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

Application Note

Toxicology

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Abstract

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

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

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

Agilent Technologies

Introduction

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

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

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

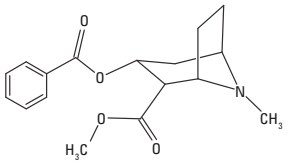
This work also represents the combined collaboration of three application chemists at Agilent and three professional forensic toxicologists. Some 50 samples, calibrators, and blanks were prepared: the postmortem samples by RTI International and the DUID samples by the University of Miami. Over three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

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

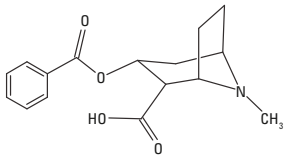
One mL of whole blood was used for each sample, with five point calibration curves generated for quantification of real case samples. Compounds analyzed in postmortem and DUID blood are shown in Figures 1a and 1b, respectively.

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

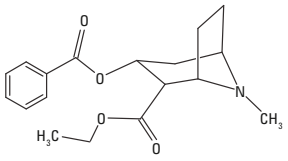
The LC conditions were consistent among all five LC/MS instruments using the same mobile phases, columns, column temperature, flow rate, and autosampler temperature. In fact, most of the work was done using two LC systems on carts moved between the various instruments.



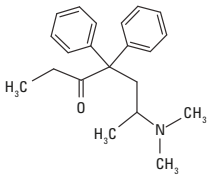
Cocaine, M+H$^+$ = 304.1543
$C_{16}H_{19}NO_4$



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

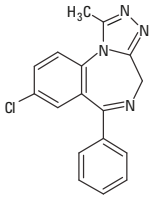


Cocaethylene, M+H$^+$ = 318.1700
$C_{18}H_{23}NO_4$

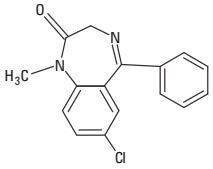


Methadone, M+H$^+$ = 310.2165
$C_{21}H_{27}NO$

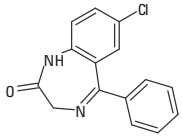
Figure 1a. Structures, chemical formulas, and exact masses of the protonated forms of the compounds analyzed in postmortem blood.



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



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



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

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

Experimental

Sample Preparation

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

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

Table 1. Calibration Levels for Quantification of Each Compound

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

LC/MS Method Details

LC Conditions (used with all MS analyzers)

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

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

Column temperature: 50 °C

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

Flow rate: 0.25 mL/min

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

Gradient:

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

Stop time: 10 min
Post run: 2 min

Common MS Conditions (related to ionization source)

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

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

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

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

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

Individual MS Conditions (related to analyzer)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results and Discussion

Single Quadrupole Mass Spectrometer

Postmortem Blood

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

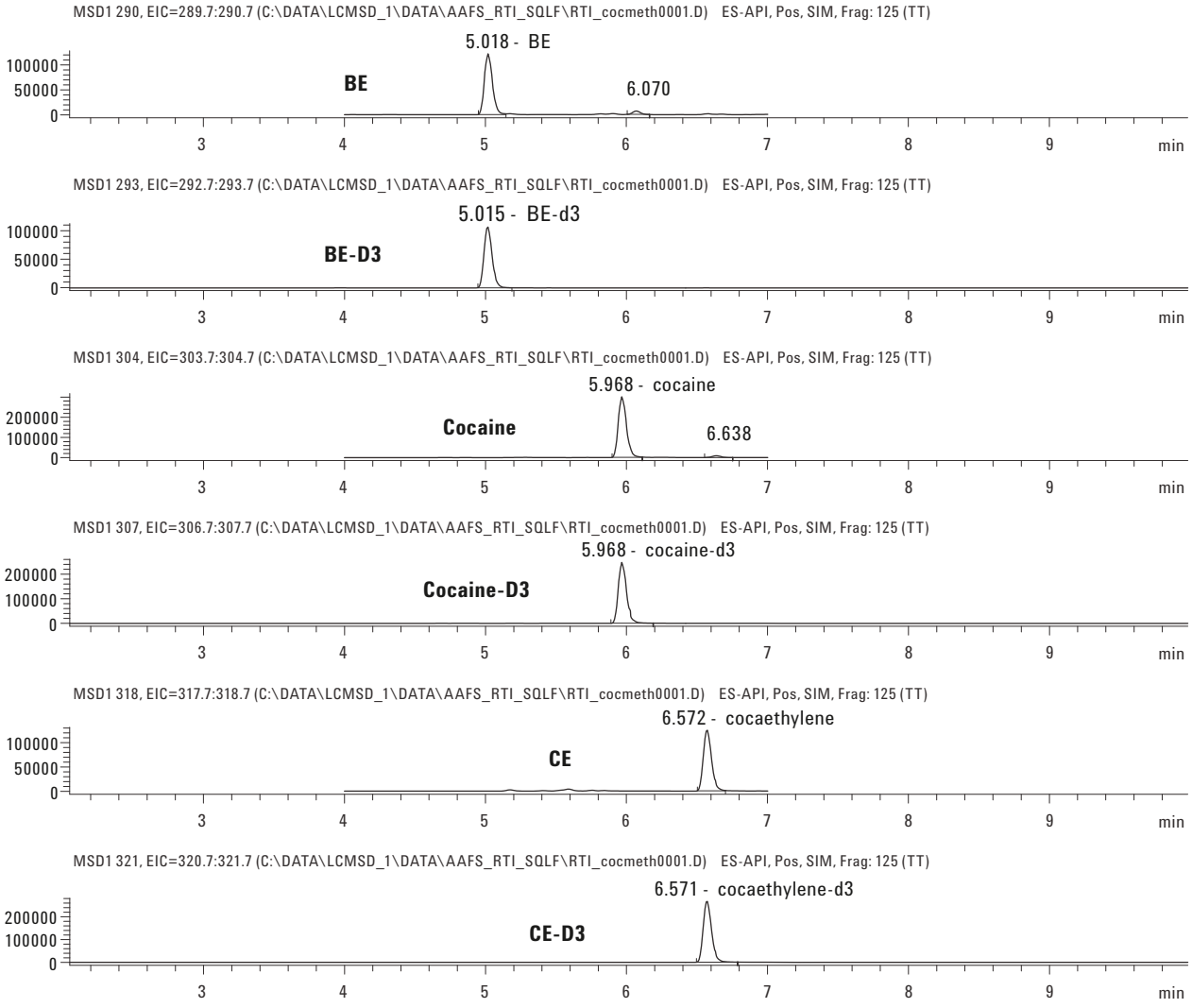


Figure 2. Compound chromatograms at the lowest calibrator of 25 ng/mL (BE and cocaine) and 10 ng/mL (CE) obtained using selected ion monitoring.

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

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

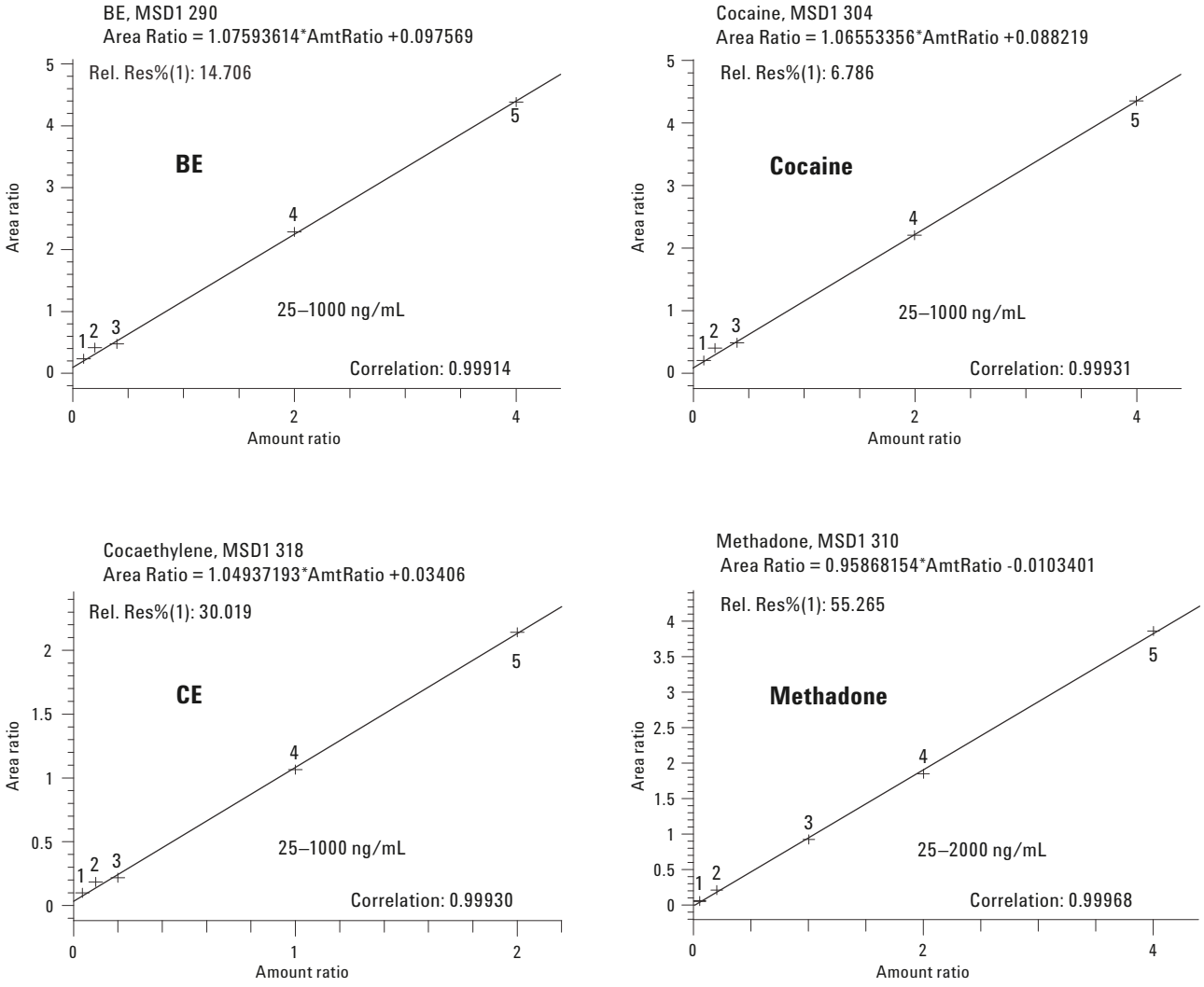


Figure 3. Calibration curves for compounds analyzed in postmortem samples: BE and cocaine (25 to 1,000 ng/mL); CE (10 to 500 ng/mL); and methadone (25 to 2,000 ng/mL).

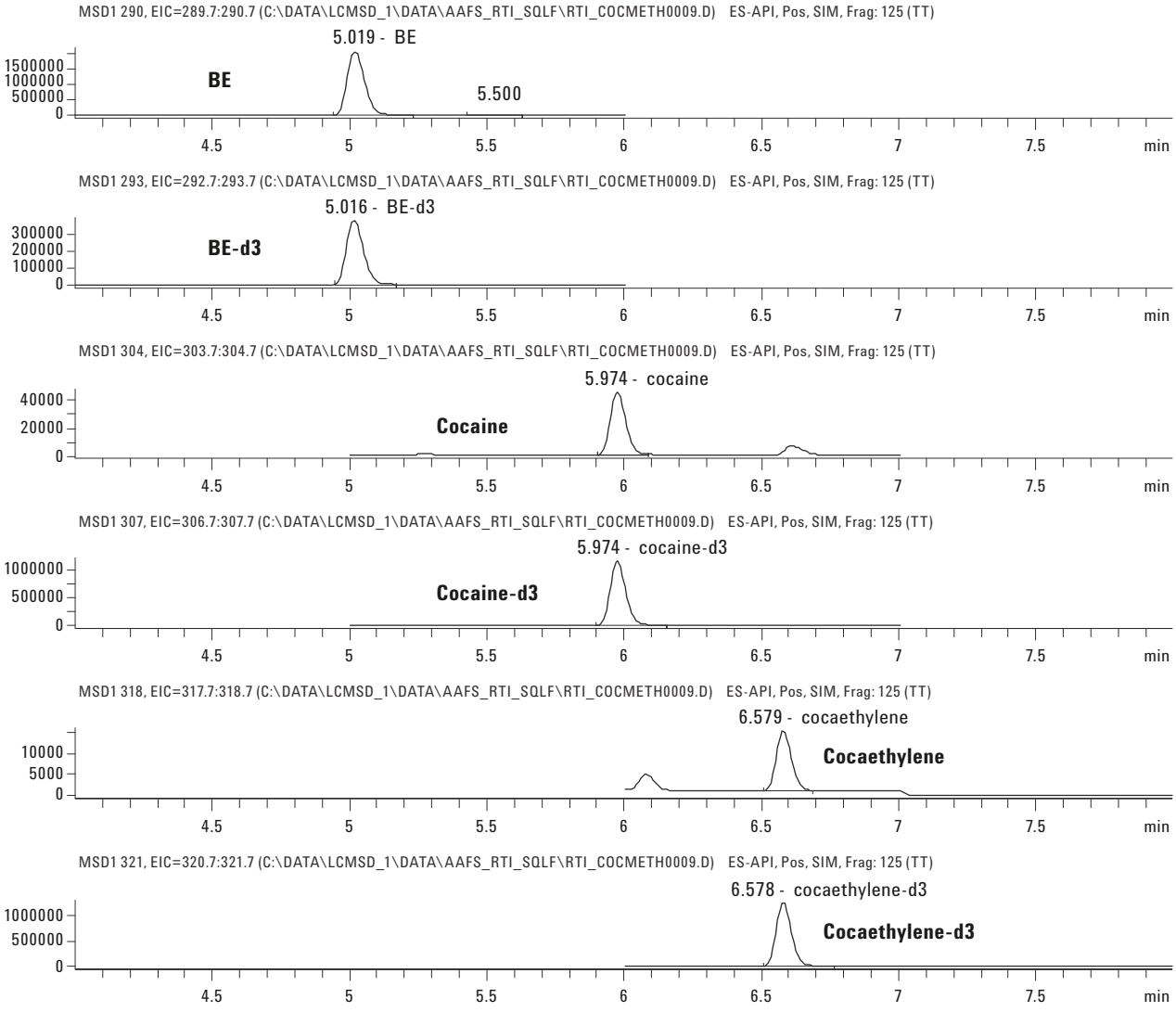


Figure 4. Postmortem cocaine case sample: BE 1,253 ng/mL; cocaine 8.8 ng/mL; and CE 2.7 ng/mL.

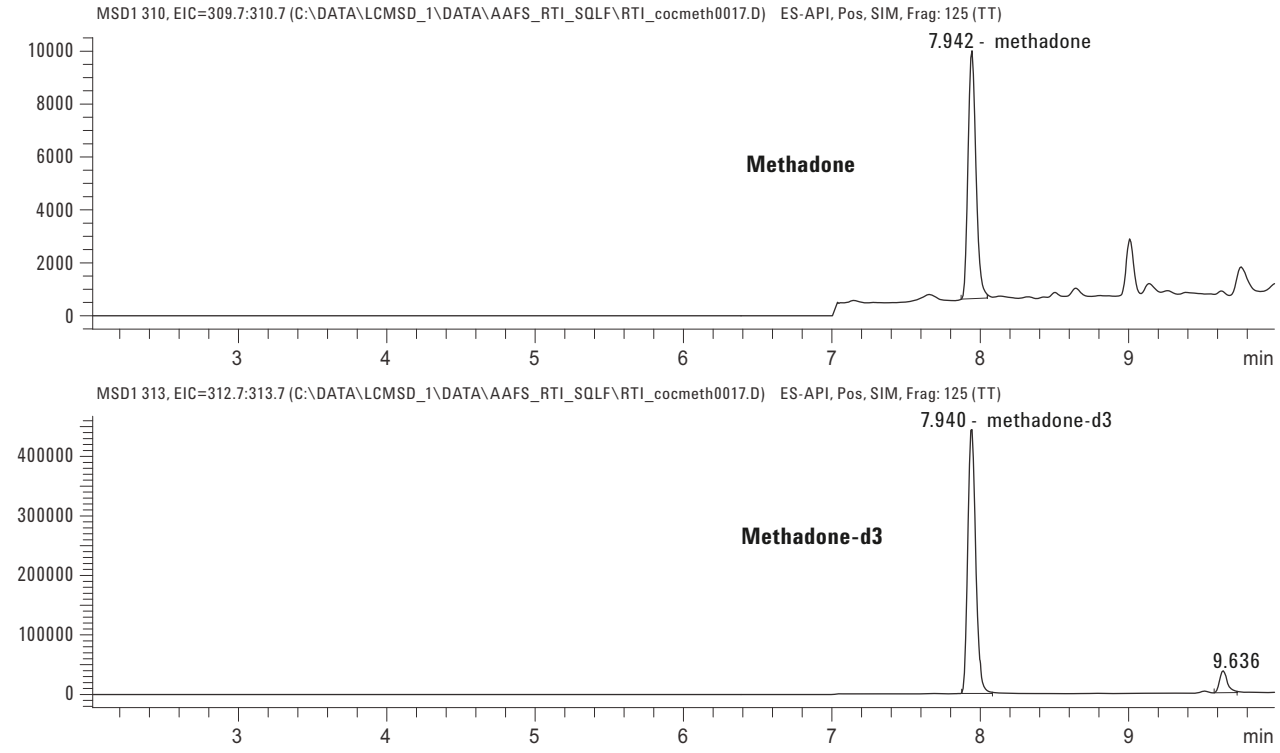


Figure 5. Postmortem methadone low calibrator (25 ng/mL).

