Application Note

Instrument: Pegasus® BT and ChromaTOF® Sync

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A Comprehensive GC-TOFMS Metabolomics Workflow

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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.

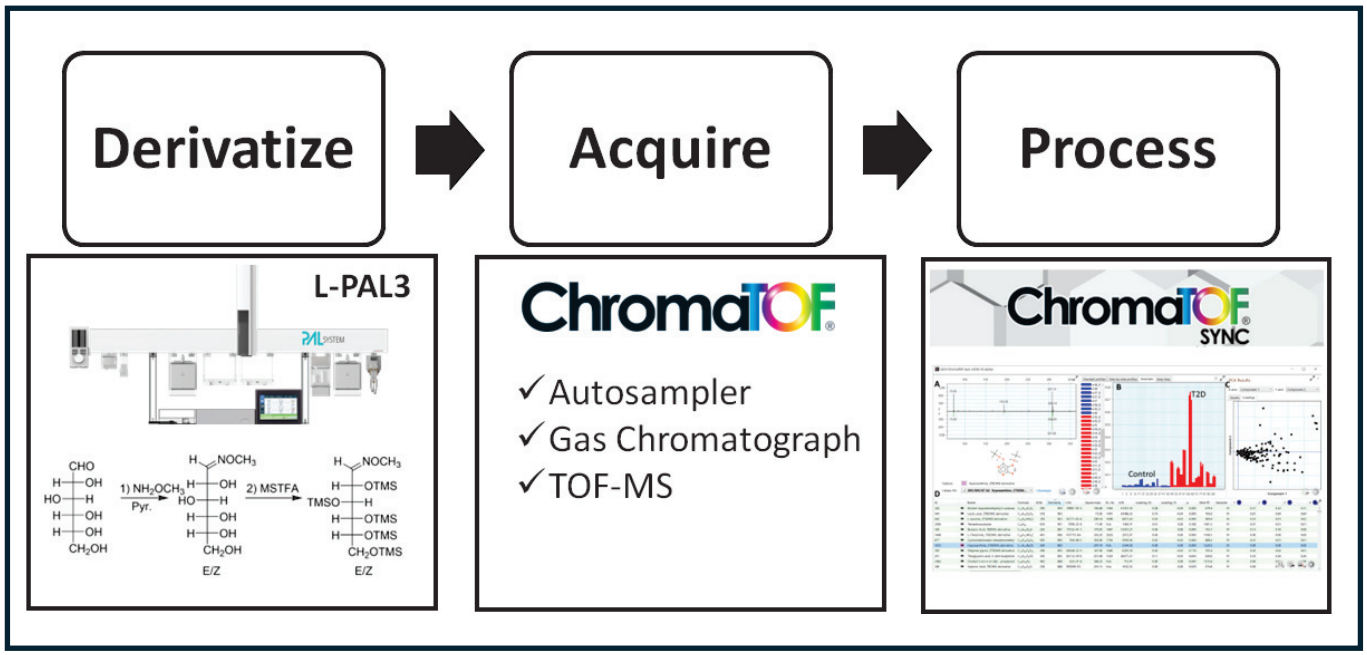


Figure 1. A complete GC-TOFMS metabolomics workflow: 1) Auto-derivatization, 2) data acquisition, and 3) statistical processing.

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 µL aliquots of plasma to 1.5 mL microtubes. Methanol (400 µL) 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 heterogenous 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 °C, P<0.1 mBar). The dry residue was derivatized using LECO's L-PAL3 autosampler by adding 80 µL of neat *N*-methyl-*N[tert*-butyldimethylsilyl trifluoroacetamide] with 1% *tert*-butyldimethylchlorosilinae (MTBSTFA w/1% TBDMCS, RESTEK, Bellefonte, PA) and heating at 75 °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 µL (Split 20:1; 250 °C)
Carrier Gas	He @ 1.4 mL/min, Constant Flow
Columns (1st Dimension)	Rxi-5MS, 30 m x 0.25 mm i.d. x 0.25 µm (Restek)
Temperature Program	50 °C (0.5 min), ramped 10 °C/min to 300 °C (10 min)
Mass Spectrometer	**LECO *Pegasus* BT**
Ion Source Temperature	250 °C
Ionization Mode	EI
Mass Range (m/z)	45-650
Acquisition Rate	10 spectra/s

