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The Metabolomic Analysis of Simvastatin-Dosed Rat Plasma by GC/Tof-MS

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Abstract

Gas chromatography coupled with time-of-flight mass spectrometry (GC/Tof-MS) has been demonstrated to be a very sensitive and reproducible technique. Accurate mass measurement matches with good dynamic range and a fast acquisition rate to make the Waters GCT Premier System a very useful tool for metabolomic studies where the elucidation of unknowns that are not found in commercially-available spectral databases is difficult.

Here, MarkerLynx XS Application Manager is employed for the analysis of simvastatin-dosed rat plasma samples using the GCT Premier System for capillary GC-MS and using both EI and CI ionization modes.

Introduction

Simvastatin is widely used as a cholesterol-lowering drug. It reduces cholesterol by inhibiting an enzyme (3-hydroxy-3-methyl-glutaryl- CoA or HMG-CoA reductase) in the liver that is necessary for the production of cholesterol. In the blood, it lowers total and LDL (“bad”) cholesterol as well as triglycerides. It also increases HDL (“good”) cholesterol. Lowering LDL and increasing HDL slows and may even reverse coronary artery disease.

In recent human clinical studies, simvastatin has also been found to be beneficial in the treatment of diseases such as Parkinson’s[1] and lung cancer.[2] The development of a metabolomic analysis method for in vivo simvastatin studies is therefore of great importance to better understand the effect of the drug on clinical markers of interest, such as cholesterol and phospholipids, in the process of interacting with the cholesterol-producing, rate-controlling enzyme HMG-CoA reductase.

Gas chromatography coupled with time-of-flight mass spectrometry (GC/Tof-MS) has been demonstrated to be a very sensitive and reproducible technique. Accurate mass measurement matches with good dynamic range and a fast acquisition rate to make the Waters GCT Premier System a very useful tool for metabolomic studies where the elucidation of unknowns that are not found in commercially-available spectral databases is difficult.

Small molecule metabolite profiling using MS is a challenging process due to the density and complexity of the data produced. The Waters MarkerLynx XS Application Manager for MassLynx Software uses multivariate statistical analysis[3] to model, analyze, and interpret complex data by comparing and discriminating them to help identify patterns quickly and effectively.

Here we report a study in which the MarkerLynx XS Application Manager is employed for the analysis of simvastatin-dosed rat plasma samples using the GCT Premier System for capillary GC-MS and using both EI and CI ionization modes.

Experimental

GC Conditions

Column:	Rxi-5ms (Restek) 30 m x 0.25 mm I.D. x 0.25 µm film
Flow rate:	1.0 mL/min Helium
Injection vol.:	1 µL, split 2:1
Injector temp.:	275 °C
Temperature program:	80 °C for 1 min, ramp to 320 °C at 12 °C/min and hold for 8 min
Solvent delay:	5 min

GC-MS Conditions

Acquisition range:	*m/z* = 40 to 800 for EI *m/z* = 90 to 900 for CI
Scan time:	0.09 sec
Interscan delay:	0.01 sec
Ionization:	EI+, CI+ using methane
Lock mass:	tris-trifluoromethyltriazine (metri)
Source temp.:	225 °C for EI and CI
Transfer line temp.:	275 °C

Sample Preparation

The plasma samples were thawed at room temperature on ice. 50 µL of methanol was added to each sample vial before speed vacuum drying to eliminate any moisture in the samples. Then, 1 mL of pyridine was added to each sample vial. After 1 min of vortexing, the sample vials were centrifuged at 2,000 RPM and the contents were allowed to settle. 500 µL off the top clean solution from each sample vial was transferred to a new sample vial and 200 µL of MSTFA + 1% TMCS was added to each new sample vial. The vials were then vortexed for 10 seconds before being placed into an air bath container for derivatization at 60 °C for 1 hour. 200 µL of each of the derivatized samples was transferred to an autosampler vial (with 400 µL low volume inserts) for GC/Tof-MS analysis.

Results and Discussion

All the samples were run in triplicate for reproducibility and stability assessment of the GC/Tof-MS system throughout the experiment.

Figure 2 shows the typical MS Total Ion Current (TIC) chromatograms from one of the simvastatin-dosed samples (triplicate injections). Very good reproducibility was obtained and maintained throughout the whole study. Figure 3 is a pair of MS TIC comparison chromatograms between a vehicle sample and a simvastatin-dosed sample. Initial inspection of these chromatograms shows the ability to differentiate samples based on their major components. Further processing using MarkerLynx XS provides more rigorous mining of the data, which not only includes these major features but also helps discover minor features that may be of greater biological significance despite their low abundance.

