Comprehensive Two-Dimensional Gas Chromatography (GCxGC)—Time-of-Flight Mass Spectrometry of Metabolic Samples Obtained from the Leaves of a Hybrid Aspen (Populus Tremula) Plant

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Key Words: GCxGC-TOFMS, Metabolomics, Deconvolution

1. Introduction

Advances in mass spectrometry have lead to a dramatic increase in the analysis of cellular proteins and metabolites. Comprehensive analysis of the metabolome (metabolomics), together with transcriptomics and proteomics, is an important step in functional genetics and system biology. Even though initially slower to be embraced than the other two "omics" approaches, the interest in metabolomics has rapidly increased.

The most popular applications of metabolomics in the area of plant systems include fingerprinting of species; genotypes or ecotypes for taxonomic or biochemical purposes; studies of the relationship between applied exogenous stimuli and the behavior of specific classes of metabolites; and comparison of the metabolite content of mutant or transgenic plants with the wild-type counterparts.

No single analytical technique can be used for complete characterization of the metabolome, and no metabolome has been completely characterized yet. Even though limited by analyte volatility, the gas chromatography-mass spectrometry (GC-MS) analytical approach to metabolomics provides good sensitivity and selectivity.

The sample complexity (300 to 500 analytes can be typically found) and the need for high sample throughput make these samples a challenge for the analyst. The additional peak capacity provided by comprehensive two-dimensional gas chromatography (GCxGC) combined with the fast acquisition rates and spectral deconvolution capabilities of the LECO Time-of-Flight (TOF) MS can increase the number of metabolites that can be characterized in a single chromatographic run.

The purpose of the analysis was to analyze and compare samples from two different leaves of five hybrid aspen plants by GCxGC-TOFMS in under 20 minutes.

2. Experimental Conditions

Sample Preparation

Dried plant extracts were obtained from hybrid aspen plants grown in a growth chamber under 18-hour photoperiods (long day) for 15 weeks. Leaves 9 and 20 counting from the top of the plant were taken from five different plants and extracted. Samples were labeled with the leaf number followed by plant number (e.g., L9 1).

The dried samples were derivatized as follows. 30 µL of methoxyamine hydrochloride (15 mg/mL) in pyridine were added to each sample. After 30 minutes of derivatization at 60°C and 16 additional hours at room temperature, the samples were trimethylsilylated for 1 hour at room temperature by addition of 30 µL MSTFA with 1% TMCS. GCxGC-TOFMS analysis was performed within 12 hours from derivatization.

Instrument

GCxGC:
Agilent 6890 GC equipped with a LECO Thermal Modulator (technology under license from Zoex Corporation)
Primary Column:
Rtx-5, 10 m, 0.18 mm id, 0.18 µm film thickness
Main Oven Program:
70°C (2 minute hold) to 320°C (5 minute hold) at 20°C/minute
Secondary Column:
DB-17, 1 m, 0.1 mm id, 0.1 µm film thickness
Secondary Oven Program:
5°C offset from the main oven
Inlet Temp: 270°C
Injection Size: 1 µl
Split Ratio: 10:1
Carrier Gas: He at a constant flow of 1 ml/minute
Modulator Temp: 30°C offset from main oven
Modulation Frequency:
3 seconds with a 0.5 second hot pulse time

MS: LECO Pegasus TOFMS
Ionization: EI at 70eV
Mass Range (u): 60 to 800
Acquisition Rate: 150 spectra/second
Source Temper: 200°C

3. Results

Each of the 20 samples was spiked prior to derivatization with a mixture of 9 internal standards. The internal standard mixture was also derivatized and analyzed separately using the conditions described above. A chromatogram of the internal standards mixture is presented in Figure 1 and the peak labels are explained in Table 1.

Table 1. Internal standards retention times and peak numbers for Figure 1.

Peak #	Analyte name	RT (sec)	
		1st dimension	2nd dimension
1	D4-Succinic Acid	353	1.12
2	D6-Salicylic acid	437	1.20
3	13C4-2-Oxoglutaric acid	461	1.21
4	D4-Putrescine	518	0.88
5	13C3-Myristic acid	548	1.04
6	13C6-Glucose	575	0.92
7	13C4-Hexadecanoic acid	608	1.04
8	13C12-Sucrose	769	1.02
9	D7-Cholesterol	865	1.79

