Small Molecule Metabolite Identifications in Diabetic Versus Non-Diabetic Urine Sample Groups Using Comprehensive Two-Dimensional Gas Chromatography Combined with Time-of-Flight Mass Spectrometry (GCxGC-TOFMS)

John Heim, LECO Corporation; Saint Joseph, Michigan USA

Key Words: Fisher Ratio, Sample Groups, Diabetes, Metabolite Profile

1. Introduction

Small molecule metabolite analysis presents challenges that historically have relied heavily upon standard quadropole GCMS utilizing targeted methods of selected ion monitoring and tandem GC/MS/MS mass spectrometric techniques. The complex nature of metabolomic samples demands analytical solutions and instrumental methods that will identify the small molecule metabolomic profile completely as well as discover significant key components of interest.

Comprehensive two-dimensional gas chromatography (GCxGC) expands the peak capacity of the chromatographic separation thereby increasing resolution and analyte characterization necessary for complex biological samples. Two orthogonal separation phases (such as nonpolar and polar) are utilized to maximize separation capacity in a single analysis. The high data density and narrow peak widths inherent to GCxGC analysis requires a detection system able to characterize the peak shape and small molecule metabolite identification. Time of flight mass spectrometry (TOFMS) offers continuous full range nonskewed mass spectral information and fast acquisition rates ideal for metabolomic identifications. The combination of TOFMS data and deconvolution algorithms facilitates trace level analyte detection that would otherwise be hidden and coeluted with other compounds in the sample.

This research conducted on the Pegasus® 4D system shows metabolomic data that illustrates the benefits of multidimensional chromatography coupled with time-of-flight mass spectrometry. Two dimensional chromatographic plots of biological samples showing increased peak capacity and structural orientation not possible in one dimensional chromatography will be highlighted. The metabolomic sample data presented shows increased analyte detectability as a result of cryo-focusing in the GCxGC process. In addition, classifications for specific chemical functional groups can be utilized as a user-defined data mining method to aid in data reduction and increase overall experimental results. Sample Groups representing normal and disease state individuals will also be discussed.

2. Research Objectives

This research study conducted on diabetic and non-diabetic subjects demonstrates the capabilities of two-dimensional chromatography combined with time-of-flight mass spectrometry to identify the metabolomic profile and show significant sample group differences by calculating Fisher Ratios in trimethylsilyl (TMS) derivatized urine samples for normal and disease state individuals.

- Demonstrate the increased detectability of GCxGC-TOFMS analysis to identify the small molecule metabolite profile in complex biological matrices
- Show the benefit of time of flight mass spectrometry to acquire the data density needed to detect trace level analytes that would otherwise be buried by coeluting compounds.
- Illustrate the Fisher Ratio calculation between diabetic and non-diabetic sample groups as a data mining tool.
- Show analyte deconvolution applied to the diabetes study GCxGC-TOFMS analysis.

3. Experimental

This research was designed to study trimethylsilyl derivatized urine samples for the small molecule metabolite profile intended to detect possible chemical variations between diabetic disease state and normal control non-diabetic subjects. Morning fast urine samples were collected from 4 subjects—2 non-diabetic normal controls, 1 type I diabetic, and 1 type II diabetic. Samples were stored under refrigeration prior to liquid/liquid extraction with methylene chloride and derivatization with N,O-bis-(Trimethylsilyl)-trifluoroacetamide (BSTFA). Six 10 mL aliquots from each subject were prepared by acidification with concentrated sulfuric acid to pH 2. 10 mL aliquots were extracted with 2 mL methlyene chloride into a 20 mL scintillation vial containing approximately 5 mg sodium sulfate. Derivatization was carried out with BSTFA by placing 200 µL of extract into a sealed 2 mL autosampler vial containing approximately 0.5 mg sodium sulfate. 30 µL of dry pyridine was added to the vial. 200 µL BSTFA was added to each vial. The samples were heated to 60°C for 1 hour and then analyzed.

GCxGC-TOFMS results were generated with a LECO Pegasus 4D time-of-flight mass spectrometer (TOFMS). The Pegasus 4D GC-TOFMS instrument was equipped with an Agilent 7890 gas chromatograph featuring a LECO two stage cryogenic modulator and secondary oven. LECO ChromaTOF® software was used for all acquisition control, data processing, Sample Group comparison, and Fisher Ratio calculations. A 30 m x 0.25 mm x 0.25 µm film thickness, Rtx-5ms (Restek Corp.), GC capillary column was used as the primary column for the GCxGC-TOFMS analysis. In the GCxGC configuration a second column (1.5 m x 0.18 mm id. x 0.18 µm film thickness, Rtx-200 (Restek Corp.), was placed inside the LECO secondary GC oven after the thermal modulator. Helium carrier gas flow rate was set to 1.5 mL/minute at a corrected constant flow via pressure ramps. The primary column was programmed with an initial temperature of 40°C for 1.00 minute and ramped at 6°C/minute to 290°C for 10 minutes. The secondary column temperature