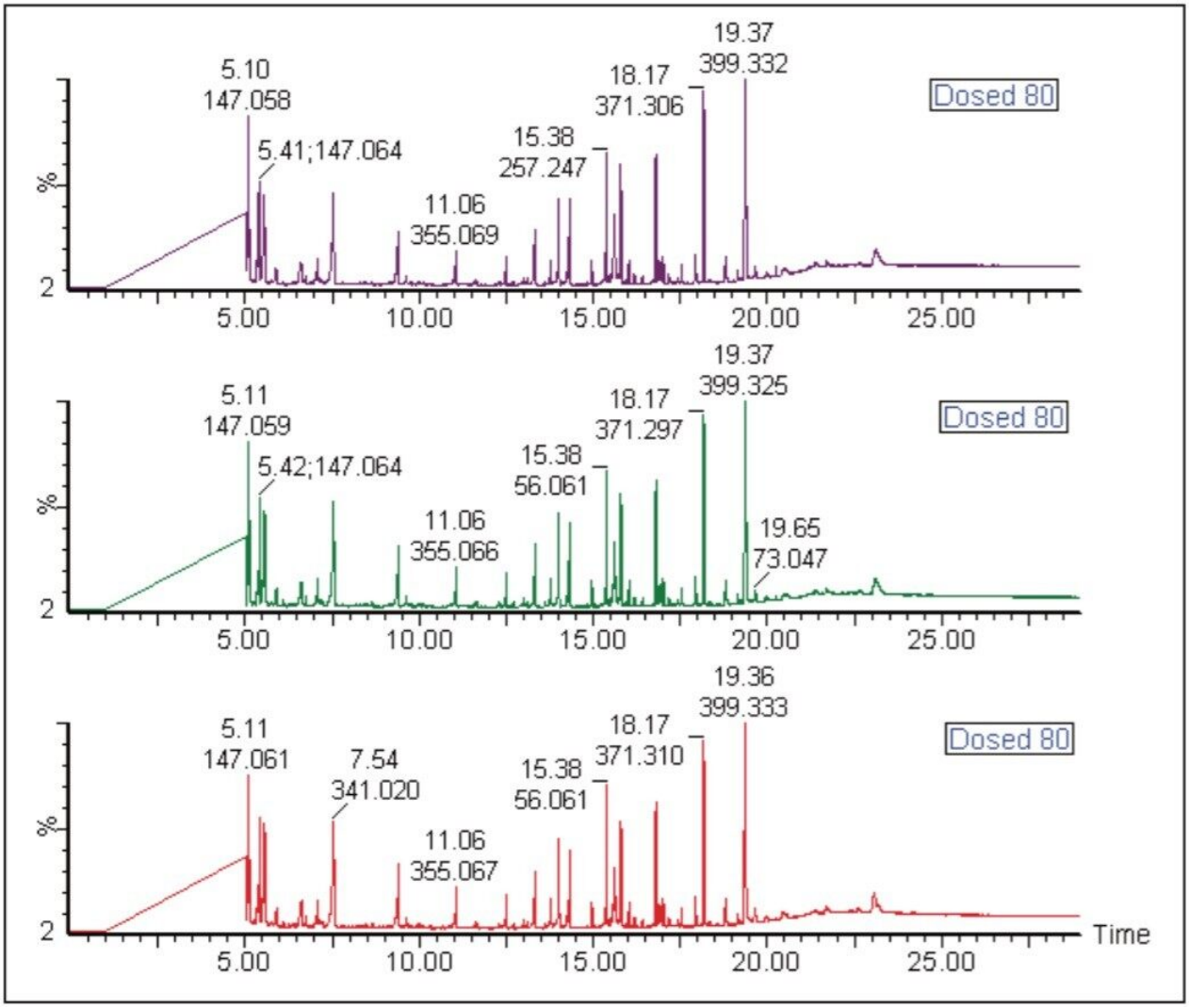


Figure 2. Representative TIC chromatograms from one of the dosed samples (triplicate injections).

