Toxicological Screening with the Agilent 6500 Series Accurate-Mass Q-TOF LC/MS and the Personal Compound Database and Library using the Broecker, Herre and Pragst Accurate Mass Spectral Library

Application Note

Forensic Toxicology

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Abstract

An application kit for general unknown screening in forensic toxicology has been developed for the Agilent hybrid quadrupole time-of-flight mass spectrome-ters (Q-TOF). This kit uses a database with over 7300 entries and a library of MS/MS spectra containing over 2400 entries of exact mass precursor and fragment ions. There are up to three spectra per compound collected at collision energies of 10, 20, and 40 eV in positive and/or negative ion mode. It can be quickly and easily used for both forensic and toxicological samples where the ability to detect, screen, and identi-fy a large number of toxic substances is necessary. The system allows the user to cre-ate custom databases containing retention times for compounds of interest. Screening with this database and library therefore, provides detection with identifica-tion using accurate mass MS/MS. A test mix is provided to demonstrate the function-ality of the kit. Examples of general methods for the determination of the test mix compounds in both blood and urine are given.

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Introduction

The screening of compounds that are of forensic toxicological concern in biological fluids is difficult not only because of the complex matrix but also the magnitude of the number of compounds sought at a wide range of concentrations of concern. For example, in the situation of an unconscious victim of apparent poisoning or overdose a "general unknown screening" or "systematic toxicological analysis" must be performed. This is the identification or exclusion of toxic compounds in the sample without any specific information. Such screening using accurate mass measurement and a large database containing compounds of toxicological and forensic concern is possible with the Agilent Forensic Toxicology Personal Compound Database [1].

Screening with the database, the analyst can detect and identify any compound ionized in the LC/MS system with a time of flight (TOF) or quadrupole time of flight (Q-TOF). Identification is based on the comparison of the exact value of the compound's neutral mono-isotopic mass with the presence of the measured mass calculated from ionic *m/z* values of all detected specified adducts within, typically, a 1 to 3 ppm mass error. In addition, the identification relies on both spacing and relative abundance of the isotopes detected. This identification only provides the molecular formula of the compound and does not distinguish isomers in the database or interfering matrix compounds. Therefore, the identification is tentative. Because there are large numbers of structural and steric isomers to a molecular formula, false positives are of continued concern and make the screening process difficult.

The addition of MS/MS spectra to the database makes it a Personal Compound Database and Library and allows comparison of positive results from the database search with the spectra in the library. It is important to note that screening with the database gives no indication that compounds not detected could be ionized and detected. In contrast, the library contains compounds that have been ionized and fragmented, so the ability to exclude compounds not detected with the library is reasonable within the confines of the experience of the analyst. Using a library of spectra in LC/MS for identification is not new [2-5]. However, the ability to obtain meaningful fragment ions indicative of a compound's structure is highly dependent on the collision energy of the collision induced dissociation (CID) process. Some compounds provide excellent structural information at low CID energy while producing only low mass ions at higher energy, whereas others provide the opposite. The collection of spectra at different energies is used to overcome this deficiency [6]. This library contains important toxicologically relevant substances kept in the laboratory of Professor Dr. Pragst and Dr. Herre. It constitutes a subset, containing all compounds that can be ionized, of those used to create an extensive UV spectral library of therapeutic and illicit drugs, pesticides, alkaloids, toxic reagents and other poisons [7-9].

Each compound's MS/MS spectrum was collected by flow injection on the Agilent 6530 Accurate-Mass Q-TOF LC/MS by S. Broecker at three collision energies of 10, 20 and 40 eV. This provides an accurate mass library to routinely match fragment ions in real samples with the expected ions for the proposed compound identification. Therefore, it can weight the presence of the ion and its measured accurate mass along with relative abundances or ion ratio's, and support the use of both a general setting for collision energy and the specific energies used in the library.

Experimental

Reagents and Chemicals

The LC/MS Toxicology Test Mix was obtained from Ultra Scientific (Agilent p/n 5190-0470). See Appendix I for a complete list of the compounds in the mixture. Each compound was at a concentration of 1 µg/mL (1 ppm). The highest purity mobile phases were used for trace analysis. Honeywell B & J LC/MS grade acetonitrile and methanol were used here. Buffers were prepared from the highest quality chemicals such as GFS doubly distilled acetic acid, formic acid and ammonium hydroxide. If solid ammonium acetate and ammonium formate is used, it should be prepared in a concentrated solution and particulates removed with 0.2-µm filters.

All data were processed with MassHunter Qualitative Analysis 3.01 with Service Pack 3.

LC/MS methods are given in the Appendices.

Sample Preparation

The test mix in solvent was prepared by dilution with methanol to the specified concentrations. Blood and urine were spiked to the concentrations of 0.5, 2, 5, 20, 50, 200, and 500 ng/mL. Morphine D_3 was added to each sample only to ensure that the retention time (RT) for early elution was stable (slight pH variations can change morphine from approximately 4 min to 3 min or less).

Urine samples

A urine sample was spiked to a final concentration of 200 ng/mL of each compound in the test mix. A 100 µL aliquot of each sample was added to 2 mL autosampler vials. The samples were each diluted with 400 µL of 10 mM ammonium acetate (NH_4Ac), pH 6.8, in water. The one to five dilution was then injected directly onto the LC/MS system.

Blood Samples

A sample preparation that would contain more matrix compounds (but would not exclude more polar compounds from the screen) used protein precipitation. Blood samples were spiked to a final concentration of 20 ng/mL and 200 ng/mL each of the test mix to whole blood. A 400-µL amount of acetonitrile was added to 100 µL of blood, and vortexed for 1 min. The samples were then centrifuged for 5 min at 13500 rpm. A 400 µL aliquot of the extract was taken to a vial and evaporated to dryness. The sample was then reconstituted in 80 µL of 35% ACN/ 65% of 0.1% formic acid (v/v).

LC/MS methods are given in the Appendices.

Appendix II gives the LC/MS method for single MS using the search feature to screen for compounds in the database

Appendix III gives the LC/MS method for targeted MS/MS using the search feature to identify compounds found in the screen by library search.

Appendix IV gives the LC/MS method for auto MS/MS using the search feature to find compounds by database and library searching.

Results and Discussion

The analysis of toxic compounds, including drugs of abuse for both prosecutable forensic and accidental cases, entails the determination of a seemingly endless number of chemical substances. The basic tenant of toxicology is "any substance is toxic at the right dose." However, experienced analysts and toxicologists have determined a significant number of compounds that would be of concern for this endeavor. The database and spectral library described here captures many of those compounds and eventually more will be added. However, there can be different constructs for analyzing samples for this large database, interrogating the data to determine the molecular formula, and identifying the structure by comparison to the accurate mass spectral library.

Two workflows are offered here with the benefits and disadvantages of each. The first workflow involves analysis using the LC/MS Q-TOF in single MS mode and performing a database search for possible compounds of concern. The resulting positive list is then examined for quality of match and those compounds that appear as possibly present are exported to a comma separated value (csv) file. With simple manipulation the list is formatted for import into a "Targeted MS/MS" list. The sample is then rerun under targeted MS/MS conditions and the resulting MS/MS spectra used to search the library for identification.

The second workflow uses Agilent's Auto MS/MS capability and provides both single MS and MS/MS in a single run. This approach, for a number of reasons, is the preferred process. However, there are some disadvantages that may require some analyses to be performed by the targeted MS/MS approach. The analyst must decide, using the specific conditions of the application, which procedure, or combination, would be "fit for purpose."

Targeted MS/MS Workflow

In the targeted MS/MS workflow, a sample of urine (for example, to perform a drug screen) is prepared by simple dilution and analyzed with the LC/MS Q-TOF in single MS mode only. This analysis can be very sensitive because the duty cycle of the instrument concentrates on only one task, collecting full spectra, single MS with more transients per spectrum. Reference ions are introduced to the system simultaneously and mass accuracy for real samples are usually better than 3 ppm. The purpose of this analysis is to detect the quasi-molecular ion in whatever adduct formation the analyst expects possible. MassHunter Qualitative Analysis software determines the mass of the neutral molecule by back calculating the adducts detected. It then compares the mass to the exact masses of the neutral molecules in the database. The exception is where an ion, as a quaternary salt, M+• or an anion M-•, is formed without an adduct. MassHunter handles this by specifying "include cations or anions." Figure 1 shows

the total ion chromatogram of a single MS LC/MS Q-TOF analysis of urine spiked at 200 ng/mL of the forensic toxicology test mix and prepared as described in the Experimental section. To find compounds in this data set, the user can choose the "Molecular Feature Extraction (MFE)." This is covered in the Quick Start Guide for using the database [10].

