Delivering the Right Results

Utilization of Comprehensive Two-Dimensional Gas Chromatography Combined with Time of Flight Mass Spectrometry (GCxGC-TOFMS) for Small Metabolite Identifications in Complex Biological Samples

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INTRODUCTION

Small molecule metabolite analysis presents challenges that historically have relied heavily upon standard quadrupole GC/MS utilizing targeted methods of selected ion monitoring and tandem GC/MS/MS mass spectrometric techniques. The complex nature of metabolomic samples demand 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 non-skewed 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 presentation will show metabolomic data that illustrates the benefits of multi-dimensional 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 will show 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 will also be discussed.

OBJECTIVES

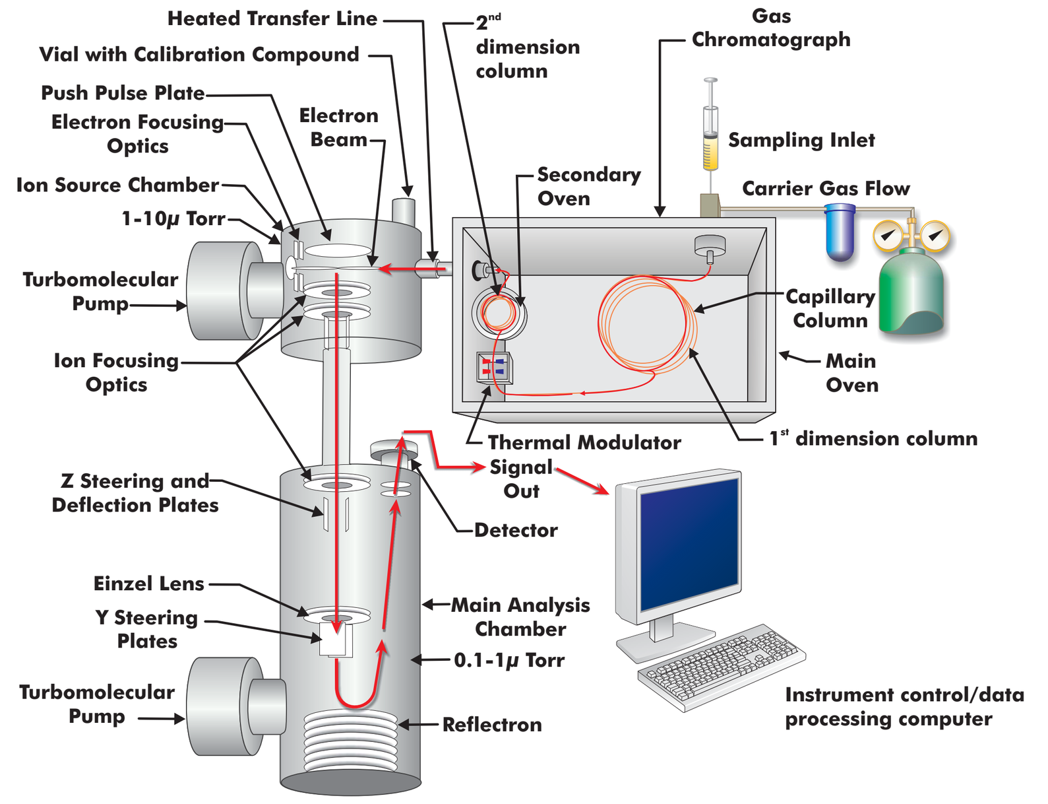
This research study conducted on diabetic and non-diabetic subjects will demonstrate the capabilities of two-dimensional chromatography combined with time-of-flight mass spectrometry to identify the metabolomic profile and show significant sample group differences by calculating Fisher Ratios in trimethylsilyl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

- ☑ Demonstrate the increased detectability of GCxGC-TOFMS analysis to identify the small 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.

