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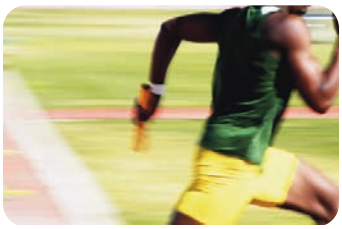
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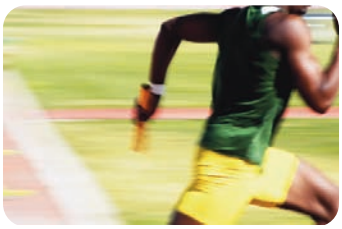


Applications by Instrumental Technique









Applications by Instrumental Technique

LC/MS/MS



Analysis of Anabolic Agents in Urine by LC/MS/MS

Application

Forensics/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

The use of anabolic substances for performance enhancement in sports seems to be a ubiquitous topic of discussion. While their use is a growing problem in high school and collegiate athletics, their use at the professional level is an ongoing controversy. It is therefore the mandate of agencies like WADA to ensure that fair competition is being maintained by monitoring the possible use of banned substances like anabolic compounds.

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).

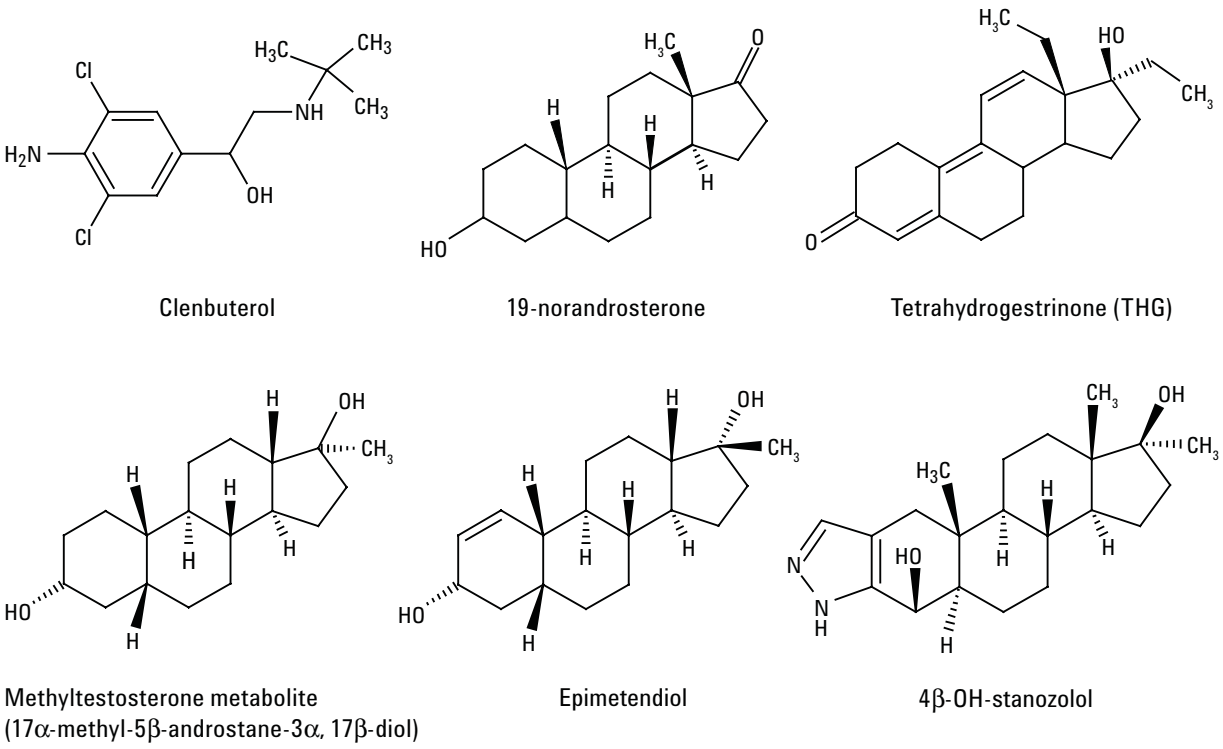


Figure 1. Structures of anabolic substances analyzed in this work.

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.

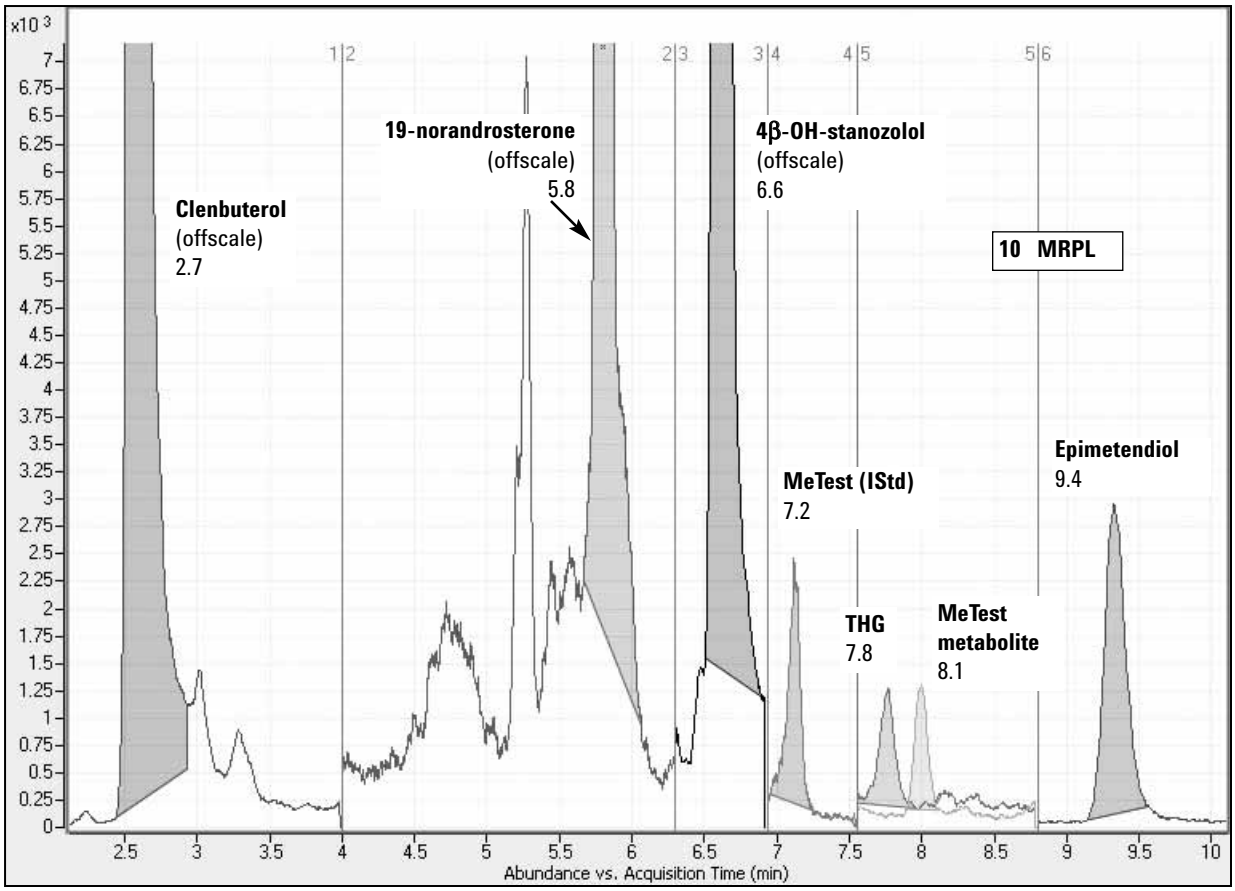


Figure 2. Chromatographic profile of 10 MRPL extract in urine.

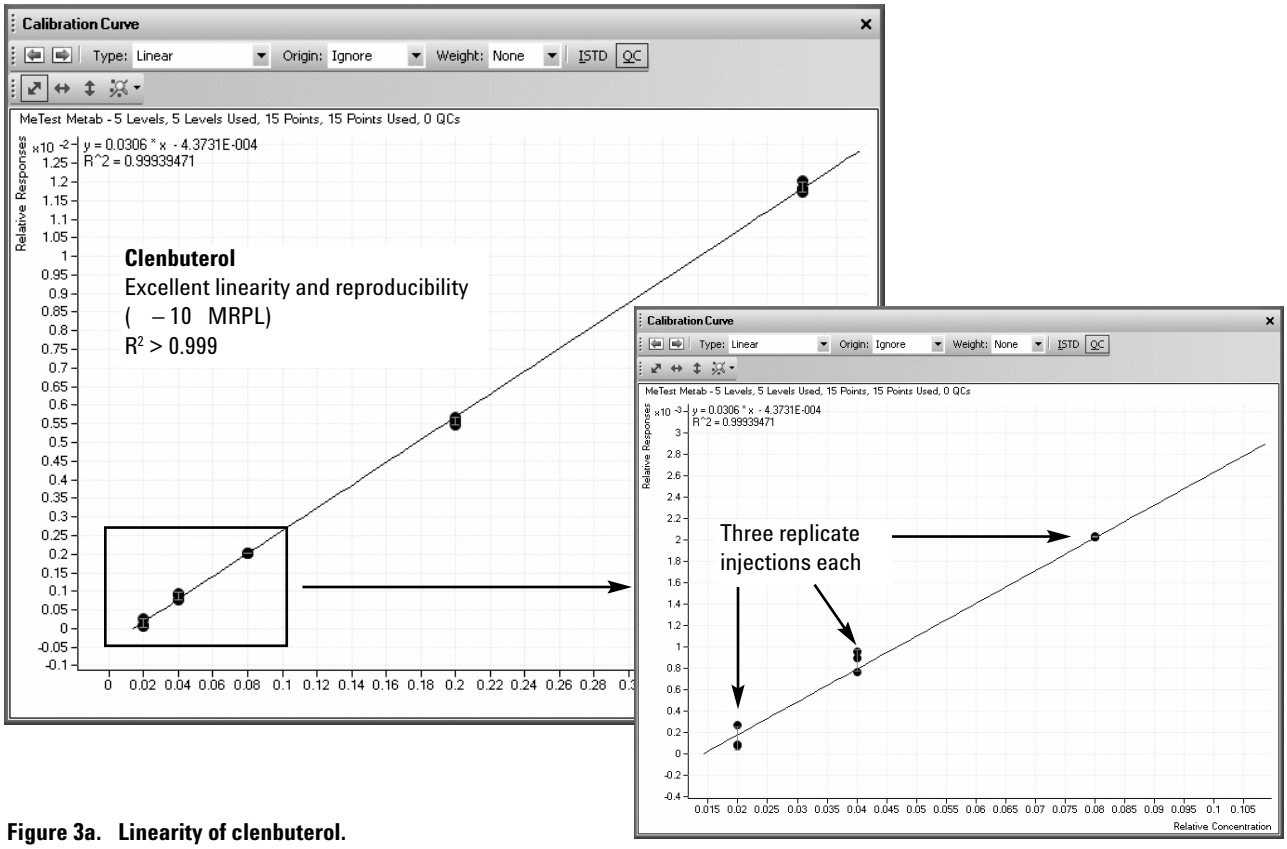


Figure 3a. Linearity of clenbuterol.

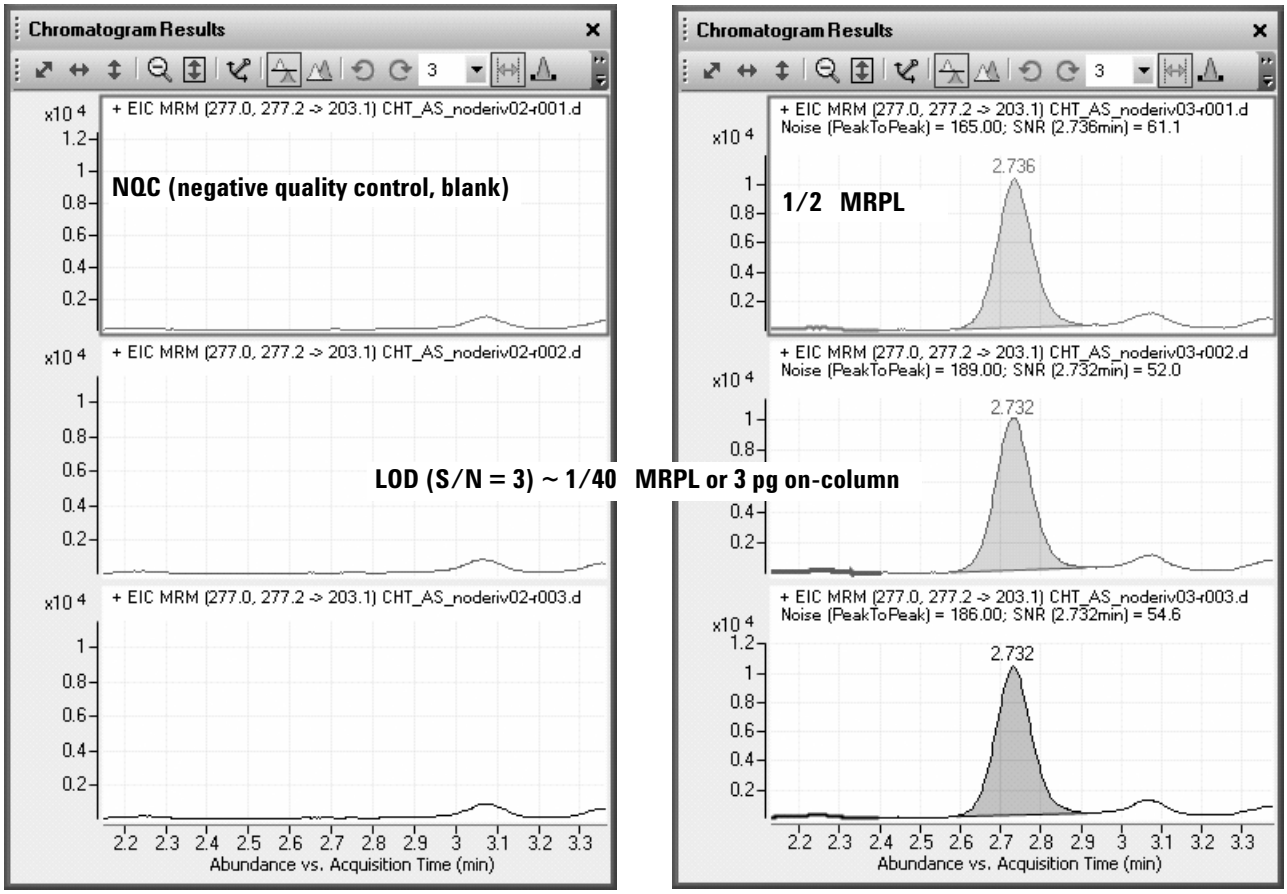


Figure 3b. Estimate of LOD for clenbuterol.

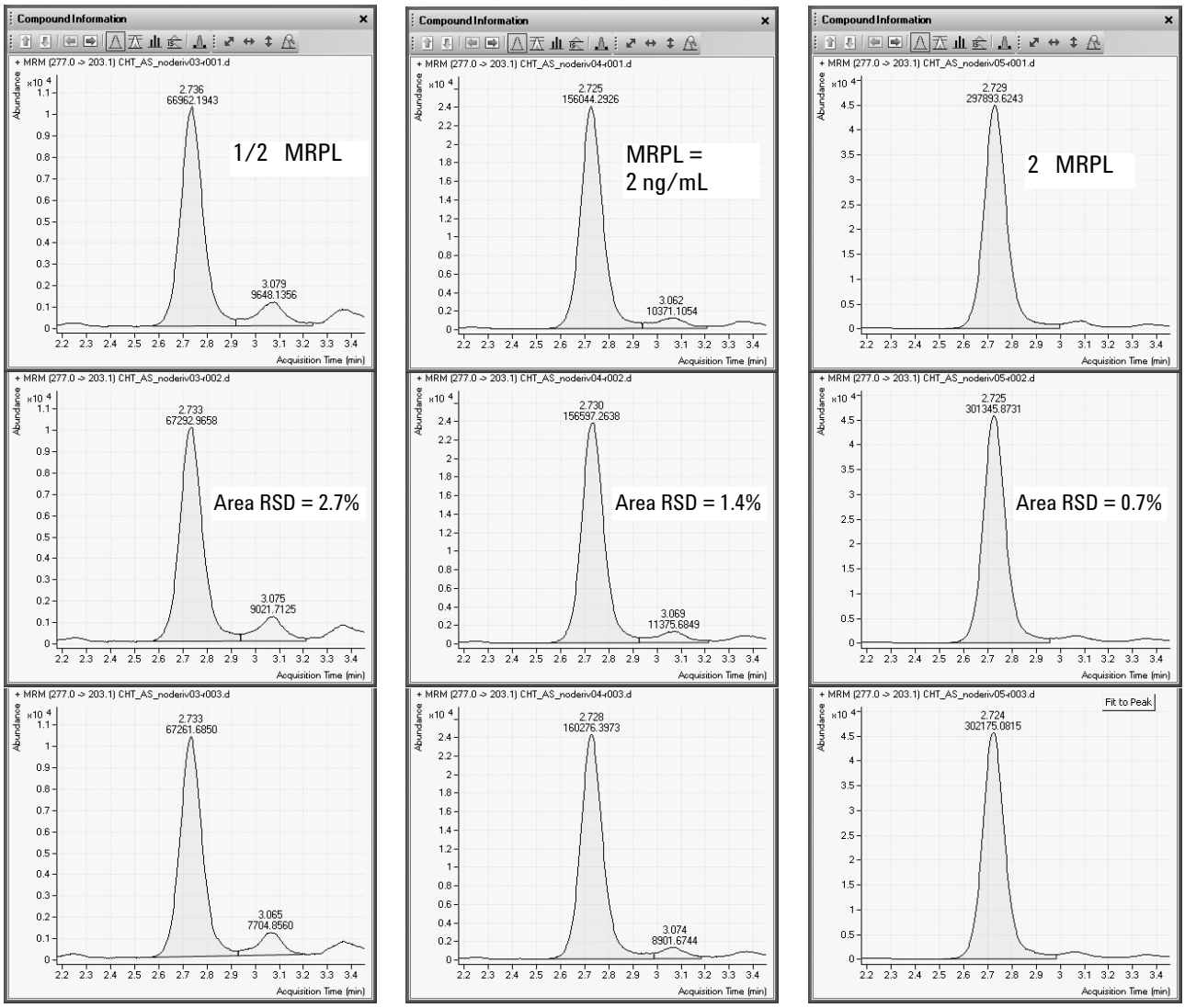


Figure 3c. Triplicate injections of the lowest three levels of clenbuterol.

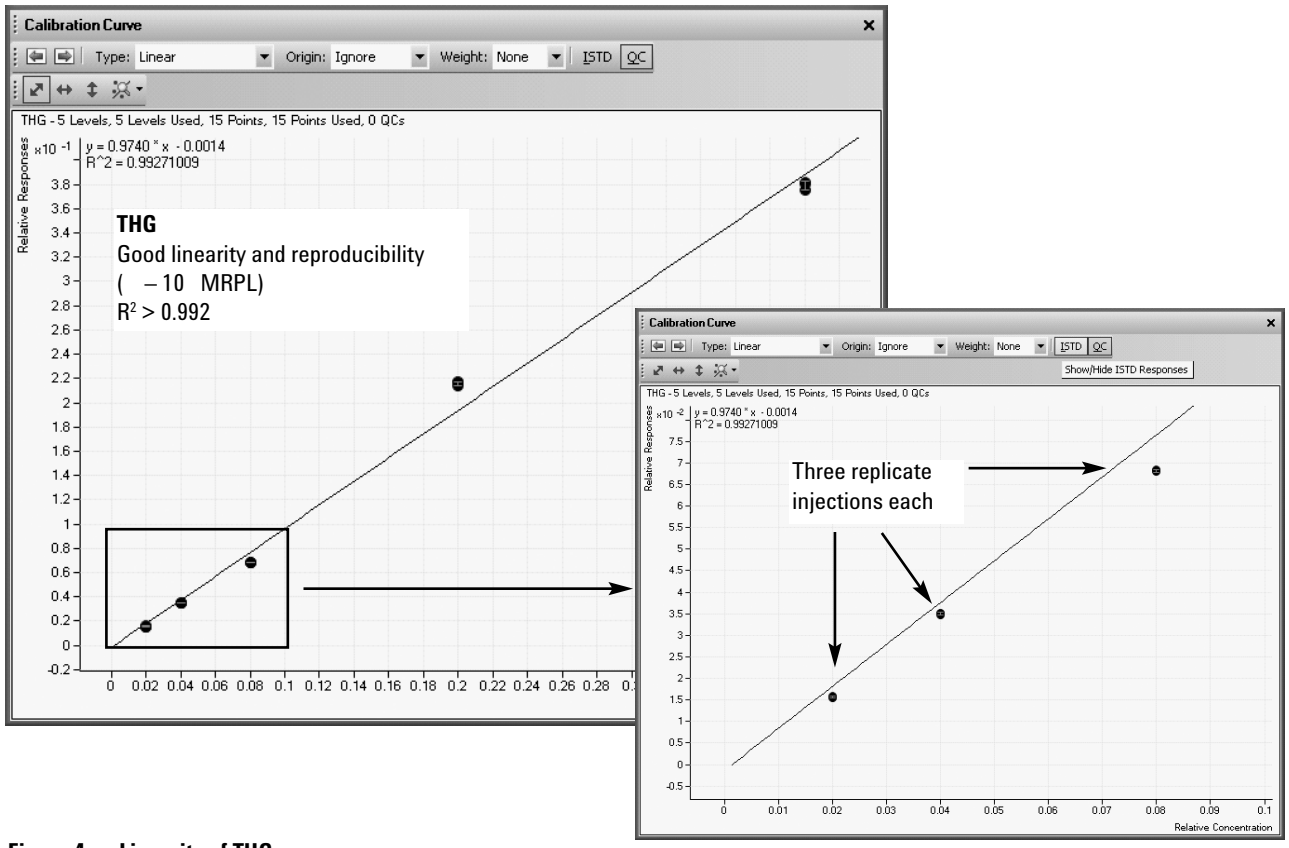


Figure 4a. Linearity of THG.

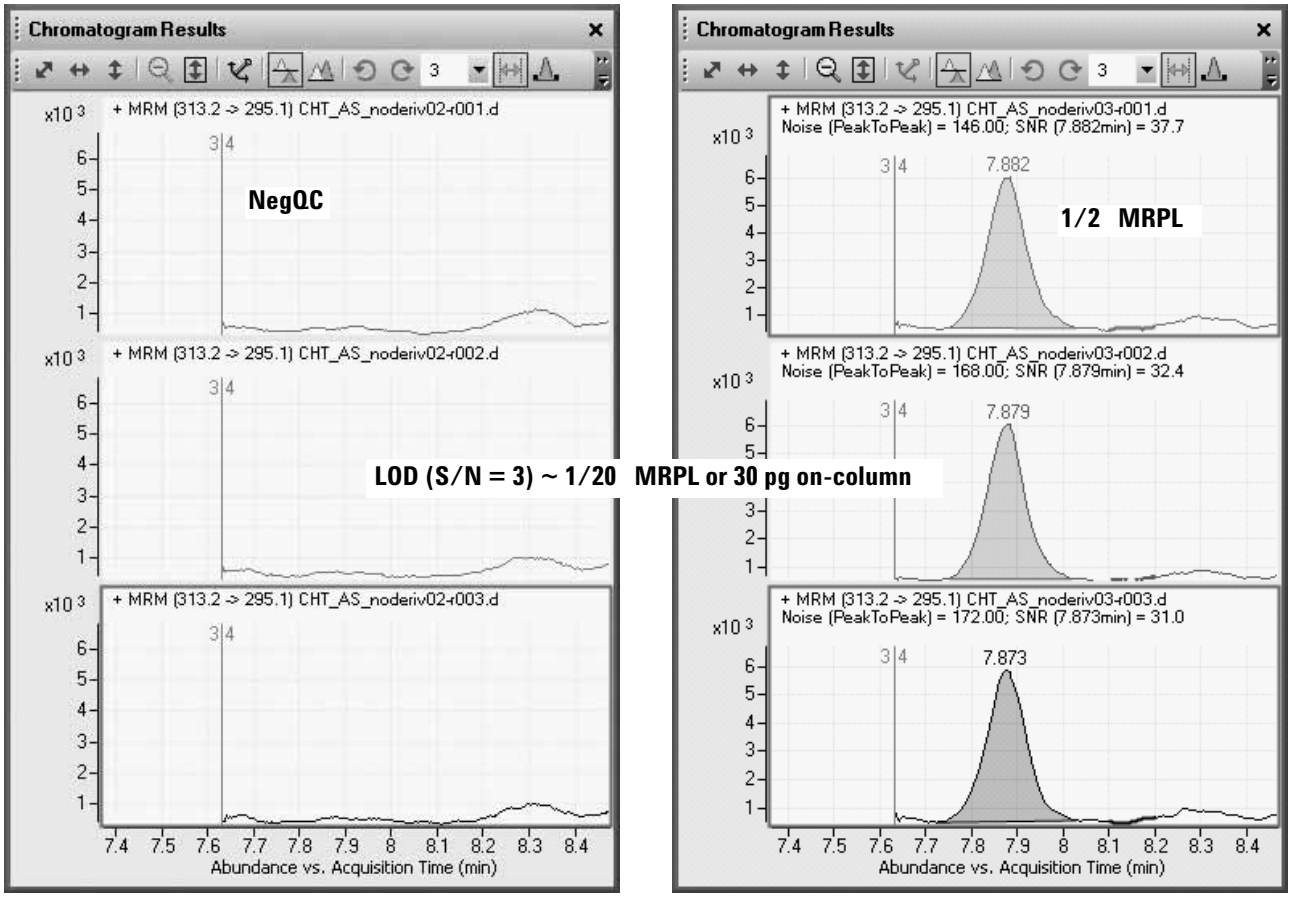


Figure 4b. Estimate of LOD for THG.

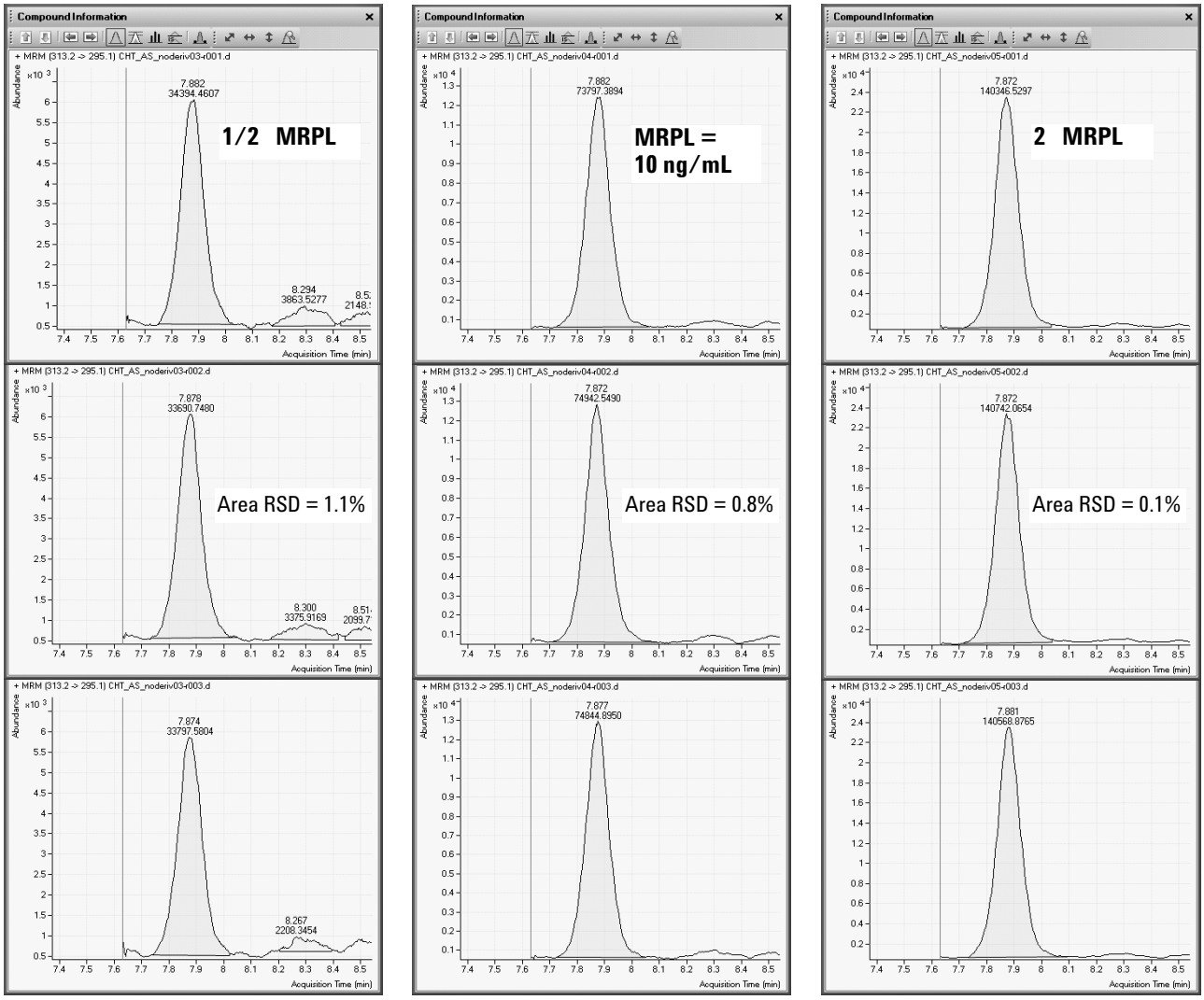


Figure 4c. Triplicate injections of the lowest three levels of THG.

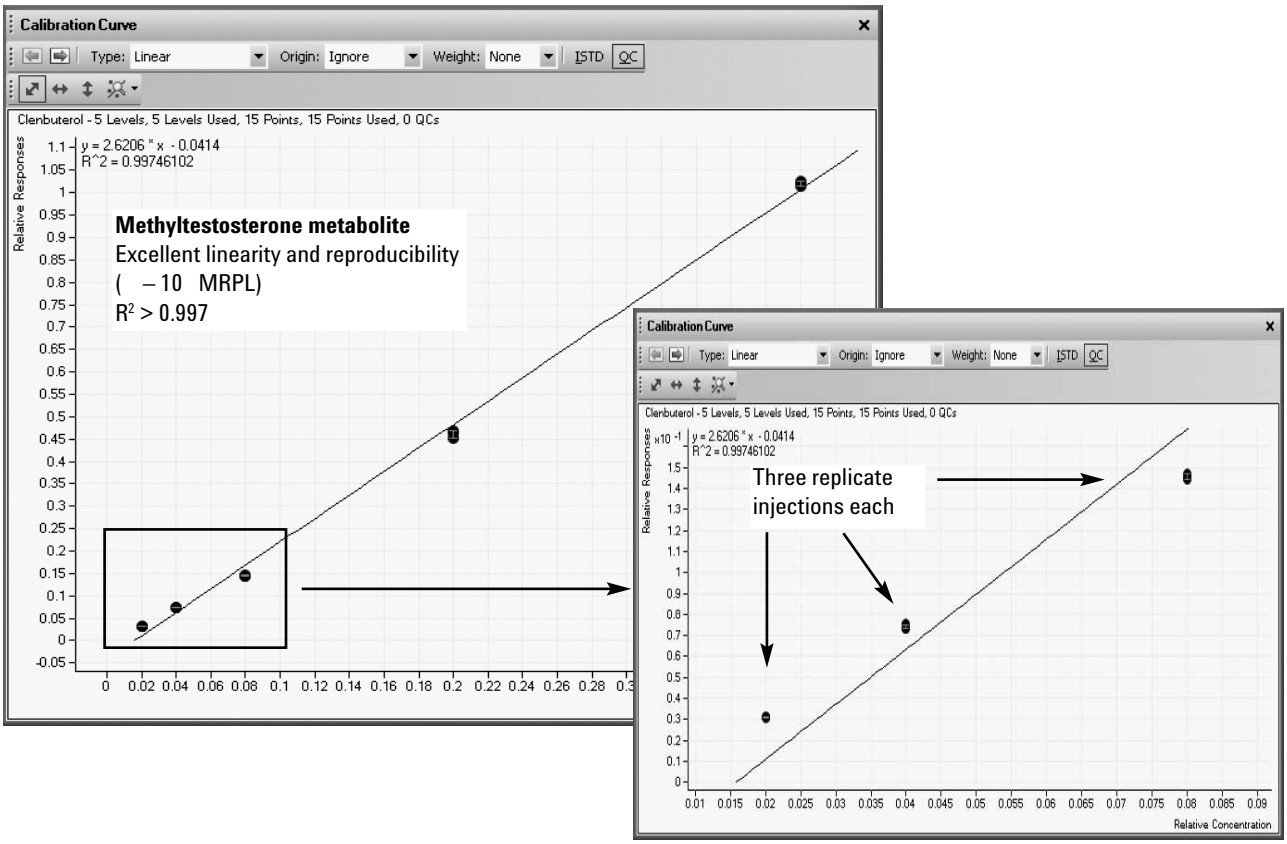


Figure 5a. Linearity of methyltestosterone metabolite.

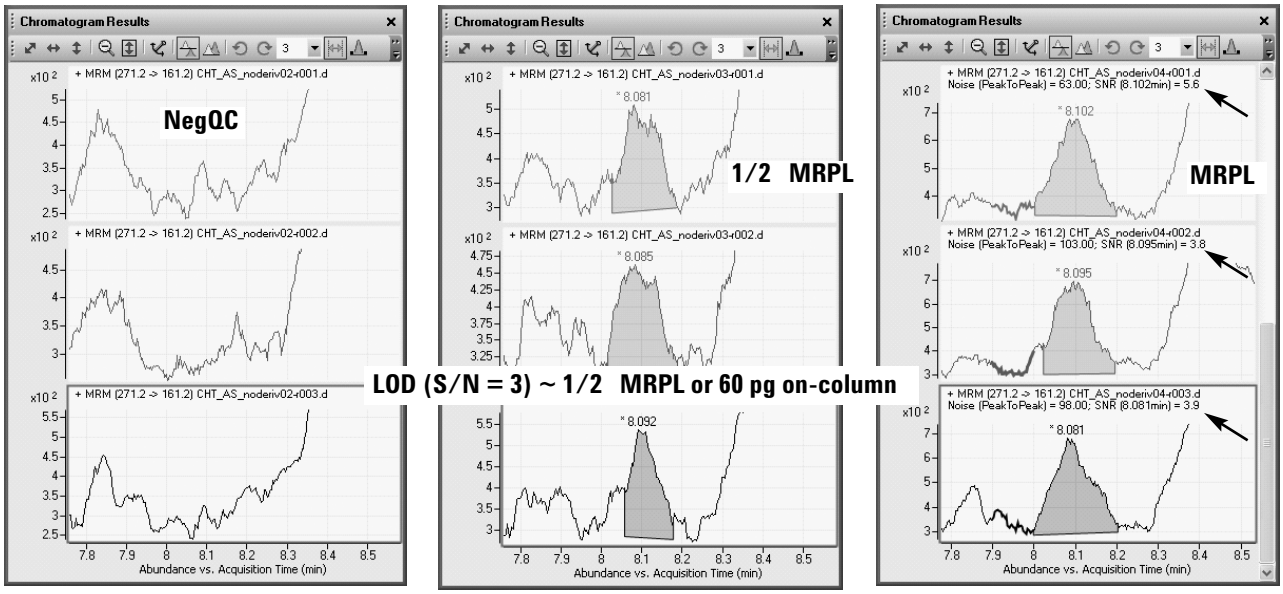


Figure 5b. Estimate of LOD for methyltestosterone metabolite.

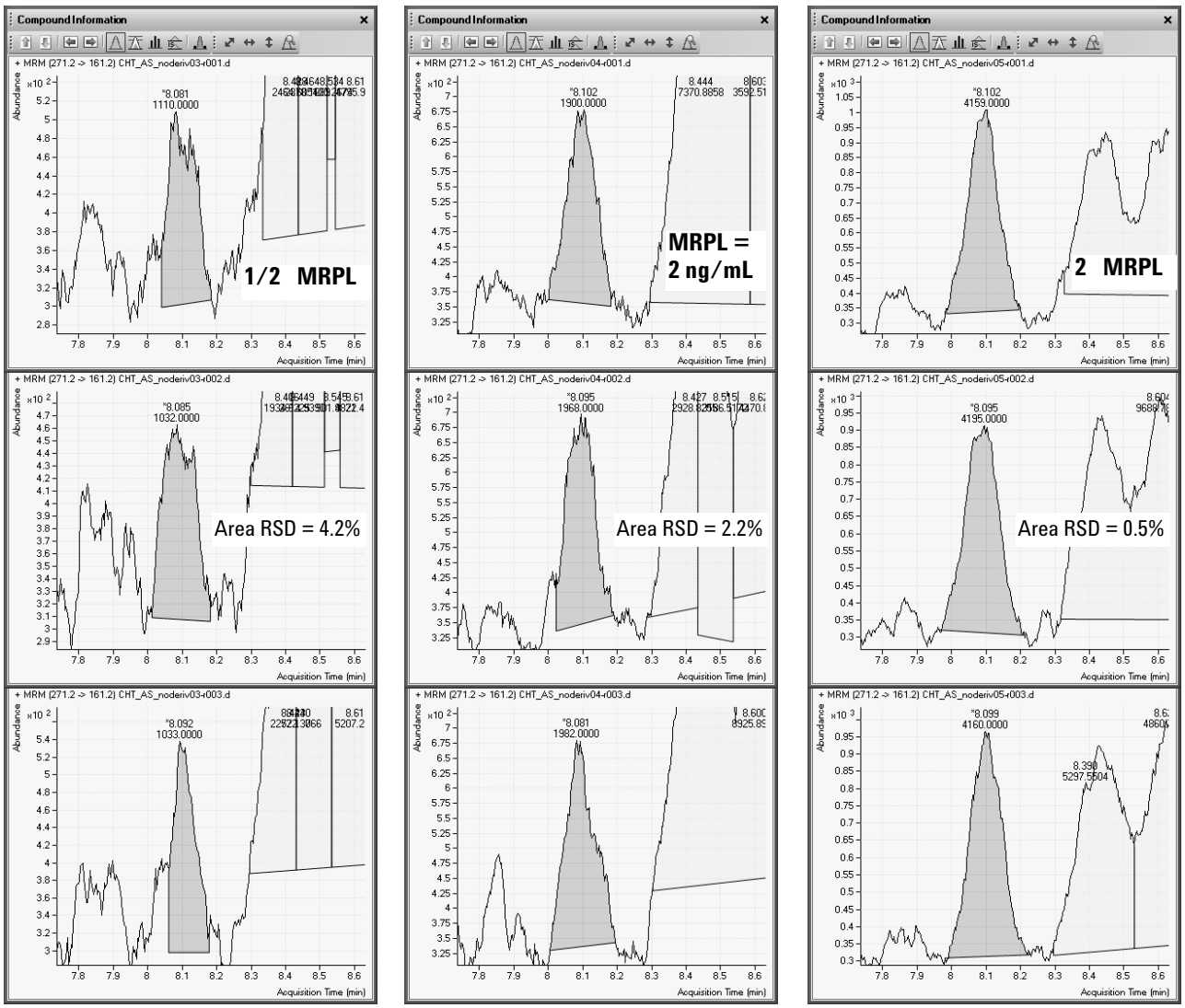


Figure 5c. Triplicate injections of the lowest three levels of methyltestosterone metabolite.

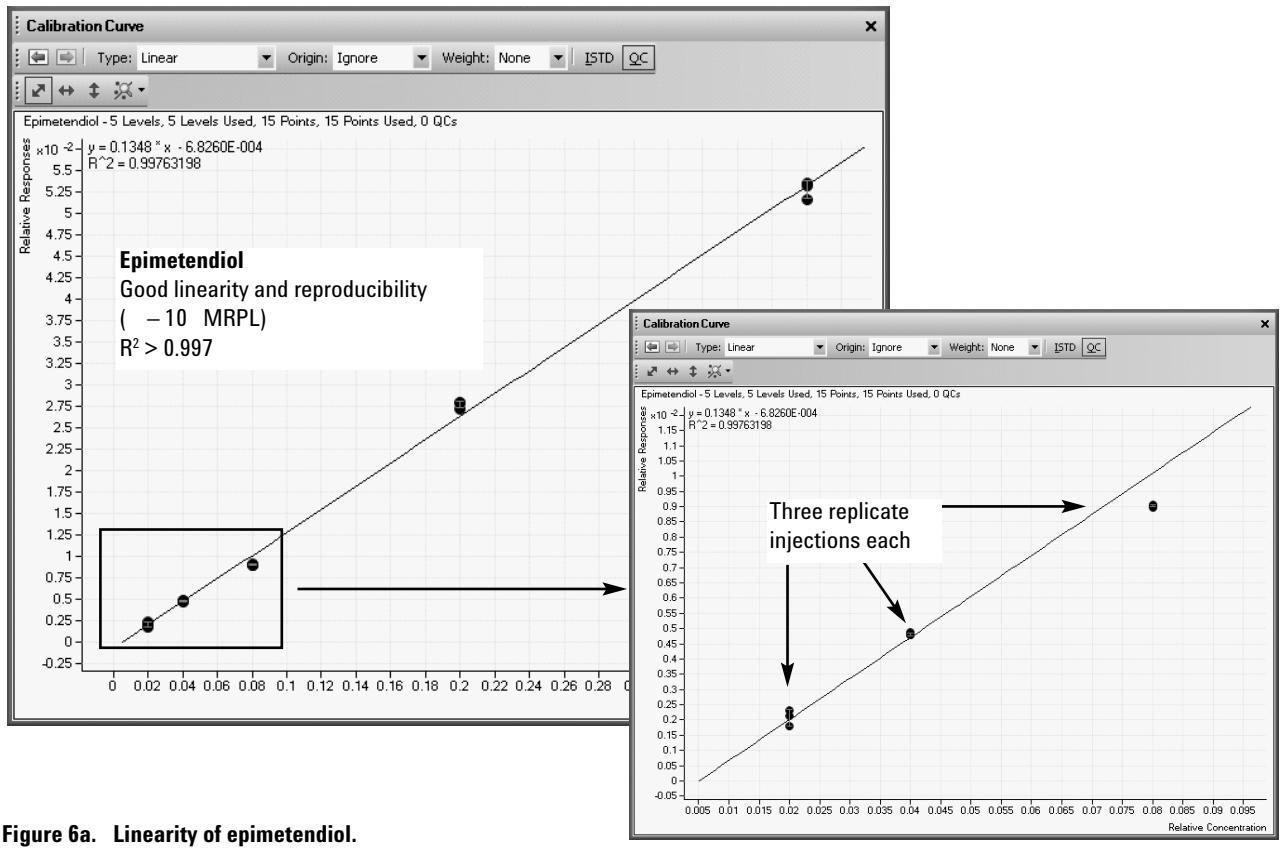


Figure 6a. Linearity of epimetendiol.

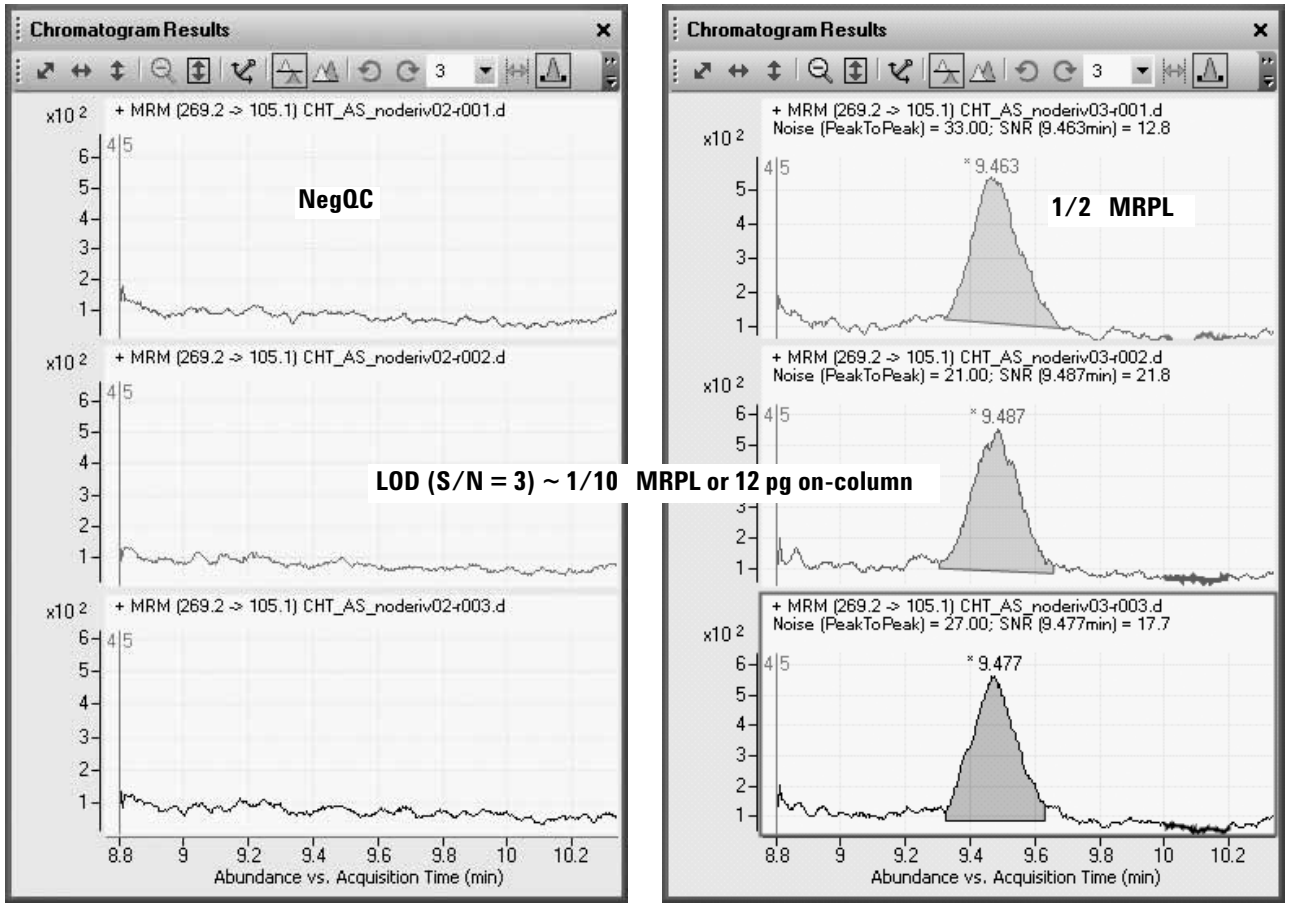


Figure 6b. Estimate of LOD for epimetendiol.

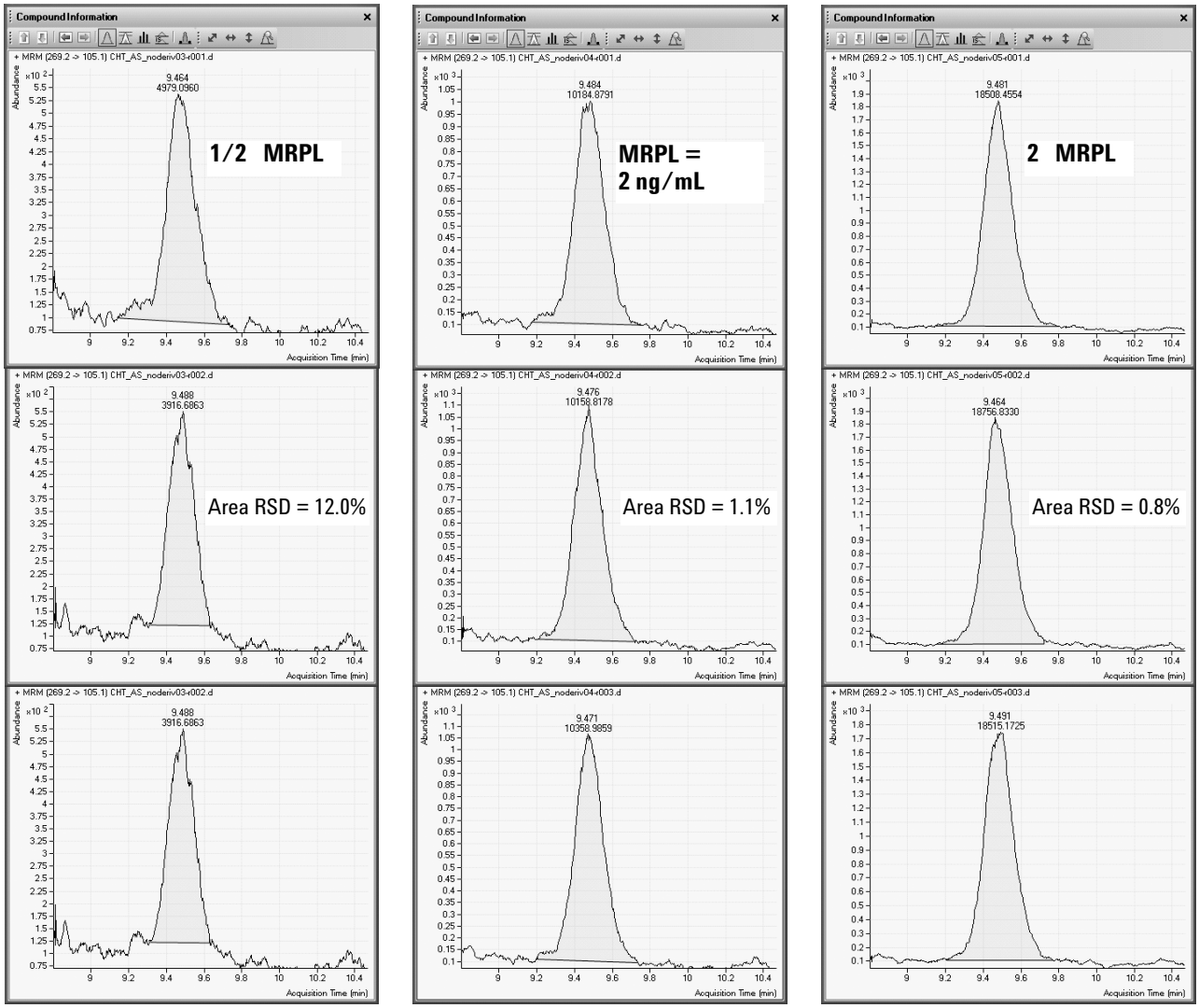


Figure 6c. Triplicate injections of the lowest three levels of epimetendiol.

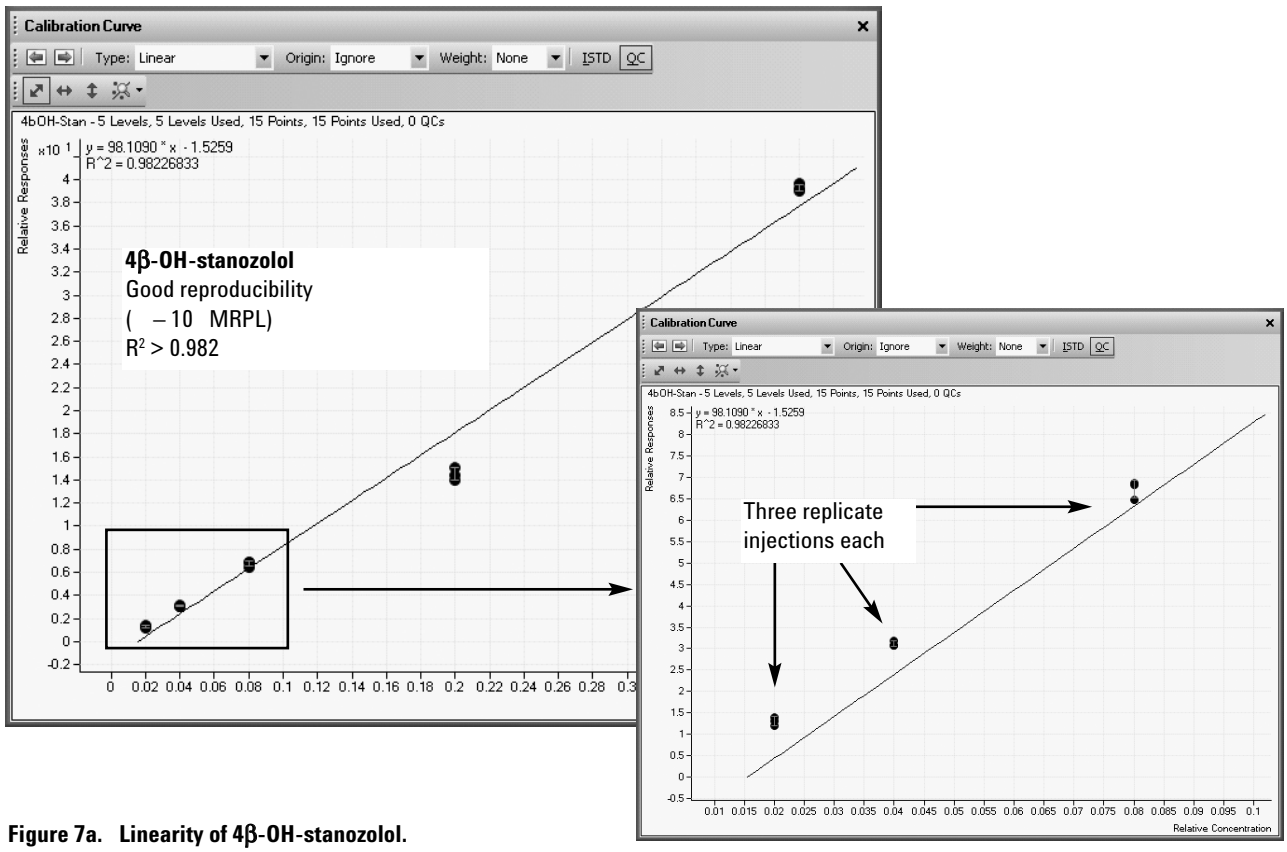


Figure 7a. Linearity of 4β-OH-stanozolol.

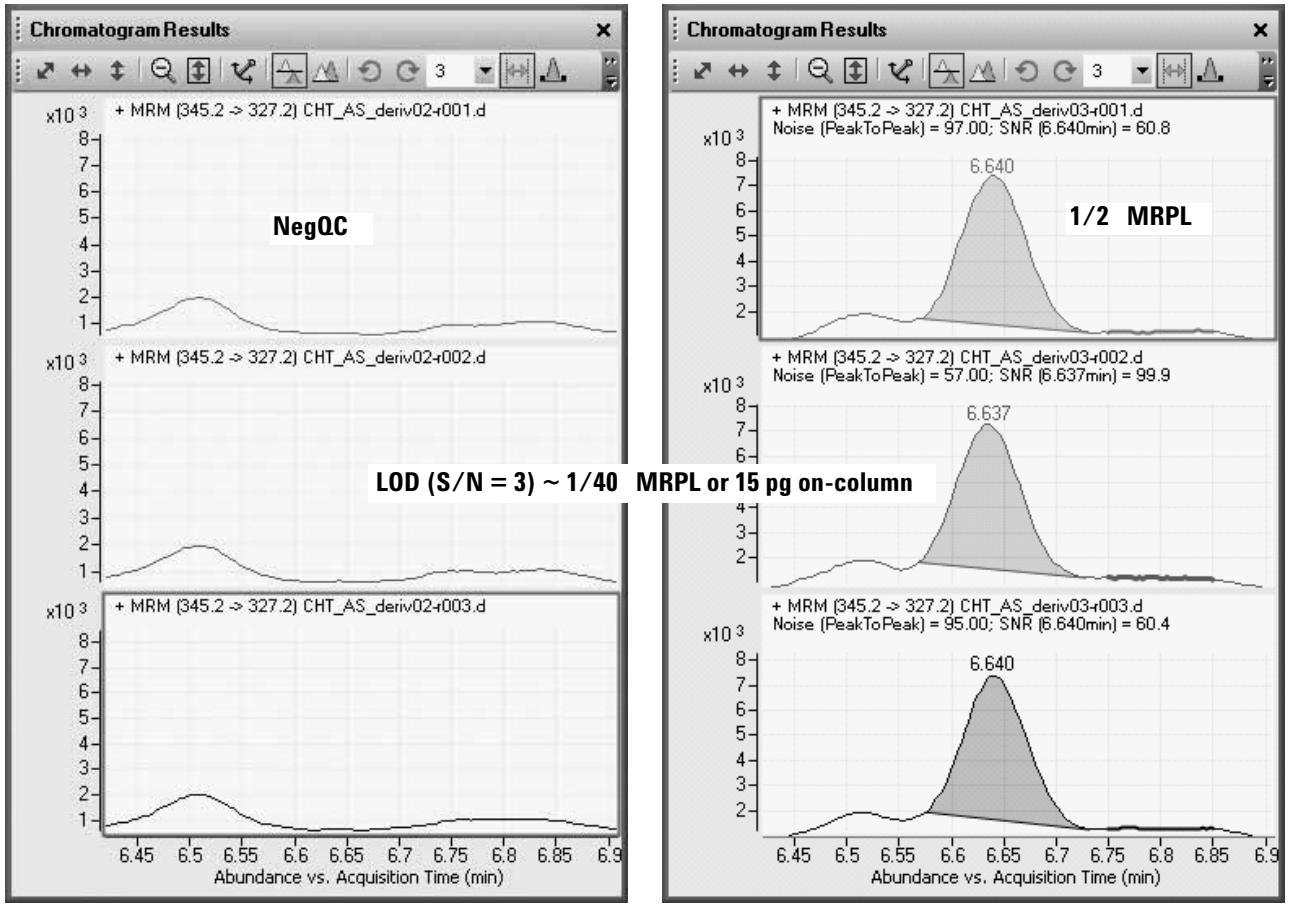


Figure 7b. Estimate of LOD for 4β-OH-stanozolol.

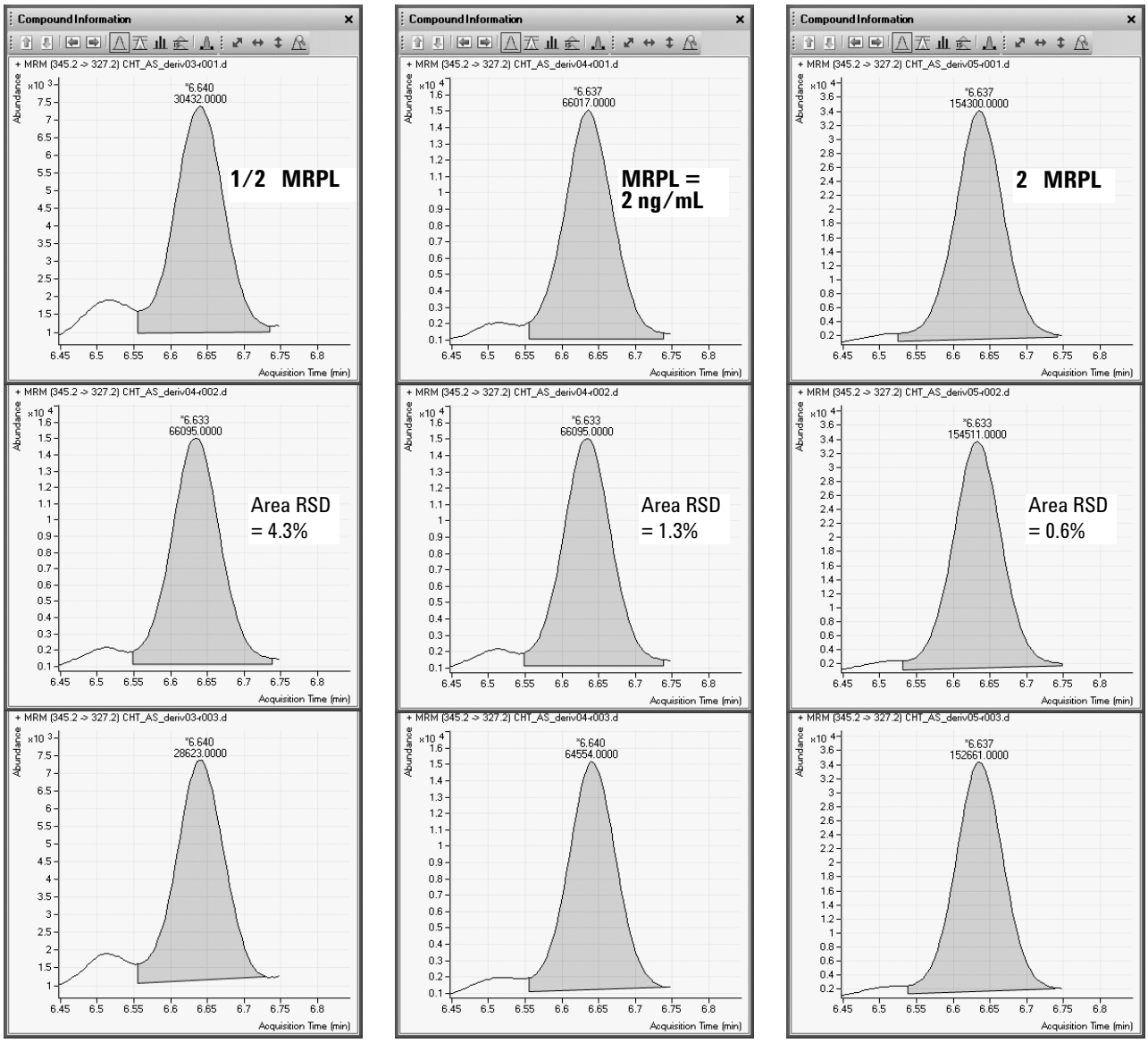


Figure 7c. Triplicate injections of the lowest three levels of 4β-OH-stanozolol.

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.

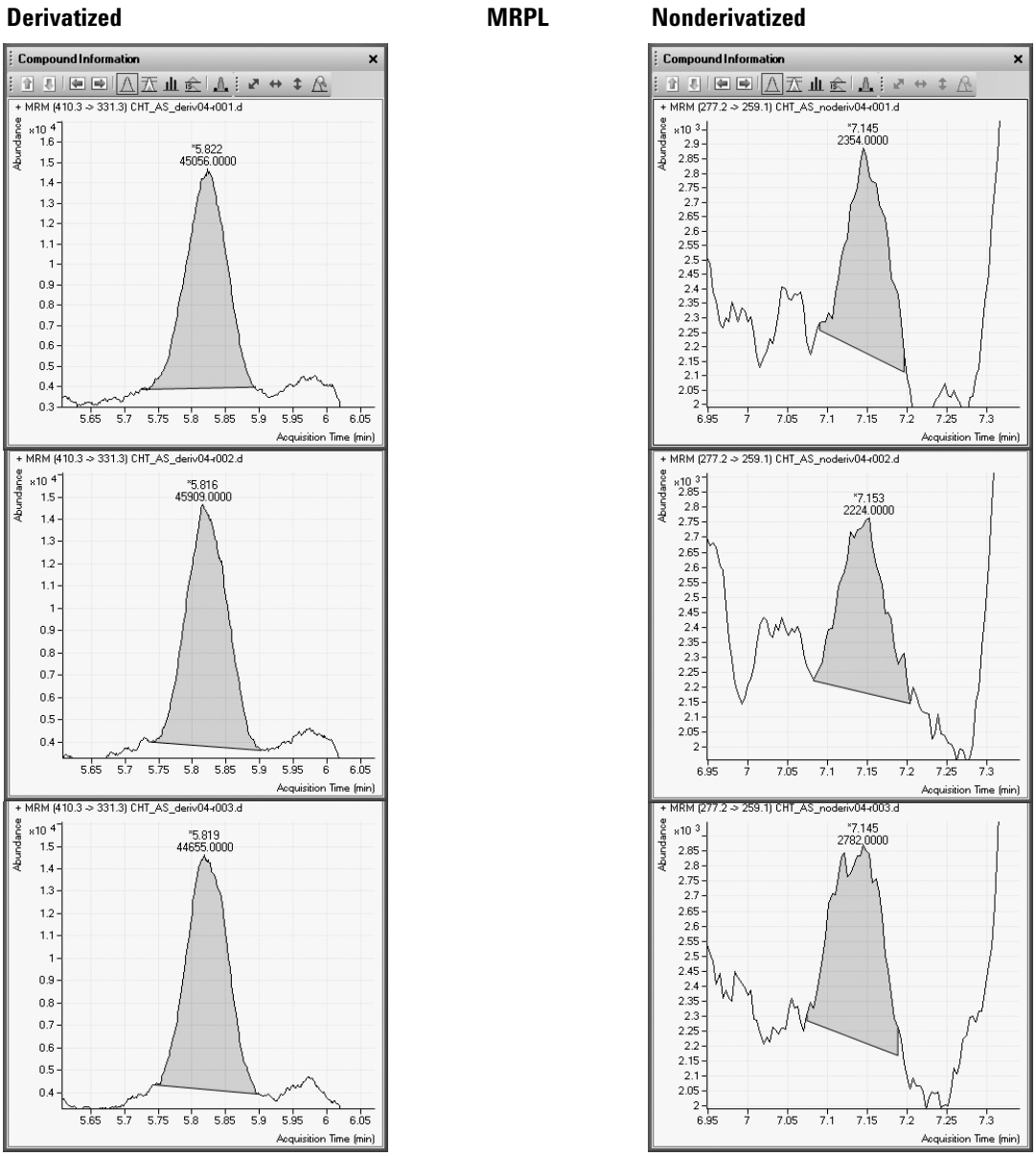


Figure 8. Comparison of signal response for the derivatized (left) versus nonderivatized forms of 19-norandrosterone.

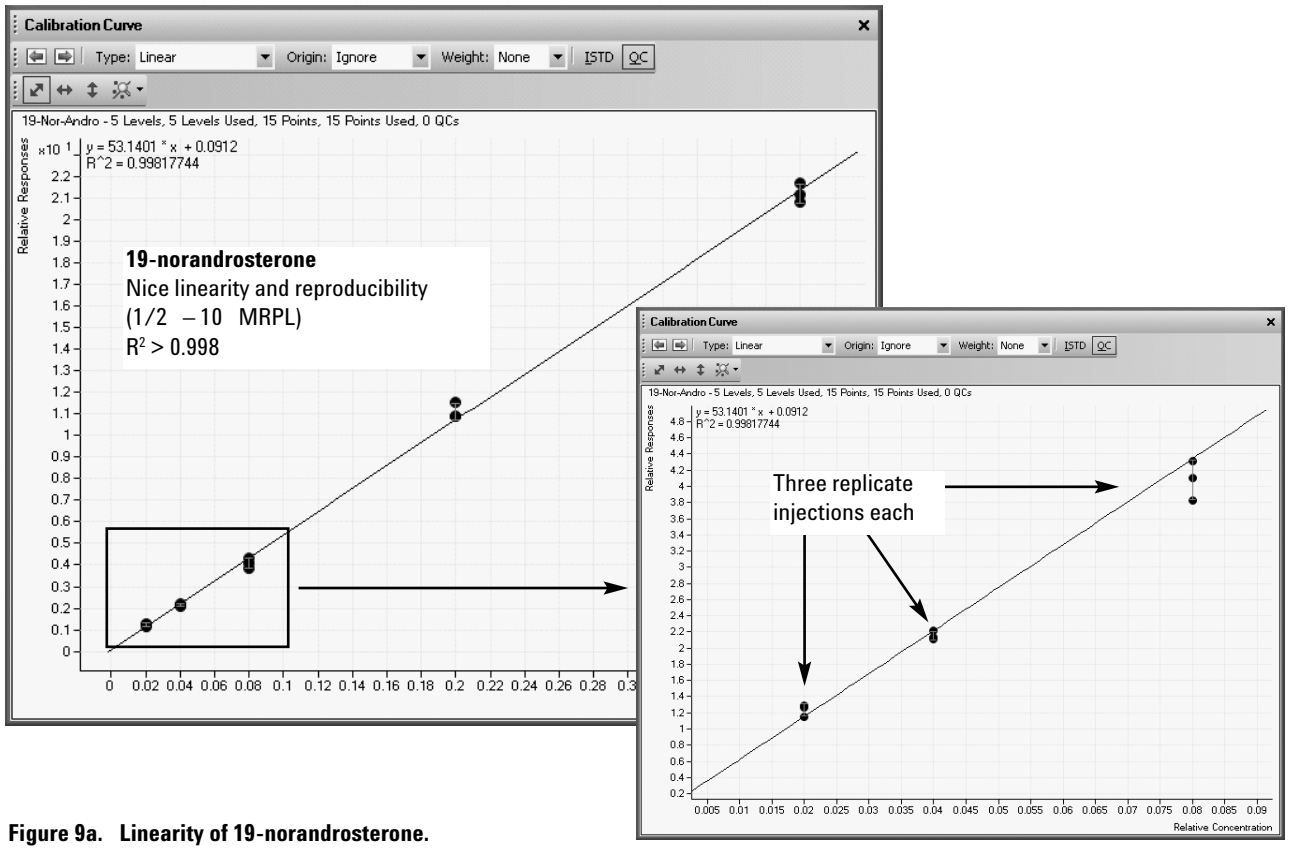


Figure 9a. Linearity of 19-norandrosterone.

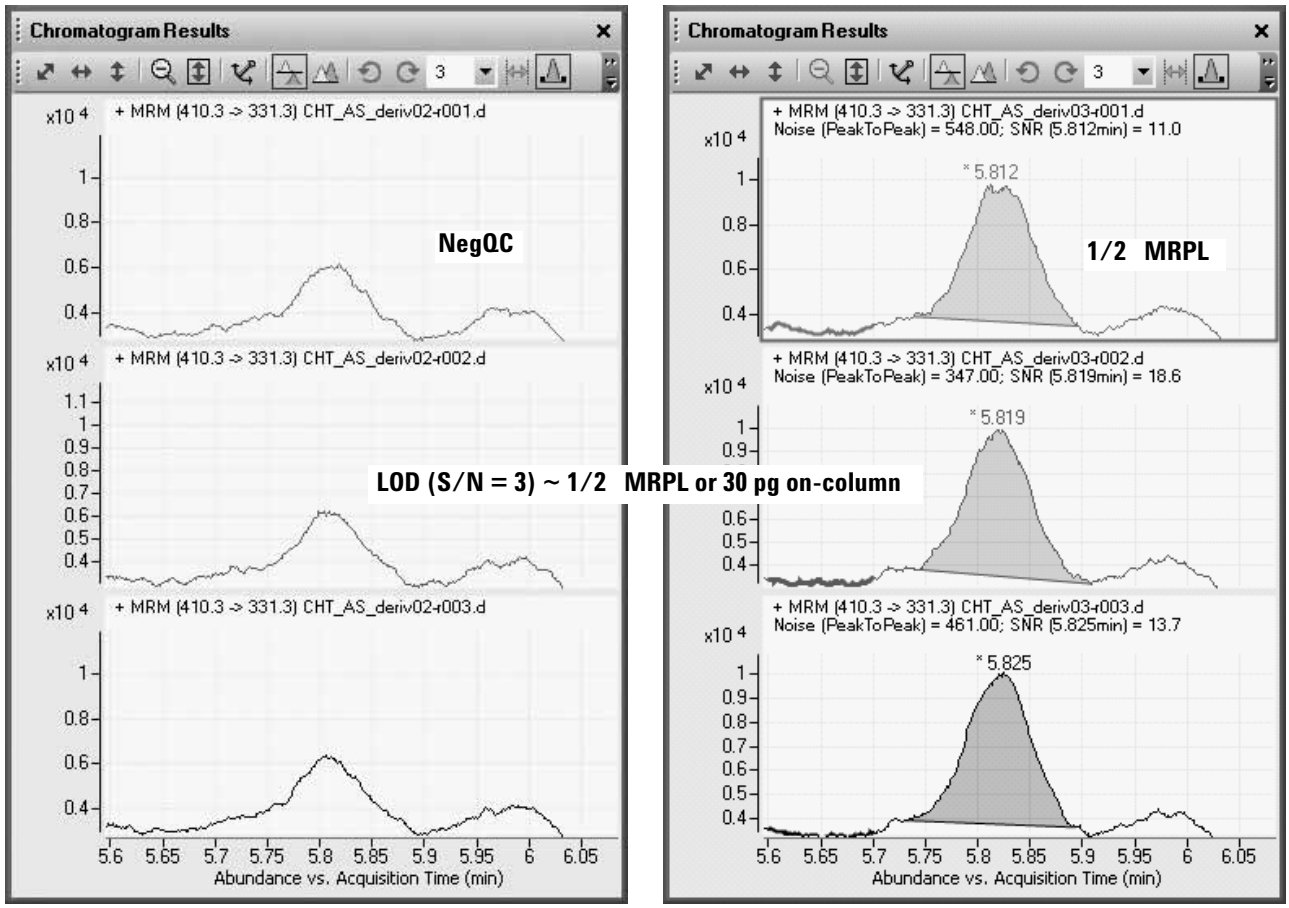


Figure 9b. Estimate of LOD for 19-norandrosterone.

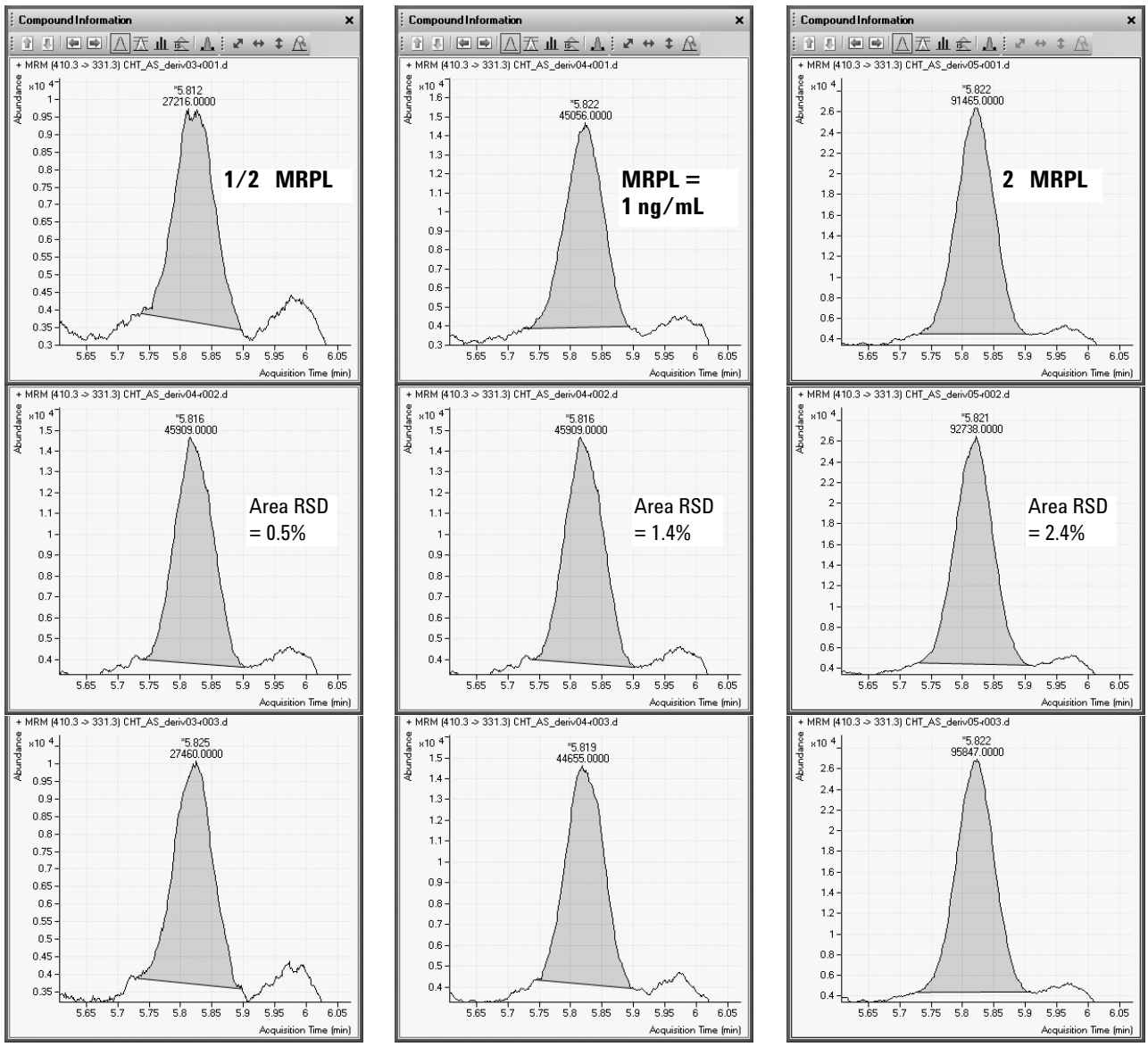


Figure 9c. Triplicate injections of the lowest three levels of 19-norandrosterone.

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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Quantitative Analysis of Amphetamine-Type Drugs by Extractive Benzoylation and LC/MS/MS

Application

Forensics

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Abstract

A fast, sensitive, and validated technique for confirming the presence of amphetamine drugs in whole blood using the Agilent G6410A Triple Quadrupole Mass Spectrometer (QQQ) is presented. Excellent linearity is demonstrated over the range of approximately 15 to 1,000 ng/mL. The amphetamine drugs analyzed in this work include amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), and methylenedioxymethamphetamine (MDMA) in blood. The drugs have been prepared using an extractive alkylation technique. The sample preparation is then followed by reverse-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 that exhibit strong central nervous system stimulant effects. Amphetamine (phenylisopropylamine) 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 applicability 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 confirmation, 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.

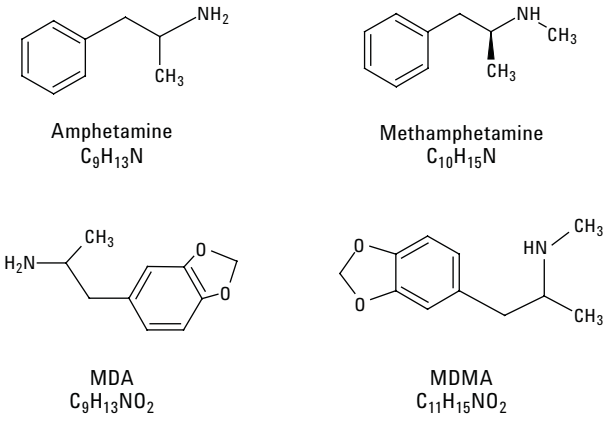


Figure 1. Structures of the compounds analyzed in this work.

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.

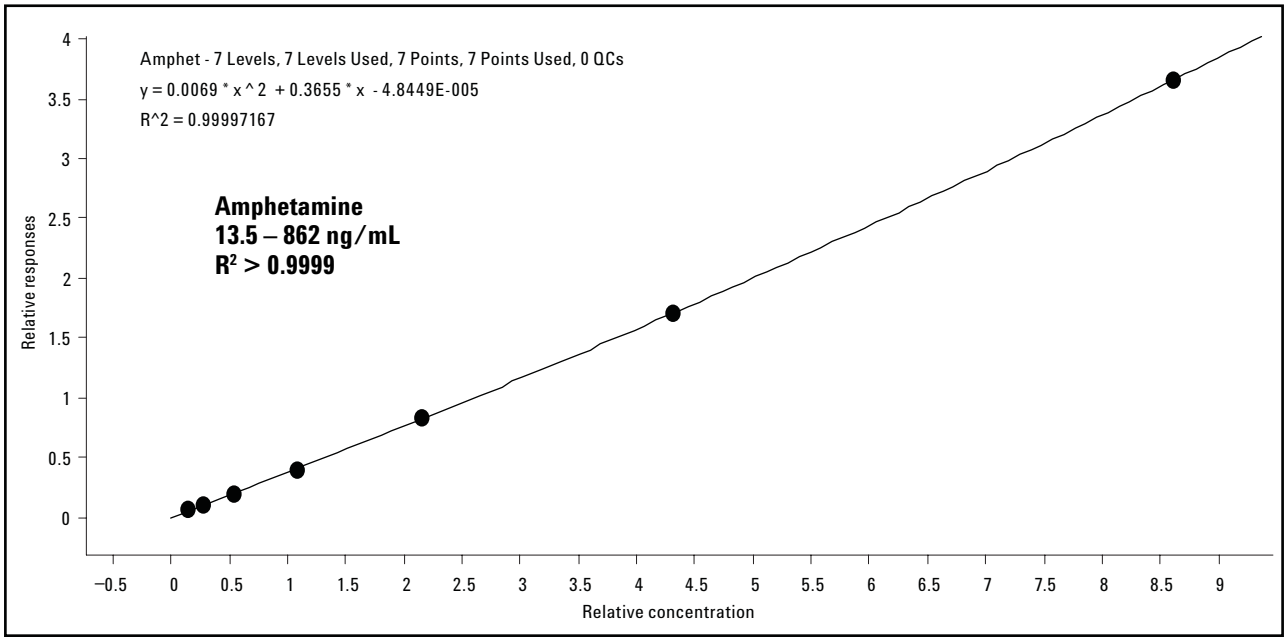


Figure 2a. Linearity of amphetamine in blood.

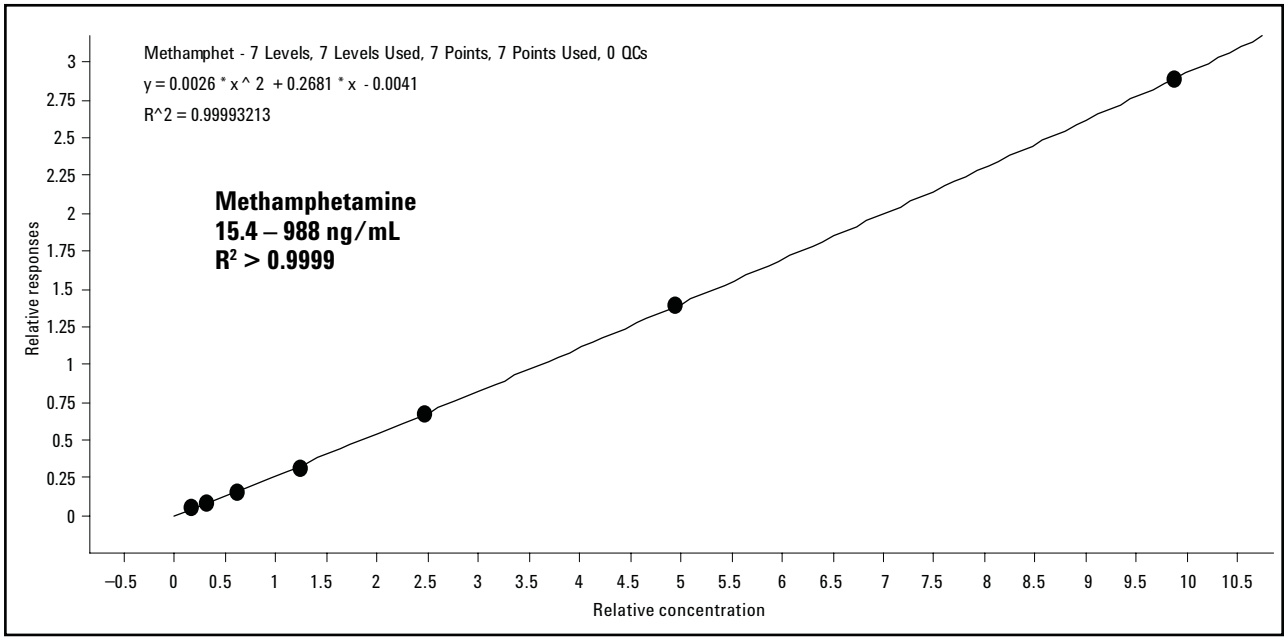


Figure 2b. Linearity of methamphetamine in blood.

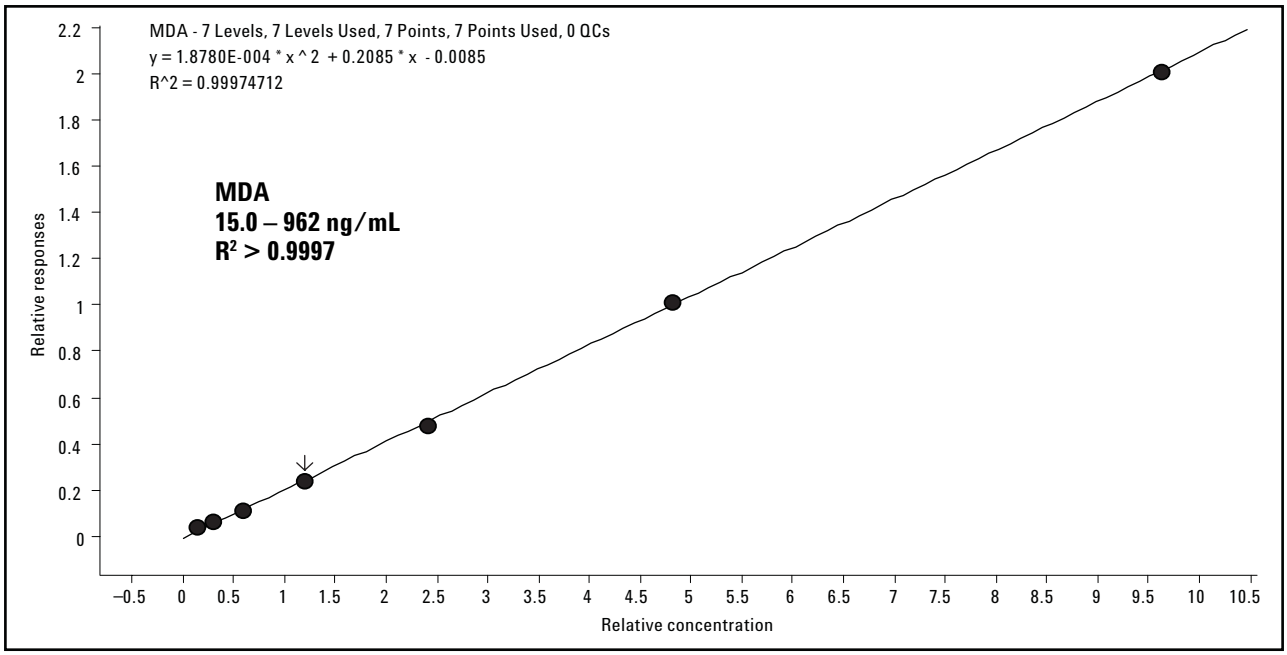


Figure 2c. Linearity of MDA in blood.

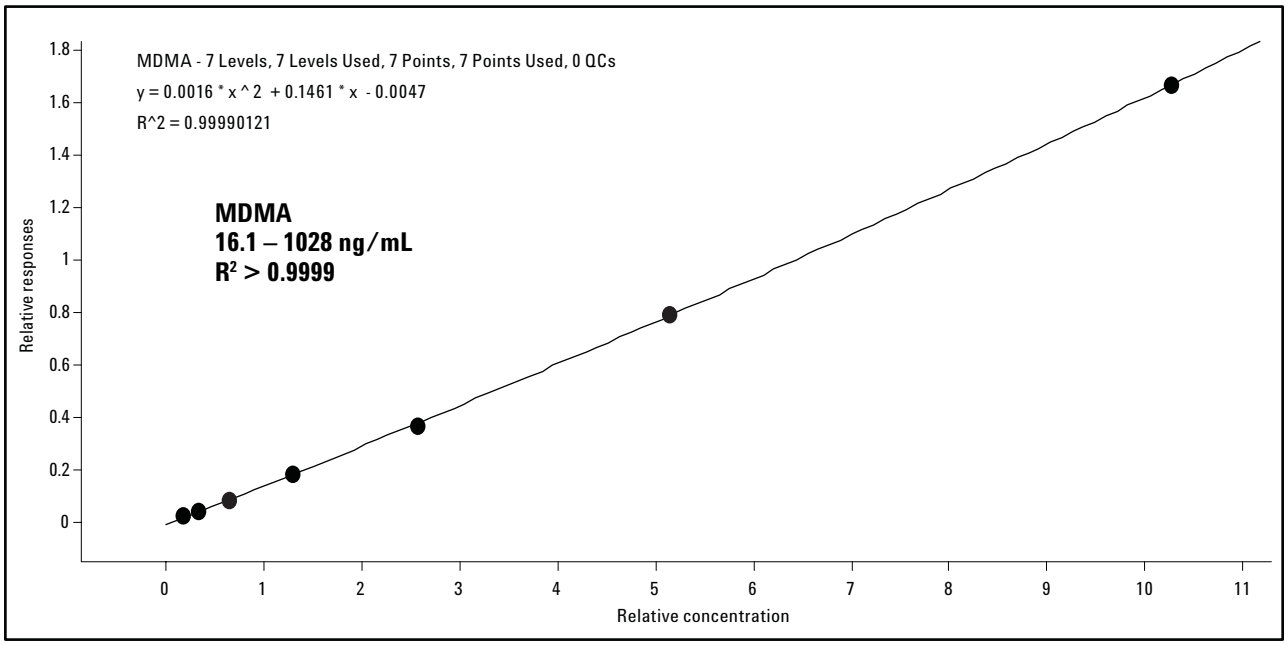


Figure 2d. Linearity of MDMA in blood.

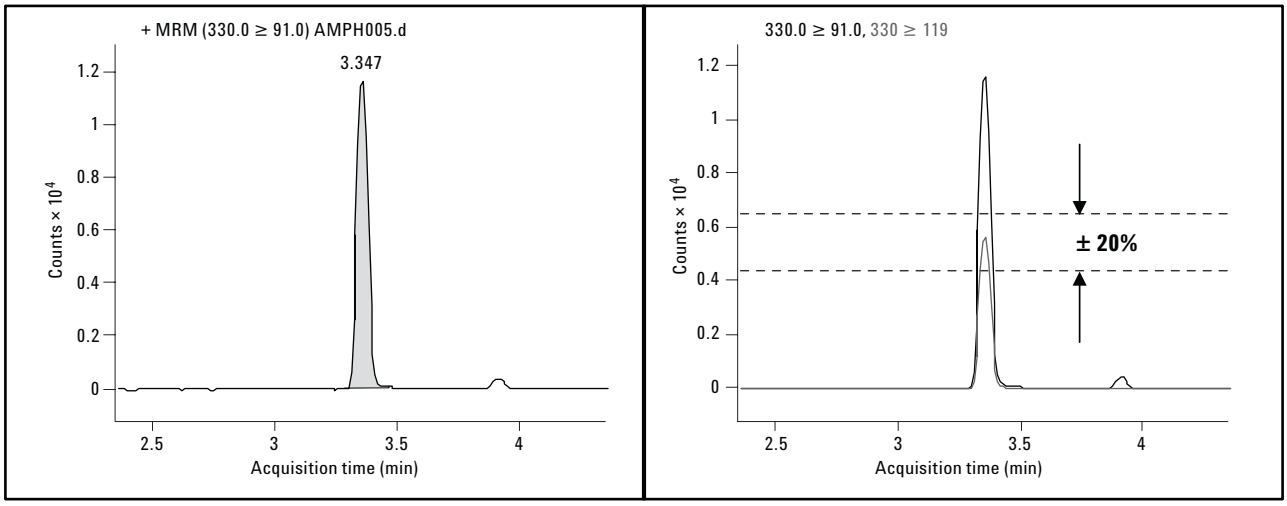


Figure 3a. Ion ratio confirmation for amphetamine in blood. Note retention time of 3.35 min.

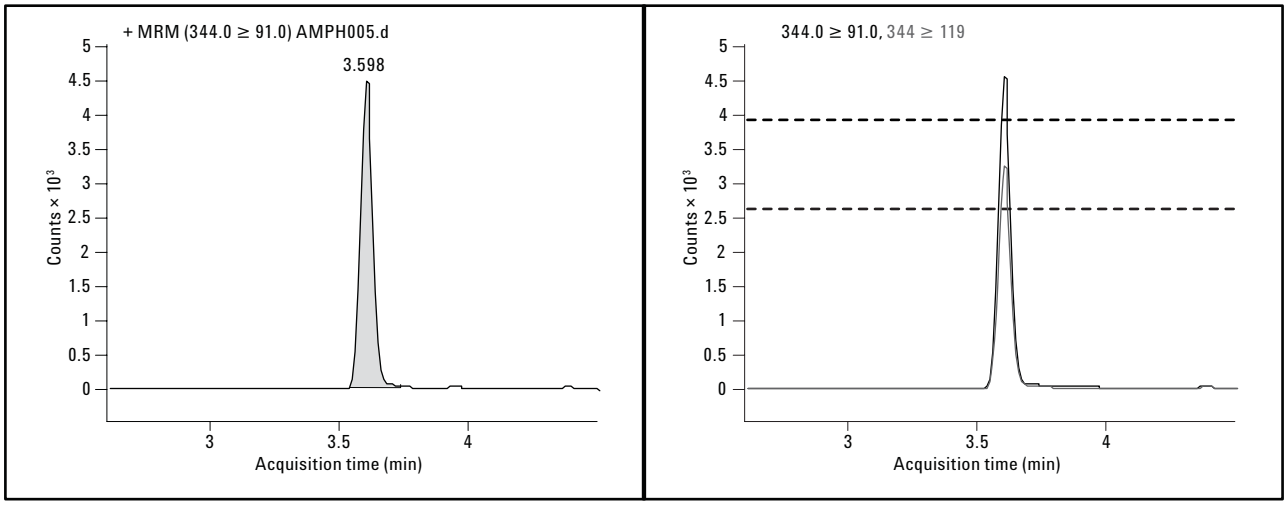


Figure 3b. Ion ratio confirmation for methamphetamine in blood. Note retention time of 3.60 min.

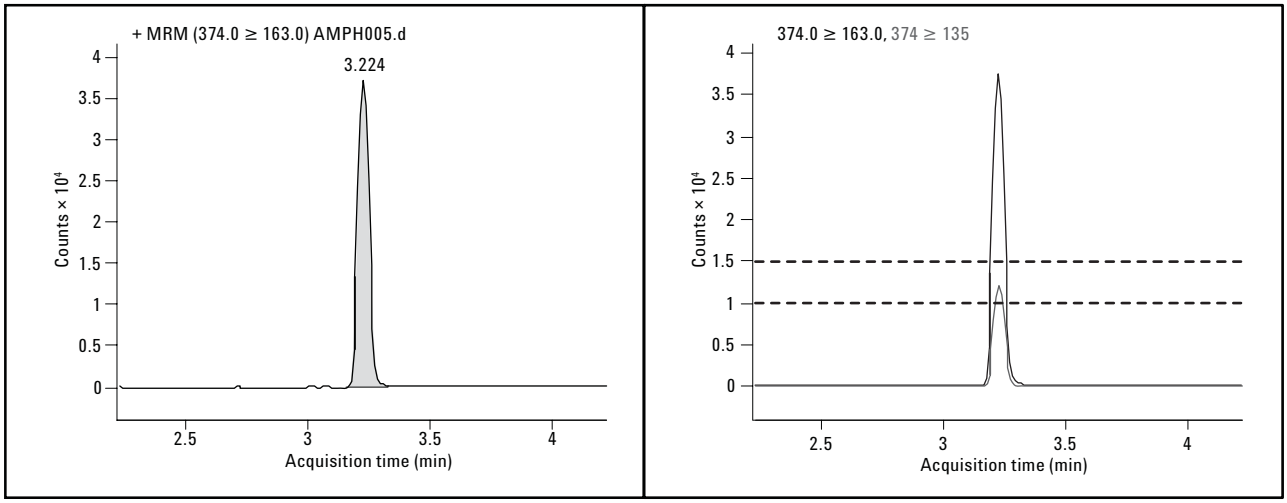


Figure 3c. Ion ratio confirmation for MDA in blood. Note retention time of 3.22 min.

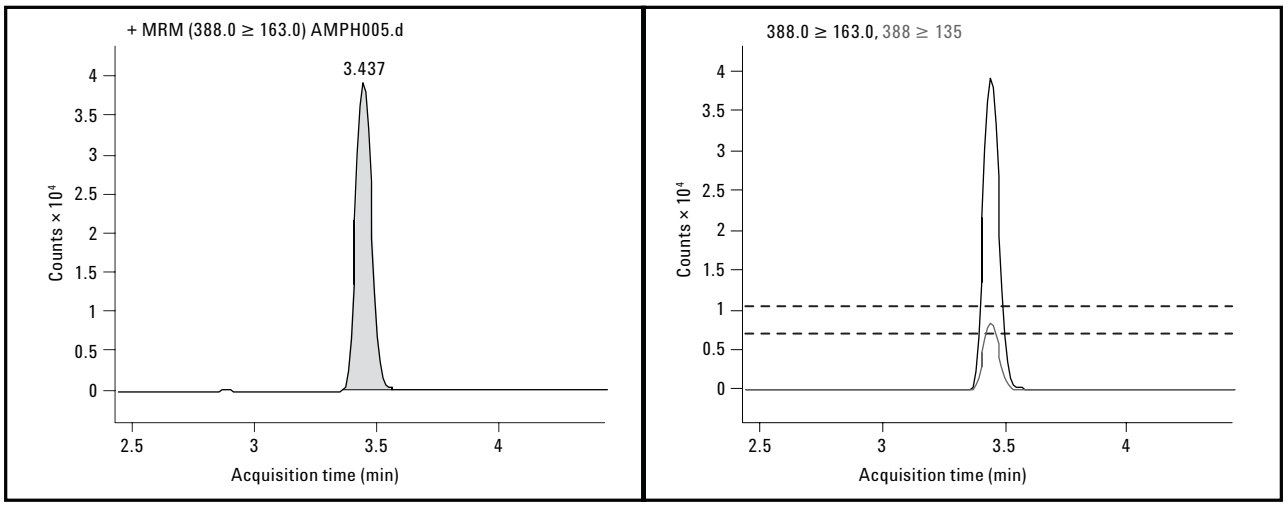


Figure 3d. Ion ratio confirmation for MDMA in blood. Note retention time of 3.44 min.

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.

Validation

1. The method is an adaptation of a published validated 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 that does not require further validation.
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

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Analysis of Benzodiazepines in Blood by LC/MS/MS

Application

Clinical and 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, including sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant [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 reasons, the analysis of benzodiazepines is of great interest to forensic and clinical 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 difficult, 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 therapeutic concentrations of each drug. Typical low and high therapeutic 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 therapeutic	High therapeutic
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 therapeutic 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.

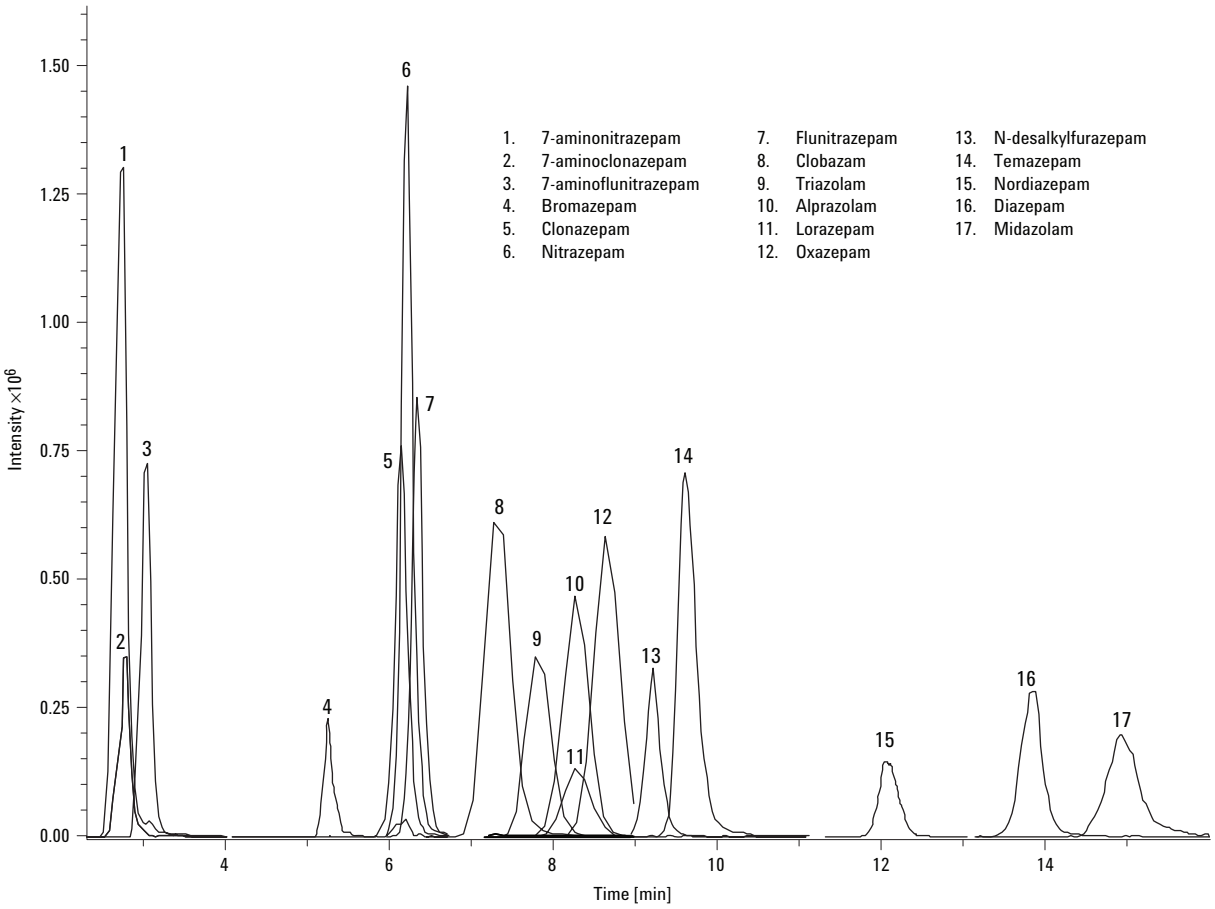


Figure 1. Overlay of principal product ion chromatograms.

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 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.

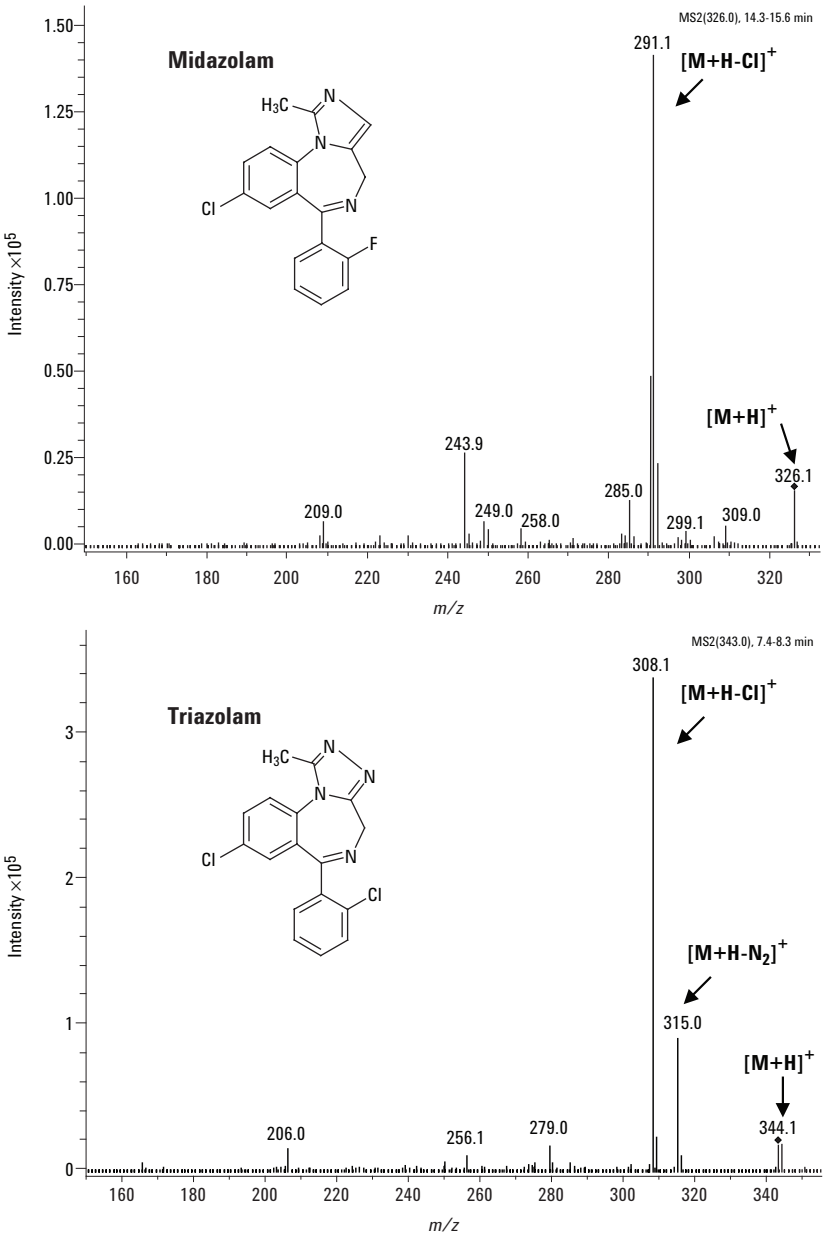


Figure 2. Structures and MS/MS spectra of midazolam and triazolam.

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.

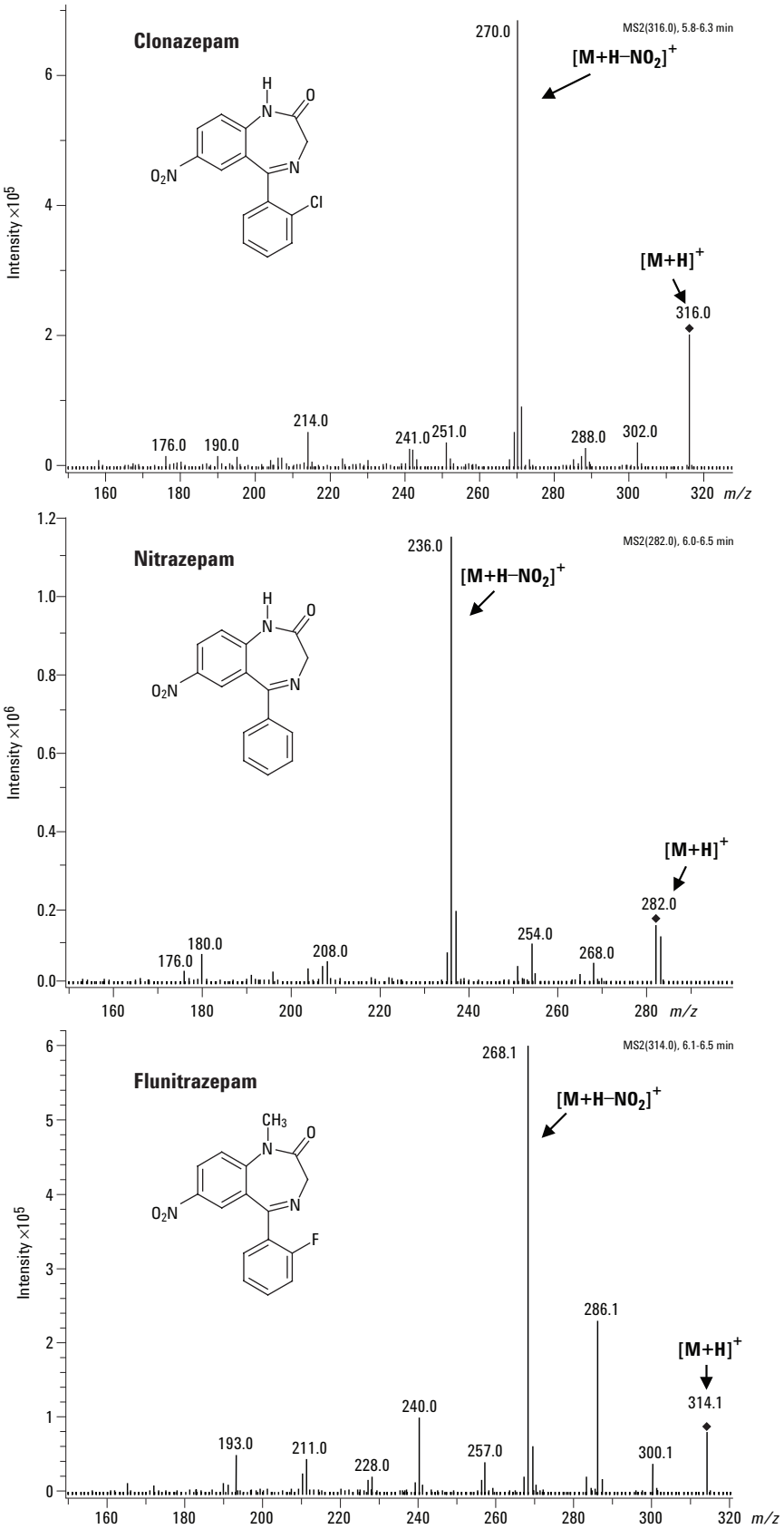


Figure 3. Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.

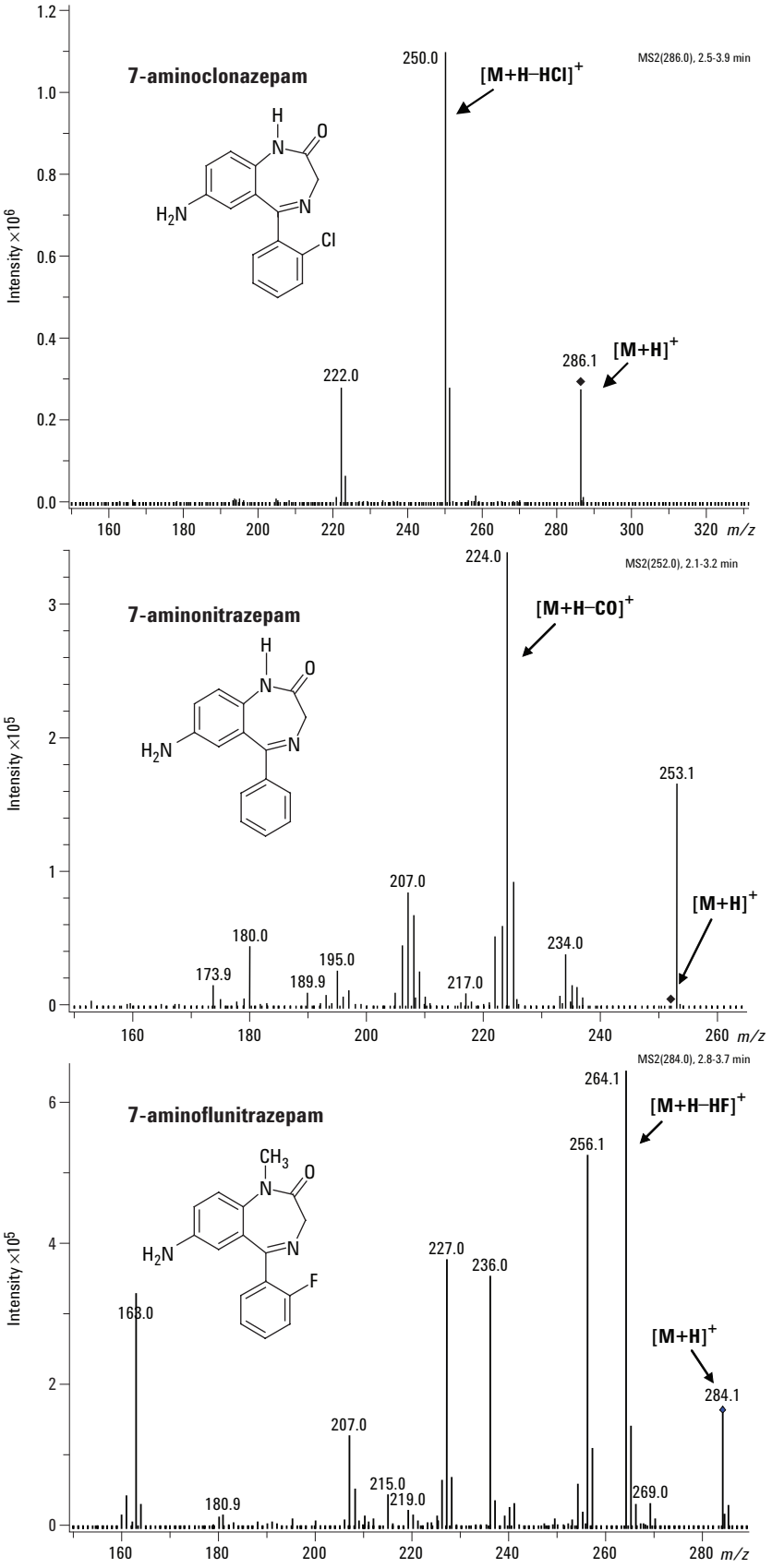


Figure 3 (continued). Structures and MS/MS spectra for clonazepam, nitrazepam, flunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 7-aminoflunitrazepam.

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.

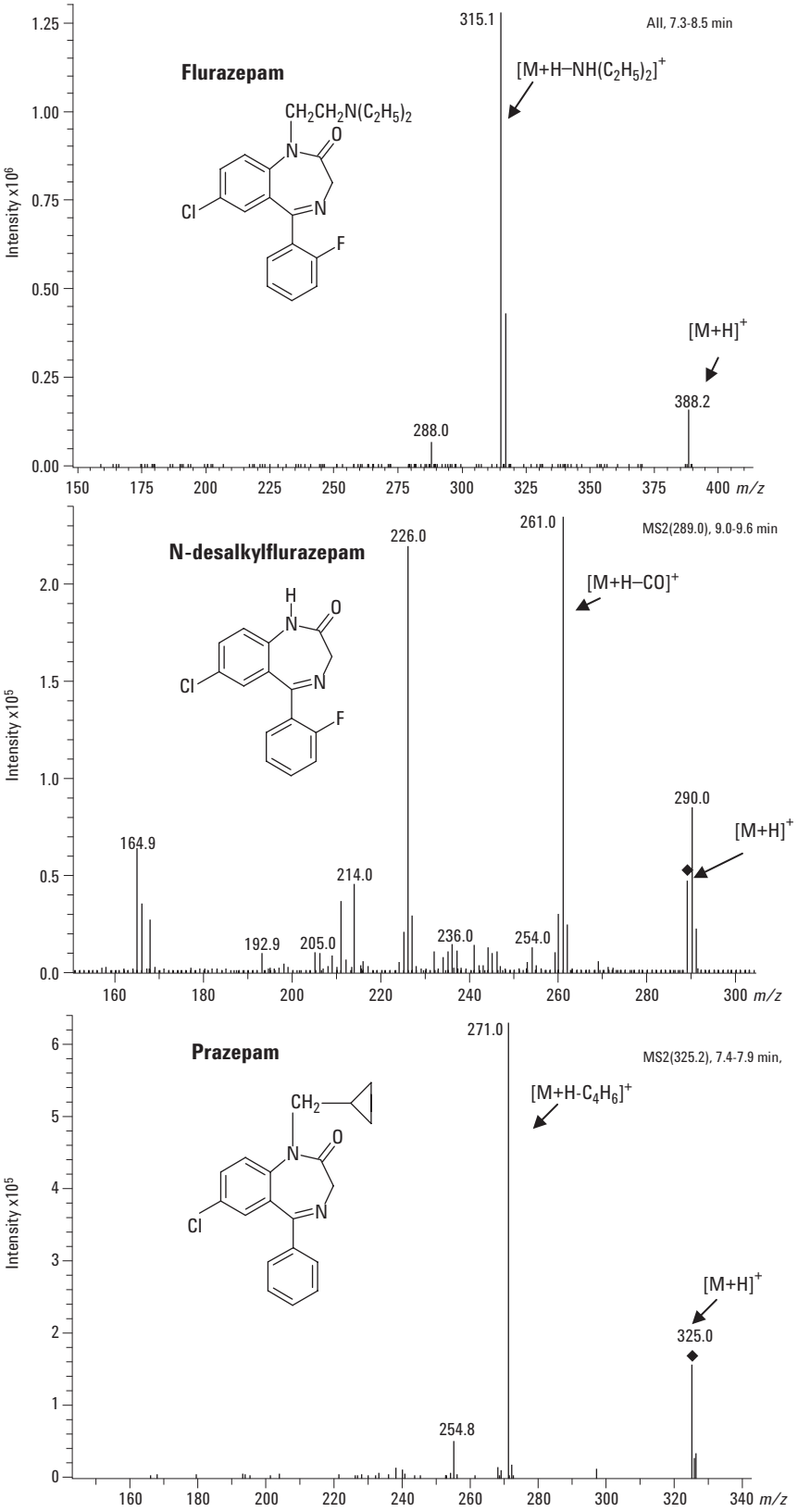


Figure 4. Structures and MS/MS spectra for flurazepam, N-desalkylflurazepam, and prazepam.

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.

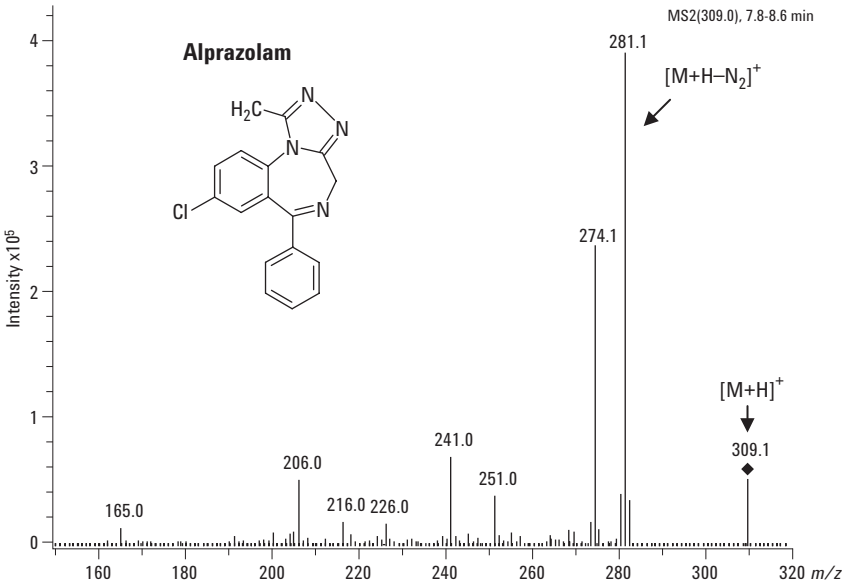


Figure 5. Structure and MS/MS spectrum of alprazolam.

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.

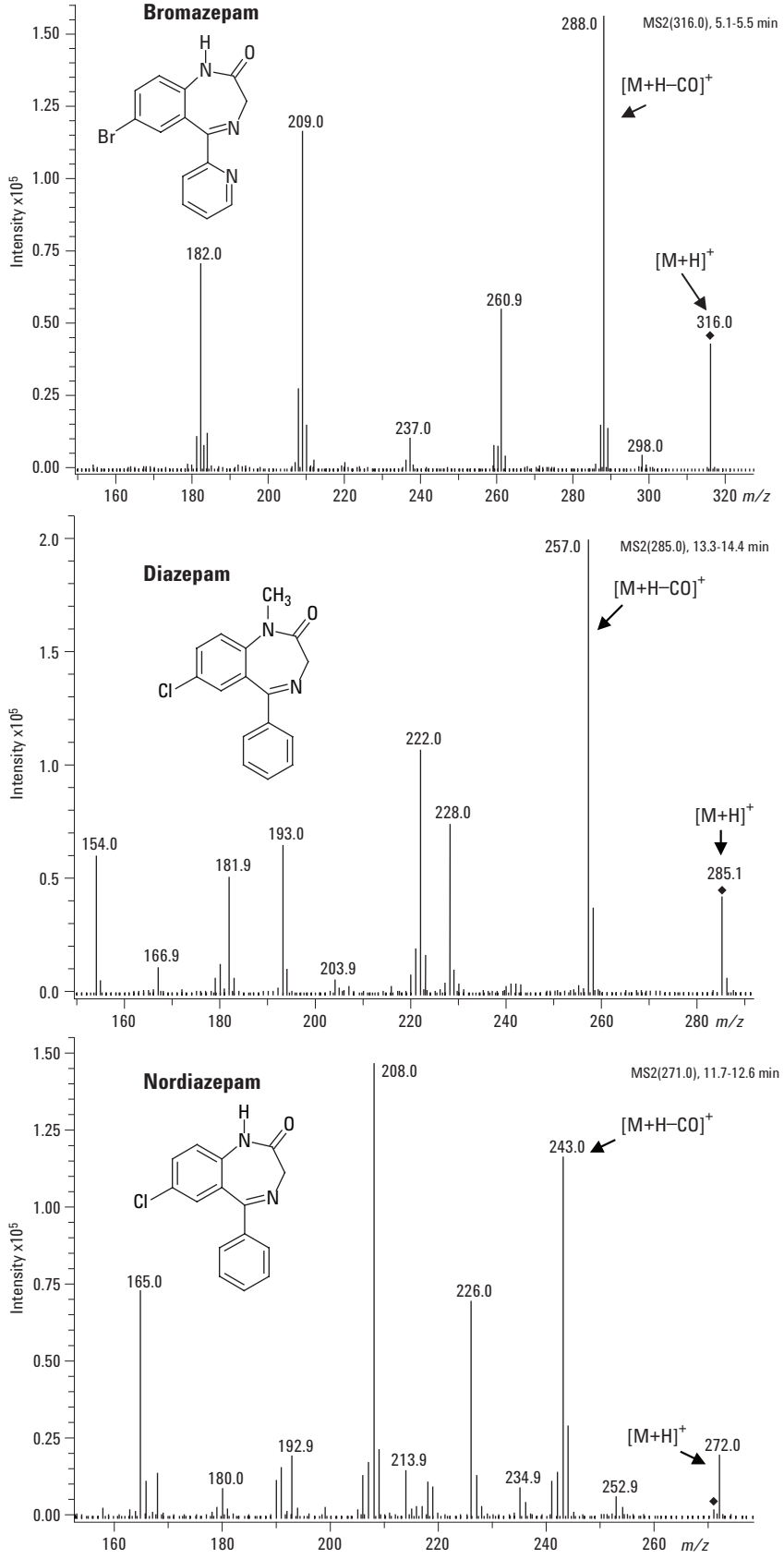


Figure 6. Structures and MS/MS spectra for bromazepam, diazepam, and nordiazepam.

Clobazam shows an extremely simple MS/MS spectrum and a unique loss of CH_2CO in Figure 7.

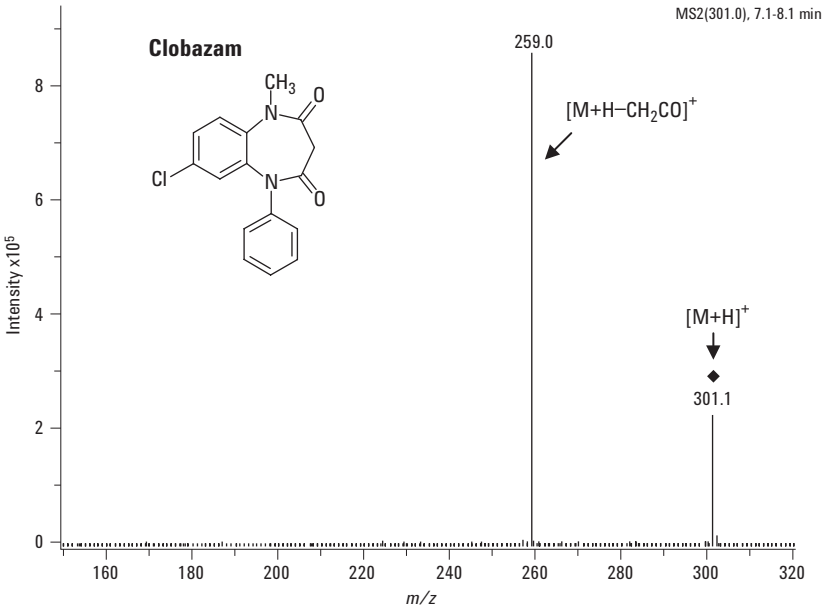


Figure 7. Structure and MS/MS spectrum for clobazam.

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.

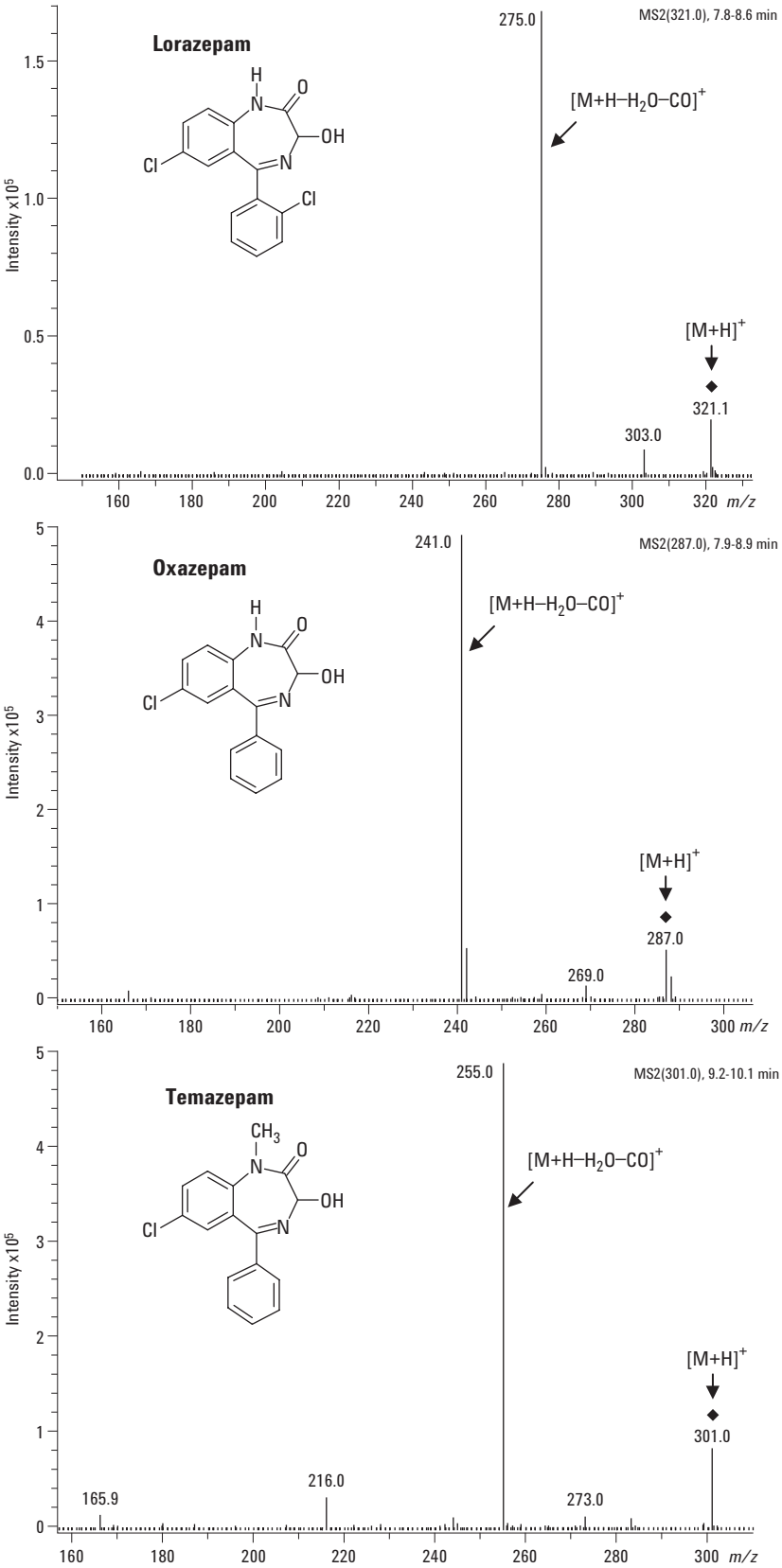


Figure 8. Structures and MS/MS spectra for lorazepam, oxazepam, and temazepam.

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 including multiple drug overdose, sexual assault victims and motor vehicle accident victims 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

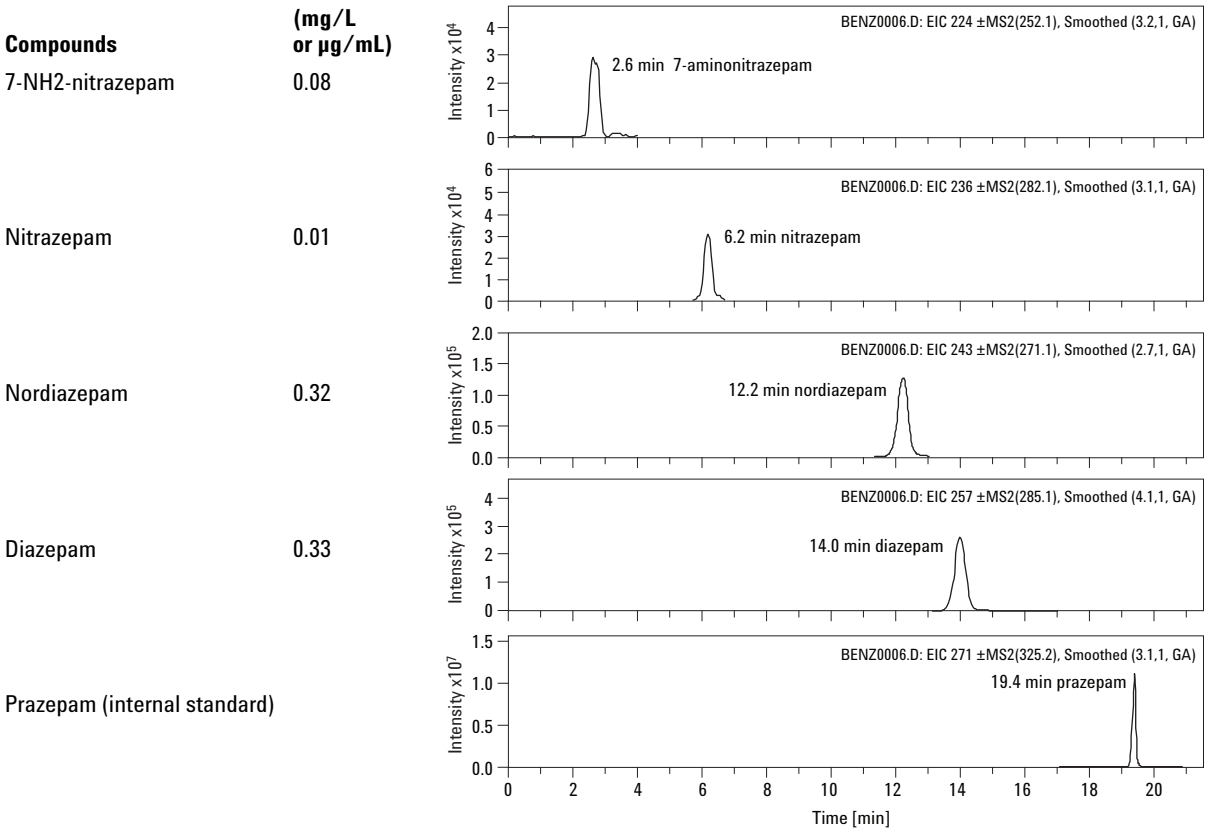


Figure 9. Ion chromatograms from Case 1 example.

