Application Note

Instrument: Pegasus® BT 4D

Analysis of Smoker's and Non-Smoker's Urine Using the Pegasus BT 4D

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Key Words: GC-MS, GC×GC-MS, GC×GC-TOFMS, Pegasus BT 4D, Smoker's Urine

Introduction

Urine is a favored biological fluid for medical testing since it is easy to obtain in large quantities and provides a window into an individual's exposure, diet, and general health. In this study, a novel analytical approach based on comprehensive two-dimensional gas chromatography-high performance time-of-flight mass spectrometry was utilized for robust identification of compounds in two urine standard reference materials (smoker's and non-smoker's urine). This rich, comprehensive GCxGC-TOFMS data can be used to visually differentiate the two urine standards as shown in Figure 1. The analytical methodology resulted in unparalleled urine-component separation and robust identification.

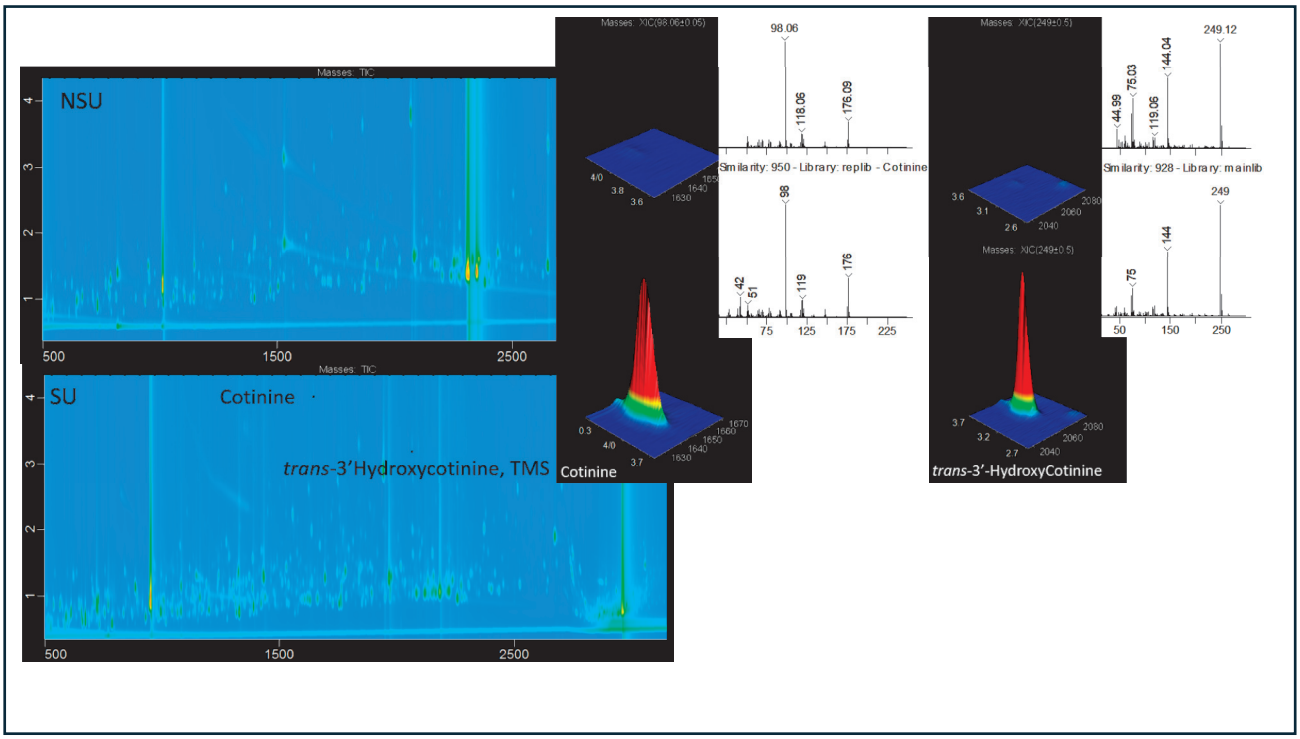


Figure 1. GCxGC-TOFMS data for non-smoker's urine (NSU, top) and smoker's urine (SU, bottom). The 3D surface plots and spectra for two analytes detected only in SU.