GCxGC-TOFMS

LECO PEGASUS® 4D GCxGC-TOFMS

Time-of-flight mass spectrometry begins as GC separated molecules enter the ion source chamber. Electron impact ionization takes place in the source chamber by an applied electron beam of -70eV. Once a molecule is ionized it may undergo fragmentation due to excessive internal energy acquired during ionization. Positive fragment ions are pushed electrically at 5000 pulses per second (5kHz) and simultaneously accelerated from the ion source by electrical potential differences between the push pulse electrode and accelerating grid electrode. Accelerated ions leave the source and enter the drift region with essentially the same kinetic energy (KE). Mass analysis by TOFMS is measured by the travel time from the source to the detector of an ion with a specific mass to charge (m/z) ratio. The travel time of an ion is proportional to $m/z^{1/2}$. In other words, the heavier the ion, the longer its time-of-flight. Accelerated ions travel through optics, steering, and focusing plates to a reflectron or ion mirror. The reflectron improves resolution by compensating for small differences in the time of flight and spread of energy in ions with the same m/z ratios. Ions continue to travel in an optimal vacuum of approximately 10^{-6} Torr to the detector. The detector type is a microchannel plate (MCP) that amplifies the ion signal by the cascading effect of electron-ion emissions. Two significant advantages of time-of-flight mass spectrometry are simultaneous sampling of all ions for each mass spectrum and fast acquisition rates up to 500 spectra/second.


Heated Transfer Line
2nd dimension column
Gas Chromatograph
Vial with Calibration Compound
Push Pulse Plate
Electron Focusing Optics
Electron Beam
Sampling Inlet
Ion Source Chamber
1-10μ Torr
Secondary Oven
Carrier Gas Flow
Turbomolecular Pump
Capillary Column
Ion Focusing Optics
Main Oven
Thermal Modulator
1st dimension column
Z Steering and Deflection Plates
Signal Out
Detector
Einzel Lens
Y Steering Plates
Main Analysis Chamber
0.1-1μ Torr
Turbomolecular Pump
Reflectron
Instrument control/data processing computer

EXPERIMENTAL

Sample Preparation

- 4 Urine samples
  - 2 Non-diabetic control subjects
  - 1 Type I diabetic
  - 1 Type II diabetic

Sample Extraction Procedure

- 10 mL aliquots were acidified to pH2 with conc. H_2SO_4
- 6 samples from each subject were extracted with 2 mL methylene chloride
- Approximately 2 mg of sodium sulfate was added to each extract

BSTFA Derivatization Procedure

- 200 µL extract was placed in a 2 mL amber glass autosampler vial containing 0.5 mg of sodium sulfate
- 30 µL of pyridine was added the vial
- 100 µL of BSTFA was placed in the vial
- Samples were heated at 60°C for 1 hour
- Derivatized samples were then analyzed by GCxGC-TOFMS on the same day as prepared

GCxGC-TOFMS Analysis Parameters

Gas Chromatograph: Agilent 7890 equipped with a LECO two-stage cryogenic modulator and secondary oven
Primary Column: 30 m x 0.25 mm id. x 0.25 µm film thickness Rtx-5ms (Restek Corp., Bellefonte, PA)
Secondary Column: 1.5 m x 0.18 mm id. x 0.20 µm film thickness Rtx-200 (Restek Corp., Bellefonte, PA)
Carrier Gas: Helium set @ 1.5 mL/min.
Injection Mode: Splitless Injection Volume: 3 µL
Inlet Temperature: 260°C
Primary Column Temperature Program: Initial temperature set @ 40°C for 1 min. ramped @ 6°C/min. to 290°C; final hold time 10 minutes
Secondary Column Temperature Program: Initial temperature set @ 50°C for 1 min. ramped @ 6°C/min. to 300°C; final hold time 10 minutes
Total Run Time: 52.67 minutes

GCxGC parameters

Column Temperature Offset: 10°C
Modulator Temperature Offset: 25°C
Modulation Period: 5 s Hot Pulse Time: 0.08 s
Cool Time Between Stages: 1.7 s

