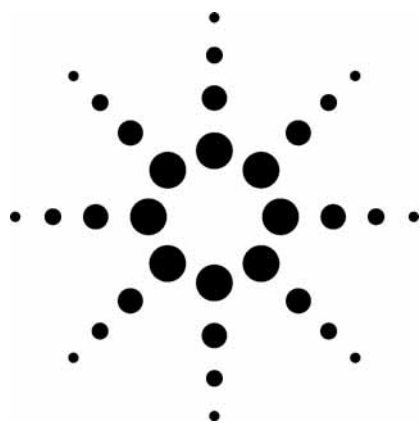


# Quantitative Analysis of Opiates in Urine Using RRHT LC/MS/MS



## 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- $\mu$ m particle size column. Both quantifier and qualifier ions are monitored for each analyte, with the requirement that the qualifier/quantifier ion ratio be within  $\pm 20\%$  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/ $\mu$ L in urine. The range for 6-MAM is 0.067 to 10 pg/ $\mu$ L. Following extraction, which corresponds to a factor of 6.78 decrease in concentration, the injected

concentrations range from 0.147 to 22.12 pg/ $\mu$ L, 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.



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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  $\pm 20\%$  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  $\pm 20\%$  is used, along with only one qualifier ion.

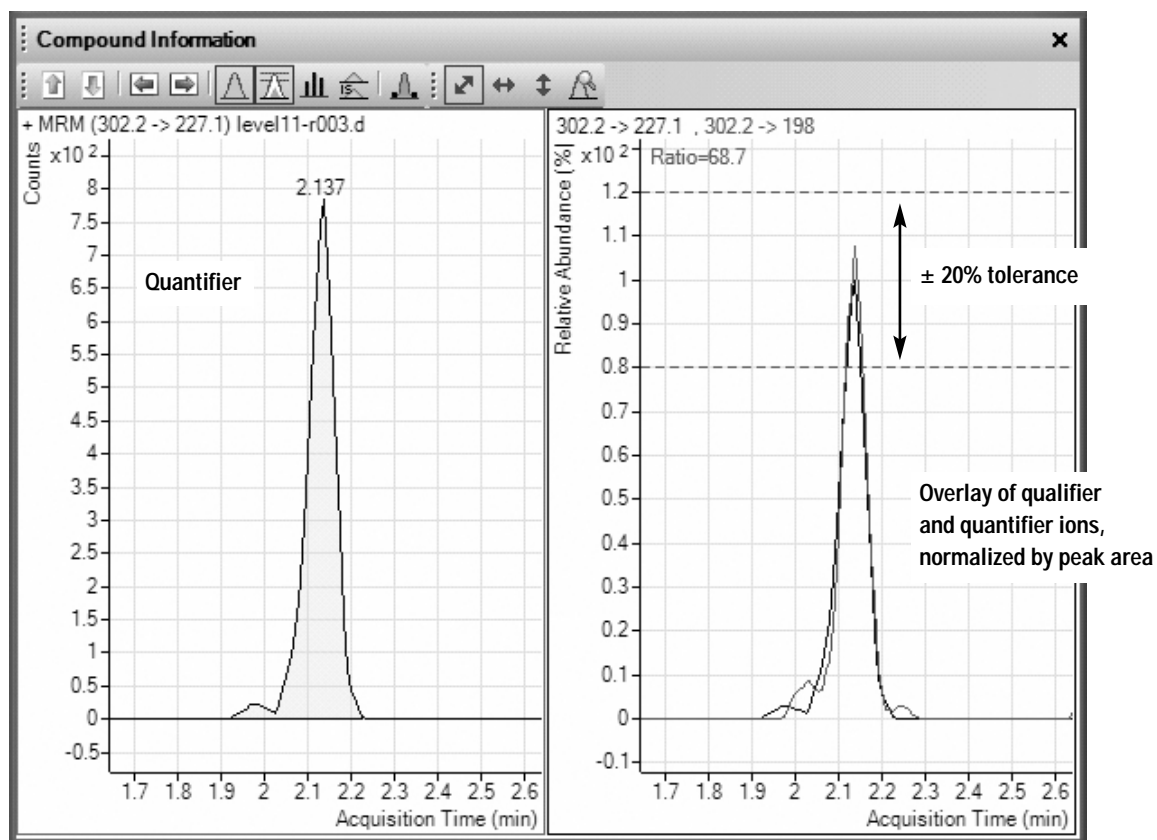


Figure 1. Qualifier/quantifier ion ratios for confirmation of oxymorphone.

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.

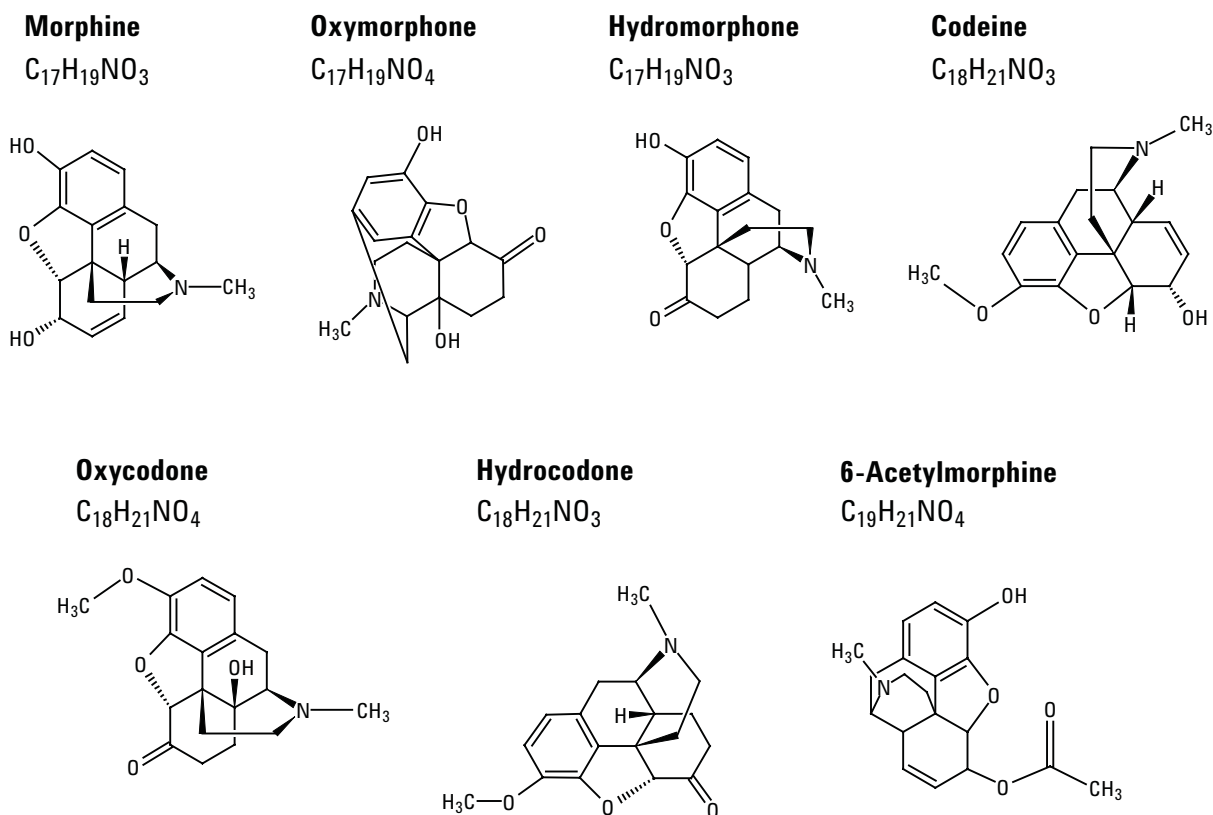


Figure 2. Structures of the opiates analyzed in this work.