Experimental

Urine standard reference materials were purchased from NIST (Organic contaminants in smoker's urine, SRM 3672; organic contaminants in non-smoker's urine, SRM 3673). A 600 µL aliquot of the individual urine standard was treated with urease (37 °C, 15 min), vortexed (2 min), and then centrifuged (12,000 g for 10 min). A 200 µL aliquot of supernatant was transferred to a 2mL GC vial and evaporated to dryness (Speed Vac). The dry material was derivatized using a two-step procedure: 1) Treatment with methoxyamine hydrochloride in anhydrous pyridine and 2) reaction with MSTFA. The derivatized samples were analyzed using both GC-TOFMS and GCxGC-TOFMS (Table 1).

Table 1. GC and GC×GC-TOFMS (*Pegasus* BT 4D) Instrument Parameters

Gas Chromatograph	**LECO GCxGC Quad Jet Thermal Modulator & L-PAL 3 Autosampler**
Injection	1µL, split 20:1 @ 280 °C
Carrier Gas	He @ 1.4 mL/min, Constant Flow
Column One	Rxi-5ms, 30 m x 0.25 mm i.d. x 0.25 µm coating (Restek, Bellefonte, PA, USA)
Column Two	Rxi-17SilMS, 0.60 m x 0.25 mm x 0.25 µm coating (Restek, Bellefonte, PA, USA)
Temperature Program	0.5 min at 50 °C, ramped 5 °C/min to 150 °C, held 1 min, ramped 2 °C/min. to 200 °C, ramped 50 °C/min to 300 °C and held 15 min. Secondary oven maintained +10 °C relative to primary oven
Modulation	4 s with temperature maintained +15 °C relative to 2nd oven
Transfer Line	300 °C
Mass Spectrometer	**LECO *Pegasus* BT 4D**
Ion Source Temperature	250 °C
Mass Range	45-600 m/z
Acquisition Rate	15 spectra/s (1D); 200 spectra/s (2D)

Results and Discussion

Analyses of urine samples resulted in contour plots or "fingerprints" (Figure 2) displaying a wide variety of different compounds including acids, diacids, amino acids, bases, fatty acids, a large assortment of monosaccharides and disaccharides, and reduced/oxidized sugars (Table 2). The average spectral similarity value for a representative set of compounds in non-smoker's urine was 883/1000.

