Evaluation of Metabolite Variation by a Pooled Sample Approach between Normal Control and Traumatic Brain Injury Mice Using GCxGC-TOFMS with Data Analysis Using a Software Driven Reference Feature

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INTRODUCTION

Metabolomic studies produce vast amounts of information which pose significant challenges for data analysis. Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) has shown great promise in its utilization to define small molecule metabolite profiles. This work employs GCxGC-TOFMS for analysis of pooled samples and data analysis by means of a software driven "Reference" feature to discover significant metabolite variations between diseased state and normal control sample pools. The pooled sample approach for trimethylsilyl-derivatized samples by GCxGC-TOFMS is a fast screening technique that can discover potential metabolite variations representing different states of health. Extracted samples from traumatic brain injured and normal control mice were derivatized with BSTFA plus 1% TMCS. A 10 µL aliquot from four diseased and four normal control samples were pooled into respective autosampler vials prior to GCxGC-TOFMS analysis. Analysis was conducted using triplicate injections of each sample pool. The data analysis strategy utilized a reference method built from the normal control pooled sample results. The "Reference" method determines the differences between the results from the brain injured and the normal control sample pools by comparison of user-defined limits for retention times, peak area, and spectral match. Peak table results list whether the reference standard components were a match, not found, or out of tolerance with the peak area percentage from reference compounds. This study presents an analytical approach using GCxGC-TOFMS and a data analysis software reference feature that can quickly screen potential metabolite variations between normal control and experimental sample pools from a traumatic brain injury study.

EXPERIMENTAL APPROACH

A comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry analysis was conducted using a LECO Pegasus® 4D GCxGC-TOFMS instrument. A total of 8 mouse brain extract samples were received, consisting of 4 control samples and 4 traumatic brain injured samples. The GCxGC-TOFMS analysis was performed in triplicate for each sample pool following derivatization.

SAMPLE PREPARATION

The pooled injured and control samples were derivatized by injecting 50 µL of BSTFA plus 1% TMCS into each sample vial. The vials were first vortexed and then heated at 80°C for 60 minutes. The vials were placed in an autosampler tray for 24 hours prior to analysis by GCxGC-TOFMS.

METHODS

GCxGC–TOFMS Analysis Parameters

- Gas Chromatograph: Agilent 7890 equipped with a LECO dual stage, quad jet thermal modulator, secondary oven and a GERSTEL MPS2 autosampler
- GC Primary Column: 30 m x 0.25 mm id. x 0.25 µm film thickness Rxi-5SilMS (Restek Corp.)
- GC Secondary Column: 1.25 m x 0.25 mm id. x 0.25 µm film thickness Rxi-17Sil-MS (Restek Corp.)
- Carrier Gas: Helium set @ 1.5 mL/min
- Injection Mode: Splitless
- Injection Volume: 1 µL
- Inlet Temperature: 275°C
- Primary Column Temperature Program: Initial temperature 50°C for 0.5 min ramped @ 5.0°C/min to 300°C, held for 6 min
- Secondary Column Temperature Program: Initial temperature 55°C for 0.5 min ramped @ 5.0°C/min. to 305°C, held for 6 min
- GCxGC Modulator Temperature Offset: 30°C
- Modulation Period: 4.0 s
- Hot Pulse: 0.5 s
- Cool Time Between Stages: 1.5 s
- Transfer Line Temperature: 280°C
- Total Run Time: 56.50 min

Mass Spectrometer: Pegasus® 4D TOFMS AnalysisParameters

- Acquisition Delay: 450 s
- Mass Range: 45–700 m/z
- Acquisition Rate: 200 spectra/s
- Ion Source Temperature: 230°C

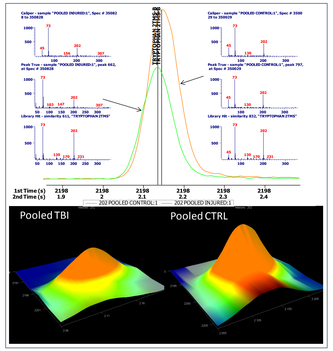

Figure 1. Figure 1 above shows the overlaid linear unique mass ion chromatogram and mass spectra for Tryptophan-2TMS in the TBI and CTRL pooled GCxGC-TOFMS analysis. The surface plots for the control and traumatic brain injury pooled samples illustrate that Tryptophan is downregulated in the TBI sample pool.

