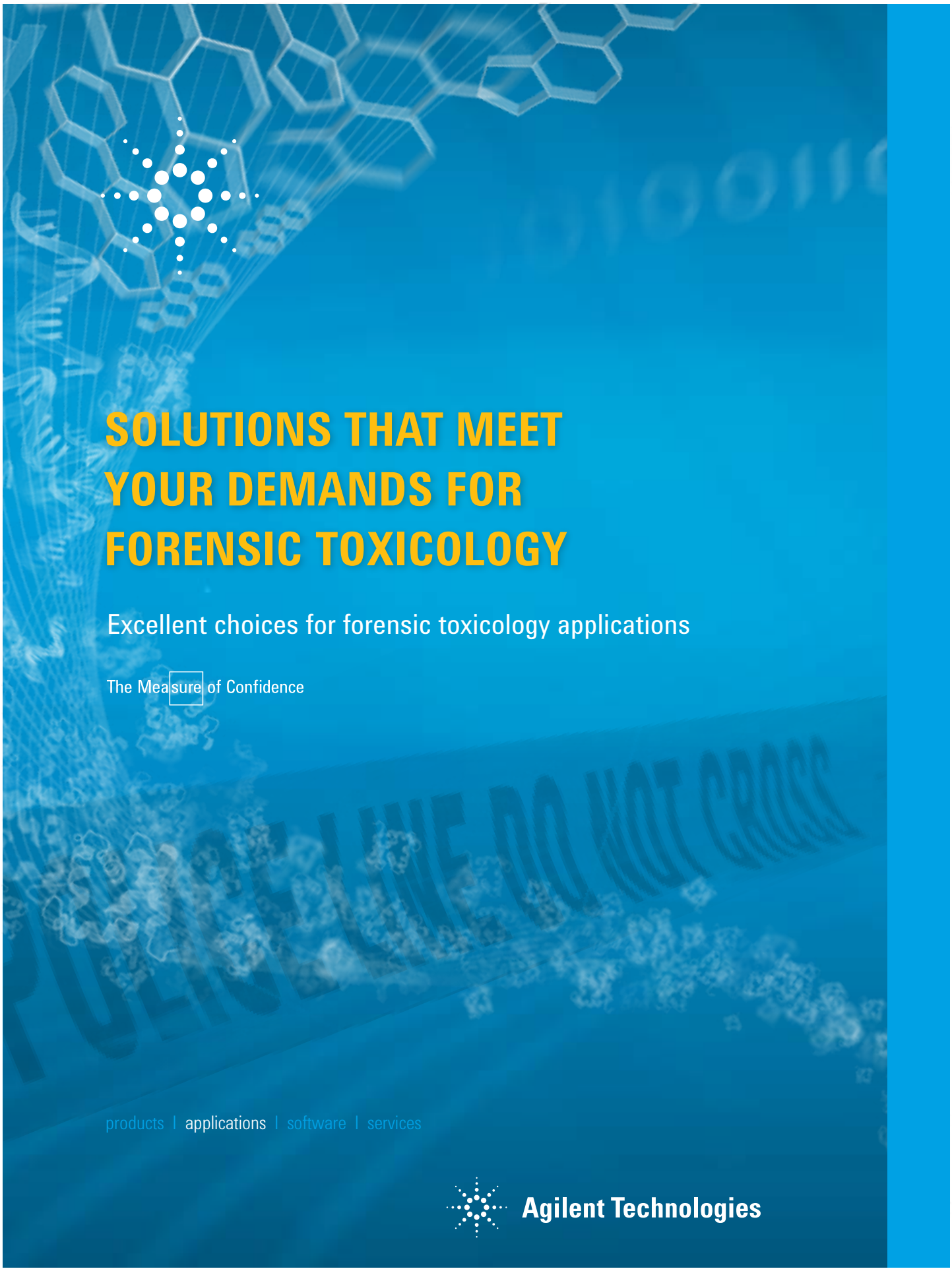

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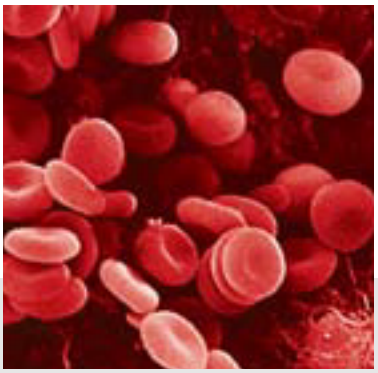

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- GC
- GC/MS
- GC/MS/MS
- LC/QQQ
- CE/MS
- ICP-OES & ICP-MS
- LC/MS
- Sample Preparation

Applications by Matrix

- Urine
- Blood/Serum/Plasma
- Oral Fluids
- Hair

Applications by Analyte

- Benzodiazepines
- Steroids
- Ethylglucuronide
- Volatiles
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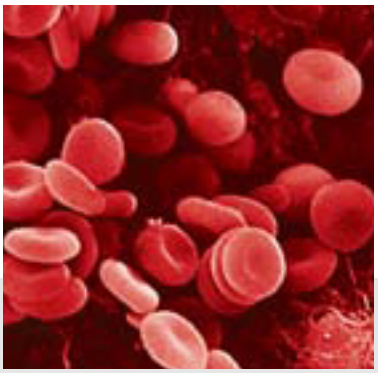

Applications by Technique

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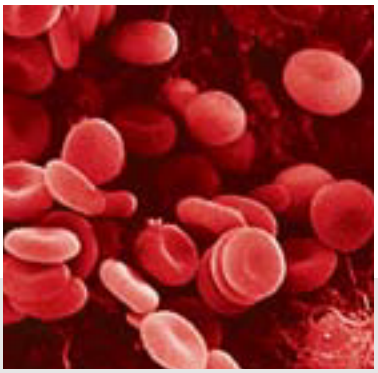

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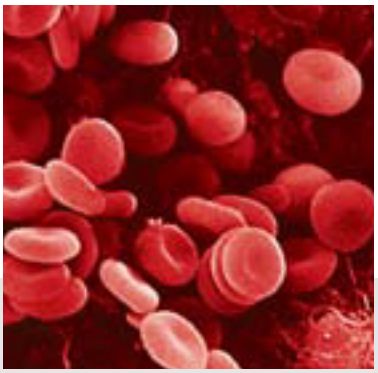
- Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler



Applications by Technique

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Volatile Organic Compounds in Serum or Plasma

Application Note

Forensic Toxicology

Author

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Introduction

The analysis of alcohols in blood (plus acetaldehyde and acetone) is demonstrated in this application note. Testing for the presence and subsequent quantification of ethanol in serum and plasma is often performed in forensic toxicology laboratories. In addition to the ethanol, analysis of other alcohols, as well as acetaldehyde and acetone, is necessary.

Ethylene glycol, for instance, is widely used as a solvent or surfactant. It is also used as a nonfreezing compound in coolants for cars. Toxic actions of ethylene glycol are the suppression of central nervous system activities and metabolic acidosis caused by glycolic acid produced from ethylene glycol. The glycolic acid is further metabolized into oxalic acid, which binds with calcium ions to form the insoluble salt; the salt precipitates in various tissues.

A sensitive and reproducible gas chromatographic method for ethanol and other volatile organic compounds in serum or plasma was developed using the polar CP-Wax 52 CB column, creating good peak shapes; this can also be seen for ethylene glycol. The method involves direct injection of the biological specimen into the GC, with little pre-treatment (plasma is mixed with internal standard solution and injected).

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Sample Preparation

The blood samples were collected in anti-coagulant (EDTA) containing tubes, closed and centrifuged for 5 minutes at 3000 RPM. 100 µL plasma was taken, mixed with 100 µL internal standard solution and stored in a closed micro sample container.

Calculation

The ethanol concentration of plasma is 1.17 times the concentration in whole blood, so the calculated value for plasma must be corrected by this factor to find the concentration in whole blood.

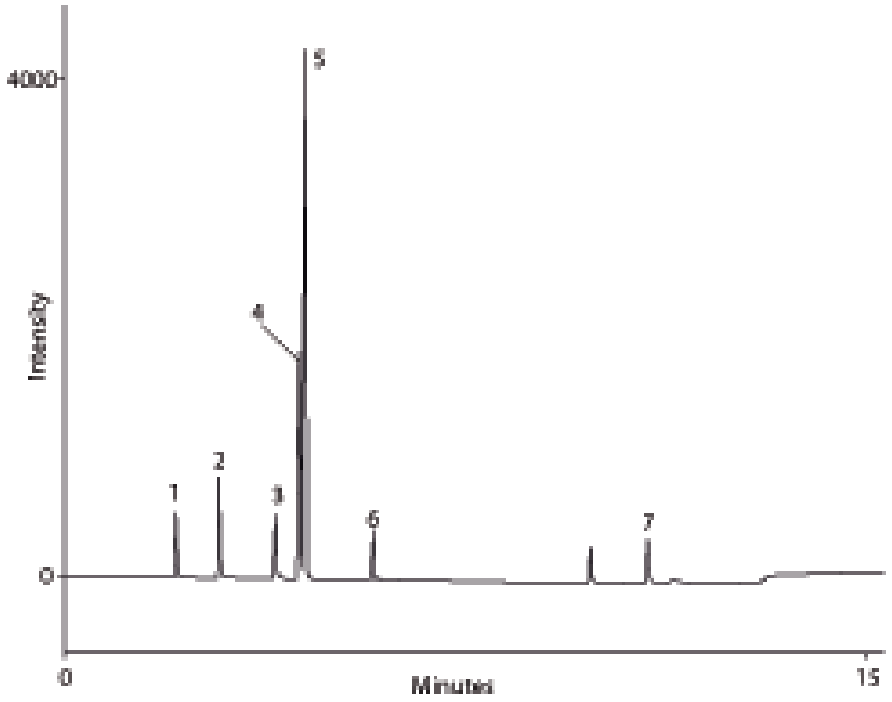
Conditions

Sample:	KKGT[1] reference sample
Column:	CP-WAX 52 CB, 0.25 mm x 30 m: 0.5 µm (part number CP8746)
Temperature:	40 °C (4 min) ⟶ 210 °C, 15 °C/min
Carrier Gas:	Nitrogen
Flow Rate:	1.46 mL/min
Pressure:	100 kPa (1.0 bar, 14 psi)
Injector:	Split, 1:25, split/splitless liner without glass wool, with carbon frits, 230 °C
Inj Vol:	0.2 µL
Sample Conc:	Acetaldehyde 0.775 g/L, Acetone 0.704 g/L, Methanol 0.652 g/L, Isopropanol 1.435 g/L, Ethanol 3.233 g/L, Ethylene glycol 0.843 g/L, 1-Propanol (I.S.) 0.333 g/L in water. Detection limit for methanol and ethanol 0.1 g/L
Detector:	FID, 250 °C

[1] Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie (Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology).

Table 1. Peak Identification for Figure 1

Peak Identification		Retention Time (min)
1	Acetaldehyde	2.07
2	Acetone	2.88
3	Methanol	3.93
4	Isopropanol	4.37
5	Ethanol	4.47
6	1-Propanol (I.S.)	5.77
7	Ethylene glycol	10.95



Analysis of organic compounds in blood plasma

Remarks

The carbon fritted liner acts as a kind of trap to prevent column contamination and is cleaned or changed regularly to prevent decreasing of the performance, typically after about 50 injections.

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Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler

Application

Forensic Toxicology

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Abstract

A G1888 Network Headspace Sampler coupled to a 6890N gas chromatograph was used for the determination of forensic blood alcohols. Standard mixtures in water were used to demonstrate the analyses. Two headspace systems, based on 0.53-mm and 0.32-mm id columns, are described. Isothermal analyses with cycle times below 5 min are easily achieved with sufficient resolution to avoid common interferences. A new automated headspace Sampler with 70-sample tray and inert flow path is introduced in this application. Total system control from the GC ChemStation is possible with new 21 CFR Part 11 compliant software specific for headspace sampling.

Introduction

Blood alcohol analysis is a widely used, high-throughput application in forensic toxicology laboratories. The use of static headspace sampling has many well known advantages for the determination of volatiles in a variety of less than ideal matrices. Blood or other biological fluids are certainly not the cleanest of matrices and, therefore, are well suited for headspace sampling. In terms of GC analysis, some of the advantages of automated headspace include reduced inlet and column main-tenance, better quantitation, limited sample prepa-ration, and increased throughput. The G1888 Network Headspace Sampler employs a completely inert flow path, uniform heated zones, and unique vent line purging capability. When taken together, these attributes lead to a reduction in carryover with improved repeatability.

Dual-column systems offer an advantage in that elution order of ethanol and some other common metabolites differ on the DB-ALC1 and DB-ALC2 stationary phases. This provides added confirmation and a potential reduction in possible inferences or co-elutions with ethanol. See Table 1 for a listing of instrument settings.

Experimental

Table 1. Instrument Conditions

0.53-mm Column System	
6890N GC	
Injection port	Split/Splitless
Temperature	250 °C
Split ratio	10:1
Carrier gas	Helium
Carrier flow	12 mL/min
Detector	FID, 300 °C
GC Oven Program	
Initial temperature	40 °C
Initial time	5 min
G1888A Headspace Sampler	
Loop size	1 mL
Vial pressure	9.0 psig
Headspace oven	70 °C
Loop temp	80 °C
Transfer line temp	90 °C
Equilibration time	10 min, high shake
GC Cycle time	4 min
Pressurization	0.2 min
Vent (loop fill)	0.2 min
Inject	0.5 min
Columns	
DB-ALC1	30 m × 0.53 mm × 3.0 µm
DB-ALC2	30 m × 0.53 mm × 3.0 µm
Guard column	0.15 m × 0.53 mm deactivated fused silica
Y splitter, deactivated Agilent part no. 5181-3398	

0.32-mm Column System	
6890N GC	
Injection port	Split/Splitless
Temperature	150 °C
Split ratio	5:1
Carrier gas	Helium
Inlet pressure	18.8 psi
Detector	FID, 300 °C
GC oven program	
Initial temperature	35 °C
Initial time	7 min
G1888A Headspace Sampler	
Loop size	1 mL
Vial pressure	11.5 psig (supplied by GC EPC Aux)
Headspace oven	60 °C
Loop temp	70 °C
Transfer line temp	80 °C
Equilibration time	15 min, high shake
GC cycle time	6 min
Pressurization	0.15 min
Vent (loop fill)	0.15 min
Inject	0.5 min
Columns	
DB-ALC1	30 m × 0.32 mm × 1.8 µm
DB-ALC2	30 m × 0.32 mm × 1.2 µm
Two-hole ferrule Agilent part no. 5062-3580	

Two columns are connected to one split/splitless injection port in both systems. This allows simultaneous injection into both columns with each connected to an flame ionization detector (FID). A glass Y connector/retention gap and two-hole ferrule are used for the 0.53-mm and 0.32-mm systems, respectively. After connection, initial experiments using n-propanol were conducted to ensure an equal split between the columns. Areas recorded on both channels agreed to within 5%.

The G1888 Headspace Sampler was interfaced to the split/splitless inlet by cutting the carrier line near the inlet weldment and then connecting a zero dead volume (ZDV) union to the headspace transfer line and inlet carrier at the weldment. The supply end of the cut carrier line is then connected to the electronic pneumatic control (EPC) carrier inlet bulkhead at the back of the G1888 Headspace Sampler. Therefore, an inlet EPC channel from the 6890N was used to control carrier flow.

Ten mL headspace vials, each with 2-mL water solution, were used throughout. The resolution check samples were prepared by adding 100 µL of a 0.1 g/dL standard to a 10-mL vial.

Results and Discussion

In this application note, the G1888 Automated Headspace Sampler was used. This sampler features an inert Siltek™ sample path for maximum inertness and minimal carryover. Sample-path

tubing, sampling needle, transfer line, and vent lines are all deactivated. Care was taken to minimize cold spots reducing the possibility of unwanted condensation. Initial setup of the blood alcohol systems first involved verification of proper column installation by checking for uniform sample split between the two columns, followed by a resolution check. To check resolution and peak symmetry, an eight-component sample (Restek #36256) was used. The resulting chromatograms are shown in Figure 1. In the United States, n-propanol, or isopropanol, are commonly used as the internal standards (ISTD) for gas chromatographic blood alcohol determinations. However, in postmortem work, methyl-ethyl ketone (MEK) is commonly used since n-propanol can be a degradation product.

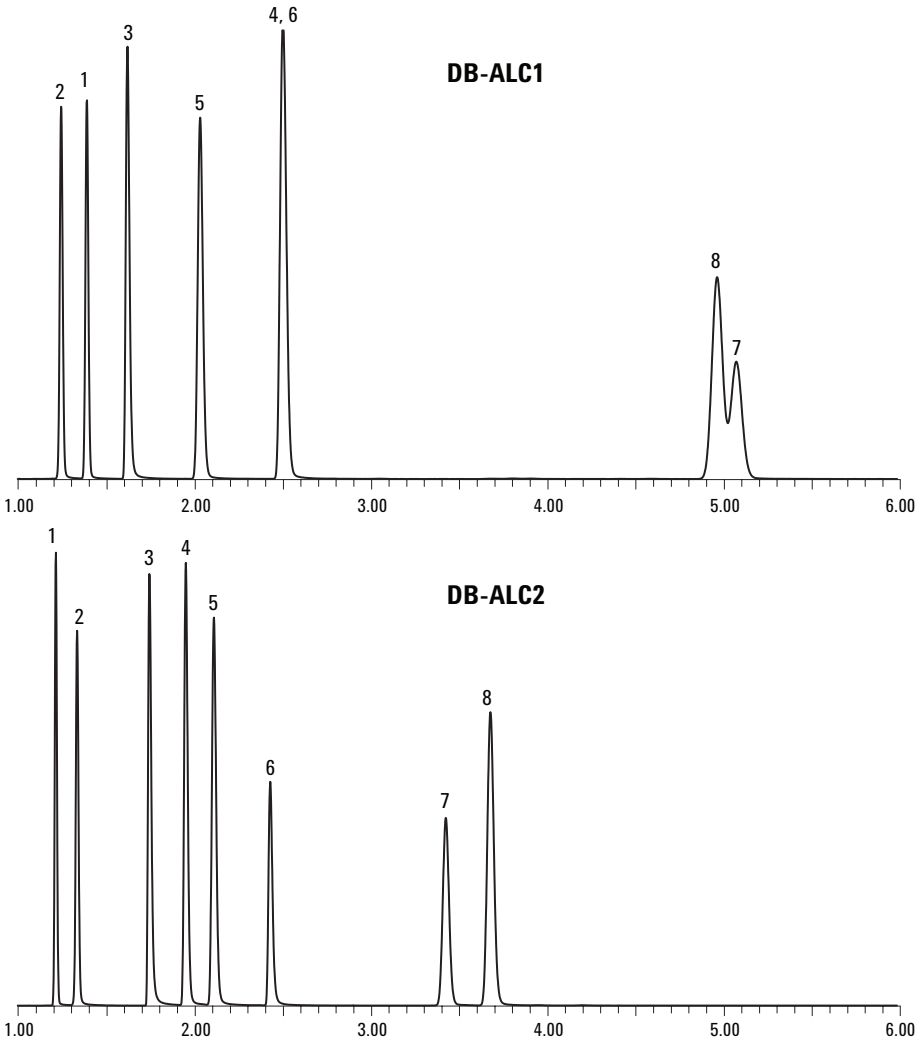


Figure 1. Resolution check standard on 0.32-mm column system at 35 °C. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. 2-propanol, 6. Acetonitrile, 7. Ethyl acetate, and 8. MEK.

A six-component mix using the wide-bore column system is shown in Figure 2.

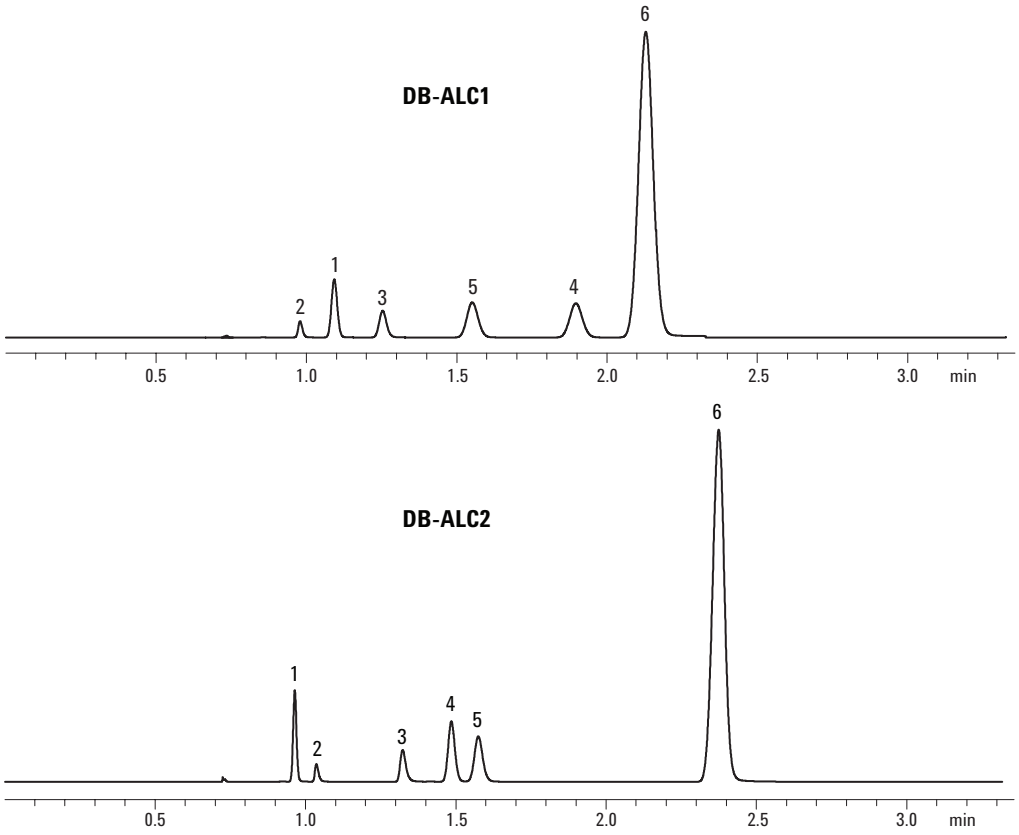


Figure 2. Chromatograms using the 0.53 mm DB-ALC1 and DB-ALC2 columns and the six-component standard at 35 °C. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, and 6. n-propanol.

Repeatability

Repeatability results for the eight-component standard and the 0.32-dual column and 0.53-dual column systems are shown in Tables 2 and 3, respectively.

Table 2. Repeatability (RSD) of the 0.32-mm Column System; 18 Runs Each of 0.1 g/dL and 0.15 g/dL Calibration Solutions. For DB-ALC1 (0.15 g/dL Runs), the Maximum k Was 0.291 and the Minimum Was 0.285.

DB-ACL-	Conc. g/dL	Acetald.	Methanol	Ethanol	Acetone	Isopropanol	n-propanol	MEK	Calibration K Factor for EtOH (RSD)
1	0.1	0.78	2.32	2.11	1.12	1.75	1.83	0.82	0.36
2	0.1	0.72	2.77	2.10	1.72	1.72	1.85	0.95	0.34
1	0.15	0.86	3.11	2.68	1.31	2.34	2.50	0.93	0.58
2	0.15	0.84	3.48	2.69	1.33	2.31	2.50	0.93	0.55

Table 3. Repeatability (RSD) of the 0.53-mm Column System; 40 Runs of a 0.1% Solution

DB-ALC-	Acetald.	Methanol	Ethanol	Acetone	Isopropanol	Acetonitrile	n-propanol	Ethyl acetate	MEK
1	1.30	1.07	1.12	0.95	0.94	0.95	0.96	2.34	2.00
2	1.22	0.99	1.01	0.96	0.90	0.83	0.93	1.70	1.13

Many laboratories use a series of replicates at the 0.15 g/dL ethanol level for calibration and assessment of system performance. Chromatograms of this mixture are shown in Figures 3A and 3B, for DB-ALC1 and DB-ALC2, respectively

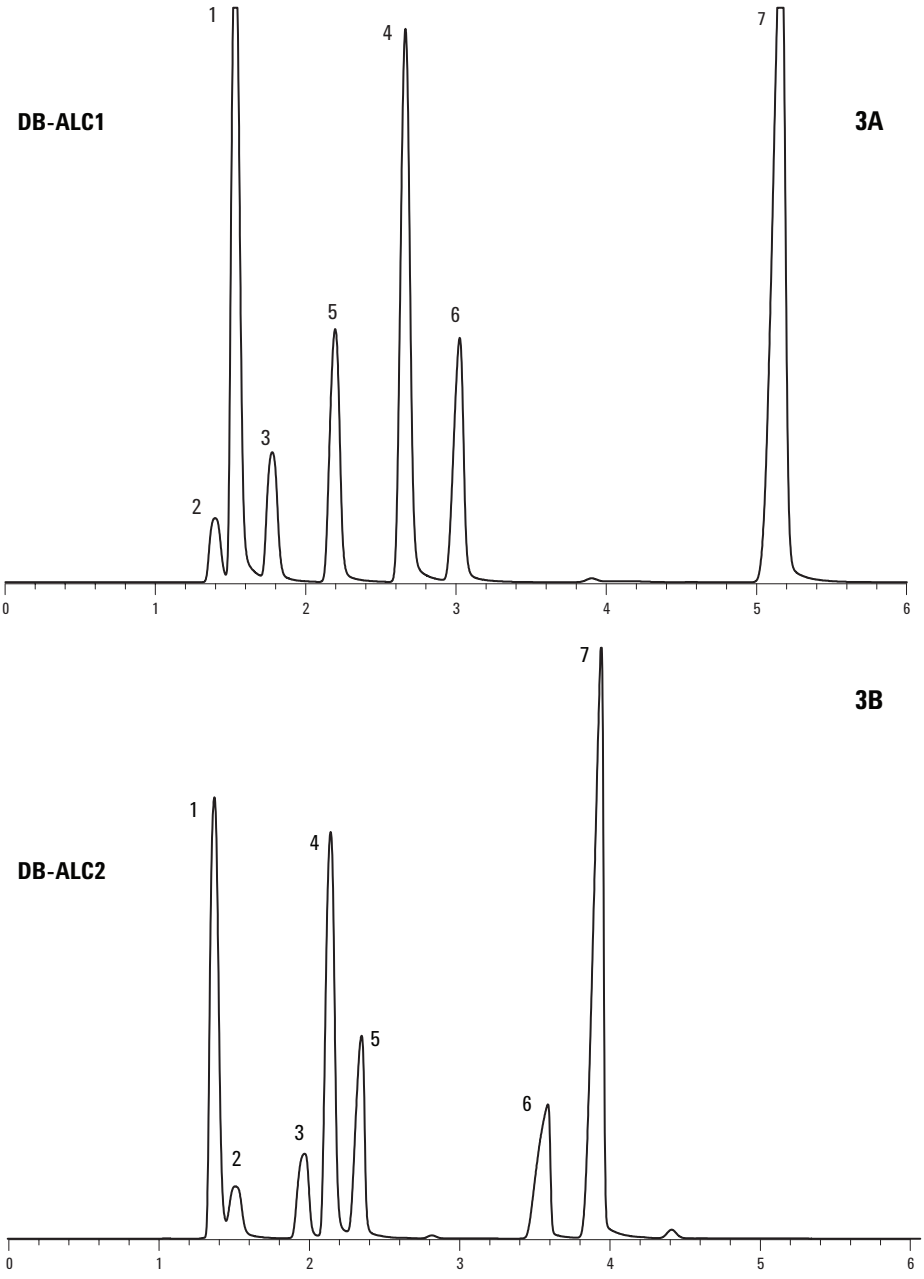


Figure 3. Blood alcohol standard at 0.15 g/dL on 0.32-mm DB-ALC1 (Figure 3A) and DB-ALC2 (Figure 3B) columns. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, 6. n-propanol, and 7. MEK.

Carryover

In blood alcohol analysis, negative or blank samples should show less than 1.0% ethanol as carryover. A 0.5% per component solution was used to demonstrate the lack of carryover, shown in Table 4. This concentration of ethanol, at several times the nominal expected level, is representative of a severe test for carryover and should show any significant weaknesses in the system. Ethanol carryover as measured by water/ISTD blank run made after 18 runs of a 0.15 g/dL standard gave a percent carryover of 0.6% (0.32-mm DB-ALC2).

A new feature of the G1888 allows users to set the vent purge time from the G1888 keyboard. This parameter is defined, as the time the vent valve is open beginning after valve injection is complete and can remain open up to a maximum of the cycle time setting. This additional purge time may provide a further reduction in carryover. The results shown in Table 4 used the default vent purge time of 30 seconds.

Table 4. Carryover Experiment. Areas are the Average of Six Consecutive Runs of a 0.5% per Component Mixture, Followed by a Water/ISTD Blank. The 0.53-mm Column System Was Used

DB-ALC1	Acetald.	Methanol	Ethanol	Acetone	Isopropanol	n-propanol
Average area	1764	7523	3268	5600	8116	1166
Blank area	31	57	47	*	*	1179
Area ratio	0.02	0.01	0.01	*	*	1.01

*Not measureable

Linearity

Flame ionization detectors are expected to show good linearity for all analytes of interest over the concentration ranges needed for blood alcohol systems. As shown in Figures 4 and 5, this has been verified for the wide-bore column system, and in Table 5 for the 0.32-mm column system. Regression coefficients for the 0.32-mm column system are indicated.

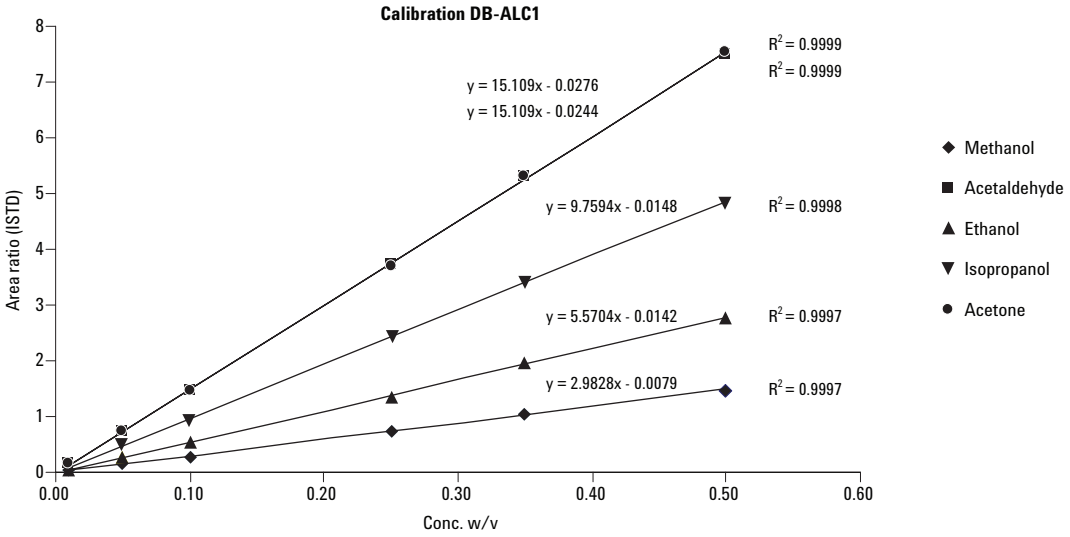


Figure 4. Calibration plots for the indicated standards using the 0.53-mm DB-ALC1 column.

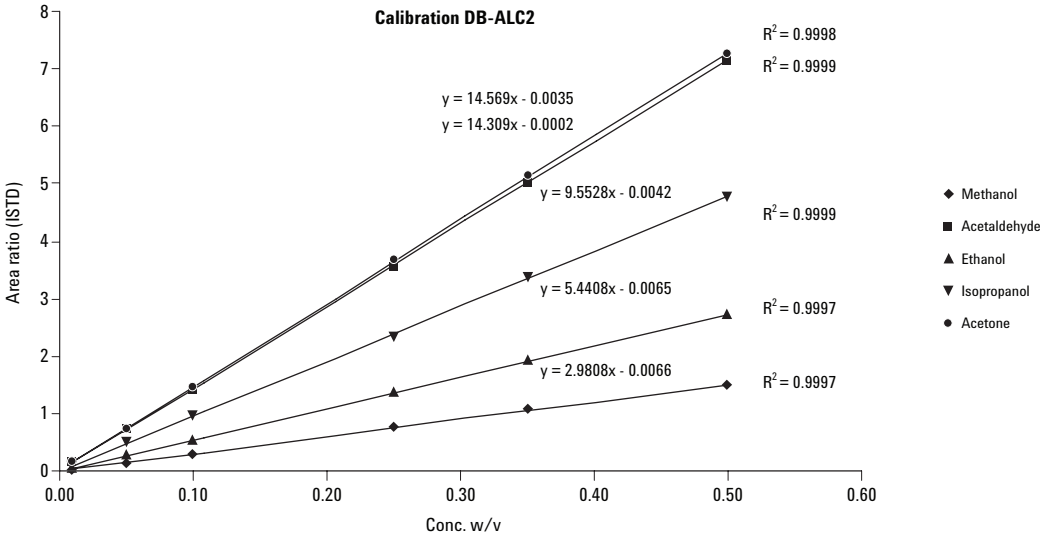


Figure 5. Calibration plots for the indicated standards using the 0.53-mm DB-ALC2 column.

Table 5. Linearity (R^2) of 0.32-mm Column System; Concentrations from 0.005 to 1.0 g/dL

DB-ALC-	Acedald.	MeOH	EtOH	Acetone	IsoPrOH.	n-PrOH.	MEK
1	0.99981	0.99946	0.99931	0.99993	0.99990	0.99983	0.99961
2	0.99985	0.99944	0.99962	0.99993	0.99990	0.99982	0.99946

System k Factor

A system k factor, or response factor, can be defined as (Area ISTD × Conc. EtOH in Std)/(Area of EtOH in Std). A system average k factor can be determined from 6 to 10 consecutive runs of a standard at 0.1 or 0.15 g/dL EtOH. The result for each sample should deviate from the average by no more than ±1.0%. See Table 2 for calibration k factor RSD's on the 0.32-mm column system. Limits are then placed on the run k factor determined for each sample. Run k values should fall within some specified allowable range established by the laboratory. Typically, ±3% of the average k is used.

The results shown in Table 6 illustrate the stability of the system for each column, after 40 runs and six different concentration levels. Calculated concentrations for run 40 differ from the initial concentration by less than 1%.

Table 6. Percent Deviation of Run 40 vs. Run 1 for Five Standards

Column	Nominal					
DB-ALC-	g/dL	MeOH	Acetald.	EtOH	IsoPrOH.	Acetone
1	0.01	0.65	–1.09	0.50	–0.08	–0.78
1	0.05	0.74	2.05	0.70	0.84	1.38
1	0.10	0.46	0.49	0.13	–0.19	–0.02
1	0.25	0.10	2.26	–0.17	0.10	1.08
1	0.35	0.23	0.00	–0.44	0.13	–0.06
1	0.50	0.27	0.73	–0.48	0.09	0.29
DB-ALC-						
2	0.01	–1.32	0.32	0.25	–0.80	–0.38
2	0.05	1.99	0.46	0.63	1.36	0.63
2	0.10	0.31	0.09	–0.40	–0.18	–0.43
2	0.25	2.15	–0.60	–0.15	1.02	0.05
2	0.35	–0.18	0.11	–0.16	–0.13	0.01
2	0.50	0.71	0.27	–0.12	0.20	–0.29

Calibration curves obtained on the first of 40 consecutive runs are based on the six concentrations shown.

European Blood Alcohols

In the European Union, blood alcohol limits are either 0.5 or 0.8 g/L, although Sweden and Norway have a stricter limit of 0.2 g/L [1]. In addition, more restrictive levels have been mandated for young drivers in many countries. Principle means of measurement of alcohol include both breath and blood, with blood testing compulsory in a few countries. Unlike the U.S., the ISTD most often used for blood testing is t-butanol. Chromatograms are shown in Figures 6A and 6B, where t-butanol was used as the ISTD. Certain potential co-elutions need to be noted, however, including t-butanol/ acetonitrile/ acetone on DB-ALC1, and t-butanol/ acetonitrile on DB-ALC2.

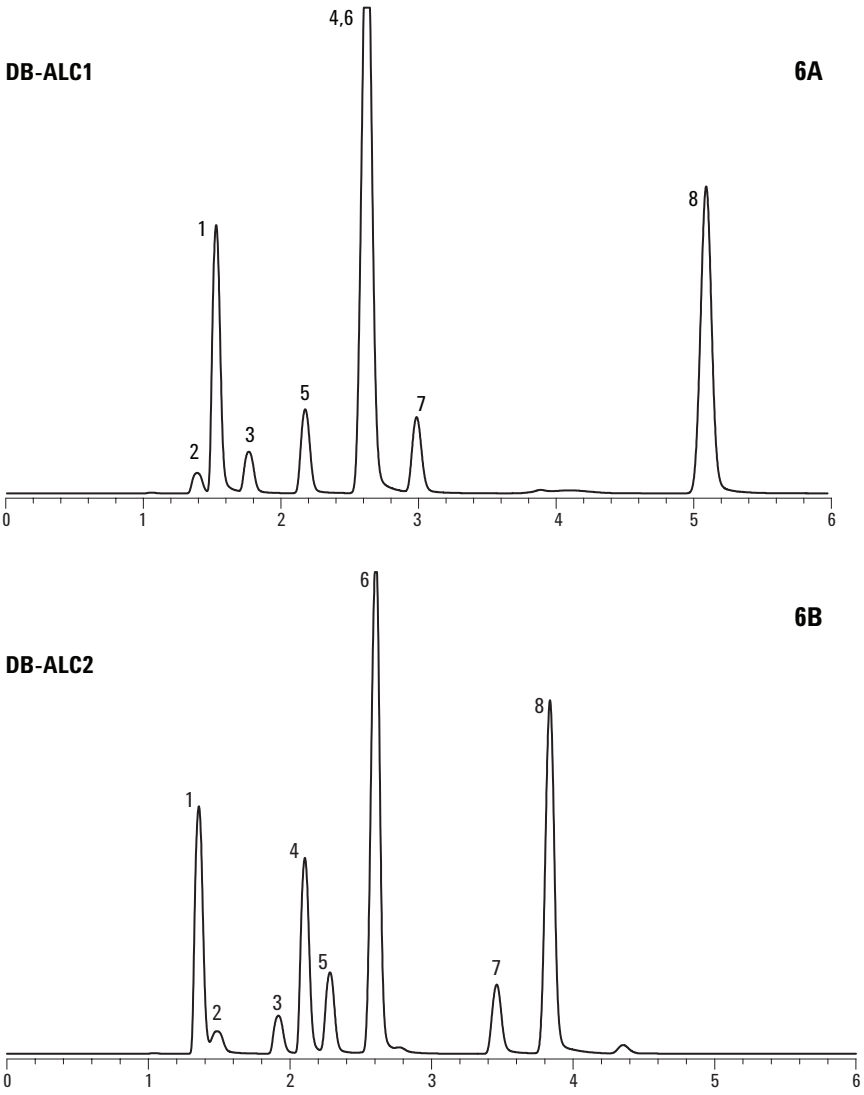


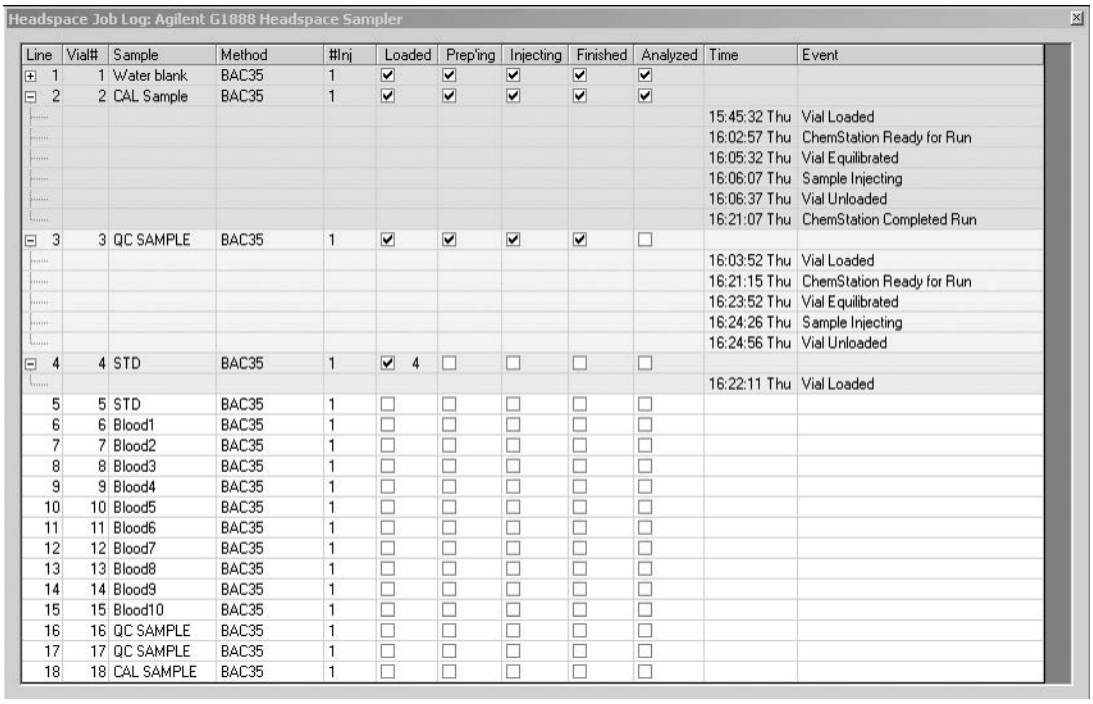
Figure 6. Blood alcohol chromatograms using t-butanol as the ISTD. Columns: 30 m × 0.32 mm DB-ALC1 (Figure 6A) and DB-ALC2 (Figure 6B). Peak identifications: 1. Acetaldeyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, 6. t-Butanol, 7. n-propanol, and 8. MEK.

Inhalants in Blood

Although not demonstrated in the work, diethyl ether, hexane, chloroform, ethyl acetate, and toluene are also separated from the standard components of blood alcohol analysis on the dual column systems [2]. A change in the chromatographic program may be needed for optimization.

ChemStation Software

A software module has been developed for the G1888 Headspace Sampler that provides complete control of all instrument parameters and also uses the same sequence table as the Agilent liquid samplers. This software is available as an add-on product to the GC ChemStation (G2922A), providing fully integrated headspace control. ChemStation revision A.09.03 or later is required. An example of the sequence log table is shown in Figure 7.



Line	Vial#	Sample	Method	#Inj	Loaded	Prep'ing	Injecting	Finished	Analyzed	Time	Event
1	1	Water blank	BAC35	1	☑	☑	☑	☑	☑		
2	2	CAL Sample	BAC35	1	☑	☑	☑	☑	☑		
										15:45:32 Thu	Vial Loaded
										16:02:57 Thu	ChemStation Ready for Run
										16:05:32 Thu	Vial Equilibrated
										16:06:07 Thu	Sample Injecting
										16:06:37 Thu	Vial Unloaded
										16:21:07 Thu	ChemStation Completed Run
3	3	QC SAMPLE	BAC35	1	☑	☑	☑	☑	☐		
										16:03:52 Thu	Vial Loaded
										16:21:15 Thu	ChemStation Ready for Run
										16:23:52 Thu	Vial Equilibrated
										16:24:26 Thu	Sample Injecting
										16:24:56 Thu	Vial Unloaded
4	4	STD	BAC35	1	☑ 4	☐	☐	☐	☐		
										16:22:11 Thu	Vial Loaded
5	5	STD	BAC35	1	☐	☐	☐	☐	☐		
6	6	Blood1	BAC35	1	☐	☐	☐	☐	☐		
7	7	Blood2	BAC35	1	☐	☐	☐	☐	☐		
8	8	Blood3	BAC35	1	☐	☐	☐	☐	☐		
9	9	Blood4	BAC35	1	☐	☐	☐	☐	☐		
10	10	Blood5	BAC35	1	☐	☐	☐	☐	☐		
11	11	Blood6	BAC35	1	☐	☐	☐	☐	☐		
12	12	Blood7	BAC35	1	☐	☐	☐	☐	☐		
13	13	Blood8	BAC35	1	☐	☐	☐	☐	☐		
14	14	Blood9	BAC35	1	☐	☐	☐	☐	☐		
15	15	Blood10	BAC35	1	☐	☐	☐	☐	☐		
16	16	QC SAMPLE	BAC35	1	☐	☐	☐	☐	☐		
17	17	QC SAMPLE	BAC35	1	☐	☐	☐	☐	☐		
18	18	CAL SAMPLE	BAC35	1	☐	☐	☐	☐	☐		

Figure 7. An example of the time stamped sequence log table window using the Agilent G1888 Headspace Sampler. A sequence log file is also created.

All major events associated with vial processing are shown with a time stamp. Software is also 21 CFR Part 11 compliant with Agilent Security Pack installed. Setup of the sampler is handled with two pull-down menus, one for setting global parameters, such as LAN address and vial size, while the other opens a dialog box to set sample sequence timing and system temperatures.

Conclusions

Key parameters for blood alcohol analysis by headspace sampling include analysis time, resolution, repeatability, and carryover. The two isothermal systems described here offer fast cycle times, typically less than 5 minutes depending on the ISTD chosen. This provides good throughput when coupled to the 70-sample tray of the G1888. The DB-ALC1 and DB-ALC2 columns also provide good resolution, separating ethanol from common interferences. Deviation of the k factors from the average system k factor is below 1.5% in the experiments described here, at concentration levels ranging from 0.01 to 0.5 g/dL.

The headspace-GC system described here will give reliable determinations of forensic ethanol levels in blood and other biological matrices. Although a single column is usually adequate, the dual column approach gives additional confirmation and separation utility without an increase in analysis time. Carryover, a common problem in high-throughput laboratories, is reduced through a combination of inert flow path, improved thermal control, and programmable vent purge. The G1888 Headspace Sampler can be a valuable addition to forensic laboratories, as the US and other countries step up the enforcement of driving under the influence (DUI) laws.

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1. Blood Alcohol Concentration Limits Worldwide, *ICAP Reports* 2002 **11**, May, www.icap.org.
2. E. Kuhn, M. Datta, and J. Ellis, “Separation and Identification of Blood Pollutants”, Agilent Technologies, publication B-0328 Rev.2, http://www.agilent.com/chem

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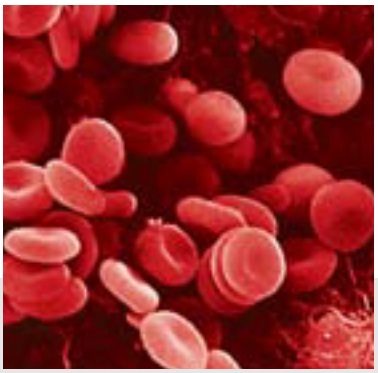
- Rapid, Robust and Sensitive Detection of 11-nor-9-Tetrahydrocannabinol-9-Carboxylic Acid in Hair
- Fast GC/MS/MS of Androgenic Anabolic Steroids in Urine Using a VF-5ms Column
- Rapid and Robust Detection of THC and Its Metabolites in Blood
- Toxicology Screening of Whole Blood Extracts Using GC/Triple Quadrupole/MS



Applications by Technique

GC/MS/MS

Rapid, Robust and Sensitive Detection of 11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid in Hair

Application Note

Forensic Toxicology/Doping Control

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Abstract

A robust method for the detection of the THCA marijuana metabolite in hair was developed with a run time of 7 min and a cycle time of 9 minutes using column switching and backflushing. The method LOD is 0.002 pg/mg and the LOQ is 0.01 pg/mg.

Introduction

Testing hair for drugs of abuse has been practiced for over 50 years, due in large part to the ability to detect drug use over a longer period of time, as compared to other biological matrices, because many drugs are well-preserved in hair. Hair testing is widely used in criminal investigations. Workplace programs include hair testing due to the ease of collection, difficulty of adulteration and longer detection times.

Marijuana is one of the drugs tested most often in forensic and drug screening applications. The parent compound, tetrahydrocannabinol (THC), is found in higher concentration in hair samples, but detection of the acid metabolite THCA (11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid) is preferred, in order to eliminate the possibility of potential environmental contamination from marijuana smoke. While guidelines for workplace hair testing have not yet been adopted by the Substance Abuse Mental Health Services Administration (SAMHSA) in the United States, a cutoff concentration for nor-9-carboxy-Δ^9-tetrahydrocannbinol as low as 0.05 pg/mg hair has been suggested, and such guidelines are a topic of additional study and analysis by this regulatory body. The Society of Hair Testing recommends a limit of quantification (LOQ) of $\leq$ 0.2 pg/mg for THCA.

Agilent Technologies

This application note describes a method developed on the Agilent 7890A GC System coupled with an Agilent 7000B Triple Quadrupole GC/MS System that provides rapid and sensitive detection of a THC metabolite in hair, using 2-D GC and negative ion chemical ionization (CI) MS/MS in the multiple reaction monitoring (MRM) mode (also called SRM, Selected Reaction Monitoring). The method is modified from a previous GC/MSD method [1] to take advantage of the lower chemical background and higher sensitivity provided by triple quadrupole MS/MS analysis. Backflush is used to increase robustness, and low thermal mass (LTM) column modules speed the chromatography process, enabling a run time of 7 min and a cycle time of 9 min. MRM MS/MS analysis on the Triple Quadrupole GC/MS System delivers excellent sensitivity, with an LOD of 0.002 pg/mg and an LOQ of 0.01 pg/mg.

Experimental

Standards and Reagents

Tri-deuterated THCA, which was used as the internal standard (100 µg/mL in methanol), and unlabelled THCA (100 µg/mL in methanol) were obtained from Cerilliant, (Round Rock, TX). The internal standard concentration in the method was 0.05 pg/mg of hair.

Methanol, acetonitrile, toluene, ethyl acetate, hexane, glacial acetic acid, and methylene chloride were obtained from Spectrum Chemicals (Gardena, CA). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Bond Elut Certify I solid-phase extraction columns (130 mg) from Agilent, Inc. (Walnut Creek, CA), or Clean Screen ZSTHC020 extraction columns (200 mg) from United Chemical Technologies, Inc. (Bristol, PA) were interchangeable for the assay. The derivatizing agents, pentafluoropropionic anhydride (PFPA) and 1,1,1, 3, 3, 3-hexafluoro-2-propanol (HFIP), were purchased from Sigma –Aldrich (St. Louis, MO) and Campbell Science (Rockton, IL), respectively.

Instruments

The experiments were performed on an Agilent 7890N GC System equipped with a multimode inlet (MMI) and an LTM System, coupled to an Agilent 7000B Triple Quadrupole GC/MS System. Two dimensional chromatography was performed using a pre-column for backflushing, two Low Thermal Mass (LTM) columns connected by a Deans Switch, and a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m × 0.15 mm × 1.2 µm DB-1 (p/n 12A-1015)
Analytical columns	
Column 1	15 m × 0.25 mm × 0.25 µm DB-1ms LTM Column Module (p/n 122-0112LTM)
Column 2	15 m × 0.25 mm × 0.25 µm DB-17ms LTM Column Module (p/n 122-4712LTM)
Injection volume	2 µL
Inlet temperature	Isothermal at 250 °C
Injection mode	0.75 minute pulsed splitless at 35 psi
Oven temperatures	
GC oven	7 minute hold at 250 °C (isothermal)
1st LTM module	50 sec hold at 100 °C
	100 °C to 210 °C at 200 °C/min
	210 °C to 267 °C at 10 °C/min
	Hold at 267 °C for 2 min
2nd LTM module	324 sec hold at 100 °C
	100 °C to 230 °C at 200 °C/min
	230 °C to 240 °C at 10 °C/min
	Hold at 240 °C for 2 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 26.6 psi; Column 2: 19.6 psi
Transfer line temp	300 °C
MS conditions	
Tune	Autotune
EMV Delta	1200 V
Acquisition parameters	NCI mode; multiple reaction monitoring (MRM)
Reagent gas	Ammonia, 35% flow
Collision gas	Argon, constant flow, 0.9 mL/min
Quench gas	Helium, constant flow, 0.5 mL/min
Solvent delay	6.2 min
MS temperatures	Source 150 °C; Quadrupole 150 °C

Sample Preparation

Samples were prepared as previously described [2]. Calibrators, controls or hair specimens (20 mg) were weighed into silanized glass tubes and washed with methylene chloride (1.5 mL). The solvent was decanted and the hair samples were allowed to dry. The internal standard, THCA-d3 (0.05 pg/mg), was added to each hair specimen. For the calibration curve, unlabelled THCA was added to the hair at concentrations of 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

Deionized water (0.5 mL) and 2N sodium hydroxide (0.5 mL) were added, and the hair was heated at 75 °C for 15 min. The sample was allowed to cool and then centrifuged (2500 rpm, 15 min). The supernatant was poured into glass tubes already containing acetic acid (1 mL), 1 M acetic acid (3 mL), and 0.1 M sodium acetate buffer (pH 4, 2 mL). The tubes were capped and mixed.

SPE columns were conditioned with hexane/ethyl acetate (75:25, v/v; 2 mL), methanol (3 mL), deionized water (3 mL), and 0.1 M hydrochloric acid (1 mL). The acidified samples were loaded onto the SPE columns and allowed to dry. The SPE columns were washed with deionized water (2 to 3 mL) and allowed to dry for 5 min. The SPE columns were washed with 0.1 M hydrochloric acid/acetonitrile (70:30 v/v; 3 mL) and allowed to dry at 30 psi for 10 min. The SPE columns were finally rinsed with hexane/ethyl acetate (75:25 v/v; 3 mL) in order to elute the THCA into silanized glass tubes.

The eluent was evaporated to dryness under nitrogen at 40 °C and reconstituted in PFPA (70 µL) and HFIP (30 µL) for derivatization. The mixture was transferred to autosampler vials with glass inserts and capped. The vials were heated at 80 °C for 20 min, then left at room temperature for 10 min. The extracts were evaporated to dryness in a vacuum oven. The samples were finally reconstituted in toluene (50 µL), for injection into the GC–MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS System parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THCA*	6.714	620→492	50	5
		620→383	50	5
THCA-d3	6.710	623→495	20	5
		623→386	20	5

*11-nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid

Results

Two Dimensional Gas Chromatography with Heart-Cutting

The use of two serial GC columns to separate background from the required peak is a well-established technology that is widely used to provide excellent separation of the analyte from matrix interferences. Once the analyte retention time on the first column has been determined, the pneumatic switch (Deans Switch) is turned on at that time to divert the flow to the second column, and turned off a short time later. This diverts a narrow, heart-cut "window" of the effluent from the first column that contains the analyte and minimal background, for further separation on the second column (Figure 1). The two columns function optimally when the stationary phases are as different as possible.

Exceptional Robustness and Speed

The unique combination of backflushing and low thermal mass (LTM) column modules make this a very robust and rapid method, compared to the traditional single column approach. Three independently programmed pressure zones are used in conjunction with three independently heated zones (Figure 1). The pre-column and the first LTM column are coated with relatively non-polar DB-1ms phase, and the second LTM column is coated with a more polar DB-17ms phase. The heart-cut window is only 0.2 min (5.5 to 5.7 min) wide.

A unique system for rapid and robust detection of THCA in hair

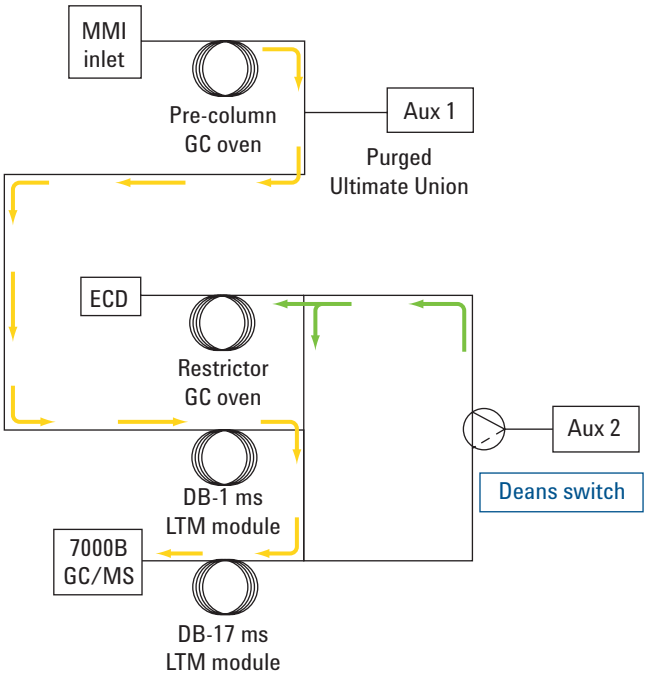

Figure 1. Schematic representation of the system used to develop the THCA method.

The precolumn and auxiliary pressure control module (AUX EPC) provides backflushing capability to protect and preserve the LTM analytical columns. The precolumn was in backflush mode with a constant pressure of 1 psi during the run. The inlet pressure pulse overrides the backflush for the initial 0.75 min. The use of backflushing prevents build-up of high-boiling compounds on the column, thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened.

This method also employs LTM column modules external to the GC oven that enable independent and optimal temperature control of the two analytical columns (Figure 2). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors, and they can be controlled from the GC software.

The end result of this unique backflushing and LTM approach is a robust method that provides excellent quantification and sensitivity (see next section) with 7 min run times and 9 min cycle times.

Unique LTM Column Modules enable rapid temperature ramping and cooling

Figure 2. Low thermal mass (LTM) column modules interfaced with the Agilent 7890A GC.

Sensitivity and Quantification

This method has a limit of detection (LOD) of 0.002 pg/mg, demonstrating excellent sensitivity that is far below the suggested cutoff of 0.05 pg/mg (Figure 3). The accuracy of quantification is also quite good, with an R^2 of 0.995, from 0.002 to 0.5 pg/mg of hair (Figure 4). The limit of quantification (LOQ) is 0.01 pg/mg, which again is more than an order of magnitude below the 0.2 pg/mg LOQ suggested guideline established by the Society of Hair Testing (Figure 5). This method also provides a compliant quantitative analysis report that includes the retention times (with limits), response level, qualifier ion ratio (with limits), and the calculated concentration. The total ion current (TIC) trace and the quantifier and qualifier MRM traces are also displayed on the report, for both the sample and the THCA-d3 internal standard (Figure 6).

LOD of 0.002 pg/mg

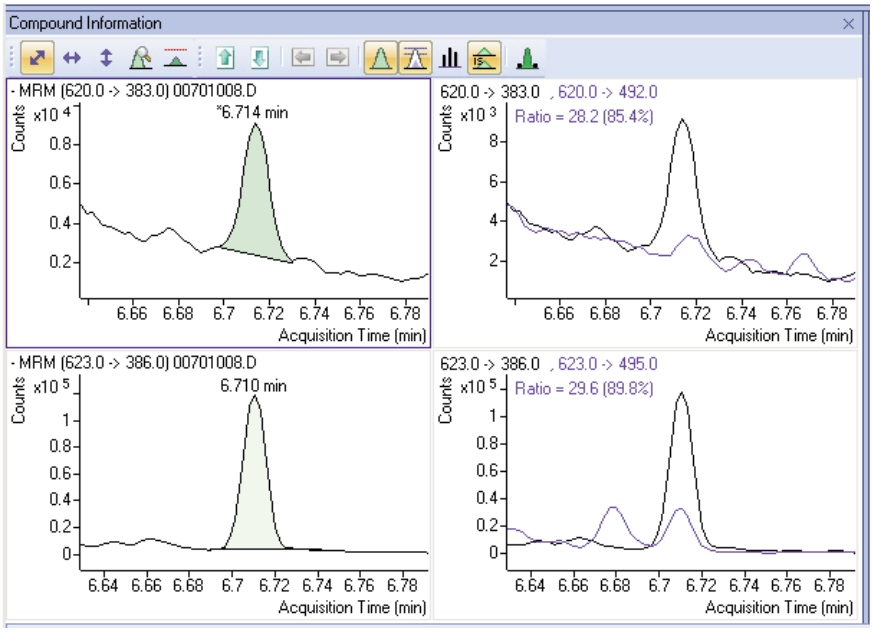

Figure 3. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.002 pg/mg LOD of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Reliable calibration

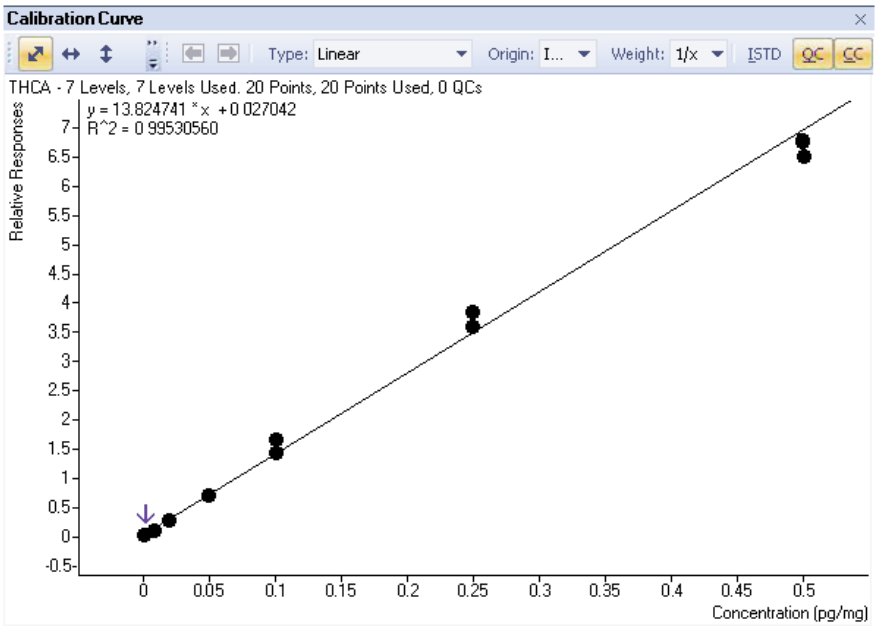

Figure 4. Calibration curve for THCA spiked into hair samples at 0.002, 0.01, 0.02, 0.05, 0.1, and 0.5 pg/mg of hair.

0.01 pg/mg LOQ

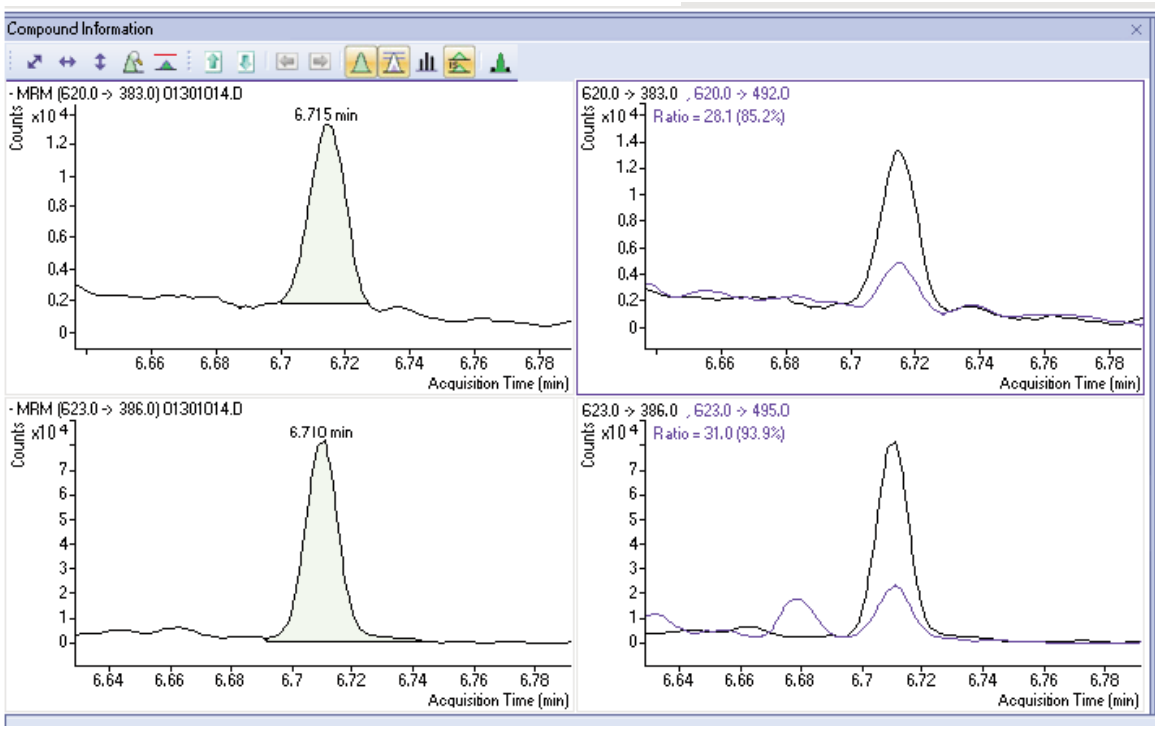

Figure 5. MRM traces for the quantifying transition (left) and both the quantifying and qualifying transitions (right) for the 0.01 pg/mg LOQ of THCA (upper panel) and the deuterated standard (lower panel) spiked into a hair sample.

Data File	01401015.D	Vial	14
Operator	DATASYSTEM01/Admin	Dillution	0.0
Acq method name		Sample information	
Acquisition date	2010-10-08 16:24	Last calib update	2010-11-28 09:34
Sample name and path	0.01 pg/mg, D:/MassHunter/GCMS/1/data/PFAA Curve Extracted/		

Compound	Signal	RT	Limits	Response	QRatio	Limits	Final conc
THCA-d3	623.0 -> 386.0	6.71		82558		35770 - 143081	
	623.0 -> 495.0			24962	30.2	23.1 - 42.9	
THCA	620.0 -> 383.0	6.71	6.38 - 7.05	10999			0.008
	620.0 -> 492.0			3908	35.5	23.1 - 42.9	

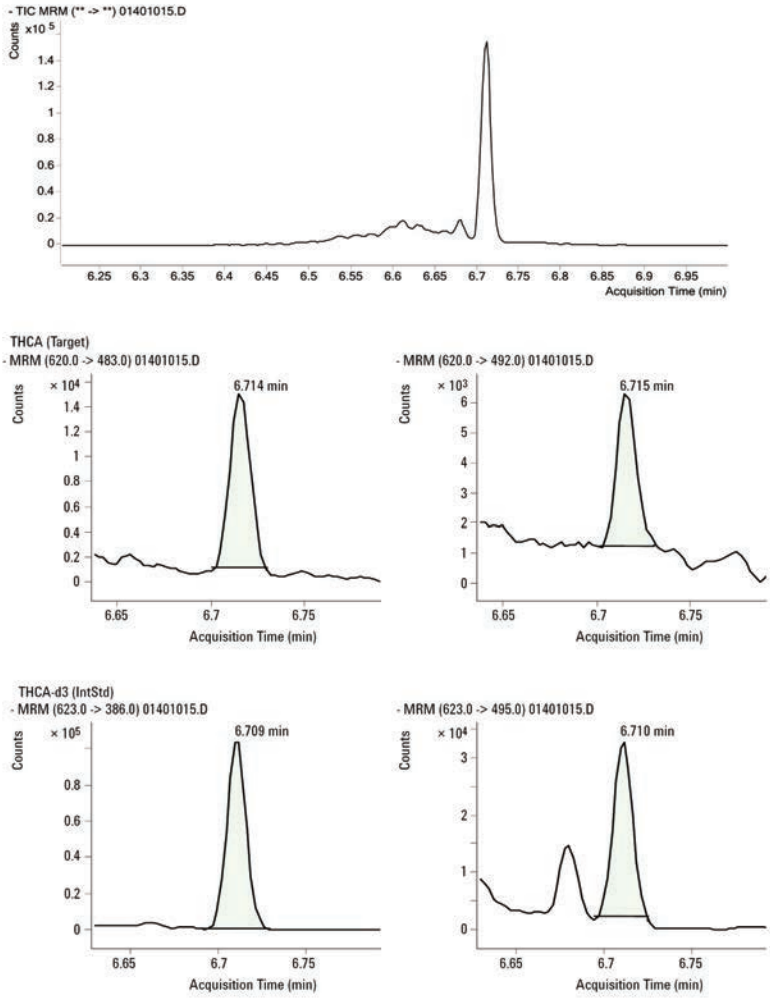

Figure 6. Quantitative Analysis Sample Report for a 0.01 pg/mg (the LOQ) sample spiked into hair.

Conclusion

The time-proven technique of heart-cutting to improve chromatographic separation is given new life in this unique method which utilizes state-of-the-art microfluidics-aided backflushing and low thermal mass column temperature ramping modules to deliver sensitive and robust detection and quantification of THCA in hair (LOD 0.002 pg/mg; LOQ 0.01 pg/mg) with run times of only 7 minutes, and cycle times of 9 minutes.

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2. C. Moore, S. Rana, C. Coulter, F. Feyerherm, H. Prest, "Application of Two-dimensional Gas Chromatography with Electron Capture Chemical Ionization Mass Spectrometry to the Detection of 11-nor-D9-Tetrahydrocannabinol-9-carboxylic acid (THCA) in Hair", J. Anal. Toxicol. 30, 171–177 (2006).

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Application Note SI-02313

Forensic Toxicology

Fast GC/MS/MS of Androgenic Anabolic Steroids in Urine Using a VF-5ms Column

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Johan Kuipers, Varian, Inc.

Introduction

The use of anabolic steroids in sport is prohibited by the World Anti-Doping Agency. Athletes are therefore subject to continuous screening for these banned substances. The analysis of large numbers of samples in a short time with a high degree of specificity is an important requirement for any screening program. The key factor is the use of a rapid gas chromatographic method in combination with a sensitive detector. This note describes a fast and sensitive method to screen 13 anabolic androgenic steroids within 12 minutes, based on a short FactorFour™ VF-5ms GC column and multiple reaction monitor (MRM) detection. This method is approximately twice as fast than a classical steroids' method analysis.

Sample Preparation

Urine (2 mL) was prepared by adding 17a-methyltestosterone as an internal standard, and the 13 compounds at concentrations of 2, 5 ng/mL. The urine sample was then buffered to pH 6 and incubated at 55 °C for one hour after the addition of 50 µL of ß-glucuronidase. The hydrolyzed urine was passed through an SPE cartridge, which was conditioned successively with methanol and water. The column was rinsed with water, 10% methanol in water, and hexane. The steroids were then eluted with methylterbutyl ether. The eluate was evaporated to dryness and subsequently derivatized with 50 µL of MSTFA/NH_4I/dithioerythritol at 60 °C for 20 minutes.

Conditions

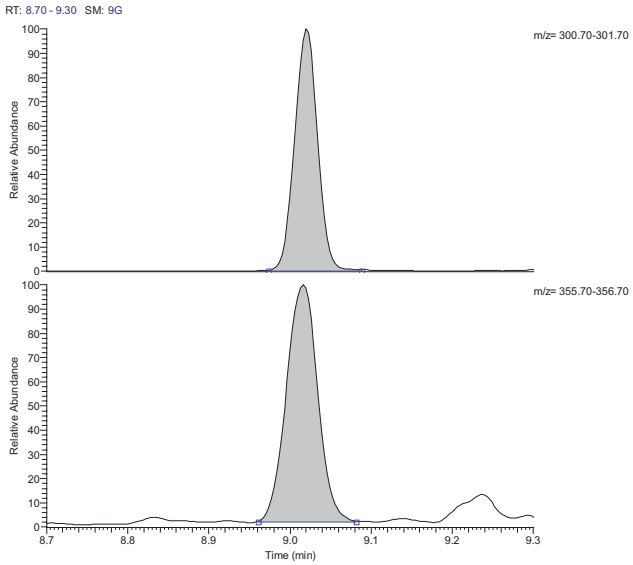
Column:	FactorFour VF-5ms, 10 m x 0.15 mm x 0.15 µm (Part no: CP9034)
Cartridge:	BondElut™ C18, 200 mg
Sample Vol:	3 µL
Carrier Gas:	0.5 mL/min Helium, constant flow
Injector:	250 °C, split ratio 1:10
Temp Gradient:	170 °C for 0.5 min, 10 °C/min to 260 °C, 50 °C/min to 320 °C (1 min)
Detector:	Triple quadripole GC, 70 eV EI Mode, ion source 250 °C

Results

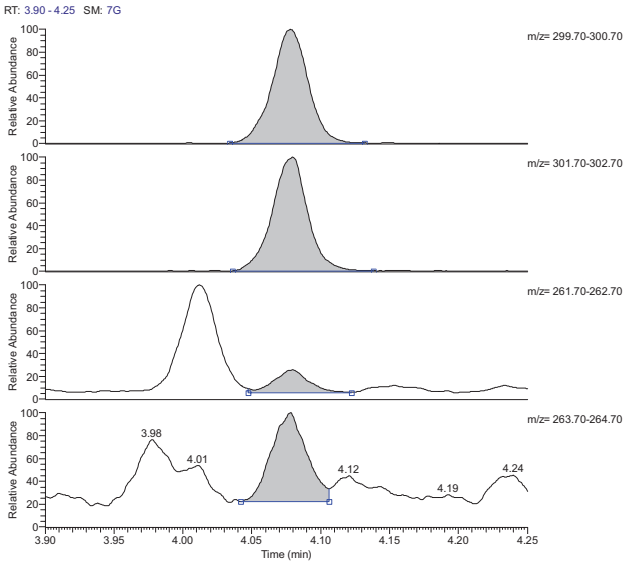
Table 1 shows the characteristics of the 13 steroids. Figure 1 shows the mass spectra obtained using the method described.

Table 1. Anabolic steroids, detection level in sample, retention time, associated precursors and daughter ions.

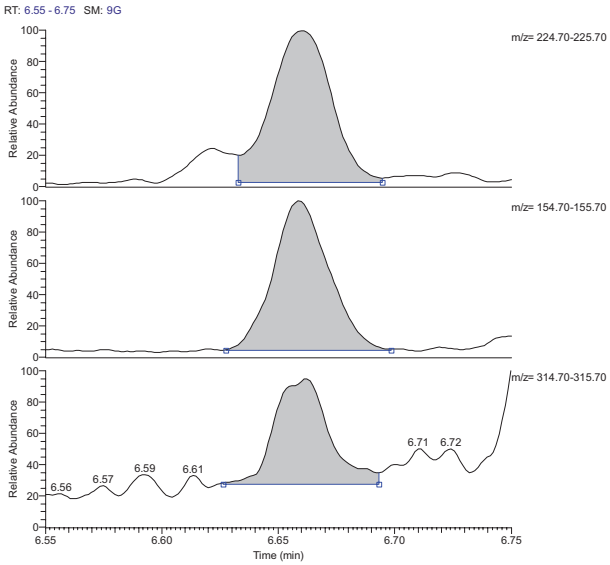
Compounds	Detection level (ng/mL)	Retention time (min)	Relative retention time	Precursor ion	Daughter ions		
17a-Methyltestosterone (ISTD)	200	9.02	-	446	301	356	
Clenbuterol	2	4.08	0.452	335	300	262	
				337	302	264	
19-Norandrosterone	2	6.66	0.738	405	225	155	315
Epimethenediol	2	6.92	0.767	358	301		
19-Noretiocholanolone	2	7.12	0.789	405	155	225	315
17-Epimethanedienone	2	8.26	0.916	444	206	339	
5a-Methyltestosterone	2	8.01	0.888	435	255	345	
5b-Methyltestostérone	2	8.07	0.895	435	255	345	
Norethandrolone metabolite	5	8.67	0.961	421	331	241	
Ethisterone	2	9.17	1.017	456	316	301	208
Bolasterone	5	9.05	1.003	460	445	355	315
Calusterone	5	9.14	1.013	460	445	355	315
6ß-Hydroxymethanedienone	2	9.74	1.080	517	229	317	281
Fluoxymesterone metabolite	5	9.33	1.034	552	495	319	
				462	337		



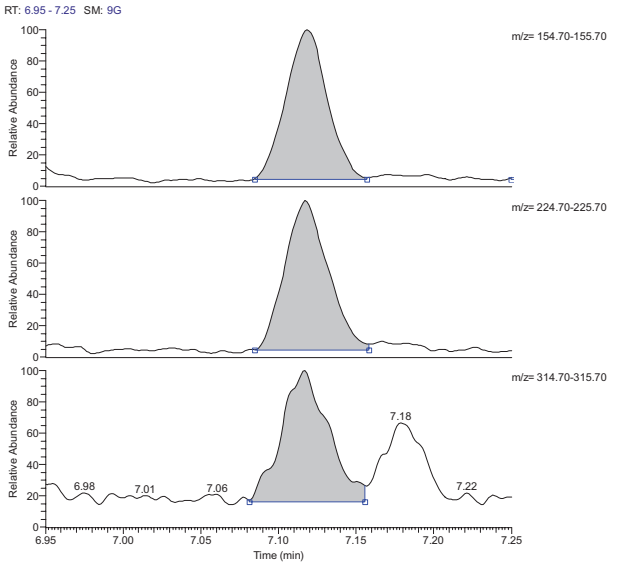
17a-Methyltestosterone (ISTD).



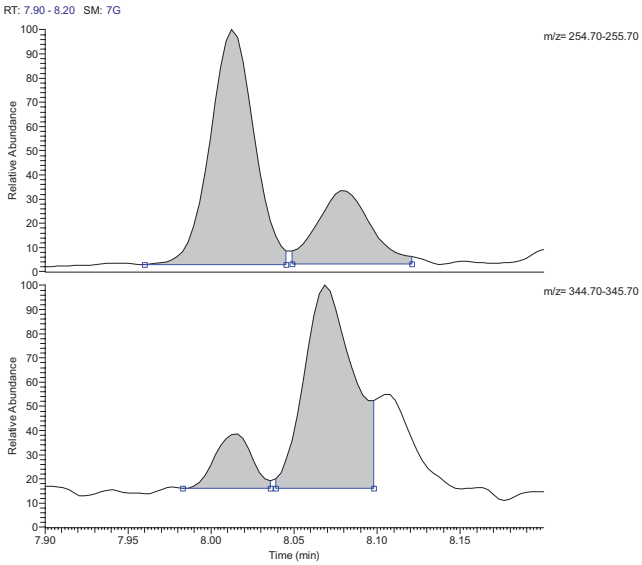
Clenbuterol.



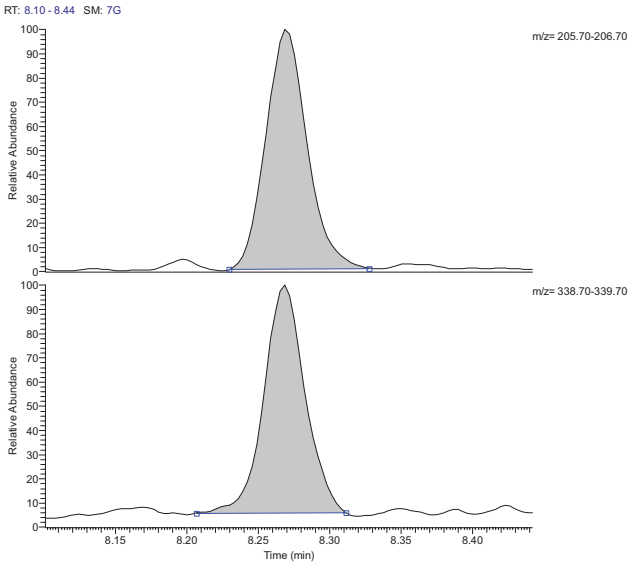
19-Norandrosterone.



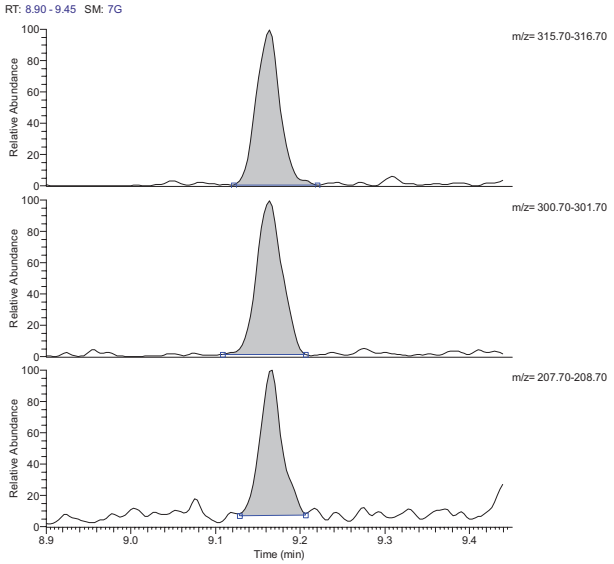
19-Noretiocholanolone.



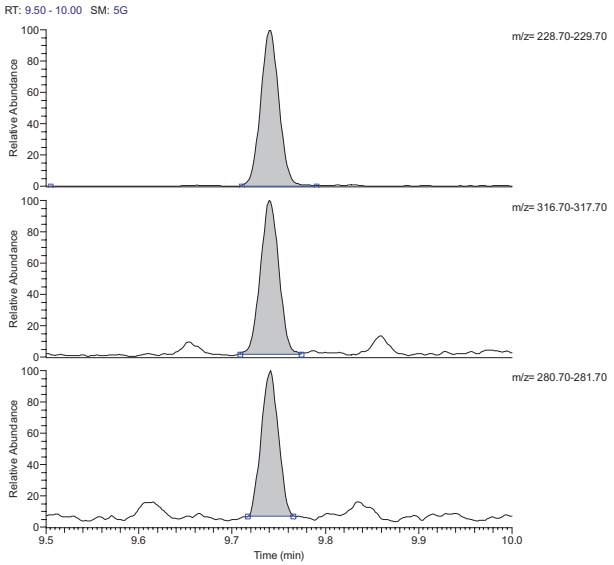
5a-Methyltestosterone, 5b-Methyltestosterone.



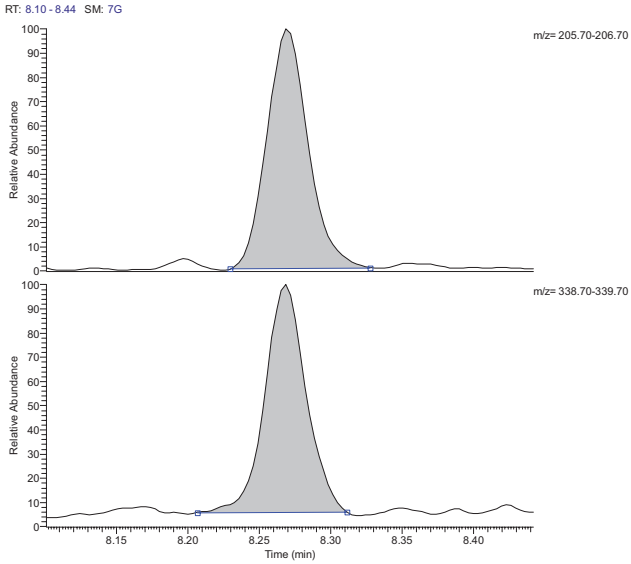
17-Epimethanedienone.



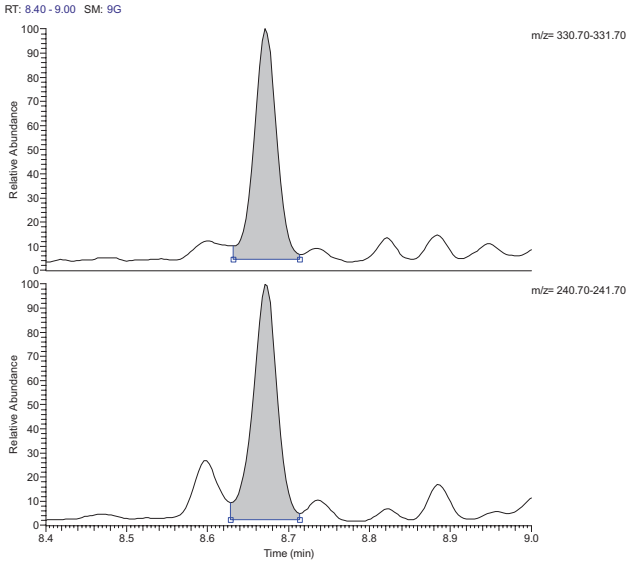
Ethisterone.



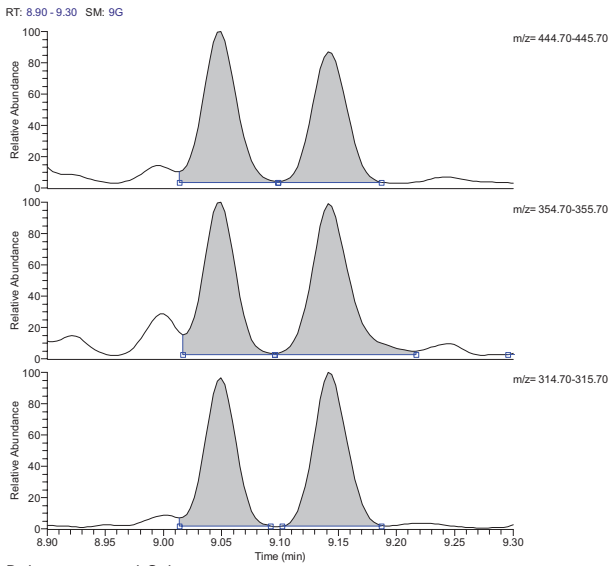
6ß-Hydroxymethanedienone.



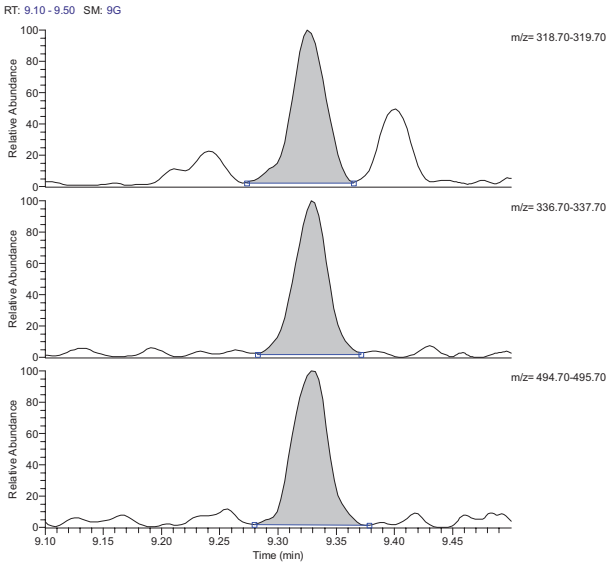
Epimethenediol.



Norethandrolone metabolite.



Bolasterone and Calusterone.



Fluoxymesterone metabolite.

Figure 1. Mass spectral information of the anabolic steroids.

Conclusion

The GC/MS/MS method described here detected ten anabolic steroids commonly tracked as banned substances using a VF-5ms capillary column. The method was optimized for a fast analysis speed, while maintaining important chromatographic separations of structurally related steroids that exhibited identical MRM fragmentation patterns. This approach permitted rapid detection of prohibited substances and delivered specific information on the compound detected.

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Application Note SI-02313

Rapid and Robust Detection of THC and Its Metabolites in Blood

Application Note

Forensic Toxicology/Doping Control

Author

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Abstract

A robust method for detection of THC and its metabolites in blood has been developed using SPE extraction and GC/MS/MS with backflushing. The dynamic range of quantification was 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA, with a run time of 6 minutes and a cycle time of 8 minutes.

Introduction

In the past decade, a great deal of research concerning the impact of cannabis use on road safety has been conducted. More specifically, studies on effects of cannabis smoking on driving performance, as well as epidemiological studies and cannabis-detection techniques have been published. As a result, several countries have adopted driving under the influence of drugs (DUID) legislation, with varying approaches worldwide. While a wide variety of bodily fluids have been used to determine the presence of cannabis, blood testing is considered the most reliable indicator of impairment. Blood testing for active tetrahydrocannabinol (THC) may also be considered by employers who wish to identify employees whose performance may be impaired by their cannabis use. Gas chromatography/mass spectrometry (GC/MS) is a standard method for detection and quantification of THC and its metabolites in blood.

One key to reliable THC testing in blood is an efficient extraction method. The use of tandem MS (MS/MS) also increases the sensitivity and reliability of quantification of THC and its metabolites in blood, due to the elimination of interferences. This application note describes a method using the High Flow Bond Elut Certify II SPE cartridge to rapidly and efficiently extract THC and its metabolites from blood. The extracts were derivatized to improve volatility and analyzed on the Agilent 7890A Triple Quadrupole GC/MS system equipped with a Low Thermal Mass Module (LTM)

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oven and backflushing. It was in turn coupled with an Agilent 7000B Triple Quadrupole GC/MS system, using MS/MS in the multiple reaction monitoring (MRM) mode to provide rapid and sensitive detection of THC and its metabolites, 11-OH-THC (11-hydoxy-Δ9-tetrahydrocannbinol) and THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid). Backflushing was used to increase robustness and speed, enabling a run time of 6 minutes and a cycle time of 8 minutes. MRM MS/MS analysis on the Triple Quadrupole GC/MS system delivers excellent results, with a dynamic range of 0.1 to 50 ng/mL.

Experimental

Standards and Reagents

Tri-deuterated THC, 11-OH-THC and THCA, which were used as internal standards (100 µg/mL in methanol), and unlabelled THC, 11-OH-THC and THCA (100 µg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). The internal standard concentrations in the method were both 10 µg/mL.

Methanol, acetonitrile, toluene, ethyl acetate, hexanes, glacial acetic acid, and methylene chloride were obtained from Sigma Aldrich (St. Louis, MO). All solvents were high-performance liquid chromatography (HPLC) grade or better, and all chemicals were ACS grade. Agilent High Flow Bond Elut Certify II solid-phase extraction columns were used for the method. The derivatizing agents, BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) were purchased from Cerilliant. Normal human whole blood stabilized with potassium oxalate and sodium fluoride was obtained from Bioreclamation (Hicksville, NY). Standards were prepared in this drug-free matrix to construct the calibration curves.

Instruments

The experiments were performed on an Agilent 7890N gas chromatograph equipped with a multimode inlet (MMI) and an LTM oven, coupled to a 7000B Triple Quadrupole GC/MS. Chromatography was performed using a pre-column for backflushing, and a Low Thermal Mass (LTM) column connected by a Purged Ultimate Union (Figure 1). The instrument conditions are listed in Table 1.

a.

Loading the sample on the pre-column

b.

Backflushing the pre-column and separation of THC and its metabolites on the primary column

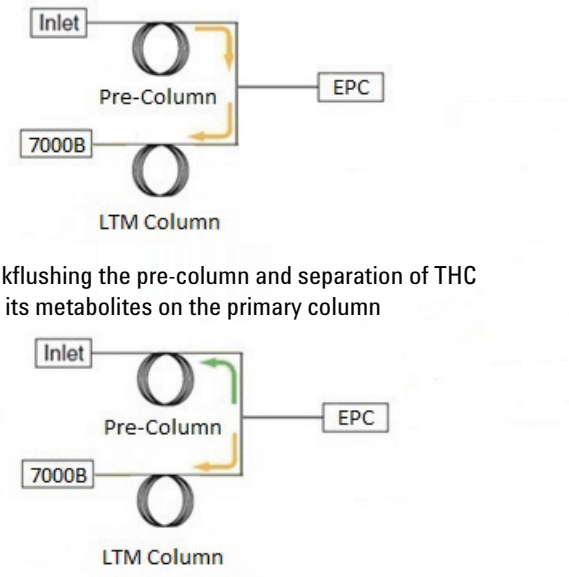


Figure 1. Schematic representation of the backflush system used to develop the method. EPC: Electronic Pneumatic Control module; 7000B: Agilent Triple Quadrupole GC/MS system

Table 1. Agilent 7890N/7000B Gas Chromatograph and Triple Quadrupole Mass Spectrometer Conditions

GC Run Conditions	
Pre-column	1 m section from a 15 m × 0.25 mm, 0.25 µm HP-5 ms Ultra Inert column (p/n 19091S-431UI)
Analytical column	15 m × 0.25 mm, 0.25 µm DB-17 ms LTM Column Module (p/n 122-4712LTM)
Injection volume	1 µL
Inlet temperature	Isothermal at 280 °C
Injection mode	0.5 min pulsed splitless at 35 psi
Oven temperatures	GC oven: 6 min hold at 280 °C (isothermal)
LTM module:	50 second hold at 100 °C 100 °C to 230 °C at 200 °C/min 230 °C to 280 °C at 10 °C/min Hold at 280 °C for 1 min
Carrier gas	Helium in constant pressure mode. Pre-column: 1 psi; Column 1: 5 psi; Column 2: 9.6 psi
Transfer line temp	300 °C
MS Conditions	
Tune	Autotune
Gain	20
Acquisition parameters	EI mode; multiple reaction monitoring (MRM)
Collision gas	Nitrogen constant flow, 1.5 mL/min
Quench gas	Helium, constant flow, 2.25 mL/min
Solvent delay	3.0 min
MS temperatures	Source 230 °C; Quadrupole 150 °C

Sample Preparation

A 2 mL blood sample containing 10 µg/mL of each internal standard (ISTD) and spiked with THC, 11-OH-THC and THCA was pipetted into a clean tube, and 4 mL of acetonitrile was added. After centrifugation at 2500 rpm for 5 minutes, the supernatant was transferred and evaporated to about 3 mL with nitrogen at 35-40 °C, and 7 mL of 0.1 M sodium acetate (pH 6.0) was added.

High Flow Bond Elut Certify II SPE columns were conditioned with 2 mL of methanol, then 2 mL 0.1 M sodium acetate buffer, pH 6.0 with 5% methanol. Cartridges were not be allowed to go to dryness prior to sample addition. The sample was drawn through the column slowly, at 1 to 2 mL/min. The column was then washed 2 mL sodium acetate buffer, pH 6.0, dried under maximum vacuum for approximately 5 minutes, then washed with 1 mL hexanes. THC was eluted under neutral conditions with 2 mL of 95:5 hexane: ethyl acetate. This was followed by a 5 mL 1:1 methanol:deionized water wash. The column was again dried under maximum vacuum for approximately 5 minutes and washed again with 1 mL hexanes. Elution of 11-OH-THC and THCA was performed with 2 mL 1% acetic acid in 75:25 hexane:ethyl acetate. The THC and the metabolite fractions were combined and dried before derivatization.

The eluent was evaporated under nitrogen at a temperature no higher than 40 °C, then reconstituted in 60 µL of toluene and 40 µL of BSTFA, 1% TMCS for derivatization. The sample tubes were capped and heated 20 minutes at 70 °C before injection into the tandem quadrupole GC/MS system.

Analysis Parameters

The Agilent Triple Quadrupole GC/MS system parameters used are shown in Table 2.

Table 2. Agilent 7000B Triple Quadrupole GC/MS System Analysis Parameters

Compound	RT (min)	MRM	Dwell time (ms)	Collision energy (EV)
THC (Δ9-Tetrahydrocannabinol)	3.5	386→303*	25	20
		386→330	27	10
		386→289	30	25
THC-d3	3.5	389→306*	10	20
		389→330	11	10
		389→292	15	25
11-OH-THC (11-hydoxy-Δ9-tetrahydrocannabinol)	4.5	371→289*	24	20
		371→305	26	15
		371→265	27	15
11-OH-THC-d3	4.5	374→292*	10	20
		374→308	12	15
		374→268	12	15
THCA (11-nor-Δ9-Tetrahydrocannabinol-9-Carboxylic Acid)	5.6	371→289*	23	15
		488→297	44	20
		488→371	29	20
THCA-d9	5.5	380→292*	15	15
		497→306	30	20
		497→380	22	20

*Target transition. All other transitions are qualifier transitions.

Results

SPE Sample Preparation with High Flow Bond Elut Certify II Columns

Screening for drugs of abuse in biological fluids requires rugged methods that provide high purification and recovery. The Bond Elut Certify was developed specifically for the rapid and effective extraction of compounds that possess both non-polar and anionic characteristics from urine and other biological matrices [1]. The mixed mode (non-polar C8 and strong anion exchange) sorbent takes advantage of non-polar, polar, and ion exchange properties to ensure rapid, reproducible, simple, and clean extraction of many drug classes. These columns enable the rapid and high recovery of THC, 11-OH-THC and THCA from whole blood.

Backflushing

Backflushing makes this a very robust and rapid method, preventing build-up of high-boiling compounds on the column and thus reducing retention time shifts, peak distortion, and chemical noise, while improving quantification. Contamination of the MS source and the resultant need for cleaning are also reduced, while the run time is shortened. The end result is a robust method that provides excellent dynamic range with 6 minute run times (not including sample prep) and 8 minute cycle times.

The suite of Agilent Capillary Flow Technology modules enables easy and rapid backflushing with minimal dead volumes for maintaining chromatographic resolution. During injection, the inlet Pneumatic Control Module (PCM) is held at an elevated pressure long enough to transfer the target analytes from the pre-column to the analytical column (Figure 1a). When backflushing, the inlet pressure is dropped to 1 psi, forcing the flow to reverse through the pre-column and out the split vent (Figure 1b). In this way, THC, 11-OH-THC and THCA are passed on to the primary column for further separation, while high-boiling compounds are swept back though the split vent.

Low Thermal Mass Modules

This method also employs a Low Thermal Mass (LTM) column module external to the GC oven that enables independent and optimal temperature control of the analytical column (Figure 1). The unique design of these modules makes it possible to employ very fast temperature ramping and rapid cooling. The LTM column modules can be added to an Agilent GC without requiring any changes in the injectors, autosamplers, or detectors.

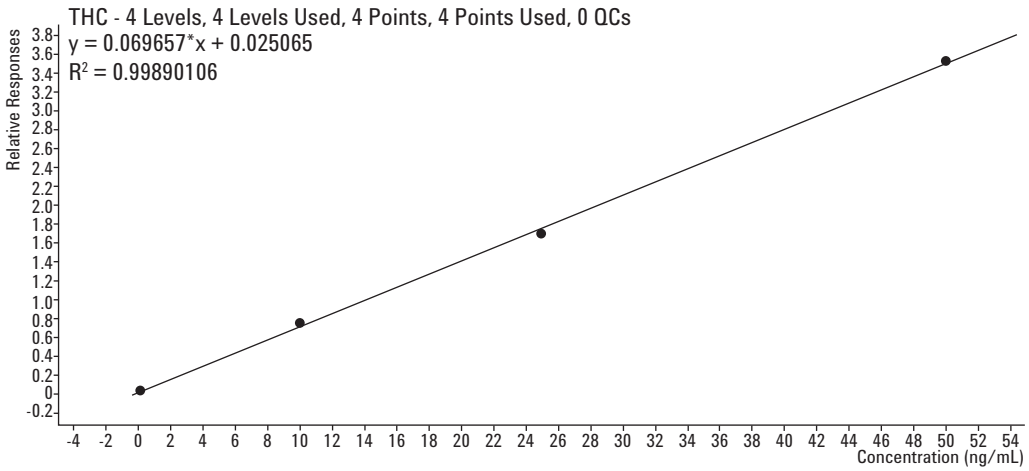
Dynamic Range

This method has a dynamic range of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA (Figure 2), which match industry norms. The accuracy of quantification is also quite good, with an R^2 of 0.999 for all three analytes.

MRM Results

Using a MassHunter forensic report template, Quantitative Analysis Sample Reports were quickly and easily prepared for THC and its two analytes (Figures 3-5), featuring a Total Ion Current (TIC) chromatogram and spectra for all of the transitions, including the internal standard. Note the lack of interference in all of the transitions, even at the lowest end of the dynamic range for each analyte.

a.



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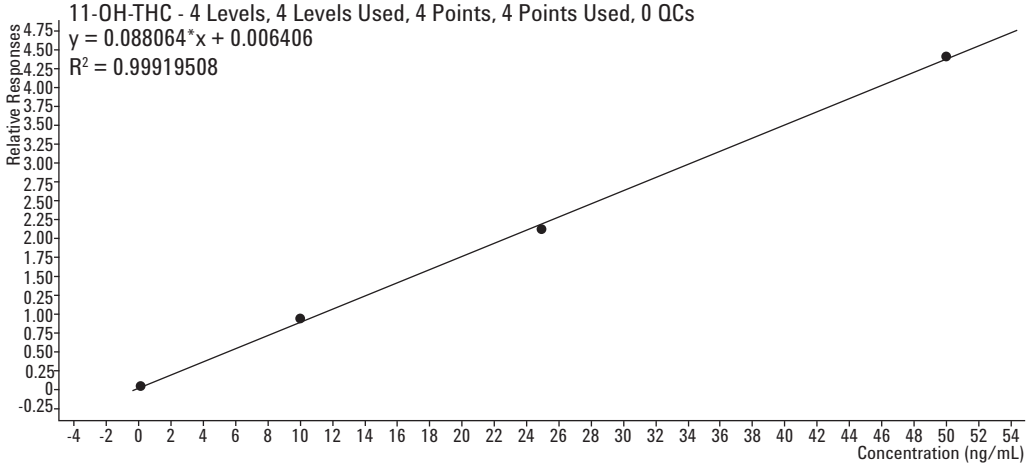

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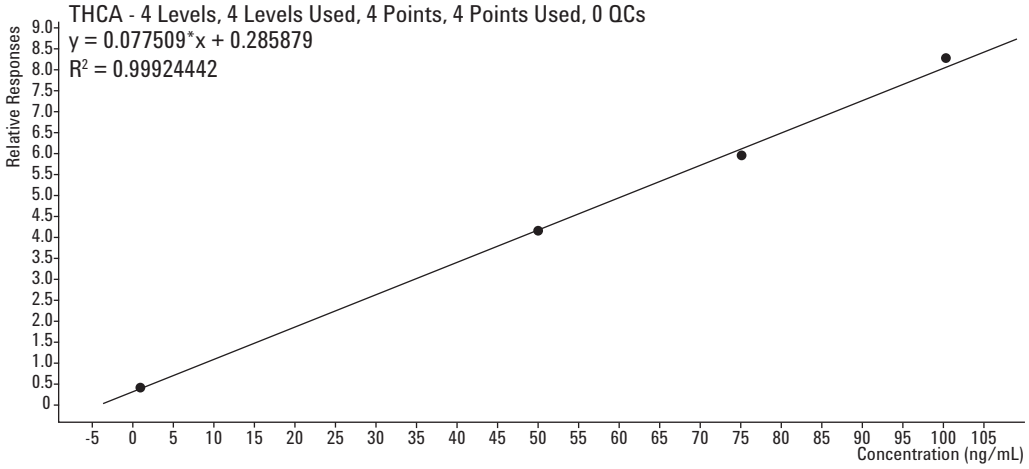
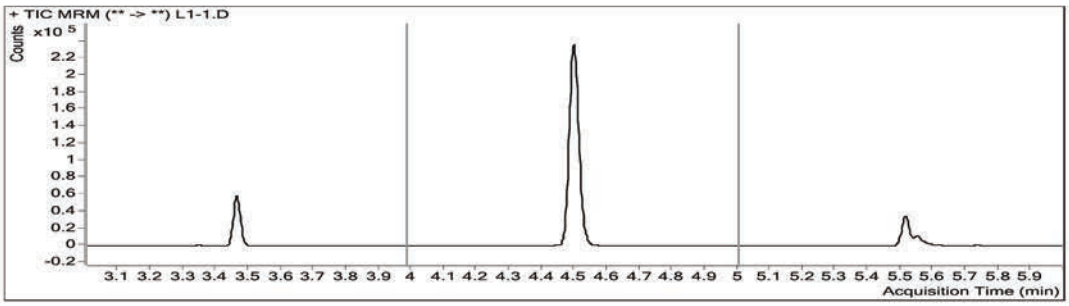


Figure 2. Calibration curves for THC (a), 11-OH-THC (b) and THCA (c) in blood. Data points were taken at 0.1, 10, 25, and 50 ng/mL for THC and 11-OH-THC, and at 1, 50, 75, and 100 ng/mL for THCA.

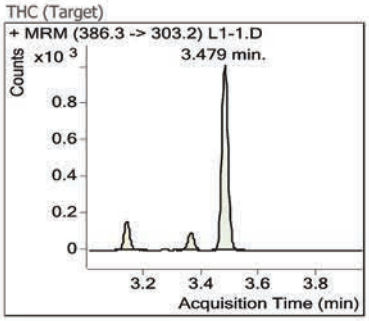
Quantitative Analysis Sample Report

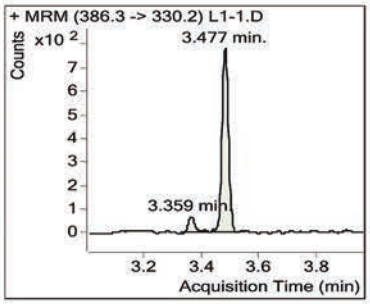
Data File	:	L1-1.D
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

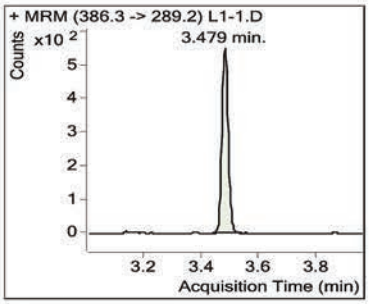
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
D3-THC	389.3 -> 306.2	3.46		37956			
	389.3 -> 330.2			32853	86.6	64.3-104.3	
	389.3 -> 292.2			18032	47.5	22.8-62.8	
THC	386.3 -> 303.2	3.48	3.29 - 3.64	1554			0.100
	386.3 -> 330.2			1237	79.7	62.4-102.4	
	386.3 -> 289.2			848	54.6	23.6-63.6	



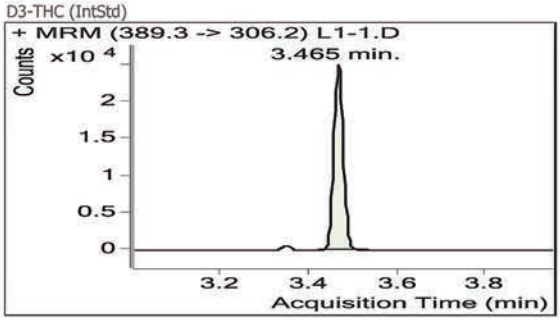
THC (Target)







D3-THC (IntStd)



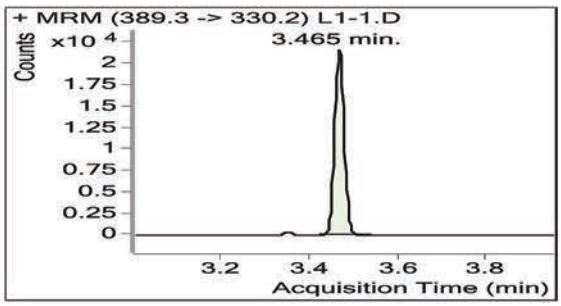


Figure 3. Quantitative Analysis Sample Report for 0.1 ng/mL of THC in blood. The RMS signal-to-noise is 175:1 with a noise region of 3.6 to 3.9 min.

Quantitative Analysis Sample Report

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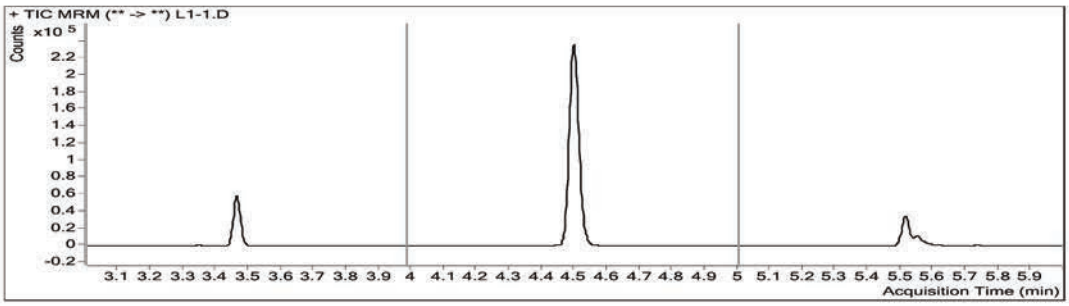
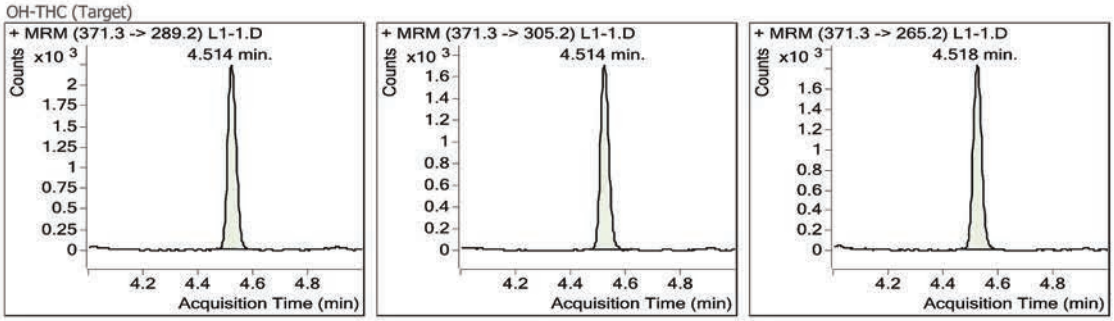
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d3-OH-THC	374.3 -> 292.2	4.5		175705			
	374.3 -> 308.2			157882	89.9	68.8-108.8	
	374.3 -> 268.3			148545	84.5	59.4-99.4	
OH-THC	371.3 -> 289.2	4.51	4.27 - 4.72	4924			0.099
	371.3 -> 305.2			3707	75.3	67.4-107.4	
	371.3 -> 265.2			4050	82.3	58.2-98.2	



OH-THC (Target)



d3-OH-THC (IntStd)

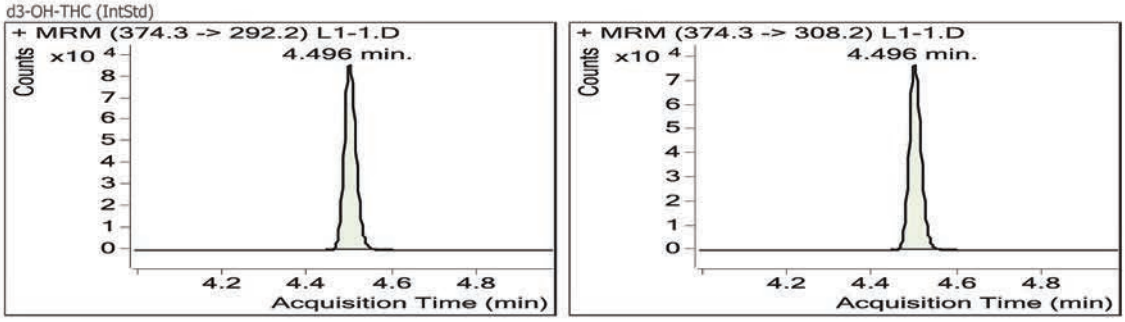


Figure 4. Quantitative Analysis Sample Report for 0.1 ng/mL of 11-OH-THC in blood. The RMS signal-to-noise is 46:1 with a noise region of 4.6 to 4.9 min.

Quantitative Analysis Sample Report

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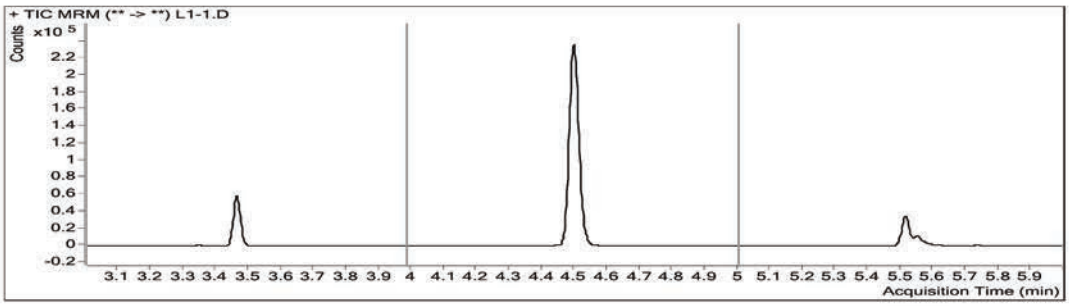
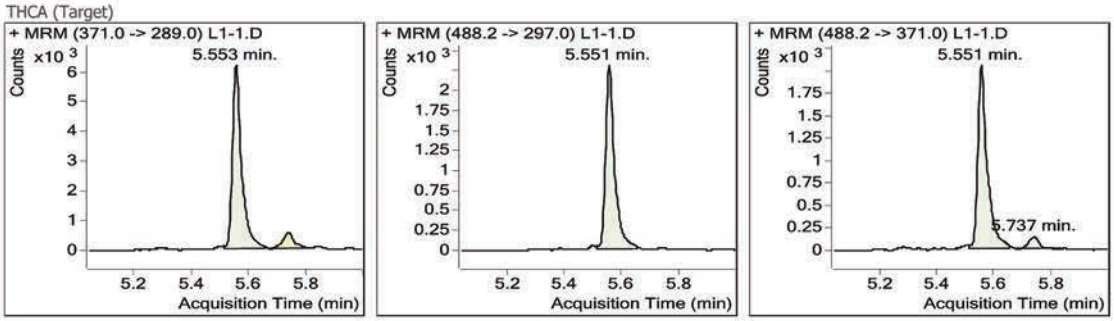
Operator	:	DATASYSTEM01\Admin
Acq Method Name	:	LTM.THC.OMRM.7000.eiex.m
Acquisition date	:	2011-04-28 18:19
Sample Name & Path	:	L1-1, D:\MassHunter\GCMS\1\data\04-28-2011-redo\
Vial	:	3
Dilution	:	0.0
Sample Info	:	
Last Calib Update	:	2011-05-05 13:57

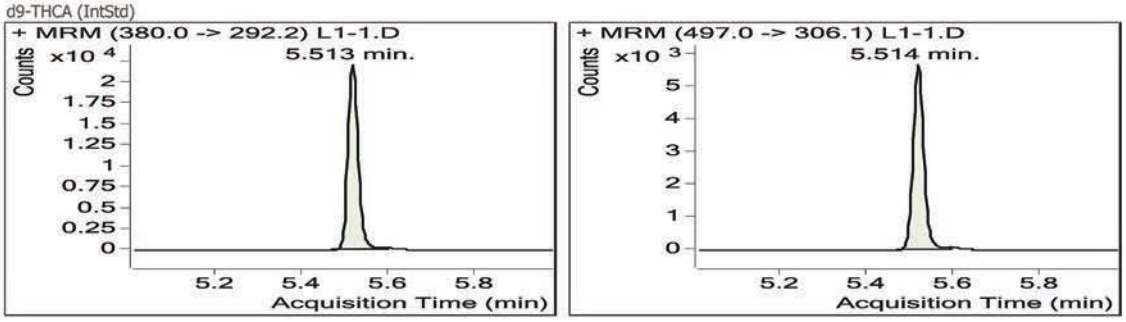
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
d9-THCA	380.0 -> 292.2	5.51		38358			
	497.0 -> 306.1			9800	25.5	5.6-45.6	
	497.0 -> 380.1			11765	30.7	12.5-52.5	
THCA	371.0 -> 289.0	5.55	5.26 - 5.82	13985			1.015
	488.2 -> 297.0			5124	36.6	9.1-49.1	
	488.2 -> 371.0			4758	34	14.4-54.4	



THCA (Target)



d9-THCA (IntStd)



DrugQuantReport_Version4-2Qualifiers.xlsx Page 6 of 15 Printed at: 2:00 PM on: 5/5/2011

Figure 5. Quantitative Analysis Sample Report for 1 ng/mL of THCA in blood. The RMS signal-to-noise is 39:1 with a noise region of 5.1 to 5.3 min.

Conclusion

Coupling the Agilent 7890N gas chromatograph utilizing an LTM system with the Agilent 7000B Triple Quadrupole GC/MS system enables a rapid and robust method for the analysis of THC and its metabolites in blood. Using the High Flow Bond Elut Certify II SPE cartridge , backflushing of the GC column, and MRM eliminate all interferences, with a resulting dynamic range of quantification of 0.1 to 50 ng/mL for THC and 11-OH-THC, and 1 to 100 ng/mL for THCA. The LTM module and backflushing facilitate rapid analysis, with a run time of 6 minutes and a cycle time of 8 minutes.

References

1. R.M Sears, Solid Phase Extraction of THD, THC-COOH and 11-OH-THC from Whole Blood, Agilent Technologies Application Note 00315.

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Agilent Technologies

Toxicology Screening of Whole Blood Extracts Using GC / Triple Quadrupole / MS

Application Note

Forensic Toxicology

Authors

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USA

Abstract

The Agilent 7000 GC / QQQ system can provide both high selectivity and high sensitivity for the analysis of drugs. Low-level detection and confirmation of large numbers of target drugs in blood extracts is possible in a single run. Combined with information from a single quadrupole screening instrument like the Agilent GC / NPD / MSD / DRS system, a much more complete picture of each sample is now possible.

Agilent Technologies

Introduction

Toxicology screening is challenging due to the need to look for large numbers of target compounds in samples that contain complex matrix interferences. GC/MS methods are widely used and accepted for this analysis. Full-scan EI methods offer many advantages for broad-range screening, such as unlimited numbers of targets, full spectrum identity confirmation, and library searching for identification of nontargets. Several recent advances in Agilent's GC/MS technology, including retention time locking (RTL), deconvolution reporting software (DRS), and capillary flow technology (CFT), have greatly improved the screening process. Samples can now be screened much more rapidly with fewer false positives and negatives [1].

Screening is usually aimed at drugs in concentrations high enough to cause intoxication or death, and GC/MS in full-scan mode usually provides sufficient sensitivity for this task. Labs routinely monitor drugs down to approximately 100 pg in matrix. For those cases where drugs need to be determined at low or trace levels, single ion monitoring (SIM) mode can be used to improve the sensitivity of the analysis. With the introduction of Agilent's SIM/scan, SIM data can be collected simultaneously with scan data, saving significant analysis time [1]. As an example, the method described in reference 1 screens for 725 compounds in SIM/scan mode with a cycle time of 9.6 minutes injection to injection. This time includes the simultaneous acquisition of scan, SIM (for 27 compounds), and NPD data.

For some drugs, however, there are limitations with SIM. Compounds present in the matrix can result in interferences that prevent detection or confirmation of trace levels of certain target analytes. For these situations, there are two main approachs to solving the problem. The first is to increase the chromatographic selectivity using Agilent's heartcutting 2D-GC technology [2]. This approach uses two columns and a Deans switch to chromatographically isolate the analyte(s) from matrix interferences. With the extremely high separation power of this technique, SIM mode can be used to detect analytes at very low levels due to the reduction in interference.

This approach is relatively simple and cost effective, but in practice, only a few analytes can be determined in one run. A second approach is to increase the mass spectral selectivity using a triple quadrupole mass spectrometer (GC/QQQ). The extremely high selectivity and sensitivity with this approach allows detection of drugs down to sub-picogram levels with minimal matrix interferences. A significant advantage is that it can be used to routinely monitor for large numbers of compounds (up to a few hundred) in a single run.

This note describes using GC/QQQ to detect low and trace levels of drugs in extracts of whole blood. The samples were previously analyzed on a system using GC/MS with SIM/scan, DRS, and simultaneous detection with a nitrogen phosphorus detector. The GC/QQQ is shown to be a powerful complement to the GC/NPD/MSD/DRS system for those cases where trace level detection and confirmation is required.

Experimental

Chemicals and Standards

Analytical reference standard solutions of the drugs in Table 1 were purchased from Cerilliant (Round Rock, TX). Calibration solutions were prepared by appropriate dilution of the reference standards in toluene. For method setup using Q1-scan mode and for product ion scans, a test solution of 1 ng/µL of the drugs was used. For calibration in MRM mode, standard solutions at 10 and 50 pg/µL were used.

Samples

Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single-step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/10th volume.

Instrumentation

Analyses were performed on an Agilent 7890 GC combined with a 7000A Triple Quadrupole MS system. The system was configured with a capillary flow technology 2-way splitter with makeup (option 889) as described in [3] to allow backflushing the column after every run. This prevents heavy matrix components from the blood extracts from fouling the column by removing them at the end of each analysis [1]. The instrumental conditions are listed in Table 2.

Several MRMs were evaluated for each analyte using the 1 ng/µL standard solution. When possible, four were identified for analysis and are listed in Table 2. Although only two are typically used for GC/QQQ analysis, four were identified in case added certainty in identification of trace analytes was desired.

The whole blood extracts were analyzed on both GC/QQQ and the GC/NPD/MSD/DRS system described in reference 1. The retention times on the GC/QQQ were precisely locked to twice those in reference 1 using Agilent's method translation and RTL software.

Table 1. MRM Parameters and MDLs

	Retention time (min)	Precursor ion	Product ion	Collision energy (EV)	Relative response	*MDL (pg)
Meperidine	5.651					
		246	172.1	10	100	0.2
		247	71	10	80	
		218	172.2	10	36	
		174	70.2	10	32	
PCP (phencyclidine)	6.497					
		200	117.2	15	100	0.1
		200	84.1	15	46	
		242	171.2	25	17	
		243	200.3	10	14	
Methadone	7.728					
		72	42	25	100	0.2
		72	44	25	4	
		223	104.9	10	3	
		178	152	25	3	
Cocaine	8.078					
		82	67	20	100	
		82	41	25	60	
		182	82	10	50	0.2
		303	82	25	20	
Codeine	8.980					
		229	214.1	10	100	2.2
		299	229	15	38	
		162	146.8	20	38	
		162	146	30	25	
Hydrocodone	9.252					
		299	242.8	10	100	1.0
		242	152.8	30	71	
		242	180.9	20	71	
		299	270.1	15	71	
THC	9.321					
		231	173.9	25	100	0.4
		299	81	20	11	
		314	81.3	30	6	
6-Acetylmorphine	9.533					
		215	42.1	30	100	50
		268	252	25	77	
Oxycodone	9.589					
		315	230.1	15	100	0.5
		315	258	10	57	
		230	215.3	10	43	
		201	186.1	25	43	
Heroin	9.970					
		327	215	15	100	0.5
		327	268	10	67	
		369	268	30	33	
		369	204	10	25	
Fentanyl	10.354					
		245	146	20	100	0.2
			189	44	20	
			202	146	10	
			189	146	5	

* Signal-to-noise ratio = 3, noise measured peak to peak

Table 2. Instrument Conditions

GC	
Agilent Technologies 7890A with autoinjector and tray	
Inlet	EPC split/splitless
Mode	Constant pressure
Injection type	Splitless
Injection volume (µL)	1.0
Inlet temperature (ºC)	280
Inlet pressure (psig)	17.8
Purge flow (mL/min)	50
Purge time (min)	0.75
Gas type	Helium
Oven	
Initial oven temperature (ºC)	100
Initial oven hold (min)	0.5
Ramp rate (ºC/min)	20
Final temperature (ºC)	325
Final hold (min)	2.5
Total run time (min)	14.25
Equilibration time (min)	0.5
Column	
Type	DB-5MS UI
Agilent part number	122-5512UI
Length (m)	15
Diameter (mm)	0.25
Film thickness (um)	0.25
Nominal initial flow (mL/min)	2.2
Outlet pressure (psig)	3.8
Column Backflushing	
2-way splitter with makeup (one port plugged)	
Restrictor length (m)	0.8
Restrictor id (mm)	0.15
Backflushing pressure (psig)	75
Backflushing temperature (ºC)	325
Backflushing time (min)	2
Triple Quadrupole MS	
Agilent Technologies 7000A	
Inert EI source, Ionization energy (EV)	70
Mode	MRM
MS1 and MS2 resolution (amu)	1.2
Collision cell nitrogen pressure (psig)	2.6
Helium quench gas pressure	6.25
Solvent delay (min)	1.4
EM voltage	Atune voltage
Quad1 and 2 temperature (ºC)	150
Source temperature (ºC)	300
Transfer line temperature (ºC)	300

Results and Discussion

Figure 1 shows the GC/QQQ TIC in MRM mode for the evaluated compounds. The compounds are not derivatized because the sample preparation for the comparison screening method from reference 1 does not use derivatization. While the amines (amphetamine, phentermine, methamphetamine, MDA, MDMA, and MDEA) all show a sizable response at 1 ng/µL, analysis at lower levels was not possible because of their loss in the chromatographic system before reaching the MS, as is well known. Trace detection of the amines would require derivatization.

With the exception of 6-acetylmorphine, the remainder of the compounds all exhibited detection limits in the low picogram range. The detection limits listed in Table 1 are calculated for a signal-to-noise ratio of three with the noise measured as peak to peak. All MDLs were measured by injecting 1 µL of a 10 pg/µL solution of the compound except for 6-acetylmorphine, for which 1 µL of 50 pg/µL was used. Figure 2 shows the response for 10 pg of heroin at the 4 MRMs listed in Table 1. This example illustrates the high sensitvity provided by the GC/QQQ.

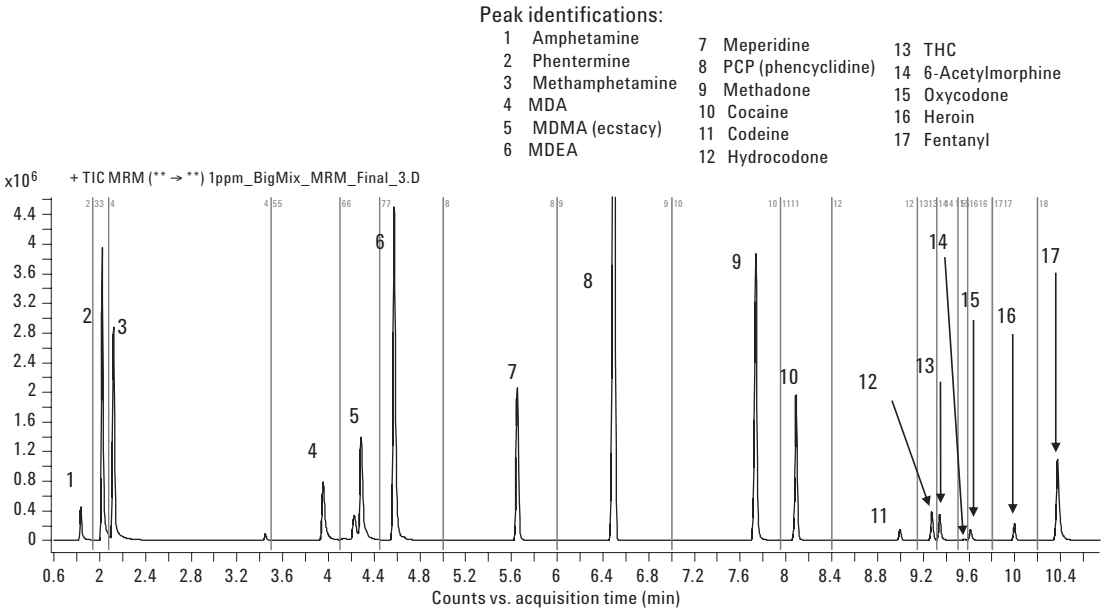

Figure 1. TIC of the Agilent 7000A Triple Quad GC/MS system in MRM mode. Standard solution of 1 ng/uL.

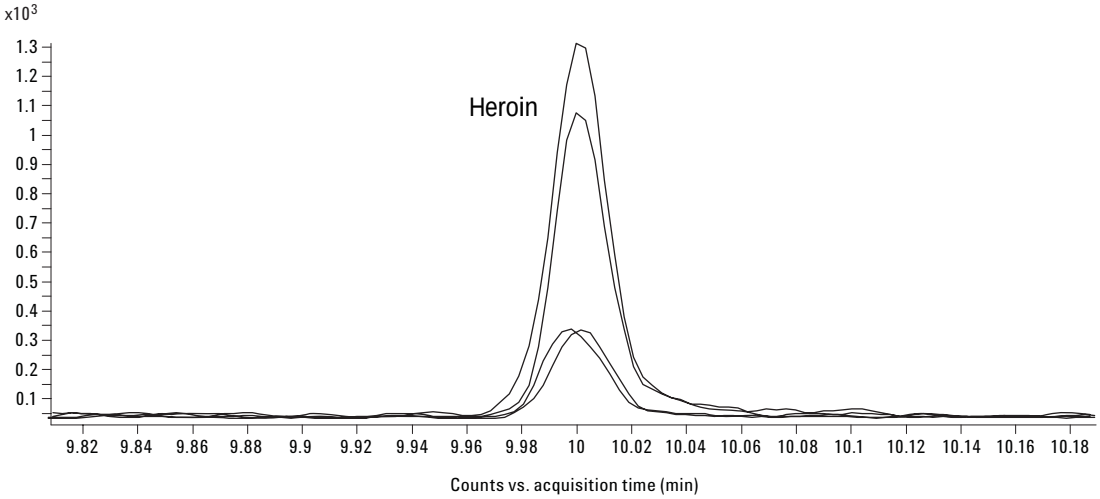

Figure 2. MRM transitions for heroin standard at 10 pg/µL. The transitions are listed in Table 1.

Figure 3 shows the extracted ion chromatograms (EICs) for codeine from the GC/NPD/MSD/DRS system scan data for whole blood extract A. The response at the codeine target ion and a corresponding peak on the NPD chromatogram at the correct retention time for codeine suggests it is present. However, confirmation with qualifier ion ratios is complicated by the low signal-to-noise ratio due to interference and the small quantity of codeine present. The deconvoluted spectrum from the DRS report only had a spectral match quality of 59 (out of 100), which is not high enough to confirm the presence of codeine.

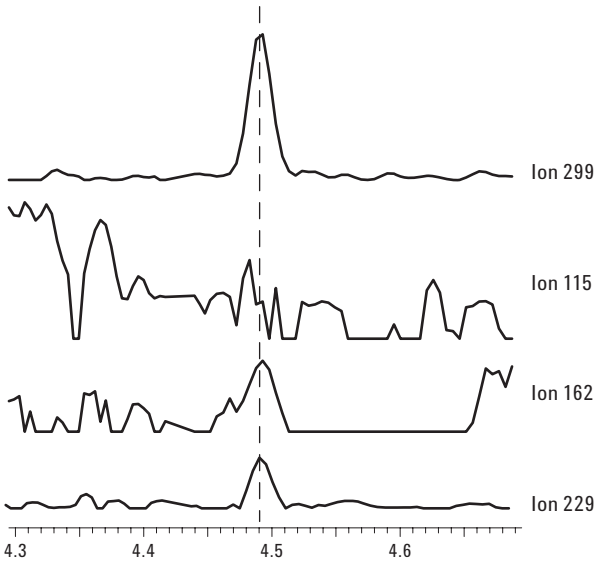

Figure 3. Codeine EICs from GC/NPD/MSD/DRS system scan data for whole blood extract A.

Figure 4 shows the corresponding GC/QQQ results for codeine in the same sample. The much higher selectivity and sensitivity afforded by GC/QQQ clearly confirm the presence of codeine in sample A. The amount detected corresponds to about 150 pg.

Detection of the powerful drug fentanyl in blood extracts is often a challenge because of the relatively small quantities of the drug administered. Confirmation is limited because there are only three ions of significant abundance. Figure 5 shows scan and SIM EICs for fentanyl from the GC/NPD/MSD/DRS system. There are only three ions and ion 189 is marginal at best due to low signal size and some interference. SIM data from SIM/scan had a much better signal-to-noise ratio, but still exhibited the same interferences on ion 189. The NPD response confirms that a nitrogen-containing compound with the same RT as fentanyl is present.

The DRS report for the sample found a marginal spectral match for fentanyl (66) at the correct RT. Based on all the information taken together, it appears that fentanyl is present in the sample.

Figure 6 shows the GC/QQQ MRMs for fentanyl in the same sample. The selectivity of MSMS detection clearly confirms its presence. The amount detected corresponds to about 150 pg.

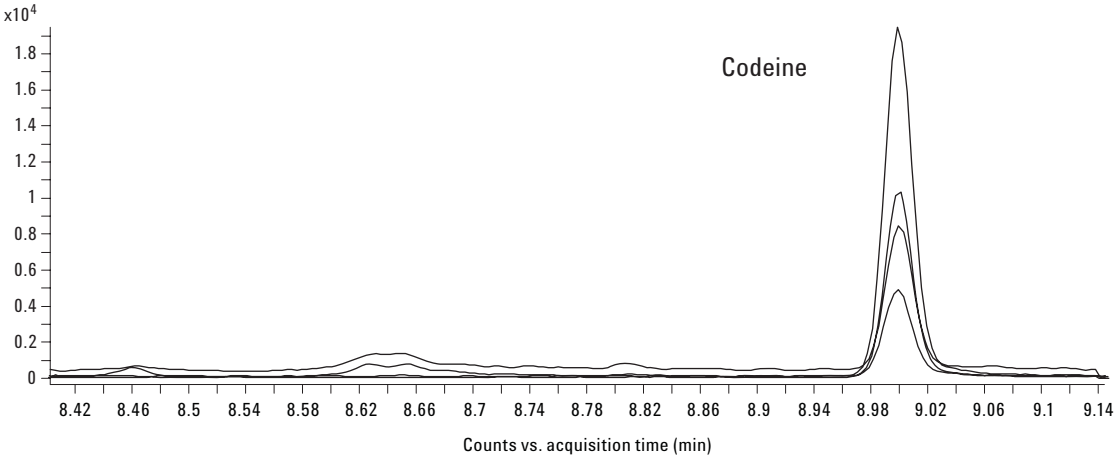

Figure 4. Codeine MRMs from GC/QQQ of whole blood extract A in Figure 3.

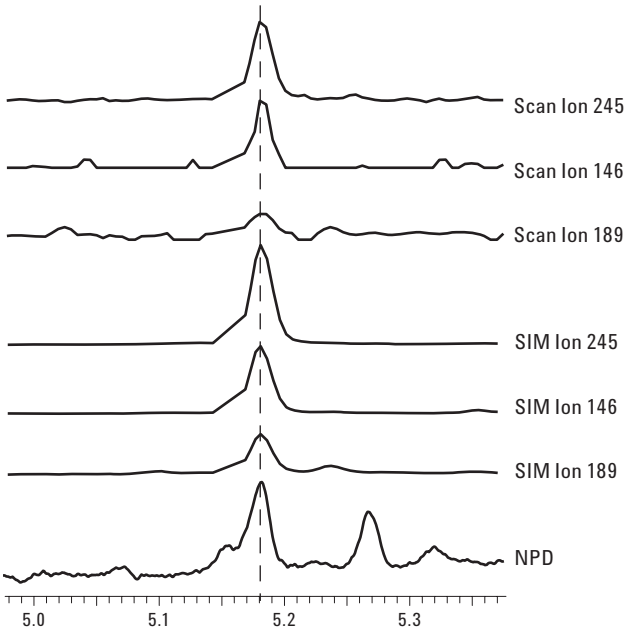

Figure 5. Fentanyl EICs and NPD response from whole blood extract B on GC/NPD/MSD/DRS system.

Figure 7. shows the scan, SIM, and NPD chromatograms for methadone in whole blood extract C from the GC/NPD/MSD/DRS system. Confirmation of methadone is complicated by the fact that its spectrum contains one large ion at a low, relatively common mass (72). The remaining ions are all small, being less than 6% relative abundance. As seen in Figure 7, the qualifier ions, especially 57, exhibit interferences. The deconvoluted spectrum had a match of 74. Note that the match quality value is dominated by the single 72 ion, so the number is artificially skewed a bit higher than normal. The data all point to methadone being present in the sample.

Figure 8 shows the GC/QQQ MRMs for methadone in sample C. The presence of methadone is clearly confirmed. The amount detected corresponds to about 170 pg.

Figure 9 shows the scan, SIM, and NPD chromatograms for oxycodone in whole blood extract B from the GC/NPD/MSD/DRS system. In this case, the amount present is relatively low at about 60 pg. Oxycodone was not reported in the DRS report because the spectral match was only 46, which is typically below the minimum match. The poor match resulted from high interferences and the small quantity of oxycodone present. In the scan EICs, the target ion and the NPD

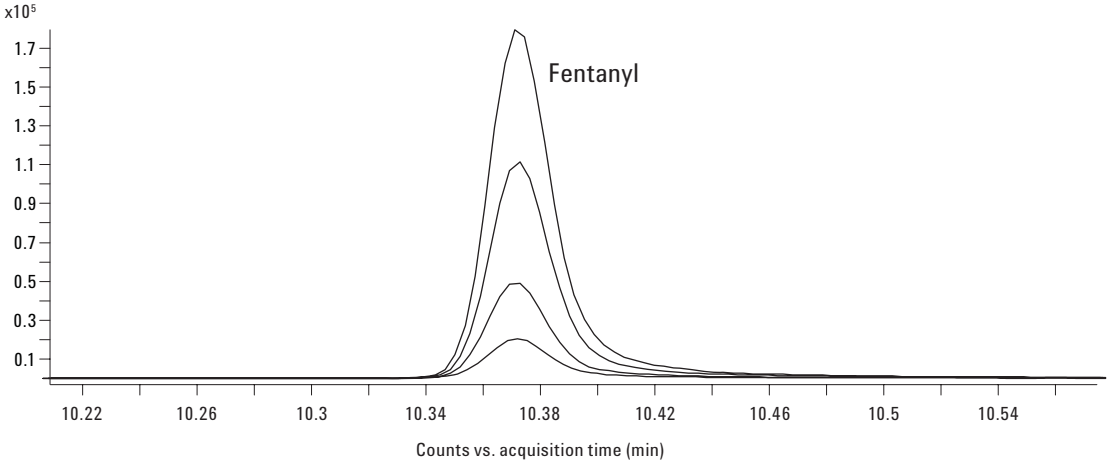

Figure 6. Fentanyl MRMs from GC/QQQ of whole blood extract B in Figure 5.

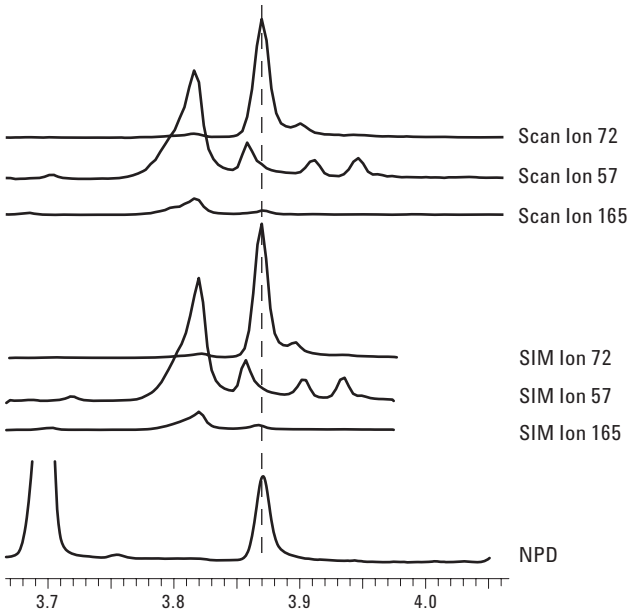

Figure 7. Methadone EICs and NPD response from whole blood extract C on GC/NPD/MSD/DRS system.

response are discernible peaks, but the two qualifiers are unusable. Note that the much higher signal-to-noise ratio provided by SIM allows a choice of ions that are too small to be used in scan mode and which have significantly higher selectivity. This is seen in the SIM chromatograms in Figure 9. The substitution of ion 316 for ion 70 now provides two clean qualifier ions with which to confirm the presence of oxycodone.

Figure 10 shows the GC/QQQ MRMs for oxycodone in the sample B. As with the previous examples, the high selectivity and sensitivity of GC/QQQ makes detection and confirmation of oxycodone straightforward.

The last example is shown in Figures 11 and 12. Figure 11 shows the scan, SIM, and NPD chromatograms for cocaine in whole blood extract A from the GC/NPD/MSD/DRS system. Note there is no indication of cocaine on either the scan or SIM chromatograms. There is what may be a very small response on the NPD, but it is too small to be significant. The GC/QQQ clearly shows the presence of cocaine in the sample at a very low level. The peak represents about 0.7 pg of cocaine, highlighting the low limits of detection available with GC/QQQ.

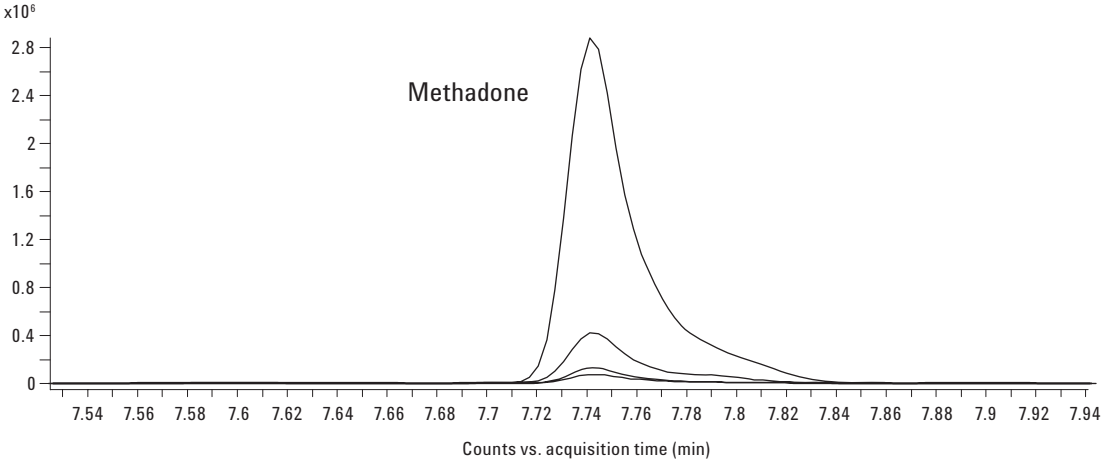

Figure 8. Methadone MRMs from GC/QQQ of whole blood extract C in Figure 7.

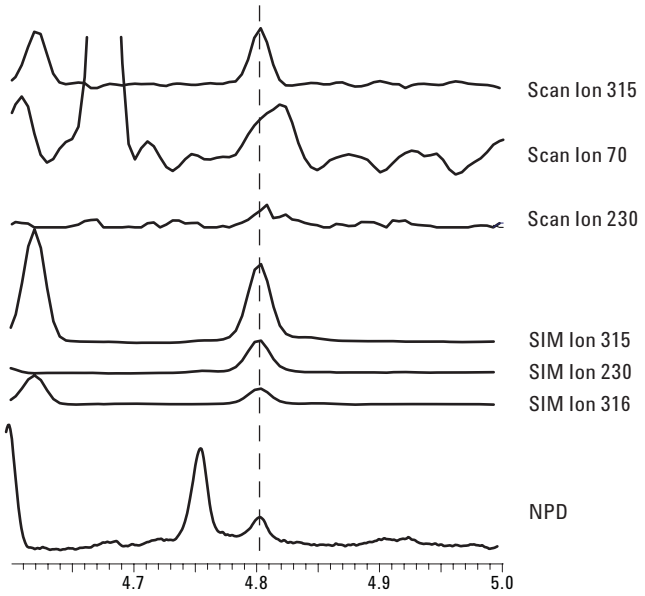

Figure 9. Oxycodone EICs and NPD response from whole blood extract B on GC/NPD/MSD/DRS system.

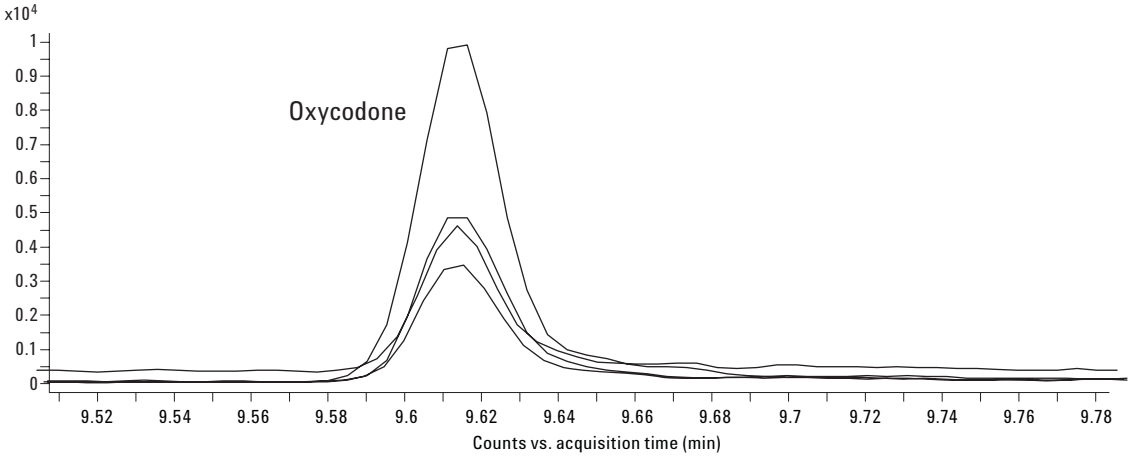

Figure 10. Oxycodone MRMs from GC/QQQ of whole blood extract B in Figure 9.

Conclusions

The Agilent 7000 GC/QQQ system provides both high sensitivity and high selectivity for the analysis of drugs. The system allows the low level detection and confirmation of large numbers of target drugs in blood extracts in a single run. When used in combination with a single quadrupole screening instrument like the Agilent GC/NPD/MSD/DRS system, a much more complete picture of each sample is now possible. The GC/NPD/MSD/DRS system provides the broadest range screen (725 compounds), full spectra and nitrogen selective detection for identifying nontarget compounds, and SIM data for lower level targets. The GC/QQQ provides routine detection and confirmation of up to a few hundred target compounds at low pg levels, even in difficult matrices.

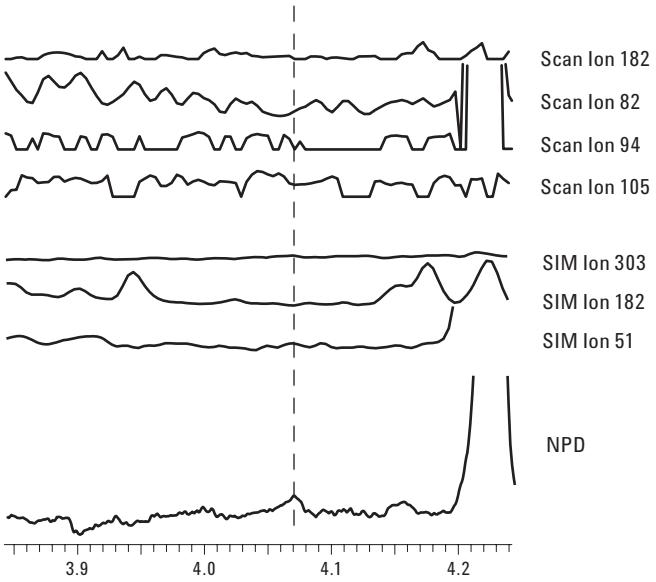

Figure 11. Cocaine EICs and NPD response from whole blood extract A on GC/NPD/MSD/DRS system.

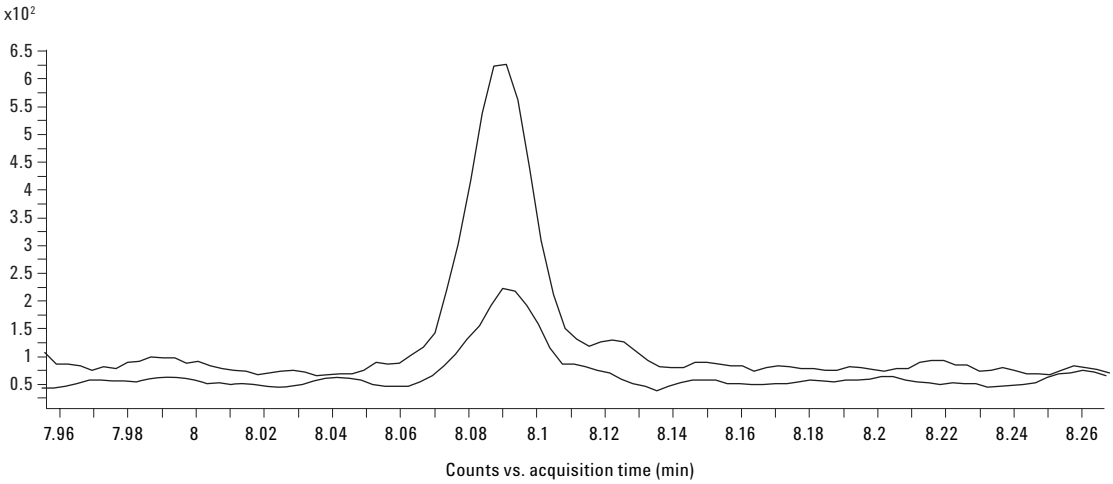

Figure 12. Cocaine MRMs from GC/QQQ of whole blood extract A in Figure 11. Top trace is MRM 182-82, bottom trace is 303-82.

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1. Bruce Quimby, "Improved Forensic Toxicology Screening Using A GC/MS/NPD System with a 725-Compound DRS Database," Agilent Technologies publication 5989-8582EN.
2. Dean F. Fritch and Bruce D. Quimby, "Confirmation of THC in Oral Fluids Using High-Resolution 2-D GC/MS," Agilent Technologies publication 5989-5668EN.
3. Chris Sandy, "Analysis of Complex Samples by GC/MS/MS – Pesticides in Marine Biota," Agilent Technologies publication 5989-9727EN.

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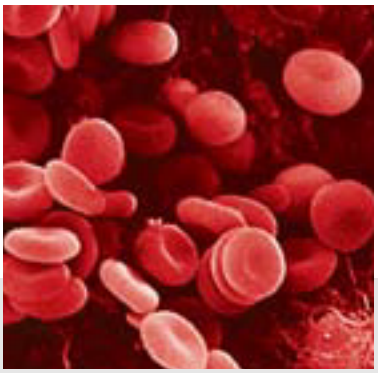
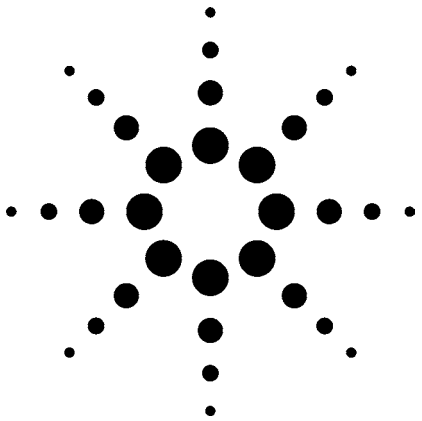


Applications by Technique

CE/MS

Analysis of Drugs by CE-MS^n

Application



Forensic Toxicology

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Abstract

CE-MS^n was demonstrated to offer the potential to increase the power of capillary electrophoresis and capillary electrophoresis electrospray ionization mass spectrometry for the identification of detected drugs. This application could be valuable in screening procedures for drugs in forensic toxicology.

Introduction

Capillary electrophoresis (CE) with ultraviolet-diode array detection (UV-DAD) is reported to be an effective tool in qualitative and quantitative drug analysis [1]. The analysis of drugs of abuse by CE coupled through electrospray ionization (ESI) to a mass spectrometer (CE-ESI-MS) is also reported [2]. Use of a mass selective detector (MSD) with an ion trap has the capability of generating MS^n data, and is expected to enhance the power of CE for drug and drug mixture analysis.

As a first step in the exploration of CE-MS in this role, it was necessary to confirm the separation capability of the technique, to test the ability of the instrument to generate meaningful MS data for drugs, and to demonstrate the building of libraries of standard data for later use.

This application note shows the feasibility of extending the CE procedure put forward by Hudson *et al* [1] to include the collection of both UV and MS^n data during a single analytical run. The analyses reported here were performed on solutions of pure drugs. A later note will explore the analysis of drugs extracted from whole blood.

Experimental

All drug analyses were done using an Agilent G1600A 3D CE coupled to an Agilent LC/MSD Trap XCT, using the G1603A CE-MS adapter kit and the G1607A CE-MS sprayer.

CE conditions

Capillary:	Bare fused silica; 50 μm diameter.
Capillary length:	21.5 cm to DAD; 84.0 cm to LC/MSD Trap
Cassette temperature:	25 °C
Run buffer:	100 mmol/L phosphate at pH 2.38
Injection:	Electrokinetic, 12.0 kV for 16.0 s
Run voltage:	Ramped 0 to 20 kV in 0.15 s; held for duration of run
Run time:	25 min
Diode array:	Wavelength: 200 nm, bandwidth 4 nm
Reference wavelength:	375 nm, bandwidth 75 nm

LC/MSD Trap conditions

Mass range mode:	Ultra Scan
Ion polarity:	Positive
Ion source type:	ESI
Drying gas temp:	130 °C
Drying gas flow:	7.00 L/min
Nebulizer:	8–12 psi
Trap drive:	27.0
Skim 1:	40.0 V
Skim 2:	–5.0 V
Octopole RF amplitude:	131.2 V
Capillary exit:	97.0 V
Scan range:	Typically 50–500
Averages:	8 spectra
Max. accumulation time:	100000 ms
ICC target:	100000
Charge control:	On
Sheath liquid:	0.5 % Formic acid in 50/50 methanol/water
Sheath liquid flow:	7.5 µL/min (0.75 mL/min split 100:1)

Auto MS parameters

Auto MS^3:	On
Threshold auto MS^3:	2500
No. of precursors:	1
Fragmentation amplitude:	1.0 V
Isolation width:	4.0 *m/z*

Seventeen drugs and an internal standard (ISTD) were chosen such that their mobilities were representative of the range of mobilities observed for 550 drugs of toxicological interest [1]. Drugs were analyzed singly and in mixtures, at a concentration of 1 mg/mL. A UV spectrum was collected for each, as well as MS, MS^2, and MS^3 data. With the instrument configuration used, it was possible to create libraries of both UV and MS data for later use. The library created was used in the Auto MS^n analysis described below.

The feature, Auto MS^n, makes it possible to collect automatically MS, MS^2, and MS^3 data (and higher orders of fragmentation as well, if necessary) on every peak with an intensity greater than some preset threshold, and then to search those data against libraries. This capability was explored through analysis of pure drug solutions.

It was observed during preliminary work that the migration time of a drug was significantly affected by the pressure of the nebulizing gas. This was further examined by analyzing a mixture of drugs at several different nebulizer pressures. While there was an increase in the observed migration rate, it was noted that this change did not affect electrophoretic mobility, and it was not studied further.

Results and Discussion

Figure 1 shows the total ion electropherogram (TIE) for the mixture of 17 drugs, each at a concentration of 1 mg/mL, plus the ISTD, that was analyzed.

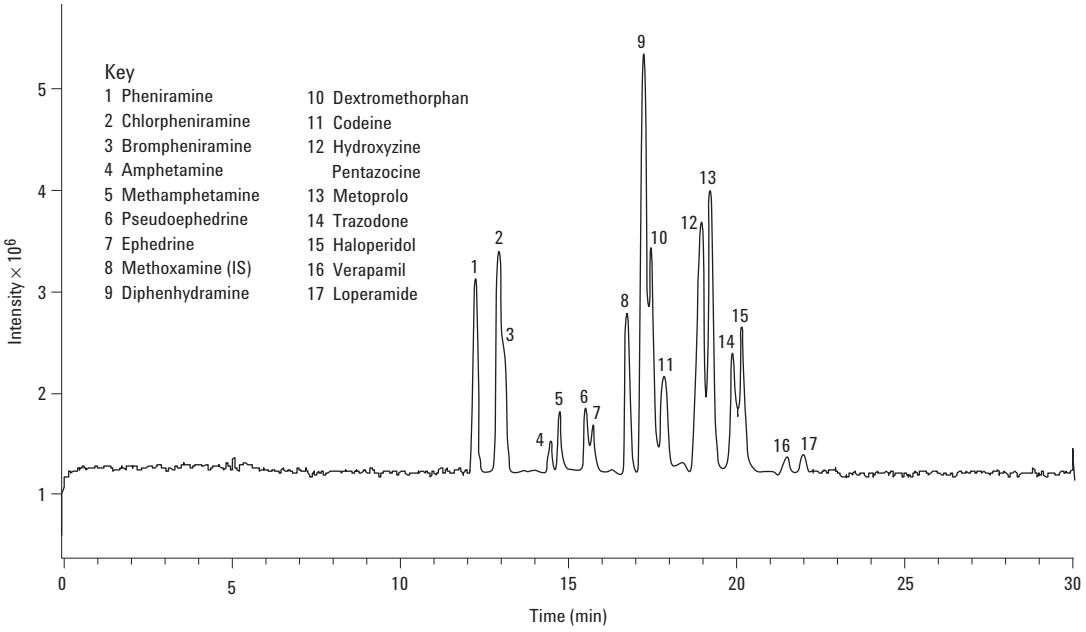

Figure 1. TIE for drug mixture.

The order of elution and degree of separation are similar to those reported by Hudson [1], except that, in the present work, hydroxyzine and pentazocine were observed to coelute. It was noted, however, that the presence of the two compounds was readily indicated by their respective mass data.

The 100 mmol/L phosphate run buffer might not be expected to provide good sensitivity with MS detection, due to suppression of ionization of the organic molecules. However, the use of 0.5% formic acid in methanol/water as sheath liquid provides the mechanism of transport from the end of the CE capillary into the MS via the droplets in which ionization occurs. With this run buffer, it is important to flush the capillary with water and air after use, and to maintain the standby drying gas temperature at 130 °C, in order to prevent plugging of the CE capillary when not in use.

Table 1 compares electrophoretic mobilities determined under the current experimental conditions with those reported by Hudson et al [1]. It should be emphasized that, while we used a capillary of the same composition and diameter as that reported previously, the pertinent lengths were quite different. Hudson *et al* used a capillary that was 60 cm to the detector; ours was 21.5 cm to the UV detector and 84 cm to the LC/MSD Trap. These differences meant that observed migration times were markedly different from those reported. The two electropherograms of Figure 2 illustrate this. Nevertheless, as Table 1 shows, electrophoretic mobilities agreed reasonably well.

Table 1. Comparison of Electrophoretic Mobilities

Drug name	Mobility* (× 10000) - Hudson (1)	Mobility* (× 10000) - present work
Pheniramine	3.287	3.235
Chlorpheniramine	3.081	3.022
Brompheniramine	3.034	2.999
Amphetamine	2.597	2.585
Methamphetamine	2.515	2.501
Pseudoephedrine	2.330	2.327
Ephedrine	2.292	2.295
Methoxamine (ISTD)	2.072	2.072
Diphenhydramine	1.985	1.990
Dextromethorphan	1.941	1.927
Codeine	1.871	1.862
Hyroxyzine	1.792	1.777
Pentazocine	1.703	1.677
Metoprolol	1.666	1.668
Trazodone	1.566	1.558
Haloperidol	1.536	1.528
Verapamil	1.391	1.381
Loperamide	1.319	1.310

*Apparent mobility of the analyte corrected for mobility of electroosmotic flow (EOF). Apparent mobility is the lL/tV where l is the capillary length to the detector (cm), L is the total capillary length (cm), t is the migration time (s) and V is the applied voltage (V). Because EOF is so low at pH 2.38, mobilities were determined relative to a reference compound, according to the method of Williams and Vigh [3].

Using amphetamine as an example of drugs analyzed in the mixture, Figure 2 shows the TIE, the UV electropherogram and the MS, MS^2, and MS^3 spectra collected on each drug.

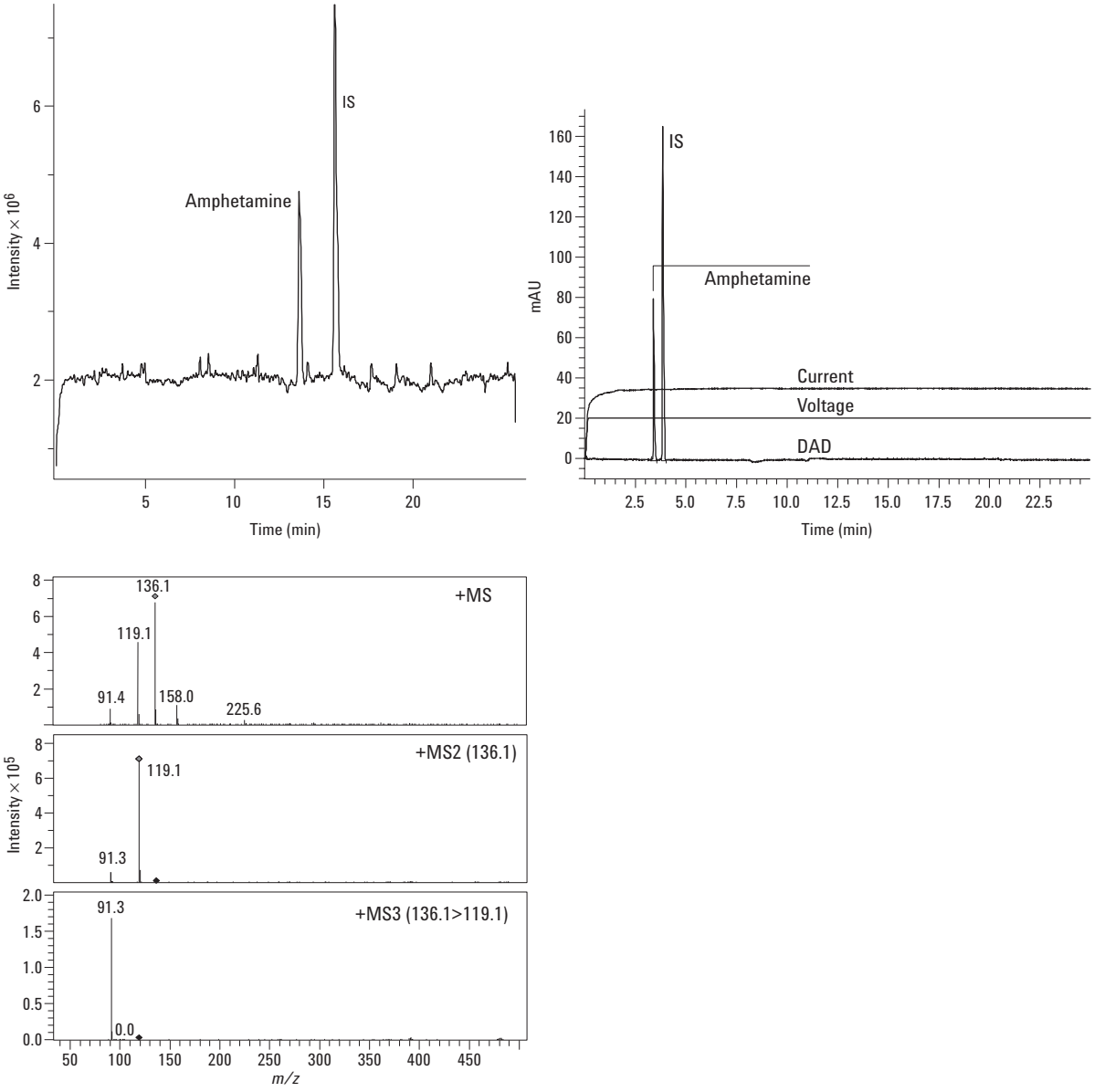

Figure 2. TIE, UV electropherogram, and MS data for amphetamine.

Figure 3 shows the result of an Auto MS^3 run on codeine, as an example. Codeine was analyzed earlier and a library entry was created for it. During the Auto MS^3 run, codeine was detected satisfactorily and identified through the MS-MS^2-MS^3 fragmentation pattern shown. Note that the fragmentation amplitude could be optimized for codeine to give greater fragmentation in the MS^2 spectrum. However, we chose a fragmentation amplitude of 1 V that would work satisfactorily for a wide variety of drugs.

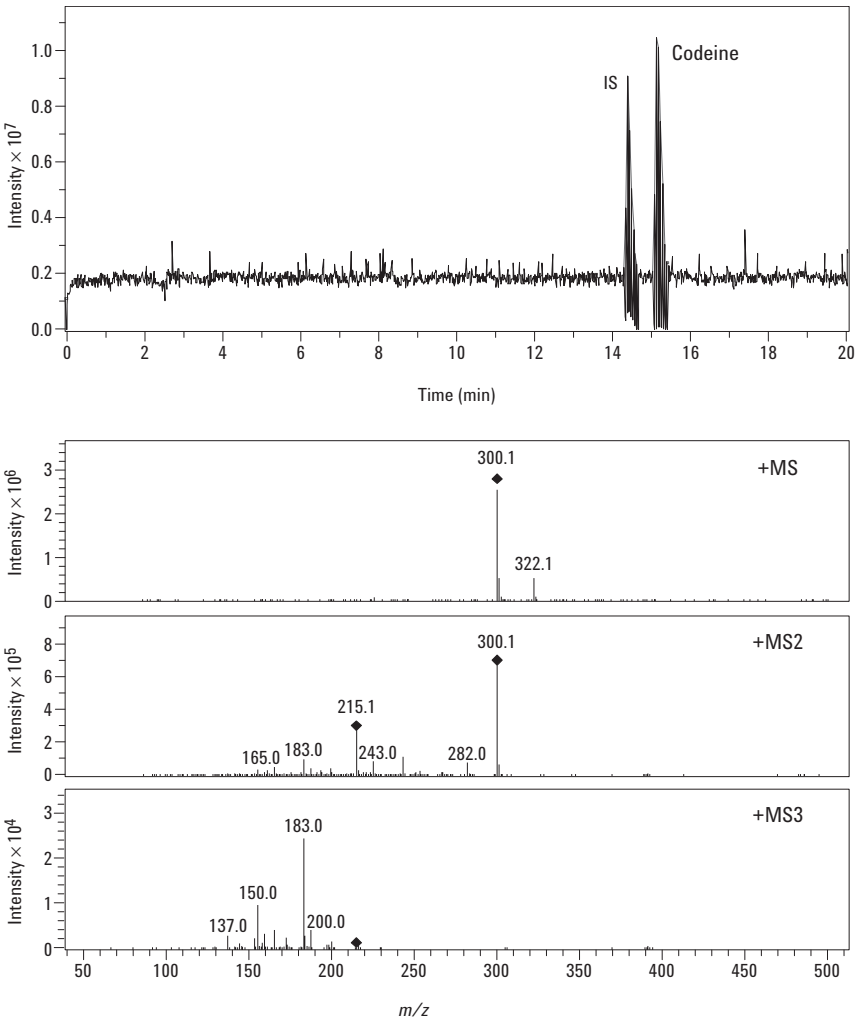

Figure 3. Auto MS data for codeine.

Conclusions

The application of CE in drug analysis was shown by Hudson et al [1]. Phan and Harrsch [2] showed the feasibility of analyzing drugs of abuse by CE-ESI-MS. We have demonstrated that CE-MS^n offers the potential to extend the power of such analyses, increasing the capability for identification of detected drugs; this application could be valuable in screening procedures for drugs in forensic toxicology.

Acknowledgement

The authors acknowledge gifts of drug standard solutions from J. Hudson, RCMP Forensic Laboratory Services, Regina, Saskatchewan, Canada.

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Forensic Toxicology Drug Screening in Blood by CE-MS^n - a Feasibility Study

Application

Forensic Toxicology

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Abstract

Preliminary observations indicate the feasibility of capillary electrophoresis-mass spectrometry (CE-MS) with the Agilent LC/MSD Trap system to be capable of detecting drugs at <20 ng/mL in whole blood, suggesting that it may be a suitable tool for screening whole blood samples for drugs. Additionally, the capability of generating MS^n data permits reliable identification of detected drugs, suggesting that both screening and identification of drugs might be accomplished in one or two injections using the same instrument system.

Introduction

Capillary electrophoresis (CE) with ultraviolet-diode array detection (UV-DAD) is reported to be an effective tool in the screening of whole blood samples for drugs of toxicological interest [1]. The first objective of any such screen is simply the detection of drugs at toxicologically significant concentrations. Since the primary focus is on detection, drug identification is often tentative at the screening stage. Rigorous identification is frequently deferred to a second analytical procedure, such as mass spectrometry (MS), often involving repeat extraction and derivatization. Analysis is more efficient if both detection and identification can be completed as part of the same analytical run or, at least, using the same instrumentation.

It was reported that CE-MS is useful in the analysis of drugs of abuse in samples of illicit drug materials [2]. An earlier application note showed that CE-MS^n is capable of separating and detecting certain drugs with sufficient sensitivity to be useful as a possible screening instrument in whole blood analysis [3]. Initial observations were made using pure drug solutions. CE-MS can only be useful in the screening of whole blood, however, if residues from the matrix do not interfere. The present application note describes the analysis of certain drugs in samples of whole blood, both spiked samples and those from actual forensic toxicology cases.

In the report of Hudson *et al* [1], it was noted that the combination of electrophoretic mobility and the UV spectrum provided an impressive degree of discrimination between analytes. This combination, however, was not claimed to represent rigorous identification. Rather, drugs tentatively identified by mobility and UV absorption would be subjected to further analysis, probably by gas chromatography/mass spectrometry (GC/MS). This note shows the feasibility of extending the CZE (Capillary Zone Electrophoresis) screening procedure put forward by Hudson et al [1] to include the collection of +MS, +MS^2 and +MS^3 data during a single screening run and the subsequent use of the MS^n data as evidence of drug identification.

Experimental

All drug analyses were done using an Agilent G1600A 3D CE coupled to an Agilent LC/MSD Trap XCT, using the G1603A CE-MS adapter kit and the G1607A CE-MS sprayer.

CE conditions

Capillary:	Bare fused silica; 50-µm diameter
Capillary length:	21.5 cm to DAD; 84.0 cm to LC/MSD Trap
Cassette temperature:	25 °C
Run buffer:	100 mmol/L phosphate at pH 2.38
Injection:	Electrokinetic, 12.0 kV for 16.0 s
Run voltage:	Ramped 0 to 20 kV in 0.15 s; held for duration of run
Run time:	25 min
Diode array:	Wavelength: 200 nm, bandwidth 4 nm
Reference wavelength:	375 nm, bandwidth 75 nm

LC/MSD Trap conditions

Mass range mode:	Ultra Scan
Ion polarity:	Positive
Ion source type:	ESI
Drying gas temp:	130 °C
Drying gas flow:	7.00 L/min
Nebulizer:	8–12 psi
Trap drive:	27.0
Skim 1:	40.0 V
Skim 2:	–5.0 V
Octopole RF amplitude:	131.2 V
Capillary exit:	97.0 V
Scan range:	Typically 50–500
Averages:	8 spectra
Max. accumulation time:	100000 µs
ICC target:	100000
Charge control:	On
Sheath liquid:	0.5 % Formic acid in 50/50 methanol/water
Sheath liquid flow:	7.5 µL/min (0.75 mL/min split 100:1)

Auto MS parameters

Auto MS^3:	On
Threshold auto MS^3:	2500
No. of precursors:	1
Fragmentation amplitude:	1.0 V
Isolation width:	4.0 *m/z*

Whole porcine blood spiked with 17 drugs, each at a concentration of 20 ng/mL of blood was previously extracted according to the procedure of Hudson [1]. Details are given in the cited reference but, briefly, basic drugs were extracted as follows: 1.0-mL whole blood + 0.2-mL concentrated ammonia + 5.0-mL 1-chlorobutane; shake; centrifuge; and evaporate to dryness. To the dry residue, add 30 µL of 10-mmol/L phosphate buffer; vortex; centrifuge briefly; transfer to sample cups and centrifuge again. Each residue was then screened by $CE\text{-}MS^n$, using the above analytical conditions. For each peak detected, MS^n data were collected.

Samples of whole human blood from three actual forensic toxicology cases were previously extracted and analyzed by ELISA, GC-NPD, GC-ECD, and CE-DAD. Drugs detected in the blood samples were identified by GC/MS. The dry residue from each of these samples was reconstituted and analyzed by $CE\text{-}MS^n$, as above.

No attempt was made to optimize separation conditions in this work. The extraction residues left over from previous analyses were simply analyzed under conditions that approximated those reported by Hudson [1]. We observed (and report here) the coelution of certain drugs that Hudson *et al* were able to separate. As shown below, how-ever, the ability to collect MS^n data makes these coelutions a less serious problem than might be supposed since the added selectivity of MS^n over-comes coelution problems observed with the less-selective DAD.

Results and Discussion

Figure 1 shows the total ion electropherogram (TIE) of the spiked porcine blood sample, along with the extracted ion electropherograms (EIEs) for each of the 17 drugs present. These data give preliminary indications on two important points: sensitivity of detection and interference from matrix.

Initially, it was thought possible that the analytical system used here would simply not be sensitive enough for a toxicological screen. From the data shown, however, it is clear that useful MS data can be collected at drug concentrations of 20 ng/mL in whole blood. It also appears that a thorough validation study would show that the limit of detection (LOD) for at least some drugs would be substantially lower than 20 ng/mL. This is probably adequate sensitivity for routine drug screening.

It was also considered possible that matrix components extracted from whole blood would overwhelm the analytical system and interfere irreversibly with collection of the MS data. Figure 1 suggests that matrix components offer no serious obstacles to MS analysis.

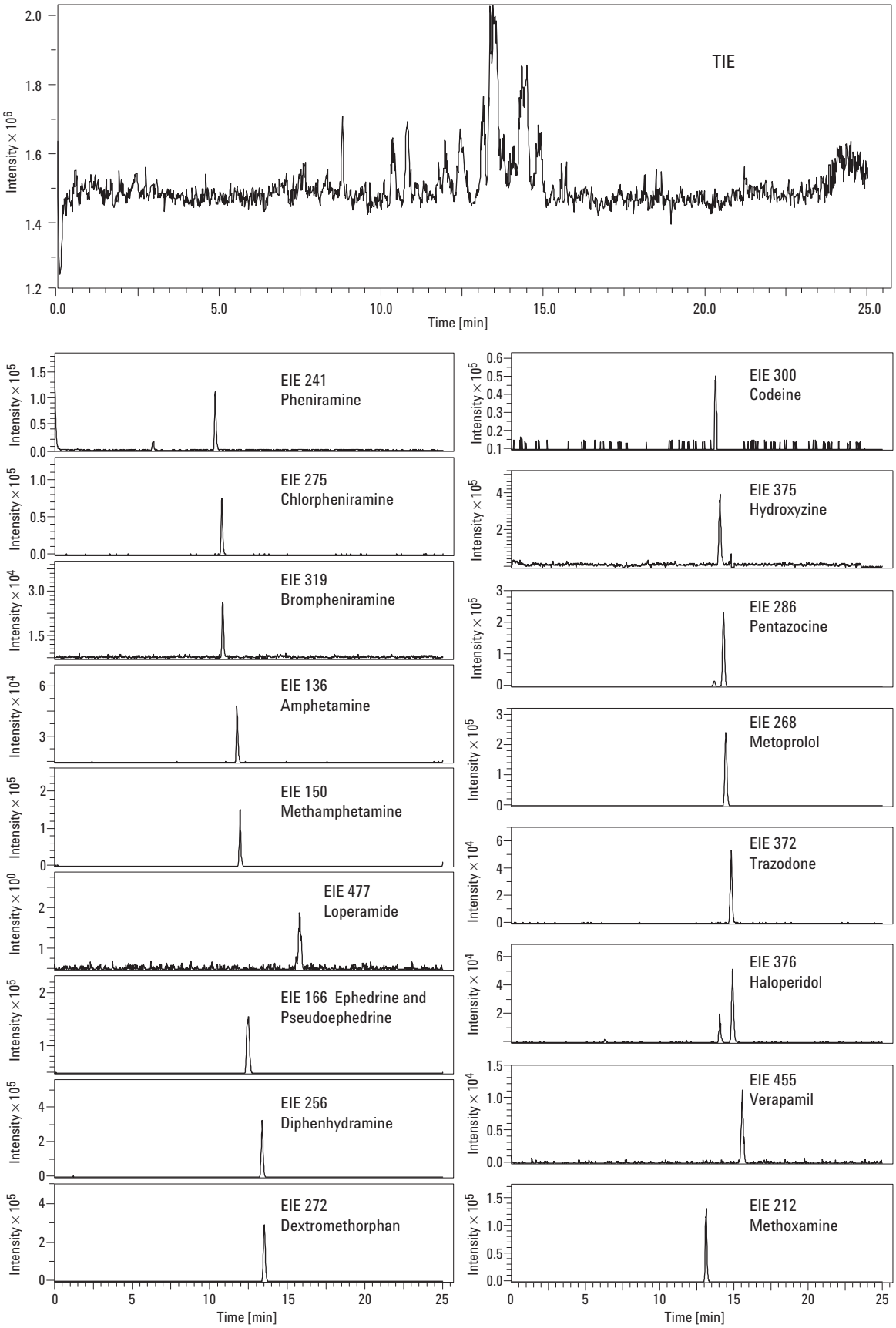

Figure 1. TIE and EIEs for extracted drug mix.

In Case 1, the blood sample was known from previous analyses to contain amphetamine, methamphetamine, and cocaine. Figure 2 shows the TIE and EIEs; Figure 3 shows Auto MS^n and library matches from the same sample.

Figure 2 shows one matrix peak (unidentified). As was suggested above, matrix interference does not appear to be a significant problem.

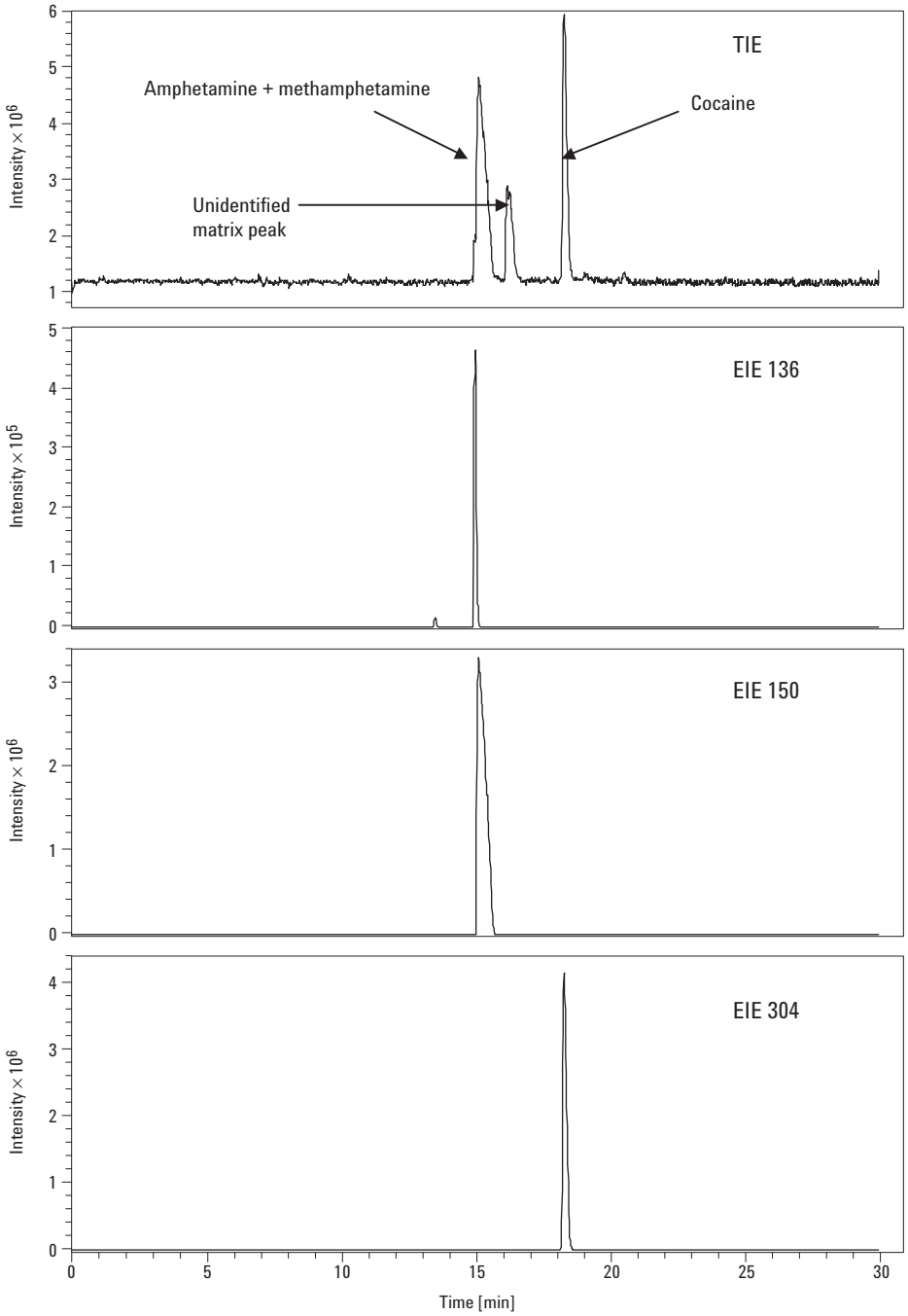

Figure 2. Case 1. Amphetamine, methamphetamine, and cocaine.

The library searches shown in Figure 3 indicate the correct identification of amphetamine, methamphetamine, and cocaine, in spite of the coelution of the former two compounds. The library search on MS^3 data from Peaks 1 and 2 (amphetamine and methamphetamine, respectively) showed no matching spectrum. The dominant ion in the library MS^3 data from both these compounds was 91.3 *m/z*. Our analysis showed a dominant ion at *m/z* 92.2, which was, of course, not interpreted as a match by the search software.

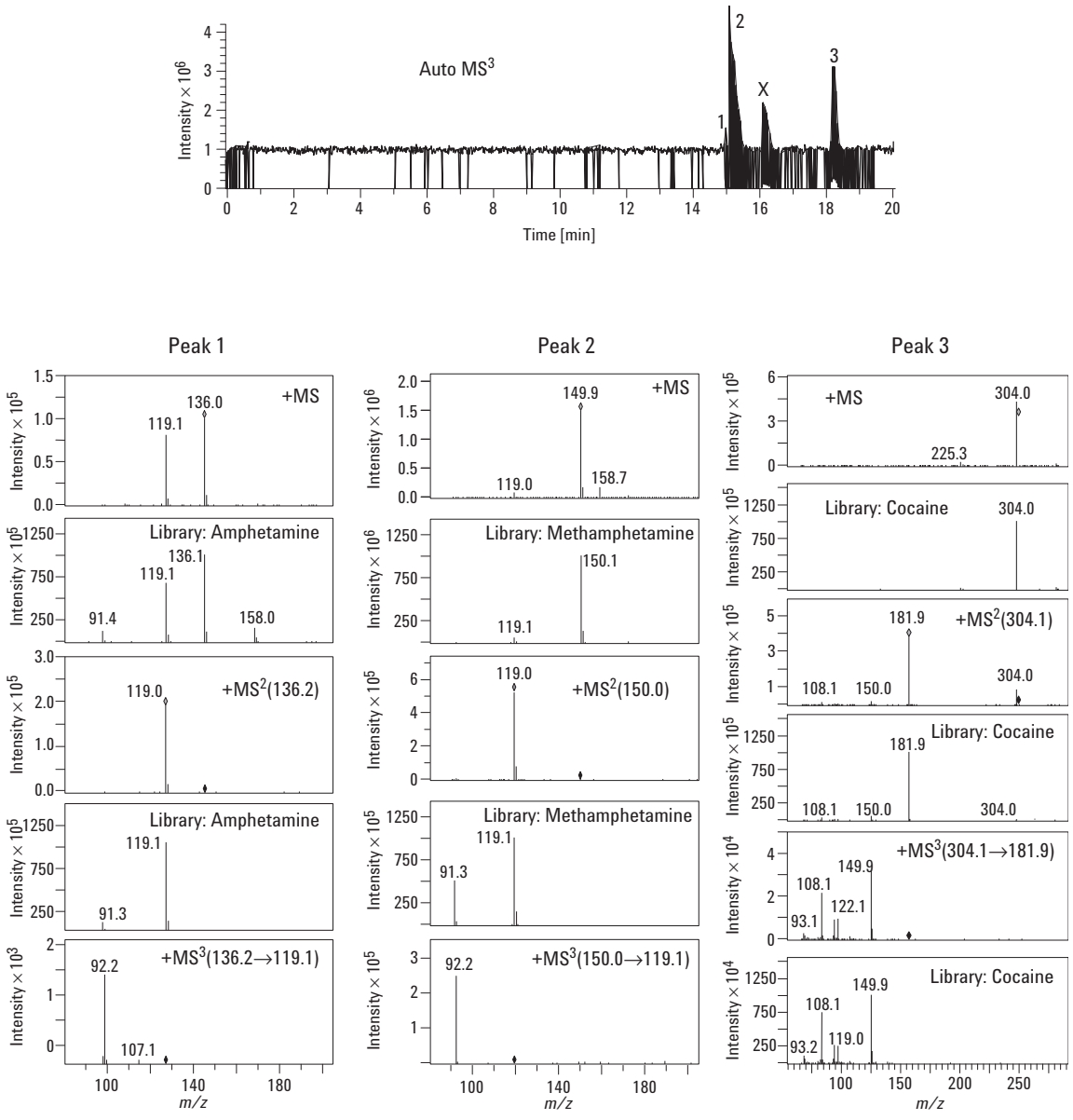

Figure 3. Case 1. AutoMS³ and library matches.

In Case 2, the blood sample was known to contain methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA). Figure 4 shows the TIE and EIEs. Figure 5 shows the Auto MS^3 and library matches from the same sample.

Figure 4 shows again the relative absence of matrix interference and the coelution of the two analytes of interest.

The library searches shown in Figure 5 identified both drugs correctly. It is interesting to note, however, that the search of MS data on MDA failed to find a match but that the correct match was found for MS^2 and MS^3 data. This was attributed to the presence of background data, such as the fragments at *m/z* 91.1 and 158.8. In the analysis of MDMA, these background fragments were removed and correct matches were found on all three searches.

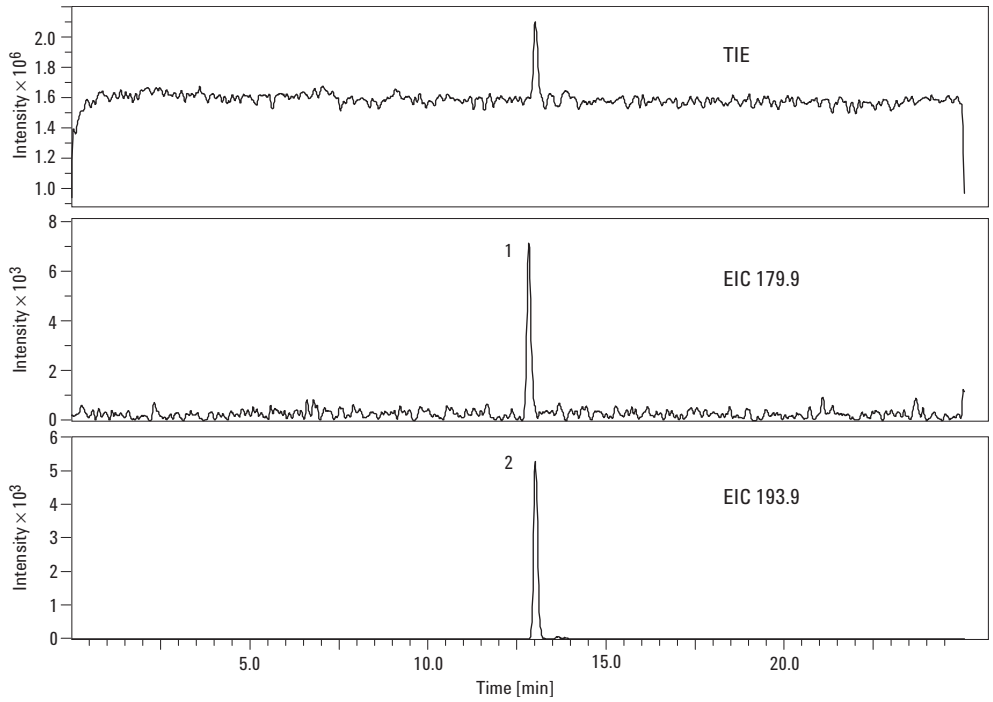

Figure 4. Case 2: MDA and MDMA.

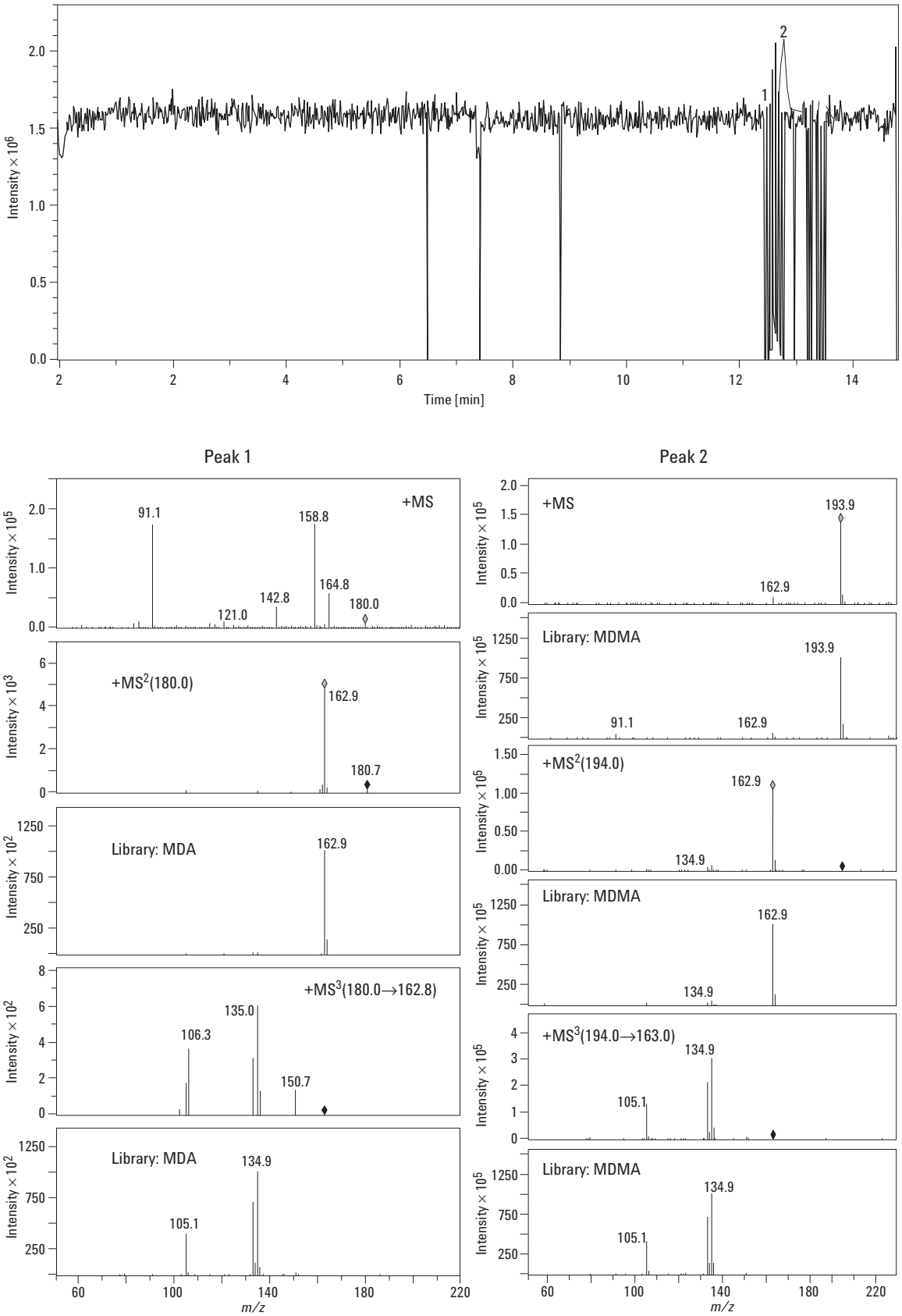

Figure 5 Case 2. Auto MS³ and library matches.

In Case 3, initial analysis of the blood sample by Hudson *et al* showed several forensically significant compounds. Quinine, cocaine, cocaethylene, bupropion, paroxetine, and lidocaine were identified by GC/MS. As before, our analysis of this sample attempted to replicate this work, given different instrument configurations. Figure 6 shows the TIE for this sample. The library matches from the Auto MS^3 analysis are shown in Table 1. There are several points to note in the following data.

First, for a variety of reasons, Peaks 2, 3, 4, and 5 were not included in Table 1. Peak 2 appeared upon later searching to be methylecgonine (see below) and Peak 3 appeared to be a matrix peak (unidentified); Peak 4 was consistent with the ISTD (methoxamine) and Peak 5 was consistent with the lidocaine metabolite, monoethylglycinexylidide (MEGX).

Second, erythrohydrobupropion and threohydrobupropion are known to be major metabolites of bupropion [4]. While Figure 6 suggests that these two compounds are potentially separable (Peaks 8 and 9), no attempt was made here to further resolve the system. As it was, the compounds were not separated by Auto MS^3 and, accordingly, Table 1 shows an entry only for Peak 8 thus indicating that the two compounds were indistinguishable on the basis of MS data. None of the metabolites of bupropion were previously identified by GC/MS.

Finally, with Peak 6, the known drug was not identified on the library search of MS data because of the presence of background fragments. As with the MDA/MDMA example in Case 2, however, identification was correct on the searches of MS^2 and MS^3 data.

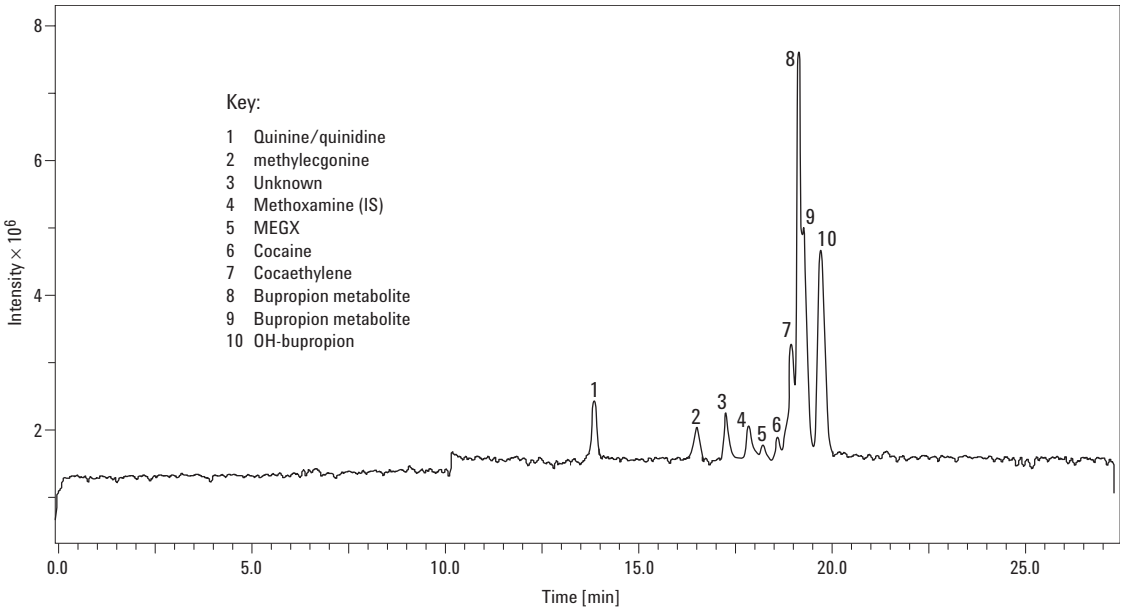

Figure 6. TIE of Case 3.

Table 1. Library Searches of Auto MS from Case 3

Identification	+MS	+MS^2	+MS^3	Fit	Rfit	Purity	Identification
Peak 1	325.2	307.1					
Library search	325.1			940	669	645	Quinine/Quinidine
		307.2		975	975	972	Quinine/Quinidine
			NC				
Peak 6	226.8 (Bkgd)	304.1	181.9	149.9			
Library search	304.1						No match
		181.9		995	995	995	Cocaine
			149.9	930	933	930	Cocaine
Peak 7	318.1	195.9	149.9				
Library search	318.1			994	992	992	Cocaethylene
		195.9		997	997	997	Cocaethylene
			149.9	997	998	997	Cocaethylene
Peak 8	241.9	167.9	150.9				
Library search	242.0			995	995	994	Amino OH bupropion*
		167.9		997	997	997	Amino OH bupropion*
			150.9	993	993	993	Amino OH bupropion*
Peak 10	256.0	237.9	138.9				
Library search	256.0			994	998	992	Hydroxybupropion
		237.9		998	998	998	Hydroxybupropion
			138.9	994	994	994	Hydroxybupropion

NC Not completed.

*Metabolites of bupropion exist in erythro- and threo- forms, not completely resolved here.

The AutoMS^3 analysis shown above did not detect certain compounds, such as bupropion, that were known to be present. Whether a particular peak is detected by AutoMS^3 or not depends upon several factors, chief among them the AutoMS^n threshold that is set and the resolution that is possible with the electrophoretic conditions used. Note that it is possible to prepare an Include List specifying precursor ions that the analyst specifically wants to search for. That was not done in this work.

However, later manual searching of the electropherogram (Figure 6) on the basis of selected masses indicated the presence of lidocaine and its metabolite, MEGX, in the region of Peak 5. Bupropion, metabolites of which were detected by AutoMS^3 in Peaks 8 and 10, appeared to be part of the unresolved complex at Peak 8. While both cocaine and the metabolite, cocaethylene, were detected by AutoMS^3, methylecgonine was not. A search based on its mass showed methylecgonine likely to be present in Peak 2. It was not detected in the AutoMS^3 analysis because we had no entry for it in our library.

Conclusions

CE-MS with the Agilent LC/MSD Trap system is capable of detecting drugs at concentrations less than 20 ng/mL in whole blood. It is, therefore, a suitable tool for screening whole blood samples for drugs as part of routine forensic toxicological analyses. In addition to relatively sensitive detection, however, the capability of generating MS^n data permits reli-able identification of detected drugs. This means that both screening and identification of drugs might be accomplished in one or two injections in a single instrument system. Finally, we show that, through the use of the Auto MS^n feature, it may be feasible to automate much of the drug screening procedure, thereby potentially increasing sample throughput significantly. We emphasize that these are preliminary observations indicating feasibility only. More development and validation work is required.

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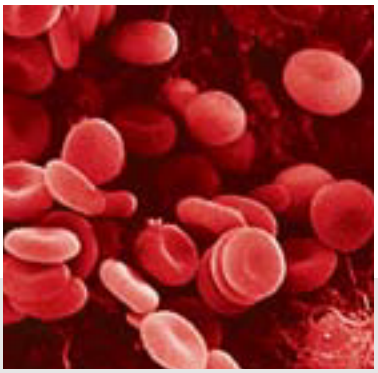
- Determination of Opiates and Metabolites in Blood Using Electrospray LC/MS
- Identification and Quantitation of Benzodiazepines and Metabolites by LC/MS
- Determination of Opioids, Cocaine, and Cocaine Metabolites by Liquid Chromatography Mass Spectrometry Using ZORBAX Eclipse Plus C18 Columns
- A Comparison of Several LC/MS Techniques for Use in Toxicology
- Analysis of Oxycodone And Its Metabolites-Noroxycodone, Oxymorphone and Noroxymorphone In Plasma By LC/MS With An Agilent ZORBAX StableBond SB-C18 LC Column
- Determination of Cocaine and Metabolites in Urine Using Electrospray LC/MS
- Screening Drugs of Abuse by LC/MS



Applications by Technique

LC/MS

Determination of Opiates and Metabolites in Blood Using Electrospray LC/MS

Application Note

Forensic Toxicology

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Montana Department of Justice, Division of Forensic Science

John M. Hughes
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Introduction

Opiates (Figure 1) are a widely abused class of drugs that can be obtained both illicitly and by prescription. The first metabolite of heroin is 6-monoacetylmorphine. It is commonly analyzed as a distinguishing marker of heroin use after an opiate-positive screening result. The well-established GC/MS method for the analysis of opiates[1] requires derivatization of these compounds. Derivatization adds variables to the analysis and can introduce aggressive derivatizing reagents into the analytical system.

Opiates and their metabolites are basic compounds that show excellent sensitivity in electrospray mass spectrometry, and can be analyzed without derivatization. The same solid-phase extraction (SPE) developed for the LC/MS analysis of plasma for clinical research studies[2] can be used for the analysis of whole blood in forensic toxicology samples. The levels of opiates found in forensic blood samples are normally high enough that the scanning mode of data acquisition can be used instead of selected ion monitoring (SIM). This allows other drugs isolated using the same sample preparation to be qualitatively identified in the same run that quantitates the opiates. The electrospray LC/MS analysis using scan mode gives accuracy and precision comparable to or better than those obtained using SIM in GC/MS.

Experimental

The Agilent 1100 Series LC/MS system included a binary pump, vacuum degasser, autosampler, thermostatted column compartment, diode-array detector, and the LC/MSD VL quadrupole mass spectrometer. The LC/MSD was used with an electrospray ionization (ESI) source. The diode-array detector was used primarily for method development purposes, although the UV spectral data obtained simultaneously with the MS data can be used for confirmation of identity of many drugs. Complete system control and data evaluation were carried out using the Agilent LC/MS ChemStation software.

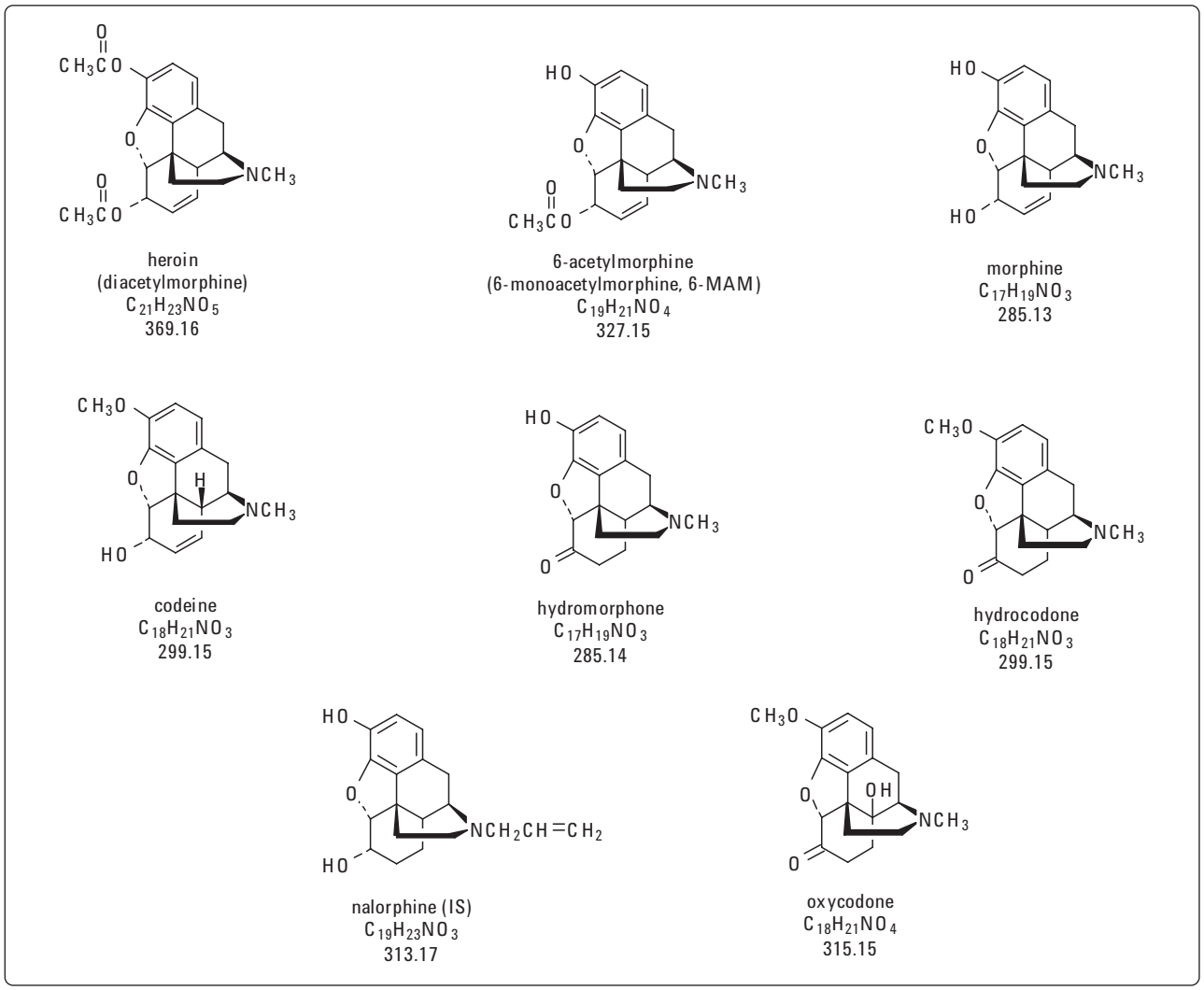

Figure 1. Opiate and internal standard structures

Analytical standards were obtained from Cerilliant Corporation (formerly Radian Analytical Products). The objective of developing a qualitative as well as a quantitative method mandated that the procedure use a non-deuterated internal standard. Nalorphine was chosen because the laboratory already had a validated protocol for opiates by GC/MS that used this internal standard.

Drug-free blood was fortified with known concentrations of the analytes and the internal standard. The tubes were capped, mixed, and incubated at 37°C for 12 hours. The sample blood (1 ml) was spiked with internal standard (to 1 mg/l), mixed, and allowed to equilibrate for 30 minutes. A 2 ml aliquot of 10 mM ammonium carbonate buffer, pH 9, was added to each sample. The samples were then mixed again and centrifuged at 3000 rpm for 10 minutes.

Clean-up SPE columns (CEC18156, United Chemical Technologies) were conditioned with 2 ml of methanol and 2 ml of deionized water, followed by 2 ml of the ammonium carbonate buffer. The supernatant was transferred to an SPE column and allowed to pass through the conditioned

column by gravity flow. The column was rinsed with 2 ml of ammonium carbonate buffer. The column bed was dried at full vacuum for five minutes, and the analytes were eluted with 3 ml of methanol using gravity flow. The eluate was evaporated to dryness with a stream of nitrogen at 40°C. The final sample residue was reconstituted in 50 µl of LC mobile phase, transferred to a 1-ml microcentrifuge tube, and centrifuged at 15,000 rpm for 2 minutes. A 10 µl aliquot was then injected for analysis by LC/MS.

It should be noted that both morphine-3 glucuronide and morphine-6 glucuronide extracted favorably with this procedure. However, because the availability of commercial standards of the various opiate glucuronide conjugates is extremely limited, hydrolysis is a potential pretreatment option. A 1 ml aliquot of blood can be treated with 100 µl of a 10000-units/ml solution (pH 4.5) of β-glucuronidase isolated from *Patella vulgata*. For analysis of unknowns, the laboratory's standard operating procedure is to hydrolyze samples if presumptive screens indicated the presence of either opiates or benzodiazepines.

In the analysis of opiates, it is important to be able to clearly distinguish the isobaric molecules (morphine/hydromorphone, codeine/hydrocodone) for accurate interpretation of results. The chromatography for this method was therefore optimized to cleanly separate the various opiates in a reasonable time. This required gradient, rather than isocratic, conditions. The column could nonetheless be re-equilibrated quickly and retention times were extremely reproducible over time.

MS parameters optimized for this analysis included fragmentor voltage (to give the most intense protonated molecule for each analyte), capillary voltage (for maximum signal), and spray chamber parameters (for maximum signal with minimum noise).

ANALYSIS METHOD

Chromatographic Conditions

Column:	Supelco Discovery HSC18, 4.6 mm x 15 cm, 3 µm
Mobile phase:	A = 0.1% formic acid in water B = methanol
Gradient:	Start with 5% B at 2 min 5% B at 10 min 90% B at 20 min 90% B
Flow rate:	0.5 ml/min
Column temp:	50°C
Injection vol:	10 µl
Diode-array detector:	Signal 214, 8 nm; reference 360, 100 nm (used for method development only)

MS Conditions

Source:	ESI
Ionization mode:	Positive
Vcap:	3000 V
Nebulizer:	40 psig
Drying gas flow:	13 l/min
Drying gas temp:	350°C
Mass range:	*m/z* 100–650
Fragmentor:	120 V
Stepsize:	0.1
Peak width:	0.12 min
Time filter:	On

Ions used for identification and quantitation:

Nalorphine (IS)	*m/z* 312
Morphine, hydromorphone	*m/z* 286
Codeine, hydrocodone	*m/z* 300
6-Acetylmorphine	*m/z* 328
Oxycodone	*m/z* 298

Results and Discussion

Recoveries for the analytes were excellent, ranging from a low of 85% for 6-acetylmorphine to a high of 100% for morphine. Figure 2 shows extracted ion chromatograms for the six opiates and the internal standard.

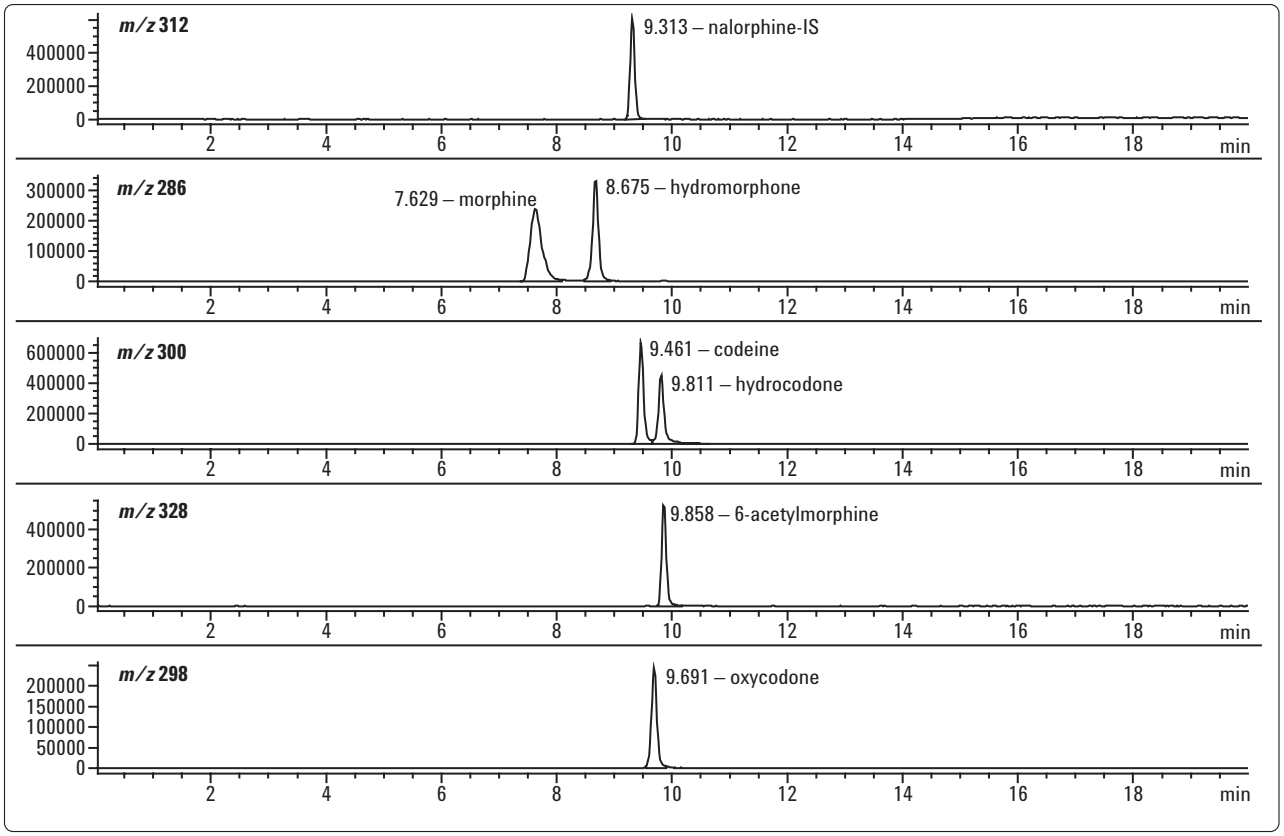

Figure 2. Extracted ion chromatograms (EICs) of opiates and internal standard

Figure 3 shows extracted ion chromatograms (EICs) for blank blood fortified with the internal standard at 1 mg/l (1000 ng/ml). Figure 4 shows extracted ion chromatograms (EICs) of control blood fortified with analytes at 0.25 mg/l (250 ng/ml).

The calibration range used for this analysis was 0.05–0.75 mg/l for all analytes. The calibration curves were linear across the calibration range without special weighting or curve treatment.

Typical calibration curves for the six analytes gave correlation coefficients (r^2) greater than 0.99 in all cases.

Quality control samples (n=10) fortified with 0.25 mg/l of each analyte gave quantitation results shown in Table 1. Coefficients of variation were typically 5% or less, and quantitation results were within 5% of the target value (within 1% or less for four of the analytes).

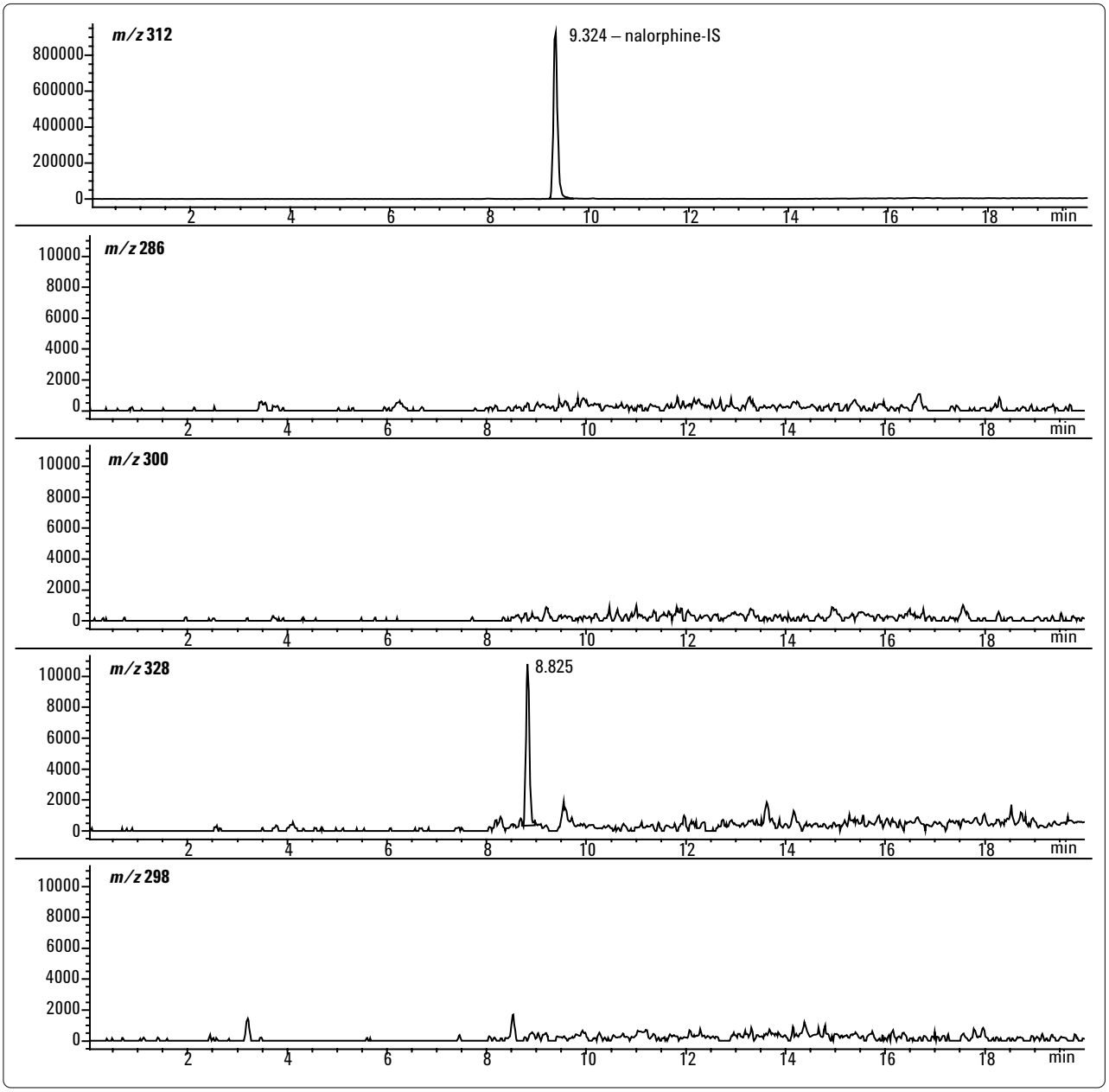

Figure 3. Extracted ion chromatograms (EICs) of blank blood fortified with internal standard at 1 mg/l (1000 ng/ml)

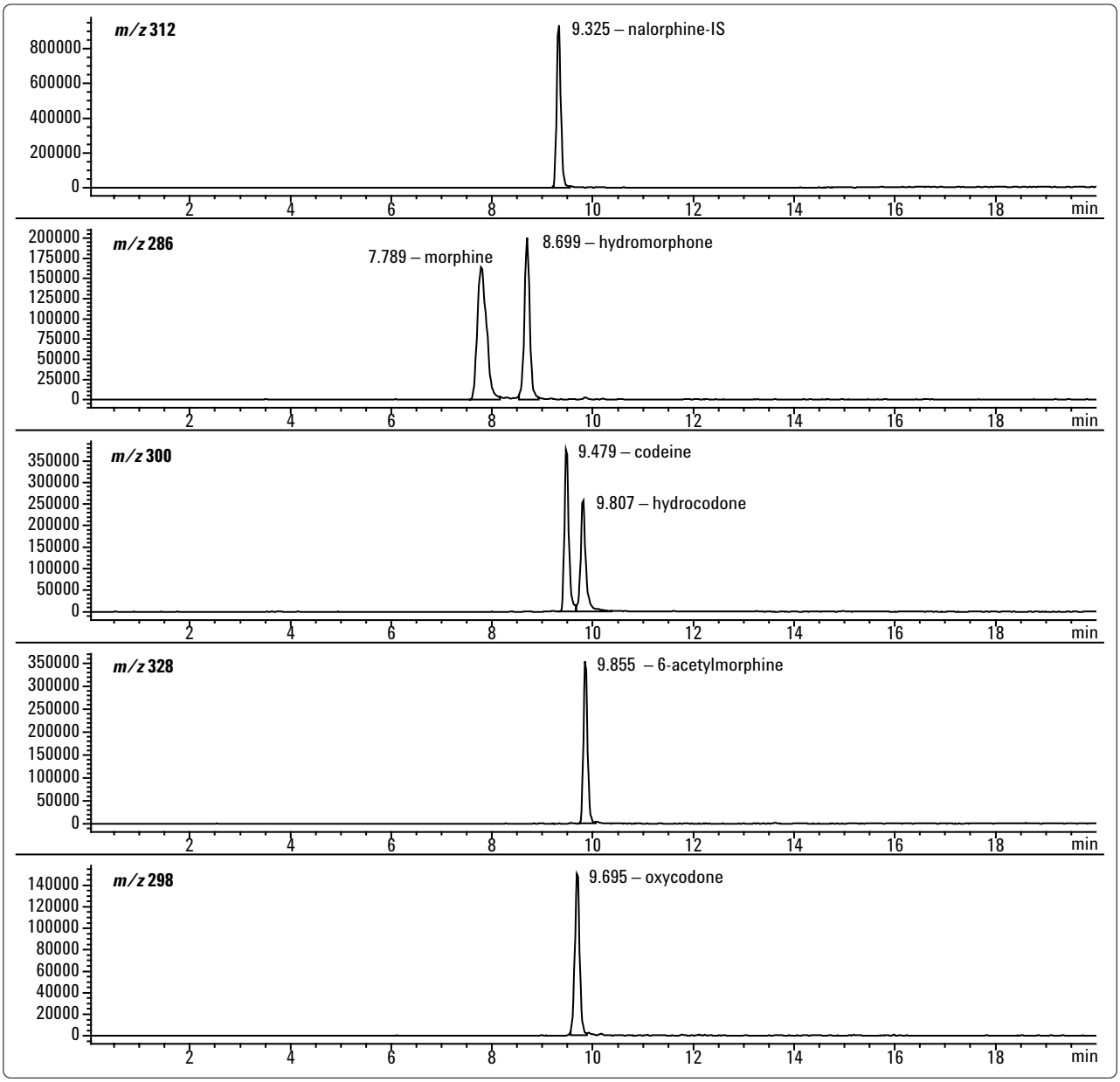

Figure 4. Extracted ion chromatograms (EICs) of control blood fortified with analytes at 0.25 mg/l

Table 1. Method accuracy and precision. Target concentrations were 0.25 mg/l

	morphine	hydromorphone	codeine	hydrocodone	6mam	oxycodone
	0.248	0.254	0.251	0.239	0.253	0.247
	0.247	0.245	0.239	0.242	0.249	0.254
	0.250	0.254	0.269	0.252	0.260	0.275
	0.267	0.249	0.246	0.245	0.230	0.264
	0.254	0.242	0.252	0.245	0.244	0.257
	0.251	0.246	0.249	0.241	0.248	0.267
	0.247	0.251	0.245	0.237	0.255	0.252
	0.258	0.259	0.256	0.250	0.258	0.263
	0.249	0.253	0.244	0.246	0.252	0.263
	0.254	0.256	0.261	0.249	0.259	0.287
mean	0.253	0.251	0.251	0.245	0.251	0.263
standard deviation	0.00942	0.00436	0.0128	0.00557	0.0128	0.0122
coefficient of variation[1]	3.729	1.737	5.102	2.276	5.117	4.638
percent error[2]	1.00%	0.36%	0.48%	–2.16%	0.32%	5.16%

[1]Coefficient of variation = (standard deviation/mean) x 100;
[2]percent error = (mean-target)/target x 100

Figure 5 shows the results for an opiate-positive blood sample from a 48-year-old female who was discovered deceased. She had an exten-sive medical history and had recently been assigned prescriptions of MS Contin (morphine sulfate) and Dilaudid. Analysis confirmed the presence of total morphine at 0.84 mg/l and total hydromorphone at 0.08 mg/l.

Another positive blood sample (Figure 6) was from a case involving a 40-year-old male discovered unconscious. LC/MS analysis of the subject's blood was positive for oxycodone at 0.23 mg/l and for codeine, which was not quantified because it was below the low calibrator (0.05 mg/l).

Analysis is also shown (Figure 7) for a third positive blood sample from a 41-year-old female found deceased. LC/MS analysis confirmed the presence of total morphine at 0.05mg/l, and clearly identified both 6-acetylmorphine and codeine at levels below the low calibrator.

The definition of the LOQs for this method is still in progress, but the sensitivity of the method reported here affords reliable quantitation down to at least 0.01 mg/l (10 ng/ml).

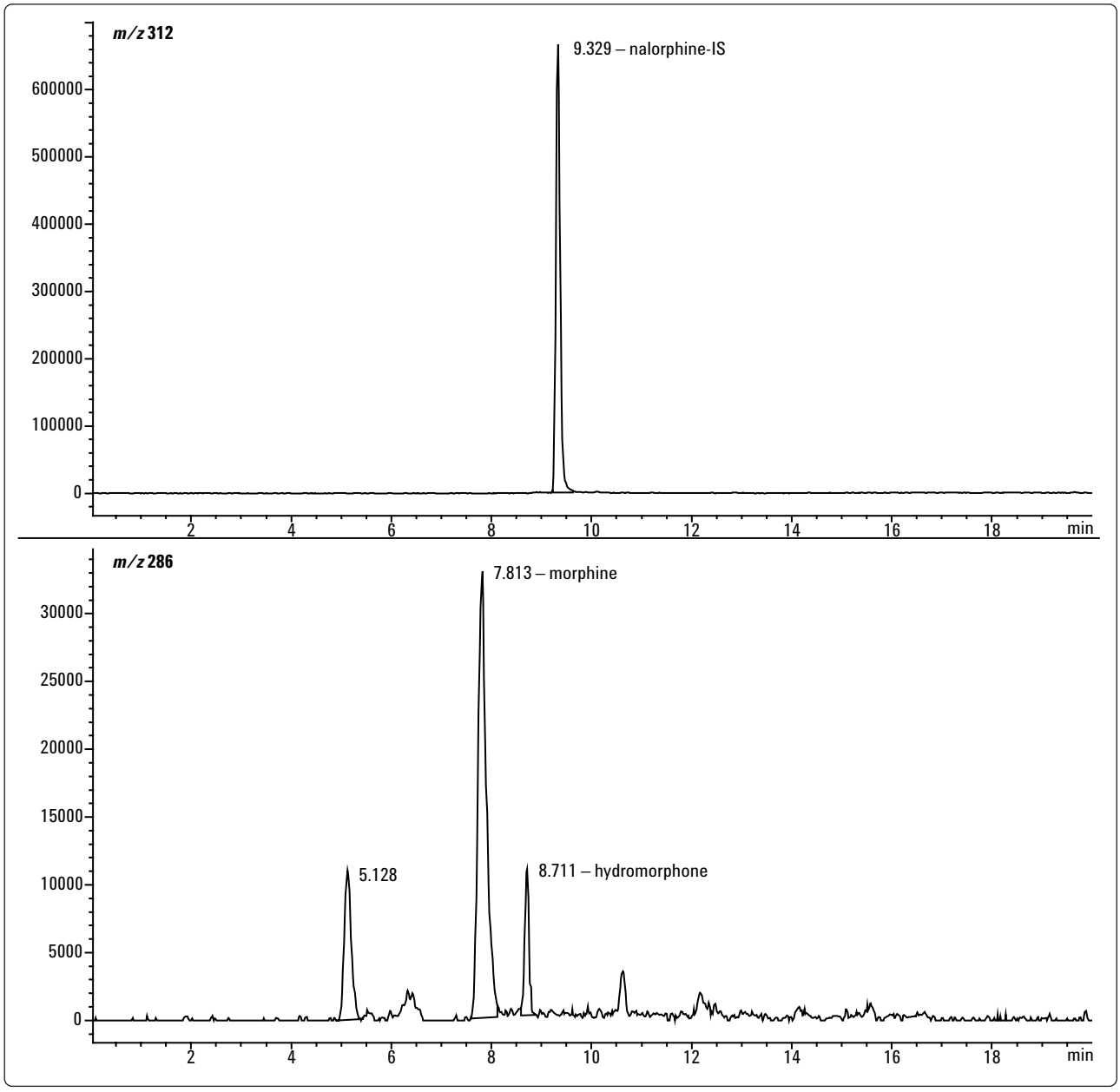

Figure 5. EICs of a positive blood sample found to contain morphine and hydromorphone

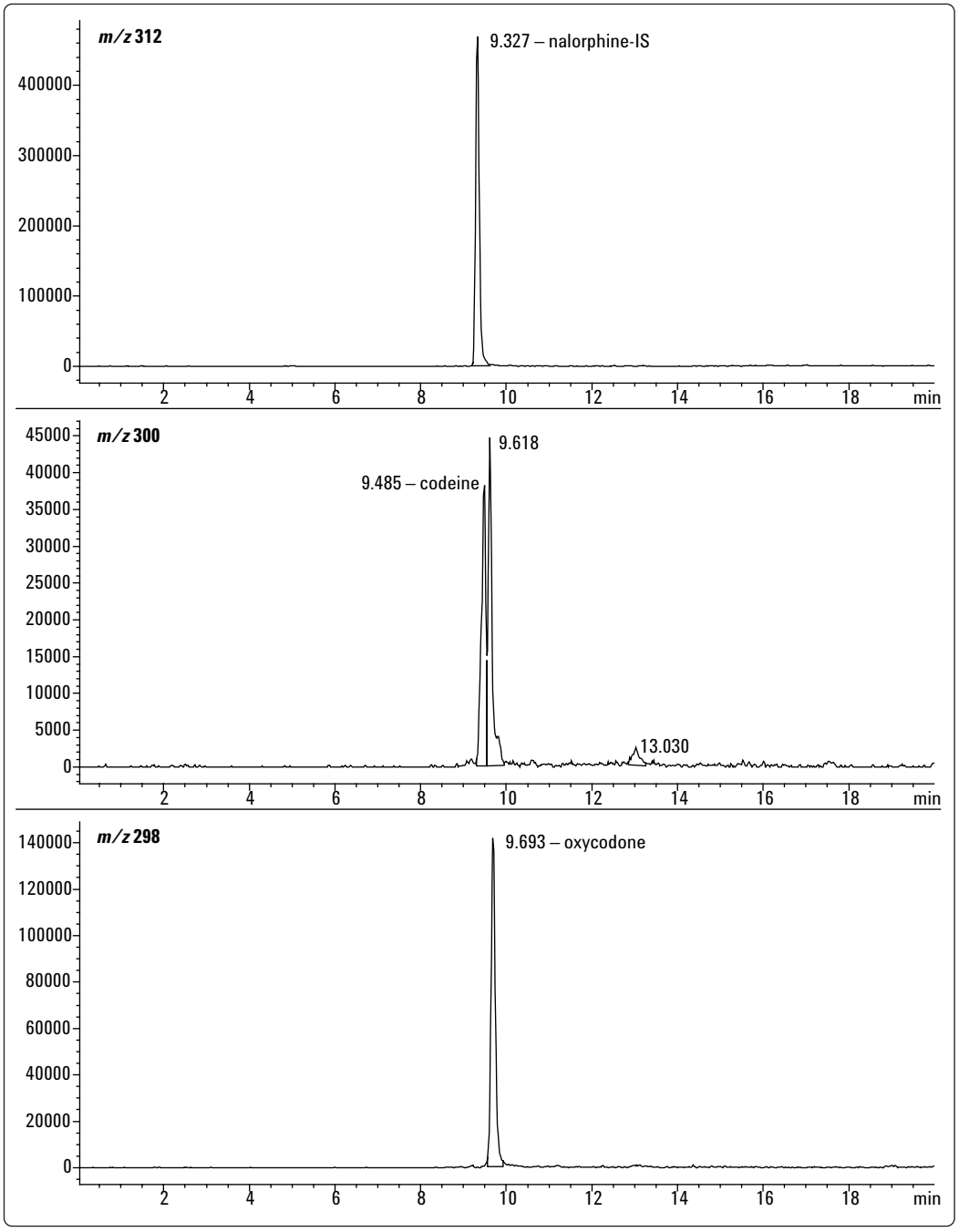

Figure 6. EICs of a positive blood sample found to contain oxycodone and codeine

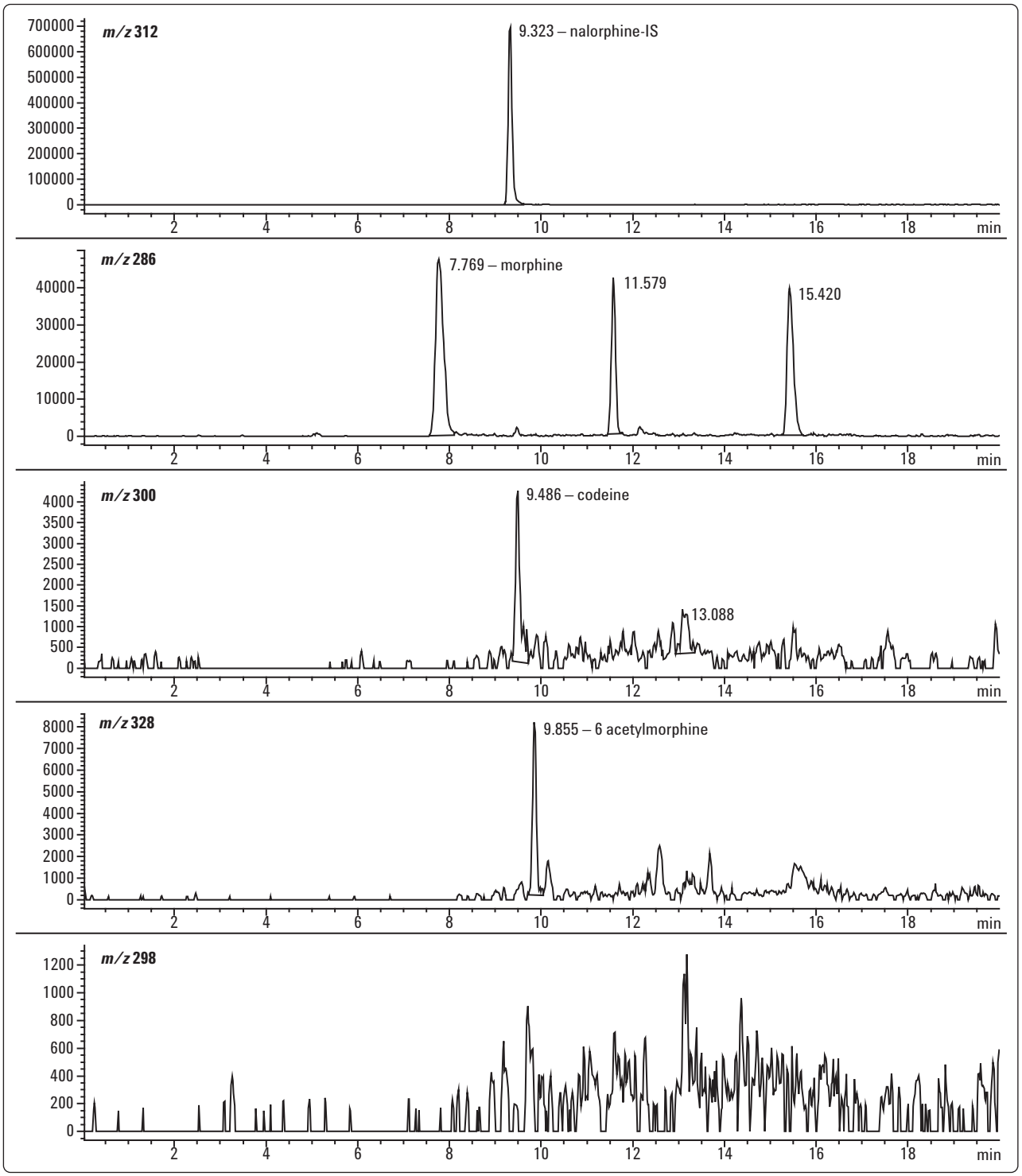

Figure 7. EICs of a positive blood sample found to contain morphine, codeine, and 6-acetylmorphine

Conclusions

The data clearly show the described electrospray LC/MS method to be suitable for routine measurements of opiates in whole blood. The assay as has a linear range of 0.05–0.75 mg/l, and the precision and accuracy of this method compare favorably to those of the well-established GC/MS methods for forensic drugs in blood. The sample preparation uses a solid phase extraction technology widely used in forensic laboratories and requires no special modifications. In compari-son to an existing GC/MS method for these ana-lytes, the LC/MS method is simpler because it does not require derivatization, which involves aggressive reagents, derivatization time, and addi-tional variability. In addition, the sensitivity of the LC/MSD VL allows the use of scan mode rather than SIM without compromising accuracy or pre-cision, making this method useful for general drug screening as well as target compound analysis.