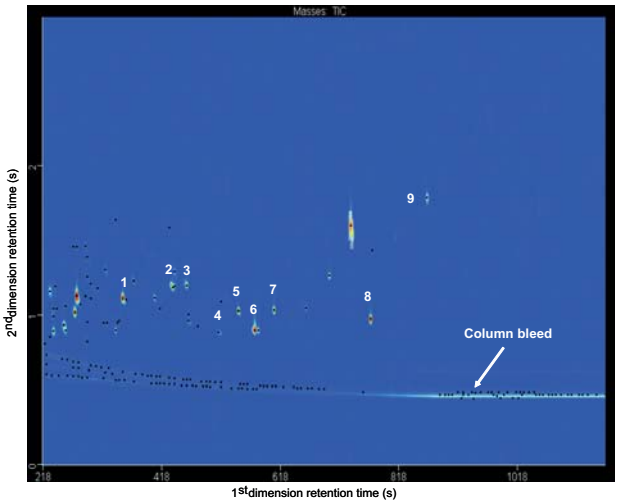

Figure 1. Total ion current (TIC) chromatogram displayed as a contour plot for the 9-component internal standards mixture. Black dots represent peak markers.

The ChromaTOF® software allows automatic comparison between multiple samples. One of the samples is selected as a reference and processed automatically under set conditions (S/N, libraries used for identification, masses used for area/height calculation, etc.). All the peaks found in this sample or only selected peaks of interest are used to build a reference table. Parameters such as S/N, area and match threshold, as well as retention time deviation and concentration tolerance are defined by the user in the reference table. The rest of the samples can then be compared against the reference sample. Quantitative comparison of the samples is done based on a one-point calibration curve.

Sample L9 1 was selected as a reference sample and processed at a S/N ratio of 100. ChromaTOF software automatically found and identified 690 peaks. Out of the total number of peaks about 200 were estimated to come from the modulation of either the column bleed or the solvent tailing. This left the number of "real" analytes found to be present in the leaf extract to more than 400. A one-dimensional analysis (Pegasus III GC-TOFMS) performed under similar conditions resulted in only 200 peaks being found after the data was processed at a S/N of 10 (reference 1).

After the initial processing, three different regions of the chromatogram were selected to demonstrate the compare algorithm and exemplify the advantages of GCxGC-TOFMS. A reference table was built containing 40 peaks present in these regions. Figures 2 to 5 show the TIC chromatogram for the entire analysis as well as the 3 regions of interest in a detailed view. Labels for the 40 peaks as well as similarities with the NIST libraries are presented in Table 2. The assigned names were taken as the first hits from the library (for some analytes, individual standards were available for confirmation). Since many of the silylated compounds present in the extracts are likely not present in the NIST library, these identifications should be regarded as tentative.

Table 2. Peak identification for Figures 3-5.

Peak #	Analyte name	NIST Similarity	RT (sec) 1st dim	RT (sec) 2nd dim
1	à-DL-Arabinopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)	778	524	1.09
2	tert-Butyl(dimethyl)silyl 2-[(tert-butyl(dimethyl)silyl]oxy)-4-methylpentanoate	678	530	1.11
3	Shikimic acid, tetrakis(trimethylsilyl)[1]	828	542	0.98
4	Citric acid, tetrakis(trimethylsilyl)[1]	946	548	1.02
5	Unknown 1[2]	571	548	1.04
6	Ethanesulfonic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	675	563	0.92
7	Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)[1]	728	575	0.92
8	D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)[1]	741	581	0.91
9	Trimethylsilyl ether of glycerol	738	584	1.36
10	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)[1]	793	599	0.89
11	D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester[1]	827	605	0.90
12	Hexadecanoic acid, trimethylsilyl ester	686	608	1.05
13	Galactaric acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, bis(trimethylsilyl) ester	816	611	0.93
14	Glucaric acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, bis(trimethylsilyl) ester	788	620	0.94
15	Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)[1]	980	632	0.92
16	1,2-Bis(trimethylsiloxy)ethane	638	643	1.08
17	Glucose oxime hexakis(trimethylsilyl)	732	643	0.90
18	Glycoside, à-methyl-trtrakis-O-(trimethylsilyl)	849	655	0.94
19	Oleic acid, trimethylsilyl ester	828	655	1.09
20	Linolenic acid, trimethylsilyl ester	904	655	1.16
21	Octadecanoic acid, trimethylsilyl ester	854	661	1.06
22	Trimethylsilyl 2-deoxy-3,4,5-tris-O-(trimethylsilyl)pentonate	742	682	0.96
23	4-Chlorophenyl benzoate	624	685	1.25
24	D-Glucose-2,3,4,5-tetrakis-O-(trimethylsilyl)-o-methyloxime-6-[bis(trimethylsilyl) pho	783	694	1.02
25	Hexanedioic acid, bis(2-ethylhexyl) ester	936	700	1.27
26	à-D-Galactopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	776	718	1.08
27	Uridine, 2',3',5'-tris-O-(trimethylsilyl)	817	718	1.33
28	à-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)	774	721	0.96
29	Unknown 2[2]	595	727	1.20
30	Mannose, 6-deoxy-2,3,4,5-tetrakis-O-(trimethylsilyl)	772	730	0.98
31	Propanetriol, 2-methyl-, tris-O-(trimethylsilyl)	738	838	1.49
32	Unknown 3[2]	599	844	1.47
33	Trimethylsilyl 2-methyl-2,3-bis[(trimethylsilyl)oxy]propanoate	652	844	1.54
34	Butanoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	618	850	1.58
35	Arachidonic acid, trimethylsilyl ester	695	865	1.79
36	d-Gluco-hexadialdose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, bis(O-methyloxime)	620	877	1.75
37	Thymol-à-d-glucopyranoside, tetrakis(O-trimethylsilyl)	632	886	2.29
38	à-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-O-(trimethylsilyl)-, cyclic m	604	889	2.38
39	Unknown 4[2]	599	889	2.46
40	Scopolin, tetra(trimethylsilyl)	622	892	2.53