Mass Spectrometer: LECO Pegasus 4D

Acquisition Delay: 250s
Mass Range: 45 - 800µ
Acquisition rate: 200 spectra/s
Ion source temperature: 230 °C
Detector Voltage: 1750 V
Electron Energy: -70 eV

GCxGC-TOFMS RESULTS

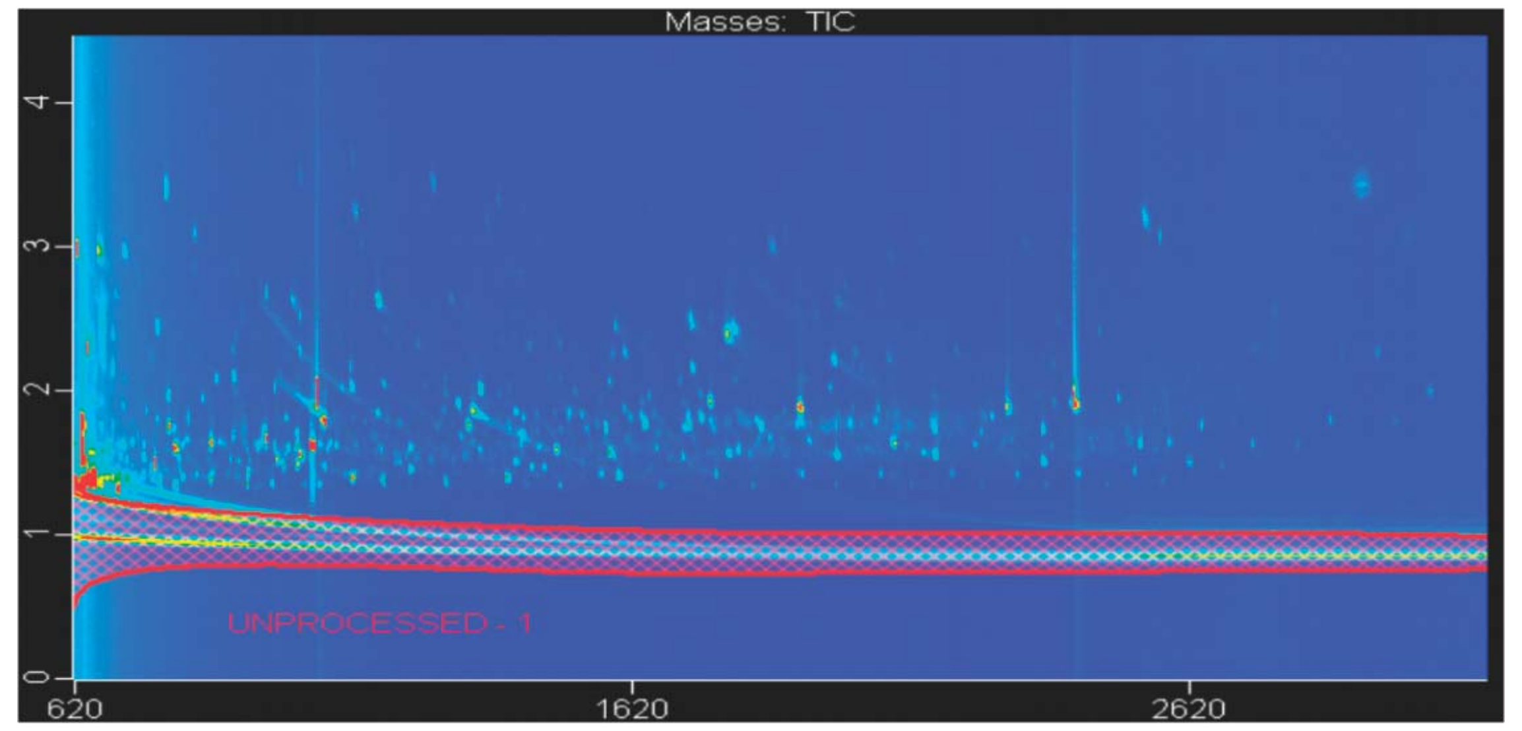


Figure 1. *Normal control subject Non-diabetic:* Contour Plot Total Ion Chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