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Identification and Quantitation of Benzodiazepines and Metabolites by LC/MS

Application

Clinical Research and Forensic Toxicology

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Abstract

A liquid chromatography/mass spectrometry (LC/MS) method for the analysis of the common benzodiazepines is described along with sample preparation suitable for blood, serum or plasma. Using the Agilent LC/MSD VL quadrupole mass spectrometer instrument in full scan mode, both spectral identification and quantitation can be carried out simultaneously. The analytical method provides a 0.02 µg/mL limit of quantitation for the analytes in blood and a correlation coefficient of better than 0.98 over three orders of magnitude. The system is sufficiently sensitive to perform this analysis in scan rather than selected ion monitoring (SIM) mode, allowing for identification of non-target compounds which may also be found in the sample.

Introduction

Benzodiazepines are an important class of drugs with a broad range of therapeutic effects, including sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant [1, 2]. Because of their wide usage, benzodiazepines have the potential for interaction with other central nervous system depressants which can result in life-threatening or impaired-driving situations. For these reasons, the analysis of benzodiazepines is of great interest to forensic and clinical research toxicologists.

Benzodiazepines have been analyzed using HPLC with UV detection [3], gas chromatography with nitrogen phosphorus and electron capture detectors [4], and gas chromatography/mass spectrometry (GC/MS) [5]. Many benzodiazepines are polar and non-volatile, making them difficult, if not impossible, to analyze with GC or GC/MS. Some of the compounds cannot be derivatized for improved chromatographic behavior. Furthermore, some of the newer benzodiazepines, like flunitrazepam, have lowered therapeutic ranges and faster clearance, and therefore require quantitation at lower levels. Liquid chromatography/quadrupole mass spectrometry is ideally suited for these com-pounds because the technique does not require derivatization, thereby saving time, expense, and experimental difficulty. The full-scan sensitivity of the Agilent liquid chromatograph/mass selective detector (LC/MSD) allows for quantitation, identification, and confirmation in a single analysis.

Experimental

The LC/MS system used in this work consisted of 1100-series vacuum degasser, binary pump, autosampler, thermostatted column compartment, diode array detector (DAD) with micro-flow cell, and LC/MSD quadrupole VL model. The DAD was used primarily for method development; however, the UV detector in series with the MS provides UV spectra which can also be used for identification when levels are sufficiently high. Complete system control and data analysis was provided by the Agilent LC/MS ChemStation.

Compounds Analyzed

Drugs	Metabolites
Alprazolam	–
Clonazepam	–
Diazepam	Nordiazepam
Flunitrazepam	7-aminoflunitrazepam
Flurazepam	Desalkylflurazepam
Halazepam	–
Temazepam	–
Triazolam	–

Flumazenil (internal standard)

Sample Preparation

Samples were prepared using liquid-liquid extraction, which is commonly used for these compounds for analysis by GC/MS. The only difference from a GC/MS method is omitting the derivitization step and reconstitution of the final sample in the LC mobile phase, rather than in a volatile solvent for GC injection.

A 1-mL volume of blood, serum, or plasma, to which 100 µL of internal standard solution (10 ng/µL) has been added, is added to 1 mL of saturated sodium borate solution, and the mixture is vortex-mixed. Ethyl acetate (4 mL) is added and mixing is carried out on a rotary shaker for 5 minutes, followed by centrifugation at 3400 rpm for 5 minutes. The upper layer is transferred to a clean tube and evaporated to dryness. The residue is reconstituted in 50 µL of the initial mobile phase and transferred to an autosampler vial.

LC/MS Method Details

LC Conditions

Instrument:	Agilent 1100 HPLC
Column:	ZORBAX XDB-C18, 150 × 4.6 mm, 3.5 µm (Agilent part number 963967-902)
Column temp:	50 °C
Mobile phase:	A = 0.1% formic acid in water B = 0.1% formic acid in methanol
Flow rate:	0.5 mL/min (optimized for this separation)
Gradient:	5% B until 2 min 90% B at 10 min, hold 8 min
Injection vol:	10 µL

MS Conditions

Instrument:	Agilent LC/MSD VL
Ionization mode:	Positive ESI
Drying gas flow:	13 L/min
Nebulizer:	40 psig
Drying gas temp:	300 °C
Scan range:	*m/z* 50–1000
V_{cap}:	3000V
Fragmentor:	120V

Results and Discussion

The sample preparation used in this method is derived from a method using GC or GC/MS. The sample preparation used for many GC or GC/MS methods can often be used for LC/MS just by omitting any derivitization step and transferring the final sample to LC mobile phase instead of using a volatile solvent. Flumazenil, a benzodiazepine antagonist not found in samples in this jurisdiction, is used as the internal standard due to the cost and availability of deuterated analogues of some analytes.

The standard VL model of the LC/MSD is quite capable of carrying out the analysis of benzodiazepines in blood. The SL model affords approximately 10x greater sensitivity if needed for other analyses, as well as multisignal capability such as alternating positive/negative mode, SIM/scan mode, and low/high fragmentation modes. The analysis is carried out in full scan acquisition mode in order to quantitate the target analytes using extracted ion chromatograms (EICs), and to alternatively confirm their identity using other ions in the spectra as well.

A moderate amount of collision-induced dissociation (CID) is used in this method by setting the Fragmentor voltage in the method to a value 50V higher than the default value of 70V which minimizes CID. This results in spectra which contain more ions than just the pseudo-molecular ion. These ions can then be used as confirming ions for EICs, as is the common practice for EI GC/MS, and the spectra can be placed in a user-created library for identification of drugs using library search of API spectra.

Figure 1 shows an overlay of the EICs of all target benzodiazepines and three common metabolites analyzed in this work.

Some typical full scan spectra used for both quantitation and confirmation are shown in Figure 2. The fragmentor voltage chosen is just high enough to produce fragment ions by CID for confirmation, while attempting to preserve significant signal for the intact molecule. For example, the *m/z* 268.1 in the spectrum of flunitrazepam arises from fragmentation of the $(M+H)^+$ ion at *m/z* 314.1.

The spectral behavior is, of course, compound-dependent. For instance, in the case of temazepam, there is more signal for the sodium adduct at *m/z* 323.1 so that ion is used for quantitation, while the fragment at *m/z* 255.0 is used for confirmation. The protonated molecular ion *m/z* 301.0 can also be used as a confirmation ion, as long as the protonated/sodiated ion ratios are constant for the analysis. Compounds with oxygen-containing functional groups can show sodium adducts as well as proton adducts; this complication can be avoided with the use of Atmospheric Pressure Chemical Ionization (APCI) in place of electrospray ionization (ESI) [6, 7].

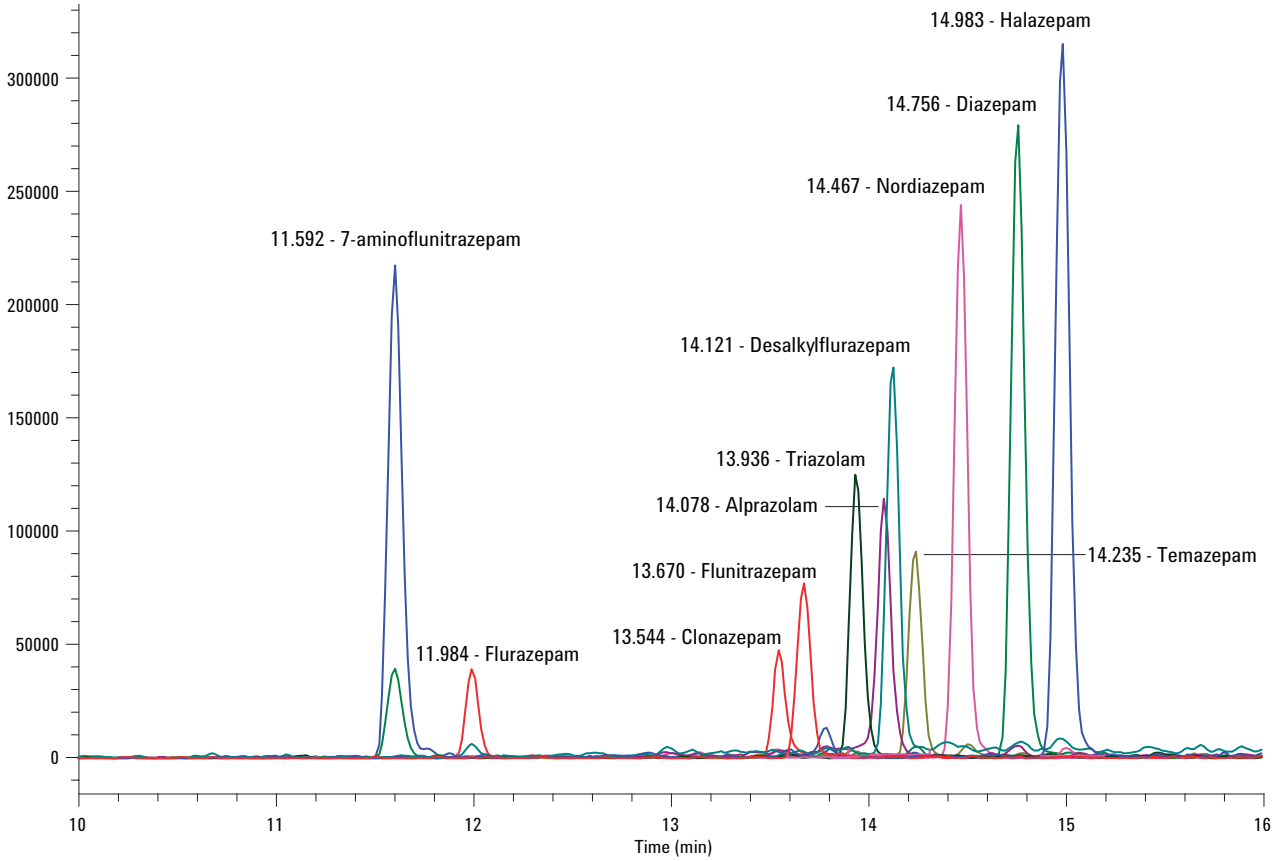

Figure 1. Overlaid EICs of 11 benzodiazepine compounds with retention times. Time axis zoomed into a time range of 10 to 16 minutes.

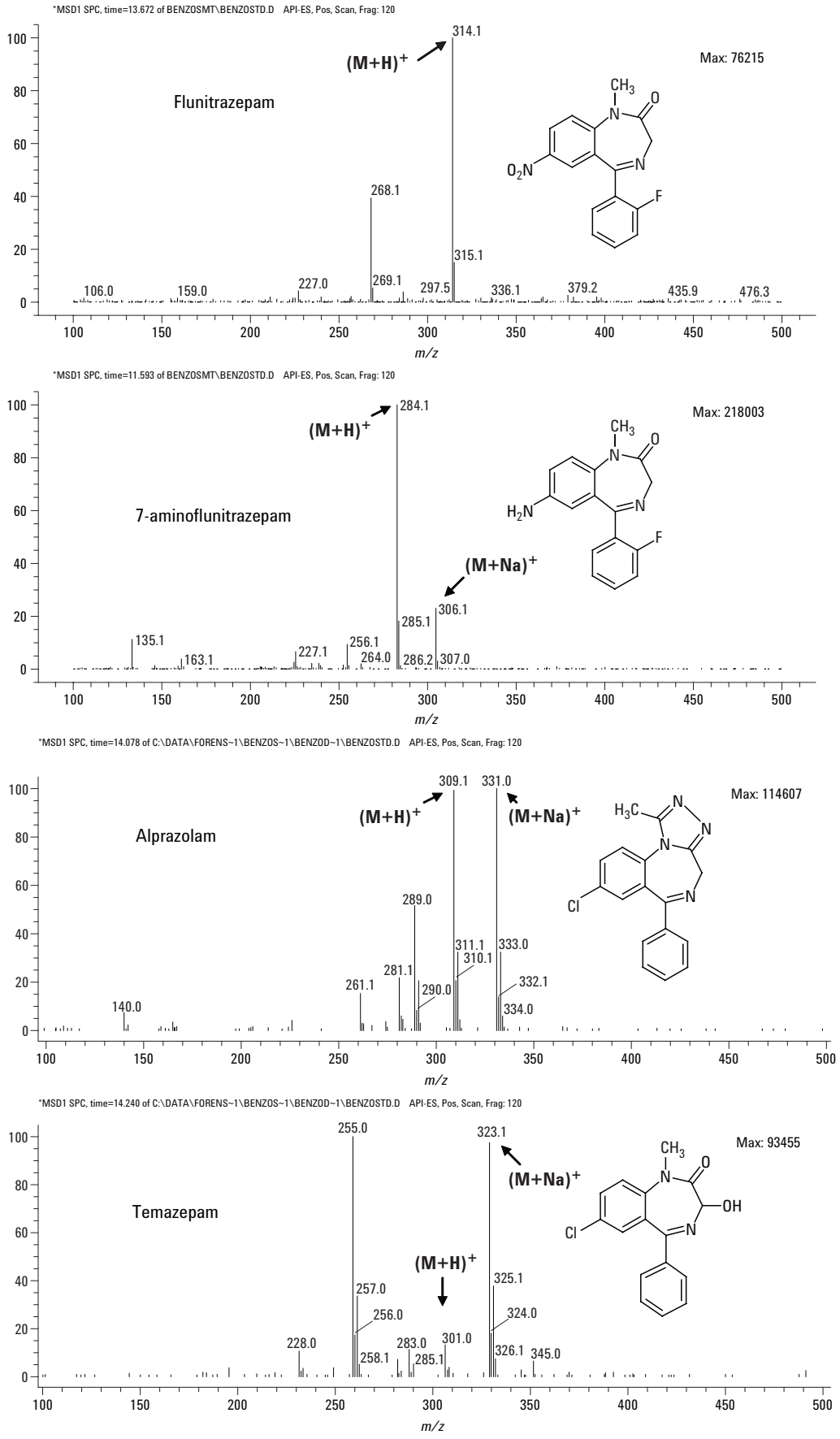

Figure 2. Typical benzodiazepine spectra showing protonated $(M+H)^+$, sodiated $(M+Na)^+$, and fragment ions.

The limit of detection (LOD) at a S/N of 3:1 is approximately 10 ng/mL using this method for most of the target compounds and this model of LC/MSD. The method as practiced at the Montana State Toxicology laboratory uses a 20 ng/mL (0.02 µg/mL) limit of quantitation (LOQ), a calibration range extending to 1000 ng/mL, and one or more qualifier ions for each analyte. Figure 3 shows extracted quantitation and confirming ions for the internal standard and temazepam in the 20 ng/mL (low) calibrator.

With the specified sample preparation and instrument conditions and a calibration range up to 1000 ng/mL, a quadratic treatment gives a better curve fit than a linear treatment for most of these analytes (r^2 >0.99). Figure 4 shows such a calibration curve for alprazolam from 5 to 2000 ng/mL. A linear fit still gives an r^2 >0.98. The curvature at the high end is undoubtedly due to the well-known phenomenon in ESI of droplets reaching a saturation limit of ions at some high analyte concentration.

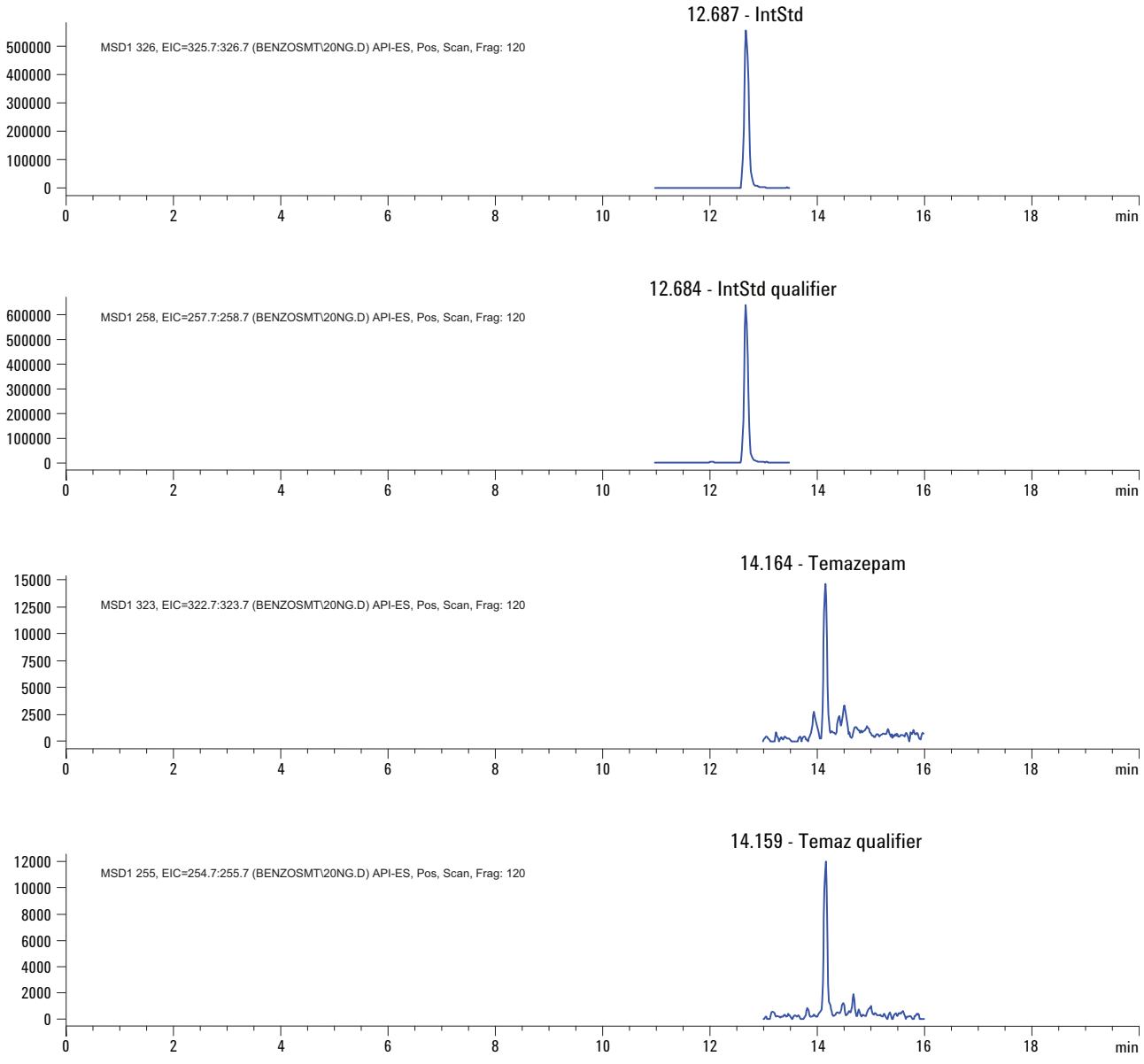

Figure 3. EICs of internal standard (flumazenil) and temazepam, at the 20 ng/mL LOQ, and their respective confirmation ions.

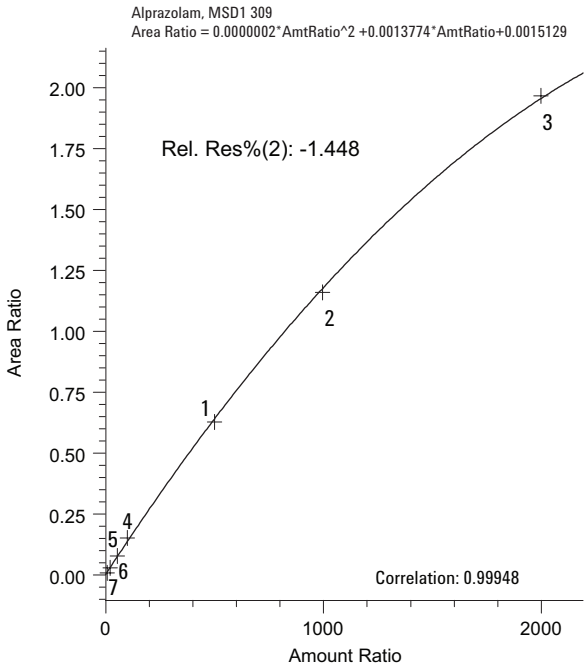

Figure 4. Calibration curve for alprazolam, 5–2000 ng/mL.

Results of an actual case sample, in which alprazolam was found at a moderate level of 128 ng/mL, are shown in Figure 5. Note the excellent chromatographic peak shape and narrow peak width. Figure 6 shows an expanded view of EICs for an impaired-driver case sample which had to be diluted 10-fold to be analyzed in the calibrated range. Blood concentration was therefore estimated to exceed 3000 ng/mL.

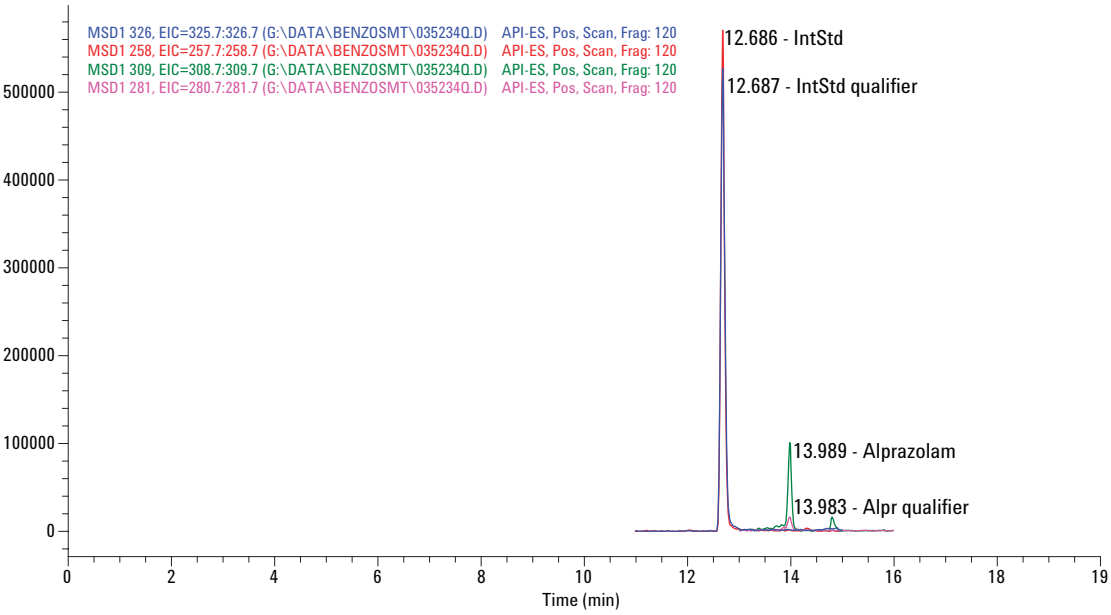

Figure 5. Case sample – alprazolam, moderate level, 128 ng/mL.

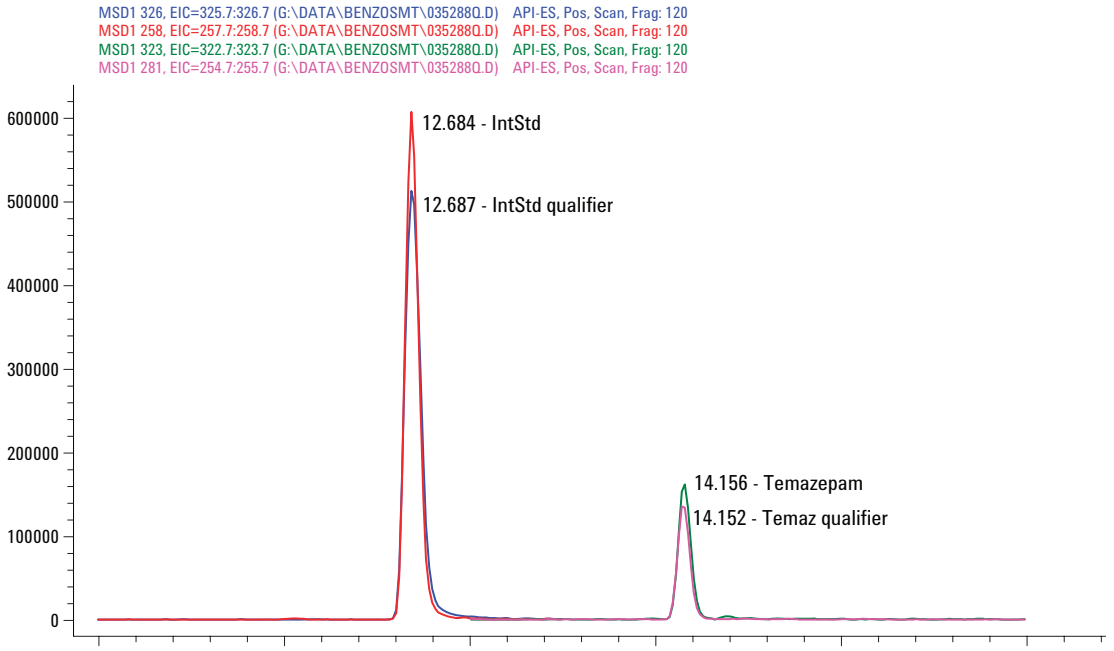

Figure 6. Case sample - temazepam, high level, 307 ng/mL (after 10x dilution to calibrated range).

Conclusions

This work demonstrates the usefulness of LC/MS for the analysis of benzodiazepines in blood. These compounds tend to be difficult to analyze by GC-based techniques, but ionize well in API-electrospray, resulting in excellent sensitivity, even in full scan mode using the lowest-cost model of LC/MSD (VL). Blood is a difficult matrix to analyze, but the results here show excellent quantitation and simultaneous identification using only 1 mL of sample and a simple liquid-liquid extraction procedure used for GC/MS, without the derivatization. The CID spectra show strong base-peak signals used for quantitation over three orders of magnitude, and CID fragment ions for ion ratio confirmation and/or library search. The method could be used in SIM and with the more sensitive LC/MSD SL model for any of the newer benzodiazepines which are found at lower levels in blood.

A rapid, reproducible method has also been published for a large number of benzodiazepines and related substances using the Agilent LC/MSD quadrupole system [6]. The method uses APCI rather than ESI, a liquid/liquid extraction procedure similar to this one, CID with greater fragmentation, and several deuterated internal standards. The publication describes the use of CID spectra and library search for identification, and includes spectra of all the analytes under both low and high fragmentation conditions.

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Determination Of Opioids, Cocaine, and Cocaine Metabolites by Liquid Chromatography Mass Spectrometry Using ZORBAX Eclipse Plus C18 Columns

Application

Forensic Toxicology

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Abstract

An improved method for the analysis of opioids, cocaine and cocaine metabolites from blood using solid phase extraction followed by LC/MS is described. An Eclipse Plus C18 column is used to separate the drugs and metabolites. The combination of excellent peak shape and resolution afforded by this column together with the sensitivity and selectivity afforded by the LC/MS allow a simple extraction without derivitization to be used to separate and quantify these drugs and metabolites in a single analysis.

Introduction

Over 20% of the blood specimens from cases submitted to the Washington State Toxicology Laboratory are positive for opiates, cocaine metabolites, or both by immunoassay screens. Drug concentrations vary widely from case to case, and the analytes appear in many different combinations. The ideal confirmatory analysis should allow determination of all available opioids, cocaine, and cocaine metabolites in a single blood specimen, with high sensitivity and a wide linear dynamic range. Until recently, gas chromatography-mass spectrometry was the industry standard for these confirmations; however, sample derivatization or even dual derivatization is required[1].

At the Washington State Toxicology Laboratory, we have employed liquid chromatography/mass spectrometry (LC/MS) with the Agilent MSD SL and the new ZORBAX Eclipse Plus C18 columns for combined analysis of opioids, cocaine, and cocaine metabolites for several thousand cases. This approach has a number of advantages over our previous GC/MS method, including simpler sample preparation, improved sensitivity, and the ability to detect a broader range of opioids in a single analysis.

Experimental

Methods

Extract

Condition Clean Screen extraction column (United Chemical Technologies, CSDAU206)

1 × 3 mL Methanol
1 × 3 mL DI Water
1 × 3 mL 0.1 M KH_2PO_4

Prepare Blood Sample

(Standards: add working standard and dry down first)
50 µL Internal Standard (ethyl morphine 2 µg/mL)
1 mL blood
3 mL 0.1 M KH_2PO_4
Vortex mix and centrifuge 2,500 rpm 15 min

Apply diluted, centrifuged blood to conditioned column at 1 to 2 mL /min

Wash Column

1 × 3 mL DI water
1 × 3 mL 0.1 N HCl
1 × 3 mL methanol
Dry 10 min at maximum vacuum

Elute

1 × 3 mL CH_2Cl_2/isopropanol/NH_4OH (72/26/2)
(Prepare fresh daily)

Evaporate @ 50° (~ 20 min) and reconstitute in 100 µL 1% acetic acid.

Chromatographic and Instrument Conditions

Instrument:	Agilent 1100 LC/MSD SL
Column:	ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 micron (Agilent PN 959993-902)
Mobile Phase:	A: 1% acetic acid B: acetonitrile Start: 3% B At 16.5 min 40% B At 17 min 40% B At 20 min 3% B At 32 min 3% B
Flow rate:	1 mL/min
Column temp.:	60 °C
Injection vol.:	2.5 µL
Needle rinse:	1% acetic acid

MS Conditions

Source:	Electrospray
Ionization mode:	Positive
Vcap:	3,000 V
Nebulizer:	40 psig
Drying gas flow:	13 L/min (nitrogen)
Drying gas temp.:	350 °C
Mass ranges:	SIM, 3 groups Group 1 (1.0 to 4.8 min) 209, 227, 284, 286, 287, 302, 462 amu Group 2 (4.8 to 8.1 min) 181, 241, 257, 268, 298, 300, 314, 316, 328 amu Group 3 (8.1 to 17 min) 168, 196, 272, 290, 291, 304, 318 amu
Fragmentor:	Groups 1 and 2: 260 V; Group 3: 220 V

Results and Discussion

Table 2 gives the retention times and ions used for the compounds in this method. Chemical structures are available in Agilent Application Note 988-4805EN[2]. Raw data files were transferred from the LC/MSD computer to a computer running the Agilent MSD Chemstation for data analysis. (Agilent LC/MS data files are fully compatible with the MSD Chemstation.) For each analyte, one of the target masses represents the pseudomolecular ion formed by proton addition (M+H). Relatively high fragmentor voltages were used in order to produce sufficient qualifier ion abundances by collision-induced dissociation. At least two masses were monitored for each compound, and the acceptable limits for ion ratios were set at ± 25%[3]. In cases where two ions were monitored for a compound, an isotopic mass (M+2) can be used as a third ion, but is not as informative as a qualifier ion representing a known fragment of the target molecule. Under these conditions, sodium adduct formation was not consistent enough to allow M+H+22 ions to be used as qualifier ions. A representative chromatogram of an extract from a control blood specimen is shown in Figure 1.

Table 3 gives the limits of detection, quantitation, and linearity for the method, along with quality control data collected over a six-month period. The laboratory policy is to set acceptance ranges for blood drug controls at ± 20% of the mean value determined in-house. Calibration curve coefficients of determination (r^2) were ≥ 0.990 for all of the routinely measured analytes. Recovery of all ana-

Table 2. Compounds Analyzed (pseudomolecular ions in bold type)

Compound	Retention time (min)	Ions monitored
Ethyl morphine (I.S.)	7.47	**314**, 257
Morphine	2.96	**286**, 227, 209
Hydromorphone	3.83	**286**, 227
Codeine	5.55	**300**, 241, 181
Oxycodone	6.18	**316**, 298, 241
6-acetyl morphine	6.4	**328**, 268
Hydrocodone	6.61	**300**, 241
Benzoylecgonine	8.62	**290**, 168
Cocaine	10.3	**304**, 272
Cocaethylene	11.94	**318**, 196
Research Compounds		
Morphine-3-glucuronide	1.93	**462**, 286
Morphine-6-glucuronide	2.8	**462**, 286
Oxymorphone	3.34	**302**, 284, 227

lytes except for morphine-3- and morphine-6-glucuronide was > 90%. Recovery of morphine glucuronides was poor (~1%). Carryover from previous injections was noted when extracts were injected without using the needle wash option, but with the needle wash incorporated into the method, carryover was eliminated at concentrations up to 10,000 ng/mL or higher.

Our methodology is based on that described by Pichini et al[4]. When we attempted to add additional opioids to their procedure, without further modification, severely asymmetric peak shapes were encountered for oxycodone, hydromorphone and hydrocodone. This problem, which has been described previously in the literature, is believed to be due to the formation of multiple adducts with mobile phase constituents[5]. Use of the high-performance Eclipse Plus columns, at a relatively high temperature (60 °C), resulted in dramatically improved peak shape for the problem analytes (Figure 1).

Analysis of as many opioids as possible in a single extract has several advantages, in addition to the obvious savings in cost and time. Potent minor active metabolites of codeine, hydrocodone, and oxycodone produced by Cytochrome P450 2D6 metabolism (morphine, hydromorphone, and oxymorphone, respectively[6]) can be monitored routinely in this procedure. Information on potent active metabolites may be helpful in assessing total opiate exposure, and may also help to differentiate acute and chronic drug exposures. Using this LC/MS method, hydromorphone can be detected in three different kinds of cases: (1) after hydromorphone administration, (2) as a potent minor

Table 3. Method Limits of Detection, Quantitation, Linearity, and Quality Control Data

Compound	LOD ng/mL	LOQ ng/mL	Upper LOL	Control conc.	CV%
Ethyl morphine (I.S.)	-	-	-	-	-
Morphine	5	5	2000	41	10%
				92	7%
Hydromorphone	1	2	400	8	8%
Codeine	5	5	2000	49	6%
				97	5%
Oxycodone	5	5	2000	45	6%
				258	4%
6-acetyl morphine	1	2	200	4	6%
Hydrocodone	5	5	2000	52	4%
				94	6%
Benzoylecgonine	25	100	5000	114	10%
				672	8%
Cocaine	5	5	2000	61	7%
				84	6%
Cocaethylene	5	5	2000	64	11%
				88	8%

metabolite of hydrocodone, and (3) as a minor metabolite after high-dose morphine administration[7].

We selected ethyl morphine as the internal standard for this method because some of the deuterated internal standards we tested fragmented to give the same ions as the homologous target compound in our single quadrupole instrument. If this method were employed with a tandem LC/MS system, multiple deuterated internal standards could be employed, which might result in even better accuracy and precision than reported here.

Oxymorphone and morphine-3- and morphine-6-glucuronides have only been analyzed on a research basis to date. Despite poor recovery, measurement of morphine-3- and morphine-6-glucuronides along with morphine appears to be valuable in differentiating some cases of acute vs. chronic drug ingestion. In one morphine-related death, a teenager took an unknown dose of an older woman's prescribed continuous-release morphine. Analysis by LC/MS revealed a post-mortem blood morphine concentration in excess of 700 ng/mL, but lower concentrations of morphine glucuronides. In contrast, post-mortem blood from terminal cancer subjects receiving chronic morphine typically contains morphine glucuronide concentrations on an order of magnitude greater than the parent drug concentration. Improved recovery of morphine glucuronides can be achieved by increasing the proportion of isopropanol in the eluting solvent in this method. Use of a simpler extraction with a hydrophobic solid-phase extraction column[2], rather than the mixed hydrophobic/cation-exchange column described in this method, gives excellent recovery of morphine glucuronides, but at the cost of increased background signal and shorter column life. An alternative extraction that may hold promise employs a polymeric solid phase column and elution with 5% ammonium hydroxide in methanol, with high recovery of morphine and its glucuronides[8].

Oxymorphone is extracted with high recovery in this method, and further work with oxymorphone is indicated because of its recent approval by the FDA as a high-potency oral opioid analgesic.[9] A number of other opioid metabolites can be mea-

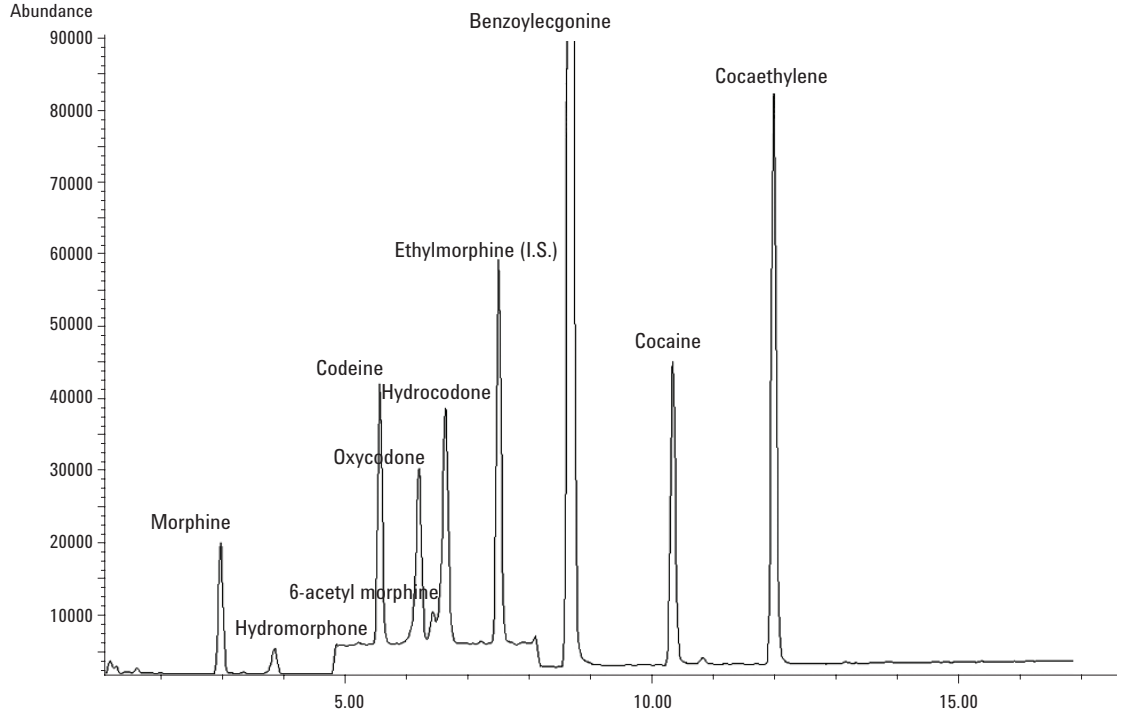

Figure 1. Total ion chromatogram of an extract of a quality control blood sample containing morphine (41 ng/mL), hydromorphone (8 ng/mL), codeine (49 ng/mL), oxycodone (45 ng/mL), 6-acetyl morphine (4 ng/mL), hydrocodone (52 ng/mL), benzoylecgonine (672 ng/mL), cocaine (61 ng/mL), and cocaethylene (64 ng/mL).

sured using this method. Hydrocodone is metabolized to hydromorphone, as previously noted, but is also metabolized to dihydrocodeine and norhydrocodone. Oxycodone is metabolized to oxymorphone, and is also metabolized to noroxycodone, alpha and beta oxycodol, noroxycodol, and other products. The choice of which metabolites to measure is complex. As mentioned previously, high-potency opioid metabolites may contribute to the effects of the parent drug, but recent data from Dr. Danny Shen's laboratory cast some doubt on this contention, at least with respect to oxycodone[10]. Even if metabolites do not contribute to the parent drug's pharmacological effects, they may be of forensic toxicological interest, for example to help distinguish acute from chronic drug use.

Another cocaine metabolite, ecgonine methyl ester, was extracted with the solid phase extraction described in this paper, but recovery was variable, possibly due to losses during the evaporation step. Because of variable recovery, quantitative analysis of ecgonine methyl ester with this methodology would require use of a deuterated internal standard.

Another potential method enhancement to this method would be the use of the nebulizer shim (Agilent part number G1946-20307), which is designed to improve ion transit into the capillary when mobile phase flow rate is high. This could result in improved assay sensitivity for this application, which uses a mobile phase flow rate of 1.0 mL/min.

Conclusions

This communication describes a comprehensive method for analysis of opioids, cocaine, and cocaine metabolites in blood, using single quadrupole LC/MS with electrospray ionization after mixed-mode solid phase extraction. The method is superior to our previous GC/MS methodology in that derivatization is not needed, limits of detection and quantitation are lower, and a broader range of opioids can be detected. In addition, by using high-performance ZORBAX Eclipse Plus C18 HPLC columns at a relatively high temperature, we were able to eliminate previously encountered problems with poor opioid peak shape.

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A Comparison of Several LC/MS Techniques for Use in Forensic Toxicology

Application Note

Forensic Toxicology

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Abstract

The analytical capabilities of various liquid chromatography/mass spectrometry (LC/MS) instruments are compared in the study of illicit and prescription drugs in blood. The blood samples analyzed include postmortem and driving under the influence of drugs (DUID). The presence of drug compounds in these samples was previously confirmed using gas chromatography/mass spectrometry (GC/MS). In this work, the LC conditions are common among the different types of mass spectrometers used. The mass spectrometers used include the single quadrupole (SQ), the time-of-flight (TOF), the ion trap (IT), the triple quadrupole (QQQ), and the quadrupole time-of-flight (QTOF). Both LC and MS instrumentation are Agilent.

In analyzing the different samples for the presence of several drug compounds, the advantages and disadvantages of each type of instrumentation are demonstrated. For example, the IT, TOF, and QTOF mass spectrometers are shown to be excellent devices for qualitative screening and identification. On the other hand, the SQ and QQQ mass spectrometers are excellent devices for quantitative targeted confirmation. And yet, the converse is somewhat true in that the TOF and QTOF instruments may also be useful for quantification, though not as sensitive as an instrument like the QQQ.

Drugs of interest in the blood samples include benzodiazepines, methadone, and cocaine metabolites.

Agilent Technologies

Introduction

Traditionally, forensic toxicology laboratories use immunoassays for screening and GC/MS for quantitative confirmation of drugs of abuse, whether illicit or prescribed. However, immunoassay is not completely specific and reagents are a significant lab expense, and GC/MS requires derivatization of samples which are polar or nonvolatile. In LC/MS, according to DeBoeck, et al [1]. "There has been an explosion in the range of new products available for solving many analytical prob-lems, particularly those applications in which nonvolatile, labile, and/or high molecular weight compounds are being analyzed."

As a result, it is becoming more and more common for forensic laboratories to be considering LC/MS for the analysis of drugs in biological samples, and not only for quantitative confirmation, but even for screening [2]. To date, LC/MS methods have been described for most of the main drug classes, including those analyzed here, like benzodiazepines, cocaine, and metabolites [3]. However, what seems to be missing from the literature is an overview of the various LC/MS techniques available and which ones are most appropriate for various tasks in the forensic toxicology laboratory.

In this work, such a comparison among LC/MS techniques is made, largely in part because Agilent has one of the broadest LC/MS portfolios of any mass spectrometry vendor. Therefore, by analyzing the same samples and calibrators and injecting them under the same LC conditions onto each mass spectrometer, fair comparisons are made to help the reader determine which instrument may be best for his or her type of application.

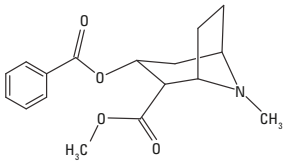
This work also represents the combined collaboration of three application chemists at Agilent and three professional forensic toxicologists. Some 50 samples, calibrators, and blanks were prepared: the postmortem samples by RTI International and the DUID samples by the University of Miami. Over three days, the samples were run on the following five different LC/MS instruments at the Agilent Technologies Center of Excellence in Wilmington, DE: SQ, IT, TOF, QQQ, and QTOF.

The postmortem blood samples from RTI are part of a project supported by NIJ Grant 2006-DN-BX-K014.

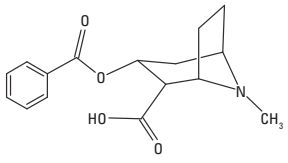
One mL of whole blood was used for each sample, with five point calibration curves generated for quantification of real case samples. Compounds analyzed in postmortem and DUID blood are shown in Figures 1a and 1b, respectively.

For the postmortem samples, cocaine, benzoylecgonine (BE), cocaethylene (CE), and methadone were analyzed, along with their deuterated D3 analogs as internal standards. For the DUID samples, alprazolam, diazepam, and nordiazepam were analyzed, along with their deuterated D5 analogs as internal standards. However, the presence of cocaine, BE, and CE in the DUID case samples was also examined.

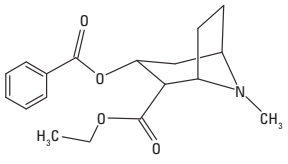
The LC conditions were consistent among all five LC/MS instruments using the same mobile phases, columns, column temperature, flow rate, and autosampler temperature. In fact, most of the work was done using two LC systems on carts moved between the various instruments.



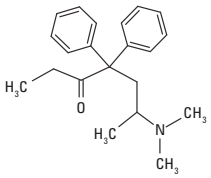
Cocaine, M+H$^+$ = 304.1543
$C_{16}H_{19}NO_4$



Benzoylecgonine, M+H$^+$ = 290.1387
$C_{17}H_{21}NO_4$

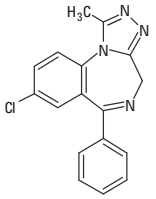


Cocaethylene, M+H$^+$ = 318.1700
$C_{18}H_{23}NO_4$

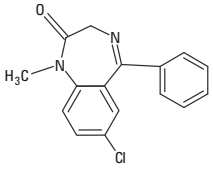


Methadone, M+H$^+$ = 310.2165
$C_{21}H_{27}NO$

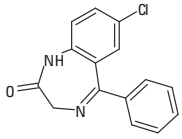
Figure 1a. Structures, chemical formulas, and exact masses of the protonated forms of the compounds analyzed in postmortem blood.



Alprazolam, M+H$^+$ = 309.0902
$C_{17}H_{13}N_4Cl$



Diazepam, M+H$^+$ = 285.0789
$C_{16}H_{14}N_2OCl$



Nordiazepam, M+H$^+$ = 271.0633
$C_{15}H_{12}N_2OCl$

Figure 1b. Structures, chemical formulas, and exact masses of the protonated forms of the compounds analyzed in DUID blood.

Experimental

Sample Preparation

Each sample size consisted of 1 mL whole blood. Solid-phase extraction cleanup (SPEware Corp., Baldwin Park, CA) appropriate for each compound analyzed was used. The postmortem samples were prepared in the RTI lab and the DUID samples were prepared at the University of Miami. Final eluates were evaporated to dryness and then shipped cold to the Agilent Center of Excellence in Wilmington, DE, where they were reconstituted in 100 µL mobile phase solvent corresponding to the starting composition of the LC gradient (5% B) just prior to analysis. The only exception to this was with the SQ, for which an additional 100 µL of mobile phase solvent was added, after it was determined that 100 µL was not enough to prevent signal saturation. As a result, the on-column injection amount was reduced by a factor of 2 for the SQ.

The five-point calibration levels for each compound are shown in Table 1. Throughout the remainder of this application note, benzoylecgonine and cocaethylene will be abbreviated as BE and CE, respectively.

Table 1. Calibration Levels for Quantification of Each Compound

Compounds, postmortem	Levels (ng/mL)
Cocaine	25, 50, 100, 500, and 1000
Benzoylecgonine (BE)	25, 50, 100, 500, and 1000
Cocaethylene (CE)	10, 25, 50, 250, and 500
Methadone	25, 100, 500, 1000, and 2000
Compounds, DUID	**Levels (ng/mL)**
Alprazolam	5, 10, 25, 100, and 500
Diazepam	25, 50, 100, 250, and 500
Nordiazepam	25, 50, 100, 250, and 500

LC/MS Method Details

LC Conditions (used with all MS analyzers)

Agilent 1200 Series binary pump SL, degasser, wellplate sampler, and thermostatted column compartment

Column: Agilent ZORBAX Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm (p/n 959764-902)

Column temperature: 50 °C

Mobile phase: A = 5 mM ammonium formate and 0.05% formic acid in water
B = 0.05% formic acid in acetonitrile

Flow rate: 0.25 mL/min

Injection volume: 5 µL (SQ, QQQ, IT); 2 µL (TOF); and 0.1 µL (QTOF)

Gradient:

Time (min)	%B
1.0	5
6.0	40
8.0	95

Stop time: 10 min
Post run: 2 min

Common MS Conditions (related to ionization source)

Mode: Positive electrospray ionization
Nebulizer: 30 psig
Drying gas flow: 10 L/min
Drying gas temperature: 350 °C
V_{cap}: 3000 V

These settings are typically the most efficient for the LC flow rate used.

Along with the ionization source, tuning of ion transfer optics and voltages in the analyzers responsible for the mass axis calibration were determined using autotune on each instrument, an automated algorithm using ions with *m/z* values in positive ESI mode corresponding to those as follows (*used for TOF and QTOF only):

118.08625, 322.04812, 622.02896, 922.00979, 1221.99064*, 1521.97148*, 1821.95231*, and 2121.93315

A calibrant solution containing these ions was automatically introduced by the autotune routine. The wide range of ion masses allows for a wide range in mass calibration as well as an optimal ion transfer for compounds being analyzed.

Individual MS Conditions (related to analyzer)

For all instruments a parameter known as the fragmentor voltage was used. This voltage may be used for the nonselective fragmentation of ions formed in the source, but in this work, it was simply used to optimally transmit each compound ion of interest from the ion source into the mass analyzer.

- *Agilent 6140A single quadrupole LC/MS system*

Acquisition settings for each compound are shown in Table 2. For all compounds analyzed in this work the fragmentor voltage was 125 V.

Table 2. Selected Ion Monitoring (SIM) Acquisition Settings for Each Compound (Detector gain shown in parentheses.)

Time (min)	Compound	SIM ion (gain)		Dwell (msec)
0.0	Cocaine	304.1	(5)	75
	Cocaine-D3	307.1		
	BE	290.1		
	BE-D3	293.1		
	CE	318.1		
	CE-D3	321.1		
7.0	Methadone	310.2		235
	Methadone-D3	313.2		
	Alprazolam	309.0	(10)	50
	Alprazolam-D5	314.0		
	Diazepam	285.0		
	Diazepam-D5	290.0		
	Nordiazepam	271.0		
	Nordiazepam-D5	276.0		

The SQ instrument was the least expensive instrument of those used in this work. It was also the easiest to use in that there was typically only one parameter, the fragmentor voltage, that needed to be optimized for each SIM experiment. As noted above, the settings for the ionization source, ion optics, and mass analyzer are already determined by the LC flow rate and by the autotune routine.

- *Agilent 6410A triple quadrupole LC/MS system*

Along with the fragmentor voltage, the collision energy (CEn) was a parameter to optimize for acquisition in the QQQ. This voltage was optimized to produce the highest response among product ions for multiple reaction monitoring (MRM). For each analyte compound, the higher response MRM was monitored for quantification and the next highest was used for confirmation as a qualifier. To confirm the presence of compounds in a sample, the peak area ratio of the qualifier versus quantifier MRM must be consistent with calibrators and within a tolerance of ± 20%. The MRM transitions are listed in Table 3. Qualifier ions and their voltages are indicated in square brackets ([ ]).

The QQQ may be operated as a scanning instrument as well, scanning as fast as 5,400 amu/sec, but this is not the most sensitive acquisition mode of the instrument. Just like the SQ, the fragmentor voltage must be optimized for each analyte ion of interest. In addition, the CEn must be optimized to maximize the responses of the quantifier and qualifier product ions. Otherwise, just like the SQ, the settings required for method development are predetermined for the ESI based on LC flow rate, and for the ion transfer optics and mass analyzer voltages based on the tuning mix ions.

Table 3. MRM Acquisition Settings for Each Compound (Qualifier ion settings in brackets, fragmentor voltage denoted as frag and collision energy denoted as CEn)

Time (min)	Compound	MRM	Frag (V)	CEn (V)	Dwell (msec)
0.0	Cocaine	304.1 > 182 [82]	130 [130]	15 [30]	40
	Cocaine-D3	307.1 > 185	130	15	
	BE	290.1 > 168 [105]	110 [110]	15 [30]	
	BE-D3	293.1 > 171	110	15	
	CE	318.1 > 196 [82]	130 [130]	15 [30]	
	CE-D3	321.1 > 191	130	15	
8.0	Methadone	310.2 > 265.1 [105]	110 [110]	15 [25]	30
	Methadone-D3	313.2 > 268	110	15	
	Alprazolam	309.0 > 205 [281.1]	170 [170]	40 [25]	
	Alprazolam-D5	314.0 > 286	170	25	
	Diazepam	285.0 > 193 [154]	170 [170]	30 [30]	
	Diazepam-D5	290.0 > 198	170 [170]	30	
	Nordiazepam	271.0 > 140 [165]	170 [170]	25 [30]	
	Nordiazepam-D5	276.0 > 213	170 [170]	30	

- *Agilent 6330A ion trap LC/MS system*

The ion trap was operated in a targeted screening mode of AutoMS(3) with an Include List of the expected compounds. The Include List consists of the *m/z* values corresponding to the expected ion masses $(M + H)^+$ of the analyte compounds. This list was the same as those shown as SIM ions in Table 2.

Operating in AutoMS(3) means that the ion trap was scanning in MS mode and when the intensity of any of the ion masses in the Include List rose above a user-defined threshold, that ion was then fragmented in full scan MS/MS mode. The instrument also looked at the intensity of the product ions and if any of them were more intense than another user-defined threshold, then that product ion would be fragmented in full-scan MS/MS/MS mode, or MS(3).

Acquiring in MS/MS/MS mode is specific to the compound structure; however, it does require enough signal in the MS/MS mode to be successful. The acquired MS/MS and MS(3) spectra are then compared to the same type of spectra in a library available from Agilent of some 400 compounds. Scoring matches are a weighted average of matching scores at the MS/MS and MS(3) levels as shown in the equation below.

$$Score' = \left(\frac{\sum_{i=1}^{M} Score \times Match}{M \times 10^6} \right)^{\frac{1}{N}} \times 1000$$

The effective score Score' is related to the individual score Score at each level of MS/MS and MS(3) matched to corresponding spectra in the library. The Score is the Fit (F), Reverse Fit (RF), and Purity (P) as calculated using the industry standard NIST-based search algorithm. The library does not contain MS spectra, so matching at that level is not carried out. Coeluting compounds can interfere with library matching at the MS level.

In the above equation, M is the number of compound spectra identified and N is the total number of spectra. Match is a parameter that may be employed to allow comparisons of different levels of MS spectra. For example, an acquired MS spectrum could be identified using an MS/MS spectrum in the library. This would correspond to a Match = 500. Since all Match parameters are set to "Forbidden," the value of Match in all instances of scoring is 1,000.

Therefore, effective scores will be expressed as Fit', RFit', and Purity'.

Fragmentation is carried out in a unique mode known as SmartFrag, which is a ramped collision energy applied over a range of 0.3 to 2.0 V, which results in producing consistent product ion spectra from one instrument to another and generates fragment ions over a wider mass range. The library spectra are also acquired using SmartFrag.

Additional acquisition parameters include Smart Parameter Settings (SPS) turned on, a scan range of 150 to 300, a Maximum Accumulation Time of 200 msec, a Smart Target of 500,000, and Averages set to 5. The SPS consists of voltages designed to optimally transmit precursor ions to the ion trap analyzer and optimally collect them in the trap itself. The Maximum Accumulation Time is the longest amount of time the ion trap will spend accumulating ions before beginning another scan or performing the fragmentation cycle on a selected precursor.

The Smart Target setting has to do with filling the ion trap to capacity but avoiding overfilling, which can result in a loss of resolution and mass assignment. Setting Averages to 5 means that 5 full scans are actually acquired and then averaged before being stored as a data scan.

Acquiring in full-scan MS/MS mode is the most sensitive acquisition of the ion trap. The ion trap can be used for quantification, but normally only if the samples are clean. This is because the ion trap collects all of the ions formed in the ion source before selecting a precursor and fragmenting it. If matrix ions are also present, then there is less room to trap the analytes of interest, thus reducing sensitivity.

As in the case of the SQ and QQQ mass spectrometers, the source settings are based on LC flow rate. The mass axis calibration is carried out using an infusion of tuning mix ions. Optimal voltages in the ion optics and mass analyzer for trapping precursor ions of interest are predetermined using the tuning mix. Method development is minimal in the AutoMS(3) mode of operation.

- *Agilent 6220 accurate-mass time-of-flight LC/MS system*

The acquisition settings include the fragmentor set to 150 V. The scanning range was *m/z* 100 to 1,000, with approximately 10,000 transients acquired per scan. A transient is one pulse, boosting a packet of ions into the TOF mass analyzer. Reference ions at *m/z* 121.0509 and 922.0098 were used for real-time calibration of each scan, updating each spectrum before it was stored in the data file.

The reference mass solution was introduced through a second sprayer and used to ensure better than 2 ppm mass accuracy in MS mode and 5 ppm in MS/MS mode on the QTOF. The second sprayer eliminates ion suppression, which might otherwise be caused by introducing the reference compounds into the LC flow prior to ionization.

The injection volume was reduced to 2 µL because the 5 µL injection volume amount used for the SQ, QQQ, and IT was found to cause either electrospray or MS detector saturation for some of the compounds in the case samples. We underestimated the sensitivity of the SQ and the TOF when initially reconstituting the samples.

Once again, because the Agilent TOF instrument shares the same ion source and ion optics as the other LC/MS instrumentation in the Agilent portfolio, method development was simplified by the fact that source settings were based on flow rate, and ion transfer optics and mass analyzer voltages were predetermined using the autotune discussed earlier. The fragmentor voltage of 150 V used in this work was an ion transfer optic setting that worked well for transferring a wide mass range of ions to the mass analyzer. The optimum fragmentor voltage varied slightly for the LC/MS systems because of slight differences in the ion optics of the five mass analyzers.

- *Agilent 6520A Quadrupole Time-of-Flight Mass Spectrometer*

The same settings were used with the QTOF as with the TOF and in an acquisition mode similar to the ion trap called AutoMS/MS. The QTOF scans *m/z* 100 to 1,000, and when an ion intensity was above a user-defined threshold, the selected ion was fragmented and a full-scan MS/MS was acquired in the mass analyzer. The collision energy was mass normalized or based on the mass of the precursor ion, assuming that the higher the precursor *m/z* the higher the collision energy required to adequately fragment it and form enough product ions to determine structure.

The same reference ions were used and also introduced through a second sprayer. Consistent with the other Agilent LC/MS instrumentation included in this work, the source settings were dependent upon LC flow rate while the ion transfer optics and mass analyzer voltages were based on an automated tuning and calibration algorithm using the ion masses listed earlier. Like the TOF, the fragmentor voltage is set to 150 V.

Results and Discussion

Single Quadrupole Mass Spectrometer

Postmortem Blood

Selected ion monitoring chromatograms for the lowest calibrator for the cocaine analytes are shown in Figure 2. For cocaine and BE, this level corresponds to 25 ng/mL, and for CE it is 10 ng/mL. Note the excellent signal-to-noise ratio (S/N) for these analytes in aged whole blood.

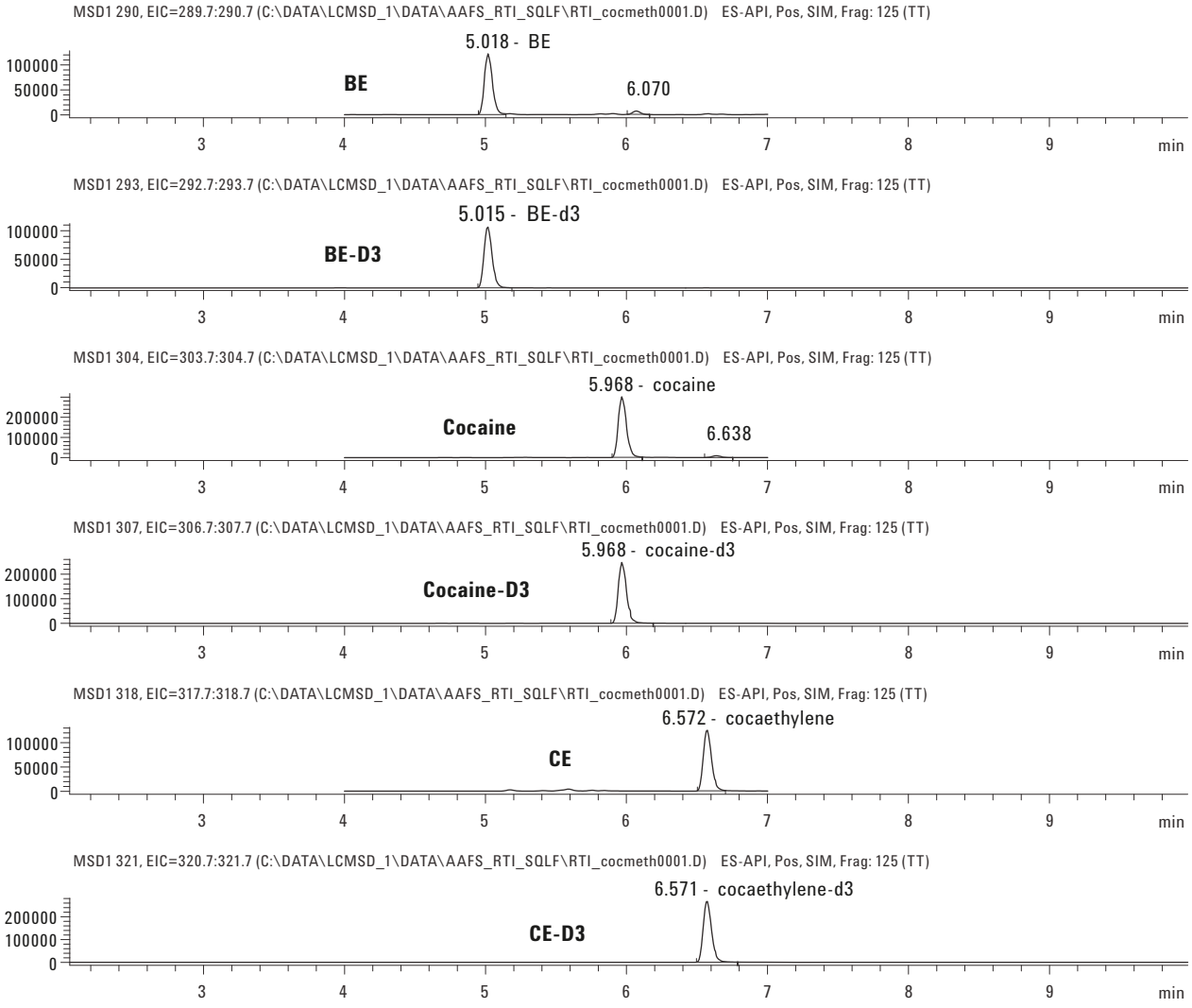


Figure 2. Compound chromatograms at the lowest calibrator of 25 ng/mL (BE and cocaine) and 10 ng/mL (CE) obtained using selected ion monitoring.

The calibration curves for each compound are shown in Figure 3, showing the calibrated range for each compound and the > 0.999 correlation coefficients. These were the ranges of quantification for each compound in any given case sample. A case sample for cocaine is shown in Figure 4, with quantification levels also displayed. Notice that all three compounds were quantified outside their calibrated ranges.

Also in the postmortem sample, methadone was analyzed. The calibration curve was shown in Figure 3, with the lowest calibrator at 25 ng/mL shown in Figure 5. The methadone case sample is shown in Figure 6.

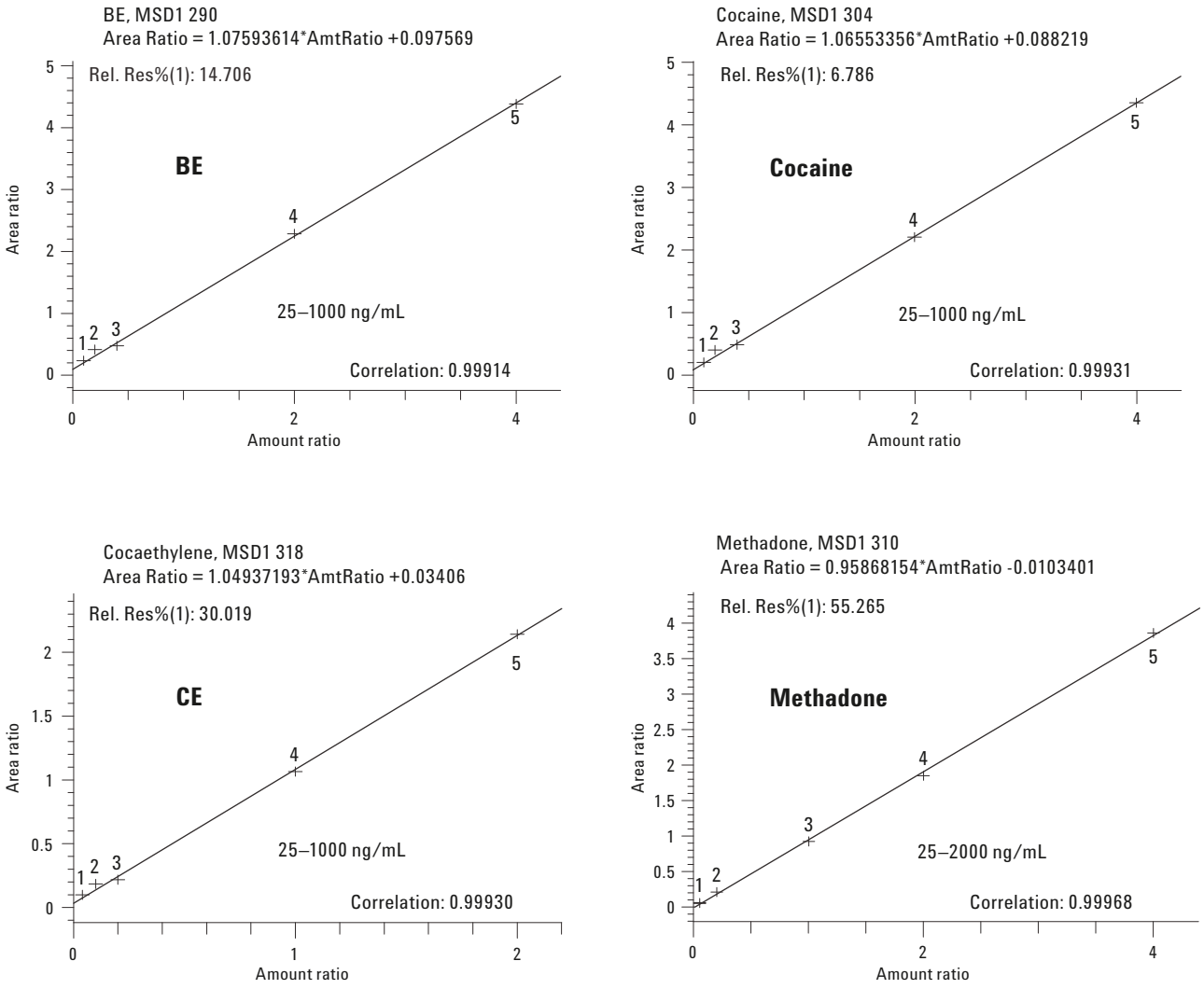


Figure 3. Calibration curves for compounds analyzed in postmortem samples: BE and cocaine (25 to 1,000 ng/mL); CE (10 to 500 ng/mL); and methadone (25 to 2,000 ng/mL).

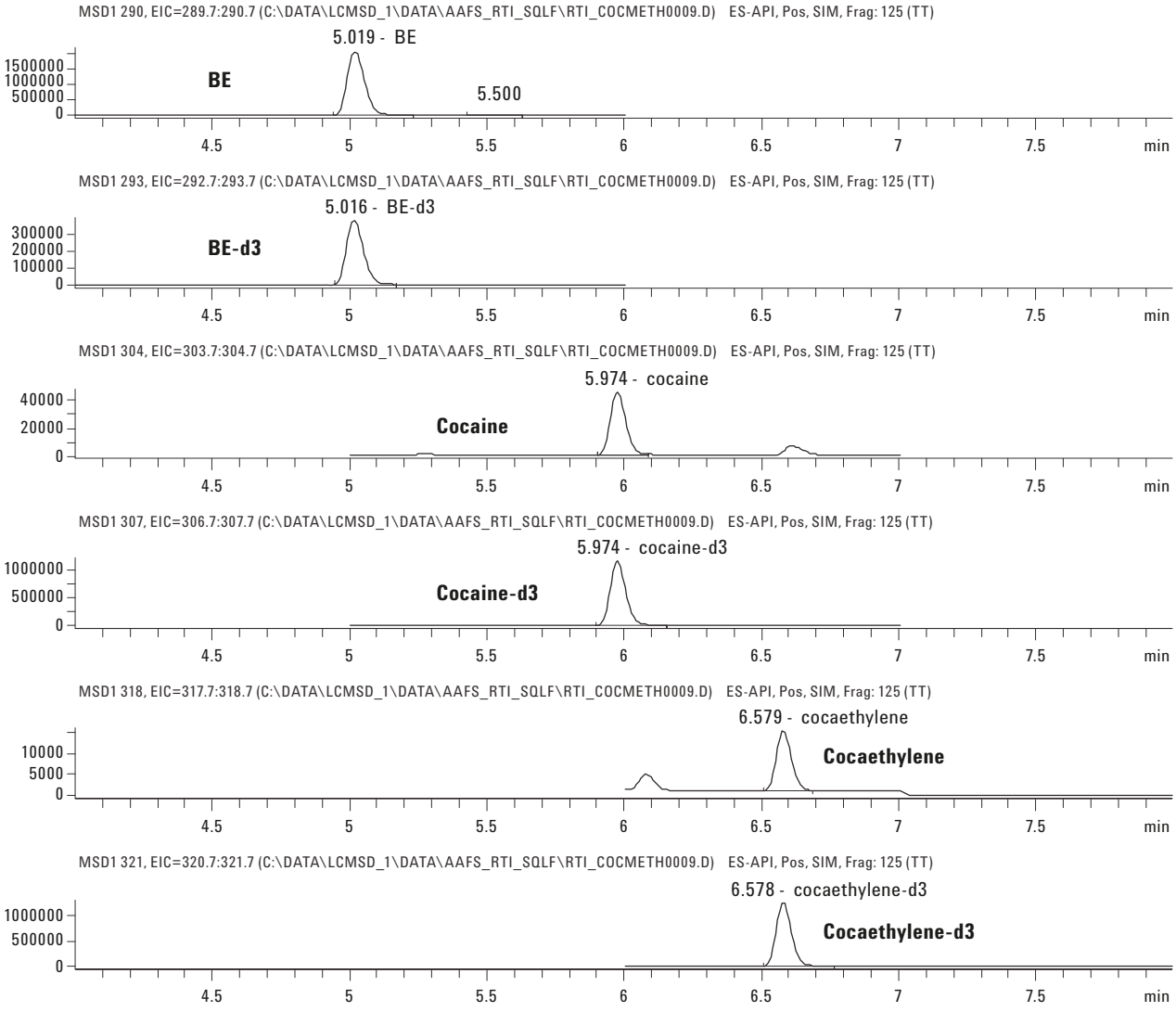


Figure 4. Postmortem cocaine case sample: BE 1,253 ng/mL; cocaine 8.8 ng/mL; and CE 2.7 ng/mL.

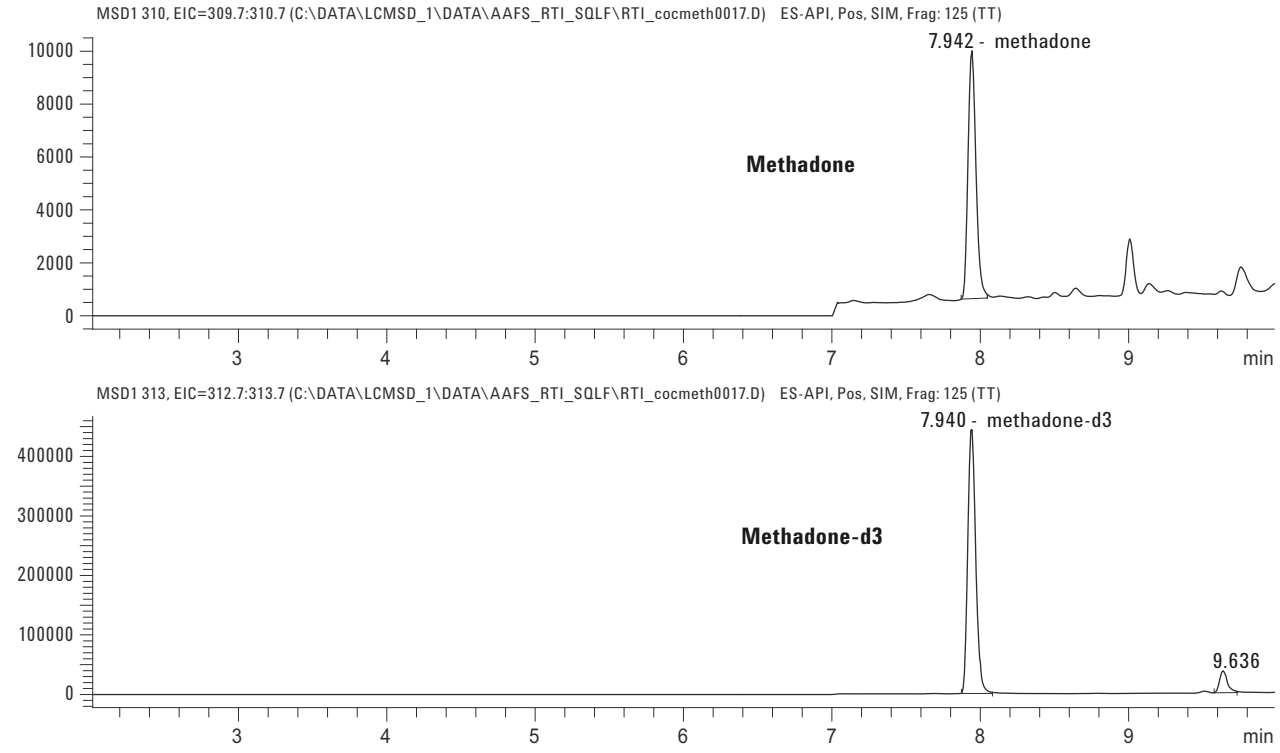


Figure 5. Postmortem methadone low calibrator (25 ng/mL).

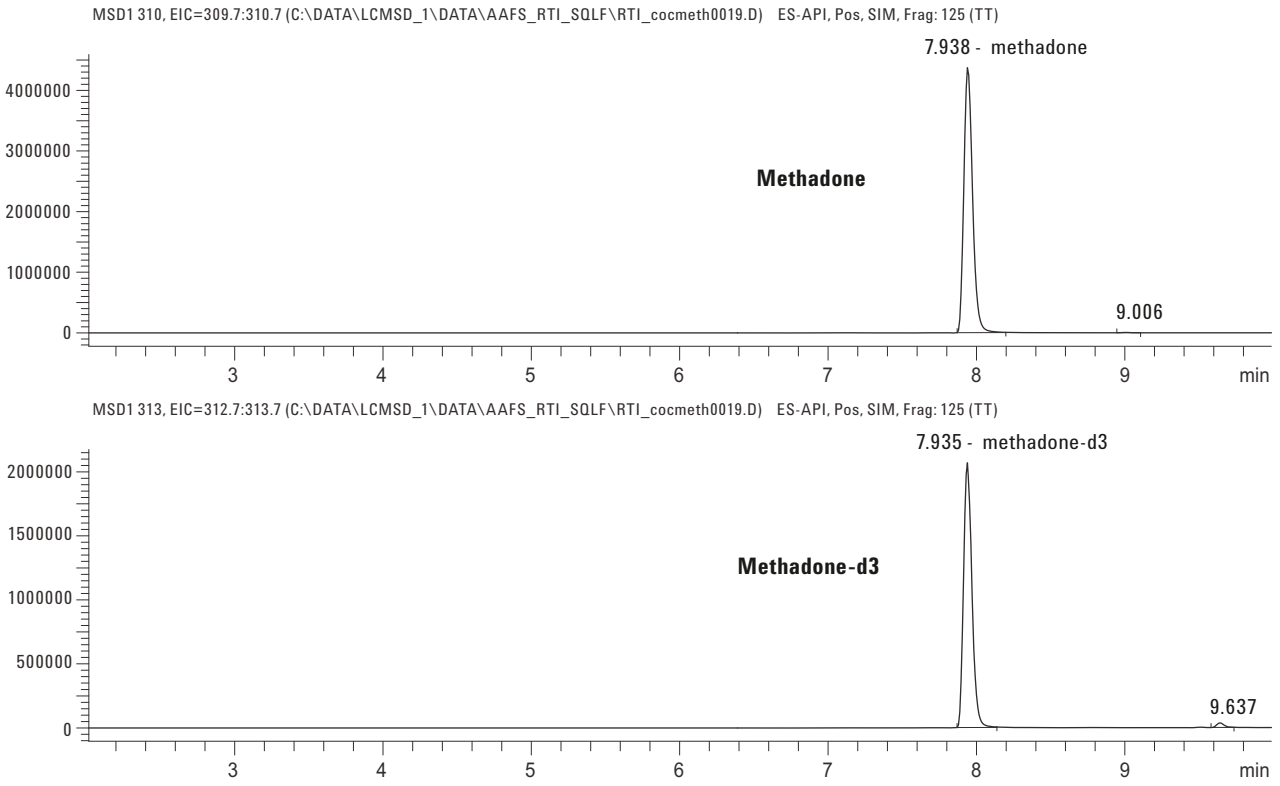


Figure 6. Postmortem methadone case sample: 1,156 ng/mL.

DUID Blood

The SIM chromatograms of the lowest level benzodiazepines are shown in Figure 7, while the calibration curves extending from 5 to 500 ng/mL are shown in Figure 8. The chromatographic result for case sample 0024 is shown in Figure 9, with the calculated quantitative results listed in Table 4.

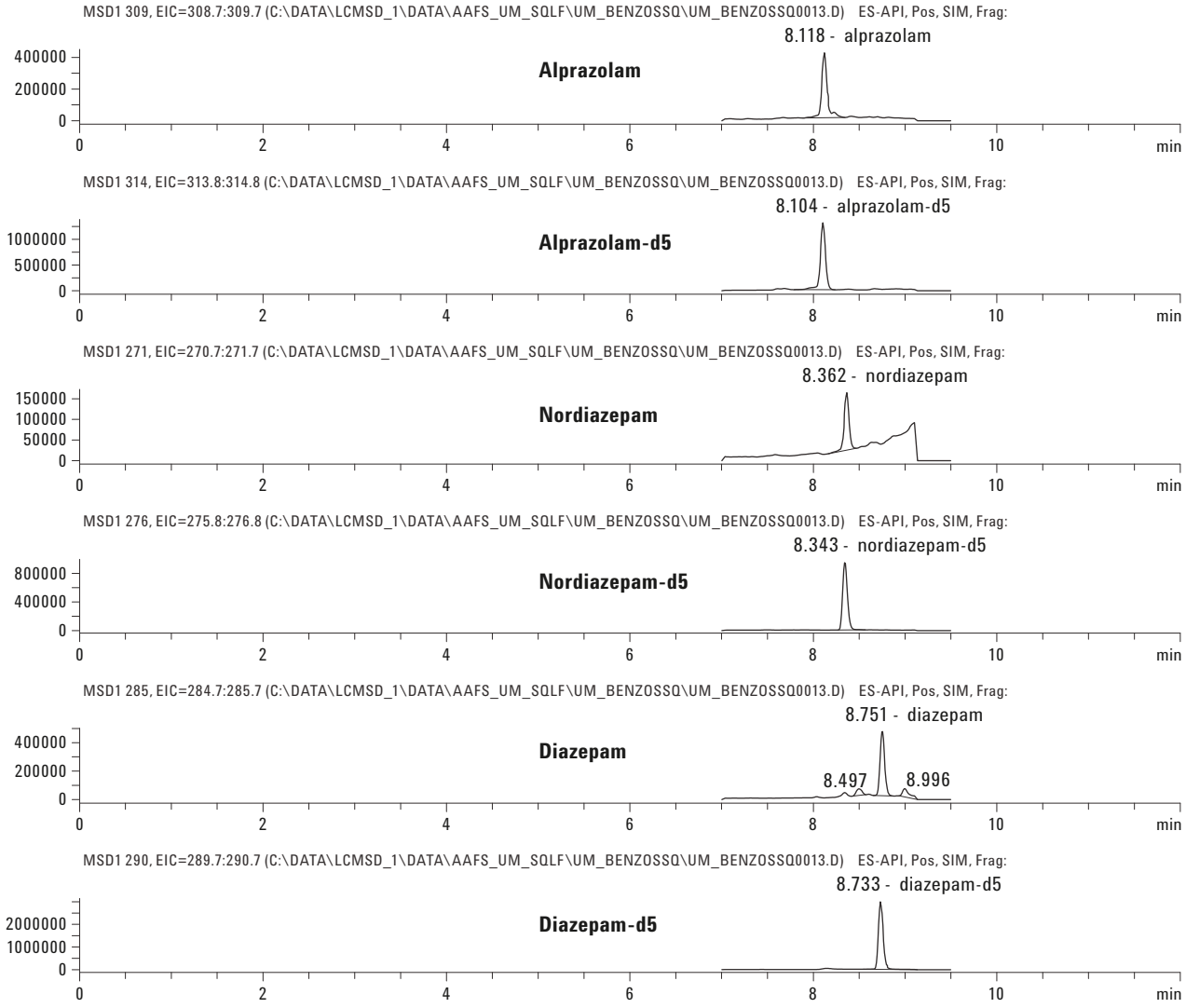


Figure 7. DUID benzodiazepines low calibrator (5 ng/mL).

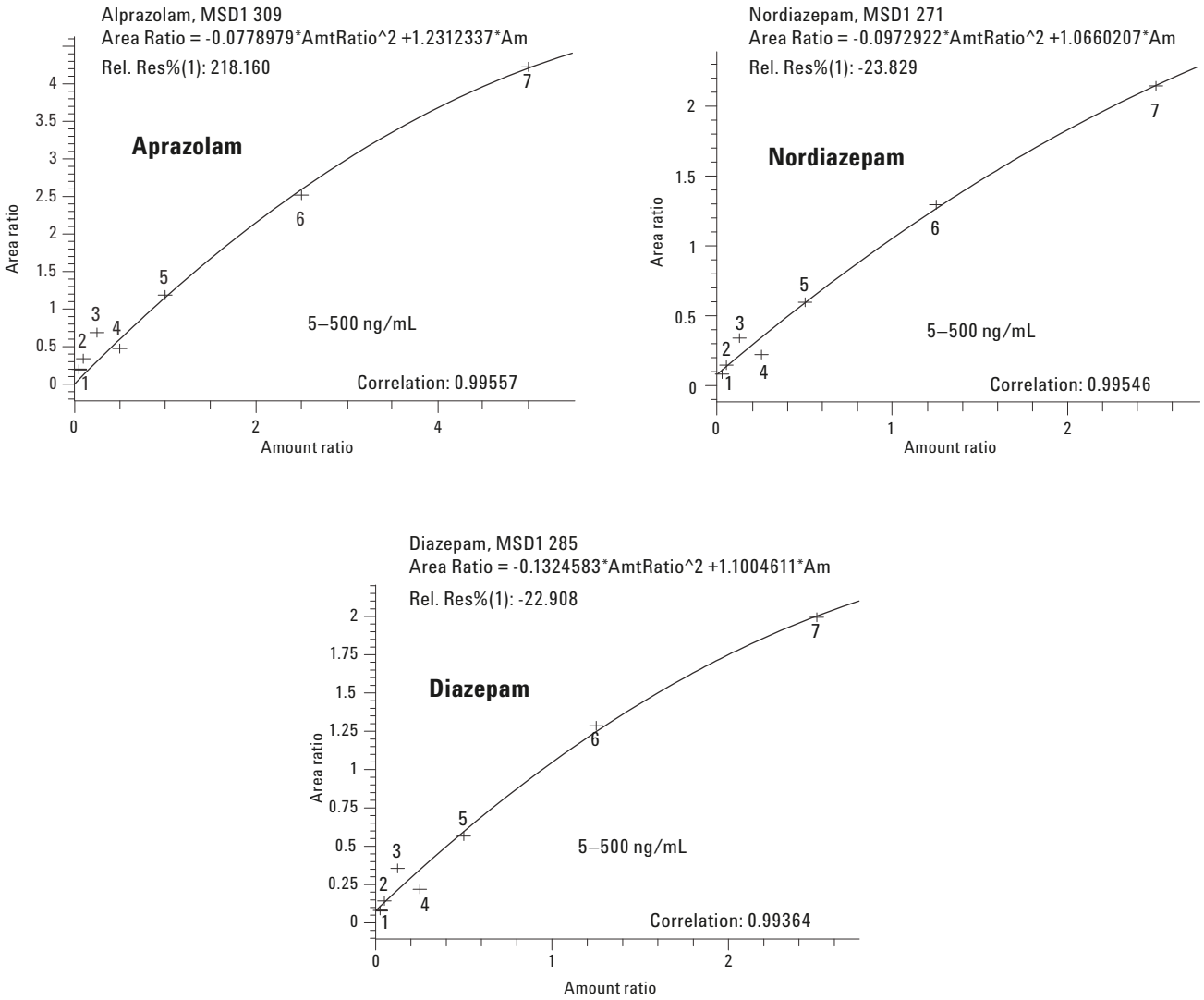


Figure 8. DUID benzodiazepines calibration (5 to 500 ng/mL). Nonlinearity is due to saturation in the electrospray ionization process and not in the MS detector.

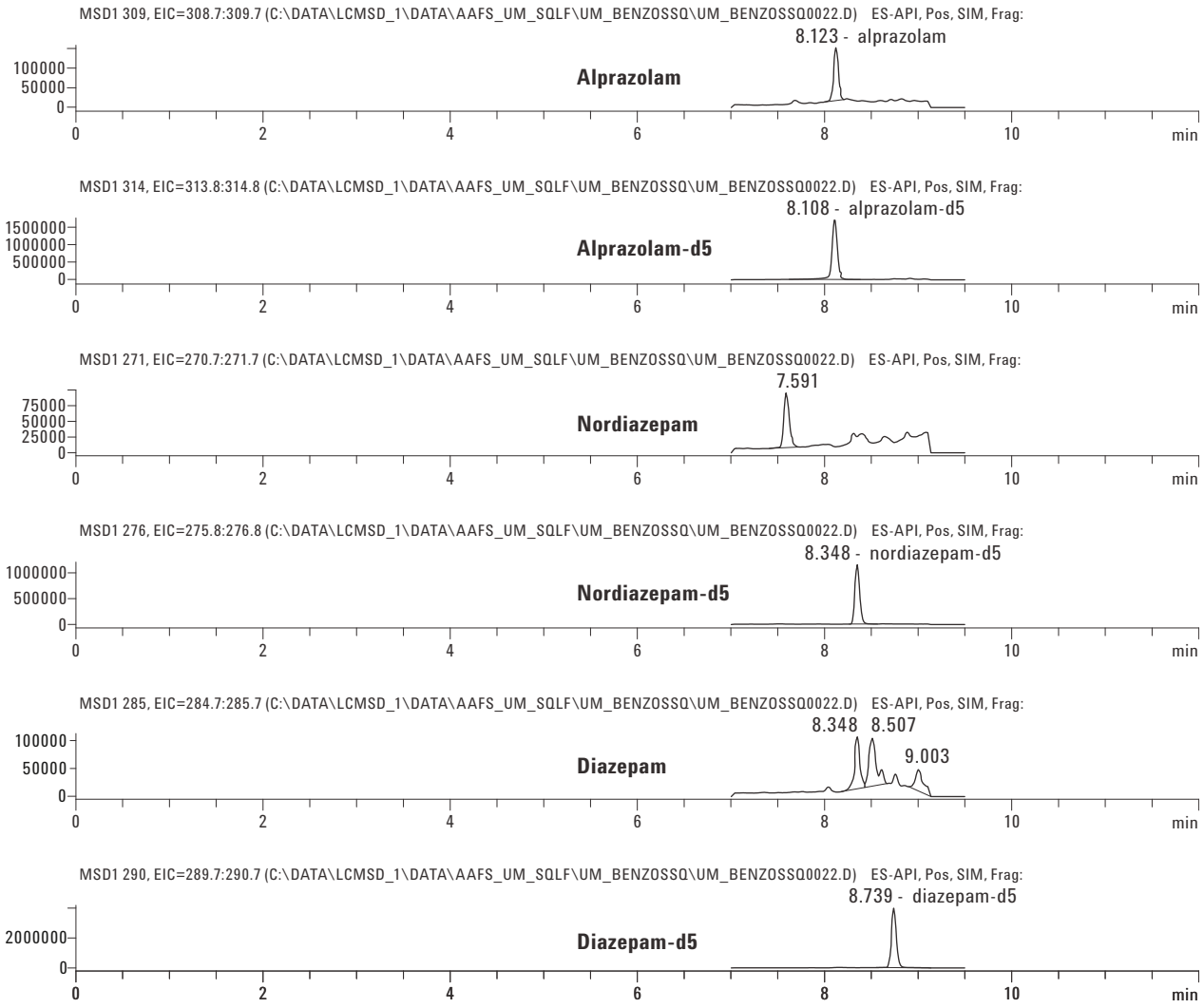


Figure 9. DUID benzodiazepines case sample 0024: alprazolam 5.6 ng/mL.

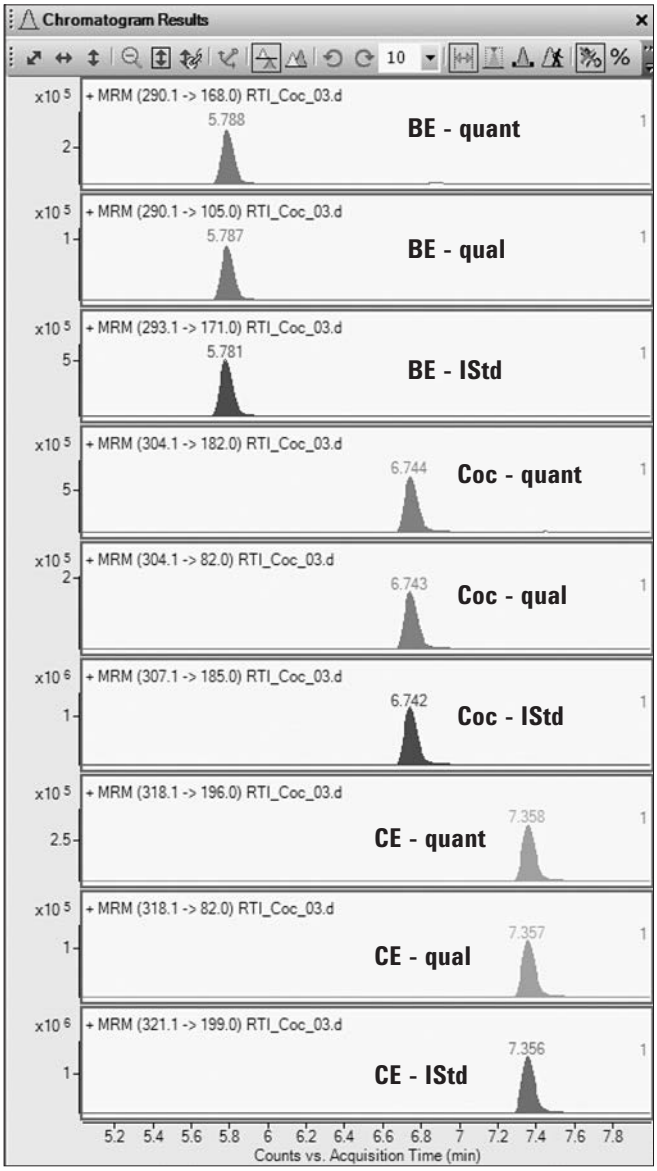
Table 4. Calculated SQ Quantification Amounts for Benzodiazepines in the Case Samples (The presence of nordiazepam and diazepam is detectable in the samples but below the range of quantification.)

DUID benzodiazepine case sample (SQ)	Calculated amounts (ng/mL)		
	Alprazolam	**Nordiazepam**	**Diazepam**
0024	5.6	< 5	< 5
0062	34.5	< 5	< 5
0083	13.6	< 5	< 5
0476	95.7	< 5	< 5
0531	67.5	< 5	< 5
0580	17.5	< 5	< 5

Triple Quadrupole Mass Spectrometer

Postmortem and DUID Blood

Multiple reaction monitoring chromatograms for a mid-level range calibrator of the cocaine metabolites are shown in Figure 10. For cocaine and BE, this level corresponds to 100 ng/mL, and for CE it is 50 ng/mL. For the analyte, both a quantifier and qualifier ion were measured and a constant ratio of the corresponding area counts is expected to be maintained for confirming the presence of compounds in samples. An example of this ratio is shown in Figure 11 with a tolerance of ± 20%. A qualifier ion for the internal standard (IStd) was not collected.



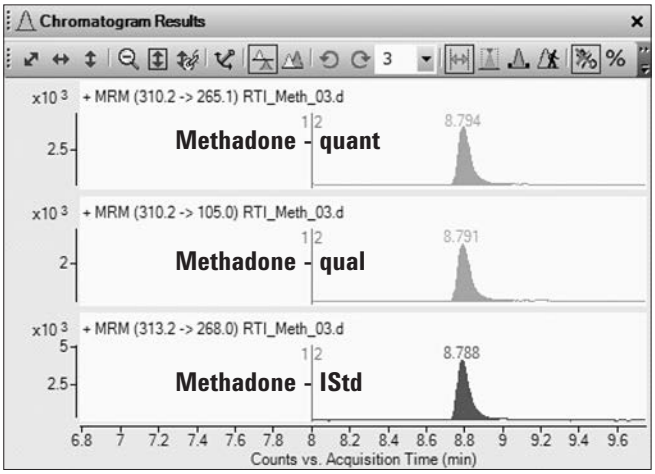


Figure 10. Compound chromatograms at the midrange level of 100 ng/mL (BE and cocaine) and 50 ng/mL (CE) obtained using multiple reaction monitoring. For each compound a quantifier, qualifier, and internal standard (IStd) ion are shown.

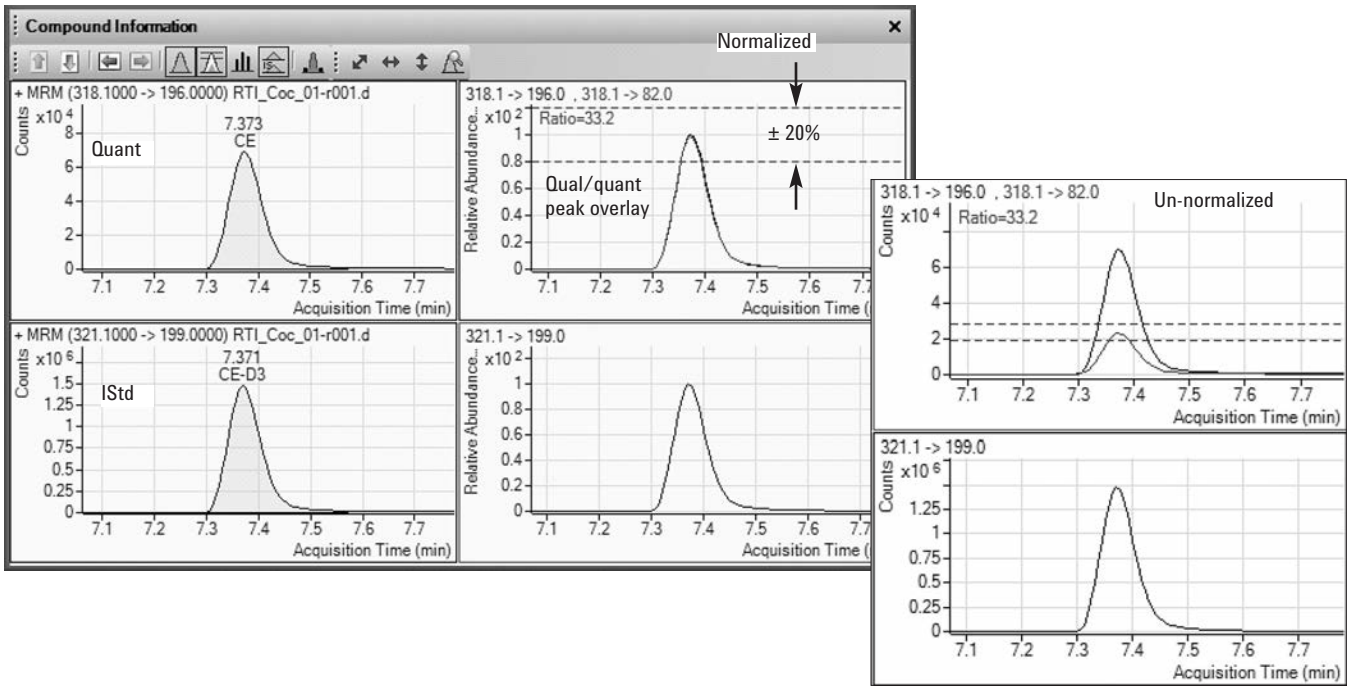


Figure 11. Qualifier peak-area ion ratios for confirmation.

On the left side of Figure 11 are shown the integrated peaks of the quantifier ion for the analyte and the IStd. Just to the right is the overlay of the qualifier ion on the quantifier ion normalized by peak areas. To the far right is shown the unnormalized overlay. The hash lines represent the ± 20% tolerance for the ion ratios.

The QQQ mass spectrometer has the unique analytical capability to both quantify and confirm in a single run. Confirmation on the SQ using at least one additional ion requires a higher fragmentor voltage to collisionally induce fragmentation. However, in an SQ this is a nonselective process and is susceptible to coeluting interferences.

The calibration curves used to quantify the postmortem samples for the presence of cocaine, CE, BE, and methadone are shown in Figure 12. These ranges and the calibrators are the same as those used for the SQ analysis.

Compound chromatograms for the DUID samples at a midrange calibration level are shown in Figure 13. As in the case of the compounds in the postmortem samples, both a quantifier and qualifier ion are measured for the analytes. The corresponding calibration curves are shown in Figure 14 and are the same as those used in the SQ analysis.

The lowest levels were injected in triplicate and the results are shown below in Table 5.

Table 5. Reproducibility Results Based on Peak Areas of Triplicate Injections at the Lowest Level of Quantification

Reproducibility at lowest level		
Compound	**Level (ng/mL)**	**% RSD response**
Cocaine	25	0.4
BE	25	1.0
CE	10	0.6
Methadone	25	0.2
Alprazolam	5	2.2
Nordiazepam	5	0.5
Diazepam	5	2.5

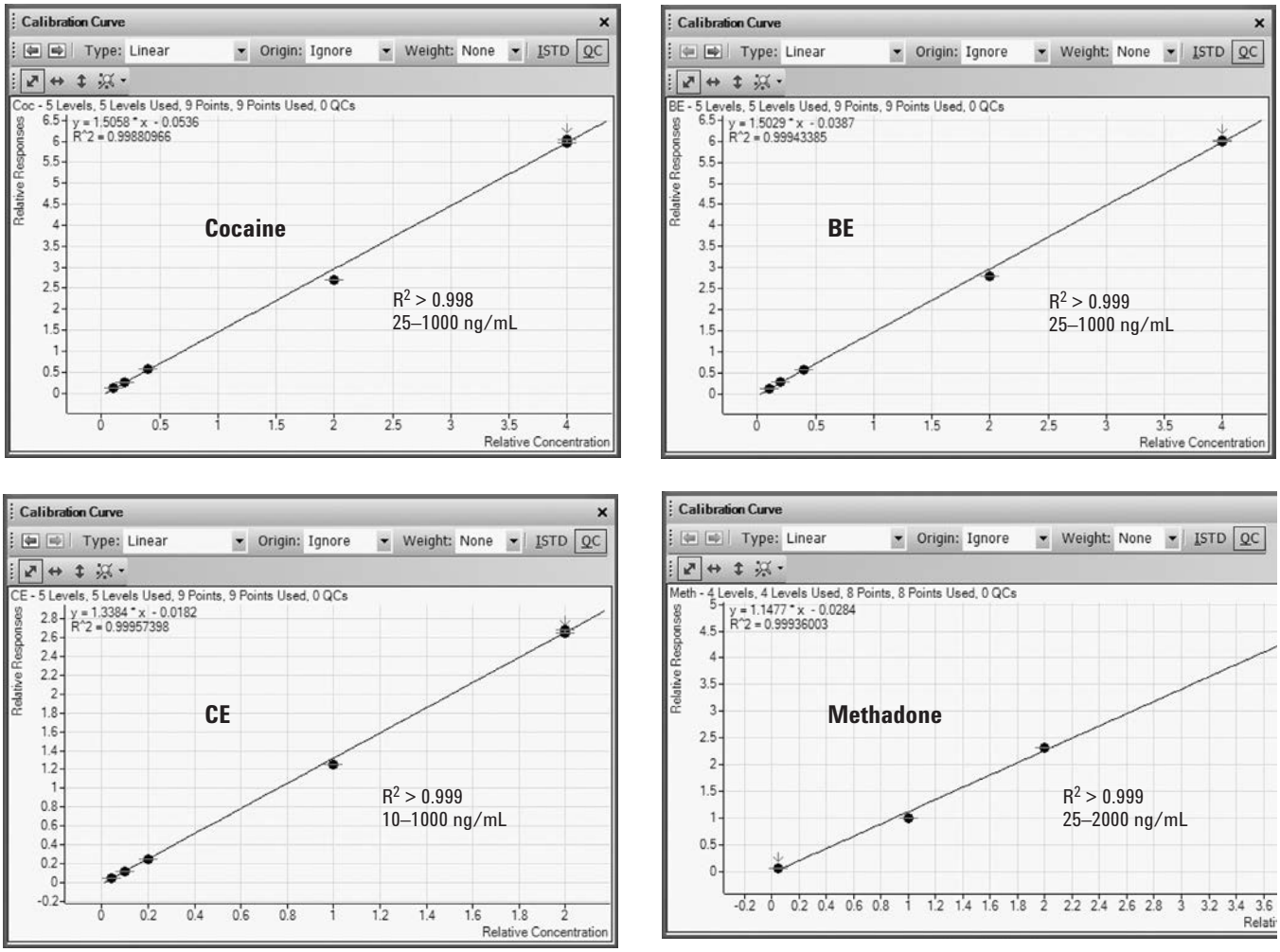


Figure 12. Linearity of compounds analyzed in postmortem samples from 25 to 1,000 ng/mL (cocaine and BE), 10 to 1,000 ng/mL (CE), and 25 to 2,000 ng/mL (methadone).

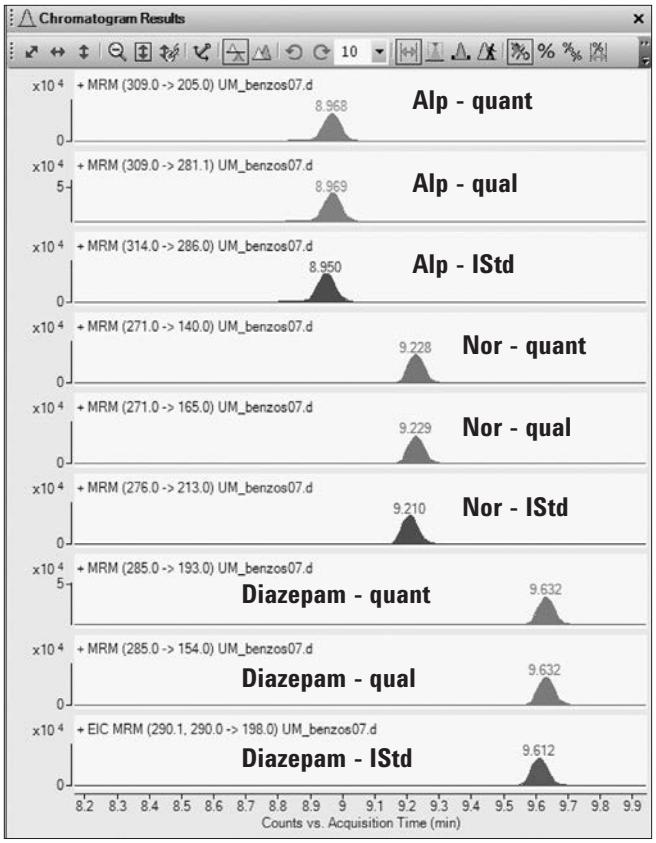


Figure 13. Compound chromatograms at the midrange level of 50 ng/mL for alprazolam, nordiazepam and diazepam. For each compound a quantifier, qualifier, and internal standard (IStd) ion are shown.

The quantification results for the case samples are shown in Table 6. Since the DUID samples were also believed to contain cocaine and metabolites, they were analyzed for these compounds as well.

Both the SQ and QQQ quantified at the lowest calibration levels, but because of the selective MS/MS capability of the QQQ, it is likely that the instrument could handle assays with less sample preparation better than the SQ.

Ion Trap Mass Spectrometer

Postmortem Blood Only

Only the postmortem samples were analyzed by the ion trap mass spectrometer as the DUID samples were depleted after analysis on the other instruments.

An attempt was made to generate calibration curves for quantification using the IT mass spectrometer. Unfortunately, there were not enough data points across the peak of about 4 seconds to get reproducible results. Peak widths of at least

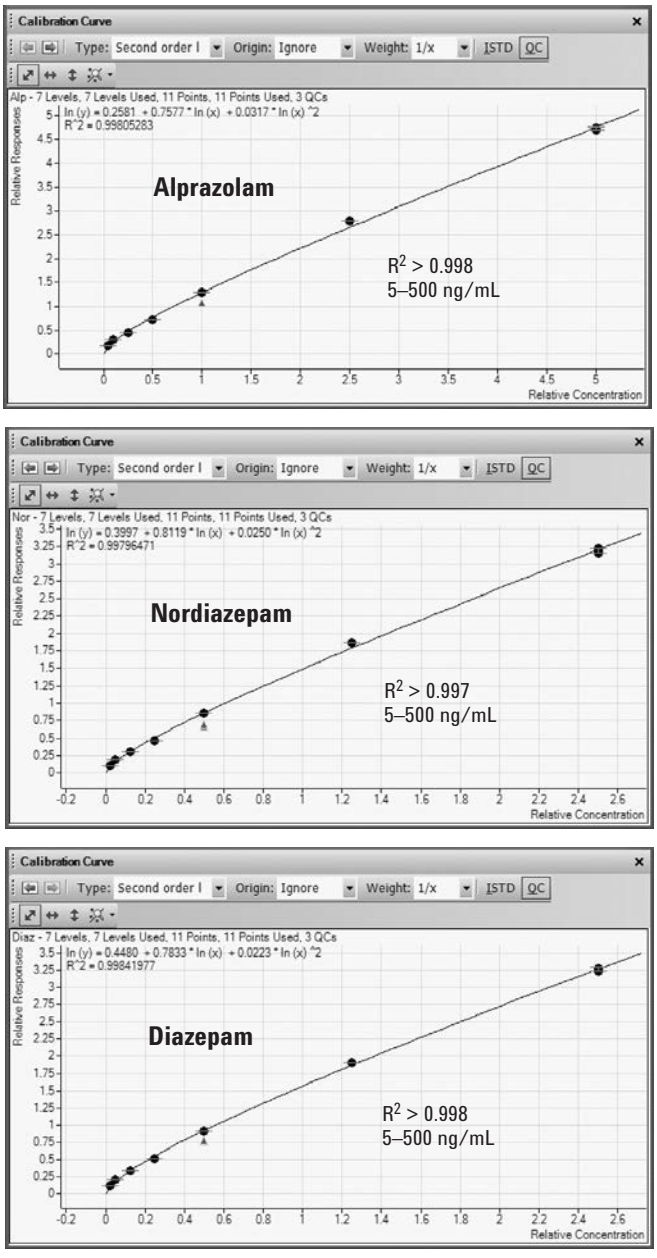


Figure 14. Postmortem benzodiazepine calibration curves for each compound are shown from 5 to 500 ng/mL.

10 seconds are typically required for quantification with an ion trap.

On the other hand, the ion trap with its full-scan MSn sensitivity allowed for identifying compounds based on their specific fragmentation patterns, also known as "fingerprints." In this work, an Agilent-created library of 400-plus compounds, containing both MS/MS and MS3 spectra, was used for identifying compounds in the postmortem and DUID case samples. An example of a library entry is shown in Figure 15 for

benzoylecgonine. The Chemical Abstracts Service Number (CAS #), chemical formula, and structure are also included in the drug library.

Table 6. QQQ Quantification Results for the Postmortem and DUID Case Samples (The hyphens represent those instances where the compounds were not detectable in the samples.)

	Calc. amounts (ng/mL)						
	Cocaine	**BE**	**CE**	**Methadone**	**Alprazolam**	**Diazepam**	**Nordiazepam**
postmortem							
Case sample - Cocaine	1.1	1448.1	0.1	–	–	–	–
Case sample - Methadone	–	–	–	1134.7	–	–	–
DUID							
Case 0024	–	699.0	286.5	93.6	0.8	–	–
Case 0062	–	25.6	37.8	390.9	36.5	–	–
Case 0083	–	9.5	1.0	1465.4	3.9	–	–
Case 0476	223.9	424.4	211.5	447903.6	96.4	–	–
Case 0531	–	123.4	1.0	1057.8	58.5	–	–
Case 0580	–	57.0	10.01	–	5.2	–	–

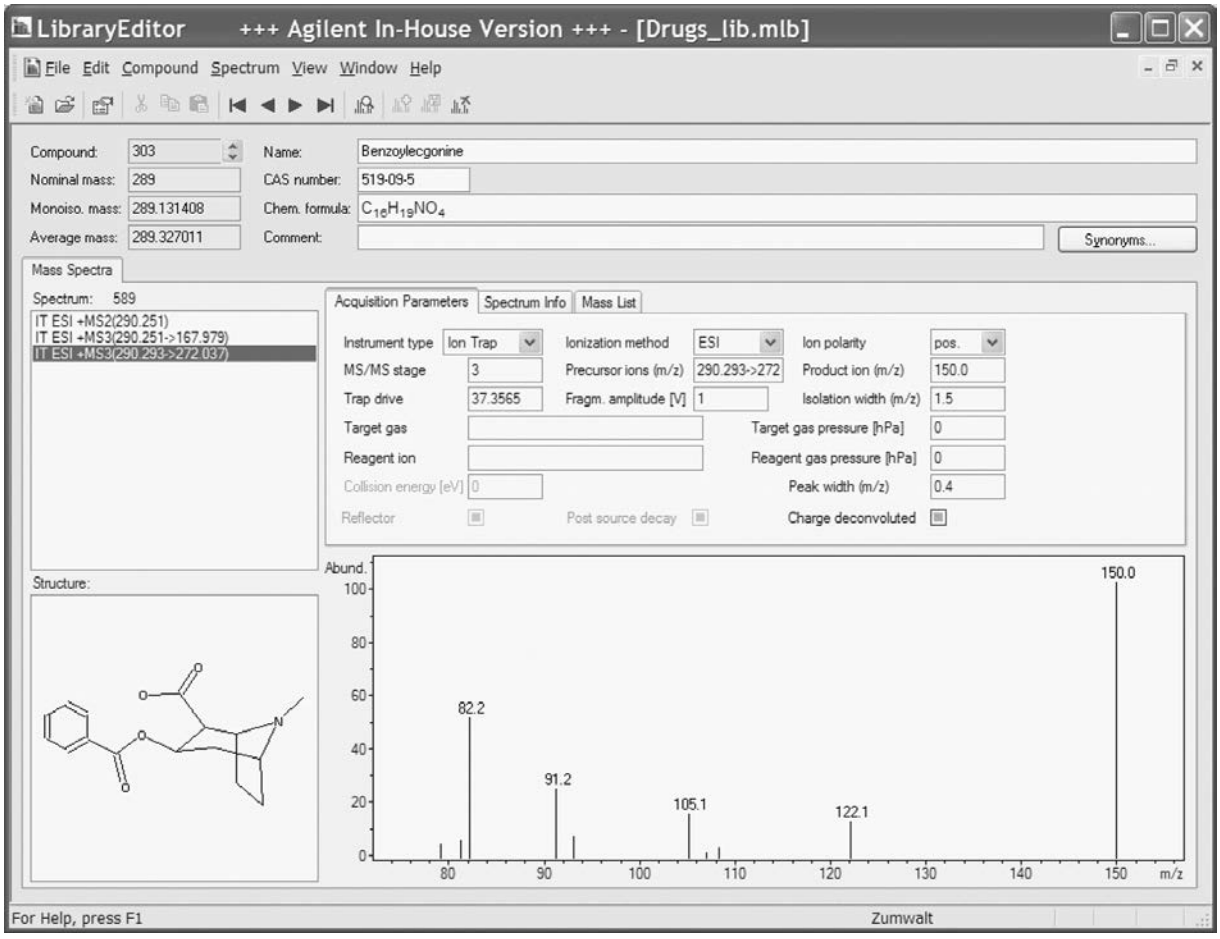


Figure 15. Library entry for benzoylecgonine includes MS/MS and MS3 spectra, CAS #, chemical formula, and structure. All spectra in library acquired using SmartFrag.

To test for required sensitivity, the lowest calibrator level for the postmortem blood analysis is shown in Figure 16. The lowest calibrator for the postmortem analysis was positively identified for the presence of BE, cocaine, CE, and methadone at the 25, 25, 10, and 25 ng/mL levels, respectively.

In the postmortem cocaine case sample, both BE and alprazolam were identified as shown in the library report of Figure 17. The presence of BE was calculated earlier by the QQQ as 1448 ng/mL. The QQQ also detected cocaine at 1.1 and CE at 0.1 ng/mL. These levels are apparently too low for adequate detection and identification by the ion trap, at least in AutoMS3 mode.

However, alprazolam was also identified, whereas the QQQ method used on the cocaine sample did not include alprazolam in its analysis. The spectral matches for BE and alprazolam are shown in Figures 18a and 18b, respectively.

Library Search Report - AutoMS(n)

Analysis Name:	DOA_RT1000013.D				
		Instrument:	Agilent 6340 Ion Trap	**Print Date:**	11/16/2007 7:54:16 AM
Method:	DOA_MZ_AUTOMS1.M	**Operator:**	Administrator	**Acq. Date:**	11/16/2007 1:28:28 AM
Sample Name:	Coc_Cal1				

#	RT [min]	MS(n) Isol. *m/z*	Compound Name	Fit'	RFit'	Purity'	Conc. (ng/mL)
1	5.3	290.4	Benzoylecgonine	1000	999	999	25
2	6.0	304.9	Cocaine	1000	1000	1000	25
3	6.7	318.3	Cocaethylene	999	995	995	10
4	7.3	310.3	Methadone	986	957	955	25

Figure 16. Library report identifying BE, cocaine, CE, and methadone in the lowest calibrator level, with known concentrations listed on the right.

Library Search Report - AutoMS(n)

Analysis Name:	DOA_RT1000019.D				
		Instrument:	Agilent 6340 Ion Trap	**Print Date:**	11/16/2007 8:28:06 AM
Method:	DOA_MZ_AUTOMS1.M	**Operator:**	Administrator	**Acq. Date:**	11/16/2007 2:45:36 AM
Sample Name:	Case Sample Coc				

#	RT [min]	MS(n) Isol. *m/z*	Compound Name	Fit'	RFit'	Purity'	Conc. (ng/mL)
1	5.0	290.4	Benzoylecgonine	962	957	932	1448
2	8.1	309.3	Alprazolam	998	974	974	Not analyzed (From QQQ)

Figure 17. Library report identifying BE and alprazolam in the postmortem cocaine case sample with the known concentration for BE as analyzed by the QQQ.

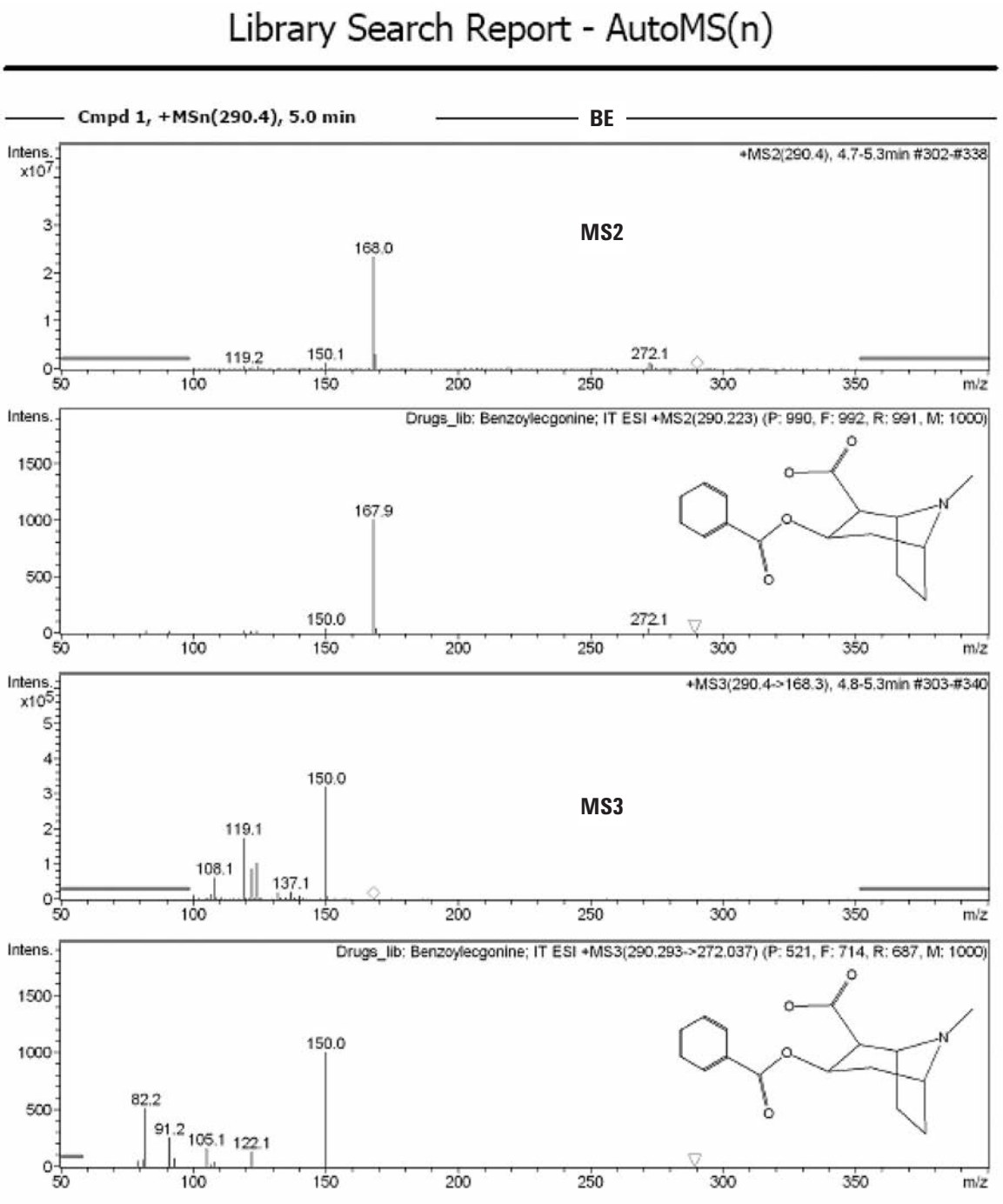


Figure 18a. Library report showing spectral matches for BE at both the MS/MS and MS3 levels in the postmortem cocaine case. Library spectra include structures.

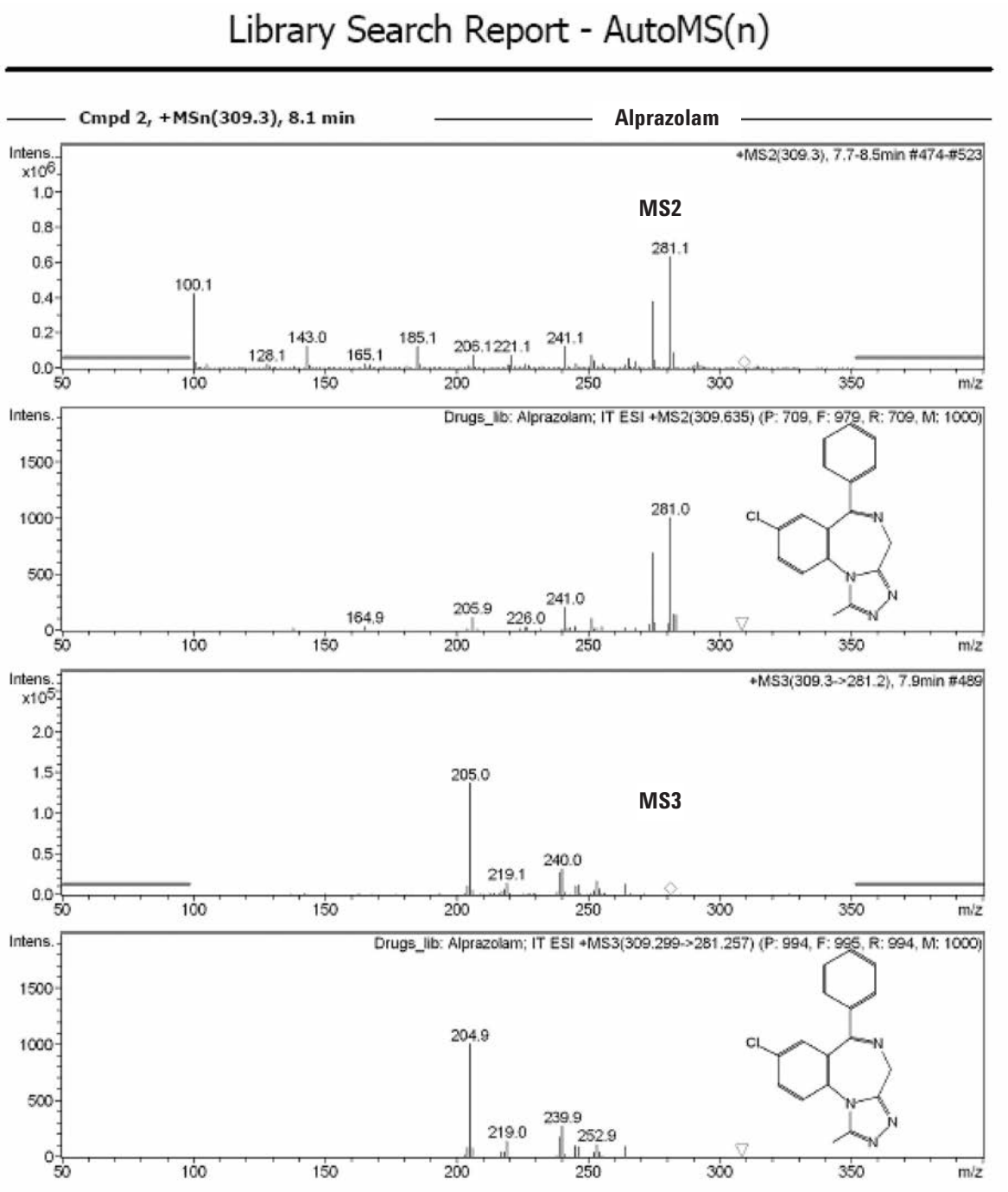


Figure 18b. Library report showing spectral matches for alprazolam at both the MS/MS and MS3 levels in the postmortem cocaine case. Library spectra include structures.

In the postmortem methadone case sample, both methadone and sertraline were identified as shown in the library report of Figure 19. The presence of methadone was calculated earlier by the QQQ as 1,135 ng/mL. The presence of sertraline was suggested by the authors from RTI and confirmed using the ion trap library.

The spectral matches for methadone and sertraline are shown in Figures 20a and 20b.

Time-of-Flight Mass Spectrometer

Postmortem Blood

The Agilent TOF instrument typically acquires mass spectra with better than 2 ppm mass accuracy. In addition, the instrument has good spectral resolution, with a specification of greater than 10,000 full-width half-maximum resolving power at *m/z* 118. This resolving power corresponds to a peak width of less than 12 mDa. In the range of the ion masses measured in this work, or around *m/z* 300, the peak widths are about 25 mDa. With such narrow peaks, extracted ion chromatograms (EICs) can be generated with extraction windows as narrow as ± 10 ppm to increase S/N for quantification similar to the SQ.

Such EICs for the lowest level calibrator of the postmortem analysis are shown in Figure 21. The mass accuracy is also represented in the EICs as the center about which the EIC of ± 10 ppm is generated. For example, cocaine has a chemical formula of $C_{17}H_{21}NO_4$, or an exact protonated ion mass $(M+H)^+$ of 304.1543. The EIC for cocaine in Figure 21 is centered about the ion mass of 304.1543, demonstrating excellent mass accuracy because the S/N is good.

Library Search Report - AutoMS(n)

Analysis Name:	DOA_METHCASE002.D				
Method:	DOA_MZ_AUTOMS1.M	**Instrument:**	Agilent 6340 Ion Trap	**Print Date:**	11/16/2007 10:15:29 AM
Sample name:	Meth Case Sample	**Operator:**	Admimistrator	**Acq. Date:**	11/16/2007 9:35:58 AM

#	RT [min]	MS(n) Isol. *m/z*	Compound Name	Fit'	RFit'	Purity'	Conc. (ng/mL)
1	7.4	310.7	Methadone	991	934	932	1135
2	8.5	307.6	Sertraline	978	989	972	No calibrator

Figure 19. Library report identifying methadone and sertraline in the postmortem methadone case sample with the known concentration for methadone as analyzed by the QQQ.

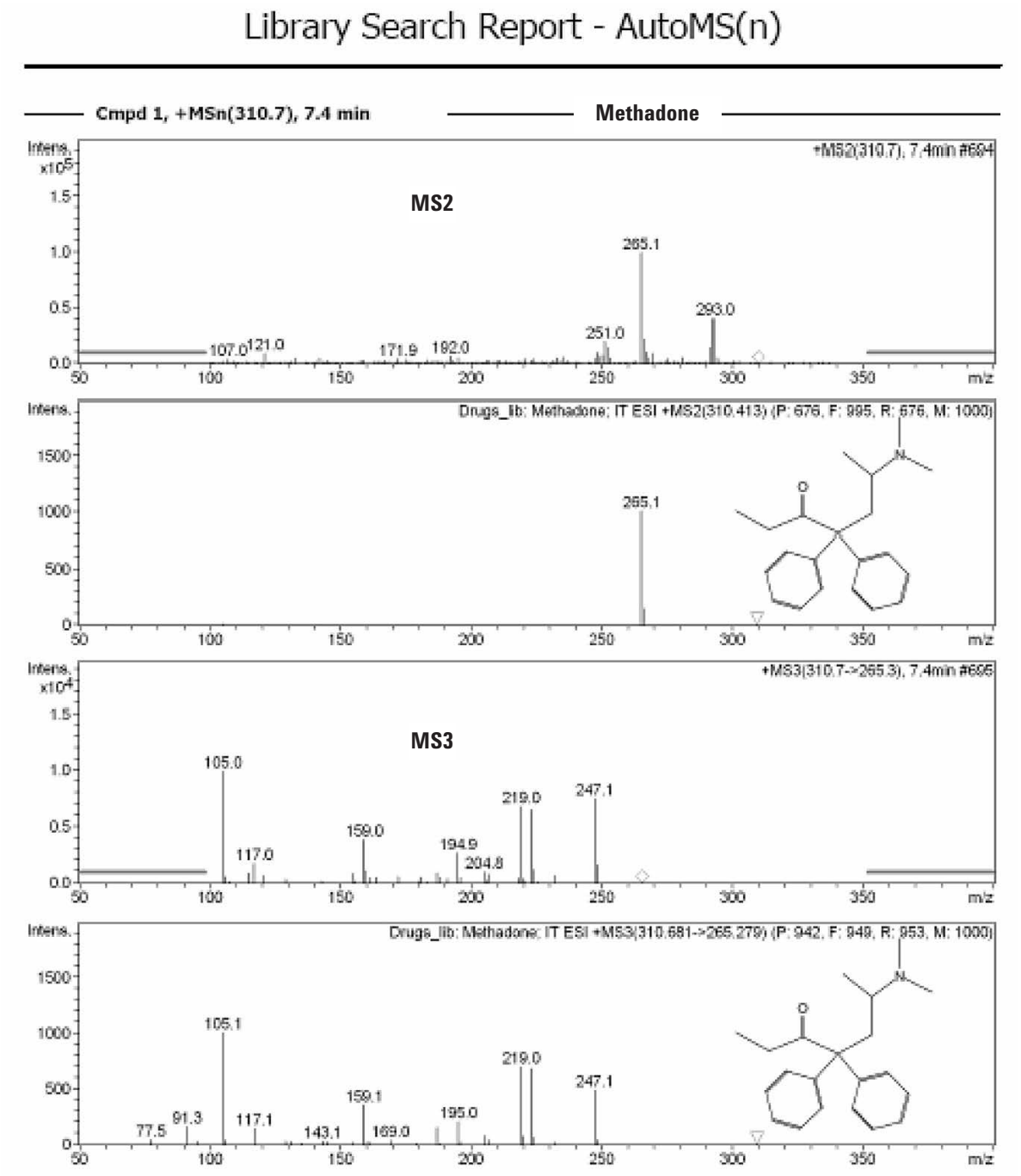


Figure 20a. Library report showing spectral matches for methadone at both the MS/MS and MS3 levels in the postmortem methadone case. Library spectra include structures.

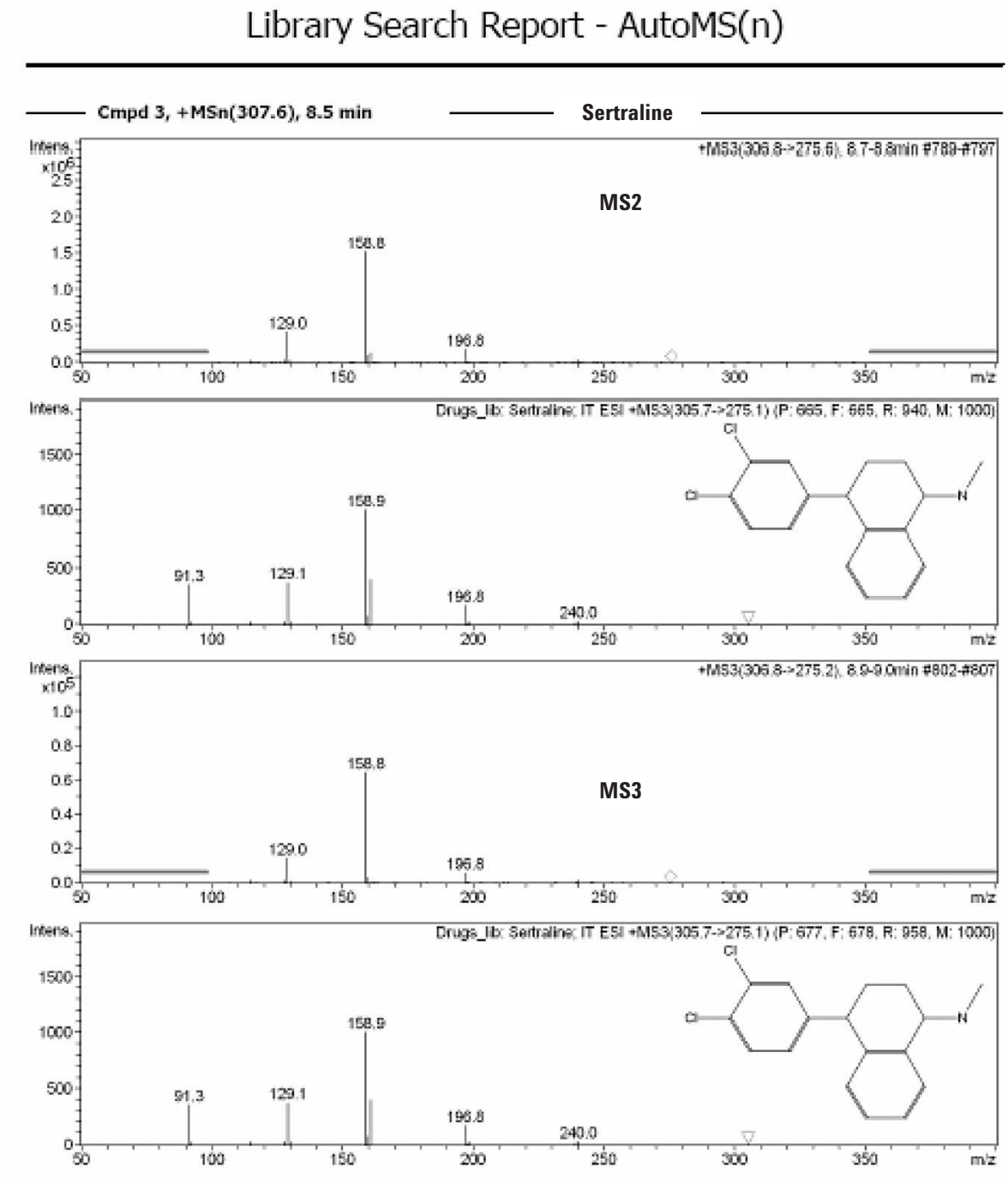


Figure 20b. Library report showing spectral matches for sertraline at both the MS/MS and MS3 levels in the postmortem methadone case. Library spectra include structures.

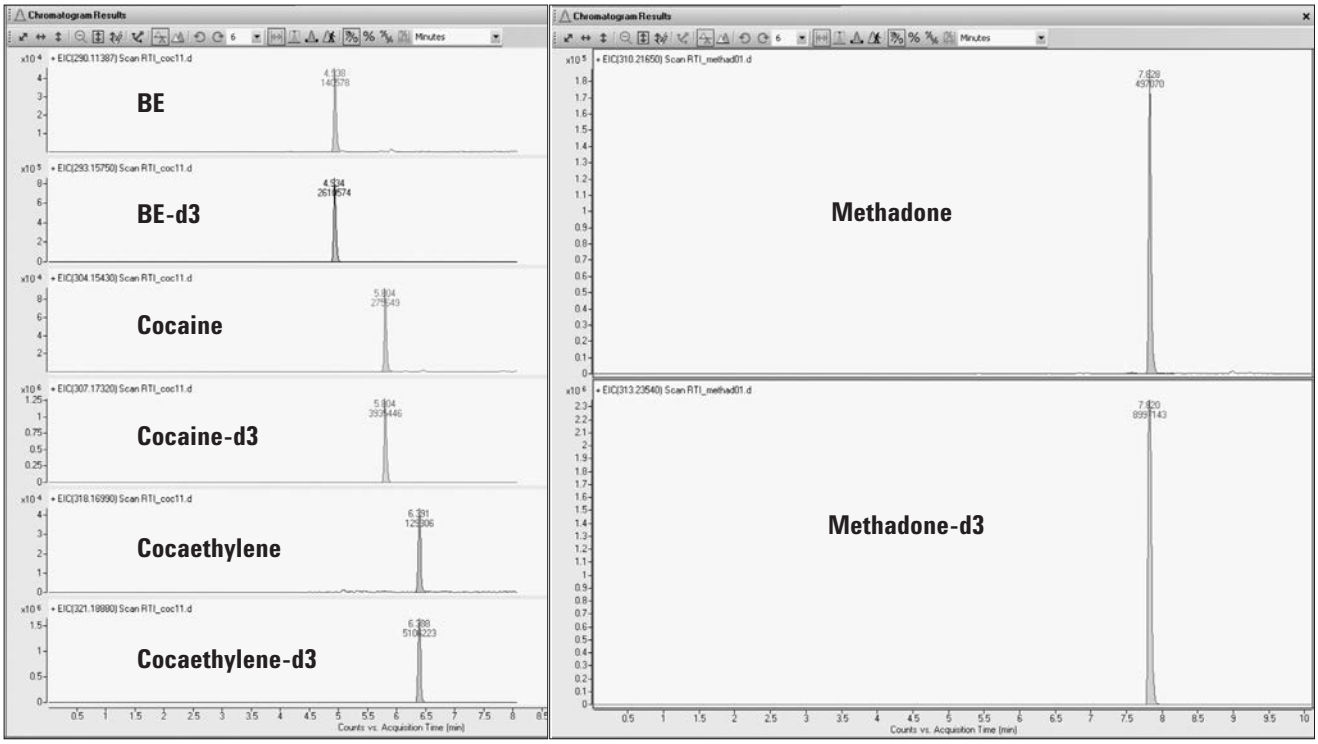


Figure 21. Extracted ion chromatograms of ± 10 ppm for the postmortem lowest level calibrator consisting of BE, cocaine, and methadone (25 ng/mL), and CE (10 ng/mL).

Calibration curves were generated over the levels given in Table 1 and displayed in Figure 22. Detector saturation was responsible for the nonlinearity seen for cocaine and CE, even with only 2 µL injected as opposed to the 5 µL used with the previous instruments. The lowest level calibrator results of Figure 21 demonstrate how sensitive the TOF instrument is. It can be seen in Figure 22 for even cocaine and CE that the lower level range is linear. The curves are still adequate for quantification, but dilutions are recommended for further work.

Subsequent quantification results of the cocaine case sample are shown in Figure 23 with BE = 1,632 ng/mL, cocaine = 12.5 ng/mL, and CE = 6.4 ng/mL. Methadone was obviously saturated at a level of at least 1,200 ng/mL as shown in Figure 24.

Postmortem Blood

For the DUID analysis, EICs for the lowest level calibrator at 5 ng/mL are shown in Figure 25. At this level all three compounds appeared to be close to their limits of quantification. The calibration curves are represented in Figure 26, extending over the ranges given in Table 1. Nonlinearity due to detector saturation is shown for nordiazepam and diazepam. The injection volumes were 5 µL and should be reduced, or at least diluted, in future work. As mentioned before, the sensitivity of the TOF was underestimated when choosing reconstitution and injection volumes.

The subsequent quantification results of the DUID case samples are tabulated in Table 7, with the chromatographic results for DUID case sample 0024 shown in Figure 27. For all case samples the presence of nordiazepam and diazepam could not be determined in any of the case samples. By contrast, the SQ could at least detect their presence, even if it was not be able to quantify them.

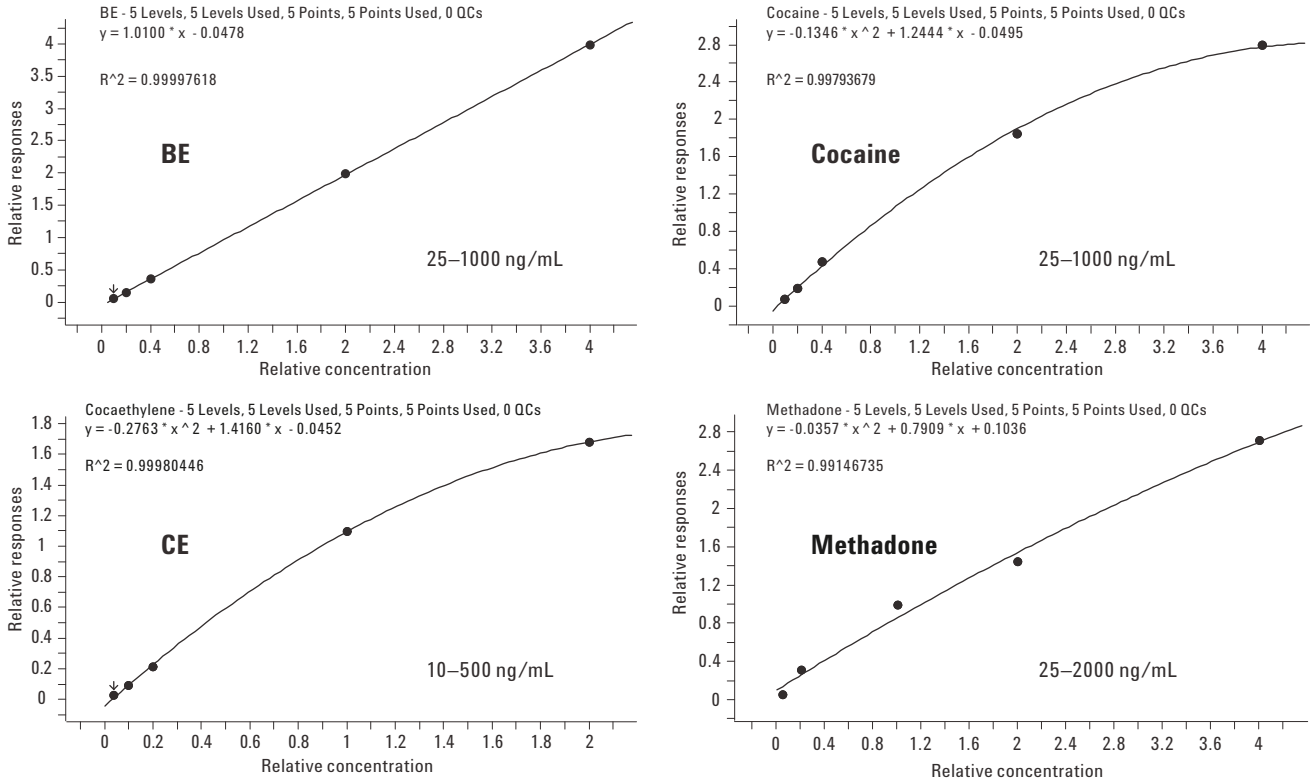


Figure 22. Calibration curves for the postmortem compounds. Detector saturation was the primary cause of the nonlinearity seen for cocaine and CE, even with only 2 uL injected.

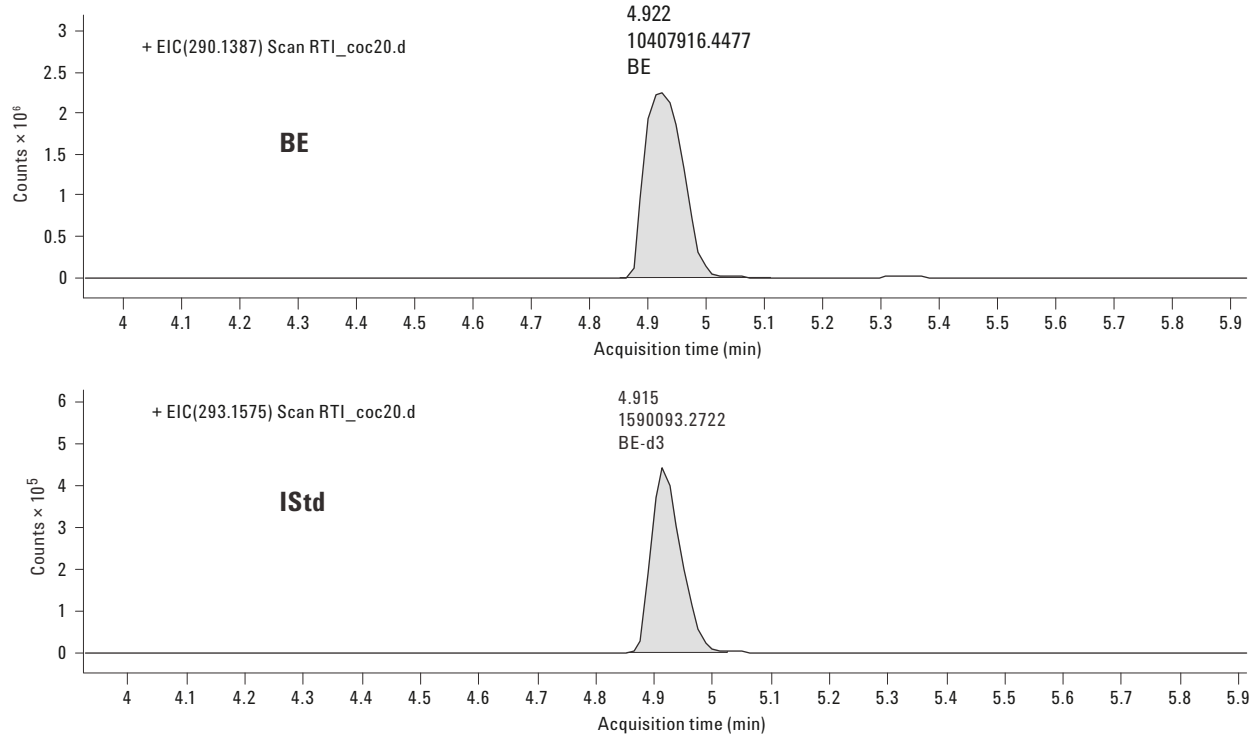


Figure 23. Cocaine case sample analyzed by TOF: BE 1,632 ng/mL; cocaine 12.5 ng/mL; and CE 6.4 ng/mL.

Continued

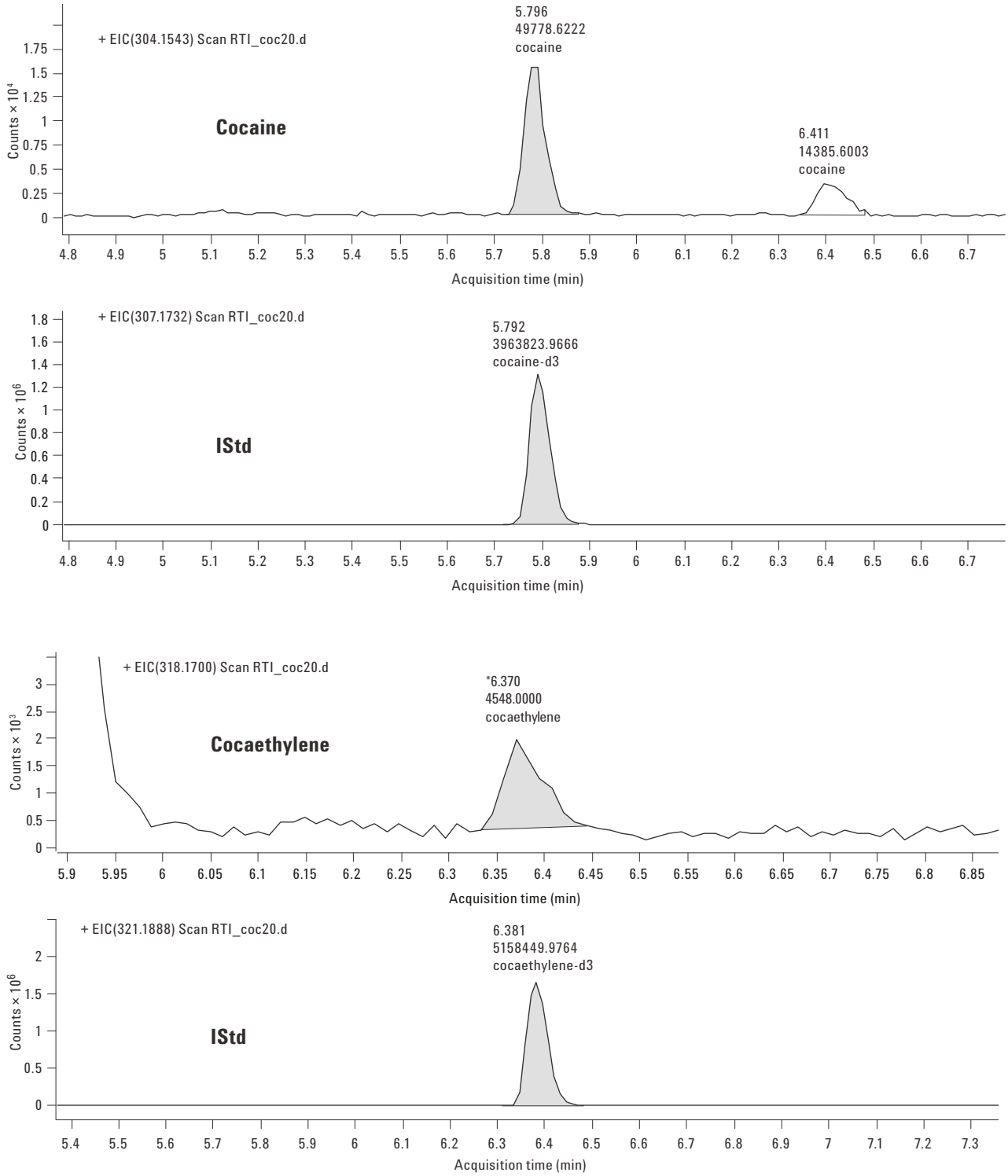


Figure 23. Cocaine case sample analyzed by TOF: BE 1,632 ng/mL; cocaine 12.5 ng/mL; and CE 6.4 ng/mL.

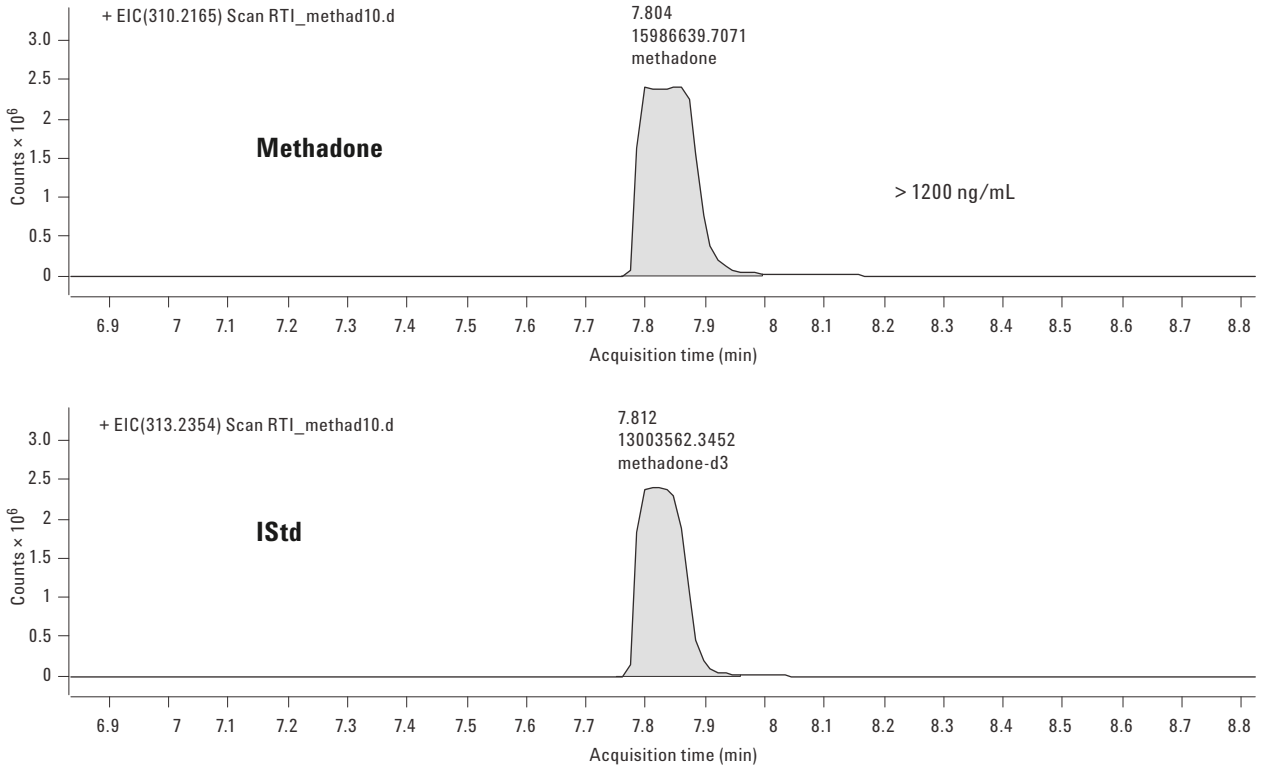


Figure 24. Methadone case sample analyzed by TOF shows detector saturation at a level greater than 1,200 ng/mL.

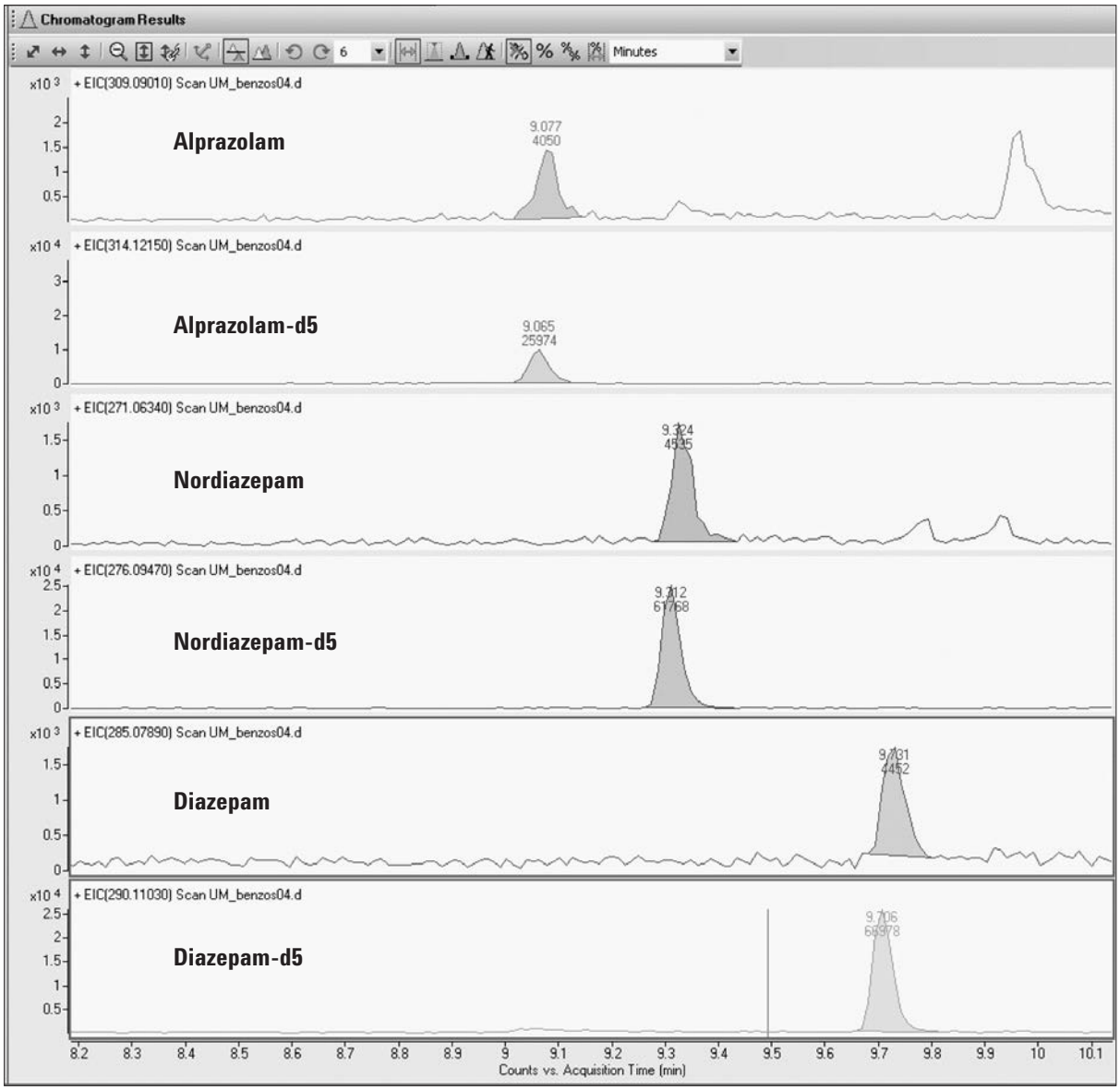


Figure 25. Extracted ion chromatograms of ± 10 ppm for the DUID lowest level calibrator consisting of alprazolam, nordiazepam, and diazepam (5 ng/mL).

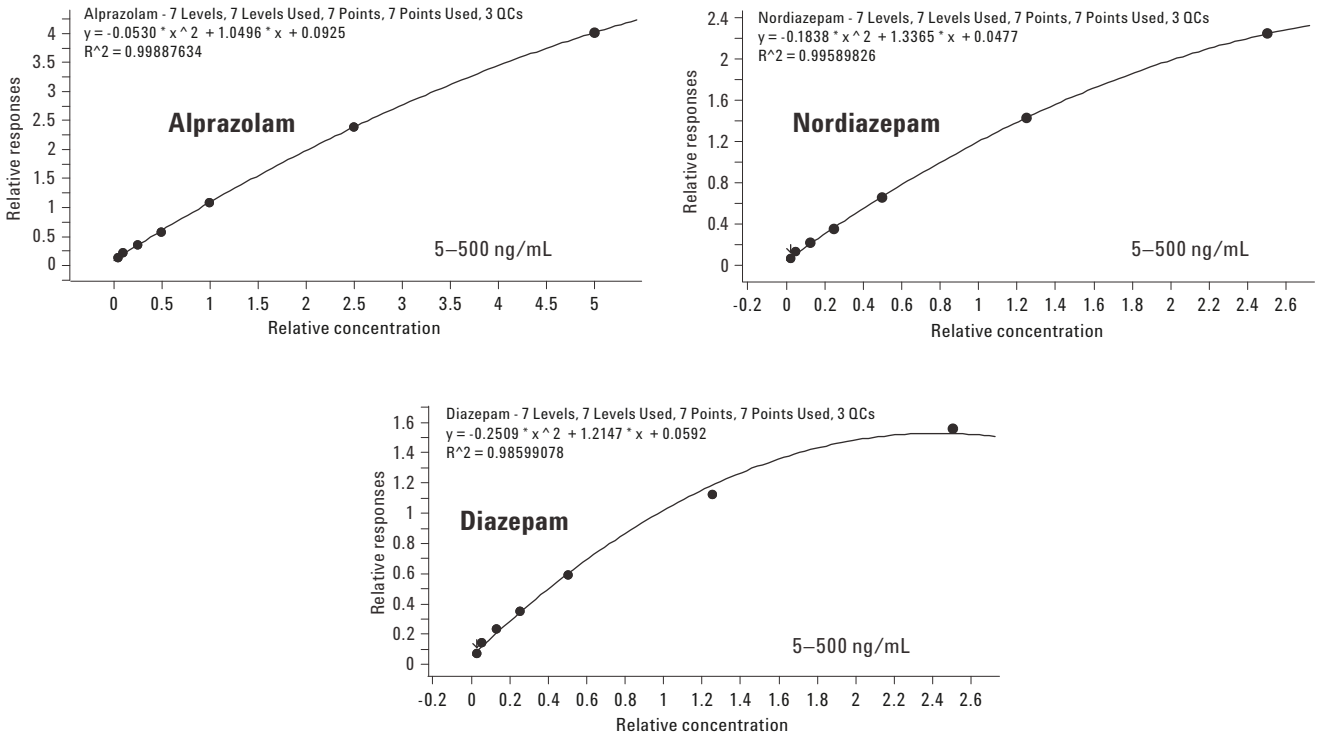


Figure 26. Calibration curves for the DUID compounds. Detector saturation was the primary cause of the nonlinearity seen for nordiazepam and diazepam.

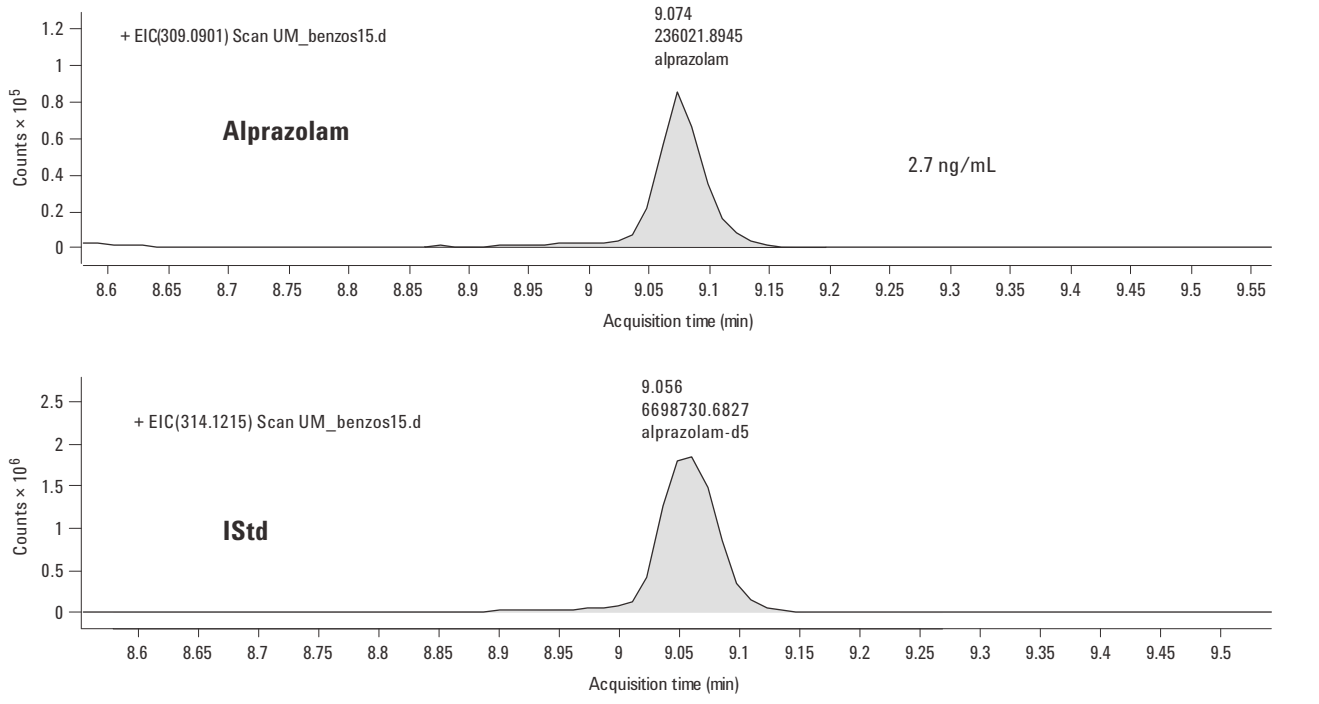


Figure 27. DUID case sample 0024 has a calculated amount of 2.7 ng/mL for alprazolam.

Table 7. Calculated TOF Quantification Amounts for Benzodiazepines in the Case Samples (The presence of nordiazepam and diazepam is not detectable in any of the samples.)

DUID benzodiazepine case sample (TOF)	Calculated amounts (ng/mL) Alprazolam	Nordiazepam	Diazepam
0024	2.7	–	–
0062	39.0	–	–
0083	7.8	–	–
0476	89.1	–	–
0531	69.2	–	–
0580	9.0	–	–

Quadrupole Time-of-Flight Mass Spectrometer

Postmortem Blood

The Agilent QTOF instrument in MS mode behaved exactly the same as the TOF. To avoid the nonlinearity effects seen in the TOF work, only 0.1 µL sample volumes were injected after observing ESI or detector saturation on the SQ and TOF instruments. Quantification was performed on the QTOF in MS mode only. Quantification may also be carried out in MS/MS mode although it is typically no more sensitive because the resolution in MS mode typically removes the effects of coeluting interferences, short of ion suppression.

As was the case with the TOF, EICs of the compounds in the lowest level calibrator for the postmortem samples are shown in Figure 28 (cocaine, BE, and CE) and Figure 29 (methadone). The EICs are generated using a window of ± 10 ppm with respect to the exact protonated masses of the compounds.

The corresponding calibration curves are shown in Figure 30 and extend over the concentration ranges given in Table 1. Linearity was good when reducing the injection volume 50-fold from 5 to 0.1 µL. Based on these calibration curves the case samples quantified as shown in Figures 31 and 32. That is, the cocaine case sample cocaine = 26.1 ng/mL, BE = 1539.6 ng/mL, and CE = 10.4 ng/mL. For the methadone case sample the calculated level of methadone was 898.1 ng/mL.

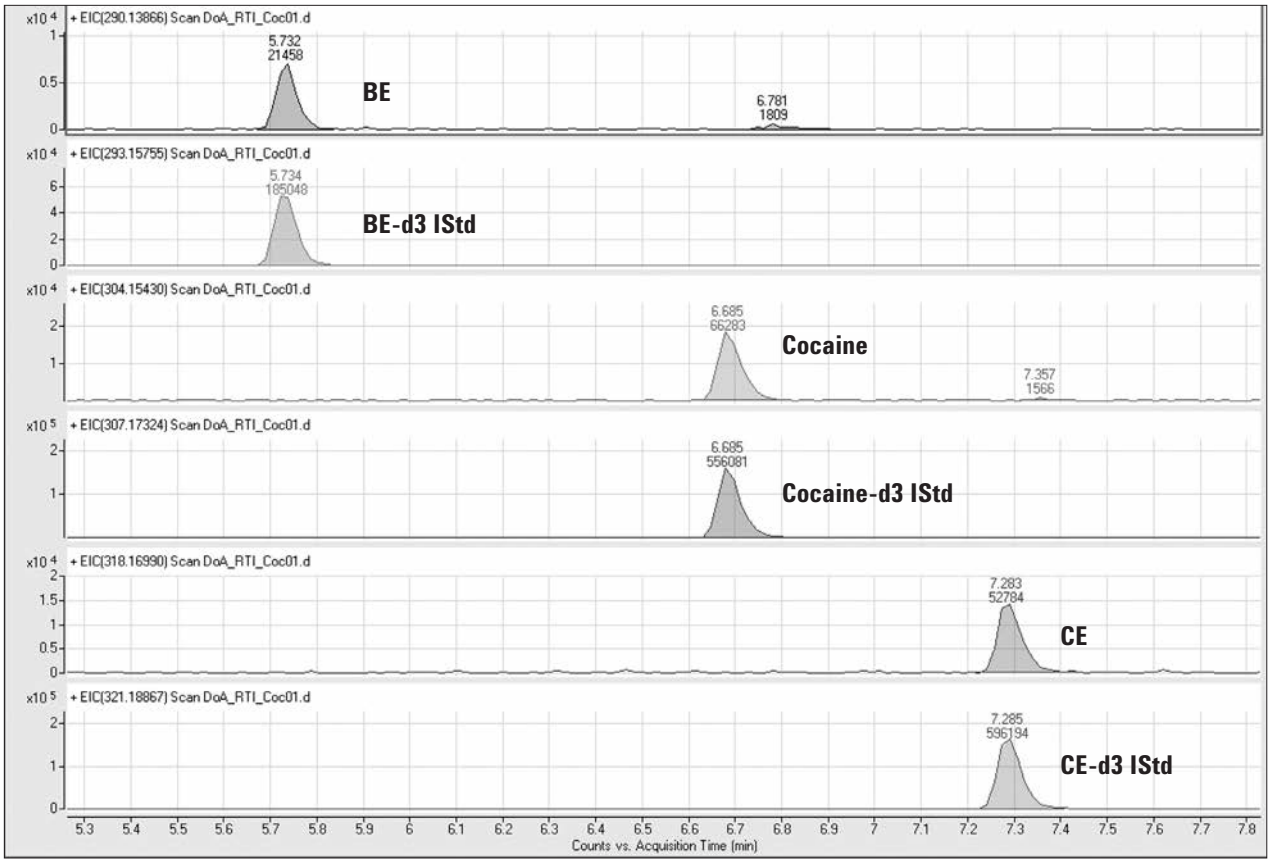


Figure 28. EICs (± 10 ppm) of lowest level calibrator in postmortem analysis: BE and cocaine (25 ng/mL); CE (10 ng/mL) at 0.1 uL injection volume.

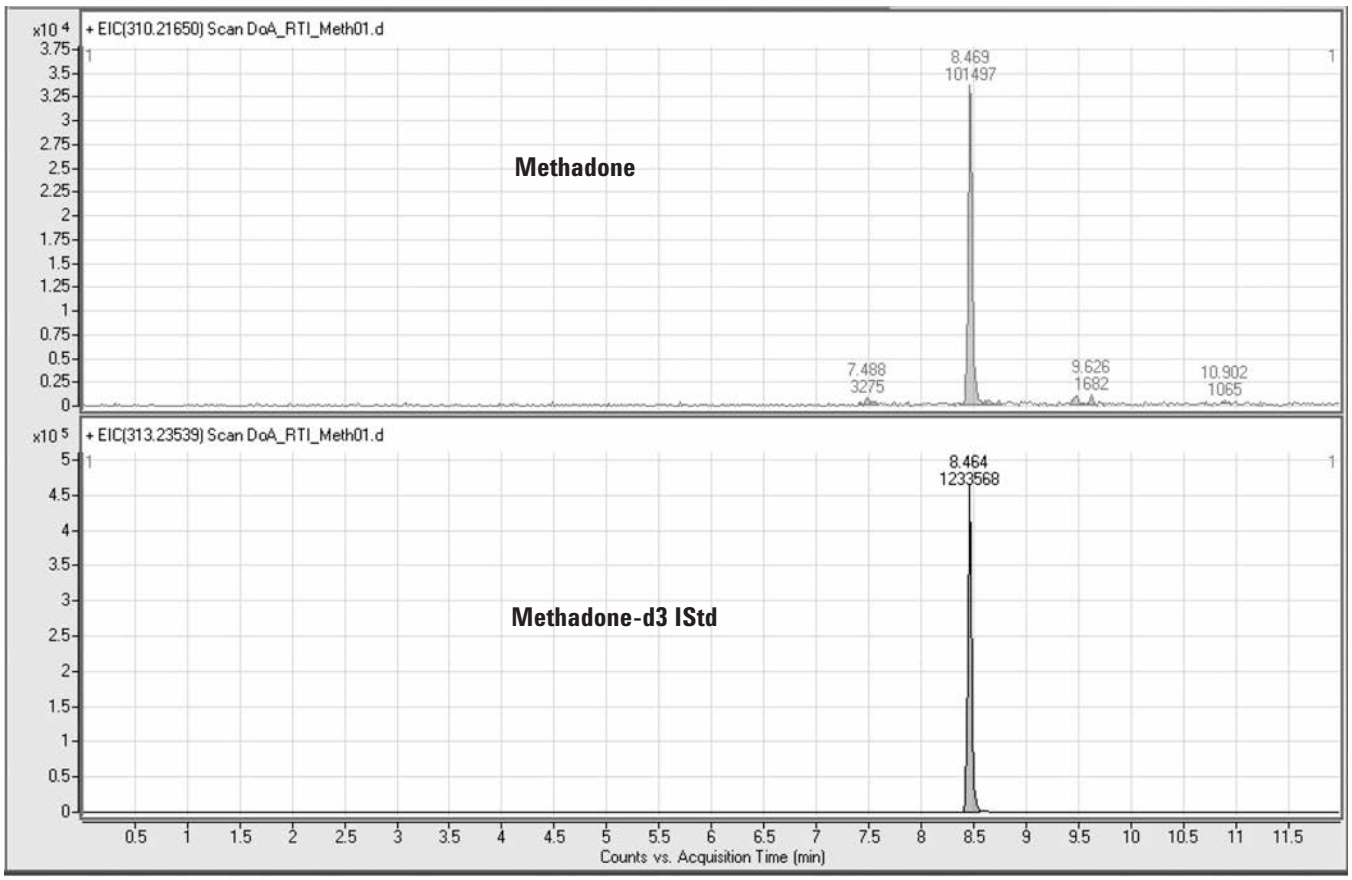


Figure 29. EICs (± 10 ppm) of methadone at 25 ng/mL in the lowest level calibrator for the postmortem analysis at a 0.1 µL injection volume.

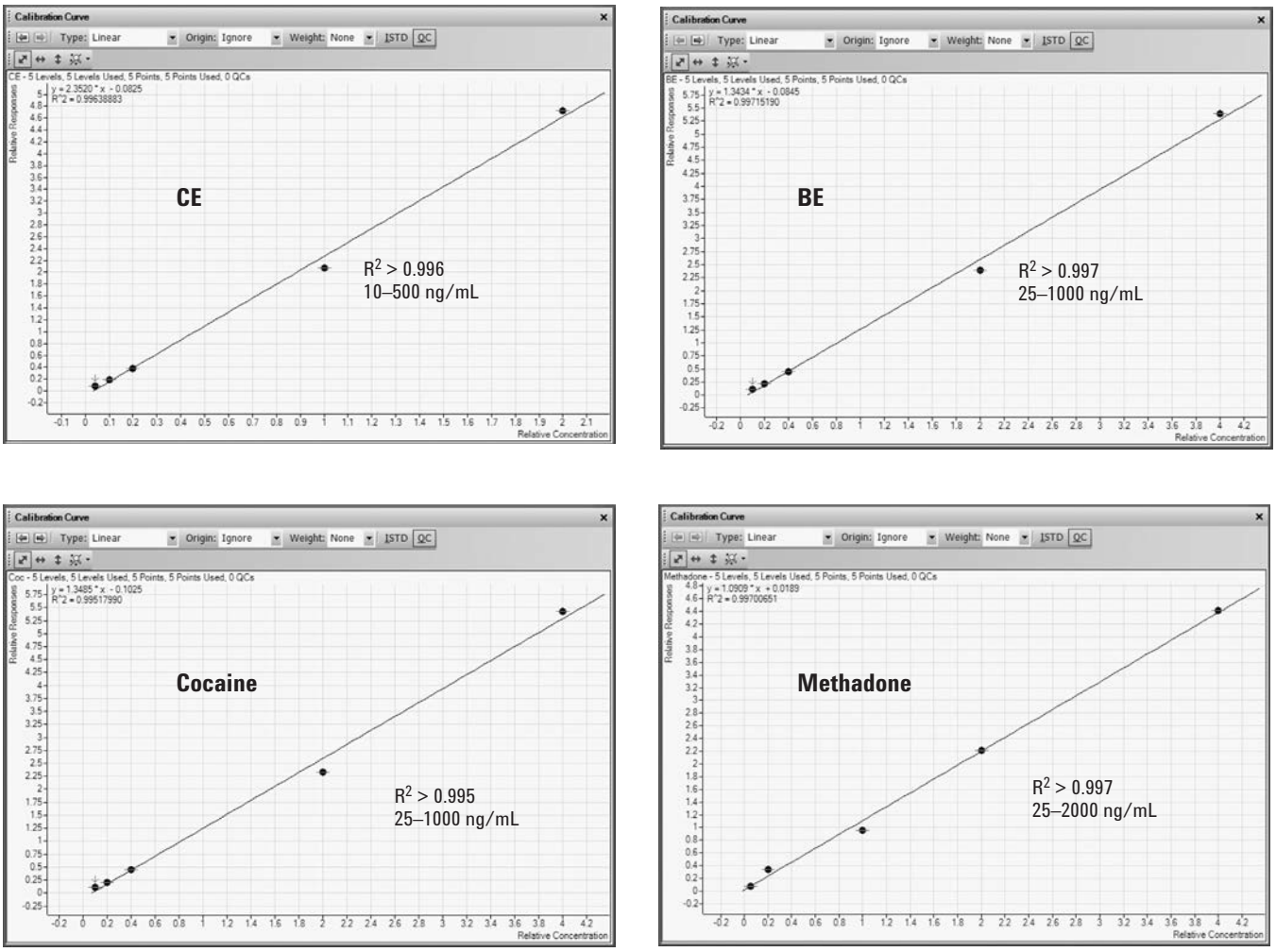


Figure 30. Calibration curves for the compounds in the postmortem analysis.

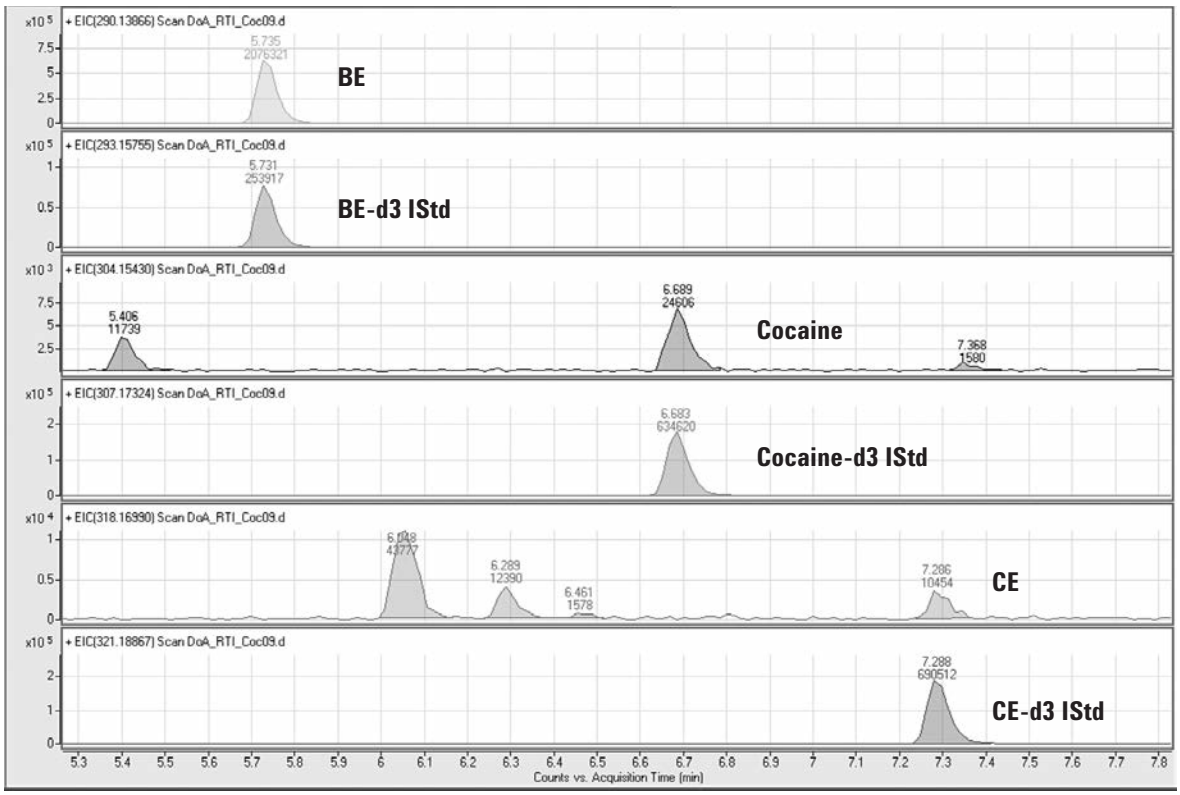


Figure 31. EICs (± 10 ppm) of cocaine case sample quantitating at BE = 1539.5 ng/mL, cocaine = 26.1 ng/mL, and CE = 10.4 ng/mL.

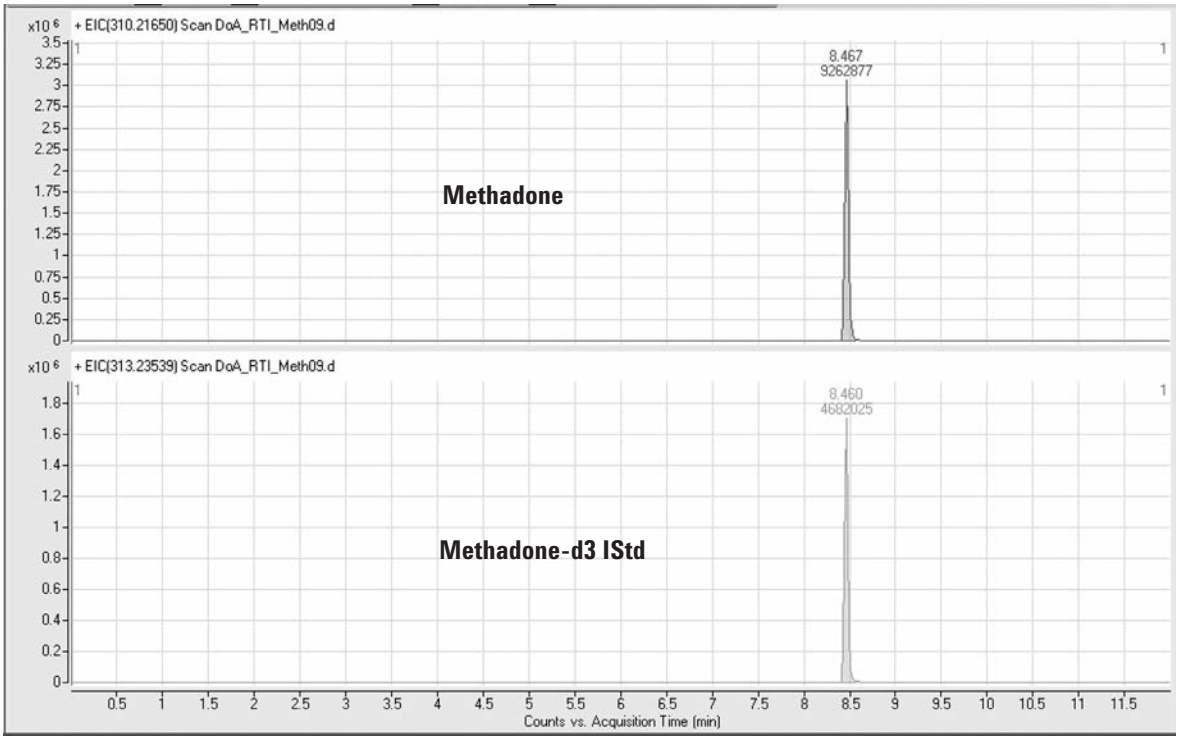


Figure 32. EICs (± 10 ppm) of methadone case sample quantitating at 898.1 ng/mL.

DUID Blood

For the DUID sample analysis by QTOF an injection volume of 0.1 µL was still used and the results for the lowest level calibrator of 5 ng/mL for alprazolam, nordiazepam, and diazepam are shown in Figure 33. The S/N looks good, suggesting that the levels of quantification could go lower.

The calibration curves for each compound ranging from 5 to 500 ng/mL are shown in Figure 34, with a calculated quantification result of 0.5 ng/mL alprazolam in case sample 0024. The other two compounds were not detectable in this sample. The results for all DUID case samples are shown in Table 8.

Table 8. Calculated QTOF Quantification Amounts in MS Mode for Benzodiazepines in the Case Samples (The presence of nordiazepam and diazepam was not detectable in any of the samples.)

DUID benzodiazepine case sample (QTOF in MS mode)	Calculated amounts (ng/mL)		
	Alprazolam	**Nordiazepam**	**Diazepam**
0024	0.5	–	–
0062	35.8	–	–
0083	3.6	–	–
0476	62.7	–	–
0531	70.9	–	–
0580	1.3	–	–

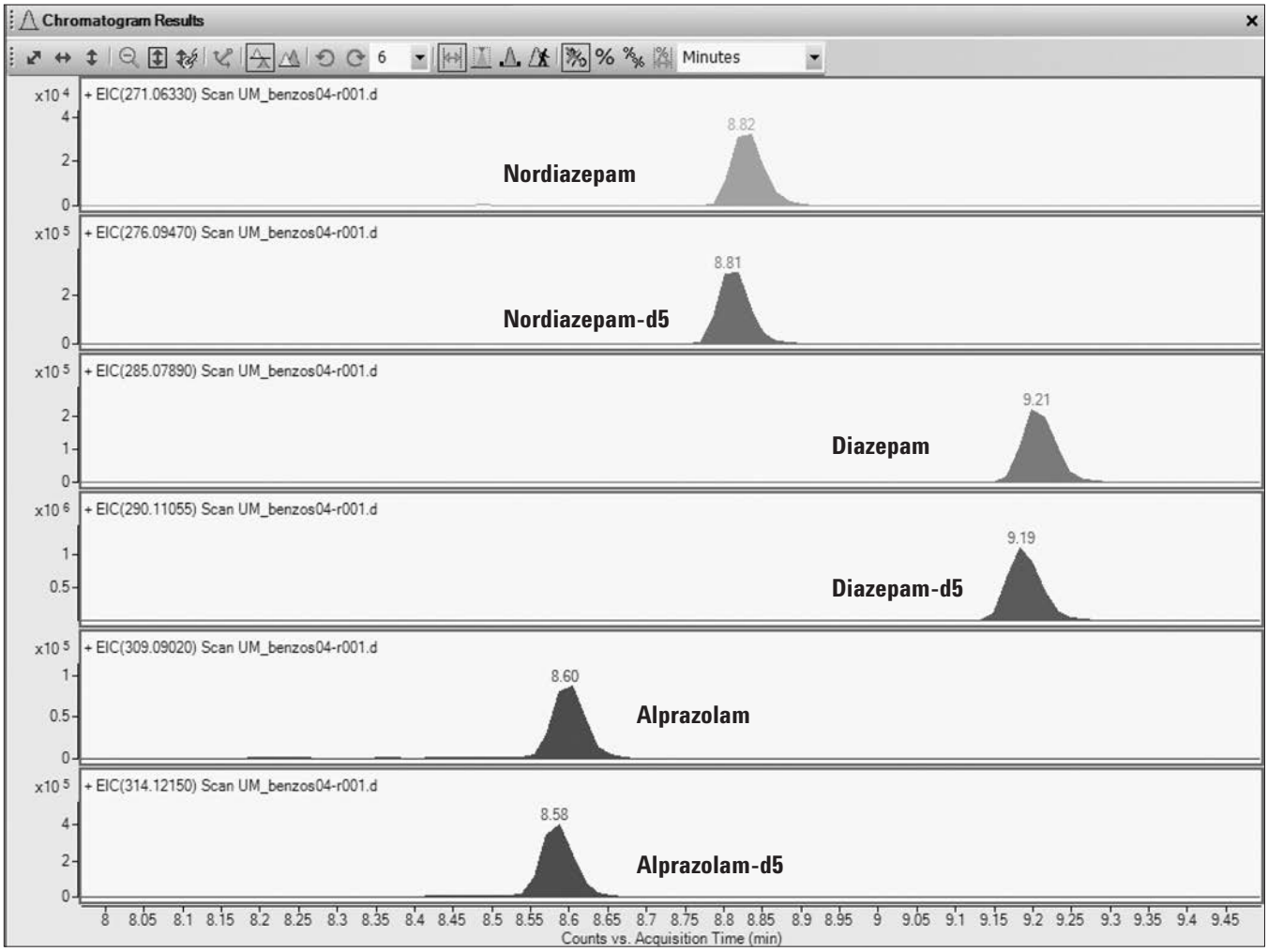


Figure 33. EICs (± 10 ppm) of lowest level calibrator at 5 ng/mL alprazolam, nordiazepam, and diazepam for DUID analysis.

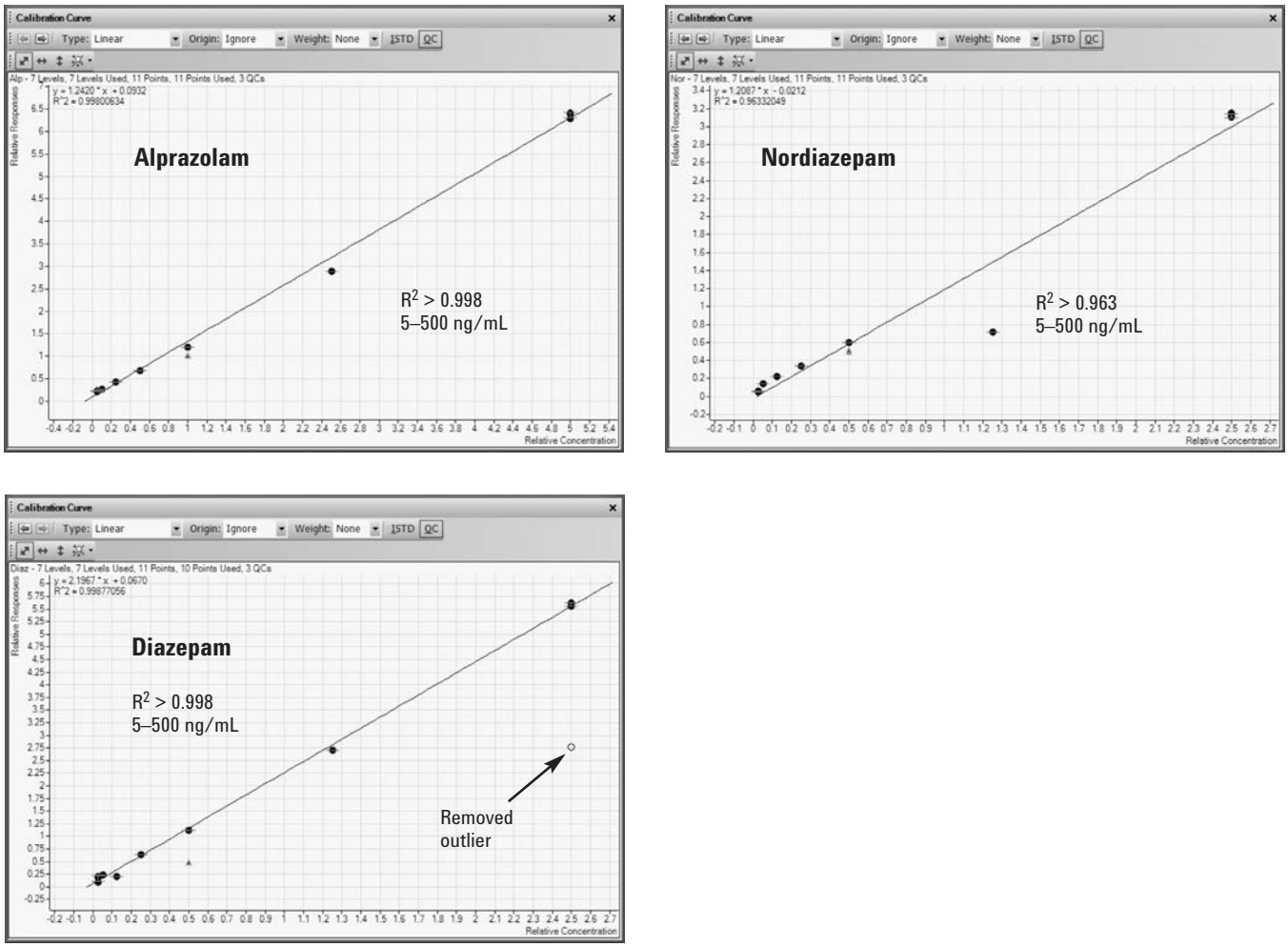


Figure 34. Calibration curves for alprazolam, nordiazepam, and diazepam in DUID analysis over 5 to 500 ng/mL concentration range.

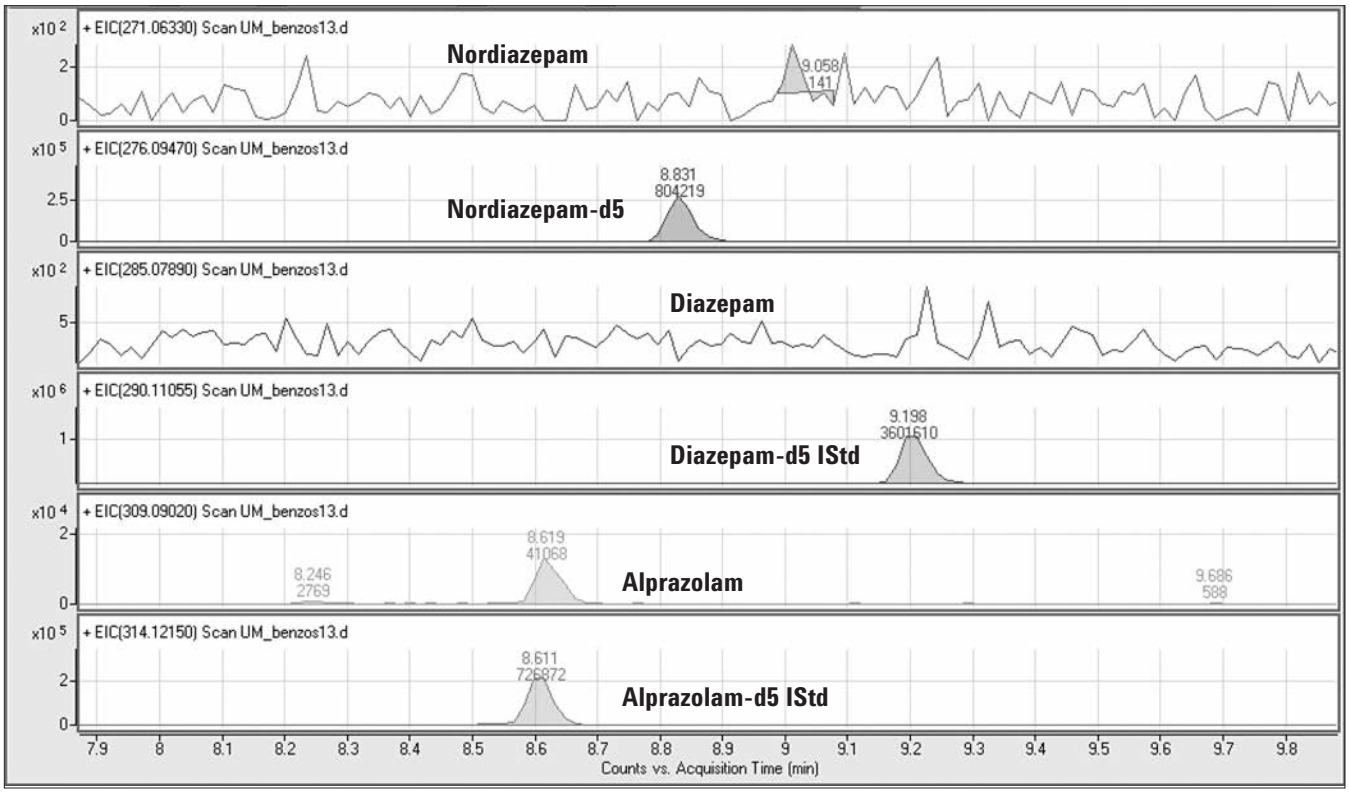


Figure 35. Calculated level of alprazolam is 0.5 ng/mL in DUID case sample 0024. Nordiazepam and diazepam were not detected.

As was the case with the TOF, identifying a sample was largely based on the mass accuracy of the instrument, which often leads to one or maybe two possible chemical formulas in the small molecule mass regime. The isotopic distribution and nitrogen rule also play a major role. For example, according to the nitrogen rule, a protonated ion of even mass must have an odd number of nitrogens in the structure. The isotopic distribution is based on natural abundances of isotopes in the molecule. All these factors play special roles in confirming the presence of compounds.

Figure 36 shows the confirmation of cocaethylene based on chemical formula and using an algorithm in the data processing software known as a molecular formula generator. The mass accuracy, isotopic distribution, and nitrogen rule are all contributing factors of the algorithm leading to confirming the presence of cocaethylene based on the derived chemical formula of $C_{18}H_{23}NO_4$.

The only dilemma would be in the fact that a chemical formula could belong to several different structures. As a result, it is generally a good idea to purchase a standard of the compound believed to be present and analyze it under the same LC conditions to determine if the resulting retention times are consistent.

Along with retention time, confidence in identifying a structure can be obtained through an accurate mass MS/MS experiment in which the chemical formula of product ions can be determined to then determine which precursor ion structure makes the most sense in generating the corresponding product ions.

The mass accuracy of the QTOF in MS mode, or TOF MS mode, is the same as the TOF, or < 2 ppm. At the MS/MS level, the mass accuracy is typically < 5 ppm. Figure 37 shows the accurate MS/MS spectrum of cocaine. The peaks in the MS/MS spectrum have good accurate mass when assigned to the likely structures shown. These product ion structures were proposed in a *Journal of Mass Spectrometry* article back in 1998 [4]. Note that the mass errors are greater than 5 ppm in the mass range below the lower mass reference ion of *m/z* 121.05058. This is partially due to S/N, or resolving analyte signal from background, as well as being outside the mass range of the reference ions. In addition, the smaller the exact mass the larger the relative mass error as the exact mass term is in the denominator of the calculation.

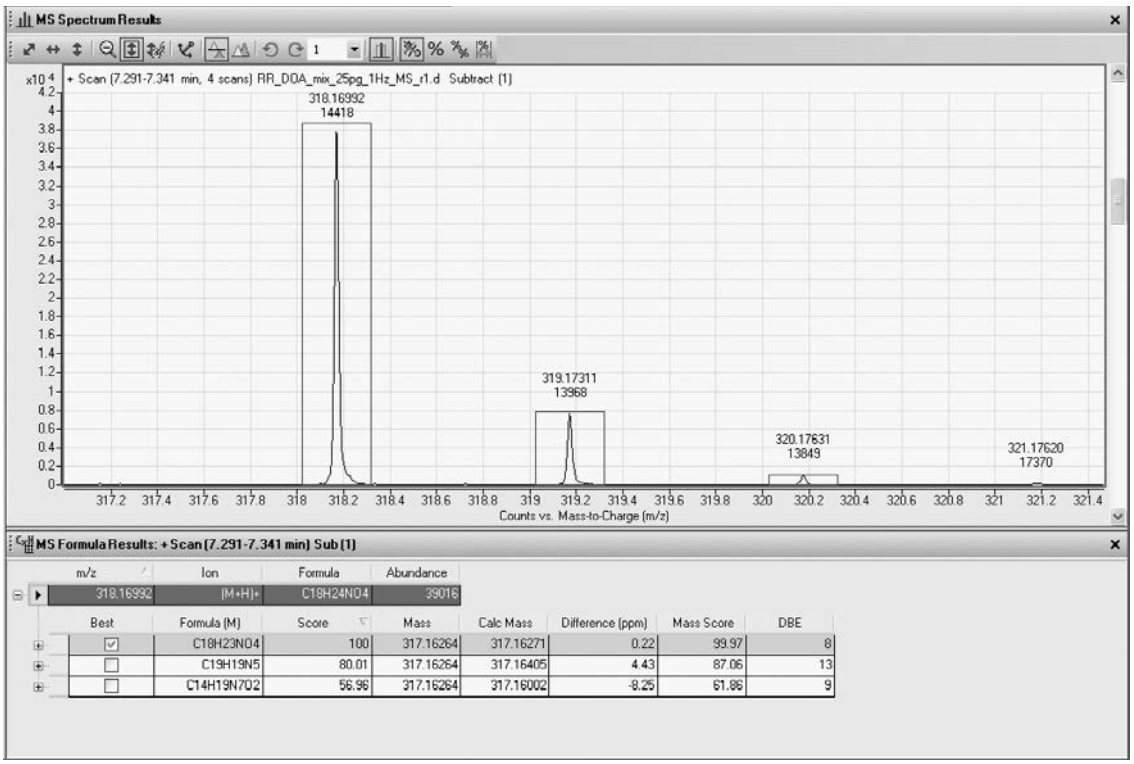


Figure 36. Confirming presence of cocaethylene using a molecular formula generator.

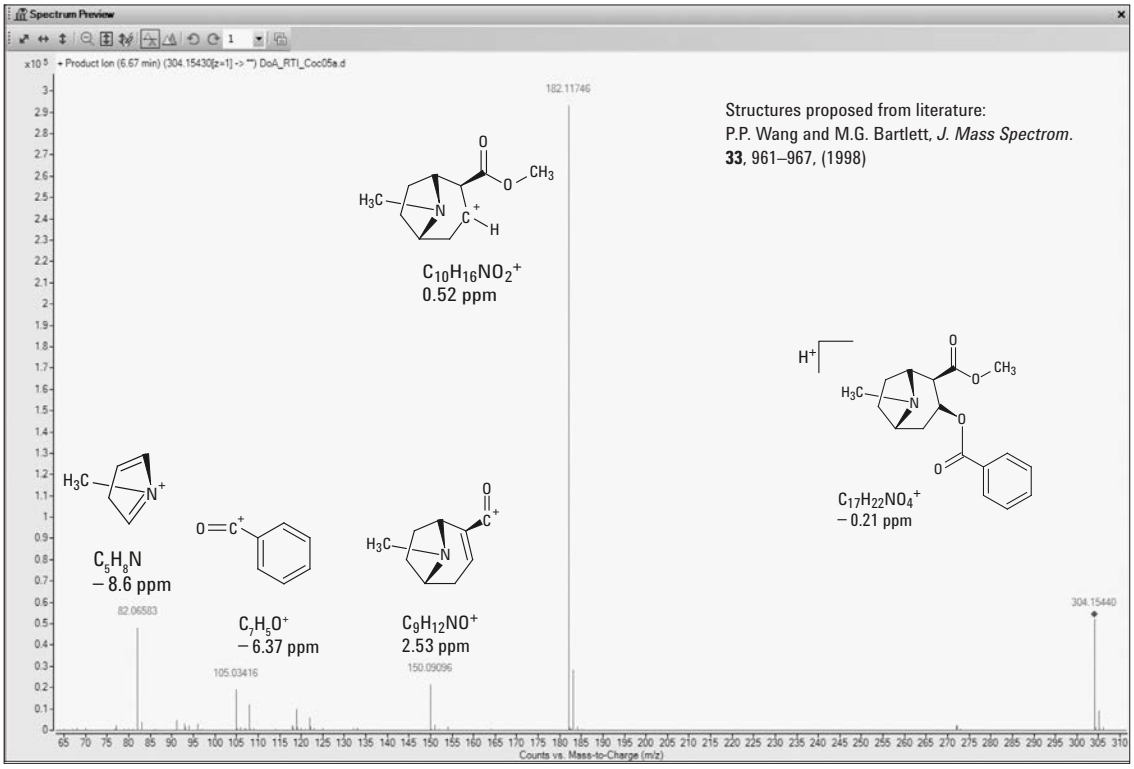


Figure 37. Targeted MS/MS of cocaine.

Conclusions

All of the instruments in this study were able to detect all the target analytes at the lowest calibration levels. For quantification, the QQQ was the best, followed by the SQ, both with good reproducibility at the lowest levels, particularly the QQQ, as shown in the results. A further benefit to using a QQQ for this kind of analysis was that it reduced sample preparation as compared to the SQ. The most sensitive mode of operation for the SQ is SIM and for the QQQ it is MRM. The primary use for both of these instruments in forensic toxicology is quantification.

The ion trap was sensitive in full-scan MS/MS and MS3 modes, but can be hampered by the presence of coeluting interferences, not making it the best choice for quantification. For reproducible quantification, peak widths on the order of 10 seconds are typically required, which are more than twice as wide as those acquired in this work using modern sub-2-micron Rapid Resolution LCs and columns.

Both the TOF and QTOF had decent sensitivity in their ability for quantification by processing narrow EICs in the MS and MS/MS modes, respectively. However, in this work, quantification with the QTOF was carried out in MS mode, which for many applications has been found to be as sensitive as MS/MS, probably because the resolving power in the MS mode is good at distinguishing analytes of interest from coeluting interferences.

For qualitative work with the purpose of identifying compounds, the ion trap, with excellent sensitivity in MS/MS and MS3 modes, does a nice job at identifying compounds based on a library. For example, the compound sertraline was found in the methadone case sample. Using a full-scan spectral library for identification is analogous to NIST-based library searching in GC/MS.

The TOF and QTOF instruments use accurate mass in full-scan MS and MS/MS modes to identify compounds not in libraries. In fact, compound identification with both of these instruments can be carried out using an accurate mass database containing compound names, chemical formula, exact masses, and retention times, if known. However, for this work, such a database was not needed as the set of compounds to be analyzed was already known.

The QTOF is the ultimate instrument for the analysis of unknown compounds, taking advantage of accurate mass at both the MS and MS/MS levels. Determining a chemical formula at the MS level doesn't necessarily indicate a particular structure. Like an ion trap, the QTOF produces a fingerprint of the compound structure by producing a full-scan MS/MS product ion spectrum. Accurate mass at the selective MS/MS level determines chemical formula of the fragments, both product ions and neutral losses, to indicate what substructures can subsequently lead back to the identification of a particular compound.

All instruments were easy to use with minimal method development, with perhaps the exception of the QQQ, which needed both the fragmentor and collision energy to be optimized for each MRM transition. However, the source settings are based on LC flow rate and the ion transfer optics and mass analyzer voltages are all taken care of with the automated tuning and calibration procedures available in each instrument.

References

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Acknowledgments

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Analysis Of Oxycodone And Its Metabolites-Noroxycodone, Oxymorphone and Noroxymorphone In Plasma By LC/MS With An Agilent ZORBAX StableBond SB-C18 LC Column

Application Note

Pharmaceutical

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Abstract

Oxycodone and its oxidative metabolites (noroxycodone, oxymorphone and noroxymorphone) are analyzed by high performance liquid chromatography/mass spectrometry (HPLC/MS), coupled with chromatographic separation by an Agilent ZORBAX Rapid Resolution High Throughput (RRHT) StableBond SB-C18 column. The method utilizes an ammonium acetate/acetonitrile gradient, with detection by mass spectrometer in electrospray mode with positive polarity. Spiked human plasma samples undergo solid phase extraction prior to LC/MS analysis. This method provides good linearity ($R^2 > 0.9900$) and reproducibility (< 10% difference between duplicates) for all compounds, while increasing productivity with a fast, efficient analysis and minimal solvent usage.

Agilent Technologies

Introduction

Oxycodone was developed in 1916 as an opioid analgesic medication. Today, oxycodone is a Schedule II drug in the US, which means, while it has proven medical uses, it is still considered highly addictive. Figure 1 shows oxycodone and its metabolic scheme, yielding noroxycodone, oxymorphone and noroxymorphone (a secondary metabolite)[1]. Because pain is subjective and metabolic rates differ from person to person, it can be difficult to determine appropriate dosages of oxycodone. One must find the balance between alleviating pain and causing adverse side effects, such as constipation, dizziness, drowsiness, headache, nausea, sleep-lessness, vomiting and weakness [2]. The key to achieving

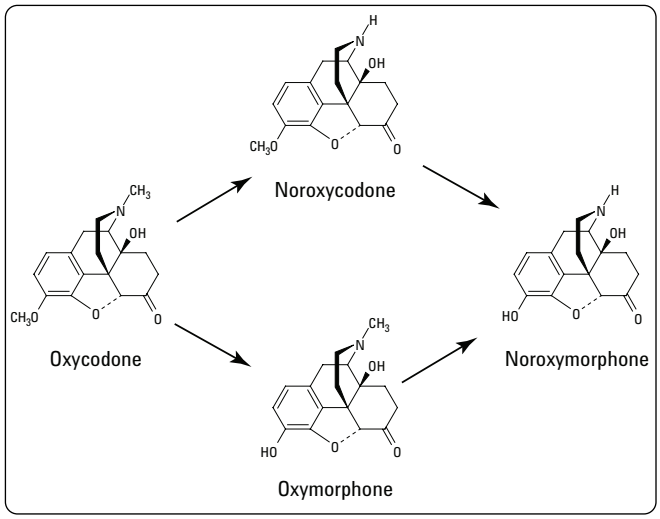

Figure 1. Metabolic scheme of oxycodone to noroxycodone, oxymorphone and noroxymorphone.

this balance is by monitoring the rate of metabolism of oxycodone to its metabolites. Extensive metabolisers require higher concentrations of oxycodone in plasma to achieve the therapeutic effects, while poor metabolisers may experience toxicity due to slow drug clearance and excessive plasma concentration. Due to the nature of this drug, it is no surprise that there is a need to qualify and quantify oxycodone and its metabolites in a variety of matrices.

Liquid chromatography coupled with mass spectrometry (LC/MS) is ideal for the detection of oxycodone and its metabolites. These alkaloid compounds can be analyzed via electrospray mass spectrometry without derivatization [3]. Additionally, mass spectrometry allows for a sensitive analysis, especially in a complex matrix such as urine, blood, hair or anywhere else one might look for drug residues.

Experimental

An Agilent 1100 Series HPLC/MS was used for this work:

- G1312A Binary Pump. Mobile phase A: 20 mM ammonium acetate, pH 4.0 and B: acetonitrile. Flow rate was 0.300 mL/min. Hold 5% B for 2.33 minutes, then increase B from 5% to 20% from 2.33 to 4.33 minutes, stop time is 6 minutes, and post time is 4 minutes.
- G1367A Wellplate Autosampler (ALS). Injection volume was 5.0 µL, with needle wash in flushport for 5 seconds with water/acetonitrile (50:50).
- G1316A Thermostated Column Compartment (TCC). Temperature was 30 °C.
- G1956B Mass Spectrometer (MS) was operated in atmospheric pressure ionization electrospray mode with positive polarity. Ion 288 *m/z* was monitored for noroxymorphone, 302 *m/z* for oxymorphone and noroxycodone, 316 *m/z* for oxycodone, and 322 *m/z* for d6-oxycodone (internal standard). Spray chamber gas temperature was 350 °C at 12 L/min.
- ChemStation version B.01.01 was used to control the HPLC/MS and process the data.

An Agilent ZORBAX Narrow Bore Rapid Resolution High Throughput (RRHT) StableBond SB-C18, 2.1 mm × 50 mm, 1.8-µm column (Agilent p/n 827700-902) was used for this chromatographic separation.

Acetonitrile, ammonium acetate, methanol, methylene chloride, isopropanol and ammonium hydroxide were purchased from Fisher. Boric acid was purchased from Baker. Standard solutions of oxycodone, noroxycodone, oxymorphone and noroxymorphone in methanol were purchased from Cerilliant, concentrations were 1 mg/mL for oxycodone, noroxycodone and oxymorphone, and 0.1 mg/mL for noroxymorphone. A composite sample was then made by combining 25 µL aliquots of oxycodone, noroxycodone and noroxymorphone, 2.5 µL of oxymorphone and 25 mL of methanol.

Matrix samples were prepared by spiking 1 mL of clean human plasma with various concentrations of the composite sample. Metabolites were extracted from plasma by solid phase extraction (SPE); SPE bonded phase was a non-end capped mixed-mode sorbent: octyl (C8) and benzenesulfonic acid (SCX). Cartridges were conditioned with 2 mL methanol, followed by 2 mL deionized water. Each spiked plasma sample was diluted with 1.5 mL borate buffer, pH 8.9, loaded into the SPE cartridge, then washed with 2 mL deionized water, 1 mL 10 mM ammonium acetate, pH 4 and 2 mL methanol, and finally eluted with 3 mL methylene chloride/isopropanol/ ammonium hydroxide (80:20:2). Samples were dried under air at 60 °C, and then reconstituted in 60 µL of 10 mM ammonium acetate, pH 4/acetonitrile (95:5).

Results and Discussion

At pH 4, the StableBond SB-C18 stationary phase (a non-end capped type B silica) demonstrates excellent selectivity with a well buffered mobile phase. The non-end capped bonded phase provides more varied selectivity for polar compounds, like oxycodone and its metabolites (bases), than end capped phases due to additional interactions with exposed silanol groups. These interactions can be controlled and optimized by altering mobile phase conditions. The small 1.8-µm particle size allows for superior resolution and efficiency over 3.5 or 5 µm particles. Additional benefits of this column are the short 50-mm length and the small internal diameter (id), 2.1 mm. The short column allows for increased productivity with faster analysis times, while the small ID allows for prudent solvent usage.

Figure 2 shows extracted ion chromatograms (EIC) of a human plasma sample, previously determined to be free of oxycodone and its metabolites, that has been spiked with 50 ng/mL oxycodone, 50 ng/mL noroxycodone, 5 ng/mL oxymorphone, 5 ng/mL noroxymorphone and 40 ng/mL d6-oxycodone (an internal standard), and then extracted by SPE. Despite being in a complex sample matrix (plasma), the chromatograms are well resolved for each of the five

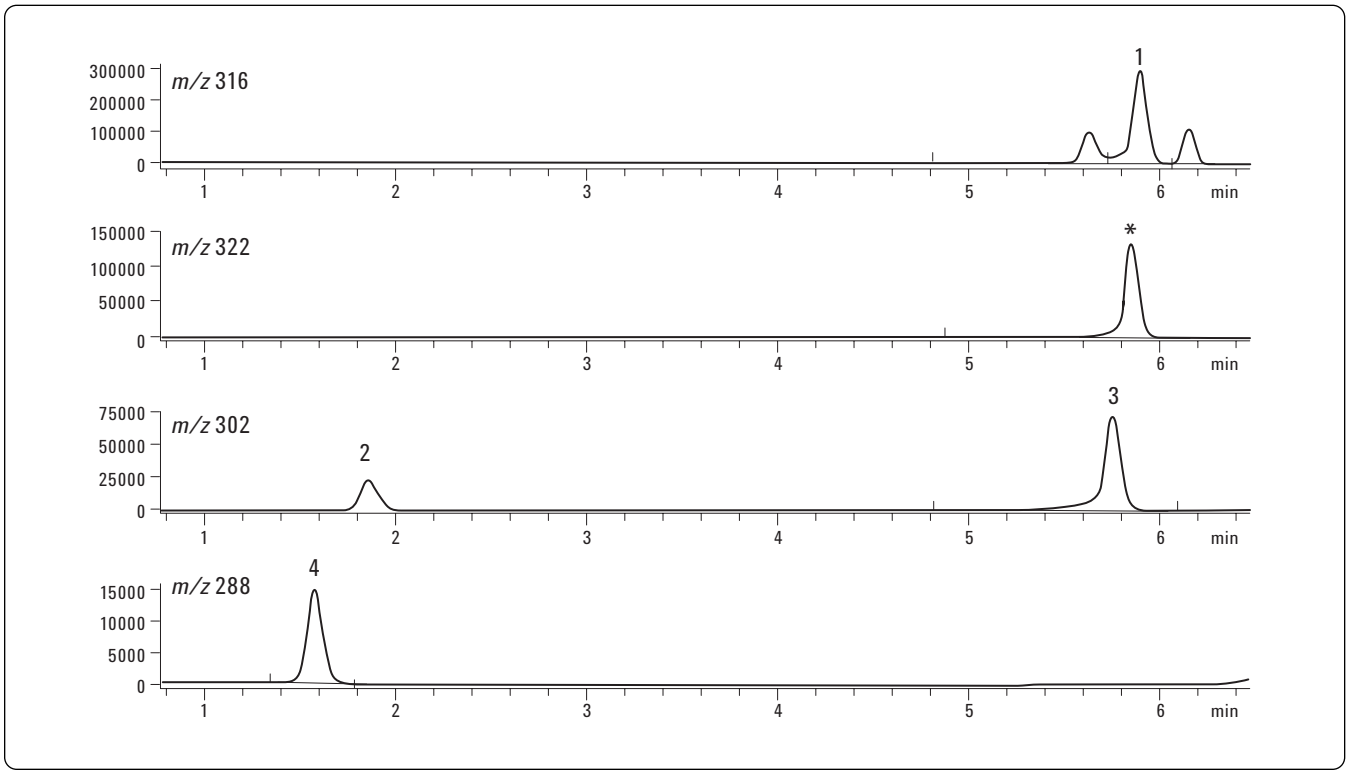

Figure 2. Human plasma sample spiked with 50 ng/mL oxycodone (1) and noroxycodone (3), 5 ng/mL oxymorphone (2) and noroxymorphone (4), and 40 ng/mL internal standard, d6-oxycodone (*). Sample was extracted by SPE, then analyzed by LC/MS with an Agilent ZORBAX StableBond SB-C18 column. The extracted ion chromatograms are shown.

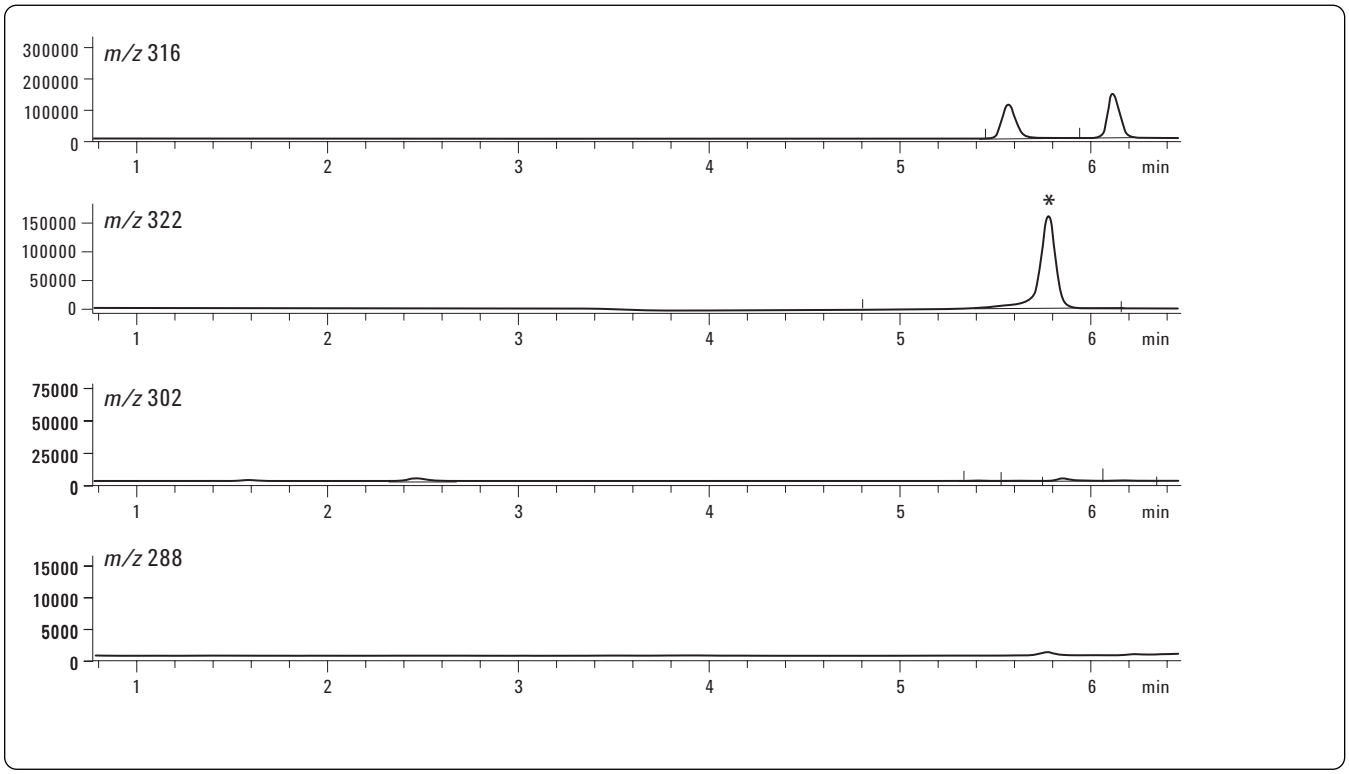

Figure 3. Human plasma sample, free from oxycodone and its metabolites, spiked with 40 ng/mL internal standard, d6-oxycodone (*). Sample was extracted by SPE, then analyzed by LC/MS with an Agilent ZORBAX StableBond SB-C18 column. The extracted ion chromatograms are shown.

compounds. In the extracted ion chromatogram for *m/z* 316, two additional peaks elute. As shown in Figure 3, an EIC for a blank plasma sample, these two peaks appear to be part of the plasma matrix.

Good linearity is found for all compounds with R^2 >0.9900 over the concentration range of 2 to 50 ng/mL for oxycodone and noroxycodone, and 0.2 to 5 ng/mL for oxymorphone and noroxymorphone. The limit of detection/quantification is 0.5 ng/mL for oxycodone, 1 ng/mL for noroxycodone, and 0.2 ng/mL for both oxymorphone and noroxymorphone with an Agilent 1100 Series LC/MS. Reproducibility is good with less than a 10% difference between each duplicate sample set over the aforementioned concentration range.

Conclusion

Oxycodone and its metabolites are successfully analyzed by LC/MS with an Agilent ZORBAX RRHT StableBond SB-C18 column over a suitable range. This column selection provides an efficient, rapid analysis for increased productivity, while keeping solvent usage to a minimum. For all compounds, calibration curves show good linearity, with sensitive and reproducible results in a complex or dirty matrix, such as plasma.

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Determination of Cocaine and Metabolites in Urine Using Electrospray LC/MS

Application Note

Drug Testing

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Abstract

A rapid, simple, and sensitive electrospray LC/MS method has been developed for the quantitative analysis of cocaine and benzoylecgonine in urine using electrospray with the Agilent 1100 LC/MSD system. Urine samples were extracted using solid phase extraction cartridges, and the drug and metabolite were analyzed without derivatization using an isocratic separation and selected ion monitoring (SIM).

Introduction

Two metabolites, namely benzoylecgonine (BE) and norcocaine, are frequently analyzed as markers of cocaine use. The well-established GC/MS analysis of cocaine and BE requires derivatization of the metabolite. Derivatization adds additional variables from the derivatization process and can also introduce aggres-sive derivatizing reagents into the analytical system. These basic molecules show excellent sensitivity in electrospray mass spectrometry, and the analysis of cocaine and both metabolites can be carried out without a derivatization step. The same solid-phase extraction (SPE) developed for the GC/MS analysis can be used for the LC/MS analysis.

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Materials and Methods

The Agilent 1100 Series system included a binary pump, vacuum degasser, autosampler, thermostatted column compartment, diode-array detector, and an LC/MSD. The LC/MSD was used with the electrospray ionization (ESI) source. The diode-array detector was used during method development only. Complete system control and data evaluation was carried out using the Agilent ChemStation for LC/MS.

Sample Preparation and Extraction

Drug-free urine was fortified with known concentrations of the analytes for preparation of standard curves. Control samples were fortified with known concentrations of the analytes prepared from separate lots of stock solutions. Clean-Screen SPE columns (ZSDAU020, United Chemical Technologies) were conditioned with 3 mL of methanol and 3 mL of Milli-Q water, followed by 1 mL of 100 mM phosphate buffer, pH 6. Urine (1 mL) was mixed with 1 mL of the phosphate buffer, spiked with deuterated internal standards (cocaine-d3 and benzoylecgonine-d3) and loaded on the conditioned column. The column was sequentially washed with 2 mL of Milli-Q water, 2 mL of 100 mM HCl, and 3 mL of methanol. The column bed was dried at full vacuum for five minutes, and the analytes were eluted with 3 mL of dichloromethane/isopropanol/ammonium hydroxide (78/20/2). The eluate was evaporated to dryness with a stream of air at 40°C. The final sample residue was reconstituted in 50 µL of LC mobile phase, and 20 µL was injected for analysis by LC/MS.

Results and Discussion

In the analysis of cocaine metabolites, it is important to be able to distinguish the isobaric BE and norcocaine to allow accurate interpretation of results. The chromatography for this method was therefore optimized to separate BE from norcocaine, and isocratic conditions were found which allow for rapid analysis without column re-equilibration. Figure 1 shows the separation of cocaine, norcocaine and BE using these conditions.

MS parameters which were optimized for this analysis included fragmentor voltage (to give the most intense protonated molecule for each analyte), capillary voltage (for maximum signal), and spray chamber parameters (for maximum signal with minimum noise).

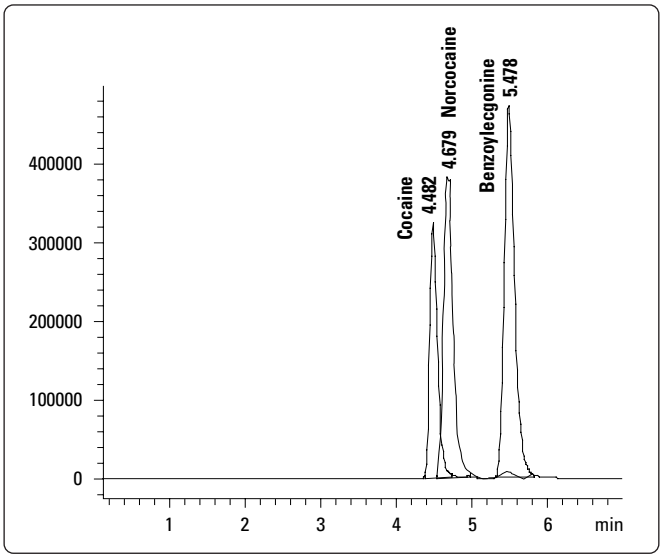

Figure 1. Isocratic separation of cocaine, norcocaine and BE.

Chromatographic Conditions	
Column:	Metasil Basic 3 µm, 3 × 150 mm (Metachem)
Mobile phase:	A = 0.1% formic acid in water
	B = methanol
Isocratic:	51% B
Flow rate:	0.2 mL/min
Column temp:	40°C
Injection vol:	20 µl
Diode-array detector:	signal: 234, 8 nm; reference: 360, 100 nm
MS Conditions	
Source:	ESI
Ionization mode:	positive
Vcap:	1500 V
Nebulizer:	20 psig
Drying gas flow:	10 L/min
Drying gas temp:	300°C
SIM ions:	*m/z* 290.1 (BE and norcocaine)
	m/z 293.1 (BE-d3)
	m/z 304.1 (cocaine)
	m/z 307.1 (cocaine –d3)
Peak width:	0.10 min
Time filter:	On
Fragmentor:	70 V

Figure 2 shows the extracted ion chromatograms (EICs) for blank urine fortified with the internal standards. Figure 3 shows the EICs for a urine standard fortified at 25 ng/mL.

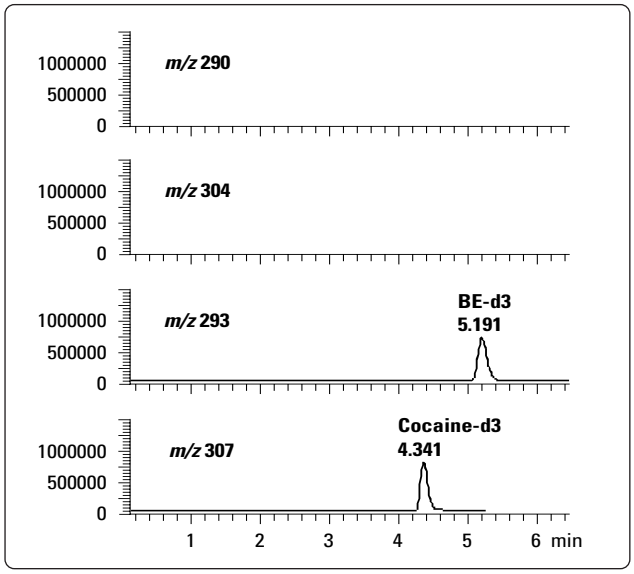

Figure 2. Extracted ion chromatograms of blank urine extract.

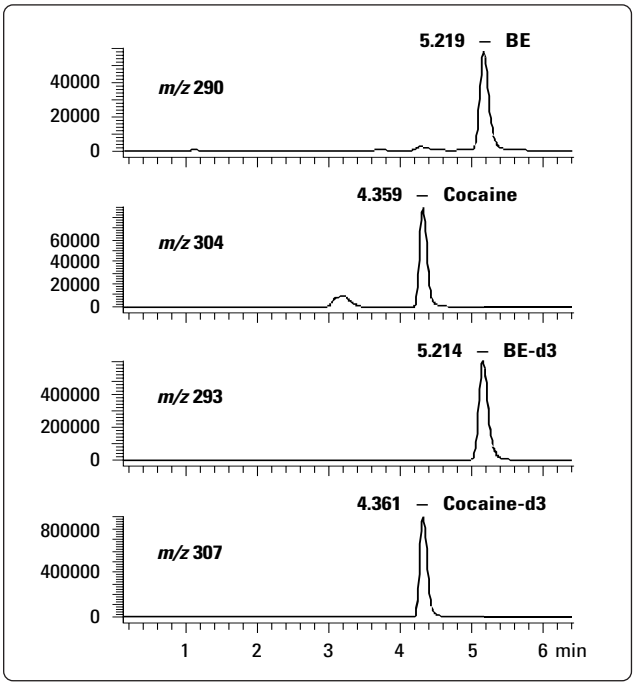

Figure 3. Extracted ion chromatograms of fortified urine extract (25 ng/mL).

The calibration range used for this analysis was 25–1000 ng/mL for both cocaine and BE. The calibration curves were linear across the calibration range without special weighting or curve treatment. Figure 4 shows typical calibration curves for cocaine and BE, with correlation coefficients (r^2) greater than 0.99 (0.99925 for cocaine and 0.99491 for BE).

Figure 5 shows the EICs of a positive urine sample found to contain 640 ng/mL cocaine and approximately 2700 ng/mL BE. The BE quantitation is an estimate, as the concentration is above the calibrated range of the method. Note that norcocaine can be clearly identified because it is chromatographically separated from benzoylecgonine which has the same mass.

Quality control samples fortified with 50 ng/mL and 150 ng/mL of each analyte gave quantitation results within 12% of the target concentration for cocaine and 3% for BE (see Table 1). Coefficients of variation were 7.1% and 5.1% for cocaine and BE respectively as shown in Table 1.

Table 1. Method accuracy and precision. Target concentrations were 50 ng/mL for cocaine and 150 ng/mL for BE.

	Cocaine	BE
	48.25	146.47
	47.06	155.69
	47.41	158.97
	46.21	148.50
	38.80	147.29
	40.89	146.57
	41.38	167.06
	42.68	159.81
Mean	44.085	153.795
Std Dev	3.570	7.734
C.V.*	7.1%	5.1%

*coefficient of variation = (mean/target)*100

These results compare well with an established GC/MS assay in which intra-assay coefficients of variation were less than 7% for both analytes when tested at 10, 25, 100, and 200 ng/mL.[1] The GC/MS assay gave quantitation results within 4% of the target concentration for cocaine and 5% for BE.

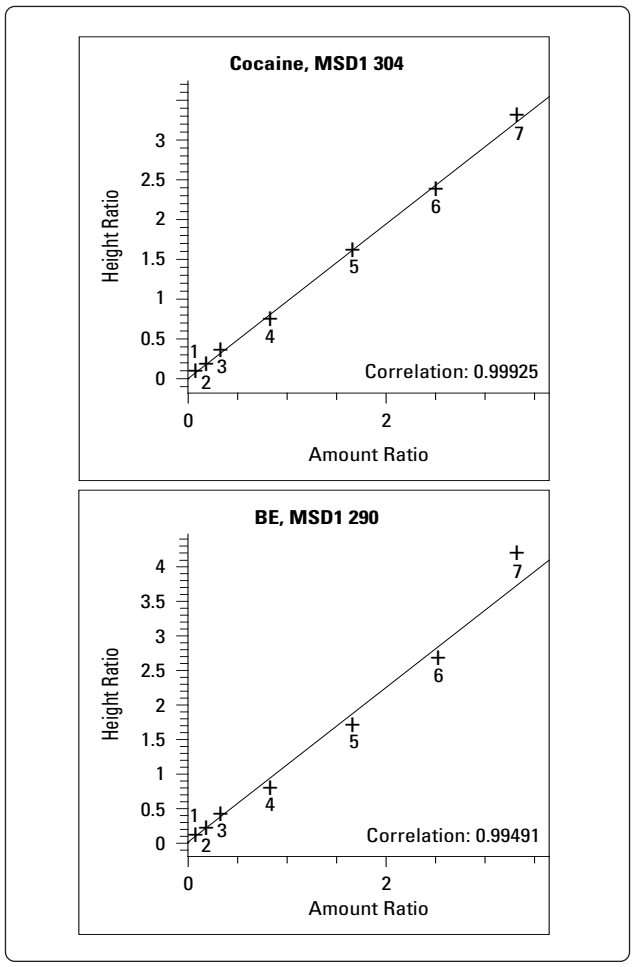

Figure 4. Calibration curves for cocaine and BE.

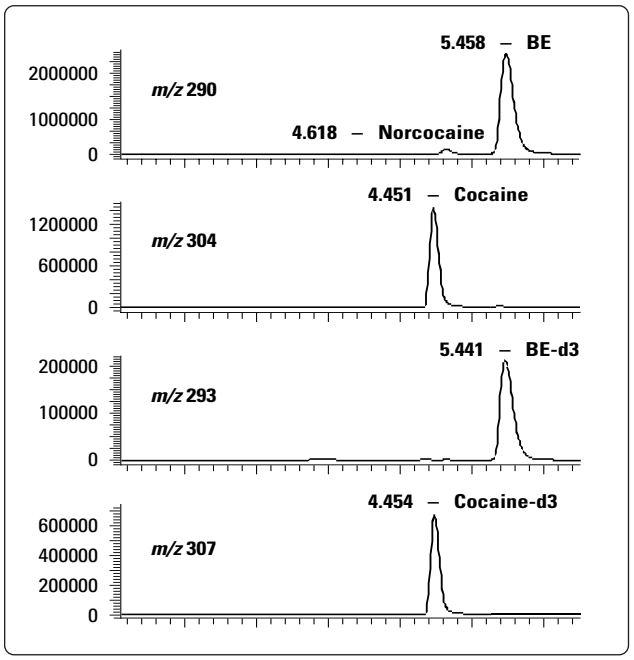

Figure 5. Extracted ion chromatograms from the extract of a positive urine sample.

Conclusions

This note describes an electrospray LC/MS method suitable for routine measurements of cocaine, BE and norcocaine in urine. The assay has a linear range of 25–1000 ng/mL and the precision and accuracy of this method compare favorably to those of the well-established GC/MS method for cocaine and BE. The sample preparation uses previously-described solid phase extraction technology widely used in forensic laboratories and requires no special modifications. In comparison to an existing GC/MS method for these analytes, the LC/MS method is simpler because it does not require derivatization, which involves aggressive reagents, derivatization time, and additional variability. In addition, the overall cycle time for one analysis is shorter for the LC/MS method than for the GC/MS method. This LC/MS method offers several advantages over traditional GC/MS assays with comparable quality of data.

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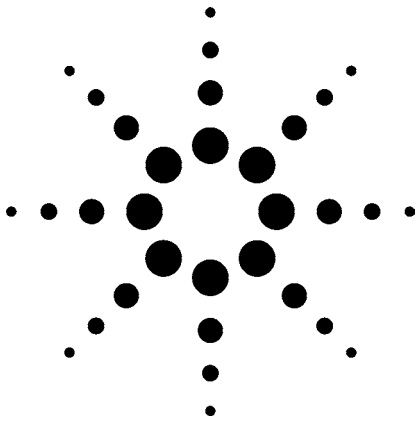
Screening Drugs of Abuse by LC/MS

Technical Overview



Forensic Toxicology

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Abstract

High through-put screening of drugs of abuse is performed at St. Olav Hospital by LC/MS. Over a million analyses per year are now made. Typically done by immunoassay, this overview describes the procedures for using this highly selective and quantitative LC/MS methodology. In addition, the advantages of using LC/MS (lower cut-offs, no false positives, etc.) are discussed.

Introduction

Today it is mandatory to be able to identify and quantify substances of abuse in biological material. Such methods were developed and are applicable for almost any possible biological matrix.

Traditionally, screening is done by immunology, which is fast and simple, but can be expensive (reagent costs), and normally determines groups of compounds, not specific analytes. Due to its lack of specificity, very often positives must be confirmed, normally by gas chromatography/mass spectrometry (GC/MS). In drug screening, immunology gives a result as “positive” or “negative”, with reference to a certain predetermined cut-off level. Cut-off values for immunoassays are fixed due to optimization of quantity, and tend to be relatively high to avoid bias from interferences. As a result, this gives a high number of false negatives that may have consequences.

