

# Quantitation of Opiates to Low ng/mL Levels in Urine for Forensic Use Using an Affordable, High-Resolution, Accurate-Mass Mass Spectrometer

Kristine Van Natta, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

## Key Words

Q Exactive Focus, opiates, morphine, codeine, hydromorphone, hydrocodone, oxycodone, TraceFinder, forensic toxicology, drugs of abuse, PRM, parallel reaction monitoring

## Goal

To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer as a quantitative platform for HPLC-MS analysis of opiates in human urine to low ng/mL levels for forensic toxicology.

## Introduction

Forensic toxicologists need an economical instrument capable of both screening a large number of compounds and quantifying smaller panels to industry-established limits. Here we present a method for quantitation of six opiates—morphine, codeine, hydromorphone, hydrocodone, oxycodone, and oxycodone—in human urine down to low ng/mL levels. This work was performed on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer.

## Methods

### Sample Preparation

Samples were processed by enzymatic hydrolysis followed by urine dilution. Briefly, an aliquot of urine was spiked with stable-isotope-labeled internal standards and incubated with  $\beta$ -glucuronidase enzyme. The resulting mixture was centrifuged and further diluted before an aliquot was analyzed by gradient HPLC and a Q Exactive Focus MS. Calibrators and controls were prepared by spiking compounds into blank synthetic urine in the range of 1 to 5000 ng/mL.

### Liquid Chromatography

Gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with OAS autosampler (Figure 1). Mobile phases consisted of 10 mM ammonium acetate with 0.1% formic acid in water and methanol (Fisher Chemical brand) for solvents A and B, respectively. The column used was a Thermo Scientific™ Accucore™ PFP, 2.6  $\mu$ m particle size, 50 x 2.1 mm fused core (p/n 17426-052130). The



Figure 1. Q Exactive Focus MS with UltiMate 3000 RSLC HPLC pump and UltiMate 3000 OAS autosampler.

gradient was run from 0 to 70% mobile phase B over 3.3 minutes followed by a column wash at 100% B and re-equilibration to starting conditions. The total run time was 5.3 minutes.

### Mass Spectrometry

Compounds were detected on a Q Exactive Focus MS equipped with a Thermo Scientific™ Ion Max™ source and a heated electrospray ionization (HESI-II) sprayer. Data was acquired in parallel reaction monitoring (PRM) mode. In this mode, a single precursor ion is selected in the quadrupole with an isolation width of 3.0  $m/z$  and fragmented in the HCD cell. The resulting MS/MS product ions are detected in the Orbitrap detector at a resolution of 35,000.

### Method Evaluation

The method precision and accuracy were evaluated by running a calibration curve and quintuplicate replicates of quality controls on three different days. Additionally, internal-standard response was assessed in 58 donor samples obtained from a collaborator laboratory and compared to a sample prepared in water to determine matrix effects.

## Data Analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. Two product ions were selected as the quantifying and confirming ions for each compound. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification and ion ratio confirmation. Because the entire MS/MS spectrum was collected, multiple confirming ions could be chosen. Figure 2 shows a representative MS/MS spectrum for oxymorphone, highlighting the quantifying and confirming ions with corresponding reconstructed chromatograms.

