

Instrument: Pegasus® BT and ChromaTOF® Sync

A Comprehensive GC-TOFMS Metabolomics Workflow

LECO Corporation; Saint Joseph, Michigan USA

Key Words: Diabetes, Time-of-Flight Mass Spectrometry, ChromaTOF Sync

Introduction

The chronic disease diabetes is a result of irregular glucose metabolism resulting in hyperglycemia due to either a deficiency of insulin secretion, insulin resistance, or both.¹ Diabetes has reached epidemic levels, affecting over 420 million adults worldwide. This number is expected to increase to about 630 million by the year 2045.² Type-2 diabetes mellitus (T2DM) accounts for 90% of all diabetes cases. It is a major cause of blindness, kidney failure, heart attacks, and lower limb amputations. It is critical to diagnose diabetes in its early stages to initiate proper treatments for disease mediation or prevention. Early disease prediction can be facilitated by the implementation of a metabolomics method for the identification of T2DM biomarkers. The approach requires the generation of comprehensive data that can be statistically processed to identify key metabolites that can be associated with diseased individuals. This application note describes a complete workflow for the automated derivatization of plasma extracts from diseased and normal subjects, data acquisition using Gas Chromatography (GC) coupled to Time-of-Flight Mass Spectrometry (TOFMS), and processing with ChromaTOF Sync for the detection of candidate disease biomarkers.

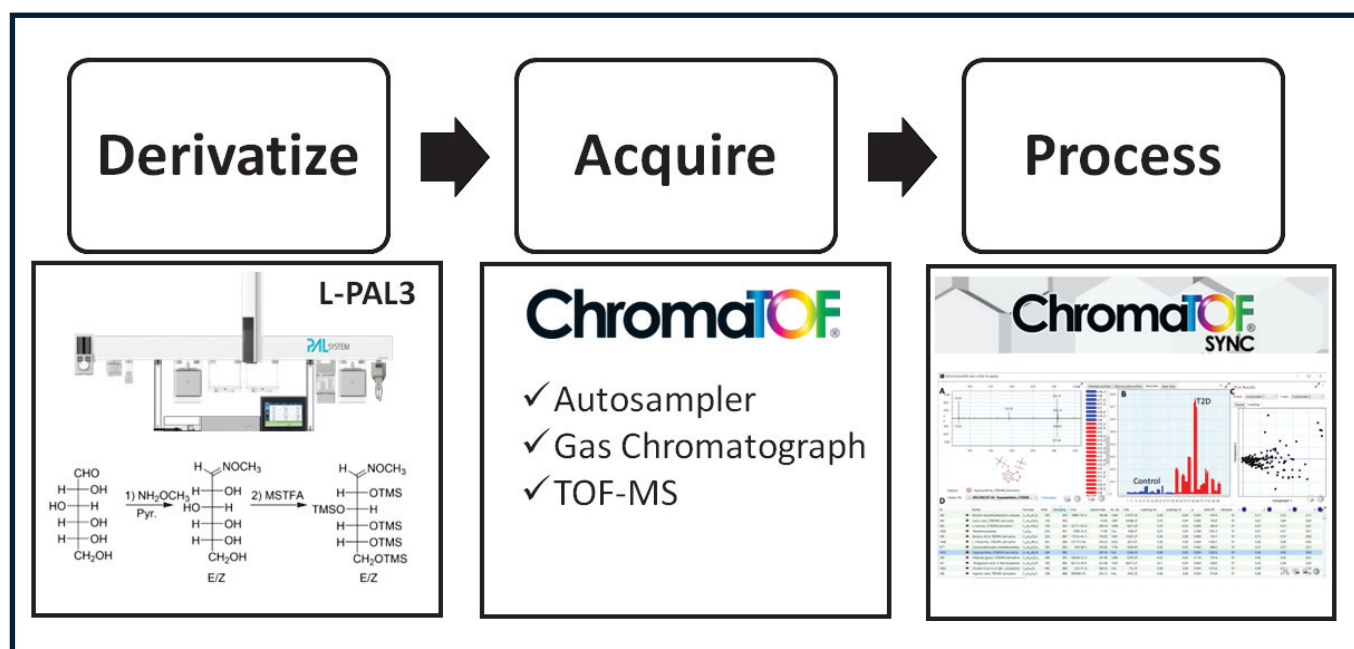


Figure 1. A complete GC-TOFMS metabolomics workflow: 1) Auto-derivatization, 2) data acquisition, and 3) statistical processing.