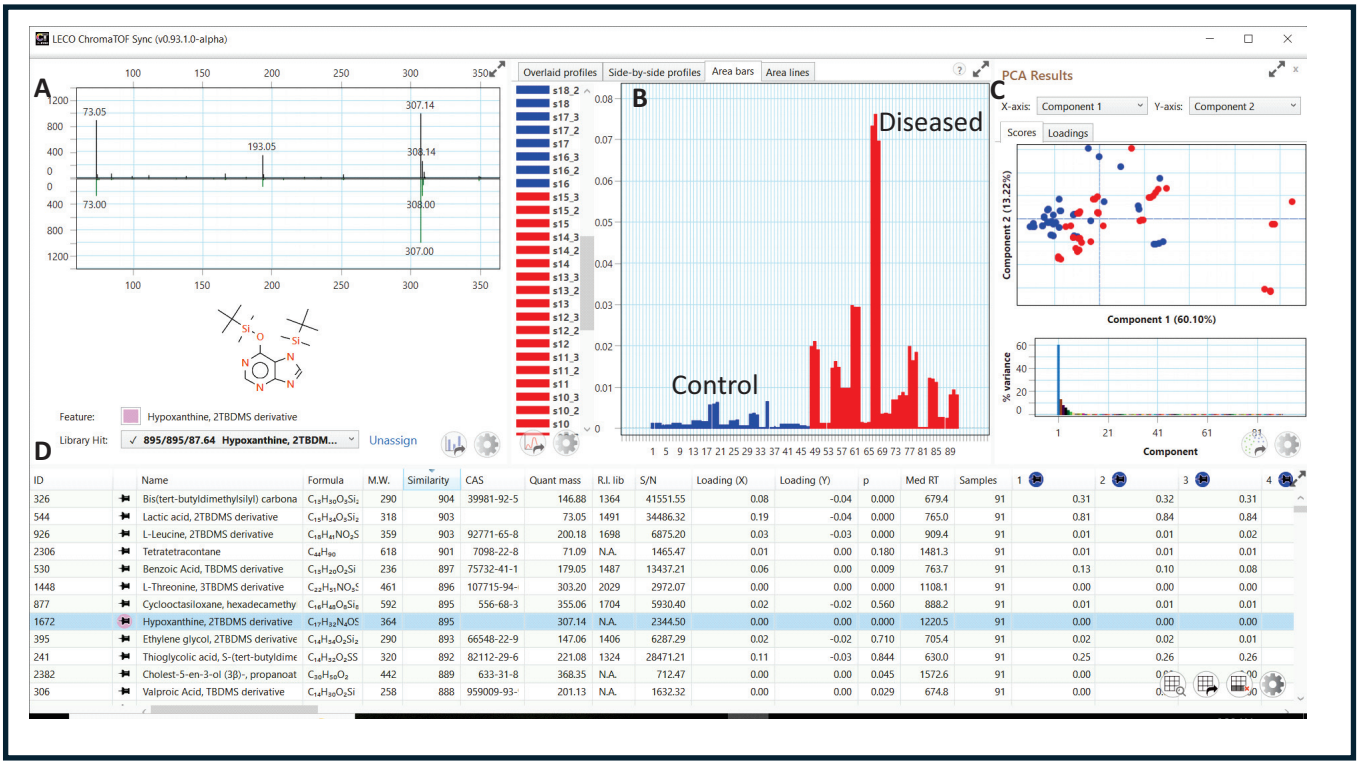
Results and Discussion

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).



