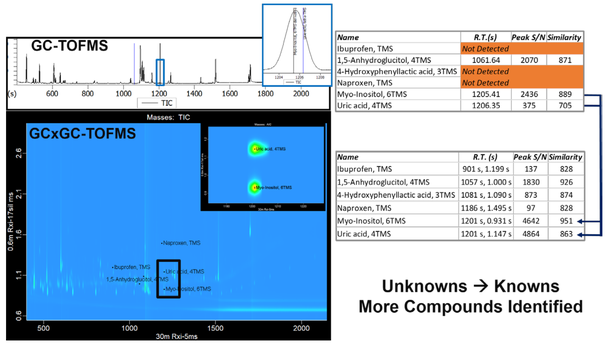


Figure 5: GCxGC-TOFMS contour plot and surface plot expansion (inset).



Name	R.T.(s)	Peak S/N	Similarity
Ibuprofen, TMS	Not Detected		
1,5-Anhydroglucitol, 4TMS	1061.64	2070	871
4-Hydroxyphenyllactic acid, 3TMS	Not Detected		
Naproxen, TMS	Not Detected		
Myo-Inositol, 6TMS	1205.41	2436	889
Uric acid, 4TMS	1206.35	375	705

Name	R.T. (s)	Peak S/N	Similarity
Ibuprofen, TMS	901 s, 1.199 s	137	828
1,5-Anhydroglucitol, 4TMS	1057 s, 1.000 s	1830	926
4-Hydroxyphenyllactic acid, 3TMS	1081 s, 1.090 s	873	874
Naproxen, TMS	1186 s, 1.495 s	97	828
Myo-Inositol, 6TMS	1201 s, 0.931 s	4642	951
Uric acid, 4TMS	1201 s, 1.147 s	4864	863

Figure 6: Comparison of GC- (top) and GCxGC-TOFMS (bottom) results. The enhanced chromatographic resolution in transitioning from 1D to 2D results in an improvement in S/N and similarity scores (upper/lower data tables).

Unknowns → Knowns
More Compounds Identified

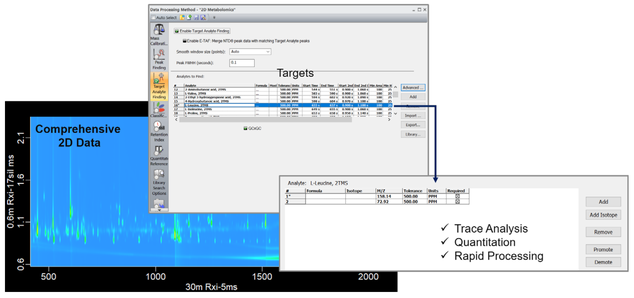


Figure 7: Target Analyte Finding (TAF) data processing method with metabolite list and target ions with tolerances.

GCxGC-TOFMS Target Analyte Processing

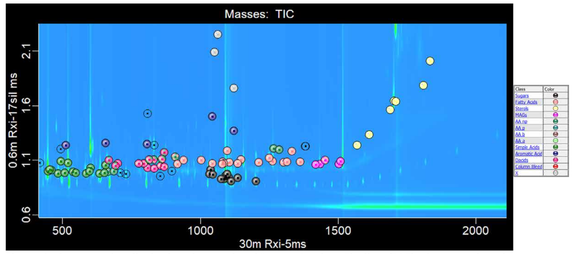
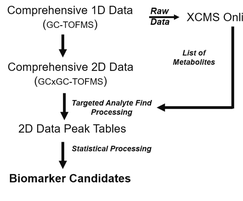


Figure 8: Contour plot displaying the results of Target Analyte Finding (TAF) of comprehensive GCxGC-TOFMS data. The color coded bubbles represent different classes of compounds identified.

Table 2: TAF results table listing targeted metabolites, 2D retention times and areas.