A drug screen by liquid chromatography/mass spectrometry (LC/MS) gives a quantitative determination of specific analytes, with known accuracy and precision, within a range of concentrations from 50–100,000 ng/mL. This allows variable cut-off levels for different purposes within the calibrated range. An argument can be made that if a compound is not included in the LC/MS screen it will be missed, and that immunoassay will give a positive in that case because it is a general screen. However, confirmation will show it to be a false positive because the GC/MS confirmation is also a targeted list.

Screening by LC/MS is a new approach compared to immunoassay. LC/MS is also fast, but provides results for specific compounds, not groups. This is important, for example, where a benzodiazepine is legally prescribed, but where a second non-prescribed benzodiazepine is abused. There is no way to account for this with immunoassay; however, intake of other "nonprescribed" benzodiazepines is easily detected by LC/MS screening. A similar argument can be applied to amphetamines and other groups of drugs. As an example, LC/MS screening of amphetamines can differentiate between the fol-lowing analytes: amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA or Ecstasy), methylenediox-amphetamine (MDA), and ephedrine.

LC/MS methods for "new" drugs on the street can be quickly developed, validated, and implemented into the assay within a few days. In the example of amphetamines, other related drugs such as cathinone can be easily and quickly added to the screen. This is not the case for immunoassay, where development of kits for new analytes is a challenging and time consuming procedure. LC/MS is flexible, reliable, and highly sensitive (low nanogram range). As part of its flexibility, note that systems used for other purposes, such as therapeutic drug monitoring (TDM), can also can used for drugs of abuse screening and vice versa [1]. This system flexibility and versatility is an impor-tant feature of the platform and is important both for logistics and maintenance.

LC/MS at St. Olav Hospital

This overview describes the successful use of LC/MS systems at St. Olav Hospital in routine service doing high-volume drug screens from 1998 to the present. Figure 1 shows the increase in the number of analyses performed each year during the period of 1996 to 2003. The first LC/MS was put in service in 1998 and the methodology was fully employed by 1999. The number of analyses for 2004 will approach 1,000,000. Note that because of the graph's scale, the increase in serum analyses cannot be read, but the number of serum determinations is increasing. Serum analyses are approaching 60,000 for this year. Each DOA analysis represents a determination equivalent to an immunoassay for a group of drugs (benzodiapenes, amphetamines, etc.). The actual LC/MS analysis determines specific compounds, and if charted by the compounds analyzed, the total number of analyses would be much higher. LC/MS screening is now performed as a routine service in a restricted area in compliance with national and international guidelines using quality control systems securing all aspects of sample handling, preparation, analysis, and reporting.

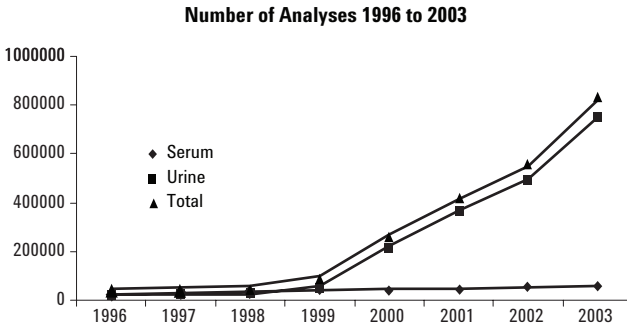

Figure 1. Number of analyses per year for drugs-of-abuse (DOA) from 1996 to 2003. Note that the first LC/MS was purchased in 1998 and LC/MS screening was fully deployed by 1999. The number of analyses represents each group of compounds equivalent to an analysis by immunoassay. In actuality, the analyses are comprised of determinations of individual drugs. The number of determinations made is much greater than indicated by this chart.

Methodology

The LC/MS platform is used for a wide variety of samples ranging from medical treatment of abuse, legal actions and forensic toxicology. Several types of these samples must be confirmed. Because GC/MS is still the accepted "gold standard" confirmation technique for legal action, this is the methodology used here. However, the use of LC/MS screening strongly reduces the number of GC/MS confirmations, a fact that saves both time and money. Comparison of GC/MS and LC/MS results show close to 100%accordance, which means no false positives. In the future, a high-throughput technology such as liquid chromatography/tandem mass spectrometry (LC/MS/MS) in full scan mode, as obtained by ion trap technology, may demonstrate the potential for performing fast confirmation. In combination with LC/MS screening, such a technique would make possible screening and confirmation in less than an hour for single samples, and within a few hours for a larger series of samples.

The systems for DOA screening and TDM use the Agilent 1100 LC/MSD quadrupoles. Presently,

24 instruments are used for these activities. All instruments (both for DOA and TDM) are equipped identically with four mobile phase constituents, using a quaternary system with methanol, acetonitrile, ammonium acetate, and formic acid. For DOA, only two columns are needed, a short C18 and a short CN. This simple strategy gives unique flexibility between instruments and very efficient backup capacity. Finally, the simplified inventory of mobile phases and columns makes fast method development easier.

Amphetamines as an Example

As an example, amphetamines are determined with a short CN column with an isocratic mobile phase (ammonium acetate and acetonitrile). Figure 2 shows amphetamine, methamphetamine, MDA, MDMA, and ephedrine with d_3-amphetamine as the internal standard (ISTD). Target ions and qualifiers are used, and the mass spectrometer is operated using electrospray ionization (ESI). The qualifier ions are obtained by collision-induced dissociation (CID) in the ion transport region of the atmospheric pressure ionization (API) interface, commonly known as "up-front or in-source CID." Note that little chromatographic separation is achieved with the fast run time. However, the single quadrupole mass spectrometer provides sufficient selectivity to separate each compound with quantitative accuracy. The qualifier ions provide additional selectivity to assure confidence in the determination of each compound. With liquid-liquid extraction of the urine samples, sufficient clean up is achieved for the analysis. Even though fast chromatography is used, there is sufficient retention for each of the analytes to be moved from the void of the column.

For complete screening of all categories of drugs of abuse, both ESI and APCI (atmospheric pressure chemical ionization) must be employed. An example of a complete group of DOA compounds best analyzed by APCI is the benzodiazepines. Some of the compounds in this category do respond well to ESI, but others do not. All do respond well to APCI. In this method, a short C18 column with gradient conditions using a mix of methanol, formic acid, and ammonium acetate, provides the best results in a relatively short time.

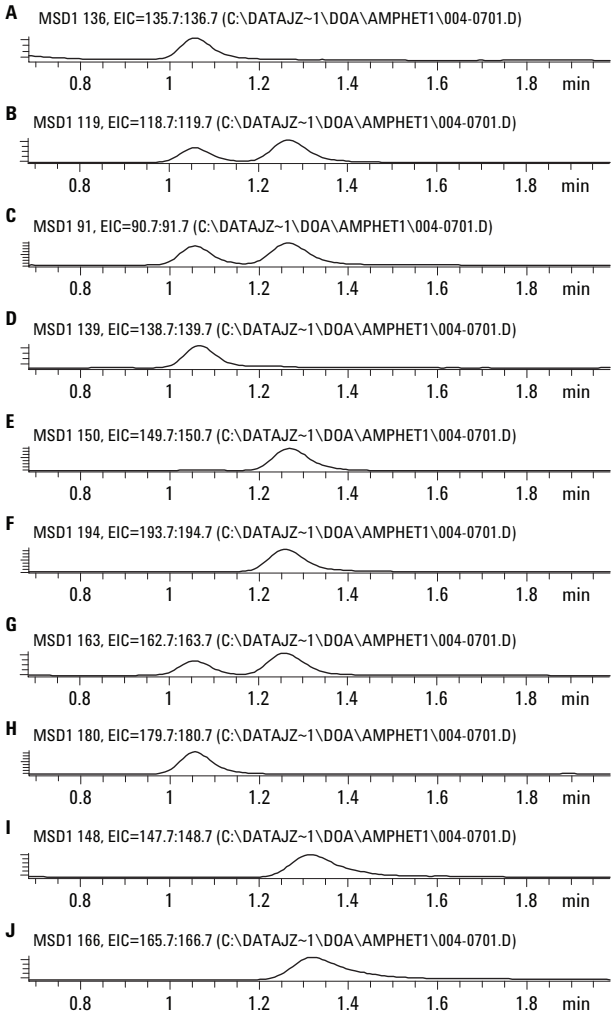

Figure 2. Selected ion monitoring (SIM) chromatograms of amphetamine screen at 100 ng/mL. The panels are A) amphetamine, B) amphetamine and methamphetamine qualifier ion, C) amphetamine and methamphetamine qualifier ion, D) ISTD, E) methamphetamine, F) MDMA, G) MDMA and MDA qualifier ion, H) MDA, I) ephedrine, and J) ephedrine qualifier ion.

Quality Assurance/Quality Control

To obtain the highest quality results, processes must be in place to assure that the instruments are running properly and that all extractions and analyses are done correctly. This assurance is provided by both internal (prepared in the laboratory) and external (obtained by sources outside the laboratory) quality control samples. Every batch of samples analyzed contains the internal quality control samples at concentrations covering the range of concern for the analytes. Table 1 shows typical results obtained for these QC samples. These QC results indicate not only the quality of the determination of each specific target compound, but their concentration as well.

Table 1. Internal QC Results for Some DOA

	QC50	QC100	QC500	QC2000
Amphetamine	48	97	517	2039
Methamphetamine	58	109	533	2049
MDMA	59	112	537	2029
MDA	50	98	517	2140
Ephedrine	56	107	512	2067
Morphine	53	105	526	1993
Codeine	53	108	511	2102
Methadone	53	104	507	2018
Benzoylecognine	59	112	503	2119
Phencyclidine (1/10)	5	10	50	204

Conclusions

The laboratory at St. Olav Hospital routinely analyzed 800,000 DOA urine samples and 30,000 TDM serum samples in 2003, using 24 LC/MS systems. This year the number is approaching 1 million analyses, taking into consideration that, for example, the amphetamine group (with five analytes) is only counted as a single analysis. This is also the case for the benzodiazepines (six analytes) as well as the opiates (four analytes). The accounting scheme is mainly for administrative reasons and for easier comparison with immunology-based laboratories. Twelve systems are set up using ESI and 12 systems using APCI and the instrument configurations are flexible enough to perform both DOA analysis and TDM analyses. The DOA screens include amphetamines, benzodiazepines, opiates, methadone, buprenorphine, PCP, cocaine and its metabolite, barbiturates, and others.

The procedures and the instrumentation briefly described here allow this laboratory to perform these analyses both in a cost-effective way and with the highest quality results possible. In addition, the laboratory uses 10 GC/MS instruments, both for confirmation and unknown compound identification. It should be emphasized that this combination of LC/MS and GC/MS instruments comprise a very strong analytical platform, especially for forensic toxicology. The laboratory performs several thousand analyses per year for these cate-gories. Biological concentrations of specific drugs with secure identification and fast results is of great importance to make assessments toward the dose and state of a subject. This analytical platform for these determinations requires a significant initial capital investment, but the return in both efficiency and medical quality of the results provide justifiable benefits.

Reference

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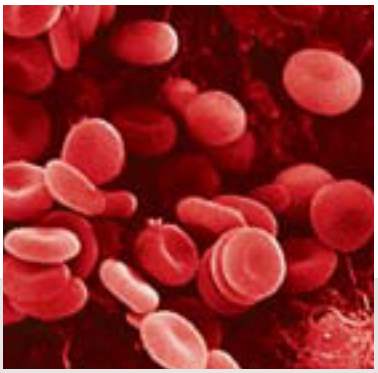

Applications by Technique

LC/TOF & LC/QTOF

Quantitative Analysis of Opiates in Urine Using Accurate Mass LC/MSD TOF

Application Note

Forensic Toxicology

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Abstract

Urine samples were quantitatively analyzed at the 6 ng/mL level using liquid chromatography/mass selective detector time-of-flight. The advantage of accurate mass measurement to enhance selectivity is presented. The instrumental detection limit is 2 pg on-column with a signal/noise ratio of 5:1.

Introduction

Until now quantitative time-of-flight (TOF) has not been broadly applicable. This application demonstrates that the Agilent liquid chromatography/mass selective detector time-of-flight (LC/MSD TOF) can routinely quantify compounds at low levels in matrices important to the forensic scientist. Both direct injection of urine and solid phase extraction (SPE) are performed to demonstrate the robustness, sensitivity, and selectivity of the LC/MSD TOF.

Experimental

Sample preparation

Direct injection samples were spiked at the specified concentrations with no further handling. Accubond II Evidex SPE Cartridges (part number 188-2946) were used as per extraction protocol for opiates (see step-by-step instructions that comes with cartridges). Five milliliters of either blank or spiked urine was treated with 0.5 mL concentrated HCl, 0.75 mL 10 N NaOH, and then adjusted to pH 6.5-7.5 with 2.5 mL 0.5 M phosphoric acid. The heating step was not included because acid hydrolysis of glucuronides were not expected. After conditioning, this solution was loaded onto the cartridge, rinsed, and then eluted with the prescribed solution of methylene chloride/isopropanol/ammonium hydroxide. The eluant was taken to dryness with nitrogen (no heat) and then reconstituted in 0.5 mL 40:60 water:acetonitrile.

Instrument

Agilent 1100 Series LC/MSD TOF with Agilent 1100 binary pump and well plate autosampler

Table 1 Experimental Conditions

LC Conditions	
Column	ZORBAX XDB-C18, 2.1 mm × 50 mm, 3.5 µm P/N 971700-902
Mobile Phases	A: Acetonitrile with 0.1 % formic acid B: Water with 0.1 % formic acid
Gradient	35% to 95% A in 5 min, then to 100% in 6 min
Flow rate:	0.35 mL/min

MS Conditions

Standard autotune conditions with calibrant delivery system providing constant low flow of ~2 µM purine and HP-921 calibrant to dual ESI for continuous auto-calibration

Results

Shown in Figure 1 (upper panel) is the total ion chromatogram (TIC) and in the lower panel, overlaid extracted ion chromatograms (EICs) for morphine, codeine, and acetylmorphine, in a direct injection of urine at 300 ng/mL. The EIC has a mass window of 20 ppm (~ ±0.002 u). Accurate mass spectra for these opiates is given in Figure 2. Table 1 shows the quantitative results obtained with direct injection. Table 2 shows the results obtained with the solid phase extraction (SPE).

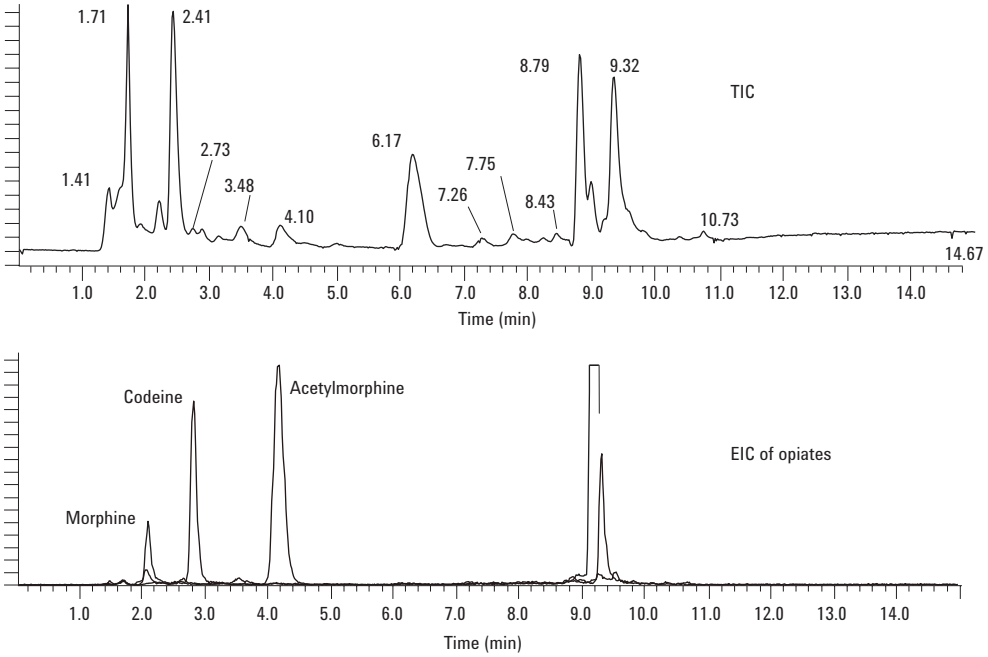

Figure 1. The upper panel shows the TIC of a direct urine injection spiked with 300 ng/mL of each opiate. The lower panel shows the EIC of each compound.

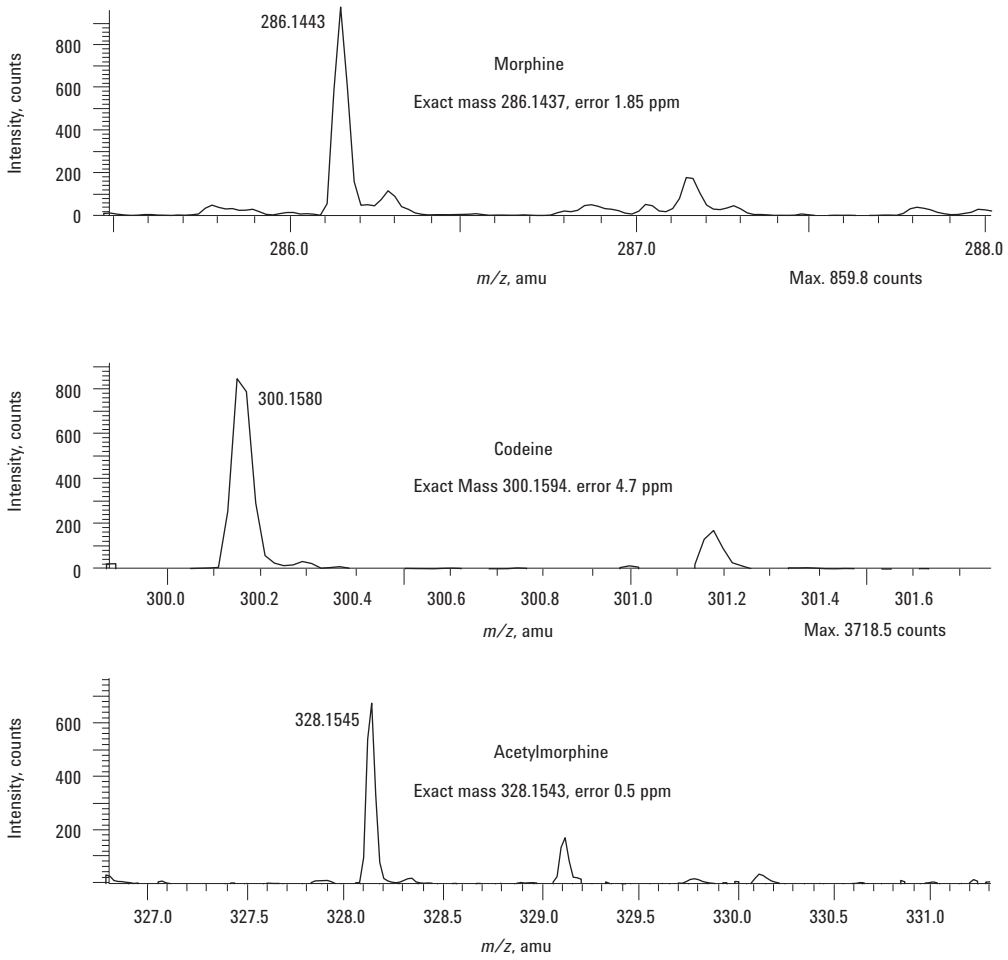

Figure 2. Mass spectra of M+H ions for opiates showing both mass resolution and mass accuracy at 2 pg on-column.

Table 2. Quantitative results (in ng/mL) of spikes at 1000 ng/mL and 300 ng/mL obtained by LC/MSD TOF direct injection of urine.

Urine direct injection (Spike 1000 ng/mL)				Urine direct injection (Spike 300 ng/mL)			
	Morphine	**Codeine**	**Acetylmorphine**		**Morphine**	**Codeine**	**Acetylmorphine**
	241	446	715		66.7	93.8	176
	222	402	653		78.5	94.8	203
	238	426	683		73.7	93	199
	195	338	687		73.7	96.5	201
	200	351	588		76.6	94.3	185
Mean	219.2	392.6	665.2	**Mean**	73.8	94.5	192.8
SD	21.2	46.8	48.4	**SD**	4.5	1.3	11.8
RSD (%)	**9.7**	**11.9**	**7.3**	**RSD (%)**	**6.1**	**1.4**	**6.1**

These are typical concentration and cut-off range of immunoassay. Note that difference between spiked value and measured concentration represents degree of ion suppression at source.

Table 3. Quantitative results of spikes at 6 ng/mL and 60 ng/mL obtained by LC/MSD TOF with Accubond Evidex SPE sample preparation.

Accubond Evidex 5 mL Urine (Spike 6 ng/mL) Expected Conc. 60 pg/µL				Accubond Evidex 5 mL Urine (Spike 60 ng/mL) Expected Conc. 600 pg/µL			
	Morphine	**Codeine**	**Acetylmorphine**		**Morphine**	**Codeine**	**Acetylmorphine**
	6.97	8.62	3.74		508	499	182
	8.56	9.57	4.21		567	543	193
	10	8.41	4.03		525	504	183
	9.24	8.5	3.81		521	502	191
	7.07	8.15	3.48		595	532	193
	9.46	8.99	3.5		591	532	192
	7.66	8.91	3.79		582	540	196
Mean	8.4	8.7	3.8	**Mean**	555.6	521.7	190.0
SD	1.2	0.5	0.3	**SD**	36.6	19.2	5.4
RSD (%)	**14.4**	**5.3**	**6.9**	**RSD (%)**	**6.6**	**3.7**	**2.8**

Difference in spiked value and measured concentration represents both recovery of SPE method and ion suppression (if any).

Conclusions

The data shown demonstrates the ability of LC/MSD TOF to confirm - with accurate mass measurement, and quantify- with selective narrow mass window.

- Direct injection of urine shows the robustness of the LC/MSD TOF.
- Typical clean-up (SPE) shows excellent sensitivity.
- High-mass resolution and accuracy (of every spectrum) provides the selectivity for reduction of chemical noise for quantitation and confirmation.

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Agilent Technologies

An Application Kit for the Screening of Samples for Analytes of Forensic Toxicological Interest using TOF or Q-TOF LC/MS with a Personal Forensic Toxicology Database

Application Note

Forensic Toxicology

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Abstract

A Forensic Toxicological screening application kit has been developed for use with the Agilent TOF and Q-TOF Mass Spectrometers which contains an accurate mass database with a content of around 6700 analytes. The aim of the MassHunter Personal Forensic Toxicology Database Kit is to provide a user with a sufficient starting point for the analysis of samples for which the ability to detect and identify from a large array of forensic toxicological analytes is necessary. The combined system allows the user to create custom databases containing retention times of compounds of interest for smaller and more specific suites of analytes according to specific requirements. A test mix containing analytes of forensic interest, to demonstrate the functionality of the MassHunter Personal Forensic Toxicology Database Kit, together with an example of a general screening method for common drugs of abuse is provided.

Agilent Technologies

Introduction

The application of high definition accurate mass spectrometers, such as time-of-flight (TOF) and quadrupole time-of-flight (Q-TOF), to screening, discovery and confirmation in the areas of forensic toxicology has become more desirable given the indiscriminant and non-targeted nature of their full spectral data capture. Indeed, given the highly accurate and sensitive mass measurement of modern TOF and Q-TOF instruments (sub 2-ppm mass accuracy, pg on-column sensitivity and high resolution) in combination with powerful software data mining tools, post acquisition screening techniques are easier to perform reliably with a higher number of analytes in one analytical method. The lists of potential toxins are large and typically depend on the area of analytical focus such as workplace drug testing, doping control, post-mortem toxicology, or explosives.

Accurate single-stage mass spectrometry (MS) mass measurements identify monoisotopic adducts to a high confirmatory degree, and databases can be built to accommodate various suites of forensic toxicological analytes of interest. They are obtained from both TOF and Q-TOF LC/MS instru-ments. In contrast LC/MS/MS with a triple quadrupole MS in its most sensitive mode, multi-reaction monitoring (MRM), provides targeted screening and confirmation only.[1]

This application note describes the Agilent MassHunter Personal Forensic Toxicology Database Kit for Forensic Toxicological Screening and Identification which contains the accurate mass (AM) details for around 6700 analytes of forensic toxicological interest. The content was gathered upon advice from many leading institutions and knowledge bases world-wide and contains information such as common names, monoisotopic mass, compound formulas, CAS & Chemspider IDs, chemical structure and in most cases the IUPAC nomenclature. In addition to accurate mass, the ability to add retention time for a chromatographic method to every analyte for extra search confirmation is a built-in functionality of the MassHunter Personal Compound and Library (PCDL) program interfaces. This allows accurate mass retention time (AMRT) data mining routines. Furthermore, an analyst can use the database content 'as is' for non-targeted screening or create smaller custom and more targeted databases from the read-only supplied database. Custom databases can be edited by changing entries, adding, and deleting entries and semi-automatically updating retention times for particular analytes and methods. [2] The analyst can create as many custom databases with LC-dependent retention times as needed.

This application note describes the typical use of the MassHunter Personal Forensic Toxicology Database Kit through a few analytical screening work flow examples.

Experimental

The analysis results outlined in this application note were obtained using an Agilent 6230 Time-of-Flight LC/MS coupled to an Agilent 1200 SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter Workstation equipped with the [G6855AA] MassHunter Personal Forensic Toxicology Database Kit.

Sample preparation

An ampoule from the LC/MS Toxicology Test Mix [p/n 5190-0470] which is included in the MassHunter Personal Forensic Toxicology Database Kit [G6855AA] was opened and 10 µL of the 1 µg/mL (1 ppm) solution was diluted to a concentra-tion of 100 ng/mL (100 ppb) using 990 µl of pure LC/MS grade methanol to create a clean solvent standard for method checkout purposes.

Table 1 outlines the composition of the LC/MS Toxicology Test Mix [p/n 5190-0470] which is intended to cover a wide and representative range of forensic analyte classes.

Table 1. LC/MS Toxicology Test Mix components (1 µg/ml)

Compound Name	Formula	Mass
3,4-Methylendioxyamphetamine (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
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
Methylendioxymethamphetamine (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

Reagents and chemicals

Burdick & Jackson LC/MS grade acetonitrile together with de-ionized water (locally produced 18.1 MΩ) were used for mobile phases. Buffers were freshly prepared using a high purity source of formic acid and ammonium formate.

Instrument settings and MS acquisition method parameters

LC conditions

Column:	Zorbax Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm [p/n - 959764-902]
Column Temperature:	60 °C
Mobile Phase	A: 5 mM NH_4 formate/0.01% Formic acid in water B: 0.01% formic acid in acetonitrile
Flow Rate:	0.5 ml/min

Gradient program:

Time	A	B	Flow rate
Initial	90%	10%	0.5 ml/min
0.5 min	85%	15%	0.5 ml/min
3.0 min	50%	50%	0.5 ml/min
4.0min	5%	95%	0.5 ml/min
6.0min	5%	95%	0.5 ml/min

Injection volume:	1 µL (with 5 second needle wash in flushport)
Analysis time:	6.0 min
Post Time:	2.0 min
Overall Cycle time:	8.0 min

6230 TOF MS conditions

Source conditions:

Electrospray AP-ESI (using Agilent Jet Stream Technology):

Positive ionization polarity	
Sheath gas temperature and flow:	380°C, 12 L/min
Nozzle voltage:	500 V
Drying gas temperature and flow:	320°C, 8 L/min
Nebulizer gas pressure:	27 psi
Capillary voltage:	3750 V
Fragmentor voltage:	150 V

Electrospray AP-ESI:

Positive ionization polarity	
Drying gas temperature and flow:	350°C, 12 L/min
Nebulizer gas pressure:	30 psi
Capillary voltage:	2000 V
Fragmentor voltage:	150 V

MS acquisition method parameters:

Reference ion mass enabled:	121.050873, 922.009798
Acquisition mode:	MS1
Minimum mass value:	50 *m/z*
Maximum mass value:	1050 *m/z*
Scan rate:	3 Hz

All other instrument operating parameters were taken care of by Agilent's autotune functionality and subsequent mass calibration using standard settings.

Results and discussion

Fast and easy start up with Agilent LC/MS Toxicology Test Mix

The LC/MS Toxicology Test Mix [p/n 5190-0470] is included in the MassHunter Personal Forensic Toxicology Database Kit [G6855AA] to rapidly implement the method and verify that acquisition and data analysis methodology is correctly set up. The LC/MS Toxicology Test Mix contains a representative range of components from 25 forensic analyte classes. (See Table 1). MS screening depends on accurate mass results from the TOF or Q-TOF. Therefore, the use of appropriate reference ions as outlined in the 'Experimental conditions' section obtains the most accurate results.

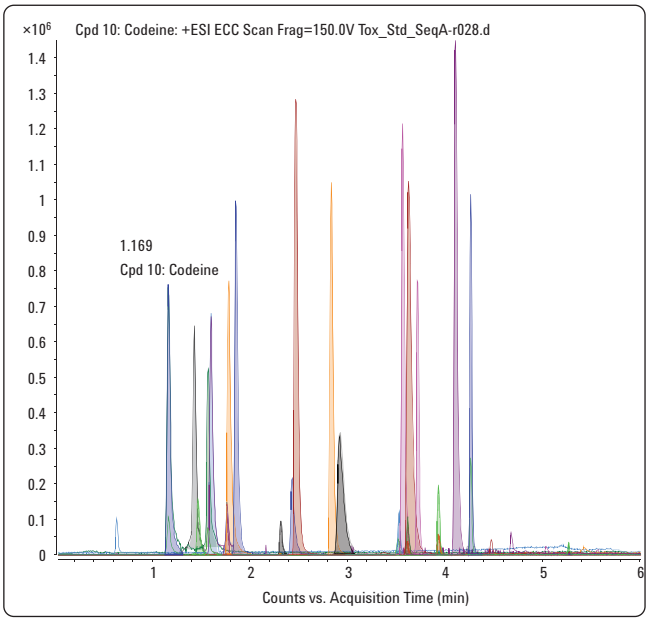


Figure 1. Extracted compound chromatogram of LC/MS Toxicology Test Mix.

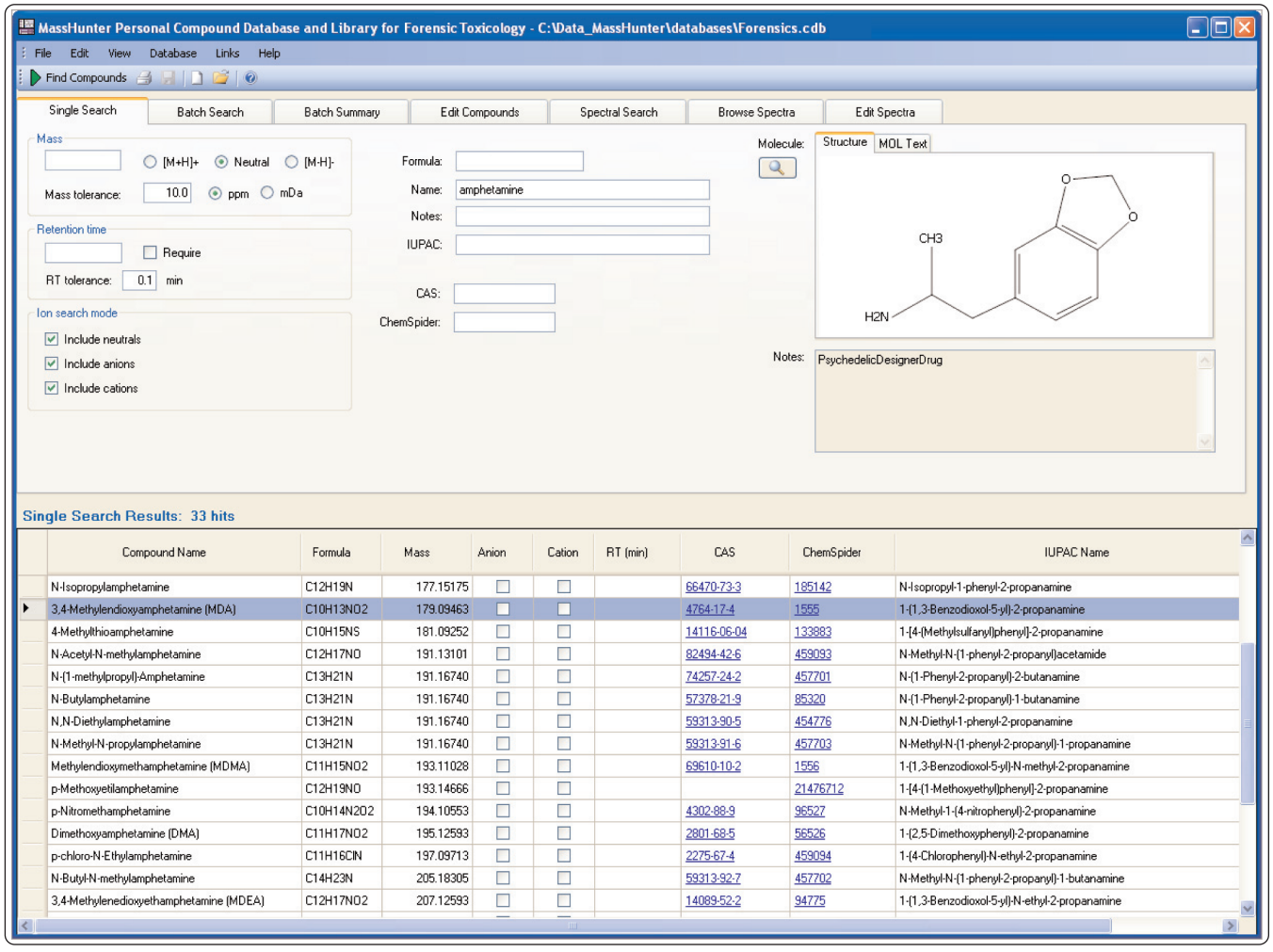
In compliance with the methodology outlined in the experimental section, a 1-µL injection of the 100 ng/mL LC/MS Toxicology Test Mix equates to a 100 pg on-column injection amount. Figure 1 shows an overlay of the expected extracted compound chromatograms for the LC/MS Toxicology Test Mix. A standard method is included for TOF and Q-TOF as part of the MassHunter Personal Forensic Toxicology Database Kit. These can be loaded so that all conditions are correct and the user can reproduce the analysis.

These methods are acquisition only methods and correspond to the instrument configuration as outlined in the experimental section of this application note. Appropriate settings must be manually input if a different instrument configuration is used. Similar results will demonstrate that the system is working properly.

Personal Compound Database and Library (PCDL) Software interface

Outline

An 'open database' dialog box appears after invoking the PCDL interface from the desktop icon. It is best to choose the pre-installed Forensic.cdb from the MassHunter\database directory. Figure 2 illustrates the single search view of the software interface. The screen shows a list of search results for 'amphetamine'. There are seven views available to the user, however, for the scope of this application note, only the first four (tabs to the left) that are directly applicable to AMRT functionality will be described. These views are switched on this flat user interface by clicking on the appropriate tab: Single Search, Batch Search, Batch Summary, or Edit Compounds.



Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name
N-Isopropylamphetamine	C12H19N	177.15175	☐	☐		66470-73-3	185142	N-Isopropyl-1-phenyl-2-propanamine
3,4-Methylendioxyamphetamine (MDA)	C10H13NO2	179.09463	☐	☐		4764-17-4	1555	1-(1,3-Benzodioxol-5-yl)-2-propanamine
4-Methylthioamphetamine	C10H15NS	181.09252	☐	☐		14116-06-04	133883	1-[4-(Methylsulfanyl)phenyl]-2-propanamine
N-Acetyl-N-methylamphetamine	C12H17NO	191.13101	☐	☐		82494-42-6	459093	N-Methyl-N-(1-phenyl-2-propanyl)acetamide
N-(1-methylpropyl)-Amphetamine	C13H21N	191.16740	☐	☐		74257-24-2	457701	N-(1-Phenyl-2-propanyl)-2-butanamine
N-Butylamphetamine	C13H21N	191.16740	☐	☐		57378-21-9	85320	N-(1-Phenyl-2-propanyl)-1-butanamine
N,N-Diethylamphetamine	C13H21N	191.16740	☐	☐		5931 3-90-5	454776	N,N-Diethyl-1-phenyl-2-propanamine
N-Methyl-N-propylamphetamine	C13H21N	191.16740	☐	☐		5931 3-91-6	457703	N-Methyl-N-(1-phenyl-2-propanyl)-1-propanamine
Methylendioxymethamphetamine (MDMA)	C11H15NO2	193.11028	☐	☐		69610-10-2	1556	1-(1,3-Benzodioxol-5-yl)-N-methyl-2-propanamine
p-Methoxyetilamphetamine	C12H19NO	193.14666	☐	☐			21476712	1-[4-(1-Methoxyethyl)phenyl]-2-propanamine
p-Nitromethamphetamine	C10H14N2O2	194.10553	☐	☐		4302-88-9	96527	N-Methyl-1-(4-nitrophenyl)-2-propanamine
Dimethoxyamphetamine (DMA)	C11H17NO2	195.12593	☐	☐		2801-68-5	56526	1-(2,5-Dimethoxyphenyl)-2-propanamine
p-chloro-N-Ethylamphetamine	C11H16ClN	197.09713	☐	☐		2275-67-4	459094	1-(4-Chlorophenyl)-N-ethyl-2-propanamine
N-Butyl-N-methylamphetamine	C14H23N	205.18305	☐	☐		5931 3-92-7	457702	N-Methyl-N-(1-phenyl-2-propanyl)-1-butanamine
3,4-Methylenedioxyethamphetamine (MDEA)	C12H17NO2	207.12593	☐	☐		14089-52-2	94775	1-(1,3-Benzodioxol-5-yl)-N-ethyl-2-propanamine

Figure 2 Single Manual Search view of the PCDL software interface.

Any field or combination of fields in the upper portion of the Single Search tab (Figure 2.) can be used to manually search the loaded database. Table 2 lists all available search fields from the PCDL single search view. The powerful search algorithm also handles partial names (eg. 'amph' will return all database entries containing this letter string.)

Note: To view the entire contents of the loaded database, a single search invoked with all empty search fields will allow the user to display the entire database content.

Table 2. All available search fields for PCDL single search.

Search Fields Available (Single Search View)	Value
Mass	Measured mass (*m/z*)
Retention time	(minutes)
Formula	Empirical Formula
Name	Common name of compound (or part thereof)
Notes	Compound class or description
IUPAC	IUPAC or commonly recognized compound name
CAS	Unique CAS number
ChemSpider	Unique ChemSpider ID

Workflow A. Manual (Single Mass Search)

Using PCDL Program

Single search would normally be used manually by obtaining a measured mass from a measured or observed spectrum in MassHunter Qualitative Analysis program and typing it in to the mass search field. Figure 3 illustrates this manual application of the MassHunter Qualitative Analysis program and PCDL single search capability for observed masses.

In this example, a compound peak was identified in MassHunter Qualitative Analysis program from positive polarity TOF data, the spectrum was extracted, and the observed mass of 244.205770 *m/z* was searched against the PCDL database (including cations) for [M+H]+ adducts using a mass tolerance of 10 ppm.

The search returns an accurate mass match with phencyclidine (PCP) and with a mass deviation (or delta mass) of 0.85 ppm between the measured and theoretical database values.

More detailed information of single search capability can be found in Agilent G6855AA MassHunter Personal Forensic Toxicology Database and Kit Quick Start Guides [3,4].

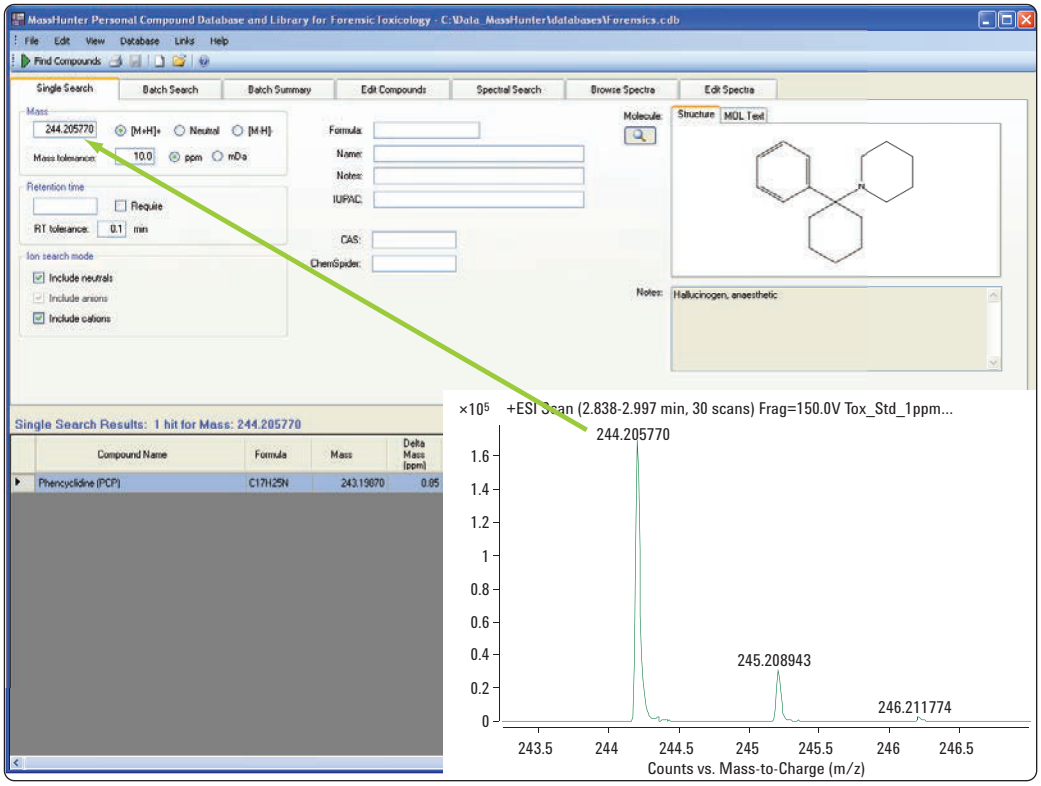


Figure 3. Manual search of observed mass.

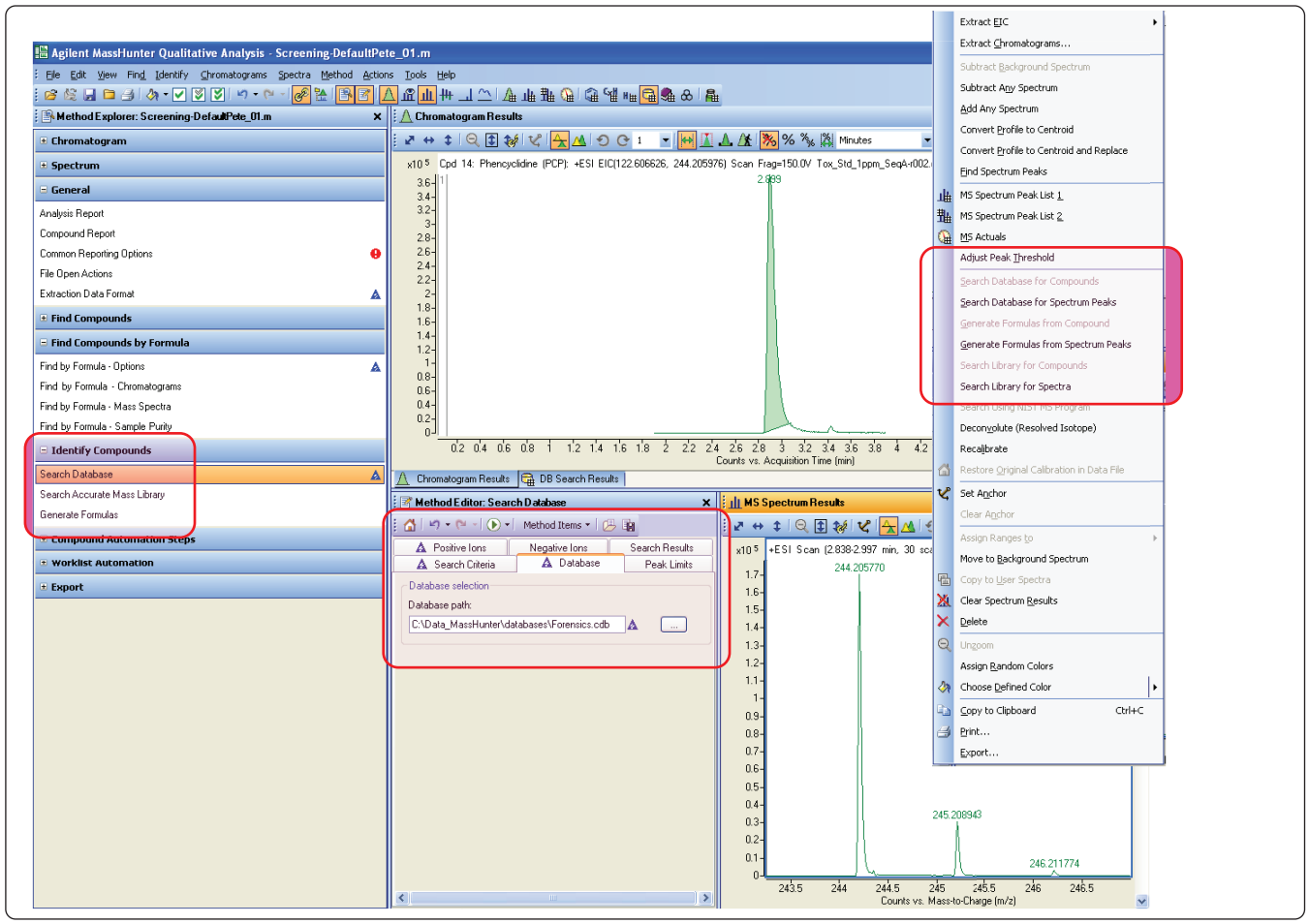


Figure 4a. Manual Search of observed mass using MassHunter Qualitative Analysis program.

Single manual search of database using MassHunter Qualitative Analysis program.

To obtain a seamless single spectral peak database search via MassHunter Qualitative Analysis program, the database must be specified in the qualitative analysis method editor. Compatible software versions are B.03.01 or higher. Figures 4a through 4d illustrate the settings used for this example.

Figure 4a shows the typical MassHunter Qualitative Analysis program view containing the chromatographic peak in question together with its manually extracted spectrum. On the left side of the screen shot, the 'Identify Compounds' method explorer options have been expanded and the 'Search Database' method editor was selected. In the method editor, the required AMRT database was specified as 'forensic.cdb'.

Figure 4b shows the mass tolerance window and the search criteria that can be selected, such as 'mass only' or 'mass with retention time'.

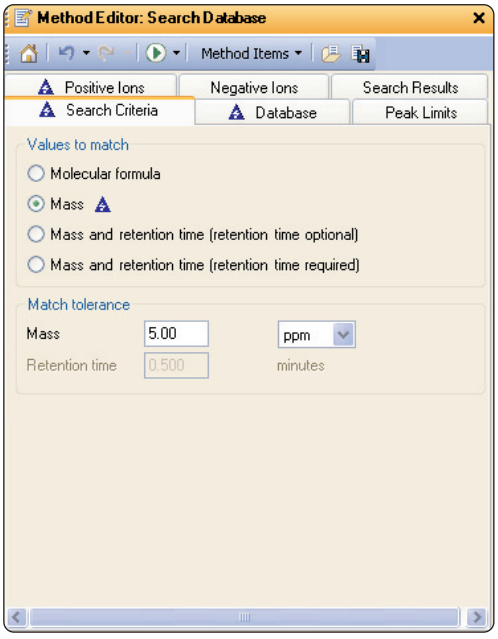


Figure 4b. Manual Search Criteria Settings.

Figure 4c illustrates more adduct and charge state options required for the database search.

Right-click in the spectrum window and a shortcut menu appears against the specified AMRT database (Figure 4a.) This menu has various options including 'Search database for spectrum peaks'. Selection of this option automatically invokes the database search. In Figure 4d the spectrum peak has been identified as PCP, with 0.87 ppm mass deviation and a spectral combined score of 99.36 out of 100 indicating extra confirmation of identity.

To calculate this score, three distinct score components were considered: Mass Match, Abundance Match, and Spacing Match with values of 99.61, 98.61, and 99.79, respectively. These are individually displayed in Figure 4d.

For trustworthy results, the software scores the database matches based on the similarity of each of the isotopic masses (Mass Match), isotope ratios (Abund Match), isotope spacing (Spacing Match), and optionally the retention time (RT Match).

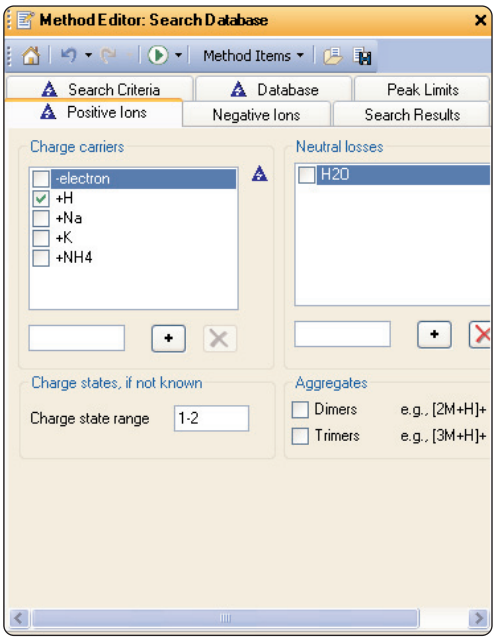


Figure 4c. Manual Search Adduct Selection.

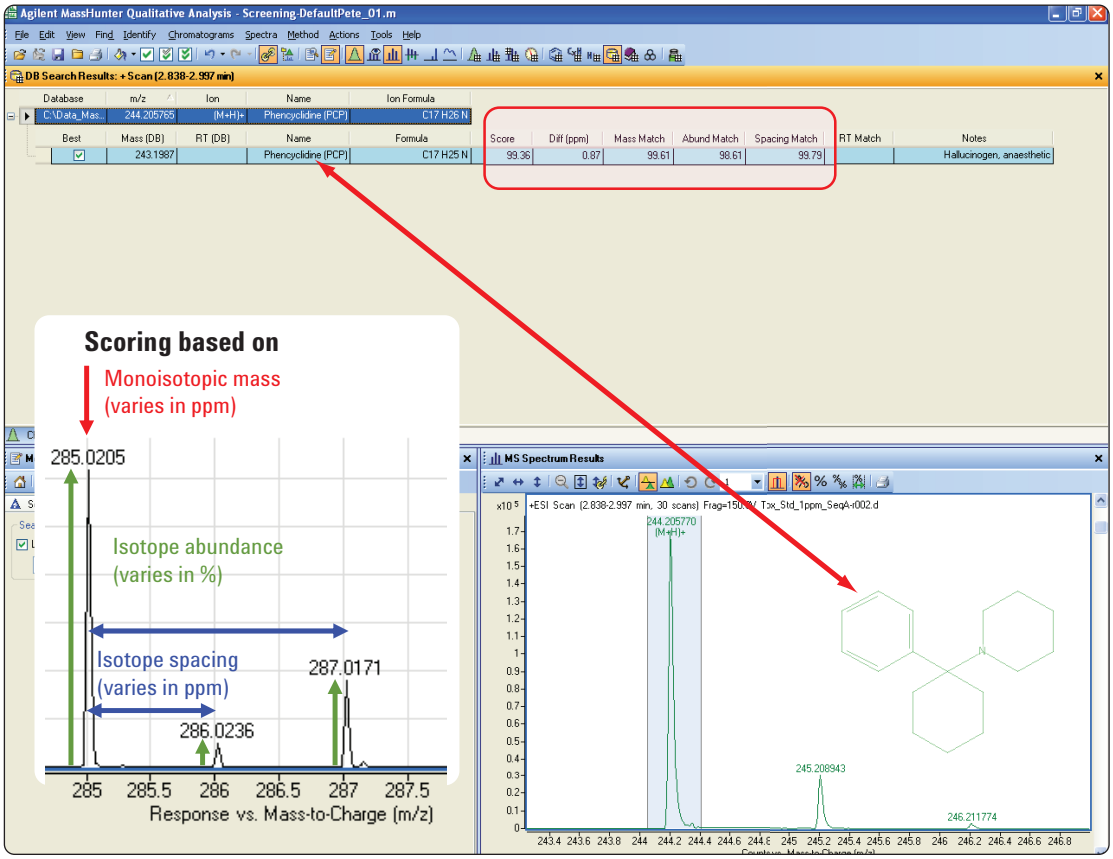


Figure 4d. Manual Database Search Results using MassHunter Qualitative Analysis program.

Isotope spacing is another important component of the scoring algorithm. The mass spacing from the M to the M+1 and M+2 isotopes can be measured with low-ppm accuracy. Any small mass shifts affect all isotopes equally, so this measurement is independent of overall mass axis shifts. This is outlined graphically in Figure 4d.

In this example, a single AMRT database result of phencyclidine (PCP) was returned, together with its structure which is optionally overlaid on the peak spectrum as shown in Figure 4d and can be displayed if selected in the reporting options.

More detailed information about MassHunter Qualitative analysis program database searching can be found in the MassHunter Qualitative Analysis Program Help Files or user guides [5].

Workflow B. Data mining using 'Molecular Feature Extractor' (MFE)

Batch PCDL searches (tabs 2 & 3) are designed for database searching and identification using an accurate mass list created from an automated data mining algorithm such as the Agilent Molecular feature extractor (MFE.) Such algorithms are extremely powerful, especially with complex data derived from difficult sample matrices, such as blood extracts. For the remainder of this application note, only batch searches invoked from inside the MassHunter Qualitative Analysis program interface will be outlined and described. For information on how to perform batch searches within the PCDL interface, please refer to the PCD application note [2].

Data mining algorithms such as MFE automatically search and 'mine' complex sets of single-stage MS data to determine and distinguish most likely and 'real' compound peaks from continuous background interferences. Combinations of adducts can be selected as part of the compound identification protocol to provide added assurance of compound validity.

Other data mining algorithms such as 'find by MS/MS' and 'find by Targeted MS/MS' are integral options included as part of the MassHunter Qualitative Analysis program software. The algorithms are dependent on the mode of operation and nature of the instrument being used. 'Find by Formula' compound search routines are described in the 'Workflow C' section of this application note.

For illustrative purposes, the LC/MS Toxicology Test Mix was analyzed under the conditions outlined in the experimental section. The data file was loaded into MassHunter Qualitative Analysis program. The 'Find by Molecular Feature' method editor was opened under the method explorer in the 'Find Compounds' section (see Figures 5a & 5b).

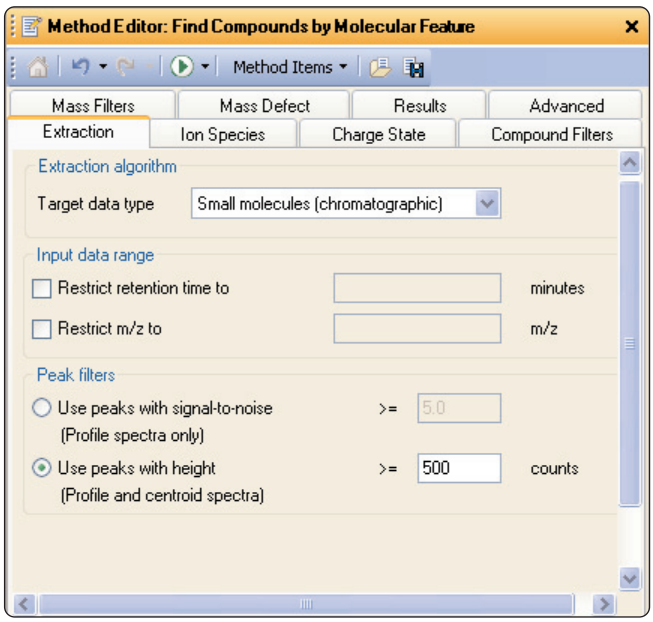


Figure 5a. MFE extraction parameters.

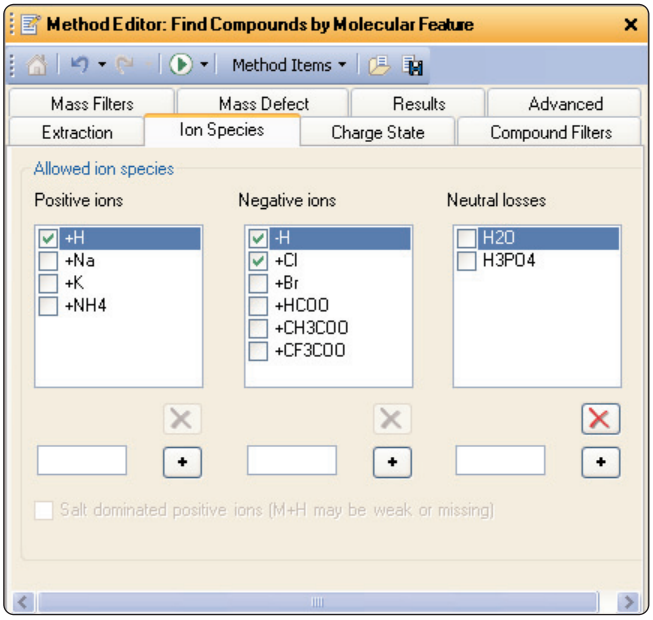


Figure 5b. MFE ion species setup.

A very aggressive setting of absolute peak height threshold (>500 counts) was used in this example (see Figure 5a), together with the small molecules algorithm (chromatographic) which yielded over 3000 possible compound hits. By raising this threshold amount, less abundant analytes may remain undetected. Conversely with a higher threshold the number of potential false positives are greatly reduced. Only [M+H]+ adducts were searched in this instance, however,

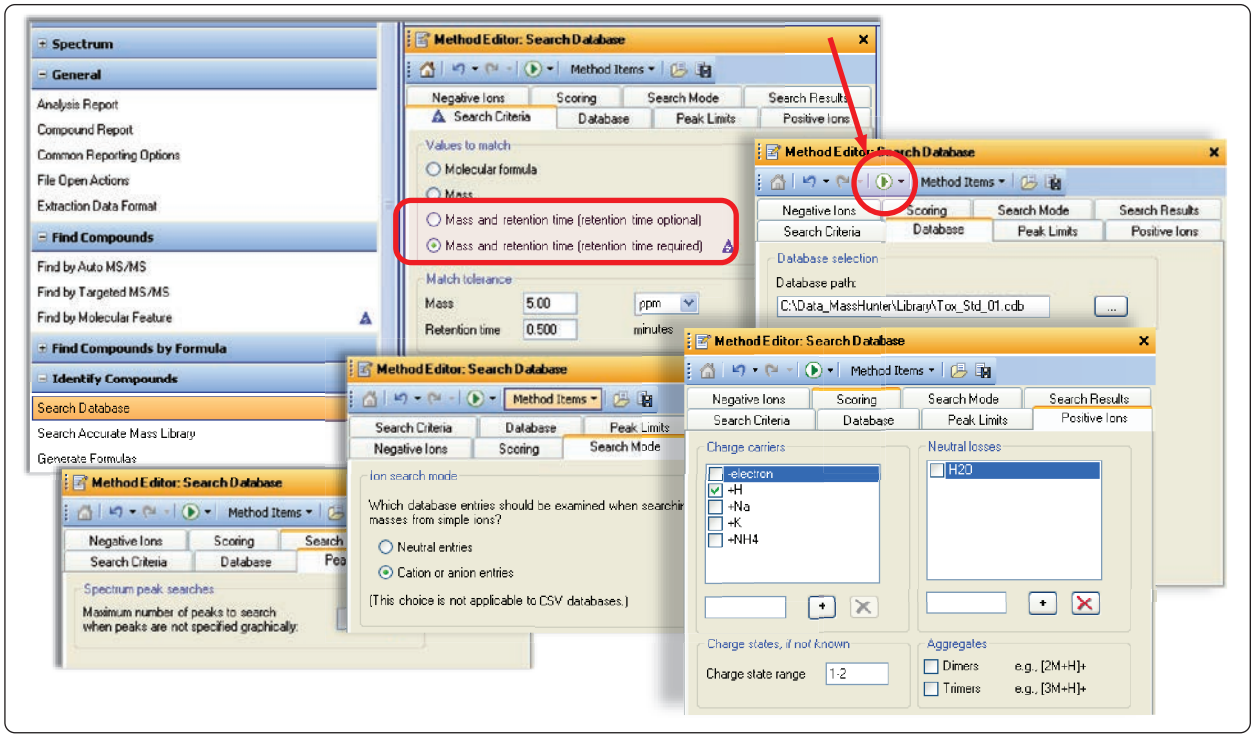


Figure 6. MFE compound database search settings.

further confidence could have been sought (see Figure 5b) by choosing additional adducts such as Na+ and NH4+.

No compound, mass filters or mass defect filters were specified for this search and a maximum charge state of 1 was specified in the MFE method setup. The next step after MFE search was to specify the forensic AMRT database (see Figure 6) in the identify compound/search database method editor, highlight all of the MFE-found compounds and search each compound against its content. A mass and retention time (RT) match was specified, since RT database values had already been pre-determined by analyzing individual standards and inserted into a customized compound database.

Figure 7 illustrates the results obtained from the MFE operation invoked by pressing the green 'process' button highlighted in the title bar of the MFE method editor (Figure 6).

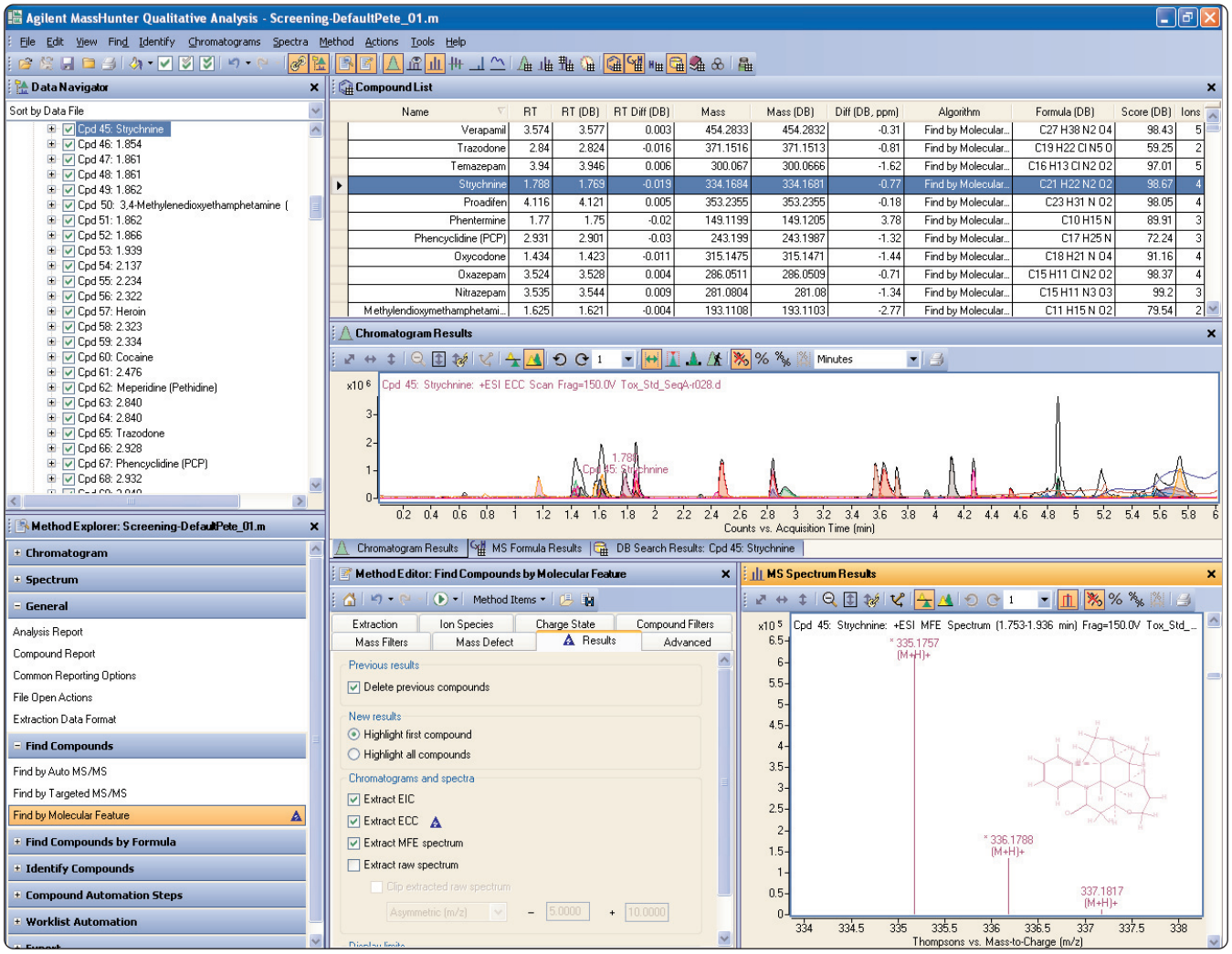


Figure 7. MFE compound database search results using MassHunter Qualitative Analysis program.

These results are detailed in Table 3 and show that all 25 compounds of the LC/MS Toxicology Test Mix were identified for this sample injection. This confirms that the data analysis settings for the find and identify steps are appropriate for the identification process. Many of the 3000+ compounds identified by MFE did not find any PCDL matches as expected and the data analysis option of excluding non-positives was used to report only the database hits.

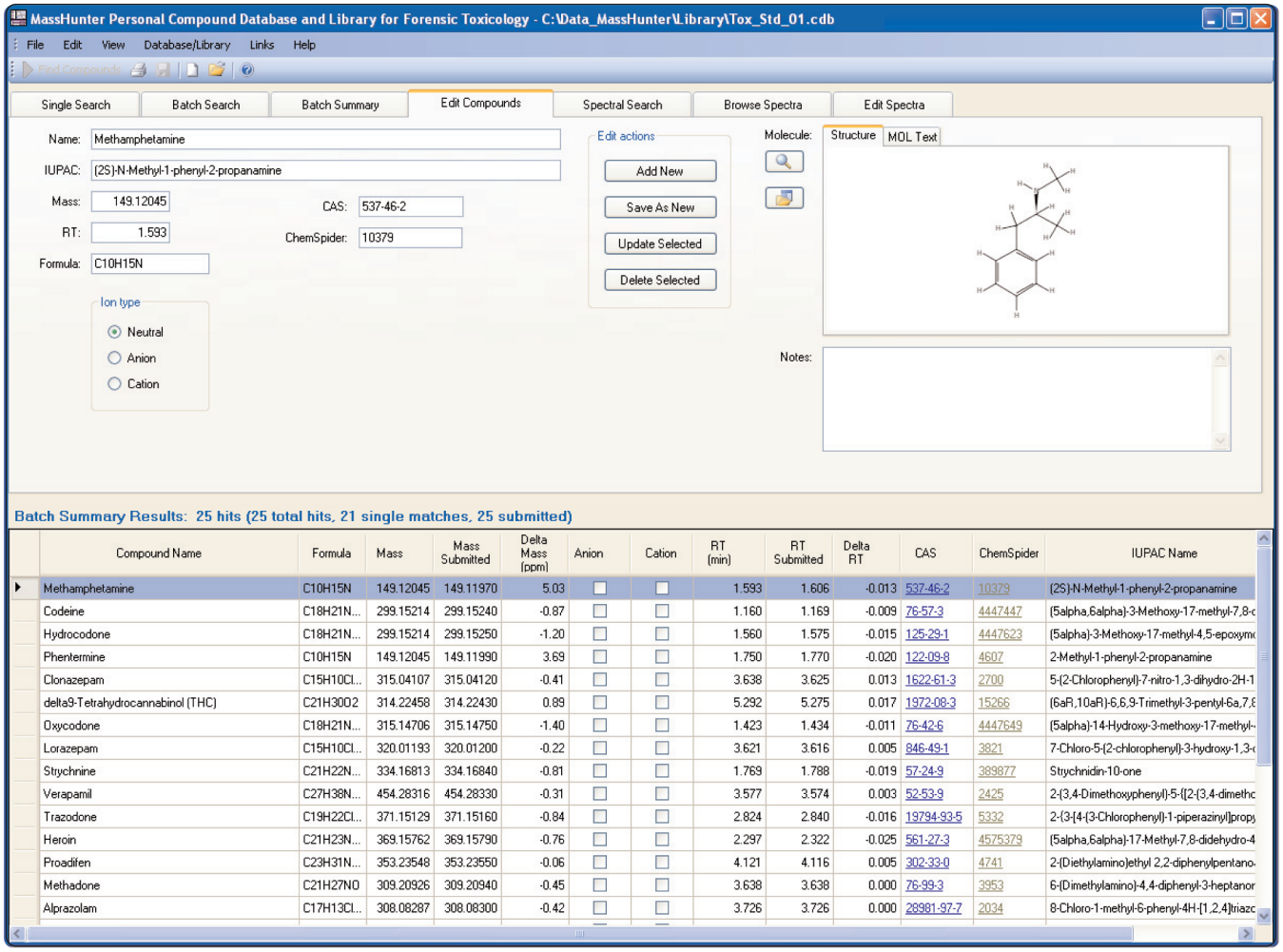
Isobaric compounds such as codeine/hydrocodone and methamphetamine/phentermine were also correctly identified and distinguished automatically, by using the retention capability of the PCDL database and by inputting the predetermined retention time of each analyte for this chromatographic methodology as outlined in the Agilent G6855AA MassHunter Personal Forensic Toxicology Database Quick Start Guide [3].

Table 3. MFE compound and database search results.

Name	RT	RT (DB)	RT Diff (DB)	Mass	Mass (DB)	Diff (DB, ppm)	Formula (DB)	Score (DB)
Verapamil	3.574	3.577	0.003	454.2833	454.2832	-0.31	$C_{27}H_{38}N_2O_4$	98.43
Trazodone	2.84	2.824	-0.016	371.1516	371.1513	-0.81	$C_{19}H_{22}ClN_5O$	59.25
Temazepam	3.94	3.946	0.006	300.067	300.0666	-1.62	$C_{16}H_{13}ClN_2O_2$	97.01
Strychnine	1.788	1.769	-0.019	334.1684	334.1681	-0.77	$C_{21}H_{22}N_2O_2$	98.67
Proadifen	4.116	4.121	0.005	353.2355	353.2355	-0.18	$C_{23}H_{31}NO_2$	98.05
Phentermine	1.77	1.75	-0.02	149.1199	149.1205	3.78	$C_{10}H_{15}N$	89.91
Phencyclidine (PCP)	2.931	2.901	-0.03	243.199	243.1987	-1.32	$C_{17}H_{25}N$	72.24
Oxycodone	1.434	1.423	-0.011	315.1475	315.1471	-1.44	$C_{18}H_{21}NO_4$	91.16
Oxazepam	3.524	3.528	0.004	286.0511	286.0509	-0.71	$C_{15}H_{11}ClN_2O_2$	98.37
Nitrazepam	3.535	3.544	0.009	281.0804	281.08	-1.34	$C_{15}H_{11}N_3O_3$	99.2
Methylendioxymethamphetamine (MDMA)	1.625	1.621	-0.004	193.1108	193.1103	-2.77	$C_{11}H_{15}NO_2$	79.54
Methamphetamine	1.606	1.593	-0.013	149.1197	149.1205	4.82	$C_{10}H_{15}N$	81.88
Methadone	3.638	3.638	0	309.2094	309.2093	-0.61	$C_{21}H_{27}NO$	99.67
Meperidine (Pethidine)	2.477	2.456	-0.021	247.1577	247.1572	-1.7	$C_{15}H_{21}NO_2$	97.91
Lorazepam	3.616	3.621	0.005	320.012	320.0119	-0.19	$C_{15}H_{10}Cl_2N_2O_2$	98.27
Hydrocodone	1.575	1.56	-0.015	299.1525	299.1521	-1.2	$C_{18}H_{21}NO_3$	85.2
Heroin	2.322	2.297	-0.025	369.1579	369.1576	-0.63	$C_{21}H_{23}NO_5$	98.97
Diazepam	4.272	4.275	0.003	284.072	284.0716	-1.36	$C_{16}H_{13}ClN_2O$	58.97
delta9-Tetrahydrocannabinol (THC)	5.275	5.292	0.017	314.2243	314.2246	0.94	$C_{21}H_30O_2$	94.83
Codeine	1.169	1.16	-0.009	299.1524	299.1521	-0.72	$C_{18}H_{21}NO_3$	72.49
Cocaine	2.44	2.418	-0.022	303.1475	303.1471	-1.29	$C_{17}H_{21}NO_4$	98.03
Clonazepam	3.625	3.638	0.013	315.0412	315.0411	-0.42	$C_{15}H_{10}ClN_3O_3$	98.72
Alprazolam	3.726	3.726	0	308.083	308.0829	-0.33	$C_{17}H_{13}ClN_4$	96.77
3,4-Methylenedioxyethamphetamine (MDEA)	1.862	1.846	-0.016	207.1263	207.1259	-1.8	$C_{12}H_{17}NO_2$	97.4
3,4-Methylendioxyamphetamine (MDA)	1.474	1.473	-0.001	179.095	179.0946	-2.23	$C_{10}H_{13}NO_2$	86.15

Customized databases with user-added retention times One of the benefits of the Agilent Personal Forensic Toxicology Database is that it can be saved to a user customized form. To create a read-write customizable database the user selects New Database from the PCDL File menu. The PCDL program then allows selection of an existing database and the naming of a new database. A description can also be given. When 'Create' is selected, the database with the new name contains all the entries of the selected database. In this way multiple custom or smaller, more targeted databases can be created depending on the analytes of interest. A technical note on the Pesticide PCD [2] shows how users can run standards with unique chromatographic conditions and easily update or insert retention times in their custom database.

Customizing and updating PCDL AMRT compound data is accomplished by using tab 4 (from left) of the PCDL program interface. This is shown in Figure 8, where the options of 'Add New', 'Save as New', 'Update Selected' and 'Delete Selected' are clearly present. When 'Allow Editing' is activated from the 'Database/Library' pull-down menu, any of the displayed information fields in the users' custom database can be changed, added to or deleted. Furthermore, the ability to insert '*.mol' molecular diagrams to any new database entry is possible from the 'Edit Compounds' tab.



Compound Name	Formula	Mass	Mass Submitted	Delta Mass (ppm)	Anion	Cation	RT (min)	RT Submitted	Delta RT	CAS	ChemSpider	IUPAC Name
Methamphetamine	C10H15N	149.12045	149.11970	5.03			1.593	1.606	-0.013	537-46-2	10379	(2S)-N-Methyl-1-phenyl-2-propanamine
Codeine	C18H21N...	299.15214	299.15240	-0.87			1.160	1.169	-0.009	76-57-3	4447447	(5alpha,6alpha)-3-Methoxy-17-methyl-7,8-d
Hydrocodone	C18H21N...	299.15214	299.15250	-1.20			1.560	1.575	-0.015	125-29-1	4447623	(5alpha)-3-Methoxy-17-methyl-4,5-epoxym
Phentermine	C10H15N	149.12045	149.11990	3.69			1.750	1.770	-0.020	122-09-8	4607	2-Methyl-1-phenyl-2-propanamine
Clonazepam	C15H10Cl...	315.04107	315.04120	-0.41			3.638	3.625	0.013	1622-61-3	2700	5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1
delta9-Tetrahydrocannabinol (THC)	C21H30O2	314.22458	314.22430	0.89			5.292	5.275	0.017	1972-08-3	15266	(6aR,10aR)-6,6,9-Trimethyl-3-pentyl-6a,7,8
Oxycodone	C18H21N...	315.14706	315.14750	-1.40			1.423	1.434	-0.011	76-42-6	4447649	(5alpha)-14-Hydroxy-3-methoxy-17-methyl-
Lorazepam	C15H10Cl...	320.01193	320.01200	-0.22			3.621	3.616	0.005	846-49-1	3821	7-Chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-d
Strychnine	C21H22N...	334.16813	334.16840	-0.81			1.769	1.788	-0.019	57-24-9	389877	Strychnidin-10-one
Verapamil	C27H38N...	454.28316	454.28330	-0.31			3.577	3.574	0.003	52-53-9	2425	2-(3,4-Dimethoxyphenyl)-5-[[2-(3,4-dimethc
Trazodone	C19H22Cl...	371.15129	371.15160	-0.84			2.824	2.840	-0.016	19794-93-5	5332	2-[3-[4-(3-Chlorophenyl)-1-piperazinyl]propy
Heroin	C21H23N...	369.15762	369.15790	-0.76			2.297	2.322	-0.025	561-27-3	4575379	(5alpha,6alpha)-17-Methyl-7,8-didehydro-4
Proadifen	C23H31N...	353.23548	353.23550	-0.06			4.121	4.116	0.005	302-33-0	4741	2-(Diethylamino)ethyl 2,2-diphenylpentano-
Methadone	C21H27NO	309.20926	309.20940	-0.45			3.638	3.638	0.000	76-99-3	3953	6-(Dimethylamino)-4,4-diphenyl-3-heptanor
Alprazolam	C17H13Cl...	308.08287	308.08300	-0.42			3.726	3.726	0.000	28981-97-7	2034	8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazc

Figure 8. Edit Compounds PCDL interface tab.

Workflow C. Data mining using 'Find by Formula' (FBF)

The 'Find by Formula' data-mining algorithm of the MassHunter Qualitative Analysis program uses a pre-defined empirical formula (or list of formulae) to search TOF and Q-TOF (MS) data files for evidence that peaks may be present. The PCDL-format databases can also be specified as the list of empirical formulae. Depending on the size and content of the database, FBF can take slightly longer than the MFE approach. However, FBF is highly accurate and sensitive especially at very low analyte concentration levels.

Figure 9 illustrates the results screen displayed after a 'Find by Formula' search has been undertaken using the LC/MS Toxicology Test Mix data file. All 25 compounds were matched with accurate mass, abundance and isotopic spacing in a combined score (shown) together with retention time. The DA method editor settings used for this FBF analysis are shown in Figure 10, where 'Tox_std_01.cdb' was a custom PCDL-format database.

When reporting the results, FBF assesses the chromatographic peak shape and isotopic match scores and returns the best match, even if there are several peaks displayed in the extracted compound chromatogram of similar mass.

Additional adducts [M+Na]+, [M+NH4]+ and [2M+H]+ were used during this FBF data screen. The extra information is displayed in the spectrum view and results table to provide added confirmatory evidence. Figure 9 shows the Temazepam spectrum which displays both [M+H]+ and [M+Na]+ adducts.

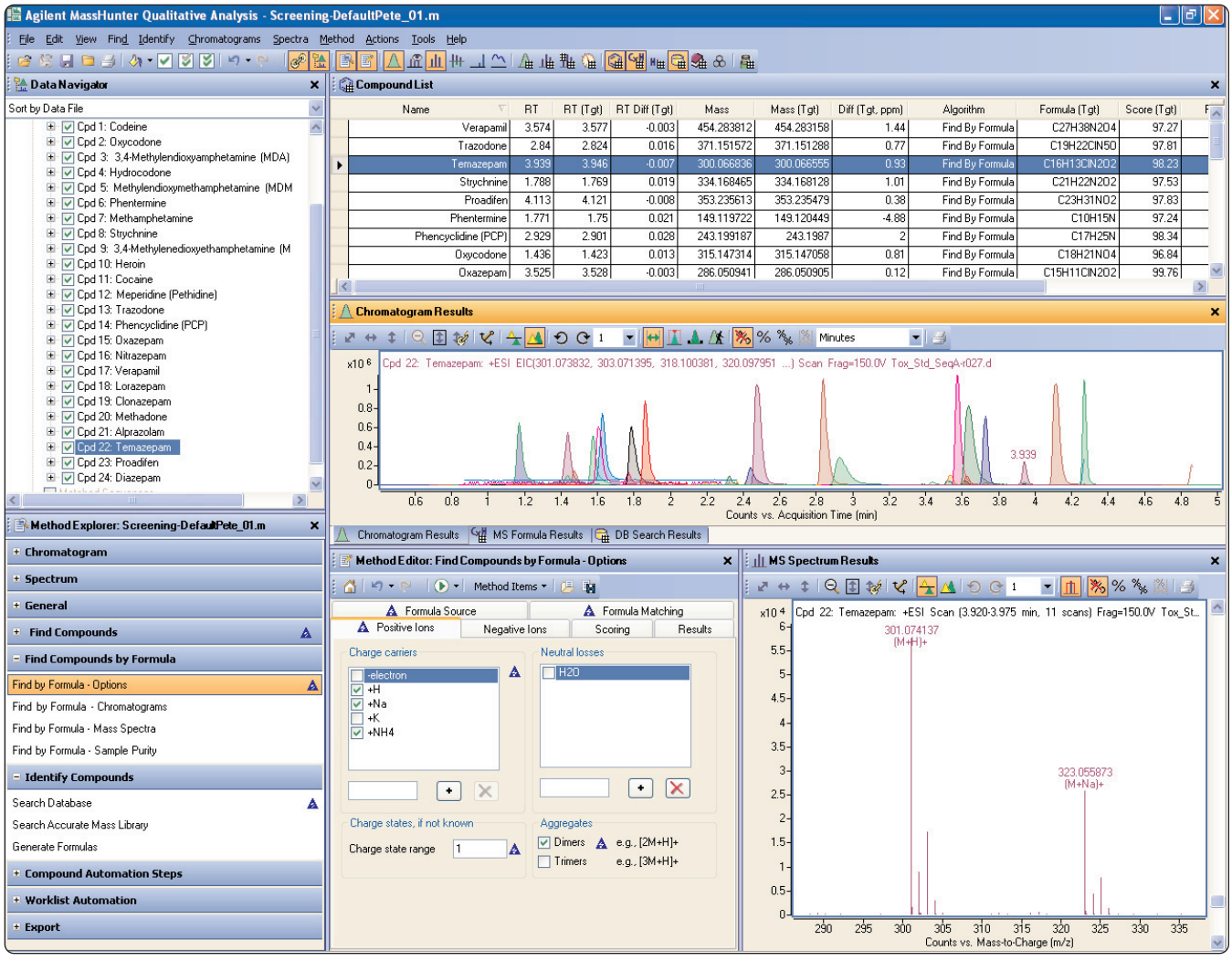


Figure 9. Find By Formula Database search results, MassHunter Qualitative Analysis program.

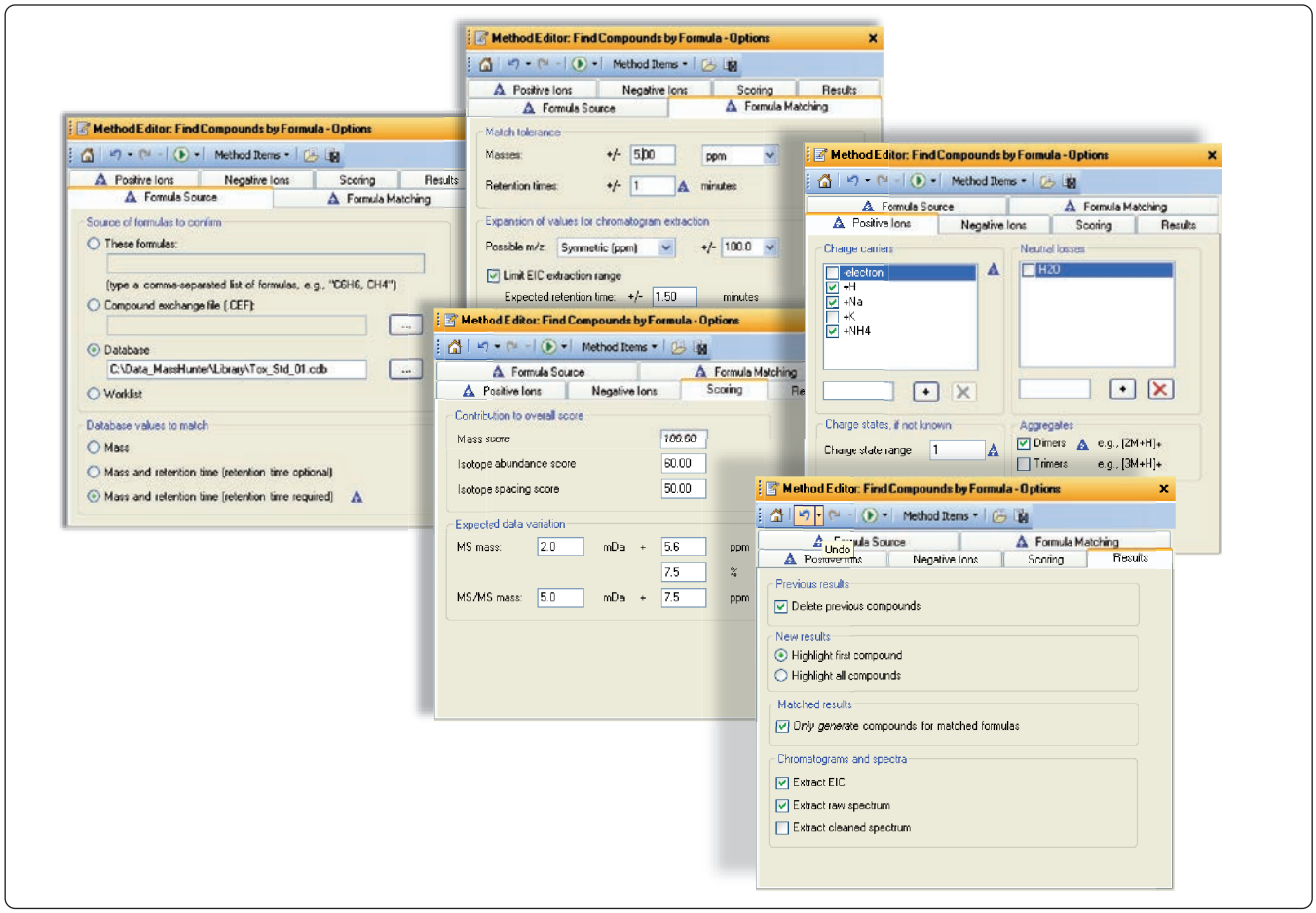


Figure 10. Find By Formula Database search - Method editor settings.

More in-depth information can be obtained from MassHunter Qualitative Analysis program Help files or Agilent MassHunter Workstation Software Qualitative Analysis Familiarization Guide [5].

Reporting

Manual, MFE and FBF database searching all use the identical method of compound reporting options in the MassHunter Qualitative Analysis program software interface. Figure 11 details the reporting options which are based upon the standard compound report template 'CompoundReportWithIdentificationHits.xlsx'. Under the General section of the method explorer, the 'Common reporting options' link opens the corresponding method editor pane, shown on the left side of Figure 11. MassHunter Qualitative Analysis program treats search algorithm data and database searches as compound-centric data. Therefore, to report the results the appropriate compound report template must be chosen. In this example, the correct report template is displayed.

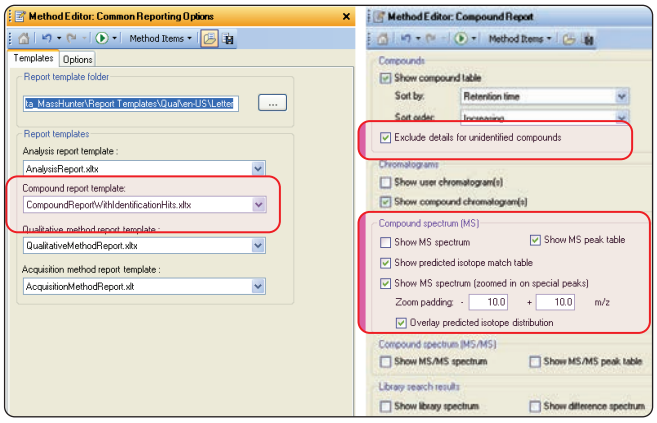


Figure 11. Common compound reporting options for Manual/MFE/FBF PCDL Searches.

More specific content can then be specified by choosing the information required for the Forensic Toxicology screen report using the 'Compound Report' options of the method editor (shown on the right in Figure 11).

Decisions about the report content are decided here. For example, if the check box for 'Exclude Details for Unidentified Compounds' is activated, then only positive PCDL identifications will be reported. The option to report compound extracted chromatograms, individual MS spectra, or summary results and individual compound tables is also determined from the compound report method editor.

Once all the correct settings have been achieved for the reporting of results, the green button (circled in Figure 12) activates the 'printing dialogue' window which gives various options for directing the output of the data file results. The user can choose to send results directly to a specified printer or save the results in excel format or public distribution format (pdf). Alternatively, the results report can be processed by choosing the 'Print Compound Report' option from the drop-down 'File' menu.

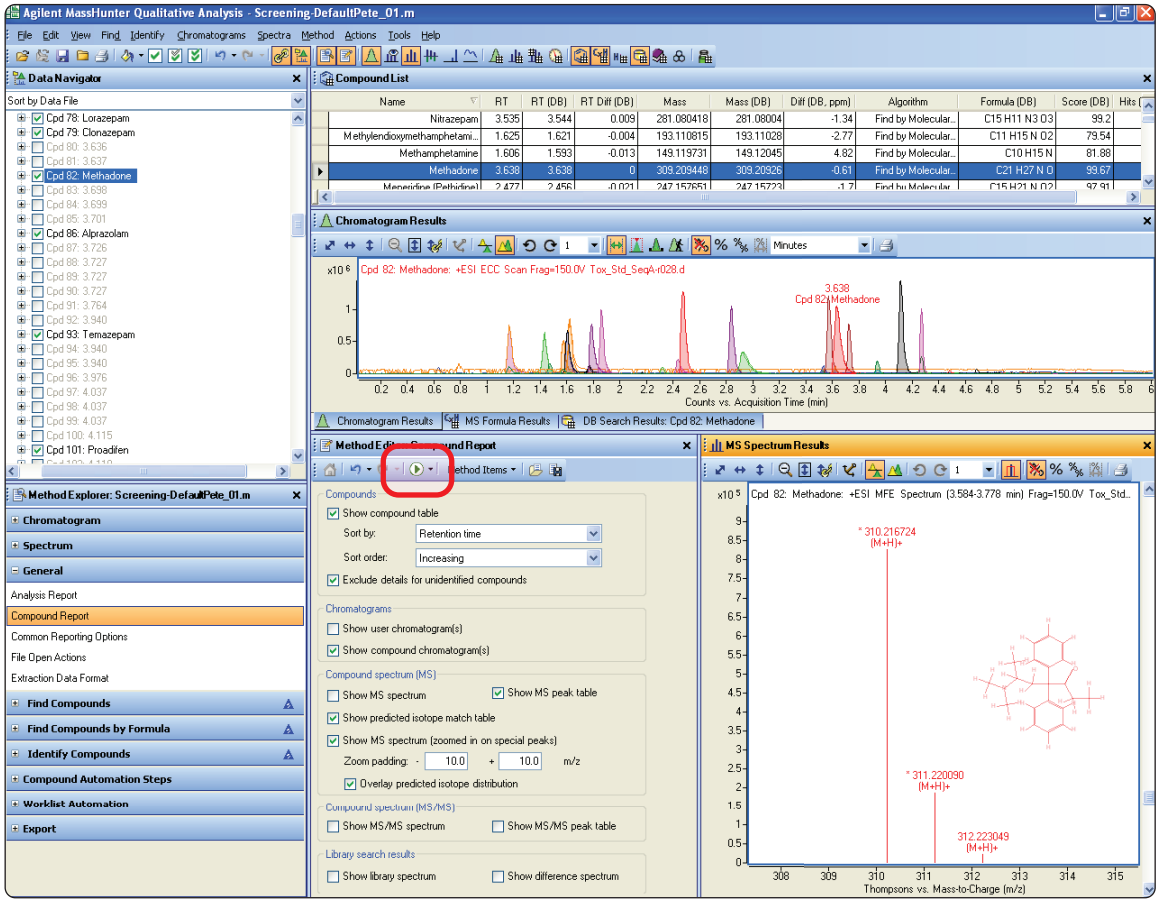


Figure 12. Compound Reporting for Manual/MFE/FBF PCDL Searches.

Figure 13 illustrates a typical report summary front page for the LC/MS Toxicology Test Mix.

Qualitative Compound Report

Data File	Tox_Std_SeqA-r028.d	**Sample Name**	Tox_Std_100pg
Sample Type	Sample	**Position**	Vial 2
Instrument Name	SEIKO-90500107	**User Name**	Pete Stone
Acq Method	PStone_Tox_Std_01.m	**Acquired Time**	7/17/2009 9:41:03 PM
IRM Calibration Status	Success	**DA Method**	Screening-DefaultPete_01.m
Comment			

Compound Table

Compound Label	RT	Mass	Name	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 10: Codeine	1.169	299.1524	Codeine	C18 H21 N O3	-0.72	2
Cpd 17: Oxycodone	1.434	315.1475	Oxycodone	C18 H21 N O4	-1.44	1
Cpd 23: 3,4-Methylendioxyamphetamine (MDA)	1.474	179.095	3,4-Methylendioxyamphetamine (MDA)	C10 H13 N O2	-2.23	1
Cpd 29: Hydrocodone	1.575	299.1525	Hydrocodone	C18 H21 N O3	-1.2	2
Cpd 34: Methamphetamine	1.606	149.1197	Methamphetamine	C10 H15 N	4.82	2
Cpd 39: Methylendioxymethampheta mine (MDMA)	1.625	193.1108	Methylendioxymethampheta mine (MDMA)	C11 H15 N O2	-2.77	1
Cpd 41: Phentermine	1.77	149.1199	Phentermine	C10 H15 N	3.78	2
Cpd 45: Strychnine	1.788	334.1684	Strychnine	C21 H22 N2 O2	-0.77	1
Cpd 50: 3,4-Methylenedioxyethamphetami ne (MDEA)	1.862	207.1263	3,4-Methylenedioxyethamphetami ne (MDEA)	C12 H17 N O2	-1.8	1
Cpd 57: Heroin	2.322	369.1579	Heroin	C21 H23 N O5	-0.63	1
Cpd 60: Cocaine	2.44	303.1475	Cocaine	C17 H21 N O4	-1.29	1
Cpd 62: Meperidine (Pethidine)	2.477	247.1577	Meperidine (Pethidine)	C15 H21 N O2	-1.7	1
Cpd 65: Trazodone	2.84	371.1516	Trazodone	C19 H22 Cl N5 O	-0.81	1
Cpd 67: Phencyclidine (PCP)	2.931	243.199	Phencyclidine (PCP)	C17 H25 N	-1.32	1
Cpd 73: Oxazepam	3.524	286.0511	Oxazepam	C15 H11 Cl N2 O2	-0.71	1
Cpd 74: Nitrazepam	3.535	281.0804	Nitrazepam	C15 H11 N3 O3	-1.34	1
Cpd 76: Verapamil	3.574	454.2833	Verapamil	C27 H38 N2 O4	-0.31	1
Cpd 78: Lorazepam	3.616	320.012	Lorazepam	C15 H10 Cl2 N2 O2	-0.19	1
Cpd 79: Clonazepam	3.625	315.0412	Clonazepam	C15 H10 Cl N3 O3	-0.42	1
Cpd 82: Methadone	3.638	309.2094	Methadone	C21 H27 N O	-0.61	1
Cpd 86: Alprazolam	3.726	308.083	Alprazolam	C17 H13 Cl N4	-0.33	1
Cpd 93: Temazepam	3.94	300.067	Temazepam	C16 H13 Cl N2 O2	-1.62	1
Cpd 101: Proadifen	4.116	353.2355	Proadifen	C23 H31 N O2	-0.18	1
Cpd 110: Diazepam	4.272	284.072	Diazepam	C16 H13 Cl N2 O	-1.36	1
Cpd 243: delta9-Tetrahydrocannabinol (THC)	5.275	314.2243	delta9-Tetrahydrocannabinol (THC)	C21 H30 O2	0.94	1

Compound Label	Name	RT	Algorithm	Mass
Cpd 10: Codeine	Codeine	1.169	Find by Molecular Feature	299.1524

Figure 13. Output Report from MFE/Database search.

Worklist Automation:

Once the analyst or operator has decided on the correct settings for all aspects of the data mining routines, the PCDL search options and reporting options (outlined in this application note) can be saved to one convenient data analysis method. This method can be used for repetitive and consistent data manipulation from week to week. This is achieved by choosing the 'Save As' option from the drop-down 'Method' menu in the MassHunter Qualitative Analysis program interface. This method will then open as the default DA method when the MassHunter Qualitative Analysis program is started until another DA method is saved or loaded.

An added advantage to saving reprocessing options is the 'Worklist Automation' functionality built into the MassHunter Qualitative Analysis program. Figure 14 outlines the setup of Worklist automation and specifically addresses a routine that would automatically interrogate a data file using MFE and PCDL database search followed by reporting of results to the specified printer or data file location.

In this example, a list of automatic data analysis steps are defined in order of operation, as they would be undertaken manually.

First, the sample data file is loaded, and all previous results (if any) are cleared. Next, the 'Find by MFE' routine according to the saved DA method setup is performed with the compound results searched against the PCDL database specified in the DA method. Finally, any results are automatically sent to a final report, the format of which has been determined and also saved to the DA method.

Two further steps must be performed to run such a worklist automation routine automatically during sample data acquisition.

First, the DA analysis method and the Worklist Automation routine must be saved into the acquisition method by using the 'Save As' option from the 'Method' menu and selecting the MassHunter acquisition method name. Once 'OK' is

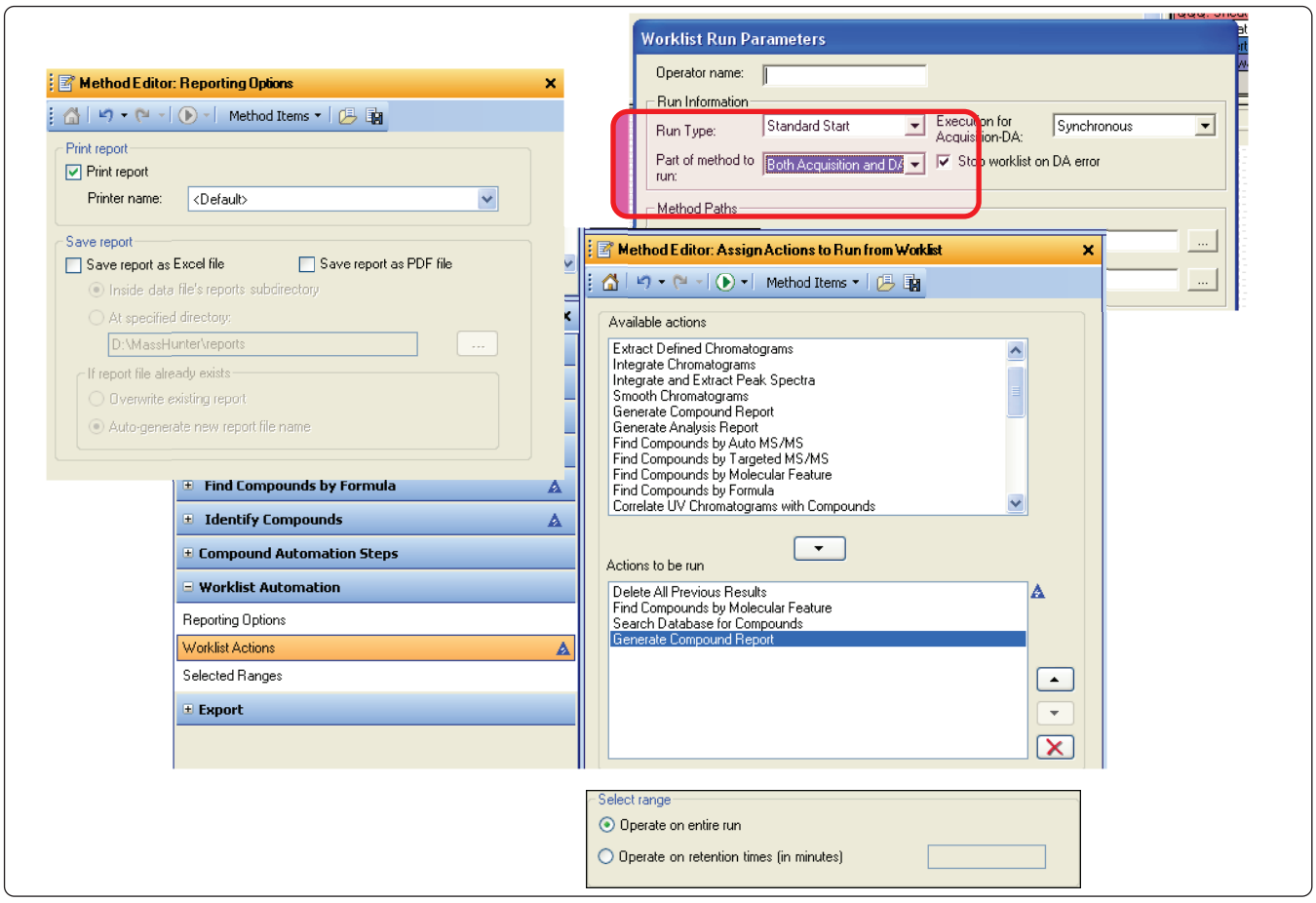


Figure 14. Worklist automation method setup.

selected, the data analysis method becomes an integral part of the Acquisition method.

Finally, to automatically perform Worklist Data Analysis during data acquisition, the 'Worklist Run Parameters' window must be opened from the 'Worklist' Menu of MassHunter Acquisition software. Figure 14 shows a screen capture of this window with the settings highlighted so that the DA routine will operate 'Parts of method to Run - Both Acquisition and DA'. The data analysis has the option to be run 'Synchronously' or 'Asyncronously'.

Conclusions

The Agilent MassHunter Personal Forensic Toxicology Database Kit has been developed to provide comprehensive screening of samples for both targeted and non-targeted approaches. The database includes accurate mass data for around 6700 compounds of potential interest and gives the user flexibility in its use.

The MassHunter Personal Forensic Toxicology Database Kit offers:

- Fast and easy startup of complex analyses
- A comprehensive database of around 6700 compounds including
  - Chemical structures, formulas and exact masses
  - Direct Chemical Internet links to PUBCHEM and ChemSpider
  - IUPAC names
  - The ability to create MS/MS spectral libraries
  - Complete customization with additions/deletions of retention time for chromatographic conditions developed by the user
- Results can be searched from within the PCDL software interface or directly from the MassHunter Qualitative Analysis program.
- Results can be data-mined with powerful searching tools, such as the Molecular Feature Extractor and Find by Formula
- Searches of the database can be partially or completely automated using MassHunter Qualitative Analysis program and the MassHunter Acquisition Worklist

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Development of a Screening Analysis by LC Time-Of-Flight MS for Drugs of Abuse

Application Note

Forensic Toxicology

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Abstract

The screening for drugs of abuse in human samples is reliant on the accuracy of drug screening. Currently, the most common method of this analysis is a straight-forward immunoassay technique, which although allowing for a rapid turnaround of screening samples, involves a slower confirmatory test of derivatization and detection by gas chromatography/mass spectrometry (GC/MS).

This application note presents the potential for the Agilent Time-of-Flight Mass Spectrometer (LC/MSD TOF) for use as both a screening and a confirmation tool in one analytical run of 30 minutes.

Introduction

Today, many drugs of choice are derived directly from natural substances with the most common being *cannabis*. An extensive review of the illicit drug market in 25 major U.S. cities is provided in the Office of National Drug Control Policy Document “Pulse Check” [1].

Over the last 100 years, the physiological effects of many of the current illicit drugs were evaluated and reviewed, resulting in their subsequent banning. During this time, new drugs were developed, many finding wide acceptance within the medical community for the treatment of specific ailments. Unfortunately, the undesirable side effects of addiction or long-term abuse were often associated with the use of these drugs. The opiate class of drugs is an excellent example of one such class, as they are highly addictive and subject to abuse.

Interest in the analysis of drugs of abuse covers many areas, all with different concerns in the results obtained. Some of the areas of significance include:

- Workplace screening
- Forensic pathology
- Accident investigation
- Crime scene investigation

Today, screening of drugs of abuse is performed through a variety of methods, with the most common lab-based technique being an Enzyme Multiple Immunoassay Test (EMIT), with a confirmatory analysis by GC/MS, if required. This immunoassay technique allows for screening to be performed and reported in as little as 2 hours, yet more commonly a 36–48 hour turnaround time is required. A further disadvantage of the EMIT technique is that it lacks the specificity to identify anything more than the class of drug detected.

The current analytical confirmatory technique of GC/MS was developed in order to achieve the sensitivity and specificity required to accurately determine the exact type and level of the drug compound, within the class indicated by the immunoassay technique. In order to achieve this detection, many of the drugs require derivatization to ensure adequate volatility and/or thermal stability required for GC analysis. See Table 1.

Table 1. National Institute of Drug Analysis Compound Class and Detection Limit Summary

Compound class	Detection limits (ng/mL)	Confirmation
Amphetamines	1000	EMIT/GC/MS
Barbiturates	300–3000	EMIT/GC/MS
Cocaine	300	EMIT/GC/MS
Methadone	300	EMIT/GC/MS
Opiates	300	EMIT/GC/MS
Phencyclidine	25	EMIT/GC/MS
Propoxyphene	300	EMIT/GC/MS
Benzodiazepines	300	EMIT/GC/MS
Methaqualone	300	EMIT/GC/MS
Cannabinoids	50	EMIT/GC/MS

Recently published Agilent application notes have shown the potential of LC/MS for the screening analysis of drugs of abuse using a single quadrupole instrument [2, 3]. Numerous other publications discuss selected drugs of abuse, or drug classes, illustrating the potential for the technique to one day replace GC/MS as either the confirmatory tool or as both the screen-ing and confirmatory tool in one analysis.

Accurate mass measurement, such as that provided by the Agilent LC/MSD TOF, greatly increases the confidence of identification because it inherently limits the possible number of candidate compounds. The better the precision and accuracy of the mass measurement, the fewer the number of compounds theoretically possible for a given accurate mass. This is particularly useful for the analysis of samples from a variety of sources, each with their own potential interferences, such as those encountered with explosives residue analysis.

This application note provides an overview of the power of the Agilent TOF mass spectrometer for the screening and confirmation analysis of drugs of abuse. The TOF mass spectrometer provides accurate mass determinations (<3 ppm) with good linearity, proving its use as an excellent tool for the detection, confirmation, and quantitation of different drug classes. The method used here is not intended to represent one that will determine the lowest possible level of any one particular analyte or class of analytes, but rather is a procedure that could be expanded to cover a wider range of components used in screening analyses.

The compounds studied and their molecular formulas are shown in Table 2.

Table 2. Compounds Included in Study

Compound	Molecular formula	Drug class
α-hydroxyalprazolam	$C_{17}H_{13}N_4OCl$	Benzodiazepine
7-Aminoclonazepam	$C_{15}H_{12}N_3OCl$	Benzodiazepine
Diazapam	$C_{16}H_{13}N_2OCl$	Benzodiazepine
Oxazepam	$C_{15}H_{11}N_2O_2Cl$	Benzodiazepine
Temazepam	$C_{16}H_{13}N_2O_2Cl$	Benzodiazepine
7-Aminoflunitrazepam	$C_{16}H_{14}N_3OF$	Benzodiazepine
7-Aminonitrazepam	$C_{15}H_{13}N_3O$	Benzodiazepine
dl-11-nor-9-carboxy-δ-9-THC	$C_{21}H_{28}O_4$	Cannabinoid
Codeine	$C_{18}H_{21}NO_3$	Opiate
Morphine 3β-d-glucuronide	$C_{23}H_{27}NO_9$	Opiate
6-acetylmorphine	$C_{19}H_{21}NO_4$	Opiate
EDDP perchlorate	$C_{20}H_{24}NO_4Cl$	Opiate
(+)-ephedrine	$C_{10}H_{15}NO$	Stimulant
Fenfluramine	$C_{12}H_{16}NF_3$	Stimulant
dl-MBDB:HCL	$C_{12}H_{18}NO_2Cl$	Stimulant
(±) BDB Hydrochloride	$C_{11}H_{16}NO_2Cl$	Stimulant
dl-MDEA	$C_{12}H_{17}NO_2$	Stimulant
dl-MDA	$C_{10}H_{13}NO_2$	Stimulant
dl-MDMA	$C_{11}H_{15}NO_2$	Stimulant
dl-Methamphetamine	$C_{10}H_{15}N$	Stimulant
dl-Amphetamine	$C_9H_{13}N$	Stimulant
Phentermine	$C_{10}H_{15}N$	Stimulant
(+)-Psuedoephedrine	$C_{10}H_{15}NO$	Stimulant
(–)-Cotinine	$C_{10}H_{12}N_2O$	Other
4'-Hydroxynordiazepam	$C_{15}H_{11}ClN_2O_2$	Benzodiazepine
Nordiazepam	$C_{15}H_{11}N_2OCl$	Benzodiazepine
Flunitrazepam	$C_{16}H_{12}N_3O_3F$	Benzodiazepine
Flurazepam	$C_{21}H_{23}N_3OClF$	Benzodiazepine
Desalkylflurazepam	$C_{15}H_{10}N_2OClF$	Benzodiazepine
(–)-δ-9-THC	$C_{21}H_{30}O_2$	Cannabinoid
(±)-11-hydroxy-δ-9-THC	$C_{21}H_{30}O_3$	Cannabinoid
Cocaine	$C_{17}H_{21}NO_4$	Cocaine
Benzoylecgonine	$C_{16}H_{19}NO_4$	Cocaine
Buprenorphine	$C_{29}H_{41}NO_4$	Opiate
Morphine	$C_{17}H_{19}NO_3$	Opiate
Normorphine	$C_{16}H_{17}NO_3$	Opiate
Meperidine	$C_{15}H_{21}NO_2$	Opiate
Normeperidine	$C_{14}H_{19}NO_2$	Opiate
dl-Methadone	$C_{21}H_{27}NO$	Opiate
EMPD	$C_{19}H_{21}N$	Opiate
Naloxone	$C_{19}H_{21}NO_4$	Opiate
Oxycodone	$C_{18}H_{21}NO_4$	Opiate
LSD	$C_{20}H_{25}N_3O$	Hallucinogen
Iso-LSD	$C_{20}H_{25}N_3O$	Hallucinogen
(±)-phenylpropanolamine:HCL	$C_9H_{13}NO$:HCl	Stimulant
Fluoxetine:HCL	$C_{17}H_{18}F_3NO$:HCl	Prosac
GHB	$C_4H_7O_3Na$	Other
(–)-Nicotine	$C_{10}H_{14}N_2$	Other

Methodology

The work undertaken in this study was performed on an Agilent 1100 system consisting of:

Binary pump
Standard auto-sampler
Thermostated column compartment
Diode Array Detector (DAD)
G1969 LC/MSD TOF.

Instrument Conditions

Pump

Time (min)	% Water (0.1% formic acid)	% Methanol (0.1% formic acid)	Flow rate (mL/min)
0	90	10	0.4
4	90	10	0.4
22	0	100	0.4
29	0	100	0.4
29.6	90	10	0.4
30	90	10	0.4

Post time:	5 minutes
Total run time:	35 minutes
Injection volume:	10 µL, with needle wash
Column temperature:	30 °C
Column:	ZORBAX SB-AQ, 150 mm × 2.1 mm × 3.5 µm

MS Detection

Ionization	**ESI Positive**
Gas temp	350 °C
Drying gas	10 L/min
Nebulizer pressure	40 psig
Capillary V (+ve)	3500 V

MS Conditions

Scan *m/z* range	100–1000
Fragmentor	125 V
Storage mode	Profile
Skimmer	60 V
Oct RF	200 V

Reference Mass Introduction with LC-TOF

The Agilent TOF MS uses a reference mass in the generation of reliable high level accurate mass. The electro-spray source for the TOF is a unique dual spray assembly that allows the simultaneous constant introduction of a reference mass component.

The reference mix 1 used in these experiments consists of 2 mL of purine and 0.8 mL of HP-0921. This mixture was prepared in 1 L of 90:10 methanol:water to better represent the mobile phase.

The control software enables the use of the following reference masses:

Positive Ion Detection
121.050873
922.009798

Analysis of Drugs of Abuse by LC-TOF MS

An overwhelming advantage of using TOF MS for the trace level detection of any component is the confirmatory information that is provided through accurate mass. An example of this mass accuracy is shown in Table 3, where the observed masses for each component are detected, and their deviation from the theoretical masses for the adduct are shown.

The ability to closely match the expected mass and the observed mass provides the analyst with a higher level of confidence in the assignment given to a chromatographic peak. In the screening for components such as drugs, this additional confidence is of great importance. This capability also allows the possibility of using this technique as a screening tool for a wide range of components.

Table 3. Theoretical Accurate Mass, Observed Mass and Mass Error

Compound	Monoisotopic mass	Retention time	Adduct	Observed mass	Adduct accurate mass	Mass error (ppm)
α-Hydroxyalprazolam	324.0778	17.76	$[M+H]^+$	325.0852	325.0850	0.41
7-Aminoclonazepam	285.0669	13.32	$[M+H]^+$	286.0739	286.0741	–0.93
Diazapam	284.0716	19.15	$[M+H]^+$	285.0796	285.0789	2.39
Oxazepam	286.0509	17.4	$[M+H]^+$	287.0579	287.0581	–0.98
Temazepam	300.0666	18.2	$[M+H]^+$	301.0741	301.0738	0.89
7-Aminoflunitrazepam	283.1121	15.3	$[M+H]^+$	284.1191	284.1093	–0.94
7-Aminonitrazepam	251.1059	8.82	$[M+H]^+$	252.1134	251.1131	1.04
dl-11-nor-9-carboxy-δ-9-THC	344.1988	21.38	$[M+H]^+$	345.2061	345.2060	0.18
Codeine	299.1521	5.5	$[M+H]^+$	300.1592	300.1594	–0.73
Morphine 3 β-d-glucuronide	461.1686	1.7	$[M+H]^+$	462.1764	462.1758	1.17
6-Acetylmorphine	327.1471	8.9	$[M+H]^+$	328.1542	328.1543	–0.41
EDDP perchlorate	377.1394	15.42	$[M-O_4Cl]^+$	278.1909	278.1903	2.06
(+)-Ephedrine	165.1154	2.46	$[M+H]^+$	166.1225	166.1226	–0.85
Fenfluramine	231.1235	12.9	$[M+H]^+$	232.1303	232.1307	–1.98
dl-MBDB:HCL	243.1026	10.63	$[M-Cl]^+$	208.1337	208.1332	2.37
(±) BDB hydrochloride	229.087	9.5	$[M-Cl]^+$	194.1181	194.1175	2.81
dl-MDEA	207.1259	9.6	$[M+H]^+$	208.1332	208.1332	–0.03
dl-MDA	179.0946	4.9	$[M+H]^+$	180.1019	180.1191	–0.03
dl-MDMA	193.1103	6.4	$[M+H]^+$	194.1174	194.1175	–0.08
dl-Methamphetamine	149.1204	3.85	$[M+H]^+$	150.1281	150.1277	2.49
dl-Amphetamine	135.1048	3.05	$[M+H]^+$	136.1125	136.112	3.11
Phentermine	149.1204	5.34	$[M+H]^+$	150.1278	150.1277	0.49
(+)-Psuedoephedrine	165.1154	2.76	$[M+H]^+$	166.1231	166.1226	2.76
(–)-Cotinine	176.095	2.56	$[M+H]^+$	177.1023	177.1022	0.34
4'-Hydroxynordiazepam	286.0509	14.23	$[M+H]^+$	287.0582	287.0581	0.06
Nordiazepam	270.056	18.1	$[M+H]^+$	271.0634	271.0632	0.49
Flunitrazepam	313.0863	18.1	$[M+H]^+$	314.0924	314.0935	–3.65
Flurazepam	387.1514	15.23	$[M+H]^+$	388.1591	388.1586	1.17
Desalkylflurazepam	288.0466	18.2	$[M+H]^+$	289.0535	289.0538	–1.19
(–)-δ-9-THC	314.2246	22.31	$[M+H]^+$	315.2328	315.2318	2.99
(±)-11-hydroxy-δ-9-THC	330.2195	21.07	$[M+H]^+$	331.2267	331.2267	–0.22
Cocaine	303.1471	12.6	$[M+H]^+$	304.1545	304.1543	0.54
Benzoylecgonine	289.1314	12.1	$[M+H]^+$	290.1386	290.1386	–0.29
Buprenorphine	467.3036	16.11	$[M+H]^+$	468.3107	468.3108	–0.29
Morphine	285.1365	2.2	$[M+H]^+$	286.1438	286.1437	–0.1
Normorphine	271.1208	2	$[M+H]^+$	272.1286	272.1281	1.7
Meperidine	247.1572	12.4	$[M+H]^+$	248.1652	248.1645	2.8
Normeperidine	233.1416	12.5	$[M+H]^+$	234.1493	234.1488	1.9
dl-Methadone	309.2093	16.72	$[M+H]^+$	310.2166	310.2165	0.19
EMPD	263.1674	16.4	$[M+H]^+$	264.1754	264.1746	2.7
Naloxone	327.1471	5.74	$[M+H]^+$	328.1541	328.1543	–0.72
Oxycodone	315.1471	7.2	$[M+H]^+$	316.1547	316.1543	1.15
LSD	323.1998	14.7	$[M+H]^+$	324.2073	324.207	0.8
Iso-LSD	323.1998	14.55	$[M+H]^+$	324.2078	324.207	2.34
(±)-Phenylpropanolamine:HCL	187.0764	1.95	$[M-Cl]^+$	152.1069	152.1069	–0.59
Fluoxetine:HCL	345.1107	16.3	$[M-Cl]^+$	310.1412	310.1413	1.85
GHB	126.0293	1.72	$[M+H]^+$	127.0369	127.0365	2.63
(–)-Nicotine	162.1157	1.6	$[M+H]^+$	163.1233	163.1229	1.99
2-oxo-3-hydroxy-LSD	355.1896	n.d.			Not detected in +veESI	

A greater than two-fold increase in sensitivity for many components is seen with the narrowing of the mass-extraction window. Figure 1 shows the reduction in noise that is observed with the extraction of a smaller mass range for flunitrazepam, commonly known as Rohypnol, a date-rape drug. The ability of TOF-MS to accurately determine the presence of components such as Rohypnol at low levels may assist with investigations into reported abuse of the illicit substance, and prove to be a critical factor in confirmation when dealing with complex matrices.

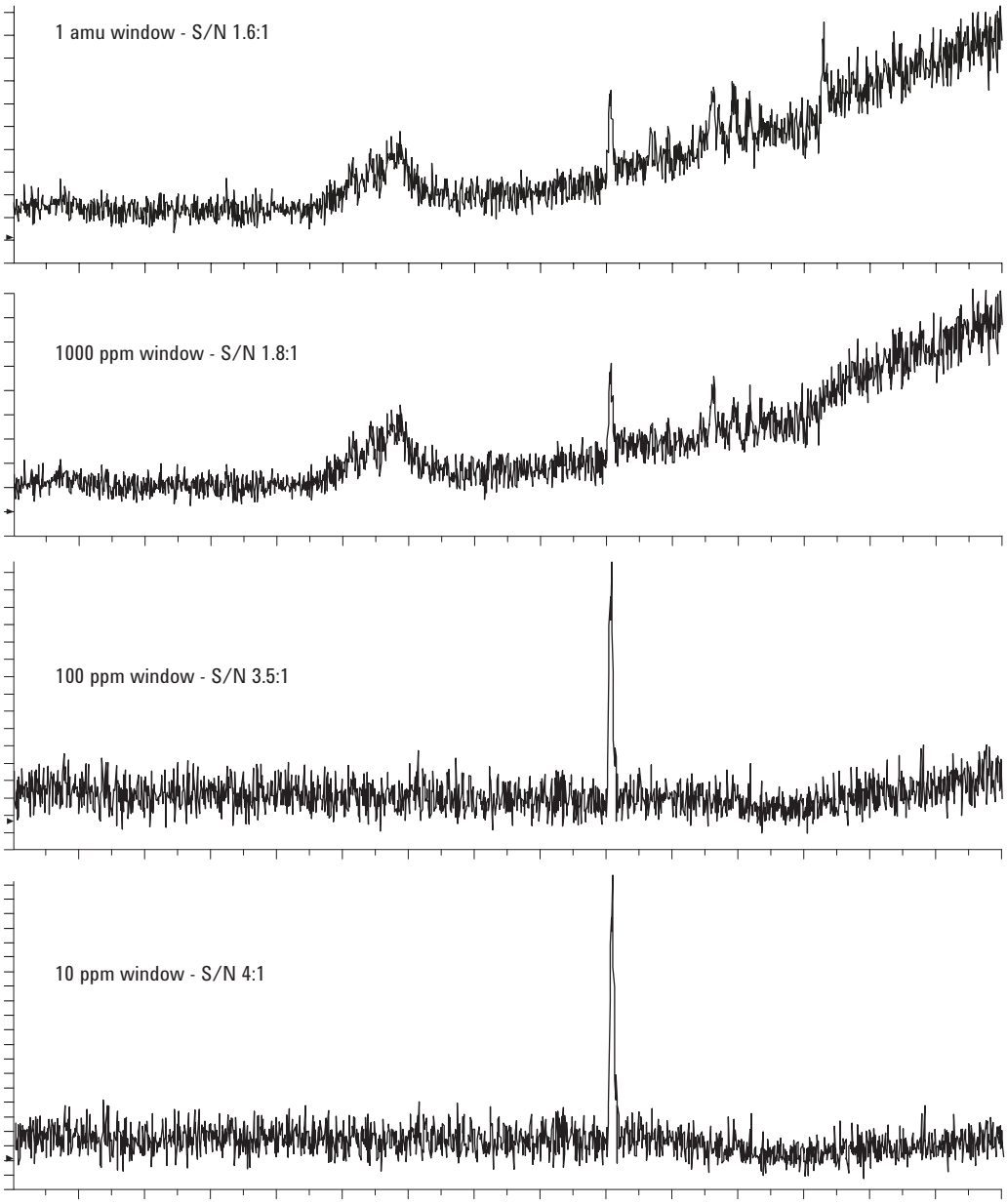


Figure 1. Effect of extracted ion range on noise – 1 ng/mL flunitrazepam.

TOF Linearity

TOF-MS has traditionally been considered as unsuitable for quantitation due to the use of time-to-digital conversion of data. The Agilent TOF MS uses analog-to-digital conversion, allowing for far better quantitative data than the alternative technology of time-to-digital conversion. Several of the components analyzed by TOF were tested for linearity as part of this study. Figures 2–5 show the linearity of four selected components, most displaying linearity over three orders of magnitude from 1 ng/mL to 1000 ng/mL. However, some components, such as δ-9-THC and fluoxetine, only exhibit a narrower linear range, a result of their ionization behavior (Figures 6 and 7). Nevertheless, regression values of over 0.999 were seen for each of these components.

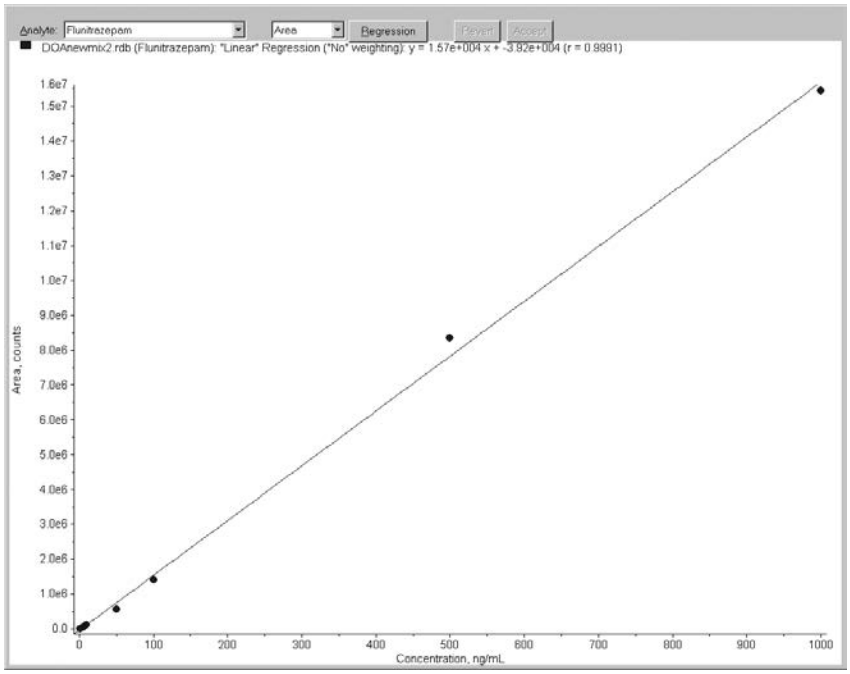


Figure 2. Calibration curve for flunitrazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

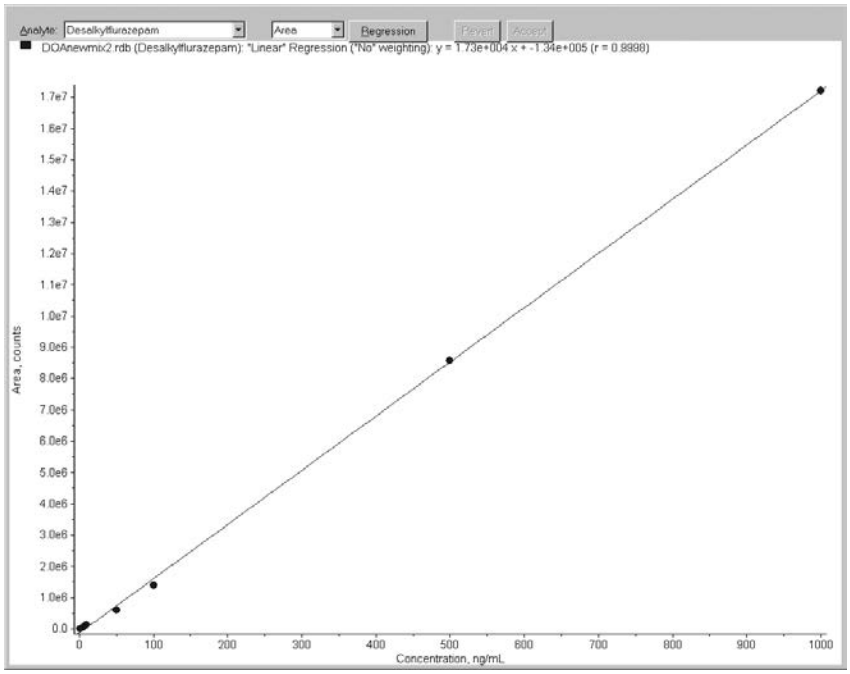


Figure 3. Calibration curve for desalkylflurazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

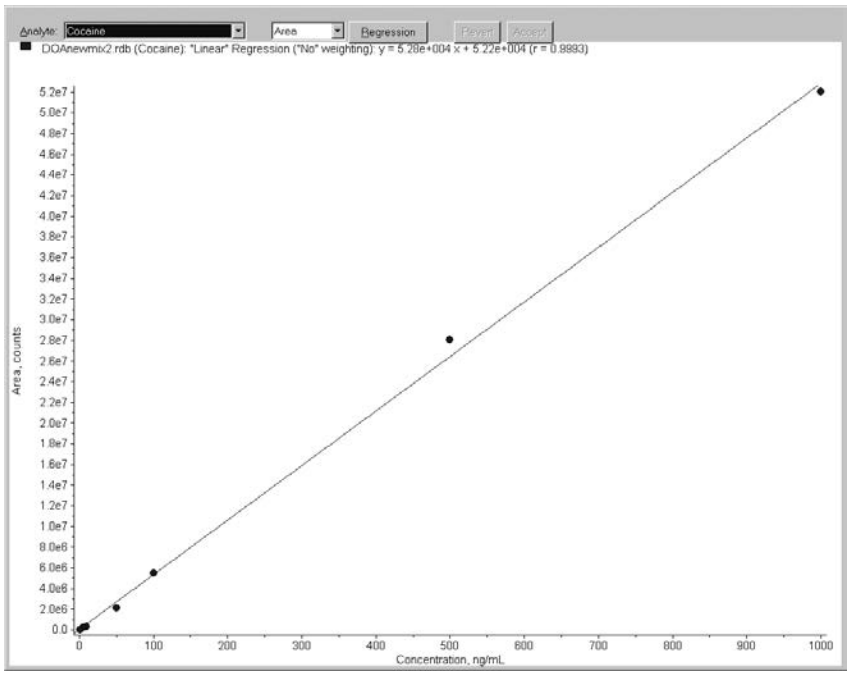


Figure 4. Calibration curve for cocaine from 1 ng/mL to 1000 ng/mL with TOF-MS.

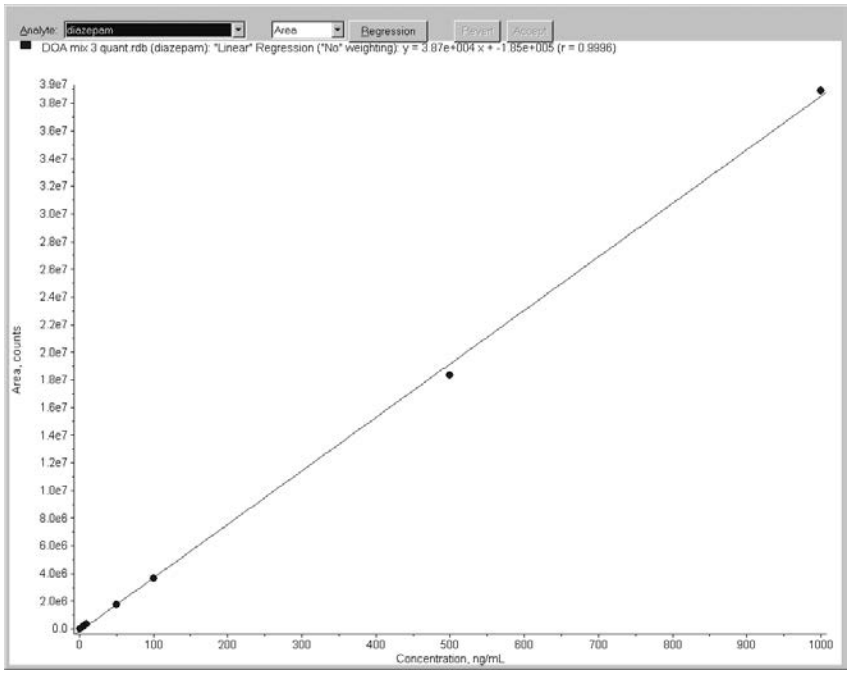


Figure 5. Calibration curve for diazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

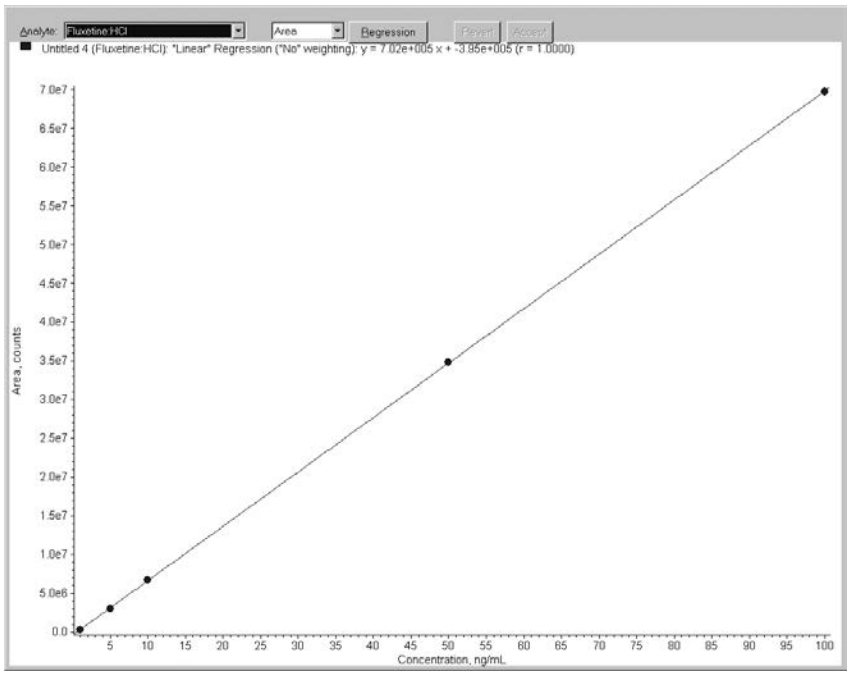


Figure 6. Calibration curve for (–)-δ-9-THC from 1 ng/mL to 100 ng/mL with TOF-MS.

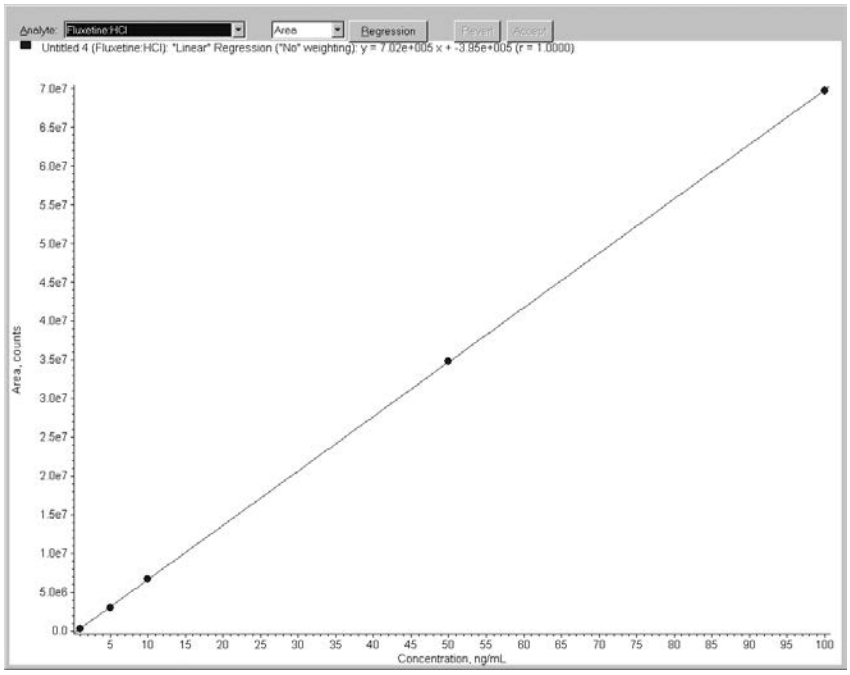
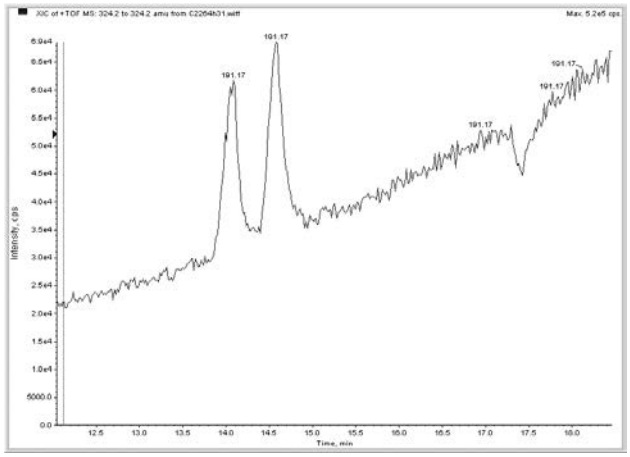
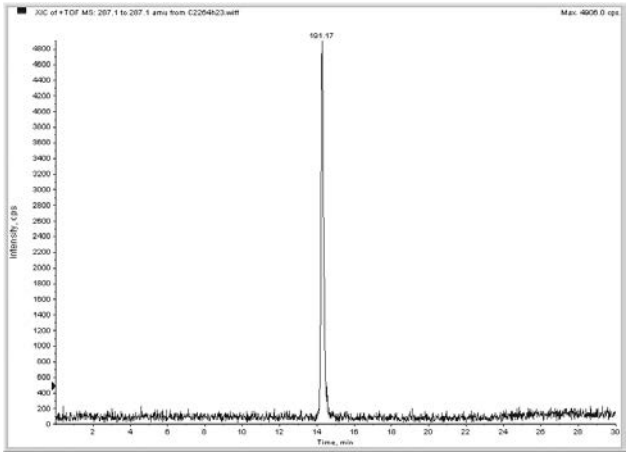


Figure 7. Calibration curve for fluoxetine from 1 ng/mL to 100 ng/mL with TOF-MS.

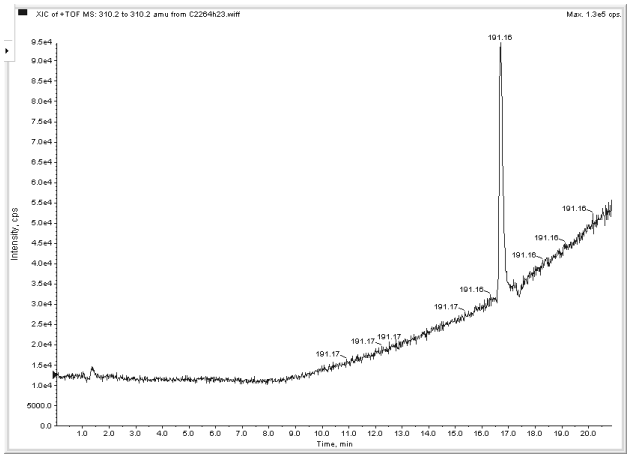
Chromatogram examples for four components at 1 ng/mL are shown below in Figure 8 with 10 ppm extraction windows.



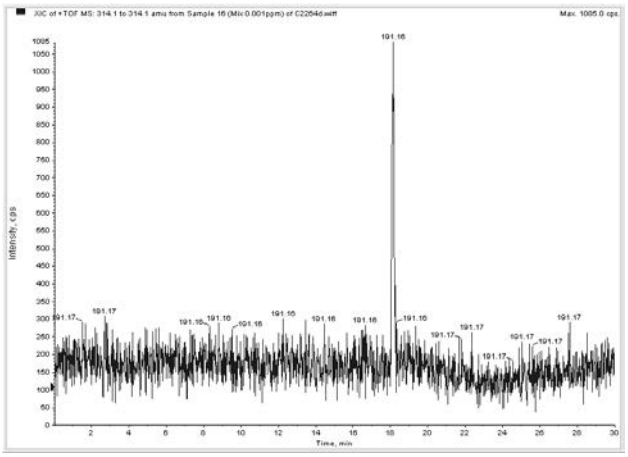
1 ng/mL iso-LSD and LSD (respectively)



1 ng/mL 4′-hydroxynordiazepam



1 ng/mL dl methadone



1 ng/mL flunitrazepam (Rohypnol)

Figure 8. Ten ppm extraction window of 1 ng/mL solutions of four drugs of abuse.

Table 4 summarizes the limits of detection (LOD) for each of the components analyzed under this method. Note that while the method has not been optimized for any one component, it is designed to provide a broad screening tool for the analysis of drugs of abuse.

Table 4. LOD for Components by LC/MSD TOF

Component	Accurate mass	LOD (ng/mL)
α-Hydroxyalprazolam	325.085	5
7-Aminoclonazepam	286.0741	1
Diazapam	285.0789	1
Oxazepam	287.0581	5
Temazepam	301.0738	5
7-Aminoflunitrazepam	284.1093	5
7-Aminonitrazepam	251.1131	1
dl-11-nor-9-carboxy-δ-9-THC	345.206	5
Codeine	300.1594	50
Morphine 3β-d-glucuronide	462.1758	5
6-Acetylmorphine	328.1543	10
EDDP perchlorate	278.1903	1
(+)-Ephedrine	166.1226	20
Fenfluramine	232.1307	1
dl-MBDB:HCL	208.1332	5
(±) BDB Hydrochloride	194.1175	10
dl-MDEA	208.1332	10
dl-MDA	180.1191	50
dl-MDMA	194.1175	50
dl-Methamphetamine	150.1277	20
dl-Amphetamine	136.112	50
Phentermine	150.1277	20
(+)-Psuedoephedrine	166.1226	20
(–)-Cotinine	177.1022	20
4'-Hydroxynordiazepam	287.0581	0.5
Nordiazepam	271.0632	0.5
Flunitrazepam	314.0935	1
Flurazepam	388.1586	1
Desalkylflurazepam	289.0538	1
(–)-δ-9-THC	315.2318	1
(±)-11-Hydroxy-δ-9-THC	331.2267	1
Cocaine	304.1543	1
Benzoylecgonine	290.1386	5
Buprenorphine	468.3108	1
Morphine	286.1437	10
Normorphine	272.1281	10
Meperidine	248.1645	1
Normeperidine	234.1488	1
dl-Methadone	310.2165	1
EMPD	264.1746	1
Naloxone	328.1543	5
Oxycodone	316.1543	20
LSD	324.207	1
Iso-LSD	324.207	1
(±)-Phenylpropanolamine:HCL	152.1069	1
Fluoxetine:HCL	310.1413	1
GHB	127.0365	20
(–)-Nicotine	163.1229	5
2-oxo-3-hydroxy-LSD	Not detected in +veESI	ND

Detection of Drugs of Abuse in Bodily Fluids

Urine is the matrix of choice for the detection of drugs of abuse in areas such as workplace screening, and was therefore chosen to evaluate the LC/TOF MS method developed. To present a "worst case scenario", neat urine was spiked for this analysis and run directly with no sample clean-up. This would not normally be done; however, it was used as an illustration of the method's ability to provide a quick screening result without cleanup.

A further discussion of a solid phase extraction (SPE) sample preparation method that may be considered can be found in Agilent Technologies application note 5989-2260EN [4].

In the first instance, neat and spiked urine was scanned for both cocaine and benzoylegonine, a metabolite of cocaine (Figures 9 and 10). Recoveries of the spiked samples (100 ng/mL – representing a level lower than the traditional EMIT screen) were both approximately 100%.

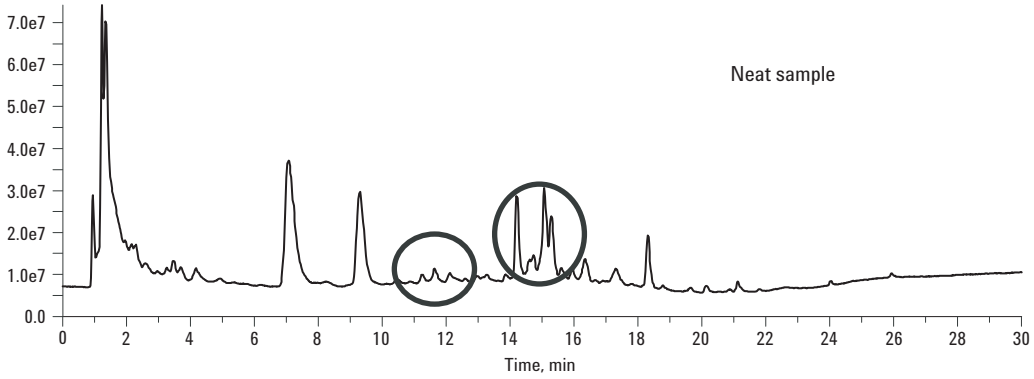


Figure 9. TIC of Blank Urine – Injected neat – circle shows expected retention time of analytes of interest.

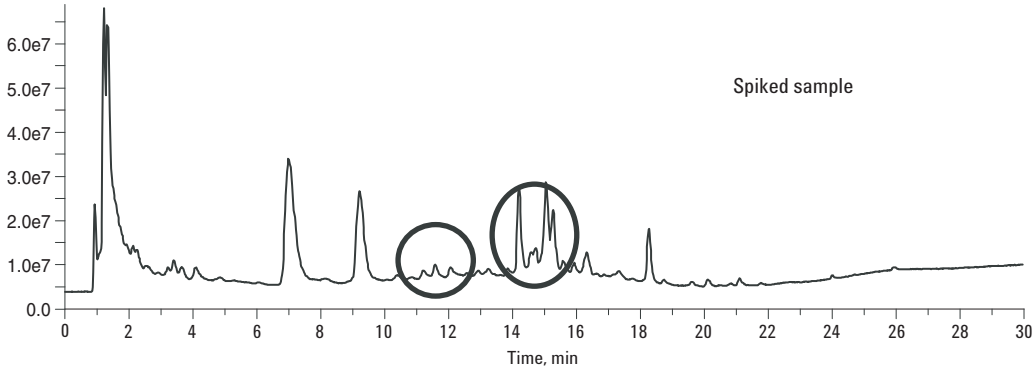


Figure 10. TIC of Neat Urine with 100 ng/mL spike of cocaine, benzoylecognine, flunitrazepam, and 7-aminoflunitrazepam.

It can be seen from Figures 11 and 12 that the ability to narrow the mass extraction window greatly reduces the noise for a given mass, and with retention time information can provide a high level of confidence in the assignment of a component.

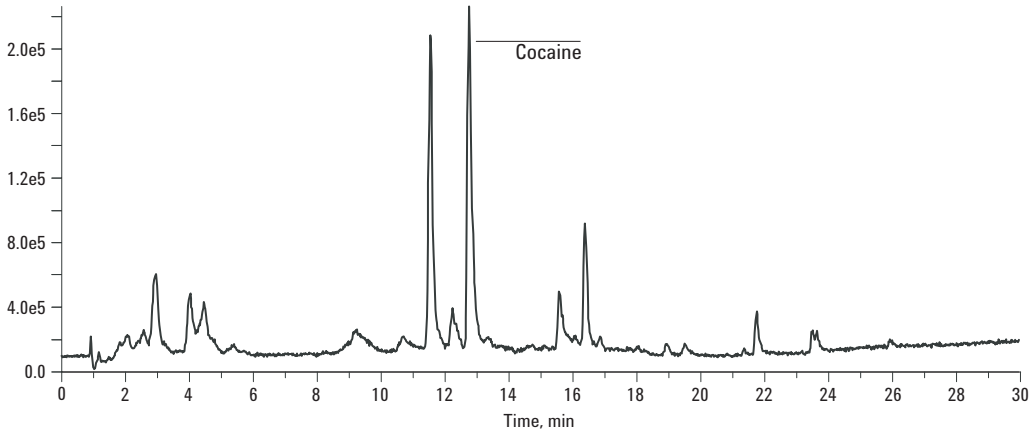


Figure 11. One amu extraction window of scanned target mass 304.1543 – cocaine.

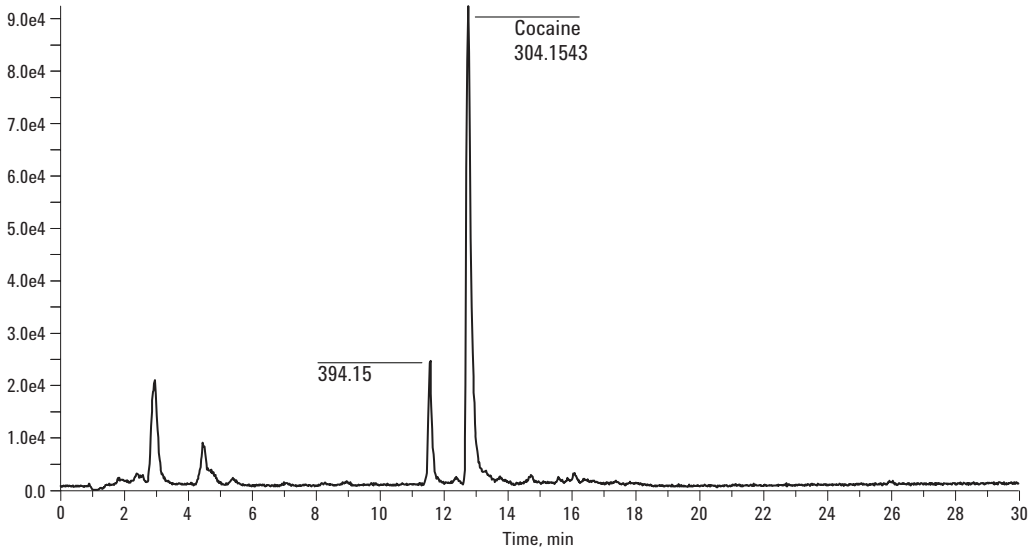


Figure 12. Ten ppm extraction window of scanned target mass 304.1543 – cocaine.

In the instance with Figure 12, the larger peak at approximately 12.9 minutes shows an excellent match with cocaine (mass error of ~0.2 ppm), while the earlier peak at 11.5 minutes has a mass of 394.15 and a fragmentation product in the extraction window (Figures 13 and 14).

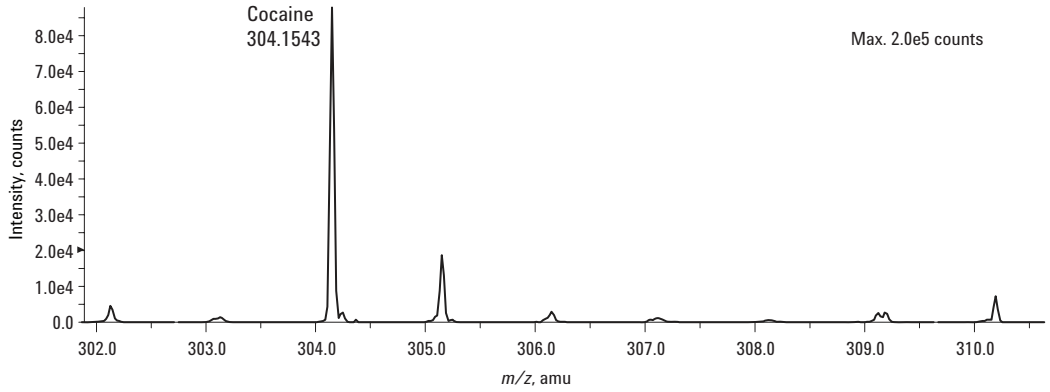


Figure 13. Confirmation of mass of cocaine.

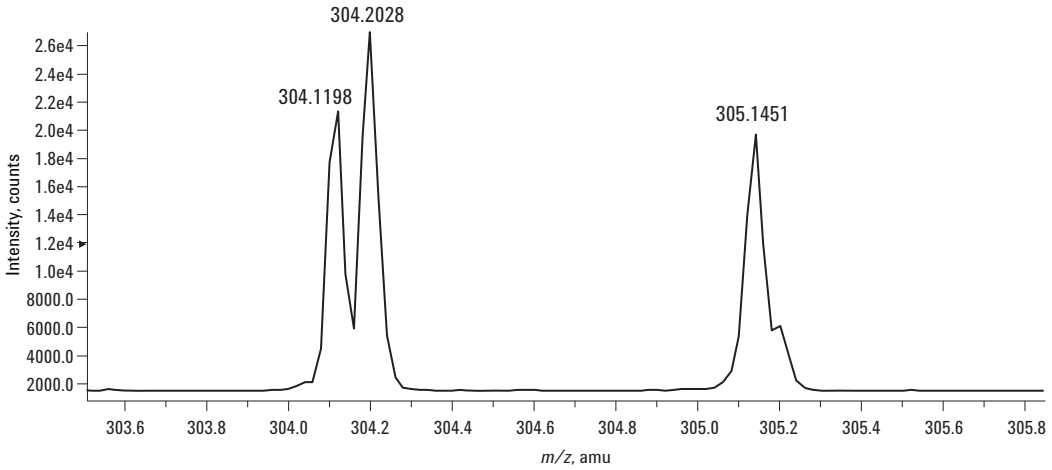


Figure 14. Mass spectrum 304 – 305 amu for peak at 11.9 minutes – no match of mass for cocaine.

A second drug that was of particular interest was flunitrazepam, another substance used as a date rape drug (Rohypnol), and one of its metabolites, 7-aminoflunitrazepam. Again, these components were spiked at 100 ng/mL into neat urine and injected directly. The recoveries achieved for these two components were approximately 40%, which is likely a result of ion suppression in the source. However, due to the excellent detection limits possible, even with up to 60% suppression of the signal, a clear peak can be seen for both compounds at 100 ng/mL (a level well below the EMIT screening reporting limits) when extracting a narrow mass window (Figures 15 and 16).

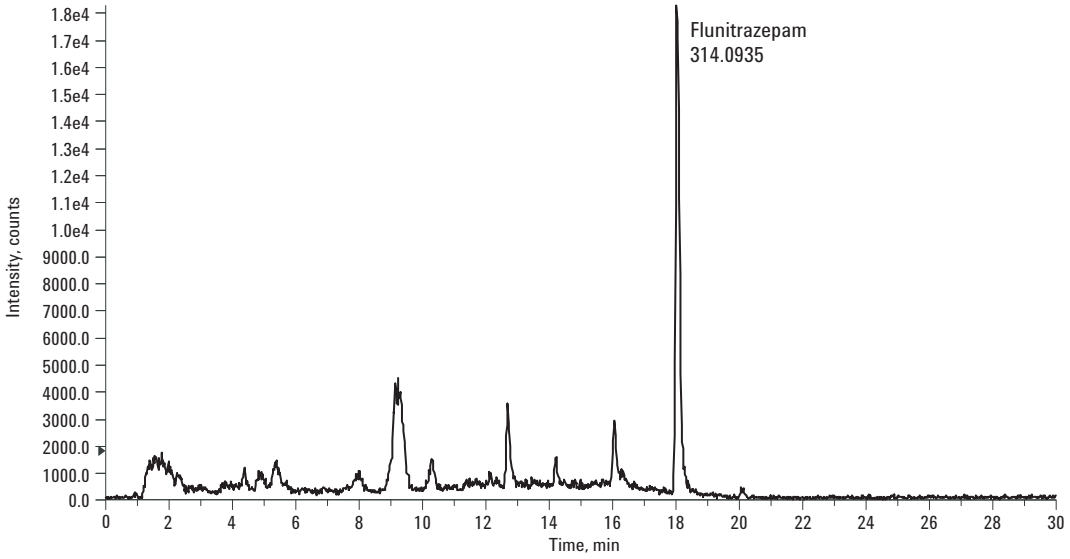


Figure 15. Ten ppm extraction window of scanned target mass 314.0935, flunitrazepam in neat urine. Retention time helps confirm presence at 18.1 minutes.

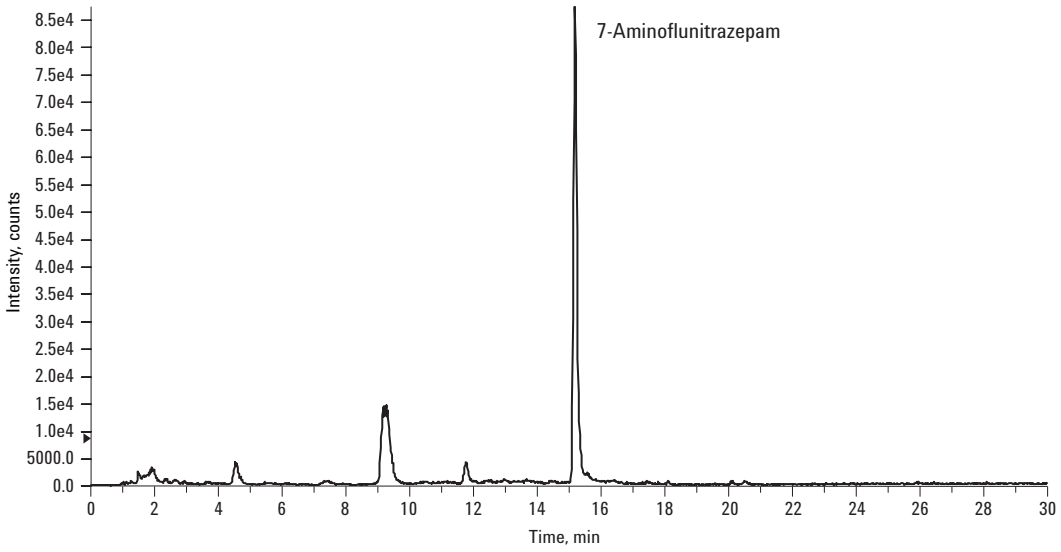


Figure 16. Ten ppm extraction window of scanned target mass 284.1191, 7-aminoflunitrazepam in neat urine. Retention time helps confirm presence at 15.3 minutes.

The second common matrix encountered in the screening of drugs of abuse is blood and plasma. To test the method when analyzing plasma, a sample was spiked with desalkylflurazepam. Sample preparation was again kept to a minimum, with a simple acetonitrile precipitation performed on the sample prior to injection. The effect of the mass extraction window on the detection of peaks, seen in Figures 17 to 20, shows the removal of interferences from the spiked plasma sample.

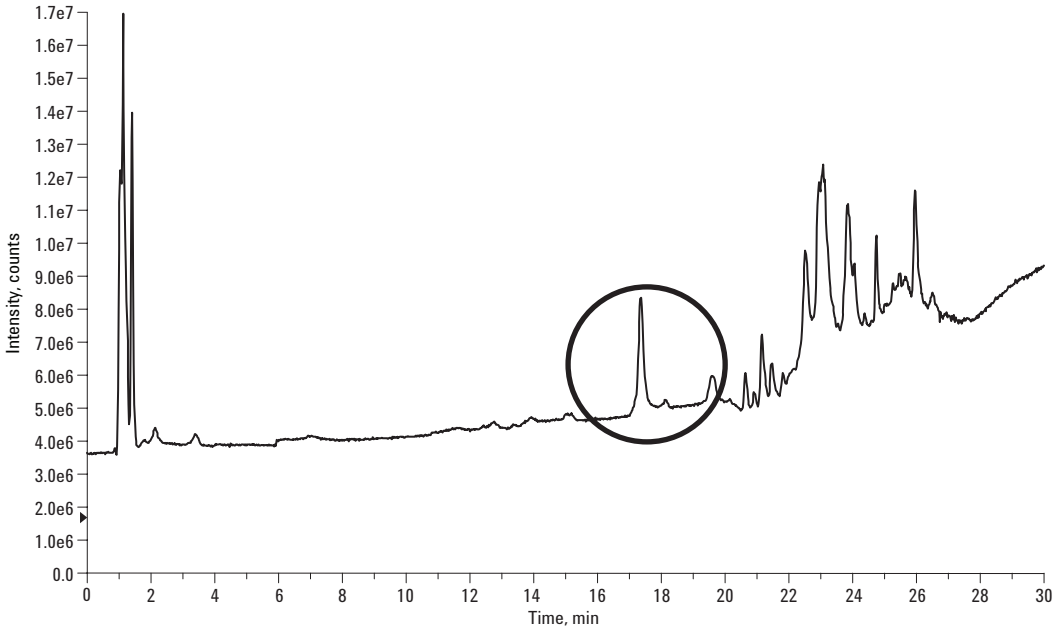


Figure 17. TIC of unspiked plasma.

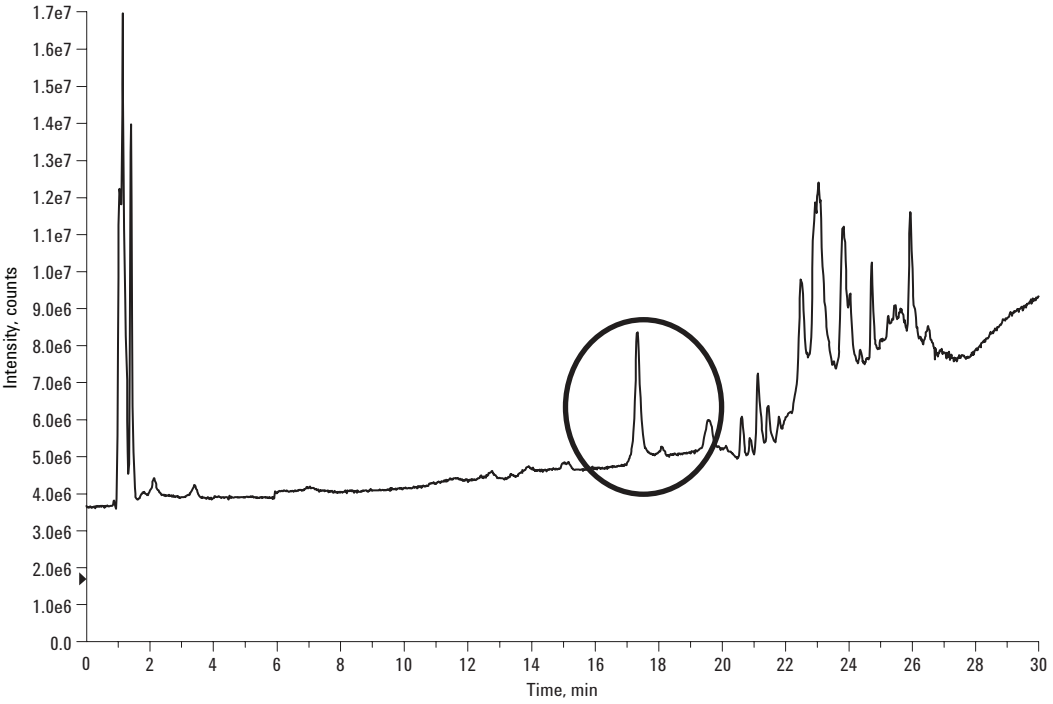


Figure 18. TIC of plasma spiked with 200 ng/mL of desalkylflurazepam.

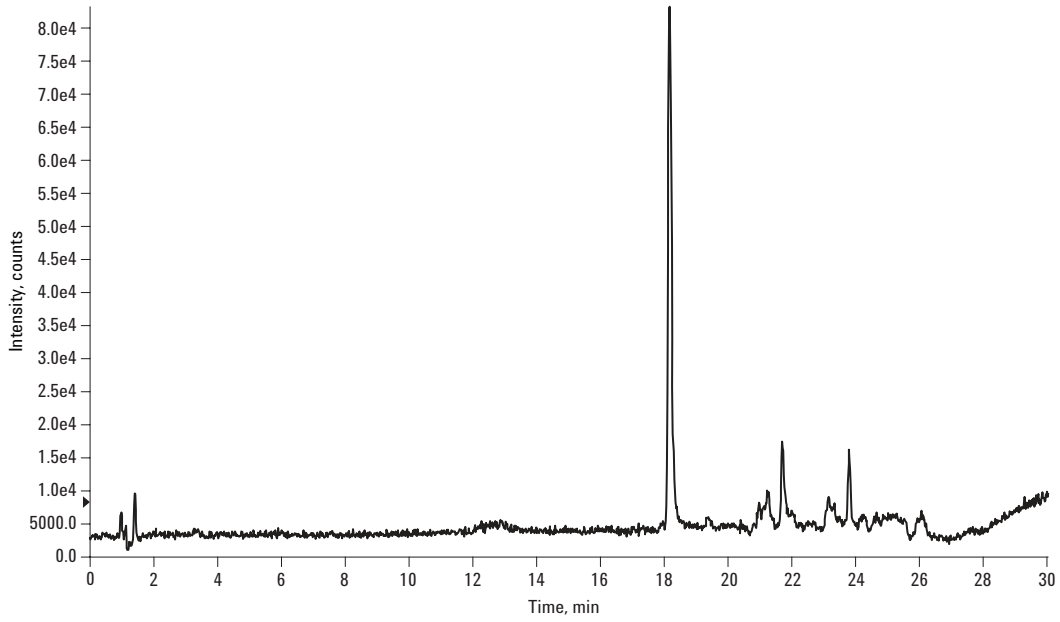


Figure 19. Mass extraction window (0.1 amu) of spiked plasma sample (346 ppm mass window).

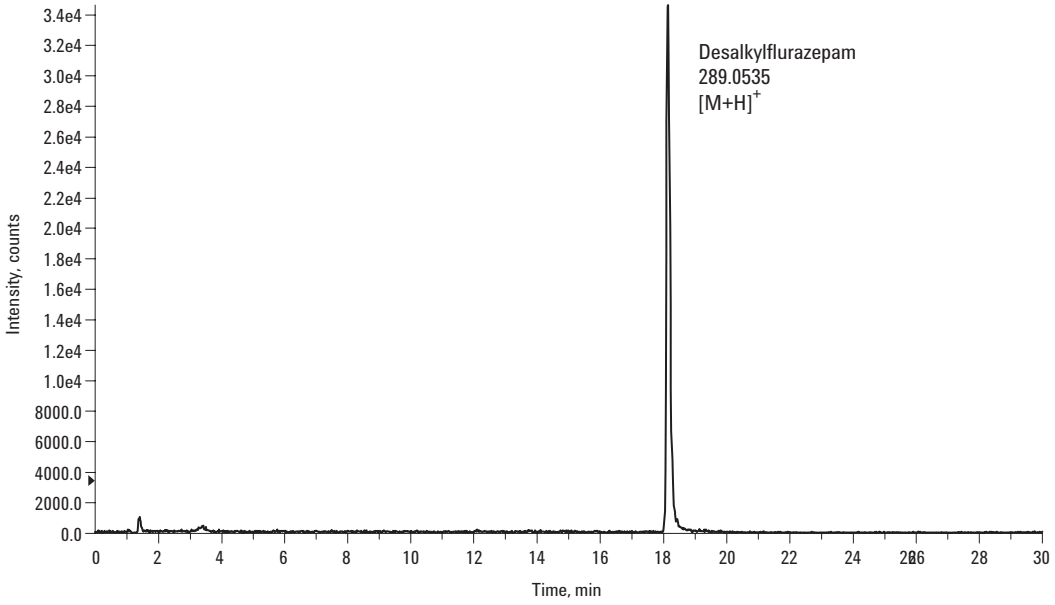


Figure 20. Ten ppm (0.003 amu) mass extraction window of spike plasma – note minimal noise.

Analysis of Coronial Samples – Using a Screener Database

The instrumentation and software provides the user the ability to create a screener database for all components they wish to automatically screen for. The minimum requirement for this database is the empirical formula and name for the component of interest, although the inclusion of a retention time will assist with confidence in the confirmation and reduce analysis time.

Several samples were acquired from the local coronial office to test the procedure that was developed. These samples were provided as butyl chloride extracts of blood samples obtained from deceased persons, for screening using the developed method.

The coronial samples supplied were screened using a database created from the 48 components analyzed under this method.

Sample Preparation

Samples were obtained from 1-mL blood volumes, liquid-liquid extracted with 6–8 mL of butyl chloride following centrifugation. Organic layer evaporated to dryness and then reconstituted in 100-µL mobile phase for a final 10-fold concentration.

Sample 1

Sample 1 was known to contain amphetamine, codeine, diazepam, and nordiazepam from the previous analysis performed at the coronial office. In addition to the four previously reported components, the screen also indicated the presence of

- Nicotine
- Cotinine
- Acetylmorphine
- Ephedrine
- Methamphetamine
- Pseudoephedrine

The total ion chromatogram (TIC) for this sample is shown in Figure 21.

An excerpt of the screen report is shown in Figure 22 with the details for cotinine. For each component included in the compound database, the screening report displays the extracted ion chromatogram, spectra of detected peak, and enlarged spectra of the target mass. This is accompanied by a summary table with the mass and retention time error. In this instance, an excellent match is seen for both retention time and mass.

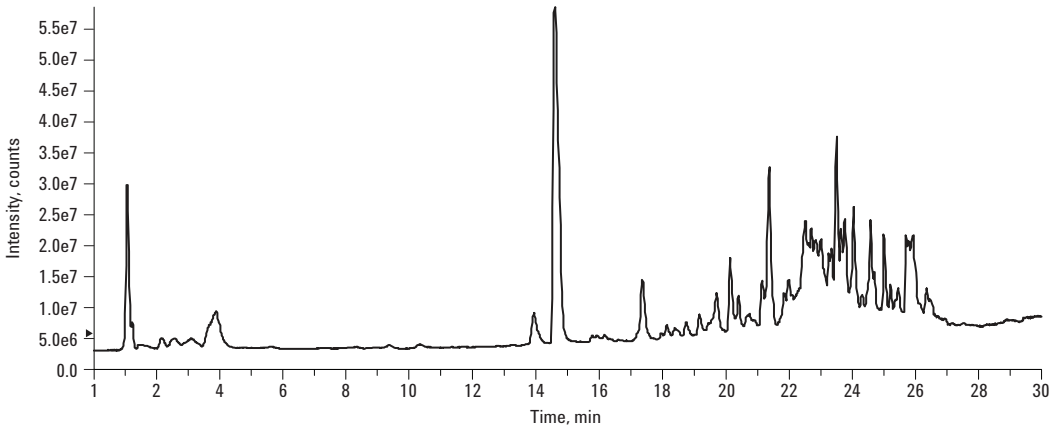
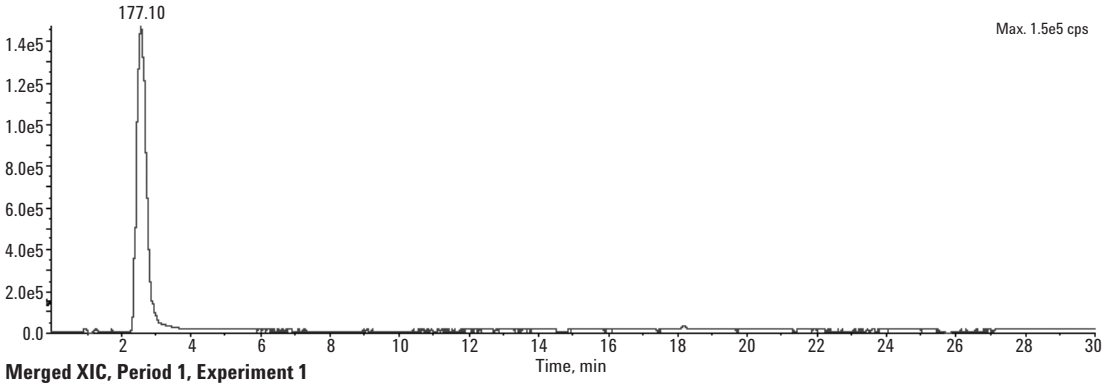
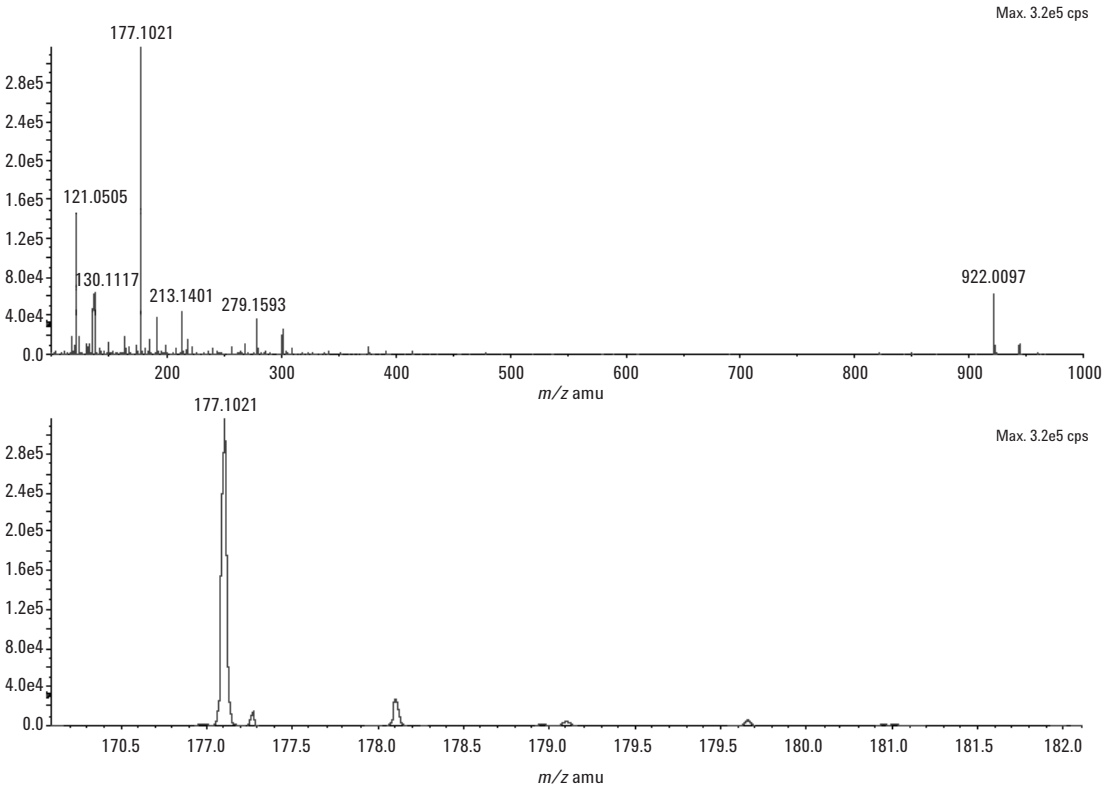


Figure 21. TIC of Coronial Sample 1.



Merged XIC, Period 1, Experiment 1



Formula	Compound name	Mass	Peak RT (min)	Peak area	Description	
$C_{10}H_{12}N_2O$	(-)-colinine	176.09496	2.57	2.78259 E7	_	
Species	**Abundance (counts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**	**Ret. time error (min)**
$[M + H]^+$	316885.14	177.10224	177.10215	-0.00009	-0.51	0.01

Figure 22. Excerpt from screener report of coronial Sample 1 showing confirmation of the presence of cotinine.

The results obtained from the analysis of Sample 1 suggest the deceased was a smoker, with the presence of both the nicotine and cotinine in the sample.

Sample 2

Sample 2 was known to contain citralopram, codeine, doxylamine, and tramadol from the previous analysis at the coronial office. In addition to the four previously reported compounds, screening with the database further showed the presence of

- Diazepam
- 6-acetylmorphine
- MDMA
- Methampethamine
- Cotinine
- Meperidine
- nicotine

A TIC of the sample is shown in Figure 23. It could again be inferred that this subject was a smoker.

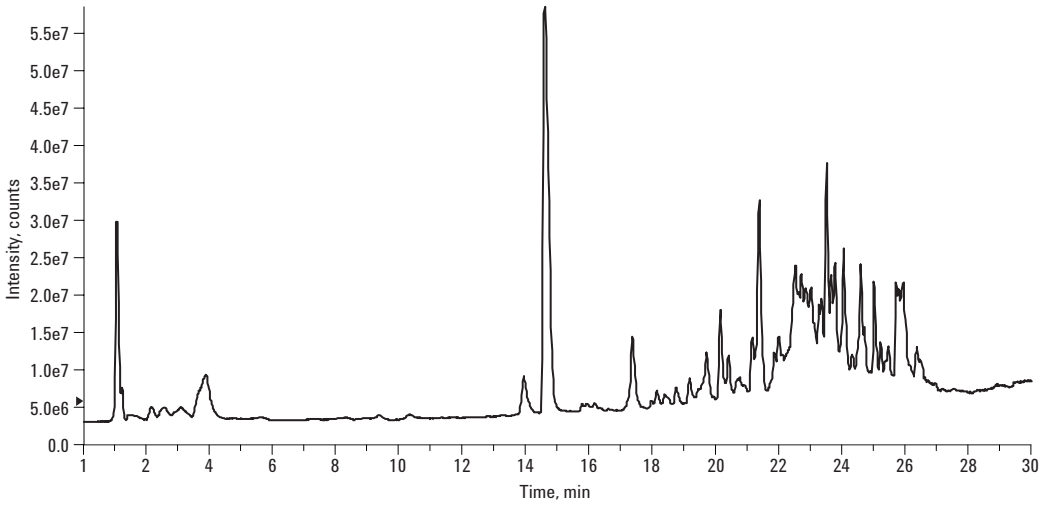
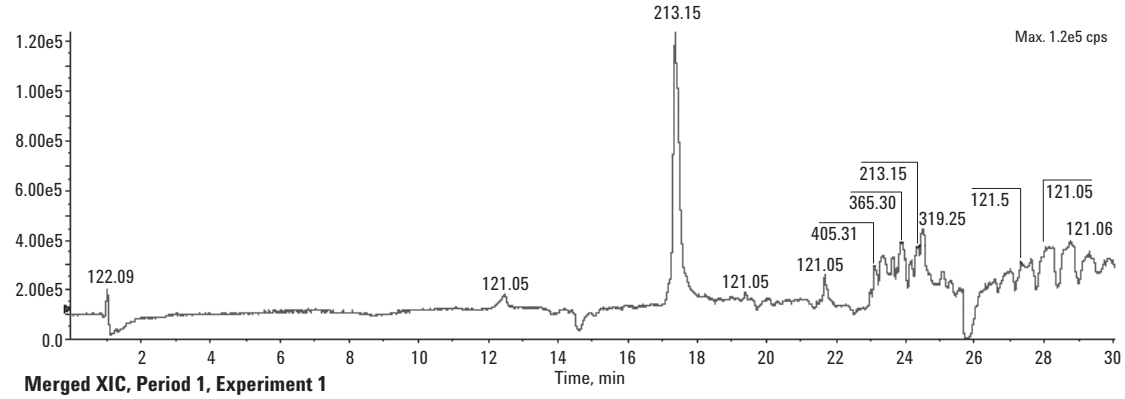
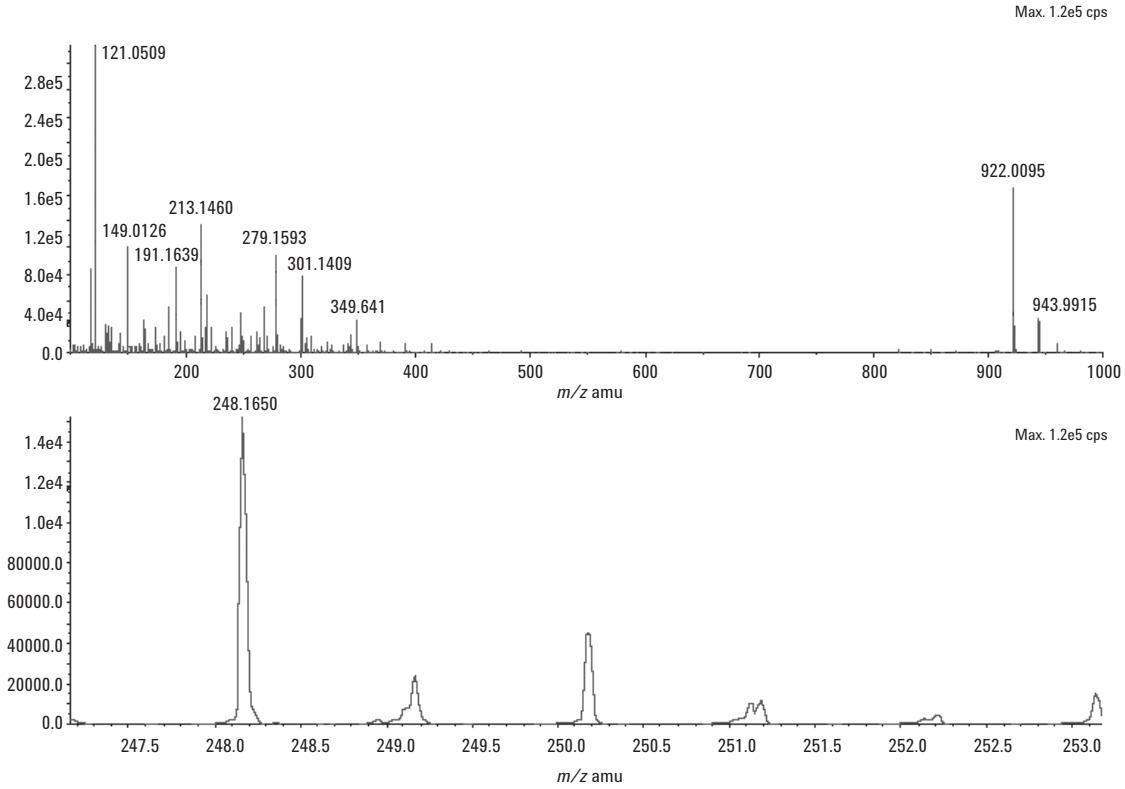


Figure 23. TIC of coronial Sample 2.

An excerpt of the screener report, in this instance for the confirmation of meperidine, is shown in Figure 24. Again, an excellent match to both retention time and mass can be seen.





Formula	Compound name	Mass	Peak RT (min)	Peak area	Description	
$C_{15}H_{21}NO_2$	Meperidine	247.15723	12.47	4.20401 E5	_	
Species	**Abundance (counts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**	**Ret. time error (min)**
$[M + H]^+$	15313.85	248.16451	248.16498	0.00047	1.90	0.07

Figure 24. Excerpt from screener report indicating presence of meperidine.

Conclusions

The analysis of drugs of abuse is important in many different areas. Current ana-lytical techniques use a two-step screening and confirmation procedure to achieve the required specificity and sensitivity required. This applica-tion note has investigated 48 of the more common drugs of abuse and their applicability for determi-nation through LC-TOF MS. It is not intended to be a comprehensive study of all possible components, but provides an excellent launching pad for the inclusion of the full gamut of possibilities

This application note shows the potential of the Agilent LC-TOF-MS as a single tool for both screen-ing and confirmatory analysis, with quantitative information, often at levels below those currently analyzed for today. As a final example of the power of this technique, real-life coronial samples were evaluated under a screening protocol, with an additional seven components other than those previously reported by the coronial office detected.

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2. Kolbjorn Zahlsen, Trond Aamo, and Jerry Zweigenbaum, “Screening Drugs of Abuse by LC/MS”, Agilent Technologies, publication 5989-1541EN www.agilent.com/chem
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Agilent Technologies

Database and Library Searching for Screening Toxins and Drugs-of-Abuse

The First Accurate Mass MS/MS Library for Forensic Toxicology Using the Agilent 6500 Series Accurate Mass Q-TOF LC/MS

The Broecker, Herre, & Pragst Personal Compound Database and Library virtually eliminates false positives and provides confident identification without standards.

Screening and identifying the large number of compounds that are of concern to forensic scientists and toxicologists is a formidable undertaking. The Agilent 6500 Accurate Mass Q-TOF LC/MS with the Forensic Toxicology Personal Compound Database and Library (PCDL) can screen and identify both the parent compound and resulting metabolites. There are over 7500 compounds in the database and over 2600 of them contain MS/MS spectra. Any of the Agilent Q-TOF LC/MS instruments can collect high resolution MS and MS/MS spectra with mass accuracies better than 3 ppm even for MS/MS fragments. Samples can be run and the database and library searched using Auto MS/MS and MassHunter Qualitative Analysis, which are powerful data mining tools that positively identify compounds with accurate mass of both precursor and fragment ion information.

Auto MS/MS precursor ions trigger MS/MS spectra to be collected under user defined conditions. All single MS ions detected are mined to determine if they represent compounds and if they do are searched against the database of compounds using exact molecular weight and the possible adducts. The MS/MS spectra are then searched for library matches and identified with both a forward and reverse score. Direct graphic and tabular inspection of the matches can be made. The power of the high quality data collected, data mining approaches, and the library allow a difficult task to be completed in hours versus days, with the confidence of a direct match from Agilent instrument to instrument.

Key Benefits

- Agilent 6500 Series Accurate Mass Q-TOF LC/MS provides the sensitivity needed with full spectra to determine toxins or drugs present in bodily fluids
- The Broecker, Herre & Pragst PCDL provides the greatest number of relevant compounds for screening and identification
- The database contains over 7500 compounds and metabolites with accurate mass MS/MS spectra for more than 2600 of them
- The library can identify a large number of compounds quickly
- False positives are virtually eliminated with confident identification of accurate MS/MS library search results
- Comprehensive workflows meet the needs of the specific analysis: Auto MS/MS for rapid screening Targeted MS/MS for focused analysis

Our measure is your success.

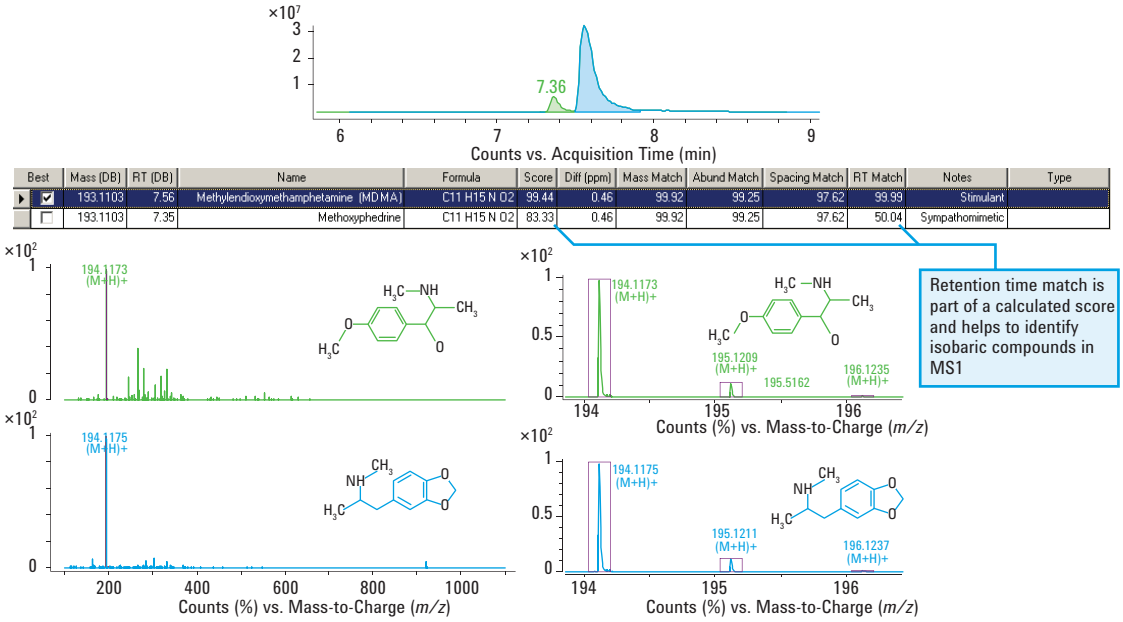

Figure 1: Single MS accurate mass data provides molecular formula but cannot determine isomers.

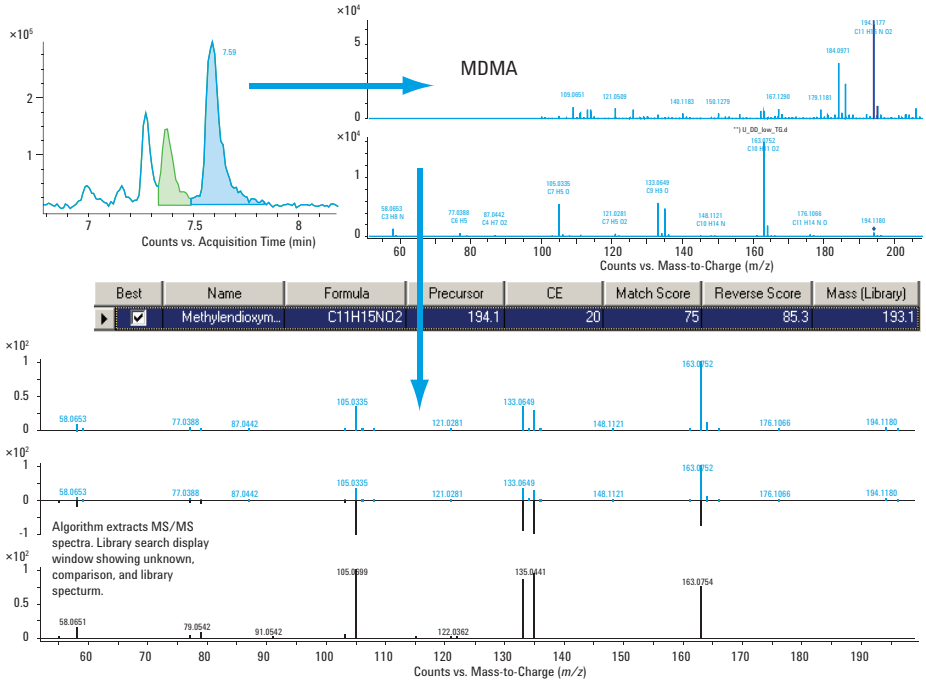

Figure 2: Detection of methoxyphedrine and MDMA isomers not distinguishable with a database search only without standards and retention time. With library, MDMA is readily identified.

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Agilent Technologies

Accurate Mass Measurement for Analyzing Drugs of Abuse by LC/Time-of-Flight Mass Spectrometry

Technical Overview

Forensic Toxicology

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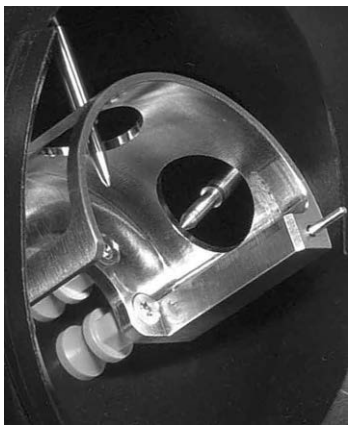
USA

In the world of forensic analysis, the unambiguous identification of chemical substances is crucial. The new Agilent 1100 liquid chromatography/ mass selective detector Time of Flight (LC/MSD TOF) system can provide important information for the forensic analyst towards identification and confirmation.

The 1100 LC/MSD TOF provides mass measurements with the accuracy and resolution to distinguish empirical formulas and separate interfering compounds with the same nominal mass. Figure 1

The new Agilent 1100LC/MSD TOF system



Dual-electrospray source

Agilent Technologies

shows 14 basic drugs of abuse (DA) separated using the LC/MSD TOF; Table 1 gives the identity of these compounds along with their retention times (RTs). Chromatographic separation is not complete in some cases, as indicated in the total ion chromatogram (TIC) between 3.3 and 4.0 minutes. However, examination of extracted ion profiles for each of the $[M+H]^+$ ions shows that each compound can be identified without interference. In the case of the isomers, hydrocodone and codeine, the extracted ion chromatogram shown in Figure 2 clearly demonstrates that what the mass spectrometer cannot do, chromatography can. These data also demonstrate the high degree of mass measurement reproducibility achieved within a chromatographic run. The averaged mass spectrum across the chromatographic peak of these two isomers is identical, as shown in Figure 3.

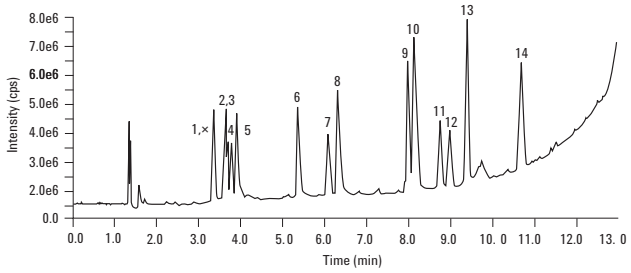


Figure 1. TIC of basic DA. LC/ES-MS TOF separation of 14 basic DA used as targeting compounds in toxicological sample screening.

Conditions

Column	ZORBAX Eclipse C18 XDB, 150 × 4.6 mm id, 5 µm
Part number	993967-902
Agilent 1100 binary pump flow	0.9 mL/min
Solvents	A = 0.1% Formic acid in water B = 0.1% Formic acid in acetonitrile
Gradient	10% B to 20% B in 1 min, then to 70% B in 11 min, then to 100% in 12 min; run time = 13 min
Agilent 1100 wellplate autosampler injection volumes	From 0.1 µL to 10 µL

Table 1. Compounds* and RT as Found in Figure 1.

Peak Number	RT (min)	Compound
1	3.37	Hydrocodone
x	3.40	Unknown with M + H = 166, 1221
2	3.64	Oxycodone
3	3.70	Amphetamine
4	3.81	Codeine
5	3.94	Methamphetamine
6	5.41	Cocaine
7	6.12	CE
8	6.36	PCP
9	8.04	Propoxyphene
10	8.18	Methadone
11	8.81	Alprazolam
12	9.00	Nordiazepam
13	9.46	Methaqualone
14	10.86	Diazepam

*Special thanks to Lucas Zarwell of the Washington D.C. Medical Examiners Office for providing the reference material.

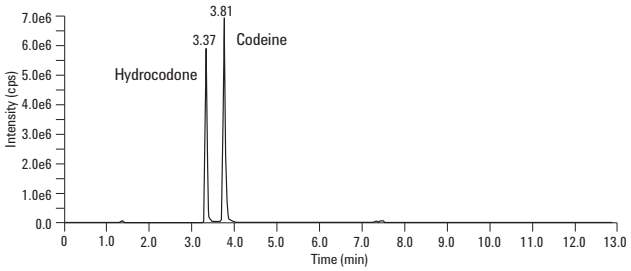


Figure 2. Extracted ion chromatogram. Ion chromatogram of *m/z* 300.0–300.2 extracted from the data shown in Figure 1. Isomers are chromatographically separated, facilitating their identification.

Because the exact mass of isomers is the same (meaning they have the same empirical formula), the mass spectrometer cannot distinguish between them. On the other hand, compounds with the same nominal mass that have different atoms will be distinguished by an accurate mass measurement. When this happens, mass resolution (M/ΔM) from >4000 (at *m/z* 200) to >10000 (at *m/z* 2722), is sufficient to resolve most nominal mass coeluting compounds. Resolution of ~7000 for *m/z* 300 in Figure 3 shows the high level of separation between the C12 and C13 isotopes.

The Agilent 1100 LC/MSD TOF system provides accurate mass measurement through highly stable electronics, a flight tube constructed with a very low coefficient of thermal expansion, and a design that maximizes consistent measurement of flight

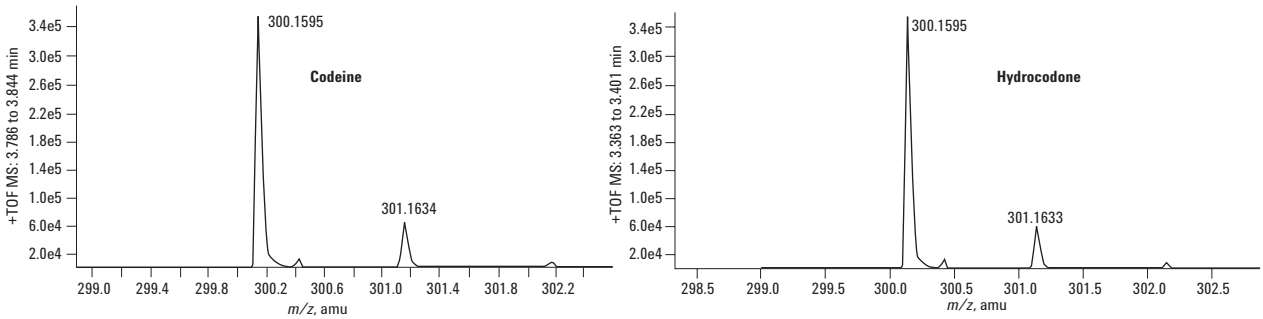


Figure 3. Mass spectra of narcotic isomers. Spectra of codeine and hydrocodone isomers representing an average spectra taken across the chromatographic peak.

time. This includes algorithms designed to minimize the error involved in calculating the center of the distribution of the ions' flight times. The Agilent calibrant delivery system (CDS) and a continuously infused reference material, a lockmass, ensure that every spectrum that is collected is automatically calibrated and stored. The reference material, compounds used in our patented calibration solution, though any reference material is acceptable, is infused at both a low concentration and low flow through the second of a dual-electrospray source. This assures that effects like ion suppression will not be caused by the lockmass. In addition, the use of analog-to-digital conversion provides a dynamic range of almost three orders of magnitude. Table 2 shows the mass accuracy achieved from 50 pg of these compounds to 50-ng injected on-column. Accuracy of better than 5 ppm is achieved for amphetamine and better than 2 ppm for oxycodone. Note that for low mass measurements, the number of possible empirical formulas is far less and a 5-ppm range is more than sufficient. At a higher mass, the possibilities increase and a lower range for error is needed to provide confirmation or suggest a reasonable empirical formula to aid the identification of an unknown.

Table 2. Accurate Mass Measurements vs. Concentration of Some DA in Reference Material Using Targeted Automatic Search of Empirical Formula

Peak Number	**3**	**5**	**1**	**4**	**2**
Compound	Amphetamine	Methanphetamine	Hydrocodone	Codeine	Oxycodone
Nominal *(m/z)*	136.10	150.10	300.15	300.15	316.15
Conc. (ng-injected)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)
50.00	–4.97	–2.41	0.94	0.94	1.32
25.00	3.53	–2.49	1.17	1.60	0.27
5.00	–4.71	–3.01	0.37	0.37	0.04
5.00	–4.53	–3.03	0.47	0.37	–0.16
5.00	4.53	–3.05	0.30	0.30	–0.75
2.50	5.00	–2.78	1.60	1.60	–0.42
0.50	–4.23	–2.20	1.34	1.90	0.04
0.50	–5.01	–2.48	1.11	1.27	–0.33
0.25	–5.70	–2.69	0.95	1.27	–1.16
0.05	5.00	–5.42	3.60	2.26	0.53

Figure 4 shows a response vs. concentration plot for codeine. The highly-linear response indicates this instrument can also be used for quantitative analysis. Note that the 50-ng injection was excluded, with this compound and others, because of detector saturation. For these compounds at saturated concentrations, accurate mass measurement was made at the edges of the chromatographic peak with an automated script. Also, a detection limit was not set and the 50-pg injection was made as an arbitrary low standard. The LC/MSD TOF specification for reserpine is 10 pg at a signal to noise ratio of 10:1. With the instrument's high mass resolution and seamless auto-calibration of every spectrum collected, selectivity of the extracted ion is increased.

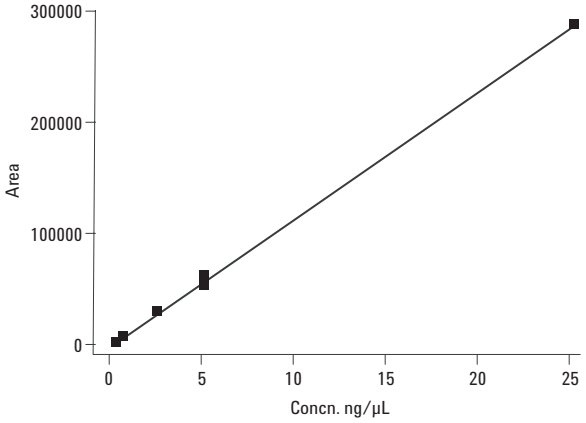


Figure 4. Codeine linearity. Plot of codeine extracted ion (ms/300.0–300.2) chromatographic peaks measured from 50-pg injected on-column to 25-ng on-column. TOF detector saturated at 50 ng.

Conclusion

The new Agilent LC/MSD TOF provides routine and seamless accurate mass measurement for confirmation of these drugs of abuse. Very high sensitivity is achieved and, with the TOF detection, all data are “full scan,” allowing compounds that are not targeted to be detected. The system offers a wide dynamic range capable of providing accurate mass measurements across that range without having to match lock-mass signal intensity with analyte intensity. Finally, a linear response is achieved within a concentration range below detector and electrospray saturation.

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Screening and Confirmation of Anabolic Steroids Using Accurate Mass LC/MS

Application Note

Forensic Toxicology

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Abstract

The use of the Agilent LC/MSD TOF, an orthogonal-axis time-of-flight (oa-TOF) mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source, is described for the analysis of four anabolic steroids and one internal standard, in urine extracts at a 1–2 ng/mL concentration. The high degree of mass accuracy (<3 ppm) of the TOF instrument allows for both excellent confirmation by empirical formula determination and quantitation using extracted ion chromatograms (EICs). The accurate mass capability of the LC/MSD TOF produces EICs with sufficiently narrow mass widths to exclude most chemical noise contributions. For increased chromatographic resolution and speed, a C18 column with a 1.8-micron particle size was employed.

Introduction

The use of anabolic substances for performance enhancement in sports is receiving increasing attention [1, 3]. Their use is a problem in some professional sports, and a growing problem in high school and collegiate athletics, and an area of doping in which new compounds are continually being introduced (for example, BALCO/THG in the USA and issues with nutritional supplements). The typical doping control analysis for anabolic steroids and related substances in urine includes screening by gas chromatography/mass spectrometry (GC/MS) [2], followed by confirmation of the screening result using high-resolution magnetic sector GC/MS in EI mode [4]. Because of the high initial and operational costs of high-resolution magnetic sector instruments, alternative techniques for the confirmation of screening results are being explored [5].

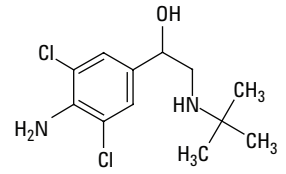
Tandem GC/MS is an alternative to high resolution GC/MS, because tandem GC/MS is an established technique for drug confirmation, and because selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) acquisitions with ion ratio calculations provide high confidence in the identification. Liquid chromatography/mass spectrometry (LC/MS) has also been used as an alternative confirmation technology with the advantages that it uses a different mode of chromatographic separation and different ionization techniques [6].

Accurate-mass API LC/MS has significant advantages because it provides not only the same spectral information, such as isotope ratios and presence of heteroatoms as previous methods, but also allows for the highly specific detection of the intact molecule and confirmation of the empirical formula. Approximately 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily detected or confirmed using GC/MS, but are amenable to LC/MS [7]. The analysis of many of these compounds is further complicated because they must be detected and confirmed at a 2-ng/mL concentration or lower in urine [the Minimum Required Performance Level (MRPL) for a laboratory, in the World Anti-Doping Agency (WADA) program]. This application note describes the results of using an Agilent LC/MSD TOF instrument for detection and confirmation of a number of analytically challenging anabolic substances at the WADA MRPL.

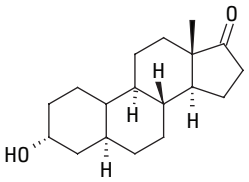
The anabolic substances analyzed in this work and their structures are shown in Figure 1.

Most of these analytes, including the internal standard (ISTD), showed better sensitivity using APCI than electrospray ionization (ESI). This might be expected for these relatively non-polar analytes that contain no basic groups. APCI is also less susceptible to ion suppression from co-eluting endogenous materials, and tends to give simpler spectra than ESI (free of complicating adducts such as Na^+ and K^+).

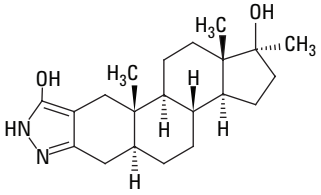
One complication of APCI can be the loss of water from the initially-formed protonated molecule due to either the thermal effect of the heated vaporizer, or to the ionization driven by the corona discharge of APCI. For some compounds, water loss is even observed in the milder ESI mode which involves ionization in the liquid phase and less contact of the analyte with heat. However, the mass



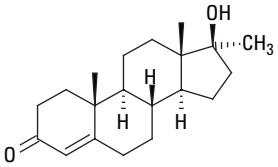
Clenbuterol



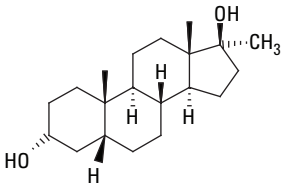
19-norandrosterone



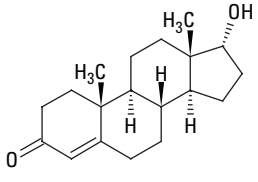
3'-OH stanozolol



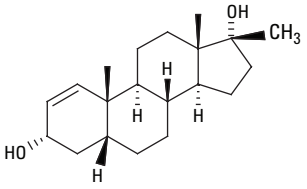
Methyltestosterone
(internal standard)



Methyltestosterone
metabolite
(17α-methyl-5β-androstane-3α,17β-diol)



Epitestosterone



Epimetendiol
(methandrostenolone
metabolite)

Figure 1. Compounds analyzed.

measurement of water-loss ions still retains its mass accuracy and the ion ratios for the $[M+H]^+$ and the subsequent water-loss ions are reproducible with this system. It is interesting to note that more water loss occurred with the later-eluting steroids rather than with the more polar early-eluting compounds.

Experimental

Sample Preparation

The sample preparation method used in this study was developed at the Center for Human Toxicology, Sports Medicine Research and Testing Laboratory, for screening of steroids in urine by GC/MS. The same sample preparation was used for the LC/MS analysis, except derivatization was unnecessary. The internal standard (20 µL of 10 ng/µL methyltestosterone) was added to 3 mL of urine, followed by 1 mL of 0.15M sodium acetate, pH 5. This solution was vortexed-mixed and then transferred to an Extrelut-3 column (Merck, VWR catalog number 48219-494, pkg of 100) connected in-line to an amino SPE column (J. T. Baker, VWR catalog number JH7088-3, pkg of 50) also containing 1 g of sodium sulfate. After an 8-minute delay, the columns were eluted with 9 mL of diethyl ether into a 13 × 100 mm silanized conical glass tube. For LC/MS, the final extracts were simply evaporated to dryness with a stream of nitrogen at 40°C. The tubes were capped and sent by overnight courier to the Agilent laboratory (Pleasanton, CA) where they were stored at –10°C while awaiting analysis. The residues were reconstituted in 100 µL of the initial mobile phase just prior to analysis.

LC/MS Method Details

The API-TOF system consisted of an Agilent 1100 LC system (vacuum degasser, binary pump, wellplate autosampler, thermostatted column compartment, and diode array UV-VIS detector), interfaced to a G1969A LC/MSD TOF mass spectrometer. The mass spectrometer was operated with either the orthogonal ESI or APCI sources. The instrument was autotuned weekly using the automatic built-in calibrant delivery system and Agilent-developed calibrant compounds. The mass axis was calibrated daily using the same mix and an automatic calibration routine. Spectra were internally mass-corrected in real time using an automatically-introduced reference mass solution containing two known compounds (purine and calibrant HP-921) bracketing the mass range of interest at *m/z* 121.050873 and 922.009798, respectively. The optimized LC, MS, and APCI conditions are shown in Table 1.

Table 1. LC/MS Conditions for the Analysis of Anabolic Substances in Urine

LC Conditions	
Column:	Agilent ZORBAX RRHT SB-C18 2.1 × 50 mm, 1.8 µm (Agilent part number 822700-902)
Mobile phase:	A = 0.1% Formic acid/water B = Methanol
Flow rate:	0.4 mL/min
Col temp:	55°C
Gradient:	55% B, hold 5 min 55% to 75% B from 5 to 9 minutes
Analysis time:	14 min
Post-time:	5 min
Injection volume:	4 µL
MS Conditions	
Ionization mode:	Positive APCI (final method)
Capillary voltage:	3500 V
Vaporizer temp:	450°C
Corona current:	4 µA
Nebulizer:	60 psig
Drying gas flow:	5 L/min
Drying gas temp:	350°C
Scan:	*m/z* 100–1000, 10,000 transients/scan (0.89 sec/scan)
Reference masses:	121 and 922 (added post-column at 5 µL/min, 10 µM solution)
Fragmentor:	150 V [no collision-induced-ionization (CID)]
Skimmer:	60 V (default)
Octopole RF:	250 V (default)

Results and Discussion

Accurate-mass API-TOF LC/MS is commonly used for empirical formula determination and confirmation of naturally-occurring and synthetic molecules. The instrument used here was specifically designed to be as easy to use as a quadrupole GC/MS or LC/MS, by virtue of features such as automatic tuning and calibration, automatic reference mass correction, and improved instrument stability resulting from its mechanical and electronic design. The instrument has a mass resolution of approximately 7000 in the *m/z* range of the anabolic steroids, a routine mass accuracy of 3 ppm or less, and operates in full scan mode. Full scan has the advantage over MRM-MS/MS of not being a target analysis acquisition. Therefore, additional compounds can be detected without modifying the acquisition method or developing and optimizing specific MS/MS parameters.

Initial studies using unextracted standards of these and other steroids with ESI demonstrated that the instrument could measure the *m/z* of the $[M+H]^+$ ion to 3 ppm accuracy or less under routine unattended operation. However, the analytes included in this work showed better sensitivity using APCI. Theoretically, APCI might have been predicted to be the ionization mode of choice because the analytes tested are relatively nonpolar, and many contain no readily ionizable functional groups. Also, APCI is less susceptible to ion suppression from coeluting endogenous materials and tends to give simpler spectra than ESI (free of complicating adducts such as Na^+ and K^+).

Figure 2 shows the base peak chromatogram of an unextracted standard equivalent to 16 ng/mL, if extracted from a 3-mL urine sample. An objective of this method development was to obtain separation of the target analytes in less than 15 minutes. Although this was achieved, it was unexpectedly challenging due to the range of polarities from clenbuterol to epimetendiol. In future work we plan to evaluate additional LC columns to improve the separation between epitestosterone and the internal standard while maintaining a reasonable analysis time.

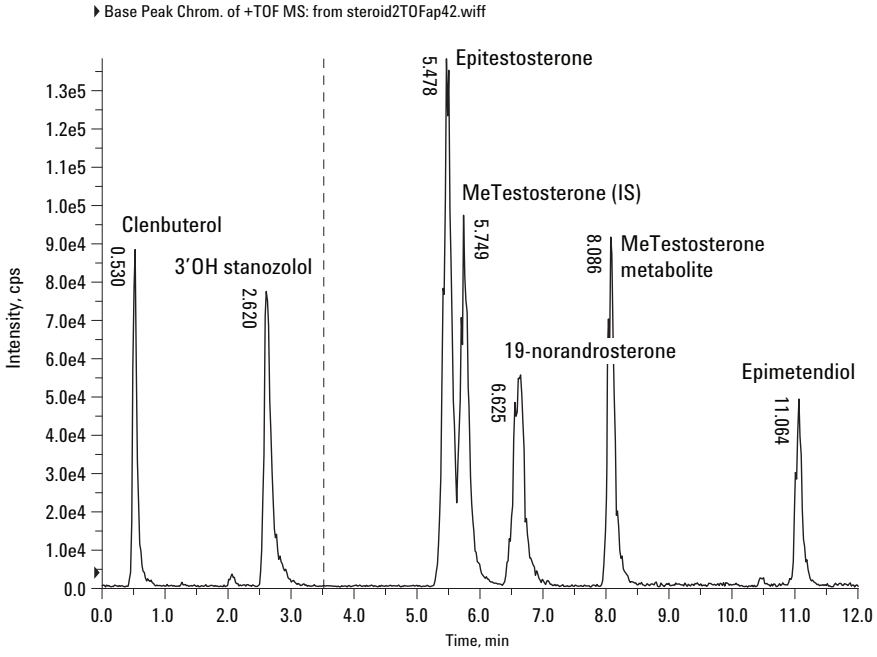


Figure 2. Base peak chromatogram of anabolics mix (unextracted standard equivalent to 16-ng/mL urine).

The vaporizer temperature and corona current were optimized for the best sensitivity across the range of analytes. In these studies, no in-source collision induced dissociation (CID) was used but in-source CID will be evaluated in future studies as a mechanism for generating ions for potential ion ratio calculations. The detection limits for these compounds were also improved by optimizing the number of transients/scan while maintaining a sufficient number of scans to accurately describe each chromatographic peak. In addition, increasing the photomultiplier voltage by only 50 V improved the detection limits without a significant increase in background noise.

An example of the typical performance of the LC/MSD TOF is shown in Figure 3 for the compound epitestosterone. The APCI spectrum shows both the $[M+H]^+$ protonated molecule and the less abundant $[M+H-18]^+$ water-loss ion. Applying the formula for mass accuracy shown in the Figure demonstrates a mass error of –1.04 ppm for the measurement of the $[M+H]^+$ ion.

The inset in Figure 3 also shows a mass resolution of 6890 measured for the $[M+H]^+$, and the large separation between the $[M+H]^+$ ion and the ^{13}C isotope peak at *m/z* 290. The resolution (R) at *m/z* 289.2159 can be calculated by dividing the *m/z* value by the full width at half maximum (FWHM or $w_{1/2}$) in Da. That is, $R = M/w_{1/2}$ where M = 289.2159 and ΔM is the width of the mass peak at half maximum, or half of its intensity. In this case, $w_{1/2}$ = 0.042 Da, so that R = 289.2159/0.042 = 6890. The accurate mass and ion ratio for the ^{13}C peak also have to match for the empirical formula calculated from the $[M+H]^+$ to be a correct assignment.

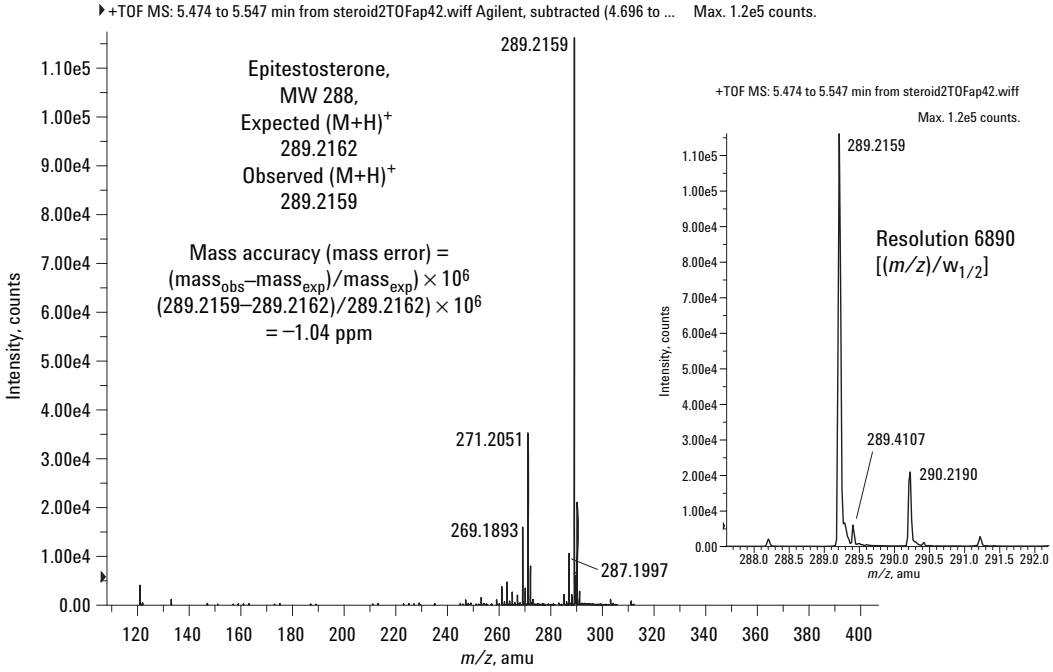


Figure 3. Typical APCI spectrum and TOF performance for epitestosterone, $[M+H]^+$ = 289.2162. Also shown is a water-loss fragment at *m/z* 271.2051.

Although there was some water loss observed in the spectrum of epitestosterone (the *m/z* 271 ion), for 19-norandrosterone [Figure 4], the most prominent ions in the spectrum are those at *m/z* 259.2049 and 241.1949. These represent the loss of one and two water molecules, respectively, from the protonated molecule. The loss of water was steroid-dependent and could not be entirely eliminated even by using ESI or by adjusting source conditions such as voltages and solvent-evaporation parameters.

Also note in Figure 4 the presence of the reference mass ions at 121 and 922, used to calibrate the mass axis for every scan in order to maintain mass accuracy throughout the acquisition. These ions are two compounds added automatically post-column as a dilute solution using a separate LC pump and a zero-dead-volume mixing tee at the MS system's inlet filter. In this case, the compounds are purine and HP-921 (a member of the Agilent API calibrant series), which are supplied with the LC/MSD TOF in a reference mass kit. Only a few thousand counts of reference mass are necessary to automatically calibrate each spectrum in real time. The instrument automatically reports any failure to find the reference masses on the screen and in a log file.

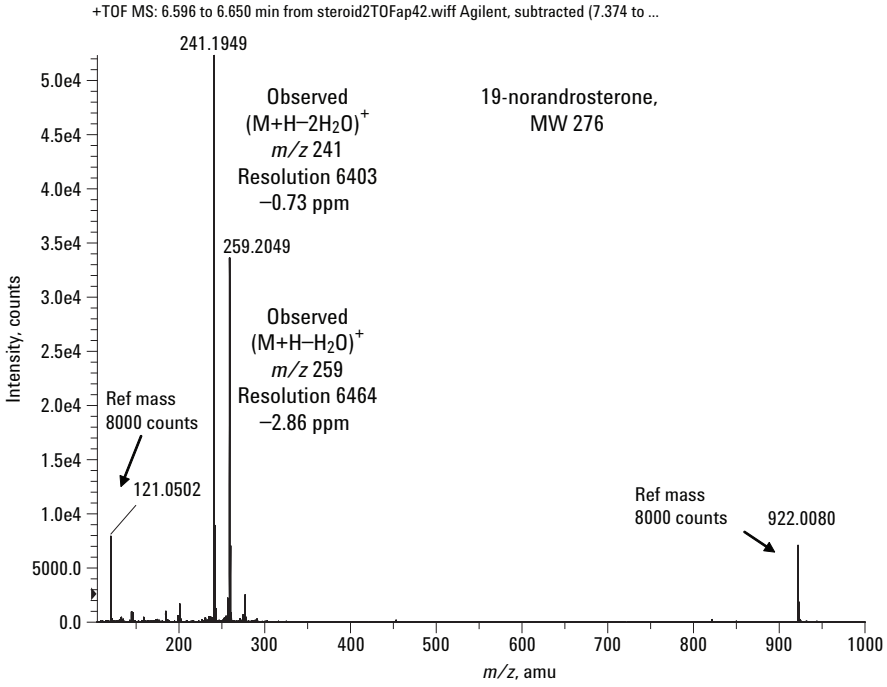


Figure 4. Typical APCI spectrum for 19-norandrosterone, MW 276, showing two water-loss ions. Reference masses at *m/z* 121 and 922 also shown.

A base peak chromatogram of an extracted steroid-free control urine fortified with epitestosterone, 19-norandrosterone, methyltestosterone metabolite and epimetendiol at 50 ng/mL, and 66 ng/mL of the methyltestosterone ISTD, is shown in Figure 5. Clenbuterol and 3'-hydroxystanozolol are not shown in the Figure because they were poorly recovered with the extraction procedure used at this stage of the method development. Because of the reduced recovery, these analytes are not shown in the subsequent Figures. Although epitestosterone and the internal standard appear unresolved chromatographically in the base peak chromatogram, they were resolved by their extracted ion chromatograms (EICs).

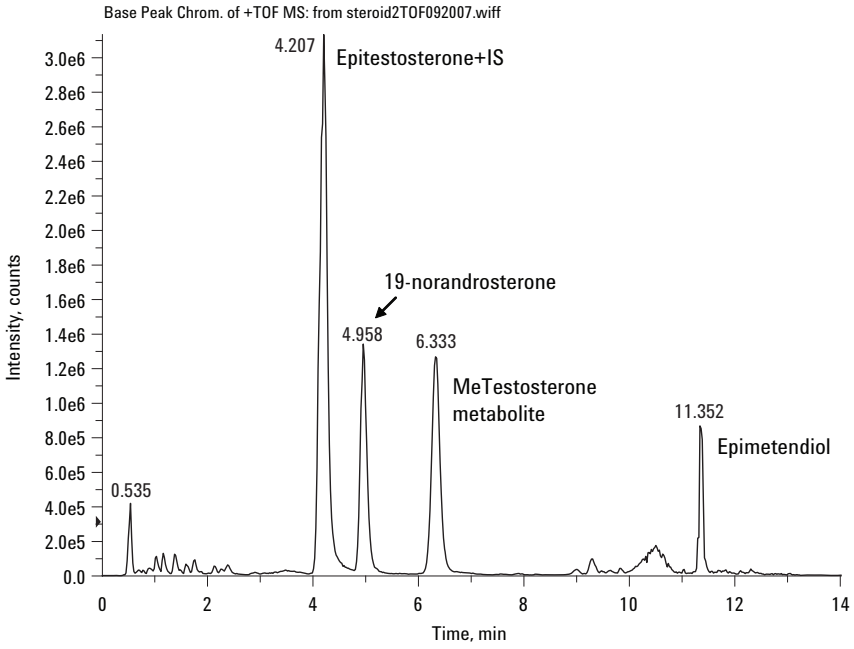


Figure 5. Extract of 50 ng/mL urine. Analytes are visible in base peak chromatogram.

Figure 6 shows the EICs from an extract of urine that was fortified with 2 ng/mL of epitestosterone, methyltestosterone metabolite and epimetendiol, and 1 ng/mL (the MRPL) of 19-norandrosterone. As shown, the EICs had 50,000–100,000 count intensities and excellent signal-to-noise, which permitted easy peak detection and location of spectra. For quantitation, one can take advantage of the high degree of mass accuracy with the TOF by designating narrow mass widths for the EICs. In this study, we used mass widths of 1 mDa (~3 ppm) for the EICs. For example, for 19-norandrosterone detection, the $[M+H - 2H_2O]^+$ ion at 241.1949 was selected, and the EIC mass window was (241.1944 to 241.1954). By using the TOF's resolving power for selectivity, nearly all of the chemical noise was eliminated and the S/N vastly improved.

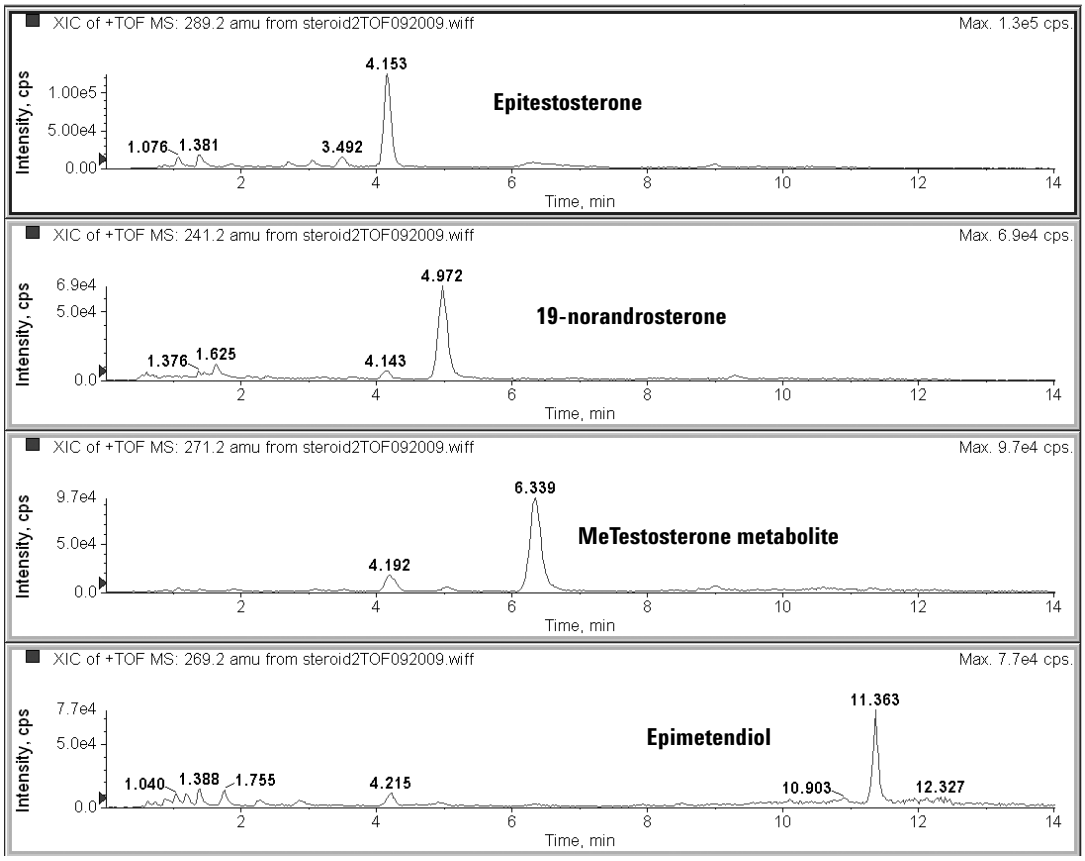


Figure 6. Extract of 2 ng/mL urine (1 ng/mL 19-norandrosterone). Analytes detected using accurate mass EICs (1 mDa width).

Figure 7 shows the spectra corresponding to the extracted analytes shown in Figure 6. Note the mass accuracy results that were calculated automatically by the TOF's data analysis method. These spectra were obtained from an estimated 240 pg of each analyte on-column, assuming 100% recovery (120 pg for 19-norandrosterone). Even at such a low concentration and in a urine extract, the mass accuracy was better than 2 ppm for each analyte. This demonstrates that there were no matrix interferences with the mass measurements even with simple and fast chromatography.

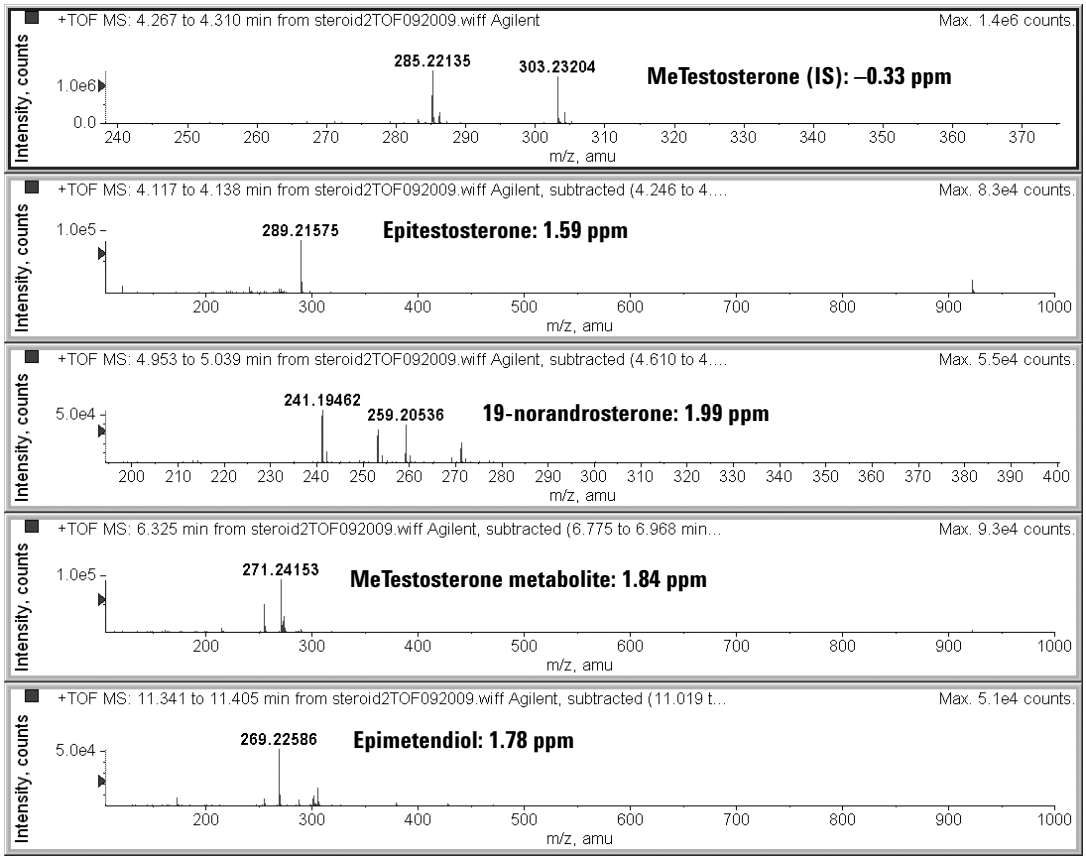


Figure 7. Spectra from 2 ng/mL urine extract (1 ng/mL 19-norandrosterone) (240/120 pg on-column) [see Figure 6].

One final experiment was performed to estimate the detection limit for the internal standard, epitestosterone, 19-norandrosterone, methyltestosterone metabolite, and epimetendiol using the developed LC/MSD TOF method. Serial dilutions of an unextracted standard were prepared and analyzed down to the concentration corresponding to 0.16 ng/mL extracted from 3 mL of urine. A 4-µL injection corresponding to 20 pg on-column was analyzed. The accurate-mass (1 mDa width) EICs are shown in Figure 8 and still exhibit excellent signal-to-noise.

It is possible that the method could be further improved by using 3.5-µm columns, rather than the 1.8-µm particle column used in this study, which would allow for larger injection volumes. The choice of columns would depend on the need for more sensitivity with larger injections, but might sacrifice the speed and resolution achieved with the 1.8-µm column.

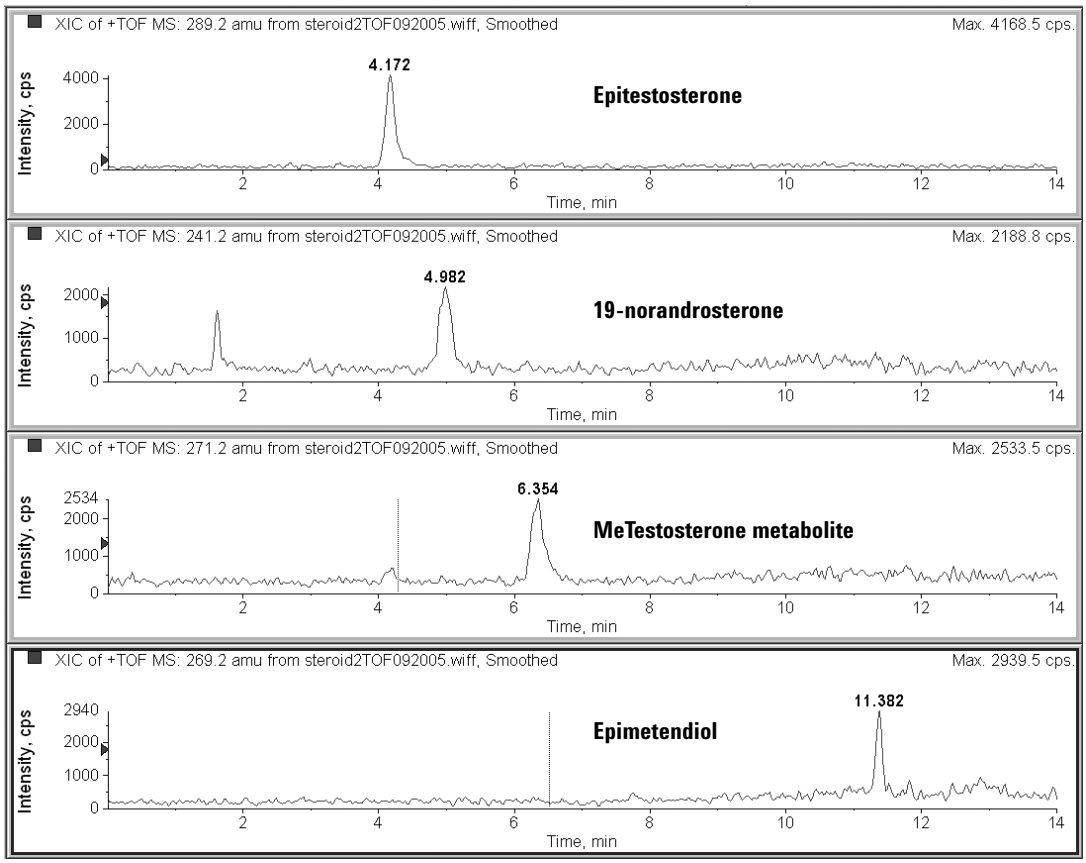


Figure 8. Accurate-mass EICs (1 mDA width) from 0.16-ng/mL unextracted standard (20 pg on-column).

Conclusions

Using a standard sample preparation method developed for GC/MS screening, but without derivatization, an LC/MS method for an easy-to-use, bench top API-TOF instrument readily detected a representative group of anabolic substances at 1–2 ng/mL concentrations in urine. This sensitivity was achieved by capitalizing on the resolution of the LC/MSD TOF that allowed for accurate mass analysis, EICs with extremely narrow mass windows, and enhanced S/N. The analysis consumed only 4 µL of the 100 µL of reconstituted extract, which allowed for re-analysis, analysis of replicates, or different analyses from a single extraction. The use of "accurate-mass EICs" (that is, EICs with an *m/z* width of 1 mDa) allowed for specific detection of the target steroids in a complex sample. The spectra obtained at these low ng/mL concentrations all showed mass errors of less than 2 ppm.

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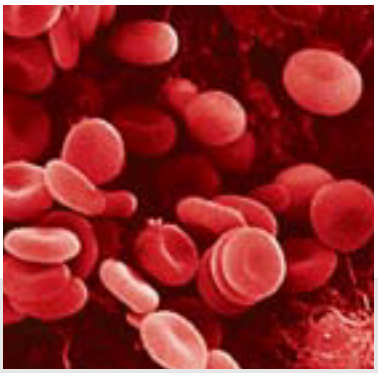

Applications by Technique

GC/MS

Confirmation of THC in Oral Fluids Using High-Resolution 2-D GC/MS

Application Note

Forensic Toxicology

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Abstract

Oral fluids are being used as an alternative matrix to urine for drug testing as it is considered to be less invasive and much more difficult to adulterate than urine samples. Samples of oral fluid are typically screened by an ELISA immunoassay method. Those found to be positive must be further analyzed with a mass spectrometry confirmation technique.

The confirmation technique must be able to detect the drugs of abuse (DOA) in oral fluids down to concentrations lower then those used in urine testing. For example, tetrahydrocannabinol (THC) must be measured down to 0.5 ng/mL of diluted oral fluid when collected with the Intercept® oral fluid collector. The analysis is complicated by interferences from the complex sample matrix. For these samples, techniques like gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) and liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) have been used. The higher level of selectivity afforded by a secondary mass spectral step is used to overcome interference problems.

GC/MS has the required sensitivity to confirm THC but lacks the selectivity over matrix interferences. This application describes a two-dimensional (heart cutting) GC/MS system where THC is heartcut from a nonpolar DB-1ms column to a polar DB-17ms column. An air-cooled focusing trap is used to improve resolution and sensitivity. Coupling high-resolution two-dimensional (2-D) GC with a standard benchtop quadrupole gas chromatography/mass selective detector (GC/MSD) provides the required selectivity and sensitivity for THC confirmations.

Introduction

GC/MS with a quadrupole mass spectrometer is a widely used analytical technique. The selectivity, sensitivity, cost effectiveness, and ability to use library searching for identification have made this the instrument of choice for many years. There are some types of samples, however, where matrix interferences prevent sucessful analysis of the desired analytes. For these samples, techniques like GC/MS/MS and LC/MS/MS have been used. The higher level of selectivity afforded by a secondary mass spectral step is used to overcome interference problems. For many analyses, especially those with a limited number of analytes, the use of a two-dimensional (heart cutting) GC with a standard quadrupole MS can be a simpler and less expensive alternative.

This application describes a two-dimensional (heart cutting) GC/MS system. The instrument configuration is a standard quadrupole GC/MS

system to which a Deans switch and air-cooled focusing trap have been added. The first GC column is typically a nonpolar DB-1ms and the second column a polar DB-17ms. Upon injection into the GC, the analytes separate on the first column. The Deans switch is time programmed to heart cut the elution time range of the analyte(s) from the first column onto the second column, where they are focused by the air-cooled trap. Upon thermal desorption in the second column, the analytes are further separated from the matrix compounds that co-eluted with them on the first column. The focusing trap is used to improve both resolution and sensitivity. The two-dimensional (2-D) GC separation is used in place of a secondary mass spectrometric operation. At the end of analyte elution, the carrier gas in the column can be reversed to backflush unwanted heavy sample components out the split vent in the inlet. This saves analysis time and reduces the need for column trimming and replacement. Since only a small portion of the injected sample enters the MS ion source, source cleaning is reduced as well. For limited numbers of analytes (typically five or fewer), high-resolution 2-D GC/MS can be a suitable alternative to MS/MS techniques.

