A Novel Screening Workflow for Nitazene Analogs using LC-QQQ Precursor Ion Scan Acquisition

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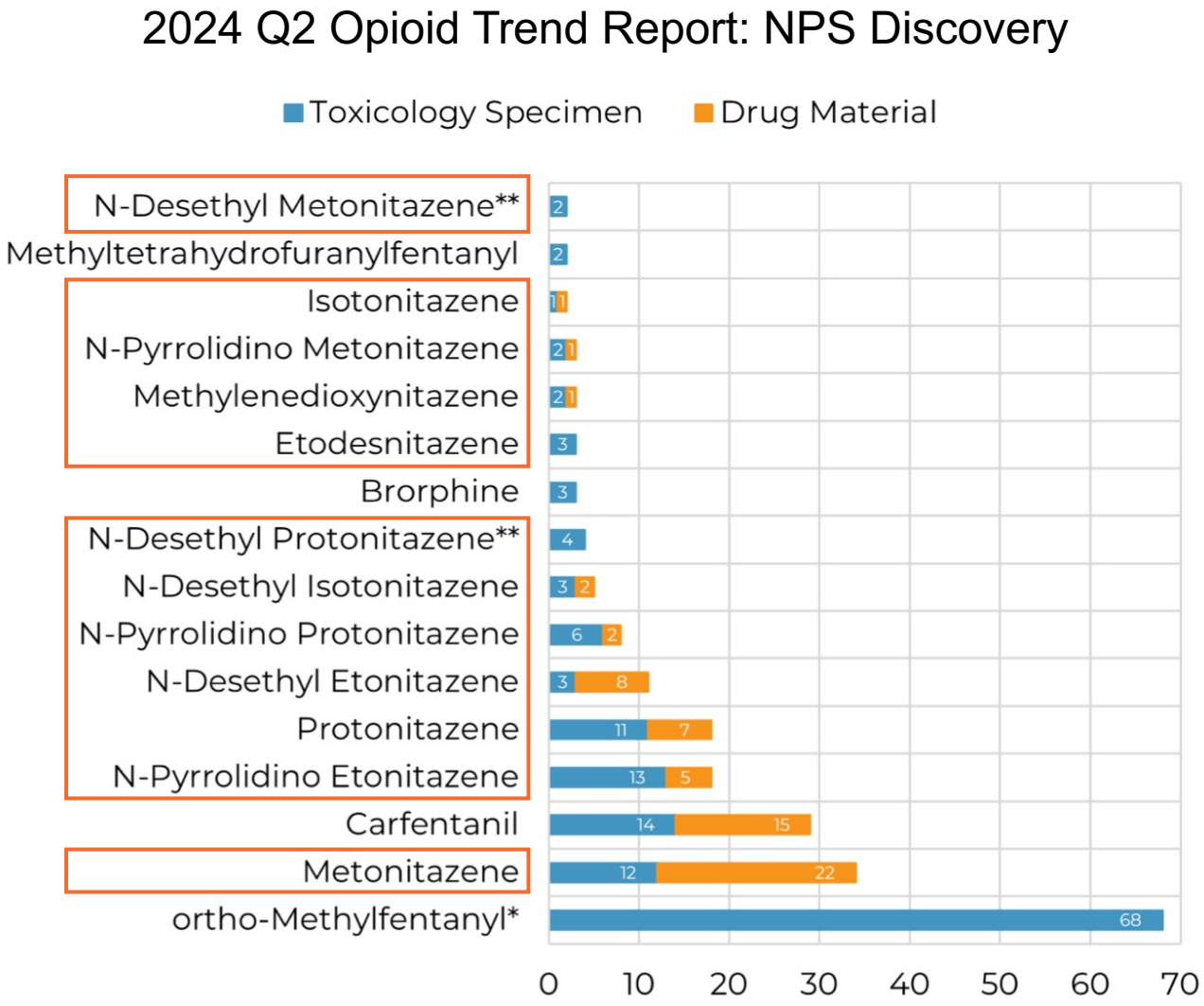
Disclosure

The authors declare no conflict of interest or financial disclosures.

Nitazenes and the Opioid Crisis

- 15 unique nitazene monographs published by NPS Discovery since 2019
- Over 40 different nitazene standards commercially available
- Rapid life cycles of analytes
  - Current trend: ring-substitutions of previously reported analogs
    - e.g., N-pyrrolidino protonitazene



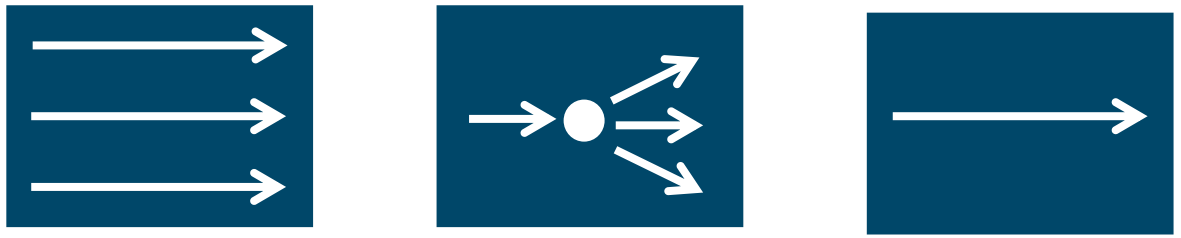
12 Nitazenes in Q2

https://www.cfsre.org/images/trendreports/2024-Q2-NPS-Opioids-Trend-Report.pdf

The Need for New Methods

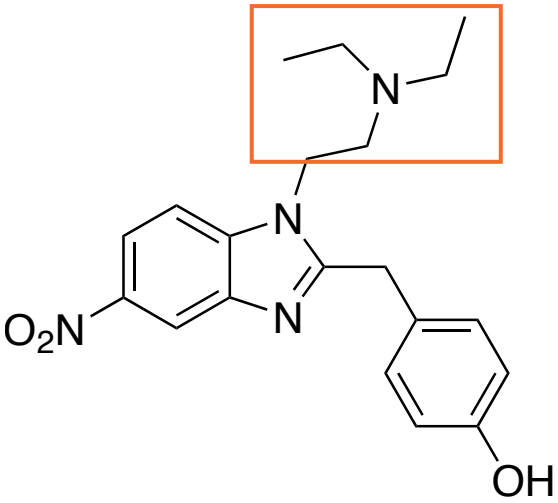
- ELISA
  - No commercially available nitazene kits
  - No known cross-reactivity with currently available kits
- LC-QTOF-MS
  - High capital costs
  - Increased maintenance
  - Time-consuming data processing

- LC-QQQ
  - Already available in many labs
  - Lower costs (install, maintenance, training)
  - Precursor ion scan (PIS)
    - Uses characteristic fragment ions
    - Previously applied to fentanyl analogs