Experimental

Control (15) and diseased (15) samples were procured from BioIVT (Westbury, NY; www.bioivt.com) for the development and evaluation of the comprehensive metabolomics workflow. Plasma donors included females and males from different age groups. The patients with T2DM also had comorbidities such as hypertension, kidney disease, hyperlipidemia, and cardiovascular disease. Sample extraction was performed by transferring 100 μ L aliquots of plasma to 1.5 mL microtubes. Methanol (400 μ L) was added to the plasma samples forming a cloudy solution. Each sample was then vortexed for one minute, centrifuged for 10 minutes (5,000 RPM), and the supernatant of the resulting heterogeneous mixture was transferred to a 2 mL GC vial. The uncapped vials were placed in a Speed Vac and dried at room temperature under reduced pressure for 2 hours. The vials were then transferred to a lyophilizer and dried for 30 minutes ($T = -50\text{ }^{\circ}\text{C}$, $P < 0.1\text{ mBar}$). The dry residue was derivatized using LECO's L-PAL3 autosampler by adding 80 μ L of neat *N*-methyl-*N*-(*tert*-butyldimethylsilyl trifluoroacetamide) with 1% *tert*-butyldimethylchlorosilane (MTBSTFA w/1% TBDMCS, RESTEK, Bellefonte, PA) and heating at 75 $^{\circ}\text{C}$ for 45 minutes. The derivatized samples were analyzed in triplicate by GC-TOFMS (LECO Pegasus BT), using the instrument parameters listed (Table 1). The data files were processed and analyzed with ChromaTOF Sync and BT software.

Table 1. Instrument Parameters

Gas Chromatograph	Agilent 7890 & L-PAL 3 Autosampler
Injection	1.0 μ L (Split 20:1; 250 $^{\circ}\text{C}$)
Carrier Gas	He @ 1.4 mL/min, Constant Flow
Columns (1 st Dimension)	Rxi-5MS, 30 m x 0.25 mm i.d. x 0.25 μ m (Restek)
Temperature Program	50 $^{\circ}\text{C}$ (0.5 min), ramped 10 $^{\circ}\text{C}/\text{min}$ to 300 $^{\circ}\text{C}$ (10 min)
Mass Spectrometer	LECO Pegasus BT
Ion Source Temperature	250 $^{\circ}\text{C}$
Ionization Mode	El
Mass Range (m/z)	45-650
Acquisition Rate	10 spectra/s

Results and Discussion

Plasma sample data acquisition using gas chromatography high-performance time-of-flight mass spectrometry resulted in rich comprehensive data. The files were processed initially with ChromaTOF Sync. ChromaTOF Sync processing involved peak finding, data alignment, database comparisons, and statistical analysis (i.e., Principal Component Analysis). The data processing and analysis resulted in the annotation of several compounds that differentiated the control and T2DM plasma samples ($p < 0.01$). Candidate biomarkers included branched amino acids, short-chain hydroxy-substituted carboxylic acids, and purine metabolites (Table 2). These compounds have been previously targeted as potential diabetes biomarkers or monitors of ketoacidosis by various research groups.³⁻⁵

Table 2: Annotated Potential T2DM Biomarkers

Metabolite	p-value
Leucine	0.000
Isoleucine	0.000
Valine	0.018
Uric Acid	0.000
3-Hydroxybutyric acid	0.002
Hypoxanthine	0.000

ChromaTOF Sync processing results include the names, formulas, retention times, quant masses, and P-values of the metabolites in the plasma samples (Figure 2). The areas for selected metabolites (e.g., hypoxanthine) are displayed in the middle of the figure. The corresponding numerical area values are listed in the table below (D). In addition, the figure includes the spectral similarity score for the selected metabolite, as well as observed and library spectra (A). Finally, ChromaTOF Sync includes a Principal Component Analysis (PCA) feature for sample comparison (C).

