An Application Kit for the Screening of Samples for Analytes of Forensic Toxicological Interest using LC/QQQ MS/MS with a Dynamic MRM Transition Database

Application Note

Forensic Toxicology

Author

Peter JW Stone
Agilent Technologies Inc
5301 Stevens Creek Blvd
Santa Clara, CA, 95051
USA

Abstract

A forensic toxicology screening application kit has been developed for use with the Agilent 6400 Series triple quadrupole (QQQ) LC/MS systems which contains a database of optimized MRM transitions for approximately 200 analytes of interest. The database content is mainly focused on controlled substances and drugs of abuse. The aim of this application kit is to provide a user with a solid starting point for building forensic analysis methods where the ability to measure a large array of analytes is necessary. Typical results obtained from such a method created by using the database are described using serial dilutions of a test mix containing analytes of forensic interest.

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Introduction

Lists of potential toxins and analytes of forensic interest can be extremely large and typically depend on the area of analytical screening focus (for example, workplace drug testing, doping control, postmortem toxicology, explosive residues, and so forth). Often, the concentration levels of such target analytes are challenging and low, which can be further impacted by a complex sample matrix or the quantity of sample obtained.

The most sensitive liquid chromatography/mass spectrometry (LC/MS) screening or quantitation techniques are those based around triple quadrupole (QQQ) LC/MS/MS instruments, where a second stage of MS (post fragmentation from a collision cell) acts as an effective method of eliminating background chemical noise that is not associated with the target precursor and fragment ions. This technique is commonly referred to as Multiple Reaction Monitoring (MRM.) Instruments using each quadrupole as targeted mass filters in this manner are an effective and widely accepted technique for forensic toxicology studies of challenging sample matrices and concentration levels.

QQQ MS instruments, however, operate by focusing a finite amount of time on only one MRM transition before the next MRM transition is selected in turn. Once the complete list of target MRM transitions has been monitored, then the MRM list is repeated or cycled until the end of the chromatographic analysis or until a new retention time segment begins that contains different MRM transitions. The amount of finite time given to any specific MRM transition is referred to as dwell time and can be uniquely specified for every MRM transition.

The chromatographic consideration with regard to dwell time and overall MRM cycle time is one of peak width or resolution, normally referred to as full width at half maximum (FWHM). Statistically, higher numbers of data points measured across a chromatographic peak will provide more accurate and reproducible results. This means that the overall cycle time of the MRM target list must be sufficiently low to achieve this, relative to the particular chromatography used. Furthermore, each MRM transition dwell time must be high enough to output ion statistics of high quality and precision.

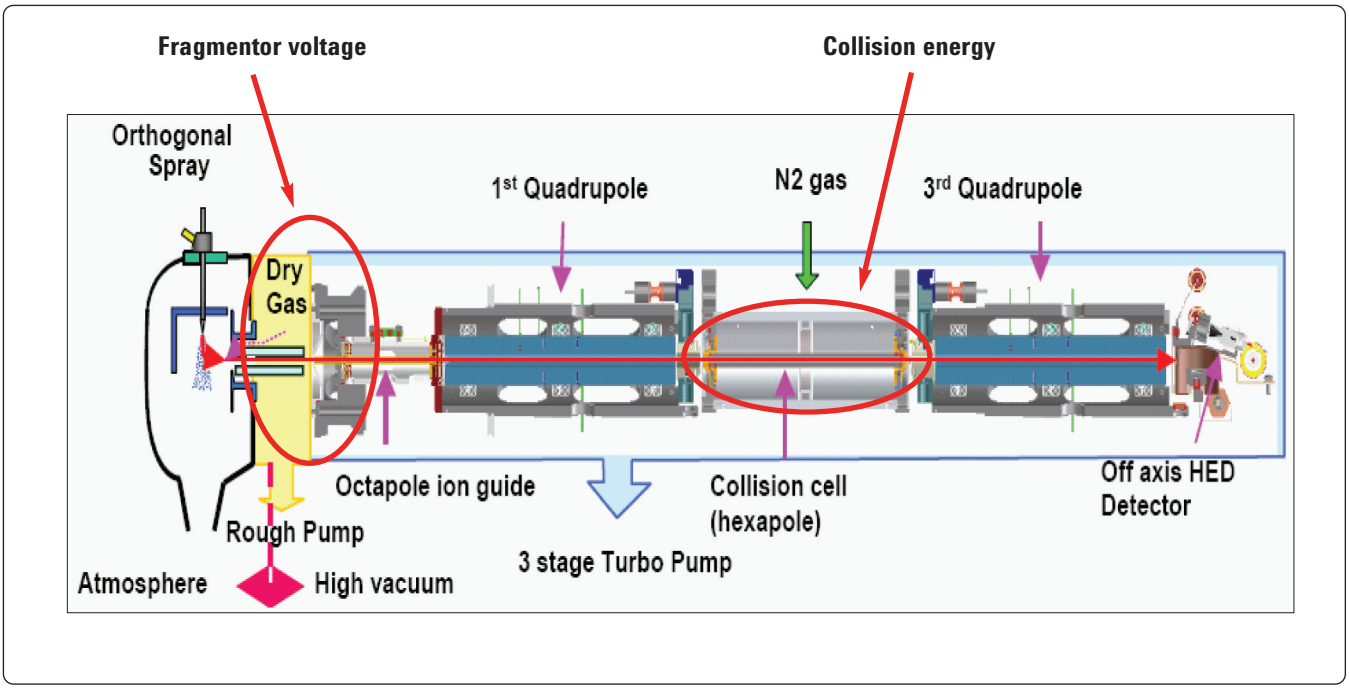

Figure 1. Two key optimized MRM transition settings.