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- $\mu$ m 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 optimization 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/ $\mu$ L, and a factor of 15 times lower for 6-MAM. These samples were then processed using the following procedure:

1. Start with 250- $\mu$ L sample size
2. Add 500  $\mu$ L sodium acetate buffer
3. Add 20  $\mu$ L glucuronidase
4. Add 75  $\mu$ L 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  $\mu$ L de-ionized water
8. Vortex and spin down
9. Place 200  $\mu$ L 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/ $\mu$ L concentration in urine has an actual concentration of 147 fg/ $\mu$ L 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/ $\mu$ L. With a 5- $\mu$ L 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  $\times$  50 mm, 1.8- $\mu$ m particle size  
(PN: 822700-902)

Column temp: 50 °C

Mobile phase: A = water  
B = acetonitrile

Flow rate: 0.4 mL/min

Injection volume: 5  $\mu$ L

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<sub>cap</sub>: 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/ $\mu$ L (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).

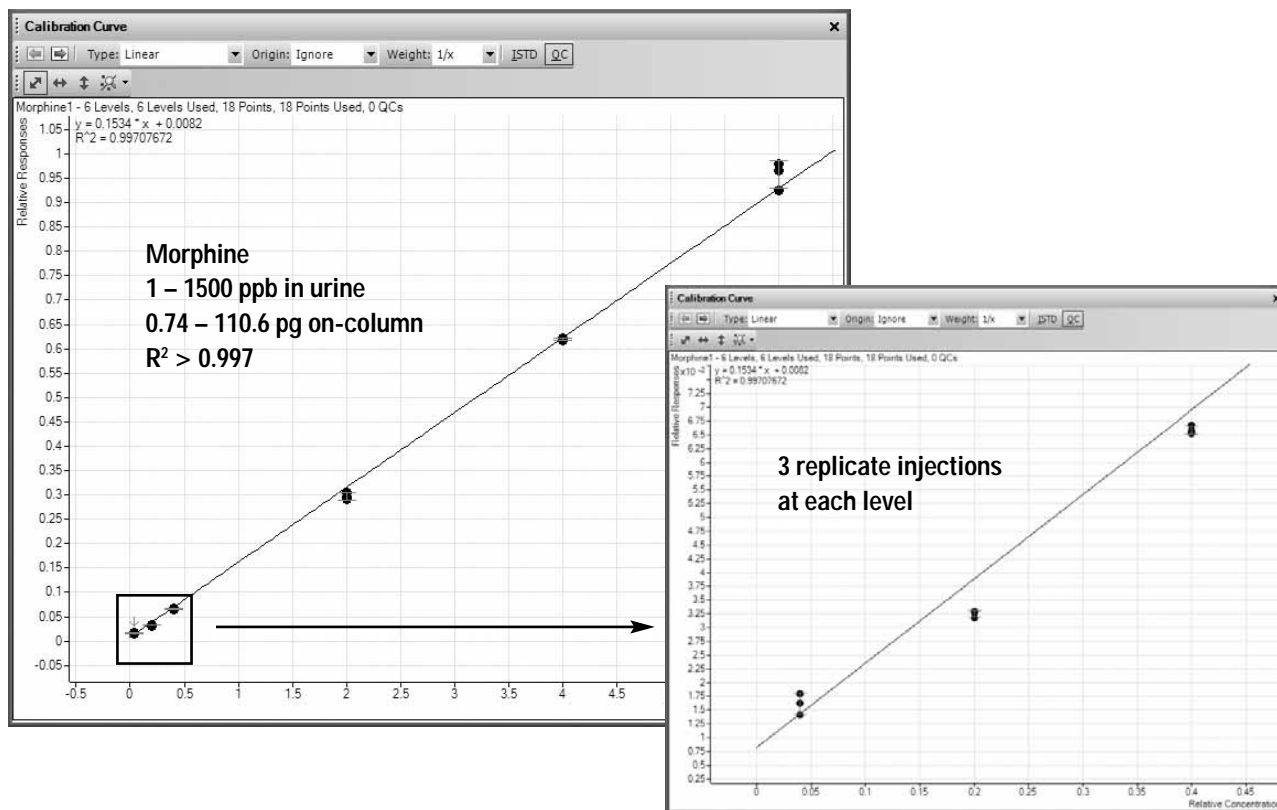


Figure 3A. Linearity of morphine in urine. Injection concentration range = 147 ppt – 22 ppb.

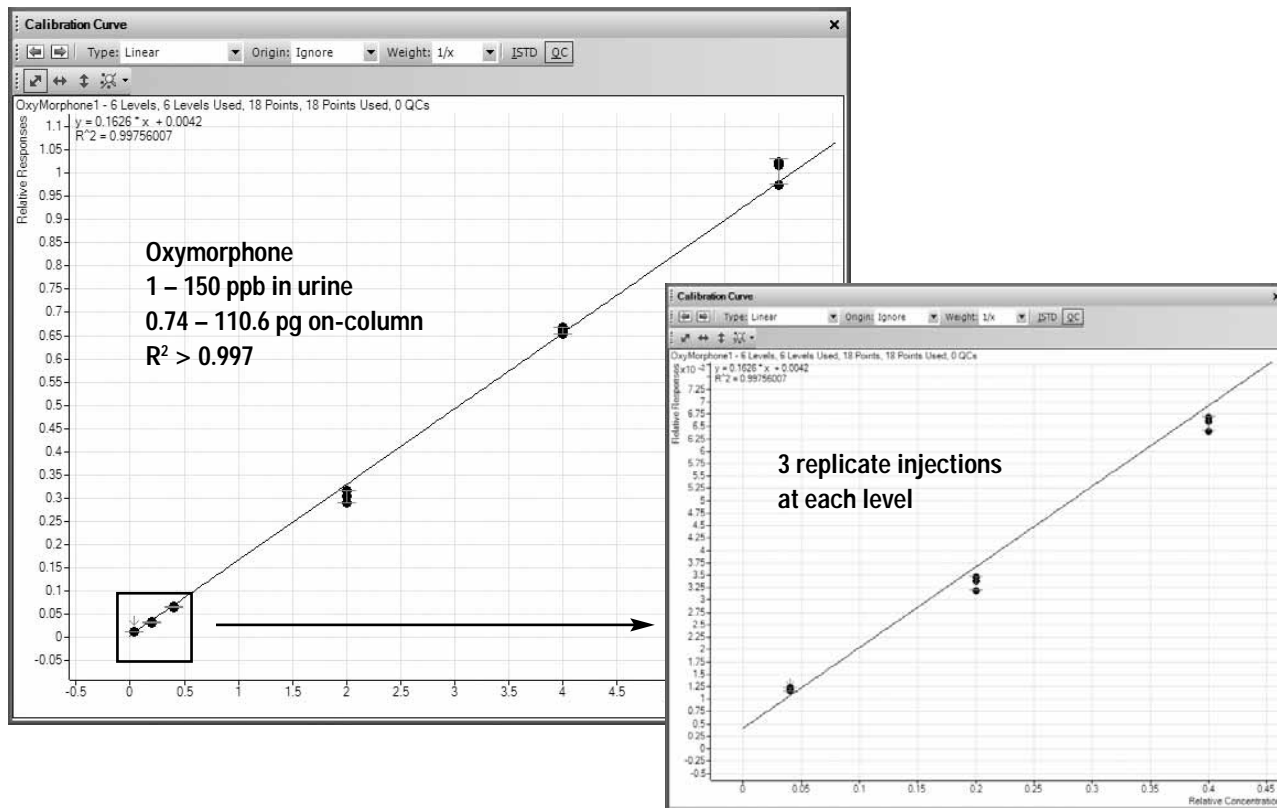


Figure 3B. Linearity of oxymorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