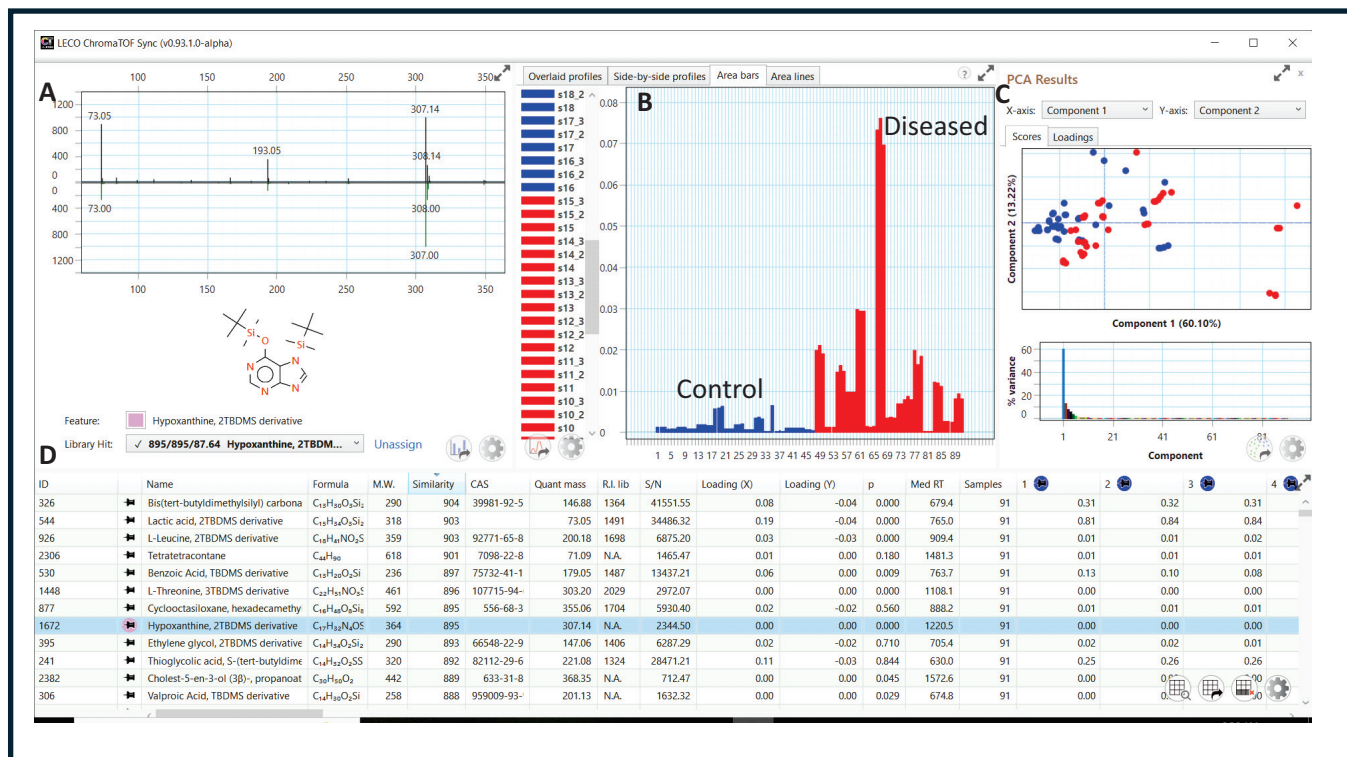


Figure 2. ChromaTOF Sync results for the comparison of T2DM and control human plasma. The display includes the spectral library search results for the selected metabolite hypoxanthine (A), a bar chart with hypoxanthine in control and diseased samples (B), a PCA plot (C), and tabulated results for the metabolites in the samples (D).

ChromaTOF Sync is a powerful standalone processing tool; however, metabolite annotation is significantly improved through a combination of ChromaTOF Sync and ChromaTOF BT software. In this workflow, data files were also processed using ChromaTOF BT software for confident compound characterization. ChromaTOF BT software has powerful deconvolution capabilities that result in improved spectral data for superior database matching and mass delta calculations. Automated processing resulted in the annotation of several metabolites in the plasma extracts including acids, diacids, amino acids, fatty acids, and sterols. The average spectral similarity score for the representative set of compounds listed in Table 3 was 820/1000.