[1] *Identification confirmed by single standard injection.*
[2] *Similarities with NIST Library lower than 600.*

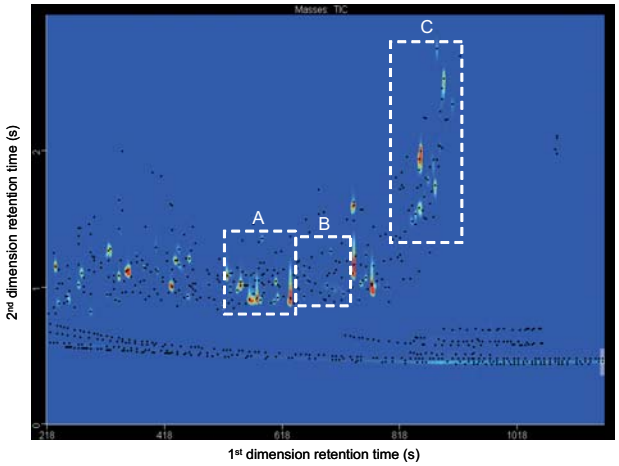

Figure 2. TIC chromatogram of sample L9 1 (reference sample).

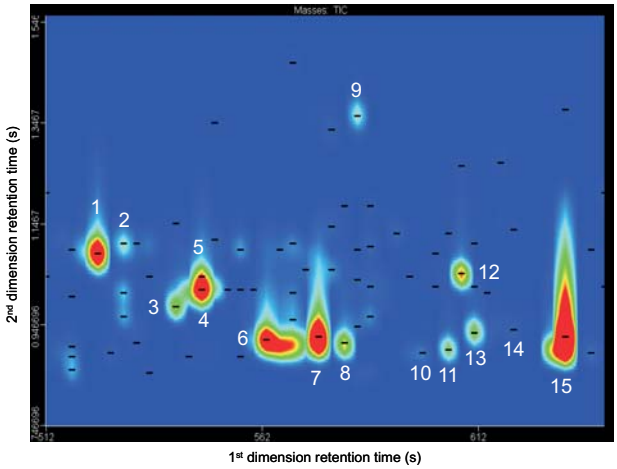

Figure 3. TIC chromatogram in region A of Figure 2.

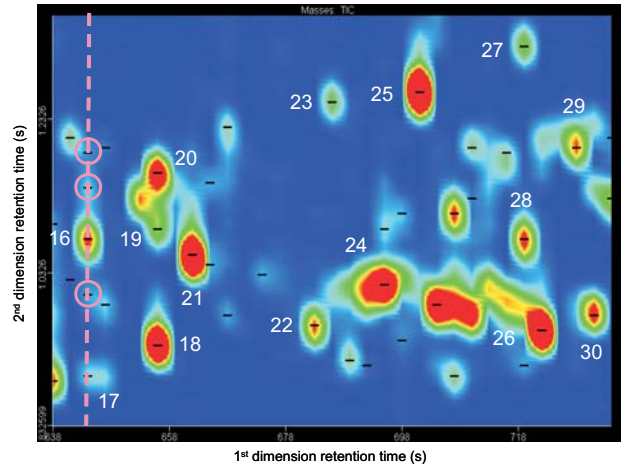

Figure 4. TIC chromatogram in region B of Figure 2.