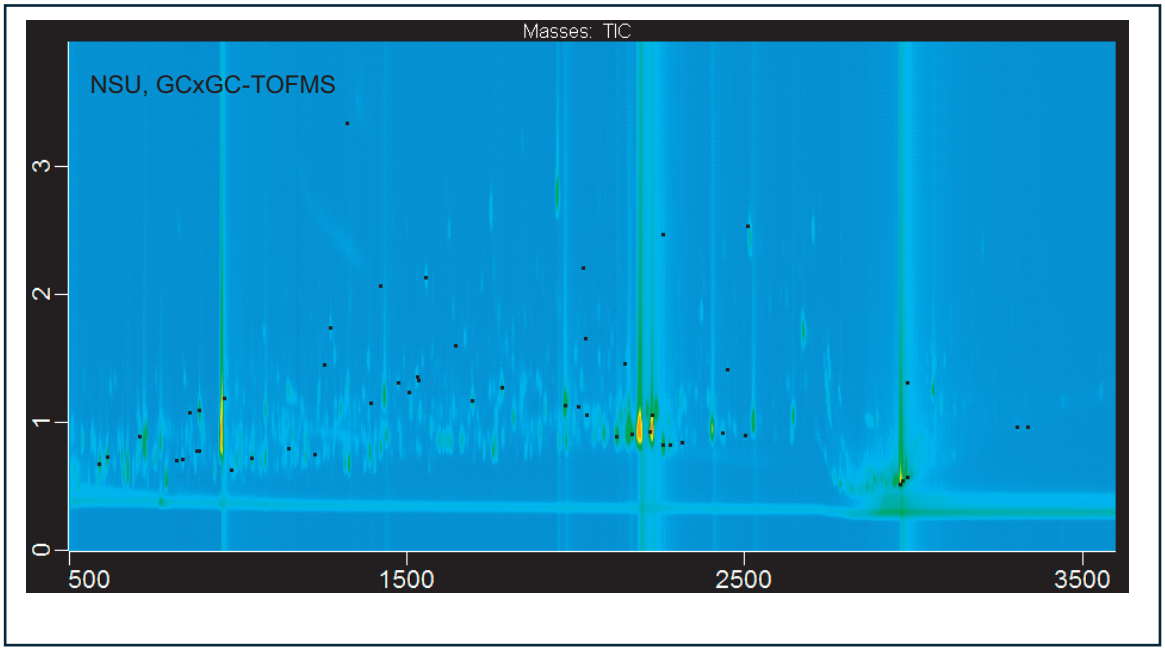


Figure 2. Contour plot with peak markers for representative compounds in NSU.

Table 2. Representative list of compounds in NSU with retention times and spectral similarity values

Name	*R.T. (s)*	*Similarity*
Lactic Acid, 2TMS	588.007, 0.683	933
Glycolic acid, 2TMS	612.009, 0.736	915
Oxalic acid, 2TMS	708.017, 0.894	927
2-Methyl-3-hydroxybutyric acid, 2TMS	816.025, 0.707	949
3-Hydroxyisovaleric acid, 2TMS	836.027, 0.714	912
Guaiacol, TMS	856.028, 1.085	805
4-Hydroxybutanoic acid, 2TMS	876.03, 0.780	805
2-Methyloctanoic acid, TMS	884.031, 0.781	813
Benzoic Acid, TMS	884.031, 1.104	932
Niacin, TMS	960.037, 1.189	912
1,2,3-Butanetriol, 3TMS	980.038, 0.634	935
Glyceric acid, 3TMS	1040.04, 0.729	920
Malonic acid, 3TMS	1148.05, 0.798	902
3-Aminoisobutyric acid, 3TMS	1228.06, 0.754	823
Anthranilic acid, TMS	1256.06, 1.461	924
Pyroglutamic acid, TMS	1272.06, 1.745	949
Malic acid, 3TMS	1284.06, 0.847	922
Uracil	1320.07, 3.345	916
3-Hydroxybenzoic acid, 2TMS	1392.07, 1.161	860
Trigonelline TMS	1420.07, 2.076	863
3-Hydroxyphenylacetic acid, 2TMS	1472.08, 1.315	937
4-Hydroxybenzoic acid, 2TMS	1504.08, 1.247	877
4-Hydroxybenzeneacetic acid, 2TMS	1528.08, 1.360	904
Vanillyl alcohol, 2TMS	1532.08, 1.328	823
Furoylglycine, TMS	1556.08, 2.135	930
Vanillylmandelic acid, 3TMS	1644.09, 1.607	851
Levoglucosan, 3TMS	1692.1, 1.174	919
Aconitic acid, (E)-, 3TMS	1780.1, 1.276	831

Name	*R.T. (s)*	*Similarity*
Citric acid, 4TMS	1968.12, 1.140	882
Methylcitric acid, 4TMS	2004.12, 1.127	814
Adenine, 2TMS	2020.12, 2.210	840
m-Coumaric acid, 2TMS	2028.12, 1.664	790
1,5-Anhydrohexitol, 4TMS	2032.12, 1.066	826
D-Fructose, MOx, 5TMS	2120.13, 0.898	908
L-Ascorbic acid, 2-O-methyl-3,5,6-tris-O-TMS	2144.13, 1.464	794
d-Galactose, (1E)-MOx, 5TMS	2164.13, 0.916	933
d-Galactose, (1Z)-MOx, 5TMS	2216.14, 0.937	909
d-Glucose, (1Z)-MOx, 5TMS	2224.14, 1.064	844
D-Mannitol, 6TMS	2256.14, 0.829	915
1H-Indole-2-acetic aciid,2TMS	2256.14, 2.475	924
D-Sorbitol, 6TMS	2276.14, 0.835	934
Myo-Inositol, 6TMS	2312.14, 0.849	923
D-Gluconic acid, 6TMS	2432.15, 0.923	910
Palmitic Acid, TMS	2448.16, 1.416	923
Scyllo-Inositol, 6TMS	2500.16, 0.903	938
Kynurenic Acid, 2TMS	2508.16, 2.538	852
N-Acetyl-D-glucosamine, MOx (anti), 4TMS	2604.17, 1.306	850
N-Acetyl-D-glucosamine, MOx (syn), 4TMS	2624.17, 1.316	869
Stearic acid, TMS	2808.18, 0.555	912
xanthurenic acid, 3TMS	2824.19, 0.586	807
D-Lactose, MOx, 8TMS (isomer 2)	2956.2, 0.524	916
Maltose, 8TMS (isomer 2)	2964.2, 0.556	891
D-(+)-Cellobiose, MOx, 8TMS (isomer 2)	2980.2, 0.581	873
Tryptophan, 4TMS	2980.2, 1.315	805
Maltose, 8TMS , isomer 1	3304.22, 0.971	840
Sucrose, 8TMS	3336.23, 0.977	846