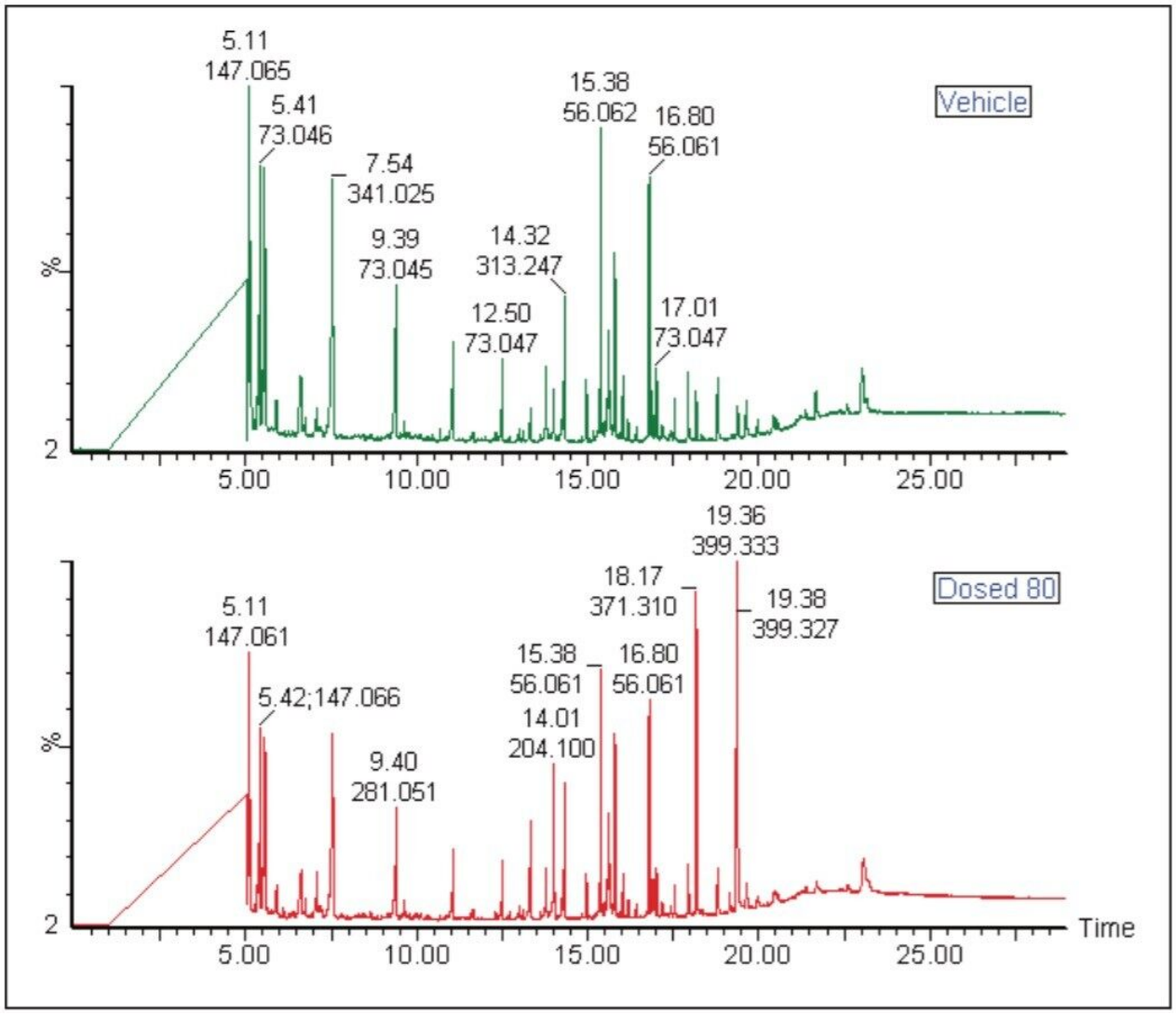


Figure 3. TIC chromatogram comparison between a vehicle sample and a simvastatin-dosed sample.

MarkerLynx XS integrates and aligns chemical and biological MS data points and converts them into Exact Mass Retention Time (EMRT) pairs. Those EMRT pairs can then be used for multivariate statistical analysis, such as principle component analysis (PCA-X), partial least-squares to latent structures data analysis (PLS-DA), and orthogonal PLS data analysis (OPLS-DA) to visualize and interpret the information rich and complex MS data.[4]

There are five vehicle (control) samples, 25 lower-dosed (80 mg/kg) samples and 15 higher-dosed samples (88 mg/kg) included in this study. In total, 4191 EMRT pairs were found by MarkerLynx XS. Figure 4 shows an OPLS-DA model’ s 3D score plot for vehicle samples (Group 1 in light green) and the lower dosed samples (Group 2 in blue). Significant differences between those two groups are clearly illustrated in the plot.