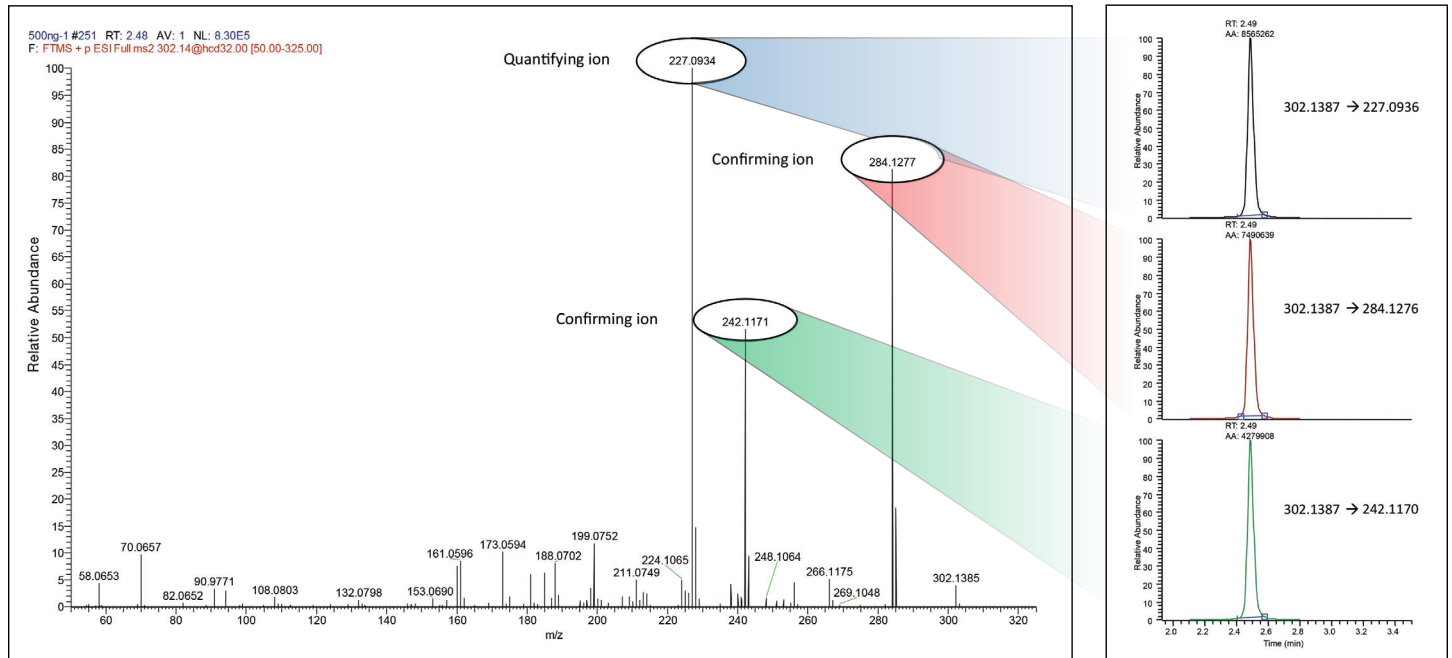


Figure 2. Representative fragmentation spectrum for oxymorphone obtained from a 500 ng/mL calibrator, highlighting the quantifying and confirming ions and showing corresponding chromatograms reconstructed with 5 ppm mass accuracy.

## Results

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20%, ion ratios within defined tolerance (tolerance dependent upon actual ratio), and quality controls within 20% RSD as well as meeting the above two requirements. Using these criteria, limits of quantitation for codeine, oxycodone, and oxymorphone were determined to be 2.5 ng/mL. For morphine, hydrocodone, and hydromorphone, the limit was 5 ng/mL. Tables 1 and 2 show the inter- and intra-assay statistics, respectively, for quality controls for all compounds in this method. Limited matrix effects were observed. The average recovery across 58 donor urine samples obtained from a collaborator laboratory ranged from 69% to 81% for the six internal standards evaluated. Figure 3 shows a combined chromatogram for analytes at their respective LOQs, and Figure 4 shows chromatograms for each compound with confirming ion ratio at its LOQ. Figure 5 shows representative calibration curves for all compounds. Figure 6 shows representative chromatograms with ion ratio confirmation for donor samples.

Table 1. Inter-assay precision and bias.

	Codeine	Hydrocodone	Hydromorphone	Morphine	Oxycodone	Oxymorphone
<b>5 ng/mL</b>						
% RSD	3.82	3.67	3.89	7.54	2.78	3.43
% Bias	-4.11	7.20	5.28	0.18	-6.19	-2.04
<b>10 ng/mL</b>						
% RSD	4.13	6.06	2.79	4.35	3.35	2.78
% Bias	-7.00	-3.47	-0.20	-6.15	-5.24	-3.60
<b>100 ng/mL</b>						
% RSD	3.00	6.13	2.03	2.28	2.58	1.62
% Bias	7.43	5.51	-0.16	0.98	5.34	0.26
<b>1000 ng/mL</b>						
% RSD	3.63	4.21	1.35	2.52	1.99	2.29
% Bias	6.07	3.64	3.53	4.67	5.44	2.86

Table 2. Intra-assay precision.

