Exploration of Ethanol-Induced Liver Disease Using High Performance GC-TOFMS and Robust Statistical Analysis

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Introduction

The differential analysis of metabolites in samples from diseased, treated, or control animals is generally referred to as metabolomics. It is of critical importance to various disciplines of science. Combining rugged analytical techniques with robust statistical tools offers a great opportunity in these studies. Here, the "polar" extracts from liver tissue of ethanol- treated mice are explored using GC-TOFMS. Data are collected from two phenotypes using GC-TOFMS with deconvolution for analyte identification. Robust algorithms are applied to align, background subtract, and quantitatively compare the sample sets to identify analytes which are present at modulated levels. The benefits of the algorithms, exploration of parameters within them, and the impact on the findings are explored. Hundreds of analytes are identified and compounds of varying classes are evaluated for modulation with disease state. This study presents a standardized approach for metabolomic GC-TOFMS analysis consisting of a recommended sample preparation procedure, instrument parameters to generate consistent and reliable results, and data interpretation methods to identify modulated analytes. This method was developed using amino acid and fatty acid standards, as well as NIST certified human plasma. Sample preparation included complete sample extract drying using lyophilization (Labconco Freezone1). Post-acquisition processing of the data using advanced statistical software for alignment and comparative statistics leverages the capabilities of the analysis.

Standardized Experimental Approach

SAMPLE PREPARATION AND DERIVATIZATION

Samples

- Animals were from diseased (ethanol treated with alcohol-induced liver disease) and control (non-diseased) animals
- Five control animals and five treated (ethanol) animals
- Weighed portions of livers were homogenized and extracted using methanol
- Extracts were obtained from the tissue of normal control and diseased animals
- Triplicate portions were used in the study
- Extracts were provided by Prof. X. Zhang, University of Louisville

Drying

- Dry samples at ambient temperature in SpeedVac (Savant) for approximately 3 hours
- Lyophilize samples overnight to remove final moisture (12 to 16 hours) (Labconco Freezone1)

Methoximation

- Methoximate overnight (12 hours) at 30°C with 10 µL N-methylhydroxyamine HCl (20 mg/mL) in anhydrous pyridine

Silylation

- Derivatize with 40 µL *MSTFA* +1% TMCS for 30 min at 37°C
- Transfer to an amber GC autosampler vial with glass insert and analyze.

Methods

GC-TOFMS METABOLOMICS METHOD

• Gas Chromatograph:	Agilent 7890 and a GERSTEL MPS2 Autosampler
• GC Column:	30 m x 0.25 mm id. x 0.25 µm film thickness Rxi-5SilMS (Restek Corp.)
• Carrier Gas:	Helium set @ 1.5 mL/min
• Injection Mode:	Split 20:1
• Injection Volume:	1 µL
• Inlet Temperature:	260°C
• Temperature Program:	Initial temperature 40°C for 0.5 min ramped at 5.0°C/min to 295°C held for 5 min
• Transfer Line Temp:	275°C
• Total Run Time:	56.50 min

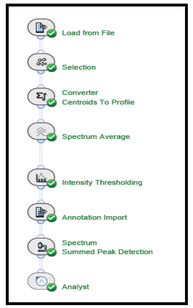
Mass Spectrometer: Pegasus® HT
TOFMS Analysis Parameters

• Acquisition Delay:	600 s
• Mass Range:	35 to 600 m/z
• Acquisition Rate:	20 spectra/s
• Ion Source Temperature:	230°C

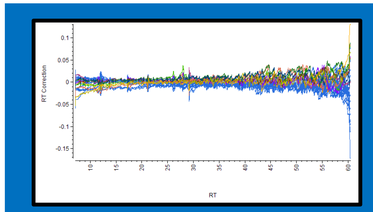
Statistical Analysis

Statistical analysis including alignment, peak finding, and population comparison was performed using a *beta* version of Expressionist MSX (Genedata). The work flow employed is shown below. Data were imported at netCDF files from the entire GCMS analysis.

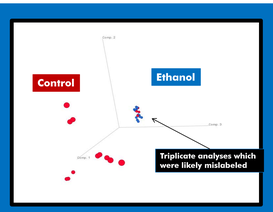
LECO Pegasus HT GC-TOFMS



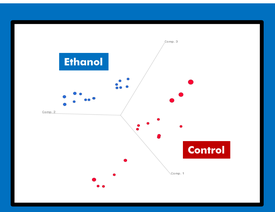
Statistical Outputs



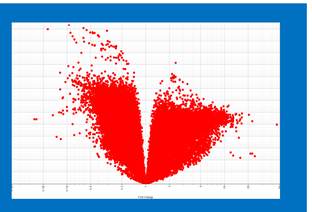
Retention Time Alignment



Statistical Analysis - PCA



Statistical Analysis - PLS



Statistical Analysis (Features) - Anova

Metabolite Comparison Examples (Control Vs. Diseased) Correlating With Ethanolic Liver Disease (>250 Metabolites Per Sample; >400 Condensed; More Than 100 Modulated)

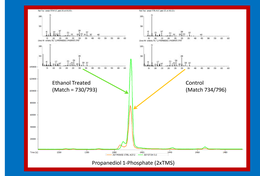


Figure 1. Comparative Chromatograms from Propanediol-1-Phosphate (2xTMS).