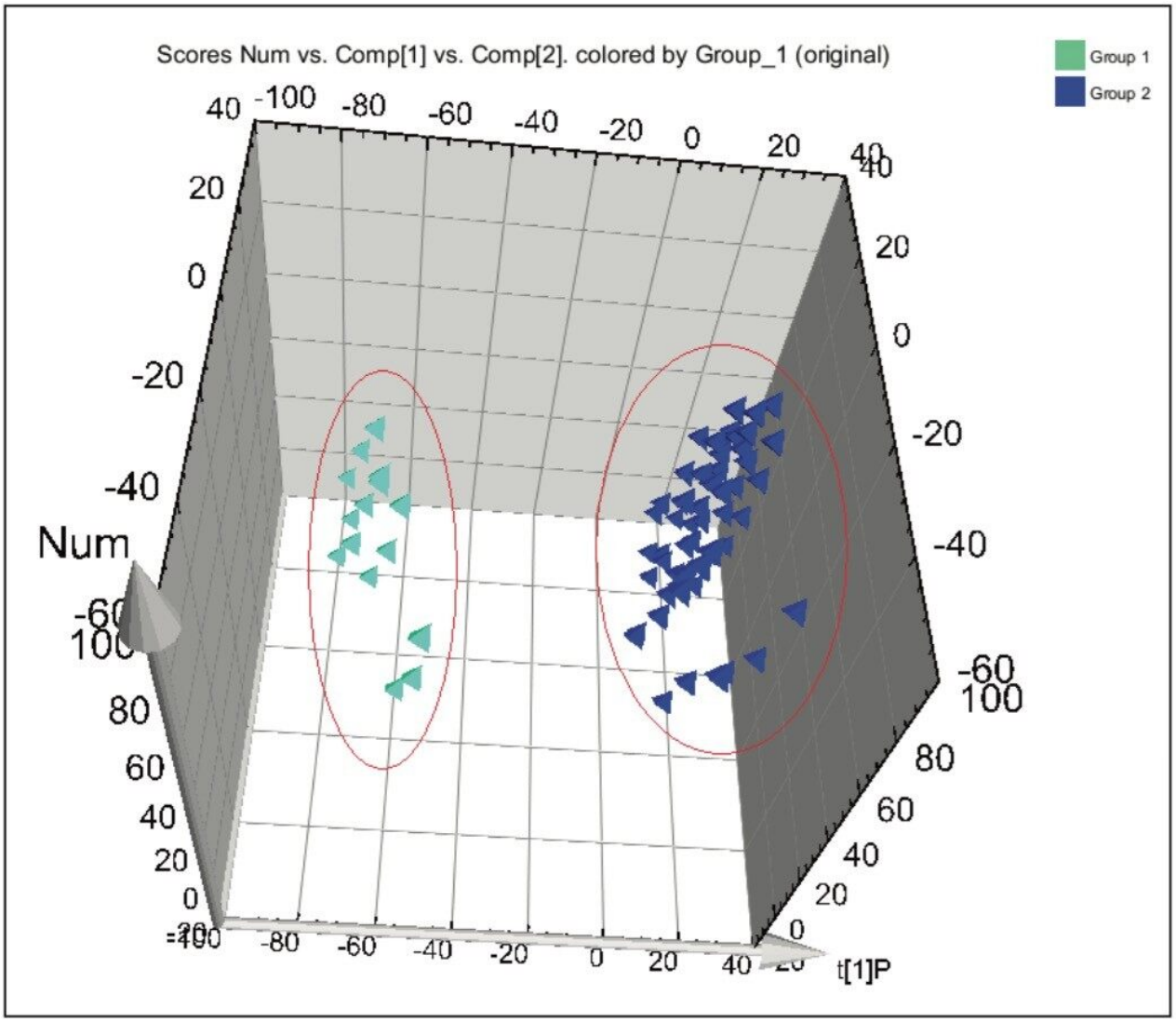


Figure 4. OPLS-DA model 3D score plot showing group difference between vehicle samples (light green, Group 1) and simvastatin-dosed samples (blue, Group 2)

From the S-plot of Figure 5, some of the most important EMRTs are tabulated and plotted in Figure 6. In the upper portion of the table, the measured intensities and factors of change are based on the average of the measured values for each EMRT in the group. The lower portion of Figure 6 shows plotted comparison columns of the most important EMRTs from those two groups of samples. Those plots simplify the process of extraction and pin-pointing significant markers from very complex datasets.