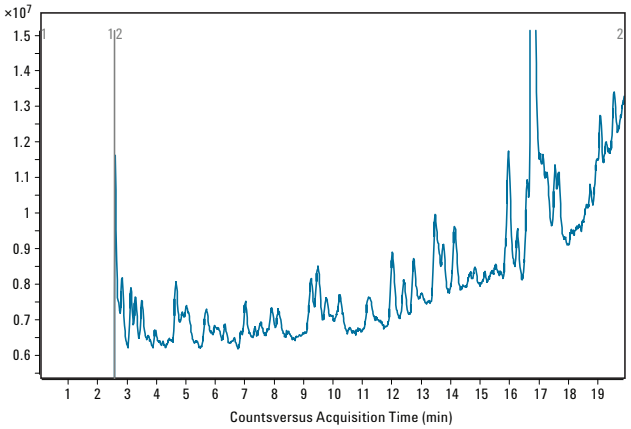


Figure 1. Single MS of urine sample diluted 1:5 with 10 mM ammonium acetate in water.

If searching the entire database, it is much faster to use MFE. It is important to review the example data before making MFE settings and then use appropriate settings in the "Extraction" tab of MFE so that unwanted background ions or chemical noise are not included in the results. For these data, we use "peaks with heights above 5,000 counts." In the compounds filter tab, the relative height is set to 2.5% and the absolute height to 25,000 counts. In addition, only the H+ adduct is sought and no other filtering is used. Once the compounds are found, they are searched against the database using the criteria of mass and 5 ppm as the mass accuracy tolerance. Note that although we typically obtain better than 3 ppm mass accuracy, this is a relative measure. The lower the mass of a molecule, such as amphetamine, the more likely the error in ppm will be higher. It is important to remember that with lower masses, even with a higher ppm error, the number of possible hits and molecular formulas are also reduced. The data can be evaluated directly from MassHunter Qualitative Analysis and the "Compound List" provides a host of information. In addition, the quality of the match of both the database and library search results can be shown. However, many analysts will want to automate data analysis by creating a method with "Worklist Automation Steps." This is outlined in the library Quick Start Guide.[11] A printed report with automation using the "Compound Report" and the report template "CompoundReportWithIdentification Hits.xltx"are provided. The results of the database search are given in Table 1. It is important to note that with a large database, isomers and closely related isobaric compounds are present. All the compounds spiked into the sample were detected. The summary results given in Table 1 show only the first entry in the database. Note the last column (DB Hits) shows multiple hits for many entries. Table 2 shows details (also given in the full report) for two positives and each has a number of isomers; eight hits for one and seven hits for the other. In this example, the first is actually meperidine and the second is proadifen. These are distinguished with the library search. However, depending on what type of isomer is present their spectra may or may not make a distinction. Typically, diasteriomers are not discriminated by their spectra. However, there is a slight difference in the spectra of codeine and hydrocodone and they are distinguished by the library seach results. Note that neither of these compounds are listed in Table 1 but metopon is listed twice (an isomer of codeine and hydrocodone). Examination of the detected compounds in the database screen shows strychnine. However, bufezolac, an isomer of strychnine could have been listed. This shows the importance of the library and the need to search spectra in a large database of compounds. Regardless of the isomer shown in the table, so long as the protonated adduct of that mass is included in the targeted MS/MS analysis as a precursor, the spectrum will be collected and the library search will identify the correct compound.

Table 1. Compound Report for Single MS Screen with Find Compounds using Molecular Feature Extraction (Filtered with Database) and Identify Compounds with Database Search.

Compound Label	RT	Mass	Name	DB formula	Tgt mass	Diff (ppm)	DB formula	DB diff (ppm)	Hits (DB)
Cpd 1: Adefovir	3.125	273.0621	Adefovir	$C_8H_{12}N_5O_4P$	273.0627	-2.04	$C_8H_{12}N_5O_4P$	2.04	1
Cpd 2: Filenadol	3.15	265.1321	Filenadol	$C_{14}H_{19}NO_4$	265.1314	2.71	$C_{14}H_{19}NO_4$	-2.71	2
Cpd 3: 5,5-Dipropylbarbituric acid	3.19	212.116	5,5-dipropylbarbituric acid	$C_{10}H_{16}N_2O_3$	212.1161	-0.25	$C_{10}H_{16}N_2O_3$	0.25	8
Cpd 4: Theophylline	3.292	180.0655	Theophyline	$C_7H_8N_4O_2$	180.0647	4.1	$C_7H_8N_4O_2$	-4.1	2
Cpd 5: Pseudoecgonine	3.518	185.1058	Pseudoecgonine	$C_9H_{15}NO_3$	185.1052	3.08	$C_9H_{15}NO_3$	-3.08	3
Cpd 6: Bendazac-	3.667	282.0993	Bendazac-	$C_{16}H_{14}N_2O_3$	282.1004	-4.18	$C_{16}H_{14}N_2O_3$	4.18	2
Cpd 8: N-Methyl-1-phenylethylamine	4.423	135.1054	N-Methyl-1-phenythethylamine	$C_9H_{13}N$	135.1048	4.44	$C_9H_{13}N$	-4.44	5
Cpd 9: Enprofylline	4.656	194.0811	Enprofyline	$C_8H_{10}N_4O_2$	194.0804	3.87	$C_8H_{10}N_4O_2$	-3.87	2
Cpd 10: Safrole	4.724	162.0687	Safrole	$C_{10}H_{10}O_2$	162.0681	3.74	$C_{10}H_{10}O_2$	-3.74	1
Cpd 11: 3,4-Methylendioxyamphetamine (MDA)	4.729	179.0951	3,4-Methylendioxy-amphetamin (MDA)	$C_{10}H_{13}NO_2$	179.0946	2.82	$C_{10}H_{13}NO_2$	-2.82	10
Cpd 12: Ortetamine	4.914	149.121	Ortetamine	$C_{10}H_{15}N$	149.1204	3.91	$C_{10}H_{15}N$	-3.91	7
Cpd 13: Ethyldibunate	5.026	348.1761	Ethyldibunate	$C_{20}H_{28}O_3S$	348.1759	0.44	$C_{20}H_{28}O_3S$	-0.44	1
Cpd 14: Safrole	5.037	162.0685	Safrole	$C_{10}H_{10}O_2$	162.0681	2.41	$C_{10}H_{10}O_2$	-2.41	1
Cpd 15: Methylendioxymeth-amphetamine (MDMA)	5.047	193.111	Methylendioxy-methamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.1103	3.73	$C_{11}H_{15}NO_2$	-3.73	12
Cpd 16: Safrole	5.679	162.0685	Safrole	$C_{10}H_{10}O_2$	162.0681	2.89	$C_{10}H_{10}O_2$	-2.89	1
Cpd 17: Pentalamide	5.681	207.1266	Pentalamide	$C_{12}H_{17}NO_2$	207.1259	3.41	$C_{12}H_{17}NO_2$	-3.41	9
Cpd 18: Dicyclopentadiene	5.885	132.0937	Dicyclopentadiene	$C_{10}H_{12}$	132.0939	-1.23	$C_{10}H_{12}$	1.23	1
Cpd 19: Ortetamine	5.887	149.1209	Ortetamine	$C_{10}H_{15}N$	149.1204	2.9	$C_{10}H_{15}N$	-2.9	7
Cpd 20: Oxycodone	6.014	315.1476	Oxycodone	$C_{18}H_{21}NO_4$	315.1471	1.64	$C_{18}H_{21}NO_4$	-1.64	3
Cpd 21: Ufenamate	6.014	337.1296	Ufenamate	$C_{18}H_{18}F_3NO_2$	337.129	1.9	$C_{18}H_{18}F_3NO_2$	-1.9	2
Cpd 22: Netilmicin	6.875	475.2994	Netilmicin	$C_{21}H_{41}N_5O_7$	475.3006	-2.48	$C_{21}H_{41}N_5O_7$	2.48	1
Cpd 23: 6-monoacetyl-morphine	6.975	327.1476	6-monoacetyl morphine	$C_{19}H_{21}NO_4$	327.1471	1.65	$C_{19}H_{21}NO_4$	-1.65	6
Cpd 24: Metopon	7.037	299.1527	Metopon	$C_{18}H_{21}NO_3$	299.1521	1.97	$C_{18}H_{21}NO_3$	-1.97	6
Cpd 25: Metopon	7.549	299.1528	Metopon	$C_{18}H_{21}NO_3$	299.1521	2.05	$C_{18}H_{21}NO_3$	-2.05	6
Cpd 26: Strychnine	8.16	334.1686	Strychnine	$C_{21}H_{22}N_2O_2$	334.1681	1.52	$C_{21}H_{22}N_2O_2$	-1.52	2
Cpd 27: Cetraxate	8.486	305.1632	Cetraxate	$C_{17}H_{23}NO_4$	305.1627	1.52	$C_{17}H_{23}NO_4$	-1.52	2
Cpd 28: Dioxamate	9.056	287.2096	Dioxamate	$C_{15}H_{29}NO_4$	287.2097	-0.29	$C_{15}H_{29}NO_4$	0.29	1
Cpd 29: Nadolol	9.062	309.1945	Nadolol	$C_{17}H_{27}NO_4$	309.194	1.74	$C_{17}H_{27}NO_4$	-1.74	2
Cpd 30: Trocimine	9.209	307.1791	Trocimine	$C_{17}H_{25}NO_4$	307.1784	2.39	$C_{17}H_{25}NO_4$	-2.39	3
Cpd 31: Pseudococaine	9.283	303.1475	Pseudococaine	$C_{17}H_{21}NO_4$	303.1471	1.53	$C_{17}H_{21}NO_4$	-1.53	7
Cpd 32: Nadolol	9.397	309.1945	Nadolol	$C_{17}H_{27}NO_4$	309.194	1.75	$C_{17}H_{27}NO_4$	-1.75	2
Cpd 33: Prodilidine	9.786	247.1581	Prodilidine	$C_{15}H_{21}NO_2$	247.1572	3.4	$C_{15}H_{21}NO_2$	-3.4	10
Cpd 34: Nadolol	10.077	309.195	Nadolol	$C_{17}H_{27}NO_4$	309.194	3.35	$C_{17}H_{27}NO_4$	-3.35	2
Cpd 35: Phencyclidine (PCP)	10.183	243.1992	Phencyclidine (PCP)	$C_{17}H_{25}N$	243.1987	2.24	$C_{17}H_{25}N$	-2.24	1
Cpd 36: Heroin	10.256	369.158	Heroin	$C_{21}H_{23}NO_5$	369.1576	1.07	$C_{21}H_{23}NO_5$	-1.07	5
Cpd 38: Nitrazepam	11.081	281.0808	Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08	2.64	$C_{15}H_{11}N_3O_3$	-2.64	3
Cpd 40: Alprazolam	12.005	308.0823	Alprazolam	$C_{17}H_{13}ClN_4$	308.0829	-1.98	$C_{17}H_{13}ClN_4$	1.98	1
Cpd 41: Coroxon	12.005	346.0376	Coroxon	$C_{14}H_{16}ClO_6P$	346.0373	0.83	$C_{14}H_{16}ClO_6P$	-0.83	2
Cpd 42: Tolnidamine	12.412	300.067	Tolnidamine	$C_{16}H_{13}ClN_2O_2$	300.0666	1.47	$C_{16}H_{13}ClN_2O_2$	-1.47	5
Cpd 43: Levomethadone	12.749	309.21	Levomethadone	$C_{21}H_{27}NO$	309.2093	2.4	$C_{21}H_{27}NO$	-2.4	5
Cpd 44: Mazindol	13.471	284.0721	Mazindol	$C_{16}H_{13}ClN_2O$	284.0716	1.51	$C_{16}H_{13}ClN_2O$	-1.51	4
Cpd 45: Proheptazine	13.483	275.1891	Proheptazine	$C_{17}H_{25}NO_2$	275.1885	2.15	$C_{17}H_{25}NO_2$	-2.15	6
Cpd 47: Verapamil	13.759	454.2831	Verapamil	$C_{27}H_{38}N_2O_4$	454.2832	-0.04	$C_{27}H_{38}N_2O_4$	0.04	5
Cpd 49: Eprazinone	15.977	380.245	Eprazinone	$C_{24}H_{32}N_2O_2$	380.2464	-3.76	$C_{24}H_{32}N_2O_2$	3.76	1
Cpd 50: Tributylphosphate	16.528	266.1652	Tributylphosphate	$C_{12}H_{27}O_4P$	266.1647	2	$C_{12}H_{27}O_4P$	-2	3
Cpd 51: Linolenic acid	16.63	278.2237	Linolenic acid	$C_{18}H_{30}O_2$	278.2246	-3.06	$C_{18}H_{30}O_2$	3.06	2
Cpd 52: Linolenic acid	16.936	278.2241	Linolenic acid	$C_{18}H_{30}O_2$	278.2246	-1.59	$C_{18}H_{30}O_2$	1.59	2
Cpd 53: Picrolichenic acid	16.958	442.198	Picrolichenic acid	$C_{25}H_{30}O_7$	442.1992	-2.55	$C_{25}H_{30}O_7$	2.55	1
Cpd 54: Amicetin	17.315	618.3027	Amicetin	$C_{29}H_{42}N_6O_9$	618.3013	2.21	$C_{29}H_{42}N_6O_9$	-2.21	1
Cpd 55: Motretinide	17.554	353.2355	Motretinide	$C_{23}H_{31}NO_2$	353.2355	0.05	$C_{23}H_{31}NO_2$	-0.05	7
Cpd 56: Pyrethrin I	17.828	328.2039	Pyrethrin I	$C_{21}H_{28}O_3$	328.2038	0.1	$C_{21}H_{28}O_3$	-0.1	3
Cpd 57: Cinitapride	17.916	402.2258	Cinitapride	$C_{21}H_{30}N_4O_4$	402.2267	-2.15	$C_{21}H_{30}N_4O_4$	2.15	1
Cpd 58: Cinchophen	18.755	249.0789	Cinchophen	$C_{16}H_{11}NO_2$	249.079	-0.13	$C_{16}H_{11}NO_2$	0.13	2
Cpd 59: Doxapram	18.755	378.2309	Doxapram	$C_{24}H_{30}N_2O_2$	378.2307	0.45	$C_{24}H_{30}N_2O_2$	-0.45	4
Cpd 60: delta9-Tetrahydrocannabinol (THC)	19.14	314.2244	delta9-Tetrahydro-cannabinol (THC)	$C_{21}H_{30}O_2$	314.2246	-0.47	$C_{21}H_{30}O_2$	0.47	4
Cpd 61: Palmitamide	19.585	255.2565	Palmitamide	$C_{16}H_{33}NO$	255.2562	1.2	$C_{16}H_{33}NO$	-1.2	1
Cpd 62: Oleamide	19.738	281.2718	Oleamide	$C_{18}H_{35}NO$	281.2719	-0.09	$C_{18}H_{35}NO$	0.09	3