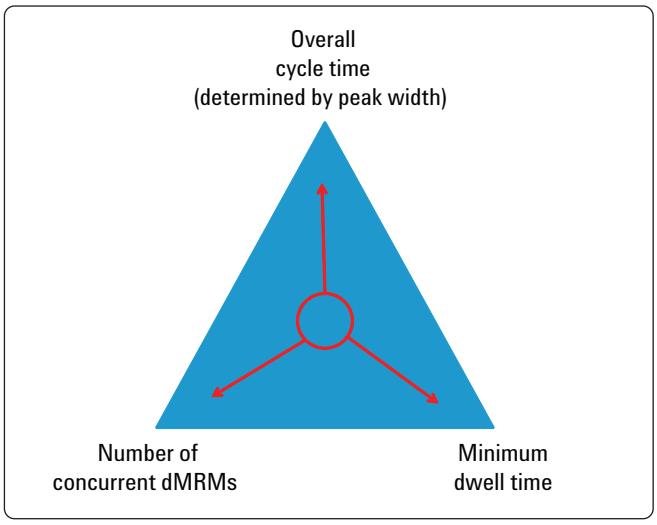

Figure 2. Compromise between cycle time, peak width, dwell time and number of MRM transitions.

Therefore, compromise between cycle time, dwell time and ultimately the total number of MRM transitions is often required especially with larger suites of analytes in a target screen assay (Figure 2). For this reason, Agilent Technologies introduced Dynamic MRM (dMRM) [1] functionality on the Agilent 6400 Series QQQ LC/MS system. Dynamic MRM is a technique where each ion transition has an associated retention time window (delta RT) where it is dynamically switched on and off without impacting a constant data cycle time. Since the complete list of ion transitions is unlikely to be cycled through at any given chromatographic retention time, then the result is normally higher dwell time for every transition and higher data quality when compared to normal MRM methods. Figure 3 graphically illustrates the Dynamic MRM principle.

Herein are described the results obtained from an analysis method using the Agilent MassHunter Forensics and Toxicology Dynamic MRM Database Kit (G1734AA) with optimized MRM transitions from the database inserted directly into the acquisition method. More detailed instruction on the creation of such methods are outlined in the G1734AA

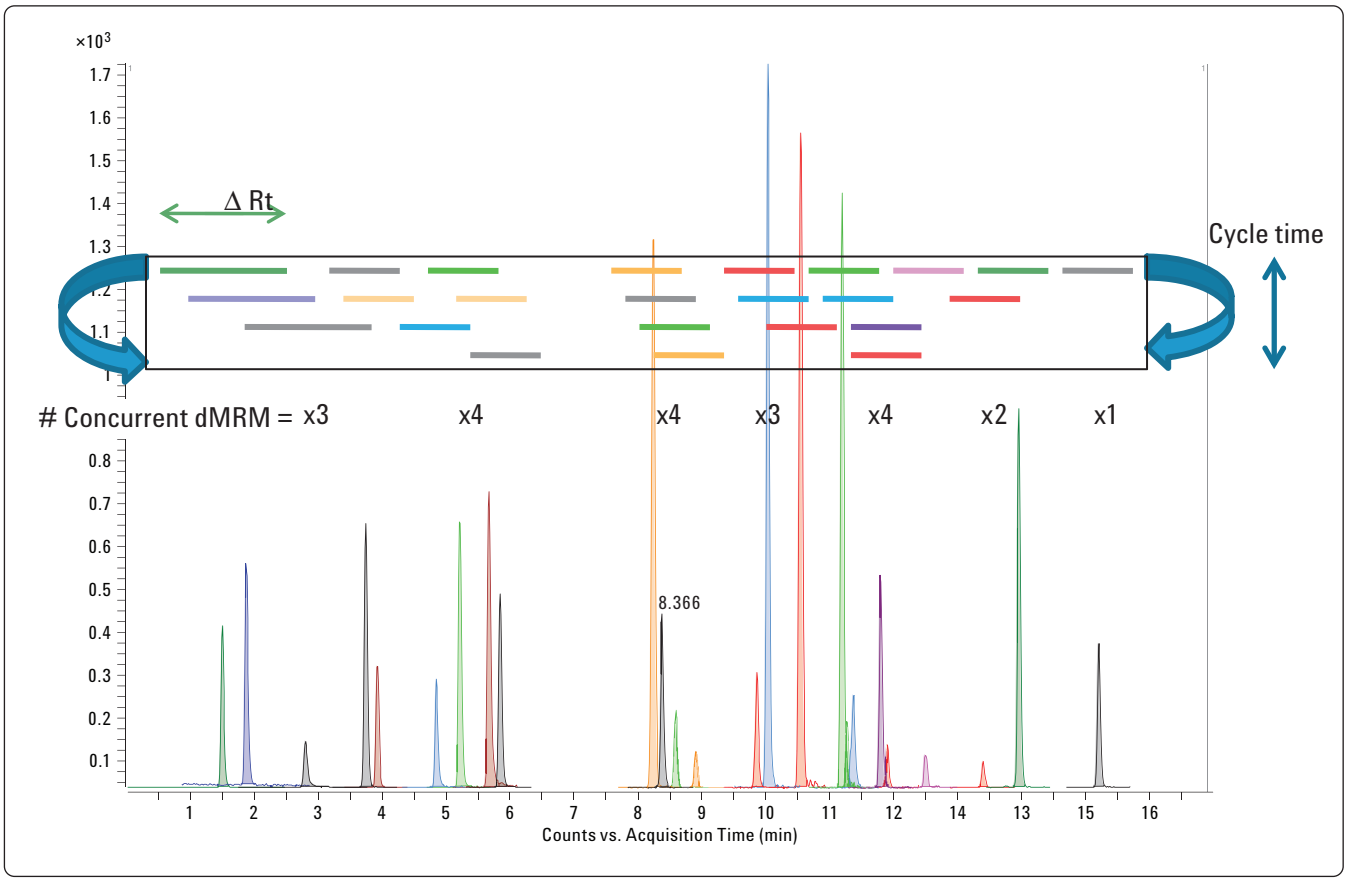

Figure 3. Illustration of Dynamic MRM principle.