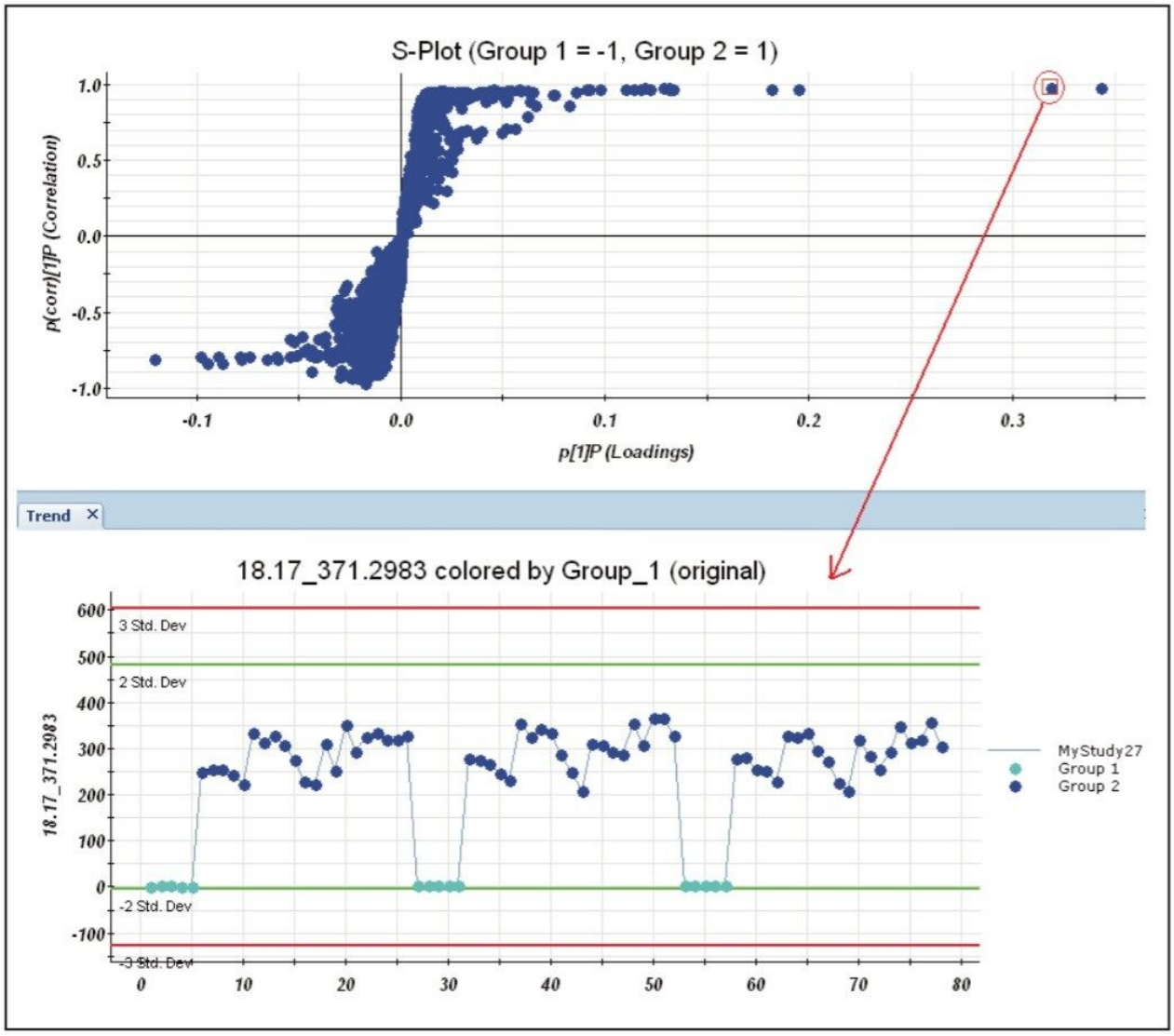


Figure 5. OPLS-DA model's S-plot. The points are Exact Mass /Retention time pairs (EMRTs) plotted by covariance (X-axis) and correlation (Y-axis) values.

	1	2	3	4	5	6	7	8
1	Primary I	Retention	Mass	p[1]P	p(corr)[1]P	Factor of	Group 1	Group 2
2	15.38_73.	15.38	73.032	-0.0951724	-0.831135	1.6	81.1408	49.7755
3	16.80_28	16.8	285.2785	-0.098315	-0.791749	1.5	105.717	70.3337
4	15.38_23	15.38	239.2364	-0.0898805	-0.786348	1.5	85.3496	55.3186
5	18.17_37	18.17	371.2983	0.318334	0.97757	81.0	3.63107	294.269
6	15.38_25	15.38	257.2481	-0.121028	-0.805681	1.6	145.531	92.3052
7	19.36_39	19.36	399.3285	0.342835	0.979876	73.7	4.69147	345.717
8	15.38_12	15.38	129.0908	-0.0880648	-0.83454	1.6	69.9341	42.9006

