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Quantitative LC-MS/MS Combining Confident Identification with *Scheduled* MRM™, Fast Polarity Switching, UHPLC Time Scales

AB SCIEX QTRAP® 4500 LC/MS/MS System

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Introduction

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for screening and confirmation of drugs of abuse in forensic samples. As the demand to monitor the ever increasing number of drugs continues to rise, so too does the need to detect and quantify these compounds in a single run. In addition, as the number of samples requiring analysis increases, it is also necessary to use shorter analysis times.

The AB SCIEX QTRAP® 4500 LC/MS/MS System incorporates proven mass spectral detection technology to face these analytical challenges. The unparalleled speed of MRM detection is made possible by advanced eQ™ electronics and the Qurved LINAC® collision cell to take advantage of ultra fast LC without compromising data quality. These in combination with The *Scheduled* MRM™ algorithm and fast polarity switching allow for the best data quality covering the broadest range of drugs possible. In addition MS/MS spectra can be acquired in the same run to enable compound identification with highest confidence based on mass spectral library matching. These advances make it possible to monitor extremely large panels of analytes within a very short time with high confidence in identification.

Here we present an LC-MS/MS method utilizing the Scheduled MRM™ algorithm in combination with fast polarity switching and acquisition of MS/MS spectra for compound identification through mass spectral library searching. The method was successfully applied to quantify and identify drug compounds from spiked urine samples.

Figure 1. AB SCIEX QTRAP® 4500 LC/MS/MS System

Key Technology Features of the AB SCIEX QTRAP® 4500 System

- The Turbo V™ source with Curtain Gas interface to reduce chemical noise and allows the use of up to 5 mL/min flow rates.
- QJet® Ion guide captures and focuses ions into the high vacuum chamber using a combination of gas dynamics and RF fields; capturing more of the free jet expansion and significantly increases overall sensitivity over skimmer designs.
- Qurved LINAC® collision cell permits greatly reduced pause and dwell times without a loss in sensitivity allowing multi-target analyses.

- Exceptional triple quadrupole and Linear Accelerator Trap (QTRAP®) sensitivity allows identification, characterization, confirmation, and quantitation of low abundance analytes with a high degree of confidence in a single experiment.

In addition, advanced software tools like *Scheduled* MRM™ algorithm intelligently uses information of retention times to automatically optimize MRM dwell time of each MRM transition and total cycle time of the experiment resulting in highest data quality. To further increase confidence in analytical results QTRAP® technology is used to automatically acquire fast and sensitive MS/MS spectra in Enhanced Product Ion (EPI) mode and search them against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive results.[1-3]

Method Details

- Each 50 µL Spiked urine sample was combined with 20 µL of internal standard spiking solution and β-Glucuronidase hydrolysis performed. Sample diluent was added to give an overall 4.4 times dilution of each sample.
- LC separation was achieved on a Shimadzu UFLCXR system with a Phenomenex Kinetex 2.6 C18, 100 Å, 50 x 3.00 mm column

To demonstrate the technology of the QTRAP® 4500system, three experiments were performed as follows.

Experiment One:

- A 15 min gradient of water and methanol with ammonium formate buffer at a flow rate of 0.4 mL/min. The injection volume was set to 10 µL.
- The AB SCIEX QTRAP® 4500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe.
- A total of 95 transitions in positive and 18 transitions in negative polarity were monitored with an MRM pause time of 3 ms.
- The *Scheduled* MRM™ algorithm was used with an MRM detection window of 90 s and a target scan time of 0.7 s in Analyst® 1.6 Software.
- A settling time of 50 ms was used for polarity switching.

Experiment Two

- A 15 min gradient of water and methanol with ammonium formate buffer at a flow rate of 0.4 mL/min. The injection volume was set to 10 µL.
- The AB SCIEX QTRAP® 4500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe.
- A total of 508 transitions (2 transitions per compound) in positive polarity were monitored with an MRM pause time of 3 ms.
- The *Scheduled* MRM™ algorithm was used with an MRM detection window of 30 s and a target scan time of 0.2 s in Analyst® 1.6 Software.
- For increased confidence in compound identification EPI spectra at a scan speed of 10000 Da/s were acquired using a dynamic fill time for optimal MS/MS quality.
- EPI spectra were generated using standardized Collision Energy (CE) of ±35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently on the compound's fragmentation efficiency.
- MS/MS spectra were searched against the iMethod™ Forensic Library version 2.1.