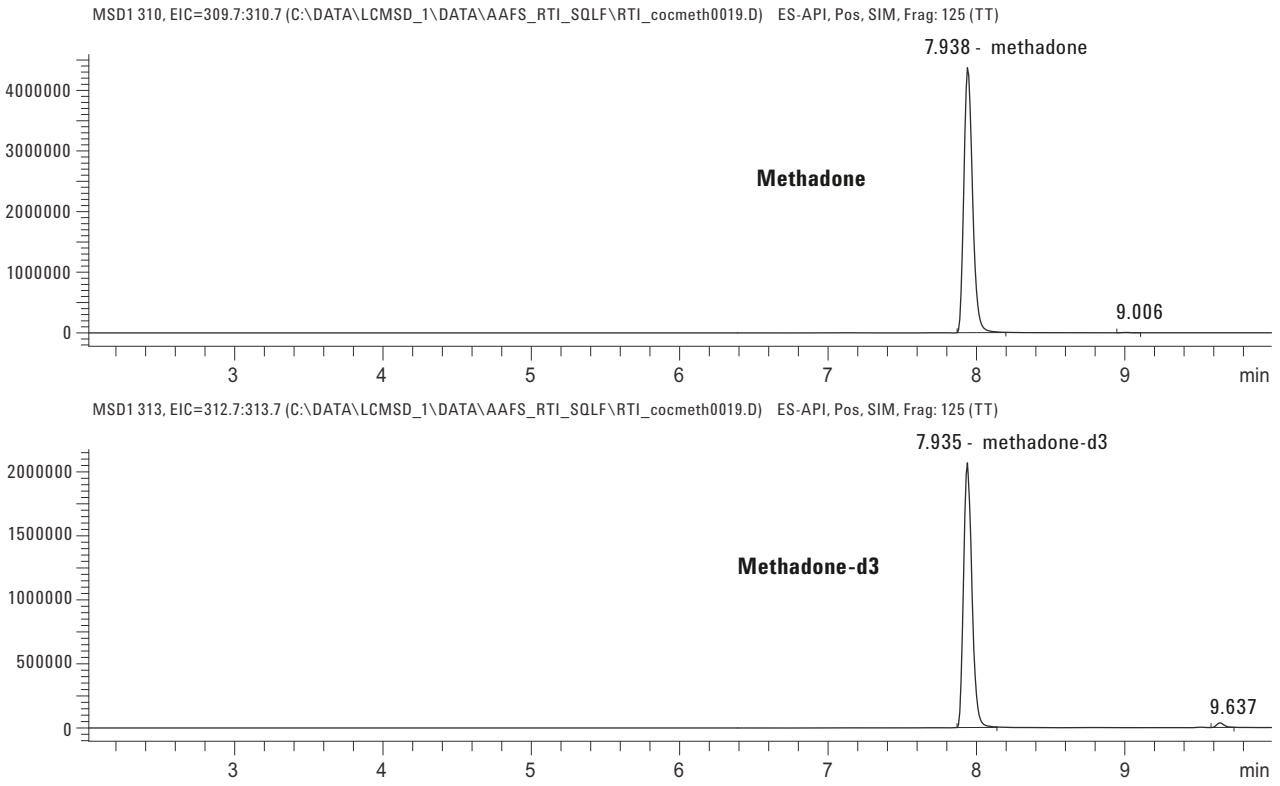


Figure 6. Postmortem methadone case sample: 1,156 ng/mL.

DUID Blood

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

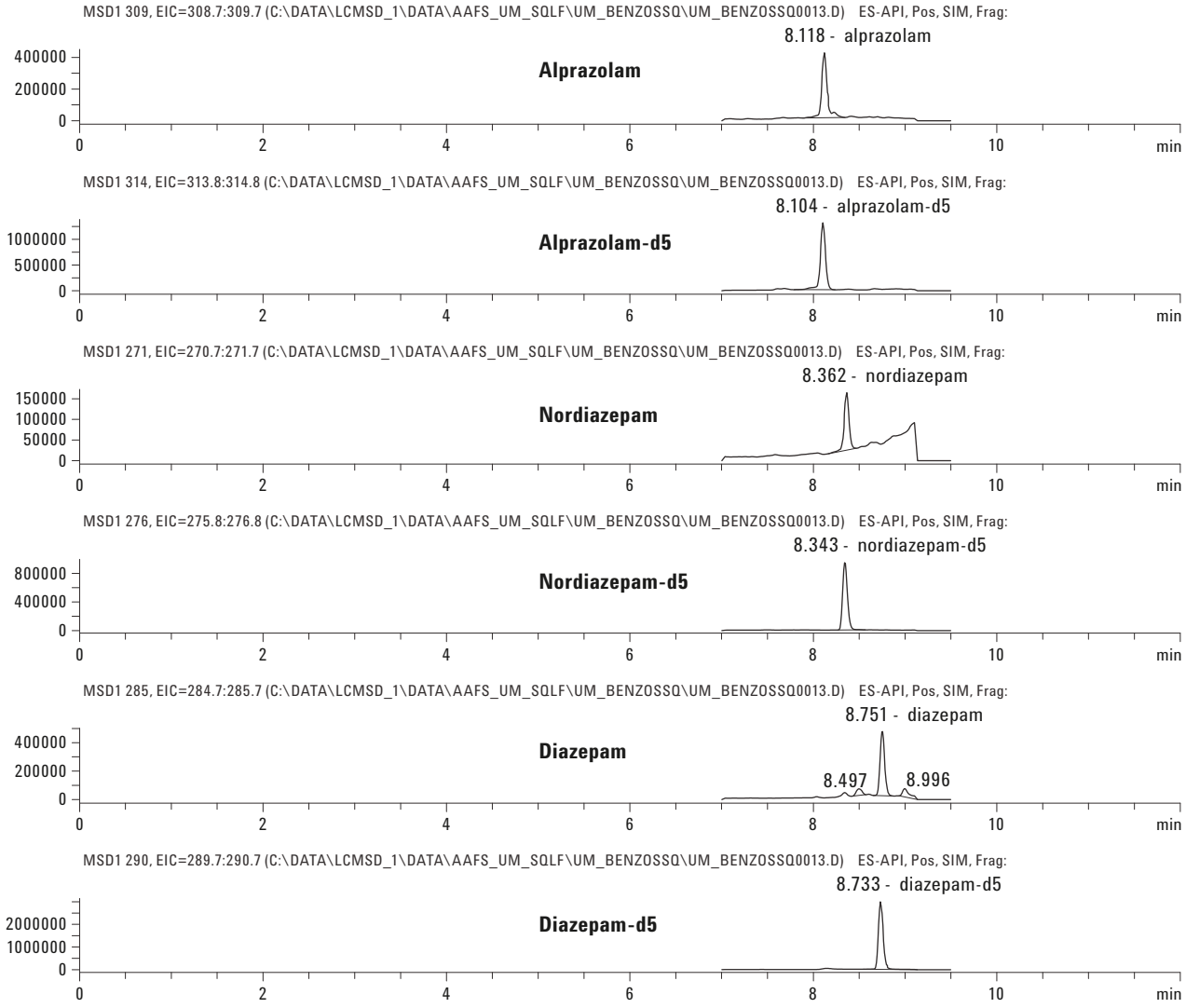


Figure 7. DUID benzodiazepines low calibrator (5 ng/mL).

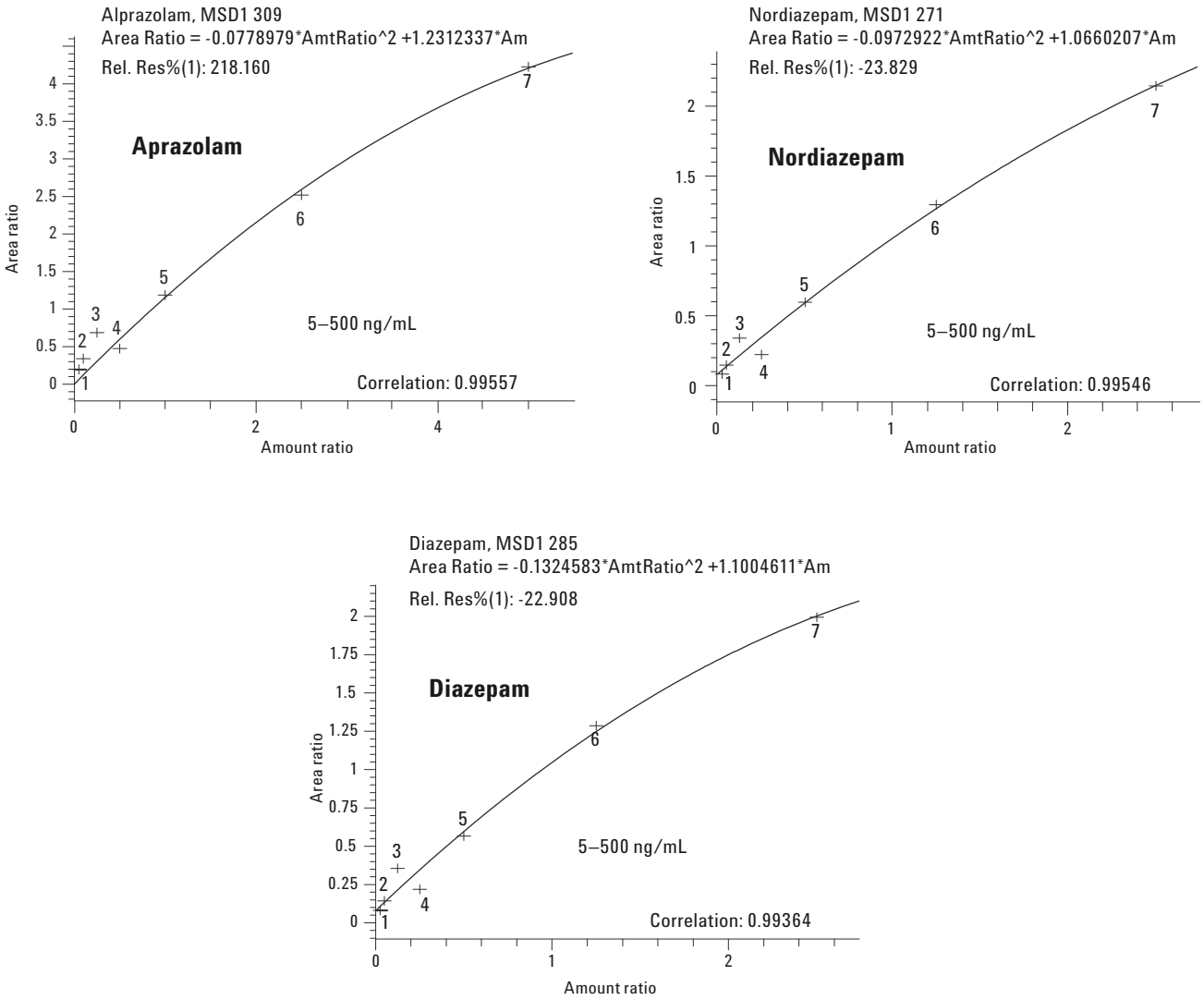


Figure 8. DUID benzodiazepines calibration (5 to 500 ng/mL). Nonlinearity is due to saturation in the electrospray ionization process and not in the MS detector.

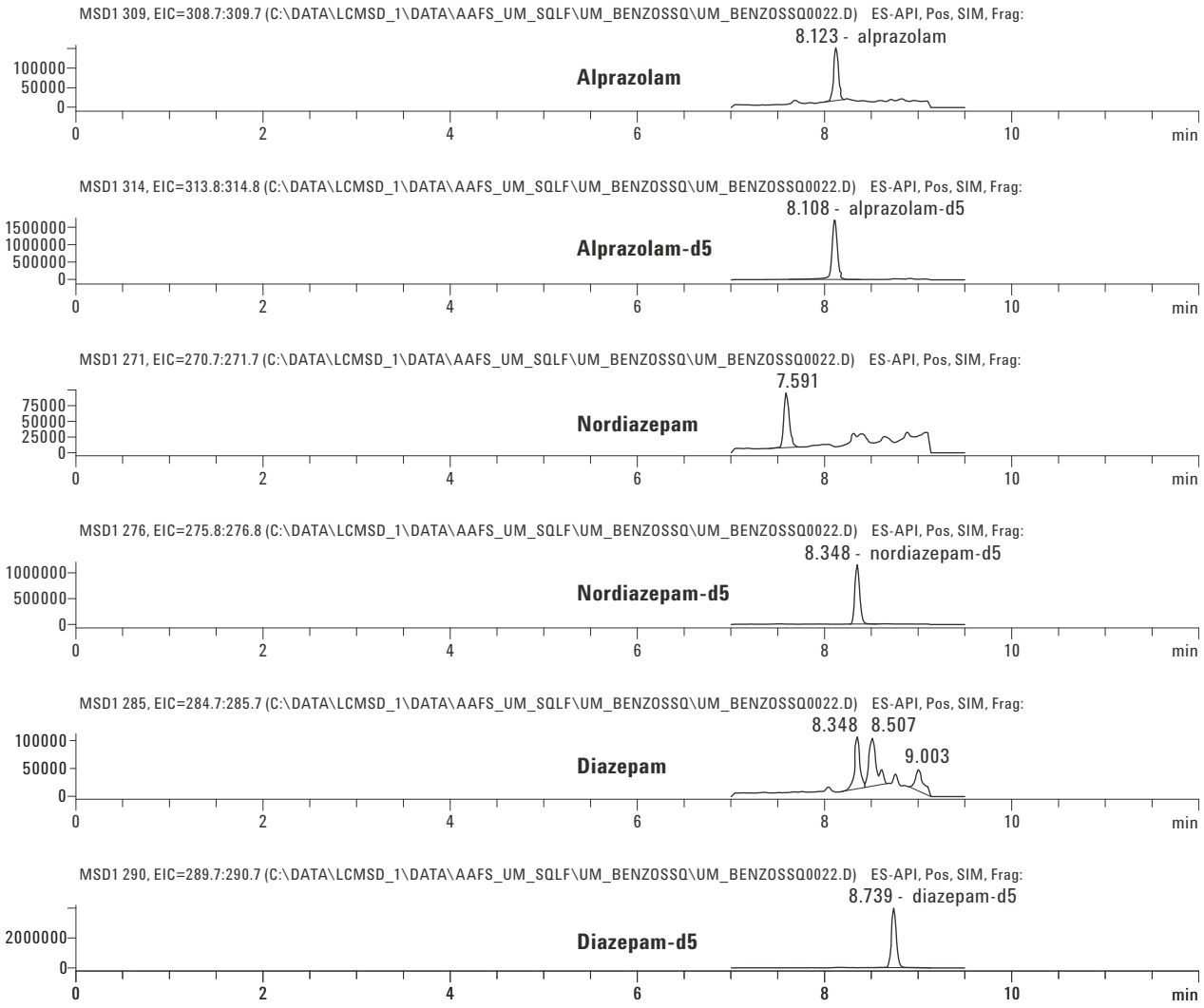


Figure 9. DUID benzodiazepines case sample 0024: alprazolam 5.6 ng/mL.