program was set to an initial temperature of 50°C for 1.00 minute and then ramped at 6°C/minute to 300°C with a 10 minute hold time. The thermal modulator was set to +25°C relative to the primary oven and a modulation time of 5 seconds was used. The MS mass range was 45-800 m/z with an acquisition rate of 200 spectra/second. The ion source chamber was set to 230°C and the detector voltage was 1750V with an electron energy of -70eV.

4. Results and Discussion

Results of the diabetic profile study between diabetic and non-diabetic subjects are shown in Figures 1 through 3 below by the total ion chromatograms depicted as contour plots. These chromatographic examples visually illustrate the peak differences between sample types as well as highlight the benefits GCxGC-TOFMS offer which include increased peak capacity, improved analyte detectability, and enhanced resolution. On average over 1000 peaks were found per sample with a signal to noise ratio of 100 for this study. The red cross hatched area in each contour plot is an unprocessed region developed in the Classifications feature of ChromaTOF software which eliminates unwanted background peaks.

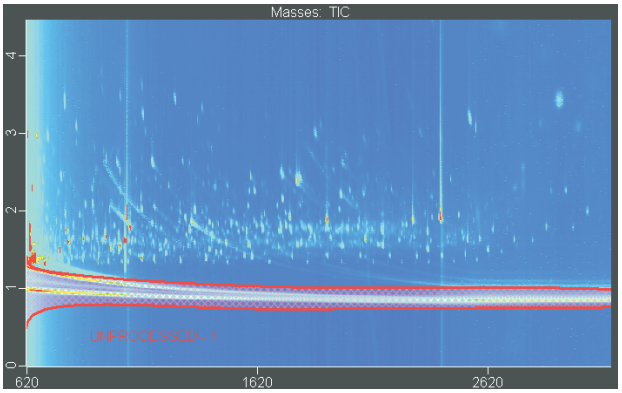

Figure 1. Normal control subject non-diabetic: Contour Plot Total Ion Chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

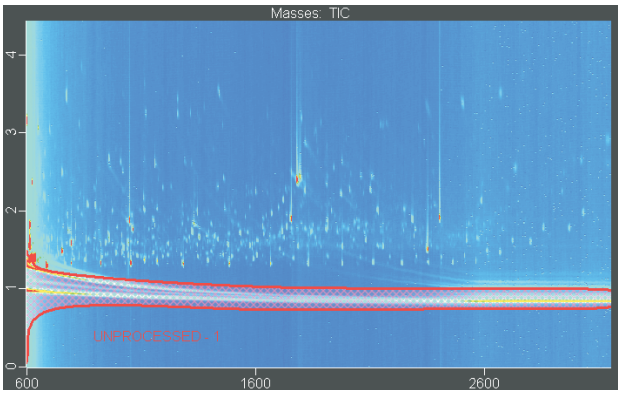

Figure 2. Disease state subject Type I diabetic: Contour Plot Total Ion Chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

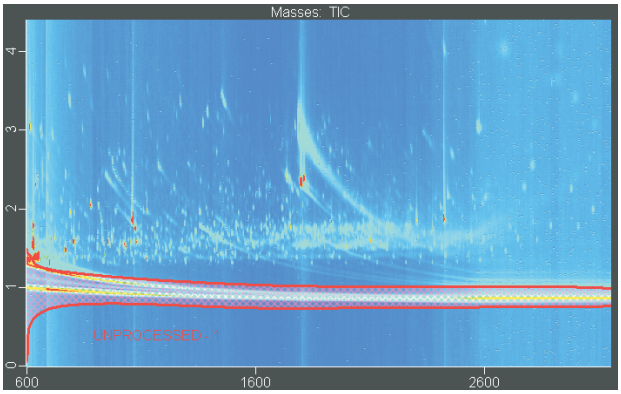

Figure 3. Disease state subject Type II diabetic: Contour Plot Total Ion chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

True Signal Deconvolution® Example

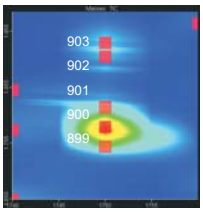

Figure 4. Contour plot showing 5 peak markers over what appears to be 2 resolved peaks.