Experiment Three.

- A 6 min gradient of water and methanol with ammonium formate buffer at a flow rate of 2.2 mL/min. The injection volume was set to 10 µL.
- The AB SCIEX QTRAP® 4500 system was operated with Turbo V™ source and Electrospray Ionization (ESI) probe.
- A total of 256 transitions in positive mode were monitored with an MRM pause time of 3 ms.
- The *Scheduled* MRM™ algorithm was used with an MRM detection window of 30 s and a target scan time of 0.1 s in Analyst® 1.6 Software.
- For increased confidence in compound identification EPI spectra at a scan speed of 10000 Da/s were acquired using a dynamic fill time for optimal MS/MS quality.
- EPI spectra were generated using standardized Collision Energy (CE) of ±35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently on the compound's fragmentation efficiency.
- MS/MS spectra were searched against the iMethod™ Forensic Library version 2.1.

Results

Scheduled MRM™ with Fast Polarity Switching

The *Scheduled* MRM™ algorithm uses knowledge of the retention time of each analyte so that each MRM transition is only monitored using a short time window. This means that each MRM transition is not monitored throughout the whole length of the LC run; instead, at any one point in time, the number of concurrent MRM transitions are significantly reduced resulting in much higher duty cycles for each analyte. The software calculates the maximum dwell times for the co-eluting compounds while still maintaining the desired cycle time for best signal-to noise (S/N), accuracy and reproducibility by maintaining the same, or even improving the number of points across the peak. As a result, *Scheduled* MRM™ allows the monitoring of many more MRM transitions in a single acquisition without compromising data quality.

The enhanced version of the *Scheduled* MRM™ algorithm offered in Analyst® 1.6 software also allows combining MRM scheduling with fast polarity switching to further extend the panel of compounds by covering substances with a wider range of chemical properties. Figure 2 shows the detection of drugs of abuse extracted from urine by monitoring 113 MRM transitions in positive and negative polarity using the *Scheduled* MRM™ algorithm and fast polarity switching.

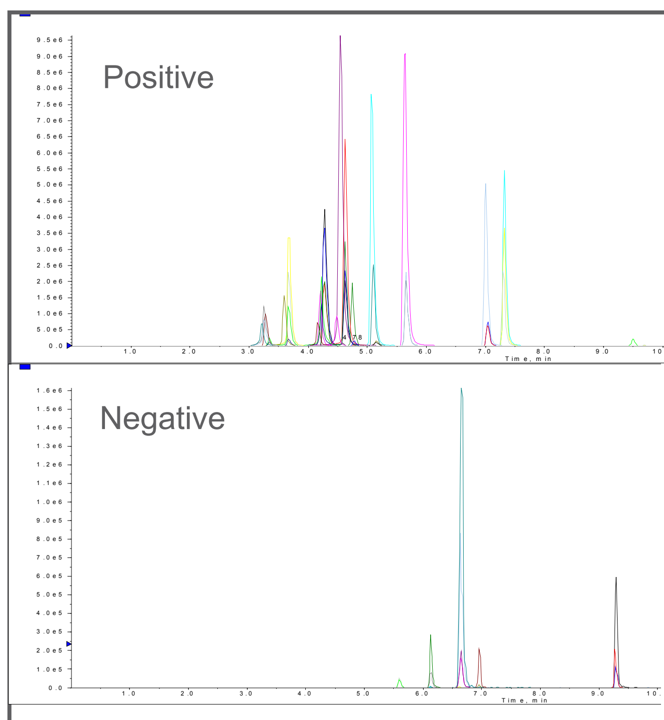


Figure 2 Detection of drugs of abuse extracted from urine by monitoring 113 MRM transitions in positive and negative polarity using the *Scheduled* MRM™ algorithm and fast polarity switching.

Quantitative Performance

The developed LC-MS/MS method using *Scheduled* MRM™ and fast polarity switching delivered excellent quantitative data. Calibration spiked urine standards were injected over various ranges depending on the compound. Example calibration curves are shown in Figures 3 and 4.