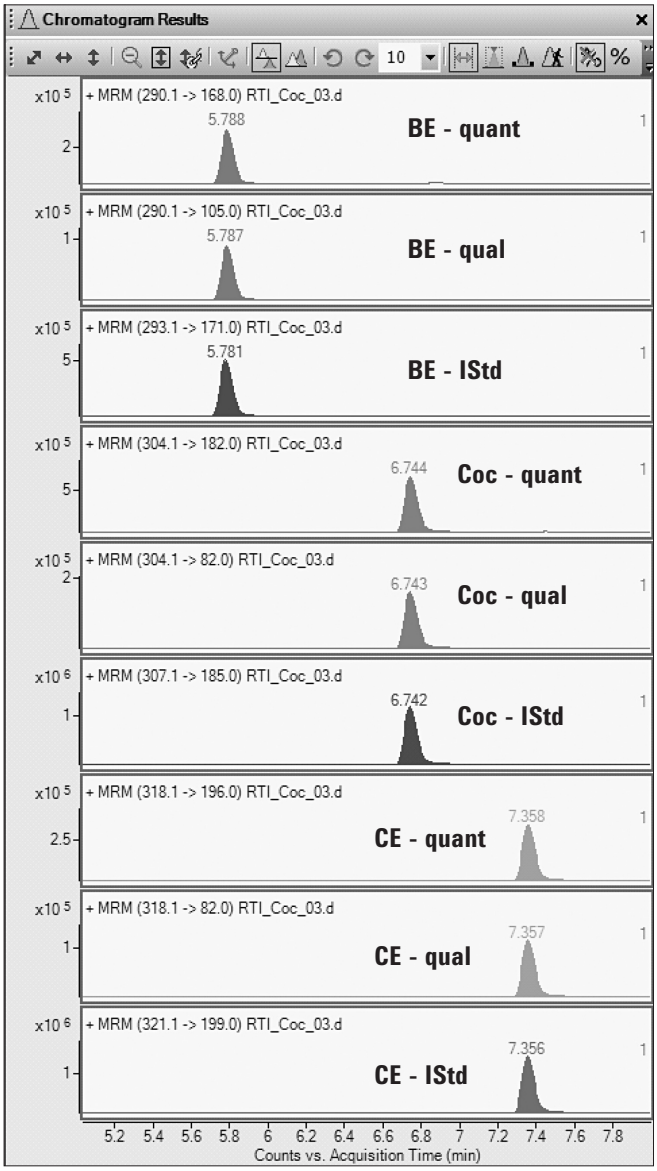
Table 4. Calculated SQ Quantification Amounts for Benzodiazepines in the Case Samples (The presence of nordiazepam and diazepam is detectable in the samples but below the range of quantification.)

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

Triple Quadrupole Mass Spectrometer

Postmortem and DUID Blood

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



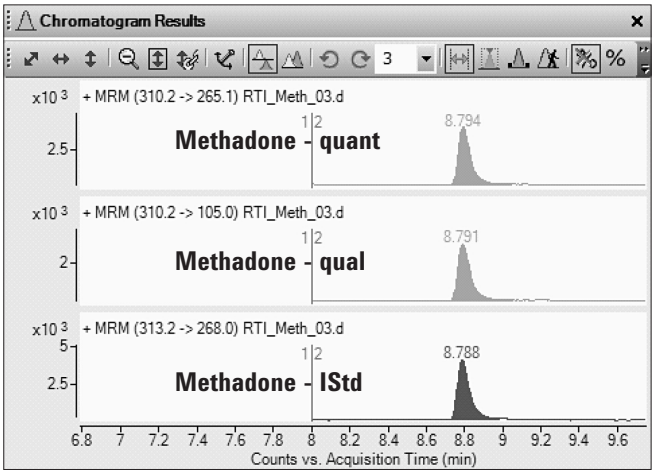


Figure 10. Compound chromatograms at the midrange level of 100 ng/mL (BE and cocaine) and 50 ng/mL (CE) obtained using multiple reaction monitoring. For each compound a quantifier, qualifier, and internal standard (IStd) ion are shown.

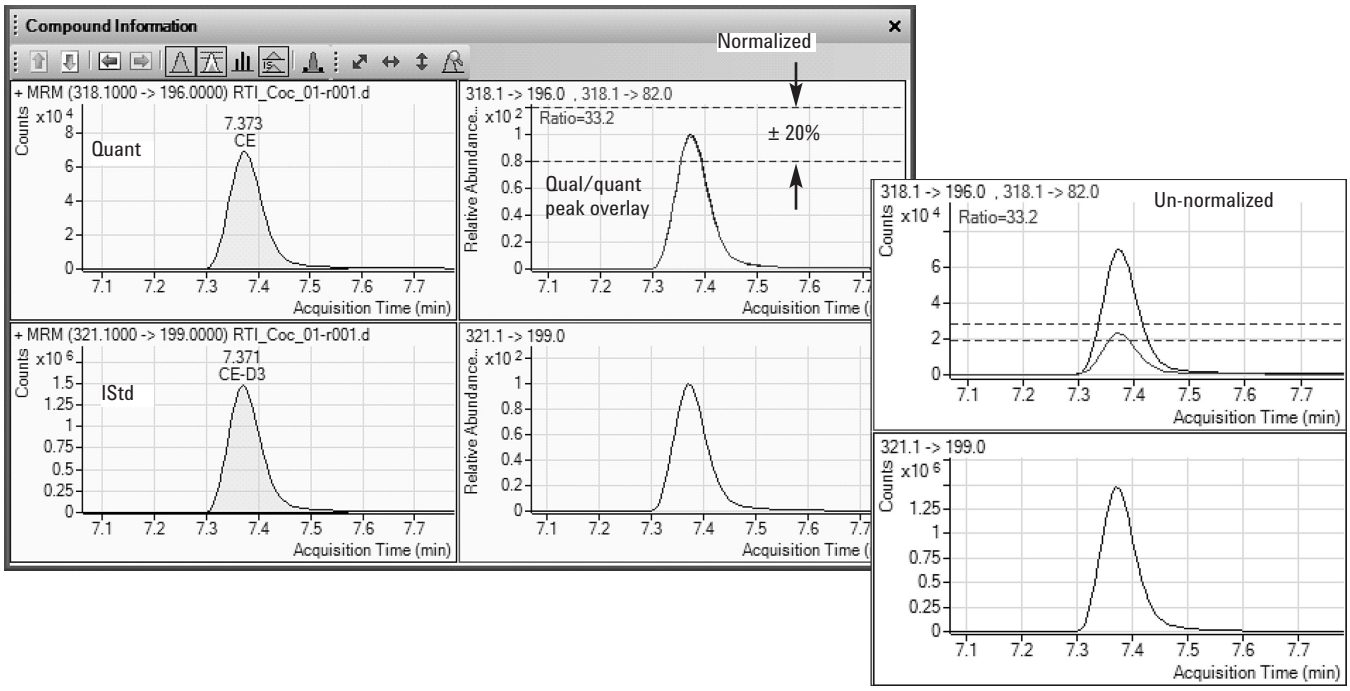


Figure 11. Qualifier peak-area ion ratios for confirmation.

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

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

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

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

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

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

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

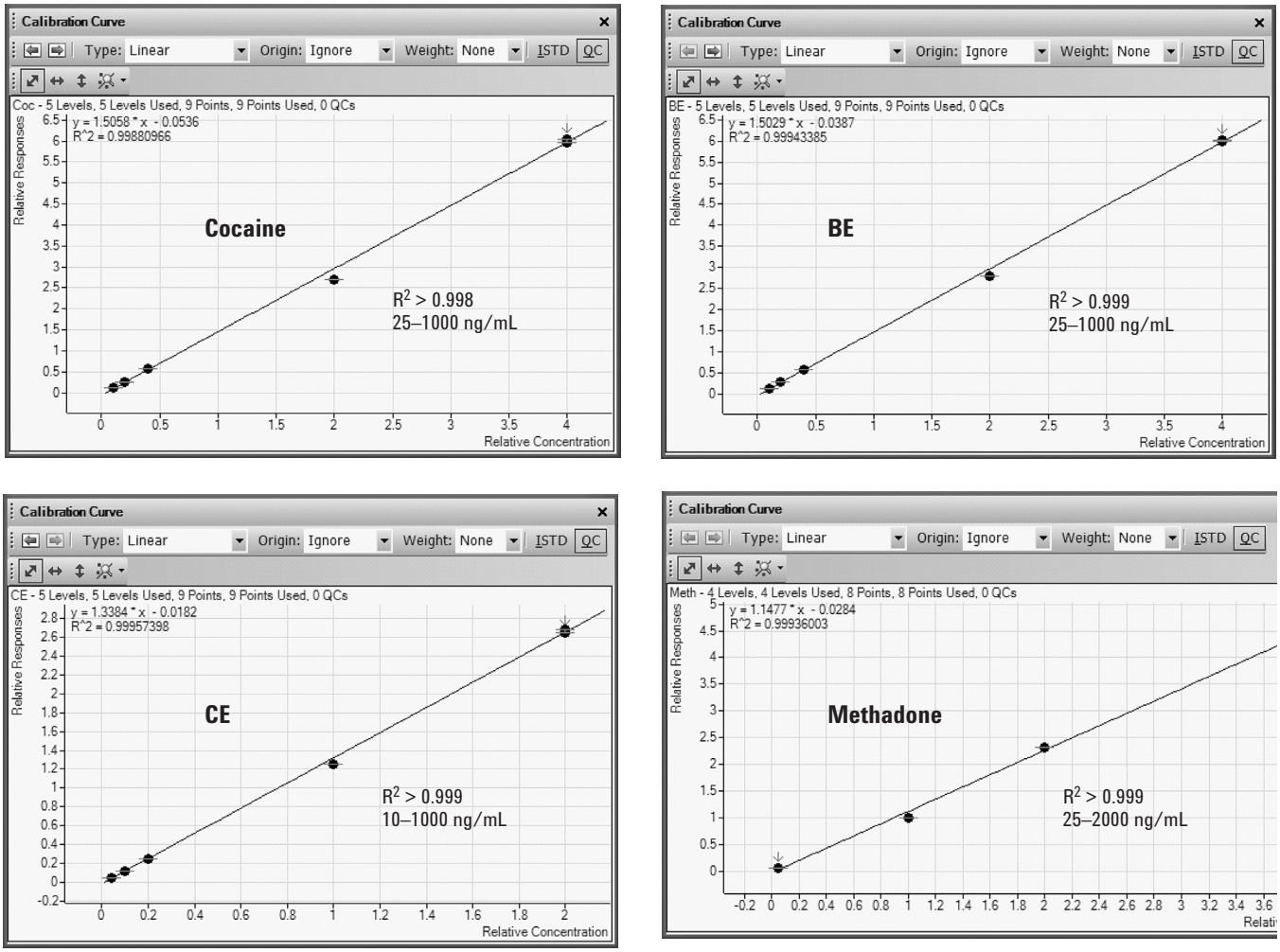


Figure 12. Linearity of compounds analyzed in postmortem samples from 25 to 1,000 ng/mL (cocaine and BE), 10 to 1,000 ng/mL (CE), and 25 to 2,000 ng/mL (methadone).

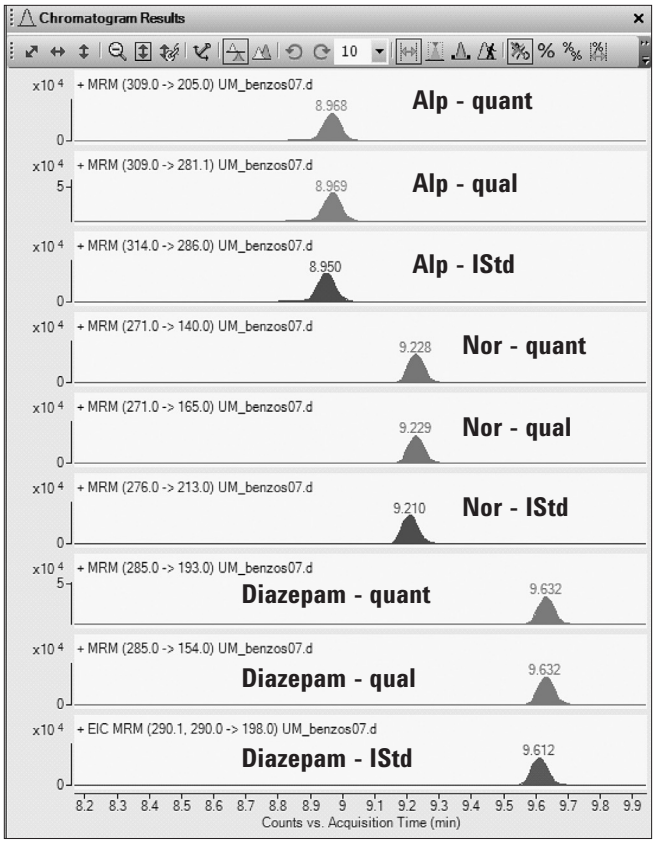


Figure 13. Compound chromatograms at the midrange level of 50 ng/mL for alprazolam, nordiazepam and diazepam. For each compound a quantifier, qualifier, and internal standard (IStd) ion are shown.

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

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

Ion Trap Mass Spectrometer

Postmortem Blood Only

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

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

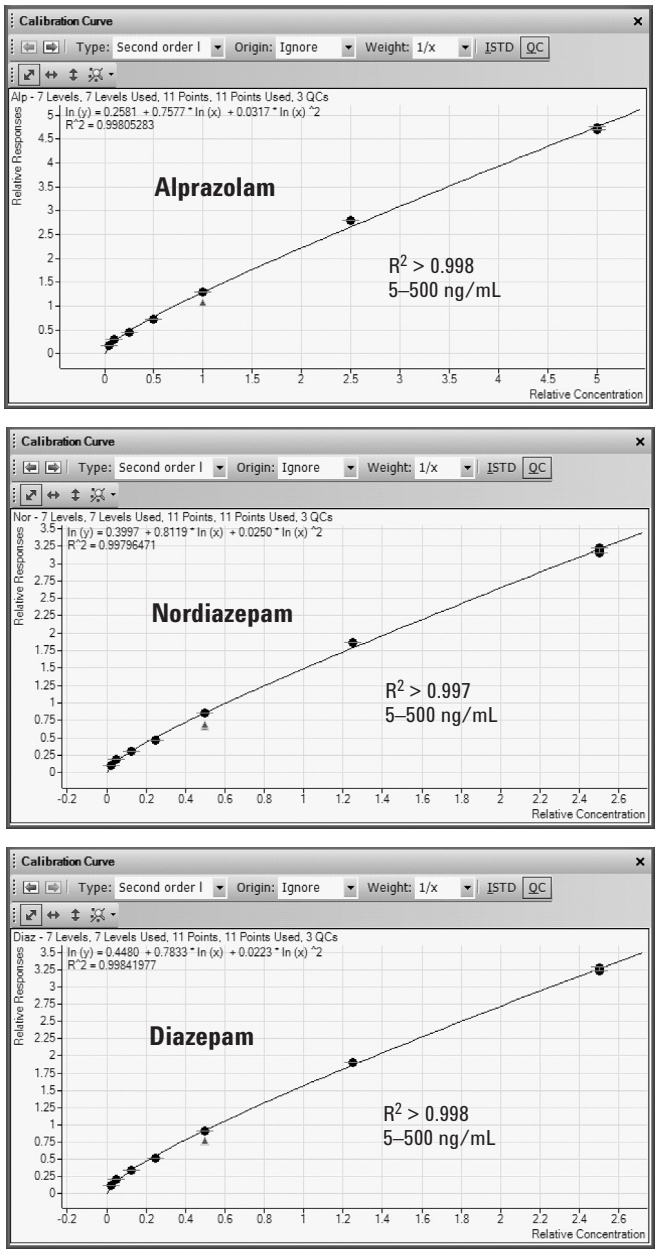


Figure 14. Postmortem benzodiazepine calibration curves for each compound are shown from 5 to 500 ng/mL.

