Smoker's and Non-Smoker's Urine Comparison Using Comprehensive Two-Dimensional Gas Chromatography High Performance Time-of-Flight Mass Spectrometry

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Introduction

Urine is a favored biofluid for diagnostic testing (Urinalysis) because it is non-invasive and large volumes are easily obtained. In addition, urine is relatively free from interfering proteins and lipids, and it tends to "hold" high concentrations of drugs and metabolites over extended periods of time. Modern, routine clinical tests include the determination of specific gravity, measurement of glucose, nitrates, etc.

In this study, a novel analytical approach was utilized for the effective characterization of compounds in two standard reference materials, NIST smoker's and non-smoker's urine.

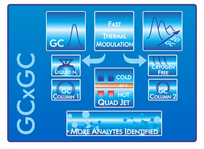
Objectives

- Implement the use of enhanced, comprehensive two-dimensional gas chromatography (GCxGC) for the separation of compounds in urine
- Use a benchtop, high performance time-of-flight mass spectrometer and powerful processing software to quickly and confidently identify compounds in urine
- Use software tools to compare smoker's and non-smoker's urine

Sample Preparation

1) 600 µL of urine treated with urease (37 °C, 15 min)
2) The mixture was vortexed (2 min) and then centrifuged (12,000 g for 10 min)
3) 200 µL of supernatant was transferred to a 2mL GC vial and evaporated to dryness (Speed Vac)
4) The dry material was derivatized using a two-step procedure
   - Methoximation (20 µL of MEOX, 80 °C, 30 min)
   - Silylation (75 µL MSTFA, 80 °C, 30 min)

Technology



LECO Pegasus® BT 4D and ChromaTOF® Brand Software

Table 1. Instrument acquisition parameters

Gas Chromatograph	**Agilent 7890, LECO Dual Stage Quad Jet Modulator & L-PAL 3 Autosampler**
Injection	1µL, Split 20:1; 280 °C
Carrier Gas	He @ 1.4 ml/min, Constant Flow
Columns (1st Dimension) (2nd Dimension)	Rxi-5 MS, 30 m x 0.25 mm i.d. x 0.25 *µm* (Restek, Bellefonte, PA, USA) Rxi-17 Sil MS 0.6 m x 0.25 mm i.d. x 0.25 µm (Restek, Bellefonte, PA, USA)
Temperature Program	50 °C (0.50 min), ramped 5 °C/min to 150 °C (1.01 min), ramped 2 °C/min to 200 °C, ramped 50 °C/min to 300 °C (15 min) 2nd oven maintained +10 °C relative to primary oven
Modulation	4s with temperature maintained +15 °C relative to secondary oven
Mass Spectrometer	**LECO Pegasus BT 4D**
Ion Source Temperature	250 °C
Ionization Mode	EI
Mass Range (m/z)	45-600
Acquisition Rate	15 spectra/s (1D); 200 spectra/s (2D)

Why GCxGC for Urine Analysis?

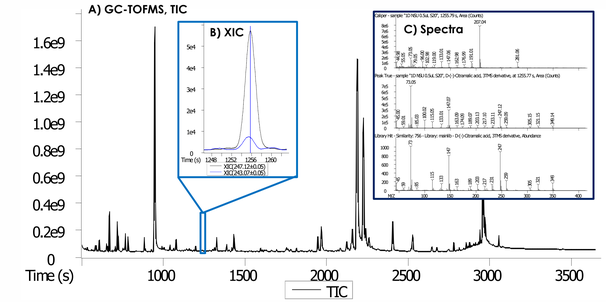


Figure 1. A) GC-TOFMS, TIC of Non-smoker's urine (NSU), B) eXtracted Ion Chromatogram (XIC) showing coeluting parabanic acid and D-(-)-citramalic acid, and C) corresponding Caliper, Peak True and Library Spectra.