The detection of drugs of abuse in oral fluids serves as a good example of where high-resolution 2-D GC/MS can be used.

Oral fluid is increasingly being used as an alternative matrix to urine in testing for recent drug exposure and impairment. The technique offers several advantages, including ease of collection, minimization of adulteration, and lowering costs for collections, scheduling, and lost time.

One challenge presented by oral fluid testing is in the confirmation of positive screen results. For example, confirmation is required down to 0.5 ng/mL of THC in oral fluid. The determination of THC at this level is complicated by interferences from nondrug compounds in the matrix that chromatographically overlap with analytes and contain ions with the same m/z values. Due to this problem, techniques such as LC/MS/MS and GC/MS/MS are often used for confirmation. This application demonstrates that high-resolution 2-D GC/MS can be used to analyze for THC in oral fluids. The extremely high chromatographic resolution afforded by the 2-D approach resolves matrix interferences from the THC. This results in detection levels comparable to MS/MS techniques.

Experimental

GC/MSD Configuration

The GC/MSD configuration used is shown in Figure 1. The system comprises:

G1540N	6890N Network GC System with options:
	652 Oral fluids analysis kit (includes Cryo-Trap, PCM and 15M DB-1MS, 15M DB-17MS columns)
	112 Split/splitless with EPC (112)
	201 MSD interface (201)
	211 Capillary FID with EPC (211)
	888 Microfluids dean switch
	002 (240 V fast oven power supply) or 003 (198 to 231 V fast oven power supply)
G3243A	5975B inert MSD/DS perf turbo EI bundle
G3397A	Ion gauge/controller for use with 5975 MSD
G2913A	7683B Autoinjector module
G2614A	7683 Autosampler tray module

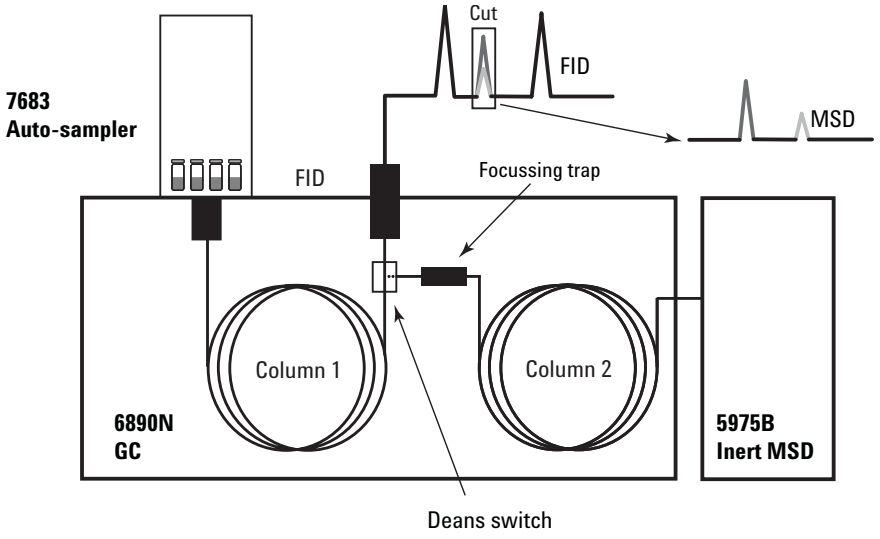

Figure 1. Hardware configuration of 2-D heartcutting GC/MSD system.

The gas chromatograph and mass spectrometer operating conditions are listed in Table 1.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC	
Agilent Technologies 6890N 7683 autoinjector and tray	
Autoinjector	
Sample washes	0
Sample pumps	0
Injection volume (µL)	4
Syringe size (µL)	10
Preinjection solvent A washes	0
Preinjection solvent B washes	0
Post-injection solvent A washes	10
Post-injection solvent B washes	10
Viscosity delay (s)	2
Plunger speed	Slow
Preinjection dwell (min)	0
Post-injection dwell (min)	0
Front Inlet	
Type	EPC split/splitless
Mode	Constant pressure
Inlet temp (°C)	250
Injection type	Pulsed splitless
Pulse pressure (psig)	45
Pulse time (min)	0.5
Purge time (min)	1
Purge flow (mL/min)	50
Pressure, nominal (psig)	26.59
Gas saver	Off
Gas type	Helium
Back Inlet	
Type	PCM/focusing trap
Initial temp (°C)	300
Initial time (min)	5.3
Ramp rate 1 (°C/min)	799
Final temp 1 (°C)	100
Final hold 1 (min)	2
Ramp rate 2 (°C/min)	799
Final temp 2 (°C)	300
Final hold 2 (min)	10
Oven	
Voltage (VAC)	240
Initial oven temp (°C)	130
Initial oven hold (min)	0.5
Ramp rate 1 (°C/min)	35
Final temp 1 (°C)	250
Final hold 1 (min)	0
Ramp rate 2 (°C/min)	10
Final temp 2 (°C)	280
Final hold 2 (min)	2.5
Equilibration time (min)	0.5
Column 1	
Inlet	Split/splitless (front)
Type	DB-1 ms
Agilent part number	122-0112
Length (m)	15
Diameter (mm)	0.25
Film thickness (µm)	0.25
Column 2	
Inlet	Deans switch (back)
Type	DB-17 ms
Agilent part number	122-4712
Length (m)	15
Diameter (mm)	0.25
Film thickness (µm)	0.25
FID	
Temperature (°C)	250
Hydrogen flow (mL/min)	50
Air flow (mL/min)	450
Mode: Constant makeup flow	Constant makeup flow
Nitrogen makeup flow (mL/min)	45
Data rate (Hz)	10
Deans Switch	
FID restrictor length (m)	0.31
FID restrictor id (mm)	0.10
Carrier gas supply	PCM of cryotrap
Deans pressure (psig)	19.60
THC cut time start (min)	6.33
THC cut time end (min)	6.44
MSD	
Agilent technologies	5975B inert MSD
Solvent delay (min)	4
Tune file	Atune.U
Mode	SIM
EM voltage	Atune voltage
Quad temp (°C)	150
Source temp (°C)	230
Transfer line temp (°C)	280
Acquisition mode	SIM
Dwell time (msec)	10
THC-TMS SIM ions	371, 386, 303
THC-D3-TMS SIM ions	374, 389, 303
Post-Run Backflush Conditions	
Post time (min)	3
Oven temperature (°C)	300
Column 1 pressure (psig)	1.0
Column 2 pressure (psig)	65

Oral Fluid Sample Collection and Preparation

Oral fluid samples were collected from 20 volunteers in a drug clinic using the Intercept® oral fluid collector (OraSure Technologies). Collected samples consisted of ~400 µL of saliva diluted with 800 µL of preservative buffer. Samples were screened for THC using the Intercept Micro-Plate EIA Screen from OraSure Technologies, Inc. (OTI). Samples found positive for THC were prepared as TMS derivatives as described below and analyzed with the 2-D GC/MSD system.

Calibration standards were prepared with the same procedure as oral fluid samples except that Oral Fluid Diluent (OTI) was used to simulate the sample matrix.

Sample preparation for 2-D GC/MSD consists of:

- Dilute 400 µL sample in 4 mL of 50 mM phosphoric acid
- Add deuterated THC internal standard (2 ng/mL)
- Wash column with 500 µL methanol
- Add diluted sample to column (Varian SPEC DAU 30 mg)
- Wash with 2 mL 50/50 (methanol/water)
- Dry 2 minutes
- Elute with 1mL 78/20/2 (methylene chloride/isopropanol/ammonia)
- Dry and derivatize with 25 µL BSTFA+1%TMCS at 70 °C for 15 minutes
- Add 25 µL acetonitrile

Standards of THC and deuterated THC-D3 were purchased from Cerilliant. BSTFA+1%TMCS was purchased from Pierce.

Deans Switch Operation

The Deans switch is a fluidic device used to heart cut peaks from the first column to the second. For analyses involving trace levels of drug analytes detected with a mass spectrometer, severe requirements are placed on components directly in the sample path. These requirements are: absence of any air leaks; inertness of surfaces contacted by samples; minimum dead volume; ease of use; and reliability over time. The Agilent microfluidic Deans switch meets all these requirements. A more detailed description of the device and its application is given in reference 1.

Figure 2a. shows a diagram of the Deans switch with the solenoid valve turned off. With the valve in this position, the effluent from the DB-1 ms column is pushed through the restrictor to the FID. When the solenoid valve is turned on as in Figure 2b, the column effluent is now pushed to the DB-17 ms column. Heart cutting a peak from the first column to the second is thus accomplished by time programming the solenoid valve to turn on just before elution of the peak and turn off just after elution of the peak.

A second use of the Deans switch is to backflush the first column. At the end of the run, the oven temperature is raised to 300 °C, the pressure in the split/splitless inlet is dropped to 1 psig, and the PCM pressure is raised to 65 psig. This change in inlet pressures causes the flow of carrier gas to reverse through column 1. This reverse flow

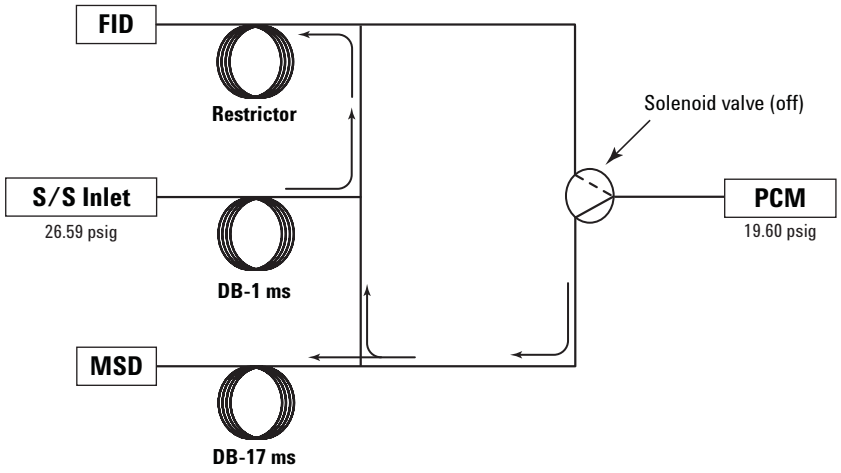

Figure 2a. Deans switch valve in no cut position. Column 1 effluent goes to FID.

backflushes any heavy materials at the head of column 1 out through the split vent trap. Backflushing increases the life of the column and results in cleaner chromatographic baselines. The backflushing mode is shown in Figure 2c.

Temperature Ramps and Cut Times

In determining the parameters for a 2-D chromatographic method, the oven temperature program is established first. The initial oven temperature is chosen to be the highest value that does not result in broadened, misshapen analyte peaks. Standards prepared at 10 ng THC/milliliter were run with initial temperatures ranging from 100 °C to 150 °C while monitoring the peak shape on the FID (no cut). For this method, 130 °C was found to perform well. The temperature ramp from 130 °C up to 250 °C was chosen to be the highest possible that would not cause oven control warnings. A ramp of 35 °C/minute was chosen. To increase the resolution of the THC from other matrix components on the first column, the oven ramp is reduced to 10 °C per minute about two minutes before elution of the THC. After elution of the THC the temperature is held isothermal at 280 °C.

Figure 3a shows the first column FID chromatogram of a sample. A large number of matrix components are clearly evident. Figure 3b is an expanded view of the elution region of THC. Also shown in Figure 3b is the chromatogram from a 10

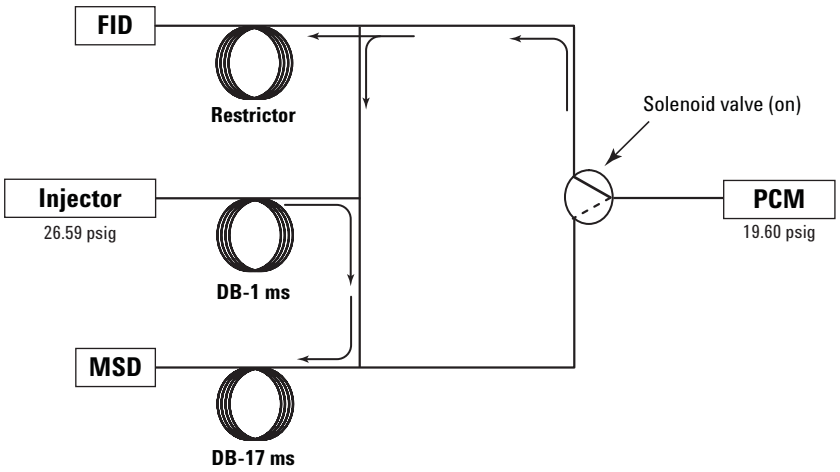

Figure 2b. Deans switch valve in cut position. Column 1 effluent goes to column 2.

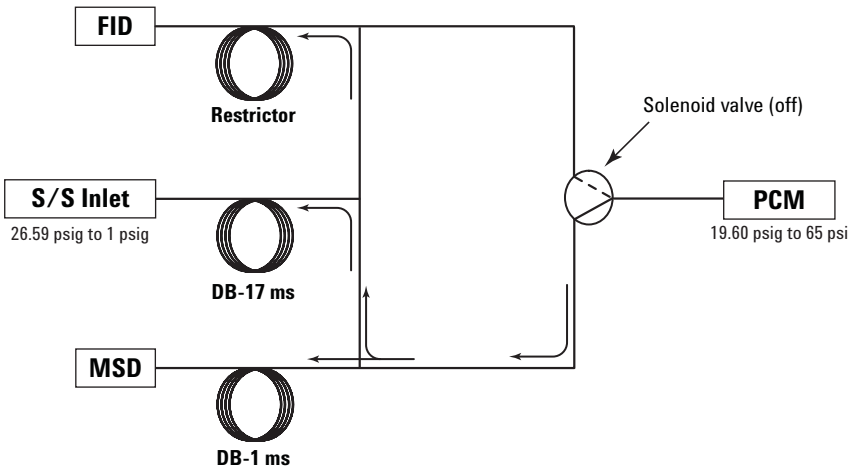

Figure 2c. Backflushing column 1. After the last analyte elutes from column 2, program inlet pressure down to 1 psig and program the PCM to 60 psi to backflush heavies out split vent.

ng/mL THC standard. The cut time for THC is chosen to start immediately before the THC peak and end immediately after it. In this example the cut time range was 6.33 minutes to 6.44 minutes.

After the cut time is determined, the focusing trap temperature program is chosen. The trap is initially held at 300 °C (that is, no trapping) and is programmed to cool to 100 °C at about one to two minutes before the cut time. This is to ensure that the trap is at 100 °C when the cut is made. After the cut is finished, the trap is then programmed back to 300 °C at its maximum rate (799 °C per minute). This desorbs the trapped components. Normally, desorption is set to start at about 0.2 minutes after the end of the cut. In this example, desorption starts approximately 1 minute after the cut.

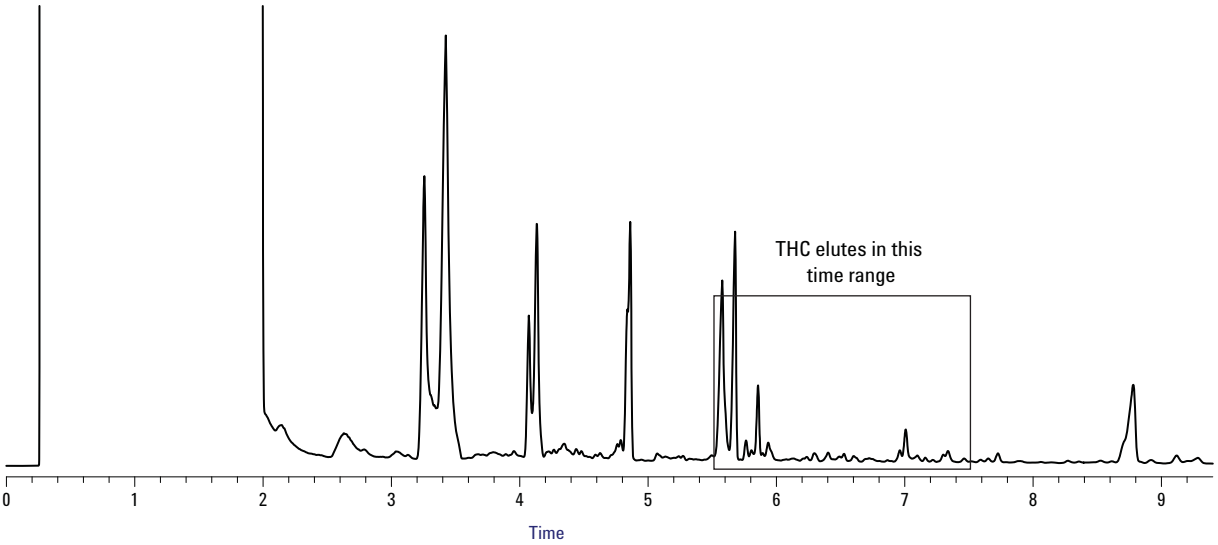

Figure 3a. First column FID chromatogram of a sample showing complexity of matrix (no cut to second column).

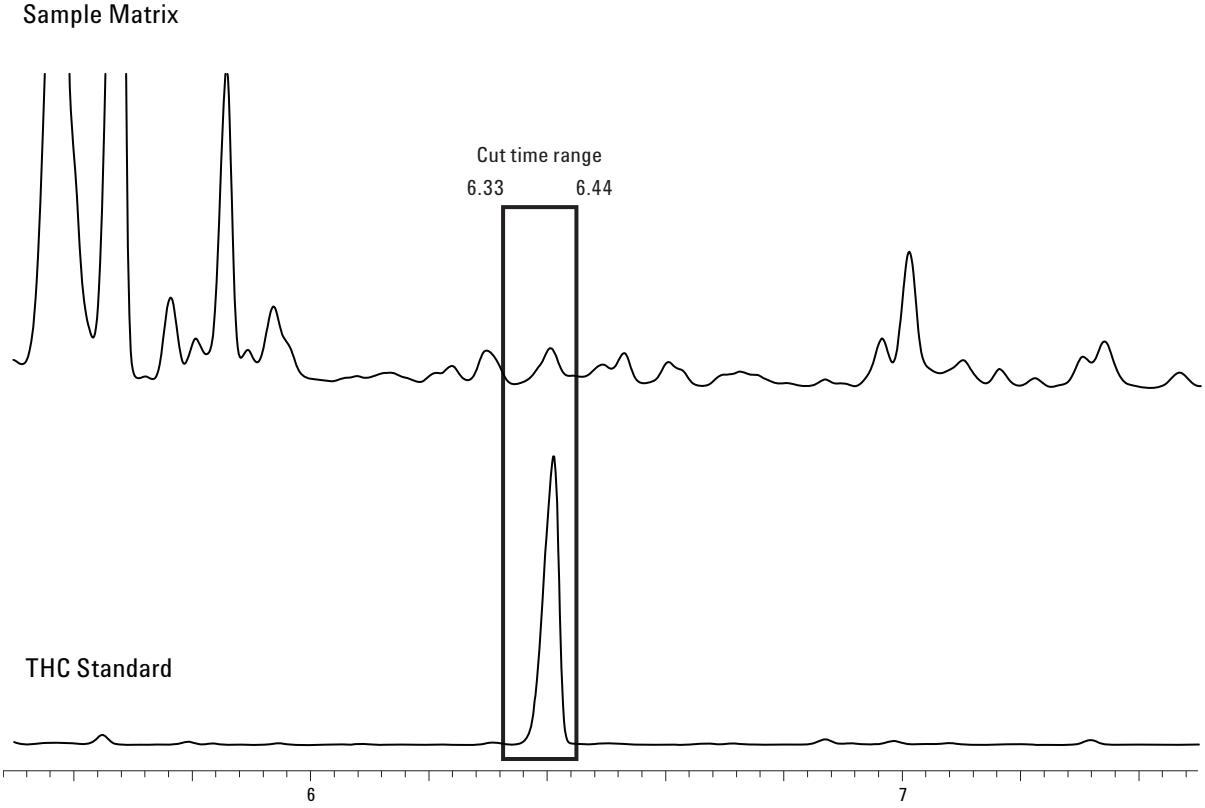

Figure 3b. Expanded view of THC elution range from Figure 3a.

Figure 4 is a timing diagram showing the relationship between the trap temperature, the oven temperature, and the Deans switch valve.

Results and Discussion

Figure 5a shows the SIM chromatograms for the internal standard (THC-D3) spiked at 2 ng/mL. The retention time of the internal standard is very close to that of THC, being only 0.002 minutes earlier. Since the retention times are so close, cut times chosen for the THC will also work well for the internal standard.

Figure 5b shows the SIM chromatograms from an unextracted THC standard prepared at the cutoff level of 0.5 ng/mL. The cutoff level is the concentration of THC in a saliva sample below which the sample is considered to be negative. The sample in Figure 5b is prepared by derivatizing the THC directly without going through the sample cleanup procedure. It is used as a reference for measuring the recovery from the sample preparation procedure.

A cutoff level standard prepared in surrogate saliva and taken through the entire sample preparation procedure is shown in Figure 5c. Comparison of the response here to that of the unextracted standard in 5b shows the recovery to be 70% or greater. It also shows that there are no significant sample preparation artifacts in the retention time range of the THC that would interfere with quantitation at the cutoff level.

A sample found to be positive for THC is shown in Figure 5d. The measured concentration of 0.64 ng/mL is just above the cutoff level. There are no significant interferences evident in the retention time region of the THC.

A negative sample is shown in Figure 5e. The concentration of THC was found to be below the lowest calibration level and was estimated to be 0.17 ng/mL THC.

Figure 6 shows the THC calibration curve from the MSD ChemStation. Acceptable linearity was found over the calibration range of 0.2 to 32 ng/mL of THC. The plot in Figure 6 shows the range bracketing the cutoff level.

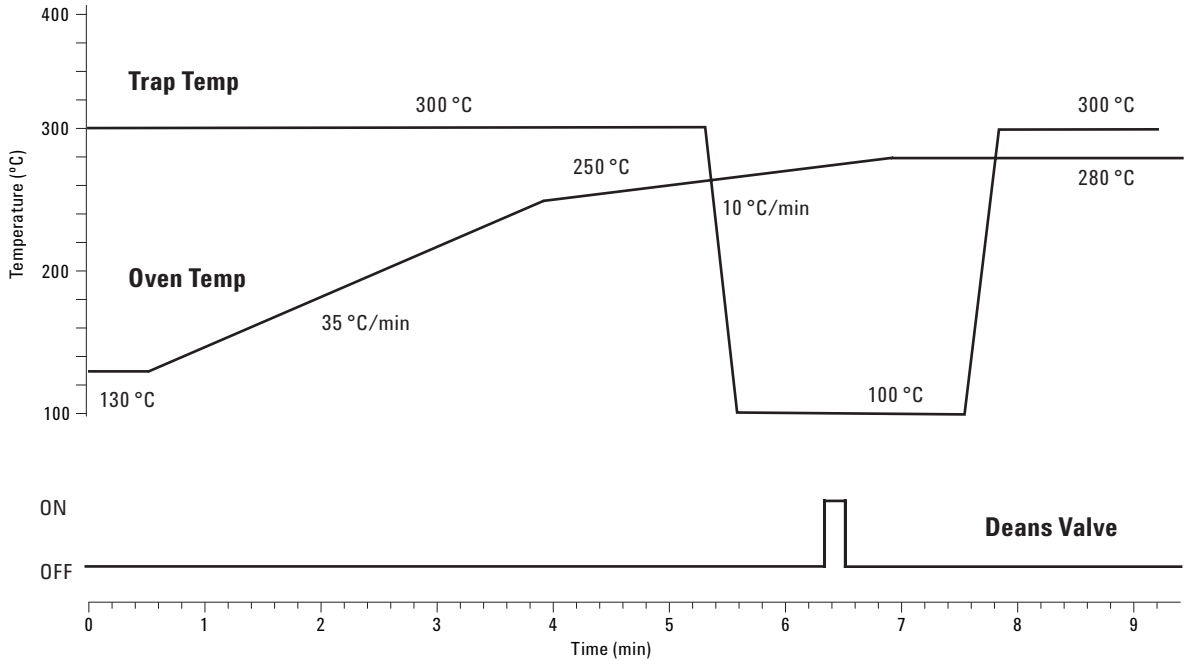

Figure 4. Temperature and valve timing for THC analysis.

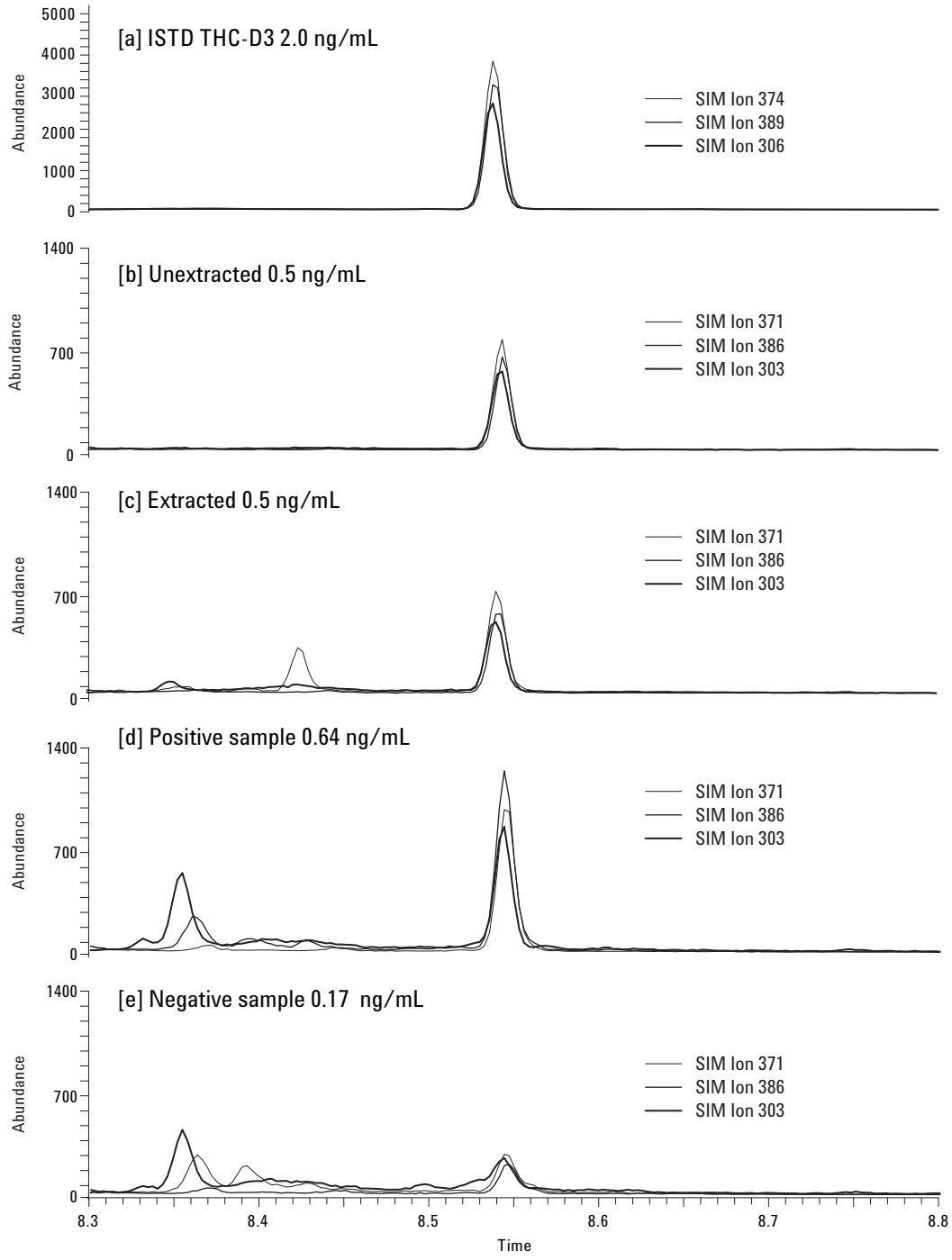

Figure 5. SIM ion chromatograms from a) internal standard THC-D3 at 2 ng/mL, b) unextracted THC cutoff at 0.5 ng/mL, c) extracted THC cutoff at 0.5 ng/mL, d)positive THC sample at 0.64 ng, and e) negative sample at 0.17 ng/mL.

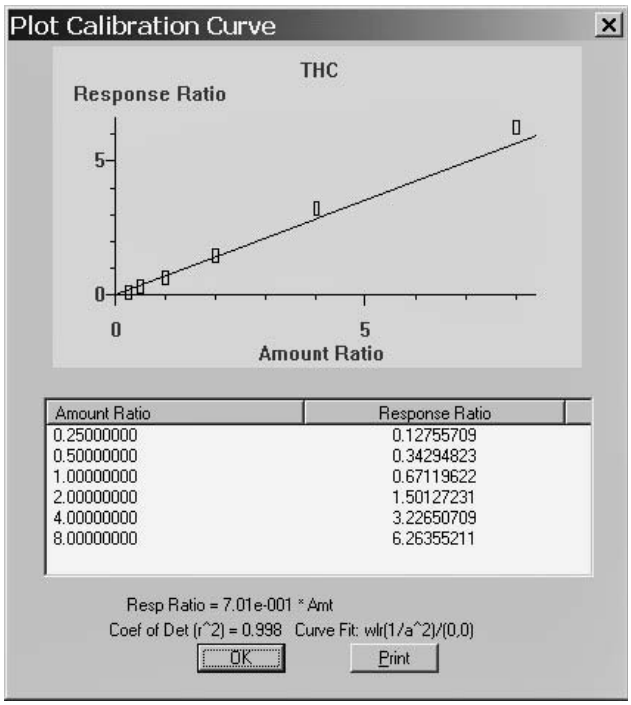

Figure 6. THC calibration curve.

The analytical performance results for the method are summarized in Table 2. The data are based on three runs at each level. The row LOD is the limit of detection. Row LOQ is the limit of quantitation. The column labeled Mean is the average concentration measured for three runs of a standard at that level when calculated using the calibration curve. The column STDEV is the standard deviation from mean, and %CV is the percent coefficient of variation. The column %Accuracy represents the agreement between the known and measured values, with 100% being perfect agreement.

Table 2. Calibration Results for THC

	ng/mL	Mean*	STDEV	%CV	%Accuracy
LOD (0.2X)	0.1	0.22	0.03	12.86	220.00
LOQ (0.4X)	0.2	0.29	0.01	3.94	146.67
Cutoff (X)	0.5	0.46	0.08	16.58	91.33
2X	1	1.03	0.04	4.23	103.00
4X	2	1.95	0.04	2.07	97.67
8X	4	4.08	0.17	4.17	102.08
Pos control	2	1.86	0.06	2.99	93.00
Recovery			70 %		
Linearity			0.2–32 ng/mL		
Carryover			None up to 32		

*3 runs

Table 3 presents the results of comparing the EIA screening results of with those of the 2-D GC/MS method. In the left-hand section in Table 3 is a matrix showing the distribution of results for the 20 samples. Four samples were found to be positive by both techniques as shown in the upper left-hand quadrant of the matrix. One sample was found to be negative by GC/MS but positive by EIA. Since GC/MS is considered to be the reference technique, the sample is considered to be a false positive for EIA. The lower left-hand quadrant shows that no samples were found to be positive with GC/MS and negative with the EIA. That is, no false negatives were found for EIA. The remaining 15 samples were found to be negative by both tech-niques.

Table 3. Comparison of EIA and 2-D GC/MS Results

		GC/MS +	GC/MS –
EIA	+	4	1
	–	0	15

	Deans switch	Package insert
N	20	200
Sensitivity	100%	97.62%
Specificity	93.75	91.67
Confirm cutoff	0.5	0.5

The right-hand portion of Table 3 represents a comparison of the sensitivity and specificity of the GC/MS method with the EIA screening technique. For this comparison, sensitivity is defined as the number of true positives divided by the sum of the true positives and the false negatives. True positives are defined as those samples that were found to be positive by both techniques. Specificity is defined as the number of true negatives divided by the sum of the true negatives and the false positives.

The data in the rightmost column of Table 3 is from a study of 200 samples provided with the package insert for the Intercept EIA kit. The present results show good agreement between the 2-D GC/MS technique and the 200-sample study used for the package insert.

Conclusions

High-resolution 2-D GC/MS can be used as a confirmatory technique for oral fluid drug screening for THC. Samples collected in Intercept® collectors had no major interferences that prevented analysis. Suitable results were obtained with 0.4 mL sample volume corresponding with the "withdrawn" SAMHSA Guideline cutoff of 2 ng/mL THC.

Note: The cutoff of 0.5 ng/mL THC was used in this application to compensate for the dilution of oral fluid that occurs when collecting samples with the Intercept collector, which contains a preservative buffer.

Sensitivity and specificity data between Intercept EIA kits and 2D GC/MS on 20 subjects from a drug clinic were similar to that published in the package inserts.

References

1. James D. McCurry, "Using a New Gas Phase Micro-Fluidic Deans Switch for the 2-D GC Analysis of Trace Methanol in Crude Oil by ASTM Method D7059," Agilent Technologies publication 5989-1840EN

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Improved Forensic Toxicology Screening Using A GC/MS/NPD System with a 725-Compound DRS Database

Application

Forensic Toxicology

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Abstract

Laboratories that perform toxicology screens are challenged by the requirement to look for large numbers of target compounds in samples that contain complex matrix interferences. GC/MS methods are widely used and accepted for this analysis. Full-scan EI methods offer many advantages for broad-range screening, such as unlimited numbers of targets, full-spectrum identity confirmation, and library searching for identification of nontargets. With recent advances in GC/MS technology, there are several opportunities to substantially increase the number of targets screened for and simultaneously reduce the time required per sample.

With the system described here, samples are screened for 725 compounds using Agilent's G1674AA Forensic Toxicology DBL. Data review time is substantially reduced using Agilent Deconvolution Reporting Software. Post-run bakeout of heavy-matrix compounds is replaced with column backflushing, which is faster and reduces system maintenance. Run time is reduced by using a fast GC run (9.75 min injection to injection) and simultaneously collecting scan, SIM, and NPD data. The scan data is deconvoluted and used to identify any of the 725 target compounds. SIM data is used to look for select low-level compounds not detectable in scan mode. The nitrogen response of the NPD is used to highlight nontarget nitrogen compounds and identity confirmation and can be used for quantitation if needed. Using extracts of whole blood samples, the system finds all the compounds detected by the conventional method in significantly less time.

Introduction

GC/MS screening methods play an important role in the forensic toxicology laboratory. With the continuing emer-gence of new drugs and toxins, the list of target compounds to be screened can easily number in the hundreds. For those compounds that are compatible with GC, GC/MS in full-scan mode with electron impact ionization (EI) is well suited for the task. The technique offers several advantages:

- It uses straightforward, reliable, and familiar instrumentation.
- Any number of targets can be monitored.
- The target list is not limited by the number of MRMs like MS/MS techniques.
- Years later, archived full-scan data can be examined for new targets.
- Identity confirmation is based on full spectra.
- Nontarget unknown compounds can be identified by searching spectra against NIST and other industry standard libraries.
- Ionization suppression due to matrix is much less of a problem than with LC/MS techniques.

While GC/MS methods offer the above advantages, there are limitations with the conventional approach. As the number of target compounds in the screen increases, the size of tasks involved in the development, maintenance, and application of the methods grows very rapidly. These considerations often limit the scope of screening methods used in forensic toxicology labs.

GC/MS methods are typically developed to analyze between 10 and 100 individual compounds. A target compound is deemed to be present if the target ion and two or three qualifier ions with specific abundance ratios fall within a defined retention time window. The identity of the target may be further confirmed by comparison of the scan at the apex of the peak with a library reference spectrum.

Matrix interferences are usually minimized by optimizing a combination of the sample preparation, GC, and MS parameters. For methods that deal with only a few matrix types, the ions chosen for identification purposes can be selected such that they are minimized in the matrix. With a limited number of targets addressed by the method, recalibration of response factors, retention times, and qualifier ion abundance ratios can be accomplished with the injection of a few calibration mixtures.

Screening methods for very large numbers of targets in varying and complex matrices offer a new set of challenges for the method developer. When screening for hundreds of targets, several factors must be addressed:

- Use of sample preparation to reduce matrix interferences is now limited because rigorous cleanup steps may unintentionally remove targets. This reduced level of cleanup can result in significantly higher levels of matrix interferences to contend with.
- Recalibration of response factors, retention times, and qualifier abundance ratios is difficult because of the large number of targets.
- The methods may be deployed in multiple laboratories without ready access to standards for all of the targets.
- The time required for data review of hundreds of targets in complex matrices can become unmanageably large.
- Even with a very large database of targets, it is possible that important compounds not in the target list could be present in a sample.

In recent years, several techniques have become available to help address the above set of challenges. Retention time locking (RTL) produces retention times that precisely match from instrument to instrument and those in a database [1]. This eliminates the need for recalibration of the individual retention times and timed events like SIM groups. The introduction of reliable and inert Capillary Flow Technology (CFT) splitters allows for the simultaneous collection of mass spectral and nitrogen/phosphorus detector (NPD) data [2]. The NPD chromatogram highlights nitrogen-containing compounds, including those not in the MS target list. It is useful in confirming the presence of a nitrogen-containing target compound and can serve as an alternative means of quantitation.

The introduction of the synchronous SIM/Scan feature allows for the simultaneous acquisition of both full-scan and SIM data from the same injection [2, 3]. The scan data can be used for screening the full list of targets in the database, while the SIM data looks for a high-priority subset of compounds (like fentanyl) down to very low levels.

One of the most significant tools developed for reducing the time required for data review is Agilent's Deconvolution Reporting Software (DRS) [4]. It uses advanced computational techniques (deconvolution) to extract the spectra of targets from those of overlapped interference peaks. It then compares the extracted spectrum with a library to determine if the target is present. If desired, hits can be confirmed by also searching against the main NIST MS reference library. The entire process is automated and provides a major time savings in data interpretation. The use of DRS also substantially reduces the number of both false positives and false negatives.

Since DRS uses the entire spectrum instead of just four ions, DRS can often correctly identify a target in the presence of interferences where the typical approach would fail. Also, since it uses the entire spectrum for identification instead of precise target/qualifier ion ratios, frequent updating of the ratios is not necessary. This is useful for targets that are rarely encountered but are still screened for.

This application describes the combination of the above techniques with a new database of 725 compounds, the Agilent G1674AA Forensic Toxicology DBL, to be used for screening purposes. The DBL contains:

- RTL methods for DB-5MS and DB-35MS columns

- Spectral libraries for DRS and the MSD ChemStation
- Preconfigured RTL methods for multiple speeds with run times of 30, 15, 10, 7, or 5 minutes, depending on hardware configuration
- Methods for both MSD direct connection (vacuum) and Capillary Flow Technology splitters (3.8 psig).
- Three quant databases included for each method:
  - Target and qualifiers are the biggest four ions.
  - Ions are optimized to give the best signal-to-noise ratio versus column bleed and background.
  - Ions are optimized to give the best signal-to-noise ratio versus common fatty acids found in blood.

The names of all the compounds in the database are listed in the appendix at the end of this application. Compounds in the DBL include drugs and select breakdown products, TMS derivatives, and acetyl derivatives. For those compounds entered as derivatives, in general, primary and secondary amino (including aliphatic and aromatic) compounds are acetylated. Hydroxyl groups (alcohols/phenols/carboxylic acids, etc.) are converted to TMS derivatives with BSTFA. Compounds having multiple functionalities (for example, phenylpropanolamine, which has a primary aliphatic amine and an alcohol) were acetylated with no further derivatization.

Methods are provided for two stationary phases to allow two-column confirmation and the ability to run other methods that require the same column on the same hardware. In general, the DB-5MS methods are preferred because the final oven temperature is lower.

The chromatographic conditions chosen for development of the database are general in nature and are compatible with the analysis of other compounds beyond those in the table. Since no one target list, no matter how large, can satisfy every lab's needs, new compounds can be added to the screen.

The retention times for compounds in the database are provided for both the column connected directly to the MSD and for the column outlet pressure at 3.8 psig using a CFT splitter. This was done to ensure that the retention times observed during sample analysis would closely match those in the database regardless of the instrument configuration.

The chromatographic conditions for the database were chosen to be compatible with Agilent's method translation technique. Constant-pressure mode was used in the GC inlet so that method translation could be used to precisely time-scale the methods for faster operation [5]. Provided with the Agilent Forensic Toxicology DBL are the files to run the analysis at precisely twofold (2x), threefold (3x), fourfold (4x), and sixfold (6x) faster than the primary database (1x). The choice of speed is determined by the degree of chromatographic resolution desired and the hardware capabilities of the GC/MSD system to be used.

For systems with a 120 V GC oven, an MSD with diffusion pump, and the column connected directly into the MSD, only 1x or 2x methods can be used. The 3x, 4x, and 6x methods require the fast oven (240 V) and performance turbopump because column flow rates exceed 2 mL per minute. Performance electronics are also preferred for the same methods. The 6x methods require both a 240 V oven and the oven "pillow" accessory to attain the 60 °C/min ramp rate. Note that use of the pillow requires that the MSD, inlet, and NPD (if used) be located in the back GC positions.

Three different versions of each method set are provided based upon the choice of ions used in the quant database. A method using the largest four ions in a compound's spectrum is supplied. The target ion is the ion with the largest abundance. The three qualifiers are the next three largest ions assigned in order of decreasing abundance. These method sets are provided for legacy reasons, and are used in some more advanced approaches.

The drawback of the largest four-ion approach is that, in some cases, the signal-to-noise performance suffers. For example, if the biggest ion for a compound is 207 and the stationary phase has its largest bleed ion at 207, the signal-to-noise ratio at that mass can be significantly reduced. The same problem is seen with low masses such as 44, where CO_2 and other background gases can result in interferences and increased noise. To reduce this problem, a second method set is provided where ions chosen for the quant database are selected to give best signal-to-noise ratios relative to column bleed and background gases. These are the methods that would normally be used, as they typically give best overall performance.

A third method type is provided where the choice of ions has been optimized for samples having large amounts of fatty acids typically seen in blood samples. These methods give the best signal-to-noise

ratios in high fatty-acid matrices. They are not the best choice for samples having low levels of interfering fatty acids.

Experimental

System Configuration

The system configuration used is shown in Figure 1. The GC is an Agilent 7890A (G3440A).

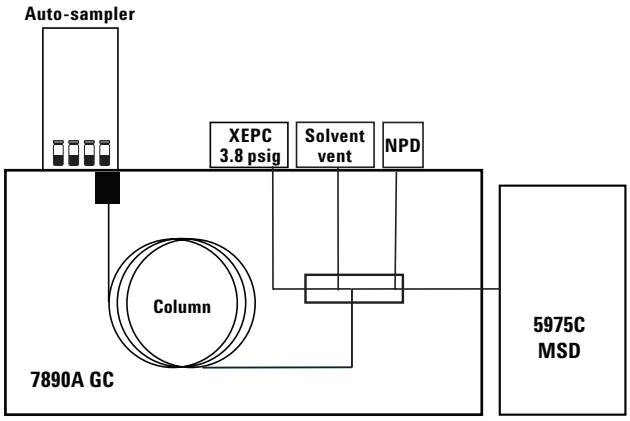

Figure 1. GC/MS/NPD system configuration used for screening blood extracts.

Key components are:

Fast Oven The primary 1x method uses a 30-m column with a 10 °C/min ramp rate and only requires the 120 V oven. With the 7890A 240 V oven (option 002), the screening method can be run up to 4 times faster using a 15-m column. If the 240 V GC is further equipped with options 199 and 202 (puts split/splitless injection port and MSD interface in the back of the oven) and uses the G2646-60500 oven insert accessory, the speed can be increased to 6 times faster (60 °C/min) with a custom length 10-m column. If an NPD is used with a splitter, option 299 places it in the back of the oven for use with the pillow.

NPD The 7890A Option 251 is a nitrogen phosphorus detector. The signal from the NPD is collected, stored, and processed by the MS ChemStation simultaneously with the MS data. NPD detectors are highly selective and exhibit very sensitive response to nitrogen and phosphorus compounds, with detection limits in the low picogram range. The NPD data can be used in several ways. Nontarget nitrogen (and phosphorus) compounds are highlighted for the data reviewer. The presence of a response at the retention time of an identified compound can be used to support confirmation of identity. The response on the NPD can be used for quantitative analysis, but only after calibration with a standard, as the response factors are compound dependent and can vary with compound class. The NPD bead is incompatible with halogenated solvents and excess silanizing reagents. If these are to be used with an NPD, the splitter setup should have solvent venting capability.

Capillary Flow Technology Splitters Agilent offers two different column effluent splitters that can be used with the 7890A for this application. Option 889 is a two-way splitter that divides the effluent of the column between the MSD and the NPD. The 7890A Option SP1 (7890-0363) does the same, but adds solvent venting capability as well. The devices are based on diffusion bonded plate technology combined with metal column ferrules to make inert, easy-to-use, leak-free, high-temperature splitters. The splitters use Auxillary EPC for constant pressure makeup (7890A Option 301). The Auxillary EPC makeup can be pressure programmed at the end of the run to higher pressure, while at the same time the inlet pressure is lowered to near ambient. This causes the flow in the column to reverse direction, backflushing heavy materials out the split vent of the inlet. Backflushing significantly reduces analysis times for samples that contain high-boiling matrix components and reduces both column head trimming and frequency of MSD source cleaning [6]. The Aux EPC also allows column changing and maintenance without venting the MSD.

For methods that use solvents compatible with the NPD and do not have silanizing reagent in the samples, the standard two-way splitter can be used. If halogenated (or other NPD incompatible) solvents or silanizing reagents are used, then the two-way splitter with solvent vent, 7890A Option SP1 (7890-0363), should be used to protect the NPD bead. This is the configuration used here.

MSD System The 5975C Inert MSD with performance turbo (G3243A) or 5973N Inert MSD with Performance Electronics and performance turbo (G2579A) EI MSD is used. These configurations provide faster full-scan rates while maintaining sensitivity. The scan rates are compatible with the narrower peaks generated by fast chromatography. The performance turbo pump is required to handle the higher flows associated with systems using splitters. It is also required for the faster versions (3x, 4x, and 6x) of the screening method with vacuum outlet (column connected directly to MSD). The standard turbo pump can be used for the slower 1x and 2x vacuum outlet versions of the method. Both the performance and standard turbos are compatible with backflushing. Backflushing cannot be done on systems with a diffusion pump.

Synchronous SIM/Scan The D.02.00 (or higher) revision of the Agilent MSD ChemStation is used because it supplies the synchronous SIM/Scan feature. SIM/Scan operates by collecting SIM data every other cycle and scan data on alternate cycles throughout the entire chromatogram. As with conventional SIM methods, not all 725 targets can be monitored in a single run due to the required time separation between SIM groups. In general, the acquisition of SIM data is set up to collect high-priority targets at very low levels. Examples would be fentanyl and phencyclidine.

DRS Software (G1716AA) Spectral deconvolution of the MS data enables identification of analytes in the presence of overlapped matrix peaks [4, 7]. This significantly reduces chromatographic resolution requirements, which allows detection of targets in higher levels of matrix or can be used with fast chromatography to shorten analysis times. DRS utilizes the AMDIS deconvolution program from NIST, originally developed for trace chemical weapons detection in complex samples. DRS presents the analyst with three distinct levels of compound identification: (1) ChemStation, based on retention time and four-ion agreement; (2) AMDIS, based on "cleaned spectra" full ion matching and locked retention time; and (3) NIST05a search using a 163,000-compound library.

G1674AA Forensic Toxicology DBL This supplies the mass spectral library, method, and DRS files for the 725 compound screening methods.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC	
Agilent Technologies 7890A with autoinjector and tray	
Inlet	EPC split/splitless
Mode	Constant pressure
Injection type	Splitless
Injection volume	1.0 µL
Inlet temperature	280 °C
Liner, Agilent dual-taper deactivated	P/N 5181-3315
Pressure, nominal	14.9 psig
RT locking compound	Proadifen (SKF-525a)
RT locking time	4.285 min
Purge flow	50 mL/min
Purge mode	Switched
Purge time	0.4 min
Gas type	Helium
Inlet backflush pressure	1 psig
Oven	
Voltage (VAC)	240*
Initial oven temperature	100 °C
Initial oven hold	0.25 min
Ramp rate	40 °C/min
Final temperature	325 °C
Final hold	1.25 min
Total run time	7.13 min
Equilibration time	0.5 min
Backflush time	0.5 min
Backflush temperature	325 °C
Column	
Type	DB-5MS
Agilent part number	Custom
Length	10 m
Diameter	0.25 mm
Film thickness	0.25 µm
Nominal initial flow	2.52 mL/min
Outlet pressure	3.8 psig
2-Way Splitter w/Solvent Vent	
7890A SP-1, num. 7890-0363	
MSD restictor length	0.69 m
MSD restictor diameter	0.15 mm
NPD restictor length	0.36 m
NPD restictor diameter	0.15 mm
Split ratio MSD:NPD	1.4:1
Solvent vent time range	0–0.75 min
Splitter pressure during run	3.8 psig
Splitter pressure during backflush	76 psig
NPD	
Hydrogen flow	3 mL/min
Air flow	60 mL/min
Nitrogen makeup flow	8 mL/min
Temperature	300 °C
MSD	
Agilent Technologies 5975 or 5973 inert with performance electronics	
Vacuum pump	Performance turbo
Tune file	Atune.U**
Mode	SIM/scan
Solvent delay	0.7 min
EM voltage	Atune voltage
Low mass	40 amu
High mass	570 amu
Threshold	0
TID	Off
Sampling	1
Quad temperature	180 °C
Source temperature	300 °C
Transfer line temperature	300 °C

*Injection port and MSD interface in back positions and G2646-60500 oven pillow

**Gain normalized, 1x

Instrument Operating Parameters

Data Acquisition

The instrument operating parameters used (unless noted otherwise) are listed in Table 1.

DB-5MS was chosen as the stationary phase for the current system. The final temperature required to elute the last compound in the screen is 325 °C instead of 345 °C as required with DB-35MS. This results in shorter run times and longer column life.

The method parameters were chosen to give the best trade-off between chromatographic resolution and sample throughput. For the blood samples analyzed here, the 4x method gave adequate resolution with an relatively short run time. Although the 4x method can be run on a standard 15-m column, a 10-m column was chosen because it gives very similar resolution with a lower column flow rate.

Time was also saved by using backflushing instead of post-run column baking to remove heavy sample matrix compounds. Backflushing is more effective, faster, and does not send the heavy materials and column bleed into the NPD and MSD source. With the current configuration, all heavy materials were removed from the column with a 0.5-minute backflush. The shorter column length (10 m) results in a reduced backflushing time compared to the 15-m column.

The 4x method can be run with a 240 V oven without the pillow accessory. The pillow was used here because it somewhat decreases the cooldown time of the oven and reduces the amount of electricity consumed by the instrument.

Further reduction in the cycle time of the instrument was achieved by using the overlapped injection setting in the autoinjector. With this feature, the autoinjector prepares the next sample for injection and has the syringe ready while the oven is cooling down from the current injection. This feature can save approximately 1 minute in cycle time, depending on the injection parameters used.

The simultaneous acquisition of SIM, scan, and NPD

Table 2. SIM Groups Used in SIM/Scan Mode

SIM Group (number)	Start Time (min)	Compound	RT (min)	Target (amu)	Q1 (amu)	Q2 (amu)
1	0	Amphetamine	0.900	44	91	65
2	0.97	Methamphetamine	1.050	58	91	65
3	1.5	Methylenedioxyamphetamine(MDA)	1.978	136	135	51
4	2.06	Methylenedioxymethamphetamine(MDMA)	2.147	58	135	77
4		Ecgonine methyl ester	2.222	94	82	96
4		Ethylecgonine	2.223	94	82	96
5	2.52	Meperidine	2.826	246	218	247
6	2.96	Ketamine	3.138	180	182	209
6		Phencyclidine	3.249	243	242	200
6		Tramadol	3.389	58	263	59
7	3.64	Methadone	3.866	72	57	165
7		Dextromethorphan	3.895	271	212	270
8	3.98	Cocaine	4.042	182	82	94
8		Cocaethylene	4.175	196	82	94
9	4.53	Diazepam	4.598	258	286	257
9		Tetrahydrocannabinol	4.666	299	300	231
9		6-Acetyl-morphine	4.773	268	327	328
10	4.85	Oxycodone	4.801	315	230	115
10		Temazepam	4.922	271	273	272
10		Diacetylmorphine	4.992	310	268	327
10		Fentanyl	5.177	245	146	189
11	5.25	Zolpidem	5.332	235	236	219
11		Clonazepam-M (amino-)	5.433	285	258	286
12	5.53	Alprazolam	5.630	308	279	280
12		Zaleplon	5.695	305	263	248
13	5.8	Zopiclone	5.905	112	99	139
13		Lysergide (LSD)	6.000	323	324	222

(all dwell times 5 msec)

data save a substantial amount of time compared to acquiring them in separate runs. The compounds and corresponding SIM groups monitored are listed in Table 2. Because the peaks in the 4x method are relatively narrow, the dwell times for SIM ions were set to 5 milliseconds.

By using the above time-saving steps, the cycle time from injection to injection is 9.6 minutes.

Data Analysis

Based on experience with analyzing 50 blood extracts, a data analysis scheme evolved that incorporated the DRS, SIM and NPD data.

The resulting data review scheme consisted of the following:

- Deconvolution results were generated with DRS and reviewed to determine compounds present. The AMDIS minimum match factor was set to 50. Any compounds with match factors less than 65 or retention time differences greater then 4 seconds were considered suspect (for example, not present unless other data like target/qualifier ratios supported presence). For suspect identifications, the NPD signal was inspected to see if there was a corresponding response of the same peak shape and retention time. If the suspect compound is nitrogen containing (as the vast majority of the compounds in the table are), NPD response provided evidence supporting the presence of the compound.
- Compounds identified by AMDIS but not found by the MSD ChemStation because of out-of-range qualifiers were manually inspected in QEdit. Quantitation was forced if AMDIS indicated an acceptable spectral and retention time match.
- A separate ChemStation data analysis method was used to review the SIM results for the 27 compounds listed in Table 2. Since SIM can detect compounds lower than can be confirmed with spectral data, identification relied on target/qualifier ion ratios and NPD data.
- The NPD trace was examined to find any larger peaks that did not correspond to identified targets. The deconvoluted spectra at the retention time of these peaks were searched against the NIST 05a library. As a practical matter, uncorrelated small NPD peaks were not pursued as they are numerous and the signal-to-noise ratio of the corresponding scan data is too small to be useful.

Except where otherwise indicated, the 4x method supplied with the ions optimized against column bleed was used for ChemStation data analysis . The approximate response factors supplied with the method were adjusted using a standard of 5 ng/uL of proadifen (the locking compound). The responses of all compounds in the quant database were multiplied by the factor required to make the calculated result for the proadifen standard equal 5 ng/µL. This allows the concentration of an identified target to be estimated if the compound has not been individually calibrated.

The approximate response factors supplied with the method are only intended to give a rough estimate of the concentration of uncalibrated analytes. Since valid quantitation requires recent recalibration of response factors on the specific instrument used for analysis, the estimated concentration should never be used to report quantitative results. The error in these values can easiliy be a factor of 10 or higher. The purpose of the estimated values is to give an approximate amount that can be used to guide standard preparation for quantitative calibration of the compound, if needed. Individual calibration should be used for all reported analytes.

The SIM data analysis method for the 27 compounds was constructed using the target and first two qualifier ions from the 4x fatty acid optimized method. This was to minimize interference from the matrix in the blood samples.

The peak recognition windows used in the MSD ChemStation were set to ± 0.150 minute for the scan data, ± 0.075 for the SIM data, and ± 6 seconds in AMDIS. These values were found to be sufficiently wide enough to allow for some retention time drift, yet narrow enough to minimize the number of false positives.

For comparison purposes, the data were also analyzed with two conventional data review approaches.

The first approach is the standard quantitation software, where the EIC of the target ion for each compound in the quant database is extracted and integrated. If a peak is detected within the peak recognition time window, the ratios of the qualifiers to the target are measured. Several optional forms of reporting are available. The reports used here were 1) report only compounds with a peak detected in the target ion EIC and that have all qualifiers within the acceptable range for ratios, and 2) report all compounds with a peak detected in the target ion EIC, regardless of qualifier status. The results of a report can then be reviewed in QEdit, where the EICs of the extracted target and qualifier

ions are overlayed for ease of inspection. The reference spectrum for the compound and the apex spectrum for the quant peak being examined are also displayed. Based on inspection of the EICs and spectra, the reviewer can include or exclude the compound from the report.

The second data review approach was to use the ChemStation Screener software. This is almost identical to QEdit, except that it also reports a cross-correlation value (XCR) of the apex spectrum for peak versus the reference library. The XCR value is an indication of spectral match quality and can be used as an additional parameter with which to locate targets. Screener has report options similar QEdit, and the same two types were used here. Note that Screener is a qualitative tool; compounds identified in Screener must then be quantified in QEdit.

Samples

Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/10th volume.

Results and Discussion

Figure 2A shows the chromatographic results from one of the blood extracts, the simultaneously acquired scan, SIM, and NPD signals. The traces make the sample look deceptively simple. Figure 2B shows the same Scan TIC and NPD signals with the scales expanded. More than 400 individual compounds are in these chromatograms when low-level responses are included.

The data from the sample were reviewed with the conventional approaches. The first report with the standard quantitation software listed compounds where all qualifier-to-target ratios were within the rather generous 50% relative limits used here. Without manual review of the 28 compounds reported, 22 were false positives; that is, they were not really present. Of the 11 target compounds actually in the sample, this report only found six of them, leaving five as false negatives.

As this situation is not uncommon, it is usually necessary to have all compounds reported that have a response at the target ion, regardless of the qualifier ratio status. These “maybes” must then be manually reviewed in QEdit. Since the integrator must be set to capture very small peaks, there are large numbers of reponses due to integration of baseline

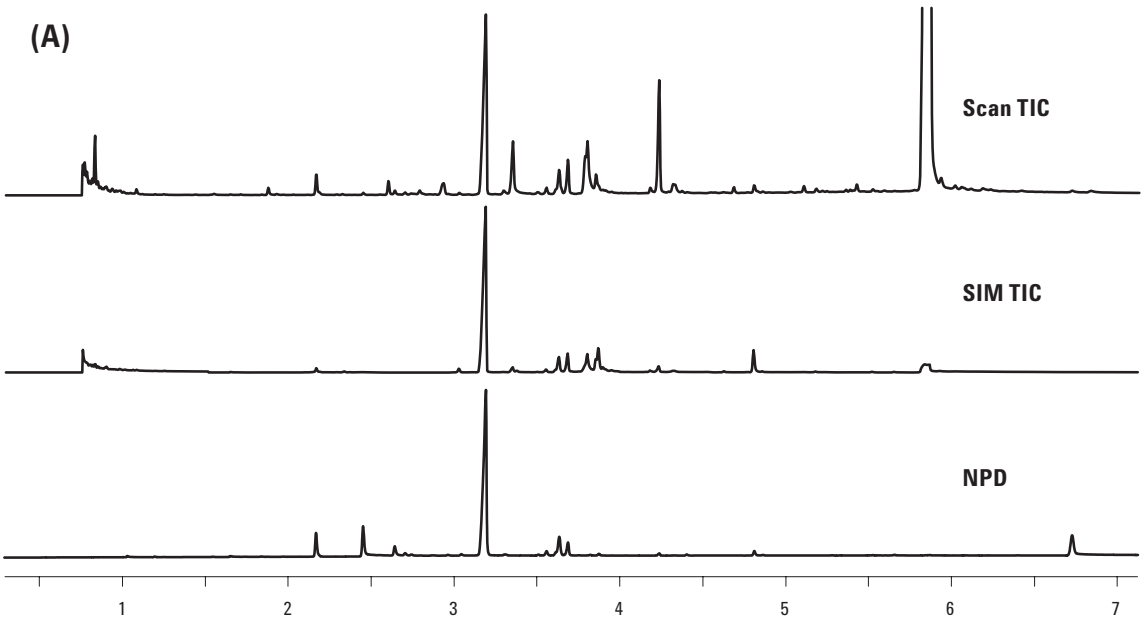

Figure 2A. Chromatograms of scan, SIM, and NPD signals from analysis of blood extract.

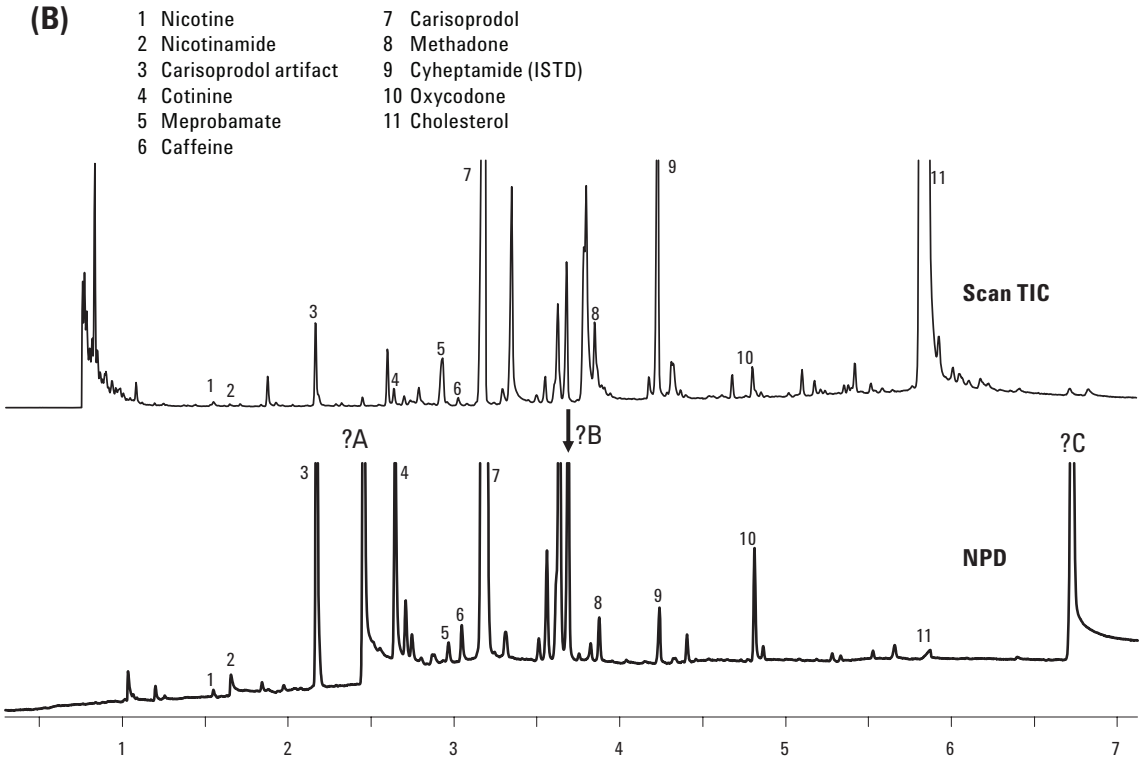

Figure 2B. Expanded scale chromatograms of scan TIC and NPD signals from analysis of blood extract. (continued)

noise. For the sample here, 367 compounds were reported found (that is, there was a response at the target ion). Of those, 356 were false positives. All 11 compounds actually present were found, so there were no false negatives. Thus, to avoid false negatives, the reviewer must manually evaluate 367 compounds to find the 11 present.

The data from the sample were then evaluated with the ChemStation Screener software. As expected, Screener reports based only on ion target/qualifier ion ratios gave very similar results to QEdit. The only way to avoid false negatives is to evalute hundreds of target ion responses to find the 11 actually present.

In an attempt to reduce the number of false positives requiring evaluation, the Screener report listing all 273 compounds with a target ion response was sorted by the XCR in descending order. Several of the compounds actually present were clustered near the top of the list. However, the target actually present with the lowest XCR value was the 162nd compound in the list. This result suggests that XCR improves the likelihood of correctly locating target compounds, but will still result in false negatives without close inspection of all of the compounds with a target ion response.

For the types of samples discussed here, correctly identifying the targets present with the conventional approach is one of the most time-consuming steps in the entire analytical process. This is why the use of deconvolution and DRS is so useful.

When this same sample was evaluated with the DRS software, 12 compounds were reported by AMDIS with a match factor for the deconvoluted spectrum greater than 50 and with retention times within 6 seconds of the locked retention time. After reviewing the 12 listed compounds, one was removed because its match factor was too low. All 11 compounds actually present were identified, with only one false positive included. The entire DRS and review process to correctly locate the targets actually present required about 5 minutes instead of more than an hour using either the QEdit target only or Screener approaches. With the compounds present in the sample identified by DRS, the final report was generated after using QEdit to quantify the targets.

MSD Deconvolution Report
Sample Name: CA5995
Date File: C:\msdchem\1\Appnote\FT5_4 x 10m_SamplesSimScan\CA5995_mss.D
Date/Time: 11:39 AM Wednesday, Apr 2 2008

The NIST library was not searched for the compounds that were found in the AMDIS target library.

			Agilent	AMDIS	
R.T.	CAS #	Compound Name	ChemStation Amount (~ng)	Match	R.T. Diff. sec.
1.539	54115	Nicotine	0.03	59	–0.5
1.6446	98920	Nicotinamide	0.27	93	–0.9
2.1631	999401024	Carisoprodol artifact	64.87	93	–0.5
2.6367	486566	Cotinine	1	96	–0.4
2.928	57534	Meprobamate	4.11	99	0.0
3.033	58082	Caffeine	0.04	82	–0.5
3.1832	78444	Carisoprodol	127.4	96	1.0
3.8653	76993	Methadone	0.39	74	–0.1
4.2279	7199293	Cyheptamide	22.5	98	0.1
4.8014	76426	Oxycodone	2.37	82	0.0
5.850	57885	Cholesterol	922.73	97	3.4

Figure 3. DRS report for the analysis in Figure 2.

Figure 3 shows the DRS report for the sample. For each compound identified, the retention time (R.T.), Chemical Abstracts number (CAS#), and compound name are listed. A line is generated in the report if a compound is found by the Agilent ChemStation, AMDIS, or both.

The report shows that a compound has been determined as present by the Agilent ChemStation if a value appears in the Agilent ChemStation Amount column. This means that the identification criteria set in the DATA ANALYSIS section of the method have been met. Typically the criteria are that the target ion is present (and integrated) and all three qualifier ions are present in ratios that fall within the percent uncertainty values for that compound. The compound would also appear here if the data reviewer manually forced integration of the target ion.

The match value listed under the AMDIS column is the degree to which the extracted (deconvoluted) spectrum of the peak at that RT matched the spectrum in the AMDIS target library. The higher this number (out of a possible 100), the better the spectra agree. The column "R.T. Diff. sec." lists the difference in seconds between the observed RT and that in the AMDIS target library. The lower this number, the better the RTs agree.

An optional third feature of the report is the NIST search column (not shown). The NIST column lists the reverse match quality of the extracted spectrum compared with the NIST main library spectrum with the same CAS#. With the present setup, there are a large number of compounds for which a CAS# is not available. The Forensic Toxicology DBL contains some contrived CAS#s that would not be found in the NIST library. In the present analysis, the NIST search feature is therefore turned off.

Also shown in the NPD trace in Figure 2B are three peaks labeled ?A, ?B, and ?C. These three relatively large peaks are not in the target list of 725 compounds. The deconvoluted spectra corresponding to each of the three NPD responses were found in AMDIS and searched against the main NIST library. Peak ?A was identified as tributyl phosphate, a phosphorus compound commonly found as a sample handling artifact. Peak ?B was identified as 10,11-dihydrodibenz(b,f)(1,4)oxazepin-11-one. It was later found to be a second internal standard added during sample preparation. Peak ?3 remains unidentified. It is not in the NIST 05a Library (the best hit was only a 38 match) and it appears in many samples.

It is instructive to go through the identification of some of the compounds in the report and look at

the details of the identifications made. Oxycodone was readily identified because it had a high match quality in the AMDIS column and a very small retention time difference. Figure 4A shows the extracted ion chromatograms (EIC) as seen in QEdit. All the ions are clearly visible without interference and the ratios of the qualifier ions to the target are within the acceptable range. Also shown are the SIM ion EICs. They also are clearly visible without interference and the ratios of the qualifier ions to the target are within the acceptable range. The bottom trace from the NPD in Figure 4A shows a response with the same shape and at the same time as the oxycodone response in the mass traces. Figure 4B compares the deconvoluted spectrum found at the oxycodone retention time with the target library reference spectrum of oxycodone. The match is very good, with a match factor of 82. Oxycodone was an easy identification with all parameters clearly pointing to its presence.

Figure 5 shows a situation that is a bit more challenging. The compound here is methadone, whose spectrum has one large ion at 72; the remaining ones are very small. The EICs in Figure 5A are from

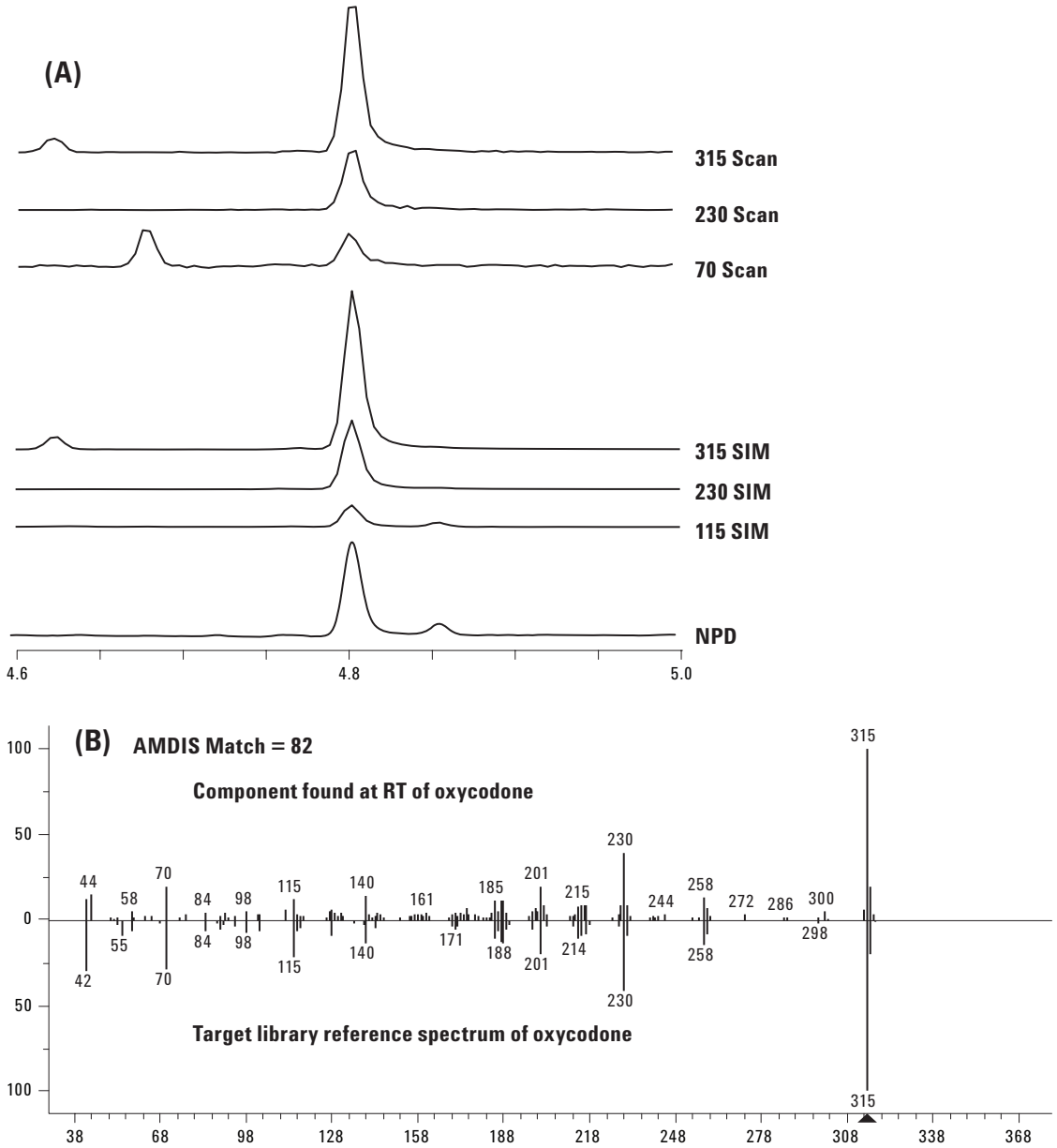

Figure 4. (A) Oxycodone response in SIM, scan, and NPD signals collected simultaneously. (B) Comparison of deconvoluted oxycodone spectrum with target library reference spectrum.

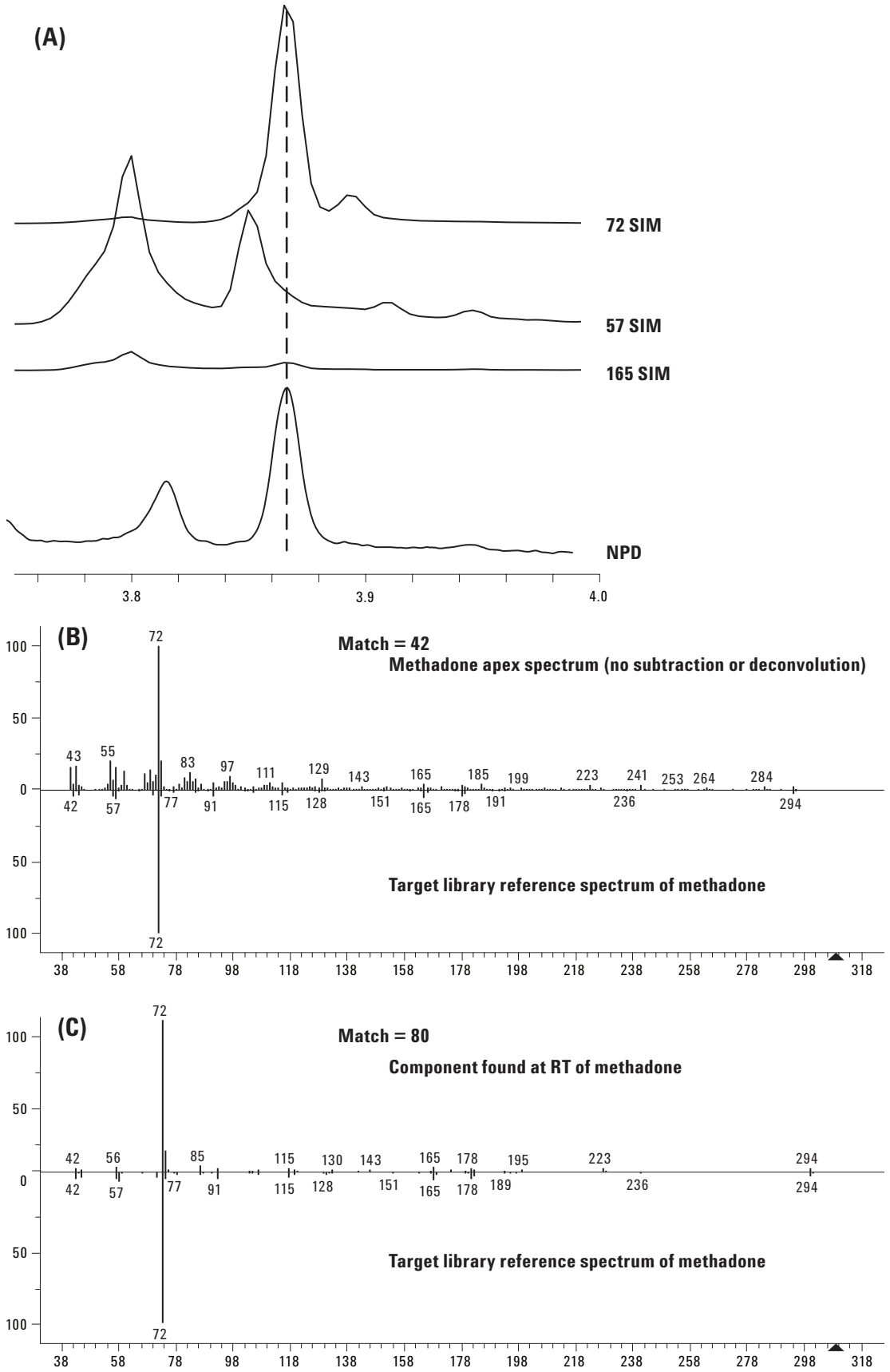

Figure 5. **(A) Methadone SIM and NPD chromatograms.**
(B) Comparison of reference spectrum with methadone spectrum without subtraction or deconvolution.
(C) Methadone deconvoluted spectrum searched against target library.

the SIM data. The traces from the scan data were identical (except of course with a lower signal-to-noise ratio). While there is a clear peak at the target ion, the middle qualifier (57) has a significant interference from the overlapping octadecanoic acid peak. With only the EIC data, the identification is questionable due to the loss of one of the qualifiers to interference. The NPD response shown below the SIM traces does support the fact that there is a nitrogen-containing compound at that retention time.

Figure 5B shows the apex spectrum at the methadone peak without subtraction or deconvolution compared with the target library reference spectrum. The match quality is unacceptably poor at 42 due to the interference of the octadecanoic acid peak. While the 72 ion is clearly visible, the other methadone ions are obscured. In Figure 5C the deconvoluted spectrum from the methadone retention time is compared with the reference. Deconvolution successfully removed the octadecanoic acid interference, and now the match quality is 80, clearly indicating the presence of methadone in the sample. The indication of methadone is also supported by two of the three ions being clearly present and in the correct ratio as well as an NPD response with the same retention time and peak shape.

Although caffeine is not a particularly high-priority target compound, the example shown in Figure 6 is

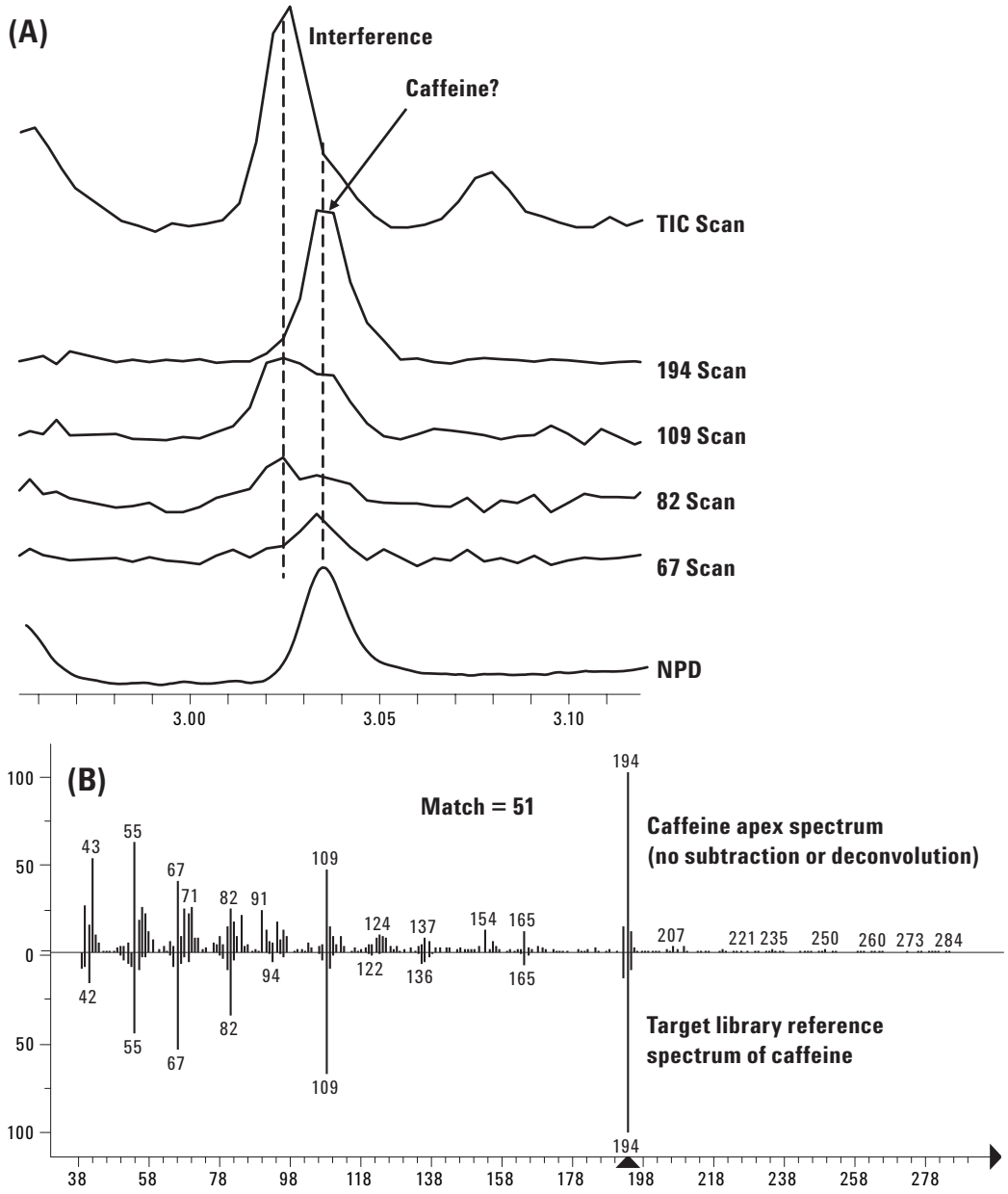

Figure 6. (A) TIC, scan EICs, and NPD signals for caffeine.
(B) Caffeine spectrum without subtraction or deconvolution shows interference from matrix compound.

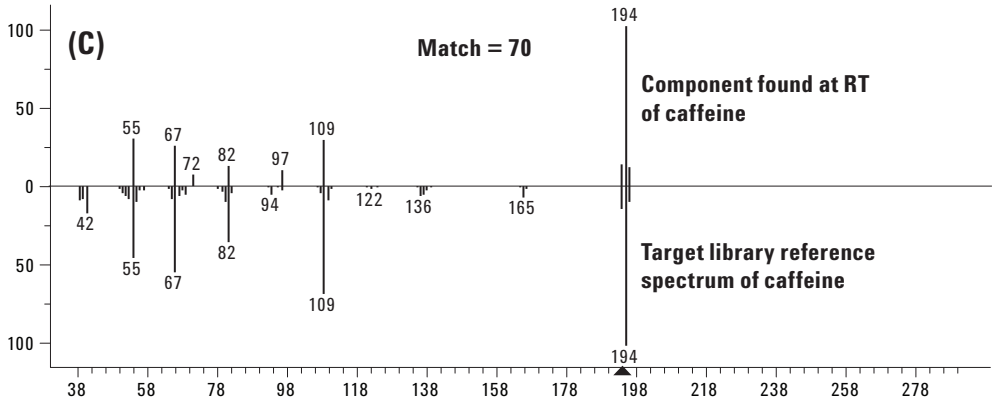

Figure 6C Caffeine deconvoluted spectrum searched against target library. (continued)

instructive. The caffeine, if present, is at a very low level as seen from the low signal-to-noise ratio of the four scan EICs shown in Figure 6A. Two ions, 109 and 82, also have interference problems from a large overlapping peak, as shown in the TIC trace at the top. The NPD trace does indicate a nitrogen-containing compound with the same peak shape and retention time as caffeine. The interfering peak was identified as 6,10,14-trimethyl-2-pentadecanone by searching the deconvoluted spectrum against the NIST main library. This compound also shares ions 109 and 82 with caffeine, resulting in the interference.

Figure 6B shows the apex spectrum of the caffeine peak without subtraction or deconvolution. When compared to the reference spectrum of caffeine, the match quality is poor, at only 51. Figure 6C shows the deconvoluted spectrum at the caffeine retention time compared to the reference spectrum and now the match quality is significantly improved to 70. This example demonstrates that the deconvolution process works even on small peaks with a low signal-to-noise ratio.

The example in Figure 7 is taken from a different sample and its purpose is to show the limits of deconvolution compared to the limits of the conventional approach. They are in fact similar because both approaches are limited by the same thing: signal-to-noise ratio. Figure 7A shows the scan and SIM EICs and the NPD trace for alprazolam. In the scan data, three of the four ions are barely visible and the fourth is lost in the noise. The SIM data clearly show a peak present at the alprazolam retention time and the ratios are in the correct range. The NPD also shows a response at the same retention time and with a similar shape. Figure 7B shows the deconvoluted spectrum compared to the NIST 05a library spectrum of alprazolam. The match factor is only 57.5. The match is marginal because AMDIS could only find a fraction of the alprazolam ions due to the extremely low level of the compound. This again illustrates that the target/qualifier approach using scan data and deconvolution begin to fail at about the same signal-to-noise ratio. In this example, the SIM data and NPD data are very helpful. If only the scan data were available for this sample, the identification of alprazolam would be doubtful and probably not reported. Taken with the SIM data in the correct ratios and the supporting evidence of the NPD response, a much stronger case can be made that alprazolam is indeed present, although at a very low level.

The last example is from a sample containing extraordinarily high levels of fatty acid interferences. These are clearly visible in Figure 8A. In QEdit, the presence of meprobamate was indicated with the peak shown at 3.007 minutes in Figure 8B. Although the ratios of the qualifiers to the target ion were within the relatively wide windows used here, the identification was doubtful. Examination of the EICs shows what looks like multiple peaks at the retention time that QEdit found. The retention time was also farther away (+ 0.080 minute) from the expected retention time of 2.928 minutes than is typically seen with the method. Also, there is no clear peak shape evident in the four traces at the 3.007 retention time. Based on these results alone, meprobamate looks like a false positive.

The EIC traces shown were from the column bleed optimized method. The use of 83 as the target ion clearly has interference problems with the high-level of fatty acids in this sample. When the method with

fatty acid optimized ions was used, the picture became a bit clearer. In this method, ion 62 is used as the target because of its significantly lower degree of interference. Looking at the trace for ion 62 in Figure 8, the peak now appears at 2.948 and is much closer to the expected retention time at 2.928 minutes. While the response at ion 62 looks a bit more like a real peak, the other ions in the fatty acid optimized method were still questionable due to the degree of interference, suggesting that it still may be a false positive. The NPD trace (not shown) did not resolve the question, as there were NPD peaks near 2.928 and 3.007 minutes.

The question was easily settled using the new A.04 release of DRS software. This version allows you to import into QEdit the AMDIS extracted peak profile from the deconvolution data and overlay it with the QEdit EICs. It also imports the deconvoluted spectrum for comparison with the QEdit-subtracted spectrum and the library reference spectrum. These capabilities simplify the review process by showing the deconvolution information inside of QEdit. Inspection of the AMDIS extracted peak profile relative to the EICs of the scan data shows that in fact the response at the target found with the fatty acid optimized method is indeed meprobamate. The

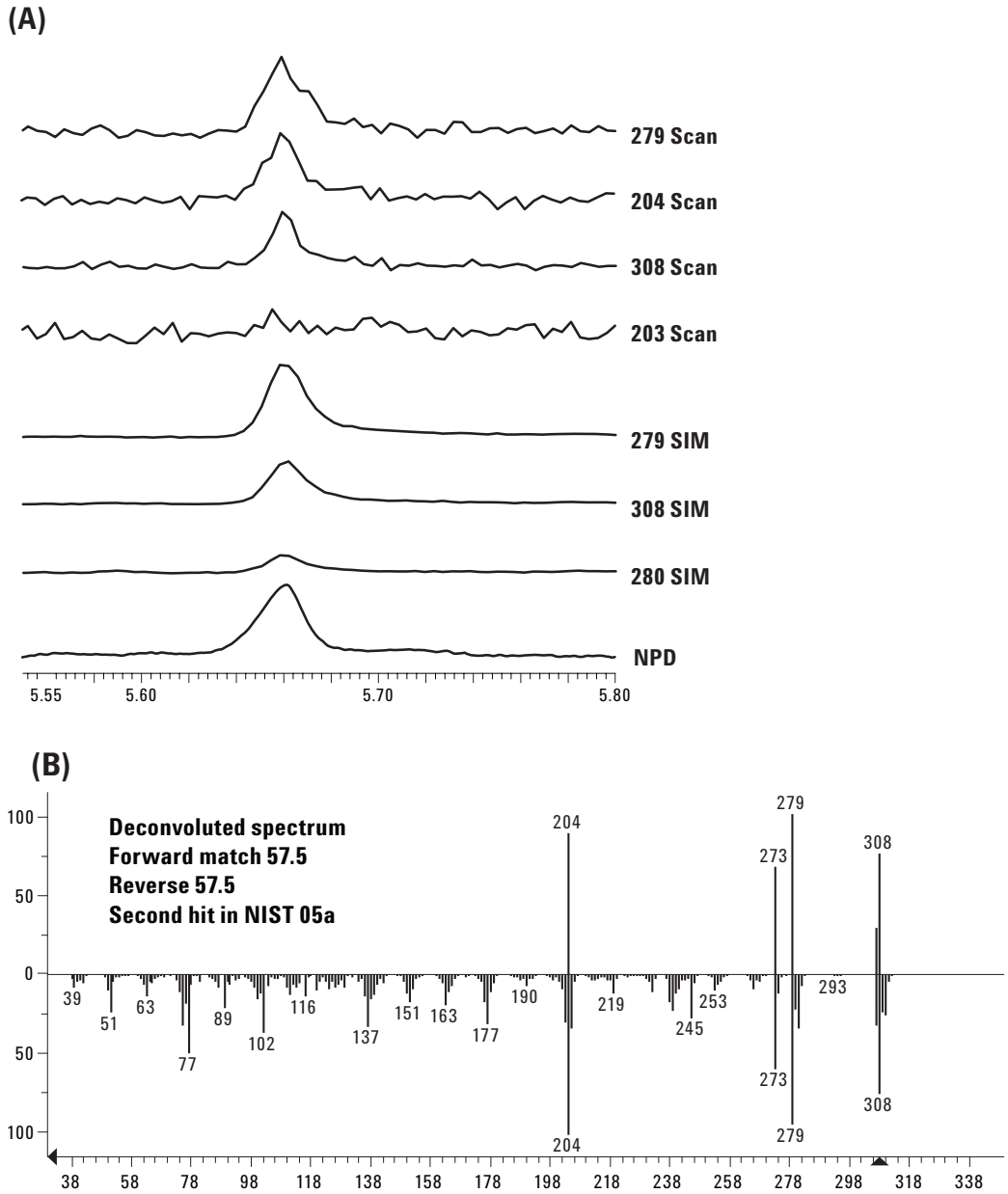

Figure 7. (A) Alprazolam response on SIM, scan, and NPD signals.
(B) Alprazolam deconvoluted spectrum searched against NIST 05a library.

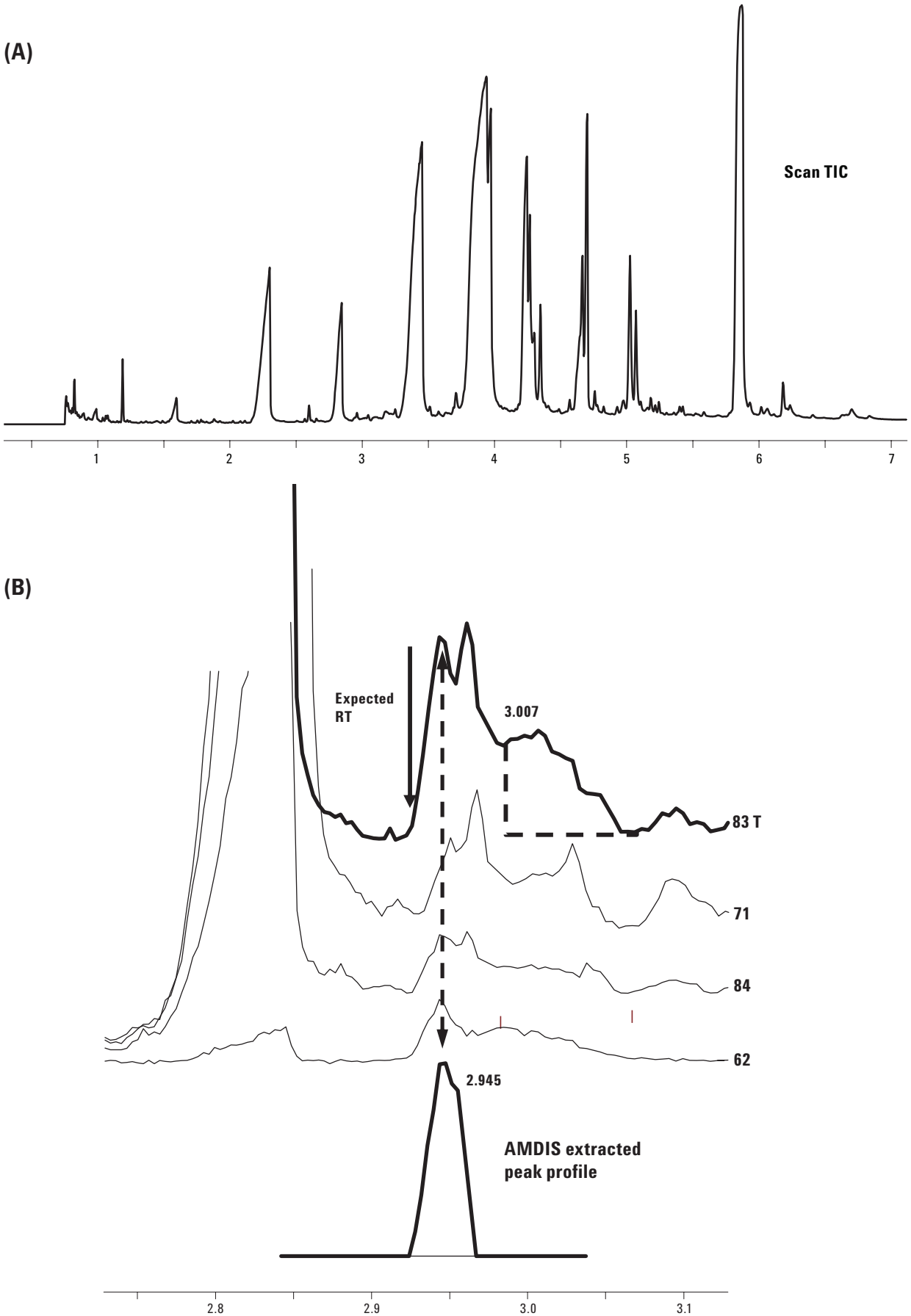

Figure 8. **(A) Scan TIC chromatogram of sample with high levels of fatty acids.**
(B) Scan EICs from bleed optimized method overlayed with AMDIS extracted peak profile.

(C)

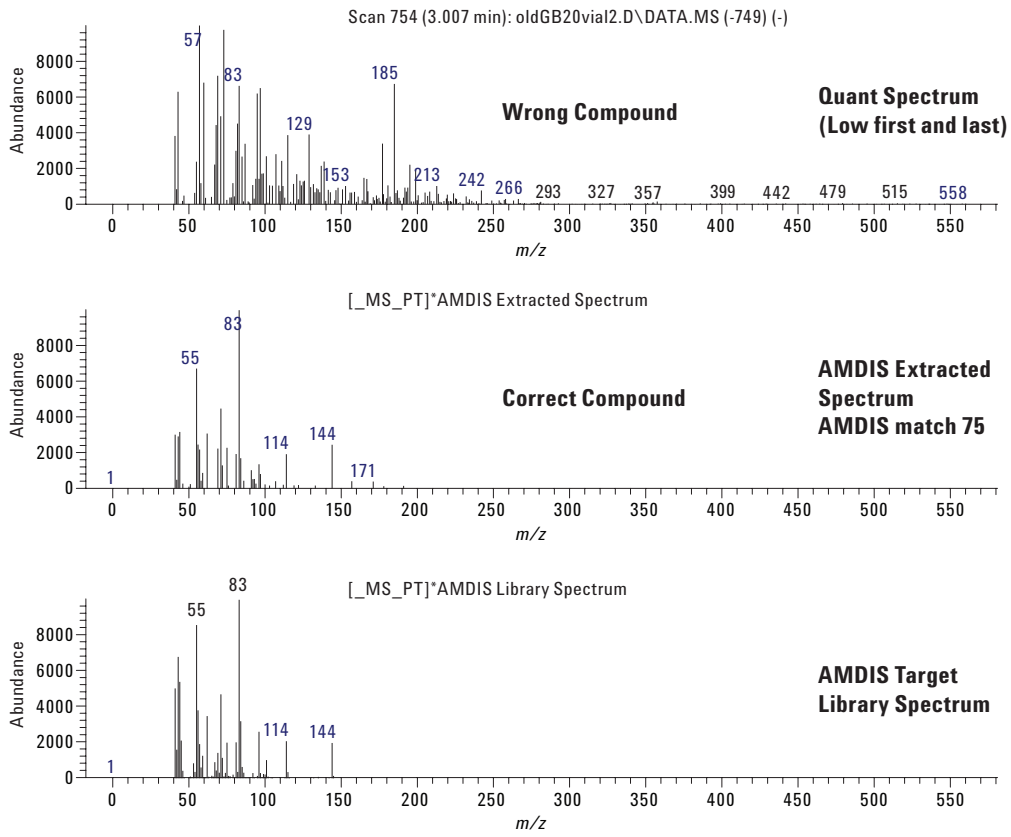

Figure 8C. Three meprobamate spectra presented in QEdit for comparison during data review using DRS A.04. (continued)

AMDIS extracted peak profile looks very similar to the peak profile in ion 62. If desired, the AMDIS extracted peak profile can be integrated for quantitation if the target ion has interference problems.

The best confirmation is provided by the deconvoluted spectrum. In Figure 8C are the three spectra presented in QEdit for comparison. The three spectra shown here were from the bleed optimized method. This method had incorrectly chosen the 3.007 peak as possibly being meprobamate, where the topmost spectrum is the spectrum at 3.007 minutes minus the spectrum five scans before, as the method uses “lowest first and last” as the subtraction method. Since the peak was found at the wrong retention time, the spectrum is of the wrong compound and of course does not match that of meprobamate. When searched against the NIST main library, meprobamate was not in the top 100 hits.

The middle spectrum is the deconvoluted component found by AMDIS. It has a match factor against the reference spectrum, shown in the bottom, of 75, confirming the presence of meprobamate. This example shows the utility of deconvolution in determining the presence of compounds that could easily be missed with the conventional approaches.

Conclusions

The system described here offers several advantages for screening toxicology samples. The advantages derive from a combination of techniques that result in both faster and more accurate screening results.

- Retention time locked target database of 725 compounds for screening with MS (G1674AA Forensic Toxicology DBL)

- CFT splitter – Use the NPD with MS data for added confirmation, find nontarget suspect compounds, and alternate quantitation
- SIM/Scan – Acquire SIM data on high-priority targets simultaneously with scan data. Saves time by eliminating need to run samples in both modes.
- DRS – Automated deconvolution increases accuracy of target identification, even in the most challenging matrices. The reduction of data interpretation from more than an hour to less than 10 minutes is especially useful.
- Fast chromatography using shorter columns, faster ovens, and backflushing to greatly reduce run times.

There is considerable advantage to using a single system that combines all of the techniques discussed. However, adding any of the above separately or in different combinations can also provide advantages. The most significant improvement can be gained by using DRS. The time savings in the data review step easily justifies the effort required to implement it.

References

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Appendix

Compound name	CAS number*
10,11-Dihydro-10-hydroxycarbazepine	999402-02-7
10,11-Dihydro-10-hydroxycarbazepine TMS	999423-02-8
10,11-Dihydrocarbamazepin	003564-73-6
5-Amino-2-chloropyridine	005350-93-6
5-Methoxy-dipropyltryptamine	999001-02-4
6-Acetyl-morphine	002784-73-8
6-Acetyl-morphine TMS	999155-02-1
7-Aminoflunitrazepam	034084-50-9
7-Aminoflunitrazepam TMS	999176-02-2
7-Hydroxyamoxapine	037081-76-8
8-Methoxyloxapine	070020-54-1
Acepromazine	000061-00-7
Acetaminophen	000103-90-2
Acetaminophen 2TMS	055530-61-5
Acetanilide	000103-84-4
Adiphenine	000064-95-9
Adiphenine-M/artifact (ME)	003469-00-9
Alfentanil	071195-58-9
Allobarbital	000052-43-7
Allopurinol TMS	999178-02-8
Alphaprodine	000077-20-3
Alphenal	000115-43-5
Alprazolam	028981-97-7
Alprenolol TMS	999381-02-1
Alverine	000150-59-4
Amantadine	000768-94-5
Amantadine AC	999127-02-5
Ambroxol	018683-91-5
Ambroxol 2AC	999341-02-5
Aminoglutethimide	000125-84-8
Aminopyrine	000058-15-1
Amitriptyline	000050-48-6
Amlodipine AC	999299-02-4
Amobarbital	000057-43-2
Amobarbital 2TMS	999179-02-1
Amoxapine	014028-44-5
Amoxapine AC	999128-02-8
Amphetamine	000060-15-1
Amphetamine AC	999107-02-7
Ampyrone	000083-07-8
Ampyrone AC	000083-15-8
Ampyrone-2AC	999240-02-7
Anhydroecgonine methyl ester	043021-26-7
Anileridine	000144-14-9
Anisindione	000117-37-3
Antazoline	000091-75-8
Antazoline AC	999408-02-5
Antipyrine	000060-80-0
Apomorphine 2TMS	074841-68-2
Aprobarbital	000077-02-1
Aprobarbital 2TMS	999180-02-8
Atenolol formyl artifact	999459-02-8
Atomoxetine	083015-26-3
Atomoxetine AC	999257-02-2
Atovaquone	953233-18-4
Atovaquone TMS	999409-02-8
Atropine	000051-55-8
Atropine TMS	055334-03-7
Azacyclonol	000115-46-8
Azatadine	003964-81-6
Barbital	000057-44-3
BDMPEA	066142-81-2
BDMPEA AC	999357-02-7
BDMPEA formyl artifact	999378-02-8
Bemegride	000064-65-3
Benzocaine	000094-09-7
Benzoylecgonine	000519-09-5
Benzoylecgonine TMS	999462-02-1
Benzphetamine	000156-08-1
Benzquinamide	000063-12-7
Benztropine	000086-13-5
Benzydamine	000642-72-8
Benzylpiperazine	002759-28-6
Benzylpiperazine AC	999129-02-1
Betahistine	005579-84-0
Betahistine AC	999439-02-0
Betaxolol	063659-18-7
Betaxolol formyl artifact	999436-02-1
Biperiden	000514-65-8
Bisacodyl	000603-50-9
Bisoprolol	066722-44-9
Bromazepam	001812-30-2

* Compounds for which a real CAS number could not be found were given a contrived one beginning with 999. These are not real CAS numbers.

Compound name	CAS number
Bromazepam TMS	999158-02-0
Bromdiphenhydramine	000118-23-0
Bromocriptine breakdown	025614-03-3
Bromperidol	010457-90-6
Brompheniramine	000086-22-6
Brucine	000357-57-3
Buclizine	000082-95-1
Bupivacaine	002180-92-9
Buprenorphine	052485-79-7
Buprenorphine TMS	999159-02-3
Bupropion	034911-55-2
Buspirone	036505-84-7
Butabarbital	000125-40-6
Butabarbital 2TMS	052988-92-8
Butacaine	000149-16-6
Butalbital	000077-26-9
Butalbital 2TMS	052937-70-9
Butethal	000077-28-1
Butorphanol	042408-82-2
Butorphanol TMS	100013-72-3
Caffeine	000058-08-2
Canrenone	000976-71-6
Canrenone TMS	999413-02-4
Cantharidin	000056-25-7
Carbamazepine	000298-46-4
Carbamazepine-M (formyl-acridine)	999243-02-6
Carbinoxamine	000486-16-8
Carbromal-M/artifact	999196-02-0
Carisoprodol	000078-44-4
Carisoprodol artifact	999401-02-4
Cathinone AC	999485-02-8
Celecoxib	169590-42-5
Cetirizine methanol adduct	083881-46-3
Cetirizine TMS	999183-02-7
Chlophedianol	000791-35-5
Chlophedianol TMS	999464-02-7
Chloramphenicol 2TMS	021196-84-9
Chlorcyclizine	000082-93-9
Chlordiazepoxide	000058-25-3
Chlordiazepoxide artifact (desoxo)	999197-02-3
Chlormezanone	000080-77-3
Chlormezanone artifact	999245-02-2
Chloroamphetamine	000064-12-0
Chloroamphetamine AC	999414-02-7
Chlorophenylpiperazine	038212-33-8
Chlorophenylpiperazine AC	999486-02-1
Chloroprocaine, 2-	000133-16-4
Chloroquine	000054-05-7
Chlorpheniramine	000132-22-9
Chlorphenisin	000104-29-0
Chlorphentermine	000461-78-9
Chlorphentermine AC	999130-02-8
Chlorpropamide artifact-2	999246-02-5
Chlorprothixene	000113-59-7
Chlorzoxazone	000095-25-0
Cholesterol	000057-88-5
Cholesterol TMS	001856-05-9
Cinnarizine	000298-57-7
Cisapride	081098-60-4
Citalopram	059729-33-8
Clemastine	015686-51-8
Clemizole	000442-52-4
Clenbuterol	037148-27-9
Clenbuterol AC	999360-02-0
Clobazam	022316-47-8
Clofibrate	000637-07-0
Clomipramine	000303-49-1
Clonazepam	001622-61-3
Clonazepam TMS	999184-02-0
Clonazepam-M (amino-)	004959-17-5
Clonazepam-M (amino) - TMS	999175-02-9
Clonidine	004205-90-7
Clonidine 2AC	999131-02-1
Clonidine AC	999132-02-4
Clopidogrel	113665-84-2
Clozapine	005786-21-0
Clozapine AC	999133-02-7
Cocaethylene	000529-38-4
Cocaine	000050-36-2
Codeine	000076-57-3
Codeine TMS	074367-14-9
Colchicine	000064-86-8

Compound name	CAS number
Colchicine breakdown	999532-02-4
Coniine	000458-88-8
Coniine AC	999361-02-3
Cotinine	000486-56-6
Cyclandelate	000456-59-7
Cyclandelate TMS	999442-02-3
Cyclizine	000082-92-8
Cyclobenzaprine	000303-53-7
Cyclophosphamide	000050-18-0
Cyclophosphamide -HCL	999379-02-1
Cyheptamide	007199-29-3
Cyproheptadine	000129-03-3
Dapsone	000080-08-0
Debrisoquine AC	999415-02-0
Desalkylflurazepam AC	999298-02-1
Desethyllidocaine (MegX)	999044-02-9
Desethyllidocaine AC (MegX)	999263-02-4
Desipramine	000050-47-5
Desipramine AC	999108-02-0
Desmethylclomipramine	000303-48-0
Desmethylclomipramine AC	999134-02-0
Desmethylclozapine	006104-71-8
Desmethyldoxepin (cis)	999516-02-8
Desmethyldoxepin (cis) AC	999517-02-1
Desmethyldoxepin (trans)	001225-56-5
Desmethyldoxepin (trans) AC	999443-02-6
Desmethylselegiline	999072-02-5
Desmethylselegiline AC	999147-02-3
Desmethylsertraline	091797-58-9
Desmethyltramadol, O-	999018-02-9
Desmethyltramadol, O- 2TMS	999444-02-9
Desmethyltrimipramine	999019-02-2
Desmethyltrimipramine AC	999445-02-2
Dextromethorphan	000125-71-3
Diacetylmorphine	000561-27-3
Diazepam	000439-14-5
Dichlorophene	000097-23-4
Dichlorophene TMS	999237-02-4
Diclofenac -H2O	999200-02-1
Diclofenac TMS	999222-02-5
Dicyclomine	000077-19-0
Diethyltryptamine	000061-51-8
Dihydrocodeine	000125-28-0
Dihydroxy-4-methylcoumarin, 7, 8 - TMS	999236-02-1
Diiodohydroxyquin	000083-73-8
Diltiazem	042399-41-7
Dimethadione	000695-53-4
Diphenadione	000082-66-6
Diphenhydramine	000058-73-1
Diphenidol	000972-02-1
Diphenidol TMS	999417-02-6
Diphenoxylate	000915-30-0
Diphenylpyraline	000147-20-6
Disopyramide	003737-09-5
Donepezil	120014-06-4
Dothiepin	000113-53-1
Doxapram	000309-29-5
Doxepin (cis)	999515-02-5
Doxepin (trans)	001668-19-5
Doxylamine	000469-21-6
Dyphylline	000479-18-5
Dyphylline TMS	999446-02-5
Ecgonine methyl ester	106293-60-1
Ecgonine methyl ester TMS	999162-02-6
Efavirenz	154598-52-4
Efavirenz AC	999489-02-0
Efavirenz TMS	999505-02-1
Emetine	000483-18-1
Encainide	999034-02-5
Ephedrine	000299-42-3
Ephedrine 2AC	055133-90-9
Epinephrine AC	999111-02-3
Ergonovine AC	999447-02-8
Estazolam	029975-16-4
Ethacrynic Acid TMS	999227-02-0
Ethambutol AC	999261-02-8
Ethamivan	000304-84-7
Ethinamate	000126-52-3
Ethopropazine	000522-00-9
Ethosuximide	000077-67-8
Ethotoin	000086-35-1
Ethyl-2-malonamide, 2-	068692-83-1

Compound name	CAS number
Ethyl-2-malonamide, 2- TMS	999418-02-9
Ethylamphetamine	000457-87-4
Ethylamphetamine AC	999148-02-6
Ethylecgonine	999037-02-4
Ethylecgonine TMS	999448-02-1
Ethylmorphine	000076-58-4
Ethylmorphine TMS	999221-02-2
Etodolac TMS	999212-02-1
Etofylline	000519-37-9
Etofylline TMS	077630-35-4
Etomidate	033125-97-2
Eucatropine Isomer 1	999038-02-7
Eucatropine Isomer 1 TMS	999278-02-3
Eucatropine Isomer 2	999277-02-0
Eucatropine Isomer 2 TMS	999518-02-4
Felbamate artifact 1	999250-02-1
Felbamate artifact 2	999251-02-4
Felbamate artifact 3	999252-02-7
Felodipine	072509-76-3
Felodipine-M/artifact (dehydro-)	999296-02-5
Fenfluramine	000458-24-2
Fenfluramine AC	999139-02-5
Fenoprofen	031879-05-7
Fenoprofen TMS	999310-02-0
Fentanyl	000437-38-7
Finasteride	098319-26-7
Flavoxate	015301-69-6
Flavoxate-M/artifact (HOOC-) ME	999279-02-6
Flecainide	054143-55-4
Flecainide AC	999140-02-2
Flumazenil	078755-81-4
Flunarizine	052468-60-7
Flunitrazepam	001622-62-4
Fluoxetine	054910-89-3
Fluoxetine AC	999141-02-5
Flupenthixol	002709-56-0
Flupentixol TMS	999387-02-9
Fluphenazine	000069-23-8
Fluphenazine TMS	999280-02-3
Fluphenazine-M (ring)	000092-30-8
Flurazepam	017617-23-1
Flurazepam-M (desalkyl-)	002886-65-9
Flurazepam-M (HO-ethyl-)	020971-53-3
Flurbiprofen	005104-49-4
Flutamide	013311-84-7
Flutamide TMS	999467-02-6
Fluvoxamine	054739-18-3
Fluvoxamine AC	999262-02-1
Furazolidone	000067-45-8
Furosemide 2TMS	999214-02-7
Gemfibrozil	025812-30-0
Gemfibrozil AC	999389-02-5
Glutethimide	000077-21-4
Griseofulvin	000126-07-8
Guaifenesin	000093-14-1
Guaifenesin 2TMS	107966-19-8
Guanethidine	000055-65-2
Haloperidol	000052-86-8
Harmaline	000304-21-2
Harmaline AC	999301-02-9
Harmine	000442-51-3
Hexobarbital	000056-29-1
Hexobarbital TMS	999469-02-2
Hexylresorcinol	000136-77-6
Hexylresorcinol 3TMS	999422-02-5
Homatropine	000087-00-3
Homatropine TMS	999282-02-9
Hydrastine	000118-08-1
Hydrocodone	000125-29-1
Hydromorphone	000466-99-9
Hydromorphone enol 2TMS	999513-02-9
Hydromorphone TMS	221209-08-1
Hydroxychloroquine AC	999512-02-6
Hydroxyethylflurazepam TMS	999204-02-3
Hydroxyloxapine, 8-	999053-02-0
Hydroxyzine	000068-88-2
Hydroxyzine AC	999113-02-9
Ibuprofen	015687-27-1
Ibuprofen TMS	999165-02-5
Iminostilbene	000256-96-2
Imipramine	000050-49-7
Indomethacin TMS	999318-02-4

Compound name	CAS number
Isocarboxazid	000059-63-2
Isometheptene AC	999265-02-0
Isoniazid	000054-85-3
Isoniazid 2AC	999266-02-3
Isoniazid AC	999254-02-3
Isoproterenol 2TMS	999424-02-1
Isoxsuprine	000395-28-8
Isoxsuprine TMS	999319-02-7
Ketamine	006740-88-1
Ketamine AC	999114-02-2
Ketoprofen TMS	999320-02-4
Ketorolac TMS	999215-02-0
Ketotifen	034580-13-7
Lamotrigine	084057-84-1
Lamotrigine 2AC	999255-02-6
Laudanosine	020412-65-1
Levallorphan	000152-02-3
Levallorphan TMS	999321-02-7
Levetiracetam	102767-28-2
Levorphanol	000077-07-6
Levorphanol TMS	999223-02-8
Lidocaine	000137-58-6
Loratadine	079794-75-5
Lorazepam	000846-49-1
Lorazepam 2TMS	999202-02-7
Lorcainide	059729-31-6
Lormetazepam	000848-75-9
Loxapine	001977-10-2
Ly170222	999123-02-3
Lysergide (LSD)	000050-37-3
Maprotiline	010262-69-8
Maprotiline AC	999366-02-8
Mazindol	022232-71-9
MBDB	100031-29-2
MBDB AC	999142-02-8
Mecamylamine	000060-40-2
Meclizine	000569-65-3
Meclofenamic acid TMS	999322-02-0
Medazepam	002898-12-6
Mefenamic acid TMS	999324-02-6
Mefloquine	053230-10-7

Compound name	CAS number
Memantine	019982-08-2
Memantine AC	999115-02-5
Meperidine	000057-42-1
Mephenesin	000059-47-2
Mephenesin 2TMS	999325-02-9
Mephentermine	000100-92-5
Mephentermine AC	999143-02-1
Mephenytoin	000050-12-4
Mephobarbital	000115-38-8
Mepivacaine	000096-88-8
Meprobamate	000057-53-4
Mescaline	000054-04-6
Mescaline AC	999511-02-3
Mescaline formyl artifact	999284-02-5
Mesuximide-M (nor)	001497-17-2
Metaproterenol AC	999391-02-5
Metaxalone	001665-48-1
Metaxalone AC	999116-02-8
Methadone	000076-99-3
Methadone-M (EDDP)	999058-02-5
Methamphetamine	000537-46-2
Methamphetamine AC	999117-02-1
Methapyrilene	000091-80-5
Methaqualone	000072-44-6
Metharbital	000050-11-3
Metharbital TMS	999186-02-6
Methazolamide	000554-57-4
Methcathinone AC	999300-02-6
Methcathinone-M (HO-) 2AC	005650-44-2
Methdilazine	001982-37-2
Methimazole	000060-56-0
Methimazole AC	999368-02-4
Methocarbamol 2TMS	999285-02-8
Methohexital	000151-83-7
Methohexital TMS	999425-02-4
Methotrimpeprazine	000060-99-1
Methoxyverapamil	016662-47-8
Methsuximide	000077-41-8
Methylaminorex, 4-	029493-77-4
Methylaminorex, 4- 2AC	999508-02-0
Methylaminorex, 4- AC	999510-02-0

Compound name	CAS number
Methylenedioxyamphetamine AC	999479-02-6
Methylenedioxyamphetamine (MDA)	004764-17-4
Methylenedioxyethylamphetamine	014089-52-2
Methylenedioxyethylamphetamine AC	999481-02-6
Methylenedioxymethamphetamine AC	999480-02-3
Methylenedioxymethamphetamine (MDMA)	042542-10-9
Methylephedrine	000552-79-4
Methylephedrine AC	999370-02-4
Methyl-nicotine	999065-02-0
Methylphenidate	000113-45-1
Methylphenidate AC	999144-02-4
Methylphenobarbtial	999509-02-3
Methylprimidone	059026-32-3
Methylprimidone 2TMS	999286-02-1
Methyprylon	000125-64-4
Metoclopramide	000364-62-5
Metoclopramide AC	999145-02-7
Metoprolol 2AC	999306-02-4
Metronidazole	000443-48-1
Metronidazole TMS	999450-02-1
Mexiletine	031828-71-4
Mexiletine AC	999146-02-0
Mianserin	024219-97-4
Mianserin-M (nor-)	999015-02-0
Mianserin-M (nor-) AC	999364-02-2
Midazolam	059467-70-8
Mirtazapine	061337-67-5
Moclobemide	071320-77-9
Molindone	007416-34-4
Morphine	000057-27-2
Morphine 2TMS	055449-66-6
Muconic acid TMS	999166-02-8
N,N-Dimethyl-5-methoxy-tryptamine	001019-45-0
N,N-Dimethyltryptamine	000061-50-7
Nabumetone	042924-53-8
N-Acetylprocainamide	999070-02-9
Nadolol 3TMS	999287-02-4
Nalbuphine	020594-83-6
Nalbuphine 2TMS	999167-02-1
Nalidixic acid	000389-08-2
Nalidixic acid TMS	999238-02-7

Compound name	CAS number
Nalorphine	000062-67-9
Nalorphine 2TMS	999473-02-8
Naloxone	000465-65-6
Naloxone TMS	999427-02-0
Naltrexol, beta-	999406-20-9
Naltrexol, beta- 2TMS	999405-02-6
Naltrexol, beta- 3TMS	999520-02-4
Naltrexone	016590-41-3
Naltrexone 2TMS	999328-02-8
Naltrexone 3TMS	999523-02-3
Naltrexone TMS	999522-02-0
Naproxen ME	999295-02-2
Naproxen TMS	074793-83-2
Nevirapine	129618-40-2
Nevirapine TMS	999451-02-4
Niclosamide	000050-65-7
Nicotinamide	000098-92-0
Nicotine	000054-11-5
Nifedipine	021829-25-4
Nikethamide	000059-26-7
Nimodipine	066085-59-4
Nimodipine-M/artifact	999340-02-2
Nitrazepam	000146-22-5
Nitrazepam TMS	999288-02-7
Nomifensine	024526-64-5
Nomifensine AC	999371-02-7
Noralfentanil	061086-18-8
Noralfentanil AC	999150-02-6
Norchlordiazepoxide	016300-25-7
Norchlordiazepoxide AC	999525-02-9
Norchlordiazepoxide breakdown	999524-02-6
Norchlordiazepoxide breakdown AC	999372-02-0
Norclozapine 2AC	999135-02-3
Norclozapine AC	999136-02-6
Norcodeine	000467-15-2
Norcodeine 2AC	999118-02-4
Nordiazepam	001088-11-5
Nordiazepam TMS	999207-02-2
Norepinephrine 2AC	999119-02-7
Norepinephrine 3AC	999528-02-8
Norfenfluramine	001886-26-6

Compound name	CAS number
Norfenfluramine AC	999120-02-4
Norfentanyl	999076-02-7
Norfentanyl AC	999272-02-5
Norfluoxetine	999077-02-0
Norfluoxetine AC	999121-02-7
Norketamine	999078-02-3
Norketamine AC	999494-02-9
Normeperidine	000077-17-8
Normeperidine AC	999122-02-0
Normetanephrine AC	999373-02-3
Normethsuximide TMS	999429-02-6
Noroxycodone	057664-96-7
Noroxycodone AC	999495-02-2
Norpropoxyphene	999079-02-6
Norpropoxyphene breakdown 1	999530-02-8
Norpropoxyphene breakdown 2	999531-02-1
Norpropoxypheneamide	999080-02-3
Norpseudoephedrine	000492-41-1
Norpseudoephedrine AC	999081-02-6
Norpseudoephedrine artifact	999478-02-3
Nortriptyline	000072-69-5
Nortriptyline AC	999151-02-9
Norvenlafaxine	130198-38-8
Norverapamil	067018-85-3
Norverapamil AC	999488-02-7
Olanzapine	132539-06-1
Opipramol TMS	999226-02-7
Orphenadrine	000083-98-7
Ortho-cotinine	999083-02-2
Oxazepam	000604-75-1
Oxazepam 2TMS	999168-02-4
Oxcarbamazepine	028721-07-5
Oxprenolol 2AC	999374-02-6
Oxybutynin	005633-20-5
Oxycodone	000076-42-6
Oxycodone enol 2TMS	999514-02-2
Oxycodone TMS	221209-10-5
Oxymorphone	000076-41-5
Oxymorphone 2TMS	999521-02-7
Oxymorphone TMS	999208-02-5
Papaverine	000058-74-2

Compound name	CAS number
Paramethadione	000115-67-3
Pargyline	000555-57-7
Paroxetine	061869-08-7
Paroxetine AC	999124-02-6
Pemoline	002152-34-3
Pentachlorophenol	000087-86-5
Pentazocine	000359-83-1
Pentazocine TMS	100013-72-2
Pentobarbital	000076-74-4
Pentobarbital 2TMS	052937-68-5
Pentoxifylline	006493-05-6
Pentylenetetrazole	000054-95-5
Pergolide	066104-22-1
Perphenazine TMS	999291-02-0
Phenacemide	000063-98-9
Phenacetin	000062-44-2
Phenacetin AC	999496-02-5
Phenacetin TMS	999504-02-8
Phenazopyridine	000094-78-0
Phenazopyridine AC	999303-02-5
Phencyclidine	000077-10-1
Phencyclidine artifact	000771-98-2
Phendimetrazine	000634-03-7
Phenelzine AC	999304-02-8
Phenindione	000083-12-5
Pheniramine	000086-21-5
Phenmetrazine	000134-49-6
Phenmetrazine AC	999090-02-7
Phenobarbital	000050-06-6
Phenobarbital 2TMS	052937-73-2
Phenolphthalein	000077-09-8
Phenolphthalein 2TMS	999292-02-3
Phenoxybenzamine	000059-96-1
Phensuximide	000086-34-0
Phentermine	000122-09-8
Phentermine AC	999152-02-2
Phenylacetamide	000103-81-1
Phenylbutazone	000050-33-9
Phenylbutazone artifact	999338-02-2
Phenylbutazone artifact TMS	999198-02-6
Phenylbutazone TMS	074810-87-0

Compound name	CAS number
Phenylephrine 3AC	999091-02-0
Phenylethylamine, beta-	000064-04-0
Phenylethylamine, beta AC	999343-02-1
Phenylpropanolamine	999498-02-1
Phenylpropanolamine AC	999092-02-3
Phenyltoloxamine	000092-12-6
Phenytoin	000057-41-0
Phenytoin 2TMS	063435-72-3
Pilocarpine	000092-13-7
Pindolol	013523-86-9
Pindolol formyl artifact	999458-02-5
PMA TMS	999172-02-0
p-Methoxyamphetamine	000064-13-1
Prazepam	002955-38-6
Prilocaine	000721-50-6
Primidone	000125-33-7
Probenecid TMS	999294-02-9
Procainamide	000051-06-9
Procaine	000059-46-1
Prochlorperazine	000058-38-8
Procyclidine	000077-37-2
Procyclidine artifact (dehydro-)	999460-02-5
Procyclidine TMS	999454-02-3
Promazine	000058-40-2
Promethazine	000060-87-7
Propantheline bromide	000050-34-0
Propiomazine	000362-29-8
Propofol	002078-54-8
Propoxur	000114-26-1
Propoxur-M/artifact	999393-02-1
Propoxyphene	000469-62-5
Propylamphetamine	051799-32-7
Propylamphetamine AC	999302-02-2
Protriptyline	000438-60-8
Protriptyline AC	999273-02-8
Pseudoephedrine	000090-82-4
Pseudoephedrine 2AC	999500-02-6
Pseudoephedrine formyl artifact	999483-02-2
Psilocin 2TMS	999192-02-8
Psilocybin 3TMS	999193-02-1
Pyrazinamide	000098-96-4
Pyrilamine	000091-84-9
Pyrimethamine	000058-14-0
Quetiapine	999097-02-8
Quetiapine TMS	999527-02-5
Quinacrine	000083-89-6
Quinidine	000056-54-2
Quinine	000130-95-0
Ramelteon	999274-02-1
Reboxetine	098769-81-4
Ritodrine 3TMS	999218-02-9
Rofecoxib	162011-90-7
Ropivacaine	132112-35-7
Salbutamol 3TMS	999394-02-4
Salicylamide	000065-45-2
Salicylamide 2TMS	055887-58-6
Salicylic acid 2TMS	003789-85-3
Salicylic acid ethylester	000118-61-6
Salicylic acid methylester	000119-36-8
Scopolamine	000051-34-3
Scopolamine TMS	999194-02-4
Secobarbital	000076-73-3
Secobarbital 2TMS	052937-71-0
Selegiline	014611-51-9
Selegiline-M (HO-) AC	999482-02-9
Sertraline	079617-96-2
Sertraline AC	999125-02-9
Sertraline-M (nor-) AC	999109-02-3
Sildenafil TMS	999213-02-4
SKF-525a	000302-33-0
Strychnine	000057-24-9
Sufentanil	056030-54-7
Sulfadiazine	000068-35-9
Sulfadimethoxine	000122-11-2
Sulfamethazine	000057-68-1
Sulfamethazine AC	999501-02-9
Sulfamethoxazole	000723-46-6
Sulfanilamide	000063-74-1
Sulfapyridine	000144-83-2
Sulfathiazole	000072-14-0
Sulfinpyrazone	000057-96-5
Tacrine	000321-64-2

Compound name	CAS number
Talbutal	000115-44-6
Tamoxifen	010540-29-1
Temazepam	000846-50-4
Temazepam artifact-2	020927-53-1
Temazepam TMS	035147-95-6
Terbinafine	091161-71-6
Terfenadine TMS	999220-02-9
Teriflunomide AC	999502-02-2
Tetracaine	000094-24-6
Tetrahydrocannabinol	001972-08-3
Tetrahydrocannabinol TMS	999529-02-1
Tetrahydrozoline	000084-22-0
Tetrahydrozoline AC	999398-02-6
Thebaine	000115-37-7
Theobromine	000083-67-0
Theophyline	000058-55-9
Thiamylal	000077-27-0
Thiethylperazine	001420-55-9
Thiopental	000076-75-5
Thioridazine	000050-52-2
Thonzylamine	000091-85-0
Ticlopidine	055142-85-3
Tiletamine	014176-49-9
Timolol TMS	999399-02-9
Tocainide	041708-72-9
Tocainide AC	999375-02-9
Tolazoline	000059-98-3
Topiramate artifact (-SO_2NH)	020880-92-6
Topiramate breakdown	097240-79-4
Tramadol	027203-92-5
Tramadol TMS	999336-02-6
Tranylcypromine	000155-09-9
Tranylcypromine AC	999305-02-1
Trazodone	019794-93-5
Triamterene	000396-01-0
Triazolam	028911-01-5
Trifluoperazine	000117-89-5
Triflupromazine	000146-54-3
Trihexyphenidyl	000144-11-6
Trimeprazine	000084-96-8
Trimethobenzamide	000138-56-7
Trimethoprim	000738-70-5
Trimipramine	000739-71-9
Tripelenamine	000091-81-6
Triprolidine	000486-12-4
Tropacocaine	000537-26-8
Tryptamine	000061-54-1
Tryptamine 2AC	999352-02-2
Tryptamine AC	999353-02-5
Tryptophan, D- AC	999519-02-7
Valproic acid	000099-66-1
Venlafaxine	093413-69-5
Venlafaxine TMS	999173-02-3
Verapamil	000052-53-9
Vigabatrin AC	999376-02-2
Warfarin	000081-81-2
Warfarin artifact	000122-57-6
Warfarin TMS	036307-79-6
Xanthinol TMS	999239-02-0
Xylazine	007361-61-7
Yohimbine	000146-48-5
Yohimibine TMS	999457-02-2
Zaleplon	151319-34-5
Zolazepam	031352-82-6
Zolpidem	082626-48-0
Zomepirac -CO_2	999355-02-1
Zonisamide	068291-97-4
Zonisamide AC	999354-02-8
Zopiclone	043200-80-2
Zotepine	026615-21-4

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Analysis of Drugs of Abuse by GC/MS using the Ultra Inert Inlet Liners with Wool

Application Note

Forensic Toxicology

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Abstract

With efficient deactivation on glass wool, the Ultra Inert liners with wool provide excellent inertness, homogeneous sample mixing and evaporation, non-volatile residue trapping, and column and detector protection for drugs of abuse screening.

Introduction

GC inlet liners are the centerpiece of the inlet system where the sample is vaporized, mixed with the carrier gas, and introduced to the capillary column. Inlet liners with wool are widely used because the wool promotes homogenous sample mixing and better quantitation. Wool provides a large surface area which aids the vaporization of liquid samples. It also acts as a trap to collect non-volatile residue in the sample, thus protecting the GC column from the negative impact of sample matrix. Wool liners also reduce sample loss by preventing sample droplets from reaching the bottom of the inlet before vaporization. Agilent MS certified liners with glass wool provide excellent performance for general application purposes. However, for specific applications of active compounds analysis, liners with superior inertness are required to achieve the most reliable results.

GC/MS screening methods are important in forensic toxicology laboratories. With the con-tinuing emergence of new drugs and toxins, the list of target compounds to be screened can number in the hundreds. For those compounds that are compatible with GC, using GC/MS in full-scan mode with electron impact ionization (EI) is well suited for the task [1]. Samples for screening usually require minimal sample preparation, or even no clean-up, to preserve target analytes. However, heavy-matrix samples, such as plasma or urine extracts, deteriorate the performance of the analytical column and detector, resulting in short column life and frequent MS source maintenance. Therefore, it is beneficial to use inlet liners with wool to protect the entire GC/MS system.

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However, if liners with wool are poorly deactivated, they can cause the adsorption or decomposition of target analytes for basic drugs of abuse. As shown in Figure 1, those drugs usually contain hetero atoms, which strongly interact with the free silanol groups (Si-OH) in glass [2]. The resulting compound adsorption and decomposition causes chromatographic problems such as broad or distorted peaks, tailing peaks, ghosting phenomena, and low responses. Liners with glass wool magnify these negative effects due to the large surface area of glass wool and difficulty of complete deactivation. A properly and efficiently deactivated inlet liner with glass wool is imperative for satisfactory chromatography with accurate and reproducible responses for these forensic/toxicology applications.

Agilent's Ultra Inert liner deactivation process significantly improves the efficacy and robustness of glass wool deactivation. The surface area is deactivated thoroughly. For the first time, liners with glass wool can analyze basic drugs of abuse using GC/MS.

The liners with wool were evaluated using Agilent Forensic/Toxicology analyzer checkout standards, including 28 popular and difficult basic drug compounds. These compounds cover the retention range from early to late eluting compounds, and contain different categories of drugs including amphetamins, alkaloids, and benzodiazpines. Figure 1 shows the chemical structures for some of the analytes. All liner tests were conducted using a GC/MS system with simultaneous collection of scan and SIM data. A 5 µg/mL standard was used for chromatographic evaluation. A 500 ng/mL standard (10× dilution) was used to assess the repeatability of injections over 50 injections.

Experimental

Chemicals and Reagents

The Agilent GC/MS Forensic Toxicology analyzer checkout mixture standard (p/n 5190-0471) was used to evaluate the performance of Ultra Inert liners with glass wool. HPLC grade Toluene and Methanol was purchased from Honeywell B&J (Muskegon, MI, USA), and Acetonitrile (AcN) was purchased from Sigma-Aldrich (St Louis, MO, USA). An Internal Standard (IS) was purchased from AccuStandard (New Haven, CT, USA), containing 0.5 mg/mL of Acenaphthene-D_{10}, Phenanthrene-D_{10}, Triphenylphosphate, Chrysene-D_{12}, and Perylene-D_{12} in Acetone.

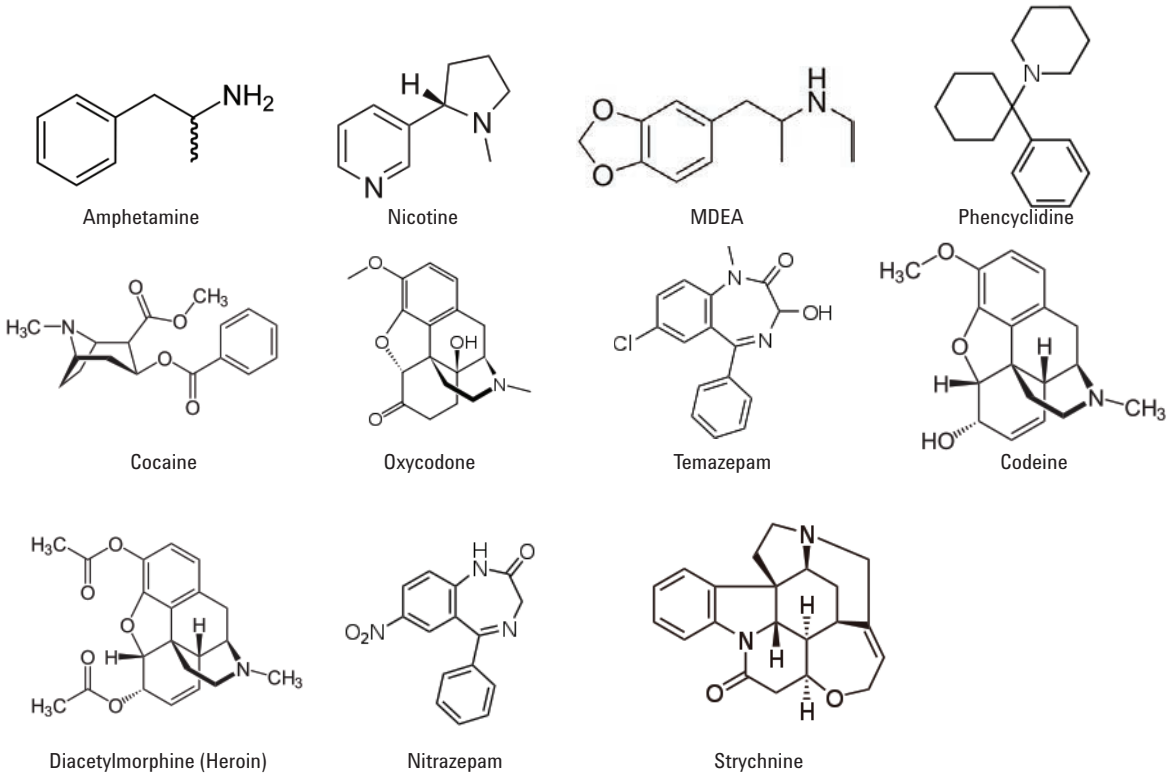

Figure 1. Chemical structure of selected basic drugs.

Solutions and Standards

The original checkout standard sample was made in a 90/5/5 Toluene/MeOH/AcN solution. A 90/5/5 Toluene/MeOH/AcN blank solvent mixture was prepared by combining 90 mL of Toluene, 5 mL of MeOH and 5 mL of AcN, and was used as reagent blank. The 5 µg/mL original standards were directly used for injection, and were diluted 10 times with blank solvent to 500 ng/mL solution. 4 µL of IS stock solution was spiked to 1 mL of standard solution, when necessary, to generate a concentration of 2 µg/mL for IS in the sample.

Instrumentation

All testing was done on an Agilent 7890A GC system equipped with a 7683B autosampler and a 5975C MSD.
Table 1 lists the instrument conditions. Table 2 lists flow path consumable supplies. Table 3 list the Selected Ion Monitoring (SIM) conditions for 28 target analytes.

Table 1. Instrumental conditions for Agilent GC/MS system used for basic drug compounds test

GC	Agilent 7890A Series
Autosampler	Agilent 7683B, 5 µL syringe (p/n 5181-5246), 1 µL injection volume
	Preinj solvent A (90/5/5 Toluene/MeOH/AcN) washes: 1 Sample pumps: 3 Postinj solvent B (90/5/5 Toluene/MeOH/AcN) washes: 3
Carrier gas	Helium, constant pressure
Inlet	Splitless mode: 280 °C
Purge flow	50 mL/min, switched mode, hold for 0.75 min
Inlet pressure	18.7 psi (RT locked) during run, 1.0 psi during back flush
RT locking	Proadifen (SKF-525a) @ 8.569 min
Oven profile	100 °C for 0.5 min, to 325 °C at 20 °C/min, hold 2.5 min
Post run	2 min at 325 °C
Capillary Flow Technology	Purged Ultimate Union (p/n G3182-61580) used for back flushing the analytical column and inlet
Aux EPC gas	Helium plumbed to Purge Ultimate Union
Aux pressure	4 psi during run, 75 psi during back-flushing
Analytical column	DB-5MSUI, 15 m × 0.25 mm, 0.25 µm (p/n 122-5512UI)
Connections	Inlet to Purged Ultimate Union (p/n G3182-61580)
Restrictor	Inert Fused Silica tubing, 0.65 m × 0.15 mm (p/n 160-7625-5)
Connections	Between Purged Ultimate Union and the MSD
MSD	Agilent 5975C inert with performance electronics
Vacuum pump	Performance turbo
Mode	Scan/SIM
Tune file	Atune.u
EM voltage	Atune voltage
Transfer line temp	300 °C
Source temp	300 °C
Quad temp	150 °C
Solvent delay	1.4 min
Scan mass range	40 – 570 amu

Table 2. Flow Path Supplies

Vials	Amber screw cap (p/n 5182-0716)
Vial caps	Blue screw cap (p/n 5182-0717)
Vial inserts	150 µL glass w/ polymer feet (p/n 5183-2088)
Septum	Advanced Green Non-Stick 11 mm (p/n 5183-4759)
Ferrules	0.4 mm id, 85/15 Vespel/graphite (p/n 5181-3323)
O-rings	Non-stick liner O-ring (p/n 5188-5365)
Capillary Flow Technology	Purged Ultimate Union (p/n G3182-61580) Internal nut (p/n G2855-20530) SilTite metal ferrules, 0.10-0.25 mm id (p/n 5188-5361)
Inlet seal	Gold plated inlet seal with washer (p/n 5188-5367)
Inlet liners	Agilent Ultra Inert deactivated single taper splitless liner with wool (p/n 5190-2293)

Table 3. SIM acquisition conditions used for 28 basic drug compounds by GC/MS

Analytes (Peak no. on chromatogram)	SIM *	RT (min)	Collection window (min)
Amphetamine (1)	**44**, 91	1.77	1.4 – 2.7
Phentermine (2)	**58**, 134	1.96	
Methamphetamine (3)	**58**, 91	2.08	
Nicotine (4)	**84**, 133	3.06	2.7 – 3.6
Methylenedioxyamphetamine (MDA) (5)	**44**, 135	3.92	3.6 – 5.0
Methylenedioxymethamphetamine (MDMA) (6)	**58**, 135	4.27	
Methylenedioxyethylamphetamine (MDEA) (7)	**72**, 135	4.57	
Meperidine (8)	**71**, 247	5.63	5.0 – 7.0
Phencyclidine (9)	**200**, 242	6.49	
Methadone (10)	**72**, 57	7.72	7.0 – 8.9
Cocaine (11)	**182**, 82	8.10	
Prodifen (SKF-525a) (12)**	**86**, 99	8.57	
Oxzepam (13)	**239**, 267	8.73	
Codeine (14)	**299**, 162	9.01	8.9 – 9.5
Lorazepam (15)	**239**, 274	9.08	
Diazepam (16)	**256**, 283	9.22	
Hydrocodone (17)	**299**, 242	9.29	
Tetrahydrocannabiol (18)	**231**, 314	9.36	
Oxycodone (19)	**315**, 230	9.63	9.5 – 10.4
Temazepam (20)	**271**, 273	9.87	
Flunitrazepam (21)	**312**, 286	9.96	
Diacetylmorphine (Heroin) (22)	**327**, 369	10.02	
Nitrazepam (23)	**253**, 206	10.62	10.4 – 11.6
Clonazepam (24)	**314**, 286	10.94	
Alprazolam (25)	**279**, 308	11.32	
Varapamil (26)	**303**, 304	12.03	11.6 – 14.0
Strychnine (27)	**334**, 335	12.18	
Trazodone (28)	**205**, 70	12.96	

* Ions in Bold were quantifiers, and the other ions were qualifiers.

** Prodifen was used for the RT locking.

A back-flushing system was used because it shortens analysis times for samples that contain high-boiling matrix interferences, reduces column head trimming, and reduces frequency of MSD source cleaning [3,4]. The instrument configuration is similar to the configuration shown in Figure 1B in the previous setup [4], except no retention gap was used for this application. Retention time locking (RTL) was used to eliminate recalibration of individual retention times and timed events such as SIM groups [5].

Results and Discussion

The purpose of these tests was to evaluate the Ultra Inert deactivated liners with wool for screening analysis of drugs of abuse by GC/MS. The Agilent Forensic Toxicology analyzer checkout standard was used for the evaluation (Table 3). The feasibility of using Ultra Inert liners with wool was determined by chromatographic evaluation, liner to liner reproducibility, and multi-injections repeatability. In parallel, liners with wool from multiple sources were tested for comparison.

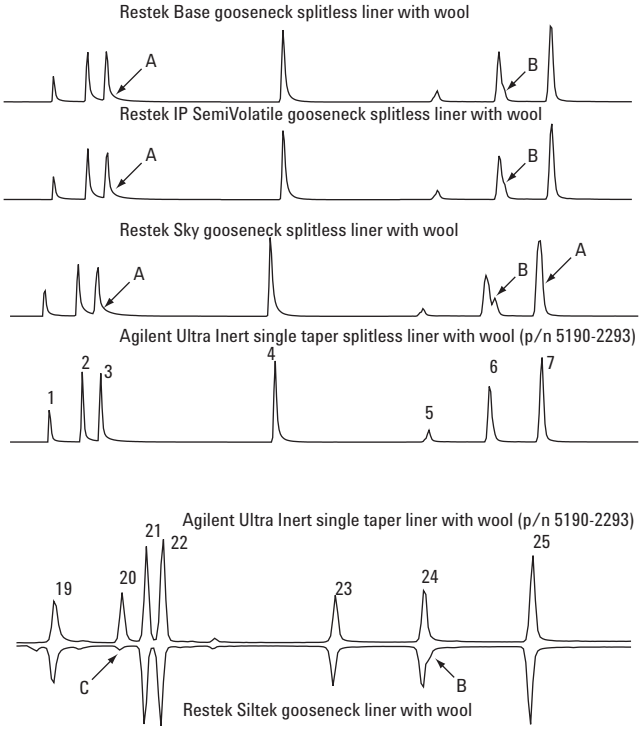

Figure 2. Chromatographic problems for drug of abuse compounds shown on GC/MS SIM chromatograms when using other equivalent liners and their comparison with chromatograms obtained by Ultra Inert liners with wool. See Table 3 for peaks identification and Table 1 for instrument conditions. 5 ng checkout standards on column. A) Broad or distorted peak, B) ghosting shoulder, C) poor sensitivity

Chromatographic performance

The adsorption or decomposition of basic drug compounds may cause various chromatographic problems including broad, distorted peaks, peak tailing, ghost peaks, and loss of sensitivity. All of these problems were observed in liners tests using the checkout standard. Peak shape problems usually occurred for early eluting compounds, such as Phentermine, Methamphetamine, MDA, and MDMA. The late eluting compounds, such as Temazepam, can disappear due to the loss of sensitivity. Figure 2 shows problematic chromatograms obtained using similar liners compared to chromatograms obtained using Ultra Inert liners with wool. As seen in Figure 2, with 5 ng on column, other liner deactivations cause chromatographic problems such as broad or distorted peaks and significant loss of response. However, the corresponding chromatograms with Agilent Ultra Inert deactivated liners show better peak shape and typically higher responses. Figure 3 shows a full chromatogram of 5 ng checkout standard on column using Agilent Ultra Inert splitless liner with wool by GC/MS. Figure 3 shows that Ultra Inert liners with wool provide the best chromatogram for all of analytes tested, even though there is small peak tailing or broadening observed for certain compounds. Six replicates of Ultra Inert liners were tested, each providing similar chromatographic performance, indicating excellent liner to liner reproducibility. The satisfactory chromatograms obtained by Ultra Inert liners demonstrate that the Ultra Inert liner deactivation process provides sufficient liner and glass wool inertness to prevent drugs of abuse from adsorption and decomposition.

1. Amphetamine, 2. Phentermine, 3. Methamphetamine, 4. Nicotine, 5. MDA, 6. MDMA, 7. MDEA, 8. Meperidine, 9. Phencyclidine, 10. Methadone, 11. Cocaine, 12. SKF-525a, 13. Oxazepam, 14. Codeine, 15. Lorazepam, 16. Diazepam, 17. Hydrocodone, 18. Tetrahydrocannabinol, 19. Oxycodone, 20. Temazepam, 21. Flunitrazepam, 22. Heroin, 23. Nitrazepam, 24. Clonazepam, 25. Alprazolam, 26. Verapamil, 27. Strychnine, 28. Trazodone.

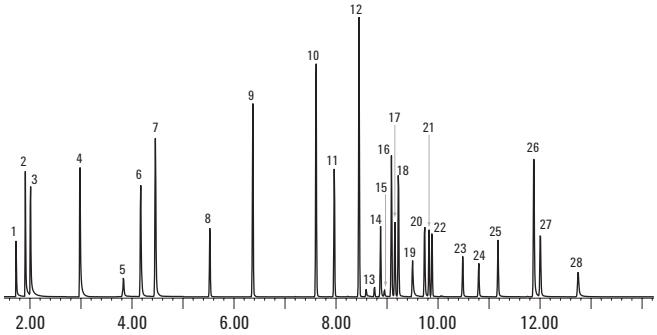

Figure 3. Chromatogram of forensic toxicology analyzer checkout standard (5 ng checkout standards on column) using Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293) by GC/MS. See Table 1 for instrument condition. Satisfactory peaks shape achieved for all of analytes

Liner to liner reproducibility

To quantitatively evaluate the liner to liner reproducibility, six Ultra Inert liners from four different lots were tested. 5 µg/mL and 500 ng/mL samples spiked with 2 µg/mL IS were used. Twelve sensitive compounds were selected for evaluation. The Response Factors (RFs) were calculated for each concentration level. The average RF values were evaluation criteria for the liner to liner reproducibility test. See Table 4. The results show excellent liner to liner performance consistency with less than 7% RSD, except for Temazepam with 11.7%, across six liners from four different lots.

*Table 4. Liner to Liner Reproducibility: 12 sensitive basic drug compounds average RF (5 µg/mL and 500 ng/mL) and RSD values for six replicates of UI deactivated liners with wool (p/n 5190-2293) **

Compounds	Liner 1 (Lot 1)	Liner 2 (Lot 1)	Liner 3 (Lot 1)	Liner 4 (Lot 2)	Liner 5 (Lot 3)	Liner 6 (Lot 4)	Mean RF	RSD
Methamphetamine (3)	0.875	0.876	0.882	0.940	0.955	0.904	0.905	3.8
MDMA (6)	0.807	0.789	0.783	0.848	0.874	0.841	0.824	4.4
Phencyclidine (9)	0.494	0.510	0.494	0.488	0.509	0.521	0.503	2.5
Cocaine (11)	0.636	0.645	0.647	0.637	0.660	0.668	0.649	2.0
Oxazepam (13)	0.050	0.055	0.052	0.055	0.062	0.057	0.055	7.6
Codeine (14)	0.096	0.098	0.095	0.090	0.099	0.102	0.097	4.2
Oxycodone (19)	0.073	0.071	0.070	0.076	0.082	0.080	0.075	6.5
Temazepam (20)	0.101	0.121	0.115	0.088	0.096	0.104	0.104	11.7
Heroin (22)	0.097	0.099	0.096	0.095	0.100	0.102	0.098	2.7
Nitrazepam (23)	0.038	0.032	0.037	0.034	0.037	0.036	0.036	6.3
Clonazepam (24)	0.035	0.035	0.034	0.032	0.034	0.033	0.034	3.5
Trazodone (28)	0.061	0.065	0.064	0.058	0.060	0.064	0.062	4.4

$$^{*}\ RF = \frac{\text{Peak Area}_{\text{Analyte}} \times \text{Concentration}_{\text{Internal Standard}}}{\text{Peak Area}_{\text{Internal Standard}} \times \text{Concentration}_{\text{Analyte}}}$$

Injection repeatability and deactivation stability

Multi-injection repeatability and deactivation stability were tested by continuously injecting 1 µL of 0.5 µg/mL standard samples for 50 injections. Data was collected and RF values were calculated every 10 injections. RSD values were calculated over 50 injections. Table 5 shows the RSD value for all of the basic drug analytes with 0.5 ng on column.

A 0.5 ng on column concentration was used for this repeatability test since low level concentrations show greater deviation contributions than high concentration samples. Higher responses of analytes could hide some deviation impact and generate better repeatability. Twenty-two of 28 analytes have excellent repeatability for 50 injections of standard solution with less than 20% RSD. 5 of 28 analytes have relatively high RSD (between 20 – 25%), but still should be acceptable at the level of 0.5 ng on column. Temazepam is a very difficult compound and extremely sensitive to the liner inertness.

Table 5. Deactivation stability: 50 injections repeatability (%RSD) for Agilent Ultra Inert deactivated liners with wool (p/n 5190-2293) for all of tested basic drug compounds with 0.5 ng of standard on column. (n = 3)

Compound	RSD (%) over 50 injections	Compound	RSD (%) over 50 injections
Amphetamine	0.3	Lorazepam	20.9
Phentermine	1.1	Diazepam	3.7
Methamphetamine	1.5	Hydrocodone	3.7
Nicotine	2.3	Tetrahydrocannabinol	8.5
MDA	3.7	Oxycodone	22.2
MDMA	2.2	Temazepam	59.9
MDEA	2.0	Flunitrazepam	8.7
Meperidine	1.9	Heroin	10.7
Phencyclidine	15.6	Nitrazepam	11.2
Methadone	3.4	Clonazepam	12.0
Cocaine	7.8	Alprazolam	13.1
Prodifen	4.4	Verapamil	15.4
Oxazepam	20.4	Strychnine	11.0
Codeine	20.5	Trazodone	23.6

As shown in Figure 2, when an inefficient deactivated liner was used the response of Temazepam (5 ng on column) can almost disappear. Compared to other similar liners, Agilent Ultra Inert liner with wool generated highest RF for Tempazepam, which is clearly shown in Figure 4. This indicates that Agilent Ultra Inert liners with wool provide the best inertness compared to competitor's equivalent liners.

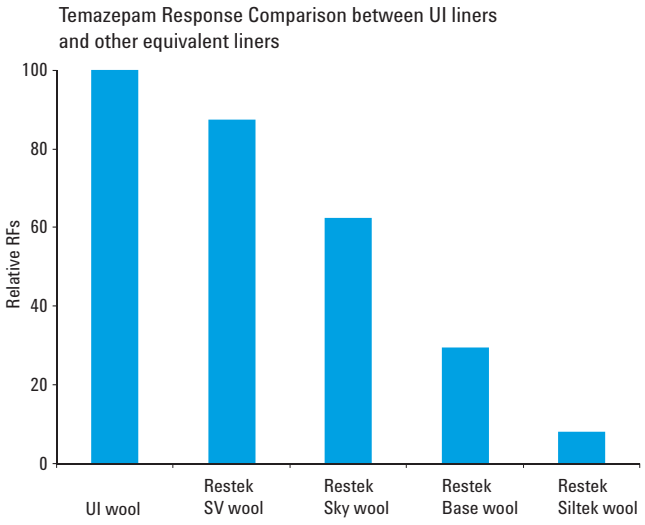

Figure 4. Sensitive compound response (Temazepam) comparison for Ultra Inert liner with wool (p/n 5190-2293) and other equivalent liners. RF calculation was based on the average RF of 0.5 ng and 5 ng standard on column. Ultra Inert liner average RF value was set to 100% and other liners average RF values were scaled.

The response of Temazepam decreased with more samples were injected, thus generated high RSD over injections. This phenomenon was observed for all of the liners tested, and the response decrease can be even worse for other liners. When Temazepam is a target analyte and the interested concentration is at ppb level, it is strongly recommend that an Ultra Inert liner with wool should be used for no more than 30 samples.

Real matrix sample analysis

Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/10th volume. Figure 5 shows the chromatogram of 2 ppm matrix spiked sample using Agilent Ultra Inert liner with wool by GC/MS, which is satisfactory for both early eluted compounds' peak shape and late eluted compounds' sensitivity. There are some minor interference peaks from matrix showing up.

Conclusion

Agilent Ultra Inert liners with wool have shown excellent inertness for the analysis of basic drugs of abuse. Ultra Inert liners with wool provide satisfactory chromatography for the selected popular and difficult basic drug compounds. The liner to liner performance shows excellent reproducibility with an average of 5% RSD for these active compound RF values. With efficient and robust deactivation of the wool, Agilent Ultra Inert liners with wool provide excellent inertness for forensic and toxicology screening. The benefits provided by liners with wool such as homogeneous sample mixing and evaporation, non-volatile residue trapping, and column and detector protection, are gained without compromise of chromatography or sensitivity of active analytes. Ultra Inert liners with wool are an excellent choice for screening analysis for drugs of abuse.

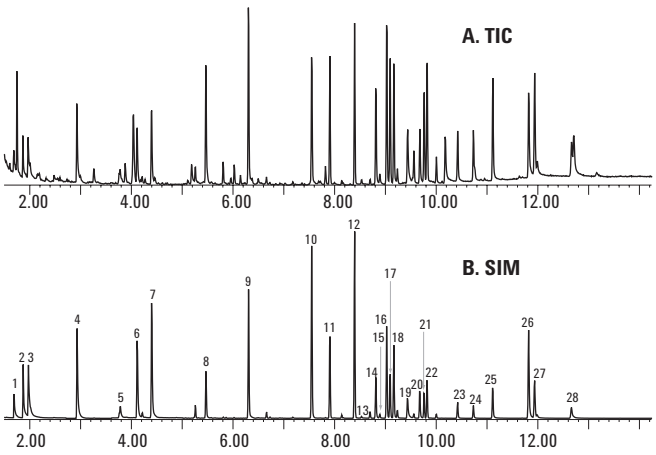

Figure 5 Chromatogram of forensic toxicology analyzer checkout standard (2 ng on column) with whole blood matrix using Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293) by GC/MS. Refer to Table 1 for instrument condition, and Table 3 for peaks identification. A) Full scan chromatogram, B) SIM chromatogram. Satisfactory peaks shape and response achieved for all of analytes.

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[4] P. Wylie and C. Meng, "A Method for the Trace Analysis of 175 Pesticides Using the Agilent Triple Quardrupole GC/MS/MS", Agilent Technologies publication 5990-3578EN

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Agilent Technologies

Rapid Forensic Toxicology Screening Using an Agilent 7890A/NPD/5975C/DRS GC/MSD System

Application Brief

Bruce Quimby

Laboratories that perform toxicology screens on forensic samples are challenged by the requirement to analyze large numbers of samples containing complex matrix interferences. The system described here addresses these demands by combining fast GC to reduce the run time; simultaneous collection of scan, SIM, and NPD data in one shorter run; backflushing to prevent heavy matrix components from fouling the detectors; and Deconvolution Reporting Software (DRS) to simplify data interpretation. The scan data is deconvoluted and used to identify any of 278 target compounds. SIM data is used to look for select low-level compounds not detectable in scan mode. The nitrogen response of the NPD is used to highlight nontarget compounds, identity confirmation, and can be used for quantitation if needed. Using an extract of a whole blood sample, the system finds all the molecules detected by the conventional method in significantly less time.

Highlights

- DRS simplifies data interpretation, especially in dirty samples.
- Simultaneous collection of SIM, scan, and NPD signals saves time.
- The 7890A GC/MS High Speed Oven Accessory provides high programming rates, even with 120 V service.
- Backflushing reduces ghost peaks in high matrix samples.

Experimental and Results

The Forensic Toxicology GC/MSD RTL Database of 277 compounds was downloaded from Agilent's Web site and converted for use with DRS. The method was scaled to precisely two times faster using Agilent's Method Translation software. Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single-step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/6th volume. Extracts were analyzed using the conditions in Table 1. The simultaneously acquired chromatograms for scan, SIM, and the NPD for one of the samples is shown in Figure 1. The 245 target ion for fentanyl shown is one of 13 SIM ions monitored. This example is particularly challenging because of the high levels of matrix interferences as seen in the scan TIC. The drug compounds present were identified using a combination of 1) full-spectrum searching of the deconvolved spectra against the target library (AMDIS), 2) target and qualifier ion ratios in the MSD ChemStation, and 3) response on the NPD.

Compounds identified by AMDIS deconvolution but not found by the MSD ChemStation because of out-of-range qualifiers were manually inspected in QEdit. Quantitation was forced if AMDIS indicated an acceptable spectral and retention time match and if there was a corresponding NPD response.

The SIM data was used to screen for several compounds (see Table 1) that are often at levels too low to be detected in scan mode. In this sample, fentanyl was found present at a low level in the scan data and confirmed with the SIM responses. The signal-to-noise ratio of the SIM target ion was 10 times greater than that of the scan.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC	Agilent Technologies 7890A		
Inlet	EPC split/splitless		
Mode	Splitless, 2 µL injected		
Inlet temp	280 °C		
Pressure	24.15 psig, retention time locked to oxycodone at 5.505 min		
Purge flow	50 mL/min		
Purge time	1 min		
Gas saver	Off		
Gas type	Helium		
Liner	Agilent splitless inlet liner, single-taper, Part # 5181-3316		
Oven	**240 V**		
Oven ramp	°C/min	Next °C	Hold min
Initial		120	1.00
Ramp 1	40	320	2.50
Total run time	8.5 min		
Equilibration time	0.5 min		
Backflush time	2.0 min		
Backflush temp	320 °C		
Column	Agilent Technologies DB-17 ms, Part # 123-4712		
Length	15.0 m		
Diameter	0.32 mm		
Film thickness	0.25 µm		
Mode	Constant pressure		
Outlet	2-way splitter with solvent vent		
Splitter pressure	3.8 psi during acqusition, 75 psi during backflush with inlet set to 1.0 psi during backflush		
Splitter restrictors	MSD:1.44 m × 0.18 mm id × 0.18 µm film DB-17 ms (Part # 121-4722). NPD:0.75 m of same		
Solvent venting	0 to 1.40 min		
NPD	Capillary NPD with EPC, option 251		
Gas flows	Hydrogen 3.0 mL/min, air 60 mL/min, nitrogen makeup 12 mL/min		
NPD temp	310 °C		
MSD	Agilent Technologies 5975C, Performance Turbo		
Solvent delay	None (solvent vented with splitter)		
EM voltage	Tune voltage		
Mode	SIM/scan		
Scan	42-550 amu, sampling: 2[1]		
SIM ions	Group 1 (PCP) 84, 186, 200, 242; Group 2 at 4.5 min, (norfentanyl butyl derivative, 6-acetylmorphine, heroin, fentanyl) 42, 82, 83, 146, 158,189, 231, 245, 268, 284, 310, 327, 369; Group 3 at 6.5 min (LSD) 221, 323, 181, 207; all dwell times 10 msec		
Quad temp	150 °C		
Source temp	280 °C		
Transfer line temp	280 °C		

Table 3 shows the DRS report for the sample in Figure 1. The report lists the compounds quantitated by the MSD ChemStation and identified by deconvolution. The quantitative results are rough approximations, as the response factors used here were only average responses for screening purposes. Note that there are several nondrug compounds in the target library that are detected as well.

The spectra of peaks found on the NPD that did not correspond to targets were searched against the NIST and Pfleger libraries for identification. The peak on the NPD in Figure 1 labeled with a question mark was not a target compound. Search results of the spectrum indicated it was cyheptamide (later found to be an internal standard added in sample preparation).

For comparison, the sample in Figure 1 was analyzed in the same way but with the 1x method for reference. All drugs found with the original 1x method were found with the 2x method.

The use of the two-way splitter with solvent venting allows the solvent peak (and any other unwanted peaks) to be vented before reaching the detectors. This helps extend the useful life of the NPD bead. The device also allows backflushing at the end of the run. As seen in Figure 1, there are large matrix peaks that elute after the last target compound. Backflushing quickly removes these compounds, saving time and reducing detector and column maintainence.

The significant time savings available with the method described here vs. the original method where three separate runs of scan, SIM, and NPD are needed to access the same information are shown in Table 2.

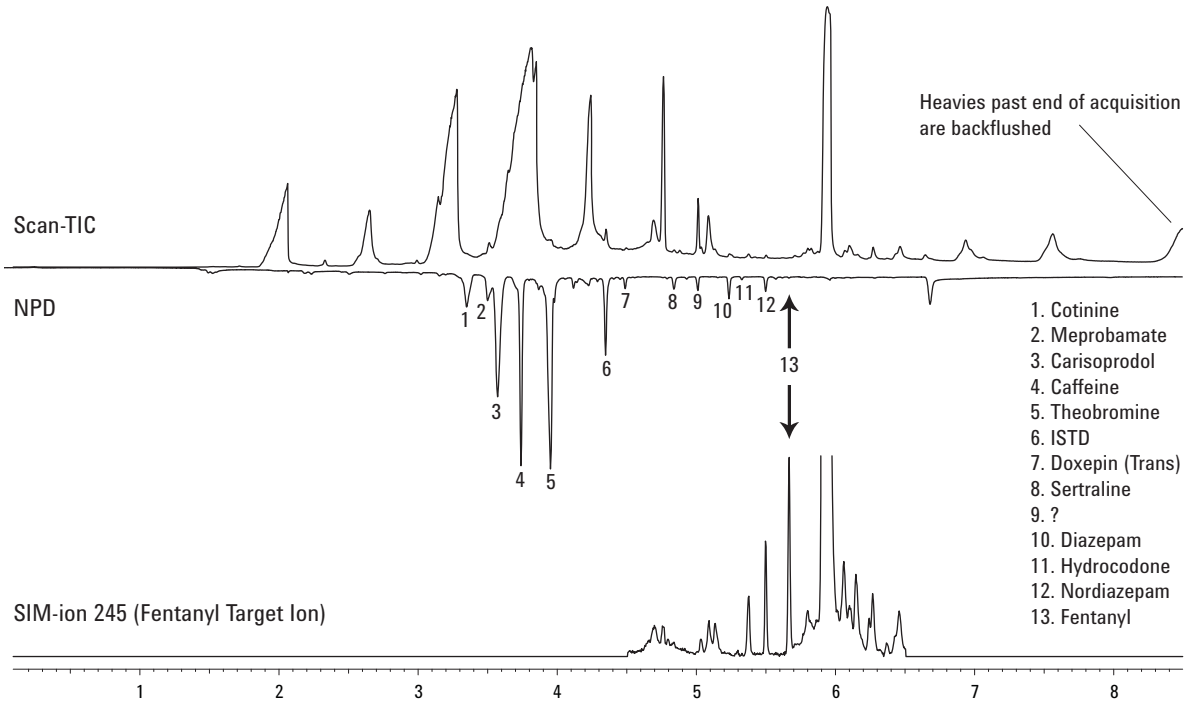

Figure 1. Chromatograms from screen of whole blood sample.

Table 2. Time Savings Using the Agilent 7890A-5975C

	Typical 6890 1X	7890A 2X	Minutes Saved
Run time without matrix bake-out, includes equib	17	8.5	8.5
Run time with matrix bake-out 6890 or Splitter 7890A	24	10.5	13.5
Cool down time from 320 to 120	2.3	1.6	0.7
Autosampler time, 7890A with overlap	1	0.1	0.9
Acquiring scan, SIM, and NPD signals separately vs. simultaneously	81.9	12.2	69.7

Time savings > 85%
Not including time saved using DRS

Table 3. DRS Report from Screen of Whole Blood Sample

			Agilent	AMDIS		NIST	
R.T.	Cas #	Compound Name	ChemStation Amount (~ng)	Match	R.T. Diff sec.	Reverse Match	Hit Num.
2.1124	nc06	DIETHYLPROPRION		61	-3.9		
2.6530	nc013	TETRADECANOIC ACID	44.17	99	-1.1		
3.3558	nc046	COTININE	1.94	78	0.9		
3.5188	nc060	MEPROBAMATE	1.79	74	-2.2		
3.5792	nc063	CARISOPRODOL	1.88	85	-0.1		
3.745	nc079	CAFFEINE	1.43				
3.958	nc089	THEOBROMINE	5.46	75	1.8		
4.3516	nc0123	10,11-DIHYDRO-DIBENZ[B,F] [1,4]OXAZEPIN-	9.25	98	-0.1		
4.494	nc0133	DOXEPIN(TRANS)	1.45				
4.4940	nc0131	DOXEPIN(CIS)		67	3.0		
4.7654	nc0155	BIS[2-ETHYLHEXYL] PHTHALATE	138.58	94	0.4		
4.8420	nc0158	SERTRALINE[2]	0.54	88	-0.1		
5.087	nc0178	DOXYLAMINE METABOLITE	0.21				
5.238	nc0187	DIAZEPAM	0.45	92	0.3		
5.3285	nc0191	HYDROCODONE	0.1	62	0.2		
5.5009	nc0198	NORDIAZEPAM	0.41	62	0.0		
5.6695	nc0210	FENTANYL	0.1	64	0.8		
5.7097	nc0218	GAMMA-TOCOPHEROL	0.94	81	0.5		
5.8750	nc0226	VITAMEN E	0.81	90	2.7		
5.930	nc0233	CHOLESTEROL	65.83	98	0.2		

Conclusions

Significant time savings can be realized in the screening of forensic toxicology samples with the system described. The cycle time required per sample is reduced 85%. Data interpretation time is also reduced with the use of DRS.

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Clenbuterol and Norandrosterone Analysis by Positive Chemical Ionization with the 5973N MSD

Application Note

Drug Testing

Bernhard Wuest, *Agilent Technologies*

Introduction

The structure and functionality of banned anabolic steroids are similar to the male hormone testosterone.

After extraction and derivatization, anabolic steroid samples are usually analyzed with high-resolution capillary gas chromatography (HRCGC) and mass selective detection (MSD). Exclusive use of GC/MS in electron ionization (EI), selected ion monitoring (SIM) mode can result in misidentification and poor quantification due to interfering compounds in the matrix that have the same *m/z* value.

Using positive chemical ionization (PCI) with ammonia as reagent gas can improve both selectivity and sensitivity. The work described here with the 5973 GC/MSD demonstrates the advantages of positive chemical ionization (PCI) over electron ionization (EI) and as well as the stability, reliability and robustness of the complete GC/MSD system. System stability and reliability derive from precise control of the reagent gas with a digital mass flow controller, and from dedicated temperature control of the ion source. In this study, urine samples were analyzed to determine the presence of clenbuterol and norandrosterone (see Figure 1).

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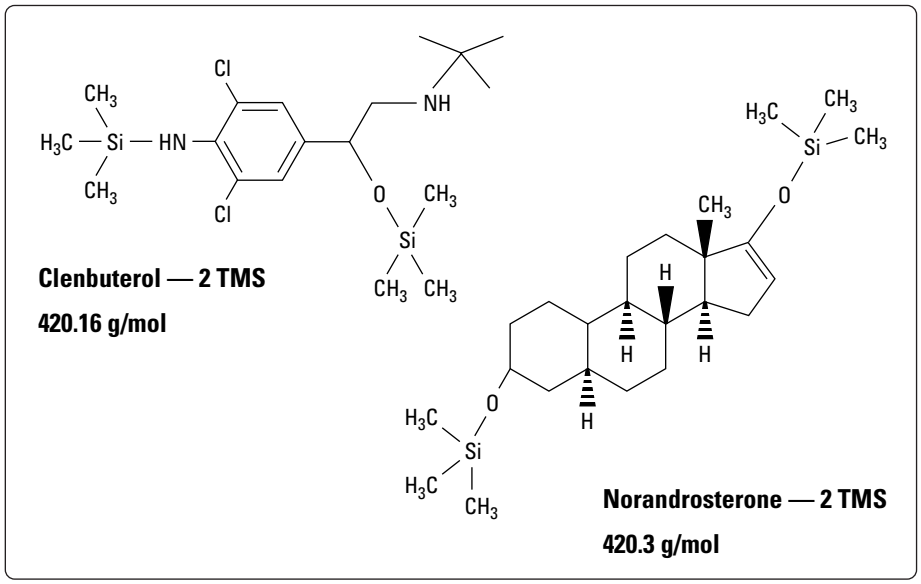

Figure 1. Structure of clenbuterol and norandrosterone derivatives.

Experimental

The instruments used for this analysis were a 6890 gas chromatograph with a 5973 mass spectrometer. A series of 120 injections of actual urine samples was made using chemical ionization (ammonia). Every sixth and seventh injection consisted of a 10- and 2-ng/ml standard, respectively.

Oven temperature program:	180°C .(1 min), 5°C/min, 300°C .(5 min)
Inlet liner:	Single-tapered deactivated with a small amount of glass wool (Agilent Part No: 5062-3587)
Injection volume:	2 µl
Split:	8:1
Column:	HP5 MS 30 m × 0.25 mm × 0.33 µm, 1.2 ml/min constant flow
MS mode:	Selected ion monitoring
EM offset:	400 V above tune
SIM mode:	Low resolution, 150 msec dwell time
Chemical ionization:	Ammonia, 1 ml/min

Results and Discussion

The EI and PCI spectra are shown in Figures 2 and 3. The PCI spectra show the molecular ion (M+1) for the TMS derivatives of the two compounds. The compounds are distinguished not only by their spectra, but also by their retention times. Positive chemical ionization provides a much cleaner total ion chromatogram than electron ionization (see Figure 4). Single-ion chromatograms were used to locate the compounds.

The short-term stability for a standard is shown in Figure 5 in which the single-ion chromatograms for eight runs are overlaid. The plot in Figure 6 provides an indication of the reproducibility of the analysis; a slight decrease in response is normal.

To demonstrate the stability with real samples, 120 injections of urine samples were run along with standards at two concentrations. The long-term stability of the system is shown in Figure 7 in which ion chromatograms for norandrosterone from eight runs are overlaid. Figure 8 shows the long-term stability with an excellent RSD of 8.5% during the run sequence. There is a slight increase for clenbuterol a result of better system inertness. The decrease in norandrosterone is due to normal liner degradation after 120 injections.

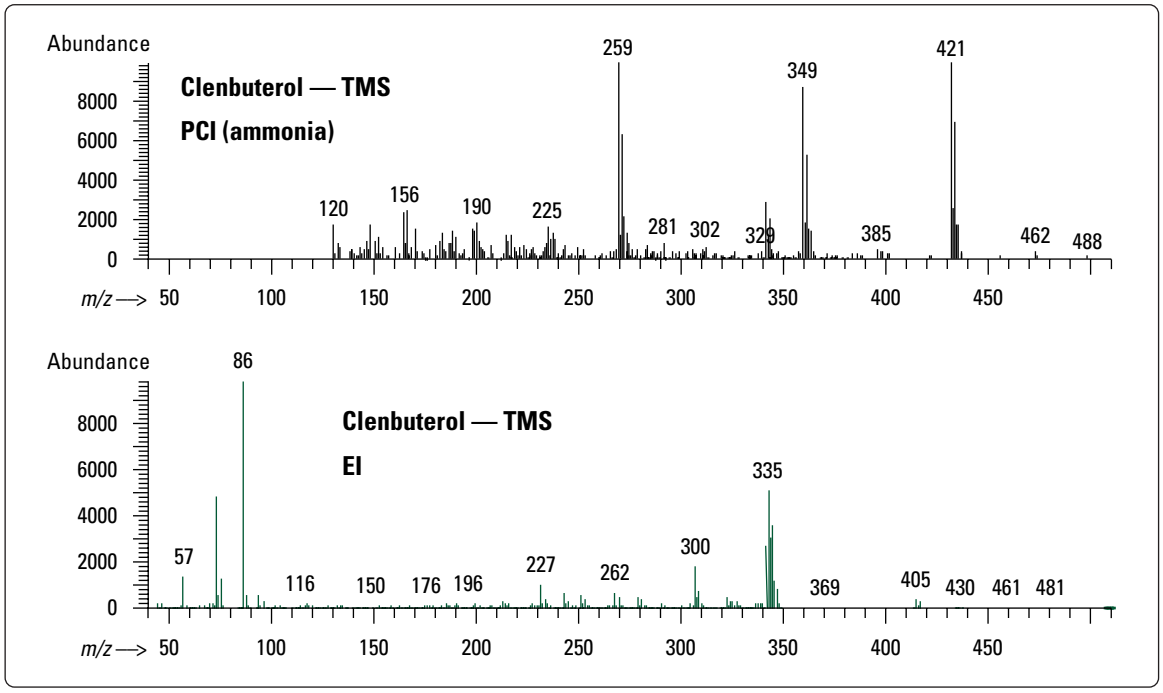

Figure 2. EI and PCI (ammonia) spectra for clenbuterol — TMS.

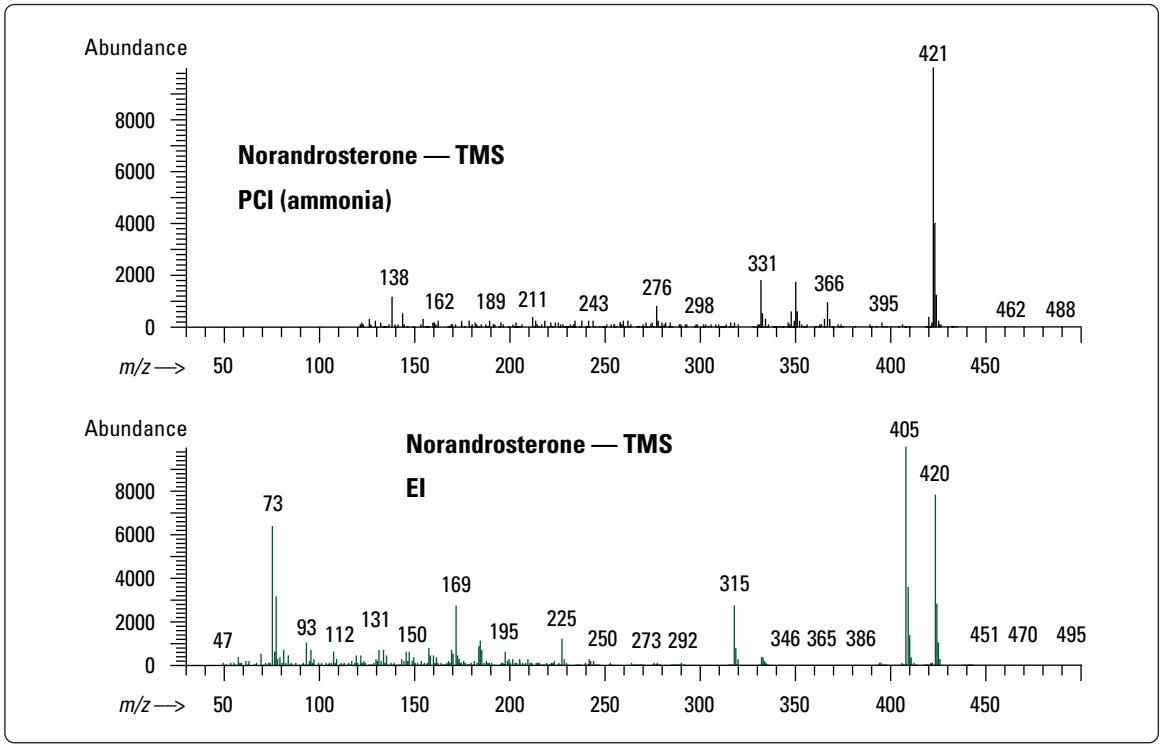

Figure 3. EI and PCI (ammonia) spectra for norandrosterone — TMS.

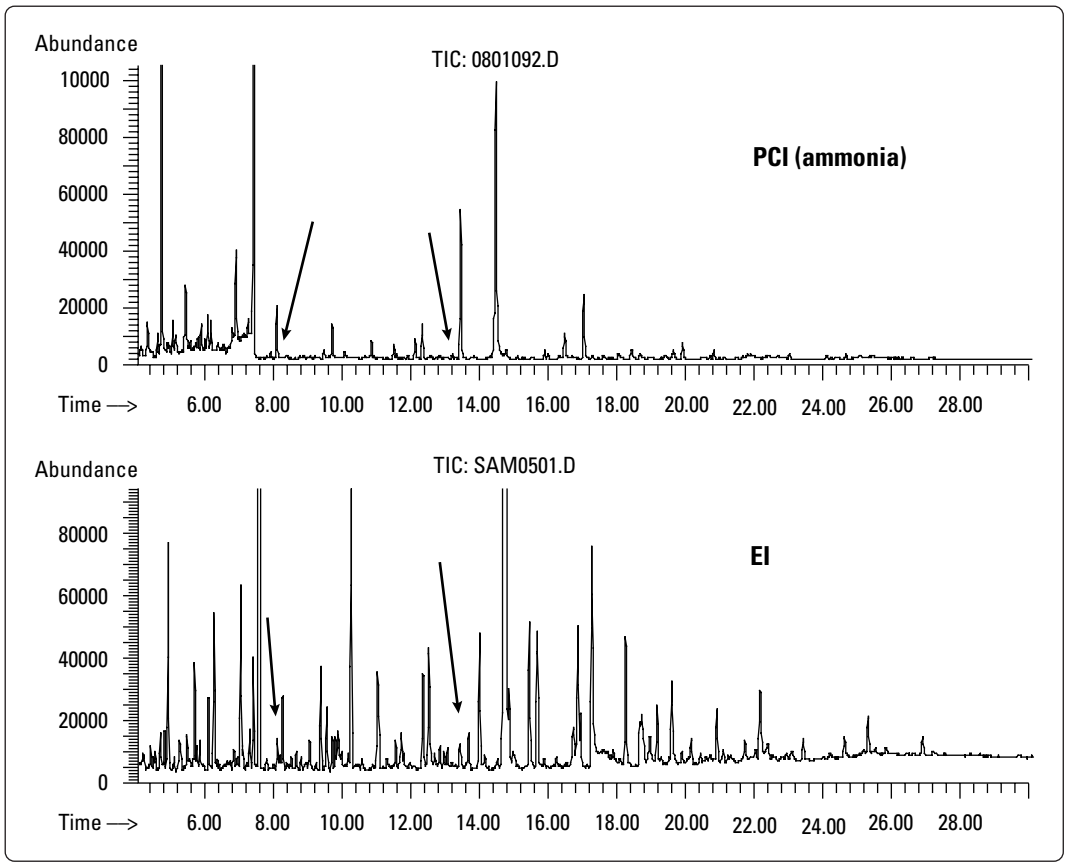

Figure 4. Total ion chromatograms for EI and PCI (ammonia).

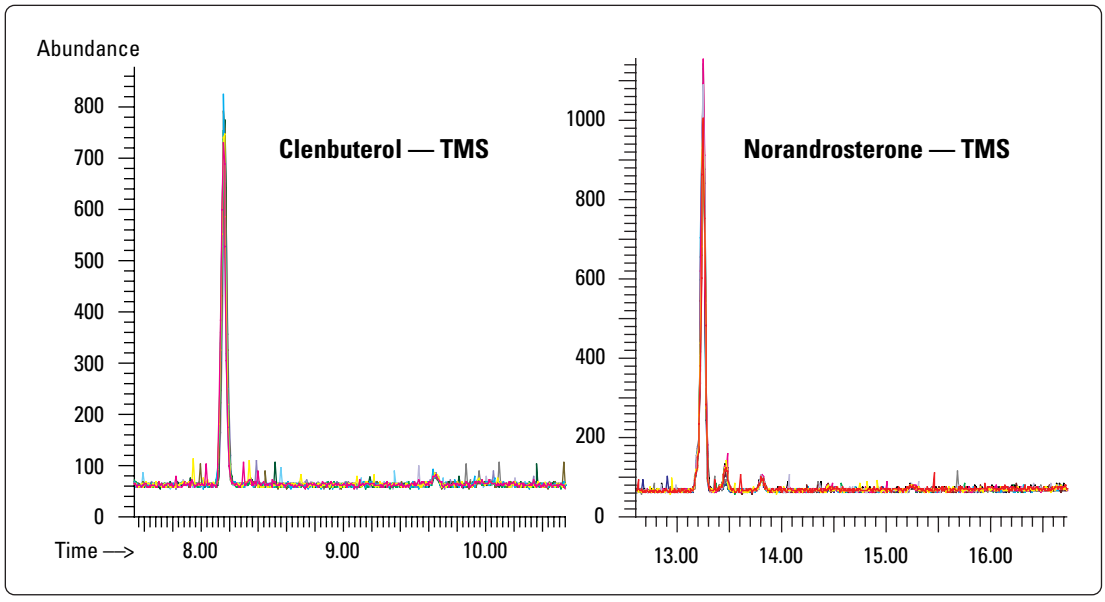

Figure 5. Short-term stability for standards.

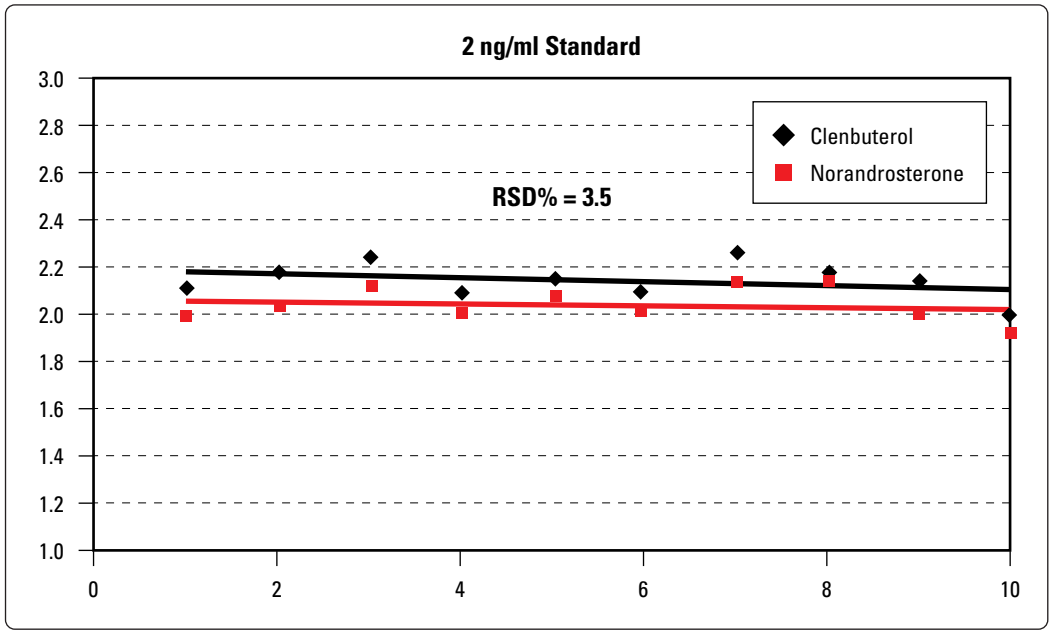

Figure 6. Short-term stability for standards.

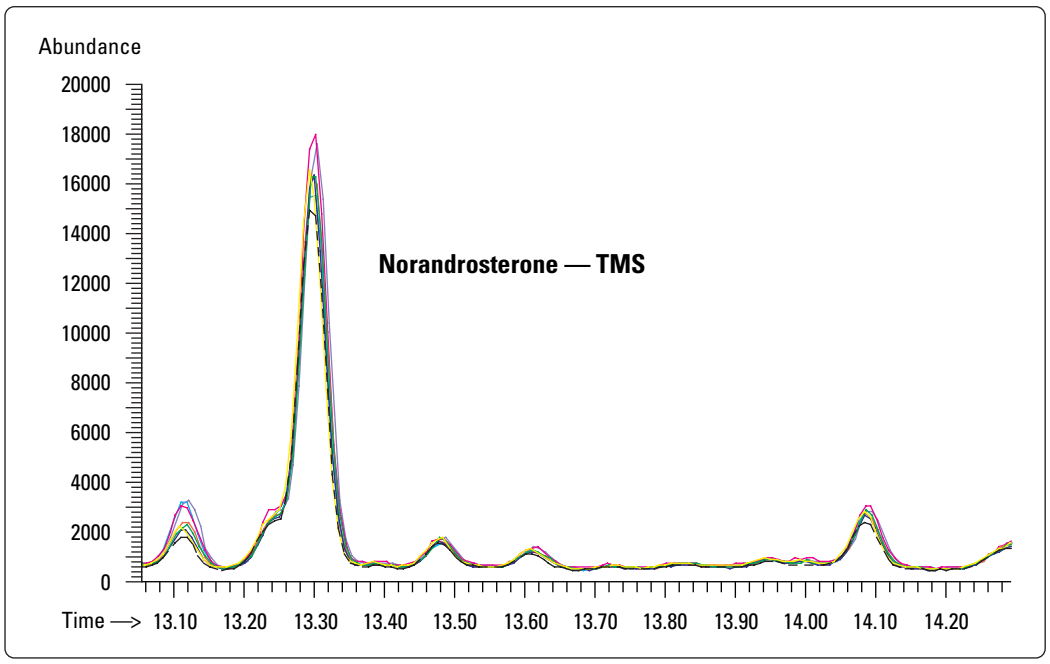

Figure 7. Long-term stability after injections of urine samples.

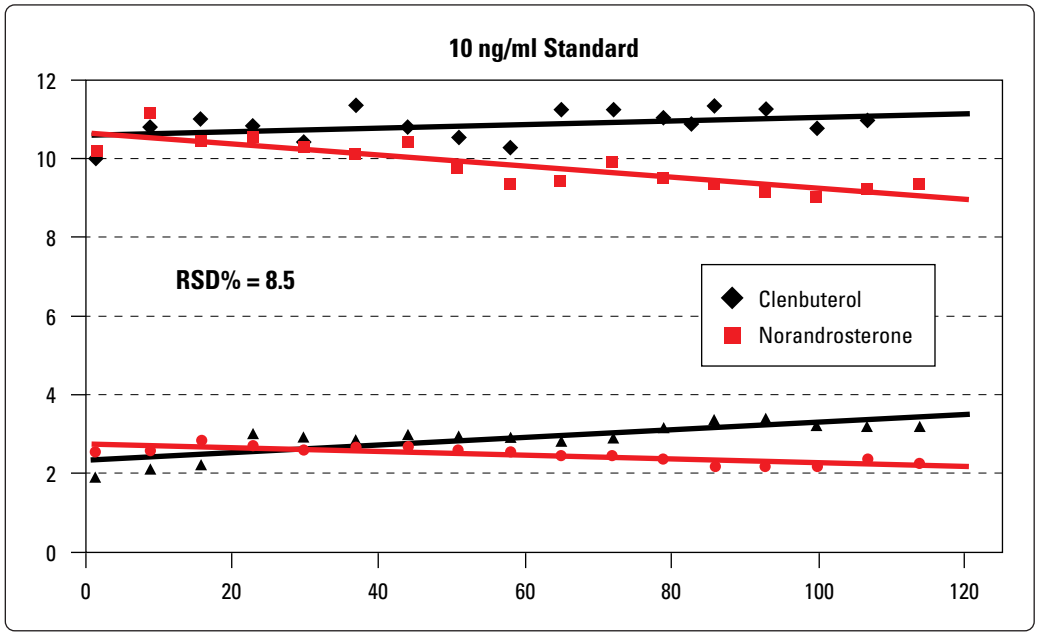

Figure 8. Long-term stability for clenbuterol and norandrosterone.

Conclusion

In the analysis of steroids by positive chemical ionization, it is necessary that the GC/MS system provide the following.

- Precise control of GC carrier gas
- Accurate, reproducible oven temperature ramping
- Stable and controllable ion-source and quadrupole temperatures
- Precise and stable CI reagent gas control

The 5973 GC/MSD uses a patented nonstainless-steel ion source that gives very stable results relative to those obtainable with stainless steel sources. Combined with a low-background flow system that uses ultraclean parts, the 5973 is capable of detecting compounds at low levels. It is concluded that the 6890 GC and 5973 GC/MSD provide robust, sensitive, and reliable detection of clenbuterol and norandrosterone in urine samples.

Authors

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Detection of Cannabinoids in Oral Fluid Using Inert Source GC/MS

Application Note

Forensic Toxicology

Authors

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Abstract

Oral fluid is being considered as an alternative to urine in many forensic arenas. In general, the concen-tration of drugs in oral fluid is much lower than in urine, so sensitive extraction and analytical procedures are required. Tetrahydrocannabinol (THC) is the active ingre-dient in marijuana. Since it is generally smoked, the constituents of the plant material, as well as the active ingredient, may be present in oral fluid specimens collected for the purposes of drug testing. An ana-lytical procedure for the simultaneous determination of the pyrolytic precursor Δ^9-tetrahydrocannabinolic acid A (THCA-A, 2-carboxy-THC), tetrahydrocannabinol (THC), cannabinol (CBN), and cannabidiol (CBD) in human oral fluid specimens using an Agilent 5975 GC/MS with an inert source is presented. The method achieves the required sensitivity for the detection of tetrahydro-cannabinol (THC), cannabinol (CBN), cannabidiol (CBD), and the pyrolytic precursor 2-carboxy-THC in oral fluid specimens taken from a habitual marijuana smoker. While these drugs have been detected in other matrices, the increasing utility of saliva for drug analysis makes development of laboratory procedures necessary and timely.

Introduction

Tetrahydrocannabinol (THC) is the active ingredient in marijuana, which is generally administered via smoking. While THC is the main psychoactive ingredient in the marijuana plant, other reports have shown that some of the effects may be in combination with at least one other constituent of the plant, cannabidiol (CBD). Various cannabinoids have been analyzed in plasma, blood, and urine, but their detection in the more esoteric matrices, such as sweat, oral fluid, and hair, has only recently been addressed.

Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside and in workplace testing. Several publications have reported the presence of THC in saliva using various collection devices. However, the presence of other cannabinoids, such as cannabinol (CBN) and cannabidiol (CBD) in the marijuana plant material, and therefore possibly in the oral fluid sample collected, has not been reported previously and may be of importance for screening and confirmatory assays. Further, Δ^9-tetrahydrocannabinolic acid A (THCA-A, 2-carboxy-THC) is the main pyrolytic precursor to tetrahydrocannabinol. The decarboxylation of 2-carboxy-THC to the active THC during smoking converts only approximately 70% of the precursor to the active form, so the potential presence of 2-carboxy-THC in oral fluid specimens was considered. While blood and urine are more commonly used for these test profiles, oral fluid is increasing in popularity as an alternative matrix

due to its ease of collection, difficulty of adulteration, and improving sensitivity of analytical techniques. One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any quantitative results meaningless without further manipulation in the laboratory. Further, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used.

This work employed Immunalysis Corporation's QUANTISAL oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the drugs from the collection pad into the transportation buffer was determined in order to increase confidence in the quantitative value. The extracts were analyzed using a standard single quadrupole Agilent GC/MS 6890-5975 instrument, with a limit of quantitation of 0.5 ng/mL.

Experimental

Oral Fluid Collection Devices

Quantisal devices for the collection of oral fluid specimens were obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (± 10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal collection devices, and drug concentrations detected were adjusted accordingly. Since 4 mL of specimen is available for analysis, the single quadrupole Agilent GC/MS 6890-5975 instrument is sufficiently sensitive to meet the proposed regulations, using only 1 mL of the total specimen. However, it should be noted that if alternate collection devices that collect much smaller volumes of oral fluid are used, then a Deans switch microfluidic mechanism may need to be used to achieve the necessary sensitivity.

Standards and Reagents

- Tri-deuterated THC for use as an internal standard as well as unlabeled THC, CBN, and CBD were purchased from Cerilliant (Round Rock, TX). 2-carboxy-THC was purchased from Lipomed (Cambridge, MA).
- Trace N 315 solid phase extraction columns were purchased from SPEWare (San Pedro, CA).
- The derivatizing agent, N,O-Bis (trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS), was from Pierce (Rockford, IL).

Internal Standard Concentration

THC 40 ng/mL

Sample Preparation for Chromatographic Analysis

- 1 mL Quantisal specimen (equivalent to 0.25 mL of oral fluid)
- Add internal standard (40 ng/mL)
- Add 0.1 M sodium acetate buffer (pH 4.5; 1 mL)
- Condition SPE columns: methanol (0.5 mL), 0.1 M acetic acid (0.1 mL)
- Add samples
- Wash columns:
  - Deionized water:0.1 M acetic acid (80:20; 1 mL)
  - Deionized water:methanol (40:60; 1 mL)
- Dry columns under nitrogen (30 psi; 2 min).
- Elute: hexane:glacial acetic acid (98:2; 0.8 mL)
- Evaporate to dryness under nitrogen

GC/MS Conditions

Instrument:	Agilent 6890 GC 5975 MSD; inert source; 220/240V oven
Detection mode:	Electron impact
Column:	DB-5 MS, 0.25 mm id, 0.25-µm film thickness, 15-m length
Injection temperature:	250 °C
Purge flow:	50 mL/min for 1 min
Carrier gas:	Helium
Injection mode:	Splitless
Injection volume:	2 µL
Mode of operation:	Constant flow at 1.5 mL/min
Transfer line:	280 °C
Quadrupole:	150 °C
Ion source:	230 °C
Dwell time:	50 ms
Oven program:	125 °C for 0.5 min; ramp at 40 °C/min to 250 °C; hold 1.3 min ramp at 70 °C/min to 300 °C
Retention times:	Deuterated THC: 4.27 min; THC 4.28 min; cannabidiol 3.88 min; cannabinol 4.61 min; 2-c-THC 5.66 min

Ions Monitored

Drug	Ions monitored
THC	Deuterated (d3) **374.3**, 389.3; Unlabeled THC **371.2**, 386.2, 303.1
CBN	**367.3**, 382.2, 310.1
CBD	**390.1**; 301.2
2-carboxy-THC	**487.3**, 488.2, 489.2

Quantitative ions in bold type

Derivatization

Reconstitute in ethyl acetate (30 µL); add BSTFA +1% TMCS (20 µL); transfer to autosampler vials; cap; incubate (60 °C/15 min).

Results and Discussion

One of the issues associated with oral fluid analysis is recovery of drug from a collection pad if a device is used. Extraction efficiency of the collection system for these drugs was determined. Six synthetic oral fluid specimens fortified with all the cannabinoids at a concentration of 4 ng/mL were prepared. The collection pad was placed into the samples until 1 mL had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector. The pad was then transferred to the Quantisal buffer, capped, and stored overnight to simulate transportation to the laboratory. The following day, the pads were removed with a serum separator, and an aliquot of the specimen was analyzed as described. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

	THC	CBD	CBN	2-carboxy-THC
Mean drug recovery (%)	89.2 ± 9.0	71.9 ± 19.1	79.7 ± 7.8	78.2 ± 11.8

GC/MS Method Evaluation

The analytical methods were evaluated according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates (n = 6) over a period of four days.

Analyte	LOQ (ng/mL)	Linear equation	Correlation r^2	Ion ratio range (%)
THC	0.5	y = 0.0266x + 0.00273	0.998	386/371:69.7–104.5 303/371:44.0–66.0
CBN	0.5	y = 0.138x + 0.0022	0.999	382/367:7.4–11.2 310/367:5.7–8.5
CBD	1	y = 0.0271x + 0.00178	0.998	301/390:17.1–25.7
2-carboxy-THC	1	y = 0.0571x + 0.0195	0.998	488/487:31.7–47.5 489/487:11.0–16.6

	THC CV (%)		CBN CV (%)		CBD CV (%)		2-c-THC CV (%)	
Concentration	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
1 ng/mL	0	4.8	5.26	15.3	7.07	6.08	5.73	15.2
2 ng/mL	0	2.53	2.21	2.41	2.82	3.12	10.3	8.3
4 ng/mL	1.39	1.46	5.96	4.20	4.08	4.52	7.03	8.5
8 ng/mL	0.68	1.77	4.66	5.58	1.66	6.84	2.99	2.25

Precision: Inter-day (n = 4) and intra-day (n = 6) precision for the determination of cannabinoids in oral fluid.

Specificity: Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays.

Authentic Specimens

The method was applied to specimens taken from an authentic user. The subject willingly consented to sample collection; he had been a marijuana smoker for over 20 years. For the purpose of this study, he remained marijuana free for five days before smoking. The initial specimen was negative for the four cannabinoids. Samples were collected almost immediately after the subject smoked (5 min), then at intervals of 30 minutes and 1, 2, 12, 24, 36, and 48 hours after smoking. Parent THC was detectable at concentrations well above over 2000 ng/mL in the 5-minute and 30-minute samples, apparently due to excessive oral cavity contamination by THC. The parent drug was detected for 24 hours, and 2-carboxy-THC was identified for up to 16 hours after intake. Cannabidiol was detected only in the specimens from 5 minutes and 30 minutes after smoking and at a concentration of 5 ng/mL. Cannabinol was measurable for only 2 hours (Figure 1).

An extracted ion chromatogram of the sample collected 1 hour after smoking is presented in Figure 2. The extracted ions for cannabidiol were not included since there was no CBD present in the specimen.

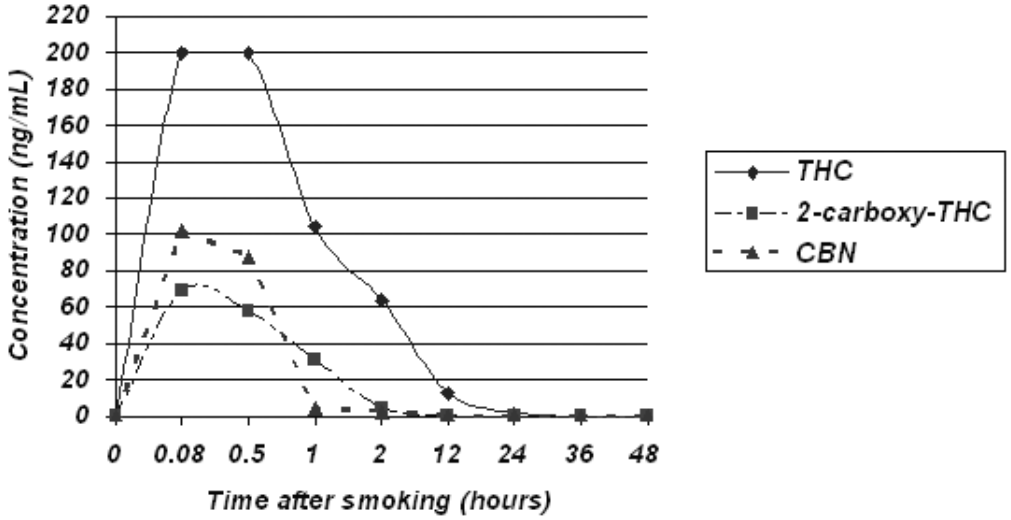

Figure 1. Cannabinoids in oral fluid following marijuana smoking.

Conclusions

The procedure described is suitable for the routine detection and confirmation of THC, CBN, and 2-carboxy-THC in oral fluid using the Quantisal oral fluid collection device and an Agilent single quadrupole GC/MSD.

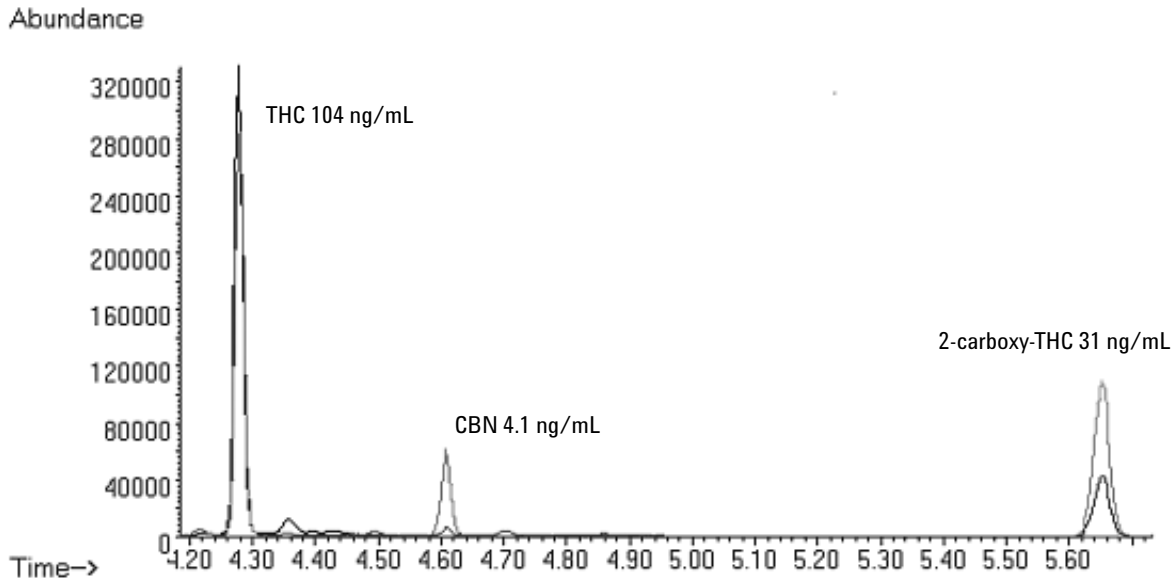

Figure 2. Oral fluid specimen collected 1 hour after marijuana smoking.

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Agilent Drug Analysis Solution

Fast Drug Analysis in Whole Blood

Forensic Toxicology

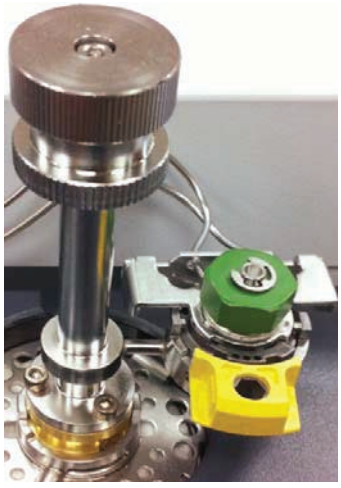
The Agilent 5975T Low Thermal Mass (LTM) GC/MSD, together with a Thermal Separation Probe (TSP) and DRS software, provide fast, accurate data analysis of blood analysis. The system provides a fast, accurate solution for liquid and solid samples with complex matrix.

Drugs abuse detection is always an important project in forensic area. Forensic toxicologists are routinely confronted with the difficult problem of detecting and quantitating a wide range of drugs in cases of fatal doses of drugs in whole blood because of the drugs universality and blood complexity. However because most drugs and their metabolites are among the structures which can be analyzed by GC/MS, the 5975 LTM GC/MSD may be used for the analysis of many drugs.

The rugged high performance Agilent 5975T LTM GC/MSD is easily transported to onsite locations or used in a lab. With its quick ramp heating oven rate and fast cooling cycle, the instrument with a LTM column provides an ultra-fast sample cycle. The Thermal Separation Probe (TSP) not only greatly reduces sample preparation time, it helps protect the entire instrument from matrix contamination.

Key Benefits

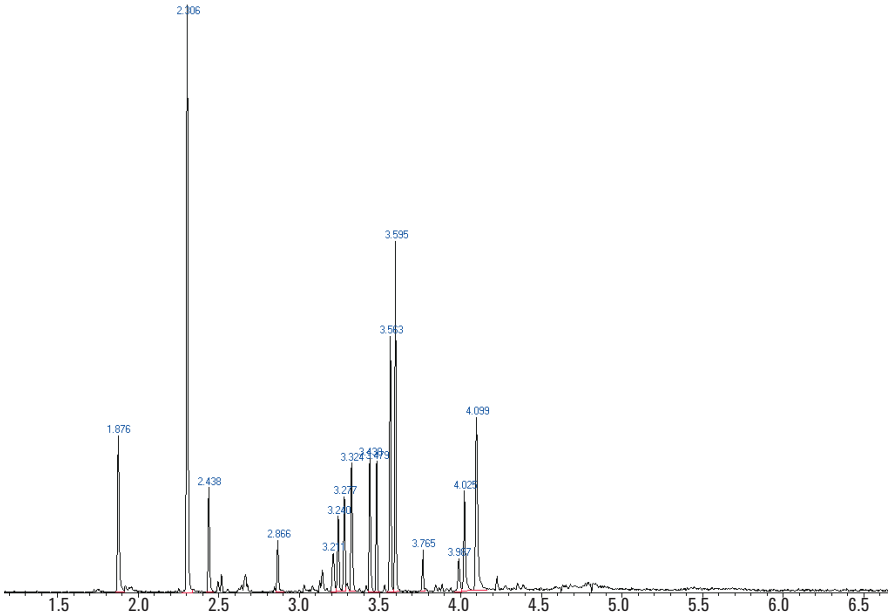
- The Agilent 5975T Low Thermal Mass (LTM) GC/MS provides rapid temperature ramps and cool down
- The Agilent TSP minimizes sample preparation time for fatal doses of drugs in whole blood
- provides quick data handling method for extracting targets from complex matrix backgrounds



The Measure of Confidence

Agilent Technologies

Blood Analysis Using an Agilent 5975T + TSP



Peak identification

1.8765	Barbital
2.3049	Amobarbital
2.4384	Secobarbital
3.1265	Cocaine
3.2050	1-Piperidinepropanol, α-cyclopentyl-α-phenyl-
3.2407	Promethazine
3.2788	SKF525
3.3232	Oxazepam
3.4365	Lorazepam
3.4782	Diazepam
3.563	Chlorpromazine
3.5645	Chlorprothixene
3.5950	Chlordiazepoxide
3.9862	Papaverine
4.024	Clozapine
4.0243	Clonazepam
4.0971	Estazolam

Figure 1. TIC of drugs standards based on fast moving method.

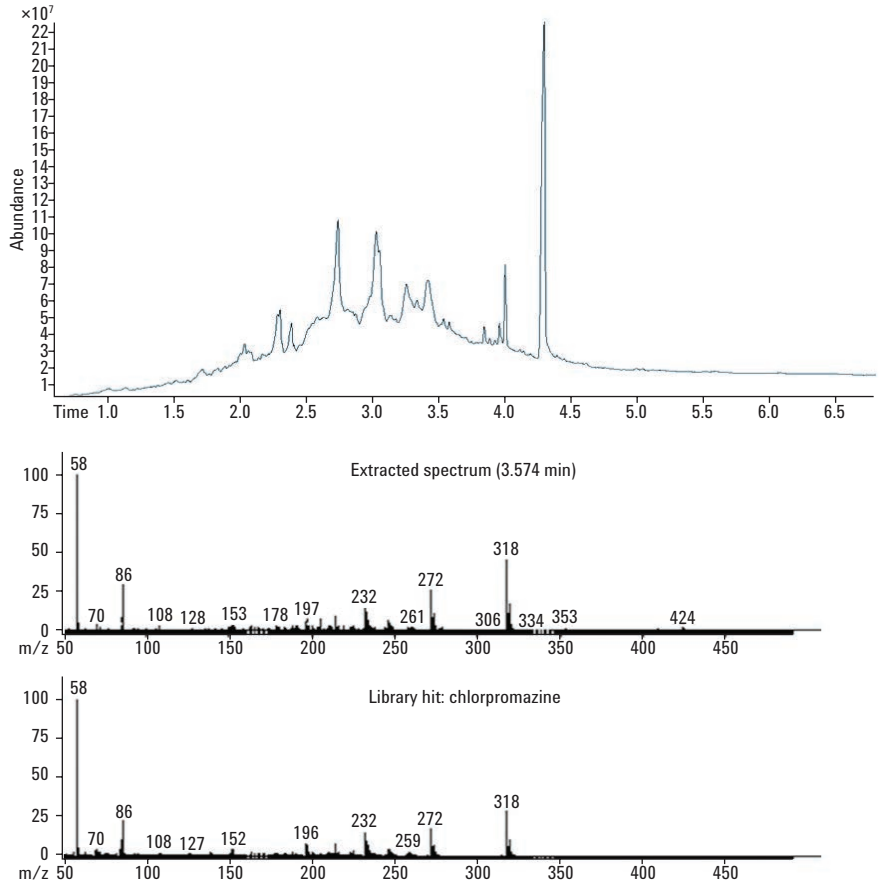


Figure 2. Drug identification by direct blood sample injection with an Agilent 5975T + TSP.

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application

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Gas Chromatography / Mass Spectrometry | 5973 MSD

Application of Electron Capture Negative Chemical Ionization for the detection of the "Date Rape" Drug Flunitrazepam

Forensic Toxicology

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Introduction

Flunitrazepam is a benzodiazepine marketed by Hoffman-La Roche under the brand name of Rohypnol®. Although the drug is available by prescription in Europe and Latin America, there is no legal use of this drug in the United States. The U.S. Department of Justice Drug Enforcement Agency has classified flunitrazepam as a Schedule I drug, which prohibits possession or trafficking in the U.S. without special exemption.

Analyzing hair is becoming a widely-applied approach to surveying for drugs of abuse. Hair offers several advantages over urine or blood testing. For example, the collection of hair samples is simpler and less invasive than the collection of urine or blood samples and issues of adulteration are minimal with hair. Hair tends to provide a longer documentation period of drug use, of the order of several months, because many compounds tend to remain very stable in hair. On the other hand, the difficulty with hair analysis is that the matrix is fairly recalcitrant to digest, and concentrations tend to be very low. As a result, sensitivity must be in the picogram per milligram range.

Metabolism of flunitrazepam produces the 7-aminoflunitrazepam metabolite via reduction of the nitro-group (Figure 1) as one of the three major metabolites (the others being norflunitrazepam and 7-acetamidoflunitrazepam). Since these compounds, like other benzodiazepines, contain electophilic groups such as halogens, nitrogen, and aromatic rings, they are good candidates for detection using electron capture negative ionization (ECNI).

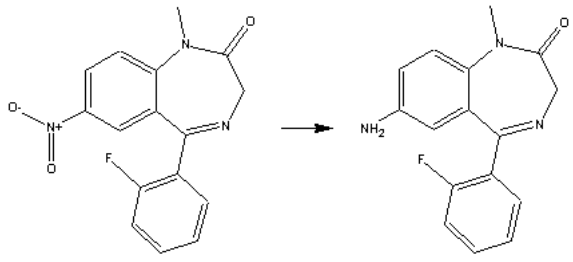

Figure 1. Flunitrazepam [5-(2-fluorophenyl)-1,3 dihydro-1-methyl-7-nitro-1,4-benzodiazepine-2-one] (left) and the analyzed metabolite, 7-aminoflunitrazepam [1-methyl-5(-2-fluorophenyl)-7-amino-1,4-benzodiazepin-2-one] (right)

Experimental

Sample Preparation

A 50-mg hair sample was pulverized using steel balls then transferred to 3 ml of methanol. Flunitrazepam-d_7 and 7-aminoflunitrazepam-d_7 were added as recovery surrogates at 100 pg/mg and 20 pg/mg, respectively, before one hour of sonication. The methanol was then decanted off and 3 ml of 0.1 N HCl is added to the hair and the mixture incubated at 55°C overnight. The supernate was then separated off by centrifugation and combined with the methanol, 1 ml of 1.93 M glacial acetic acid and 9 ml of deionized water. The sample was then added to a pre-conditioned mixed-mode solid-phase extraction column. After drying, the column was eluted with a mixture of dichloromethane, isopropanol, and ammonium hydroxide (78:20:2 v/v/v). The eluate was evaporated under dry nitrogen. The residue was redissolved in 50 µl of ethyl acetate and transferred to an autosampler vial then evaporated to dryness. Heptafluorobutyric acid anhydride (HFBA) was added, the sample incubated at 60°C for 30 minutes and then evaporated to remove excess derivatization reagent. The sample was reconstituted in 25 µl of ethyl acetate. The flunitrazepam was analyzed underivatized and the 7-aminoflunitrazepam was derivatized by the heptafluorobutyric acid anhydride (HFBA).

Instrumental Parameters

The Agilent Technologies 6980/5973 GC/MSD with CI option was operated in the electron capture negative ionization selected ion monitoring mode (SIM) with methane buffer gas. Flunitrazepam was monitored at m/z 313, the 7-aminoflunitrazepam metabolite at m/z 459, and the hepta-deuterated parent and metabolite surrogates at 320 and 466 m/z, respectively.

A more detailed discussion of the extraction and instrumental method parameters will be published shortly.[1]

Results

Figure 2 shows the intense response in electron capture negative ionization SIM of small amounts of flunitrazepam and metabolite extracted from hair on the 5973 MSD. Notice that the background is relatively free of interferences despite the complicated nature of the hair matrix.

Figure 3 and Figure 4 show the linearity of the ECNI response ratio for the parent and metabolite relative to their corresponding surrogates versus the hair concentration over two orders of magnitude. The additional fluorines on the derivatized 7-aminoflunitrazepam greatly enhances the ECNI response over that of the parent.

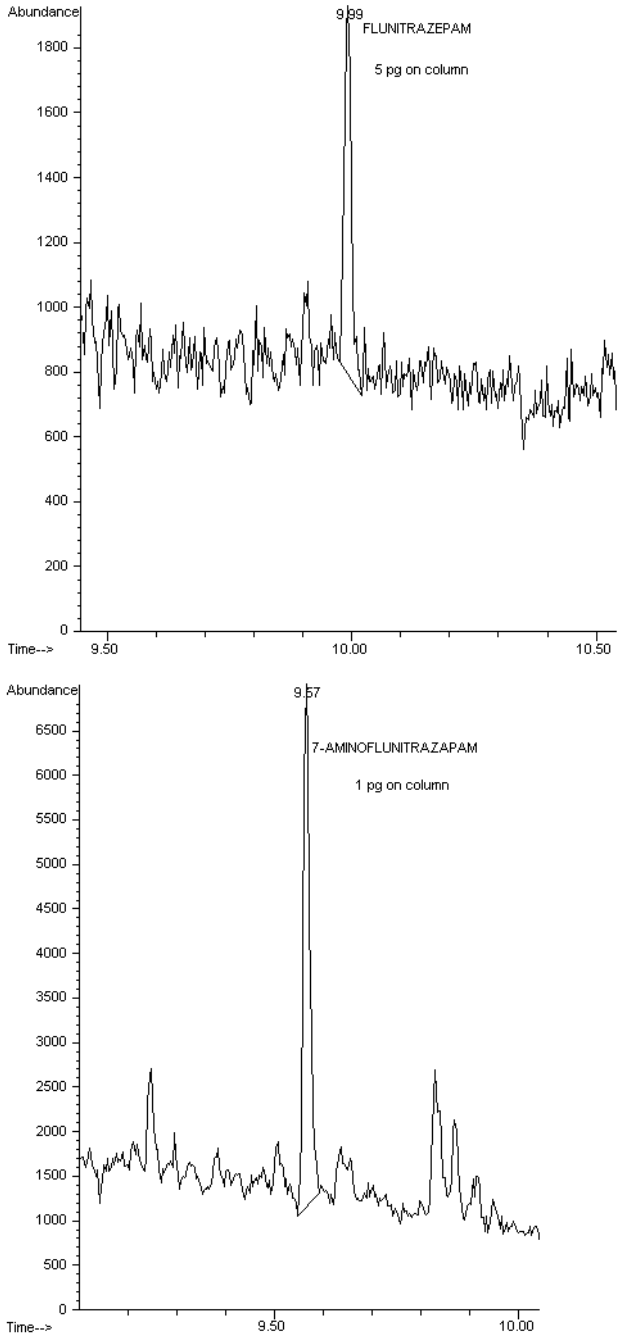

Figure 2. ECNI-SIM chromatograms of 1 pg injected of the derivatized 7-aminoflunitrazepam (lower panel), and 5 pg injected of flunitrazepam (upper panel), both extracted from hair

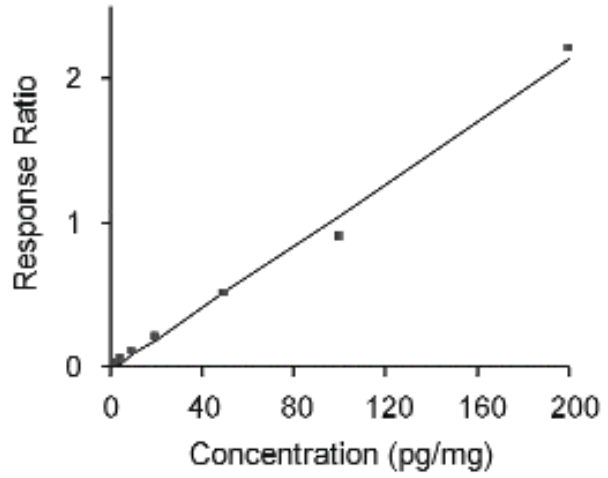

Figure 3. Relative response ratio versus concentration in hair for flunitrazepam from 2.5 to 200 pg/mg, r^2 = 0.996

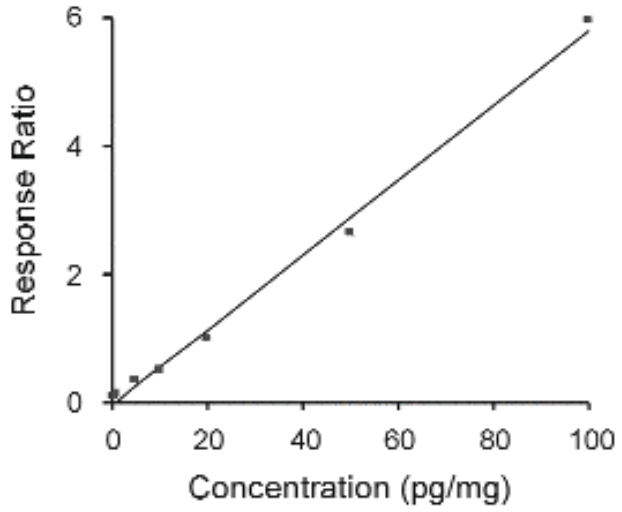

Figure 4. Relative response ratio versus concentration in hair for 7-aminoflunitrazepam from 500 fg/mg to 100 pg/mg, r^2 = 0.998

A study of the intraday and interday accuracy and precision of the method shows very good reliability with high relative accuracy and high relative precision (Table 1 and 2).

Application of the technique to a postmortem hair sample taken from a 70-year old man who died from an overdose indicated parent compound in the hair at a concentration just below the quantitation limit, but a very high concentration of the metabolite (Figure 5). The results are interesting for two reasons: (1) only 9 mg of hair was available, which is 5 times less than is typically analyzed, and (2) the hair was gray.

A low detection limit for the metabolite is also obtained in urine extracts using ECNI SIM. Figure 6 shows results for 7-aminoflunitrazepam in urine at 10 pg/ml which is near the limit of quantitation of the technique. The literature cites methods that have limits of detection using electron impact mass spectrometry with SIM of approximately 10 ng/ml.[2] This improvement of 1000 fold in sensitivity greatly extends the period during which Rohypnol® can be detected.

Table 1. Accuracy and Precision of Flunitrazepam Hair Preparations

Target concentration (pg/mg)	15	80
Intraday study (number of replicates)	N = 4	N = 4
Mean measured concentration (std. dev.)	15.86 (0.75)	71.12 (5.82)
Coefficient of variation as percent	4.73 %	8.18 %
Relative accuracy as percent	5.73 %	–11.10 %
Interday study (number of replicates)	N = 13	N = 13
Mean measured concentration (std. dev.)	14.58 (1.31)	70.40 (4.82)
Coefficient of variation as percent	8.98 %	6.85 %
Relative accuracy as percent	–2.80 %	–12.00 %

Table 2. Accuracy and Precision of 7-Aminoflunitrazepam Hair Preparations

Target concentration (pg/mg)	3	40
Intraday study (number of replicates)	N = 4	N = 4
Mean measured concentration (std. dev.)	2.81 (0.26)	36.31 (3.72)
Coefficient of variation as percent	9.25 %	10.25 %
Relative accuracy as percent	–6.33 %	–9.23 %
Interday study (number of replicates)	N = 14	N = 14
Mean measured concentration (std. dev.)	2.93 (0.28)	38.21 (3.39)
Coefficient of variation as percent	9.56 %	8.87 %
Relative accuracy as percent	–2.33 %	–4.48 %

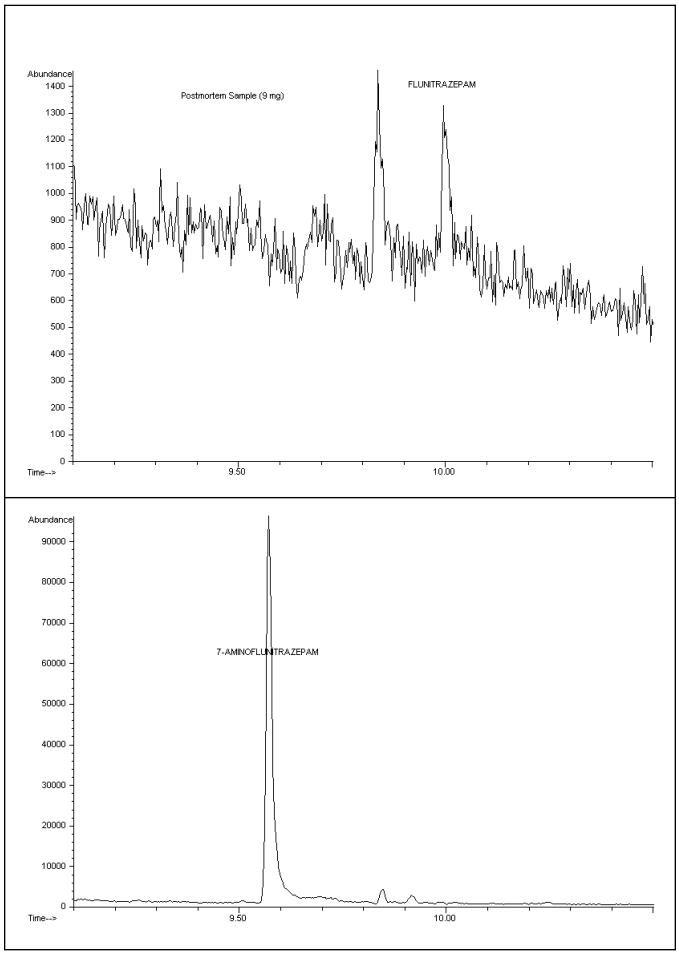

Figure 5. Flunitrazepam (upper panel) and derivatized metabolite (lower panel) in 9 mg postmortem hair sample

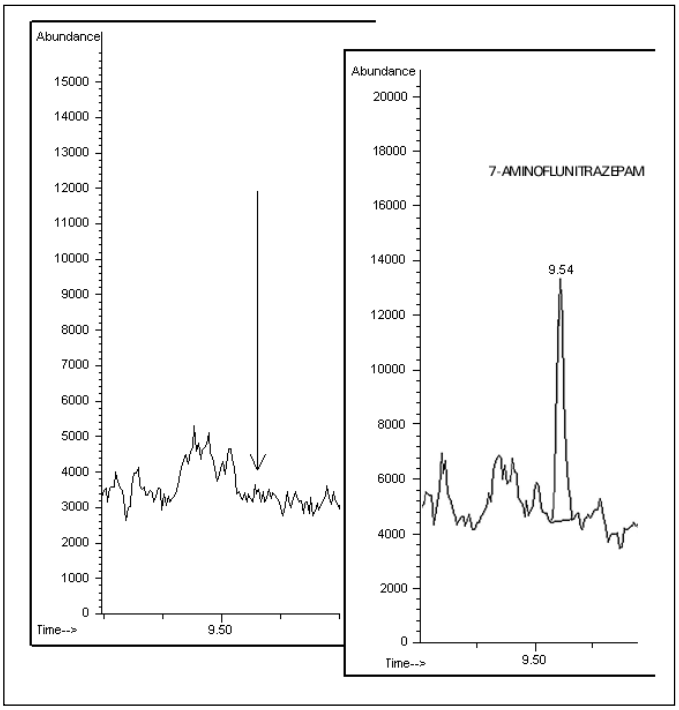

Figure 6. ECNI-SIM chromatograms of urine blank (left panel) and 7-aminoflunitrazepam at 10 pg/ml in urine (right panel)

References

1. Negrusz, A., et al., *Highly sensitive micro-plate enzyme immunoassay screening and NCI-GC-MS confirmation of flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair.* Journal of Analytical Toxicology, accepted.

2. Lin, Z. and O. Beck, *Procedure for verification of flunitrazepam and nitrazepam intake by gas chromatographic-mass spectrometric analysis of urine.* Journal of Pharmaceutical and Biomedical Analysis, 1995. **13**: p. 719–722.

Acknowledgements

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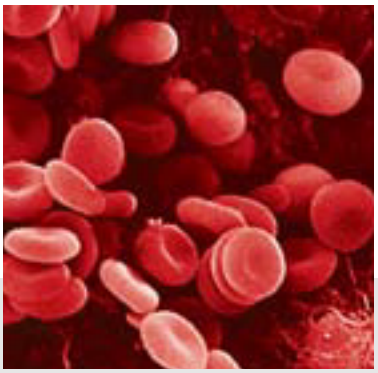

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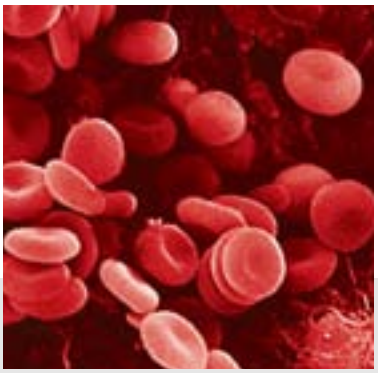

Applications by Technique

LC/QQQ

Analysis of Benzodiazepines in Blood by LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A sensitive and selective method for the simultaneous screening and identification of 13 benzodiazepines and 5 metabolites in human blood using the Agilent LC/MSD Trap is described. The method uses liquid-liquid extraction followed by reverse-phase LC/MS/MS (liquid chromatography/tandem mass spectrometry). The technique is suitable for screening analysis and high-confidence identification of the analytes at their lowest reported therapeutic concentrations using only 500 µL of blood and the original model ("Classic") of the Agilent LC/MSD Trap. The method has been successfully applied in forensic cases involving low concentrations of benzodiazepines.

Introduction

Benzodiazepines are an important class of drugs with a broad range of therapeutic effects [1]. Because of their wide usage, benzodiazepines have the potential for interaction with other central nervous system depressants which can result in life-threatening or impaired-driving situations. Benzodiazepines are now among the most commonly-prescribed drugs, which increases their potential for addiction and abuse, and often they are found in combination with other drugs in drug-related fatalities or drug-facilitated sexual assault cases [2]. For these rea-sons, the analysis of benzodiazepines is of great interest to forensic toxicologists.

Screening of these compounds has been problematic since immunoassays are often not sufficiently specific or sensitive enough for low-dosage benzodiazepines, especially in blood. Benzodiazepines have been analyzed using gas chromatography/nitrogen phosphorus detector (GC/NPD) [3], gas chromatography/electron capture detector (GC/ECD) [3], and gas chromatography/mass spectrometry (GC/MS) [4, 5]. Many benzodiazepines are polar and thermally labile, making them diffi-cult, if not impossible, to analyze with GC or GC/MS without derivatization. Some of the compounds cannot be derivatized for improved chromatographic behavior.

Screening for benzodiazepines can also be carried out using HPLC with UV detection [6], but this technique lacks both the sensitivity and specificity required for forensic applications. Furthermore, some of the newer benzodiazepines, like flunitrazepam, have much lower therapeutic ranges and faster clearance, and therefore require identification at lower levels.

Liquid chromatography/mass spectrometry (LC/MS) is ideally suited for this family of compounds because the technique does not require derivatization, thereby saving time, expense, and experimental difficulty. This class of compounds also ionizes well in either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) modes, and therefore can be easily detected at µg/mL levels and below. A large number of benzodiazepines and related substances have also been analyzed using the Agilent single quadrupole liquid chromatography/mass selective detector (LC/MSD) in APCI mode and selected ion monitoring (SIM) mode [7]. The full scan sensitivity of the Agilent LC/MSD Trap allows for both identification/confirmation and quantitation in a single analysis, and the multiple reaction monitoring (MRM) mode provides for more specific detection in a complex matrix such as blood.

In this work 13 benzodiazepines and 5 metabolites [Table 1] are analyzed in a single run of approximately 20 minutes. This application note is derived from work carried out in the Australian laboratory and previously published in reference 8.

Table 1. Compounds Analyzed

Benzodiazepines	Metabolites
Alprazolam	–
Bromazepam	–
Clobazam	–
Clonazepam	7-aminoclonazepam
Diazepam	Nordiazepam
Flunitrazepam	7-aminoflunitrazepam
Flurazepam	N-desalkylflurazepam
Lorazepam	–
Midazolam	–
Nitrazepam	7-aminonitrazepam
Oxazepam	–
Temazepam	–
Triazolam	–
Prazepam (internal standard)	

Experimental

Sample Preparation

Reference solutions of each analyte were combined, diluted in water and added to drug-free blood, along with the internal standard, to prepare calibrators at low, medium and high concentrations of each drug. Typical low and high concentrations are shown in Table 2. The extraction method is the same as used for screening of these drugs by GC/ECD and GC/MS, with any derivatization step omitted and the final residue dissolved in the initial mobile phase rather than in a typical GC solvent.

Table 2. Concentration Ranges for Analytes (mg/L or µg/mL)

Benzodiazepine/ metabolite	Low concentration	High concentration
Alprazolam	0.01	0.1
Bromazepam	0.08	0.2
Clobazam	0.1	1
Clonazepam	0.03	0.08
7-aminoclonazepam	0.03	0.14
Diazepam	0.05	2
Nordiazepam	0.05	2
Flunitrazepam	0.005	0.02
7-aminoflunitrazepam	0.002	0.02
Flurazepam	0.0005	0.028
N-desalkylflurazepam	0.04	0.15
Lorazepam	0.02	0.3
Midazolam	0.08	0.25
Nitrazepam	0.03	0.2
7-aminonitrazepam	0.03	0.2
Oxazepam	0.15	2
Temazepam	0.3	1
Triazolam	0.002	0.02

To 0.5-mL blood in a glass screw-top tube was added 50 µL of freshly prepared internal standard working solution (5 µg/mL in water). To this tube was added 1.75 mL of 4.5% ammonia solution and 10 mL of 1-chlorobutane, and the contents rolled on a mechanical mixer for 10 minutes. After centrifuging, the solvent was drawn off, transferred to a clean glass tube and evaporated to dryness in a Jouan centrifugal evaporator. The residue was dissolved in 100 µL of mobile phase.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consisted of an Agilent 1100-series vacuum degasser, binary pump, autosampler, thermostatted column compartment, diode array detector (DAD) with microflow cell, an LC/MSD Trap "Classic" (model

G2445A, equivalent in performance to the current VL model), and a G1947A APCI source. Complete system control and data analysis was provided by the Agilent LC/MS ChemStation.

LC/MS Method Details

LC Conditions

Column:	Agilent ZORBAX Eclipse XDB-C8, 150 × 4.6 mm, 5 µm (p/n 993967-906)
Mobile phase:	A = 20 mM ammonium formate, pH 9 in water
	B = methanol
Flow rate:	0.7 mL/min
Gradient:	60% B until 15 min 100% B at 16 min 100% B to 21 min Post-time (column re-equil): 5 minutes (sufficient for reproducible retention times)
Injection vol:	5 µL

MS Conditions

Source:	Positive APCI
Nebulizer:	60 psig
Vaporizer:	400 °C
Drying gas flow:	5 L/min
Drying gas temp:	350 °C
V_{cap}:	3000 V
Corona:	4 µA
Scan:	*m/z* 150–400
Averages:	2
SPS settings:	Target mass *m/z* 300
	Compound stability 60% (Skim 1: 24 V, Cap exit offset: 69 V)
	Trap drive 100% (resulting value 27)
Precursor isolation width:	2.5 amu
Cutoff:	45% (113–175 *m/z* for these compounds)
MRM:	Eight time segments as shown in Table 3

Results and Discussion

Discussion

Both ESI and APCI were evaluated for the analysis of these compounds. Generally, both gave good sensitivity at the low concentration levels needed for the method. However, both flurazepam and lorazepam showed poor response in ESI with the mobile phase which gave the best chromatographic separation in a reasonable time. Because APCI has also been demonstrated to be less susceptible to matrix suppression effects than ESI, and because both flurazepam and lorazepam showed better sensitivity with APCI, it was chosen as the preferred ionization method.

Various mobile phase compositions were evaluated, with the objective being a best compromise among a simple LC method, reasonably short run time, and maximizing chromatographic resolution of the analytes. The choices included isocratic and gradient methods using either 20-mM ammonium formate at pH 3 or pH 9, or 0.1% formic acid. Once APCI was chosen as the preferred ionization method, methanol became the organic component of choice over acetonitrile, as it provides better sensitivity in APCI and does not build up carbon deposits on the APCI corona needle. Acetonitrile has higher gas phase basicity than methanol; since ionization in APCI occurs in the gas phase (rather in the liquid phase as in ESI), acetonitrile can compete with analyte molecules for the available protonation “work”.

The basic aqueous phase with methanol as the organic component was found to give the best separation, chromatographic peak shape, and sensitivity for this analysis. The ZORBAX Eclipse C8 column is stable at the effective pH of this mobile phase for extended periods of time. The C8 stationary phase proved to be sufficiently retentive even for the polar metabolites; C18 would have required longer run times.

Reconstituting the sample extracts in the initial mobile phase, a recommended practice for HPLC, was found to give better peak shapes and therefore better sensitivity than using simply methanol/water. This is especially important for the early-eluting polar analytes.

Prazepam was chosen as a suitable internal standard because of its structural similarity to the other analytes and because it is not prescribed in Australia.

The optimum fragmentation amplitude for each analyte was determined by infusing a 5-µg/mL solution of a single compound into the MS/MS, and increasing the fragmentation amplitude until the precursor ion intensity was reduced to 10%–20% of its major product ion response. The resulting value was used in the data acquisition method as shown in Table 3.