Name	R.T. (s)	Area	Name	R.T. (s)	Area	Name	R.T. (s)	Area
Lactic Acid, 2TMS	446 s, 1.005 s	15307843067	L-Methionine, 2TMS	829 s, 1.150 s	116146521	L-Tyrosine, 3TMS	1105 s, 1.085 s	177794300
Hexanoic acid, TMS	454 s, 1.025 s	15322252	L-Aspartic acid, 3TMS	832 s, 1.030 s	3356604	D-Mannitol, 6TMS	1111 s, 0.915 s	38627940
Glycolic acid, 2TMS	460 s, 1.015 s	80842344	L-5-Oxoproline, , 2TMS	832 s, 1.240 s	4815248660	3-Hydroxybutiric acid, 2TMS	1120 s, 1.375 s	512142
L-Alanine, 2TMS	464 s, 0.985 s	462566060	Erythritol, acid, 4TMS	833 s, 0.960 s	2000406	Glucopyranose, 5TMS	1131 s, 0.945 s	276702138
Butanoic acid, 2 (methoxymino)- 3-methyl-, TMS	493 s, 1.095 s	13622033	L-Cysteine, 3TMS	856 s, 1.050 s	12124921	Palmitic Acid, TMS	1135 s, 1.075 s	1217644
2-Hydroxybutyric acid, 2TMS	508, 0.990 s	18205950	Creatinine enol, 2TMS	859 s, 1.135 s	92805218	Palmitelaidic acid, TMS	1147 s, 1.110 s	17566506
Levulinic acid, TMS	511 s, 1.240 s	428778	Pentanedioic acid, 2-hydroxy, 3TMS	868 s, 1.045 s	940599	Myo-Inositol, 6TMS	1201 s, 0.931 s	112559586
Oxalic acid, 2TMS	520 s, 0.985 s	78453934	Pentanedioic acid, 2-oxo-,2TMS MOX	868 s, 1.175 s	8610200	Margarate, TMS	1218 s, 1.085 s	5003105
3-Hydroxybutyric acid, 2TMS	538 s, 1.000 s	88630520	L-Ornithine, 3TMS	895 s, 1.005 s	13090017	Oleic Acid, (Z)-, TMS	1249 s, 1.125 s	116038276
2-Aminobutyric acid, 2TMS	547 s, 0.995 s	79490932	Phenylalanine, 2TMS	907 s, 1.135 s	108232725	Stearic acid, TMS	1261 s, 1.095 s	44688072
L-Valine, 2TMS	586 s, 0.985 s	139449790	Dodecanoic acid, TMS	916 s, 1.070 s	13068309	Tryptophan, 3TMS	1264 s, 1.210 s	24687640
2-Ethyl-3-hydroxypropionic acid, 2TMS	599 s, 0.995 s	3890773	Asparagine, 3TMS	937 s, 1.100 s	120243201	Xanthosine, 4TMS	1285 s, 1.200 s	2553020
4-Hydroxybutyrate acid, 2TMS	601 s, 1.035 s	8320894	L-Glutamine, 3TMS	1003 s, 1.105 s	1035895292	L-Cystine, 4TMS	1300 s, 1.085 s	2097898
L-Leucine, 2TMS	634 s, 0.985 s	230656845	L-Ornithine, 4TMS	1033 s, 0.980 s	56448880	Arachidonic acid, TMS	1312 s, 1.090 s	511285
L-Isoleucine, 2TMS	652 s, 0.990 s	83917355	Citric acid, 4TMS	1036 s, 1.020 s	221423396	Arachidic acid, TMS	1330 s, 1.185 s	6177398
L-Proline, 2TMS	655 s, 0.980 s	928573200	Myristic acid, TMS	1042 s, 1.075 s	35015194	Arachidic acid, TMS	1340 s, 1.090 s	10512923
Benzeneacetic acid, TMS	655 s, 1.765 s	537815	Hypoxic acid, TMS	1042 s, 1.505 s	6827209	Uridine, 3TMS	1378 s, 1.230 s	1786668
Urea, N,N'-bis(trimethylsilyl)-	664 s, 0.800 s	260315445	D-Fructose 1, 5TMS MOX	1045 s, 0.970 s	5222597	2-Palmitoylglycerol, 2TMS	1417 s, 1.065 s	33950455
Succinic acid, 2TMS	667 s, 1.035 s	21832327	D-Fructose 2, 5TMS MOX	1075 s, 0.935 s	116178769	1-Monopalmitin, 2TMS	1432 s, 1.070 s	115285362
Glyceric acid, 3TMS	683 s, 1.005 s	42813133	4-Hydroxyphenyllactic acid, 3TMS	1078 s, 1.030 s	370562	Behenic acid, TMS	1458 s, 1.105 s	1364481
Fumaric acid, bis-TMS	691 s, 1.055 s	8891312	4-Hydroxyphenyllactic acid, 3TMS	1081 s, 1.060 s	2936110	2-Monostearin, 2TMS	1501 s, 1.070 s	66790109
Nonanoic acid, TMS	700 s, 1.075 s	19826092	D-Galactose (1), 5TMS MOX	1084 s, 0.955 s	89729752	1-Monostearoylglycerol, 2TMS	1507 s, 1.095 s	4623066
Serine, 3TMS	709 s, 0.990 s	109659036	Pentadecanoic acid, TMS	1084 s, 1.075 s	7581403	ß-Tocopherol, TMS	1567 s, 1.245 s	396713
L-Threonine, 3TMS	730 s, 0.980 s	158957560	D-Galactose (2), 5TMS MOX	1092 s, 0.955 s	194365532	D-Tocopherol, TMS	1612 s, 1.340 s	1084349
Decanoic acid, TMS	775 s, 1.075 s	9667180	Sedoheptulose, 6TMS MOX	1095 s, 0.965 s	207354223	ß-Tocopherol, TMS	1667 s, 1.565 s	5286900
5-Aminovaleric acid, 2TMS	793 s, 1.075 s	5656882	D-Glucose 1, 5TMS MOX	1073 s, 0.940 s	496733883	Cholesterol, TMS	1702 s, 1.645 s	240537185
Benzoic acid, 2,5-dimethyl-, TMS	805 s, 1.255 s	5071920	L-Lysine, 4TMS	1096 s, 0.960 s	64866588	Cholestan-3-ol, TMS	1708 s, 1.640 s	1652256
Malic acid, 3TMS	808 s, 1.030 s	13738253	L-Histidine, 3TMS	1096 s, 1.190 s	113896968	Dehydrocholic acid, 2TMS	1807 s, 1.790 s	5444685
Adipic acid, 2TMS	814 s, 1.020 s	2264952	D-Glucose 2, 6TMS MOX	1102 s, 0.990 s	258338306	ß-Sitosterol, TMS	1831 s, 2.010 s	3138005