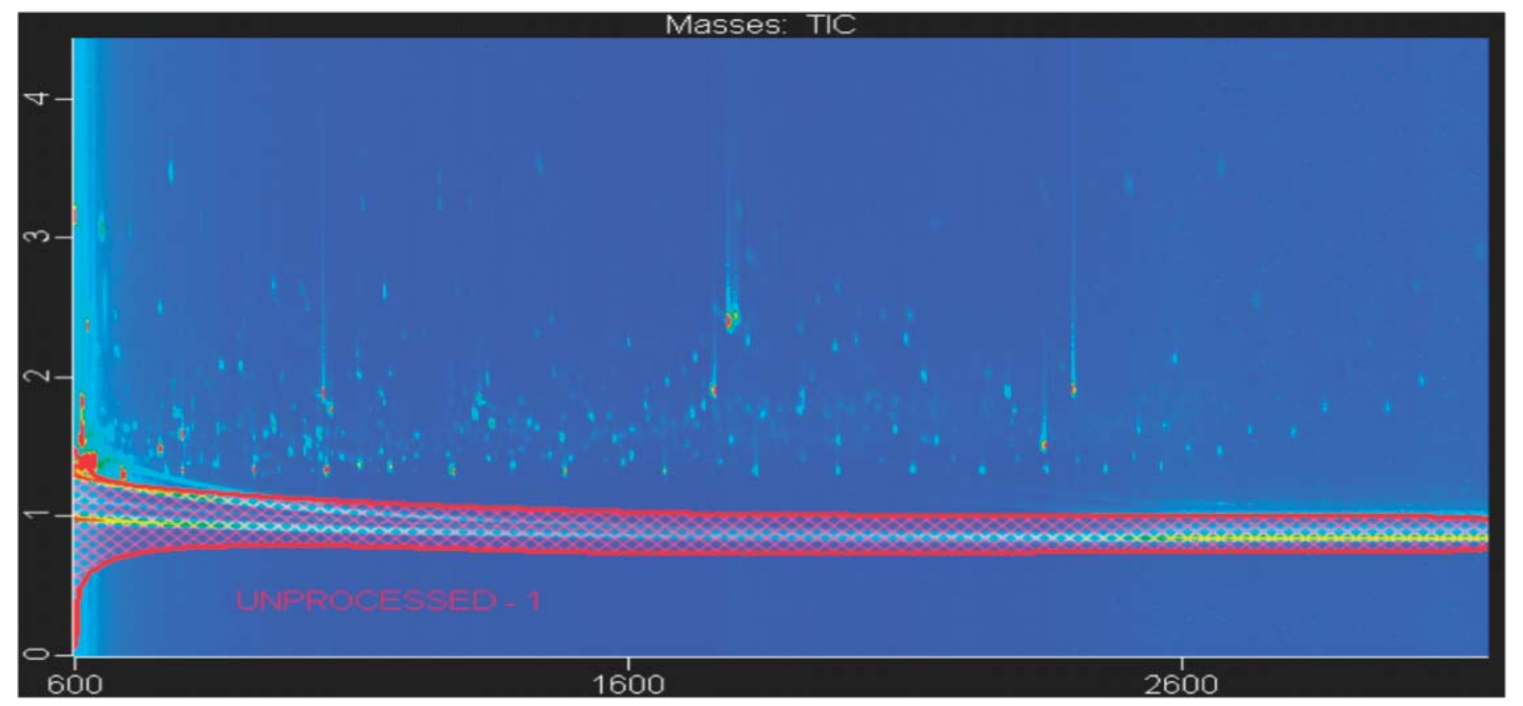


Figure 2. *Diseased state subject Type I diabetic:* Contour Plot Total Ion Chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