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

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

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

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

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

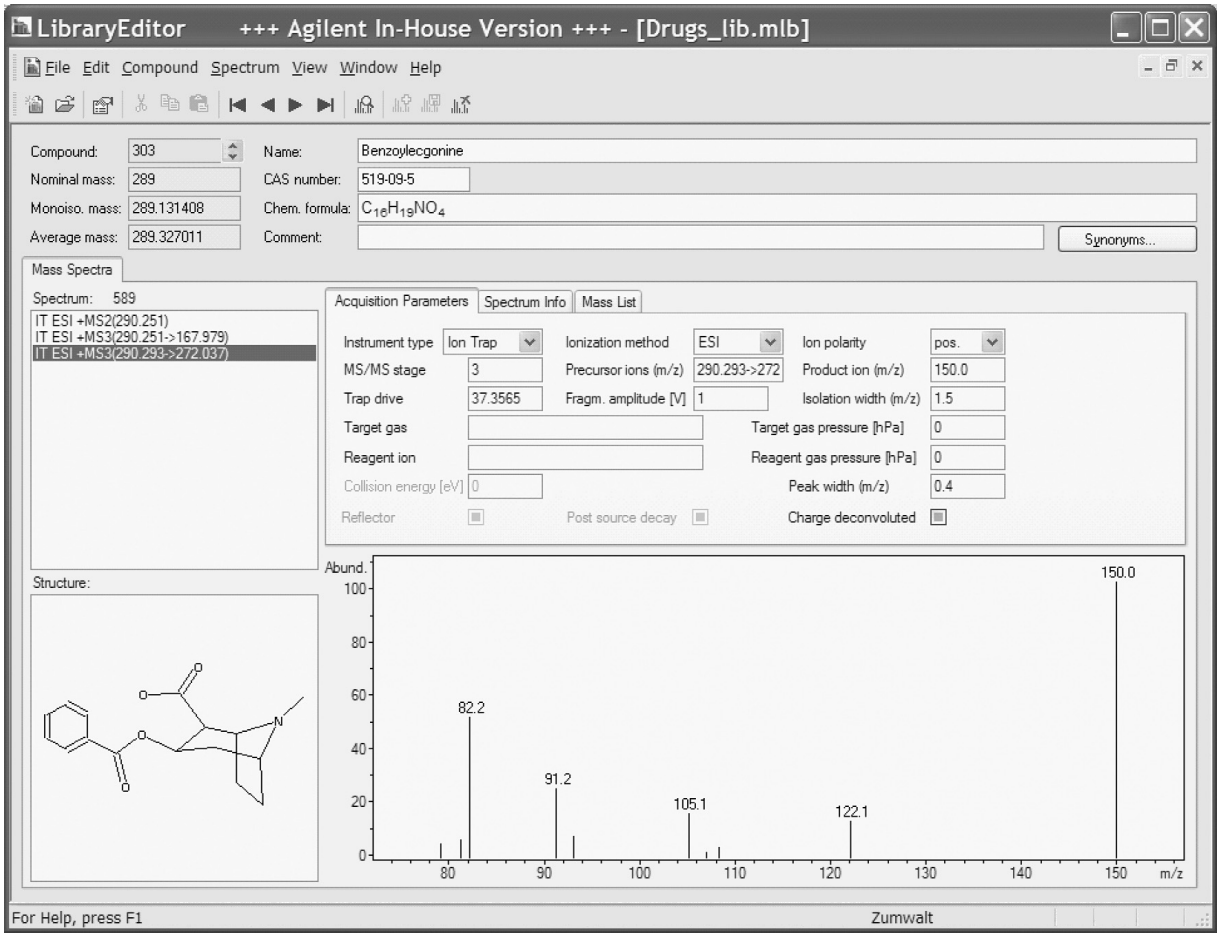


Figure 15. Library entry for benzoylecgonine includes MS/MS and MS3 spectra, CAS #, chemical formula, and structure. All spectra in library acquired using SmartFrag.

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

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

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

Library Search Report - AutoMS(n)

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

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

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

Library Search Report - AutoMS(n)

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

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

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

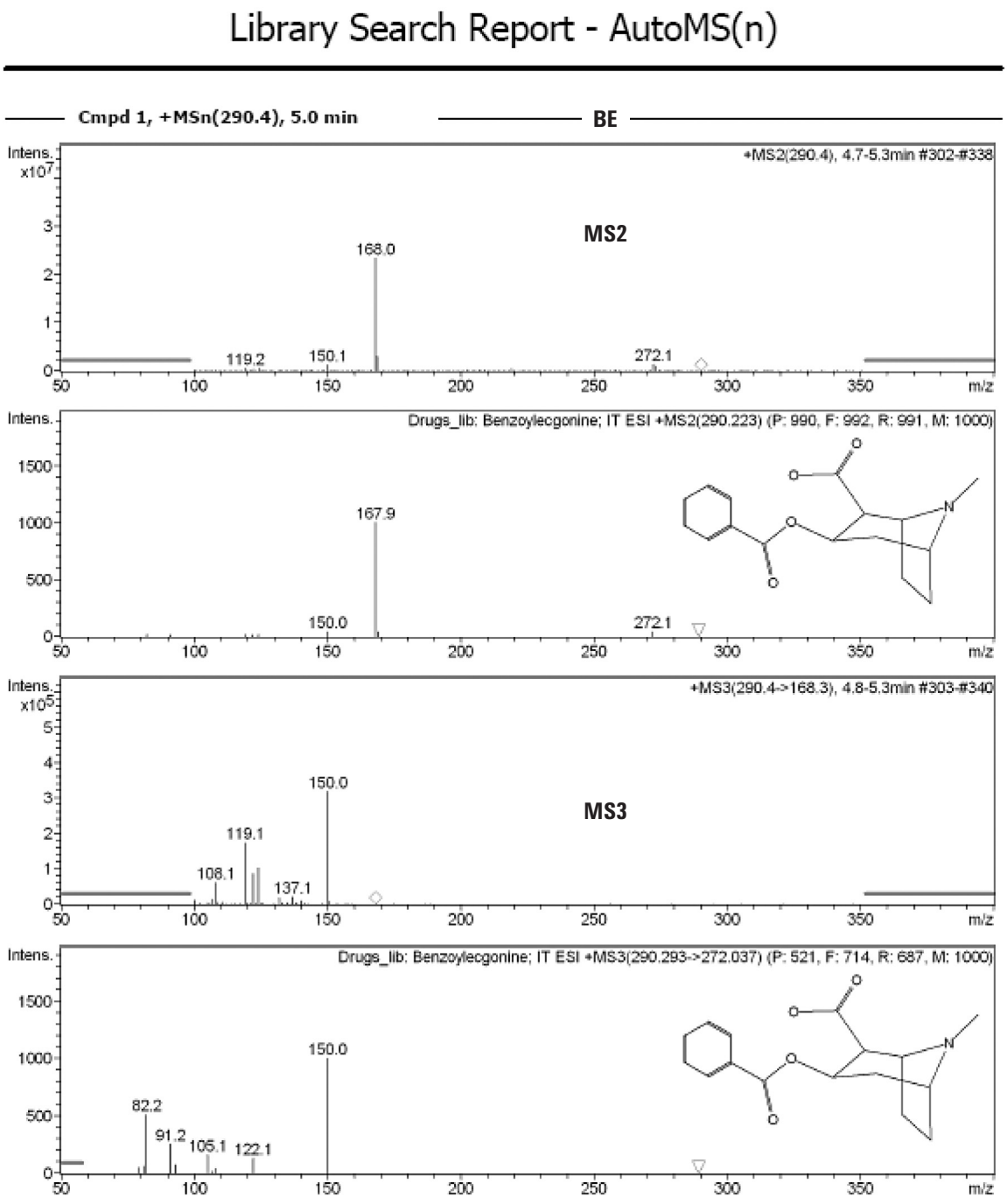


Figure 18a. Library report showing spectral matches for BE at both the MS/MS and MS3 levels in the postmortem cocaine case. Library spectra include structures.

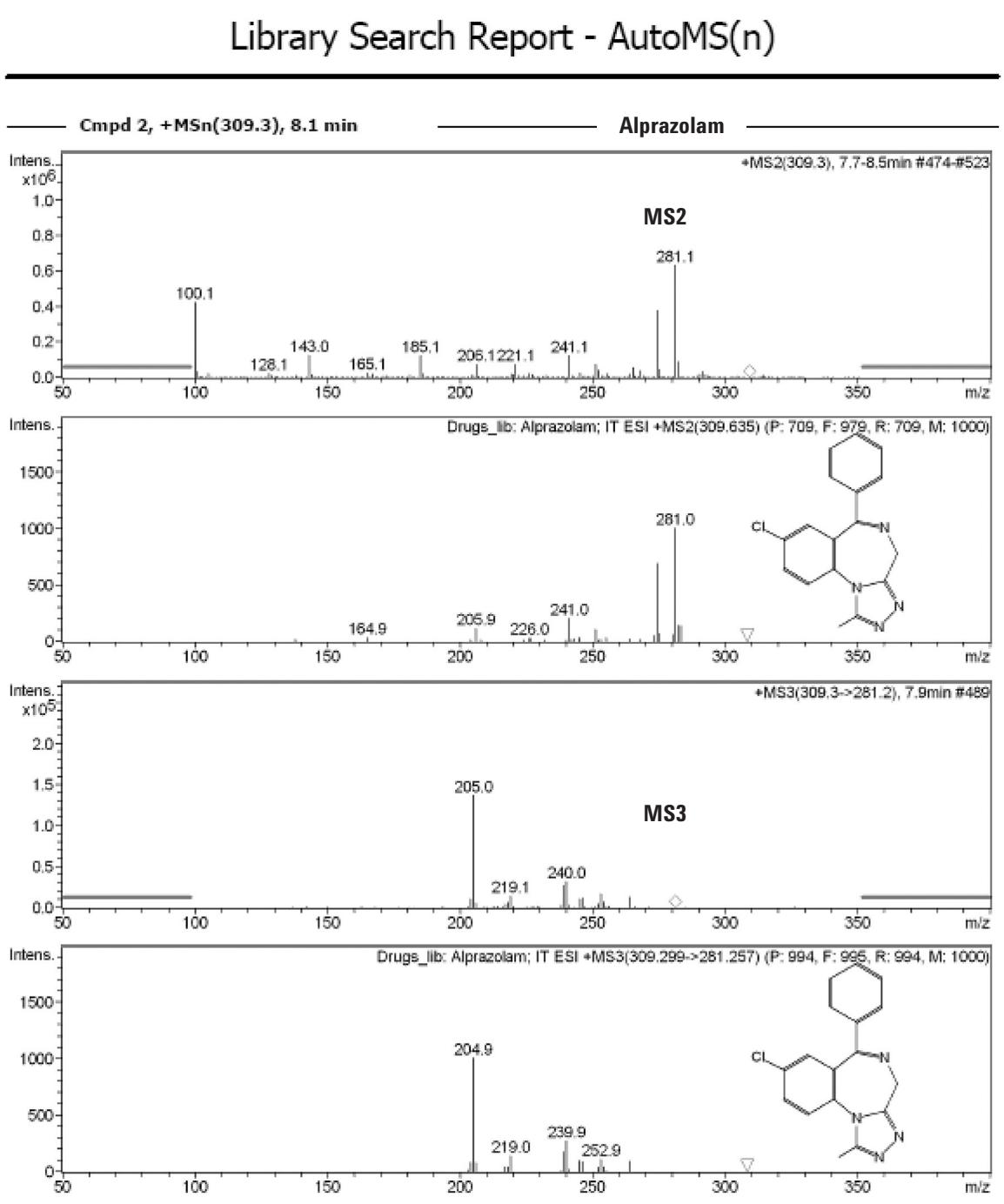


Figure 18b. Library report showing spectral matches for alprazolam at both the MS/MS and MS3 levels in the postmortem cocaine case. Library spectra include structures.

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

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

Time-of-Flight Mass Spectrometer

Postmortem Blood

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

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

Library Search Report - AutoMS(n)

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

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

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

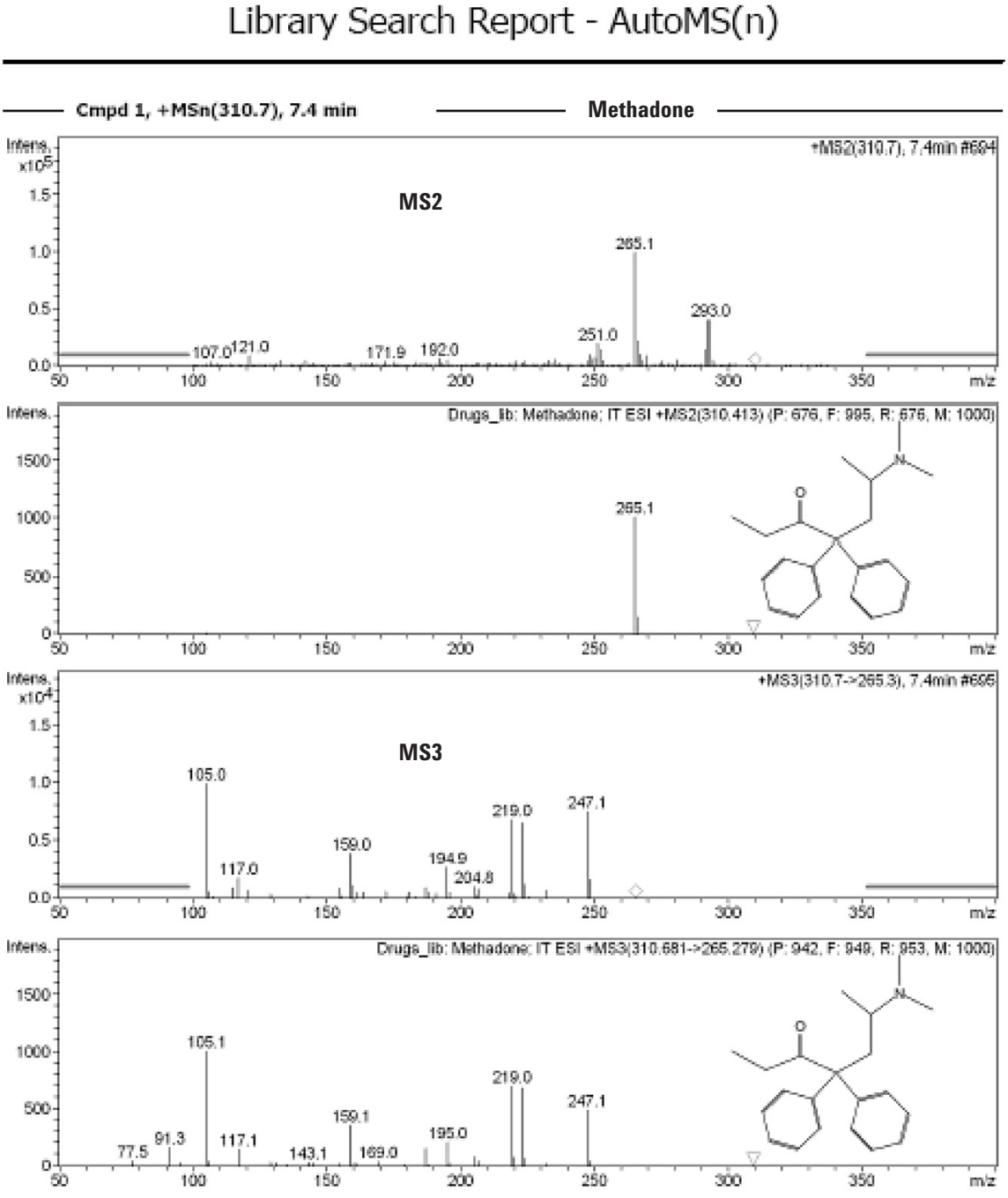


Figure 20a. Library report showing spectral matches for methadone at both the MS/MS and MS3 levels in the postmortem methadone case. Library spectra include structures.