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

Compound identification was accomplished through automated peak find and deconvolution, spectral similarity searches of large, well-established databases, mass Δ calculations, and retention index filtering. For example, the spectral similarity values for methylmalonic acid and adipic acid were 778 and 854/1000 respectively (Figure 3). Further confidence for the identification of the acids was achieved through the comparison of the absolute value of their mass delta values ($|M\Delta| = 0.02$ Da). Retention index filtering was also applied during Peak Find processing resulting in an average absolute value ($|\Delta RI_{ave}| =$) of 0.54 for a representative set of diacids in NSU (Table 3).

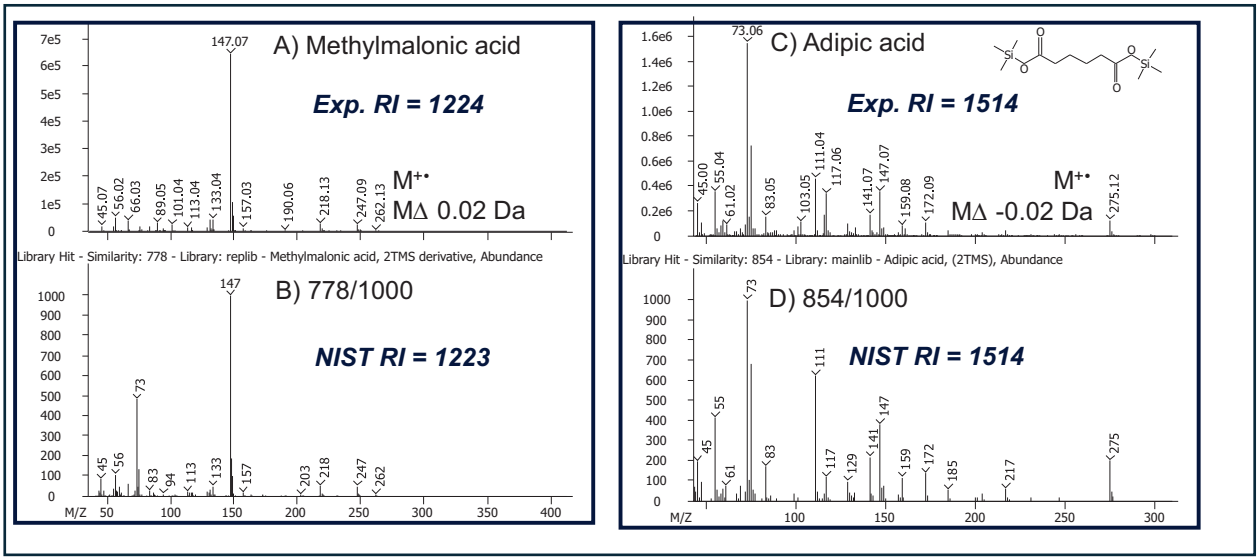


Figure 3. NSU GCxGC-TOFMS Peak True spectra, library mass spectra, RI (Experimental and NIST), and Mass Δ Values for methylmalonic acid (A/B) and adipic acid (C/D).