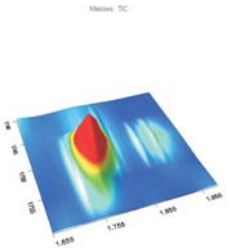

Figure 5. Surface plot showing second dimension separation of 5 compounds.

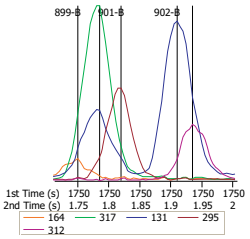

Figure 6. Extracted ion chromatogram of the unique masses for each of the 5 analytes found.

Peak #	Name
899	Lycopene
900	Azelaic acid, bis(TMS) ester
901	1,4-Benzenedicarboxylic acid, bis(TMS) ester
902	Benzene, (1-isocyanato-2-propenyl)-
903	Bicyclo[5.3.0]decan-2-one, -9-(1-phenyl-1TMS-methylene)-

Table 1. Peaks 899 – 903 with library matched identifications.

Figures 4 through 6 above illustrate the ability of two dimensional chromatography to separate compounds that would otherwise be coeluted in the first dimension. Figure 6 shows the unique masses for the 5 analytes demonstrating the ability of time of flight mass spectrometry and deconvolution algorithms to identify closely eluting components.

Statistical Comparisons with Sample Groups and Fisher Ratios

The Sample Groups option feature in ChromaTOF software allows the user capability to view statistical comparisons from groups of samples. This software feature aligns the data for the groups of samples specified in Sample Groups. For example, by aligning data between different peak tables, the software can match peaks from the sample groups based on criteria such as peak area and relative standard deviation. Statistical comparisons can then be calculated using the software to facilitate evaluation and assessment between sample groups. Fisher ratios can be calculated in ChromaTOF software. The Fisher ratio method uses an indexing scheme to discover the unknown chemical differences among known classes of complex samples.

Table 2. A partial Sample Group Compound Table is illustrated above showing the top 25 compounds with the highest Fisher Ratio values. These compounds represent the most variation between the diabetic and non-diabetic sample group.

Compound Table

Analyte	Name	1st Dimension Time (s)	Count	Area	Area	Area	Mass	Fisher Ratio
		Average		Min	Max	Average		
462	Androst-5-en-17-one, 3-[(trimethylsilyl)oxy]-, (3á)-	2584.17	6	9431.390	65665.630	44607.287	230	15941
383	Tetradecanoic acid, trimethylsilyl ester	1785	8	15086.470	561860.256	346812.605	117	15596
146	2,2,4,4-Tetramethyloctane	749.583	12	0.000	474332.916	87103.083	57	14245
150	Cyclohexanone, 3,3,5-trimethyl-	755	7	0.000	1113325.18	765774.793	83	7841.9
286	Linolenic acid, trimethylsilyl ester	1301.25	8	113913.787	1150196.10	277511.070	122	7365.1
446	Oleamide, N-trimethylsilyl-	2297.14	7	38675.367	221466.098	135901.404	144	7026.4
126	Tris(trimethylsilyl)borate	695	11	0.000	51070159.8	7422081.72	221	6435.1
376	Azelaic acid, bis(trimethylsilyl) ester	1742.73	11	0.000	5143686.37	1243390.72	73	5834.5
445	Dehydroabietic acid, trimethylsilyl ester	2288.57	7	0.000	113369.257	83759.426	239	5823.0
32	Ethylbis(trimethylsilyl)amine	621.875	8	0.000	10392.466	1299.058	187	5294.2
384	1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl-	1797.5	10	1021186.16	6793564.20	4474224.96	194	5224.6
413	Theobromine TMS derivative	1910	6	28939.131	285852.728	184138.559	237	4991.4
212	Silanamine, N,N'-methanetetrayllbis[1,1,1-trimethyl-	997.5	10	75680.892	485806.980	181001.020	171	4446.3
424	4áH,5à-Eremophil-1(10)-ene, 11-(trimethylsiloxy)-	1975.83	6	1292.377	56101.258	29685.258	232	4368.8
476	Cholesterol trimethylsilyl ether	2937.5	6	16373.853	60407.108	36682.274	129	4141.3
264	1,4-Naphthalenedione, 2-acetyl-	1196.25	8	8391.214	189341.769	132142.428	200	4091.0
378	Glycine, N-benzoyl-N-(trimethylsilyl)-, trimethylsilyl ester	1756.11	9	496953.646	15493603.3	7387460.48	105	4074.4
322	Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester	1462.08	12	188217.862	771219.402	333935.157	263	3916.5
352	13,13-Dimethyl-3,6,9-trioxa-13-silatetradecan-1-ol	1645	9	0.000	1372386.42	386378.224	175	3899.0
92	Heptane, 3-chloro-3-methyl-	664.5	10	12269.119	771404.282	252399.817	83	3892.8
385	Tetradecanoic acid, trimethylsilyl ester	1794.58	12	0.000	1249229.28	321744.340	117	3824.6
268	2,4,4-Trimethyl-1-pentanol	1219.09	11	303141.947	4442860.17	1078983.80	57	3582.5
46	Carbamic acid,(trimethylsilyl),(trimethylsilyloxy)	627.5	10	2970521.26	177790930	41375840.3	206	3485.2
141	Benzene, 1-methyl-2-(1-methylethyl)-	735	8	0.000	110477.325	72870.348	119	3482.6
390	1H-Purine-2,6-dione, 3,7-dihydro-3,7-dimethyl-	1818.33	6	572599.250	710574.008	647561.595	180	3294.0