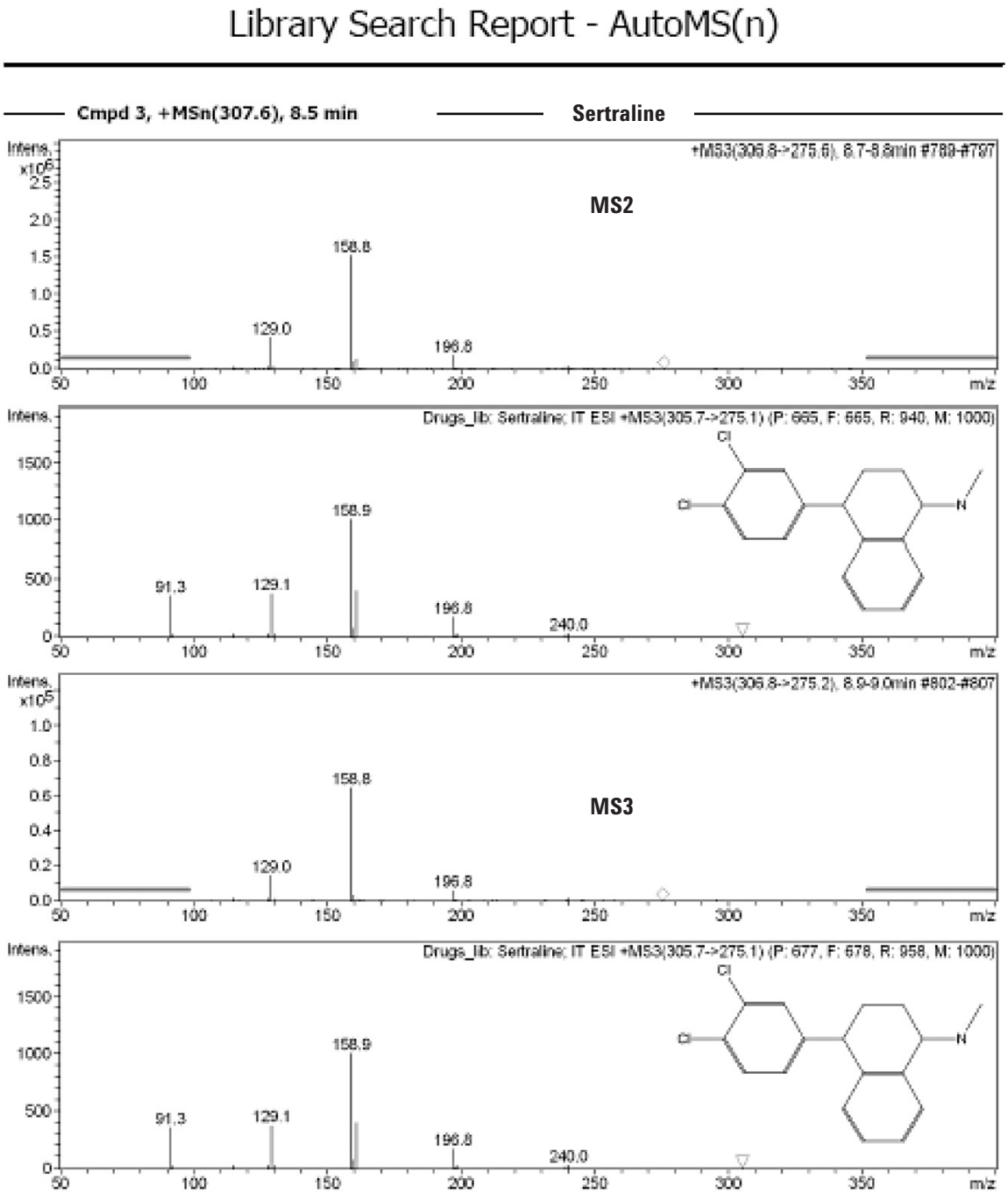


Figure 20b. Library report showing spectral matches for sertraline at both the MS/MS and MS3 levels in the postmortem methadone case. Library spectra include structures.

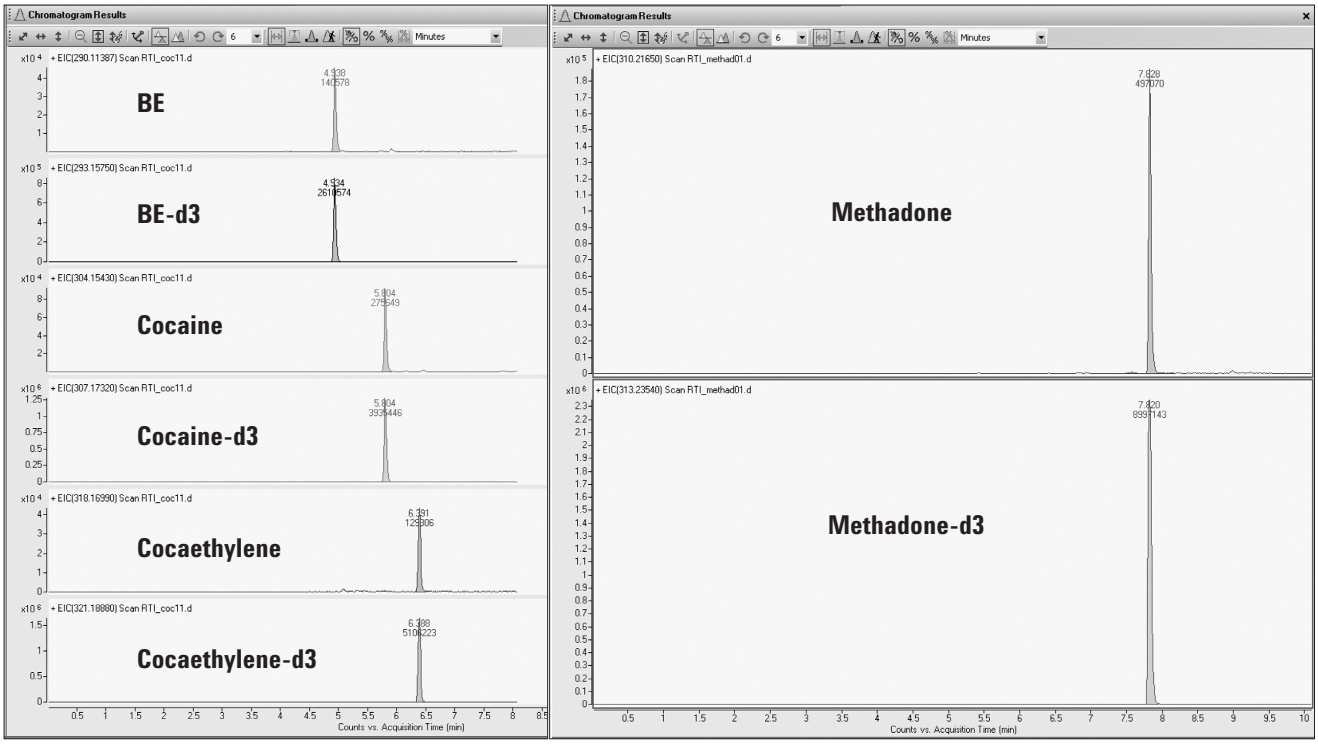


Figure 21. Extracted ion chromatograms of ± 10 ppm for the postmortem lowest level calibrator consisting of BE, cocaine, and methadone (25 ng/mL), and CE (10 ng/mL).

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

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

Postmortem Blood

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

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

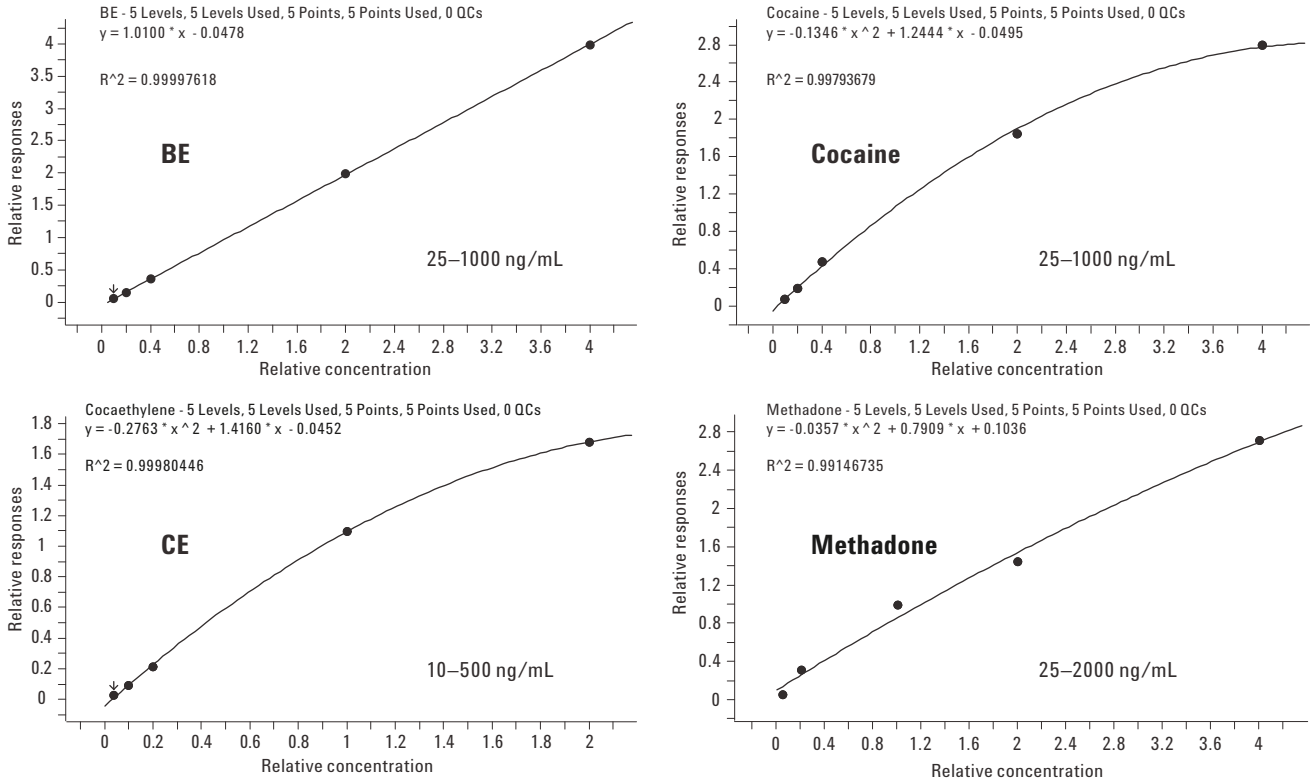


Figure 22. Calibration curves for the postmortem compounds. Detector saturation was the primary cause of the nonlinearity seen for cocaine and CE, even with only 2 uL injected.

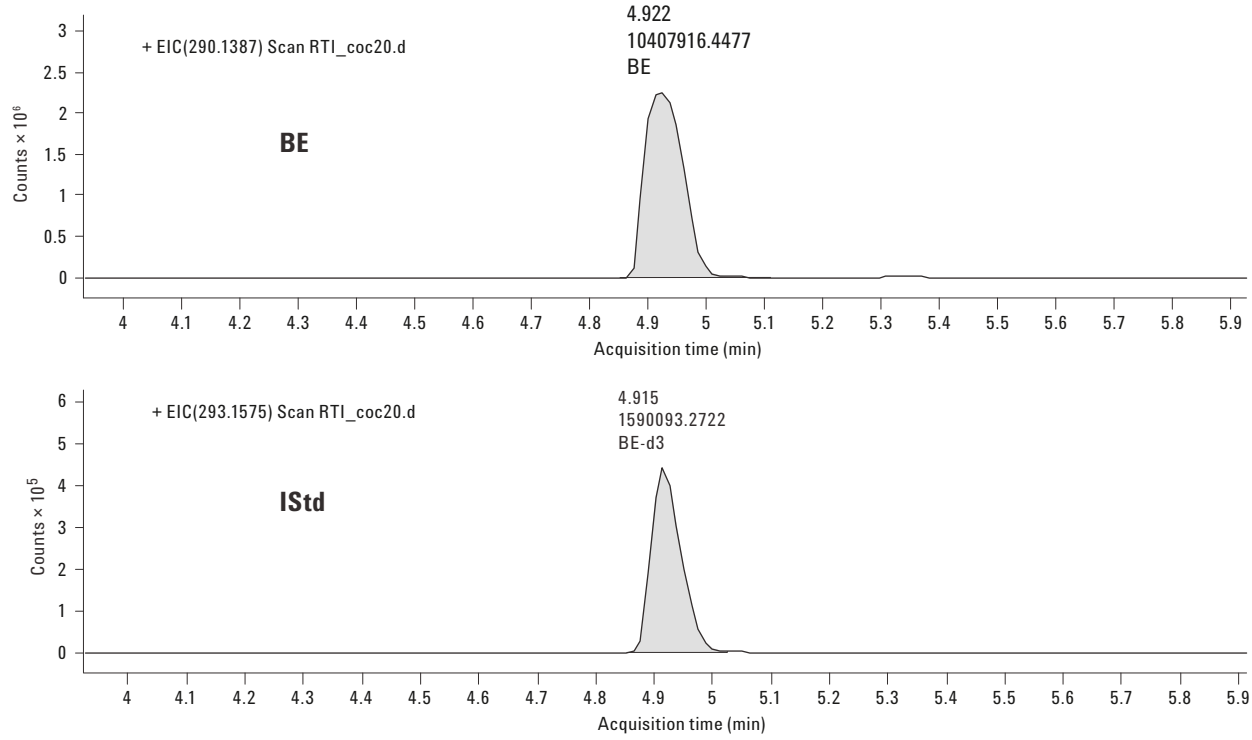


Figure 23. Cocaine case sample analyzed by TOF: BE 1,632 ng/mL; cocaine 12.5 ng/mL; and CE 6.4 ng/mL.

Continued

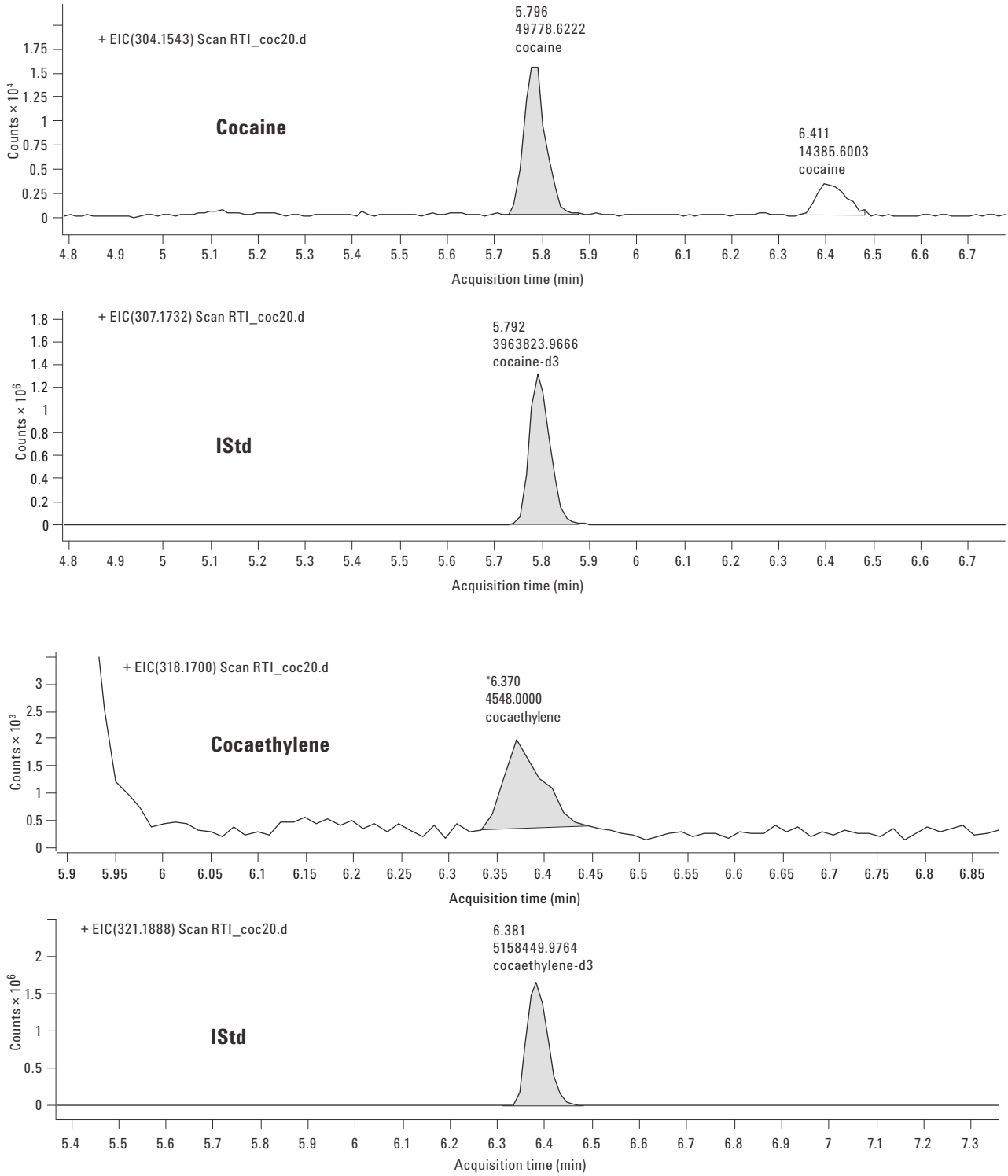


Figure 23. Cocaine case sample analyzed by TOF: BE 1,632 ng/mL; cocaine 12.5 ng/mL; and CE 6.4 ng/mL.