Table 3. Data Acquisition Parameters for MRM

Group number [min]	Benzodiazepine/ metabolite	RT (min)	Precursor ion $[M+H]^+$	Major Product ion (*m/z*)	Fragmentation amplitude (V)	Fragmentation width (*m/z*)
1 [1.00–4.00]	7-aminonitrazepam	2.6	252	224	2.00	10
	7-aminoclonazepam	2.8	286	250	2.50	10
	7-aminoflunitrazepam	3.1	284	264	1.88	10
2 [4.00–5.70]	Bromazepam	5.3	316	288	1.92	10
3 [5.70–6.70]	Clonazepam	6.1	316	270	2.00	10
	Nitrazepam	6.2	282	236	1.86	10
	Flunitrazepam	6.3	314	268	1.90	10
4 [6.70–8.80]	Clobazam	7.3	301	259	3.37	40
	Flurazepam	7.6	388	315	2.60	40
	Triazolam	7.8	343	308	3.57	40
	Alprazolam	8.3	309	281	4.67	40
	Lorazepam	8.3	321	275	2.98	40
	Oxazepam	8.6	287	241	3.32	40
5 [8.80–11.10]	N-desalkylflurazepam	9.2	289	261	4.57	40
	Temazepam	9.6	301	255	3.72	40
6 [11.10–13.00]	Nordiazepam	12.1	271	243	1.88	10
7 [13.00–17.00]	Diazepam	13.9	285	257	1.90	10
	Midazolam	14.9	326	291	2.05	10
8 [17.00–21.00]	Prazepam	19.4	325	271	1.90	10

In spite of the extremely fast scan speed of the Agilent LC/MSD Trap (up to 26,000 amu/s for current models), configuring the MRM method to repetitively step through all of the precursor ions and MS/MS scans for all 18 analytes plus internal standard would result in unacceptably long cycle times and insufficient data points per second to properly define and quantitate each analyte. Therefore the analysis is set up in time-programmed segments in which MRM occurs for a few analytes at a time in a given portion of the chromatogram, as shown in Table 3.

A time delay may be observed during switchover between groups in which a large number of analytes are being monitored if using manual Fragmentation Cut-off values. This delay can be averted by setting the Cut-off selection in all groups (found under Fragmentation in the MS/MS section of the Trap Control window) to an appropriate percentage value of the precursor mass, or to "Default", rather than setting a manual value for each analyte. The original version of this method used a manual cut-off value of 150 *m/z* for each MRM, which resulted in such a delay. Improved sensitivity for these analytes is obtained by setting the Cut-off to 45% rather than the default 27%. This essentially causes the trap to focus on the *m/z* regions where the major product ions of these analytes are found, not trapping lower mass ions less useful for identification and quantitation.

The results of optimization of MS/MS acquisition can be seen by examining the chromatograms in Figure 1 which shows the overlaid principal product ion chromatograms of all the analytes for the analysis described here.

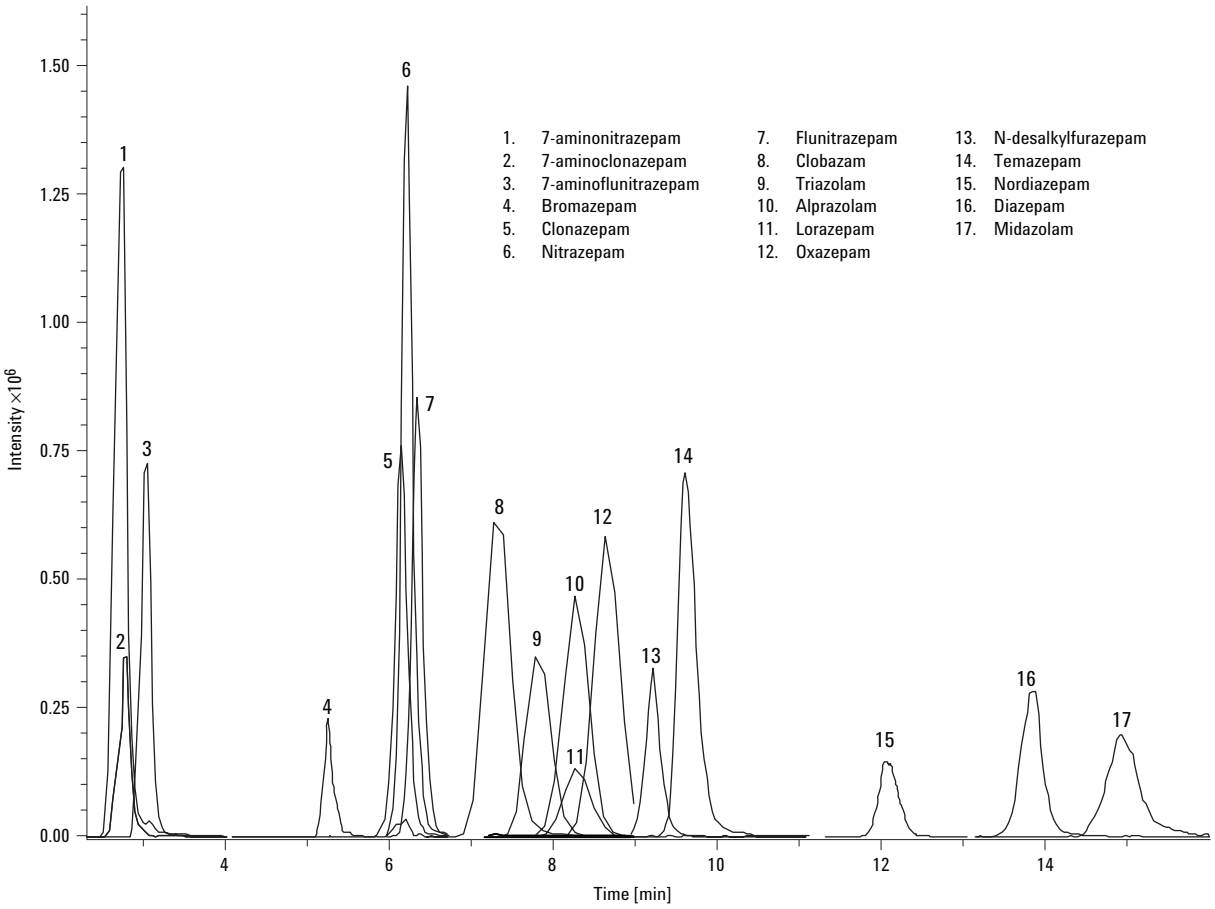

Figure 1. Overlay of principal product ion chromatograms.

The first group, which extends from 1.0 to 4.0 minutes, includes the MRM analysis of three compounds, which practically co-elute. The second group only covers one compound, while the third group covers another three compounds. The fourth group covers six compounds, which reduces the effective duty cycle for analyzing each compound. Whether or not the compounds are eluting, the MRM is cycling through six different precursor ions. However, the chromatographic peak widths are large enough that sufficient data points are produced for each analyte.

The product ion spectra are acquired in full scan mode which allows the MS/MS spectra to be added to a user library as an automated aid to screening and compound identification. Such a library is in use in this laboratory and others in the forensic toxicology field. For screening a larger number of drugs, the AutoMS^n mode of analysis can produce both MS and MS/MS (even MS/MS/MS, or MS^3) spectra which can be searched against a library of spectra created using identical MS^n parameters from authentic standards.

Switching on the SmartFrag option may offer some advantages for qualitative analyses where spectral reproducibility and an abundance of product ions are primary concerns. Switching off the SmartFrag option to maximize the intensity of fewer product ions will assist low level quantitative analyses.

Figures 2–8 show the structures and MS/MS spectra for the analytes under the conditions of the method. The spectra are grouped to illustrate some common losses and the interesting change in fragmentation behavior that can occur with a relatively small change in structure.

Figure 2 shows MS/MS spectra of the chlorine-containing midazolam and triazolam which lose chlorine under these conditions. Triazolam with the five-membered nitrogen-containing ring also shows a major fragment ion corresponding to ring-opening and loss of diatomic nitrogen.

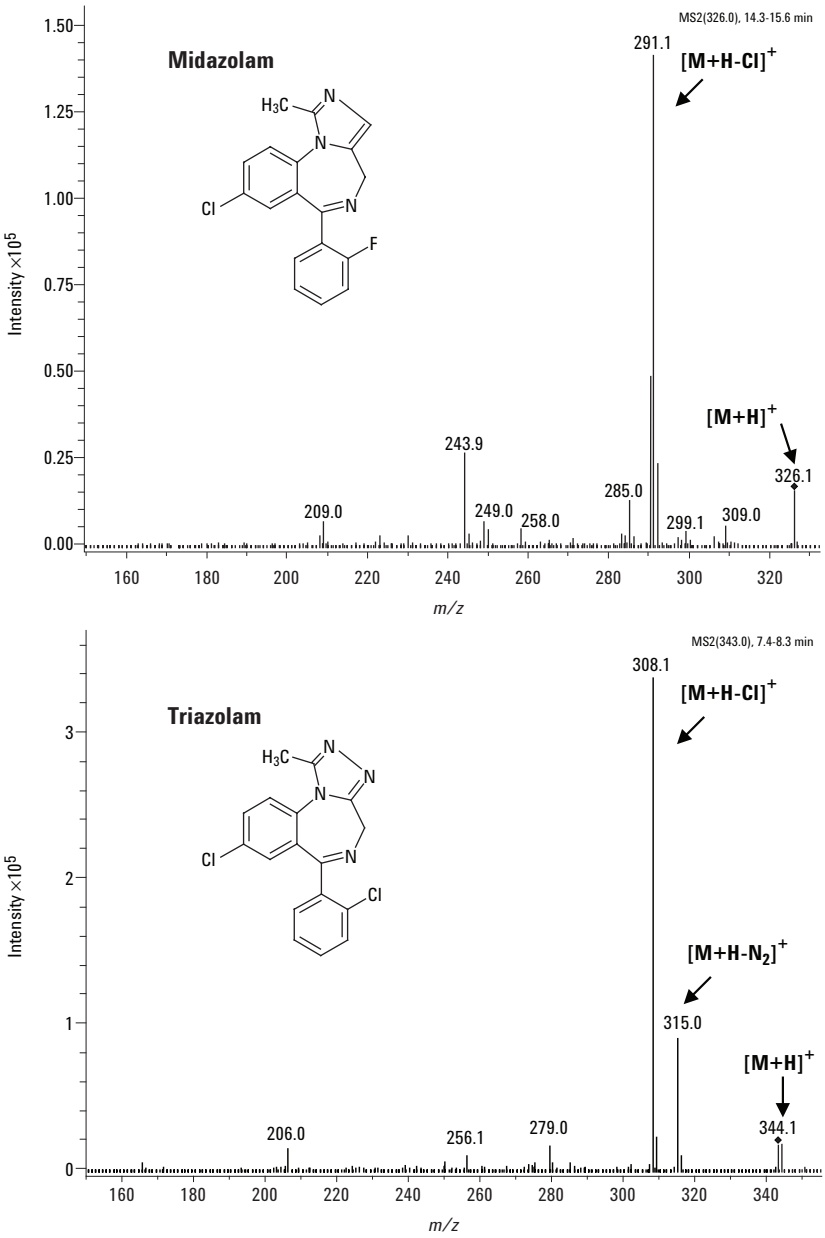

Figure 2. Structures and MS/MS spectra of midazolam and triazolam.

Figure 3 shows three benzodiazepines whose base peak in the MS/MS spectrum corresponds to loss of NO_2. The figure also shows the spectra of the metabolite of each parent drug in which the nitro group has been reduced to an amino group. In each case, the structural change gives rise to a different fragmentation: 7-aminoclonazepam loses HCl; 7-aminonitrazepam loses CO, apparently via opening of the 7-membered ring; and 7-amino-flunitrazepam loses HF. Note the similarity in structures for the analytes with HCl and HF losses.

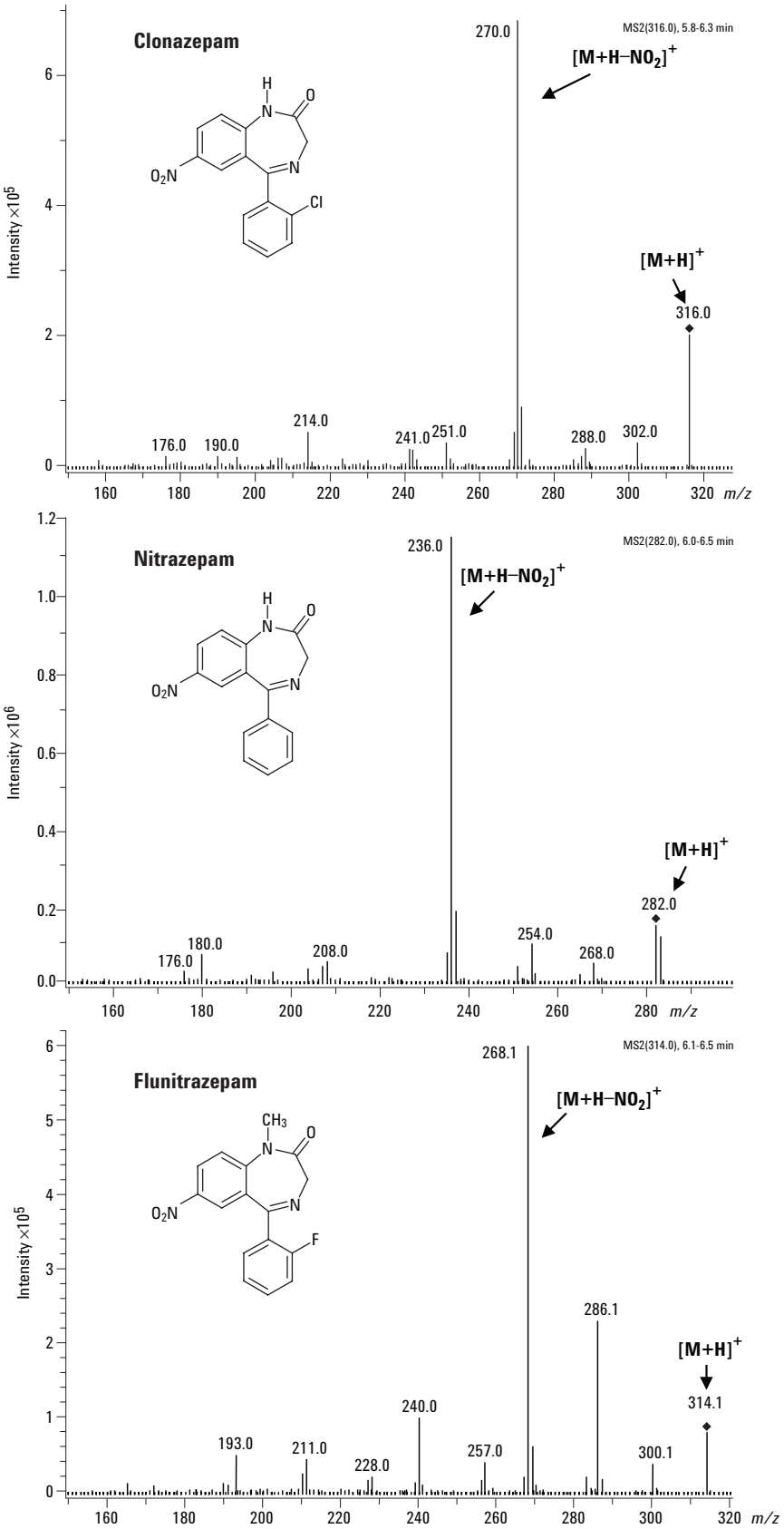

Figure 3. Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.

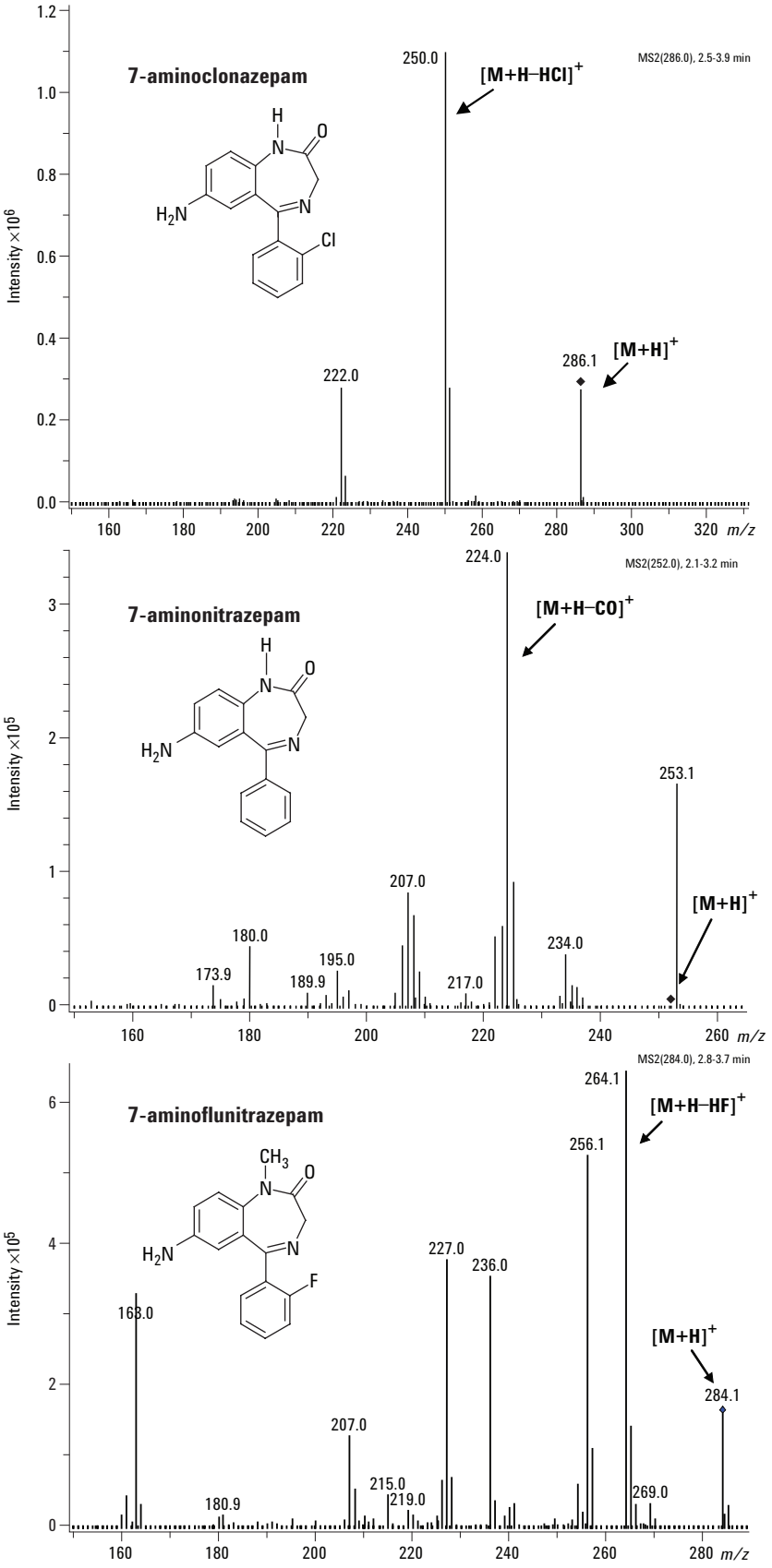

Figure 3 (continued). Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.

Flurazepam and prazepam lose alkyl groups as shown in Figure 4, and the desalkylflurazepam metabolite which has lost the entire alkylamino substituent develops a different fragmentation behavior with the major ion corresponding to loss of CO.

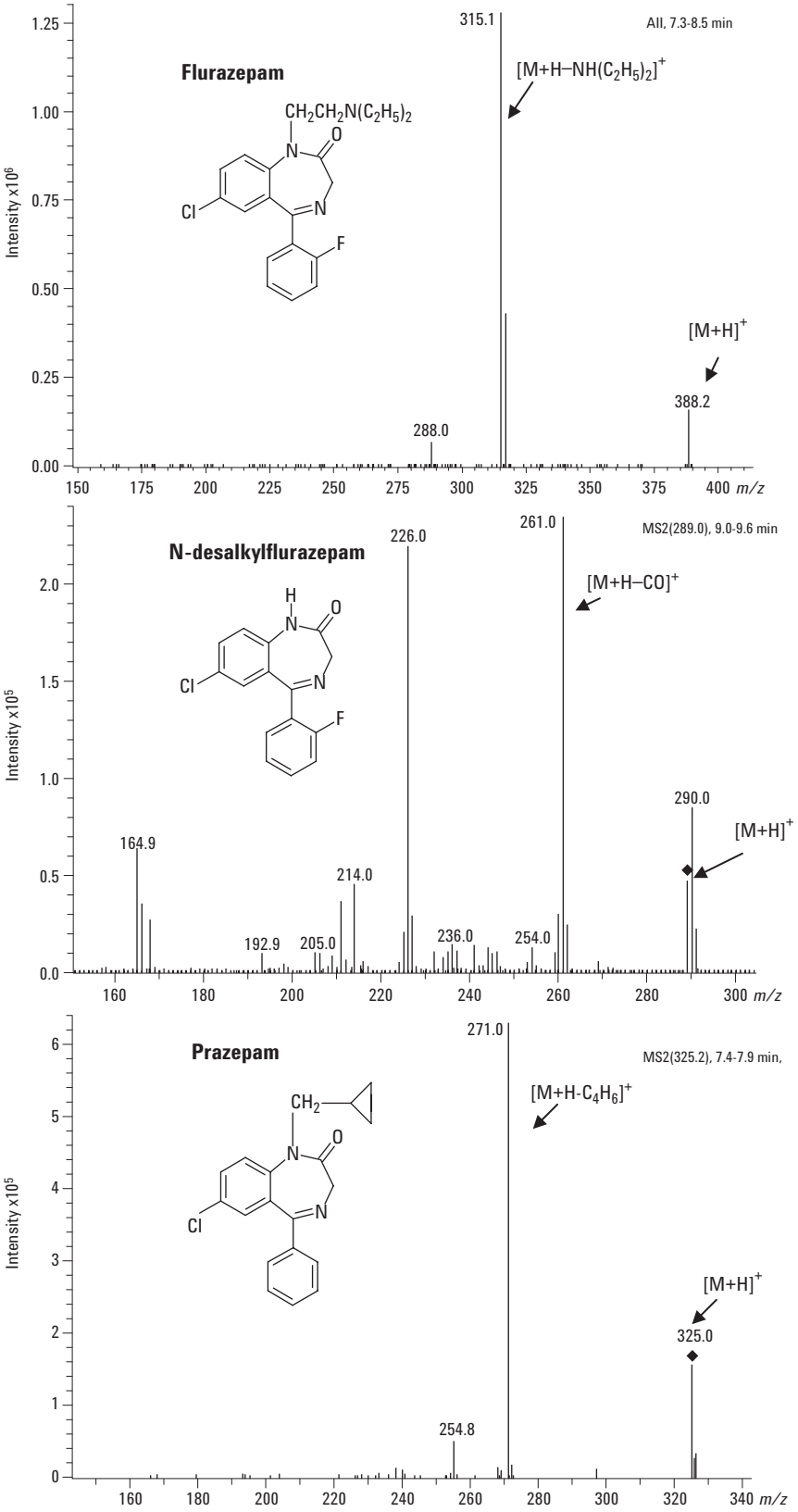

Figure 4. Structures and MS/MS spectra for flurazepam, N-desalkylflurazepam, and prazepam.

Figure 5 shows the loss of N_2 from alprazolam under these conditions. Its structure is very similar to that of triazolam (Figure 2) which also loses N_2, presumably also from the five-membered ring.

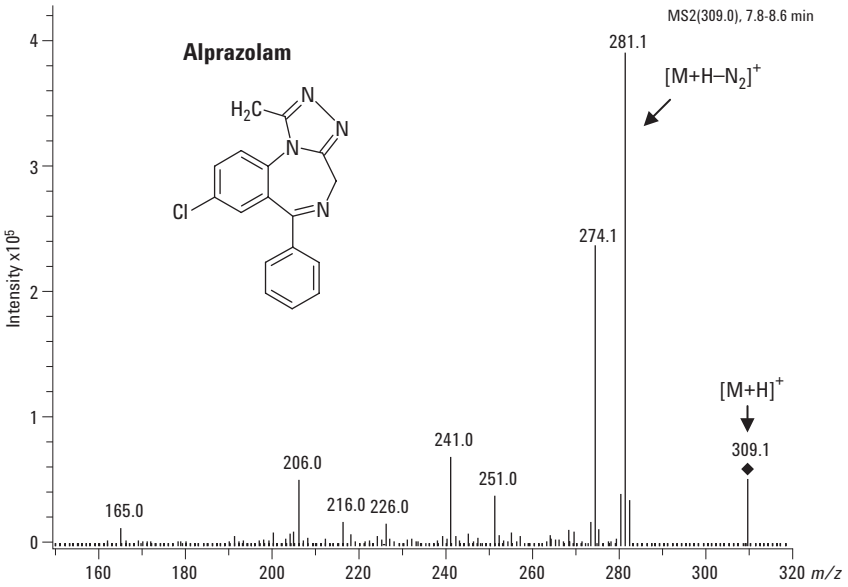

Figure 5. Structure and MS/MS spectrum of alprazolam.

Figure 6 shows several more benzodiazepines which lose the elements of CO, like N-desalkylflurazepam in Figure 4. It is interesting that all three lose CO from the 7-ring even though they all have a halogen in the 7-position of the fused benzene ring. Notice the HX loss from 7-aminoclonazepam and 7-aminoflunitrazepam (Figure 3) where a halogen is on the 2'-position of the non-fused benzene ring.

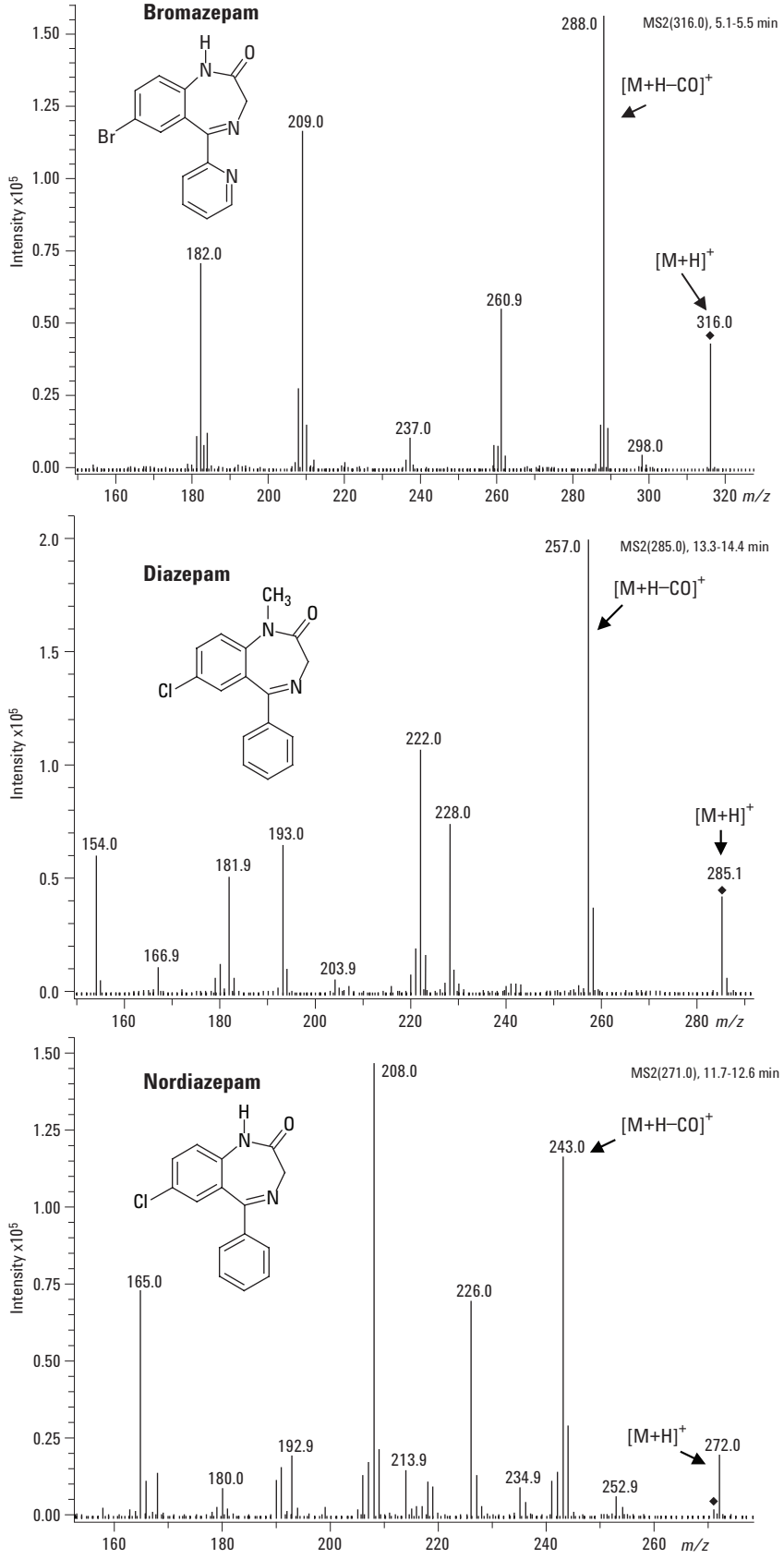

Figure 6. Structures and MS/MS spectra for bromazepam, diazepam, and nordiazepam.

Clobazam shows an extremely simple MS/MS spectrum and a unique loss of CH_2CO in Figure 7.

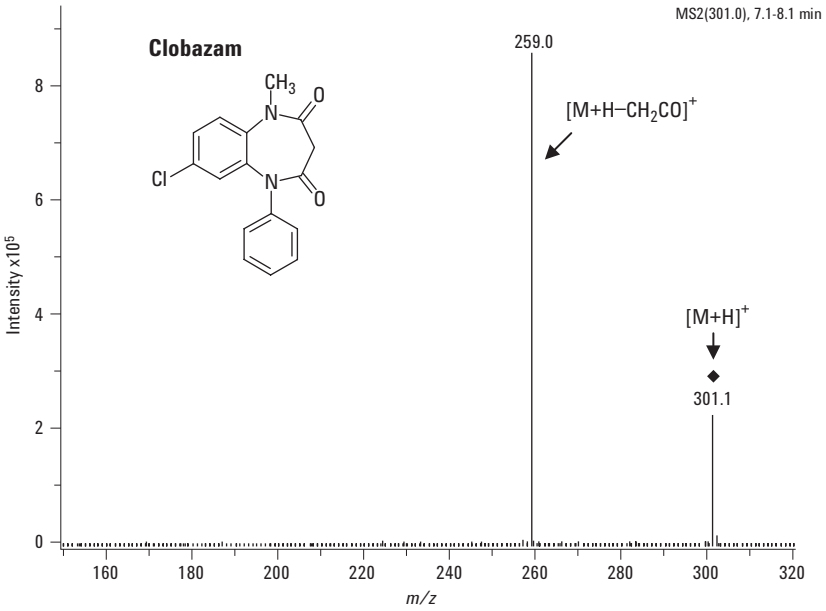

Figure 7. Structure and MS/MS spectrum for clobazam.

Figure 8 shows the MS/MS spectra of the remaining benzodiazepines which are obtained using an important feature of the Agilent LC/MSD Trap. On initial examination of MS/MS spectra during method optimization, lorazepam, oxazepam, and temazepam were found to have the major product ion to be the result of loss of the elements of water. As this is not the most specific loss one might prefer for identification purposes, a more information-rich MS/MS spectrum was obtained for each using the following technique. By increasing the fragmentation window from 10 amu to 40 amu (±20 amu centered on the precursor ion mass), fragmentation energy is applied to both the $[M+H]^+$ and the $[M+H–H_2O]^+$ ions, and the resulting MS/MS spectra are much more specific for identification.

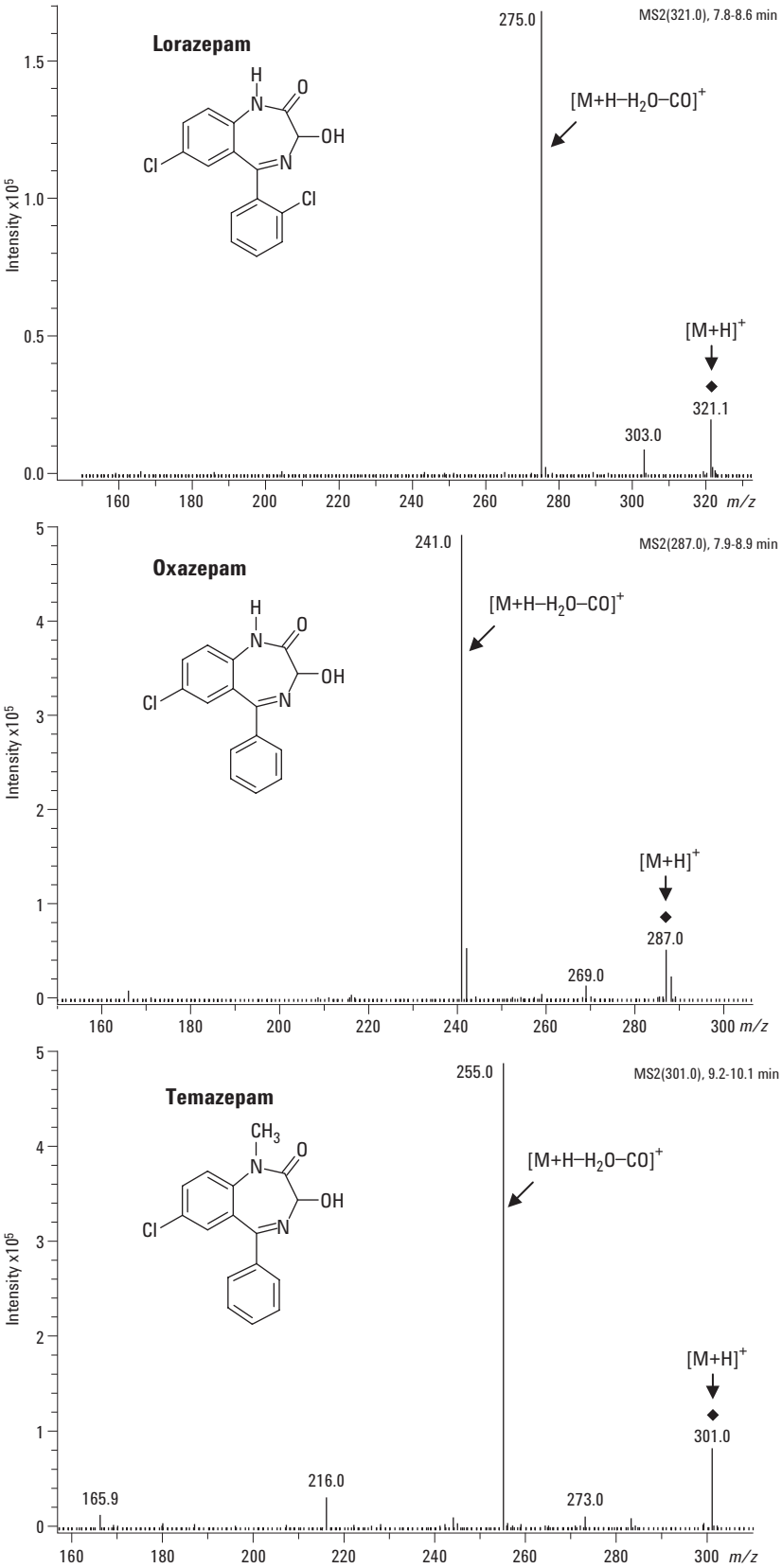

Figure 8. Structures and MS/MS spectra for lorazepam, oxazepam, and temazepam.

For example, with temazepam, fragmentation can result in the water-loss ion at *m/z* 283.0 and the water-loss plus CO loss ion at *m/z* 255.0. If the fragmentation energy is applied not only to the pseudomolecular ion at *m/z* 301.0, but also to the water-loss ion by using a fragmentation window of 40 amu, the intensity of the *m/z* 255.0 is improved, resulting in better detection and identification.

Application to Forensic Cases

Blood extracts from a wide variety of case types have been analyzed by this LC/MS/MS procedure. A number of benzodiazepines have been identified using this screening method, and are subsequently quantified by GC/ECD or HPLC-UV. A selection of the cases and their blood drug concentrations are shown in Table 4.

The ion chromatograms from the blood sample in Case 1, which provide the detection of nitrazepam, 7-aminonitrazepam, diazepam, nordiazepam and prazepam, are shown in Figure 9.

Table 4. Case Examples of Drugs and their Concentrations in Blood

Case	Benzodiazepine/ metabolite	Concentration mg/L
1	Nitrazepam	0.01
	7-aminonitrazepam	0.08
	Diazepam	0.33
	Nordiazepam	0.32
2	Diazepam	0.06
	Clonazepam	0.009
	7-aminoclonazepam	0.02
3	Bromazepam	0.40
4	Alprazolam	0.006
	Diazepam	0.05
	Nordiazepam	0.01
5	Diazepam	0.58
	Nordiazepam	0.66
	Oxazepam	0.04
	Temazepam	0.12
6	Clobazam	0.10

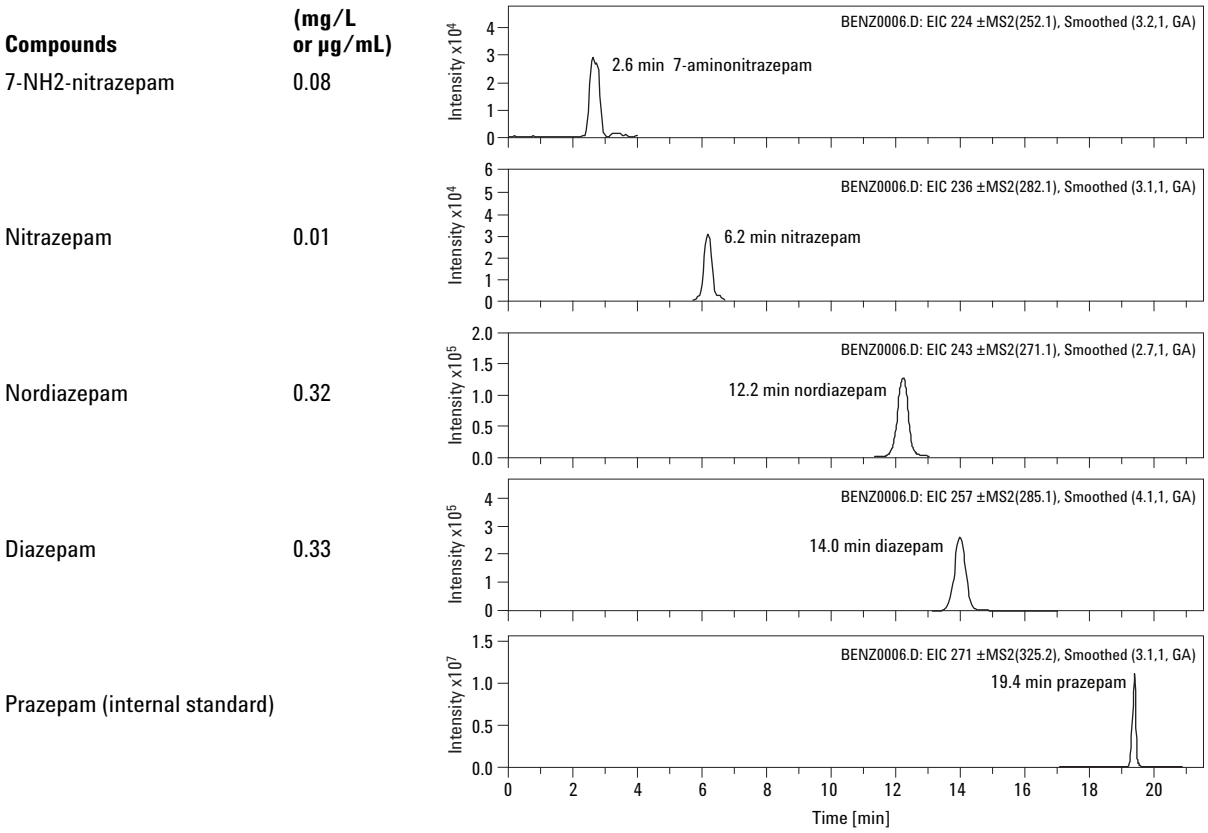

Figure 9. Ion chromatograms from Case 1 example.

Polypharmacy in such cases is not uncommon, and the method can easily detect, confirm and quantify multiple benzodiazepines and metabolites in a single analysis, as illustrated by this case.

Typical ion chromatograms from a blank blood sample are shown in Figure 10. Note that in Case 4 (see Table 4) it was possible to detect 0.006 mg/L (6 ng/mL) of alprazolam while still obtaining a clear identification with a full-scan MS/MS spectrum. Case 4 contains the lowest level of benzodiazepine detected in these cases, that of alprazolam at a level of 6 µg/L (6 ng/mL).

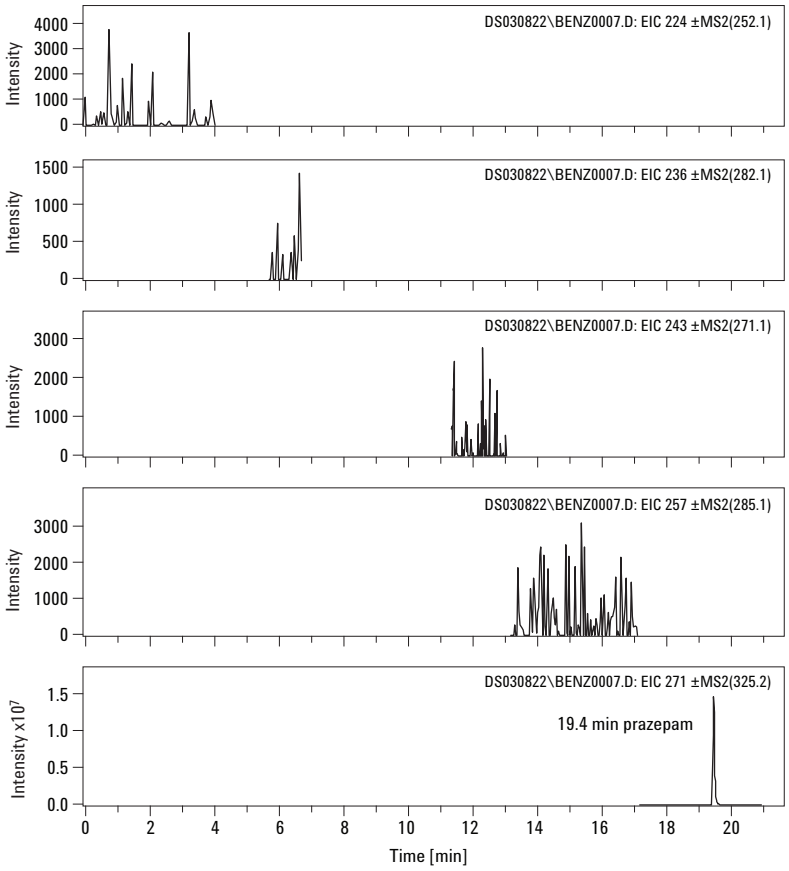

Figure 10. Ion chromatograms from blank blood with internal standard.

Conclusions

The LC/MS/MS method described here provides a single procedure for the identification of a wide range of benzodiazepines available for medical use in Australia and their metabolites, with a simple adaptation of an existing GC/MS sample preparation procedure, and without the need for derivatization. The MS/MS spectra provide a high-confidence identification of the drugs. The technique is suitable for screening analyses and confirmation of identity of the benzodiazepines at their lowest reported therapeutic concentrations using only 500 µL of blood. The data in Table 4 and Figure 9 illustrate that the procedure is able to identify concentrations of benzodiazepines in casework samples. Low concentrations of various benzodiazepines have been rapidly and successfully identified in forensic cases.

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Acknowledgements

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Agilent Technologies

Quantitative Analysis of Ethylglucuronide in Urine Using the Agilent 1200 RRLC and 6410 Triple Quadrupole Mass Spectrometer

Application Note

Forensic Toxicology

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Abstract

An Agilent 1200 Series Rapid Resolution Liquid Chromatography (RRLC) system is interfaced to a 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of a G1948B Electrospray Ionization Source (ESI), operated in negative ion mode, to confirm the presence of ethylglucuronide (EtG), a chemical biomarker for detecting recent alcohol use. The calibration range used in this work is 250 ng / mL to 2,500 ng / mL of EtG in urine. With 10-µL injections on a ZORBAX Eclipse XDB-C18 column, 3 × 250 mm (5-µm particle size) at an isocratic flow rate of 800 µL / min, excellent reproducibility and linearity is demonstrated. A retention time for EtG of 2.2 minutes makes this analysis a fast quantitation method.

Introduction

Ethylglucuronide (EtG) is a minor metabolite of ethanol that can be used as a direct biomarker of recent alcohol use. EtG is formed in the liver via glucuronidation, less than 0.1% of an ingested dose of ethanol is converted to EtG. EtG may be detected in urine up to 72 to 96 hours following ethanol ingestion which is considerably longer than the12- to 14- hour detection window of ethanol in urine. In addition to the narrow window of detection, another disadvantage of using urinary ethanol is the formation of ethanol via fermentation. This is a potential problem in monitoring alcohol use in individuals with diabetes. EtG is not formed via fermentation; it is found in urine only after hepatic exposure to alcohol. Concentrations of EtG in urine samples collected from humans range from undetectable in nonalcohol users to levels in excess of one million ng/mL in chronic users.

Many zero tolerance alcohol treatment programs use 100 ng/mL as a positive cut-off value as a marker of alcohol consumption. One problem with the 100 ng/mL positive cut-off results from incidental exposure to alcohol via over-the-counter pharmaceutical agents, alcohol-containing mouthwashes, hand sanitizers, food products, cosmetics, etc., which may result in EtG levels in excess of 100 ng/mL. To obviate this situation, many labora-

tories and treatment programs elect to use higher positive cut-off values of either 250 or 500 ng/mL. This eliminates positive EtG results from incidental exposure to alcohol but also decreases the detection window of alcohol consumption. STERLING Reference Laboratories utilizes a 250 ng/mL cut-off value for most programs.

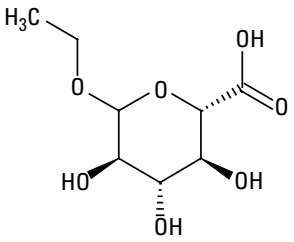
A further increase in specificity comes with the use of a tandem MS/MS mass spectrometer for analysis. The QQQ provides this capability by selecting the EtG precursor ion and generating product ions that are specific to its structure. The more intense product ion is then used for quantitation while the less intense ion is used as a qualifier for confirming the presence of EtG by maintaining a particular ion ratio with the quantitation ion throughout the batch of calibration standards, quality controls (QCs), and samples. To account for the effects of sample extraction recovery and matrix effects, an internal standard is added and analyzed with analogous requirements for confirmation.

EtG is water soluble and stable, but thermally labile, making it a difficult molecule to analyze by GC/MS without derivatization. It is also a carboxylic acid and particularly amenable to electro-spray ionization, forming a de-protonated ion in solution.

In this work the analytical range for EtG is 250 to 2,500 ng/mL. Ten urine samples are analyzed for the presence of EtG, and three QCs at 211, 383, and 1,594 ng/mL are included. As more than adequate sensitivity for this analysis is available using the Agilent 6410 QQQ mass spectrometer, 10-fold dilutions in water (0.1 % formic acid) are made to reduce column contamination. It should be noted that there is still adequate sensitivity to allow for a 20- to 25-fold dilution.

Based on the derived calibration curve, the quantitative accuracies of these controls are 94, 94, and 98%, respectively. Furthermore, of the 10 samples analyzed, seven are determined to be positive, or having levels above the 250 ng/mL cutoff.

The structure of EtG is shown in Figure 1.



EtG
$[M–H]^-$ = 221.1

Figure 1. Structure of ethylglucuronide and associated deprotonated *m/z*.

Experimental

Sample Preparation

An EtG standard and its deuterated analog (D5) are obtained from Sterling Reference Labs (SRL) at concentrations of 10 and 0.1 mg/mL in methanol, respectively. Dilutions of the standard are made up in water with 0.1% formic acid (v/v). The resulting concentrations of the calibration level standards are 250, 1,000, and 2,500 ng/mL. Unfiltered control urine samples and quality controls (QCs) are also obtained from SRL. The three QC samples are known to be 211, 383, and 1,594 ng/mL. The level of the internal standard in all samples is 500 ng/mL. To 50 µL of each dilution standard, QC, and sample is added 450 µL of the D5 internal standard.

LC / MS Method Details

LC Conditions

Agilent 1200 Series binary pump, degasser, thermostatted wellplate sampler, and thermostatted column compartment

Sample temperature:	10 °C
Needle wash:	(50:50 methanol/water) – flush port 3 seconds
Column:	Agilent ZORBAX XDB-C18, 3.0 mm × 250 mm, 5 µm (p/n: 990967-302)
Column temperature:	45 °C
Mobile phase (isocratic):	90:10 of 0.25% formic acid in water/methanol
Flow rate:	0.8 mL/min;
Injection volume:	10 µL
Stop time:	4 min

MS Conditions

Mode:	Negative ESI using the Agilent G1948B ionization source
Nebulizer:	60 psig
Drying gas flow:	13 L/min
Drying gas temperature:	350 °C
V_{cap}:	3,500 V

The MRM transitions with settings for optimal sensitivity are given in Table 1.

Table 1. MRM Transitions Acquired Using the 6410 QQQ Mass Spectrometer

Compound	Transition	Fragmentor (V)	Collision energy (V)	Dwell time (msec)
EtG – quantifier	221.0 > 85.0	140	12	200
EtG – qualifier	221.0 > 75.0			
D5 – EtG (IStd) – quantifier	226.0 > 85.0			
D5 – EtG (IStd) – qualifier	226.0 > 75.0			

Resolution (FWHM): Q1 = 0.7 amu Q2 = 0.7 amu

In addition, a blank is used throughout the analysis to show that there is no carryover. The blank is prepared by mixing 50 µL of water with 0.1% formic acid with 450 µL of the internal standard.

Diluting the samples and QCs 10-fold using the internal standard reduces the amount of system contamination of the unfiltered urine matrix. In addition, because injecting unfiltered urine can cause column degradation, it is recommended by SRL to wash the column with 100% organic at least once per day.

Results and Discussion

The resulting calibration curve for this work is shown in Figure 2. An excellent correlation coefficient of $R^2 > 0.999$ is derived with conservative data fit settings of linear type, ignored origin, and no weighting. The quantitative accuracies of the three QCs are 94% (211 ng/mL) 94% (383 ng/mL) and 98% (1,594 ng/mL). Based on an injection volume of 10 µL and previously stated dilution in mobile phase 1:10, the on-column injection amount corresponding to the analytical range is 250 pg (250 ng/mL) to 2.5 ng (2,500 ng/mL). No saturation or nonlinearity is observed.

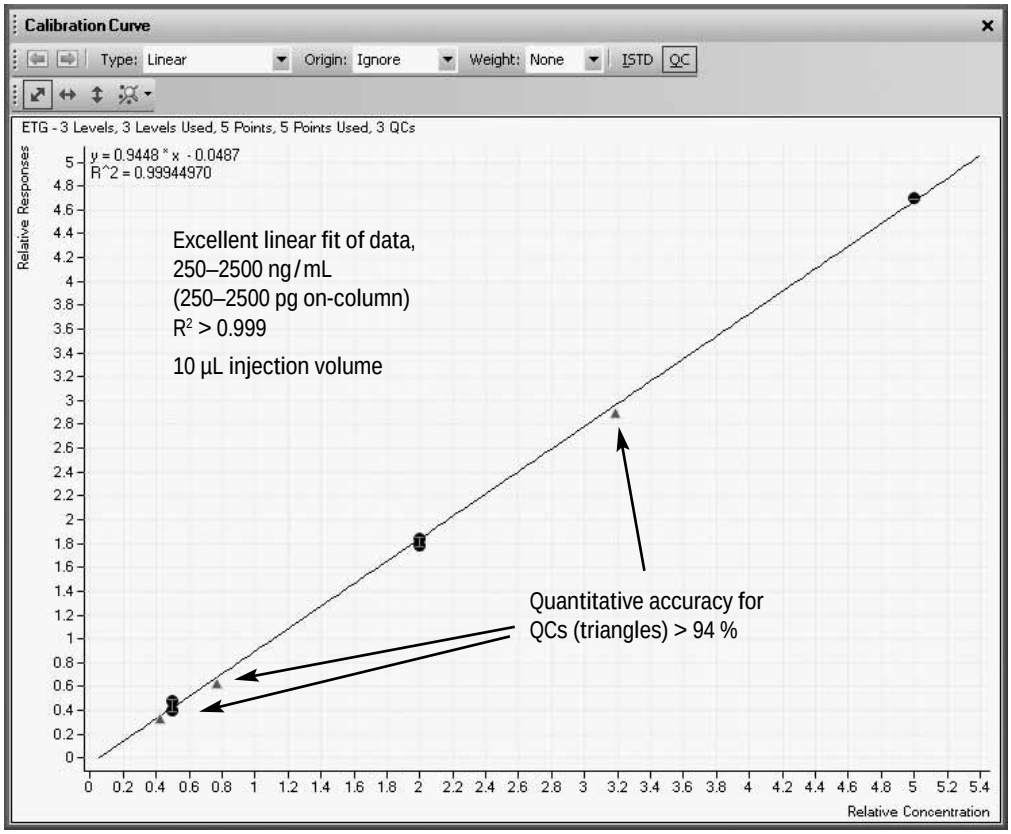


Figure 2. Excellent linearity over the 250 to 2,500 ng/mL analytical range. Curve fit settings of linear type, ignore origin and no weighting used. Based on dilution by internal standard and 10-µL injection volume, the corresponding on-column injection range is 250 to 2,500 pg.

For confirmation, a qualifier peak area ion ratio is derived for both the EtG analyte and the D5-EtG internal standard using one of the calibration level standards. This ratio is then applied to all samples with an acceptance tolerance of ± 20%. For example, since the derived qualifier/quantifier ion ratio for the EtG analyte is 84%, all qualifier/quantifier ion ratios must be within ± 20% of 84%, or an area ratio range of 67 to 101%. This is likewise applied to the internal standard. All samples quantitated within the calibration range satisfy this criteria. See Figure 3.

The integration results are tabulated for all samples in Table 2. Note that urine samples 1, 4, and 8 ("Sample1," "Sample2," and "Sample8") are considered negative because their calculated concentrations all fall below the quantitation curve lower limit of 250 ng/mL. The other seven samples either fall within the quantitation range of 250 to 2,500 ng/mL

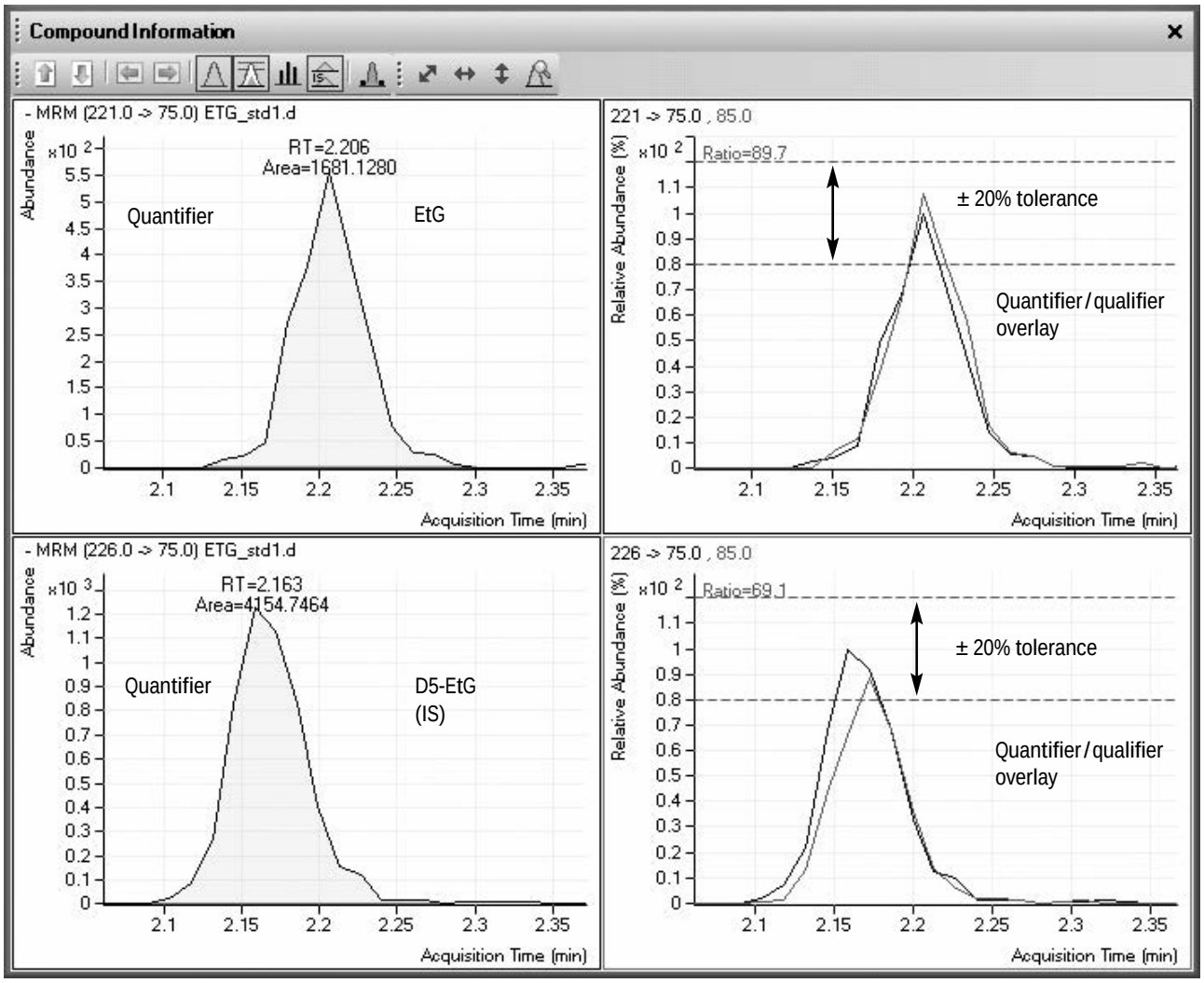


Figure 3. Confirming presence of EtG in sample based on qualifier ion ratio shown here for one of the 250 ng / mL calibration standard injections. Normalized overlay of qualifier and quantifier ions, based on area counts, shown on the right.

or are very positive and could be diluted and re-analyzed. Note also that all of the samples that quantitate above 250 ng/mL, including the QC1, satisfy the qualifier ion ratio of 0.84 ± 20% for both the analyte EtG and the D5-EtG internal standard.

As previously mentioned, the accuracies of quantitation for the quality control samples are 94% for both QC1 and QC2 and 98% for QC3.

Finally, the injection of blanks, which contain internal standards (IStds) only, are included to demonstrate that there is no significant carryover in this analysis.

Conclusions

The EtG compound quantitates very well in negative electrospray ionization mode. Excellent linearity over the analytical range is demonstrated with a correlation coefficient of linearity of $R^2 > 0.999$. The data is conservatively fit using a linear type, no inclusion of the origin, and no weighting. The QC samples have very good quantitative accuracies of at least 94% and seven of the 10 urine samples are confirmed as positive. All samples quantitated above the lower limit of 250 ng/mL satisfy the qualifier ion ratio criteria for both the EtG analyte and the D5-EtG internal standard. This work represents a good example of the ability of the QQQ to provide quantitation and confirm the presence of EtG in urine based on the specificity of tandem MS/MS.

Table 2. Integration Results for All Samples

			EtG				D5-EtG		
Type	**Level**	**Exp. conc.**	**RT**	**Calc. conc.**	**Accuracy**	**Ratio**	**RT**	**Resp.**	**Ratio**
Cal - 250	1	250	2.2	240	96.0	89.7	2.2	4154.7	69
Cal - 250	1	250	2.2	280	111.8	81.0	2.2	3914.7	82
Cal - 1000	2	1000	2.2	972	97.2	81.9	2.2	3325.7	79
Cal - 1000	2	1000	2.2	999	99.9	87.2	2.2	4032.8	83
Cal - 2500	3	2500	2.2	2510	100.4	81.2	2.2	4409.6	83
Blank			2.2	29*			2.2	4509.8	79
QC1 - 211	4	211	2.2	199	94.4	84.2	2.2	4416.0	79
QC2 - 383	5	383	2.2	358	93.5	82.7	2.2	4457.6	80
QC3 - 1594	6	1594	2.2	1559	97.8	90.3	2.2	4223.3	80
Blank				Not found			2.2	3983.9	88
Sample1			2.3	183		22.7	2.2	3809.3	77
Sample2			2.2	27865		82.9	2.2	3594.1	105
Sample3			2.2	81139		84.6	2.2	4089.2	82
Sample4			2.2	105		45.6	2.2	3491.7	85
Sample5			2.2	783269		84.7	2.2	1776.3	97
Sample6			2.2	5904		78.2	2.2	3253.2	85
Sample7			2.2	256		86.2	2.2	4876.2	80
Sample8			2.3	142		37.0	2.2	3890.6	88
Sample9			2.2	1428		85.1	2.2	4543.0	84
Sample10			2.2	370		67.5	2.2	2896.0	85

* Approximately 1% carryover.

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Determination of Benzodiazepines in Urine and Blood Using Rapid Resolution Liquid Chromatography/Triple Quadrupole Mass Spectrometry

Application Note

Forensic Toxicology

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of 14 benzodiazepines and six metabolites in urine and blood, using the Agilent 6410 Triple Quadrupole Mass Spectrometer in electrospray mode, is described. For the urine samples, preparation included treatment with β-glucuronidase in authentic samples. For the blood samples, preparation included precipitation of the red blood cells with acetonitrile followed by solid phase extraction, evaporation of the final eluent to dryness, and reconstitution in mobile phase for injection into the LC/MS/MS system.

To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion, which is required to be present within a specific ratio to the primary ion for acceptable identification. The unique features of the Agilent software allow the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis

Introduction

Benzodiazepines are the most commonly prescribed class of drugs in the USA [1]. They are commonly detected in incidents of sexual assault, driving under the influ-ence of drugs (DUID), and often in combination with other medications [2,3]. Hegstad et al. pub-lished a procedure using LC/MS/MS for the detec-tion of some benzodiazepines in urine, including 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, alpha-hydroxyalprazolam, oxazepam, 3-OH-diazepam, and nordiazepam [4]. Following a single dose of lorazepam (2.5 mg), Kintz et al. were able to detect greater than 5 ng/mL of lorazepam in urine for up to 96 hours [5]. After the administration of a single oral dose of bromazepam (6 mg) and clonazepam (2 mg), Cheze et al. reported the bromazepam concentration above 5 ng/mL for 60 hours; 7-aminoclonazepam was detectable for at least 144 hours [6].

Blood is generally collected following traffic safety incidents, and it is also the main biological specimen analyzed following autopsy. However, the detection of benzodiazepines, particularly in blood, is not without difficulty, since the concentrations present, especially following therapeutic use, can be low. Several publications have addressed the issue of their analysis in plasma or serum; however, few have attempted the detection in whole blood. Gunnar et al. [7] determined several benzodiazepines in whole blood using extraction, derivatization, and GC/MS analysis.

An excellent publication by Laloup et al. reported the screening of urine, blood, and hair using tandem LC mass spectrometry for 26 benzodiazepines and metabolites [8]. While the authors list a primary transition and a qualifying ion for each drug, the authors noted that a second injection was required for further confirmation of positive samples monitoring two transitions per compound. Using the Agilent system, the monitoring of the qualifying ion and calculation of its ratio to the intensity of the primary transition is an integral part of the software package.

Experimental

Sample Preparation

Standards and Reagents

- Deuterated internal standards: D5-diazepam; D5-temazepam; D5-alprazolam D7-7-aminoflunitrazepam, D4-clonazepam, as well as unlabeled drug standards: 7-aminoflunitrazepam; 7-aminoclonazepam; 7-aminonitrazepam; α-OH-alprazolam; α-OH-triazolam; desalkylflurazepam, bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam, oxazepam, nordiazepam, temazepam were purchased from Cerilliant (Round Rock, TX).
- Mixed-mode solid-phase extraction columns (Clin II) were purchased from SPEWare (San Pedro, CA).
- All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Standards (prepared in methanol)

- Internal standard mix: D7-7-aminoflunitrazepam; D5-alprazolam; D4-clonazepam; D5-temazepam; D5-oxazepam; D5-diazepam (1,000 ng/mL)
- Unlabeled drugs: 7-aminoflunitrazepam; 7-aminoclonazepam; 7-aminonitrazepam; α-OH-alprazolam; α-OH-triazolam; desalkylflurazepam; bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam, oxazepam, nordiazepam, temazepam

Extraction Procedure–Urine

Deuterated internal standard (100 µL) was added to urine (1 mL) and mixed.

Calibration Curve:

Negative:	100 µL of deuterated stock solution (1,000 ng/mL)
10 ng/mL:	100 µL of deuterated stock solution (1,000 ng/mL) 10 µL of 1,000 ng/mL stock solution
25 ng/mL:	100 µL of deuterated stock solution (1,000 ng/mL) 25 µL of 1,000 ng/mL stock solution
50 ng/mL:	100 µL of deuterated stock solution (1,000 ng/mL) 50 µL of 1,000 ng/mL stock solution
100 ng/mL:	100 µL of deuterated stock solution (1,000 ng/mL) 100 µL of 1,000 ng/mL stock solution

A 2 M sodium acetate buffer (pH 5.0; 0.1 mL) was added, and for authentic specimens, α-glucuronidase (50 µL) was also added. The mixture was heated for 3 hours at 45 °C. Following centrifugation (10 min; 2,500 rpm), 0.1 M sodium phosphate buffer (pH 6.0, 1 mL) was added to the decanted upper layer supernatant. Extraction tubes were placed onto the vacuum manifold and conditioned with methanol (3 mL), deionized water (3 mL), and 0.1 M phosphate buffer (pH 6.0, 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL), 0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry. Hexane was allowed to flow through the column (1 mL). Finally, the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi/37 °C) and reconstituted in methanol[1] (50 µL) for analysis.

[1]Since this work was completed it was found that reconstituting in water worked even more consistently than methanol.

Extraction Procedure–Blood

Acetonitrile (1 mL) was added to whole blood (1 mL). A mix of deuterated internal standards (100 µL; 50 ng/mL) was added and the sample was mixed, then centrifuged (20 min; 2,500 rpm). The supernatant was decanted and 0.025 M sodium phosphate buffer (pH 2.7; 1.5 mL) was added.

Calibration Curve:

Negative:	50 µL of deuterated stock solution (1,000 ng/mL)
5 ng/mL:	50 µL of deuterated stock solution (1,000 ng/mL) 50 µL of 100 ng/mL stock solution
10 ng/mL:	50 µL of deuterated stock solution (1,000 ng/mL) 10 µL of 1,000 ng/mL stock solution
25 ng/mL:	50 µL of deuterated stock solution (1,000 ng/mL) 25 µL of 1,000 ng/mL stock solution
50 ng/mL:	50 µL of deuterated stock solution (1,000 ng/mL) 50 µL of 1,000 ng/mL stock solution
100 ng/mL:	50 µL of deuterated stock solution (1,000 ng/mL) 100 µL of 100 ng/mL stock solution

Extraction tubes were placed onto the vacuum manifold and conditioned with methanol (3 mL), deionized water (3 mL), and 0.1 M phosphate buffer (pH 6.0; 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL), 0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry. Hexane was allowed to flow through the column (1 mL). Finally, the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi / 37 °C) and reconstituted in methanol[2] (50 µL) for analysis.

[2]Since this work was completed it was found that reconstituting in water worked even more consistently than methanol.

Analytical Procedure–Both Urine and Blood

The 7-amino metabolites of flunitrazepam, nitrazepam, and clonazepam eluted from the analytical column rapidly, even though the flow rate was 0.2 mL/min. Optimization of the gradient and flow rate were attempted but did not give acceptable chromatography for the three metabolites. Subsequently, a separate method was implemented, lasting only 3.5 min and monitoring only those three metabolites. The chromatography and sensitivity were greatly improved by separating the two methods.

Both assays employed the Agilent 6410 LC Triple Quadrupole Mass Spectrometer (LC/MS/MS) incorporating an Agilent 1200 Series LC pump; ZORBAX Eclipse XDB C18 4.6 × 50 mm × 1.8-µm analytical column (Agilent PN: 922975-902); and an injection volume of 5 µL. Although the author (CM) obtained good results using the 4.6 mm i.d. column, the 2.1 mm i.d. column with 1.8 um-particle size is normally recommended by Agilent for increased sensitivity at the flow rates used.

The mass spectrometric parameters are shown in Table 1, qualifier ions in parentheses.

Benzodiazepines (except 7-amino metabolites)**:**

Column temperature:	35 °C	
Solvent flow rate:	0.2 mL/min	
Mobile phase:	A = 20 mM ammonium formate (pH = 8.6) B = acetonitrile	
Isocratic:	50% B	
	Time (minutes)	Flow rate (mL/min)
	0	0.2
	6.5	0.2
	8	1
	10	0.2
Post time:	4.5 min	

7-Amino Metabolites Only:

Column temperature:	45 °C
Solvent flow rate:	0.6 mL/min
Mobile phase:	A = 20 mM ammonium formate (pH = 8.6) B = acetonitrile
Isocratic:	35% B
Stop time:	3.5 min

Mass Spectrometer Conditions:

Operation:	Electrospray positive mode	
	7-Amino metabolites	Other benzodiazepines
Gas temperature:	350 °C	300 °C
Gas flow (N_2):	6 L/min	6 L/min
Nebulizer pressure:	20 psi	15* psi
Capillary voltage:	4000 V	4500 V

* At LC flow rates of 0.6 mL/min, nebulizer pressure settings as high as 50 psi are recommended for stable ion spray.

Table 1a. Acquisition Parameters: 7-Amino Metabolites

Compound	Start time (min)	Precursor ion	Product ion	Fragment voltage (V)	CE (V)
Segment 1					
D7-7-Aminoflunitrazepam	0	291	263	120	25
7-Aminoclonazepam	0	286	222 (121)	200	25 (25)
7-Aminonitrazepam	0	252	121 (208)	120	30 (35)
7-Aminoflunitrazepam	0	284	226 (256)	160	30 (25)

Table 1b. Acquisition Parameters: Benzodiazepines

Compound	Start time (min)	Precursor ion	Product ion	Fragment voltage (V)	CE (V)
Segment 1					
Bromazepam	0	316	288 (209)	160	20 (30)
Segment 2					
D4-Clonazepam	4.1	320	274	120	25
Clonazepam	4.1	316	270 (214)	120	25 (35)
α-Hydroxyalprazolam	4.1	325	297 (216)	120	30 (35)
α-Hydroxytriazolam	4.1	359	331 (176)	120	25 (25)
Lorazepam	4.1	321	275 (229)	140	25 (35)
Nitrazepam	4.1	282	236 (180)	160	25 (35)
D5-Alprazolam	4.1	314	286	160	25
Alprazolam	4.1	309	281 (274)	160	25 (30)
Chlordiazepoxide	4.1	300	283 (227)	120	15 (30)
D5-Oxazepam	4.1	292	246	120	20
Oxazepam	4.1	287	241 (269)	120	20 (20)
Triazolam	4.1	343	308 (239)	120	35 (35)
Segment 3					
Flunitrazepam	5.4	314	268 (239)	160	30 (35)
Midazolam	5.4	326	291 (249)	200	30 (40)
D5-Temazepam	5.4	306	260	120	25
Temazepam	5.4	301	255 (177)	120	35 (40)
Desalkylflurazepam	5.4	289	226 (261)	160	30 (25)
Nordiazepam	5.4	271	140 (165)	160	30 (30)
Segment 4					
5-Diazepam	7.2	290	262	160	25
Diazepam	7.2	285	257 (222)	160	25 (25)
Flurazepam	7.2	388	315 (288)	160	25 (25)

* ( ) qualifier ions; qualifier ratios must be within 20% of calibration point

LC/MS/MS Method Evaluation

The analytical method was evaluated according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates (n = 5) over a period of 5 days. The slope of the calibration curve was not forced through the origin. The equation of the calibration curves and correlation coefficients (R^2) are shown in Tables 2a (urine) and 2b (blood); the inter-day precision and accuracy of the assay are shown in Tables 3a and 3b, respectively. In addition, the intra-day precision and accuracy of the assay are shown in Tables 4a and 4b, respectively. The assay was robust, precise, and accurate at the selected level of 25 ng/mL and was linear over the range 5 to 100 ng/mL. The precision for all drugs was less than 20% both intra-day and inter-day, with most benzodiazepines showing a variation of less than 10%. One exception was 7-amnionitrazepam in urine, which showed a 24.4% variation over five

replicates. The limit of quantitation for all drugs was 5 ng/mL. Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays.

Figure 1a shows a typical calibration curve for lorazepam in urine ($R^2 > 0.998$). Figure 1b shows a typical calibration curve for midazolam, with a correlation coefficient greater than 0.999.

Table 2a. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Benzodiazepines in Urine

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-Aminoflunitrazepam	Y = 0.0210x – 0.0481	0.9985	69.4 (55.4–83.2)
7-Aminonitrazepam	Y = 0.5293x – 0.2512	0.9990	8.6 (6.9–10.3)
7-Aminoclonazepam	Y = 0.0523x – 0.1647	0.9959	84.5 (67.6–101.4)
α-Hydroxyalprazolam	Y = 0.0019x – 0.0053	0.9997	40.4 (32.3–48.5)
α-Hydroxytriazolam	Y = 0.000971x – 0.0024	0.9996	92 (73.6–110.45)
Alprazolam	Y = 0.0117x + 0.00063	0.9998	15.8 (12.6–18.9)
Bromazepam	Y = 0.0035x + 0.0095	0.9948	59.4 (47.5–71.25)
Chlordiazepoxide	Y = 0.0064x + 0.0284	0.9982	80.2 (64.1–96.2)
Clonazepam	Y = 0.0121x – 0.0342	0.9997	24.5 (19.5–29.3)
Desalkylflurazepam	Y = 0.0027x + 0.023	0.9986	26.7 (21.3–32)
Diazepam	Y = 0.0116x +0.0166	0.9996	82.5 (66–99)
Flunitrazepam	Y = 0.0025x – 0.000311	0.9994	49.4 (39.5–59.2)
Flurazepam	Y = 0.1291x + 0.2849	0.9993	13.6 (10.8–16.3)
Lorazepam	Y = 0.0104x – 0.0457	0.9981	34.2 (27.3–41)
Midazolam	Y = 0.0117x + 0.0149	0.9997	31.4 (25–37.6)
Nitrazepam	Y = 0.015x + 0.0176	0.9948	20 (34.9–52.3)
Nordiazepam	Y = 0.0032x + 0.0139	0.9998	65.8 (52.6–78.9)
Oxazepam	Y = 0.0079x – 0.0123	0.9999	24.3 (19.4–29.1)
Temazepam	Y = 0.0062x + 0.0011	0.9998	31 (24.8–37.2)
Triazolam	Y = 0.0076x + 0.0522	0.9983	92.1 (73.7–110.5)

Table 2b. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Benzodiazepines in Blood

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-Aminoflunitrazepam	Y = 0.0199x – 0.0196	0.9997	73.3 (58.6–88)
7-Aminonitrazepam	Y = 0.525x – 0.2845	0.9985	7.3 (5.8–8.7)
7-Aminoclonazepam	Y = 0.0403x – 0.0429	0.9996	97.8 (78.2–117.3)
α-Hydroxyalprazolam	Y = 0.001x – 0.0016	0.9989	41.0 (32.8–49.2)
α-Hydroxytriazolam	Y = 0.00033x + 0.00065	0.9985	90.4 (72.3–108.5)
Alprazolam	Y = 0.0124x – 0.0092	0.9999	15.0 (12–18)
Bromazepam	Y = 0.0029x – 0.0128	0.9940	59.2 (47.4–71.1)
Chlordiazepoxide	Y = 0.0136x + 0.0708	0.9833	78.9 (63.1–94.7)
Clonazepam	Y = 0.0113x – 0.0332	0.9980	25.2 (20.2–30.3)
Desalkylflurazepam	Y = 0.0029x + 0.0006	0.9996	26.6 (21.3–31.9)
Diazepam	Y = 0.0105x – 0.0197	0.9992	83.3 (66.6–100)
Flunitrazepam	Y = 0.00083x + 0.00084	0.9989	49.7 (39.8–59.7)
Flurazepam	Y = 0.1303x + 0.1446	0.9994	13.8 (11.0–16.6)
Lorazepam	Y = 0.0153x – 0.0538	0.9971	35.1 (28.1–42.2)
Midazolam	Y = 0.0142x – 0.0088	0.9986	31.8 (25.4–38.2)
Nitrazepam	Y = 0.0273x + 0.0974	0.9951	42.7 (34.2–51.3)
Nordiazepam	Y = 0.0048x + 0.0058	0.9980	65.5 (52.4–78.6)
Oxazepam	Y = 0.009x – 0.0136	0.9997	23.6 (18.9–28.4)
Temazepam	Y = 0.0063x – 0.0041	0.9999	30.6 (24.5–36.7)
Triazolam	Y = 0.0032x + 0.00091	0.9966	92.7 (74.2–111.3)

Table 3a. Inter-Day Precision and Accuracy (25 ng/mL Control Specimens; n = 5) for Benzodiazepines in Urine

Drug	Mean recovery (ng/mL)	SD	Precision (%)	Accuracy (%)
7-Aminoclonazepam	25.18	3.15	12.5	99.29
7-Aminoflunitrazepam	23.92	1.55	6.47	104.52
7-Aminonitrazepam	23.52	2.14	9.09	106.29
α-Hydroxyalprazolam	24.8	1.74	7.02	100.81
α-Hydroxytriazolam	24.94	2.21	8.85	100.24
Alprazolam	25.5	0.81	3.16	98.04
Bromazepam	27.1	1.63	6.02	92.25
Chlordiazepoxide	25.3	1.35	5.32	98.81
Clonazepam	24.86	0.84	3.37	100.56
Desalkylflurazepam	26.16	0.3	1.13	95.57
Diazepam	25.02	1.01	4.04	99.92
Flunitrazepam	25.2	0.31	1.22	99.21
Flurazepam	25.64	1.4	5.46	97.5
Lorazepam	23.8	1.85	7.76	105.04
Midazolam	25.58	0.98	3.83	97.73
Nitrazepam	26.84	1.11	4.15	93.14
Nordiazepam	26.26	0.65	2.46	95.2
Oxazepam	24.94	0.55	2.19	100.24
Temazepam	25.4	0.34	1.34	98.43
Triazolam	27.16	1.96	7.23	92.05

Table 3b. Inter-Day Precision and Accuracy (25 ng/mL Control Specimens; n = 5) for Benzodiazepines in Blood

Drug	Mean recovery (ng/mL)	SD	Precision (%)	Accuracy (%)
7-Aminoclonazepam	26.3	1.46	5.54	105.2
7-Aminoflunitrazepam	24.84	1.05	4.24	99.36
7-Aminonitrazepam	25.1	1.57	6.27	100.4
α-Hydroxyalprazolam	24.62	0.88	3.56	98.48
α-Hydroxytriazolam	25.7	1.39	5.41	102.8
Alprazolam	24.56	0.42	1.72	98.24
Bromazepam	26.14	2.9	11.1	104.56
Chlordiazepoxide	25.26	4.03	15.94	101.04
Clonazepam	24.32	0.85	3.51	97.28
Desalkylflurazepam	25.54	0.53	2.06	102.16
Diazepam	24.84	0.59	2.39	99.36
Flunitrazepam	24.82	1.49	5.99	99.28
Flurazepam	26	1.05	4.04	104
Lorazepam	24.82	0.53	2.12	99.28
Midazolam	24.72	1.41	5.7	98.88
Nitrazepam	28.32	2.73	9.65	113.28
Nordiazepam	25.86	0.62	2.41	103.44
Oxazepam	24.32	0.89	3.67	97.28
Temazepam	24.72	0.41	1.65	98.88
Triazolam	25.8	3.41	13.22	103.2

Table 4a. Intra-Day Precision (n = 5) for Benzodiazepines in Urine

Drug	Mean recovery (ng/mL)	SD	Precision (%)
7-Aminoclonazepam	27.36	2.84	10.4
7-Aminoflunitrazepam	24.74	0.57	2.31
7-Aminonitrazepam	28.26	6.9	24.4
α-Hydroxyalprazolam	23.9	2.74	11.47
α-Hydroxytriazolam	23.6	3.16	13.4
Alprazolam	26.26	0.74	2.83
Bromazepam	23.5	3.93	16.7
Chlordiazepoxide	23.2	1.49	6.42
Clonazepam	25.9	0.29	1.13
Desalkylflurazepam	26.2	1.06	4.03
Diazepam	25.78	0.82	3.18
Flunitrazepam	25.42	0.79	3.13
Flurazepam	26.88	1.09	4.05
Lorazepam	24.78	0.47	1.9
Midazolam	25.8	0.74	2.86
Nitrazepam	27.62	1.76	6.37
Nordiazepam	25.28	0.47	1.77
Oxazepam	25.28	0.92	3.64
Temazepam	25.42	0.36	1.43
Triazolam	27.24	2.2	8.09

Table 4b. Intra-Day Precision (n = 5) for Benzodiazepines in Blood

Drug	Mean recovery (ng/mL)	SD	Precision (%)
7-Aminoclonazepam	24.02	1.57	6.52
7-Aminoflunitrazepam	23.82	1.35	5.67
7-Aminonitrazepam	28.64	1.04	3.86
α-Hydroxyalprazolam	24.36	1.77	7.28
α-Hydroxytriazolam	24.66	3.35	13.57
Alprazolam	24.6	0.33	1.35
Bromazepam	27.38	4.24	15.5
Chlordiazepoxide	25.52	2.69	10.54
Clonazepam	23.84	0.34	1.41
Desalkylflurazepam	26.96	2.32	8.61
Diazepam	24.96	1.82	7.29
Flunitrazepam	24.54	4.37	17.8
Flurazepam	25.74	0.55	2.12
Lorazepam	17.66	2.38	13.48
Midazolam	23.74	1.53	6.43
Nitrazepam	30.52	2.88	9.45
Nordiazepam	27.28	2.76	10.1
Oxazepam	23.84	0.6	2.51
Temazepam	25.04	0.53	2.12
Triazolam	26.02	4.17	16.02

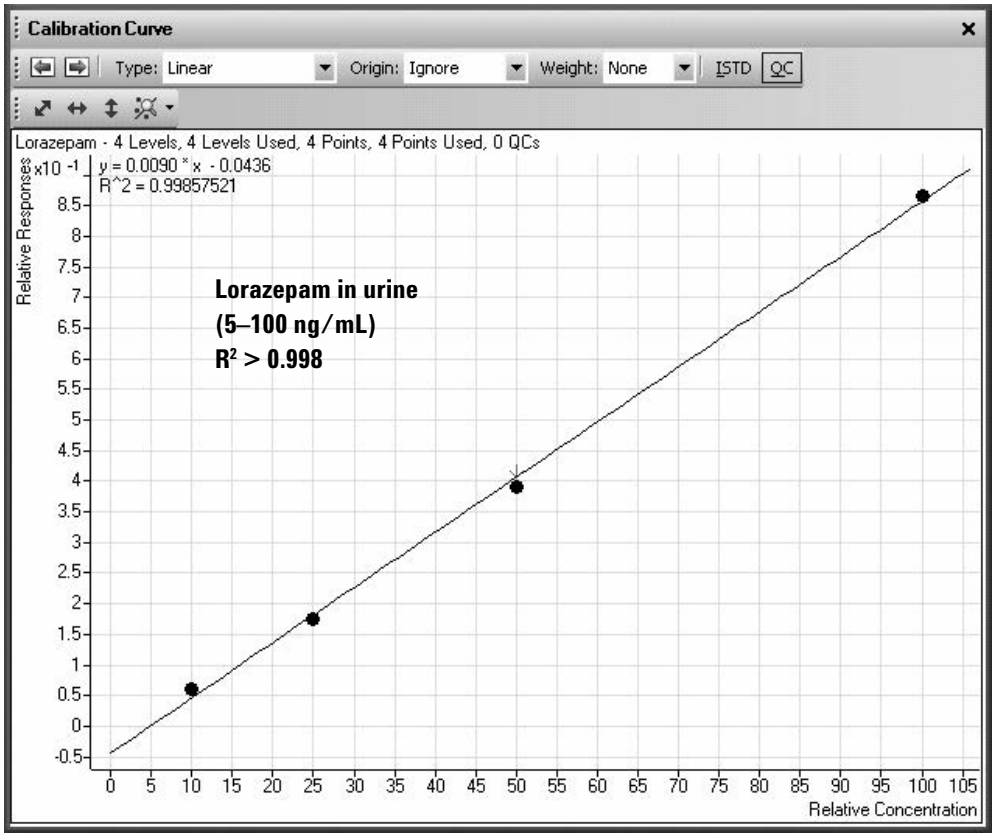

Figure 1a. Calibration curve for lorazepam in urine (5, 10, 25, 50, and 100 ng/mL).

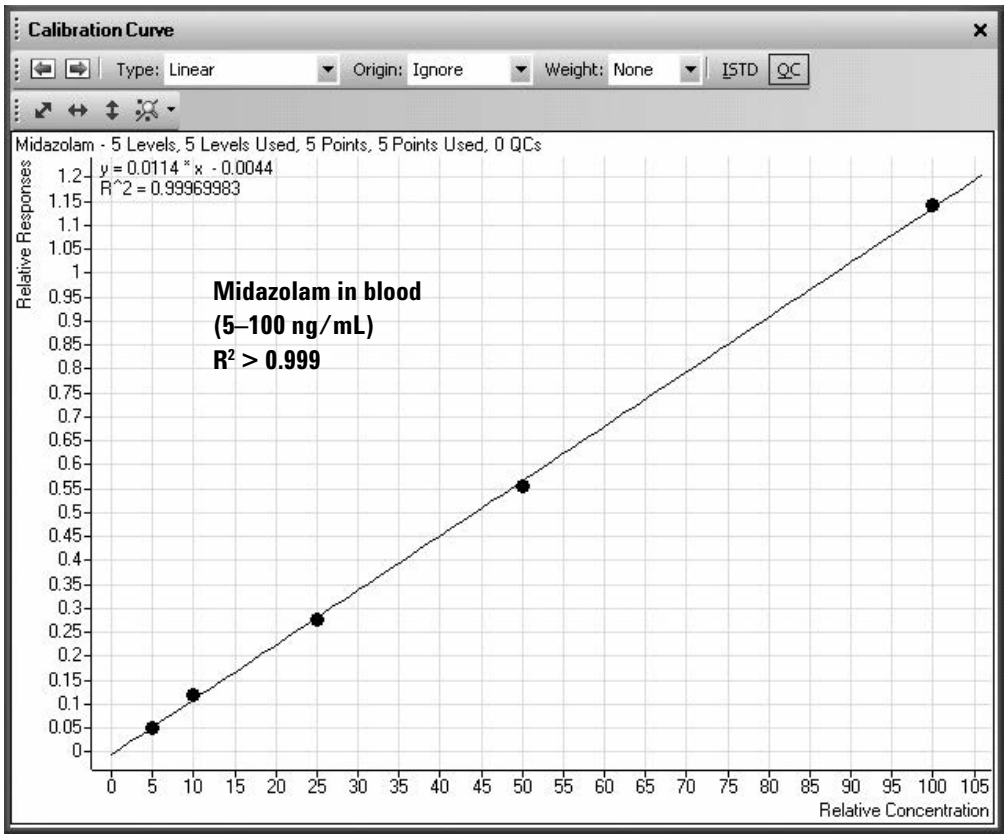

Figure 1b. Calibration curve for midazolam in blood (5, 10, 25, 50, and 100 ng/mL).

Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines and six metabolites in urine and blood. The chromatographic separation produced by the small-particle analytical column allowed separation of the peaks in each group segment (Figures 2a and 2b, respectively). The metabolites 7-aminonitrazepam, flunitrazepam, and clonazepam showed poor chromatography when analyzed on this LC program, so they were analyzed separately in a fast run (3.5 min).

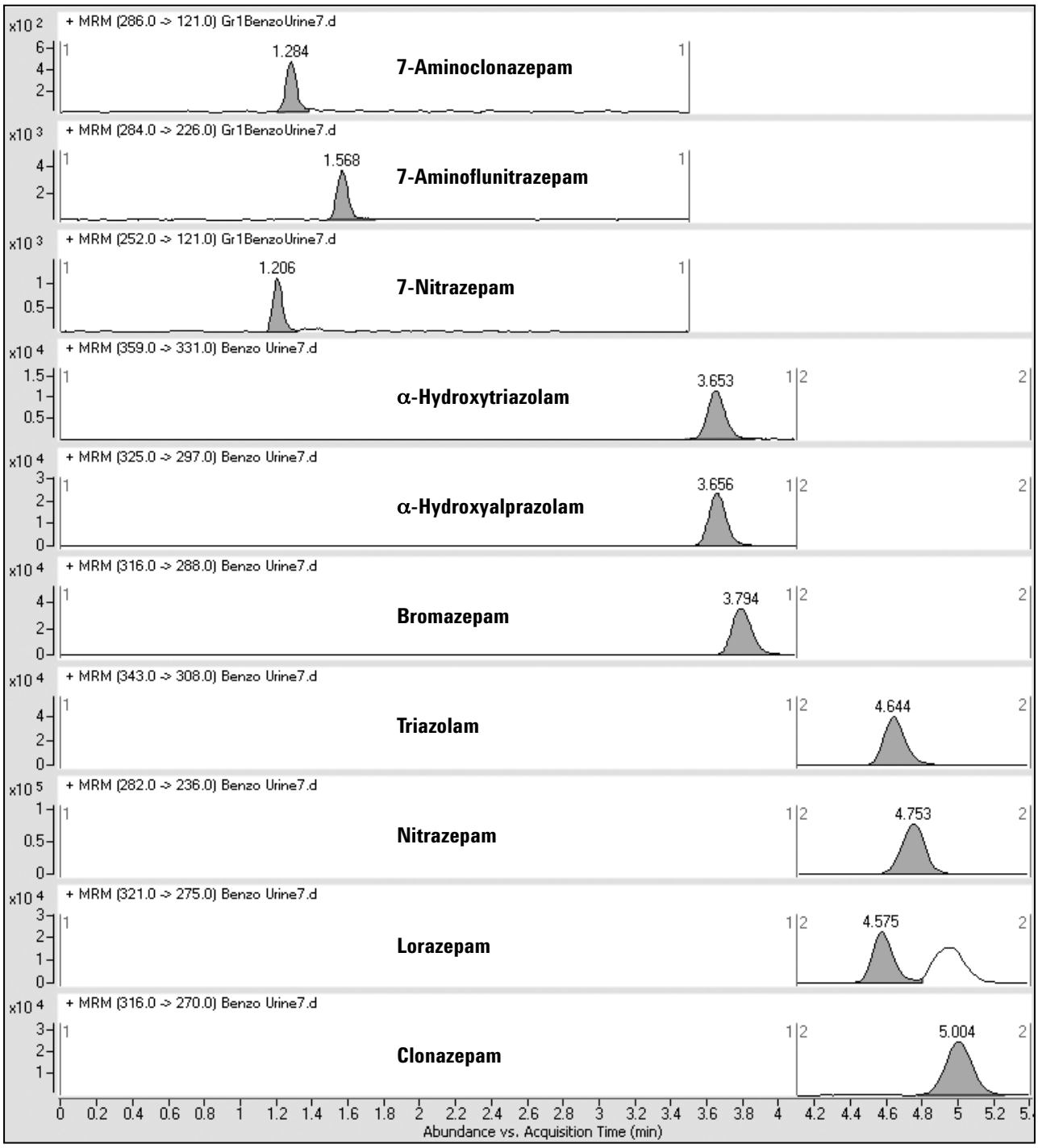

Figure 2a. Benzodiazepines extracted from urine (25 ng/mL): primary transitions, for clarity, internal standards not shown.

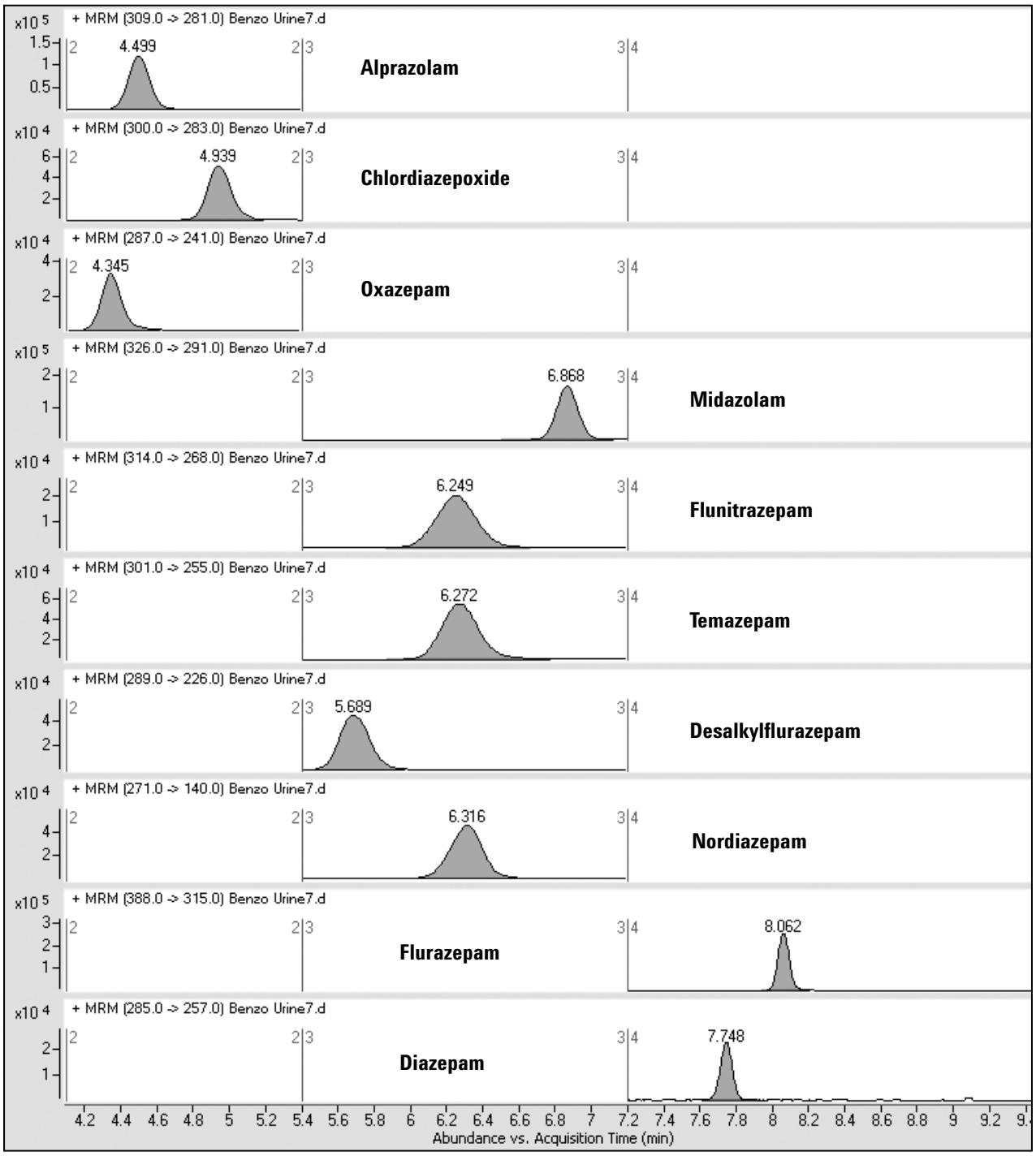

Figure 2a. Benzodiazepines extracted from urine (25 ng/mL): primary transitions, for clarity, internal standards not shown. (continued)

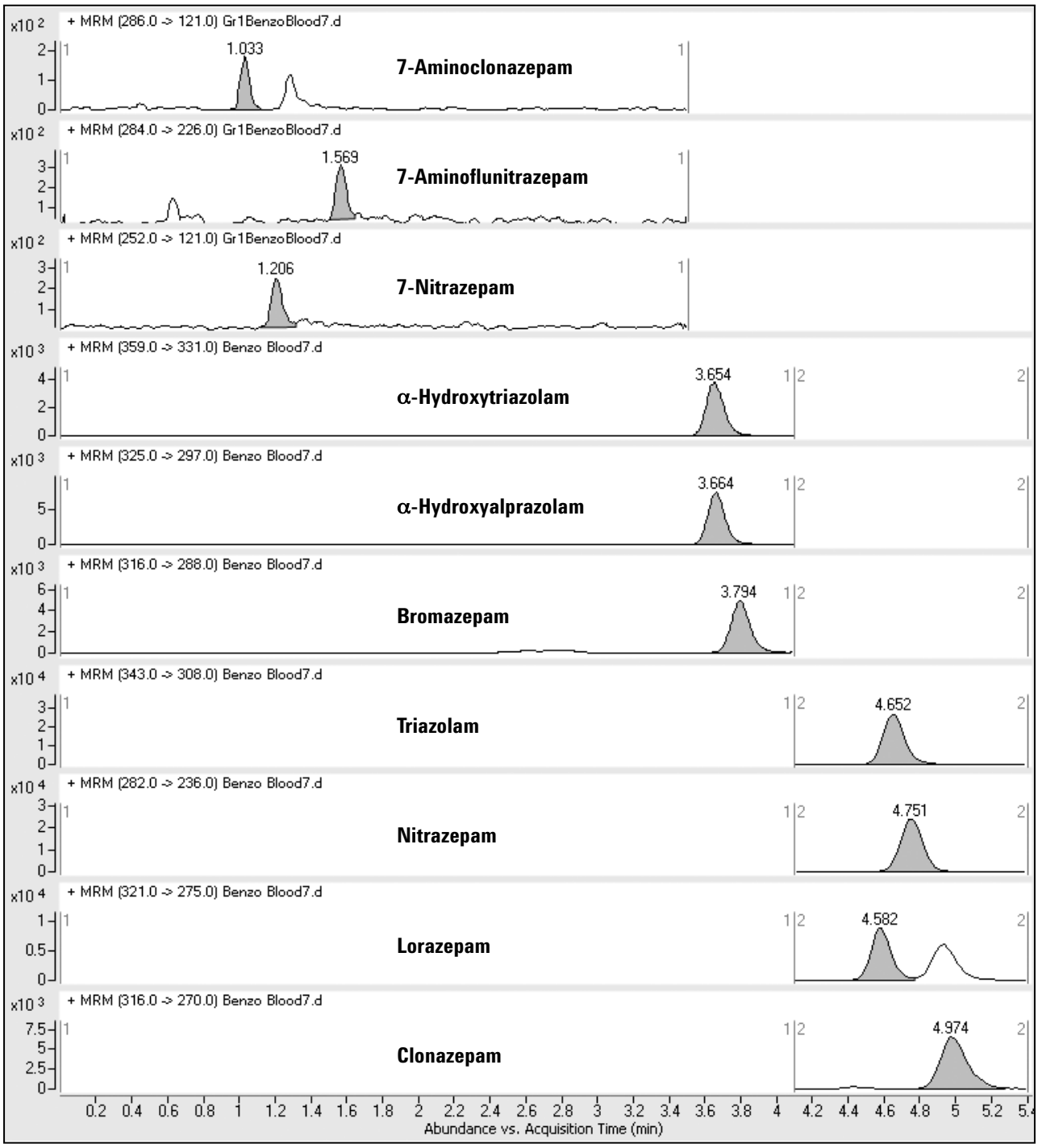

Figure 2b. Benzodiazepines extracted from blood (25 ng/mL): primary transitions, for clarity, internal standards not shown.

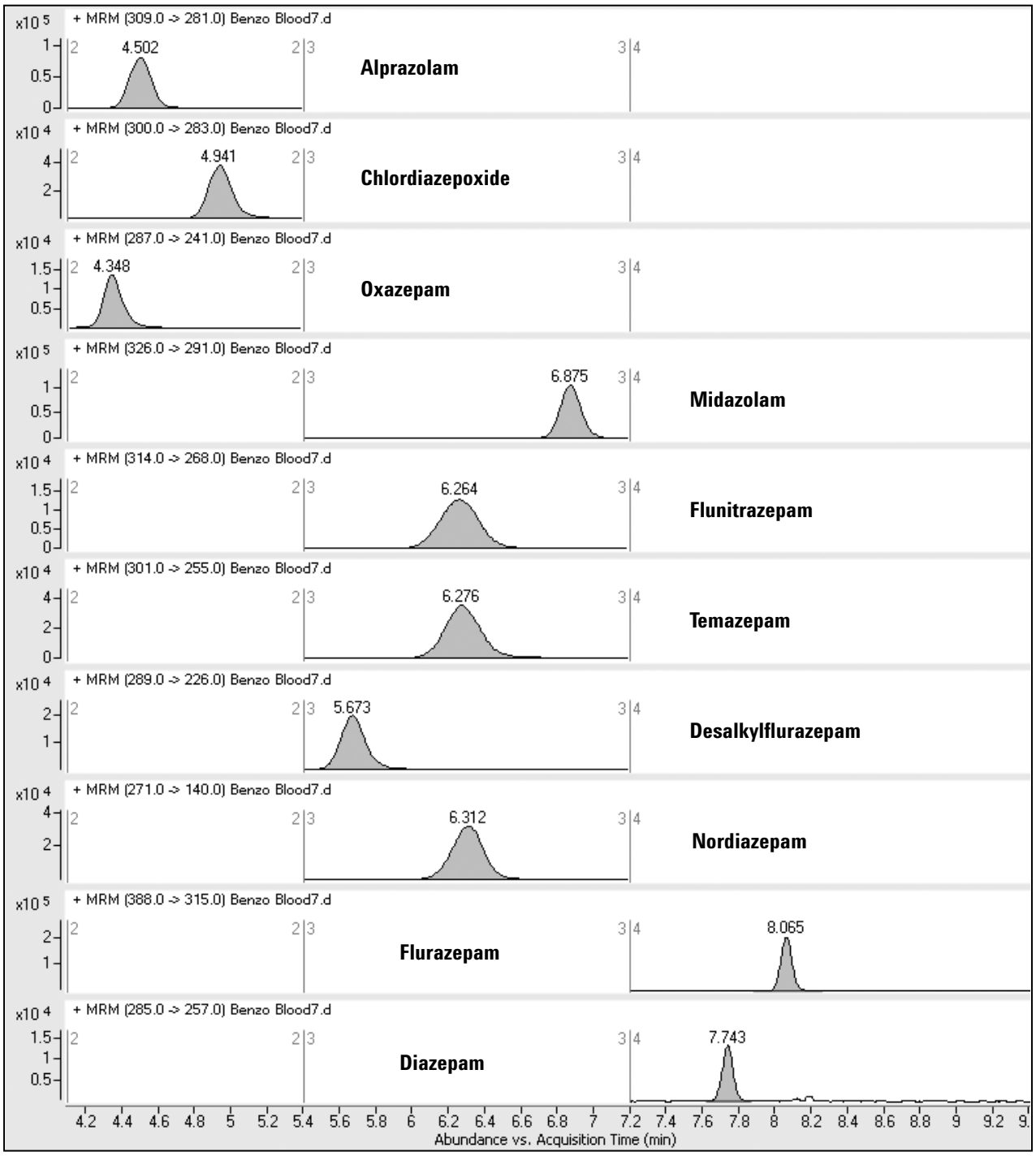

Figure 2b. Benzodiazepines extracted from blood (25 ng/mL): primary transitions, for clarity, internal standards not shown. (continued)

The software provided with the instrument is unique in its ability to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, the identification is rejected. This is an additional feature of the triple quadrupole mass spectrometer, which is extremely important in forensic analysis, where court challenges to laboratory data are frequent.

Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in urine. The software plots the ratio in the chromatographic window, so the operator is able to assess positiveness visually using the "uncertainty" band imposed by the software (Figure 3a: urine; Figure 3b: blood).

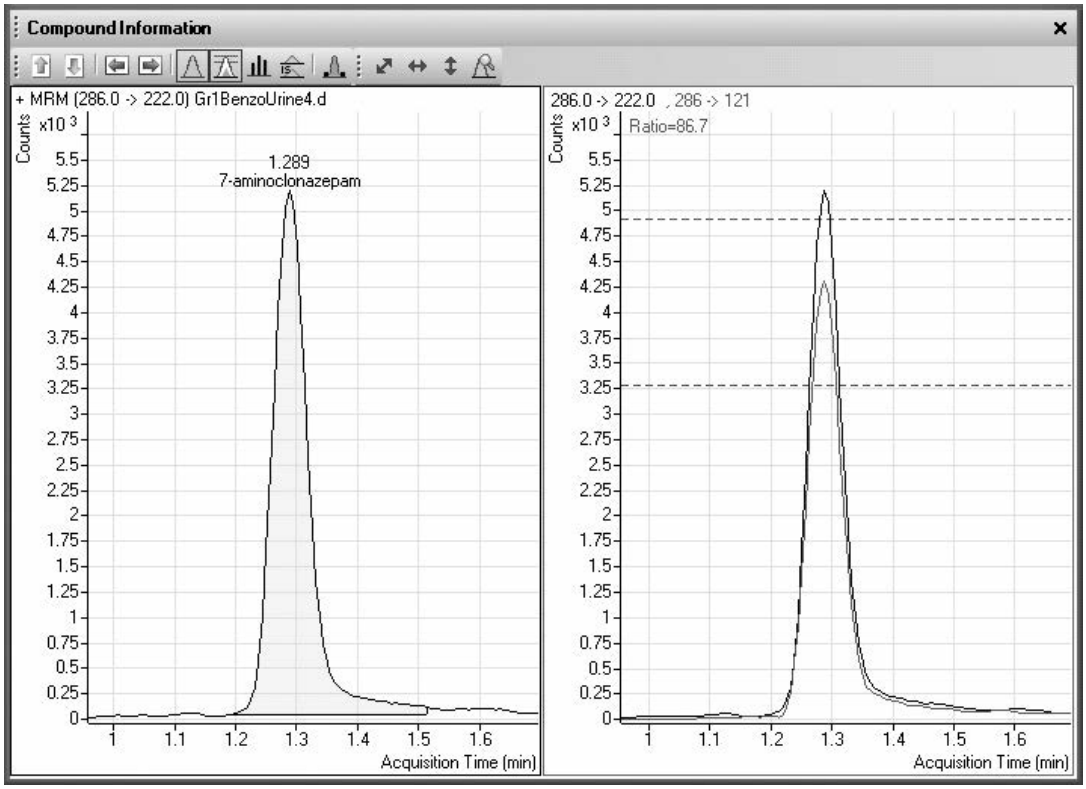

Figure 3a. 7-Aminoclonazepam extracted from urine (50 ng/mL) showing qualifying ion (normalized by area) and acceptable ratio 86.7 with ± 20% tolerance (range: 69.4–104.0).

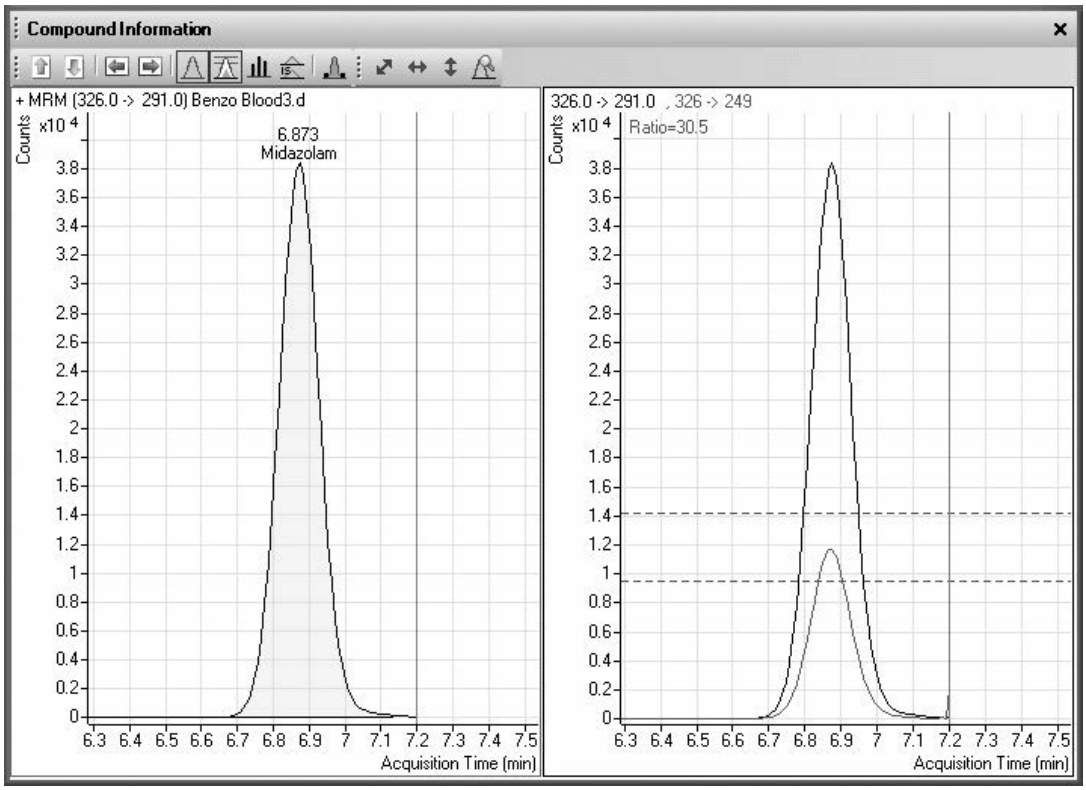

Figure 3b. Midazolam extracted from blood (10 ng/mL) showing qualifying ion and acceptable ratio 30.5 with ± 20% tolerance (range: 24.4–36.6).

Conclusions

The procedure described is suitable for the detection of benzodiazepines in urine using an Agilent Technologies triple quadrupole LC/MS/MS system. To our knowledge, this is the first method where the intensity of qualifying transitions are required to be within a specific ratio compared to the primary transition.

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Determination of Benzodiazepines in Oral Fluid Using LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of 14 benzodiazepines in oral fluid, using the Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) in electrospray mode, is described. Sample preparation includes solid-phase extraction, evaporation of the final eluent to dryness, and reconstitution in mobile phase for injection into the LC/MS/MS system. To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion, which is required to be present within a specific ratio to the primary ion for acceptable identification. The unique features of the Agilent software allow the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis.

Introduction

Benzodiazepines are the most commonly prescribed class of drugs in the USA [1]. They are commonly detected in incidents of driving under the influence of drugs (DUID), often in combination with other medications [2,3]. Oral fluid is becoming increasingly used as a specimen in many areas of forensic interest, including collection at the roadside during traffic stops. Its ease of collection, difficulty of adulteration, and applicability to routine testing has promoted its use as a valid test specimen. However, the detection of benzodiazepines in particular in oral fluid is not without difficulty since the saliva:plasma ratio for most of the drug class is low.

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any quantitative results meaningless without further manipulation in the laboratory [4,5]. Further, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used [6].

This work employs the Quantisal oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the benzodiazepines from the collection pad into the trans-

portation buffer is determined, in order to increase confidence in the quantitative value.

Several publications have addressed the issue of the analysis of benzodiazepines in oral fluid. Quintela et al. [7] determined nine benzodiazepines in neat oral fluid using an LC/MS procedure. They included lormetazepam and tetrazepam, which were not in our profile; however, clonazepam, chlordiazepoxide, nordiazepam, temazepam, oxazepam, flurazepam, and nitrazepam were not included.

A recent publication from Oiestad et al reported the screening of oral fluid using tandem LC mass spectrometry for several drugs, including benzodiazepines [8]. They analyzed fenazepam and some benzodiazepine metabolites, which we did not include (see below); but they did not include the commonly prescribed drugs triazolam, temazepam, midazolam, flurazepam, or chlordiazepoxide. Smink et al. [9] analyzed urine and oral fluid for 33 benzodiazepines using LC/MS/MS. With the exception of diazepam, where a limit of quantitation (LOQ) of 0 ng/mL was reported, the lower limit of quantitation for the other analytes was significantly higher than in our application. In their study, five oral fluid samples were found to be positive; two for oxazepam (concentrations of 18 and 1,659 ng/mL) and three for alprazolam (concentrations of 5, 6, and 9 ng/mL).

In our research, we did not include the metabolites such as 7-aminoflunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, α-hydroxy alprazolam, α-hydroxytriazolam, or desalkylflurazepam because the parent drug is more often in higher concentration than metabolites in oral fluid. We did, however, include metabolites such as nordiazepam, temazepam, lorazepam, and oxazepam as they can be prescribed as individual drugs.

Experimental

Materials and Methods

Oral Fluid Collection Devices

Quantisal devices for the collection of oral fluid specimens are obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (± 10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal collection devices, and drug concentrations detected were adjusted accordingly.

Standards and Reagents

Deuterated internal standards: D5-diazepam; D5-temazepam; D5-alprazolam and D4-clonazepam, as well as unlabeled drug standards: bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam, oxazepam, nordiazepam, temazepam were purchased from Cerilliant (Round Rock, TX). Mixed-mode solid-phase extraction columns (CSDAU020) were purchased from United Chemical Technologies (Bristol, PA)

All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Calibrators and Controls

Calibration standards and controls were prepared from synthetic oral fluid and diluted with Quantisal transportation buffer. Throughout the development of the assay, multiple Quantisal collection devices were selected from different lots. In this experiment, the drug concentration used to fortify the synthetic oral fluid was adjusted according to the dilution factor for all calibration standards and controls. In this way, the final result obtained from the instrument did not need to be recalculated for dilution factors. For each analysis, a four-point calibration curve (1, 10, 20, and 40 ng/mL) was run with each batch; the internal standard concentration was 100 ng/mL.

Extraction Procedure

Quantisal buffer (1 mL) was measured and the calibration curve was prepared at the following concentrations:

Negative:	100 µL of deuterated stock solution (100 ng/mL)
0.5 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 12.5 µL of 10 ng/mL stock solution
1 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 5 µL of 10 ng/mL stock solution
10 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 25 µL of 100 ng/mL stock solution
20 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 50 µL of 100 ng/mL stock solution
40 ng/mL:	100 µL of deuterated stock solution (100 ng/mL) 100 µL of 100 ng/mL stock solution

Sodium phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to the buffer and the samples were mixed. Extraction tubes were placed onto the vacuum manifold and conditioned with methanol (3 mL), deionized water (3 mL), and 0.1 M phosphate buffer (pH 6.0, 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL) and 0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry. Hexane was allowed to flow through the column (1 mL). Finally, the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi /37 °C) and reconstituted in water (50 µL) for analysis.

Drug Recovery from the Collection Pad

Extraction efficiency of the collection system for benzodiazepines was determined. Oral fluid was fortified with all the drugs at the concentration of 10 ng/mL (n = 6). A collection pad was placed into the fluid until the volume adequacy indicator turned blue, showing that 1 mL (±10%) of oral fluid had been absorbed. The pads were placed into the Quantisal buffer (3 mL), capped, and allowed to remain at room temperature overnight to simulate transportation to the laboratory. The following day, the pads were removed and an aliquot (1 mL) of the specimens was analyzed according to the described procedures.

Analytical Procedure

Instrument: Agilent 1200 Series RRLC; 6410 LC Triple Quadrupole Mass Spectrometer

LC Conditions

Column:	ZORBAX Eclipse XDB C18 4.6 x 50 mm x 1.8 µm (PN: 922795-902) A 2.1-mm id column is optimal for a 0.2 mL/min flow rate, but a 1 mL/min column flush is used at the end of the run.
Column temperature:	35°C
Injection volume:	5 µL
Solvent flow rate:	0.2 mL/min
Isocratic pump program:	A = 20 mM ammonium formate (pH = 8.6) B = Acetonitrile 50:50 v,v
Time (minutes)	Flow rate (mL/min)
0	0.2
6.5	0.2
8	1
10	0.2
Post time:	4.5 min

Mass Spectrometer Conditions

Operation:	Electrospray ESI positive mode using Agilent G1948B ESI source
Gas temperature:	300 °C
Gas flow (N_2):	6 L/min
Nebulizer pressure:	15 psi (pressure of 30 to 40 psi recommended)
Capillary voltage:	4,500 V

The precursor and product ions, along with optimized fragmentor and collision energy (CE) voltages, are shown in Table 1. Values pertaining to qualifier ions are in parentheses.

Table 1. Benzodiazepine Acquisition Parameters

Compound	Precursor ion	Product ion	Fragmentor (V)	CE (V)
Segment 1 (time = 0 min)				
Bromazepam	316	288 (209)	160	20 (30)
Segment 2 (time = 4.1 min)				
D4-Clonazepam	320	274	120	25
Clonazepam	316	270 (214)	120	25 (35)
Lorazepam	321	275 (229)	140	25 (35)
Nitrazepam	282	236 (180)	160	25 (35)
D5-Alprazolam	314	286	160	25
Alprazolam	309	281 (274)	160	25 (30)
Chlordiazepoxide	300	283 (227)	120	15 (30)
D5-Oxazepam	292	246	120	20
Oxazepam	287	241 (269)	120	20 (20)
Triazolam	343	308 (239)	120	35 (35)

Table 1. Benzodiazepine Acquisition Parameters (Collision energy abbreviated as CE) (continued)

Compound	Precursor ion	Product ion	Fragmentor (V)	CE
Segment 3 (time = 5.4 min)				
Flunitrazepam	314	268 (239)	160	30 (35)
Midazolam	326	291 (249)	200	30 (40)
D5-Temazepam	306	260	120	25
Temazepam	301	255 (177)	120	35 (40)
D5-Nordiazepam	276	140	120	30
Nordiazepam	271	140 (165)	160	30 (30)
Segment 4 (time = 7.2 min)				
D5-Diazepam	290	262	160	25
Diazepam	285	257 (222)	160	25 (25)
Flurazepam	388	315 (288)	160	25 (25)

LC/MS/MS Method Evaluation

The analytical method was evaluated according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates over a period of 5 days. The results are presented in Table 2. The slope of the calibration curve was not forced through the origin. The precision of the assays was excellent, with both within-day and between-day variations (CV) being below 7% for all drugs. The limit of quantitation for all drugs was 0.5 ng/mL of neat oral fluid, equivalent to 0.125 ng per mL of buffer solution.

Table 2A. Slope of Calibration Curve and Correlation Coefficient

Analyte	Equation	Correlation (R^2)
Alprazolam	Y = 0.0298x + 0.0114	0.9995
Bromazepam	Y = 0.0096x – 0.0129	0.9909
Chlordiazepoxide	Y = 0.0146x – 0.0032	0.9998
Clonazepam	Y = 0.0278x – 0.0108	0.9991
Diazepam	Y = 0.0305x – 0.0004	0.9996
Flunitrazepam	Y = 0.007x – 0.0002	0.9999
Flurazepam	Y = 0.2984x – 0.0024	0.9993
Lorazepam	Y = 0.0189x – 0.008	0.9986
Midazolam	Y = 0.0156x – 0.0143	0.9960
Nitrazepam	Y = 0.0551x + 0.018	0.9987
Nordiazepam	Y = 0.011x – 0.0013	0.9999
Oxazepam	Y = 0.0228x – 0.0065	0.9996
Temazepam	Y = 0.0149x – 0.0034	0.9998
Triazolam	Y = 0.0225x + 0.0073	0.9995

Table 2B. Inter-Day Precision (10 ng/mL control specimens; n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)	Acuracy (%)
Alprazolam	9.48	0.19	2.03	105.49
Bromazepam	9.72	0.66	6.8	102.88
Chlordiazepoxide	10.08	0.23	2.26	99.21
Clonazepam	9.44	0.3	3.14	105.93
Diazepam	9.84	0.59	6.04	101.63
Flunitrazepam	9.84	0.5	5.11	101.63
Flurazepam	9.84	0.49	5.01	101.63
Lorazepam	8.88	0.33	3.68	112.61
Midazolam	9.18	0.54	5.94	108.93
Nitrazepam	10.48	0.115	1.42	95.42
Nordiazepam	9.9	0.32	3.27	101.01
Oxazepam	9.94	0.3	3.07	100.6
Temazepam	10	0.3	3	100
Triazolam	9.86	0.25	2.55	101.42

Table 2C. Intra-Day Precision (n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Alprazolam	9.64	0.27	2.80
Bromazepam	10.08	0.62	6.13
Chlordiazepoxide	10.14	0.68	6.71
Clonazepam	9.18	0.39	4.25
Diazepam	9.48	0.69	7.29
Flunitrazepam	9.94	0.46	4.64
Flurazepam	9.74	0.68	6.95
Lorazepam	9.24	0.34	3.64
Midazolam	9.26	0.30	3.29
Nitrazepam	10.40	0.46	4.41
Nordiazepam	9.84	0.36	3.71
Oxazepam	9.58	0.40	4.20
Temazepam	10.12	0.39	3.85

Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays. Figure 1 shows a typical calibration curve for alprazolam, with a correlation coefficient of 0.9995. The recovery of the various benzodiazepines from the collection system is shown in Table 3.

Table 3. Percentage Recovery of Benzodiazepines from Oral Fluid Collection System Following Overnight Incubation at Room Temperature (fortified at 10 ng/mL; n = 6)

Drug	Mean recovery (%)	CV (%)
Alprazolam	86.76	8.85
Bromazepam	88.42	14.01
Chlordiazepoxide	89.41	6.33
Clonazepam	88.10	2.97
Diazepam	82.82	4.42
Flunitrazepam	85.10	4.46
Flurazepam	81.57	2.85
Lorazepam	83.44	2.52
Midazolam	81.48	5.32
Nitrazepam	90.17	3.64
Nordiazepam	83.28	3.80
Oxazepam	84.65	2.82
Temazepam	84.19	2.96
Triazolam	85.45	8.71

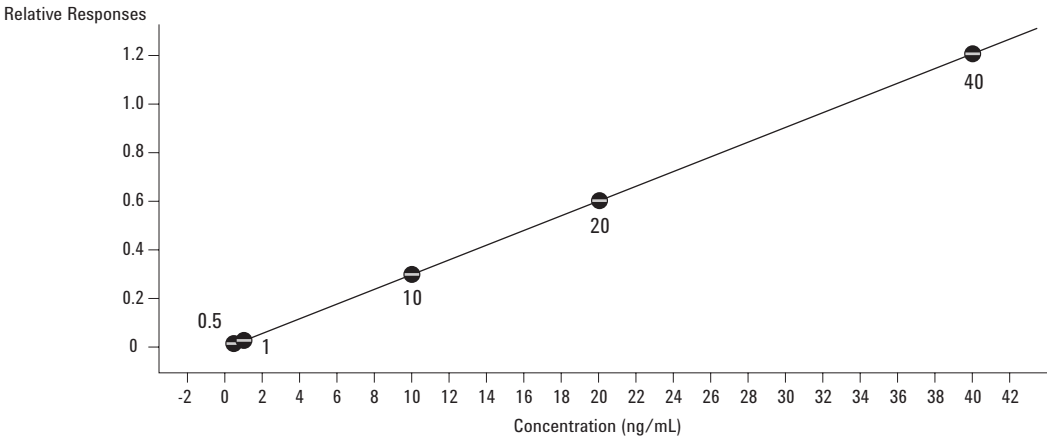

Figure 1. Calibration curve for alprazolam in oral fluid (0.5, 1, 10, 20, and 40 ng/mL).

Results and Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines in oral fluid at an extremely low concentration, as is required for these drugs. The chromatography afforded by the small-particle analytical column allowed separation of the peaks in each of the four group segments (Figure 2).

Further, the Agilent software is unique in its ability to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, the identification is rejected. This is an additional feature of the QQQ mass spectrometer, which is extremely important in forensic analysis, where court challenges to labo-

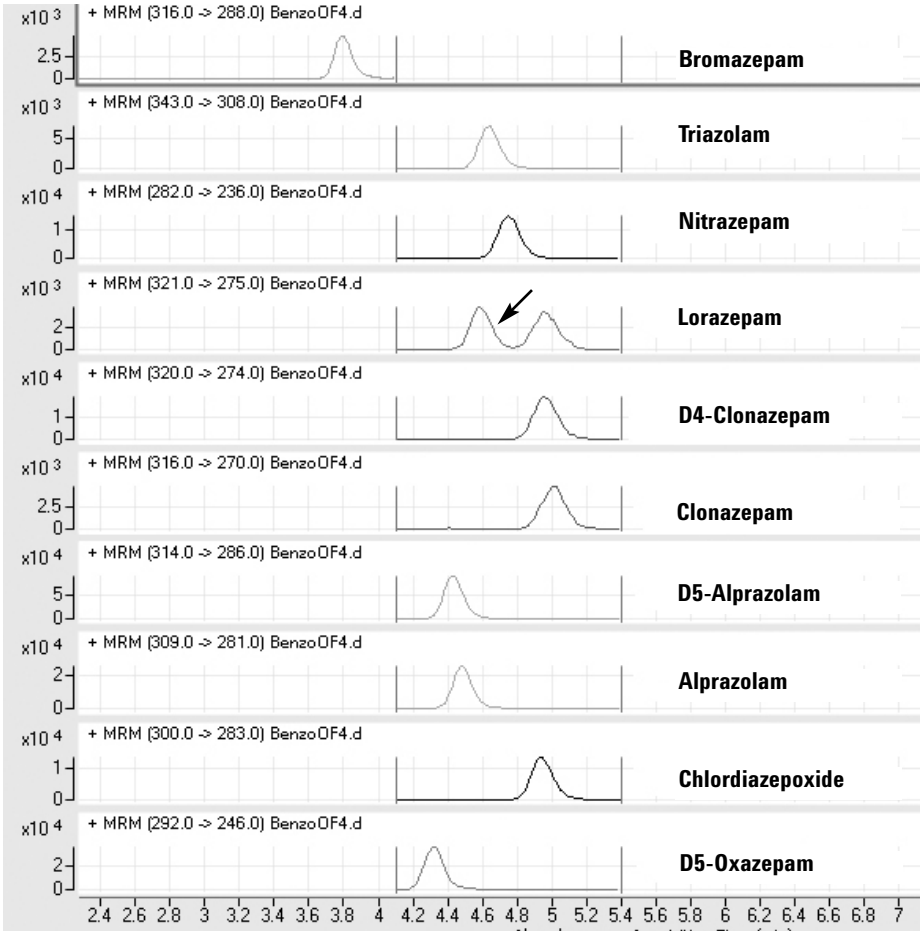

Figure 2. Primary transitions for benzodiazepines in oral fluid.

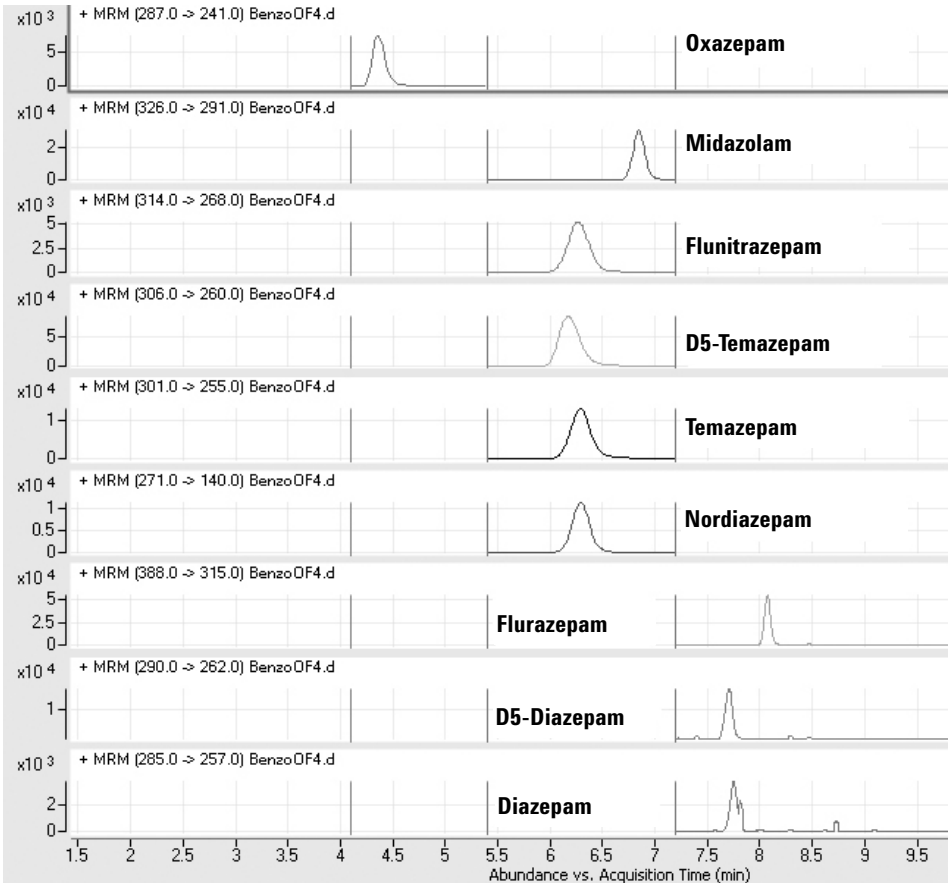

Figure 2. Primary transitions for benzodiazepines in oral fluid. (continued)

ratory data are frequent. Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in oral fluid. The software plots the ratio in the chromatographic window, so the operator is able to assess positivity visually (Figure 3).

Conclusions

The procedure described is suitable for the detection of benzodiazepines in oral fluid using an Agilent Technologies QQQ LC/MS/MS system. The sensitivity of the assay is a significant improvement over other methods. This is the first method that includes qualifying ions for the identification

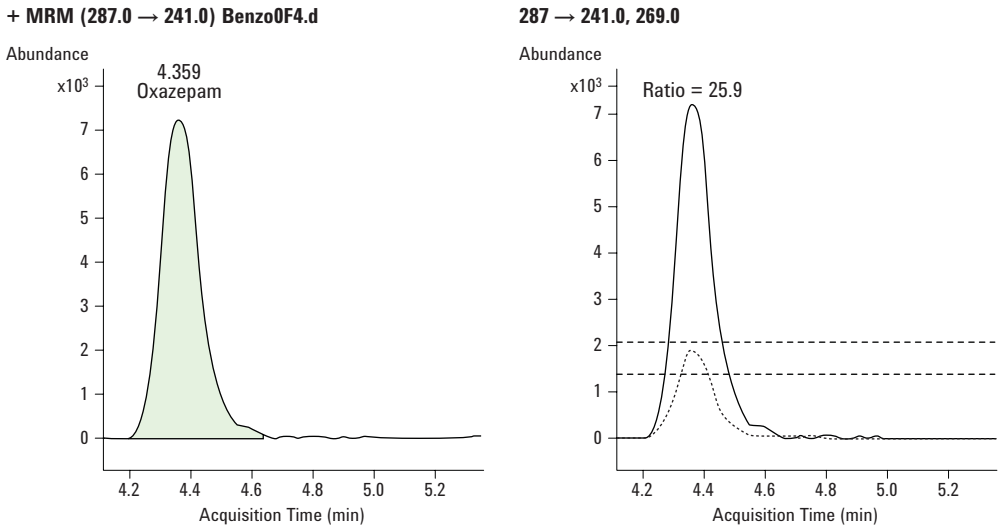

Figure 3. Oxazepam extracted from oral fluid (10 ng/mL).

of benzodiazepines at low concentration in oral fluid, and is in routine use in our laboratory.

Author's note: This work has been accepted for publication in the *Journal of Analytical Toxicology*.

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Determination of Cocaine, Benzoylecgonine, Cocaethylene, and Norcocaine in Human Hair Using Solid-Phase Extraction and Liquid Chromatography with Tandem Mass Spectrometric Detection

Application Note

Forensic Toxicology

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Abstract

A quantitative analytical procedure for the determination of cocaine, benzoylecgonine, cocaethylene, and norcocaine in hair has been developed and evaluated. The hair samples were washed, incubated, and any drugs present were quantified using mixed-mode solid-phase extraction and liquid chromatography with tandem mass spectrometric detection in positive atmospheric pressure chemical ionization mode. For confirmation, two transitions were monitored and one ion ratio was determined, which was within 20% of that of the known calibration standards. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion limited the sensitivity of the assay, particularly for benzoylecgonine; however, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. Even with simultaneous monitoring, the concentrations proposed by the United States federal guidelines for hair analysis were achieved. The limits of quantitation were 50 pg/mg; the limit of detection was 25 pg/mg. The intra-day precision of the assays at 100 pg/mg (n = 5) was 1.3%, 8.1%, 0.8%, and 0.4%; inter-day precision 4.8%, 9.2%, 15.7%, and 12.6% (n = 10) for cocaine, benzoylecgonine, cocaethylene, and norcocaine, respectively. The methods were applied to both proficiency specimens and to samples obtained during research studies in the USA.

Introduction

Cocaine (COC) and its metabolites are included in the proposed United States federal regulations for hair analysis. The suggested cut-off concentration for the metabolites is 50 pg/mg, which is difficult to achieve routinely using electron impact gas chromatography-mass spectrometry (GC/MS) [1,2]. This may be due to the inability to derivatize cocaethylene (CE) to improve its response; the coelution of norcocaine (NC) and CE, or potentially similar ions for the derivatives of NC and benzoylecgonine (BZE). Procedures have been developed to approach the proposed detection requirements, including positive chemical ionization GC/MS [3], and GC with tandem mass spectrometry [4].

There are two publications describing the analysis of COC and its metabolites in hair using LC/MS/MS in atmospheric pressure chemical ionization (APCI) mode, in a similar manner to our approach [5,6]. The first of these analyzes only COC and BZE, but more importantly, both procedures monitor only one transition in the multiple reaction-monitoring mode (MRM). Recently, several authors have focused on the need for the monitoring of a second transition, allowing the ratio between the abundance of the primary and secondary ions to be calculated and establishing more

confidence in the final result. Maralikova and Weinmann [7] note that guidelines for confirmatory analysis using LC/MS/MS have not yet been established and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs. Johansen and Bhatia [8] describe the analysis of COC and its metabolites in whole blood and urine using LC/MS/MS, focusing on the establishment of identification criteria based on two MRM transitions, their ratio, and retention time. This is particularly important in assays that include compounds with similar molecular weights and chemical properties, since the same product ion is often present.

Using these suggestions for tandem mass spectrometry, we developed and evaluated a procedure using LC/MS/MS for the analysis of COC and its metabolites in hair in order to provide additional confidence in the generated result. The method was applied to specimens received by our laboratory from proficiency programs and research studies. Structures of the compounds are shown in Figure 1.

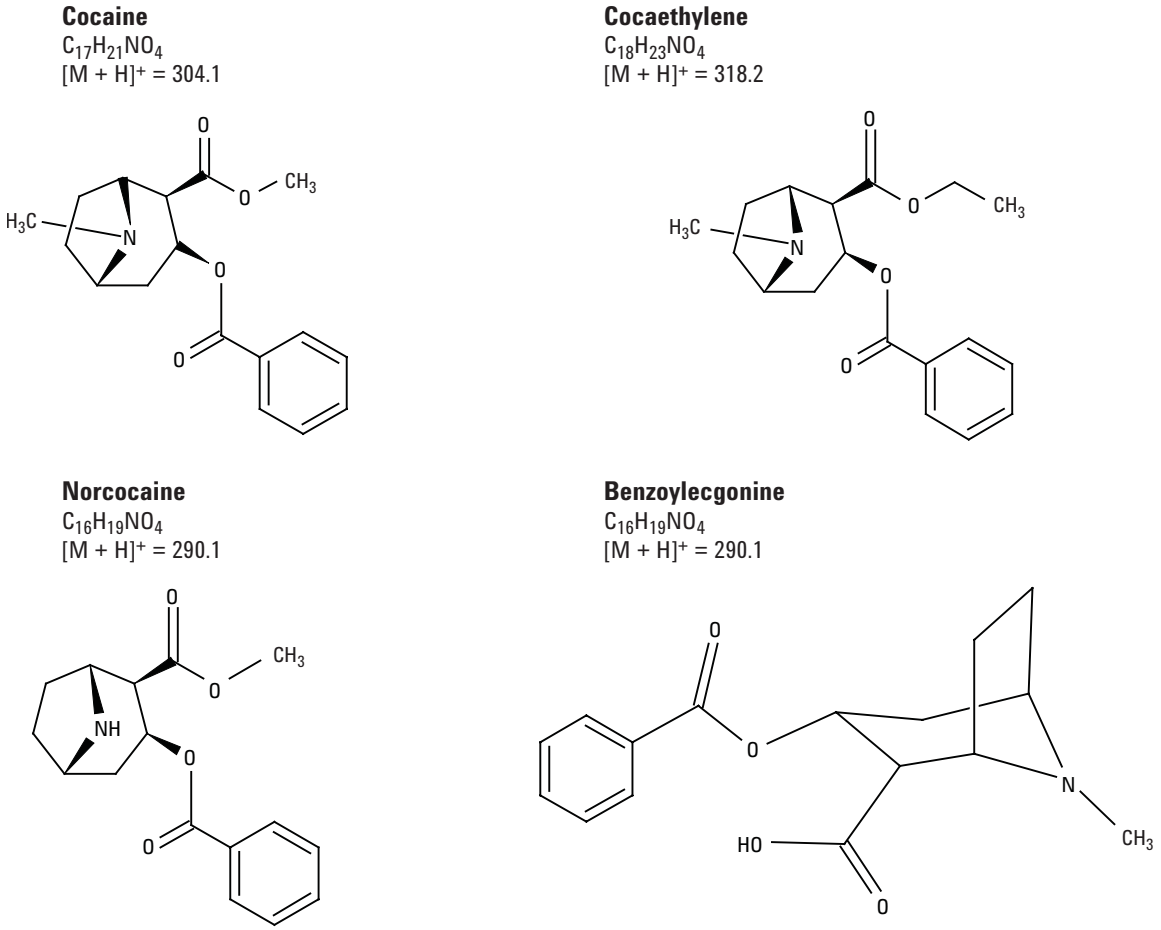

Figure 1. Structures of cocaine and metabolites analyzed in this work.

Experimental

Sample Preparation

Standards and Reagents

Deuterated internal standards (BZE-d3, COC-d3, NC-d3, and CE-d8) as well as unlabeled drug standards for each of the drugs were obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare, (San Pedro, CA). All solvents were HPLC grade or better, and all chemicals were ACS grade.

Calibrators

For the chromatographic calibration standards, a working solution containing deuterated internal standards was prepared in methanol at a concentration of 200 ng/mL. Unlabeled drug standards were prepared in methanol at the same concentration. All the working solutions were stored at –20 °C when not in use. For each batch, eight calibration standards were prepared in drug-free hair (10 mg). Drug concentrations of 25, 50, 100, 200, 500, 1,000, 2,000 and 10,000 pg/mg of hair were prepared (internal standard concentration: 1,000 pg/mg).

Sample Preparation for Chromatographic Analysis

An aliquot of hair (10 mg) was briefly rinsed with methylene chloride (1.5 mL) to remove hair treatments such as mousse, spray, gels, etc., and allowed to dry. The hair was cut into small pieces and internal standard was added (50 µL). 0.025 M phosphate buffer (pH 2.7; 1.5 mL) was added and the hair was sonicated at 75 °C for 2 hours. The buffer was decanted into clean glass tubes and 0.1 M sodium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control, or hair specimen. The mix was centrifuged for 10 min to ensure that no hair strands were applied to the solid-phase extraction column. Solid-phase mixed-mode extraction columns (Clin II, 691-0353T) were placed into a positive pressure manifold. Each column was conditioned with methylene chloride: methanol: ammonium hydroxide (78:20:2 v,v 2 mL), ethyl acetate (2 mL), methanol (2 mL), and 0.1 M hydrochloric acid (1 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (2 mL), 0.1 M hydrochloric acid (2 mL), methanol (2 mL), and ethyl acetate (2 mL). The columns were allowed to dry between washes under nitrogen pressure (30 psi; 2 min). The drugs were finally eluted using freshly prepared methylene chloride: methanol: ammonium hydroxide (78:20:2 v,v 3 mL). The extracts were evaporated to dryness under nitrogen at 40 °C and reconstituted in methanol (50 µL).

Data Analysis

Calibration using deuterated internal standards was calculated using linear regression analysis over a concentration range of 25 to 10,000 pg/mg for all drugs. Peak area ratios of target analytes and their respective deuterated standards were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a 1/x weighting and was not forced through the origin.

Selectivity

Drug-free hair specimens were obtained from volunteers and extracted and analyzed according to the described procedures in order to assess interference from extraction or matrix, or potential ion suppression. Ion suppression is not as prevalent using APCI as it is in electrospray mode. In addition, interferences from commonly encountered drugs were added to the drug-free hair specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 20,000 pg/mg: morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, desmethyltramadol, fentanyl, gamma-hydroxybutyrate (GHB), tetrahydrocannabinol (THC), 9-carboxy-THC, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, phencyclidine, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, nitrazepam, triazolam, α-hydroxytriazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital, and phenobarbital.

Linearity and Sensitivity

The linearity of the assays was established with eight calibration points, excluding the drug-free matrix. The sensitivity of the method was deter-

mined by establishing the limit of quantitation (LOQ), defined as the lowest concentration detectable with a signal-to-noise (S:N) ratio of at least 10 and retention time within 0.2 minutes of the calibration standard. The limit of detection (LOD) was determined from the lowest concentration detectable with an S:N ratio of at least 3.

Precision

Inter- (between day) and intra-day (same day) precision of the assays was determined at the calibration point of 100 pg/mg for all drugs. Intra-day data were obtained from five analyses performed on one day; inter-day data were obtained by analyzing a total of 10 specimens over 5 days (2 samples per day for 5 days; n = 10).

Stability

The stability of the drug extracts at a concentration of 50 pg/mg was determined by allowing the autosampler vials to remain in the liquid chromatographic chamber for 48 hours, after which time they were re-analyzed. The unit was maintained at 4 °C. The responses were compared to those achieved on the first day of analysis.

Application to Authentic Specimens

As part of various ongoing research studies, our laboratory receives hair specimens for research purposes as well as proficiency specimens.

LC/MS Method Details

LC Conditions

Agilent 1200 Series binary pump, degasser, thermostat-controlled wellplate sampler, and thermostatted column compartment.

Column:	Agilent ZORBAX XDB-C18, 4.6 x 50 mm, 1.8 µm (p/n 922975-902)
Column temperature:	40 °C
Mobile phase:	A = 20 mM ammonium acetate (pH 6.4) B = methanol
Flow rate:	0.9 mL/min
Injection vol:	2 µL

Gradient:

Time (min)	%B	Flow (mL/min)	
0.0–1.5	30	0.9	
4.5	55	1	
5	60	1	Stop time: 7 min
7	75	1	Post time: 6 min

Needle wash (75:25 methanol/water): flush port 2 seconds

MS Conditions

Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ)

Mode:	Positive APCI using the Agilent G1947B ionization source
Vaporizer temperature:	400 °C
Drying gas flow:	5 L/min
Drying gas temperature:	350 °C
Nebulizer:	50 psig
V_{cap}:	4500 V
Corona needle:	4 µA
Resolution (FWHM):	Q1 = 2.5 amu; Q2 = 0.7 amu

Dwell time for all MRM transitions = 50 msec

Two transitions were selected and optimized for each drug using flow injection analysis. One parameter requiring optimization is the fragmentor voltage, which is located between the ion source and the QQQ mass analyzer. This voltage needs to be optimized for maximum transfer of the precursor ions into the first quadrupole of the mass analyzer. For all compounds this value was determined to be 120 V.

Table 1 shows the optimized collision energy voltages for each precursor ion (M + 1) to produce the quantifier and qualifier product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ± 20% in order to meet the criterion for a positive confirmation. The ion ratio for each drug was

Table 1. MRM Mode Parameters (Values for qualifiers in parentheses)

Segment	Compound	Transition	Collision Energy (V)
1 (0 min)	Benzoylecgonine	290.3 > 168.3 (105.3)	15 (15)
	D3-Benzoylecgonine	293.3 > 171.4	20
2 (3.2 min)	Not used		
3 (4 min)	Cocaine	304.3 > 182.3 (82.2)	20 (25)
	D3-Cocaine	307.3 > 185.3	20
4 (4.9 min)	Cocaethylene	318.3 > 196.4 (82.2)	25 (25)
	D8-Cocaethylene	326.3 > 204.4	20
	Norcocaine	290.3 > 168.3 (136.3)	15 (25)
	D3-Norcocaine	293.3 > 171.4	15

determined at the concentration level of 100 pg/mg.

Results and Discussion

Method Development

The development of simple LC/MS/MS assays for the detection of COC and its metabolites in hair is reported. While these drugs have been detected in hair, the increasing utility of LC/MS/MS in laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying ion is reported for the first time for COC hair analysis, and is necessary for the improved confidence in the identification of the analyte. An example is shown in Figure 2.

Method Evaluation

The chromatographic procedures developed for COC, BZE, CE, and NC were evaluated according to accepted protocols. The limit of quantitation for each drug and calibration curve data were determined as described in the Experimental section. Linearity was obtained with an average correlation coefficient for all the drugs of $R^2 > 0.99$ over the concentration range from 25 to 10,000 pg/mg of hair. An example is shown for CE in Figure 3.

Table 2 shows the mean correlation, equation of the slope of the calibration curve, and the qualifying ratio between the transitions monitored. The low intensity of the second transition for BZE (6.7 to 10%) limited the sensitivity of the method for that particular drug; however, the importance of having a qualifying transition was considered to be of greater importance in forensic identification than sensitivity.

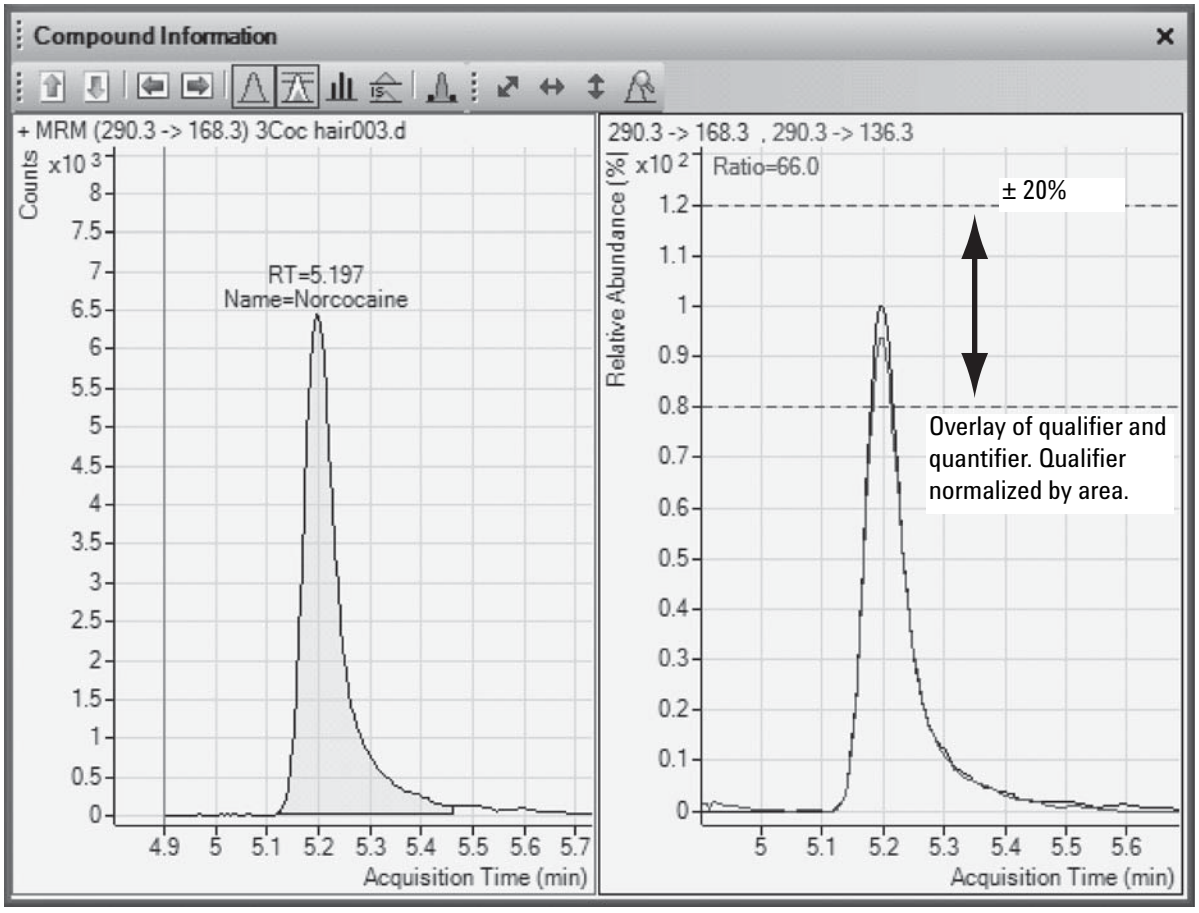

Figure 2. Ion ratio confirmation for norcocaine at 100 pg/mg level.

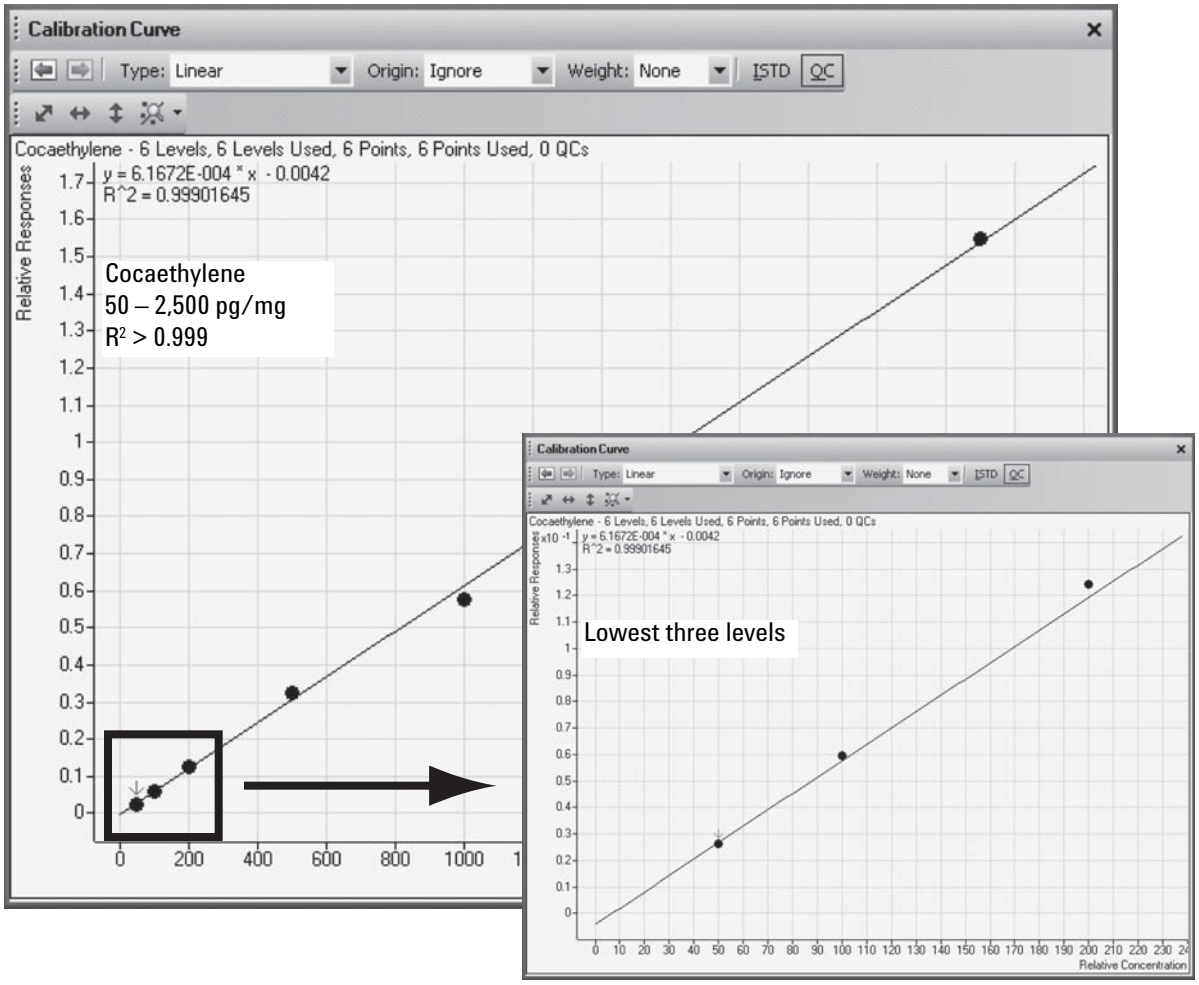

Figure 3. Linearity of cocaethylene with lowest levels detailed to show excellent accuracy.

Table 2. Mean Correlation, Equation of the Slope of the Calibration Curve and the Qualifying Ratio Between the Transitions Monitored

Drug	Mean correlation (n = 3)	Equation for calibration curve	Allowable range of intensity for qualifying ion
Benzoylecgonine	0.9989	y = 0.00116x	6.7–10%
Cocaine	0.9995	y = 0.00106x	37.8–56.8%
Cocaethylene	0.9987	y = 0.00061x	49.3–74%
Norcocaine	0.9992	y = 0.00096x	52.8–79.2%

Hair specimens collected from drug-free individuals showed no interference with any of the assays, which was not unexpected, since it is unlikely these drugs are similar to endogenous substances in hair. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Experimental section. No chromatographic interference was observed in the channels of these transitions.

An example of an extracted hair specimen at a concentration of 50 pg/mg is shown in Figure 4. The inter-day and intra-day precision of the assays was determined using replicate analyses as described. For BZE, COC, CE, and NC, the inter-day precision was 9.2%, 4.8%, 15.7%, and 12.6%, respectively (n = 10). For same-day precision (n = 5), the values were 8.1%, 1.3%, 0.8 %, and 0.4%, respectively. Finally, the stability of the drugs

in the collection system and the stability of the extracts were assessed. The extracts were stable for at least 2 days when kept in the instrument rack inside the autosampler, which was maintained at 7 °C. There was less than a 5% difference in the quantitation of the extracts after 48 hours.

Authentic Specimens

The procedures were applied to proficiency specimens received into the laboratory. The performance was excellent, with all quantitation being within 10% of the group mean identified by the program administrators.

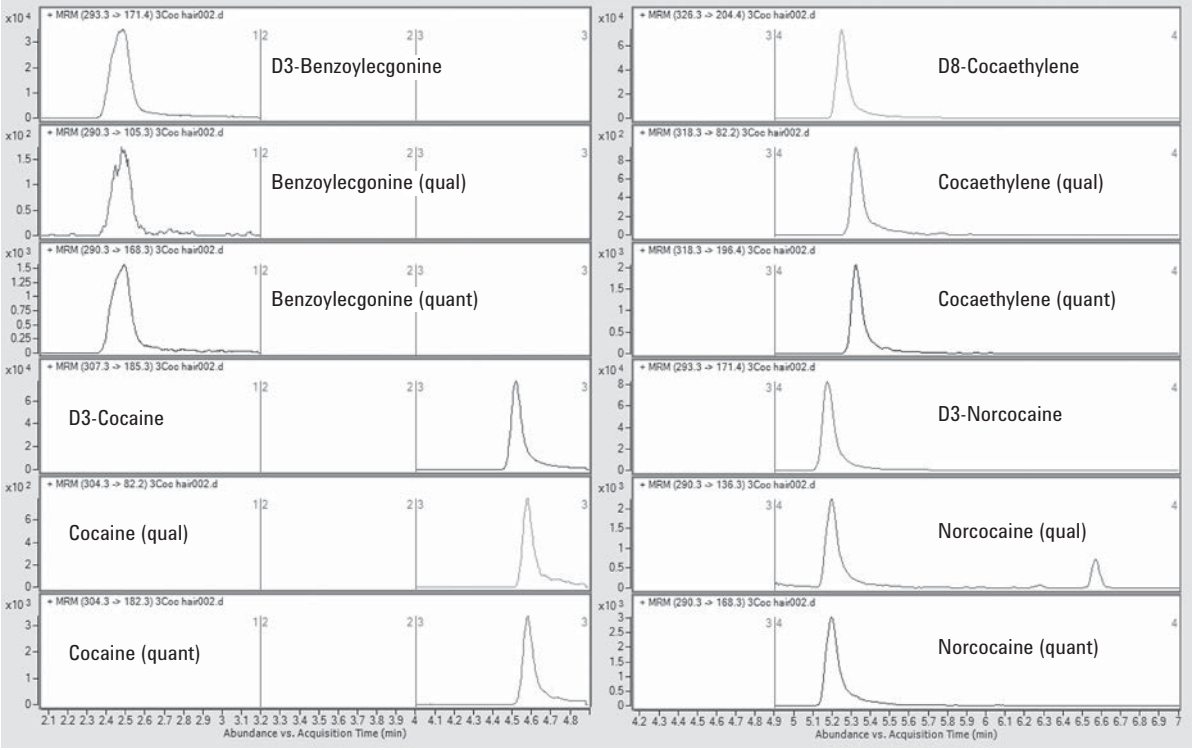

Figure 4. Chromatographic profile of all compounds analyzed in hair at the 50 pg/mg level.

Conclusions

The determination of COC, BZE, CE, and NC in hair is described. The LC/MS/MS procedure is reproducible, robust, and precise. The assay includes the monitoring of a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for definitive identification to be made. The method is easily incorporated into routine forensic laboratory testing.

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The Analysis of Benzodiazepines in Hair Using RRHT LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A quantitative analytical procedure for the determination of benzodiazepines and metabolites in hair has been developed and evaluated. The hair samples were washed, incubated, and any drugs present were quantified using mixed mode solid-phase extraction and liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) in positive electrospray ionization mode. The liquid chromatography is carried out on a ZORBAX Rapid Resolution High Throughput (RRHT) C18 column, which has a 1.8-µm particle size.

For confirmation, two transitions were monitored and one ion ratio was determined, which was within 20% of that of the known calibration standards. The range of concentration analyzed for each compound was 50 to 1,000 pg/mg hair. The intra-day precision of the assays at 100 pg/mg (n = 5) was as low as 1.75% for 7-aminoclonazepam, and as high as 11.8% for a-OH-alprazolam. Inter-day precision (once each day for five days) ranged from as low as 2.55% for diazepam to as high as 13.4% for 7-aminoclonazepam.

To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion, which is required to be present within a specific ratio to the primary ion for acceptable identification. The unique features of the Agilent software allow the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis.

Introduction

Benzodiazepines are frequently prescribed. They exert an additive effect when used in con-junction with alcohol or other drugs, and are subject to abuse. In particular, health-care professionals have higher rates of abuse with benzodiazepines and opiates than other drugs [1]. Using hair as a biological specimen allows a more historical perspective on the drug use of an individual, depending upon the length of the hair tested, compared to blood or urine, and may be a useful specimen for inclusion in the testing of medical professionals seeking to regain licensing or who are subject to frequent testing.

In 2003, Scott and Nakahara showed the incorporation of eight benzodiazepines into hair [2], while others have reported single drugs for example in cases of drug-facilitated sexual assault [3]. Miller et al recently reported the detection of nine benzodiazepines in hair using immunoassay and LC/MS/MS and their application to authentic spec-

imens. The concentration of drugs found in the hair samples ranged from 30 to well over 200 pg/mg for diazepam [4].

We report the detection of 14 benzodiazepines and 5 metabolites in hair. The procedure includes the simultaneous monitoring of a qualifying ion, which is required to be present within a specific ratio to the primary ion for acceptable identification. The features of the Agilent software allow the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. In some cases, monitoring a qualifying transition may inhibit the sensitivity of the assay, but the additional confidence in the result is a critical factor in forensic analysis. The limit of quantitation was 50 pg/mg of hair; the intra-day precision of the assays (n = 5) ranged from 1.75 % for 7-aminoclonazepam to 11.78% for α-hydroxyalprazolam; and the inter-day precision ranged from 2.55% for diazepam to 13.37% for 7-aminoclonazepam (n = 5).

As these compounds have been analyzed in blood and urine in another Agilent application note (5989-7072EN) the reader is referred to that application note for illustrated structures of these compounds.

Experimental

Sample Preparation

Solvents and Reagants

All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Standards (purchased from Cerilliant, Round Rock, TX)

Internal standard mix: D7-7-aminoflunitrazepam; D5-alprazolam; D4-clonazepam; D5-temazepam; D5-oxazepam; D5-nordiazepam; D5-diazepam (100 ng/mL)

Unlabeled drugs: 7-aminoflunitrazepam; 7-aminoclonazepam; 7-aminonitrazepam; α-OH-alprazolam; α-OH-triazolam; desalkylflurazepam; bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam; oxazepam; nordiazepam; temazepam

Extraction Procedure

For each calibration level used for quantitation, an aliquot of hair (10 mg) was briefly rinsed with methylene chloride (1.5 mL) to remove hair treatments such as mousse, spray, gels, etc., and allowed to dry. The hair was cut into small pieces and both analyte and deuterated internal standard were added as shown below.

Calibration curve:

Negative:	50 µL of deuterated stock solution (100 ng/mL)
50 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 5 µL of 100 ng/mL stock solution
100 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 10 µL of 100 ng/mL stock solution
500 pg/mg:	50 µL of deuterated stock solution (100 ng/mL) 50 µL of 100 ng/mL stock solution
1 ng/mg:	50 µL of deuterated stock solution (100 ng/mL) 100 µL of 10 ng/mL stock solution

Deuterated internal standard (50 µL) was also added to proficiency samples used in the evaluation study.

Add hair extraction buffer (0.025 M phosphate buffer, pH 2.7; 1.5 mL); mix

Sonicate (2 hrs; 75°C); decant liquid

Add 0.1 M sodium phosphate buffer (pH 6.0, 1 mL); vortex

Place extraction tubes (CSDAU020) onto the vacuum manifold

Condition each column:

methanol (3 mL)
deionized water (3 mL)
0.1 M phosphate buffer (pH 6.0, 2 mL)

Important: Do not allow the column bed to go dry.

Pour sample through column. Dry.

Rinse each column with:

Deionized water (3 mL),
0.1 M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL)
Dry column; wash column with hexane (1 mL)

Elute drugs: ethyl acetate + 2% ammonium hydroxide (2 mL)
Evaporate to dryness under nitrogen (20 psi/ 37 °C)
Reconstitute in water (50 µL); transfer to autosampler vials; cap

Analytical Procedure

Instrument: Agilent 1200 Series RRLC; 6410 LC Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX RRHT Eclipse XDB C18, 4.6 mm x 50 mm x 1.8 µm (PN: 922975-902)

The 7-amino metabolites of flunitrazepam, nitrazepam, and clonazepam eluted from the analytical column rapidly, even though the flow rate was 0.2 mL/min. Optimization of the gradient and flow rate was attempted but did not give acceptable chromatography for the three metabolites. Subsequently, a separate method was implemented, lasting only 3.5 min and monitoring only those three metabolites. The chromatography and sensitivity were greatly improved by separating the two methods. Although the author (CM) obtained good results using the 4.6-mm id column, the 2.1-mm id column with 1.8-µm particle size is normally recommended by Agilent for increased sensitivity at the flow rates used.

7-amino metabolites only:

Column temperature:	45 °C
Solvent flow rate:	0.6 mL/min
Mobile phase:	A = 20 mM ammonium formate, **pH 8.6** B = acetonitrile - Isocratic, 35% B
Stop time:	3.5 min
Post time:	Off

Benzodiazepines (except 7-amino metabolites):

Column temperature:	35 °C
Solvent flow rate:	0.2 mL/min (initial)
Mobile phase:	A = 20 mM ammonium formate, **pH 8.6** B = acetonitrile - Isocratic, 50% B

Time (minutes)	Flow rate (mL/min)
0	0.2
6.5	0.2
8	1
10	0.2

Stop time = 10 min; Post time = 5 min

MS Conditions:

Operation:	Electrospray ESI positive mode	
	7-amino metabolites	Other benzodiazepines
Gas temperature:	350 °C	300 °C
Gas flow (N_2):	6 L/min	6 L/min
Nebulizer pressure:	20 psi	50 psi
Capillary voltage:	4000 V	4500 V

The multiple reaction monitoring (MRM) transitions are shown in Table 1. For all compounds, the first quadrupole, for the precursor ion, is operated at low resolution, or full width half maximum (FWHM) equal to 2.5 amu. The last quadrupole, for the product ion, is operated at unit resolution, or FWHM = 0.7 amu.

Retention times are given as used in the quantitation method. The two parameters requiring optimization for each compound include the fragmentor (Frag) voltage and the collision energy (CE), expressed in units of voltage. The fragmentor is part of the ion transfer optics located between the ion source and the mass analyzer, responsible for transferring the precursor ion mass of the specified compound. This parameter is optimized for each compound using flow injection analysis (FIA) of the corresponding standard in which the fragmentor voltage is varied with each injection and the voltage for the optimal response is determined.

Once the fragmentor voltage is optimized, the collision energy voltages are determined for which an optimal response of both the quantifier and the qualifier ions are obtained. The quantifier ion corresponds to the product ion that has the best signal response overall. The qualifier ion corresponds to the second most-intense product ion and is used for confirmation based on its peak area ratio versus that of the quantifier ion.

Table 1. Multiple Reaction Monitoring (MRM) Ttransitions for the Benzodiazepines Analyzed in the Work

Compound	RT (min)	MRM transition	Frag (V)	CE (V)
7-amino metabolites only:				
D7-7-aminoflunitrazepam	1.102	291 > 263	120	25
7-aminoclonazepam	0.94	286 > 222 (121)	200	25 (25)
7-aminonitrazepam	0.95	252 > 121 (208)	120	30 (35)
7-aminoflunitrazepam	1.104	284 > 226 (256)	160	30 (25)
Remaining benzodiazepines: Segment 1 (0.0 min)				
α-OH-triazolam	3.71	359 > 331 (176)	120	25 (25)
α-OH alprazolam	3.72	325 > 297 (216)	120	30 (35)
Bromazepam	3.85	316 > 288 (209)	160	20 (30)
Segment 2 (4.1 min)				
D5-oxazepam	4.40	292 > 246	120	20
Oxazepam	4.44	287 > 241 (269)	120	20 (20)
D5-alprazolam	4.57	314 > 286	160	25
Alprazolam	4.63	309 > 281 (274)	160	25 (30)
Lorazepam	4.67	321 > 275 (229)	140	25 (35)
Triazolam	4.79	343 > 308 (239)	120	35 (35)
Nitrazepam	4.85	282 > 236 (180)	160	25 (35)
Chlordiazepoxide	5.07	300 > 283 (227)	120	15 (30)
D4-clonazepam	5.07	320 > 274	120	25
Clonazepam	5.12	316 > 270 (214)	120	25 (35)
Segment 3 (5.6 min)				
D5-temazepam	6.34	306 > 260	120	25
Temazepam	6.43	301 > 255 (177)	120	35 (40)
Flunitrazepam	6.44	314 > 268 (239)	160	30 (35)
Nordiazepam	6.46	271 > 140 (165)	160	30 (30)
Midazolam	7.05	326 > 291 (249)	200	30 (40)
Segment 4 (7.4 min)				
D5-diazepam	7.78	290 > 262	160	25
Diazepam	7.83	285 > 257 (222)	160	25 (25)
Flurazepam	8.08	388 > 315 (288)	160	25 (25)

* ( ) qualifier ions; qualifier ratios must be within 20% of calibration point

LC/MS/MS Method Evaluation

The analytical method was evaluated according to standard protocols, whereby the linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates (n = 5) over a period of 5 days. The slope of the calibration curve was forced through the origin. The typical equations of the calibration curves and correlation coefficients (R^2) are shown in Table 2; the inter-day precision and accuracy of the assay are shown in Table 3. In addition, the intra-day precision and accuracy of the assay are shown in Table 4. The assay was robust, precise, and accurate at the selected level of 100 pg/mg and was linear over the range 50 to 1,000 pg/mg. The precision for all drugs was less than 20% both within day and between days, with most benzodiazepines showing a variation of less than 10%.

Figure 1 shows a typical calibration curve for oxazepam in urine ($R^2 > 0.9996$).

Table 2. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Benzodiazepines in Hair

Analyte	Equation	Correlation (R^2)	Qualifying ratio (20% range)
7-aminoflunitrazepam	y = 0.0013x	0.9984	69.4 (55.5–83.3)
7-aminonitrazepam	y = 0.0112x	0.9678	8.6 (6.9–10.3)
7-aminoclonazepam	y = 0.0027x	0.9978	84.5 (67.6–101.4)
α-hydroxyalprazolam	y = 0.0001x	0.9992	51.7 (41.4–62.0)
α-hydroxytriazolam	y = 0.000073x	0.9964	95.5 (76.4–114.6)
Alprazolam	y = 0.001x	0.9999	15.6 (12.5–18.7)
Bromazepam	y = 0.00035x	0.9974	61.3 (49.0–73.6)
Chlordiazepoxide	y = 0.0004x	0.9996	91.3 (73.0–109.6)
Clonazepam	y = 0.0015x	0.9999	30.1 (24.1–36.1)
Diazepam	y = 0.0012x	0.9987	76.0 (60.8–91.2)
Flunitrazepam	y = 0.00038x	0.9946	56.5 (45.2–67.8)
Flurazepam	y = 0.0011x	0.9998	11.9 (9.5–14.3)
Lorazepam	y = 0.00005x	0.9832	34.5 (27.6–41.4)
Midazolam	y = 0.00064x	0.9994	31.2 (25.0–37.4)
Nitrazepam	y = 0.00026x	0.997	47.7 (38.1–57.2)
Nordiazepam	y = 0.00036x	0.9955	59.6 (47.7–71.5)
Oxazepam	y = 0.001x	0.9996	26.0 (20.8–31.2)
Temazepam	y = 0.00045x	0.9987	39.1 (31.3–46.9)
Triazolam	y = 0.00036x	0.9998	75.2 (60.2–90.2)

Table 3. Inter-Day Mean, Standard Deviation (SD), Precision (CV), and Accuracy (100 pg/mg Control Specimens; n = 5) for Benzodiazepines in Hair

Analyte	Mean	SD	CV (%)	Accuracy (%)
7-aminoflunitrazepam	103.38	13.80	13.35	96.73
7-aminonitrazepam	93.72	11.54	12.31	106.70
7-aminoclonazepam	101.50	13.57	13.37	98.52
α-hydroxyalprazolam	105.56	3.23	3.06	94.73
α-hydroxytriazolam	106.38	3.91	3.67	94.00
Alprazolam	97.70	6.77	6.93	102.35
Bromazepam	98.78	5.42	5.49	101.24
Chlordiazepoxide	95.24	9.07	9.52	105.00
Clonazepam	101.66	5.59	5.50	98.37
Diazepam	100.38	2.56	2.55	99.62
Flunitrazepam	100.52	12.24	12.18	99.48
Flurazepam	96.98	11.44	11.80	103.11
Lorazepam	107.72	12.38	11.50	92.83
Midazolam	97.18	6.38	6.57	102.90
Nitrazepam	107.90	7.03	6.51	92.68
Nordiazepam	106.14	5.25	4.95	94.22
Oxazepam	100.28	11.33	11.30	99.72
Temazepam	97.56	4.66	4.78	102.50
Triazolam	103.52	10.82	10.45	96.60

Table 4. Intra-Day Mean, Standard Deviation, Precision, and Accuracy (100 pg/mg Control Specimens; n = 5) for Benzodiazepines in Hair

Analyte	Mean	SD	CV (%)	Accuracy (%)
7-aminoflunitrazepam	99.78	5.43	5.44	100.22
7-aminonitrazepam	107.73	12.28	11.40	92.83
7-aminoclonazepam	110.58	1.94	1.75	90.43
α-hydroxyalprazolam	93.24	10.99	11.78	107.25
α-hydroxytriazolam	97.00	5.13	5.29	103.09
Alprazolam	97.72	4.28	4.38	102.33
Bromazepam	93.00	7.13	7.66	107.53
Chlordiazepoxide	91.36	7.00	7.66	109.46
Clonazepam	92.98	5.32	5.72	107.55
Diazepam	102.32	3.70	3.62	97.73
Flunitrazepam	106.24	4.87	4.59	94.13
Flurazepam	87.98	4.98	5.66	113.66
Lorazepam	99.86	5.39	5.40	100.14
Midazolam	94.52	6.79	7.18	105.80
Nitrazepam	104.48	6.63	6.35	95.71
Nordiazepam	107.38	5.32	4.96	93.13
Oxazepam	91.62	9.29	10.14	109.15
Temazepam	93.66	3.12	3.33	106.77
Triazolam	107.80	5.05	4.68	92.76

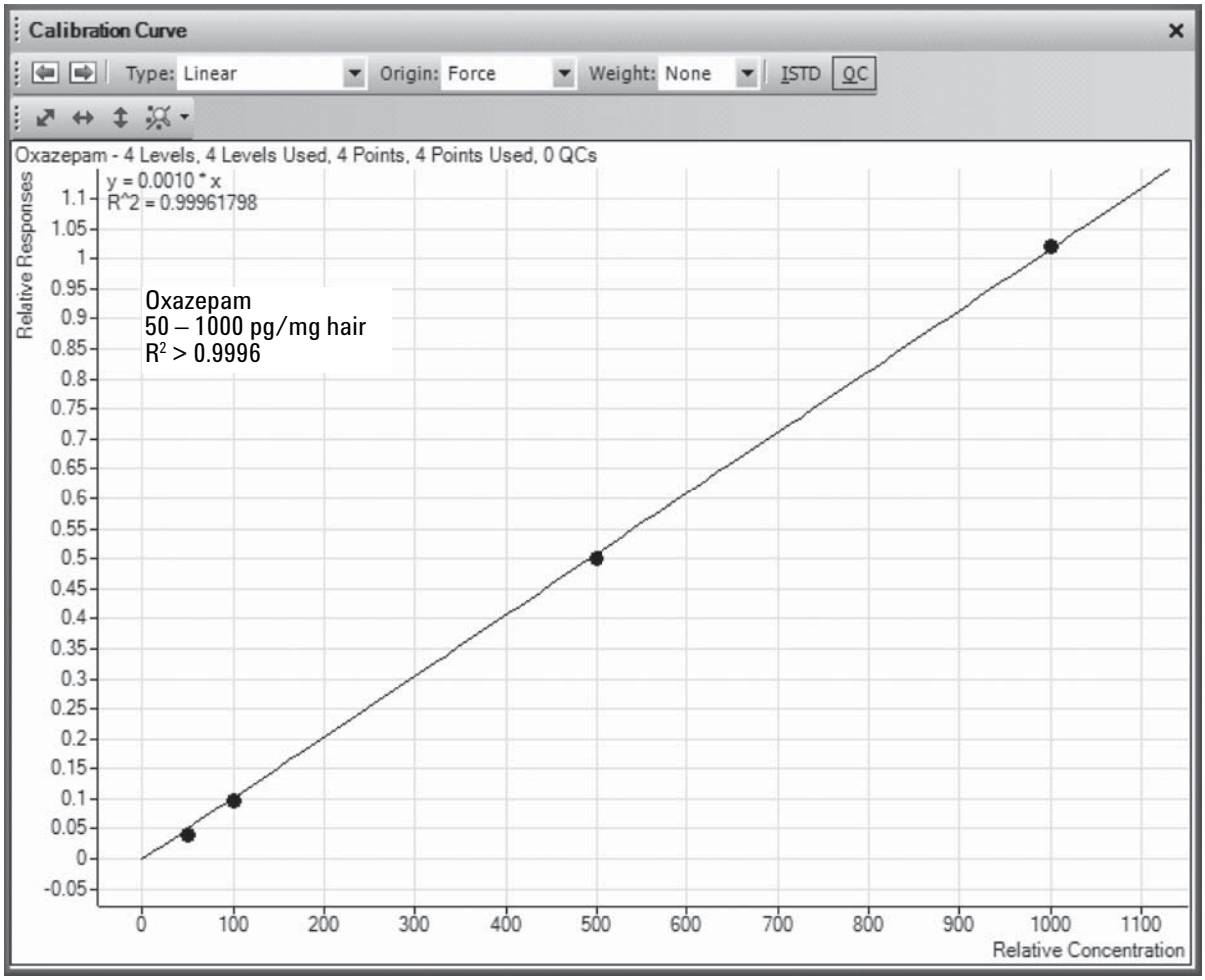

Figure 1. Calibration curve for oxazepam using a linear fit, forced origin, and no weighting.

Results and Discussion

The Agilent instrumentation allowed the rapid determination of 14 benzodiazepines and 5 metabolites in hair. The chromatographic separation produced by the small-particle analytical column allowed separation of the peaks in each group segment (Figure 2). The metabolites 7-aminonitrazepam, flunitrazepam, and clonazepam showed poor chromatography when analyzed on this LC program, so they were analyzed separately in a fast run (3.5 min).

In Figure 3 is shown the confirmation of midazolam in hair at the 50 pg/mg level. The requirement for confirmation used in this work is that the peak area ratio of the quantifier and the qualifier ions must be within a tolerance of ± 20% of the expected ratio. For this calibration level the expected ratio is 31%, which is within the tolerance of the 35% found.

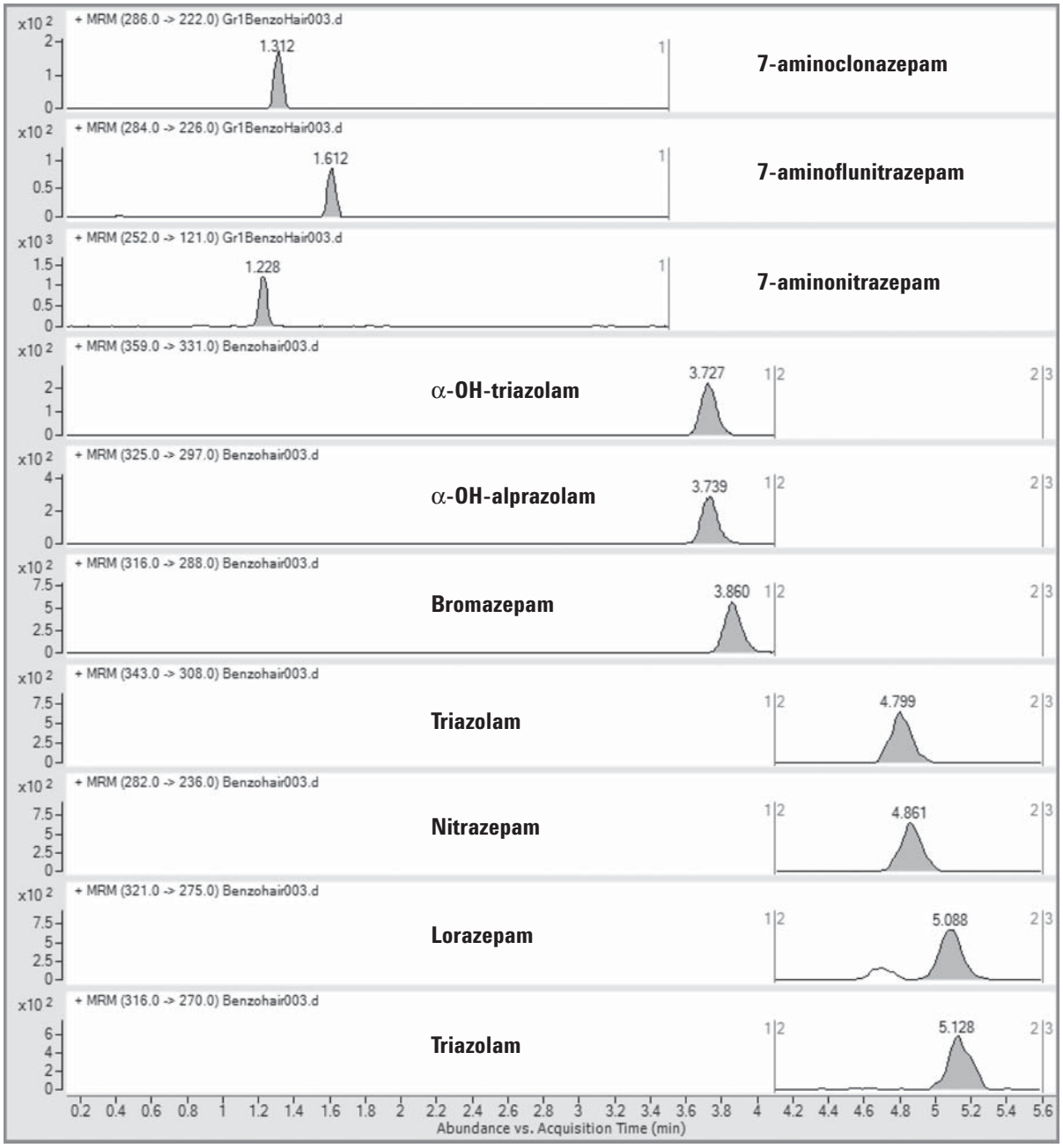

Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers).

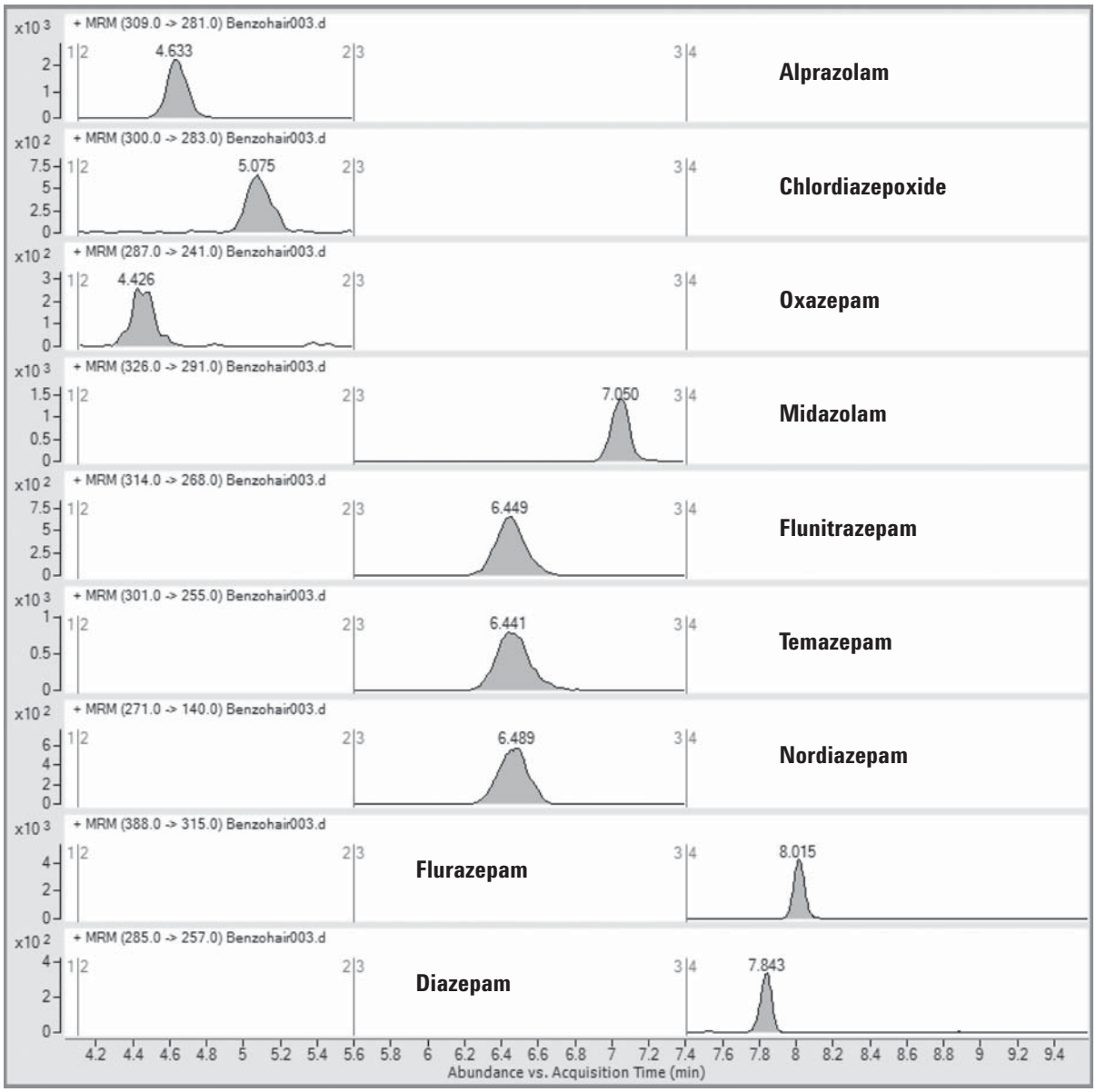

Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers). (continued)

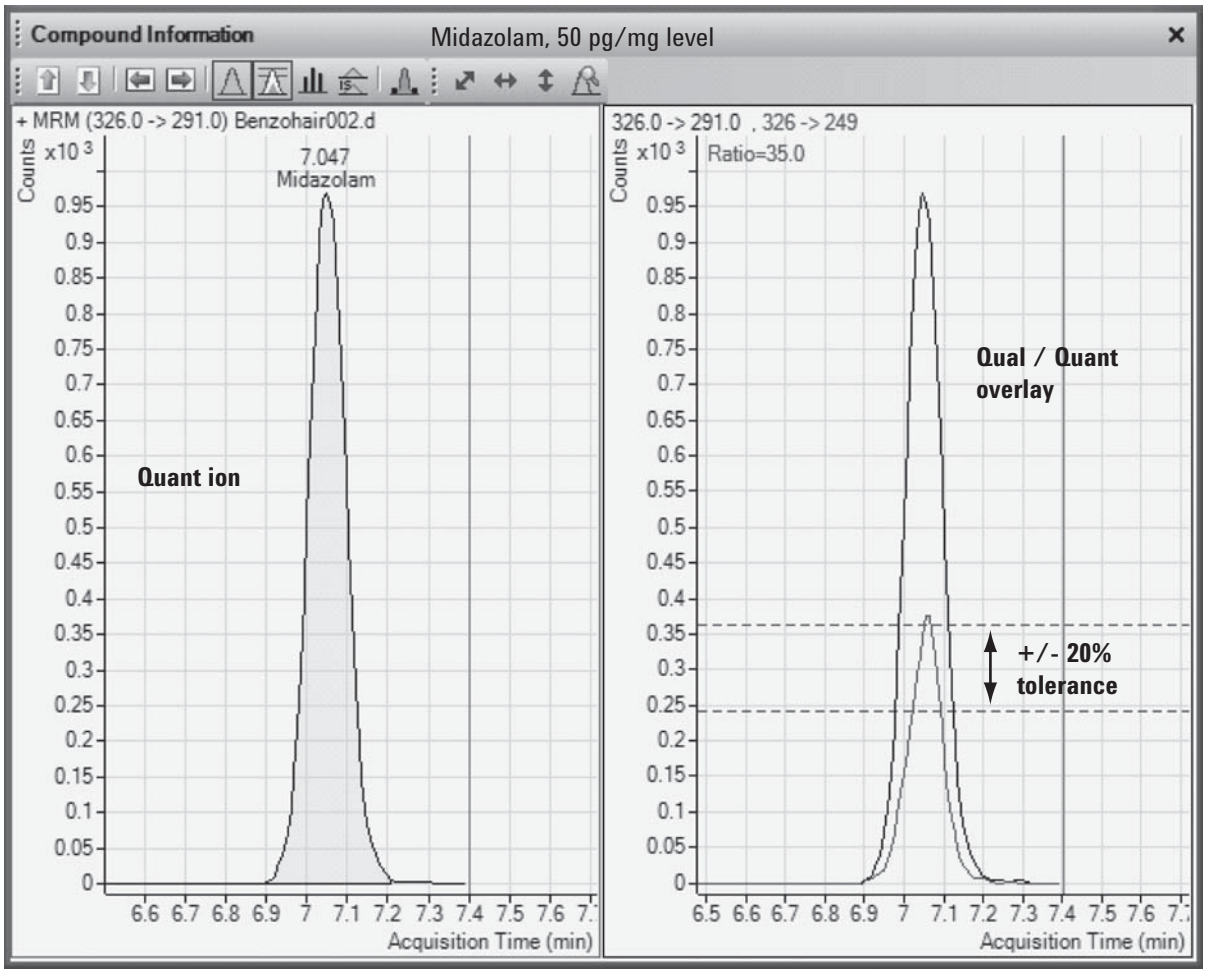

Figure 3. Confirming presence of midazolam using qualifier to quantifier ion peak area ratio.

Conclusions

The procedure described is suitable for the detection of benzodiazepines in hair using an Agilent Technologies triple quadrupole LC/MS/MS system. To our knowledge, this is the first method in which the intensity of qualifying transitions are required to be within a specific ratio compared to the primary transition.

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Quantitative Analysis of Amphetamine-Type Drugs by Extractive Benzoylation and LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A fast, sensitive technique for confirming the presence of amphetamine drugs in whole blood using the Agilent G6410A Triple Quadrupole Mass Spec-trometer (QQQ) is presented. Excellent linearity is demon-strated over the range of approximately 15 to 1,000 ng/mL. The amphetamine drugs analyzed in this work include amphetamine, methamphetamine, methyl-enedioxyamphetamine (MDA), and methylenedioxymeth-amphetamine (MDMA) in blood. The drugs have been prepared using an extractive alkylation technique. The sample preparation is then followed by reversed-phase LC/MS/MS using a 1.8-µm particle size C18 column for high chromatographic resolution with a high-speed separation. As a result, elution times for both analytes and internal standards are all less than or equal to 3.6 minutes.

Introduction

Amphetamines are a group of sympathomimetic drugs. Amphetamine (phenylisopropy-lamine) is the parent drug in this class to which all others are structurally related. Other drugs in the class include: ephedrine, pseudoephedrine, methylamphetamine, phentermine, fenfluramine, chlorphentermine, MDA, and MDMA (“Ecstasy”). The LC/MS/MS method used in this work has applica-bility to the quantitative analysis of amphetamine-like drugs in both ante- and post-mortem blood and urine samples and post-mortem liver and viscera samples. Primary and secondary aliphatic amines react with pentafluorobenzoyl chloride in alkaline conditions to form the respective amides. The method utilizes this reaction and the principle of extractive alkylation to isolate the products formed by these drugs from blood or urine.

The drugs are quantified by electrospray liquid chromatography/tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS-MRM). For purposes of quantitation, each drug analyte has a quantitative product ion monitored. For con-firmation, each analyte has an additional product ion, known as the qualifier ion, monitored. The overall ion ratio of the qualifier to the quantifier ions is fixed to a method-determined value and applied to all samples for confirming the presence of compounds. The tolerance for acceptance of this ratio is ± 20%.

For the associated D5 internal standards, only a quantifier ion is monitored because confirmation is not required.

The compounds' structures are shown in Figure 1.

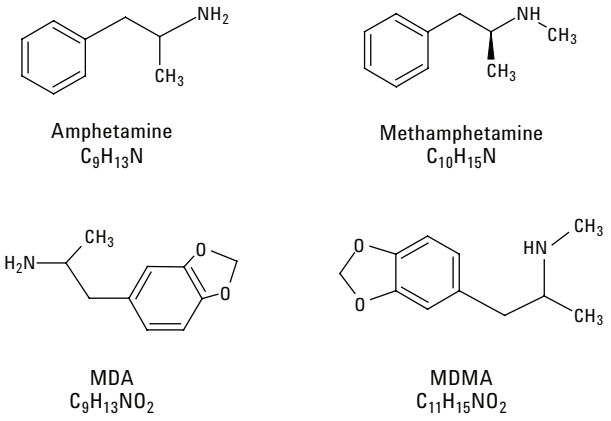

Figure 1. Structures of the compounds analyzed in this work.

Experimental

Reagents (Sigma-Aldrich, Castle Hill, NSW, Australia)

1. 5% Pentafluorobenzoyl chloride (PFBCl) (Prepare fresh by pipetting 0.25 mL PFBCl into 5 mL butyl chloride.)
2. Triethanolamine/cyclohexane (TEA/CH) (Pipette 0.5 mL of TEA/CH into a 500-mL measuring cylinder. Make final volume of 500 mL with cyclohexane. Mix and allow phases to separate.)
3. Ammonia buffer (Place 100 mL of water in a beaker. Dissolve ammonium chloride until a saturated solution is obtained. Adjust to pH 9.4 with concentrated ammonia solution.)
4. Anhydrous sodium sulphate

Standards (Cerilliant, Round Rock, TX, USA)

1. Standard reference solutions of target drugs in methanol made from solid material. The actual amounts vary slightly from one analyte compound to another and are reflected in the concentration ranges reported later. The standards are diluted in methanol and added to the blood to achieve the concentration range of approximately 15 to 1,000 ng/mL.
2. Internal Standards – 10-µg/mL mixture of D5-amphetamine, D5-methylamphetamine, D5-MDMA, and D5-MDA. The standards are purchased from commercial suppliers and are obtained as sealed ampoules, each containing approximately 100 µg of drug in 1 mL of methanol.
3. The response factor(s) is determined by addition of the standards to blood at concentrations that bracket the expected range of significant analytical results. For blood this should be equivalent to concentrations of 0.05, 0.1, 0.25, 0.5, and 1 µg/mL. A blank must be included in each analytical batch.

Sample Preparation

1. Transfer 0.2 mL blood into a 15-mL disposable test tube and dilute to 1 mL with water. Add 5 mL of the TEA/CH solution spiked with the D5-amphetamine standards mixture to a concentration of 50 nanograms per 5 mL, 0.2 mL of ammonia solution, and 0.01 mL of freshly prepared 5% PFBCl solution. Alternatively, the blood can be more conveniently sampled and diluted with the aid of an autodiluter (Hamilton Microlab Series 500) using a 0.2 to 1 mL dilution program.
2. The standard reference solutions are treated as above, with 0.2 mL of blank blood added to the diluted standards.
3. Vortex for 3 minutes, heat at 60 °C for 10 minutes, then centrifuge (see Note 4).
4. Remove the organic phase, dry by passage through a Pasteur pipette packed with anhydrous sodium sulfate, and evaporate to dryness.
5. Reconstitute the residue in 100 µL of methanol, transfer to a low-volume autosampler vial, seal, and then analyze by LC/MS/MS-MRM.

Notes:

1. The IStd (internal standard) quantity described above is equivalent to 250 ng/mL and is appropriate for concentrations in the range 10 to 1,000 ng/mL.
2. The internal standard chosen for analytes where no deuterated analogue is available must match the chemical nature of the analyte, that is, a primary amine is used for a primary amine and a secondary amine for a secondary amine.

3. An emulsion may occur during vortex mixing. It may be broken by stirring with a Pasteur pipette and recentrifuging.
4. For amphetamine, methylamphetamine, MDMA, and MDA, the reaction will proceed without the requirement for heating. If ephedrine is also to be quantitated, the heating step must be included.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consisted of an Agilent 1200 Series vacuum degasser, binary pump, autosampler, thermostatted column compartment, the Agilent G6410A Triple Quadrupole Mass Spectrometer (QQQ), and the G1948B electrospray ionization source (ESI). System control and data analysis were provided by the Agilent MassHunter B.01.01 software. Detailed LC and MS conditions are shown below.

LC/MS Method Details

LC Conditions

Column:	Agilent ZORBAX XDB-C18, 4.6 × 50 mm, 1.8 µm (p/n 922975-902)		
Column temp:	60 °C		
Mobile phase:	A = Ammonia buffer (pH = 9), see Reagents B = Methanol		
Flow rate:	0.7 mL/min		
Gradient:	Time (min)	%B	
	0 – 0.2	50	
	3.0 – 4.0	100	Post run time = 1 min.
	4.1 – 6.0	50	
Injection vol:	2 µL		

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	50 psig
Drying gas flow:	6 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 Resolution:	Unit, 0.7 amu (FWHM)
Q2 Resolution:	Unit, 0.7 amu (FWHM)

MRM settings are shown in Table 1. Note that the fragmentor voltage and dwell time for each MRM is fixed for all transitions at 140 V and 40 msec, respectively.

Table 1. MRM Settings for the Compounds Analyzed in This Work (For confirmation, the qualifier ions are also shown in parentheses.)

Compound	Precursor ion	Product ion (qualifier)	Collision Energy
Amphetamine	330	119 (91)	15
D5-Amphetamine	335	124	15
Methylamphetamine	344	119 (91)	15
D5-Methylamphetamine	349	121	15
MDMA	388	163 (135)	20
D5-MDMA	393	165	20
MDA	374	163 (135)	20
D5-MDA	379	168	20

Results and Discussion

The linearity for each compound over the range of approximately 15 to 1,000 ng/mL is shown in Figures 2a through 2d. Note that a quadratic curve fit is applied. There is no weighting and the origin is ignored. The coefficient of determination (R^2) for all four curve fits is excellent at greater than 0.999. As the second-order coefficients are all less than 0.007, see Figures 2a through 2d, making extremely low contributions to the curve fits, the resulting curves can be considered linear for all intents and purposes.

For confirming the presence of the compounds the peak area ratio of the qualifier to quantifier ions must fall within a ± 20% tolerance of an expected value derived during method development. All samples within the batch, including calibrators and quality controls (QCs), must meet this criterion or they are considered negative.

An example of the ion ratio confirmation for each compound is shown in Figures 3a through 3d.

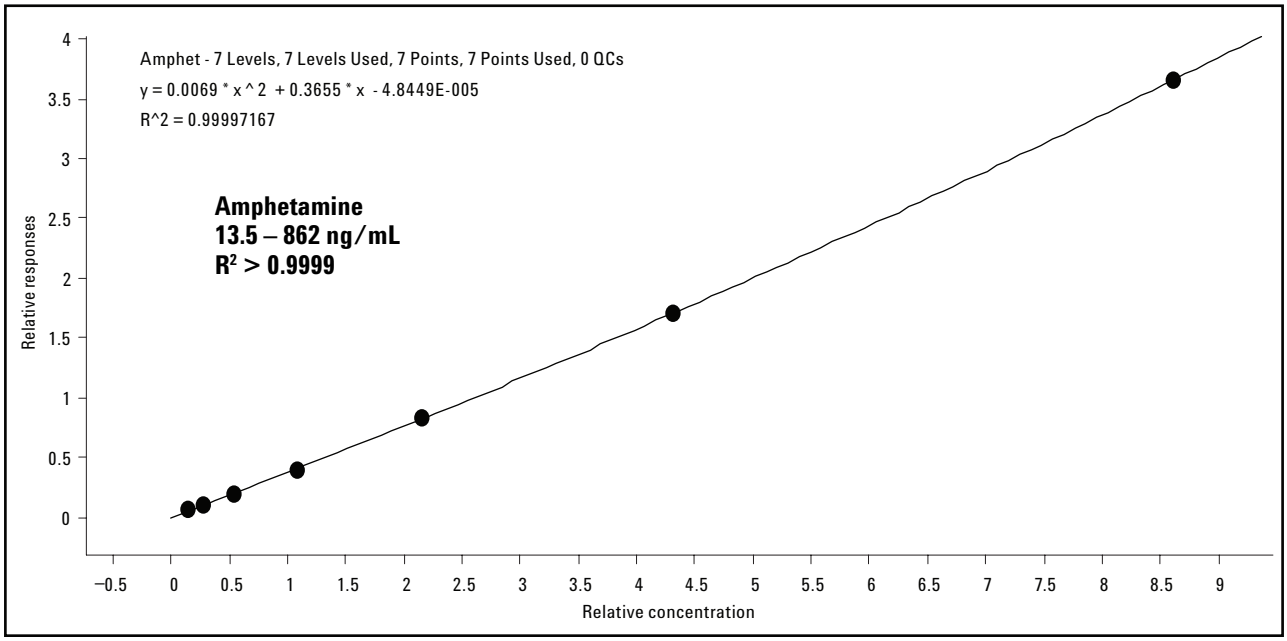

Figure 2a. Linearity of amphetamine in blood.

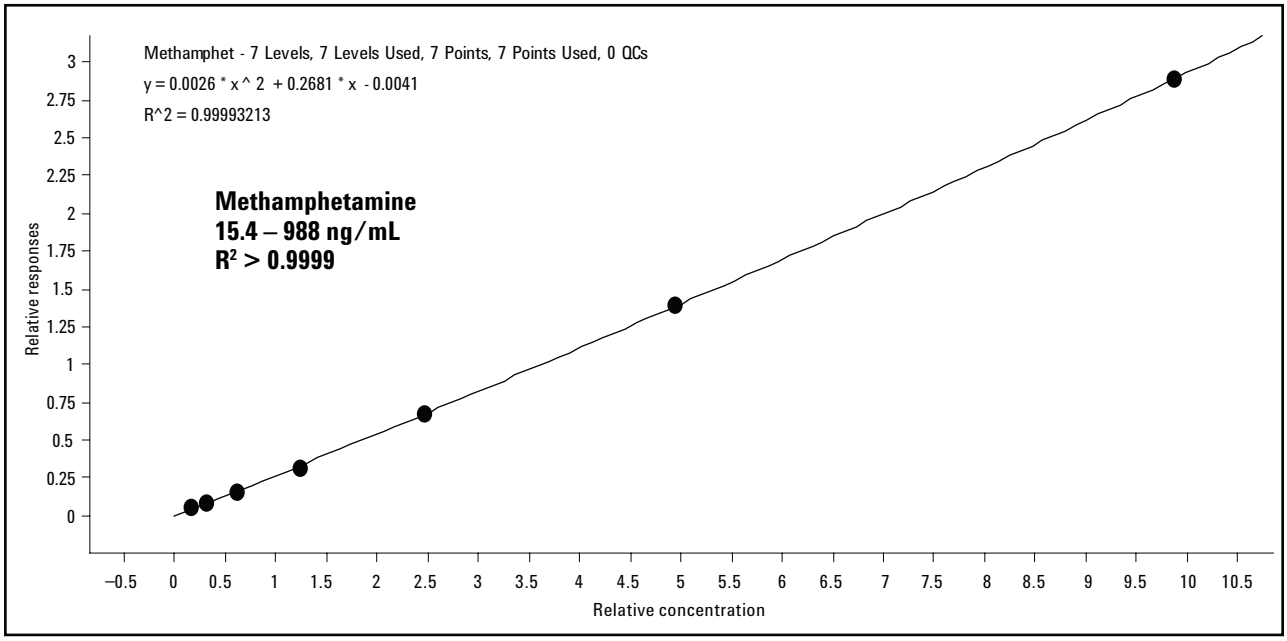

Figure 2b. Linearity of methamphetamine in blood.

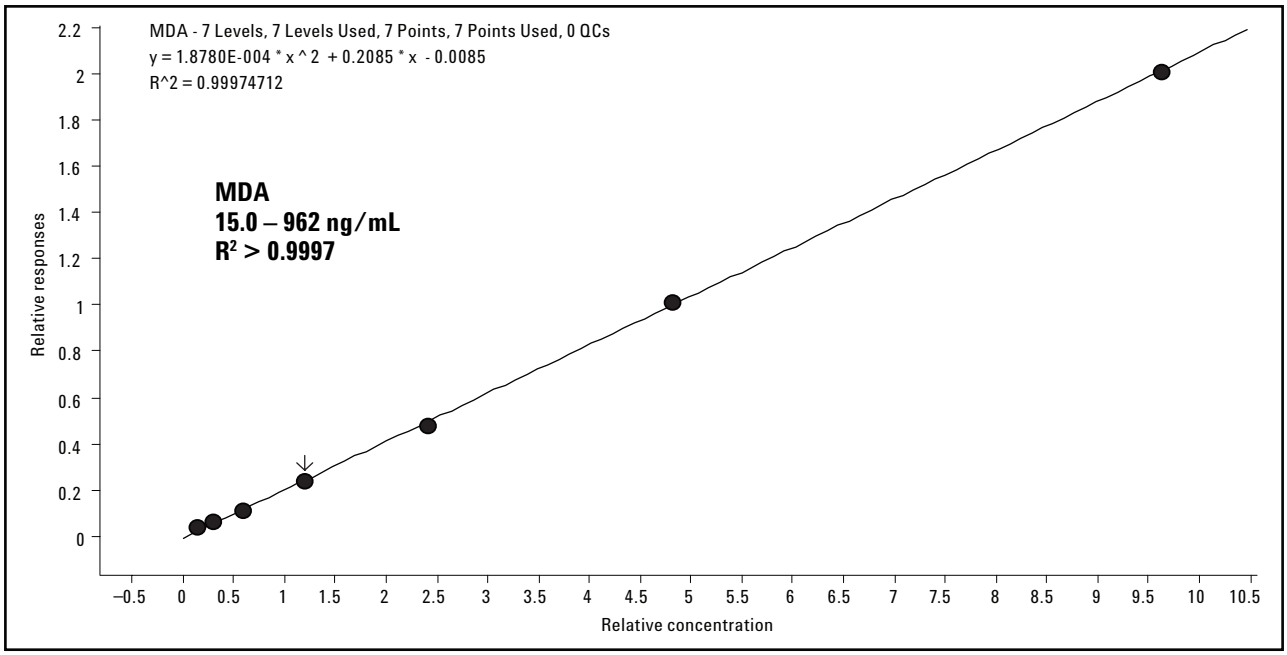

Figure 2c. Linearity of MDA in blood.

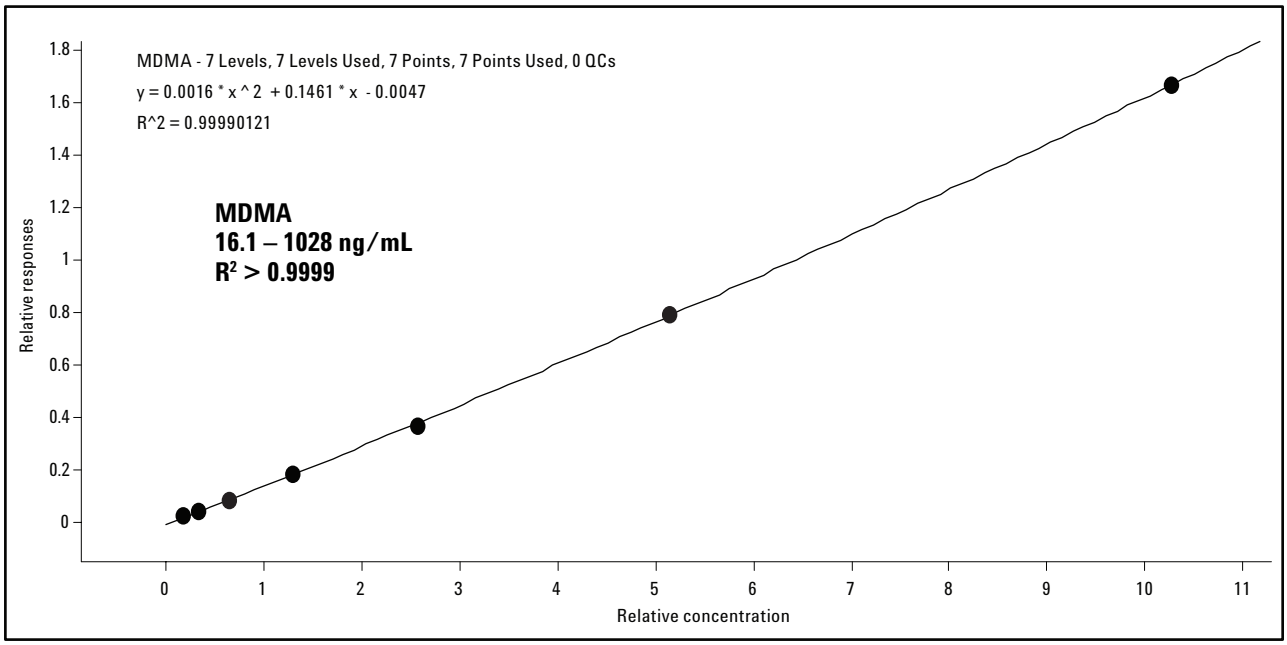

Figure 2d. Linearity of MDMA in blood.

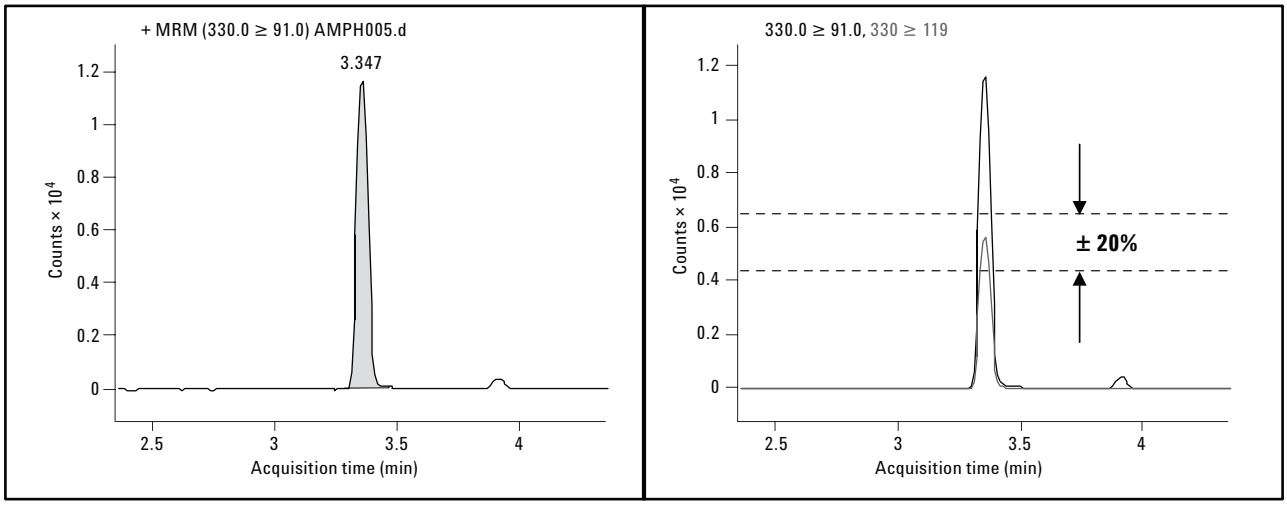

Figure 3a. Ion ratio confirmation for amphetamine in blood. Note retention time of 3.35 min.

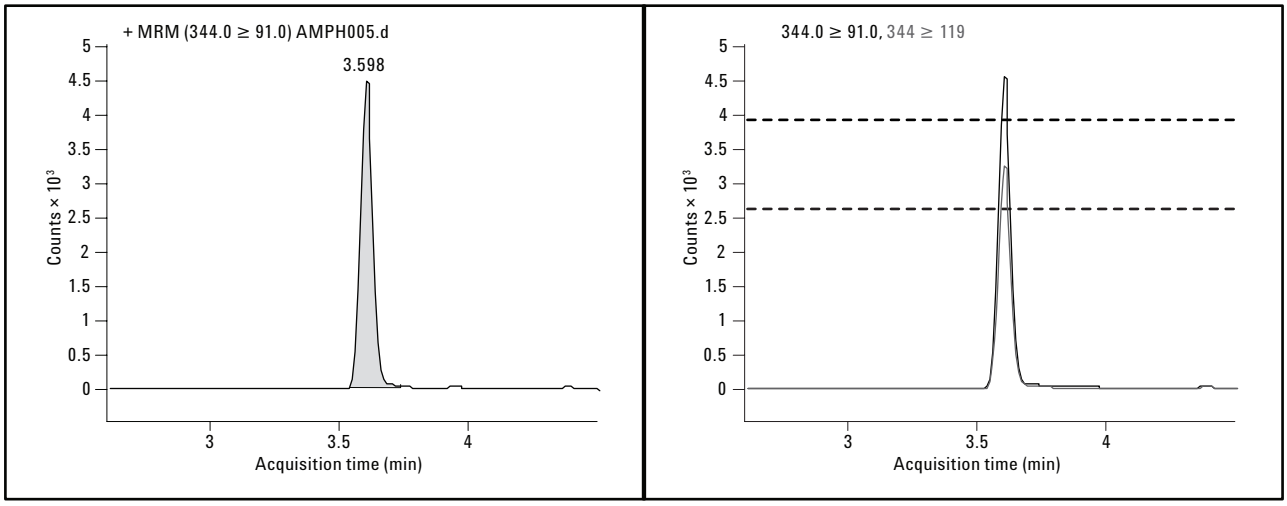

Figure 3b. Ion ratio confirmation for methamphetamine in blood. Note retention time of 3.60 min.

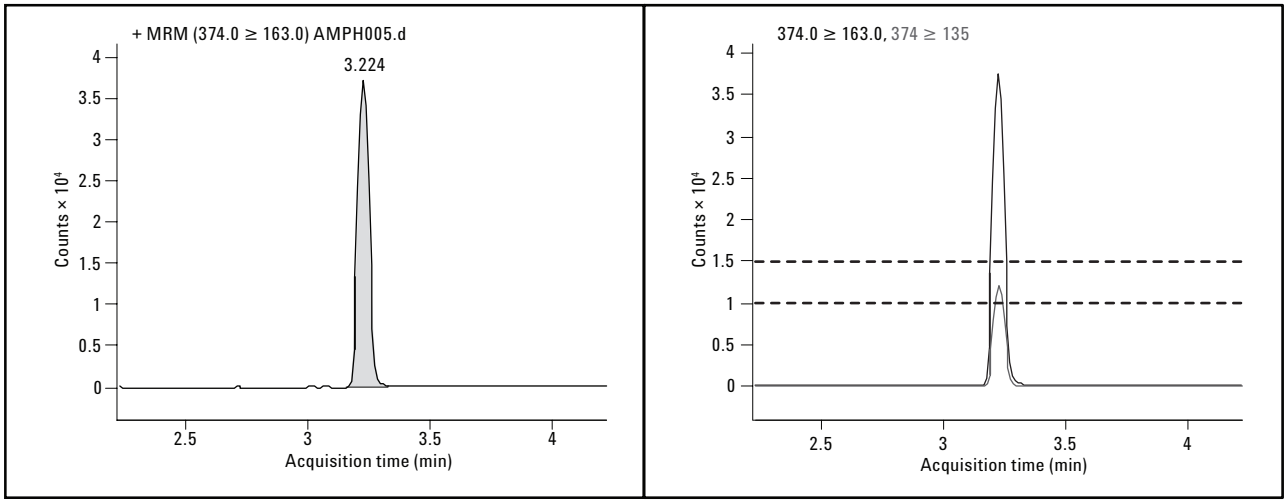

Figure 3c. Ion ratio confirmation for MDA in blood. Note retention time of 3.22 min.

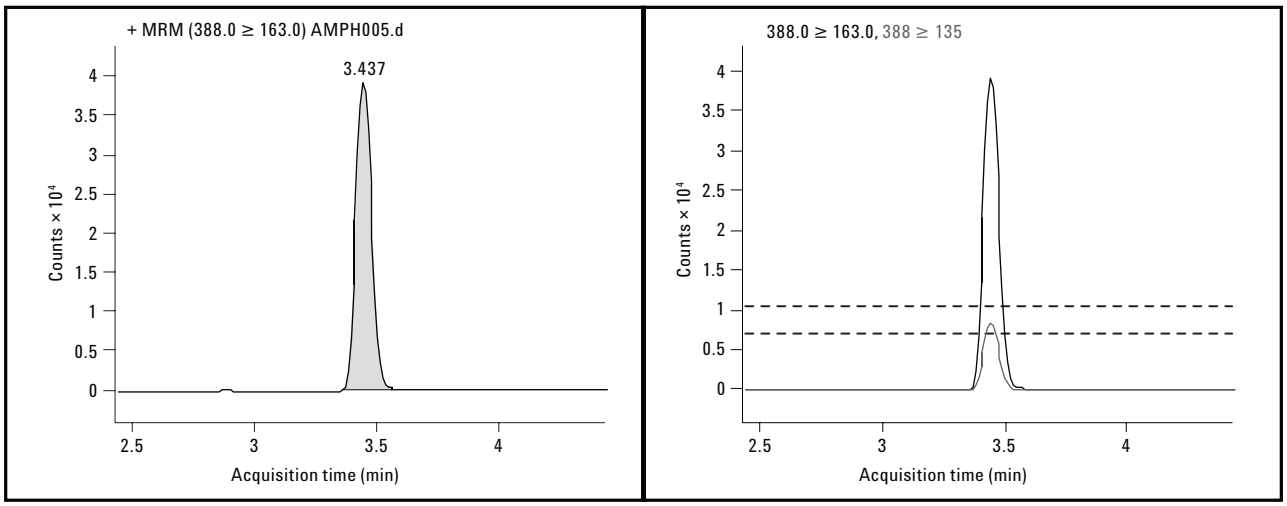

Figure 3d. Ion ratio confirmation for MDMA in blood. Note retention time of 3.44 min.

Also carried out was a study of the reproducibility of amphetamine (amp) and methamphetamine (meth) at two different concentration levels in blood. The results are tabulated below in Tables 2a and 2b in which 10 replicate injections at the 0.5 and 0.25 µg/mL in blood concentration levels are each made. The resulting peak area percent relative standard deviation (%RSD) relative response values of amphetamine and methamphetamine, with respect to the D5 IStd, at the 0.5 µg/mL level are 0.48 and 0.89, respectively. At the 0.25 µg/mL level the corresponding values are 1.12 and 2.27, respectively.

Method Evaluation

1. The method is an adaptation of a published method and an "in-house" GC-MS method that has been subject to extensive validation. The use of LC/MS/MS-MRM detection is an established technique.
2. Within-run precision has been established by statistical analysis of replicate samples.
3. Known concentrations of amphetamine and methylamphetamine from commercially available control samples and interlaboratory proficiency trials have been successfully analyzed by the method.

Table 2a. Reproducibility of Amphetamine and Methamphetamine in Blood at the 0.5 µg/mL Level

Injection number	Amp (area cts * 1000)	D5-Amp (area cts * 1000)	Relative response	Meth (area cts * 1000)	D5-Meth (area cts * 1000)	Relative response
1	918	1844	0.498	1060	1600	0.663
2	933	1887	0.494	1077	1599	0.674
3	938	1875	0.500	1087	1620	0.671
4	949	1904	0.498	1076	1627	0.661
5	948	1909	0.497	1082	1648	0.657
6	949	1911	0.497	1081	1641	0.659
7	967	1924	0.503	1109	1650	0.672
8	980	1963	0.499	1132	1689	0.670
9	986	1969	0.501	1128	1678	0.672
10	1006	2011	0.500	1145	1720	0.666
		Std dev	0.002		Std dev	0.006
		%RSD	0.484		%RSD	0.889

Table 2b. Reproducibility of Amphetamine and Methamphetamine in Blood at the 0.25 µg/mL Level

Injection number	Amp (area cts * 1000)	D5-Amp (area cts * 1000)	Relative response	Meth (area cts * 1000)	D5-Meth (area cts * 1000)	Relative response
1	236	966	0.244	167	515	0.324
2	243	957	0.254	173	506	0.336
3	247	972	0.254	173	513	0.342
4	246	972	0.253	166	518	0.324
5	246	973	0.253	175	516	0.338
6	245	978	0.251	173	514	0.335
7	250	994	0.252	176	512	0.342
8	248	989	0.251	178	526	0.348
9	254	1004	0.253	175	536	0.333
10	253	1005	0.252	179	536	0.334
		Std dev	0.003		Std dev	0.008
		%RSD	1.126		%RSD	2.270

4. A calibration curve is established on an analytical batch basis by addition of a range of concentrations of standard amphetamines to blank blood or urine. The method has been shown to be linear in the concentration range of 15 to 1,000 ng/mL. For results greater or less than this range, the result should be reported as "greater than" or "less than." Alternatively, report the result as approximate or the sample may be reanalyzed with the standard range extended to include the concentration encountered.
5. The uncertainty of the method determined from control data and precision studies is 10% at the 95% confidence level.

Conclusions

The LC/MS/MS method described here provides a procedure for the quantitation and confirmation of multiple drugs of abuse in whole blood with very fast analysis times. The multiple reaction monitoring of several fragmentation transitions is carried out not only for quantitation using designated quantifying ions, but also for confirmation using designated qualifier ions. Using the Agilent C18 column with 1.8-µm particle size allows for excellent resolution and peak shape at a relatively high flow rate of 700 µL/min for a 4.6-mm id column and an ESI interface. Less than 1% RSD relative response is shown for both amphetamine and methamphetamine at the 0.5 µg/mL level in blood.

Acknowledgments

A special thanks to Agilent colleague John M. Hughes for very valuable review and comments.

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Detection of Phencyclidine in Human Oral Fluid Using Solid Phase Extraction and Liquid Chromatography with Tandem Mass Spectrometric Detection

Application Note

Forensic Toxicology

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Abstract

An analytical procedure for the determination of phencyclidine in oral fluid has been developed and evaluated using liquid chromatography with tandem mass spectral detection, following initial screening with enzyme-linked immunosorbent assay. The oral fluid samples were collected using the Quantisal™ device, and any drugs present were quantified using mixed mode solid-phase extraction followed by mass spectrometric detection in positive atmospheric pressure chemical ionization mode. For confirmation, two transitions were monitored and one ratio determined, which had to be within 20% of that of the known calibration standard. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion has the potential of limiting the sensitivity of the assay. However, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. The limit of quantitation was 5 ng/mL; the intraday precision of the assay was 3.04% (n = 5); interday precision was 3.35% (n = 5). The percentage recovery of phencyclidine from the oral fluid collection pad was 81.7 % (n = 6). The methods were applied to both proficiency specimens and to samples obtained during research studies in the USA.

Introduction

Oral fluid is increasing in popularity as an alternative matrix to blood or urine for standard drug testing due to its ease of collection, difficulty of adulteration, and the improving sensitivity of analytical techniques. Phencyclidine (PCP) is included in the proposed United States federal regulations for saliva drug testing in the workplace, and the suggested cut-off concentration is 10 ng/mL of neat oral fluid. Surprisingly, there are no published procedures for the determination of PCP in oral fluid using liquid chromatography with tandem mass spectrometry (LC/MS/MS). However, there is one method for its analysis in rat plasma [1]. Other methods for the determination of PCP in blood [2], urine [3], hair [4], and meconium [5] have been reported, which incorporate the more standard gas chromatography-mass spectrometry instrumentation.

There are publications describing the analysis of various other drugs of abuse in oral fluid using LC/MS/MS in APCI mode, in a similar manner to our approach; however, many of these procedures monitor only one transition in the multiple-reaction monitoring mode (MRM). Recently,

several authors have focused on the need to monitor a second transition, allowing the ratio between the abundance of the primary and secondary ions to be calculated and establishing more confidence in the final result. Maralikova and Weinmann noted that guidelines for confirmatory analysis using LC/MS/MS have not yet been established, and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs [6].

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any quantitative results meaningless without further manipulation in the laboratory [7]. Furthermore, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used [8].

The work presented here employed the Quantisal™ oral fluid collection device, which collected a known amount of neat oral fluid. The recovery efficiency of PCP from the collection pad into the transportation buffer was determined in order to increase confidence in the quantitative value. The stability of the drugs in the buffer at room temperature and at 4 °C was studied, as well as the stability of extracted oral fluid specimens.

We have evaluated a procedure for the determination of PCP in oral fluid that provides forensic defensibility for the generated result in terms of specimen volume, drug recovery from the collection pad, and LC/MS/MS with two monitored transitions. The method is applied to specimens received into our laboratory from proficiency programs and research studies.

The structure of PCP is shown in Figure 1.

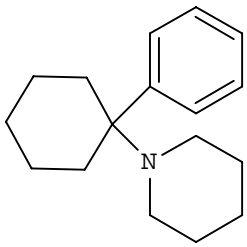

Figure 1. Structure of phencyclidine (PCP).

Experimental

Sample Preparation

Oral Fluid Collection Devices
Quantisal™ devices for the collection of oral fluid specimens are obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (± 10%) is collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal™ collection devices, and drug concentrations detected are adjusted accordingly.

Standards and Reagents
The Phencyclidine Direct ELISA kit (Catalog #208) was obtained from Immunalysis Corporation (Pomona, CA) and used for screening the oral fluid samples. For confirmatory procedures, pentadeuterated internal standard (phencyclidine-d5) as well as unlabeled drug standard were obtained from Cerilliant (Round Rock, TX). Solid phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare, (San Pedro, CA). All solvents were HPLC grade or better, and all chemicals were ACS grade.

Calibrators
For the chromatographic calibration standards, a working solution for the deuterated internal standard was prepared in methanol at a concentration of 250 ng/mL. Unlabeled drug standard was prepared in methanol at the same concentration. All the working solutions were stored at –20 °C when not in use. For each batch, four calibration standards were prepared in synthetic oral fluid (1 mL), then transportation buffer from the Quantisal™ collection device was added (3 mL). Drug concentrations of 5, 10, 20, and 40 ng/mL of neat oral fluid equivalents were prepared (internal standard concentration: 20 ng/mL).

Screening Assay
Enzyme linked immunosorbent assay (ELISA) technology is based upon the competitive binding to antibody of enzyme-labeled antigen and unlabeled antigen in proportion to their concentration in the reaction well. The oral fluid specimens were screened according to the manufacturer's instruc-

tions, which recommended cut-off concentrations of 10 ng/mL for phencyclidine; of neat oral fluid equivalents. A standard curve consisting of a drug-free negative oral fluid specimen and drug-free oral fluid specimens spiked at 50% and 200% of the recommended cut-off concentrations was analyzed with every batch. The optimal sample size as suggested by the manufacturer was 10 µL. The sample volume was pipetted directly from the collection device into the microplate. Specimens screening positively using ELISA were carried forward to confirmation using the described procedure.

Sample Preparation for Chromatographic Analysis

An aliquot (1 mL) from the Quantisal™ collection device, equivalent to 0.25 mL of neat oral fluid, was removed and internal standard (20 µL) was added. 0.1 M sodium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control, or oral fluid specimen. Solid-phase mixed mode extraction columns (Clin II, 691-0353T) were placed into a positive pressure manifold. Each column was conditioned with methanol (2 mL), and 0.1 M phosphate buffer (pH 6.0; 2 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (1 mL), 0.1 M acetate buffer (pH 4; 1 mL), methanol (1 mL), and ethyl acetate (1 mL). The columns were allowed to dry under nitrogen pressure (30 psi; 2 min). The drugs were finally eluted using freshly prepared ethyl acetate/ammonium hydroxide (98:2 v,v; 2 mL). The extracts were evaporated to dryness under nitrogen and reconstituted in 70:30 v/v of 20 mM ammonium formate (pH 6.4) and methanol (40 µL).

Analytical Procedure

Instrument:	Agilent 1200 Series RRLC; 6410 LC Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX Eclipse XDB C18, 4.6 mm x 50 mm x 1.8 µm, (p/n 822795-902)
Column temperature:	40 °C
Solvent flow rate:	0.6 mL/min
Mobile phase:	A = 20 mM ammonium formate, pH 6.4 B = methanol
Injection volume:	5 µL

Gradient:

Time (minutes)	%B	Flow rate (mL/min)
0	25	0.9
1.5	30	0.9
4.5	55	1
5	60	1
7	75	1

Stop time = 7 min; Post time = 3 min

MS Conditions:

Operation:	Positive APCI mode
Gas temperature:	350 °C
Gas flow (N_2):	5 L/min
Nebulizer pressure:	50 psi
Capillary voltage:	4500 V

The multiple reaction monitoring (MRM) transitions are shown in Table 1. Derived retention times are also given. For all transitions the first quadrupole, for the precursor ion, is operated at wide resolution, or full width half maximum (FWHM) equal to 2.5 amu. The last quadrupole, for the product ions, is operated at unit resolution, or FWHM equal to 0.7 amu. Finally, the dwell time for each transition is 75 msec.

Table 1. Multiple Reaction Monitoring (MRM) Transitions for Phencyclidine and Its Deuterated Analog (D5), Used as the Internal Standard (IStd)

Compound	RT (min)	MRM transition	Frag (V)	CE (V)
PCP	6.1	244.3 > 91.2 (86.2)	40	25 (25)
PCP-D5	6.1	249.3 > 164.3	40	15

* ( ) qualifier ions; qualifier ratios must be within 20% of calibration point

Results and Discussion

Data Analysis

Calibration using deuterated internal standard was calculated using linear regression analysis over a concentration range of 5 to 40 ng/mL. Peak area ratios of the target analyte and the internal standard were calculated using MassHunter software (Agilent). The data were fit to a linear least-squares regression curve with no weighting and was not forced through the origin.

Method Development

The development of a simple LC/MS/MS assay for the detection of phencyclidine in oral fluid is reported. While these drugs have been detected in oral fluid, the increasing utility of LC/MS/MS in

laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying ion is reported for the first time for the determination of PCP in oral fluid analysis and is necessary for the improved confidence in the identification of the analyte.

Method Evaluation

The chromatographic procedure developed for PCP was evaluated according to accepted protocols. The limit of quantitation was 5 ng/mL and was determined as described in the Experimental section. Linearity was obtained with an average correlation coefficient for all the drugs of > 0.99 over the dynamic range from 5 to 40 ng/mL of oral fluid. The mean correlation for the calibration curve was R^2 = 0.99644 (n = 6) with an average slope equation of y = 0.1531x, where x = concentration of PCP and the relative response, y, = peak area response of the drug/peak area response of the internal standard. An example of one of the calibration curves is shown in Figure 2.

Method of Confirmation

Two product ions from fragmentation of PCP were monitored. The most intense (*m/z* = 91.2) was used for quantitation. The least intense of the two (*m/z* = 86.2) was used as a qualifier for ion ratio confirmation. That is, the ratio of the two peak areas must have been consistent, and within a tolerance of ± 20%, to be considered acceptable. The allowable qualifying ratio for the intensity of the second transition is 59.6% to 89.5% (± 20% of 0.74) and applied across all batches. An example at the lowest calibration level of 5 ng/mL is shown in Figure 3.

Recovery and Interference

The recovery of PCP from the collection pad using the Quantisal™ device was determined to be 81.67% (SD 1.17; n = 6). Oral fluid specimens collected from drug-free individuals showed no interference with any of the assays, which was not unexpected, since it is unlikely that these drugs

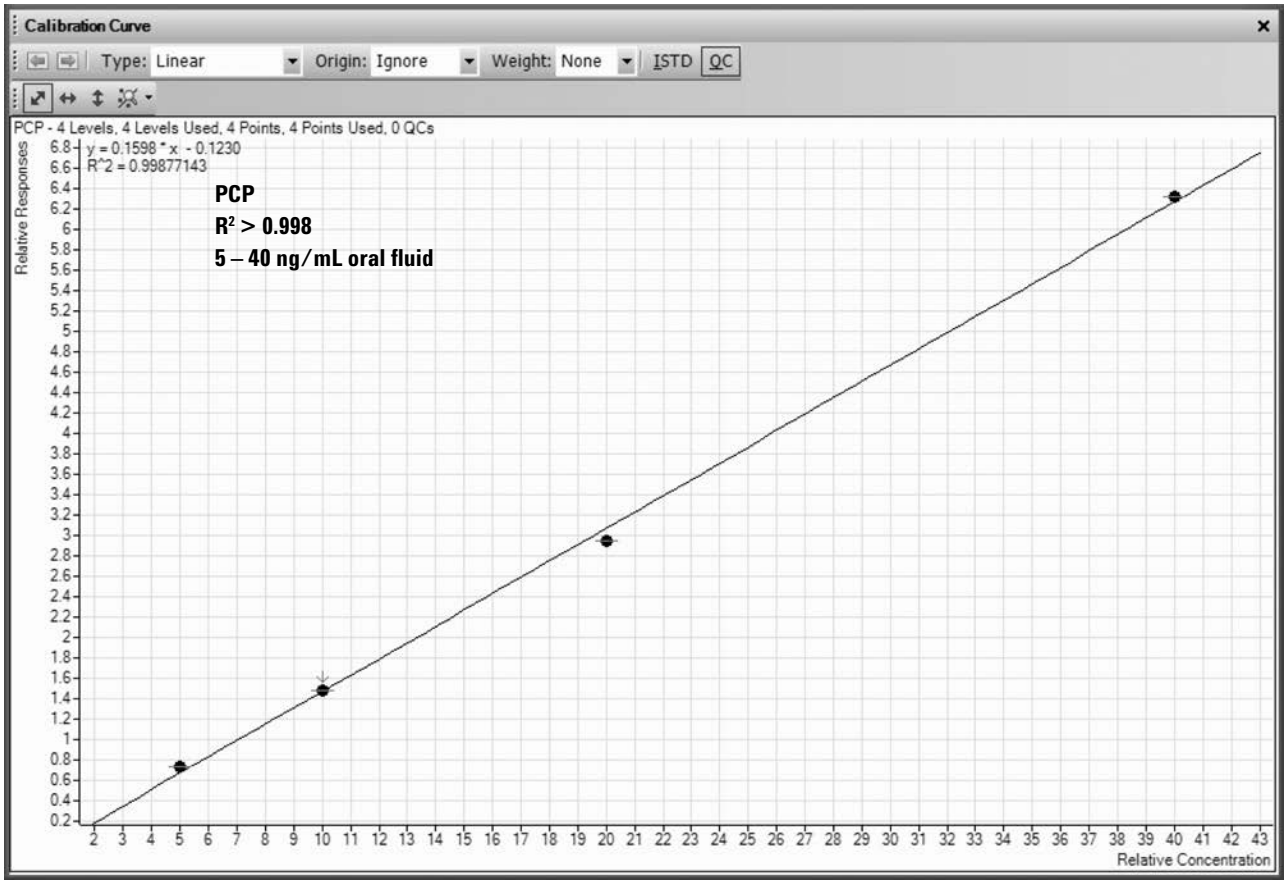

Figure 2. Linearity of PCP.