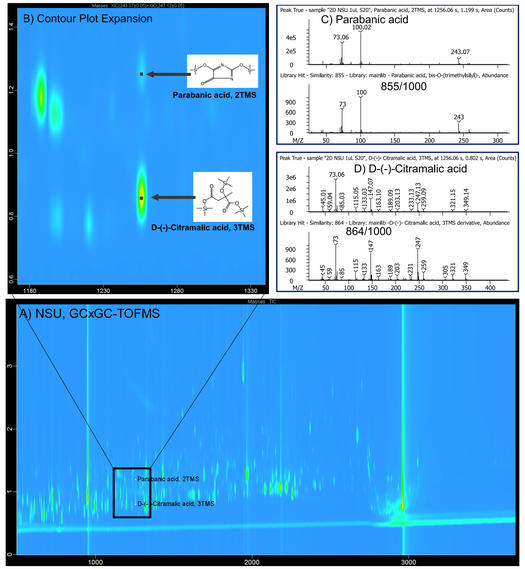


Figure 2. A) GCxGC-TOFMS Contour Plot for NSU and B) Plot expansion displaying separated parabanic acid, and D-(-)-citramalic acid. Improved Peak True and Library spectra for the chromatographically resolved acids.

Table 2. Comparison of GC and GCxGC-TOFMS spectral similarity values for some acids in NSU (Unknowns → Knowns)

GC-TOFMS

Name	R.T. (s)	Similarity
D-(-)-Citramalic acid, 3TMS	1255,77	756
Parabanic acid, 2TMS	Not Found	
Kojic acid, 2TMS	1264,51	570
Quinolinic acid, 2TMS	1721,83	326
Orotic Acid, 3TMS	1778,33	660
Homovanillic Acid, 2TMS	1814,9	449
Hippuric acid, TMS	1947,01	649
Vanillylmandelic acid, 3TMS	2102,38	681
Pantothenic acid, 3TMS	2369,86	537
Caffeic acid, 3TMS	2702,75	533

GCxGC-TOFMS

R.T. (s)	Similarity	Mass Δ (Da)
1256,06, 0,802	864	N/A
1256,06, 1,199	855	0,01
1264,06, 1,020	818	-0,01
1720,1, 2,088	941	0,01
1776,1, 1,357	807	0,02
1812,1, 1,726	895	0,01
1944,12, 2,811	953	0,02
2096,13, 1,537	860	0,02
2364,15, 1,395	912	N/A
2696,18, 1,615	865	0,02

Delivering the Right Results

Confident Characterization: Similarity, Retention Index, and Mass Δ

Spectral Similarity

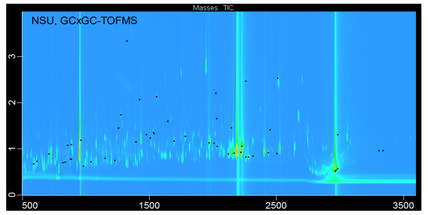


Figure 3. Contour plot with peak markers for representative compounds in NSU: acids, diacids, fatty acids, amino acids, monosaccharides, disaccharides, etc.

Table 3. Representative list of compounds in NSU with retention times and spectral similarity values