ID	Name	Formula	M.W.	Similarity	CAS	Quant mass	R.I. lib	S/N	Loading (X)	Loading (Y)	p	Med RT	Samples	1	2	3	4
326	Bis(tert-butyldimethylsilyl) carbona	$C_{13}H_{30}O_3Si_2$	290	904	39981-92-5	146.88	1364	41551.55	0.08	-0.04	0.000	679.4	91	0.31	0.32	0.31	
544	Lactic acid, 2TBDMS derivative	$C_{15}H_{34}O_3Si_2$	318	903		73.05	1491	34486.32	0.19	-0.04	0.000	765.0	91	0.81	0.84	0.84	
926	L-Leucine, 2TBDMS derivative	$C_{18}H_{41}NO_2S$	359	903	92771-65-8	200.18	1698	6875.20	0.03	-0.03	0.000	909.4	91	0.01	0.01	0.02	
2306	Tetratetracontane	$C_{44}H_{90}$	618	901	7098-22-8	71.09	N.A.	1465.47	0.01	0.00	0.180	1481.3	91	0.01	0.01	0.01	
530	Benzoic Acid, TBDMS derivative	$C_{13}H_{20}O_2Si$	236	897	75732-41-1	179.05	1487	13437.21	0.06	0.00	0.009	763.7	91	0.13	0.10	0.08	
1448	L-Threonine, 3TBDMS derivative	$C_{22}H_{51}NO_3S$	461	896	107715-94-	303.20	2029	2972.07	0.00	0.00	0.000	1108.1	91	0.00	0.00	0.00	
877	Cyclooctasiloxane, hexadecamethy	$C_{16}H_{48}O_8Si_8$	592	895	556-68-3	355.06	1704	5930.40	0.02	-0.02	0.560	888.2	91	0.01	0.01	0.01	
1672	Hypoxanthine, 2TBDMS derivative	$C_{17}H_{32}N_4OS$	364	895		307.14	N.A.	2344.50	0.00	0.00	0.000	1220.5	91	0.00	0.00	0.00	
395	Ethylene glycol, 2TBDMS derivative	$C_{14}H_{34}O_2Si_2$	290	893	66548-22-9	147.06	1406	6287.29	0.02	-0.02	0.710	705.4	91	0.02	0.02	0.01	
241	Thioglycolic acid, S-(tert-butyldime	$C_{14}H_{32}O_2SS$	320	892	82112-29-6	221.08	1324	28471.21	0.11	-0.03	0.844	630.0	91	0.25	0.26	0.26	
2382	Cholest-5-en-3-ol (3β)-, propanoat	$C_{30}H_{50}O_2$	442	889	633-31-8	368.35	N.A.	712.47	0.00	0.00	0.045	1572.6	91	0.00	0.0	00	
306	Valproic Acid, TBDMS derivative	$C_{14}H_{30}O_2Si$	258	888	959009-93-	201.13	N.A.	1632.32	0.00	0.00	0.029	674.8	91	0.00	0.0	00	