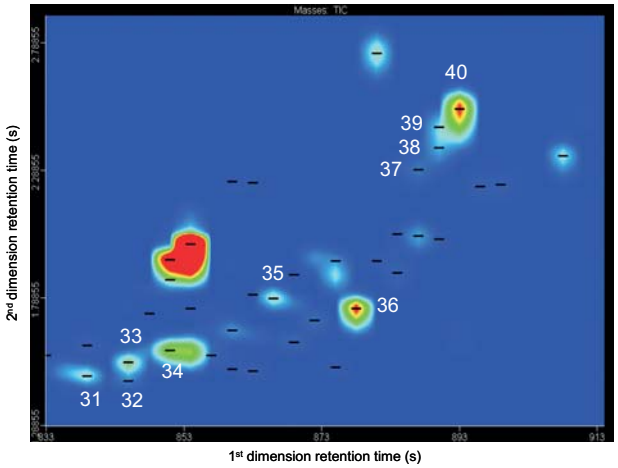

Figure 5. TIC chromatogram in region C of Figure 2.

After the reference table was built using sample L9 1, the rest of the samples (L9 2-5) were compared against it. Since the exact concentration of the analytes was not known, the concentration of all analytes in sample L9 1 was arbitrarily set to 100%. With the exception of components 39 and 40 all other compounds were found in all the samples at various concentration levels. Results obtained with the compare algorithm are summarized in Table 3. While more than 30% of the analytes showed an increase in concentration from plant 1 to plant 5, 30% showed the opposite trend and 20% showed an almost constant concentration across the 5 different samples. For 7 of the analytes, a general trend in variation of the concentration could not be established. Larger differences could be seen when extracts from leaves 9 and 20 of the same plant (samples L9 1 and L20 1) were compared to each other.

The gain in separation obtained by the use of GCxGC-TOFMS can be clearly seen in Figures 2-5. For example, if retention time in the second dimension is projected on the x-axis for peaks 16, 17, and the three peaks circled in pink (Figure 4), it is easy to see that if only the Rtx-5 column (first column) was used, these five peaks would be completely co-eluting. The addition of the secondary DB-17 column allows the five analytes to be separated as can be seen in Figure 6.

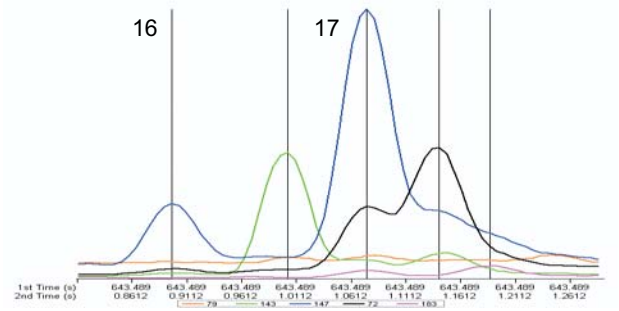

Figure 6. Unique masses for peaks eluting during a single modulation period (see the pink line in Figure 4) showing increased separation by the use of GCxGC-TOFMS.

Table 3. Sample comparison results (analyte names can be found in Table 2).

Peak #	IS used	Concentration				
		L9 1	L9 2	L9 3	L9 4	L9 5
1	D6-Salicylic acid	100	78	128	98	103
2	D6-Salicylic acid	100	32	134	66	27
3	D6-Salicylic acid	100	116	120	171	139
4	D6-Salicylic acid	100	112	146	101	92
5	13C6-Glucose	100	89	123	100	105
6	13C6-Glucose	100	181	125	222	142
7	13C6-Glucose	100	37	92	121	149
8	13C6-Glucose	100	69	97	112	124
9	13C6-Glucose	100	215	87	138	118
10	13C6-Glucose	100	73	73	59	117
11	13C4-Hexadecanoic acid	100	64	73	66	67
12	13C4-Hexadecanoic acid	100	97	108	123	133
13	13C4-Hexadecanoic acid	100	201	112	77	99
14	13C4-Hexadecanoic acid	100	117	134	123	157
15	13C4-Hexadecanoic acid	100	128	106	149	139
16	13C4-Hexadecanoic acid	100	158	124	246	131
17	13C4-Hexadecanoic acid	100	171	119	116	172
18	13C4-Hexadecanoic acid	100	28	35	61	96
19	13C4-Hexadecanoic acid	100	128	188	155	191
20	13C4-Hexadecanoic acid	100	121	129	99	150
21	13C4-Hexadecanoic acid	100	81	96	90	104
22	13C4-Hexadecanoic acid	100	132	114	129	94
23	13C4-Hexadecanoic acid	100	219	139	187	150
24	13C4-Hexadecanoic acid	100	101	102	87	73
25	13C4-Hexadecanoic acid	100	47	95	98	105
26	13C4-Hexadecanoic acid	100	63	33	28	16
27	13C4-Hexadecanoic acid	100	19	98	43	57
28	13C4-Hexadecanoic acid	100	107	229	253	214
29	13C12-Sucrose	100	78	66	48	50
30	13C12-Sucrose	100	68	87	68	69
31	13C12-Sucrose	100	98	64	81	73
32	D7-Cholesterol	100	78	91	156	146
33	D7-Cholesterol	100	145	100	79	90
34	D7-Cholesterol	100	91	86	136	112
35	D7-Cholesterol	100	71	107	102	82
36	D7-Cholesterol	100	85	85	127	99
37	D7-Cholesterol	100	0	81	132	149
38	D7-Cholesterol	100	0	0	0	0
39	D7-Cholesterol	100	0	636	1060	1228
40	D7-Cholesterol	100	55	116	188	221