RESULTS

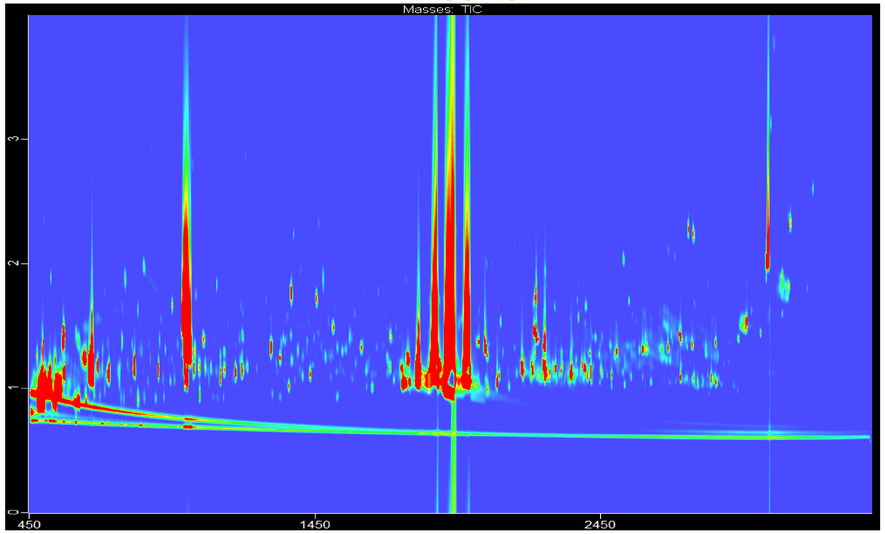

Figure 2. The two-dimensional chromatogram contour plot shown above is the GCxGC-TOFMS analysis of one of the TMS derivatized pooled control samples from the traumatic brain injury experimental study. The 2D contour plot highlights the capability of comprehensive two-dimensional chromatography to provide peak capacity and resolution that is not possible with a one-dimensional separation.

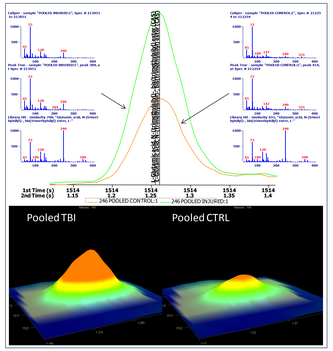

Figure 3. Figure 3 above shows the overlaid linear unique mass ion chromatogram and mass spectra for Glutamic acid-3TMS in the TBI and CTRL pooled GCxGC-TOFMS analysis. The surface plots for the control and traumatic brain injury pooled samples illustrate that Glutamic acid is upregulated in the TBI sample pool.

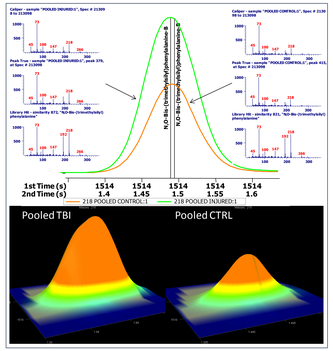

Figure 4. Figure 4 above shows the overlaid linear unique mass ion chromatogram and mass spectra for Phenylalanine-2TMS in the TBI and CTRL pooled GCxGC-TOFMS analysis. The surface plots for the control and traumatic brain injury pooled samples illustrate that Phenylalanine is upregulated in the TBI sample pool.

METABOLITE VARIATION DISCOVERY (CTRL vs. TBI)

Table 1 shown below is a peak list of sixteen targeted metabolites that were determined to show either a match or variance between the control "Reference" and traumatic brain injury pooled sample analysis. The list is comprised of mainly trimethylsilyl derivatized amino acids, as well as several trimethylsilyl derivatized sugars. The table records in the Type column whether the analyte is a "Match" or "Out of Tolerance". The user defines the area "Tolerance Percent" to be used for the reference method. This reference method used a 50% tolerance as the user-defined criteria. This means that a peak area between 50 and 150%, compared to the analyte reference peak area, will return a "Match" result. Any peak area found below 50% or above 150% are returned as "Out of Tolerance" with the reference compound. The concentration column is a direct peak area percentage comparison to the specified reference analyte.

An "Untargeted Reference" can also be utilized as a useful data processing feature for metabolite biomarker discovery. The normal control sample was employed to develop a second "Untargeted Reference" and applied successfully to reveal metabolites exhibiting high variance compared to the traumatic brain injury sample pool. Results from the "Untargeted Reference" data processing indicate 169 analytes found "Out of Tolerence", 212 analytes found as a "Match", and 7 analytes recorded as "Not Found".

Amino acids such as glutamate, taurine, and aspartate are known to show increased levels in traumatic brain injured individuals. The "Reference" peak list of metabolites illustrates the capability of the pooled sample approach coupled with comprehensive GCxGC-TOFMS analysis to detect and identify potentially important biomarkers for disease metabolite profiles.

Table 1. Peak List.