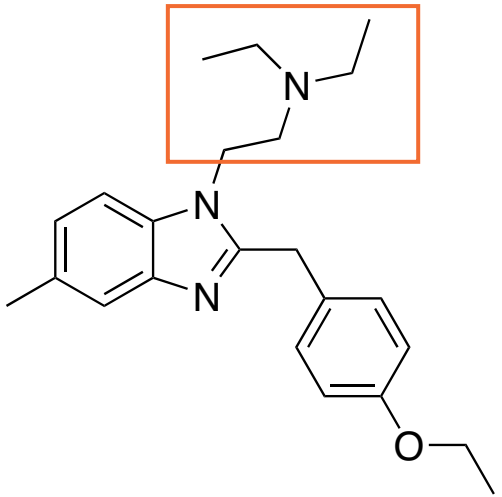


Quadrupole 1: Scanning | Collision Cell | Quadrupole 3: Targeted *m/z*

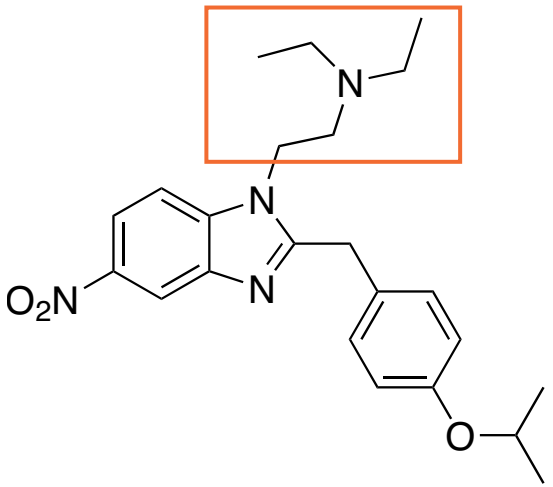
Scope



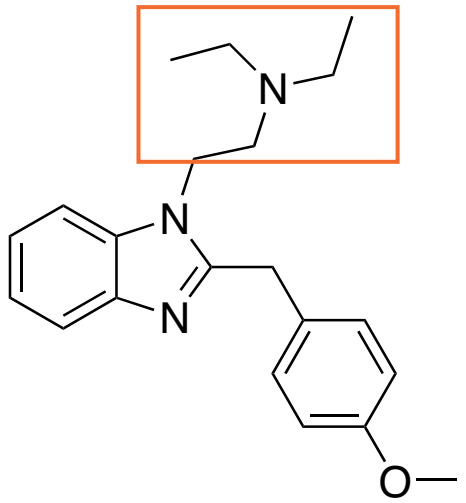
4'-OH nitazene
MW: 368.40



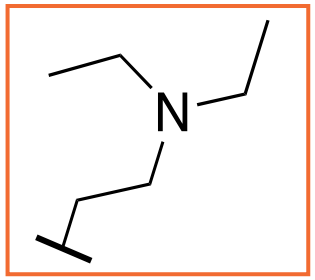
5-methyl Etodesnitazene
MW: 365.50



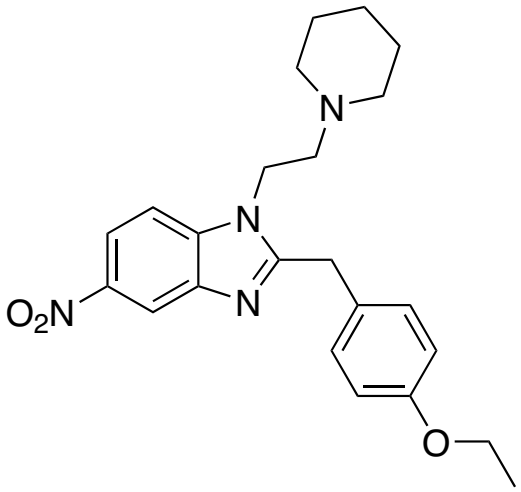
Isotonitazene
MW: 410.23



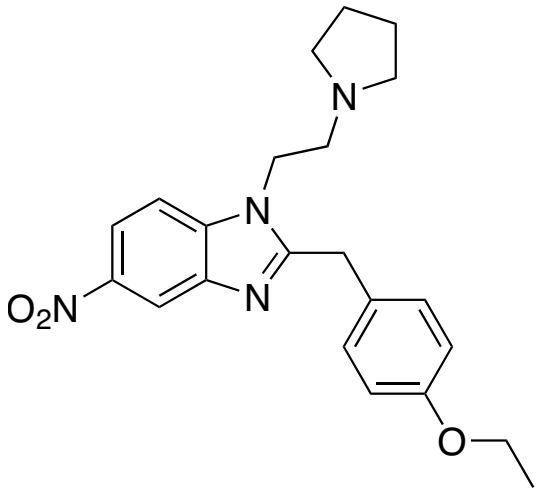
Metodesnitazene
MW: 337.21



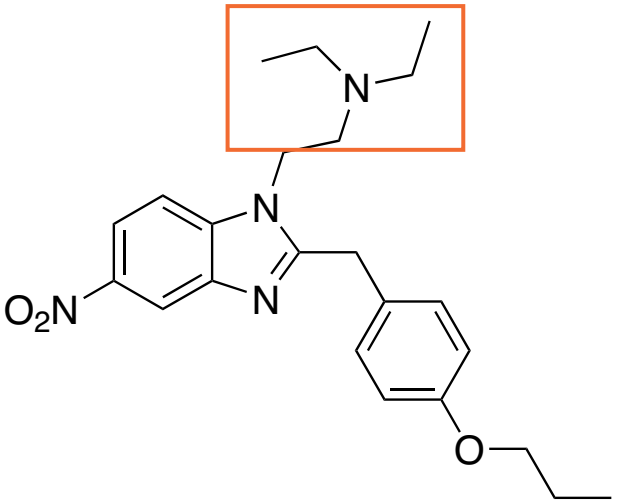
***m/z* 100, 72**



N-Piperidinyl Etonitazene
MW: 408.50

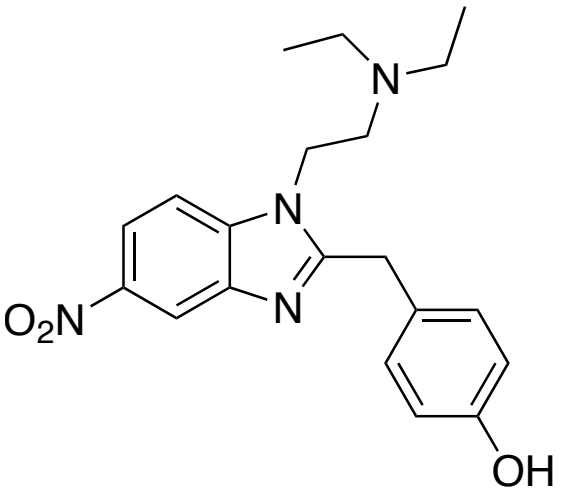


N-Pyrrolidino Etonitazene
MW: 394.20



Protonitazene
MW: 410.23

Scope



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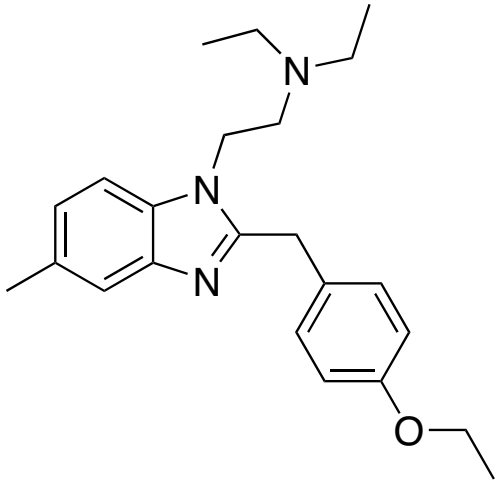

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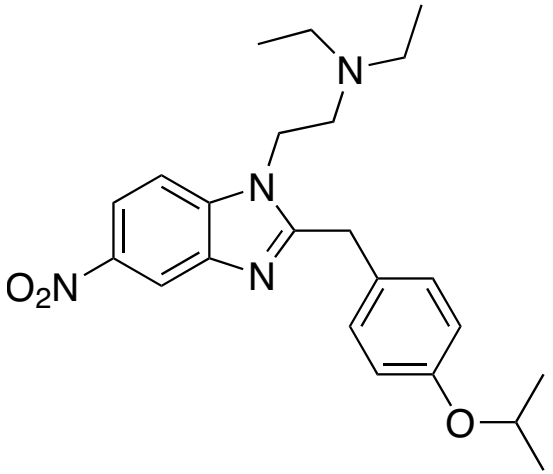

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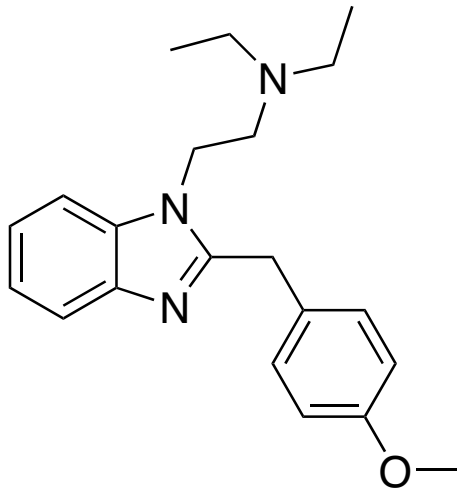