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).

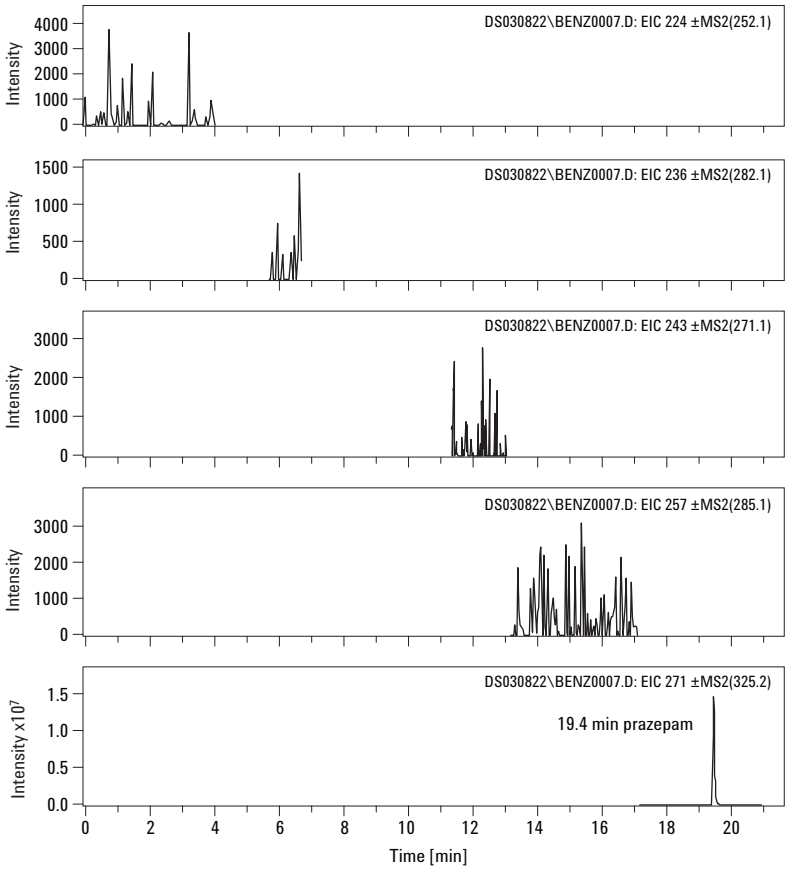


Figure 10. Ion chromatograms from blank blood with internal standard.

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 therapeutic and sub-therapeutic concentrations of benzodiazepines in casework samples. Low concentrations of various benzodiazepines have been rapidly and successfully identified in forensic cases.

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The Analysis of Benzodiazepines in Hair Using RRHT LC/MS/MS

Application

Forensics

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Abstract

A quantitative analytical procedure for the determination of benzodiazepines and metabolites in hair has been developed and validated. 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 sedative, anti-anxiety, hypnotic, and muscle-relaxants. They exert an additive effect when used in conjunction with alcohol or other drugs, and are subject to abuse and show potential for addiction. 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 validation 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 Validation

The analytical method was validated 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

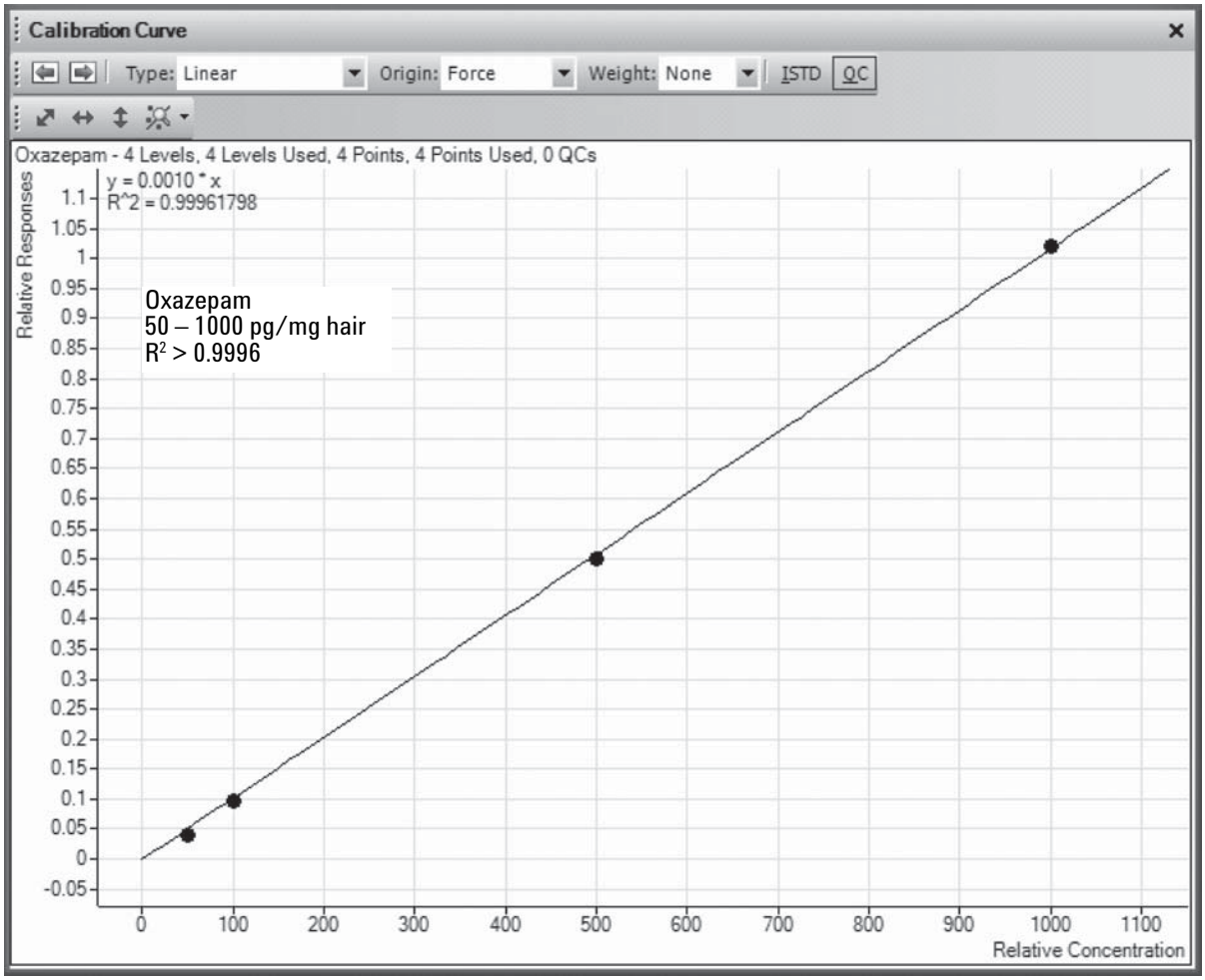


Figure 1. Calibration curve for oxazepam using a linear fit, forced origin, and no weighting.

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.

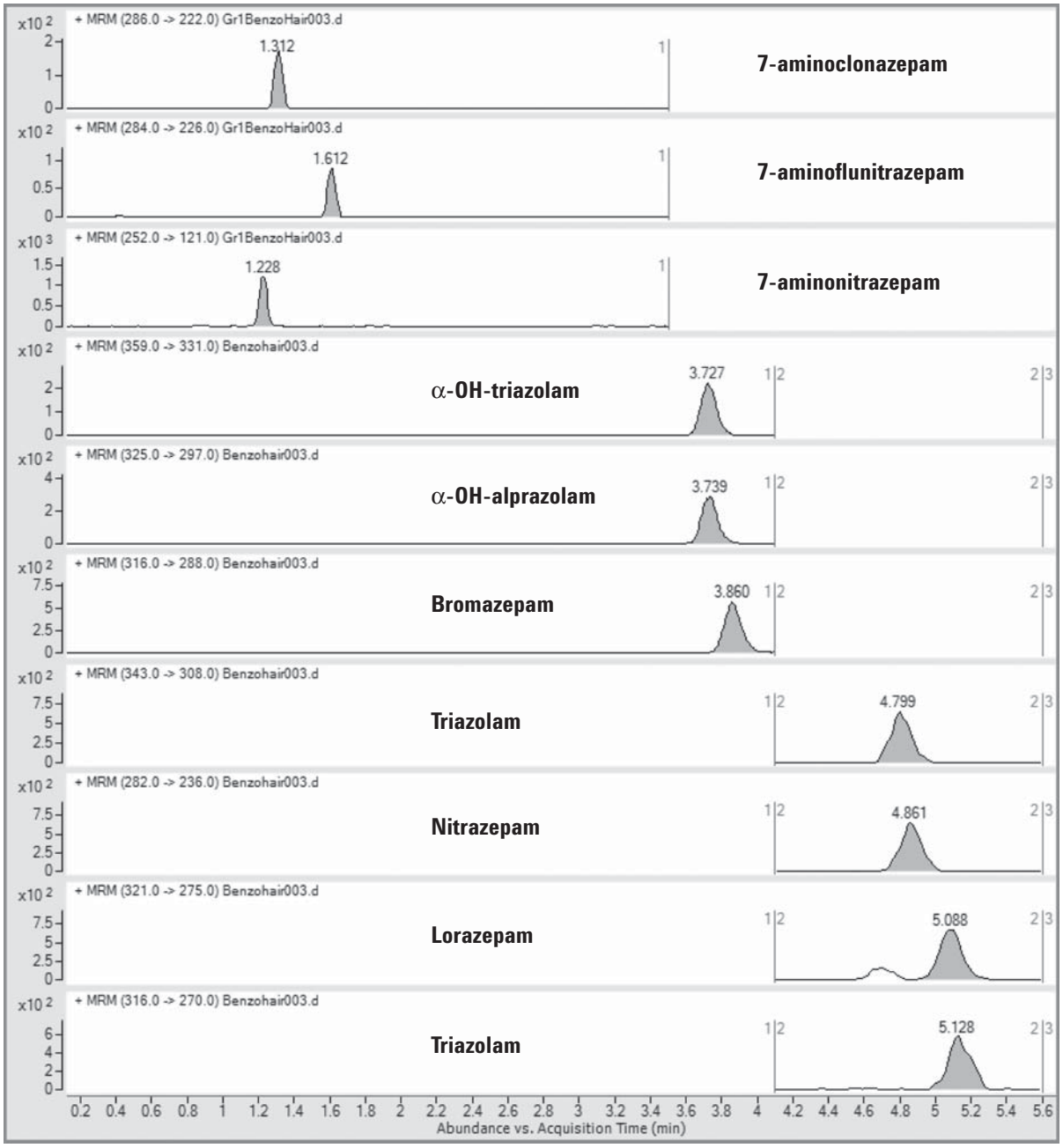


Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers).

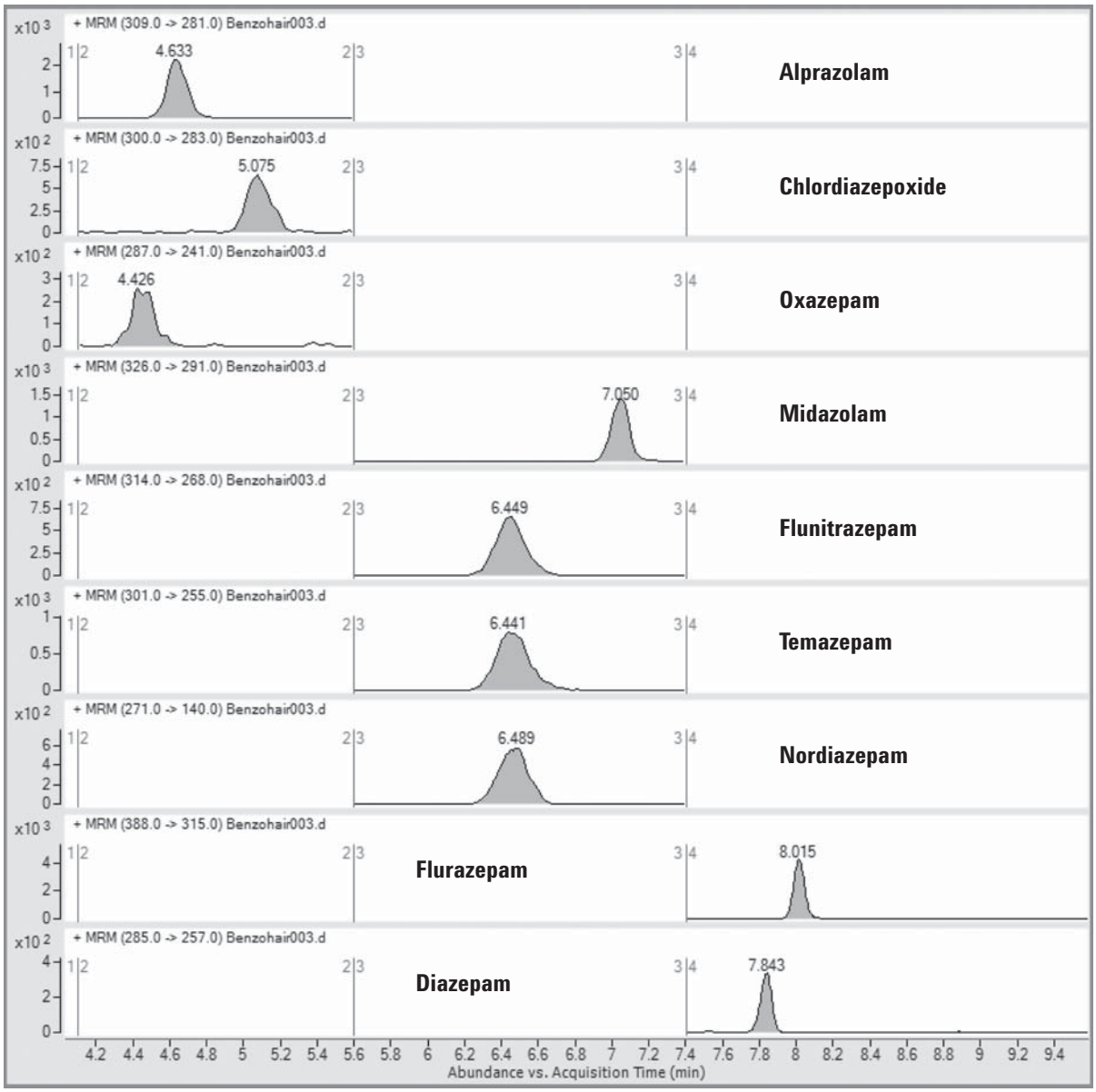


Figure 2. Benzodiazepines extracted from hair (100 pg/mg): primary transitions (quantifiers). (continued)

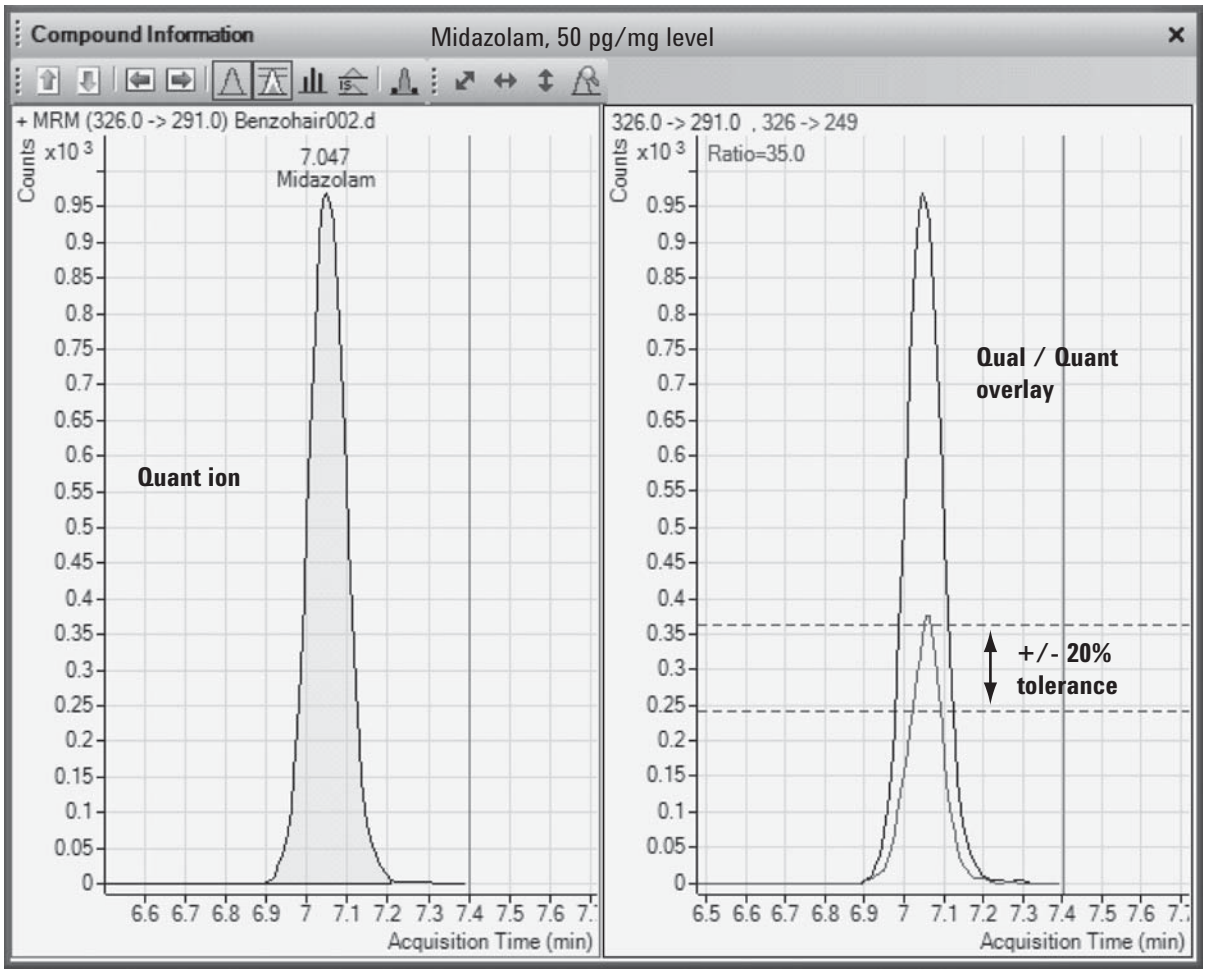


Figure 3. Confirming presence of midazolam using qualifier to quantifier ion peak area ratio.

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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Determination of Benzodiazepines in Oral Fluid Using LC/MS/MS

Application

Forensics

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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 for the treatment of anxiety and insomnia, particularly in the elderly [1]. They are also used as muscle relaxants and anticonvulsants. 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 and clinical 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 Validation

The analytical method was validated 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 validation 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

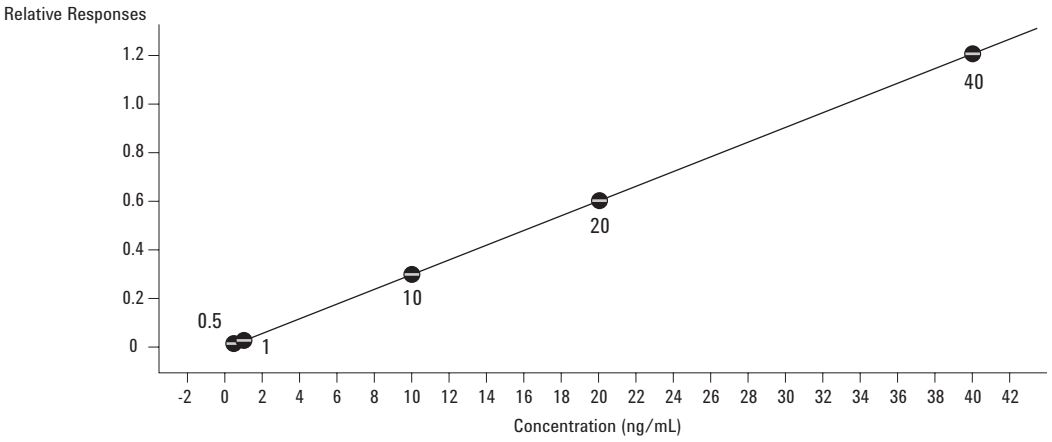


Figure 1. Calibration curve for alprazolam in oral fluid (0.5, 1, 10, 20, and 40 ng/mL).

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-

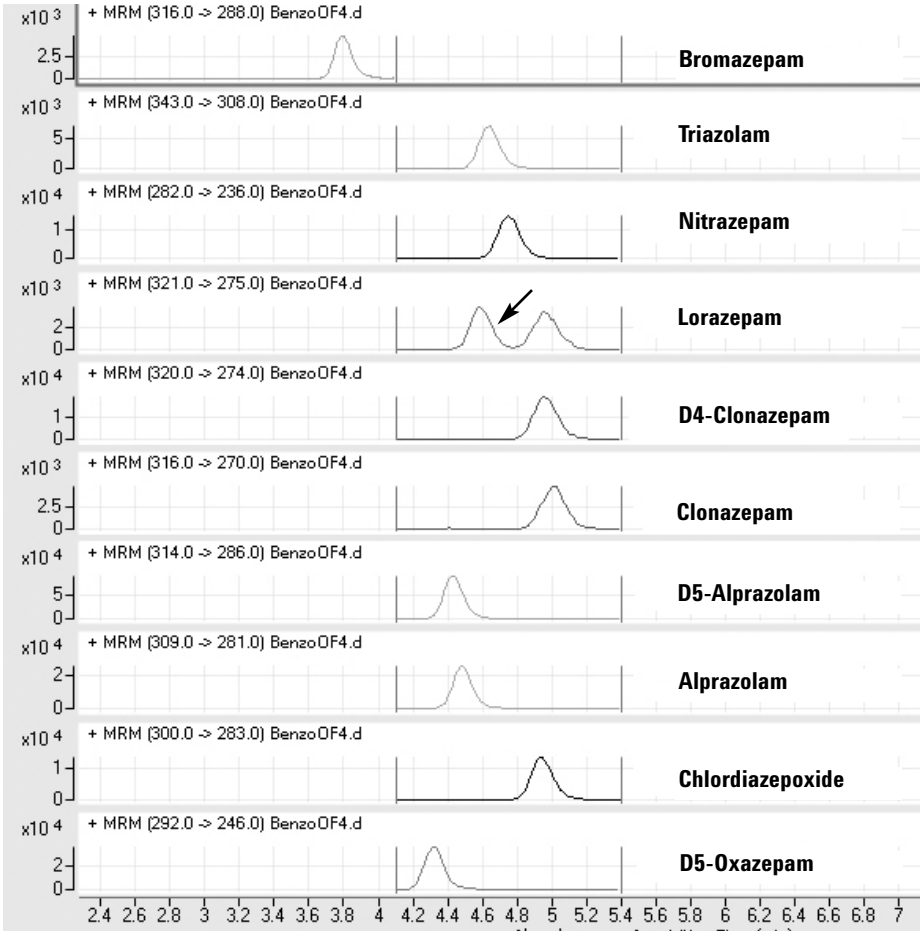


Figure 2. Primary transitions for benzodiazepines in oral fluid.

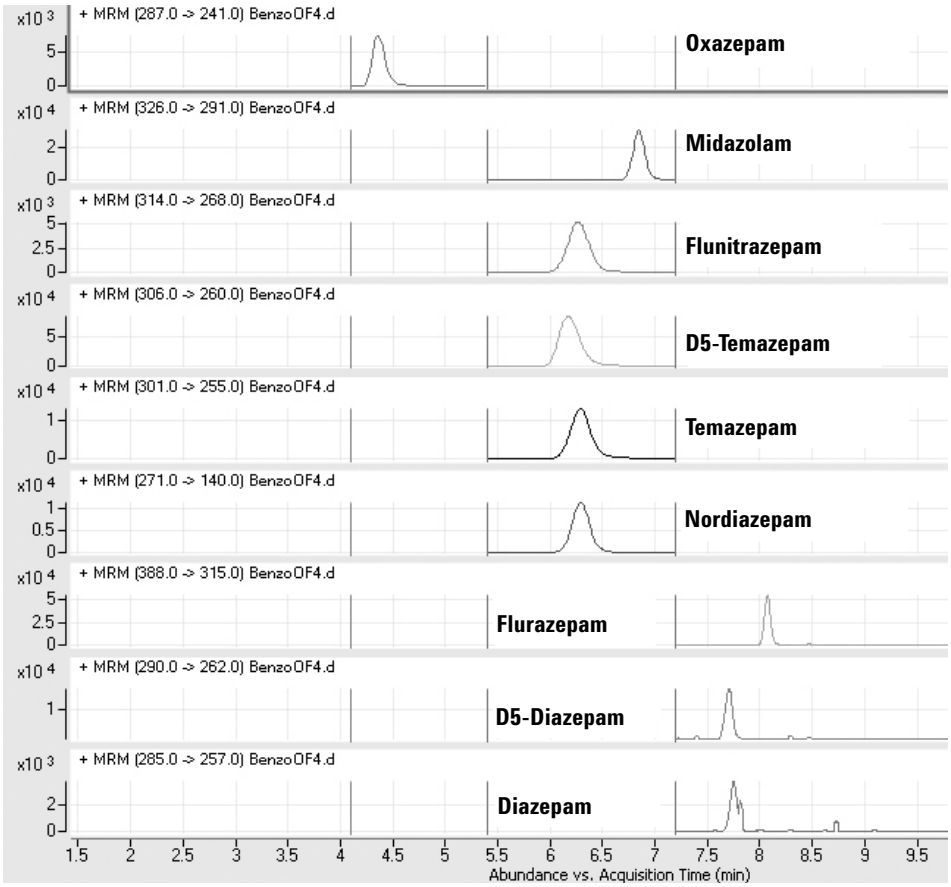


Figure 2. Primary transitions for benzodiazepines in oral fluid. (continued)

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

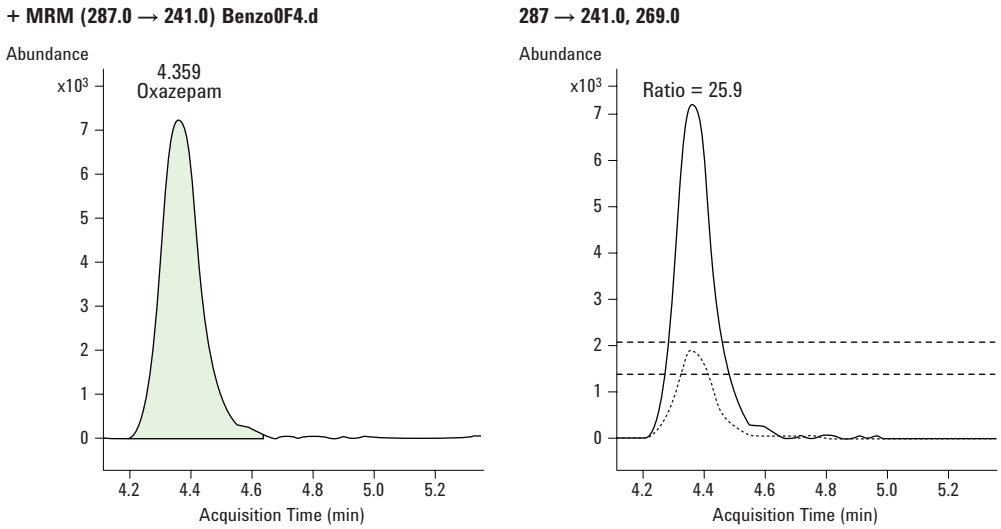


Figure 3. Oxazepam extracted from oral fluid (10 ng/mL).

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 Benzodiazepines in Urine and Blood Using Rapid Resolution Liquid Chromatography/Triple Quadrupole Mass Spectrometry

Application

Forensics

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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 for the treatment of anxiety and insomnia, particularly in the elderly [1]. They are also used as muscle relaxants and anticonvulsants and are commonly detected in incidents of sexual assault, driving under the influence of drugs (DUID), and often in combination with other medications [2,3]. Hegstad et al. published a procedure using LC/MS/MS for the detection 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 Validation

The analytical method was validated 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

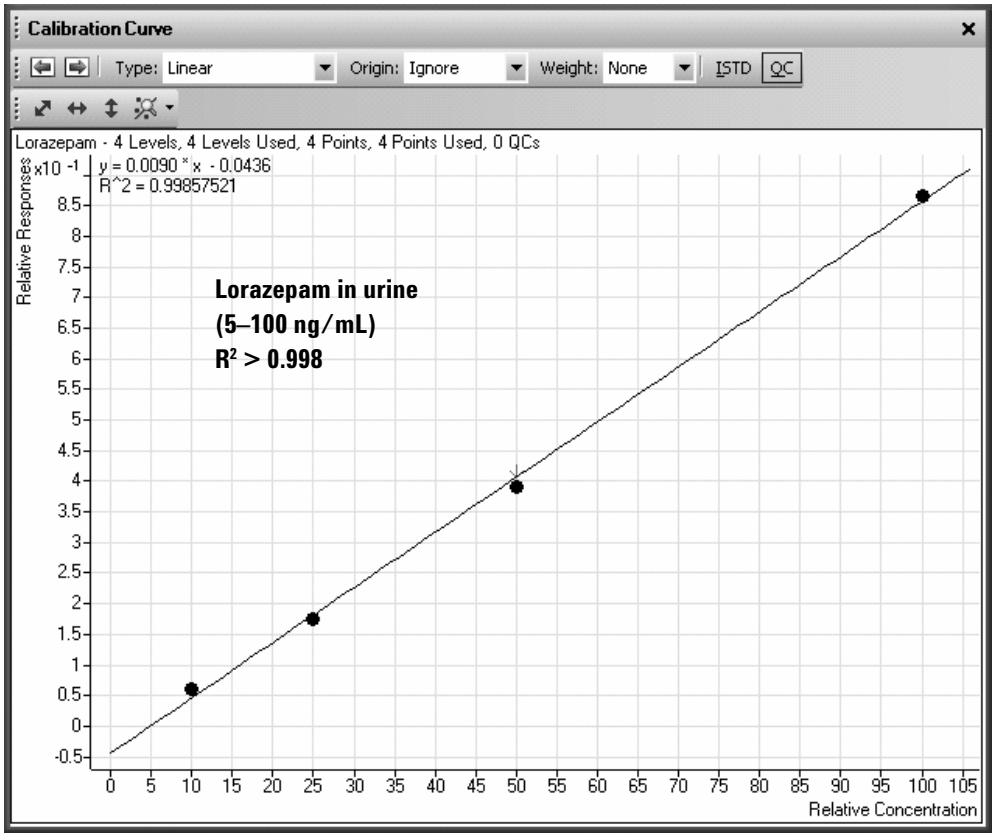


Figure 1a. Calibration curve for lorazepam in urine (5, 10, 25, 50, and 100 ng/mL).

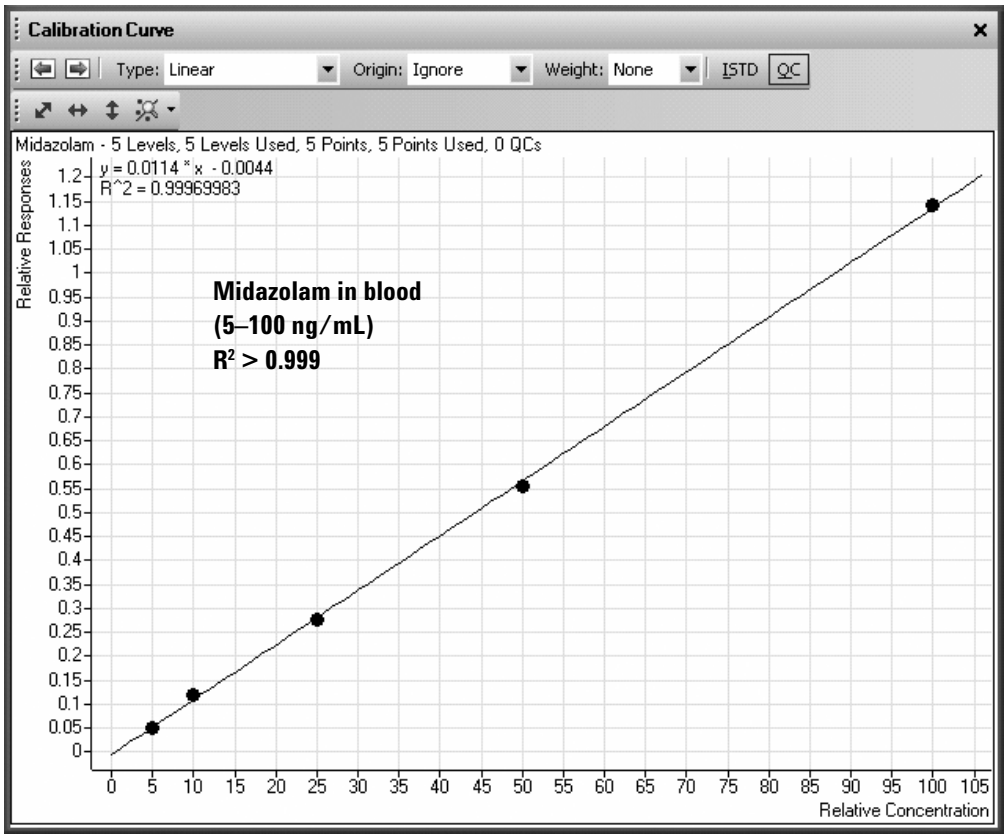


Figure 1b. Calibration curve for midazolam in blood (5, 10, 25, 50, and 100 ng/mL).

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).

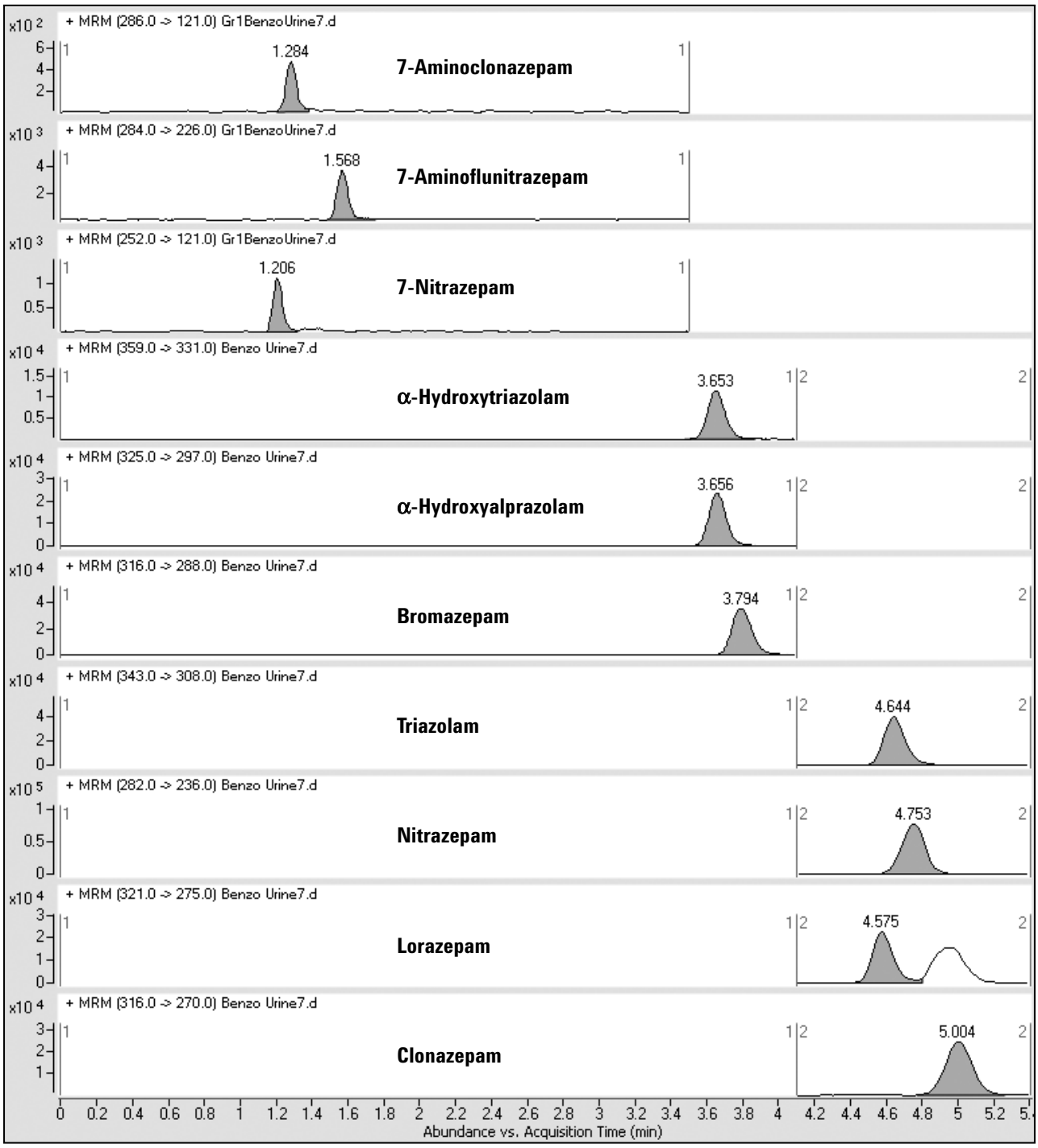


Figure 2a. Benzodiazepines extracted from urine (25 ng/mL): primary transitions, for clarity, internal standards not shown.

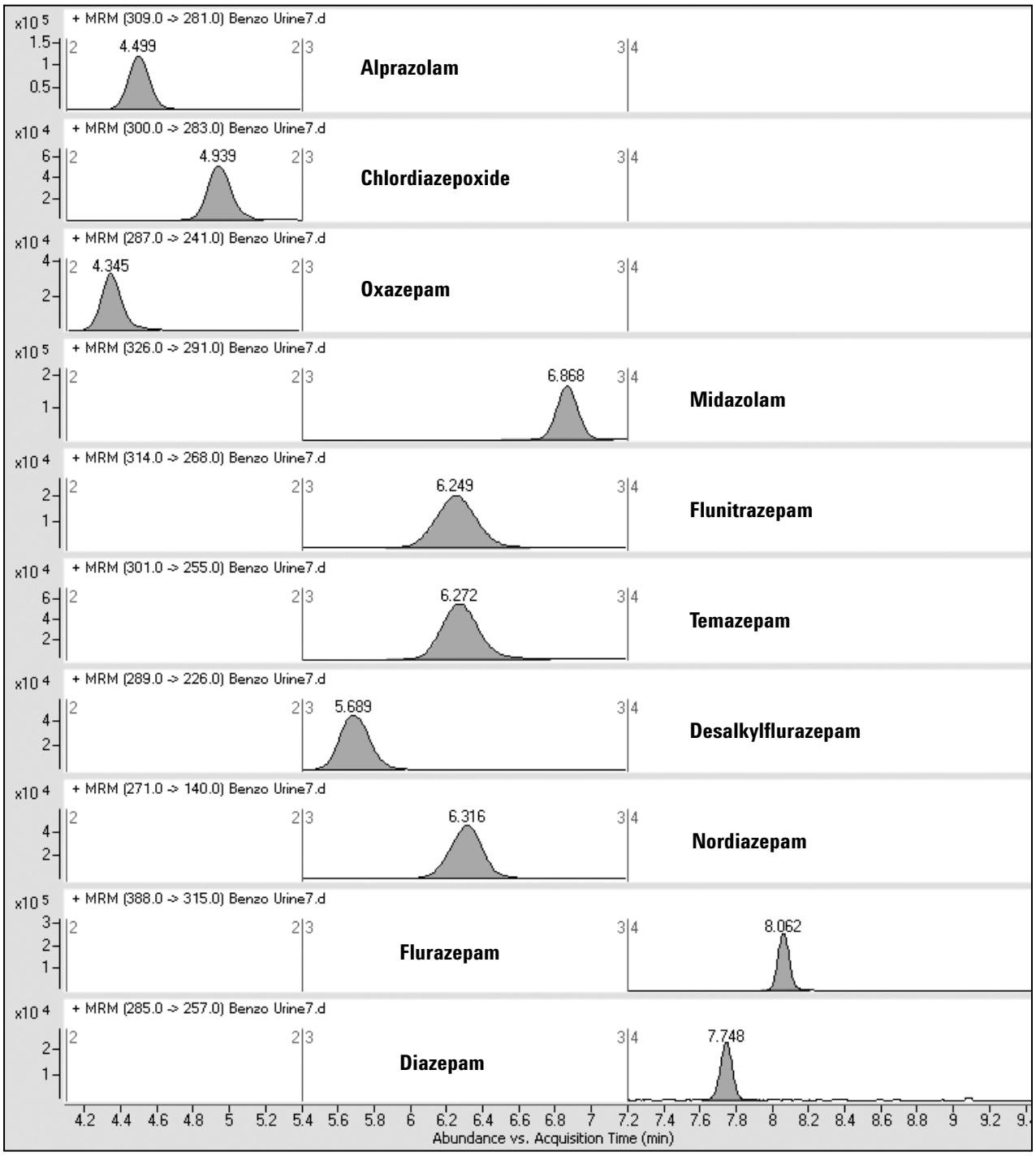


Figure 2a. Benzodiazepines extracted from urine (25 ng/mL): primary transitions, for clarity, internal standards not shown. (continued)

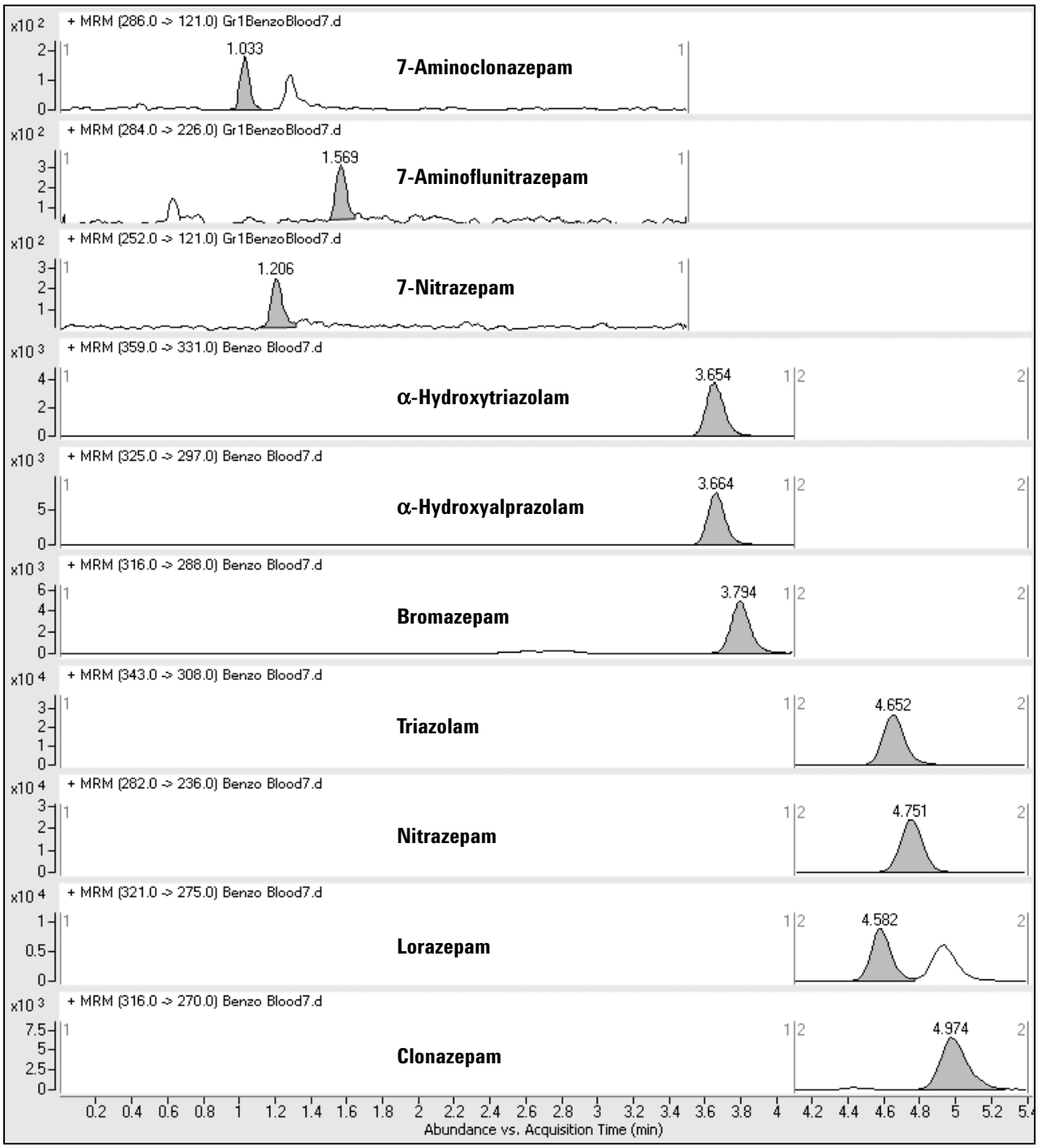


Figure 2b. Benzodiazepines extracted from blood (25 ng/mL): primary transitions, for clarity, internal standards not shown.

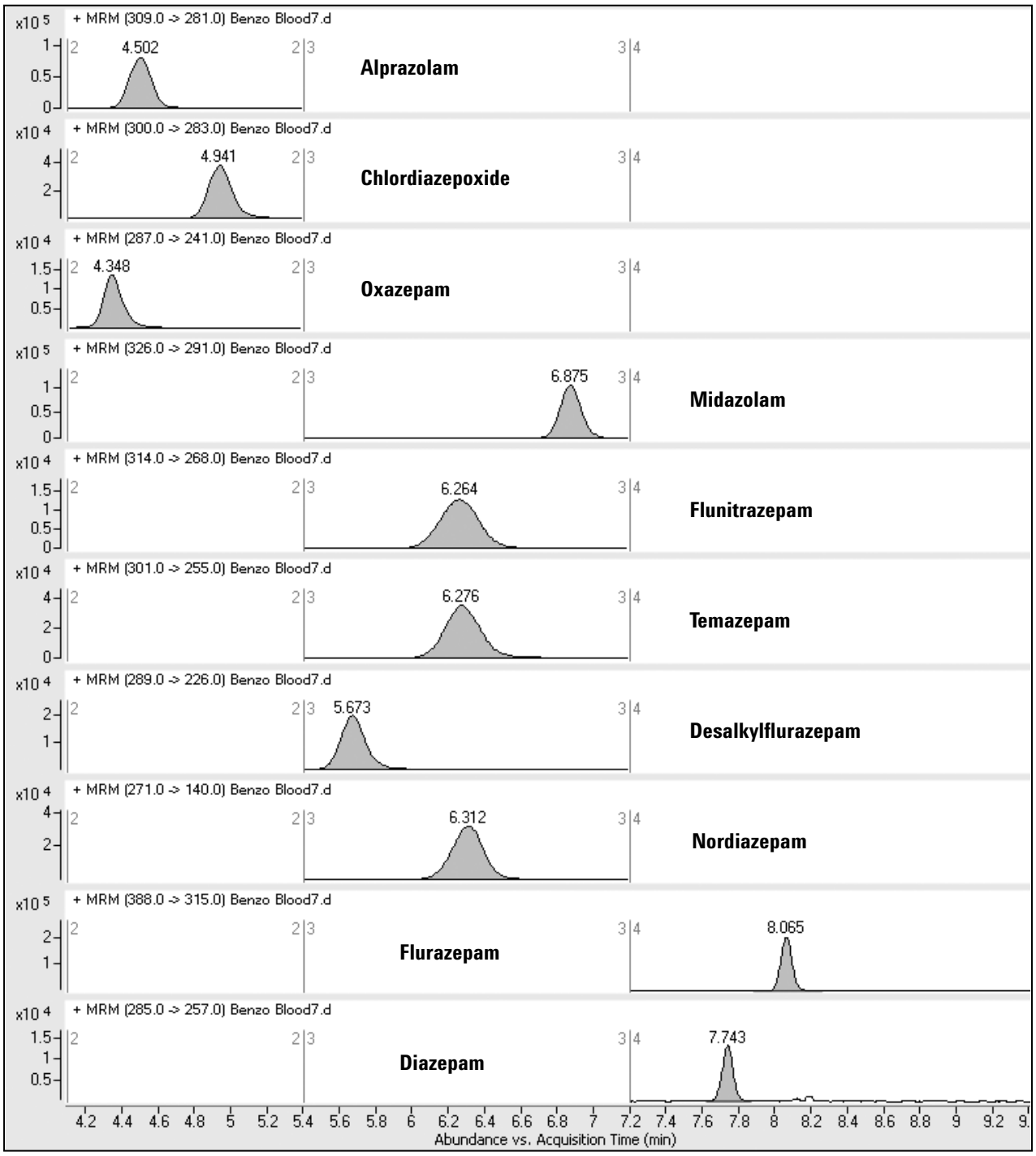


Figure 2b. Benzodiazepines extracted from blood (25 ng/mL): primary transitions, for clarity, internal standards not shown. (continued)

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).

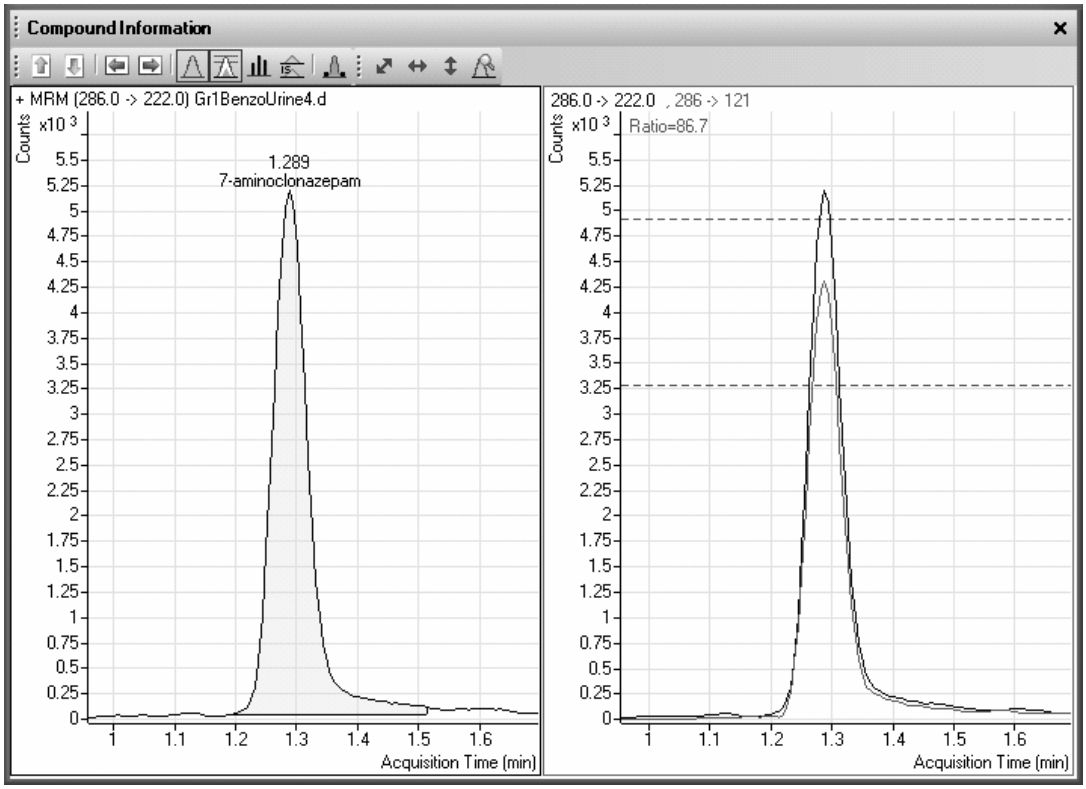


Figure 3a. 7-Aminoclonazepam extracted from urine (50 ng/mL) showing qualifying ion (normalized by area) and acceptable ratio 86.7 with ± 20% tolerance (range: 69.4–104.0).

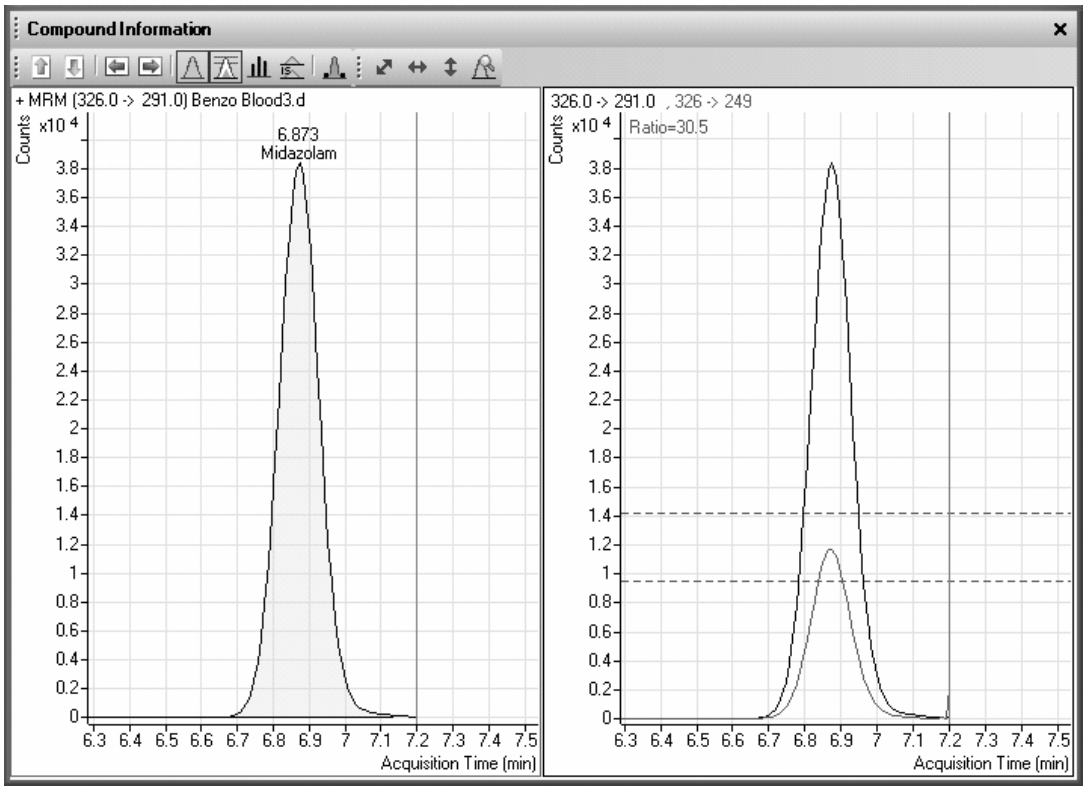


Figure 3b. Midazolam extracted from blood (10 ng/mL) showing qualifying ion and acceptable ratio 30.5 with ± 20% tolerance (range: 24.4–36.6).

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 Buprenorphine, Norbuprenorphine, and Their Glucuronides in Urine Using LC/MS/MS

Application

Forensics

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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 for the treatment of chronic pain, and in heroin addiction, as an alternative to methadone. It is metabolized to norbuprenorphine, and both species undergo extensive conjugation with glucuronide before urine excretion. The simultaneous determination of buprenorphine, norbuprenorphine, and methadone has recently been published [1]. Liquid chromatographic methods for the detection of buprenorphine in urine have predominantly been directed towards the free drug following 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 patients. 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 Validation

The analytical method was validated 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.

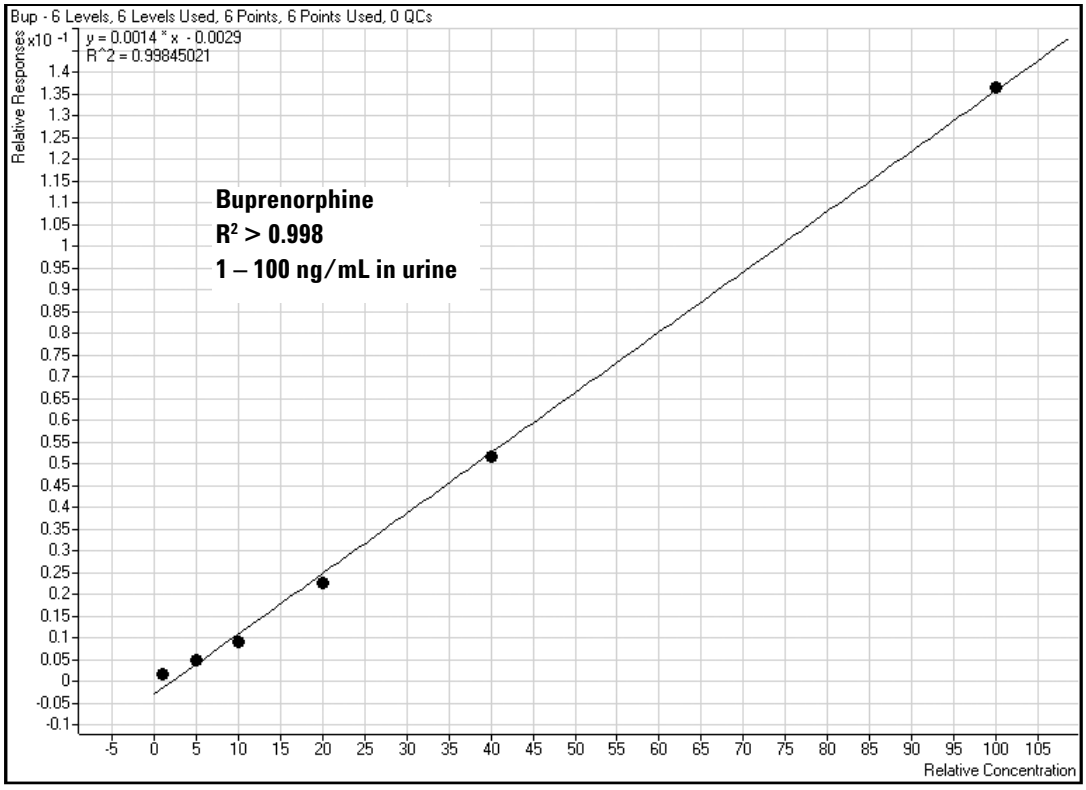


Figure 1. Calibration curve for free buprenorphine in urine.

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).

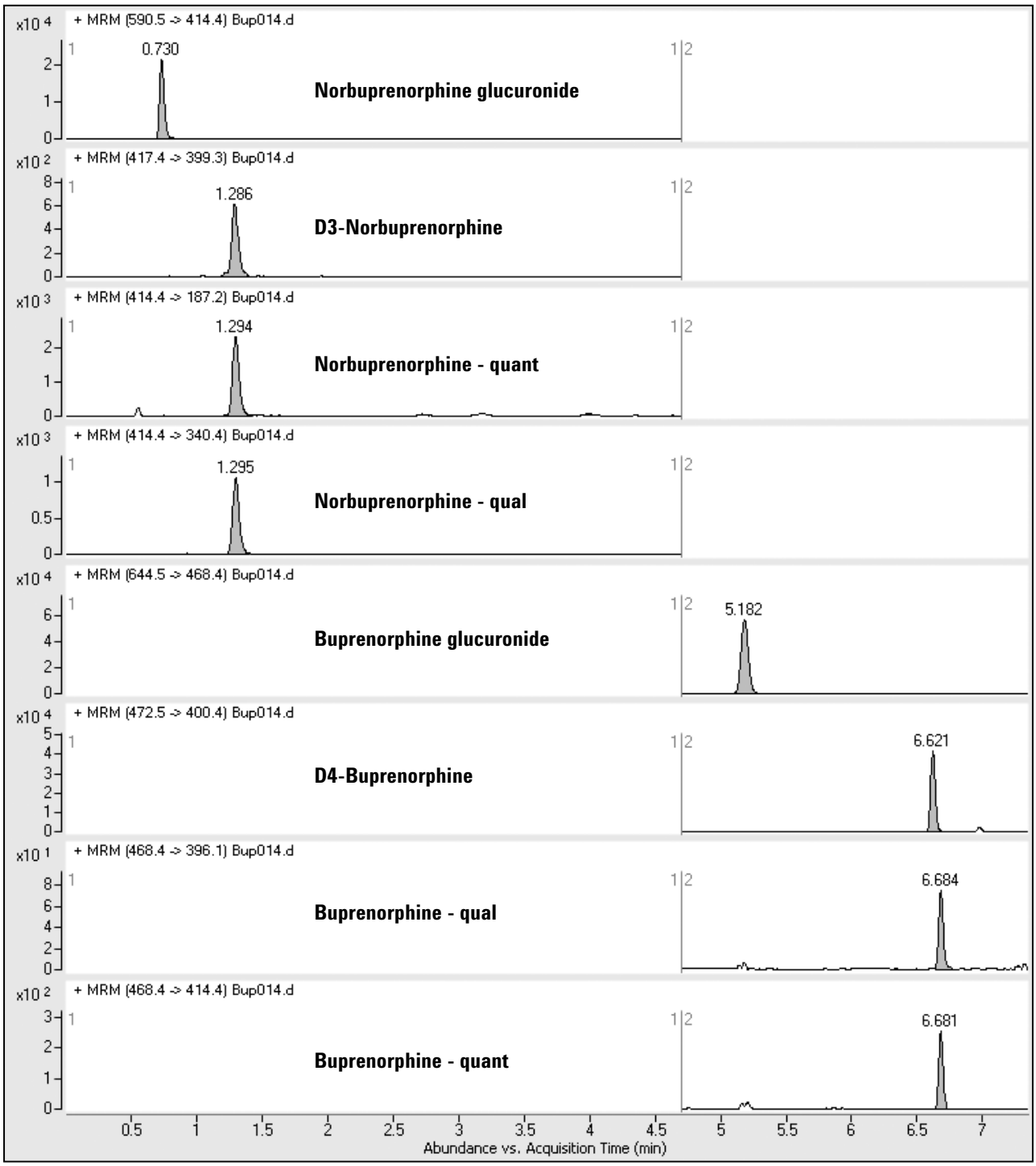


Figure 2. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide extracted from authentic urine specimen.

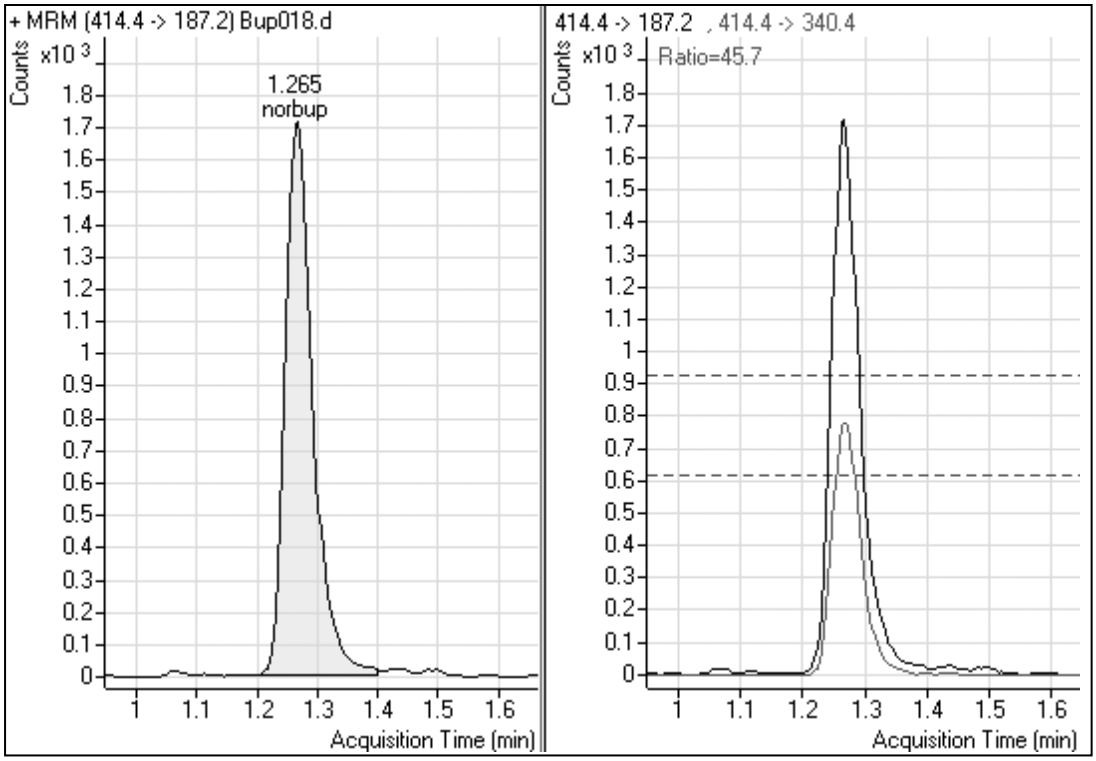


Figure 3. Free norbuprenorphine (98 ng/mL) in urine: quantitation ion at left and overlay of quantitation ion with qualifier ion at right.

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. The method is in routine use in our laboratory.

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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

Forensics

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Abstract

A quantitative analytical procedure for the determination of cocaine, benzoylecgonine, cocaethylene, and norcocaine in hair has been developed and validated. 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 validated 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.

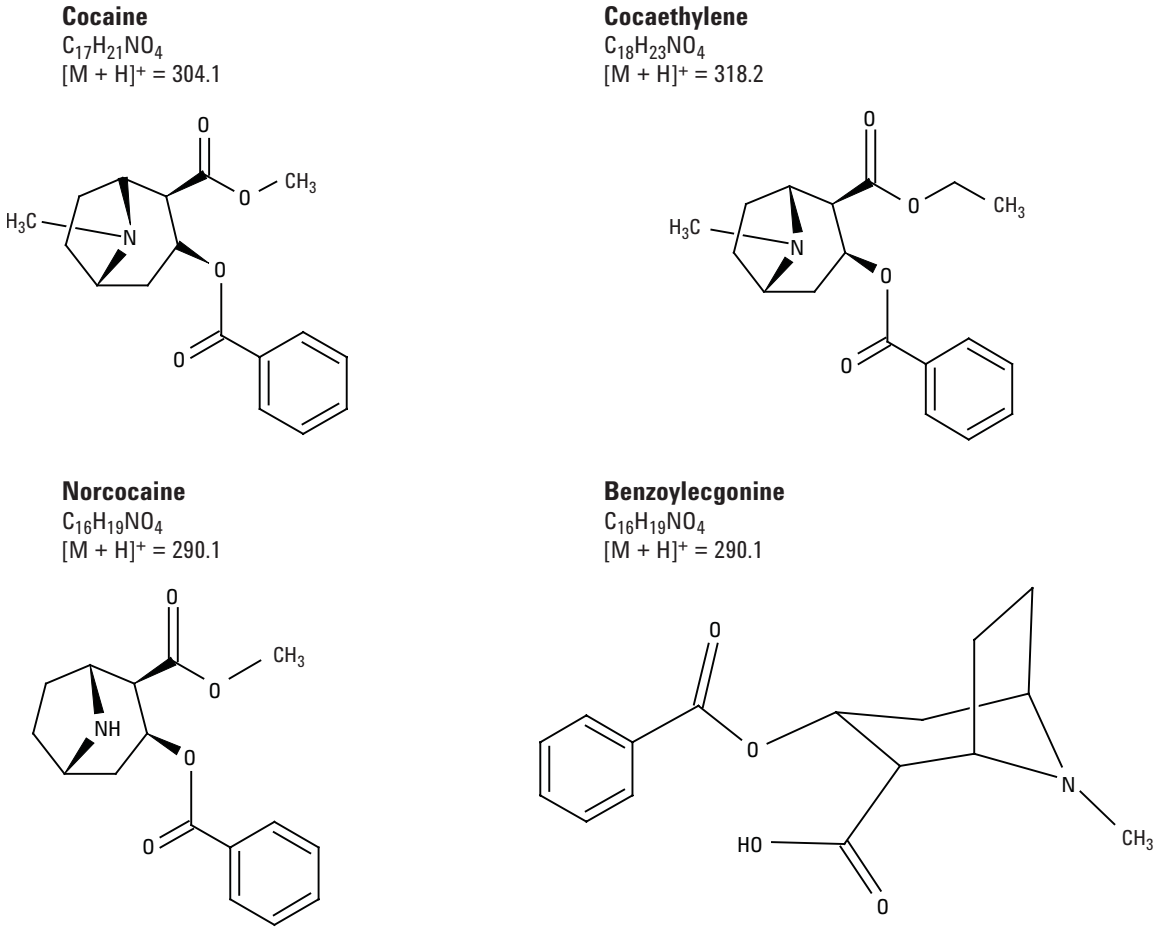


Figure 1. Structures of cocaine and metabolites analyzed in this work.

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 Validation

The chromatographic procedures developed for COC, BZE, CE, and NC were validated 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.

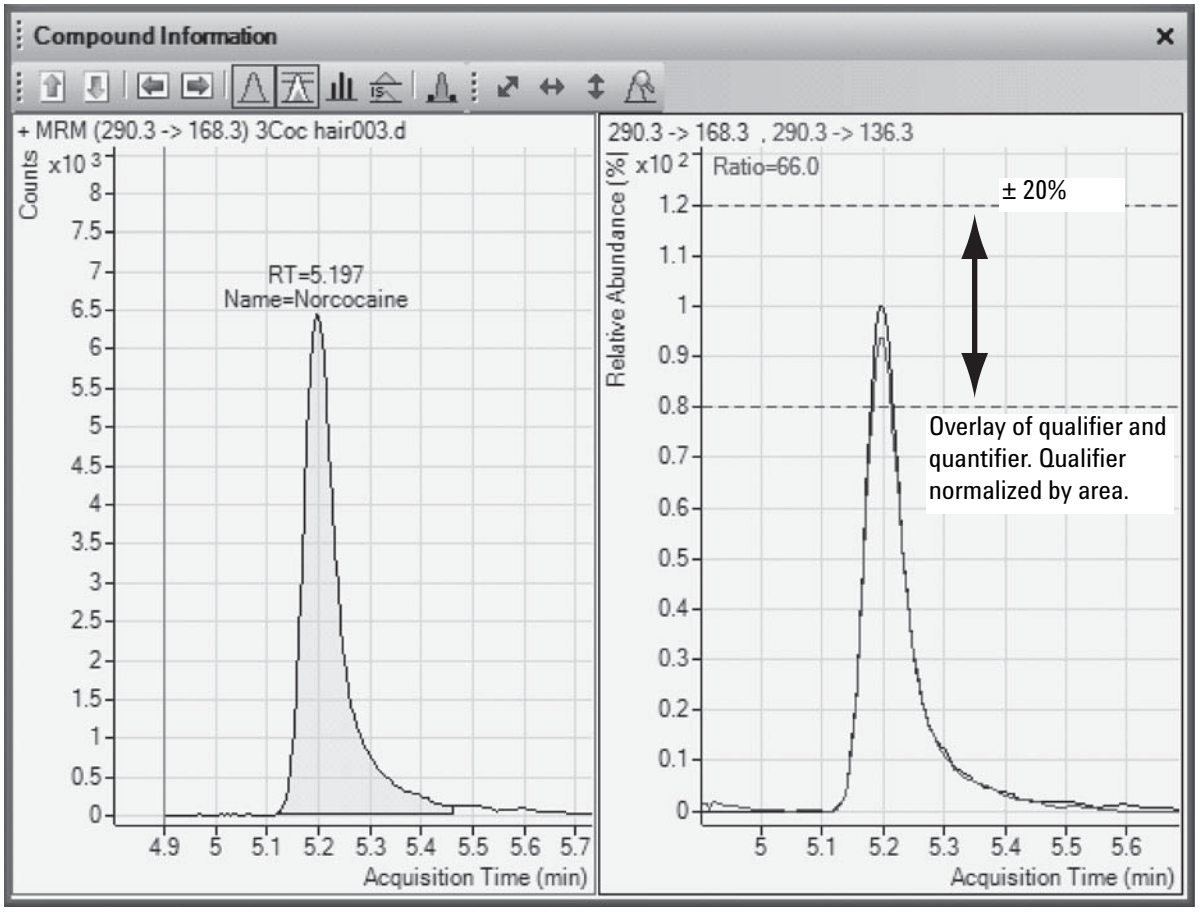


Figure 2. Ion ratio confirmation for norcocaine at 100 pg/mg level.

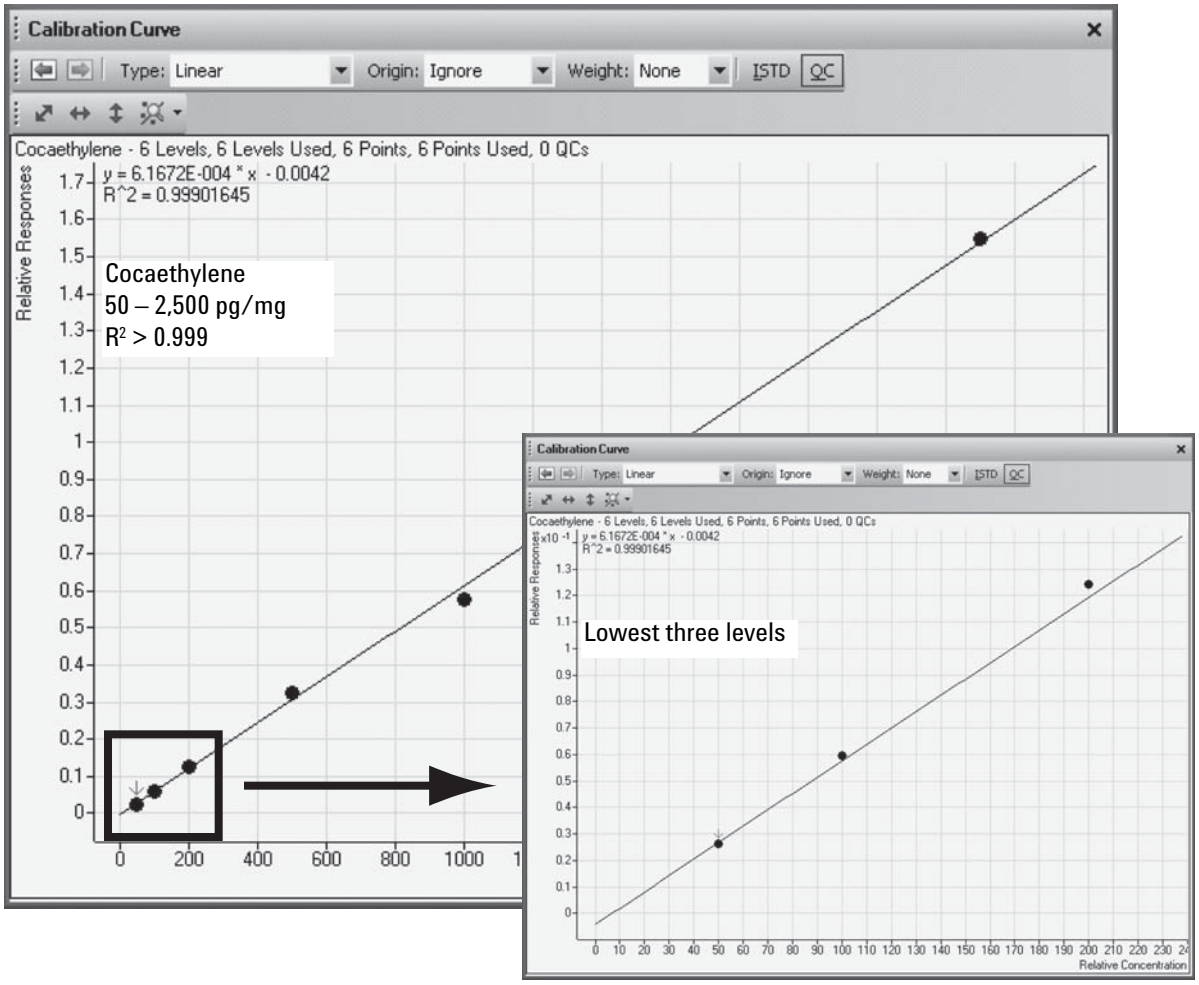


Figure 3. Linearity of cocaethylene with lowest levels detailed to show excellent accuracy.

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.

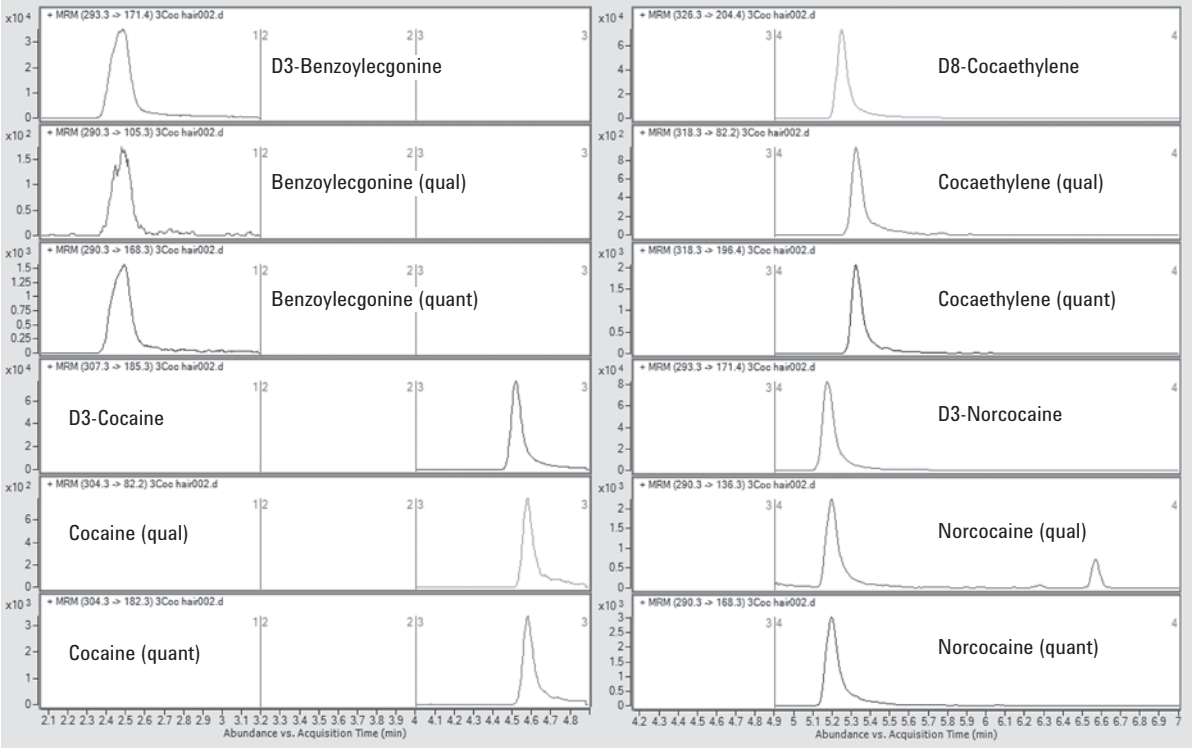


Figure 4. Chromatographic profile of all compounds analyzed in hair at the 50 pg/mg level.

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 laboratory testing.

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Agilent Technologies

Rapid Analysis of Drugs of Abuse by LC/Triple Quadrupole Mass Spectrometry

Application Note

Forensics

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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.

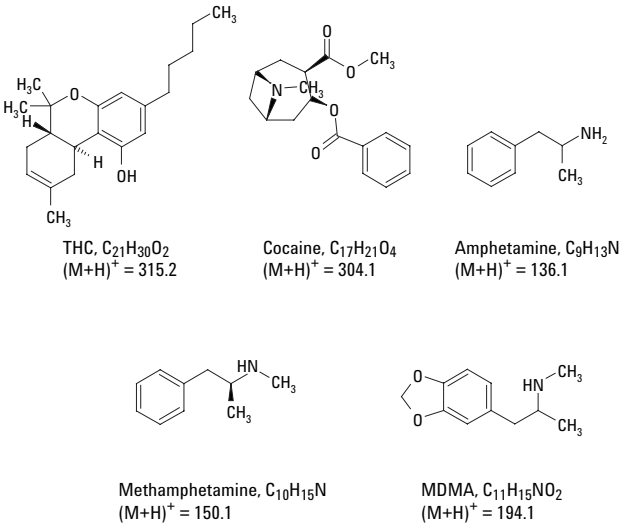


Figure 1. Target compound structures, and their molecular ion masses.

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.

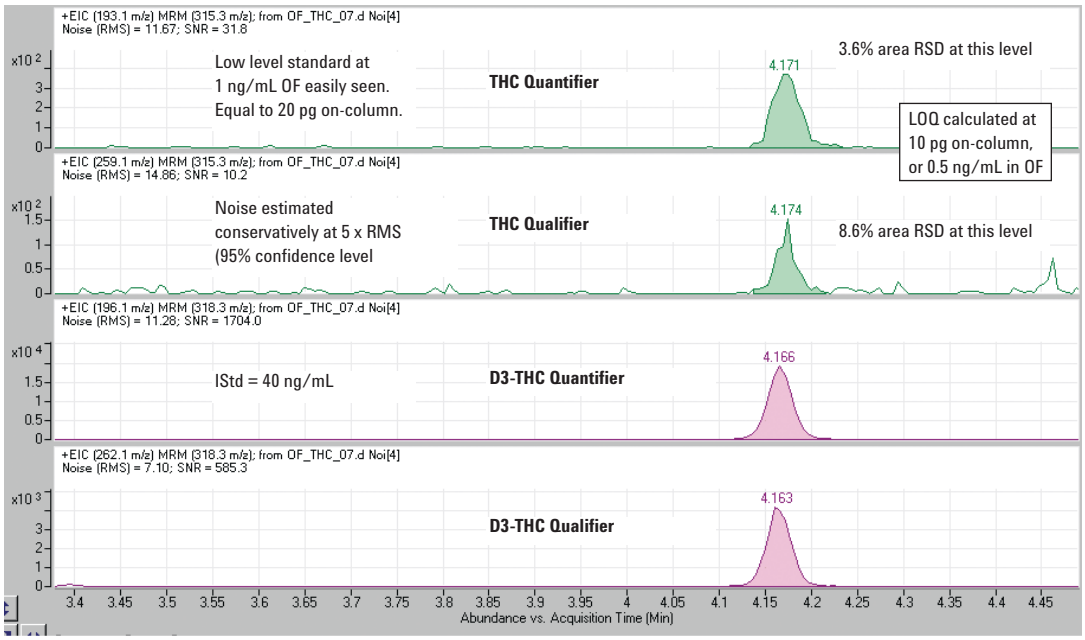


Figure 2. Product ion chromatograms for THC and D3-THC. Generation of chromatograms and integration of peaks is automated with opening of data file by the Agilent Qualitative Analysis software. Peak elution times less than 4.2 minutes. No smoothing applied.

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.

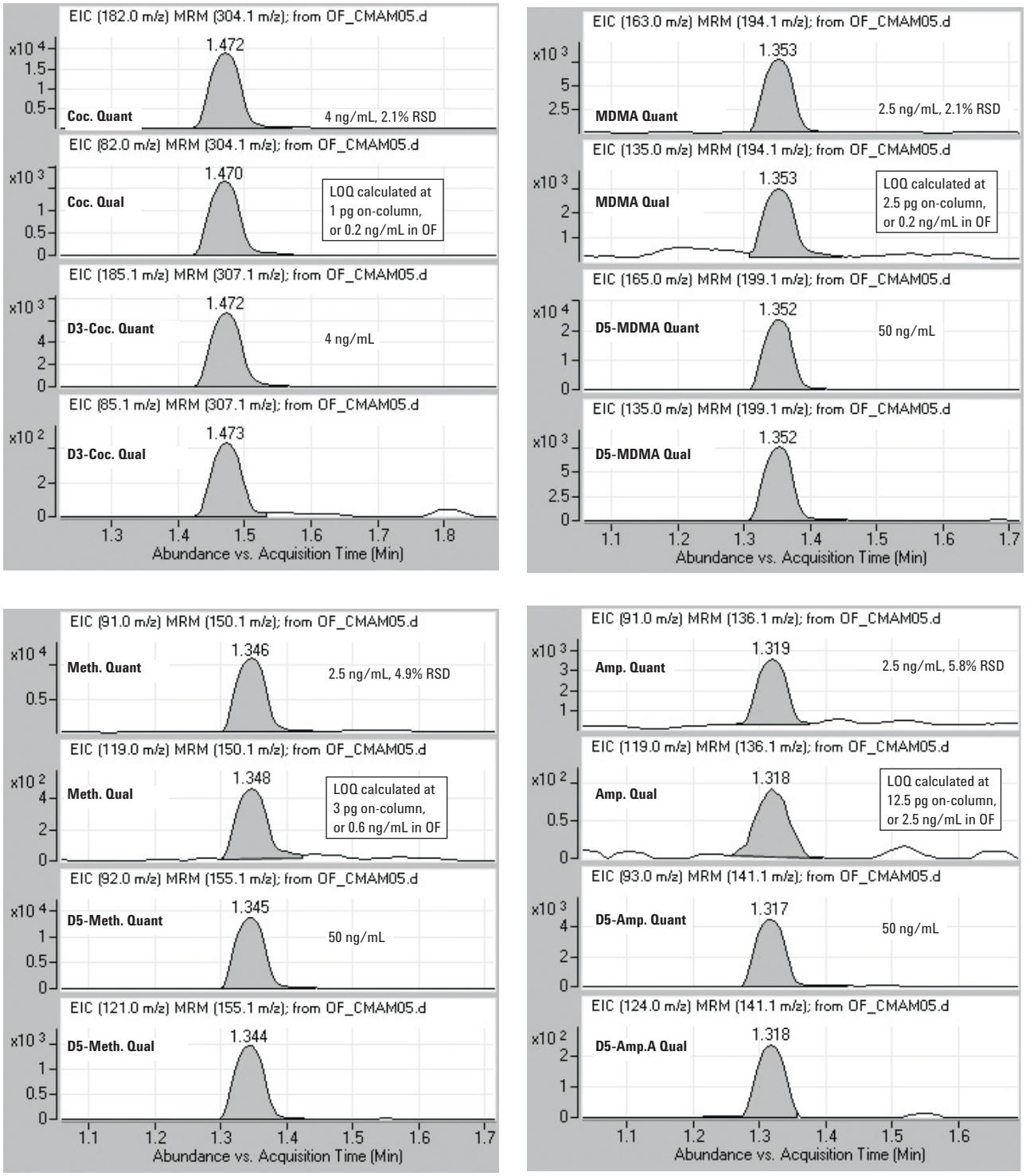


Figure 3. Product ion chromatograms for lowest level standard containing cocaine, D3-cocaine, MDMA, D5-MDMA, amphetamine, D5-amphetamine, methamphetamine, and D5-methamphetamine. Peak elution times less than 1.5 minutes. No smoothing applied.

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.

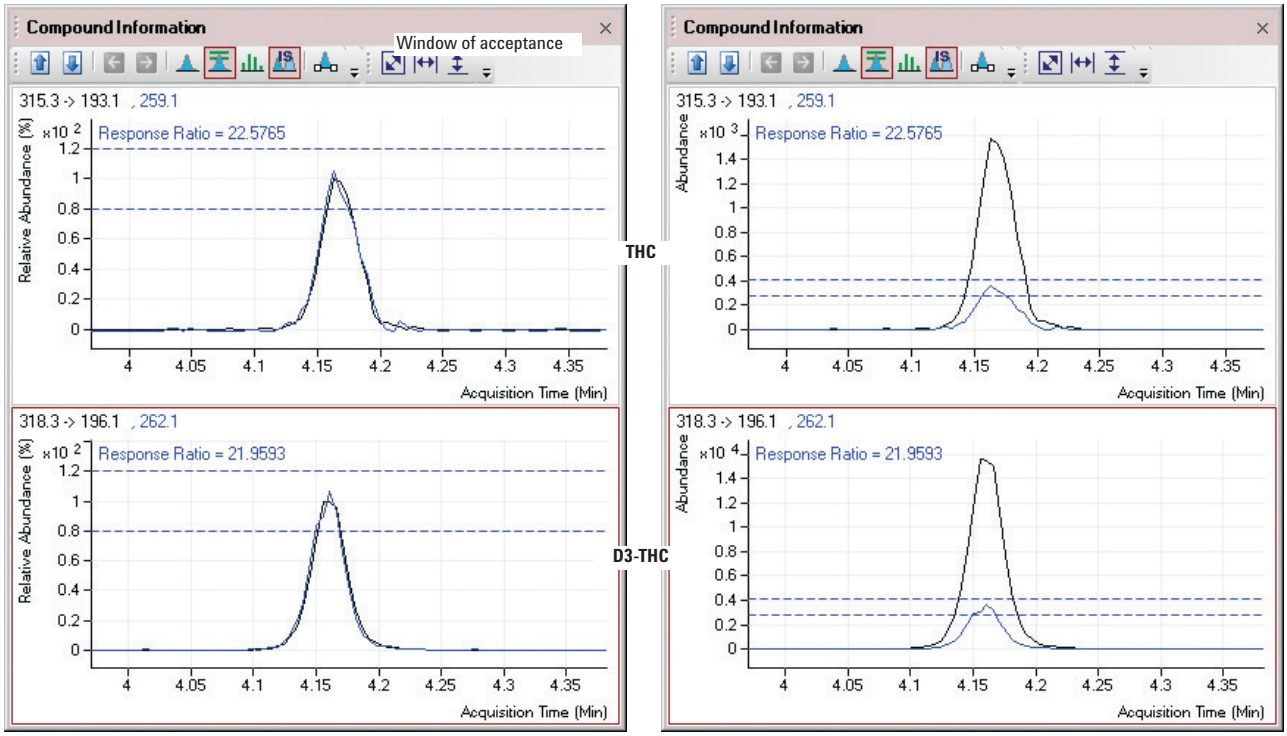


Figure 4. **For confirmation of THC, the qualifier ion area must be 22% that of the quantifier ion area and within a window of ±20% of that value, or from 17.6% to 26.4% overall. The two ways to display this for fast confirmation in the Quantitative Analysis software is normalized by area (left) and 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.

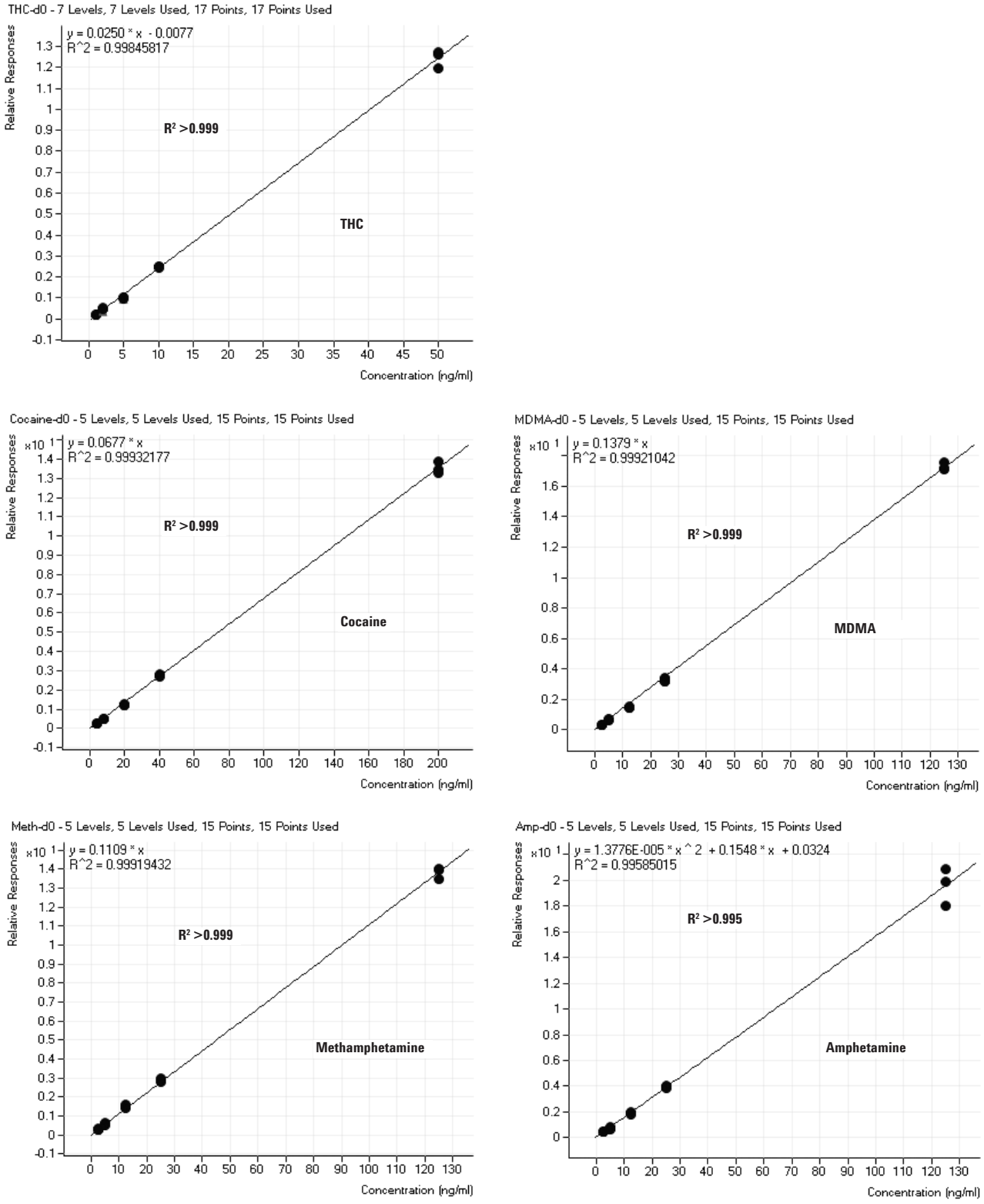


Figure 5. Calibration curves for each DOA using a linear line fit with no weighting and no origin treatment.

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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Quantitative Analysis of Ethylglucuronide in Urine Using the Agilent 1200 RRLC and 6410 Triple Quadrupole Mass Spectrometer

Application

Forensics

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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 alcoholics.

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 and has an overall positivity rate of approximately 20%. EtG detection has been shown to be a valuable tool in programs where abstinence from alcohol use is a strict requirement. As with all markers of substance abuse, EtG levels should not be used as the sole criteria of alcohol use, but should be evaluated in concert with other factors by trained counselors and professionals.

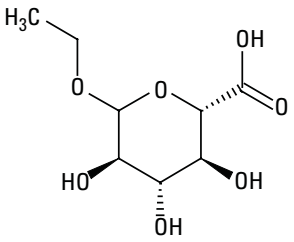
A further increase in specificity comes with the use of a tandem MS/MS mass spectrometer for analysis. The QQQ provides this capability by selecting the EtG precursor ion and generating product ions that are specific to its structure. The more intense product ion is then used for quantitation while the 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 patient 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 electrospray 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 patient 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.

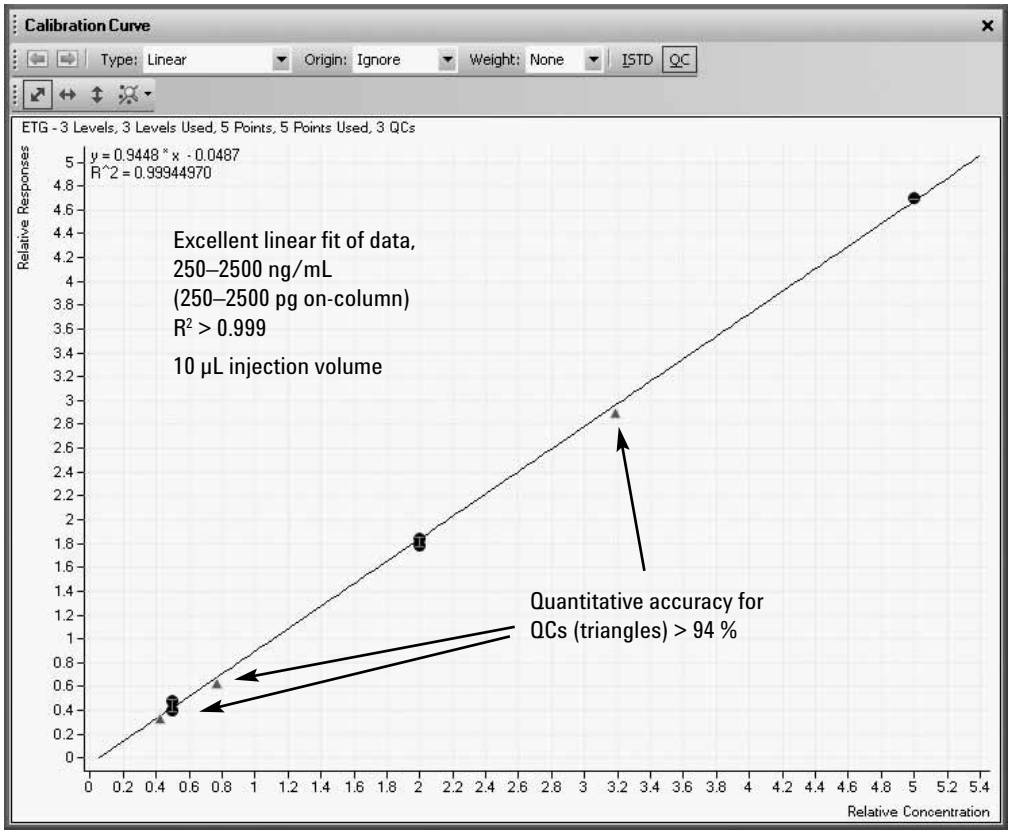


Figure 2. Excellent linearity over the 250 to 2,500 ng/mL analytical range. Curve fit settings of linear type, ignore origin and no weighting used. Based on dilution by internal standard and 10-µL injection volume, the corresponding on-column injection range is 250 to 2,500 pg.

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

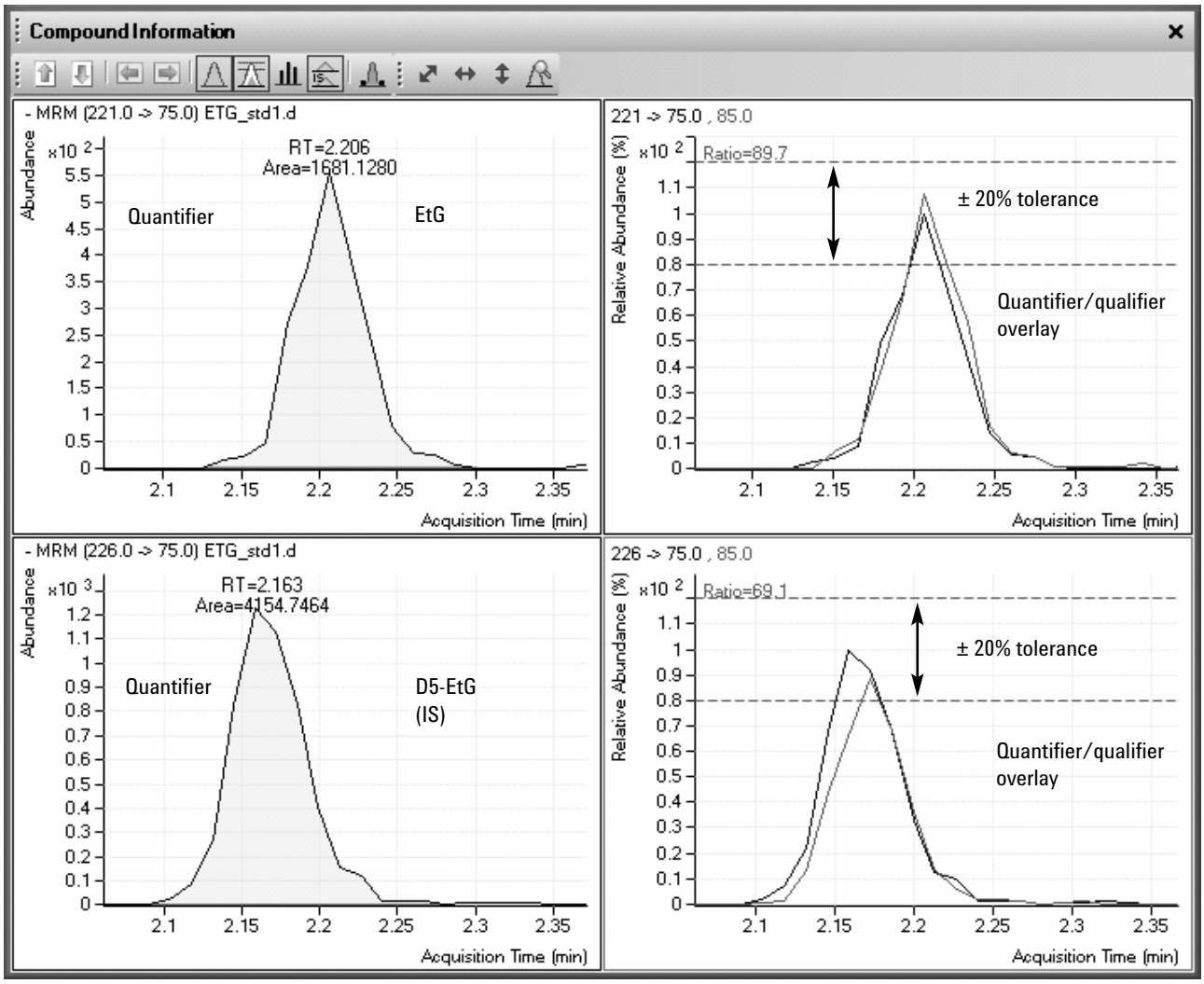


Figure 3. Confirming presence of EtG in sample based on qualifier ion ratio shown here for one of the 250 ng/mL calibration standard injections. Normalized overlay of qualifier and quantifier ions, based on area counts, shown on the right.

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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Agilent Technologies

Using LC/Triple Quadrupole Mass Spectrometry for a Fast Quantitation of Immunosuppressants in Blood

Application

Clinical Toxicology

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Abstract

A fast and sensitive technique for confirming the presence of immunosuppressants in human blood, using the Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) is presented. The QQQ provides good quantitative sensitivity without requiring sample cleanup beyond a simple protein precipitation step. Chromatographic elution times for all three compounds analyzed (tacrolimus, cyclosporin A, and sirolimus) are less than 3 minutes using a 3.5-µm particle size Agilent cyano column at a mobile phase flow rate of 450 µL/min. Using water and acetonitrile, both with 0.1 % acetic acid, the best sensitivity is achieved in analyzing the sodiated adducts $[M + Na]^+$ of sirolimus, tacrolimus, and everolimus [internal standard], and the deprotonated $[M - H]^-$ form of cyclosporin A. Limits of detection [S/N = 3:1] as low as 800 fg injection amounts are achieved.

Introduction

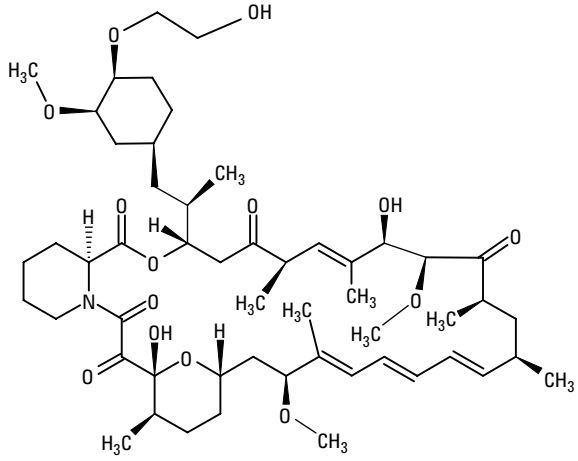
Immunosuppressants like sirolimus and the calcineurin inhibitors cyclosporin and tacrolimus are drugs typically used for treatment of organ transplant recipients. The drugs are used to suppress the immune system of the patient and thus reduce the risk of organ transplant rejection. Because of their narrow therapeutic concentration range and potential for toxicity, accurate drug monitoring of these compounds in blood concentrations is required.

To achieve the required limits of quantitation (tacrolimus and sirolimus < 1 ng/mL) with high-throughput analysis, the common practice of analysis involves large-volume injections (> 0.1 mL) onto solid phase extraction cartridges (on-line SPE) using one LC pump for sample loading, clean-up, and trapping, followed by a second pump to elute the trapped compounds onto an analytical column for chromatographic separation [1]. In such a configuration, analysis turnaround times may be as low as 2.5 minutes [2, 3]. However, when comparing the performance of different system configurations, especially with regards to sensitivity, the actual on-column injection amount

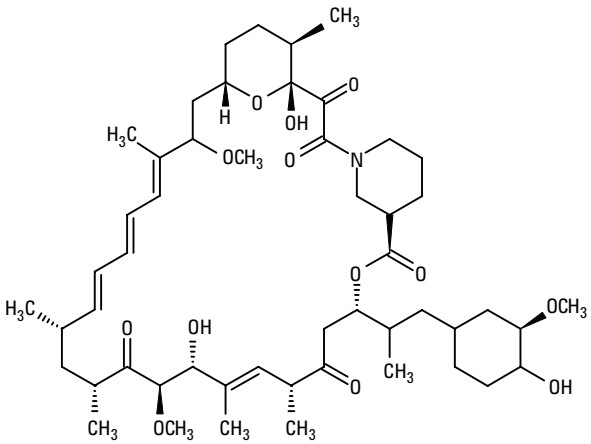
is an important consideration. Such a consideration helps the analyst to decide how much of the blood sample needs to be analyzed and to what degree the sample extraction process must be carried out.

For example, the author (Christians) may inject 100 µL of supernatant following a simple protein precipitation step from whole blood, containing 1 ng/mL of an immunosuppressant compound, which corresponds to an on-column injection amount of 20 pg. In this work, 40 µL of supernatant is injected onto an LC system in which SPE is not implemented. As a result, the sample quantities being examined here are less than commonly analyzed, and the additional process of sample cleanup using SPE is bypassed.

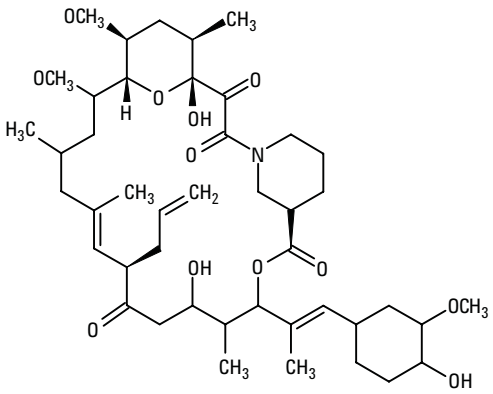
Compounds Analyzed



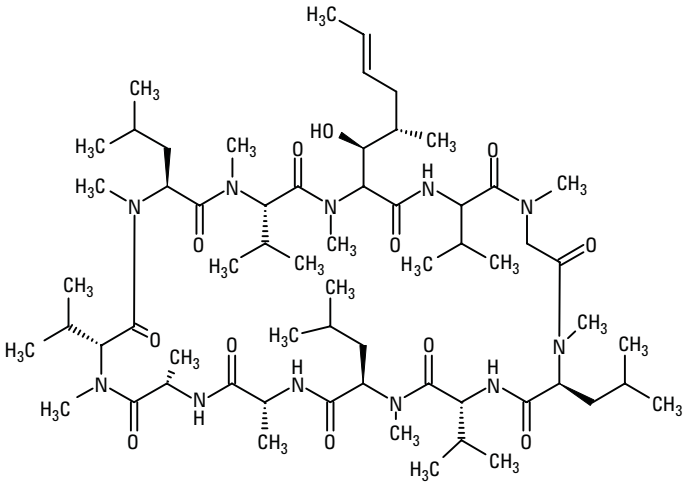
Internal standard:
Everolimus, $C_{53}H_{83}NO_{14}$
$[M + Na]^+ = 980.6$



Sirolimus, $C_{61}N_{79}NO_{13}$
$[M + Na]^+ = 936.5$



Tacrolimus, $C_{44}H_{69}NO_{12}$
$[M + Na]^+ = 826.5$



Cyclosporin A, $C_{62}H_{111}N_{11}O_{12}$
$[M - H]^- = 1200.8$

Experimental

Sample Preparation

The compounds analyzed in this work include tacrolimus, sirolimus, and cyclosporin A, which have the structures shown below.

Immunosuppressants are each spiked into 200 µL whole human blood. Extraction begins with the addition of 800 µL of 0.2 M $ZnSO_4$/MeOH (30:70 v/v). After centrifugation, approximately 800 µL of supernatant is transferred to a sample vial to be injected into an LC/QQQ system. The concentration of the internal standard, everolimus, is 100 ng/mL.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consisted of an Agilent 1200 Series vacuum degasser, binary pump, well-plate autosampler, thermostatted column compartment, the Agilent 6410 Triple Quadrupole Mass Spectrometer, and an electrospray ionization (ESI) source. System control and data analysis were provided by the Agilent MassHunter QQQ Control Console and Qualitative and Quantitative Data Analysis software programs. LC and MS conditions are shown in LC/MS Method Details.

The objective of the method development was to obtain a fast and sensitive analysis for quantifying immunosuppressants in blood. For speed, different solvents, flow rates, and column parameters were optimized while maintaining good chromatographic resolution and peak symmetry. It was found that a simple solvent system using water, acetonitrile, and acetic acid at an isocratic flow at 450 µL/min on a 3.5-µm particle size CN column would sufficiently elute the compounds in times very competitive with most techniques available in LC/MS. The chromatography conditions reflected the earlier work of P. Hatsis and D. A. Volmer, reducing the chance of ion suppression between one analyte and another. [4]

Also during the method development involving different LC solvents, the multiple reaction monitoring (MRM) signal responses for protonated $[M + H]^+$, deprotonated $[M - H]^-$, and sodiated $[M + Na]^+$ molecular ions were compared. Since the sodiated form generated the highest response for sirolimus, tacrolimus, and everolimus, positive ion mode was run during the elution of the compounds, which were all less than 2.3 minutes. The remaining compound, cyclosporin A, responded best for the deprotonated form $[M - H]^-$ so that the QQQ polarity was switched to negative mode during the later elution of cyclosporin A.

LC/MS Method Details

LC Conditions

Agilent 1200 Series binary pump SL, well-plate sampler, thermostatted column compartment, inline filter 0.5 µm between needle seat capillary and injector valve.

Column:	Agilent ZORBAX XDB-CN, 2.1 × 150 mm, 3.5 µm (p/n 930990-905)
Guard column:	Agilent ZORBAX XDB-CN, 2.1 × 12.5 mm, 5 µm (p/n 821125-935)
Column temperature:	50 °C
Mobile phase:	A = 0.1% acetic acid in water B = 0.1% acetic acid in acetonitrile (ACN)

Amount of sodium present in both mobile phases can vary from lab to lab. To increase likelihood of sodium adduct formation of the analytes, one may add as much as 50 µM sodium acetate.

Flow rate:	0.45 mL/min
Isocratic:	0–3 min @ 52% B 100% B, 3.1–4.0 min
Stop time:	3.0 min
Post-run time:	1.0 min
Injection vol:	40 µL

It should be noted that the post-run time of 1 min is used for a column wash at 100% B. However, as noted by Hatsis and Volmer in reference 4, column washes need only be performed every 50 injections.

MS Conditions

Mode:	Positive ESI using the Agilent G1948A ionization source
Nebulizer:	40 psig
Drying gas flow:	10 L/min
Drying gas temperature:	350 °C
V_{cap}:	4,000 V
Q1 resolution:	2.5 amu
Q2 resolution:	0.7 amu
MRM:	Transitions for tacrolimus, sirolimus, and cyclosporin A shown in Table 1. Fragmentor (Frag), collision energy (CE), and dwell times included.

Table 1. Data Acquisition Parameters for MRM Transitions

Compound	Precursor ion	Product ion (*m/z*)	Frag (V)	CE (V)	Dwell (msec)
Segment 1 (0–2.25 min)					
Tacrolimus	826.4 $[M + Na]^+$	616.2	200	40	150
Sirolimus	936.4 $[M + Na]^+$	409.2	360	60	150
Everolimus (IS)	980.7 $[M + Na]^+$	389.4	360	60	150
Segment 2 (2.25–3 min)					
Cyclosporin A	1200.7 $[M - H]^-$	1088.6	260	30	250

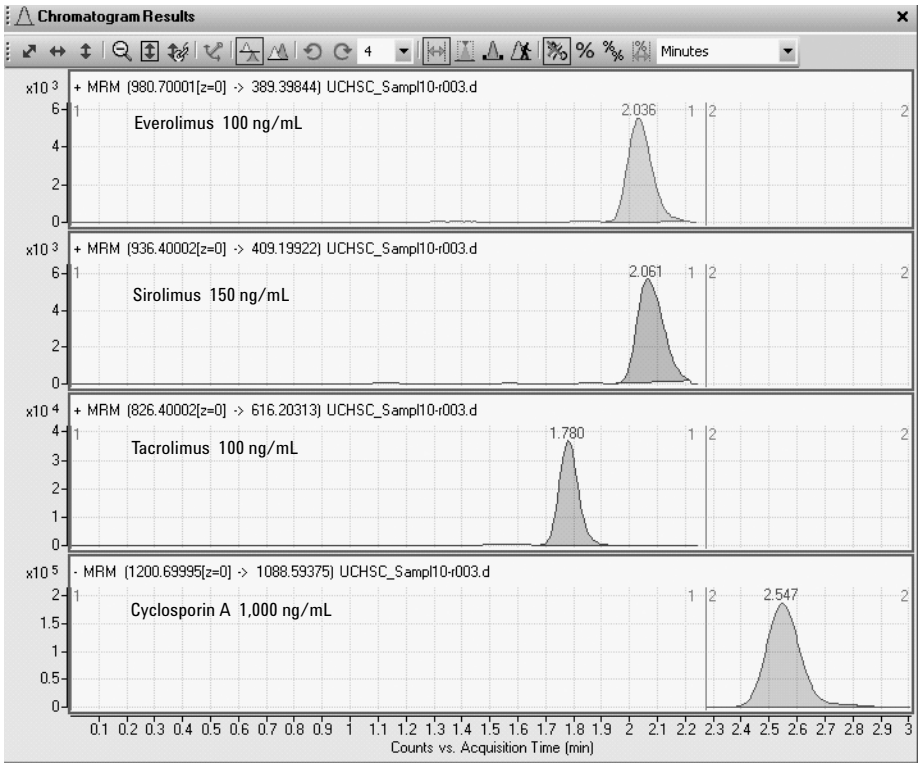


Figure 1. Chromatographic elution profiles of the highest concentration containing all four immunosuppressant compounds. Note the segment division between positive (0 to 2.25 min) and negative (2.25 to 3.0 min) polarities.

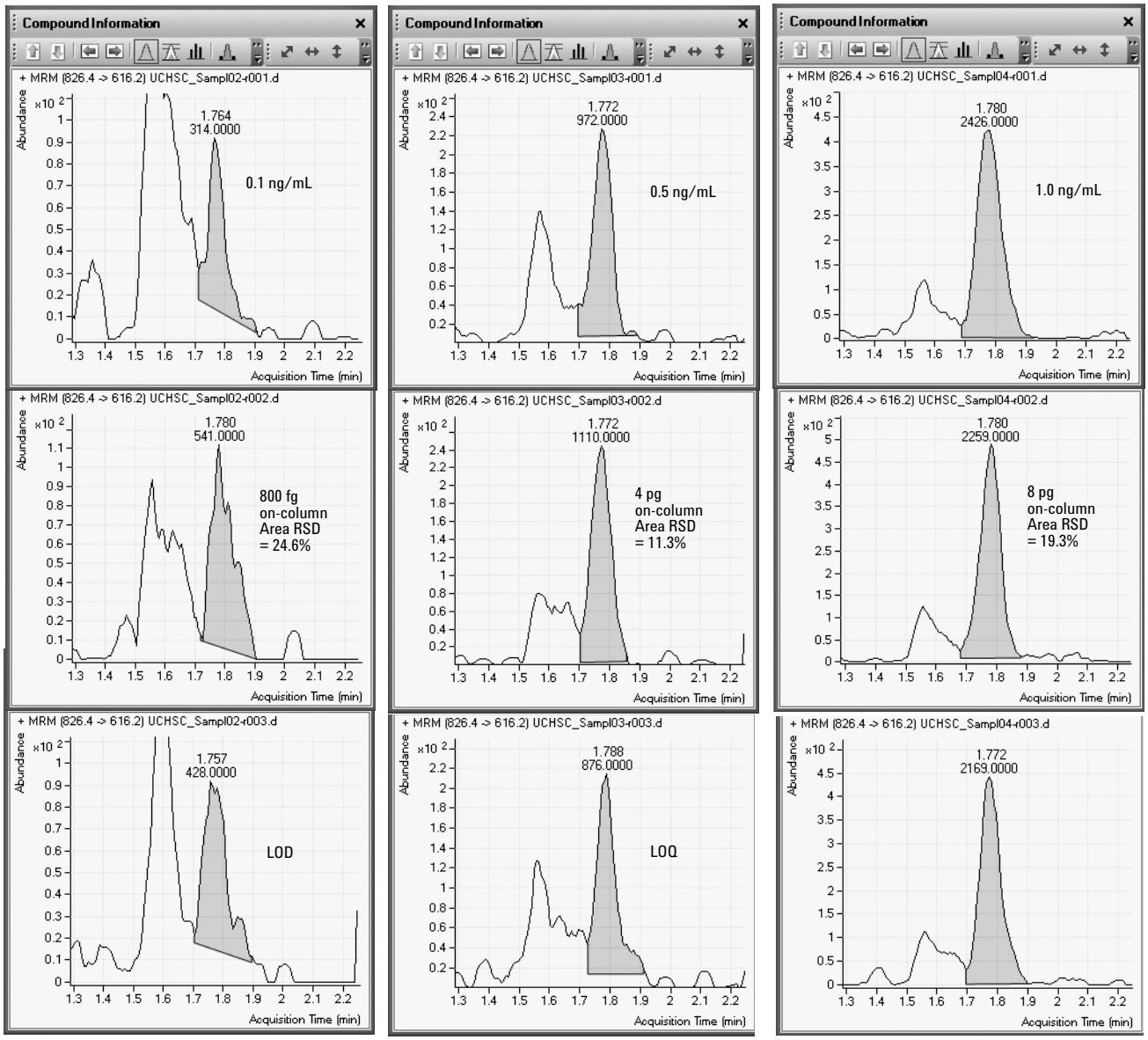


Figure 2. The integrated peaks of the lowest three levels for tacrolimus (triplicate injections).

Results and Discussion

In this work, therapeutic ranges for immunosuppressant dosage measurements are considered to be 1 to 50 ng/mL for both tacrolimus and sirolimus, and 10 to 1,000 ng/mL for cyclosporin A. A chromatogram of the highest level containing all four compounds is shown in Figure 1. Limits of quantitation (LOQs) are defined to be those levels in which peak area count reproducibility among three or more injections has a percent relative standard deviation (% RSD) of 20% or less. The results of tacrolimus are shown in Figure 2. The triplicate injections of the lowest three levels are included. The LOQ is determined to be 0.5 ng/mL and the LOD, based on the obvious presence of the peaks in Figure 2 and the correspondence of their integrated areas with the linearity of Figure 3, is determined to be 0.1 ng/mL. The linearity over three orders of magnitude for tacrolimus is shown in Figure 3, with a coefficient $R^2 > 0.997$. Note that the retention time for tacrolimus is only 1.8 minutes.

The analysis is repeated for sirolimus and cyclosporin A in Figures 4 to 7. The excellent linearity over three orders of magnitude is noteworthy. Moreover, as in the case of tacrolimus, the curve fit settings of linear, ignore origin, and no weighting also work well for sirolimus, resulting in a coefficient $R^2 > 0.999$. For the linearity of cyclosporin A the coefficient became $R^2 > 0.997$ by forcing the origin to be included.

The retention times for sirolimus and cyclosporin A are only 2.0 and 2.5 minutes, respectively. The limit of detection for sirolimus is 0.1 ng/mL and the limit of quantitation is 0.5 ng/mL. Furthermore, although the peaks at the lowest concentration are

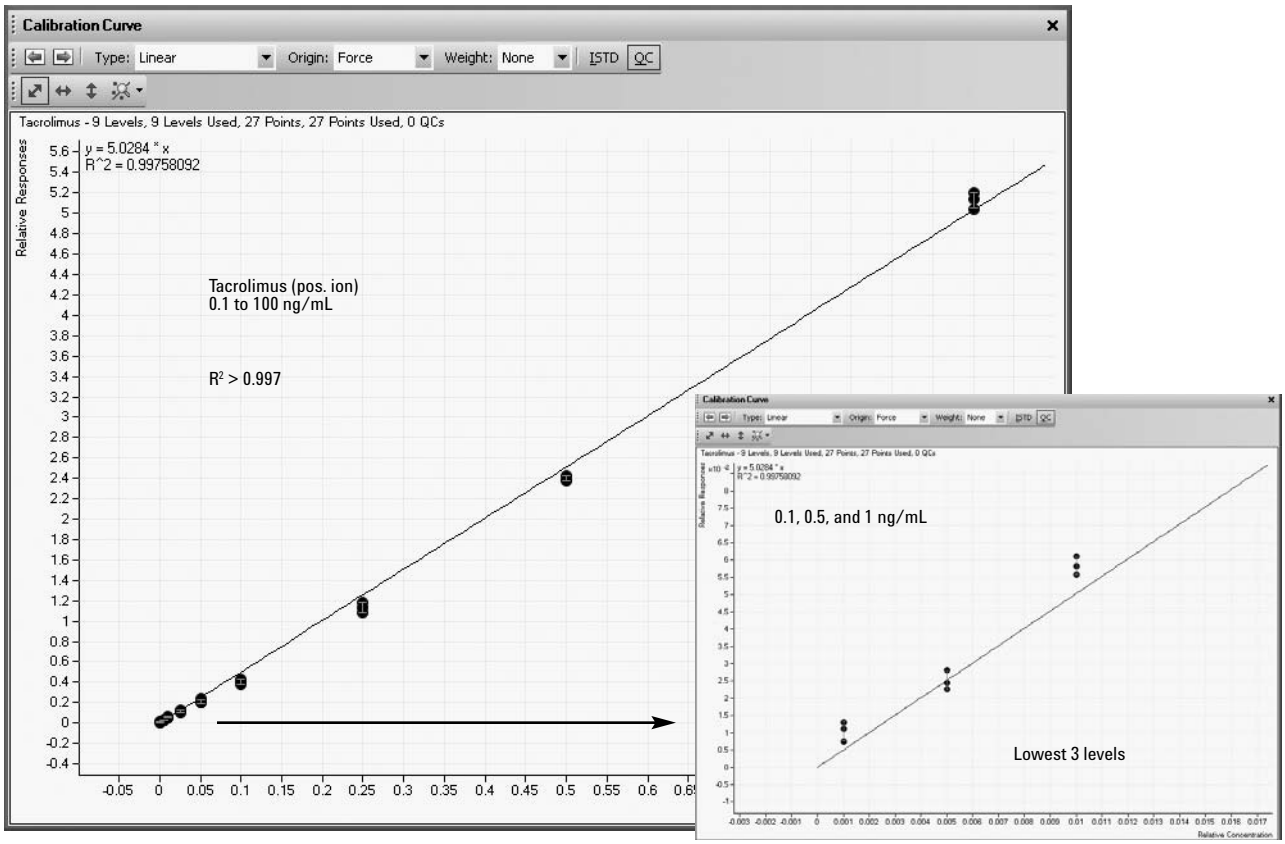


Figure 3. Linearity of tacrolimus over three orders of magnitude. Region of lowest three levels expanded.

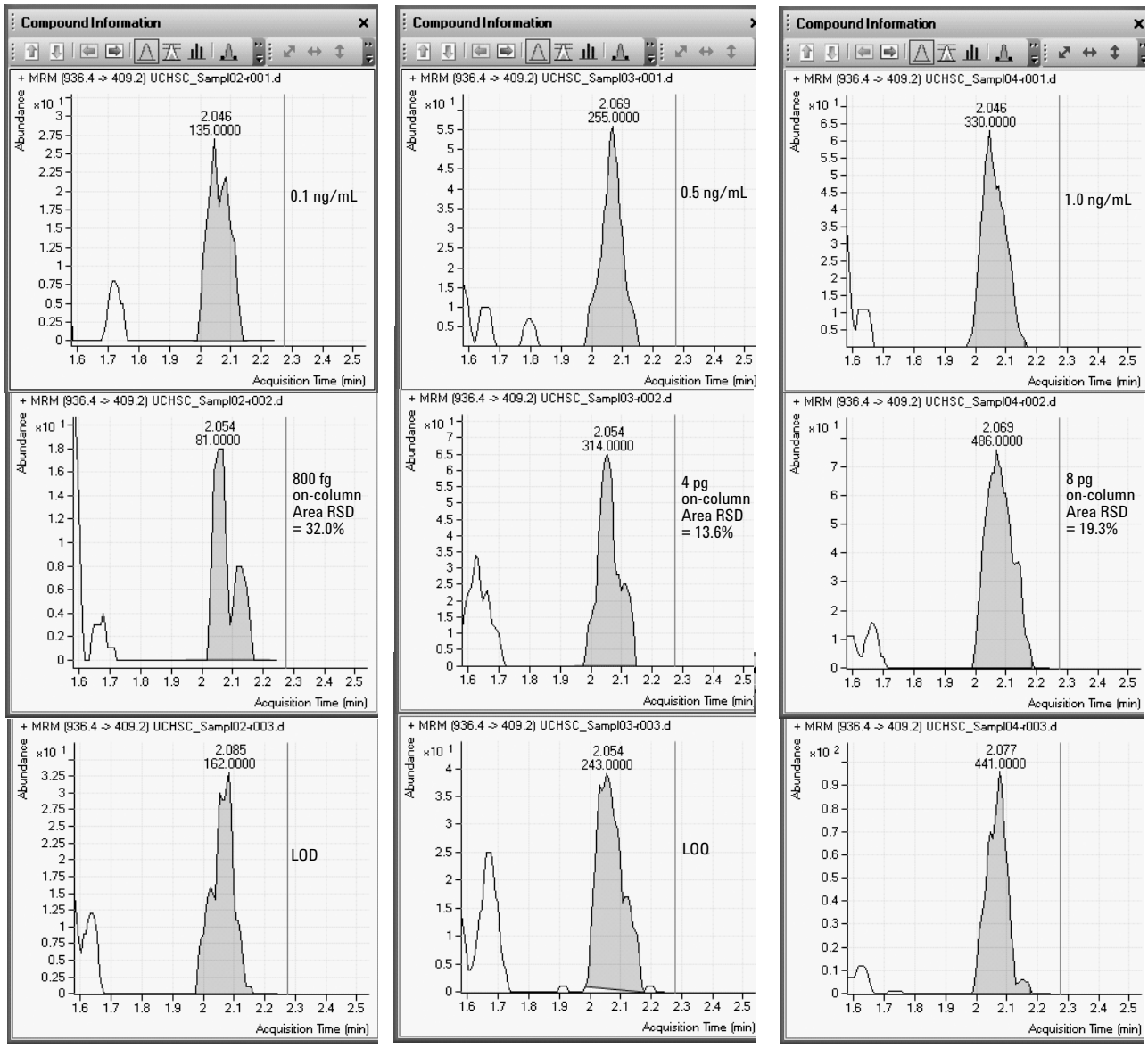


Figure 4. The integrated peaks of the lowest three levels for sirolimus (triplicate injections).

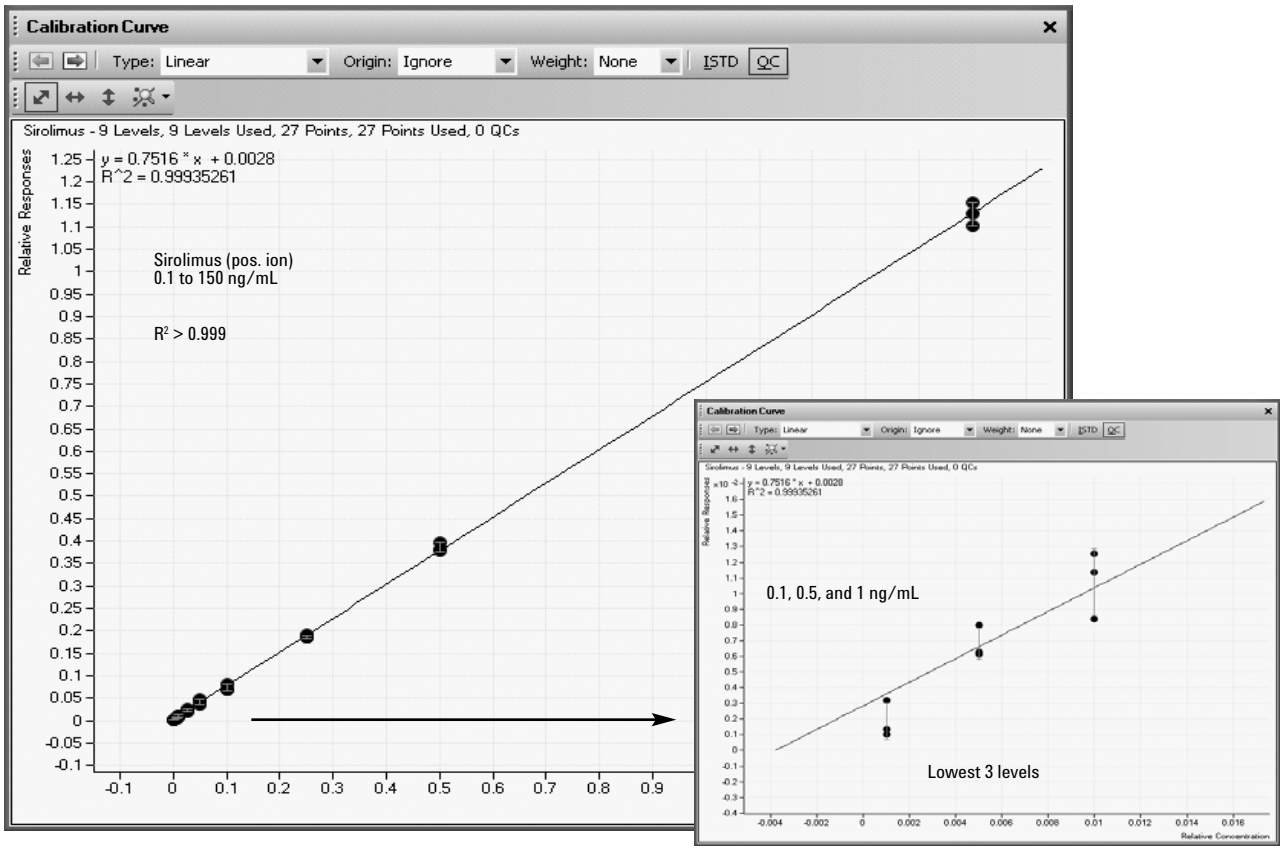


Figure 5. Linearity of sirolimus over three orders of magnitude. Region of lowest three levels expanded.