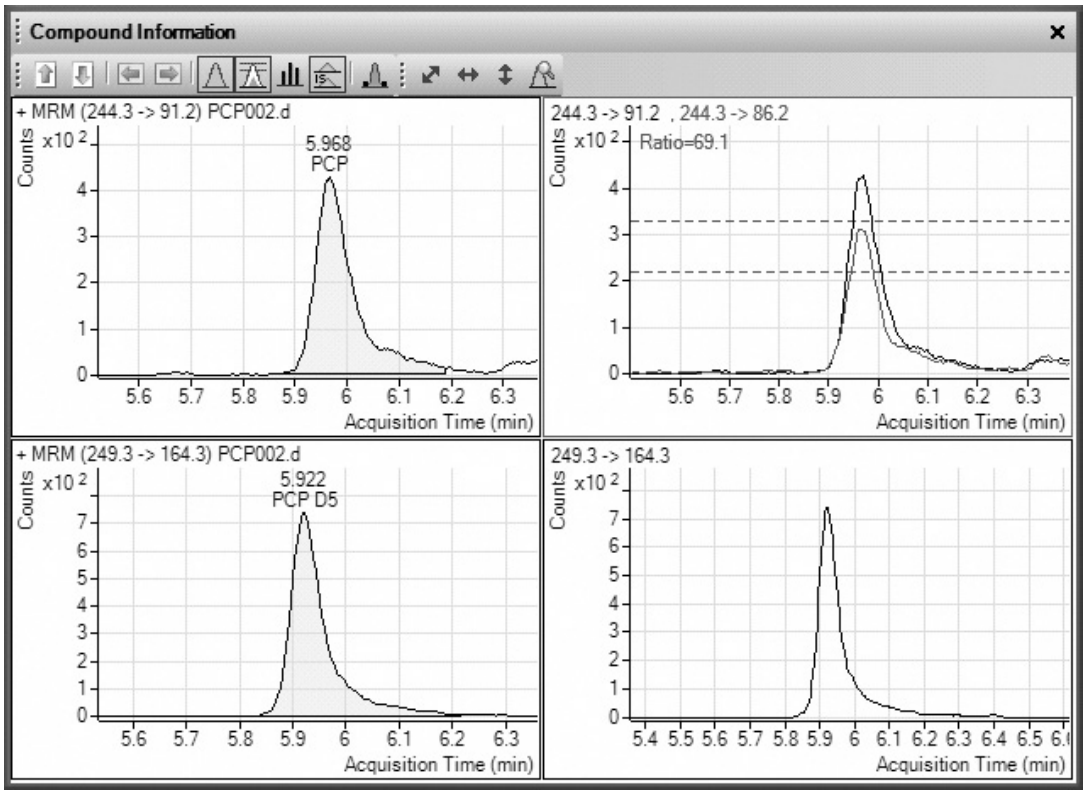

Figure 3. Confirming the presence of PCP using quant/qual ion ratios. In this example, the ratio of the lowest calibration level of 5 ng/mL is 0.69, which is within 20% of 0.74.

are similar to endogenous substances in oral fluid. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Experimental section. No chromatographic interference was observed in the channels of these transitions. Since the oral fluid was diluted during collection and the drugs are extracted using a specific solid-phase procedure, ion suppression of any significance was not observed.

Precision, Accuracy, and Stability

The accuracy of the assay was determined as described and the results are shown in Table 2. The procedure was very accurate, with a maximum variation of –6.5% from the fortified level at the cut-off concentration. The interday (between-day) and intraday (same-day) precision of the assay was determined using replicate analyses as described. The interday precision was 3.35% (n = 5); intraday precision was 3.04% (n = 5). Finally, the stability of the drugs in the collection system and the stability of the extracts were assessed. The extracts were stable for at least 2 days when kept in the instrument rack inside the autosampler, which was maintained at 4 °C. There was less than a 5 % difference in the quantitation of the extracts after 48 hours.

Table 2. Interassay Accuracy from Six Analytical Runs

Nominal concentration	5 ng/mL	10 ng/mL	20 ng/mL	40 ng/mL
Assay run #1	4.7	9.5	21	39
2	5.4	9.0	19	40
3	5.3	9.2	19	40
4	5.6	9.2	18	38
5	5	9.8	18	42
6	5	9.4	21	39
Mean (ng/mL)	5.1	9.3	19.8	40
Accuracy (%)	3.3	–6.5	–3.3	–0.83

Table 3. Intraday and Interday Reproducibility Monitoring the 10 ng/mL Control Level

Nominal concentration	Interday (n = 5)	Intraday (n = 5)
	9.5	10
	9.0	10.8
	9.2	10.7
	9.2	10.7
	9.8	10.5
Mean (ng/mL)	9.34	0.54
Std Dev.	0.31	0.32
Accuracy (%)	3.35	3.04

Authentic Specimens

The procedures were applied to proficiency specimens received into the laboratory. The performance was excellent, with all quantitation being within 10% of the group mean identified by the program administrators. An example of an authentic oral fluid specimen at a concentration of 14.7 ng/mL is shown in Figure 4.

Conclusions

The determination of PCP in oral fluid is described. The LC/MS/MS procedure is reproducible, robust, and precise. The assay includes the monitoring of a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for definitive identification to be made. The method is easily incorporated into routine forensic laboratory testing.

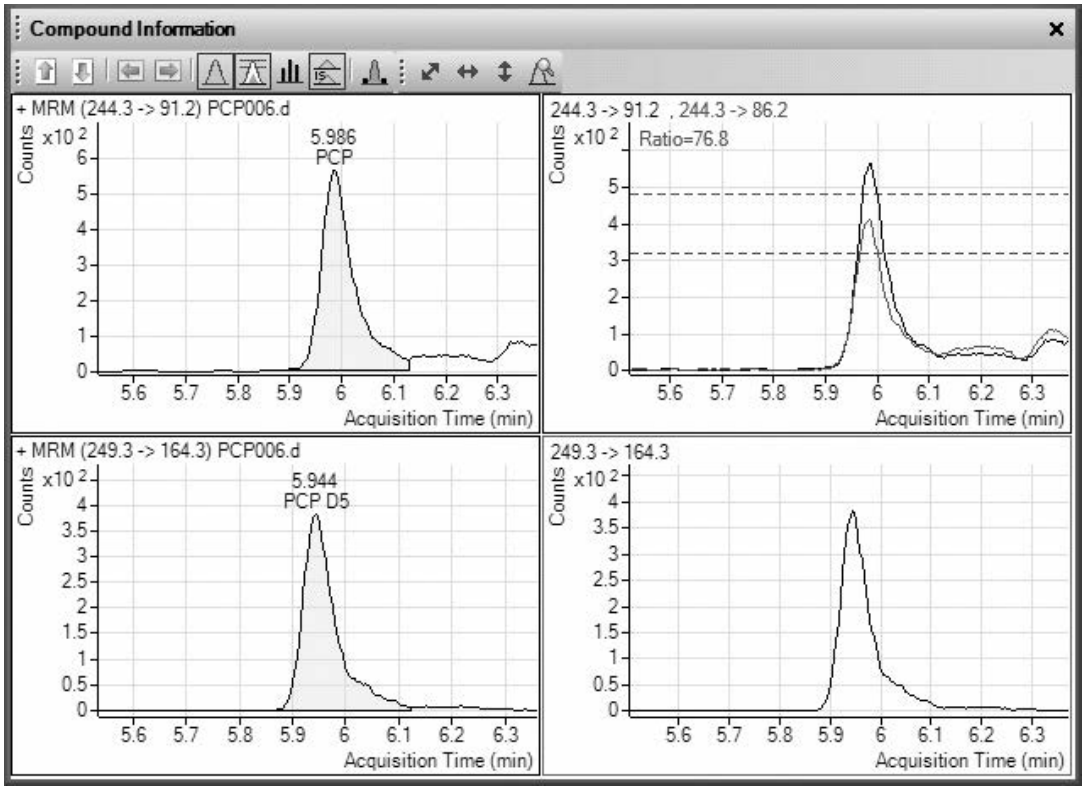

Figure 4. Confirming the presence of PCP using quant/qual ion ratios in an actual volunteer sample at a level of 14.8 ng/mL.

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Analysis of Cannabinoids and Amphetamines in Serum by RRLC / Triple Quadrupole Mass Spectrometry Using a Multimode Ion Source

Application Note

Forensic Toxicology

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Abstract

LC / MS / MS is a useful analytical technique for the analysis of amphetamines and cannabinoids in biological matrices. Amphetamines ionize well in electrospray ionization (ESI), whereas cannabinoids exhibit better sensitivity with atmospheric pressure chemical ionization (APCI). Using a 1.8-µm particle size RRHT column for the LC separation, the Agilent G1978B multimode ion (MMI) source was utilized in order to achieve a balanced response for both compound classes in a single analysis. The presented method exhibits good within-day and day-to-day reproducibility. The coefficients of variation ranged from 3 to 15%; most of the coefficients were in the 5 to 10% range.

Introduction

Driving after consumption of cannabis and amphetamines, including their methylene-dioxy-derivatives methylenedioxymethampheta-mine (MDMA) and methylenedioxyethylampheta-mine (MDE), was sanctioned by the German Road Traffic Act in 1998. Since then, the number of toxicological analyses of serum for Δ9-tetrahydro-cannabinol (THC) or amphetamine derivatives has increased enormously. Therefore, forensic laboratories need analytical methods that can handle a large number of samples in a relative short time. An appropriate technique to meet these needs is LC/MS/MS.

Amphetamines are basic polar compounds and ionize well in electrospray ionization (ESI), whereas the relatively nonpolar cannabinoids exhibit better sensitivity with atmospheric pressure chemical ionization (APCI) (Figure 1). To use the optimum ionization technique for each class of drug in a single run, the Agilent G1978B multimode ion (MMI) source (Figure 2) was evaluated in order to achieve a balanced response for both compound classes. The MMI source can operate in either ESI or APCI modes or in “mixed” mode, which is simultaneous ESI and APCI. The choice and parameters of ionization mode can be time-programmed during the run.

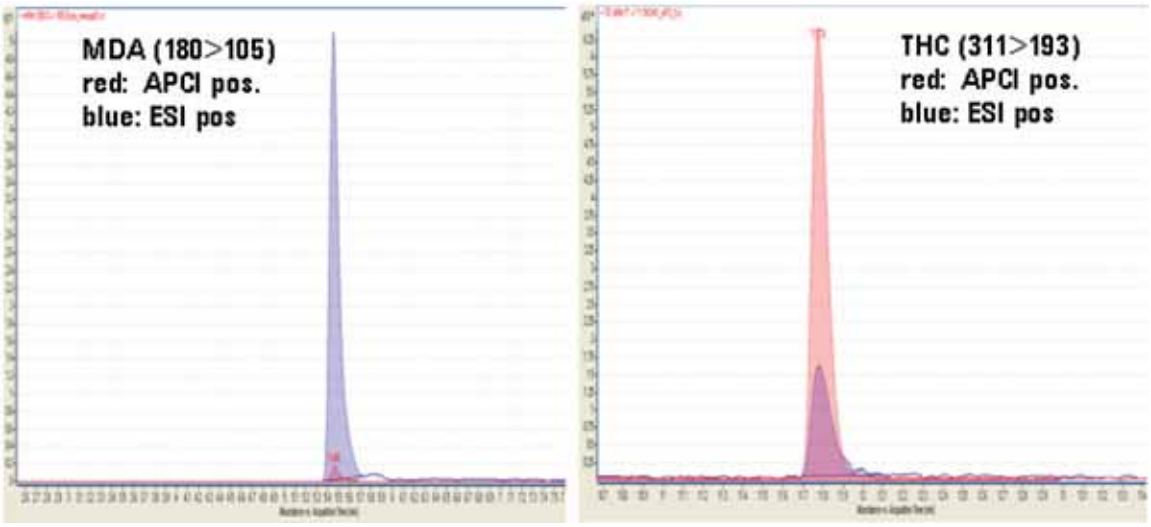

Figure 1. Comparison of the MDA and THC response in ESI and APCI modes.

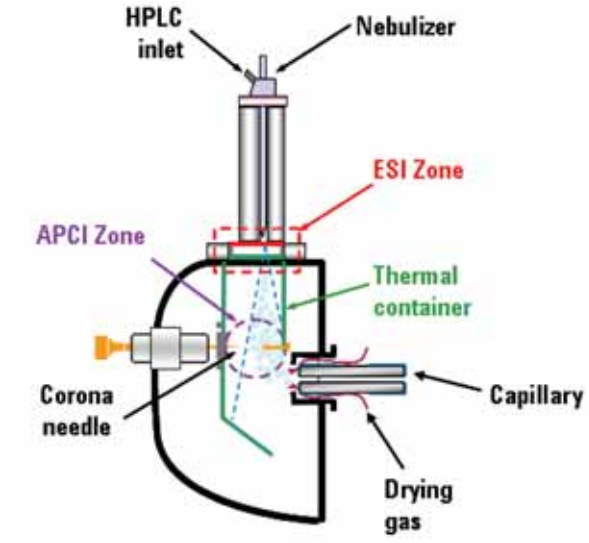

Figure 2. Design of the multimode source.

Experimental

Reagents

All solvents and reagents were analytical grade. Methanol, acetone, acetic acid, dichloromethane, 2-propanol, and ammonia were purchased from E. Merck (Darmstadt, Germany) or from Sigma-Aldrich (Deisenhofen, Germany). Solid-phase extraction columns were purchased from Mallinckrodt Baker (Griesheim, Germany), and all drug standard solutions and deuterated compounds were purchased from Cerilliant (Austin, TX).

Sample Preparation

A 1-mL sample of serum was diluted with 6 mL of phosphate buffer (0.1 M, pH 6). Then 50 µL of the internal standard mixture was added (1 ng/µL each of methanolic solution of D_{11}-amphetamine, D_{11}-methamphetamine, D_5-MDA, D_5-MDMA, D_6-MDE, and D_9-THC-COOH and 0.1 ng/µL each of D_3-THC and D_3-THC-OH). The sample was mixed for 3 minutes and the mixture was centrifuged at 3,000 rpm for 10 minutes. Solid-phase extraction was either automated, using the Caliper RapidTrace SPE workstation, or was done manually, using a vacuum manifold.

The supernatant was applied to a solid-phase extraction column (Bakerbond SPE C18, 500 mg), which had been conditioned by flushing with 2 × 3 mL of methanol and 2 mL of water. The column was rinsed with 2 × 2 mL water, 2 × 2 mL water/methanol (80:20; v/v), and 1 mL of 0.1 M acetic acid. The column was dried for 10 minutes.

The elution was carried out in two steps. First the cannabinoids were eluted with 3 mL of dichloromethane/acetone (50:50; v/v), followed by elution of amphetamines, opiates, and cocaine/metabolites with 3 mL of dichloromethane/2-propanol/ammonia (40:10:2; v/v/v). Both extracts were evaporated under a slight stream of nitrogen at 30 °C, reconstituded in 0.1 mL methanol, and added together. This combined SPE fraction was diluted with water (ratio 1:4) to improve the chromatographic peak shape.

LC / MS / MS Method

The LC/MS/MS consisted of an Agilent 1200 Rapid Resolution liquid chromatograph and an Agilent G6410A Triple Quadrupole mass spectrometer. Different ZORBAX columns were evaluated in combination with different solvents, flow rates, and column parameters to optimize the speed of the analysis while maintaining a good chromatographic resolution.

The best results were obtained with a 1.8-µm particle size ZORBAX SB-C18 column (2.1 × 100 mm) using a water/acetonitrile gradient (both containing 0.1% formic acid). The detailed LC conditions are listed in Table 1.

Table 1. LC Method

Column	Zorbax RRHT SB-C18 (2.1 mm id × 100 mm, 1.8 µm) p/n 828700-902
Column temperature	70 °C
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Flow rate	0.6 mL/min
Gradient	10% B at 0 min 10% B at 2 min 95% B at 8 min 95% B at 11min 10% B at 11.5min
Stop time	15 min
Post time	None
Injection volume	10 µL (sample diluted with water 1:4 to improve peak shape)

In addition to the standard ESI and APCI sources, an Agilent G1978B Multimode source was coupled to the mass spectrometer. The MMI source was operated in mixed mode (ESI and APCI simultaneously in positive polarity) or alternatively in pure ESI and APCI modes, switching between these ionization techniques based on a chromatographic time scale. The optimized source parameters are shown in Table 2.

Determination of the optimal MRM transitions for both analytes and internal standards was carried out by flow injection analysis of the single components at concentration levels around 1 µg/mL. See Table 3.

Results and Discussion

Four possible MMI modes were investigated (Figure 3). Using an MMI method that begins in ESI mode and switches to APCI mode after five minutes resulted in the best overall compound responses. The LC method was not fully optimized for speed (Table 1) because the vaporizer temperature is changed from 175 to 250 °C after the switch of the ionization mode, and that change requires some short time before the cannabinoids elute. The total run time, including the re-equilibration time of the column at starting gradient conditions, was 15 minutes.

Table 2. Optimized MMI-Parameters

MMI mode	Neb. press (psi)	Drying gas flow (L/min)	Drying gas temp (°C)	Charging voltage (V)	Capillary voltage (V)	Vaporizer temp (°C)	Corona current (µA)
Mixed	40	5	300	2000	2000	200	2
ESI	60	5	300	2000	2000	175	0
APCI	20	5	300	2000	2000	250	4

Table 3. Data Acquisition Parameters for the MRM Transitions

Compound	RT (min)	Precursor $(M-H)^+$	Frag (V)	CE (V)	Product ion (*m/z*)	CE (V)	Product ion 2 (*m/z*)
Amphetamine	1.3	136	100	15	91	10	119
D_{11}-Amphetamine	1.3	147	100	15	127	15	97
MDA	1.4	180	100	15	105	15	135
D_5-MDA	1.4	185	100	10	168	15	138
Methylamphetamine	1.5	150	100	15	91	10	119
D_{11}-Methylamphetamine	1.5	161	100	15	127	15	97
MDMA	1.9	194	100	10	163	15	135
D_5-MDMA	1.9	199	100	15	165	10	135
MDE	2.6	208	100	15	135	15	147
D_6-MDE	2.6	214	100	15	166	15	136
THC-OH	7.9	331	110	30	193	30	201
D_3-THC-OH	7.9	334	110	20	316	25	196
THC-COOH	8.1	345	110	30	193	30	299
D_9-THC-COOH	8.1	354	120	22	308	25	196
THC	8.6	315	110	30	193	30	259
D_3-THC	8.6	318	100	30	196	30	105

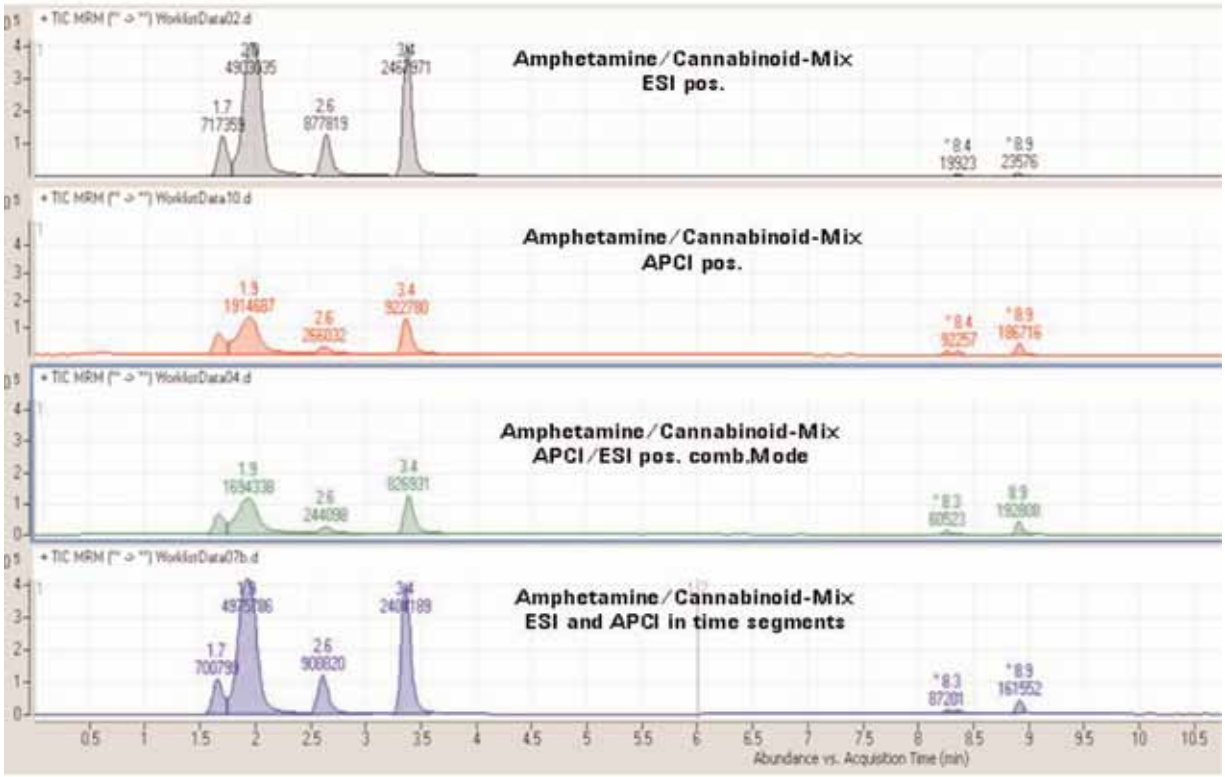

Figure 3. Comparison of the different MMI modes.

Method Evaluation

The LC/MS/MS method was evaluated for the detection and quantification of THC, THC-OH, THC-COOH, amphetamine, methamphetamine, MDA, MDMA, and MDE in serum. The evaluation of the method was carried out according to Peters et al [1] and the German Society of Toxicology and Forensic Chemistry (GTFCh). The method evaluation was performed by using a Microsoft Excel-based validation program (VALISTAT [2]). Drug-free serum was used as a blank matrix for the evaluation measurements.

Seven calibration standards were prepared. The different calibration levels were obtained by spiking the blank serum with 50 µL of methanolic solutions containing appropriate amounts of the analytes. The calibration levels are shown in Table 4.

Table 4. Calibration Range for Amphetamines and Cannabinoids in Serum Samples

Compound	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
Amphetamine	0	10	20	40	60	80	100	500
Methamphetamine	0	10	20	40	60	80	100	500
MDA	0	10	20	40	60	80	100	500
MDMA	0	10	20	40	60	80	100	500
MDE	0	10	20	40	60	80	100	500
THC	0	0.5	1	2	3	4	5	25
THC-OH	0	0.5	1	2	3	4	5	25
THC-COOH	0	5	10	20	30	40	50	250

A seven-point calibration curve for each compound was obtained by measuring of the calibration standards in six replicate injections. The calibrations were linear in the range tested and the correlation coefficients were > 0.98 for all compounds. The S/N calculations for calibration standard Cal 3, which represents the limit of quantitation, were based on peak-to-peak noise definition and no smoothing was applied. All quantifier and qualifier ions of the amphetamines and cannabinoids can be easily detected, even when diluting the methanol-reconstituted SPE fractions with water (ratio 1:4) to improve the chromatographic peak shape (Figure 4).

Intra-assay and inter-assay precision data were obtained from two analyses in a series performed on eight different days at two concentration levels (low, high). The intra-assay precision (within-day reproducibility) is defined as the mean value of the eight coefficients of variation (CV) from the two measurements carried out on one day. Inter-assay precision (day-to-day reproducibility) is the coefficient of variation from the average of the eight mean values of the two measurements carried out on one day. The intra-assay coefficients of variation ranged from 2.9 to 15.3 % (Table 5). The day-to-day coefficients of variation ranged from 3.4 to 15.3 %

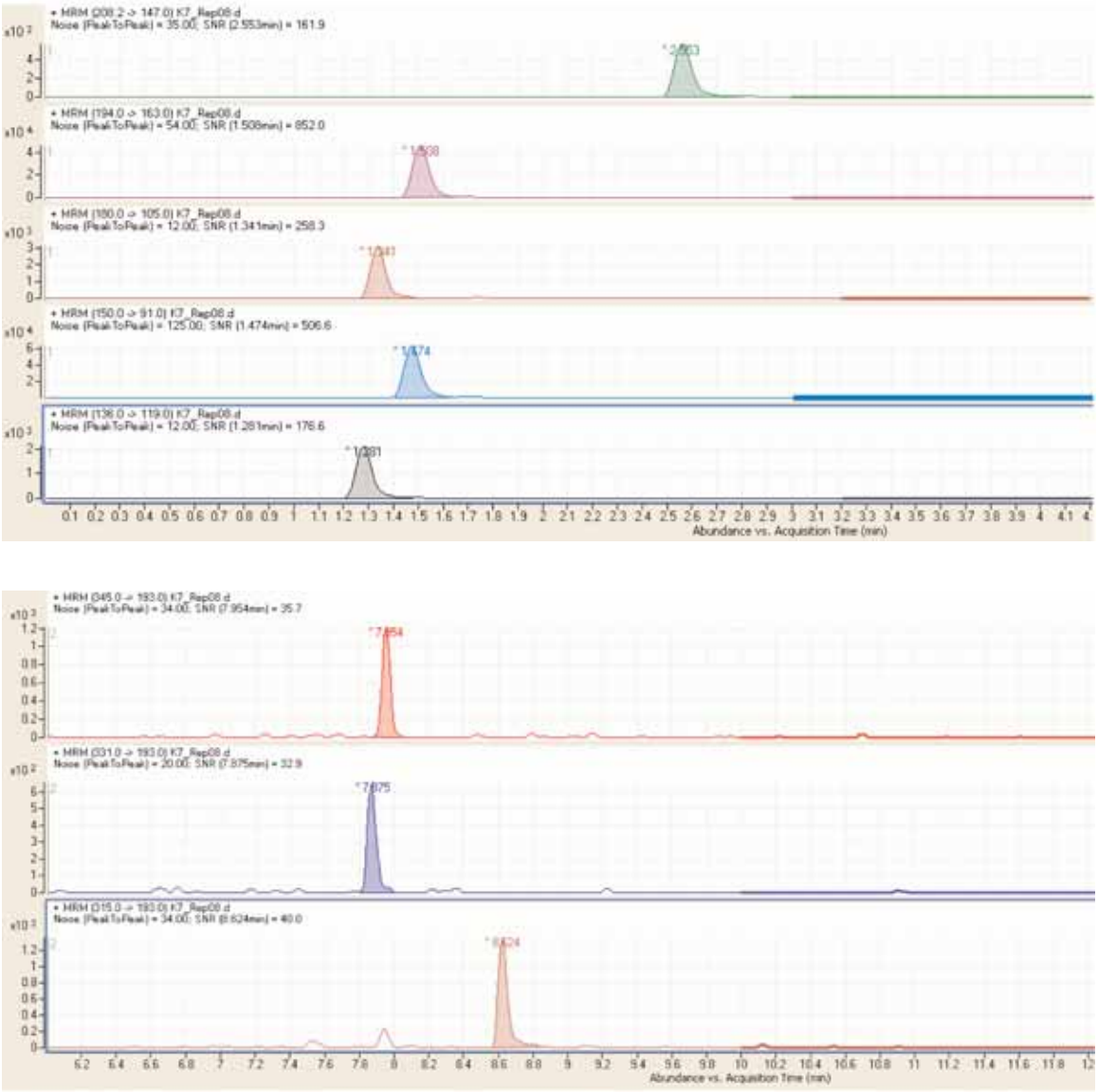

Figure 4. S/N calculation for standard Cal 3.

Table 5. Inter-Assay and Intra-Assay Precision at Two Concentration Levels (Cal 3 and Cal 7)

	Intra-assay precision in %		Inter-assay precision in %	
Compound	**CalStandard 3**	**CalStandard 7**	**CalStandard 3**	**CalStandard 7**
Amphetamine	4.3	2.9	4.9	5.6
Methamphetamine	4.7	4.7	6.1	5.5
MDA	7.6	4.2	8.4	4.6
MDMA	5.9	3.2	5.9	3.4
MDE	8.5	5.2	8.5	5.6
THC	9.5	5.8	10.0	6.3
THC-OH	8.4	8.7	11.6	8.7
THC-COOH	15.3	5.4	15.3	5.5

Conclusions

The use of a 1.8-µm particle size RRHT column for the LC separation provides a faster analysis (cycle time 12 min) than GC/MS (cycle time 45 min). Due to the polarity differences of the two compound classes, the use of the multimode ion source allows the detection of the eight compounds with an optimal response for each compound (switching the ionization mode on a time-based scale leads to the best results). In comparison to the established GC/MS method, the RRLC/QQQ method shows a higher sensitivity and selectivity (considering an injection volume of 1 µl in GC/MS and 10 µl in LC/MS/MS with a dilution factor of 4). The presented method exhibits good within-day and day-to-day reproducibility. The coefficents of variation ranged from 3 to 15%; most of the coefficients were in the 5 to 10% range.

In the future, other drugs of abuse (opiates like morphine, 6-acetylmorphine, and codeine as well as cocaine and its metabolites) will be included in this RRLC/QQQ method. Also, the use of online SPE will be evaluated.

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Screening of Corticosteroids in Urine by Positive Atmospheric Pressure Chemical Ionization LC/MS/MS

Forensic Toxicology

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Introduction

Corticosteroids are a class of components often abused and misused in sport. They are very potent drugs in the treatment of inflammations and asthma [1]. Corticosteroids can have an effect on the nervous system and can improve an athlete's ability to concentrate and perform in endurance and power events [2,3]. To prevent their misuse, the anti-doping governing bodies are restricting the use of corticosteroids. Systemic use of corticosteroids is forbidden in all circumstances. However, when medically necessary, local and intra-articular injections or dermatological preparations are allowed under the approval of a therapeutic use exemption [4].

The samples collected for doping control are mainly urine samples because large sample volumes can be collected in a non-invasive way. Therefore, the abuse of corticosteroids is analyzed using urine samples.

A simple and sensitive LC/MS/MS method for the screening of 17 corticoster oids is described below. The method is able to detect corticosteroids from the doping control urine samples at 20 ng/mL – below the WADA minimum required performance level (MRPL), which is 30 ng/mL.

Instrumentation

- Varian ProStar ™430 AutoSampler
- Varian ProStar ™210 Solvent Delivery Modules
- Varian 1200L LC/MS equipped with Atmospheric Pressure Chemical Ionization (APCI) source
- Harvard Syringe Pump model 11

Materials and Reagents

- Standards of corticosteroids, from Sigma-Aldrich, USA
- Methanol, gradient grade for liquid chromatography, from Merck, Germany
- Water supplied by a Simplicity 185 ultrapure water system, from Millipore, Great Britain
- α-glucuronidase from E. Coli K12, from Roche Diagnostics, Germany
- All other chemicals ar e pro analysis or HPLC grade

Sample Pr eparation

The samples are prepared by a standard procedure for steroids. A 2 mL urine sample is transferred in a tube. A 40 µL aliquot of a 10 ppm desoximetasone (internal standard) solution, 1 mL phosphate buffer 0.8M pH 7.0 and 25 µL beta-glucuronidase are added, the mixture is vigorously vortexed and kept for 1 hour at 50 °C for enzymatic hydrolysis. (The enzymatic hydrolysis step is needed since the corticosteroids are mainly excreted in a conjugated form with the glucuronic acid). Adding 750 µL of 20% buffer K_2CO_3/ $KHCO_3$ (1:1), which brings the pH around 9, stops the hydrolysis. Next, 5 mL of tertbutylmethylether is added, and the mixture shaken for 15 min. After centrifugation, the organic layer is transferred to another tube and evaporated to dryness. The remaining residue is dissolved in 100 µL mobile phase (20:80, solvent A / solvent B). Then, 10 µL is injected in LC/MS/MS [5].

HPLC Conditions

Column	ChromSep SS 100x2.0 mm with guard column ChromSep OmniSpher 3 C18 (Varian Part No. CP27839)
Solvent A	0.1% acetic acid : 5 mM ammonium acetate in water (v/v)
Solvent B	Methanol

LC Program	Time (min:sec)	%A	%B
	0:00	70	30
	0:30	70	30
	1:00	50	50
	16:00	30	70
	17:00	30	70
	17:06	70	30
	22:00	70	30

Flow	0.25 mL/min
Mixer	250 µL
Injection V olume	10 µL
Injection Solvent	20% solvent A / 80% solvent B

MS Parameters

Ionization Mode	APCI negative
Collision Gas	1.5 mTorr Argon
Housing	50 °C
API Drying Gas	12 psi at 150 °C
API Nebulizing Gas	58 psi at 400 °C
Auxiliary Gas	17 psi
Scan Time	1 - 1.7s
SIM Width	0.7 amu
Corona current	5 µA
Shield	600 V
Capillary	Tuned Values
Detector	1500 V

Scan parameters

No.	RT (min)	Corticosteroid	Capillary (V)	Precursor Ion	Product Ion	CE (V)
1	5.5	Triamcinolone	-30	453.2	345 363	23 12
2	7.3	Prednisone	-30	417.2	327 357	18 8
3	7.6	Cortisone	-25	419.2	329	16
4	8.7	Prednisolone	-20	419.2	329 295	16 36.5
5	8.7	Hydrocortisone	-25	421.2	331	19
6	10.4	Flumethasone	-40	469.2	379 305	19 41
7	10.8	Betamethasone + Dexamethasone	-40	451.2	361 307	19 33
8	11.1	Triamcinolone acetonide	-25	493.2	375 413	14 22
9	11.1	Fludrocortisone acetate	-35	481.2	349 341	25.5 21
10	11.2	Metilprednisolone	-25	433.2	343 309	17.5 37
11	11.3	Bechlomethasone	-55	467.2	377 341	14 21.5
12	11.8	Flunisolide	-55	493.2	375 357	14 21
13	12.0	Fluorometolone	-40	435.2	59 355	12 16
14	12.1	Flurandrenolide	-35	495.2	377 359	14 20
ISTD	12.6	Desoximetasone	-10	435.2	355	16
15	15.5	Fluocinolone acetonide acetate	-55	553.2	375 355	18 24
16	15.6	Budesonide	-50	489.2	357 339	13.5 19
17	17.9	Fluticasone propionate	-50	559.2	413 433	22.5 15.5

The scan method is divided in 3 segments of acquisition:

- 0 - 9.5 min 7 transitions Scan time 1s
- 9.5 - 15 min 17 transitions Scan time 1.7s
- 15 - 20 min 6 transitions Scan time 1s

Results and Discussion

In order to develop the MS parameters, 10 ppm solutions of each corticosteroid were prepared in a 20% buffer A / 80% methanol mixture. The mixture was meant to mimic the mobile phase that would elute with the compound of interest in an actual LC/MS analysis. The 10 ppm solutions were directly injected in the APCI with a syringe pump at a 50 µL/min rate. First, the most appropriate precursor ion was selected from the parent scan, and the capillary voltage was optimized for its highest abundance. Second, the product ions were selected and the collision energies optimized by the MS/MS breakdown automatic procedure.

Two MS/MS product ions, instead of one, are used to monitor each corticosteroid in order to better eliminate the false positives. Only cortisone and hydrocortisone, which are endogenous corticosteroids, are monitored with one ion. Three ions were not used in order not to increase the scan time. The confirmation of the positive sample can be done by a confirmation method specific for the suspected corticosteroid monitoring at least three of its MS/MS transitions.

Particular care was taken to separate the prednisolone from cortisone. The two corticosteroids have the same molecular masses, and cortisone gives an abundant peak on the transition (-) 419.2>329 of prednisolone. The triamcinolone acetonide-flunisolide and fluorometholone-desoximethasone pairs also share their transitions, but are separated by their retention times. The epimeres betamethasone and dexamethasone have similar retention times and mass spectra, and they could not be separated in the LC/MS conditions described.

Figure 1 (page 3) shows the LC/MS analysis of a blank urine sample spiked with 20 ng/mL of each corticosteroid (except cortisone and hydrocortisone, which are endogenous) and 200 ng/mL internal standard. There are no matrix interferences, and the abundances and signal/noise ratios are satisfactory for all compounds of interest even with a standard gain of the detector. In order to increase the reliability of the result, the confirmation of a positive sample can be done with the detector set on high gain.

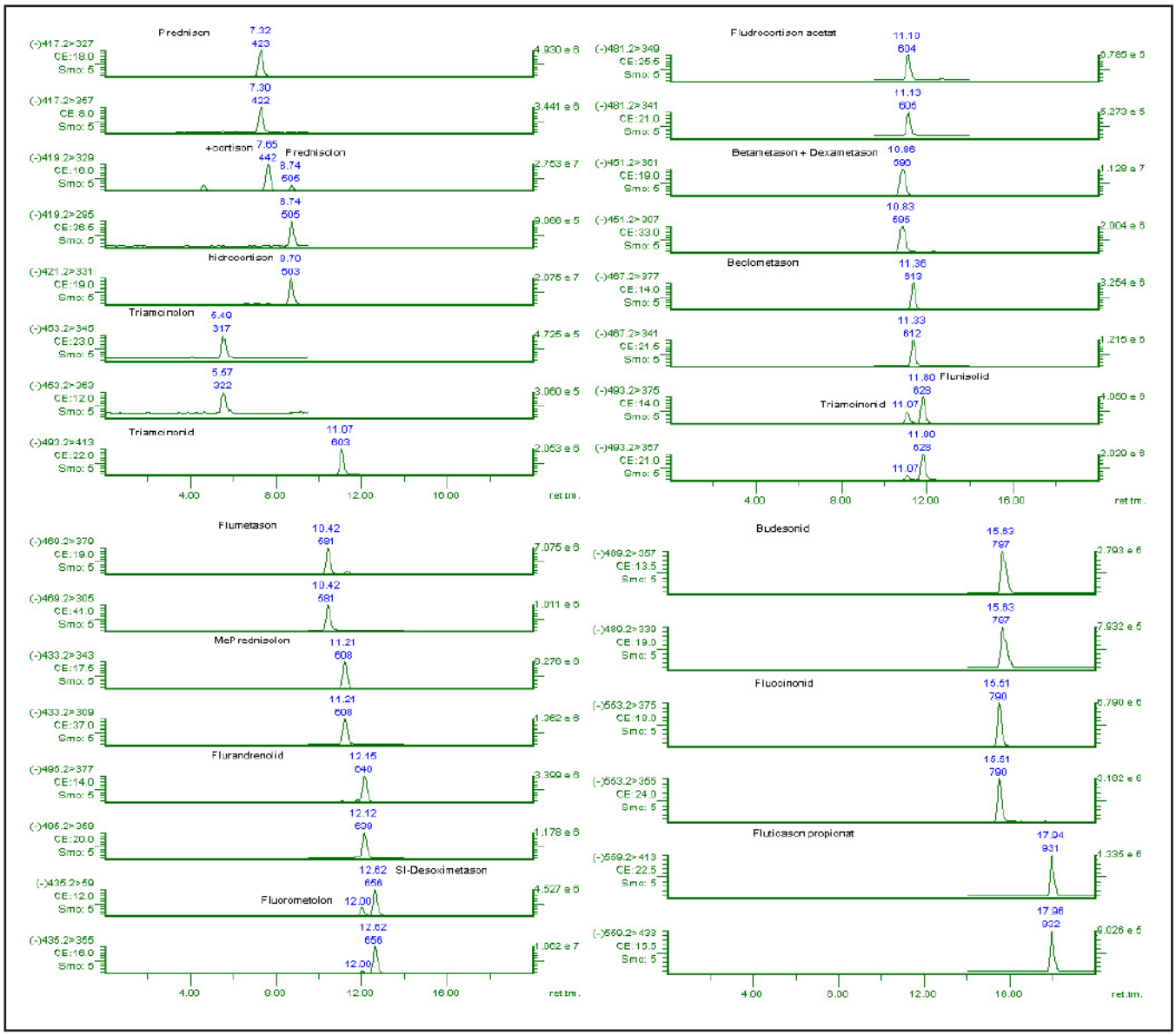

Figure 1. Analysis of a blank urine sample spiked with 20 ng/mL corticosteroids

Conclusion

The LC/MS/MS method described in this application note is simple and sensitive. In one run this method can screen for 17 corticosteroids and easily detect them below the WADA's MRPL, 30 ng/mL. The Varian 1200L system proved to be an essential tool for a doping control laboratory.

References

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2. Polettini, A.; Marrubini Bouland, G.; Montagna, M.J.; J. Chromatogr. B 1998; 713:339
3. Cummiskey, J.; Glucocorticosteroids in Doping in Sport, Concerted Action in the Fight Against Doping in Sport (CAF-DIS), Dublin, 2002.
4. 2005 WADA Prohibited List
5. Deventer, K.; Delbeke, F.T., Rapid Commun. Mass Spectrom. 2003; 17:2107-2114

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LC/MS Application Note

Number 13
February 2004

Analysis of (±)-11-Nor-9-Carboxy-Delta-9-THC in Urine by Negative Ion Electrospray LC/MS/MS

Forensic Toxicology

J. Beck and C. Schmidt, Varian, Inc.

Introduction

The compound that gives the "high" from smoking marijuana is tetrahydrocannabinol or THC. Forensic Toxicology labs are often asked to analyze urine samples for the presence of one of the metabolites of THC, most frequently the carboxy form.

Traditionally, samples are screened for THC metabolites by immunoassay and confirmed using GC/MS. GC/MS, while the current standard for THC metabolite testing, requires time consuming sample derivatization prior to analysis. LC/MS provides the same specificity and sensitivity without the need for a derivatization process.

A simple, high throughput LC/MS/MS method is described here for the detection and quantitation of (±)-11-nor-9-carboxy-delta-9-THC in urine.

Instrumentation

- Varian ProStar 410 AutoSampler
- Varian ProStar 210 Solvent Delivery Modules
- Varian 1200L LC/MS/MS equipped with ESI source

Materials and Reagents

- Standard solution: 0.1 mg/mL (±)-11-Nor-9-Carboxy-Delta-9-THC (Catalog No. T-006), from Cerilliant Corp., Texas, USA.
- Internal standard (IS) solution: 500 ng/mL (±)-11-Nor-9-Carboxy-Delta-9-THC-d9 in methanol, a gift from Norchem Drug Testing, Flagstaff, AZ.
- Test samples: samples containing various amounts of (±)-11-Nor-9-Carboxy-Delta-9-THC, also gifts from Norchem Drug Testing, Flagstaff, AZ.
- All other chemicals are reagent grade or HPLC grade.

Sample Preparation

Serial dilutions of the standard solution of carboxy-THC (THCC) were prepared in deionized water. The concentrations of the samples ranged from 1 ng/mL (1 ppb) to 1000 ng/mL. A 50 µL aliquot of the internal standard solution was added to 1 mL of each sample. A 20 µL aliquot was injected directly onto the column for analysis.

Test samples from Norchem Drug Testing were prepared in 1:4 dilutions of pooled urine with deionized water.

HPLC Conditions

Column	Varian Pursuit Diphenyl 3 µm, 50 x 2 mm (Varian Part No. A3041-050X020)			
Solvent A	deionized water			
Solvent B	methanol			
LC Program	Time (min:sec)	%A	%B	Flow (mL/min)
	0:00	60	40	0.2
	0:30	60	40	0.2
	1:00	5	95	0.2
	3:00	5	95	0.2
	3:01	60	40	0.2
	6:30	60	40	0.2
Injection Volume	20 µL			

MS Parameters

Ionization Mode	ESI negative
Collision Gas	2.0 mTorr Argon
API Drying Gas	25 psi at 325 °C
API Nebulizing Gas	51 psi
Scan Time	0.5 sec
SIM Width	0.7 amu
Needle	-4200V
Capillary	-30V
Detector	1620V

Scan Parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
(±)-Carboxy-THC	343.5	299	17.5
(±)-Carboxy-THC-d9	352.5	308	20.5

Results and Discussion

The LC gradient for this analysis results in a retention time of 4.6 minutes for the THCC analyte and the IS peak. Chromatograms for the 1 ng/mL sample and 25 ng/mL IS (20 pg and 500 pg on column, respectively) are shown in Figure 1. This level is 50 times below the proposed drug cutoff level for the initial immunoassay screen published by the Substance Abuse and Mental Health Services Administration (SAMHSA)[1] and 15 times below the proposed cutoff level for the GC/MS confirmatory test.

The LC/MS/MS method described here is linear from 1 ppb to 1000 ppb as shown in Figure 2. Each calibration standard was run in triplicate and the three data points were averaged.

A series of test samples ranging from 1 ng/mL to 800 ng/mL were run on the 1200L LC/MS/MS. The calculated and actual values are shown in Table 1.

A representative chromatogram for the Norchem Drug Testing samples is shown in Figure 3. At the 1 ng/mL LOQ level, no interference is observed, demonstrating the specificity of the LC/MS/MS method.

Conclusion

The LC/MS/MS method presented in this application note is very simple and sensitive. The method eliminates the need for a time-consuming derivatization step which can take an hour or more. The Varian 1200L LC/MS/MS can be a powerful tool in forensic toxicology laboratories offering significant cost and time savings.

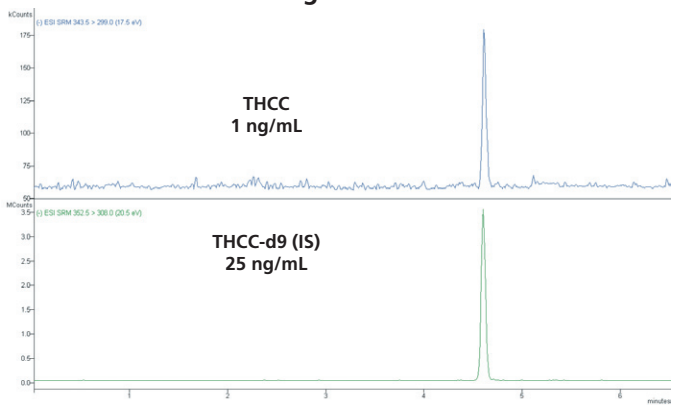

Figure 1. Signal-to-noise (300:1 RMS) is excellent at the lowest calibration level of 1 ng/mL for THCC.

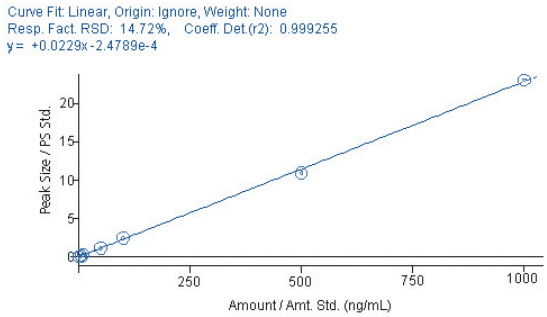

Figure 2. Seven calibration levels for THCC (1, 5, 10, 50, 100, 500, and 1000 ng/mL) with 25 ng/mL internal standard.

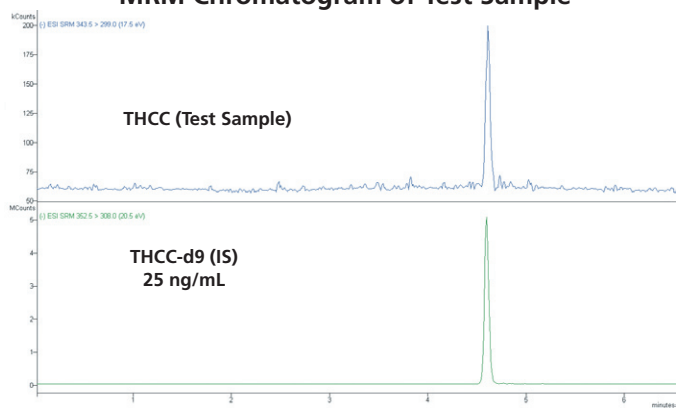

Figure 3. For the THCC test sample in diluted urine at 1 ng/mL, the calculated value based on the calibration curve is 1.1 ng/mL.

Results of LC/MS/MS Study of THCC

Sample ID	Calculated Amount (ng/mL)	Actual Amount (ng/mL)
F	1.1	1.0
E	5.4	5.5
G	10.9	11.0
D	15.3	15.0
C	106.0	100.0
B	411.0	400.0
A	802.0	800.0

Table 1. The calculated results correspond very well to the actual concentration of the spiked samples provided by Norchem Drug Testing.

Acknowledgement

The authors would like thank Dr. A. Fischinger, Norchem Drug Testing, Flagstaff, AZ for kindly supplying technical advice and the THCC test samples for this study.

Reference

1. http://workplace.samhsa.gov/ResourceCenter/DT/FA/GuidelinesDraft4.htm

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Quantitative Analysis of Opiates in Urine Using RRHT LC / MS / MS

Application Note

Forensic Toxicology

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Abstract

An Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) is used to analyze several opiates in urine. A simple isocratic liquid chromatography elution is carried out to detect all seven analytes and their respective internal standards in less than 3.5 minutes using Rapid Resolution High-Throughput liquid chromatography with a ZORBAX C18, 2.1 × 50 mm, 1.8-µm particle size column. Both quantifier and qualifier ions are monitored for each analyte, with the requirement that the qualifier / quantifier ion ratio be within ± 20% for confirming their presence in samples. Except for 6-acetylmorphine (6-MAM), all calibration standards are extracted in matrix and range from 1 to 150 pg / µL in urine. The range for 6-MAM is 0.067 to 10 pg / µL. Following extraction, which corresponds to a factor of 6.78 decrease in concentration, the injected concentrations range from 0.147 to 22.12 pg / µL, or 147 ppt to 22.12 ppb. For 6-MAM, this corresponds to 9.8 ppt to 1.5 ppb. All compounds show very good linearity (R^2 > 0.99).

Introduction

Opiates are drug compounds that may be obtained both legally as prescription medication or illegally. For several reasons, including therapeutic drug monitoring, driving under the influence of drugs, and workplace drug testing, these compounds are commonly analyzed, particularly in urine due to ease of sample availability and volume. For testing in the area of forensics it is often necessary to provide additional confirmation of the presence of these compounds beyond their quantitative values exceeding defined cutoff values.

The triple quadrupole mass spectrometer (QQQ) provides the most sensitive form of quantitation by acquiring the signal corresponding to the highest response product ion (quantifier) from the fragmentation of the analyte precursor ion. This transition is known as multiple reaction monitoring (MRM). However, by acquiring additional signal corresponding to the next highest product ion (qualifier), enough information may be considered available for confirmation, particularly if the ratio of signal between the two product ions is consistent between the calibration standards and the unknown samples. Using the QQQ to acquire MRM signals for both the quantifier and qualifier ions can result in both quantitation and confirmation simultaneously.

The Agilent MassHunter software includes user-definable ion ratio confirmation in the quantitative analysis program as shown in Figure 1. The default tolerance for confirmation is ± 20% of the derived ion ratio, but this may be customized for the particular user. Additionally, up to four different product ions may be used as qualifiers. In this work, the default value of ± 20% is used, along with only one qualifier ion.

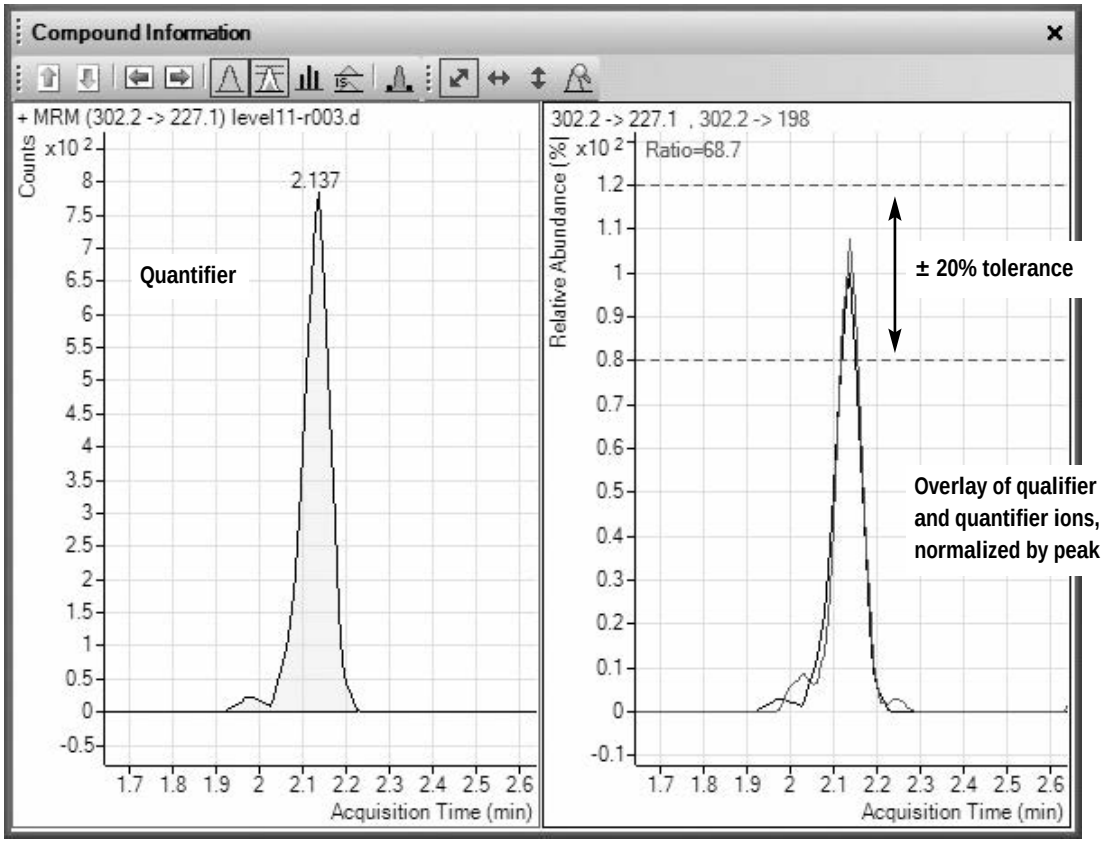


Figure 1. Qualifier / quantifier ion ratios for confirmation of oxymorphone.

Several opiates in urine, including morphine, oxymorphone, hydromorphone, codeine, oxycodone, hydrocodone, and 6-acetylmorphine (6-MAM), a metabolite of heroin, are analyzed in this work. The corresponding structures are shown in Figure 2. A deuterated chemical analog for each compound is included to account for extraction efficiency and matrix interference. A qualifier ion for each internal standard is not necessary and is therefore not analyzed.

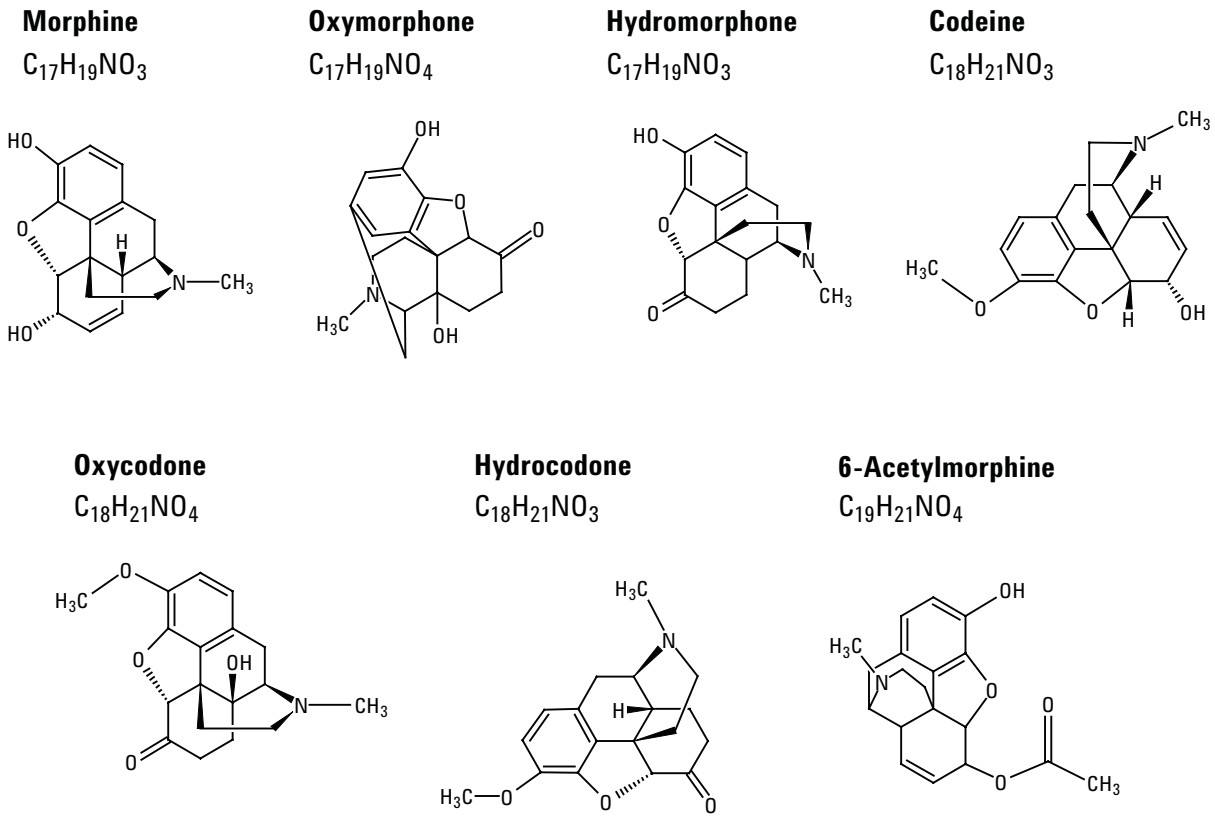


Figure 2. Structures of the opiates analyzed in this work.

This work uses a gradient LC analysis consisting of only water and acetonitrile (no modifiers) to elute all analytes and corresponding internal standards in less than 3.5 min on a Rapid Resolution High-Throughput (RRHT) LC column with a 1.8-µm particle size. The complete cycle time from one injection to the next is about 8 minutes. The compounds are analyzed using an electrospray ionization source in positive ion mode. Parameters associated with this ion source, like drying gas, are standard for the LC flow rate of 0.4 mL/min, in which the samples are introduced into the mass spectrometer.

Voltage settings for maximum ion transfer between the ion source and the mass analyzer components of the QQQ instrument are set using the autotune capability of the instrument to optimize signal intensity, resolution, and mass assignment across a wide mass range. One parameter requiring optimization for each analyte is the fragmentor voltage, which is located in the ion transfer optics between the ion source and the mass analyzer. This optimization results in the maximum response of the precursor ion of interest incident upon the first quadrupole of the QQQ mass analyzer. The fragmentor voltage of 110 V worked best for all analytes.

Once this is done, the optimal collision energy for fragmenting the precursor to form the highest possible response of a product ion is obtained. The mass spectrometer method development is now complete for the quantifier ion. Repeat optimizaton of the collision energy for the second most-abundant product ion and both MRM transitions are thus derived for one compound. Both steps in optimization may be carried out by flow injection analysis.

Experimental

Sample Preparation

Urine samples spiked with the opiate compounds were provided at the following labeled concentrations: 1, 5, 10, 50, 100, and 150 pg/µL, and a factor of 15 times lower for 6-MAM. These samples were then processed using the following procedure:

1. Start with 250-µL sample size
2. Add 500 µL sodium acetate buffer
3. Add 20 µL glucuronidase
4. Add 75 µL of internal standard mixture at 500 ng/mL concentration (de-ionized water)
5. Vortex
6. Incubate at 60 °C for 20 minutes
7. Add 850 µL de-ionized water
8. Vortex and spin down
9. Place 200 µL of supernatant in sample vial

All prepared samples provided by customer.

This procedure dilutes the samples by a factor of 6.78 so that a 1 pg/µL concentration in urine has an actual concentration of 147 fg/µL for injection. Upon addition of internal standards and extraction, the starting concentrations in urine now correspond to the following concentrations for injection: 0.147, 0.737, 1.47, 7.37, 14.7, and 22.12 pg/µL. With a 5-µL injection volume (see LC Conditions), this range then corresponds to 0.737, 3.685, 7.35, 36.85, 73.5, and 110.6 pg on-column. For 6-MAM, all of these values are a factor of 15 lower.

LC / MS Method Details

LC Conditions

Agilent 1200 Series binary pump, degasser, wellplate sampler, and thermostatted column compartment

Column:	Agilent ZORBAX SB-C18, 2.1 × 50 mm, 1.8-µm particle size (PN: 822700-902)
Column temp:	50 °C
Mobile phase:	A = water B = acetonitrile
Flow rate:	0.4 mL/min
Injection volume:	5 µL

Gradient:

Time (min)	%B	
0	2	
4	40	Stop time: 6.1 min
4.1	90	Post time: 2.0 min
6	90	
6.1	2	

Needle wash (25:75 water/methanol)–flush port 10 seconds

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	60 psig
Drying gas flow:	11 L/min
Drying gas temp:	350 °C
V_{cap}:	2000 V

Resolution (FWHM): Q1 = 0.7; Q2 = 0.7
Dwell time for all MRM transitions = 50 msec
Fragmentor voltage for all transitions = 110 V

The MRM transitions for each compound are listed in Table 1 by retention time. Those product ions in parentheses are used as qualifiers. The retention times are included. Note that 6-MAM, or 6-monoacetylmorphine, is abbreviated as 6-MAM.

Table 1. MRM Mode Parameters for Opiates

Segment	Compound	Transition	Collision energy (V)	Retention time (min)
1 (0 min)	D3-morphine	289.2 > 152.1	75	1.851
	Morphine	286.2 > 152.1 (128.0)	75 (73)	1.862
	D3-oxymorphone	305.2 > 230.1	33	2.138
	Oxymorphone	302.2 > 227.1 (198.0)	33 (55)	2.146
	D3-hydromorphone	289.2 > 157.1	50	2.379
	Hydromorphone	286.2 > 185.0 (157.0)	33 (50)	2.385
2 (2.65 min)	D3-codeine	303.2 > 152.0	75	2.908
	Codeine	300.2 > 152.0 (115.0)	75 (85)	2.912
	D3-oxycodone	319.2 > 244.1	30	3.109
	Oxycodone	316.2 > 241.0 (256.0)	30 (27)	3.120
	D6-6-MAM	334.2 > 165.1	40	3.161
	6-MAM	328.2 > 165.0 (211.0)	40 (27)	3.168
	D3-hydrocodone	303.2 > 199.1	28	3.245
	Hydrocodone	300.2 > 199.0 (128.0)	28 (73)	3.249

Results and Discussion

The calibration curves for all seven compounds are shown in Figures 3A through 3G, including expanded views of the lowest three levels. All calibration curves are generated using a linear fit, no inclusion of the origin, and a 1/x weighting. All curves have linearity coefficients of at least 0.99 and show good reproducibility and accuracy at the lowest levels. One exception is 6-MAM, which only showed signal for two of the three injections at the lowest level (49 fg on-column). However, the corresponding concentration in urine is 0.067 pg/µL (0.067 ng/mL), which is much lower than the 10 ng/mL confirmatory cutoff level for workplace testing proposed by the U.S. Substance Abuse Mental Health Services Administration (SAMHSA).

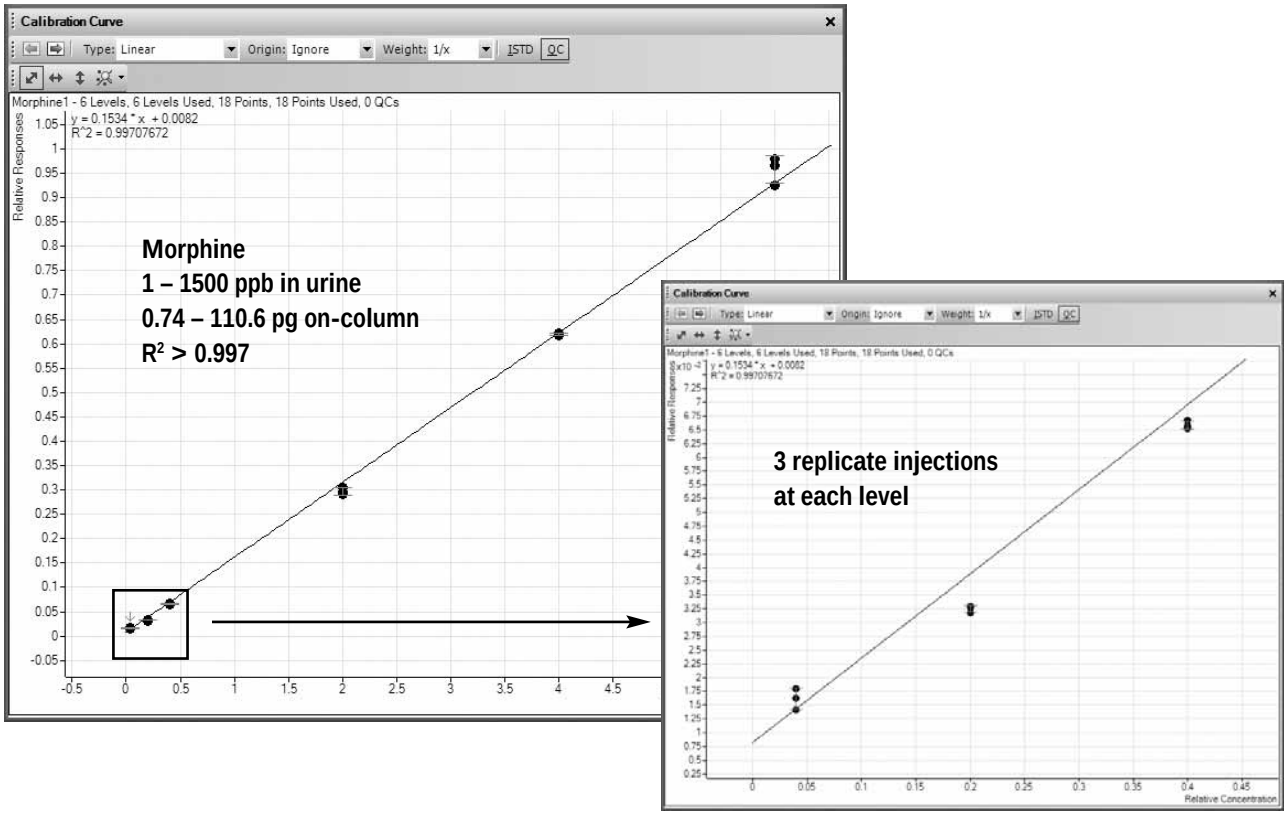


Figure 3A. Linearity of morphine in urine. Injection concentration range = 147 ppt – 22 ppb.

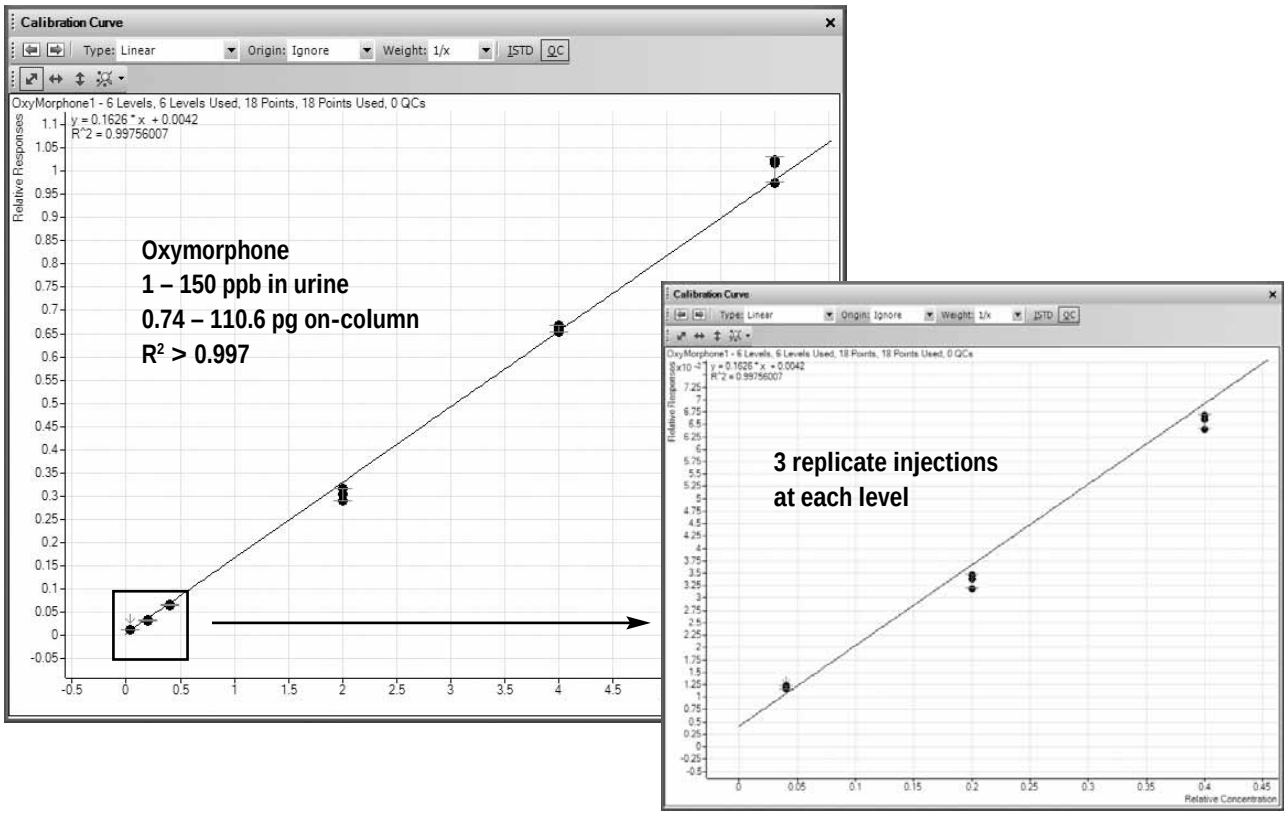


Figure 3B. Linearity of oxymorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

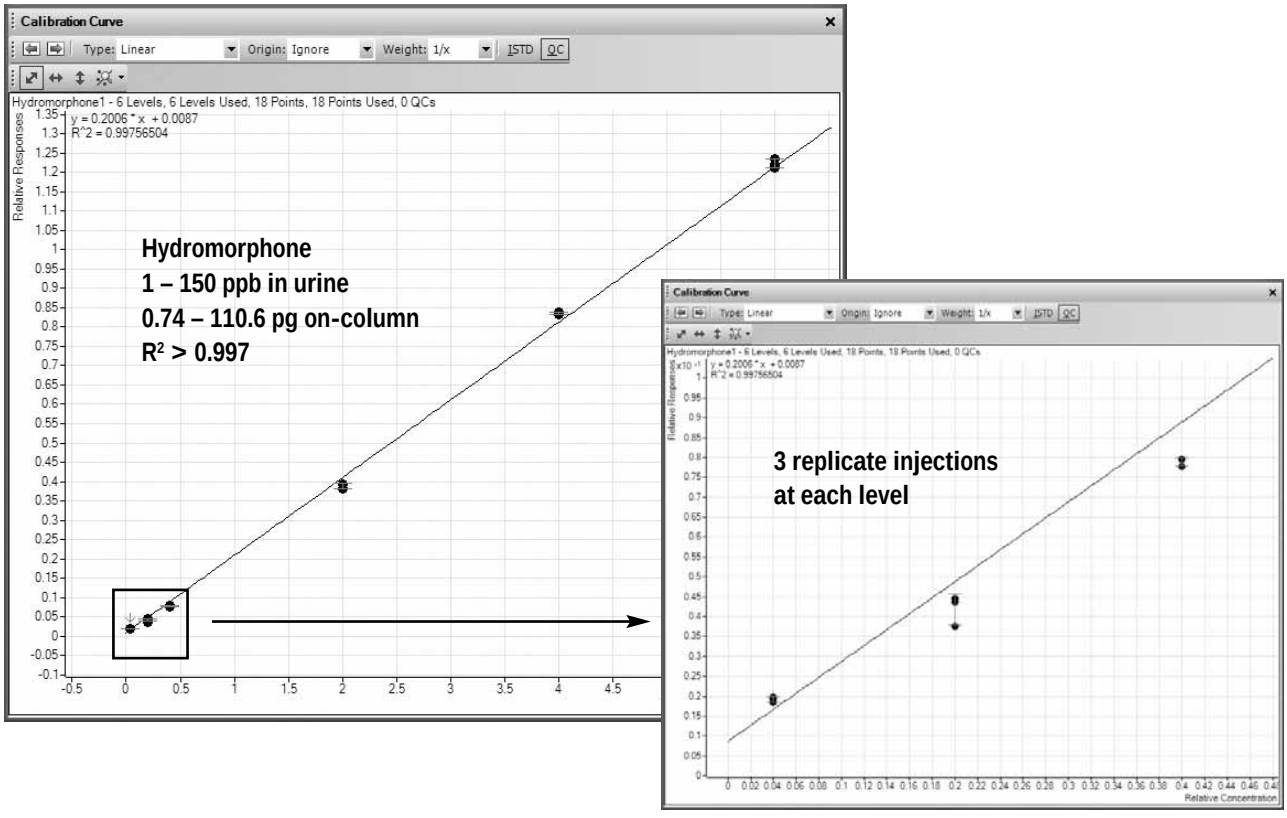


Figure 3C. Linearity of hydromorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

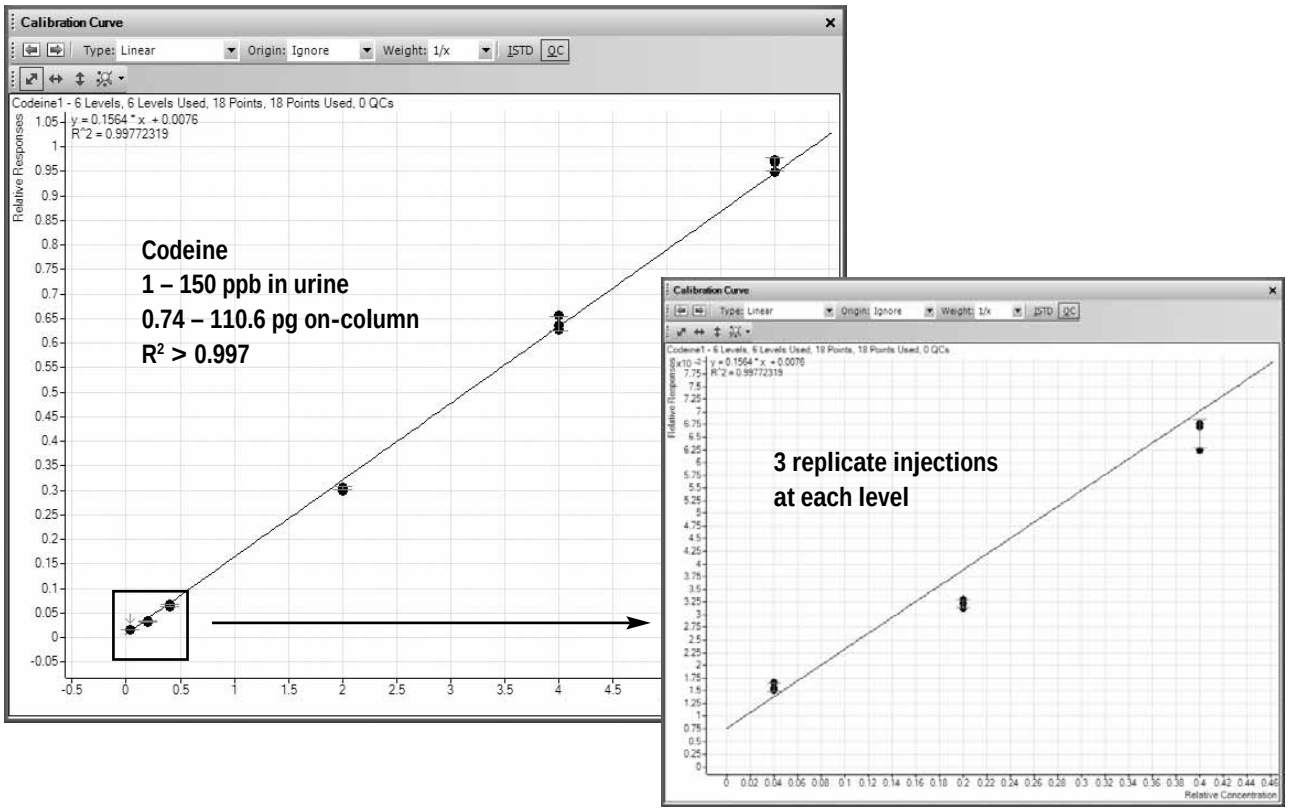


Figure 3D. Linearity of codeine in urine. Injection concentration range = 147 ppt – 22 ppb.

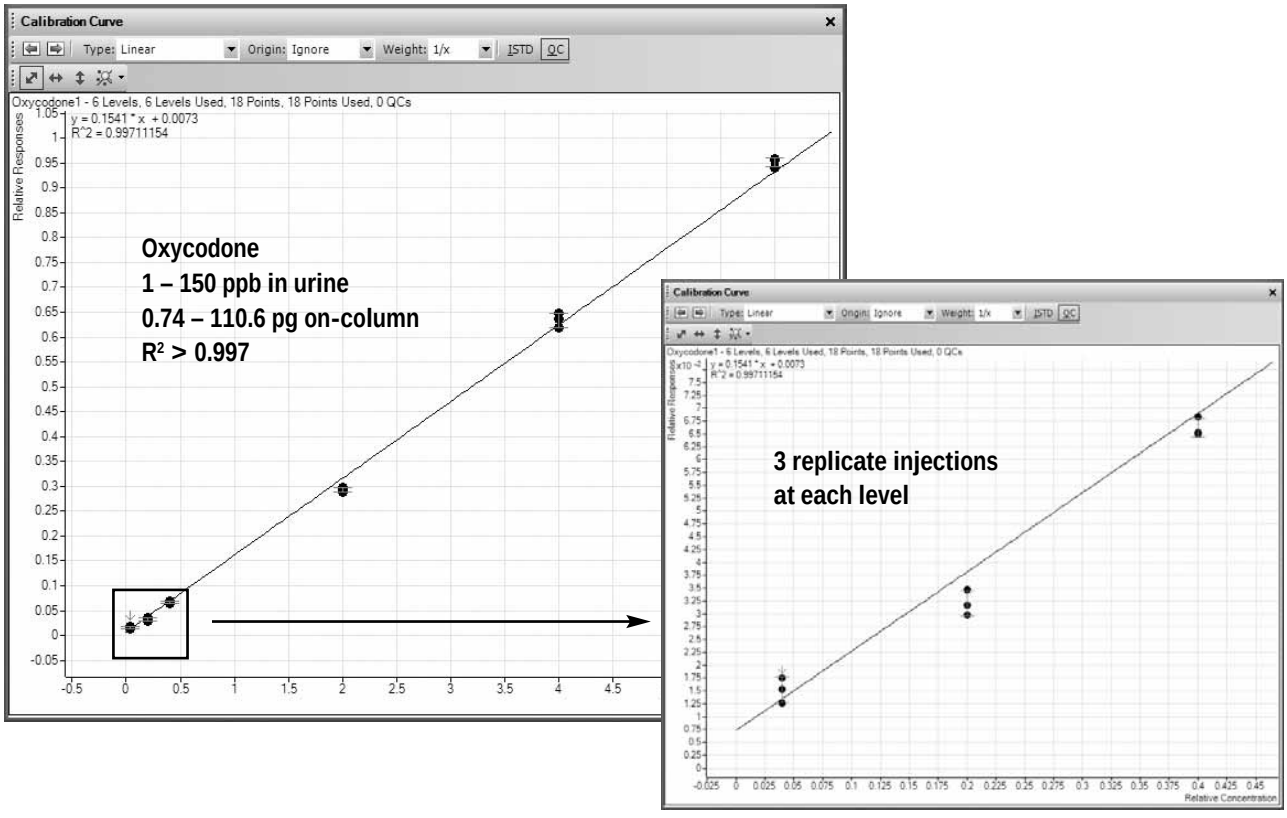


Figure 3E. Linearity of oxycodone in urine. Injection concentration range = 147 ppt – 22 ppb.

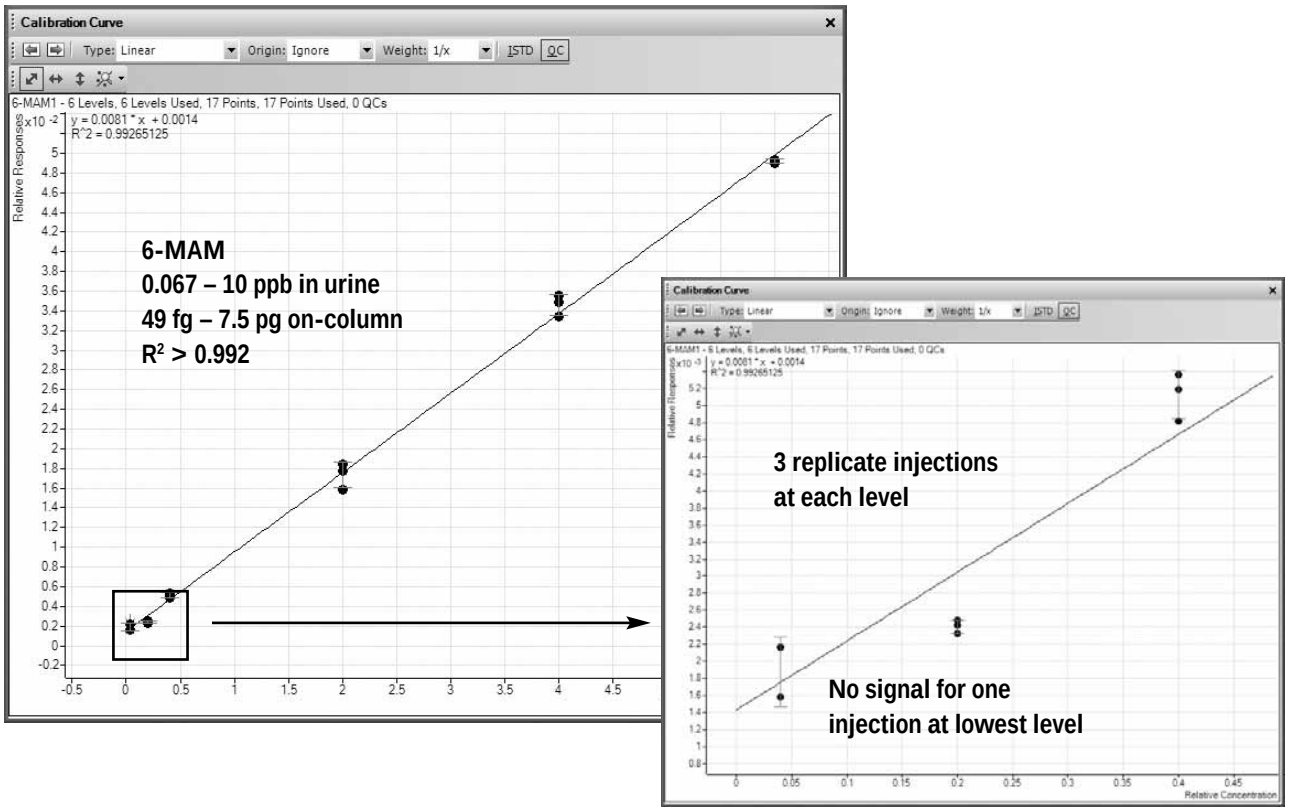


Figure 3F. Linearity of 6-MAM in urine. Injection concentration range = 9.8 ppt – 1.5 ppb.

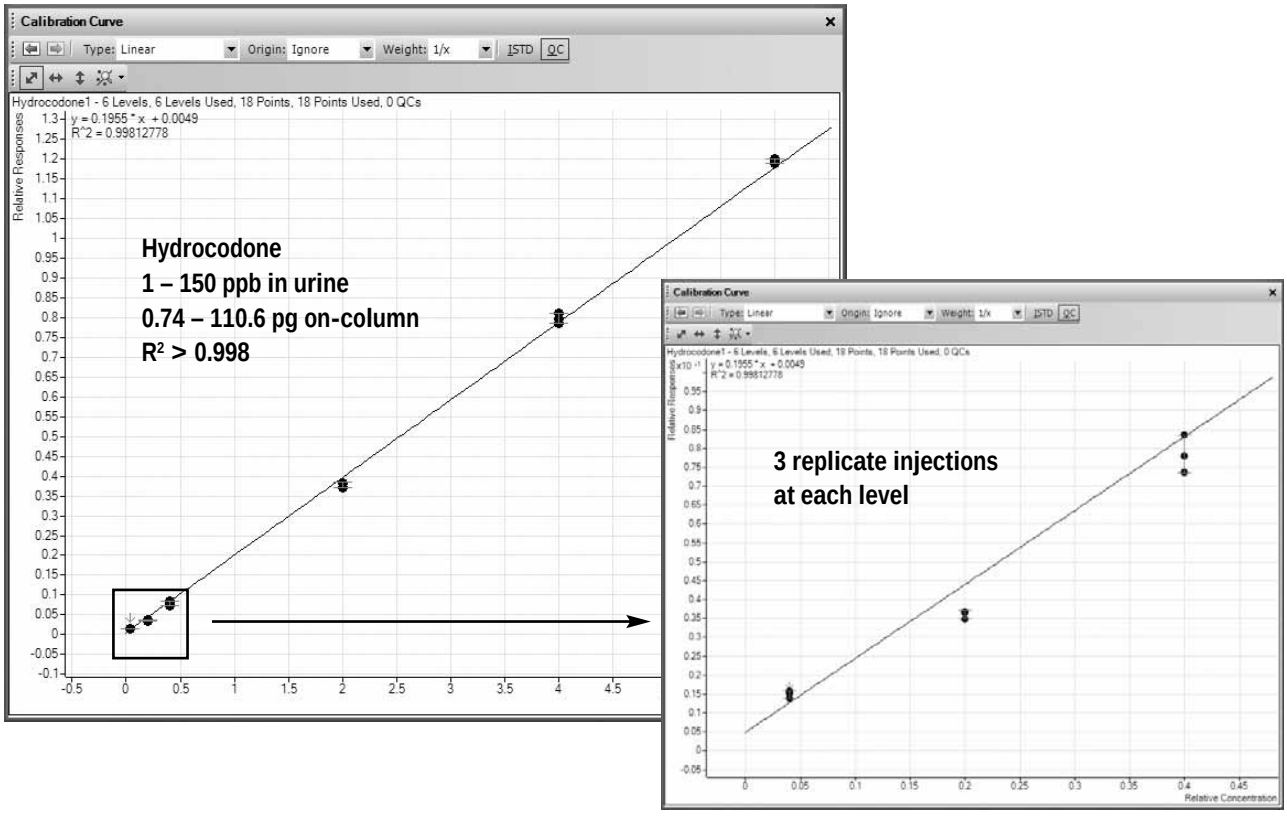
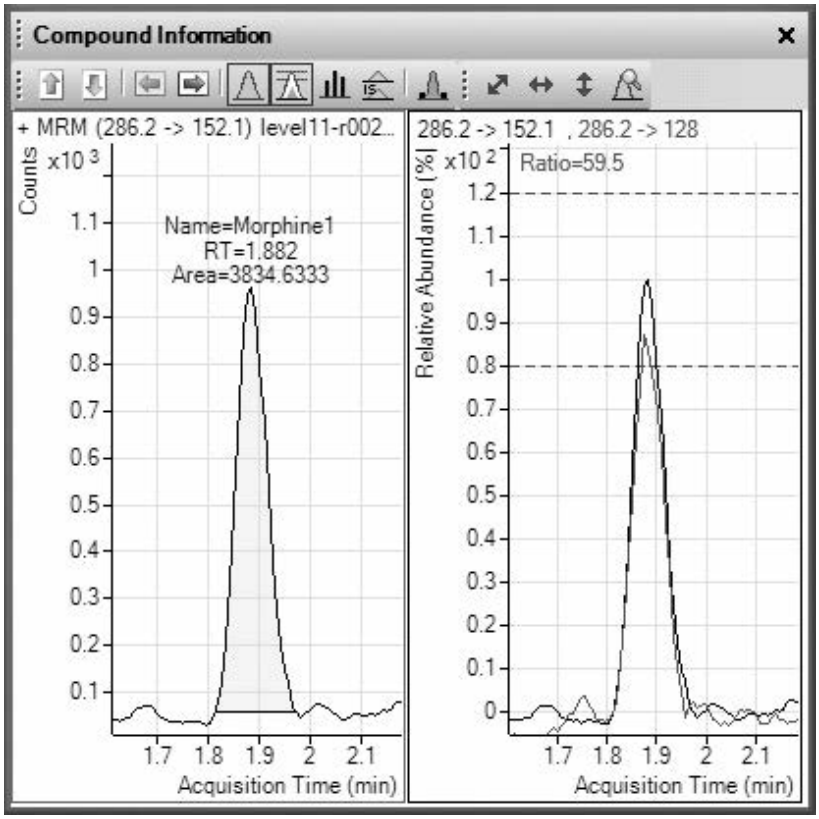


Figure 3G. Linearity of hydrocodone in urine. Injection concentration range = 147 ppt – 22 ppb.

Confirmation is carried out by examining the qualifier/quantifier ion ratio and making sure it stays within ± 20% of the determined value for each analyte. For example, after optimizing the MRM transitions for both product ions of morphine, it is automatically determined by the MassHunter Quantitative Analysis that the ratio of the qualifier peak to that of the quantifier should be 0.7%, or 70%. Applying a ± 20% tolerance to this ratio means that all calibration standards and samples analyzed in this batch should have a ratio of 0.56 to 0.84 in order to confirm the presence of morphine. The lowest calibration levels that consistently satisfy the confirmation requirement for each analyte are shown in Figures 4A through 4G.

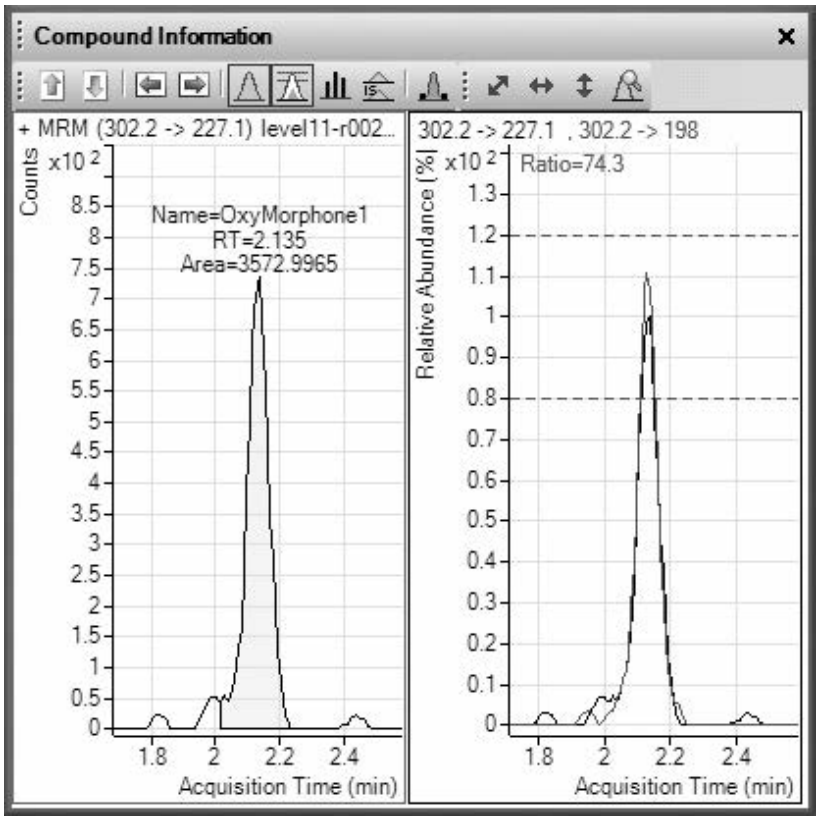
Note that with the exception of oxycodone and 6-MAM, the confirmation ion ratio for all analytes is satisfied at the corresponding lowest calibration levels of 1 pg/µL in urine. For oxycodone and 6-MAM, the lowest levels are 5 and 0.3 pg/µL, respectively.

Limits of detection (shown in Figures 5A through 5G) are also determined for this work using the quantifier ion of each analyte and based on a visual determination of peak-to-peak signal-to-noise ratio of at least 3:1 and a peak area %RSD (percent relative standard deviation) of 30 or less. The results for all analytes except oxycodone and 6-MAM are based on eight 1-µL injections at 147 fg on-column each. These correspond to original concentrations in urine of 1 pg/µL. For oxycodone, the LOD is determined from the triplicate 5-µL injections of the calibration level corresponding to 1 pg/µL (see Figure 5E). Like oxycodone, the LOD of 6-MAM is seen at a 5-µL injection, but of the 0.067 pg/µL level. However, only two of the three injections had signal so an area %RSD was not calculated. These values are further tabulated in Table 2.



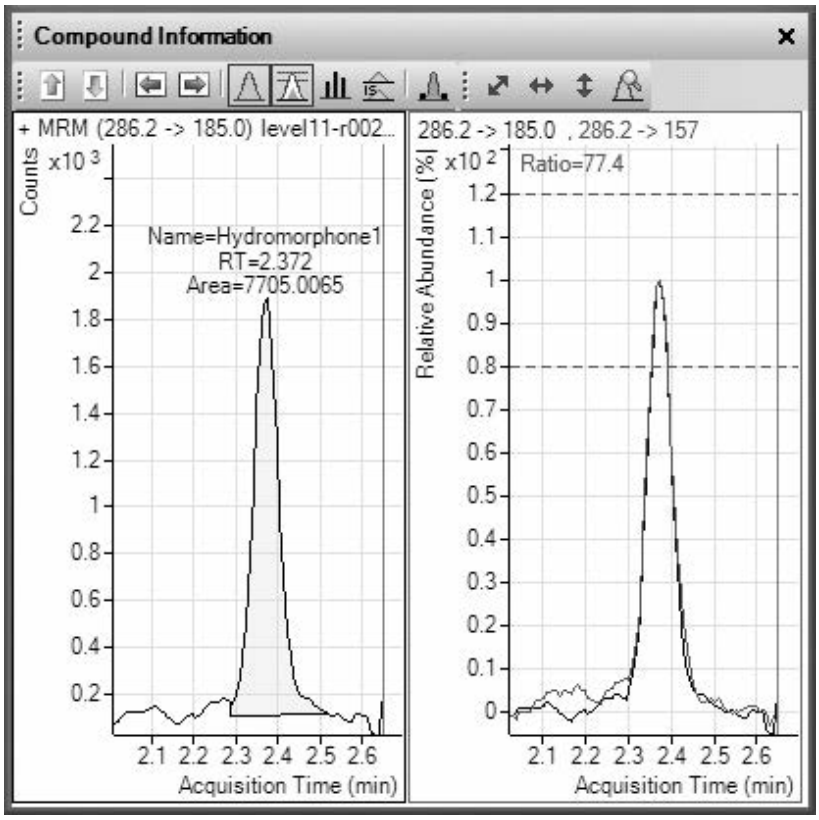
0.74 fg on-column

Figure 4A. Confirmation of morphine at 1 pg / µL (147 fg / µL).



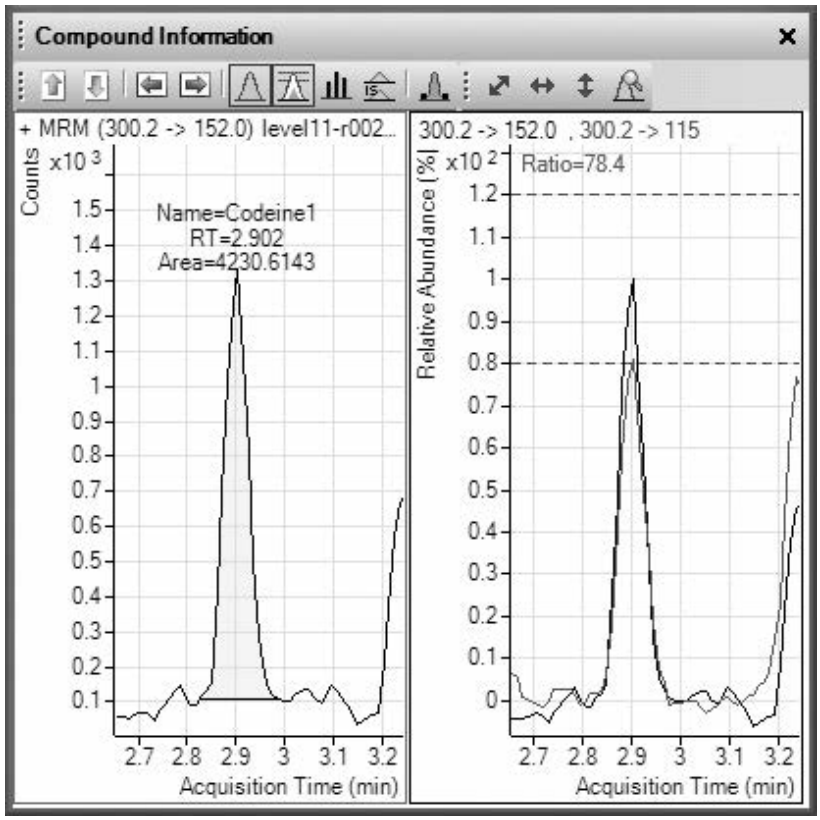
0.74 fg on-column

Figure 4B. Confirmation of oxymorphone at 1 pg / µL (147 fg / µL).



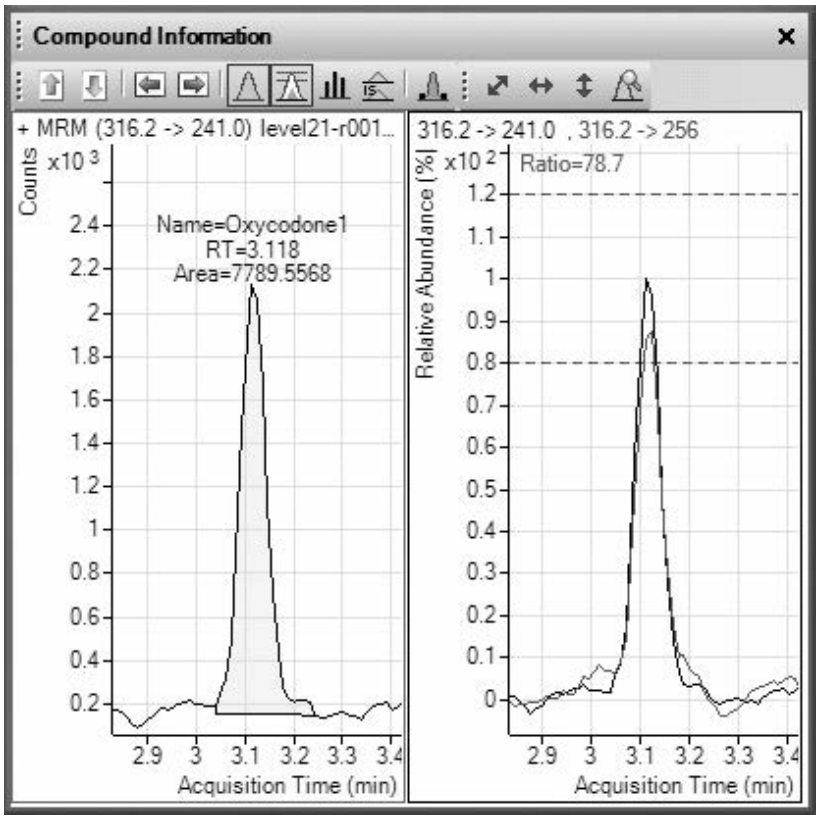
0.74 fg on-column

Figure 4C. Confirmation of hydromorphone at 1 pg / µL (147 fg / µL).



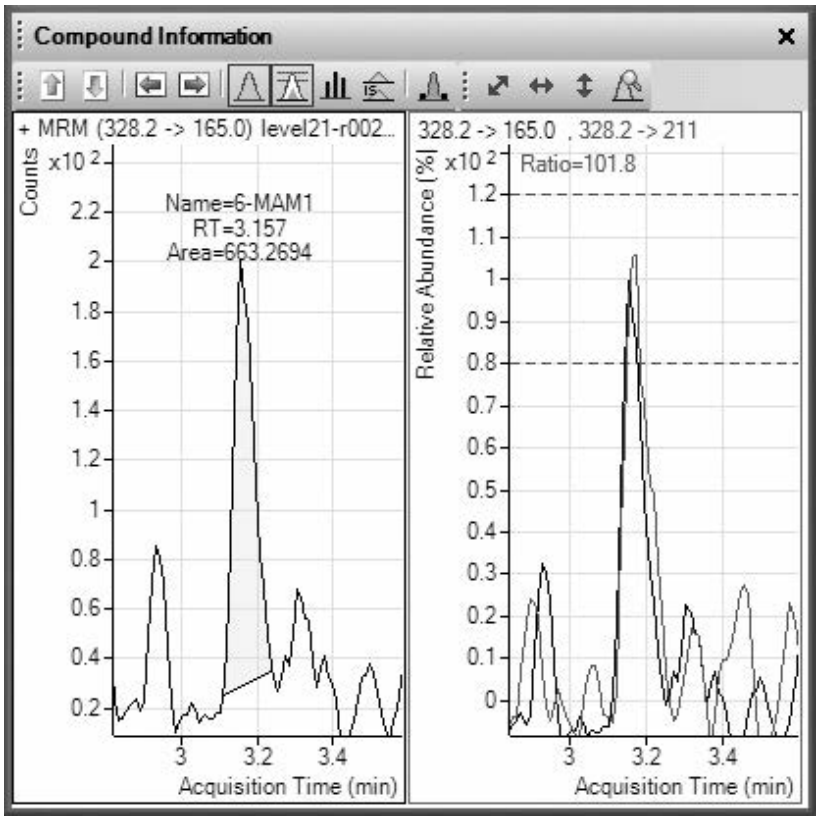
0.74 fg on-column

Figure 4D. Confirmation of codeine at 1 pg / µL (147 fg / µL).



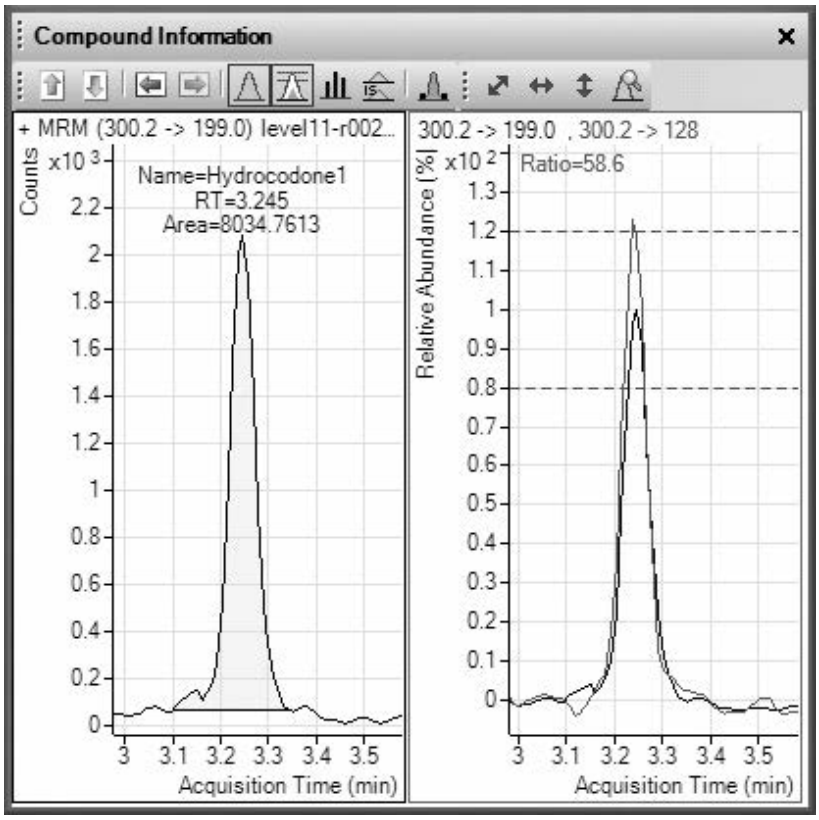
3.7 pg on-column

Figure 4E. Confirmation of oxycodone at 5 pg / µL (737 fg / µL).



245 fg on-column

Figure 4F. Confirmation of 6-MAM at 0.3 pg / µL (49 fg / µL).



0.74 pg on-column

Figure 4G. Confirmation of hydrocodone at 1 pg / µL (147 fg / µL).

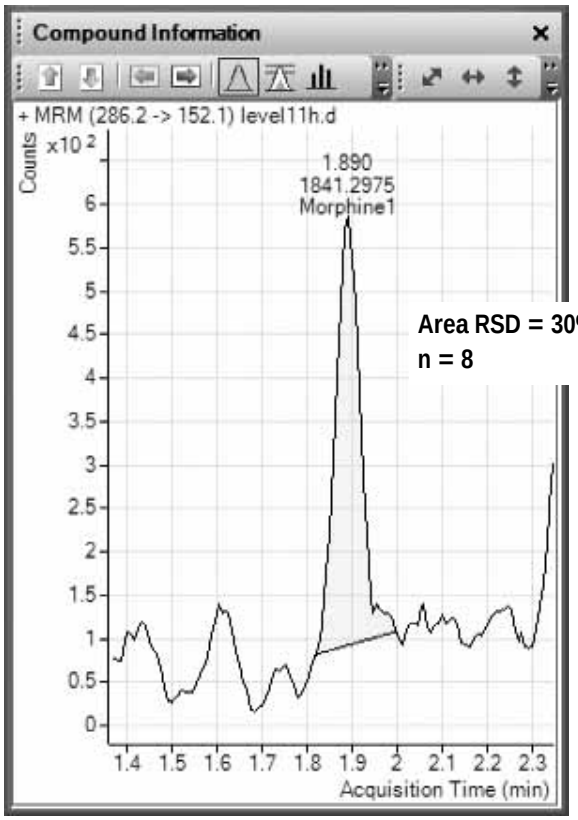


Figure 5A. LOD of morphine at 147 fg on-column.

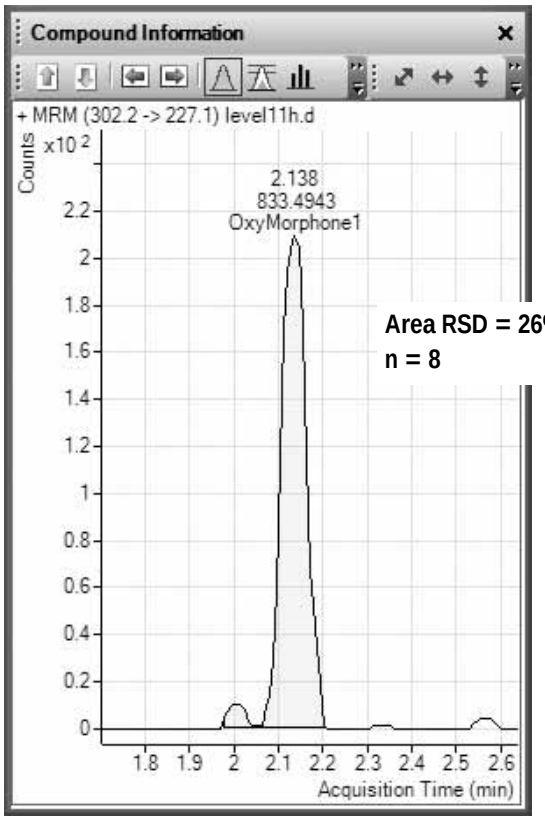


Figure 5B. LOD of oxymorphone at 147 fg on-column.

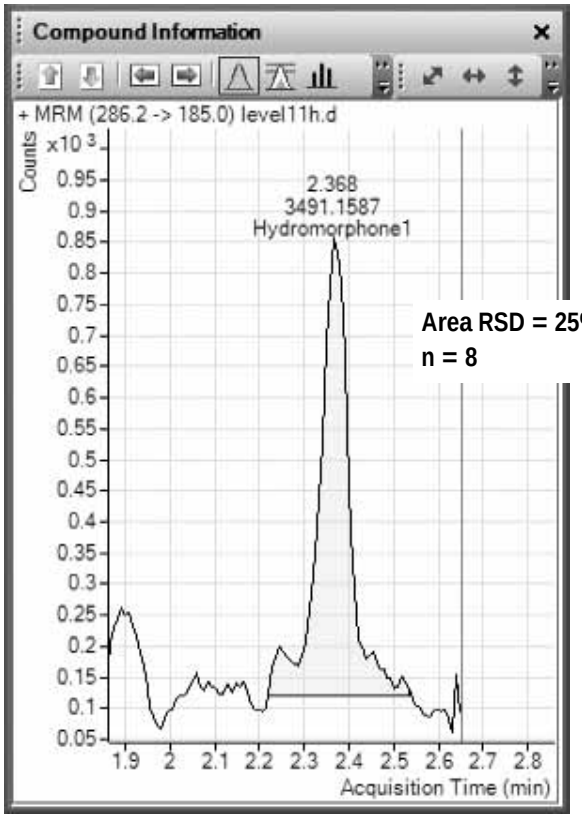


Figure 5C. LOD of hydromorphone at 147 fg on-column.

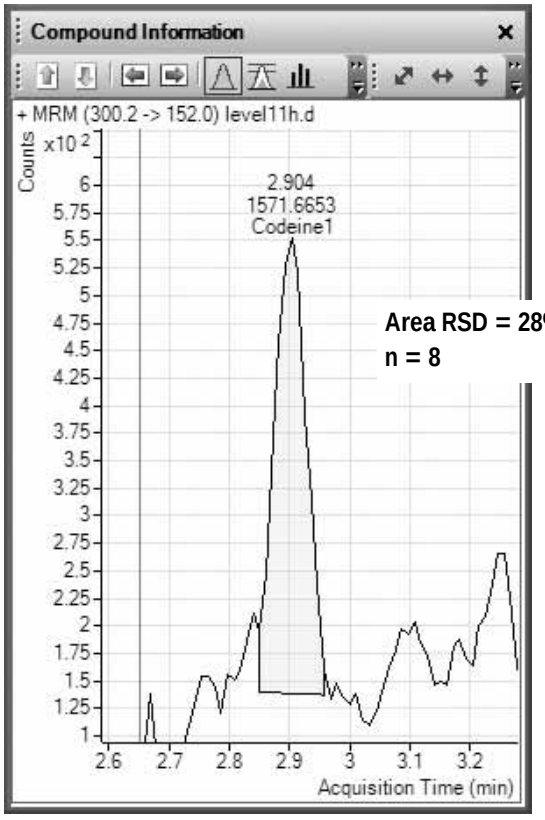


Figure 5D. LOD of codeine at 147 fg on-column.

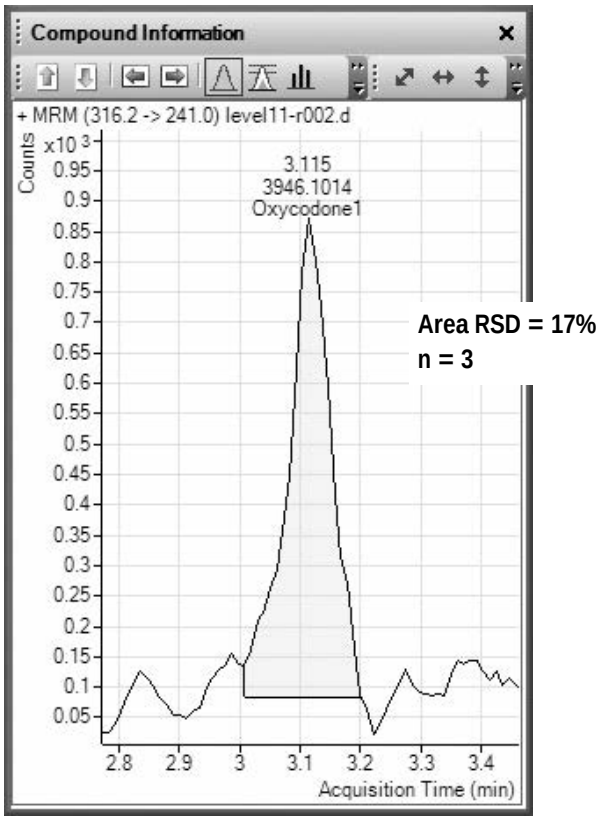


Figure 5E. LOD of oxycodone at 737 fg on-column.

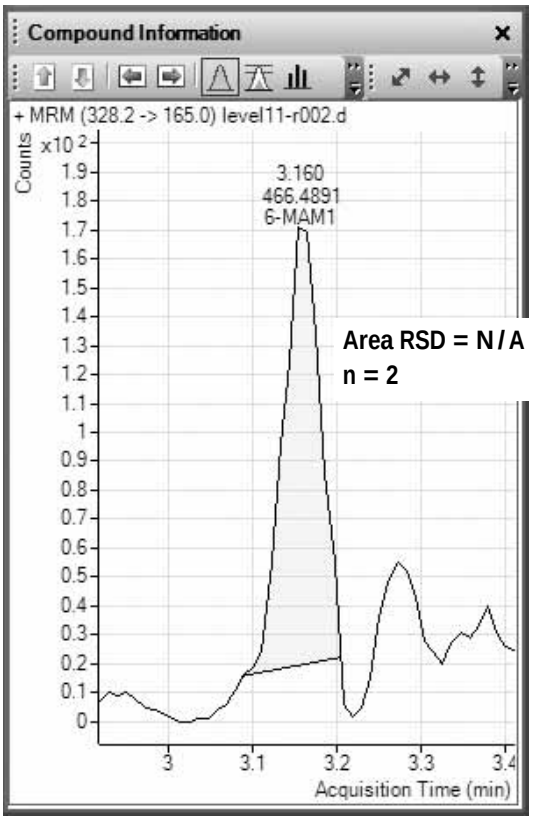


Figure 5F. LOD of 6-MAM at 49 fg on-column. Peak area %RSD not applicable because only two of three injections contained signal.

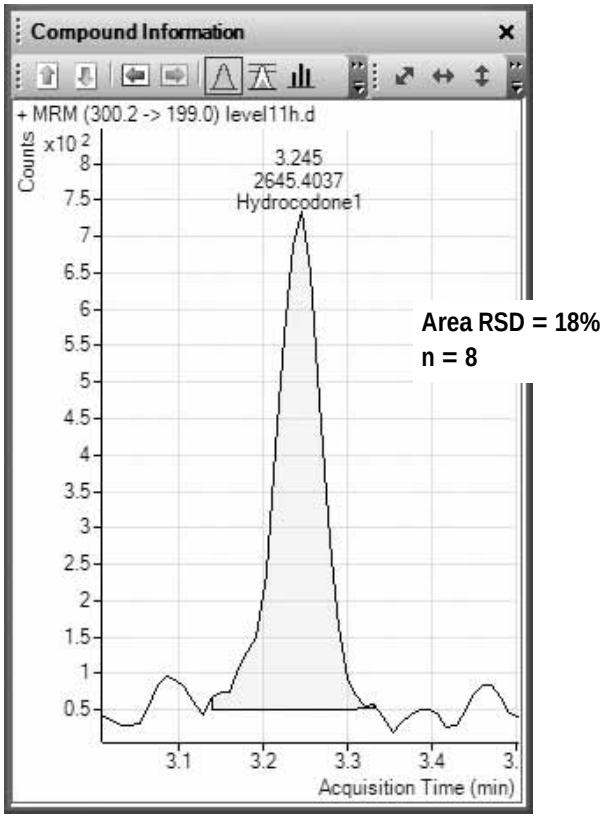


Figure 5G. LOD of hydrocodone at 147 fg on-column.

Table 2. Determined Limits of Detection (LODs) in Urine for Each Analyte

Analyte	LOD (fg on-column)
Morphine	147
Oxymorphone	147
Hydromorphone	147
Codeine	147
Oxycodone	737
6-MAM	49
Hydrocodone	147

Conclusions

Opiates are successfully analyzed in the presence of urine. Good linearity ($R^2 > 0.99$) is obtained for all compounds over two orders magnitude in concentration range, which is 1 to 150 ppb for all analytes except 6-MAM; for 6-MAM this range is 0.067 to 10 ppb. After processing the samples and considering the 5-µL injection volume, this range corresponds to 0.74 to 110.6 pg on-column (49 fg to 7.5 pg for 6-MAM). The calibration curve fitting is carried out with no inclusion of the origin, a linear fit, and a 1/x weighting. At the lowest levels very good reproducibility and accuracy is demonstrated. Limits of detection are less than 1 pg on-column for all analytes. The Agilent 6410 QQQ is an excellent instrument for sensitive quantitation in a relatively dirty matrix.

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For more information on our products and services, visit our Web site at www.agilent.com/chem.

For more details concerning this application, please contact Michael Zumwalt at Agilent Technologies, Inc.

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For Forensic Use.

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Agilent Technologies

VARIAN

LC/MS Application Note

Number 10.r1
December 2003

Rapid Screening of Amphetamine Drugs in Urine by Positive Ion Electrospray LC/MS/MS

Forensic Toxicology

Z. Yang and S. Sadjadi, Varian, Inc.

Introduction

Amphetamine drugs are often abused and misused. Forensic toxicology and doping laboratories are frequently asked to analyze for the presence of amphetamines in urine. Urine samples are most common because large sample volumes can be collected non-invasively. These drugs generally remain detectable in urine for two to three days longer than in blood. For most forensic applications, initial screening is done by immunoassay with presumptive positive samples confirmed by a second, more specific method such as gas chromatography/mass spectrometry (GC/MS).

A simple and sensitive LC/MS/MS method is described below for high throughput identification and quantitation amphetamine drugs in urine. A rapid and effective solid-phase extraction (SPE) procedure using Focus™ was used to extract amphetamines from urine samples.

Instrumentation

- Varian ProStar 410 AutoSampler
- Varian ProStar 210 Isocratic Solvent Delivery Modules
- Varian 1200L LC/MS equipped with ESI source

Materials and Reagents

- Standard solutions: 1.0 mg/mL ((±)-Amphetamine, (±)-Methamphetamine, 1S,2R(+)-Ephedrine, (±)-MDMA, (±)-MDA and (±)-MDEA), from Cerilliant Corp., Texas, USA.
- Internal standard (IS) solutions: 0.1 mg/mL ((±)-Amphetamine-D5, (±)-Methamphetamine-D5, 1S,2R(+)-Ephedrine-D3 HCl, (±)-MDA-D5, (±)-MDMA-D5 and (±)-MDEA-D5), from Cerilliant Corp., Texas, USA.
- All other chemicals are reagent grade or HPLC grade.
- Focus™ Solid Phase Extraction Cartridges (Varian Part No. A5306021).
- In-house vacuum or vacuum pump (Varian Part No. WL2012B01).
- Vac Elut 20 Manifold with the standard Glass Basin (Varian Part No. 12234505) and Collection Rack for 13 x 75 mm test tubes (Varian Part No. 12234507).

Sample Preparation

A 100 µL aliquot of a 500 ng/mL deuterated internal standards solution was transferred into individually labeled tubes (double blank tube was urine only). To each tube, a 1 mL of urine sample followed by 0.1 mL of 0.1 N KOH solution was added and mixed by vortex.

The mixture was loaded onto the sorbent bed of an activated 3 mL Focus cartridge pretreated with 1 mL of methanol followed by a 1 mL deionized water wash under gentle vacuum of1 to 2 in. Hg. Next, the sorbent bed was washed with 2 x 1 mL acetonitrile/water (10:90, v/v) under gentle vacuum.

The analyte was collected in a 2 mL autosampler vial by eluting with 2 x 100 µL elution solvent (acetonitrile/methanol/water/formic acid (22:68:9:1, v/v) under gentle vacuum. The sorbent bed was then flushed with 600 µL of water under vacuum to wash off the elution solvent and dilute the sample for injection. A 10 µL aliquot was injected directly for analysis.

HPLC Conditions

Column	MonoChrom MS 5 µm, 50 x 2 mm (Varian Part No. A2080050X020)			
Mixer	250 µL static mixer			
Solvent A	0.2% formic acid:10mM NH_4OAc in water (v/v)			
Solvent B	acetonitrile/methanol (1:1, v/v)			
LC Program	Time (min:sec)	%A	%B	Flow (mL/min)
	0:00	75	25	0.25
	6:00	75	25	0.25
Injection Volume	10 µL			
Injection Solvent	acetonitrile/methanol/water/formic acid (5.5:17:77.25:0.25, v/v)			

MS Parameters

Ionization Mode	ESI positive
Collision Gas	2.0 mTorr Argon
API Drying Gas	30 psi at 380 °C
API Nebulizing Gas	59 psi
Scan Time	1.8 sec
SIM Width	0.7 amu
Needle	5000V
Shield	600V
Capillary	30V
Detector	1800V

Scan Parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
(±)-Amphetamine	136	91	14.0
	136	119	6.5
(±)-Amphetamine-D5	141	96	12.5
(±)-Methamphetamine	150	91	17.0
	150	119	9.0
(±)-Methamphetamine-D5	155	92	16.5
1S,2R(+)-Ephedrine	166	117	17.0
	166	148	10.0
1S,2R(+)-Ephedrine-D3	169	151	9.5
(±)-MDA	180	105	20.5
	180	163	9.0
(±)-MDA-D5	185	168	9.0
(±)-MDMA	194	135	19.0
	194	163	10.0
(±)-MDMA-D5	199	165	10.5
(±)-MDEA	208	135	18.0
	208	163	11.5
(±)-MDEA-D5	213	163	12.0

MRM Chromatograms of Amphetamines

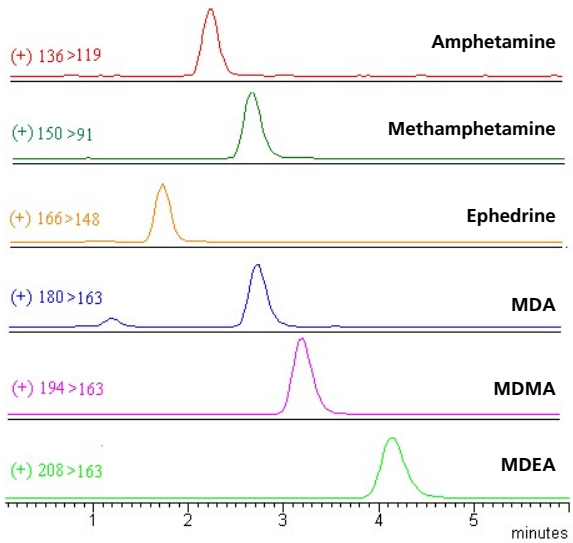

Figure 1. Good separations with short run time and no matrix interferences. Sample: spiked 50 ng/mL in urine.

Example of a Tox Report for Methamphetamine

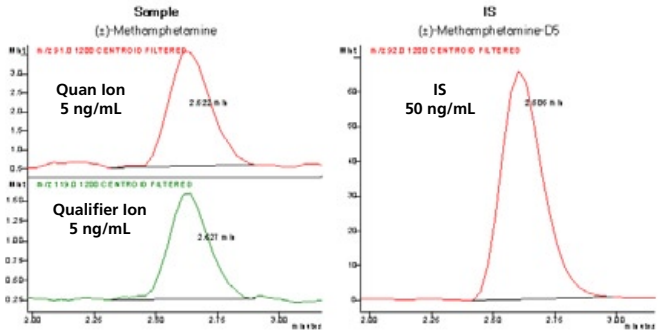

Figure 2. The positive identification was confirmed by retention time matching of the Quan ion with the confirmatory qualifier ion. The IS was used to measure and calculate recovery. Also, the IS was used to provide additional confirmation by retention time as a reference marker.

Example of a Standard Calibration Curve for Methamphetamine

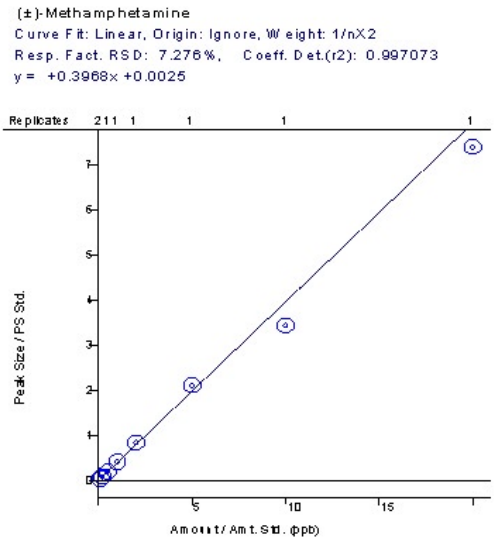

Figure 3. Eight calibration levels (5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL) standard with 50 ng/mL internal standard.

Example of Breakdown Curve for Methamphetamine

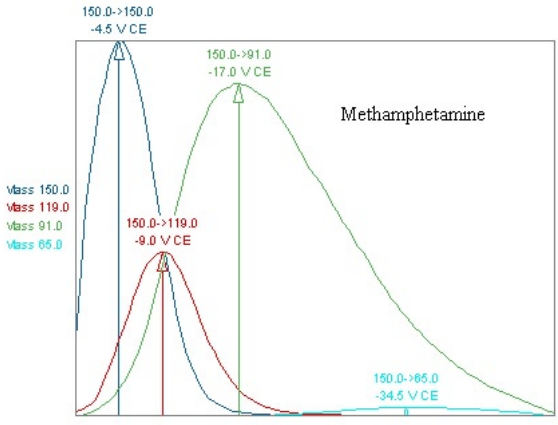

Figure 4. In this typical MS breakdown curve, methamphetamine gives two intense product ions, 150>91 and 150>119.

Results and Discussion

The LC method used a six-minute run cycle time with the first peak at 1.65 minutes and the last peak at 4.12 minutes (Figure 1). The two product ions for each analyte can be quantitatively analyzed at the level of 5 ng/mL in urine (Figure 2, about 50 pg on-column). This level is 50 times below the proposed drug cutoff levels published by the Substance Abuse and Mental Health Services Administration (SAMHSA).[1] Eight concentration levels were used to generate the calibration curves for the standard. The linearity of the detector response and the response factor-Relative Standard Deviation (rf-RSD) are excellent (Table 1, Figure 3).

The recovery of the drugs from urine was > 85%. The eluent from the Focus cartridge can be injected directly into LC/MS system without derivatization, evaporation, and reconstitute steps. The 96-well format Focus can be used for automation and high-throughput screening.

Only two product ions were used for this analysis because amphetamine and methamphetamine only give two intense product ions (Figure 4, Table 2) while ephedrine, MDA, MDMA, and MDEA produce multiple intense product ions (Table 2). Run-to-run retention time is very reproducible with a <1.4% RSD. Two product ions with a retention time match can be strong evidence for positive identification of amphetamine drugs (Figure 2). Both the urine double blank and the blank with IS show no interference of the analysis at low quantitation level (LQL). For the standard calibration curve, the LQL is 5 ng/mL and upper quantitation level (UQL) is 1000 ng/mL. This LC/MS/MS method is very sensitive and can be possibly adapted to other body fluid analysis for amphetamines, such as sweat and oral fluid which have confirmatory drug cutoff levels of 25 ng/mL and 50 ng/mL, respectively.

Conclusion

The LC/MS/MS method described in this application note is simple and sensitive. This method can quantitatively analyze amphetamine drugs at 50 times below the drug cutoff levels in urine. The Varian SPE and 1200L LC/MS/MS system demonstrated excellent performance for the urinalysis of amphetamines. The system can be a useful tool for forensics toxicology and doping laboratories.

Reference

1. http://workplace.samhsa.gov/ResourceCenter/DT/FA/GuidelinesDraft4.htm

*ng/mL=ppb

Combined Results of LC/MS Study of Amphetamines

	Retention Time		Curve Parameters				Drug Cutoff Levels in Urine	
Drug Name	min	RSD (%)	R^2	rf-RSD (%)	LQL (ng/mL)	S/N (5 ng/mL)	Initial (ng/mL)	Confirmatory (ng/mL)
Amphetamine	2.16	0.91	0.995	7.96	5	163	500	250
Methamphetamine	2.57	0.94	0.997	7.29	5	1242	500	250
Ephedrine	1.65	0.94	0.999	3.79	5	1031	500	250
MDA	2.67	1.22	0.999	9.39	5	521	500	250
MDMA	3.14	1.24	0.999	1.86	5	729	500	250
MDEA	4.12	1.40	0.999	4.84	5	440	500	250

Table 1. Run-to-run retention time over 13 injections was very reproducible. The linearity of the detector response and the response factor-RSD are excellent. The LQL of this method is 50 times below the proposed drug cutoff levels as published by the SAMHSA.

Summary of Breakdown Data by Ion Transition, Ion Intensity, and Collision Energy (V)

Amphetamine	Methamphetamine	Ephedrine	MDA	MDMA	MDEA
136>91, 100%, -14.5	150>150, 100%, -4.5	166>148, 100%, -10.0	180>163, 100%, -9.0	194>163, 100%, -10.0	208>163, 100%, -11.5
136>136, 64.26%, -4.5	150>91, 88.54%, -17.0	166>166, 75.85%, -4.0	188>188, 50.46%, -4.0	194>194, 95.26%, -4.5	208>208, 89.31%, -4.0
136>119, 63.06%, -7.5	150>119, 43.65%, -9.0	166>117, 15.39%, 17.0	180>105, 32.34% -20.5	194>105, 33.3%, -22.0	208>105, 31.77%, -23.0
136>65, 3.99%, -32.5	150>65, 2.26%, -34.5	166>115, 14.5%, -24.0	180>133, 29.25%, -16.0	194>135, 29.93%, -19.0	208>135, 29.97%, -18.0
		166>133, 13.68%, -18.5	180>135, 29.5%, -16.0	194>133, 29.74%, -18.5	208>133, 29.11%, -18.0
		166>91, 8.82%, -29.0	180>77, 6.83%, -32.0	194>77, 6.34%, -35.5	208>103, 8.03%, -33.0

Table 2. Amphetamine and methamphetamine only give two intense product ions while ephedrine, MDA, MDMA, and MDEA produce multiple intense product ions.

These data represent typical results.
For further information, contact your local Varian Sales Office.

An Application Kit for the Screening of Samples for Analytes of Forensic Toxicological Interest using LC/QQQ MS/MS with a Dynamic MRM Transition Database

Application Note

Forensic Toxicology

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Abstract

A Forensic Toxicological screening application kit has been developed for use with the Agilent 6400 Series triple quadrupole (QQQ) LC/MS systems which contains a database of optimized MRM transitions for approximately 200 analytes of forensic and toxicological interest. The database content is mainly focused on controlled substances and drugs of abuse. The aim of this application kit is to provide a user with a solid starting point for building analysis methods where the ability to screen for a large array of forensic toxicological analytes is necessary. Typical results obtained from such a method created by using the database are described using serial dilutions of a test mix containing analytes of forensic interest.

Agilent Technologies

Introduction

Lists of potential toxins and analytes of forensic interest can be extremely large and typically depend on the area of analytical screening focus (for example, workplace drug testing, doping control, postmortem toxicology, explosive residues, and so forth). Often, the concentration levels of such target analytes are challenging and low, which can be further impacted by a complex sample matrix or the quantity of sample obtained.

The most sensitive liquid chromatography/mass spectrometry (LC/MS) screening or quantitation techniques are those based around triple quadrupole (QQQ) LC/MS/MS instruments, where a second stage of MS (post fragmentation from a collision cell) acts as an effective method of eliminating background chemical noise that is not associated with the target precursor and fragment ions. This technique is commonly referred to as Multiple Reaction Monitoring (MRM.) Instruments using each quadrupole as targeted mass filters in this manner are an effective and widely accepted technique for forensic and toxicological studies of challenging sample matrices and concentration levels.

QQQ MS instruments, however, operate by focusing a finite amount of time on only one MRM transition before the next MRM transition is selected in turn. Once the complete list of target MRM transitions has been monitored, then the MRM list is repeated or cycled until the end of the chromatographic analysis or until a new retention time segment begins that contains different MRM transitions. The amount of finite time given to any specific MRM transition is referred to as dwell time and can be uniquely specified for every MRM transition.

The chromatographic consideration with regard to dwell time and overall MRM cycle time is one of peak width or resolution, normally referred to as full width at half maximum (FWHM). Statistically, higher numbers of data points measured across a chromatographic peak will provide more accurate and reproducible results. This means that the overall cycle time of the MRM target list must be sufficiently low to achieve this, relative to the particular chromatography used. Furthermore, each MRM transition dwell time must be high enough to output ion statistics of high quality and precision.

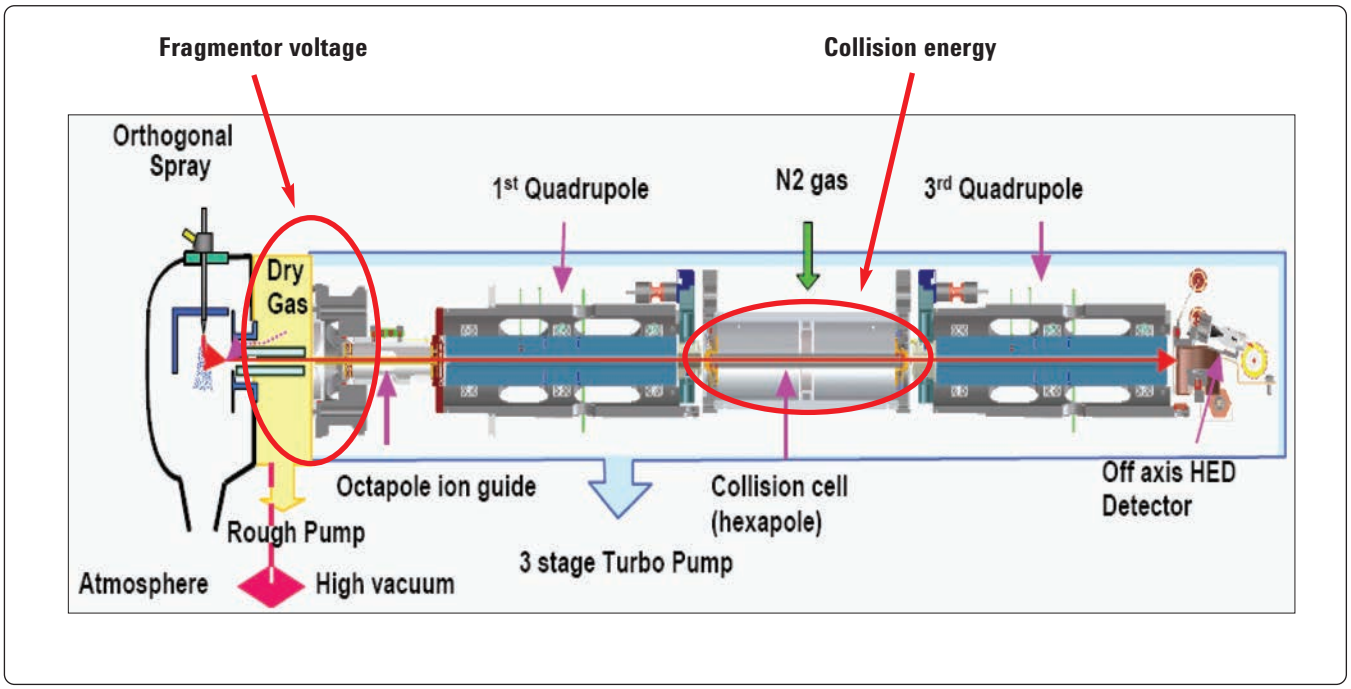

Figure 1. Two key optimized MRM transition settings.

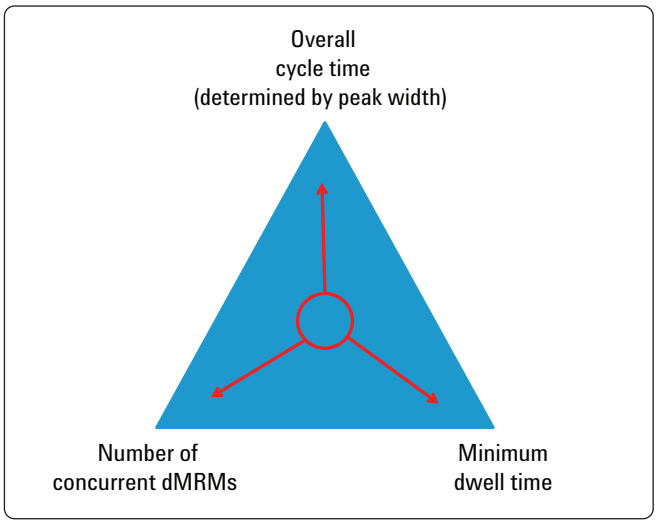

Figure 2. Compromise between cycle time, peak width, dwell time and number of MRM transitions.

Therefore, compromise between cycle time, dwell time and ultimately the total number of MRM transitions is often required especially with larger suites of analytes in a target screen assay (Figure 2). For this reason, Agilent Technologies introduced Dynamic MRM (dMRM) [1] functionality on the Agilent 6400 Series QQQ LC/MS system. Dynamic MRM is a technique where each ion transition has an associated retention time window (delta RT) where it is dynamically switched on and off without impacting a constant data cycle time. Since the complete list of ion transitions is unlikely to be cycled through at any given chromatographic retention time, then the result is normally higher dwell time for every transition and higher data quality when compared to normal MRM methods. Figure 3 graphically illustrates the Dynamic MRM principle.

Herein are described the results obtained from an analysis method using the Agilent MassHunter Forensic Toxicology Dynamic MRM Database Kit (G1734AA) with optimized MRM transitions from the database inserted direct-ly into the acquisition method. More detailed instruction on the creation of such methods are outlined in the G1734AA

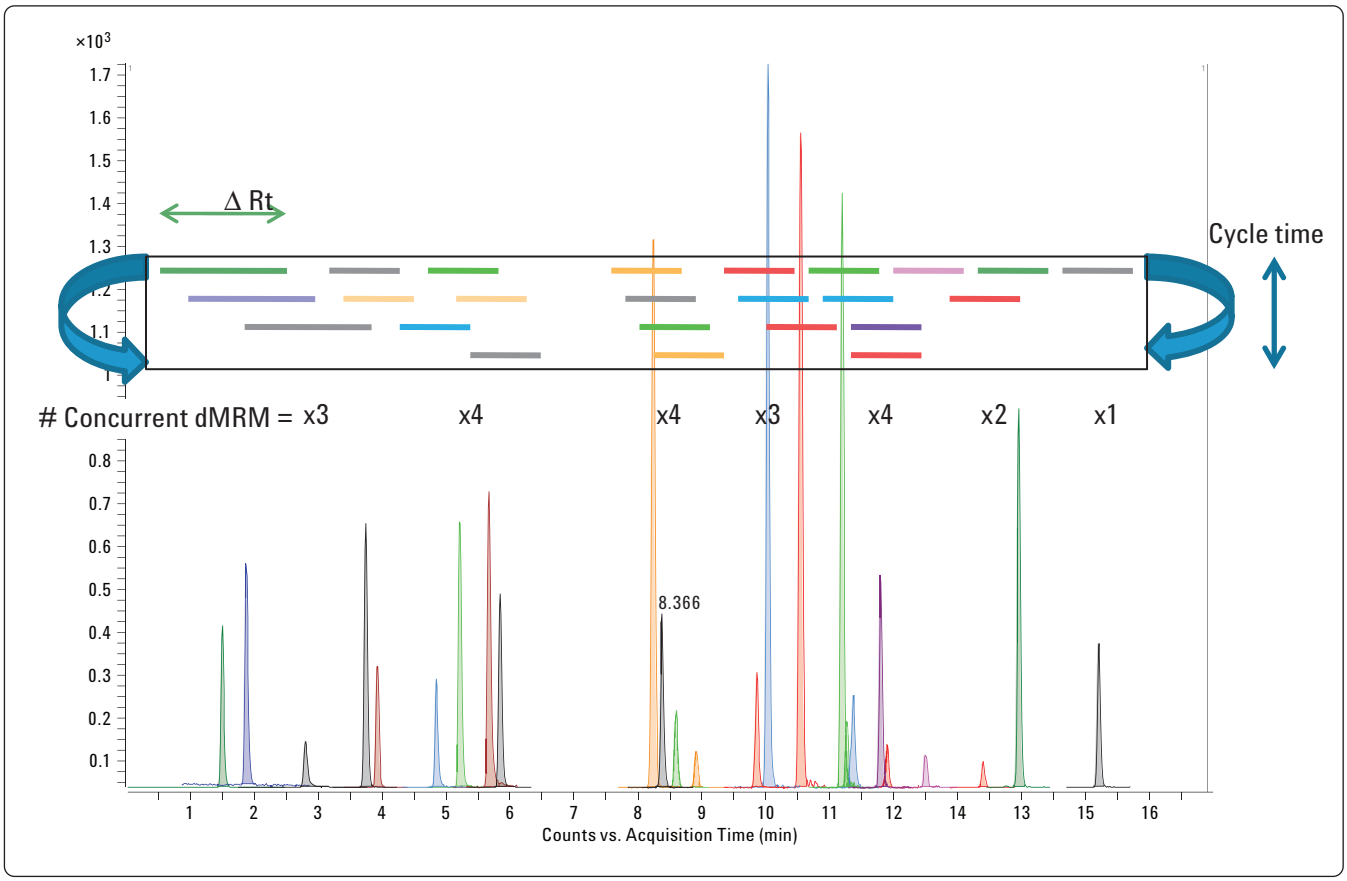

Figure 3. Illustration of Dynamic MRM principle.

MassHunter Forensic Toxicology Dynamic MRM Database Kit Quick Start Guide [2]. Confirmatory evidence was obtained by using the two most abundant MRM transitions for use as quantifier and qualifier ions, the ratio of which are indicative of the analyte of interest. This application note aims to describe typical results using an LC/MS Forensic Toxicology Test Mix.

Experimental

The analysis results outlined in this application note were obtained using an Agilent 6460 QQQ LC/MS coupled to an Agilent 1200SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system equipped with the MassHunter Optimizer program (Rev. B.02.01) and the [G1734AA] forensic toxicology Dynamic MRM application kit.

Sample Preparation

An ampoule from the LC/MS Forensic Toxicology Test Mix [p/n 5190-0470] which is included in the Forensic and Toxicology application kit [G1734AA] was opened and 100 µL of the 1 µg/mL (1ppm) solution was diluted to a concentration of 10 ng/mL (10 ppb) using 9.9 mL of pure LC/MS grade methanol to create a clean solvent standard for method checkout purposes.

Appropriate serial dilutions from the original LC/MS Forensic Toxicology Test Mix were created for the purposes of quantitation. These are listed in Table 1.

Table 1. Dilution Series of LC/MS Forensic Toxicology Test Mix

Data File	Type	Level	Vol. (uL)	Conc.	Units
LCMS_Forensic and Toxicology Test Mix 10fg.d	Cal	1	1	10	fg on-column
LCMS_Forensic and Toxicology Test Mix 25fg.d	Cal	2	1	25	fg on-column
LCMS_Forensic and Toxicology Test Mix 50fg.d	Cal	3	1	50	fg on-column
LCMS_Forensic and Toxicology Test Mix 100fg.d	Cal	4	1	100	fg on-column
LCMS_Forensic and Toxicology Test Mix 250fg.d	Cal	5	1	250	fg on-column
LCMS_Forensic and Toxicology Test Mix 500fg.d	Cal	6	1	500	fg on-column
LCMS_Forensic and Toxicology Test Mix 1pg.d	Cal	7	1	1000	fg on-column
LCMS_Forensic and Toxicology Test Mix 5pg.d	Cal	8	1	5000	fg on-column
LCMS_Forensic and Toxicology Test Mix 10pg.d	Cal	9	1	10000	fg on-column
LCMS_Forensic and Toxicology Test Mix 25pg.d	Cal	10	1	25000	fg on-column
LCMS_Forensic and Toxicology Test Mix 50pg.d	Cal	11	1	50000	fg on-column

Table 2 outlines the composition of the LC/MS Toxicology Test Mix [p/n 5190-0470] which is intended to cover a wide and representative range of forensic analyte classes.

Table 2. LC/MS Forensic Toxicology Test Mix Components (1 µg/mL)

Compound Name	Formula	Mass
3,4-Methylendioxyamphetamine (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
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
Methylendioxymethamphetamine (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

Reagents and Chemicals

Burdick & Jackson LC/MS grade acetonitrile together with deionized water (locally produced 18.1 MΩ) were used for mobile phases. Buffers were freshly prepared using a high purity source of formic acid and ammonium formate.

Instrumentation

LC Conditions

Column:	Agilent Zorbax Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm [p/n - 959764-902]
Column temperature:	60 °C
Mobile phase	A: 5 mM NH_4 formate/0.01% Formic acid in water B: 0.01% formic acid in acetonitrile
Flow rate:	0.5 mL/min

Gradient program:

Time (min)	A (%)	B (%)	Flow rate mL/min
Initial	90	10	0.5
0.5	85	15	0.5
3.0	50	50	0.5
4.0	5	95	0.5
6.0	5	95	0.5

Injection volume:	1 µL (with 5 second needle wash in flushport)
Analysis time:	6.0 min
Post time:	2.0 min
Overall cycle time:	8.0 min

6460 QQQ LC/MS Conditions

Source Conditions:
Electrospray AP-ESI (using Agilent Jet Stream Technology):

Positive ionization polarity

Sheath gas temperature and flow:	380 °C, 12 L/min
Nozzle voltage:	500 V
Drying gas temperature and flow:	320 °C, 8 L/min
Nebulizer gas pressure:	27 psi
Capillary voltage:	3750 V
Fragmentor voltage:	150 V

6410 QQQ LC/MS Conditions
(Results not included in this application note.)

Source Conditions:
Electrospray AP-ESI:

Positive ionization polarity

Drying gas temperature and flow:	350 °C, 12 L/min
Nebulizer gas pressure:	30 psi
Capillary voltage:	2000 V
Fragmentor voltage:	150 V

All other instrument operating parameters were taken care of by Agilent's autotune functionality and subsequent mass calibration using standard settings.

Dynamic MRM Acquisition Method Parameters

Table 3. Dynamic MRM Method Conditions

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Codeine	–	300.2	Unit	165.1	Unit	158	45	1.11	0.4	Positive
Codeine	–	300.2	Unit	58.1	Unit	158	29	1.11	0.4	Positive
Oxycodone	–	316.2	Unit	298.1	Unit	143	17	1.285	0.4	Positive
Oxycodone	–	316.2	Unit	256.1	Unit	143	25	1.285	0.4	Positive
δ-Amphetamine	–	136.1	Unit	119.1	Unit	66	5	1.296	0.4	Positive
δ-Amphetamine	–	136.1	Unit	91	Unit	66	17	1.296	0.4	Positive
MDA	–	180.1	Unit	163	Unit	61	5	1.332	0.4	Positive
MDA	–	180.1	Unit	105	Unit	61	21	1.332	0.4	Positive
Hydrocodone	–	300.2	Unit	199	Unit	159	29	1.4	0.4	Positive
Hydrocodone	–	300.2	Unit	128	Unit	159	65	1.4	0.4	Positive
Methamphetamine	–	150.1	Unit	119	Unit	92	5	1.45	0.4	Positive
Methamphetamine	–	150.1	Unit	91	Unit	92	17	1.45	0.4	Positive
MDMA	–	194.1	Unit	163	Unit	97	9	1.468	0.4	Positive
MDMA	–	194.1	Unit	105	Unit	97	25	1.468	0.4	Positive
Strychnine	–	335.2	Unit	184	Unit	195	41	1.629	0.4	Positive
Strychnine	–	335.2	Unit	156	Unit	195	53	1.629	0.4	Positive
MDEA	–	208.1	Unit	163	Unit	107	9	1.735	0.4	Positive
MDEA		208.1	Unit	105	Unit	107	25	1.735	0.4	Positive
Heroine		370.2	Unit	268.1	Unit	149	37	2.256	0.4	Positive
Heroin		370.2	Unit	165	Unit	149	61	2.256	0.4	Positive
Cocaine		304.2	Unit	182.1	Unit	138	17	2.376	0.4	Positive
Cocaine		304.2	Unit	77	Unit	138	61	2.376	0.4	Positive
Meperidine		248.2	Unit	220.1	Unit	128	21	2.419	0.4	Positive
Meperidine		248.2	Unit	174.1	Unit	128	17	2.419	0.4	Positive
Trazodone		372.2	Unit	176	Unit	159	25	2.797	0.4	Positive
Trazodone		372.2	Unit	148	Unit	159	37	2.797	0.4	Positive
PCP		244.2	Unit	91	Unit	86	41	2.876	0.4	Positive
PCP	–	244.2	Unit	86.1	Unit	86	9	2.876	0.4	Positive
Oxazepam	–	287	Unit	269	Unit	150	12	3.53	0.4	Positive
Oxazepam	–	287	Unit	241	Unit	150	20	3.53	0.4	Positive
Nitrazepam	–	282.1	Unit	236.1	Unit	148	25	3.542	0.4	Positive
Nitrazepam	–	282.1	Unit	180	Unit	148	41	3.542	0.4	Positive
Verapamil	–	455.3	Unit	165	Unit	158	37	3.554	0.4	Positive
Verapamil	–	455.3	Unit	150	Unit	158	45	3.554	0.4	Positive
Methadone	–	310.2	Unit	265.1	Unit	112	9	3.61	0.4	Positive
Methadone	–	310.2	Unit	105	Unit	112	29	3.61	0.4	Positive
Lorazepam	–	321	Unit	275	Unit	102	21	3.626	0.4	Positive
Lorazepam	–	321	Unit	194	Unit	102	49	3.626	0.4	Positive
Alprazolam	–	309.1	Unit	281	Unit	179	25	3.727	0.4	Positive
Alprazolam	–	309.1	Unit	205	Unit	179	49	3.727	0.4	Positive
Temazepam	–	301.1	Unit	255.1	Unit	117	29	3.941	0.4	Positive

Table 3. Dynamic MRM Method Conditions (continued)

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Temazepam	–	301.1	Unit	177	Unit	117	45	3.941	0.4	Positive
Proadifen	–	354.2	Unit	167	Unit	153	29	4.088	0.4	Positive
Proadifen	–	354.2	Unit	91.1	Unit	153	45	4.088	0.4	Positive
Diazepam	–	285.1	Unit	193	Unit	169	45	4.268	0.4	Positive
Diazepam	–	285.1	Unit	154	Unit	169	25	4.268	0.4	Positive
THC	–	315.2	Unit	193.2	Unit	150	20	5.277	0.4	Positive
THC	–	315.2	Unit	123.3	Unit	150	30	5.277	0.4	Positive

Results and discussion

Fast and easy startup with Agilent Test Mix

In order to rapidly implement and verify that acquisition and data analysis methodology is correctly set up, the LC/MS Forensic Toxicology Test Mix [p/n 5190-0470] is included in the Forensic Toxicology Dynamic MRM application kit [G1734AA] which contains a representative range of forensic analyte classes of 25 components (Table 2).

To create a method from first principles, the required transitions are selected from the database browser window (Figure 4). Once each selection has been made, the transitions are transferred to the acquisition method by clicking the 'Import' button to the bottom right of the browser window. An example of an acquisition method is illustrated in Figure 5.

Detailed information on this operation is contained in the MassHunter Forensic Toxicology Dynamic MRM Database Kit Quick Start Guide [2].

Using the methodology outlined in the experimental section, a 1-uL injection of the 10 ng/mL LC/MS Forensics Toxicology Test Mix equates to a 10 pg on-column injection amount. Figure 6 illustrates a typical overlay of extracted compound chromatograms for the test mix. A prepared method for QQQ is included in the application kit. When this method is loaded all conditions are correct and the user is able to reproduce the analysis.*

*These methods are acquisition-only and correspond to the instrument configuration as outlined in the experimental section of this application note. Appropriate settings must be manually input if a different instrument configuration is used. Similar results will demonstrate that the system is working properly.

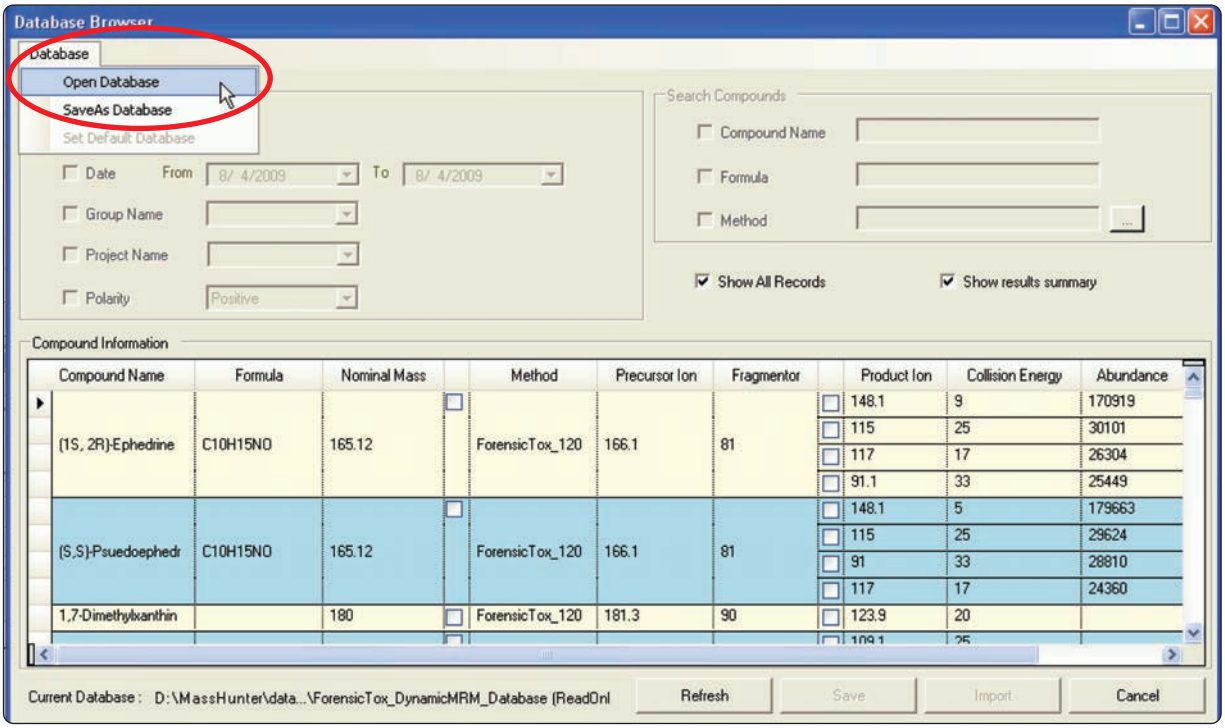

Figure 4. Compound MRM database browser containing 200 forensic analytes.

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragmentor	Collision Energy	Ret Time (min)	Delta Ret Time	Polarity
Alprazolam	☐	309.1	Unit	281	Unit	179	25	3.715	1	Positive
Cocaine	☐	304.2	Unit	182.1	Unit	138	17	2.358	1	Positive
d-Amphetamine	☐	136.1	Unit	91	Unit	66	17	1.278	1	Positive
Diazepam	☐	285.1	Unit	154	Unit	169	25	4.269	1	Positive
Heroin	☐	370.2	Unit	165	Unit	149	61	2.236	1	Positive
Hydrocodone	☐	300.2	Unit	199	Unit	159	29	1.38	1	Positive
Lorazepam	☐	321	Unit	275	Unit	102	21	3.61	1	Positive
MDA	☐	180.1	Unit	163	Unit	61	5	1.311	1	Positive
MDEA	☐	208.1	Unit	163	Unit	107	9	1.72	1	Positive

Dynamic MRM Parameters

Cycle Time 500 ms

Figure 5. Scan segments table with Dynamic MRM transitions imported database browser.

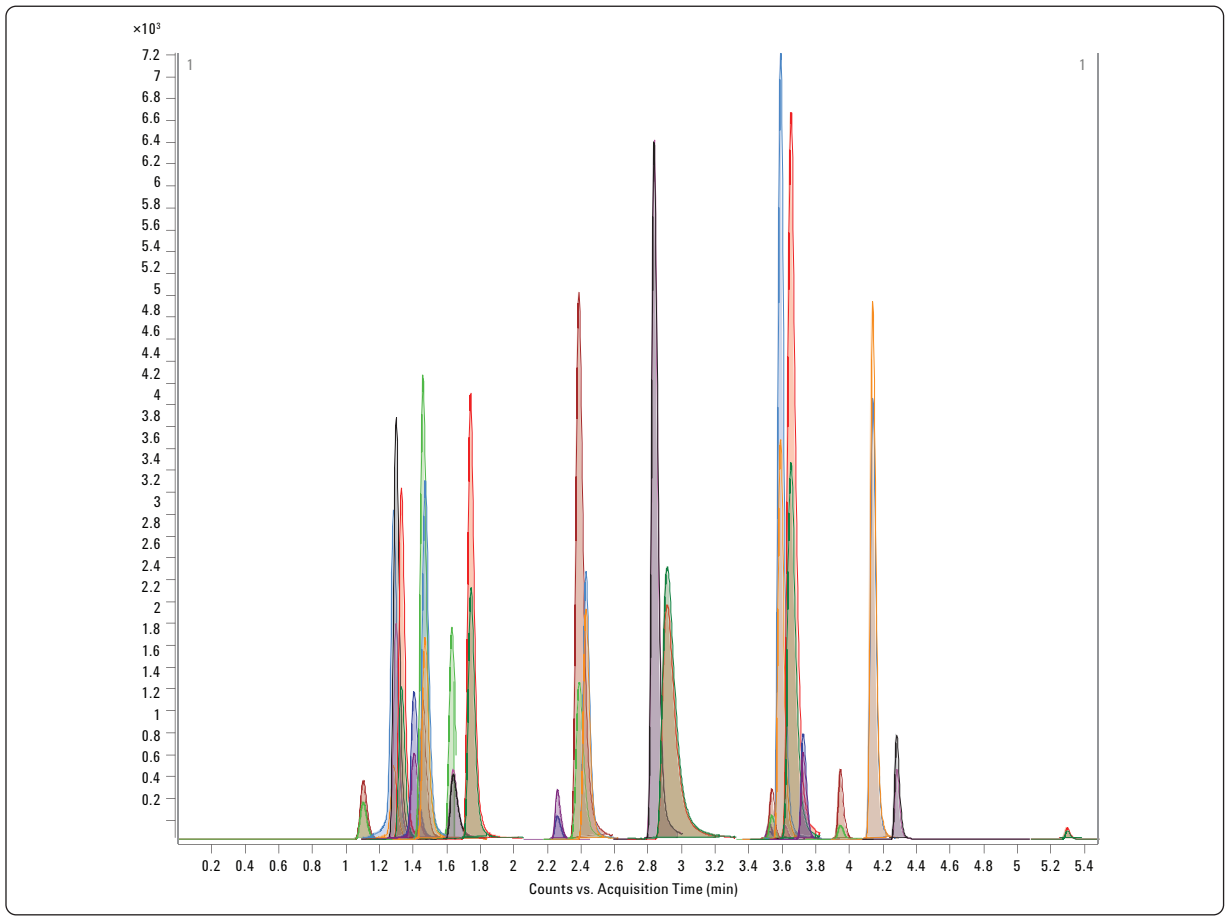

Figure 6. Example LC/MS Forensic Toxicology test mix 10 pg on-column extracted ion chromatogram (overlay).

Quantitative analysis and standard curves

By using a Dynamic MRM acquisition method, the series of LC/MS Forensic Toxicology Test Mix dilutions (Table 1) were analyzed according to the procedure outlined in the experimental section. All 50 Dynamic MRM transitions were used and Table 4 summarizes the results for the limits of detection and linearity of each component in the 25-component test mix.

Table 4. Limits of Detection and Calibration Linearity Results

Compound Name	Limit of Detection (fg on-column)	Linearity Correlation
3,4-Methylendioxyamphetamine (MDA)	50	0.99817
3,4-Methylenedioxyethamphetamine (MDEA)	10	0.99743
Alprazolam	50	0.99755
Clonazepam	100	0.99501
Cocaine	10	0.99755
Codeine	50	0.99841
δ9-Tetrahydrocannabinol (THC)	50	0.99869
Diazepam	10	0.99896
Heroin	25	0.99863
Hydrocodone	25	0.99493
Lorazepam	100	0.99601
Meperidine (Pethidine)	10	0.99687
Methadone	10	0.99666
Methamphetamine	10	0.98750
Methylendioxymethamphetamine (MDMA)	25	0.99217
Nitrazepam	25	0.99712
Oxazepam	250	0.99544
Oxycodone	50	0.99804
Phencyclidine (PCP)	25	0.99659
Phentermine	50	0.99898
Proadifen	<5	0.99772
Strychnine	50	0.99496
Temazepam	25	0.99751
Trazodone	<5	0.99777
Verapamil	<5	0.99787

Figures 7 through 10 illustrate the calibration curves through the range of 10-50000 fg on-column for six of the analytes from the LC/MS Forensic Toxicology Test Mix.

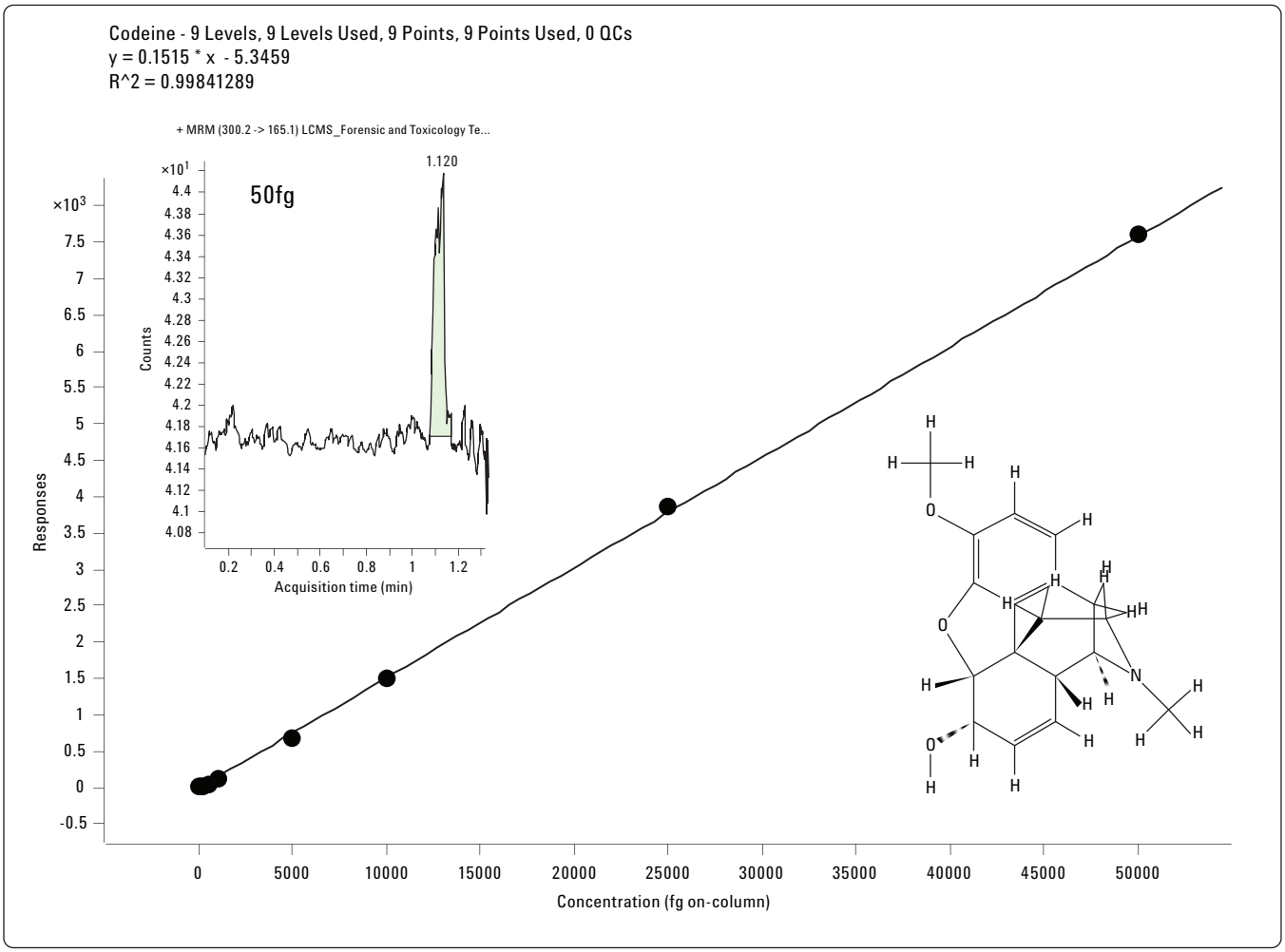

Figure 7. Calibration curve and LOD chromatogram, codeine.

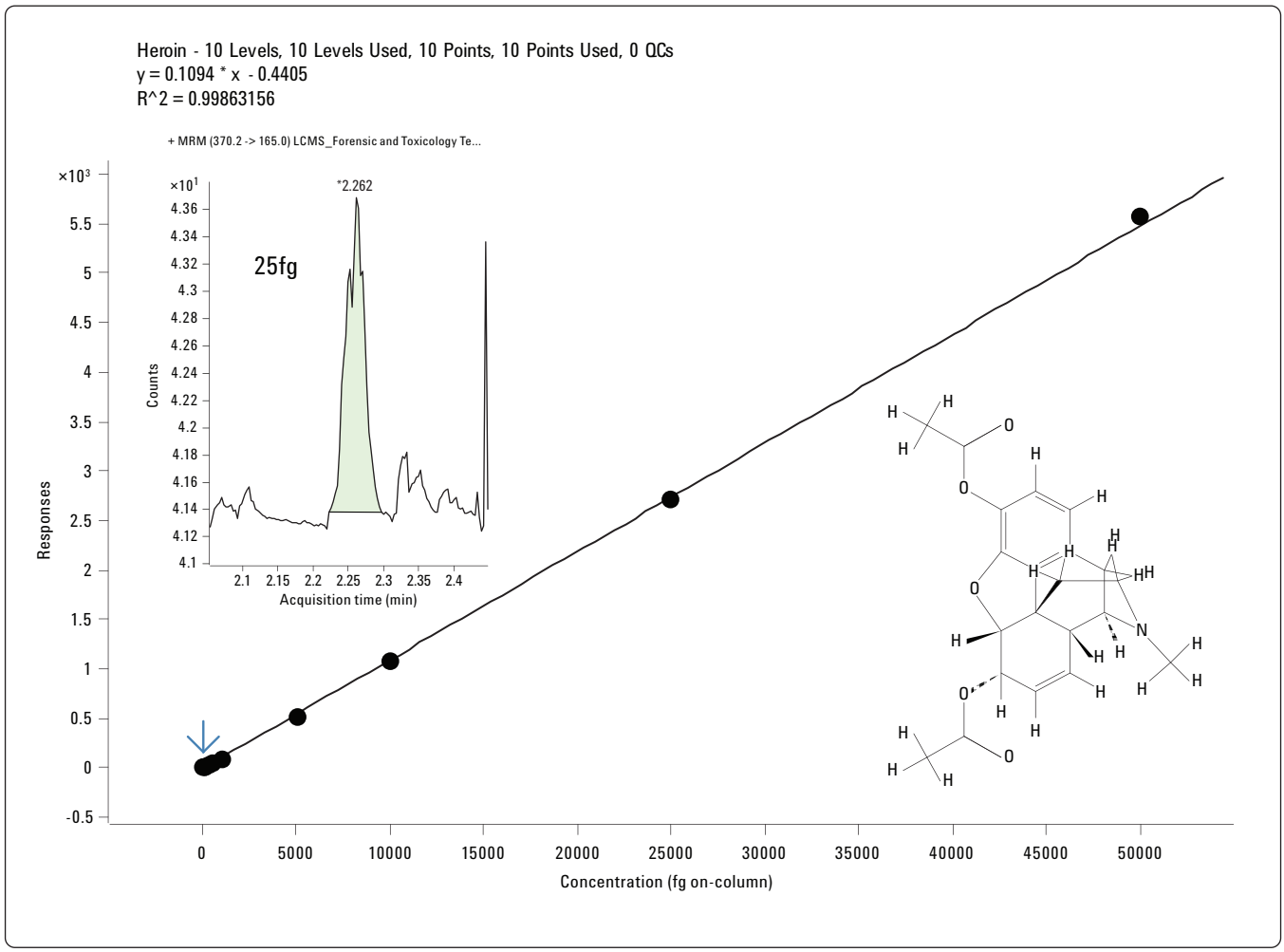

Figure 8. Calibration curve and LOD chromatogram, heroin.

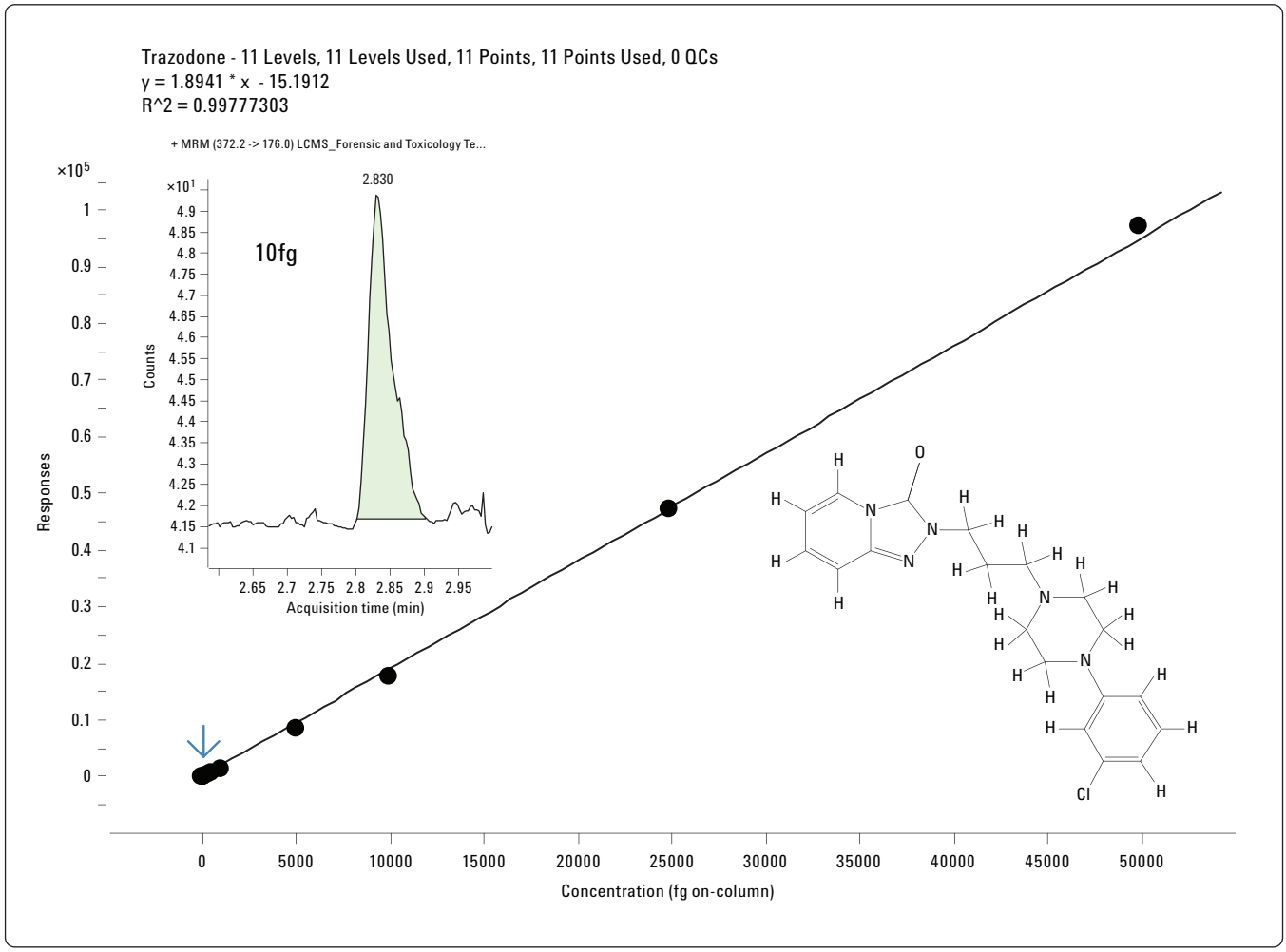

Figure 9. Calibration curve and LOD chromatogram, trazodone.

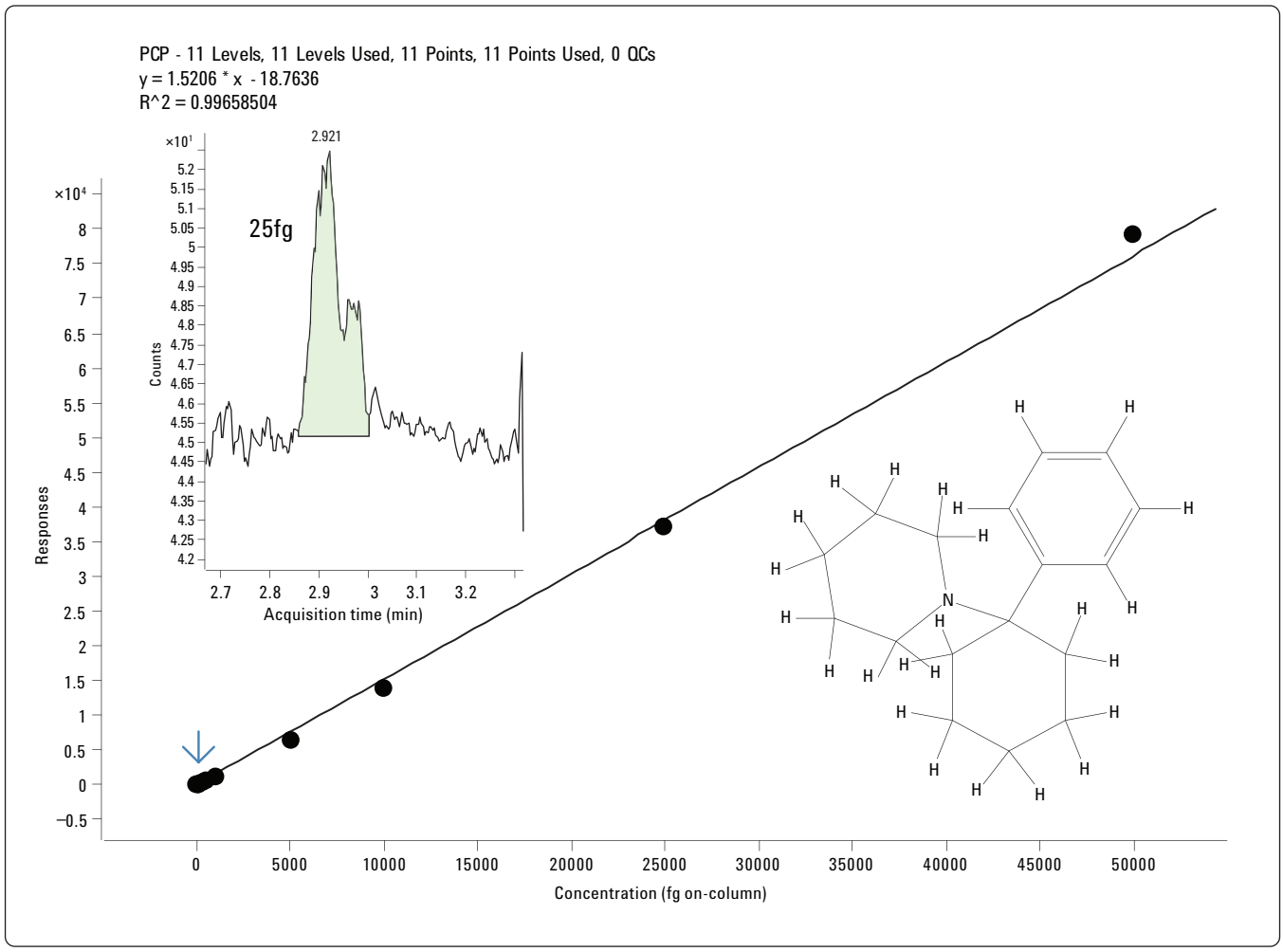

Figure 10. Calibration curve and LOD chromatogram, phencyclidine (PCP).

Conclusions

The Agilent MassHunter Forensic Toxicology Dynamic MRM Database Kit provides a user with faster method development capability for 200 forensic analytes with up to 4 MRM transitions for each. These methods can be used equally for screening or for more focused and dedicated analyte quantitation dependant on specific needs.

This application note briefly outlines the type of results that could be obtained by using database optimized MRM parameters with the appropriate chromatography conditions and MS ion source settings.

The kit offers:

- Fast and easy startup of complex analyses.
- An optimized MRM transition database of approximately 200 forensic compounds.
- Completely customizable with additional optimized transitions to the database.
- Example chromatography with ready to use methods inclusive of test sample and chromatography column.
- Automatic re-optimization of transition parameters using the MassHunter Optimizer program for particular instrument conditions and method revalidation.

References

1. "New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses," Agilent application note publication 5990-3595EN.
2. "Agilent G1734AA MassHunter Forensics and Toxicology Dynamic MRM Database Kit Quick Start Guide." Agilent Technologies publication 5990-4265EN

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For Forensic Use.

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Agilent Technologies

Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Forensic Toxicology

Authors

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Agilent Technologies, Inc.

Introduction

Benzodiazepines are a large class of drugs and include compounds such as diazepam (Valium), chlordiazepoxide (Librium), oxazepam (Serax), lorazepam (Ativan), alprazolam (Xanax), clonazepam (Clonopin) and others. 1,4-benzodiazepines, like diazepam, nordiazepam and temazepam, are metabolized and excreted as oxazepam and oxazepam glucuronide. The nitrobenzodiazepines, like clonazepam and flunitrazepam,aremetabolizedtoa7-aminometabolitein urine. Flurazepam is rapidly desalkylated.

Quantitative analysis of benzodiazepines in urine by LC/MS can be diffi cult due to the high level of matrix components. Organic salts as well as pigments and proteins cause ion suppression and the loss of signal intensity. Bond Elut Plexa PCX SPE products are a new addition to the Plexa family based on a polymeric cation exchanger. Plexa PCX products use a generic and simplifie d method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantifi cation of basic compounds.

In addition, Plexa PCX products offer faster and highly reproducible fl ow rates, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX products exhibit significantlyreducedionsuppressionbecausetheirhighlypolar, hydroxylated surfaces are entirely amide-free. Therefore, the particle exterior minimizes strong binding of proteins and phospholipids. An LC/MS/MS method is presented for the quantitative determination of benzodiazepines and their target metabolites in human urine specimens with Plexa PCX tubes. Hydrolysis may also be necessary by adding 5000 units of b-glucuronidase to a 1 M acetic acid (pH=3.8) buffered urine sample. The sample is vortexed and incubated for 2 hours at 60 °C prior to extraction.

Agilent Technologies

Materials and Methods

Table 1. SPE Reagents and Solutions

2% Formic Acid	Add 2 mL of concentrated formic acid to 100 mL of DI water
Methanol	Reagent grade or better
50% Methanol	Add 50 mL of methanol to 50 mL of DI water
5% Ammonia in Methanol	Add 5 mL of concentrated ammonia to 100 mL of methanol
Bond Elut Plexa PCX 30 mg 3 mL tube (part number 12108303)	

Table 2. SPE Method

Sample Pre-treatment	1 mL human urine. Dilute 1:2 with 2% formic acid.
Condition	1. 1 mL CH_30H 2. 1 mL H_2O
Wash 1	2 mL 2% formic acid
Wash 2	2 mL 50% CH_3OH in water
Elution	1 mL 5% NH_3 in methanol

All samples are evaporated to dryness and reconstituted in 200 µL of 50:50 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient:
t = 0-1 min 40% A : 60% B
t = 2.0-4.30 min 20% A : 80% B
t = 4.31-5.30 min 40% A : 60% B

Flow Rate: 0.2 mL/min

Column: Pursuit XRs$^{Ultra\ 2.8}$ C18, 100 x 2.0 mm (part number A7511100X020)

Table 3: MS Conditions

Transition ions and collision energy were:

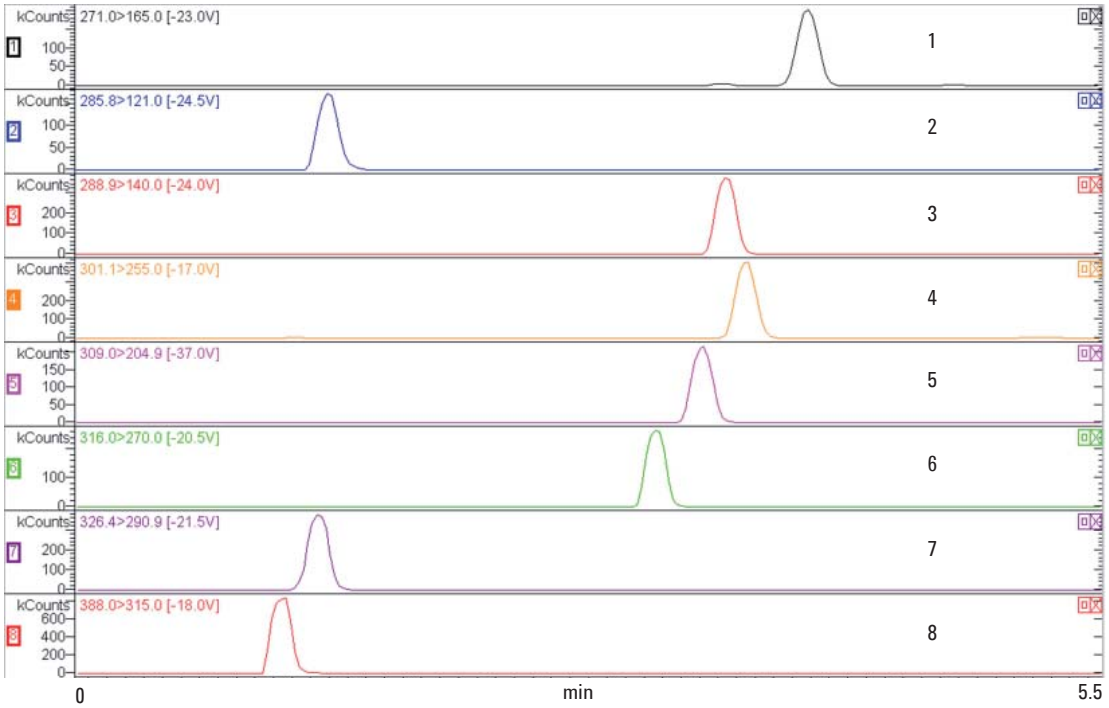
Compound	Q1	Q3	CE
Clonazepam	316.0	270.0	16.5 V
7-Aminoclonazepam	285.8	121.0	24.5 V
Flurazepam	388.0	315.0	18.0 V
Desalkylflurazepam	288.9	140.0	24.0 V
Midazolam	326.4	290.9	21.5 V
Alprazolam	309.0	204.9	37.0 V
Flunitrazepam	314.0	268.0	21.0 V
7-Aminoflunitrazepam	284.1	135.0	22.0 V
Chlordiazepoxide	300.3	227.0	19.5 V
Diazepam	285.0	222.0	20.5 V
Lorazepam	321.0	274.9	18.0 V
Oxazepam	286.8	241.0	16.5 V
Nordiazepam	271.0	165.0	23.0 V
Temazepam	301.0	255.0	17.0 V

Capillary: 70 V
Dry Gas Temperature: 350 °C, 30 psi
CID: Argon
Polarity: Negative

The procedure describes a method for extracting and determining fourteen different benzodiazepines in human urine. The Limit of Detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 1st order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 4, the extractions produced very reproducibly high recoveries.

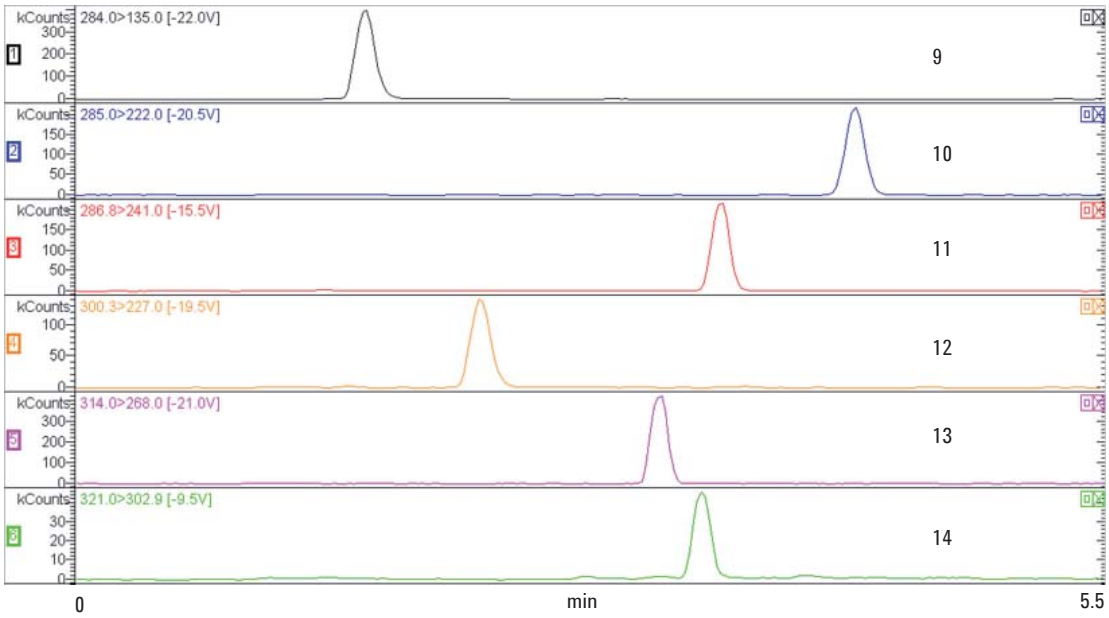
Table 4. Analyte relative recoveries

Analyte	% Rec (1 ng/mL)	% RSD	% Rec (100 ng/mL)	% RSD
Clonazepam	116	13	103	7
7-Aminoclonazepam	102	10	99	2
Flurazepam	117	14	106	8
Desalkylflurazepam	115	13	99	6
Midazolam	108	13	110	4
Nordiazepam	113	15	107	7
Alprazolam	113	17	110	8
Flunitrazepam	107	16	101	3
7-Aminoflunitrazepam	112	18	95	9
Chordiazepoxide	119	15	92	10
Diazepam	111	12	99	8
Temazepam	118	4	97	8
Lorazepam	102	14	94	10
Oxazepam	113	10	97	5



Peak Identification
1. Nordiazepam
2. 7-Aminoclonazepam
3. Desalkylflurazepam
4. Temazepam
5. Alprazolam
6. Clonazepam
7. Midazolam
8. Flurazepam

Figure 1a. Chromatograms of a 100 ng/mL urine extract (peaks 1-8)



Peak Identification
9. 7-Aminoflunitrazepam
10. Diazepam
11. Oxazepam
12. Chlordiazepoxide
13. Flunitrazepam
14. Lorazepam

Figure 1b. Chromatograms of a 100 ng/mL urine extract (peaks 9-14)

Conclusions

Bond Elut Plexa PCX products are a useful tool for high throughput SPE applications, which require analysis at low analyte levels, need validated reproducibility, and that must be quickly implemented with minimal method development. Bond Elut Plexa products meet these requirements and are therefore highly recommended for forensic toxicology work.

With Bond Elut Plexa PCX, a generic drug extraction protocol can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent. Polar interferences and proteins are washed away with an acidic, aqueous solution. A wash with 50% aqueous methanol is possible without significant loss of analytes. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, ammoniated methanol is used to disrupt the cation exchange interaction, resulting in the elution of the benzodiazepines.

Flow rate is fast because Bond Elut Plexa PCX particles have much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent tube-to-tube reproducibility. Bond Elut Plexa tubes are therefore a useful tool for high throughput SPE applications, which require analysis at low analyte levels, validated reproducibility and quick implementation, with minimal method development.

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Want to evaluate your methods and be running billable urine confirmations using LC/MS/MS in under 60 days?

Recent changes to the Mandatory Guidelines for Federal Workplace Drug Testing Programs now allow the use of LC/MS/MS for urine drug confirmations. In addition, several new target drugs have been added to the panel (MDA, MDMA, MDEA), several cut-off concentrations have been adjusted, minimum requirements for interference testing have been specified for amphetamines & opiates, and there is a new requirement specifying a minimum of 10 data points across a peak.

Agilent Technologies has partnered with a NLCP-certified laboratory to develop and evaluate a set of sample preparation and LC/MS/MS methods to meet the updated guidelines. The methods were developed to provide reliable sensitivity & specificity comparable to or better than the corresponding GC/MS methods. They also feature fast quantitative data analysis, reporting with GC/MS-like ion ratios and well documented procedures that can be easily learned by GC/MS trained personnel.

The resulting methods, which have been previously validated, use the same column and only two mobile phase combinations so that all 5 drug classes can be analyzed on a single instrument without hardware or mobile phase changes.

Compounds

- Amphetamine
- Methamphetamine
- MDA
- MDMA
- MDEA
- Cocaine Metabolite (BE)
- Marijuana Metabolite (cTHC)
- Phencyclidine (PCP)
- Morphine
- Codeine
- 6-Acetylmorphine

Performance Examples

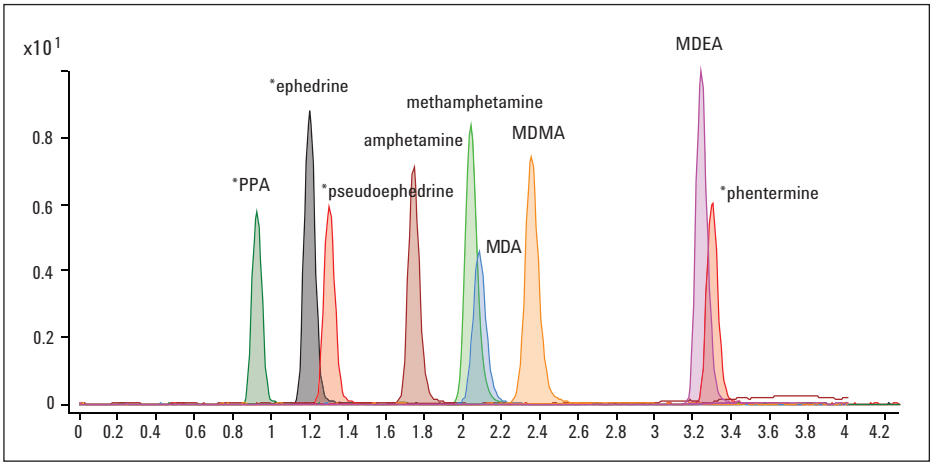

Figure 1: Separation of amphetamines from potential interferences*

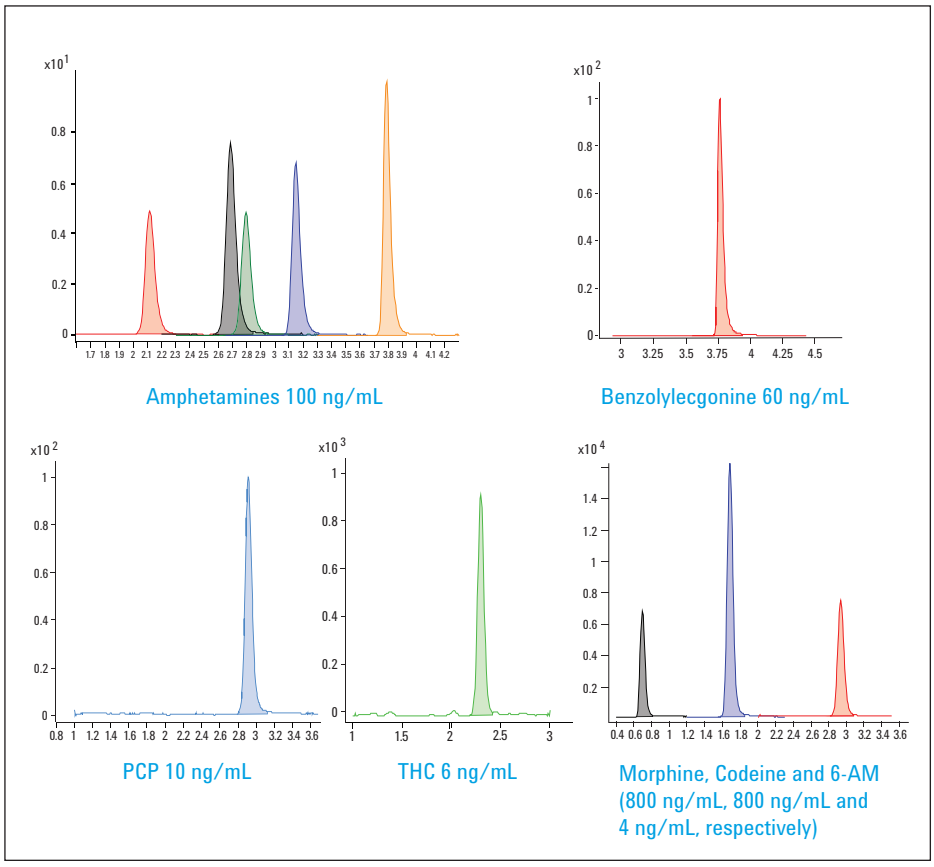

Figure 2: NIDA-5 drugs at 40% of their respective cut-off concentrations

Key Benefits

- LC/MS/MS methods that meet or exceed the new 2010 SAMHSA guidelines
- Standard operating procedures (SOP) including instrument parameters and sample preparation for all 5 drug classes
- Methods that have been evaluated in an NLCP-certified workplace drug testing laboratory
- All 5 drug classes can be run on a single instrument without mobile phase changes
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Agilent Technologies

Analysis of Anabolic Agents in Urine by LC/MS/MS

Application Note

Forensic Toxicology/Doping Control

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Abstract

The use of the Agilent 1200 Series liquid chromatography (LC) system coupled to the 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of the G1948B electrospray ionization (ESI) source is demonstrated in the analysis of anabolic substances in urine. The high degree of sensitivity of the QQQ instrument allows for excellent quantitation and linearity for meeting Minimum Required Performance Levels (MRPLs) for each compound as specified by the World Anti-Doping Agency (WADA). For increased chromatographic resolution and speed, a 2.1 mm id C18 column with a 3.5-µm particle size is employed. The seven compounds, including a designated internal standard, all elute within 10 minutes at a flow rate of 0.4 mL/min.

Introduction

Traditionally, doping control analysis for anabolic substances, including steroids, in urine includes screening by derivatization and GC/MS [1], followed by confirmation of the presumptive positive using high-resolution magnetic sector GC/MS in EI mode [2]. The high purchase and operational costs of high-resolution magnetic sector instruments make alternative techniques like LC/MS attractive for confirming the presence of the banned compounds.

More than 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily analyzed using GC/MS but are amenable to LC/MS. The analysis of some of these compounds is very challenging, as they must be detected and confirmed at MRPLs of 2 ng/mL or lower in urine.

This work describes the results of using the Agilent LC/QQQ instrument for detection and confirmation of a number of anabolic substances at the

WADA MRPL or, more specifically, covering the 1/2 × – 10 × MRPL range. The anabolic compounds analyzed in this work are listed in Table 1 along with their MRPLs.

Some previous work [3] used the TOF to analyze these compounds and found that accurate mass could be used for both screening and confirmation. However, the QQQ is more specific with MS/MS, increasing the confidence in confirmation and quantitating compounds of interest.

Table 1. Minimum Required Performance Levels (ng/mL of urine)

Compound	MRPL
Clenbuterol	2
19-norandrosterone	1
4β-OH-stanozolol	10
Tetrahydrogestrinone (THG)	10
Methyl testosterone metabolite (MeTest metabolite) or 17α-methyl-5β-androstane-3α,17β-diol	2
Epimetendiol	2
Methyl testosterone – Internal Standard	NA

In this study all compounds are steroids except for clenbuterol.

The structures of the compounds analyzed in this work are shown in Figure 1. Based on the results of work presented elsewhere [4], a derivatizing agent is used on these samples, but only reacts with 19-norandrosterone to improve sensitivity. The derivatizing agent, known as Girard's Reagent P (Sigma Aldrich, St. Louis, MO), reacts with ketone groups to form a quaternary amine, which is more easily ionized by ESI.

Experimental

Sample Preparation

The anabolic agents and their metabolites are purchased from Sigma Aldrich (St. Louis, MO), Steraloids (Newport, RI), and the National Measurement Institute (Sydney, Australia). Girard's Reagant P (GRP) is purchased from Sigma Aldrich and β-glucuronidase is purchased from Roche (Indianapolis, IN).

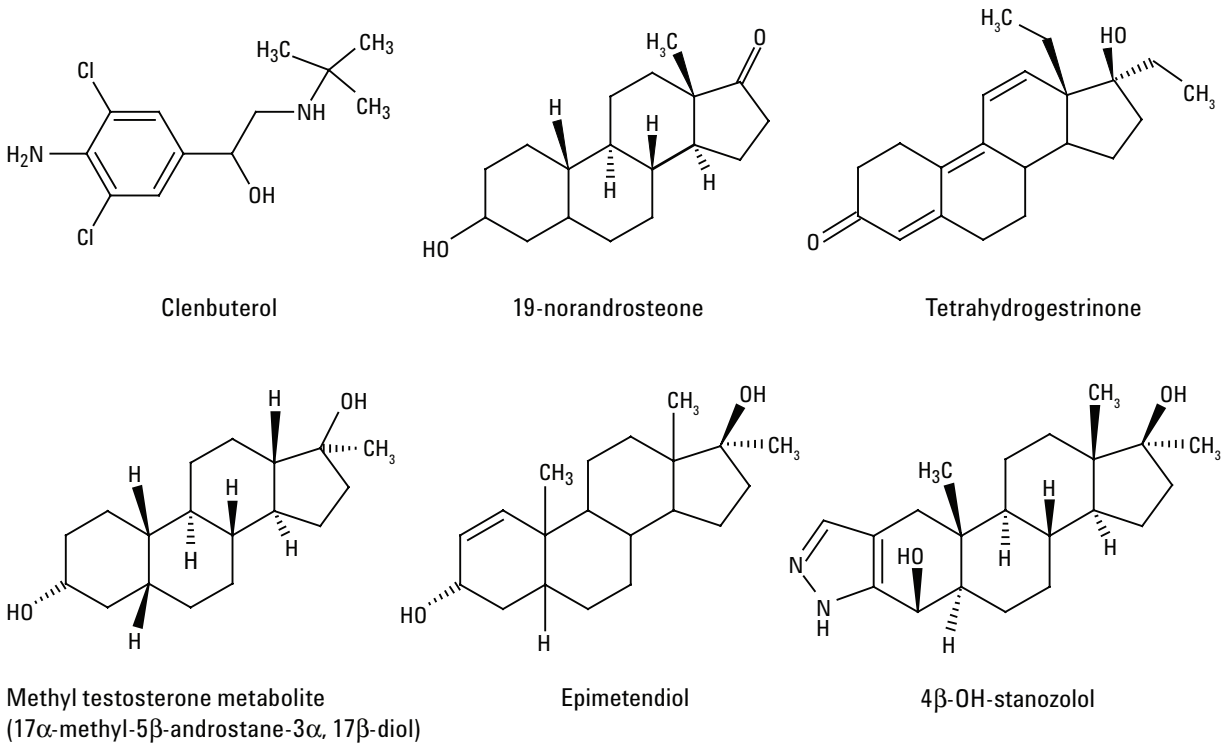

Figure 1. Structures of anabolic substances analyzed in this work.

To 3 mL urine negative control sample, 1 mL 0.8 M potassium phosphate buffer, pH 7.0, is added. A further 25 µL β-glucuronidase is added and then the mixture is incubated at 50 °C for one hour. A 750-µL mixture of 20% (w/v) $K_2CO_3/KHCO_3$ (1:1) mixture is then added. Extract with methyl-t-butyl ether and then remove and dry the organic extract.

The same extraction procedure used for GC/MS screening is employed except that the compounds are not derivatized as usual for GC/MS analysis. Rather, the samples are dried and then reconstituted in 100 µL of LC mobile phase.

As part of the reconstitution step for LC/MS/MS analysis, 20 µL methanol, followed by 8 µL of 1M GRP in 50 mM ammonium acetate buffer, pH 4.2, is added. Incubation at room temperature for one hour is then followed by LC/MS/MS analysis.

Of the compounds analyzed, only the 19-norandrosterone is reactive with the GRP derivative. This compound has been problematic in LC/MS/MS analysis and the GRP improves sensitivity.

The MeTest internal standard has a fixed concentration of 10 ng/mL.

LC/MS Method Details

LC Conditions

Agilent 1200 Series binary pump SL, wellplate sampler, thermostatted column compartment, inline filter 0.5 µm between needle seat and injector valve.

Column:	Agilent ZORBAX XDB-CN 2.1 × 100 mm, 3.5 µm (p/n 961764-905)
Column temp:	50 °C
Mobile phase:	A = 0.1% formic acid in water B = 0.1% formic acid in methanol
Flow rate:	0.4 mL/min; injection vol: 2 µL

Gradient:

Time (min)	%B
0–1	5
3	15
3.01	40
12	50
15	95

Stop time = 15 min; Post-run time = 3 min.

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	40 psig
Drying gas flow:	9 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 resolution:	0.7 amu
Q2 resolution:	0.7 amu

MRM transitions shown in Table 2. Chromatographic retention times (RTs), fragmentor (Frag), collision energy (CE), and dwell times are included. Time segments in which the MRM transitions are implemented are also noted.

Results and Discussion

The chromatographic elution profile of all compounds at their equivalent 10 × MRPL is shown in Figure 2. The responses vary quite significantly among the compounds and the background interference from the matrix is evident.

Concentration levels ranging from 1/2 × to 10 × MRPL are run in triplicate injections. The results for clenbuterol are shown in Figures 3a to 3c. Linearity over this range has a correlation coefficient of $R^2 > 0.999$ using the most conservative

Table 2. Data Acquisition Parameters for MRM Transitions

Compound	RT (min)	MRM	Frag (V)	CE (V)	Dwell (msec)
Segment 1 (0–4.0 min)					
Clenbuterol	2.74	277.0 > 203.1	100	15	200
Segment 2 (4.0–6.3 min)					
19-norandrosterone	5.82	410.3 > 331.3	130	30	75
Segment 3 (6.3– 6.93 min)					
4β-OH-stanozolol	6.64	345.2 > 327.2	140	15	200
Segment 4 (6.93–7.55 min)					
MeTest (IStd)	7.19	303.2 > 97.1	140	25	75
Segment 5 (7.55–8.8 min)					
THG	7.88	313.2 > 295.1	150	15	100
MeTest metabolite	8.08	271.2 > 161.2	110	20	100
Segment 6 (8.8–12.0 min)					
Epimetendiol	9.47	269.2 > 105.1	90	20	200

curve fit settings of linear, ignored origin, and no weighting. A closer look at the reproducibility of the lowest three level replicates is included in Figure 3a. The limit of detection (LOD), which is defined here as being a peak-to-peak signal-to-noise (S/N) ratio of 3:1, the S/N of the lowest level (1/2 × MRPL) is measured first. Then the same factor that is applied to this S/N, in order to obtain a S/N of 3:1, is also applied to the lowest level.

For example, in Figure 3b the S/N is nearly 60:1 for all three injections at the 1/2 × MRPL. A factor of 20 is applied to achieve 3:1 so that the LOD is 1/20th the concentration of this level, or 1/40 × MRPL.

To determine the on-column injection amount it should be noted that the original sample corresponds to 3 mL of urine. Since the MRPL of clenbuterol is 2 ng/mL, according to Table 1, then the 1/2 × MRPL contains 3 ng clenbuterol in the 3 mL urine sample. Following extraction and evaporating to dryness, this 3 ng of clenbuterol is reconstituted in 100 µL of LC mobile phase. Of this volume, 2 µL is injected. Therefore, the on-column injection amount of clenbuterol at the 1/2 × MRPL corresponds to 2/100 × 3 ng = 60 pg. The LOD is therefore 1/20 × 60 pg, or about 3 pg on-column.

The LOD for clenbuterol is given in Figure 3b. Note that the negative quality control (NQC) is also shown as evidence that the calculated S/N is justifiable.

Figure 3c shows the replicate injections at the lowest three levels.

The results for THG, MeTest metabolite, epimetendiol, and 4β-OH-stanozolol are shown in Figures 4, 5, 6, and 7, respectively.

As can be seen from Figure 5a, the 1/2 x MRPL does not appear to be a limit of detection because an S/N of 3:1 does not seem possible. However, in comparison to the matrix blank (NegQC) this level is certainly detectable. For this reason, including the fact that the 1/2 × MRPL replicate injections are at the lowest end of the range investigated and linear with the curve fit, the 1/2 × MRPL of the MeTest metabolite is considered the LOD.

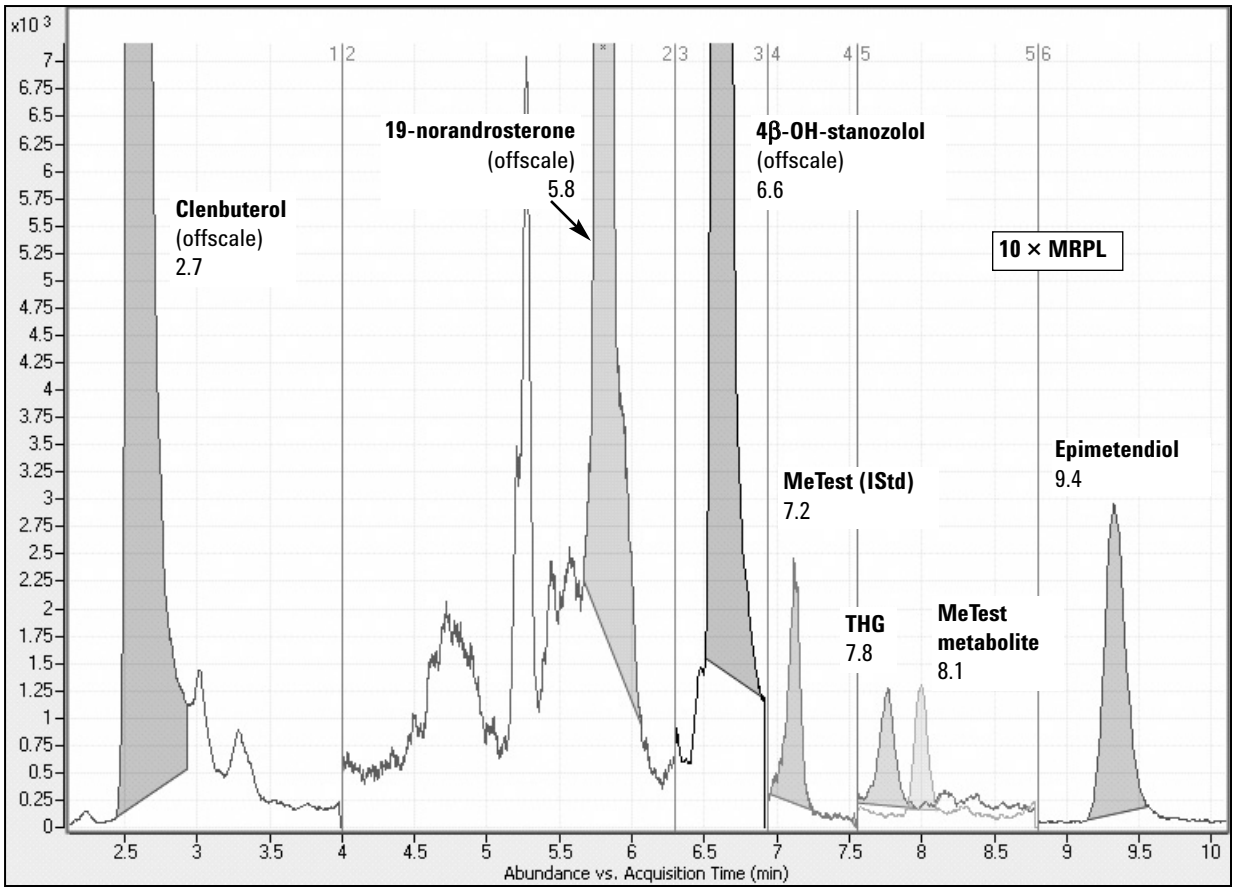

Figure 2. Chromatographic profile of 10 × MRPL extract in urine.

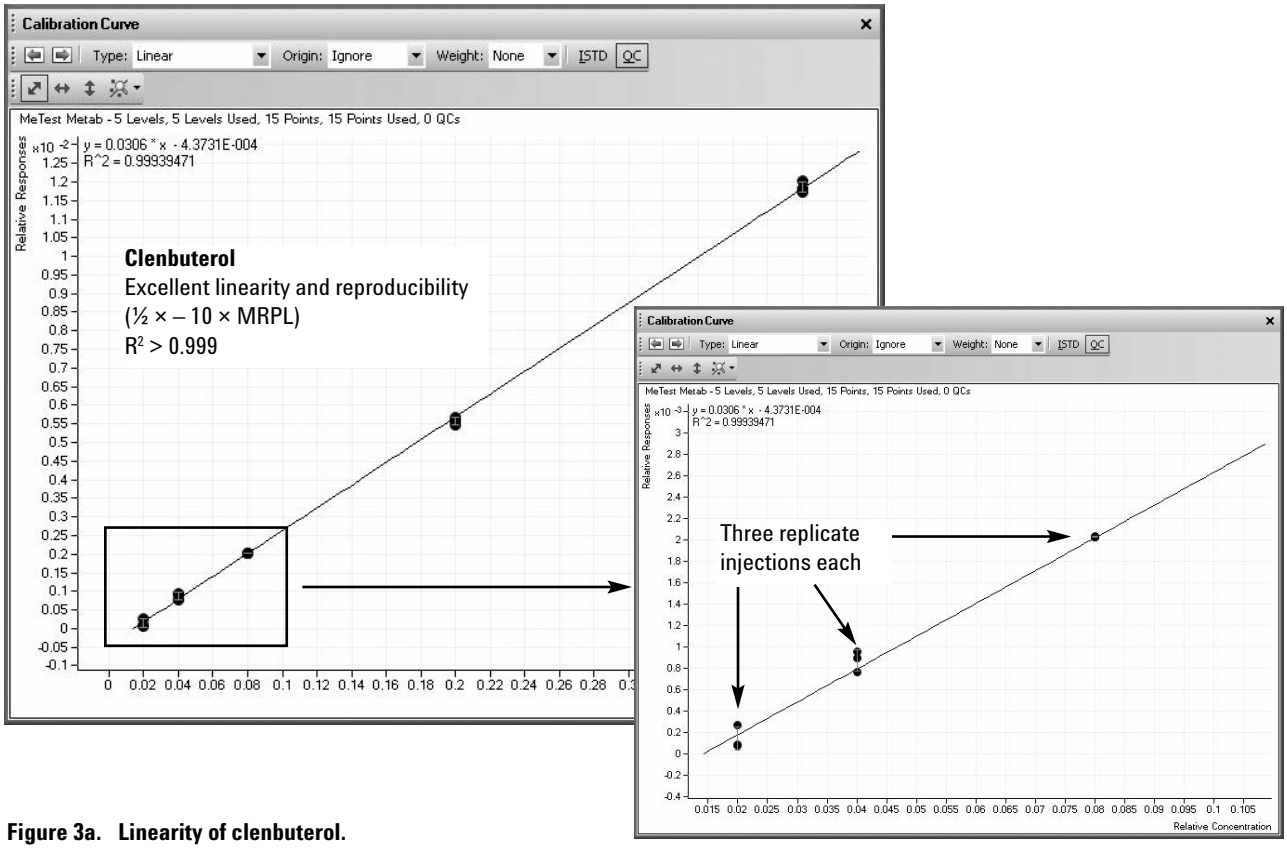

Figure 3a. Linearity of clenbuterol.

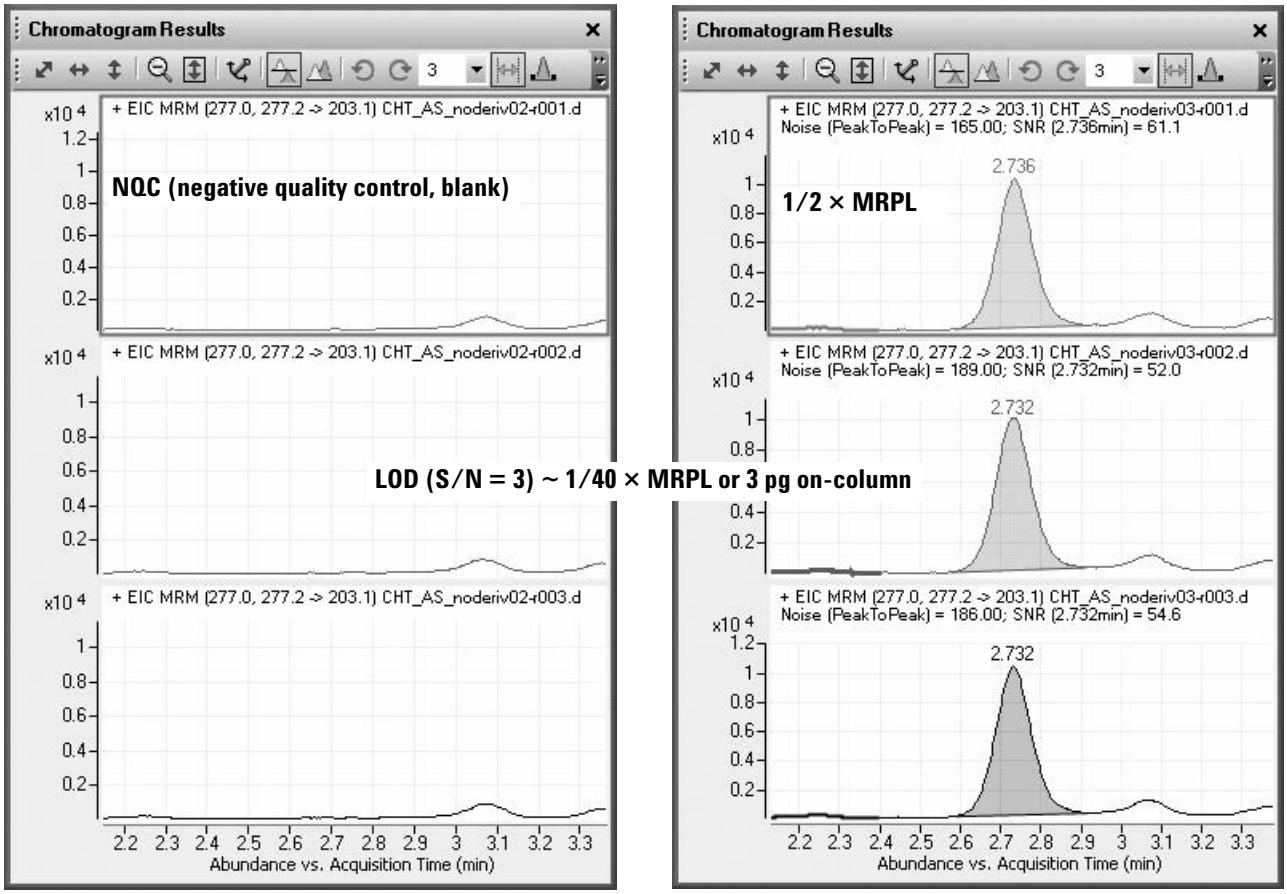

Figure 3b. Estimate of LOD for clenbuterol.

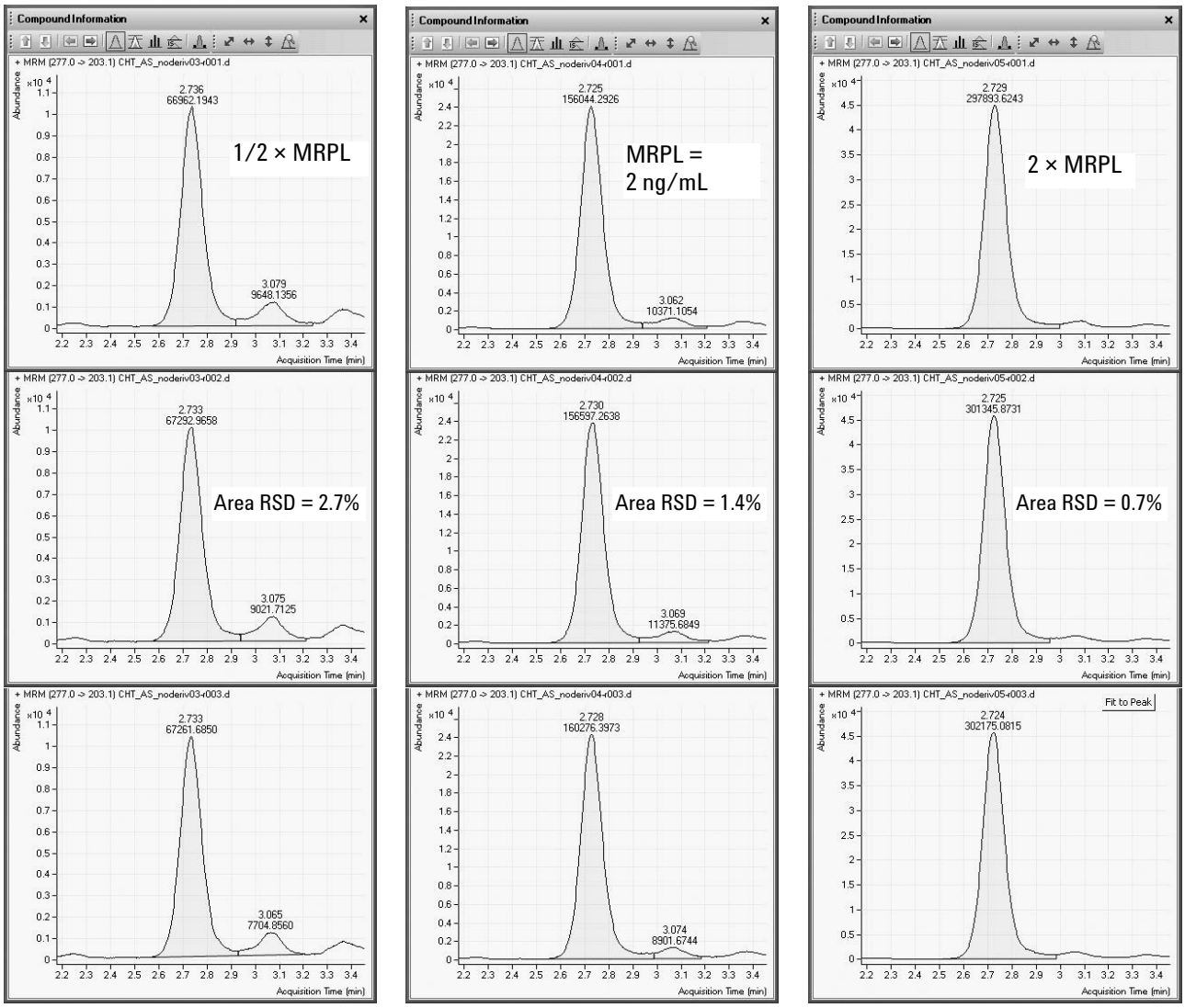

Figure 3c. Triplicate injections of the lowest three levels of clenbuterol.

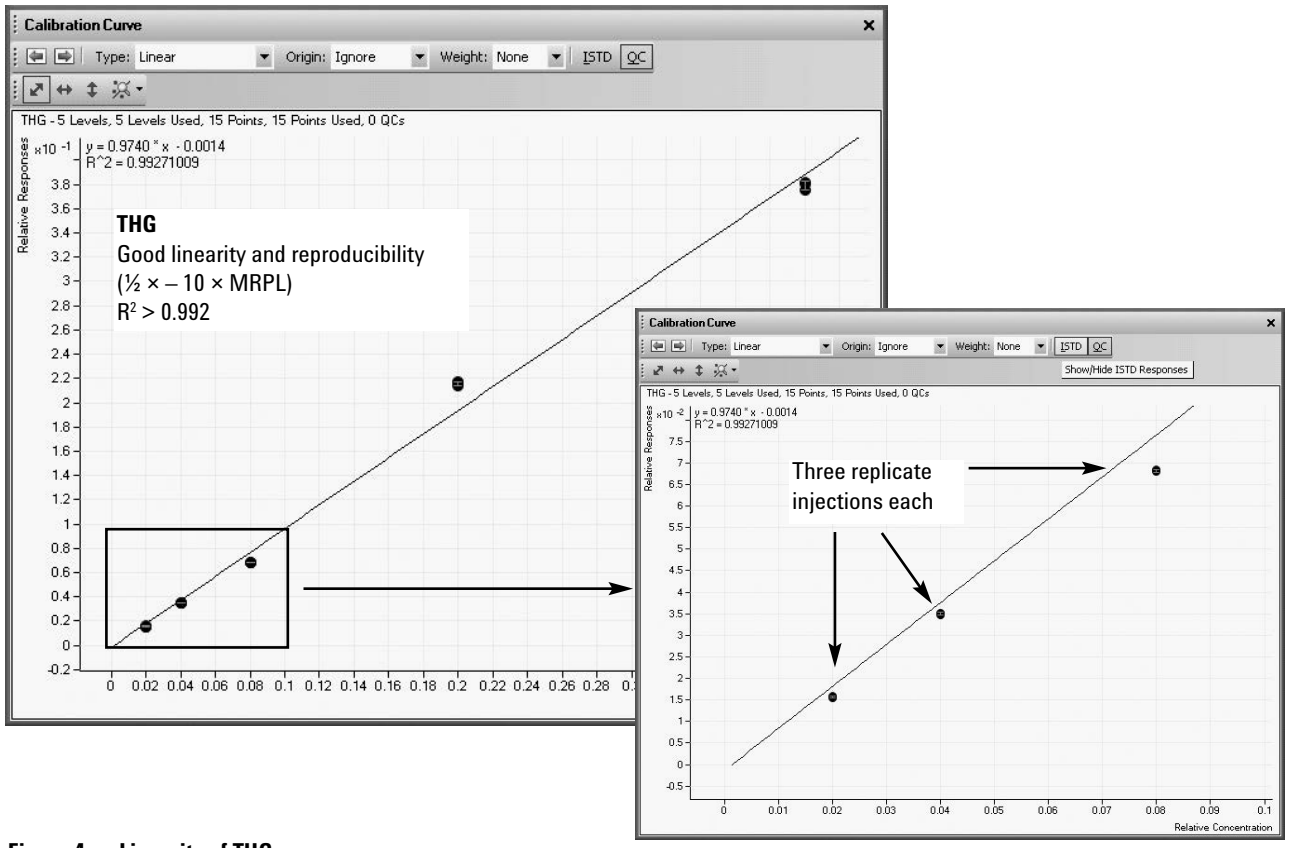

Figure 4a. Linearity of THG.

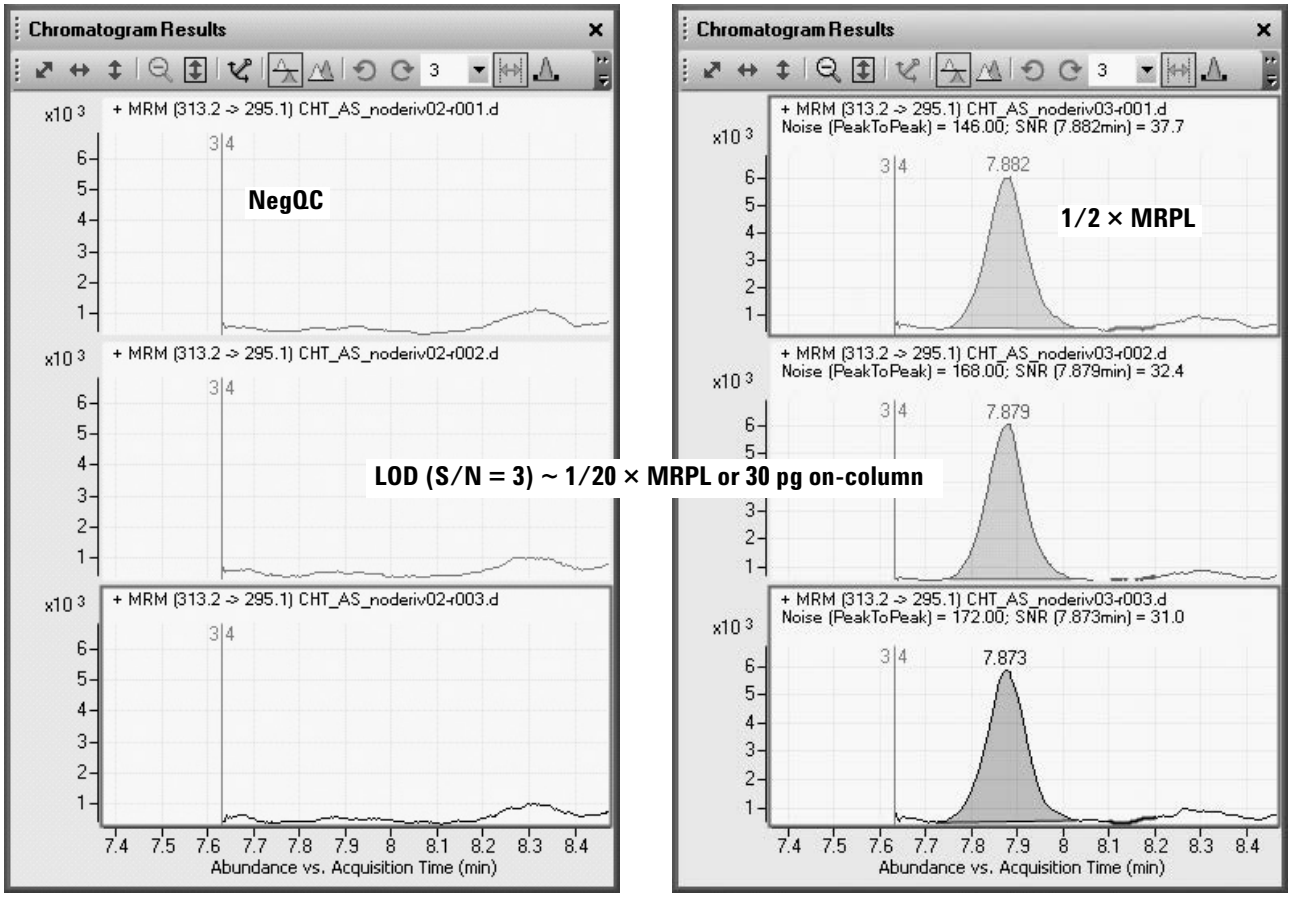

Figure 4b. Estimate of LOD for THG.

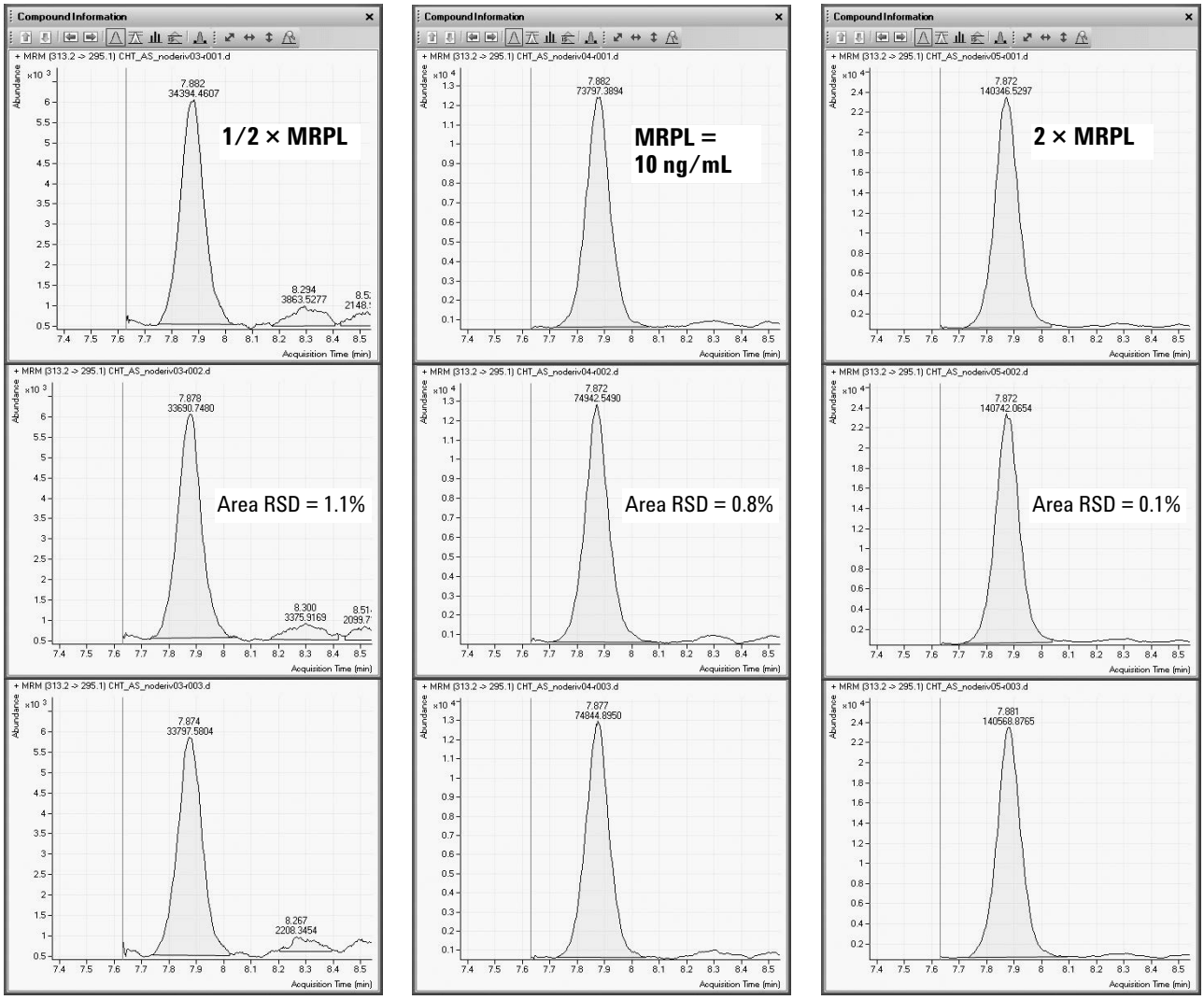

Figure 4c. Triplicate injections of the lowest three levels of THG.

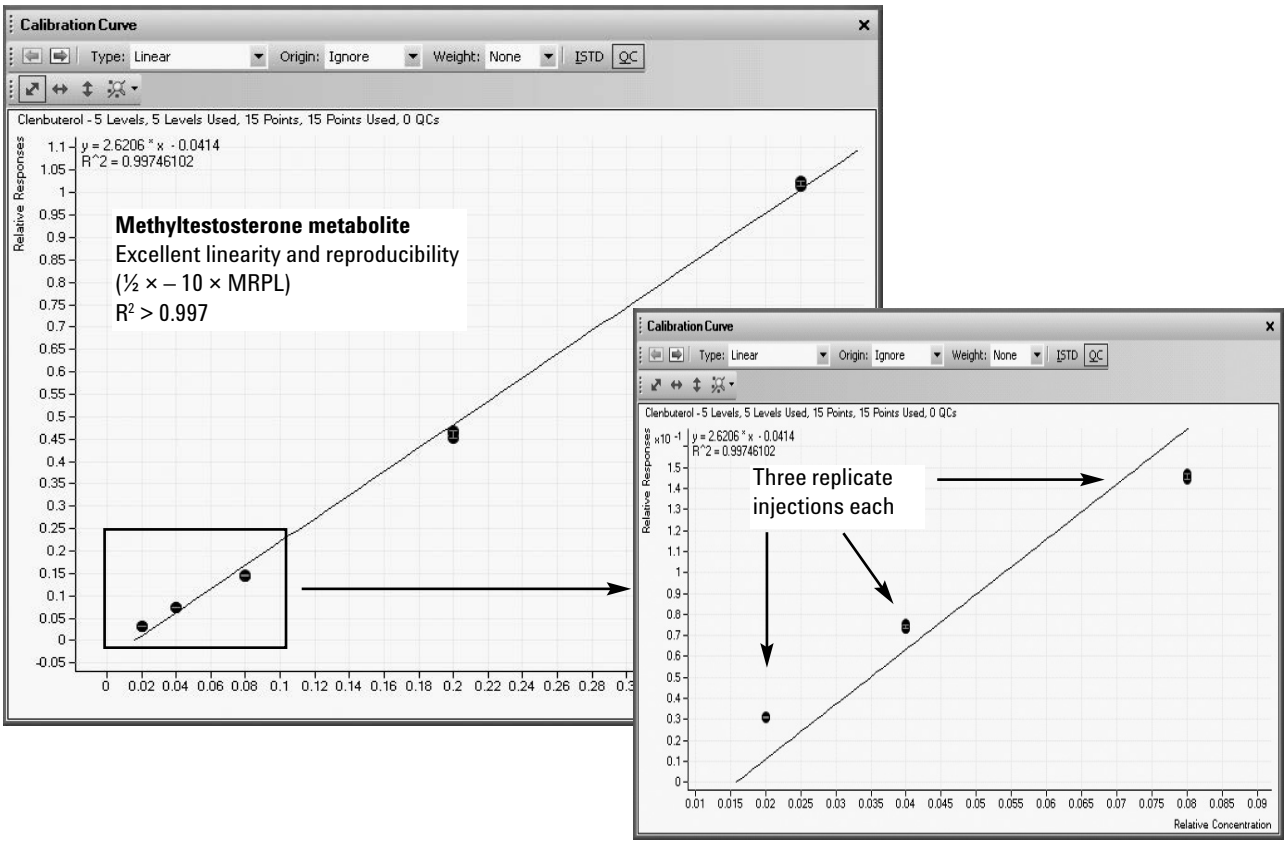

Figure 5a. Linearity of methyltestosterone metabolite.

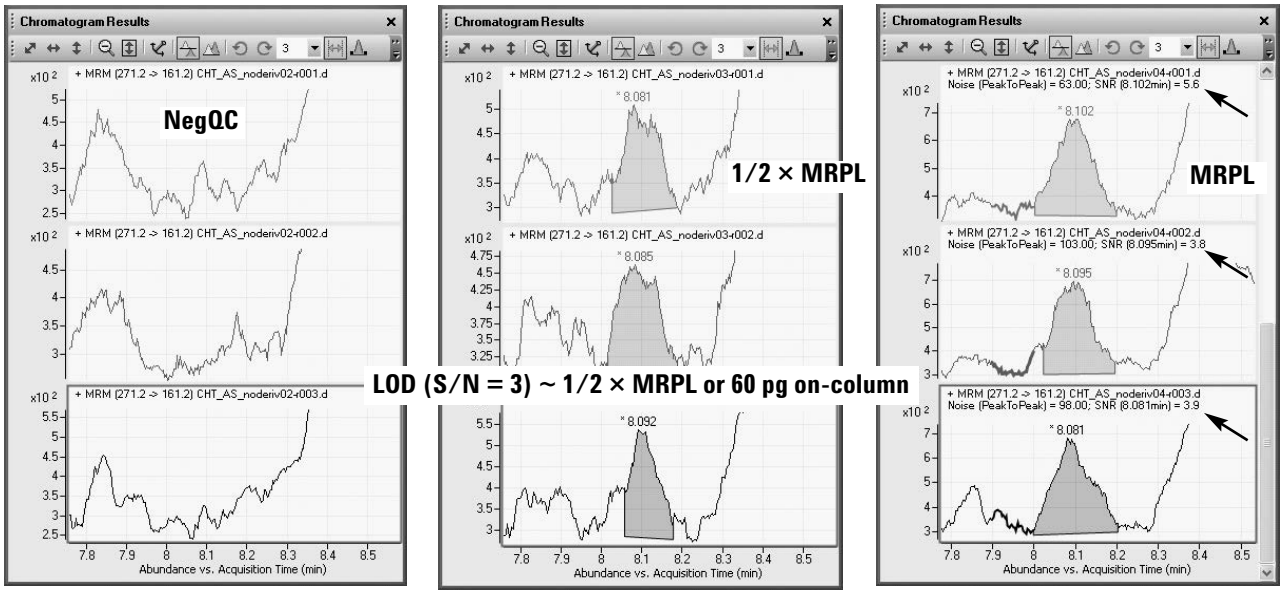

Figure 5b. Estimate of LOD for methyltestosterone metabolite.

