

Rapid Workflows for Triple Quadrupole LC-MS/MS Screening

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1. Introduction:

The rapid emergence of novel psychoactive substances (NPS) has reshaped the drug landscape and placed new demands on toxicology testing. Traditional immunoassay-based screens, while long used as a first line approach, cannot keep pace with shifting drug trends because assay development is slow. In contrast, MS-based screening assays can be adapted quickly to incorporate new analytes, making them better suited for the dynamic environment. As toxicology laboratories face rising sample volumes, screening procedures are essential for limiting the number of specimens requiring resource intensive confirmatory testing. An effective screening method must be fast, inexpensive, and simple to execute while confidently excluding most negative samples without increasing the risk of false negatives.

2. Objectives:

To develop an LCMS method for screening with:

- ≤ 5 minute total loop time
- Polarity switching
- 40 analytes including the NPS carfentanil, xylazine, protonitazene, and metonitazene
- Chromatographic separation of isobars morphine and hydromorphone and codeine and hydrocodone
- Demonstrated specificity and sensitivity in synthetic urine with no sample preparation
- Compatibility with the Shimadzu Forensic Toxicology Database

3. Methods:

41 analytes for drugs of abuse, including isobars such as morphine and hydromorphone as well as codeine and hydrocodone, were spiked into synthetic urine, injected without dilution, and separated on a Shim-pack Scepter PFPP-120 column (1.9 µm, 2.1 x 50 mm) with 2 mM ammonium formate and 0.002% formic acid as additives in water (mobile phase A) and methanol (mobile phase B) utilizing gradient elution. The gradient ramps from 55-100% B over 2.5 minutes. The compounds were analyzed by positive and negative mode electrospray ionization (ESI) with an acquisition time of 2.4 minutes on a Shimadzu LCMS-8050RX. Three screening acquisition methods were compared: MRM Spectrum Mode, triggered Product Ion Scans, and MRM. The RX probe was used at a position of +5 mm with a Focus Voltage of 1 kV.

Table 1. LC-MS/MS Acquisition Parameters	
LC Parameters	
Column	Shim-pack Scepter PFPP-120 1.9 µm, 2.1 x 50 mm
Mobile Phase A	Water
Mobile Phase B	Methanol
Additives	2mM ammonium formate 0.002% formic acid
Flow Rate	0.4 mL/min
Run Time	5 min
Gradient	See Figure 1
MS Parameters	
Nebulizing Gas	3.0 L/min
Heating Gas	15.0 L/min
Interface Temp.	350 °C
Drying Gas	3.0 L/min
DL Temp.	150 °C
Heat Block Temp.	300 °C
Acquisition Mode	MRM Spectrum Mode Triggered Product Ion Scan



Figure 2. Using a +5 mm probe position and RX probe with focus voltage allows robustness running untreated matrix.

3.1 RX Probe Position and Focus Voltage

The Shimadzu ESI probe allows the position of the ESI spray cone to move horizontally closer or further from the orthogonally placed desolvation line (DL). This allows robust performance in dirty matrices by moving the spray cone physically farther from the DL, allowing matrix materials to spray down into the source without entering the DL. This distance from the DL can reduce sensitivity; however, the RX focus electrode can apply a voltage to push ions of interest towards the DL without propelling uncharged and oppositely charged matrix ions toward the DL. This voltage allows the RX probe to maintain sensitivity even at distant probe positions, combining the best of both robustness and sensitivity for the analysis.

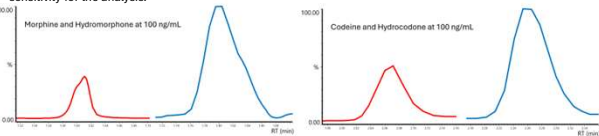


Figure 4. Chromatographic separation of isobars at the highest concentration (100 ng/mL).

3.2 Acquisition Methods: MRM Spectrum Mode, Triggered Product Ion Scans, and MRM

Three screening acquisition methods were evaluated: MRM Spectrum Mode, Triggered Product Ion Scans, and traditional two MRM acquisition. MRM Spectrum mode, as shown in Figure 5 below, is an MRM based method that collects up to 15 MRMs per compound and displays the merged MRM spectrum to be used for library matching. Triggered Product Ion Scans (Figure 6) use a single MRM event to determine whether or not to trigger a product ion scan at three different collision energies for an analyte of interest. These product ion scans are merged and used for library matching. For comparison, a method utilizing two MRMs, a quantifier and qualifier ion, was used with the merged MRMs searched against the library as well (Figure 7).