MassHunter Forensics and Toxicology Dynamic MRM Database Kit Quick Start Guide [2]. Confirmatory evidence was obtained by using the two most abundant MRM transitions for use as quantifier and qualifier ions, the ratio of which are indicative of the analyte of interest. This application note aims to describe typical results using an LC/MS Forensics and Toxicology Test Mix.

Experimental

The analysis results outlined in this application note were obtained using an Agilent 6460 QQQ LC/MS coupled to an Agilent 1200SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system equipped with the MassHunter Optimizer program (Rev. B.02.01) and the [G1734AA] Forensics and Toxicology Dynamic MRM Database Kit.

Sample Preparation

An ampoule from the LC/MS Forensics and Toxicology Test Mix [p/n 5190-0470] which is included in the Forensics and Toxicology Dynamic MRM Database Kit [G1734AA] was opened and 100 µL of the 1 µg/mL (1ppm) solution was diluted to a concentration of 10 ng/mL (10 ppb) using 9.9 mL of pure LC/MS grade methanol to create a clean solvent standard for method checkout purposes.

Appropriate serial dilutions from the original LC/MS Forensics and Toxicology Test Mix were created for the purposes of quantitation. These are listed in Table 1.

Table 1. Dilution Series of LC/MS Forensics and Toxicology Test Mix

Data File	Type	Level	Vol. (uL)	Conc.	Units
LCMS_Forensics and Toxicology Test Mix 10fg.d	Cal	1	1	10	fg on-column
LCMS_Forensics and Toxicology Test Mix 25fg.d	Cal	2	1	25	fg on-column
LCMS_Forensics and Toxicology Test Mix 50fg.d	Cal	3	1	50	fg on-column
LCMS_Forensics and Toxicology Test Mix 100fg.d	Cal	4	1	100	fg on-column
LCMS_Forensics and Toxicology Test Mix 250fg.d	Cal	5	1	250	fg on-column
LCMS_Forensics and Toxicology Test Mix 500fg.d	Cal	6	1	500	fg on-column
LCMS_Forensics and Toxicology Test Mix 1pg.d	Cal	7	1	1000	fg on-column
LCMS_Forensics and Toxicology Test Mix 5pg.d	Cal	8	1	5000	fg on-column
LCMS_Forensics and Toxicology Test Mix 10pg.d	Cal	9	1	10000	fg on-column
LCMS_Forensics and Toxicology Test Mix 25pg.d	Cal	10	1	25000	fg on-column
LCMS_Forensics and Toxicology Test Mix 50pg.d	Cal	11	1	50000	fg on-column

Table 2 outlines the composition of the LC/MS Forensics and Toxicology Test Mix [p/n 5190-0470] which is intended to cover a wide and representative range of forensic analyte classes.

Table 2. LC/MS Forensics and Toxicology Test Mix Components (1µg/mL)

Compound Name	Formula	Mass
3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107
Cocaine	$C_{17}H_{21}NO_4$	303.14706
Codeine	$C_{18}H_{21}NO_3$	299.15214
delta9-Tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458
Diazepam	$C_{16}H_{13}ClN_{2O}$	284.07164
Heroin	$C_{21}H_{23}NO_5$	369.15762
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723
Methadone	$C_{21}H_{27}NO$	309.20926
Methamphetamine	$C_{10}H_{15}N$	149.12045
Methylendioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091
Oxycodone	$C_{18}H_{21}NO_4$	315.14706
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.1987
Phentermine	$C_{10}H_{15}N$	149.12045
Proadifen	$C_{23}H_{31}NO_2$	353.23548
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316

Reagents and Chemicals

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

Instrumentation

LC Conditions

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

Gradient program:

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

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

6460 QQQ LC/MS Conditions

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

Positive ionization polarity

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

6410 QQQ LC/MS Conditions
(Results not included in this application note.)

Source Conditions:
Electrospray AP-ESI:

Positive ionization polarity

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

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

Dynamic MRM Acquisition Method Parameters

Table 3. Dynamic MRM Method Conditions

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Codeine	–	300.2	Unit	165.1	Unit	158	45	1.11	0.4	Positive
Codeine	–	300.2	Unit	58.1	Unit	158	29	1.11	0.4	Positive
Oxycodone	–	316.2	Unit	298.1	Unit	143	17	1.285	0.4	Positive
Oxycodone	–	316.2	Unit	256.1	Unit	143	25	1.285	0.4	Positive
δ-Amphetamine	–	136.1	Unit	119.1	Unit	66	5	1.296	0.4	Positive
δ-Amphetamine	–	136.1	Unit	91	Unit	66	17	1.296	0.4	Positive
MDA	–	180.1	Unit	163	Unit	61	5	1.332	0.4	Positive
MDA	–	180.1	Unit	105	Unit	61	21	1.332	0.4	Positive
Hydrocodone	–	300.2	Unit	199	Unit	159	29	1.4	0.4	Positive
Hydrocodone	–	300.2	Unit	128	Unit	159	65	1.4	0.4	Positive
Methamphetamine	–	150.1	Unit	119	Unit	92	5	1.45	0.4	Positive
Methamphetamine	–	150.1	Unit	91	Unit	92	17	1.45	0.4	Positive
MDMA	–	194.1	Unit	163	Unit	97	9	1.468	0.4	Positive
MDMA	–	194.1	Unit	105	Unit	97	25	1.468	0.4	Positive
Strychnine	–	335.2	Unit	184	Unit	195	41	1.629	0.4	Positive
Strychnine	–	335.2	Unit	156	Unit	195	53	1.629	0.4	Positive
MDEA	–	208.1	Unit	163	Unit	107	9	1.735	0.4	Positive
MDEA		208.1	Unit	105	Unit	107	25	1.735	0.4	Positive
Heroine		370.2	Unit	268.1	Unit	149	37	2.256	0.4	Positive
Heroin		370.2	Unit	165	Unit	149	61	2.256	0.4	Positive
Cocaine		304.2	Unit	182.1	Unit	138	17	2.376	0.4	Positive
Cocaine		304.2	Unit	77	Unit	138	61	2.376	0.4	Positive
Meperidine		248.2	Unit	220.1	Unit	128	21	2.419	0.4	Positive
Meperidine		248.2	Unit	174.1	Unit	128	17	2.419	0.4	Positive
Trazodone		372.2	Unit	176	Unit	159	25	2.797	0.4	Positive
Trazodone		372.2	Unit	148	Unit	159	37	2.797	0.4	Positive
PCP		244.2	Unit	91	Unit	86	41	2.876	0.4	Positive
PCP	–	244.2	Unit	86.1	Unit	86	9	2.876	0.4	Positive
Oxazepam	–	287	Unit	269	Unit	150	12	3.53	0.4	Positive
Oxazepam	–	287	Unit	241	Unit	150	20	3.53	0.4	Positive
Nitrazepam	–	282.1	Unit	236.1	Unit	148	25	3.542	0.4	Positive
Nitrazepam	–	282.1	Unit	180	Unit	148	41	3.542	0.4	Positive
Verapamil	–	455.3	Unit	165	Unit	158	37	3.554	0.4	Positive
Verapamil	–	455.3	Unit	150	Unit	158	45	3.554	0.4	Positive
Methadone	–	310.2	Unit	265.1	Unit	112	9	3.61	0.4	Positive
Methadone	–	310.2	Unit	105	Unit	112	29	3.61	0.4	Positive
Lorazepam	–	321	Unit	275	Unit	102	21	3.626	0.4	Positive
Lorazepam	–	321	Unit	194	Unit	102	49	3.626	0.4	Positive
Alprazolam	–	309.1	Unit	281	Unit	179	25	3.727	0.4	Positive
Alprazolam	–	309.1	Unit	205	Unit	179	49	3.727	0.4	Positive
Temazepam	–	301.1	Unit	255.1	Unit	117	29	3.941	0.4	Positive