Maximum %RSD from Three Runs	5 ng/mL	10 ng/mL	100 ng/mL	1000 ng/mL
Codeine	5.09	4.56	3.61	3.34
Hydrocodone	3.55	5.60	6.38	4.25
Hydromorphone	3.16	1.87	2.41	1.90
Morphine	8.25	4.52	2.77	3.64
Oxycodone	3.27	3.27	2.85	2.31
Oxymorphone	4.28	3.04	1.78	2.73

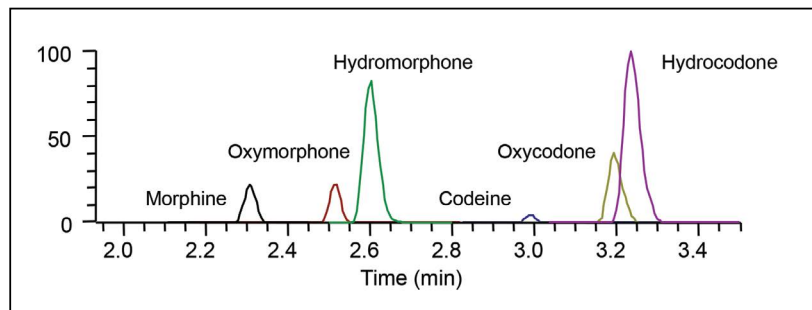
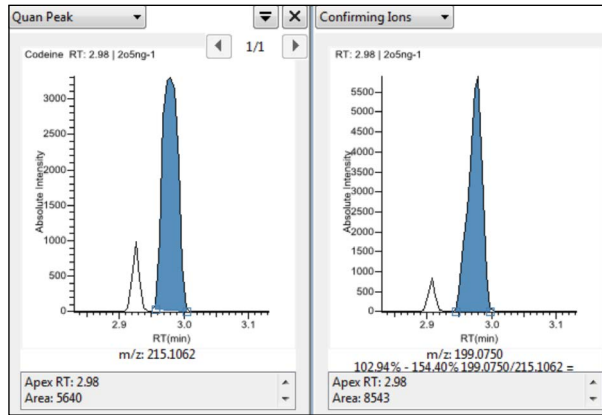
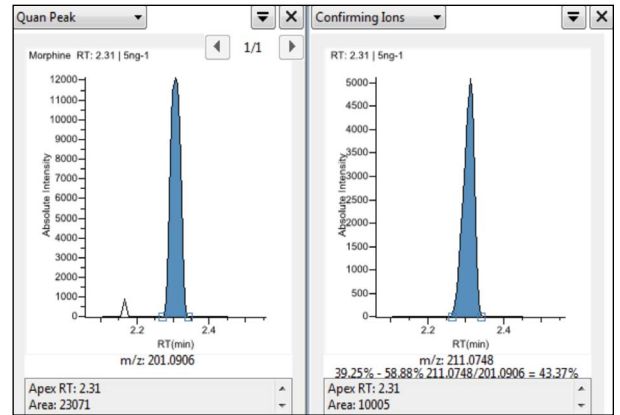
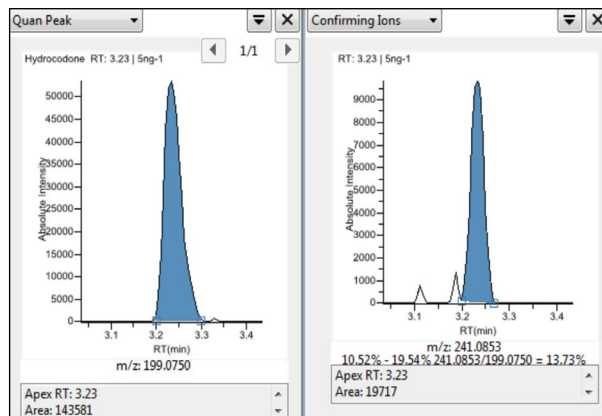
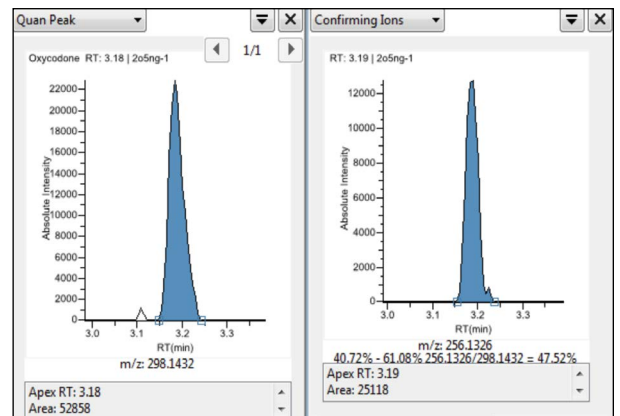