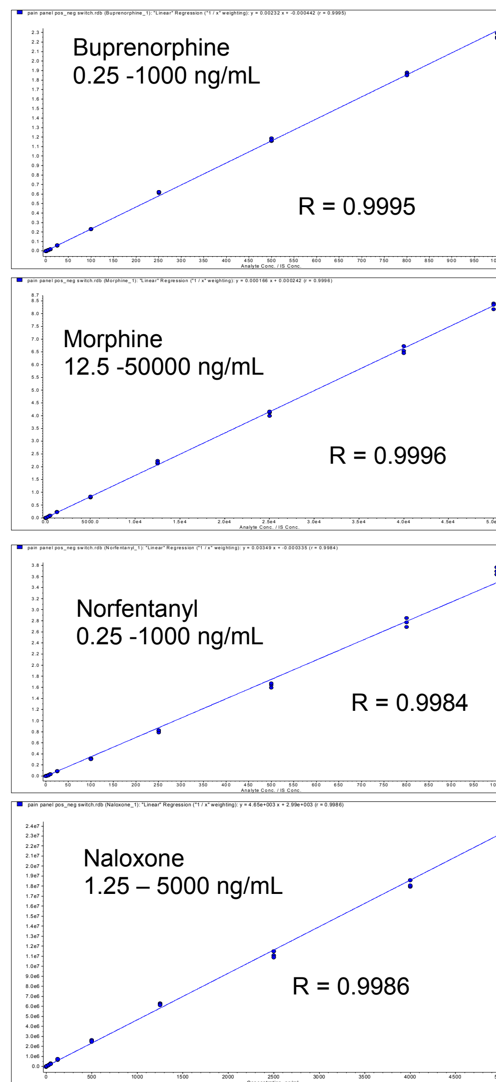


Figure 3. Example calibration lines of the quantifier MRM transitions in positive mode

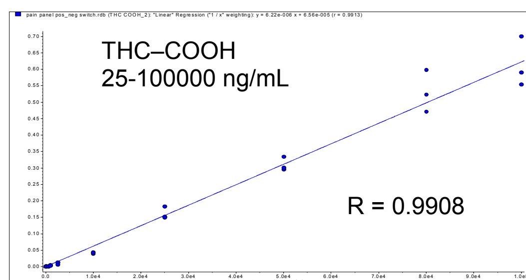


Figure 4. Example calibration line of the quantifier MRM transitions in negative mode

Accuracy between 80 and 120% were achieved for all targeted compounds over the entire calibration range. The coefficients of variation (%CV) were typically found to be much below 10%.

These excellent quantitative results highlight the advantage of combining *Scheduled* MRM™ with fast polarity switching for a comprehensive multi-target quantitative screen.

Scheduled MRM™ with acquisition of MS/MS spectra for compound identification through mass spectral library searching

Despite the high selectivity of MRM detection, there is always a risk of false positive findings due to interfering matrix signals. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, it has been reported that relying only on MRM ratios for identification can result in a significant number of false positive results for compound identification, especially if the targeted analytes have a low fragmentation efficiency (many low intensity product ions).[4-6] For improved accuracy, identification can be performed using full scan MS/MS experiments and library searching to compare the unknown with a standard spectrum. Here dependent MS/MS spectra are acquired in the Enhanced Product Ion (EPI) mode of the QTRAP® 4500 system after being triggered from a *Scheduled* MRM™ Information Dependant Acquisition (IDA) survey scan. The rapidly collected high quality MS/MS data is used in mass spectral library searching to increase the confidence of detection. Example spectra and library search Purity Score values using an MS/MS library search algorithm are shown in Figures 5 and 6 for samples with very low analyte concentrations.

Quantification can be performed in the same run allowing for both quantification and qualitative data to be collected simultaneously. Figure 7 shows example calibration curves created from the same run for the two compounds identified from the library hits in figures 5 and 6. The QTRAP® 4500 system high scan speeds allow identification through the traditional ion ratio determinations by monitoring two transitions per compound but at the same time enables the collection of high quality MS/MS data, in EPI mode, to use in mass spectral library searching. Figure 8 shows the quality of Extracted Ion Chromatogram peaks are maintained for accuracy and precision for quantification while at the same time monitoring two transitions per compound and acquiring MS/MS data that return high purity scores when searched against the mass spectral library for increased confidence in detection and identification.