Table 3. Dynamic MRM Method Conditions (continued)

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Temazepam	–	301.1	Unit	177	Unit	117	45	3.941	0.4	Positive
Proadifen	–	354.2	Unit	167	Unit	153	29	4.088	0.4	Positive
Proadifen	–	354.2	Unit	91.1	Unit	153	45	4.088	0.4	Positive
Diazepam	–	285.1	Unit	193	Unit	169	45	4.268	0.4	Positive
Diazepam	–	285.1	Unit	154	Unit	169	25	4.268	0.4	Positive
THC	–	315.2	Unit	193.2	Unit	150	20	5.277	0.4	Positive
THC	–	315.2	Unit	123.3	Unit	150	30	5.277	0.4	Positive

Results and discussion

Fast and easy startup with Agilent Test Mix

In order to rapidly implement and verify that acquisition and data analysis methodology is correctly set up, the LC/MS Forensics and Toxicology Test Mix [p/n 5190-0470] is included in the Forensics and Toxicology Dynamic MRM Database Kit [G1734AA] which contains a representative range of forensic analyte classes of 25 components (Table 2).

To create a method from first principles, the required transitions are selected from the database browser window (Figure 4). Once each selection has been made, the transitions are transferred to the acquisition method by clicking the 'Import' button to the bottom right of the browser window. An example of an acquisition method is illustrated in Figure 5.

Detailed information on this operation is contained in the MassHunter Forensics and Toxicology Dynamic MRM Database Kit Quick Start Guide [2].

Using the methodology outlined in the experimental section, a 1-uL injection of the 10 ng/mL LC/MS Forensics and Toxicology Test Mix equates to a 10 pg on-column injection amount. Figure 6 illustrates a typical overlay of extracted compound chromatograms for the test mix. A prepared method for QQQ is included in the application kit. When this method is loaded all conditions are correct and the user is able to reproduce the analysis.*

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

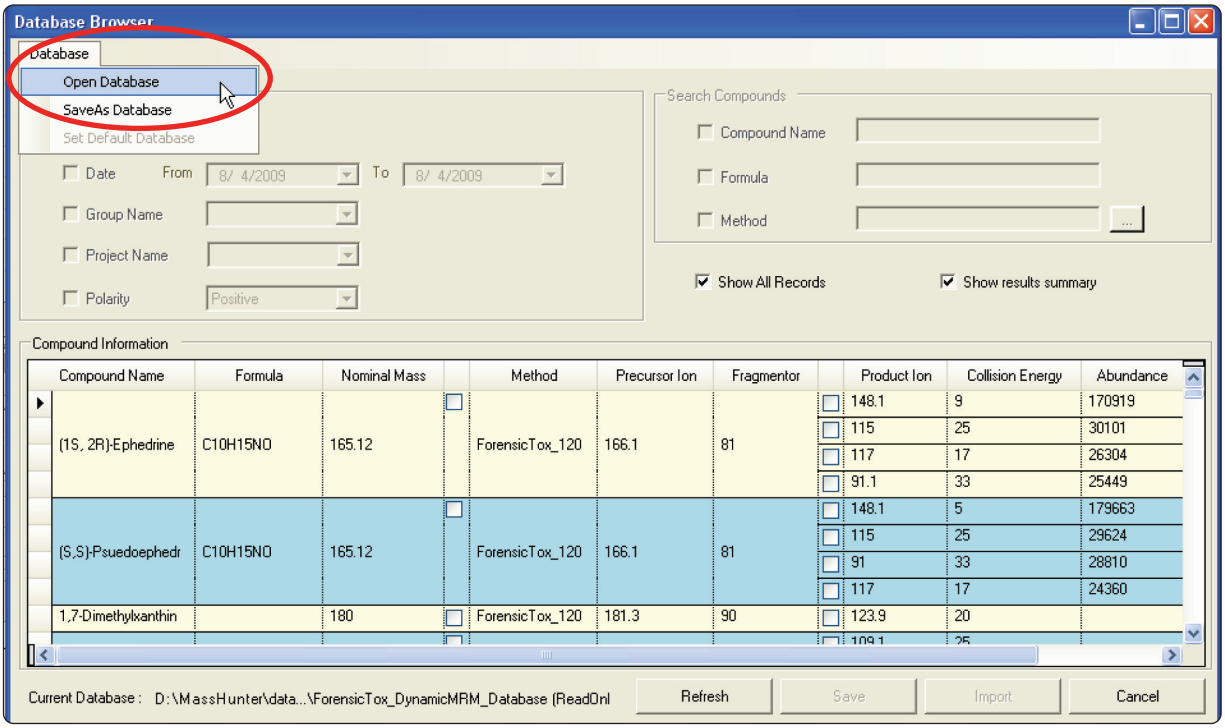

Figure 4. Compound MRM database browser containing 200 forensic analytes.