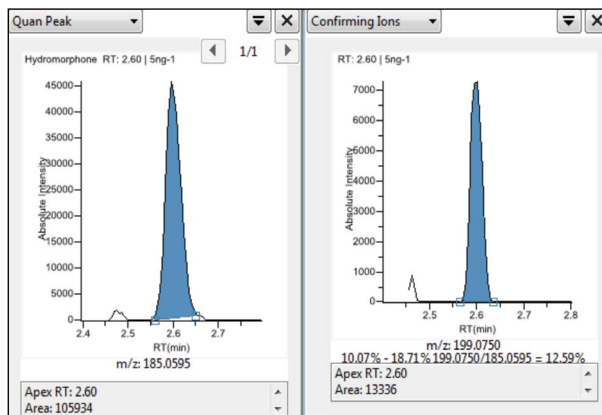
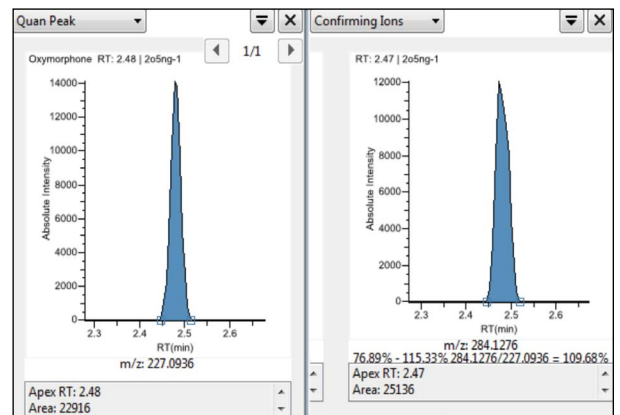
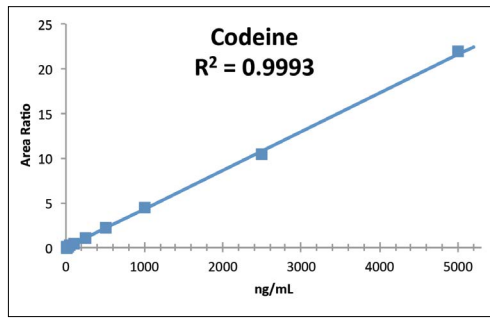


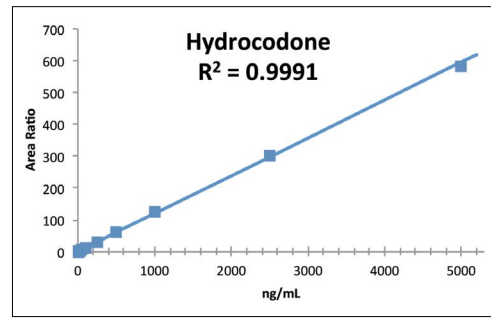
Figure 3. Chromatograms extracted from MS<sup>2</sup> spectra obtained from a confirmation PRM experiment for six opiates at their respective LOQs (2.5 ng/mL for codeine, oxycodone, and oxymorphone, and 5 ng/mL for hydrocodone, hydromorphone, and morphine) in hydrolyzed and diluted urine.