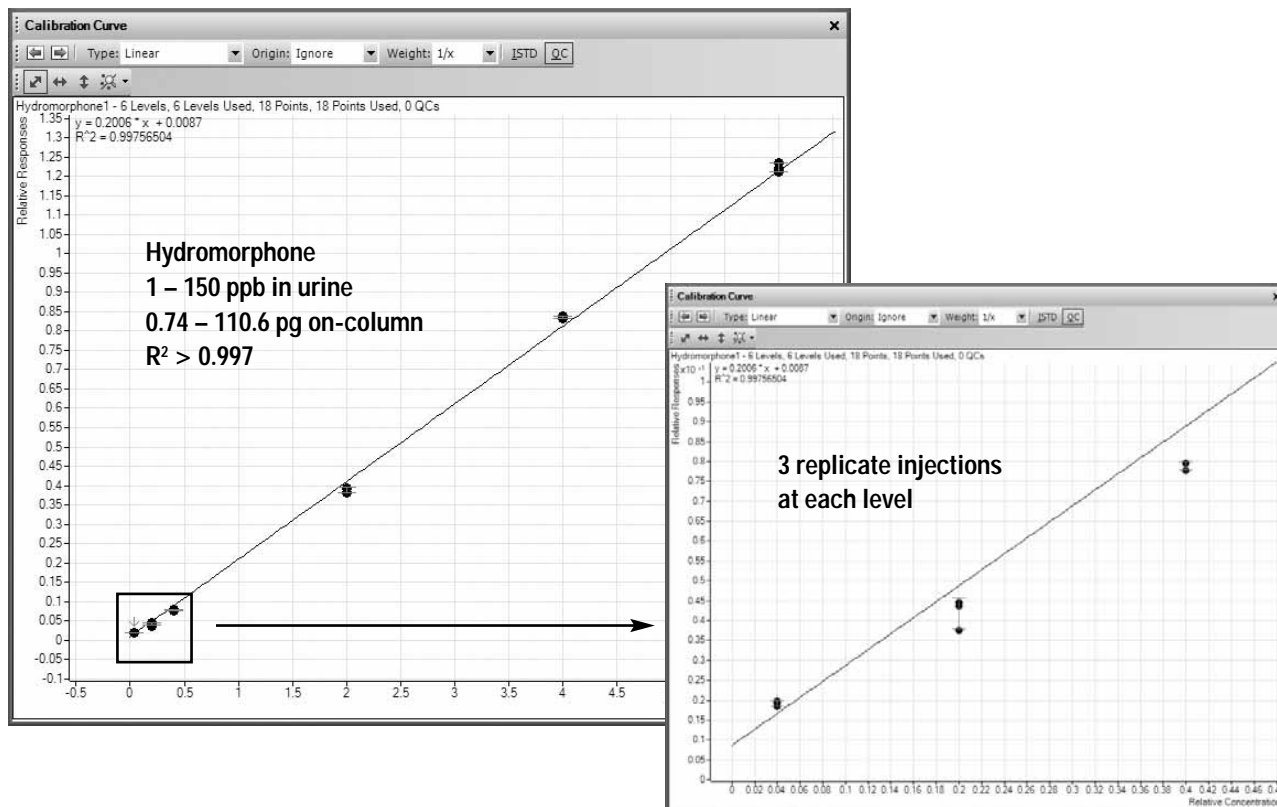


Figure 3C. Linearity of hydromorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

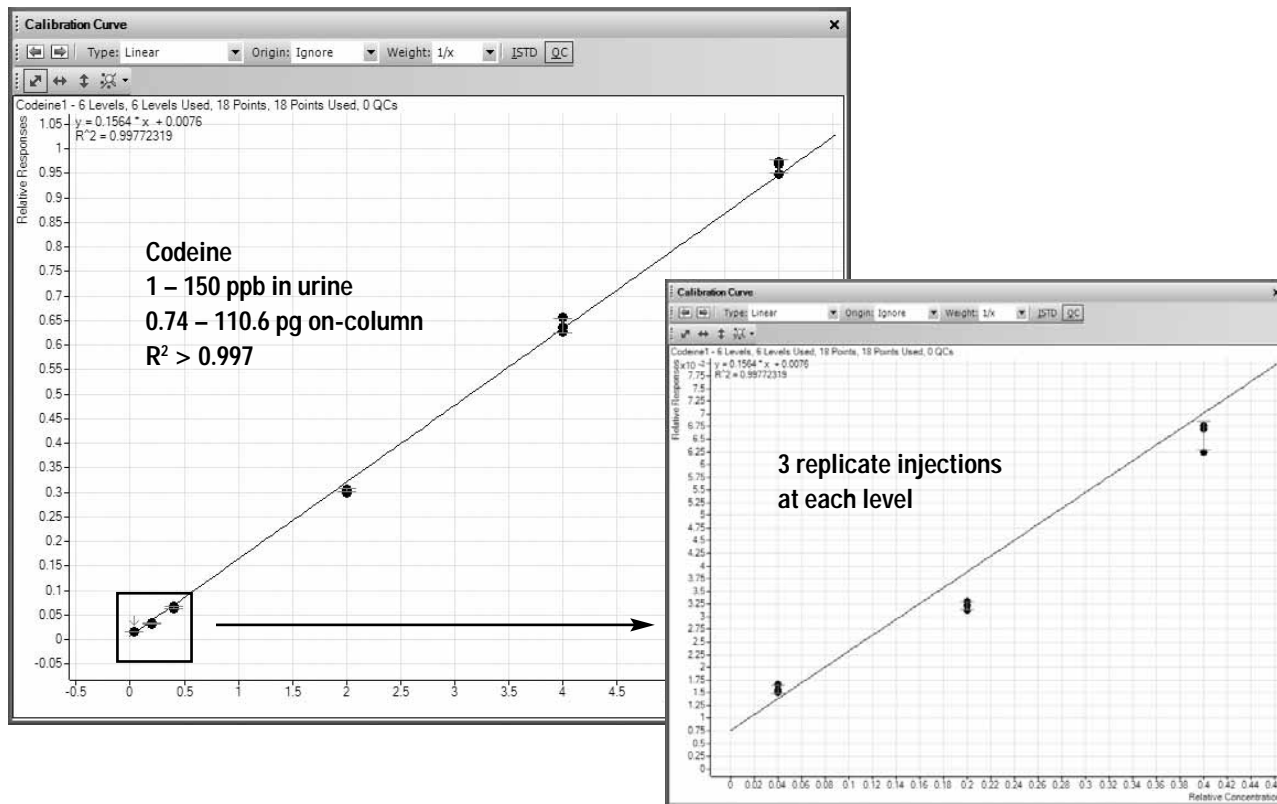


Figure 3D. Linearity of codeine in urine. Injection concentration range = 147 ppt – 22 ppb.