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragmentor	Collision Energy	Ret Time (min)	Delta Ret Time	Polarity
Alprazolam	☐	309.1	Unit	281	Unit	179	25	3.715	1	Positive
Cocaine	☐	304.2	Unit	182.1	Unit	138	17	2.358	1	Positive
d-Amphetamine	☐	136.1	Unit	91	Unit	66	17	1.278	1	Positive
Diazepam	☐	285.1	Unit	154	Unit	169	25	4.269	1	Positive
Heroin	☐	370.2	Unit	165	Unit	149	61	2.236	1	Positive
Hydrocodone	☐	300.2	Unit	199	Unit	159	29	1.38	1	Positive
Lorazepam	☐	321	Unit	275	Unit	102	21	3.61	1	Positive
MDA	☐	180.1	Unit	163	Unit	61	5	1.311	1	Positive
MDEA	☐	208.1	Unit	163	Unit	107	9	1.72	1	Positive

Dynamic MRM Parameters

Cycle Time 500 ms

Figure 5. Scan segments table with Dynamic MRM transitions imported database browser.

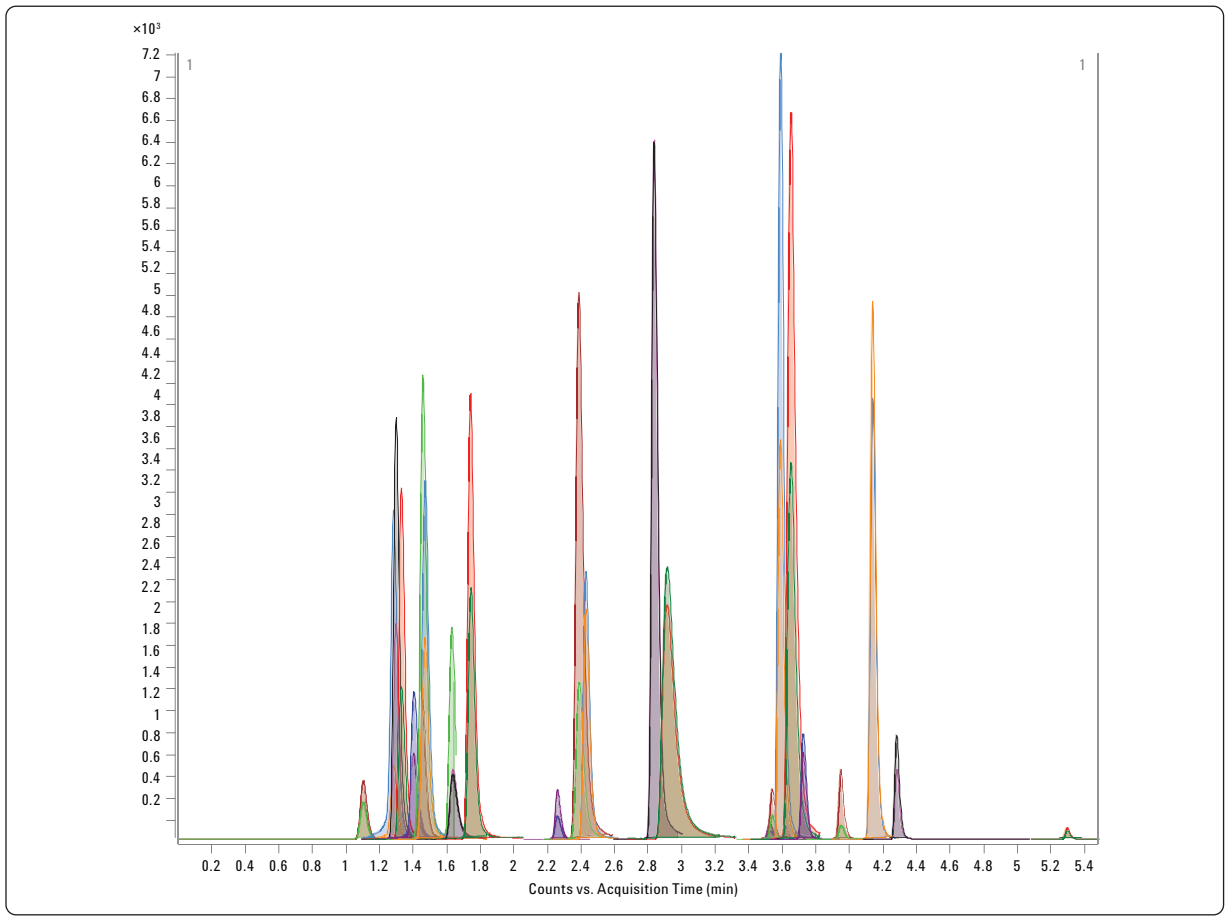

Figure 6. Example LC/MS Forensics and Toxicology test mix 10 pg on-column extracted ion chromatogram (overlay).

Quantitative analysis and standard curves

By using a Dynamic MRM acquisition method, the series of LC/MS Forensics and Toxicology Test Mix dilutions (Table 1) were analyzed according to the procedure outlined in the experimental section. All 50 Dynamic MRM transitions were used and Table 4 summarizes the results for the limits of detection and linearity of each component in the 25-component test mix.