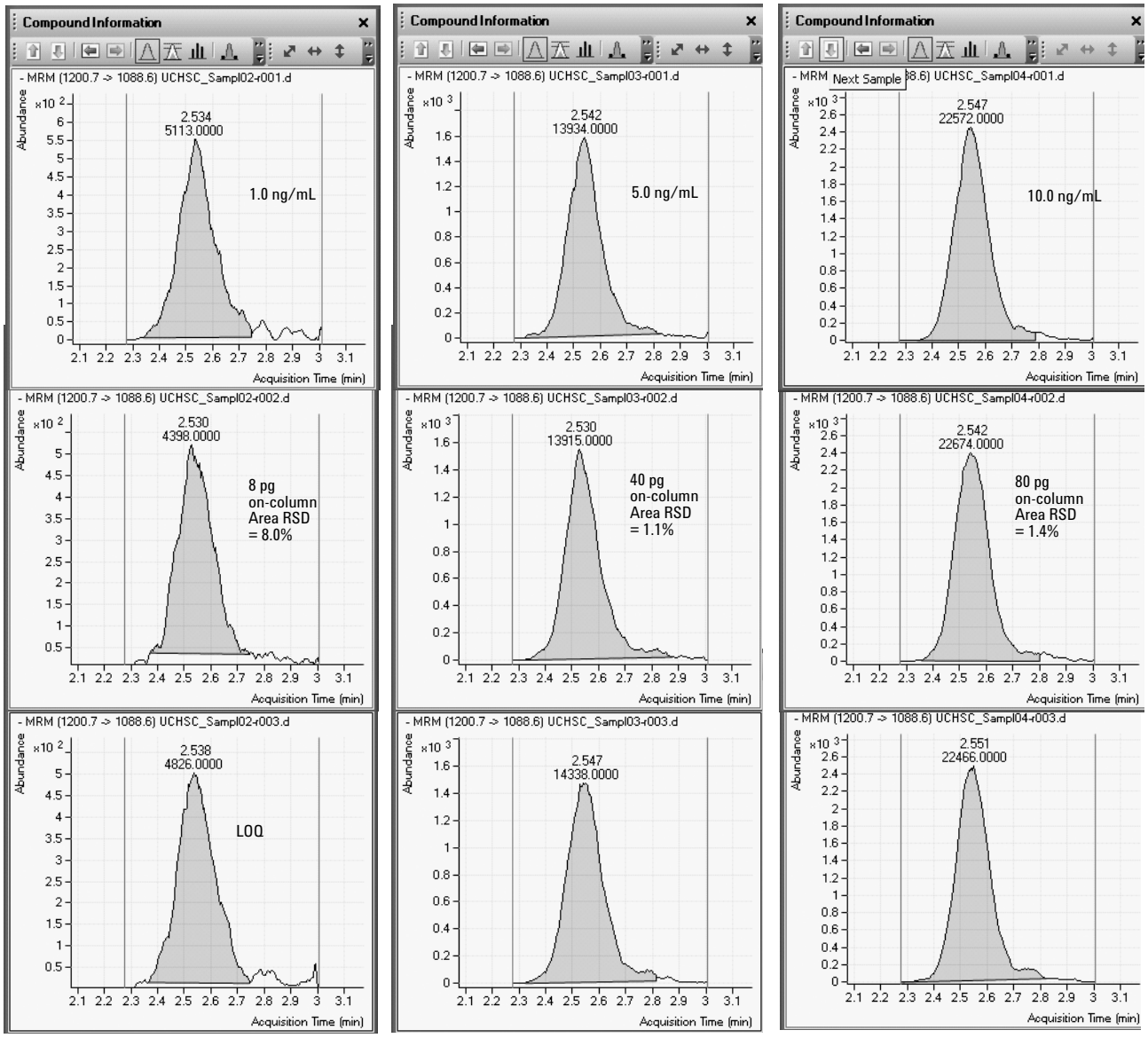


Figure 6. The integrated peaks of the lowest three levels for cyclosporin A (triplicate injections).

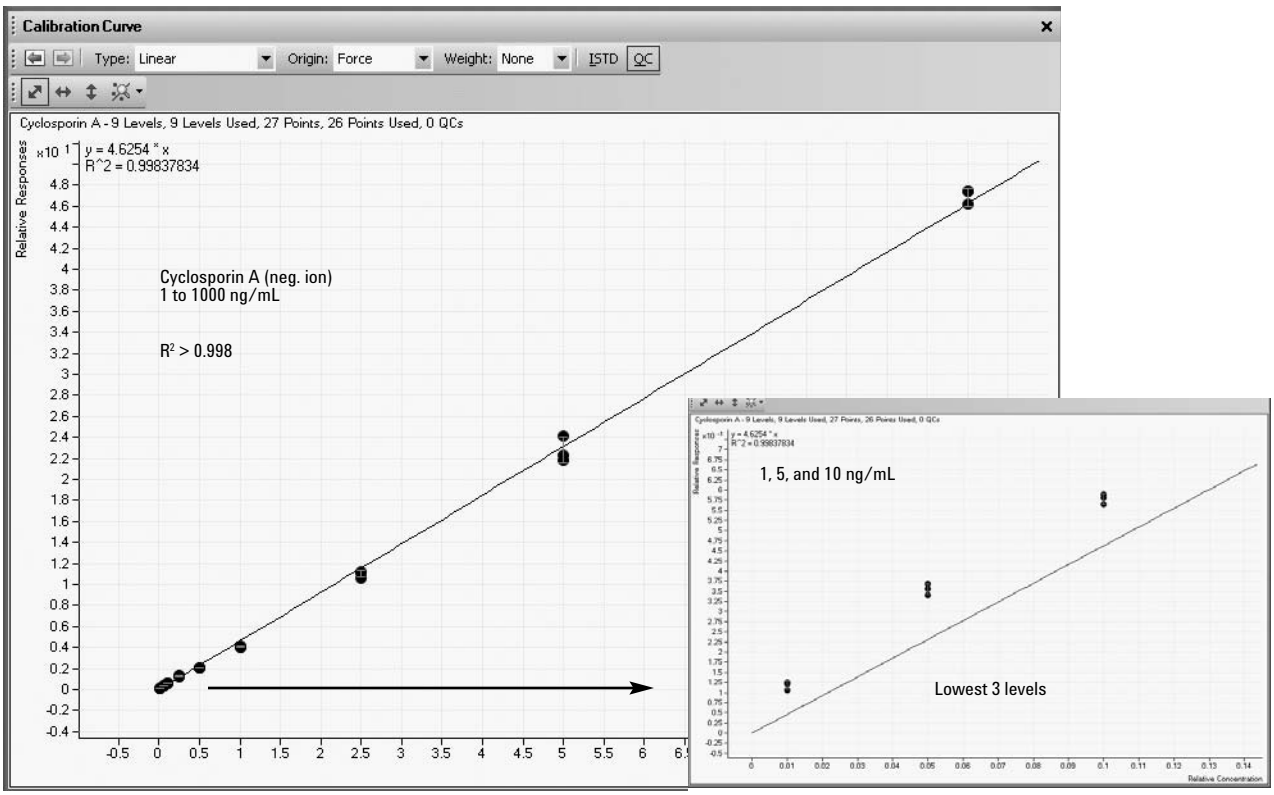
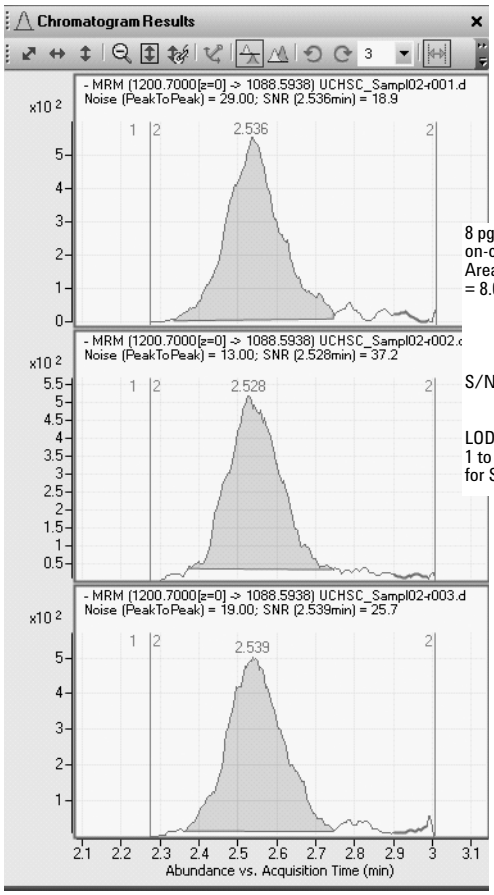


Figure 7. Linearity of cyclosporin A over three orders of magnitude. Region of lowest three levels expanded.



8 pg
on-column
Area RSD
= 8.0%

S/N ~ 20

LOD estimated at
1 to 2 pg on-column
for S/N = 3.

Figure 8. Calculation of LOD for cyclosporin A based on concentration corresponding to S/N = 3.

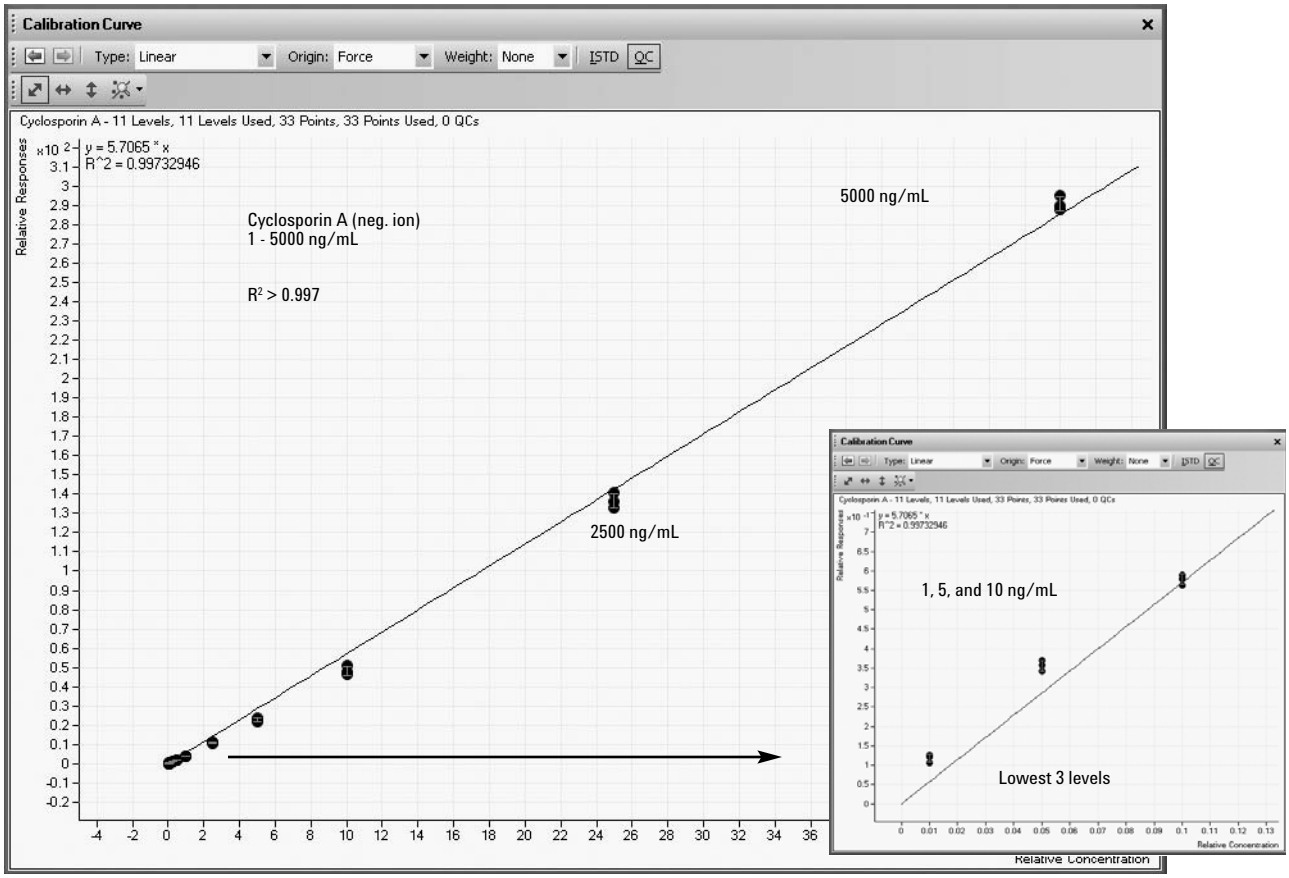


Figure 9. Two additional levels containing cyclosporin A are run showing excellent linearity over nearly 4 orders of magnitude. Accuracy of lowest three levels still very good.

obviously at trace levels, their peak areas still integrate well.

As shown in Figure 6 the LOQ requirement for cyclosporin A is easily satisfied at the 1.0 ng/mL level. However, the LOD for cyclosporin A requires a closer look. See Figure 8 for a calculation of the signal-to-noise ratio for the triplicate injections of the lowest level for cyclosporin A, 1 ng/mL. The average S/N is about 20:1. This means that the limit of detection is about 1 to 2 pg on-column.

Since levels for cyclosporin A can be even higher, it is important to know if the QQQ mass spectrometer can maintain linearity for higher concentrations like 2,500 and 5,000 ng/mL. In Figure 9 we can see that not only is linearity preserved with a coefficient $R^2 > 0.997$, but the lowest three levels maintain their accuracy.

Conclusions

The LC/MS/MS method described here provides a reference for on-column sensitivity of the most commonly analyzed immunosuppressants: sirolimus, tacrolimus, and cyclosporine A. The analysis of sodium adducts for tacrolimus and sirolimus and the deprotonated form of cyclosporin A appear to provide the best sensitivity. The sample preparation includes only a simple protein-precipitation step. Linearity coefficients of more than 0.997 and limits of detection as low as 800 fg on-column are also obtained. All compounds elute in less than 3 minutes, making this method an excellent candidate for high-throughput analysis of immunosuppressants in blood. Finally, the cyano column gives nice resolution at the flow rate of 450 µL/mn.

References

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Quantitative Analysis of Opiates in Urine Using RRHT LC/MS/MS

Application

Forensics

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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 commonly used for sedation and pain relief and may be obtained both legally as prescription medication or illegally. Their abuse can often lead to addiction. 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.

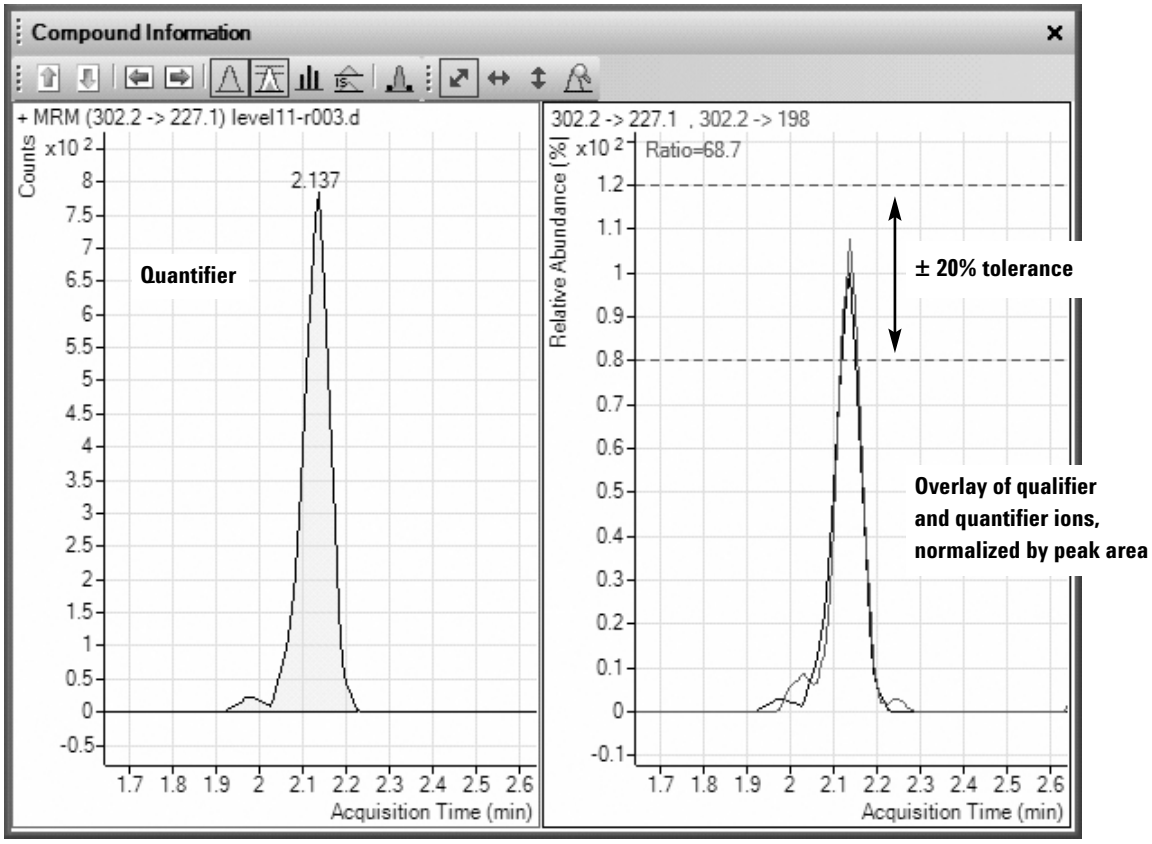


Figure 1. Qualifier/quantifier ion ratios for confirmation of oxymorphone.

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.

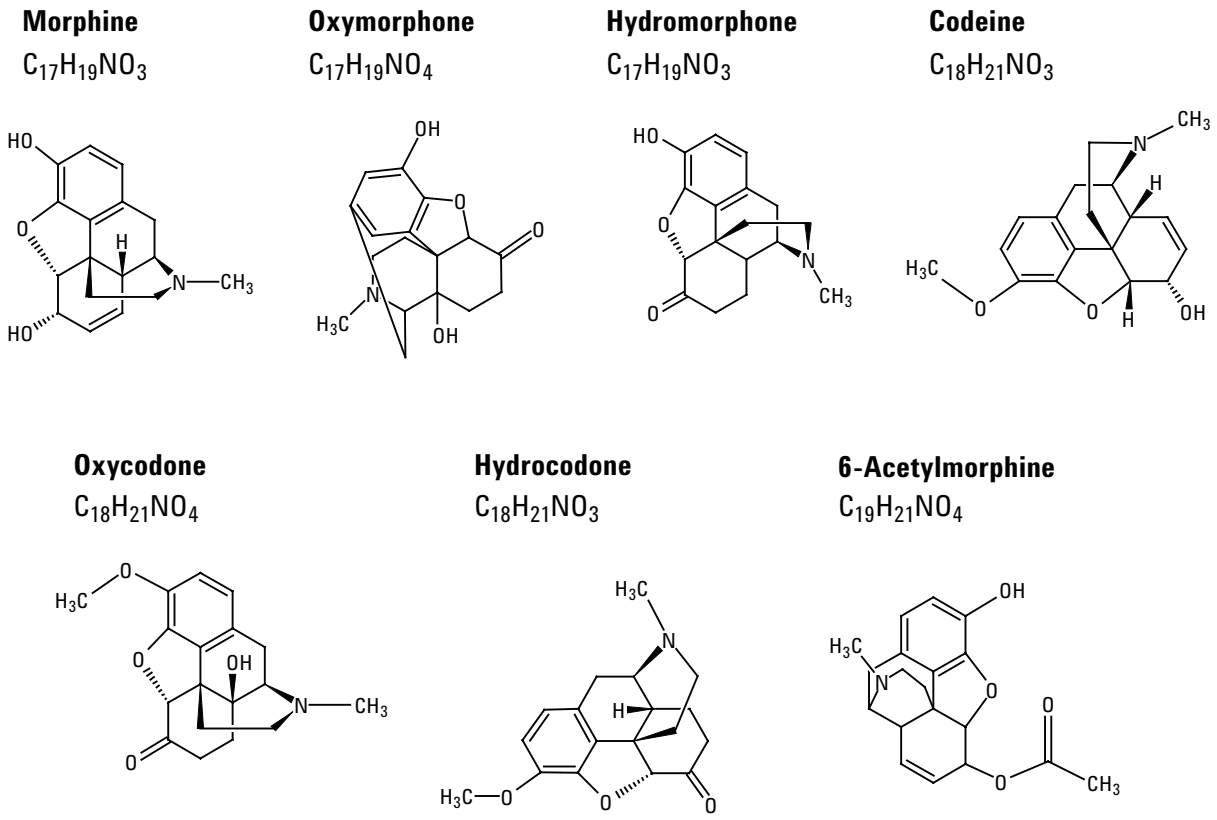


Figure 2. Structures of the opiates analyzed in this work.

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).

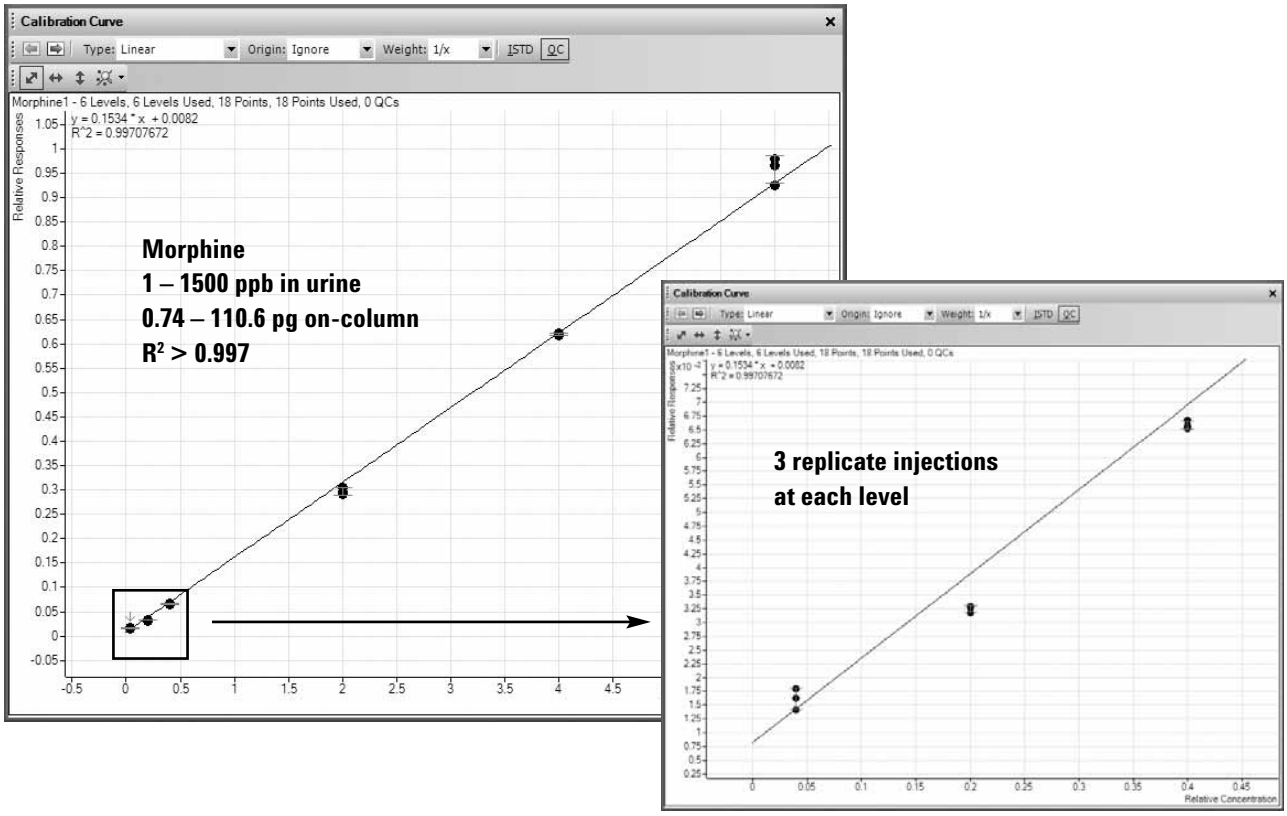


Figure 3A. Linearity of morphine in urine. Injection concentration range = 147 ppt – 22 ppb.

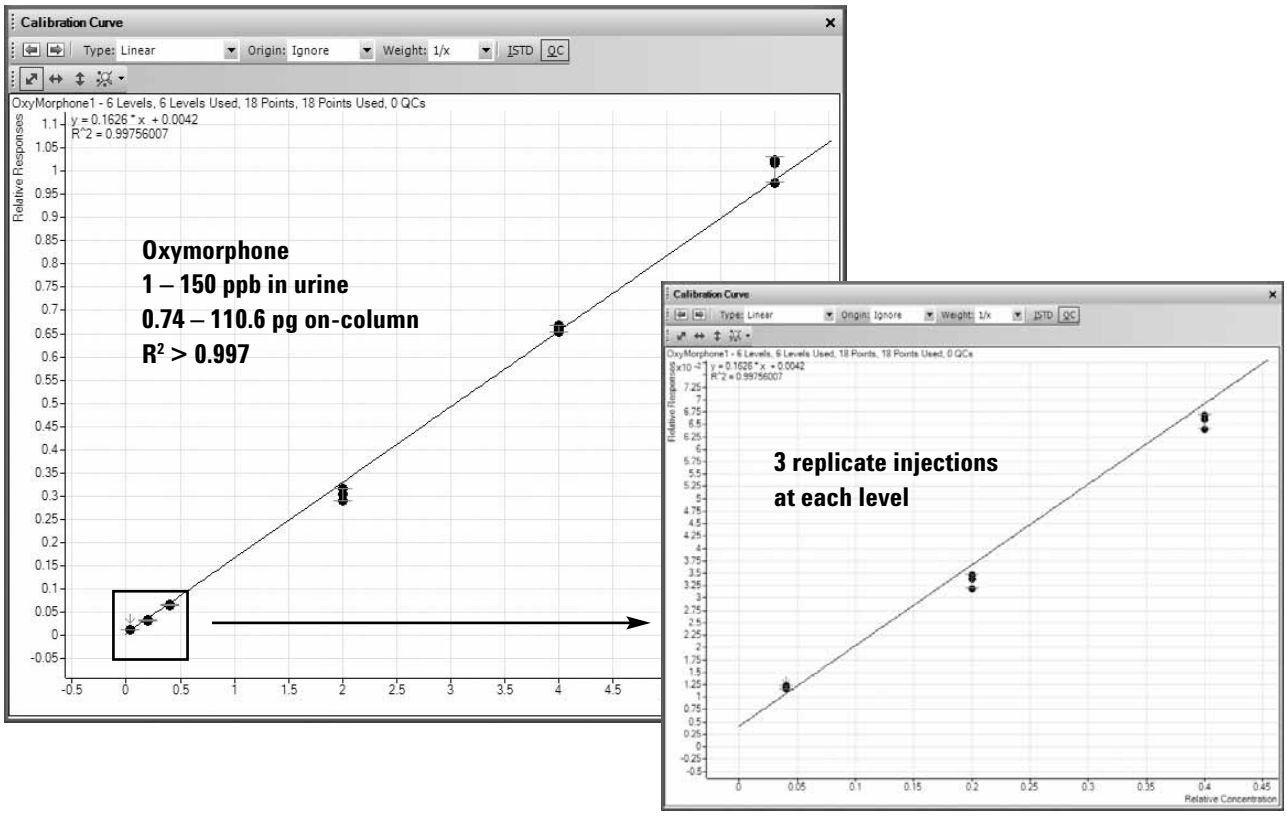


Figure 3B. Linearity of oxymorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

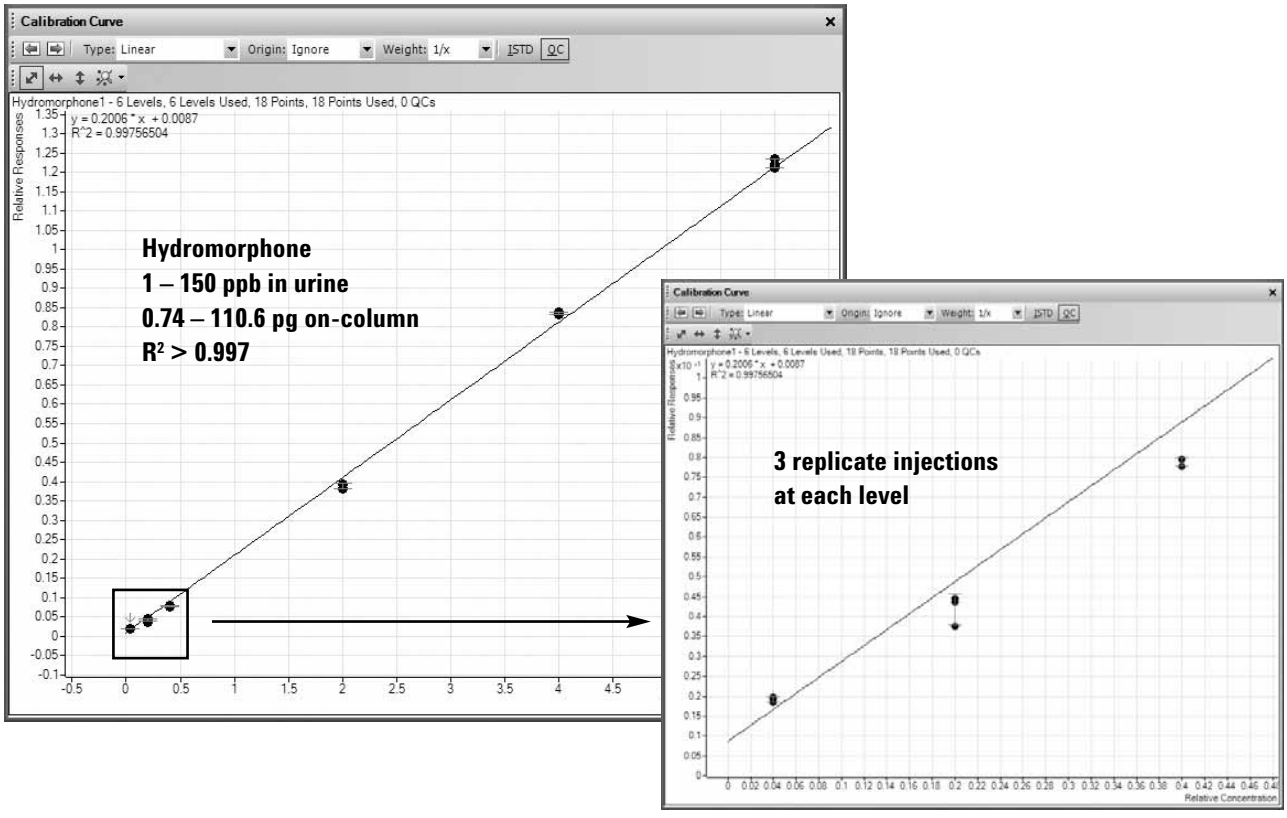


Figure 3C. Linearity of hydromorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

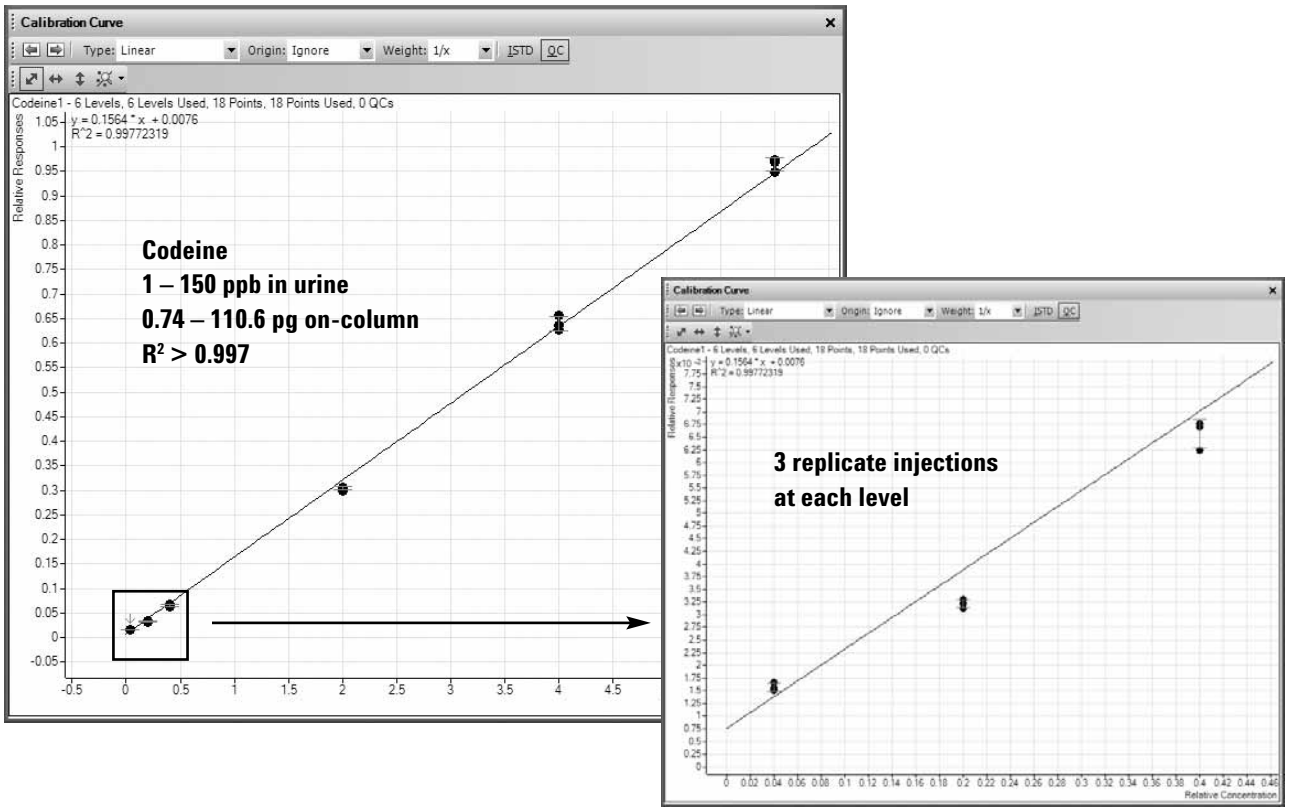


Figure 3D. Linearity of codeine in urine. Injection concentration range = 147 ppt – 22 ppb.

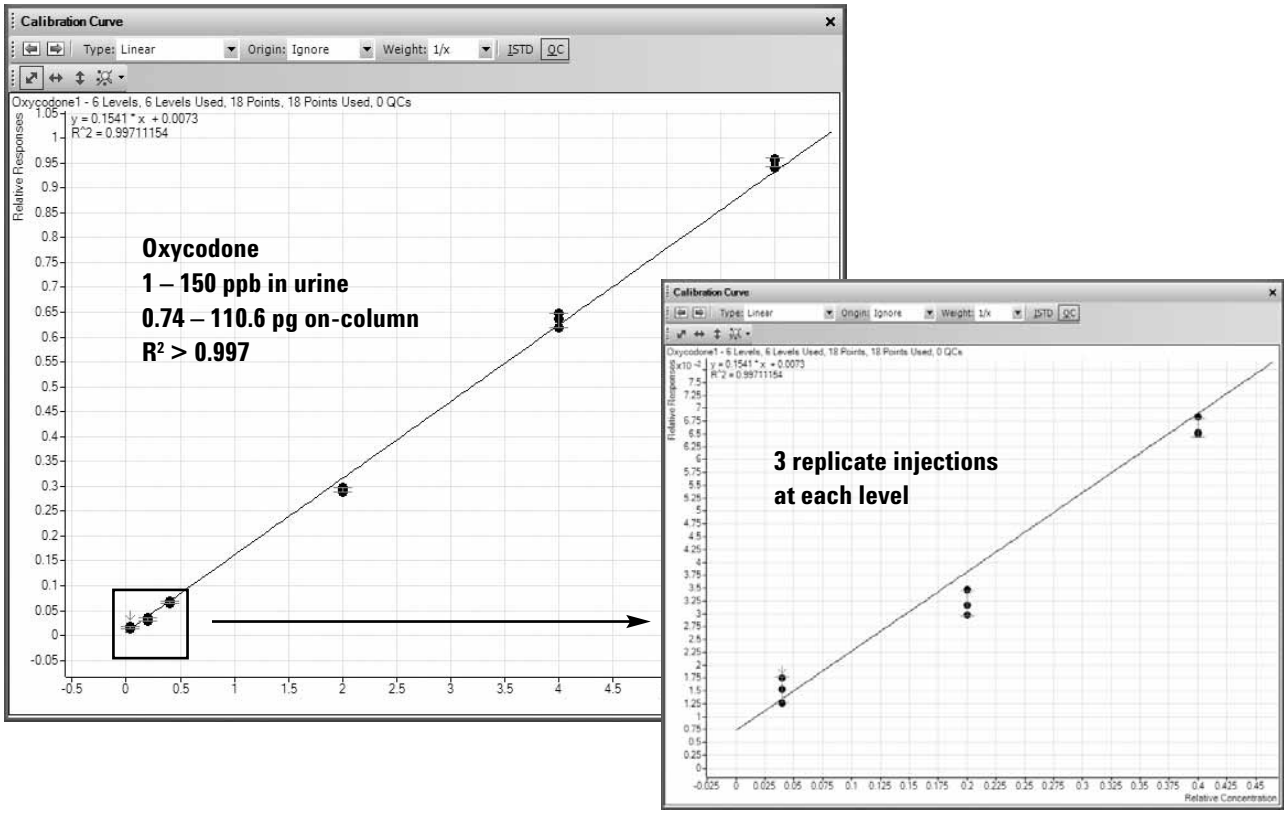


Figure 3E. Linearity of oxycodone in urine. Injection concentration range = 147 ppt – 22 ppb.

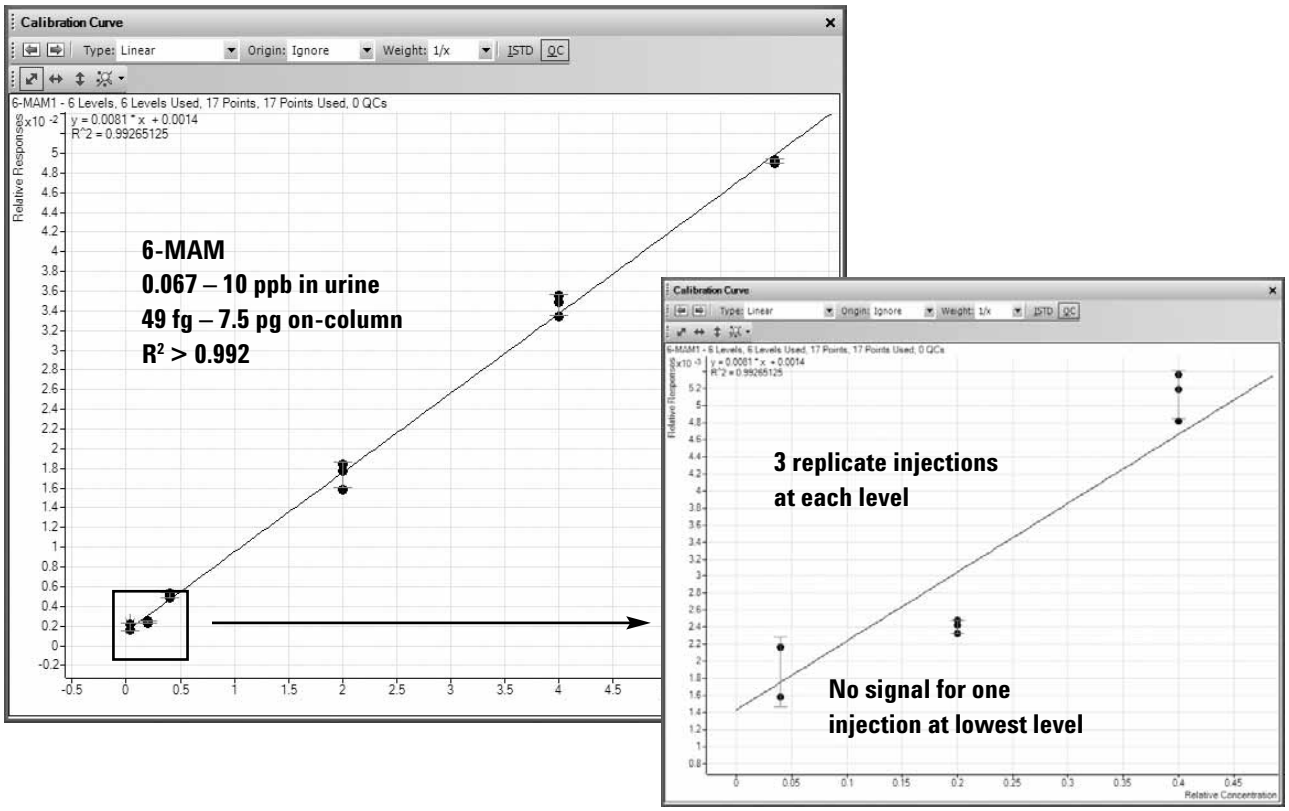


Figure 3F. Linearity of 6-MAM in urine. Injection concentration range = 9.8 ppt – 1.5 ppb.

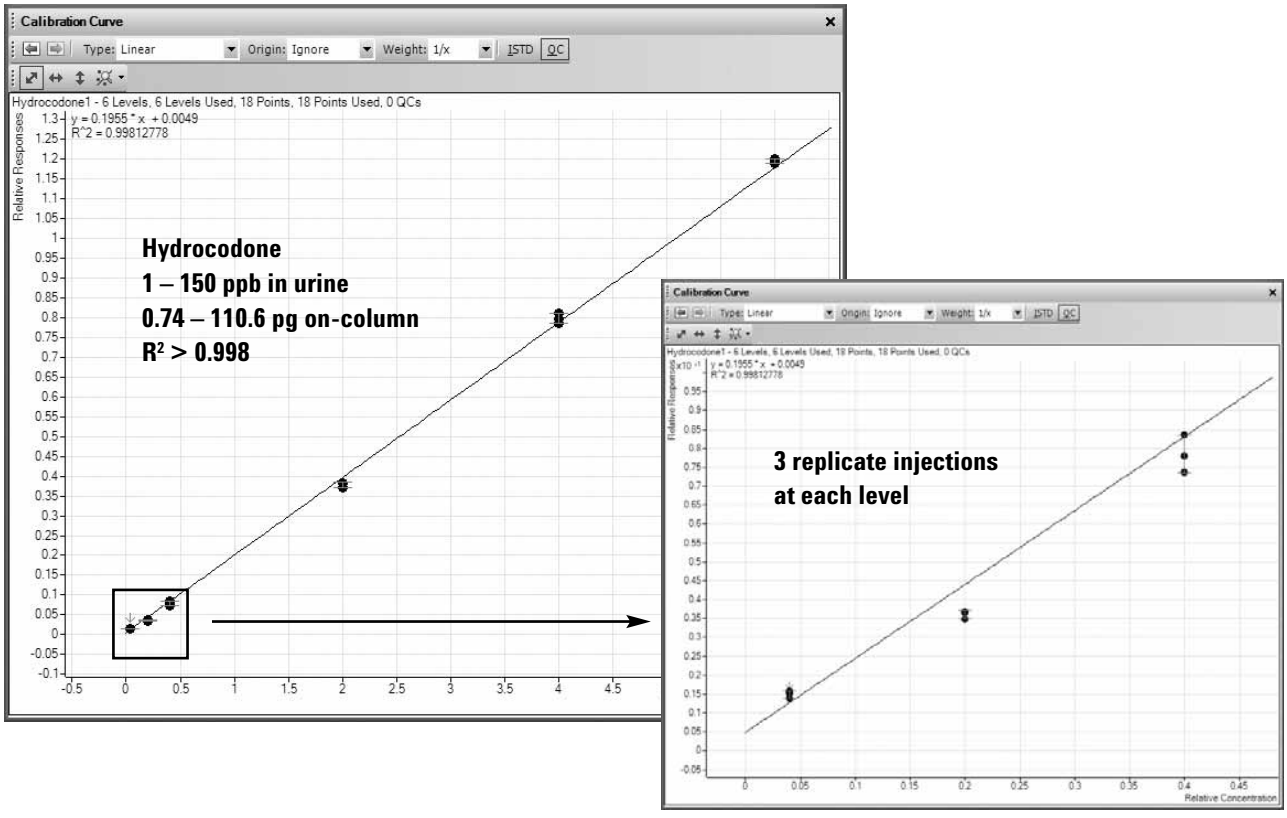
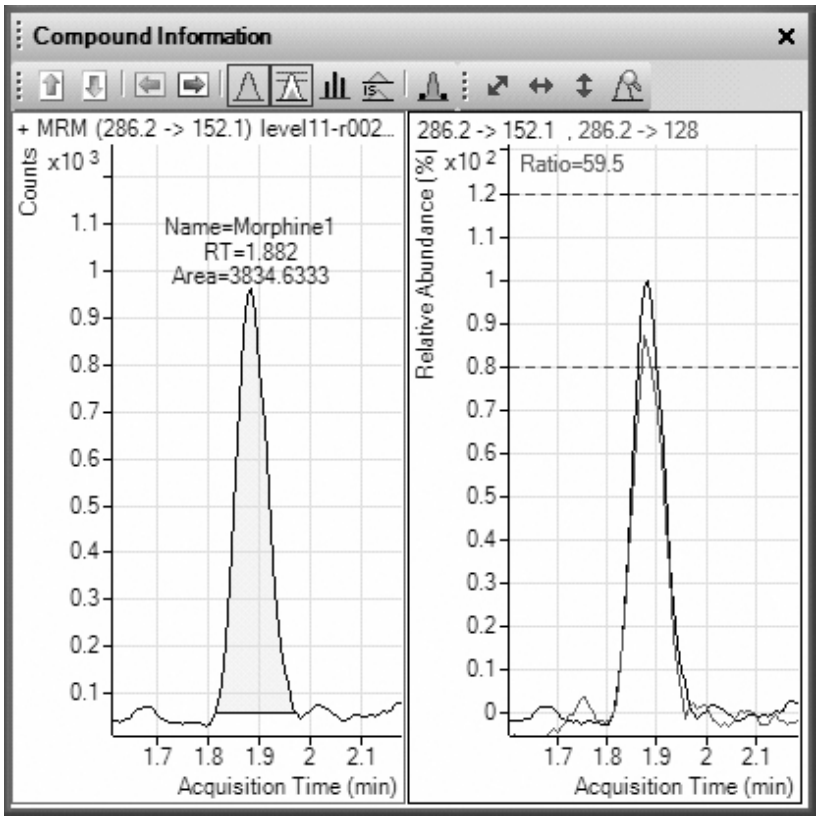


Figure 3G. Linearity of hydrocodone in urine. Injection concentration range = 147 ppt – 22 ppb.

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.

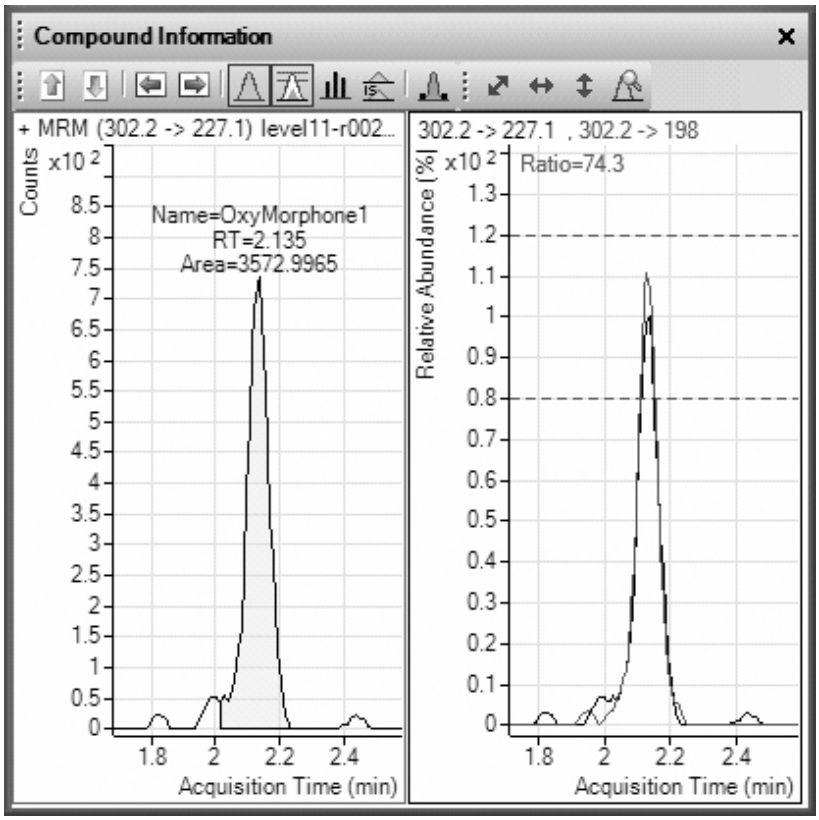
Note that with the exception of oxycodone and 6-MAM, the confirmation ion ratio for all analytes is satisfied at the corresponding lowest calibration levels of 1 pg/µL in urine. For oxycodone and 6-MAM, the lowest levels are 5 and 0.3 pg/µL, respectively.

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.



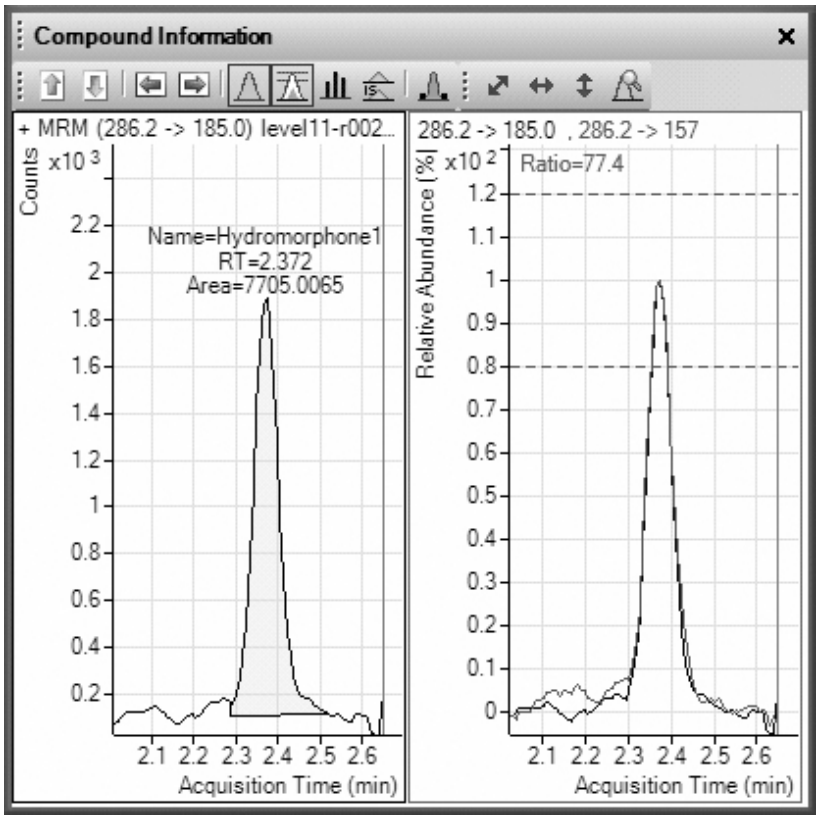
0.74 fg on-column

Figure 4A. Confirmation of morphine at 1 pg/µL (147 fg/µL).



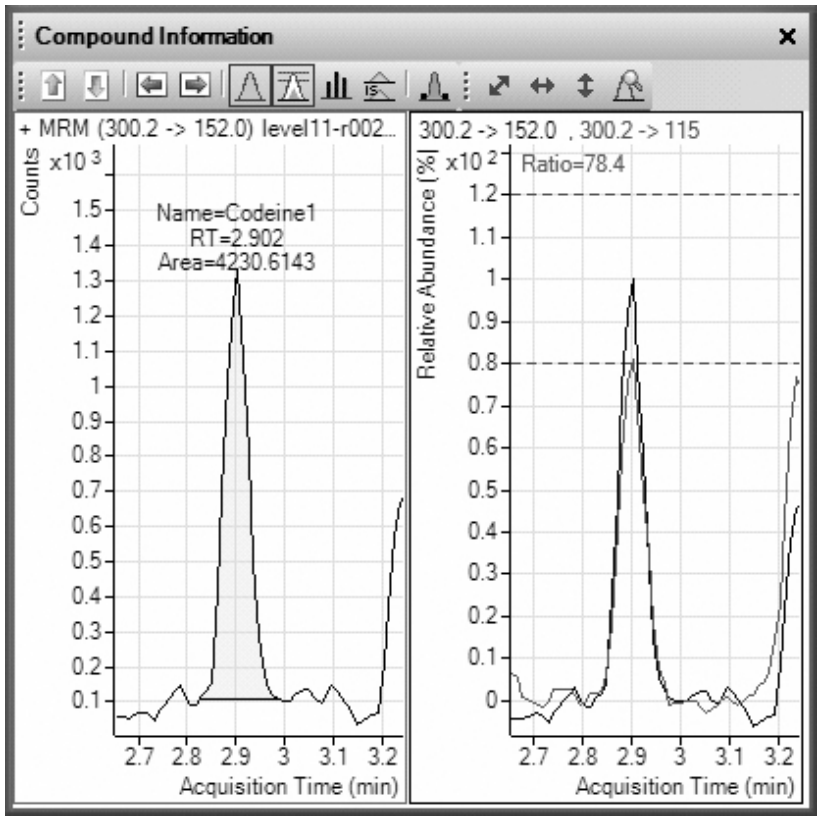
0.74 fg on-column

Figure 4B. Confirmation of oxymorphone at 1 pg/µL (147 fg/µL).



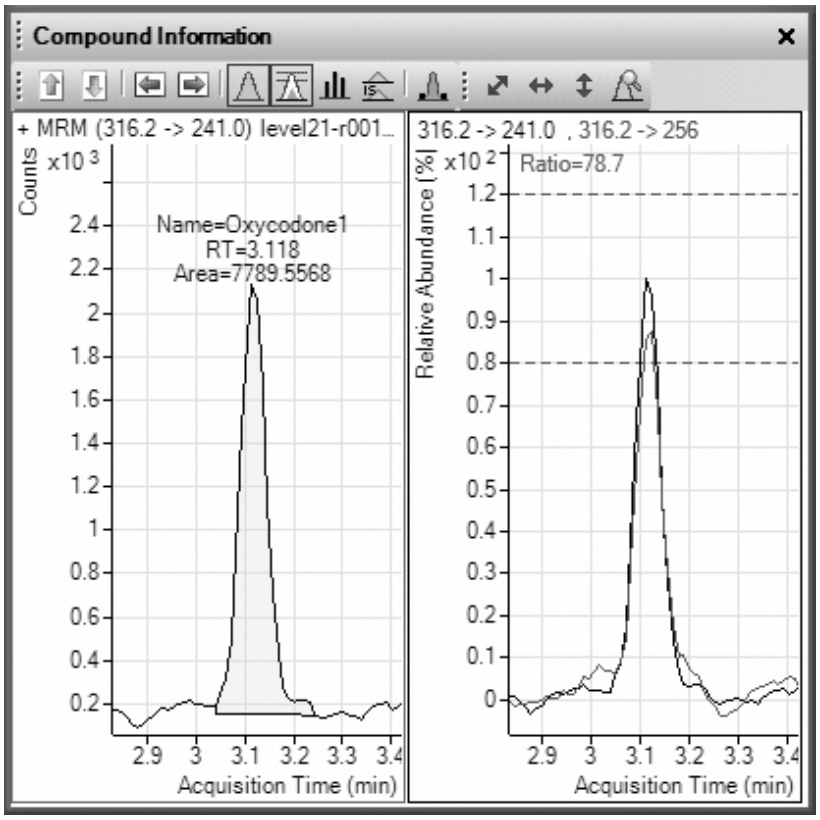
0.74 fg on-column

Figure 4C. Confirmation of hydromorphone at 1 pg/µL (147 fg/µL).



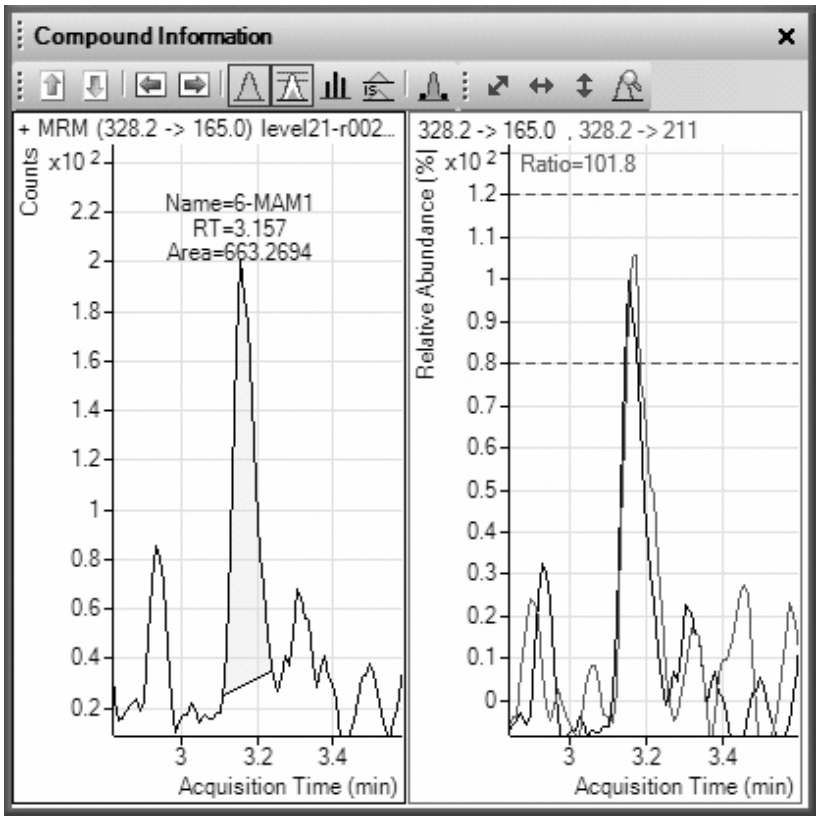
0.74 fg on-column

Figure 4D. Confirmation of codeine at 1 pg/µL (147 fg/µL).



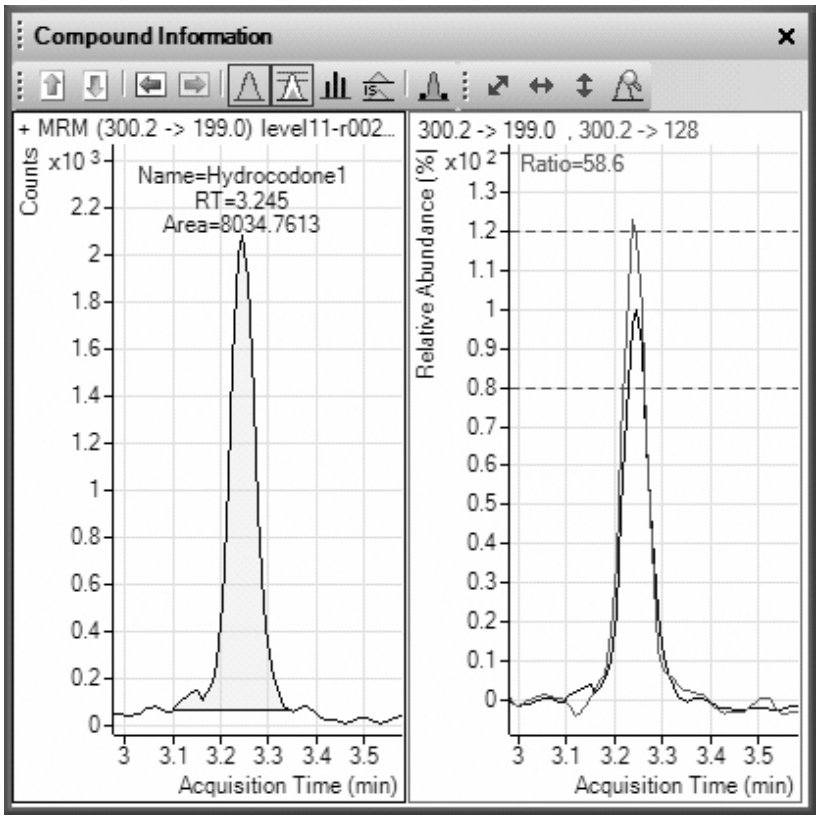
3.7 pg on-column

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).

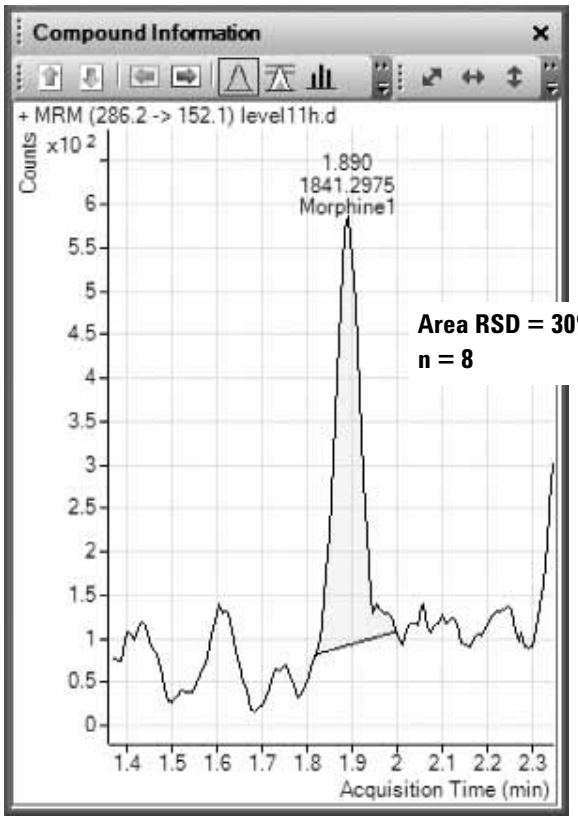


Figure 5A. LOD of morphine at 147 fg on-column.

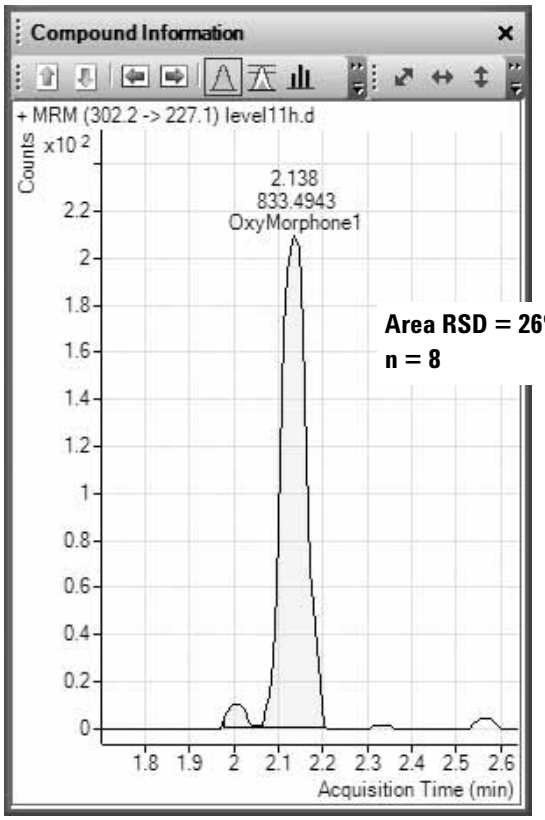


Figure 5B. LOD of oxymorphone at 147 fg on-column.

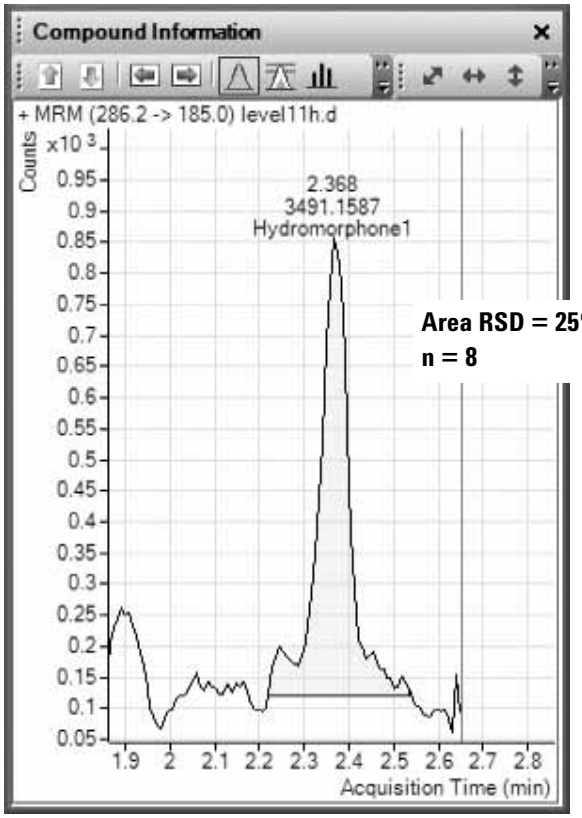


Figure 5C. LOD of hydromorphone at 147 fg on-column.

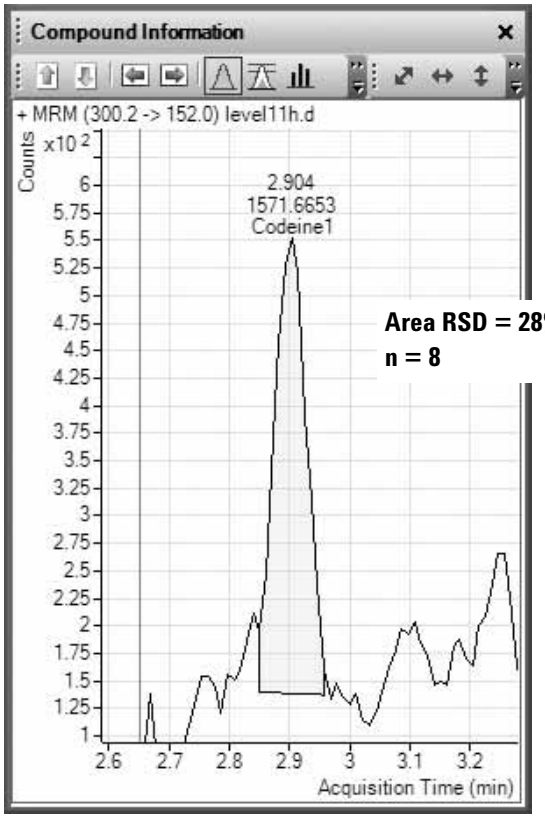


Figure 5D. LOD of codeine at 147 fg on-column.

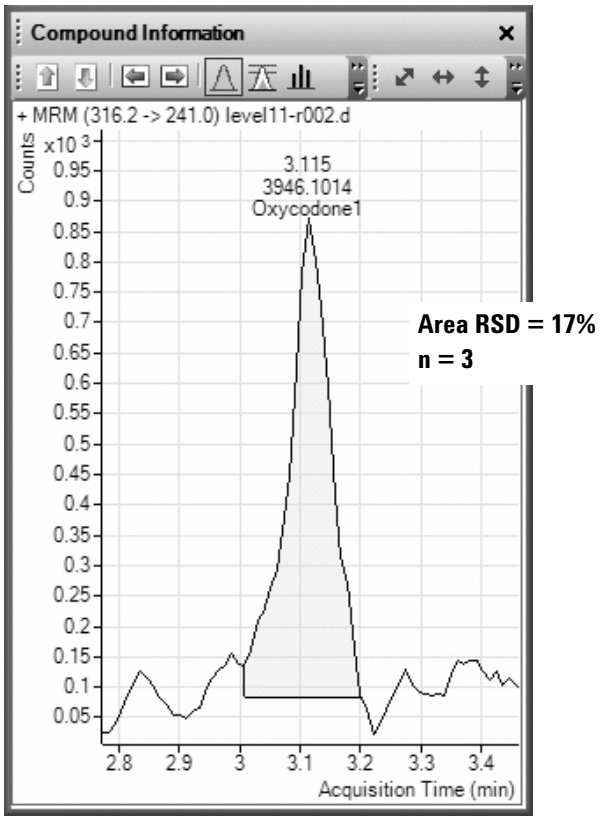


Figure 5E. LOD of oxycodone at 737 fg on-column.

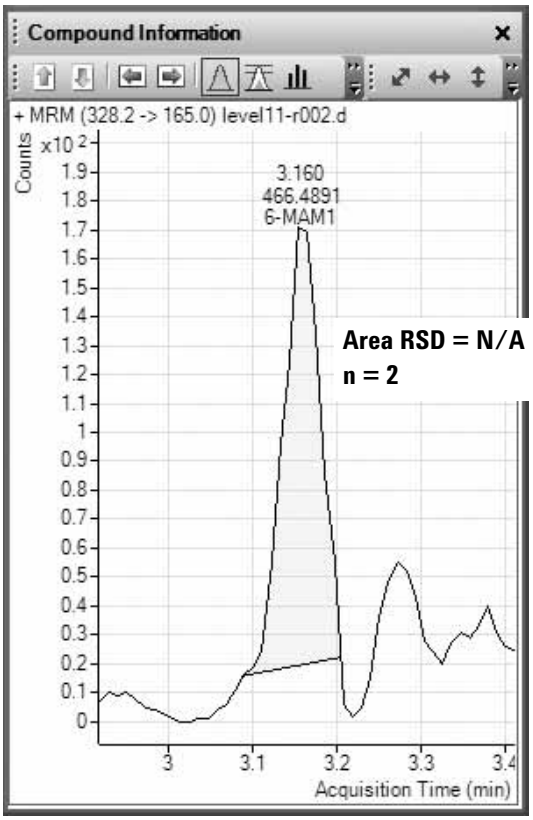


Figure 5F. LOD of 6-MAM at 49 fg on-column. Peak area %RSD not applicable because only two of three injections contained signal.

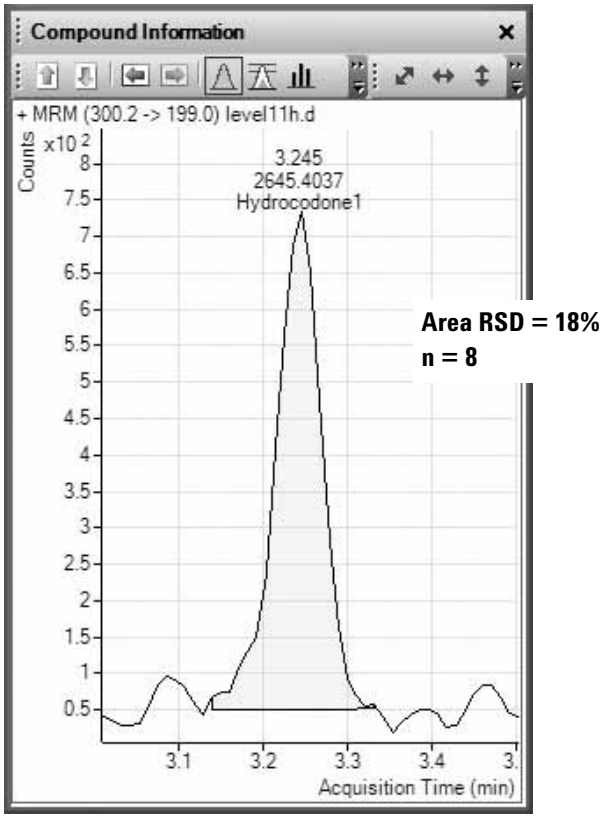


Figure 5G. LOD of hydrocodone at 147 fg on-column.

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

Application

Forensics

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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. Death investigations and drug-impaired driving arrests account for the majority of these cases, with a smaller number of sexual assault cases also encountered. 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 diverted and 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 patients 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-

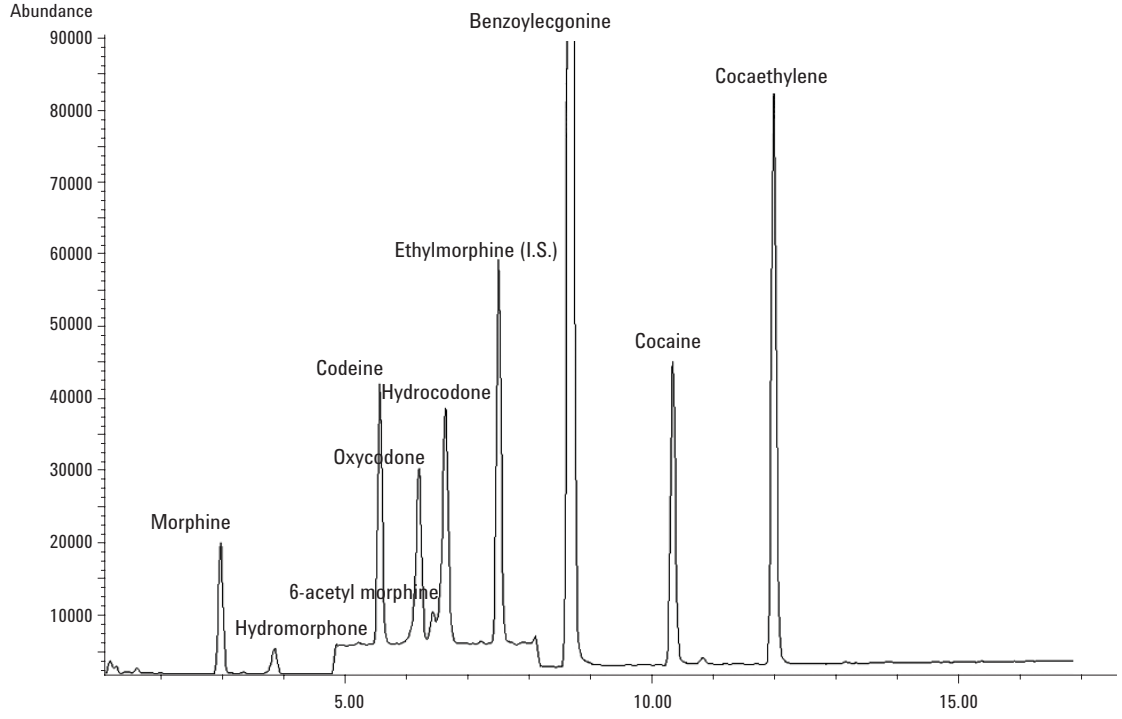


Figure 1. Total ion chromatogram of an extract of a quality control blood sample containing morphine (41 ng/mL), hydromorphone (8 ng/mL), codeine (49 ng/mL), oxycodone (45 ng/mL), 6-acetyl morphine (4 ng/mL), hydrocodone (52 ng/mL), benzoylecgonine (672 ng/mL), cocaine (61 ng/mL), and cocaethylene (64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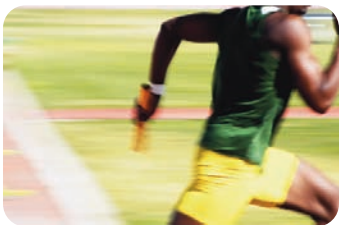
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Applications by Instrumental Technique

LC/TOF/MS



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

Application

Forensics

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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 an avowed problem in some professional sports, a growing problem in high school and collegiate athletics, and an area of doping in which new compounds are continually being introduced (for example, the BALCO/THG controversy 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

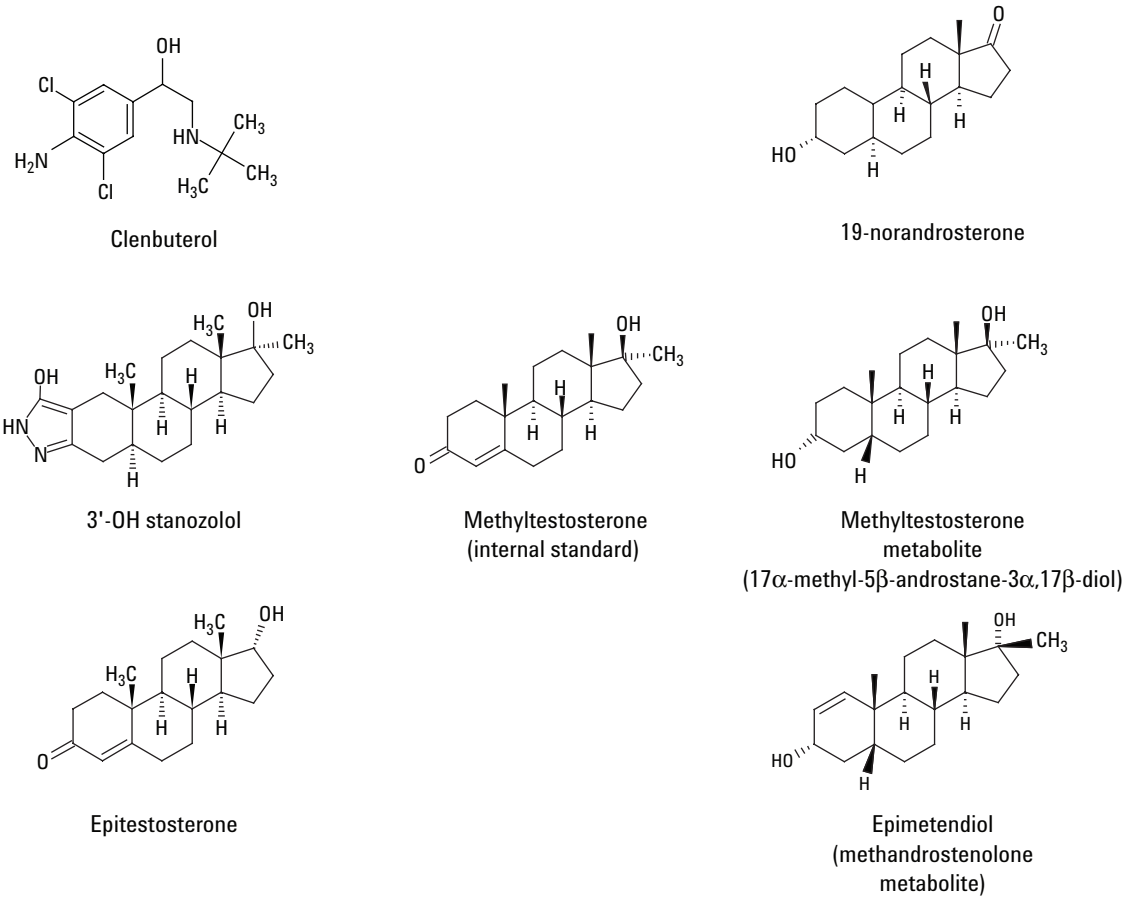


Figure 1. Compounds analyzed.

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.

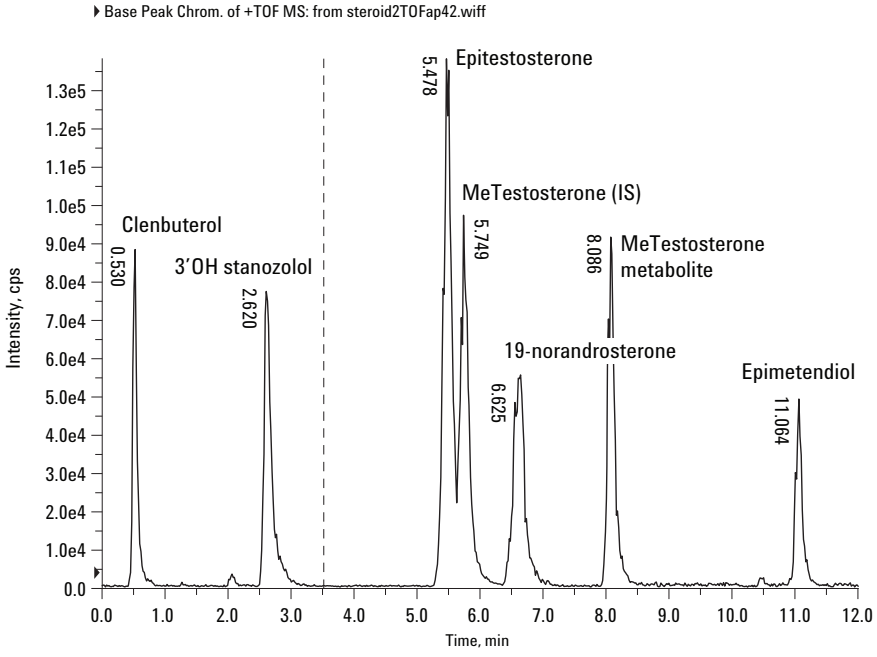


Figure 2. Base peak chromatogram of anabolics mix (unextracted standard equivalent to 16-ng/mL urine).

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.

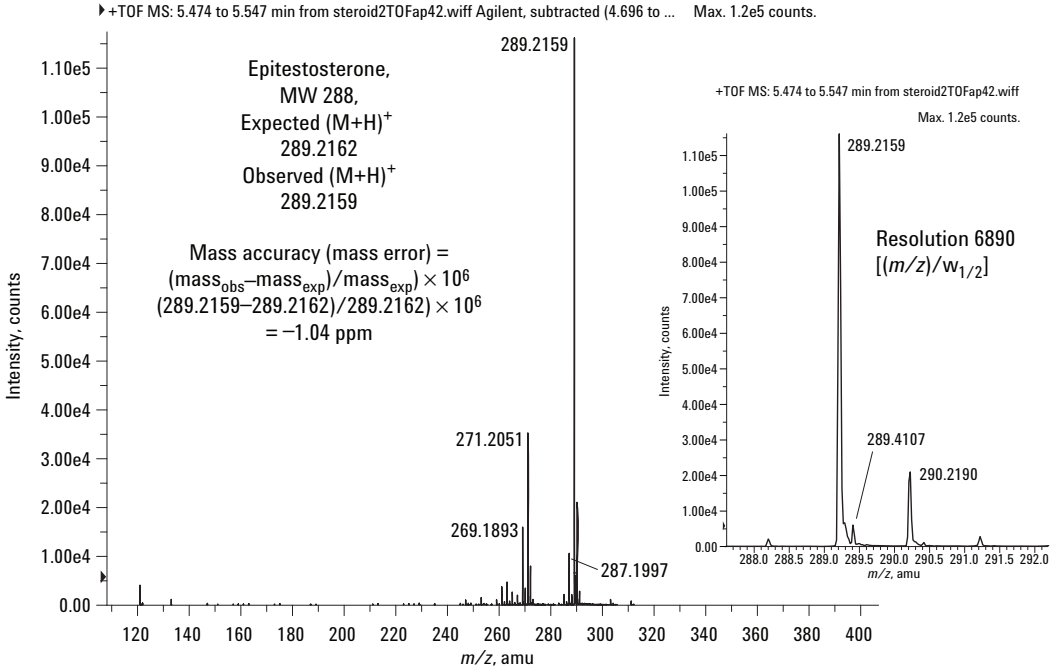


Figure 3. Typical APCI spectrum and TOF performance for epitestosterone, $[M+H]^+$ = 289.2162. Also shown is a water-loss fragment at *m/z* 271.2051.

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.

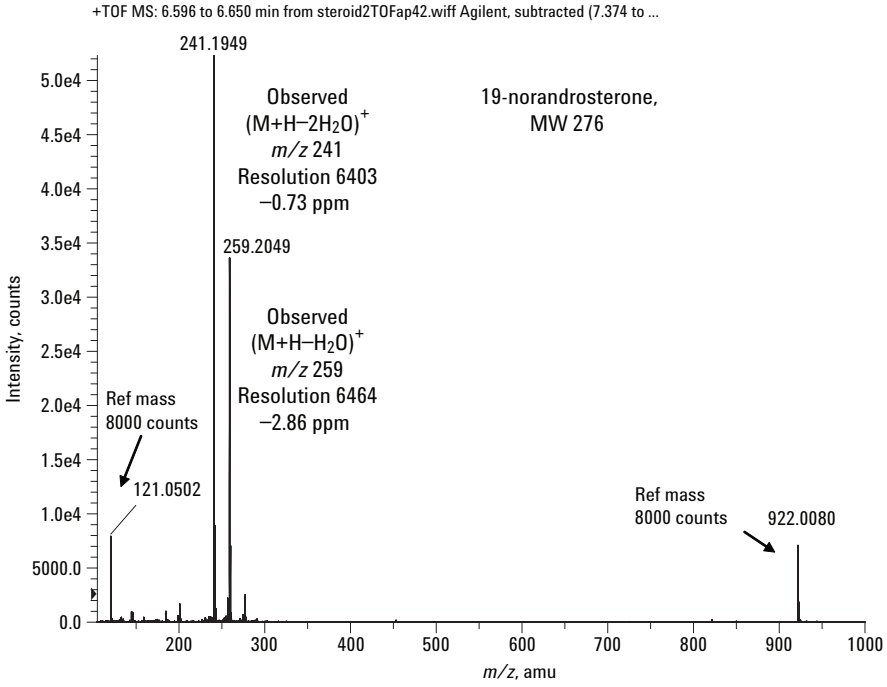


Figure 4. Typical APCI spectrum for 19-norandrosterone, MW 276, showing two water-loss ions. Reference masses at *m/z* 121 and 922 also shown.

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).

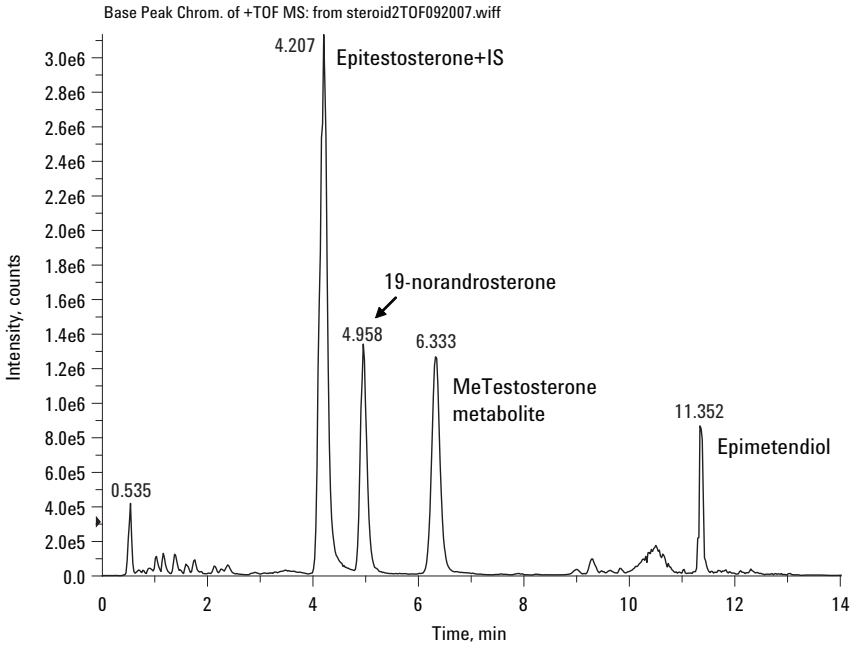


Figure 5. Extract of 50 ng/mL urine. Analytes are visible in base peak chromatogram.

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.

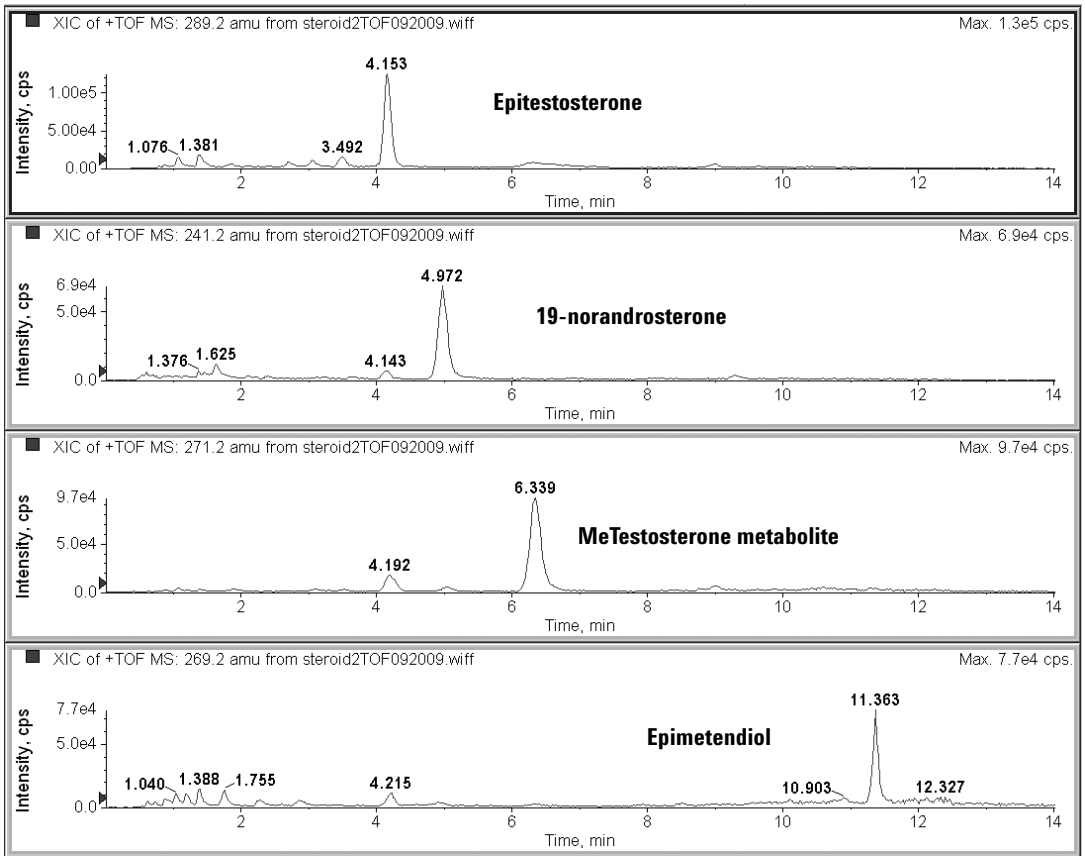


Figure 6. Extract of 2 ng/mL urine (1 ng/mL 19-norandrosterone). Analytes detected using accurate mass EICs (1 mDa width).

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.

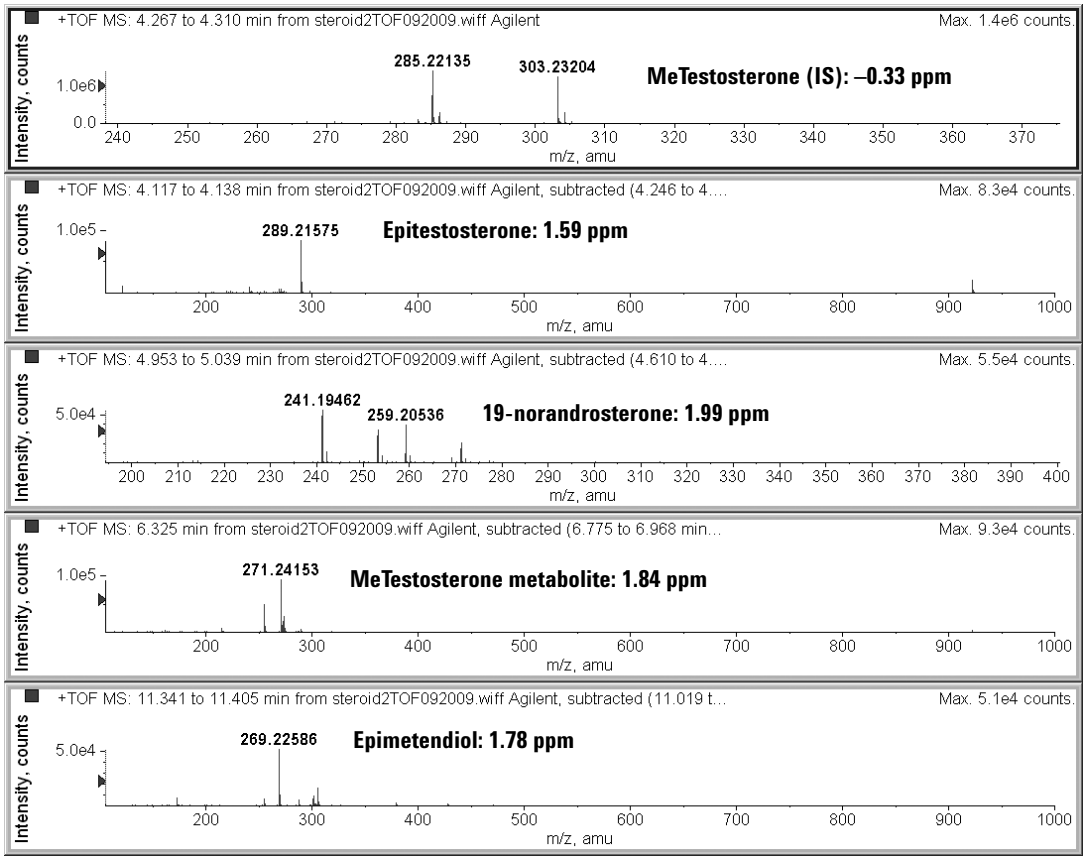


Figure 7. Spectra from 2 ng/mL urine extract (1 ng/mL 19-norandrosterone) (240/120 pg on-column) [see Figure 6].

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.

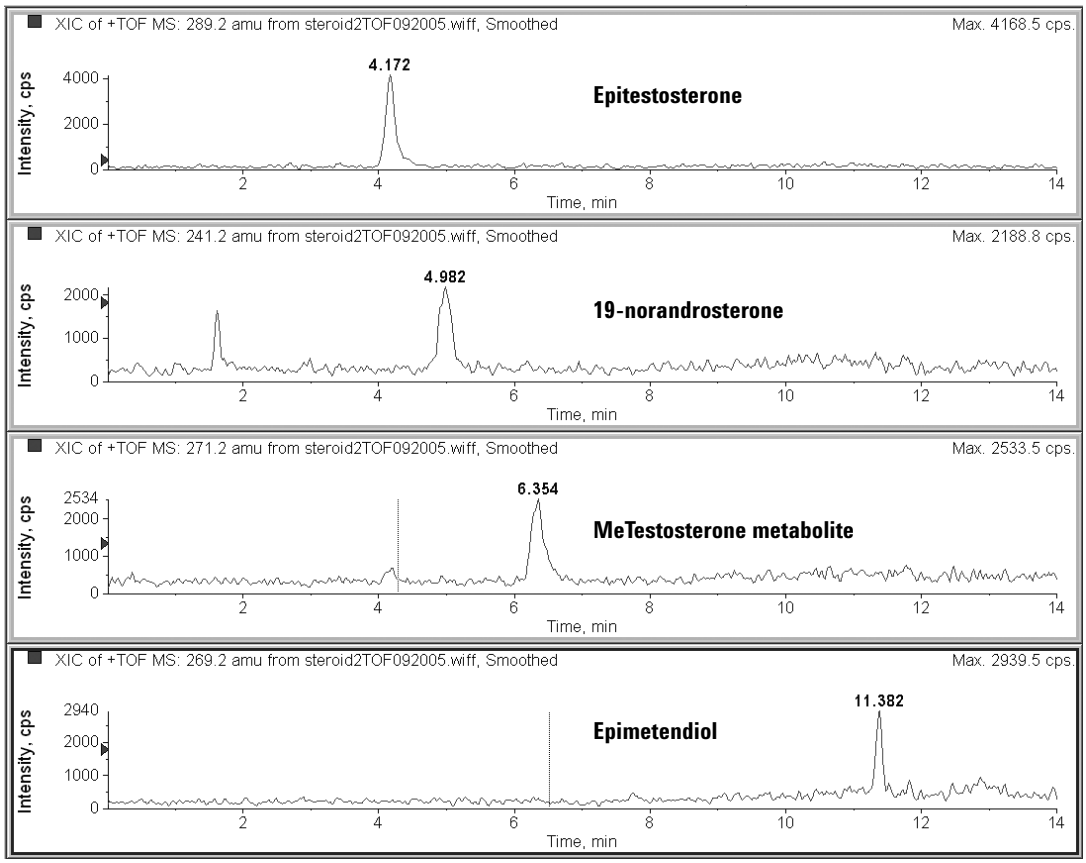


Figure 8. Accurate-mass EICs (1 mDA width) from 0.16-ng/mL unextracted standard (20 pg on-column).

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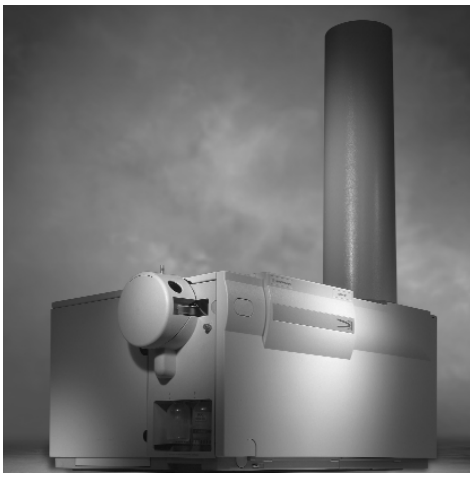
Accurate Mass Measurement for Analyzing Drugs of Abuse by LC/Time-of-Flight Mass Spectrometry

Technical Overview

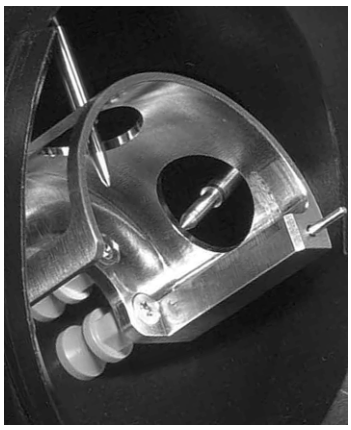
Forensic

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USA



The new Agilent 1100LC/MSD TOF system



Dual-electrospray source

In the world of forensic analysis, the unambiguous identification of chemical substances is crucial. For substance abuse, this is simply a matter of providing scientific evidence indicating innocence or guilt in the possession and/or sale of controlled substances. For toxicology, this can include treatment of poison victims, identification of adulterants in tampered products, and crimes involving substance abuse. In addition, toxicological screens of postmortem samples can provide scientific evidence supporting the commission of a crime. To protect the innocent and to prosecute the guilty, both the identification of unknown compounds and the confirmation of suspect compounds are imperative. 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

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.

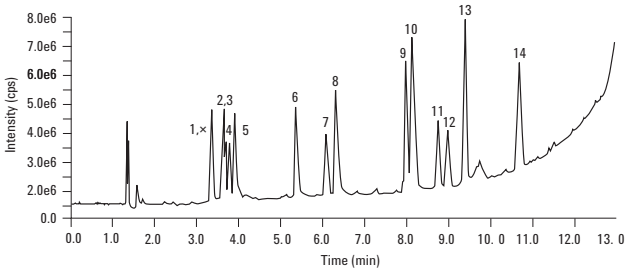


Figure 1. TIC of basic DA. LC/ES-MS TOF separation of 14 basic DA used as targeting compounds in toxicological sample screening.

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.

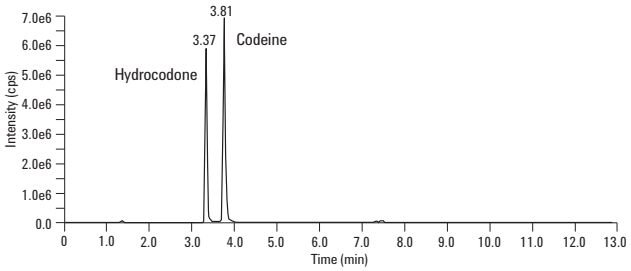


Figure 2. Extracted ion chromatogram. Ion chromatogram of *m/z* 300.0–300.2 extracted from the data shown in Figure 1. Isomers are chromatographically separated, facilitating their identification.

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

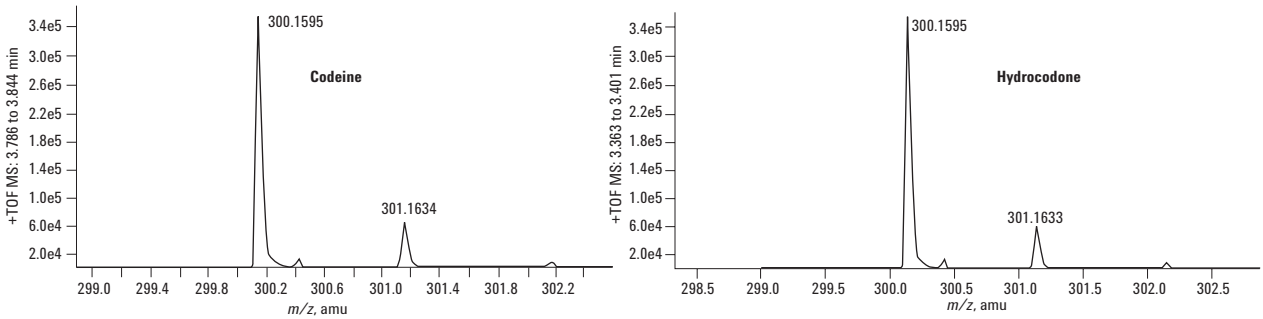


Figure 3. Mass spectra of narcotic isomers. Spectra of codeine and hydrocodone isomers representing an average spectra taken across the chromatographic peak.

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 Nominal *(m/z)*	Amphetamine 136.10	Methanphetamine 150.10	Hydrocodone 300.15	Codeine 300.15	Oxycodone 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.

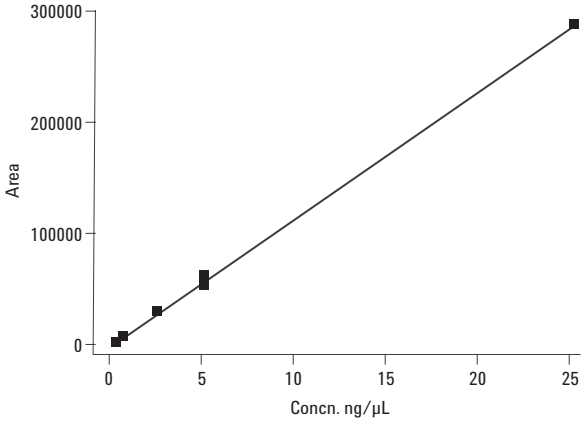


Figure 4. Codeine linearity. Plot of codeine extracted ion (ms/300.0–300.2) chromatographic peaks measured from 50-pg injected on-column to 25-ng on-column. TOF detector saturated at 50 ng.

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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High Resolution Quantitative Analysis of ß$_2$-Agonist in Human Urine by LC/MS Using the Agilent LC/MSD TOF

Application

Forensics

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Abstract

A liquid chromatography/mass spectrometry (LC/MS) method was developed for the sensitive qualitative and quantitative analysis of clenbuterol, mabuterol, bambuterol, and ractopamin to demonstrate the utility of an orthogonal-axis electrospray time-of-flight mass spectrometer (oa-ESI-TOF) for doping analysis. The limit of quantitation for these compounds was 2 ng/mL in human urine, well below control levels. In addition, the ability to measure exact mass, to accuracies better than 3 ppm at all levels and in sample matrix was demonstrated.

Introduction

Beta$_2$-agonists such as clenbuterol, mabuterol, bambuterol, and other sympathomimetic agents are commonly used for the treatment of pulmonary diseases such as bronchial asthma. In 1992, sympathomimetics were added to the list of prohibited substances by the World Anti-Doping Agency (IOC). They are classified as stimulants and anabolic agents with specific regulations in terms of competition and out-of-competition testing.

A LC/MS method was developed for the sensitive, qualitative and quantitative analysis of clenbuterol, mabuterol, bambuterol, and ractopamine to demonstrate the utility of an oa-ESI-TOF mass spectrometer for doping analysis.

Experimental

All LC/MS experiments were performed using an Agilent LC/MSD TOF mass spectrometer coupled to an Agilent 1100 Series LC system. The TOF was operated with an orthogonal electrospray source in positive ion mode. A gradient method was used for chromatography. Clenpenterol was used as an internal standard (ISTD) for all analytes. Method conditions are given in Table 1.

Table 1. Experimental Conditions

HPLC	**Agilent 1100**
Column	ZORBAX XDB-C18, 50 mm × 2.1 mm × 3.5 µm
Solvents	A: Water + 5 mM of NH_4OAc + 0.1% of acetic acid B: Acetonitrile + 0.1%-acetic acid
Gradient	5% A, 0–7 min; 80% A, 7–9 min
Flow rate:	0.3 mL/min
Injection:	20 µL out of 1000 µL
Agilent LC/MSD TOF system	
Ionization mode:	Positive ESI
Nebulizer pressure:	45 psi
Drying gas flow:	11 L/min
Drying gas temperature:	350 °C
Full scan mode	100 amu–1000 amu
Automatic reference	Ion 122 and Ion 922
Quantitation	
Bambuterol	EIC of 368.20750 - 368.22959, Ret-Time: 6.3 min
Clenbuterol	EIC of 277.07858 - 277.09520, Ret-Time: 5.8 min
Mabuterol	EIC of 311.10392 - 311.12258, Ret-Time: 6.45 min
Ractopamine	EIC of 302.16600 - 302.18413, Ret-Time: 5.35 min
Clenpenterol (ISTD)	EIC of 291.09381 - 291.11128 + EIC of 293.09080 - 293.10839, Ret-Time: 6.4 min
Calibration curves:	All weighted 1/x

Sample Preparation

Samples were prepared according to the protocol established by the Institute of Biochemistry, German Sport University [1]. Using this protocol, to a volume of 5 mL of urine, 300 µL of 10 M hydrochloric acid and 250 ng of clenpenterol were added; and then the sample was incubated at 80 °C for 45 min. After cooling to ambient temperature, 5 mL of t-butyl methyl ether was added, the mixture was shaken for 15 min, centrifuged at 620*g* for 5 min, and the organic layer was discarded. To the remaining aqueous phase, 0.65 mL of 5M aqueous KOH, 1 mL of t-butanol, 500 mg of a mixture of K_2CO_3 and $NaHCO_3$ (2:1, w/w), 2 g of NaCl, and 5 mL of t-butyl methyl ether were added. The mixture was shaken for 15 min and centrifuged at 620*g* for 10 min. The organic layer was transferred to a fresh tube and evaporated to dryness at 50 °C, under vacuum. The dry residue was dissolved in 1 mL of 0.06 M HCl, transferred to HPLC vials, and 20 µL injected into the LC/MS system.

Chemicals

Hydrochloric acid 32% (for example, Merck, 100319)

t-Butanol

Potassium hydroxide, 85%

Potassium carbonate

Sodium hydrogencarbonate, anhydrous

Sodium chloride, (Merck)

t-Butyl methyl ether, distilled before use, (KMF, St. Augustin, Germany)

Sodium acetate, anhydrous, (Sigma)

Results and Discussion

Several ß$_2$-agonists need to be identified in urine samples for doping control in sports. The structures of the compounds used to demonstrate this methodology are given in Figure 1. A liquid-liquid extraction was used to extract the ß$_2$-agonists from the urine matrix. The TOF mass spectrometer was used for identification with accurate mass measurement and to quantify the analytes under control. This instrument operates only in full-scan mode, allowing detection of any ionizable compound in the mass range. No special method setup was necessary for the mass spectrometer. This allows for the addition of more analytes without changing the acquisition method.

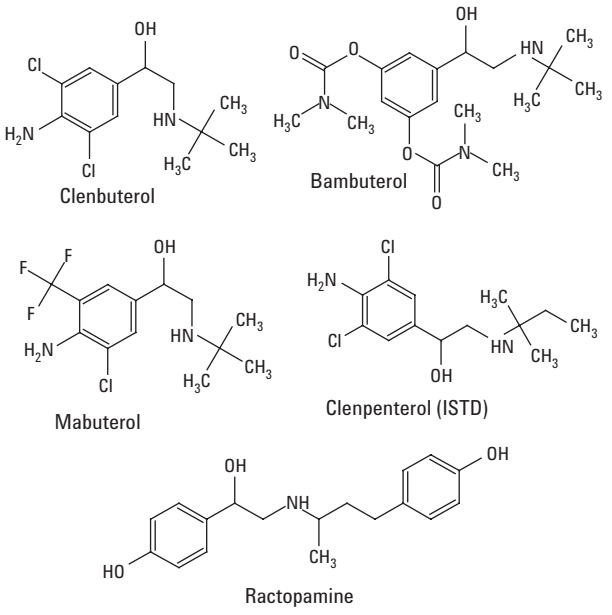


Figure 1. Chemical structures of the investigated ß^2-agonists.

Quantitation was performed by selecting the quasi-molecular ion to create extracted ion chromatograms (EICs) for each analyte and ISTD. Figure 2 displays example EICs from a 2-ng/mL spiked urine sample. Figure 3 shows calibration curves based on spiked urine samples as well as the statistics in Table 2. The IOC list of prohibited substances in sports gives the limit of detection (LOD) for some of the ß$_2$-agonists. Clenbuterol and salbutamol are referred to as anabolic agents. For the latter, a threshold of 1 µg/mL is established. The capability shown here is well below those levels.

Identification was performed by using an automated empirical formula search routine. Added to the runtime work list, this allows for an automated empirical formula calculation, post-acquisition, reported in html, and a comma-separated value spreadsheet for further manipulation. Figure 4 displays a plot showing the calculated mass accuracy for standards as well as spiked urine samples. It shows that the system is able to routinely measure mass accuracy to better than 3 ppm in both standards and sample matrix.

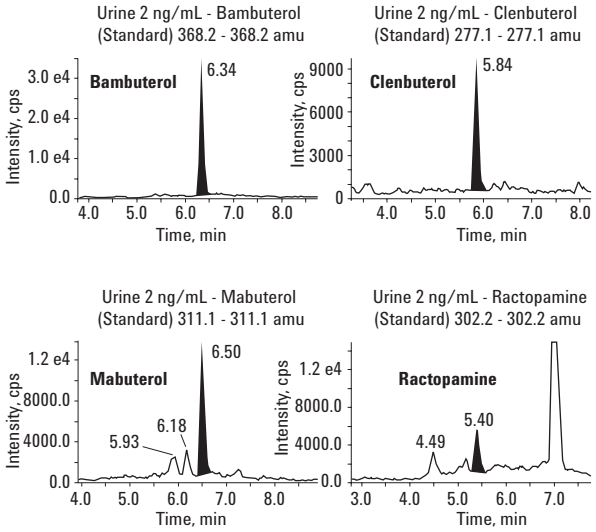


Figure 2. Representative EICs of a spiked urine sample at the 2 ng/mL level.

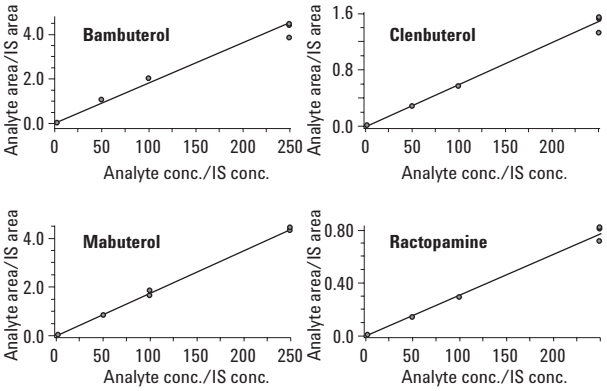


Figure 3. Calibration curves based on spiked urine samples (2 ng/mL-250 ng/mL).

Table 2. Calibration Curve Statistics

Compound	Expected conc. ng/mL	No. of values	Low ng/mL	High ng/mL	Mean ng/mL	Standard Deviation	%CV	%Accuracy
Bambuterol	2	5	1.59	1.67	1.65	0.035	2.104	82.40
	50	5	58.11	58.56	58.36	0.185	0.318	116.72
	100	5	110.50	111.93	111.27	0.640	0.575	111.27
	250	5	211.46	247.25	232.40	17.934	7.717	92.96
Mabuterol	2	5	2.02	2.09	2.06	0.026	1.243	102.90
	50	5	48.72	49.11	48.92	0.188	0.385	97.85
	100	5	95.62	108.17	98.39	4.807	4.886	98.39
	250	5	250.48	256.32	253.69	2.674	1.054	101.47
Clenbuterol	2	5	2.05	2.17	2.09	0.051	2.455	104.50
	50	5	47.65	48.77	48.24	0.422	0.876	96.49
	100	5	96.27	98.05	97.25	0.715	0.736	97.25
	250	5	226.79	263.17	254.41	15.545	6.110	101.77
Ractopamine	2	5	2.14	2.24	2.21	0.040	1.832	110.51
	50	5	44.27	45.78	45.09	0.705	1.563	90.18
	100	5	95.13	96.59	95.71	0.632	0.661	95.71
	250	5	232.60	267.43	258.99	14.825	5.724	103.60

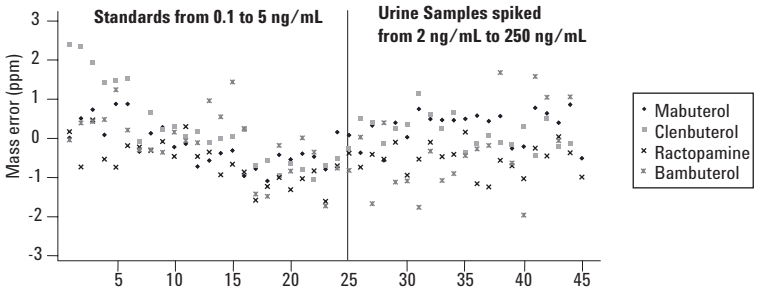


Figure 4. Mass Accuracy on an 18-hour sequence for the investigated ß$_2$-agonists.

Conclusion

A LC/MS method was developed for the qualitative and quantitative measurement of ß$_2$-agonists in human urine using the Agilent LC/MSD TOF system. The LOQ for bambuterol, clenbuterol, mabuterol and ractopamine was 2 ng/mL in human urine, well below control levels. In addition, the ability to measure exact mass, to accuracies better than 3 ppm at all levels and in sample matrix was demonstrated

Reference

1. Mario Thevis, (2003) "Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometric Screening and Confirmation Methods for ß$_2$-Agonists in Human or Equine Urine" *J. Mass Spectrom.*, **38**, 1197–1206.

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The Benefits of Achieving High Mass Accuracy at High Speed Using Agilent's TOF-MS Technology

Application Note

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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.

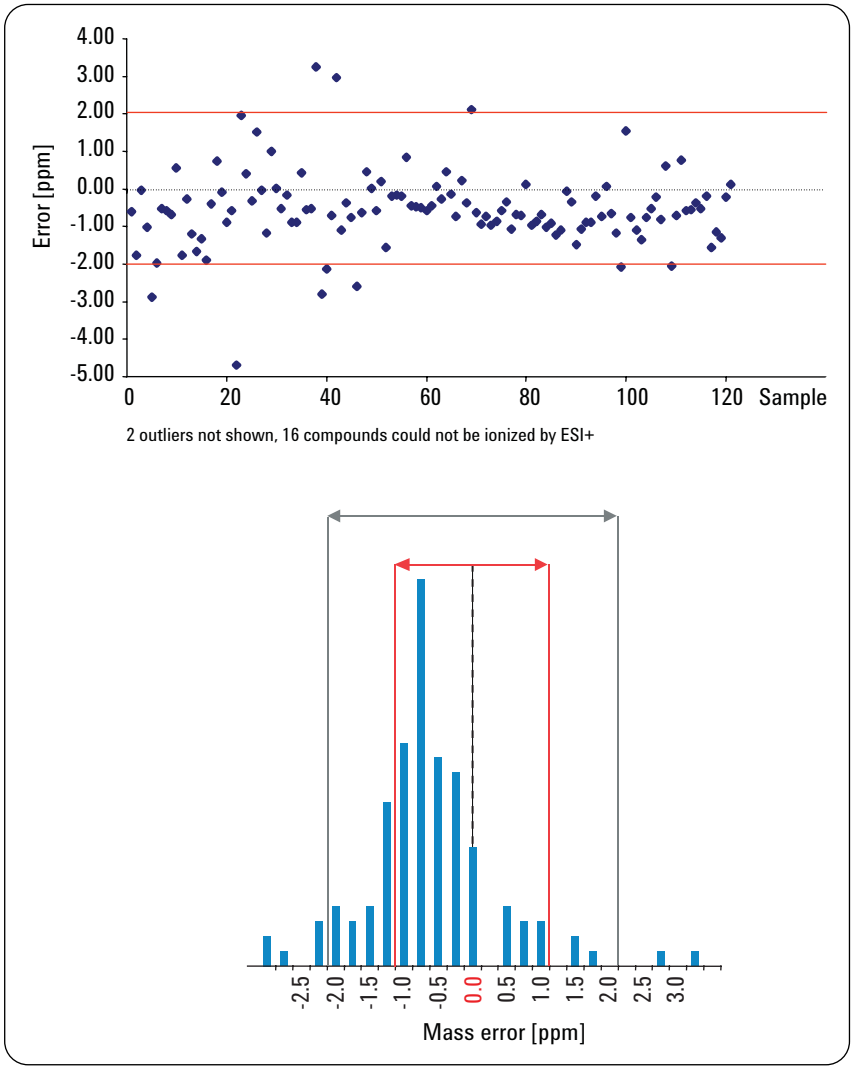


Figure 1
A) Mass accuracy errors as returned by an automatically generated report.
B) Histogram of the mass accuracy errors of the analysis of 140 real chemical library samples of a pharmaceutical company.

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).

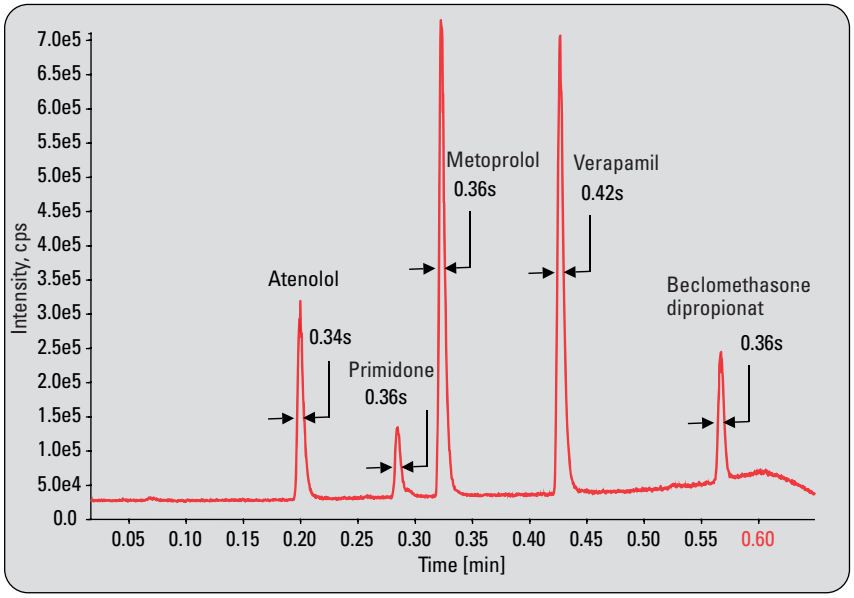


Figure 2
TIC chromatogram (40-Hz data rate of the 6210 TOF mass spectrometer, 80-Hz data rate of the DAD) with PWHH values for the TIC.

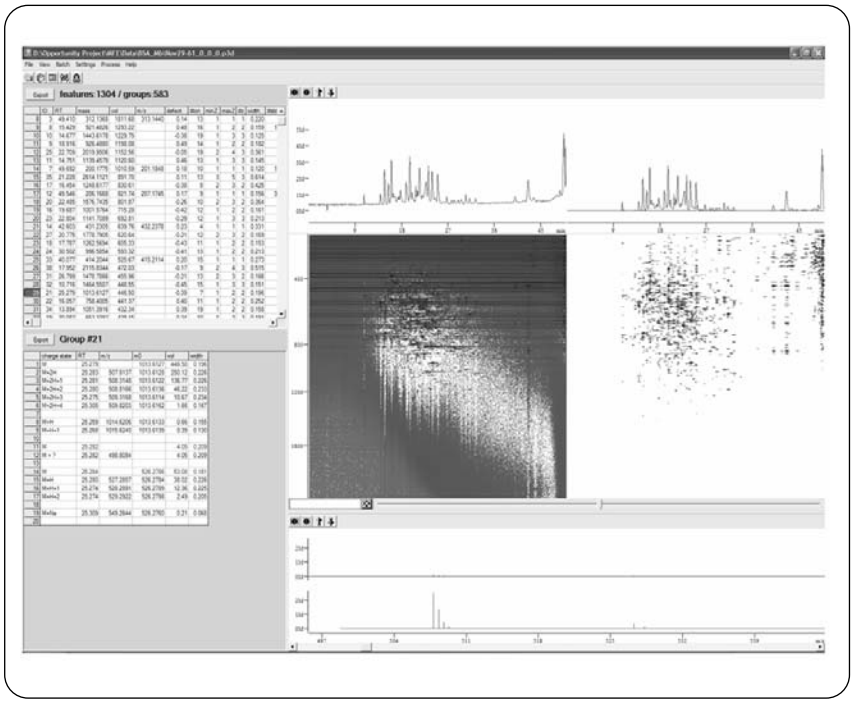


Figure 3
MassHunter software for analysis of complex samples.

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 clinical samples, metabolite stability and metabolite identification, identification of minor impurities in drugs and natural product analysis.

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

Application

Forensics

Authors

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Abstract

The screening for drugs of abuse in human samples has relevance in much of today's society; employers, police and prison officials, and forensic pathologists all rely 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

History has shown that the human race has a long-standing fascination with the consumption and experimentation of mind and body altering substances. Traditionally, all of these substances occurred naturally. Even today, many drugs of choice for this use are derived directly from natural substances. One of the most common of these, *cannabis*, is reported to have been tried on at least one occasion by up to 32% of the American population, and amongst the younger generation, 56% of high school senior students. These large numbers and the associated law enforcement issues have resulted in the decriminalization of its private use in many states and territories, but this does not diminish the potential impact on workplace performance or motor skills impairment, which may result in serious accidents. 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 banishment from society. During this time, a plethora of 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, which provided substantial improvement for the comfort of many patients, 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
- Therapeutic monitoring
- 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 and therapeutic monitoring 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 screening 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, which may have a significant impact on the life of a person, 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.

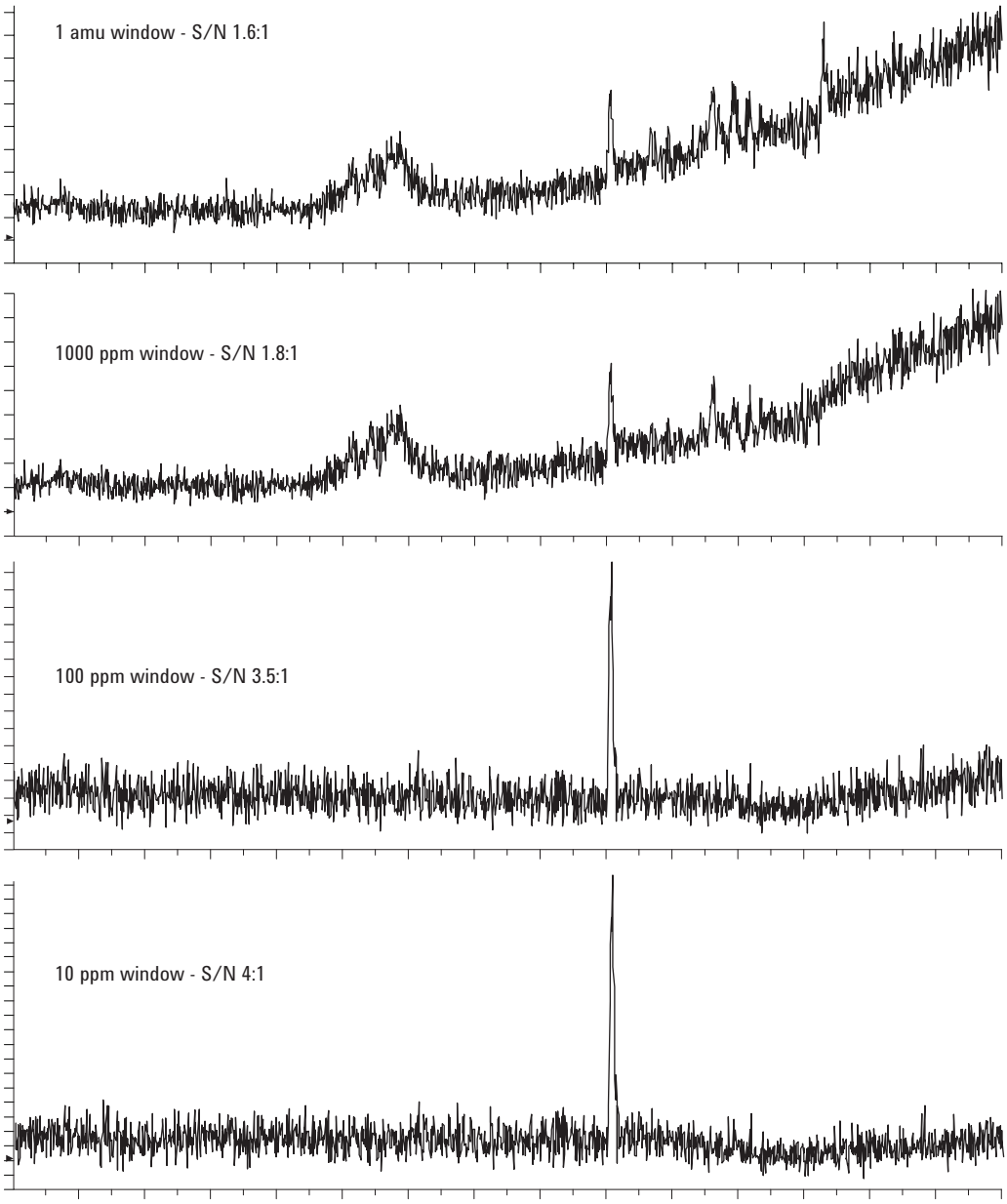


Figure 1. Effect of extracted ion range on noise – 1 ng/mL flunitrazepam.

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.

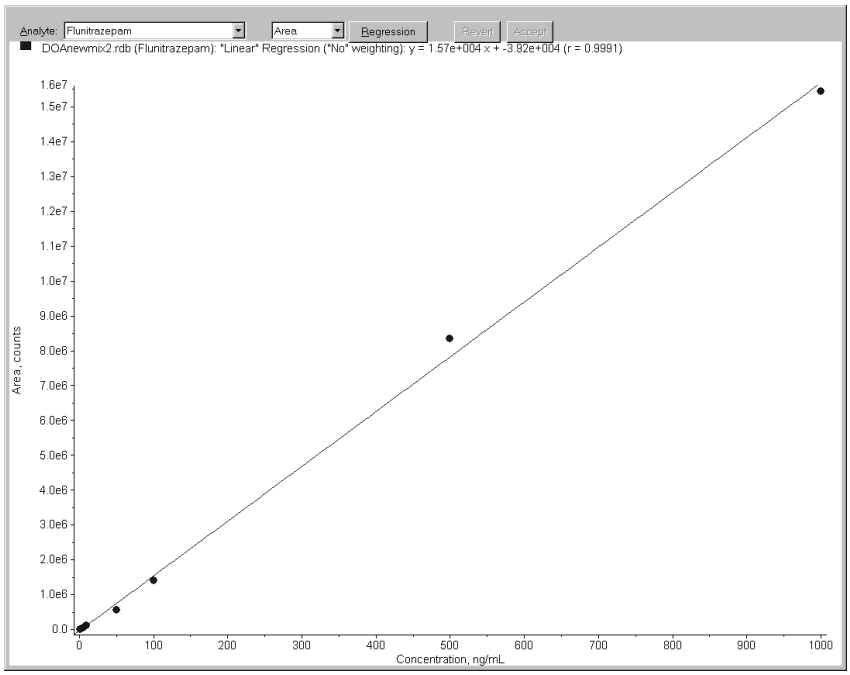


Figure 2. Calibration curve for flunitrazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

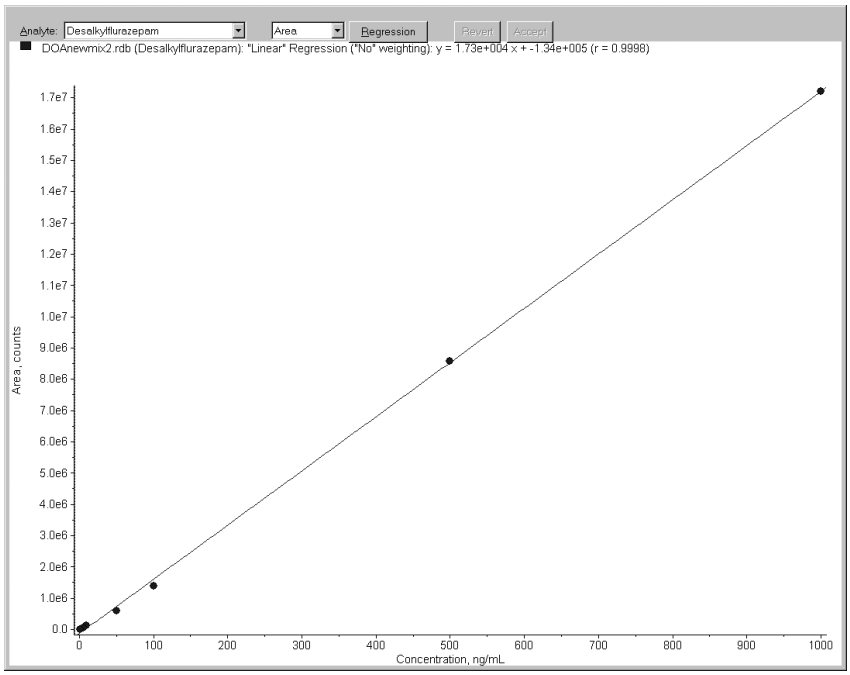


Figure 3. Calibration curve for desalkylflurazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

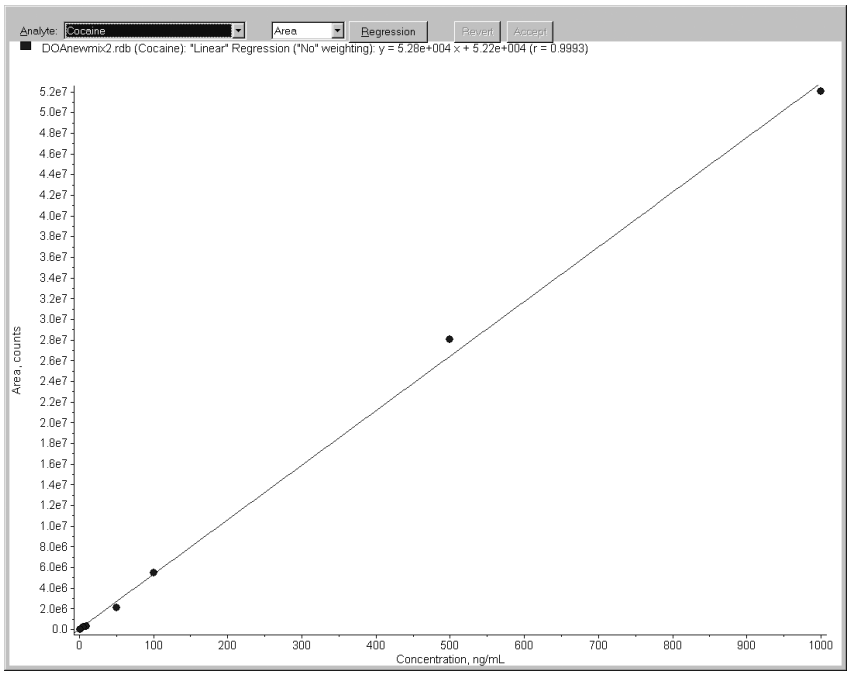


Figure 4. Calibration curve for cocaine from 1 ng/mL to 1000 ng/mL with TOF-MS.