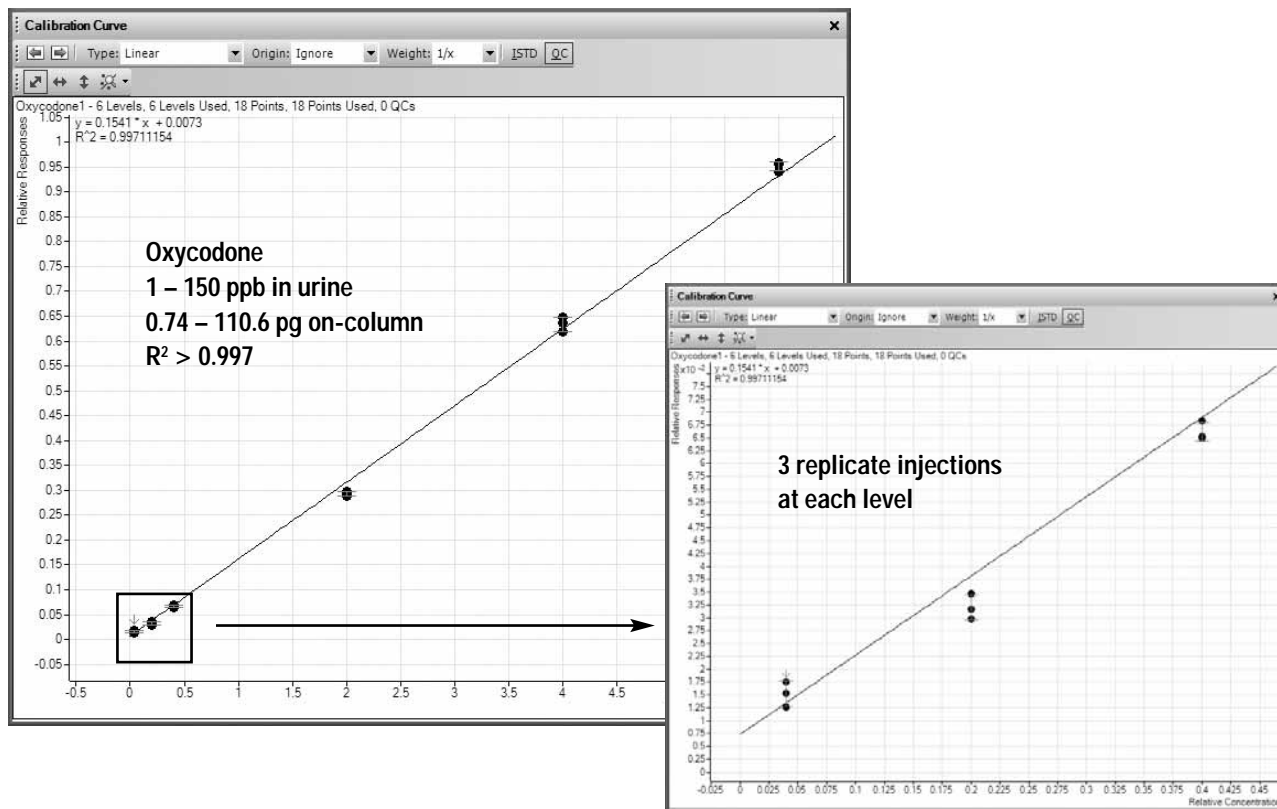


Figure 3E. Linearity of oxycodone in urine. Injection concentration range = 147 ppt – 22 ppb.

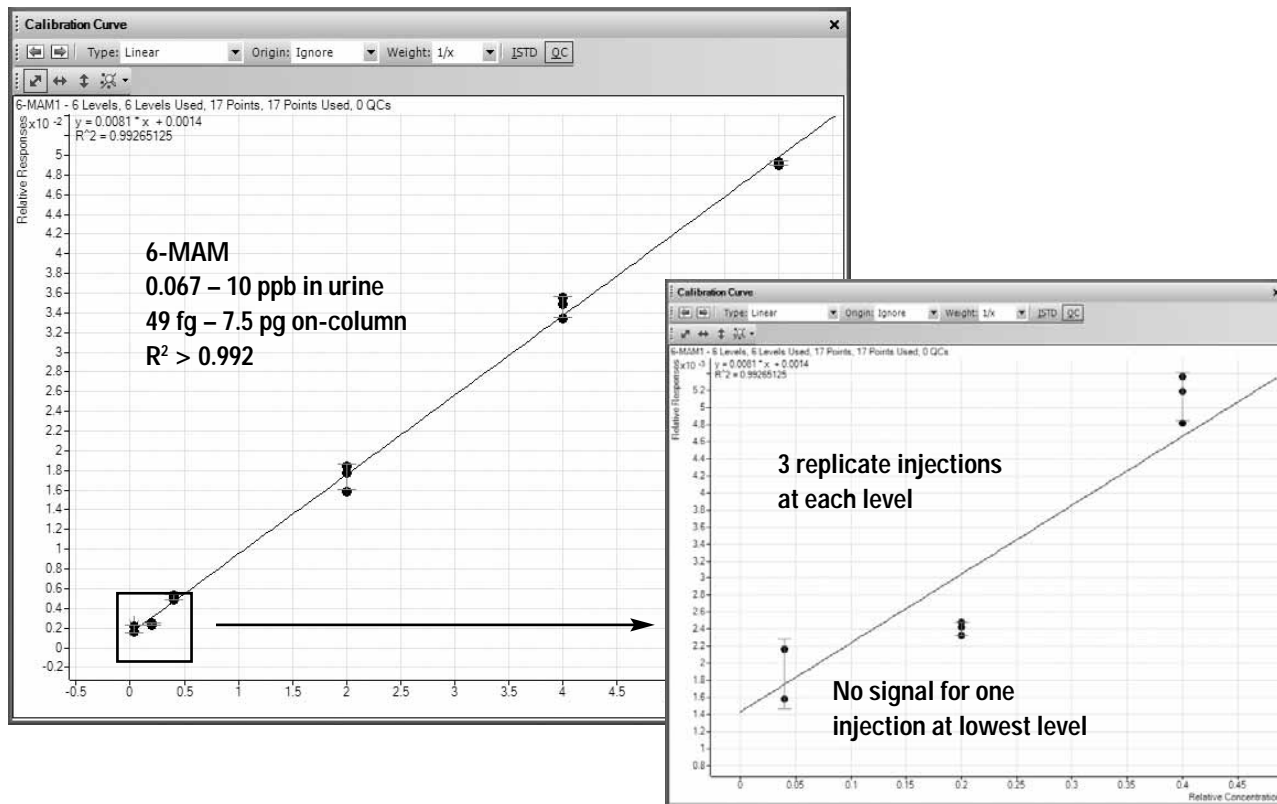


Figure 3F. Linearity of 6-MAM in urine. Injection concentration range = 9.8 ppt – 1.5 ppb.