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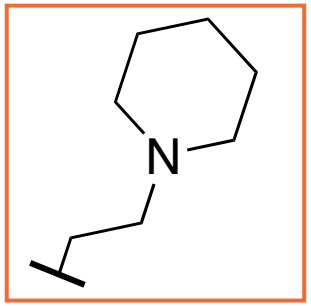
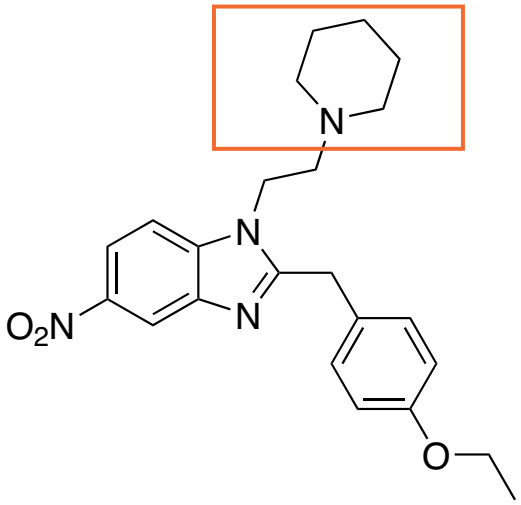


***m/z* 112**



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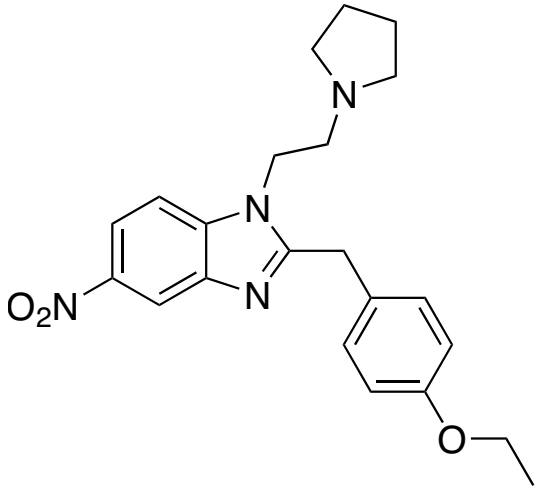

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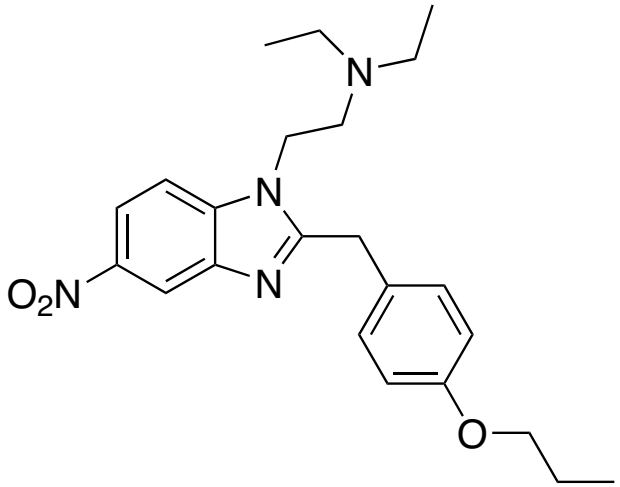
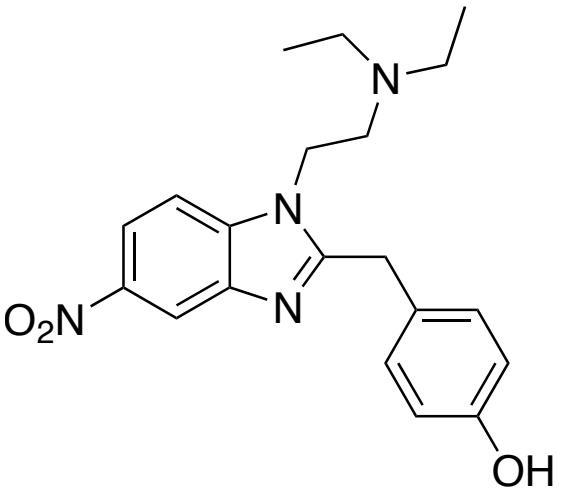


Protonitazene
MW: 410.23

Scope



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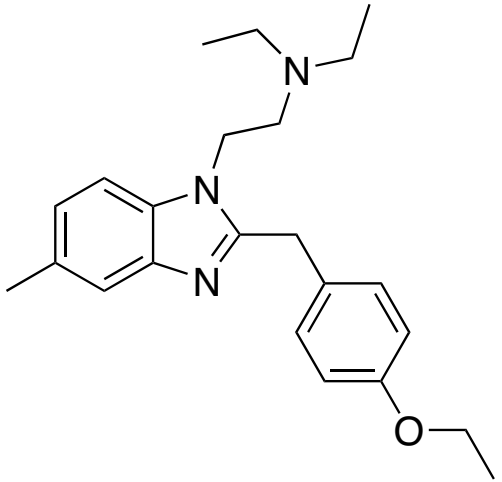

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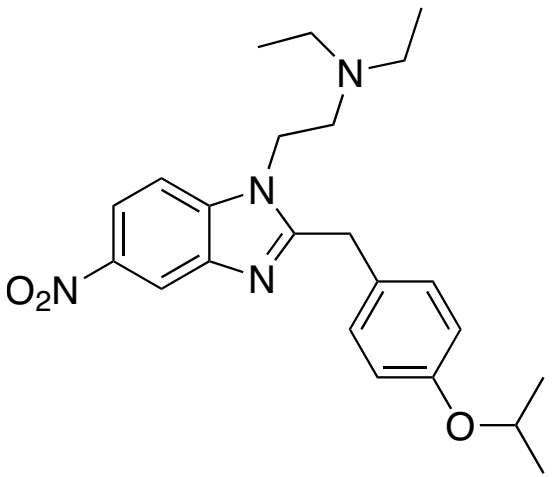
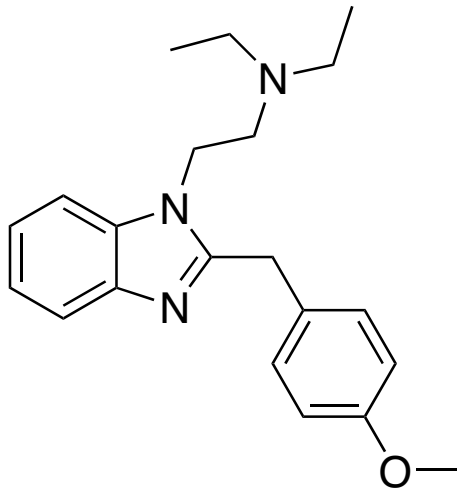
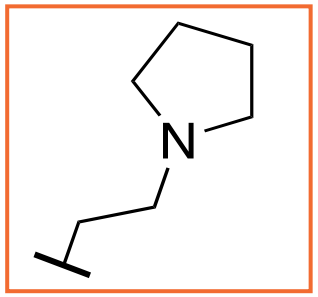