Table 2. Details of each Result in the Compound Report Where A) is the Detailed Result for the Match of Ciclafrine, Actual Isomer Present is Meperidine and B) is the Match for Alphacetylmethadol, Actual Isomer Present is Proadefin

A – Database Search Results

Compound	Hits
Ciclafrine	8

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Ciclafrine	TRUE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Ciclonicate	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
beta-Eucaine	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Cetobemidone	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Prodilidine	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Tolpronine	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Indenolol	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786
Meperidine (Pethidine)	FALSE	$C_{15}H_{21}NO_2$	247.1581	247.1572	-3.4	9.786

B – Database Search Results

Compound	Hits
Alphacetylmethadol	7

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Alphacetylmethadol	TRUE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Acetylmethadol	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Acetylmethadol	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Betacetylmethadol	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Levomoramide	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Proadifen	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554
Motretinide	FALSE	$C_{23}H_{31}NO_2$	353.2355	353.2355	-0.05	17.554

These results can be saved in an Excel spreadsheet, reviewed, and put in a form where the target *m/z* values and retention times can be imported directly into MassHunter Q-TOF acquisition for targeted MS/MS acquisition. The Quick Start Guide for the database and library described how this is done [11]. A review of the database search results will help determine what compounds are of concern, and what are not. For example, in a drug screen caffeine or nicotine should not be of concern. However, in a poisoning, a very large peak of the same compound might be cause to include it in the targeted MS/MS analysis. A problem with this approach is that the Q-TOF will not be able to handle the duty cycle for too many false positives with close retention times. False positives are typical of low level ions in complex matrices. Duty cycle is dependent on the number of single MS scans per second, the number of MS/MS scans per second and the number of compounds that overlap or co-elute in retention time. A long cycle time may cause peaks to be missed and sensitivity is impacted by the number of scans per second. As shown in Appendix II, the targeted list of *m/z* values have reasonable overlap of retention times. In addition, each compound is measured at the same collision energies used in the library, (10, 20 and 40 eV), which also increases the duty cycle and makes it difficult to detect more compounds. However, this approach (using the same energies found in the library) provides much better matches with the library spectra.

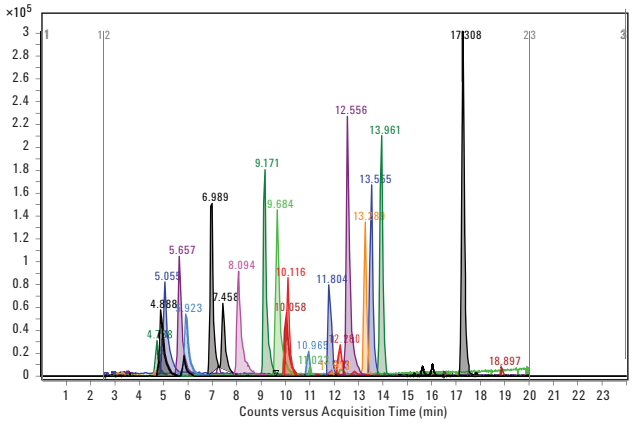


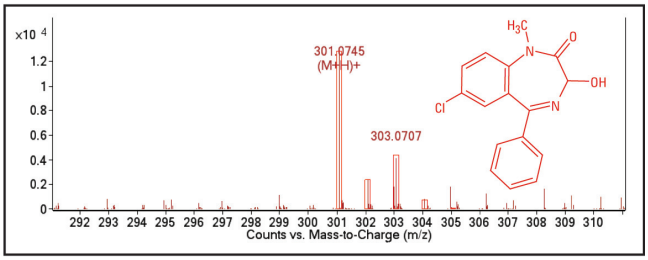
Figure 2. Extracted ion chromatogram of the urine sample for the compounds in the test mix.