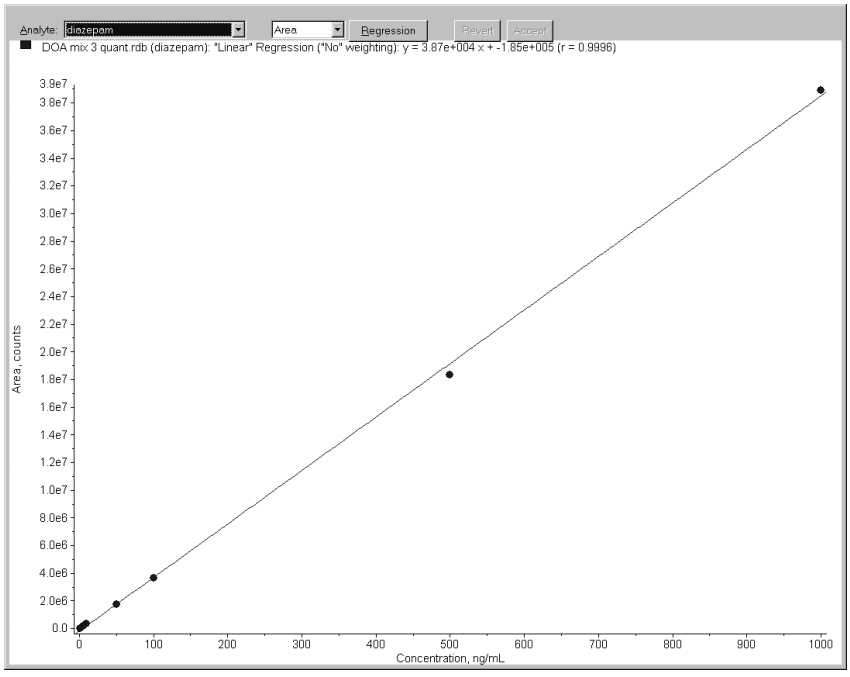


Figure 5. Calibration curve for diazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

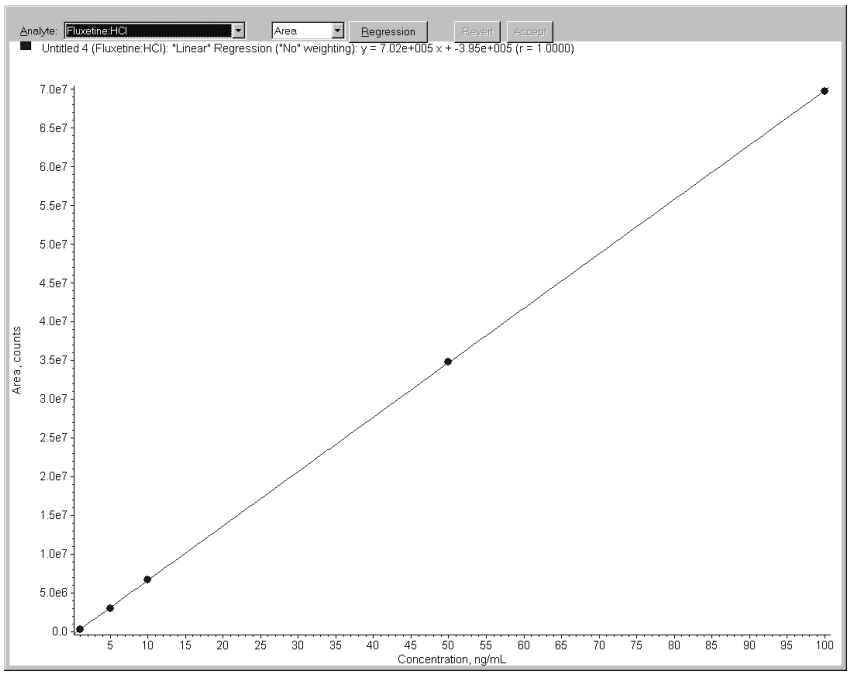


Figure 6. Calibration curve for (–)-δ-9-THC from 1 ng/mL to 100 ng/mL with TOF-MS.

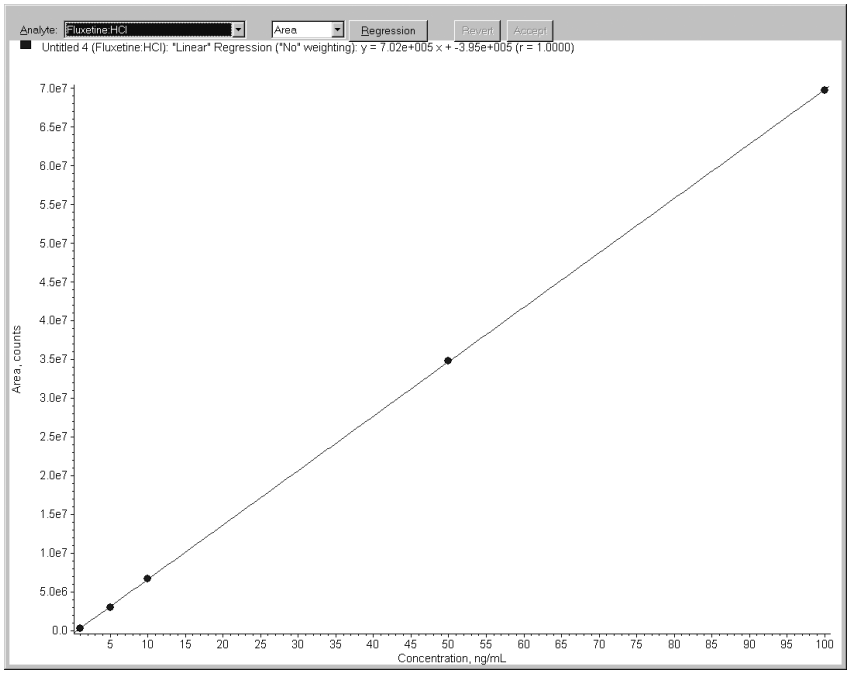
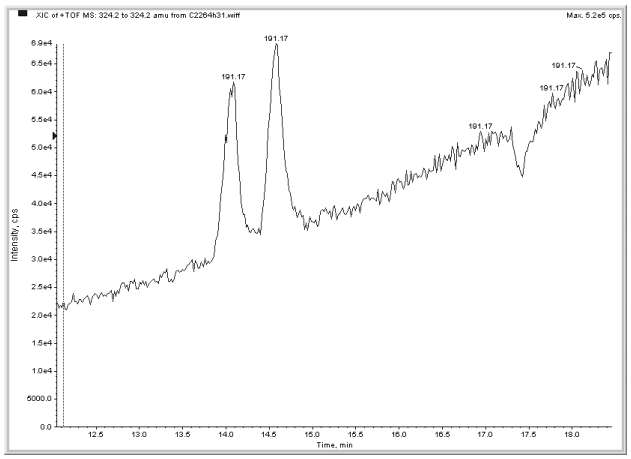
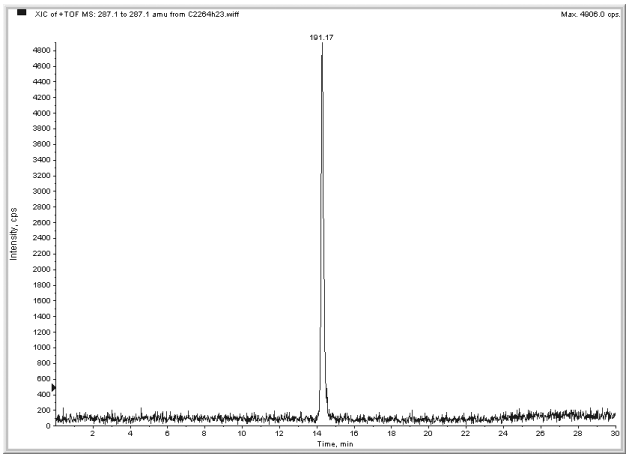


Figure 7. Calibration curve for fluoxetine from 1 ng/mL to 100 ng/mL with TOF-MS.

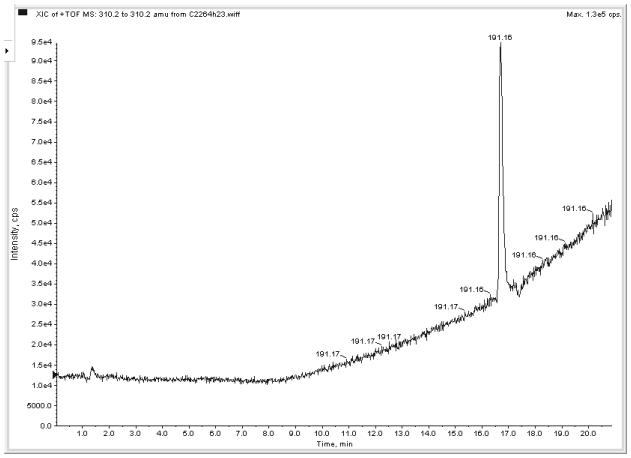
Chromatogram examples for four components at 1 ng/mL are shown below in Figure 8 with 10 ppm extraction windows.



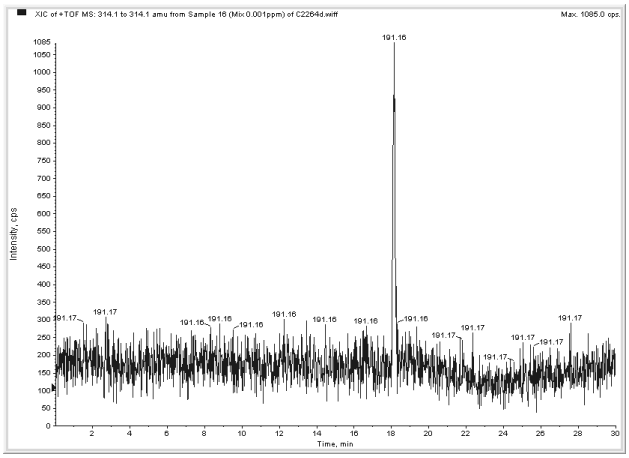
1 ng/mL iso-LSD and LSD (respectively)



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%.

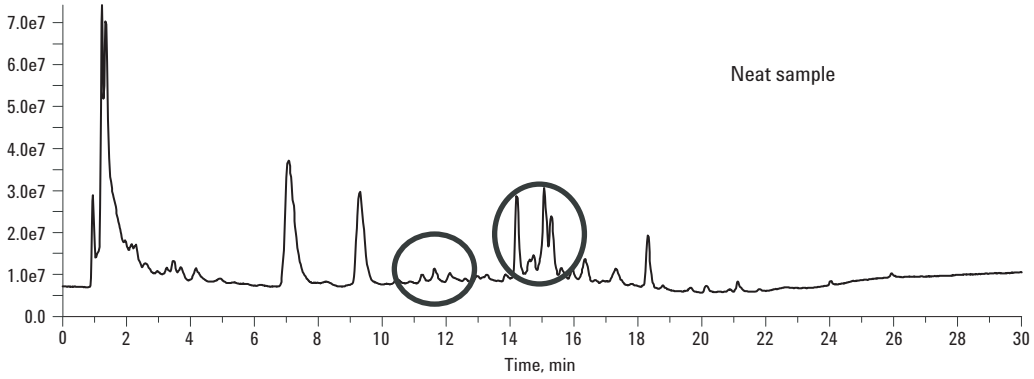


Figure 9. TIC of Blank Urine – Injected neat – circle shows expected retention time of analytes of interest.

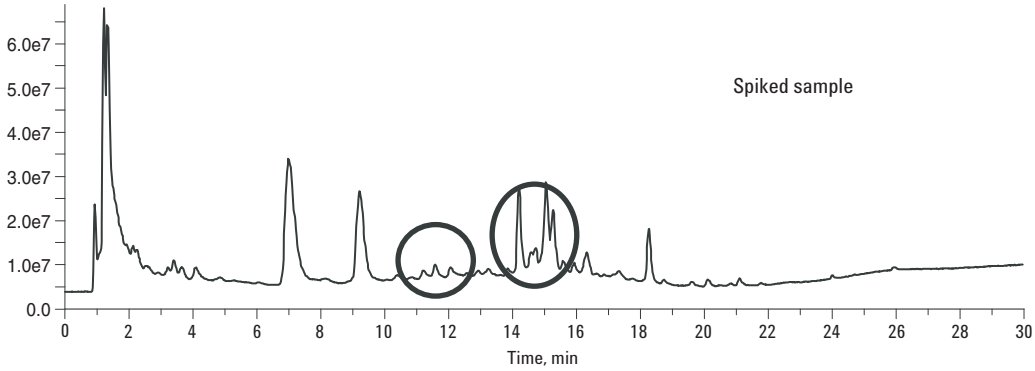


Figure 10. TIC of Neat Urine with 100 ng/mL spike of cocaine, benzoylecognine, flunitrazepam, and 7-aminoflunitrazepam.

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.

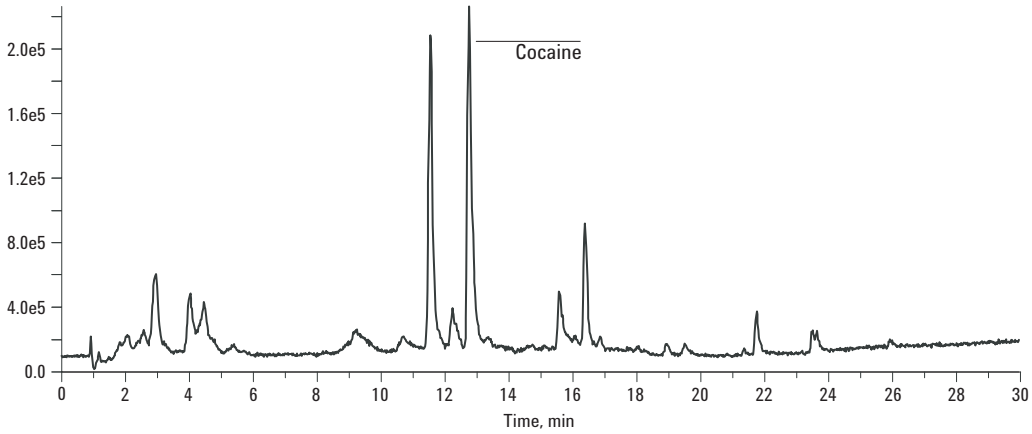


Figure 11. One amu extraction window of scanned target mass 304.1543 – cocaine.

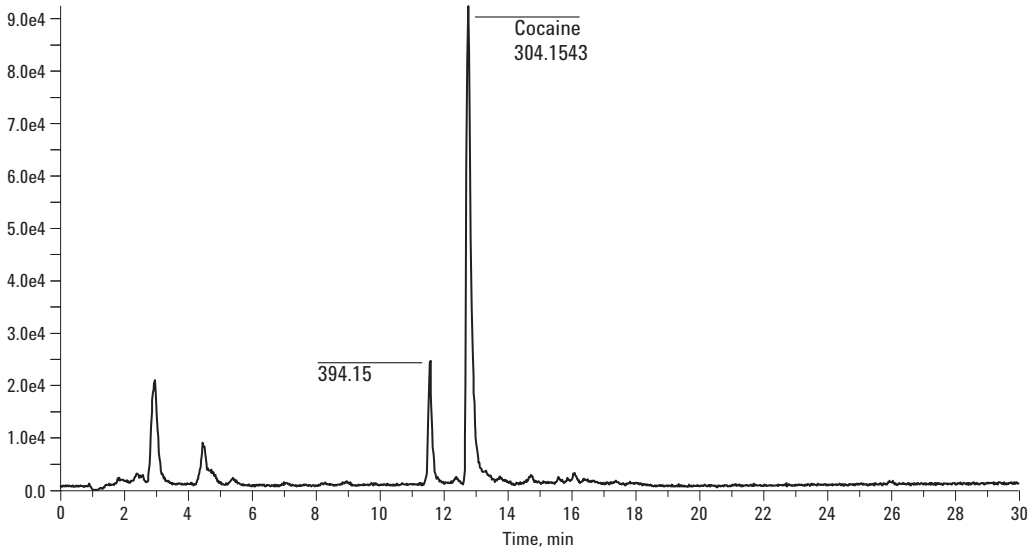


Figure 12. Ten ppm extraction window of scanned target mass 304.1543 – cocaine.

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).

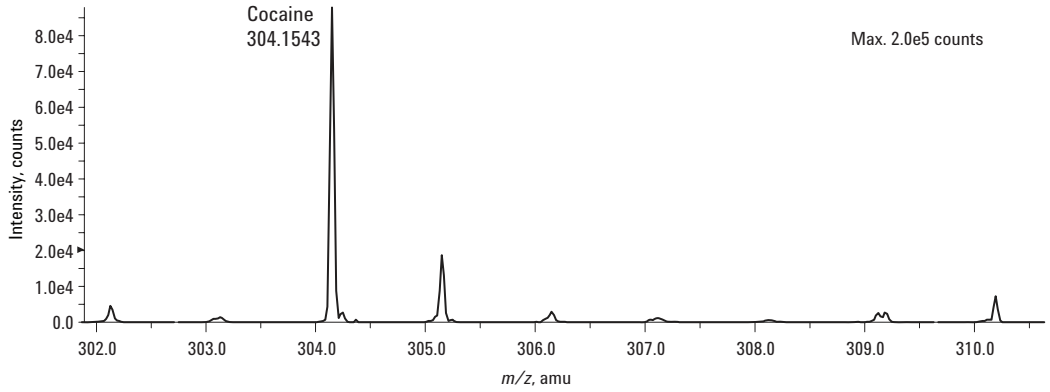


Figure 13. Confirmation of mass of cocaine.

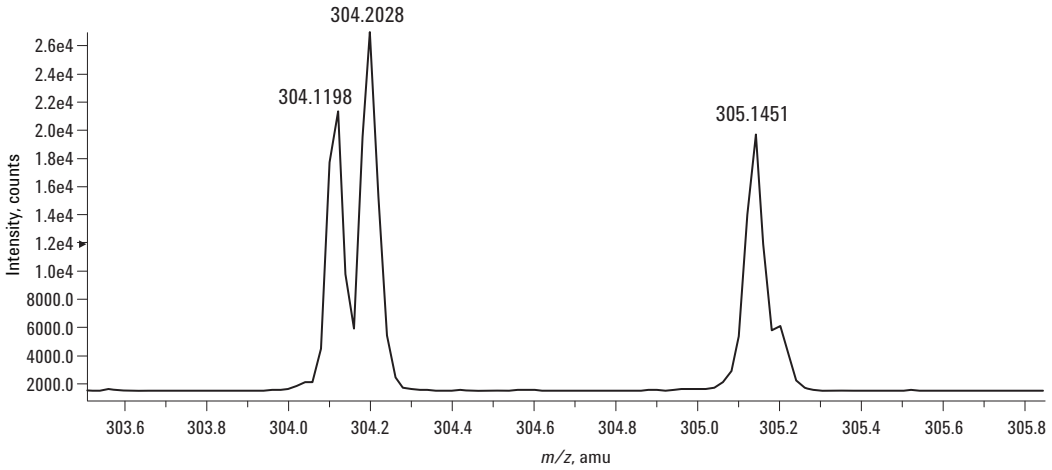


Figure 14. Mass spectrum 304 – 305 amu for peak at 11.9 minutes – no match of mass for cocaine.

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).

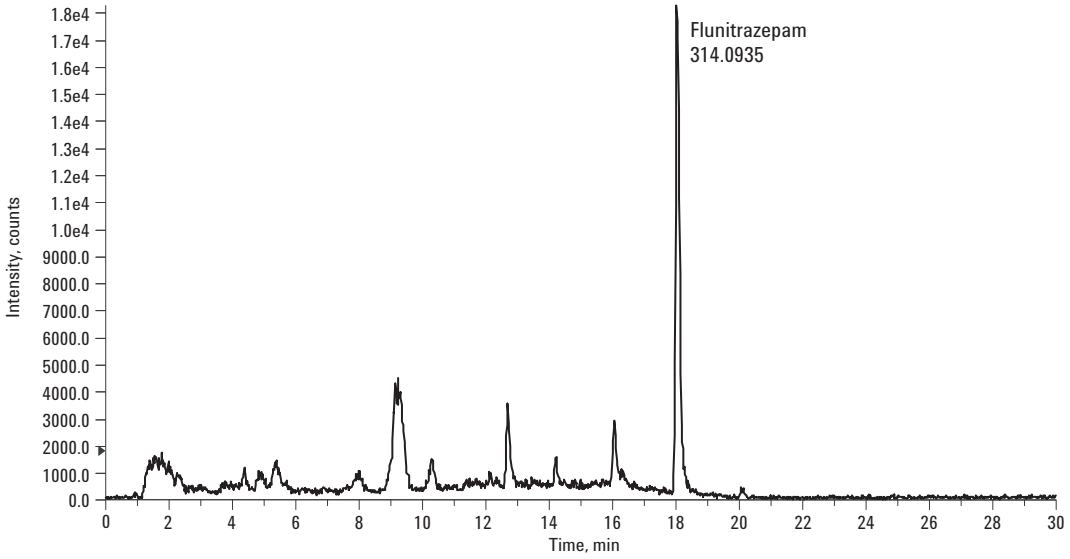


Figure 15. Ten ppm extraction window of scanned target mass 314.0935, flunitrazepam in neat urine. Retention time helps confirm presence at 18.1 minutes.

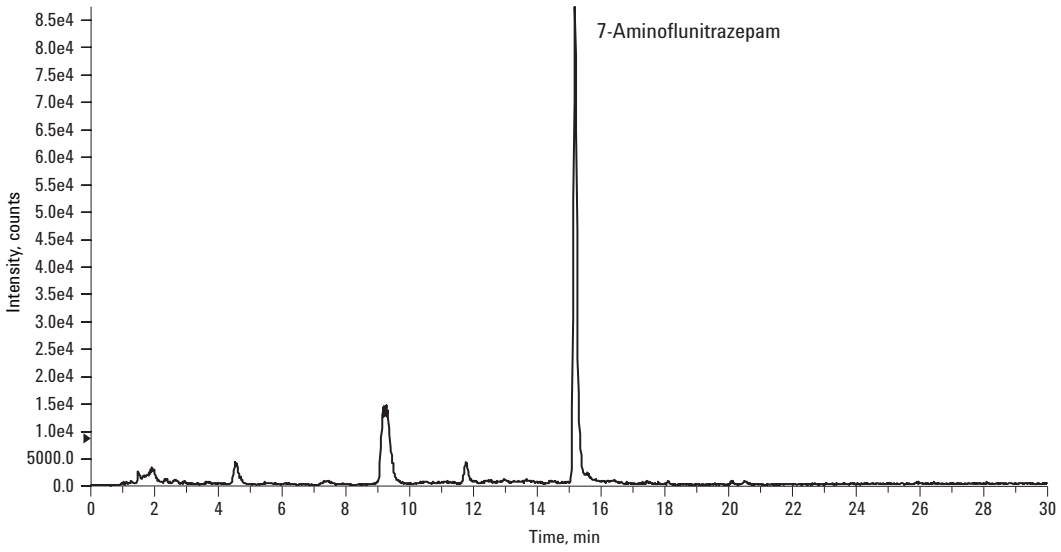


Figure 16. Ten ppm extraction window of scanned target mass 284.1191, 7-aminoflunitrazepam in neat urine. Retention time helps confirm presence at 15.3 minutes.

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.

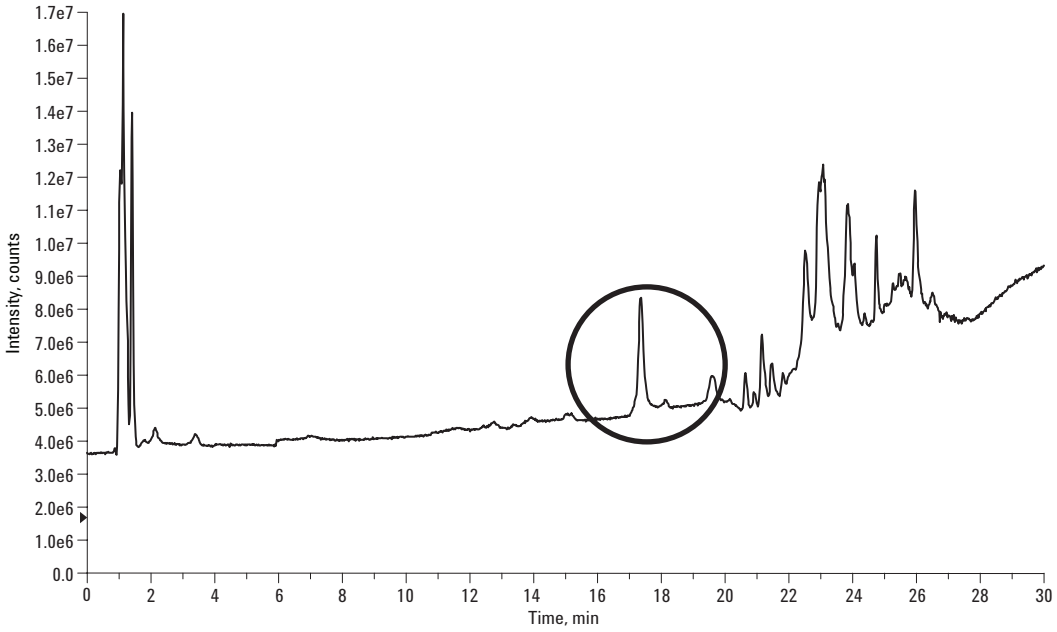


Figure 17. TIC of unspiked plasma.

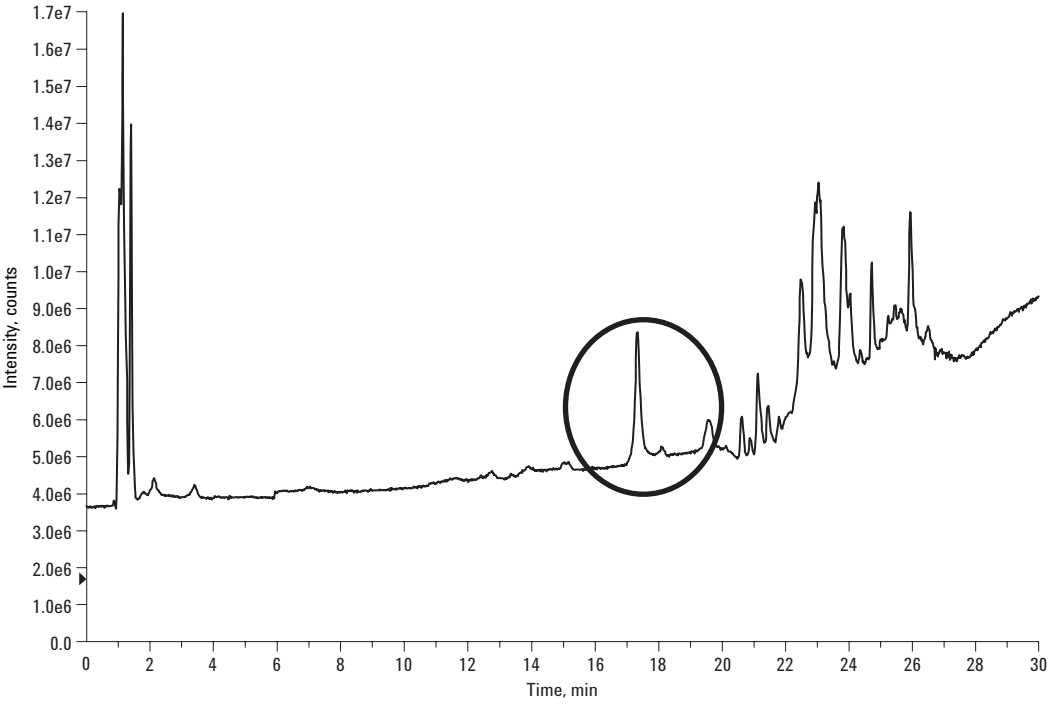


Figure 18. TIC of plasma spiked with 200 ng/mL of desalkylflurazepam.

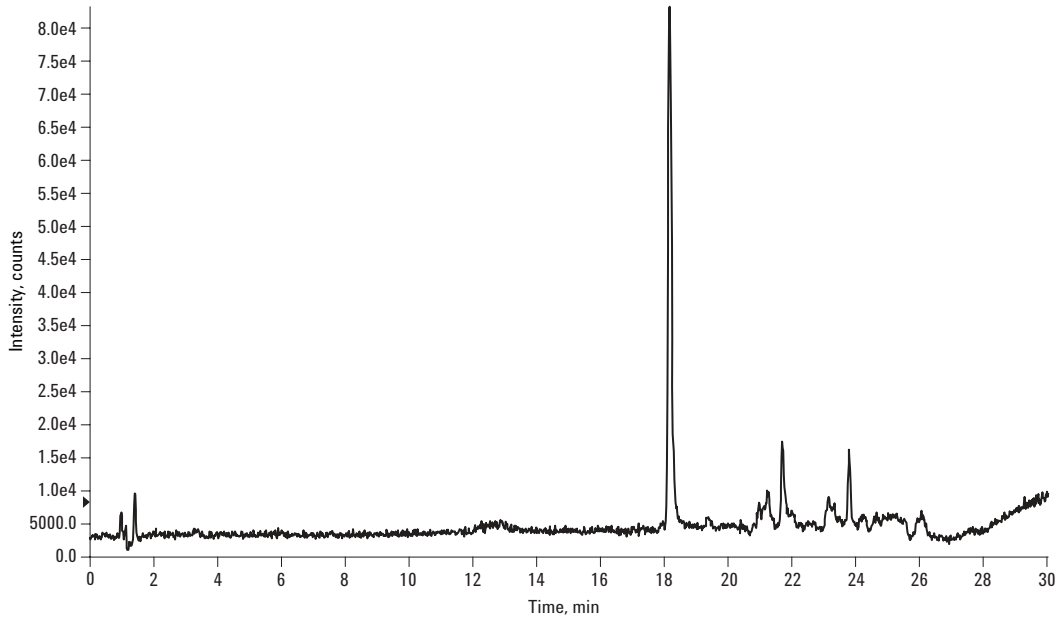


Figure 19. Mass extraction window (0.1 amu) of spiked plasma sample (346 ppm mass window).

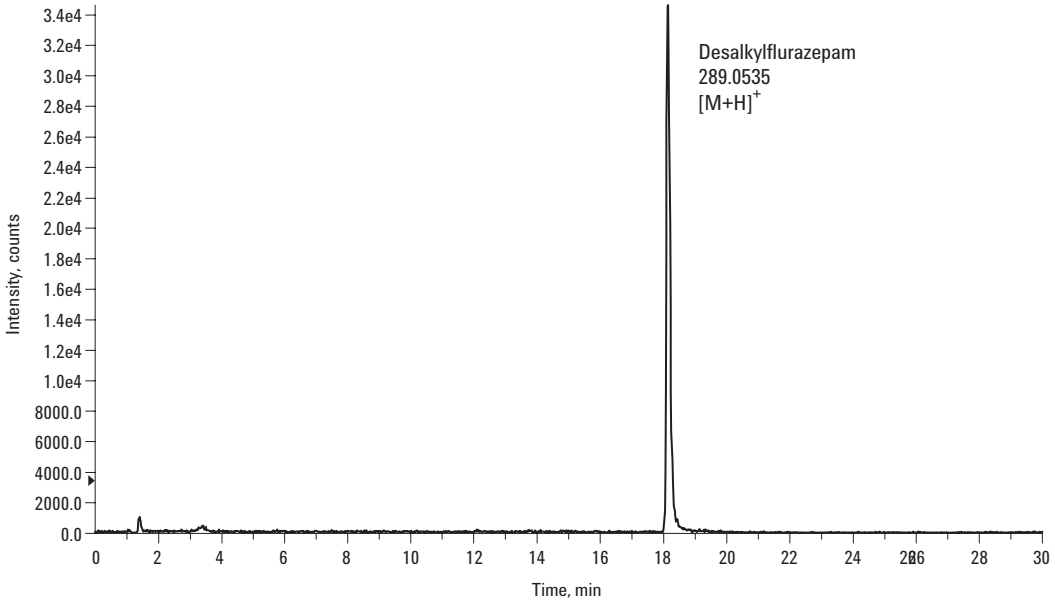


Figure 20. Ten ppm (0.003 amu) mass extraction window of spike plasma – note minimal noise.

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.

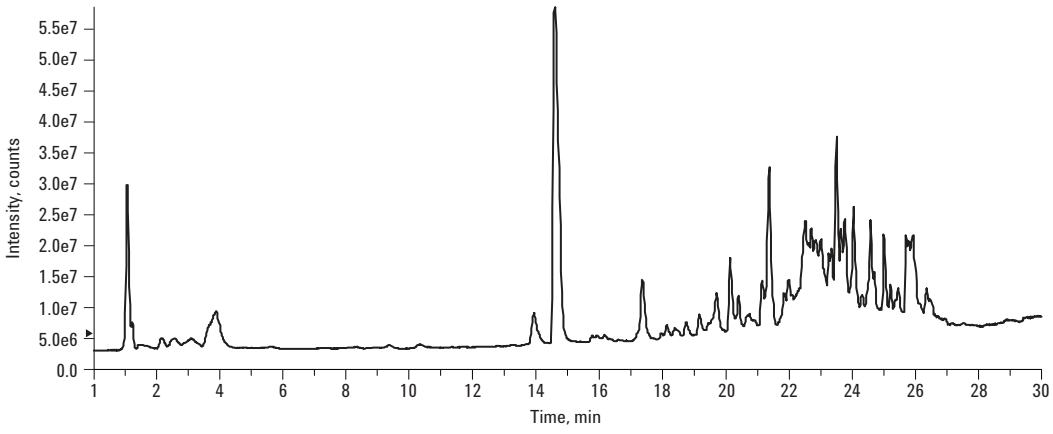
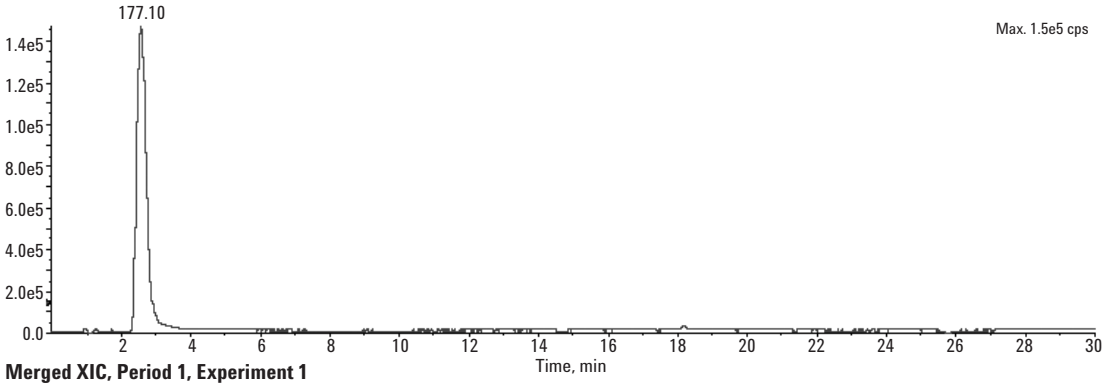
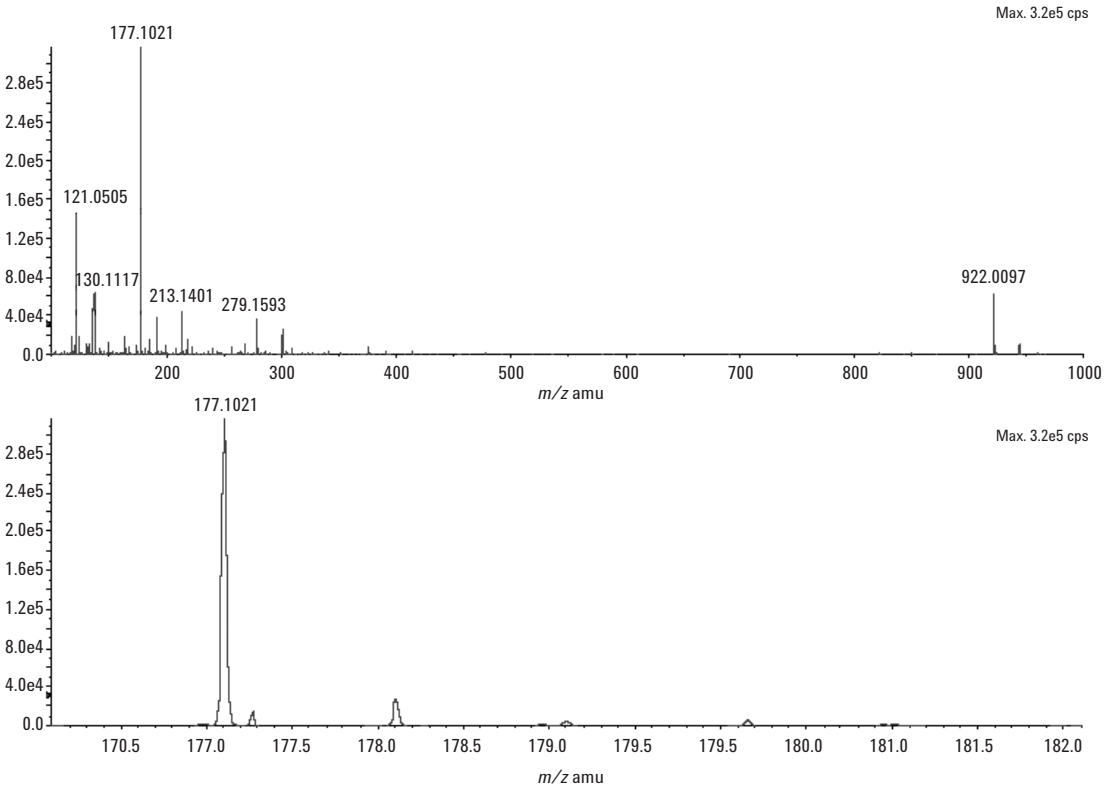


Figure 21. TIC of Coronial Sample 1.



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 patient was a smoker.

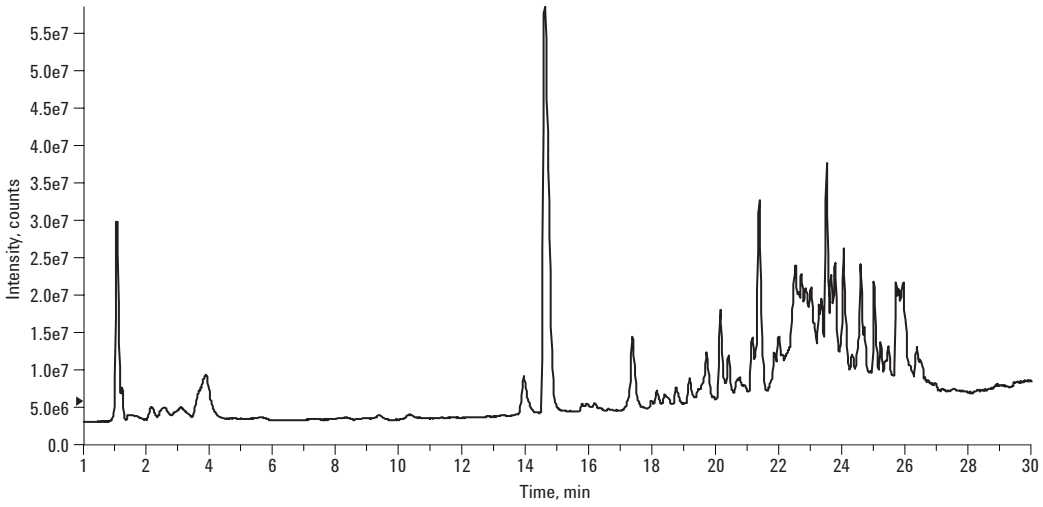
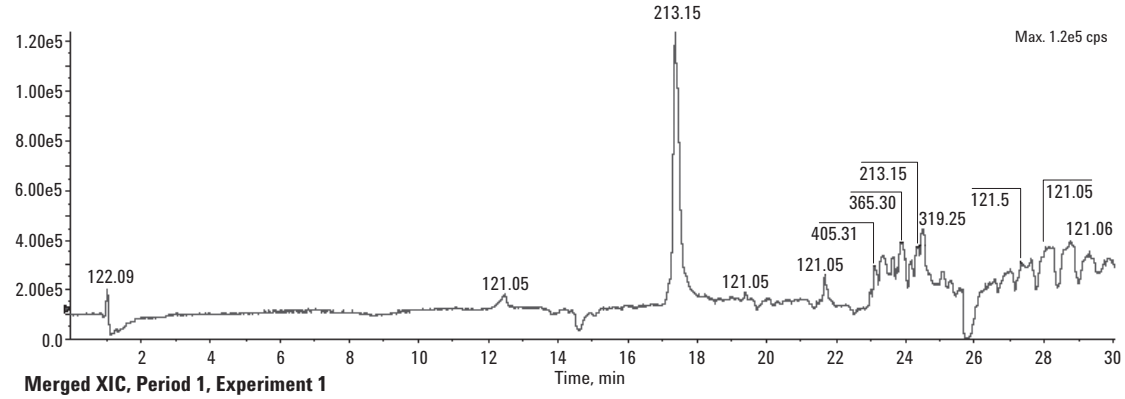
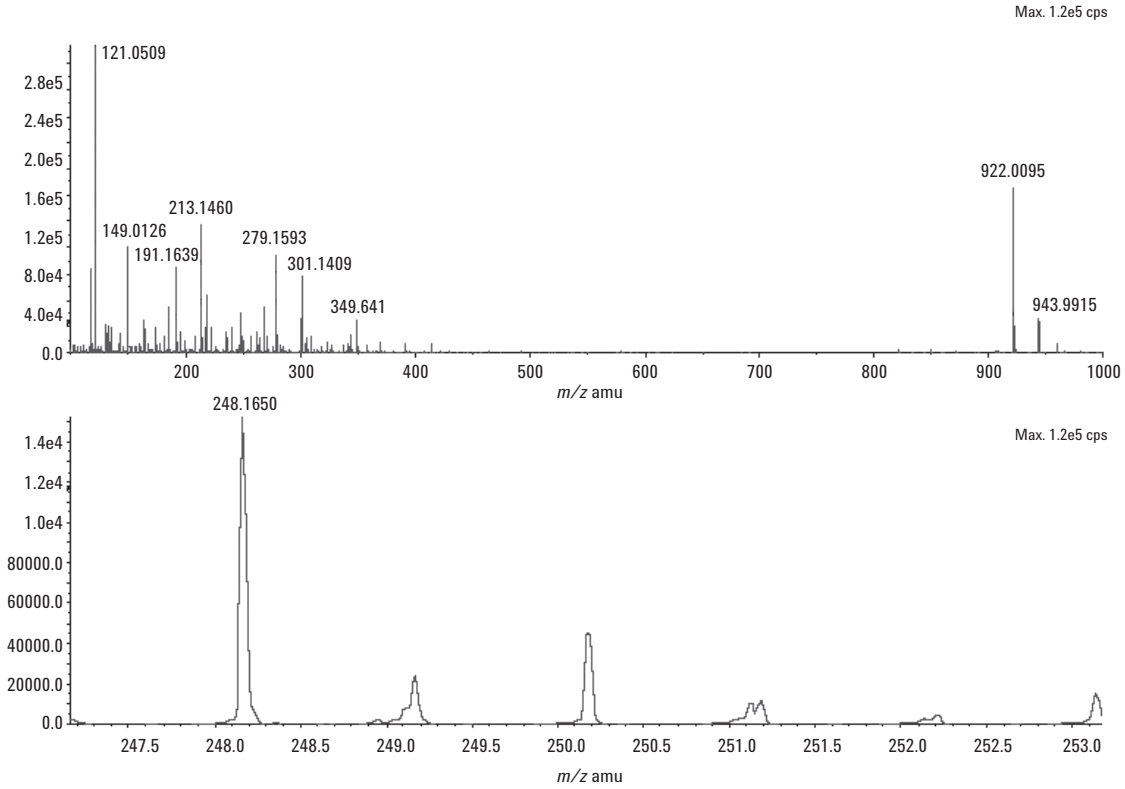


Figure 23. TIC of coronial Sample 2.

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 of our society, from law enforcement to medical monitoring. Current analytical techniques use a two-step screening and confirmation procedure to achieve the required specificity and sensitivity required. This application note has investigated 48 of the more common drugs of abuse and their applicability for determination 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 screening 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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4. Jerry Zweigenbaum, “Quantitative Analysis of Opiates in Urine Using Accurate Mass LC/MSD TOF”, Agilent Technologies, publication 5989-2206EN www.agilent.com/chem

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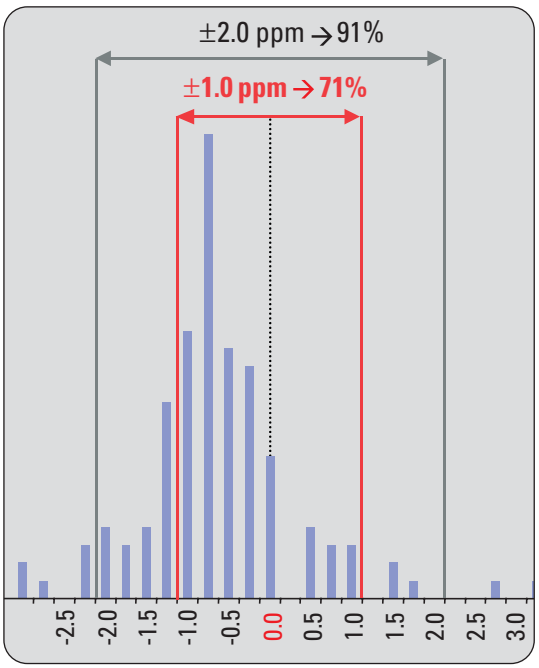
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Agilent Technologies

Agilent 1200 Series Rapid Resolution LC system and the Agilent 6210 TOF MS – Highest data content with highest throughput

Application Note



Michael Frank

Abstract

Fast and unambiguous determination of purity and identity of compounds derived from screening libraries is a common task for many analytical labs in the pharmaceutical industriy. The method of choice to determine the identity of compounds is mass spectrometry, preferably with accurate mass. As yet, data quality was usually compromised by gaining higher throughput. This Application Note demonstrates how a daily throughput of far more than 1000 samples can be achieved together with full spectral data acquisition and accurate mass information with close to FT-MS mass accuracy.

Agilent Technologies

Introduction

In the quest to achieve highest throughput in LC/MS analyses, the quality of the data is often compromised. There are certain approaches to increase the throughput of LC/MS systems. One approach is to do flow injection analysis. This probably delivers the highest possible throughput, however since no chromatographic separation occurs, the probability to loose compounds by the ion suppression effect during the ionization process is high. Orthogonal detection methods like UV detection do not succeed at all in flow injection analysis as all compound signals are overlaid. Approaches to achieve at least minimal chromatographic separation by using very short columns with 5 µm particles and ballistic gradients are an improvement in view of data quality, however, not state-of-the-art. Some manufactures have established parallel working instrumentation with a shared mass spectrometer and shared UV detector. Obviously, this also compromises data quality as the full acquisition rate of each instrument has to be shared on each LC channel[1].

With the introduction of an LC/MS system which facilitates the use of columns with sub two micron particles it is now possible to achieve short analyses times as well as high chromatographic resolution. Furthermore the system is able to acquire full UV spectral data and mass spectral data with accurate masses.

Experimental

The Agilent 1200 Series Rapid Resolution LC system is set up for alternating column regeneration (ACR)[2] using 2.1-mm id columns. The pumps are in the low delay volume configuration with an internal volume of only ca. 120 µL. All other modules are optimized for lowest delay volumes by using the low delay volume capillary kit (G1316-68744) and the alternating column regeneration kit (G1316-68721). Consequently, from the injection valve on only capillaries of 0.12 mm id are used. In the thermostatted column compartment the newly introduced low dispersion heat exchangers consisting of 1.6 µL internal volume have been used as well as the high pressure rated 2-position/10-port valve.

The instrument set-up is shown in figure 1:

- Two Agilent 1200 Series binary pumps SL with the new Agilent 1200 Series micro vacuum degasser placed between the two pumps eliminates the need for long tubing to the pumps.
- Agilent 1200 Series high performance autosampler SL.
- An Agilent 1200 Series thermostatted column compartment SL, equipped with a high pressure, 2-position/10-port valve, facilitating alternating column regeneration.
- An Agilent 1200 Series diode-array detector SL allowing a data acquisition rate of 80 Hz and equipped with a 500 nano liter flow cell with 0.12-mm id connecting capillaries.
- Agilent 6210 Time-of-Flight mass spectrometer allowing a maximum data acquisition rate of 40 Hz and equipped with a dual ESI source for parallel ionization of the analyte and a reference mixture.
- Two ZORBAX SB C18, 2.1 mm id x 50 mm, 1.8 µm columns
- As mobile phase gradient grade water with 0.1 % trifluoro acetic acid and acetonitrile with 0.08 % trifluoro acetic acid was used. No additional filtering of the solvents was made.

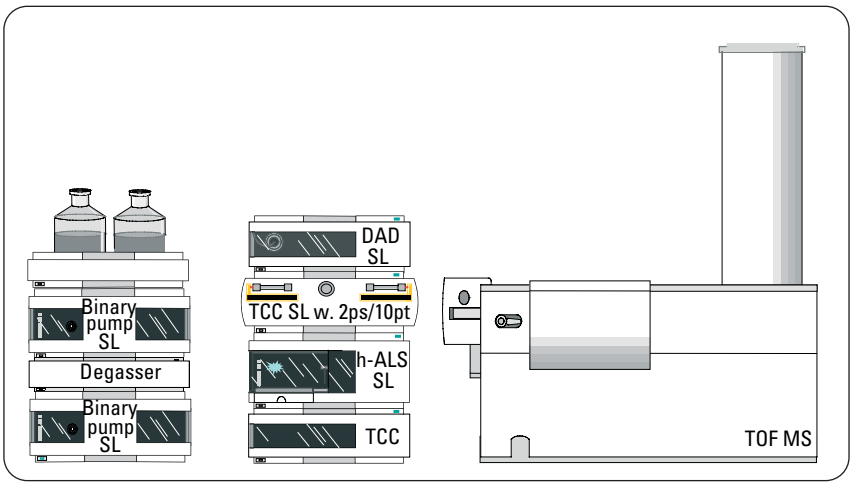


Figure 1
Agilent 1200 Series Rapid Resolution LC system with Agilent 6210 TOF-MS with low delay volume for high speed applications using 2.1-mm id columns with lengths ranging from 20 to 50 mm.

Instrument control and data acquisition was done by the Agilent TOF-software A02.01 running on a Hewlett-Packard xw 4300 workstation with an Intel dual core Pentium™ D840 CPU at 3.2 GHz.

Results and discussion

By applying elevated temperatures the viscosity of the solvent can be reduced which allows higher flow rates and therefore shorter gradient times. A maximum temperature of 80 °C was applied, which allowed a flow rate of 1.8 mL/min without hitting the pressure limit of the pump. This results in a linear velocity of approximately 11 mm/s for the 2.1 mm x 50 mm column (1.8 µm). With the help of the regeneration pump and the 2-position/10-port valve in the column compartment cycle times could be reduced significantly because one column is flushed with high organic content solvent and then re-equilibrated again with the starting composition of the gradient while on the second column the separation of a sample occurs. After this sequence the 10-port valve is switched and both columns are exchanged in the flow path. Details of alternating column regeneration and the correct setting of time points are described in another Application Note[2]. Despite the high flow rate (1.8 mL/min), the column effluent was not split prior to reaching the mass spectrometer. The standard ESI source specifies a maximum flow rate of up to 1 mL/min, however even these higher flows are tolerated if the drying gas temperature and flow rate are set to maximum and little condensation occurs. Condensation of water is practically eliminated when using ACR because equilibration is done on the column which is not connected to the detector. Generally the use of an Agilent multi mode source with a specified flow rate up to 2 mL/min even with pure water is recommended. The chromatographic conditions in table 1 were used to achieve gradient times of 0.5 min. Under these conditions, the peak capacity for the MS detection is in the range of >40 in 39 s. With the use of a 5-µm particle size column of the same dimension the peak capacity would only be half!

Data File	Sample Type	Inj Vol (µl)	Capillary	Fragmentor	Skimmer
opt_4000_215_60.wiff	Unknown	1	4000	210	60
opt_4000_215_60.wiff	Unknown	1	3000	210	60
opt_4000_215_60.wiff	Unknown	1	2000	210	60
opt_4000_215_60.wiff	Unknown	1	4000	180	60
opt_4000_215_60.wiff	Unknown	1	3000	180	60
opt_4000_215_60.wiff	Unknown	1	2000	180	60
opt_4000_215_60.wiff	Unknown	1	4000	150	60
opt_4000_215_60.wiff	Unknown	1	3000	150	60
opt_4000_215_60.wiff	Unknown	1	2000	150	60
opt_4000_215_60.wiff	Unknown	1	4000	210	40
opt_4000_215_60.wiff	Unknown	1	3000	210	40

Figure 2
Feature of the TOF software to modify the MS parameter from run to run.

Method:				
Solvent:	A = water (0.1% TFA), B = ACN (0.08% TFA)			
Temperature:	80 °C			
Flow:	1.8 mL/min			
Gradient:	0.00 min 5%B 0.50 min 90%B 0.51 min 5%B 0.65 min 5%B		**Regeneration:**	0.00 min 5%B 0.01 min 95%B 0.20 min 95%B 0.21 min 5%B 0.65 min 5%B
Stoptime:	0.65 min			no limit
Posttime:	off			off
DAD:	Wavelength:	210 nm (8), ref. off		
	Peak width:	>0.0025 min (0.05s responsetime), 80 Hz		
	Spectra:	no		
	Slit:	8 nm		
	Balance:	pre-run		
MS:	Scan range:	100-1000 m/z		
	Acquisition rate:	5, 20, 30 and 40 cycles/s		
	Data type:	profile data		
	Capillary voltage:	3000 V		
	Fragmentor:	180 V		
	Skimmer:	40V		
	Gas temperature:	350 °C		
	Gas flow:	13 L/min		
Injection volume:	1µL			
Injector:	Overlapped injection, Automatic delay volume reduction, Sample flush out factor = 10			
Valve position:	Next position			

Table 1
LC/MS method used for the data shown in figures 3-5. The method was also used to achieve the values in table 2.

The detector of the Agilent 6210 TOF MS would be saturated if the compound concentrations used here to give also significant UV signals would be injected into the MS without special settings. Saturation of the MS detector would produce incorrect results in mass determination. The solution is to intentionally desensitize the TOF MS. This can be done quite easily by applying the functionality of the TOF software to alter the MS parameters from one run to the other,

simply by adding one or more "MS-parameter" columns to the worklist (figure 2). Select "add columns" from the worklist and then chose "MS-parameter" and the desired parameter. As the reference mixture is also affected by these settings, the concentration of the reference mixture was increased. Only the capillary voltage, the fragmentor voltage and the skimmer voltage were varied. The optimal conditions determined by this approach can be found in the method parameters in table 1.

In figure 3 the total ion chromatogram and the UV chromatogram achieved with conditions above (80 Hz DAD, 30 Hz TOF data acquisition rate) is shown for a five-component sample (58 ng/µL atenolol, 85 ng/µL primidon, 62 ng/µL metoprolol, 125 ng/µL verapamil and 75 ng/µL beclomethasone-dipropionat). The peaks of the total ion chromatogram are inherently broader than the peaks of the UV chromatogram because of additional extra column volume from the flow cell and also from connecting the capillary between the UV detector and ESI interface. But as can be seen in figure 3, the additional peak broadening of the MS peaks is only minor. The peak widths at half height of the MS peaks obtained under the highest data acquisition rate (40 Hz) are shown in figure 4 with values from as little as 0.34 to 0.42 s. The chromatograms shown in figure 5 were produced under the same chromatographic conditions, but with different data acquisition rates of the time-of-flight MS. The peak form and resolution are improved by having high data acquisition

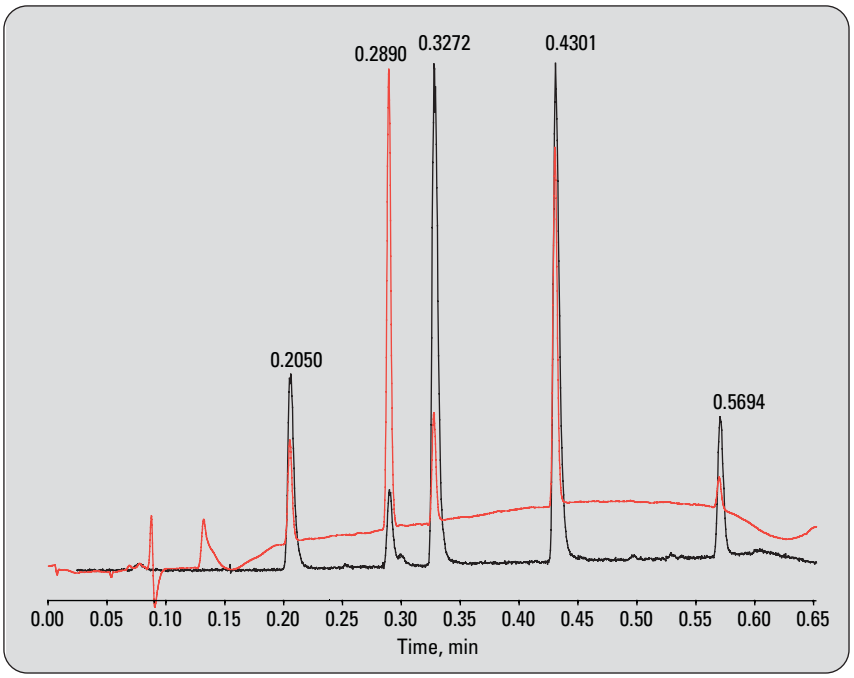


Figure 3
Comparison of corresponding peaks in the UV (red trace) and the MS detection (black trace).

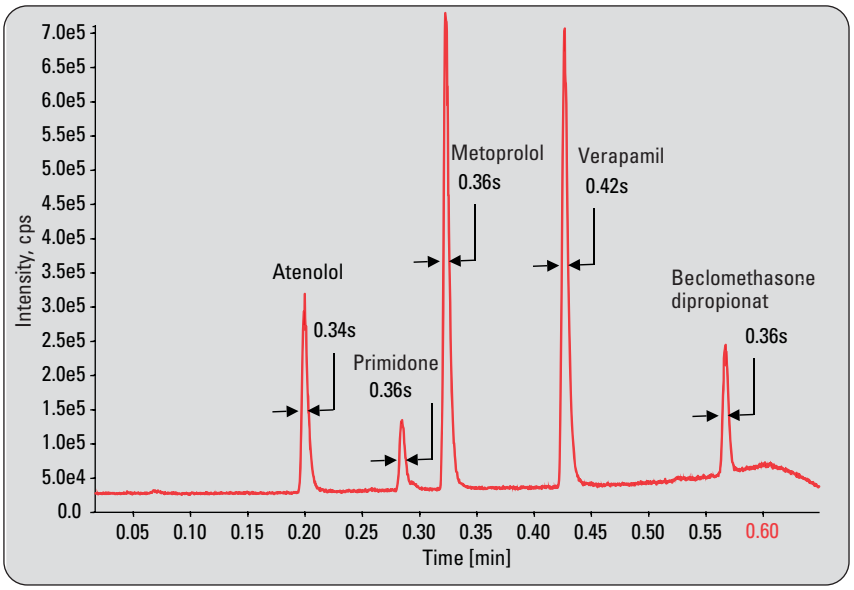


Figure 4
MS total ion chromatogram of highest speed LC-TOF-MS analysis (40 Hz TOF data acquisition rate).

rates in the MS which shows clearly in figure 5. The effect is nicely demonstrated on the little side peak next to the primidon peak – with 40-Hz data acquisition rate it is obvious that an additional compound shows up but with 5 Hz data acquisition rate this could not be differentiated from tailing of the primidon! The advantage, especially if MS quantization is necessary, is clear.

By applying the chromatographic conditions of table 1 and 80 Hz signal data acquisition of one wavelength and 30 Hz TOF centroid data, a cycle time of 49 s was achieved. The achievable cycle time is not only dependent on the used run time (that is the gradient time plus additional flush and re-equilibration times, or in Agilent terminology the stop time plus post time) but also very much dependent on the instrument overhead time. This is usually caused by communication between the data system and the individual LC/MS modules as well as the data system writing data to the hard disc and initiating certain processes. The overhead time caused by the data system can be significant if the computer's performance is not sufficient to handle the data amount or if other software programs or processes are consuming the power available. To decrease the cycle time it might be worth decreasing the amount of data acquired.

Table 2 shows the cycle times and the possible daily throughput depending on the DAD and MS settings. Since the MS data are constantly written to the hard disc during data acquisition, whereas the UV data are buffered and added to the data file after the stop time of

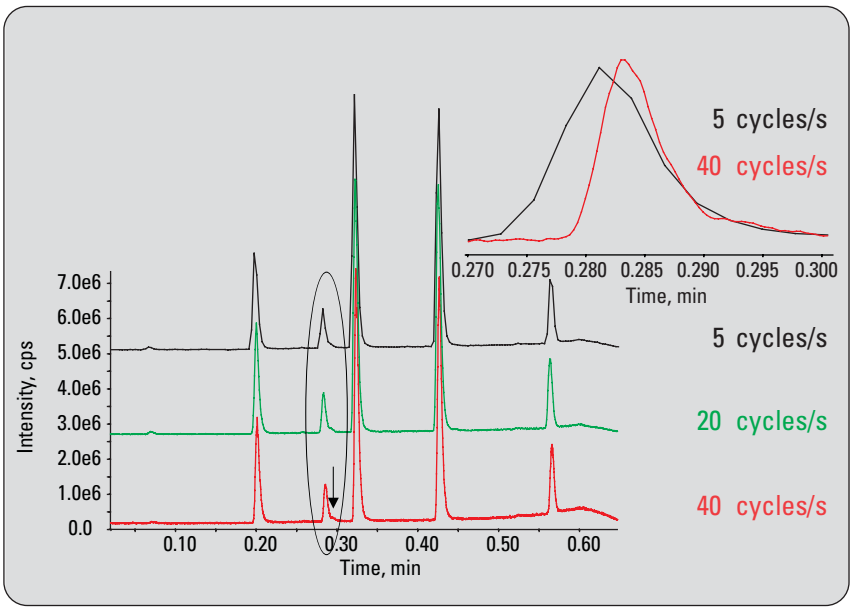


Figure 5
Total ion chromatograms recorded with varying data acquisition rates – dependence of the MS peak shape and resolution on the data acquisition rate.

DAD (80 Hz)		TOF (100 – 1000 Da)			Cycletime	Throughput
Type	Wavelength	Centroide	Profile	Data rate [Hz]	[s]	[Samples/day]
spectral	190-900 (1)		x	20	62	1394
spectral	190-900 (1)	x		20	62	1394
spectral	190-400 (2)		x	20	59	1464
spectral	190-400 (2)		x	40	59	1464
spectral	190-400 (2)		x	30	58	1490
signal	210/254		x	20	50	1728
signal	210	x		30	49	1763

Table 2
Dependence of the cycle time on the DAD and MS data acquisition settings, method stop-time was 0.65 min (39 s), pre-run balance was applied (ca. 2 s). The number in brackets for the DAD wavelength range stands for the scan width in nm.

the method, the cycle time depends more on the UV data amount than on the MS data amount. The cycle time was calculated from the time stamp each file gets assigned from the WindowsXP™ operating system after closing the file following data acquisition.

If using a TOF MS the attention is certainly focused on the accurate mass. The question may arise if the possibility to obtain low mass accuracy errors might suffer from these high speed conditions. Figure 6 shows the achieved mass accuracy errors of the analysis of 140 members of a chemical library used in a screening campaign by a pharmaceutical company. The shown error-values have been extracted from an automated empirical formula confirmation report and involved no manual interference. Sixteen of the compounds could not be ionized under positive ESI conditions and two compounds showed large mass errors of 11 and 15 ppm, probably caused by co-eluting isobaric impu-

rities. The cycle time was 90 s and was determined by a required injector program which allowed an on-line dilution of the samples directly prior to the analysis. Chromatographic conditions applied a 5-100 % water-acetonitrile (0.1 % TFA) gradient in 0.7 min at a flow rate of 1.5 mL/min and 60 °C column temperature. UV data acquisition to determine purity was done in the wavelength range of 210 to 500 nm with an acquisition rate of 80 Hz. The MS data acquisition rate was at 8 Hz to reduce the file size. The scan range was 120 – 1200 Da, capillary voltage 4000 V and the fragmentor voltage at 215 V. No ACR was applied and the flow to the MS was splitted in a 1:7.5 ratio.

More compelling is the histogram of the mass errors of these samples as shown in figure 7. More than 91 % of the ionizable compounds (outliers included) have a mass accuracy error in the range of ±2.0 ppm. Excluding the outliers even 93 % of the analyzed samples lie in-between the ±2.0 ppm range. In the ±1.0 ppm range which is FT-MS-like mass accuracy 71 % of the samples can be found (72 % excluding the outliers).

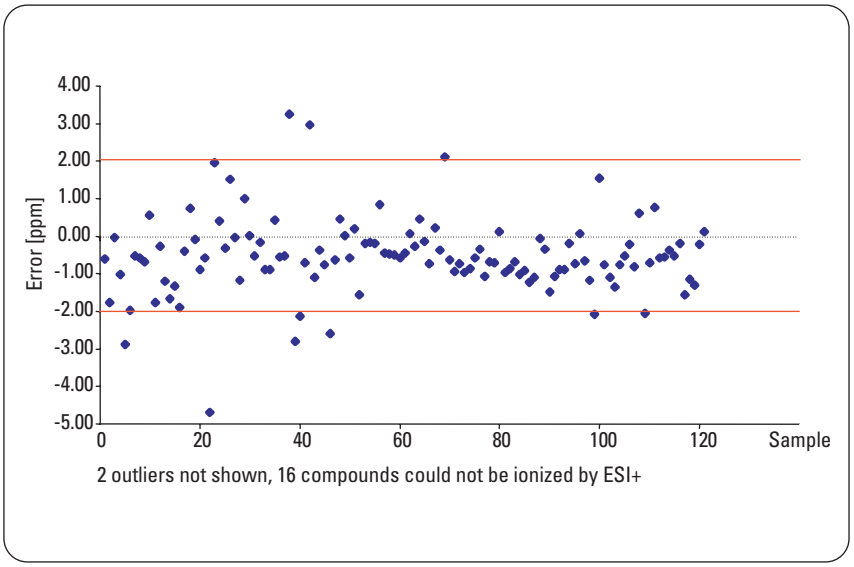


Figure 6
Mass accuracy errors of the analyses of a set of chemical library members under fast-LC conditions.

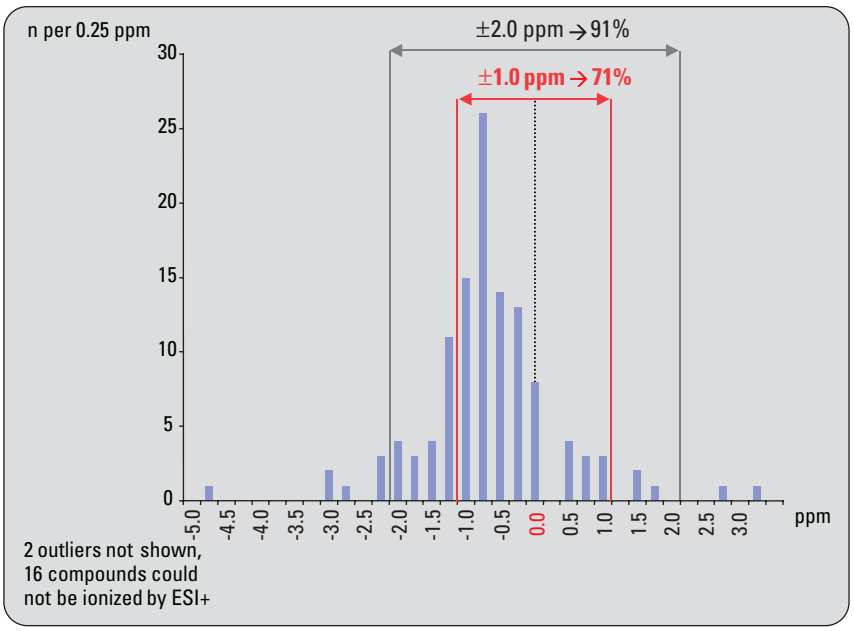


Figure 7
Histogram of the mass accuracy errors of the analyses of a set of chemical library members under fast LC conditions. The given populations of the ±1.0 ppm and ±2.0 ppm range include the outliers.

Conclusion

The Agilent 1200 Series Rapid Resolution LC system together with the Agilent 6210 Time-of-Flight mass spectrometer allows acquisition of a wealth of data to unambiguously determine the purity and identity of compounds in samples as they are typical for the high throughput analytical departments of pharmaceutical companies. In the time range of one minute high chromatographic resolution, full spectral diode-array data from 190-900 nm wavelength in a band width of 1 nm at an 80 Hz acquisition rate plus full MS spectral data from 100-1000 m/z with high acquisition rate and with an accurate mass with a mass error below ±2.0 ppm for more than 91 % of the samples could be acquired.

Using features like alternating column regeneration, overlapped injection, high temperatures, high flow rates together with highest data acquisition rates and most importantly stable and easy-to-use accurate mass, this system outperforms other high throughput LC/MS techniques used as yet in throughput and/or data quality. The linear velocities achieved were in the range of 11 mm/s and cycle times were as fast as 49 s for a run time of 41 s. Due to the columns with particle sizes of 1.8 µm, the UV peak capacities were still in the range of fifty and even the MS peak capacities were in the range of forty for a gradient time of 39 s.

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2.

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Most of the data herein was presented as a poster, titled "Non-multiplexed DAD-ToF Analysis of 1400 Samples/day" by Michael G. Frank, Edgar Naegele (Agilent Technologies, Waldbronn, Germany), Doug McIntyre (Agilent Technologies, Santa Clara, USA), Thilo A. Fligge, Stefan Buehler, Markus Christ (Boehringer-Ingelheim, Biberach, Germany), CO-1152, at the Pittcon conference 2006 in Orlando, Florida, USA.

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Analysis of Trace Residues of Explosive Materials by Time-of-Flight LC/MS

Application

Forensics

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Abstract

A key technique used in trace explosives analysis is HPLC with UV detection, following the guidelines set out in USEPA method 8330. Although sensitive for many target explosives, the method is limited by a lack of detector selectivity. This application note outlines the benefits and limitations of the use of liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) for the detection and quantitation of trace levels of these explosive residues.

Introduction

The identification of explosive residues in crime scene forensic investigation, environmental site remediation, and homeland security is an analysis of major significance to both public and regulatory authorities. The traditional and most commonly accepted method for the analysis of the nitroaromatic class of explosives is USEPA Method 8330. This method provides a sensitive UV-based analysis of 14 nitroaromatics and nitramines. However, due to the lack of selectivity provided by UV detection, confirmation of the species present requires the analysis to be performed on two analytical columns with different stationary phases.

The terrorist attacks on 9/11/2001, and subsequent attacks around the world have brought a new focus onto the identification and quantitation of explosive residues in crime scene investigation and homeland security. One of the front lines of homeland security, airport departure gates, uses sophisticated screening devices such as ion mobility spectrometers. These devices, though sensitive, face selectivity limitations in that they cannot determine the explosive species present. Additionally, terrorists are becoming increasingly erudite, as was seen in the attempt by Richard Reid in late December 2001. He used a peroxide-based explosive within his shoes, which was not detectable at trace levels using the analytical techniques commonly used for explosives analysis.

Inherent to the nature of explosive compounds is their instability, and propensity to breakdown. One of the best known and most common explosive compounds, trinitrotoluene (TNT) is reduced by bacteria to 2-amino-4,6-dinitrotoluene, (2-AMDNT) and 4-amino-2,6-dinitrotoluene (4-AMDNT); a metabolism that occurs also in plants and animals. Both of these compounds are markers for the former presence of TNT, and are also known to show severe toxicity and mutagenicity, making them important environmental markers.

An extensive search of the literature found several articles detailing the analysis of explosive materials using liquid chromatography (LC) [1-4]. However, of the 14 explosive materials of interest, very few, in particular TNT and RDX, were readily identified using mass spectrometry (MS) [5-7].

There still exists the requirement for a reliable and sensitive confirmatory technique of analysis for these explosive residues that can be performed on samples from a wide variety of sources. Liquid chromatography/mass spectrometry (LC/MS) provides an excellent tool for this analysis with the ability to couple the mass spectrometer to existing instruments performing USEPA method 8330. Furthermore, the choice of a mass selective detector (MSD) can provide confirmatory information previously required through the use of a second analytical column.

Accurate mass measurement, such as provided by the Agilent LC/MSD TOF time-of-flight mass spectrometer (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 compounds are theoretically possible. This is particularly useful when needing to analyze samples from a variety of sources, each with their own potential interferences such as those encountered with explosives residues.

This application note demonstrates the utility of the LC/MSD TOF for the determination of low level explosives. The LC/MSD TOF provides accurate mass determination (better than 3 ppm) and linearity to three orders of magnitude, and thus is an excellent tool for the detection, confirmation, and quantitation of explosive compound residues.

The explosives studied are shown in Tables 1 and 2, including the chemical structure and theoretically calculated exact mass.

Table 1. Names, Abbreviations and Molecular Formulae of Explosives Studied

#	Name	Abbreviation	CAS no.	Molecular formula
1	Hexamethylenetriperoxidediamine	HMTD	NA	$C_6H_{12}N_2O_6$
2	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine	HMX	2691-41-0	$C_4H_8N_8O_8$
3	Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	121-82-4	$C_3H_6N_6O_6$
4	1,3,5-triamino-2,4,6-trinitrobenzene	TATB	3058-38-6	$C_6H_6N_6O_6$
5	Ethylene glycol dinitrate	EGDN	628-96-6	$C_2H_4N_2O_6$
6	1,3,5-Trinitrobenzene	1,3,5-TNB	99-35-4	$C_6H_3N_3O_6$
7	1,3-Dinitrobenzene	1,3-DNB	99-65-0	$C_6H_4N_2O_4$
8	Methyl-2,4,6-trinitrophenylnitramine	Tetryl	479-45-8	$C_7H_5N_5O_8$
9	4-amino-2,6-dinitrotoluene	4A-DNT	1946-51-0	$C_7H_7N_3O_4$
10	Nitrobenzene	NB	98-95-3	$C_6H_5NO_2$
11	Nitroglycerin	NG	55-63-0	$C_3H_5N_3O_9$
12	2-amino-4,6-dinitrotoluene	2A-DNT	355-72-78-2	$C_7H_7N_3O_4$
13	2,4,6-Trinitrotoluene	TNT	118-96-7	$C_7H_5N_3O_6$
14	2,6-Dinitrotoluene	2,6-DNT	606-20-2	$C_7H_6N_2O_4$
15	2,4-Dinitrotoluene	2,4-DNT	121-14-2	$C_7H_6N_2O_4$
16	Hexanitrostilbene	HNS	19138-90-0	$C_{14}H_6N_6O_{12}$
17	2-Nitrotoluene	2-NT	88-72-2	$C_7H_7NO_2$
18	4-Nitrotoluene	4-NT	99-99-0	$C_7H_7NO_2$
19	Pentaerythritol tetranitrate	PETN	78-11-5	$C_5H_8N_4O_{12}$
20	3-Nitrotoluene	3-NT	99-08-1	$C_7H_7NO_2$
21	Triacetone triperoxide	TATP	NA	$C_9H_{18}O_6$
22	Carbamite	Carbamite	NA	$C_{17}H_{20}N2_O$

NA Not applicable

Table 2. Molecular Structures and Calculated Accurate Masses of Explosives Studied

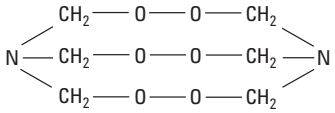
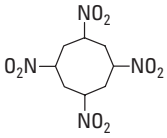
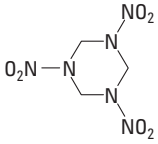
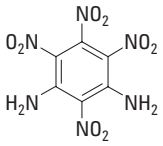
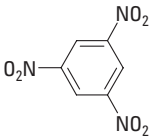
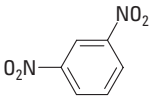
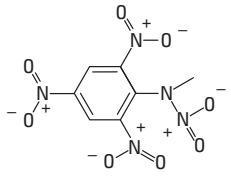
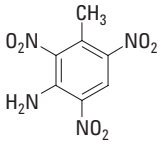
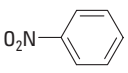
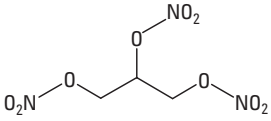
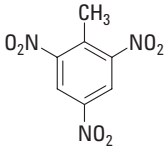
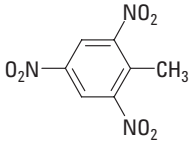
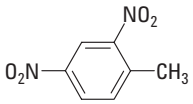
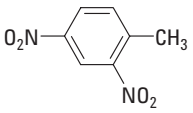
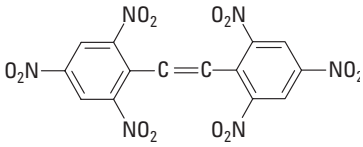
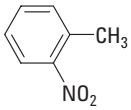
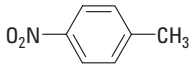
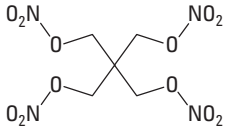
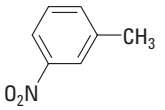
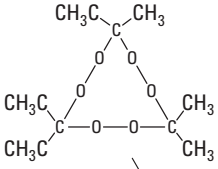
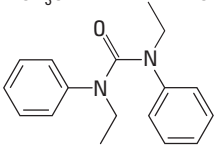
#	Name	Molecular formula	Structure	Molecular weight
1	HMTD	$C_6H_{12}N_2O_6$		208.0695
2	HMX	$C_4H_8N_8O_8$		296.0465
3	RDX	$C_3H_6N_6O_6$		222.0349
4	TATB	$C_6H_6N_6O_6$		258.0349
5	EGDN	$C_2H_4N_2O_6$		152.0069
6	1,3,5-TNB	$C_6H_3N_3O_6$		213.0022
7	1,3-DNB	$C_6H_4N_2O_4$		168.0171
8	Tetryl	$C_7H_5N_5O_8$		287.0138
9	4A-DNT	$C_7H_7N_3O_4$		197.0437
10	NB	$C_6H_5NO_2$		123.0320
11	NG	$C_3H_5N_3O_9$		227.0026

Table 2. Molecular Structures and Calculated Accurate Masses of Explosives Studied (continued)

#	Name	Molecular formula	Structure	Molecular weight
12	2A-DNT	$C_7H_7N_3O_4$		197.0437
13	TNT	$C_7H_5N_3O_6$		227.0178
14	2,6-DNT	$C_7H_6N_2O_4$		182.0328
15	2,4-DNT	$C_7H_6N_2O_4$		182.0328
16	HNS	$C_{14}H_6N_6O_{12}$		450.0044
17	2-NT	$C_7H_7NO_2$		137.0477
18	4-NT	$C_7H_7NO_2$		137.0477
19	PETN	$C_5H_8N_4O_{12}$		316.0139
20	3-NT	$C_7H_7NO_2$		137.0477
21	TATP	$C_9H_{18}O_6$		222.1103
22	Carbamite	$C_{17}H_{20}N_2O$		268.1576

Methodology

The work undertaken in this study was performed on an Agilent 1100 LC system consisting of: binary pump, autosampler, thermostatted column compartment, and the LC/MSD TOF.

LC Conditions

Solvents	Methanol and water		
Flow rate	0.9 mL/min		
Gradient	**Time (min)**	**% Methanol**	**% Water**
	0	60	40
	1	60	40
	15	92	8
	16	100	0
	18	100	0
	19	60	40
Post time	5 minutes		
Total run time	24 minutes		
Injection volume	10 µL, with needle wash		
Column temperature	40 °C		
Column	ZORBAX Extend-C18 4.6 mm × 250 mm, 5 µm p/n 770450-902		

MS Detection conditions

Ionization	APCI
Gas temperature	350 °C
Vaporizer temperature	325 °C
Drying gas flow	5 L/min
Nebulizer pressure	40 psig
PCI Corona current	4 µA
PCI Capillary voltage	4000 V
NCI Corona current	10 µA
NCI Capillary voltage	1500 V
Scan *m/z* range	70–1000
Fragmentor voltage	100 V
Storage mode	Profile
Skimmer	60 V
Oct RF	250 V

The experimental conditions listed above were optimized for sensitivity. Vaporizer temperature, drying gas temperature and flow rate, corona current, capillary voltage, and fragmentor voltage were all optimized.

A large increase in signal was observed by reducing the drying gas flow rate from 6 L/min to 5 L/min. This resulted in a 30% increase in signal area for more than 80% of the explosives under investigation.

Reference Mass Introduction with LC/MSD TOF

The Agilent LC/MSD TOF uses a reference mass in the generation of reliable accurate masses. The electrospray source for the LC/MSD TOF is a unique dual spray assembly that allows the simultaneous and constant introduction of a reference mass solution. When using APCI (atmospheric pressure chemical ionization), the reference masses must be introduced into the mobile phase post-column.

This was achieved via a low dead-volume tee connected prior to the APCI source with PEEK™ tubing. An isocratic pump was used to deliver the reference mix at a flow rate of 50 µL/min in positive ion mode and flow programmed from 70 µL/min to 150 µL/min in negative ion mode over the run time of 1 to 15 minutes. In order to ensure pulse-less reference mass introduction a rapid resolution column (ZORBAX SB-C18, 30 mm × 2.1 mm, 5 µm, part number 873700-902) was installed in the flow path, providing backpressure for the isocratic pump.

The reference mix was modified to suit the methodology, 25 µL of purine and 250 µL of HP-0921 was added to 250 mL of 90:10 methanol:water.

This enabled the use of the following reference masses:
Positive ion mode: 121.050873 and 922.009798
Negative ion mode: 119.036320, 966.000725, and 980.016375

In negative ion mode, with increasing organic mobile phase strength, the reference masses 966.000725 and 980.016375 decrease in intensity. By using the custom reference mass mix outlined above and the use of flow programming, sufficient abundance of the reference mass ions is maintained throughout the analytical run.

With the paucity of literature discussing the detection of explosives by LC/MS, the first step of development was evaluating component responses under both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) in both positive and negative ion modes. Table 3 lists the response characteristics for many of the compounds tested in this study and it clearly shows that no one ionization and detection technique is universally applicable.

Table 3. Detection Modes for Various Explosives

Compound	UV/Visible	ESI +	ESI -	APCI +	APCI -
HMTD	√	×	×	√	×
HMX	√	×	×	×	√
RDX	√	×	√	×	√
TATB	√	×	×	×	√
EGDN	×	×	×	×	P
1,3,5-TNB	√	×	√	×	√
1,3- DNB	√	×	×	×	√
Tetryl	√	×	×	×	√
4A-DNT	√	×	×	×	√
Nitrobenzene	√	×	×	×	P
Nitroglycerin	P	×	×	×	P
2A-DNT	√	×	×	×	√
TNT	√	×	√	×	√
2,6-DNT	√	×	×	×	√
2,4-DNT	√	×	√	×	√
HNS	√	×	×	×	√
2-NT	√	×	×	×	P
4-NT	√	×	×	×	P
PETN	P	×	√	×	√
3-NT	√	×	×	×	P
TATP	P	×	×	P	×
Carbamite	√	√	×	√	×

√ Good response

P Poor response

× No response

It was observed that negative APCI provided the best response for most explosives studied, and if run in positive APCI mode as well, additional components are detected. Negative APCI also has the advantage of being very selective, removing possible matrix interferences. The ability to couple UV detection prior to the mass spectrometer also provides a highly capable analysis for explosives. However, the major advantage of LC/MS over UV detection is the ability to detect the newer, more terrorist-friendly explosives such as TATP and HMTD in positive APCI mode. These peroxide explosives are reported to degrade when exposed to intense sources of UV light, such as what might be experienced in a UV detector.

A key parameter considered during the development of the method was the ability to transfer the HPLC method between different detectors. This precluded the use of nonvolatile buffers which would be detrimental to MS detection. Initial analyses investigated the use of buffers such as acetic acid, formic acid, ammonium acetate, and ammonium formate. While in many cases the chromatographic separation was improved, signal response was compromised. Using the high selectivity of the LC/MSD TOF, signal intensity was chosen as the key parameter to optimize. The addition of chloroform in APCI mode can also increase sensitivity with some explosive compounds; however, it was found in this study that the majority of compounds are best analyzed with no organic modifier present.

Various HPLC columns, mobile phase compositions, and gradients were also tested in this investigation. The conditions finally used were chosen for their selectivity, speed of analysis, and detection limits (DLs) attainable with MS.

Detection of Explosives Using the LC/MSD TOF

An overwhelming advantage of using the LC/MSD TOF for the trace level detection of any component is the confirmatory information that is provided through accurate mass measurement. An example of this mass accuracy is shown in Table 4, where observed masses and their deviations from the theoretical exact mass are shown.

The ability to closely match the expected mass and the observed mass provides the analyst with a very high level of confidence in the assignment given to a chromatographic peak.

Table 4. Theoretical Exact Mass, Observed Mass, Mass Error, and Limit of Quantitation (LOQ) Using the LC/MSD TOF

Compound	Monoisotopic mass	Adduct	Adduct mass	Observed mass	Mass error (ppm)	LOQ (µg/L)
HMTD	208.0695	$[M-H]^+$	207.0611	207.0612	0.18	30
HMX	296.0465	$[M+CHO_2]^-$	341.0447	341.0446	–0.11	10
RDX	222.0349	$[M+CHO_2]^-$	267.0330	267.0328	–1.07	0.5
TATB	258.0349	$[M-H]^-$	257.0276	257.0276	–.02	5
EGDN	152.0069	No response by TOFMS				
1,3,5-TNB	213.0022	$[M]^-$	213.0027	213.0026	–0.63	15
1,3-DNB	168.0171	$[M]^-$	168.0176	168.0175	–0.92	10
Tetryl	287.0138	$[M-NO_2]^-$	241.0214	241.0214	–0.24	5
4A-DNT	197.0437	$[M-H]^-$	196.0363	196.0362	–0.92	10
NB	123.0320	No response by TOFMS				
NG	227.0026	No response by TOFMS				
2A-DNT	197.0437	$[M-H]^-$	196.0363	196.0364	0.92	5
TNT	227.0178	$[M]^-$	227.0183	227.0178	–2.6	4
2,6-DNT	182.0328	$[M]^-$	182.0333	182.0331	–1.1	8
2,4-DNT	182.0328	$[M]^-$	182.0333	182.0331	–1.1	4
HNS	450.0044	$[M]^-$	450.0049	450.0042	–1.6	1
2-NT	137.0477	$[M-H]^-$	136.0404	136.0406	1.5	100
4-NT	137.0477	$[M-H]^-$	136.0404	136.0407	2.2	50
PETN	316.0139	$[M-NO_2 + CH_2O_2]^-$	316.0269	316.0267	–0.94	250
3-NT	137.0477	$[M]^-$	137.0482	137.0480	–1.7	5000
TATP	222.1103	Unassigned	ND	89.0597	ND	1000
Carbamite	268.1576	$[M+H]^+$	269.1659	269.1665	2.1	10

ND Not determined

A powerful result of accurate mass measurement was the ability to assign the ion formed by positive APCI of HMTD. The paper by Xu et al [8] assigned the ion observed for HMTD as being the $[M-1]^+$ species. It was not clear what the ion was and thus it was identified only as a loss of one mass unit. Using the accurate mass data obtained from the Agilent LC/MSD TOF, this ion can be assigned as the $[M-H]^+$ species, as the likely result of the multiple peroxide linkages in close association with a nitrogen atom. Note that the measured mass in Table 4 shows a loss of hydrogen. This is shown in Figure 1 below.

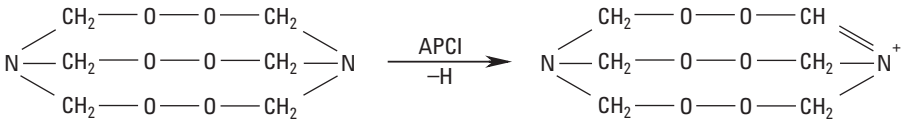


Figure 1. The theoretical positive ion formed from HMTD using APCI.

Further, a high degree of mass accuracy can increase the detection limit (DL), as noise is effectively reduced by narrowing the monitored mass range. This can be shown by observing the signal-to-noise (S/N) of RDX over a mass window of 0.1 amu (similar to what can be achieved on a single quadrupole system) and a mass window of 0.01 amu for a 1 µg/L (ppb) solution (Table 5).

Table 5. Calculated S/N for a 1 µg/L RDX at Different Mass Extraction Windows

Extracted ion range	Noise time range	Mean noise	P-P noise	Peak height	S/N (P-P)
267.0–267.1	3.509–3.692	21.4	57.0	285.5	5.0
267.03–267.04	3.509–3.692	5.2	21.0	245.5	11.7

A greater than two-fold increase in sensitivity is seen for these compounds. Figure 2 shows the reduction in noise that is observed with the extraction of a narrower mass range, a critical factor in confirmation when dealing with complex matrices.

An interesting observation that was made at higher concentrations was the dominance of a different adduct. This was a particular feature of HMX and RDX, whereby at high concentrations the adduct formed was $[M + CH_2O_2]^-$ instead of the otherwise observed $[M + CHO_2]^-$. This radical anion adduct could be explained by a charge exchange catalyzed by the very high concentration of ions/molecules in the APCI source. This split of signal would also explain the highly accurate mass measurement in spite of the high concentration that typically causes detector saturation and loss of accuracy.

Mass Accuracy with Concentration

The mass accuracy of the LC/MSD TOF was evaluated for four of the explosive compounds over a concentration range of 100,000 µg/L (100 ppm) to 1 µg/L (1 ppb) and is shown in Tables 6–9. The mass accuracy data was obtained from observing the mass spectral data at the apex of a plus/minus 1 amu extracted window of the accurate mass. The % RSD for each mass is reported and the mass error from the average mass. It should be noted that the error for the 100,000 µg/L HMX solution is for the previously mentioned $[CH_2O_2]^-$ adduct. Saturation of the detector at high concentrations is known to cause a loss of mass accuracy as shown in the results. For HMX and RDX the low concentration and low signal intensity resulted in a reduced mass accuracy as well. Higher signal intensity for the two other compounds, TNT and 2A-DNT, resulted in mass accuracy less than 2 ppm at the 1 µg/L concentration.

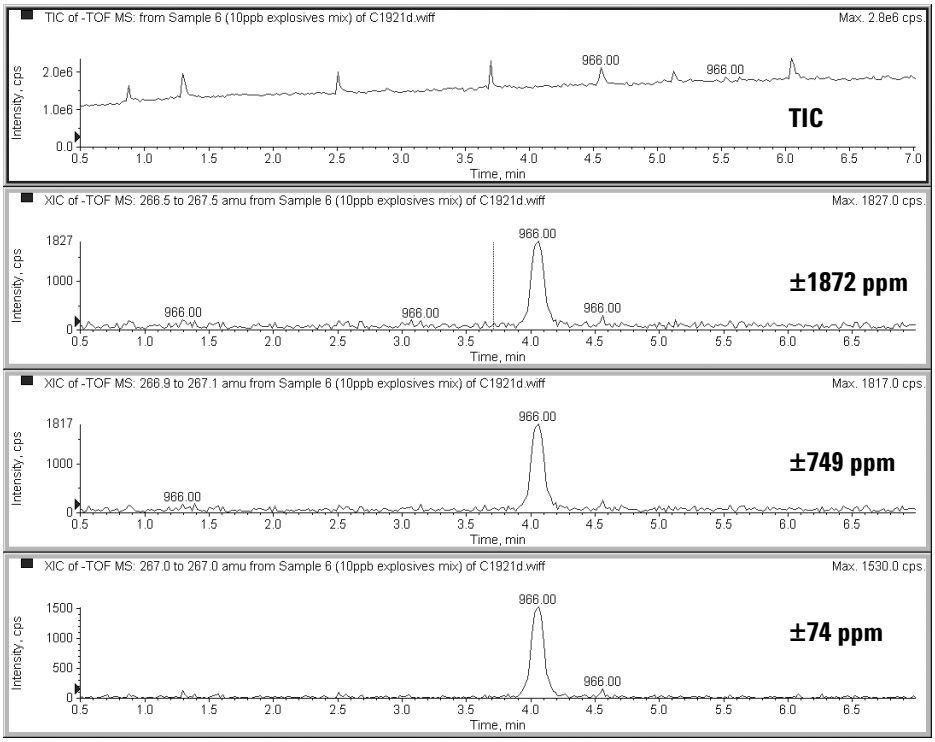


Figure 2. Effect of extracted ion range on noise of 10 µg/L RDX. The value given in each panel is the mass range extracted in parts per million (ppm) of expected exact mass of RDX.

Table 6. Mass Accuracy at Five Concentration Levels (1–100,000 µg/L) for HMX

Replicate	1	10	100	1,000	10,000	100,000
1	341.0453	341.0444	341.0441	341.0449	341.0444	342.0668
2	341.0425	341.0461	341.0444	341.0445	341.0445	342.0645
3	341.0429	341.0446	341.0441	341.0446	341.0445	342.0628
4	341.0418	341.0445	341.0443	341.0444	341.0444	342.0651
5	341.0416	341.0457	341.0447	341.0443	341.0445	342.0600
Average	341.0428	341.0451	341.0443	341.0445	341.0445	342.0638
SD	0.0015	0.0008	0.0002	0.0002	0.0001	0.0026
Error (ppm)	–5.6	1.14	–1.2	–0.62	–0.62	34.61

Table 7. Mass Accuracy at Five Concentration Levels (1–100,000 µg/L) for RDX

Replicate	1	10	100	1,000	10,000	100,000
1	267.036	267.0328	267.0345	267.0329	267.0324	267.0333
2	267.0357	267.033	267.0341	267.0331	267.0328	267.0336
3	267.0354	267.0314	267.0338	267.0331	267.0325	267.0335
4	267.0371	267.0326	267.033	267.0332	267.0327	267.0333
5	267.0297	267.0349	267.0334	267.0331	267.0322	267.0335
Average	267.0348	267.0329	267.0338	267.0331	267.0325	267.0334
SD	0.0029	0.0013	0.0006	0.0001	0.0002	0.0001
Error (ppm)	6.4	–0.69	2.7	–0.06	–2.2	1.2

Table 8. Mass Accuracy at Five Concentration Levels (1–100,000 µg/L) for TNT

Replicate	1	10	100	1,000	10,000	100,000
1	227.0174	227.0180	227.0176	227.0177	227.0185	227.0457
2	227.0178	227.0162	227.0179	227.0176	227.0184	227.0416
3	227.0184	227.0173	227.0180	227.0177	227.0183	227.0346
4	227.0173	227.0170	227.0181	227.0177	227.0183	227.0360
5	227.0197	227.0193	227.0181	227.0176	227.0184	227.0318
Average	227.0181	227.0176	227.0179	227.0177	227.0184	227.0379
SD	0.0010	0.0012	0.0002	0.0001	0.0001	0.0056
Error (ppm)	–1.2	–3.5	–2.1	–3.01	0.072	86

Table 9. Mass Accuracy at Five Concentration Levels (1–100,000 µg/L) for 2A-DNT

Replicate	1	10	100	1,000	10,000	100,000
1	196.0375	196.0364	196.0361	196.0357	196.0399	196.0859
2	196.0371	196.0366	196.0361	196.0361	196.0397	196.0819
3	196.0360	196.0369	196.0364	196.0359	196.0397	196.0786
4	196.0358	196.0358	196.0368	196.0358	196.0390	196.0799
5	196.0368	196.0364	196.0364	196.0359	196.0394	196.0770
Average	196.0366	196.0364	196.0364	196.0359	196.0395	196.0807
SD	0.0007	0.0004	0.0003	0.0001	0.0004	0.0034
Error (ppm)	1.1	0.11	0.11	–2.4	16	230

Area Repeatability

Time-of-flight mass spectrometers have traditionally had a reputation as being unsuitable for quantitation and the provision of repeatable areas.

The area repeatability for the LC/MSD TOF was investigated at multiple levels for three of the explosive components. Generally, the LC/MSD TOF showed repeatability across five runs of better than 5% RSD. However, sometimes when approaching the LOQ, this would increase to a larger error. The area repeatability for RDX, TNT, and 2A-DNT for five injections at each concentration level analyzed are shown in Tables 10–12.

Table 10. RDX Concentration (µg/L)

Replicate	1	10	100	1,000	10,000	100,000
1	426	1890	12300	154000	2540000	14100000
2	642	1780	13000	143000	2450000	15200000
3	541	1820	13300	146000	2460000	15300000
4	659	2620	14000	141000	2330000	14900000
5	508	2760	13600	149000	2130000	14700000
Average	555.2	2174	13240	146600	2382000	14840000
SD	96	475	642	5128	159593	477493
%RSD	17.42	21.86	4.85	3.5	6.7	3.22

Table 11. TNT Concentration (µg/L)

Replicate	1	10	100	1,000	10,000	100,000
1	4760	16400	127000	1730000	20700000	74800000
2	4330	16600	134000	1700000	20600000	73300000
3	4490	16500	134000	1840000	20900000	71600000
4	4200	16200	134000	1790000	20400000	71300000
5	3990	16100	132000	1830000	19600000	71200000
Average	4354	16360	132200	1778000	20440000	72440000
SD	291	207	3033	61400	502991	1569394
%RSD	6.7	1.27	2.29	3.45	2.46	2.17

Table 12. 2A-DNT Concentration (µg/L)

Replicate	1	10	100	1,000	10,000	100,000
1	2300	7820	68400	779000	9720000	27600000
2	2440	9040	64500	807000	10400000	28800000
3	2340	8910	66200	862000	10400000	30800000
4	2250	8760	65900	849000	9690000	28400000
5	2350	7830	77800	940000	10100000	29600000
Average	2336	8472	68560	847400	10062000	29040000
SD	70	598	5350	61443	348310	1219836
%RSD	3.01	7.07	7.8	7.25	3.46	4.2

TOF Linearity

The linearity of the LC/MSD TOF was investigated for a range of the components in the mixture. Of the 10 components evaluated, most exhibited a linear regression coefficient of variation of greater than 0.998. Some of the compounds displayed excellent linearity across the four orders of magnitude. A linear dynamic range for this instrument is typically two-to-three orders of magnitude. As can be seen in the repeatability results for RDX, the area response is very linear between 10 and 10,000 µg/L. The 100,000 µg/L showed saturation and the 1 µg/L showed a less than 5x decrease in signal vs the nearly 10x for the other concentrations. Figures 3 to 5 show representative calibration curves for 3 of the 10 components evaluated.

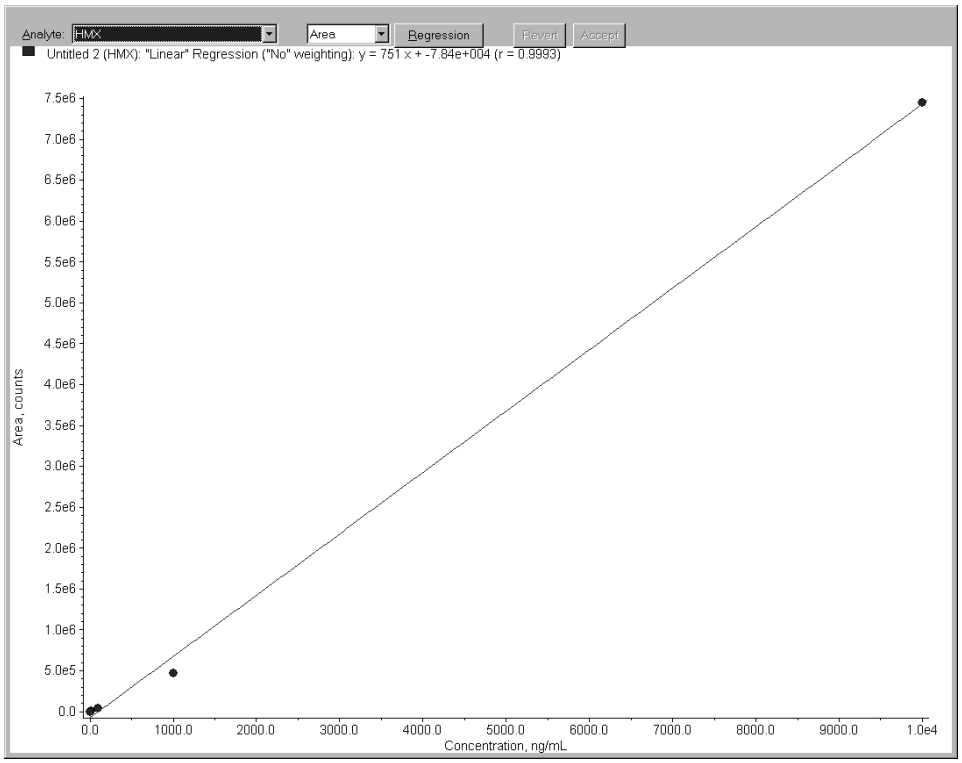


Figure 3. Calibration curve for HMX from 1 µg/L to 10,000 µg/L with MSD TOF.

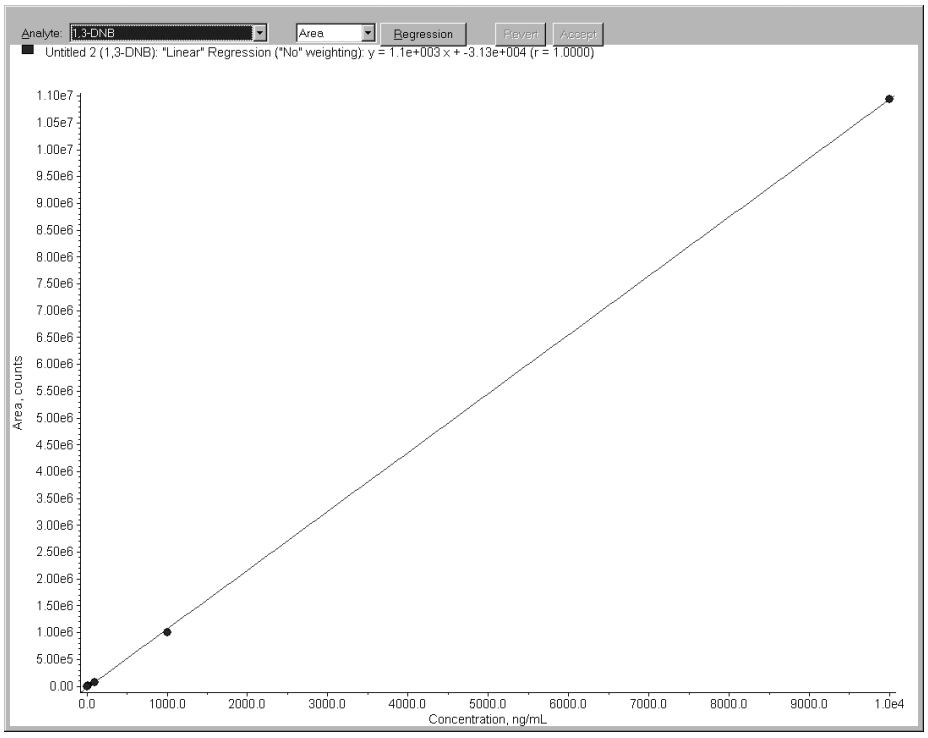


Figure 4. Calibration curve for 1,3-DNB from 1 µg/L to 10,000 µg/L with LC/MSD TOF.

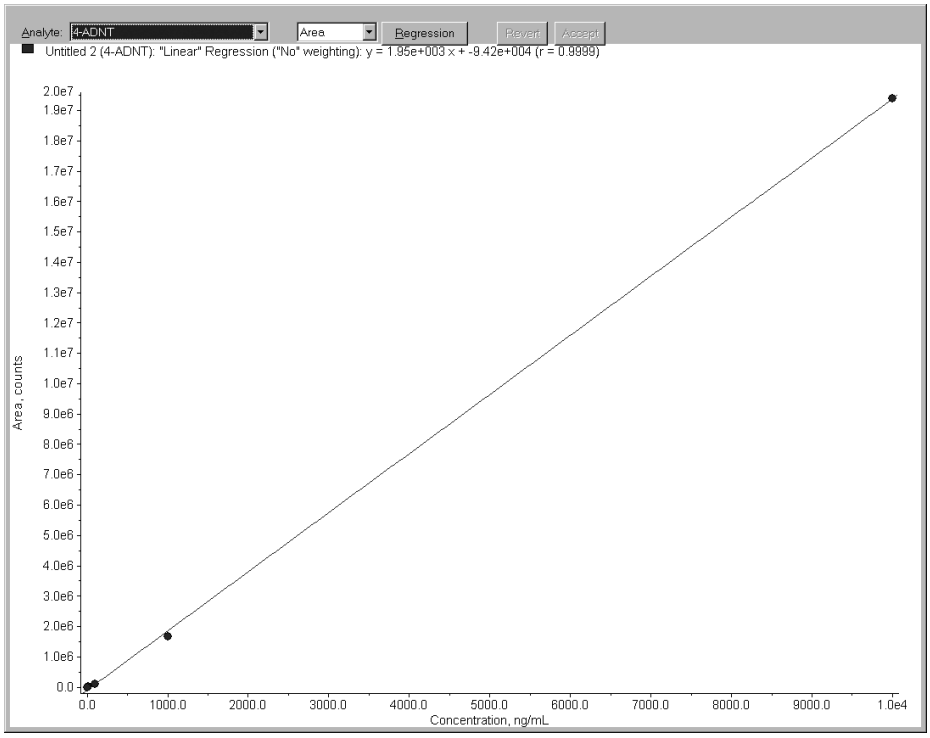


Figure 5. Calibration curve for 4A-DNT from 1 µg/L to 10,000 µg/L with LC/MSD TOF.

Chromatograms for four components are shown in Figure 6 at 10 µg/L with ±100-ppm extraction windows.

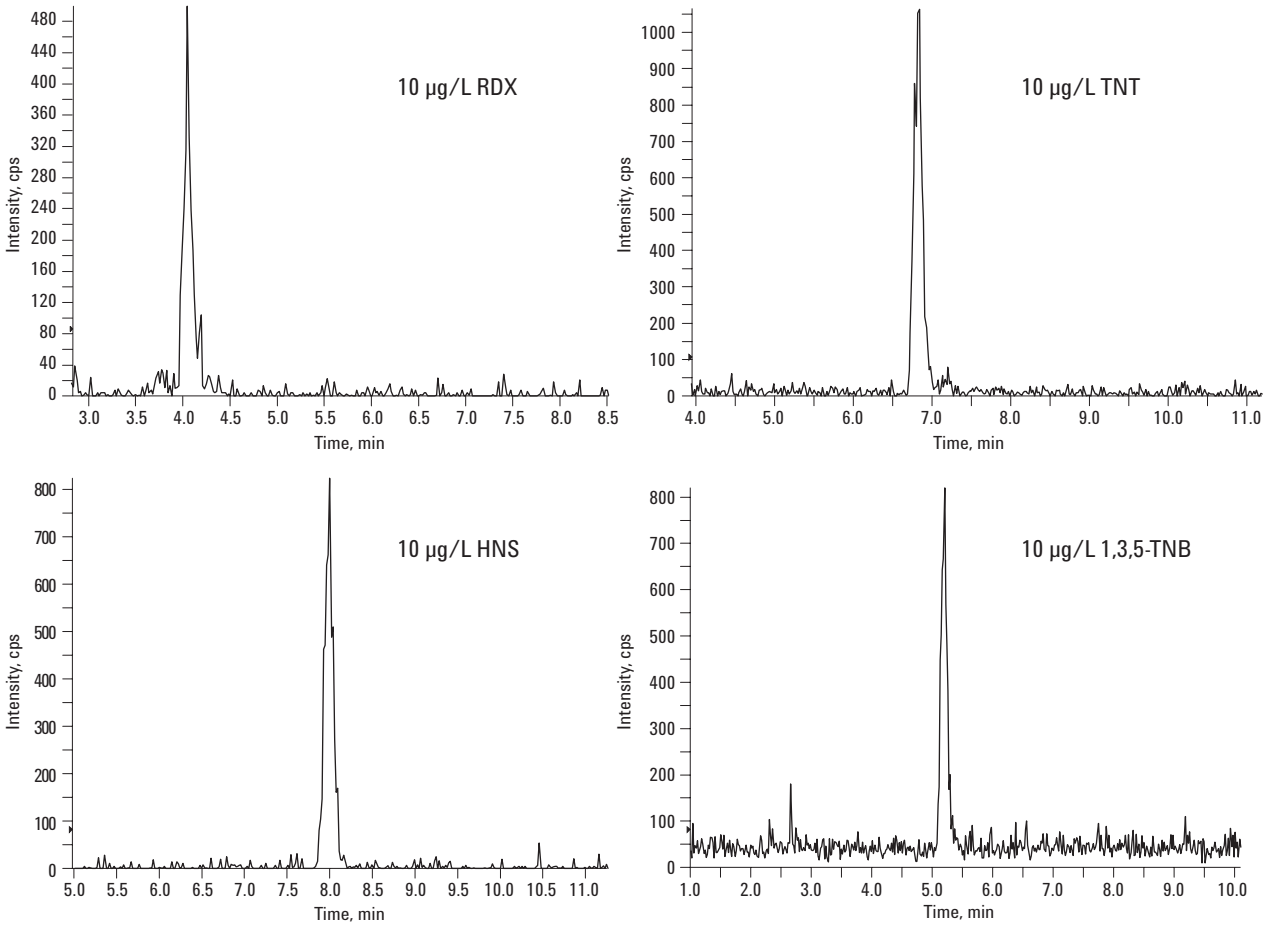


Figure 6. Representative chromatographic responses for four of the explosive compounds at the 10 µg/L concentration.

Spiked Recovery of Soil Samples

Figure 7 shows the results obtained from a soil spike of RDX.

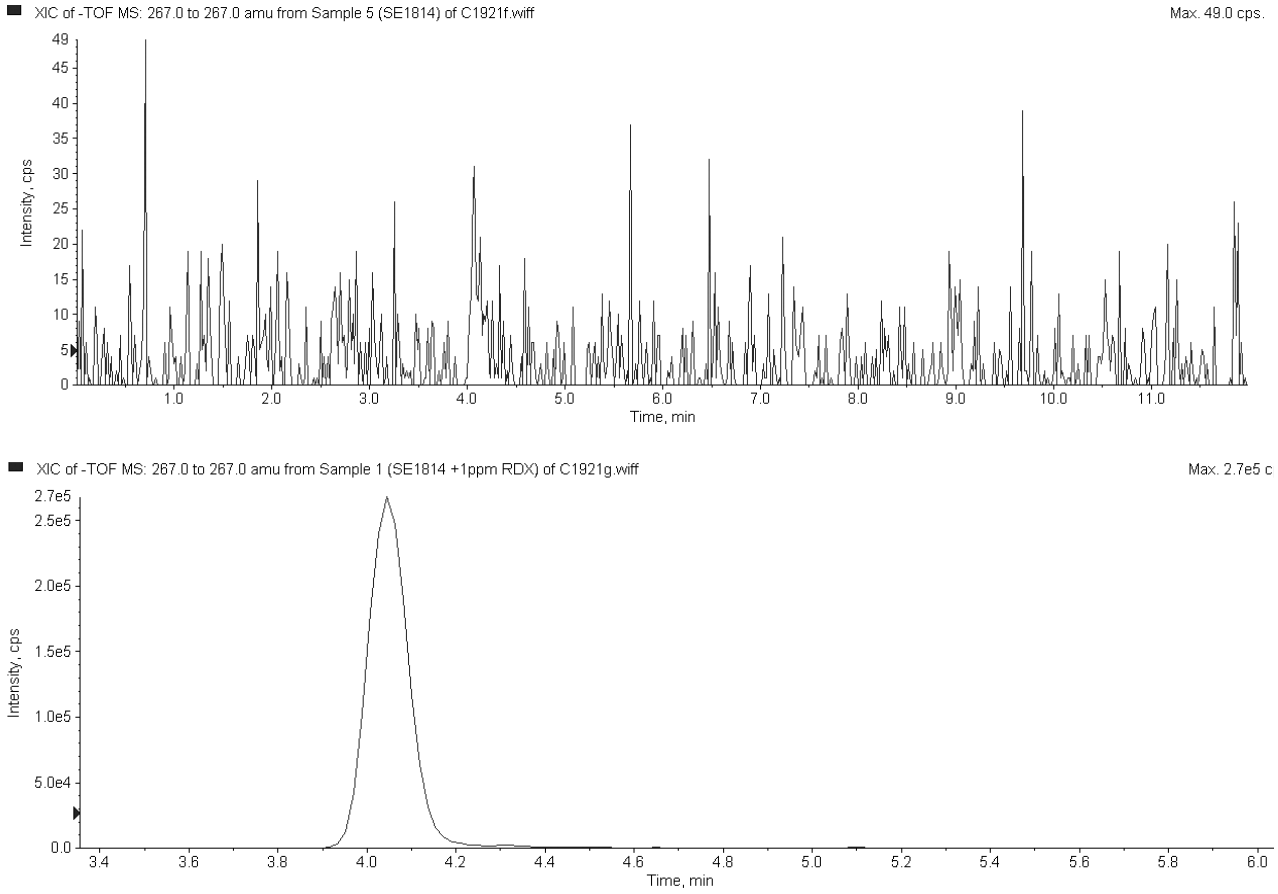


Figure 7. Soil sample SE1814 before and after spiking with 1 mg/L RDX. Extracted *m/z* 267.02-267.03

Table 13 gives the recoveries obtained when a dirty soil matrix is spiked with various explosives. The LC/MSD TOF provides a powerful tool in its ability to remove interference through the power of accurate mass measurements made at every scan.

Table 13. Spike and Recovery Levels for Three Soil Extracts

Soil sample ID	Spiked compound (amount)	Recovery
SE1814	RDX (1ppm)	115%
SE1814	RDX (0.1ppm)	120%
SE1816	2,4-Dinitrotoluene (1ppm)	75%
SE1947	1,3-dinitrobenzene (1ppm)	99%

Analysis of Crime Scene Samples

Two blind samples from archived crime evidence were analyzed with the Agilent LC/MSD TOF using the methodology developed in this study.

The first sample was treated as an unknown explosive. A small amount of material was dissolved in methanol and the resulting chromatogram in shown in Figure 8. The retention time of 6.8 minutes results in either two possibilities by retention time match, TNT or 2A-DNT (a TNT metabolite). By measuring the accurate mass of 227.0180 (Figure 9), it is a match for TNT with a radical union exact mass of 227.0183 (1.3 ppm mass error). Note that the $[M-H]^-$ ion is also observed and its measured mass of 226.0106 is only 0.18 ppm from the expected exact mass of this ion.

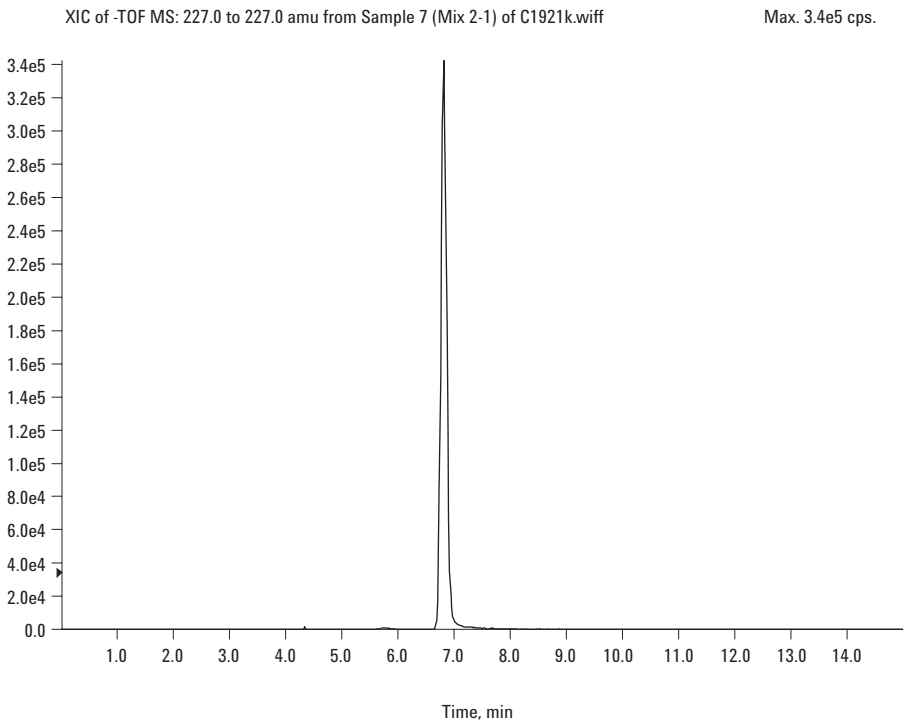


Figure 8. LC/MSD TOF Chromatogram of an unknown explosive material.

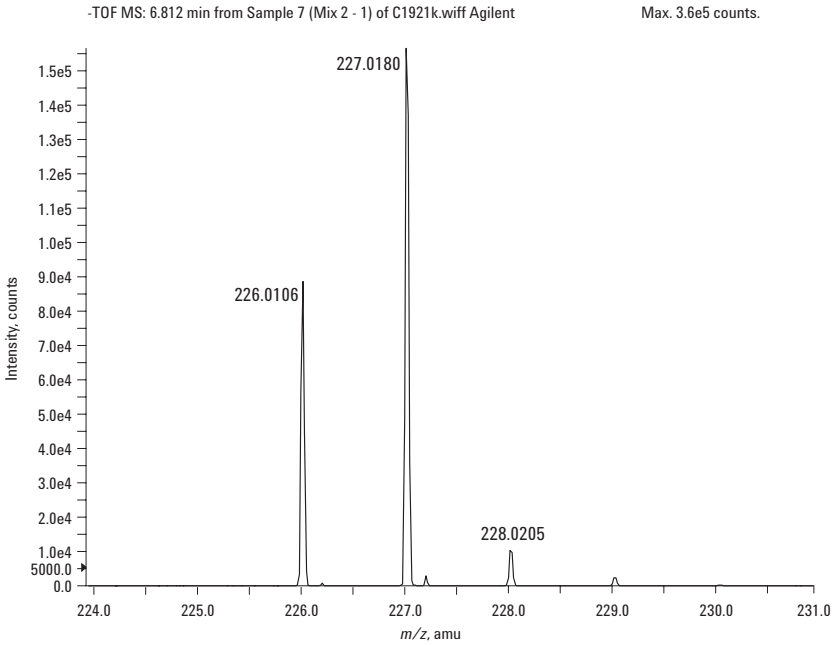


Figure 9. Mass Spectrum of an unknown explosive material.

The second sample was a soil extract to determine the possible presence of an explosive residue. By extracting all known accurate masses identified in this investigation within a 100 ppm mass window, one peak was identified at 4.0 minutes with a mass of 267.0331 *m/z*, which correlates to the presence of RDX with a mass error of 0.06 ppm for the formate adduct (Figure 10).

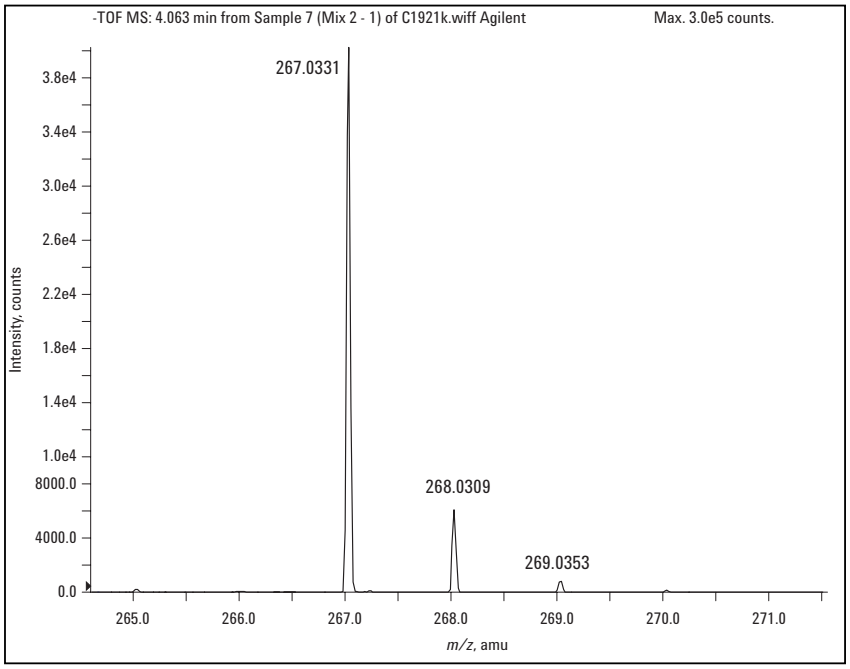


Figure 10. Confirmation of RDX in an explosive crime scene residue.

Summary

The detection of explosives has become a critical analysis in many countries from crime scene forensics to homeland security to environmental testing and remediation. The traditional method of analysis, USEPA method 8330 uses UV detection, which although for some components is sensitive, is nonselective and is prone to interference from the matrix.

The LC/MSD TOF, operated in APCI mode, has the advantage that all analyses take place in full scan mode, and hence any other components may be observed. This is coupled with a sensitivity that far exceeds UV detection as shown in Table 14. Additional confirmatory information and selectivity that is provided through the determination of the accurate mass provides a very powerful technique for the detection, identification and quantitation of explosive compounds.

This work has shown the Agilent LC/MSD TOF's ability to:

- Measure accurate masses within 3 ppm and often much better across a wide range of concentrations for many explosive compounds
- Obtain a high degree of selectivity, achieved with high resolution and accurate mass measurement at every scan
- Provide quantitative results
- Provide repeatability of response consistent with typical quantitative analysis
- Determines the identity of explosives in real samples with a high level of confidence

Table 14. LOQ for Explosives Using UV and LC/MSD TOF

Compound	UV	LC/MSD TOF
HMTD	10,000	30
HMX	1,000	10
RDX	100	0.5
TATB	1,000	5
EGDN	2,000	N.D.
1,3,5-TNB	3,000	3
1,3-DNB	500	2
Tetryl	500	5
4A-DNT	500	10
NB	800	N.D.
NG	500	N.D.
2A-DNT	500	5
TNT	200	4
2,6-DNT	400	8
2,4-DNT	400	4
HNS	500	1
2-NT	300	100
4-NT	200	50
PETN	1,000	250
3-NT	300	5000
TATP	10,000	1000
Carbamite	500	10

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

Application

Forensics

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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).

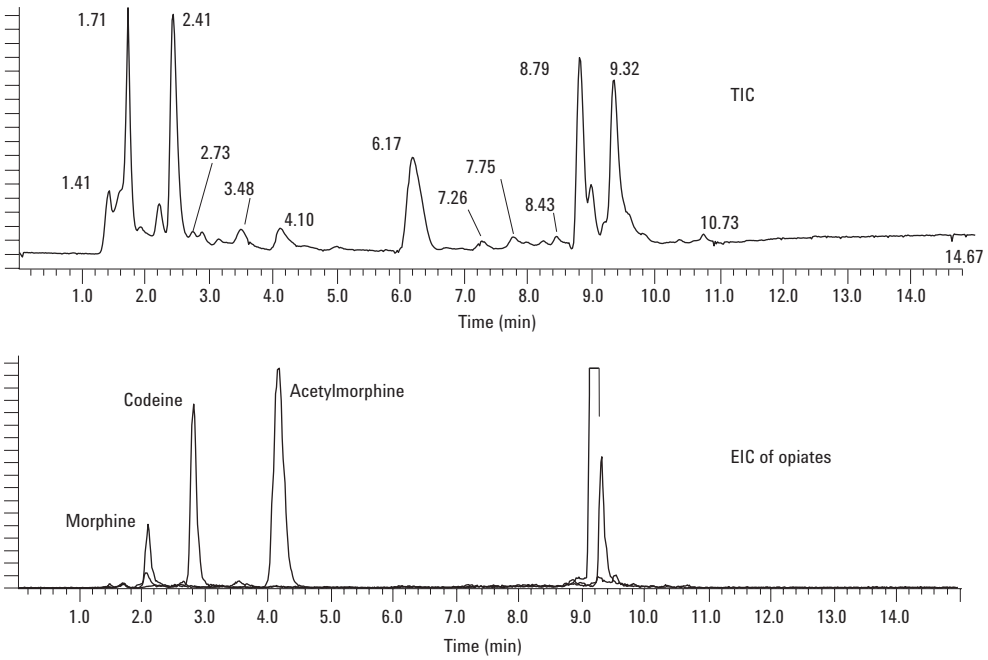


Figure 1. The upper panel shows the TIC of a direct urine injection spiked with 300 ng/mL of each opiate. The lower panel shows the EIC of each compound.

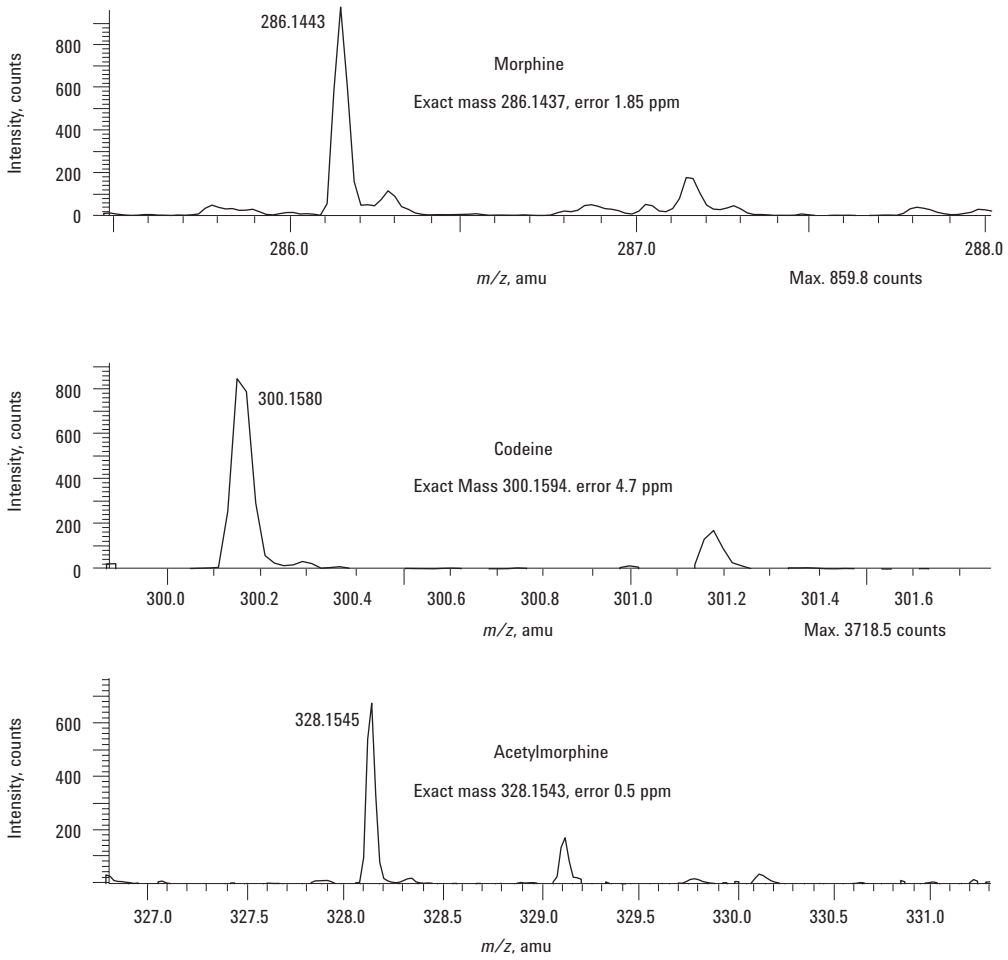


Figure 2. Mass spectra of M+H ions for opiates showing both mass resolution and mass accuracy at 2 pg on-column.

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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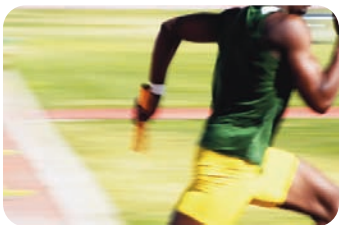
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Agilent Technologies







Applications by Instrumental Technique

LC/TRAP/MS



Detection of 23 Corticosteroids, Anabolic Steroids, and ß2-Agonist in Athens Doping Control Lab, During Athens 2004 Olympic Games Using Agilent 1100 LC/MSD Trap Mass Spectrometers

Application

Forensics

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Abstract

Anabolic steroids, corticosteroids and ß2-agonists are considered performance-enhancing drugs, intentionally regulated by most international sports agencies for the purpose of maintaining fairness in competition. Controlling the use of such drug usage among athletes should also be considered a health benefit. During the 2 weeks of the 2004 Summer Olympics in Athens, Greece, several ion trap instruments were used 24 hours per day for the analysis of extracted urine samples, meeting the World Anti-Doping Agency (WADA) guidelines for minimum required performance limits (MRPLs). In this report Agilent 1100 Series HPLCs are coupled with LC/MSD Trap SLs, each using an electrospray ionization source (ESI) for the analysis of controlled substances. The MRPLs of 10 ng/mL urine for the anabolic steroids, 30 ng/mL urine for the corticosteroids and ß2-agonists are easily met. For example, 1 ng/mL urine for triamcinolone acetonide and less than 0.5 ng/mL for the designer drug tetrahydrogestrinone (THG). Moreover, the instrument sensitivity is shown to be maintained for the entire 2 weeks even though the sample load was extensive.

Introduction

Most international sports agencies introduced drug testing by the 1970's. The use of anabolic steroids was becoming widespread, especially in strength events, but there was no way of detecting them yet. A reliable test method was finally introduced in 1974 and the International Olympic Committee (IOC) added anabolic steroids to its list of prohibited substances in 1976. This resulted in a marked increase in the number of drug-related disqualifications in the late 1970's, notably in strength-related sports such as throwing events and weightlifting.