Peak #	Name	R.T. (s)	Type	Match	Concentration	Area	Quant Masses	S/N	Library
160	l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester (CAS)	718 , 1.145	Out of Tolerance	857	1000.91	66311353	116	10898	Wiley9
207	L-VALINE, N-(TRIMETHYLSILYL)-, TRIMETHYLSILYL ESTER	898 , 1.155	Match	912	142.34	36984759	144	19923	Wiley9
269	L-Isoleucine, N-(trimethylsilyl)-, trimethylsilyl ester (CAS)	1022 , 1.165	Out of Tolerance	919	159.74	25358304	158	58704	replib
301	Serine tritms	1130 , 1.155	Out of Tolerance	913	177.04	9186513	204	18548	mainlib
311	N,O,O-Tris(trimethylsilyl)-L-threonine	1170 , 1.135	Match	955	138.15	4533576	219	18074	mainlib
343	L-Methionine, N-(trimethylsilyl)-, trimethylsilyl ester (CAS)	1366 , 1.415	Out of Tolerance	952	198.78	4814149	128	17269	Wiley9
349	L-Proline, 1-(trimethylsilyl)-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester, trans- (CAS)	1378 , 1.190	Match	915	132.17	635150	140	3412.9	Wiley9
350	L-Aspartic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester (CAS)	1386 , 1.260	Match	861	50.34	169423	232	999.94	replib
378	L-Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester (CAS)	1514 , 1.255	Out of Tolerance	790	161.82	300961	246	3261.5	mainlib
379	N,O-Bis-(trimethylsilyl)phenylalanine	1514 , 1.490	Out of Tolerance	929	203.44	9790514	218	24272	mainlib
406	L-(-)-Arabitol, pentakis(trimethylsilyl) ether	1650 , 0.995	Match	971	80.34	1579096	217	3104.5	replib
412	l-Glutamine, tris(trimethylsilyl) deriv. (CAS)	1710 , 1.420	Out of Tolerance	945	158.1	11930764	156	43725	Wiley9
644	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	2086 , 1.045	Match	890	85.51	4537371	305	32792	Wiley9
662	TRYPTOPHAN 2TMS	2202 , 2.140	Out of Tolerance	765	26.91	126332	202	13.762	Wiley9
757	Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	2446 , 1.735	Match	782	66.03	85970	217	18.131	mainlib
765	D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	2486 , 1.020	Match	942	75.7	325409	319	1922	mainlib

USING THE REFERENCE STANDARD FEATURE

A "Reference Method" is built into ChromaTOF® software from a user-created standard that is applied and compared to a sample. The purpose of a Reference Method is to determine the component differences between a sample and a reference standard within-user defined limits of retention time, peak area, and spectral match. In this study, metabolites found in the "Control" pooled sample were used to create the "Reference" standard within the software. The "Reference" was applied as part of the data processing method. The processed sample's peak table displays each compound from the reference in the Type column as either a "Match", found but "Out of Tolerance" by peak area percent, "Not Found", or "Unknown".

An example of a peak table is shown below (Table 2) for the "Reference" used for this analysis. The columns show the Compound Name, Type of Match, the Match similarity score, retention time, peak area, signal to noise, and the library used to identify the mass spectral peak.

Table 2. Peak Table.

Name	Type	Match	R.T. (s)	Area	S/N	Library
D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	Match	942	2486 , 1.020	325409	1922	mainlib
N,O-Bis-(trimethylsilyl)phenylalanine	Out of Tolerance	929	1514 , 1.490	9790514	24272	mainlib
N-Methylmaleimide	Not Found	600				mainlib

CONCLUSIONS

This work demonstrates proof of concept for a fast, efficient, and simple comparative analysis technique for screening large sample pools to distinguish metabolite variations as potential biomarkers for healthy and diseased state populations. This work investigated the use of GCxGC-TOFMS analysis of pooled normal control versus traumatic brain injury mouse brain extracts. The results from this study highlight the expanded capabilities of comprehensive two-dimensional GCxGC coupled with the continuous full-range fast acquisition rates obtainable by TOFMS. The comparisons between the control and traumatic brain injury sample pools establish this analytical approach and instrumental platform as an efficient screening tool for discovery of metabolite variation and potential biomarker discovery between pooled populations representing dissimilar states of health. The data from these analyses was processed using a "Reference" standard feature available in ChromaTOF software that enables the discovery of potential biomarkers. The "Reference" feature allows the user to define chromatographic and mass spectral criteria that enhance and facilitate biomarker discovery. The pooled sample approach coupled with the GCxGC-TOFMS platform is a valuable time and resource saving option for the comparative analysis of pooled samples from varying states of health.

For further information regarding this study contact the authors at john_heim@leco.com.

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