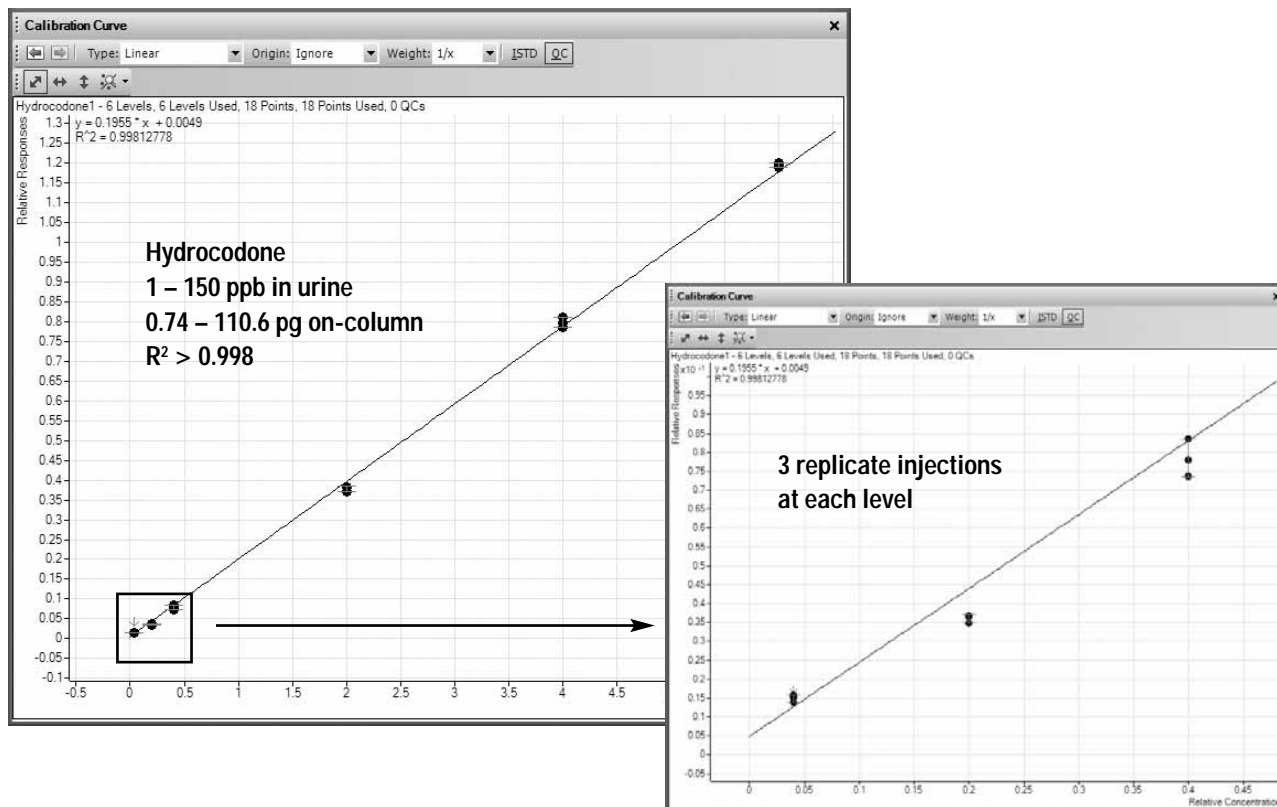


Figure 3G. Linearity of hydrocodone in urine. Injection concentration range = 147 ppt – 22 ppb.

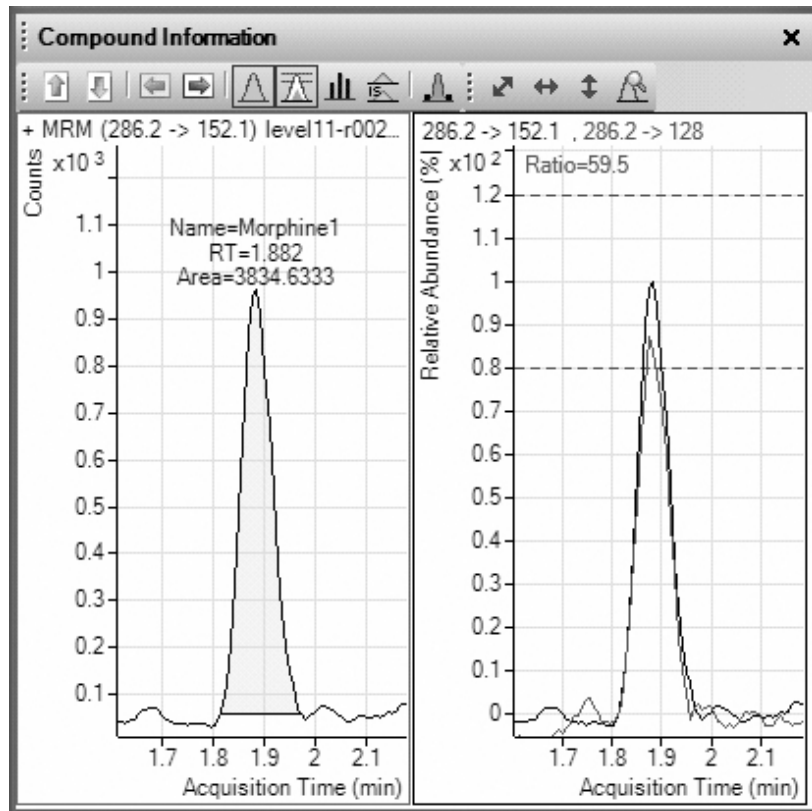


Confirmation is carried out by examining the qualifier/quantifier ion ratio and making sure it stays within  $\pm 20\%$  of the determined value for each analyte. For example, after optimizing the MRM transitions for both product ions of morphine, it is automatically determined by the MassHunter Quantitative Analysis that the ratio of the qualifier peak to that of the quantifier should be 0.7%, or 70%. Applying a  $\pm 20\%$  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/ $\mu$ L in urine. For

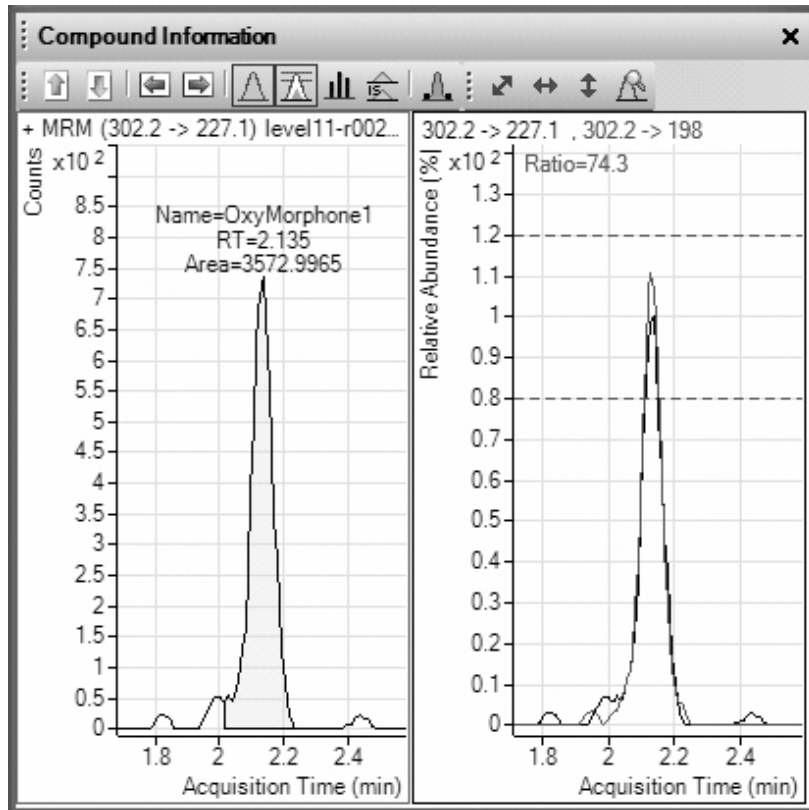
oxycodone and 6-MAM, the lowest levels are 5 and 0.3 pg/ $\mu$ L, respectively.

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- $\mu$ L injections at 147 fg on-column each. These correspond to original concentrations in urine of 1 pg/ $\mu$ L. For oxycodone, the LOD is determined from the triplicate 5- $\mu$ L injections of the calibration level corresponding to 1 pg/ $\mu$ L (see Figure 5E). Like oxycodone, the LOD of 6-MAM is seen at a 5- $\mu$ L injection, but of the 0.067 pg/ $\mu$ L 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.