Since then the list of performance enhancing drugs has expanded from anabolic steroids to include corticosteroids and ß2-agonists. Anabolic androgenic steroids are natural or synthetic substances which act in the same way as testosterone. That is, they propose the development of sex characteristics (androgenic effect) and the gradual growth of

muscle tissue (anabolic effect). Corticosteroids are the most powerful anti-inflammatory drugs available for modern medicine. Also, in large dosages these compounds can help recover an injured muscle or inflamed joint, improving athletic performance. Finally, ß2-agonists are used to treat asthma and are allowed only as inhaled medication for those who are otherwise limited by the condition.

Most of the compounds analyzed in this report are polar and thermally labile. Therefore, they are not only amenable to liquid chromatography/mass spectrometry (LC/MS) analysis, but also preferred over the more traditional technique for drug testing in urine of gas chromatography/mass spectrometry (GC/MS). Furthermore, the sensitivity required for measuring ion ratios of the compound-specific product ions, generated by MS/MS (MS2), is unparalleled in an ion trap mass spectrometer. Analyzing compounds using multiple stages of MS also eliminates the need for good chromatographic resolution, as required for ultraviolet (UV) quantitation, because co-eluting compounds are distinguishable. In addition, the specificity of MS/MS/MS (MS3) can further distinguish those compounds, which have identical precursor ion masses and similar product ion distributions. Not only does the sensitivity of the ion trap in product ion generation make it the preferred analytical technique over triple-quadrupole instruments, but their typically lower prices are also an important consideration.

In this work the patented SmartFrag capability of the LC/MSD Trap is implemented to generate reproducible fragment ion spectra at both the MS2 and MS3 stages. Using this technique, it is more likely that each precursor ion will receive exactly the energy it needs to produce an abundant and wider variety of product ions for structural confirmation. By optimizing the generation of certain product ions for each compound, a multiple reaction monitoring (MRM) technique is used for quantitation. It should also be noted that the stringent requirements for confirmation, according to the WADA guidelines, had to be met consistently for at least a 20-day period, analyzing more than 4000 samples, during which the Olympic events took place, so that linearity and reproducibility were critical to not only ensure fairness in competition, but to safeguard the reputation of the Doping Control Lab of Athens. The compounds analyzed are listed below.

Corticosteroids

Triamcinolone, 16α-hydroxyprednisolone (budesonide metabolite), cortisol, prednisolone, fludrocortisone, prednisone, cortisone, methylprednisolone, betamethasone, dexamethasone, flumethasone, beclomethasone, flunisolide, fluocortolone, triamcinolone acetonide, desonide, budesonide.

ß2-Agonist

Formoterol.

Anabolic Steroids

Trenbolone, epitrenbolone (trenbolone metabolite), gestrinone, THG, hydroxymethylformebolone (formebolone metabolite).

Internal Standard (ISTD)

Methyltestosterone

Note: THG (tetrahydrogestrinone) is a designer steroid derived from another banned steroid, gestrinone. See Figure 1.

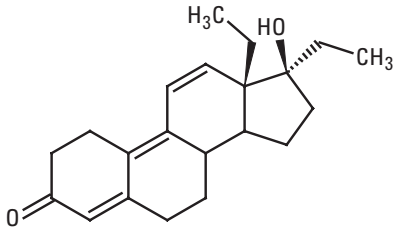


Figure 1. Designer steroid THG.

Experimental

Sample Preparation

Standard extraction procedure for GC/MS analysis is used except for the final step involving deriviatization. Instead, the sample is reconstituted in the LC mobile phase.

To a 2.5-mL urine sample is added the internal standard (methyltestosterone). An enzymatic hydrolysis is then performed with ß-glucuronidase (Helix Pomatia) at pH 7, at 50 °C for 1.5 hours. Alkaline extraction at pH 10 with 5.0 mL diethylether, using 1 g of sodium sulphate as the desalting agent. Agitation for 20 min followed by centrifugation. The ether phase is transferred to a conical tube and evaporated with nitrogen flow to dryness. Finally, the urine extract was reconstituted with 100 µL of mobile phase.

LC/MS/MS Method Details (example)

LC Conditions

Instrument:	Agilent 1100 HPLC
Column:	ZORBAX RX-C8, 2.1 mm × 50 mm, 5-µm particle size
Mobile phase:	A = 0.1% acetic acid in water B = acetonitrile
Gradient:	15% B at 1 min 40% B at 8 min 40% B at 12 min 90% B at 15 min 100% B at 17 min Post Run: 4 min
Flow rate:	0.4 mL/min
Injection vol:	10 µL

MS Conditions

Instruments:	Agilent LC/MSD Trap SLs
Ionization mode:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	50 psig
Drying gas temperature:	325 °C
V_{cap}.	3500 V
Skim 1:	15.0 V
Capillary exit:	50.0 V
Scan:	*m/z* 100–400 amu.
Normal resolution:	13,000 amu/s
Averages:	2
ICC:	On Maximum accumulation time: 20 ms Target: 30000 MRM mode: 10 segments
Qualification:	
Fragmentation amplitude:	1.0 V
SmartFrag:	On, 30%–200%
Quantitation:	
Fragmentation amplitude:	Optimized for each compound

Results and Discussion

One of the limitations of many ion trap MS systems is that fewer product ions exist because of the relatively short time precursor ions are held in the ion trap. The use of the patented SmartFrag feature of the Agilent LC/MSD Trap was central to its successful use for identification of the compounds analyzed during the 2004 Summer Olympic Games in Athens, Greece. An example of the abundant variety of product ions using SmartFrag ramped collisionally induced dissociation (CID) technique is shown in Figure 2. This technique ramps the fragmentation energy from 30% to 200% (user-adjustable) of the user-designated fragmentation voltage, one volt in this work. In this example, we applied the Smart Fragmentation feature of the LC/MSD trap to produce an optimal set of fragment rich MS/MS spectra for prednisone, as shown in Figure 2C.

Unlike traditional ion traps, the Agilent LC/MSD Trap CID voltage energy is quickly ramped over the range of energies, which results in the optimum energy for each analyte. The SmartFrag feature of the Agilent LC/MSD Trap eliminates the need for time-consuming collision voltage optimization. The analyst efficiently obtains fragment-rich, full-scan product ion spectra for confirmation of identity or structural elucidation.

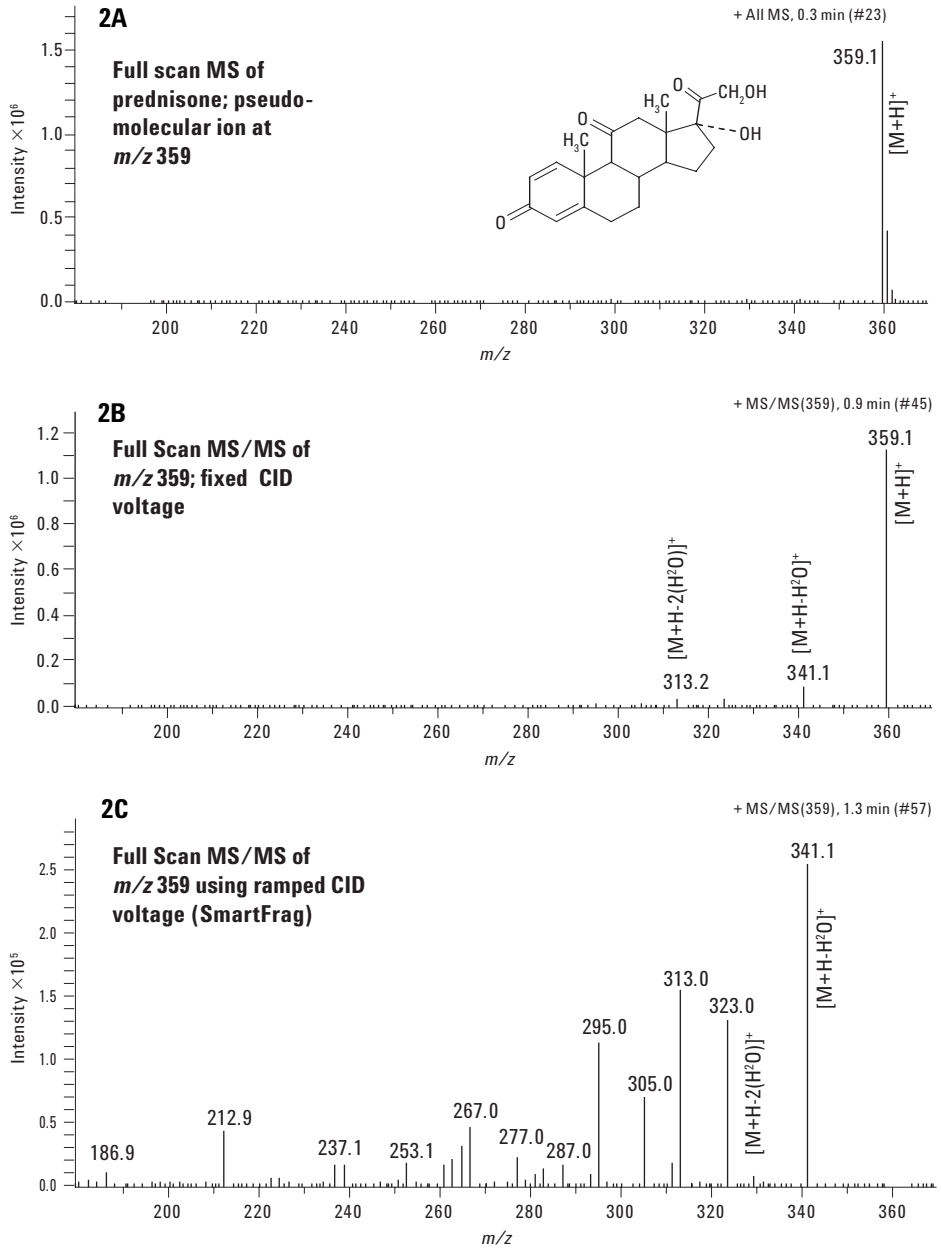


Figure 2. The usefulness of SmartFrag is demonstrated with the compound prednisone.

Derived mass spectra should produce characteristic ions for the substance's structure. Product ion abundances over 5% relative abundance, as required by WADA, offer a rapid comparison tool for the verification of the presence of a prohibited substance in an athlete sample with respect to standard quality control (QC) samples run with every sequence (blank urine samples spiked with MRPL concentrations of the prohibited substances).

An example of confirmation by identification is shown in the verification of a positive case in which an athlete was given triamcinolone acetonide for therapeutic use. Figure 3 shows the results of a library match for the sample compound mass spectrum, with background subtraction. The Fit, reverse fit (RFit), Purity (NIST algorithm) values are each greater than 950 out of a possible 1000.

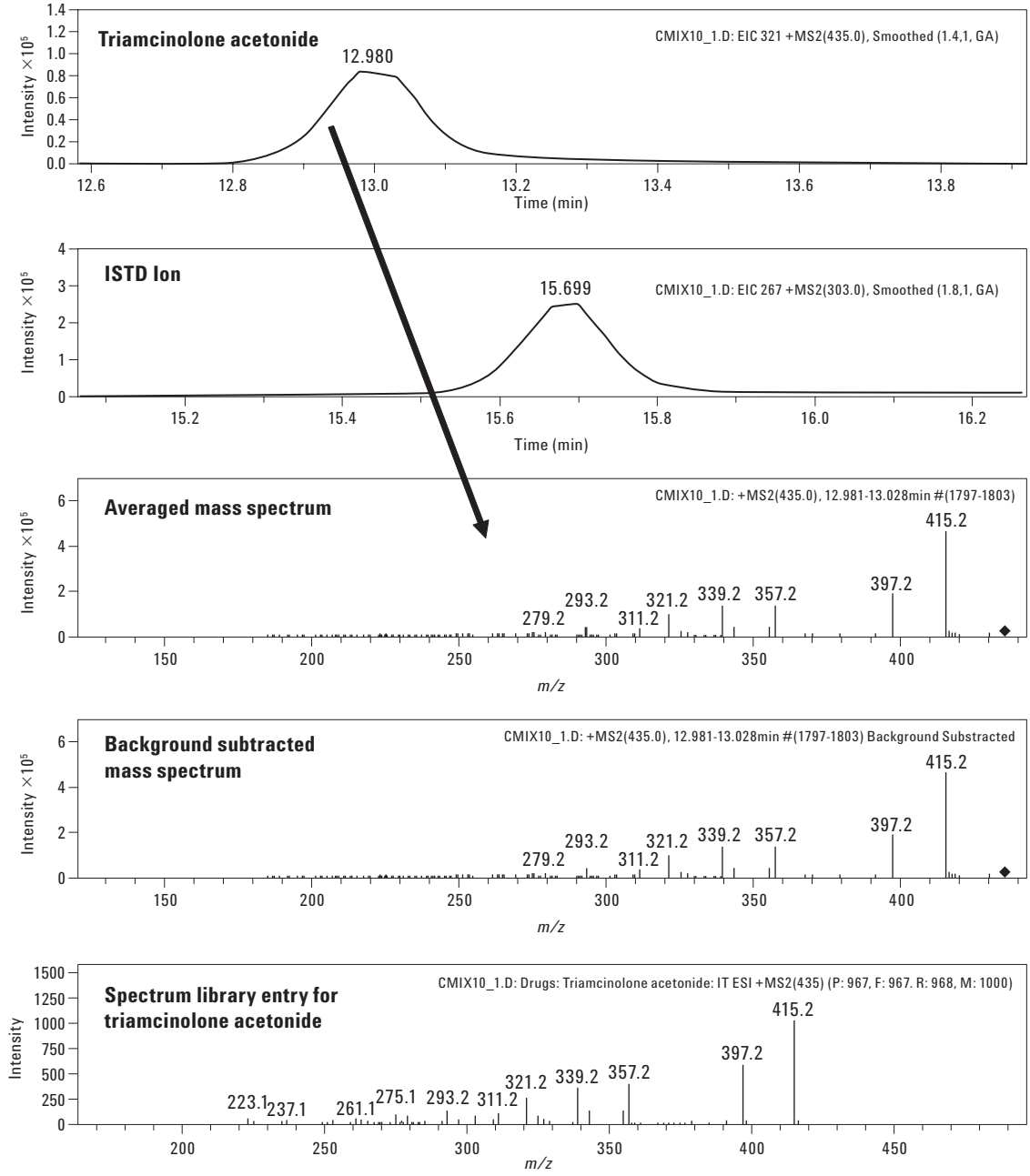


Figure 3. Positive case verification procedure - athlete sample. Library match: (P)urity = 967; (F)it = 967; and (R)everse Fit = 968.

Along with confirmation, QC samples run in every sequence were used to monitor system stability throughout the duration of testing at the Olympics. Figure 4 shows the reproducibility of quantitation with the LC/MSD Trap SL for the 20-day period. Each instrument analyzed more than 1100 samples without any routine maintenance beyond the easily accessed source chamber region.

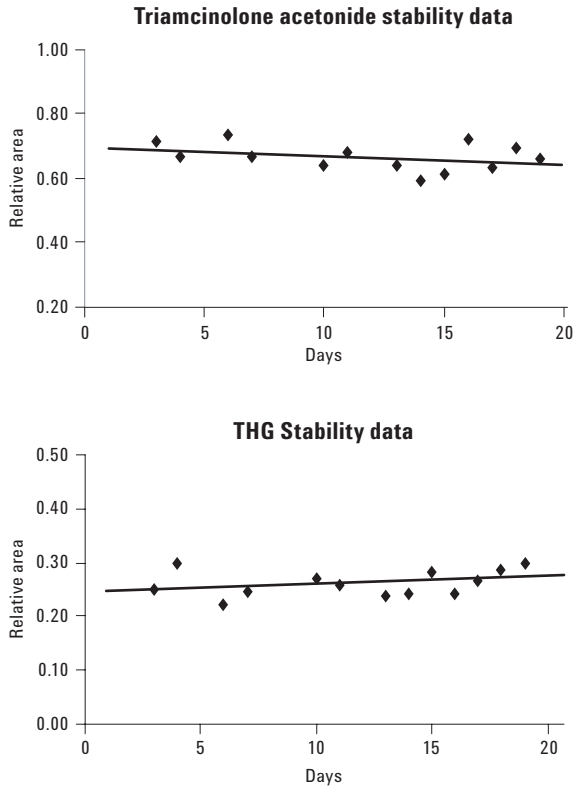


Figure 4. System stability expressed as substance area (triamcinolone acetonide and THG), divided by ISTD area (methyl testosterone).

As a demonstration of linearity, the results of testing three different compounds prior to the start of the Olympic games are shown in Figure 5. Each calibration level is calculated as the average of two identical samples, each run twice (four runs total). The Relative Response is calculated by dividing the substance area by the ISTD area. All calibration curves show a linearity coefficient, r^2, of at least 0.99, with nearly zero intercepts. Furthermore, the highest calibration point for each plot is the WADA MRPL limits, which means that system sensitivity is far below these limits.

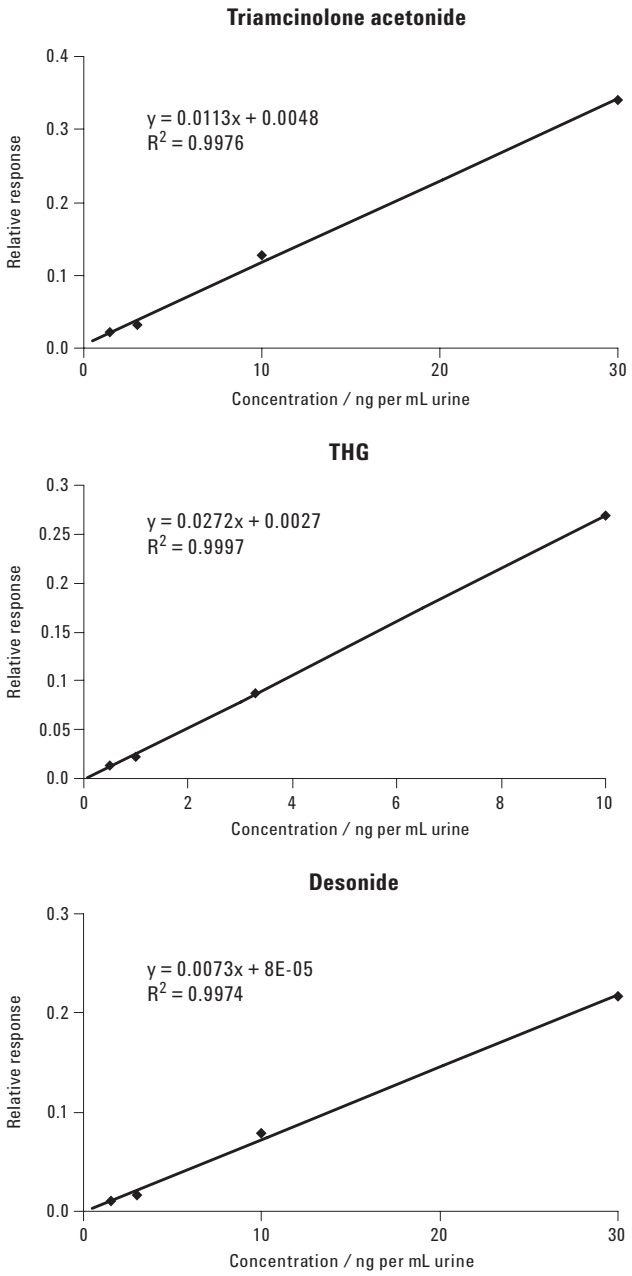


Figure 5. Demonstration of system linearity using the compounds triamcinolone, THG, and desonide.

An example printout of a QC sample is shown in Figure 6, consisting of six compounds, including the ISTD, being analyzed each with three confirmation ions in a vertical descending order of intensity. Relative to this printout, confirmation of drug testing failures can be easily identified just by visual comparison.

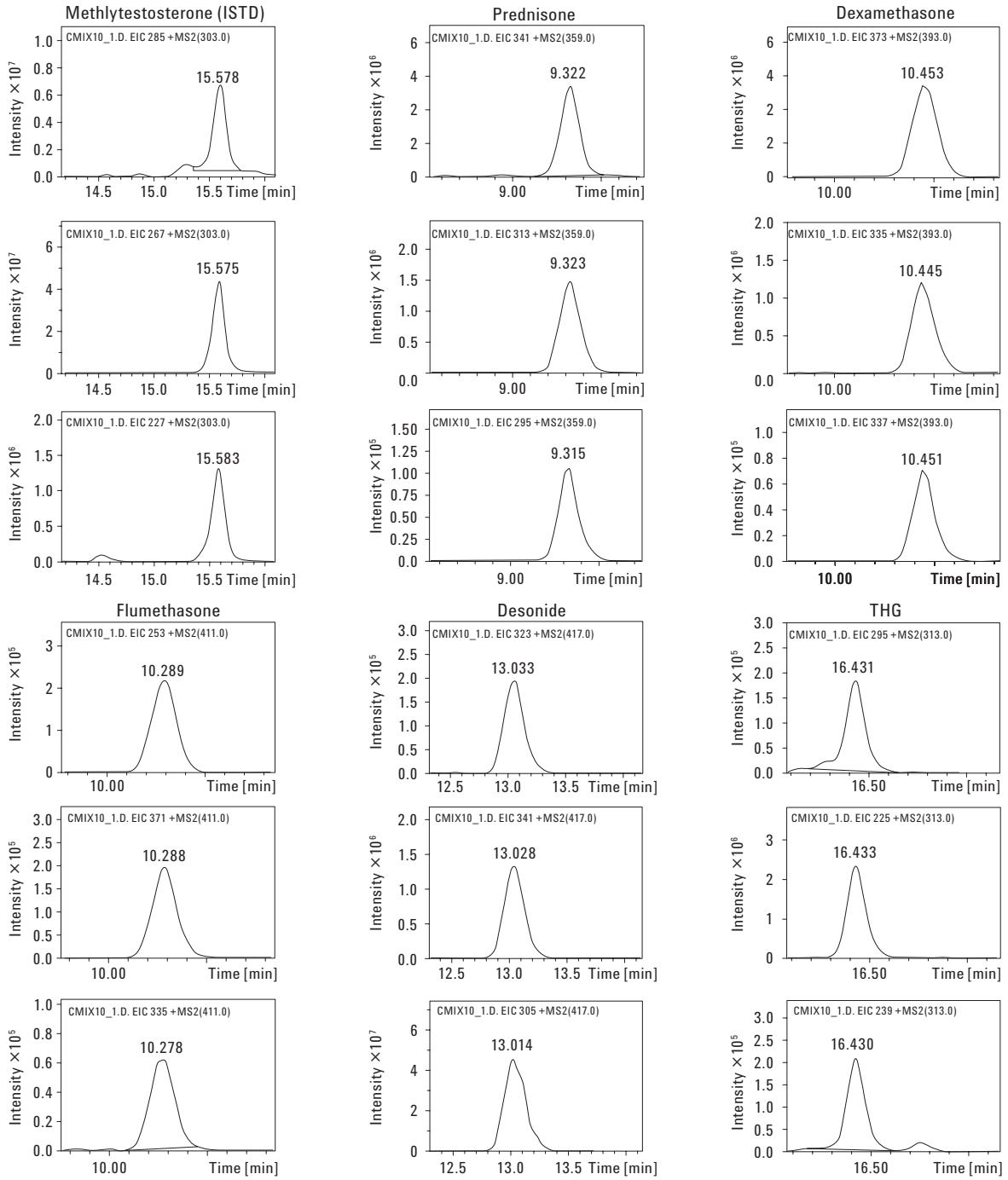


Figure 6. Screening QC sample printout.

Returning to the positive test case verification involving the athlete sample (library match shown in Figure 3), the quantitation of triamcinolone acetonide is confirmed as being below 15 ng/mL urine, but cannot be specified further for the sake of privacy. See Figure 7.

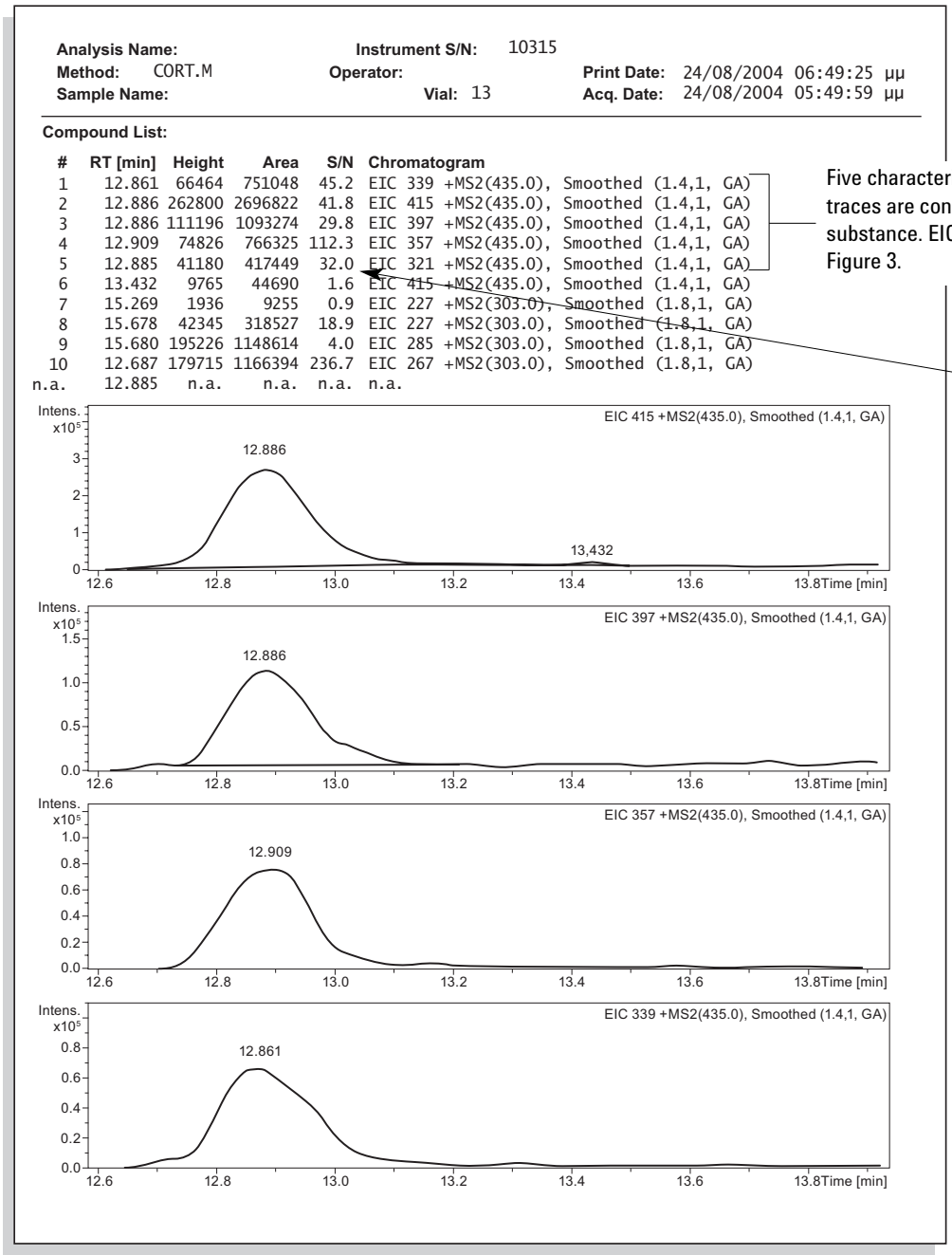


Figure 7. Positive case verification - athlete sample, by quantitation.

Conclusions

This work demonstrates the robust operation of the Agilent LC/MSD Trap SL for the purpose of testing for the presence of performance-enhancing drugs in Olympic athletes during the 2004 Summer Olympic Games in Athens, Greece. The sensitivity requirements of WADA are easily met using the full scan MS/MS capabilities of the LC/MSD Trap. Moreover, the patented SmartFrag technique involving a ramping of the fragmentation voltage being applied to the precursor ion, produces abundant and specific product ions at both the MS2 and MS3 levels for unique identification and confirmation of the presence of as many as 23 separate controlled substance compounds in urine.

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Confirming the Presence of Drugs of Abuse in Samples Using an LC/Ion Trap Mass Spectrometer and a Nearly 400 Drugs Compound Library Containing MS^2 and MS^3 Spectra

Application

Forensics/Toxicology

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Abstract

The presence of drugs of abuse is confirmed in samples analyzed in three independent labs using a library of almost 400 drugs of abuse and pharmaceutical compounds containing MS/MS (MS^2) and MS/MS/MS (MS^3) spectra. By ramping the collision energy during the fragmentation stage of analysis, reproducible product ion spectra are generated for the library. By doing this in an ion trap, the spectra represent specific pathway steps of fragmentation. In order to identify the compounds in real samples, the generation of product ion spectra in those samples is also carried out using ramped collision energy.

The process of confirmation includes the generating of compound spectra in the sample data, followed by NIST-based scoring of Fit, Reverse Fit, and Purity matching of library spectra. Searching of the MS spectra is not included because matrix interferences can result in poor scoring. Besides, the precursor ions seen in the MS scan are recorded in the MS^2 and MS^3 level scans.

Finally, an automated procedure for processing the data to generate compound spectra and search them, followed by printing, is presented. The printing of results is limited to just those compounds that are identified, but the overall scoring for each compound takes into account that multiple spectra at multiple levels of fragmentation may match spectra of different compounds in the library. It is this combination of spectral matching at the MS^2 and MS^3 levels that makes searching ion trap libraries not only a unique tool for identification, but also more reliable if a reproducible technique for generating product ions, such as a ramped collision energy, is employed.

In this work, a library has been generated by the Riverview Hospital using an Agilent LC/MSD Trap XCT mass spectrometer, operated with a ramped collision energy of 30% to 200% of the set collision energy, which in this case was 1 V. The ramped voltage of 0.3 to 2.0 V was implemented for 40 msec to generate MS^2 spectra from an isolated precursor ion. The process was repeated on an isolated product ion to produce MS^3 spectra where possible.

This library is used to confirm the presence of certain drugs in a sample analyzed by Riverview Hospital. The library is then used to identify samples at St. Olav's Hospital and Athens Doping Center that they analyzed by their respective Agilent LC/MSD Trap systems. In each case, spectral matches are shown to demonstrate the reproducible nature of generating product ion spectra on the Agilent LC/MSD Trap mass spectrometer. Implementation of automated analysis is also demonstrated.

Introduction

The ion trap mass spectrometer is known for having the most sensitivity for full-scan MS^2 acquisition. Fragment ion spectra are considered "fingerprints" of molecules because they contain product ion masses that correspond to smaller pieces of the overall structure of the molecule. Although other mass spectrometers like the QQQ and the QTOF are able to produce product ions, the process is not exactly the same, which leads to a different characteristic of fragment ion spectra. In the case of a QQQ or QTOF instrument, both of which produce true MS/MS spectra, the product ion spectra typically represent not only primary fragmentation of the precursor ion, but also secondary, tertiary, and so on. That is, after the precursor ion is fragmented while still near the entrance to the collision cell, the produced fragment ions may undergo further fragmentation before exiting toward the third quadrupole.

As a result, the final MS/MS spectrum produced from either the QQQ or QTOF will include product ions that may have come from the precursor or from other product ions. Therefore, fragmentation pathways are unknown. On the other hand, an ion trap is much more selective in fragmenting precursor ions. Following the isolation process, energy is added into the system corresponding to the frequency of oscillation of the precursor ion. The frequency of oscillation is directly related to the mass of the precursor. The precursor ion then absorbs the additional energy and fragments. This is known as resonance excitation.

However, since the produced fragment ions have different masses from the precursor, and thus have different frequencies of oscillation, they do not absorb the additional energy so they are not further fragmented. As a result, the product ions seen in the MS/MS spectrum had to come directly from the precursor ion. Further isolation of a product ion, followed by fragmentation, produces product ions that also lie on a direct pathway back to the precursor ion. Consequently, the ion trap is considered to be a softer, but more specific, MS/MS mass spectrometer than the QQQ or QTOF. Furthermore, the product ion spectra are typically cleaner in the ion trap than in the other instruments. The additional step of forming MS^3 product ions increases the specificity of the precursor ion structure, making the ion trap a unique instrument for structural elucidation.

The caveats of trying to generate MS^3 spectra is that there may not be enough precursor ion left at the MS^2 level to generate an appreciable signal at the MS^3 level or the MS^2 fragment ion may be too stable for further fragmentation. In either case, MS^3 spectra are not available.

Along with increased specificity, there are a couple more benefits to acquiring MS^3 spectra. One is that many compounds may only fragment to form water-loss ions $(M + H - H_2O)^+$, which are not very informative. A further generation of fragment ions in the MS^3 spectra may increase information content drastically. Another benefit of generating MS^3 spectra is that co-eluting, isobaric interferences may contribute product ions at the MS^2 level, which are filtered out at the MS^3 level. Therefore, MS^3 spectra are typically very clean.

The Agilent LC/MSD Trap has an additional feature that enables it to outperform its competitors by generating reproducible product ion spectra. This reproducibility from instrument to instrument is based primarily on the technique known as SmartFrag, which involves a ramping of the fragmentation energy (in volts) during the sequence of fragmentation in the ion trap. The default range of 30% to 200%, with a set value of 1 V, is typically used because 0.3 V (30%) is generally not enough to fragment most precursor ions, and 2.0 V (200%) is too much. However, using a range with such extremes means that the onset of fragmentation for any particular molecular ion will almost always be within the range.

In general, the onset of fragmentation, or the lowest voltage to produce fragments, results in the production of higher mass fragment ions. By ramping to higher voltages, smaller and smaller fragment ions are produced. The overall result is to produce fragment ions across a wide product ion mass range, which is very useful for library identification. (See Figure 1 for an example of how this works.) Furthermore, such a wide range takes into account subtle differences between ion traps in terms of geometry and buffer gas pressure in the ion trap mass analyzer. This results in the ability to generate a library of spectra on one instrument and use it for searching on other instruments.

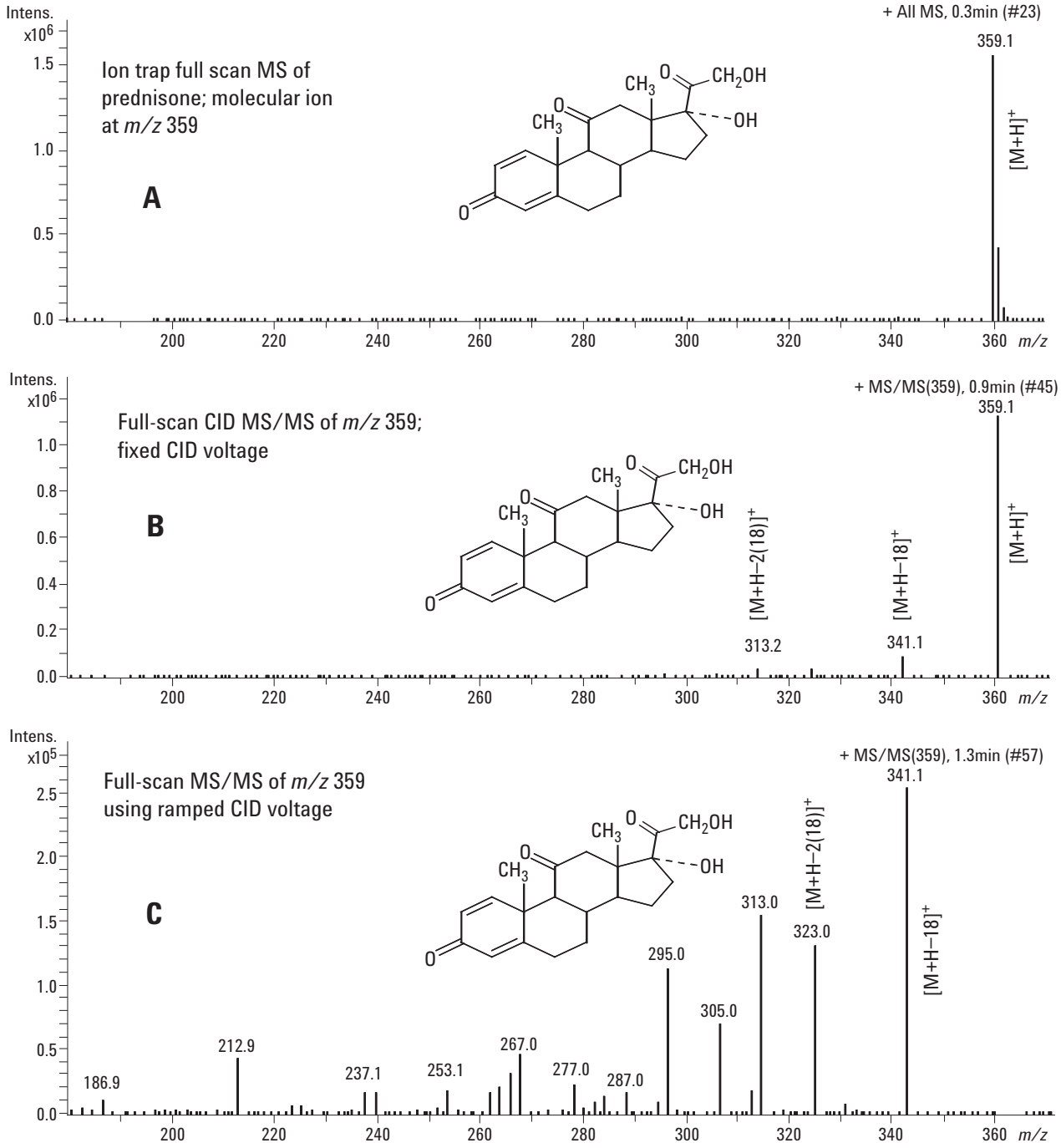


Figure 1. Illustration of using SmartFrag to generate product ion spectra of prednisone across a wide mass range. Spectrum A represents no fragmentation at all. Spectrum B represents a typical fragmentation voltage of 1 V, which produces very little fragmentation. Spectrum C is the result of implementing SmartFrag, or a ramp of 0.3 to 2.0 V, to produce fragment ions across a wide mass range. Note that no precursor ion intensity remains in Spectrum C.

Table 1. List of 386 Drug Compounds in "Drugs_lib" Library

7-Acetamidoclonazepam
7-Acetamidonitrazepam
8-Chlorotheophylline
Acebutolol
Acetamidoclonazepam
Acetaminophen
Acetazolamide
Acetohexamide
Acetylaminoglutethimide
Acetylcodeine
Acetylprocainamide
Acyclovir
Alfentanil
Alphaprodine
Alprazolam
Amantadine
Aminoclonazepam
Aminoflunitrazepam
Aminoglutethimide
Amiodarone
Amisulpiride
Amitriptyline
Amlodipine
Amobarbital
Amoxapine
Amphetamine
Anhydroecognine
Anhydroecognine methyl ester
Anileridine
Aprobarbital
Atenolol
Atomoxetine
Atropine
Azatadine
AZT
Baclofen
Bamethane
Benzoylecgonine
Benzoylecognine
Benztropine
Bosentan
Bromacil
Bromazepam
Bromodiolone
Brompheniramine
Bupivacaine
Buprenorphine
Bupropion
Butabarbital
Butalbital
Butorphanol
Butriptyline
Caffeine
Captopril
Carbamazepine
Carbamazepine epoxide
Carboxytetrahydrocannabinol
Carbromal
Carisoprodol
Celecoxib
Cephalexin
Cetirizine
Chlordiazepoxide
Chloroquine
Chlorpheniramine
Chlorpromazine
Chlorpropamide
Chlorprothixene
Cimetadine
Cimetidine
Citalopram
Clenbuterol
Clobazam
Clomipramine
Clonazepam
Clonidine
Clozapine
Cocaethylene
Cocaine
Codeine
Colchicine
Corticosterone
Cortisone
Cotinine
Cyclizine
Cyclobenzaprine
Cyclophosphamide
Cyproheptadine
Debrisoquine
Demoxepam
Desalkyldisopyramide
Desalkylflurazepam
Desipramine
Desmethylchlordiazepoxide
Desmethylclobazam
Desmethylclomipramine
Desmethyldiazepam
Desmethyldoxepin
Desmethylflunitrazepam
Desmethylmaprotiline
Desmethylmethsuximide
Desmethylnaproxen
Desmethyltrimipramine
Dexamethasone
Dextromethorphan
Diazepam
Diazoxide
Diclofenac
Diethylpropion
Dihydroergotamine
Diltiazem
Dimethoate
Dinoseb
Diphenhydramine
Diphenoxylate
Diphenylpyraline
Dipyridamole
Disopyramide
Donepezil
Dopamine
Doxepin
Doxylamine
Droperidol
Ecognine
EDDP
Emetine
Enalapril
Encainide
Ephedrine
Eprosartan
Erythromicin
Ethylmorphine
Fenazepam
Fenfluramine
Fenoprofen
Fenoterol
Fentanyl
Flecainide
Fluconazole
Fludrocortisone acetate
Flunarizine
Flunitrazepam
Fluoxetine
Flupenthixol
Fluphenazine
Flurazepam
Fluvoxamine
Furosemide
Gabapentin
Gentamicin
Gliclazide
Glipizide
Glyburide
Glyphosate
Guaifenesin
Haloperidol
Heroin
Hexobarbital
Hydrochlorothiazide
Hydrocodone
Hydrocortisone
Hydromorphone
Hydroxyalprazolam
Hydroxyamoxapine
Hydroxybupropion
Hydroxychloroquine
Hydroxydesipramine
Hydroxyhaloperidol
Hydroxyrisperidone
Hydroxytriazolam
Hydroxyzine
Ibuprofen
Imipramine
Indomethacin
Isoniazid
Isoproterenol
Itraconazole
Ketamine
Ketazolam
Ketoconazole
Ketoprofen
Ketorolac
Labetolol
Lamotrigine
Leviteracetam
Levodopa
Levorphanol
Lidocaine
Loperamide
Lorazepam
Loxapine
LSD
Maprotiline
Mazindol
MDA

Table 1. List of 386 Drug Compounds in "Drugs_lib" Library (continued)

MDA-NH_3 loss
MDEA
MDMA
Mecoprop
Mefenamic acid
Melatonin
Meperidine
Mephenytoin
Mephobarbital
Mepivacaine
Meprobamate
Mescaline
Metanephrine
Metformin
Methadone
Methamphetamine
Methapyriline
Methaqualone
Methocarbamol
Methotrexate
Methotrimeprazine
Methsuximide
Methylclonazepam
Methyldopa
Methylecognine
Methylphenidate
Methylprednisolone
Methyprylon
Metoclopramide
Metoprolol
Metronidazole
Mexiletine
Midazolam
Mirtazapine
Moclobemide
Monoacetylmorphine
Morphine
Morphine glucuronide
Nadolol
Naloxone
Naltrexol
Naltrexone
Naproxen
Nefazodone
Nicotine
Nitrazepam
Nitrofurantoin
Nomefensine
Norclozapine
Norcocaine
Norcodeine
Norfentanyl
Norfluoxetine
Norketamine
Normeperidine
Normethadone
Normorphine
Noroxycodone
Noroxymorphone
Norpropoxyphene
Nortriptyline
Olanzapine
Orphenadrine
Oxazepam
Oxbutynin
Oxcarbazepine
Oxcarbazepine metabolite
Oxprenolol
Oxycodone
Oxymorphone
Oxyphenbutazone
Papaverine
Paraquat
Paroxetine
Pemoline
Penicillamine
Pentazocine
Pentobarbital
Pentoxyfylline
Pericyazine
Perphenazine
Phenacetin
Phencyclidine
Phendimetrazine
Phenelzine
Phenformin
Pheniramine
Phenobarbital
Phentermine
Phenylbutazone
Phenylephrine
Phenylpropanolamine
Phenytoin
Pholcodine
Picloram
Pimozide
Pindolol
Pipotiazine
Piroxicam
Pizotifen
PMA
Prazepam
Prazosin
Prednisolone
Prednisone
Prilocaine
Primidone
Procainamide
Procaine
Prochlorperazine
Procyclidine
Promethazine
Propafenone
Propoxyphene
Propranolol
Protriptyline
Pseudoephedrine
Psilocin
Pyrilamine
Pyrithyldione
Quetiapine
Quinidine
Quinine
Ramipril
Ranitidine
Reboxetine
Remifentanil
Risperidone
Ritalinic acid
Rivastigmine
Salbutamol
Salicylate
Scopolamine
Secobarbital
Serotonin
Sertraline
Sibutramine
Sildenafil
Sotalol
Stanozolol
Strychnine
Sufentanil
Sulfadiazine
Sulfamethoxazole
Sulfasalazine
Sulfinpyrazone
Sulfisoxazole
Sulindac
Sulpiride
Sumatriptan
Temazepam
Terbutaline
Testosterone
Tetrabenazine
Tetracycline
Tetrahydrocannabinol
Theophylline
Thiethylperazine
Thiopental
Thioproperazine
Thioridazine
Thiothixene
Tiaprofenic acid
Timolol
Tolazamide
Tolbutamide
Topiramate
Tramadol
Tranylcypromine
Trazodone
Triamcinolone
Triamterene
Triazolam
Trifluoperazine
Trihexyphenidyl
Trimeprazine
Trimethoprim
Trimipramine
Tripelennamine
Venlafaxine
Verapamil
Vigabatrin
Warfarin
Yohimbine
Zaleplon
Zanamivir
Ziprasidone
Zolpidem
Zomepirac
Zopiclone
Zuclopenthixol

In this article, the Experimental section is divided into subsections about the formation of the library, the application of the library to samples from two different labs around the world (Riverview Hospital and Athens Doping Lab), and the implementation of identifying compounds and reporting results in an automated fashion for a sample from a third lab (St. Olav's Hospital).

Experimental

Part 1 - Creating the Library

The library used in this work includes the compounds listed in Table 1, which have been analyzed in the lab of Riverview Hospital (Coquitlam, BC, Canada) using HPLC and an LC/MSD Trap XCT mass spectrometer. The library is simply called "Drugs_lib." Liquid chromatography is useful for removing background interferences from impurities that may exist even in compound standards. However, infusion using a syringe pump is also possible, especially if the standard solution is pure.

Each compound is run chromatographically as a standard with a fast elution time of about one minute. The details of the chromatography vary a little depending upon the sample, so they are not presented here. In addition, some compounds give appreciably better spectral signal in negative electrospray ionization (ESI) mode, while the analysis of some others results in much better signal using atmospheric pressure chemical ionization (APCI), whether positive or negative. However, the majority of the compounds show the best spectral response in positive ESI, especially those that are later confirmed in the analyses of St. Olav's Hospital and Athens Doping Lab.

The most relevant acquisition settings for generating the library spectra are outlined in Table 2. An Agilent LC/MSD Trap XCT mass spectrometer is used to acquire the spectra, which consist of one MS/MS (MS^2) and at least one MS/MS/MS (MS^3) spectrum. The spectra that are specific to each transition are then averaged together over the width of the eluting compound peak to produce clean spectra for each transition. The spectra are then transferred to the library for each compound so that the corresponding library entry contains one MS^2 and one or more MS^3 spectra (Figure 2).

Table 2. Mass Spectrometer Acquisition Settings for Library Creation

Parameter	Value
Ramped collision energy	0.3–2.0 V
Precursor isolation width	4 amu
Spectra acquired	1 MS, 1 MS^2, and ≥ 1 MS^3, if possible

A few comments should be made about which spectra are used in the library. First of all, although a full-scan MS, or just MS, acquisition is made as part of deciding which precursor ion to isolate and fragment, the spectrum itself is not used in the library. The reason for this is twofold. First of all, an MS spectrum may contain co-eluting interferences that degrade the spectrum, making it difficult for use in identifying unknowns.

The identification algorithm used in the Agilent ion trap software for matching each spectrum of an unknown compound with one in the library is NIST-based. Therefore, scores for Fit (F), Reverse Fit (RFit), and Purity (P) are obtained for each spectrum match. The Fit score is directly related to the number of spectral peaks in the library compound spectrum that are also found in the unknown. The converse is true for the RFit score, which is related to the number of spectral peaks in the unknown spectrum that are also in the library spectrum.

Therefore, if the library spectrum contains more peaks than the unknown, then the Fit score will be low. This is likely in the MS spectrum if there are co-eluting interferences in the spectral acquisition for the library. If the unknown spectrum contains interferences, then the RFit score will be low. As a result, it is preferable to simply not consider the MS spectra for purposes of identification.

Secondly, the most important information in the MS spectrum, which is the precursor ion mass, is stored in the MS^2 and MS^3 spectra because they are all generated from a fragmentation pathway that begins with the precursor ion.

As shown in Figure 2, each compound entry includes an MS^2 spectrum, and usually at least one MS^3 spectrum, the CAS number, chemical formula, and an image of the structure. In addition, the mass list of each spectrum undergoes a filtering in which any m/z value with an abundance less than 10 is removed from the spectrum. The reasoning for this is that the largest m/z is normalized to 999 and an abundance of 10 represents about 1.0%. The 1.0% threshold corresponds to the possible residual signal following the isolation step. That

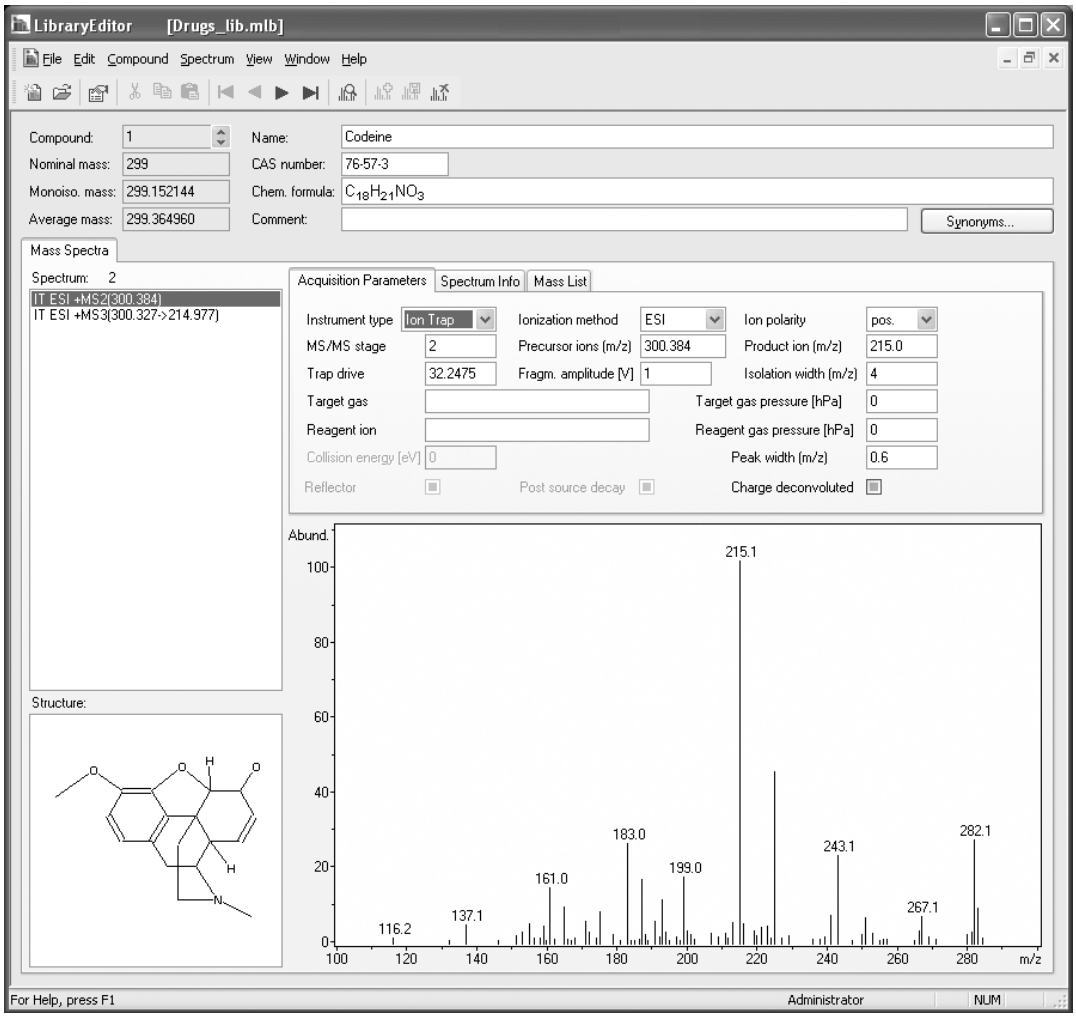


Figure 2. Library entry for cocaine compound containing one MS^2 and one MS^3 spectrum.

is, isolation of the precursor ion in an ion trap is better than 99%, but not quite 100%. Therefore, following the precursor ion isolation there may be as much as 1% of the signal remaining from an *m/z* ion outside the isolation window. Since this residual does not represent fragmentation of the isolated precursor ion, it is removed from the library spectrum.

Part 2 - Using the Library for Identification

Sample 1 (Riverview Hospital)

The first example of using this library to identify or confirm the presence of drug compounds is in a patient blood extract sample at the Riverview Hospital. Although the library spectra in Drugs_lib are obtained from standards run on the same LC/MSD Trap XCT instrument in the Toxicology Lab, the patient sample includes a blood extract matrix, which can interfere with the analyte signal as it co-elutes with the compounds of interest. Therefore, this first test of the library demonstrates the power of MS^2 to selectively remove sample matrix interferences.

The analysis of the patient sample is carried out using an "AutoMS(3)"(Figure 10) acquisition in which the signal is monitored in the MS only mode, or full- scan MS. When the intensity of any ion within the allowed selection window exceeds a minimum threshold value, that ion is then selected as a precursor ion and fragmented. Up to two precursor ions in each MS spectrum may be selected. Once the precursor ion is fragmented, any product ions exceeding a threshold for MS^3 are then fragmented. Only one precursor ion from each MS^2 spectrum may be chosen.

Since the resulting data file consists of MS, MS^2, and MS^3 spectra, a special algorithm in the software is used to generate corresponding

compounds, each of which include MS, MS^2, and MS^3 spectra related to each other by a common MS precursor ion within a time window of one minute. The time window is important because if another compound of the same *m/z* precursor ion mass in MS elutes later, it will not be confused with the earlier eluting compound.

Figure 3 shows the result of applying the special algorithm, also known as Find – Compounds | AutoMS(n), to a patient sample data file. Based on user-defined parameter settings in the algorithm, 10 compounds are found, each containing at least MS and MS^2 spectra, some containing MS^3, as shown in the figure. The next step is to identify these compounds using the Drugs_lib library, which also contains MS^2 spectra, and for most of the compounds, MS^3 spectra as well.

At this point, it should be noted that the "Identify" algorithm of the MSD Trap software is set to ignore the MS spectra of the compounds in the sample data file. Therefore, only MS^2 and MS^3 spectra of the sample will be compared with MS^2 and MS^3 spectra of the library.

The MS spectra of the sample data file are ignored for two reasons. First, the MS spectra contain co-eluting interferences, which can reduce the NIST-based scores for matching spectra. Second, the only information of importance in the MS spectrum is the precursor ion mass, which is stored in the MS^2 and MS^3 spectra anyway. If it is of interest to use adducts like sodium or potassium for identification, the MS data is still there for further investigation later.

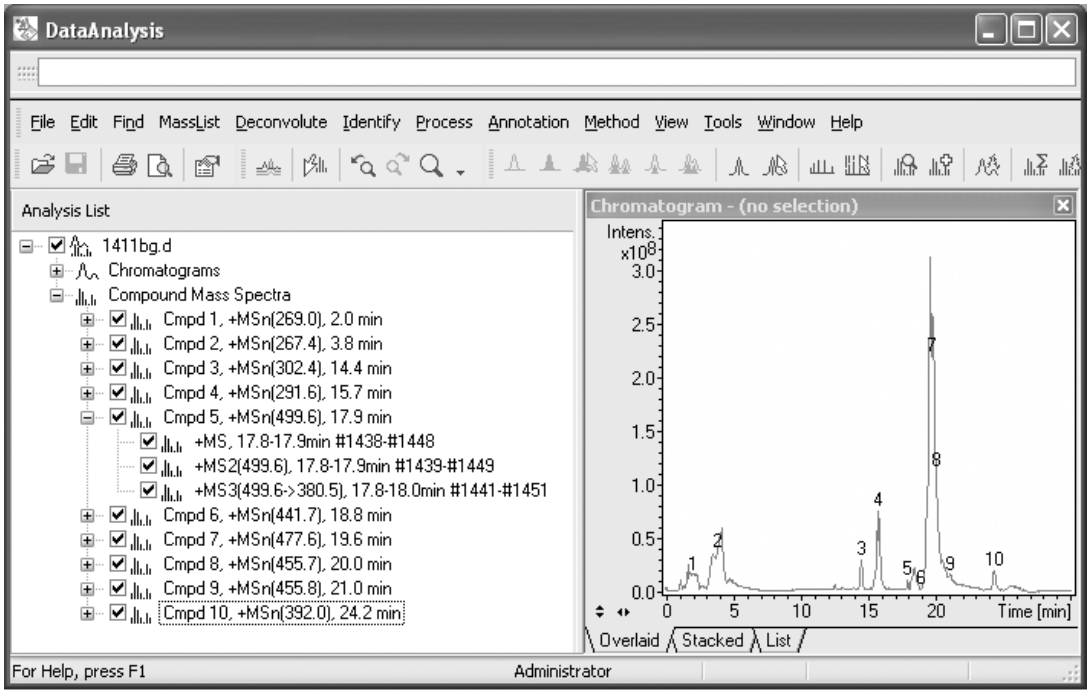


Figure 3. Ten compounds found in the patient blood extract sample data file named "1411bg." Note that the compounds found are based on user-defined parameter settings.

After identifying the compounds, any of the compounds that are not identified can be removed from the analysis list by using the Visual Basic script shown in Figure 4. By implementing this script, the number of relevant compounds is reduced from 10 to 5, as seen in Figure 5.

The five compounds are identified as shown in Figure 6. Obviously, compounds 4 and 5 are the same compound and representative of a large peak with substantial intensity spread out over more than one minute in elution.

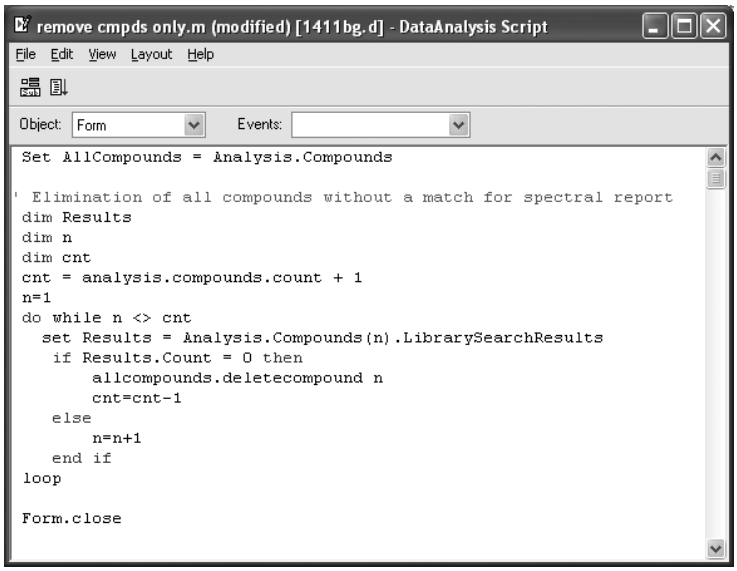


Figure 4. Visual Basic script used to remove any compounds not identified by the library. This can make reporting results more simplified.

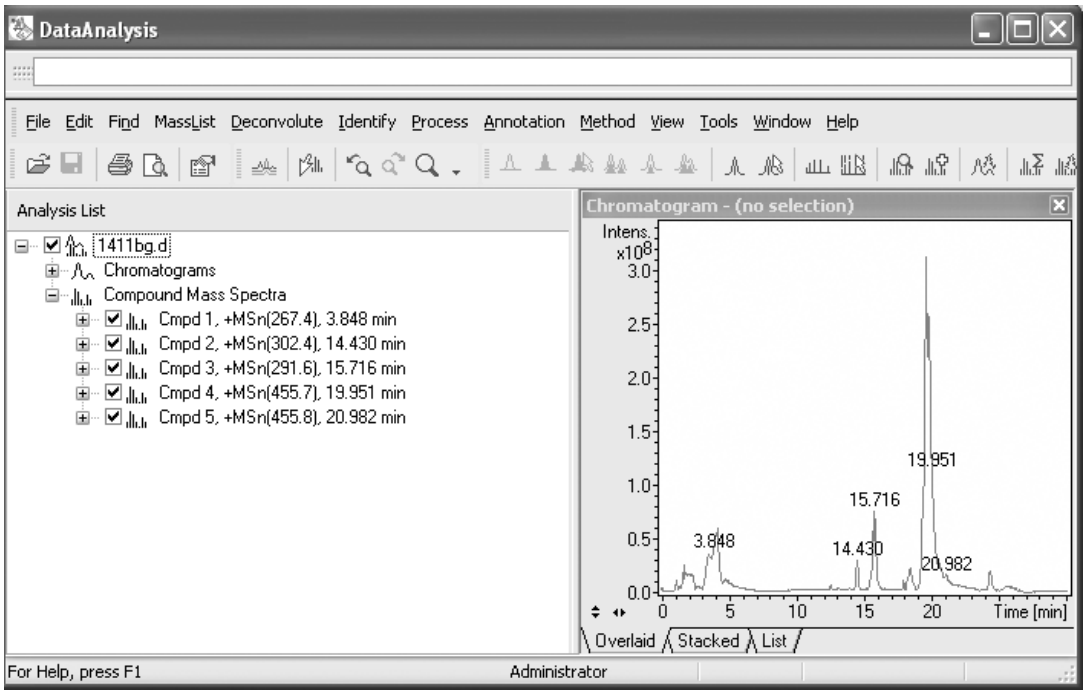


Figure 5. After searching the compound spectra of the sample data file, only five are identified with the library. The script shown in Figure 4 removes the compounds not identified.

Library Search Report - AutoMS(n)

Compound List:

#	RT [min]	MS(n) Isol. m/z	Compound Name	Fit'	RFit'	Purity'
1	3.848	267.4	Atenolol	998	998	998
2	14.430	302.4	Temazepam	998	998	998
3	15.716	291.6	Azatadine	1000	997	997
4	19.951	455.7	Verapamil	998	998	998
5	20.982	455.8	Verapamil	998	998	998

Figure 6. Compounds identified in patient blood extract sample include atenolol, temazepam, azatadine and verapamil.

The scores shown in Figure 6 (Fit', RFit', and Purity') are effective scores that take into account the matching upon multiple spectra between the samples' compounds and those in the library at both the MS^2 and MS^3 levels. As an example, consider the Fit' score for atenolol. The equation used for determining any effective score is given below.

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

Score' represents the effective score while Score represents the individual scores for each matched spectrum in the data file. The value for N is the total number of spectra and M is the number of identified spectra.

The Match score has to do with user-defined matching parameters. Looking at the results for atenolol in the data file (see Figure 7) the individual Fit scores for the identified spectra of Compound 1 are 991 for MS^2 and 1,000 for MS^3. The match values are maxima of 1,000 each because an MS^2 spectrum of the library has been matched to the MS^2 spectrum of Compound 1 and likewise for the MS^3 spectra. Had an MS^3 spectrum been matched to an MS^2 spectrum, the Match value would be 500.

The effective score for Fit, or Fit', is therefore $[(991 \times 1000 + 1000 \times 1000)/(2 \times 10^6)]^{1/2} \times 1000 = 998$, which is shown in Figure 6.

While the Fit, RFit, and Purity scores for each spectrum are from NIST-based algorithms, the handling of multiple spectral matches and different levels of MSn is unique to the MSD Trap data analysis software. This is an intelligent way of deriving an overall score, or effective score, for the compound in question.

Taking a look at the actual spectral matches, atenolol is once again used as an example. Figure 8 shows the MS^2 and MS^3 matches as seen in the remainder of the library report shown in Figure 6.

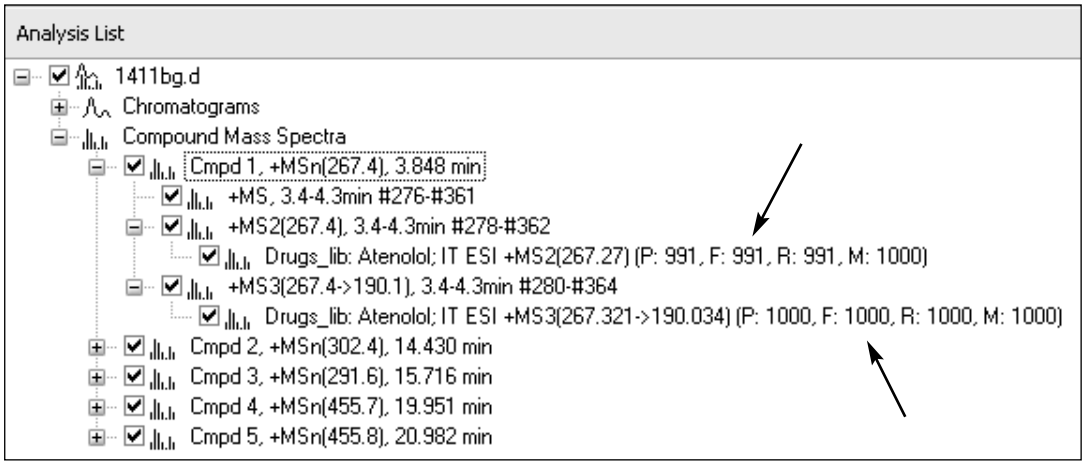


Figure 7. Individual library search scores shown for Compound 1 ("Cmpd 1"). The Fit score for the MS^2 spectrum is 991 and the Fit score for the MS^3 spectrum is 1,000, the maximum possible.

Figure 6 also shows that Compound 1 was only identified at atenolol and no other drug in the library. This demonstrates the power of using both MS^2 and MS^3 spectra for identification as well as the reproducible nature of using ramped collision energy to form fragments across a wide mass range.

Sample 2 (Athens Doping Lab)

The presence of corticosteroids in a urine extract sample is both confirmed and quantitated through targeted MS^2 with ramped collision energy, or SmartFrag. While it is better to use a specific collision energy for quantitation, the implementation of SmartFrag can still give good sensitivity in full-scan MS^2 while at the same time providing the spectra for identification.

In a particular sample known as "CMX_12," the presence of triamcinolone, corticosterone, hydrocortisone, prednisolone, prednisone, methylprednisolone, and dexamethasone is confirmed through library identification and quantitated. Figures 9A and 9B show two examples. Although no MS^3 data is acquired for this sample, the scores for the MS^2 matches are all greater than 900. However, it should be noted that the effective scores would be much closer to the maximum of 1,000 if MS^3 spectra are also acquired. The reason it is not included here is to save time for acquiring enough signal in MS^2 for quantitation.

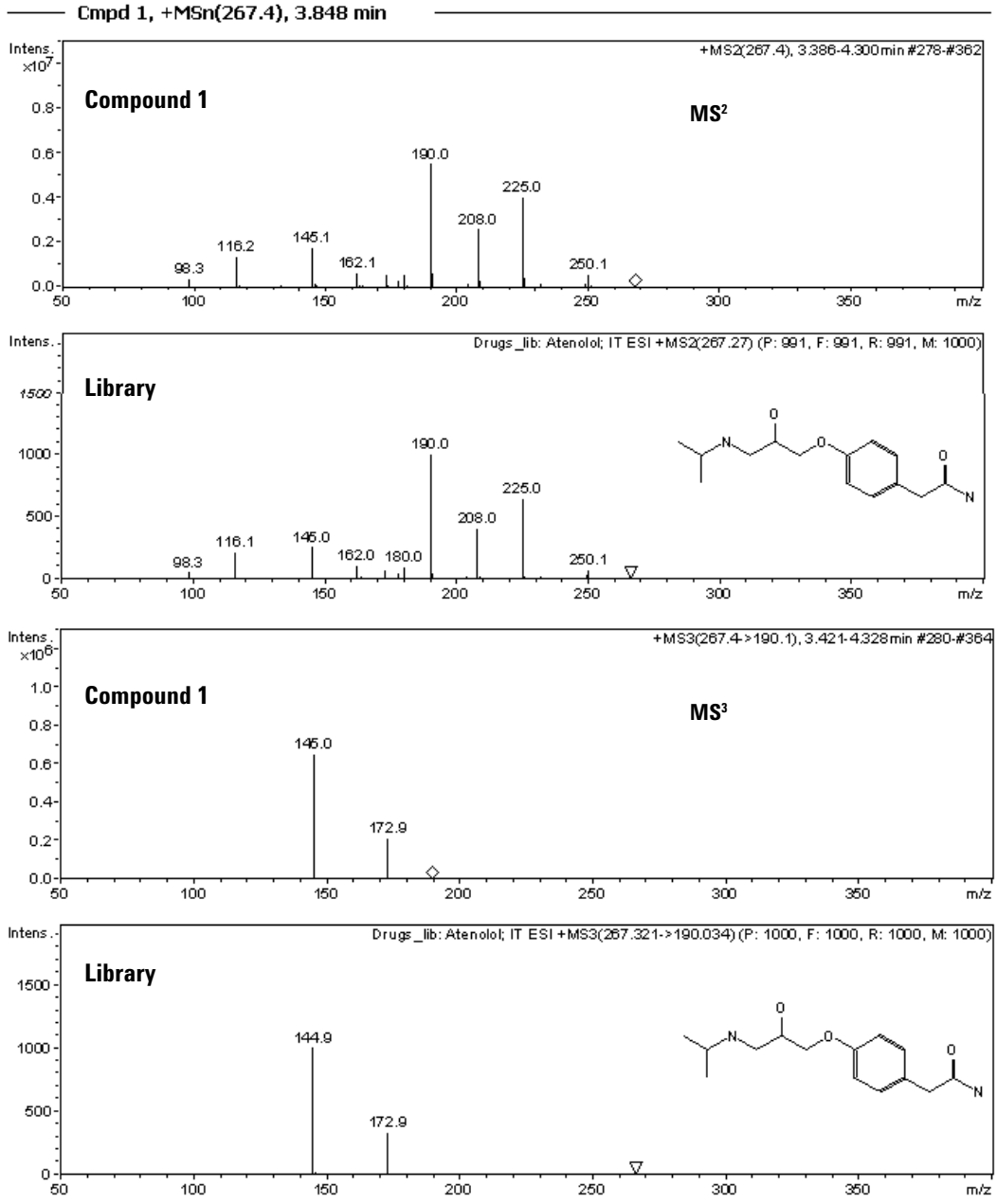


Figure 8. Matching of Compound 1 spectra to library spectra of atenolol in Drugs_lib.

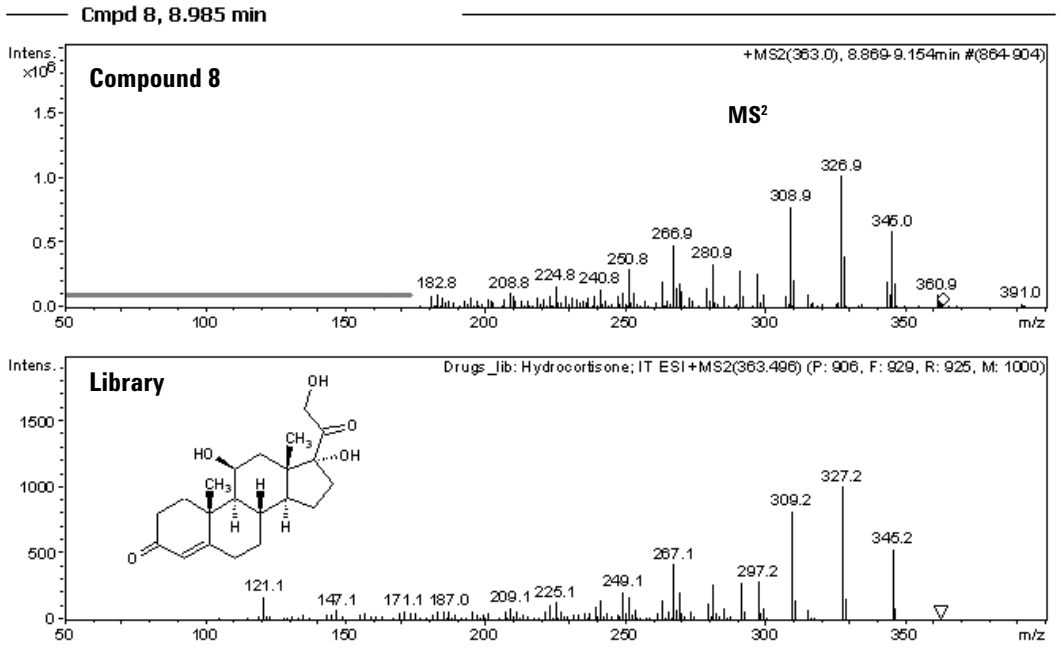


Figure 9A. Matching of Compound 8 spectra to library spectra of hydrocortisone in Drugs_lib.

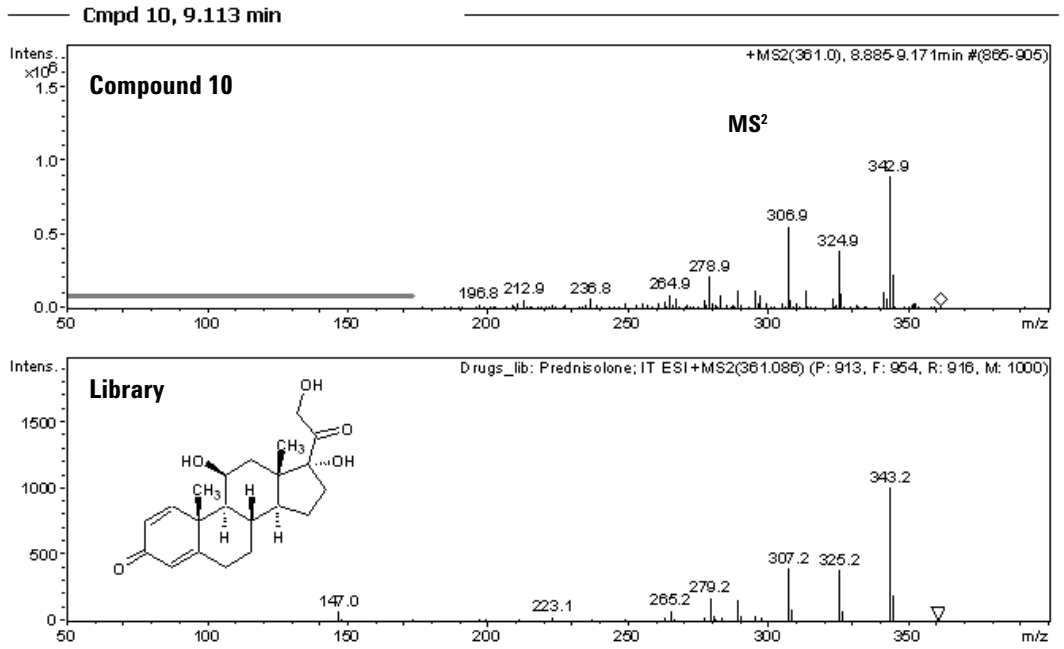


Figure 9B. Matching of Compound 10 spectra to library spectra of prednisolone in Drugs_lib.

Because there are no MS^3 spectra to also search, the effective scores reported in the library report, using the equation given above, are the same as those shown in Figures 9A and 9B. Furthermore, it should be noted that even though the mass assignments differ by as much as 0.3 amu, the spectra are remarkably similar in appearance for comparing data from an Agilent ion trap in the lab of Riverview Hospital and an Agilent ion trap in the Athens Doping Lab. In fact, the two models of ion traps are different as well. The ion trap of the Athens Doping Lab is the LC/MSD Trap SL and predecessor to the LC/MSD Trap XCT, which is at Riverview Hospital.

Part 3 – Using the Library for Automated Screening

High-throughput screening of drugs of abuse is performed in the Department of Clinical Pharmacology (DCP) at St. Olav's Hospital

(Trondheim, Norway) by LC/MS, which performs more than one million analyses per year. Traditionally, screening is performed by immunoassay, which can be expensive and less selective than mass spectrometry. The samples that are positive in the screening process are then traditionally confirmed using GC/MS, which requires derivatization as part of the sample preparation process. In such an environment, confirming the presence of compounds using an LC/MS/MS library in an automated fashion can be extremely valuable.

In this part, the results of carrying out a confirmation analysis on an Agilent LC/MSD Trap XCT mass spectrometer, located at DCP, is demonstrated. Once again, SmartFrag is used. This time, the compounds of the screened samples are analyzed by the LC/ion trap using data-dependent acquisition in which a list of precursor ions is referenced. Therefore, AutoMS(3) is once again used, but this time there is an inclusion list of *m/z* values corresponding to the presence of molecular ions being confirmed. In this way, the ion trap is monitoring the MS acquisition to see if any of these ions appear above a particular background threshold. If they do, the ion trap will isolate and fragment them. If any product ions in the MS^2 stage are above a user-defined level, then the corresponding product ions will in turn be isolated and fragmented.

The acquisition settings are shown in Figure 10. The list of ions in the Include list shown in Figure 10 is defined in Table 3.

Once data acquisition is completed for each data file in a sequence, the data file is automatically sent to the LC/MSD Trap DataAnalysis program, where a Visual Basic script is applied to first find all compounds in the data file. This is done as discussed previously (see also Figure 3) by generating all possible MS, MS^2, and MS^3 extracted ion chromatograms (EICs), locating the peaks in these EICs and then associating the spectra under these peaks with each other according to the MS-based precursor ion *m/z* from which they came. Each grouping of spectra is known as a compound.

The compound MS^2 and MS^3 spectra are then automatically searched against the Drugs_lib library database, and NIST-based scores for Fit, RFit, and Purity are determined for each spectrum relative to a library spectrum. To take advantage of the LC/MSD Trap preserving specific pathway

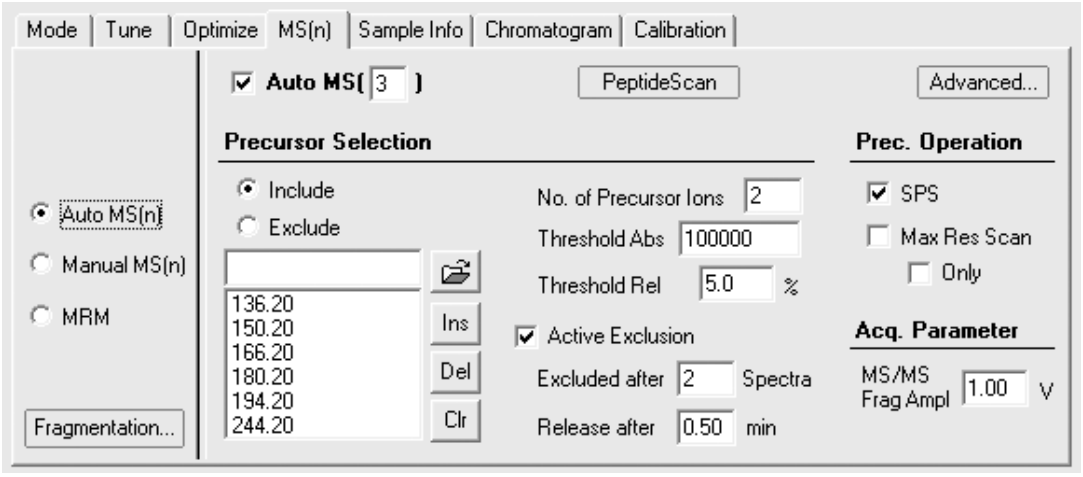


Figure 10. AutoMS(3) settings for confirmation acquisition.

Table 3. List of Precursor Ions Used for Data-Dependent MS^2 and MS^3

Precursor (*m/z*)	Compound	Precursor (*m/z*)	Compound
136.2	Amphetamine	286.2	Morphine
150.2	Methamphetamine	300.2	Codeine
166.2	Ephedrine	310.0	Methadone
180.2	MDA	314.2	Ethylmorphine
194.2	MDMA	316.2	Oxycodone
244.2	Phencyclidine	399.2	Pholcodine

information at the MS^2 level, only the MS^2 spectra of the library are searched for comparison with the MS^2 of the sample compound.

At the MS^3 level many of the product ions are the same, even though different pathways through different MS^2 product ions are followed. Therefore, the search algorithm allows for the comparison of sample compound MS^3 spectra with MS^3 library spectra where the MS^2 product ions may differ. Nevertheless, all searches are limited to comparing the same MS^2 transitions between the sample compound spectra and the library spectra, as well as comparing the corresponding MS^3 spectra, even though the MS^2 product ions may differ.

Finally, those compounds not identified are left out of the report by removing them using the automation script, as was shown in Figure 4.

A nice example of using the ion trap library is shown in the automatically generated library report of Figures 11 and 12 for a data file called "012-1901." The sample involved has been screened by an Agilent LC/MSD single quadrupole mass spectrometer, and the presence of amphetamine, methamphetamine and codeine has been detected. The codeine is quantified at a level of 6.5 µg/mL of urine, but the signal for the other two drugs is too low to measure with confidence.

The results in the report demonstrate not only the power to identify the compounds expected in the sample, but also the sensitivity to detect two other compounds not seen in the screening step: morphine and MDMA. The scores shown in the report are effective scores based on the mathematical consideration of both the MS^2 and MS^3 level matches.

Figure 12 shows good spectral matches for codeine between the sample analyzed in the St. Olav's lab and the library developed in the Riverview Hospital lab. The importance of using MS^3 spectra is demonstrated by the fact that the individual scores for the match at the MS^2 level are all just under 900, but the scores at the MS^3 level are close to 1000, so that the overall effective scores shown in Figure 11 are well over 900.

Library Search Report - AutoMS(n)

St. Olav's Hospital
Department of Clinical Pharmacology
7006 Trondheim, Norway

Analysis Name: 012-1901.D **Instrument:** LC-MSD-Trap-XCT **Print Date:** 10/12/200612:24:42 AM
Method: BAS_SB.M **Operator:** Administrator **Acq. Date:** 8/28/2006 6:02:18 PM
Sample Name: 109647

Compound List:

#	RT [min]	MS(n) Isol. m/z	Compound Name	Fit'	RFit'	Purity'
1	2.0	286.6	Morphine	975	993	971
2	2.1	194.4	MDMA	978	954	934
3	2.8	300.4	Codeine	989	990	989
4	2.8	136.0	Amphetamine	1000	1000	999
5	3.6	150.4	Methamphetamine	998	912	910

Figure 11. First page of report listing compounds identified.

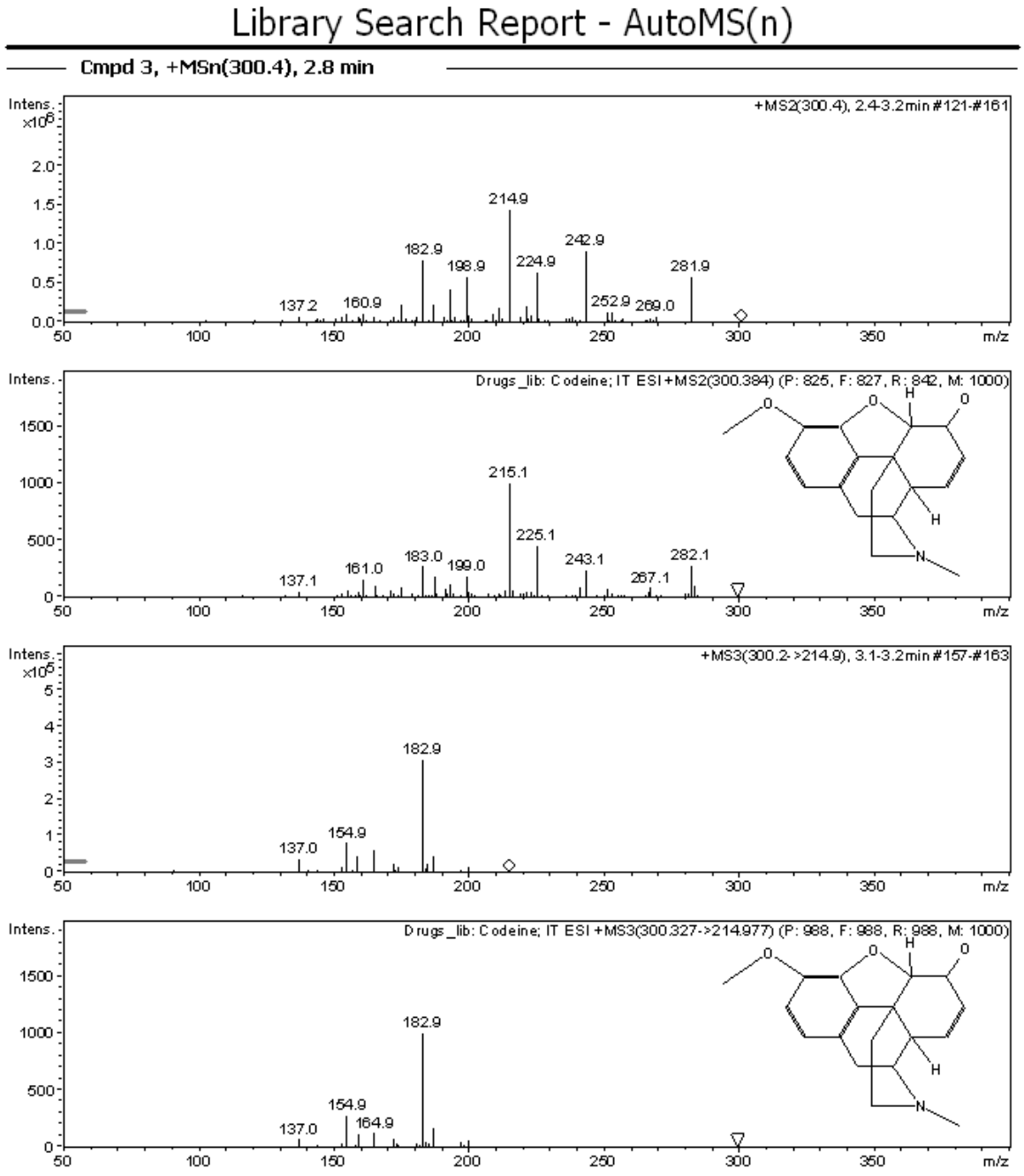


Figure 12. A page in a report showing spectral matching.

Conclusions

MS^2 and MS^3 spectra, acquired using ramped collision energy, is an effective technique for confirming the presence of compounds in a sample. MS^3 spectra increase the specificity of analysis and an overall scoring technique needs to account for matching spectra at both the MS^2 and MS^3 levels. Calculating effective scores from the individual NIST-based scores for each spectrum takes into account the overall identification of the compound of interest. To increase productivity, the data processing, compound identification, and reporting must be automated.

For More Information

For more details concerning this technical overview, please contact Michael Zumwalt at Agilent Technologies, Inc.

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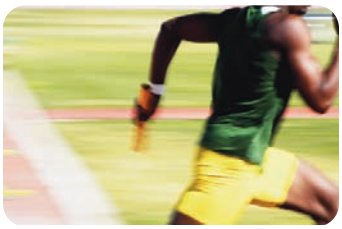
Printed in the USA
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5989-5861EN



Agilent Technologies







Applications by Instrumental Technique

LC/MS





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

Application Note

Drug Testing

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John M. Hughes
Agilent Technologies

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

Cocaine is a widely abused drug for which two metabolites, 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 aggressive 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).

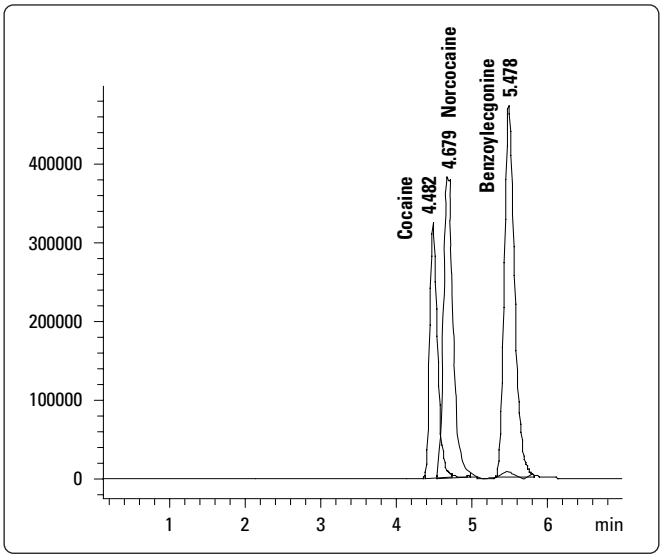


Figure 1. Isocratic separation of cocaine, norcocaine and BE.

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.

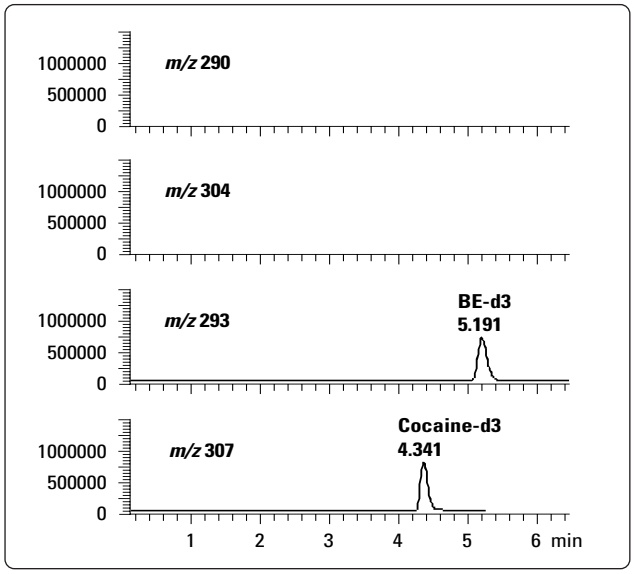


Figure 2. Extracted ion chromatograms of blank urine extract.

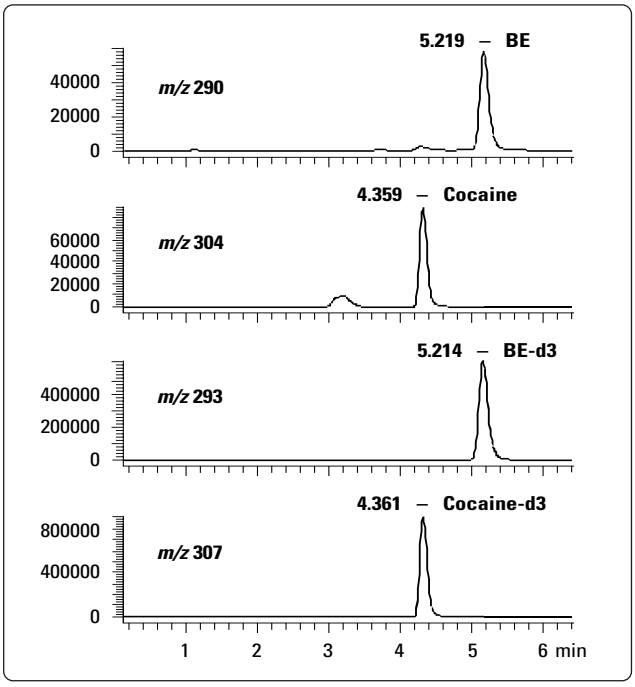


Figure 3. Extracted ion chromatograms of fortified urine extract (25 ng/mL).

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.

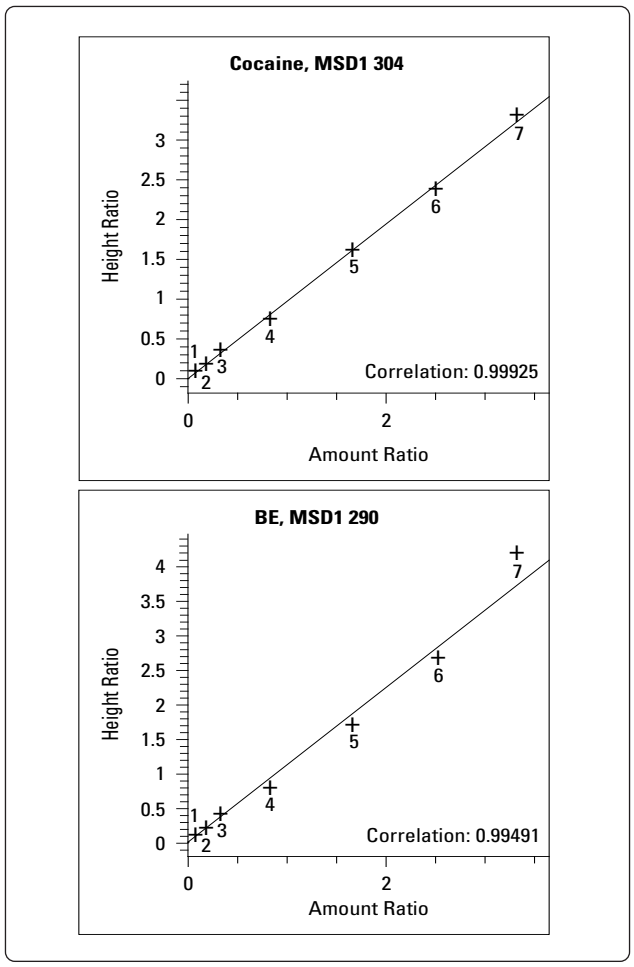


Figure 4. Calibration curves for cocaine and BE.

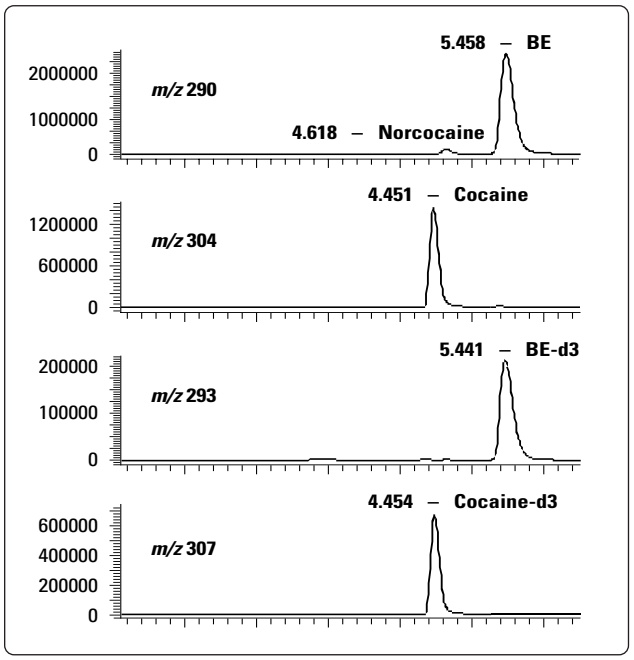


Figure 5. Extracted ion chromatograms from the extract of a positive urine sample.

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.

References

1. Crouch, D.J.; Alburges, M.E.; Spanbauer, A.C.; Rollins, D.E.; Moody, D.E.; Chasin, A.A. *Journal of Analytical Toxicology* 1995, 19, 352–358.

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Therapeutic Drug Monitoring by LC/MSD - Clozapine, an Example

Application

Pharmacology

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Abstract

Therapeutic drug monitoring is performed by LC/MS on a routine basis at St. Olav Hospital. Over 200 drugs are analyzed for both identification and quantification using this technique. This is made practical by high-throughput analysis and the use of carefully applied methodology. The method for clozapine and its metabolite, desmethylclozapine is given as an example. The advantages over immunoassay are briefly discussed.

Introduction

Therapeutic drug monitoring (TDM) has been available for many years for drugs with a narrow therapeutic index (for example, digoxin, theophylline, and lithium). Analyses for most of these drugs have been performed with immunological techniques. For many years clinicians believed that there was no need to perform TDM on a routine basis for the majority of other drugs. However, during the past few years there has been increased focus on, and a growing awareness of, the interindividual variability in response to drug exposure. This variability may be due to pharmacodynamic, pharmacokinetic, environmental (food, tobacco smoke, ethanol), psychological (insight into the disease), and compliance factors. The lack of ways to correct for many aspects of this variability, other than pure clinical judgement, has evoked a renaissance for TDM. Initially the focus of the "new" TDM was directed towards treatment of psychiatric and neurological disorders, but TDM is now expanding into other medical areas.

TDM at St. Olav Hospital

The Department of Clinical Pharmacology at St. Olav Hospital, Trondheim, routinely monitors more than 200 different drugs, almost exclusively by liquid chromatography/mass spectrometry (LC/MS). For example, there are methods available for more than 20 drugs used for treatment of schizophrenia and other psychoses. One of the drugs in this group that is routinely monitored is clozapine, which is analyzed in about 200 samples per month. Other drugs in the same group include, but are not limited to, olanzapine, quetiapine, ziprasidone, risperidone, sertindole, amisulpiride, haloperidol, perphenazine, zuklopentixol, fluphenazine, chlorpromazine, thioridazine, and chloroprotixene. Similar repertoires are also available for other groups such as antidepressants and mood stabilizers.

Clozapine

Clozapine is mainly used against therapy resistant schizophrenia. Clozapine's mechanism of action is not completely understood, but it is assumed that a combination of a weak dopamine antagonism together with combined serotonin agonism and antagonism are the most important mechanisms. Clozapine is metabolized to numerous metabolites, mainly by the cytochrome P450 (CYP1A2) enzymes, and the half-life in blood usually is within the range of 6–36 hours. Some data indicate that individuals with a high level of desmethyl-clozapine are more susceptible to develop agranulocytosis; that is a potentially lethal adverse event.

Experimental

In the TDM-service, clozapine and one of its metabolites, desmethylclozapine, are measured in serum by the Agilent Technologies 1100 LC/MSD. The LC/MS with a single quadrupole provides both the selectivity and sensitivity needed for this analysis. Both the ruggedness of the methodology and the instrumentation make it a cost effective process for continuous operation. The use of in-house instrument engineers and meticulous documentation of maintenance operations along with the records obtained from operation 24 hours a day, 7 days a week provide "up-time" of greater than 95%.

All analytical results are transferred to a database. The actual concentration in the serum is interpreted by a MD (specialist in clinical pharmacology). The interpretations usually consist of an evaluation of the concentration/dose relationship in the actual sample together with evaluation of age, possible drug-drug interactions, possible adverse events if focused by the clinician, etc. versus therapy success or lack of success. New measurements are always compared with previous results in the database. The overall impression is that TDM is an extremely valuable tool for the clinician in the decision making process.

Table 1 gives the extraction procedure for the preparation of blood serum for the analysis of clozapine and its metabolite.

Table 1. Procedure to Extract Clozapine and its Metabolite from Blood Serum

Step	Action
1.	Take 0.5-mL serum (standard, QC or sample).
2.	Add 50 µL of 10-nM flurazepam [internal standard (ISTD) for clozapine].
3.	Add 50 µL of 10-nM periciazin (ISTD for desmethylclozapine).
4.	Mix (short).
5.	Add 4 mL of hexane: n-butanol: acetonitrile (93:5:2).
6.	Mix 5-min on rotary mixer.
7.	Centifuge 3000 rpm 5 min.
8.	Transfer organic phase.
9.	Evaporate under stream of air at 40 °C.
10.	Reconstitute in 50-µL methanol.
11.	Transfer to vial with 150 µL insert and cap with crimp-on cap.

Two internal standards (ISTDs) are used, flurazepam and periciazin. The structures of each are shown in Figure 1.

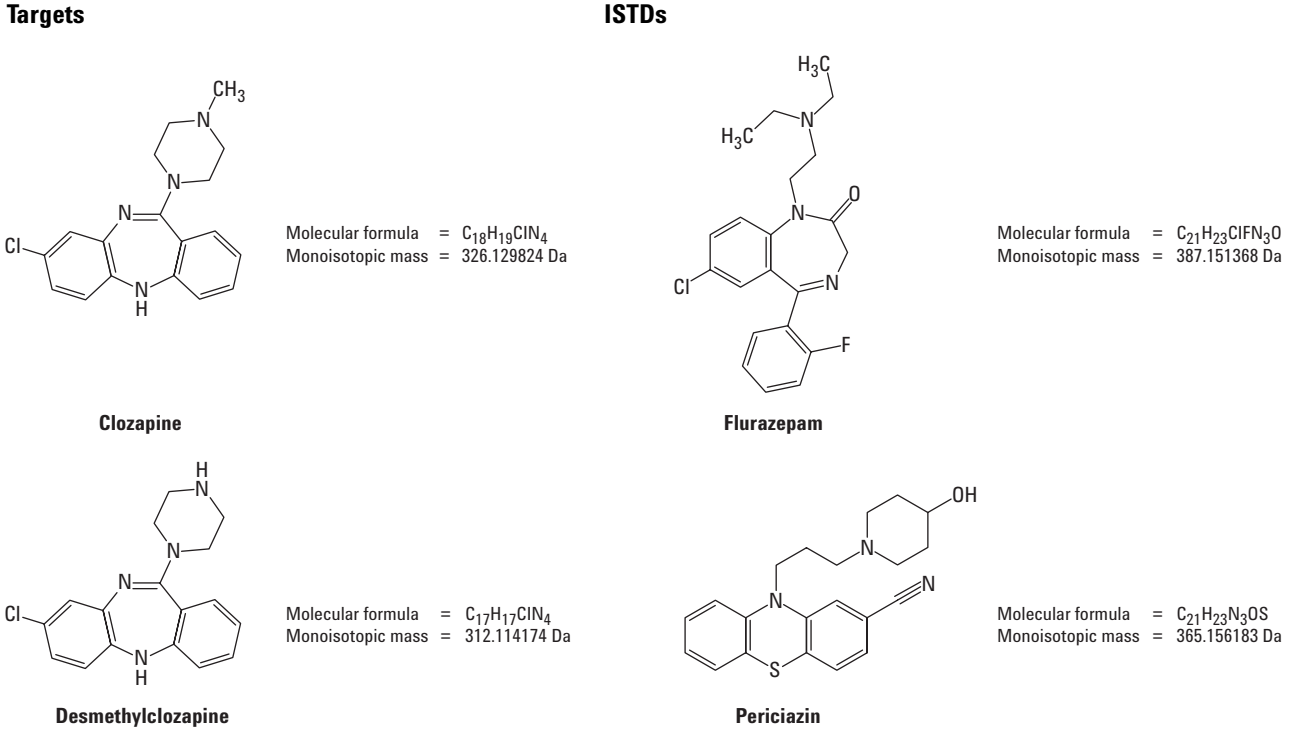


Figure 1. Structures of clozapine, desmethylclozapine and their ISTDs (shown to their right).

Instrument conditions for the analysis are shown in Table 2.

Table 2. Instrumental Conditions for the Analysis

LC/MS Analysis			
LC Conditions			
Instrument	Agilent 1100 LC/MSD with Quaternary pump and electrospray		
Column	ZORBAX SB-C18, 4.6 mm × 30 mm × 3.5 µm, p/n 833975-902		
Mobile phase	Methanol: 50-mM ammonium acetate in water (60:40)		
Flow rate	1 mL/min		
Standard automatic liquid sampler (ALS) with needle wash			
MS Conditions			
Injection Volume	2.5 µL		
Nebulizer	25 psi at 350 °C		
Drying gas	9 L/min		
	RT (min)	**Target**	**Qualifier**
Clozapine	3.0	327.0/frag 100 V	270.0/frag 150 V
Desmethylclozapine	1.3	313.0/frag 100 V	270.0/ frag 150 V
Flurazepam	2.2	388.1/frag 100 V	
Periciazin	1.9	365.5/frag 100 V	

Calibration is done with zero serum using spiked concentrations of calibration standards (clozapine and desmethylclozapine): 0, 25, 100, 500, 1000, 2000, 3000, and 4000 nM. The calibration curves use a quadratic fit (because of the wide range of concentration) with r^2 >0.999. Quality control samples are prepared with zero serum using spiked concentrations of QCs (clozapine and desmethylclozapine): 500, 500, 1000, 1000, 3000 and 3000 nM. The QCs are run randomly distributed among the samples.

Results

Figure 2 shows the selected ion chromatograms of the target compounds with qualifying ions and their ISTDs for the 25 nM standard.

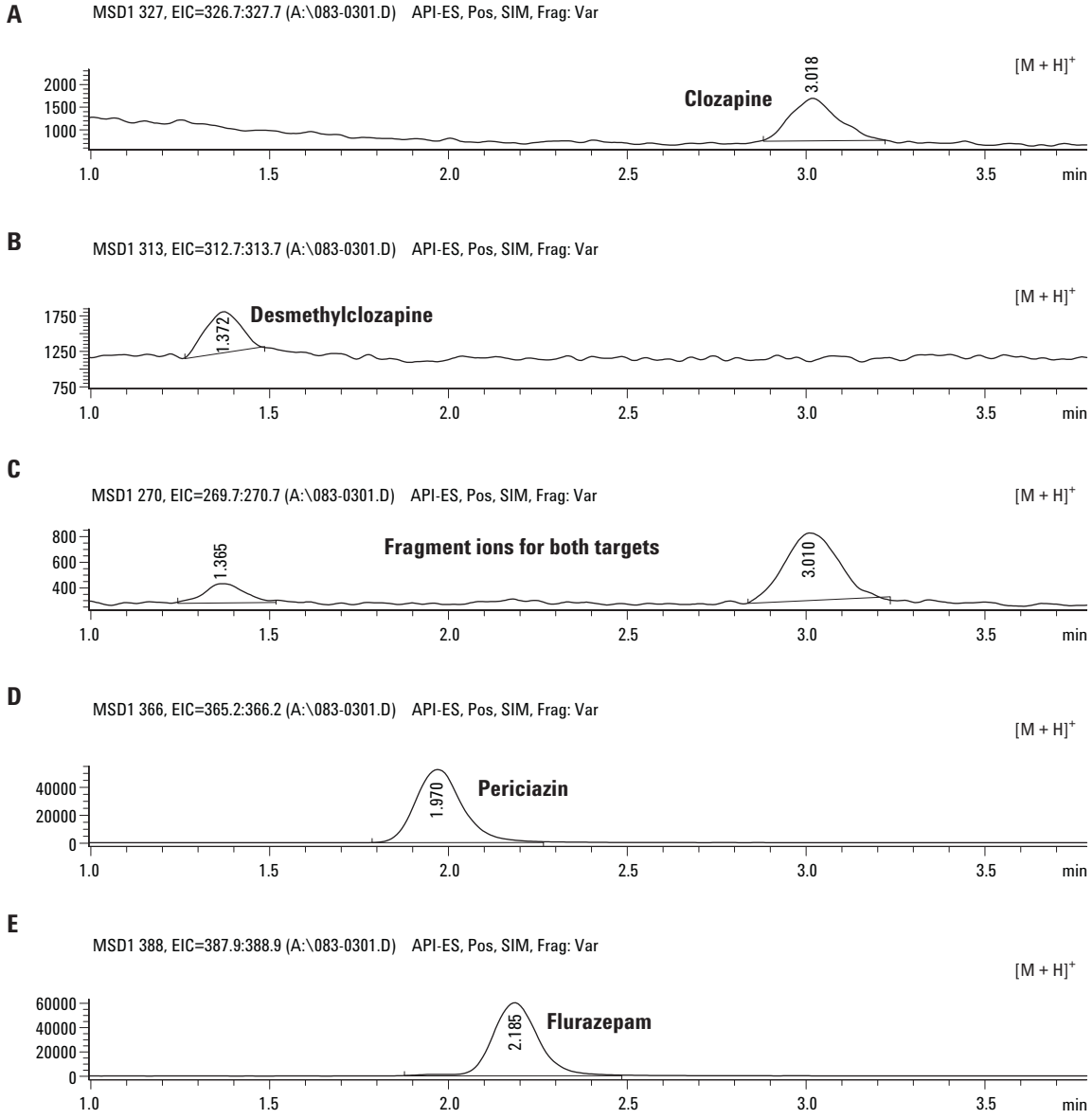


Figure 2. Selected ion chromatograms for 25 nM clozapine, desmethylclozapine, and their ISTDs. All chromatograms show the $[M+H]^+$ ions. Figure 2A is derived from clozapine, 2B from desmethylclozapine, 2C from fragments from both target compounds (after neutral loss of 1-N-methylaziridine), 2D from peraciazin, and 2E from flurazapam.

Up-front collision-induced-dissociation (CID) is sufficient to produce a qualifier ion to increase the specificity of the analysis. This is the lowest standard in serum and represents a concentration typically below normal therapeutic monitoring levels. There is variation of the serum matrix from one sample to another but that variation is not sufficient to have any impact on the analysis.

Figure 3 shows a blank containing the ISTDs and Figure 4 presents the results of a patient sample at a typical lower level (for true patient samples). In this patient sample, the clozapine was measured at 366 nM, and the metabolite at 365 nM. Note how clean the signals are with no interference. The methods demonstrated were robust for a period of 5 years.

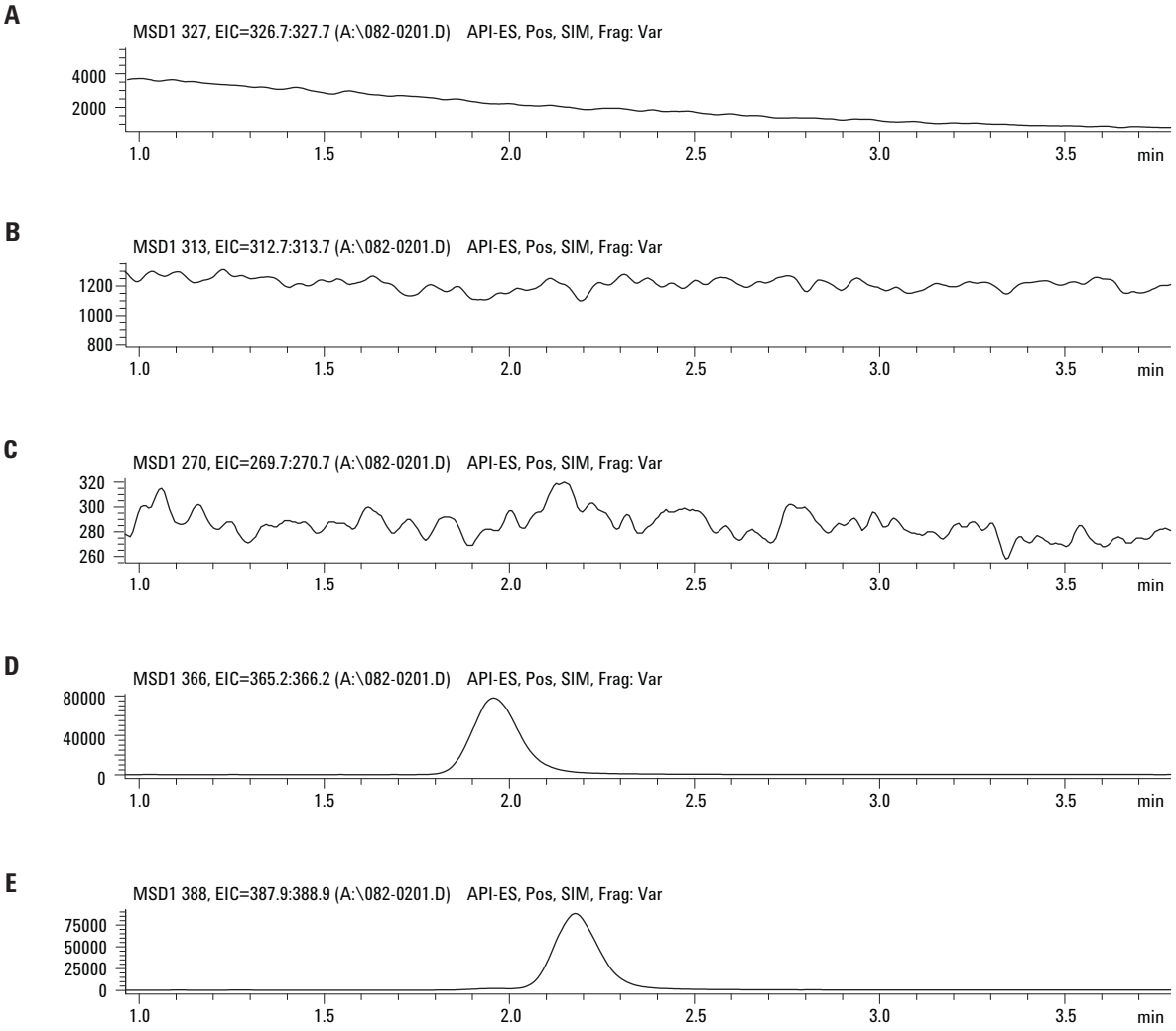


Figure 3. Extracted ion chromatograms (EIC) of blank clozapine in blood serum with ISTDs.

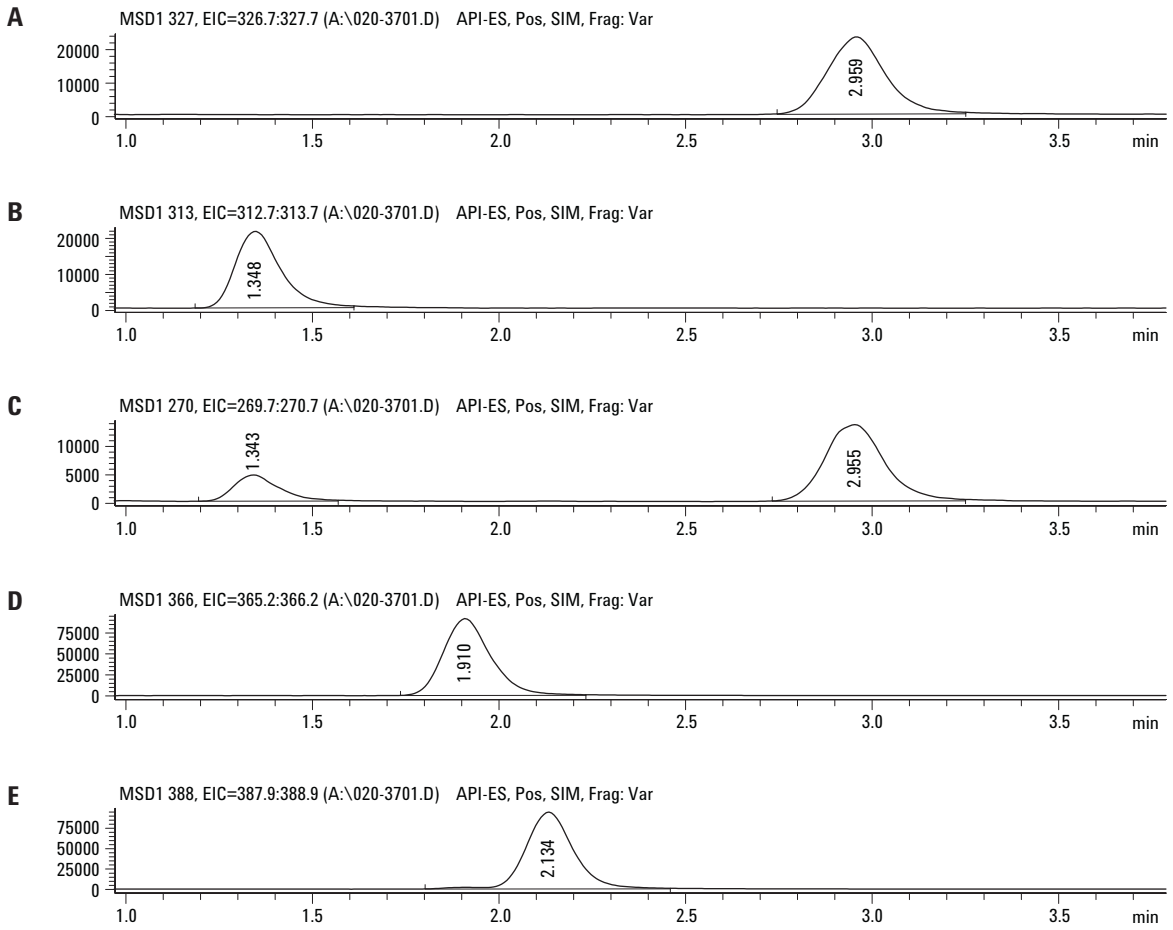


Figure 4. EIC of patient sample at a typical low level.

Reproducibility

True day-to-day reproducibility with different instruments and different operators is obtained through the evaluation of the QC samples. These are shown in Table 3, as a 3-month rolling average.

Table 3. True Reproducibility of Analysis (3-month rolling average)

	25 nM	500 nM	3000 nM
Clozapine	6.1%	6.1%	7.8%
Desmethylclozapine	9.1%	8.0%	7.6%

Table 4 shows a 1 day example of the QC results at the different levels.

Table 4. True Reproducibility of Analysis (1 day example)

	500	1000	3000
Clozapine	596	1068	3361
	543	1068	2935
Desmethylclozapine	462	1026	3345
	534	992	2904

Once per month QC samples from Heatcontrol/ Cardiff Bioanalytical Services Ltd. are analyzed and a report generated. A 12-month control chart of the Bias Index Score (BIS) is shown in Figure 5. The BIS is defined as the difference of the laboratory's measurement from the consensus mean scaled in terms of a chosen common coefficient of variation for all participants. If the exact coefficient of variation is used, a BIS value of 300 corresponds to 3 standard deviations (SDs). External quality control samples are used for any TDM analyte that is available.

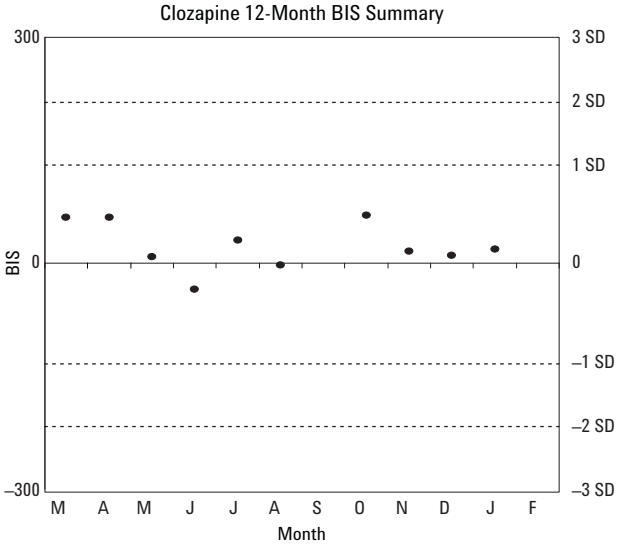


Figure 5. Control chart for external quality sample.

Conclusion

Through the use of well developed and validated methodology, carefully documented procedures followed by highly skilled and trained operators (technicians or engineers), and rigorous QC incorporated into good laboratory practices, this lab routinely performs therapeutic drug monitoring, clozapine as an example, with LC/MS running 24 hours a day, 7 days a week. For clozapine, typical serum levels for patients range from 450 nM to 3000 nM.

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

Application Note

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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 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.

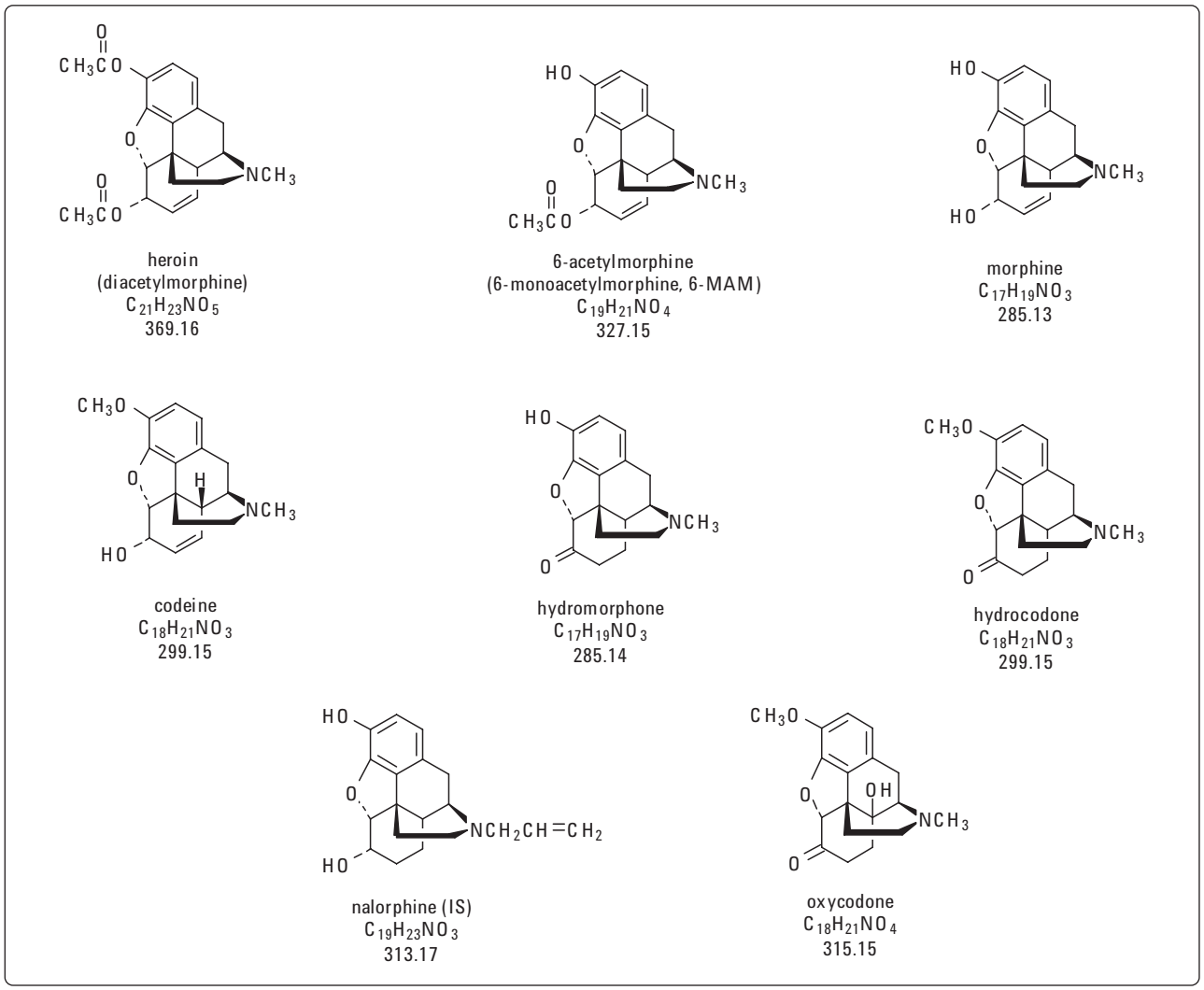


Figure 1. Opiate and internal standard structures

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.

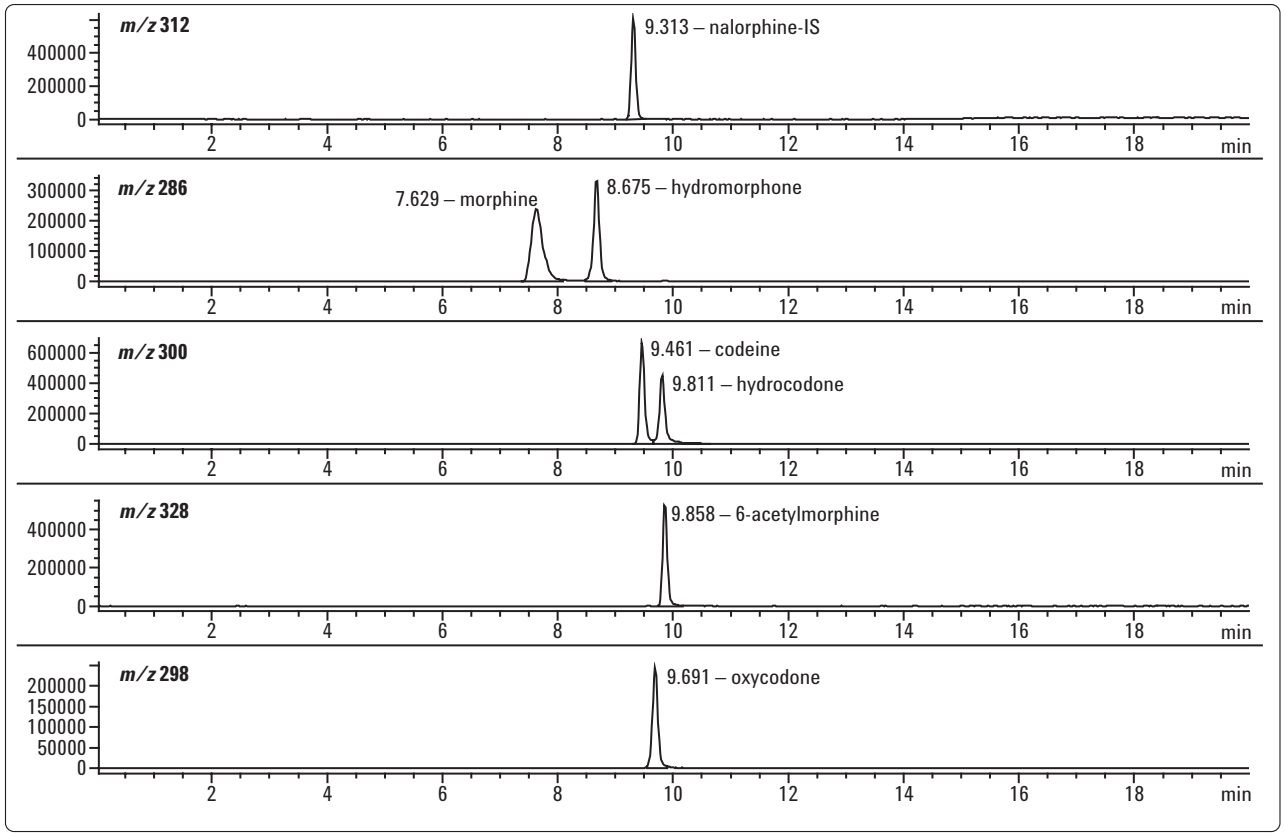


Figure 2. Extracted ion chromatograms (EICs) of opiates and internal standard

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).

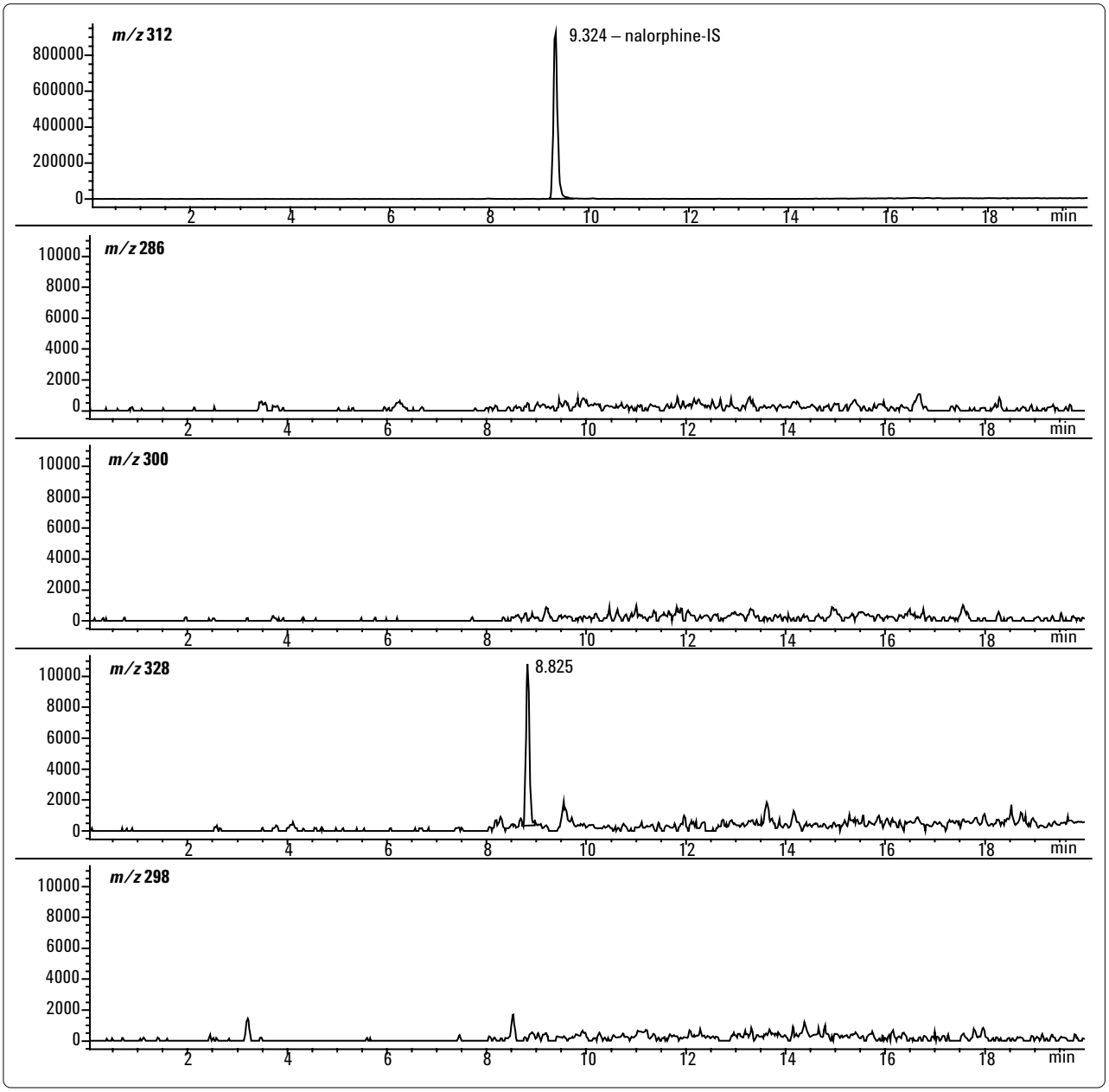


Figure 3. Extracted ion chromatograms (EICs) of blank blood fortified with internal standard at 1 mg/l (1000 ng/ml)

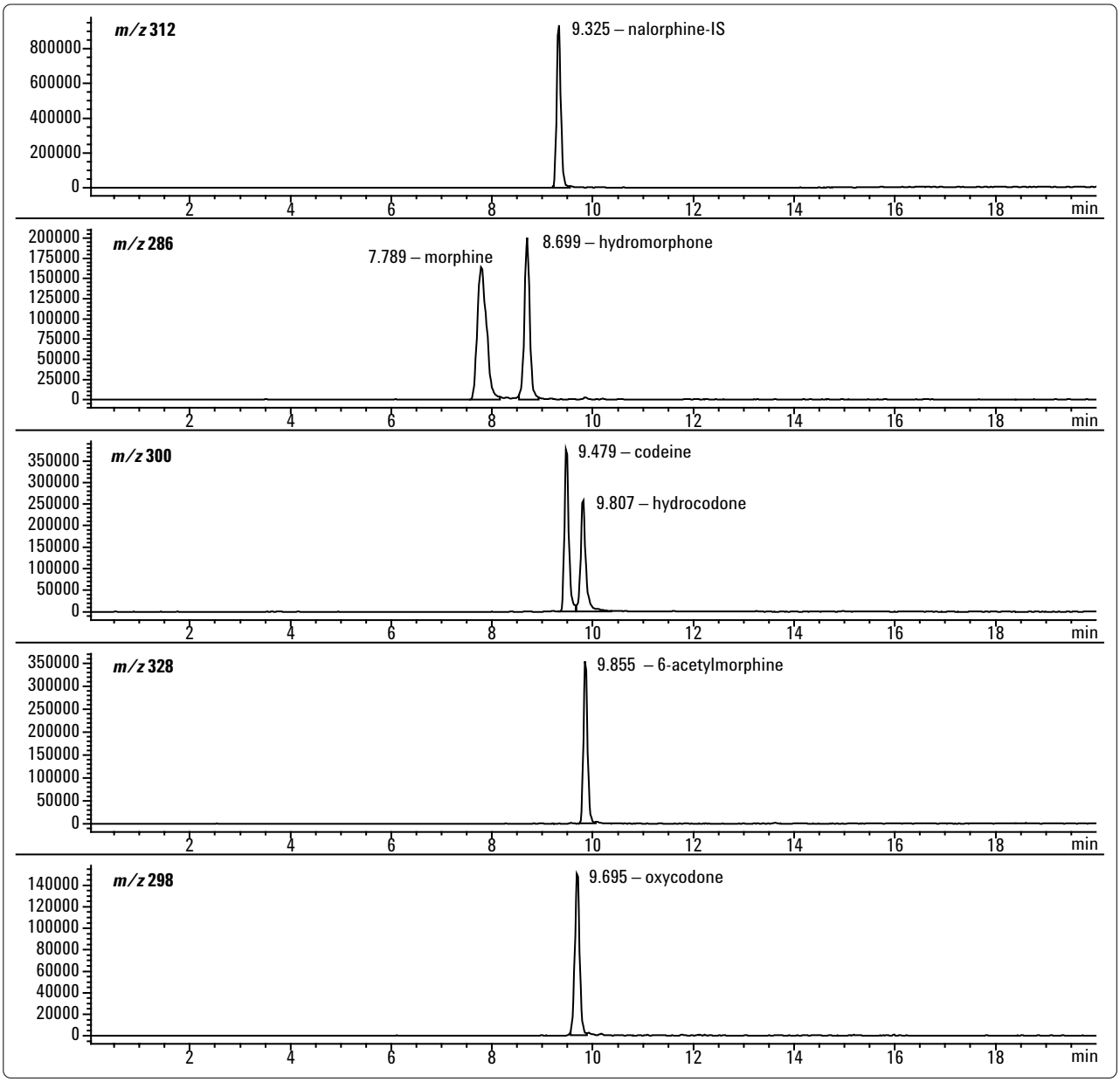


Figure 4. Extracted ion chromatograms (EICs) of control blood fortified with analytes at 0.25 mg/l

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 in her bed. She had an extensive 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 in a friend's apartment with oxycontin in his pocket. He suffered from hepatitis C and had a history of drug abuse. 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 in a motel room. A syringe was found in her hand and small packages of narcotics were discovered in the toilet. 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).