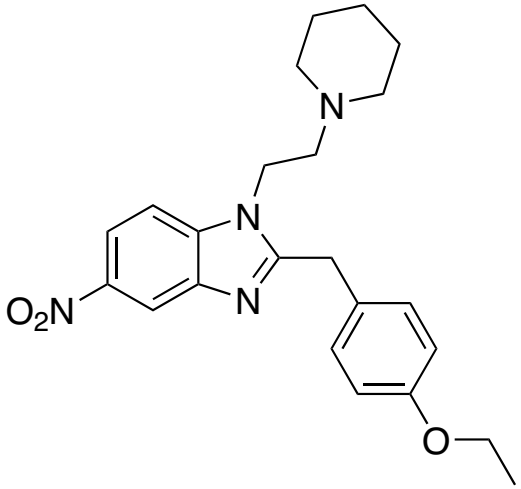
Isotonitazene
MW: 410.23



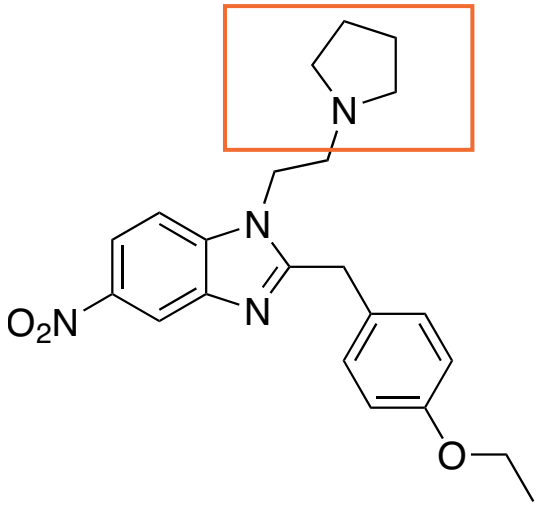
Metodesnitazene
MW: 337.21



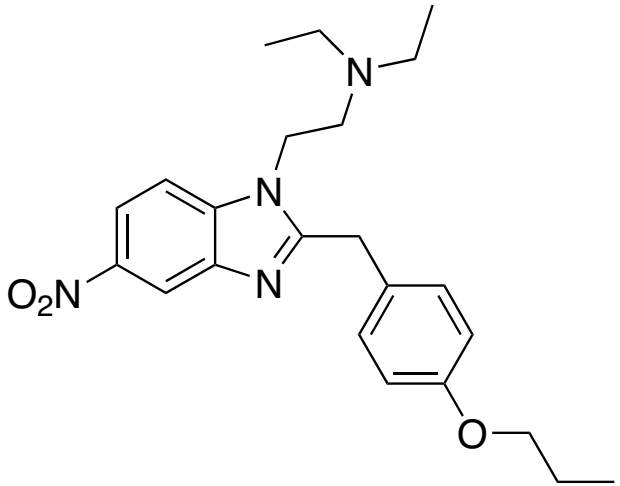
***m/z* 98**



N-Piperidinyl Etonitazene
MW: 408.50



N-Pyrrolidino Etonitazene
MW: 394.20



Protonitazene
MW: 410.23

Extraction and LC Methodology

Prep
- 0.5 mL whole blood

Add
- 50 µL NH_4OH
- 1 mL borate buffer

Add
- 3 mL 1-chlorobutane (*N*-butyl chloride)

Mix
- 15 min rotation

Isolate
- 10 min centrifuge (4000 rpm)
- Transfer organic to clean conical tubes

Dry
- Dry (40˚C) under N_2 (~12 mins)

Recon
- Reconstitute (200 µL) 90:10 mobile phase

LC Conditions	
Mobile Phases	A: 0.1% formic acid with 5 mM ammonium formate in deionized water
	B: 0.1% formic acid in acetonitrile
Column	Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7µm) + matching guard
Gradient	0.25 min hold at 10% B 10% B → 25% B over 1 min 25% B → 50% B over 3 mins 50% B → 90% B over 0.75 min (2 min hold) 90% B → 10% B
Injection Volume	5 µL
Column Temperature	35˚C
Flow Rate	0.4 mL/min

MS/MS Methodology

MS tested and optimized parameters:

- Scan range (*m/z*)
- Scan time (ms)
- Fragmentor (V)
- Collision energy (V)
- Cell acceleration voltage (V)
- MS2 Res

ESI+ Source Conditions	
Drying Gas	350˚C 11 L/min
Sheath Gas	400˚C 12 L/min
Nebulizer	40 psi
Capillary	3500 V
Nozzle	0 V

Monitored Product Ions	
m/z 72 and 100	Metodesnitazene 4'-OH nitazene 5-methyl etodesnitazene Isotonitazene Protonitazene
m/z 98	N-pyrrolidino etonitazene
m/z 112	N-piperidinyl etonitazene
m/z 104	Metodesnitazene-D_4

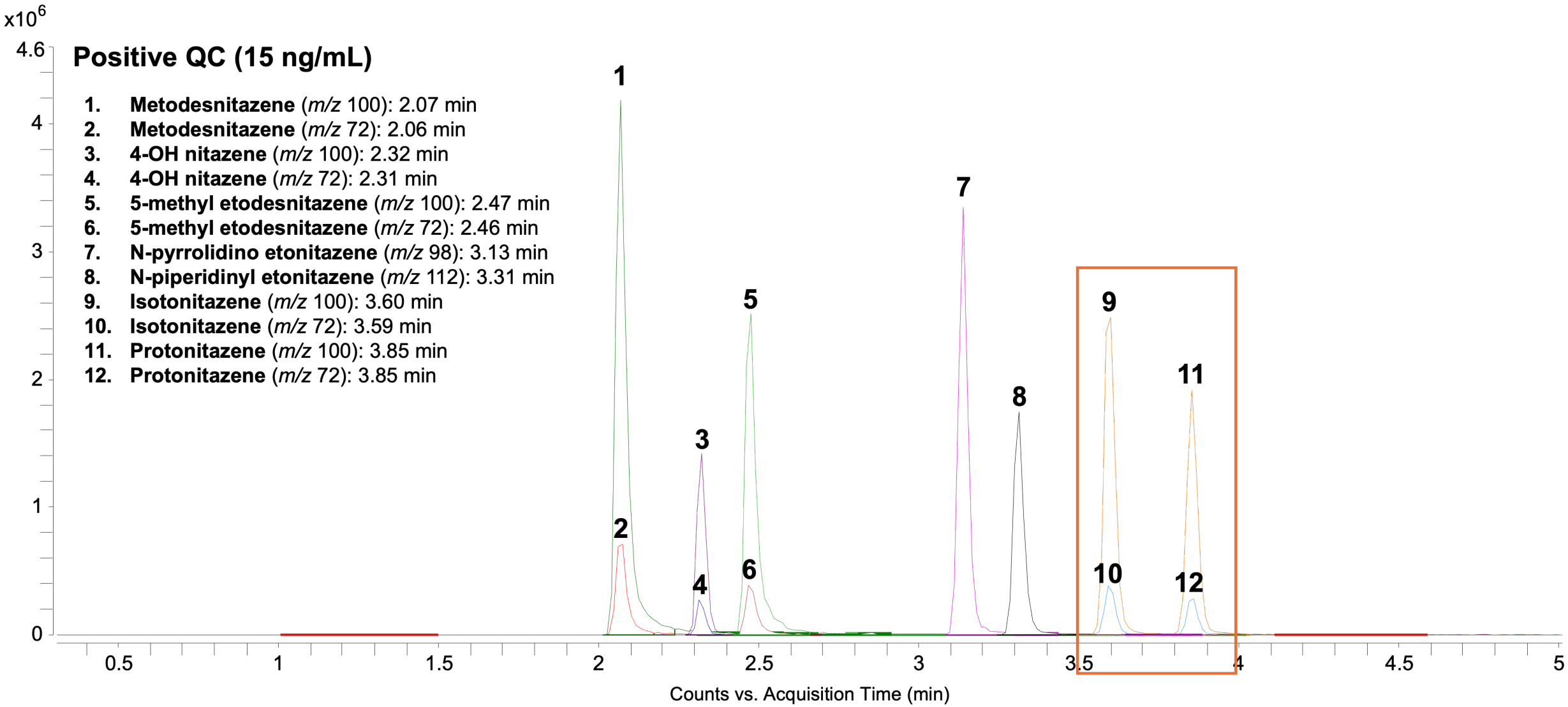
Method Development Results

MS tested and optimized parameters:

Product Ion (*m/z*)	Scan Range (*m/z*)	Scan Time (ms)	Fragmentor (V)	CE (V)
72.1	300-450	175	125	50
98.0	300-450	175	130	25
100.1	300-450	175	125	20
112.0	300-450	175	130	25
104.1 (ISTD)	300-350	150	120	20