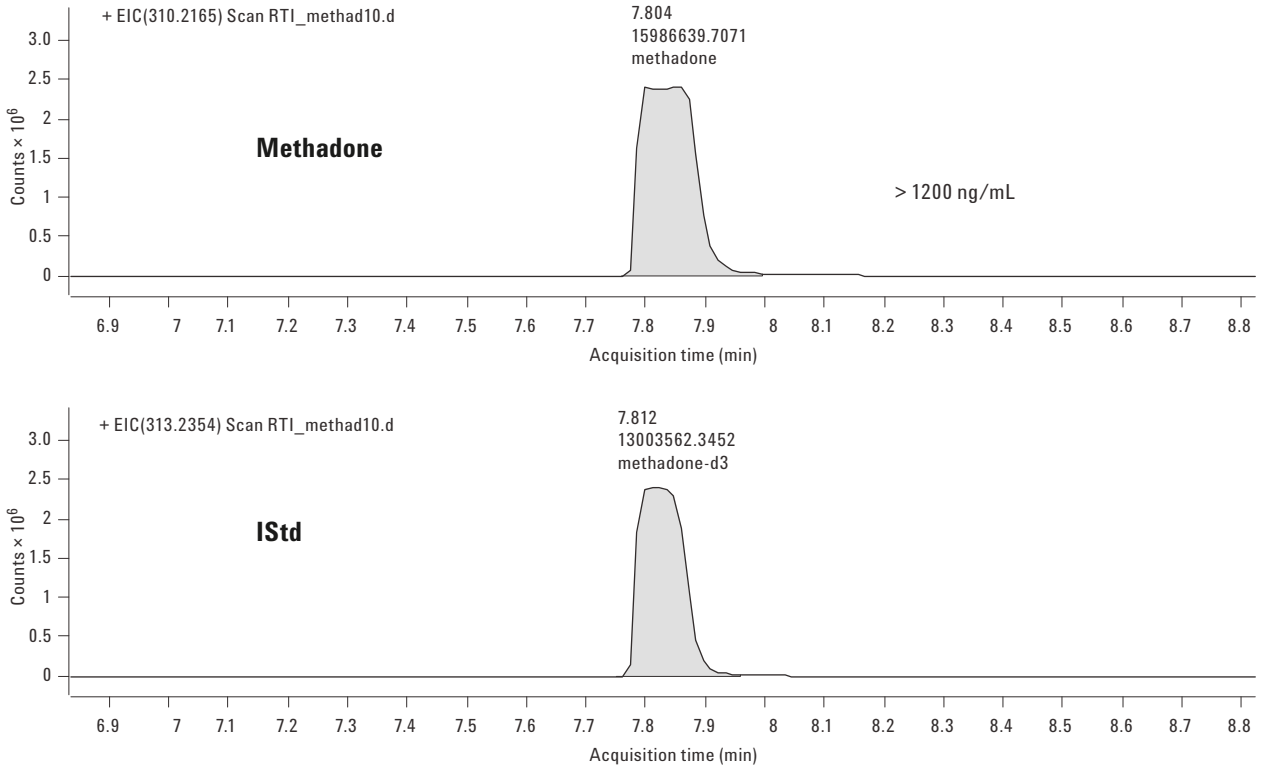


Figure 24. Methadone case sample analyzed by TOF shows detector saturation at a level greater than 1,200 ng/mL.

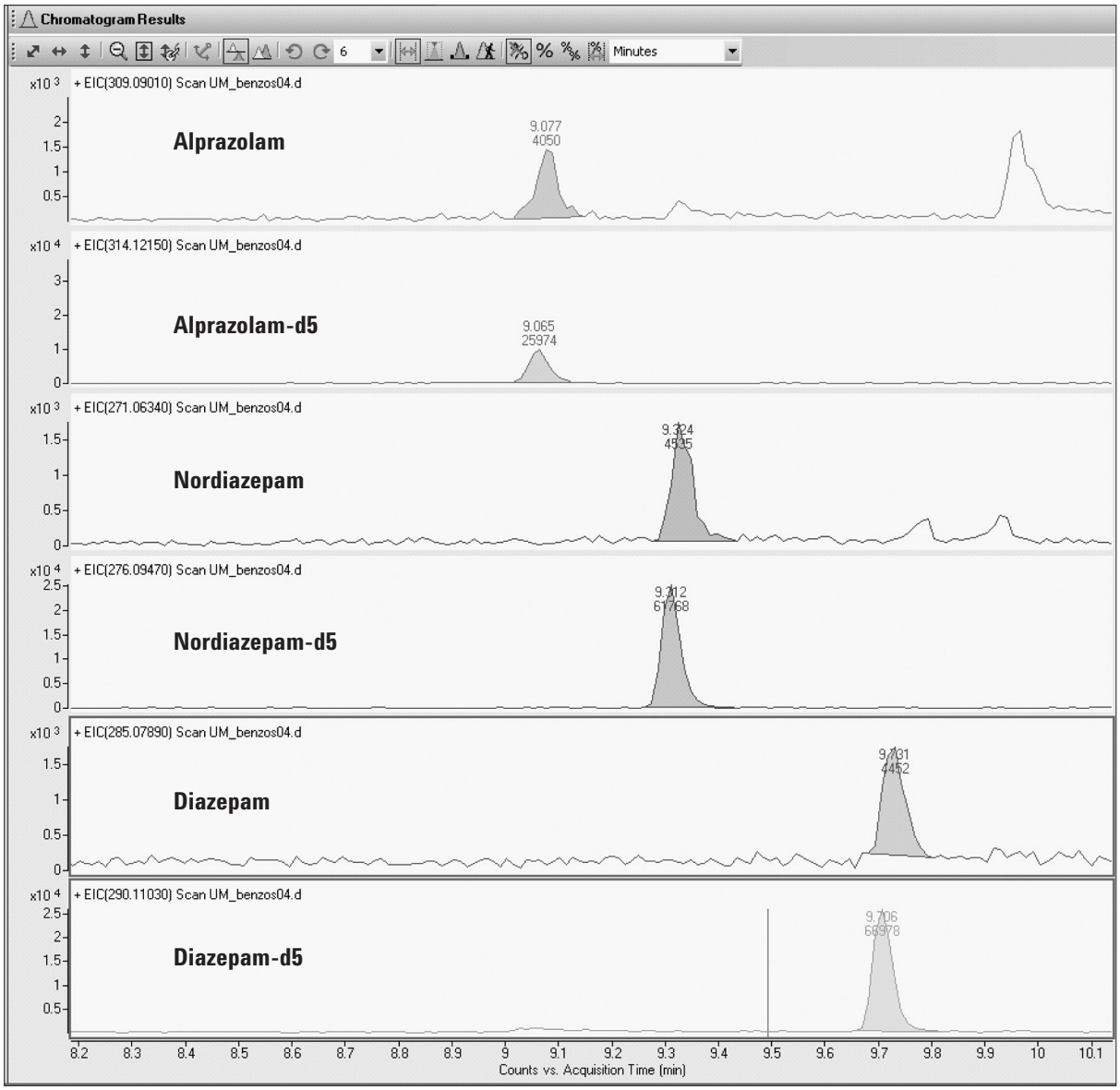


Figure 25. Extracted ion chromatograms of ± 10 ppm for the DUID lowest level calibrator consisting of alprazolam, nordiazepam, and diazepam (5 ng/mL).

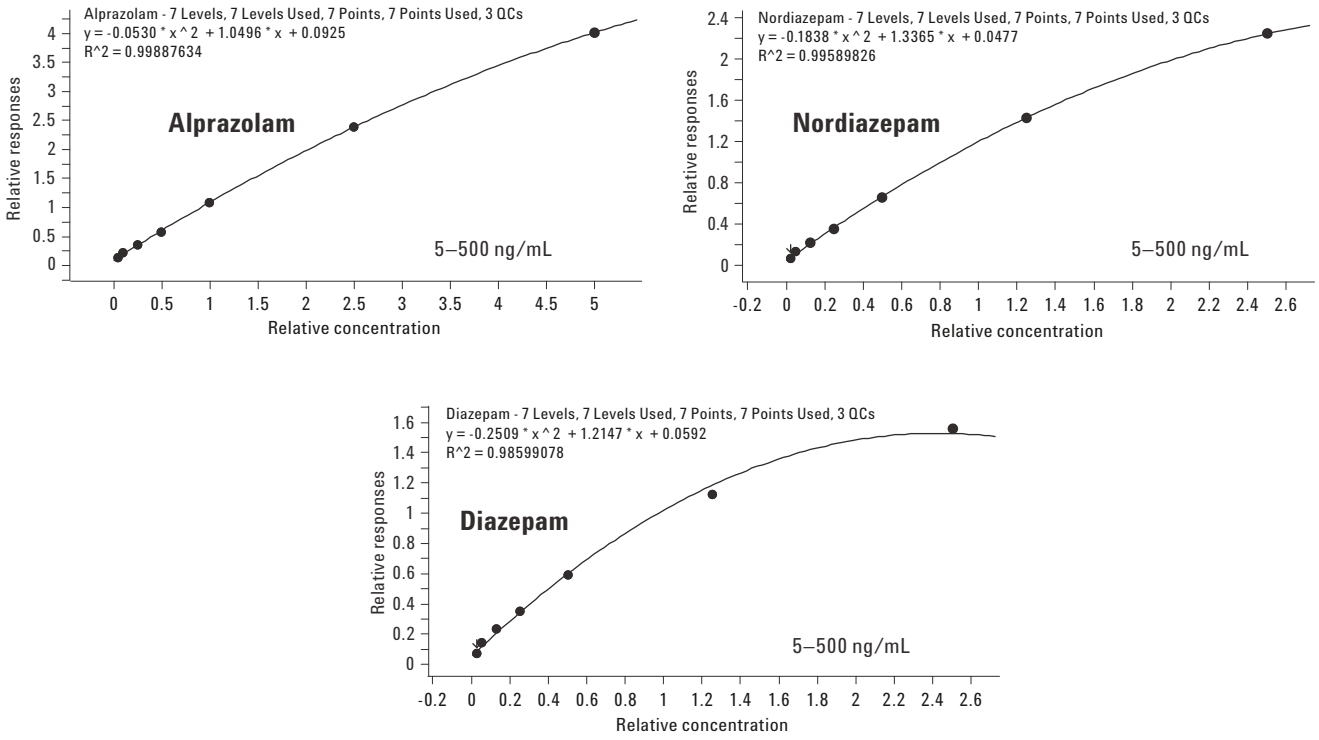


Figure 26. Calibration curves for the DUID compounds. Detector saturation was the primary cause of the nonlinearity seen for nordiazepam and diazepam.

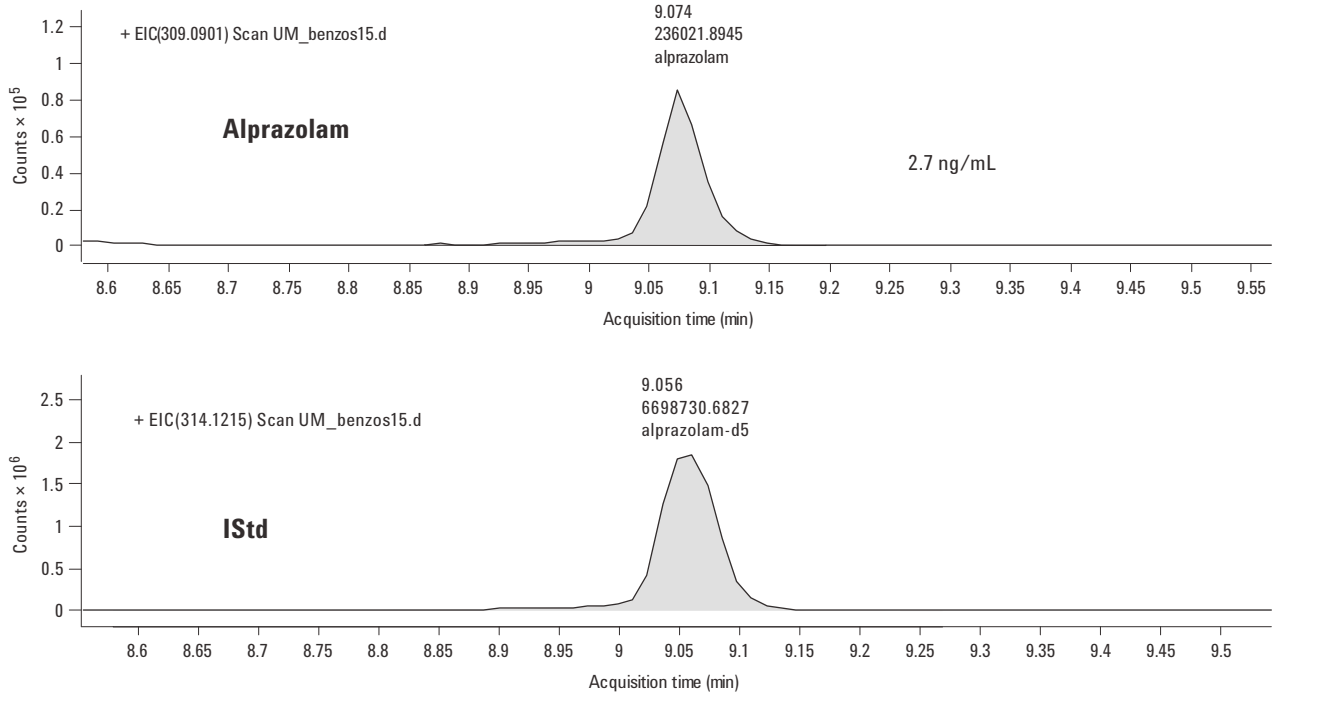


Figure 27. DUID case sample 0024 has a calculated amount of 2.7 ng/mL for alprazolam.

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

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

Quadrupole Time-of-Flight Mass Spectrometer

Postmortem Blood

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

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

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

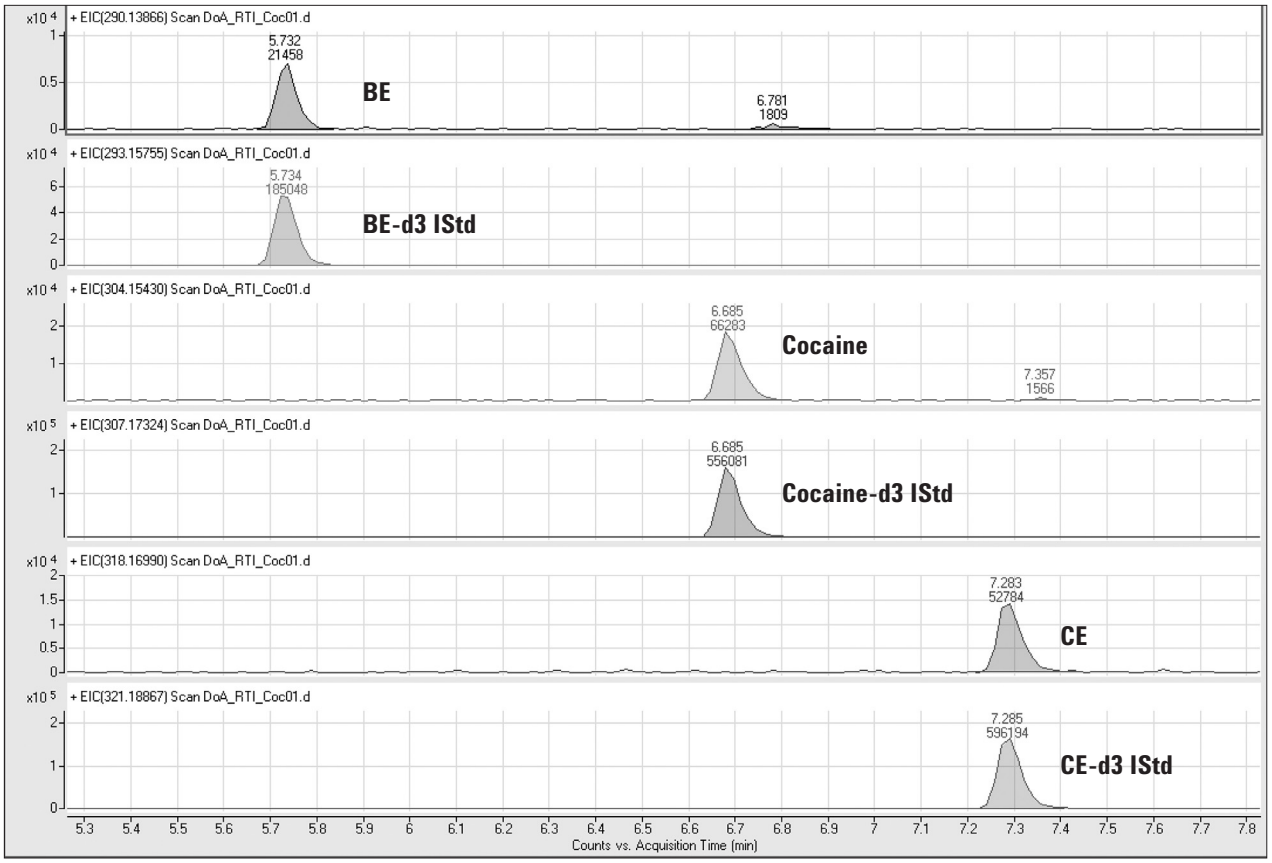


Figure 28. EICs (± 10 ppm) of lowest level calibrator in postmortem analysis: BE and cocaine (25 ng/mL); CE (10 ng/mL) at 0.1 uL injection volume.