Table 4. Limits of Detection and Calibration Linearity Results

Compound Name	Limit of Detection (fg on-column)	Linearity Correlation
3,4-Methylendioxyamphetamine (MDA)	50	0.99817
3,4-Methylenedioxyethamphetamine (MDEA)	10	0.99743
Alprazolam	50	0.99755
Clonazepam	100	0.99501
Cocaine	10	0.99755
Codeine	50	0.99841
δ9-Tetrahydrocannabinol (THC)	50	0.99869
Diazepam	10	0.99896
Heroin	25	0.99863
Hydrocodone	25	0.99493
Lorazepam	100	0.99601
Meperidine (Pethidine)	10	0.99687
Methadone	10	0.99666
Methamphetamine	10	0.98750
Methylendioxymethamphetamine (MDMA)	25	0.99217
Nitrazepam	25	0.99712
Oxazepam	250	0.99544
Oxycodone	50	0.99804
Phencyclidine (PCP)	25	0.99659
Phentermine	50	0.99898
Proadifen	<5	0.99772
Strychnine	50	0.99496
Temazepam	25	0.99751
Trazodone	<5	0.99777
Verapamil	<5	0.99787

Figures 7 through 10 illustrate the calibration curves through the range of 10-50000 fg on-column for six of the analytes from the LC/MS Forensics and Toxicology Test Mix.

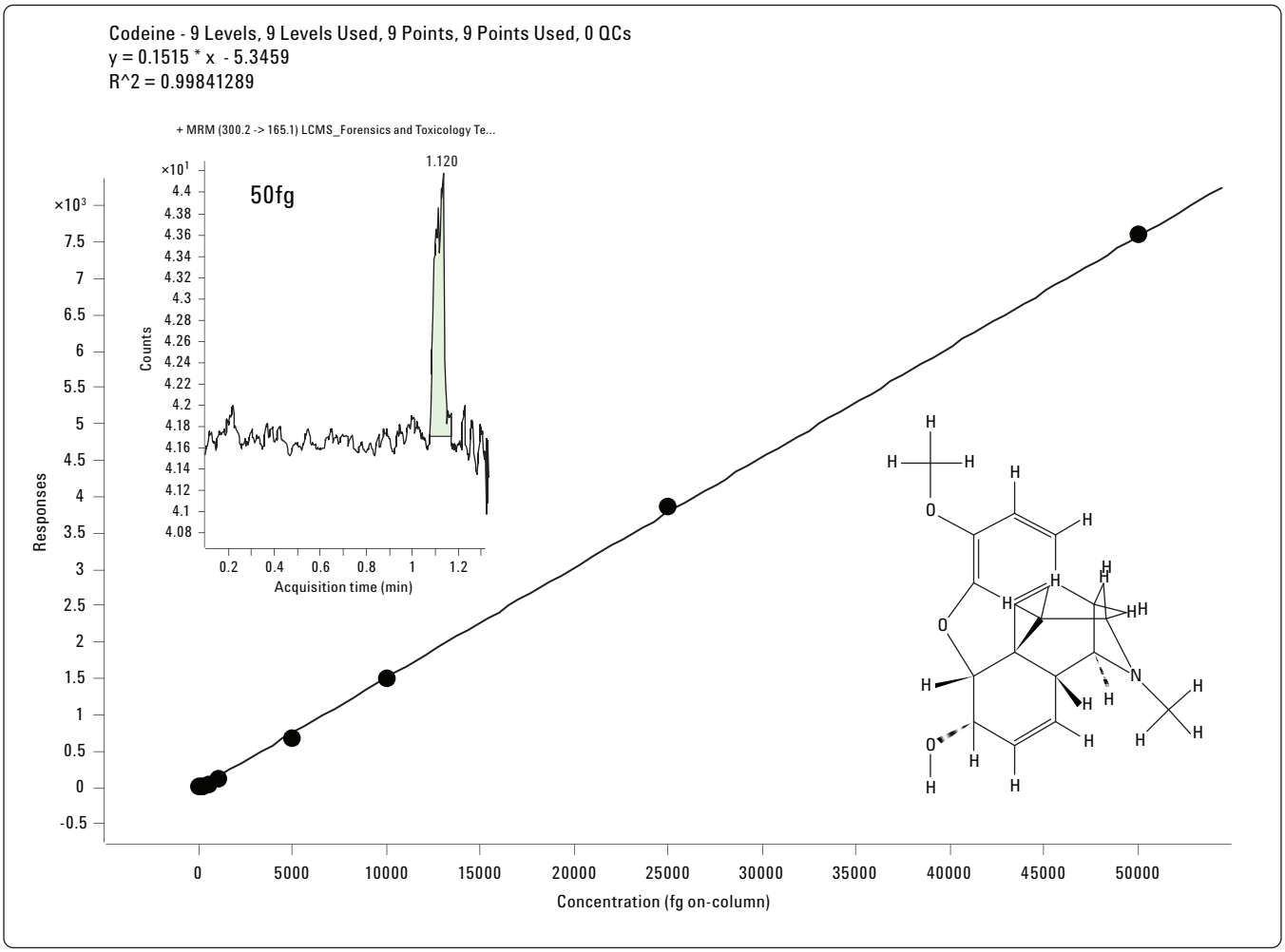

Figure 7. Calibration curve and LOD chromatogram, codeine.