Table 3: Representative Compounds in a Control Sample

Name	Formula	R.T. (s)	Similarity	Name	Formula	R.T. (s)	Similarity
Neophytadiene	C ₂₀ H ₃₈	520	725	Glycerol, 3TBDMS	C ₂₁ H ₅₀ O ₂ Si ₃	1024	908
Carbamic acid, N,N-dimethyl-, TBDMS	C ₉ H ₂₁ NO ₂ Si	525	957	Dodecanoic acid, TBDMS	C ₁₈ H ₃₈ O ₂ Si	1025	866
3,3-Dimethylacrylic acid, TBDMS	C ₁₁ H ₂₂ O ₂ Si	560	746	Erythrono-1,4-lactone, 2TBDMS	C ₁₆ H ₃₄ O ₄ Si ₂	1027	865
3-Methyl-2-ketobutyric acid, TBDMS	C ₁₁ H ₂₂ O ₃ Si	564	753	L-Aspartic acid, 3TBDMS	C ₂₂ H ₄₉ NO ₄ Si ₃	1181	825
3-Pyridinol, TBDMS derivative	C ₁₁ H ₁₉ NOSi	587	792	L-Pyroglutamic acid, 2TBDMS	C ₁₇ H ₃₅ NO ₃ Si ₂	1067	887
Maltol, TBDMS	C ₁₂ H ₂₀ O ₃ Si	603	766	Glyceric acid, 3TBDMS	C ₂₁ H ₄₈ O ₄ Si ₃	1069	786
2-Butoxyethanol, TBDMS	C ₁₂ H ₂₈ O ₂ Si	604	743	Salicylic acid, 2TBDMS	C ₁₉ H ₃₄ O ₃ Si ₂	1076	839
Phenol, TBDMS	C ₁₂ H ₂₀ OSi	605	801	L-Cysteine, 3TBDMS	C ₂₁ H ₄₉ NO ₂ Si ₃	1208	715
Hexanoic acid, TBDMS	C ₁₂ H ₂₆ O ₂ Si	611	923	N-Acetylaspartic acid, 2TBDMS	C ₁₈ H ₃₇ NO ₅ Si ₂	1080	715
Levulinic acid, TBDMS	C ₁₁ H ₂₂ O ₃ Si	665	835	L-Glutamic acid, 3TBDMS	C ₂₃ H ₅₁ NO ₄ Si ₃	1242	842
m-Cresol, TBDMS	C ₁₃ H ₂₂ OSi	685	936	L-Threonine, 3TBDMS	C ₂₃ H ₅₁ NO ₃ Si ₃	1108	900
Tiglic acid, TBDMS	C ₁₁ H ₂₂ O ₂ Si	723	750	2-Hydroxybenzeneacetic acid, 2TBDMS	C ₂₀ H ₃₆ O ₃ Si ₂	1112	706
2-Octenoic acid, TBDMS	C ₁₄ H ₂₈ O ₂ Si	759	718	Malic acid, 3TBDMS	C ₂₂ H ₄₈ O ₅ Si ₃	1119	701
Benzoic Acid, TBDMS	C ₁₃ H ₂₀ O ₂ Si	764	948	Myristoleic acid, TBDMS	C ₂₀ H ₄₀ O ₂ Si	1136	812
Lactic acid, 2TBDMS	C ₁₃ H ₂₄ O ₃ Si ₂	765	871	L-Lysine, 3TBDMS	C ₂₄ H ₅₆ N ₂ O ₂ Si ₃	1295	795
Glycine, 2TBDMS	C ₁₄ H ₃₃ NO ₂ Si ₂	796	736	Myristic acid, TBDMS	C ₂₀ H ₄₂ O ₂ Si	1141	942
Glycolic acid, 2TBDMS	C ₁₄ H ₃₀ O ₃ Si ₂	776	862	L-Phenylalanine, 2TBDMS	C ₂₃ H ₃₉ NO ₂ Si ₂	1147	878
Phenylacetic acid, TBDMS	C ₁₄ H ₂₂ O ₂ Si	792	851	Hippuric acid, 2TBDMS	C ₁₅ H ₂₃ NO ₃ Si	1148	905
L-Alanine, 2TBDMS	C ₁₅ H ₃₅ NO ₂ Si ₂	799	903	4-Hydroxybenzoic acid, 2TBDMS	C ₁₉ H ₃₄ O ₃ Si ₂	1160	784
Oxalic acid, 2TBDMS	C ₁₅ H ₃₀ O ₄ Si ₂	807	894	9,12-Octadecadienoic acid (Z,Z)-, TBDMS	C ₂₄ H ₄₆ O ₂ Si	1332	774
Uracil, 2TBDMS	C ₁₆ H ₃₂ N ₂ O ₂ Si ₂	842	708	Pentadecanoic acid, TBDMS	C ₂₁ H ₄₄ O ₂ Si	1195	827