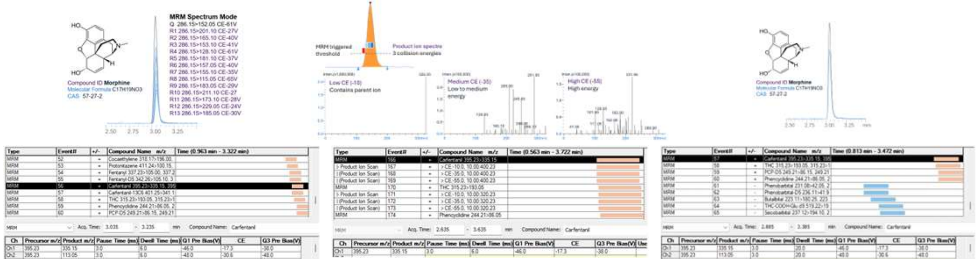


Figure 5. MRM Spectrum Mode has up to 15 MRMs per analyte allowing higher specificity even in complex matrices

Figure 6. Triggered Product Ion Scans use an MRM threshold triggering product ion scans for quantitation and library matching

Figure 7. Traditional 2 MRM method maximizes quantitation data quality with compromises in specificity and library matching

4. Results:

The method achieved complete chromatographic separation of isobaric compounds, enabling identification of all targeted analytes across workflows. Table 2 presents information for all analytes across methods including the lowest concentration at which the analyte was identified as the top library match, the lowest concentration at which the minimum number of MRMs were observed, or Product Ion Scans were triggered, as well as calibration curve information, allowing informed evaluation of relative sensitivity and selection of an appropriate screening workflow. Overall MRM Spectrum Mode offered the greatest combination of selectivity and sensitivity in synthetic urine. Library searches were conducted against the complete Shimadzu Forensic Toxicology Database, but retention time information was not utilized for library matches because the chromatography used does not match the Database chromatography. Library entries were made for Xylazine, Protonitazene, Metonitazene, and THC-COOH-Glu from solvent spiked solutions at 10 ng/mL.

Using MRM Spectrum Mode:

- All analytes were identified as the top library match at concentrations from 0.001 ng/mL to 1 ng/mL without retention time information.
- All analytes (except the barbiturates) were identified with 3 (or the maximum number) of MRMs at concentrations ranging from 0.001 ng/mL to 1 ng/mL.
- Quantitation ranges were sufficient for informing dilution for confirmation.

4.1 Results: Library Matching

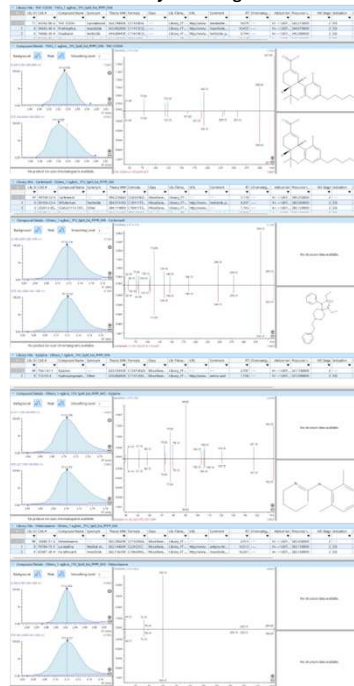


Figure 8. Merged MRM Spectra and library matches for THC-COOH, Carfentanil, Xylazine, and Metonitazene at 1 ng/mL collected with the MRM Spectrum Mode method.

Table 2. Method performance information for all analytes across methods. All concentrations are in ng/mL.