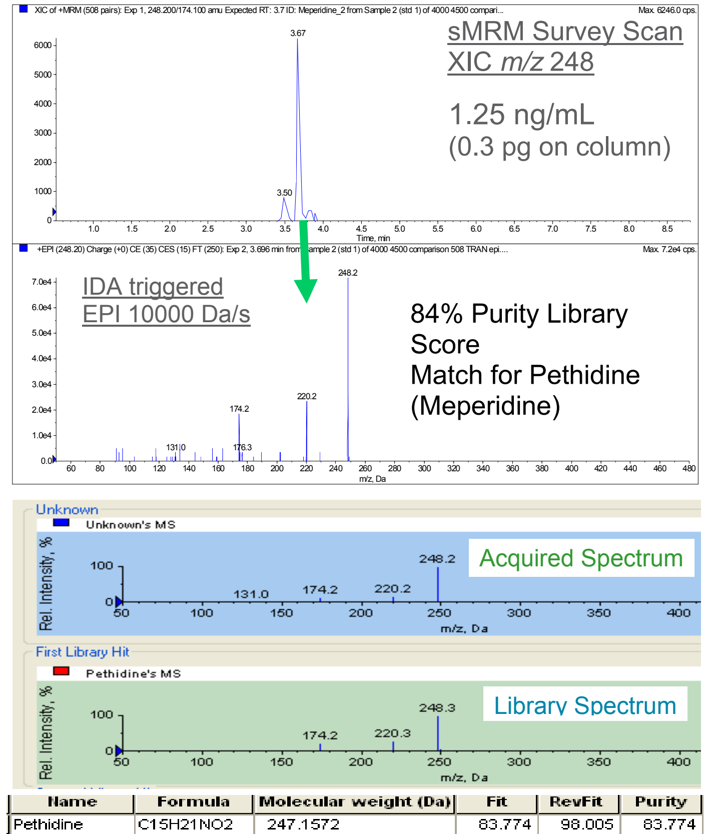
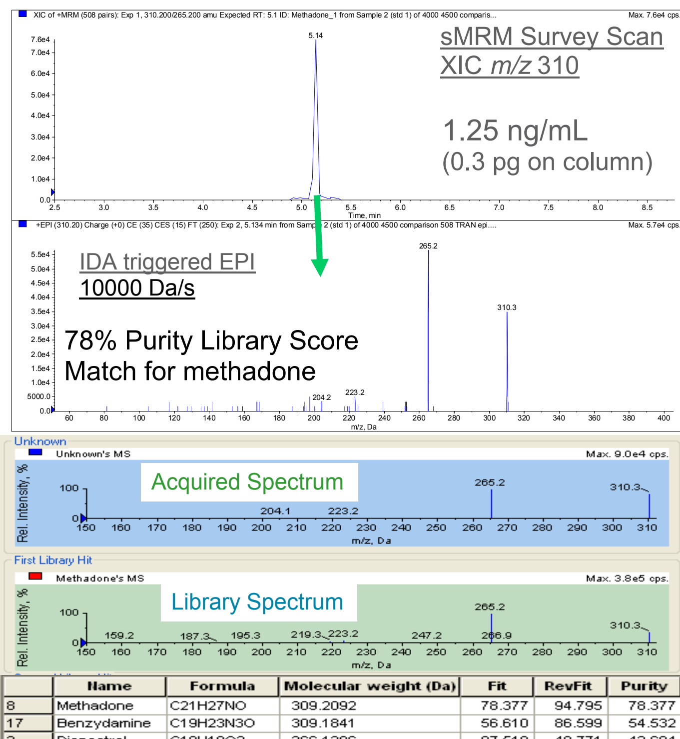


Figure 5. Spike urine sample (extract 4.4x dilution) screened for drugs of abuse with MS/MS library search results for additional confidence in compound identification. Good match to library spectrum at low concentration with an 84 % purity score for meperidine.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity
8	Methadone	C21H27NO	309.2092	78.377	94.795	78.377
17	Benzydamine	C19H23N3O	309.1841	56.610	86.599	54.532

Figure 6. Spike urine sample (extract 4.4x dilution) screened for drugs of abuse with MS/MS library search results for additional confidence in compound identification. Good match to library spectrum at low concentration with a 78% purity library score match for methadone

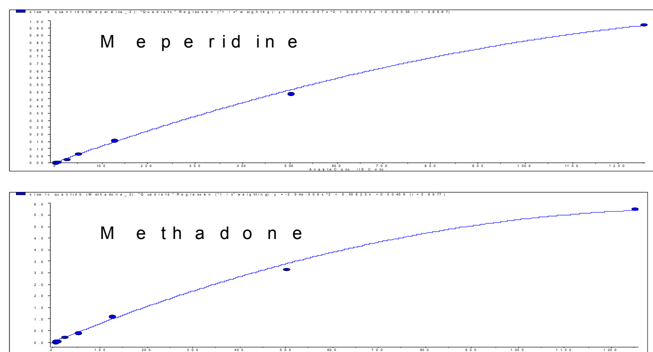


Figure 7. Example calibration curves generated from the same run in which qualitative information was obtained for confident identification through library searching. Linear range of 1.25-1250 ng/mL.