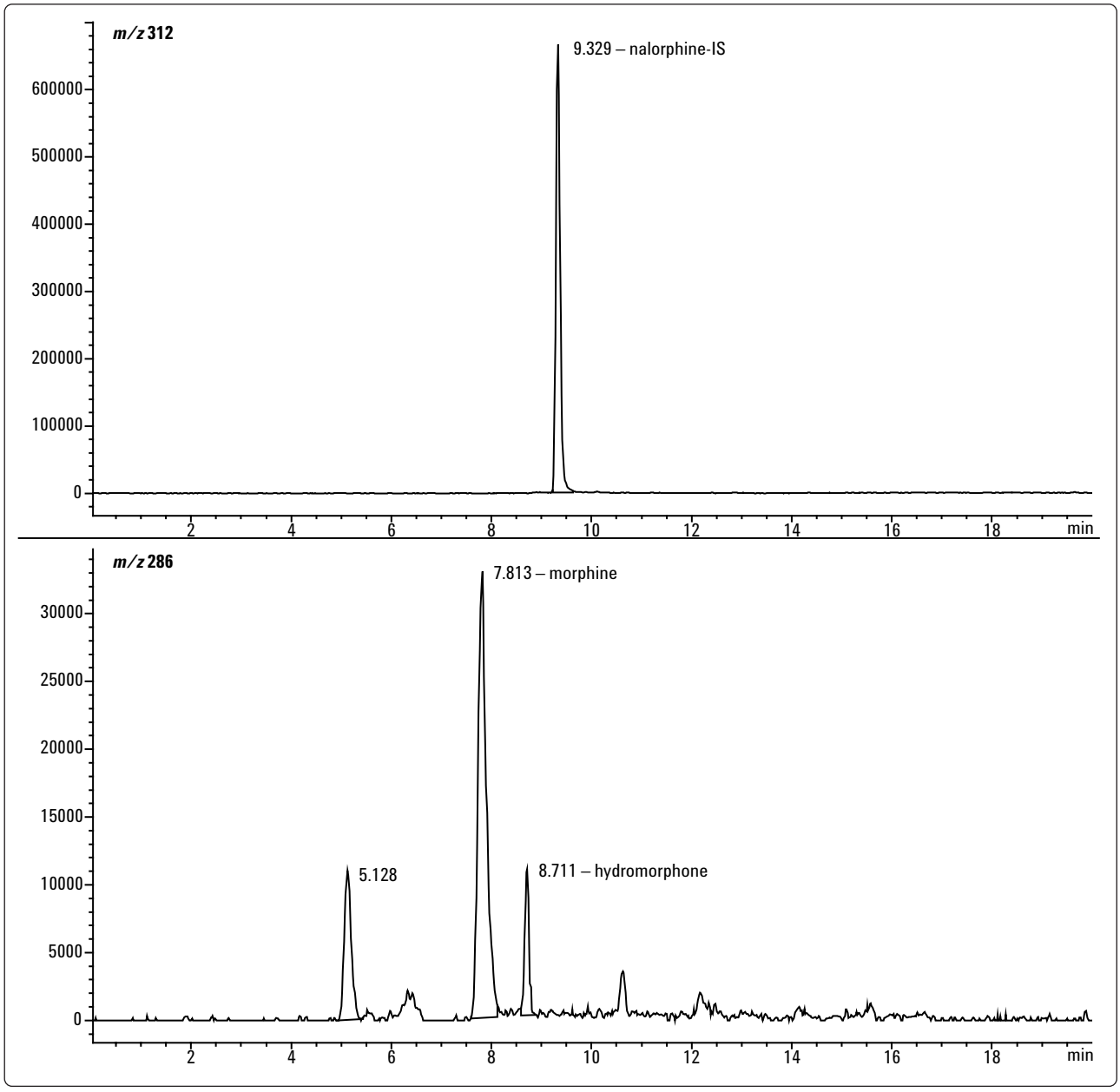


Figure 5. EICs of a positive blood sample found to contain morphine and hydromorphone

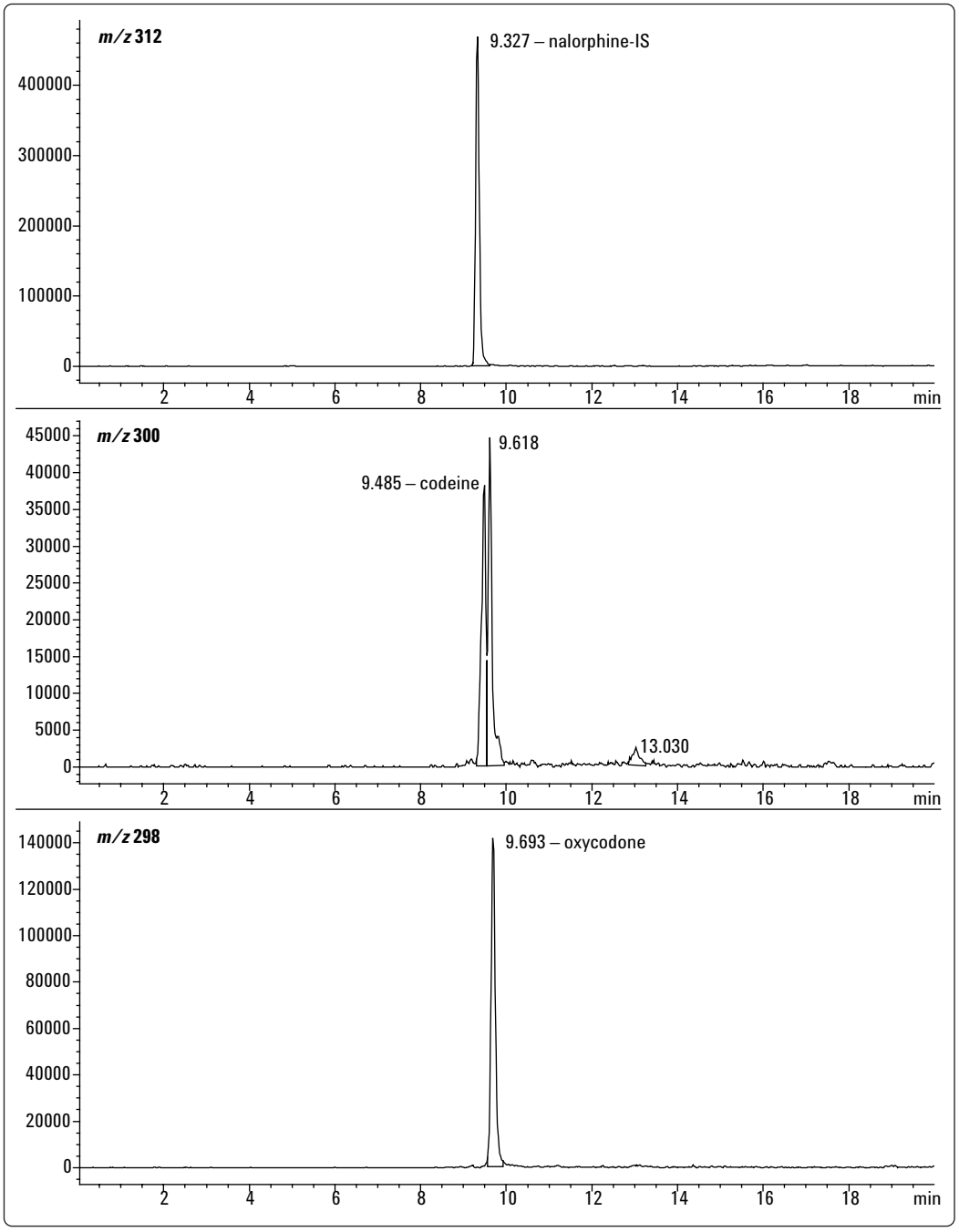


Figure 6. EICs of a positive blood sample found to contain oxycodone and codeine

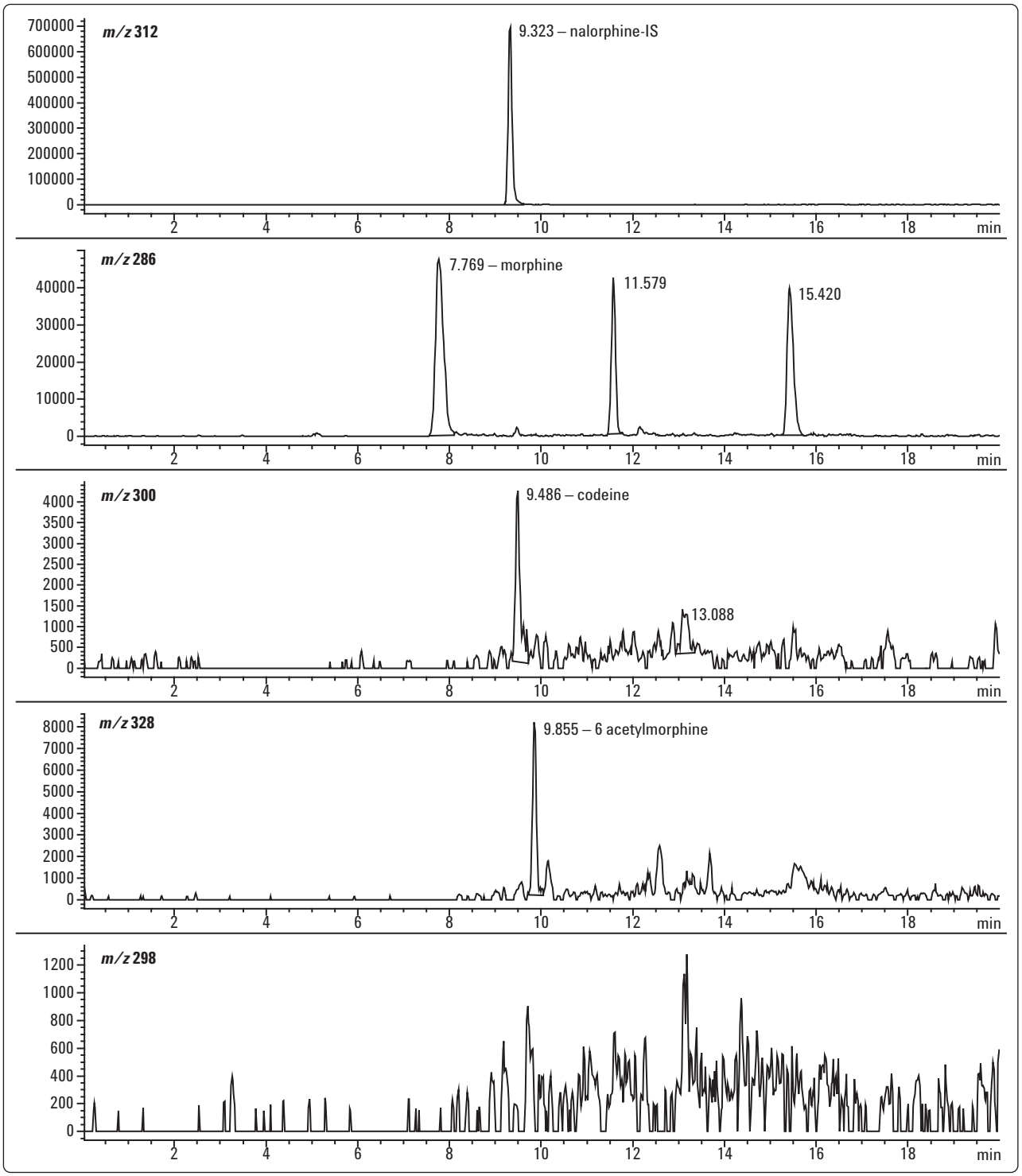


Figure 7. EICs of a positive blood sample found to contain morphine, codeine, and 6-acetylmorphine

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 validated thus far 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 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 sensitivity of the LC/MSD VL allows the use of scan mode rather than SIM without compromising accuracy or precision, making this method useful for general drug screening as well as target compound analysis.

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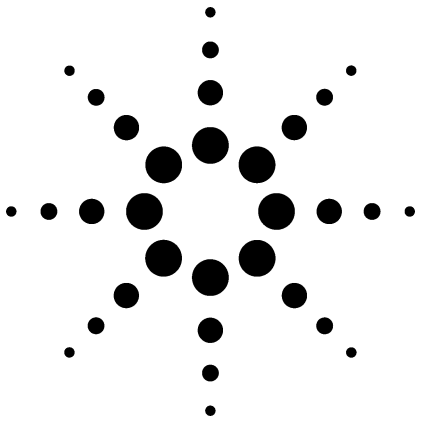
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Screening Drugs of Abuse by LC/MS

Technical Overview



Forensic

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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

Substance abuse is increasing in almost every country, and is associated with criminality, health hazards, accidents, and fatalities. Communities, therefore, face a significant resource investment in their efforts to reduce or stop this abuse. Most substance abuse is illegal. Symptoms and signs are unspecific, and substance abuse is difficult to identify outside the boundary of clinical criteria. This, combined with the fact that substance abuse under many circumstances leads to legal actions, makes it 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 clinical and societal 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 the patient is legally prescribed one benzodiazepine but may abuse a second benzodiazepine. 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 following 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 important 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.

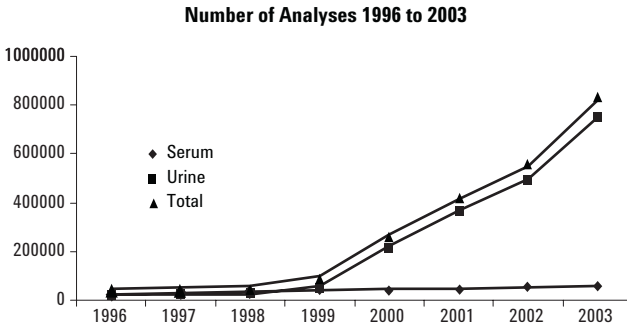


Figure 1. Number of analyses per year for drugs-of-abuse (DOA) from 1996 to 2003. Note that the first LC/MS was purchased in 1998 and LC/MS screening was fully deployed by 1999. The number of analyses represents each group of compounds equivalent to an analysis by immunoassay. In actuality, the analyses 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/negative sanctions, forensics, and clinical 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.

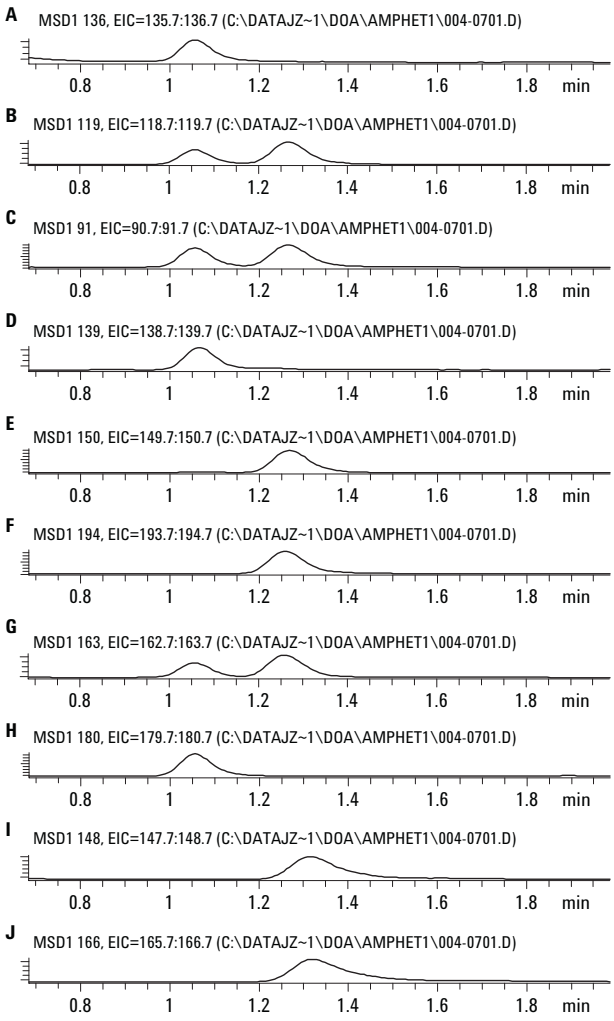


Figure 2. Selected ion monitoring (SIM) chromatograms of amphetamine screen at 100 ng/mL. The panels are A) amphetamine, B) amphetamine and methamphetamine qualifier ion, C) amphetamine and methamphetamine qualifier ion, D) ISTD, E) methamphetamine, F) MDMA, G) MDMA and MDA qualifier ion, H) MDA, I) 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 and clinical toxicology. The laboratory performs several thousand analyses per year for these categories. Biological concentrations of specific drugs with secure identification and fast results is of great importance for the clinicians to make assessments toward the dose and clinical state of a patient. 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

1. Kolbjørn Zahlsen, Trond Aamo, and Jerry Zweigenbaum, “Therapeutic Drug Monitoring by LC/MSD - Clozapine, an Example”, Agilent Technologies, publication 5989-1267EN, www.agilent.com/chem

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Simultaneous Measurement of Sirolimus and Tacrolimus Concentrations in Blood by Semi-Automated Extraction and Liquid Chromatography-Electrospray Ionization Mass Spectrometry

Application

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Abstract

The immunosuppressants sirolimus and tacrolimus are used in transplantation medicine to suppress the body's rejection response. An improved and sensitive method for their analysis is described in which they are first isolated from whole-blood specimens by solid phase extraction and then analyzed by liquid chromatography and electrospray-mass spectrometry. For both drugs, response is linear over the range 0.5 to 120 µg/L, and between-day CVs are about 16% at 1.5 µg/L and 3% at 48 µg/L.

Introduction

Sirolimus and tacrolimus are used in transplantation medicine to suppress the body's rejection response. Monitoring their blood concentrations has been deemed essential because of their narrow effective concentration range and potential for toxicity. Immunochemical assays for sirolimus and tacrolimus suffer from imprecision, inaccuracy, and low sensitivity. Combined immunosuppressant therapy at lower doses requires accurate analysis at the low detection limits (LOD) of <1 µg/L.

This application note describes an improved method in which sirolimus and tacrolimus are first isolated from whole-blood specimens using a solid phase sorbent, extracted, and then analyzed by liquid chromatography (LC) and electrospray-mass spectrometry (ESI-MS). Both drugs are measured using the same extraction and liquid chromatography/mass spectrometry (LC/MS) conditions. Automation of extraction and LC/MS analysis increases sample throughput and the productivity of multiple analysts. The method's advantages include low reagent costs and the ability to measure sirolimus and tacrolimus simultaneously. Performance studies and daily use of the method in our laboratory for over 6 months have validated the integrity and usefulness of the method.

Chemical Structures

The chemical structures of the target compounds and the internal standard, ascomycin, appear in Figure 1.

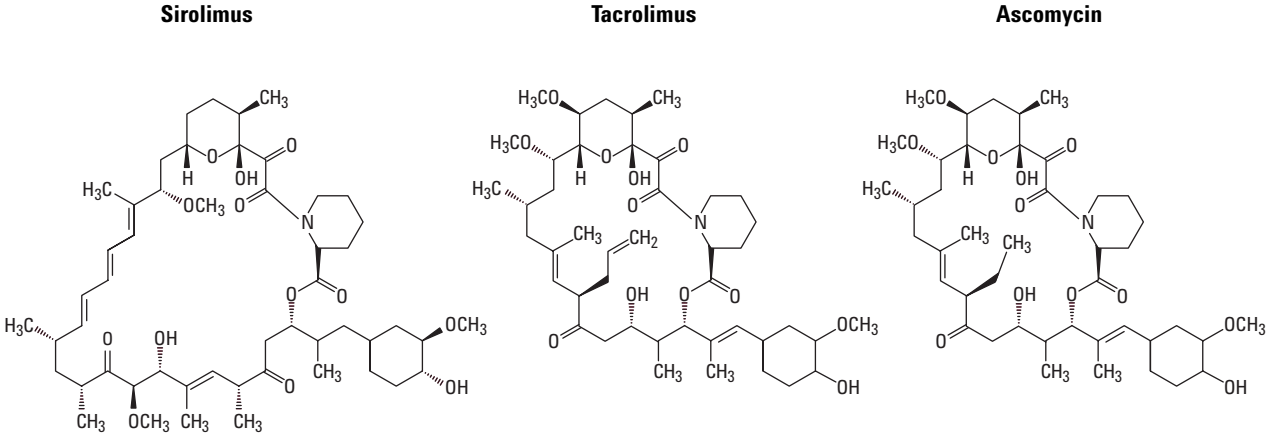


Figure 1. Chemical structures for sirolimus, tacrolimus and ascomycin (internal standard).

Method

The assay comprises three distinct steps:

1. Precipitation of whole-blood proteins
2. Automated extraction of sirolimus, tacrolimus, and the internal standard, ascomycin from the clear supernatant
3. Automated LC/MS analysis of the extract

The assay uses four solutions:

Solution A: 50 mL of 35 g/dL zinc sulphate heptahydrate in water, 500 mL acetonitrile, 500 mL water.

Solution B: 10/90 acetonitrile/water (v/v)

Solution C: 15/85 acetonitrile/water (v/v)

Solution D: 55/45 acetonitrile/water (v/v)

1. Combine 0.5 mL of blood with 1.5 mL of Solution A in 13 × 100 mm glass tube.
2. Vortex. Wait 10 minutes. Add 1 mL water. Centrifuge. Decant.
3. Transfer supernate to 10 × 75 mm glass tube and take sample tube to the Gilson ASPEC XL4 [1].
4. First prime the SPE column [Empore SDB-XC, 1 mL SPE cartridge] with 0.5 mL acetonitrile and then with 0.5 mL Solution B.
5. Add 2.5 mL of protein-free supernate.
6. Wash with 0.5 mL Solution C.
7. Add 1.5 mL of Solution D and elute into empty autosampler vial.
8. Transfer vial to the Agilent 1100 LC/MS.

LC Parameters

- Analytical column: Restek Allure C18, 30 mm long × 3.2 mm id
- Mobile phase conditioning column: Prepacked (50 µ silica) Alltech Presat cartridge kit, 250 mm × 4.6 mm, placed between pump and injector. This column provides sodium to the mobile phase to promote sodium adduct formation of sirolimus and tacrolimus and slows degradation of the analytical column.
- Mobile phase: acetonitrile/water, 90/10 (v/v)
- Flow rate: 0.5 mL/min
- Temperature: 75 °C
- Run time: 1.0 min/injection
- Injection volume: 5 µL

MS Parameters

Individual parameters were adjusted to obtain optimum signal by flow injection analysis (FIA) of drug substance in mobile phase.

- Nebulizer pressure: 13 psig
- Drying gas flow and temperature: 12 L/min and 350 °C
- V_{cap}: 5300 V
- Fragmentor voltage ramped from 155 to 200 V
- Gain 5, varies with instrument

Compound	SIM ion *m/z*	Fragmentor voltage
Sirolimus	936.5	185
Tacrolimus	826.5	155
Tacrolimus fragment	616.4	200
Ascomycin	814.5	155
Ascomycin fragment	604.4	200

ESI-CID Positive Ion Mass Spectra

The most abundant ion arises from the positively charged sodium adduct. Fragmentor voltage was adjusted to produce fragment ions via collision-induced dissociation (CID). See Figure 2.

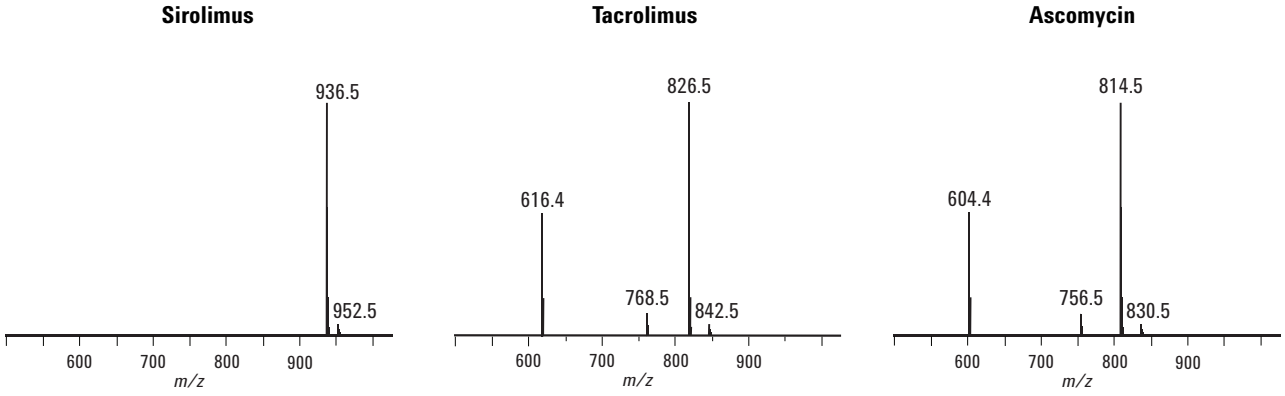


Figure 2. Positive ion mass spectra for the sodium adducts of the target compounds.

Results

Figure 3 demonstrates that both sirolimus and tacrolimus can be analyzed simultaneously, sensitively, and rapidly.

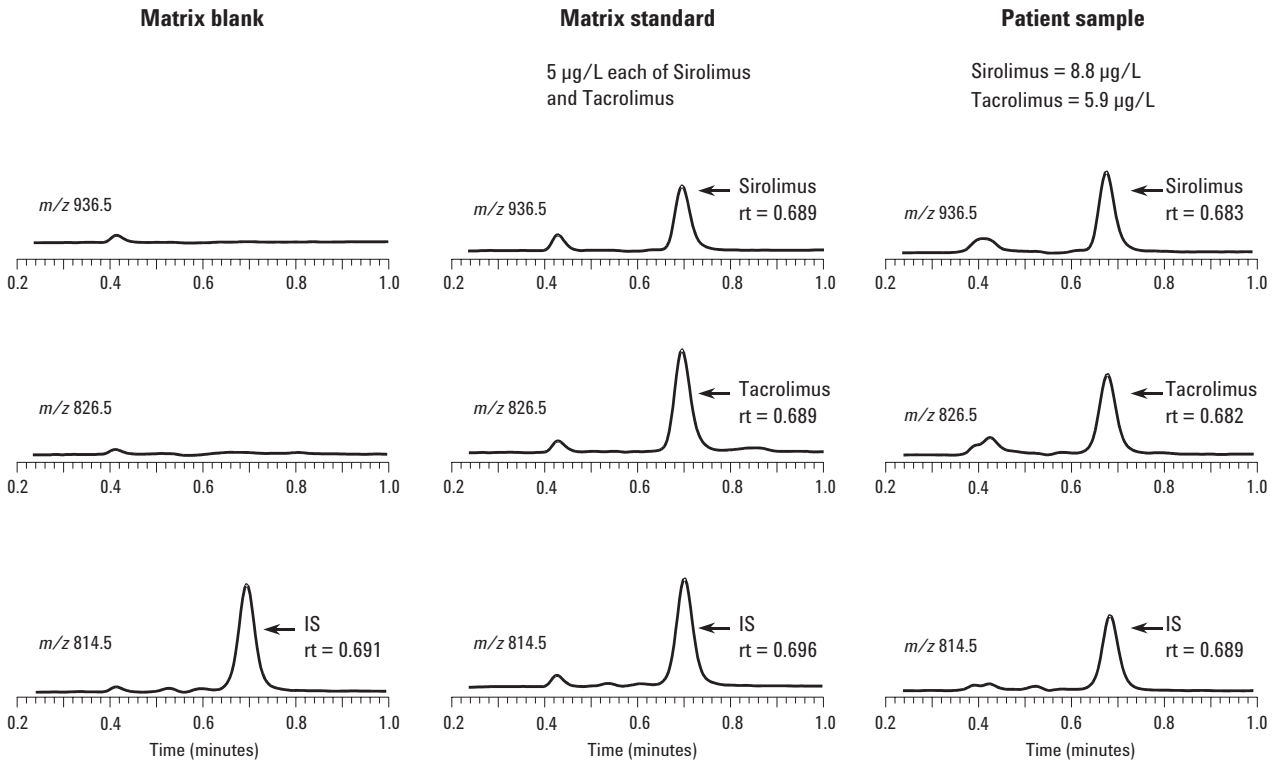


Figure 3. Extracted ion chromatograms of target and reference compounds derived from three reference-containing samples; extract of blank blood, extract of spiked blood, and extract of patient blood.

Precision

Table 1 lists Within-run and Between-day precision data for both sirolimus and tacrolimus at five different concentration levels, increasing from 1 to about 49 µg/L.

Table 1. Method Precision Data

	Sirolimus		Tacrolimus	
	Within-run	**Between-day**	**Within-run**	**Between-day**
N (for all levels)	10	18	10	18
Mean, µg/L	1.03	0.95	1.12	1.95
SD, µ/L	0.094	0.146	0.103	0.185
CV %	9.2	15.4	9.2	17.6
Mean, µg/L	4.44	4.5	4.15	4.83
SD, µ/L	0.183	0.194	0.259	0.240
CV %	4.1	4.3	6.2	4.9
Mean, µg/L	9.63	9.56	9.84	9.87
SD, µ/L	0.249	0.391	0.142	0.323
CV %	2.6	4.1	1.6	3.3
Mean, µg/L	20.5	19.89	19.48	20.18
SD, µ/L	0.828	0.666	0.484	0.573
CV %	4.0	3.3	2.5	2.8
Mean, µg/L	46.14	47.5	48.1	49.0
SD, µ/L	1.01	1.51	1.18	1.20
CV %	2.2	3.2	2.2	2.5

Recovery

Recoveries were obtained using the technique of standard additions.

	n	Concentration range	Recovery
Sirolimus	6	2.7–63 µg/L	82–108%
Tacrolimus	6	3.0–61 µg/L	88–107%

Limit of Detection (Quantitation)

Sirolimus	0.38 µg/L ± 0.09 µg/L
Tacrolimus	0.57 µg/L ± 0.19 µg/L

Interference Study

No interference was seen from metabolites of sirolimus and tacrolimus, nor from cyclosporin or its metabolites. We have noted interference from compounds leached from the red-stoppered Vacutainer™ collection tubes. Whole blood should not be transferred to this tube after being collected in EDTA.

Calculations

Analytical values were calculated from peak height by automated internal standard method.

Linearity

Figure 4 illustrates the excellent linearity obtained by this method for sirolimus and tacrolimus over the studied range.

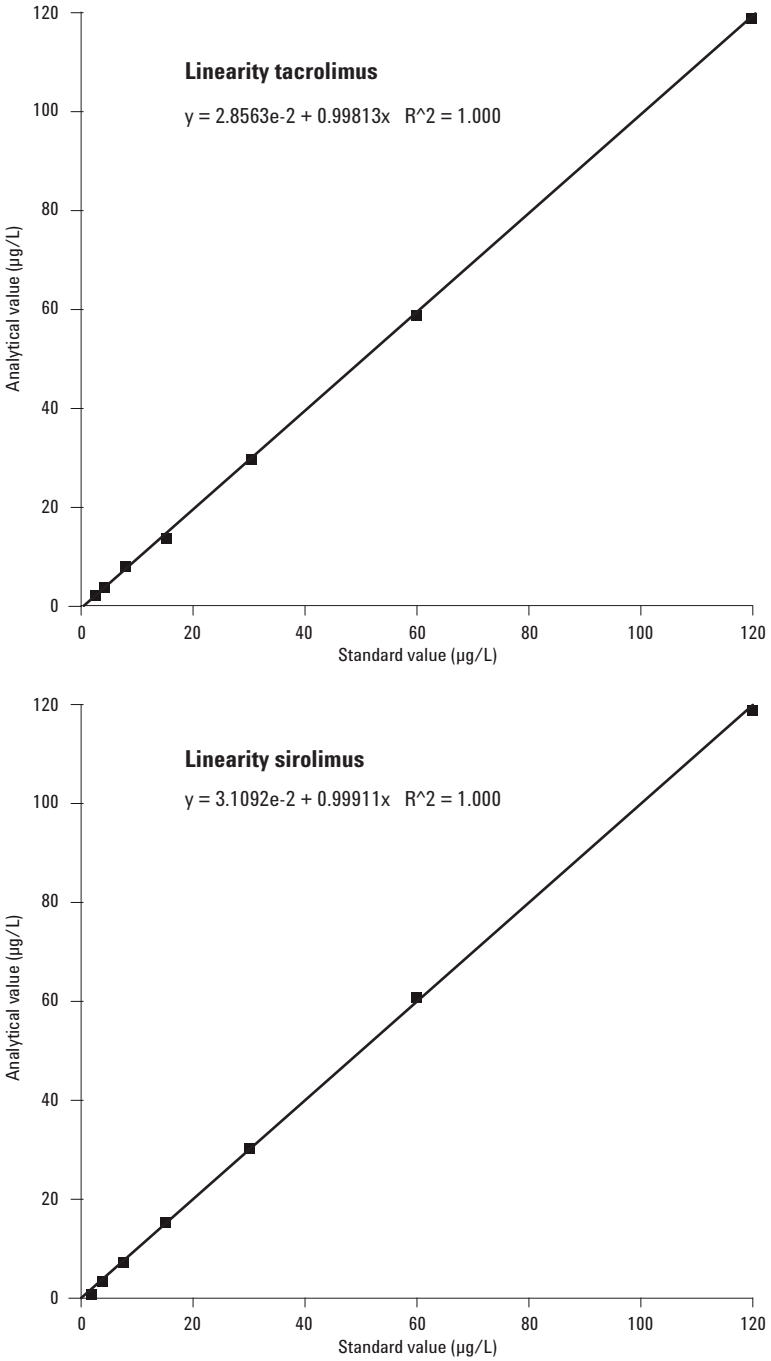


Figure 4. Linearity plots for sirolimus and tacrolimus.

Method Comparison

Samples were analyzed by our method and sent to reference labs for analysis (LC/MS for tacrolimus and LC/MS/MS for sirolimus). Plots of our results vs. reference lab results (Figure 5) show excellent correlation over a wide range of concentrations.

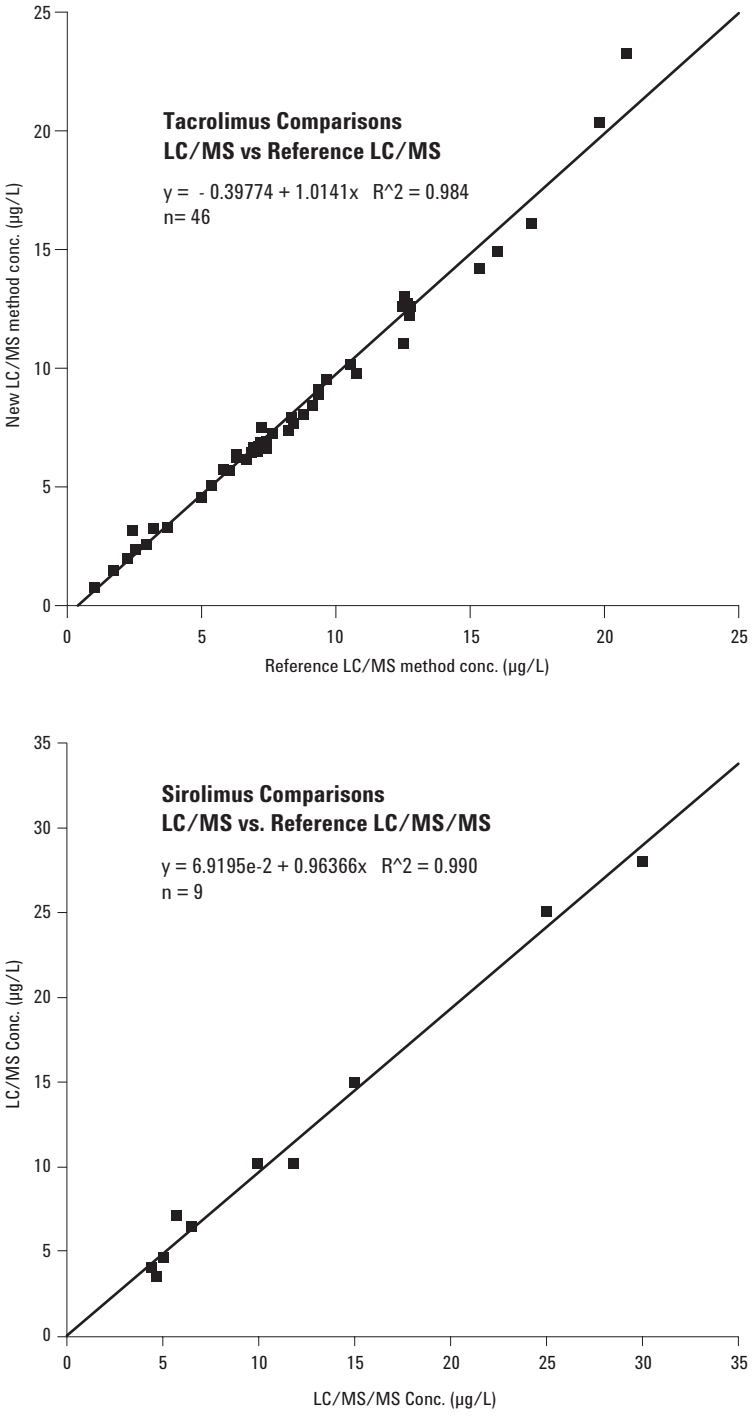


Figure 5. Method comparison plots for sirolimus and tacrolimus.

An example of the use of this method appears in Figure 6.

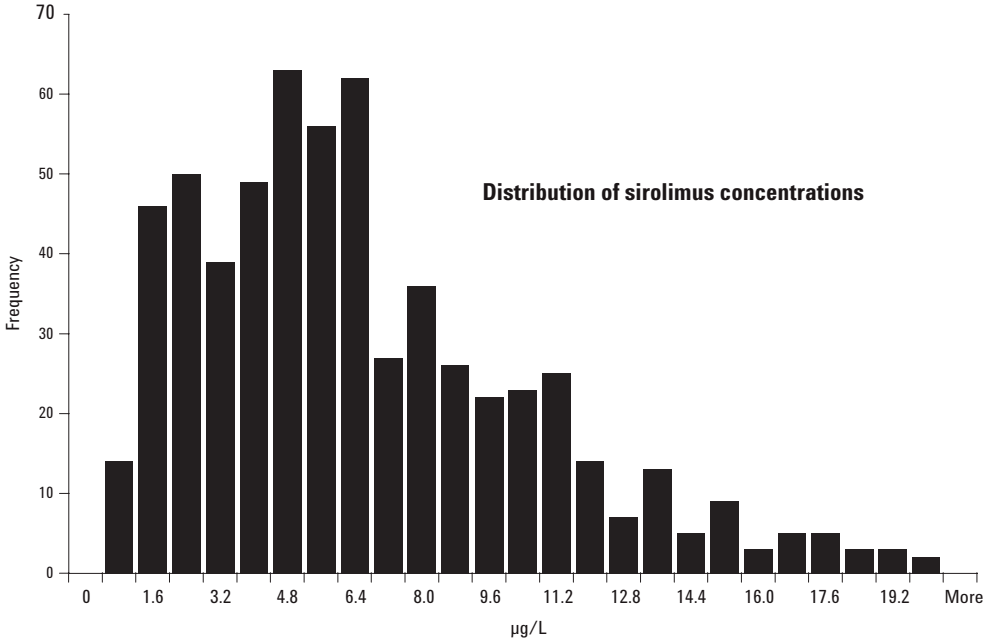


Figure 6. Distribution of observed sirolimus concentrations among clinic patients.

Conclusions

- Sirolimus and tacrolimus are measured simultaneously using semi-automated extraction and LC/MS.
- Method performance data validate that single analyzer LC/MS instrumentation can satisfactorily be used in place of the more costly LC/MS triple quadrupole.
- Minimal maintenance of the LC/MS is needed because of the cleanliness of sample extracts.
- For both drugs, linearity is from 0.5 µg/L up to at least 120 µg/L; between-run precision CVs are 15%–17% at concentrations 1–2 µg/L and 2.5%–4.9% over a concentration range of 4–49 µg/L; recovery is 82%–108%.
- This assay has proven its utility for daily therapeutic monitoring of sirolimus and tacrolimus in our clinical laboratory over the past 6 months.

Reference

1. G. L. Lensmeyer and M. A. Poquette, Therapeutic Monitoring of Tacrolimus Concentrations in Blood: Semi-Automated Extraction and Liquid Chromatography-Electrospray Ionization Mass Spectrometry *Therapeutic Drug Monitoring* 2001:23;239-249.

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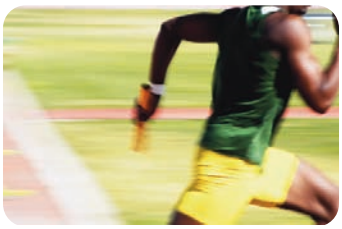
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Applications by Instrumental Technique

LC



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

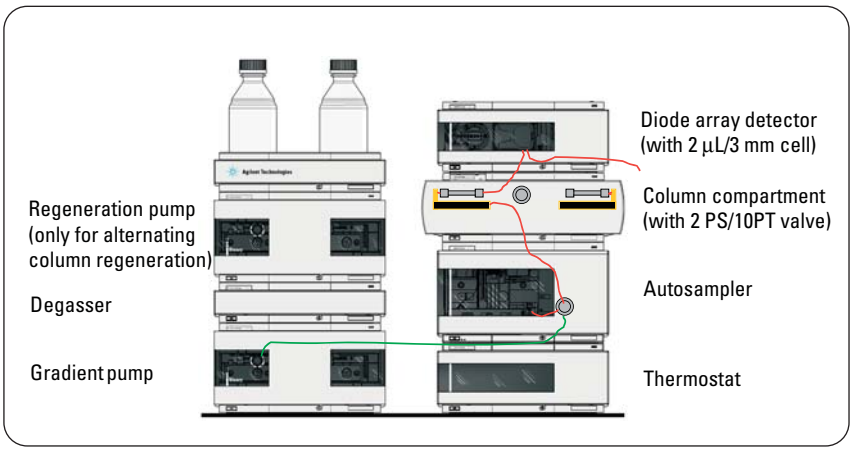


Figure 1
System setup with low delay volume for high speed applications using 2.1-mm id columns with lengths from 20 to 50 mm.

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.

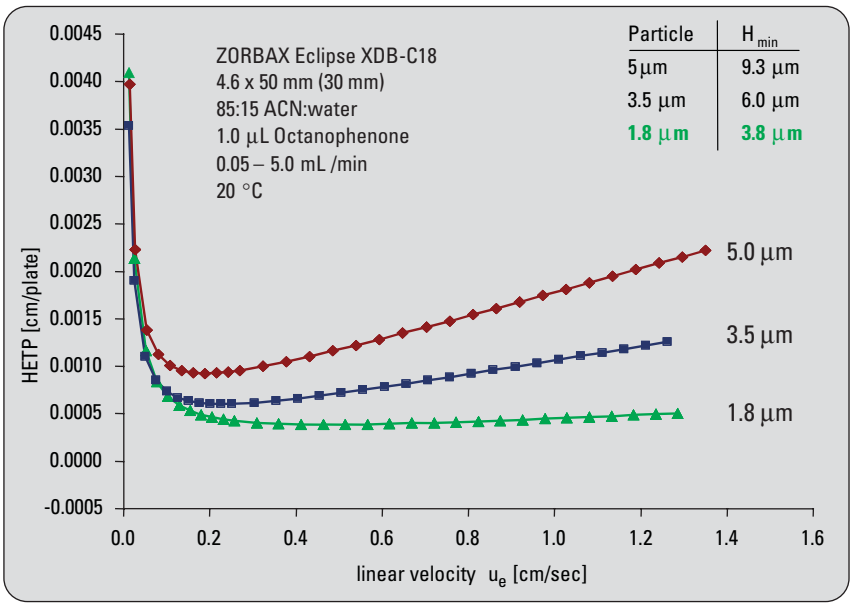


Figure 2
Van Deemter curves of columns packed with 1.8 µm, 3.5 µm and 5.0 µm particles.

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).

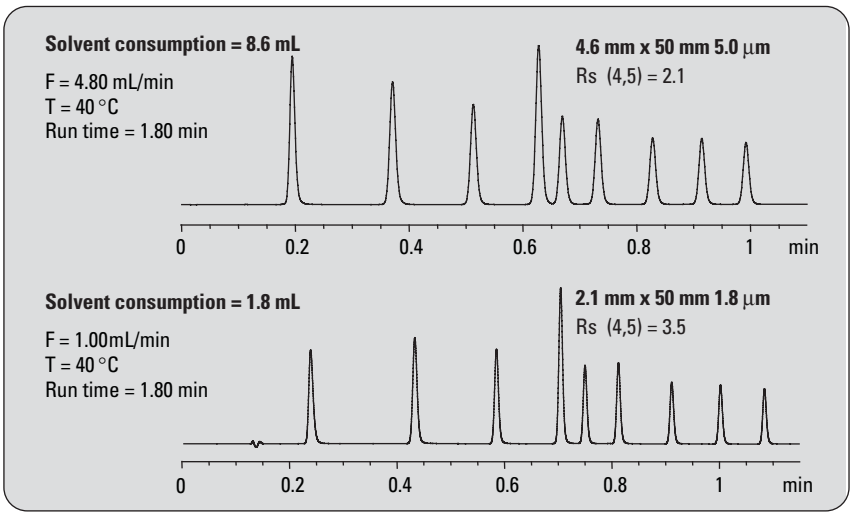


Figure 3
Analysis with 1.8-µm particle column vs. 5.0 µm particle column.

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)

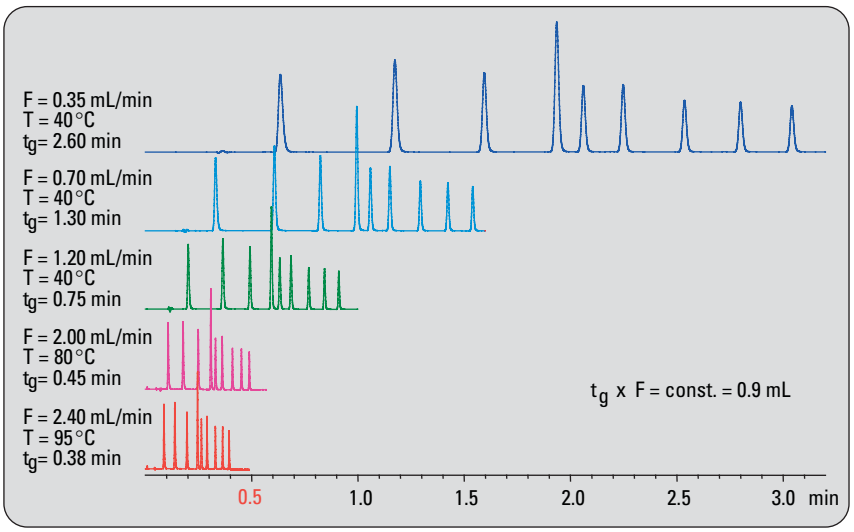


Figure 4
Increasing separation speed by increasing temperature and flow rate while decreasing gradient time.

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

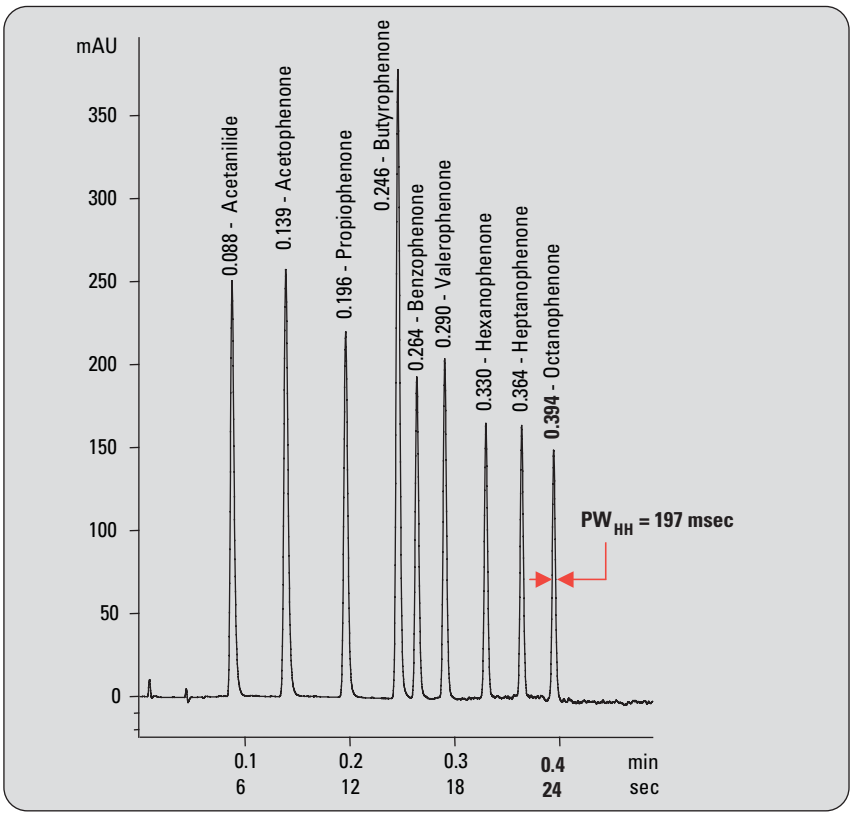


Figure 5
Separation of a nine compound mixture under ultra fast conditions.

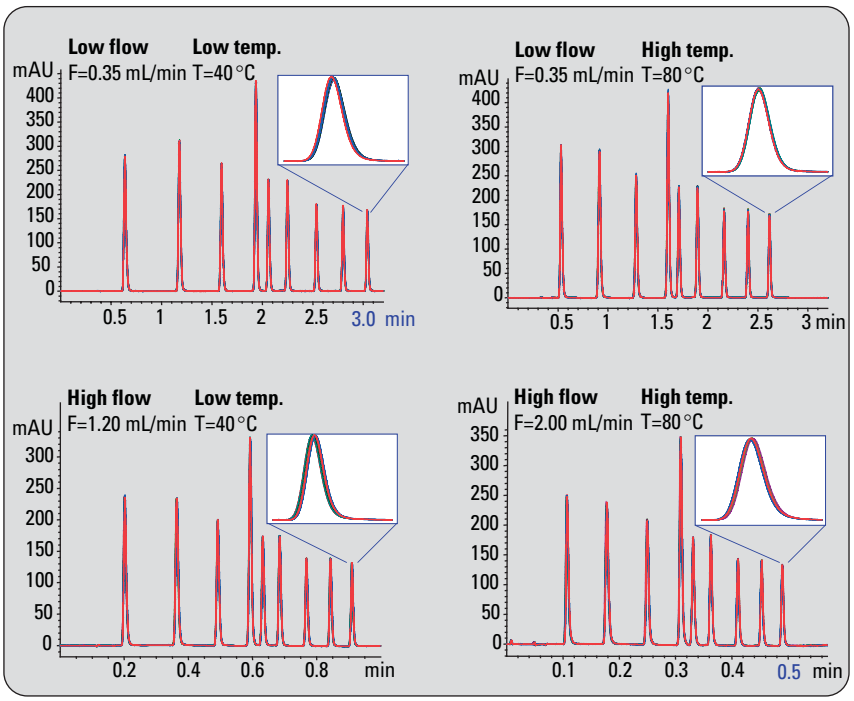


Figure 6
Overlaid chromatograms of the repeated analysis of a 9 compound mixture under various conditions.

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.

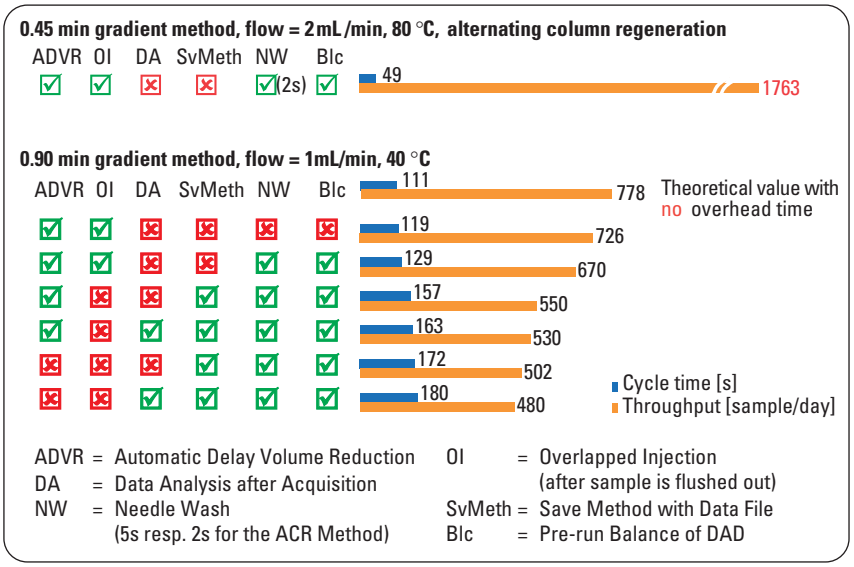


Figure 7
Cycle time and daily throughput optimization.

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

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.

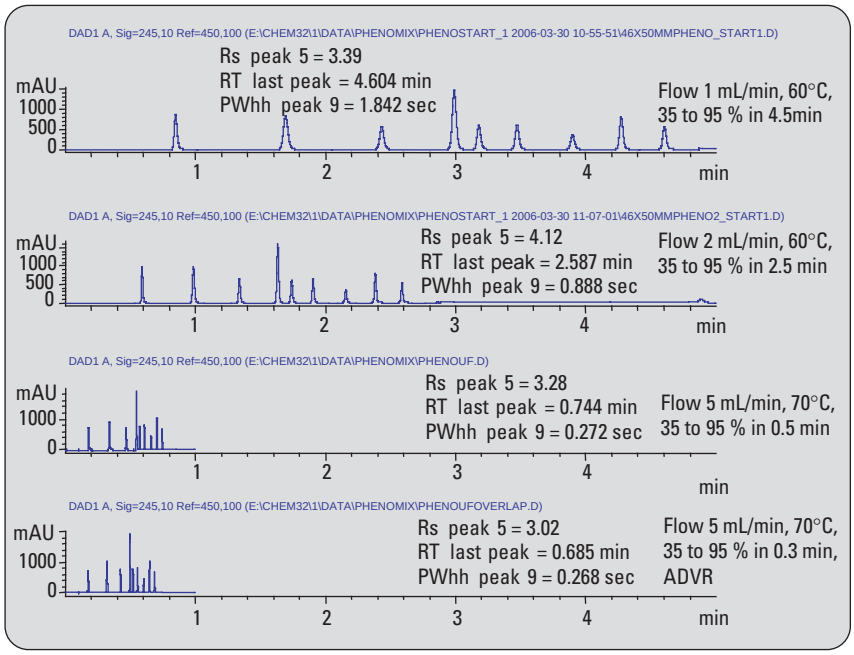


Figure 1
Method development of an ultra fast LC method.

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.

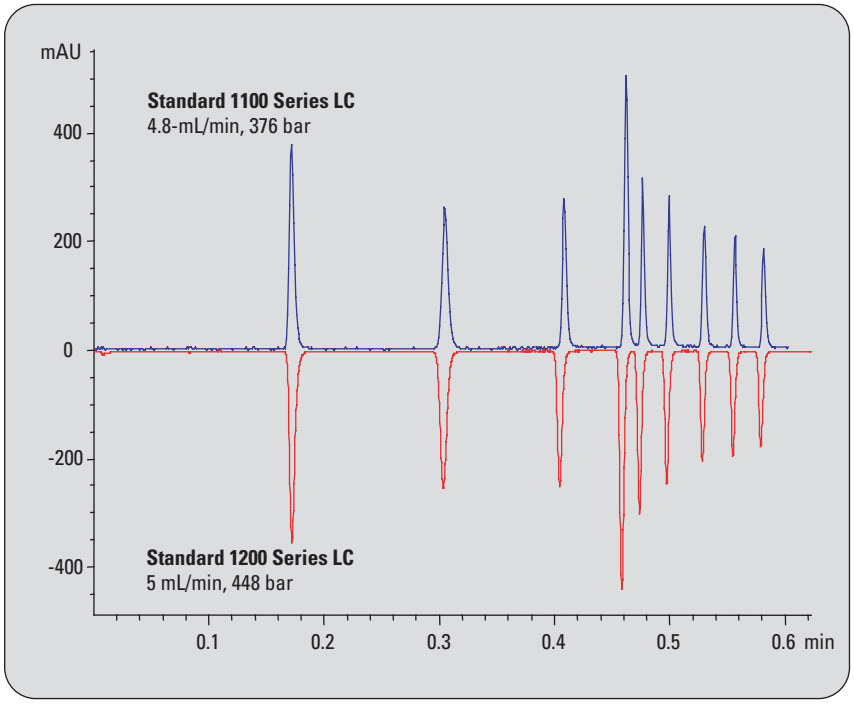


Figure 2
Standard Agilent 1200 Series RRLC system vs. Agilent 1100 Series LC system: analysis of phenone mix on 4.6-mm ID column packed with 1.8-µm particles.

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.

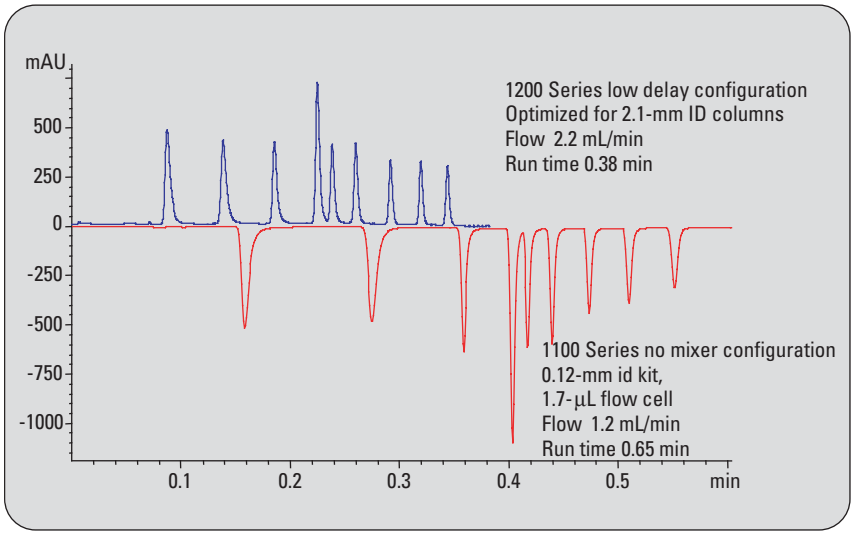


Figure 3
Analysis performed with a 2.1-mm ID column with the optimized Agilent 1200 Series RRLC system and the optimized Agilent 1100 Series LC system using automated delay volume reduction for both systems.

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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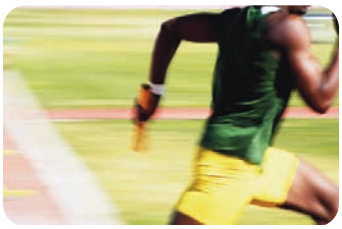
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Agilent Technologies







Applications by Instrumental Technique

GC/MS



Detection of Cannabinoids in Oral Fluid Using Inert Source GC/MS

Application

Forensics

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Abstract

Oral fluid is being considered as an alternative to urine in many forensic and clinical arenas. In general, the concentration of drugs in oral fluid is much lower than in urine, so sensitive extraction and analytical procedures are required. Tetrahydrocannabinol (THC) is the active ingredient in marijuana, which is the most widely used illegal recreational drug in the USA. 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 analytical 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 tetrahydrocannabinol (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, the mostly widely used illegal drug in the USA. Generally, it is administered via smoking, resulting in euphoria and hallucinations. 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 Validation

The analytical methods were validated 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

Concentration	THC CV (%) Intra	THC CV (%) Inter	CBN CV (%) Intra	CBN CV (%) Inter	CBD CV (%) Intra	CBD CV (%) Inter	2-c-THC CV (%) Intra	2-c-THC CV (%) 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, a 46-year-old male 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.

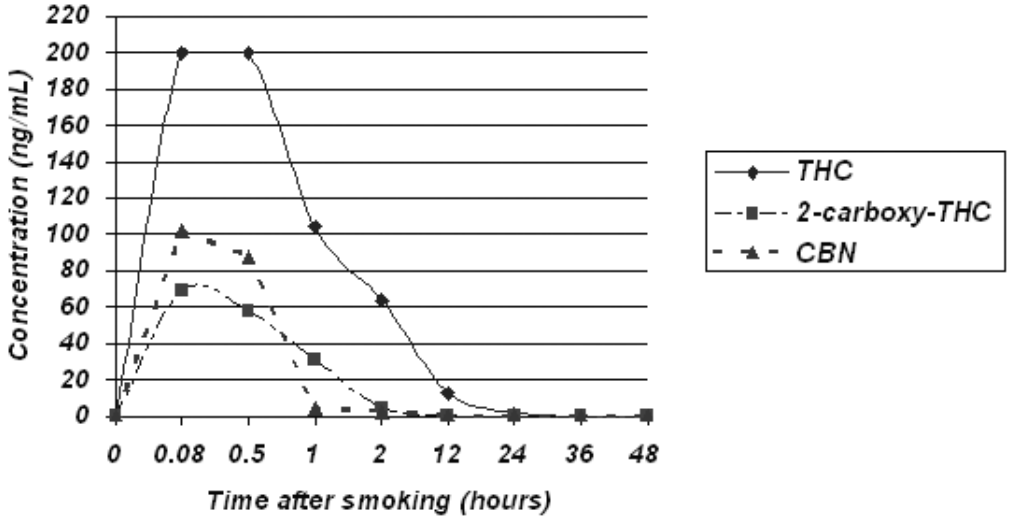


Figure 1. Cannabinoids in oral fluid following marijuana smoking.

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.

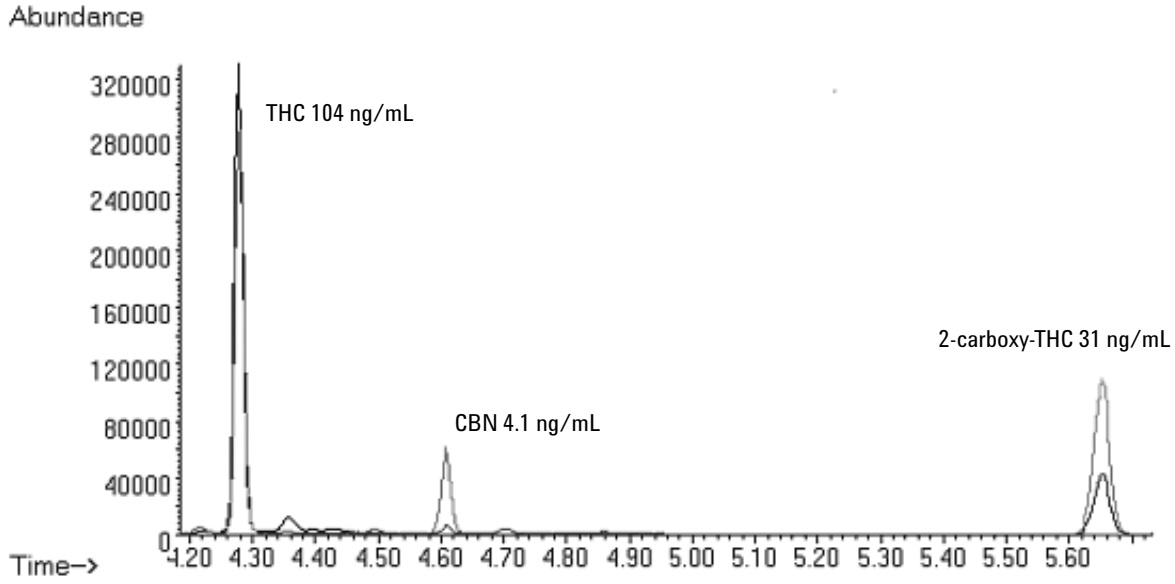


Figure 2. Oral fluid specimen collected 1 hour after marijuana smoking.

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Agilent Technologies

Combined EI and CI Using a Single Source

Technical Overview

Chris Sandy

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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.

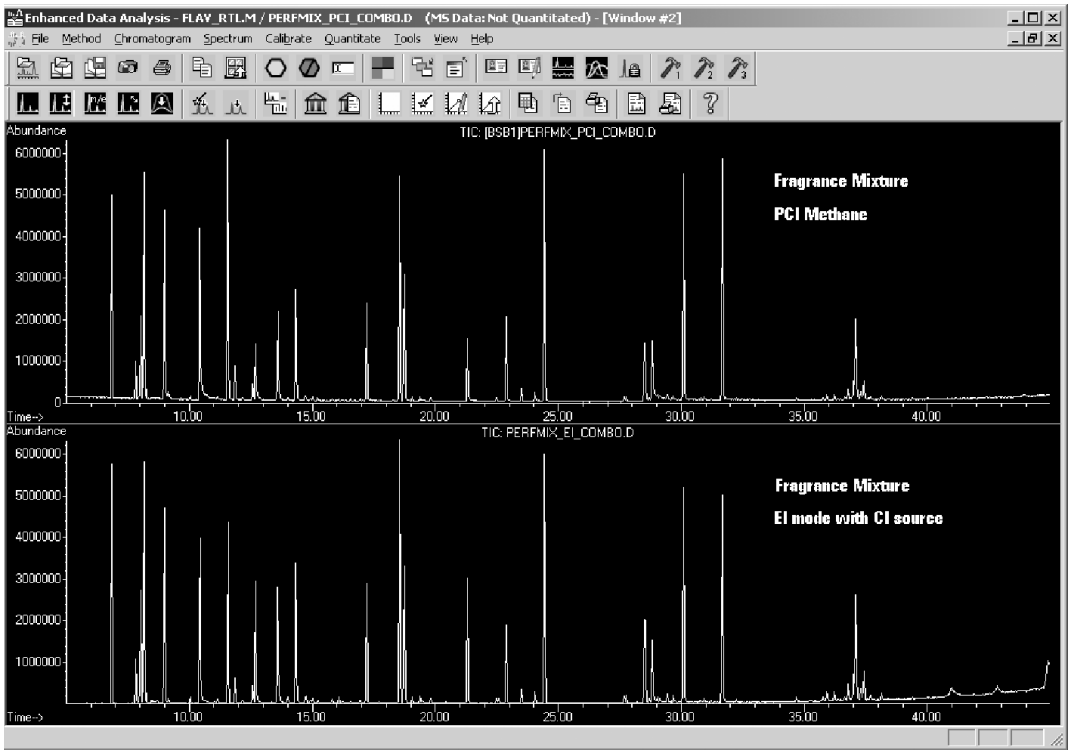


Figure 1. PCI and EI total ion chromatograms using the CI source.

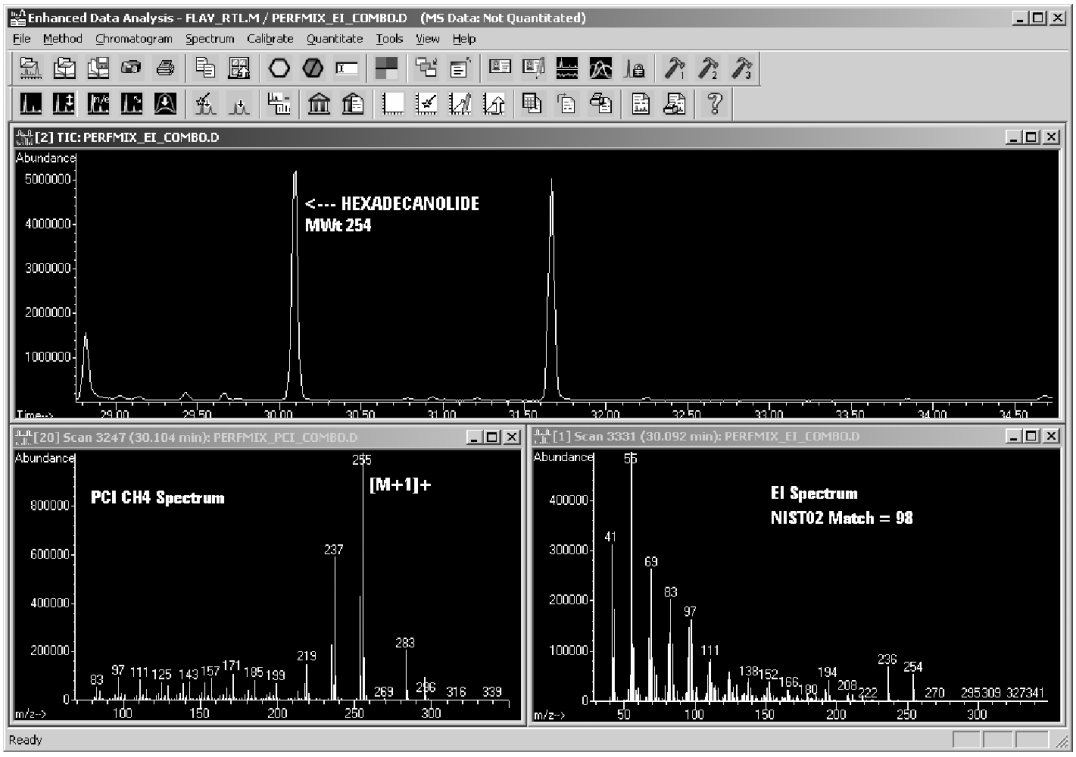


Figure 2. PCI and EI spectra for Hexadeconolide.

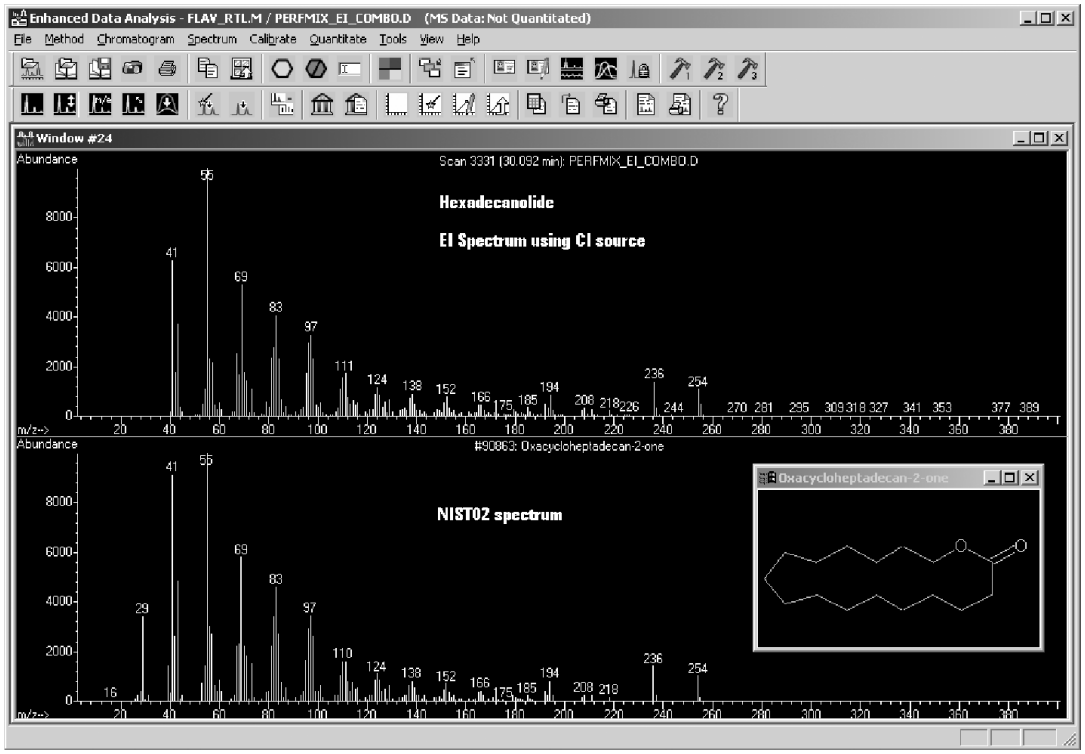


Figure 3. Acquired EI spectrum compared to the NIST02 library reference spectrum.

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

Enhanced Reliability of Forensic Drug Testing Using Retention Time Locking

Application

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, 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.

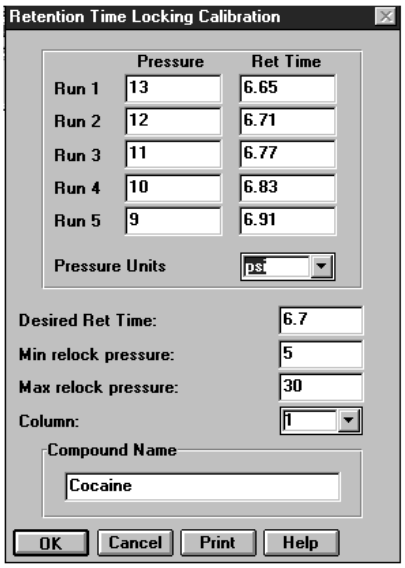


Figure 1. Preparation of retention time locking calibration using the Agilent GC ChemStation.

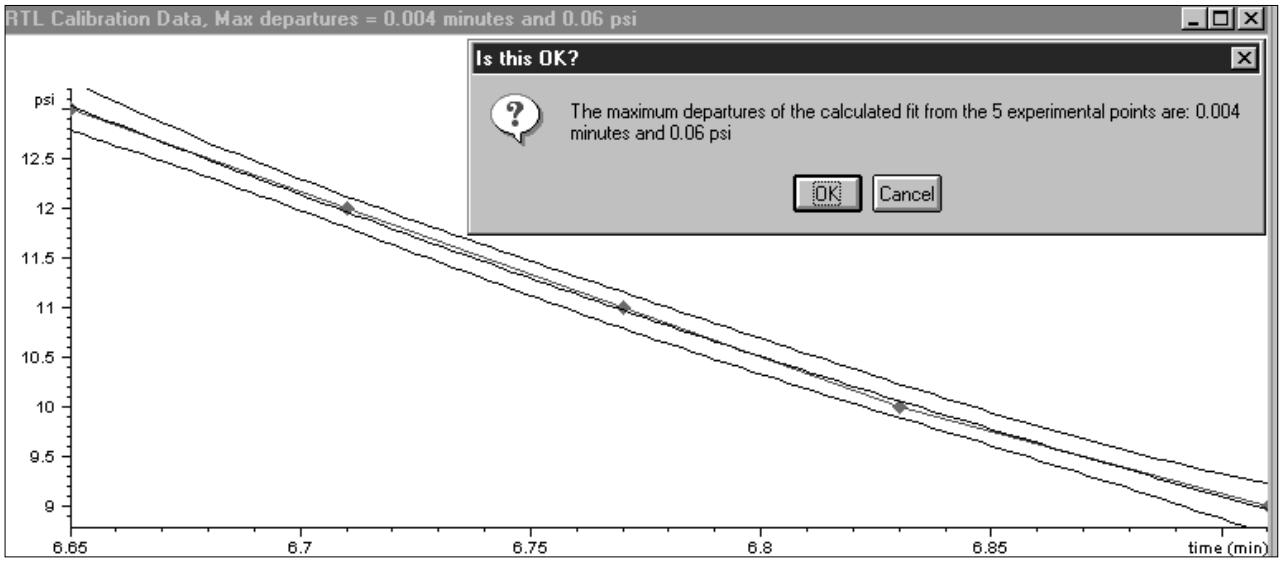


Figure 2. RT vs. P relationship for retention time locking.

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

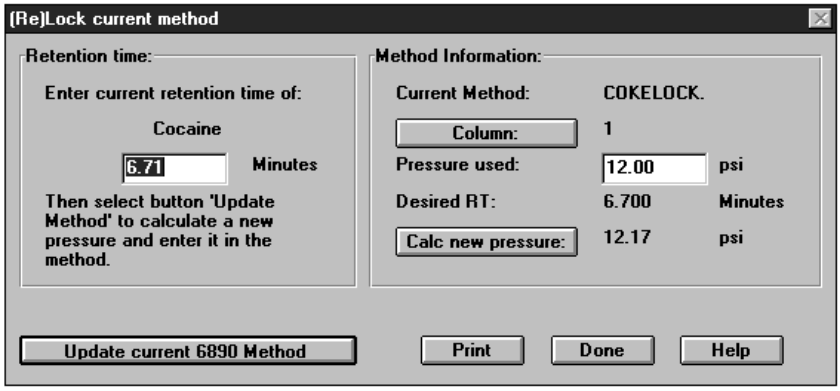


Figure 3. Column pressure recommendation for retention time locking and method update (as found on the RTL software for the GC ChemStation).

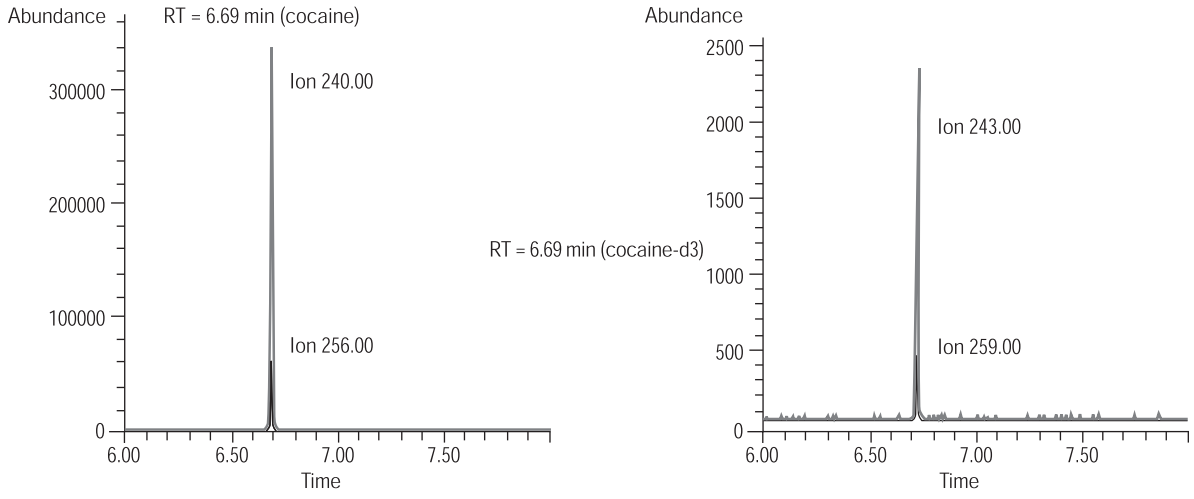


Figure 4. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System I (6890/5972 GC-MSD)

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

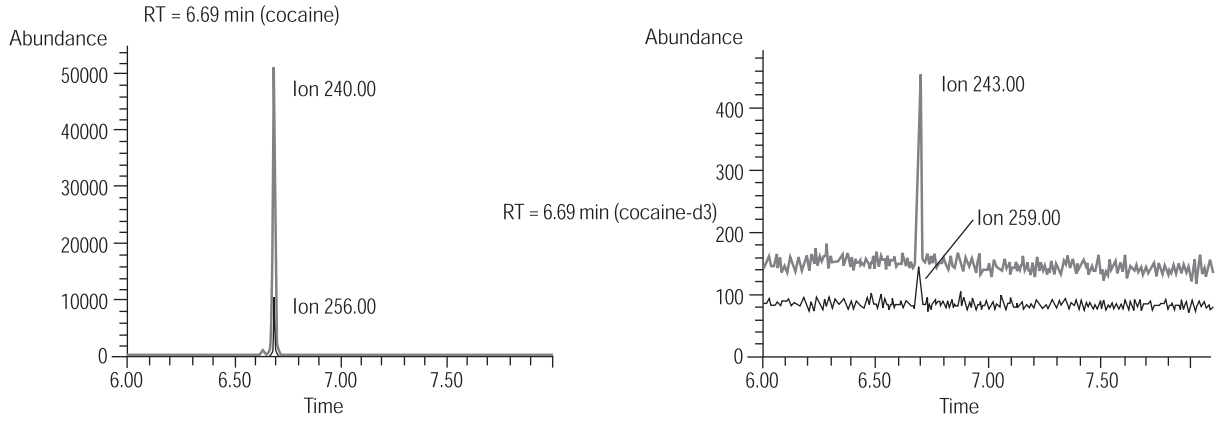


Figure 5. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System III (5890/5972 GC-MSD).

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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Agilent Technologies
Innovating the HP Way

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

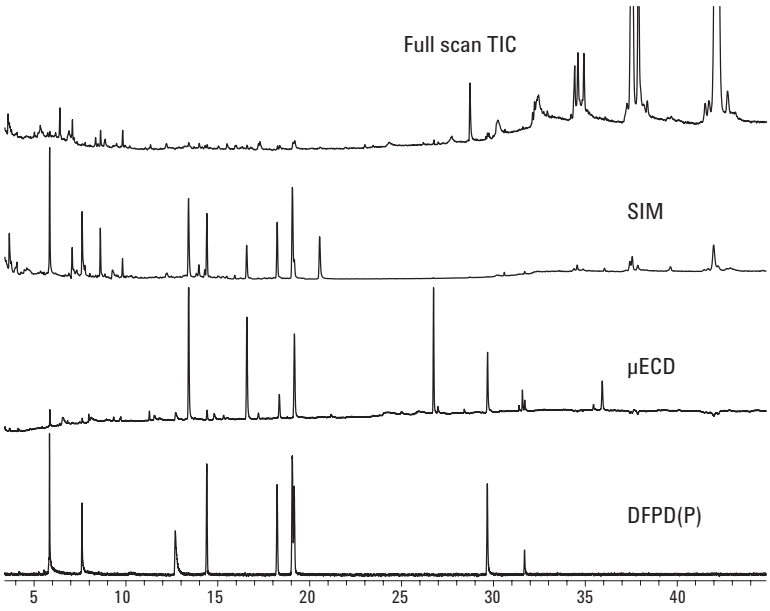


Figure 1. Four chromatograms collected simultaneously from a single injection of a milk extract.

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.

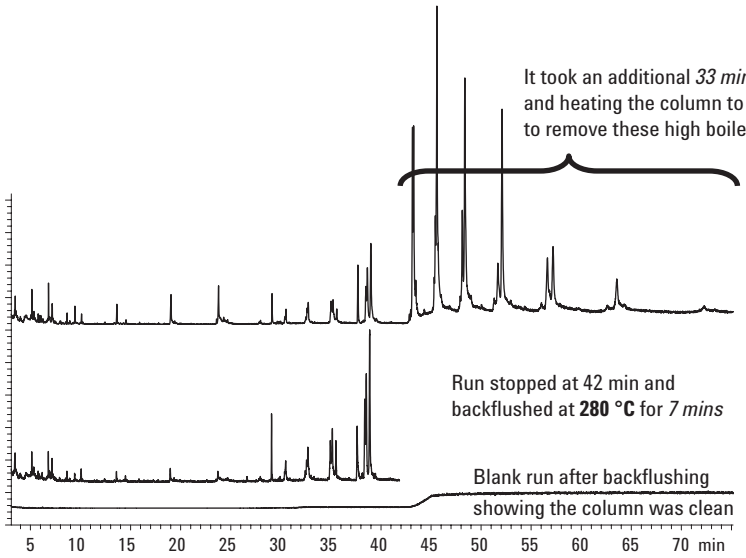


Figure 2. Three total ion chromatograms comparing the results with and without backflush.

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The 5973N inert MSD: Using Higher Ion Source Temperatures

Application

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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.

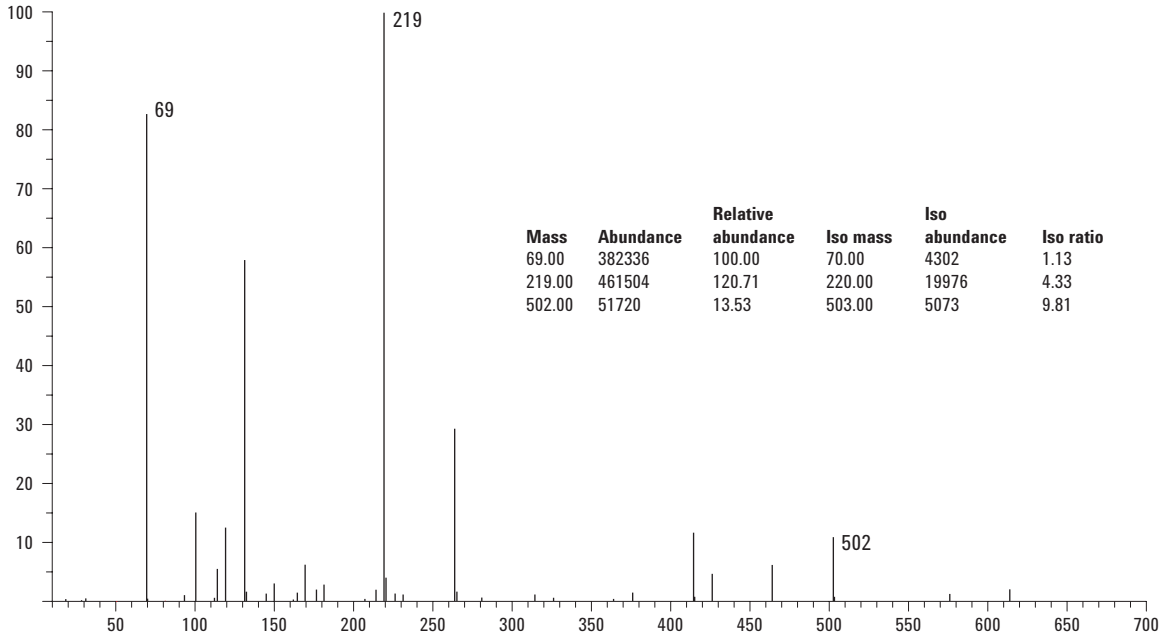


Figure 1. Autotune results for an ion source temperature of 230 °C.

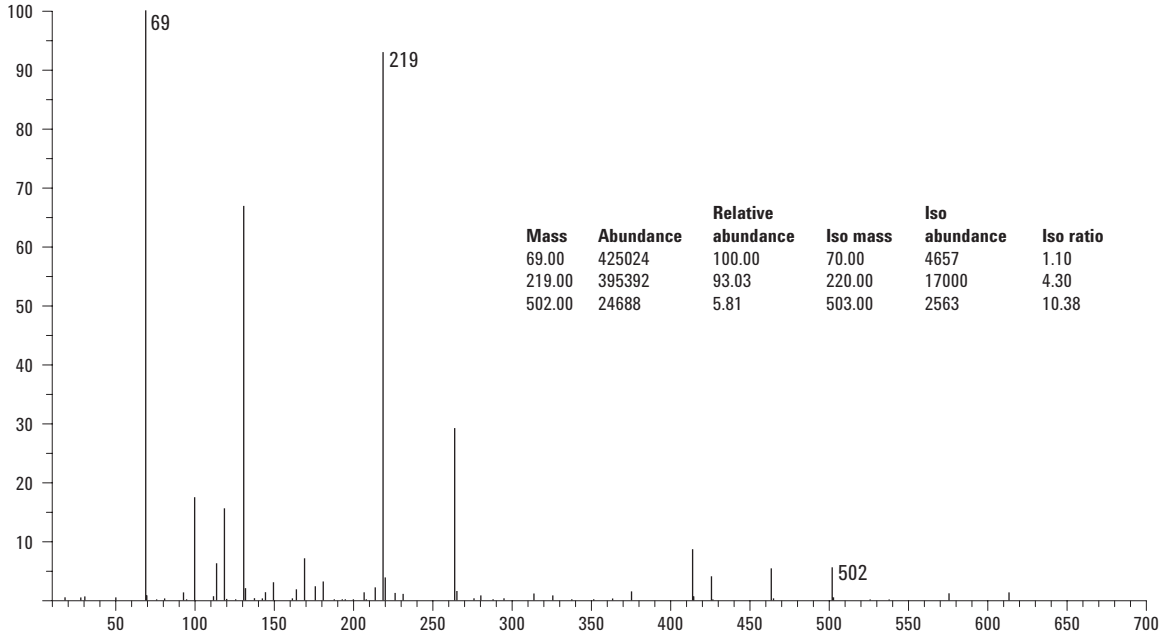


Figure 2. Autotune results for an ion source temperature of 300 °C.

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.

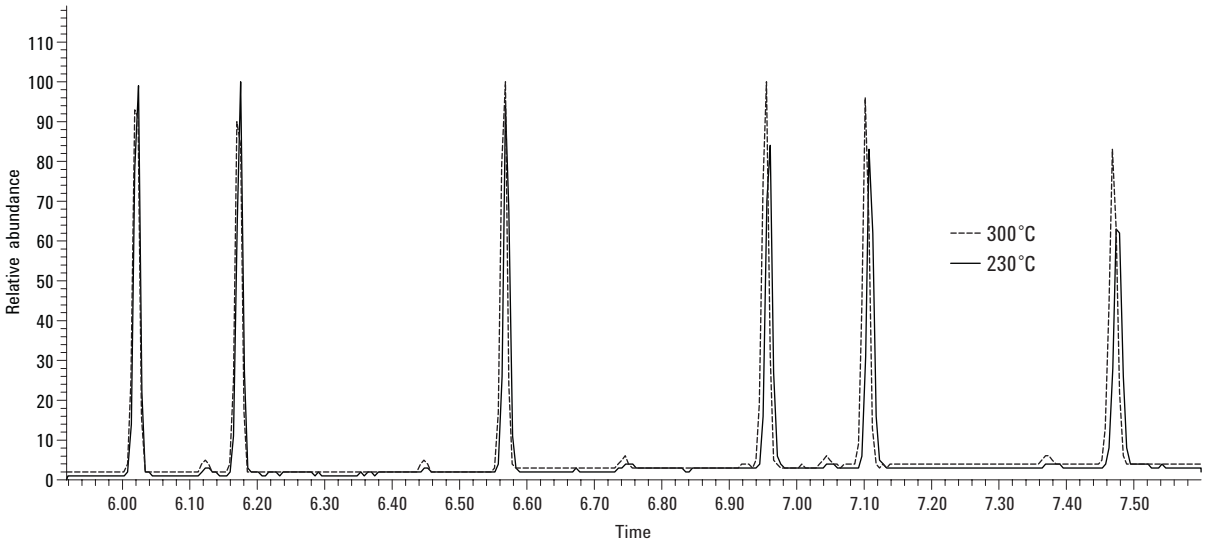


Figure 3. Overlaid RTICC of six PCBs acquired in full-scan (50–505 amu) at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram, the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

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.

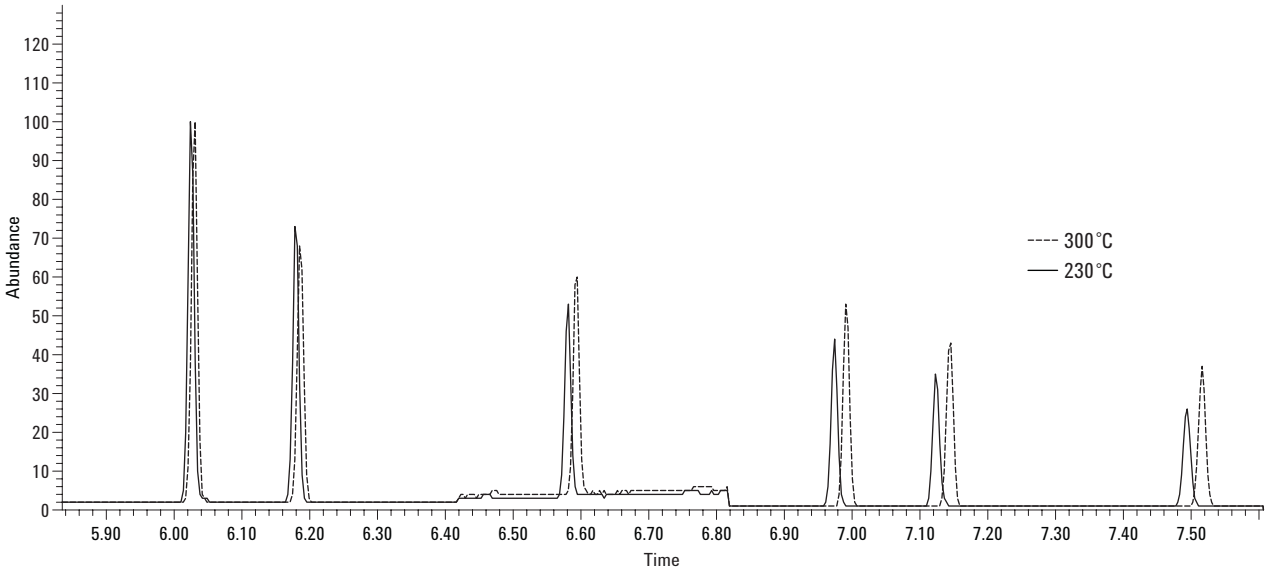


Figure 4. Overlaid RTICC of six PCBs acquired in SIM at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

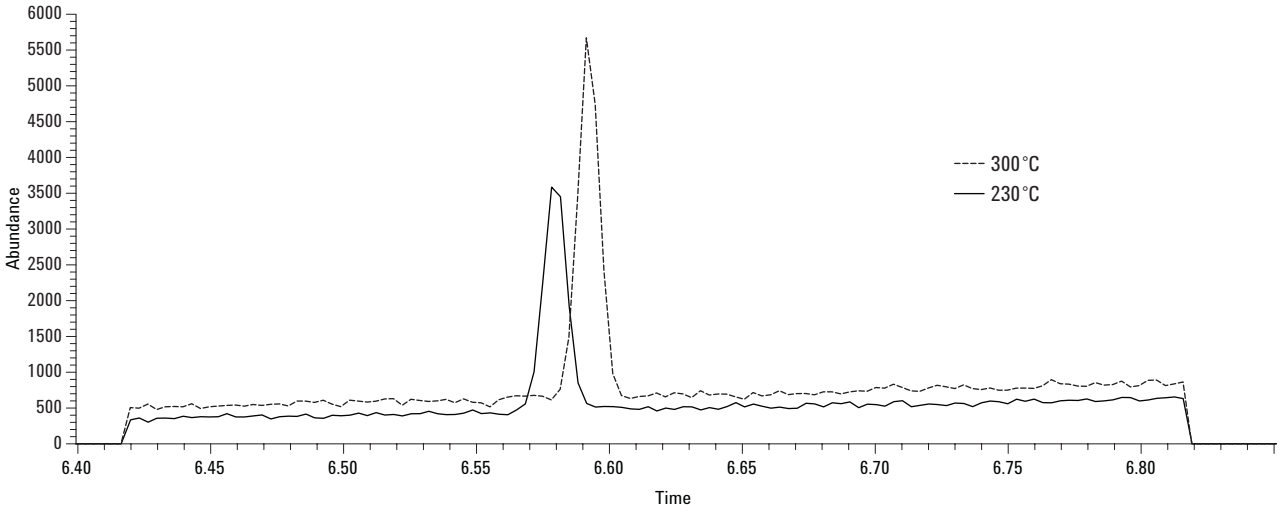


Figure 5. Overlaid extracted ion-current chromatograms of one ion (M-70) for the pentachlorobiphenyl acquired in SIM at source temperatures of 230 °C and 300 °C.

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.

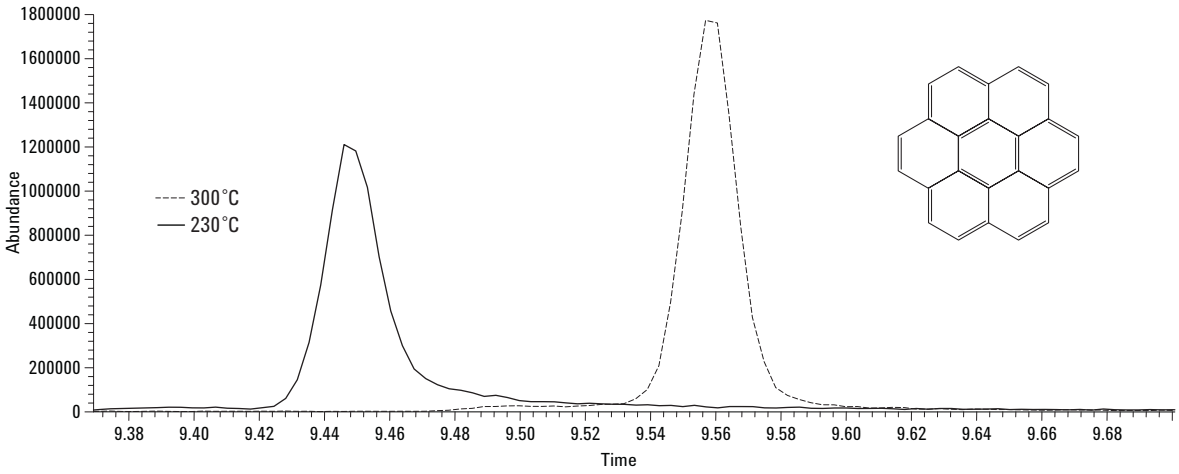


Figure 6. Overlaid extracted ion-current chromatograms of one ion (*m/z* 300) for coronene acquired in full scan at source temperatures of 230 °C, and 300 °C.

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.

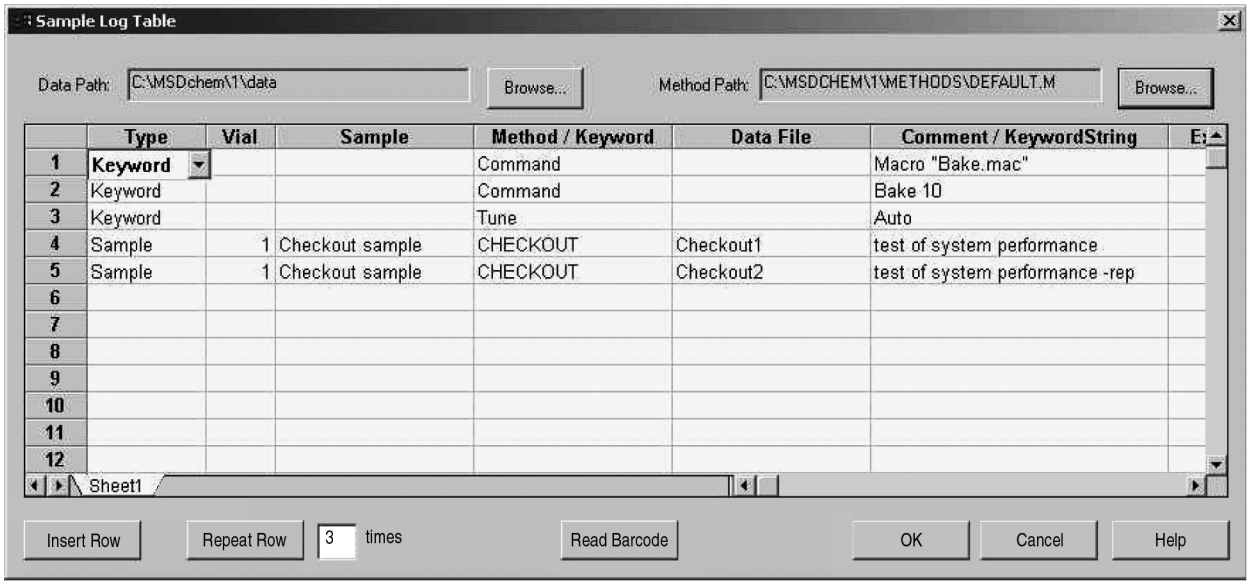


Figure 7. Pumpdown sequence table using source bakeout.

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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Agilent Technologies

Improving the Effectiveness of Method Translation for Fast and High Resolution Separations

Application

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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.

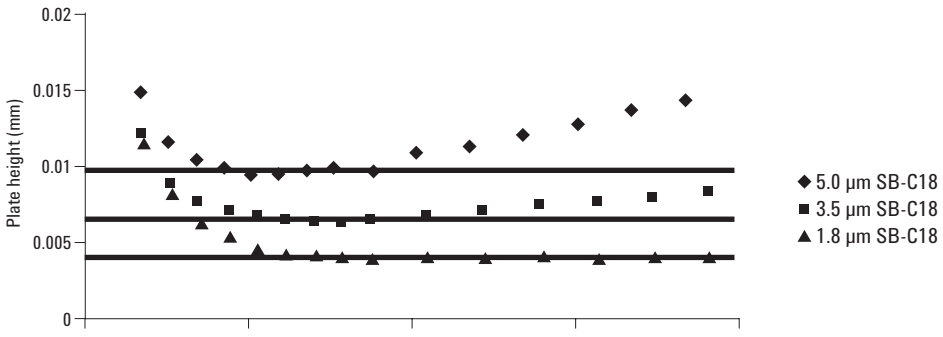
$$\text{Inj. vol.}_{\text{col. 1}} \times \left[\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}}\right] = \text{Inj. vol.}_{\text{col. 2}} \quad \text{(eq. 2)}$$

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

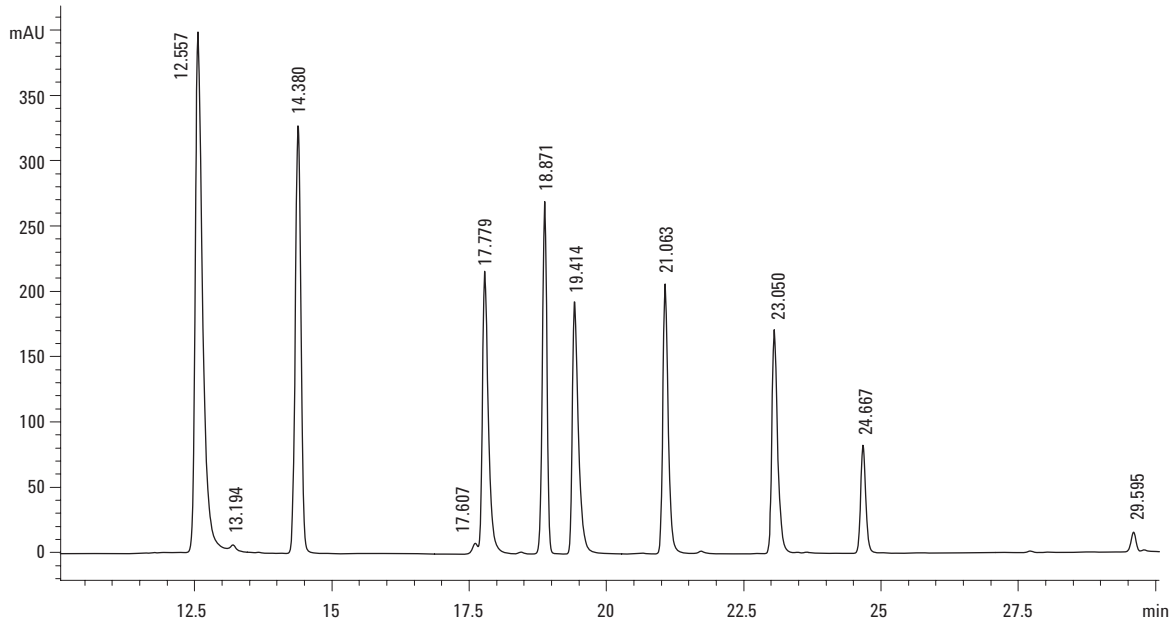
Standard mixture of chlorinated phenoxy acid herbicides, 100 µg/mL in methanol

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

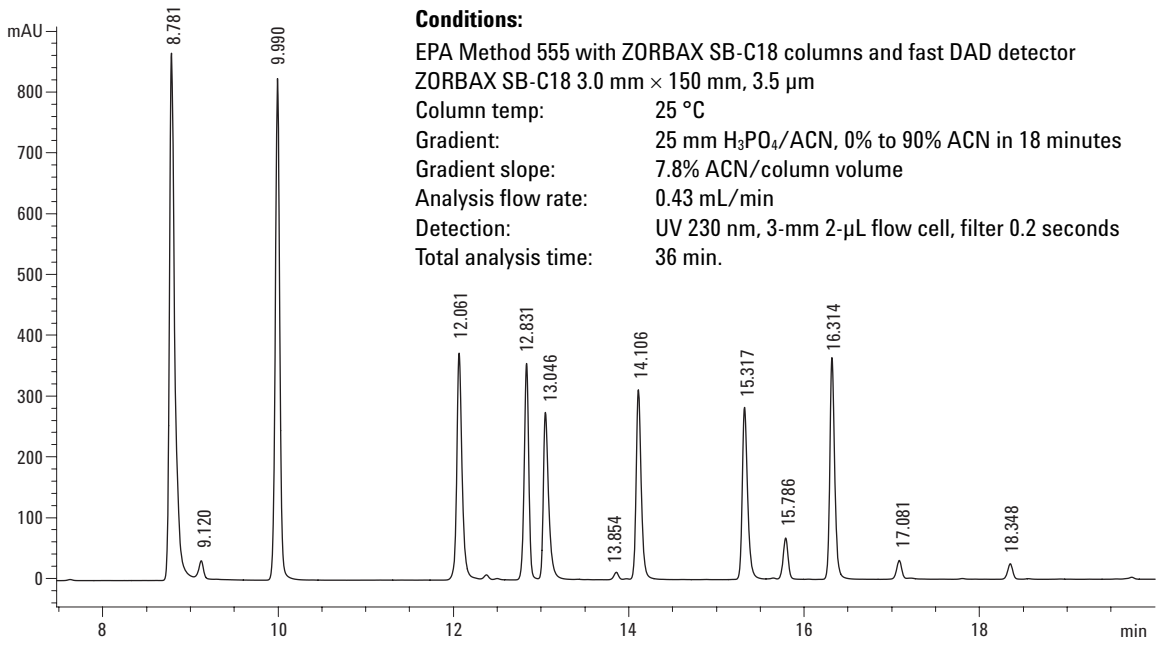
EPA Method 555 with ZORBAX SB-C18 columns and fast DAD detector
ZORBAX SB-C18 4.6 mm × 250 mm, 5 µm

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.

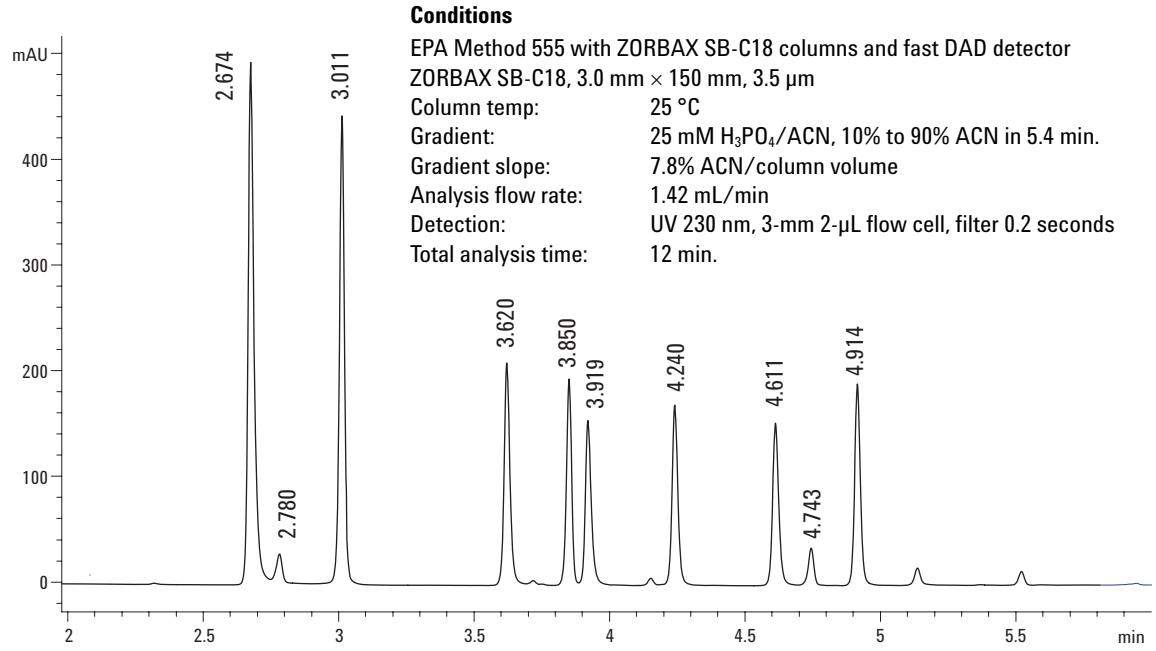


Figure 4. High speed gradient separation of herbicides on 3.0 150 mm, 3.5-µm ZORBAX SB-C18.

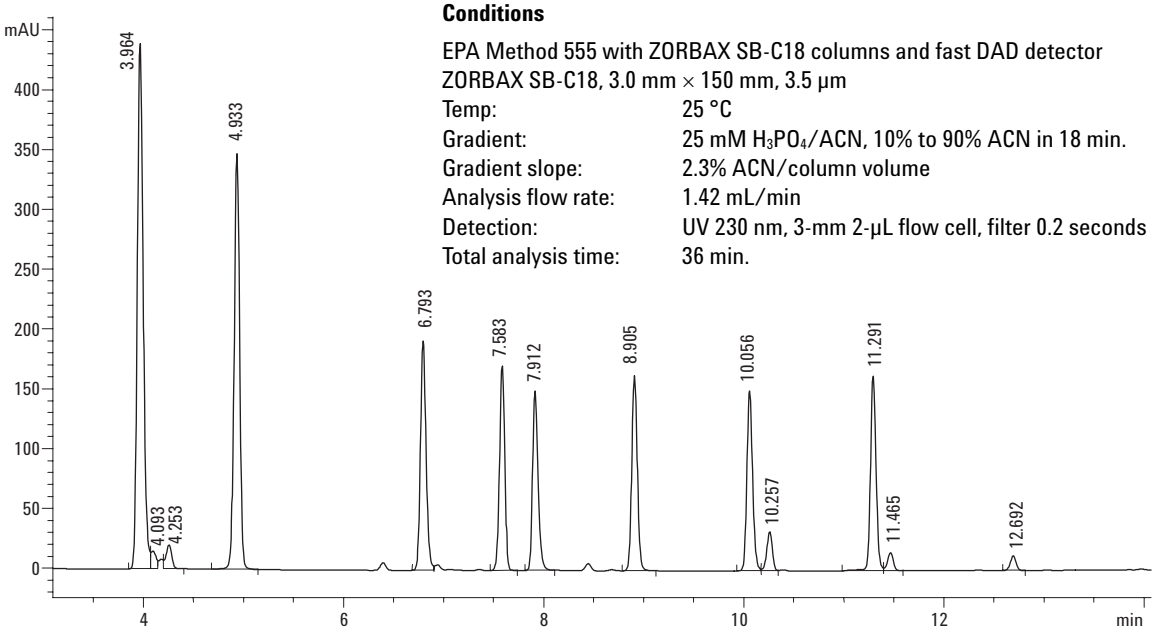


Figure 5. Reduced slope gradient separation of herbicides on 3.0 150 mm, 3.5-µm ZORBAX SB-C18.

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.

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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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Confirmation of Pain Medications in Oral Fluid Using Inert Source GC/MS

Application

Forensics

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Abstract

Oral fluid is being considered as an alternative to urine in many forensic and clinical arenas for the detection of prescription medications, which may be diverted and abused by medical professionals. Since "Med-Pro" testing panels are now widespread, the analysis of these medications in the less-invasive oral fluid is an attractive alternative for routine monitoring. In general, the concentration of drugs in oral fluids is much lower than in urine, so sensitive extraction and analytical procedures are required. There are no specific "cut-off" concentrations recommended for oral fluid analysis for these drugs, so procedures were developed to the limit of quantitation. The Agilent 5975 GC/MS with an inert source achieves the required sensitivity for the detection of meperidine, tramadol, propoxyphene, and oxycodone in oral fluid. 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

Various laboratories currently offer "Medical Professional" drug test panels, which as the name implies, are targeted at the detection of prescription medications as well as the more common drugs of abuse. Standard prescription medication drug test panels include meperidine (Pethidine, Demerol), tramadol (Ultram), propoxyphene (Co-proxamol) and oxycodone (Percocet, Oxycontin).

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.

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.

Standards and Reagents

- Deuterated internal standards: Cis-tramadol d4, meperidine-d4, oxycodone-d6, propoxyphene-d5, and unlabeled drug standards: meperidine, tramadol, oxycodone, and propoxyphene were purchased from Cerilliant (Round Rock, TX).
- Clin II solid phase extraction columns (Part #691-0353T) were purchased from SPEWare (San Pedro, CA).
- Derivatizing agents: N,O-Bis (trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS), and N-methyl-N-trimethylsilyltrifluoroacetamide +1% trimethylchlorosilane (MSTFA +1% TMCS) were from Pierce (Rockford, IL).

Internal Standard Concentration

Meperidine, propoxyphene, and tramadol 250 ng/mL; oxycodone 200 ng/mL

Sample Preparation for Chromatographic Analysis

- 1 mL Quantisal specimen (equivalent to 0.25 mL of oral fluid)
- Add internal standard
- For meperidine, tramadol, and oxycodone, add 0.1 M sodium phosphate buffer (pH 6.0; 1 mL)
- For propoxyphene, add 0.05 M sodium hydrogen carbonate buffer (pH 8.0; 1 mL)
- Condition SPE columns: methanol (2 mL), 0.1 M phosphate buffer (pH 6.0; 2 mL)
- Add samples
- Wash columns:
  - deionized water (1 mL)
  - 0.1 M acetate buffer (pH 4; 1 mL)
  - methanol (1 mL)
  - ethyl acetate (1 mL)
- Dry columns under nitrogen (30 psi; 2 min)
- Elute: freshly prepared ethyl acetate: ammonium hydroxide (98:2 v,v; 2 mL)
- Evaporate to dryness under nitrogen

No Derivatization

Propoxyphene: Reconstitute in ethyl acetate (50 µL); transfer to autosampler vials

Meperidine: Meperidine does not derivatize since there are no active hydrogen sites available for reaction; however, the extract was reconstituted in ethyl acetate (20 µL). BSTFA + 1% TMCS (20 µL) were added, capped, and heated (50 °C/20 min). The addition of a silanizing reagent to the extract improved stability for the extract and produced markedly better chromatography of meperidine.

Derivatization

Tramadol: Reconstitute in ethyl acetate (25 µL); add BSTFA +1% TMCS (25 µL); transfer to autosampler vials, cap, and incubate (70 °C/20 min).

Oxycodone: Reconstitute in 1% hydroxylamine HCl in pyridine solution (50 µL) and incubate (45 °C/30 min). Add MSFTA + 1% TMCS (50 µL), cap, and incubate (65 °C/20 min).

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 Programs

Propoxyphene:	60 °C for 1 min; ramp 30 °C/min to 200 °C; hold 0.2 min; ramp 80 °C/min to 250 °C
Meperidine:	50 °C; ramp at 30 °C/min to 280 °C
Tramadol:	65 °C for 1 min; ramp 40 °C/min to 200 °C; ramp 15 °C/min to 230 °C; ramp 100 °C/min to 290 °C
Oxycodone:	100 °C for 0.5 min; ramp 10 °C/min to 270 °C

Ions Monitored

Drug	Ions monitored					
Propoxyphene	Deuterated (d5)	**213.2**,	198.1;	**208.2**,	193.1,	179.1
Meperidine	Deuterated (d4)	**251.2**,	222.2;	**247.2**,	218.2,	172.2
Tramadol	Deuterated (d4	**339.3**,	324.2;	**335.3**,	245.2,	320.2
Oxycodone	Deuterated (d6)	**480.3**,	391.3;	**474.3**,	385.3,	459.3

Quantitative ions in bold type

Retention Times

	Minutes
Propoxyphene:	5.5
Meperidine:	5.1
Tramadol:	5.4
Oxycodone:	16.0

Results and Discussions

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. Oral fluid was fortified with all three drugs at the concentration of 25 ng/mL for tramadol and meperidine, 20 ng/mL for oxycodone, and 10 ng/mL propoxyphene.

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 (n = 6).

	Meperidine	Oxycodone	Tramadol	Propoxyphene
Mean drug recovery (%)	86.7	96.6	87.7	92.0

GC/MS Method Validation

The analytical methods were validated 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 five days. The slope of the calibration curve was forced through the origin.

Analyte	Limit of quantitation (ng/mL)	Equation (mean SD)	Correlation (r^2)	Linearity range (ng/mL)
Meperidine	10	y = 0.0196x	0.999	10–100
Propoxyphene	5	y = 0.0392x	0.999	5–300
Oxycodone	10	y = 0.0132x	0.997	10–80
Tramadol	10	y = 0.0190x	0.999	10–100

Inter-day Precision (n = 10)

Drug	Expected concentration (ng/mL)	Observed concentration (mean ±SD) (ng/mL)	Precision (%)
Meperidine	25	25.32 ± 0.628	2.48
Propoxyphene	10	10.12 ± 0.36	3.59
Oxycodone	20	20.74 ± 1.202	5.80
Tramadol	25	25.65 ± 0.627	2.44

Inter-day Precision (n = 5)

Drug	Expected concentration (ng/mL)	Observed concentration (mean +-SD) (ng/mL)	Precision (%)
Meperidine	25	25.72 ± 0.60	2.33
Propoxyphene	10	9.8 ± 0.23	2.39
Oxycodone	20	23.08 ± 1.75	7.61
Tramadol	25	25.88 ± 0.25	1.00

Commonly encountered drugs were extracted and analyzed at high concentrations and found not to interfere with the assays. Figure 1 shows tramadol (1175 ng/mL) in oral fluid from an authentic specimen.

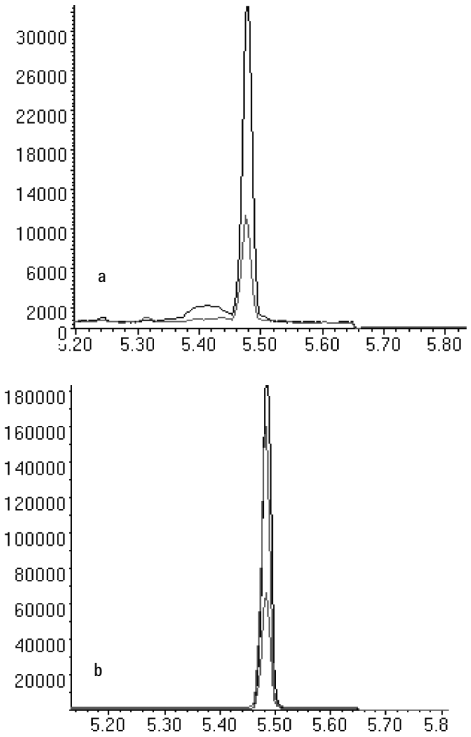


Figure 1. Oral fluid specimen collected using the Quantisal device and analyzed using the described procedure.

a) D4-tramadol: Ions 339.3; 324.3

b) Tramadol 335.3, 320.3, 245.2

Conclusions

The procedures described are suitable for the detection of these pain medications in oral fluid using an Agilent Technologies single quadrupole GC/MS system with an inert source. The methods are in routine use for the measurement of drug concentrations in oral fluid.

Reference

S. Rana, C. Moore, A. Agrawal, C. Coulter, M. Vincent, and J. Soares. Determination of propoxyphene in oral fluid. *J Anal Toxicol* 2006; 30(8): 516-518.

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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.

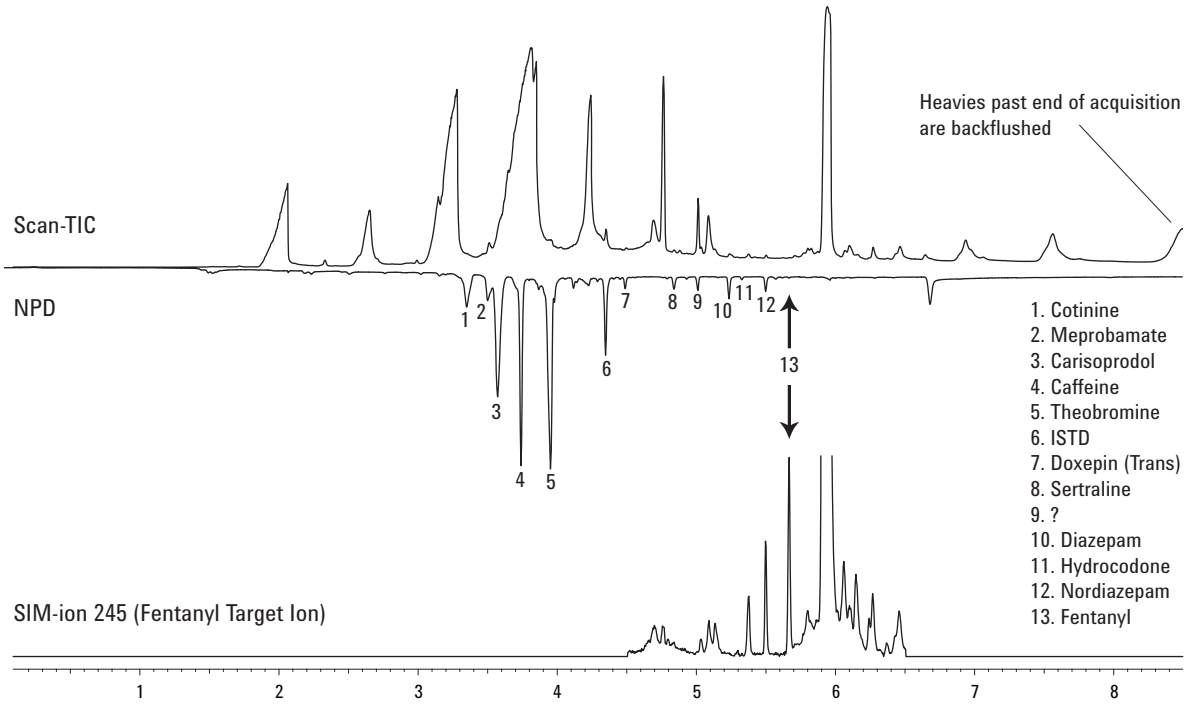


Figure 1. Chromatograms from screen of whole blood sample.

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 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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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

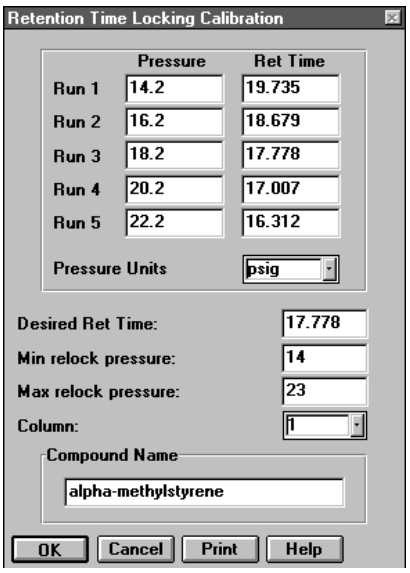


Figure 1. Dialog box used for entering retention time locking calibration data

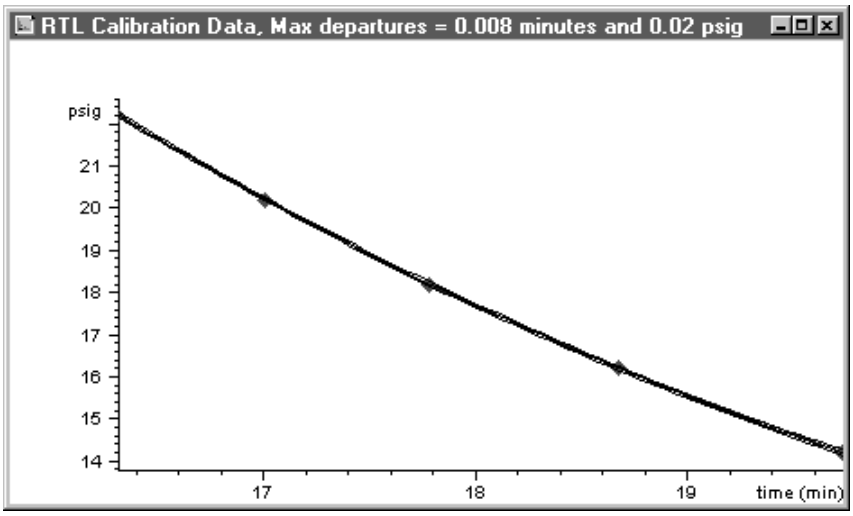


Figure 2. Plot of calibration data as displayed by RTL software

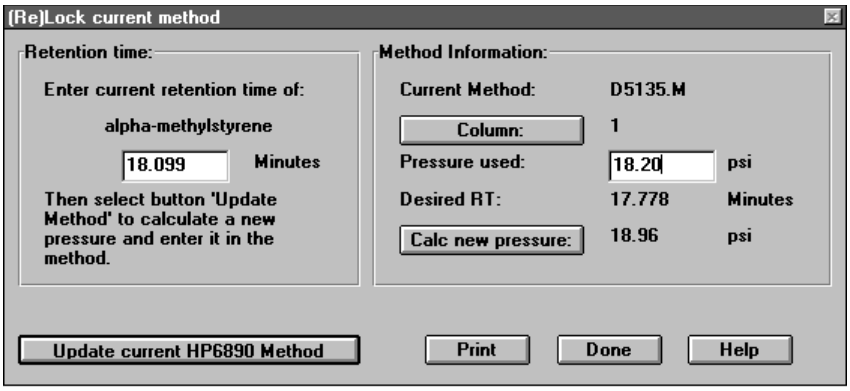


Figure 3. Dialog box used to calculate locking pressure and update the 6890 method

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

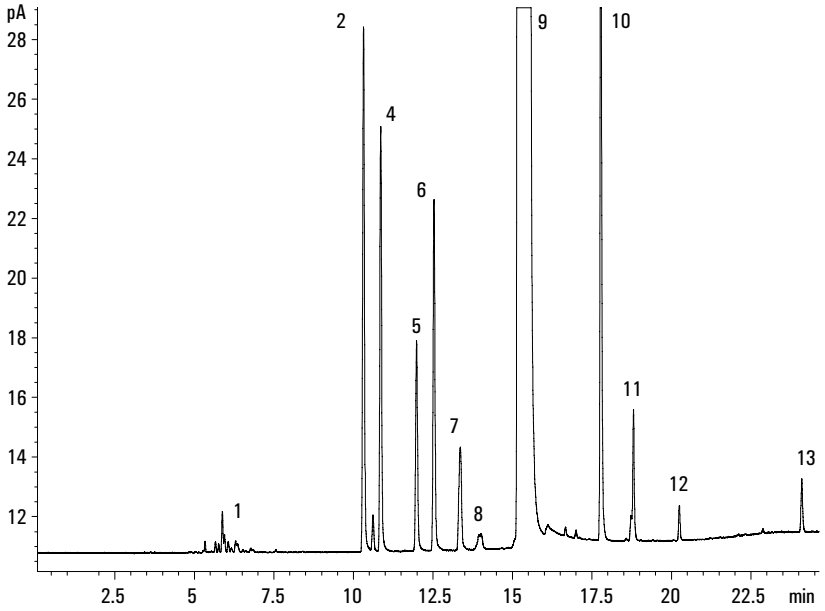


Figure 4. Styrene sample run on GC system 1 at 18.2 psi initial pressure, constant flow mode

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		

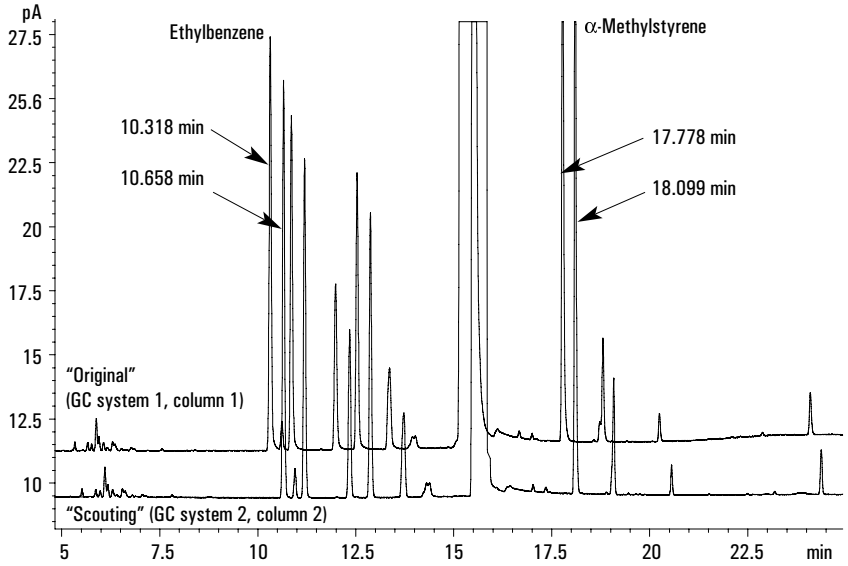


Figure 5. Comparison of original chromatogram on GC system 1 with GC system 2 before retention time locking

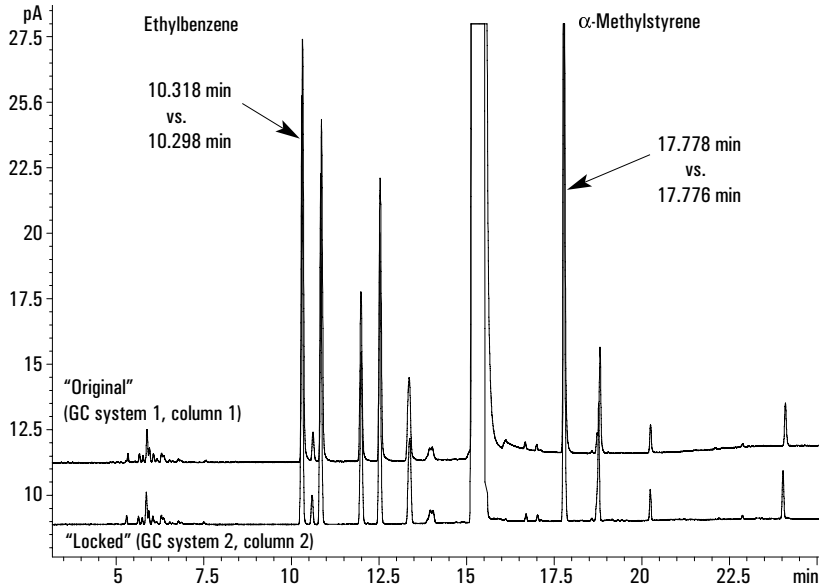


Figure 6. Comparison of original chromatogram on GC system 1 with GC System 2 after retention time locking

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

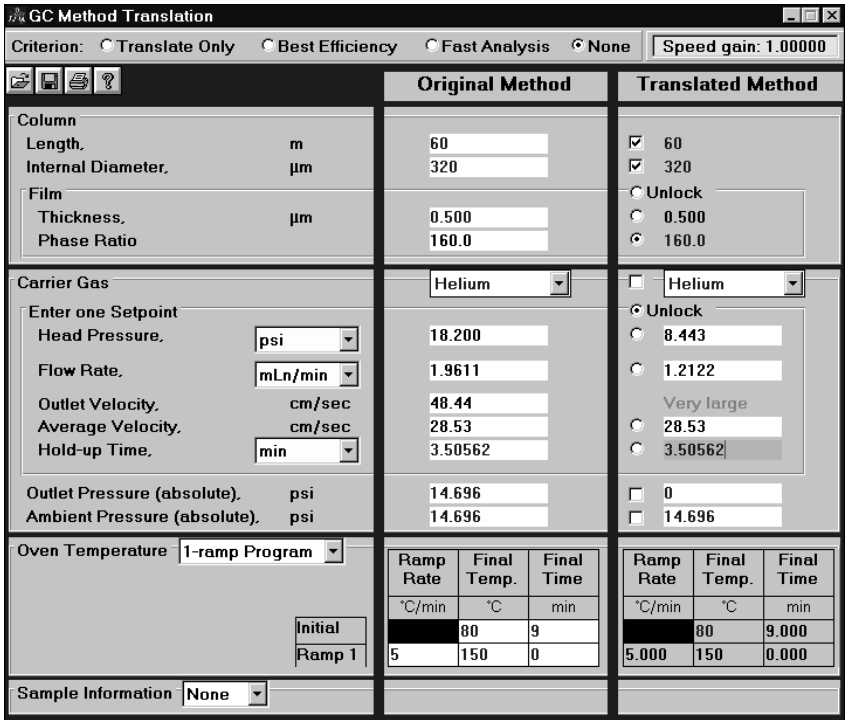


Figure 7. Method translation software provides scaled conditions for GC systems with different configurations

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.