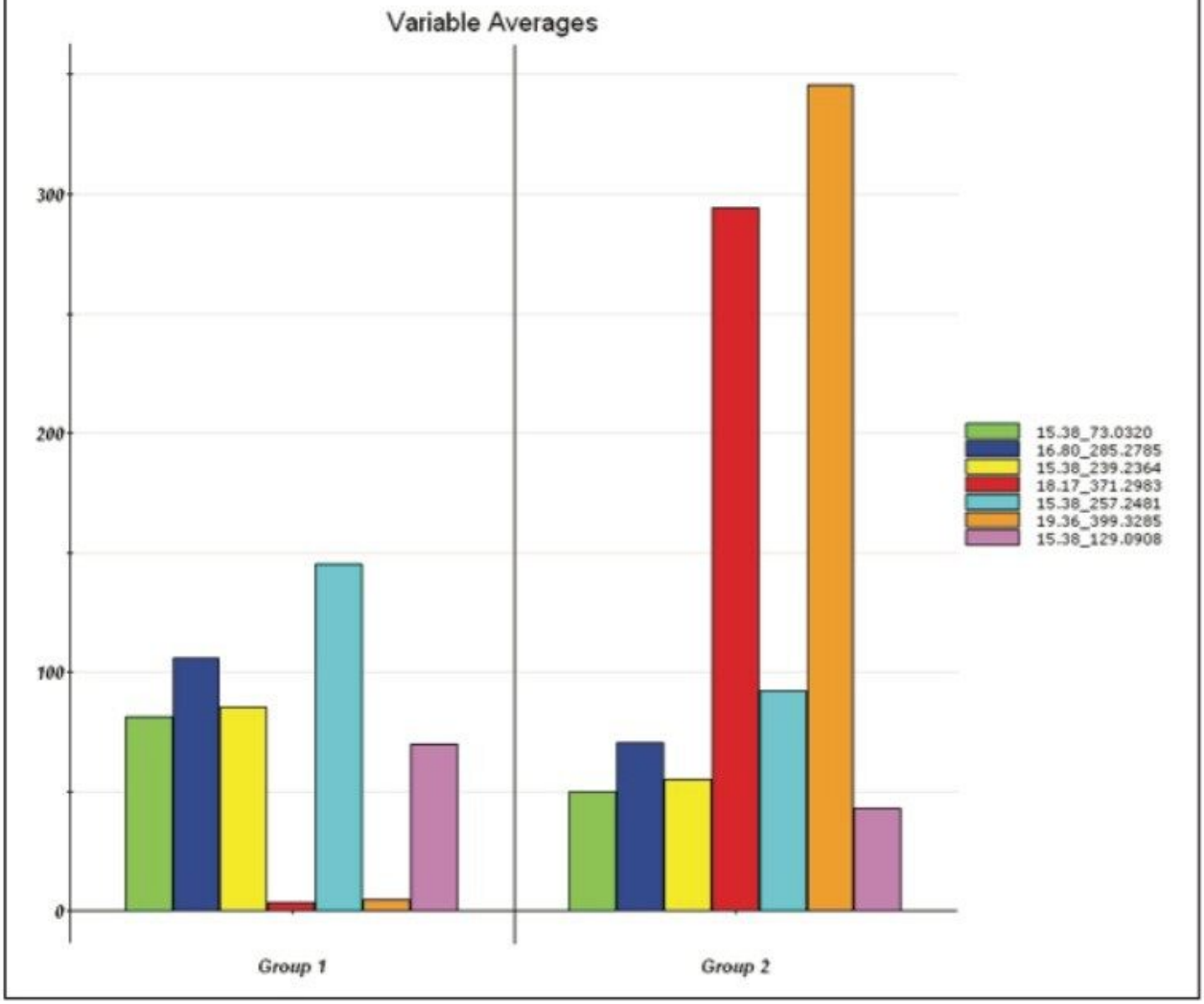


Figure 6. Tabulated listing of some of the most important EMRTs (upper). The measured intensities and factor of change are based on the average of the measured values for each ERMT in the group. Plotted columns of the most important EMRTs (lower).

The upper portion of Figure 5 is an OPLS-DA model’ s S-plot for the same data sets as in Figure 4. The points are EMRTs plotted by covariance (X-axis) and correlation (Y-axis) values. The upper right quadrant of the S-plot shows those EMRT pairs that are elevated in the dosed group, while the lower left quadrant shows EMRT pairs elevated in the vehicle group. The farther along the X-axis the greater the contribution to the variance between the groups, while, the farther along the y-axis the higher the reliability of the analytical result. The lower portion of Figure 5 illustrates a plot for the same EMRT pair point (*m/z* 371.2983 at RT 18.17 in this case) from both the vehicle and lower-dosed samples. The difference of this EMRT pair (point) is significant between the vehicle samples and the lower-dose samples.

In addition, the identification of those elevated points, putative biomarkers, can be easily verified by using the spectra searching against existing databases. Figure 7 shows the NIST 2008 EI library searching results from the spectrum extracted from the EMRT pair of *m/z* 371.2983 at 18.17 min. The top hit from the database is Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy] propyl ester. It is suggested that it came from phospholipids broken down in the derivatization process. The EI spectrum profile matching with the spectrum from the NIST database was excellent with a score of 890 and a probability score of 97%. The peak of *m/z* 371.2983 is found to be a major fragment from the precursor ion of *m/z* 474.3561, with which the formula of $C_{25}H5_4O_4Si_2$ can be confirmed from elemental composition analysis by exact mass (with a mass accuracy of 1.7 ppm in this case, Figure 8). The precursor ion of *m/z* 474.3561 is also supported by the CI data acquired separately but under the same GC conditions. A list of other compounds identified in the same fashion from the elevated EMRT points is shown in the table in Figure 9.