Scheduled MRM™ with acquisition of MS/MS spectra for compound identification through mass spectral library searching using Ultra Fast LC with Accelerated MRM Detection

The new AB SCIEX QTRAP® 4500 LC/MS/MS System allows the use of very short dwell and pause times to monitor MRM transitions. This feature is especially important when UHPLC is combined with MS/MS to screen and confirm for a larger panel of analytes. The developed method was applied to detect 256 compounds in a chromatographic run of less than 6 minutes. Figure 9 shows examples of the resulting triggered EPI spectra that were generated with high enough quality and used in library searching to confidently identify the drugs spiked into the urine sample even when monitoring a large panel of drugs using Fast LC conditions that included a six minute LC gradient, 2.2 mL/min flow rate, ~5 sec wide peaks.

Summary

The new QTRAP® 4500 LC/MS/MS System is a powerful tool for ultra fast multi-analyte quantitation and identification in forensic samples. The high speed scanning capabilities of the new 4500 series systems permit the use of very short dwell times and pause times, resulting in the ideal detector for the ultra-fast LC methods being employed in today's modern laboratories. Analysis times can be reduced, therefore sample throughput increased, while maintaining data quality (sensitivity, reproducibility, linear dynamic range, no crosstalk). This unique LC-MS/MS method utilizing the *Scheduled* MRM™ algorithm in combination with fast polarity switching and acquisition of MS/MS spectra for compound identification allows for the analysis of the ever increasing number of compounds that are required to be monitored in a single method. The method was successfully used to quantify and identify drugs of abuse in forensic samples covering a broad range of chemical properties, including the acquisition of positive and negative polarity spectra from the same injection.