Figure 2 shows the extracted ion chromatograms (EIC) of the urine spike for all the compounds in the test mix run with the targeted MS/MS acquisition. The data was processed using the "Find Compounds" function by "Find by Targeted MS/MS" to obtain each compound's MS/MS spectrum. The compound list was then processed using the "Identify Compounds" function by "Search Accurate Mass Library." In addition, single MS spectra are collected with the targeted MS/MS. It is these spectra that contain the reference ions

used to correct for drift and obtain good mass accuracy in the MS/MS spectra. Again using the same report template the information shown in Figure 3 for temazepam shows the results obtained with the single MS. These results include the graphic isotope comparison with the "best" formula and a table with the same. Figure 4 shows the MS/MS spectra given in the report with the search scores for each spectrum. The scores are the average of both forward and reverse scores if both were selected in the search criteria. Figure 5 shows the spectral difference comparison for the temazepam spectrum in the sample collected at 20 eV as compared to the same collision energy of the library spectrum. Note that less intense ions are missing in the sample spectrum probably due to the duty cycle required to collect all energies. In contrast, if there were more ions in the sample spectrum than that of the library, there may be a co-eluting isobaric compound.

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 66: Temazepam	Temazepam	301.0745	12.387	Targeted MS/MS	300.0672

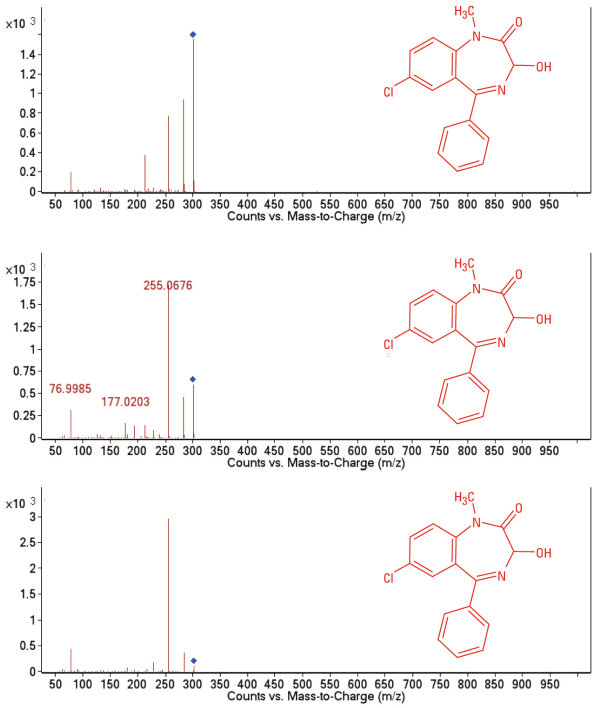
MS Zoomed Spectrum



Predicted Isotope Match Table

Isotope	*m/z*	Calc *m/z*	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	301.0745	301.0738	-2.07	100	100	63.03	62.91
2	302.0764	302.077	1.73	18.2	18.27	11.47	11.5
3	303.0707	303.0714	2.25	32.65	33.98	20.58	21.38
4	304.0767	304.0742	-8.12	6.31	6.01	3.98	3.78
5	305.0801	305.0768	-10.84	1.22	0.65	0.77	0.41
6	306.0876	306.0794	-26.78	0.27	0.05	0.17	0.03

Figure 3. Database search results of urine sample from single MS analysis for temazepam. Note the excellent mass accuracy for the A+1 and A+2 isotopes. The higher level isotopes are very weak signals and it would not be expected to obtain high mass accuracy for these very low abundance ions.



Library Search Results

Best	CAS	Match Score	Weight (Library)	Formula
FALSE	846 - 50 - 4	86.6	300.1	$C_{16}H_{13}ClN_2O_2$
TRUE	846 - 50 - 4	89.3	300.1	$C_{16}H_{13}ClN_2O_2$
FALSE	846 - 50 - 4	78.2	300.1	$C_{16}H_{13}ClN_2O_2$

Figure 4. Library search results of urine sample for temazepam. The top spectrum was collected at 10 eV, the middle at 20 eV and the bottom at 40 eV. The table shows the match score for the spectra at each collision energy and simply labels the "best" as the one with the highest score.

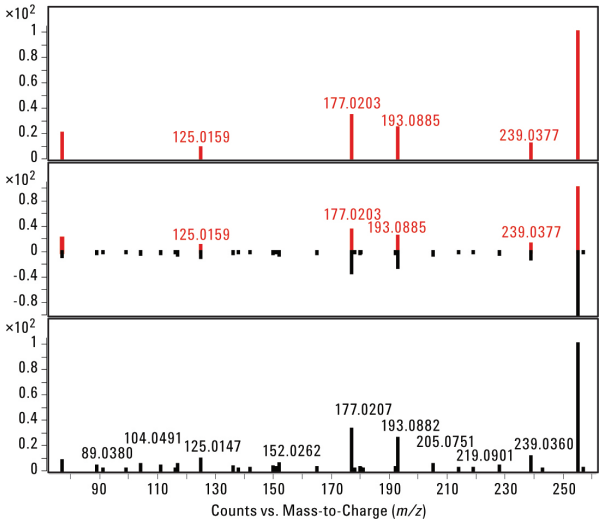


Figure 5. Spectral comparison of library versus urine sample of tamezepam. The top spectrum is of the sample, the bottom spectrum is of temazepam in the library, and the middle shows the mirrored difference of the two. Both the library and sample spectra were collected at 20 eV.

Blood samples were prepared at 20 ng/mL and 200 ng/mL with a protein precipitation sample preparation. At the 20 ng/mL level not all compounds in the test mix were detected using molecular feature extraction and the database search. Using "Find by Formula", more of the compounds in the test mix could be found. However, this approach is very slow and uses a much reduced database of suspected compounds. This is reasonable if the most common drugs of abuse are used to create a custom database. In addition, matrix compounds are a significant part of the TIC of a single MS analysis of the spike, as shown in Figure 6. Figure 7 shows the extracted ion chromatograms of some of the test mix compounds detected in this sample. The spectrum in Figure 8 is that of the peak for codeine and shows excellent mass accuracy. A Find by Formula search result (from the compound list in MassHunter Qualitative Analysis) is shown in Figure 9. The identification of THC is incorrect because we know the retention time is 19.1 min. The Find by Formula algorithm did find a matching adduct ion as shown in Figure 10 and what appears to be a peak at the retention time specified. If there was not a standard available and this was submitted for targeted MS/MS analysis the false positive would be confirmed as not present. In contrast, compounds that are detected are confirmed by targeted MS/MS. Figure 11 shows the identification of codeine by library search with the correct retention time that is in the targeted MS/MS window. The comparison of the spectrum from the sample and the library is given in Figure 12. Note that not all low level ions are present but there are enough to make a good match. This is significant as both codeine and hydrocodone are isomers and their spectra are similar but different enough for the library to distinguish them.

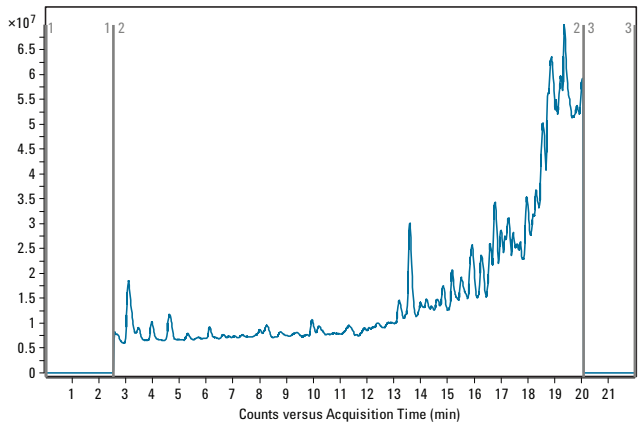


Figure 6. TIC of blood sample.

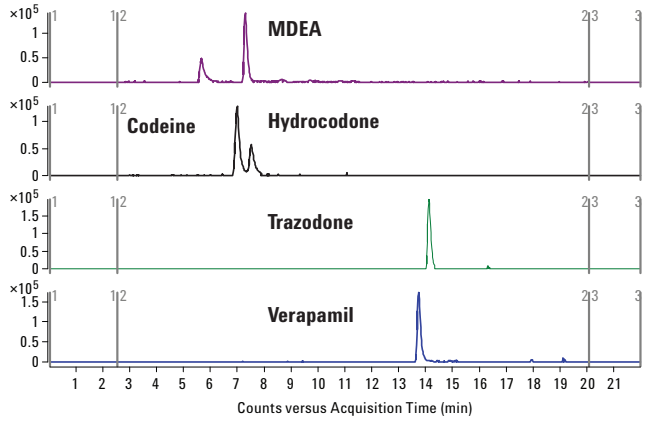


Figure 7. Extracted ion chromatograms (20 ppm extraction window) of blood sample spiked at 20 ng/mL.