Name	R.T. (s)	Similarity	Name	R.T. (s)	Similarity
Lactic Acid, 2TMS	588.007, 0.683	933	Citric acid, 4TMS	1968.12, 1.140	882
Glycolic acid, 2TMS	612.009, 0.736	915	Methylcitric acid, 4TMS	2004.12, 1.127	814
Oxalic acid, 2TMS	708.017, 0.894	927	Adenine, 2TMS	2020.12, 2.210	840
2-Methyl-3-hydroxybutyric acid, 2TMS	816.025, 0.707	949	m-Coumaric acid, 2TMS	2028.12, 1.664	790
3-Hydroxyisovaleric acid, 2TMS	836.027, 0.714	912	1,5-Anhydrohexitol, 4TMS	2032.12, 1.066	826
Guaiacol, TMS	856.028, 1.085	805	D-Fructose, MOX, 5TMS	2120.13, 0.898	908
4-Hydroxybutanoic acid, 2TMS	876.03, 0.780	805	L-Ascorbic acid, 2-O-methyl-3,5,6-tris-O-TMS	2144.13, 1.464	794
2-Methylacetoacetic acid, TMS	884.031, 0.781	813	d-Galactose, (1E)-MOX, 5TMS	2164.13, 0.916	933
Benzoic Acid, TMS	884.031, 1.104	932	d-Galactose, (1Z)-MOX, 5TMS	2216.14, 0.937	909
Niacin, TMS	960.037, 1.189	912	d-Glucose, (1Z)-MOX, 5TMS	2224.14, 1.064	844
1,2,3-Butanetriol, 3TMS	980.038, 0.634	935	D-Mannitol, 6TMS	2256.14, 0.829	915
Glyceric acid, 3TMS	1040.04, 0.729	920	1H-Indole-2-acetic acid,2TMS	2256.14, 2.475	924
Malonic acid, 3TMS	1148.05, 0.798	902	D-Sorbitol, 6TMS	2276.14, 0.835	934
3-Aminoisobutyric acid, 3TMS	1228.06, 0.754	823	Myo-Inositol, 6TMS	2312.14, 0.849	923
Anthranilic acid, TMS	1256.06, 1.461	924	D-Gluconic acid, 6TMS	2432.15, 0.923	910
Pyroglutamic acid, TMS	1272.06, 1.745	949	Palmitic Acid, TMS	2448.16, 1.416	923
Malic acid, 3TMS	1284.06, 0.847	922	Scyllo-Inositol, 6TMS	2500.16, 0.903	938
Uracil	1320.07, 3.345	916	Kynurenic Acid, 2TMS	2508.16, 2.538	852
3-Hydroxybenzoic acid, 2TMS	1392.07, 1.161	860	N-Acetyl-D-glucosamine, MOX (anti), 4TMS	2604.17, 1.306	850
Trigonelline TMS	1420.07, 2.076	863	N-Acetyl-D-glucosamine, MOX (syn), 4TMS	2624.17, 1.316	869
3-Hydroxyphenylacetic acid, 2TMS	1472.08, 1.315	937	Stearic acid, TMS	2808.18, 0.555	912
4-Hydroxybenzoic acid, 2TMS	1504.08, 1.247	877	Xanthurenic acid, 3TMS	2824.19, 0.586	807
4-Hydroxybenzeneacetic acid, 2TMS	1528.08, 1.360	904	D-Lactose, MOX, 8TMS (isomer 2)	2956.2, 0.524	916
Vanillyl alcohol, 2TMS	1532.08, 1.328	823	Maltose, 8TMS (isomer 2)	2964.2, 0.556	891
Furoylglycine, TMS	1556.08, 2.135	930	D-(+)-Cellobiose, MOX, 8TMS (isomer 2)	2980.2, 0.581	873
Vanillylmandelic acid, 3TMS	1644.09, 1.607	851	Tryptophan, 4TMS	2980.2, 1.315	805
Levoglucosan, 3TMS	1692.1, 1.174	919	Maltose, 8TMS , isomer 1	3304.22, 0.971	840
Aconitic acid, (E)-, 3TMS	1780.1, 1.276	831	Sucrose, 8TMS	3336.23, 0.977	846

$\bar{x}$ **Similarity = 883/1000**

Mass Δ, Retention Index

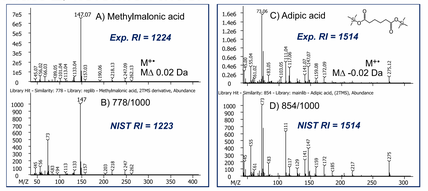


Figure 4. NSU GCxGC-TOFMS Peak True spectra, library mass spectra, RI (Experimental and NIST calculated) and Mass Δ values for methylmalonic acid (A/B), and adipic acid (C/D).