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*
Neophytadiene	$C_{20}H_{38}$	520	725
Carbamic acid, N,N-dimethyl-, TBDMS	$C_9H_{21}NO_2Si$	525	957
3,3-Dimethylacrylic acid, TBDMS	$C_{11}H_{22}O_2Si$	560	746
3-Methyl-2-ketobutyric acid, TBDMS	$C_{11}H_{22}O_3Si$	564	753
3-Pyridinol, TBDMS derivative	$C_{11}H_{19}NOSi$	587	792
Maltol, TBDMS	$C_{12}H_{20}O_3Si$	603	766
2-Butoxyethanol, TBDMS	$C_{12}H_{28}O_2Si$	604	743
Phenol, TBDMS	$C_{12}H_{20}OSi$	605	801
Hexanoic acid, TBDMS	$C_{12}H_{26}O_2Si$	611	923
Levulinic acid, TBDMS	$C_{11}H_{22}O_3Si$	665	835
m-Cresol, TBDMS	$C_{13}H_{22}OSi$	685	936
Tiglic acid, TBDMS	$C_{11}H_{22}O_2Si$	723	750
2-Octenoic acid, TBDMS	$C_{14}H_{28}O_2Si$	759	718
Benzoic Acid, TBDMS	$C_{13}H_{20}O_2Si$	764	948
Lactic acid, 2TBDMS	$C_{15}H_{34}O_3Si_2$	765	871
Glycine, 2TBDMS	$C_{14}H_{33}NO_2Si_2$	796	736
Glycolic acid, 2TBDMS	$C_{14}H_{32}O_3Si_2$	776	862
Phenylacetic acid, TBDMS	$C_{14}H_{22}O_2Si$	792	851
L-Alanine, 2TBDMS	$C_{15}H_{35}NO_2Si_2$	799	903
Oxalic acid, 2TBDMS	$C_{14}H_{30}O_4Si_2$	807	894
Uracil, 2TBDMS	$C_{16}H_{32}N_2O_2Si_2$	842	708
a-Hydroxybutyric acid, TBDMS	$C_{16}H_{36}O_3Si_2$	814	900
DL-Glyceraldehyde, 2TBDMS	$C_{15}H_{34}O_3Si_2$	819	839
3-Hydroxypropionic acid, 2TBDMS	$C_{15}H_{34}O_3Si_2$	828	809
Nonanoic acid, TBDMS	$C_{15}H_{32}O_2Si$	832	881
Urea, 2TBDMS	$C_{13}H_{32}N_2OSi_2$	878	786
3-Hydroxybutyric acid, 2TBDMS	$C_{16}H_{36}O_3Si_2$	838	912
2-Hydroxyisovalerate, 2TBDMS	$C_{17}H_{38}O_3Si_2$	846	794
2-Aminobutanoic acid, TBMS	$C_{16}H_{37}NO_2Si_2$	848	884
Decanoic acid, TBDMS	$C_{16}H_{34}O_2Si$	899	724
Benzenepropanoic acid, TBDMS	$C_{15}H_{24}O_2Si$	870	704
Benzophenone-*d10 (ISTD)*	$C_{11}H_{16}N_2O$	872	637
(±)-2-Phenylpropanoic Acid, TBDMS	$C_{15}H_{24}O_2Si$	875	754
L-Valine, 2TBDMS	$C_{17}H_{39}NO_2Si_2$	882	797
Succinic acid, 2TBDMS	$C_{16}H_{34}O_4Si_2$	948	705
1-Dodecanol, TBDMS	$C_{18}H_{40}OSi$	968	752
L-Leucine, 2TBDMS	$C_{18}H_{41}NO_2Si_2$	909	912
Isoleucine, 2TBDMS	$C_{18}H_{41}NO_2Si_2$	930	885
3,4-Dimethylbenzoic acid, TBDMS	$C_{15}H_{24}O_2Si$	934	854
3-methyl-2-oxopentanoic acid, 2TBDMS	$C_{18}H_{38}O_3Si_2$	946	738
L-Proline, 2TBDMS	$C_{17}H_{37}NO_2Si_2$	955	852
L-Serine, 3TBDMS	$C_{21}H_{49}NO_3Si_3$	1090	725