Table 3. Comparison of experimental and NIST RI values for diacids in NSU

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, 0.942	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

The increase of confidently identified compounds in urine standards was a direct result of transitioning from GC-TOFMS to high performance GCxGC-TOFMS, which yields cleaner spectra and thus improved spectral similarity scores as shown in Table 4 (516/1000 to 877/1000). This is a 170% improvement in average spectral similarity. The trend is evident by comparing the 1D and 2D data for parabinic acid which is not found in the 1D data since it perfectly coelutes with citramalic acid, but is detected and identified in the 2D data with a spectral similarity of 855/1000. It is clearly evident from these examples that the enhanced chromatographic resolution of GCxGC improves acquired mass spectral data and increases similarity scores resulting in a transformation of 1D unknowns into known compounds.

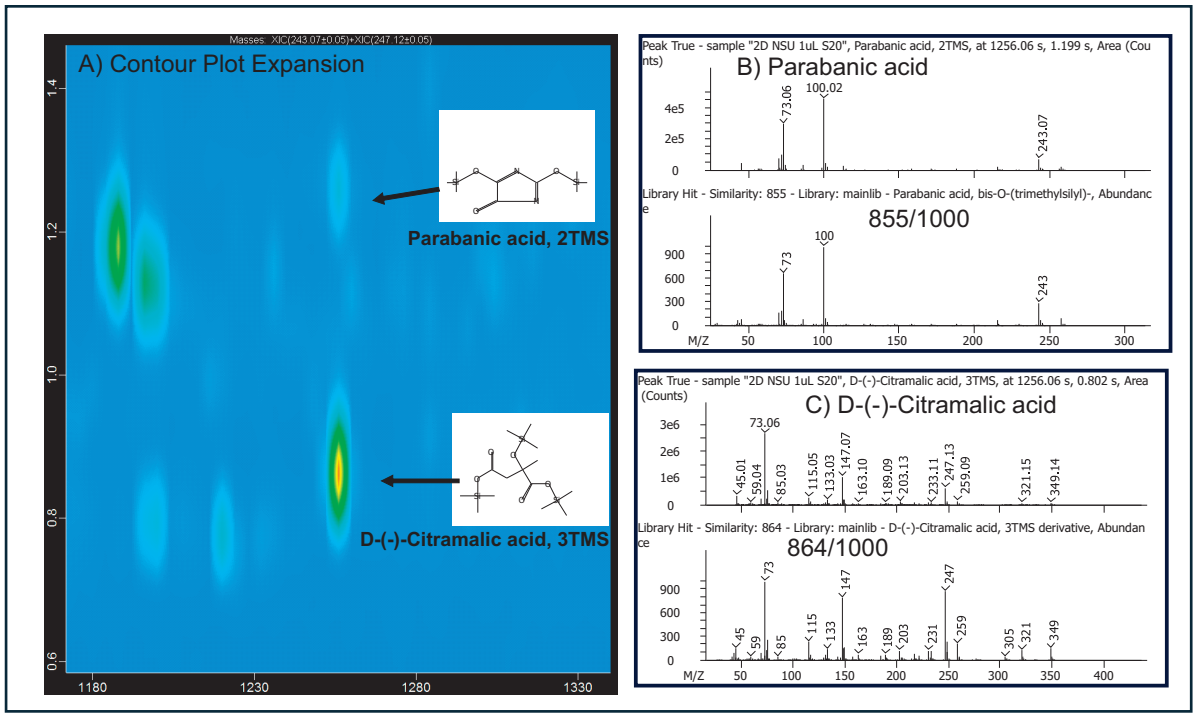
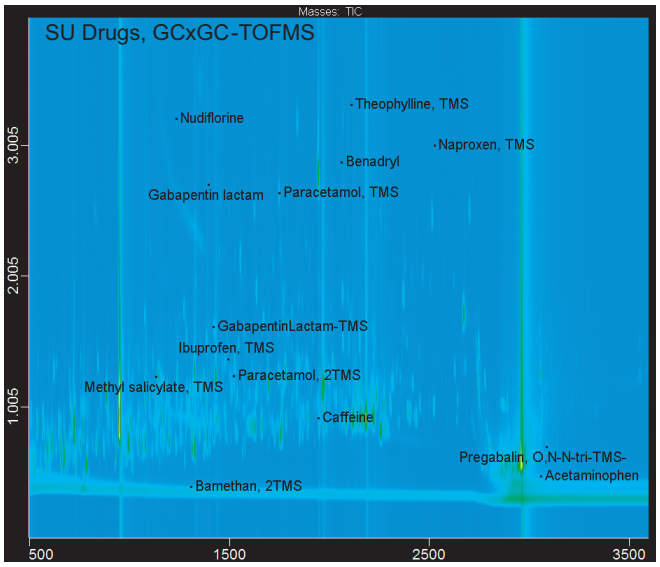