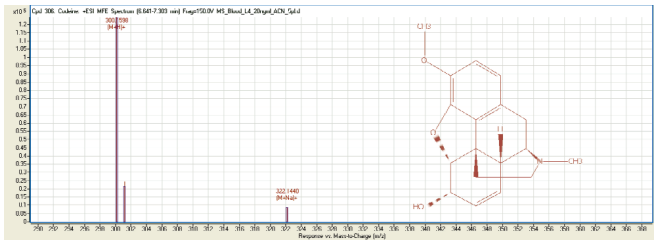


Figure 8. Single MS spectrum of codeine, find compound by Molecular Feature and identify by database search.

Show/Hide	Cpd	Name	File	RT	Base Peak	Mass (Tgt)	Diff (Tgt, ppm)
☑	42...	Codeine	MS_Blood_L4_2...	6.988	295.2267	299.1521	2.32
☑	42...	Hydrocodone	MS_Blood_L4_2...	6.988	295.2267	299.1521	2.32
☑	42...	Temazepam	MS_Blood_L4_2...	12.397	309.2839	300.0666	0.8
☑	42...	Cocaine	MS_Blood_L4_2...	9.271	311.3186	303.1471	3.46
☑	42...	Alprazolam	MS_Blood_L4_2...	11.983	233.2084	308.0829	0.93
☑	42...	Methadone	MS_Blood_L4_2...	11.255	327.2981	309.2093	-3.71
☑	42...	delta9-Tetrahydrocannabinol (THC)	MS_Blood_L4_2...	10.561	219.2076	314.2246	1.48

Figure 9. Result of Find by Formula where THC is misidentified with the wrong retention time.

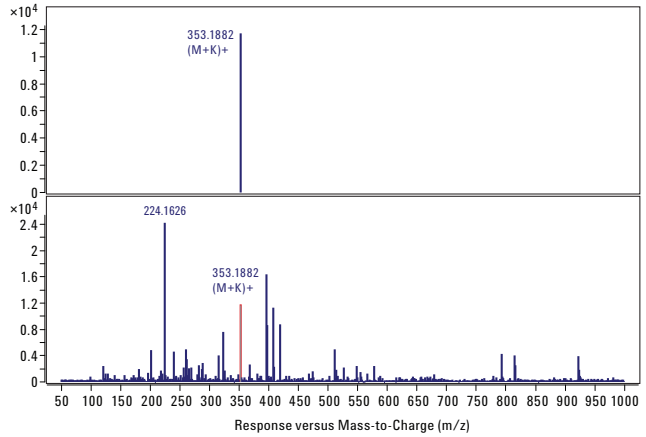


Figure 10. Extracted compound spectrum of incorrectly identified THC (above) with full spectrum (below). Note that no isotopes are present and a targeted MS/MS of a potassium adduct would not produce a meaningful spectrum. The resulting spectrum of this precursor ion would show that it is not THC.

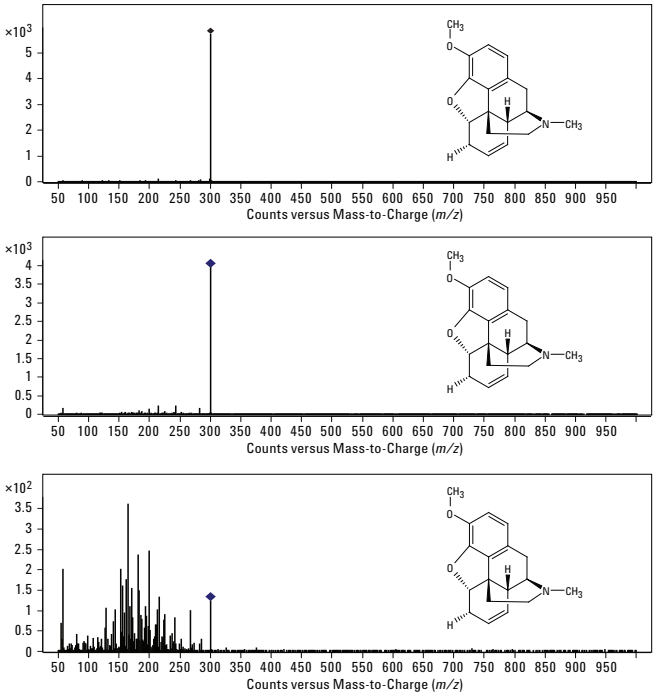
Table 3. Compound Report for Auto MS/MS of Urine Sample

Compound Table

Compound Label	RT	Name	DB Formula	Hits (DB)
Cpd 36: Diaveridin	3.273	Diaveridin	$C_{13}H_{16}N_4O_2$	1
Cpd 43: Theophylline	3.426	Theophylline	$C_7H_8N_4O_2$	1
Cpd 80: Amphetamine	4.48	Amphetamine	$C_9H_{13}N$	2
Cpd 84: Caffeine	4.584	Caffeine	$C_8H_{10}N_4O_2$	2
Cpd 90: 3,4-Methylenedioxyamphetamine (MDA)	4.759	3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	2
Cpd 98: Caffeine	4.951	Caffeine	$C_8H_{10}N_4O_2$	1
Cpd 104: Methamphetamine	5.08	Methamphetamine	$C_{10}H_{15}N$	3
Cpd 110: Naphazoline	5.421	Naphazoline	$C_{14}H_{14}N_2$	2
Cpd 119: 3,4-Methylenedioxyethamphetamine (MDEA)	5.772	3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	2
Cpd 123: Methamphetamine	5.915	Methamphetamine	$C_{10}H_{15}N$	4
Cpd 124: Oxycodone	6.01	Oxycodone	$C_{18}H_{21}NO_4$	1
Cpd 147: 6-monoacetyl-morphine	6.979	6-monoacetyl-morphine	$C_{19}H_{21}NO_4$	1
Cpd 152: Codeine	7.161	Codeine	$C_{18}H_{21}NO_3$	2
Cpd 164: Codeine	7.643	Codeine	$C_{18}H_{21}NO_3$	2
Cpd 166: Naphazoline	7.69	Naphazoline	$C_{14}H_{14}N_2$	2
Cpd 185: Vidarabin	8.674	Vidarabin	$C_{10}H_{13}N_5O_4$	2
Cpd 194: Vidarabin	9.107	Vidarabin	$C_{10}H_{13}N_5O_4$	2
Cpd 198: Cocaine	9.314	Cocaine	$C_{17}H_{21}NO_4$	1
Cpd 199: Cocaine	9.32	Cocaine	$C_{17}H_{21}NO_4$	1
Cpd 212: Mirtazapine	9.955	Mirtazapine	$C_{17}H_{19}N_3$	1
Cpd 220: Heroin	10.314	Heroin	$C_{21}H_{23}NO_5$	1
Cpd 228: Nitrazepam	11.103	Nitrazepam	$C_{15}H_{11}N_3O_3$	1
Cpd 231: Clonazepam	11.217	Clonazepam	$C_{15}H_{10}ClN_3O_3$	1
Cpd 243: Alprazolam	11.988	Alprazolam	$C_{17}H_{13}ClN_4$	1
Cpd 257: Temazepam	12.426	Temazepam	$C_{16}H_{13}ClN_2O_2$	1
Cpd 259: Benzoylprop-ethyl	12.752	Benzoylprop-ethyl	$C_{18}H_{17}Cl_2NO_3$	6
Cpd 261: Methadone	12.874	Methadone	$C_{21}H_{27}NO$	2
Cpd 272: Diazepam	13.442	Diazepam	$C_{16}H_{13}ClN_2O$	1
Cpd 300: Trazodone	14.185	Trazodone	$C_{19}H_{22}ClN_5O$	1
Cpd 334: Oxeladin	15.835	Oxeladin	$C_{20}H_{33}NO_3$	1

Show/Hide	Cpd	Name	File	RT	Base Peak
☑	10	Oxycodone	TMSMS_FbF_Bl...	5.999	241.1077
☑	17	Codeine	TMSMS_FbF_Bl...	7	165.0682
☑	18	Strychnine	TMSMS_FbF_Bl...	8.151	335.1738
☑	27	Meperidine (Pethidine)	TMSMS_FbF_Bl...	9.774	70.0648
☑	42	Nitrazepam	TMSMS_FbF_Bl...	11.074	180.0816

Figure 11. Library search result of targeted MS/MS for the spiked blood sample spiked at 20 ng/mL.



Library Search Results

Best	CAS	Match Score	Weight (Library)	Formula
TRUE	76 - 57 - 3	93.2	299.2	$C_{18}H_{21}NO_3$
FALSE	125 - 29 - 1	92.3	299.2	$C_{18}H_{21}NO_3$
FALSE	76 - 57 - 3	90.7	299.2	$C_{18}H_{21}NO_3$
FALSE	125 - 29 - 1	83.5	299.2	$C_{18}H_{21}NO_3$

Figure 12. Library search results for codeine in blood sample from targeted MS/MS.