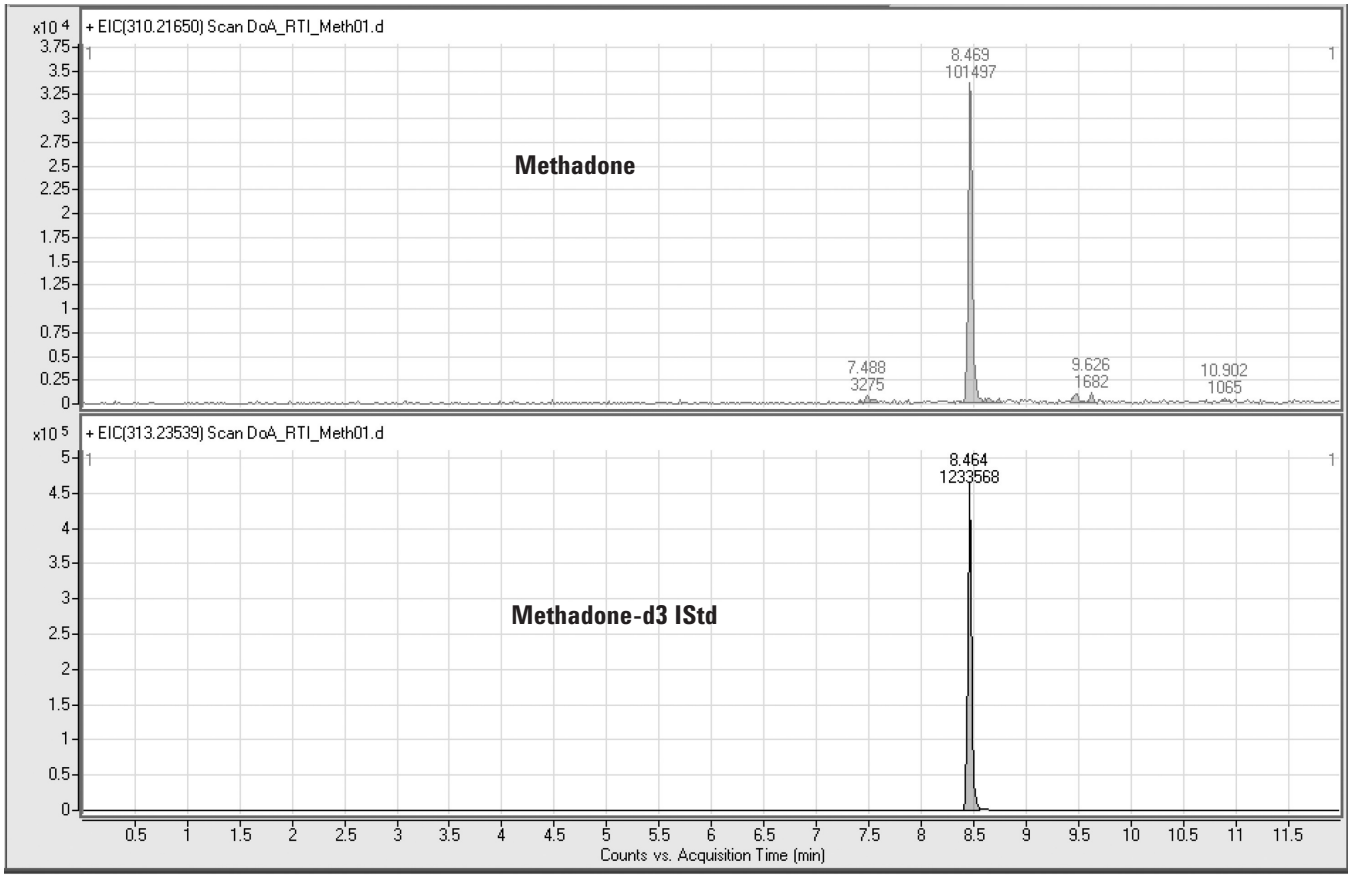


Figure 29. EICs (± 10 ppm) of methadone at 25 ng/mL in the lowest level calibrator for the postmortem analysis at a 0.1 µL injection volume.

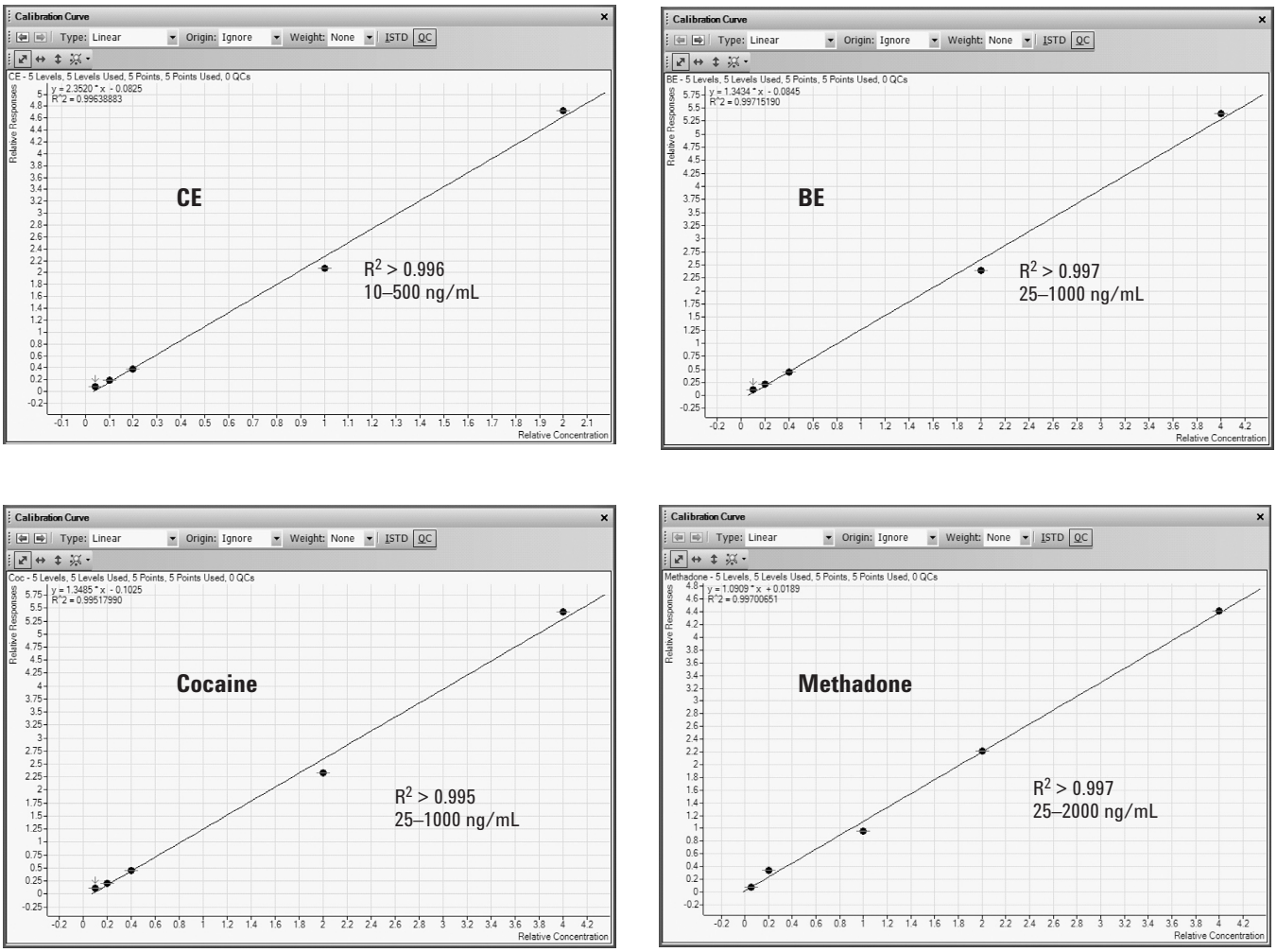


Figure 30. Calibration curves for the compounds in the postmortem analysis.

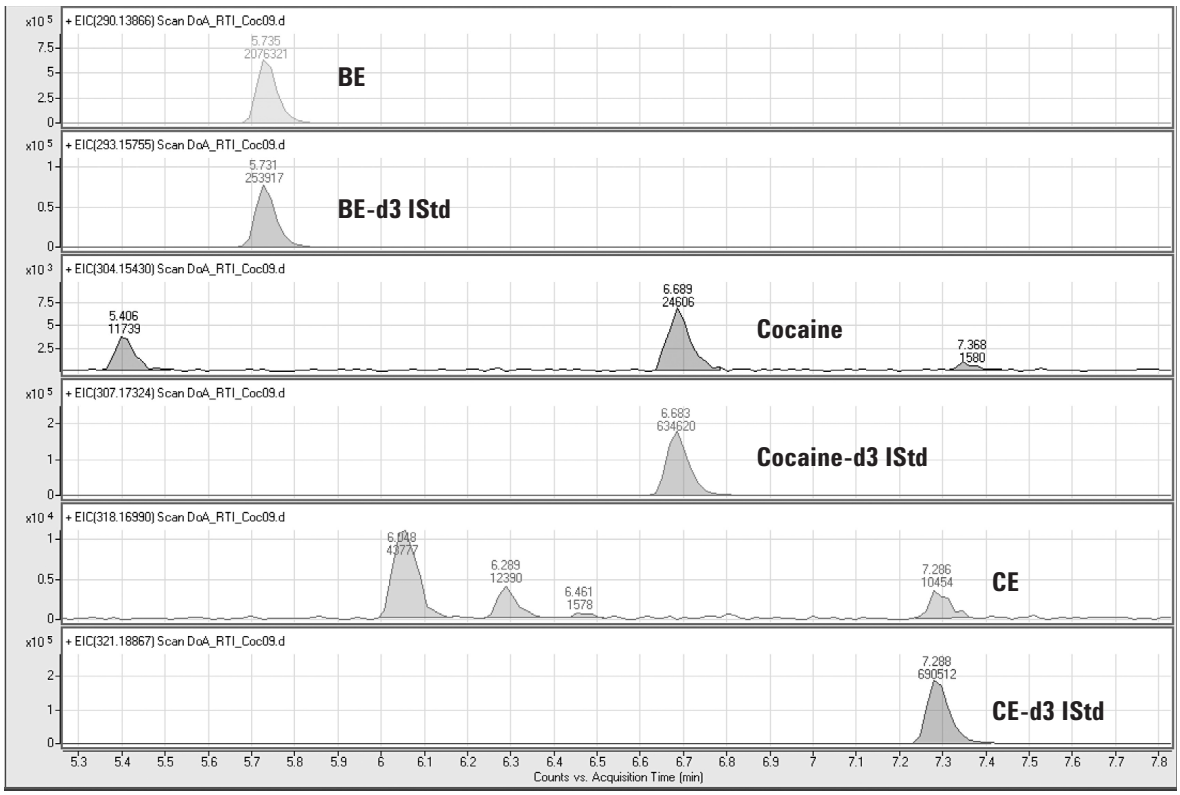


Figure 31. EICs (± 10 ppm) of cocaine case sample quantitating at BE = 1539.5 ng/mL, cocaine = 26.1 ng/mL, and CE = 10.4 ng/mL.

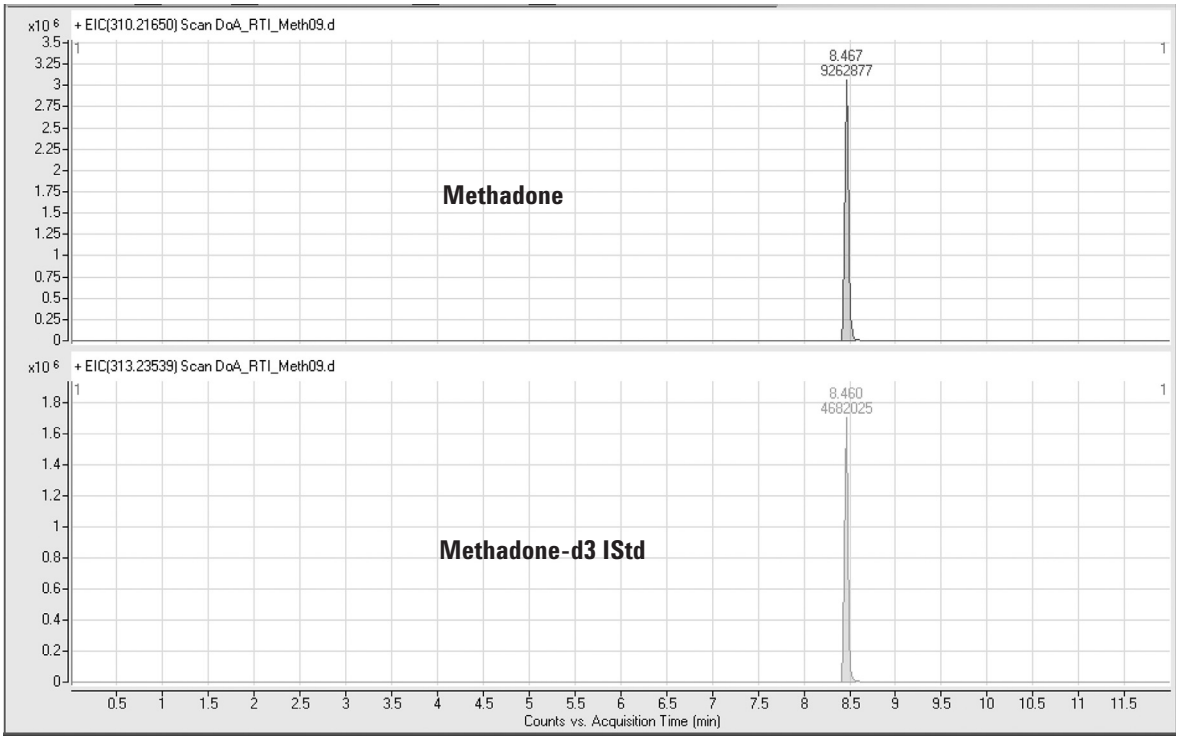


Figure 32. EICs (± 10 ppm) of methadone case sample quantitating at 898.1 ng/mL.