Figure 4. Chromatograms showing quantifying and confirming ions with ion ratio at LOQ for each compound in this method.

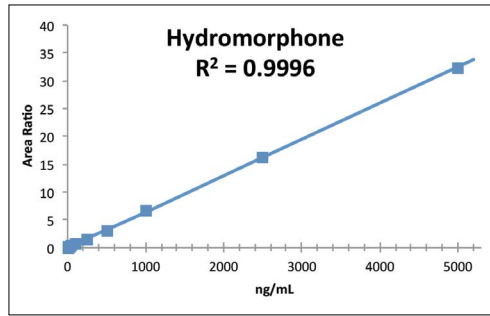
**Codeine LOQ = 2.5 ng/mL****Morphine LOQ = 5 ng/mL****Hydrocodone LOQ = 5 ng/mL****Oxycodone LOQ = 2.5 ng/mL****Hydromorphone LOQ = 5 ng/mL****Oxymorphone LOQ = 2.5 ng/mL**



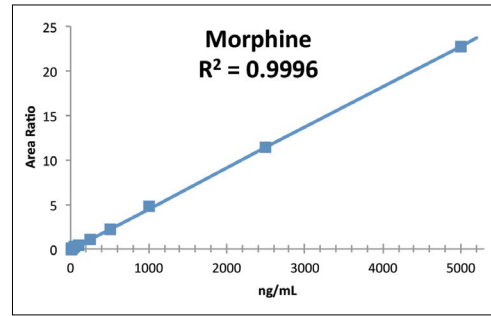
ng/mL	%Diff
5000	1.20
2500	-3.69
1000	3.55
500	-1.29
250	-1.53
100	7.11
50	2.83
25	-5.20
10	-5.64
5	6.42
2.5	-3.76



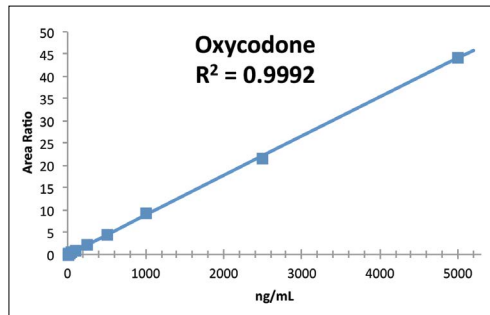
ng/mL	%Diff
5000	-2.24
2500	1.63
1000	5.46
500	1.86
250	-1.18
100	9.49
50	8.09
25	-10.1
10	-6.88
5	-6.10



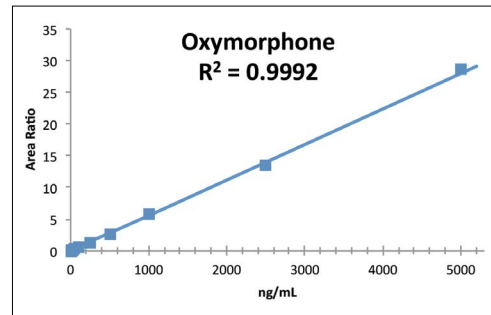
ng/mL	%Diff
5000	-0.390
2500	0.580
1000	3.72
500	-2.77
250	-7.48
100	0.510
50	0.750
25	-4.42
10	0.280
5	9.22



ng/mL	%Diff
5000	-0.140
2500	0.260
1000	4.09
500	-5.75
250	-4.14
100	-1.16
50	3.62
25	-7.44
10	-6.89
5	5.70