a-Hydroxybutyric acid, TBDMS	C ₁₆ H ₃₆ O ₃ Si ₂	814	900	L-Histidine, 3TBDMS	C ₂₄ H ₅₁ N ₃ O ₂ Si ₃	1396	737
DL-Glyceraldehyde, 2TBDMS	C ₁₅ H ₃₄ O ₃ Si ₂	819	839	Hypoxanthine, 2TBDMS	C ₁₇ H ₃₂ N ₄ O ₅ Si ₂	1220	910
3-Hydroxypropionic acid, 2TBDMS	C ₁₅ H ₃₄ O ₃ Si ₂	828	809	Arachidonic Acid, TBDMS	C ₂₆ H ₄₆ O ₂ Si	1406	829
Nonanoic acid, TBDMS	C ₁₅ H ₃₂ O ₂ Si	832	881	Isonanillic acid, 2TBDMS	C ₂₀ H ₃₆ O ₄ Si ₂	1232	719
Urea, 2TBDMS	C ₁₃ H ₃₂ N ₂ O ₂ Si ₂	878	786	9-Hexadecenoic acid, (Z)-, TBDMS	C ₂₂ H ₄₄ O ₂ Si	1238	886
3-Hydroxybutyric acid, 2TBDMS	C ₁₆ H ₃₆ O ₃ Si ₂	838	912	Palmitic acid, TBDMS	C ₂₂ H ₄₆ O ₂ Si	1247	944
2-Hydroxyisovalerate, 2TBDMS	C ₁₇ H ₃₈ O ₃ Si ₂	846	794	Doconexent, TBDMS	C ₂₈ H ₄₆ O ₂ Si	1491	796
2-Aminobutanoic acid, TBMS	C ₁₆ H ₃₇ NO ₂ Si ₂	848	884	Uric acid, 4TBDMS	C ₂₉ H ₆₀ N ₄ O ₃ Si ₄	1517	792
Decanoic acid, TBDMS	C ₁₆ H ₃₄ O ₂ Si	899	724	Margaric acid, TBDMS	C ₂₃ H ₄₈ O ₂ Si	1297	880
Benzenepropanoic acid, TBDMS	C ₁₅ H ₂₄ O ₂ Si	870	704	L-Glutamine, 3TBDMS	C ₂₃ H ₅₂ N ₂ O ₃ Si ₃	1317	795
Benzophenone-d10 (ISTD)	C ₁₁ H ₁₆ N ₂ O	872	637	Lignoceric acid, TBDMS	C ₃₀ H ₆₂ O ₂ Si	1608	763
(±)-2-Phenylpropanoic Acid, TBDMS	C ₁₅ H ₂₄ O ₂ Si	875	754	Oleic Acid, (E)-, TBDMS	C ₂₄ H ₄₈ O ₂ Si	1334	948
L-Valine, 2TBDMS	C ₁₇ H ₃₃ NO ₂ Si ₂	882	797	Vaccenic acid, (E)-, TBDMS	C ₂₄ H ₄₈ O ₂ Si	1337	808
Succinic acid, 2TBDMS	C ₁₆ H ₃₄ O ₄ Si ₂	948	705	Stearic acid, TBDMS	C ₂₄ H ₅₀ O ₂ Si	1345	950
1-Dodecanol, TBDMS	C ₁₈ H ₄₀ OSi	968	752	2-Hydroxyhippuric acid, 2TBDMS	C ₂₁ H ₃₇ NO ₄ Si ₂	1374	842
L-Leucine, 2TBDMS	C ₁₈ H ₄₁ NO ₂ Si ₂	909	912	3,5-Dihydroxybenzoic acid, 3TBDMS	C ₂₅ H ₄₈ O ₄ Si ₃	1402	710
Isoleucine, 2TBDMS	C ₁₈ H ₄₁ NO ₂ Si ₂	930	885	Citric acid, 4TBDMS	C ₃₀ H ₆₄ O ₇ Si ₄	1408	813
3,4-Dimethylbenzoic acid, TBDMS	C ₁₅ H ₂₄ O ₂ Si	934	854	L-Tyrosine, 3TBDMS	C ₂₇ H ₅₃ NO ₃ Si ₃	1417	793
3-methyl-2-oxopentanoic acid, 2TBDMS	C ₁₈ H ₃₈ O ₃ Si ₂	946	738	L-Tryptophan, 3TBDMS	C ₂₉ H ₅₄ N ₂ O ₂ Si ₃	1509	829
L-Proline, 2TBDMS	C ₁₇ H ₃₇ NO ₂ Si ₂	955	852	Cholesta-3,5-diene	C ₂₇ H ₄₄	1573	889
L-Serine, 3TBDMS	C ₂₁ H ₄₉ NO ₃ Si ₃	1090	725	Cholesterol, TBDMS	C ₃₃ H ₆₀ OSi	1865	935