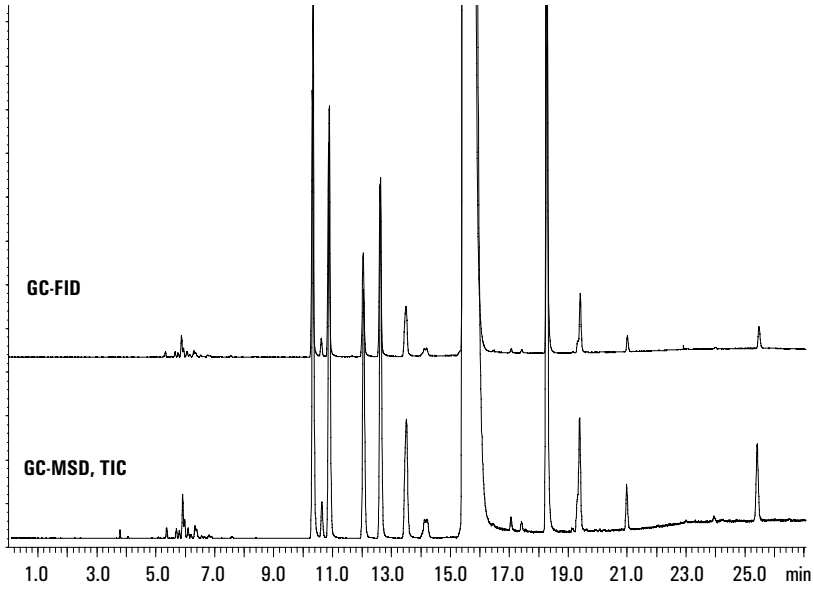


Figure 8. Comparison of chromatogram on GC system 1 with GC-MSD system after retention time locking, Constant Pressure Mode

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

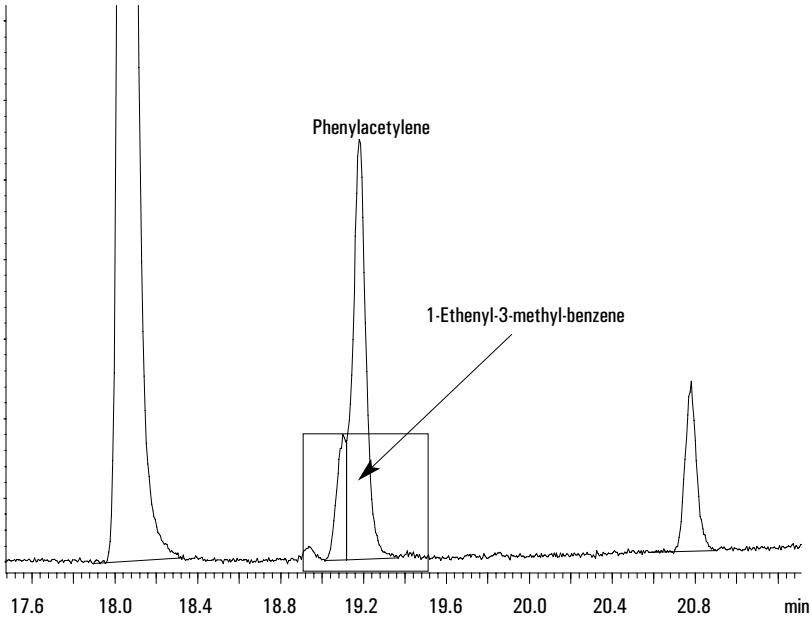


Figure 9. GC-MSD identification of impurity in shoulder of phenylacetylene peak

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

Application

Forensics

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Abstract

Oral fluids are being used as an alternative matrix to urine for drug testing related to employment, drug treatment, parole, and suspicion of driving under the influence of drugs. Oral fluid 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

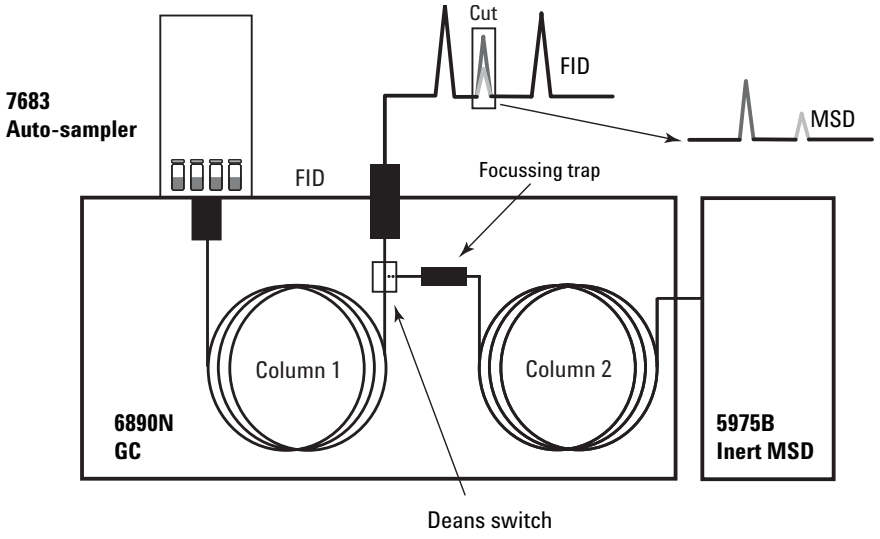


Figure 1. Hardware configuration of 2-D heartcutting GC/MSD system.

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 patients 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

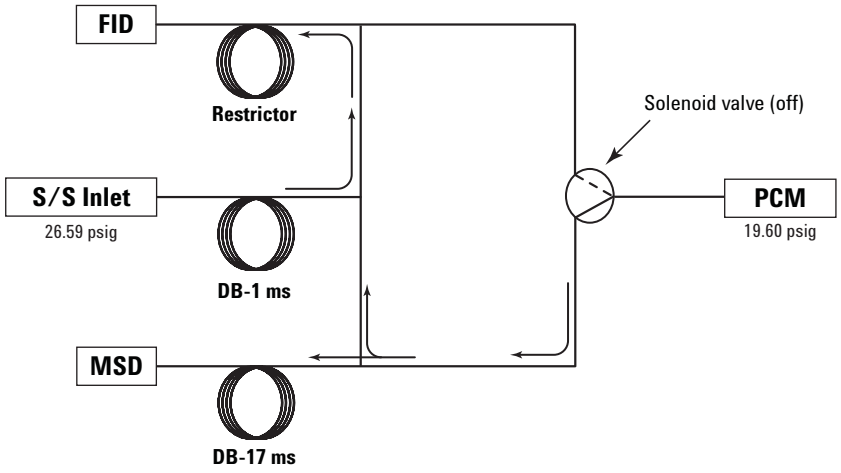


Figure 2a. Deans switch valve in no cut position. Column 1 effluent goes to FID.

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 patient 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

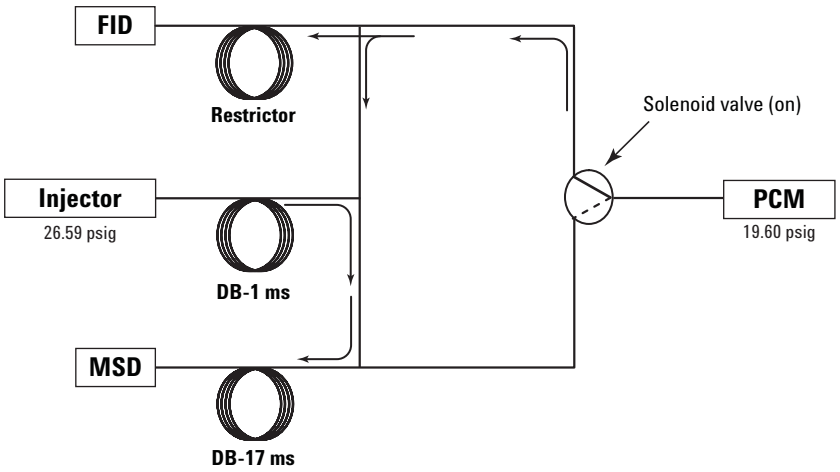


Figure 2b. Deans switch valve in cut position. Column 1 effluent goes to column 2.

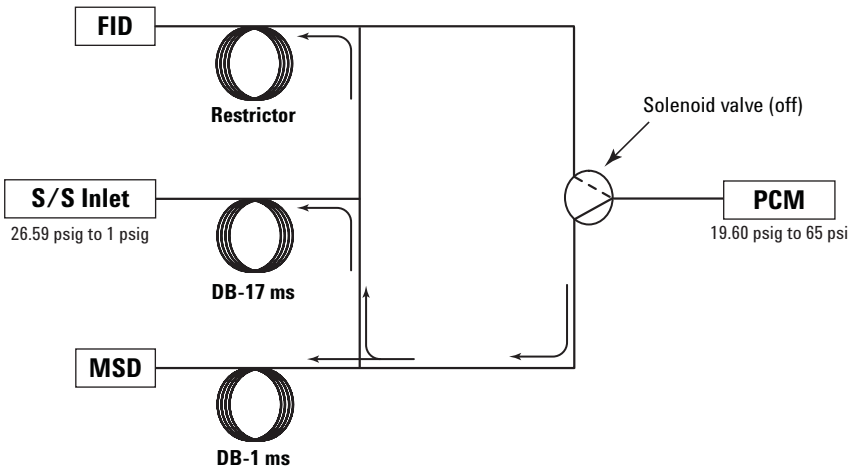


Figure 2c. Backflushing column 1. After the last analyte elutes from column 2, program inlet pressure down to 1 psig and program the PCM to 60 psi to backflush heavies out split vent.

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.

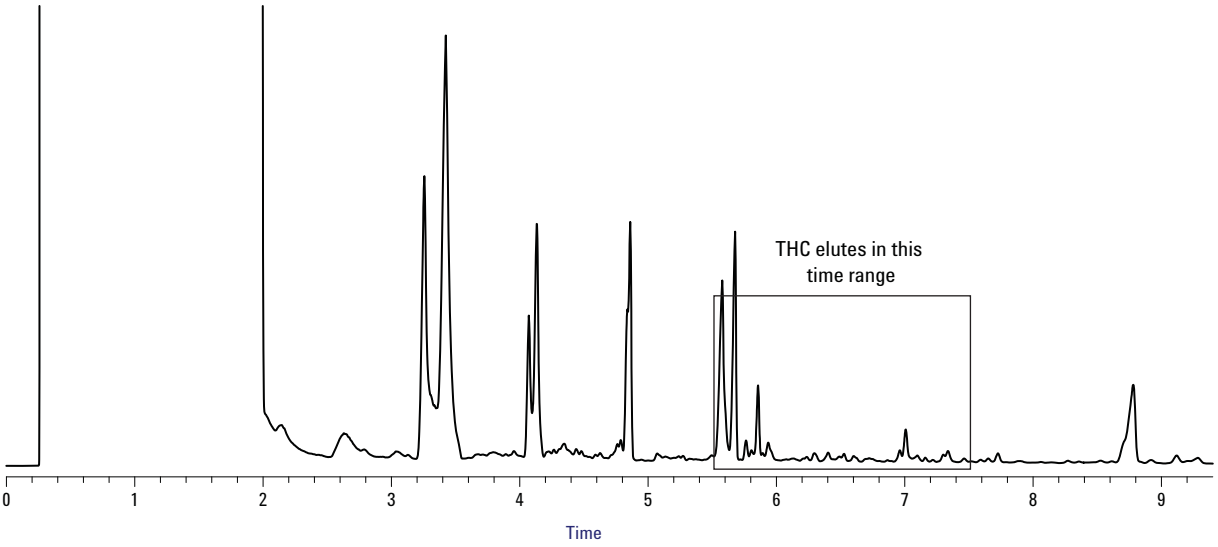


Figure 3a. First column FID chromatogram of patient sample showing complexity of matrix (no cut to second column).

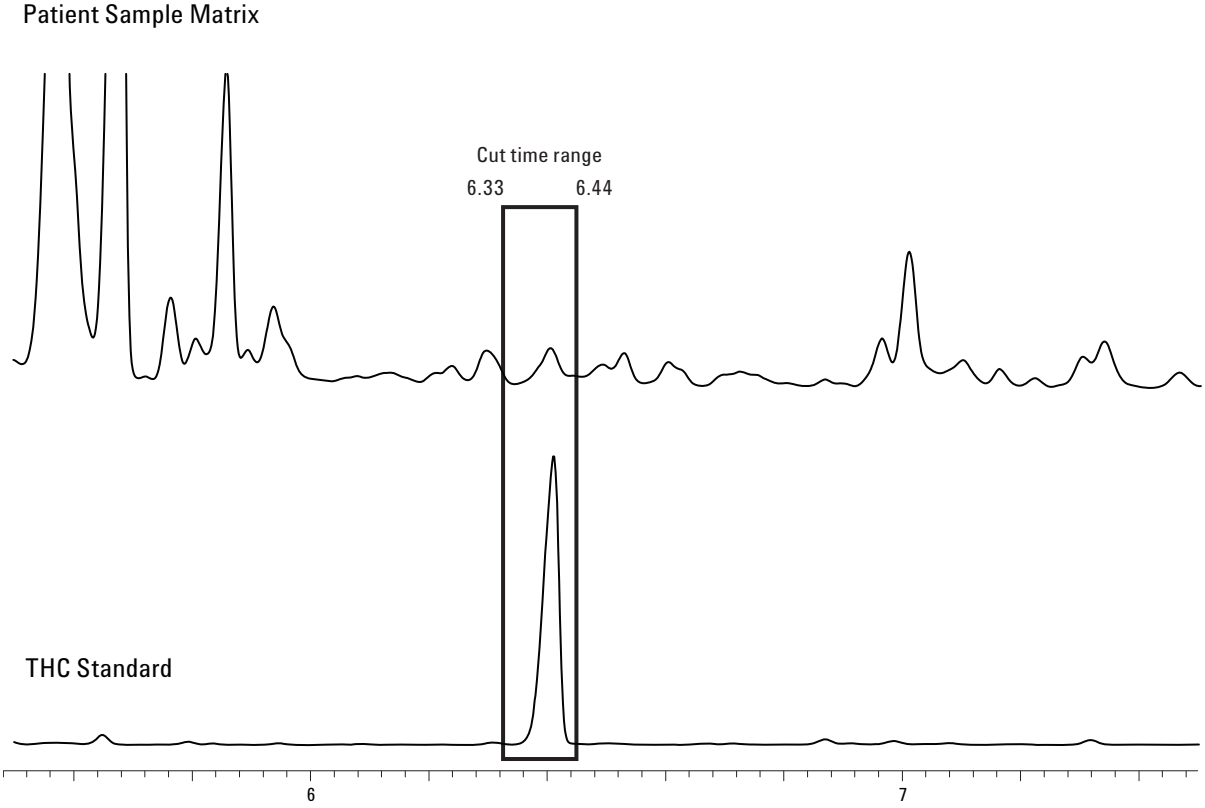


Figure 3b. Expanded view of THC elution range from Figure 3a.

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 patient 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 patient 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.

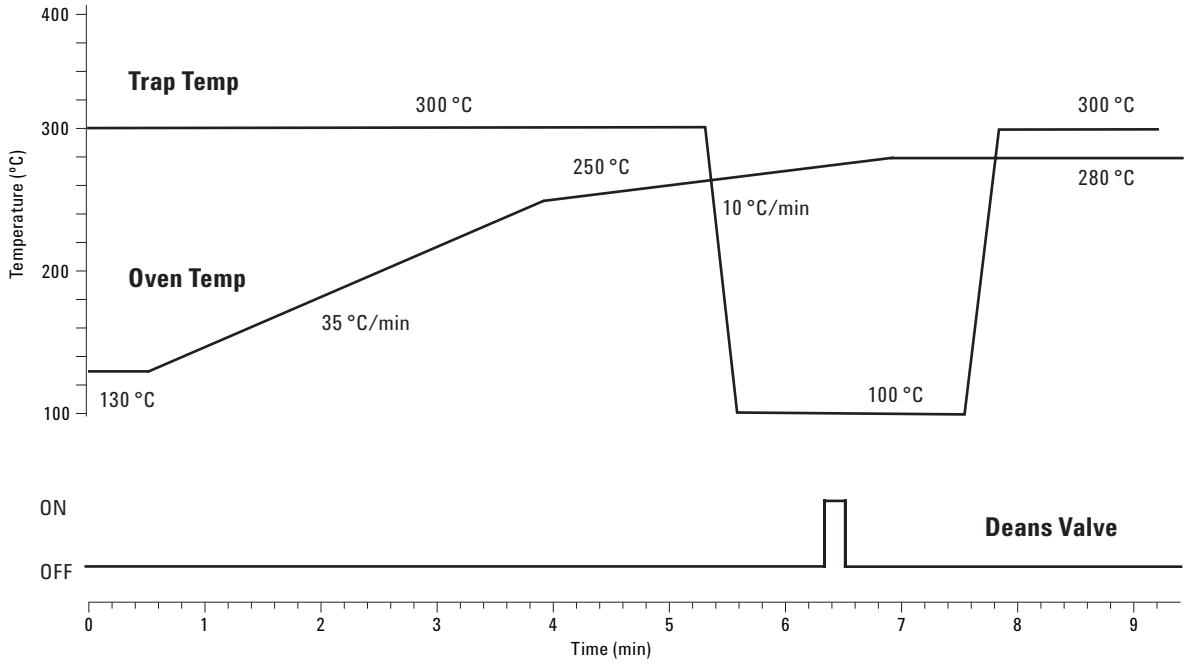


Figure 4. Temperature and valve timing for THC analysis.

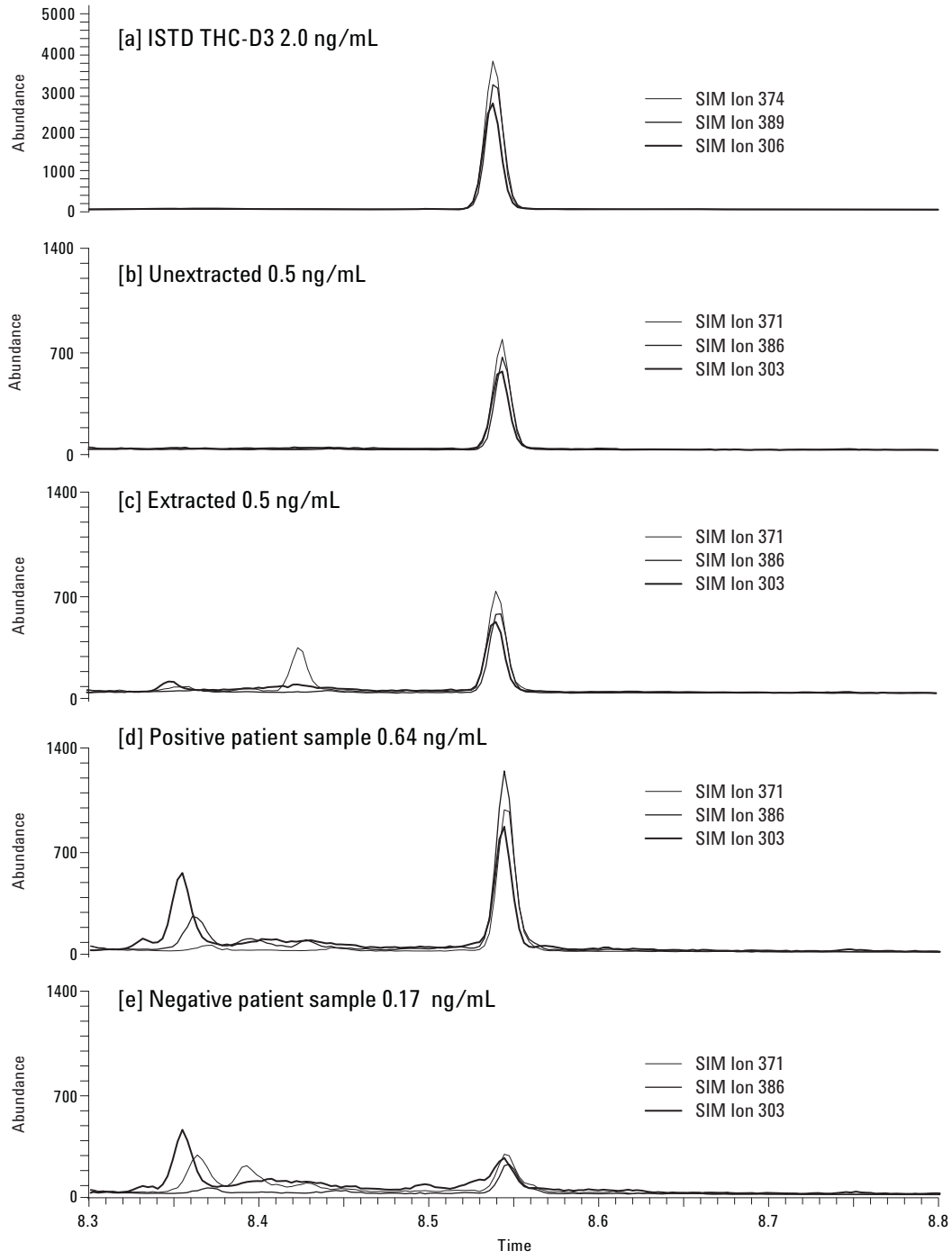


Figure 5. SIM ion chromatograms from a) internal standard THC-D3 at 2 ng/mL, b) unextracted THC cutoff at 0.5 ng/mL, c) extracted THC cutoff at 0.5 ng/mL, d)positive patient THC sample at 0.64 ng, and e) negative patient sample at 0.17 ng/mL.

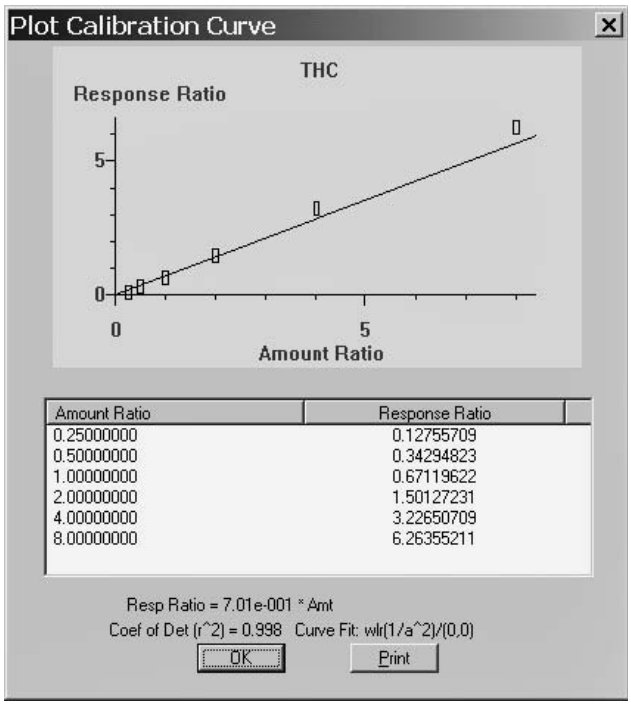


Figure 6. THC calibration curve.

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 patient 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 techniques.

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 patient 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.

Patient sensitivity and specificity data between Intercept EIA kits and 2D GC/MS on 20 patients 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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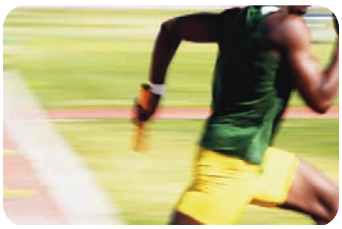
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Applications by Instrumental Technique

GC



Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler

Application

Forensics

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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 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 maintenance, better quantitation, limited sample preparation, 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.

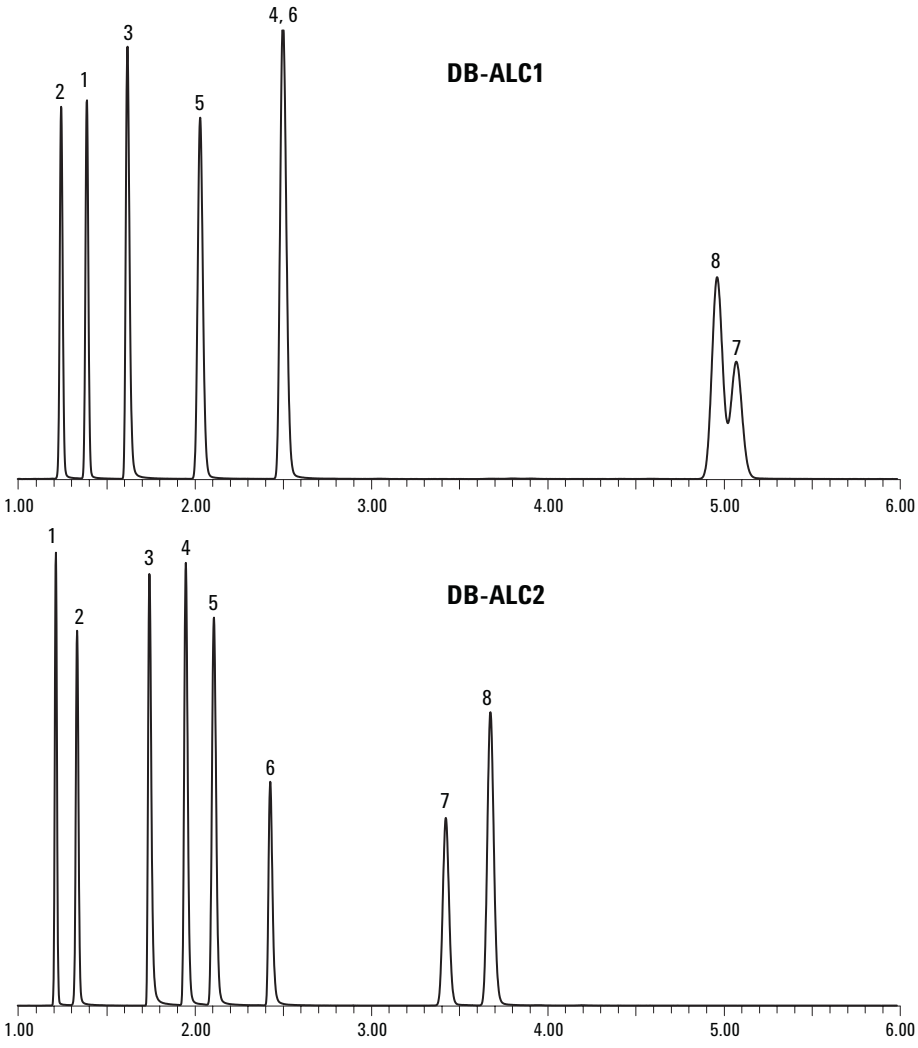


Figure 1. Resolution check standard on 0.32-mm column system at 35 °C. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. 2-propanol, 6. Acetonitrile, 7. Ethyl acetate, and 8. MEK.

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

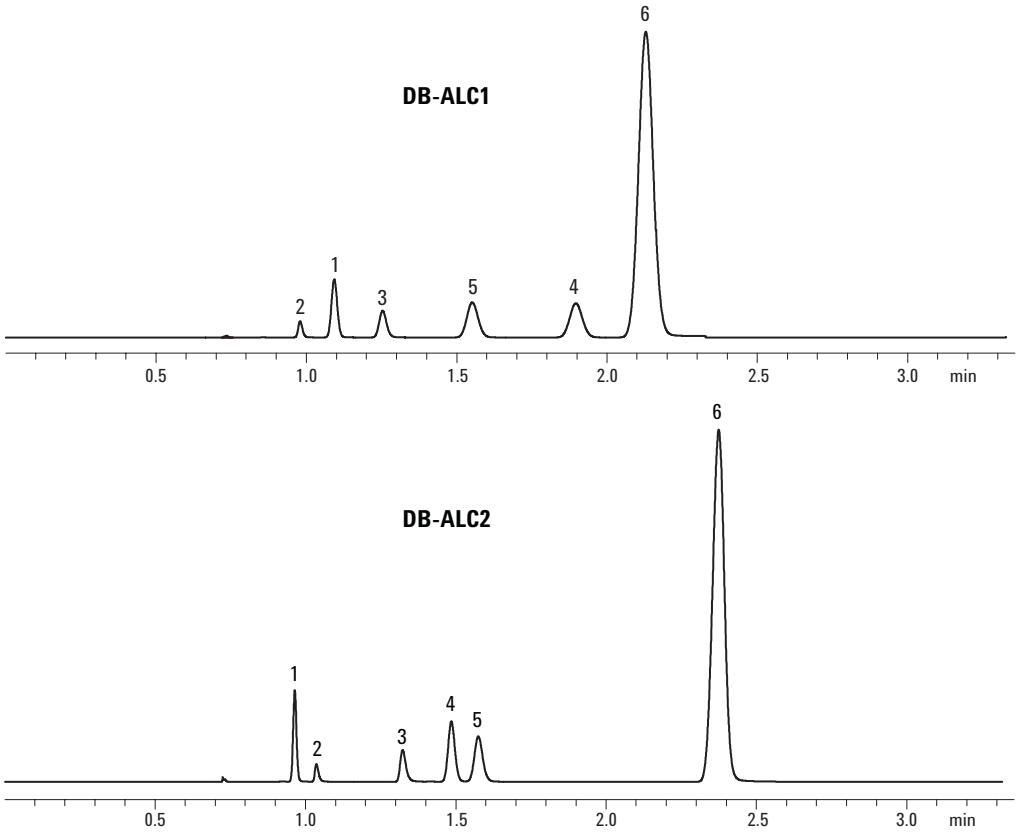


Figure 2. Chromatograms using the 0.53 mm DB-ALC1 and DB-ALC2 columns and the six-component standard at 35 °C. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, and 6. n-propanol.

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

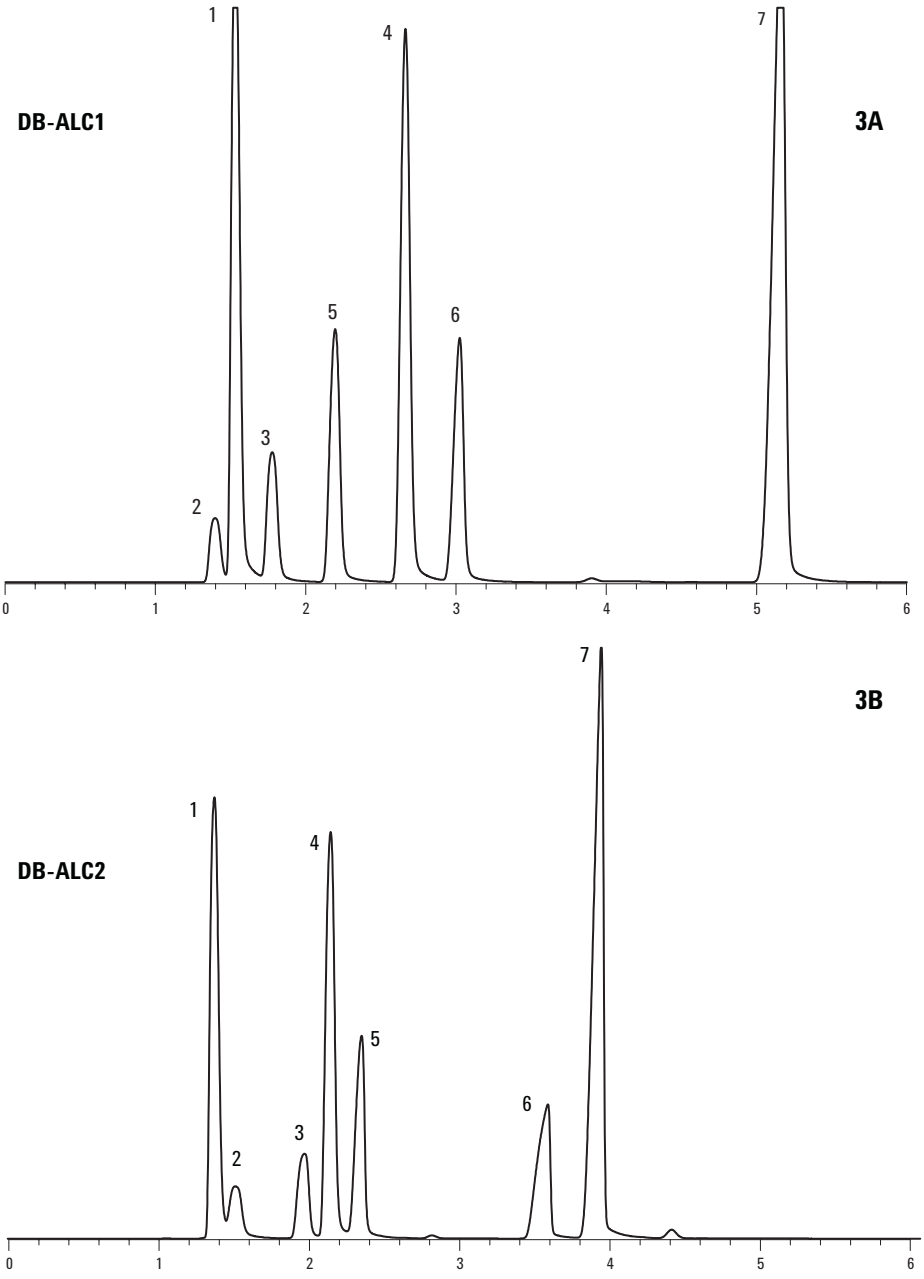


Figure 3. Blood alcohol standard at 0.15 g/dL on 0.32-mm DB-ALC1 (Figure 3A) and DB-ALC2 (Figure 3B) columns. Peak identifications: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, 6. n-propanol, and 7. MEK.

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.

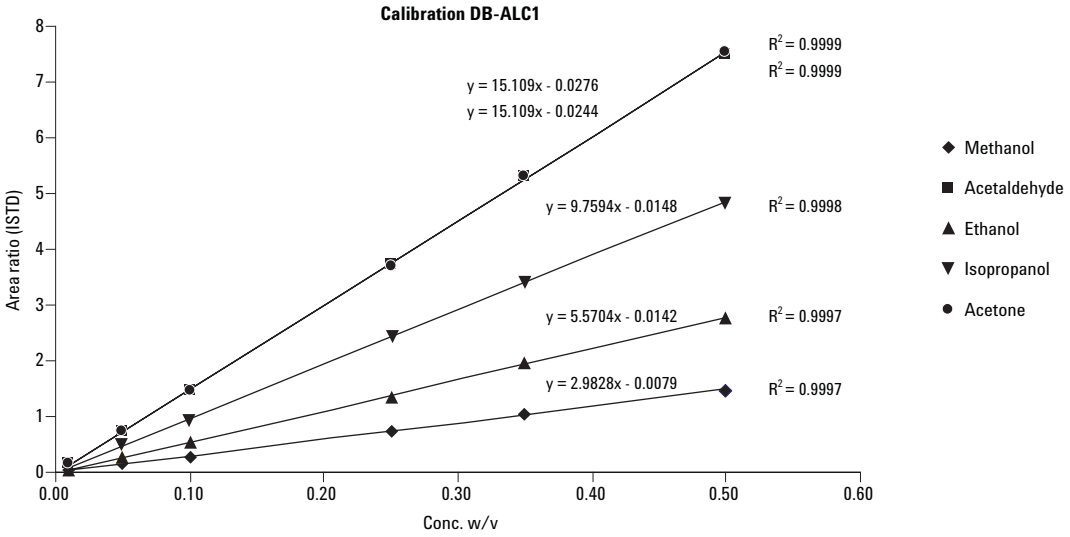


Figure 4. Calibration plots for the indicated standards using the 0.53-mm DB-ALC1 column.

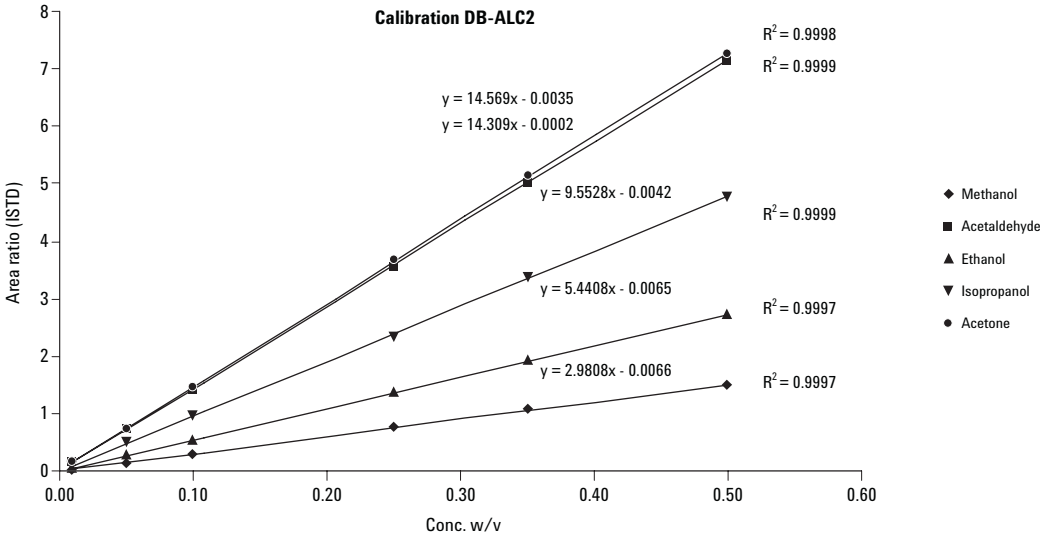


Figure 5. Calibration plots for the indicated standards using the 0.53-mm DB-ALC2 column.

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.

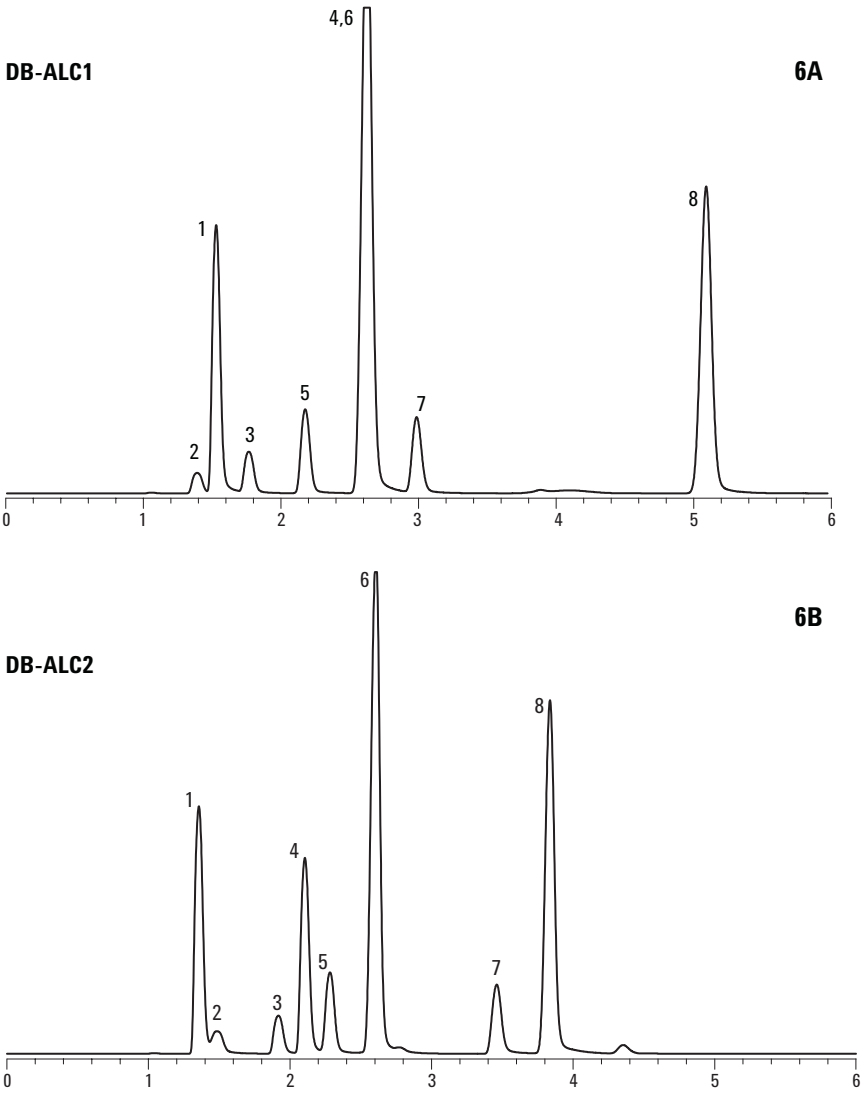


Figure 6. Blood alcohol chromatograms using t-butanol as the ISTD. Columns: 30 m × 0.32 mm DB-ALC1 (Figure 6A) and DB-ALC2 (Figure 6B). Peak identifications: 1. Acetaldeyde, 2. Methanol, 3. Ethanol, 4. Acetone, 5. Isopropanol, 6. t-Butanol, 7. n-propanol, and 8. MEK.

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.

Headspace Job Log: Agilent G1888 Headspace Sampler

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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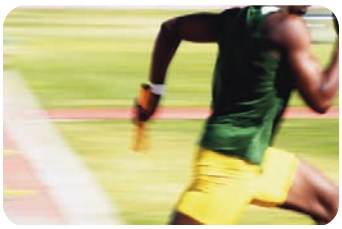
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Applications by Instrumental Technique

ICP/MS



The Use of Collision/Reaction Cell ICP-MS for the Simultaneous Determination of 18 Elements in Blood and Serum Samples

Application

Clinical

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UK

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 clinical 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 clinical sample types has presented clinical 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 from patients 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 clinical 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 volumes, as well as providing isotopic information and the possibility of employing isotope dilution mass spectrometry (IDMS) as a high-caliber reference 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 sufficiently 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-effects 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 patients 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 clinical 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 clinical analysis due to the presence of many of the elements that are usually used in environmental applications at ng/mL levels in clinical 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 clinical sample types at ng/mL levels, which can be seen here after sample 8.

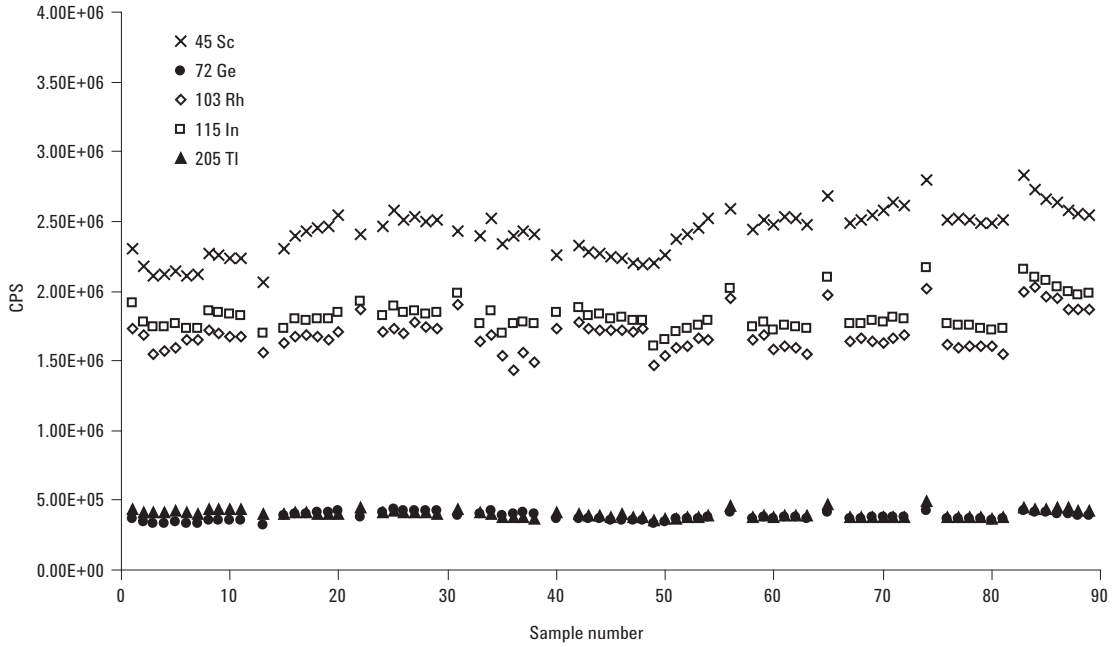


Figure 1. Variation of the IS signals in standard mode throughout the 10-hour run.

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.

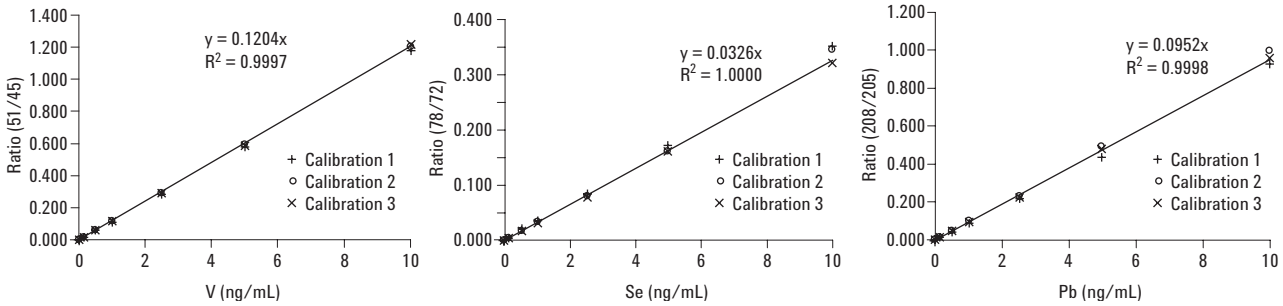


Figure 2. Linearity of overlaid calibration curves for V, Se, and Pb, showing stability of the external calibration approach throughout a 90-sample sequence.

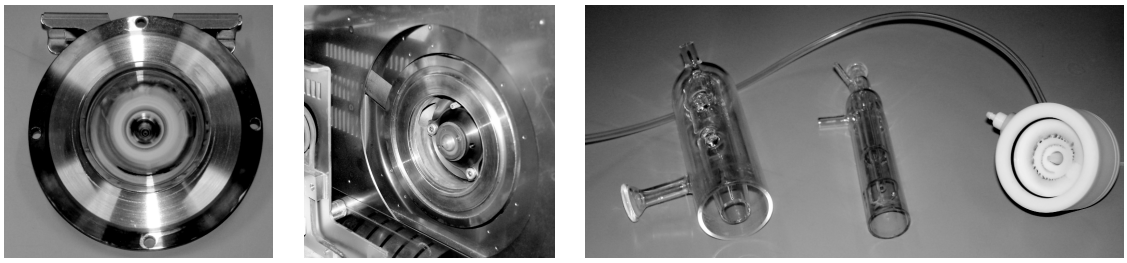


Figure 3. Photos of the interface and sample introduction system after a 90-sample run. Both the sampler and skimmer cones show minor matrix deposits. The 2.5-mm injector torch used was relatively deposit-free. The blood deposits on the spray chamber and the nebulizer block were removed using a sodium 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 clinical 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.).

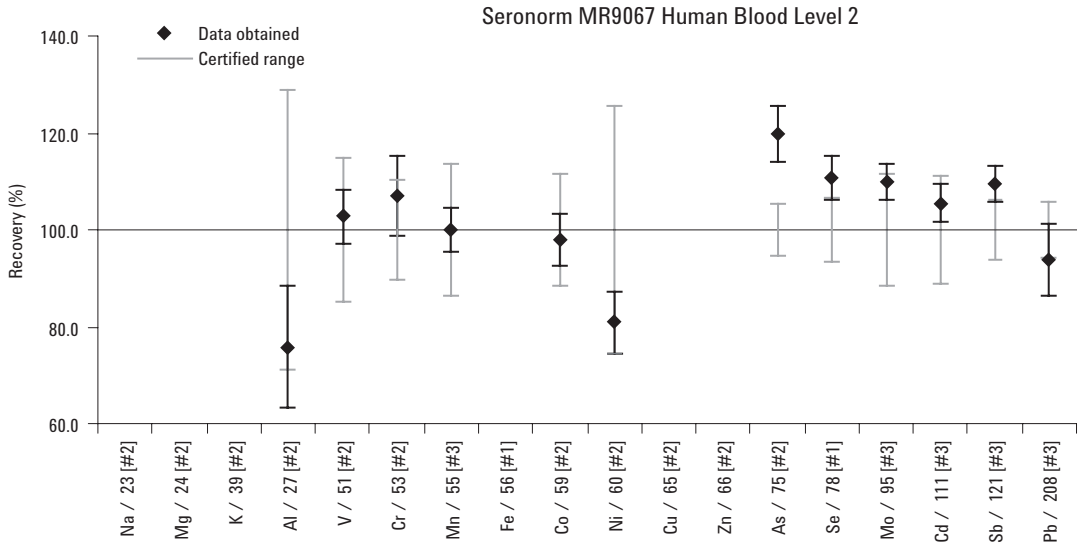
Table 3. Certified Concentrations for the Analytes of Interest in the SRM NIST 1598 and the Reference Material Seronorm MR9067. Method DLs Calculated Back to the Undiluted Sample are Given for Comparative Purposes.

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

a)



b)

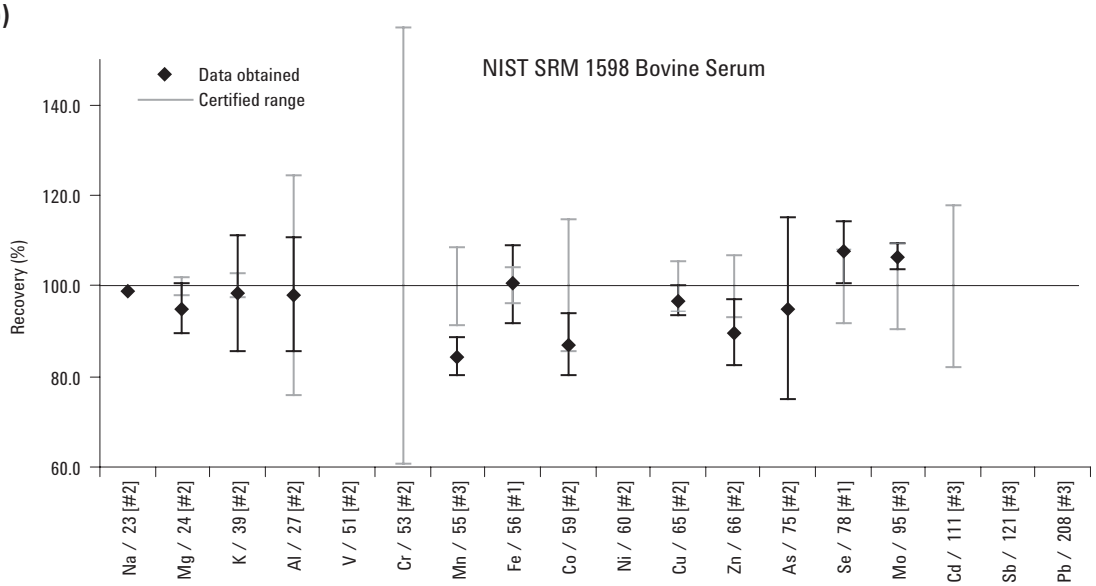


Figure 4. Data obtained expressed relative to the certified data for a) Human blood Seronorm MR9067 and b) Bovine serum SRM NIST 1598. Errors bars represent expanded uncertainties for data obtained and certified ranges for the reference materials. The numbers after the isotopes indicate the tune step 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.

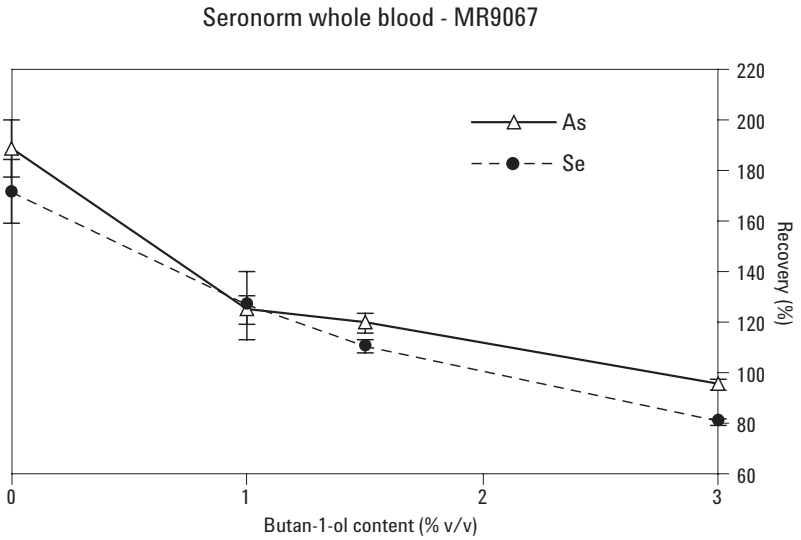


Figure 5. Recovery data for As and Se in Seronorm whole blood (Level 2) with varying levels of butan-1-ol addition to the diluent.

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 clinical 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 clinical 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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Methods for the Forensic Analysis of Adhesive Tape Samples by LA-ICP-MS

Application

Forensics

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Abstract

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) offers great potential as a highly discriminatory technique for the analysis of forensic samples of adhesive tape. Four tape samples and a polyethylene standard were analyzed in this study. By ablating through multiple layers of alternating tape and adhesive glue, an elemental pattern that is unique to that specific tape can be obtained. Being able to present data clearly and unambigiously in a court of law is another consideration for the forensic scientist. Fortunately, LA-ICP-MS data can be presented using various plotting techniques, each designed to discriminate samples with similar visual, physical, and chemical characteristics. These attributes, combined with low levels of detection and high precision, explain the increasing acceptance of LA-ICP-MS for forensic investigation of tapes.

Introduction

Adhesive tape samples may be presented as crime scene evidence from various types of criminal activities: drugs, explosives, stolen articles, documents, etc. In such cases, the forensic scientists may be requested to compare the tape encountered at the crime scene with that found with a suspect or suspects. Traditional techniques for the analysis of tape include visual methods, Fourier transform infrared (FT-IR) for layers analysis, and x-ray fluorescence (XRF) for elemental analysis. Tapes from the same batch, from different manufacturers, of different color and/or morphology, can be discriminated effectively using these methods in many cases. However, for "in-type" discrimination (same brand, different batch and/or same color and matrix), a more rigorous chemical approach is necessary [1].

Standard techniques for the trace elemental analysis of these materials (polyethylene, polypropylene, acetate polymers) typically include time-consuming digestion procedures and hazardous waste by-products. Complete digestion and good trace element recoveries are not always guaranteed.

LA-ICP-MS is an alternative method offering many advantages over standard dissolution techniques. This application note will describe a procedure for the analysis, interpretation, and quantification of these sample types. Though this is a forensic application, there are clear benefits of this technique for environmental concerns.

Instrumentation

All the analyses for these experiments were undertaken using an Agilent 7500s ICP-MS. Solid sampling was achieved by introducing a stream of particles generated *in-situ* by direct coupling of a short ultraviolet (UV) laser with the sample surface into the ICP using a stable flow of argon gas. The laser system used was a New Wave Research (Fremont, CA) UP-213AI Nd:YAG operating at the 5th harmonic frequency (213 nm). Operating parameters for each experiment are given in Table 1. For more information on LA-ICP-MS, refer to application note 5989-1565EN [2].

Operating Parameters

Table 1. LA-ICP-MS Operating Conditions

Polyethylene standard			
Laser		**ICP-MS**	
Line ablation		RF Power:	1200 W
Spot size:	100 µm	Plasma gas:	14 L/min
Line length:	350 µm	Carrier gas:	0.8 L/min
Power:	1.2 mJ	Acquisition:	Time Resolved Analysis (TRA)
Stage speed:	20 µm/s	Integration:	50 ms
Pulse frequency:	10 Hz	Masses:	21
		Acquire time	180 s
Adhesive tape			
Laser		**ICP-MS**	
Spot ablation		Same as standard	
Spot size:	250 µm		
Power:	2.2 mJ		
Pulse frequency:	10 Hz		

Experimental

Calibration of the LA-ICP-MS was carried out using the following standard from Institute for Reference Materials and Measurements, Geel, Belgium.

BCR SRM 680: Trace elements in polyethylene

Adhesive tape samples were acquired from multiple sources. Two samples (P377R, red and P366B, blue) were supplied by VHG Labs, Inc., Manchester, NH. They were part of a group of industrial QC samples sent to the lab for digestion and subsequent aqueous analysis. Tan packing tape brand A and brand B, and 3M and Scotch black electrical tape were purchased at Walgreens, Fremont, CA. Both the tan adhesive tape samples and the black electrical tape samples were visually identical, but produced by two different manufacturers.

Both the polyethylene standard (BCR SRM 680) and the tape samples were attached to a petrographic slide (Figure 1) and placed in the standard UP sample cell for analysis. Tape samples were cut directly from the parent role. For sampling, at least 10 layers of tape were removed as a section from each roll. All tape samples were ablated continuously (250-µm spot) through multiple, alternating layers of the base polymer and sticky adhesive as illustrated in Figure 2. The data was imported into Glitter™ data reduction software for both qualitative and quantitative analysis.

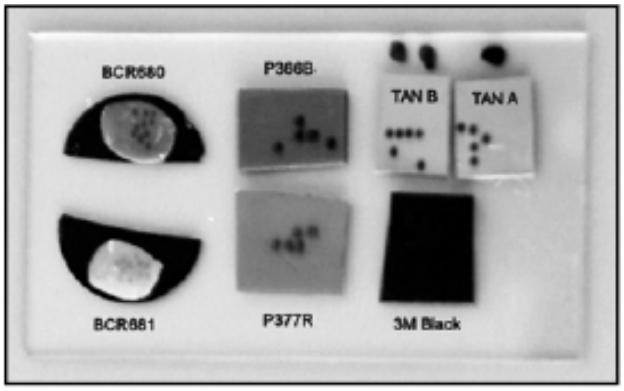


Figure 1. BCR standards and adhesive tape mounted on petrographic slide showing ablation craters.

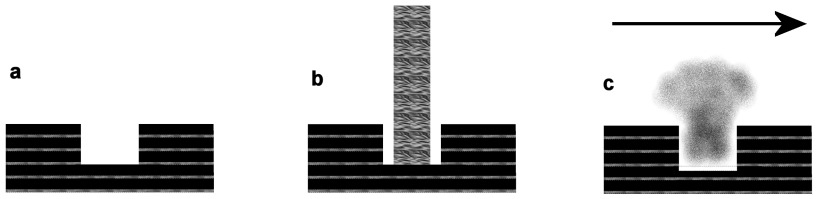


Figure 2. Graphical representation of the ablation process on layered tape samples. a) Layered tape sample after repetitive ablation b) generation of laser plume and c) subsequent removal of laser aerosol within an argon carrier gas stream (arrow).

Results

The six tape samples and a polyethylene standard (BCR SRM 680) were analyzed. Five repetitive analyses for each sample and standard were performed. The time-resolved layer analysis (Figure 3) was evaluated to determine the most appropriate way to integrate the data. By ablating through multiple layers of alternating tape and adhesive glue, a unique elemental pattern can be visualized. These elemental "wave-forms" appear to be interlaced or "out of phase" with one another. One set of elements (Al, Mn, Co, and Sb) appears to be associated with the tape backing material, as their signal rises immediately after the start of the ablation cycle. The second set of elements (Cr, Zr, La, Ce, and Pb) appears to be associated with the adhesive glue, as their signals trail the first set by 10 seconds. The lines continue out of phase for the remainder of the ablation cycle. These data were integrated over the entire period of ablation using the ^{13}C profile as a reference. The trace element concentrations for the P366B and P377R samples were calculated using the BCR SRM 680 (Table 2), and elemental relationships were characterized using a stacked bar graph (Figure 4).

In a previous study (Dobney et al) [1], tape samples were acid digested and the aqueous aerosol analyzed. It was determined that the polymeric base material (PP, PE, and PVC) was difficult to get into solution, was more prone to acid-based matrix interferences, and was chemically less interesting than the adhesive glue. Therefore, trace elements were quantified in the adhesive glue only. By ablating through multiple layers, it is possible to integrate the adhesive elements independently from the elements in the tape backing (data not shown) without any of the difficulties inherent with aqueous digestion.

Ternary plots compare the relationship between three components in a system. Each corner of the plot represents 100% of the labeled component. A data point in the center of the plot signifies that the sample is of equal composition for all three constituents. Ternary plots, more technical in nature compared to bar charts, can discriminate different sample types from one another as well as display sample reproducibility. Tight clustering of sample types describes good sampling precision and data reliability. Figure 5a describes the relationship between ^{27}Al, ^{121}Sb, and ^{137}Ba for the six tape samples characterized here. Although good separation is possible for four of the six tape samples, further discrimination (Figure 5b) is necessary to discriminate the remaining two Tan tape samples by changing the parameters of the ternary plot. In this way, clear separation may be accomplished between samples that are visually identical.

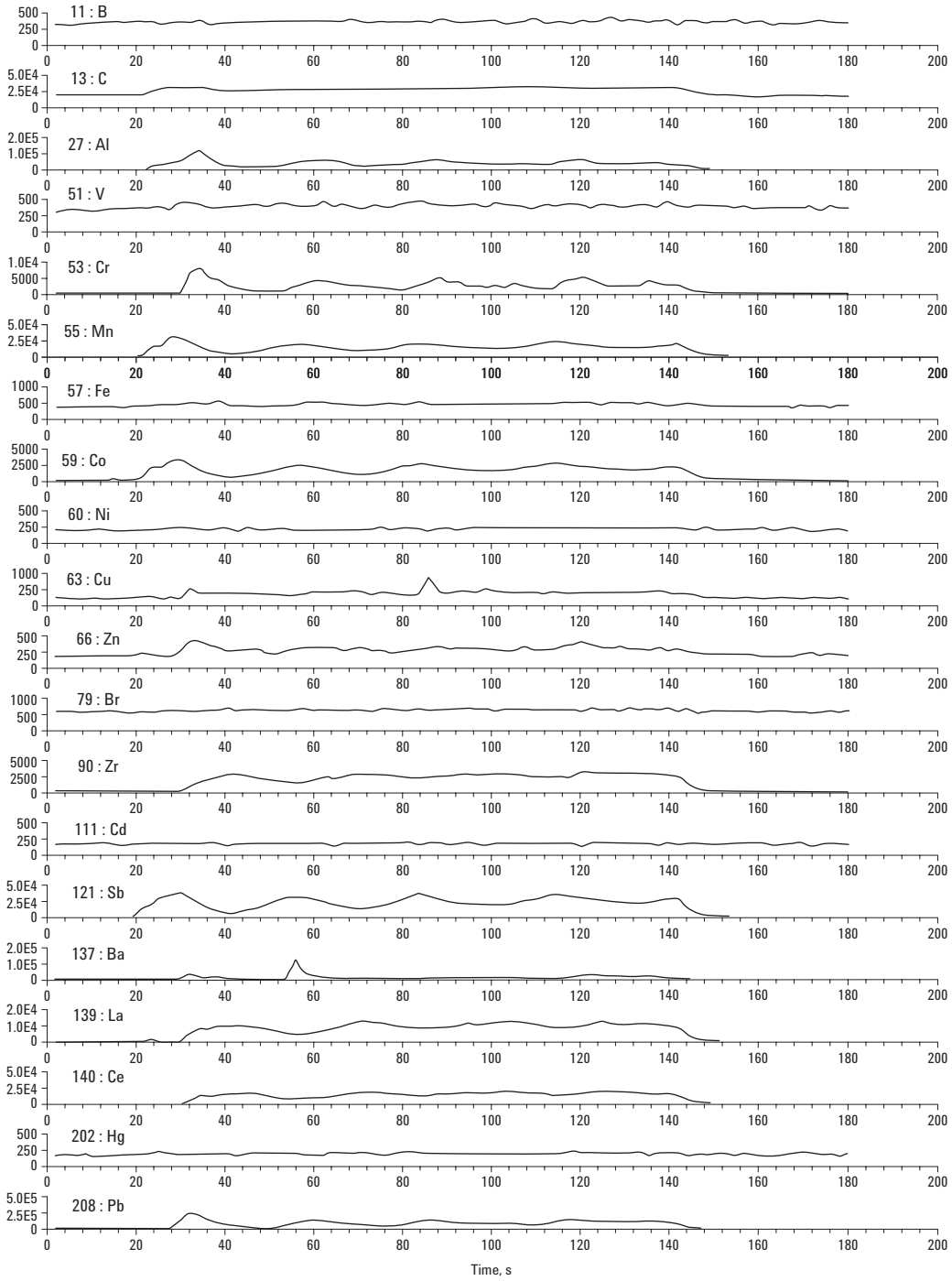


Figure 3. Time resolved layer analysis of colored (blue and red) electrical adhesive tape. Agilent ChemStation time resolved output format of a multi-element profile through successive tape layers. Notice how certain elemental signatures have delayed rise times. Elements within the tape matrix and the adhesive matrix are "out of phase" with respect to each other. Al, Mn, Co, and Sb rise with the onset of the ablation start point (t + 20s). Cr, Zr, La, Ce, and Pb first rise approximately 10 seconds later.

Table 2. Quantitative Analysis of Trace Elements in Polyethylene (BCR SRM 680), and the Two Tape Samples (P366B and P377R). Quantitative Data Was Reduced Using Glitter Data Reduction Software.

BCR

Element	Mean	SD	Agreement %
Al 27	51.4	0.4	100.7
Cr 53	114.5	0.1	99.9
Cu 63	118.4	1.9	99.5
Br 79	798.9	8.8	98.9
Cd 111	135.2	12.8	96.0
Sb 121	6.3	0.2	101.1
Ba 137	2639.0	132.8	97.1
Hg 202	24.4	0.7	96.5
Pb 208	107.3	3.2	99.7

P366B

Element	Mean	SD
Al 27	585.2	25.2
Cr 53	2.5	0.7
Cu 63	1016.7	32.5
Br 79	17.2	3.3
Cd 111	18.3	1.1
Sb 121	1162.2	22.1
Ba 137	9.8	2.7
Hg 202	1.8	0.3
Pb 208	2.2	0.7

P377R

Element	Mean	SD
Al 27	611.7	77.8
Cr 53	215.1	29.2
Cu 63	4.2	0.7
Br 79	30.9	15.4
Cd 111	22.9	4.3
Sb 121	1139.8	90.8
Ba 137	877.3	87.4
Hg 202	3.2	1.0
Pb 208	3067.8	397.2

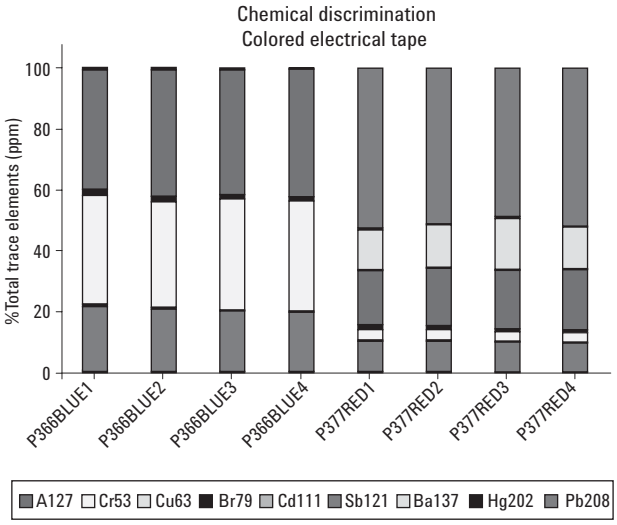


Figure 4. Stacked bar plot of two polypropylene tapes (blue and red) from the same manufacturer. The data in the chart is derived from Table 2.

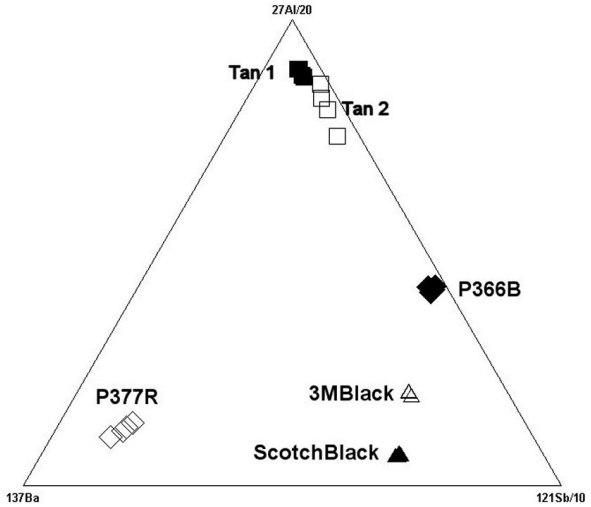


Figure 5a. Ternary Plot of Adhesive tape data (integrated counts per second). Ternary plots are an effective way to discriminate subtle differences in sample populations, especially when multiple data point display is desirable.

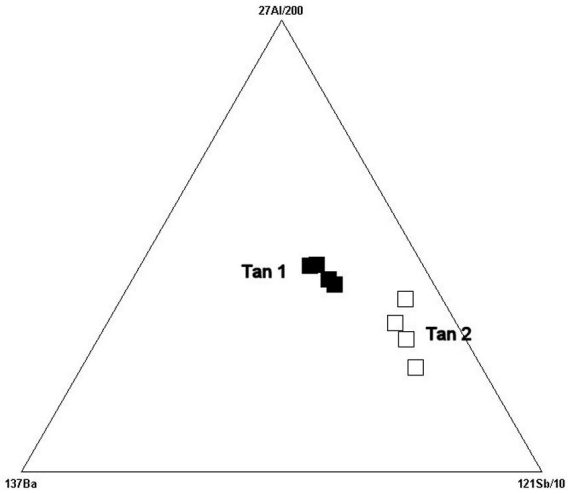


Figure 5b. By changing the parameters of ternary plots, it is possible to further discriminate subtle differences in chemistry between visually identical samples.

Conclusion

LA-ICP-MS is an *in-situ* analytical method capable of sampling through layered materials. Through the direct analysis of adhesive tape, three-dimensional chemical characterization is possible. Preliminary evidence suggests that there is an advantage to analyzing both the substrate and the adhesive in these samples.

Through the implementation of various plotting techniques, it is possible to discriminate samples with similar visual, physical, and chemical characteristics. By combining powerful, *in-situ* micro analysis, low levels of detection, high precision, and clear and easily understandable diagrams, LA-ICP-MS is becoming an increasingly important weapon in the arsenal of forensic science.

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Analysis of Forensic Glass Samples by Laser Ablation ICP-MS

Application

Forensics

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Abstract

Physical evidence is often distributed widely when a crime is being committed. The smaller these suspect materials are, the more likely they will be transported from the crime scene undetected. When glass is shattered, the fragments created can be less than a few hundred microns (<0.2 mm). These fragments can become attached to clothing and embedded in shoes, "tagging" the criminal with a unique marker. However, as the major and minor elemental composition of modern glass is becoming more difficult to discriminate using traditional methods, new instrumentation is needed capable of resolving differences in the trace elemental profiles of similar glasses. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was evaluated and found to provide the accuracy, sensitivity and spatial resolution necessary for this application.

Introduction

Traditional methods of forensic glass analysis include the determination of a number of physical properties, including refractive index (RI), wet chemistry, scanning electron microscopy (SEM), x-ray fluorescence (XRF), and optical microscopy [1]. Although these techniques offer a high degree of differentiation with traditional glass, modern glass has a greater degree of chemical and physical similarity. The major and minor elemental composition and RI values of these new materials are becoming more difficult to discriminate. The histograms in Figures 1a and 1b show RI values for flat glass extracted from an FBI database for the periods of 1964 to 1979 and 1980 to 1997 respectively [2]. Comparison of the two charts clearly shows the reduced opportunity for intersample discrimination using this technique. Although the major and minor elemental composition of these glasses are very similar and therefore difficult or impossible to discriminate, using traditional methods of characterization, these glasses may have trace elemental signatures which are distinguishable by LA-ICP-MS.

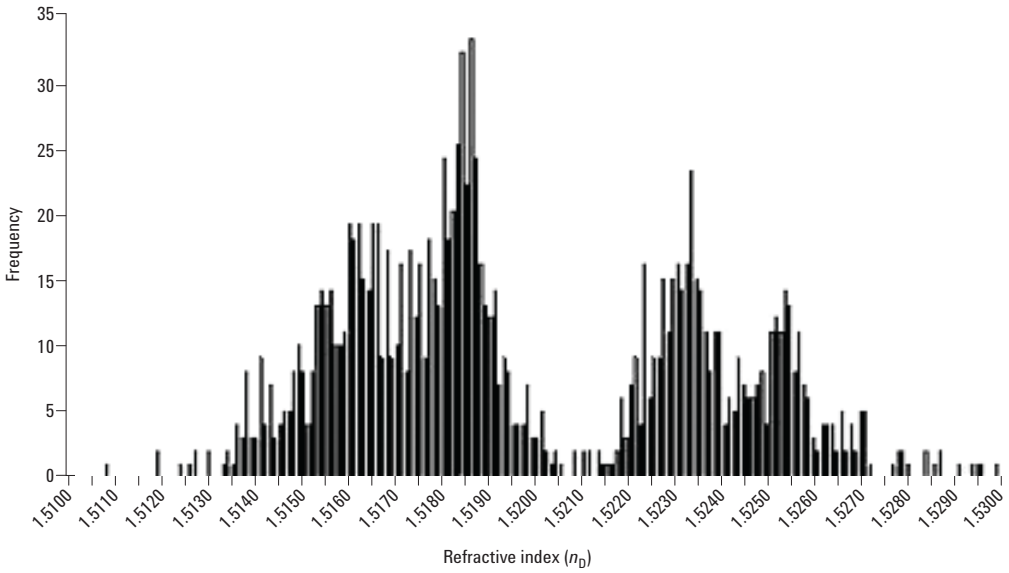


Figure 1a. Distribution of RI values from FBI database of flat glasses, 1964 to 1979.

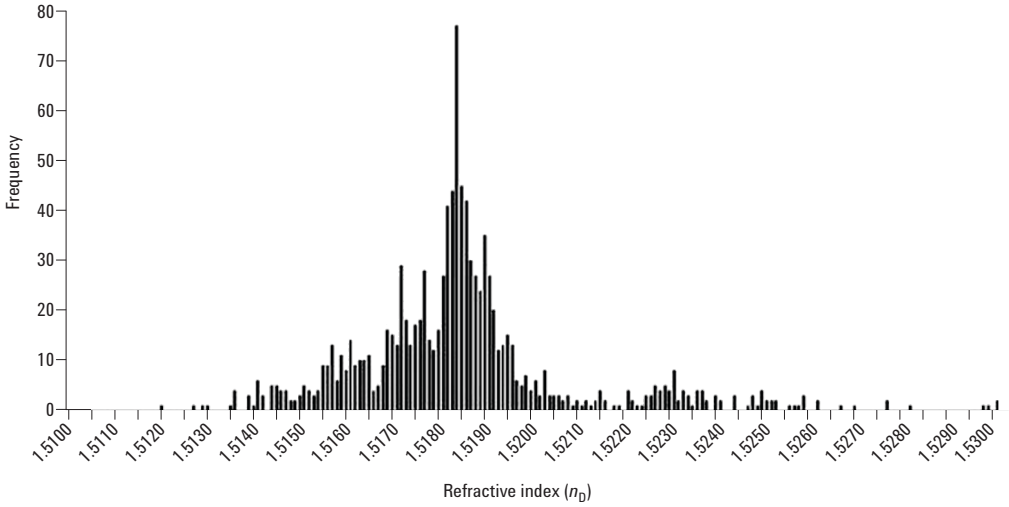


Figure 1b. Distribution of RI values from FBI database of flat glasses, 1980 to 1997.

Instrumentation

All the analyses for these experiments were undertaken using an Agilent 7500s ICP-MS. Solid sampling was achieved by introducing a stream of particles generated *in-situ* by direct coupling of a short ultraviolet (UV) laser with the sample surface into the ICP using a stable flow of argon gas. The laser system used was a New Wave Research (Fremont, CA) UP-213AI Nd:YAG operating at the 5th harmonic frequency (213 nm). Operating parameters for each experiment are given in Table 1. For more information on LA-ICP-MS, see Reference 3.

Operating Parameters

Glass Fragments

Table 1. LA-ICP-MS Operating Conditions

Laser		ICP-MS	
Line ablation		RF Power:	1200 W
Spot size:	100 µm	Plasma gas:	14 L/min
Line length:	350 µm	Carrier gas:	0.8 L/min
Power:	2 mJ	Acquisition:	Time Resolved Analysis (TRA)
Stage speed:	20 µm/s	Integration:	10 ms
Pulse frequency:	10 Hz	Masses:	36
		Acquire time	114 s

Experimental

Calibration of the LA-ICP-MS was carried out using the following standard, obtained from National Institute of Science and Technology (NIST), USA:

NIST SRM 612: 50 µg/g nominal trace element concentration.

Matrix elements: Si (SiO_2), Na (Na_2O), Ca (CaO), Al (Al_2O_3)

NIST soda lime glass standards (620, 621 and 1831) were used as surrogates for float glass (flat, clear glass) samples. It was therefore possible to check the accuracy and the precision of the calculated values by comparing them with the certified values given for the major elements (Table 2). Each sample was placed in a separate, sealed plastic bag and shattered. The small fragments (0.5 mm to 2 mm) were attached to a petrographic slide using double-sided graphite tape (Figure 2). This process was repeated for all the surrogates, as well as the three headlamp samples.

Table 2. NIST SRM 612 Major and Trace Multi-Element Results

Element	Na 23	Mg 24	Al 27	Ca 44	Ti 47	Cr 52	Mn 55	Fe 57	Ni 60	Zn 66
Mean, ppm	10.4%	79.07	1.1%	8.7%	49.5	39.8	38.5	57.7	38.8	38.6
SD	0.2%	2.51	0.0%	0.1%	2.52	0.30	0.49	2.99	2.02	1.50
%RSD	1.9	3.2	0.5	1.1	5.1	0.8	1.3	5.2	5.2	3.9
%Agreement	100.3	102.1	105.6	101.3	103.0	99.6	100.2	102.5	100.8	101.7
Element	**Rb 85**	**Sr 88**	**Y 89**	**Zr 90**	**Mo 95**	**Ba 38**	**Ce 140**	**Hf 178**	**Pb 208**	**Th 232**
Mean ppm	32.1	77.0	38.7	36.3	38.7	38.0	38.5	34.5	36.2	36.9
SD	0.42	0.94	0.95	0.97	0.81	0.60	0.53	1.01	5.06	1.15
%RSD	1.3	1.2	2.4	2.7	2.1	1.6	1.4	2.9	14.0	3.1
%Agreement	101.5	101.1	101.0	100.8	101.0	100.6	100.5	99.2	92.9	99.1

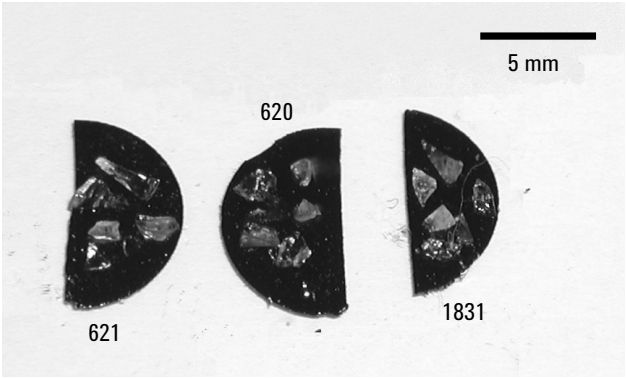


Figure 2. Sample mounting of glass fragments.

NIST 612 standard glass was used as a means of calibration and was analyzed repeatedly throughout the analysis procedure, bracketing each sample set. Each sample analysis was 115 seconds and consisted of a 20-second blank delay, a 60-second laser sampling period, followed by a 35-second washout period. Six repetitive data acquisitions over two separate lines were collected for each sample. The data was imported into Glitter™ data reduction software (Macquarie University - GEMOC). Analyte and blank regions were defined within the Signal Selection Screen (Figure 3) and quantitative values were determined. The mean and standard deviation (SD) for each sample was then calculated (Table 3a).

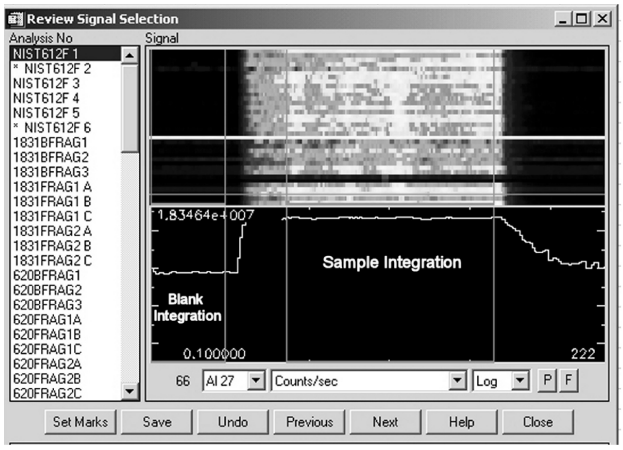


Figure 3. Signal selection screen, Glitter data reduction software. Traditionally used in geochronology, forensic data benefits from the ability of this software to enable easy isolation of changing data sets within a heterogeneous sample matrix. Each sample has its own associated blank, reducing memory effects.

Table 3a. Glass Data Obtained From the Analysis of Standard Glass Fragments
Unless Otherwise Noted all Data is in µg/g (ppm)

Element	NIST 620				NIST 621				NIST 1831			
	Mean (ppm)	SD (ppm)	RSD (%)	Agreement (%)	Mean (ppm)	SD (ppm)	RSD (%)	Agreement (%)	Mean (ppm)	SD (ppm)	RSD (%)	Agreement (%)
Na 23	10.8%	0.33%	3.1	100.7	9.3%	0.39%	4.2	98.0	9.8%	0.33%	3.4	100.9
Mg 24	3.1%	0.05%	1.5	140.2	0.2%	0.003%	1.5	136.1	2.9%	0.038%	1.3	137.3
Al 27	1.0%	0.02%	1.8	107.3	1.6%	0.01%	0.6	108.8	0.66%	0.004%	0.6	102.7
Ca 44	5.0%	0.09%	1.8	99.0	7.7%	0.11%	1.4	100.2	5.7%	0.064%	1.1	96.6
Ti 47	105	2.245	2.1	97.1	86	1.28	1.5	102.7	118	1.77	1.5	103.4
Cr 53	2.02	0.297	14.7	–	3.97	0.29	7.2	–	2.13	0.19	9.1	–
Mn 55	13.9	0.261	1.9	–	17.9	0.31	1.7	–	12.8	0.15	1.2	–
Fe 57	203	3.310	1.6	–	210	2.04	1.0	–	397	5.89	1.5	–
Ni 60	0.49	0.049	10.0	–	1.80	1.13	62.8	–	0.57	0.21	37.3	–
Zn 66	6.7	0.265	3.9	–	2.76	0.17	6.1	–	8.4	0.61	7.3	–
Rb 85	5.3	0.197	3.7	–	38.2	1.23	3.2	–	6.03	0.16	2.6	–
Sr 88	286	4.709	1.6	–	106	1.44	1.4	–	89.9	1.21	1.3	–
Y 89	2.99	0.043	1.5	–	2.63	0.05	1.9	–	2.05	0.04	1.8	–
Zr 90	198	4.291	2.2	–	62.7	0.96	1.5	120.9	39.5	0.93	2.3	–
Mo 95	0.19	0.022	11.6	–	2.34	0.13	5.7	–	0.18	0.01	6.9	–
Ba 138	22.5	0.156	0.7	–	84.7	7.41	0.9	–	30	0.54	1.8	–
Ce 140	2.50	0.036	1.4	–	2.09	0.03	1.4	–	4.35	0.08	1.8	–
Hf 178	4.30	0.106	2.5	–	1.51	0.02	1.1	–	0.97	0.03	3.5	–
Pb 208	1.97	0.138	7.0	–	14.5	0.96	6.6	–	1.94	0.10	5.1	–
Th 232	0.40	0.002	0.6	–	0.62	0.00	0.4	–	0.60	0.01	0.9	–

Table 3b. Glass Data Obtained from Headlight Fragments
Unless Otherwise Noted all Data is in µg/g (ppm)

Element	Fragment Sample A (Sylvania Headlamp H6024CB)			Fragment Sample B (Sylvania Headlamp H4656)			Fragment Sample C (Sylvania Headlamp 5006)		
	Mean (ppm)	SD (ppm)	RSD (%)	Mean (ppm)	SD (ppm)	RSD (%)	Mean (ppm)	SD (ppm)	RSD (%)
Na 23	3.5%	0.09%	2.64	34.9%	0.54%	1.54	3.59%	0.08%	2.36
Mg 24	41.78	0.37	0.88	70.6	0.96	1.36	62.8	0.55	0.88
Al 27	1.2%	0.04%	3.10	1.0%	0.01%	1.00	1.04%	0.01%	0.70
Ca 44	153	29.23	19.12	221	14.59	6.60	200	34.29	17.16
Ti 47	71	5.19	7.31	46.3	0.97	2.10	44.5	0.71	1.60
Cr 53	1.26	0.29	23.08	2.19	0.36	16.39	2.13	0.26	12.15
Mn 55	2.67	0.06	2.07	1.29	0.10	8.10	1.20	0.03	2.34
Fe 57	96	1.85	1.93	234	4.15	1.78	237	4.23	1.78
Ni 60	0.43	0.06	13.61	0.32	0.05	15.09	0.27	0.08	27.36
Zn 66	1.44	0.13	9.02	1.01	0.10	9.80	0.89	0.16	18.42
Rb 85	0.38	0.01	3.81	0.38	0.01	2.43	0.40	0.01	3.60
Sr 88	4.08	0.11	2.58	5.16	0.10	2.00	3.95	0.10	2.44
Y 89	9.42	1.27	13.50	0.92	0.03	2.84	0.86	0.03	3.38
Zr 90	5099	711.55	13.95	119	4.62	3.88	97	5.95	6.12
Mo 95	3.28	0.11	3.37	0.69	0.08	11.48	0.53	0.06	11.87
Ba 138	4.42	0.09	1.97	1.86	0.05	2.52	1.82	0.04	2.10
Ce 140	3.09	0.15	5.02	3.79	0.06	1.58	3.57	0.06	1.71
Hf 178	113	15.85	14.01	2.96	0.13	4.29	2.23	0.15	6.76
Pb 208	0.41	0.03	6.21	0.42	0.01	2.46	0.36	0.02	5.67
Th 232	1.74	0.22	12.71	0.29	0.01	2.08	0.25	0.01	2.93

Results

Discrimination of Clear Glass Fragments

Three sets of automobile headlamp fragments and three sets of NIST soda lime glass standard fragments were chosen as forensic sample surrogates for this study. All glass samples were colorless to the naked eye. Time resolved data was imported directly into Glitter data reduction software from the Agilent 7500s ICP-MS ChemStation software. Blank and sample integration areas were defined within the Signal Selection screen (Figure 3) and elemental concentrations were calculated using NIST 612 as the multi-element standard (Table 2). Though the glass fragments were typically <1 mm, elemental recoveries for the NIST certified values were very good and RSDs were <3% for many elements.

NIST soda-lime glass standards 620 (flat glass), 621 (container glass), and 1831 (sheet glass) were used to emulate samples. The good agreement between the certified values and the returned values support the efficacy of the method used. Though the Mg values are consistently high by approximately 40%, the data suggests that this is likely due to a problem with the calibration standard either because of an inhomogenous distribution of the element, or even possibly variation in the certified value. In this study, the value for Mg in NIST 612 was defined as 77.44 µg/g, 1σ 30.15 µg/g (Pearce, et al 1997) [4]. Another study (Gao, et al 2002) published the NIST 612 Mg value as 64 µg/g, 1σ 6 µg/g [5].

Forensic data must be presented in the most accurate and clearly understandable format. Jurors with little or no scientific background must be able to decipher subtle chemical differences between evidentiary materials. Consequently, we have presented our glass data in two discriminating formats: numerically and stacked bar graphs (Tables 3a and 3b and Figure 5). Stacked bar graphs are extremely effective in comparing different multi-component data sets. We have therefore included the quantitative mean values with 1σ SD (Tables 3a and 3b).

Like gel electrophoresis, banding patterns within an elemental data set are easy to visualize and differentiate. Stacked bar charts can clearly characterize the elemental nature of a unique sample type. Notice the clear and even banding pattern of NIST 612 (first bar Figure 5). In NIST 612, all elements with the exception of Sr (76 ppm) are nominally at equal concentration (50 ppm), which the banding pattern clearly portrays. The NIST glass serves not only as a quantitative standard, but also describes the effectiveness of the stacked bar chart in its ability to compare trace element constituents.

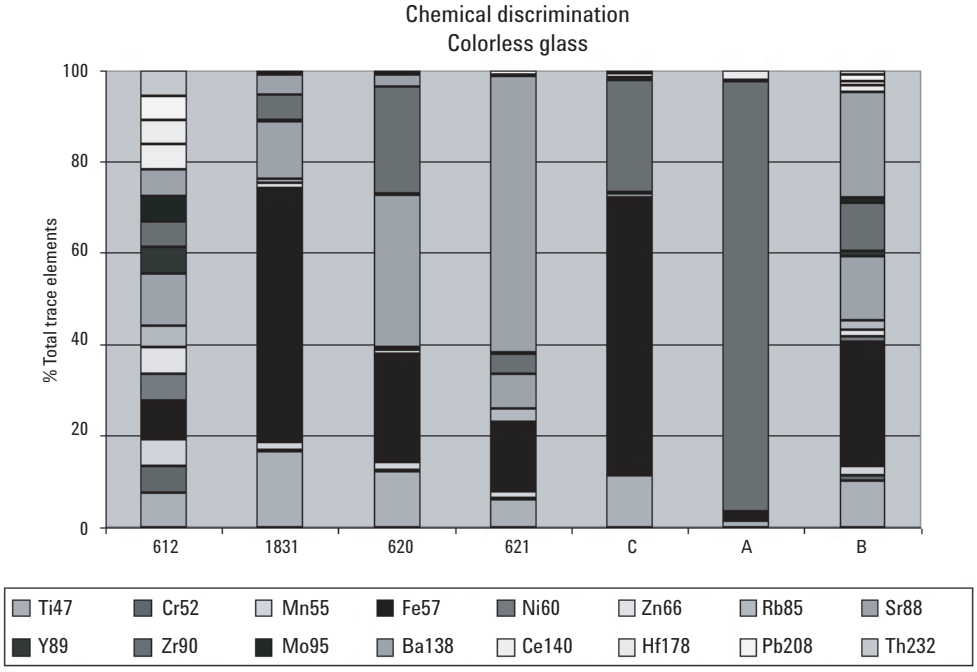


Figure 5:. The mean data from Table 3 is presented in a stacked bar chart format. This visual representation of the data aids data presentation in terms of clarity and relative simplicity.

Conclusion

LA-ICP-MS is an effective tool for the analysis of forensic glass samples. This technique is particularly useful in overcoming the limitations associated with very small sample types or samples composed of chemically inert materials.

Colorless glass fragments, indistinguishable to the naked eye and chemically identical at the ppm level, may be discriminated with good accuracy and precision, even at sub-millimeter dimensions. Due to the micro-destructive nature of this technique, forensic samples characterized by this method may also be available to alternative analysis if confirmation is required.

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Analysis of Gunshot Residue by ICP-MS

Agilent 4500 ICP-MS

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Abstract

ICP-MS was successfully utilized for elemental analysis of gunshot residues (GSR). The concentrations of antimony, barium, and lead were determined from the GSR collection swab extract solutions. The capabilities of semiquantitative analysis were also demonstrated.

Introduction

The elemental analysis of GSR is being used as one of the tools in interpretation of the criminal event. Some answers to the question of "accepted uniqueness"[1] of GSR particles can be given by the determination of lead, antimony and barium with additional information provided by the determination of copper, zinc, and iron. Determination of antimony, barium, and lead from the hands of a suspect was originally performed by the Dermal Nitrate (paraffin cast) technique with diphenylamine used as the testing reagent. This technique detects nitrites in GSR. However, this technique also detects nitrites originating from other sources, such as urine, matches, fertilizer, and some pharmaceuticals. Thus, the diphenylamine test produces numerous false positives and has been abandoned as a means of detecting GSR[2]. The elemental analysis techniques which replaced the Dermal Nitrate method were neutron activation analysis (NAA) and graphite furnace atomic absorption spectrometry (GFAAS). Both of these techniques suffered from several limitations, especially as a tool for routine, rapid analysis of GSR samples. In the past several years, inductively coupled plasma mass spectrometry (ICP-MS) has gained wide acceptance for trace and ultratrace analysis of liquid and solid samples in variety of application fields, including judicial and regulatory arenas.

Experimental

GSR samples were collected from the hands of a person who had fired a 9 mm semiautomatic gun (Glock) with 9 mm ammunition (Federal Hydra Shok). The shooting was conducted outdoors, gun was handled two-handed, and the samples were collected approximately 40 minutes after the shooting. GSR samples and calibration solutions were placed on Q-tip cotton swabs (a pair of swabs for each sample and standard), placed in 15-mL polypropylene screw-top tubes and dried overnight. Sample preparation consisted of adding of 10.0 mL a 10% (v/v) nitric acid (Fisher Scientific, Optima grade) into each tube, recapping and vortexing for about 1 minute. The nitric acid solution was spiked with 50 µg/L each of indium (In) and bismuth (Bi) as internal standards[3]. The tubes with caps removed were placed in an oven set at 80°C for 2 hours. Solutions were mixed again, and centrifuged for 5 minutes for extract separation. The extract solution was transferred by pipetting into another polypropylene tube and analyzed.

The solutions were analyzed in unattended mode, employing the ASX-500 (CETAC) autosampler and the Agilent ChemStation software feature allowing for sequential analysis of the samples. The additional QA/QC software can be applied, to monitor the quality requirements of the analysis.

Table 1: Instrument Parameters

Agilent 4500 Series ICP-MS with ASX-500 Autosampler	
RF Power	1210 W
Nebulizer	cross-flow
Cones	Nickel
Sampling depth	8.4 mm
Plasma gas	16.0 L/min
Auxiliary gas	1.0 L/min
Carrier gas	1.16 L/min

The operating conditions for Agilent 4500 series ICP-MS instrument are listed in Table 1. Table 2 shows the acquisition parameters employed in quantitative analysis of the swabs.

Table 2: Acquisition Parameters Used for Quantitative Analysis

Monitored Masses	115, 118, 121, 123, 137, 138, 206, 207, 208, 209
Detector Mode	auto
Int.Time/point	0.1 s
Int. Time/mass	0.3 s
Number of Points/mass	3
Number of Repetitions:	3
Total Acquisition Time	16 s

Quantitation of element concentration was made using ^{115}In as internal standard for Sb and Ba. ^{209}Bi was used as the internal standard for lead (represented by the sum of its three major isotopes: ^{206}Pb + ^{207}Pb + ^{208}Pb). Lead has four naturally occurring isotopes at masses 204, 206, 207 and 208. Three major isotopes (206, 207, and 208), being products of different radio-decay processes, may vary in abundance, depending upon the source of lead. To minimize errors caused by the difference in isotopic distribution, the sum of the signals measured at three major isotopes is used and represented as the lead value. This approach was adapted from the USEPA methods utilizing ICP-MS for determination of lead in environmental samples[4]. Twelve-point calibration curves were created for all analytes. The calibration standard concentrations are listed in Table 3. The internal standard mix contained indium, and bismuth added at 0.05 µg level to all swabs.

The calibration curves for 121Sb, 138Ba and 208Pb are shown in Figure 1 through Figure 3, respectively.

Table 3: Calibration Standards for GSR Analysis (12-point Calibration)

Standard	Sb (µg)/swab	Ba (µg)/swab	Pb (µg)/swab
Blank (S-0)	0	0	0
S-1	0.01	0.05	0.05
S-2	0.02	0.10	0.10
S-3	0.03	0.15	0.15
S-4	0.04	0.20	0.20
S-5	0.05	0.25	0.25
S-6	0.10	0.50	0.50
S-7	0.15	0.75	0.75
S-8	0.20	1.00	1.00
S-9	0.50	2.50	2.50
S-10	1.00	5.00	5.00
S-11	2.00	10.0	10.0

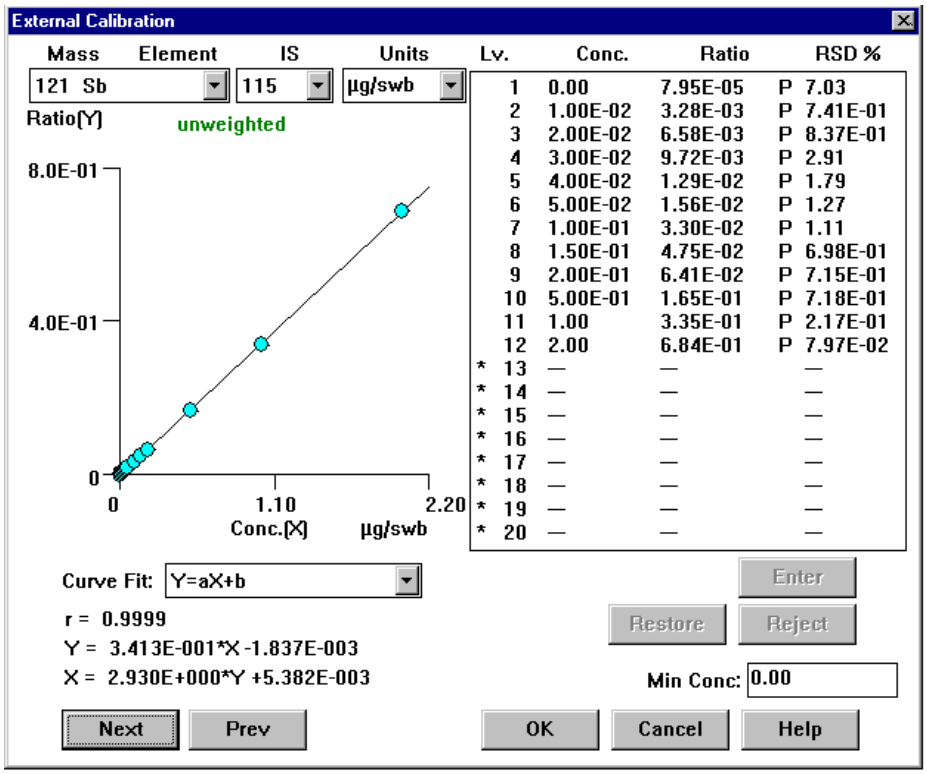


Figure 1. Calibration Curve: Antimony

The semiquantitative analysis of the samples was performed to demonstrate the unique capability of ICP-MS in providing fast and reliable information for over 70 elements, which can be present in the sample. This additional information can be used for further "fingerprinting" of GSR, similarly to the methods used in other forensic applications[5]. The acquisition parameters used in semiquantitative analysis are presented in Table 4.

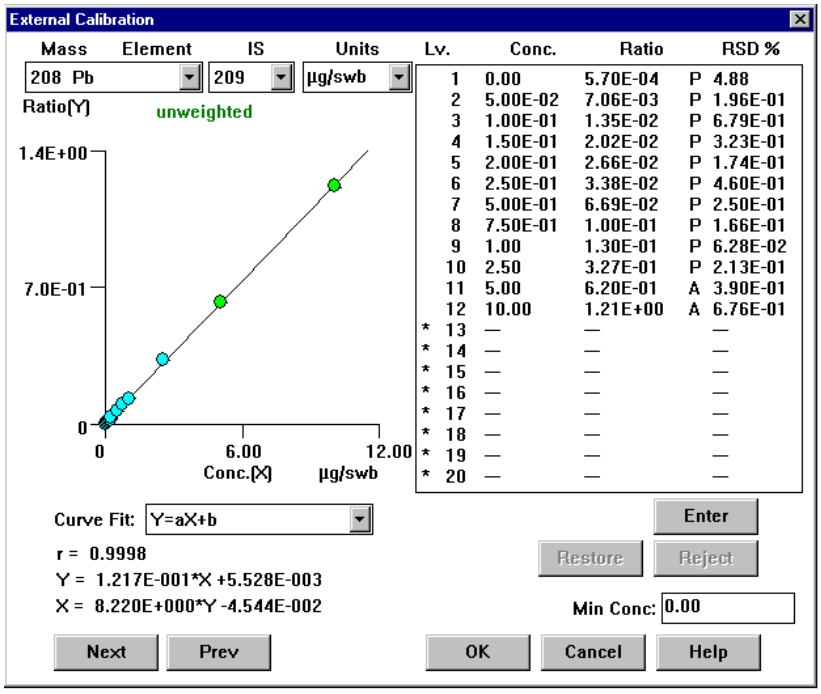


Figure 2. Calibration Curve: Barium

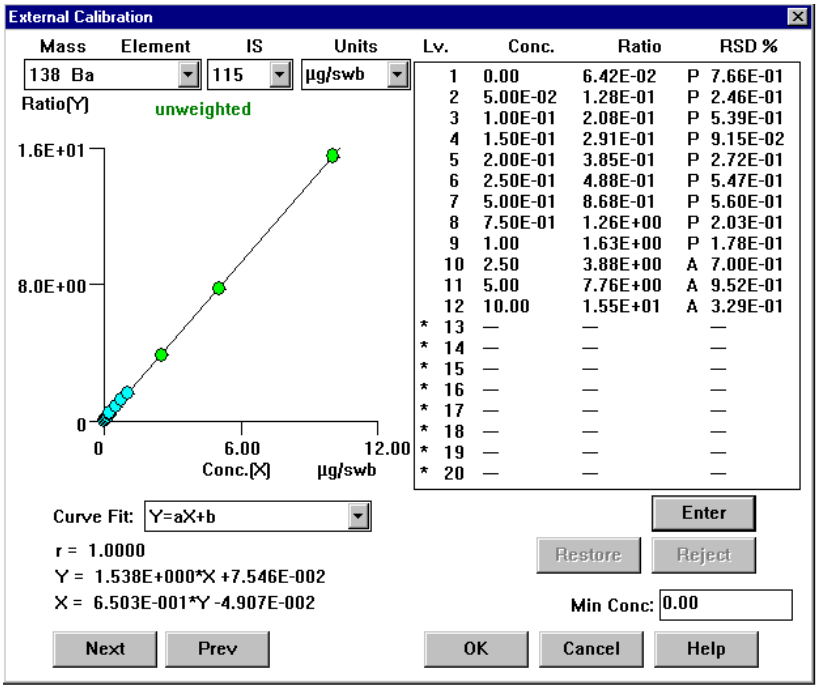


Figure 3. Calibration Curve: Lead

Table 4: Acquisition Parameters Used for Semiquantitative Analysis

Mass Ranges	6-11, 23-29, 39, 43-75, 77-78, 82-209, 232-238
Number of Masses	185
Detector Mode	auto
Int.Time/point	0.1 s
Int. Time/mass	0.6 s
No. of Points/mass	6
No. of Repetitions	1
Total Acq. Time	137 s

Results

Four samples were analyzed for the determination of Sb, Ba and Pb. They were swaps from the left hand palm, left hand's bottom, right hand palm, and right hand's bottom. They are labeled LP, LB, RP, and RB, respectively.

The results of the quantitative analysis of those four samples are presented in Table 5.

Table 5. Quantitative Analysis of GRS Samples

Sample	121Sb (µg)	138Ba (µg)	208Pb (µg)
LP	1.26	4.09	5.33
LB	0.27	0.91	1.50
RP	2.20	8.18	11.1
RB	0.12	0.40	0.91
Swab blank	0.007	<0.001	<0.001

The mass range, which includes the analytes of interest, is practically interference-free and monitoring either of the masses (121 or 123) for antimony will give the same results, especially since their relative abundances are almost equal. The isotope 121 was selected for reporting antimony results. For barium the two most abundant isotopes are 137 (11.2%) and 138 (71.7%). The latter offers more than six-fold higher signal, however it can

suffer from unpredictable elemental interferences from lanthanum and cerium. Most likely those interferences will be negligible in GRS samples, so the choice of isotope 138 is recommended. For routine analysis, only one isotope of antimony and one isotope of barium need to be quantified. As mentioned before, the lead value is calculated from a sum of signals collected at masses 206, 207 and 208 and represented as a value for isotope 208.

Figure 4 exemplifies the use of the semiquantitative analysis to verify that the signal measured at mass 138 was associated with barium. The software allows for measurement of the signals generated at the large mass range and subsequently fits a template corresponding to the natural abundance of the barium isotopes over the resulting signals. It is clear that there is a perfect fit between the experimental and theoretical values, thus the signal measured at mass 138 is a consequence of barium present in the solution.

Conclusions

ICP-MS was proven to be rapid and reliable analytical method for the determination of antimony, barium and lead in gun shot residue samples. The total analysis time (without sample preparation) was little over 2 minutes, with the actual data acquisition time equal to 16 seconds.

There are additional possible applications of ICP-MS for the evaluation of the origin of the GSR by the determination of other elements like copper, nickel and silver, and measurement of the isotopic ratios of lead.

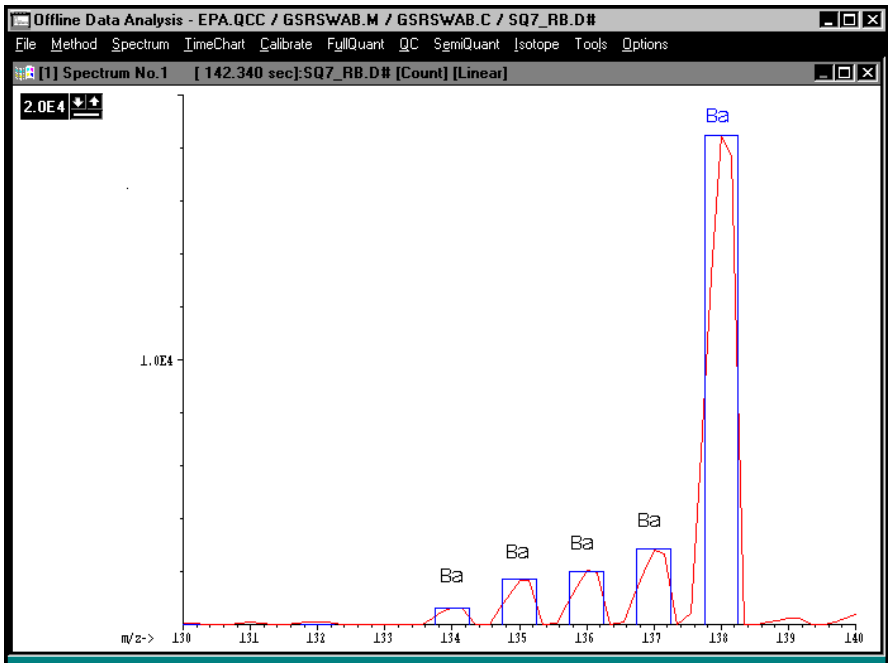


Figure 4. Isotopes of Barium: Semiquantitative Analysis

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[4] US Environmental Protection Agency, EPA Method 200.8, Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma Mass Spectrometry, Version 5.4, 1994

[5] E. Bakowska, HP Application Brief, September 1998, Publ.No. (23) 5968-1953E

This application bulletin demonstrates feasibility of concept. Additional development and/or validation may be required for routine use.

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Introduction to Laser Ablation ICP-MS for the Analysis of Forensic Samples

Application

Forensics

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Abstract

Forensic scientists require reliable methodologies capable of determining the origin of inorganic materials found at the scene of a crime. Linking these materials to a suspect or suspects can result in the vital evidence needed to secure a successful prosecution. Due to the great variety, shape, and size of forensic material, there is a need for a flexible analytical tool capable of analyzing the trace element content of solid samples directly. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) enables identification and comparison of physical evidence, discriminating elemental and isotopic differences at the part per billion (ppb) level. In contrast to aqueous analysis, where significant amounts of material need to be destroyed in the analytical process, LA-ICP-MS is a micro-destructive technique. Often the total volume of sample ablated with this technique is <1 µg; sustaining the essential integrity of the original evidence, which may be extremely small, and enabling further measurements to corroborate the results.

Introduction

Today's quadrupole ICP mass spectrometers enable the analysis of elements across the periodic table at very high scanning speeds and with very low detection limits. Typically samples are introduced into an ICP-MS by aspirating a solution of the sample. Often liquid samples require little preparation, but without a solid sampling accessory, solid samples need to be dissolved. This process is time consuming and often requires the use of acid dissolution reagents and additional sample preparation apparatus. Adding hazardous chemicals, such as hydrofluoric acid to dissolve the sample, can give rise to matrix-based interferences forming in the plasma. Hazardous chemicals are also a potential source of contamination. In contrast, combining ICP-MS with the direct solid-sample introduction technique of laser ablation (LA) requires minimal sample preparation. LA-ICP-MS provides an excellent and relatively nondestructive technique for elemental analysis of forensic samples that are difficult to digest, or where small fragments or inclusions must be analyzed. LA-ICP-MS is particularly amenable to time-resolved analysis (TRA); enabling direct comparison of samples in three dimensions. Combining such flexible data handling capabilities with *in-situ* solid sampling enhances discriminating power; strengthening the analyst's ability to determine the similarities and differences within large data sets.

LA-ICP-MS

LA-ICP-MS is widely used to determine elements directly in solid samples with minimal sample preparation. It is a highly sensitive multi-element technique with a wide analytical dynamic range from the part per trillion (ppt) to the part per million (ppm) level in the solid. For this study, a Merchantek UP-213 (New Wave Research. Inc, USA) LA system was coupled to an Agilent 7500s ICP-MS. A schematic of the LA system is shown in Figure 1.

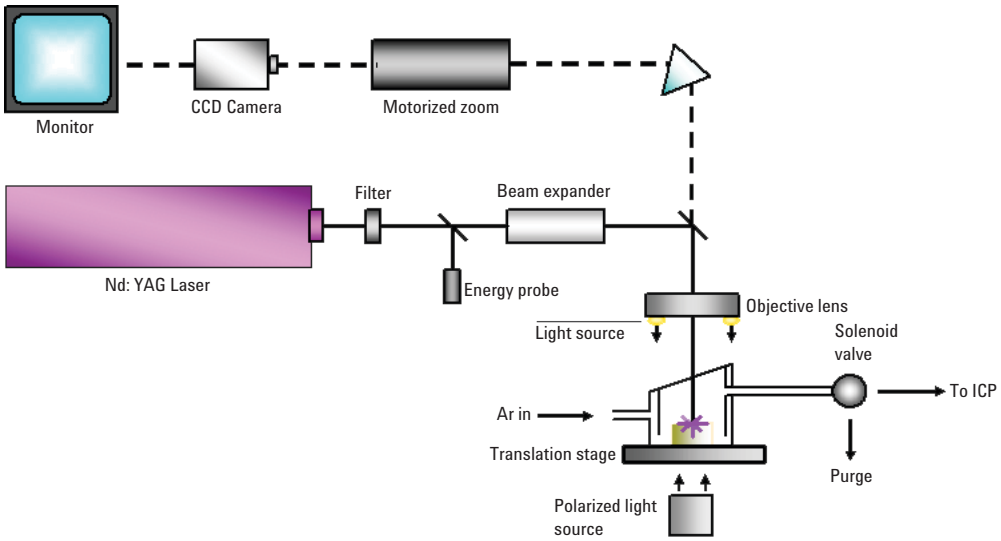


Figure 1. Schematic of Nd: YAG LA system (5th harmonic - 213 nm) for ICP-MS.

The sample surface is irradiated with deep-UV (213 nm) output from a frequency-quintupled Nd:YAG (neodymium doped yttrium aluminum garnet crystal) laser. The high-intensity pulsed ultraviolet (UV) beam is focused onto the sample surface in an ablation chamber or cell, which is purged with argon. The UV beam diameter can be accurately set by 12 software-controlled apertures to produce variable "spot" sizes from <5 µm to 300 µm depending on the application. The high-power, short-wavelength 213-nm laser couples directly with the sample matrix, with high absorption efficiency, reducing or eliminating plasma induced fractionation. The resultant laser-induced aerosol is then transported to the ICP in an argon carrier gas stream where it is decomposed, atomized and ionized, before extraction into the mass spectrometer vacuum system for analysis. Calibration is typically undertaken using a well-characterized synthetic solid material, such as NIST 612 Trace Elements in Glass or other suitable solid standard reference material (SRM).

Sample Analysis Using LA-ICP-MS

Generally, the ICP-MS is optimized by tuning the system during continuous ablation of a suitable SRM; examples of reference materials for glass and tape are given in Table 1. The Agilent ICP-MS can be optimized automatically using the AutoTune function of the Agilent ChemStation software. Often, tuning parameters for LA analysis are similar to those used for solution analysis. Tune parameters can be saved in a separate file for recall at a later date. If a SRM is available for the matrix being analyzed, it can be used to generate semi-quantitative response factors which are automatically stored in the ChemStation software. The sample can then be analyzed using a matrix element as the internal standard (IS). If an SRM is analyzed, the concentration of the IS is given and quantification is straightforward. However, for unknown samples, typical IS examples include the use of ^{13}C in polymer analysis and minor matrix isotopes in materials such as ceramics, stainless steel, and borosilicate glass, where the stoichiometry of the sample is known. While it is ideal to match the matrix of the standard to the sample, good semiquantitative data can be obtained for a wide range of matrices using a single set of response factors. This is because of the uniform response of the 7500 Series ICP-MS across the mass range, and the fine aerosol generated by the UV laser, which is more completely decomposed in the plasma, reducing matrix effects.

Table 1. Details of Forensic Standard Reference Materials

	Glass	Tape
Standard	NIST SRM 612: 50/µg/g nominal trace element concentration	BCR SRM 680: Trace elements in polyethylene
Matrix elements	Si (SiO_2), Na (Na_2O), Ca (CaO), Al (Al_2O_3)	
Source	National Institute of Standards and Technology, USA	Institute for Reference Materials and Measurements, Geel, Belgium

Software Controlled Operation

The LA software can be fully integrated into the Agilent ICP-MS ChemStation software for ease of setup and operation of the LA and ICP-MS. All laser parameters (for example, laser energy, frequency, purge valve position, sample viewing, stage positioning, and ablation pattern) can be controlled via the ICP-MS ChemStation PC. A high-magnification video system enables a full color, high-resolution image of the sample to be viewed directly on the ICP-MS monitor in real time, see Figure 2. The computer-controlled zoom feature and electronically-generated cross hairs aid sample positioning and can also be used to measure the size of any inclusions directly on the screen. Laser parameters can then be set accordingly, and during data acquisition, the laser power meter reading can be monitored on screen.

Forensic Applications

It is the task of the analyst to generate evidence based on trace elemental fingerprinting that can prove or disprove the source of the material. As a consequence, forensic materials presented for analysis by LA-ICP-MS could be anything from strands of hair to fibers of clothing.

For example, the glass used in the headlights and windows of automobiles is often unique to a manufacturer, and the elemental profile can be used to identify the marque, brand, or even year of manufacture of the vehicle. Trace element content offers far better discrimination than the traditional refractive index (RI) method. LA-ICP-MS provides a fast and simple means of characterizing glass fragments found on a suspect's clothing or at the location of an accident, without time-consuming sample preparation. Although the major and minor elemental composition of these glasses are very similar, and therefore are difficult or impossible to discriminate using traditional methods of characterization, these glasses may have trace elemental signatures (Figure 3) which enable accurate evaluation of differences by LA-ICP-MS.

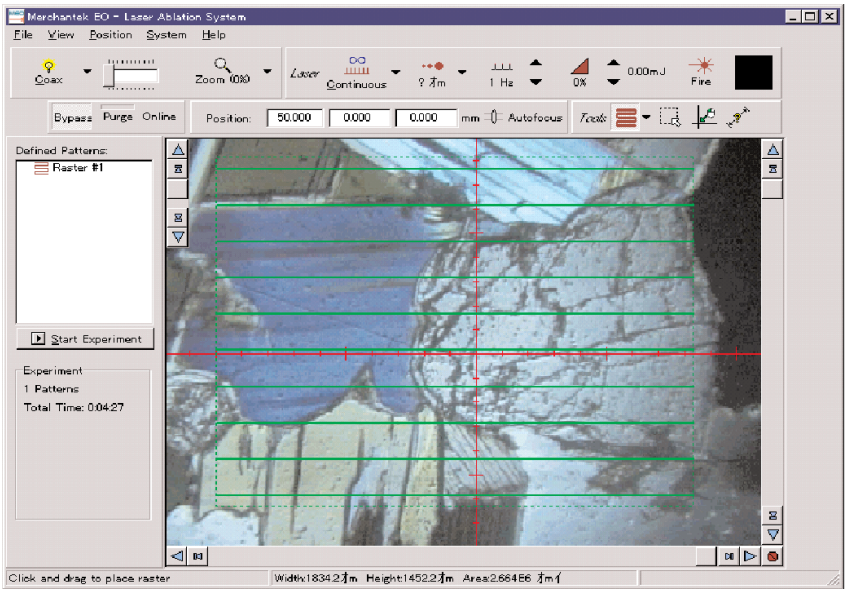


Figure 2. Screen capture showing a full color, high-resolution image of the sample.

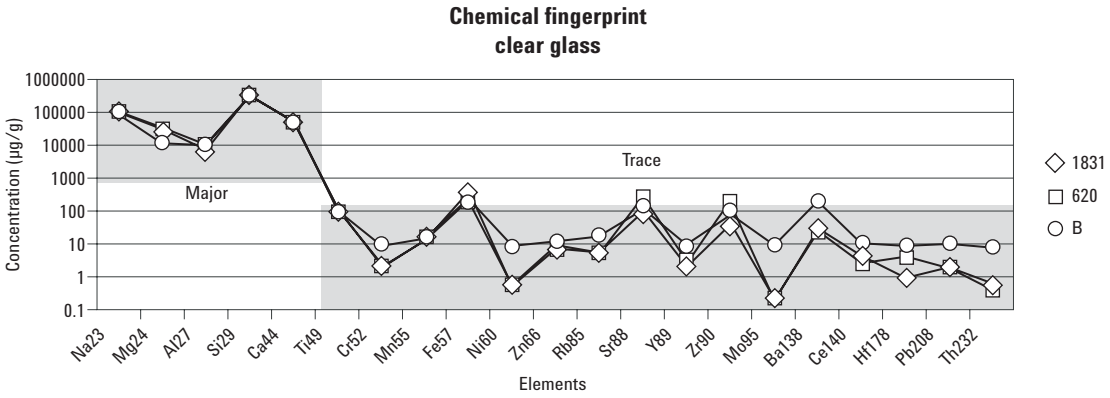


Figure 3. Elemental signature of clear glass.
The major elemental composition of these three glasses is similar (Na, Al, Si and Ca) while the trace elemental composition ≤100 µg/g (Cr, Ni, Rb, Sr, Y, Zr, Mo, Ba, Ce, Hf, Pb, Th) displays significant differences. Mg is an exception and at high concentration is often used as a discriminating element.

LA-ICP-MS can also be applied to other samples such as identifying inks on suspect documents, or element profiles of other scene of crime debris, including multi-layer paints, coatings on glass, bulk polymers, plastic bags, tape, and automobile parts. Figure 4 illustrates a sample of ballpoint pen ink after analysis using LA-ICP-MS. The ablated portion of the ink is clearly visible on the right side of the photograph.

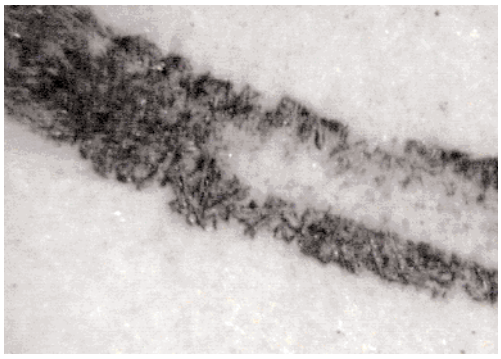


Figure 4. Magnified photograph of ballpoint pen ink sample after LA-ICP-MS sampling.

Data Manipulation

The data generated from LA-ICP-MS can be manipulated in real-time to enable the user to view the results of an analysis within seconds of data acquisition. Various optional software packages are available including:

- Glitter™ data reduction software
  Macquarie University - GEMOC [1]

GLITTER is an acronym for GEMOC Laser ICP-MS Total Trace Element Reduction. In addition to real-time on-line data reduction, GLITTER features a variety of plotting options, linked graphics and analysis tables, for simple presentation of the results. The ability to visualize results can aid users of forensic evidence in their understanding of the data.

- TriPlot Ternary plotting software
  Todd Thompson Software [2]

TriPlot produces a triangular plot of three variables that are plotted on the left, right and bottom sides of an equilateral triangle. Ternary plots are an effective way to discriminate subtle differences in sample populations, especially when multiple data point display is desirable as shown in the example in Figure 5.

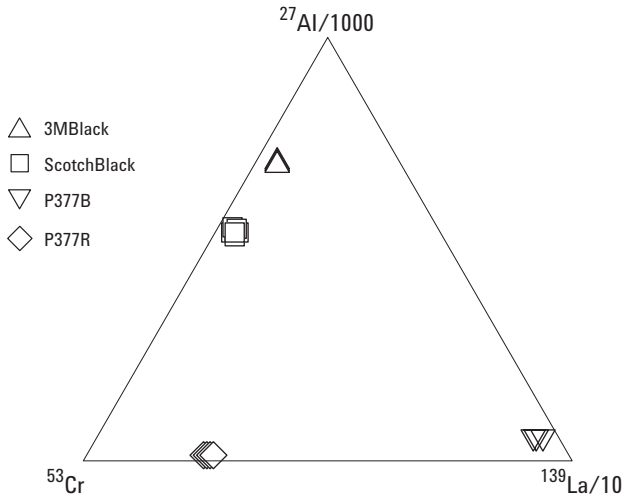


Figure 5. Ternary plot of adhesive tape data (integrated counts per second).

Conclusions

LA-ICP-MS is an effective tool for the analysis of a wide variety of forensic samples. This technique is particularly effective in overcoming the limitations associated with very small sample types or samples composed of chemically inert materials. The definitive "fingerprint" produced by LA-ICP-MS based on elemental and isotopic ratio data is used to qualify or disqualify the source of physical evidence. Often a clear visual representation of the data is produced using a suitable plotting program making it easier to discriminate samples.

Reference

1. Glitter™ data reduction software, Macquarie University - GEMOC
   http://www.es.mq.edu.au/GEMOC
2. TriPlot Ternary plotting sofware, Todd Thompson Software
   www.home.earthlink.net/~baedke/triplot

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Agilent Technologies

Semiquantitative Analysis of Glass Fragments using Laser Ablation ICP-MS

Application Brief

Forensic

Abstract

The 4500 ICP-MS equipped with a laser ablation system (LSX-100) was utilized to characterize and identify glass fragments from various sources. Rapid, semiquantitative analysis of the samples resulted in unique elemental "fingerprint" patterns that were used for sample identification. The method requires almost no sample preparation and sample consumption is limited. As a result, the remaining sample can be used to perform further tests if necessary.

Agilent Technologies
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Introduction

When examining a glass fragment in a criminal case, the point in question is the identification or exclusion of the glass source. Historically, forensic comparison of glass samples has been limited to the comparison of physical properties of known and questioned samples, principally by the measurements of refractive index and density values. Due to advances in glass manufacturing technology, the range of the refractive indices of modern glass is narrowing, thereby potentially resulting in an increase of false positives[1]. In this study, an LSX-100 laser ablation system was connected to a standard 4500 ICP-MS enabling the direct multielement analysis of glass fragments. The elemental composition of a glass sample is a combination of major components, minor elements intentionally added to molten glass to enhance its physical properties, and trace levels of other elements which were present as contamination in raw materials.

Methods of elemental analysis are gaining popularity as forensic tools. The main disadvantage of many of these techniques for the analysis of glass is the required sample preparation: digestion/dissolution of the samples in HF. This sample preparation method is not only time consuming and requires extra safety precautions, but is also a destructive method, which in many cases may not be acceptable. LA-ICP-MS eliminates the need for extensive sample preparation, provides excellent detection limits, offers unmatched elemental coverage, and exhibits a wide dynamic range. An additional benefit of LA-ICP-MS is that the amount of sample used for a single determination is negligible and leaves the remaining sample available for further tests, if required.

Experimental

The 4500 ICP-MS was optimized using an NIST SRM 614 glass standard. The operating conditions for both the 4500 ICP-MS and the LSX-100 laser ablation system are listed in Table 1. Six glass samples of known origin were analyzed. The samples were washed with double distilled water, sonicated for 1 minute, and dried with isopropyl alcohol. The whole sample preparation process was less than 3 minutes. Three samples represented a class of flat glass: picture frame glass, window glass and a Pyrex glass from the laboratory. Three automotive glasses were also analyzed: 1991 Geo Metro, 1996 Dodge Avenger, and unidentified glass from the scene of an automobile accident.

Elemental ratios were used in developing the fingerprint patterns in order to eliminate variations in laser focus and in the extent of laser interaction with the sample surface. Another advantage to the use of elemental ratios is that quantitative calibration of the ICP-MS instrument is not required. However, if some estimate of the concentration were desired,

4500 ICP-MS	
RF Power	1.3kW
Interface	Ni cones
Plasma Gas	16 L/min
Auxiliary Gas	1.0 L/min
Carrier Gas	1.16 L/min
Sampling Depth	7 mm
Integration Time	0.6 sec/mass
LSX-100 Laser Ablation System	
Mode	Q-switched
Laser Power	1.5 mJ TEM00
Defocus	0.6 mm
Repetition Rate	20 MHz
Laser Scan Speed	0.03 mm/sec

Table 1.
Instrumental Parameters

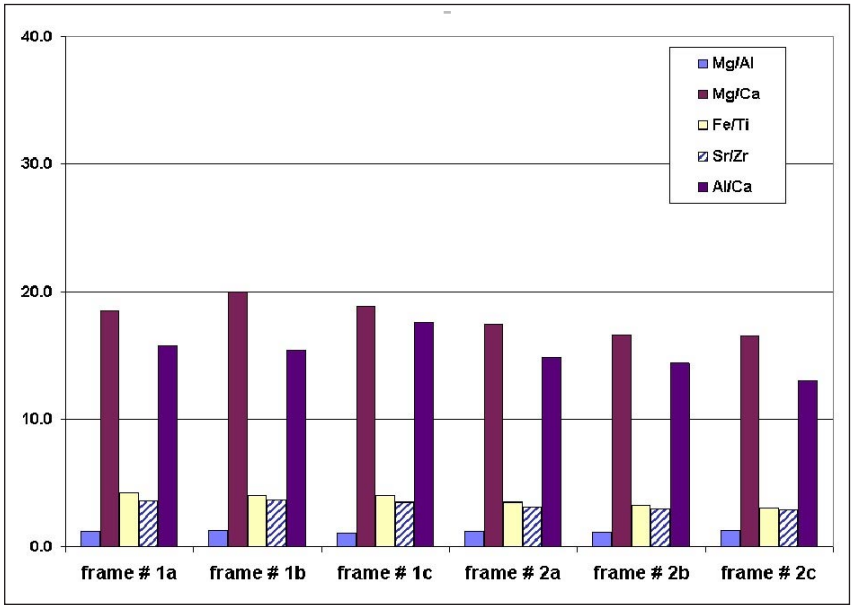


Figure 1.
Sample-to-Sampe and Intra-Sample Reproducibility

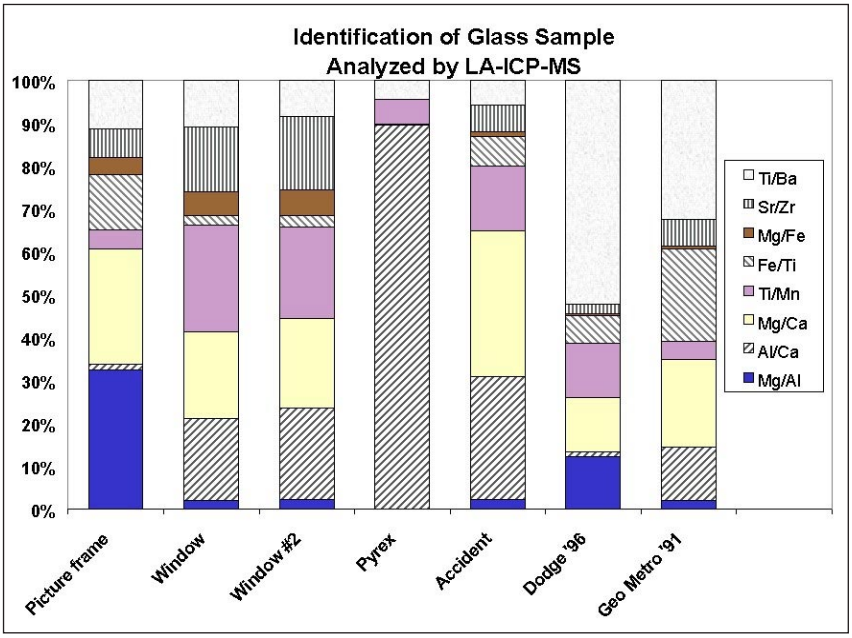


Figure 2.
The Graphical Representation of the Elemental Composition of Glass Samples

then the semiquantitative analysis feature of the 4500 ICP-MS software allows for the determination of over 70 elements during a single analysis in approximately 2 minutes.