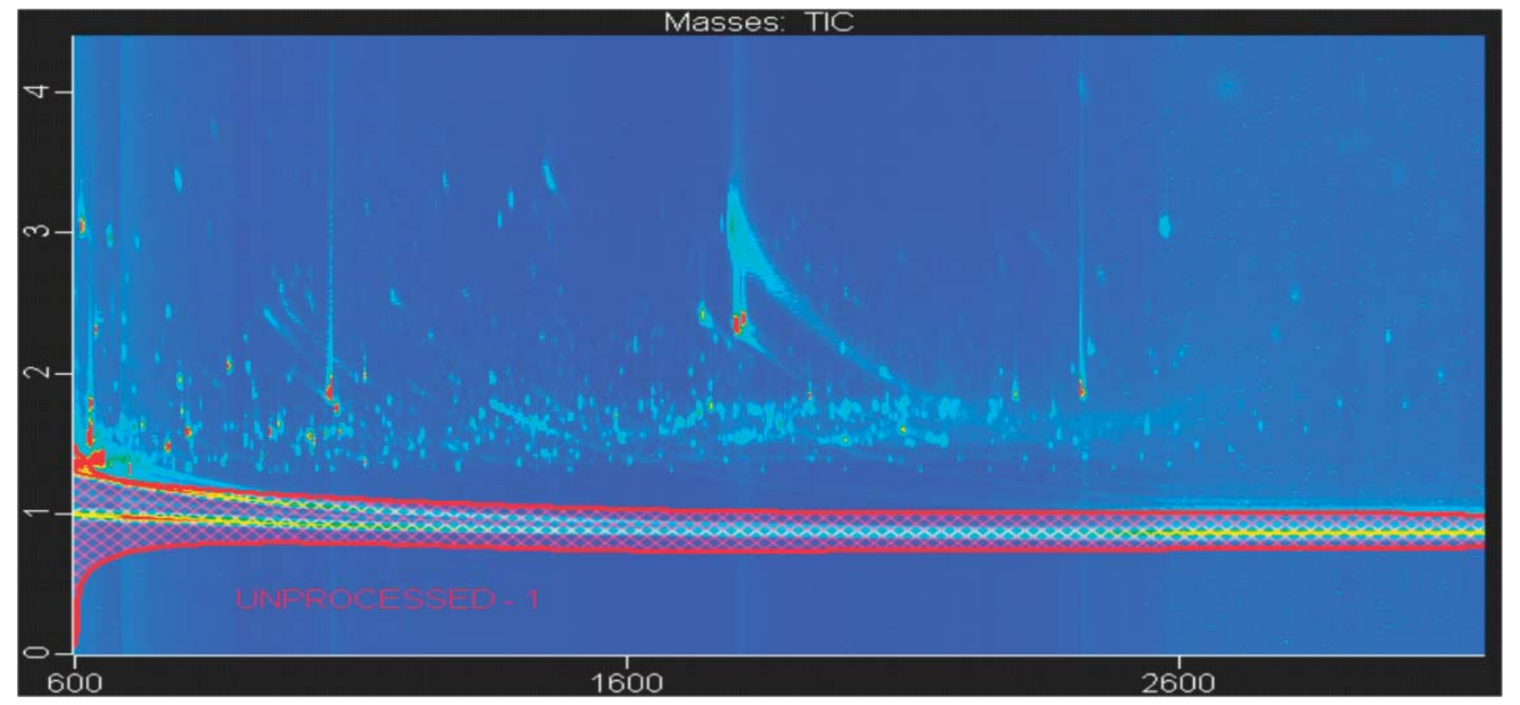


Figure 3. *Diseased state subject Type II diabetic:* Contour Plot Total Ion Chromatogram of TMS-derivatized urine sample showing the small molecule metabolite profile.

Results of the Diabetic Profile study between diabetic and non-diabetic subjects are shown in the figures above by the total ion chromatograms depicted as contour plots. These chromatographic examples visually illustrate 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.

STATISTICAL COMPARE/FISHER RATIO CALCULATION

The Statistical Compare option feature in the ChromaTOF software allows the user the ability to view statistical comparisons from groups of samples. This software feature aligns the data for the groups of samples specified in Statistical Compare. 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 comparisons among sample groups. Fisher Ratios can be calculated by the ChromaTOF software. The Fisher Ratio method uses an indexing scheme to discover the unknown chemical differences among known classes of complex samples.