Auto MS/MS Workflow

The Auto MS/MS workflow is not only more efficient, it also helps the MS/MS duty cycle. It is more efficient because both single MS screening and MS/MS identification are done in one run. Auto MS/MS relies on the detection of ions above a threshold that triggers the MS/MS using that ion as the precursor. Background ions and compounds in the blank can have an adverse affect on this acquisition mode. Therefore, the cleaner the system the better. Settings such as how many ions to perform MS/MS as precursor in one cycle as well as active exclusion after x number of spectra are important parameters because they determine the duty cycle in a dynamic way for this mode of operation. In addition, active exclusion before x min sets the time in which an ion is released from exclusion so that MS/MS can be performed on it again. These settings can determine whether an ion is selected or missed for MS/MS and depending on the matrix and concentration whether good quality spectra are collected (for example, near the top of the peak or near the baseline).

With a well-tuned system that has low level background ions and contaminants, appropriate settings can produce the best results. Figure 13 shows the extracted compound chromatograms of the compounds detected with “Find Compounds” by “Find by Auto MS/MS”. A compound report with the same template used for the targeted MS/MS is shown in Table 3. In this report, the first hit should be the one best matching the spectral library search. Therefore, this combines both screening and identification, reducing false positives and providing definitive information for the identification result. Figure 14 shows an example of the additional information that can be given in the compound report. Figure 15 is the direct comparison of the spectrum of MDA with the library spectrum. This approach uses both the library search and the database search so that the precursor ion can be compared to the database and the spectrum compared to the library in one analysis.

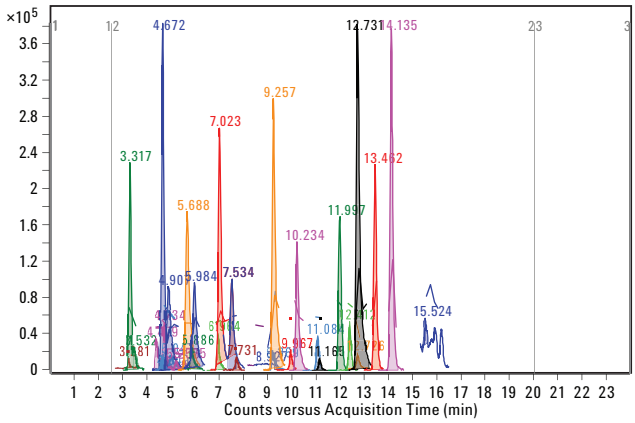
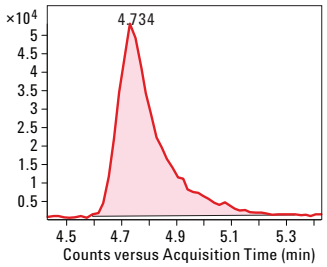


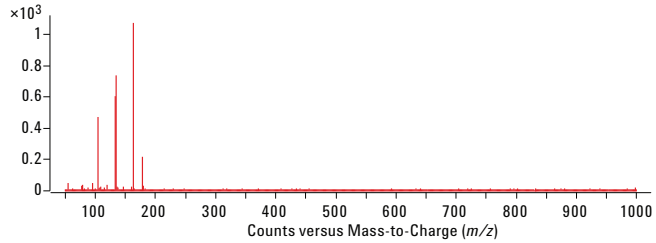
Figure 13. Extracted compound chromatograms (ECC) for the compounds detected in the urine sample by Auto MS/MS.

Compound Label	Name	*m/z*	RT	Algorithm
Cpd 90: 3,4- Methylendioxy amphetamine (MDA)	3,4- Methylendioxy amphetamine (MDA)	180.1022	4.759	Auto MS/MS

Compound Chromatogram



MSMS Spectrum



Library Search Results

Best	CAS	Match Score	Weight (Library)	Formula
TRUE	4764-17-4	79.6	179.1	$C_{10}H_{13}NO_2$
FALSE	4764-17-4	79.3	179.1	$C_{10}H_{13}NO_2$

Figure 14. Details of positively identified compound in urine sample for Auto MS/MS. The chromatogram is of the single MS spectra. Note that there are only two MS/MS spectra collected making data acquisition more efficient and thus allowing collection of more MS/MS spectra of different precursor ions.

It should be noted that the collision energy is 14.8 eV and careful examination of the Auto MS/MS method in Appendix

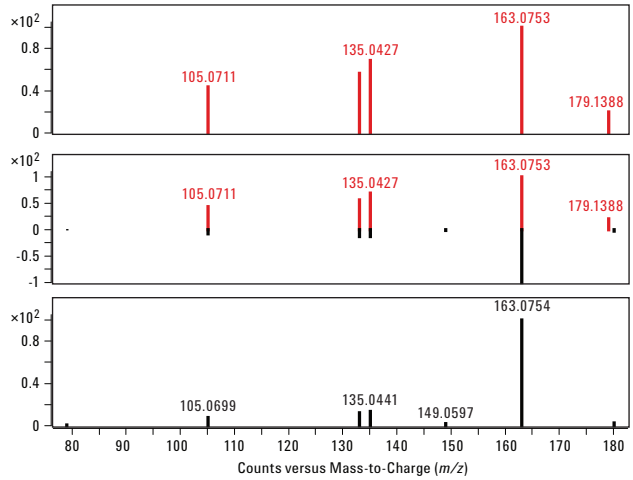


Figure 15. Spectrum from Auto MS/MS of MDA in the urine sample (top) as compared to the library spectrum (bottom). The difference is shown in the center.

III shows that the energy is set using a linear calculation based on the *m/z* of the ion. The library search compares the spectra in the library that were collected near the energy of the sample spectrum. If the determined energy does not produce significant fragments, the workflow demands that the sample be run again with targeted MS/MS at a higher collision energy. This can be optimized by quick examination of the spectra in the library for that compound. If the highest quality spectrum is obtained at 20 or 40 eV then that energy should be used for the rerun. If, as can happen for some compounds, the energy is too high providing only a few low mass fragments, the energy is reduced in the targeted MS/MS rerun. A high quality match in this analysis then constitutes a definitive identification. (Note that unambiguous identification would require a high quality match of retention time and spectra against a standard run under the same conditions).

For the Auto MS/MS workflow, the question remains whether this is a better approach for nontargeted screening and identification for the lower level blood sample without any prior knowledge of what might be in the sample. Figure 16 shows the extracted compound chromatograms only for the positives found by both the database search and the library search by Auto MS/MS. In Table 4 it is observed that some of the test mix compounds are identified. Figure 17 shows the quality of results for the database search of the Auto MS/MS of codeine. Figure 18 gives the spectrum of the sample as compared to that of the one in the library. Again the library spectrum is selected as closest in energy to the Auto MS/MS spectrum. Note that all the compounds were found by both the targeted MS/MS and Auto MS/MS procedures for a spiked blood sample at 200 ng /mL.

Table 4. Compound Report for Auto MS/MS of Blood Sample

Compound Table

Compound Label	RT	Mass	Abund	Name	MGF Formula	MFG Diff (ppm)	DB Formula	Hits (DB)
Cpd 4: Vidarabin	2.695	267.1228	6148	Vidarabin	$C_9 H_{18} F N_3 O_5$	0.97	$C_{10}H_{13}N_5O_4$	1
Cpd 35: Theophylline	3.329	180.0661	33064	Theophylline	$C_4 H_9 F N_4 O_3$	-1.51	$C_7H_8N_4O_2$	1
Cpd 91: Caffeine	4.797	194.0849	409991	Caffeine	$C_{13} H_{10} N_2$	–2.37	$C_8H_{10}N_4O_2$	2
Cpd 159: 6-monoacetyl-morphine	6.938	327.1468	9404	6-monoacetyl-morphine	$C_9 H_{19} F_2 N_7 O_4$	–0.48	$C_{19}H_{21}NO_4$	1
Cpd 161: Codeine	7.012	299.1516	19589	Codeine	$C_{18} H_{21} N O_3$	1.75	$C_{18}H_{21}NO_3$	2
Cpd 180: Hydrocodone	7.572	299.1524	10960	Hydrocodone	$C_{18} H_{21} N O_3$	–0.91	$C_{18}H_{21}NO_3$	2
Cpd 204: Strychnine	8.235	334.1686		Strychnine	$C_{18} H_{26} Cl F_3$	–3.26	$C_{21}H_{22}N_2O_2$	1
Cpd 246: Cocaine	9.304	303.1472	17740	Cocaine	$C_{17} H_{21} N O_4$	–0.54	$C_{17}H_{21}NO_4$	1
Cpd 331: Alprazolam	12.007	308.0813	28076	Alprazolam	$C_{16} H_{17} Cl O_4$	0.7	$C_{17}H_{13}ClN_4$	1
Cpd 455: Phenazocine	15.008	321.2288		Phenazocine	$C_{17} H_{30} F_3 N O$	–2.72	$C_{22}H_{27}NO$	1

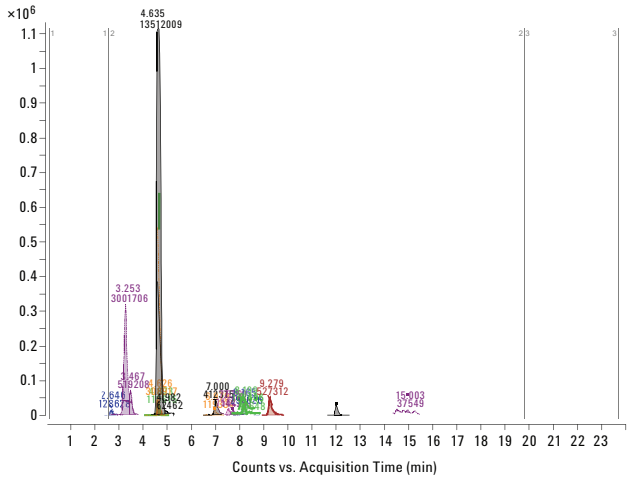


Figure 16. Extracted compound chromatogram of the low level blood sample for positive hits from the database and library.