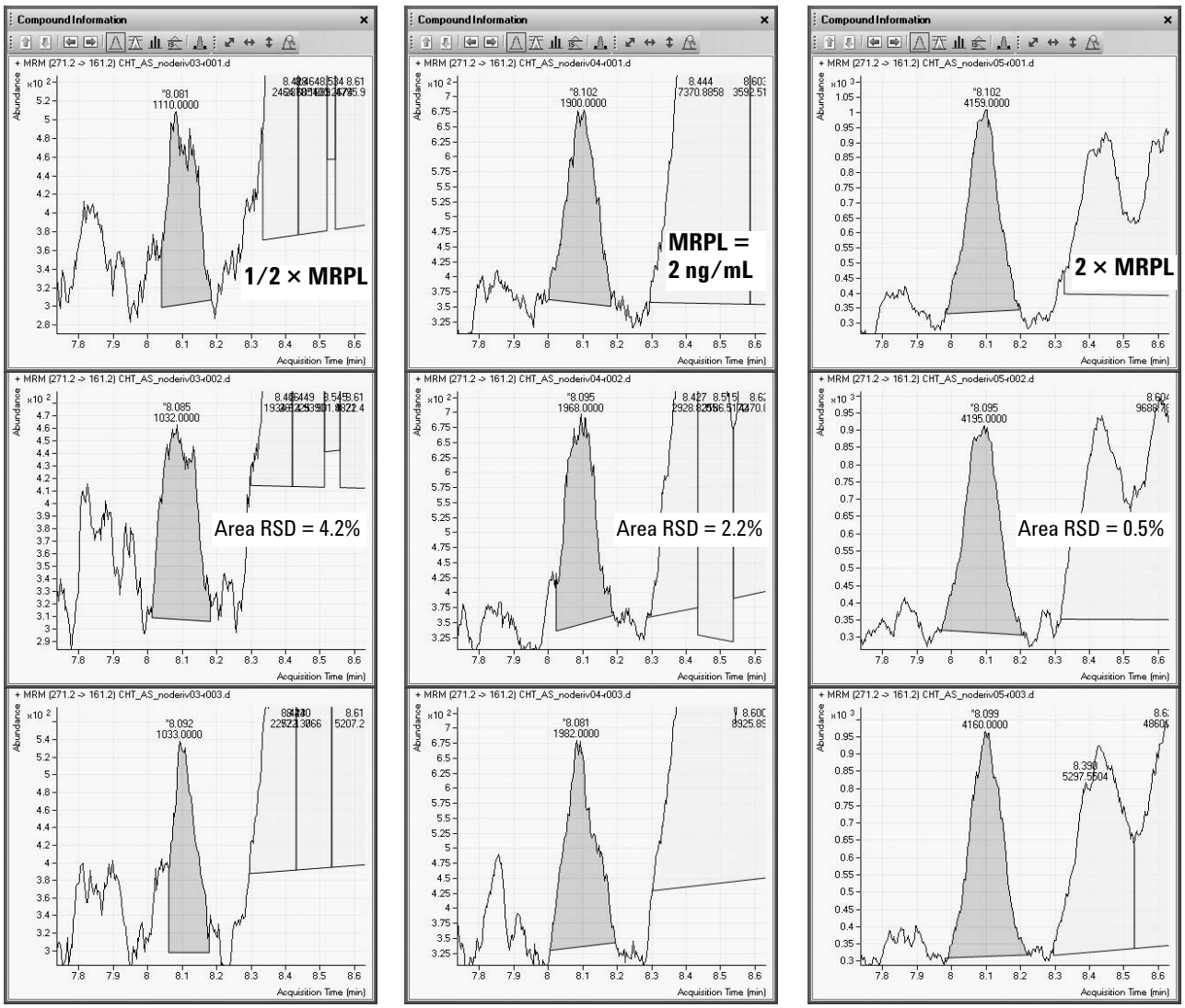

Figure 5c. Triplicate injections of the lowest three levels of methyltestosterone metabolite.

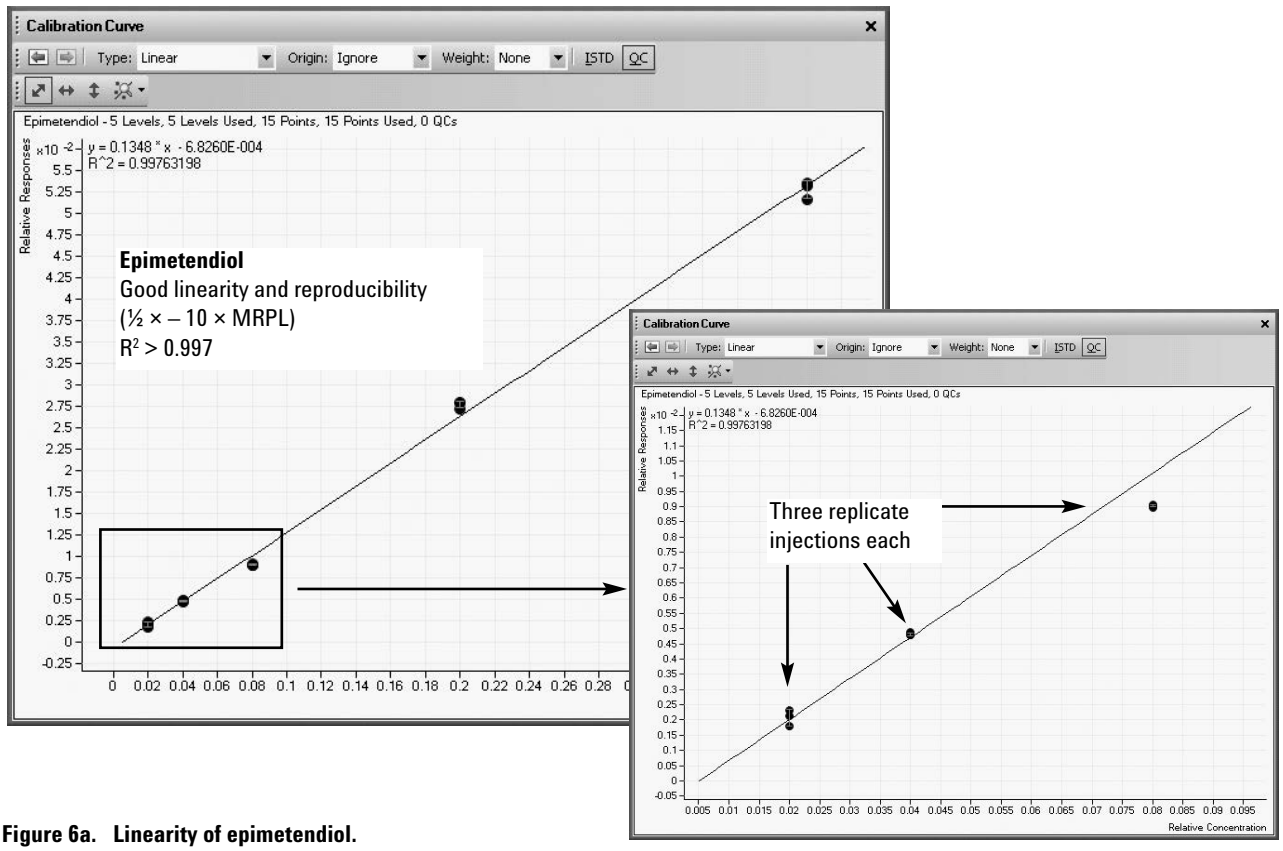

Figure 6a. Linearity of epimetendiol.

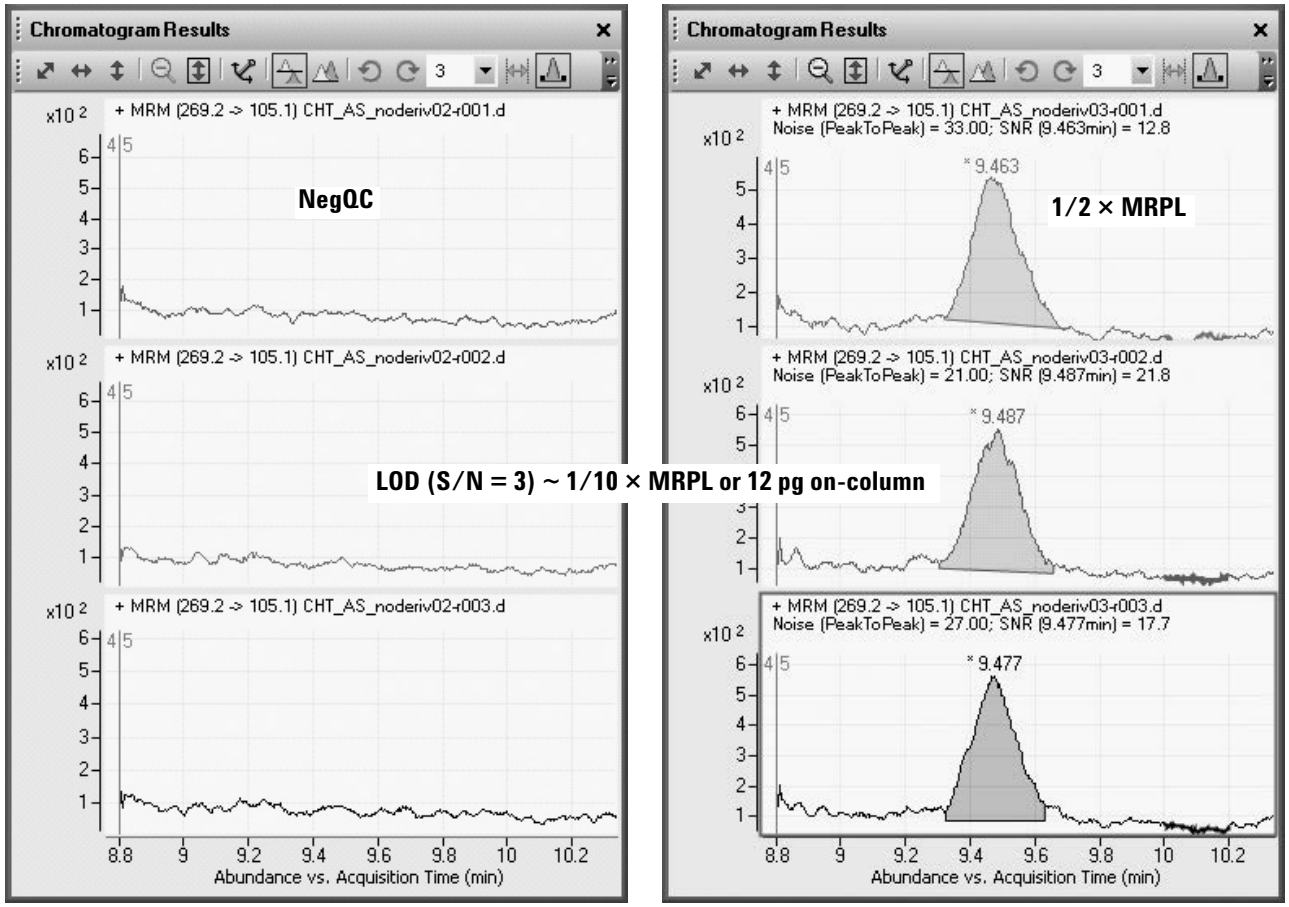

Figure 6b. Estimate of LOD for epimetendiol.

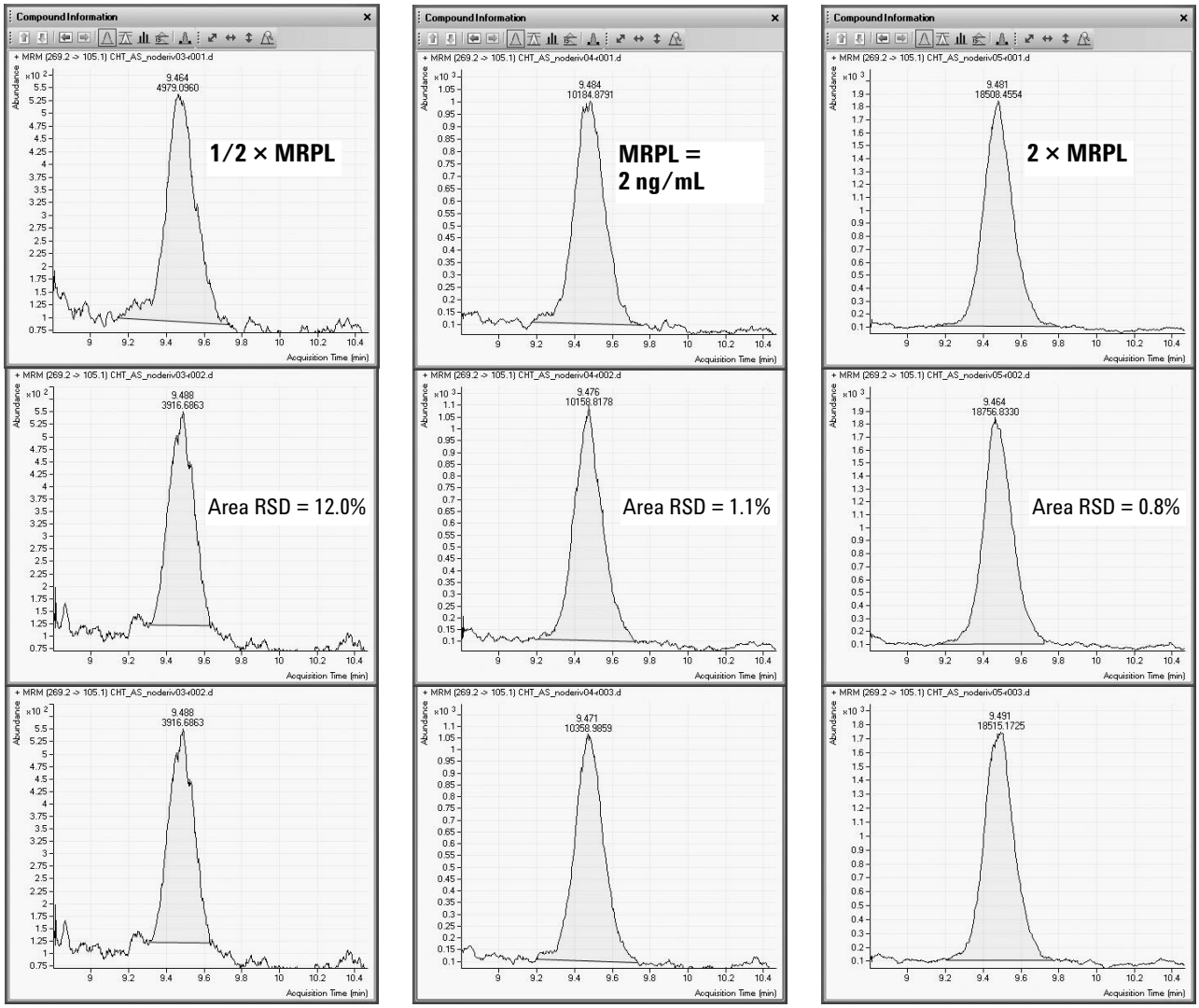

Figure 6c. Triplicate injections of the lowest three levels of epimetendiol.

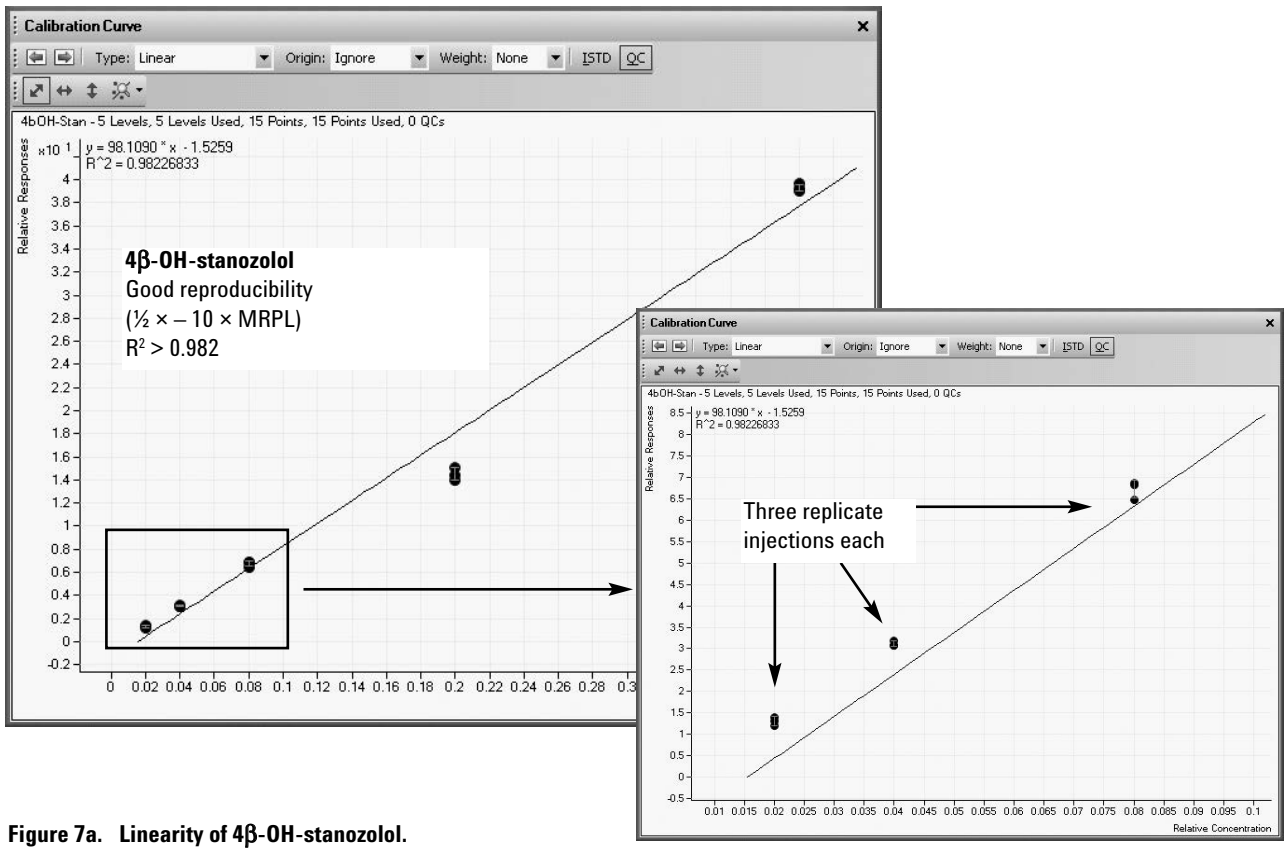

Figure 7a. Linearity of 4β-OH-stanozolol.

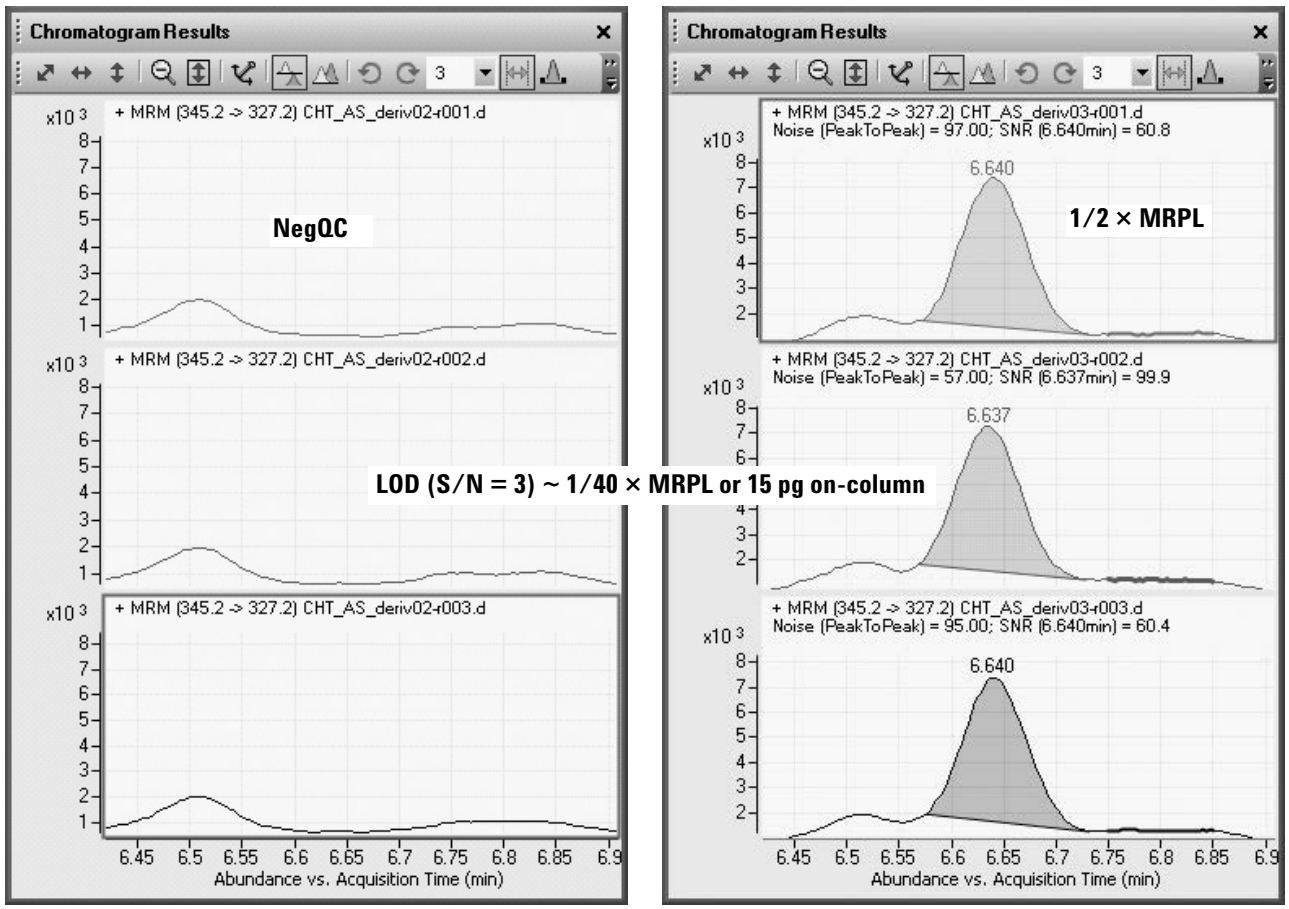

Figure 7b. Estimate of LOD for 4β-OH-stanozolol.

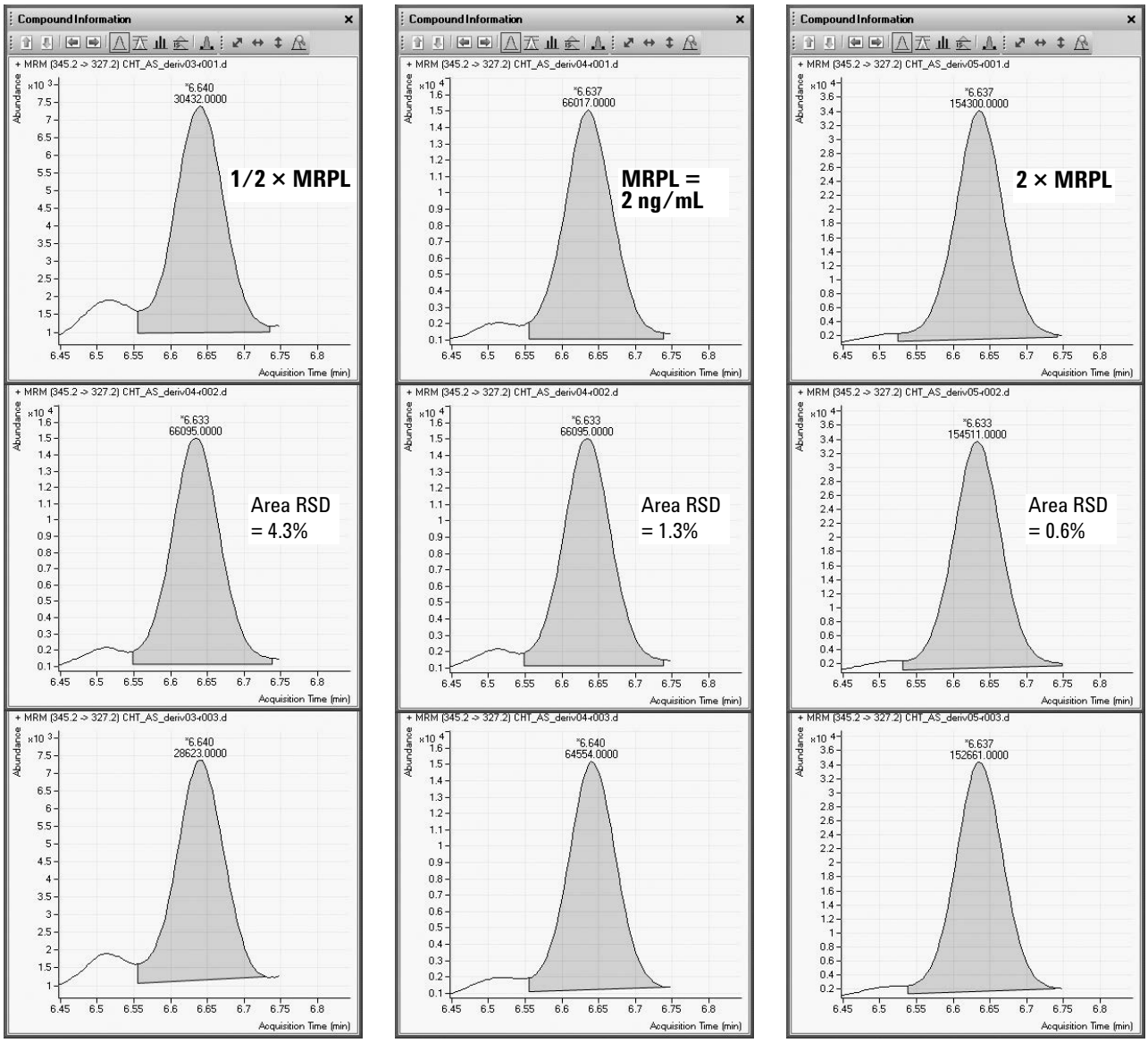

Figure 7c. Triplicate injections of the lowest three levels of 4β-OH-stanozolol.

In Figure 8 the reason for using the GRP derivative is shown by comparing the sensitivity of analyzing the 19-norandrosterone with and without the derivative.

Figures 9a to 9c show the linearity, LOD, and the lowest three level replicate injections for 19-norandrosterone. In Figure 9b we see noticeable signal in the negative quality control. However, this signal definitely comes from the matrix itself as it is not seen in the solvent blank.

The results for all compounds are summarized in Table 3.

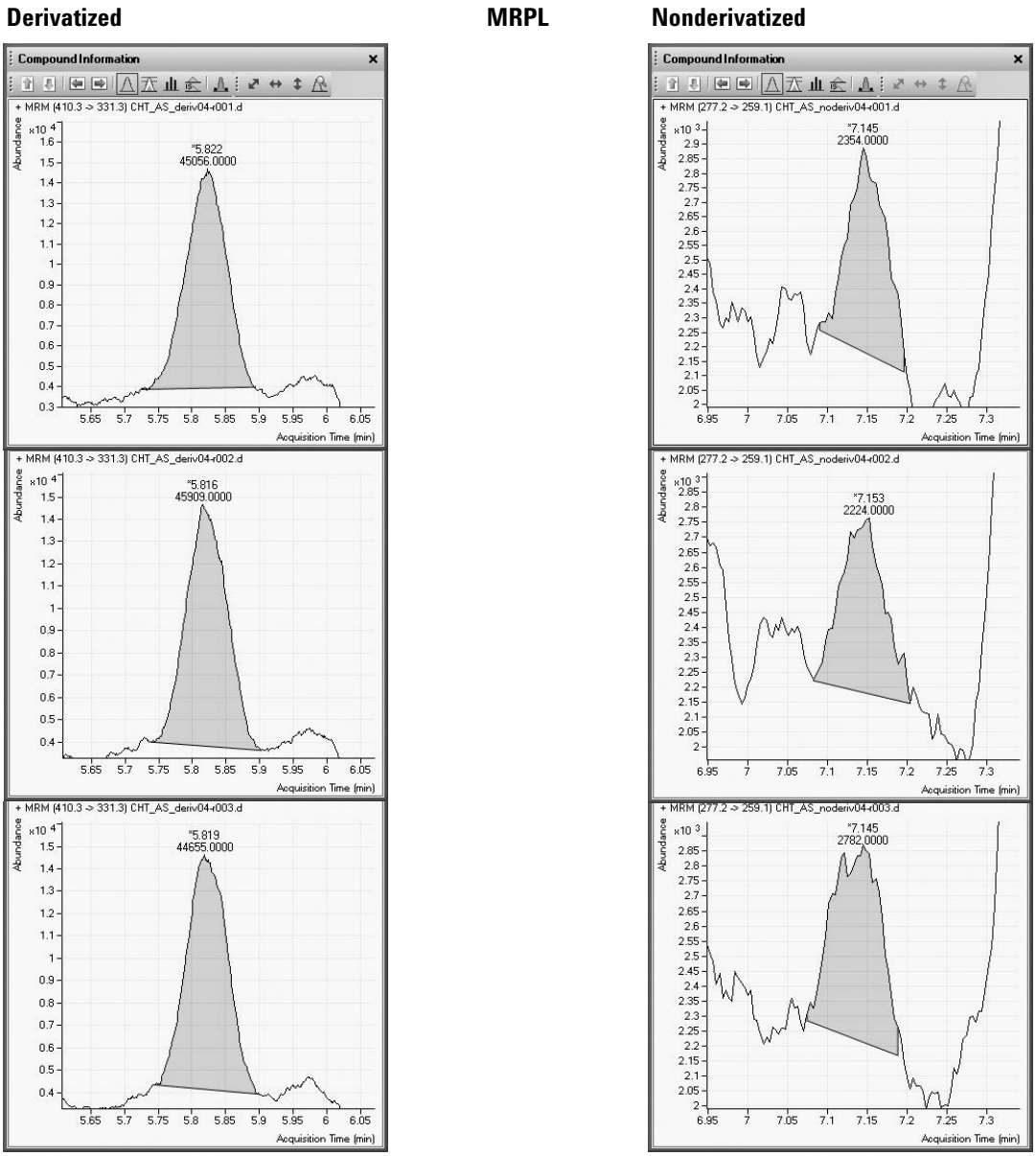

Figure 8. Comparison of signal response for the derivatized (left) versus nonderivatized forms of 19-norandrosterone.

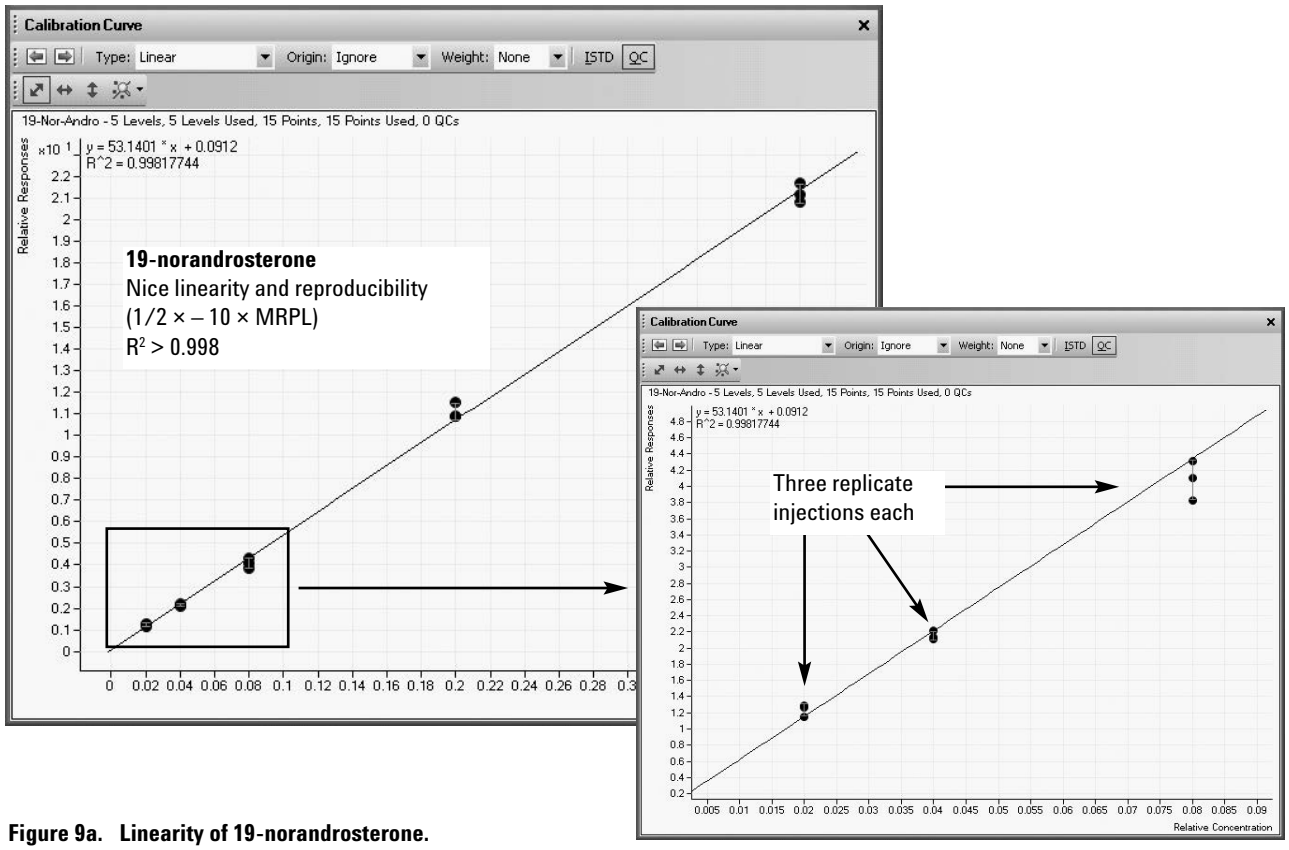

Figure 9a. Linearity of 19-norandrosterone.

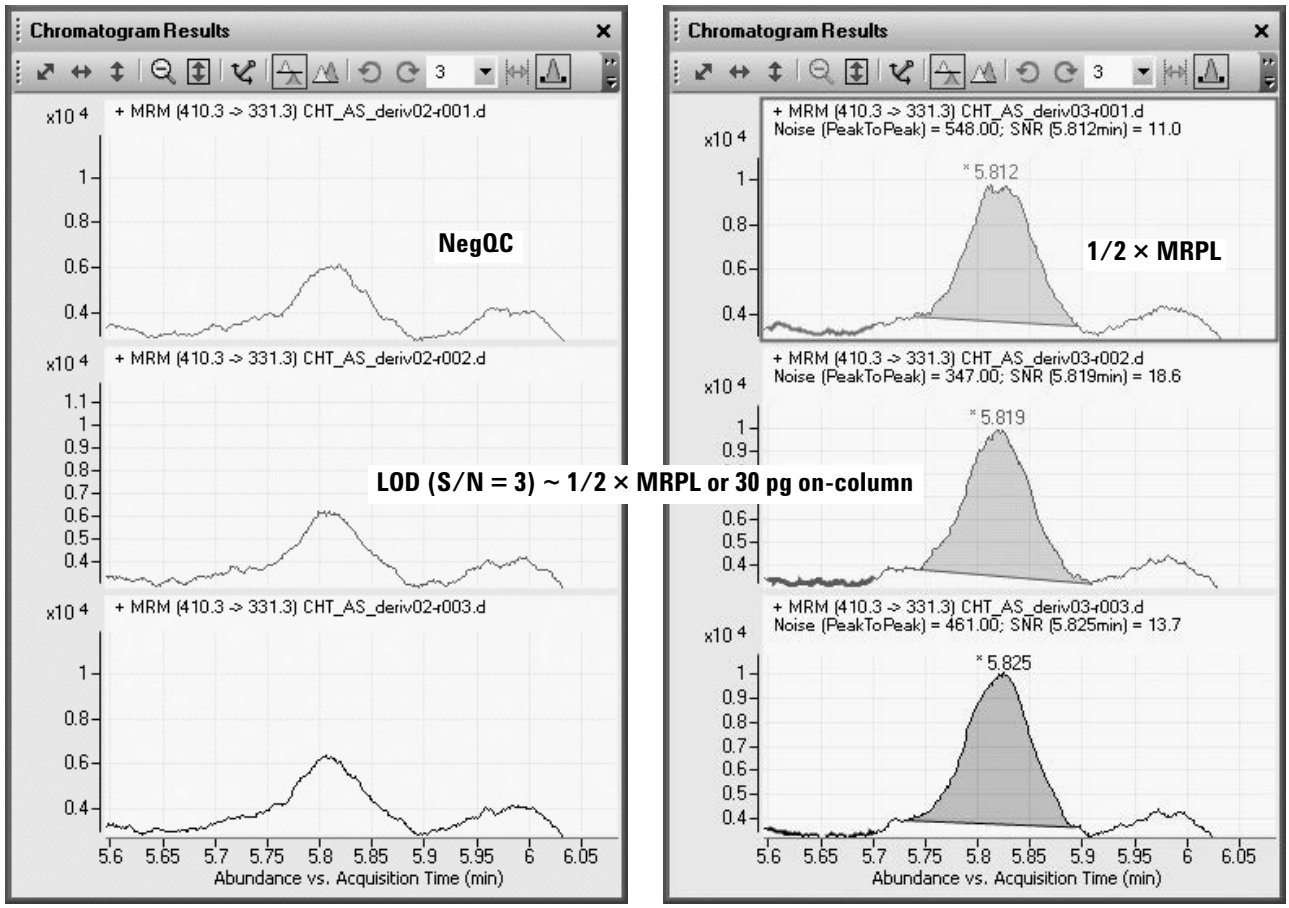

Figure 9b. Estimate of LOD for 19-norandrosterone.

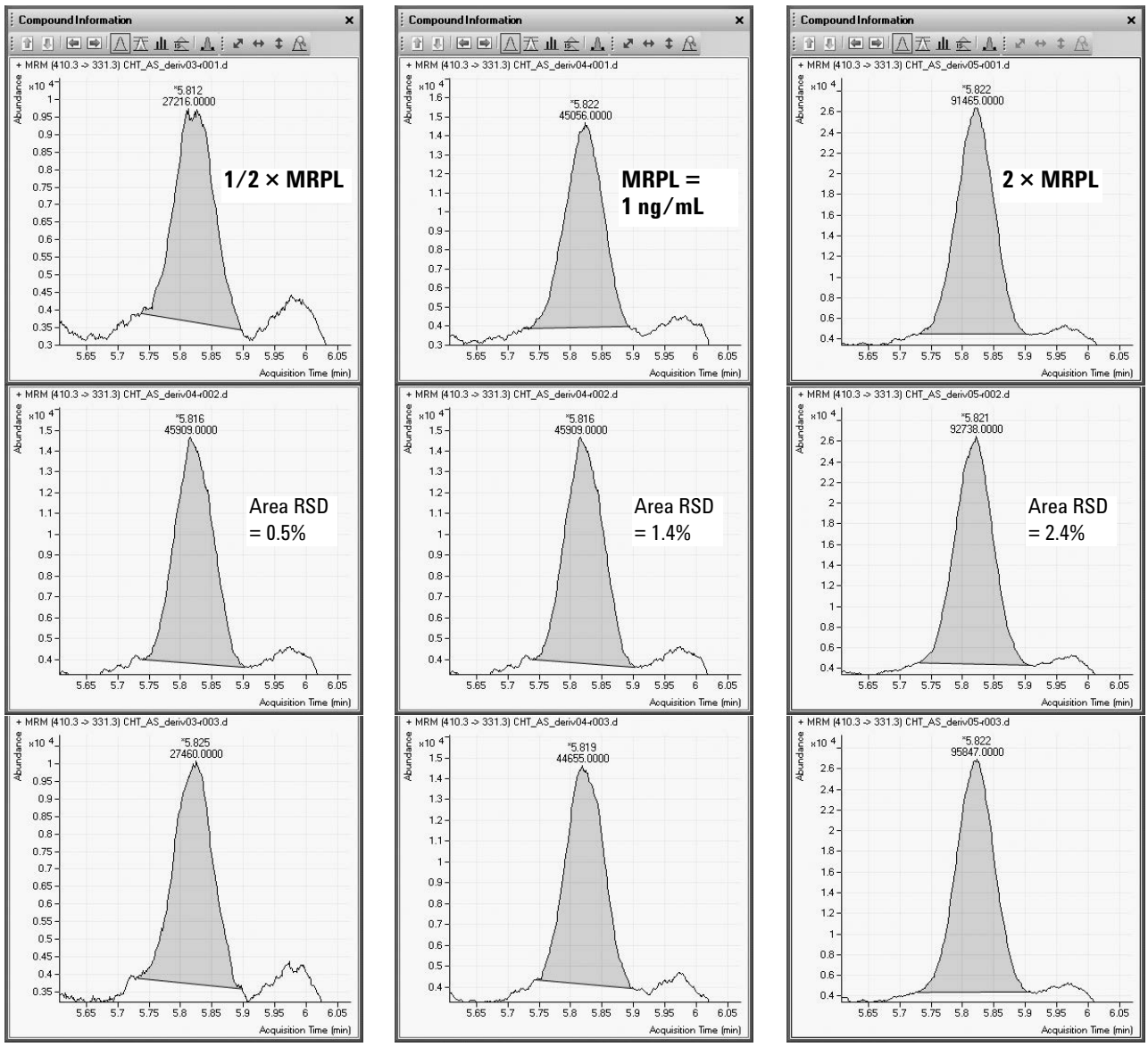

Figure 9c. Triplicate injections of the lowest three levels of 19-norandrosterone.

Table 3. Linearity, Reproducibility, and Calculated Sensitivity for All Compounds Analyzed

Compound	Linearity R^2	% RSD at 1/2 × MRPL	LOD on-column (pg)	LOD MRPL (×)
Clenbuterol	> 0.999	1.1	3	1/40
THG	> 0.992	1.1	30	1/20
MeTest metabolite	> 0.997	4.2	60	1/2
Epimetendiol	> 0.997	12.0	12	1/10
4β-OH-stanozolol	> 0.982	4.3	15	1/40
19-norandrosterone	> 0.998	0.5	30	1/2

Conclusions

The analysis of anabolic substances in urine can be difficult and may require the sensitivity of a triple quadrupole mass spectrometer as seen in this work. Linearity over the range of 1/2 × to 10 × MRPL for each compound is demonstrated and shown to be very good, especially for clenbuterol, which has a correlation coefficient of more than 0.999. The liquid chromatography in this work only uses solvents of water and methanol, with the addition of formic acid for a simple gradient. Limits of detection at levels lower than the minimum required performance levels are demonstrated with percent relative standard deviations of peak areas ranging from 12.0% to as low as 0.5%. The addition of Girard's Reagent P solution shows a marked improvement in sensitivity for the 19-norandrosterone compound.

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Determination of Buprenorphine, Norbuprenorphine, and Their Glucuronides in Urine Using LC/MS/MS

Application Note

Forensic Toxicology

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of buprenorphine, its metabolite norbuprenorphine, and their glucuronides in urine using the Agilent 6410 Triple Quadrupole Mass Spectrometer in electrospray mode is described. Sample preparation included dilution of the urine samples in deionized water for direct injection into the LC/MS/MS system. Since the glucuronides are monitored in the same assay as the free drugs, no hydrolysis or extraction was necessary. To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion for the parent drug, which is required to be present within a specific ratio to the primary ion for acceptable identification (± 20%). The Agilent MassHunter software allows the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis.

Introduction

Buprenorphine is a member of the opioid family of drugs. It is metabolized to norbuprenorphine, and both species undergo extensive conjugation with glu-curonide before urine excretion. The simultaneous determination of buprenorphine, norbuprenor-phine, and methadone has recently been published [1]. Liquid chromatographic methods for the detection of buprenorphine in urine have predomi-nantly been directed towards the free drug follow-ing hydrolysis, centrifugation, and/or extraction [2–4]. However, in 2003, Kronstrand et al. were the first to report on the detection of both free and conjugated compounds in urine using LC/MS/MS, noting that a low concentration of 20 ng/mL of free compounds seemed appropriate for the testing of volunteers. They improved the detection limit by hydrolyzing the specimens and subjecting them to solid phase extraction [5].

In this work, we present a rapid method, sensitive to 1 ng/mL of urine, for the detection of buprenorphine, norbuprenorphine, and their glucuronides in urine involving simple dilution of authentic urine samples with deionized water. Two transitions per compound are monitored for the free drugs and one transition for the glucuronides. The monitoring of the qualifying ion and calculation of its ratio to the intensity of the primary transition are integral parts of the software package and necessary for forensic identification.

The method is simple, sensitive, and rapid, with all analytes being determined in less than 8 minutes.

Experimental

Materials and Methods

Standards and Reagents

D4-Buprenorphine (D4-BUP); D3-Norbuprenorphine (D3-NBUP); BUP; NBUP; BUP glucuronide; and NBUP glucuronide were purchased from Cerilliant (Round Rock, TX). All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Internal standard mix: D4-BUP; D3-NBUP (1,000 ng/mL)

Unlabelled drugs: BUP, NBUP, BUP glucuronide, NBUP glucuronide

Extraction Procedure–Urine

To urine (0.1 mL), add deionized water (0.35 mL) and 0.1 mL internal standard (1 µg/mL)

Calibration Curve:

a)	Negative:	0.1 mL D4-BUP; D3- NBUP
b)	1 ng/mL:	0.1 mL D4-BUP and D3-NBUP 10 µL of BUP, NBUP, and their glucuronides (100 ng/mL)
c)	5 ng/mL:	0.1 mL D4-BUP and D3-NBUP 5 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
d)	10 ng/mL:	0.1 mL D4-BUP and D3-NBUP 10 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
e)	20 ng/mL:	0.1 mL D4-BUP and D3-NBUP 20 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
f)	40 ng/mL:	0.1 mL D4-BUP and D3-NBUP 40 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
g)	100 ng/mL:	0.1 mL D4-BUP and D3-NBUP 100 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)

Analytical Procedure

Instrument:	Agilent 1200 Series RRLC; 6410 Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX Eclipse XDB C18 4.6 mm × 50 mm × 1.8 µm (PN: 922795-902)
Dimensions:	4.6 mm × 50 mm × 1.8 µm
Column temp:	40 °C
Injection volume:	5 µL
Solvent flow rate:	0.8 mL/min

Pump Program:

Time (minutes)	% 20 mM ammonium formate (A)	% Methanol (B)
0	40	60
2.5	40	60
5	0	100
8.5	0	100
10	40	60
Post time:	3 min	

Mass Spectrometer Conditions:

Operation:	Electrospray (ESI) positive mode
Gas temperature:	300 °C
Gas flow (N_2):	6 L/min
Nebulizer pressure:	50 psi
Capillary voltage:	4500 V
Dwell Time:	50 ms

The MRM transition settings are shown in Table 1. The NBUP and BUP have both quant and qual (in parenthesis) product ions.

Table 1. Buprenorphine Acquisition Parameters

Compound	Precursor ion	Fragment ion	RT (min)	Fragment voltage (V)	CE (V)
Group 1					
D3-NBUP	417.4	399.3	1.16	240	40
NBUP 3 gluc	590.5	414.4	0.73	240	40
NBUP	414.4	340.4	1.17	240	35
		(187.2)	1.17	240	40
Group 2					
D4-BUP	472.5	400.4	6.62	240	45
BUP 3 gluc	644.5	468.4	5.21	240	40
BUP	468.4	414.4	6.68	240	35
		(396.1)	6.68	240	55

( ) Qualifier ratios must be within 20% of calibration point

LC/MS/MS Method Evaluation

The analytical method was evaluated according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates over a period of 4 days. The slope of the calibration curve was not forced through the origin. The equation of the calibration curves and correlation coefficients (R^2) are shown in Table 2; the precision and accuracy of the assay are shown in Table 3. The assay was robust, precise, and accurate at the selected point of 10 ng/mL and was linear over the range of 5 to 100 ng/mL. The precision for all drugs was less than 20% both within day and between days, with most showing a variation of less than 10%. The limit of quantitation was 5 ng/mL; the limit of detection was 1 ng/mL.

Figure 1 shows a typical calibration curve for buprenorphine, with a correlation coefficient of 0.9984.

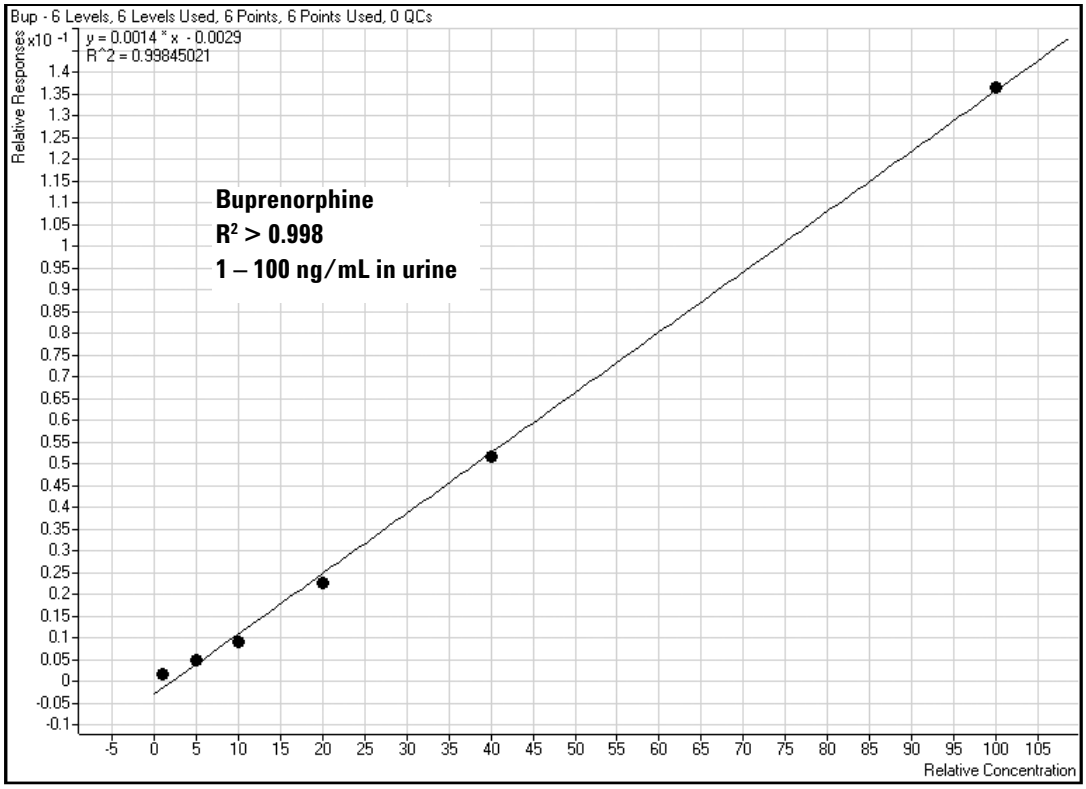

Figure 1. Calibration curve for free buprenorphine in urine.

Table 2. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Buprenorphine and Related Compounds in Urine

Drug	Calibration equation	Correlation coefficient (R^2)	Acceptable qualifier ratio (20%)
Buprenorphine	Y = 0.0065x – 0.005	0.9984	35.4 (28.3–42.5)
Norbuprenorphine	Y = 0.0068x – 0.0036	0.9995	44.9 (35.9–53.9)
Buprenorphine 3 glucuronide	Y = 0.0226x – 0.0064	0.9927	
Norbuprenorphine 3 glucuronide	Y = 0.0013x – 0.0039	0.9948	

Table 3. Inter-Day Precision (10 ng/mL Control Specimens; n = 10)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Buprenorphine	10.74	1.38	12.85
Norbuprenorphine	10.08	1.36	13.51
Buprenorphine glucuronide	12.68	2.41	19.02
Norbuprenorphine glucuronide	11.1	1.84	16.55

Intra-Day Precision (n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Buprenorphine	10.22	0.58	5.64
Norbuprenorphine	8.76	0.57	6.54
Buprenorphine glucuronide	10	0.8	7.04
Norbuprenorphine glucuronide	8.98	0.61	6.75

Discussion

The instrumentation allowed the rapid determination of buprenorphine, norbuprenorphine, and their glucuronides at low concentration, as is required for these drugs. The chromatographic separation produced by the small particle analytical column allowed separation of the peaks in each group segment (Figure 2). The software provided with the instrument is able to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, the identification is rejected. This is an additional feature of the triple quadrupole mass spectrometer, which is extremely important in forensic analysis where court challenges to laboratory data are frequent. Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in blood. The software plots the ratio in the chromatographic window, so the operator is able to assess positivity visually using the “uncertainty” band imposed by the software (Figure 3).

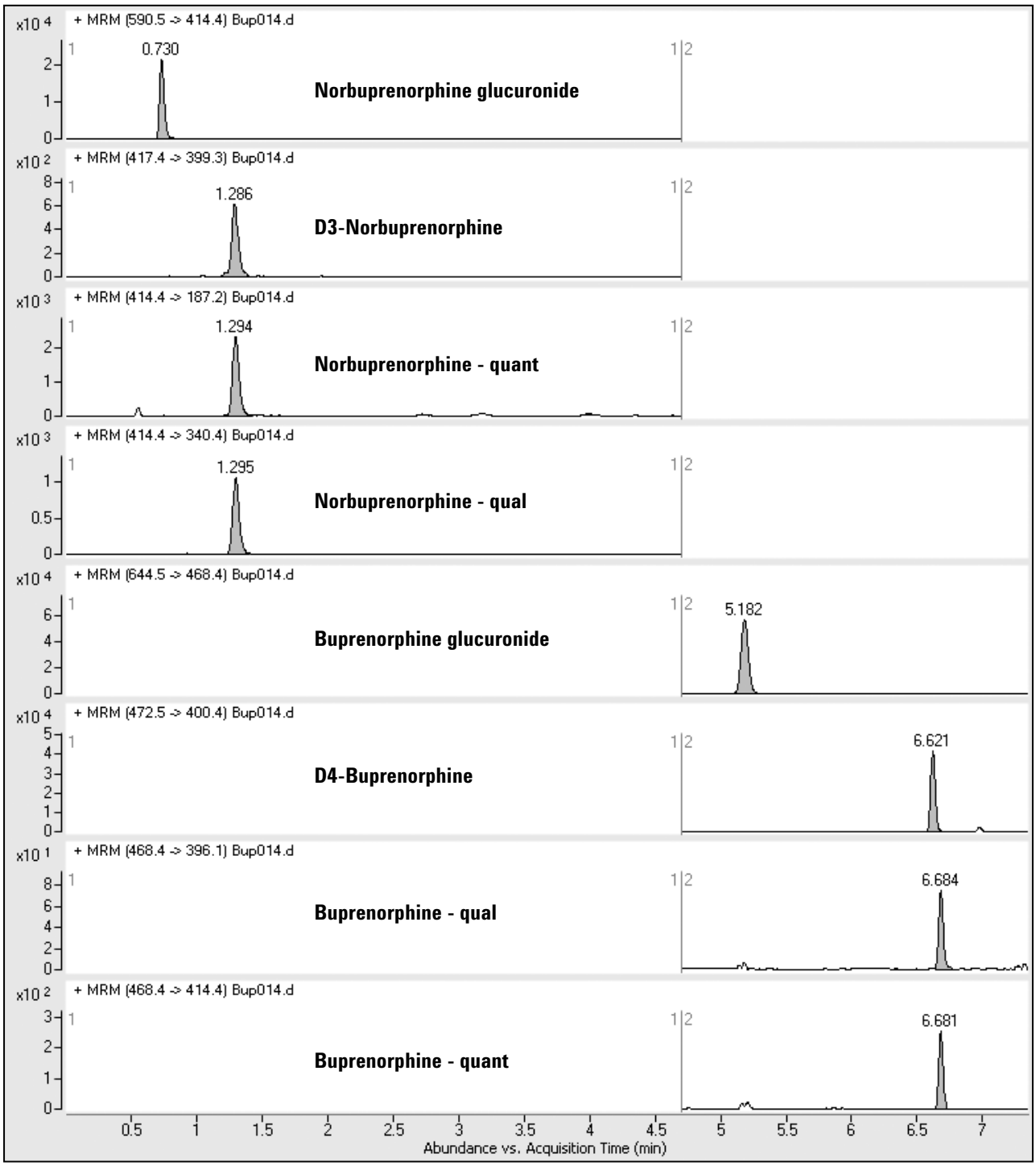

Figure 2. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide extracted from authentic urine specimen.

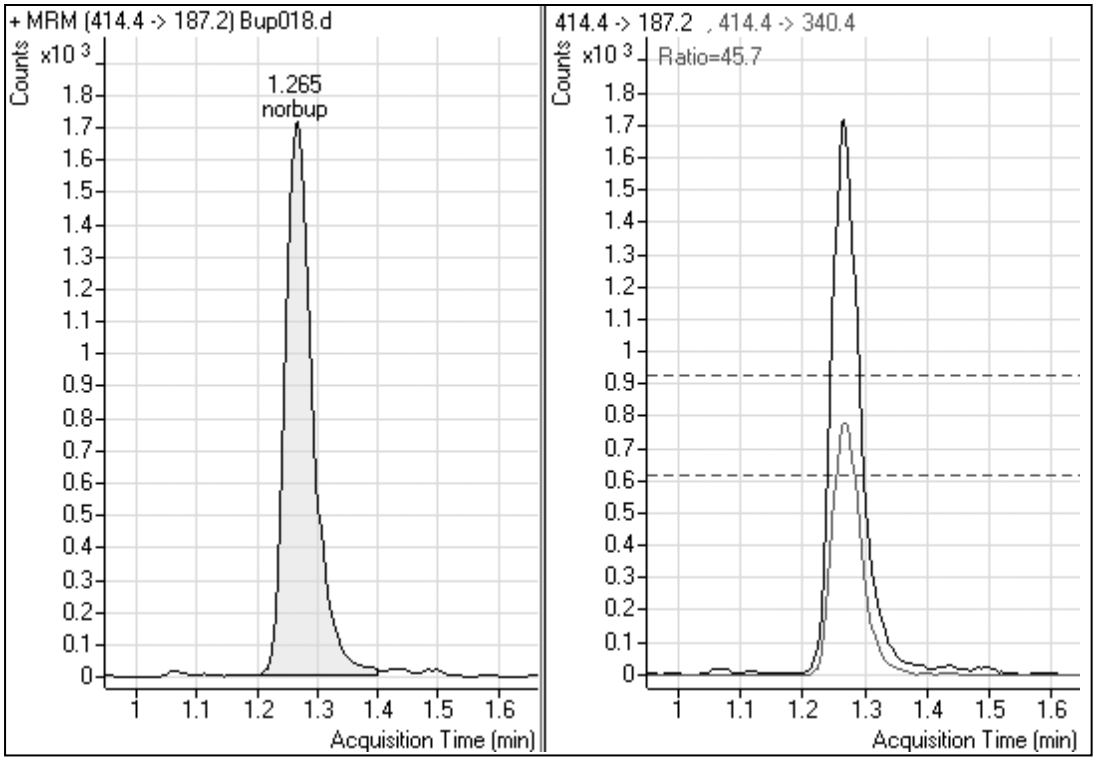

Figure 3. Free norbuprenorphine (98 ng/mL) in urine: quantitation ion at left and overlay of quantitation ion with qualifier ion at right.

Conclusions

The procedure described is suitable for the detection of buprenorphine and norbuprenorphine glucuronides in urine, without need for hydrolysis or extraction using an Agilent Technologies 6410 Triple Quadrupole LC/MS/MS system. This is the first method, which includes qualifying ions required to be present within a specific ratio, for the identification of buprenorphine and norbuprenorphine at low concentration in urine.

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Rapid Analysis of Drugs of Abuse by LC/Triple Quadrupole Mass Spectrometry

Application Note

Forensic Toxicology

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Abstract

A fast, sensitive and reproducible technique for confirming the presence of drugs of abuse (DOA) in oral fluids (OF) using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ) is presented. The sensitivity of the QQQ easily meets the cutoff levels required by the United States Substance Abuse and Mental Health Services Administration (SAMHSA) for workplace testing. The DOA analyzed in this work include THC, cocaine, amphetamine, methamphetamine, and MDMA ("Ecstasy") in OFs, which have been prepared using solid phase extraction (SPE). The sample preparation is then followed by reverse-phase LC/MS/MS using a 1.8-µm, C18 column for high-chromatographic resolution with high-speed separation. As a result, elution times for both analytes and internal standards are less than 4.2 minutes for THC, and less than 1.5 minutes for the remaining drugs. The technique is applied successfully to the quantification of quality controls.

Introduction

In 2004, the United States SAMHSA, proposed a new rule that would allow Federal agencies to use sweat, saliva, and hair in Federal drug testing programs that now only test urine [1]. This initiative effectively confirmed the analysis of oral fluids as a viable test matrix for the determination of drug levels in humans in the workplace, which is logically extended to other areas of testing including police checkpoints for possible driving while under the influence of drugs (DUID) violations.

Confirming the presence of DOA in OF using liquid chromatography/tandem mass spectrometry (LC/MS/MS) provides a faster analysis than gas chromatography/mass spectrometry (GC/MS) because the sample derivatization step, usually required for GC/MS analysis, is bypassed without sacrificing required levels of sensitivity. The use of a C18 column with 1.8-µm particle size for liquid chromatography (LC) results in nicely resolved, symmetric peaks at high flow rates. The multiple reaction monitoring (MRM) capability of the QQQ allows for the highly selective MS/MS analysis of coeluting analyte compounds and their corresponding internal standards, along with monitoring more abundant product ions for quantification and less abundant product ions as qualifier ions for confirmation. The MRM provides for highly specific detection in a complex matrix such as OF.

In this work five DOA are analyzed in two separate runs of less than 4.2 minutes for THC (tetrahydrocannabinol) and less than 1.5 minutes for cocaine, amphetamine, methamphetamine, and MDMA (3,4-methylenedioxymethamphetamine). The sensitivity requirements set forth by SAMHSA for these drugs are easily met. The corresponding cutoff levels are shown in Table 1.

Table 1. SAMHSA Cutoff Levels for Drugs of Abuse

Compound	Cutoff level (ng/mL of OF)
THC	2
Cocaine	8
Amphetamine	50
Methamphetamine	50
MDMA	50

Experimental

Sample Preparation

For each sample, 1 mL of OF is collected using the FDA-approved Quantisal™ collection device, which is then dissolved in 3 mL of a proprietary buffer solution already contained in the sample collection device. One mL of this sample is used for further analysis, which corresponds to 250 µL of OF. For the quality control (QC) samples, reference solutions of each analyte are added to drug-free OF, along with the internal standard (ISTD) at low and medium concentrations of each drug. To the unknown samples only internal standards are added, and for the calibration standards the prescribed levels of analytes and ISTDs are added after the extraction.

The extraction method is the same as used for analysis of these drugs by GC/MS, with any derivatization step omitted and the final residue dissolved in the initial mobile phase rather than in a typical GC solvent.

To the OF/buffer aliquot 2 mL of 0.1 M potassium phosphate buffer is added and then vortexed. The SPE (part number 691-0353T, SPEWare, San Pedro, CA), is conditioned with 0.5 mL of methanol for THC, and 3 mL of methanol for cocaine, etc., followed by 100 µL of 0.1 M acetic acid for THC, and 2 mL of 0.1 M phosphate buffer for cocaine, etc. The SPE is performed by adding the sample to the SPE column followed by successive washes, which include methanol and deionized water, followed by 98:2 hexane:acetic acid for THC, 78:20:2 CH_2Cl_2/IPA/NH_4OH for cocaine, or 2% NH_4OH in ethyl acetate for amphetamine, methamphetamine, and MDMA.

After evaporating the sample to dryness, it is reconstituted in the initial LC mobile phase (0.1% formic acid/water). For the calibration standards, analytes, ISTDs, and mobile phase are added to make 1-mL volumes.

Note that the objective of this work was to test QQQ instrument capability and not the quality of the extraction procedure. Therefore, it was decided that spiking blank OF extracts with both reference and ISTDs after the extraction process would eliminate the variability of sample recovery. However, QCs were spiked with both analytes and ISTDs before the extraction, and the unknown samples were only spiked with ISTDs before the extraction.

Compounds Analyzed

The target compounds and their molecular ion masses are given in Figure 1.

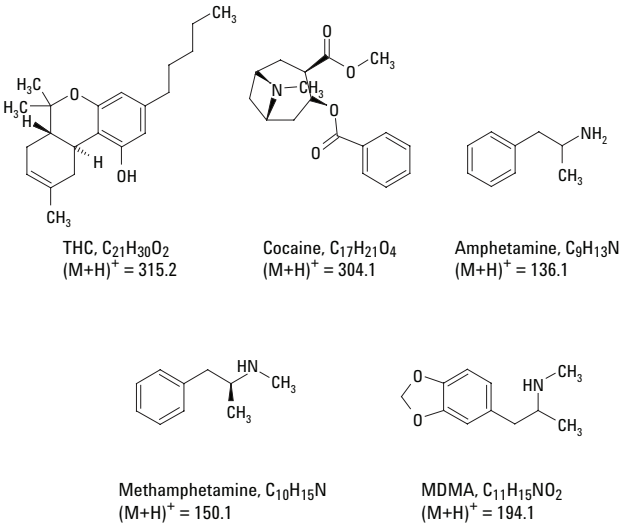

Figure 1. Target compound structures, and their molecular ion masses.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consists of an Agilent 1100-series vacuum degasser, binary pump, well-plate autosampler, thermostatted column compartment, the Agilent G6410AA Triple Quadrupole Mass Spectrometer, and an electrospray ionization source (ESI). System control and data analysis is provided by the Agilent QQQ Control (R&D version), Qualitative and Quantitative Data Analysis software programs. Detailed LC and MS conditions are shown below.

The objective of the method development was to obtain a fast and sensitive analysis for quantifying and confirming the presence of drugs of abuse in oral fluids. For speed, while maintaining good chromatographic resolution and peak symmetry, different solvents, flow rates, and column parameters were optimized. It was found that not only would a simple solvent system using water, acetonitrile, and formic acid, work very well, but a very fast 1-minute gradient on a 1.8-µm particle

size C18 column would elute the compounds in times very competitive with most techniques available in GC/MS as well as LC/MS.

LC Conditions

Column:	Agilent ZORBAX SB-C18, RRHT 2.1 × 50 mm, 1.8 µm (p/n 822700-902)
Column temp:	40 °C
Mobile phase:	A = 0.1% Formic acid in water B = 0.1% Formic acid in acetonitrile
Flow rate:	0.5 mL/min
Gradient:	5% B at 0 min 95% B at 1 min 95% B at 6 min Post run time = 2.5 min
Injection vol:	80 µL (THC); 20 µL (for cocaine, etc)

MS Conditions

Mode:	Positive ESI using the Agilent G1948A ionization source
Nebulizer:	40 psig
Drying gas flow:	10 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 Resolution:	0.7 amu (FWHM)
Q2 Resolution:	0.7 amu (FWHM)
Collision energy:	23 V (THC); 5 V (all other analytes)
MRM:	4 transitions for THC; 16 transitions for cocaine, amphetamines, methamphetamines, and MDMA as shown in Table 2

LC/MS Method Details

Determination of the optimal MRM transitions for both quantifier and qualifier ions was carried out by infusing the individual standards at concentration levels around 1 ng/µL. The quantifier ion was chosen as the most abundant product ion and the qualifier ion was chosen as the second-most abundant product ion.

At the time of this writing, the preliminary version of software only allowed one collision energy and one time segment for the entire chromatographic run. Therefore, a single fragmentation energy of 23 V was used for all transitions of for THC and ISTD, and 5 V was used for all of the transitions of the cocaine, etc., compounds and their associated ISTDs, even though these settings were not optimal for each transition. In addition, MRM transitions were monitored continuously throughout the entire run. As a result, while the data shown here satisfies the requirements of SAMHSA, even better sensitivity should be achievable with optimization of collision energy and time programming of MRM events.

Table 2. Data Acquisition Parameters for MRM Transitions

Compound	RT (min)	Pseudo-molecular ion $(M+H)^+$	Quantitation product ion (*m/z*)	Qualifier product ion (*m/z*)
THC	4.2	315.3	193.1	259.1
D3-THC	4.2	318.3	196.1	262.1
Cocaine	1.5	304.1	182.0	82.0
D3-cocaine	1.5	307.1	185.1	85.1
Amphetamine	1.3	136.1	91.0	119.0
D5-amphetamine	1.3	141.1	93.0	124.0
Methamphetamine	1.3	150.1	91.0	119.0
D5-methamphetamine	1.3	155.1	92.0	121.0
MDMA	1.4	194.1	163.0	135.0
D5-MDMA	1.4	199.1	165.0	135.0

Results and Discussion

The chromatograms corresponding to one-half the cutoff value for THC, or 1 ng/mL, are shown in Figure 2. This level is easily seen and the on-column injection amount corresponds to 20 pg. The area reproducibility among three injections is 3.6%. The root-mean-squared (RMS) signal-to-noise (S/N) is estimated conservatively as five times the RMS S/N. This corresponds to a S/N value of 32:1. The limit of quantitation (LOQ) is about half this value, which corresponds to 0.5 ng/mL, and was confirmed by injecting smaller volumes.

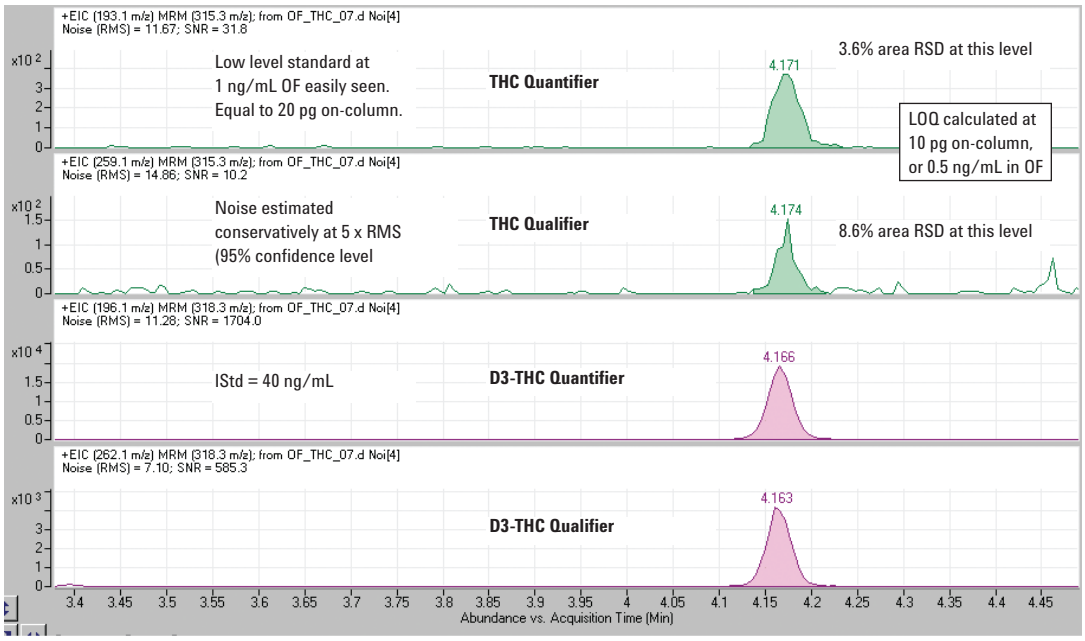

Figure 2. Product ion chromatograms for THC and D3-THC. Generation of chromatograms and integration of peaks is automated with opening of data file by the Agilent Qualitative Analysis software. Peak elution times less than 4.2 minutes. No smoothing applied.

In Figure 3, and using the same reasoning for THC, the LOQs for cocaine (coc), MDMA, methamphetamine (meth), and amphetamine (amp) are estimated to be 0.2, 0.5, 0.6, and 2.5 ng/mL in OF, respectively.

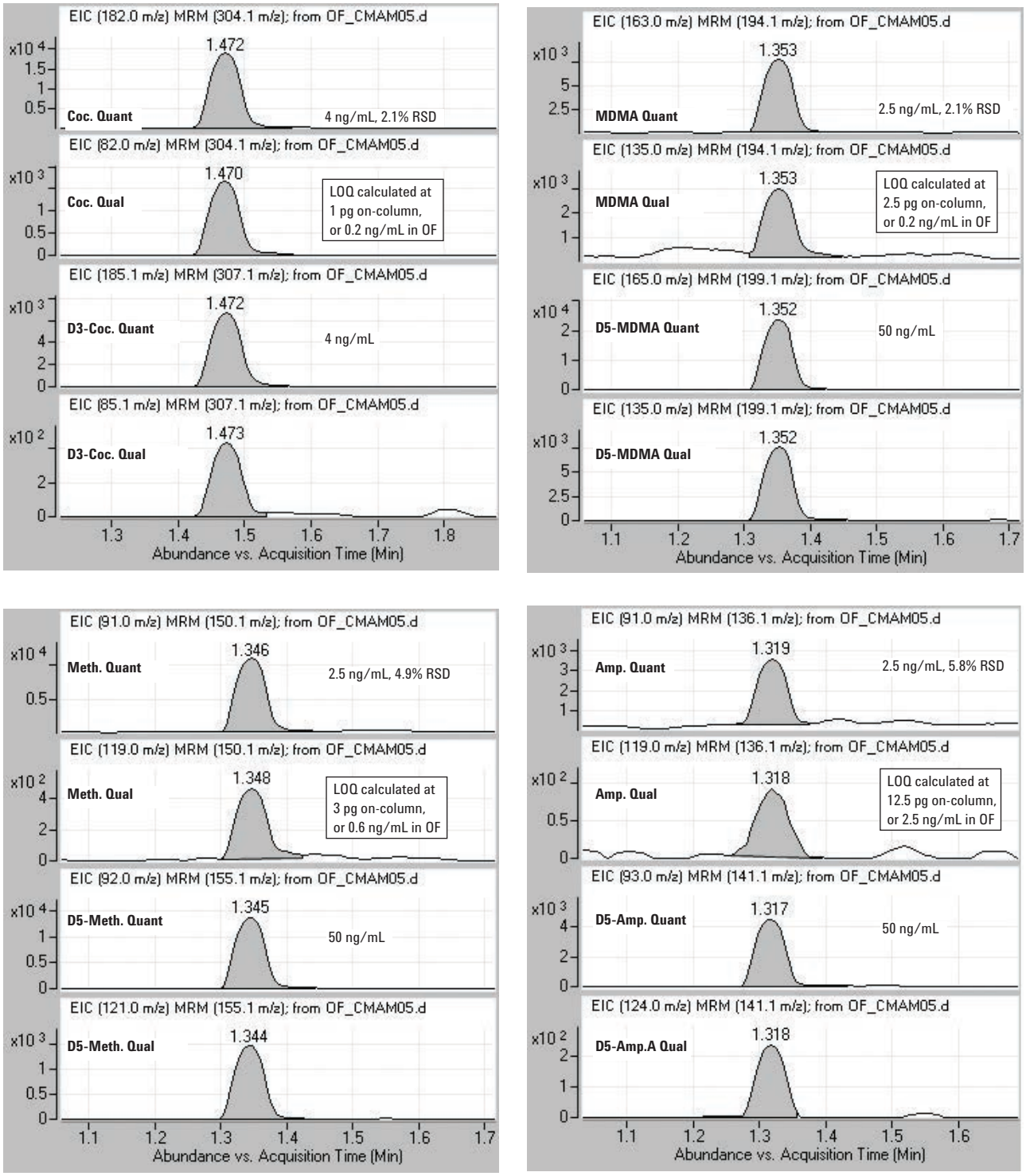

Figure 3. Product ion chromatograms for lowest level standard containing cocaine, D3-cocaine, MDMA, D5-MDMA, amphetamine, D5-amphetamine, methamphetamine, and D5-methamphetamine. Peak elution times less than 1.5 minutes. No smoothing applied.

Along with the quantifier ions for each of the compounds and associated ISTDs, the qualifier ions are also shown in Figure 4. The requirement for each qualifier ion is that its measured area falls within a range of specified ratios with respect to the area of the quantifier ion. For example, with the THC qualifier ion, as determined experimentally by the Agilent G6410AA instrument, the ratio of its measured area to that of the THC quantifier ion should be 22%. Applying a window of acceptance that is ±20% gives an overall range of 17.6% to 26.4%. As long as the ratio of the areas falls within this range, the acceptance criteria for confirmation is met. For all THC compounds, both calibration standards and QCs, this criteria was satisfied. A similar criteria was established for the ISTD.

For the remaining compounds, the qualifier ion area ratio criteria were established as 4% for cocaine, 9% for MDMA, 95% for methamphetamine, and 26% for amphetamine. As was the case for THC, criteria were established for the associated ISTDs as well. All calibration standards and QCs met these criteria.

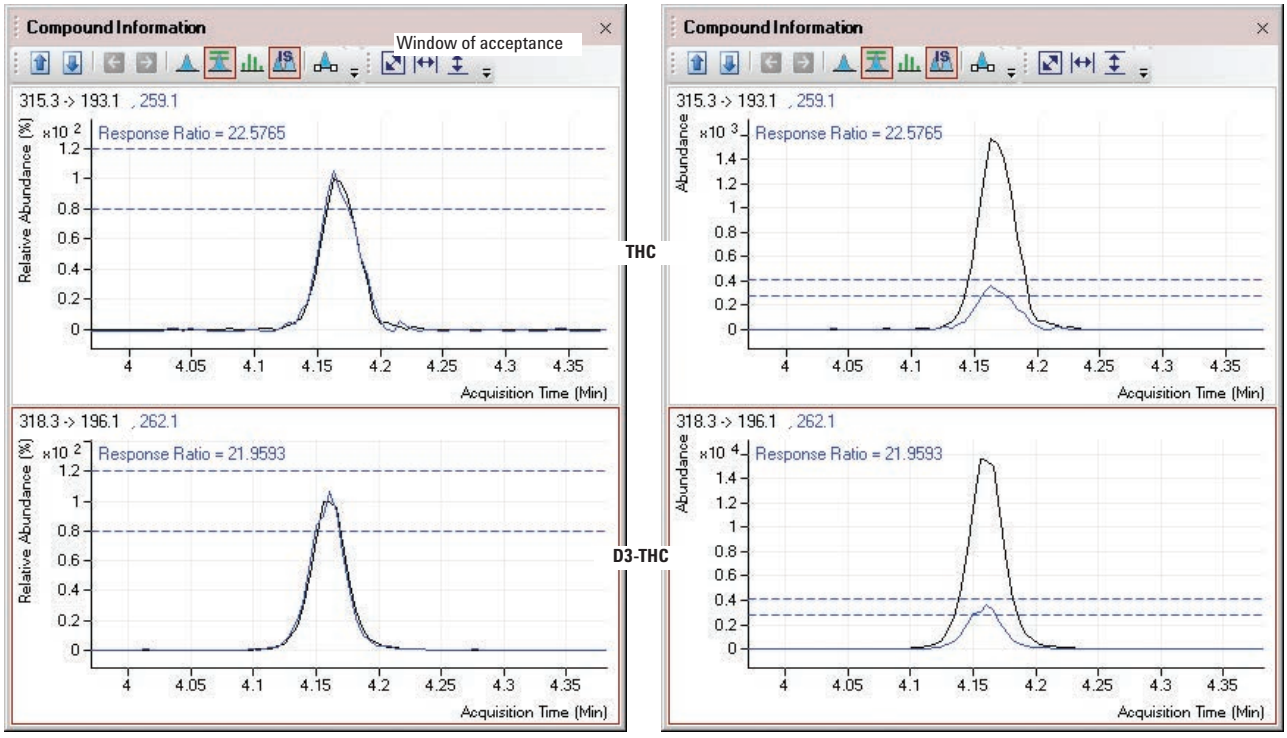

Figure 4. **For confirmation of THC, the qualifier ion area must be 22% that of the quantifier ion area and within a window of ±20% of that value, or from 17.6% to 26.4% overall. The two ways to display this for fast confirmation in the Quantitative Analysis software is normalized by area (left) and un-normalized (right), both of which show the overlap of the qualifier ion on the quantifier ion. If the ion ratio is outside the window of acceptance, the integrated area of qualifier ion will be shaded blue, but transparently to still observe overlap.**

The calibration curves generated for all compounds are shown in Figure 5. The most conservative fitting options are used to generate the line; that is, a linear fit with no weighting and no origin treatment. Each line is based on calibration levels extending across nearly two orders of magnitude.

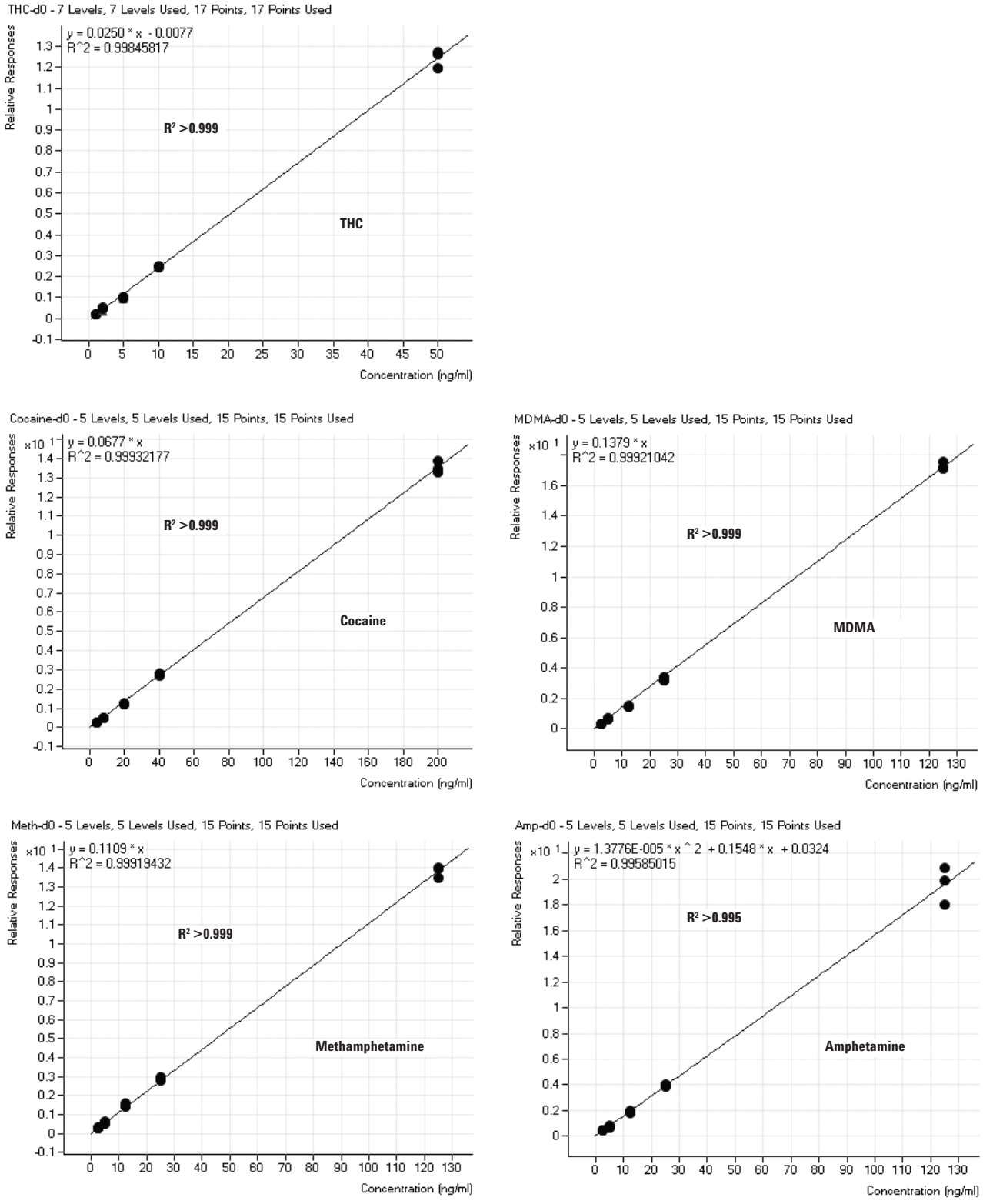

Figure 5. Calibration curves for each DOA using a linear line fit with no weighting and no origin treatment.

The reproducibility for THC is shown in Table 3, and as expected, the %RSD values are lower for higher concentrations. The %RSD is calculated from the area counts for three repeat injections.

Table 3. Reproducibility for THC

Level (ng THC/mL OF)	%RSD
1	3.6
2	2.5
5	2.3
10	1.0
50	1.7

Based on the calibration curves, the QC samples and unknowns are quantified as shown in Table 4. Also shown are the expected amounts of the QCs as prepared by Immunalysis Corporation and the unknown sample THC as measured by GC/MS.

Table 4. Measured Levels of QC and Unknown Samples

Sample	Expected concentration (ng/mL)	Measured concentration (ng/mL)	Accuracy (%)
THC QC1	2	1.81	9.5
THC QC2	5	4.21	16.0
THC Unknown	10*	9.39	6.1
Coc QC1	8	7.51	6.1
Coc QC2	8	7.68	4.0

* Measured by GC/MS

Further Work

Other work has shown that the analysis of THC using atmospheric pressure chemical ionization (APCI), and even atmospheric pressure photoionization (APPI), are more sensitive techniques than ESI [2]. At the time of this writing, the G6410AA Triple Quadrupole Mass Spectrometer instrument was still in its prototype stage and did not support the Agilent G1948A APCI Source, or the Agilent G1978A Multimode Source, which includes simultaneous ESI and APCI capability. Using the APCI Source for the THC could lead to better sensitivity and using the Multimode Source could allow for the analysis of the cocaine, MDMA, methamphetamine, and amphetamine compounds in ESI mode during the first 2 minutes of the run, and the switching to APCI for the remainder of the run when the THC elutes.

As mentioned earlier in this note, the capability to use optimal fragmentation voltages for each MRM transition would lead to an increase in sensitivity. Nevertheless, the G6410A easily meets SAMHSA requirements even without optimization of collision energies or ionization modes.

Conclusions

The LC/MS/MS method described here provides procedures for identification of multiple DOAs in OF with very fast analysis times. Sensitivity levels required by SAMHSA are met for workplace testing, and MRM of several fragmentation transitions are carried out not only for quantitation using designated quantifying ions, but also for confirmation using designated qualifier ions. Using the Agilent C18 column with 1.8-µm particles allows for excellent resolution and peak shape at a relatively high flow rate of 500 µL/min for a 2.1-mm id column and an ESI interface.

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For more details concerning this note, please contact Michael Zumwalt at Agilent Technologies, Inc.

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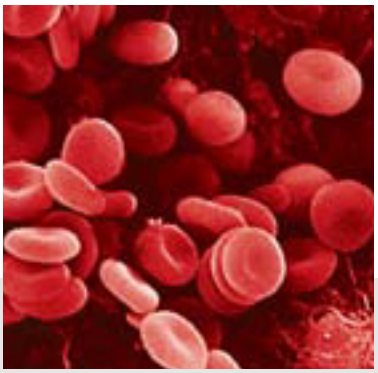
- Analysis of Blood Serum on the Liberty Series II ICP OES with the Axially-Viewed Plasma
- The Use of Collision/Reaction Cell ICP-MS for the Simultaneous Determination of 18 Elements in Blood and Serum Samples
- Sensitive, High-Throughput Analysis of Lead in Whole Blood using the Agilent 7500cx ICP-MS with ISIS-DS



Applications by Technique

ICP-OES & ICP-MS

The Use of Collision/Reaction Cell ICP-MS for the Simultaneous Determination of 18 Elements in Blood and Serum Samples

Application Note

Clinical Research

Authors

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Abstract

This study describes the development of a robust high-throughput analytical method for the determination of 18 elements (15 trace elements and 3 electrolytes) in blood and serum samples using an Agilent Technologies 7500ce collision/reaction cell ICP-MS system. The only sample preparation necessary was dilution using an alkaline diluent containing ammonia, EDTA Triton X-100, and butan-1-ol. Instrument calibration was performed using external calibration with internal standardization. The performance of the method exceeded a previously-used magnetic sector HR-ICP-MS method by at least a factor of three in terms of sample throughput and matched the precision and detection limits of that method.

Introduction

The analysis of metals in biological fluids such as whole blood, serum, and urine has been used for many years to provide information on toxicity, work-place exposure, and nutrient availability, and as a diagnostic tool for a number of ailments. The fact that many trace metals are present at variable and often low concentrations (sub ng/mL range) in different sample types has presented clinical research analysts with a variety of challenges. In addition, matrix components, such as organic compounds, proteins, or electrolyte salts that may interfere with the analysis of trace elements, are often present at elevated levels (mg/mL or above). The matrix to be analyzed, the amount of sample that can be taken and the means of sampling may also impose restrictions. Sufficient volumes of urine can normally be obtained with noninvasive techniques, whereas the collection of whole blood or serum samples usually involves use of needle and syringe and generally yields smaller sample volumes (often only µL or mL) for analysis. The analysis technique employed should therefore provide the following capabilities: sufficiently low detection limits (DLs), ability to overcome matrix related interferences, sufficient linearity to measure a wide concentration range in unknown samples, simultaneous multi-elemental determinations, and ability to cope with small sample volumes.

Analysis of biological sample matrices by inductively coupled plasma mass-spectrometry (ICP-MS) is becoming more widespread since ICP-MS meets a number of the above requirements, namely very low DLs for many trace metals (sub ng/mL), relative freedom from interferences, simultaneous multi-elemental determination, and suitability for small sample vol-umes, as well as providing isotopic information and the possibility of employing isotope dilution mass spectrometry (IDMS) as a high-caliber refer-ence calibration technique. When analyzed by ICP-MS, many of the elements of interest suffer from mass spectral interferences derived from the sample matrix. Before the development of suffi-ciently sensitive collision/reaction cell (CRC) quadrupole ICP-MS instruments, matrix-based spectral interferences were overcome by the use of sector field or high-resolution ICP-MS (HR-ICP-MS)[1] or by non-mass spectroscopic techniques such as atomic fluorescence (AF) [2] or atomic

absorption spectroscopy (AAS) [3]. Another way of overcoming matrix-ef fects is the use of sample digestion using concentrated acids or ashing techniques [4]. These techniques can be expensive, time-consuming, and/or less suitable for high sample throughput.

In our laboratory, magnetic sector HR-ICP-MS (Element 1, ThermoFinnigan) was used [1, 5] for monitoring post- and pre-operation samples from subjects with metal-on-metal hip replacements. After 1:20 dilutions of blood and serum samples with approximately 0.7-mM ammonia, 0.01-mM EDTA, and 0.07% (v/v) Triton X-100 or 1:15 dilutions of urine samples with 1% HNO_3, the elements such as Al, V, Co, Cr, Mo, Ni, and Ti were analyzed. The main drawbacks of this technique were cost, practicality, and duration of instrument set-up, as well as instrument down-time and matrix tolerance during analytical runs containing more than ~30 blood or serum samples.

Objectives

The aim of this work was to develop a robust ICP-MS methodology based on CRC quadrupole ICP-MS (CRC-ICP-MS), capable of measuring a wide range of elements in a single analysis after only a simple dilution of the samples.

A simple dilution of the samples was selected as the preferred sample preparation method, as acid digestion techniques can increase the sample turnaround time, cost, and the potential for contamination.

In order to achieve the required sample throughput of up to 100 samples per batch, the quantitation method had to be based on external calibration. Minimal instrument drift was therefore paramount in order to reduce the need for frequent recalibration or drift correction.

The target elements included the trace metals Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Sb, Se, V, and Zn as well as the electrolytes K, Mg, and Na.

The sensitivity achieved needed to match previously obtained DLs using HR-ICP-MS in our laboratory of 0.2 ng/mL (for example, Co, Mo) and 1.0 ng/mL (Ni) in the undiluted samples.

Sample Preparation

All samples, standards, and quality control (QC) materials were diluted 20-fold using a solution containing approx. 0.7-mM ammonia, 0.01-mM EDTA, and 0.07% (v/v) Triton X-100. Butan-1-ol was added to the diluent as a carbon source at 1.5% (v/v) in order to improve matrix matching between standards and samples and thereby increase the accuracy for analytes such as As and Se, whose ionization behaviors in the plasma are affected by the carbon content [6]. In order to keep the chemistry of the sample introduction system stable throughout the run, the diluent was also used for pre- and post-analysis rinse functions. Commonly used rinse acids such as HNO_3, even at dilute levels of 1%, can lead to coagulation or precipitation of sample matrix components and result in tubing or nebulizer blockages.

The selection of internal standard (IS) elements and the IS concentration is very important. The choice of elements is often restricted in analysis due to the presence of many of the ele-ments that are usually used in environmental applications at ng/mL levels in biological samples. Blood and serum samples were analyzed in semi-quantitative mode to determine the most suitable IS elements, that is, those which were not present or present at the lowest levels in relative terms. For elements that were present in the samples, such as Sc, the concentration of the IS was added at such a level that the contribution from Sc in the sample to the total ^{45}Sc signal would be negligible. The chosen IS elements (Sc, Ge, Rh, In, and Tl) were added to the diluent at a concentration of 20 ng/mL. Addition of the IS in this way negated possible mixing problems if online addition of the IS via a T-piece was used.

Instrumentation

An Agilent 7500ce Octopole Reaction System (ORS) ICP-MS was used in three different gas modes: hydrogen, helium, and standard or no-gas mode. The ICP-MS conditions and the isotopes, integration times and gas modes for the multi-elemental determination are given in Tables 1 and 2. Quantitation on all isotopes was performed using the three central points of the spectral peaks.

A 100-µL/min PFA microflow nebulizer was used and sample uptake and washout times were reduced using the larger diameter peristaltic pumps of the Integrated Sample Introduction System (ISIS). The pump speed was set at 0.1 rps during the analysis and washout in order to minimize overloading of the sample introduction system and the plasma with matrix components. The torch was equipped with a 2.5-mm diameter injector and the Shield Torch system was used. Nickel (Ni) cones were used at all times.

The total acquisition time per sample was 208 s. This included the sequential loading of the H_2, He, and Std tune files and a 40 s equilibration and stabilization time between the different gas modes. Each sample/standard solution was analyzed sequentially in all gas modes before the autosampler probe moved to the next sample. After each sample, the autosampler probe was rinsed for 5 s using 5% HNO_3 and the sample introduction system was then rinsed using the diluent for 30 s.

Table 1. ICP-MS Parameters Used in the Different Gas Modes

	H_2	He	Std
Rf Power (W)	1500	1500	1500
Carrier gas (L/min)	0.87	0.87	0.87
Make up gas (L/min)	0.17	0.17	0.17
Spray chamber temp (°C)	2	2	2
Gas flow (mL/min)	4	4	Not used

Table 2. Analysis Parameters for the Analytes of Interest

Analyte	Isotope monitored (*m/z*)	Integration time per mass (s)	Internal standard used (*m/z*)	Gas mode used
Na	23	0.3	45	He
Mg	24	0.3	45	He
Al	27	3.0	45	He
K	39	0.3	45	He
V	51	1.5	45	He
Cr	53	3.0	45	He
Mn	55	0.9	45	Std
Fe	56	0.3	45	H_2
Co	59	1.5	45	He
Ni	60	1.5	45	He
Cu	65	0.9	72	He
Zn	66	0.3	72	He
As	75	1.5	72	He
Se	78	1.5	72	H_2
Mo	95	1.5	103	Std
Cd	111	1.5	115	Std
Sb	121	0.9	115	Std
Pb	Sum of 206, 207 and 208	0.9	205	Std

Method Performance and Robustness

The stability of the proposed methodology was tested by running blood and serum samples in a sequence over a 10-hour period (a total of 90 samples, including calibration standards and QC checks) and monitoring the behavior of IS elements, calibration slopes, and check standards.

Instrument Stability - Signal Variation for IS Elements

Typical signal variation for the IS elements of choice (Sc, Ge, Rh, In, Tl) was 4.8%–9.3% in hydrogen mode, 5.5%–8.2% in helium mode, and 6.7%–10.0% in standard mode. This was assessed during a 90-sample sequence of blood and serum samples. Figure 1 shows the variation for the IS elements throughout the 10-hour run. Sc is present in some sample types at ng/mL levels, which can be seen here after sample 8.

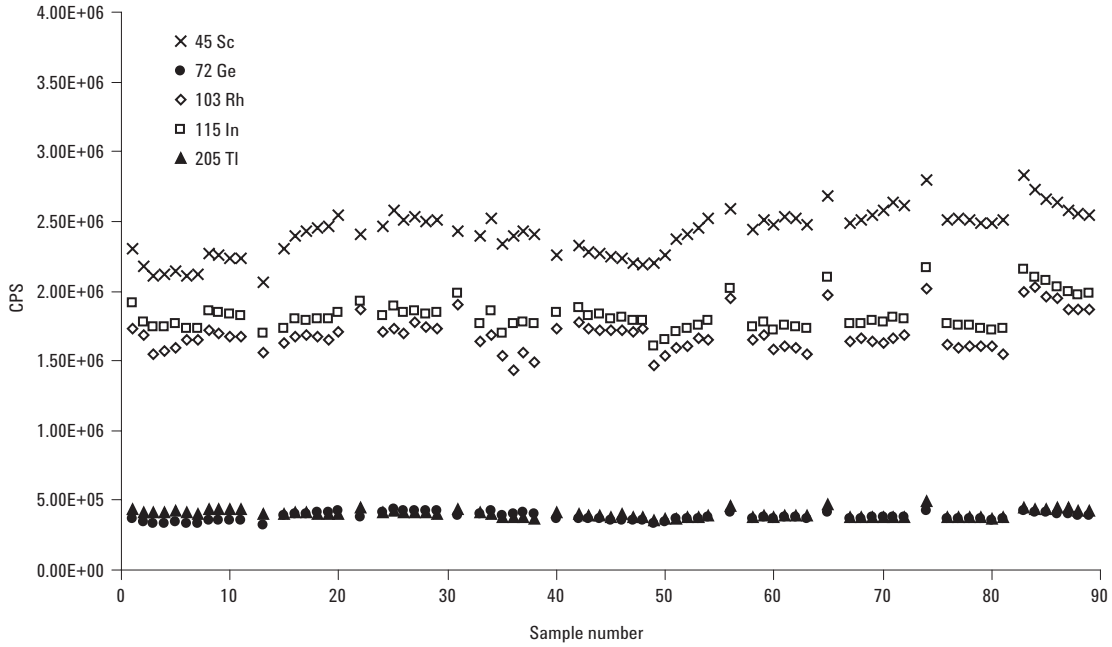

Figure 1. Variation of the IS signals in standard mode throughout the 10-hour run.

Calibration Repeatability and Linearity

Overlaying calibration curves from the beginning, middle, and end of the 10-hour run assessed the robustness of the calibration technique. The correlation coefficients for the mean slope of three calibrations for V, Se, and Pb (Figure 2) during a 10-hour sequence ranged from 0.9997 to 1.0000 and indicate the robustness of the method with these matrices. The calibration coefficients for all elements measured were generally better than r^2 >0.9900.

Check Standards

Check standards at 1 ng/mL level were analyzed throughout the run after every nine samples for the trace metals and were within 10% of the expected value for the elements tested.

Effects of Sample Matrix on the Sample Introduction System

Using dilution factors of 20-fold or less for analysis of these matrices by HR-ICP-MS lead to frequent problems with the sample introduction system, especially blocking of the torch injector. When using quadrupole ICP-MS as described above, dilution factors of 15- and 10-fold could be used without detrimental effects on the sample introduction system (Figure 3) or instrument performance. Reagent blanks were monitored after the analytical run, and no significant deterioration in the DLs or increase in the background levels was observed.

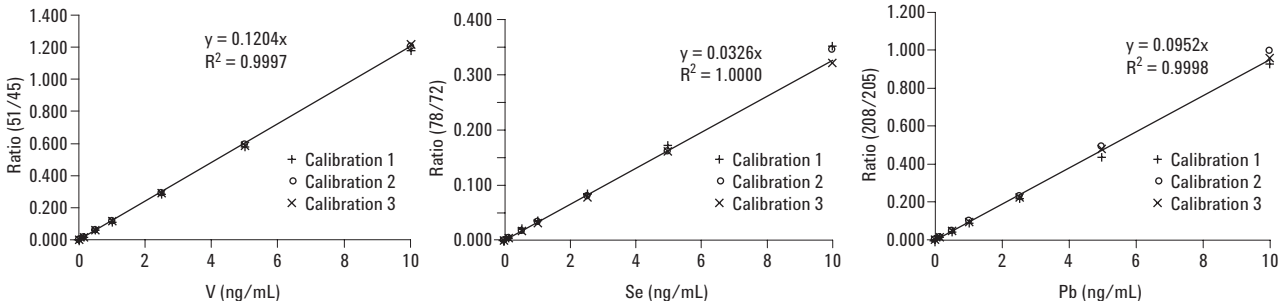

Figure 2. Linearity of overlaid calibration curves for V, Se, and Pb, showing stability of the external calibration approach throughout a 90-sample sequence.

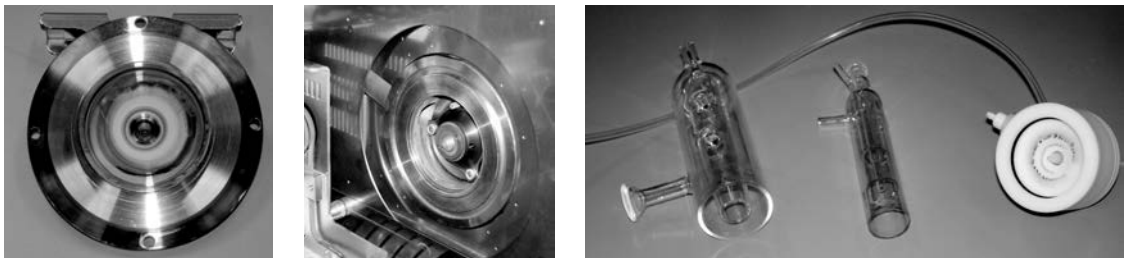

Figure 3. Photos of the interface and sample introduction system after a 90-sample run. Both the sampler and skimmer cones show minor matrix deposits. The 2.5-mm injector torch used was relatively deposit-free. The blood deposits on the spray chamber and the nebulizer block were removed using a sodium hypochlorite solution.

Analysis of Certified Reference Materials (CRMs)

Multiple sub-samples (n=4) of the certified reference material NIST SRM 1598 Bovine serum and the reference material Seronorm MR9067 (whole human blood, level 2) were diluted 20-fold as described above and analyzed using the conditions described in Tables 1 and 2. These materials were chosen because they represented different biological matrices and contained a wide range of analytes of interest ranging in concentration levels from sub ng/mL to mg/mL. Levels for the same analytes often varied by more than an order of magnitude between the two materials. Certificate data for both materials as well as method DLs (calculated back to the undiluted sample and based on 3 s of the blank concentration) for the method proposed here are shown in Table 3.

The analytical data for both materials were converted to percent recovery data relative to the certified or indicative values and are shown in Figure 4a) and b). The combination of the reference materials chosen for this study provided certified values with uncertainty estimates for all of the elements determined except for Na, where only an indicative value was available. The recovery for Na compared to the indicative value was 99.0%, and the data for the remaining elements measured fell within the uncertainty range for either one or both of the reference materials. Where the certificate values were not achieved (for example, V, Cr, and Cd), the certified concentrations in SRM 1598 were below the DL for the method. Na, As, Ni, and Pb are quoted as indicative values only in SRM 1598 (Table 3.).

Table 3. Certified Concentrations for the Analytes of Interest in the SRM NIST 1598 and the Reference Material Seronorm MR9067. Method DLs Calculated Back to the Undiluted Sample are Given for Comparative Purposes.

Trace elements	NIST SRM 1598 Bovine serum (ng/g)	Seronorm MR9067 human blood level 2 (ng/mL)	DL (ng/mL)
Al	3.7±0.9	39–71	0.8
As	0.2*	10.6–11.8	0.1
Cd	0.089±0.016	4.8–6.0	0.1
Co	1.24±0.016	4.6–5.8	0.1
Cr	0.14±0.08	5.1–6.3	1.0
Cu	720±40	NA	0.4
Fe	2550±100	NA	19
Mn	3.78±0.32	10.1–13.3	0.1
Mo	11.5±1.1	5.3–6.7	0.1
Ni	0.7*	5.1–8.6	0.2
Pb	0.6*	373–417	0.1
Sb	NA	25–28	0.5
Se	42.4±3.5	114–130	0.2
V	0.06*	3.1–4.2	0.1
Zn	890±60	NA	3.0
Major elements	**(µg/g)**		**(ng/mL)**
K	196±5	NA	100
Mg	20.0±0.4	NA	1.5
Na	3000*	NA	5.0

*Is indicative value only

NA Not applicable

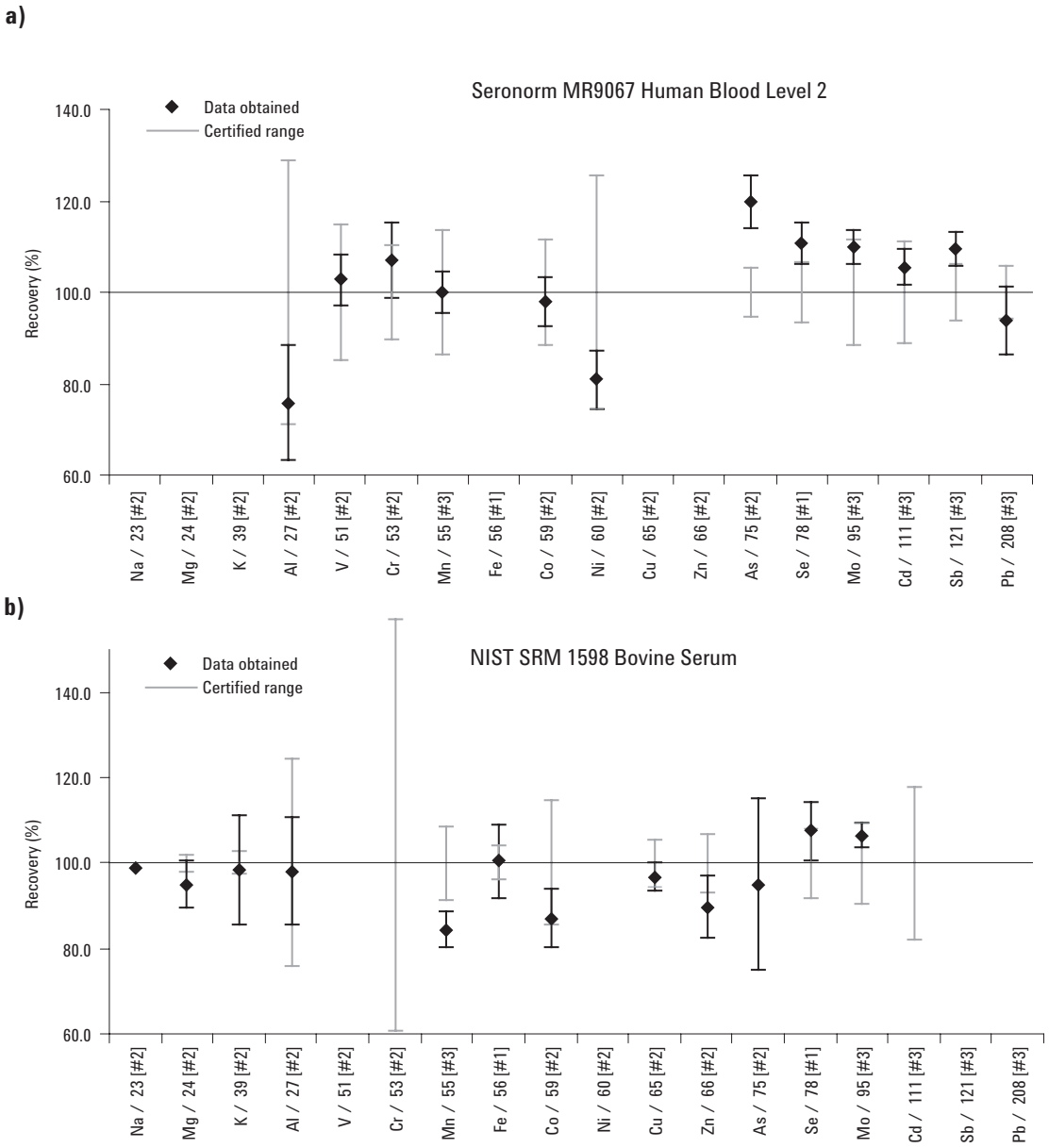

Figure 4. Data obtained expressed relative to the certified data for a) Human blood Seronorm MR9067 and b) Bovine serum SRM NIST 1598. Errors bars represent expanded uncertainties for data obtained and certified ranges for the reference materials. The numbers after the isotopes indicate the tune step used (#1 = H_2, #2 = He, #3 = Std).

Importance of Matrix-Matching and Choice of IS Elements

The data for As and Se in MR 9067 are slightly high compared to the certified mean value, and this could be due to a higher carbon content in this matrix. When increasing the level of butan-1-ol in the diluent from 0% to 3% v/v, recoveries for these analytes decreased and approached 100% (Figure 5). When no butan-1-ol was added to the diluent, recoveries for As and Se were significantly higher than the mean certified values (by 94% and 72% respectively) in comparison to recoveries obtained with butan-1-ol addition at 1.5% (v/v). A complete matrix match was achieved for both samples by using the standard addition technique for As and Se in both reference materials. Recoveries for Se were 95.8% and 99.9% in NIST SRM 1598 and Seronorm MR9067 respectively, and 102.6% for As in Seronorm MR9067. Figure 5 also indicates that the effect of the carbon addition on both elements is slightly different.

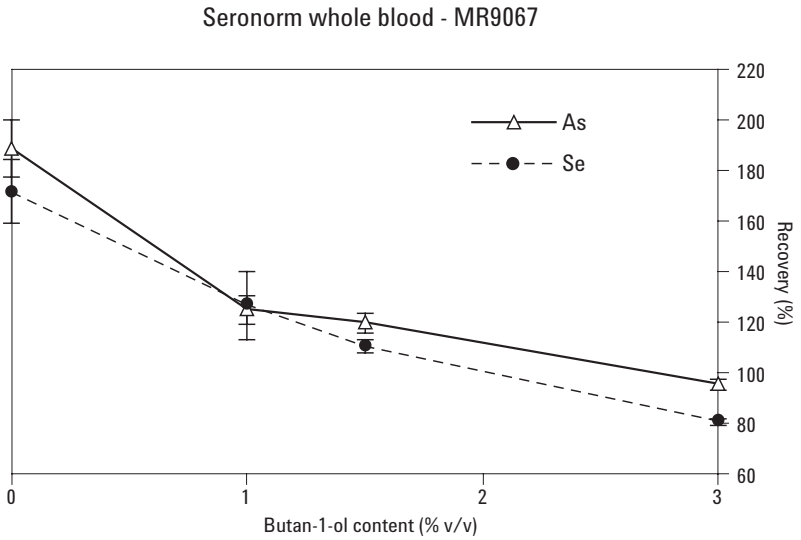

Figure 5. Recovery data for As and Se in Seronorm whole blood (Level 2) with varying levels of butan-1-ol addition to the diluent.

According to the data obtained here, an addition of 3% would be best for As (mean recovery of 95.4 ±2.3%), whereas the ideal volume of butan-1-ol addition for this sample and dilution level for Se is closer to 2%.

For such elements where the ionization is affected by matrix components in the plasma, it is therefore imperative to obtain a good level of matrix matching for the greatest accuracy. If this is not possible, for example if the carbon levels in different samples vary significantly, it may be better to use a different sample preparation procedure such as closed-vessel microwave digestion in order to destroy the organic carbon matrix. However, this can significantly increase the sample turn-around time for large sample batches.

Spike Recovery Data

Spike recovery experiments were performed on both materials for the trace metal analytes at 2–4 different levels with concentrations ranging from 2–5 times of the original analyte concentrations. The only exception to this spiking regime was Fe in MR9067, for which no certificate or indicative value was available before the analysis and where the spike concentration added (20 ng/mL) was not sufficiently high above the determined sample concentration (400 ±5 µg/mL) to give meaningful recovery data. The mean data for all spike levels for the trace metal analytes are shown in Table 4.

Spike recoveries for all elements fell within 100 ±20%, and all except Fe, Se and Mo were within 100 ±10%. The high Se recoveries are thought to be due to the fact that the matrix matching for carbon content consisted of only 1.5% butan-1-ol. High recoveries for Mo were also observed when the samples were analyzed by HR-ICP-MS, and this effect is currently under closer investigation.

Table 4. Mean Spike Recovery Data Obtained for Both Reference Materials

	NIST SRM 1598 bovine serum	Seronorm MR9067 human blood level 2
100% ±5%	Al, V, Cr, Mn, Cu, Zn,Cd, Sb, Pb	Al, V, Cr, Mn, Co,Ni, Cu, Cd
100% ±10%	Co, Ni, As	Zn, As, Sb, Pb
100% ±15%	Fe, Se	Se
100% ±20%	Mo	Mo

Conclusions

A robust CRC-ICP-MS method was developed that is capable of high sample throughput (up to 100 samples per batch) for a large suite of elements in difficult biological matrices after simple dilution. The method robustness was demonstrated by minimal signal drift during analytical sequences of 10-hour duration, negating the need for frequent recalibration. The method DLs achieved matched those of a previously used HR-ICP-MS method. Further improvements in method DLs can be achieved by reducing the dilution levels of the biological matrices, which is possible due to the robustness of the sample introduction system. Good agreement within the uncertainty of certificate values was achieved for all of the target analytes in both reference materials where certified data were available across concentration levels ranging from ng/mL–mg/mL level. Spike recoveries for all elements fell within 100 ±20%, and all except Fe, Se, and Mo were within 100 ±10%.

Acknowledgements

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Sensitive, High-Throughput Analysis of Lead in Whole Blood using the Agilent 7500cx ICP-MS with ISIS-DS

Application Note

Forensic Toxicology

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Abstract

The analysis of biological samples has become a key application of ICP-MS. Of the matrices typically analyzed in laboratories, whole blood presents some specific challenges, due to the high matrix levels, and its tendency to coagulate when mixed with the acids that are commonly used for ICP-MS sample preparation. Prior to analysis, whole blood requires solubilization, typically using a highly basic diluent to prevent this coagulation. The key requirements for routine analysis of whole blood are sensitivity, simplicity, robustness in complex matrices, long term stability and high sample throughput. This application note describes a rapid (52 sec/sample) analysis of approximately 300 whole blood samples using the Agilent 7500cx ICP-MS fitted with an Integrated Sample Introduction System-Discrete Sampling (ISIS-DS) accessory. The performance demonstrates superb long term stability, with a sub-ppb method detection limit for the analysis of lead in whole blood.

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Introduction

Although much stricter regulations have been implemented on the use of lead, it still finds its way into many consumer products [1]. As a result of its potent toxicity, emphasis has been placed on its analysis in biological fluids.

For the analysis of Pb in whole blood, minimal sample handling is critical in order to minimize contamination. A highly robust and stable instrument is essential to minimize signal suppression and drift due to the complex sample matrix. Furthermore, forensic laboratories typically require the highest possible sample throughput in order to cope with large numbers of samples generated during routine blood-lead screening. Currently, many forensic laboratories still use graphite furnace atomic absorption (GFAA), and anodic striping voltammetry (ASV) for the analysis of lead in whole blood [2]. Although both techniques may achieve the required sensitivity (10 µg/dL), they are lacking in speed and ease-of-use. ICP-MS, with discrete sampling is a simpler, faster method, and better suited to this application. In addition to increasing sample throughput, the ISIS-DS reduces the total amount of sample matrix to which the ICP-MS interface is exposed. This provides improved long term stability with this type of complex sample matrix. As a result, instrument maintenance is reduced, further increasing overall sample throughput. The ISIS-DS is fully integrated with the Agilent 7500 (and 7700) Series ICP-MS instruments and is controlled by the instrument's operating software.

Configuring the ISIS-DS is simple, since it consists essentially of a switching valve and sample loop. The ICP-MS is tuned for typical robust plasma conditions providing a highly reproducible and accurate analysis.

Experimental

Instrument parameters were optimized to normal robust plasma conditions with oxide levels ~1% (CeO^+/Ce^+) (Table 1).

Table 1. ICP-MS Operating Parameters

Instrument parameters	No gas mode
Forward power (W)	1550
Sample depth (mm)	8
Carrier gas (L/min)	0.85
Makeup gas (L/min)	0.15
Extract 1 (V)	0
ISIS loop length (cm)	50
ISIS loop id (mm)	0.8
ISIS loop volume (µL)	250
ISIS stabilization time (sec)	20

Samples were supplied by the California Department of Public Health (CADPH) and analyzed according to the CADPH method that specifies a 50x dilution of the whole blood. The high matrix tolerance of the Agilent 7500cx ICP-MS allows whole blood to be analyzed routinely at a 10x dilution and many labs take that approach. However, in compliance with the CADPH method, a 50x dilution was applied for this work. The samples consisted of the following: base blood, 1 ppb spike base blood, 1 ppb CCV, CCB (diluent only), and the following CADPH Standard Reference Materials (SRM); low blood QC (4.98 ± 0.17 µg/dL Pb where 1 µg/dL = 10 ppb), medium blood QC (9.66 ± 0.12 µg/dL Pb), and high blood QC (19.03 ± 0.29 µg/dL Pb) samples. These samples were analyzed repeatedly for a total of approximately 300 analyses. Calibration standards were not matrix-matched and consisted of a blank, 0.01, 0.05, 0.1, and 1 µg/dL Pb, yielding an instrument detection limit of 3.09×10^{-4} µg/dL (3.1 ppt) (Figure 1).

Calibration standards were prepared in an NH_4OH, EDTA, 1-butanol, Triton X-100 diluent (2% NH_4OH, 4% 1-butanol, 0.1% EDTA, 0.1% Triton X-100).

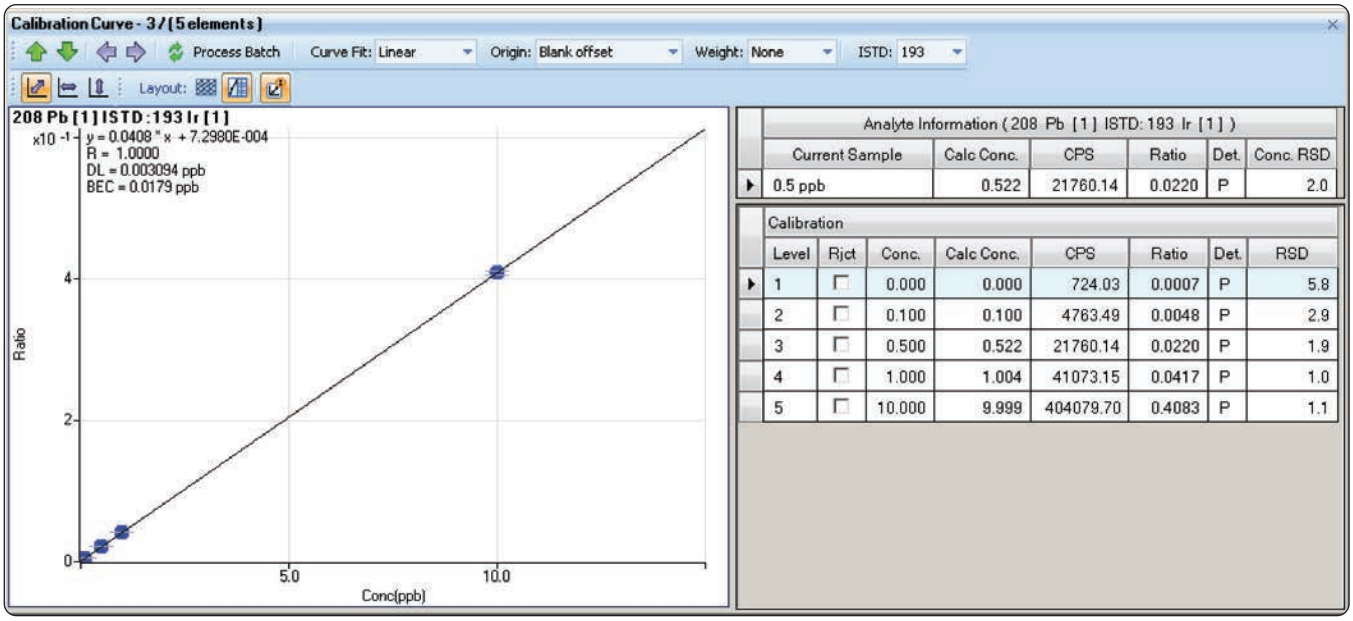

Figure 1. Calibration curve and table.

Data and Results

Sensitivity and Precision

To determine the method sensitivity and precision for Pb, seven replicates of the 0.01 µg/dL standard were acquired and the standard deviation was multiplied by 3.14 (99% confidence limits for student t-test) to give the measured on-instrument detection limit (DL). Table 2 shows the concentration and standard deviation (SD) used to calculate the resulting on-instrument detection limit of 5.4 x 10^{-4} µg/dL (5.4 ppt). In-sample method detection limits would require correction for the sample prep dilution factor, which in this case was 50x. However, Agilent standard procedure specifies 10x, which would result in a MDL of 54 ppt.

Table 2. Precision and Measured Detection Limits for Lead

Date	Time	Sample	Measured Pb Concentration (ppb)	Measured Pb Concentration (µg/dL)
10/13/2009	12:24 PM	0.01 µg/dL	0.0997	0.00997
10/13/2009	12:24 PM	0.01 µg/dL	0.0985	0.00985
10/13/2009	12:25 PM	0.01 µg/dL	0.0968	0.00968
10/13/2009	12:26 PM	0.01 µg/dL	0.1001	0.01001
10/13/2009	12:27 PM	0.01 µg/dL	0.0985	0.00985
10/13/2009	12:29 PM	0.01 µg/dL	0.0952	0.00952
10/13/2009	12:30 PM	0.01 µg/dL	0.0972	0.00972
Standard Deviation			0.001734	0.0001734
On-instrument Detection Limit			5.445×10^{-3}	5.445×10^{-4}

Whole Blood Results

Three CADPH SRMs, spike base blood, and Continuing Calibration Verification/Blanks (CCV/CCB) were repeatedly analyzed, totaling 301 individual analyses. There were over 40 analyses per sample, with the exception of the CCV/CCB pair, which was analyzed after every ten analytical runs. The entire analysis took 259 minutes, resulting in a sample-to-sample run time of 52 seconds. Table 3 details the sample results.

Reference values for the SRM samples are listed in Table 4. Note that the sample concentration as injected into the ICP-MS ranged from approximately 0.099 to 0.381 µg/dL (~1-4 ppb), illustrating the ability of the Agilent 7500cx ICP-MS to accurately measure low analyte concentrations in a complex matrix.

Internal Standard (ISTD) Recoveries

The long term instrument stability can be demonstrated by monitoring ISTD recovery verses time. Figure 2 details the ISTD recoveries for the entire analytical run. Both ^{103}Rh and ^{193}IR are plotted here, though only ^{193}IR was used for all calculations. Control limits (dotted lines) were set at 85% to 105%. ISTD stability was excellent through the entire run with no significant drift observed. In addition, ISTD suppression due to the 50x whole blood matrix was minimal, demonstrating the robustness of the Agilent 7500cx ICP-MS. The slightly elevated points visible in the plot are due to the small increase in nebulization efficiency when the non-matrix matched QC samples (CCB and CCV) were measured.

Table 3. Results for Whole Blood Samples. All Samples Were Diluted 50x Except CCV/CCB.

Sample name	Sample number (n)	Ave Pb concentration (µg/dL)	Standard deviation	% RSD	% Recovery
Base Blood	52	0.004	0.0003	6.09	NA*
Base Blood Spike (1 ppb)	45	0.097	0.0011	1.20	97
CCB	26	0.0002	0.00010	46.5	NA*
CCV	26	0.099	0.0014	1.36	99
Low Blood SRM	45	4.911	0.0687	1.40	99
Medium Blood SRM	44	9.696	0.1136	1.18	100
High Blood SRM	44	18.947	0.2231	1.18	100

**NA-not applicable*

Table 4. Reference Values for the CADPH Standard Reference Materials

SRM	Value (undiluted)	Value (50x dilution)
Low Blood SRM	4.98 ± 0.17 µg/dL	0.0996 µg/dL
Medium Blood SRM	9.66 ± 0.12 µg/dL	0.1932 µg/dL
High Blood SRM	19.03 ± 0.29 µg/dL	0.3806 µg/dL

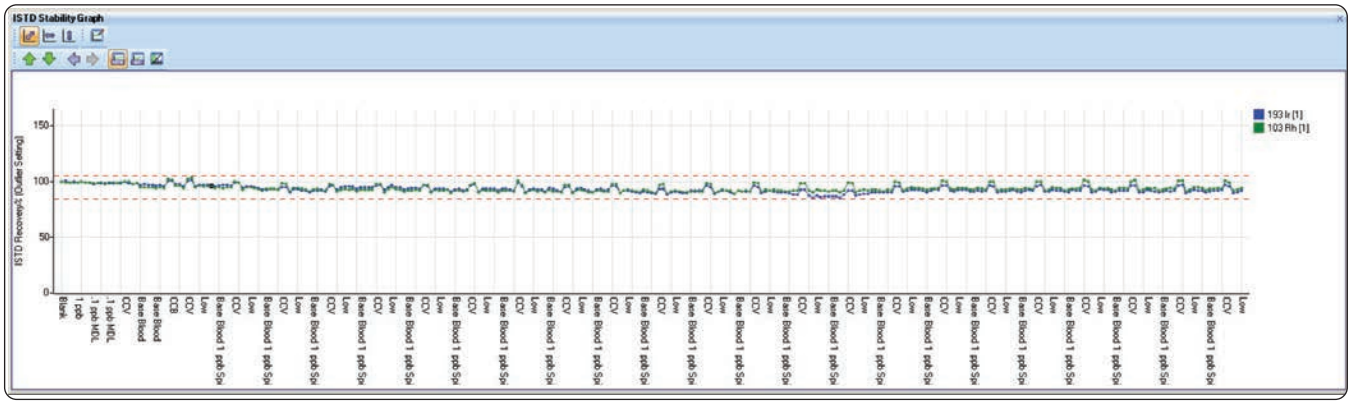

Figure 2. ISTD recoveries (due to space limitation, not every sample name is displayed on the X-axis).

Conclusions

High-throughput, whole blood analysis presents several challenges for ICP-MS. Rapid sample handling, high sensitivity, excellent long term stability, and high tolerance to complex matrices are all critical to a successful analysis. The Agilent 7500cx ICP-MS with ISIS-DS allows for rapid (52 sec) sample-to-sample analyses with minimal to no carryover and superb sensitivity and long term stability throughout a sequence of more than 300 samples. The highly robust plasma of the Agilent 7500cx ICP-MS eliminates the need for matrix-matched standards and blanks, further simplifying the analysis.

References

1. Centers for Disease Control and Prevention, National Center for Environmental Health online resource, US, www.cdc.gov/nceh/lead
2. C40-A Analytical Procedures for the Determination of Lead in Blood, 06/01/2001, Clinical and Laboratory Standards Institute (CLSI), US, www.clsi.org

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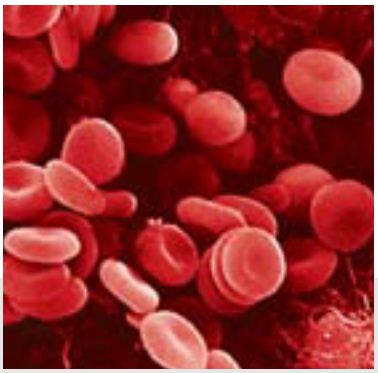
- Extraction of Acidic Drugs from Plasma with Polymeric SPE
- Extraction of Basic Drugs from Plasma with Polymeric SPE
- Fractionation of Acidic, Neutral and Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX
- Extraction of Non-Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX
- Extraction of Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX
- Quantitative Analysis of Amphetamine-Type Drugs by Extractive Benzoylation and LC/MS/MS
- Analysis of Oxycodone And Its Metabolites-Noroxycodone, Oxymorphone and Noroxymorphone In Plasma By LC/MS With An Agilent ZORBAX StableBond SB-C18 LC Column



Applications by Technique

Sample Preparation

Extraction of Acidic Drugs from Plasma with Polymeric SPE

Application Note

Pharmaceuticals

Authors

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Introduction

Acidic drug extraction from biofluids often poses unique challenges for the bioanalytical chemist. While basic drugs are routinely extracted by means of cation exchange solid phase extraction (SPE), the related approach for acids (anion exchange) often fails. The reason is that naturally occurring ions (phosphate, citrate, various sulfates, and other larger anions) present in blood and other biofluids, are likely to retain on anion-exchange sorbents and interfere with extraction of acidic analytes. This effect is less pronounced in cation exchange SPE of basic analytes because endogenous cations are typically limited to Group 1 and 2 metals such as sodium and potassium, which are considerably smaller, more polar, and therefore less likely to retain by ion exchange, or interfere in the extraction.

An alternative to anion exchange of acidic analytes is a nonpolar extraction. For optimal extraction using this retention mode, the analytes should be neutralized (protonated) at the SPE load step by applying the sample under acidic conditions.

Because the nonpolar retention mode in SPE is less selective than ion-exchange, the possibility of interferences and ion suppression effects in LC/MS analysis should be considered for these types of extractions.

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Bond Elut Plexa, a unique polymeric SPE phase, is an alternative for the extraction of acidic analytes. A gradient of polarity on the polymer surface shunts small analytes, including neutralized acids, to the more hydrophobic center of the polymer bead where they are retained. Because the particle surface is highly polar and entirely amide-free, binding of proteins on the polymer surface is minimized, resulting in cleaner samples and reduced ion suppression. The procedure described here provides a simple and effective SPE method for the extraction of acidic drugs from human plasma.

Materials and Methods

SPE reagents and solutions

1% formic acid	Add 10 µL concentrated formic acid to 1 mL DI H_2O
Methanol	Reagent grade or better
5% methanol	Add 5 mL methanol to 95 mL DI H_2O
Bond Elut Plexa	10 mg 96 well plate (p/n A4969010)

SPE method

Sample	100 µL human plasma
Pretreat	Dilute with 300 µL 1% formic acid
Condition	1. 500 µL CH_3OH
	2. 500 µL H_2O
Wash	500 µL 5% CH_3OH in H_2O
Elute	500 µL CH_3OH

All samples evaporated to dryness and reconstituted in 100 µL of 80:20 5 mM ammonium formate: CH_3OH.

LC/MS performed – ESI, drying gas @ 250 °C, 25 psi in negative ionization mode

LC conditions

Mobile phase

A	5 mM ammonium formate
B	Methanol

LC gradient program

Time (min)	%B
0:00	40
0:15	40
1:00	80
3:00	80
4:30	40

Column

Type	Pursuit XRs C18 3 µm, 50 × 2.0 mm (p/n A6001050X020)
Flow rate	0.2 mL/min

Results and Discussion

The Limit of Quantitation (LOQ) of the combined SPE and LC/MS/MS analysis was 5.0 ng/mL. The internal standard for the application was 100 ng/mL naproxen. Recoveries were calculated from a second order regression with RSD values based on a sampling of n = 6. Excellent recoveries were achieved (Table 1), demonstrating good retention and elution, as well as minimal ion suppression. Response for all compounds evaluated was linear up to 3 orders of magnitude from 5.0 ng/mL to 5.0 µg/mL with correlation coefficients all above 0.995. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6 at each concentration). Figure 1 shows the chromatograms of the extracts at 50 ng/mL. As shown in Table 1, the extractions produced reproducibly high recoveries.

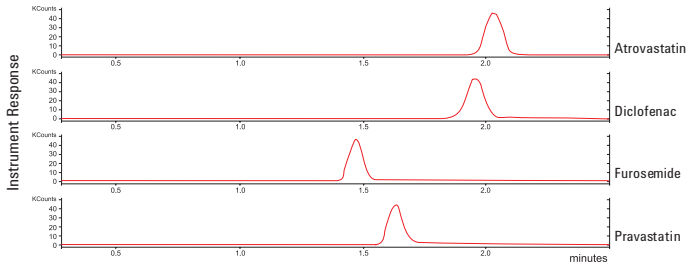

Figure 1. Chromatograms of a 50 ng/mL human plasma extract.

Table 1. High Recoveries of Acidic Drugs with Bond Elut Plexa

Drug	log P	pKa	2 µg/mL %Recovery	%RSD	5 µg/mL %Recovery	%RSD	R^{2*} 5.0 ng/mL to 5000 ng/mL
Atorvastatin	6.3	4.5	91	10	100	9	0.9967
Diclofenac	4.2	4.2	97	6	100	5	0.9995
Furosemide	1.5	4.7	95	5	100	2	0.9983
Pravastatin	2.6	4.6	95	8	100	7	0.9986

* Second-order regression used to calculate correlation coefficient (R^2)

Conclusions

As shown in Figure 1 and Table 1, extraction of acidic drugs on the general-purpose SPE product Bond Elut Plexa provides a viable alternative to mixed-mode and other complicated ion exchange sorbents. Using a simple method with no buffers in the eluant, good recoveries with high reproducibility are achieved for a variety of acidic compounds spanning a range of polarities from log P 1.5 to 6.3. Improved analytical sensitivity and reproducibility arise from the performance features built directly into the polymeric sorbent, so the SPE methodology can remain simple. Bond Elut Plexa is recommended for high-throughput assays where method development time must be minimized without compromising data quality or reproducibility.

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Agilent Technologies

Extraction of Basic Drugs from Plasma with Polymeric SPE

Application Note

Pharmaceuticals

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USA

Introduction

Bioanalytical solid phase extraction (SPE) has been dominated by polymeric sorbents in recent years. The ease-of-use, good flow, and resistance to effects of drying relative to silica-based sorbents make polymeric sorbents an obvious choice for high volume, high throughput assays requiring quick validation and minimal method development.

Because the method validation process is time consuming and requires high quality data, SPE methods that are fast, yet produce good recoveries with high reproducibility, are desirable. To the extent that the SPE process is streamlined without compromising data integrity, method validation can be simplified and shortened. Bond Elut Plexa minimizes method development with simple and effective methods and improves analytical sensitivity and reproducibility with an advanced polymeric structure that minimizes binding of large biomolecules to the surface, with the end result of simplifying and streamlining the SPE process.

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Materials and Methods

SPE reagents and solutions

2% ammonium hydroxide	Add 20 µL concentrated ammonium hydroxide to 1 mL DI H_2O
Methanol	Reagent grade or better
5% methanol	Add 5 mL methanol to 95 mL DI H_2O
Bond Elut Plexa	10 mg 96 well plate (p/n A4969010)

SPE method

Sample	100 µL human plasma
Pretreat	Dilute with 300 µL 2% NH_4OH
Condition	1. 500 µL CH_3OH 2. 500 µL H_2O
Wash	500 µL 5% CH_3OH in H_2O
Elute	500 µL CH_3OH

All samples evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% formic acid: CH_3OH aq.

LC/MS performed – ESI, drying gas @ 400 °C, 30 psi

LC conditions

Mobile phase

A	0.1% Formic acid
B	Methanol

LC gradient program

Time (min)	%B
0:00	40
0:15	40
1:00	80
3:00	80
4:30	40

Column

Type	Pursuit XRs C18 3 µm, 50 × 2.0 mm (p/n A3001050X020)
Flow rate	0.2 mL/min

Results and Discussion

The procedure described provides a simple and effective SPE method for the extraction of basic or neutral drugs from human plasma. The Limit of Quantitation (LOQ) of the combined SPE and LC/MS/MS analysis was 1.0 ng/mL. The internal standard for the application was 50 ng/mL quetiapine.

Recoveries were calculated from a second order regression with RSD values based on a sampling of n = 6. Excellent recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995 (n = 6). To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). Figure 1 shows the chromatograms of the extractions at 100 ng/mL. As shown in Table 1, the extractions produced reproducibly high recoveries.

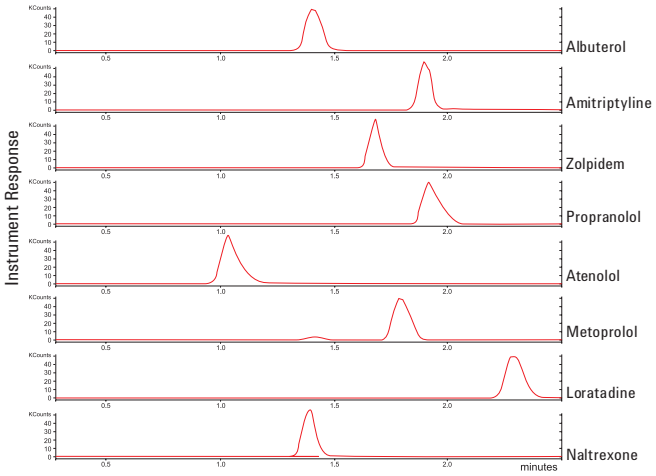

Figure 1. Chromatograms of a 100 ng/mL human plasma extract.

Table 1. High Recoveries of Basic Drugs with Bond Elut Plexa

Drug	log P	pKa	0.5 µg/mL %Recovery	%RSD	1.0 µg/mL %Recovery	%RSD
Albuterol	1.3	10.3	95	5	100	2
Amitriptyline	4.6	9.4	100	10	100	4
Zolpidem	3.9	6.2	100	8	103	2
Propranolol	3.6	9.5	102	6	101	6
Atenolol	1.3	9.6	97	4	101	4
Metoprolol	1.3	10.8	100	5	100	5
Loratadine	5.2	4.9	97	5	95	3
Naltrexone	1.8	9.2	103	11	100	4

Conclusions

Bond Elut Plexa is a useful tool for high-throughput SPE applications that require analysis at low analyte levels, need validated reproducibility, and must be quickly implemented with minimal method development. A single method for basic analytes covers a broad range of analyte polarites and delivers reproducibly high recoveries. Bond Elut Plexa is therefore highly recommended for bioanalytical work in pharmaceutical clinical research trials, including contract research.

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Agilent Technologies

Fractionation of Acidic, Neutral and Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Clinical Research

Authors

William Hudson and
Andrea Junker-Buchheit
Agilent Technologies, Inc.

Introduction

Bioanalytical SPE has been dominated by polymeric sorbents in recent years. The ease-of-use, good flow, and resistance to effects of drying relative to silica-based sorbents make polymeric sorbents an obvious choice for high volume, high throughput assays requiring quick validation and minimal method development. Mixed mode polymers are often preferred among polymeric sorbents for basic drugs which take advantage of the cation exchange properties for an efficient extraction. In some drug studies the analyst may need to extract multiple drug classes in a single extract due to limited sample size. A mixed mode polymer is an effective way to analyze multiple drug classes in a single plasma sample. Acidic and neutral drugs can be retained on the hydrophobic portion while basic drugs interact with the sorbent's cation exchange properties. Each drug class can then be fractioned off the sorbent using organic solvents and changing the pH to elute the compounds of interest.

Bond Elut Plexa PCX is a new addition to the Plexa family and uses a mixed mode polymer cation exchange technique. This advanced SPE sorbent retains neutral and acidic compounds from biofluids via hydrophobic interactions and concentrates basic analytes due to ion-exchange capabilities. A single method is sufficient to fractionate different classes of compounds at high recoveries in clean extracts. Acidic and neutral compounds are eluted in a neutral fraction, while basic compounds elute in a basic fraction.

Plexa PCX significantly reduces ion suppression because its highly polar, hydroxylated surface is entirely amide-free. The particle exterior minimizes protein access to the pore structure and avoids strong binding of phospholipids ensuring reduced ion suppression. A simple method utilizing the new Plexa PCX was developed for the extraction of acidic, neutral and basic drugs in human plasma.

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Materials and Methods

Table 1. SPE Reagents and Solutions

2% Phosphoric Acid	Add 20 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_3 Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	100 µL human plasma. Dilute 1:3 with 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Load	Sample with the drug mixture at the flow rate of 1 mL/min
Wash	500 µL 2% formic acid
Elution 1 (acids, neutral)	500 µL methanol:acetonitrile (1:1, v/v)
Elution 2 (bases)	500 µL 5% NH_3 methanol:acetonitrile (1:1, v/v)

All samples evaporated to dryness and reconstituted in 100 µL of 5 mM ammonium formate (acids and neutrals), or 100 µL of 80:20 0.1% Aq formic acid: CH_3OH (bases).

Results and Discussion

Acids

LC Conditions - Acids and Neutrals

Mobile Phase: A: 5 mM Ammonium Formate
B: Methanol

Gradient: t = 0 min 60% A: 40% B
t = 0-1 min 20% A: 80% B
t = 2-3 min 60% A: 40% B

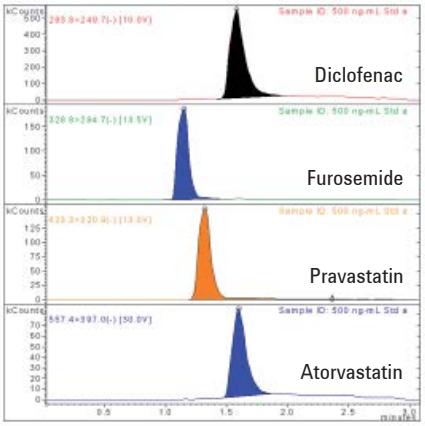
Column: Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)

MS Conditions Acids

Compound	Q1	Q3	CE
Atorvastatin	557.4	397.0	30.0V
Diclofenac	293.8	249.7	10.0V
Furosemide	328.8	284.7	13.5V
Pravastatin	423.3	320.9	13.0V

Capillary = 80 V, Dry gas temp = 350 °C, 30 psi, CID = Argon

Polarity: Negative



Chromatograms of a 50 ng/mL extract

Acid analytes are retained on Plexa PCX via hydrophobic interaction at a pH below their pKa values. The limit of detection (LOD) of the combined solid phase extraction and LC-MS-MS analysis was 1.0 ng/mL. Recoveries were calculated from a 1st order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 5.0 µg/mL with correlation coefficients all above 0.999.

To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 3, the described generic SPE protocol yields reproducibly high recoveries.

Table 3. Analyte Relative Recoveries - Acids

			0.5 µg/mL		1.0 µg/mL	
	log P	pKa	Rec %	RSD	Rec %	RSD
Diclofenac	4.2	4.2	101	4	103	6
Furosemide	1.2	3.9	104	3	96	2
Pravastatin	2.6	4.7	95	4	106	6
Atorvastatin	6.3	4.5	100	4	103	5

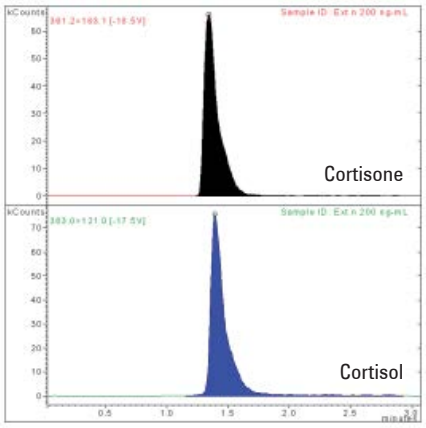
Neutrals

MS Conditions - Neutrals

Compound	Q1	Q3	CE
Cortisone	361.2	163.1	-18.5V
Cortisol	363.2	121.0	-17.5V

Capillary = 80 V, Dry gas temp = 350 °C, 30 psi, CID = Argon

Polarity: Positive



Chromatograms of a 50 ng/mL extract

Neutral compounds have a similar retention behavior as non-dissociated acid compounds and are therefore eluted in the neutral fraction. The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 5.0 µg/mL with correlation coefficients all above 0.998.

To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 4, the extractions according to the generic protocol with Plexa PCX produced reproducibly high recoveries.

Table 4. Analyte Relative Recoveries - Neutrals

			0.5 µg/mL		1.0 µg/mL	
	log P	pKa	Rec %	RSD	Rec %	RSD
Cortisone	1.5	N/A	93	4	97	6
Cortisol	1.5	N/A	101	4	101	4

Bases

LC Conditions - Bases

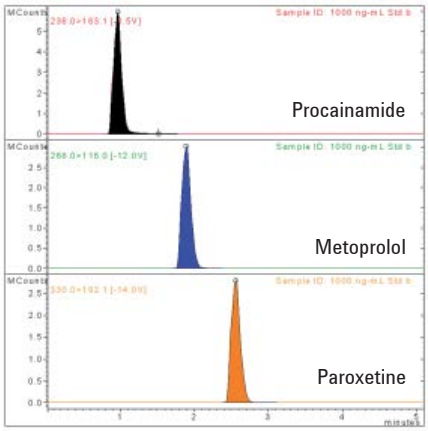
Mobile Phase:	A:	0.1% Formic Acid
	B:	Methanol
Gradient:	t = 0 min	80% A : 20% B
	t = 0-2 min	20% A : 80% B
	t = 3.5-5 min	80% A : 20% B
Column:	Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)	

MS Conditions - Bases

Compound	Q1	Q3	CE
Procainamide	236.0	163.1	-8.5V
Metoprolol	268.0	116.0	-12.0V
Paroxetine	330.0	192.1	-14.0V

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon

Polarity: Positive



Chromatograms of a 50 ng/mL extract

Basic analytes from human plasma samples are retained by the cation exchange interactions with the sorbent and elute separately utilizing an ammoniated solvent system. The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from

1.0 ng/mL to 5.0 µg/mL with correlation coefficients all above 0.999. To demonstrate reproducibility, samples were analyzed at two different concentrations (n = 6). As shown in Table 5, reproducibly high recoveries were obtained according to the generic standard protocol.

Table 5. Analyte Relative Recoveries - Bases

			0.5 µg/mL		1.0 µg/mL	
	log P	pKa	Rec %	RSD	Rec %	RSD
Procainamide	1.3	9.2	100	5	98	3
Metoprolol	1.9	9.6	94	4	92	6
Paroxetine	3.4	9.9	94	5	99	4

Conclusions

With Bond Elut Plexa PCX, a generic protocol for drug extraction from plasma can be applied to analytes which belong to different chemical classes of drugs. Under acidic conditions, charged basic analytes bind to the cation exchange groups of the sorbent whereas the neutralized acidic and neutral compounds are retained in the more hydrophobic center of the polymer bead. As the non-polar retention mode in SPE is less selective than ion exchange, the polar interferences and proteins as well as ion suppression effects in LC/MS analysis must be minimized by a wash step with an acidic, aqueous solution. An elution with 50% methanol:acetonitrile is sufficient to achieve high recoveries and a clean extract for the acidic and neutral compounds. Finally, a mixture of organic solvents with ammonia is used to disrupt the cation exchange interaction, resulting in the elution of the basic drugs.

Plexa PCX particles have much narrower particle size distribution creating more consistent interstitial paths. The consistent Plexa particle size results in superior flow characteristic across the 96-well plate and excellent well-to-well reproducibility. Automated 96-well technology is simplified opening new opportunities to maximize efficiency. Bond Elut Plexa PCX is a useful tool for high-throughput SPE applications which require analysis at low concentration levels, validated reproducibility and quick implementation. Minimal method development is needed with a wide range of different compounds. Plexa PCX is highly recommended for multiple compounds in bioanalytical work and systematic toxicological analysis.

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Agilent Technologies

Extraction of Non-Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Clinical Research

Authors

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Andrea Junker-Buchheit
Agilent Technologies, Inc.

Introduction

Bioanalytical methods for pharmaceutical analysis require quick and easy method development and validation to reduce bottlenecks in drug development. Biological samples can be complicated to analyze due to proteins, peptides, salts, phospholipids and other endogenous compounds. Sample clean-up is necessary to remove these inferences without significant loss of the target analytes. Solid phase extraction utilizing simplified methodologies for routine analysis are the techniques of choice.

Bond Elut Plexa PCX is a new addition to the Plexa family and uses a polymer cation exchange technique. Plexa PCX utilizes a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes resulting in improved analytical performance and sensitivity in the quantitation of basic compounds. In addition, faster and highly reproducible flow rates are the norm, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX significantly reduces ion suppression because its highly polar, hydroxylated surface is entirely amide-free. The particle exterior excludes proteins and avoids strong binding of phospholipids. Thus, efficient removal of phospholipids from plasma is ensured. A simple generic method was developed for the extraction and analysis of non-polar basic compounds in human plasma.

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Materials and Methods

Table 1. SPE Reagents and Solutions

2% Phosphoric Acid	Add 20 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_3 Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	100 µL human plasma. Dilute 1:3 with 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Load	Sample with the drug mixture at the flow rate of 1 mL/min
Wash 1	500 µL 2% formic acid
Wash 2	500 µL acetonitrile:methanol (1:1, v/v)
Elution	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient:
t = 0 min 80% A : 20% B
t = 0-2 min 20% A : 80% B
t = 3.5-5 min 80% A : 20% B

Column: Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Ranitidine	315.0	176.0	-21.0V
Propranolol	260.1	116.0	-17.5V
Amitriptyline	278.1	233.0	-17.0V
Loratadine	383.1	337.0	-31.0V

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon

Polarity: Positive

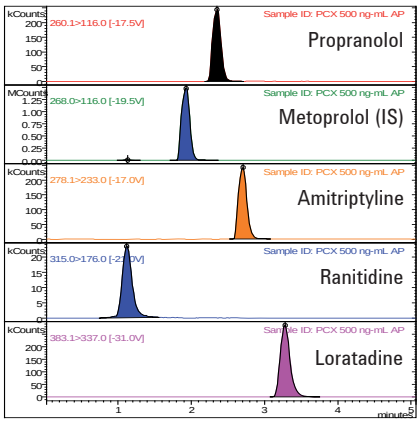

Figure 1. Chromatograms of a 50 ng/mL extract

This LC/MS method describes the quantitative determination of non-polar basic compounds in human plasma using Bond Elut Plexa PCX for SPE (Figure 1). The Limit of Detection (LOD) of the solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6. Excellent recoveries were achieved, demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.999.

To demonstrate reproducibility, samples were analyzed at two different concentrations (n = 6). As shown in Table 3, reproducibly high recoveries were obtained according to the generic standard protocol.

Table 3. Recoveries of non-polar basic compounds from human plasma

Analyte	log P	pKa	% Rec (500 ng/mL)	% RSD[2]	% Rec (1000 ng/mL)	% RSD[2]
Ranitidine	1.9	8.2	101	5	94	6
Propranolol	3.6	9.5	97	7	92	4
Amitriptyline	4.6	9.4	95	5	91	5
Loratadine	5.2	9.3	100	4	91	4

[1]Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.
[2]RSD = standard deviation/average recovery x 100; n = 6.

Conclusions

With Bond Elut Plexa PCX, it is possible to use a single method for the extraction of non-polar basic analytes from plasma that delivers reproducibly high recoveries. Under acidic conditions, the charged analyte binds to the cation-exchange groups of the sorbent (see Table 3 for pKa). Polar interferences and proteins are washed away with an acidic, aqueous solution. A neutral wash with relatively strong solvents, such as 50% methanol:acetonitrile, is possible without loss of analyte. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, a mixture of organic solvents with ammonia is used to disrupt the cation exchange interaction, resulting in the elution of the basic drugs.

Flow rate over the 96-well plate is fast because Plexa PCX particles have much smaller interstitial paths with no fines to cause blockages, resulting in high well-to-well reproducibility. Automated 96-well technology is convenient which opens new opportunities to maximize efficiency. Bond Elut Plexa PCX is therefore a useful tool for high-throughput SPE applications which require analysis at low analyte levels, need validated reproducibility, and that must be quickly implemented with minimal method development. It is highly recommended for bioanalytical work in pharmaceutical clinical research trials, including contract research.

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Agilent Technologies

Extraction of Polar Basic Drugs from Plasma with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Clinical Research

Authors

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Agilent Technologies, Inc.

Introduction

Basic pharmaceutical drugs are ideal for a cation exchange sorbent. Analytes are easily charged in an acidic solution and readily interact with the ion exchange function of the sorbent. Polar basic compounds can be problematic for reverse phase sorbents due to their poor hydrophobic interaction and water solubility.

Bond Elut Plexa PCX is a new addition to the Plexa family and uses a polymeric cation exchange technique. Plexa PCX uses a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantification of basic compounds.

In addition, Plexa PCX offers faster and highly reproducible flow rates, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX significantly reduces ion suppression because its highly polar, hydroxylated surface is entirely amide-free. The particle exterior minimizes strong binding of proteins and phospholipids. Efficient removal of phospholipids from plasma is ensured. A simple generic method was developed for the extraction of polar basic drugs in human plasma.

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Materials and Methods

Table 1. SPE Reagents and Solutions

2% Phosphoric Acid	Add 20 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_3 Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonia to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	100 µL human plasma. Dilute 1:3 with 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Load	Sample with the drug mixture at the flow rate of 1 mL/min
Wash 1	500 µL 2% formic acid
Wash 2	500 µL acetonitrile:methanol (1:1, v/v)
Elution	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient:
t = 0 min 80% A : 0% B
t = 0-2 min 20% A : 80% B
t = 3.5-5 min 80% A : 20% B

Column: Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Albuterol	240.1	148.0	-23.5V
Lamotrignine	256.0	256.0	-5.0V
Atenolol	267.0	145.0	-34.0V
Sumatriptan	296.1	201.1	-14.0V

Capillary = 25 V, Dry gas temp = 400 °C, 30 psi, CID = Argon

Polarity: Positive

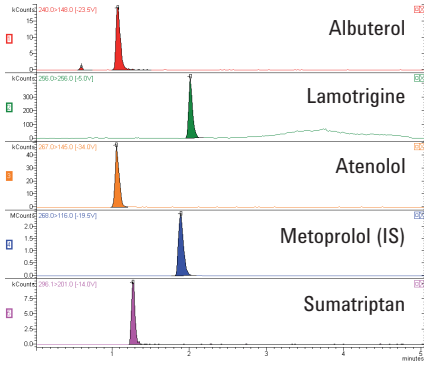

Figure 1. Chromatograms of a 50 ng/mL extract

This LC/MS method describes the quantitative determination of polar basic compounds in human plasma using Bond Elut Plexa PCX for SPE (Figure 1). The limit of detection (LOD) of the solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 2nd order regression with RSD values based on a sampling of n = 6.

Excellent recoveries were achieved, which demonstrated good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.999. To demonstrate reproducibility, samples were analyzed at two different concentrations (n = 6). As shown in Table 3, reproducibly high recoveries were obtained according to the generic standard protocol.

Table 3. Recoveries of polar basic compounds from human plasma

Analyte	log P	pKa	% Rec (500 ng/mL)	% RSD[2]	% Rec (1000 ng/mL)	% RSD[2]
Sumatriptan	0.96	9.6	95	5	97	4
Atenolol	1.30	9.6	94	3	91	2
Albuterol	1.30	10.3	95	5	100	7
Lamotrigine	1.50	5.7	92	3	97	4

[1]Recoveries calculated as % of signal intensity of an extracted sample compared to that calibration curve.
[2]RSD = standard deviation/average recovery x 100; n = 6.

Conclusions

With Bond Elut Plexa PCX, a generic drug extraction protocol from plasma can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent (see Table 3 for pKa). Polar interferences and proteins are washed away with an acidic, aqueous solution. A neutral wash with relatively strong solvents, such as 50% methanol:acetonitrile, is possible without any loss of analyte. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, a mixture of organic solvents with ammonia is used to disrupt the cation exchange interaction, resulting in the elution of the basic drugs.

Flow rate all over the 96 well plate is fast because Plexa PCX particles have a much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent well-to-well reproducibility. Automated 96 well technology is easily possible, which opens up new opportunities to maximize efficiency. Bond Elut Plexa PCX is therefore a useful tool for high throughput SPE applications, which require analysis at low analyte levels, validated reproducibility and quick implementation, with minimal method development. It is therefore highly recommended for bioanalytical work in pharmaceutical clinical research trials, including contract research.

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Analysis of Blood Serum on the Liberty Series II ICP OES with the Axially-Viewed Plasma

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Andrew Ryan

Introduction

The role of major, minor and trace levels of elements in human health has been an important area of scientific research.

The advent of atomic absorption (AA) techniques and the development of the graphite tube atomizer (GTA) has provided the means for accurate determination of all levels of many elements in human body fluids. An advantage of the graphite furnace is the small sample consumption in the determination of trace levels. Disadvantages of flame AA are that releasing agents or modifiers are necessary and careful control of the flame stoichiometry is important to overcome chemical interferences [1]. While the atomic absorption technique offers adequate performance, in most cases it is a single element technique and is therefore slow.

The inductively coupled plasma mass spectrometer (ICP-MS) offers rapid, highly sensitive, multi-element determinations. The high sensitivity of ICP-MS means that samples can be diluted to give a reasonable working volume. Dilution is also required for ICP-MS because of limitations imposed by the sample matrix. Typically with ICP-MS, an upper total dissolved solids (TDS) limit of 0.2% in the solution should not be exceeded to ensure continuous operation for an extended period [2]. At TDS levels in excess of this limit, unacceptable levels of signal instability are commonly experienced.

Inductively coupled plasma optical emission spectrometry (ICP-OES) also offers rapid, multi-element determinations. The sensitivity of ICP-OES is lower than that of either ICP-MS or AA-GTA, but ICP-OES can handle higher levels of TDS than ICP-MS and is much faster than AA-GTA. Since ICP-OES is able to analyze samples with higher TDS, more concentrated solutions can be prepared allowing trace elements to be measured. A disadvantage of ICP-OES for the determination of trace elements is that sample volumes will often be small and sample consumption for ICP-OES is

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typically about 1–2 mL/min. The use of a microconcentric nebulizer (MCN) is a convenient way to reduce the sample consumption. Such nebulizers are obtainable from several suppliers. For example, Glass Expansion Pty Ltd supply MCNs with free aspiration uptake rates ranging from 100 to 800 µL/min. A Glass Expansion MCN with a free aspiration uptake rate of 400 µL/min was used in this work.

This report describes the analysis of blood serum using standard quantitative calibration with aqueous standards. Viscosity effects of the blood serum solutions were corrected using scandium (361.384 nm—ionic line) as an internal standard. Major, minor and trace elements were determined in a single analysis.

A major element in blood serum is sodium, which is an easily ionized element (EIE) and has been reported to cause ionization interference when present in reasonably high levels. Ionization interference tends to cause a reduction in signal intensity with increasing concentration of EIE and the effect is prominent at interferent concentrations at or above 100 mg/L. The atomic lines of Na, K and to a lesser extent Ca (422.673 nm) exhibit signal enhancement with increasing concentrations of EIE. The effect can be easily minimized or eliminated on a radially-viewed ICP-OES by adjusting the viewing height. For the more sensitive axially-viewed ICP-OES, many reports of interferences due to EIE have been described [3–5]. Reducing the nebulizer pressure and increasing the RF power has been reported to reduce ionization interference on the axially-viewed ICP-OES [3]. Scandium as an internal standard has also been found to compensate for part of the signal depression [4,5]. Generally, when analyzing samples that contain high levels of EIE, it is recommended that all standards have similar levels of EIE added (matrix matching).

An alternative is to saturate the plasma with a high concentration of another EIE such as cesium. Therefore, the effect of adding cesium as an ionization buffer to the standards and samples was also investigated.

Cesium was chosen as an ionization buffer as it has a low energy of ionization, is not very sensitive by ICP-OES and, therefore, spectral interference is generally not a problem. Cesium chloride is available in a very pure form and does not build up in the torch injector tube as readily as other alkali salts.

The accuracy and validity of the method was assessed by the use of Nycomed Pharma "Seronorm Trace Elements Serum batch no. 311089".

Experimental

Instrumental

An Agilent Liberty Series II ICP-OES with the axially-viewed plasma was used for the analysis.

The Liberty Series II ICP features a 40 MHz free running RF generator, a 0.75 m Czerny-Turner monochromator with a 1800 grooves/mm holographic grating used in up to four orders. The resolution of the optical system ranges from 0.018 nm in the 1st order to 0.006 nm in the 4th order.

The instrument was controlled with a Digital Equipment Corporation (DEC) Venturis computer with an Intel Pentium processor and Agilent Plasma 96 software running under Microsoft Windows 95 operating system.

The instrument operating conditions are listed in Table 1.

Table 1. Instrument Operating Conditions

Power	1.0 kW
Plasma gas flow	15.0 L/min
Auxiliary gas flow	1.5 L/min
Spray chamber type	Glass cyclonic
Torch	Standard axial torch with 2.3 mm id injector
Nebulizer	High flow microconcentric nebulizer (Glass Expansion Pty Ltd), free aspiration uptake rate 400 µL/min
Nebulizer pressure	300 kPa
Pump tube	Inlet - PVC, orange-green, 0.38 mm id Cs solution inlet, orange-blue, 0.25 mm id Outlet - PVC, black-black, 0.76 mm id
Pump speed	15 rpm
Sample uptake rate	160 µL/min
Integration time	3 seconds for Ca, Cu, Fe, K, Mg, Na, P, S and Zn 5 seconds for Al and Mn
No. of replicates	3
Sample delay time	20 seconds
Stabilization time	15 seconds
Fast pump	On
Upward curvature limit	125%
Background correction	Polynomial plotted background for Ca, Cu, Fe, K, Mg, Na, P, S and Zn
	Offpeak background correction for Al and Mn
	0.015 nm left of peak
	0.015 nm right of peak
PMT voltage	650 V

For the determination of sulfur, an Auxiliary Gas Module-2 (AGM-2) is required. The AGM-2 provides a nitrogen purge for the monochromator to extend the working wavelength range from 189 nm down to 175 nm. The default grating order was used for all lines with the exception of the Al 396.152 nm line where the order was changed from 1st to 2nd order because of the presence of spectral interference from the blood serum matrix.

Standard Preparation

Aqueous standards were prepared from Custom-Grade Multi-element Solution Var Cal 2 (Inorganic Ventures, Inc.) and from 1,000 mg/L and 10,000 mg/L single element standards (Spectrosol, BDH Chemicals). The standards were made up in 18 MΩ Milli-Q water with 1% v/v high purity HNO_3 (Mallinckrodt, AR SELECT PLUS) and 0.01% v/v Triton X100 prepared from a 1% w/v Triton X100 solution. Scandium was added to each solution as an internal standard with a final concentration of 0.5 mg/L.

The following calibration standards were prepared.

Table 2. Calibration Standards

Standard No.	Concentration (mg/L)
Standard 1	20 µg/L Al, Cu, Fe, Mn and Zn 1.3046 mg/L P 6.6752 mg/L S
Standard 2	100 µg/L Al, Cu, Fe, Mn and Zn 6.5228 mg/L P 33.376 mg/L S
Standard 3	0.4 mg/L Ca and K 0.1 mg/L Mg 10 mg/L Na
Standard 4	2 mg/L Ca and K 0.5 mg/L Mg 50 mg/L Na
Standard 5	10 mg/L Ca and K 2.5 mg/L Mg 250 mg/L Na (for Na 330.237 nm line only)

Rinse and calibration blank solutions were prepared from 18 MΩ Milli-Q water with 1% HNO_3 and 0.01% Triton X100

Sample Preparation

Solutions were prepared from Seronorm Trace Elements Serum, batch no. 311089.

The serum was reconstituted by removing the screw cap and carefully lifting the rubber stopper—without removing it completely. Air was allowed to enter the vial through the grooves on the lower part of the stopper. The stopper was removed and 3.00 mL of 18 MΩ Milli-Q water was added. Care must be taken when removing the stopper to avoid loss of dried material. The vial was closed and allowed to stand for 30 minutes. The contents were completely dissolved by swirling gently. Shaking of the vial will result in the formation of foam. Long term contact between the liquid and the rubber stopper should be avoided, particularly for the determination of zinc or aluminium, to prevent contamination from the rubber stopper.

Three solutions with dilution factors of 5, 20 and 100 were prepared in 1% HNO_3 and 0.01% Triton X100.

Scandium was added to each solution as an internal standard with a final concentration of 0.5 mg/L.

For the study of the effect on the addition of cesium as an ionization buffer, 2% w/v Cs as CsCl was added online to all solutions by pumping the solution into a "T" piece just before the nebulizer. The optional three channel pump was utilized with one channel used to introduce the cesium solution. It is possible to add the internal standard to the cesium solution instead of each individual solution.

Results and Discussion

Wavelength Selection

Wavelength selection was based on the sensitivity of the line and the concentration of elements in each of the solutions. For most lines, spectral interference did not appear to be a major problem.

Ionic and atomic lines were selected for Ca and Mg so the effect of ionization interference on the two emission line types could be observed. Any variation in the results would also show the presence of spectral interference.

The K 766.490 nm line is known to be subject to spectral interference from Mg, so the K 769.896 nm line was also selected. The concentration of K in this sample was approximately 8.5 times that of Mg, and consequently the Mg spectral interference on the K 766.490 nm line was expected to be negligible. This expectation was confirmed when similar results were found for K at both lines.

For Cu, the 324.754 nm or 327.396 nm emission lines are generally used, although the 327.396 nm line is preferred. For both Cu lines, a small OH emission line from the aqueous matrix is observed and is more prominent with the axially-viewed plasma than with the radially-viewed plasma. The OH emission line is not resolved from the 324.754 nm line, which is used in the 2nd order (default or recommended setting), whereas the OH emission line is almost completely resolved from the 327.396 nm line, which is used in the 1st order.

Figures 1 and 2 show the wavelength scans for the Cu 327.396 nm and 324.754 nm emission lines in blood serum diluted by a factor of 20.

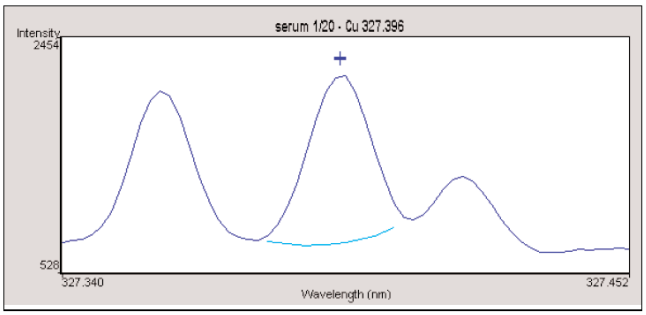

Figure 1. Wavelength scan for Cu 327.396 nm in the 1st order.

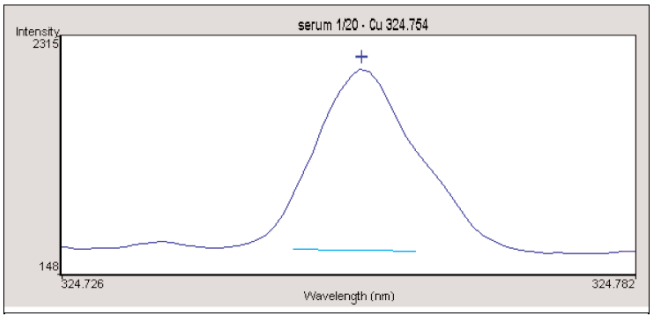

Figure 2. Wavelength scan for Cu 324.754 nm in the 2nd order.

The peak to the left of Cu (327.396 nm) is Sc and causes no problems as it is completely resolved. The tail of the OH peak does contribute slightly to the signal of the Cu (327.396 nm) but this has been successfully corrected using polynomial plotted background correction.

There is also the option of using the 327.396 nm line in the 2nd order where the resolution will be improved by a factor of 2. The sensitivity of the line is only reduced by approximately 30% when using it in second order and the peaks are completely resolved.

The Cu 327.396 nm line in the 1st order was selected for the analysis.

Blood Serum Analysis

The analysis consisted of a single Seronorm Trace Elements Serum, batch no. 311089, that was diluted 100-fold for the determination of Ca, Mg, Na and K, 20-fold for the determination of Ca, Mg, Na, K, Cu, Fe, P, S and Zn and 5-fold for the determination of Al and Mn. The elements Ca, Mg Na and K were determined in the 100 and 20-fold dilution blood serum solutions because any variation in the result would indicate the presence of ionization interference.

The analysis was repeated with the addition of Cs as an ionization buffer. The Cs was added by pumping CsCl solution (2% w/v Cs) into a "T" piece just before the nebulizer.

Blood serum contains high levels of sodium and the potential for ionization interference is high. Matrix matching, such as having equal amounts of Na in all solutions, would mean that any signal enhancement or suppression because of ionization interference would be the same for all solutions. To measure major, minor and trace levels of elements in blood would then require multiple analyses because blood serum solutions of various dilution factors would be necessary with matrix matched standards to be prepared for each. The aim of this work was to show that the effect of ionization interference could be overcome, and therefore allow all levels of elements to be determined in a single analysis.

Figures 3–10 represent the calibration graphs for the standard solutions displayed in Table 2, with and without the addition of cesium. Standards 3, 4 and 5 contained varying levels of Ca, K and Na. Sodium was present in concentrations high enough for ionization interference to have considerable influence on the signals of the other elements in the standard solutions.

Figures 3– 6 show the effect of the varying levels of ionization interference, because of the varying EIE concentration between solutions, on the atomic lines of K (766.490 nm and 769.896 nm), Na (589.592 nm and 330.237 nm) and Ca (422.673 nm) as signal enhancement has produced upward curvature of the calibration. The addition of Cs nullified the effect of the varying levels of ionization interference, producing a more linear calibration. Adding Cs instead of matrix matching allows all elements to be determined in a single analysis because sample solutions with varying dilution factors, and varying concentrations of EIE, can be analyzed.

An ionic line for Ca (317.933 nm) was also used and upward curvature of the calibration was not found. The calibration for Mg (285.213 nm) atomic line exhibited little, if any, upward curvature as did the remaining atomic and ionic analyte lines. This is consistent with other reports [4,5] that the atomic lines of group I and to a lesser extent, group II elements,

exhibit signal enhancement with increasing levels of EIE. The atomic lines of other elements and all ionic lines tend to exhibit signal suppression by EIE but the effect is not as severe.

In the Plasma 96 software, the maximum % error of the slope of the calibration, which is set in the calibration page of the method editor, only sets the limit of downward curvature for each specific element. The maximum % error of upward curvature is set from the switches registry (\\Varian\ICPAES\Run\Switches.reg) and is applied to all elements. The upward curvature limit was set to 125% and the calibration failed if the slope at the top of the curve was more than 125% of the slope at the bottom of the curve.

Without the addition of cesium, the upward curvature for the atomic lines of Na, K and Ca exceeded the limits and the calibration failed for these lines.

With the addition of cesium, the effect of ionization interference was reduced and all elements calibrated successfully.

Even though the calibrations for the atomic lines of Ca, K and Na failed when cesium was not added, the maximum upward curvature limit was increased post-run so that the calibrations would pass. By so doing, a comparison of the results with and without the addition of cesium could be made.

Note that Figures 3–10 show the effect of Cs on the linearity of the calibration and not the effect on the intensity of the analyte peak.

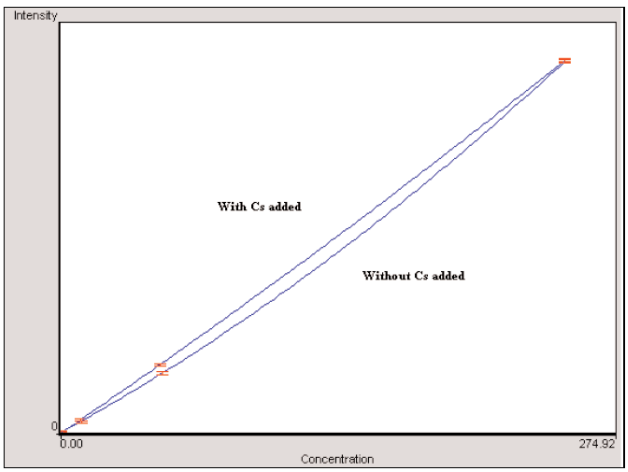

Figure 3. Calibration graph for Na 330.237 nm (atomic) with and without the addition of cesium.

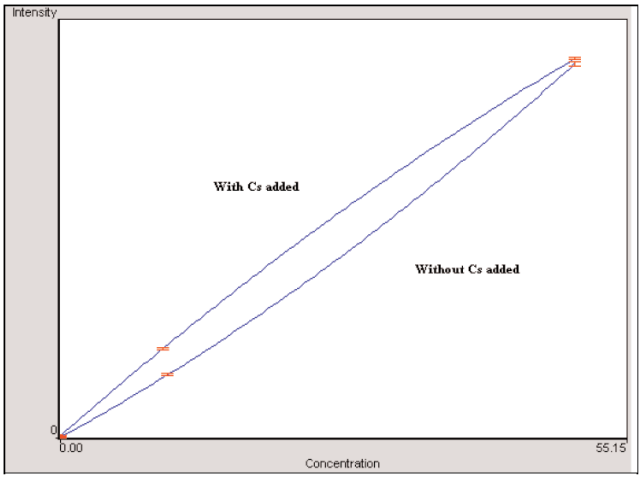

Figure 4. Calibration graph for Na 589.592 nm (atomic) with and without the addition of cesium.

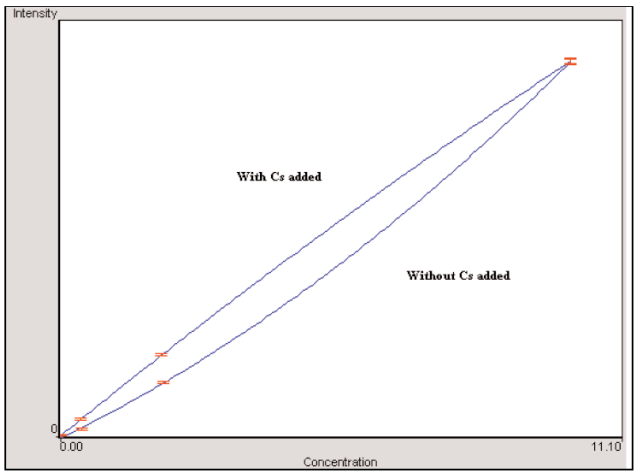

Figure 5. Calibration graph for K 766.490 nm (atomic) with and without the addition of cesium.

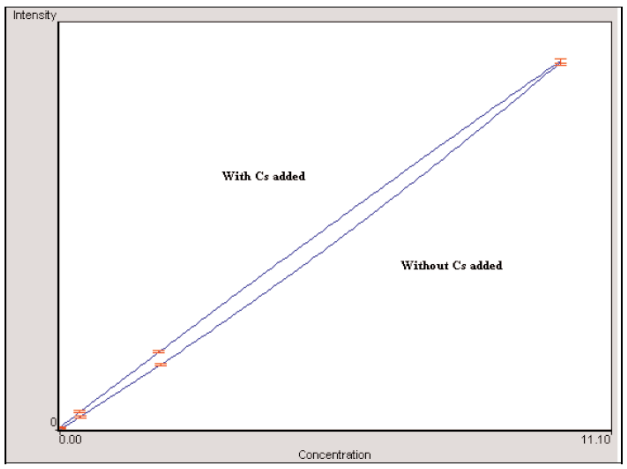

Figure 6. Calibration graph for Ca 422.673 nm (atomic) with and without the addition of cesium.

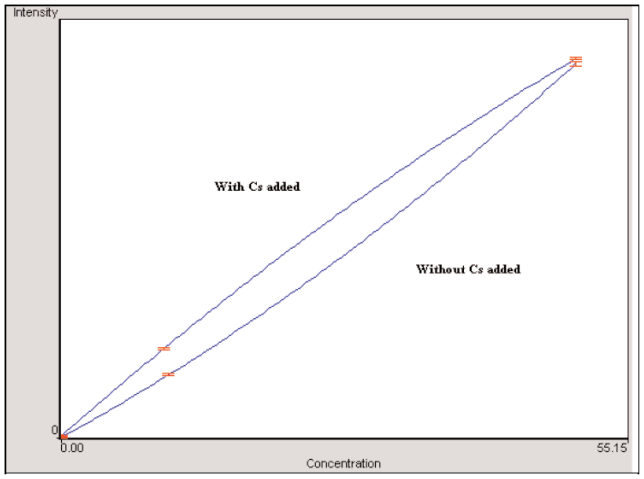

Figure 7. Calibration graph for Ca 317.933 nm (ionic) with and without the addition of cesium.

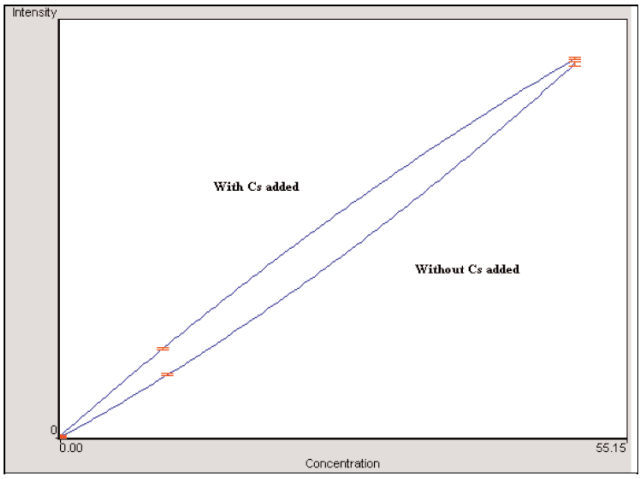

Figure 9. Calibration graph for Mg 285.213 nm (atomic) with and without the addition of cesium.

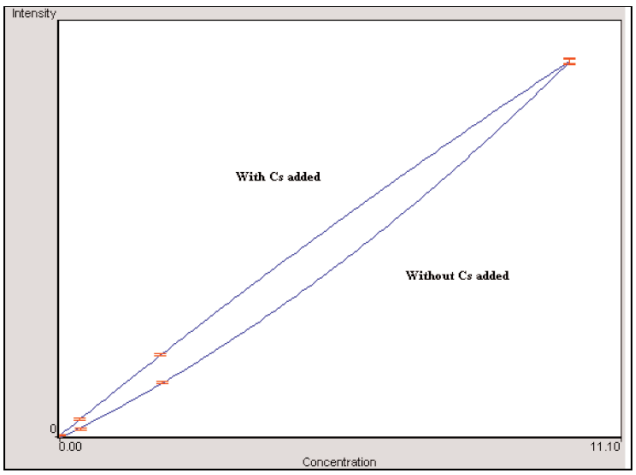

Figure 8. Calibration graph for Mg 279.553 nm (ionic) with and without the addition of cesium.

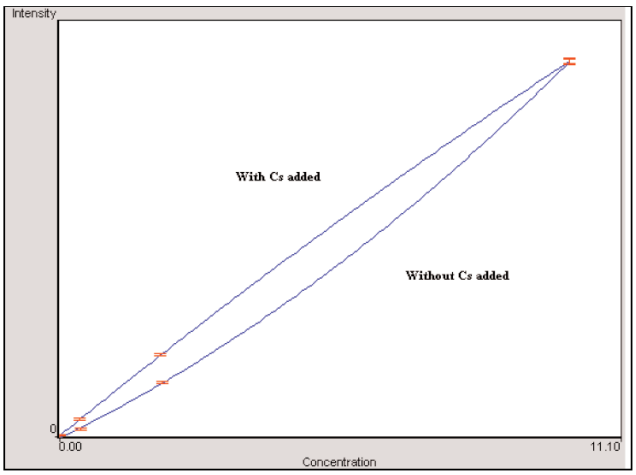

Figure 10. Calibration graph for Cu 327.396 nm (atomic) with and without the addition of cesium.

The mean results of the triplicate analyses for the determination of elements in blood serum with and without the addition of 2% w/v Cs are listed in Table 3.

Table 3. Results of the Blood Serum Analysis With and Without the Addition of Cesium

Element	Wavelength (nm)	Blood serum dilution factor	Measured value without Cs (mg/L)	Measured value with Cs (mg/L)	Certified value (mg/L)
Ca	317.933	100	94.9 ± 2.0	95.1 ± 0.8	94
Ca	317.933	20	96.4 ± 2.0	95.4 ± 1.7	94
Ca	422.673	100	94.1 ± 0.9	93.9 ± 0.7	94
Ca	422.673	20	103.2 ± 0.6	95.7 ± 1.5	94
Mg	279.553	100	19.0 ± 0.1	20.0 ± 0.2	20
Mg	279.553	20	19.3 ± 0.3	20.0 ± 0.1	20
Mg	285.213	100	19.3 ± 0.3	19.7 ± 0.2	20
Mg	285.213	20	19.9 ± 0.3	19.6 ± 0.1	20
Na	330.237	100	3101 ± 7	3151 ± 45	3080
Na	330.237	20	3314 ± 60	3307 ± 6	3080
Na	589.592	100	3305 ± 49	3166 ± 40	3080
Cu	327.396	20	1.19 ± 0.01	1.24 ± 0.01	1.27
Fe	259.940	20	1.19 ± 0.02	1.28 ± 0.04	1.3
K	766.940	100	151.2 ± 3.0	163.8 ± 2.6	168
K	766.940	20	162.8 ± 0.02	167.0 ± 0.8	168
K	769.896	100	154.5 ± 4.0	168.6 ± 3.1	168
K	769.896	20	164.3 ± 3.4	170.7 ± 0.7	168
P	213.618	20	75.5 ± 0.8	75.5 ± 1.1	–
S	180.731	20	1077 ± 6	1112 ± 17	–
Zn	213.856	20	1.51 ± 0.03	1.58 ± 0.03	1.50
Al	396.152	5	0.090 ± 0.005	0.088 ± 0.012	0.093
Mn	257.610	5	0.0073 ± 0.0003	0.0077 ± 0.0004	0.0073

The effect of ionization interference, particularly on the EIE such as K, Na and Ca, is clearly visible from the results displayed in Table 3. Without the addition of Cs, variations in the measured results were found for the atomic lines of Ca (317.933 nm), Na (330.237 nm) and K (766.940 nm and 769.896 nm). The results for the 100-fold dilution blood serum solution were lower than those measured in the 20-fold dilution blood serum solution because of the higher level of EIE in the latter, particularly Na.

The effect of ionization interference on different line types was observed for the Ca atomic line (422.673 nm) and Ca ionic line (317.933 nm). From Figures 6 and 7, it can be seen that ionization interference had considerable effect on the calibration of the Ca (422.673 nm) line while the Ca (317.933 nm) line remained unaffected. This is reflected in the results of Table 3 with varying results found for Ca (422.673 nm) and similar results found for Ca (317.933 nm) at the different dilution factors, without the addition of Cs. When Cs was added, the measured values were similar for both Ca lines and both diluted solutions.

In comparison, the effect of ionization interference on the Mg (279.553 nm) ionic line and Mg (285.213 nm) atomic line was small, but still present. Similar results were obtained for both the ionic and atomic lines of Mg at the different dilution factors, although slight enhancement of the Mg (285.213 nm) line in the more concentrated solution was observed. The addition of cesium appeared to improve accuracy of the result for the Mg (279.553 nm) ionic line, suggesting a small amount of signal suppression due to ionization interference.

Some signal depression was also observed for Cu and Fe, although it was not severe. With the addition of cesium, the determined concentrations were closer to the certified values.

With the addition of cesium, Na still appears to be affected by ionization interference when the levels of EIE are high. This is observed for the Na (330.237 nm) as a higher result was found for the more concentrated blood serum solution. It would therefore be recommended that blood serum be diluted by a least a factor of 100 when repeating the analysis to determine Na.

The determination of sodium was repeated with a 1000-fold dilution of the blood serum. Standards containing 1 and 5 mg/L Na only were prepared and the Na 589.592 nm line was used. No internal standard or ionization buffer was added. Triplicate analyses were done and the average result was 3181 mg/L in the original sample. The analysis was repeated using a standard high flow concentric nebulizer and the average result was 3170 mg/L. Both these results are similar to that obtained for Na (330.237 nm and 589.592 nm) in the 100-fold dilution blood serum solution with cesium being added.

The measured concentrations of Zn and the trace elements Mn and Al without the addition of cesium were similar to the certified values. With the addition of cesium, a slight increase in the measured concentrations of Zn and Mn were found. Although the results for Zn and Mn were slightly higher, they still compare well with the certified values.

No certified value was available for P and S in the blood serum batch that was used for the analysis. The measured results for P and S were however, very close to the certified values of another Seronorm Trace Elements Serum batch. The certified concentrations of the other elements for both serum batches varied only slightly and, therefore, the same could be assumed for P and S. These two elements did not appear to be affected by the presence of EIE as similar results were found with and without the addition of Cs.

Long Term Stability

No extensive evaluation of the long term stability was done with the microconcentric nebulizer. It appeared to operate well with no blockage being evident. Blood serum solutions diluted by a factor of 5 were aspirated continuously for periods of more than 30 minutes with no blockage being observed. Blood serum diluted 2-fold appeared to cause no problems.

A single long term stability run was done by continuously aspirating a 20-fold dilution blood serum solution and measuring the signal for a number of elements at intervals. The reproducibility of the measurements for Ca, Cu, Fe, Mg, Na, S and Zn over one hour ranged between 0.6 and 1.0 %RSD.

The replicate precision using a 3 second integration time and measuring 3 replicates ranged between 0.1 and 1.7 %RSD for all elements.

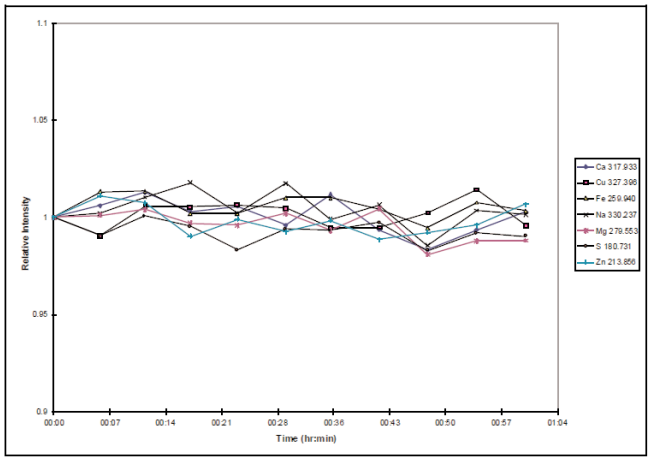

Figure 11. Signal stability over one hour for a 20-fold dilution blood serum solution.

Summary

The concentrations of major, minor and trace levels of elements in blood serum were determined in a single analysis on the Liberty Series II with the axially-viewed plasma.

Aqueous calibration solutions were used and the scandium internal standard successfully corrected for the varying viscosity of the sample. Scandium also exhibits signal suppression because of ionization interference and therefore compensates for part of the signal suppression of the other elements.

The addition of cesium as an ionization buffer considerably reduced the effect of ionization interference and the need for dilution, allowing both major, minor and trace constituents to be measured in a single analysis.

With the addition of cesium, all measured values were in very good agreement with the certified values for the Seronorm Trace Elements Serum sample, confirming the accuracy of the method.

The microconcentric nebulizer performed very well with no blockage ever occurring during the analysis of the blood serum. Sensitivity of the microconcentric nebulizer with an uptake rate of 160 µL/min was estimated as approximately half that of the standard high flow concentric nebulizer operating at an uptake rate of 1.5 mL/min. The sensitivity could have been improved by increasing the uptake rate but 160 µL/min appeared to be a good comprise between sufficient sensitivity, particularly for Al, and low sample consumption.

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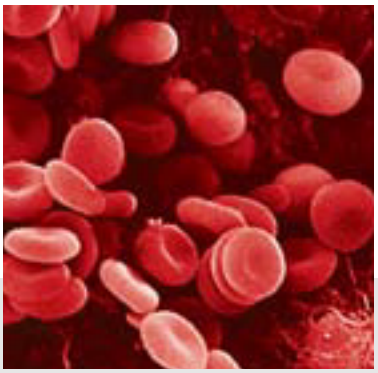

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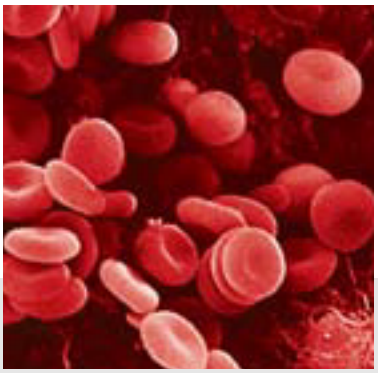

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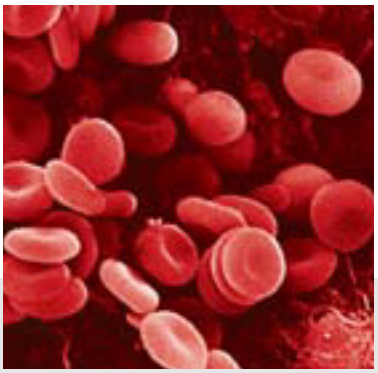

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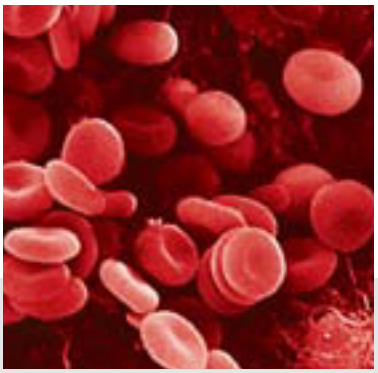

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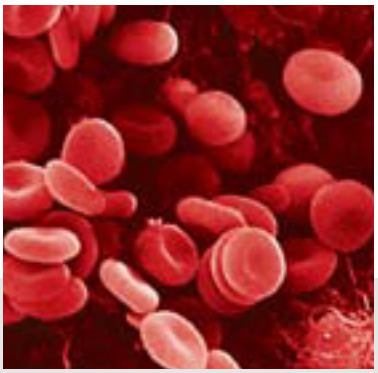

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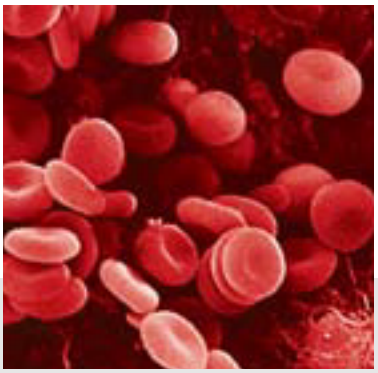

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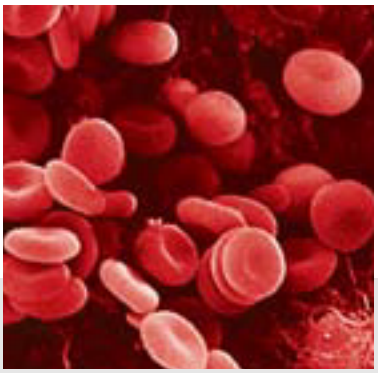

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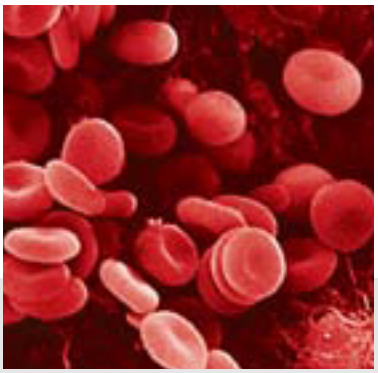

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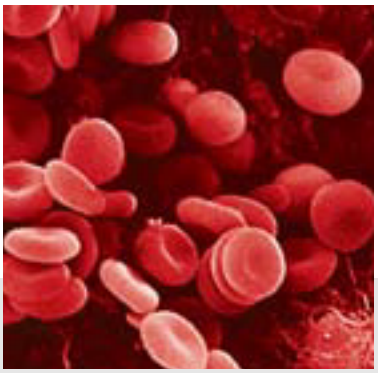

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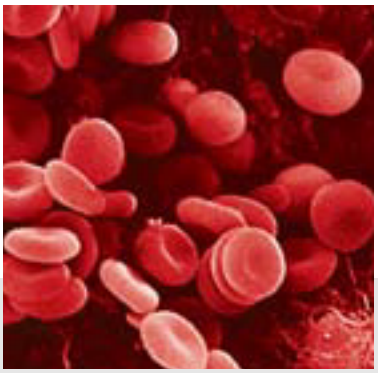

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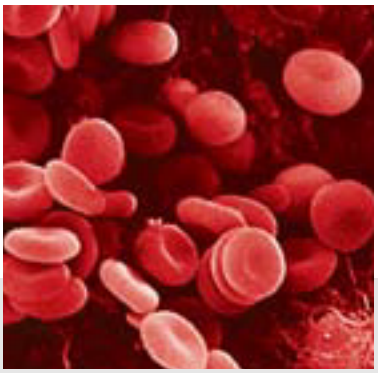

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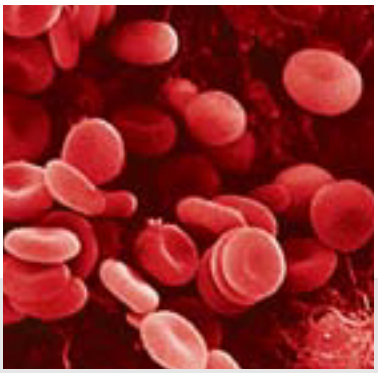

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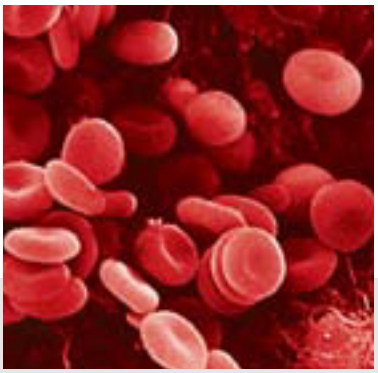
- Toxicology Screening of Whole Blood Extracts Using GC/Triple Quadrupole/MS
- Rapid Analysis of Drugs of Abuse by LC/Triple Quadrupole Mass Spectrometry
- Screening Drugs of Abuse by LC/MS



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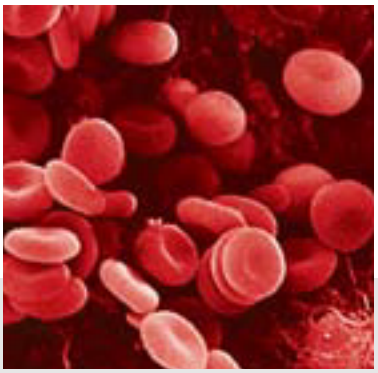
- Detection of Cannabinoids in Oral Fluid Using Inert Source GC/MS



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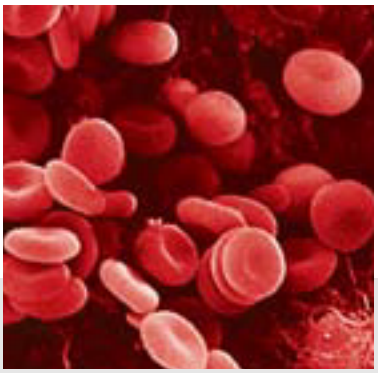

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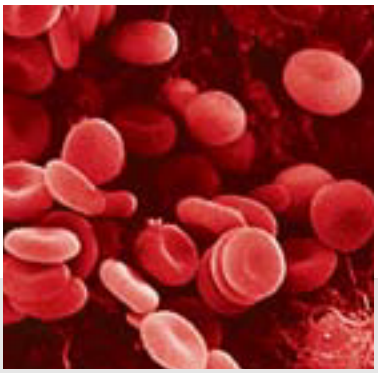
- Determination of Opiates and Metabolites in Blood Using Electrospray LC/MS



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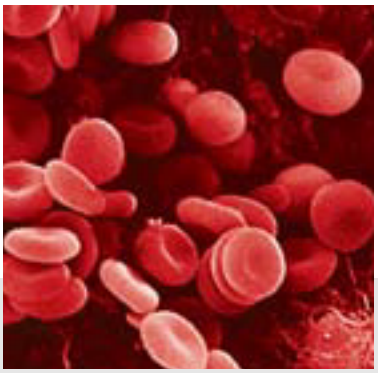

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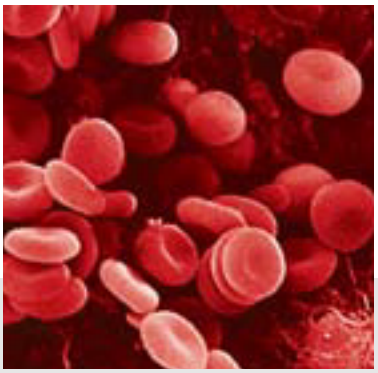

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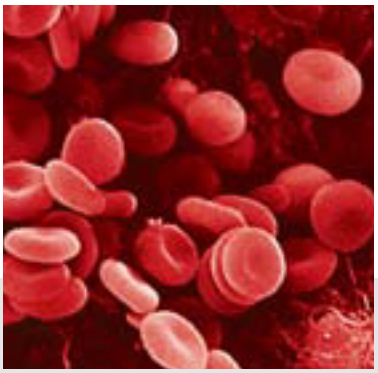

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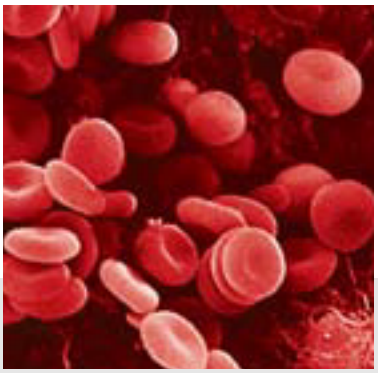

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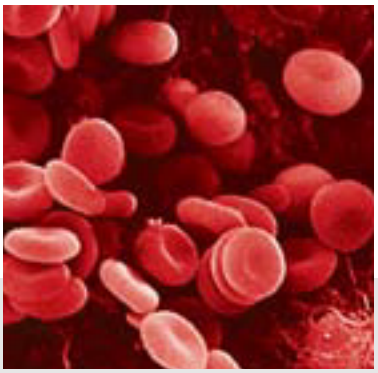

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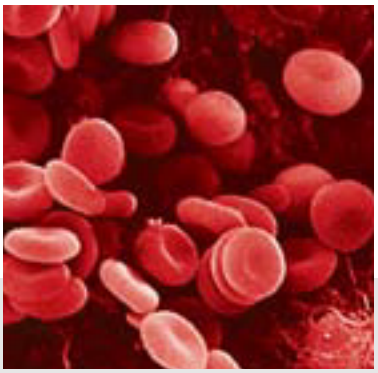

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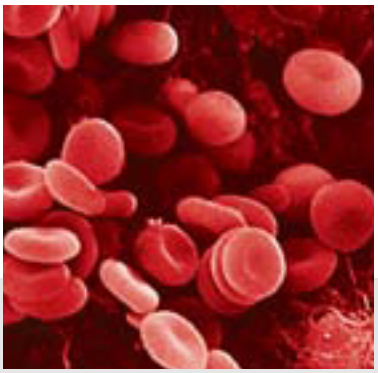

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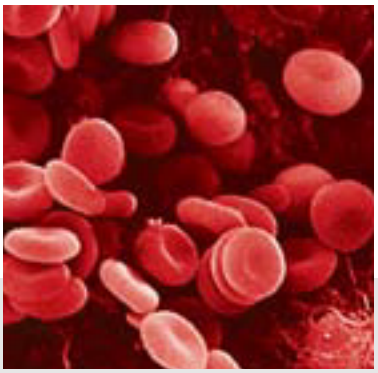

Productivity Tools

Improving Productivity and Extending Column Life with Backflush

Application Brief

Chin-Kai Meng

All Industries

A previous application note [1] has shown that multiple GC signals and MS signals can be acquired from a single sample injection. When a 3-way splitter is connected to the end of a column, column effluent can be directed proportionally to two GC detectors as well as the MSD. This multi-signal configuration provides full-scan data for library searching, SIM data for quantitation, and element selective detector data for excellent selectivity and sensitivity from complex matrices.

The system used in this study consists of a 7683ALS, a 7890A GC with split/splitless inlet, 3-way splitter, μECD, dual flame photometric detector (DFPD), and a 5975C MSD. Figure 1 shows four chromatograms from a single injection of a milk extract. The synchronous SIM/scan feature of the 5975C MSD provides data useful for both screening (full scan data) and quantitation (SIM data). DFPD provides both P and S signals without the need to switch light filters.

Noticeably in the full scan TIC in Figure 1, a significant number of matrix peaks were observed after 32 minutes. It is not uncommon to add a "bake-out" oven ramp to clean the column after analyzing complex samples. The bake-out period is used to quickly push the late eluters out of the column to be ready for the next injection. Therefore, it is common to use a higher oven temperature than required for the analysis and an extended bake-out period at the end of a normal

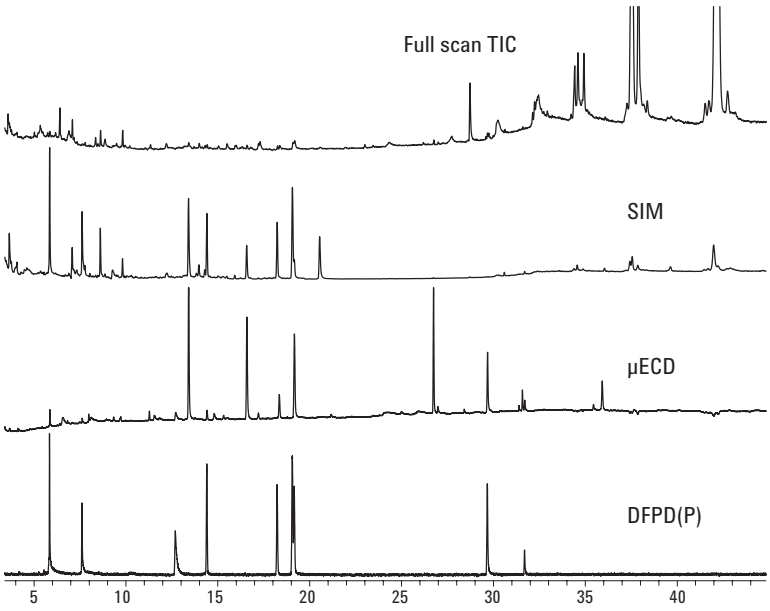

Figure 1. Four chromatograms collected simultaneously from a single injection of a milk extract.

Highlights

- Backflush – a simple technique to remove high boilers from the column faster and at a lower column temperature to cut down analysis time and increase column lifetime.
- The milk extract example shows that a 7-minute 280 °C backflush cleaned the column as well as a 33-minute 320 °C bake-out. The cycle time was reduced by more than 30%.
- Using backflush, excess column bleed and heavy residues will not be introduced into the MSD, thus reducing ion source contamination.

over program to clean out the column, which adds to the cycle time and shortens the column lifetime. Adding the bake-out period to the milk extract analysis, additional matrix peaks were observed even up to 72 minutes, while target compounds already eluted before 42 minutes. This means that 30 minutes were lost in productivity for each injection.

Backflush [2] is a simple technique to drastically decrease the cycle time by reversing the column flow to push the late eluters out of the inlet end of the column. Late eluters stay near the front of the column until the oven temperature is high enough to move them through the column. When the column flow is reversed before the late eluters start to move down the column, these late eluters will take less time and at a lower oven temperature to exit the inlet end of the column.

There are many benefits in using backflush:

- Cycle time is reduced (no bake-out period, cooling down from a lower oven temperature)
- Column bleed is reduced (no high-temperature bake-out needed), resulting longer column life
- Ghost peaks are eliminated (no high boilers carryover into subsequent runs)
- Contamination that goes into the detector is minimized, which is especially valuable for the MSD (less ion source cleaning)

Figure 2 shows three total ion chromatograms from the Agilent 7890A GC/5975C MSD. The top chromatogram is a milk extract analysis with all the target compounds eluted before 42 minutes (over program goes to 280 °C). However, an additional 33-minute bake-out period at 320 °C was needed to move the high boilers out of the column. This bake-out period was almost as long as the required time to elute all target compounds. The middle chromatogram is the same milk extract analysis stopped at 42 minutes with a 7-minute backflush post-run at 280 °C added to the analysis. The bottom chromatogram is a blank run after the backflushing was completed. The blank run shows that the column was very clean after backflushing. The example shows that a 7-minute backflush cleaned the column as well as a 33-minute bake-out.

The milk extract example in Figure 2 illustrates the backflush technique in reducing cycle time and column bleed. The cycle time was reduced by more than 30% and the column was kept at 280 °C, without going to the bake-out temperature of 320 °C. A column effluent splitter or QuickSwap is required to do the backflush.

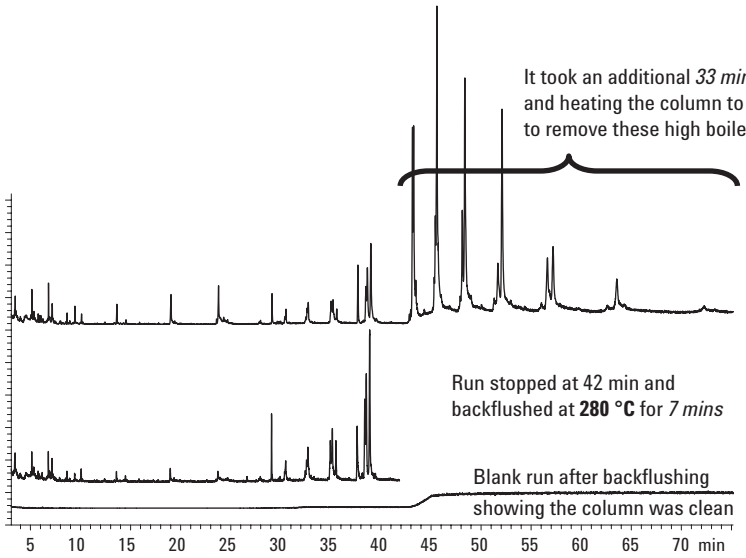

Figure 2. Three total ion chromatograms comparing the results with and without backflush.

References

1. Chin-Kai Meng and Bruce Quimby, "Identifying Pesticides with Full Scan, SIM, μECD, and FPD from a Single Injection," Agilent Application Note, 5989-3299EN, July 2005.
2. Matthew Klee, "Simplified Backflush Using Agilent 6890 GC Post Run Command," Agilent Application Note, 5989-5111EN, June 2006.

Acknowledgement

Milk extract is courtesy of Dr. Steven Lehotay from USDA Agricultural Research Service in Wyndmoor, Pennsylvania, USA.

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Improved Data Quality Through Automated Sample Preparation

Application Note

Authors

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Abstract

Sample preparation tasks can be extremely time-consuming and are often prone to errors, leading to poor reproducibility and accuracy. Many of these tasks, such as calibration curve generation, sample dilution, internal standard addition, or sample derivatization are performed daily, requiring significant resources as well. The Agilent 7696 Sample Prep WorkBench can perform many common sample prep tasks with better accuracy and precision than most manual methods, while using significantly fewer reagents and requiring less time from the operator. To demonstrate this, three sample preparation tasks were adapted for use on the Agilent 7696 Sample Prep WorkBench and yielded the same, if not better, results than the manual methods for accuracy and precision.

Agilent Technologies

Introduction

The Agilent 7696 Sample Prep WorkBench can perform many sample preparation tasks for either gas chromatographic (GC) or liquid chromatographic (LC) analyses. The Agilent 7696 Sample Prep WorkBench consists of two liquid dispensing modules, a single vial heater capable of reaching 80 °C, a single vial mixer, and barcode reader (Figure 1). This enables dilutions/aliquoting, liquid addition, heating for derivatization or digestion, liquid/liquid extractions, and sample mixing. Individual racks can also be heated and/or cooled. This sample preparation instrument can perform tasks with the same accuracy and precision as the Agilent 7693A Automatic Liquid Sampler only in an offline setting instead of on top of a GC [1]. Many sample preparation tasks such as sample dilution, calibration curve standard generation, and sample derivatization within both fields can be time consuming and resource intensive. Automating these procedures with the Agilent 7696 Sample Prep WorkBench therefore is beneficial in many ways.

Figure 1. The Agilent 7696 Sample Prep WorkBench.

A side-by-side comparison of manual and automated methods was performed for three common sample prep applications to demonstrate the improved data quality achieved through automated sample preparation. Sample dilution, calibration curve standard generation, and derivatizations were performed with success on the Agilent 7696 Sample Prep WorkBench.

Experimental

Three common sample preparation tasks were performed with the Agilent 7696 Sample Prep WorkBench. First, sample dilutions and internal standard additions were performed for analysis by both GC and LC. For the GC samples, 50 µL each of isooctane and a standard solution containing four analytes were added to an empty 2-mL autosampler vial. Additionally 0.5 µL of an internal standard solution (ISTD) containing three analytes was added to the vial. The solution was mixed using the onboard mixer before transferring the vials to a GC for analysis. The samples for LC followed a similar procedure. To an empty 2-mL autosampler vial, 187.5 µL of acetonitrile, 62.5 µL of a pesticide standard, and 125 µL of an ISTD were added. The sample was mixed before being transferred to an LC for analysis. For both of these sample dilutions, n=10.

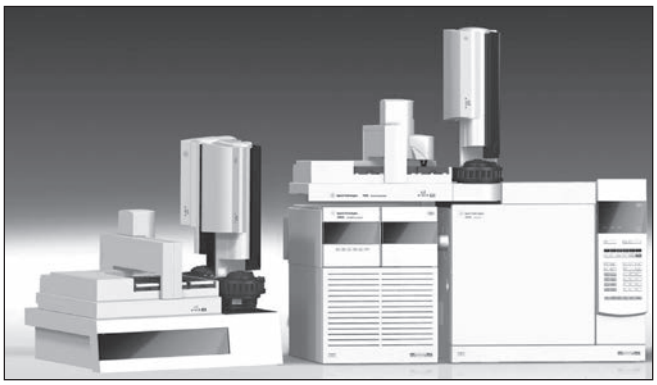

Figure 2. The Agilent 7696 Sample Prep WorkBench with a gas chromatograph and mass spectrometer.

Second, generic calibration curves for the GC were made in triplicate via linear dilution both manually in 10-mL volumetric flasks and with the Agilent 7696 Sample Prep WorkBench. To make the standards manually, small amounts of hexane was added to six clean, dry 10-mL volumetric flasks. Varying amounts of a stock solution containing five analytes at 5 mg/mL, ranging from 0.1 to 1 mL, were added using serological pipets. The flasks were diluted to the mark with hexane to yield concentrations of 50, 100, 200, 300, 400, and 500 ppm. For the automated method, 100 µL of hexane was added to six empty 2-mL autosampler vials. Again, varying amounts of the stock solution, ranging from 1 to 10 µL, was added to the vials yielding approximately the same concentrations.

Figure 3. The Agilent 7696 Sample Prep WorkBench with a liquid chromatograph.

Third, derivatization of fatty acids via silylation reaction was performed. For the manual prep, 100 µL of a silylating reagent was added to approximately 0.5 mL of a free fatty acid solution using an automatic pipettor. The solutions were heated to 70 °C using a heated block. The same derivatization was performed with the Agilent 7696 Sample Prep WorkBench using the single vial heater.

Results and Discussion

GC and LC Sample Dilution

For the 10 samples diluted for GC and LC analysis, the dispensed solvent, standard solution, and ISTD, was measured gravimetrically to determine the reproducibility of the dispensing action. Dispensing 50 µL with a 250 µL syringe results in a 0.5% relative standard deviation (RSD) for the 10 samples measured by weight. The samples were diluted within 1% accuracy, determined from the peak areas. The ISTD exhibited a slightly higher RSD. Dispensing 0.5 µL with a 25 µL syringe resulted in an RSD of 2% for the 10 samples. If a smaller syringe had been used to dispense the ISTD, a lower RSD, closer to that obtained when dispensing the solvent and standard, would have resulted. The added ISTD did not affect the accuracy of the diluted sample (Figure 4).

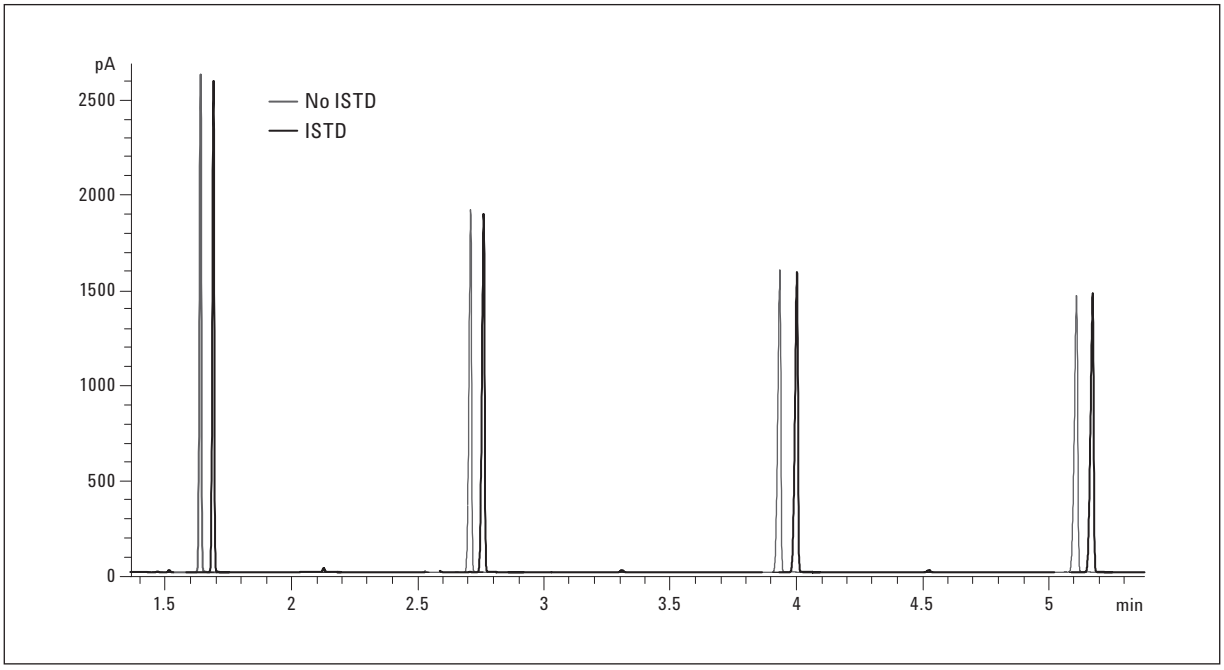

Figure 4. GC chromatograms (slightly offset) are shown for a standard solution dispensed and diluted with and without an ISTD added. No difference in peak areas are observed.

For the 10 samples diluted for LC analysis, similar results were obtained. Dispensing all three volumes with a 250 µL syringe resulted in a RSD of <0.5%, determined gravimetrically. By examining the peak areas after analysis, the dilutions were found to be accurate within 2% (Figure 5).

Calibration Curve Standard Preparation

Three sets of standards were made both manually and with the Agilent 7696 Sample Prep WorkBench. Comparing the three standard sets on the same plot highlighted the increased reproducibility of the Agilent 7696 Sample Prep WorkBench (Figure 6). While each individual curve yielded R^2 values of 0.999, when plotted together the R^2 value was reduced to 0.934 for the manually prepared standards. In contrast, the three curves prepared by the Agilent 7696 Sample prep WorkBench also yielded R^2 values of 0.999 for the individual curves, but when plotted together, the R^2 value was only reduced to 0.997.

Additionally, the relative response factor (RRF) was calculated for each set of standards. Calculating the RSD of the RRFs provides a measure of linearity and reproducibility. The individual calibration curves yielded good RSDs (<5%), demonstrating linear relationships. However, when comparing the three calibration curves together the superiority of the 7696 Sample Prep WorkBench made standards is evident. The average RSD of the RRFs for the three curves made manually was 16%; the three calibration curves made with the 7696 Sample Prep WorkBench gave an average RRF RSD of 4%.

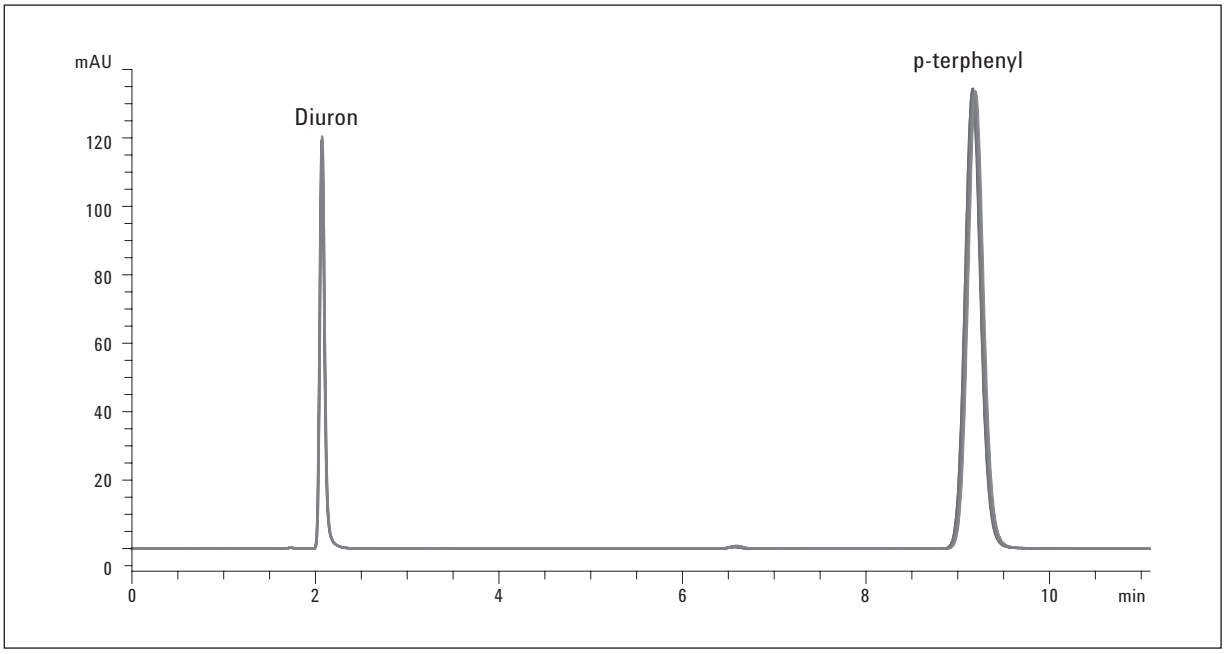

Figure 5. LC Chromatograms are shown for a diluted pesticide standard with an ISTD added. Excellent reproducibility was observed for the five samples shown.

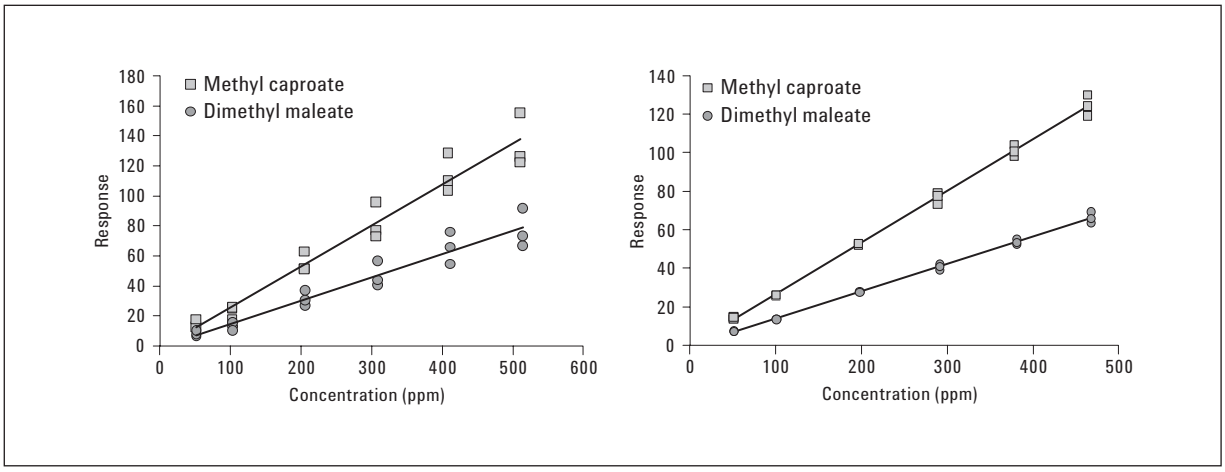

Figure 6. Two calibration curves are shown for two representative analytes. The curves on the right, prepared with the Agilent 7696 Sample Prep WorkBench, are visibly more reproducible than the curves made manually on the left.

Fatty Acid Derivatization

For sample derivatization, identical results were obtained whether the sample was derivatized manually or with the Agilent 7696 Sample Prep WorkBench. For a set of four fatty acids, no discrimination was observed in either method when derivatizing with a silylating reagent (Table 1). However, as seen with other sample preparation tasks, the Agilent 7696 Sample Prep WorkBench is more reproducible in its liquid delivery. The RSD from the peak areas for the three samples prepared manually 0.9%. The RSD for the three samples prepared with the Agilent 7696 Sample Prep WorkBench was 0.7%.

Table 1. After normalizing the fatty acid peak areas to myristic acid, no discrimination was observed from automating the derivatization

Analyte	Ratio-manual	Ratio-automated
Capric acid	0.92	0.92
Capric acid	1.2	1.2
Myristic acid	1.0	1.0
Palmitic acid	1.1	1.1

Conclusions

The three sample preparation tasks presented in this application note highlight the increased reproducibility achieved by automation with the Agilent 7696 Sample Prep WorkBench. Sample dilutions are accurate and reproducible, calibration curve standards are more linear with fewer errors, and sample derivatizations can be performed without analyte discrimination. However, additional benefits can be reaped through sample prep automation with the Agilent 7696 Sample Prep WorkBench.

By automating calibration curve standard preparation, solvent and reagent usage is significantly reduced. Instead of using >60 mL of solvent to make up standards in 10-mL flasks, only 600 µL of solvent was used, excluding the wash vials. This can result in substantial cost savings for laboratories. Additionally, calibrations curve standards required approximately half the time to complete with the Agilent 7696 Sample Prep WorkBench, compared to making up the standards manually. While the other automated sample prep tasks require the same amount of time to complete as the manual methods, the Agilent 7696 Sample Prep WorkBench frees the operator to perform other tasks, such as experiment design or data analysis.

Overall there are many benefits to sample prep automation with the Agilent 7696 Sample Prep WorkBench. While freeing personnel to perform other tasks and reduced solvent usage are important, the largest benefit comes from the reproducibility and accuracy achieved with this system. The automated methods showed better reproducibility and accuracy with fewer errors, thereby improving the quality of the data.

Reference

1. Susanne Moyer, Dale Synder, Rebecca Veeneman, and Bill Wilson, "Typical Injection Performance for the Agilent 7693A Autoinjector," Agilent Technologies Publication 5990-4606EN.

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Agilent Technologies

Retention Time Locking: Concepts and Applications

Application

Gas Chromatography

December 1997

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Abstract

The concepts and applications of retention time locking (RTL) are described. RTL simplifies the process of transferring methods from chromatographic instrument to chromatographic instrument, column to column, and detector to detector. The analysis of impurities in styrene according to ASTM D 5135 is used to demonstrate the efficacy of the approach. Using RTL, the retention times matched within an average of 0.16% (0.02–0.03 minute) in constant pressure modes.

Key Words

Retention time locking, method validation, styrene analysis, ASTM D 5135, capillary gas chromatography, laboratory productivity

Introduction

Retention time is the fundamental qualitative measurement of chromatography. Most peak identification is performed by comparing the retention time of the unknown peak with that of a standard. It is much easier to identify peaks and validate methods if there is no variation in the retention time of each analyte.

However, shifts in retention time occur frequently. Routine maintenance procedures such as column trimming alter retention times. In a multi-instrument laboratory running duplicate methods, the retention times for each instrument will differ from each other, even when run under nominally identical conditions. These differences in retention times mean that each instrument must have a separate calibration and integration event table, making it time-consuming to transfer methods from one instrument to another. Differences in retention time also complicate comparison of data between instruments and over time.

Retention time locking (RTL) is the ability to very closely match chromatographic retention times in any Agilent 6890 gas chromatograph (GC) system to those in another 6890 GC system with the same nominal column.

There are several subtle effects that combine to cause retention time differences between similarly configured GC systems. Columns of the same part number can vary slightly in length, diameter, and film thickness.

GC pneumatics can have small variations in the actual inlet pressure applied at a given setpoint. The actual temperature of the GC oven also has minute but real deviations from the indicated value. The sum of these and other effects result in the observed retention time differences between similarly configured GC systems.

The pneumatics and oven temperature control of the 6890 GC have advanced the state of the art in GC hardware accuracy and precision. Agilent's advances in fused silica capillary column technology have resulted in highly reproducible column-to-column retention characteristics. With these advances, retention time precision for a given peak in a single GC setup is usually better than 0.01 minute. However, even with these advances in columns and instrument hardware, the sum of the effects mentioned above can cause retention time differences between identically configured GC systems of as much as 0.4 minute.

It would be impractical to control all of the instrument and column variables to a degree where retention time differences between similarly configured GC systems are removed. There is, however, a means of greatly reducing these differences. By making an adjustment in the inlet pressure, the retention times on a given GC setup can be closely matched to those of a similarly configured GC system. RTL is based on this principle. The process of RTL is to determine what adjustment in inlet pressure is necessary to achieve the desired match in retention times. Agilent RTL software (G2080AA), which integrates into the Agilent GC ChemStation (version A.05.02 or later), provides the tool required to determine the correct inlet pressure quickly and simply.

There are several advantages gained by using RTL in the laboratory. Peak identification becomes easier and more reliable. It is easier to compare data both between instruments and over time. Comparison of data when using different detectors for analyte identification is simplified. Transferring methods from instrument to instrument or laboratory to laboratory is easier because calibration time windows normally will not require readjustment. Validation of system performance is easier. With "locked" GC methods, the development and use of retention time data bases for unknown identification is much more straightforward.

To maintain a locked method, RTL should be performed whenever:

- The column is changed or trimmed
- The method is installed on a new instrument
- A detector of different outlet pressure is used
- System performance is validated
- Troubleshooting chromatographic problems

To lock a given method for the first-time or for the reasons below, one must first develop a retention time versus pressure (RT vs. P) calibration.

Even when using columns with the same part number (same id, stationary phase type, phase ratio, and same nominal length), separate/different locking calibration curves are needed when using:

- Systems with different column outlet pressures (FID/atmospheric, MSD/vacuum, AED/elevated)
- Columns differing from the "nominal" length by more than 15% (e.g., due to trimming)
- Systems where the predicted locking pressure falls outside the range of the current calibration

A specific solute (usually one found in the normal method calibration standard) must be chosen and then used for both developing the locking calibration and locking all future systems. The solute, or target peak, should be easily identifiable, symmetrical, and should elute in the most critical part of the chromatogram. Solutes that are very polar or subject to degradation should be avoided.

Once the target solute has been chosen and all other chromatographic parameters of the method have been determined, five calibration runs are performed. The runs are made at conditions identical to the nominal method except that four of the runs are made at different pressures. The pressures used are typically:

- Target pressure – 20%
- Target pressure – 10%
- Target pressure (nominal method pressure)
- Target pressure + 10%
- Target pressure + 20%

The retention time of the target compound is determined for each run. The resulting five pairs of inlet pressures and corresponding retention times are entered into the ChemStation software to generate an RTL calibration file.

Figure 1 shows the dialog box used to enter the calibration data. After the data is entered, a plot is displayed, as shown in figure 2. The maximum departure of the fitted curve from the data is given for both time and pressure. If the fit is acceptable, the retention time versus pressure calibration is stored and becomes part of the GC

method. This calibration need only be generated once. Subsequent users of the method can use this calibration when running the method on a similar instrument setup, regardless of location.

To relock a system or lock a new one:

1. Set up the method conditions and run a standard containing the target compound.
2. Enter the actual retention time of the target compound into the "(Re)Lock current method" dialog box (see figure 3).
3. Update the 6890 method with the new calculated pressure, and save the method.
4. Validate the retention time lock by injecting the standard at the new pressure, and compare the retention time obtained to the desired retention time.
5. Repeat steps 2 to 4, if necessary.

A Note on Constant Flow versus Constant Pressure Modes of EPC Operation

Many GC chromatographers prefer to use the "constant flow mode" of EPC operation. In this mode, inlet pressure increases automatically to maintain constant outlet flow rate as the oven temperature increases during the run. Constant flow mode reduces run time and ensures that flow-sensitive detectors see a constant column effluent flow.

The "constant pressure" mode of EPC operation is also popular. In this mode, the pressure remains constant during the run (outlet flow will decrease as temperature increases). For those wishing to reduce run time in constant pressure mode, a higher pressure can be chosen. For

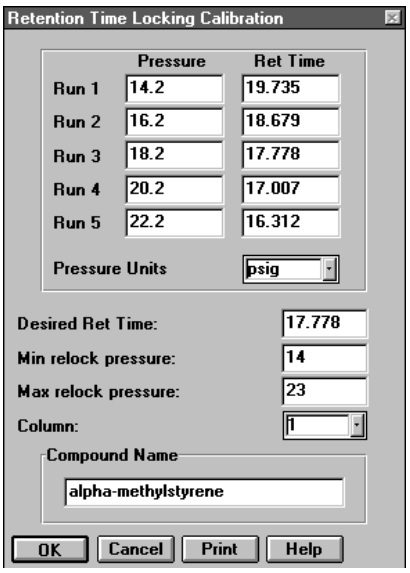

Figure 1. Dialog box used for entering retention time locking calibration data

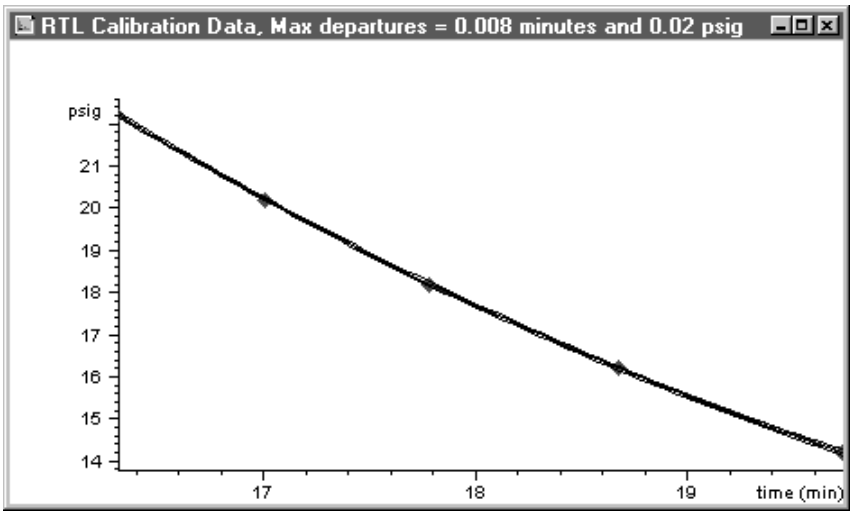

Figure 2. Plot of calibration data as displayed by RTL software

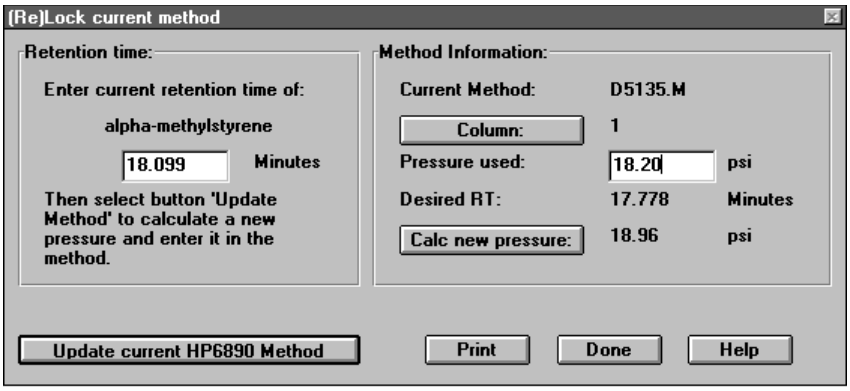

Figure 3. Dialog box used to calculate locking pressure and update the 6890 method

flow-sensitive detectors, one can set "constant column flow + makeup" via the 6890 keyboard or ChemStation. In this mode, the makeup flow is increased as the column flow decreases to keep the sum of the two constant.

The underlying theory of RTL predicts that constant pressure mode of EPC provides the closest matching of retention times. If one desires to compare data from systems with very different configurations, such as GC/FID to GC/MSD, it is best to use constant pressure mode. As can be seen from the styrene analysis data herein, retention time matching between systems of the same configuration (GC/FID, in this case) is still quite good in the constant flow mode.

This application note shows the use of RTL to lock retention times between multiple chromatographic instruments, columns, and detector types and demonstrates RTL in both constant flow and constant pressure modes.

Experimental

Two 6890 Series GC systems were used. Each system was equipped with:

- Electronic pneumatics control (EPC)
- Split/splitless inlet (250 °C, He carrier gas, split 80:1)
- Automatic liquid sampler
- GC ChemStation (version A.05.02)
- Flame ionization detector (FID)
- 60 m × 0.32 mm, 0.5 mm HP-INNOWax column (part no. 19091N-216)
- Temperature program: 80 °C (9 min), 5 °C/min to 150 °C

The inlet pressures/flows used are indicated with each chromatogram.

A third 6890 Series GC was also used. This system was equipped with an Agilent 5973 mass selective detector (MSD) and was used for peak identification. The GC-MSD chromatographic parameters used were the same as the GC systems noted above except for the inlet pressures as indicated.

Results and Discussion

GC-FID to GC-FID Locking

Figure 4 shows the original chromatogram (GC system 1) obtained from running a styrene sample under the conditions specified in ASTM D 5135.[1] Many of the typical impurities found in styrene are found here. The phenylacetylene peak represents about 60 ppm. The peaks are identified in table 1.