Processing & Statistical Analysis



Compounds	CIRR patients (24) vs HCC patients (39)		
	p value	adjusted p-value (FDR=0.05)	FC (HCC/CIRR)
3-Hydroxybutyric acid, 3TMS*	0.00137	0.00520	-1.64
3-Indoleacetic acid, 2TMS	0.00211	0.00779	1.69
4-Hydroxyphenyllactic acid, 3TMS*	0.00000	0.00000	4.59
Arachidonic acid, TMS*	0.00004	0.00028	-1.45
Butanoic acid, 2-(methoxymino)-3-methyl-, TMS	0.00130	0.00589	-1.22
Cholestan-3-ol, TMS	0.00004	0.00028	2.46
Citric acid, 4TMS	0.00000	0.00000	2.82
D-Fructose 2, 5TMS MOX	0.00003	0.00024	2.40
Decanoic acid, TMS	0.00003	0.00024	1.47
Dodecanoic acid, TMS	0.00292	0.00972	1.51
Erythritol, acid, 4TMS	0.00005	0.00028	2.52
Glyceric acid, 3TMS	0.00515	0.01598	1.20
Glycine, 3TMS	0.00855	0.02332	1.08
Glycolic acid, 2TMS*	0.00000	0.00001	1.69
L-Isoleucine, 2TMS *	0.00000	0.00001	-1.53
L-Leucine, 2TMS*	0.00049	0.00234	-1.44
L-Methionine, 2TMS	0.00010	0.00055	1.99
L-Ornithine, 3TMS	0.00720	0.02159	1.47
L-Ornithine, 4TMS	0.00492	0.01581	1.17
L-Threonine, 3TMS	0.01219	0.03325	1.19
L-Tyrosine, 3TMS*	0.00000	0.00000	1.94
L-Valine, 2TMS*	0.00004	0.00028	-1.41
Malic acid, 3TMS	0.00997	0.02803	1.21
Myristic acid, TMS	0.00216	0.00779	1.51
Palmitelaidic acid, TMS	0.00003	0.00024	2.53
Phenylalanine, 2TMS	0.00000	0.00006	1.55
Succinic acid, 2TMS	0.00081	0.00362	1.41
α-Tocopherol, TMS*	0.00017	0.00083	-1.69
ß-Sitosterol, TMS	0.00232	0.00805	1.63

* Significant also in the HCC QC (11) vs CIRR patients (24) comparison

Figure 9: Data processing strategy (Left), and 2D data statistical results showing 29 significant metabolites (Probability Value, p<0.05 and False Discovery Rate, FDR<5%) in the comparison of CIRR and HCC patients (Right).

Summary

- Acquired & Processed GC and GCxGC-TOFMS Data for HCC & CIRR Patients
- Compound Characterization
  - Spectral Similarity
  - Mass Delta Calculations
- 1D à 2D; More Compounds Identified
- TAF was performed on the GCxGC-TOFMS data for the *preparation of matrix file for further processing*
- Statistical analysis of data facilitated identification of HCC biomarker candidates

Acknowledgements

NIH grants U01CA185188 and R01GM123766 (PI: Habtom Ressom, Georgetown University).