Semiquantitative analysis requires quantitative calibration with a single standard and a minimum of only three elements which are not necessarily the analytes of interest. Approximate concentrations for all remaining elements in the periodic table can than be determined. Of course, true quantitative analytical results can also be obtained, if required.

Two different pieces of glass from each source were analyzed in triplicate. From all the elements, which were determined, 9 were identified as providing a distinct elemental fingerprint for the glasses examined: aluminum, barium, calcium, iron, magnesium, manganese, strontium, titanium and zirconium. The results, given in Figure 1, show excellent reproducibility between replicate analysis of a sample of glass from a picture frame (replicates a, b, and c) as well as between two different fragments of the same glass (frame #1 and frame #2). The results of the analysis of the six glasses are shown in Figure 2. Note that each sample has a distinct, identifiable elemental fingerprint pattern. A second fragment of the window glass (Window #2) was analyzed to once again confirm the unique nature of the fingerprint pattern and the reproducibility of the analysis.

Reference

[1] J.A. Buscaglia, Analytical Chimica Acta, 288 (1994) 17-24

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Performance Characteristics of the Agilent 7500ce - The ORS Advantage for High Matrix Analysis

Part 1 of a 3 part series on Environmental Analysis

Application

Environmental Analysis

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Abstract

The Agilent 7500ce ICP-MS was specifically designed and optimized for the analysis of trace metals in high matrix samples including environmental, clinical, geological, and others. The 7500ce uses enhanced Octopole Reaction System (ORS) technology for improved sensitivity and robustness over previous generation inductivity coupled plasma mass spectrometry (ICP-MS) instruments. This application note outlines the theory of interference removal using the ORS, the design enhancements employed, and the typical performance of the Agilent 7500ce.

Introduction

This application note is Part One of a three part series on environmental analysis using the Agilent 7500ce ICP-MS system. Part Two is an application note demonstrating the ability of the Agilent 7500ce ICP-MS system to measure trace elements in drinking water, at substantially below regulated levels, under challenging real-world conditions [1].

Part Three is an application note covering the analysis of various high matrix environmental samples using the Agilent 7500ce ICP-MS [2].

This application note details the advances in ion optics and octopole reaction system (ORS) design that were incorporated into the 7500ce. These advances came about as a result of extensive testing and development of its predecessor (Agilent 7500c) with difficult, high-matrix samples. The design goals of the 7500ce were:

- Develop an ICP-MS system specifically to meet the needs of analytical laboratories to analyze unknown, variable, high-matrix samples, which are currently depending on inductively coupled plasma-optical emission spectroscopy (ICP-OES), graphite furnace atomic absorption (GFAA), and hydride and cold vapor techniques, in addition to ICP-MS.
- Maintain the simple, effective interference removal characteristics of the ORS - successfully introduced in the 7500c.
- Improve the overall sensitivity to allow ultra-trace analysis of mercury (Hg) and other low level elements, which were previously difficult in some very high matrix sample types.

These goals were achieved through enhancements in the sample introduction system, interface, ion optic, and ORS regions of the instrument. In common with all the other models in the 7500 Series, the 7500ce uses highly efficient 27 MHz plasma coupled to a low-flow nebulizer and cooled-spray chamber to minimize plasma and interface matrix effects. This approach has been

successfully used in all Agilent ICP-MS instruments since the 4500 Series in 1994, but recent enhancements with the development of a new digitally driven, all solid-state RF generator have further increased plasma robustness. This serves to reduce metal oxide interferences, as evidenced by a very low CeO^+/Ce^+ ratio of <1.5% (<0.8% in He cell gas mode). Following the successful strategy of the 7500c, all ion lenses with the exception of the octopole are outside the high vacuum region and can be serviced without venting the mass analyzer. This design greatly reduces downtime for routine system maintenance. The 7500ce maintains a linear, axial flow of ions from the sampler and skimmer cones through a pair of on-axis extraction lenses, enhancing ion transmission and reducing the effects of matrix accumulation on the extraction lenses. Borrowed from the successful 4500 and 7500a systems, the 7500ce uses a simplified Omega lens to eliminate photons and neutrals from the ion beam before entering the octopole. Unlike older photon stop designs, the Omega lens eliminates photons and neutrals while maintaining high ion transmission, particularly at low masses. After the Omega lens, ions enter the octopole reaction cell, which is now located on-axis to the quadrupole and detector, further enhancing ion transmission. The redesigned ion lens and ORS provide improved ion transmission without compromising the tight control of ion energy, which is essential for efficient interference removal by energy discrimination (ED).

Figure 1 compares the 7500c and 7500ce configurations, highlighting the simplification in the ion trajectory that has led to the improved performance specifications of the 7500ce. Enhancements in software designed specifically for routine high matrix analyses add additional capability and ease of use. These include the introduction of "Virtual Internal Standardization" (VIS) which allows the user to interpolate between internal standard (ISTD) response factors to create a VIS at a mass where no appropriate ISTD exists. Intelligent calibration resloping can automatically fine-tune a calibration curve, if needed, during a long sequence of high matrix samples, without the time consuming recalibration. This can be accomplished in the same process as monitoring a required continuing calibration verification (CCV).

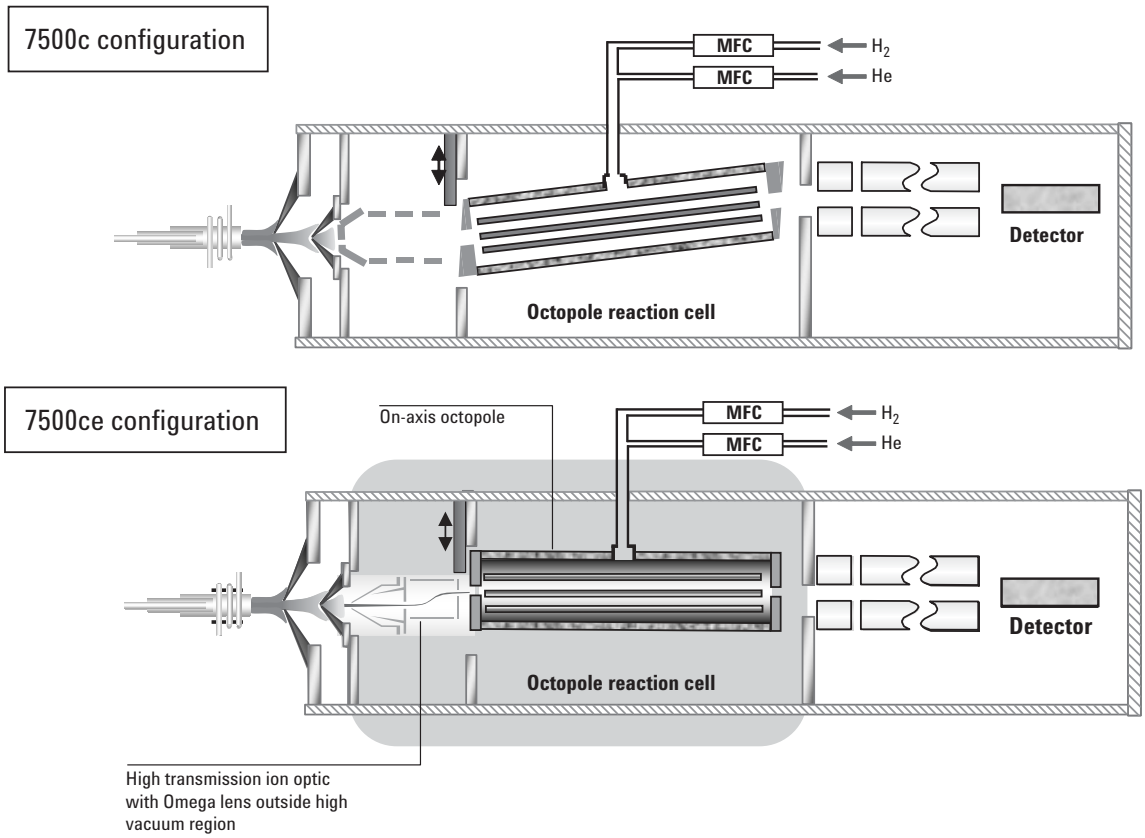


Figure 1. A comparison of the ion optic and octopole configurations between the Agilent 7500c and 7500ce ICP-MS systems.

Enhanced ORS

Like its predecessor the 7500c, the 7500ce uses collision/reaction cell (CRC) technology in the form of the ORS to remove polyatomic interferences. The use of CRC technology to reduce interferences in ICP-MS is well-documented [3]. However, until now, there have been compromises associated with the use of some designs of CRC ICP-MS for trace level, multi-element analysis in unknown or variable matrices. These compromises included poor sensitivity for low-mass analytes, poor stability, and the necessity for matrix matching of samples and standards to avoid unexpected new interferences caused by complex, sequential reaction chemistry in the cell. As a result, some CRC systems allow only the analysis of a small number of analytes under a specific set of conditions for a single sample matrix.

Numerous publications [3, 4, 5] have discussed the mechanisms for polyatomic interference removal using CRC technology including:

- Collisionally induced dissociation (CID)
- Chemical reaction
  - Charge transfer
  - Atom transfer
- Kinetic energy discrimination (KED)

Mechanism 1, CID, does not occur to any great degree with the relatively light gases typically used in the collision cell because the combined kinetic energy of the collision does not generally exceed the bond energy of the polyatomic species. In most CRC ICP-MS systems, chemical reaction mechanisms including charge transfer and atom transfer are the predominant mechanisms [4, 5]. However, in order to provide sufficient reduction of interferences, the reaction must be highly favored, which can require the use of very reactive gases for many interferences. Such gases can also react with analyte ions, so reducing sensitivity and compromising multi-element analysis, or form reaction by-products that can interfere with other analytes [4]. In this case, reaction cell conditions must be matched to a specific analyte/matrix combination and cannot be used simultaneously for multiple analytes in variable matrices. Mechanism 3, KED, relies on the fact that at the exit of the collision cell polyatomic species will possess lower kinetic energy than atomic ions at the same mass-to-charge ratio [3, 4]. This is due to the fact that collision cross sections of polyatomic ions are larger than for atomic ions, so that polyatomic species suffer more collisions with the cell gas, thus losing more of their initial energy. A bias voltage at the cell exit is then used to reject the low-energy polyatomic species, while allowing the high-energy atomic ions to enter the quadrupole for analysis and detection

Three Modes of Operation - One Set of Conditions

Table 1 lists the typical instrument conditions used for high-matrix analysis for the 7500ce. Instrument parameters are essentially the same for all three modes of operation[1]. This is because no complex procedures are required to remove newly created interferences or to avoid the reactive loss of analyte in any ORS mode.

[1]Slightly higher bias voltages are required in the octopole and quadrupole to maintain ion velocity in a pressurized collision cell compared with a nonpressurized cell. Other parameters, with the exception of the cell gas flow, are identical in all modes of operation.

Table 1. Instrument Parameters for Robust Plasma Conditions Used with the 7500ce

Instrument parameter	Normal mode	Hydrogen mode	Helium mode
RF Power	1500 W	<Same	<Same
Sample depth	8 mm	<Same	<Same
Carrier gas	0.85 L/min	<Same	<Same
Makeup gas	0.2 L/min	<Same	<Same
Spray chamber temp	2 °C	<Same	<Same
Extract 1	0 V	<Same	<Same
Extract 2	–160 V	<Same	<Same
Omega bias	–24 V	<Same	<Same
Omega lens	–0.6 V	<Same	<Same
Cell entrance	–30 V	<Same	<Same
QP focus	3 V	–11 V	<Same as H_2
Cell exit	–30 V	–44 V	<Same as H_2
Octopole bias	–7 V	–18 V	<Same as H_2
QP bias	–3.5 V	–14.5 V	<Same as H_2
Cell gas flow	0	3.0 mL/min H_2	4.5 mL/min He

The Hydrogen Reaction Mode

In hydrogen reaction mode, the ORS is pressurized using a small flow of pure hydrogen at 1–5 mL min^{-1}. Simple reactions with hydrogen remove argon-based polyatomics according to the following examples.

Charge (e^-) transfer:

$Ar^+ + H_2 \rightarrow H_2^+ + Ar$ (interference on $^{40}Ca^+$)

Proton transfer:

$Ar_2^+ + H_2 \rightarrow Ar_2H^+ + H$ (interferences on $^{78}Se^+$ and $^{80}Se^+$)

$ArO^+ + H_2 \rightarrow ArOH^+ + H$ (interference on ^{56}Fe)

Atom transfer:

$ArO^+ + H_2 \rightarrow H_2O^+ + Ar$ (interference on ^{56}Fe)

In all cases the Ar interference is removed from mass 40, 56, 78, and 80. Since Ca, Fe, and Se do not react with H_2, there is no loss of analyte signal.

$Ca^+ + H_2 \rightarrow$ No reaction

$Se^+ + H_2 \rightarrow$ No reaction

$Fe^+ + H_2 \rightarrow$ No reaction

Note that some of these reaction processes lead to new polyatomic ion species, principally hydrides of the original interference. However, these new, cell-formed species all have low energy and are removed from the ion beam using the same bias voltage, as was discussed above, under interference removal by KED.

Reaction mechanisms can be highly efficient, as evidenced by the calibration curves in Figure 2 for ^{78}Se, ^{40}Ca and ^{56}Fe, which show that all the "normal" background species are reduced significantly under a single set of cell conditions.

Figure 3 illustrates the reduction in background from Ar^+ at m/z = 40 as hydrogen flow in the cell is increased, yielding a 10^9 reduction in background. Since the reaction chemistry is specific to argon polyatomics, no signal is lost due to reaction of the analyte with hydrogen, as could occur with other more reactive gases. However, due to the specificity of reaction mode, there are numerous examples where it is not useful. For example, in samples where the matrix composition is unknown, or there are multiple polyatomic interferences at a single m/z, it is not possible to use reaction mode effectively. In this case, a more generic method of interference removal is needed.

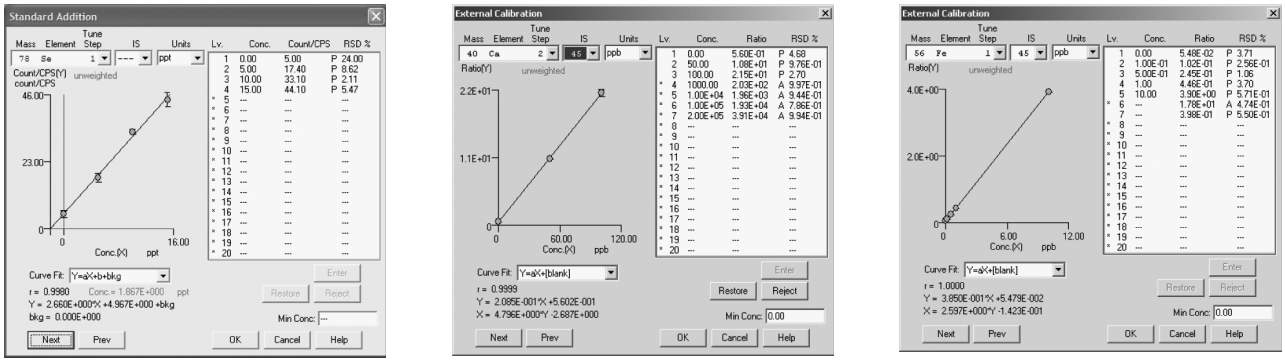


Figure 2. Calibration plots for ^{78}Se, ^{40}Ca, and ^{56}Fe under hydrogen reaction conditions.

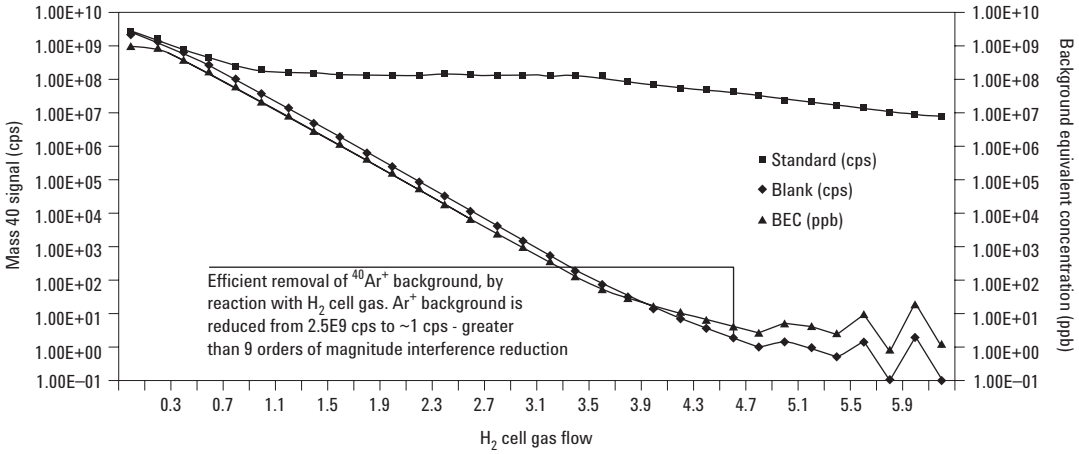


Figure 3. Reduction in background on mass 40 for calcium using hydrogen reaction mode. In this case, as the hydrogen flow is increased to about 5 mL/min, the background at mass 40 decreased from approximately 2.5 billion cps to about 1 cps, a >10^9 reduction.

The Helium Collision Mode

Helium collision mode can reduce or eliminate polyatomic interferences by one of two mechanisms; either CID or KED. Both are nonreactive mechanisms and so they do not form any new polyatomic ionic species that must be managed. CID can occur when the collision energy between the polyatomic ion and the collision gas (typically He) is sufficient to break the polyatomic bond. The result is two (usually atomic) fragments at lower mass, one of which will retain the charge of the original ion. A few common polyatomic interferences are bound weakly enough for this to occur. They include $NaAr^+$, which can interfere with the measurement of ^{63}Cu in high sodium samples and ArO^+ which interferes with iron. However, when ion energies are properly controlled, KED is the more useful of the two techniques. Kinetic energy discrimination depends on the fact that polyatomic ions are always larger in collisional cross section than monatomic ions (Figure 4), and as a result undergo more collisions and so lose more energy when traversing a pressurized collision cell. Figures 5 and 6 depict the KED process.

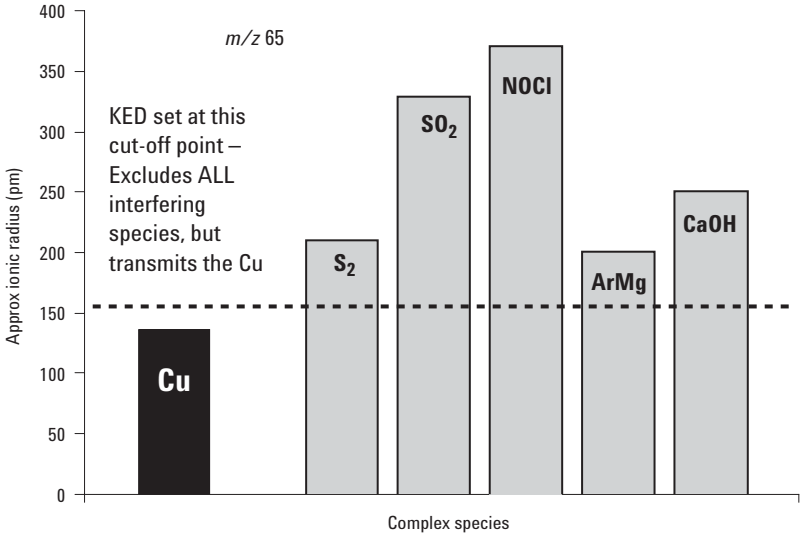


Figure 4. Comparison of approximate ionic radii (in picometers) for copper and several polyatomic species.

Figure 5 shows the greater loss of energy of the polyatomic ion relative to the atomic ion, in this case $ArCl^+$ relative to As^+. However, for KED to be effective, it must remove the polyatomic ion effectively while not significantly reducing the response of the atomic ion. This means there must be minimal overlap in ion kinetic energies between the polyatomic and atomic ions at the exit of the octopole. For this to be the case, the energy spread of incoming ions must be less than the energy difference between analyte and polyatomic interference at the octopole exit.

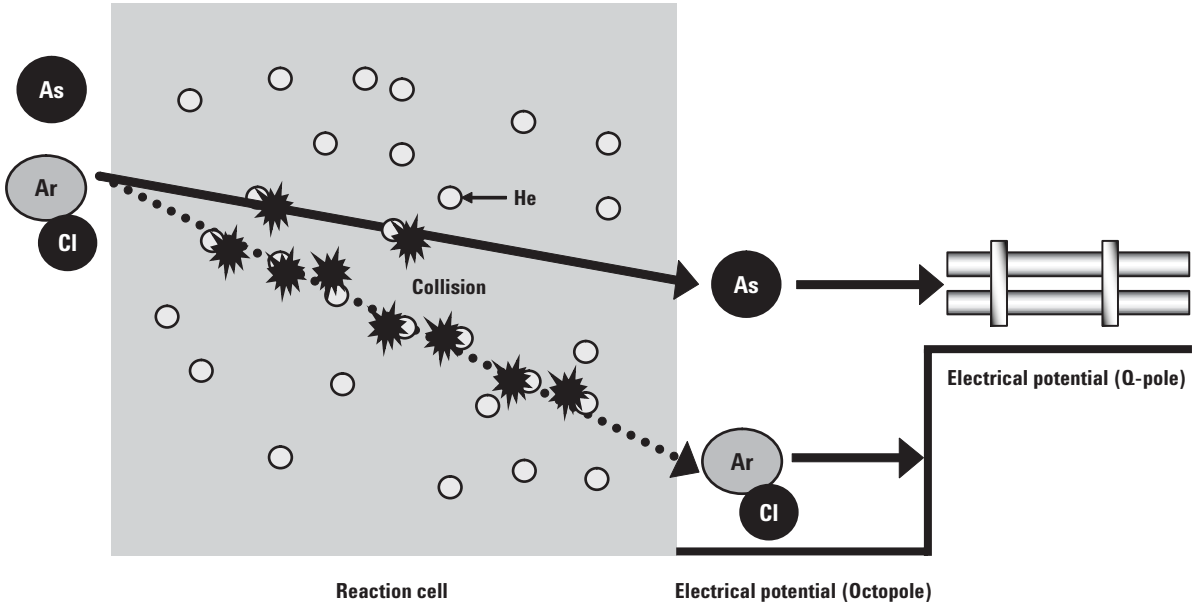


Figure 5. KED. Polyatomic species have a larger collision cross section, and so experience more energy dissipating collisions and exit the cell with lower kinetic energy. A small stopping potential between the exit of the octopole and the entrance of the quadrupole keeps the polyatomic ions from entering the quadrupole and being detected.

Only the Agilent ORS can accomplish this, as a result of the use of the ShieldTorch system, which minimizes plasma potential and eliminates secondary ionization in the interface, which would otherwise cause broadening of the ion energy spread. It is also essential to avoid band-broadening collisions induced by high extraction voltages in the high-pressure region immediately behind the skimmer cone. On the 7500ce, this is accomplished by using soft-extraction, (extract 1 operates at 0 to +5 V), as a result of which, the mean ion energy is maintained at less than 2 eV with an ion energy spread of about 0.5 eV, ideal for the KED of plasma-source polyatomic interferences.

A simplified schematic representation of ion kinetic energy and energy distribution for a typical ICP-MS system and from an Agilent 7500 ORS ICP-MS is shown in Figure 6.

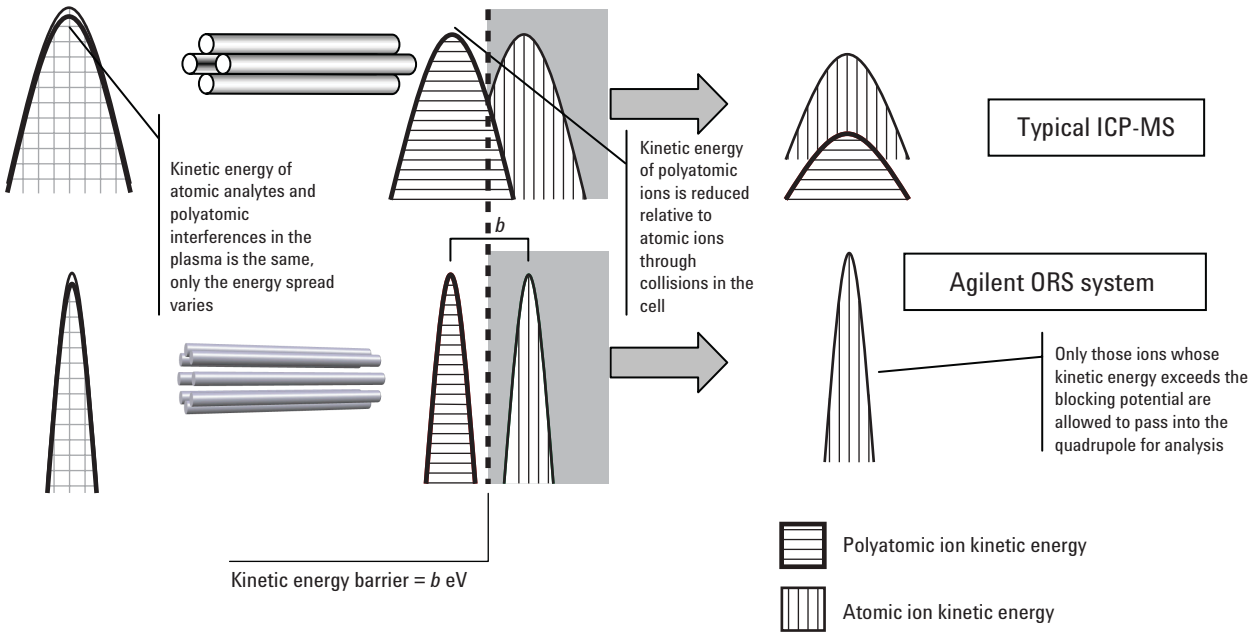


Figure 6. Simplified schematic representation of ion kinetic energy and energy distribution of a typical ICP-MS system (upper), and from an Agilent 7500 ORS ICP-MS (lower).

After multiple collisions in the CRC, in both cases the average kinetic energy of the larger polyatomic ions is decreased relative to the smaller atomic ions by *b* eV. If a kinetic energy barrier is applied to the ion beam at the exit to the collision cell that is equivalent to the kinetic energy difference, *b*, (indicated by dashed line), then any ions whose kinetic energy is lower than the barrier will be blocked. If the energy distribution of ions is larger than the difference in average energies of the ions, only partial rejection of polyatomic ions occurs accompanied by a loss of atomic ions.

While ED was described on other designs of ICP-MS systems, these systems do not have the tight control of ion energy provided by the ShieldTorch System and so the ED is only effective for reducing the very low energy polyatomics formed within the cell, typically as a result of sequential reaction chemistry, which is characteristic of the use of a highly reactive cell gas, such as NH_3. Table 2 shows the reactants and products for a number of polyatomic interferences using both hydrogen and ammonia reaction mode. As can be seen, in the presence of common matrix components such as carbon and sulfur, the use of NH_3 can create multiple new interferences, which must be removed. Avoiding the use of NH_3 eliminates the possibility of creating new, cell-formed, polyatomic cluster ions in the first place.

Reaction of hydrogen with plasma-based polyatomics such as Ar^+ is highly favored and results in elimination of the interferent. Neither hydrogen nor NH_3 are effective at removing the interference from ClO^+ on vanadium. Additionally, the use of ammonia can lead to reaction with other common matrix elements such as carbon and sulfur, creating new interferences such as HCN^+ on aluminum and $NHSH^+$ on titanium. Using the Agilent system with an inert cell gas and KED would eliminate the interference from ClO^+ on vanadium AND, for example, ArC^+ on Cr, without producing any new interferences.

An excellent test of the efficiency of interference removal can be seen in low-level calibration plots. When interferences are present, the response curve will be offset in the y direction by the magnitude of the interference, increasing the background equivalent concentration (BEC) and the detection limit (DL). When the interference is removed, the calibration curve intersects the y-axis at a point much nearer to zero with a correspondingly lower BEC and DL. Figure 7 depicts sub-ppb calibration curves for chromium and vanadium in 1% each methanol, HCl, and HNO_3, with and without the use of helium collision mode. Since KED does not depend on chemical reaction, it is independent of matrix concentration as well as composition.

Table 2. Comparison of Reaction Products for Several Possible Reactants Involving Hydrogen and Ammonia

Reactants		Interfered analytes	Products	
Ar^+	H_2	Ca^+	H_2^+	Ar
Ar_2^+	H_2	Se^+	ArH^+	Ar, H
ArO^+	H_2	Fe^+	H_2O^+, $ArOH^+$	
ClO^+	H_2	V^+	None	None
ClO^+	NH_3	V^+	None	None
HCN^+	H_2	Al^+	$HCNH^+$ (28)	H
C^+	NH_3		NH_3^+ (17)	C
			HCN^+ (27)	H_2
			$HCNH^+$ (28)	H
S^+	NH_3		$NHSH^+$ (48)	

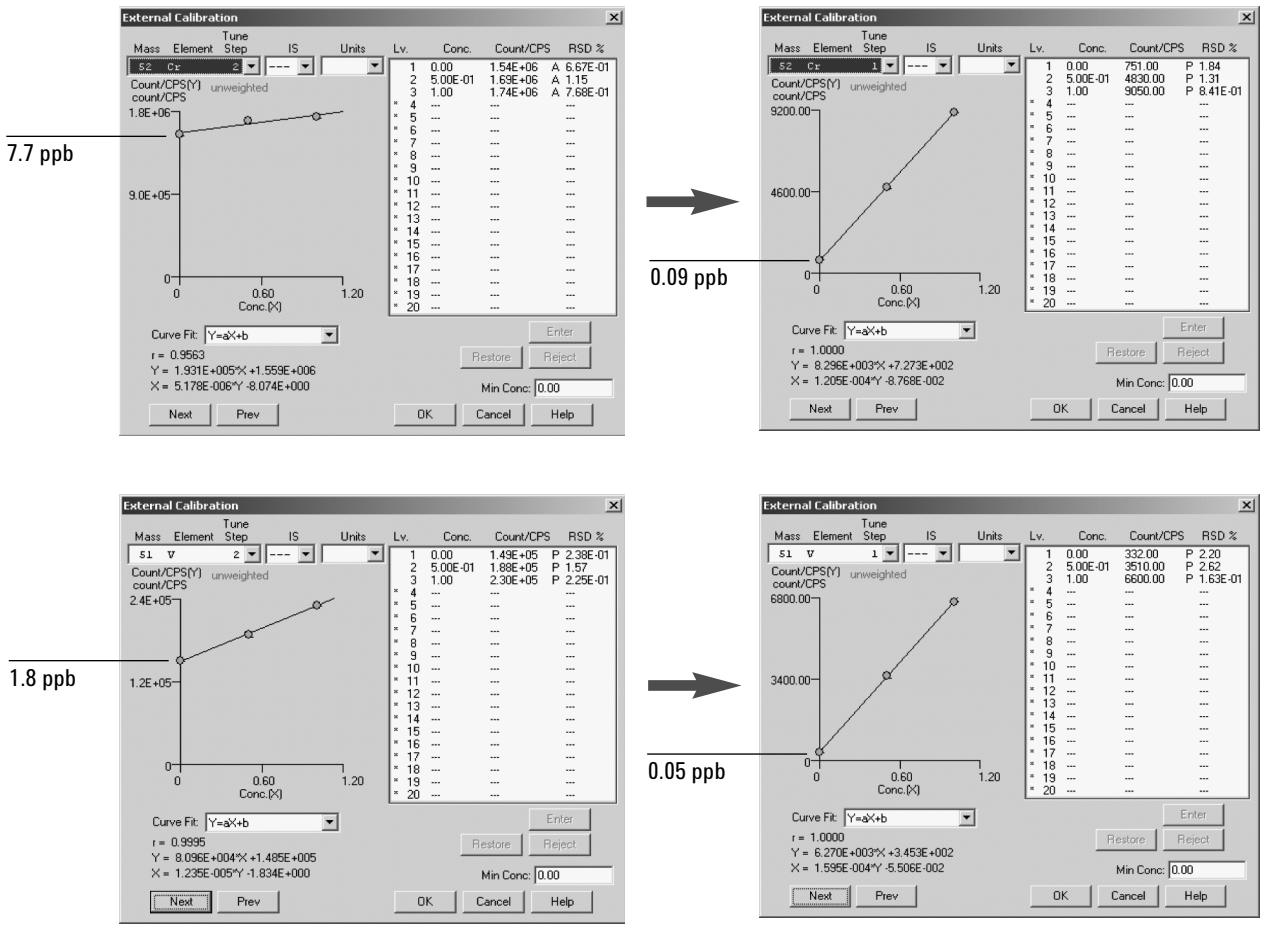


Figure 7. Calibration plots of ^{52}chromium and ^{51}vanadium in 1% nitric, 1% hydrochloric, 1% methanol showing the contribution of interferences from ArC^+ and ClO^+ in normal mode on the left and after removal of interferences by the ORS using He on the right.

Figure 8 depicts the effect of increasing HCl on the measured concentration of a 5-ppb solution of vanadium in both helium and normal (no gas) mode. Increasing the HCl from 0% to 1% causes an 80% increase in measured vanadium concentration in the no-gas mode. There is no increase in the V concentration reported for the variable sample matrix, when measured in He mode.

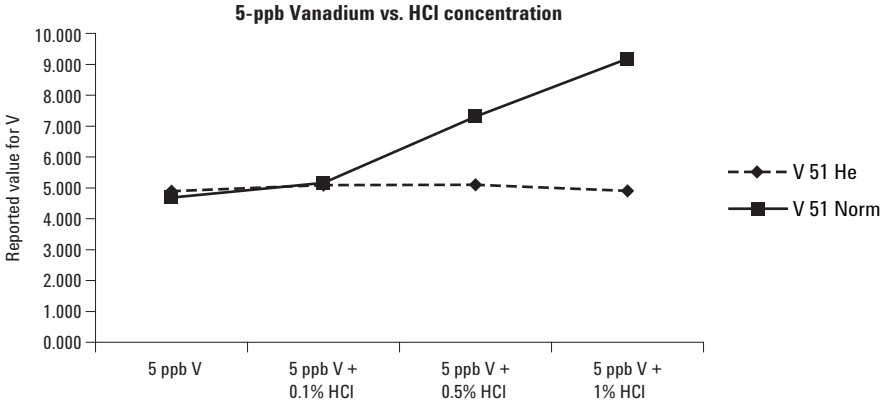


Figure 8. Effects of increasing HCl concentration on vanadium response in both normal and helium modes.

The Normal Mode

Normal mode uses the octopole as an ion guide only with no additional gas added. In this mode, the Agilent 7500ce ICP-MS operates as a conventional (non-collision cell) instrument. Because an octopole is a highly efficient ion guide as compared with a lower-order multipole, such as a hexapole or quadrupole, there are no compromises in ion transmission efficiency and the addition of a collision gas to promote collisional focusing in normal mode is not required. Because of this, the Agilent 7500ce exhibits exceptional sensitivity for uninterfered low mass elements, such as lithium, beryllium, and boron. Typically, normal mode is used only for these elements, though it is also acceptable for other elements that do not require interference removal such as lead, mercury, thallium, and uranium. Examination of Table 3 will show that the DLs for the interference-free heavy metals are essentially the same in all three modes, giving the user the flexibility to select as appropriate.

Table 3. Three Sigma Detection Limits (ppt). All Isotopes 1 s Total Integration Time Except Ca and Hg Which Were 3 s Total Integration Time

		STD Mode (No gas)		H_2 Mode (5 mL/min)		He Mode (4 mL/min)	
Mass	**Element**	**DL (3-Sigma)**	**BEC**	**DL (3-Sigma)**	**BEC**	**DL (3-Sigma)**	**BEC**
6	Li	4.99	17.09	161.491	95.83	331.32	142.58
7	Li	1.67	14.36	23.755	26.31	9.38	20.55
9	Be	0.19	0.11	6.932	2.62	7.812	2.61
11	B	5.88	47.26	83.182	128.03	48.21	107.28
23	Na	3.36	148.40	62.682	313.64	37.65	299.38
24	Mg	0.27	0.72	2.570	1.75	3.37	1.41
27	Al	3.05	50.70	8.079	5.51	37.56	53.52
31	P	418.27	12521.95	–	–	1903.62	3800.52
39	K	1347.50	47564.25	29.532	118.74	2838.73	27943.17
40	Ca	–	–	2.936	7.13	–	–
43	Ca	460.04	8520.74	129.640	121.68	191.85	742.92
44	Ca	2932.48	50407.443	102.104	121.94	48.01	352.38
45	Sc	7.95	183.06	6.446	19.41	1.34	6.46
47	Ti	4.07	40.86	32.197	19.88	4.98	3.52
49	Ti	11.49	57.33	17.535	10.46	5.69	1.70
51	V	0.40	2.52	1.309	0.73	0.42	0.19
52	Cr	5.53	212.670	19.919	68.63	3.10	22.70
53	Cr	7.98	52.87	28.504	82.65	8.70	21.60
55	Mn	1.69	25.24	1.362	4.10	4.25	12.23
56	Fe	1443.70	55093.34	5.034	20.21	53.99	451.26
57	Fe	444.36	23132.21	66.614	261.56	71.06	215.90
59	Co	0.21	2.15	0.816	0.26	0.38	0.30
60	Ni	26.523	672.20	71.224	742.27	41.70	491.67
63	Cu	1.32	37.48	20.271	20.14	6.37	68.35
65	Cu	2.70	47.46	27.845	27.76	7.52	59.76
66	Zn	2.85	9.01	1.612	1.83	1.84	2.18
69	Ga	0.30	3.03	0.273	0.19	0.82	1.04
71	Ga	1.22	7.40	0.125	0.15	1.80	2.22
72	Ge	2.60	53.69	1.448	1.14	3.32	7.55
73	Ge	3.72	32.74	5.556	2.74	7.30	6.04
75	As	23.24	660.78	14.130	14.00	10.72	65.72
78	Se	48.10	6351.29	2.396	2.556	48.93	195.45
82	Se	26.92	251.29	15.225	56.51	20.63	116.01
85	Rb	0.27	1.06	0.349	0.57	0.72	0.34
88	Sr	0.19	0.84	0.072	0.04	0.38	0.13
89	Y	0.10	0.26	0.054	0.04	0.16	0.04
90	Zr	0.07	0.09	0.709	0.18	0.15	0.06

Table 3. Three Sigma Detection Limits (ppt). All Isotopes 1 s Total Integration Time, Except Ca and Hg Which Were 3 s Total Integration Time (Continued)

		STD Mode (No gas)		H_2 Mode (5 mL/min)		He Mode (4 mL/min)	
Mass	**Element**	**DL (3-Sigma)**	**BEC**	**DL (3-Sigma)**	**BEC**	**DL (3-Sigma)**	**BEC**
93	Nb	0.12	0.20	0.68	0.53	0.15	0.05
95	Mo	0.32	0.67	14.53	4.33	0.31	0.1
101	Ru	0.60	1.40	19.27	5.74	0.28	0.09
103	Rh	0.08	0.11	4.24	1.75	0.05	0.0
105	Pd	0.30	0.27	10.11	6.39	0.33	0.15
107	Ag	0.23	0.33	1.42	1.61	0.28	0.4
111	Cd	0.56	0.83	0.32	0.20	0.86	0.54
115	In	0.07	0.11	0.05	0.03	0.08	0.05
116	Cd	0.33	0.41	0.40	0.47	0.34	0.23
118	Sn	0.24	0.43	0.55	0.54	0.91	0.73
121	Sb	0.11	0.08	0.21	0.010	0.46	0.25
125	Te	1.96	0.94	2.05	1.29	9.57	4.12
126	Te	1.12	1.64	2.08	1.67	7.33	4.27
127	I	2.02	21.73	3.57	22.30	7.71	20.41
133	Cs	0.09	0.04	0.06	0.04	0.15	0.06
137	Ba	0.22	0.20	0.38	0.16	0.9	0.38
139	La	0.17	1.94	2.49	2.46	0.66	2.14
140	Ce	0.223	2.65	2.18	3.21	0.47	2.88
141	Pr	0.11	0.25	0.12	0.28	0.12	0.31
146	Nd	0.39	0.44	0.43	0.50	0.70	0.73
147	Sm	0.22	0.17	0.11	0.04	0.58	0.24
153	Eu	0.02	0.03	0.04	0.02	0.11	0.03
157	Gd	0.17	0.14	0.15	0.05	0.35	0.22
159	Tb	0.03	0.02	0.01	0.01	0.055	0.03
161	Dy	0.18	0.15	0.05	0.05	0.23	0.17
163	Dy	0.15	0.08	0.08	0.04	0.23	0.16
165	Ho	0.04	0.00	0.02	0.01	0.05	0.02
166	Er	0.15	0.06	0.05	0.02	0.17	0.09
169	Tm	0.02	0.02	0.02	0.01	0.03	0.03
172	Yb	0.11	0.09	0.07	0.02	0.30	0.18
175	Lu	0.04	0.020	0.02	0.01	0.06	0.03
178	Hf	0.13	0.08	0.06	0.06	0.32	0.15
181	Ta	0.04	0.06	0.06	0.049	0.11	0.08
182	W	0.32	0.35	1.39	0.5	0.56	0.33
183	W	5.07	1.07	0.87	0.42	0.43	0.47
185	Re	0.12	0.07	0.07	0.05	0.12	0.08
193	Ir	0.09	0.10	0.25	0.08	0.33	0.15
195	Pt	0.14	0.17	1.94	0.52	0.22	0.18
197	Au	0.22	0.11	1.76	0.43	0.18	0.07
200	Hg	0.82	2.00	1.04	1.78	1.15	2.18
201	Hg	1.11	2.54	2.07	2.29	1.56	2.58
202	Hg	0.86	1.84	0.75	1.77	0.59	1.91
205	Tl	0.20	0.24	0.13	0.22	0.35	0.30
206	Pb	0.33	0.84	0.28	0.64	0.34	0.73
207	Pb	0.51	0.94	0.25	0.69	0.95	1.05
208	Pb	0.47	0.712	0.40	0.55	0.53	0.755
209	Bi	0.05	0.04	0.03	0.02	0.06	0.05
232	Th	0.04	0.04	0.03	0.01	0.06	0.05
238	U	0.05	0.04	0.04	0.01	0.05	0.044

Conclusions

The Agilent 7500ce ICP-MS has achieved its design goals of providing sensitive, robust, interference-free analysis of difficult, high-matrix samples. With five times the sensitivity of its predecessor, nine operating orders of dynamic range and increased matrix tolerance, it is capable of replacing both GFAA and ICP-OES instruments in addition to older generation ICP-MS systems. The 7500ce is unique in offering a single solution for multi-elemental analysis of complex and variable, high matrix samples, while allowing the operator the freedom to use simple and consistent sets of instrument conditions for almost all elements in almost any matrix.

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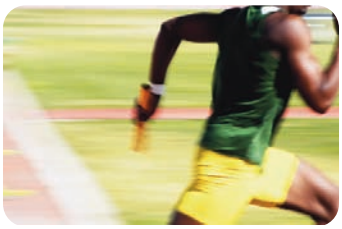
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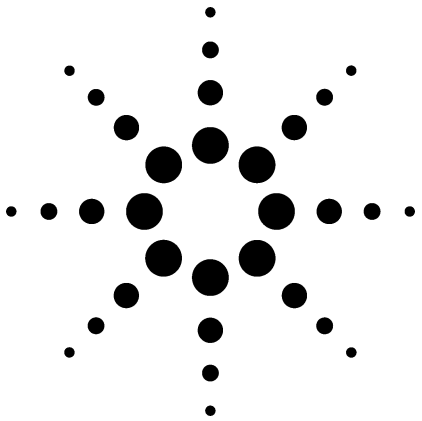
Applications by Instrumental Technique

Capillary Electrophoresis



Analysis of Drugs by CE-MS^n

Application



Forensics/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 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.

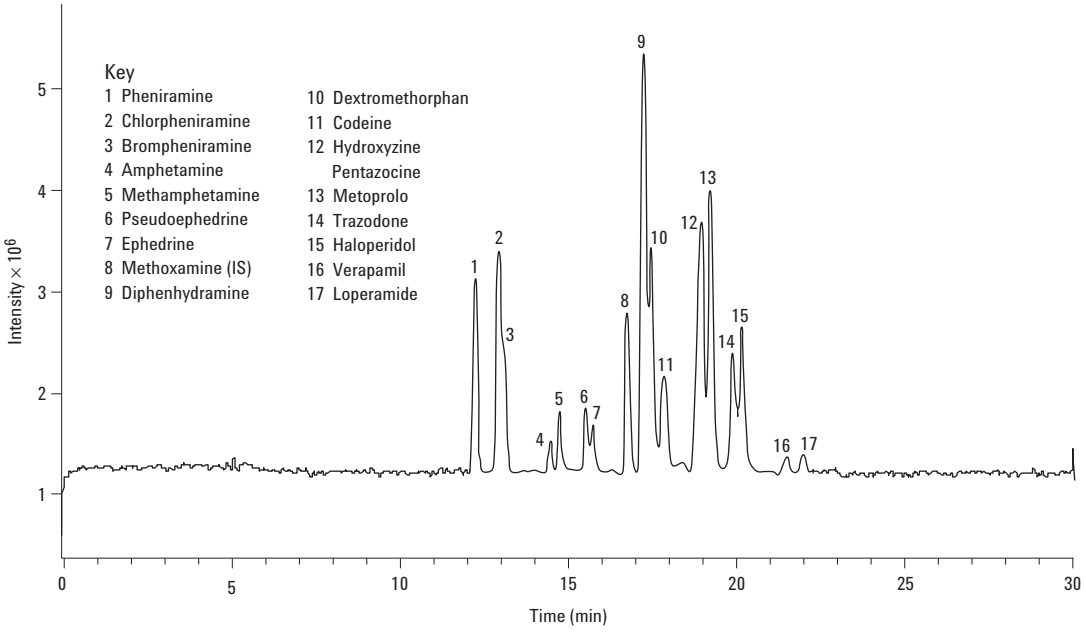


Figure 1. TIE for drug mixture.

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.

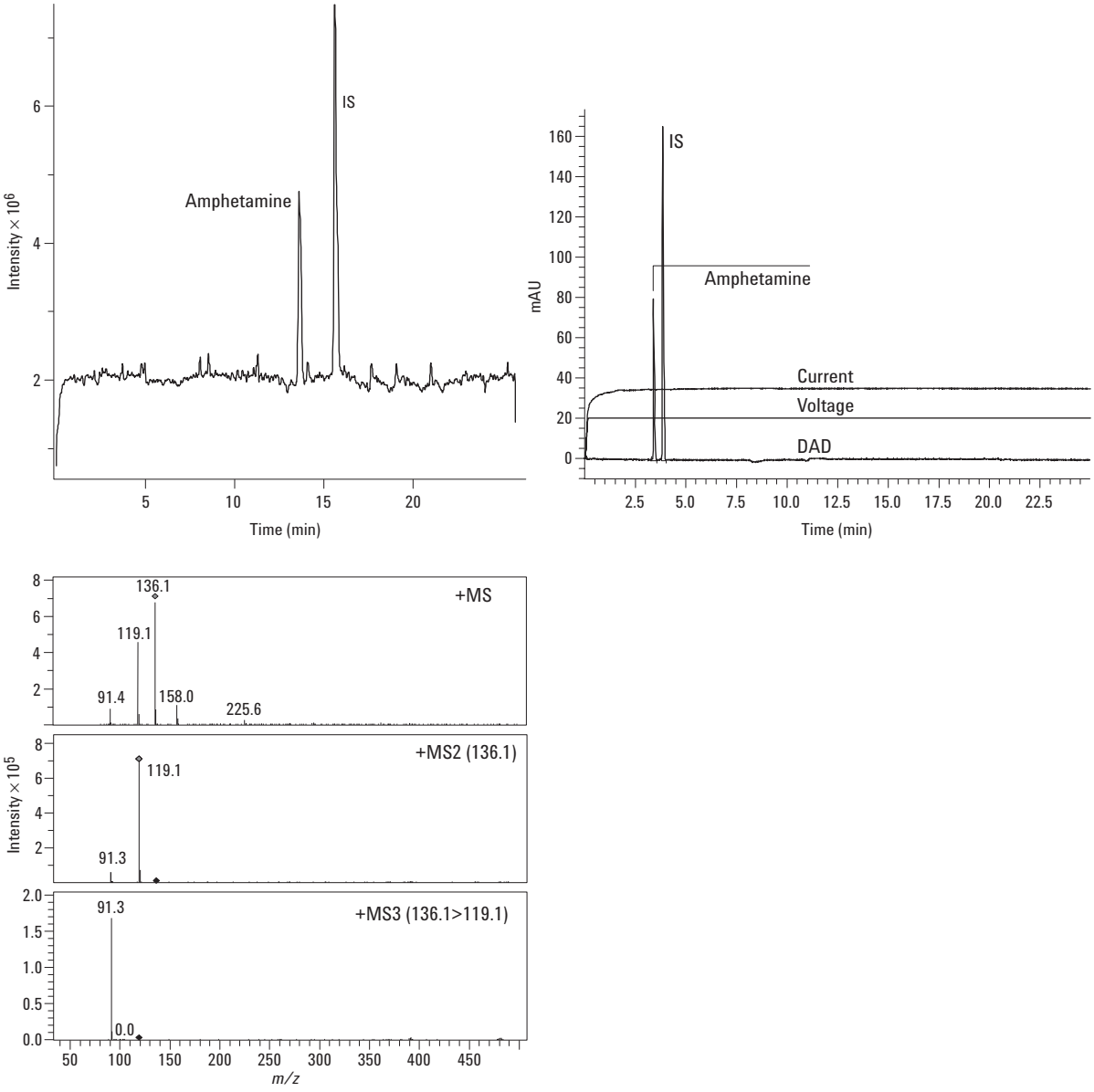


Figure 2. TIE, UV electropherogram, and MS data for amphetamine.

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.

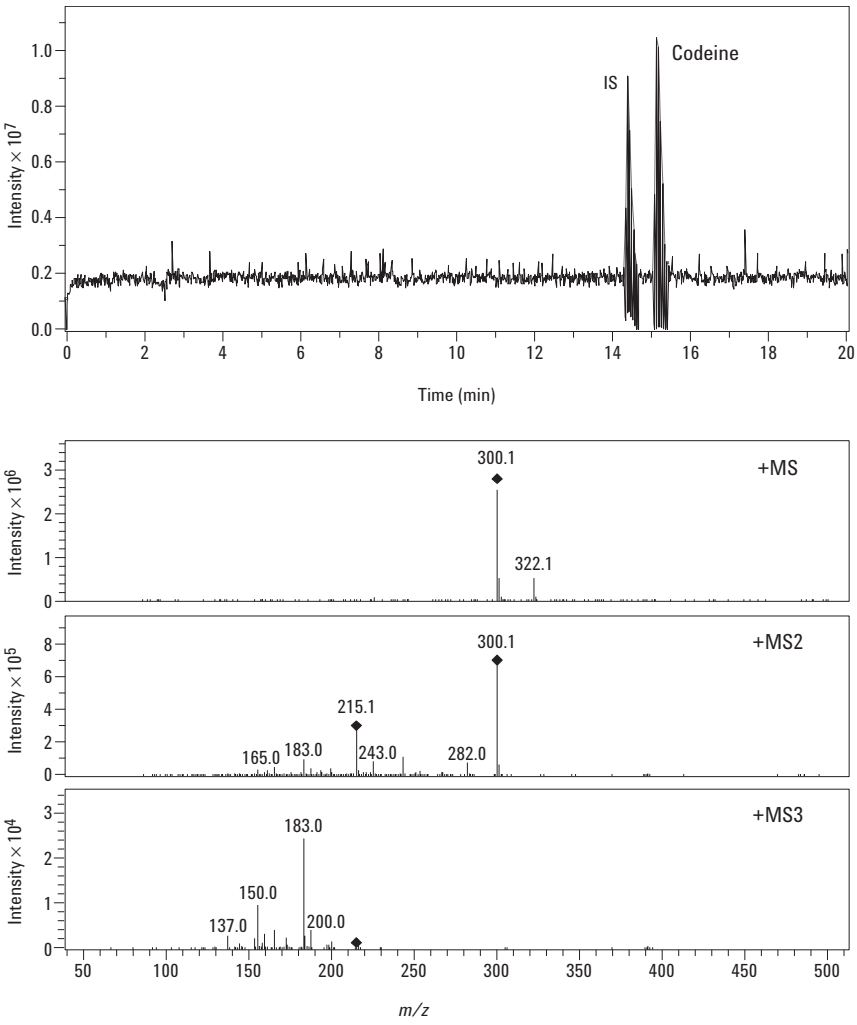


Figure 3. Auto MS data for codeine.

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 toxicology.

Acknowledgement

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

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Analysis of drugs of abuse by CE-ESI-MS

Dat T. Phan
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Forensics

Abstract
Samples of drug substances containing LSD and a mixture of methamphetamine, cocaine, and heroin, were analyzed by capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS). Both protonated molecular ions and characteristic fragment ions were observed, allowing unique identification. The data shown here demonstrate feasibility. Additional method development and/or validation may be required for routine use.

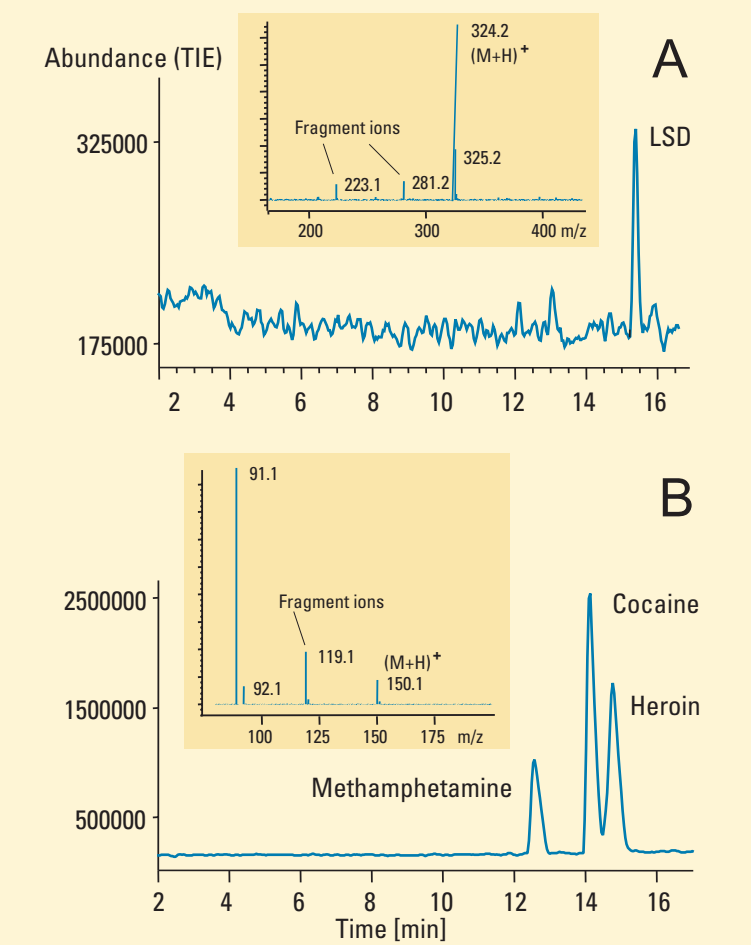


Figure 1
Total ion electropherograms and mass spectra of LSD and a mixture of methamphetamine, cocaine and herion.

Conditions

Sample
A) LSD (8 µg/mL), B) heroin, methamphetamine and cocaine (300 µg/mL)
Injection
3 sec @ 50 mbar
Capillary
bare fused silica
total length 100 cm, 50 µm id
Buffer
50 mM ammonimum acetate, pH 4.5
Voltage 25 kV
Temperature 25°C
Preconditioning
3 min flush with buffer at 1 bar
Sheath liquid
0.5 % acetic acid in 50 % acetonitrile, 5 µL/min
Nebulizing gas nitrogen, 20 psi
Drying gas nitrogen, 7 L/min, 200 °C
Acquisition
positive mode, Vcap -3.5 kV, fragmentor 100 V, step size 0.1, PW 0.25 min, time filter on
Scan range
80–500 m/z

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Experimental

The Agilent CE system with CE-MS capillary cassette was connected to an Agilent 1100 Series MSD. The MSD was fitted with electrospray ionization and orthogonal sprayer for CE-ESI-MS. Sheath liquid was delivered by an Agilent 1100 Series binary pump equipped with a 1:100 flow splitter. All instrumentation was controlled by an Agilent ChemStation.The samples were provided by a law enforcement agency specifically for these studies. Stock solutions were made in methanol and then diluted with water. The fused silica capillary was prepared by flushing for 30 min with 1 N NaOH followed by a 10-min wash with deionized water prior to insertion into the sprayer needle. Once installed the capillary was flushed with running buffer for 30 minutes.

Results

Figure 1A shows the analysis of LSD. The mass spectrum of the peak at ca. 15 minutes reveals the protonated molecular ion at 324.2 m/z. Ions with masses of 223.1 and 281.2 are characteristic fragment ions caused by collision induced dissociation (CID). At fragmentor values greater than 100 V another characteristic fragment ion at a mass of 208.2 appears (data not shown). Based on these observations, a very specific method for identification of LSD could be developed.

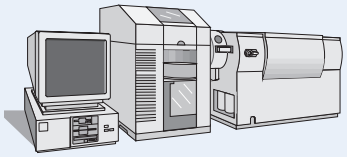
The total ion electropherogram (TIE) in Figure 1B shows the separation of a mixture of methamphetamine, cocaine and heroin. In the mass spectrum of the peak at 12.5 min the ion with mass 150.1 is the protonated molecular ion of methamphetamine. The ions at m/z 119.1 and 91.1 are fragment ions and are in greater abundance than the molecular ion. Methamphetamine undergoes CID at lower fragmentor voltages and hence is significantly fragmented at the 100 V used for this analysis.

In this experiment no fragment ions are observed for cocaine or heroin, only the protonated molecular ions at 304.1 and 370.1 m/z, respectively (mass spectra not shown). To obtain confirmatory fragmentation, and add specificity to the method for these two compounds, the fragmentor voltage would have to be increased. Alternatively a fragmentor ramp from 100 V to higher values would give fragmentation patterns for cocaine and heroin.

CE provides a selectivity that allows the analysis of these compounds using a single method. In combination with spectra and fragmentation patterns obtained from MS detection, this provides a rapid and unequivocal identification of these compounds in drug seizures.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent CE-MS Adapter Kit
- Agilent 1100 Series LC/MSD module with API Electrospray Source
- Agilent CE-ESI-MS Sprayer Kit
- Agilent ChemStation and CE-MS add-on software



Dat Phan and Peter Harrsch are senior application consultants at Agilent Technologies Company.

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

Application

Forensics, 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 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 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, however, 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 overcomes 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.

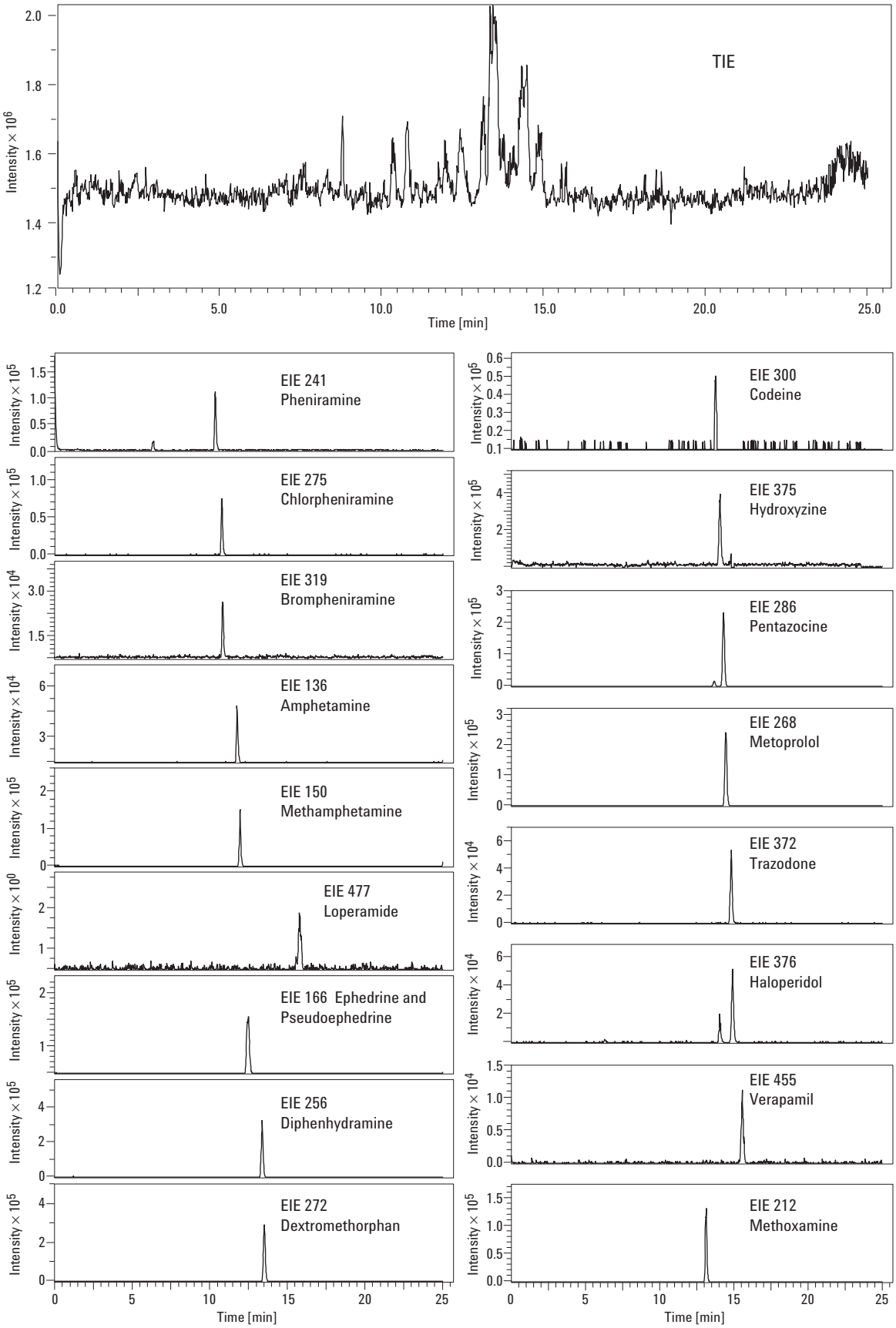


Figure 1. TIE and EIEs for extracted drug mix.

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.

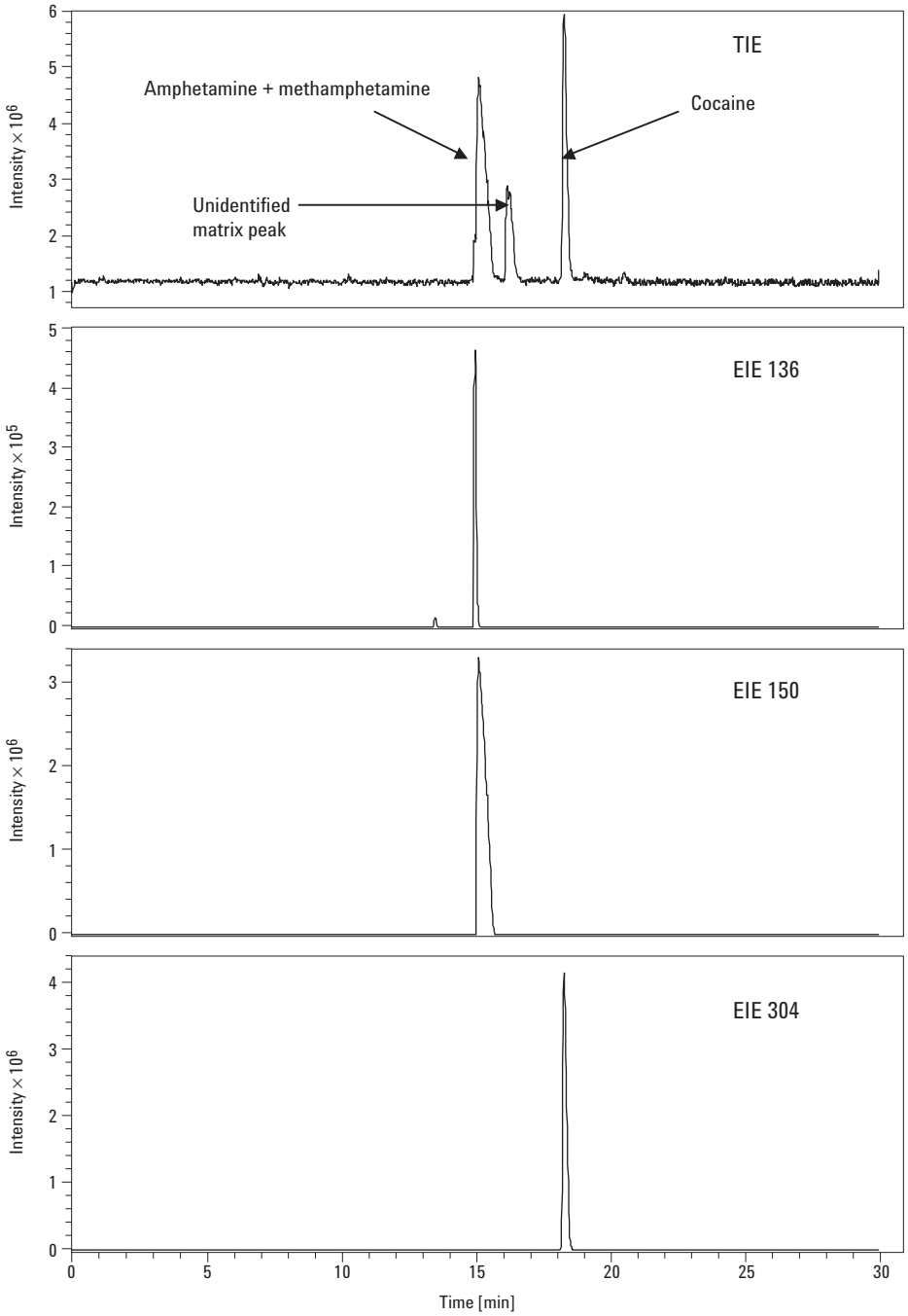


Figure 2. Case 1. Amphetamine, methamphetamine, and cocaine.

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.

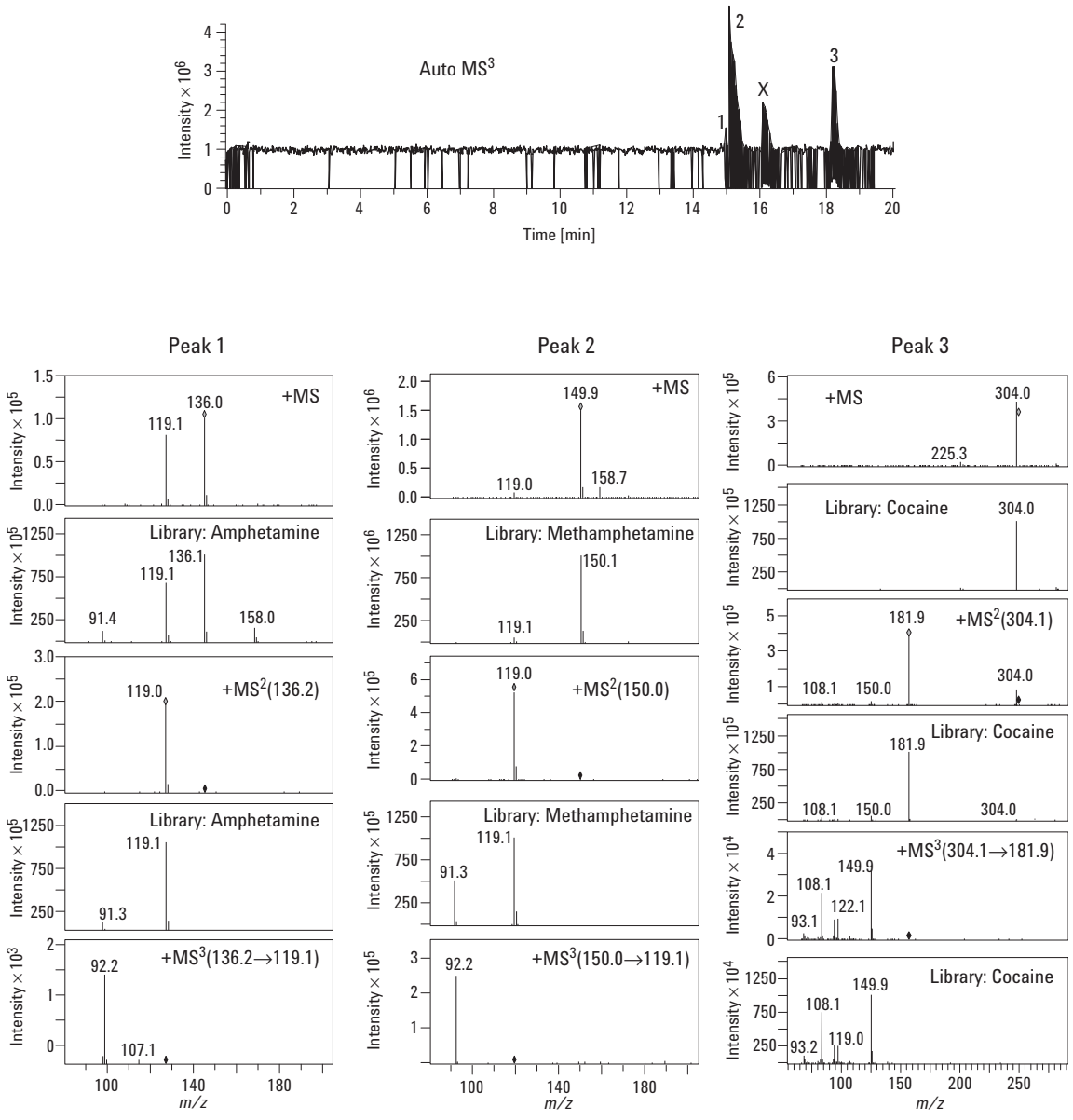


Figure 3. Case 1. AutoMS³ and library matches.

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.

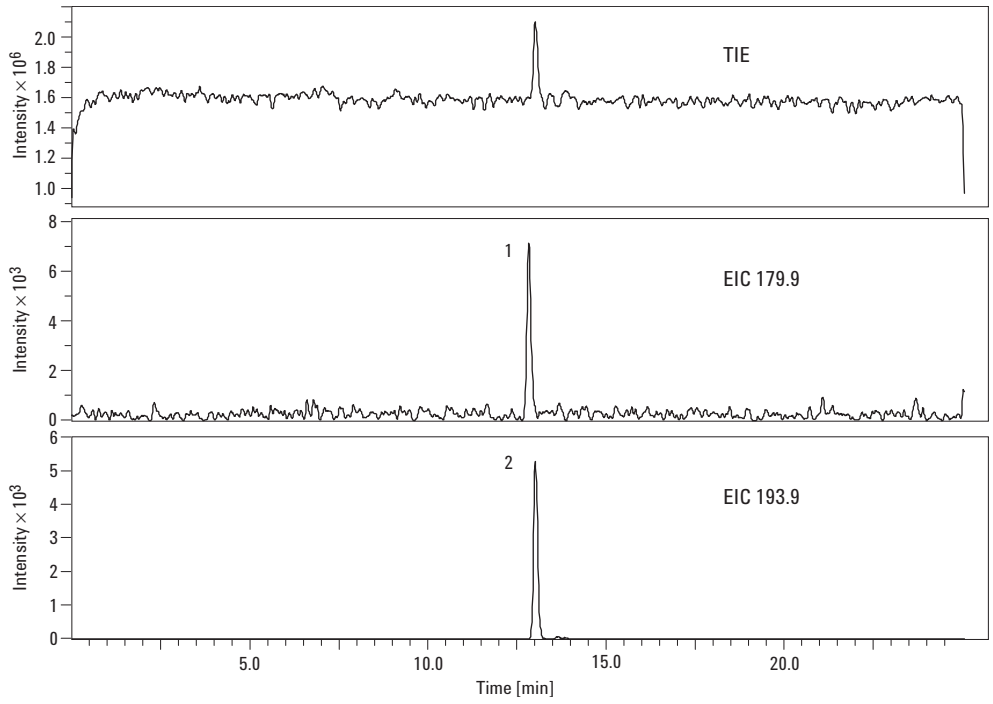


Figure 4. Case 2: MDA and MDMA.

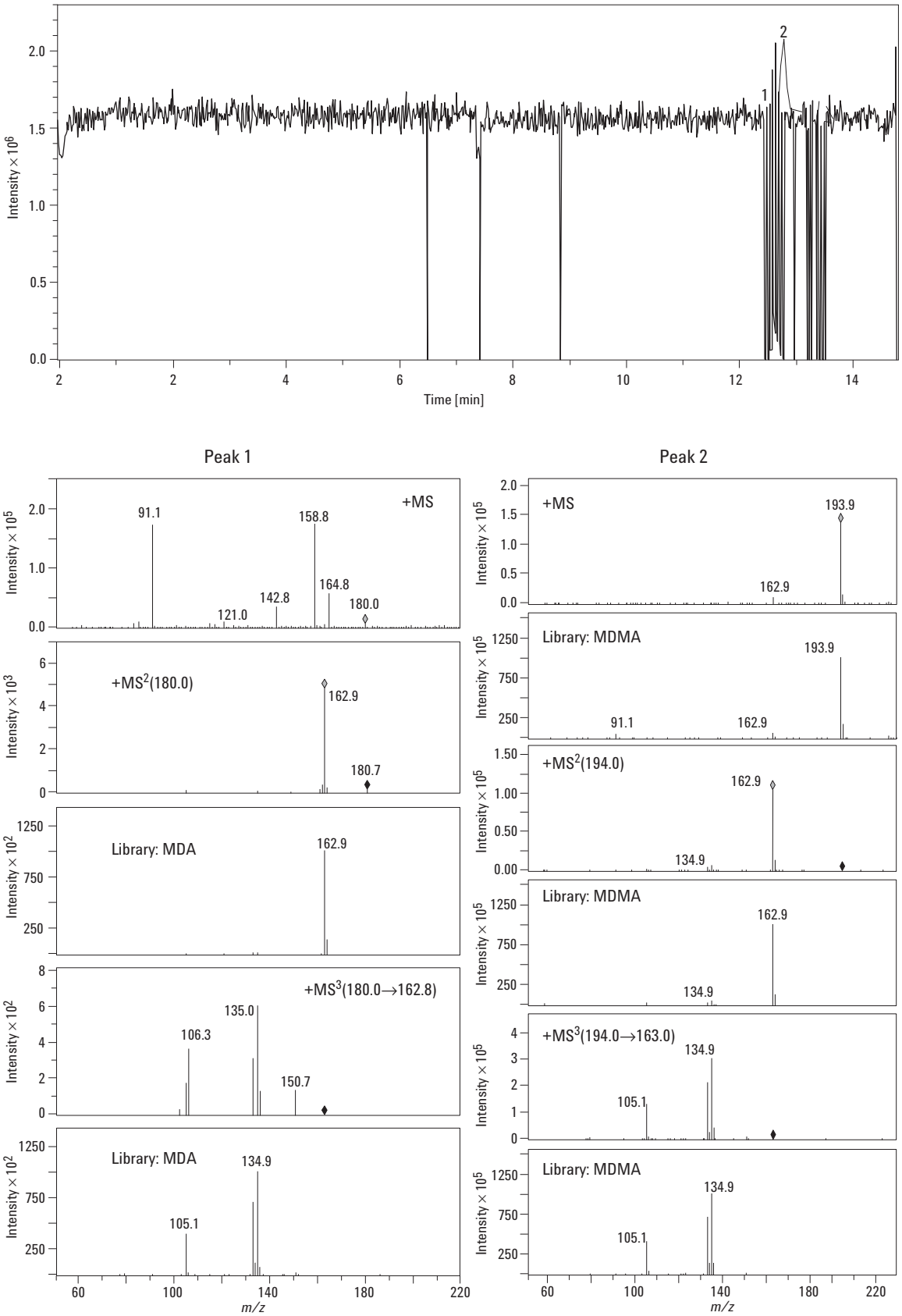


Figure 5 Case 2. Auto MS³ and library matches.

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.

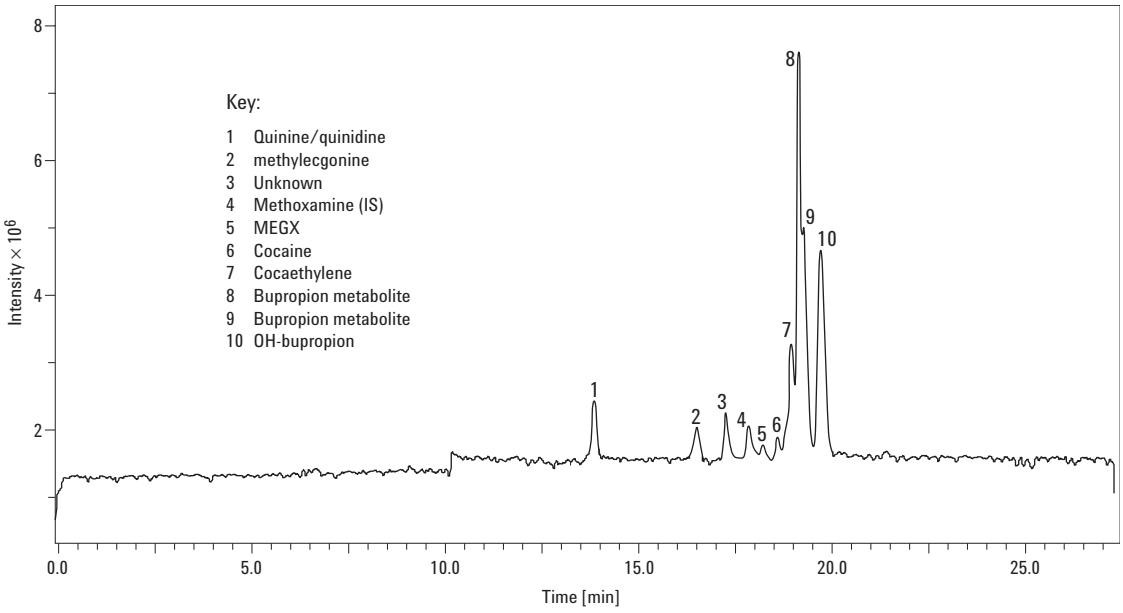


Figure 6. TIE of Case 3.

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 toxicological analyses. In addition to relatively sensitive detection, however, the capability of generating MS^n data permits reliable 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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Acknowledgements

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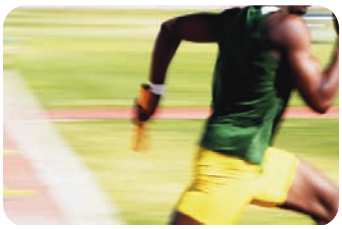
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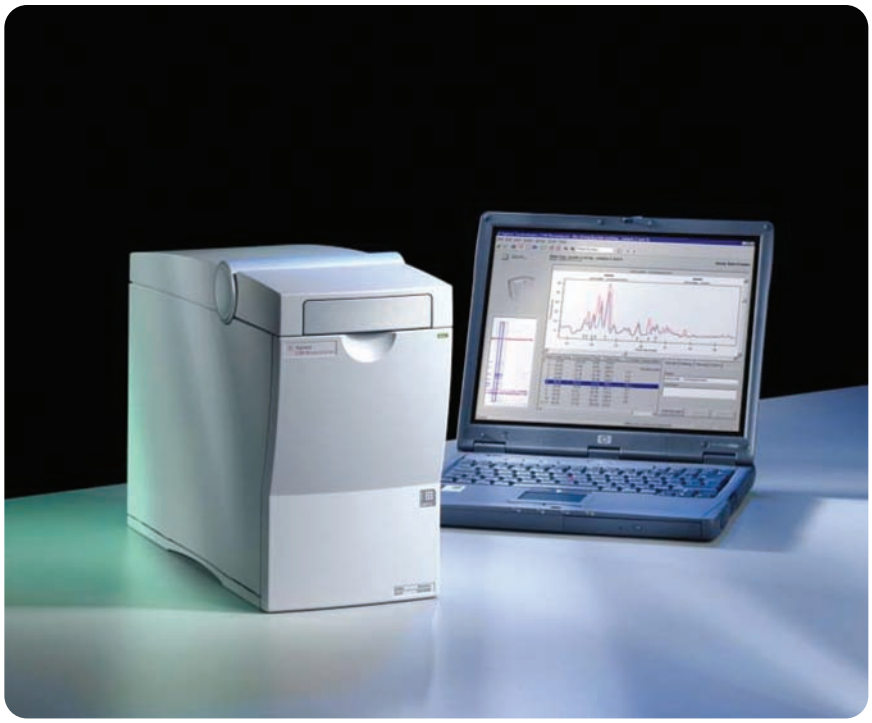






Applications by Instrumental Technique

2100 Bioanalyzer



Use of the Agilent 2100 Bioanalyzer and the DNA 500 LabChip in the Analysis of PCR Amplified Mitochondrial DNA

Application

Homeland Security/Forensics

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Abstract

Sequence analysis of PCR-amplified mitochondrial DNA (mtDNA) is quickly becoming an accepted tool for forensic testing. Establishing that these amplified products possess the proper size, purity, and concentration to be suitable for sequence analysis is a critical first step for forensic mtDNA applications. The Agilent 2100 bioanalyzer and DNA 500 LabChip have been shown to accurately and precisely measure both size and concentration of DNA fragments. In this application note, the capability of the bioanalyzer to rapidly and efficiently analyze mitochondrial DNA fragments amplified by PCR prior to sequence determination is demonstrated.

Introduction

The utility of mtDNA sequence analysis has seen enormous growth in recent years. In 1995 mtDNA analysis was certified by the Department of Defense as a reliable forensic tool [1]. Since that time, identification of American war casualties has been done primarily through mtDNA sequence analysis. The remains of victims from the September 11 terrorist attack on the World Trade Center bombing are also currently being analyzed by mtDNA sequence. In addition to these forensic applications, mtDNA is used in the study of both historical and anthropological samples. These studies include such samples as the heart of Louis XVII [2], the remains of Czar Nicholas II [3], 7000-year-old brain tissue, and the remains of a Neanderthal man.

Mitochondria are subcellular structures found in the cytoplasm of virtually all eukaryotic cells. These structures are believed to be the descendents of bacterial cells incorporated by primitive eukaryotic cells. Over time the bacterial cells developed a symbiotic relationship with their hosts and eventually became part of the hosts essential biochemical machinery. This is believed to be the reason mitochondria contain their own unique DNA, which encodes some of the proteins found in these structures.

Mitochondrial DNA was first sequenced in the lab of Fredrick Sanger in 1981. Human mtDNA is a small circular genome containing 16569 base pairs (bp). The current reference human mtDNA sequence is known as the Anderson or Cambridge reference sequence (CRS) (Genebank accession: M63933). This genome encodes for numerous polypeptides that are subunits of proteins involved in oxidative phosphorylation. Nucleotide sequences for 2 ribosomal RNAs and 22 transfer RNAs are also found in this genome. In addition to these coding sequences, the mitochondrial genome contains a noncoding region of 1100 bp known as a D-loop or control region. The D-loop contains sufficient sequence variation to be useful in human identity testing. The variation in sequence between unrelated individuals in this region ranges from

1%–3%. Most of the sequence variation is found in two sections of the control region, hypervariable region 1 (HV1) and hypervariable region 2 (HV2).

Mitochondrial DNA sequences are inherited directly from the mother. Unless a mutation has occurred, siblings and all maternal relatives have the same mtDNA sequence. Since mtDNA does not undergo recombination, maternal relatives dating back several generations can provide useful genetic samples. Such sequence information is often quite helpful in missing persons cases. In forensic cases mtDNA sequence is more often used for exclusion rather than identification of suspects, because multiple members of the same family all have the same mtDNA sequence.

Unlike the cell nucleus, which contains only one copy of genomic DNA, the cell cytoplasm may contain up to 1000 copies of mtDNA. Since each cell contains multiple mtDNA copies, only a few cells are required for sequence analysis. To carry out a sequence analysis on a mtDNA sample, it is usually necessary to extract the DNA and amplify the variable regions by polymerase chain reaction (PCR). Because the amplification process can convert even a few copies of target DNA into billions of copies, it is possible to do a sequence analysis from limited or badly degraded samples such as bone, teeth, or hair.

Analysis of PCR Amplified mtDNA

The number of PCR required to amplify the mtDNA HV regions depends on the age and condition of the sample. When mtDNA degradation is minimal, each HV region is amplified in a single PCR. These reactions yield products that are roughly 450 bp in length. Prolonged exposure to moisture, heat, and bacteria can result in significant degradation of the DNA, whereby the DNA is broken into smaller pieces. The average mtDNA fragment size in such degraded samples may be considerably shorter than 450 bp. Since the size of the amplification product cannot exceed the initial DNA target, amplifications on highly degraded samples are carried out in a series of shorter PCR steps, generally ranging in length from 100–200 bp.

Analysis of PCR amplicons has typically been done with agarose or acrylamide gels. These yield-gels require the user to run the unknown PCR sample adjacent to a set of predetermined standards of known concentration. The user attempts to match the band intensity of the PCR sample to one of the standards. The range of standard concentrations usually covers at least three orders of magnitude because it is also necessary to quantitate any secondary unintended PCR products. The accuracy of this type of analysis is minimal at best because errors in estimating concentration often exceed 100%. For this reason, the use of yield-gels to assess the ratio of target to nontarget PCR product is quite problematic.

The gel electrophoresis limitations can be overcome with the Agilent 2100 bioanalyzer, which is the first commercially available chip-based nucleic acid separation system. The Agilent 2100 bioanalyzer separates nucleic acid fragments in microfabricated channels and automates detection as well as online data evaluation. The Agilent 2100 bioanalyzer is connected to a PC for run control and automated data analysis.

Analysis of PCR products with the Agilent 2100 bioanalyzer has several important advantages compared to traditional gel electrophoresis. With a short separation channel and the application of a high electrical field, the speed of analysis is dramatically increased compared to gel electrophoresis. The instrument is equipped with a fluorescence detection system resulting in superior detection sensitivity. The prepackaged reagents and kits are used in conjunction with standardized protocols, and result in more reproducible data. These kits also help to improve the overall reproducibility between different runs, chips, and instruments. Compared to data assessment with gel-scanning systems, the amount of manual work is significantly reduced and even data analysis is performed in an automated manner. Sample and reagent consumption in the range of one to a few microliters minimizes exposure to hazardous materials and reduces the amount of waste material.

Several kits are available to analyze a variety of nucleic acid sample types. Because of its 25–500 bp size range, the DNA 500 LabChip® is well suited for the rapid determination of amplicon concentration and quality, as required for accurate mtDNA sequencing. The DNA 500 assay is capable of 5-bp resolution for 25 to 100-bp fragments, and 5% resolution for 100 to 500-bp fragments. The sizing accuracy has an error of less than 10% over this entire size range. Previous studies have shown that the

bioanalyzer is capable of detecting DNA fragments that cannot be identified on agarose gels. A comparison with gels stained with SYBR gold or ethidium bromide demonstrated that the bioanalyzer was 5 times more sensitive than SYBR gold and 25 times more sensitive than ethidium bromide staining. The bioanalyzer has consistently detected DNA at the 20 pg level [4].

The quantitative capability of the bioanalyzer is illustrated in Table 1. A DNA mass ladder containing three fragments with sizes of 100, 200, and 400 bp was used to verify quantitation accuracy. Concentrations of the ladder components were guaranteed by the supplier (Low DNA Mass™ ladder, Life Technologies, USA). For all three fragments the error in concentration measurement was less than 10% with a coefficient of variation of less than 15% [5].

Table 1. Quantitation Accuracy and Precision of the Agilent 2100 Bioanalyzer

	100 bp	200 bp	400 bp
Average [ng/µL]	0.73	1.53	3.02
Target [ng/µL]	0.80	1.60	3.20
Percent error	–8.28	–4.56	–5.62
STDV	0.08	0.11	0.20
CV	10.73	7.46	6.73

Requirements for PCR Amplified mtDNA

High quality sequencing data requires a single homogenous PCR product in a concentration range of 10–100 ng/mL. Laboratory procedures, in use in the FBI regional labs, currently stipulate that the amplified target mtDNA must be present in 10-fold excess above any unintended PCR products. Failure to meet that purity requirement means that the underlying sequence data from the secondary PCR products may render the target sequence unreadable or may even result in erroneous nucleotide base assignments. For this reason, an accurate determination of the concentrations of all the PCR products is critical in assessing the quality of the PCR sample.

Figure 1 shows an example of an amplified mtDNA sequence made from the HV1 region. Aside from a small primer dimer peak adjacent to the low molecular-weight marker, the PCR product contains only a single homogeneous 273 bp PCR product at a concentration of 44.1 ng/µL. Such a product is clearly suitable for mtDNA sequence analysis.

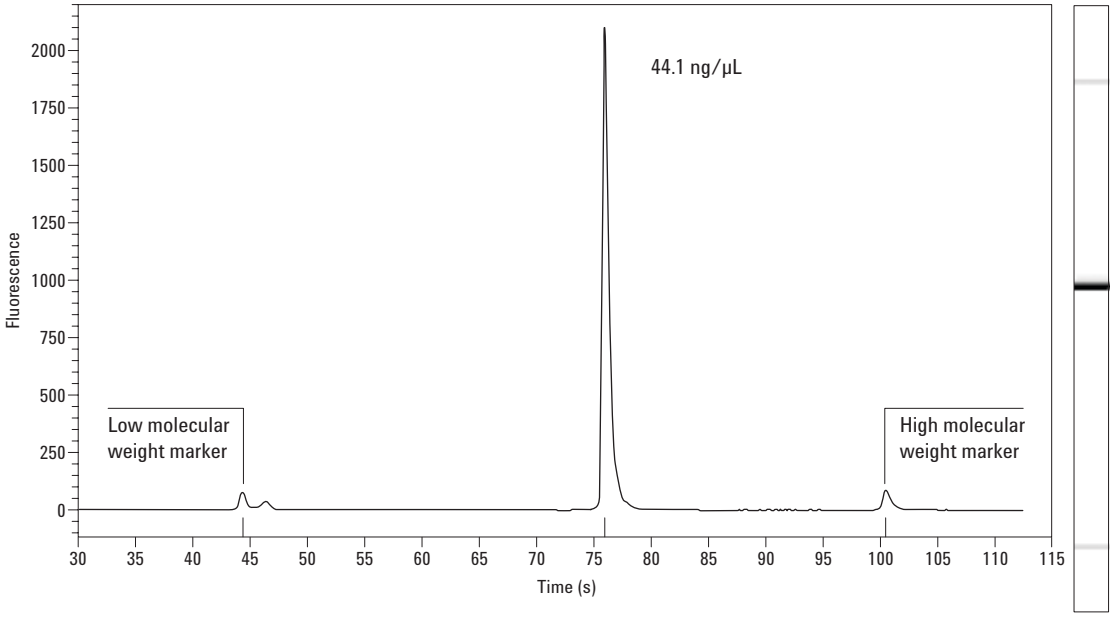


Figure 1. Electropherogram of PCR-amplified mtDNA from HV1 region analyzed on the Agilent 2100 bioanalyzer.

Figure 2 shows a mtDNA amplification product that is contaminated with a second PCR amplicon. The target PCR product is a 222 bp fragment with a concentration of 48.8 ng/µL. The unintended secondary product has a concentration of 10.2 ng/µL with a size of 69 bp. Since the concentration of the second product is greater than 10% of the target fragment, this PCR amplification would not be suitable for mtDNA sequencing under the current FBI 10:1 guideline.

Another problem frequently encountered in the amplification of mtDNA sequences occurs when a region that is rich in adenine (A) and thymine (T), is followed by a long string of guanines (G). When this sequence region is amplified the DNA strands can partially melt and then reanneal. With a long string containing n number of Gs, the reannealled DNA will occasionally lose its reference frame thereby incorporating an additional G. If this happens multiple times over the amplification process, significant concentrations of amplicons containing n+1 or n+2 Gs will be produced. This behavior is referred to as a G-stutter. When PCR products showing this G-stutter phenomenon are used in sequencing reactions, data downstream from the Gs is usually unreadable.

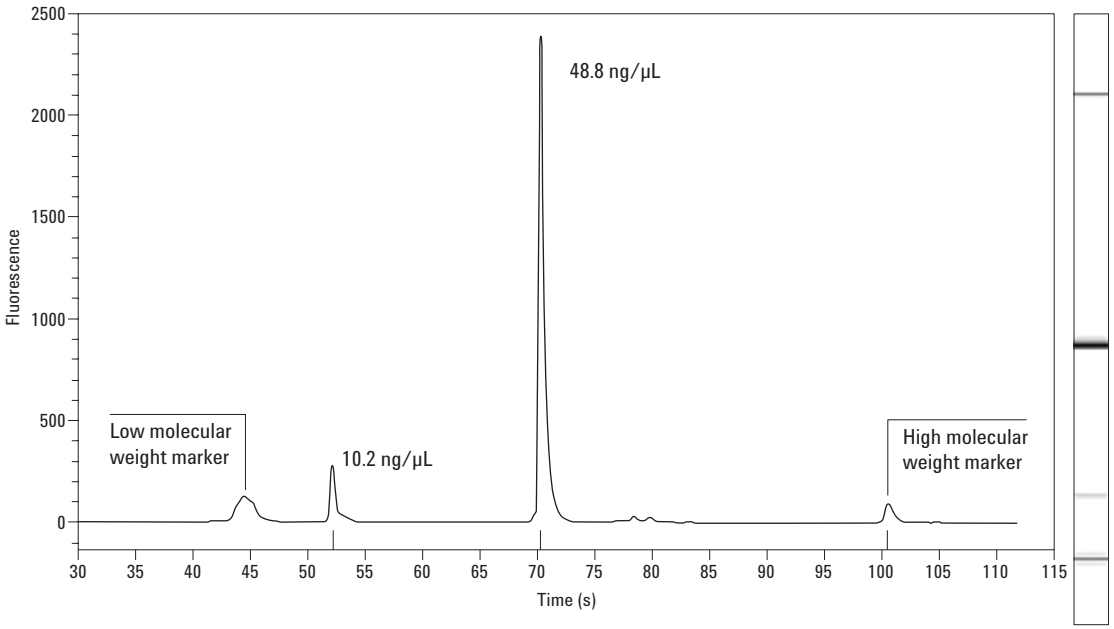


Figure 2. Electropherogram of PCR-amplified mtDNA from HV1 region showing the presence of a second unintended PCR product analyzed on the Agilent 2100 bioanalyzer.

In Figure 3A an electropherogram of a PCR amplified HV1 sequence showing this G-stutter behavior is shown. A fluorescence labeled sequencing product made from this PCR product will contain a mixed population of sequences containing fragments with n, n+1, and n+2 Gs. This mixed population will make the sequence data downstream from the G region virtually unreadable. Figure 3B shows an example of how the sequence data is degraded. A nucleotide base designation of N indicates that no sequence information could be obtained.

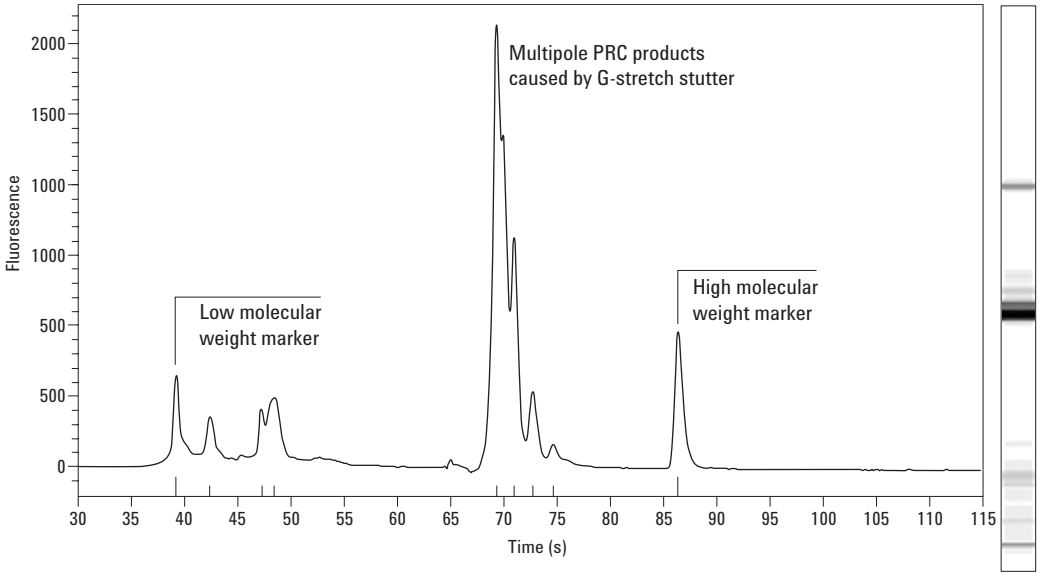


Figure 3A. Electropherogram of PCR-amplified mtDNA from the HV1 region showing the presence of multiple G-stutter products analyzed on the Agilent 2100 bioanalyzer.

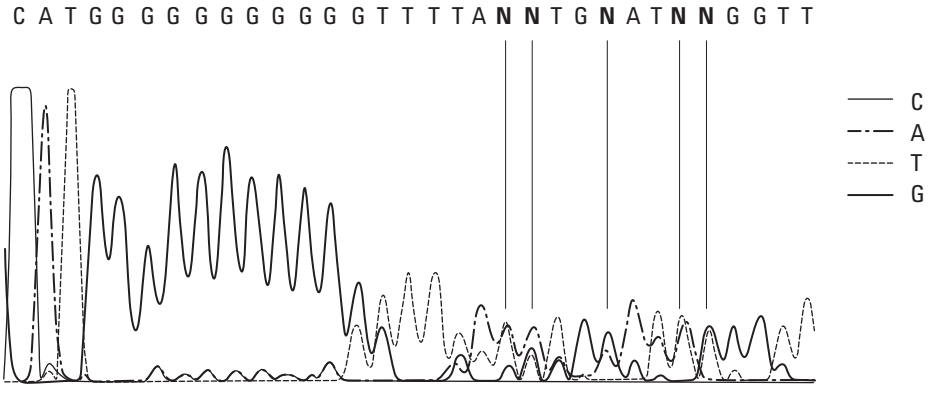


Figure 3B. Typical sequencing data output for mtDNA PCR products using amplified products with a G-stutter. Note the loss of readable sequence after the run of Gs.

In Figure 4A an electropherogram of a PCR amplification of the same HV1 sequence relatively free of artifacts is shown. This PCR amplicon is a single homogeneous product and is quite suitable for DNA sequence analysis. Figure 4B shows a section of sequence data generated from such a PCR product. Note that unlike the sequence from Figure 3B, the sequencing output downstream from the consecutive string of Gs is clear and unambiguous.

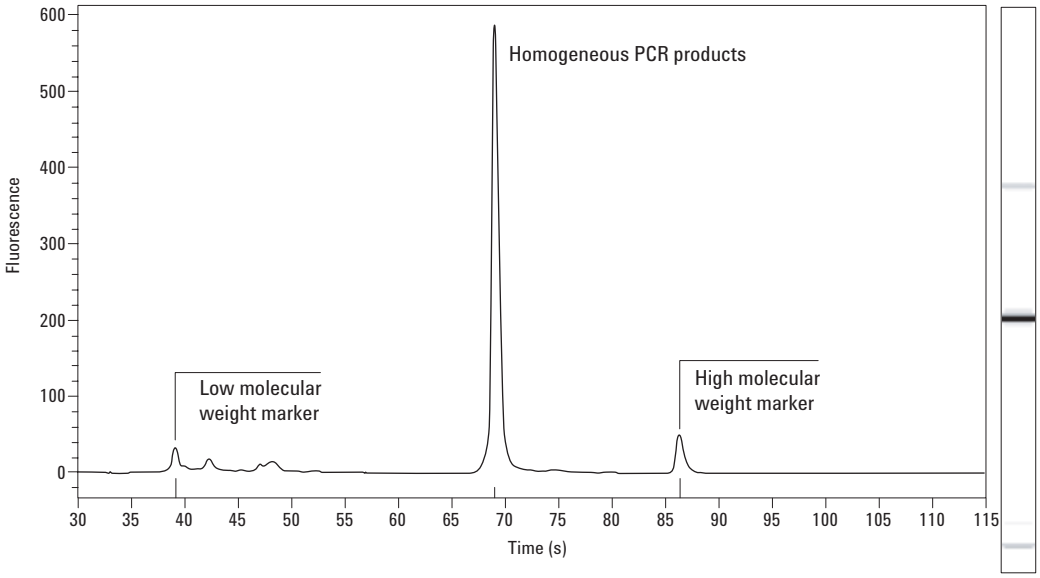


Figure 4A. Agilent 2100 bioanalyzer electropherogram of PCR amplified mtDNA sequence from the HV1 region showing a single homogeneous PCR product.

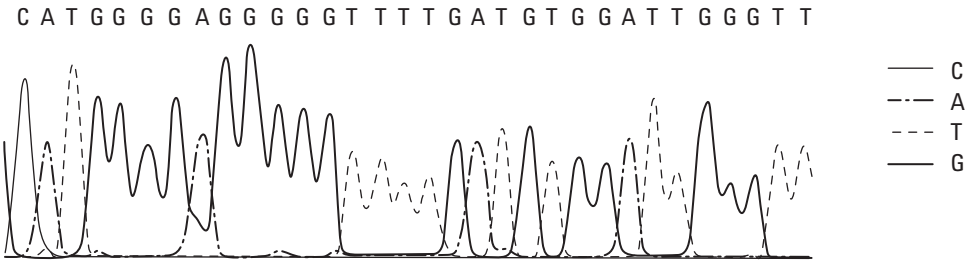


Figure 4B. Typical sequencing data output for mtDNA PCR products using amplified mtDNA with a single homogeneous product. Note that the sequencing data output shows no loss of readable sequence after the run of Gs.

Conclusion

Mitochondrial DNA analysis is quickly growing in popularity as evidenced by the proliferation of the technique within the forensic community. The Agilent 2100 bioanalyzer facilitates this analysis by rapidly providing accurate size and concentration profiles of mtDNA amplification products. Through the use of internal and external DNA markers, the Agilent 2100 bioanalyzer provides excellent quantitative analysis and sizing of PCR products. This technology enables the identification of high quality amplified mtDNA fragments and quantifies the yield of these fragments to assure reliable results in the subsequent sequencing analysis. The DNA 500 assay reliably performs DNA separations in the range of 25 to 500 base pairs. Because of the bioanalyzer's excellent sensitivity and resolution, it is capable of resolving and accurately quantitating secondary PCR products. This feature is particularly important when the user is required to demonstrate that the target mtDNA amplicon is present in 10-fold excess above any secondary unintended PCR products.

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Using the 2100 Bioanalyzer to Optimize the PCR Amplification of Mitochondrial DNA Sequences

Application

Genomics

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Abstract

In polymerase chain reaction (PCR) product analysis, a comparison of target amplicon yield to such PCR artifacts as primer-dimers and misprimed secondary products is essential in PCR assay optimization. This process requires accurate and reproducible measurement of amplified DNA products over a wide range of sizes and concentrations. The 2100 Bioanalyzer is ideally suited to provide rapid quantitative analysis of all products and byproducts in a PCR reaction, thereby facilitating the development of an optimized PCR amplification.

Introduction

Optimization of PCRs can often be a tedious and time-consuming process, particularly when the amplified segments contain long stretches of high or low melting temperature sequences. The objective of the optimization process is to minimize secondary products, such as primer-dimers and mispriming events, while maximizing the yield of the target amplicon. Analysis of the amplification products by gel electrophoresis is not particularly helpful because it is difficult to obtain accurate quantitative information about secondary PCR products. The absence of such quantitative information can delay and confound the optimization process and can even result in the final PCR reaction being run under conditions that do not produce the maximum selectivity and yield.

The 2100 Bioanalyzer is an ideal tool to address problems of PCR process optimization. Not only can secondary products be accurately and reproducibly measured, but secondary products that are similar in size to the desired target can also be resolved and quantified [1]. The ability to quantitate all of the reaction products provides the user with the opportunity to accurately characterize the mathematical relationship between specific reaction parameters and the overall efficiency of the PCR reaction. These relationships can then be used to facilitate the identification of the optimal reaction conditions.

Mitochondrial DNA (mtDNA) is a good example of a target sequence that can present a number of PCR amplification difficulties. Human mtDNA is a small (16,569 bp) circular genome containing 37 genes found in cellular cytoplasm. The mitochondrial genome contains a noncoding region of 1100 bp known as a D-loop or control region. Within the D-loop is found two sections of highly variable DNA sequence. These regions, hypervariable region 1 (HV1) and hypervariable region 2 (HV2), contain sufficient sequence variation to be useful in human identity testing [2, 3].

When a sufficient amount of intact genomic DNA (gDNA) cannot be extracted from a sample, mtDNA becomes the next analysis tool because the cytoplasm of a single cell may contain up to 1000 copies of mtDNA. MtDNA is often extracted from small or badly degraded samples such as bone, teeth, or hair and amplified by PCR. The amplified product is used for DNA sequence analysis.

Since sample degradation decreases the mean DNA fragment size, the number of PCRs required to amplify the HV regions depends on the age and condition of the sample. If the DNA degradation is minimal, each HV region can be amplified in a single PCR. Highly degraded samples require the HV regions to be amplified in a series of PCR reactions of 100 bps–200 bps. Since these samples require a variety of amplification strategies, amplification of mtDNA sequences is a relevant and important example of how the 2100 Bioanalyzer may be used to facilitate the optimization of specific PCR amplifications.

Methods

For the purpose of this study, the effect of pH, Mg concentration, and Taq polymerase activity, on the amplification efficiency of four mitochondrial DNA sequences was evaluated. To insure that variations in sample matrix did not influence the results, a bulk extraction of DNA was made at the beginning of the study. This extracted DNA was diluted and aliquoted out to provide all the DNA samples for the evaluation. The extraction protocol is described below.

DNA Preparation

1. Add 10 hairs with roots intact to 0.2 mL of extraction buffer and incubate for 3 hours at 56 °C.
2. Vortex and heat to 95 °C for 8 minutes.
3. Centrifuge mixture for 5 min at 15,000g.
4. Remove 100 µL and combine with 900-µL PBS for PCR amplification.
5. Use 5 µL of diluted extracted sample/PCR

Extraction Buffer

The components of the extraction buffer were as follows: 6.0% Chelex 100 in 0.137 M NaCl, 0.0027 M KCl, 0.010 M Na_2HPO_4, 0.0018 M KH_2PO_4, at pH 7.4.

The Invitrogen PCR Optimizer Kit was used for the PCR optimization evaluation. This kit contains 10 buffers ranging in pH from 8.5 to 9.5 and Mg levels from 1.5 to 3.5 mM. These individual reaction buffers were evaluated in the amplification reactions of four mtDNA segments. The description of the buffers is shown in Table 1.

Table 1. Buffer Composition

Component	1× Composition
Buffer A	1.5-mM $MgCl_2$, pH 8.5
Buffer B	2.0-mM $MgCl_2$, pH 8.5
Buffer C	2.5-mM $MgCl_2$, pH 8.5
Buffer D	3.5-mM $MgCl_2$, pH 8.5
Buffer E	1.5-mM $MgCl_2$, pH 9.0
Buffer F	2.0-mM $MgCl_2$, pH 9.0
Buffer G	2.5-mM $MgCl_2$, pH 9.0
Buffer H	3.5-mM $MgCl_2$, pH 9.0
Buffer I	1.5-mM $MgCl_2$, pH 9.5
Buffer J	2.0-mM $MgCl_2$, pH 9.5

In addition to the reaction buffers, the amplification reactions were also evaluated at three levels of Taq polymerase: 1, 1.5, and 2 units/reaction.

Primer Selection

The primer site selection was based on the Armed Forces DNA Identification Laboratory suggested sites for amplification of badly degraded remains [4]. In some cases the priming site was shifted slightly to better match the melting temperature, T_M, of all the primers. The primer locations are located in the HV regions of the D-loop in the mtDNA genome. Two sets of primers were used to cover HV1 and an additional two sets to cover HV2. The primer site locations are illustrated schematically in Figure 1.

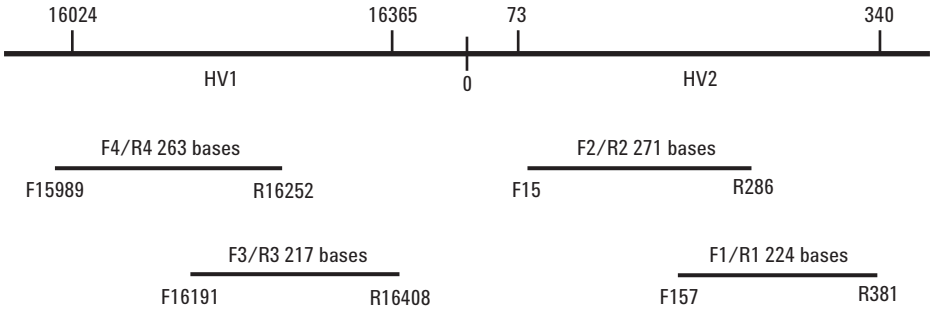


Figure 1. Primer site selection: Base position is assigned from the Anderson reference sequence [5].

Each of the four amplifications was evaluated for yield of primary target and secondary artifact products. Optimum primer concentration, 0.4 µM, and annealing temperature, 55 °C, were determined in a previous study and will not be described in this note.

Results

Electropherograms showing typical product profiles of the four mtDNA amplification reactions are shown in Figure 2.

In Figure 2A two peaks can be seen in size region of the expected target. This phenomenon is known as C-heteroplasmy and is indicative of two sequence groups with a one base variation in the length of a run of Cs within a single mtDNA sample. Figures 2B and 2C both contain single homogenous amplification products and are suitable for sequence analysis. In Figure 2D, an even more extreme case of C-heteroplasmy is seen. Sequencing of amplicons 2A and 2D would not yield any useful sequence data downstream from the C-heteroplasmy.

Since the C-heteroplasmy is the result of a mixed sequence population within a single sample, this condition cannot be improved by any of the modifications in amplifications conditions discussed in this application note. This problem is typically overcome by selecting an additional priming site downstream from the run of Cs.

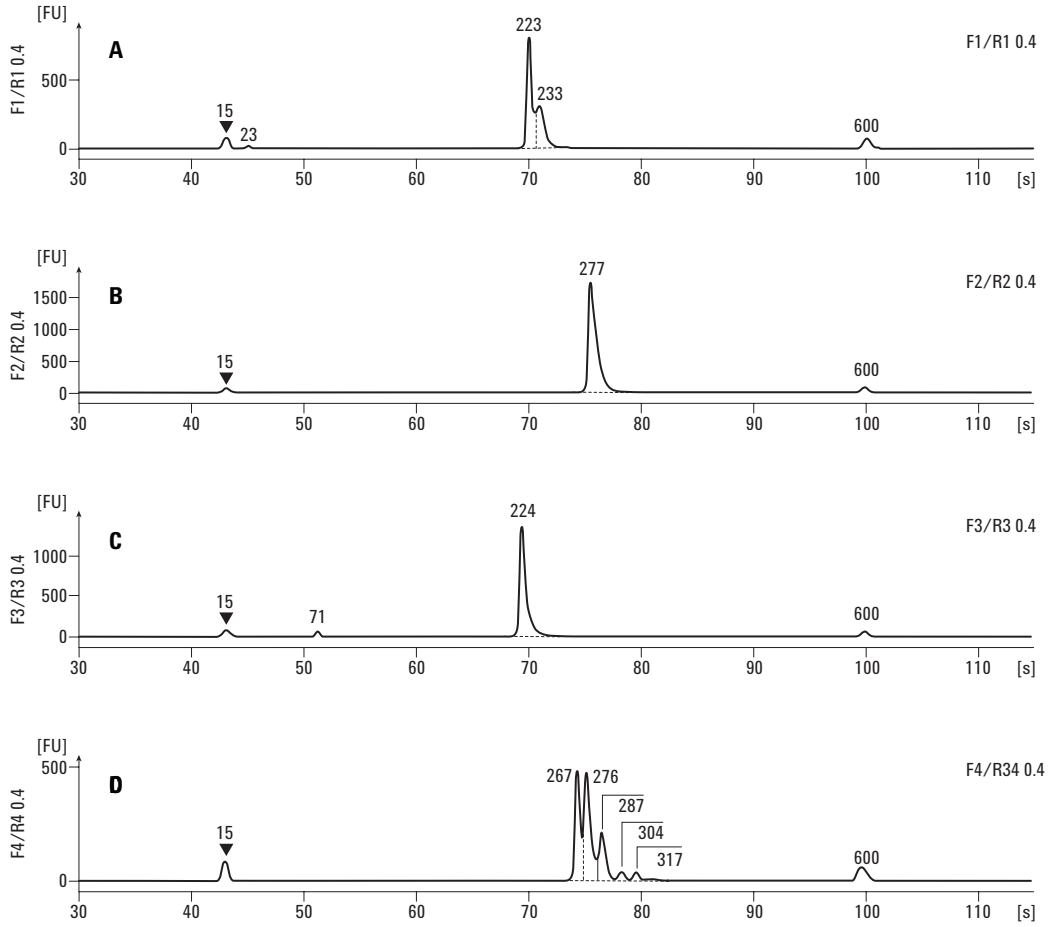


Figure 2. 2100 Bioanalyzer electropherograms of the four mtDNA amplifications; (A) F1/R1 - 224 bp, (B) F2/R2 - 271 bps, (C) F3/R3 - 217 bps and (D) F4/R4 - 263 bps.

To characterize the effect of [Mg] on amplification yield, concentrations of Mg ranging from 1.5 to 3.5 mM were examined at three pH levels, 8.5, 8.7, and 9.0. The pH 8.7 reaction buffer was prepared by mixing equal amounts of pH 8.5 and 9.0 reaction buffer at a fixed Mg level. The influence of Mg concentration on the amplification yield for the four mtDNA amplification products can be seen in Figure 3.

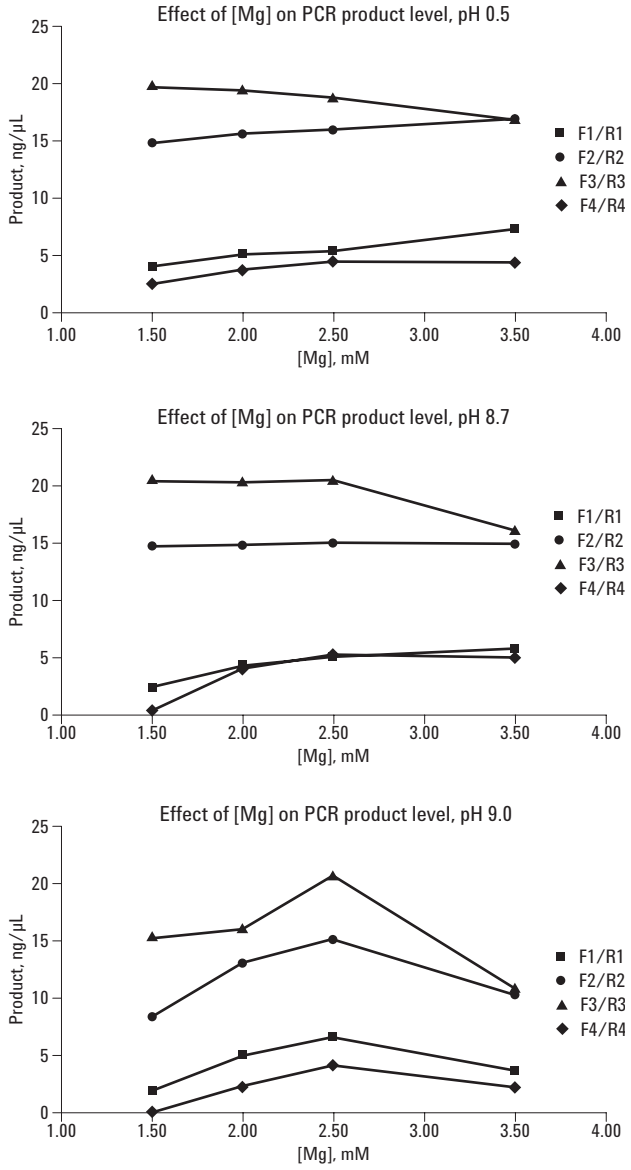


Figure 3. Influence of Mg level on product yield.

It is readily apparent from the yield graphs that the effect of Mg is both pH and sequence dependent. At pH 8.5 three of four mtDNA products show a continuous increase in yield as the Mg level is increased. For two of these amplification reactions, F3/R3 and F4/R4, the level of artifact products increases even more rapidly over the same Mg concentration range (data not shown). When the pH is raised to 8.7, only the F3/R3 and F4/R4 amplicons show increased yields with increasing Mg concentration. At pH 9.0 all four amplicons show a distinct maximum yield at 2.5-mM Mg.

The effect of pH at a single Mg level was evaluated for a pH range of 8.5 to 9.5. Figure 4 shows the effect of this pH range at a Mg level of 2.0 mM.

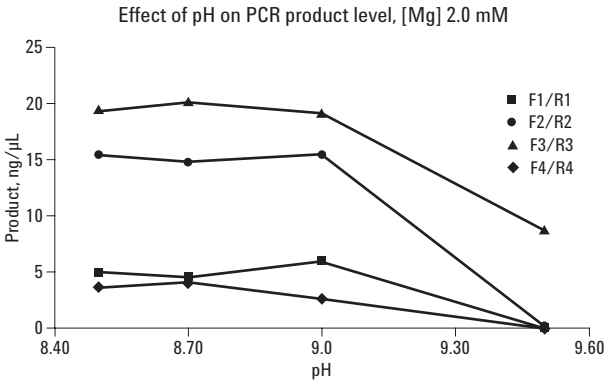


Figure 4. Reaction buffer pH level influence on product yield.

For 2.0-mM Mg the maximum yield for all four amplicons is seen at both at pH 8.5 and 8.7. A significant decrease in yield of one amplicon, F4/R4, is observed at pH 9.0. At pH 9.5 all four amplicons show a profound loss in yield.

The effect of Taq activity on the production of target and artifact amplicons was studied in buffer C, which contains 2.5-mM Mg at pH 8.5. The results are shown in Figure 5.

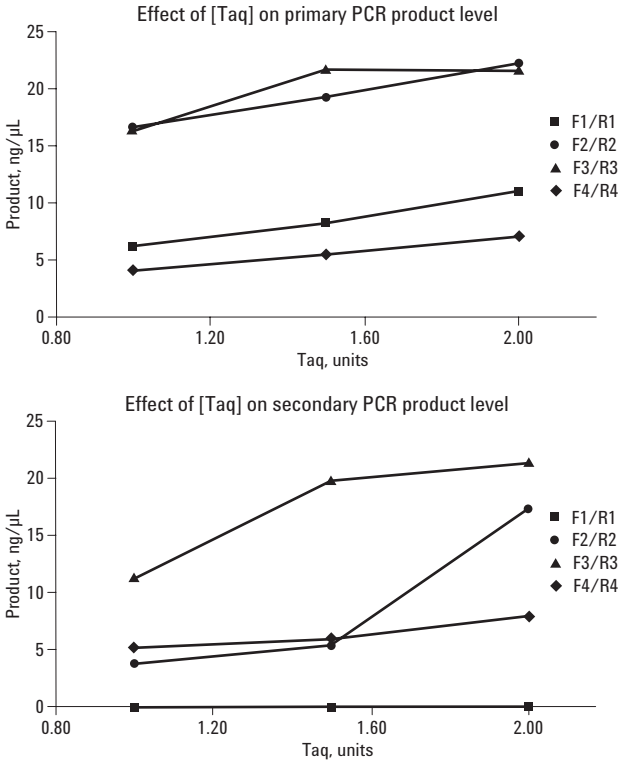


Figure 5. Influence of Taq activity on primary and secondary product yields.

The observation that target yield increases with increasing Taq activity is not surprising. However, note that the rate of artifact production increases even more rapidly for three of the amplification products. An examination of the target:artifact ratio shows that the highest ratio is obtained when only one unit of Taq is used per reaction.

Conclusion

In optimizing reaction conditions for a group of amplifications, some compromise of reaction parameters will invariably be required. This compromise requires an accurate determination of both target and artifact products for all of the PCR reactions. For the four amplicons in HV1 and HV2 of the mtDNA genome, the highest quality target amplification was produced at pH 8.7 with a Mg level of 2.0 mM, using one unit of Taq polymerase per reaction.

The Agilent 2100 Bioanalyzer facilitated this PCR optimization process by providing rapid quantitative analysis of both mtDNA amplification products and PCR artifacts. The large dynamic linear range made it possible to compare target and artifact yields even when their concentrations were more than a decade apart. Accurate characterization of the relationship between such reaction parameters as pH, Mg concentration and Taq activity, and the relative yields of target products and artifacts, made it possible to identify the reaction conditions that produce the highest relative yield of sequencing quality PCR target amplicon.

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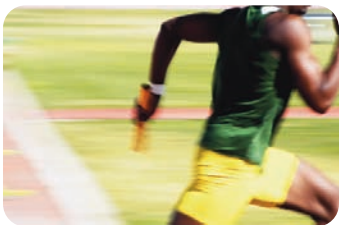
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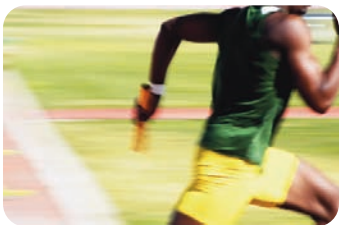




Applications by Sample Matrix





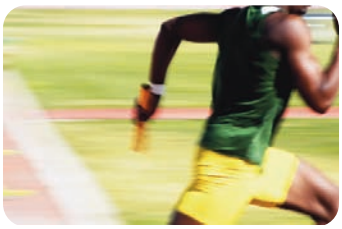


Applications by Sample Matrix

Blood





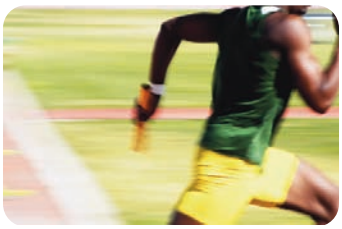


Applications by Sample Matrix

Urine





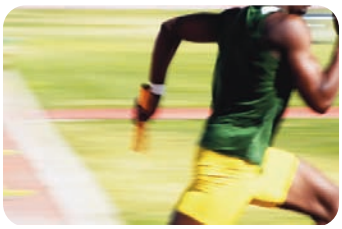


Applications by Sample Matrix

Alternative Matrices





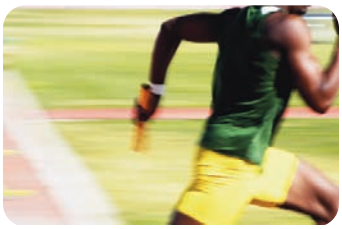


Applications by Sample Matrix

Trace Analysis





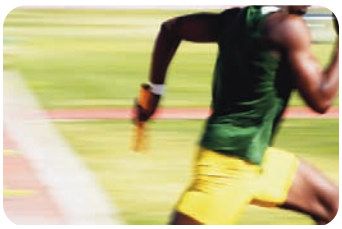


Applications by Sample Matrix

DNA



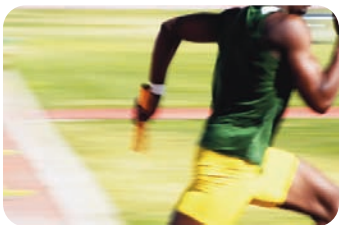




Applications by Target Analyte





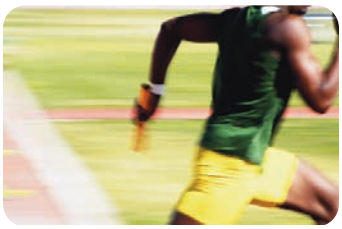


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Benzodiazepines





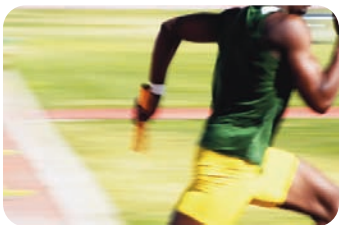


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Ethylglucuronide





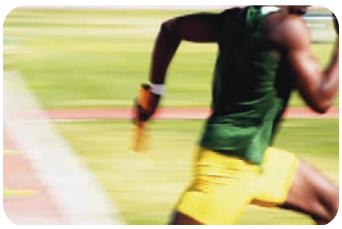


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Amphetamines





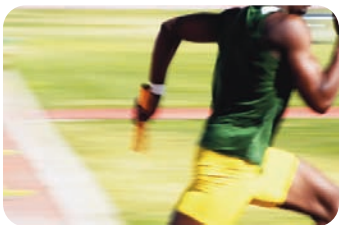


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β2-Agonists





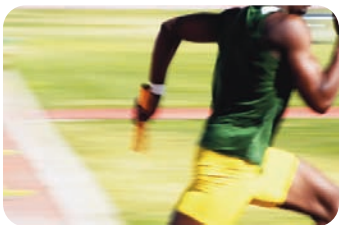


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Drugs of Abuse





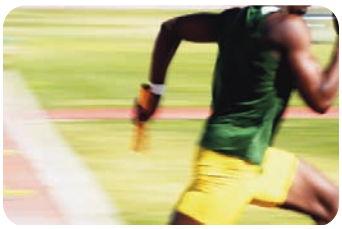


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Cannabinoids





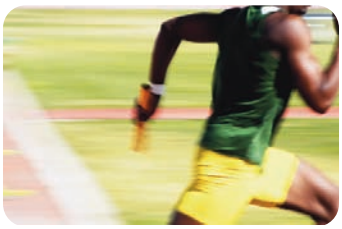


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Cocaine





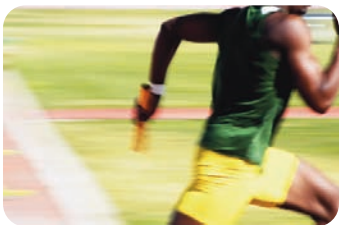


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Immunosuppressants





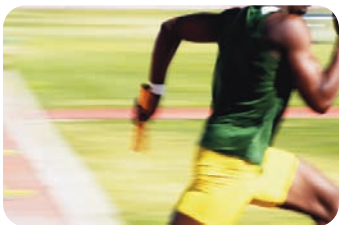


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Mitochondrial DNA





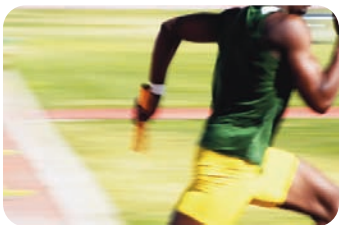


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Opiates





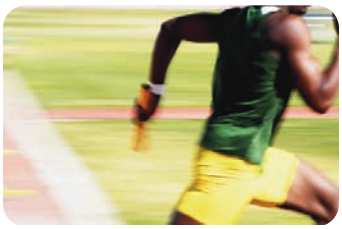


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Steroids





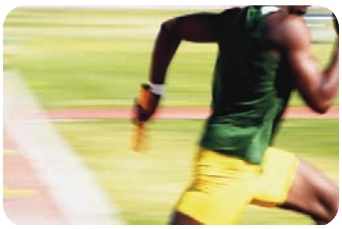


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Volatiles





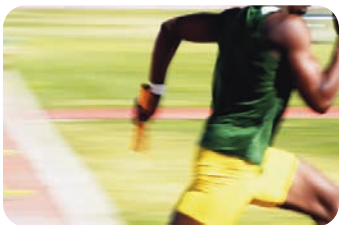


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Trace Analysis





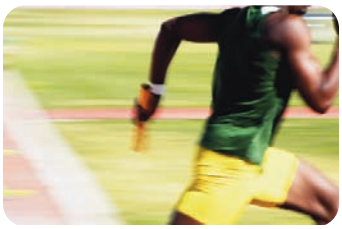


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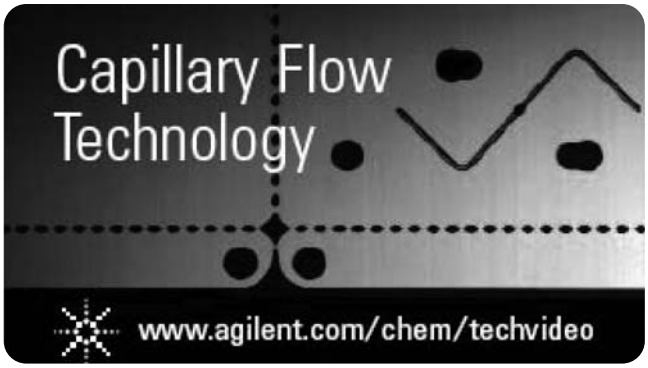
Metals Analysis







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