Name	*Formula*	*R.T. (s)*	*Similarity*
Glycerol, 3TBDMS	$C_{21}H_{50}O_3Si_3$	1024	908
Dodecanoic acid, TBDMS	$C_{18}H_{38}O_2Si$	1025	866
Erythrono-1,4-lactone, 2TBDMS	$C_{16}H_{34}O_4Si_2$	1027	865
L-Aspartic acid, 3TBDMS	$C_{22}H_{49}NO_4Si_3$	1181	825
L-Pyroglutamic acid, 2TBDMS	$C_{17}H_{35}NO_3Si_2$	1067	887
Glyceric acid, 3TBDMS	$C_{21}H_{48}O_4Si_3$	1069	786
Salicylic acid, 2TBDMS	$C_{19}H_{34}O_3Si_2$	1076	839
L-Cysteine, 3TBDMS	$C_{21}H_{49}NO_2SSi_3$	1208	715
N-Acetylaspartic acid, 2TBDMS	$C_{18}H_{37}NO_5Si_2$	1080	715
L-Glutamic acid, 3TBDMS	$C_{23}H_{51}NO_4Si_3$	1242	842
L-Threonine, 3TBDMS	$C_{22}H_{51}NO_3Si_3$	1108	900
2-Hydroxybenzeneacetic acid, 2TBDMS	$C_{20}H_{36}O_3Si_2$	1112	706
Malic acid, 3TBDMS	$C_{22}H_{48}O_5Si_3$	1119	701
Myristoleic acid, TBDMS	$C_{20}H_{40}O_2Si$	1136	812
L-Lysine, 3TBDMS	$C_{24}H_{56}N_2O_2Si_3$	1295	795
Myristic acid, TBDMS	$C_{20}H_{42}O_2Si$	1141	942
L-Phenylalanine, 2TBDMS	$C_{21}H_{39}NO_2Si_2$	1147	878
Hippuric acid, 2TBDMS	$C_{15}H_{23}NO_3Si$	1148	905
4-Hydroxybenzoic acid, 2TBDMS	$C_{19}H_{34}O_3Si_2$	1160	784
9,12-Octadecadienoic acid (Z,Z)-, TBDMS	$C_{24}H_{46}O_2Si$	1332	774
Pentadecanoic acid, TBDMS	$C_{21}H_{44}O_2Si$	1195	827
L-Histidine, 3TBDMS	$C_{24}H_{51}N_3O_2Si_3$	1396	737
Hypoxanthine, 2TBDMS	$C_{17}H_{32}N_4OSi_2$	1220	910
Arachidonic Acid, TBDMS	$C_{26}H_{46}O_2Si$	1406	829
Isovanillic acid, 2TBDMS	$C_{20}H_{36}O_4Si_2$	1232	719
9-Hexadecenoic acid, (Z)-, TBDMS	$C_{22}H_{44}O_2Si$	1238	886
Palmitic Acid, TBDMS	$C_{22}H_{46}O_2Si$	1247	944
Doconexent, TBDMS	$C_{28}H_{46}O_2Si$	1491	796
Uric acid, 4TBDMS	$C_{29}H_{60}N_4O_3Si_4$	1517	792
Margaric acid, TBDMS	$C_{23}H_{48}O_2Si$	1297	880
L-Glutamine, 3TBDMS	$C_{23}H_{52}N_2O_3Si_3$	1317	795
Lignoceric acid, TBDMS	$C_{30}H_{62}O_2Si$	1608	763
Oleic Acid, (E)-, TBDMS	$C_{24}H_{48}O_2Si$	1334	948
Vaccenic acid, (E)-, TBDMS	$C_{24}H_{48}O_2Si$	1337	808
Stearic acid, TBDMS	$C_{24}H_{50}O_2Si$	1345	950
2-Hydroxyhippuric acid, 2TBDMS	$C_{21}H_{37}NO_4Si_2$	1374	842
3,5-Dihydroxybenzoic acid, 3TBDMS	$C_{25}H_{48}O_4Si_3$	1402	710
Citric acid, 4TBDMS	$C_{30}H_{64}O_7Si_4$	1408	813
L-Tyrosine, 3TBDMS	$C_{27}H_{53}NO_3Si_3$	1417	793
L-Tryptophan, 3TBDMS	$C_{29}H_{54}N_2O_2Si_3$	1509	829
Cholesta-3,5-diene	$C_{27}H_{44}$	1573	889
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.