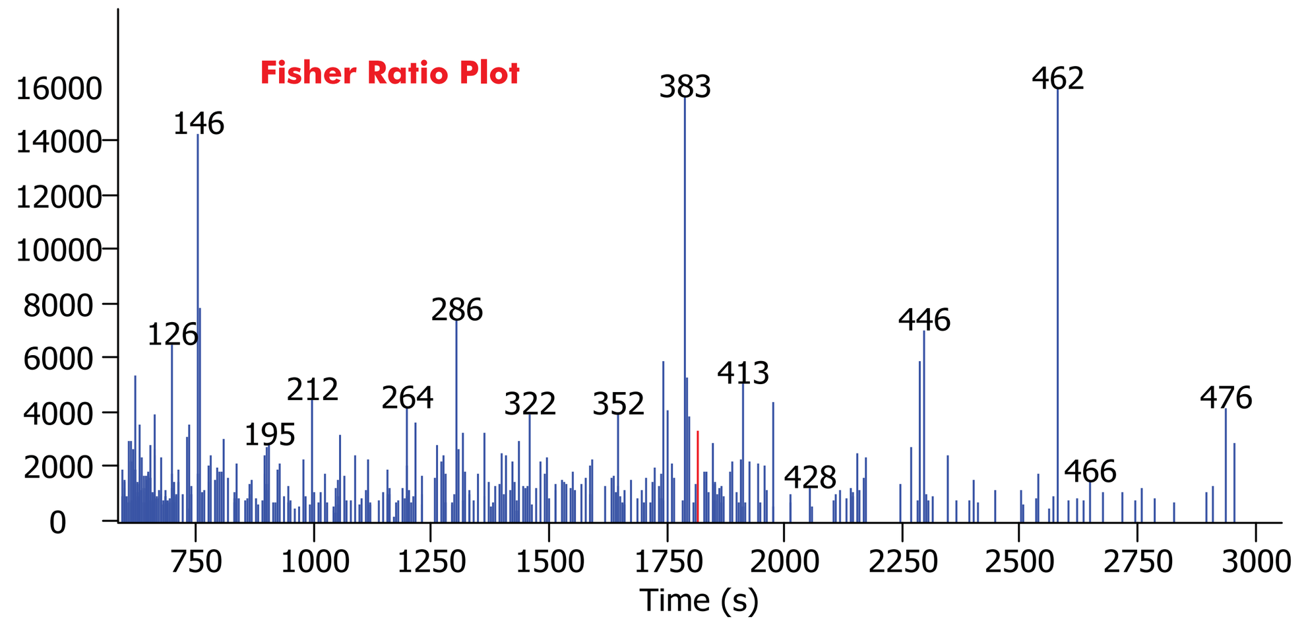


Figure 4. The Fisher Ratio plot shown above graphically represents unknown chemical differences between the normal control non-diabetic sample group and the Type I diabetic diseased state sample group.

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

Table 1. A Compound Statistics table is generated in the Statistical Compare feature of ChromaTOF software. The example above shows that Peak 390 was found in the Type I diabetic Class 1 and not found in the Normal Control Class 2.

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'-methanetetraylbis[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 partial 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.

Statistical Compare generated useful statistical information from the GCxGC-TOFMS research study conducted between non-diabetic and diabetic subjects. The Fisher Plot shown in Figure 4 shows that variation and chemical differences between sample groups can be quickly identified. The compound table (Table 3) and the Compound Statistics Table (Table 1) provide insightful information into the chemical nature and metabolite profile between disease state and non-disease state individuals.