The analytical ion chromatograms (AICs) for a diseased (red) and a control (blue) plasma are displayed, with three labeled peak markers for candidate biomarkers from the ChromaTOF Sync results (Figure. 3). The compounds include 3-hydroxybutyric, hippuric acid, and hypoxanthine. These metabolites are elevated in T2DM samples as illustrated by the extracted ion chromatograms (XICs) in Figures 4, 5, and 6. The Peak True and library match spectral data, as well as the associated spectral similarity score, are also displayed in these figures.

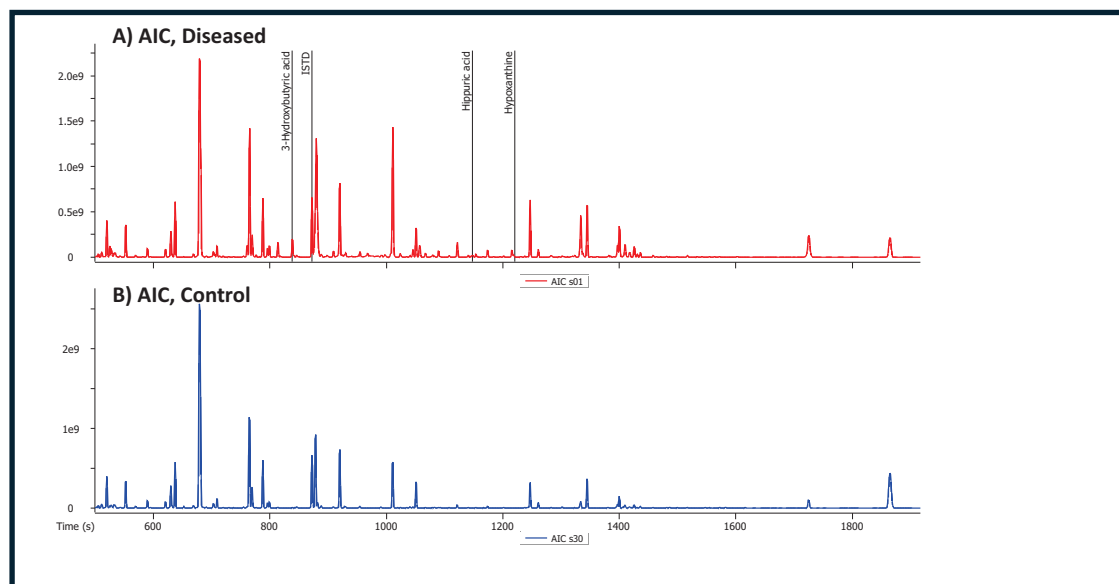


Figure 3. AICs for T2DM (A) and control (B) samples. Peak markers for 3-hydroxybutyric acid, hippuric acid, and hypoxanthine are displayed in the XIC for the diseased sample.