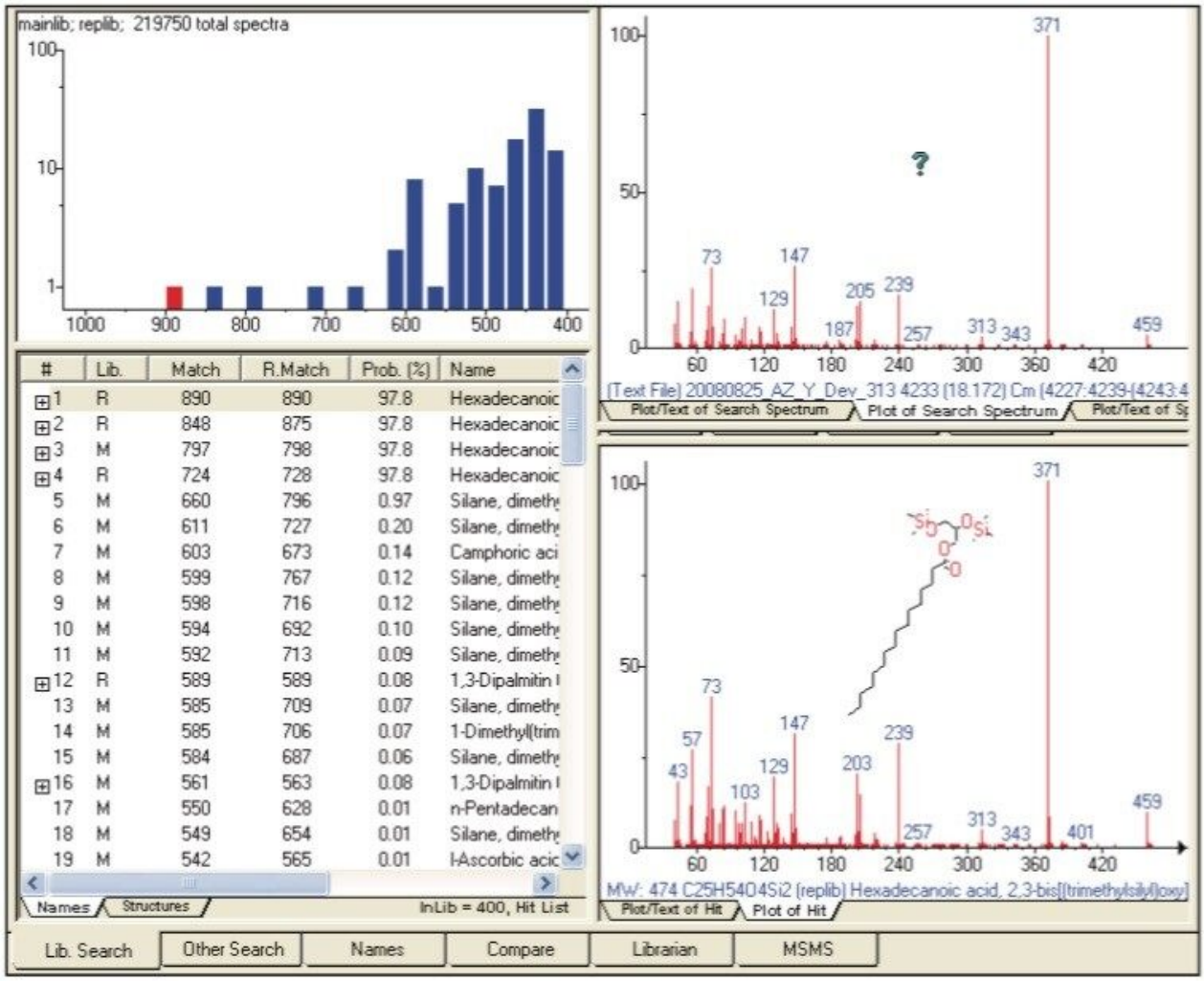


Figure 7. NIST 2008 EI library searching results from the MS spectrum.