Figure 4. A) NSU contour plot expansion displaying separated parabanic acid, and D-(-)-citramalic acid. B,C) Improved Peak true and library spectra for the chromatographically resolved acids.

Table 4. Comparison of GC and GCxGC-TOFMS spectral similarity values for acids in NSU. The separation power of GCxGC takes unknowns in 1D separations and makes them knowns.

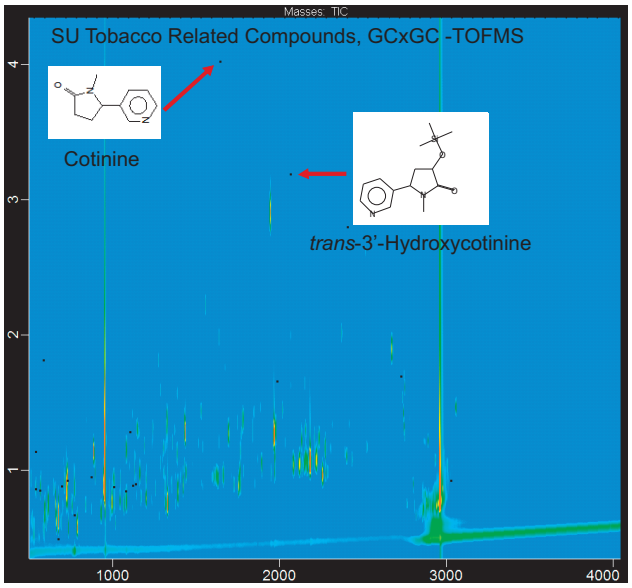
GC-TOFMS			GCxGC-TOFMS	
Name	***R.T. (s)***	***Similarity***	***R.T. (s)***	***Similarity***
D-(-)-Citramalic acid, 3TMS	1255.77	756	1256.06, 0.802	864
Parabanic acid, 2TMS			1256.06, 1.199	855
Kojic acid, 2TMS	1264.51	570	1264.06, 1.020	818
Quinolinic acid, 2TMS	1721.83	326	1720.1, 2.088	941
Orotic Acid, 3TMS	1778.33	660	1776.1, 1.357	807
Homovanillic Acid, 2TMS	1814.9	449	1812.1, 1.726	895
Hippuric acid, TMS	1947.01	649	1944.12, 2.811	953
Vanillylmandelic acid, 3TMS	2102.38	681	2096.13, 1.537	860
Pantothenic acid, 3TMS	2369.86	537	2364.15, 1.395	912
Caffeic acid, 3TMS	2702.75	533	2696.18, 1.615	865
	Ave.= 516 Unknowns		***Ave.= 877 Knowns***	

The rich comprehensive smoker's urine (SU) data was probed to identify important drug and tobacco-related classes of compounds. The characterized compounds included anti-anxiety medication, and over-the-counter medications such as ibuprofen, acetaminophen, Naproxen, and Benadryl (Figure 5). The sample also contained polyaromatic hydrocarbons, phthalate metabolites, phenols, and tobacco metabolites (Figure 6).



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 5. Contour plot and table listing drugs in SU.



Name	R.T. (s)	Similarity	Mass N (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-a-methylnaphthaleneacetic acid, 2TMS	2728.18, 1.694	897	0.01
4-Nitrophenyl-ß-D-galacturonide, 3TMS	3028.2, 0.927	736	N/A

Figure 6. Contour plot and table listing tobacco related compounds in SU.