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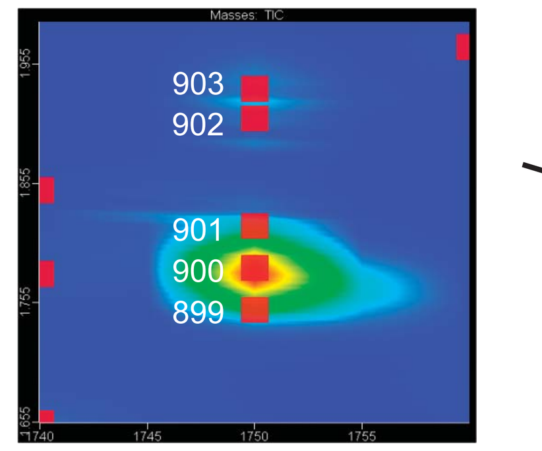

Figure 5. Contour plot showing 5 peak markers over what appears to be 2 resolved peaks.

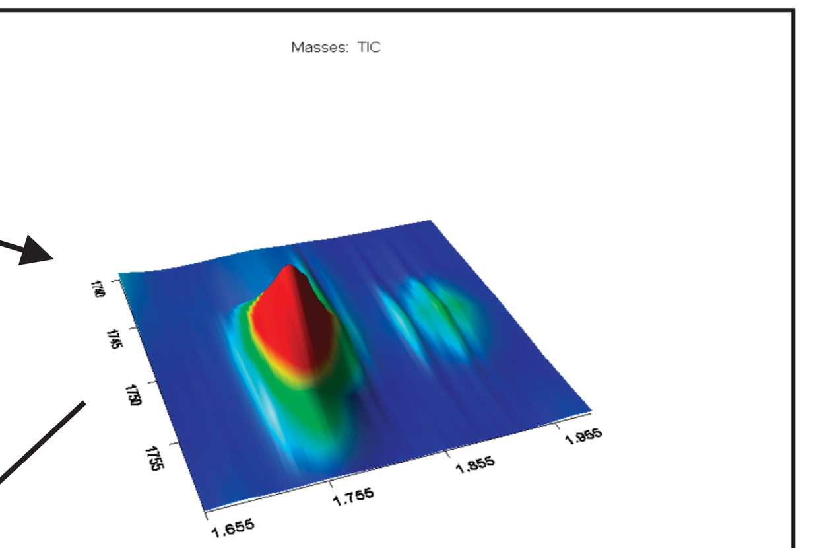

Figure 6. Surface plot showing second dimension separation of 5 compounds.

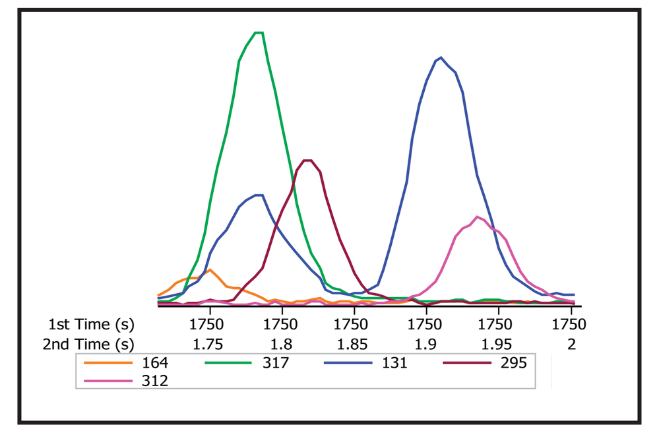

Figure 7. Extracted ion chromatogram of the unique masses for each of the 5 analytes found.

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 2. Peaks 899–903 with library matched identifications.

The figures 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.

CONCLUSIONS

The experimentation 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. 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 Statistical Compare feature of the ChromaTOF software was executed between the normal and diseased state sample data. Fisher Ratios were calculated for the diseased and non-disease 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 GCXGC-TOFMS analysis is an excellent tool for the characterization of the small molecule metabolite profile. The Statistical Compare and Fisher Ratio features allow the analyst to efficiently mine the data for key chemical differences and metabolites that will aid in the search for disease biomarkers.

For further information regarding the results obtained in this study, please contact the authors at john_heim@leco.com.