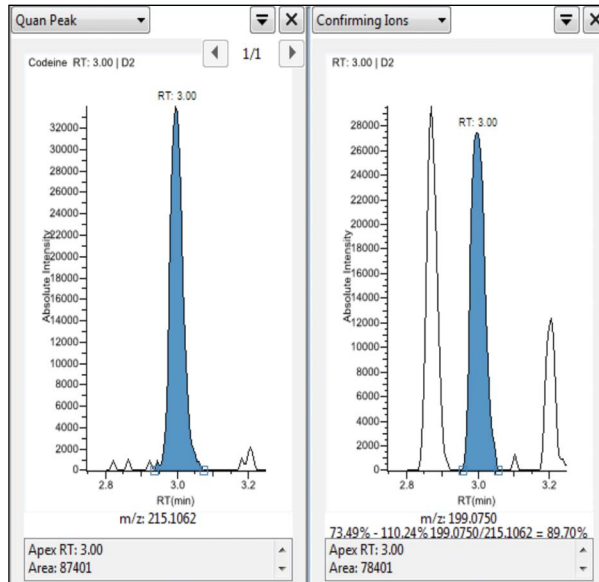
ng/mL	%Diff
5000	-0.0200
2500	-2.61
1000	6.71
500	-0.130
250	-2.87
100	4.51
50	6.80
25	-0.630
10	-4.42
5	-7.32
2.5	-4.00



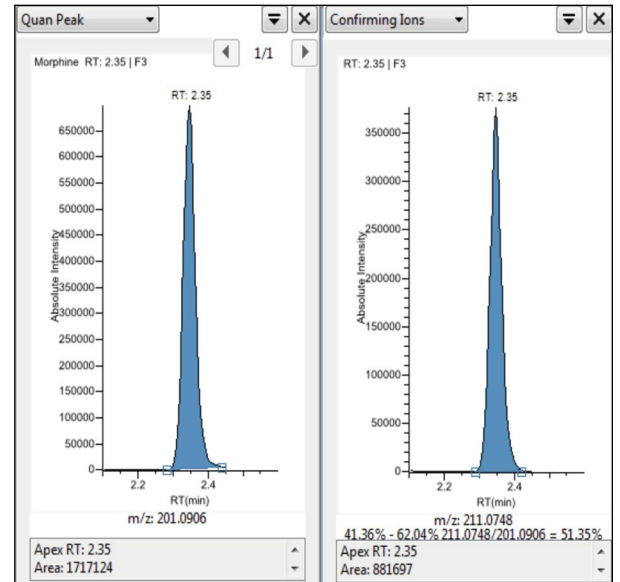
ng/mL	%Diff
5000	1.93
2500	-3.57
1000	2.06
500	-2.66
250	-7.29
100	2.72
50	3.44
25	-4.19
10	-1.57
5	1.08
2.5	8.04

Figure 6. Extracted ion chromatogram from donor sample obtained in confirmation PRM experiment.