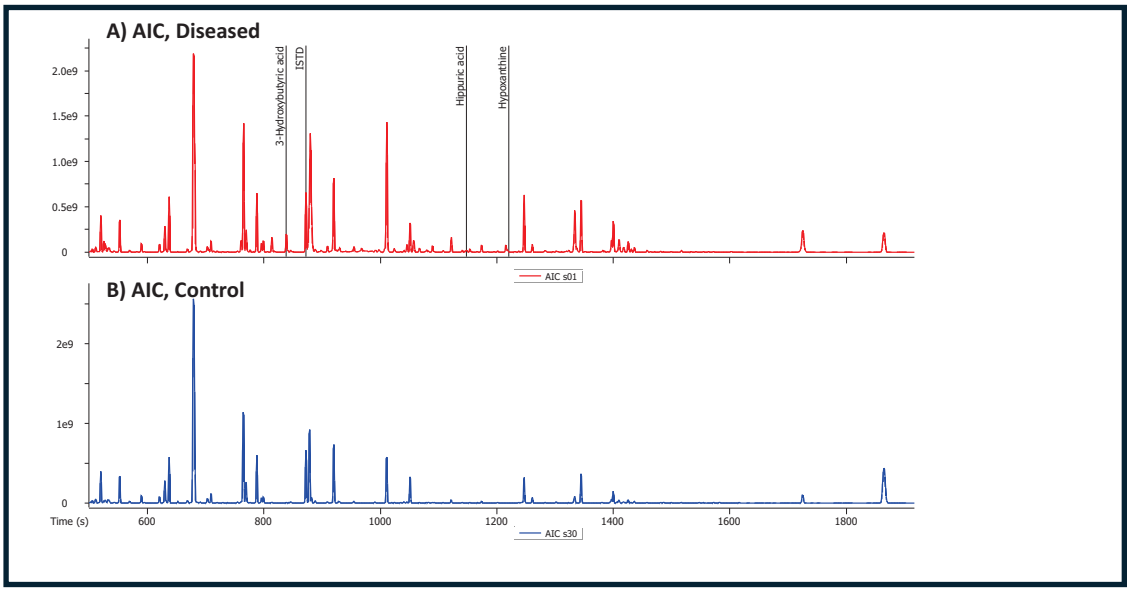


Figure 3. AICs for T2DM (A) and control (B) samples. Peak markers for 3-hydroxybutyric acid, hippuric acid, and hypoxanthine are displayed in the XIC for the diseased sample.