MS2 Res	Wide (all)
Cell accelerator voltage	3 (all)
Gain factor	2 (time segment 2 only)

Chromatography



Validation and Acceptance Criteria

ANSI/ASB 036

- Limit of detection (using reference materials)
- Interferences (matrix, ISTD, commonly encountered analytes)
- Ionization suppression/enhancement
- Carryover
- Processed sample stability

Adopted from ANSI/ASB 098 and 113

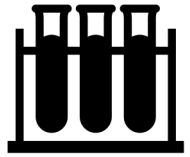
- Tolerances established for peak shape, retention time, resolution
- Minimum of 1 diagnostic ion required for identification
- No ions present >50% of the target ion abundance in the sample
  - Unless also present in positive QC

Validation Results

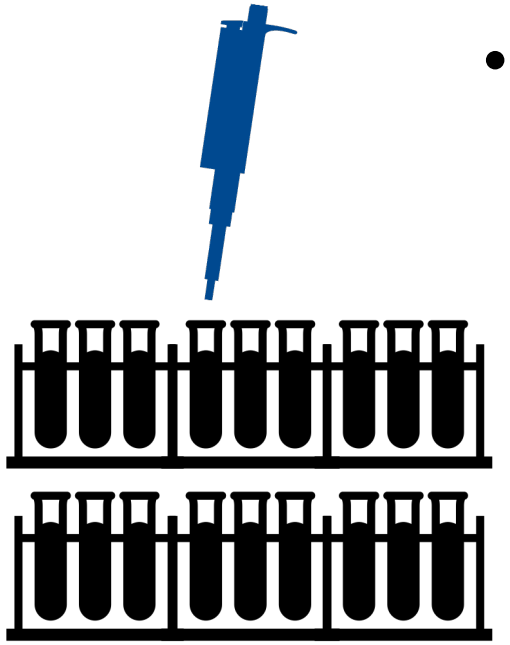
Analyte (Precursor)	Product Ions (*m/z*)	Limits of Detection (ng/mL)	Matrix Effects (%)*	Stability (hr)
Metodesnitazene	72.1	0.5	-32.1	≥ 48
	100.1	0.5	-32.7	≥ 48
4-OH nitazene	72.1	0.5	-49.1	≥ 48
	100.1	0.5	-50.5	≥ 48
5-methyl etodesnitazene	72.1	0.5	-33.2	≤ 24
	100.1	0.5	-35.0	≥ 48
Isotonitazene	72.1	0.5	-34.2	≥ 48
	100.1	0.5	-34.1	≥ 48
Protonitazene	72.1	0.5	-35.4	≥ 48
	100.1	0.5	-35.2	≥ 48
N-pyrrolidino etonitazene	98.0	0.5	-33.4	≥ 48
N-piperidinyl etonitazene	112.0	0.5	-50.9	≥ 48
Metodesnitazene-D_4	104.1	N/A	-33.0	≥ 48

*Results from matrix effects at low concentration (5 ng/mL)

Authentic and Blind Samples



- 3 samples previously confirmed for nitazenes from the CFSRE



- 20 samples prepared in-house by another analyst
  - Blinded to the extractor until after data analysis
  - Mimicked casework referenced in literature through:
    1. Differing concentrations based on potency
    2. Prevalence
    3. Combinations with other drugs of abuse
       - e.g., other opioids, novel benzos, and stimulants

Authentic and Blind Samples

Sample	Previous ID	Reported Concentration	QQQ PIS Positivity	Ions detected above LOD (*m/z*)
CFSRE 1	Metonitazene N-desethyl isotonitazene	0.6 ng/mL 2.2 ng/mL	Metonitazene* N-desethyl isotonitazene**	72, 100 72
CFSRE 2	Protonitazene	3.6 ng/mL	Protonitazene	72, 100
CFSRE 3	Protonitazene Metonitazene	1.3 ng/mL 0.8 ng/mL	Protonitazene Metonitazene*	72, 100 72, 100

*Analyte not included in method validation scope but identified with passing criteria (RT confirmed with standard)
**Analyte not included in method validation scope but presumptively identified with passing criteria

- Identification of analytes not included in initial scope of study
- No interferences that impacted accurate identification
- 100% positivity rate (for both blind and authentic samples)