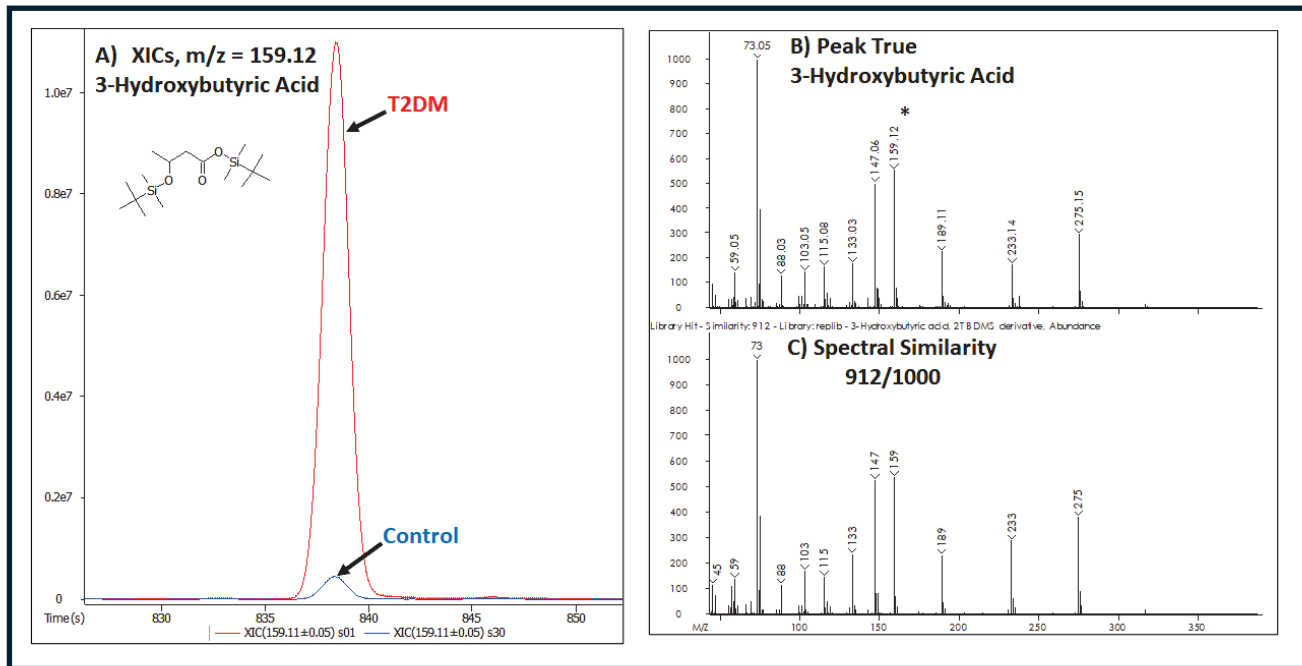


Figure 4. A) XIC (m/z = 159.12) for 3-hydroxybutyric acid in a control and T2DM sample, B) Peak True, and C) library Spectra for 3-hydroxybutyric acid.

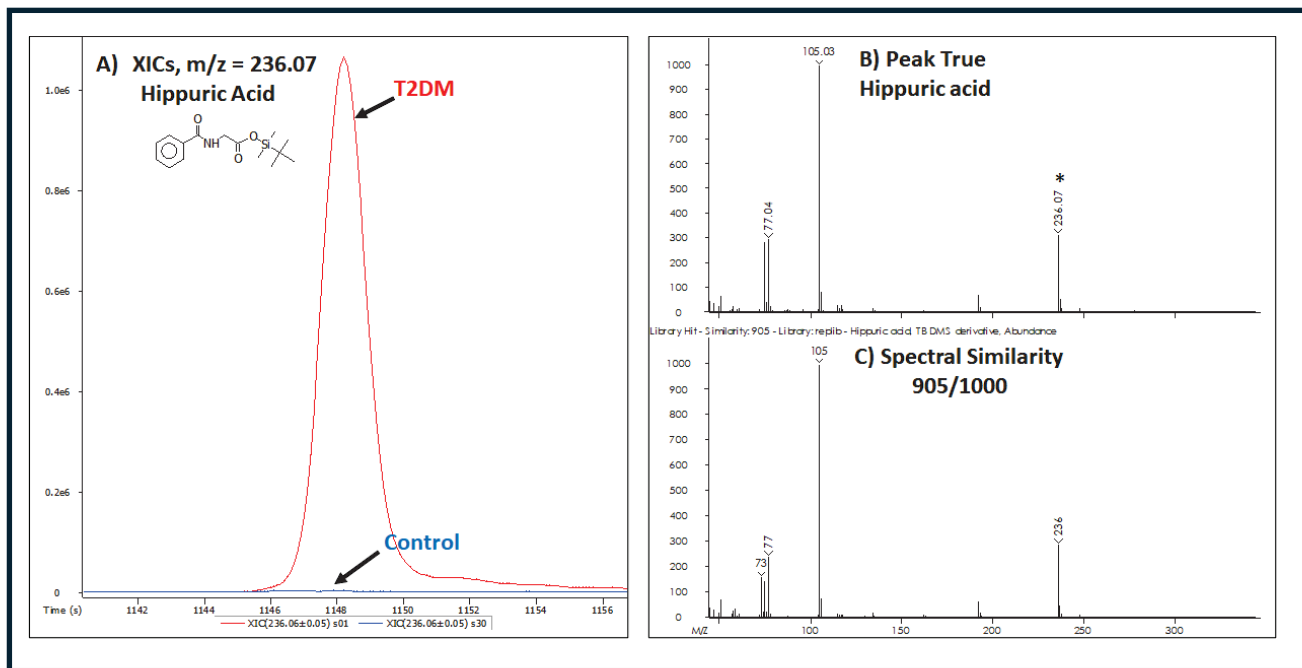


Figure 5. A) XIC (m/z = 236.06) for hippuric acid in a control and T2DM sample, B) Peak True, and C) library Spectra for hippuric acid.