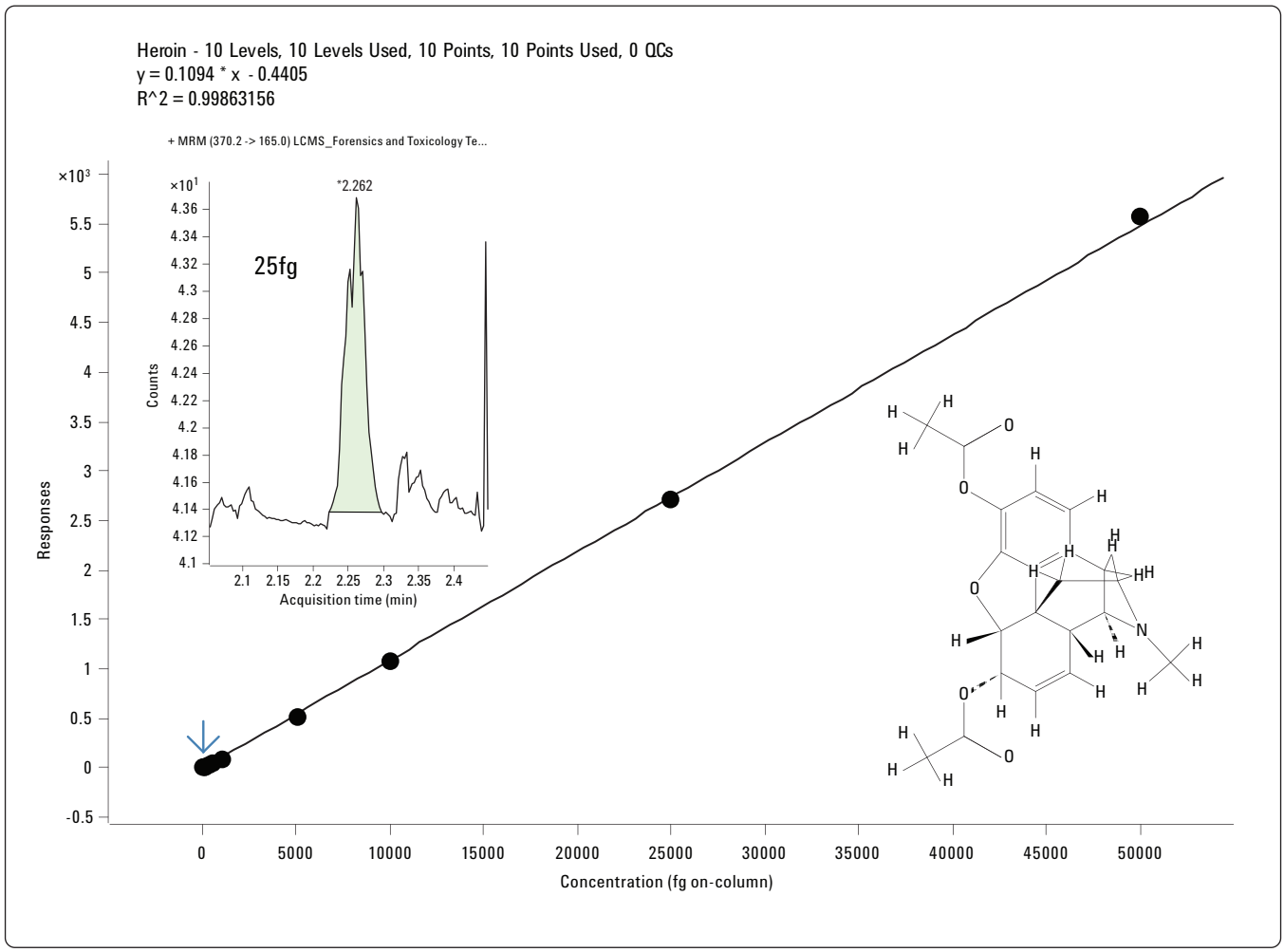

Figure 8. Calibration curve and LOD chromatogram, heroin.