The sample was then run at four other pressures to collect the five data pairs for RTL calibration. Because this method was run in constant flow mode, the pressures entered into the RTL software were the initial pressures. The α-methylstyrene peak (peak 10) was chosen as the target compound. The calibration data are shown in figure 1.

The method conditions and RTL calibration were then moved to GC system 2, a different GC and column. The sample was run at the original method inlet pressure of 18.2 psi. The chromatogram obtained using this scouting run is overlaid on the original chromatogram in figure 5. The retention times shifted about 0.3 minute on the second GC. This is a typical result obtained when trying to replicate an analysis on a second instrument or with a second column.

The retention time of α-methylstyrene was entered into the RTL software

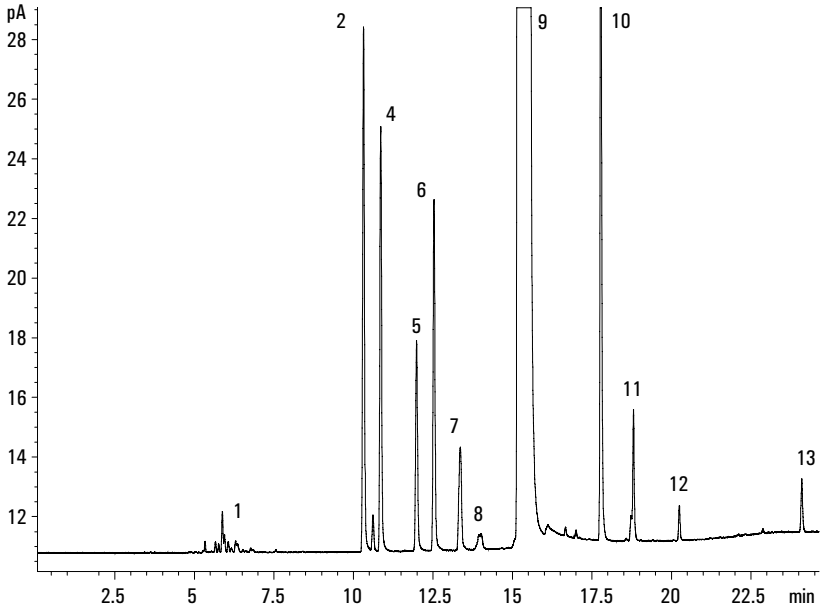

Figure 4. Styrene sample run on GC system 1 at 18.2 psi initial pressure, constant flow mode

dialog box on GC system 2, as shown in figure 3. The RTL software indicated the initial pressure should be modified from 18.2 psi to 18.96 psi. The new initial pressure was entered into the method and saved.

Figure 6 compares the chromatograms obtained from the original run and after locking retention times using the α-methylstyrene. Table 2 compares the retention times before and after using this approach. The retention times are now closely matched.

GC-FID to GC-MSD Locking

A second experiment was conducted to lock the original method from GC system 1 to the GC-MSD. This is useful for identification of unknown impurities that show up in the FID chromatogram. For example, there is a shoulder evident on the front side of the phenylacetylene peak in figure 4. It would simplify locating the impurity in the GC-MSD data if the retention times closely matched that of the GC-FID.

Because constant pressure mode is preferred when comparing data from FID and MSD systems, constant pressure mode was chosen, and the styrene sample was re-run on GC system 1 at 18.2 psi for reference.

The next step was to determine the chromatographic conditions to be used on the GC-MSD. The Agilent method translation software tool was used to calculate the conditions necessary to have the peaks elute in the identical order on the two systems.[2,3] Because the retention times need to match, the dead time and temperature program used for running the GC-MSD must be the same as the GC

Table 1. Peak Identities for Figure 4

Peak #	Name	Peak #	Name
1	Nonaromatics	8	p/m-Ethyltoluene
2	Ethylbenzene	9	Styrene
3	p-Xylene	10	α-Methylstyrene
4	m-Xylene	11	Phenylacetylene
5	i-Propylbenzene	12	β-Methylstyrene
6	o-Xylene	13	Benzaldehyde
7	n-Propylbenzene		

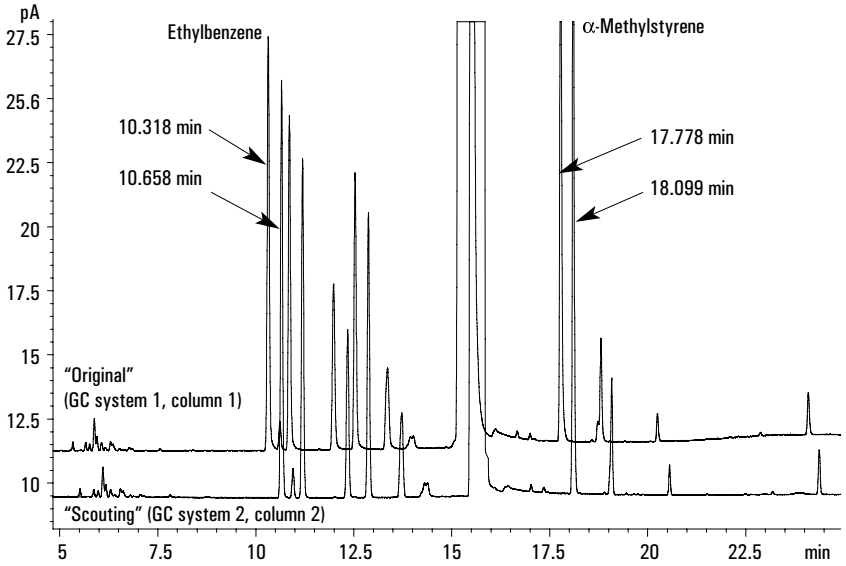

Figure 5. Comparison of original chromatogram on GC system 1 with GC system 2 before retention time locking

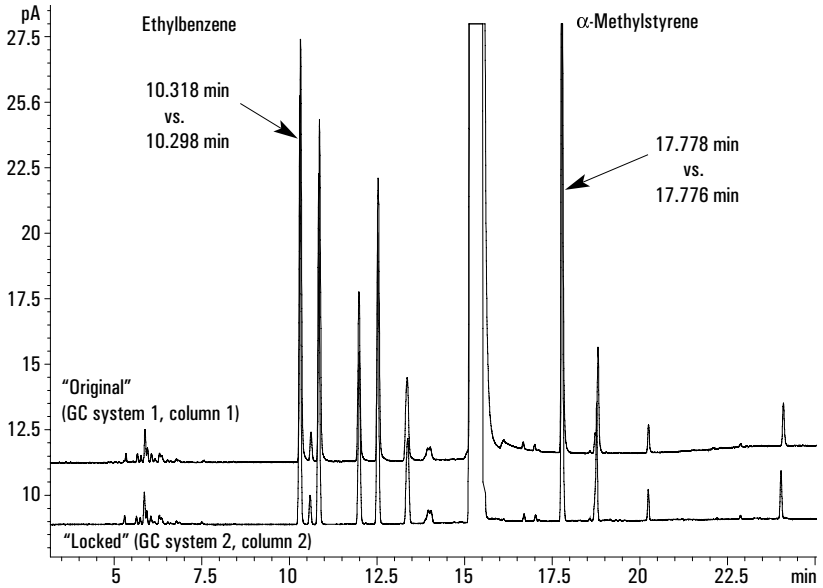

Figure 6. Comparison of original chromatogram on GC system 1 with GC System 2 after retention time locking

method. The pressure used, however, will be different due to the difference in column outlet pressure. The GC-MSD inlet pressure is calculated using the "none" mode of the method translation software (figure 7). In this mode, the holdup time between the two columns was forced to be identical to the GC-FID. This gives a speed gain of 1. The pressure calculated for use on the GC-MSD was 8.44 psi. Note that this calculated pressure is only the nominal pressure required to get similar retention times, not the exact locking pressure.

A different RTL calibration is required for GC-MSD because the outlet pressure is vacuum, and that of the FID is atmospheric pressure. Five runs were made on the GC-MSD system bracketing the 8.44 psi nominal method pressure. Because the GC-MSD used in this study was not equipped with RTL software, a dummy method was created in GC system 1 and the GC-MSD RTL calibration data was entered into it. A scouting run of the Styrene sample was made on the GC-MSD, and the α-methylstyrene retention time was used for locking. The locking inlet pressure calculated with the dummy method was 7.9 psi and was entered into the GC-MSD.

Figure 8 shows the resulting matched chromatograms from the GC-FID and GC-MSD. As seen in table 3, the retention times are now closely matched within 0.02 minute.

Figure 9 shows the MSD first choice of library search result of the impurity that created the shoulder on the front side of the Phenylacetylene peak. RTL ensured that this shoulder remained separated on the MSD system and eluted at the same time for easy comparison to the FID results.

Table 2. GC-FID Retention Times Before and After Locking for Styrene Impurities (Constant Flow Conditions). Chromatograms Shown in Figures 4, 5, and 6.

Component	Original Run GC 1/Column 1 18.2 psi	GC2–GC1 Before RTL	Scouting Run GC 2/Column 2 18.2 psi	GC2–GC1 After RTL	Locking Run GC 2/Column 2 19.0 psi
Ethylbenzene	10.318	0.340	10.658	–0.020	10.298
p-Xylene	10.616	0.333	10.949	–0.026	10.590
m-Xylene	10.858	0.337	11.195	–0.022	10.836
i-Propylbenzene	11.985	0.359	12.344	+0.005	11.990
o-Xylene	12.533	0.345	12.878	–0.012	12.521
n-Propylbenzene	13..360	0.364	13.724	–0.016	13.376
α-Methylstyrene*	17.778	0.321	18.099	–0.002	17.776
Phenylacetylene	18.806	0.275	19.081	–0.040	18.766
β-Methylstyrene	20.248	0.310	20.558	–0.006	20.242
Benzaldehyde	24.097	0.279	24.376	–0.069	24.028
Average Δ		0.326		0.028	

* Used in locking calculation

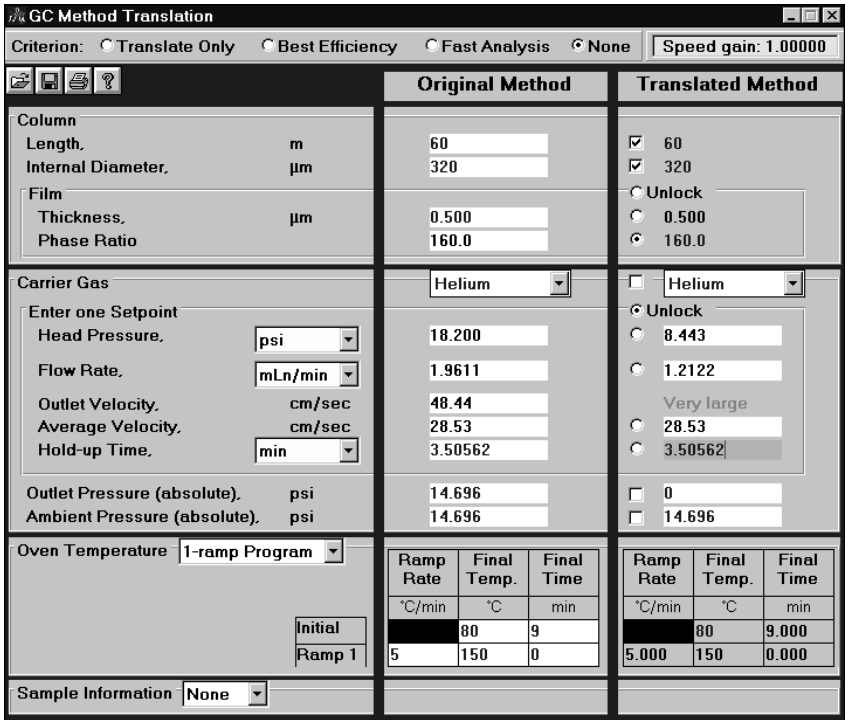

Figure 7. Method translation software provides scaled conditions for GC systems with different configurations

Conclusions

Retention time locking facilitates replicating results from instrument to instrument, from column to column, and from detector to detector by locking retention times. The retention times of a styrene sample analyzed according to ASTM D 5135 matched to within 0.06 minute after locking.

References

1. ASTM D 5135-95, "Analyses of Styrene by Capillary Gas Chromatography," Annual Book of Standards, Volume 06.04, ASTM, 100 Bar Harbor Drive, West Conshohocken, PA 19428 USA.
2. M. Klee and V. Giarrocco, "Predictable Translation of Capillary GC Methods for Fast GC" Agilent Technologies, Inc., Application Note 228-373, Publication 5965-7673E, March 1997.
3. GC Pressure/Flow Calculator for Windows, Version 2.0 and Method Translation Tool Version 2.0. Available at http://www.chem.agilent.com/servsup/usersoft/main.html.

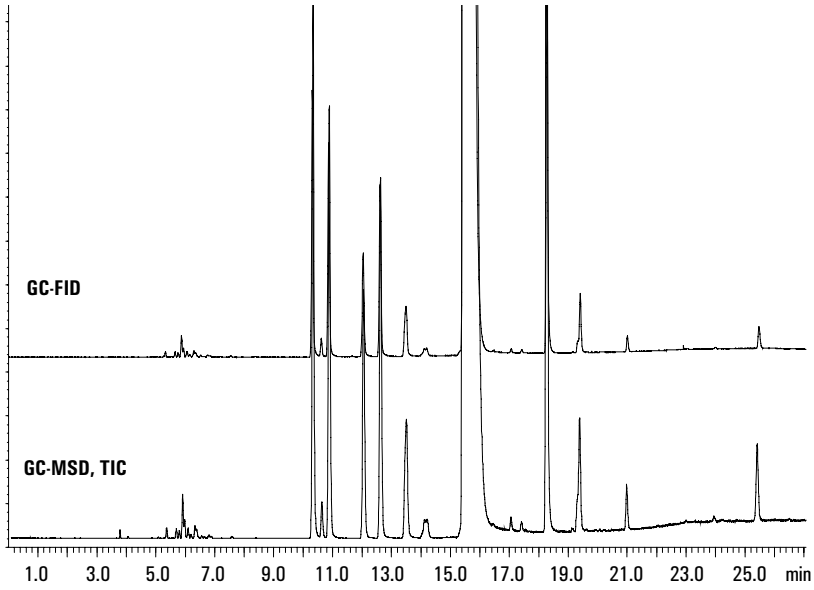

Figure 8. Comparison of chromatogram on GC system 1 with GC-MSD system after retention time locking, Constant Pressure Mode

Table 3. GC-FID vs. GC-MSD, Method Translated then Locked—Retention Times (Constant Pressure Conditions)

Component	GC-FID Original 18.2 psi	GC-MSD 7.9 psi	RT Difference min
Ethylbenzene	10.315	10.338	0.023
p-Xylene	10.620	10.642	0.022
m-Xylene	10.869	10.890	0.021
i-Propylbenzene	12.038	12.053	0.015
o-Xylene	12.613	12.630	0.017
n-Propylbenzene	13.492	13.508	0.016
a-Methylstyrene*	18.276	18.267	–0.009
Phenylacetylene	19.406	19.389	–0.017
b-Methylstyrene	21.008	20.987	–0.011
Benzaldehyde	25.475	25.415	–0.060
		Average	0.021

* Used in locking calculation

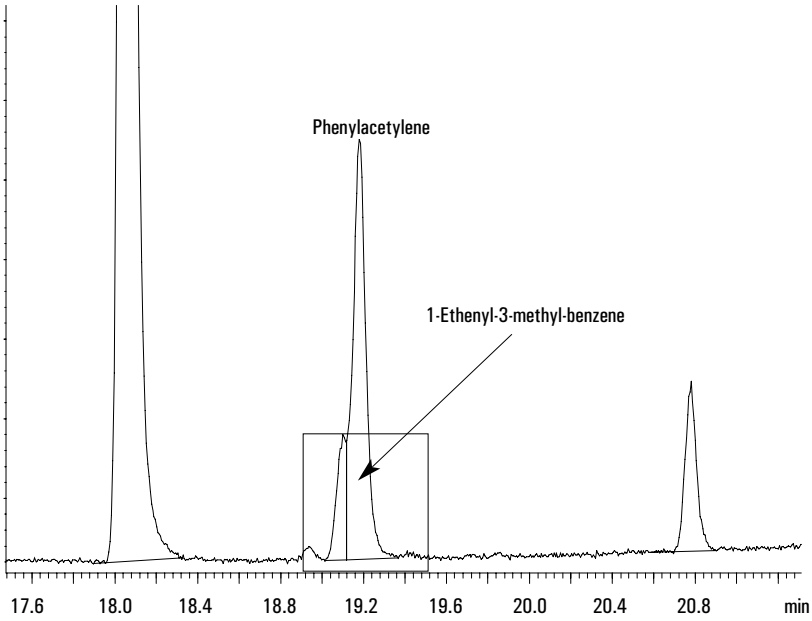

Figure 9. GC-MSD identification of impurity in shoulder of phenylacetylene peak

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Improving the Effectiveness of Method Translation for Fast and High Resolution Separations

Application Note

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Abstract

The increased availability of sub-2-micron (STM) columns and increased demand for methods friendly to mass spectrometers has led to strong trend toward conversion of existing HPLC methods to smaller diameter and smaller particle size columns. While the conversion is a simple mathematical exercise requiring the scaling flow rates, gradient times and injection volumes, many users observe less than perfect results. Here we look closely at the problem and propose calculations that improve the speed and/or resolution in a more predictable and beneficial way.

Introduction

Methods developed on older columns packed with large 5- or 10-µm particles are often good candidates for modernization by replacing these columns with smaller dimension columns packed with smaller particle sizes. The potential benefits include reduced analysis time and solvent consumption, improved sensitivity and greater compatibility with mass spectrometer ionization sources.

Simplistically, a column of 250-mm length and containing 5-µm particles can be replaced by a 150-mm length column packed with 3-µm particles. If the ratio of length to particle size is equal, the two columns are considered to have equal resolving power. Solvent consumption is reduced by L1/L2, here about 1.6-fold reduction in solvent usage per analysis. If an equal mass of analyte can then be successfully injected, the sensitivity should also increase by 1.6-fold due to reduced dilution of the peak as it travels through a smaller column of equal efficiency.

LC/MS (Liquid Chromatography/Mass Spectrometry) ionization sources, especially the electrospray ionization mode, have demonstrated greater sensitivity at lower flow rates than typically used in normal LC/UV (UltraViolet UV/VIS optical detection) methods, so it may also be advantageous to reduce the internal diameter of a column to allow timely analysis at lower flow rates. The relationship of flow rate between different column diameters is shown in Equation 1.

$$\text{Flow}_{\text{col. 1}} \times \left[\frac{\text{Diam.}_{\text{column2}}}{\text{Diam.}_{\text{column1}}}\right]^2 = \text{Flow}_{\text{col. 2}} \quad \text{(eq. 1)}$$

The combined effect of reduced length and diameter contributes to a reduction in solvent consumption and, again assuming the same analyte mass can be injected on the smaller column, a proportional increase in peak response. We normally scale the injection mass to the size of the column,

though, and a proportional injection volume would be calculated from the ratio of the void volumes of the two columns, multiplied by the injection volume on the original column.

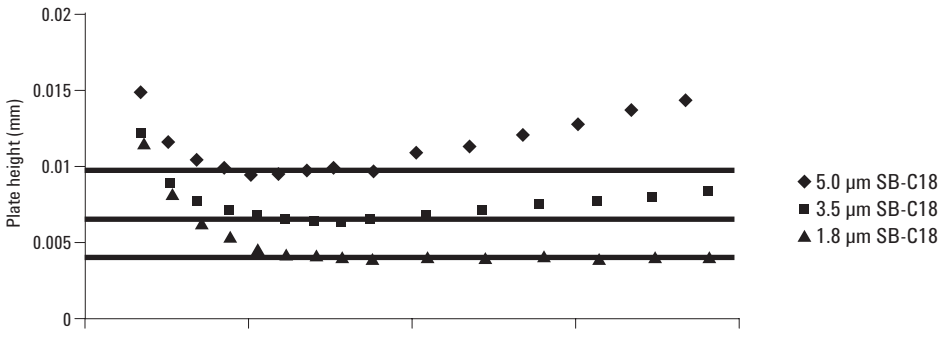
$$\text{Inj. vol.}_{\text{col. 1}} \times \left[\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}}\right] = \text{Inj. vol.}_{\text{col. 2}} \quad \text{(eq. 2)}$$

For isocratic separations, the above conditions will normally result in a successful conversion of the method with little or no change in overall resolution. If one wishes to improve the outcome of the method conversion, though, there are several other parameters that should be considered. The first of these parameters is the column efficiency relative to flow rate, or more correctly efficiency to linear velocity, as commonly defined by van Deemter [1] and others, and the second is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column.

Van Deemter observed and mathematically expressed the relationship of column efficiency to a variety of parameters, but we are most interested here in his observations that there is an optimum linear velocity for any given particle size, in a well-packed HPLC column, and that the optimum linear velocity increases as the particle size decreases. Graphically, this is often represented in van Deemter plots as shown in Figure 1, a modified version of the original plot [2].

In Figure 1 we observe that the linear velocity at which 5-µm materials are most efficient, under the conditions used by the authors, is about 1 mm/sec. For 3.5-µm materials the optimum linear velocity is about 1.7 mm/sec and has a less distinct optimum value, suggesting that 3.5-µm materials would give a more consistent column efficiency over a wider flow range. For the 1.8-µm materials, the minimum plate height, or maximum efficiency, is a broad range beginning at about 2 mm/sec and continuing past the range of the presented data. The practical application of this information is that a reduction in particle size, as discussed earlier, can often be further optimized by increasing the linear velocity which results in a further reduction in analysis time. This increase in elution speed will decrease absolute peak width and may require the user to increase data acquisition rates and reduce signal filtering parameters to ensure that the chromatographic separation is accurately recorded in the acquisition data file.

The second important consideration is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column. As column volume is reduced, peak elution volumes are proportionately reduced. If smaller particle sizes are also employed there is a further reduction in the expected peak volume. The liquid chromatograph, and particularly the areas where the analytes will traverse, is a collection of various connecting capillaries and fittings which will cause a measurable amount of bandspreading. From the injector to the detector flow cell, the cumulative dispersion that occurs degrades the column performance and results in observed efficiencies that can be far below the values that would be estimated by purely theoretical means. It is fairly typical to see a measured dispersion of 20 to 100 µL in an HPLC system. This has a disproportionate effect on the smallest columns and smallest particle sizes, both of which are expected to yield the smallest



Lin. vel.	mm/sec	1	2	3	4	5
4.6 mm	mL/min	0.7	1.4	2.1	2.8	3.5
3 mm	mL/min	0.3	0.6	0.9	1.2	1.5
2.1 mm	mL/min	0.14	0.29	0.44	0.58	0.73
1 mm	mL/min	0.033	0.066	0.1	0.133	0.166

Figure 1. van Deemter plot with various flow rates and particle sizes.

possible peak volumes. Care must be taken by the user to minimize the extracolumn volume and to reduce, where practical, the number of connecting fittings and the volume of injection valves and detector flow cells.

For gradient elution separations, where the mobile phase composition increases through the initial part of the analysis until the analytes of interest have been eluted from the column, successful method conversion to smaller columns requires that the gradient slope be preserved. While many publications have referred to gradient slope in terms of % change per minute, it is more useful to express it as % change per column volume. In this way, the change in column volume during method conversion can be used to accurately render the new gradient condition. If we think of each line of a gradient table as a segment, we can express the gradient by the following equation:

$$\%\ \text{Gradient slope} = \left[\frac{(\text{End}\% - \text{Start}\%)}{\#\text{Column volumes}} \right] \quad \text{(eq. 3)}$$

Note that the use of % change per column volume rather than % change per minute frees the user to control gradient slope by altering gradient time and/or gradient flow rate. A large value for gradient slope yields very fast gradients with minimal resolution, while lower gradient slopes produce higher resolution at the expense of increased solvent consumption and somewhat reduced sensitivity. Longer analysis time may also result unless the gradient slope is reduced by increasing the flow rate, within acceptable operating pressure ranges, rather than by increasing the gradient time.

Resolution increases with shallow gradients because the effective capacity factor, k*, is increased. Much like in isocratic separations, where the capacity term is called k', a higher value directly increases resolution. The effect is quite dramatic up to a k value of about 5 to 10, after which little improvement is observed. In the subsequent examples, we will see the results associated with the calculations discussed above.

Experimental Conditions

System

Agilent 1200 Series Rapid Resolution LC consisting of:
G1379B micro degasser
G1312B binary pump SL
G1367C autosampler SL, with thermostatic temperature control
G1316B Thermostatted column compartment SL
G1315C UV/VIS diode array detector SL, flow cell as indicated in individual chromatograms
ChemStation 32-bit version B.02.01

Columns

Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm
Agilent ZORBAX SB-C18, 3.0 mm × 150 mm, 3.5 µm

Mobile phase conditions

Organic solvent:	Acetonitrile
Aqueous solvent:	25 mm phosphoric acid in Milli-Q water

Gradient Conditions

Gradient slope:	7.8% or 2.3% per column volume, as indicated. See individual chromatograms for flow rate and time

Sample

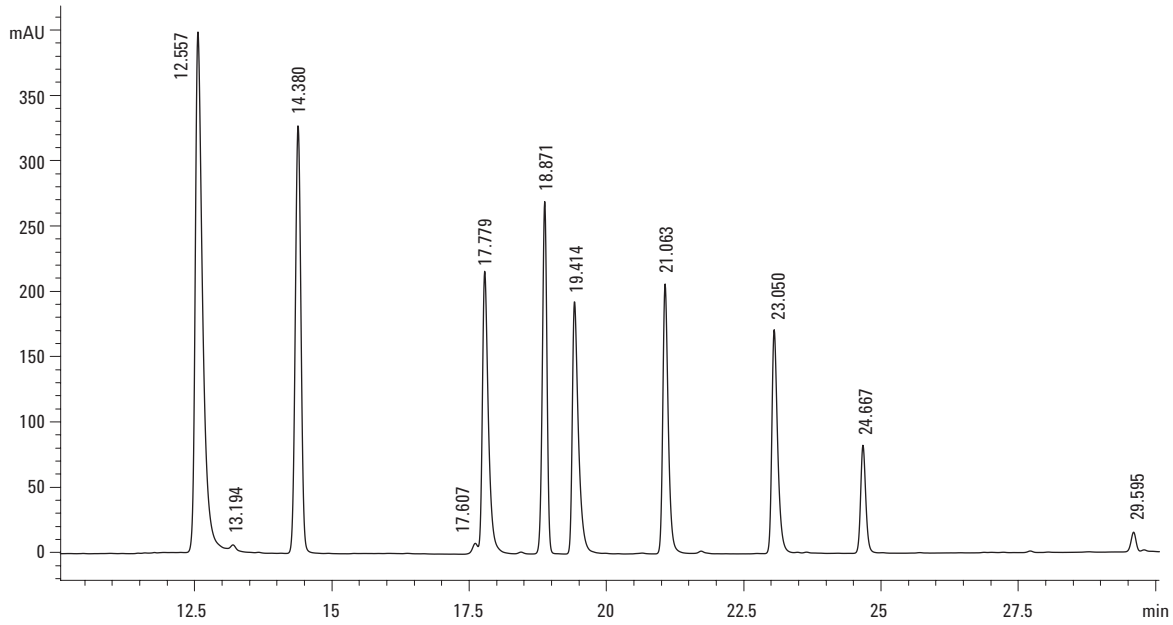
Standard mixture of chlorinated phenoxy acid herbicides, 100 µg/mL in methanol

Results

The separation was initially performed on a standard 4.6 × 250 mm, 5-µm ZORBAX SB-C18 column thermostatted to 25 °C (Figure 2) using conditions referenced in US EPA Method 555. The method was then scaled in flow and time for exact translation to a 3.0 × 150 mm, 3.5-µm column (Figure 3). Solvent consumption is reduced from 60 mL to 15.5 mL per analysis.

The separation was then re-optimized for faster separation with the identical slope, 7.8%, by increasing the flow rate from 0.43 to 1.42 mL/min, and proportionately reducing the gradient time (Figure 4). Finally, increased resolution is demonstrated by keeping the original times used in Figure 3 with the increased flow rate (Figure 5). This yields a gradient with identical time but a reduced slope of 2.3%. The increased resolution of peaks 4 and 5 is readily apparent.

The conditions in Figure 4, 7.8% slope at increased linear velocity on 3.0 × 150 mm, 3.5-µm material, yield a separation with comparable resolution to the original 4.6 × 250 mm method, but with only a 12-minute total analysis time. This is excellent for



Conditions

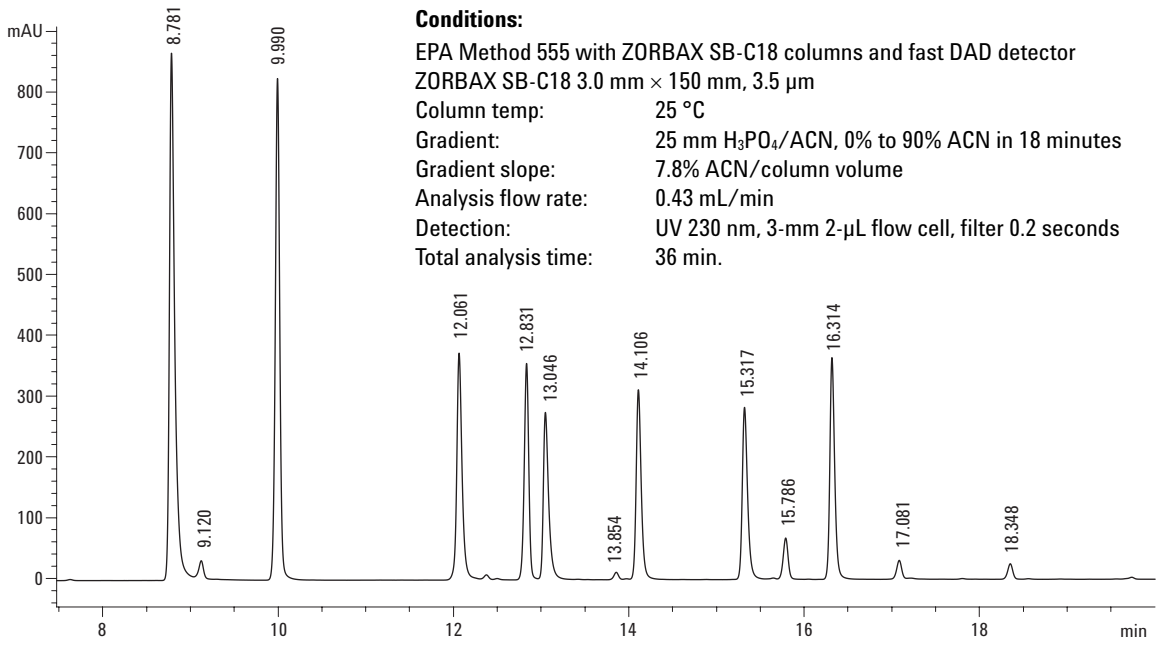
EPA Method 555 with ZORBAX SB-C18 columns and fast DAD detector
ZORBAX SB-C18 4.6 mm × 250 mm, 5 µm

Column temp:	25 °C
Gradient:	10% to 90% ACN vs. 25 mM H_3PO_4
Gradient slope:	7.8% ACN/column volume
Analysis flow rate:	1 mL/min

Group A Compounds

Total analysis time:	60 min
Detection:	UV 230 nm, 10-mm 13-µL flow cell, filter 2 seconds (default)

Figure 2. Gradient separation of herbicides on 4.6 × 250 mm 5-µm ZORBAX SB-C18.



Conditions:

EPA Method 555 with ZORBAX SB-C18 columns and fast DAD detector
ZORBAX SB-C18 3.0 mm × 150 mm, 3.5 µm

Column temp:	25 °C
Gradient:	25 mm H_3PO_4/ACN, 0% to 90% ACN in 18 minutes
Gradient slope:	7.8% ACN/column volume
Analysis flow rate:	0.43 mL/min
Detection:	UV 230 nm, 3-mm 2-µL flow cell, filter 0.2 seconds
Total analysis time:	36 min.

Figure 3. Gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

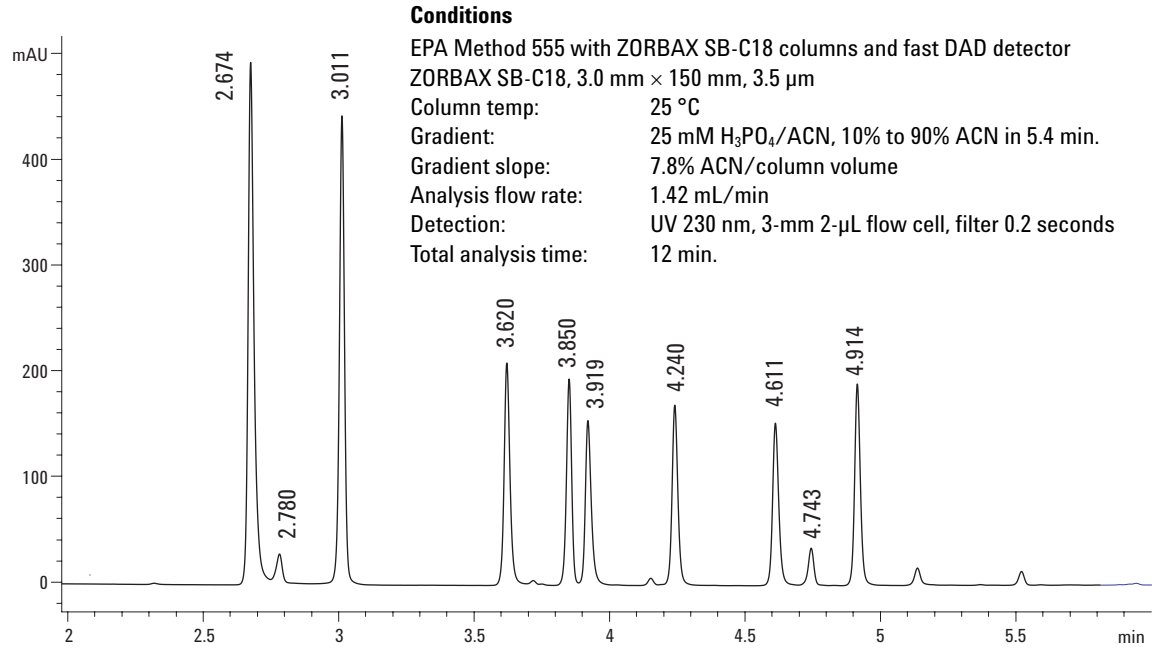


Figure 4. High speed gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

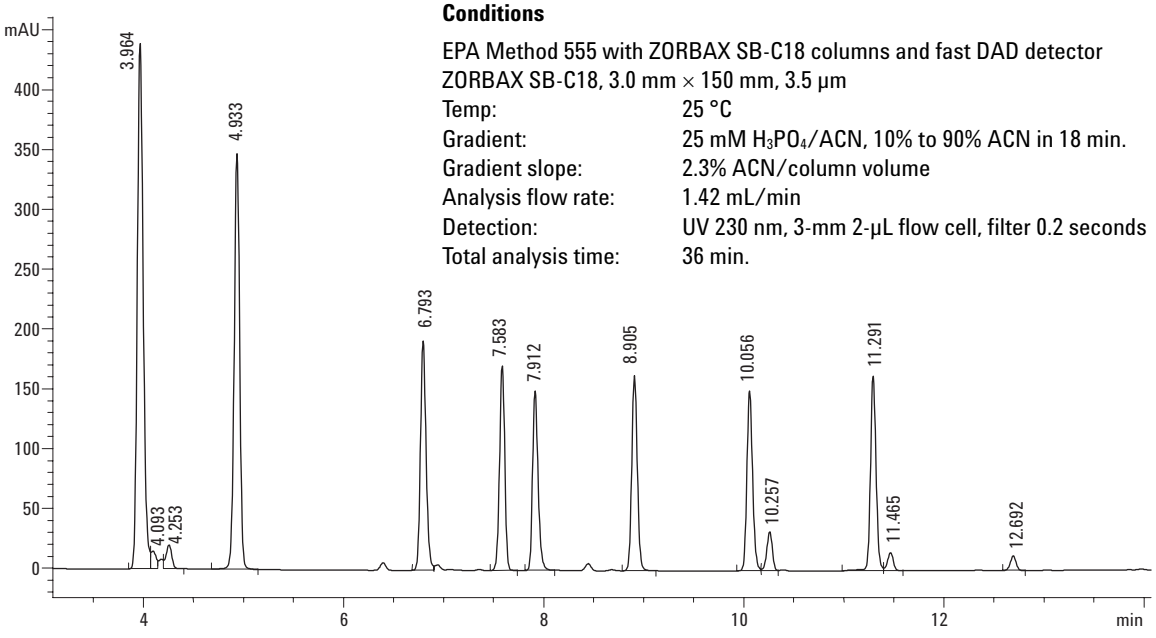


Figure 5. Reduced slope gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

high throughput screening and quantitation of a large number of samples. Figure 5, with the gradient slope reduced to 2.3%, results in a high-resolution separation with a calculated R value of 3.3 vs. the standard 3.0 × 150 mm separation value of 1.9, for the critical pair seen in Figure 5 at 7.5 to 8 minutes.

In Table 1 the column has been replaced with a low dead volume connecting union in a system fitted with 0.12-mm id capillary tubing at all points of sample contact. A 1-µL injection of dilute actone is made to determine the bandspreading contribution of the system, with various flow cells. Multiple flow cells were tested, and the average result reported, where possible. The elution volume summarizes the total volume of all tubing in the system. While the absolute volume from the 2-µL to the 13-µL flow cells is 11 µL, we observe an increase of 15 to 16 µL because of the larger diameter inlet tubing integral to the larger volume flow cells.

Table 1. Volumetric Measurements of Various Flow Cells

Flow cell	Elution volume (µL)	Half height width (µL)	5 Sigma width (µL)
New SL 2 µL 3 mm	11	5	12
Micro 6 mm 1.7 µL (n = 2)	14	6	18
Semi-micro 6 mm 5 µL (n = 2)	13	6.5	18.5
Standard 10 mm 13 µL	26	11	26
New SL 10 mm 13 µL	27	11	25

Conclusion

Careful analysis of the existing gradient conditions, coupled with an awareness of the need to accurately calculate new flow and gradient conditions can lead to an easy and reliable conversion of existing methods to new faster or higher resolution conditions. In addition, awareness of extracolumn dispersion, especially with small and high resolution columns, will ensure good column efficiency which is critical to a successful translation of the method.

References

1. J. J. van Deemter, F. J. Zuiderweg, A. Klinkenberg, Chemical Engineering Science 1956, 5, 271–289
2. The Influence of Sub-Two Micron Particles on HPLC Performance, Agilent Technologies, application note 5989-9251EN, May 2003

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Improving GC-MS Method Robustness and Cycle Times Using Capillary Flow Technology and Backflushing

Application Note

Environmental

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Abstract

This application note demonstrates the customer benefits from using Capillary Flow Technology to provide backflushing of high-boiling materials in GC and GC/MS analyses. Benefits include reduction in chromatographic cycle times, a reduction in system column maintenance, and extended GC column life. If a GC/MS system is utilized, the author has experienced an increase in the number of samples analyzed before ion source maintenance is required.

Agilent Technologies

Introduction

A critical component of the GC/MS analysis of any sample that contains large amounts of matrix material is the sample preparation. Environmental samples such as soils and sediments require not only extraction, but may also require multiple cleanup steps in order to present as clean an extract as possible for injection in to the GC/MS system.

Any remaining matrix in the sample extract can have deleterious effects on the GC sample inlet, column, and the ion source of the mass spectrometer. Traditionally, these high-boiling matrix materials are removed from the capillary column by a long bake-out period after the analytes of interest have eluted. This long bake-out process causes thermal stress to the column and also drives the matrix material towards the ion source, where it will eventually affect system performance. Moreover, should any material remain in the column after the bake-out process, it can cause loss of chromatographic peak shape and retention time shifting of target analytes. This shifting of retention time is particularly troublesome if the mass spectrometer is being used in the selected ion monitoring (SIM) mode (as with a single quadrupole GC/MS) or in the multiple reaction monitoring (MRM) mode (as with a triple quadrupole GC/MS).

This paper demonstrates how high-boiling matrix materials can be removed from the column quickly and effectively – between sample injections – by using capillary flow technology and capillary column backflushing.

Figure 1 shows a schematic diagram of the GC/MS system used. The 15-m analytical column was connected to the EPC split/splitless inlet and a capillary flow technology two-way splitter (p/n G3180B or G1540 option number 889).

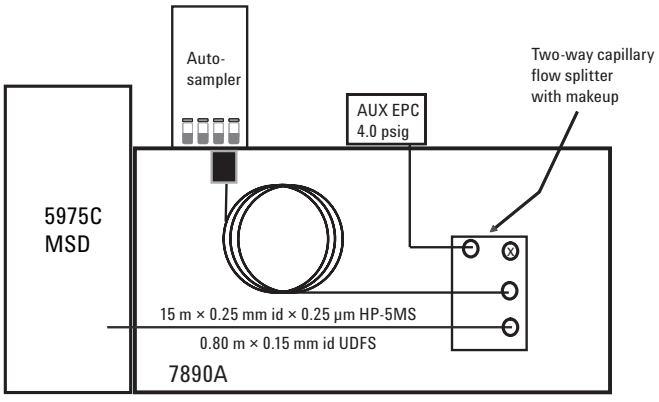

Figure 1. Schematic diagram of GC-MS system.

A short length of uncoated, deactivated fused silica (UDFS) capillary column is used as a restrictor between the splitter and the MS. Note carefully how the connections are made at the splitter. The X represents a port on the splitter plate that is closed off with a SilTite metal ferrule and stainless steel wire plug.

Backflushing in this example was accomplished during a post-run period by a combination of increasing oven temperature, reducing the inlet pressure of the analytical column, and increasing the pressure applied to the splitter plate.

Experimental

The full analytical conditions, both with and without post-run backflush set-points, are shown in Table 1.

Table 1. GC/MS Analysis Conditions

Gas chromatograph	Agilent 7890A
Columns	(1) 15.0 m × 0.25 µm id × 0.25 µm HP-5MS Ultra Inert (19091S-431SI) Inlet Front split/splitless, outlet 2-way Capillary Flow Device
	(2) 0.80 m × 0.15 mm id uncoated deactivated fused silica inlet two-way capillary flow device at 4.0 psig outlet vacuum
Carrier gas	Helium
Carrier gas mode	Constant pressure
Flow rate	17.18 psi
Injection port	EPC split/splitless
Autosampler	Agilent 7683A
Injection mode	Splitless, purge delay 0.5 min Purge flow 50.0 mL/min at 0.5 min
Injection volume	2.0 µL
Injection port liner	4 mm single-taper splitless liner (5181-3316)
Oven program °C (min)	70 (1) – 50 °C /min – 150 (0) 6 – 200 (0) – 16 – 280 (0) °C
Mass spectrometer	Agilent 5975C MSD
MS interface	280 °C
MS source	230 °C
MS quad 1	150 °C
Backflush conditions (1)	Post-run, 10 min, AUX 60 psig, oven 320 °C
Backflush conditions (2)	Post-run, 6 min, AUX 80 psig, oven 320 °C
Detection mode	EI full scan; mass range 40:550 amu
EI tune	Gain factor = 1

Results and Discussions

Experiment 1: No Backflushing Employed

In the first experiment, an extracted sediment sample was analyzed in full-scan mode to show the extent of the matrix problem. No backflushing was employed.

Before any sediment was injected, a system blank (no injection) followed by a 2-µL solvent blank was made. In the absence of the actual hexane solvent used to prepare the sediment extract, hexane that was not particularly clean was used. The TICs are shown overlaid in Figure 2, system blank in black, and solvent blank in gray. These chromatograms show that the system is free from high-boiling matrix material.

Following the blanks, a single injection of the sediment extract was made without backflushing; the TIC is shown in Figure 3. Note the very high abundance of the matrix and that when the analysis finishes, there is still a significant amount of matrix material to elute from the column.

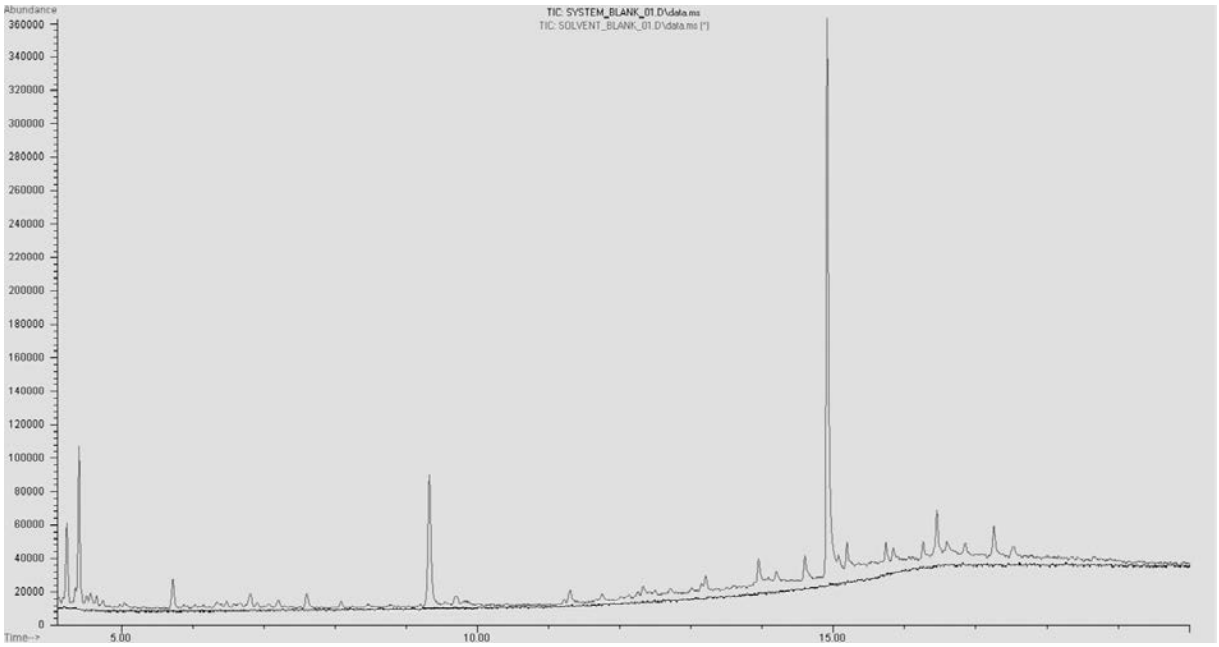

Figure 2. System blank and solvent blank TICs.

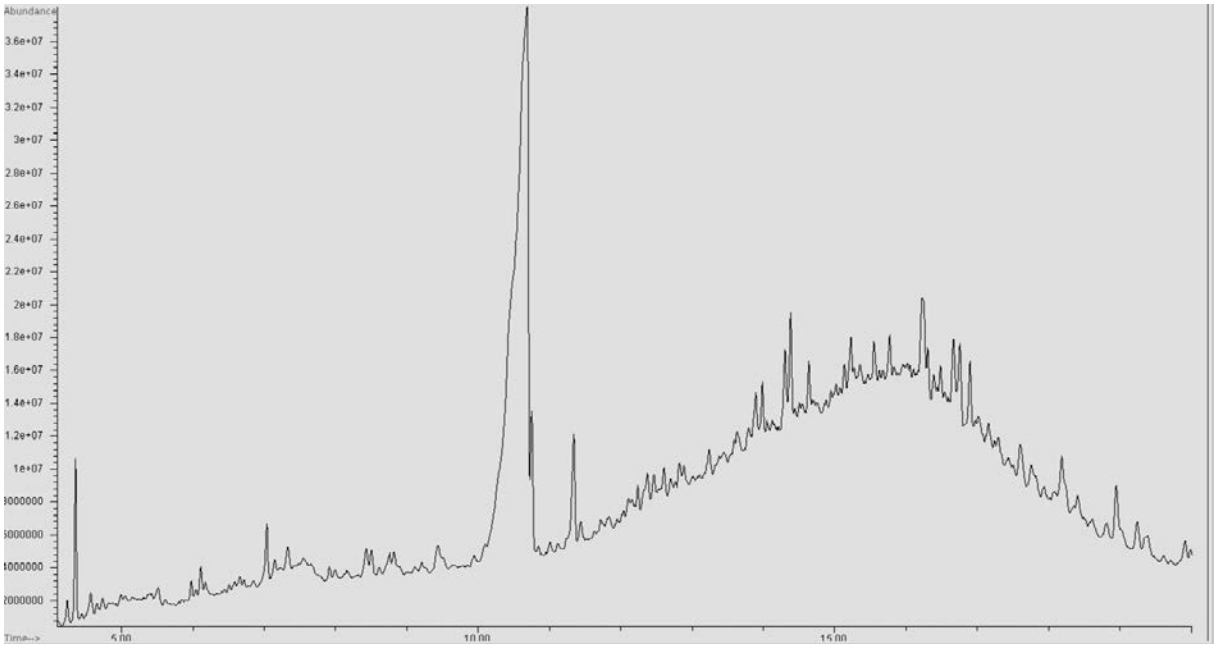

Figure 3. Sediment extract TIC.

The sediment extract injection was followed by a series of hexane blank injections. The first seven hexane blank TICs are shown overlaid in Figure 4 with the solvent blank before the sediment was injected into the GC/MS system.

Figure 5 shows that after the eighth solvent blank injection, the system has almost recovered to the level of background before the sediment sample was injected.

The original solvent blank TIC is shown in black, the eighth solvent blank TIC after the sediment injection is shown in gray.

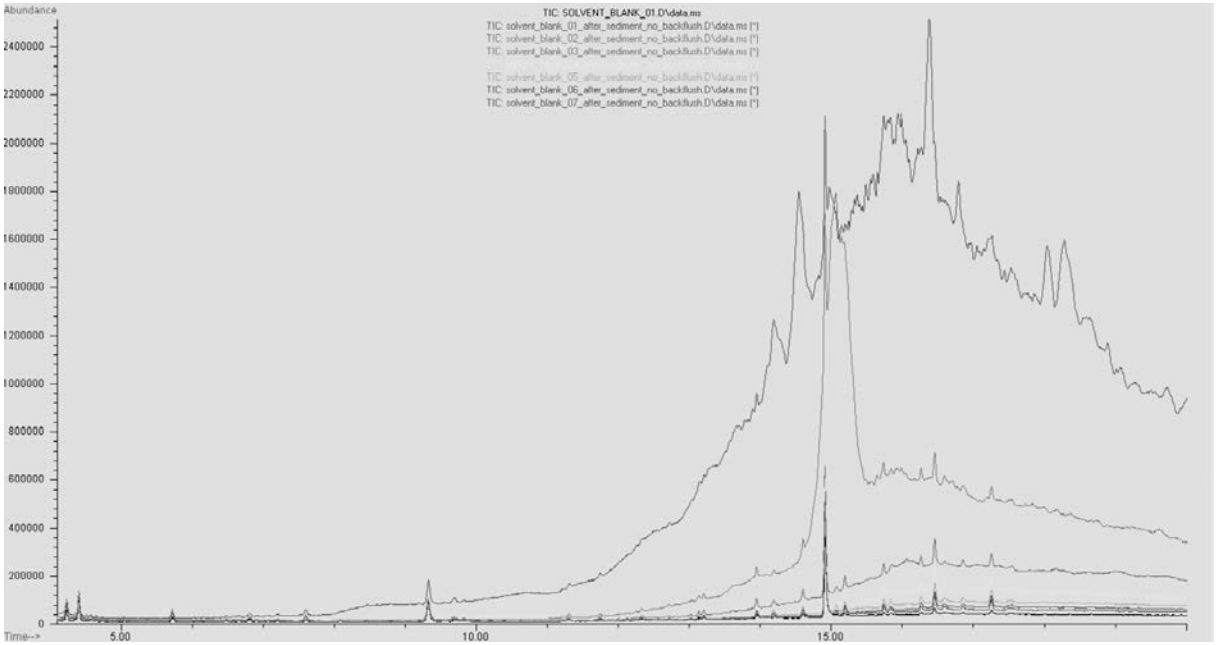

Figure 4. Successive solvent blank injections.

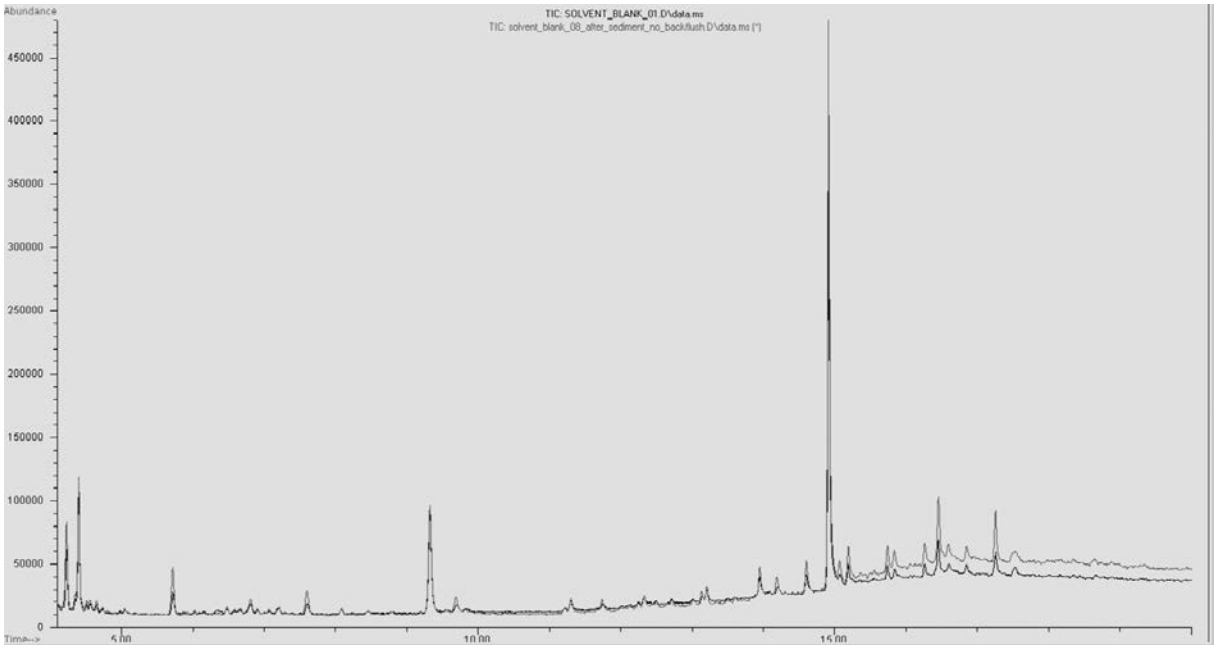

Figure 5. Eighth solvent blank and original solvent blank TICs

Experiment 2: Backflushing Employed

Backflushing was enabled during a post-run period by increasing column oven temperature, reducing the inlet pressure of the analytical column, and increasing the gas pressure applied to the splitter plate.

The 7890A instrument control software includes simple and easy-to-use screens to help set up post-run backflushing conditions. Figure 6 shows the configuration of columns and connections with the GC oven.

Figure 7 shows the actual backflushing conditions, namely the post-run oven temperature (320 °C), post-run inlet pressure for the analytical column (1 psig), post-run pressure applied to the splitter device (60 psig), and post-run time (10 minutes). The figure also shows the number of column-volumes of carrier gas that will backflush the analytical column.

Note that using the backflushing conditions shown in Figure 7 (320 °C, column pressure 1 psig, and splitter pressure 60 psig for 10 minutes), that 59.4 column volumes of carrier gas was used to backflush the column during the post-run period. This backflush time may have been more than necessary. Alternate conditions were also investigated and are presented later.

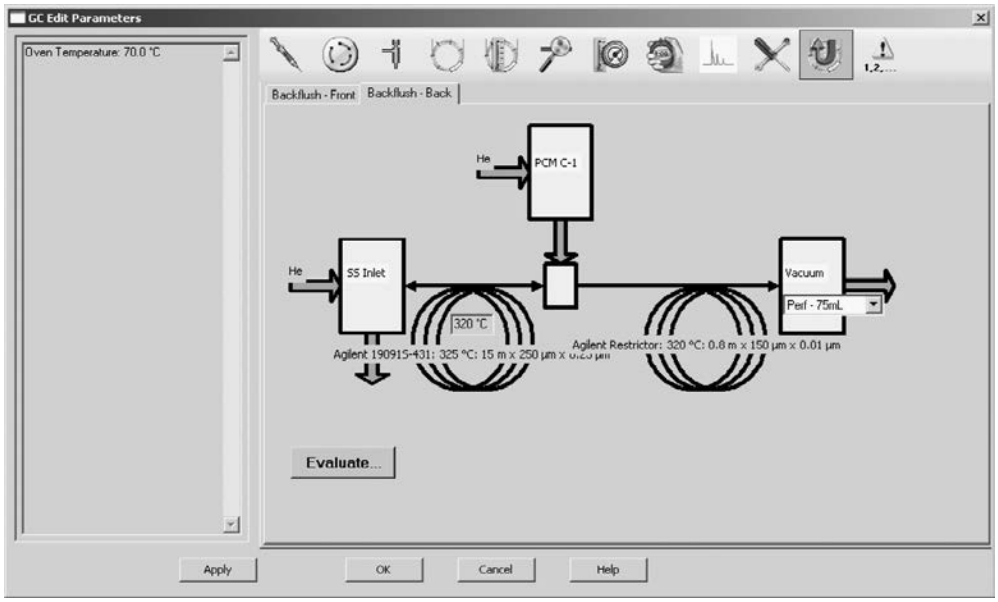

Figure 6. Post-run backflushing screen number 1.

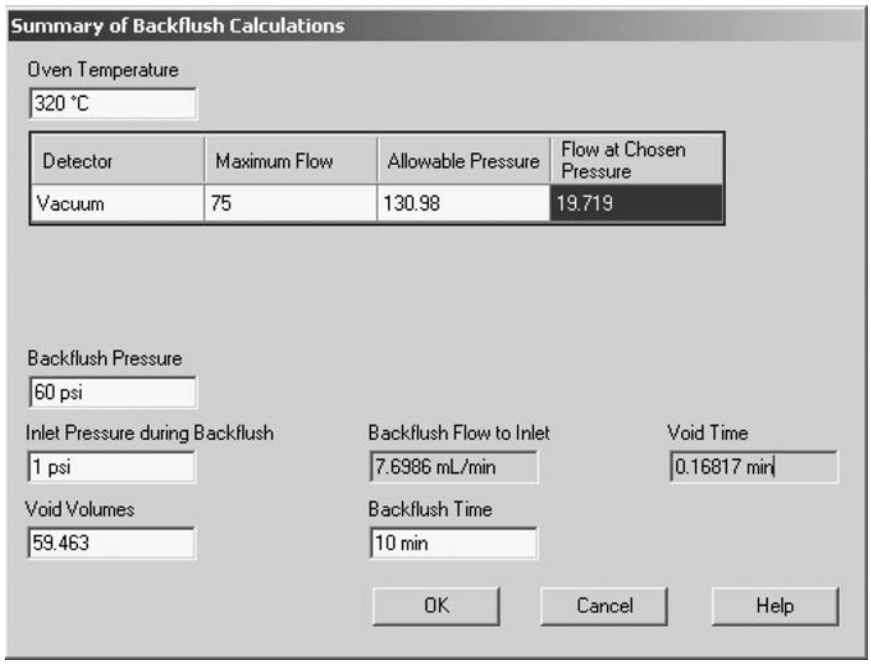

Figure 7. Post-run backflushing screen number 2.

Before applying the backflush conditions to the method the user is presented with a convenient summary of the back-flush conditions. See Figure 8.

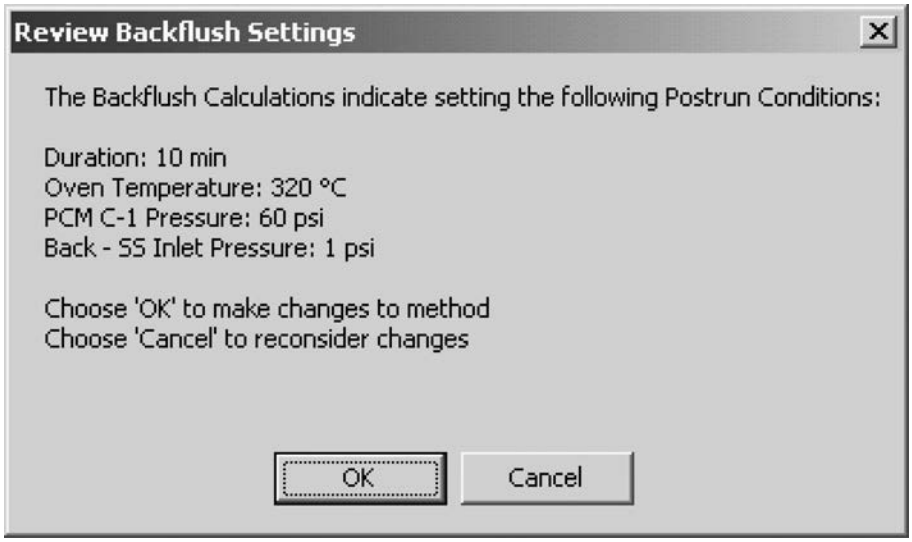

Figure 8. Post-run backflushing screen number 3.

Another injection of the sediment including backflush was made followed by a blank injection of solvent. Figure 9 shows the overlaid TIC of the original solvent blank (black) overlaid on the solvent blank after the sediment injection (gray).

No evidence of any matrix material is indicated, demonstrating that all the high-boiling matrix material had been effectively removed by backflushing.

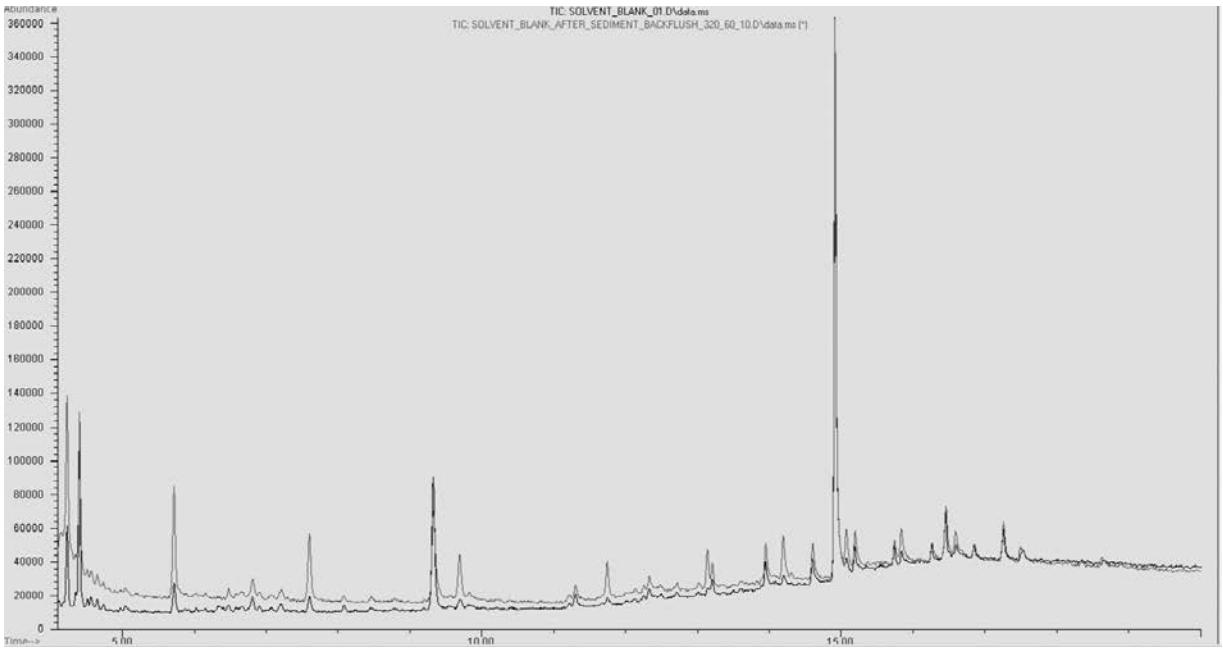

Figure 9. Original solvent blank TIC and solvent blank after sediment injection with post-run backflush (1).

Experiment 3: Backflushing Employed

In order to reduce cycle time for the method, the backflush conditions were modified by increasing the backflush pressure to 80 psig and holding for 6 minutes.

Note that using the backflushing conditions shown in Figure 10 (320 °C, column pressure 1 psig, and splitter pressure 80 psig for 6 minutes), that 46.6 column volumes of carrier gas was used to backflush the column during the post-run period.

Another injection of the sediment was made, followed by a blank injection of solvent. Figure 11 shows the overlaid TIC of the original solvent blank (black) overlaid on the solvent blank after the sediment injection (gray).

No evidence of any matrix material is indicated, demonstrating that all the high-boiling matrix material has been removed by backflushing with the more aggressive conditions as well. These conditions reduced the cycle time for this method 4 minutes compared to the backflushing conditions used in Experiment 1.

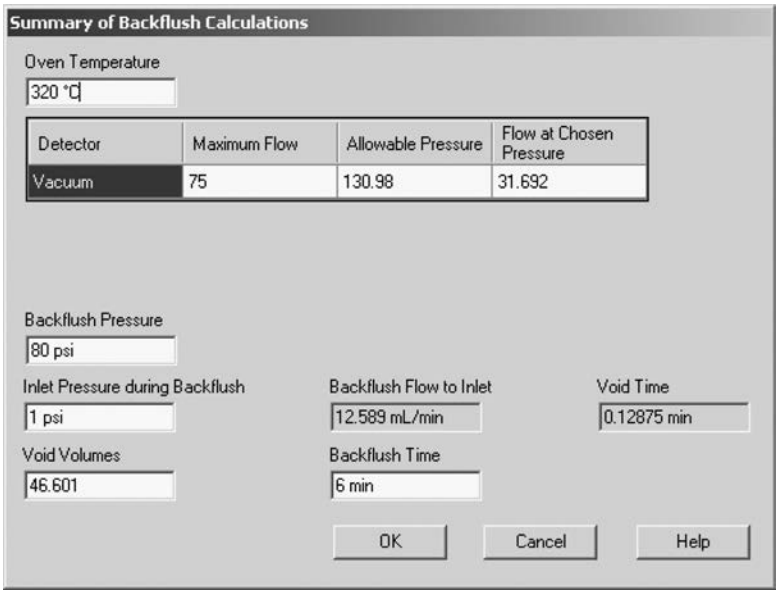

Figure 10. Post-run backflushing screen conditions number 2.

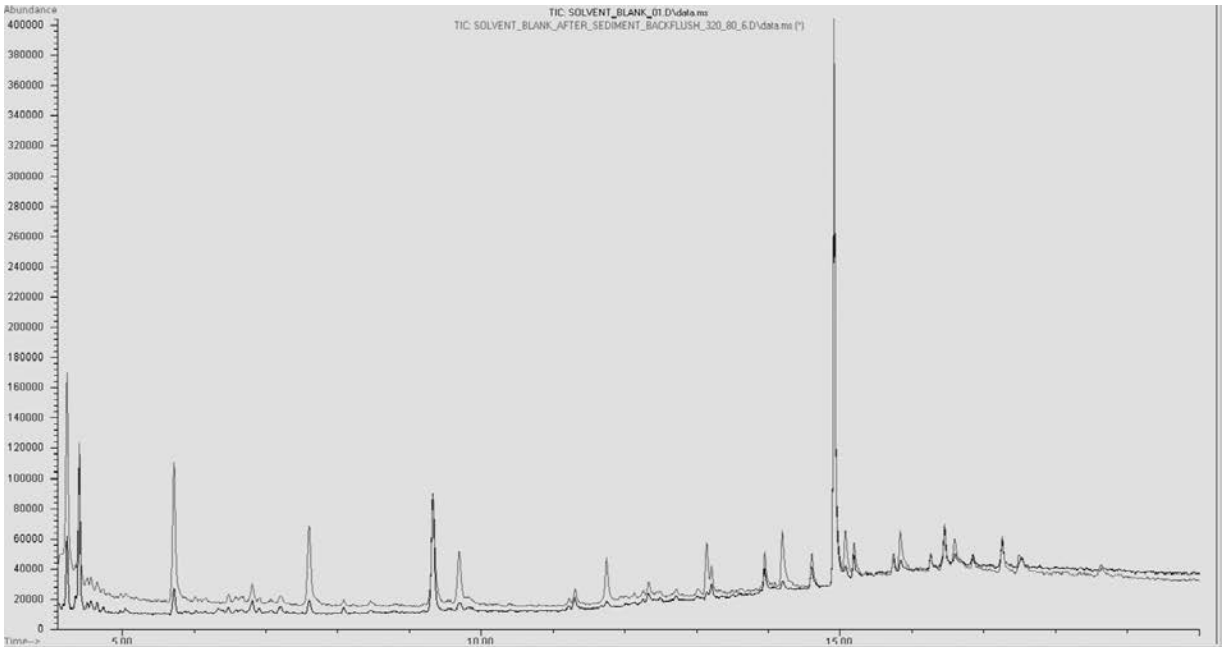

Figure 11. Original solvent blank TIC and solvent blank after sediment injection with post-run backflush (2).

Conclusions

Post-run backflushing was shown to effectively eliminate high-boiling sample matrix in a short amount of time. The major benefits of GC capillary column post-run backflushing include:

- Agilent's capillary flow technology and GC software enable easy and robust setup of GC backflushing.
- Compared to long bake-out periods with flow in the forward direction, a short period of backflushing can remove high-boiling matrix materials more effectively without contaminating the MS ion source.
- Chromatographic cycle time is reduced, columns stay clean, and the integrity of target analyte peak shapes and retention times are maintained.
- For this particular sediment extract the GC column was free of sample matrix after a backflush period of 6 minutes.
- Less system maintenance (ion source cleaning) is required.

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Agilent Technologies

The 5973N inert MSD: Using Higher Ion Source Temperatures

Application Note

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Abstract

The new 5973N inert MSD and ChemStation software (G1701DA) offers the capability of operating the ion source at higher temperatures. This feature, combined with the improved inertness of the source, can provide the user with improvements in analysis, if exploited coherently. This application note provides advice and examples of how to explore the utility of ion source temperature.

Introduction

The default ion source temperature of 230 °C is commonly applied in electron impact (EI) ionization on the 5973 MSD platforms. The new Inert Source when used with the new revision of the ChemStation software (rev. DA) allows ion source temperature to be set to a maximum of 300 °C. As with all advances, there are advantages and disadvantages in operating at higher source temperatures. This note will address several general aspects in EI operation.

Tuning

Figures 1 and 2 show the results for autotuning the Inert Source at the standard 230 °C ion source temperature and the 300 °C temperature limit of the new source (quadrupole temperature 200 °C). The higher temperature for the source produces a perfluorotributylamine (PFTBA) spectrum that shows lower abundances of the higher mass fragments, which is not entirely unexpected. The *m/z* 219 fragment has dropped to an abundance comparable to the *m/z* 69 ion and the ion at *m/z* 502 has dropped about 50%. This is to be expected as the internal energy of the calibrating gas has increased. Note, however, that the isotopic ratios are maintained.

The user should also expect to see a higher background in the higher temperature tunes. A portion of the background will be due to ions associated with column bleed. Bleed, which usually condenses in the source, now is volatized and will appear as an increase in background and baseline.

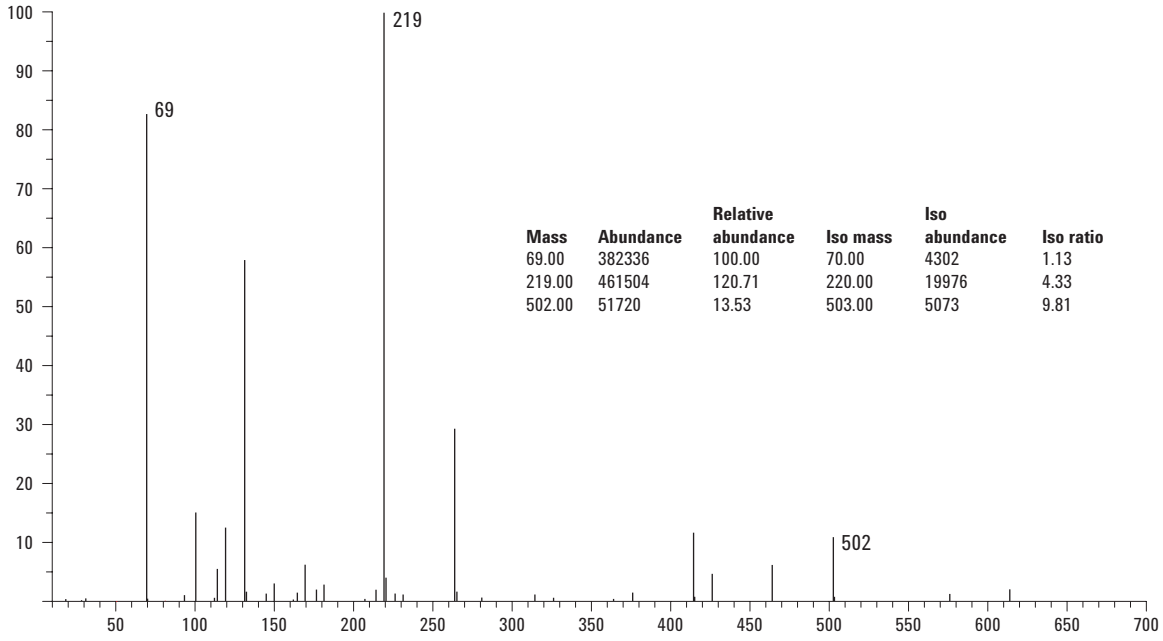

Figure 1. Autotune results for an ion source temperature of 230 °C.

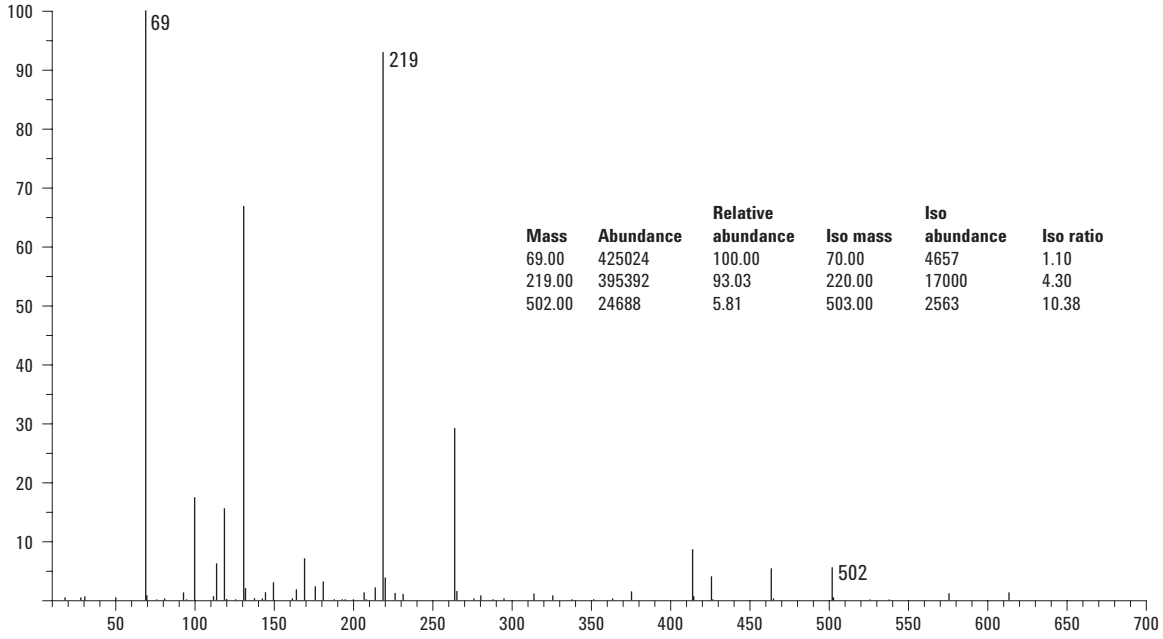

Figure 2. Autotune results for an ion source temperature of 300 °C.

Implications for Analytical Applications

Although the tuning compound showed a spectral change that favored more fragmentation, and all compounds could be expected to be influenced similarly, there are some advantages that can occur for less fragile compounds, especially those that have higher boiling points and are late eluting in GC. Analysis of the class of compounds known as "persistent organic pollutants" (POPs) is likely to benefit from higher source temperatures.

To illustrate the aspects that need to be examined, consider the six polychlorinated biphenyls (PCBs) acquired in full-scan and presented in Figure 3. The overlaid reconstructed total-ion-current chromatograms (RTICCs) suggest that the higher source temperature increases the total response for the later eluting PCBs but produces little enhancement for the early eluters. This could be due to more fragmentation and may not necessarily be useful if the increase in the RTIC is due to lower mass fragments since these lower mass ions are usually compromised by interferences. A calculation of the signal/noise (S/N) for the RTICCs shows that while there is an increase in signal at the source higher temperature, there is also an increase in the background noise and the result is a lower S/N ratio for the higher source temperature.

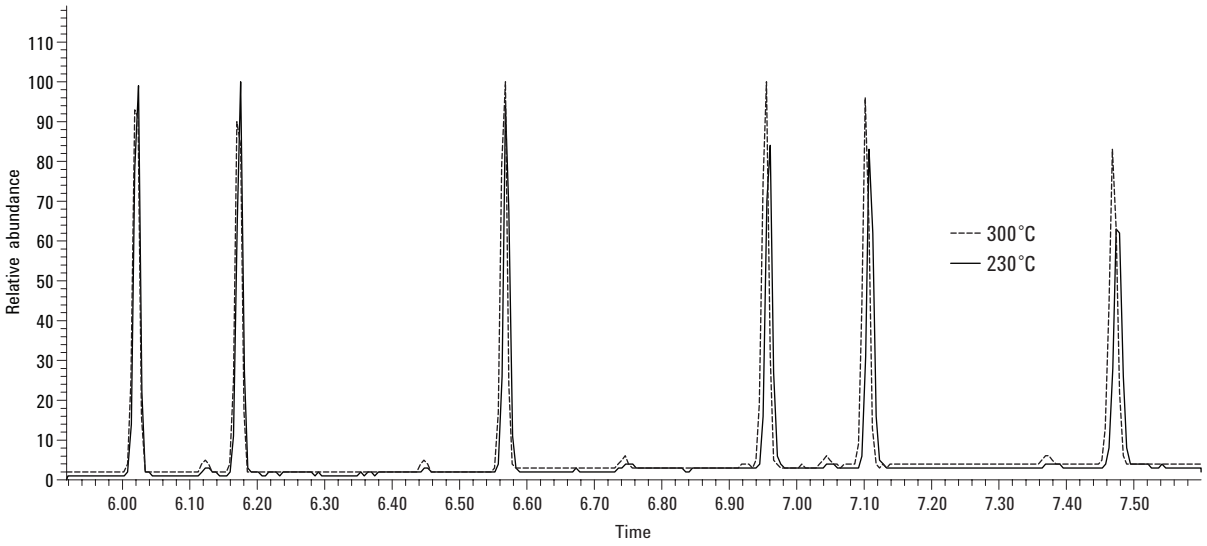

Figure 3. Overlaid RTICC of six PCBs acquired in full-scan (50–505 amu) at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram, the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

Figure 4 shows the same analytes acquired in selected-ion-monitoring mode (SIM) using three ions for each component (M, M+2 or M–2, and M–70). The same trend appears with an enhancement apparent in signal for the later eluting PCBs but little increase for the earlier PCBs. Now, however, the RTIC for the SIM acquisition does show a higher S/N ratio for these later PCBs. As opposed to the full-scan acquisition, the SIM mode acquisition at higher source temperature does increase signal for the ions of interest and, because there was no increase in background, a useful S/N increase was obtained. As always, the guiding principle that an increase in signal is only useful if it exceeds the concomitant increase in background holds. This is clearly illustrated by the third PCB, the pentachlorobiphenyl (Cl_5–B). Figure 5 shows the behavior of the signal and background for the two source temperatures for one of the pentachlorobiphenyl confirming ions. The higher source temperature raises the signal and the background for this ion of interest over the lower temperature but fortunately signal increases faster than background. In this case, the background is due to column bleed components and is unavoidable but fortunately not very intense. This may or may not be the case in sample analysis.

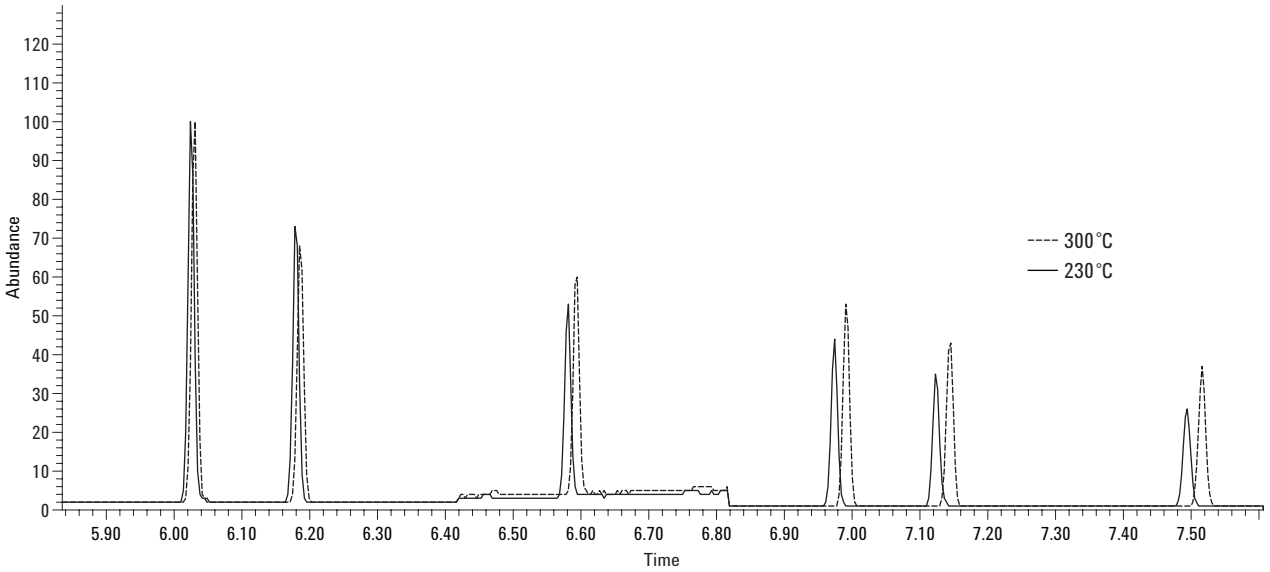

Figure 4. Overlaid RTICC of six PCBs acquired in SIM at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

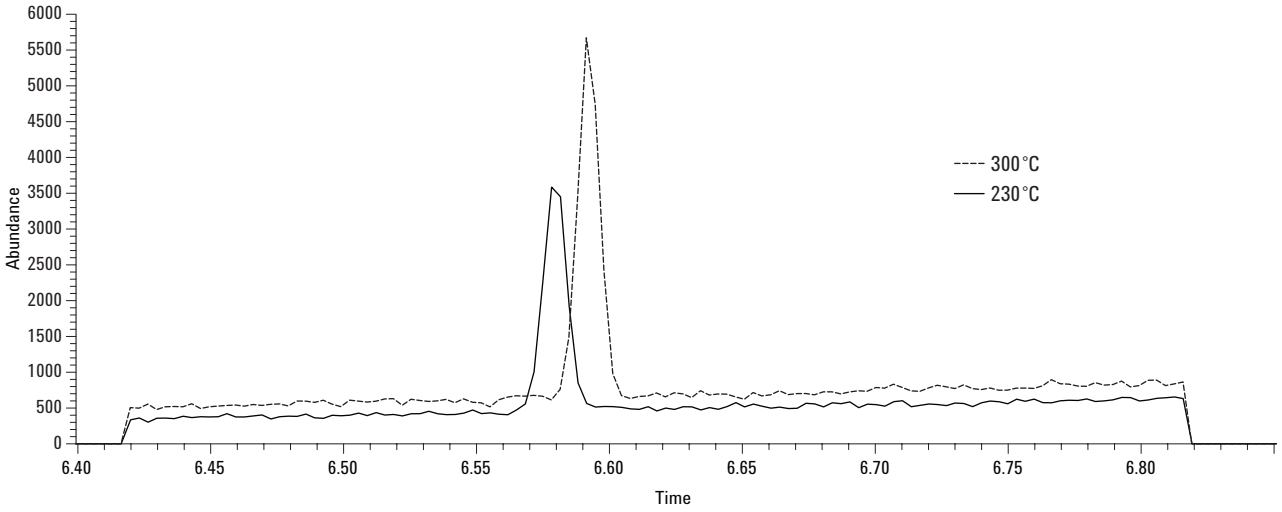

Figure 5. Overlaid extracted ion-current chromatograms of one ion (M-70) for the pentachlorobiphenyl acquired in SIM at source temperatures of 230 °C and 300 °C.

The detection limits for many late eluting, "high-boiling" compounds that will improve by implementing higher source temperatures (for example, PAHs, terphenyls, etc.). As an illustration of the enhancement for very "high-boiling" compounds, consider the 6-ring benzenoid hydrocarbon (PAH), coronene (CAS 191-07-1). This compound is difficult to determine due to low response and poor chromatography, although it is present in many sediment samples. Figure 6 shows overlaid RICCs for acquisitions of coronene at 230 °C and 300 °C. Although the peak area is the same, the enhanced Gaussian peak shape achieved at 300 °C improves detection.

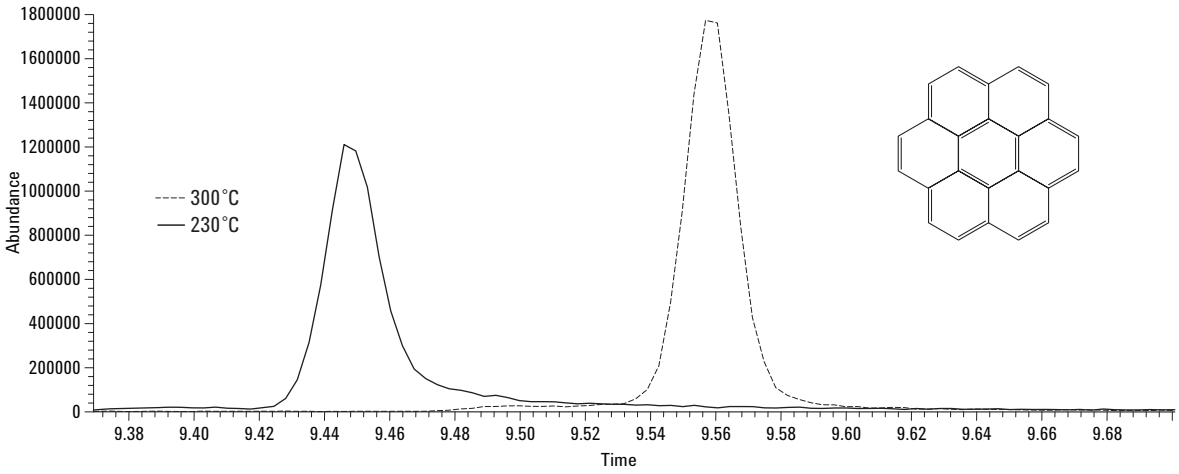

Figure 6. Overlaid extracted ion-current chromatograms of one ion (*m/z* 300) for coronene acquired in full scan at source temperatures of 230 °C, and 300 °C.

Source "Bakeout"

There may be considerable temptation to use the higher source temperature for source “cleaning” by “baking”. In other words, when the user notices a higher background in the source or a reduction in response, the ill-conceived approach of baking the source clean may come to mind. The result will be that “garbage” coating the source will be volatized further into the analyzer; the other lenses will get dirtier, as will the multiplier, etc. “Baking” is not a substitute for mechanical cleaning of the source. However, baking a source after a cleaning is a good approach and a macro that provides this option is given in Table 1. After a source has been cleaned, and the MS system pumped down and checked to be leak free, this macro can be implemented either manually or in a sequence. (Note that the temperature limits in the tune file need to be altered to 300 and 200 for source and quadrupole, respectively). Manually the bakeout is called from the command line in TOP by –

```
macro "bake.mac"  <enter>
bake 2  <enter>
```

The “2” calls for a 2 hour bakeout, and which can be set to anytime the user requires.

Copy the lines in Table 1 into Notepad and save the file as BAKE.MAC in the MSDCHEM\MSEXE directory. The “!” indicates a comment (line) which is not executed. Note that the temperature limits, which reside in the tune file, must be edited to allow the higher settings.

Table 1. ChemStation Macro for Baking the Source and Quadrupole After Source Maintenance

```
name Bake
! this macro sets the source and quad temps to their maximum and holds for a set period
parameter hours def 6                  ! default setting is 6 hours -this is customizable
msinsctl "mstemp QUAD, , , 200"        ! sets the quad temperature to bake at 200C
synchronize
msinsctl "mstemp SOURCE, , , 300"      ! sets the source temperature to bake at 300C
synchronize
SLEEP hours*60*60                      ! bakes for set period
msinsctl "mstemp QUAD, , , 150"        ! sets the quad temperature to operating temp at 150C
synchronize
msinsctl "mstemp SOURCE, , , 230"      ! sets the source temperature to operating temp at 230C
synchronize
return
```

Usually a source cleaning is executed at the end of the working day, and the system pumped down overnight for operation the next day. In this case, a "pumpdown sequence" is useful. After the system is confirmed to be leak-tight, this sequence is loaded and executed which bakes the source and quad overnight, then executes an Autotune, and then makes a few injections of a checkout standard to confirm system performance. In this way, the analyst returns the next day to review data about the system prior to beginning new analyses. An example of this is given in Figure 7.

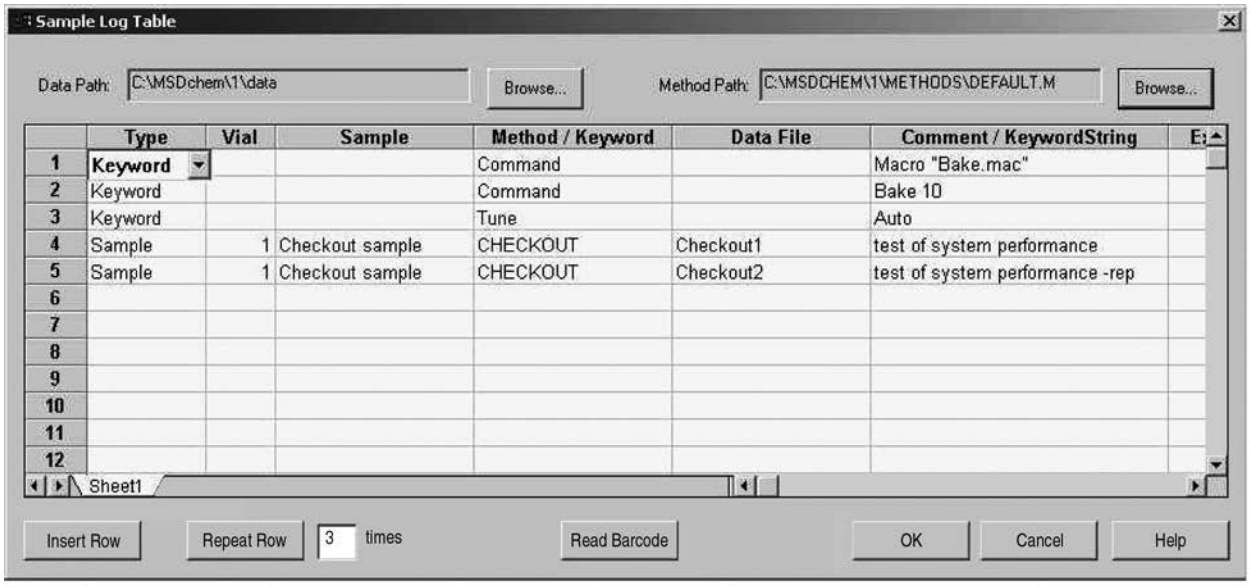

Figure 7. Pumpdown sequence table using source bakeout.

Line 1 Loads the Bake macro. Line 2 sets the bake time to 10 hours. After the bake, (Line 3) an autotune is executed. Lines 4 and 5 run the system performance method, CHECKOUT.M, on the system checkout standard. Note: after the system has been cleaned and leak-checked, the CHECKOUT.M method should be loaded, THEN this sequence should be run!

Conclusions

The increased source temperature limit available on the 5973N inert MSD can provide improved detection limits for common, late-eluting, recalcitrant compounds such as the POPs when properly applied. A requirement, that must be explored, is that the higher source temperatures do not increase compound fragmentation or reduce the intensity of the (useful) higher mass ions. These improvements are most likely to be realized in SIM acquisitions where the increased background that must result from higher source temperatures is not as likely to affect the signal.

This application note also describes a programmed bake-out of the source and quadrupole that can be automatically implemented after source cleaning. This bake-out provides a rapid lowering of the air-water background and can be used within the sequence table as part of the instrument performance checkout.

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Enhanced Reliability of Forensic Drug Testing Using Retention Time Locking

Application Note

Gas Chromatography

January 1998

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Abstract

Retention time locking (RTL) is used to lock retention times when transferring methods from chromatographic instrument to chromatographic instrument, column to column, and detector to detector. The retention times for a derivatized cocaine standard were locked when the method was translated from an Agilent 6890/Agilent 5972 GC-MSD (gas chromatograph-mass selective detector) system to other Agilent GC-MSD systems and when performing column trimming as a routine maintenance procedure on the same system. The retention times, under both circumstances, matched the original retention times within 0.01 minute after locking.

Key Words

Retention time locking, RTL, method validation, forensic drug testing, capillary gas chromatography, MSD, laboratory productivity, cocaine.

Introduction

In large drug testing laboratories, several instruments are used simultaneously to analyze or screen for cocaine and/or its derivatives. To increase laboratory productivity and improve ease of sample identification and quantitation, forensic laboratories could benefit in many ways by matching retention times of analytes and internal standards from instrument to instrument, day to day, and location to location. This is easily achieved using the G2080AA retention time locking (RTL) software tool for GC systems with electronic pneumatics control (EPC) using the Agilent GC ChemStation (version A.05.02).

RTL provides the ability to match chromatographic retention times exactly in any 6890 GC system to those in another chromatographic system with the same nominal column. The RTL software allows rapid, accurate locking of all retention times using columns of the same stationary phase and dimensions (same part number). The concepts, uses, and requirements for RTL have been discussed elsewhere.[1] This application note examines the use of RTL to prepare the retention time vs. pressure (RT vs. P) calculation for a derivatized cocaine standard sample from a method that was optimized for a 6890/ 5972 GC-MSD system. Retention times for the derivatized cocaine sample were easily locked when the same method was transferred to a 6890/Agilent 5973 GC-MSD system or an 5890/5972 GC-MSD system. In addition, retention times of target cocaine derivatives were again locked every time when column trimming was performed in the same GC system.

At the time of this study, the software used to accomplish locking was not available for the GC/ MSD system. A duplicate method was created on a separate GC ChemStation to develop the RTL relationships for the data collected from the GC/MSD systems. The RTL software residing on the GC ChemStation was also used to calculate the recommended locking pressure for the GC/MSD system based on the RT vs. P relationship and the results from the GC/MSD scouting run.

Experimental

Samples of benzoylecgonine (BE) containing cocaine and cocaine-d3 (internal standard) were prepared and analyzed in accordance with methods reported previously.[2,3]

For the analysis of trimethylsilyl derivatives of BE samples, GC systems equipped with split/splitless inlets (splitless mode) and mass selective detectors (5972 MSD or 5973 MSD) were used. A GC/MSD ChemStation was used for instrument control and data acquisition. The experimental conditions for the GC methods are given in table 1.

Table 1. Experimental Conditions

Automatic sampler	Agilent 7673B, 10-µL syringe, 1-µL injection, viscosity delay: 1 sec
Inlet	Split/splitless inlet, 250 °C, splitless mode
Carrier	Helium, constant pressure (1.2 mL/min at 150 °C), vacuum compensation: on
Column	Agilent HP-5MS, 30 m x 0.25 mm x 0.25 µm (part no. 19091S-433)
Oven	150 °C (0.5 min), 20 °C/min to 290 °C (4 min)
Detector	Direct column interface to MSD at 290 °C, autotune with 600 V above autotune voltage, SIM mode with 25-second dwell time
Software	G2070AA GC ChemStation A.05.02, G2080AA RTL software for GC ChemStation, and G1701AA MSD ChemStation A.03.00

Results and Discussion

In this study, a testing laboratory wanted to lock the retention times of the trimethylsilyl derivative of cocaine and its internal standard at 6.70 minutes on any Agilent GC-MSD system. The chemist developing the locking method first ran five analyses using different column head pressures to establish an RT vs. P relationship. The RT vs. P data were manually entered into the RTL software resident on a separate GC ChemStation. The RTL software RT vs. P calibration screen is shown in figure 1. The RT vs. P information is saved in the method and is valid for any Agilent GC system using the same method and nominal column. The result of this RT vs. P relationship is shown in figure 2. Once this relationship is developed, it can be used to lock any other system of the same configuration. To do that, the method is loaded and a scouting run is done at the method's nominal pressure.

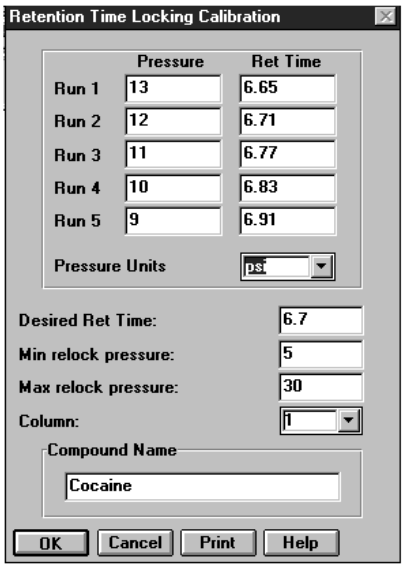

Figure 1. Preparation of retention time locking calibration using the Agilent GC ChemStation.

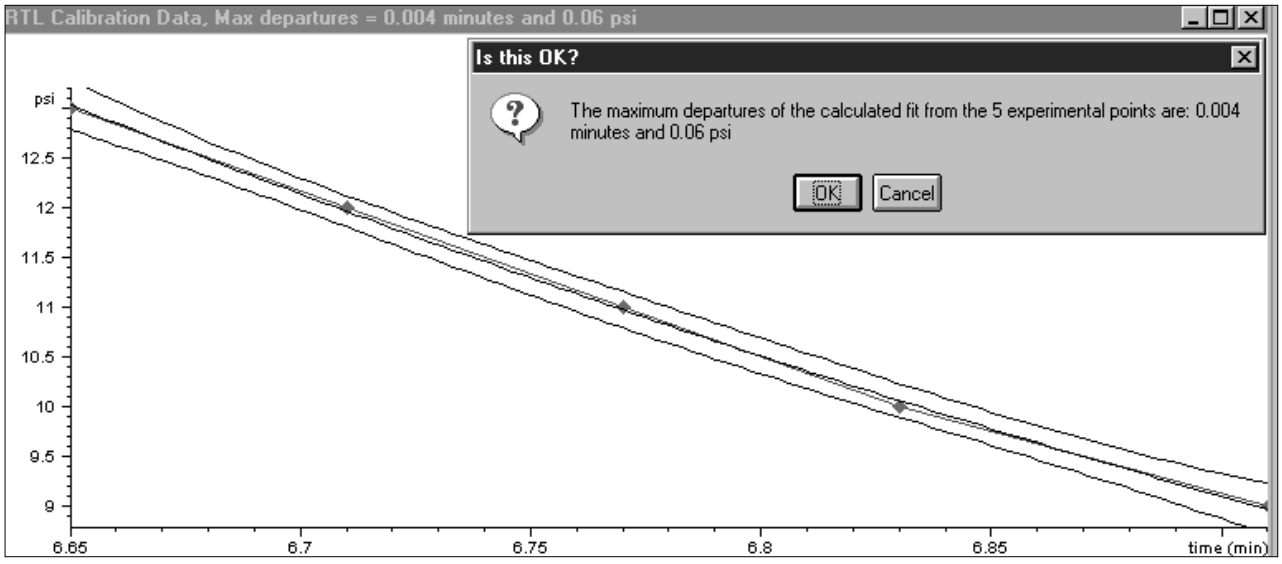

Figure 2. RT vs. P relationship for retention time locking.

On system I (6890/5972 GC-MSD), a scouting run using a head pressure of 12.00 psi yielded a retention time of cocaine at 6.71 min (see table 2). Based on this data set, the RTL software (on the separate GC ChemStation) recommended a column pressure of 12.17 psi for the method (see figure 3).

A column pressure of 12.2 psi was manually entered into the GC/MSD and the extracted ion chromatograms shown in figure 4 were produced. The RT for cocaine was found at 6.69 minutes. In the next five runs at 12.20 psi, the retention time for cocaine was reproducibly obtained at exactly 6.69 min. Extracted ion chromatograms of these runs (m/z 240/256 and 243/259) confirmed that the peak eluting at 6.69 min was indeed cocaine and its internal standard, cocaine-d3 (see figure 4).

Similarly, a good RT match was obtained on system II (6890/ 5973 GC-MSD system) shown in table 2. The same technique was used to

Table 2. Retention Time Locking When Transferring a Method to Different Systems

	Scouting Run		(Re)Lock Run	
	Pressure (psi)	Retention Time(min)	Pressure (psi)	Retention Time(min)
System I	12.00	6.71	12.20	6.69
System II	12.20	7.57	9.10	6.70
System III	12.20	7.33	18.77	6.80
			20.39*	6.69*

* Second (Re)Lock run to fine-tune the method because the experimental retention time falls outside the RT vs. P curve

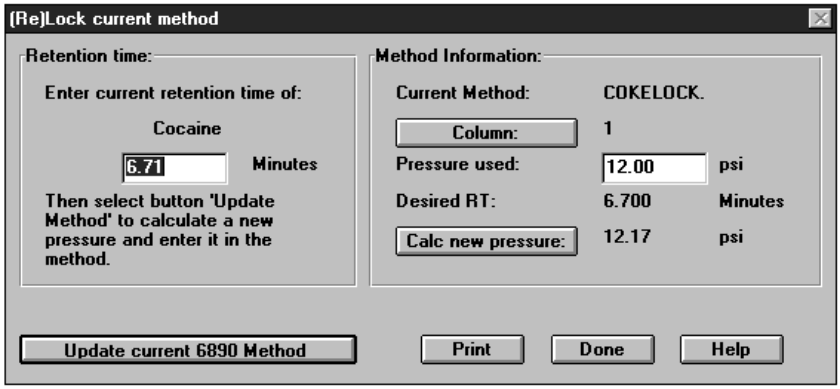

Figure 3. Column pressure recommendation for retention time locking and method update (as found on the RTL software for the GC ChemStation).

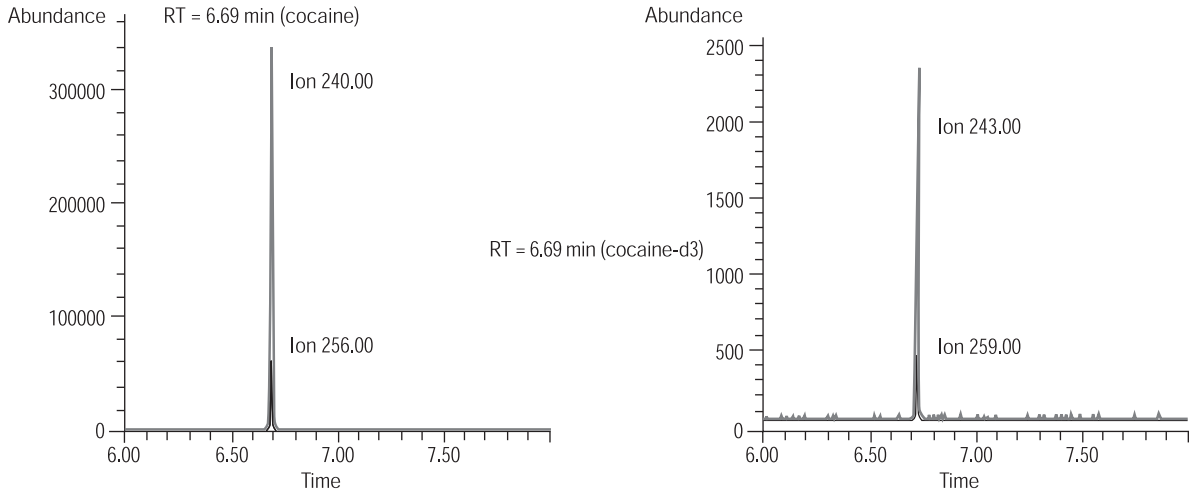

Figure 4. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System I (6890/5972 GC-MSD)

match RT on system III (5890 GC/5972 MSD system)-also shown in table 2. The results shown suggest that system III may have experienced some hardware irregularity. However, the RTL software was able to correct for minor system trouble and to provide recommendations for matching RTs after a third trial to finetune RT matching (see table 2). The recommended column head pressure of 20.39 psi was used to obtain a match for cocaine at 6.69 min for system III. The extracted ion chromatograms confirmed that the analytes that eluted at 6.69 minutes at this column head pressure of 20.39 psi on system III were cocaine and its internal standard, cocaine-d3 (see figure 5).

A routine system maintenance task such as cutting a short length from the head of the analytical column is performed regularly in testing laboratories. The RTs of cocaine were closely matched after each column trimming was performed (see table 3). Each time, a scouting run followed by a (Re)Lock run were performed and an RT of 6.70 min was achieved for cocaine.

First, cocaine was locked at 6.69 min with a pressure of 12.20 psi on system I using an Agilent HP-5MS column with a nominal length of 30 meters. When 14 inches of the column were trimmed off, a scouting run at 12.20 psi yielded an RT of 6.66 min for cocaine. To lock the system, the RTL software suggested a new column pressure of 11.48 psi. The (Re)Lock run yielded an RT of 6.70 min for cocaine (see table 3), and the system was locked again.

The column was trimmed a second time by an additional 14 inches, and a scouting run followed by a (Re)Lock run were performed. The resulting RT for cocaine was locked at 6.70 min with the recommended pressure of 11.00 psi. Finally, the column was trimmed by an additional 28 inches (for a total of 56 inches cut from the 30-meter column). Again, the RT for cocaine was easily locked at 6.70 min with a column pressure of 9.10 psi, which was recommended by the RTL software and manually entered into the GC/MSD (see table 3).

Table 3. Pressures and Retention Times after Performing System Maintenance on System I

Tasks	Scouting Run		(Re)Lock Run	
	Pressure (psi)	Retention Time (min)	Pressure (psi)	Retention Time (min)
Column length, 30 meters			12.20	6.69
Cut 14 inches (30 m - 14 in.)	12.20	6.66	11.48	6.70
Cut additional 14 inches (30 m - 28 in.)	11.50	6.66	11.00	6.70
Cut additional 28 inches (30 m - 56 in.)	11.00	6.60	9.10	6.70

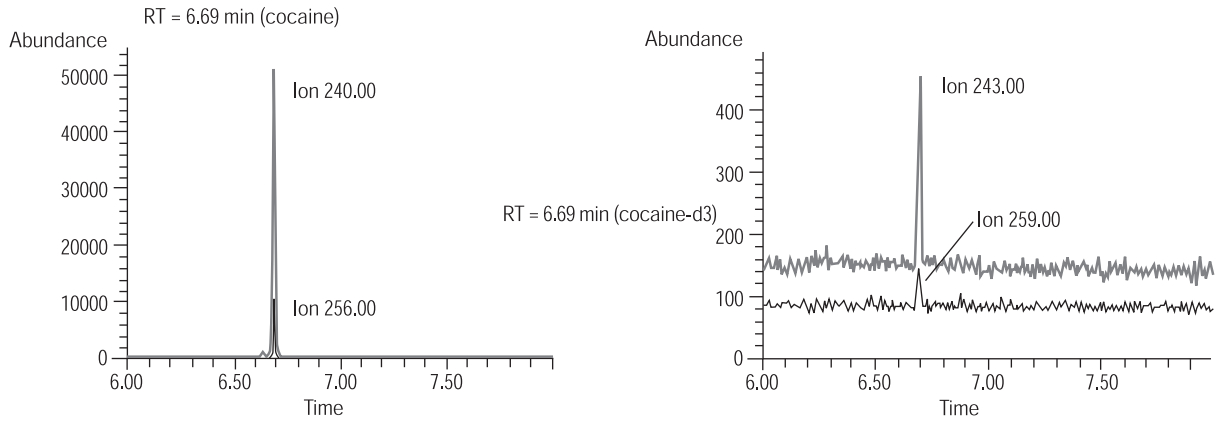

Figure 5. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System III (5890/5972 GC-MSD).

Conclusions

The G2080AA retention time locking software tool was used to lock retention times for a derivatized cocaine sample analyzed on three different GC/MSD systems and after column trimming. The retention times under these circumstances matched the original retention time within 0.01 minute. The ability to lock retention times means that one calibration can be used for multiple systems, and data from the past can be easily compared to new data.

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Innovating the HP Way

Fast and Ultra-fast Analysis with the Agilent 1200 Series Rapid Resolution LC System Compared to a Conventional Agilent 1100 Series LC System Using Sub 2-µm Particle Columns

Application Note

A. G. Huesgen

Abstract

Due to an increasing workload in many analytical laboratories, a need to develop analytical methods faster has arisen. Furthermore, developing faster methods for standard columns is critical. Faster method development for faster LC methods is a requirement that can be met with state-of-the-art LC equipment. Even though conventional LC equipment can also provide fast methods, better performance and time savings can be obtained on specially designed LC systems with wider pressure and temperature ranges and lower delay volume - predominantly with 2.1-mm ID columns, where typically lower flow rates are used than on 4.6-mm ID columns. This Application Note shows that shorter run times, shorter equilibration times, and consequently shorter cycle times and more sample throughput are obtained using the Agilent 1200 Series Rapid Resolution LC (RRLC) system.

Agilent Technologies

Introduction

Due to an increasing workload in many analytical laboratories, a need to develop analytical methods faster has arisen. Furthermore, developing faster methods for standard columns is critical. Increasingly more applications are carried out using LC/MS systems, therefore there is also a demand to use narrow-bore columns for full compatibility with most MS engines. Narrow-bore columns with an internal diameter of 2.1 mm and lower have high demands in respect to low delay volumes and dispersion volumes before and after the column. In the following experiment an example is given, showing how fast methods can be developed on an LC system taking advantage of higher pressure and temperature limits of state-of-the-art equipment. In addition, speed and performance comparisons are made between a conventional Agilent 1100 Series LC system and an Agilent 1200 Series Rapid Resolution LC system, using 4.6-mm ID columns and 2.-mm ID columns packed with 1.8-µm particles.

Experimental

An Agilent 1200 Series RRLC system was used with the following modules:

- Agilent 1200 Series binary pump SL with vacuum degasser for applications using 1.8-µm particle columns up to 150-mm length and with internal diameters from 2.1 to 4.6 mm
- Agilent 1200 Series high-performance autosampler SL for highest area precision
- Agilent 1200 Series thermostatted column compartment SL with wide temperature range from 10 degrees below ambient up to 100 °C
- Agilent 1200 Series diode-array detector SL for 80-Hz operation, including new data protection tool
- ZORBAX SB C-18 columns with different internal diameters and 50-mm length, packed with 1.8-µm particles
- Low dispersion kit for optimized conditions for 2.1-mm ID columns (Agilent part number G1316-68744)

An Agilent 1100 Series LC system was used with the following modules:

- Agilent 1100 Series binary pump with vacuum degasser
- Agilent 1100 Series well-plate autosampler
- Agilent 1100 Series thermostatted column compartment
- Agilent 1100 Series diode-array detector B
- Low dispersion kit for optimized conditions for 2.1-mm ID columns (Agilent part number 5065-9947)

Results and discussion

In the past the Agilent 1100 Series LC system was frequently used for fast and ultra-fast analysis[1]. The instrument is very well suited specifically for the analysis of compounds using short 4.6-mm ID column packed with 1.8-µm particles, and run times below one minute. Cycle times below two minutes were achieved. The Agilent 1200 Series RRLC system is a newly developed LC system with a wider pressure and temperature range, lower system delay volumes and improved noise for the DAD system. Due to these advancements, speed and performance have improved compared to an Agilent 1100 Series LC system, especially for columns with an internal diameter of 2.1 mm.

Experiments using a 4.6-mm ID column

Both instruments were set up in a standard configuration with mixers and 0.17-mm ID flow capillaries installed. Typically the same parameters can be used to optimize an LC method for speed and resolution. These parameters are flow rate, column temperature, gradient profile and other instrument-specific parameters such as switching the autosampler delay volume out of the flow path after the sample has reached the top of the column (ADVR=automatic delay volume reduction). Gradient changes can therefore reach the column much faster. A typical example of how a fast method can be developed is given in figure 1. The objective is to achieve fast cycle times and a minimum resolution of 2 for all peaks.

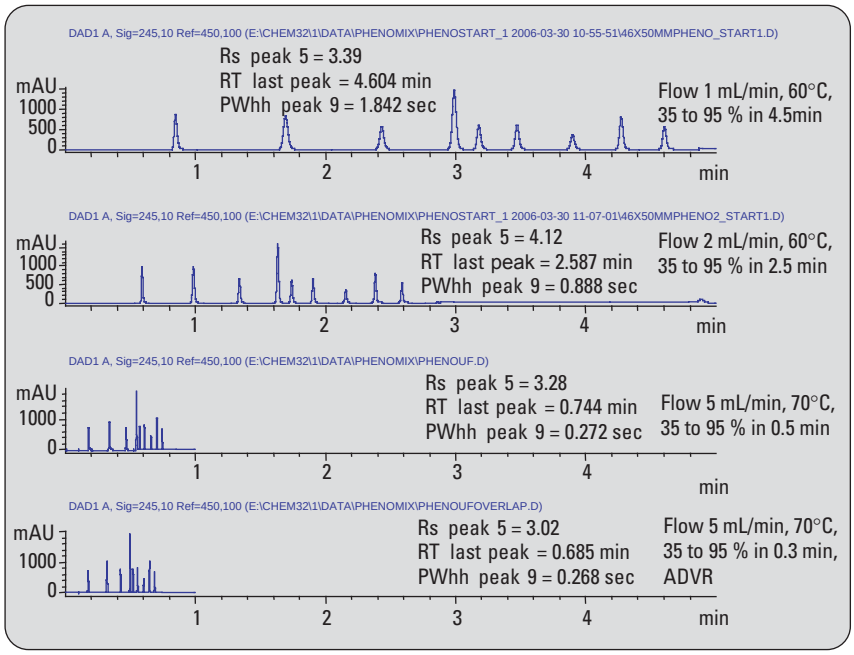

Figure 1
Method development of an ultra fast LC method.

Chromatographic conditions:

Test sample:	Set of 9 compounds; 100 ng/µL each; dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3: Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6.Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 4.6 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O + Solvent B: ACN Gradient: 35 to 95 % B using different profiles
Autosampler:	Injection volume: 1 µL Wash 5 sec for needle exterior flush out factor 20
Thermostatted column compartment:	Temperature: different temperatures
Diode array detector	B and diode-array detector SL: Signal: 245/10 nm Ref 450/100 nm

Optimization of all of the above-mentioned parameters on both systems resulted in the chromatograms shown in figure 2. The pressure limit of 400 bar on the Agilent 1100 Series LC system restricts the maximum possible flow. 5 mL/min flow was not possible, even though the column temperature was set to 80 °C, which is the upper limit for the 1100 Series column compartment. The Agilent 1200 Series RRLC system can be operated with up to 600 bar and up to 100 °C. Applying a flow rate of 5 mL/min can be done without reaching the 600 bar pressure limit at elevated temperatures. In addition, due to design changes, the noise level of the Agilent 1200 Series DAD SL has significantly improved compared to the Agilent 1100 Series DAD B.
The performance for both systems is shown in table 1.

Resolution and noise have improved with the Agilent 1200 Series RRLC system, whereas run and cycle times are comparable. The noise level of the 1200 Series RRLC system can be further reduced using the post column cooling device[2]. The device adapts the temperature of the column effluent to the temperature of the optical unit. This further reduces the noise level, especially if high flow rates and high temperatures are used. Another possibility to reduce cycle time is to enable the overlapped injection features, which is possible with both systems.

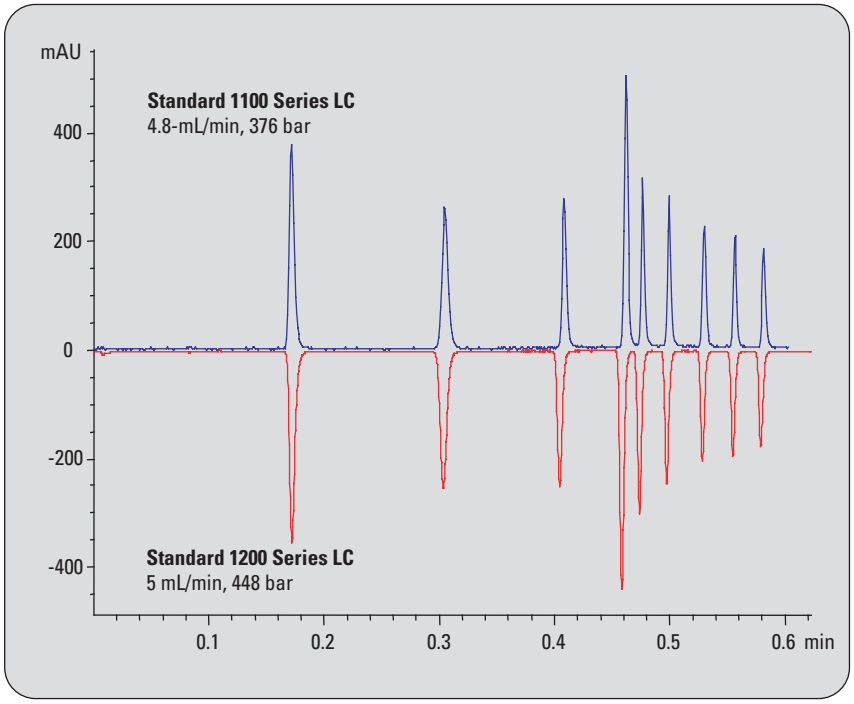

Figure 2
Standard Agilent 1200 Series RRLC system vs. Agilent 1100 Series LC system: analysis of phenone mix on 4.6-mm ID column packed with 1.8-µm particles.

Chromatographic conditions:

Test sample:	Set of 9 compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3: Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 4.6 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O, Solvent B: ACN Gradient: 35 to 95 % B in 0.3 min
Autosampler:	Injection volume: 1 µL Wash 5 sec for needle exterior, flush-out factor 20
Thermostatted column Compartment:	Temperature: 80 °C
Detector DAD B and DAD SL:	Signal: 245/10 nm Ref 450/100 nm

_Parameter	Standard 1100 Series 80 °C 4.8 mL/min	Standard 1200 Series 80 °C 5 mL/min
Flow rate	4.8 mL/min	5 mL/min
Run time	0.60 min	0.60 min
Cycle time	1 min 37 sec	1 min 37 sec
Rs Peak 5	2.22	2.30
PW1/2 peak 9	0.00378 min	0.00375 min
PW1/2 peak 1	0.00458 min	0.00486 min
Noise PtoP	6.2021mAU	0.7930 mAU
Backpressure	376 bar	448 bar
Injection volume	1 µL	1 µL
DAD data rate	20 Hz, path 10 mm	80 Hz, path 10 mm

Table 1
Performance comparison for 4.6-mm ID column.

Furthermore, column switching valves can be installed in the ovens, which provides even higher sample throughput using 2 columns for analysis. A sample is analyzed on the first column, while the second column is regenerated using a second pump. If the analysis on the first column is completed, the next injection can be immediately performed on the previously equilibrated second column.

Experiments using 2.1-mm ID column

Columns with an internal diameter of 2.1 mm and lower have high demands regarding low delay volumes and dispersion volumes before and after the column. Using columns with an internal diameter of 2.1 mm, the Agilent 1100 Series binary LC system must be optimized without using a mixer or only a mixer with a significantly smaller volume and capillaries with smaller IDs for all flow connections. Nevertheless, cycle times below 2 minutes could barely be achieved using columns packed with 1.8 µm particles and 50 mm length. This was mainly due to the pressure limitation of 400 bar for the Agilent 1100 Series LC system. In addition, the delay volume of the 1100 Series LC system is a drawback for fast run and equilibration times. With the introduction of the Agilent 1200 Series RRLC system this gap was closed. Now using narrow bore columns packed with 1.8-µm particles, run times below 0.5 min are possible, with higher flow rates and elevated temperatures. Both systems are compared using the same column and optimized instrument configurations. To allow for optimized conditions for both systems, the following set-ups were used:

Configuration of the Agilent 1100 Series LC system:

- The mixer was replaced by a short capillary with an internal diameter of 0.12 mm (Agilent part number G1312-67301)
- Seat and seat capillary were replaced by 0.12-mm ID parts (well-plate seat, Agilent part number G1367-87104, and seat capillary, Agilent part number G1313-87103)
- The capillary from the injector to the column compartment was replaced with a 0.12-mm ID capillary (Agilent part number 01090-87610)
- The 0.17-mm ID capillary from the column compartment to the column was exchanged with a capillary with an internal diameter of 0.12 mm (Agilent part number G1316-87303)
- The column was connected to the detector using the detector inlet capillary.
- A 1.7-µL cell with a path length of 6 mm was used as the detector cell.

Configuration of the Agilent 1200 Series RRLC system:

- The low delay volume configuration for the pump was set up with a 120-µL delay volume (mixer and damper were moved out of the flow path).
- Two flow capillaries were replaced with 0.12-mm ID capillaries, all included in the Agilent 1200 Series low dispersion kit (Agilent part number G1316-68744).
- The seat capillary was also replaced with a 0.12-mm ID capillary (included in kit Agilent part number G1316-68744)
- The DAD SL 2 µL flow cell with a 3-mm path length was used. The inlet capillary was directly connected to the column outlet.

The same 2.1 x 50 mm column was used for both systems. The flow rate was set so that the backpressure was close to the limit of each system. Automated delay volume reduction (ADVR) was selected in the injector setup screen for both systems. The injection volume was set to 1 µL for the Agilent 1100 Series LC system, and to 2 µL for the Agilent 1200 Series RRLC system to compensate for the lower path length of the 1200 Series 2-µL flow cell.

In figure 3 an overlay of the chromatograms obtained from both systems is shown. In table 2 the performance for both system is recorded.

The chromatograms in figure 3 clearly demonstrate the advantages of the Agilent 1200 Series RRLC system, using 2.1-mm ID columns, packed with 1.8-µm particles. Faster run times and cycle times are possible, due to the fact that higher flow rates can be obtained with the Agilent 1200 Series RRLC system. Table 2 indicates that the cycle time for the Agilent 1200 Series RRLC system is only half that of the Agilent 1100 Series LC system. In addition, the resolution of the 5th peak and also peak width at half height is significantly improved at higher flow rates.

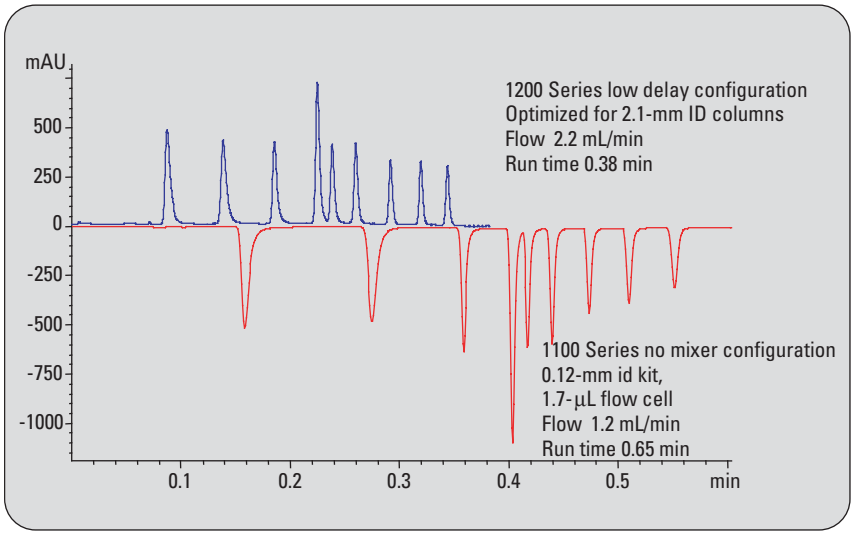

Figure 3
Analysis performed with a 2.1-mm ID column with the optimized Agilent 1200 Series RRLC system and the optimized Agilent 1100 Series LC system using automated delay volume reduction for both systems.

Chromatographic conditions:

Test sample:	Set of 9 compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3. Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 2.1 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O , Solvent B: ACN Gradient: 35 to 95 % B in 0.3 min
Autosampler:	Injection volume: 1 and 2 µL Wash 5 sec for needle exterior, flush out factor 20
Thermostatted column compartment:	Temperature: 80 and 95 °C
Detector DAD B and DAD SL:	Signal: 245/10 nm Ref 450/100 nm

Parameter	1100 Series, optimized, no mixer, ADVR, 80 °C	1200 Series, optimized, low delay volume configuration, ADVR, 95 °C
Flow rate	1.2 mL/min	2.2 mL/min
Run time	0.65 min	0.38 min
Cycle time	2 min 33 sec	1 min 16 sec
Rs Peak 5	1.86	2.15
PW1/2 peak 9	0.00556 min	0.00328 min
PW1/2 peak 1	0.00729 min	0.0049 min
Noise PtoP	0.1 mAU	0.2 mAU
Backpressure	370 bar	570 bar
Injection volume	1 µL	2 µL
DAD data rate	20 Hz, path 6 mm	80 Hz, path 3 mm

Table 2
Performance comparison using a 2.-mm ID column.

Conclusions

Faster method development for faster LC methods is a requirement that can be met with state-of-the-art LC equipment. Even though conventional LC equipment can also provide fast methods, better performance and time savings can be obtained on specially designed LC systems with wider pressure and temperature ranges. Predominantly with 2.1-mm ID columns, where typically lower flow rates are used than on 4.6-mm ID columns, an LC system like the Agilent 1200 Series RRLC system provides significantly lower delay volumes. Shorter run times and shorter equilibration times, and consequently shorter cycle times and more sample throughput are obtained.

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www.agilent.com/chem/1200rr

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Agilent Technologies

Achieving fastest analyses with the Agilent 1200 Series Rapid Resolution LC system and 2.1-mm id columns

Application Note

Michael Frank

Abstract

The need to increase the daily throughputs of LC systems is a constant desire. Now, with the Agilent 1200 Series Rapid Resolution LC system highest throughputs are possible, and in combination with the Agilent ZORBAX RRHT columns and the increased pressure and temperature range of the LC system, excellent chromatographic resolution can be achieved even at run times below one minute.

This Application Note describes the correct set-up of the instrument which is the key for optimal results with narrow bore columns, such as a 2.1 mm x 50 mm column packed with sub two micron particles. Peak capacities in the range of fifty in analysis times as short as 24 seconds and peak widths as narrow as 200 milliseconds are shown. The well-balanced use of all possible module options to achieve shortest cycle times with throughputs far beyond 1500 samples per day is described.

Agilent Technologies

Introduction

Particularly analytical service laboratories in the pharmaceutical industry, responsible for analyzing chemical libraries[1] or performing MS based quantifications of certain ADME-properties and drug metabolism studies of drug candidates[2] are faced with the challenge to increase their throughput, but also to maintain a high chromatographic resolution. In 2003 Agilent Technologies introduced sub two micron particles in their RRHT column series. Because of the small particle size, the chromatographic resolution obtainable with these columns is superior to standard particle sizes such as 3.5 µm or even 5 µm. Due to a unique silica manufacturing process, Agilent ZORBAX RRHT columns show a significantly reduced backpressure, if compared to similar column dimensions of other manufacturers. Excellent chromatographic results are achieved in a very short analysis time with the Agilent 1200 Series Rapid Resolution LC system, which facilitates an increased pressure range and flow rates from 0.05 up to 5 mL/min using column diameters ranging from 2.1-mm id up to 4.6-mm id. This Application Note will focus on 2.1-mm id columns only.
Not only are the run times of the analyses important for high throughput, but also the overhead time. The Agilent 1200 Series Rapid Resolution LC system can be optimized to achieve highest throughputs with exceptionally good overall system performance.

Experimental

An important issue when dealing with narrow bore columns, especially in gradient mode where smallest peak widths can be achieved, is to have small extra column volumes. This also includes any volumes in front of the sampling device, because any volume after the solvent mixing point will increase the time for the gradient composition to reach the column. This results in an increased run time. The Agilent 1200 Series Rapid Resolution LC system can be reconfigured within a few minutes to provide appropriate system volumes for different column ids. Here, the pumps are set-up in the low delay volume configuration with an internal volume of approximately 120 µL. All other modules are optimized for lowest delay volumes by using the low delay volume capillary kit (G1316-68744). Consequently, only capillaries of 0.12 mm id are used beyond the injection valve. In the Agilent 1200 Series thermostatted column compartment SL the newly introduced low dispersion heat exchangers with 1.6 µL internal volume were used. In some experiments, the Agilent 1200 Series Rapid Resolution LC is set up for alternating column regeneration to achieve highest throughput using the ACR-capillary kit (G1316-68721) and 2.1-mm id columns[3]. The high pressure rated 2-position/10-port valve in the thermostatted column compartment was only placed into the flow path if alternating column regeneration was used indeed.

The instrument set-up is as follows (figure 1):

- Agilent 1200 Series binary pump SL with the new Agilent 1200 Series micro vacuum degasser
- Agilent 1200 Series high performance autosampler SL
- Agilent 1200 Series thermostatted column compartment SL, equipped with a high pressure, 2-position/10-port valve, facilitating alternating column regeneration
- Agilent 1200 Series diode-array detector SL with a 2-µL/3-mm cell
- ZORBAX SB C18, 2.1 mm id x 50 mm, 1.8 µm

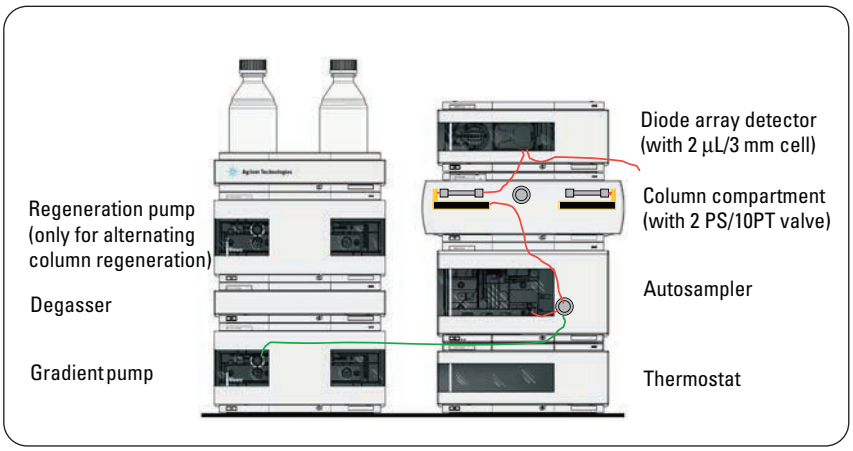

Figure 1
System setup with low delay volume for high speed applications using 2.1-mm id columns with lengths from 20 to 50 mm.

The Agilent 1200 Series binary pump SL is designed to fulfill the demands for high throughput, highest performance, optimum resolution and lowest pump ripple. The pump hardware is significantly different from the standard binary pump. In the Agilent 1200 Series binary pump SL the pressure transducer is separate from the damper which has been modified to have a lower delay volume (pressure dependent ranging from 80-280 µL). In this study the pumps were used in the low delay volume configuration without the mixer and damper in the flow path. In contrast to the standard binary pump the pump heads of the binary pump SL have an additional damping coil (500 µL volume each) to allow damping in the low delay volume configuration. This does not add to the gradient delay volume because it is before the mixing point. Anyhow, pressure ripples are also strongly suppressed by the Electronic Damping Control (EDC). The pressure range of the pump and all other modules is increased to 600 bar.

Only one sample, the so-called "phenone-mix", was used in the course of this study to keep variations low. The sample consists of nine compounds: acetanilid, acetophenone, propiophenone, butyrophenone, benzophenone, valerophenone, hexanophenone, heptanophenone and octanophenone. Unless otherwise stated, the concentration was 0.1 µg/µL for each compound except butyrophenone which was 0.2 µg/µL. The solvent was water-acetonitril 2:1.

Results and discussion

The most frequently sold particle size in chromatographic columns today is 5 µm. Of course, fast and ultra fast LC is also possible with columns packed with particles of these larger diameters – the reduced back pressure is even beneficial to allow higher flow rates. However, resolution will be sacrificed because conditions are usually far on the right side of the van-Deemter-optimum. Here, the big advantage of the RRHT columns with particles of less than 2 µm diameter is proven. The van Deemter optimum is shifted further to the right and the curve is much flatter at the onset because the "resistance of mass transfer" term is diminished (figure 2). In figure 3 the analysis on a 2.1-mm id column with 1.8-µm particles is compared to the linear scaled analysis on the same stationary phase but on 5 µm particles packed in a 4.6-mm id-column. The gain in resolution is obvious – from Rs = 2.1 up to Rs = 3.5 for the critical pair which matches the theoretically expected value of a 1.66 fold increase in resolution. Also note that there is a saving in solvent consumption of 8.6 mL in the "standard" HPLC analysis and only 1.8 mL in the ultra fast HPLC analysis.

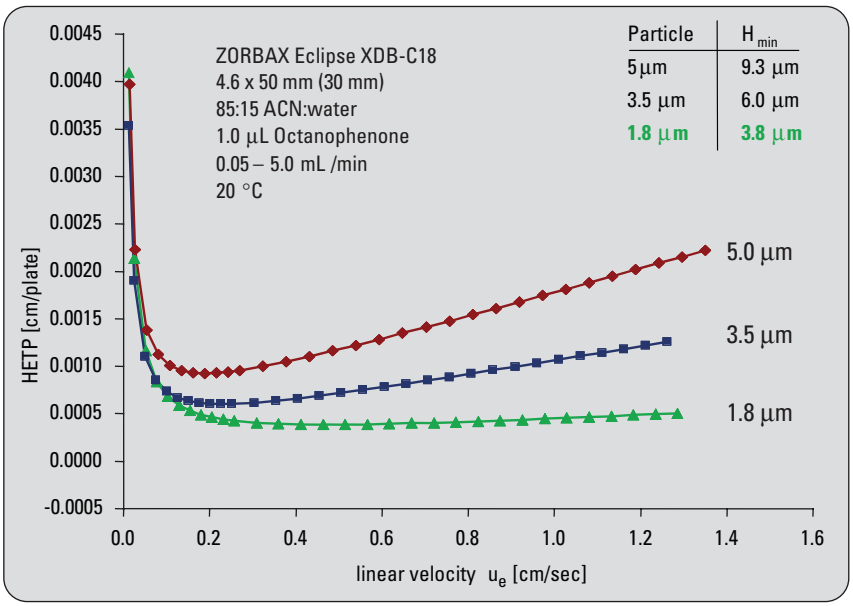

Figure 2
Van Deemter curves of columns packed with 1.8 µm, 3.5 µm and 5.0 µm particles.

For gradient separation the dependencies of the capacity factor can be expressed as:

$$k^* = 0.87 \cdot tg \cdot \frac{F}{Vm \cdot \Delta\%B \cdot S}$$

(tg = gradient time, F = flow rate, Vm = column void volume, △%B = gradient steepness, S = solvent and solute dependent factor)

If the product of the gradient time and flow rate, the so-called gradient volume, is kept constant together with all other parameters, the gradient time might be decreased while the flow rate is increased. Thus, the capacity factors of two compounds will stay constant and if no large alteration of the plate height occurs, the resolution will not change significantly, either. The final point is the big advantage of the sub two micron particles – the van-Deemter curve is nearly flat on the right side of the minimum (figure 2) and flow rates can be increased with only little increase in plate heights. However, the equation is an empirical one and deviations may occur especially under extreme conditions.

With a two-step approach, highest gradient speeds with virtually no loss or only little loss in resolution can be achieved. In the first step, start from a medium temperature and begin to increase the flow rate up to the pressure maximum. Subsequently the temperature should be increased to lower the viscosity of the solvent and then the flow rate is increased again. It may be worthwhile to check the resolution with two identical gradients but with different temperatures to see the influence of the temperature change on the resolution which may be very compound dependent. In figure 4 the result of this approach is shown. A nearly 7-fold increase in separation speed could be achieved with still baseline separation of the critical pair before meeting the pressure and temperature limit (the maximum temperature is a function of flow, temperature, number of controlled Peltier elements and of the heat capacity of the solvent used).

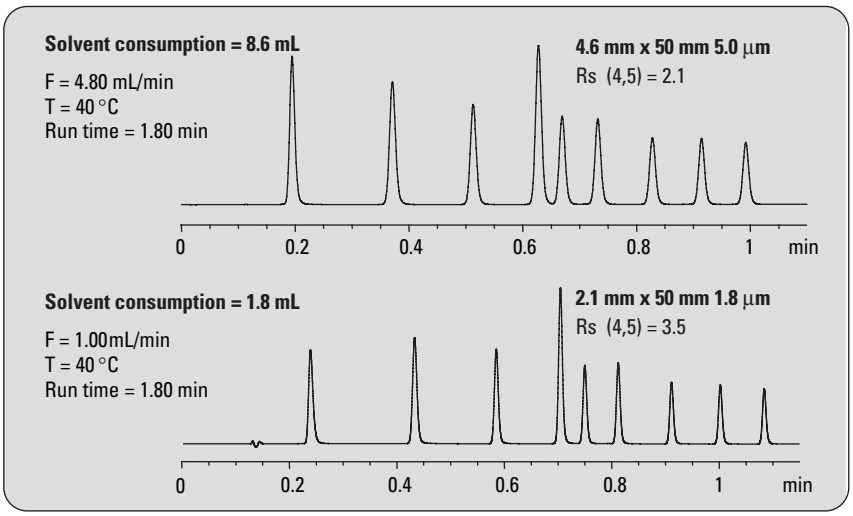

Figure 3
Analysis with 1.8-µm particle column vs. 5.0 µm particle column.

Conditions:	4.6-mm id column used on standard Agilent 1200 system	
Solvent:	A = Water, B = ACN	
Temperature:	40 °C	
Column:	**2.1 mm x 50 mm, 1.8 µm**	**4.6 mm x 50 mm, 5.0 µm**
Flow:	1.0 mL/min	4.8 mL/min (scaled from 2.1 mm col.)
Gradient:	0.00 min 35 %B	0.00 min 35 %B
	0.90 min 95 %B	0.90 min 95 %B
	1.10 min 95 %B	1.10 min 95 %B
	1.11 min 35 % B	1.11 min 35 % B
Stoptime:	1.15 min	1.15 min
Posttime:	0.70 min	0.70 min
Wavelength:	245 nm (8), ref. 450 nm (100)	245 nm (8), ref. 450 nm (80)
Peakwidth:	>0.0025 min (0.05 s res.time), 80 Hz	>0.01 min (>0.2 s), 20 Hz
Injection volume:	1 µL	5 µL (not scaled)

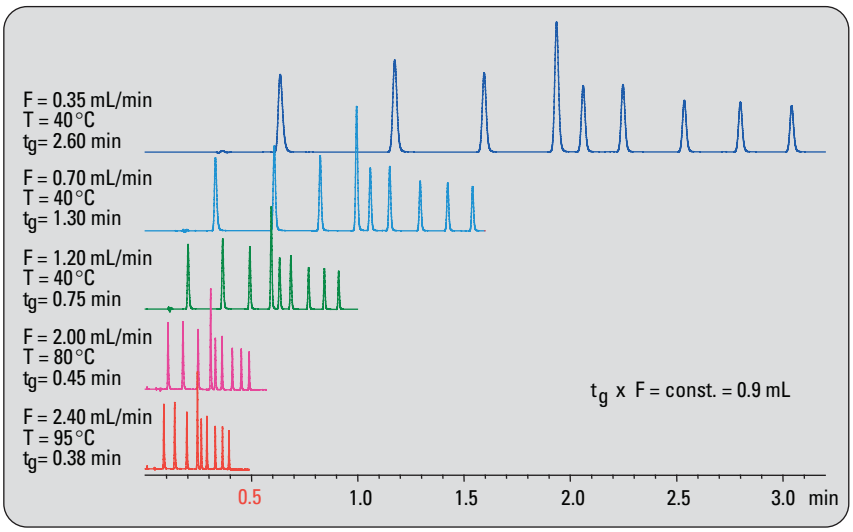

Figure 4
Increasing separation speed by increasing temperature and flow rate while decreasing gradient time.

Conditions:
Solvent: A = water, B = ACN
Temp.: 40 °C, 80 °C, 95 °C
Flow: 0.35, 0.70, 1.20, 2.00, 2.40 mL/min
Gradient: 0.00 min 35 %B
2.60 min 95 %B
3.20 min 95 %B
3.21 min 35 %B
Time values for F = 0.35 mL/min. For all other flow rates times are scaled so that (tg x F) = 0.90 mL
Stop time: 3.20 min
Post time: 2.00 min
Wavelength: 245 nm (8), Ref. 450 nm (100)
Peak width: >0.0025 min (0.05 s response time), 80 Hz

The last chromatogram is enlarged in figure 5 and reveals the details of this separation. The first peak is eluted after only five seconds and peaks with a width at half height of less than 200 ms are achievable. Within twenty-four seconds nine compounds are separated with a peak capacity in the range of fifty.

Retention time precision at highest analysis speed

High analysis speed is meaningless without precision. One basic performance criteria for HPLC pumps is the precision of gradient formation measured by the precision of retention times of repeated gradients. However, the stability of the column temperature must also be taken into consideration, because temperature fluctuations will also influence the retention times of a given sample. In table 1 and figure 6 the results from the 10-fold repeated analysis of a standard sample are listed and since the deviation between individual runs is so small, the octanophenone peak is enlarged in a separate window. This sample contains compounds that are both not retained and refer to isocratically eluted compounds found at the starting conditions of the gradient, as well as highly unpolar and strongly retained compounds. The analyses

Conditions:

Solvent:	A = Water, B = ACN
Temp.:	40 °C, 80 °C
Flow:	0.35 mL/min, 1.20 mL/min, 2.0 mL/min
Gradient:	0.00 min 35%B
	2.60 min 95%B
	3.20 min 95%B
	3.21 min 35%B
	Time values for F = 0.35 mL/min.
	For all other flow rates times are
	scaled so that (time x flow) = 0.90 mL
Stop time:	3.20 min
Post time:	2.00 min
Injection vol.:	1.0 µL

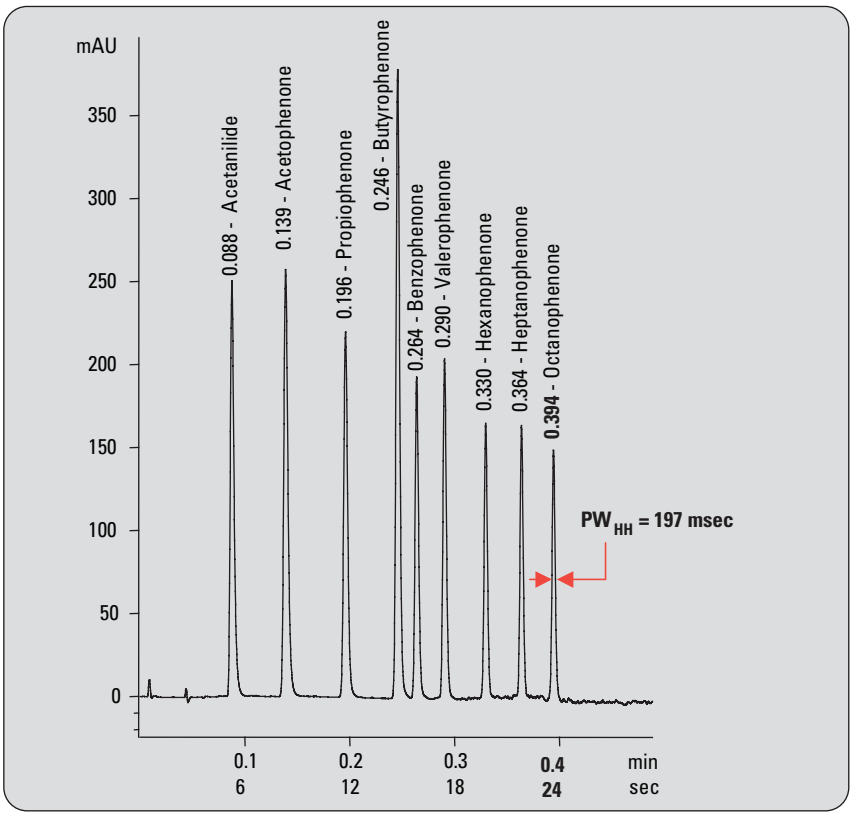

Figure 5
Separation of a nine compound mixture under ultra fast conditions.

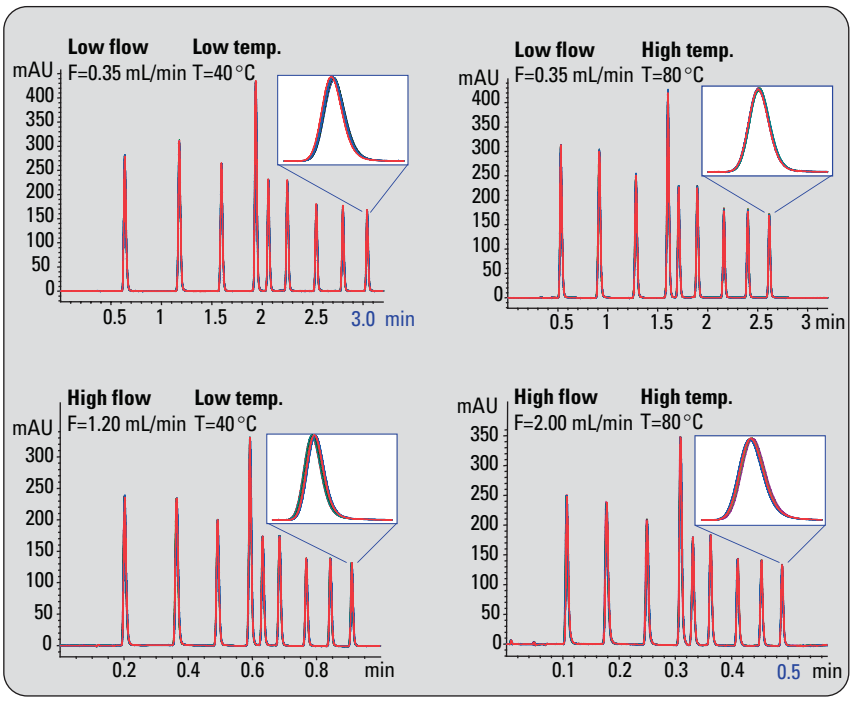

Figure 6
Overlaid chromatograms of the repeated analysis of a 9 compound mixture under various conditions.

were done at high and low flow rates as well as with high and low temperatures as in the examples shown earlier. In all cases the mean retention time precision is below 0.3 % RSD, which was the specification of the Agilent 1100 Series LC system. Of course, the results are also in line with the specifications for the new Agilent 1200 Series Rapid Resolution LC system which is < 0.07 % RSD or < 0.02 min SD, whichever is met first. At these high gradient speeds, the SD criteria are always met. The RSD criteria are also met for both fast-LC gradients of 2.6 min duration (0.35 mL/min flow rate). Even at ultra-fast gradient speeds, the retention time precisions are still below or only slightly higher than 0.1% RSD (table 1).

Improving the cycle-time

Not only is the gradient speed important when dealing with high-throughput analysis but furthermore the over all cycle time of the entire system, which is the time between two consecutive analyses. A good method to measure the cycle time is by using the time stamp the data file is assigned by the operating system of the computer. Clearly, optimizing the cycle time has some drawbacks. For example, extensive needle cleaning procedures are in contradiction with a high sampling speed. Table 2 gives an overview of important parameters influencing the cycle time. Using 1.8-µm particle size columns together with an optimized HPLC system very short run times can be achieved without sacrificing chromatographic resolution. Combining short run times together with low overhead times will result in a high daily throughput. In figure 7 the cycle time and daily throughput is shown for two

	0.35 mL/min, 40°C		0.35 mL/min, 80°C		1.20 mL/min, 40°C		2.00 mL/min, 80°C	
	SD	% RSD	SD	% RSD	SD	% RSD	SD	% RSD
Average	0.00107	0.067	0.00084	0.070	0.00048	0.098	0.00031	0.134

Table 1
Standard deviations (mAU) and %RSD (n=10) of the retention times under different chromatographic conditions in temperature and flow.

Module	Parameter	Effect on cycle time	Other effects
Pump	Low delay volume setting	Reduced retention times, run time can be shortened, reduced cycle time	Increased pressure ripple, slightly increased mixing noise if modifiers such as TFA are used.
Autosampler	Automatic Delay Volume Reduction (ADVR) – activated	Reduced delay volume, reduced retention times, run time can be shortened, reduced cycle time	Increased carry-over
	ADVR activated and Overlapped Injection (OI)	Enables parallel sampling, thus reduces the cycle time independently of the below listed settings (as long as the overall sampling speed does not exceed the gradient and post time)	Increased carry-over
	no OI – Needle Wash	Increased sampling time with increasing wash time	Reduced carry-over with longer needle wash time
	no OI – Equilibration time	Increased sampling time with increased equilibration time	Better injection precision with longer equilibration time
	no OI – Draw/Eject speed	Low speed causes increased sampling time	Low speed results in better injection precision
Column compartment	Alternating column regeneration	Saves column wash-out and equilibration time, reduces cycle time enormously	Additional hardware required, slightly increased extra column volume, slightly different retention times between columns possible
Detector	Pre-run and/or post-run balance	Increased cycle time	Baseline drifts possible if not applied
	Spectral data acquisition with high data rate, small band width and broad wavelength range large data files	Depending on computer power and additional processes running might increase cycle time because of writing speed	Reduced information content if no spectral data acquired or with lower resolution
Software	Data analysis with acquisition	Increased cycle time, depending on computer power and number of peaks	Data analysis has to be done offline is no set
	Save method with data	Slightly increased cycle time	Information is missing if method is not saved
	Execution of pre-run or post-run macros	Increased cycle time, depending on macro	Depending on macro
System	LC controlled over local network between computer and LC (and MS) only	Faster data and method transfer between computer and LC because of reduced net work traffic reduced cycle time	Additional hardware might be necessary (use independent acquisition computer)
	Number of detectors	More detectors produce a higher data amount and lower the data transfer speed, resulting in higher cycle times	More detectors higher information content

Table 2
Influence of various parameters on the overall cycle time.

different methods – both giving virtually the same resolution. The first method (0.45 min gradient) utilizes alternating column regeneration and high temperatures to allow high flow rates and speed optimized settings. A cycle time of 49 s could be achieved, resulting in a theoretical daily throughput of more than 1700 samples per day. The second method (0.90 min gradient) does not use high temperatures or alternating column regeneration and the time saving of some simple and often forgotten method options are shown. By optimizing these parameters the real cycle time gets as close to 8 s to the run time (stop time plus post time) and allows a daily throughput of more than 700 samples per day. By sub-optimal method set up this can easily drop to below 500 samples per day if options like automatic delay volume reduction, overlapped injection or offline data-analysis are not used.

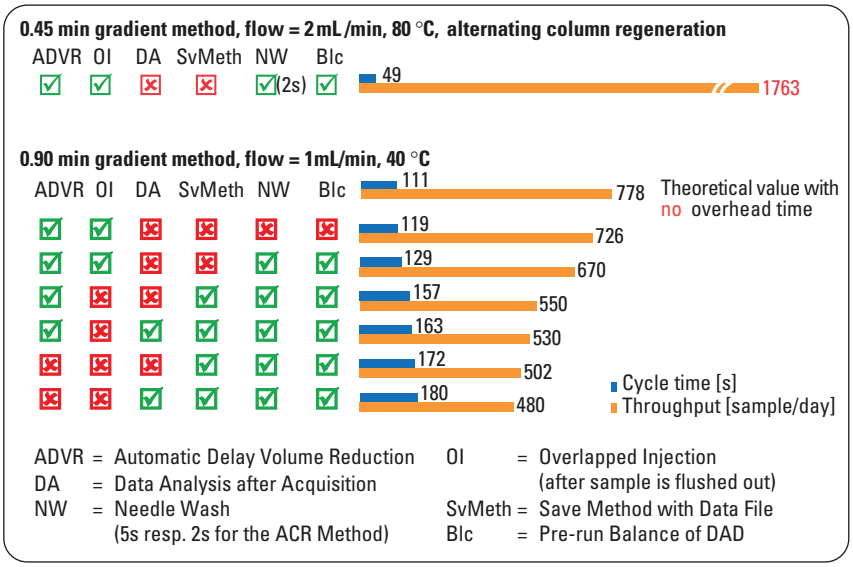

Figure 7
Cycle time and daily throughput optimization.

Chromatographic conditions:

Alternating Column Regeneration Method

Solvent:	A = Water, B = ACN	
Temp.:	80 °C	
Flow:	2.0 mL/min	
ADVR:	Yes	
Gradient:	**Gradient-Pump**	**Regeneration-Pump**
	0.00 min 35 %B	0.00 min 35 %B
	0.45 min 95 %B	0.01 min 95 %B
	0.46 min 35 %B	0.11 min 95 %B
	0.57 min 35 %B	0.12 min 35 %B
Stoptime:	0.57 min	no limit
Posttime:	off	off
Wavelength:	245 nm (8), ref. 450 nm (100)	
Peak width:	> 0.0025 min (0.05 s response time), 80 Hz	
Spectra:	none	
Injection volume:	1.0 µL	
Injector:	Overlapped injection, 2 s needle wash, sample flush-out factor = 10, draw/eject speed = 100 µL/min	
Valve:	next position	

No Alternating Column Regeneration Method

Solvent:	A = Water, B = ACN	
Temp.:	40 °C	
Flow:	1.0 mL/min	
ADVR:	Yes	No
Gradient:	0.00 min 35 %B	0.00 min 35 %B
	0.90 min 95 %B	0.90 min 95 %B
	1.10 min 95 %B	1.10 min 95 %B
	1.11 min 35 %B	1.11 min 35 %B
Stoptime:	1.15 min	*1.40 min (add. 300 µL extra column volume, increased retention times)*
Posttime:	0.70 min	0.70 min
Wavelength:	245 nm (8), ref. 450 nm (100)	
Peak width:	> 0.0025 min (0.05 s response time), 80 Hz	
Spectra:	all, 190-500 nm, BW = 1 nm	
Injection volume:	1.0 µL	
Injector:	See figure 7, 2 s equilibration time	

Conclusion

The Agilent 1200 Series Rapid Resolution LC system is a powerful tool to achieve highest chromatographic resolutions and also highest throughputs. The extended pressure range allows the usage of columns packed with stationary phases with particles sizes below 2 µm, for example, Agilent RRHT columns with particle sizes of 1.8 µm. These columns not only allow an increase in linear flow rates with virtually no loss in resolution but also have an inherently higher resolution compared to 3.5 µm or even 5.0 µm particle sizes. The possibility to switch the pump into its low delay volume configuration allows the use of the entire bandwidth of today's widely used column ids – from 4.6 mm down to 2.1 mm and even 1.0 mm. As illustrated above, the system has uncompromised performance characteristics even at highest gradient speeds.

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Agilent Technologies

Combined EI and CI Using a Single Source

Technical Overview

Chris Sandy

Agilent Technologies

Introduction

The Agilent 5973x gas chromatograph/mass selective detectors (GC/MSDs) come with sources optimized for electron ionization (EI) and chemical ionization (CI). However, there are occasions where another ionization mode is desired without changing sources. This note demonstrates the capability of acquiring high-quality EI spectra with the CI source.

Data Acquisition

An Agilent 5973 inert MSD with a CI source was set up for the experiments. The following process was used to tune the MS:

1. Perform the CI autotune at the normal methane reagent gas flow rate (typically at a mass flow controller (MFC) setting of 20%).
2. Reduce the CI flow to 2%.
3. Set the emission current to 250 µa.
4. In Manual Tune, ramp the repeller from 0–5 volts for the mass 69 ion.
5. Set the repeller voltage to the maximum value.
6. Turn off the CI gas.
7. Save tune file.
8. Associate tune file with method.

Data was acquired in positive CI (PCI) and EI modes. Figure 1 shows the CI and EI total ion chromatograms using the CI source. The major and minor peaks are easily comparable in the two chromatograms.

Figure 2 shows the CI spectrum for Hexadecanolide (MW = 254) with the expected adduct ions for methane. Note the relatively large response for the 255 ion. As expected, there is little fragmentation due to the soft ionization.

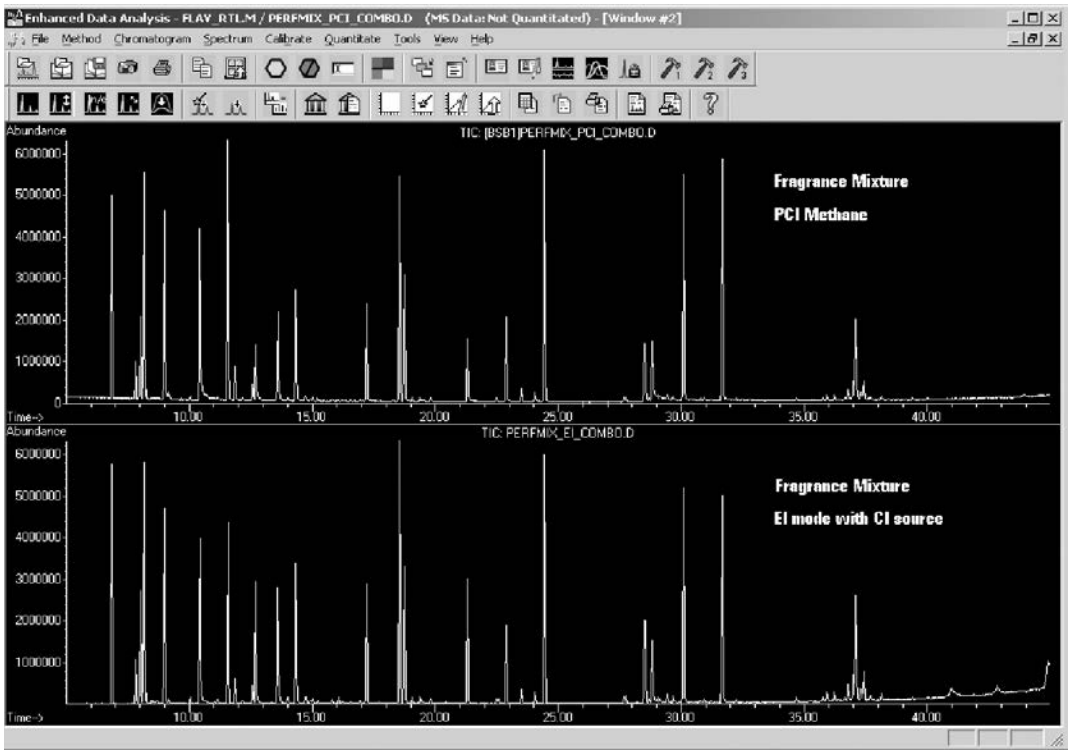

Figure 1. PCI and EI total ion chromatograms using the CI source.

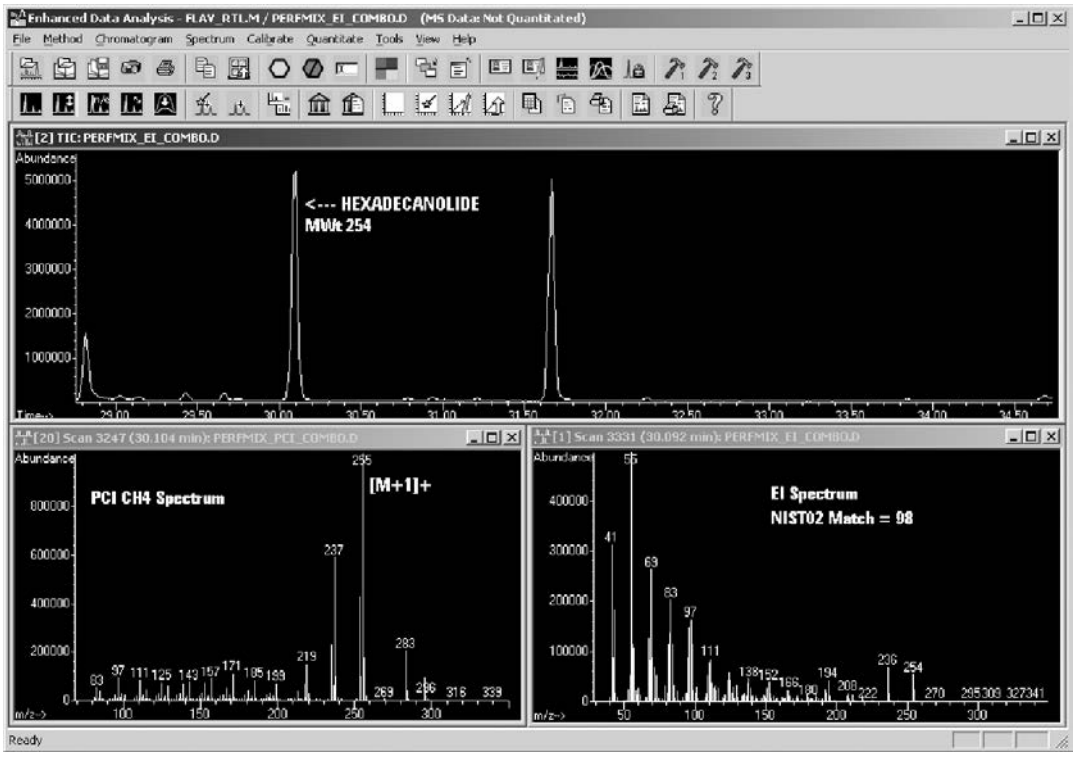

Figure 2. PCI and EI spectra for Hexadeconolide.

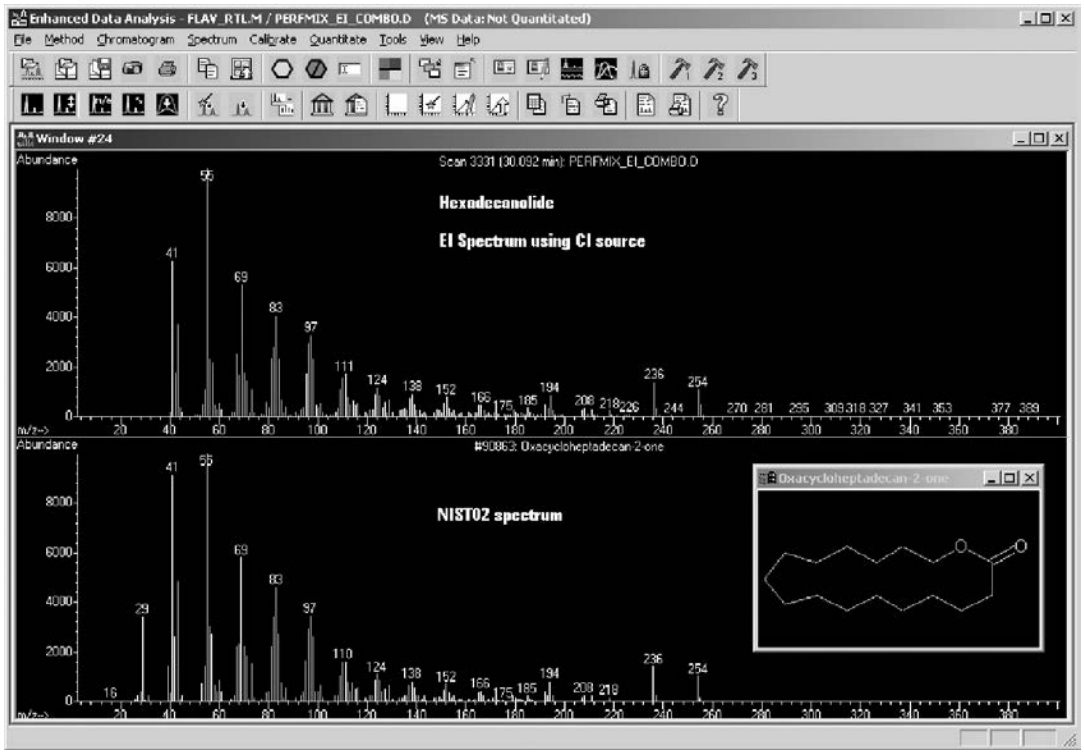

Figure 3. Acquired EI spectrum compared to the NIST02 library reference spectrum.

The EI data in Figure 3 shows much more fragmentation useful for compound identification. The response for 255 is relatively small. Using the NIST02 library, the EI reference spectra for Hexadecanolide (Oxacyclohelptadecan-2-one) was retrieved with a 98% quality match.

Summary

This data demonstrates the Agilent 5973 inert GC/MSD's ability to acquire high quality EI spectra using the CI source. The EI spectra can be searched against standard libraries for identification while the CI spectra provide molecular weight information. The ability to acquire both types of data without changing sources results in increased productivity.

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Agilent Technologies

The Benefits of Achieving High Mass Accuracy at High Speed Using Agilent's TOF-MS Technology

Application Note

Edgar Naegele

Abstract

Measuring accurate molecular mass by mass spectrometry and calculating the corresponding empirical formula is an important step in the identification process of small molecules in a variety of application fields. Depending on the accuracy of mass measurement, significant empirical formulas can be calculated in low numbers. This Application Note will discuss the benefits of using the Agilent 6210 TOF mass spectrometer in combination with the Agilent 1200 Series Rapid Resolution LC system for compound identification in various applications.

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Introduction

Reliable empirical formula confirmation necessitates setting a mass accuracy limit, which takes the acceptable uncertainty of the accurate molecular mass measurement into consideration[1]. This results in more accurate mass measurement with decreasing relative mass error and requires fewer possibilities to consider for an empirical formula (table 1).

Mass accuracy [ppm]	Empirical formulae
100	138
50	67
25	32
10	15
5	7
2	2

Table 1
Mass accuracy vs. number of calculated empirical formulae for reserpine ($C_{33}H_{40}N_2O_9$ M=608.2734; within $C_{1\text{-}100}H_{2\text{-}200}N_{0\text{-}10}O_{0\text{-}10}$).

The current generation of comparably easy-to-use and inexpensive ESI orthogonal acceleration TOF (oaTOF) instruments are capable of handling this task. This was clearly demonstrated by a comparison study of different types of MS instruments, which are used for the determination of accurate mass of small molecules[2]. Innovations in TOF technology introduced during the past several years, like the orthogonal acceleration TOF technology with an analog-to-digital (ADC) converter, made this progress possible[3]. This Application Note will demonstrate the benefits of using the Agilent 6210 time-of-flight mass spectrometer in combination with the Agilent 1200 Series Rapid Resolution LC (RRLC) system and their impact on compound identification in various applications.

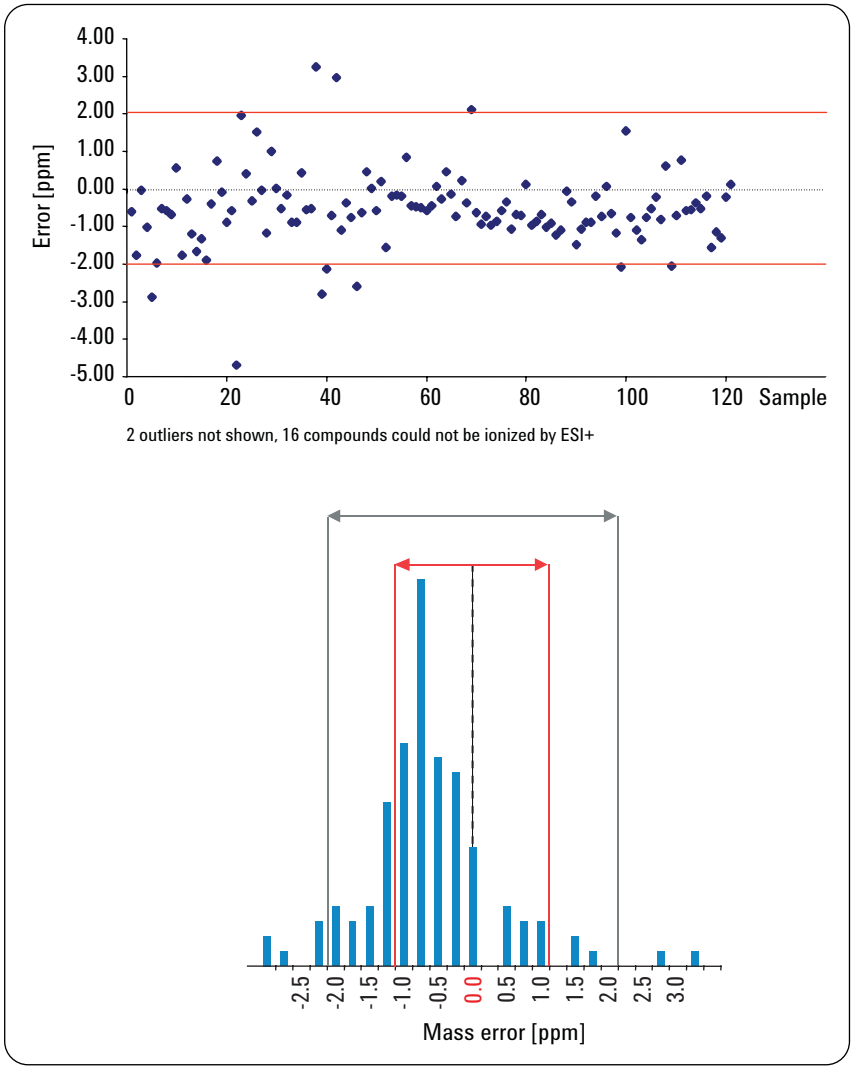

Figure 1
A) Mass accuracy errors as returned by an automatically generated report.
B) Histogram of the mass accuracy errors of the analysis of 140 real chemical library samples of a pharmaceutical company.

Results and discussion

When using a TOF mass spectrometer, attention is certainly focussed on the accurate mass. Figure 1A shows the achieved mass accuracy errors of the analysis of 140 members of a chemical library used in a screening campaign. More compelling is the histogram of these samples as shown in figure 1B. More than 71 % of the analyzed compounds have a mass accuracy error in the range of ± 1.0 ppm. This efficiency enables the chemist to narrow down the number of possible calculated empirical formulas for confirming the identity of a compound[4]. Analysis times below one

minute could be achieved, with high peak capacities above forty in just 39 seconds, both in the UV and in the MS chromatogram (figure 2) by using a method which includes alternating column regeneration, MS TOF data acquisition at 40 Hz, and DAD data acquisition at 80 Hz.

Application examples

- Analysis of complex samples with the MassHunter software, which allows extraction of molecular mass data and their detailed analysis[5] (figure 3).
- Detection and identification of minor impurities in pharmaceutical compounds generated during stability testing, production, formulation or storage of the final drug compound (Agilent publication numbers 5989-2348EN and 5989-5617EN).
- Statistical evaluation of achieved TOF mass accuracies with a real sample of less than 2 ppm (Agilent publication number 5989-3561EN).
- Simultaneous determination of metabolic stability and metabolite identification by high speed and high resolution (Agilent publication number 5989-5110EN).
- Automated screening of clinical body fluid samples for administered drugs (Agilent publica tion number 5989-5835EN).
- Identification of natural products from complex plant extracts (Agilent publication number 5989-4506EN).
- A complete overview of TOF applications is published in a compendium (Agilent publication number 5989-2549EN).

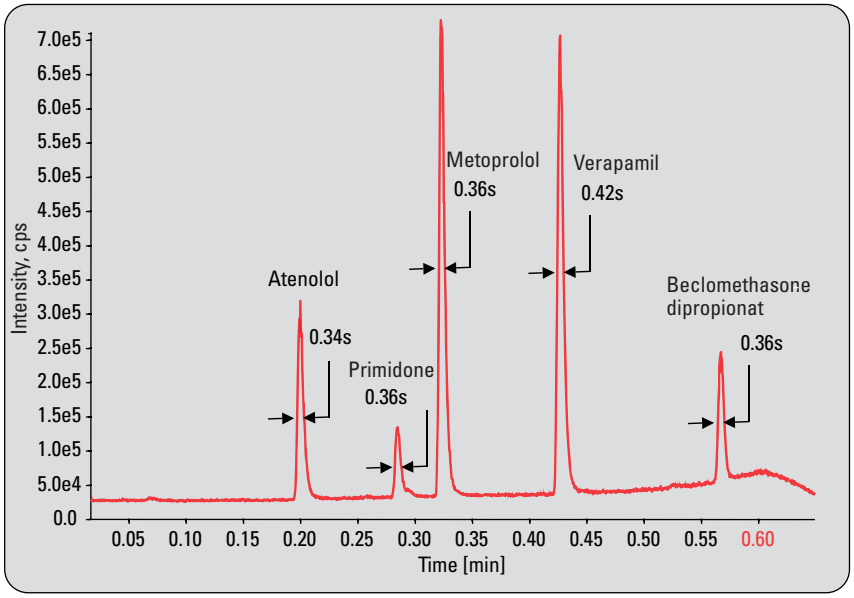

Figure 2
TIC chromatogram (40-Hz data rate of the 6210 TOF mass spectrometer, 80-Hz data rate of the DAD) with PWHH values for the TIC.

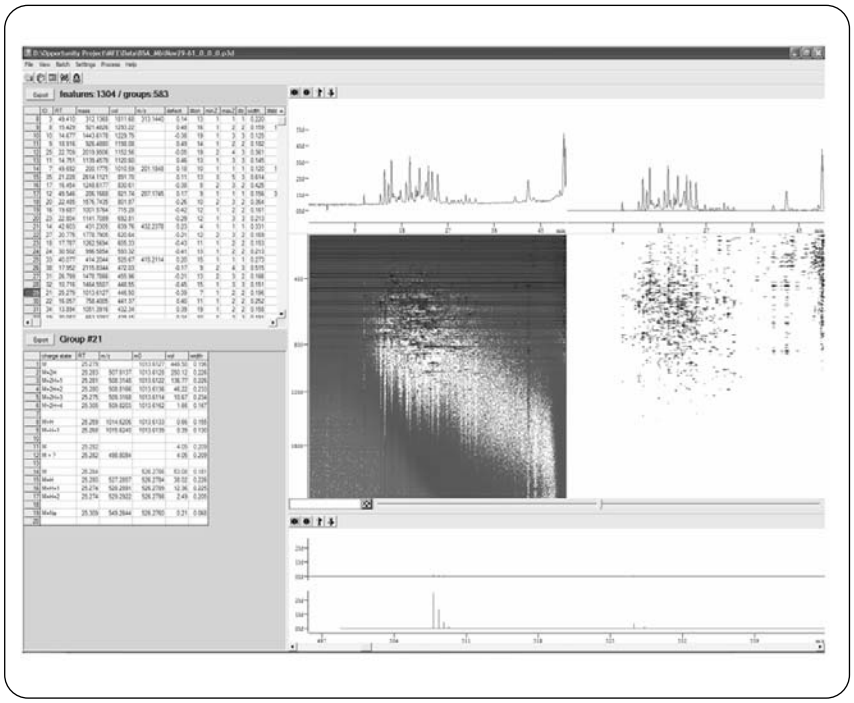

Figure 3
MassHunter software for analysis of complex samples.

Conclusion

- It is possible to rapidly acquire molecular mass data with highest mass accuracy in the single digit ppm error range with the Agilent 6210 TOF. This allows the unambiguous calculation of empirical formulas for compound confirmation.
- It is possible to measure mass differences with highest resolution with the Agilent 6210 TOF instrument. This allows the separation of compounds, which have a similar mass and distinguish between their empirical formulas.
- It is possible to acquire date with up to 40 Hz acquisition rate with the Agilent 6210 TOF. This permits the instrument to be used in ultra-fast LC separation applications.
- The principal benefits are accurate time-of-flight mass measurement, high resolution and high speed data acquisi tion, which can be used over a broad range of applications, such as library screening, screening of biological samples, metabolite stability and metabolite identification, identification of minor impurities in drugs and natural product analysis.

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4.
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5.
"Hunting the masses – Part 1: Computer aided analysis of LC/ESI-TOF data from complex natural product extracts for compound structure elucidation" *Agilent Application Note, publication number 5989-5928EN*, **2006**.

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www.agilent.com/chem/tof

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Agilent Technologies

Can "Deconvolution" Improve GC/MS Detectability?

Application Note

All Industries

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Abstract

This study uses 35 pesticides spiked in spinach extracts at the 50 ppb level to find the optimal AMDIS deconvolution settings. Additional advantages of using deconvolution versus MSD ChemStation, to find more compounds in an extract are also discussed.

The detectability of compounds in a complex matrix is significantly improved with deconvolution. This can also be viewed as better or increased sensitivity through improved selectivity versus the background.

Agilent's MSD ChemStation add-on - Deconvolution Reporting Software (DRS) runs AMDIS automatically to generate an easy-to-read quantitation report.

Agilent Technologies

Introduction

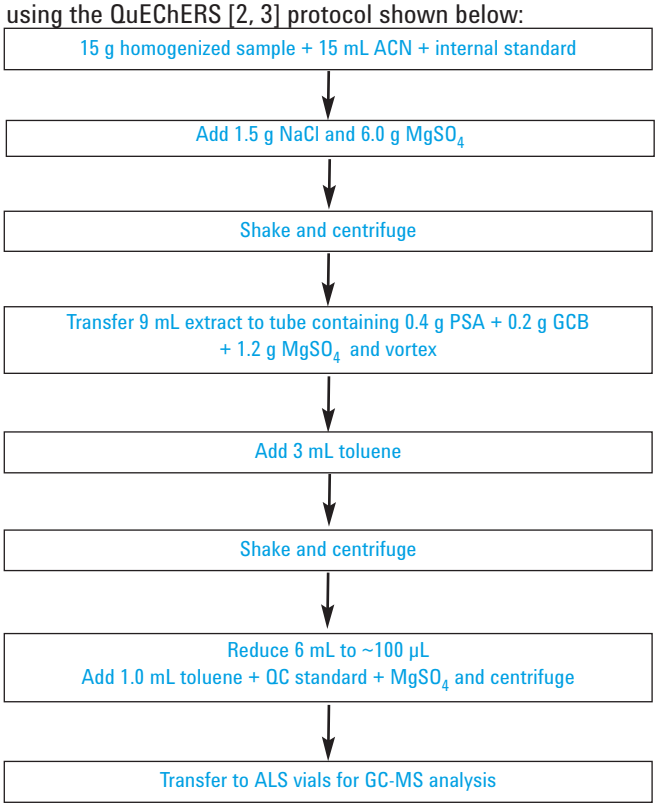
Instrument detectability is usually determined by the amount of sample injected, the responses from the detector and matrix interferences. The signal-to-noise ratio (S/N) can be used to gauge the sensitivity of an instrument in a clean sample. The presence of matrix alters this sensitivity due to a lack of selectivity between compounds of interest and background.

In a multiresidue analysis, the data reviewing process is also very important in confirming the hits found by the software and reviewing the integration and quantitation for accuracy.

Agilent Deconvolution Reporting Software (DRS) has been proven as a powerful data processing tool for finding trace compounds in complex matrices [1]. In this study, results from the Automated Mass spectral Deconvolution and Identification System (AMDIS), part of DRS is closely studied and compared to the results from ChemStation. The goal is to determine if deconvolution (DRS) can provide better results (detectability) than routine ChemStation data processing.

Experimental

Spinach extracts (see Acknowledgement) were prepared using the QuEChERS [2, 3] protocol shown below:



Thirty-five pesticides were spiked into spinach extract at 50 ppb (pg/µL).

Instrument parameters

GC: **7890A**

Autoinjector: 7693A
Retention gap: 2 m × 0.25 mm id Siltek capillary tubing
Column: HP-5MS UI (ultra inert), 15 m × 0.25 mm, 0.25 µm (from inlet to Purged Union) Agilent p/n 19091S-431 UI

Oven ramp:	Rate (°C/min)	Temp (°C)	Time (min)
Initial		100	1.6
Ramp 1	50	150	0
Ramp 2	6	200	0
Ramp 1	16	280	5

Run time: 20.933 min
Inlet: Multimode Inlet (MMI) at 17.73 psi (Retention Time Locked), constant pressure mode
RT locking: Chlorpyrifos-methyl locked to 8.297 min
Liner: Helix double taper, deactivated (Agilent p/n 5188-5398)
Injection mode: 2-µL cold splitless (fast injection)

Inlet temp. ramp:	Rate °C/min	Temp °C	Time min
Initial		50	0.01
Ramp 1	720	300	hold

Septum purge: 3 mL/min
Purged Union: 4 psi (PCM)
Split vent: 50 mL/min at 0.75 min
Gas saver: 20 mL/min after 4 min
Cryo on: Cryo use temperature 150 °C; time out at 15 min

Backflush

Postrun: 5 min
Oven: 280 °C
Purged Union: 70 psi
MMI: 2 psi
Restrictor: 0.7 m × 0.15 mm deactivated fused silica tubing (from Purged Union to MSD)

MSD: **5975C**

Solvent delay: 2.5 min
EMV mode: Gain Factor = 2
Mass Range: Full scan, 45-550
Threshold: 0
Sample number: 2 A/D Samples 4
Transfer Line: 280 °C
Source: 300 °C
Quad: 200 °C

Deconvolution

Deconvolution is a process for extracting ions from a complex total ion chromatogram (TIC), even with the target compound signal at trace levels. The software used for this technique is AMDIS developed by NIST (National Institute of Standards and Technology) [4].

As a review, let's look at the deconvolution process. AMDIS considers the peak shapes of all extracted ions and their apex retention times (RT). In this example, only some of the extracted ion chromatograms (EICs) are overlaid for clarity with the apex spectrum (Figure 1A).

Ion 160 EIC has the same RT as ions 50, 170 and 280, but has a different peak shape. Ion 185 has a different peak shape and an earlier RT. Ions 75 and 310 have similar peak shapes but they have different RTs.

Figure 1A

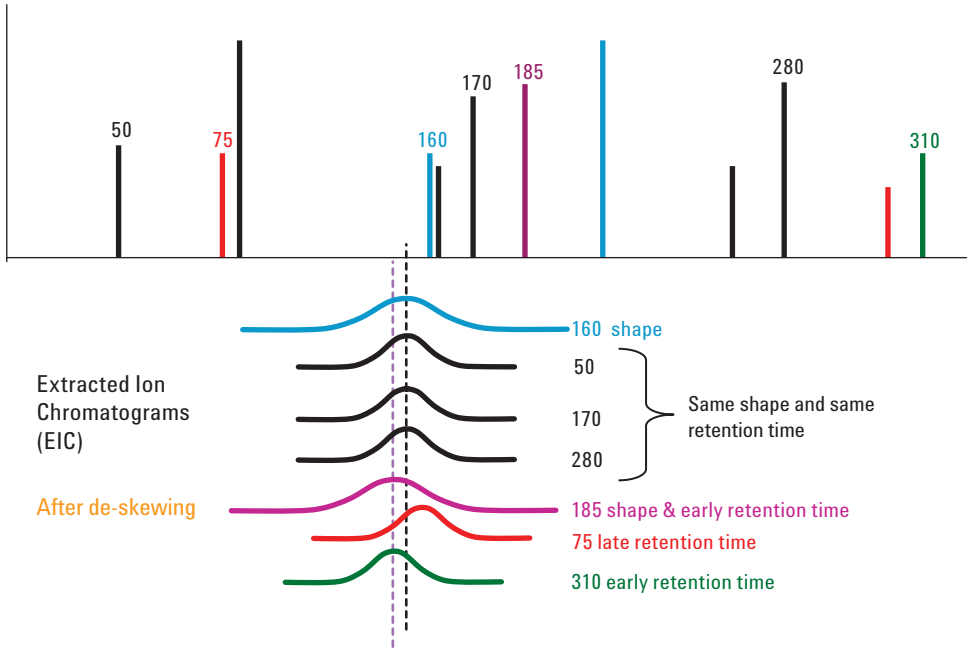


Figure 1B shows the EICs after the different peak shapes or RTs are eliminated from Figure 1A. Ions 50, 170, 280 and a few others remain.

Figure 1B

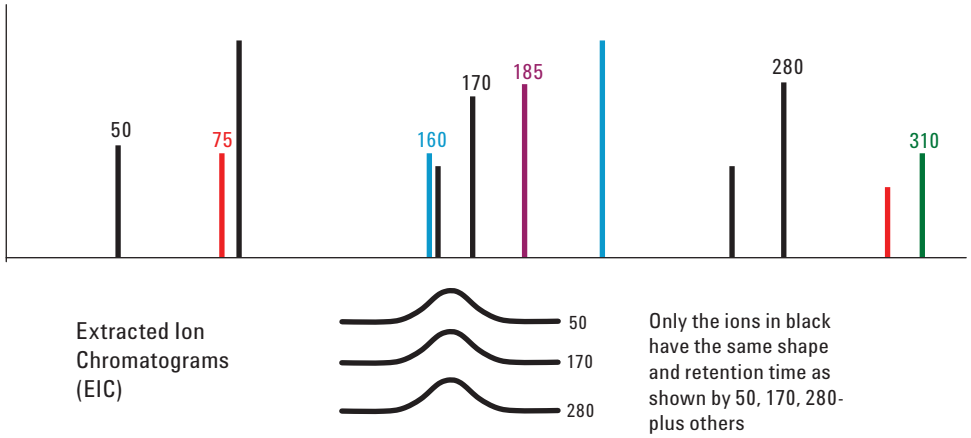


Figure 1A-1C. Simplified deconvolution process (continued).

Figure 1C shows all of the ions in black that have similar peak shapes and RTs, within the criteria set earlier by the analyst. These are grouped together and referred to as a component by AMDIS.

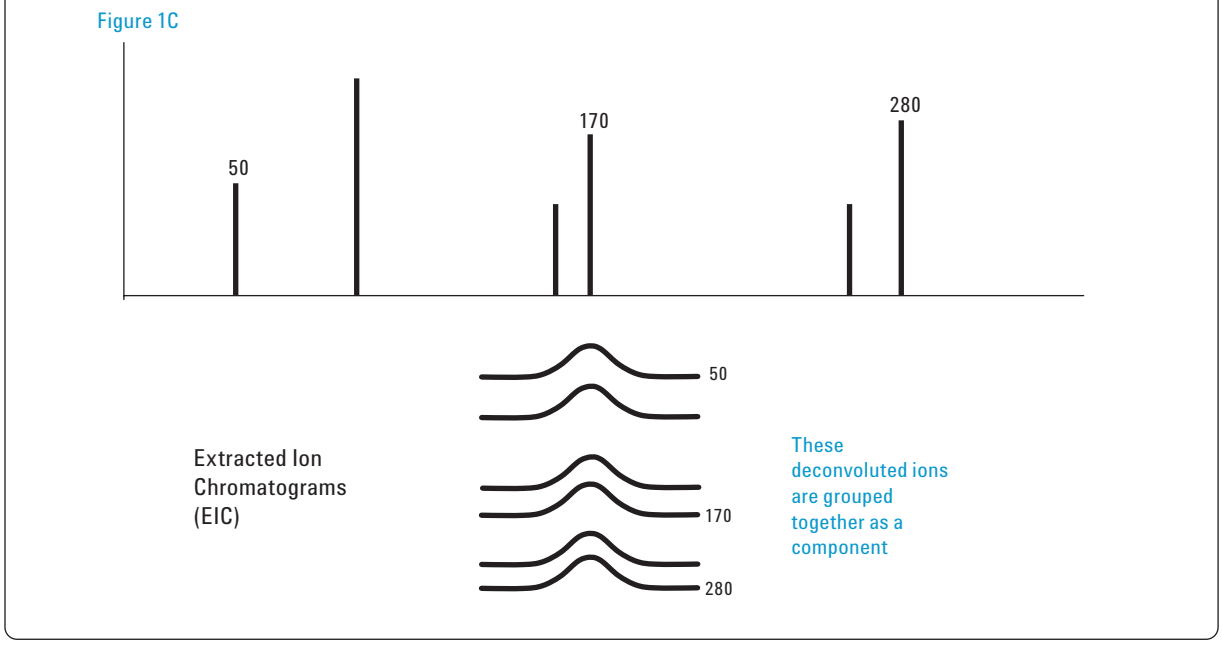


Figure 1A-1C. *Simplified deconvolution process (continued).*

Deconvolution finds the components from a complex TIC. Each component is searched against a retention time locking (RTL) library in AMDIS format. In addition to spectral matching, the locked RT can also be used as a criterion for hits. Depending on the match factor from the search, target compounds can be identified or flagged in a complex TIC. The power of deconvolution is appreciated while comparing the top two spectra in Figure 2. The raw scan or original nondeconvoluted scan is shown on top. The clean scan, that is the deconvoluted component, is shown in the middle. The bottom scan is the identified compound in the AMDIS library. Without deconvolution, the analyst would visually compare the background subtracted raw scan and library scans for confirmation. It would be very difficult, if not impossible, to say that Fenbuconazole, the target compound in this example, is present using that type of comparison.

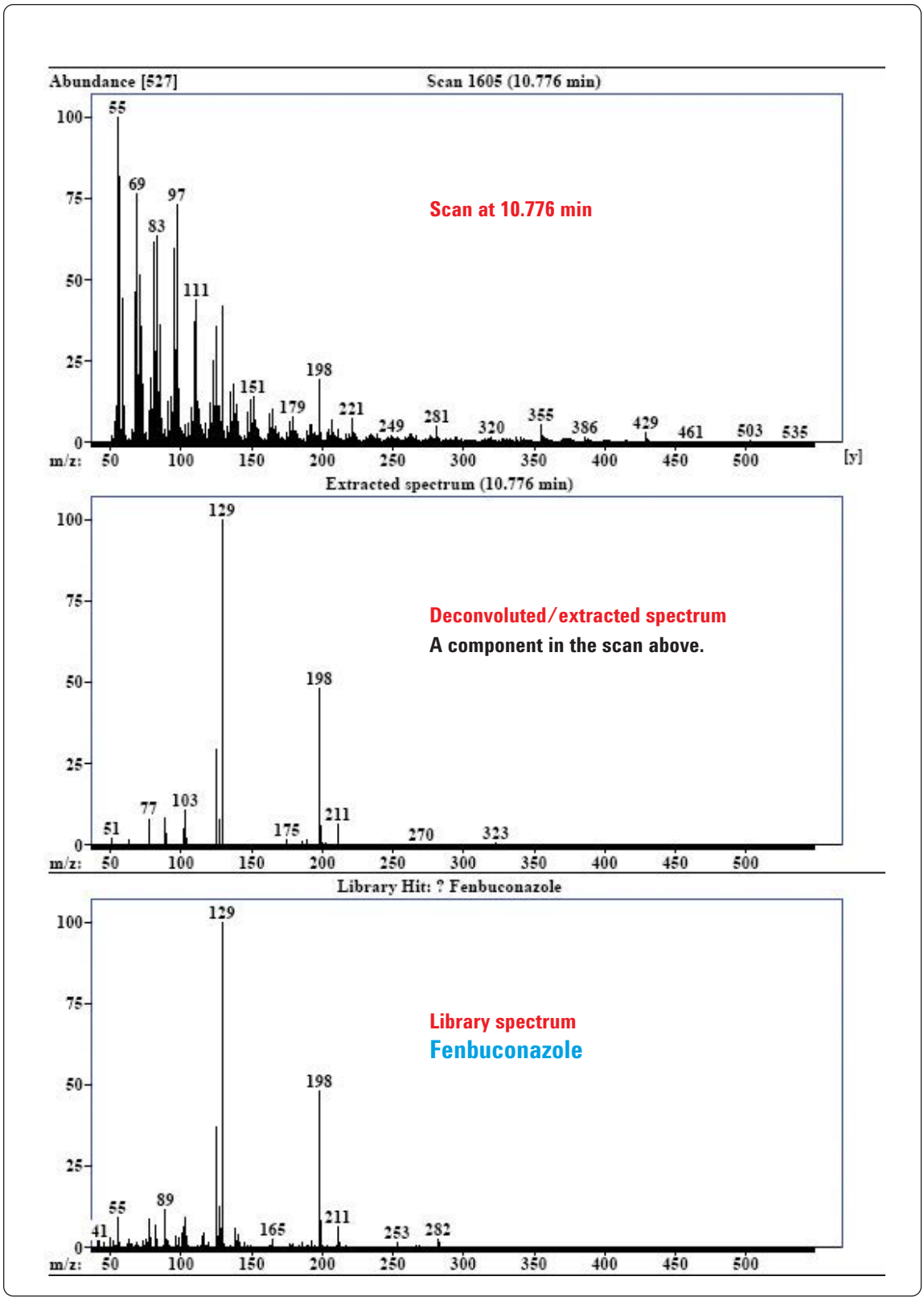


Figure 2. Comparison of raw, deconvoluted, and library spectra.

AMDIS Settings

Previous publications that discussed the power of using deconvolution to screen complex matrices, did not discuss specific AMDIS settings to define components [1, 5, 6]. In this study, several settings (that is, resolution, sensitivity, and shape requirements) are compared to find the maximum number of spiked compounds. The minimum match factor is set to 30 and the retention time window is limited to ± 30 seconds (RI window is set to 30) to qualify the hits from the retention time library search (Figure 3). The expected retention times of the compounds in the library database are obtained in acetone solvent without a retention gap. The samples in this study are in toluene solvent with a retention gap. Therefore, the retention time window is set wider than the normal 10 or 15 seconds, at ± 30 seconds.

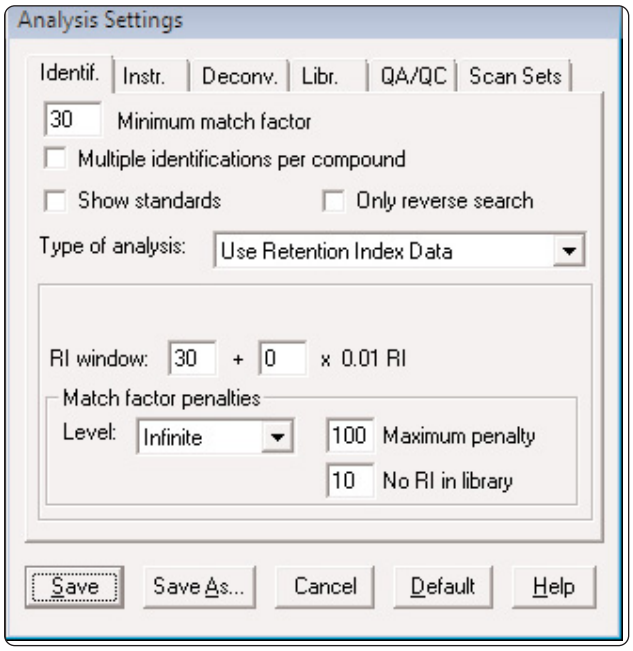


Figure 3. AMDIS identification settings.

Figures 4 and 5 describe some of the parameters in the AMDIS deconvolution tab. In this article, "1 M H M" means: adjacent peak subtraction = 1, resolution = medium, sensitivity = high, shape requirements = medium.

Settings can be optimized for chromatographic resolution, peak shape, retention time windows, acceptance criteria, and so forth. Settings can be saved to "ini" files. The chemist has control over the deconvolution and identification process by varying numerous AMDIS settings. Most of these parameter settings are not independent; so changing one parameter can affect another.

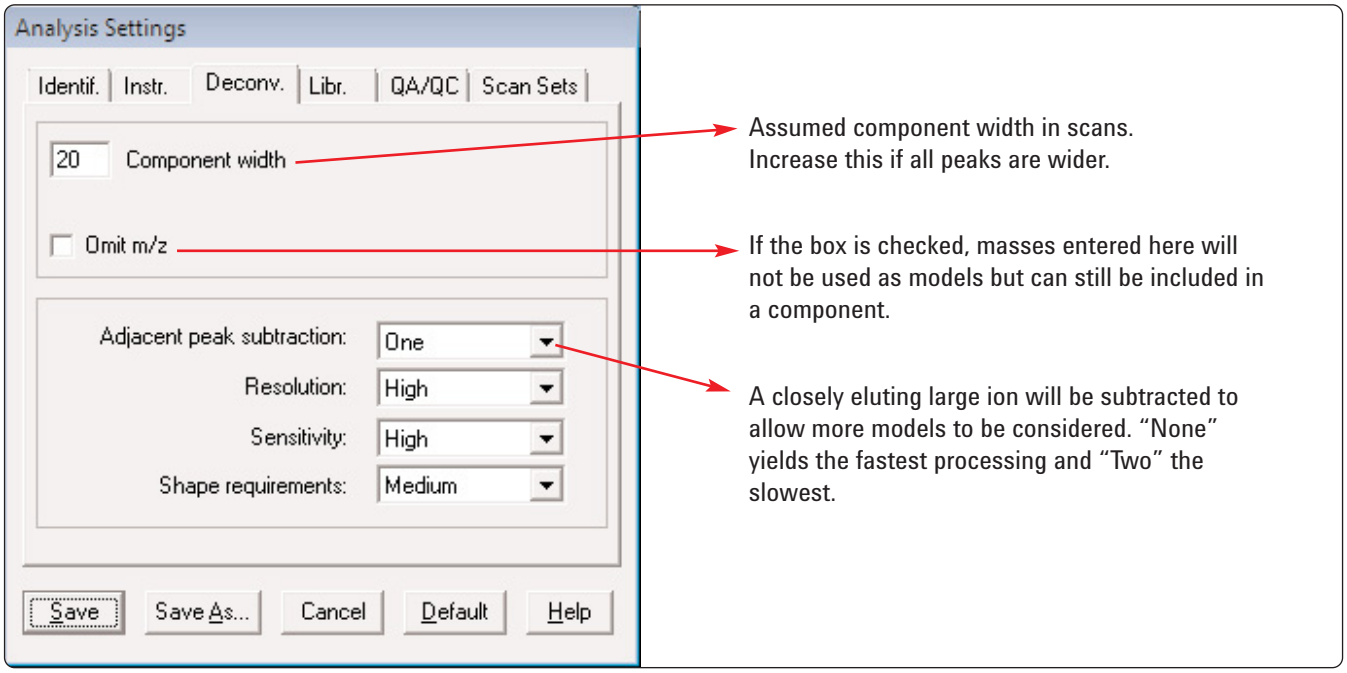


Figure 4. AMDIS deconvolution settings.

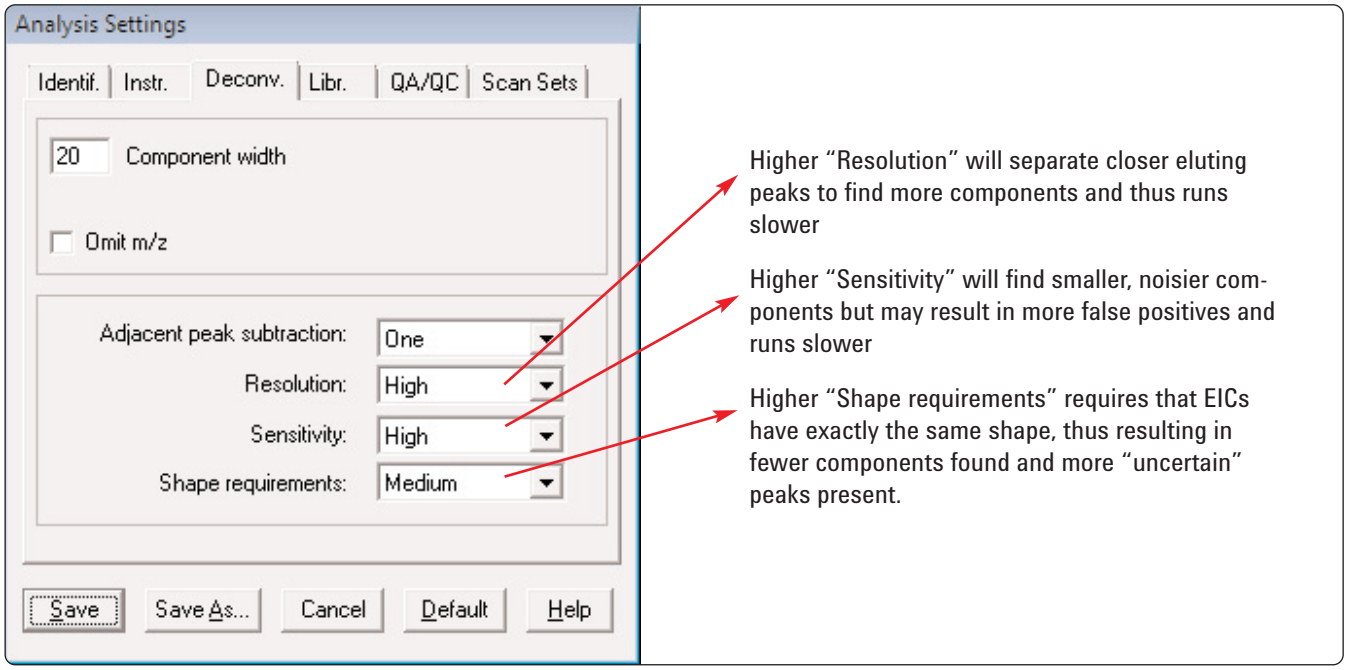


Figure 5. AMDIS deconvolution settings.

Results and Discussion

Deconvolution Settings

Figure 6 shows effects on match factors (y-axis) due to variation of adjacent peak subtraction and sensitivity across 35 pesticides (x-axis). This figure shows two things:

– The adjacent peak subtraction (1 or 2) makes little difference in match factor

– The sensitivity setting (very high and high) makes little difference in match factor

In the next few figures, the AMDIS setting is varied one at a time to observe the number of pesticides found. The reference point is the optimal setting (HHM) where the maximum number of hits were obtained.

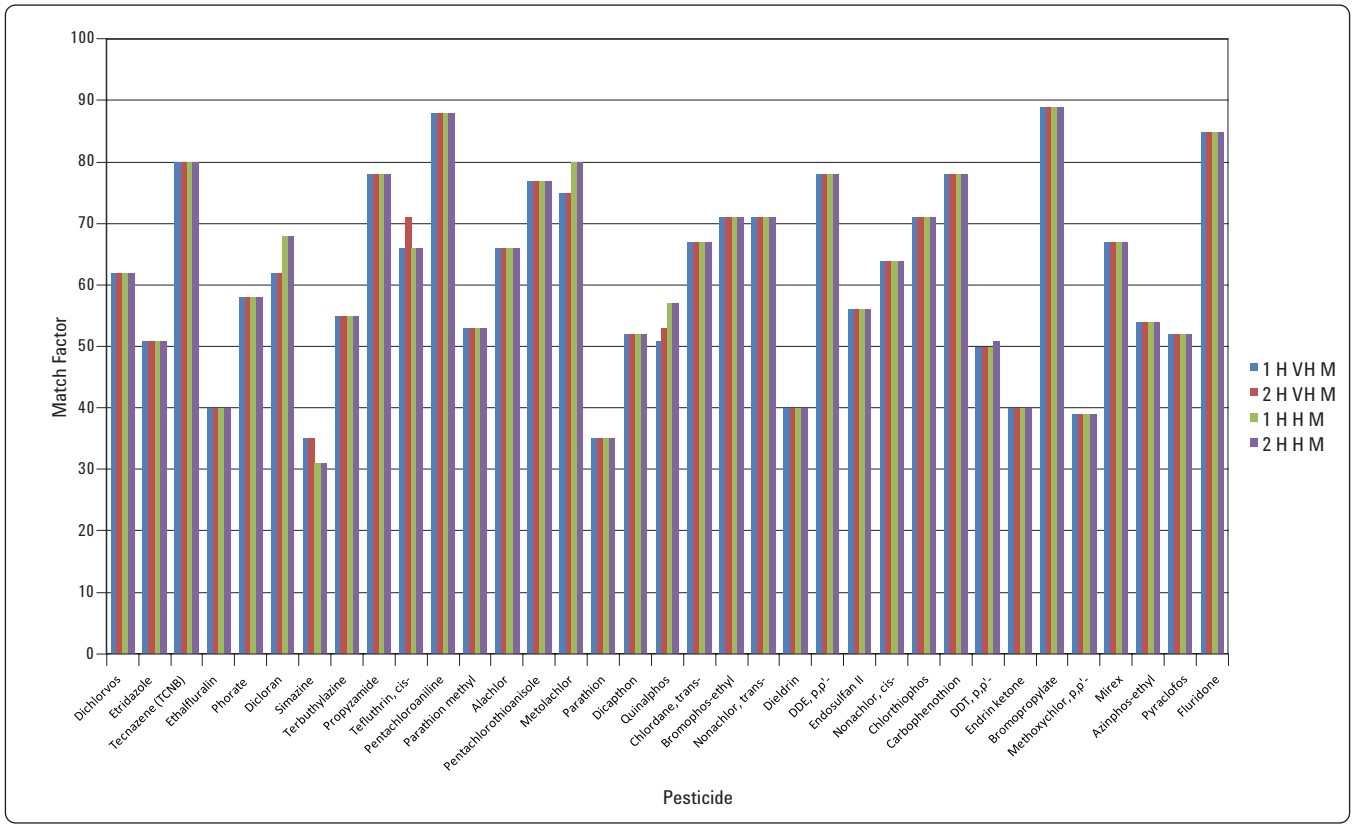


Figure 6. Comparison of match factors with four AMDIS settings.

Figure 7 shows that keeping the sensitivity and peak requirements the same, and lowering the resolution from H to M will find fewer targets. The number of targets found is in the yellow circle. A resolution setting of "low" yields even fewer targets.

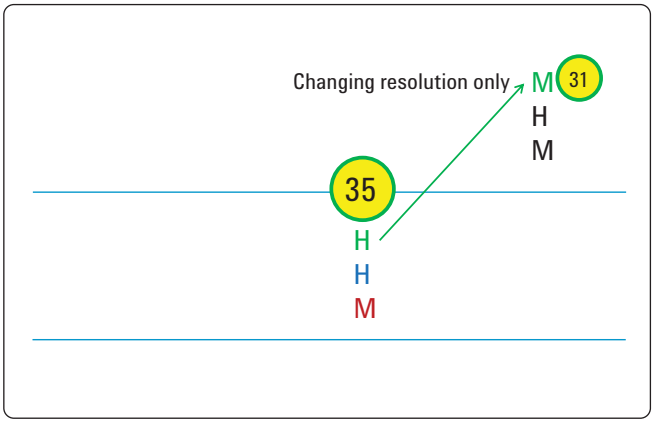


Figure 7. Number of compounds found by varying resolution.

Figure 8 shows that while keeping the resolution and peak requirement constant, lowering the sensitivity from H to M will find fewer targets. However, increasing the sensitivity from H to VH does not affect the number of targets found, similar to that in Figure 6.

Figure 9 shows that while keeping the resolution and sensitivity the same, lowering or increasing the peak shape requirement from M to L or H will find less targets.

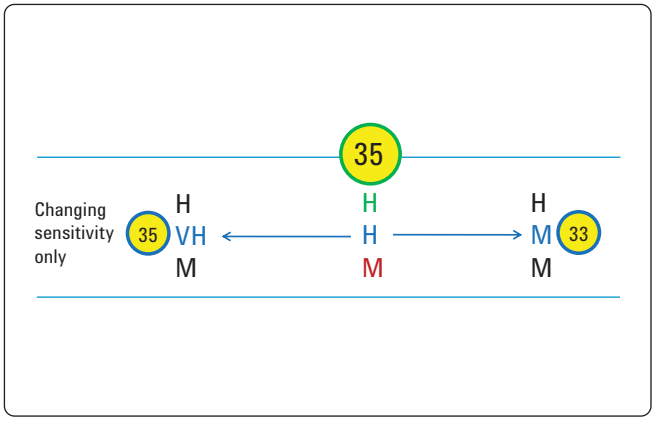


Figure 8. Number of compounds found by varying sensitivity.

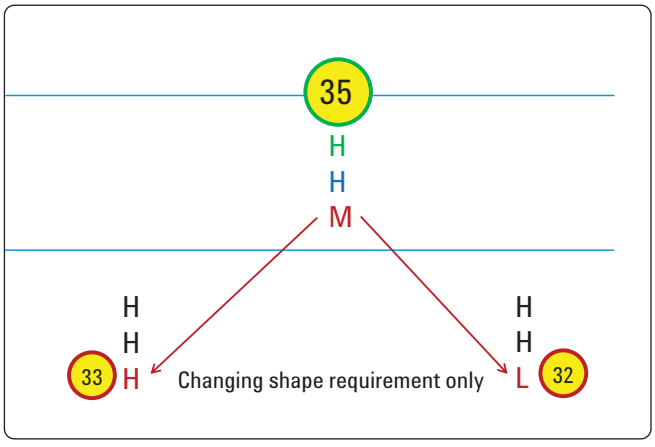


Figure 9. Number of compounds found by varying peak shape.

In addition to the number of targets found, we should look at the Average Match Factor (AMF) of all the targets found. The AMF is the number in the green triangle. Figure 10 shows that there is no significant variation in AMFs except in HHH mode (58.5) which is much lower than others (>61.6). This supports that HHM is still the optimal setting, considering processing speed and number of false positives.

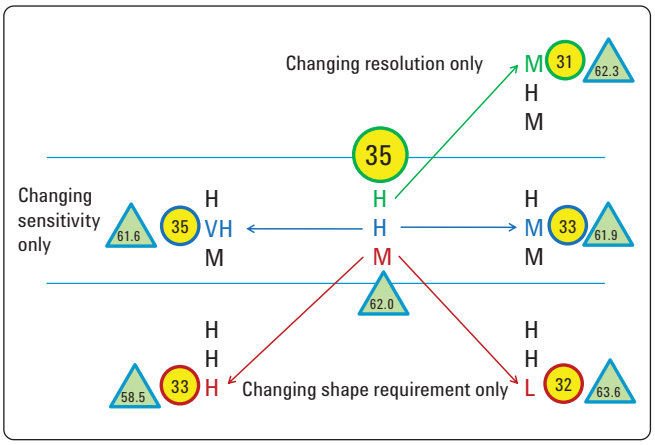


Figure 10. Comparison of average match factors with AMDIS settings.

ChemStation Quant settings

Figure 11 shows part of the "Edit Compound" screen in the MSD ChemStation. This shows the quant database for locating and confirming compounds using three ion ratios of each target analyte. The RT window is specified in the upper box and the ions and ion ratios are specified in the lower box.

As shown in Figure 11, the Extraction RT window is set to ± 0.5 min and the Qualifier Ion (Q1, Q2, and Q3), % Uncertainty is set to Absolute 50%. In ChemStation, the

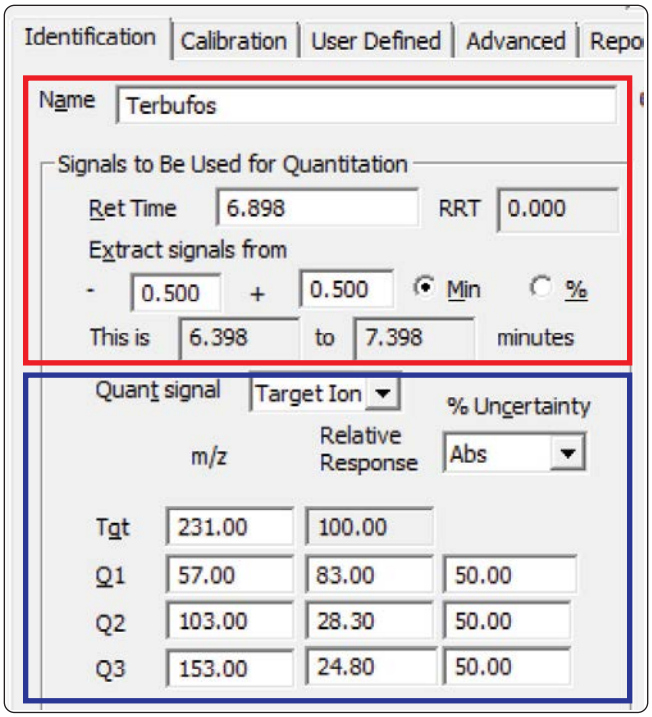


Figure 11. Target compound RT and ion setup.

target compound identification is based on four ions and three qualifier ion ratios. However, the target compound identification in AMDIS (Figure 2) was based on the full spectral library match which is more dependable.

Another key parameter in quantitation is the "Quantitation subtraction method" which is set to "Avg first and last" and not shown here.

Figure 12 is an overlay of four ions (Quant and Qualifiers) from ChemStation and the quant ion from AMDIS (in magenta).

Due to the chemical background, the four ions from ChemStation have offset and noisy baselines, which will affect the peak integration and proper quantitation results.

In comparison, the magenta trace is the deconvoluted quant ion from AMDIS. The chemical noise had been removed in the deconvolution process. It shows a flat baseline and accurate integration. There are other advantages of using deconvolution in GC/MS analysis as discussed below.

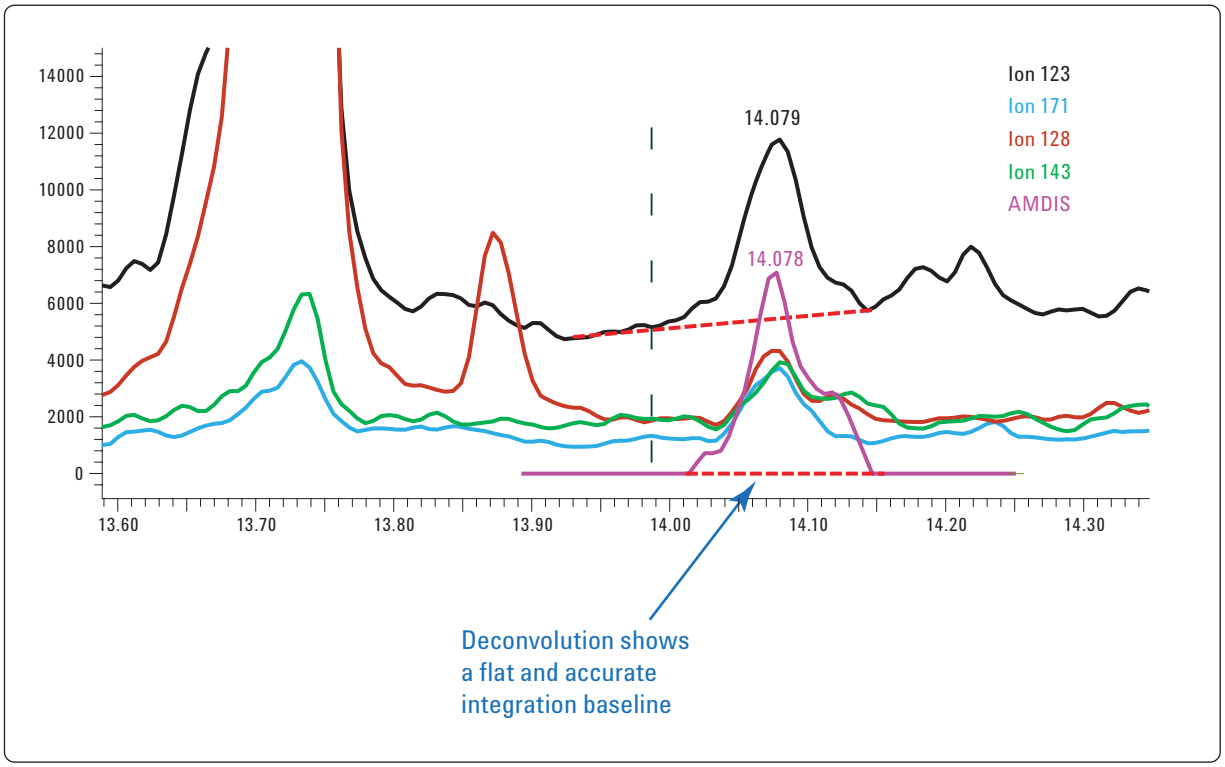


Figure 12. Target, qualifier and AMDIS deconvoluted EIC overlay.

Additional Advantages of Using Deconvolution

Finds more compounds than ChemStation does

In Figure 13, ChemStation did not integrate ion 109 (ChemStation target ion) at the expected RT, therefore, the compound was not found. AMDIS found Fonofos correctly, at 6.898 min. The qualifier ion ratios at this RT also match that required by ChemStation for identification.

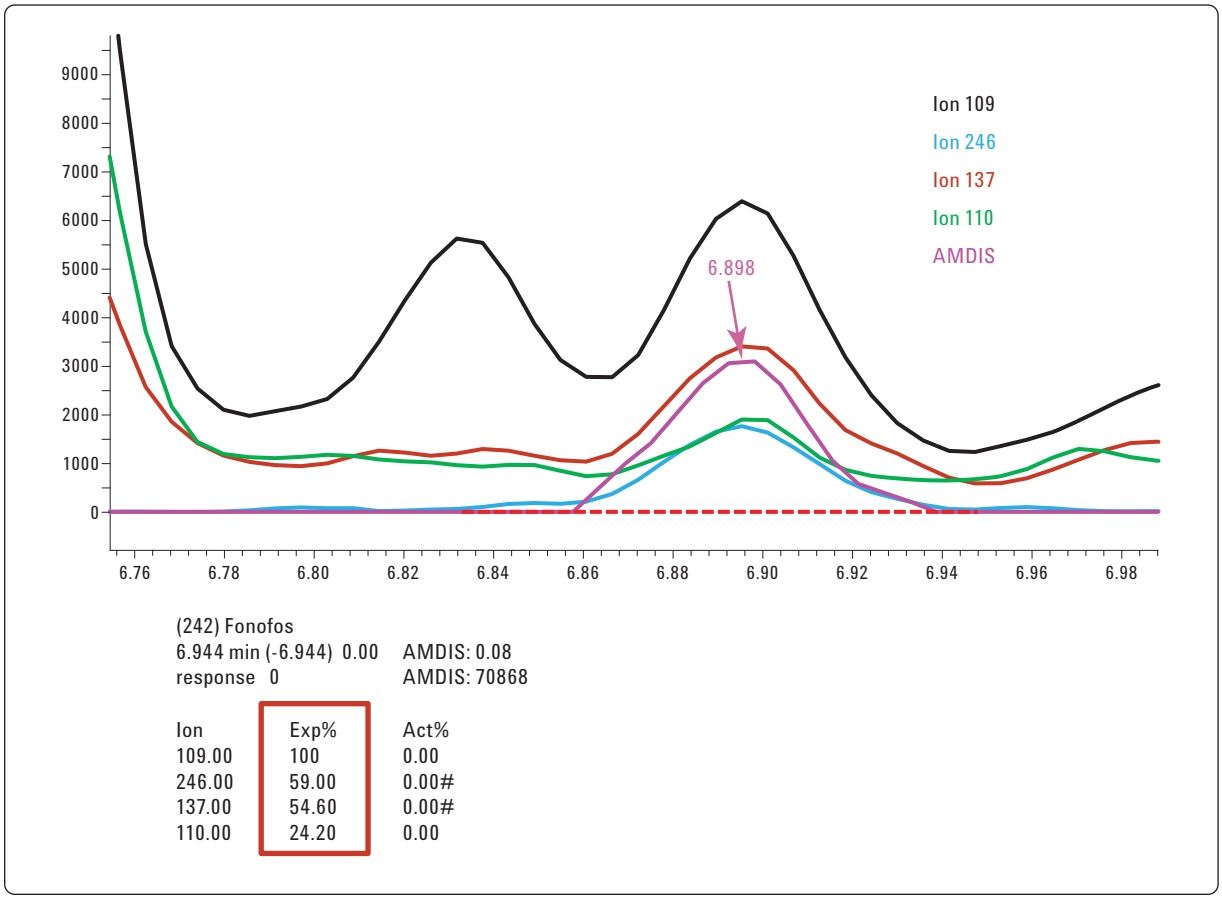


Figure 13. Target, qualifier and AMDIS deconvoluted EIC overlay.

Finds the correct peak

In Figure 14, from the size and location of the three qualifier ions, it is obvious that ChemStation picked the wrong peak (at RT = 4.067) to quantitate. However, AMDIS found a peak (at RT = 3.873) whose ion ratios are in agreement with the ChemStation qualifier ions. Again, this demonstrates that the AMDIS full-spectrum matching process is a more robust approach for identifing a compound in a complex matrix.

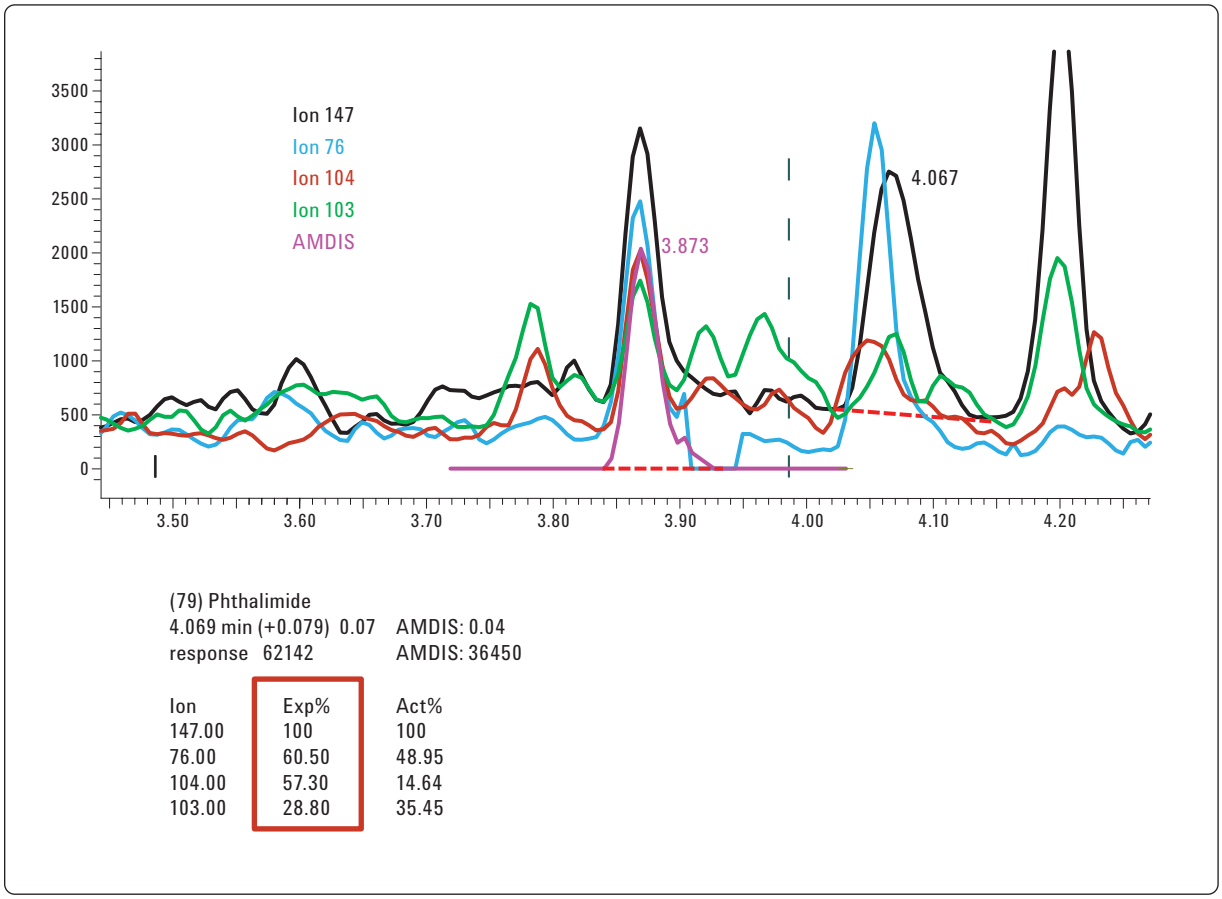


Figure 14. Target, qualifier and AMDIS deconvoluted EIC overlay.

Higher discrimination power than ChemStation

In Figure 15, the target ion (ion 235) is overwhelmed by the matrix background (shown as a large fronting peak). ChemStation was not able to differentiate the ion 235 contribution from the background or the compound; therefore it integrated the distorted peak. Due to the rising baseline, ChemStation integrated a large area of chemical background as the "target compound signal". On the other hand, AMDIS was able to deconvolute the compound signal away from the background ion and remove noise properly before the integration. This provides a more reliable quant result.

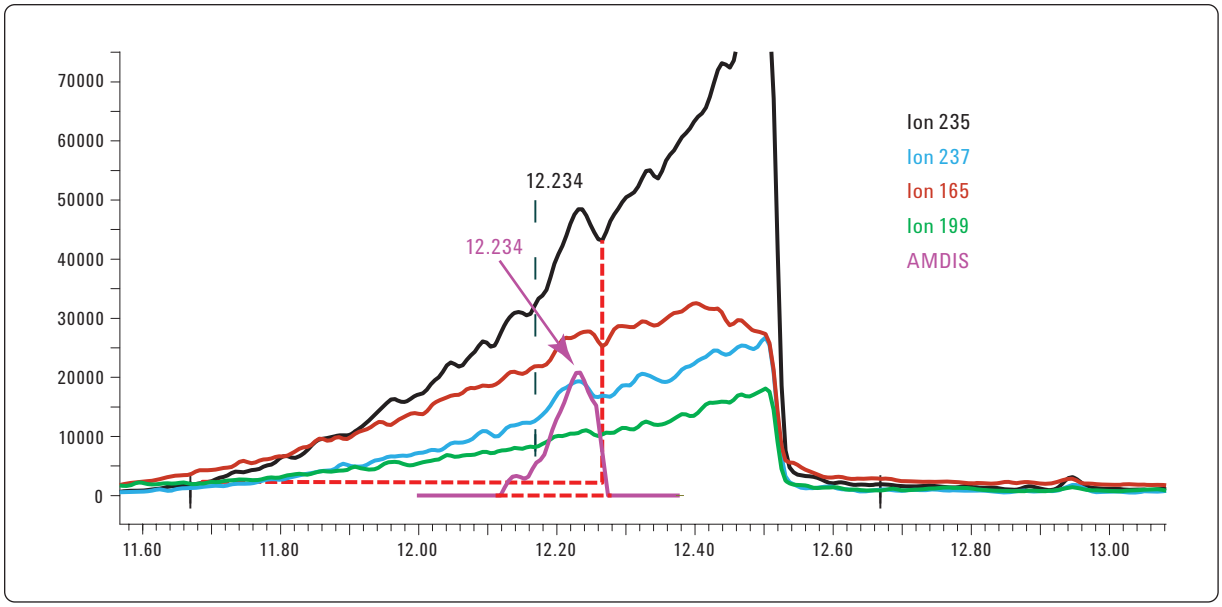


Figure 15. Target, qualifier and AMDIS deconvoluted EIC overlay.

Deconvoluted ion is noise-free, thus easier to integrate for more reliable quantitation results

In Figure 16, ChemStation and AMDIS found the same peak. Due to the noisy baseline, ChemStation drew the integration baseline (red dash line) incorrectly. Again, deconvolution removes chemical noise first, and can therefore, integrate the peak easily and reliably.

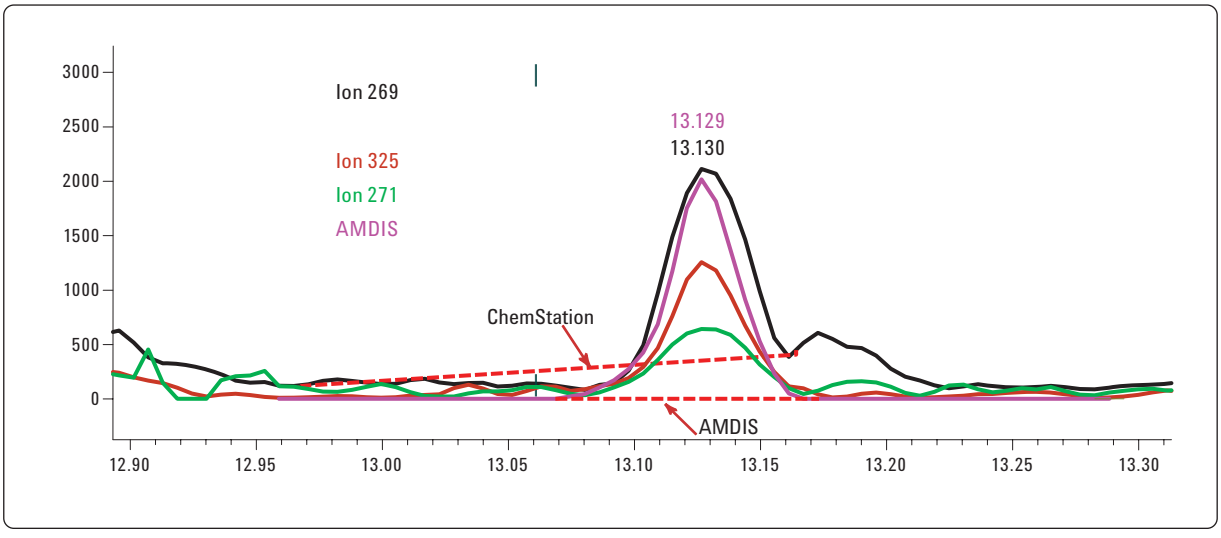


Figure 16. Target, qualifier and AMDIS deconvoluted EIC overlay.

Agilent's ChemStation add-on - Deconvolution Reporting Software (DRS) incorporates AMDIS deconvolution. Therefore, the above AMDIS advantages are automatically captured in DRS data processing which combines results from ChemStation, AMDIS, and NIST MS Search into one report.

Comparing number of compounds found between ChemStation and AMDIS

Figure 17 is a summary of the hits from ChemStation and AMDIS under four different settings, respectively. The blue bars represent the number of false positives and the red bars represent the number of actual target compounds found. On the left side of the graph, the settings of ChemStation are Ion Ratio Uncertainty. Although the absolute 30% and 50% increase the total number of compounds found, only about half of the 35 targets are found. The analyst is forced to review more hits and does not gain any additional information. The entire target list of 900+ compounds must be reviewed for false negatives. The right side of the graph shows that the four AMDIS settings gave similar results. In each case, all 35 targets were found with a reasonable number of false positives. There were no false negatives. The analyst must only review the positives, which is a significant time savings. This shows that AMDIS (DRS) is much more capable than ChemStation in finding target compounds in a complex matrix. AMDIS (DRS) provides better detectability and faster data processing.

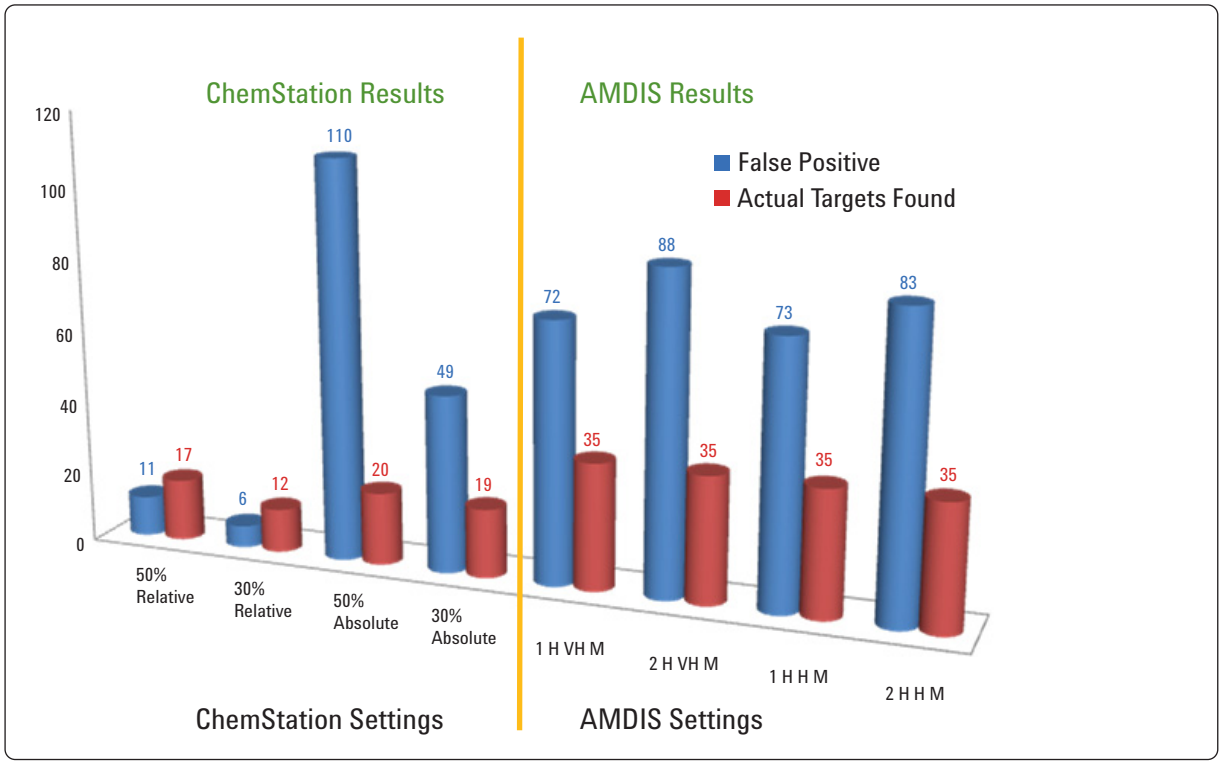


Figure 17. Overall comparison of AMDIS and MSD ChemStation compounds found.

Conclusions

- AMDIS finds more target compounds than ChemStation in a complex matrix. Deconvolution (DRS) provides a cleaned peak to integrate properly giving more reliable results.
- AMDIS did not miss any target compounds at the 50 ppb level using scan data. This minimizes the time an analyst must spend reviewing results.
- Confirmation of compounds is done in significantly less time with deconvoluted component spectra available.
- The detectability of compounds in a complex matrix is significantly improved with deconvolution. This can also be viewed as better or increased sensitivity through improved selectivity versus the background.
- Deconvolution Reporting Software (DRS) automates the deconvolution (AMDIS) process to produce an easy-to-read quantitation report.

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