Table 3. A Compound Statistics table is generated in the Sample Group feature of ChromaTOF software. The example in Table 3 shows that Peak 390, (1H-Purine-2,6-dione, 3,7-dihydro-3,7-dimethyl-) from the Compound Table (Table 2) was found in the type I diabetic (Class 1) and not found in the non-diabetic normal Control (Class 2).

Compound Stats By Class-TYP...

ID	Name	1st Dimension Time (s)	2nd Dimension Time (s)	Area	Area	S/N	Count
		Average	Average	Average	%RSD	Average	
390-1*	Class1	1818.33	2.87167	647561.595	8.541	727.086	6
390-2	Class2	0	0	0.000	0.000	0.000	0
390	Total	1818.33	2.87167	647561.595	8.541	727.086	6

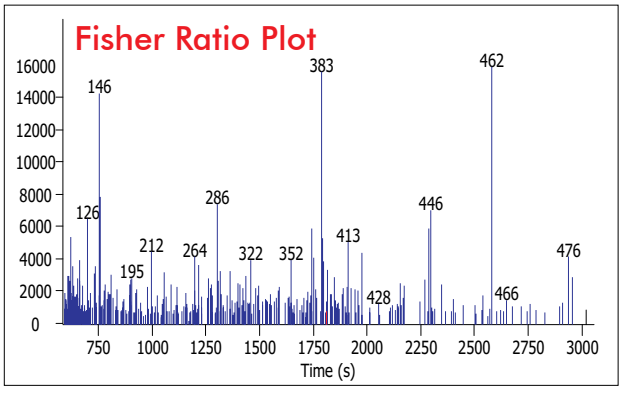

Figure 7. The Fisher Ratio plot shown above graphically represents unknown chemical differences between the normal control non-diabetic sample group and the type I diabetic disease state sample group.

The graphics representation in Figure 7 of the Fisher Ratio plot displays the components as intensity lines. Compounds with the highest Fisher Ratios and variation are shown graphically as the largest intensity values in the plot.

Delivering the Right Results

Sample Groups generated useful statistical information from the GCxGC-TOFMS research study conducted between non-diabetic and diabetic subjects. The Fisher Plot shown in Figure 7 shows that variation and chemical differences between sample groups can be quickly identified. The compound table (Table 2) and the Compound Statistics Table (Table 3) provide insightful information into the chemical nature and metabolite profile between disease state and non-disease state individuals.

5. Conclusions

The diabetic versus non-diabetic research conducted demonstrates that comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GCxGC-TOFMS) is an ideal instrument for identification of the small molecule metabolite profile in complex biological samples. GCxGC-TOFMS analysis was conducted on 6 sample replicates each from 2 non-diabetic normal control subjects and 2 diabetic diseased subjects. The contour plots represented by Figures 1 through 3 show the increased peak capacity, enhanced resolution, and improved peak detectability that comprehensive GCxGC offers. An average of over 1000 peaks were found per sample with a signal-to-noise ratio of 100 or greater. Benefits of time-of-flight mass spectrometry (TOFMS) are highlighted in Figures 5 through 7 by the deconvolution example of 5 analytes in less than 200 milliseconds. The Sample Groups feature of ChromaTOF software was executed between the normal and disease state sample data. Fisher Ratios were calculated for the diseased and non-diseased state sample groups. The Fisher Plot shows that distinct chemical differences and potential key components can be observed quickly utilizing this feature as a statistical model to aid in the data mining process as well as provide efficient and accurate information that will completely characterize the metabolite profile.

This research study confirms that the LECO Pegasus 4D GCxGC-TOFMS is an excellent tool for the characterization of the small molecule metabolite profile in complex biological samples. The Sample Groups and Fisher Ratio features within ChromaTOF allow the analyst to efficiently mine the data for key chemical differences and metabolites that will aid in the search for disease biomarkers.

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