Not surprisingly, targeted data processing (Figure 7) of NSU and SU data files demonstrated increased quantities of tobacco related compounds, such as cotinine and trans-3'-hydroxycotinine in the latter (Table 5).

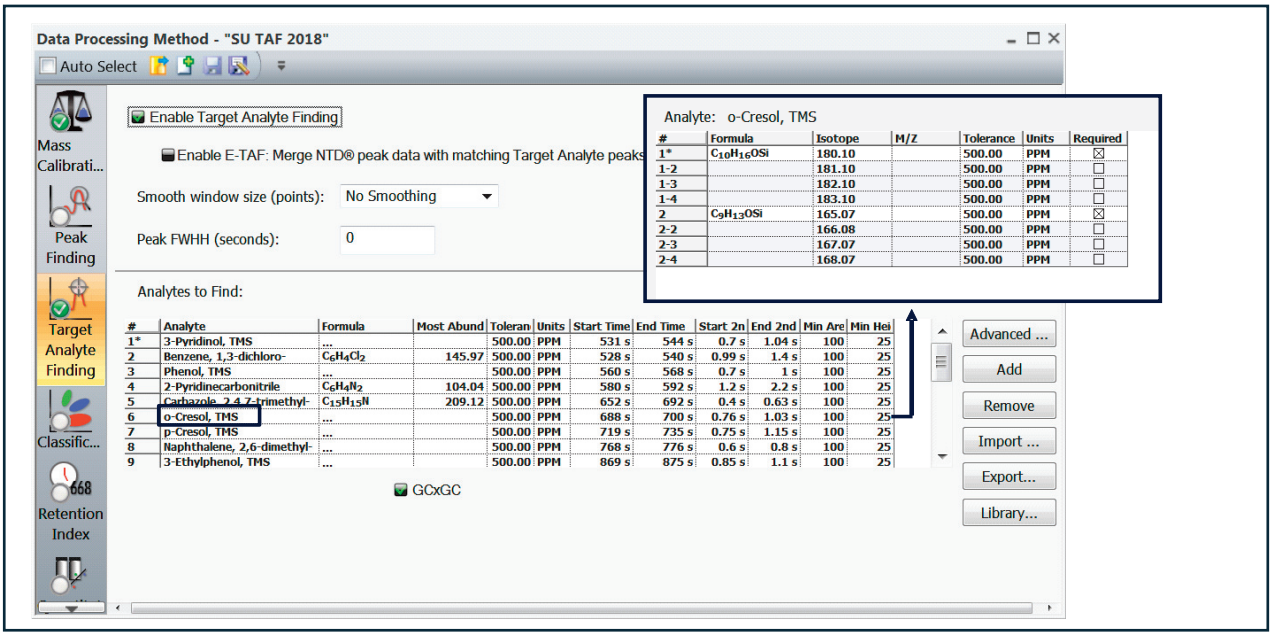


Figure 7. Target Analyte Find (TAF) processing method for rapid and robust identification of tobacco related compounds in comprehensive data files.

Table 5. TAF results for SU and NSU

Name	*R.T. (s)*	*SU Area*	*NSU Area*
3-Pyridinol, TMS	536 s, 0.868 s	371202153	310107452
Benzene, 1,3-dichloro-	536 s, 1.148 s	2650601	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, 0.702 s	569201	248416
3-Ethylphenol, TMS	872 s, 0.985 s	1525954	1013646
Catechol, 2TMS	1008 s, 0.921 s	523485419	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

Conclusion

The Pegasus BT 4D facilitated fast and confident compound identification through enhanced two-dimensional chromatographic resolution and high performance TOFMS. GCxGC-TOFMS contour plots were highly structured showing clustered classes of compounds and provided high quality spectral data that were searched against large, well-established databases. Library hits were filtered using retention index software tools and findings further supported by calculating mass delta values for molecular and fragment ions. Comprehensive data was processed via non-targeted and targeted methods. Comparison of smoker's and non-smoker's results demonstrated increased quantities of tobacco related compounds such as cotinine and trans-3'-hydroxycotinine, but also phenols, and additional nitrogen-containing compounds.

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