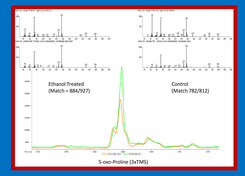


Figure 2. Comparative Analysis of 5-oxo-Proline (3xTMS).

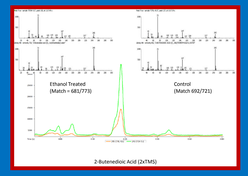


Figure 3. Comparative Analysis of 2-Butenedioic Acid (2xTMS).

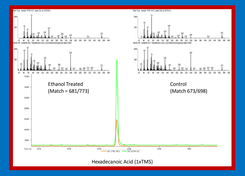


Figure 4. Comparative Analysis of Hexadecanoic Acid.

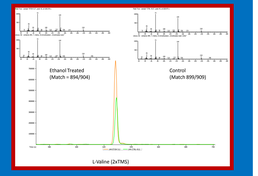


Figure 5. Comparative Analysis of Valine.

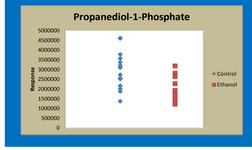
Propanediol-1-phosphate is slightly downward modulated in ethanol-treated animals. This compound is related to lactaldehyde and lactic acid metabolism. This is a potential indicator of oxidative balance.

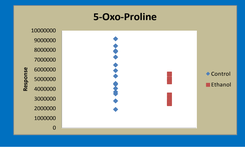
5-Oxo-Proline (pyroglutamic acid) is slightly downward modulated in ethanol-treated animals. This is statistically challenged by the high variability in the control data. This compound is a potential indicator of oxidative stress or related to protein processing. It is associated with some diseases and is related to glutathione synthesis.

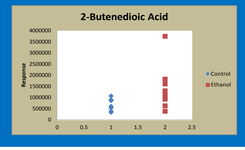
Butenedioic acid (fumaric/maleic acid) is slightly upwardly modulated in ethanol-treated animals to the extent of about 30-40% with one significant outlier. This compound (as fumaric acid) is an intermediate of the Citric Acid Cycle (TCA cycle), a potential indicator of oxidative stress or related to protein processing. In the absence of a standard we cannot distinguish fumaric and maleic acids.

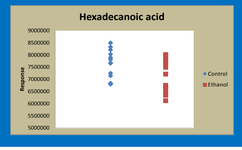
Hexadecanoic acid (palmitic acid) is downward modulated in treated animals one of the most common fatty acids. It is a key feedback inhibitor of fatty acid synthesis. This compound is correlated with increased risk of heart disease and is important in select modification of proteins for incorporation into cell membranes. There are inverse correlations between free levels and increased triacylglycerol synthesis.

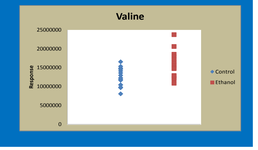
Valine is an essential amino acid and is upwardly modulated (in liver) nearly 50% (average) in ethanol-treated animals. This amino acid is able to contribute to both gluconeogenesis and fatty acid synthesis. Valine accumulation in the liver has been well-documented, and it plays a central role in protein synthesis and nitrogen balance in the liver.











Conclusions

Hundreds of metabolites are confidently identified in the extracts by comparison to NIST, Wiley, and Human Metabolome databases. After alignment, background subtraction, normalization, and feature integration using Genedata Expressionist MSX, the data can be interrogated for differences between populations. Evaluation of all samples and metabolites provides for a metaconsensus list, which can be evaluated for statistically robust modulations between populations. Genedata software provides the tools including drift correction, PCA, PLS, and Anova analysis at a feature level. These tools provide the statistical information needed to suggest that metabolites or panels of metabolites can be identified from these samples to monitor or distinguish the two phenotypes. Analyte identification is provided by interfacing with Wiley and NIST libraries through ChromaTOF® software. Among the analytes identified are fatty acids, small organic acids, amino acids, aromatics, vitamins, lipogenic compounds, and metabolites from TCA and other common metabolic pathways. Representative analytes are provided which demonstrate the capabilities of GC-TOFMS in combination with appropriate software tools. In particular, these analytes are intimately tied to lipid transport or metabolism, or oxidative stress.

Specifically, Valine and butenedioic acid are positively modulated in the ethanol-treated populations, while hexadecanoic acid, propanediol-1-phosphate, and 5-oxo-proline are negatively modulated. Each of these metabolites has been previously correlated to similar physiological conditions lending validity to the findings.

GC-TOFMS provides a robust technique for the comparison of polar metabolites from liver and provides confident, deconvolved identifications. This is demonstrated in the representative spectra provided and resultant identifications of the modulated metabolites. The application of alignment tools and robust statistical analyses in Expressionist MSX leverages the ability of GC-TOFMS to uncover significant numbers of analytes which are up or down regulated.

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

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