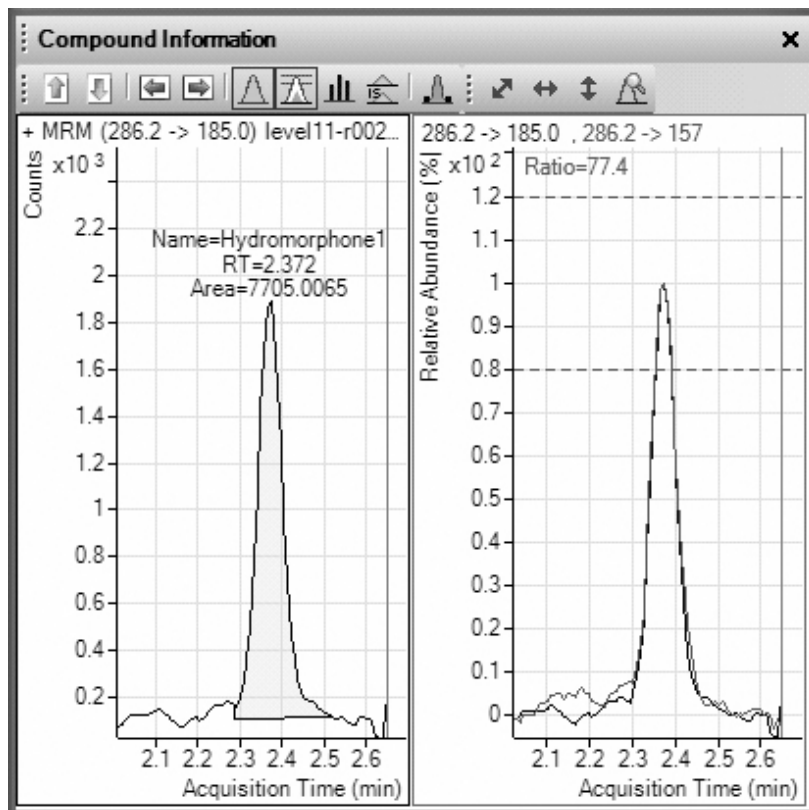
0.74 fg on-column

Figure 4A. Confirmation of morphine at 1 pg/ $\mu$ L (147 fg/ $\mu$ L).



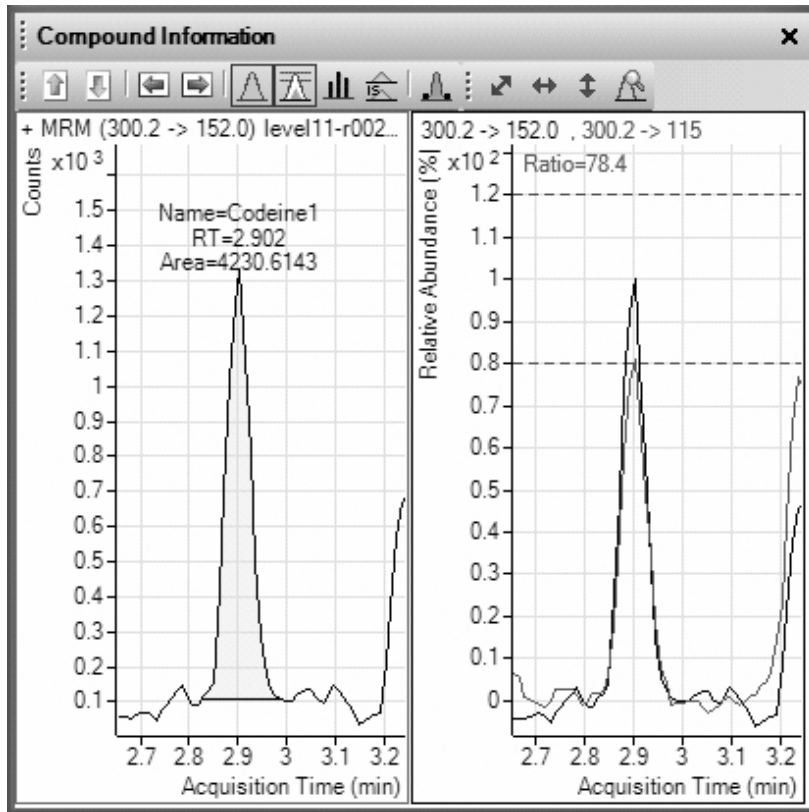
0.74 fg on-column

Figure 4B. Confirmation of oxymorphone at 1 pg/ $\mu$ L (147 fg/ $\mu$ L).



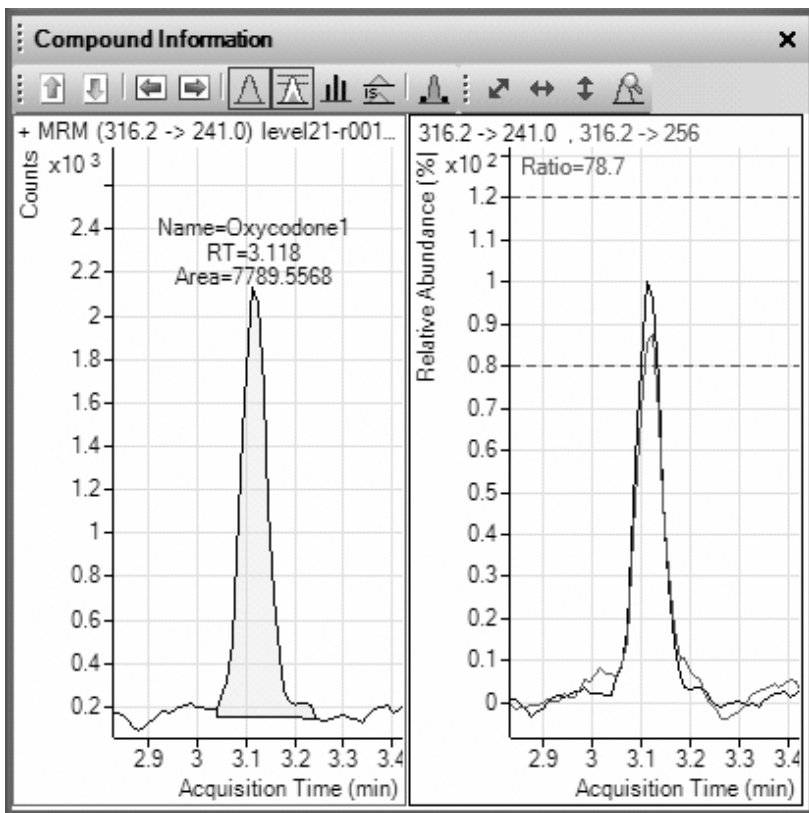
0.74 fg on-column

Figure 4C. Confirmation of hydromorphone at 1 pg/ $\mu$ L (147 fg/ $\mu$ L).



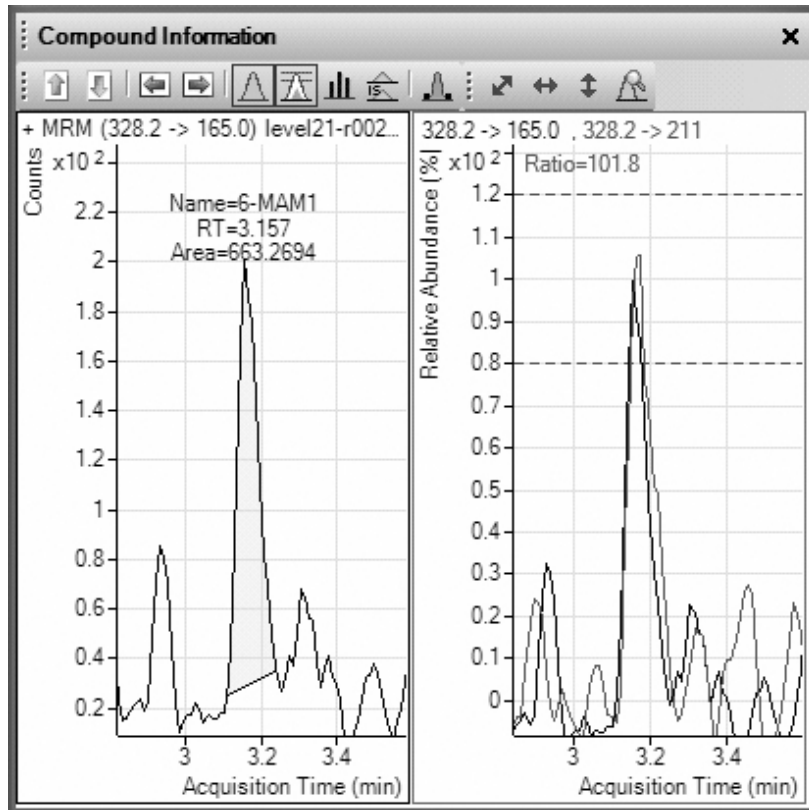
0.74 fg on-column

Figure 4D. Confirmation of codeine at 1 pg/ $\mu$ L (147 fg/ $\mu$ L).