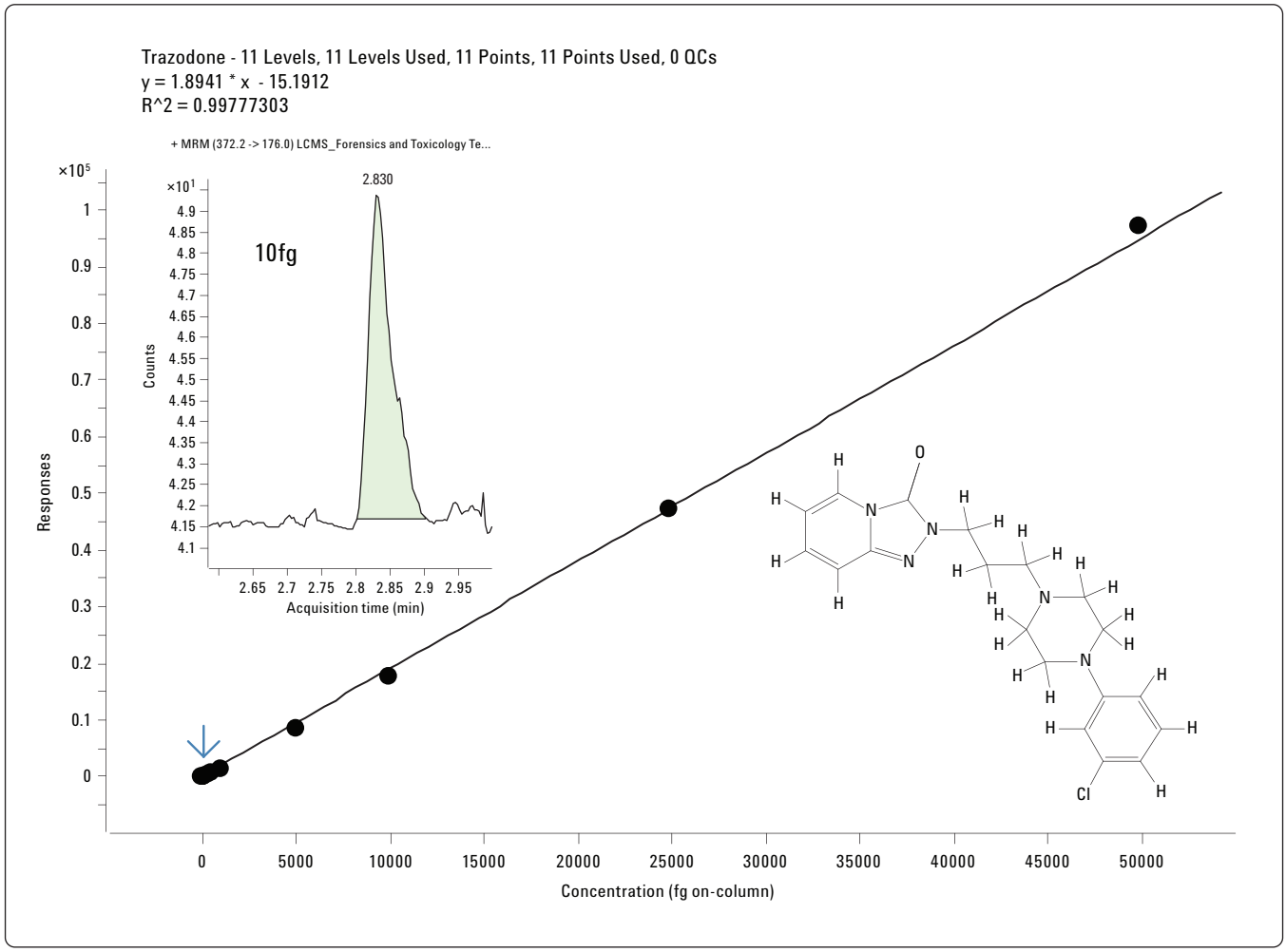

Figure 9. Calibration curve and LOD chromatogram, trazodone.

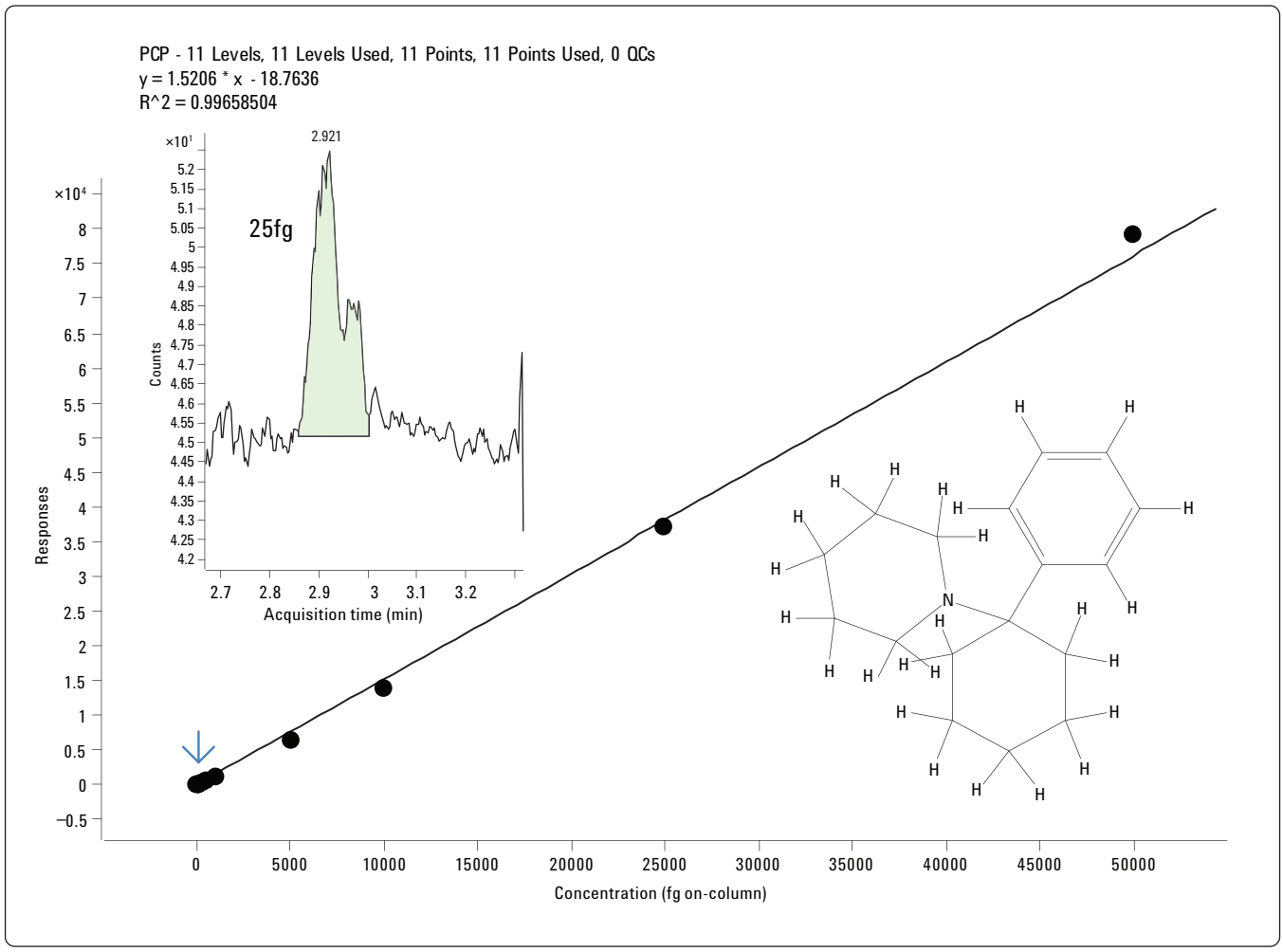

Figure 10. Calibration curve and LOD chromatogram, phencyclidine (PCP).

Conclusions

The Agilent MassHunter Forensics and Toxicology Dynamic MRM Database Kit provides a user with faster method development capability for 200 analytes with up to 4 MRM transitions for each. These methods can be used equally for screening or for more focused and dedicated analyte quantitation dependant on specific needs.

This application note briefly outlines the type of results that could be obtained by using database optimized MRM parameters with the appropriate chromatography conditions and MS ion source settings.

The kit offers:

- Fast and easy startup of complex analyses.
- An optimized MRM transition database of approximately 200 compounds.
- Completely customizable with additional optimized transitions to the database.
- Example chromatography with ready to use methods inclusive of test sample and chromatography column.
- Automatic re-optimization of transition parameters using the MassHunter Optimizer program for particular instrument conditions and method revalidation.

References

1. "New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses," Agilent application note publication 5990-3595EN.
2. "Agilent G1734AA MassHunter Forensics and Toxicology Dynamic MRM Database Kit Quick Start Guide." Agilent Technologies publication 5990-4265EN

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