Similar results can be seen for components 18-20 in the same region of the chromatogram (Figure 7). For this case, even though some of the masses are shared between peaks 19 and 20, the automated deconvolution algorithm is capable of extracting correct spectra. Similarities with the NIST library are more than 800 (1000 being the perfect match) for both components.

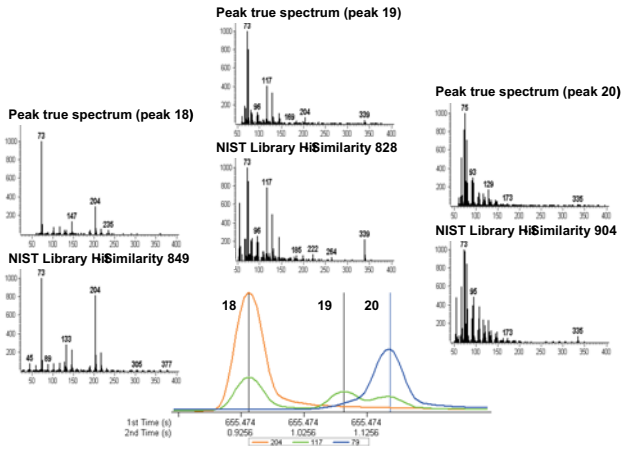

Figure 7. Increased separation by the use of GCxGC-TOFMS and spectral deconvolution results.

Figure 8 illustrates how spectral deconvolution can provide the additional analytical separation needed for finding and identification of peaks in a case where the increased peak capacity proved to be insufficient for analyte separation. Peaks 4 and 5 are completely co-eluted and visual examination of the TIC shows only one peak present in this region. Despite the severe co-elution the deconvolution algorithm correctly extracted the spectral information for the trimethylsilyl derivative of citric acid. Spectral match with the reference standard (injected individually) was 965 while similarity with the NIST library was 803.

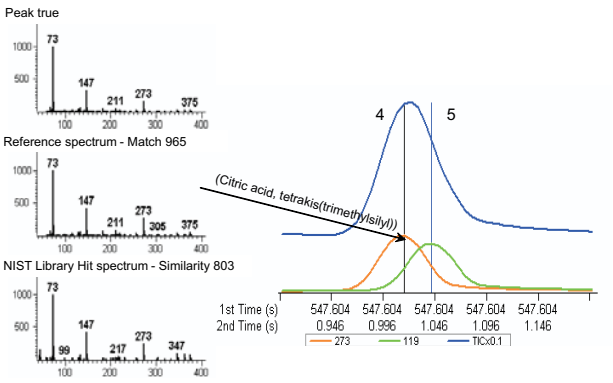

Figure 8. Spectral deconvolution results for the tetramethylsilyl derivative of citric acid (peak 4).

4. Conclusions

Ten dried extracts obtained from two different leaves of five hybrid aspen plants were derivatized and analyzed by GCxGC-TOFMS. Samples from the same leaf (leaf 9) and from the same plant (leaves 9 and 20) were compared and contrasted using the automated compare function of the ChromaTOF software. The increased separation obtained by using the GCxGC technique and the additional analytical separation gained by spectral deconvolution resulted in more than 400 peaks being found and identified.

5. References

Jonsson, P.; Gullberg, J.; Nordstrom, A.; Kusano, M.; Kowalczyk, M.; Sjostrom, M.; Moritz, T.; Anal. Chem.; 2004; ASAP Article

6. Acknowledgement

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