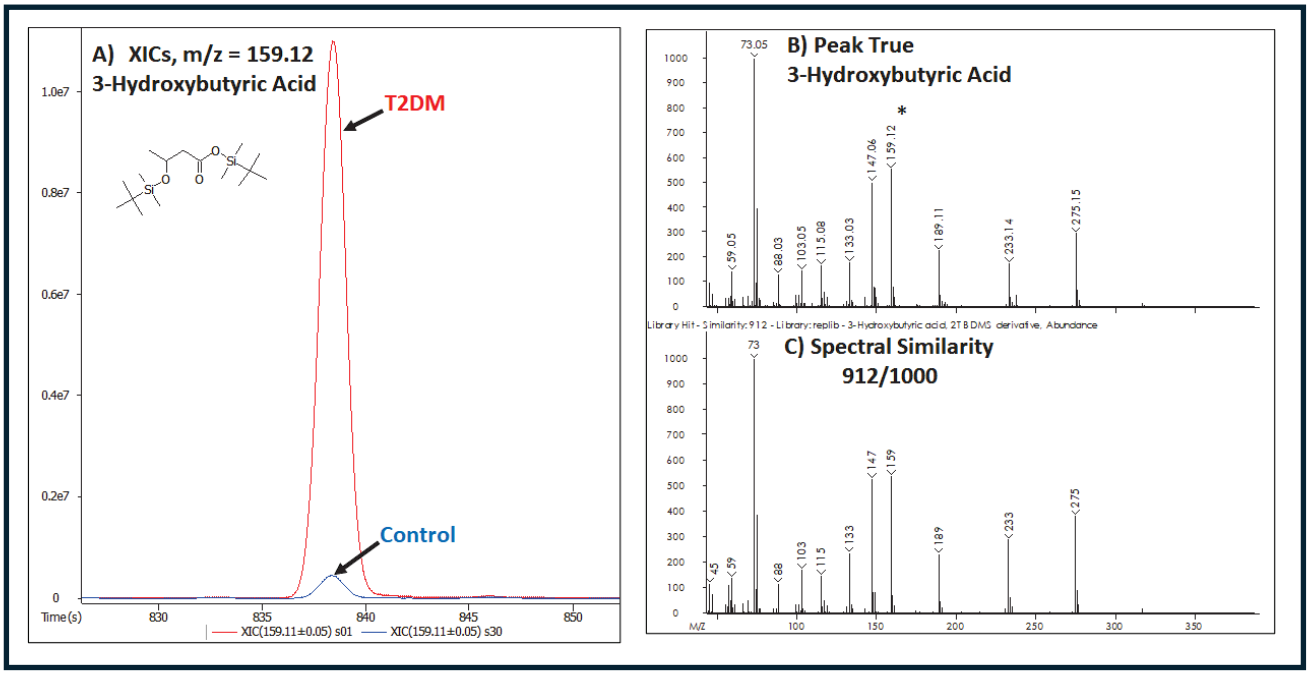


Figure 4. A) XIC (m/z = 159.12) for 3-hydroxybutyric acid in a control and T2DM sample, B) Peak True, and C) library Spectra for 3-hydroxybutyric acid.