Table 4. Comparison of experimental and NIST RI values for diacids in urine

Name	R.T. (s)	Similarity	Mass Δ (Da)	Exp RI	NIST RI
Oxalic acid, 2TMS	708.017, 0.894	927	N/A	1142	1136
Methylmalonic acid, 2TMS	844.028, 0.825	778	0.02	1224	1223
Succinic acid, 2TMS	1004.04, 0.896	885	0.01	1323	1321
Methylsuccinic acid, 2TMS	1024.04, 0.861	907	N/A	1336	1331
Fumaric acid, 2TMS	1052.04, 0.823	915	N/A	1354	1353
Itaconic acid, 2TMS	1052.04, 0.842	816	N/A	1354	1398
Methylmaleic acid, 2TMS	1064.05, 0.972	876	N/A	1362	1386
3-Methylglutaric acid, 2TMS	1172.05, 0.892	906	N/A	1432	1431
Adipic acid, 2TMS	1296.06, 0.993	854	-0.02	1514	1514
3-Methyladipic acid, 2TMS	1344.07, 1.021	849	N/A	1543	1544
2-Oxoglutaric acid, MOX, 2TMS	1380.07, 1.179	837	0.02	1564	1587
α-Hydroxyglutaric acid, 2TMS	1428.07, 0.961	898	0.03	1593	1586
Pimelic acid, 2TMS	1464.08, 1.150	933	N/A	1613	1610
Tartaric acid, 4TMS	1572.09, 0.962	878	-0.01	1669	1665
Suberic acid, 2TMS	1648.09, 1.294	933	N/A	1707	1708
Azelaic acid, 2TMS	1864.11, 1.401	928	-0.01	1807	1807

$\bar{x}$ **%Error = 0.54**

Smoker's Urine Results

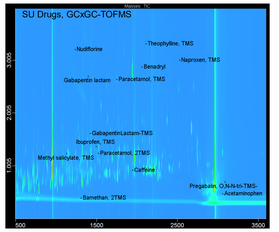


Figure 5. Drugs in smoker's urine (SU).

Table 5. Retention times, similarity values for drugs in SU

Name	R.T. (s)	Similarity	Mass Δ (Da)
Methyl salicylate, TMS	1132.05, 1.237	838	N/A
Nudiflorine	1236.06, 3.209	811	0.00
Bamethan, 2TMS	1308.06, 0.393	816	N/A
Gabapentin lactam	1396.07, 2.706	938	0.00
GabapentinLactam-TMS	1420.07, 1.613	944	0.01
Ibuprofen, TMS	1492.08, 1.372	889	0.00
Paracetamol, 2TMS	1520.08, 1.240	906	0.01
Paracetamol, TMS	1748.1, 2.638	913	0.01
Caffeine	1944.12, 0.919	871	0.00
Benadryl	2060.12, 2.873	900	N/A
Theophylline, TMS	2108.13, 3.314	793	0.01
Naproxen, TMS	2524.16, 3.003	911	0.01
Acetaminophen	3056.2, 0.477	892	-0.01
Pregabalin, O,N-N-tri-TMS-	3084.21, 0.698	759	N/A

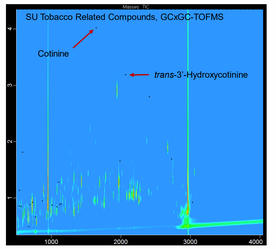


Figure 6. Tobacco related compounds in SU.