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 161: Codeine	**Codeine**	300.1589	7.012	Auto MS/MS	299.1516

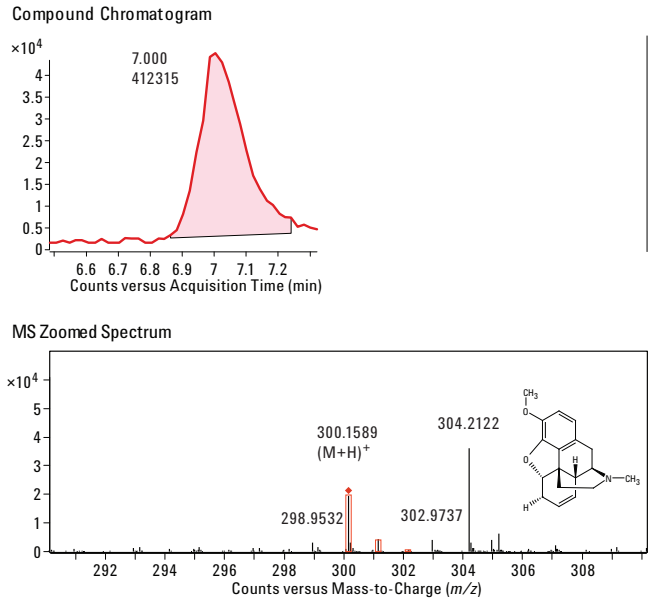


Figure 17. Auto MS/MS database results of blood sample for codeine. The database result of the precursor ion shows good mass accuracy and isotope match.

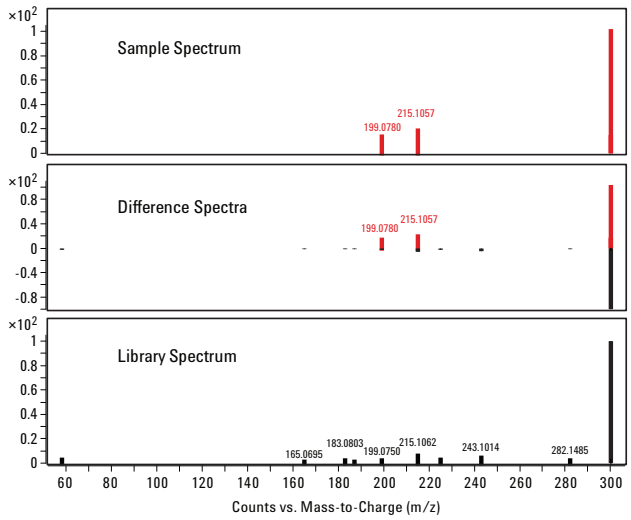


Figure 18. Auto MS/MS library comparison with blood sample spectrum (top) and library spectrum (bottom) of codeine. The difference is shown in the center panel. Again there is sufficient information in the spectrum to identify codeine and distinguish it from hydrocodone.

Conclusions

The Agilent Forensic Toxicology Accurate Mass Database has been augmented with the Broecker, Herre, and Pragst Accurate Mass Spectral Library. Urine and blood samples, spiked with the Agilent forensic toxicology test mix, shows that the database and library is a highly effective way to screen and identify thousands of compounds of concern without the need for standards. Two workflows are presented, one requiring a single MS analysis with database search followed by a targeted MS/MS of the compounds found in the screen. This workflow was shown to be comprehensive but suffers from interferences from the sample matrix especially when low levels of the compounds of interest are present.

A second workflow combining both screening and identification in one run uses the Agilent Auto MS/MS capability of the Q-TOF. This workflow requires special attention to operation and data processing settings but can be more efficient and provide more effective screening and identification especially at low levels even in the presence of heavy matrix compounds. The data presented here used MassHunter Acquisition 2.01 for the Agilent TOF and Q-TOF and MassHunter Qualitative Analysis 3.01 with Service Pack 3. In addition, the Personal Compounds Database and Library Software 3.01 was used to interrogate the database and library directly. Advances in this software and the algorithms within will make the capability of Q-TOF and database with library searching even more powerful.

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Appendix I.

Compounds in test mix at 1 µg/mL as supplied (Agilent part number 5190-0470)

Compound Name	Formula	Mass
3,4-Methylenedioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287
Amphetamine	$C_9H_{13}N$	135.10480
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107
Cocaine	$C_{17}H_{21}NO_4$	303.14706
Codeine	$C_{18}H_{21}NO_3$	299.15214
delta9-Tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458
Diazepam	$C_{16}H_{13}ClN_2O$	284.07164
Heroin	$C_{21}H_{23}NO_5$	369.15762
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723
Methadone	$C_{21}H_{27}NO$	309.20926
Methamphetamine	$C_{10}H_{15}N$	149.12045
Methylenedioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091
Oxycodone	$C_{18}H_{21}NO_4$	315.14706
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.1987
Phentermine	$C_{10}H_{15}N$	149.12045
Proadifen	$C_{23}H_{31}NO_2$	353.23548
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316

Appendix II.

LC/MS Q-TOF Conditions for Single MS Screen

Agilent 1200 Series SL LC Parameters

Column	Agilent ZORBAX Eclipse Plus C18 2.1 mm × 100 mm, 3.5 µm, p/n 959793-902
Column temperature	30 °C
Injection volume	5
Autosampler temperature	Ambient
Needle wash	5 s with methanol
Mobile phase	A = 10 mM ammonium acetate in water, pH 6.8
	B = 100% Methanol
Flow rate	0.5 mL/min
Gradient	10% B at t = 0 to 100 % B at t = 20 min, hold 1.9 min, 0.1 min return to initial conditions
Stop time	22 min
Post time	3 min

Agilent 6530 Q-TOF parameters

Time Segment #	Start Time	Diverter Valve State	Storage Mode	Ion Mode
1	0	Waste	None	ESI+Agilent Jet Stream
2	2.5	MS	Both	ESI+Agilent Jet Stream
3	20	Waste	None	ESI+Agilent Jet Stream

(Note that start time is in min, storage mode "both" indicates data is stored as centroid and profile)

Time Segment	2
Acquisition Mode	MS1
Min Range	*m/z* 50
Max Range	*m/z* 1000
Scan Rate	1 sec/scan

Source Parameters

Parameter	Value
Gas Temperature (°C)	320
Gas Flow (l/min)	8
Nebulizer (psi)	35
Sheath Gas Temp (°C)	380
Sheath Gas Flow (l/min)	11

Scan Seg #	Ion Polarity
All	Positive
Scan Segment	1

Scan Source Parameters

Parameter	Value
V_{Cap}	3000
Nozzle Voltage (V)	0
Fragmentor	150
Skimmer1	65
OctopoleRFPeak	750

Reference Masses- positive ion
121.050873 ($M+H^+$ for purine)
922.009798 ($M+H^+$ for HP-921- hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine)

Appendix III.

LC/MS Q-TOF Conditions Targeted MS/MS Identification

Agilent 1200 Series SL LC Parameters

Column	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100mm 3.5 µm, p/n 959793-902
Column temperature	30 °C
Injection volume	5
Autosampler temperature	Ambient
Needle wash	5 s with methanol
Mobile phase	A = 10 mM ammonium acetate in water, pH 6.8
	B = 100% Methanol
Flow rate	0.5 mL/min
Gradient	10% B at t = 0 to 100 % B at t = 20 min, hold 1.9 min, 0.1 min return to initial conditions
Stop time	22 min
Post time	3 min

Agilent 6530 Q-TOF parameters

Time Segment #	Start Time	Diverter Valve State	Storage Mode	Ion Mode
1	0	Waste	None	ESI+Agilent Jet Stream
2	2.5	MS	Both	ESI+Agilent Jet Stream
3	20	Waste	None	ESI+Agilent Jet Stream

(Note that start time is in min, storage mode "both" indicates data is stored as centroid and profile)

Acquisition Mode	Targeted MS2
MS/MS Min Range	50
MS Min Range	50
MS/MS Max Range	1000
MS Max Range	1000
MS/MS Scan Rate	3
MS Scan Rate	5
Max Time Between MS	0

Source Parameters

Parameter	Value
Gas Temperature (°C)	320
Gas Flow (l/min)	8
Nebulizer (psi)	35
Sheath Gas Temperature (°C)	380
Sheath Gas Flow (l/min)	11