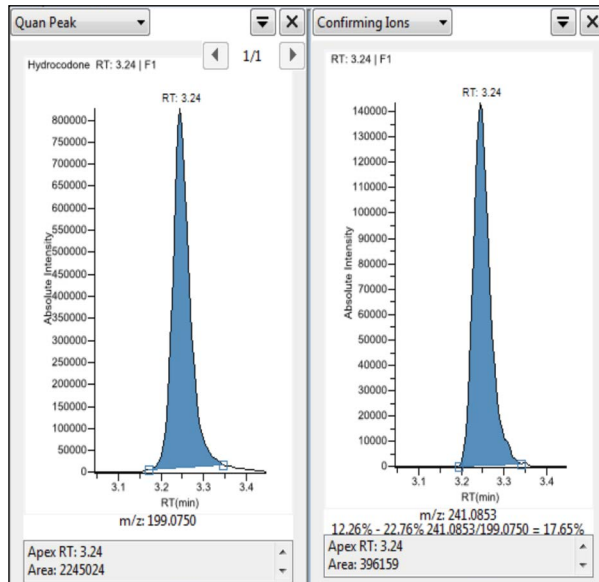
## Codeine in Donor D2, 12.1 ng/mL



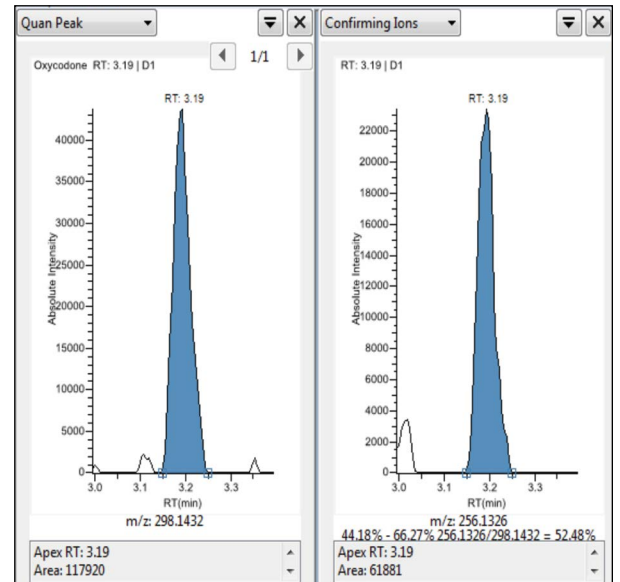
## Morphine in donor F3, 217 ng/mL



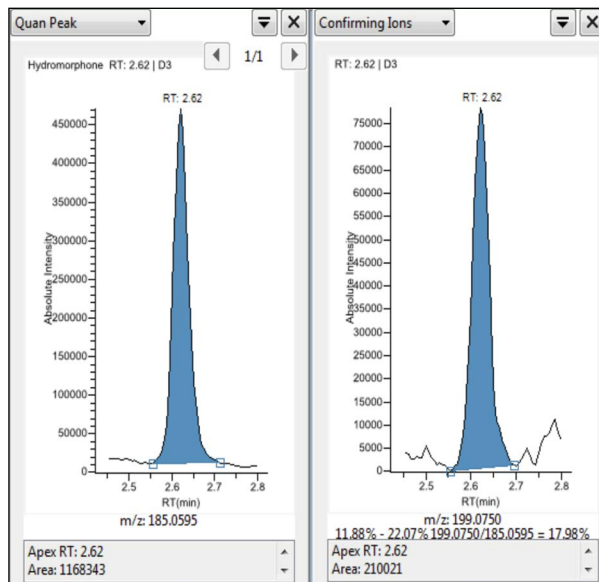
## Hydrocodone in Donor F1, 60.6 ng/mL



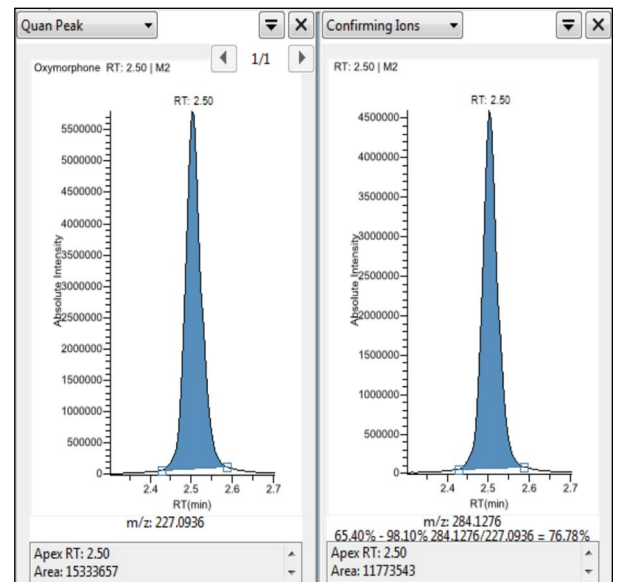
## Oxycodone in Donor D1, 4.38 ng/mL



## Hydromorphone in Donor D3, 37.1 ng/mL



## Oxymorphone in Donor M2, 698 ng/mL



## Conclusion

The Q Exactive Focus MS accurately quantitated all six opiates tested to the low ng/mL level in human urine. This new instrument gives forensic laboratories a single versatile platform capable of both screening large panels<sup>1</sup> and quantitative confirmation of specific panels that provides performance with value.

## References

1. Kozak, M.; Van Natta, K., Thermo Fisher Scientific Application Note 616: Forensic Screening for Drugs in Urine Using High-Resolution MS/MS Spectra and Simplified High-Performance Screening Software, 2014.

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