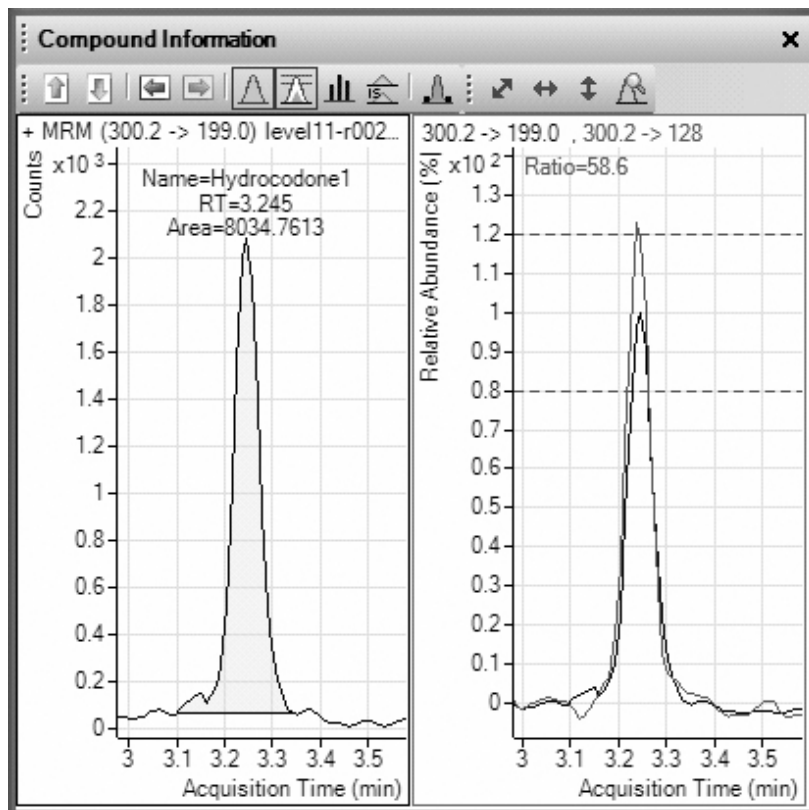
3.7 pg on-column

Figure 4E. Confirmation of oxycodone at 5 pg/ $\mu$ L (737 fg/ $\mu$ L).



245 fg on-column

Figure 4F. Confirmation of 6-MAM at 0.3 pg/ $\mu$ L (49 fg/ $\mu$ L).



0.74 pg on-column

Figure 4G. Confirmation of hydrocodone at 1 pg/ $\mu$ L (147 fg/ $\mu$ L).

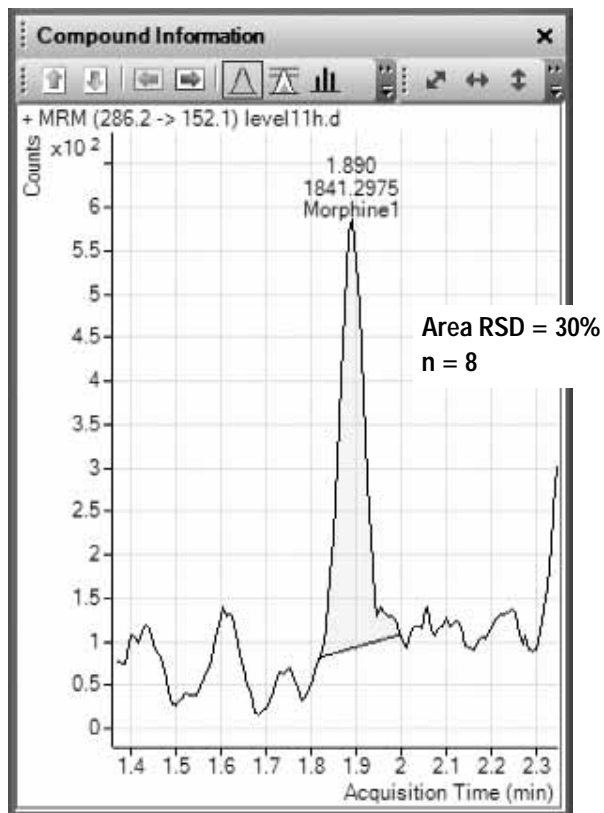


Figure 5A. LOD of morphine at 147 fg on-column.

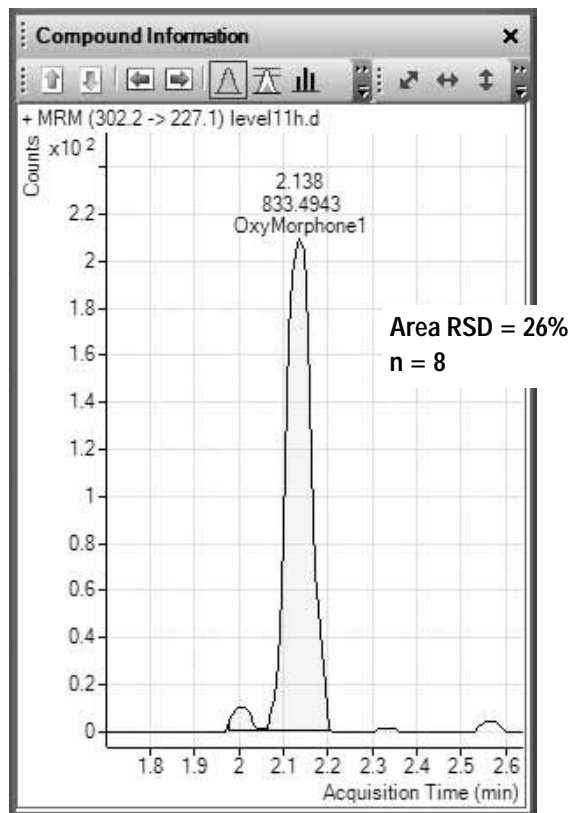


Figure 5B. LOD of oxymorphone at 147 fg on-column.