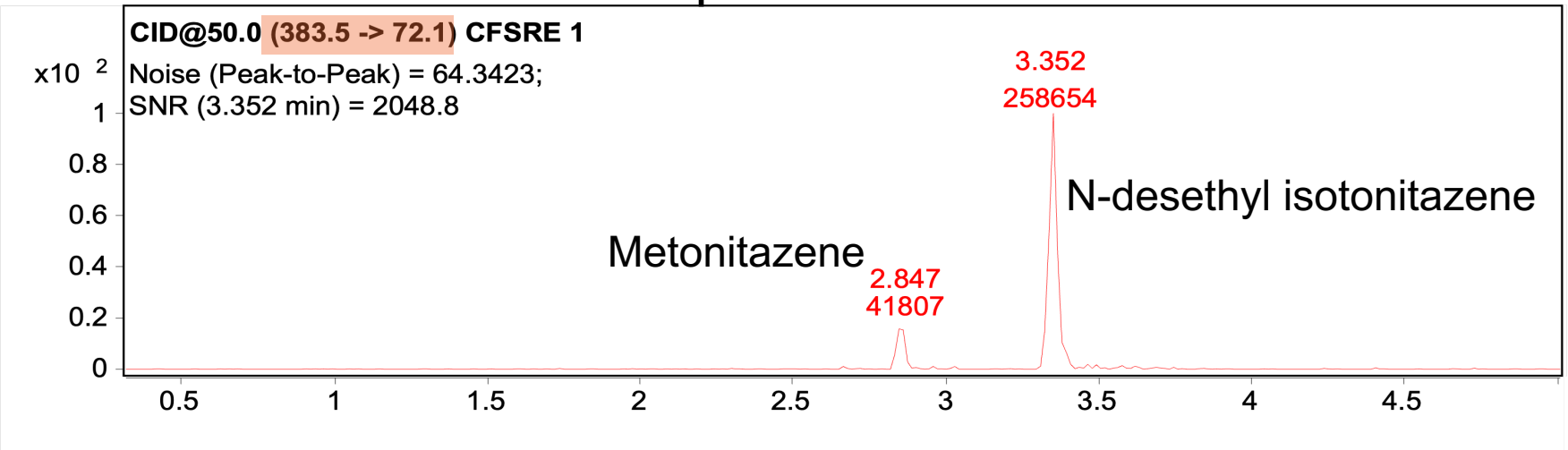
Authentic and Blind Samples

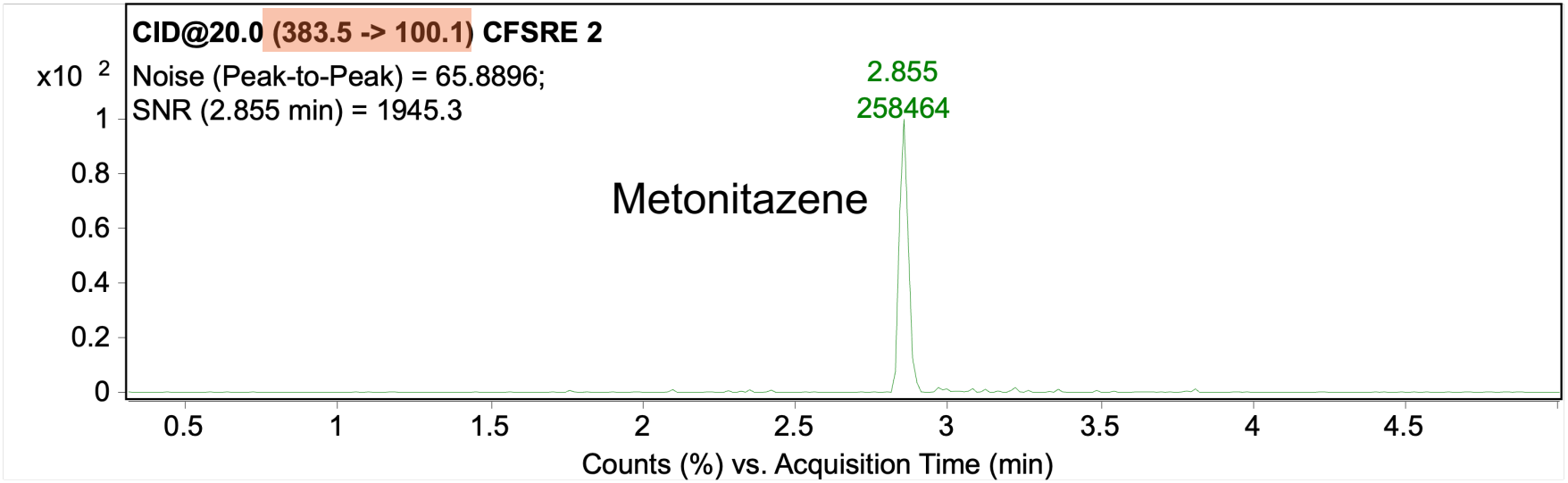
Sample	Nitazene(s) Added	QQQ ID(s)
Blind 1	Metonitazene	Metonitazene
Blind 2	N-pyrrolidino etonitazene	N-pyrrolidino etonitazene
Blind 3	5-methyl etodesnitazene	5-methyl etodesnitazene
Blind 4	Protonitazene 4-OH nitazene	Protonitazene 4-OH nitazene
Blind 5	Protonitazene	Protonitazene
Blind 6	N-piperidinyl etonitazene	N-piperidinyl etonitazene
Blind 7	Protonitazene Metonitazene	Protonitazene Metonitazene
Blind 8	None	ND
Blind 9	Isotonitazene	Isotonitazene
Blind 10	Metonitazene	Metonitazene

Sample	Nitazene(s) Added	QQQ ID(s)
Blind 11	None	ND
Blind 12	4-OH nitazene Isotonitazene	4-OH nitazene Isotonitazene
Blind 13	None	ND
Blind 14	Isotonitazene	Isotonitazene
Blind 15	None	ND
Blind 16	N-pyrrolidino etonitazene	N-pyrrolidino etonitazene
Blind 17	5-methyl etodesnitazene	5-methyl etodesnitazene
Blind 18	Metonitazene Metodesnitazene	Metonitazene Metodesnitazene
Blind 19	Metodesnitazene	Metodesnitazene
Blind 20	None	ND