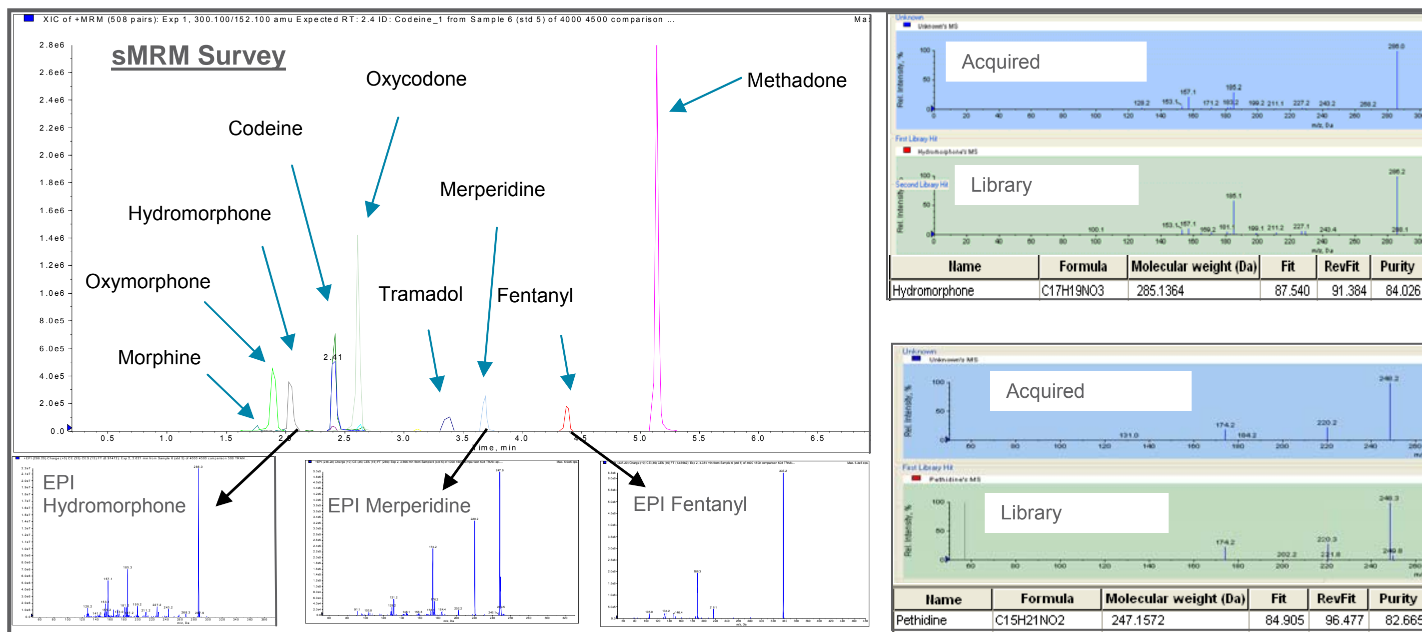


Figure 8: Spiked urine sample dilute and shoot analysis; 4.4x dilution, 508 MRM transitions monitored, 2 transitions per compound, *Scheduled* MRM™ Information Dependant Acquisition (IDA) Survey Scan with Triggered Enhanced Product Ion Spectra . Both quantitative and qualitative data is obtained from the same injection.

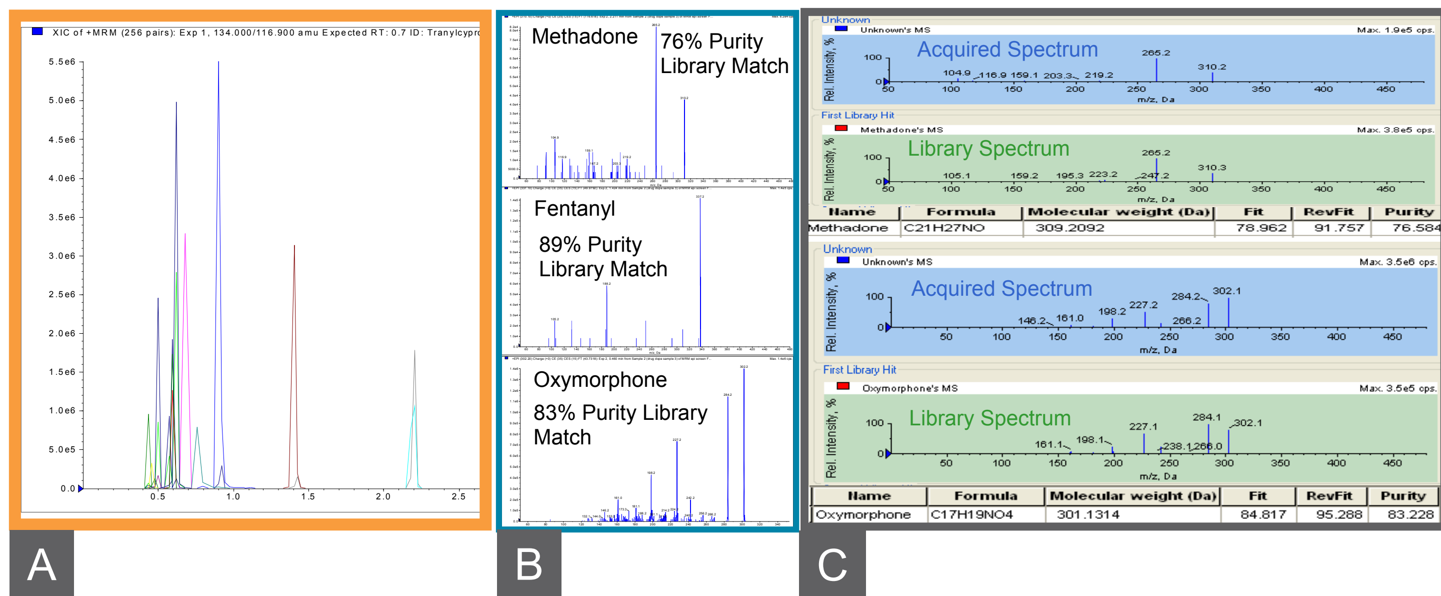


Figure 9. Confident Compound Identification through triggering of product ion spectra used in library searching on UHPLC time scales. A) *Scheduled* MRM™ Information Dependant Acquisition (IDA) survey scan used to detect compounds present in the sample and for quantitation from a 6 min LC run; monitoring 256 compounds; 2.2 mL/min flow rate; 5 sec wide peaks. B) Example IDA Triggered Product Ion Spectra; acquired using 10,000 Da/s scan rate. C) Example results of full scan MS/MS Enhanced Product Ion Spectra being used in library searching to compare the unknown with a standard spectrum increasing the confidence of detection.

References

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