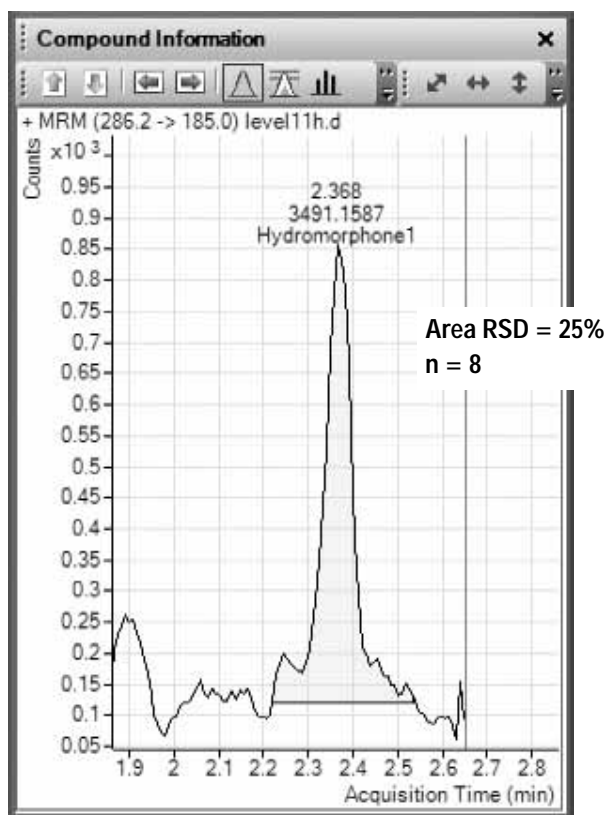


Figure 5C. LOD of hydromorphone at 147 fg on-column.

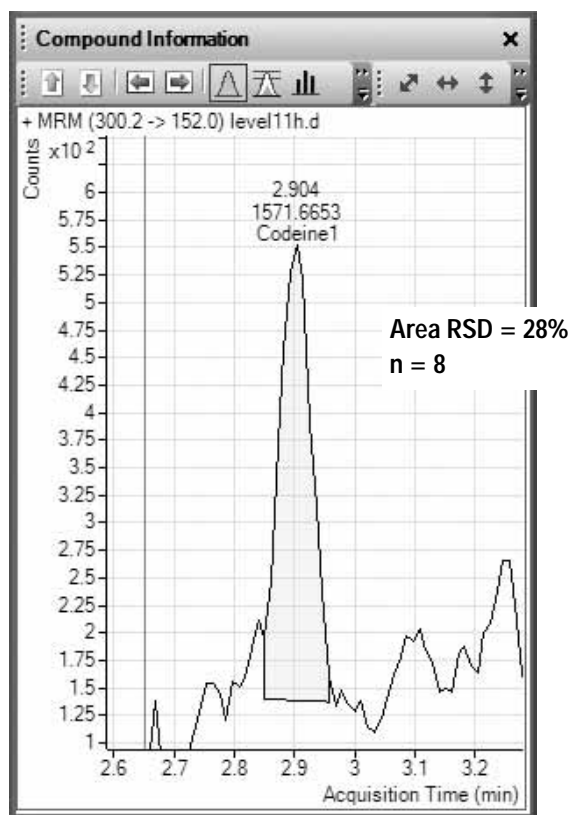


Figure 5D. LOD of codeine at 147 fg on-column.

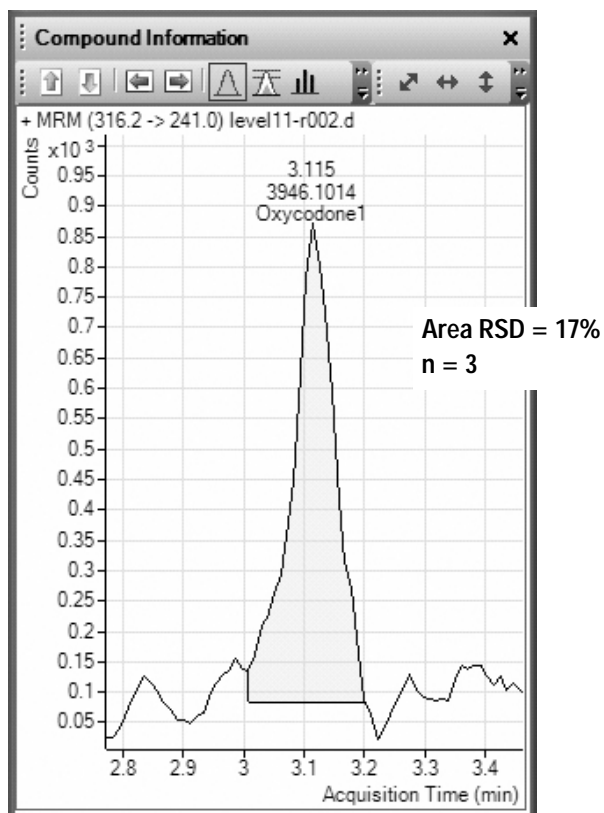


Figure 5E. LOD of oxycodone at 737 fg on-column.

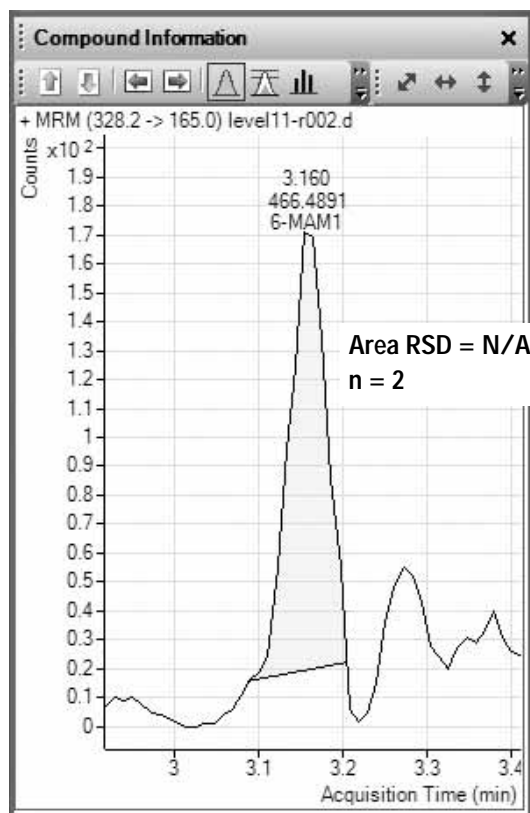


Figure 5F. LOD of 6-MAM at 49 fg on-column. Peak area %RSD not applicable because only two of three injections contained signal.

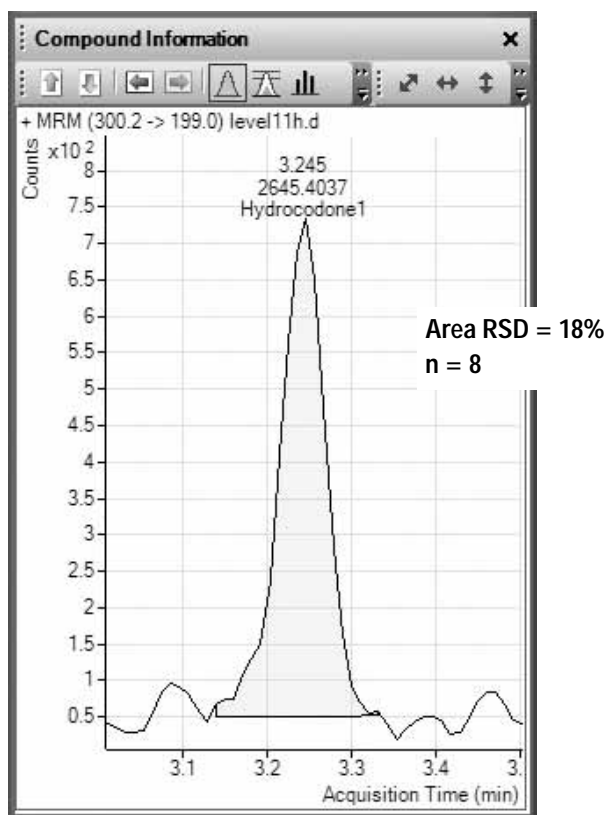


Figure 5G. LOD of hydrocodone at 147 fg on-column.

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- $\mu$ L 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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