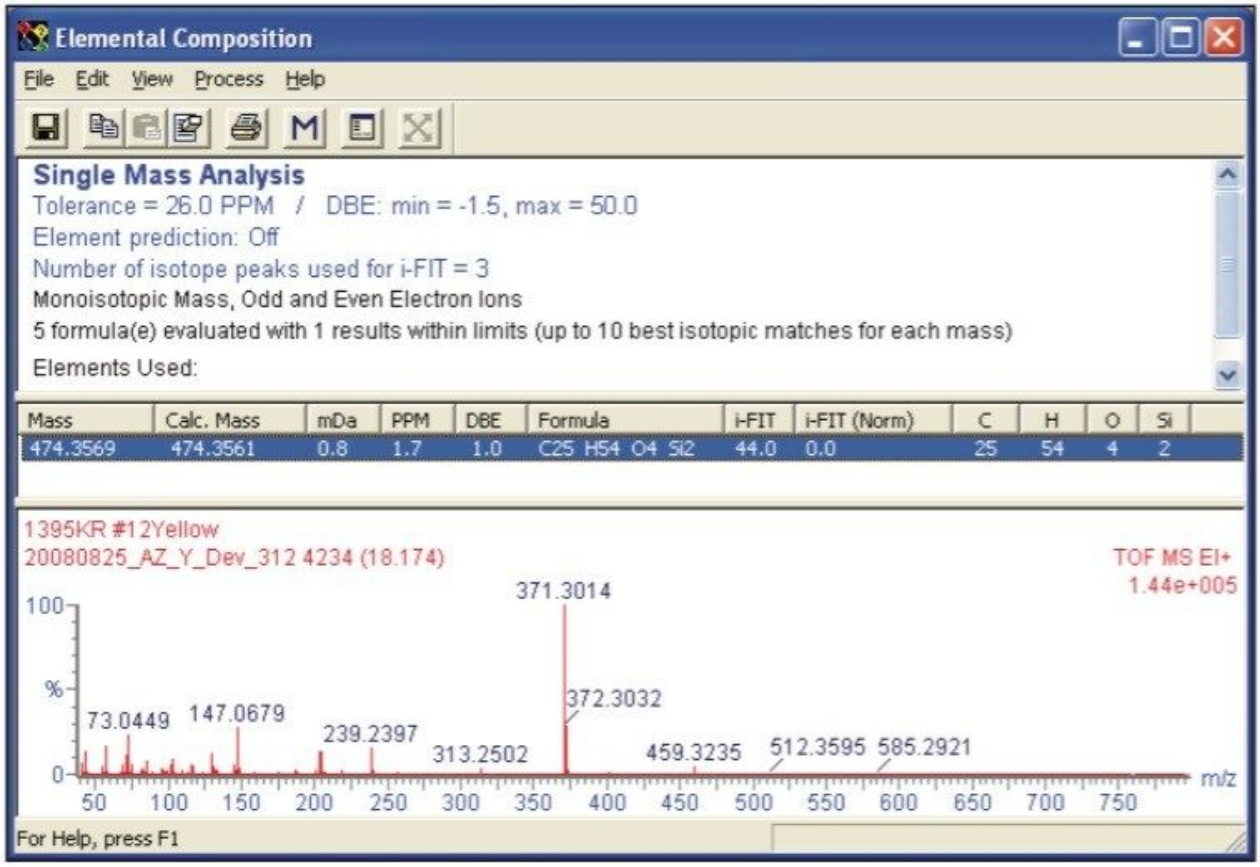


Figure 8. Elemental composition analysis for m/z 474.3561.

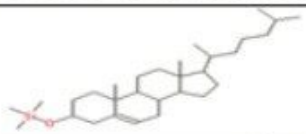
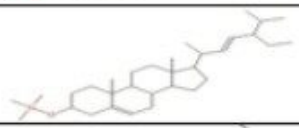
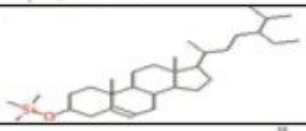
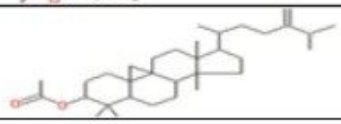
Primary ID	Name	Structure
15.38_257.2481	Hexadecanoic acid, butyl ester	
16.80_285.2785	Octadecanoic acid, butyl ester	
19.36_399.3285	Bis[trimethylsilyl]-monostearin	
14.31_313.2563	Hexadecanoic acid, trimethylsilyl ester	
15.59_352.2809	9,12-Octadecadenoic acid [z,z]-, trimethylsilyl ester	
15.63_339.2733	Oleic acid, trymethylsilyl ester	
16.87_338.3904	Tetracosane	
17.17_369.3177	Eicosanoic acid, trymethylsilyl ester	
17.54_352.4062	Pentacosane	
19.98_408.4292	Nonacosane	
20.26_368.3458	Cholesterol margarate	
20.48_427.3641	Eicosanoic acid, 2,3-bis{[trimethylsilyl]oxy} propyl ester	
20.56_422.4929	Triacontane	
21.69_458.3956	Cholesterol trymethylsilyl ether	
22.61_484.4105	Stigmasterol tri,ethylsilyl ether	
23.06_486.4254	B-Sitosterol trimethylsilyl ether	
24.13_422.3895	9,19-Cyclolanostan-3-ol, 24-methylene-, acetate, [3β]-	

Figure 9. A list of additional compounds that contribute to the variance between the groups.

Conclusion

The combination of the GCT Premier System with the MarkerLynx XS Application Manager has created a very powerful system for metabolomics studies, which is complementary to the LC-MS approach. The application of multivariate statistical tools, such as principle components analysis (PCA-X) and orthogonal PLS data analysis (OPLS-DA), enable visualizing information-rich and extremely complex biological data sets easily and in a variety of ways.

The rapid, sensitive, accurate mass acquisition of the GCT Premier, along with the ability to search spectra against an existing database, such as NIST 2008, provides researchers in the field of metabolomics with a reliable method for identifying putative markers discovered through PCA processing. For those analytes that have no reference spectrum in existing databases, accurate mass measured EI data is an invaluable asset in elucidation of unknowns.

References

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