Authentic and Blind Samples

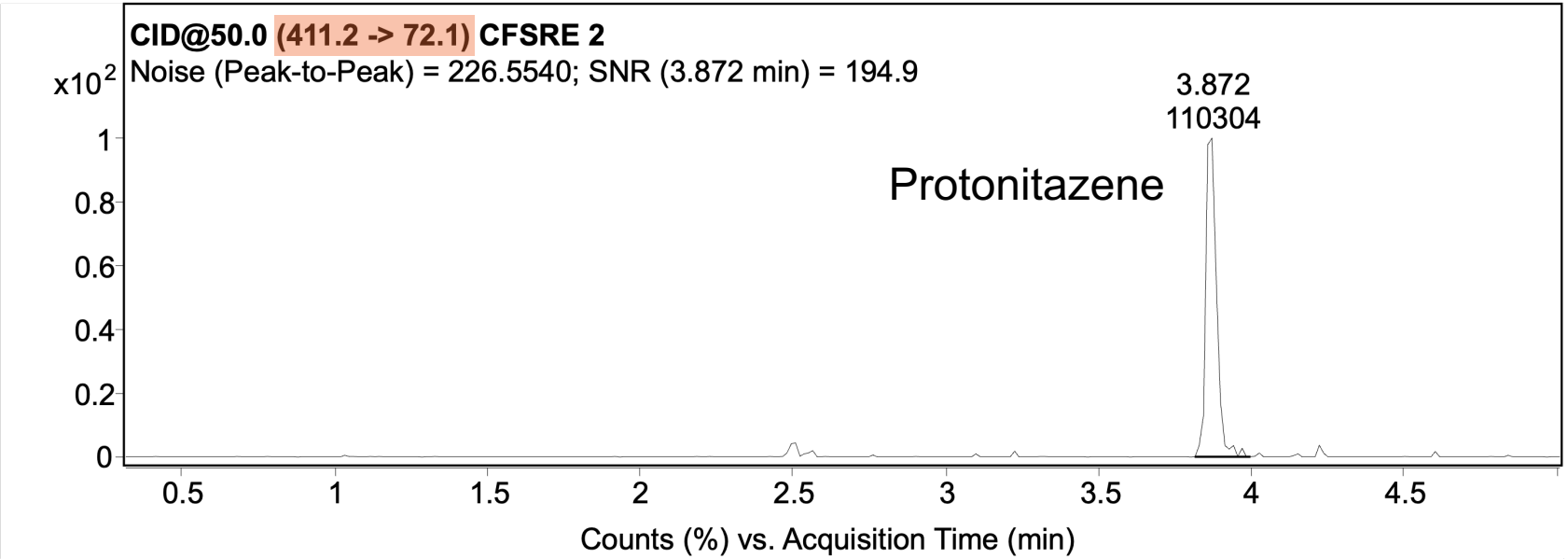
Sample: CFSRE 1

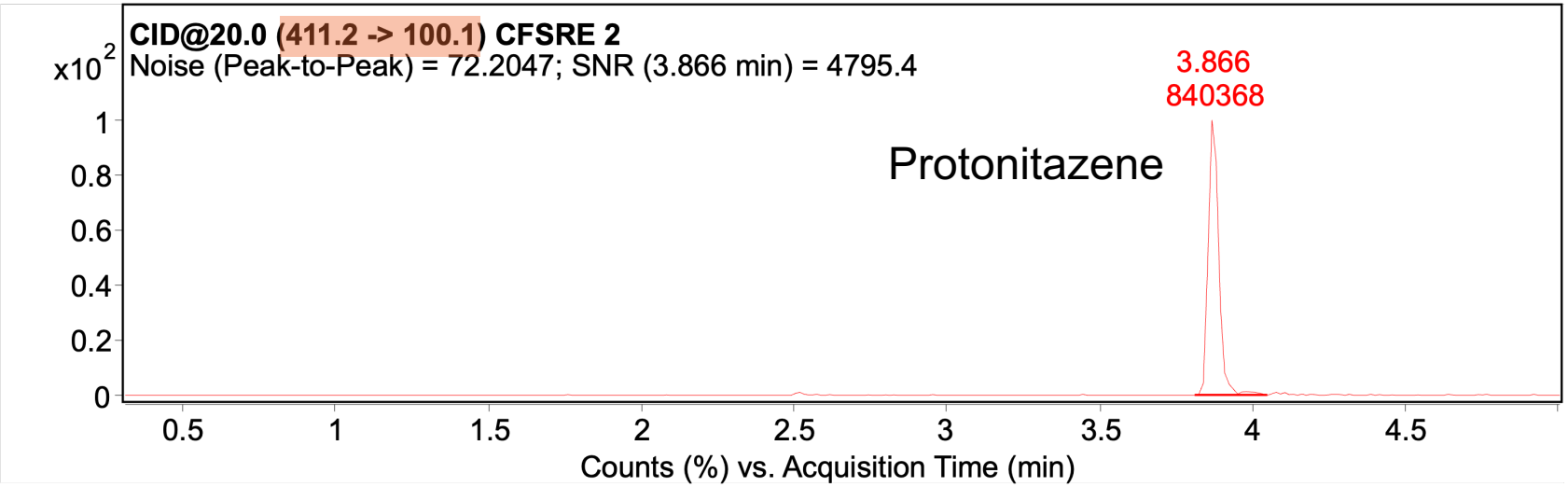




Authentic and Blind Samples

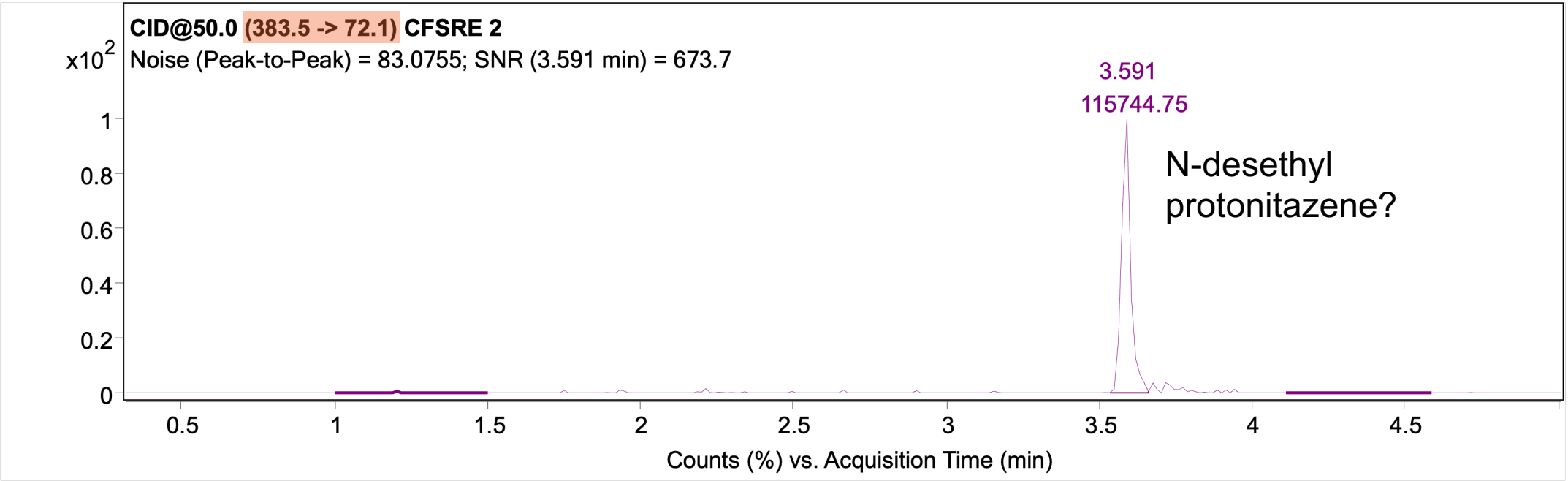
Sample: CFSRE 2



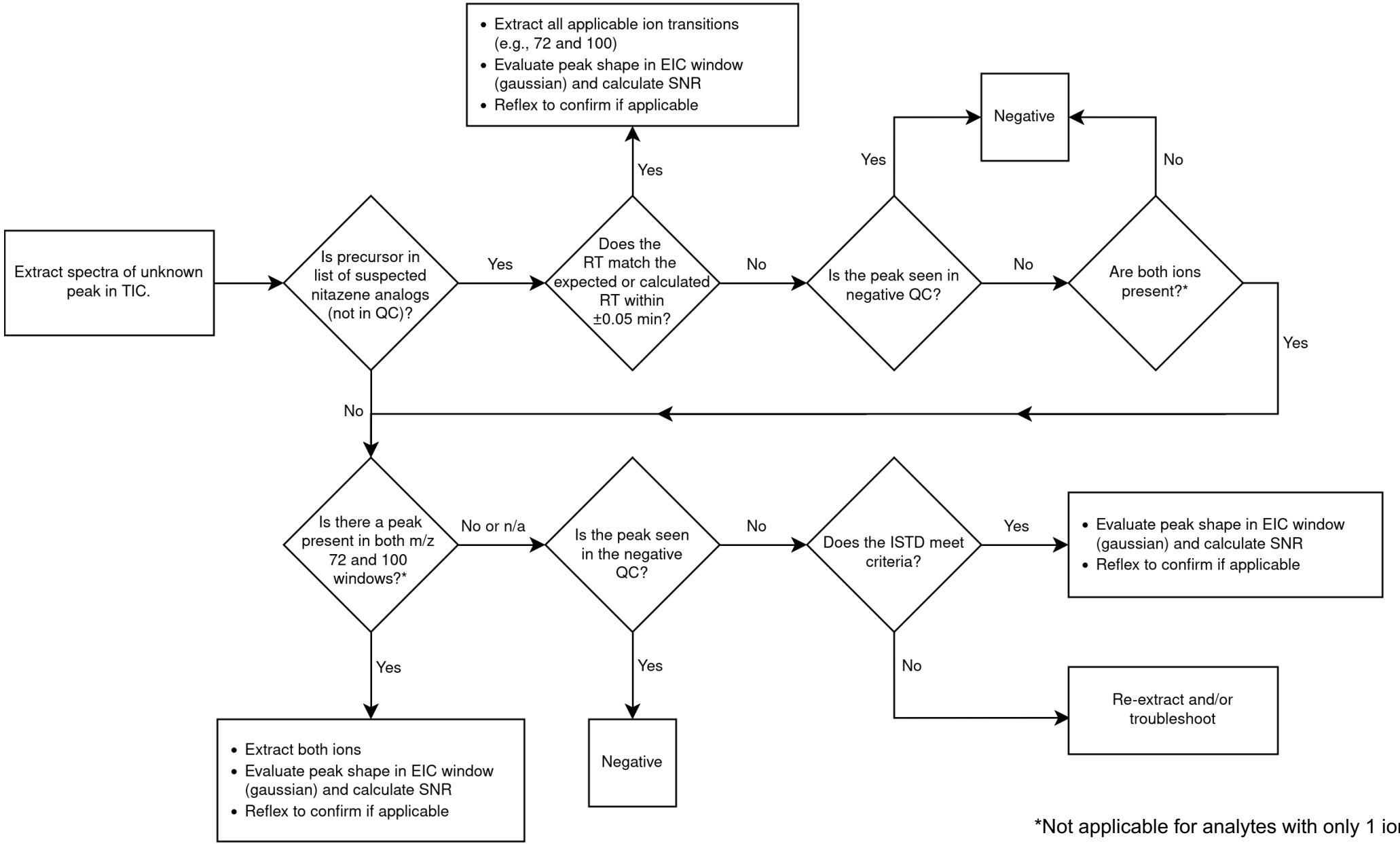


Authentic and Blind Samples

Sample: CFSRE 2



Proposed Workflow for Unknowns Analysis



*Not applicable for analytes with only 1 ion monitored

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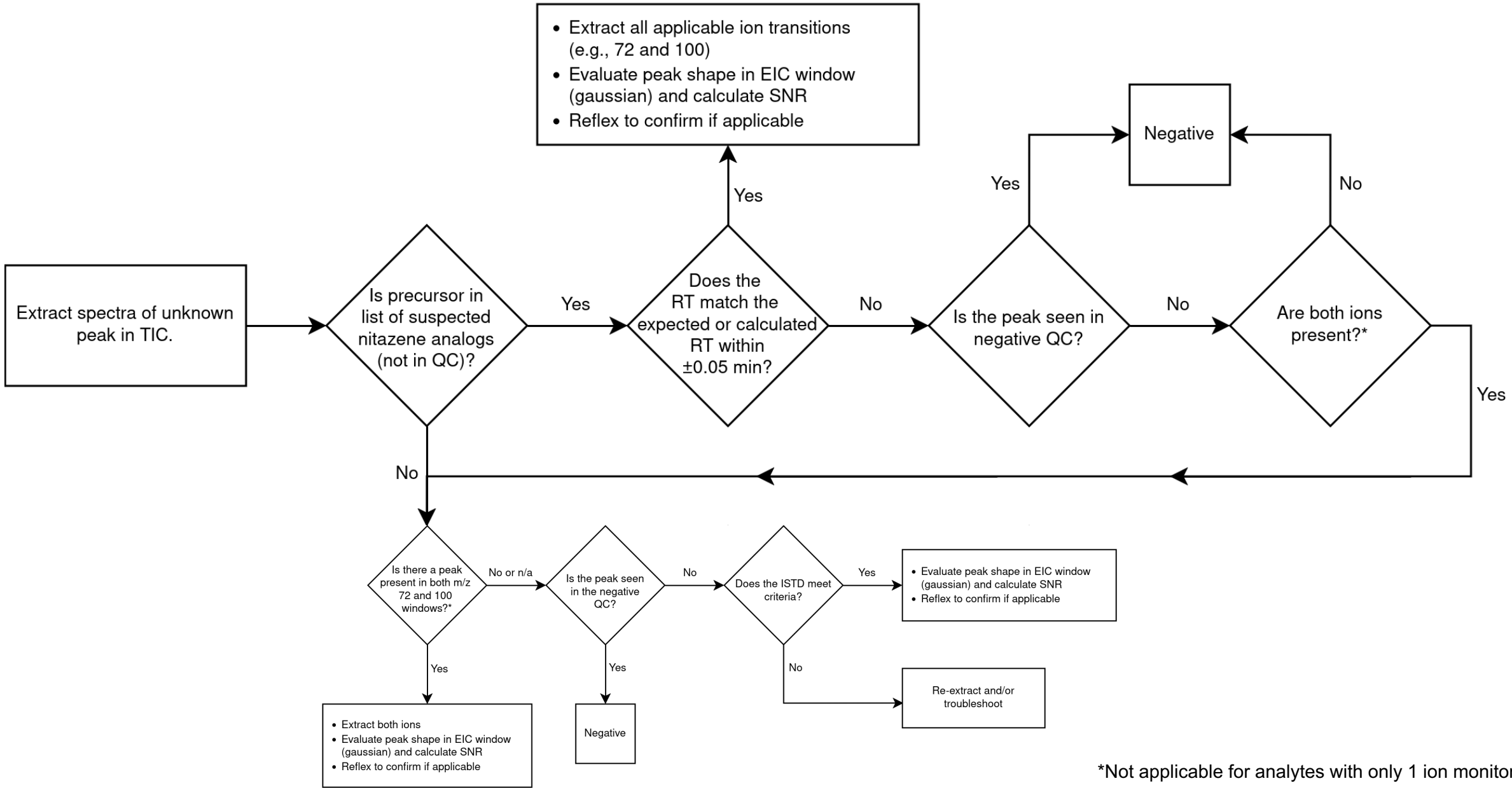

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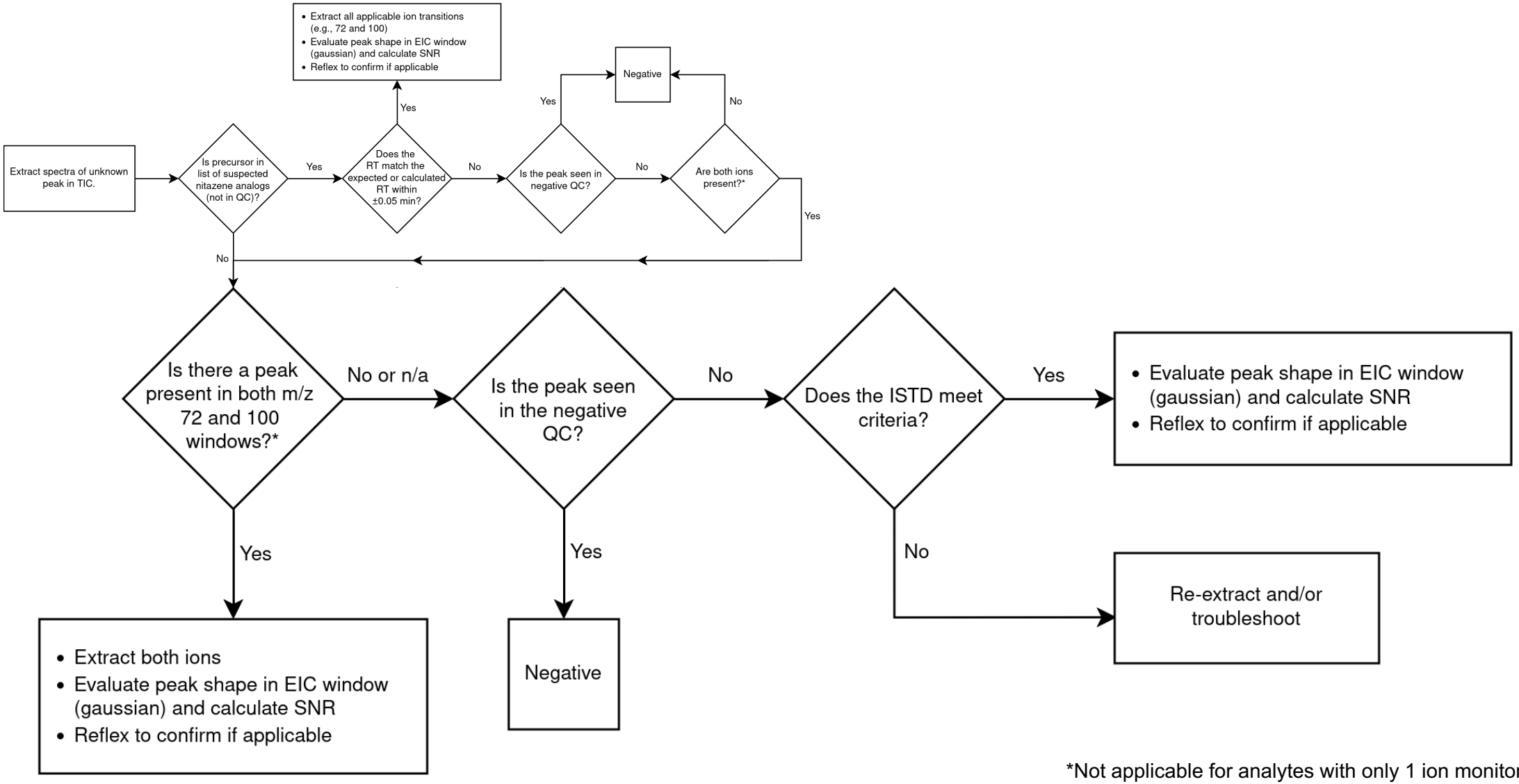

*Not applicable for analytes with only 1 ion monitored

Discussion

- Method performance was evaluated and validated using guidance from ASB standards 036, 098, and 113
  - Method allowed for forensically-relevant detection limits
  - No interferences or carryover were observed
  - Ion suppression was observed but demonstrated no impact to LODs and other critical validation parameters
  - Most analytes/ions were stable for at least 48 hours (except 5-methyl etodesnitazene ≤24)
- Previously analyzed authentic samples were reinterrogated using this method
  - Analytes not in the validation scope but previously confirmed were identified
  - Analytes not previously confirmed were presumptively identified
- 20 blind specimens were prepared with various drug combinations containing nitazenes
  - All nitazenes were correctly identified using the method described

Conclusions

- A new precursor ion scan method was successfully developed for the broad identification of nitazene analogs in whole blood
  - Laboratories can use existing in-lab instrumentation for screening of nitazene analogs
  - Reduced suspect nitazene samples sent for confirmation testing
- The method can identify previously undetected compounds that were not evaluated at initial processing
  - Potential for retrospective data analysis
  - Broader detection of characteristic fragments can help identify undescribed analytes

Limitations

- Data processing software is not well-supported for this type of analysis
  - Specific workflows are needed to accommodate analysis
- More studies are needed to further develop rigorous assessment criteria and include additional nitazene analogs
  - Especially N-desethyl analogs
- May offset limitations of ELISA, but high-resolution screening is still preferred for unknown identification
  - However, lower LODs might be achieved with QQQ and libraries are not needed for initial data interrogation

Acknowledgements

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  - Dr. Alex Krotulski
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- Colleagues
  - Dr. Britni Skillman
  - Sara Kuberski

Questions?

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