Scan Source Parameters

Parameter	Value
V_{Cap}	3000
Nozzle Voltage (V)	0
Fragmentor	150
Skimmer1	65

Targeted MSMS Table

On	Prec. *m/z*	Z	RT (min)	Delta RT	Iso. width	Collision energy
TRUE	136.1121	1	4.403	1.5	Narrow (~1.3 *m/z*)	10
TRUE	150.1277	1	4.899	1.5	Narrow (~1.3 *m/z*)	10
TRUE	150.1277	1	5.89	1.5	Narrow (~1.3 *m/z*)	10
TRUE	180.1019	1	4.717	1.5	Narrow (~1.3 *m/z*)	10
TRUE	194.1187	1	5.031	1.5	Narrow (~1.3 *m/z*)	10
TRUE	208.1344	1	5.676	1.5	Narrow (~1.3 *m/z)*	10
TRUE	244.206	1	10.158	1.5	Narrow (~1.3 *m/z*)	10
TRUE	248.1657	1	9.778	1.5	Narrow (~1.3 *m/z)*	10
TRUE	282.0885	1	11.084	1.5	Narrow (~1.3 *m/z*)	10
TRUE	285.0789	1	13.466	1.5	Narrow (~1.3 *m/z*)	10
TRUE	287.0578	1	12.027	1.5	Narrow (~1.3 *m/z*)	10
TRUE	300.1606	1	7.032	1.5	Narrow (~1.3 *m/z*)	10
TRUE	300.1606	1	7.56	1.5	Narrow (~1.3 *m/z*)	10
TRUE	301.0739	1	12.407	1.5	Narrow (~1.3 *m/z*)	10
TRUE	304.1555	1	9.281	1.5	Narrow (~1.3 *m/z*)	10
TRUE	309.0902	1	12.01	1.5	Narrow (~1.3 *m/z*)	10
TRUE	310.2166	1	12.738	1.5	Narrow (~1.3 *m/z*)	10
TRUE	315.2319	1	19.139	1.5	Narrow (~1.3 *m/z*)	10
TRUE	316.0484	1	11.167	1.5	Narrow (~1.3 *m/z*)	10
TRUE	316.1544	1	5.99	1.5	Narrow (~1.3 *m/z*)	10
TRUE	321.0188	1	12.06	1.5	Narrow (~1.3 *m/z*)	10
TRUE	335.1766	1	8.14	1.5	Narrow (~1.3 *m/z*)	10
TRUE	354.2435	1	17.551	1.5	Narrow (~1.3 *m/z*)	10
TRUE	370.1649	1	10.257	1.5	Narrow (~1.3 *m/z*)	10
TRUE	372.1597	1	14.144	1.5	Narrow (~1.3 *m/z*)	10
TRUE	455.2916	1	13.747	1.5	Narrow (~1.3 *m/z*)	10
TRUE	136.1121	1	4.403	1.5	Narrow (~1.3 *m/z*)	20
TRUE	150.1277	1	4.899	1.5	Narrow (~1.3 *m/z*)	20
TRUE	150.1277	1	5.89	1.5	Narrow (~1.3 *m/z*)	20
TRUE	180.1019	1	4.717	1.5	Narrow (~1.3 *m/z*)	20
TRUE	194.1187	1	5.031	1.5	Narrow (~1.3 *m/z*)	20
TRUE	208.1344	1	5.676	1.5	Narrow (~1.3 *m/z*)	20
TRUE	244.206	1	10.158	1.5	Narrow (~1.3 *m/z*)	20
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TRUE	285.0789	1	13.466	1.5	Narrow (~1.3 *m/z*)	20
TRUE	287.0578	1	12.027	1.5	Narrow (~1.3 *m/z*)	20
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TRUE	301.0739	1	12.407	1.5	Narrow (~1.3 *m/z*)	20
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TRUE	309.0902	1	12.01	1.5	Narrow (~1.3 *m/z*)	20
TRUE	310.2166	1	12.738	1.5	Narrow (~1.3 *m/z*)	20
TRUE	315.2319	1	19.139	1.5	Narrow (~1.3 *m/z*)	20
TRUE	316.0484	1	11.167	1.5	Narrow (~1.3 *m/z*)	20
TRUE	316.1544	1	5.99	1.5	Narrow (~1.3 *m/z*)	20
TRUE	321.0188	1	12.06	1.5	Narrow (~1.3 *m/z*)	20
TRUE	335.1766	1	8.14	1.5	Narrow (~1.3 *m/z*)	20
TRUE	354.2435	1	17.551	1.5	Narrow (~1.3 *m/z*)	20
TRUE	370.1649	1	10.257	1.5	Narrow (~1.3 *m/z*)	20
TRUE	372.1597	1	14.144	1.5	Narrow (~1.3 *m/z*)	20
TRUE	455.2916	1	13.747	1.5	Narrow (~1.3 *m/z*)	20

Targeted MSMS Table

On	Prec. *m/z*	Z	RT (min)	Delta RT	Iso. width	Collision energy
TRUE	136.1121	1	4.403	1.5	Narrow (~1.3 m/z)	40
TRUE	150.1277	1	4.899	1.5	Narrow (~1.3 m/z)	40
TRUE	150.1277	1	5.89	1.5	Narrow (~1.3 m/z)	40
TRUE	180.1019	1	4.717	1.5	Narrow (~1.3 m/z)	40
TRUE	194.1187	1	5.031	1.5	Narrow (~1.3 m/z)	40
TRUE	208.1344	1	5.676	1.5	Narrow (~1.3 m/z)	40
TRUE	244.206	1	10.158	1.5	Narrow (~1.3 m/z)	40
TRUE	248.1657	1	9.778	1.5	Narrow (~1.3 m/z)	40
TRUE	282.0885	1	11.084	1.5	Narrow (~1.3 m/z)	40
TRUE	285.0789	1	13.466	1.5	Narrow (~1.3 m/z)	40
TRUE	287.0578	1	12.027	1.5	Narrow (~1.3 m/z)	40
TRUE	300.1606	1	7.032	1.5	Narrow (~1.3 m/z)	40
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TRUE	301.0739	1	12.407	1.5	Narrow (~1.3 m/z)	40
TRUE	304.1555	1	9.281	1.5	Narrow (~1.3 m/z)	40
TRUE	309.0902	1	12.01	1.5	Narrow (~1.3 m/z)	40
TRUE	310.2166	1	12.738	1.5	Narrow (~1.3 m/z)	40
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TRUE	354.2435	1	17.551	1.5	Narrow (~1.3 m/z)	40
TRUE	370.1649	1	10.257	1.5	Narrow (~1.3 m/z)	40
TRUE	372.1597	1	14.144	1.5	Narrow (~1.3 m/z)	40
TRUE	455.2916	1	13.747	1.5	Narrow (~1.3 m/z)	40

Reference Masses- positive ion
121.050873 (M+H+ for purine)
922.009798 (M+H+ for HP-921)

Appendix IV.

LC/MS Q-TOF Conditions Auto MS/MS Screen and Identification

Agilent 1200 Series SL LC Parameters

Column	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm, 3.5 µm, p/n 959793-902
Column temperature	30 °C
Injection volume	5
Autosampler temperature	Ambient
Needle wash	5 s with methanol
Mobile phase	A = 10 mM ammonium acetate in water, pH 6.8
	B = 100% Methanol
Flow rate	0.5 mL/min
Gradient	10% B at t = 0 to 100 % B at t = 20 min, hold 1.9 min, 0.1 min return to initial conditions
Stop time	22 min
Post time	3 min

Agilent 6530 Q-TOF parameters

Segment Number	Start Time	Diverter Valve State	Storage Mode	Ion Mode
1	0	Waste	None	ESI+Agilent Jet Stream
2	2.5	MS	Both	ESI+Agilent Jet Stream
3	20	Waste	None	ESI+Agilent Jet Stream

(Note that start time is in min, storage mode "both" indicates data is stored as centroid and profile)

Time Segment	2
Acquisition Mode	AutoMS2
MS/MS Min Range	50
MS Min Range	100
MS/MS Max Range	1000
MS Max Range	1000
MS/MS Scan Rate	3
MS Scan Rate	3
Isolation Width MS/MS	Narrow (~1.3 amu)

Ramped Collision Energy

Slope	Offset
6	4

Precursor Selection

Max Precursors Per Cycle	2	Threshold (Abs)	200
Active Exclusion After # Spectra	1	Threshold (Rel)	0.01%
Precursor Scan Speed	TRUE	Active Exclusion Before 0.15 minutes	

Static Exclusion Ranges

Start *m/z*	End *m/z*
50	130
600	1700

Sort by Abundance Only
Charge State Preference
1
Unk

Source Parameters

Parameter	Value
Gas Temperature (°C)	320
Gas Flow (l/min)	8
Nebulizer (psi)	35
Sheath Gas Temperature (°C)	380
Sheath Gas Flow (l/min)	11

Scan Seg #	Ion Polarity
All	Positive
Scan Segment	1

Scan Source Parameters

Parameter	Value
V_{Cap}	3000
Nozzle Voltage (V)	0
Fragmentor	150
Skimmer1	65
OctopoleRFPeak	750

Reference Masses- positive ion
121.050873 (M+H+ for purine)
922.009798 (M+H+ for HP-921)

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