MRM Spectrum Mode										Product Ion Scans										MRM										
Analyte	Low Cal	High Cal	R ²	Curve Fit	Difference (%)	Min Conc 2 MRMs	Top Library Match	Low Cal	High Cal	R ²	Curve Fit	Difference (%)	Min Conc 1 Scan	Top Library Match	Low Cal	High Cal	R ²	Curve Fit	Difference (%)	Min Conc 2 MRMs	Top Library Match	Difference	Min Conc	Top Library Match	Difference	Min Conc	Top Library Match	Difference	Min Conc	Top Library Match
Amphetamine	0.5	100	0.99	quadratic	15%	0.001	0.001	0.5	100	0.98	quadratic	20%	0.001	0.001	0.5	100	1	linear	25%	0.001	0.001	1	1	1	1	1	1	1	1	1
Metoprolol	0.5	100	0.98	quadratic	25%	0.001	0.001	0.5	100	0.94	linear	20%	0.001	0.001	0.5	100	0.99	quadratic	20%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Nitrazepam	0.5	100	0.99	quadratic	15%	0.001	0.001	0.5	100	0.93	quadratic	30%	0.001	0.001	0.5	100	0.99	quadratic	20%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MDA	0.5	100	1.00	quadratic	10%	0.001	0.001	0.5	100	1.00	quadratic	10%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MDA	0.5	100	1.00	quadratic	10%	0.001	0.001	0.5	100	1.00	quadratic	10%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MDA	0.5	100	1.00	quadratic	10%	0.001	0.001	0.5	100	1.00	quadratic	10%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Phenacetin	0.5	100	0.98	quadratic	25%	0.1	0.1	1	100	1	quadratic	25%	10	10	1	100	1	linear	25%	1	1	1	1	1	1	1	1	1	1	1
Phenacetin	1	100	0.94	linear	25%	10 ⁻⁵	1	1	100	0.99	linear	20%	10	10	1	100	1	linear	25%	1	1	1	1	1	1	1	1	1	1	1
Nitrazepam	0.5	100	0.99	quadratic	15%	0.1	0.1	0.01	100	0.95	quadratic	60%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Nitrazepam	0.5	100	0.99	quadratic	15%	0.001	0.001	0.5	100	0.93	quadratic	15%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Chlorazepam	0.5	100	1.00	quadratic	10%	0.1	0.1	0.1	100	0.92	quadratic	15%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Torazepam	0.5	100	0.97	quadratic	25%	0.1	0.01	0.01	100	0.79	linear	80%	0.1	0.1	0.1	100	0.98	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Flunitrazepam	0.5	100	1.00	quadratic	10%	0.1	0.01	0.01	100	0.86	quadratic	80%	0.1	10 ⁻⁵	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Clonazepam	0.5	100	0.98	linear	25%	1	0.1	0.1	100	0.86	quadratic	80%	0.1	0.1	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Lorazepam	0.5	100	1.00	quadratic	10%	1	0.01	1	100	0.94	linear	80%	1	10 ⁻⁵	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Benzydiazepam	0.5	100	0.99	quadratic	15%	0.001	0.01	0.01	100	0.89	linear	15%	0.001	0.001	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Carbamazepine	0.001	0.001	0.99	linear	15%	0.001	0.001	0.001	10	0.85	linear	30%	0.001	0.1	0.001	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Nitrazepam	0.5	100	0.98	linear	35%	0.01	0.01	0.01	100	0.99	quadratic	15%	0.01	0.01	0.01	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Permethrin	0.001	0.001	0.98	quadratic	20%	0.001	0.001	0.1	100	0.83	quadratic	90%	0.1	1	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Morphine	0.5	100	1.00	quadratic	15%	0.01	0.01	0.1	100	0.83	quadratic	60%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Hydrocodone	1	100	0.95	linear	25%	0.01	0.01	0.1	100	0.83	quadratic	90%	0.1	1	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Codeine	1	100	0.99	linear	15%	0.01	0.01	0.1	100	0.98	linear	25%	0.1	0.1	1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Glycidolone	0.1	100	0.95	quadratic	25%	0.1	0.1	0.1	100	1	quadratic	10%	0.001	0.001	0.1	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Nitrazepam	0.5	100	0.98	quadratic	25%	0.1	0.1	0.1	100	0.99	quadratic	15%	0.1	0.1	0.1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Permethrin	0.001	0.001	0.99	linear	15%	0.001	0.001	0.01	100	0.89	linear	40%	0.001	0.1	0.001	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Carbamazepine	0.001	0.001	0.99	linear	15%	0.001	0.001	0.01	100	0.85	linear	40%	0.001	0.1	0.001	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Xylazine	0.001	0.001	0.99	linear	15%	0.001	0.001	0.01	100	0.85	linear	40%	0.001	0.1	0.001	100	0.99	quadratic	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Protonitazene	0.001	0.001	1.00	linear	10%	0.001	0.001	0.01	100	1	quadratic	15%	0.001	0.001																
THC-COOH	0.1	100	0.97	quadratic	25%	0.1	0.1	0.1	100	0.98	linear	15%	1	1	1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
THC-COOH	0.1	100	0.97	quadratic	25%	0.1	0.1	0.1	100	0.98	linear	15%	1	1	1	100	1	linear	25%	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

These analyses only had 2 MRMs

At least average concentration had a different top library match

Without reference times the library was unable to distinguish between