DUID Blood

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

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

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

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

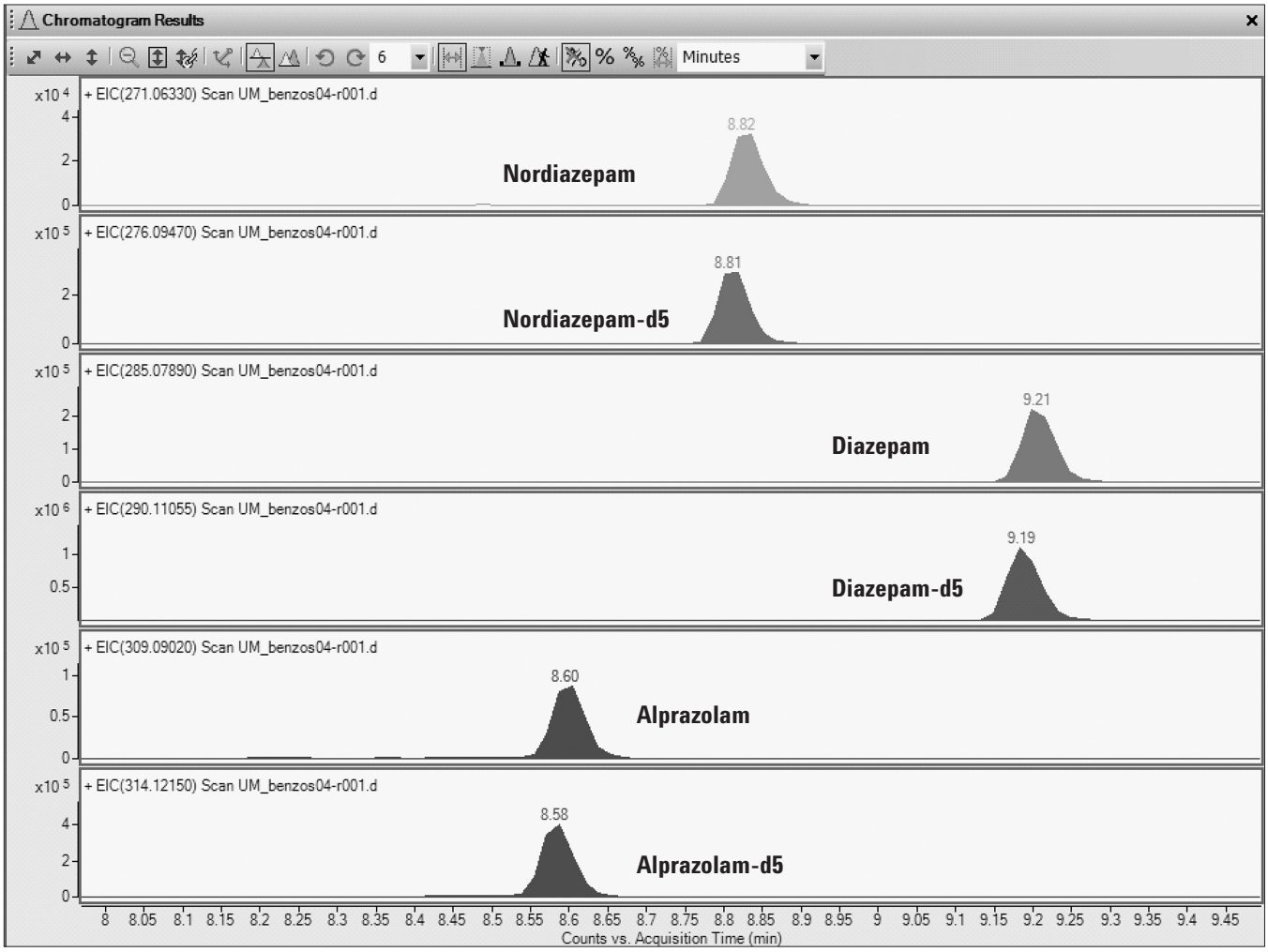


Figure 33. EICs (± 10 ppm) of lowest level calibrator at 5 ng/mL alprazolam, nordiazepam, and diazepam for DUID analysis.

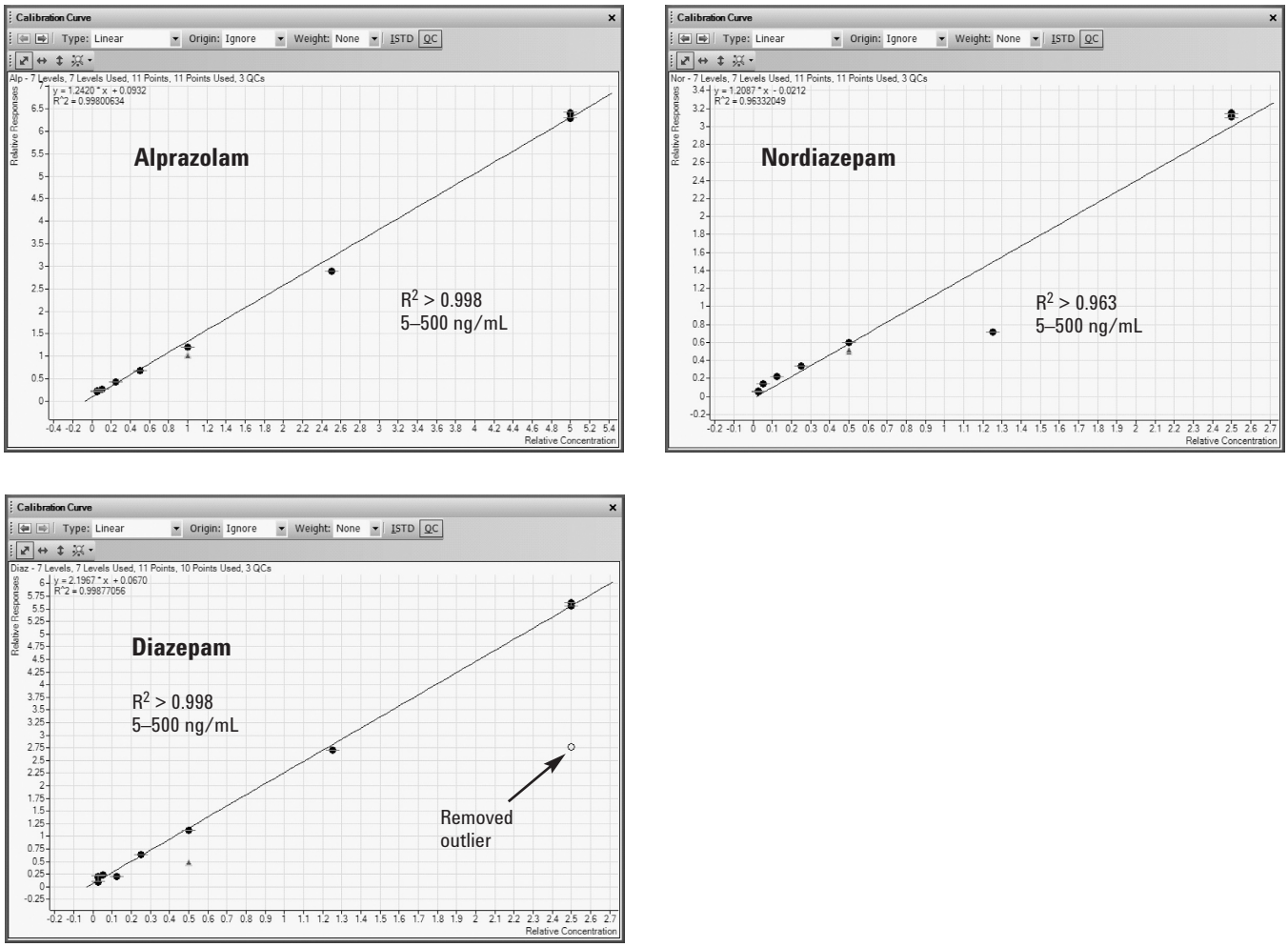


Figure 34. Calibration curves for alprazolam, nordiazepam, and diazepam in DUID analysis over 5 to 500 ng/mL concentration range.

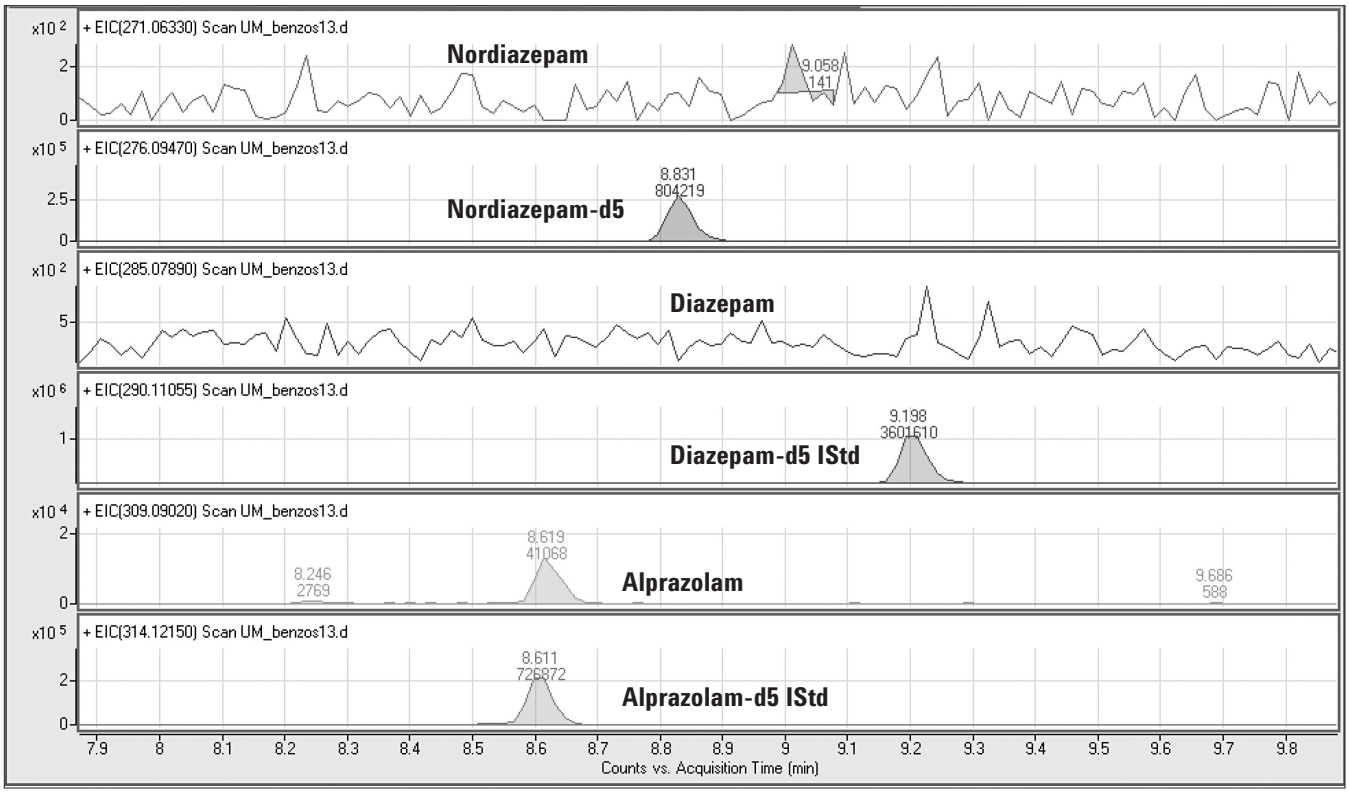


Figure 35. Calculated level of alprazolam is 0.5 ng/mL in DUID case sample 0024. Nordiazepam and diazepam were not detected.

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

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

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

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

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

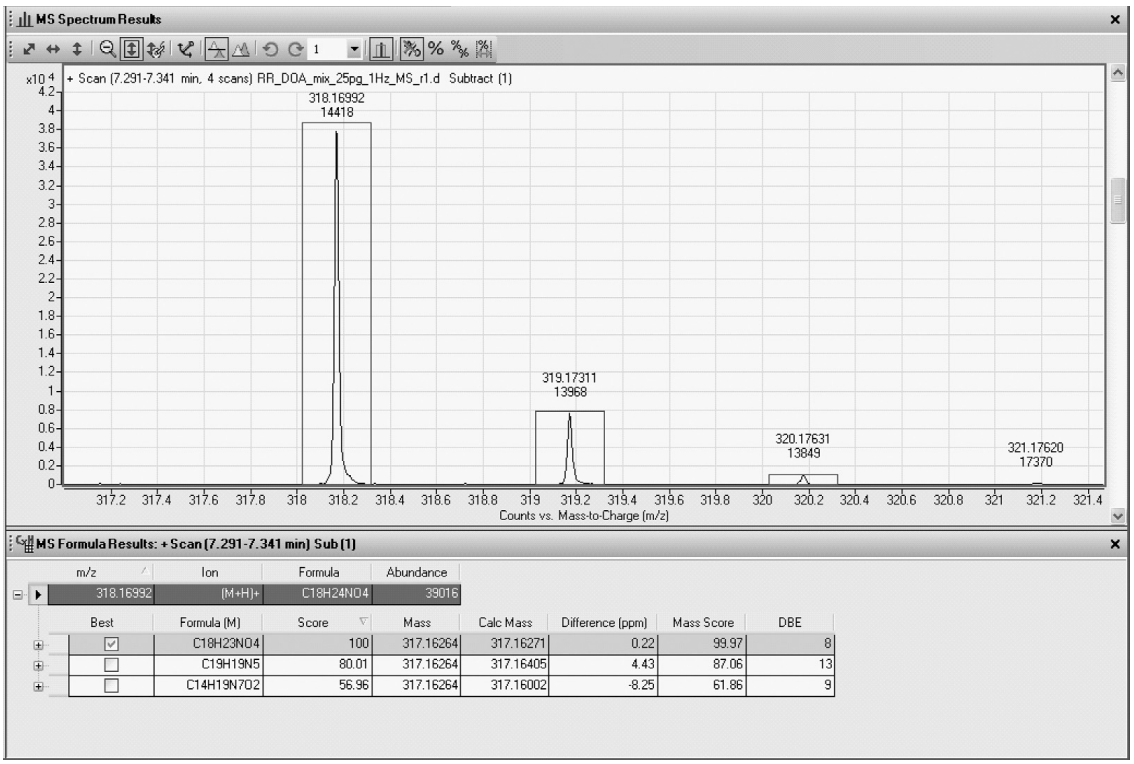


Figure 36. Confirming presence of cocaethylene using a molecular formula generator.

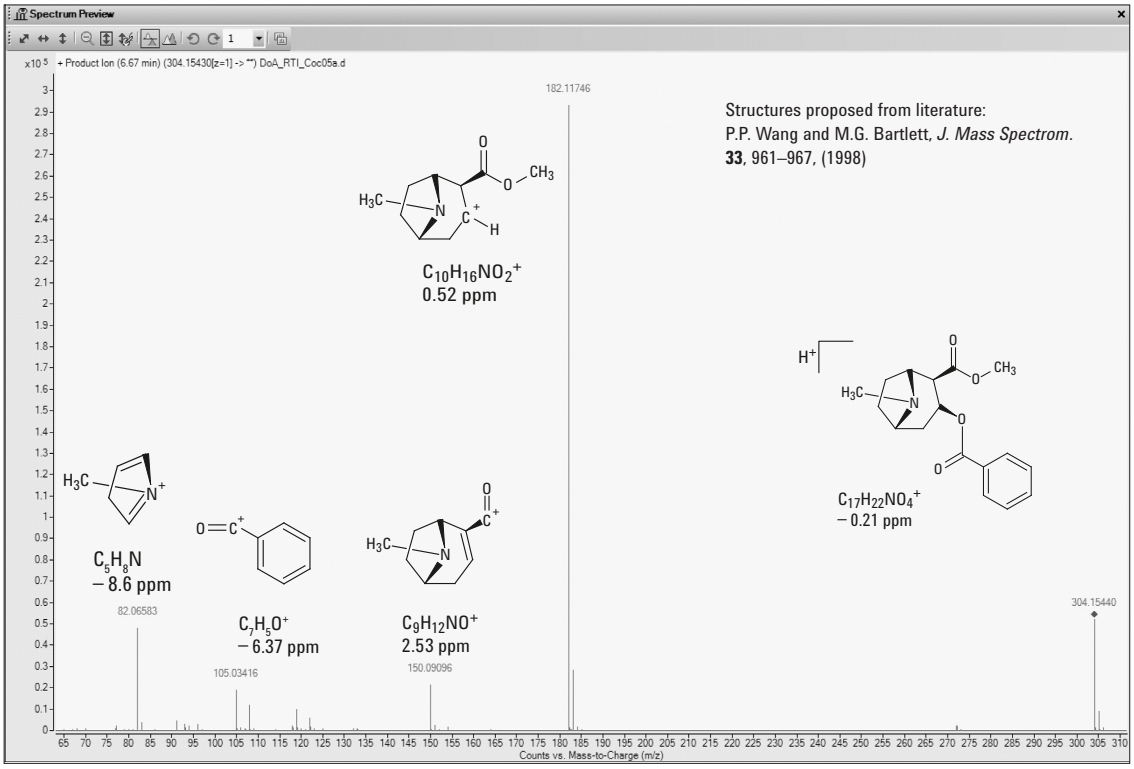


Figure 37. Targeted MS/MS of cocaine.

Conclusions

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

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

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

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

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

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

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

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