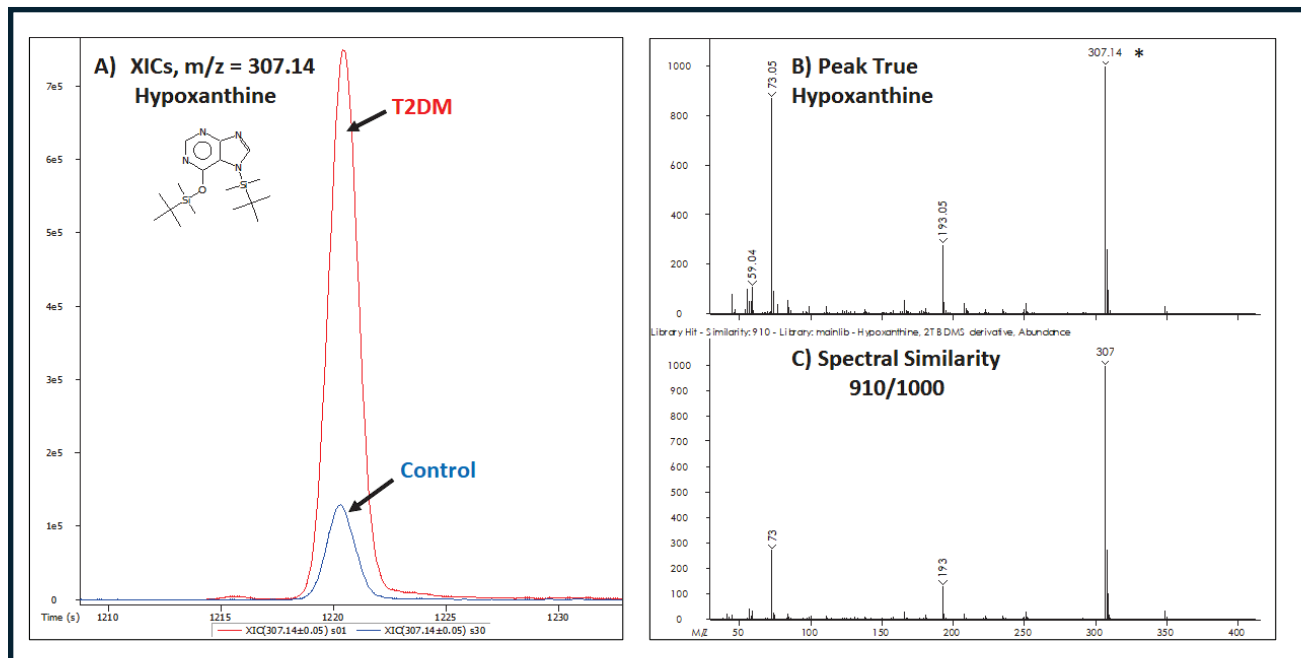


Figure 6. A) XIC (m/z = 307.14) for hypoxanthine in a control and T2DM sample, B) Peak True, and C) library Spectra for hypoxanthine.

Conclusion

A comprehensive GC-TOFMS metabolomics workflow for the annotation of potential T2DM biomarkers was developed using the LECO Pegasus BT, ChromaTOF BT, and ChromaTOF Sync software. The workflow includes automated sample preparation, data collection, and processing that features data alignment and statistical analysis. The utilization of ChromaTOF Sync together with ChromaTOF BT software for data processing resulted in the confident characterization of candidate disease biomarkers.

References

- ¹Gedela S., Rao A.A., Medicheria N.R., *International Journal of Biomedical Science* **2007**, 3(4), 229-236.
- ²Laakso M., *Molecular Metabolism* **2019**, 27, S139-S146.
- ³Long G., Yang Z., Wang L., Han Y., Peng C., Yan C., and Yan C., *BMC Endocrine Disorders*, **2020**, 20,174.
- ⁴Long L., Liu H., Wang Yan, Wang Yuming, Liu J., Zhou Z., Chu H., Zhuang P., and Zhang Y., *Journal of Chromatography B*, **2015**, 997, 96-104.
- ⁵Zhao X., Fritsche J., Wang J., Chen J., Rittig K., Schmitt-Kopplin S., Fritsche A., Haring H-H. Schleicher E.D., Xu G., and Lehmann R., *Metabolomics* **2010**, 7, 362-374.

ChromaTOF[®]|SYNC



LECO Corporation | 3000 Lakeview Avenue | St. Joseph, MI 49085 | Phone: 800-292-6141 | 269-985-5496
 info@leco.com • www.leco.com | ISO-9001:2015 Q-994 | LECO is a registered trademark of LECO Corporation.
 Pegasus, ChromaTOF are registered trademarks of LECO Corporation.