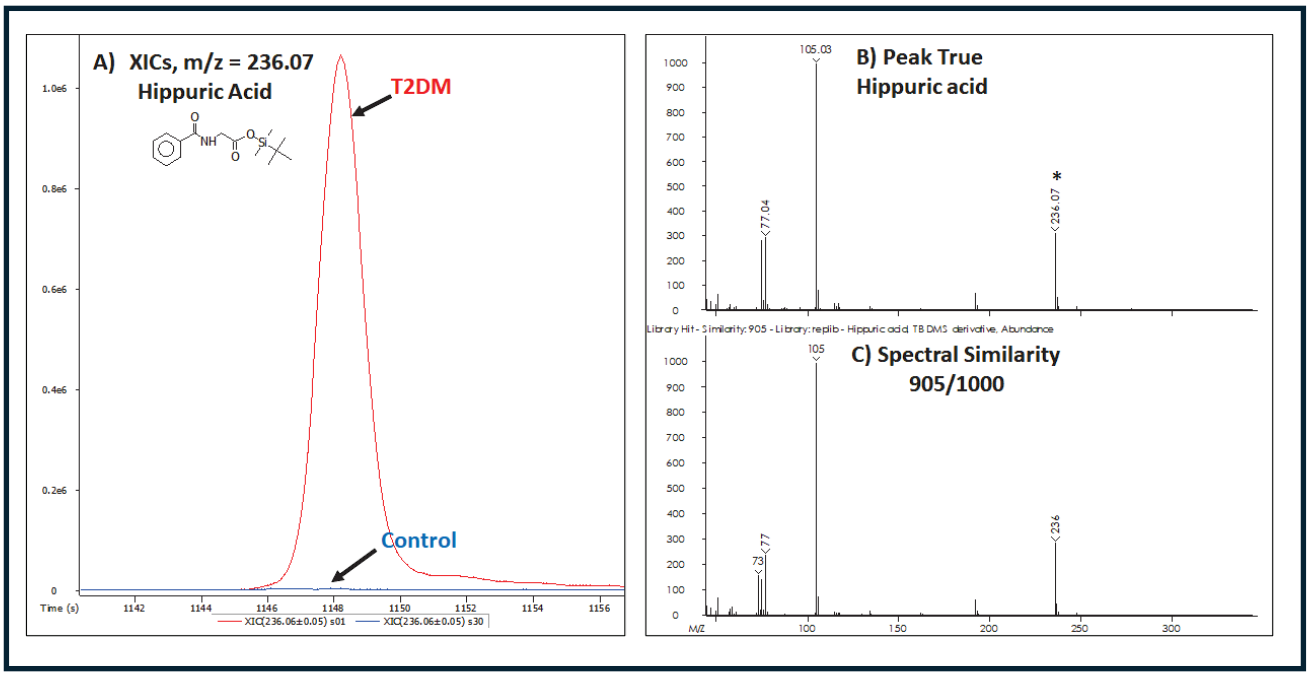


Figure 5. A) XIC (m/z = 236.06) for hippuric acid in a control and T2DM sample, B) Peak True, and C) library Spectra for hippuric acid.

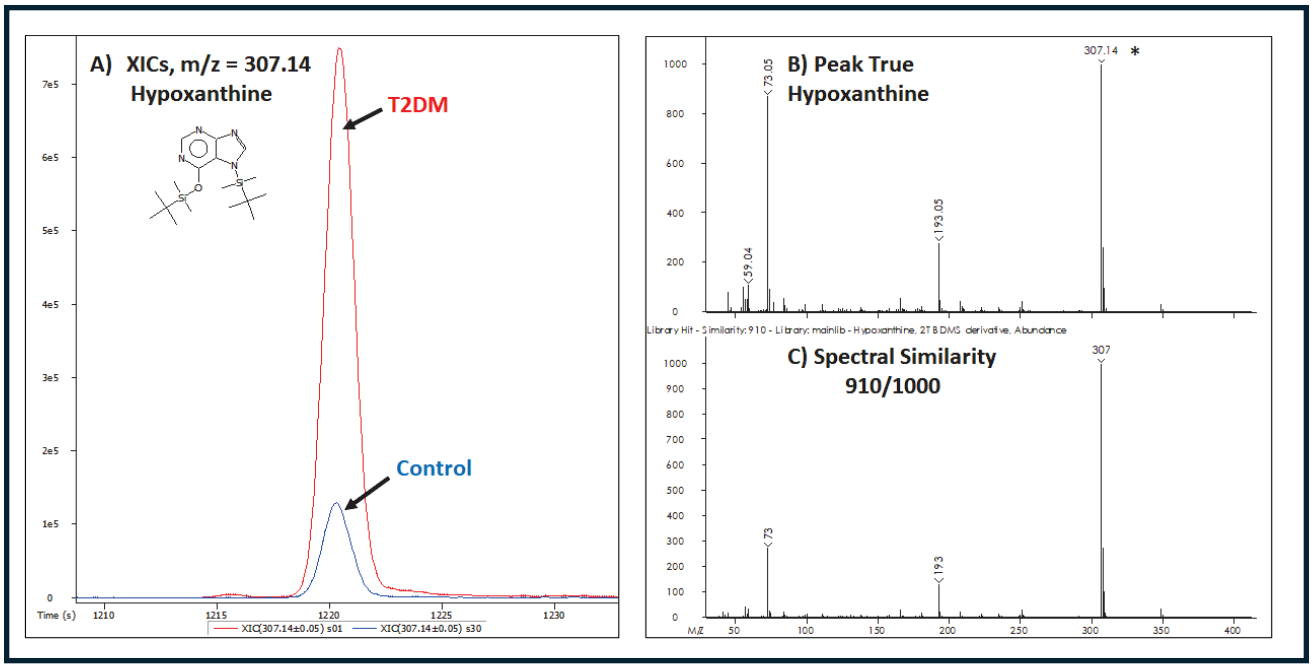


Figure 6. A) XIC (m/z = 307.14) for hypoxanthine in a control and T2DM sample, B) Peak True, and C) library Spectra for hypoxanthine.

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.

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