Table 6. Retention times, similarity values for tobacco related compounds in SU

Name	R.T. (s)	Similarity	Mass Δ (Da)
3-Pyridinol, TMS	536.003, 0.864	812	0.00
Benzene, 1,3-dichloro-	536.003, 1.142	837	0.00
Phenol, TMS	564.005, 0.857	898	0.00
2-Pyridinecarbonitrile	584.007, 1.815	898	0.00
Carbazole, 2,4,7-trimethyl-	672.014, 0.497	715	-0.08
o-Cresol, TMS	692.015, 0.886	780	0.00
3-Pyridinol, TMS	704.016, 1.044	915	0.00
p-Cresol, TMS derivative	728.018, 0.932	886	0.00
Naphthalene, 2,6-dimethyl-	772.022, 0.675	752	0.00
3-Ethylphenol, TMS	872.03, 0.957	781	0.00
Catechol, 2TMS	1008.04, 0.881	923	0.00
Pyrene, 1,9-dimethyl-	1080.05, 0.849	724	0.02
4-Cyanophenol, TMS	1104.05, 1.286	733	0.01
4-Methylcatechol, 2TMS	1120.05, 0.892	908	0.01
Hydroquinone, 2TMS	1136.05, 0.899	786	0.01
Cotinine	1648.09, 0.021	950	0.00
Theobromine	1984.12, 1.660	918	0.00
trans-3'-Hydroxycotinine, TMS	2064.13, 3.190	928	0.01
Theobromine, TMS derivative	2224.14, 0.044	725	0.00
2-Hydroxy-3-methylanthraquinone, O-TMS	2408.15, 2.800	810	0.07
6-Hydroxy-α-methylnaphthaleneacetic acid, 2TMS	2728.18, 1.694	897	0.01
4-Nitrophenyl-β-D-galacturonide, 3TMS	3028.2, 0.927	736	N/A

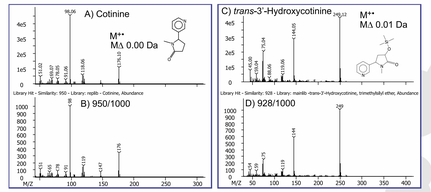


Figure 7. SU GCxGC-TOFMS Peak True spectra, library mass spectra for cotinine (A/B), and trans-3'-hydroxycotinine (C/D).

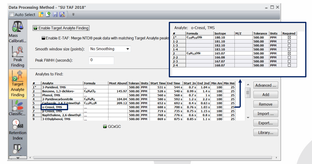


Figure 8. Target Analyte Finding (TAF) processing method for rapid and robust identification of tobacco related compounds in comprehensive data files.

Table 7. TAF processing results for SU and NSU. As expected increased quantities of tobacco related compounds were detected in smoker's urine

Name	R.T. (s)	SU Area	NSU Area
3-Pyridinol, TMS	536 s, 0.8668 s	371202153	310107452
Benzene, 1,3-dichloro-	536 s, 1.148 s	2650602	2571935
Phenol, TMS	564 s, 0.860 s	277041941	149122874
2-Pyridinecarbonitrile	584 s, 1.822 s	3093248	2124158
Carbazole, 2,4,7-trimethyl-	672 s, 0.509 s	606916	Not Detected
o-Cresol, TMS	692 s, 0.905 s	1892146	530009
p-Cresol, TMS	728 s, 0.948 s	401973973	429576716
Naphthalene, 2,6-dimethyl-	772 s, 0.703 s	560201	248416
3-Ethylphenol, TMS	872 s, 0.985 s	1525954	1013646
Catechol, 2TMS	1008 s, 0.921 s	523485415	416608899
Pyrene, 1,9-dimethyl-	1080 s, 0.896 s	1637450	886909
4-Cyanophenol, TMS	1104 s, 1.498 s	8685	8367
4-Methylcatechol, 2TMS	1120 s, 0.940 s	104959692	81921674
Hydroquinone, 2TMS	1136 s, 0.936 s	16420475	9693926
Cotinine	1648 s, 0.122 s	***23942764***	***50642***
Theobromine	1988 s, 1.789 s	9058621	17027847
trans-3'-Hydroxycotinine, TMS	2060 s, 3.305 s	***705212***	***Not Detected***
2-Hydroxy-3-methylanthraquinone, TMS	2404 s, 2.778 s	584	4222
6-Hydroxy-α-methylnaphthaleneacetic acid 2TMS	2728 s, 1.873 s	6367223	14024
4-Nitrophenyl-β-D-galacturonide, 4TMS	3032 s, 1.028 s	74974112	18560460

Summary

- The Pegasus BT 4D facilitates fast and confident compound identification through enhanced two-dimensional chromatographic resolution and high performance TOFMS.
- Robust compound identification was obtained through spectral similarity searches of large, well-established databases, mass Δ calculations and retention index filtering.
- Comprehensive data can be processed via non-targeted Peak Find or rapid Target Analyte Finding.