DOMINION DIAGNOSTICS

Evaluating Technical Suitability of High Resolution Mass Spectrometry for Simultaneous Screening and Quantification of 47 Drugs in Human Urine

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- Introduction
- Materials and Methods
- Results
- Conclusions
- Questions

Introduction

Why did we consider High Resolution Accurate Mass(HRAM) instrumentation as an alternative to immunoassay?

- Most drug screening is performed in two steps comprising an initial immunoassay screen followed by confirmation by liquid chromatography mass spectrometry.
- Monitoring various drugs of abuse using HRAM LC-MS allows for simpler sample preparation, and increased analytical specificity and selectivity.
- Decrease sample analysis cost while increasing accuracy and the number of analytes screened and quantified on a single injection using HRAM LC-MS.

Introduction

Types of Screening

EIA/Elisa	**LC MS Accurate Mass**
•Fast Cycle Time (seconds) •Low Sensitivity •Low Specificity •Pos/Neg Results Only – drug families, no individual drugs	•High Sensitivity •Very High Specificity •Screening and Quantitation are Accurate •Cycle time in minutes (not seconds)

Introduction

Types of Screening using Accurate Mass	
Full MS Scan	**Full MS Scan/MS^2**
•Retention Time	•Retention Time
•Accurate Mass of the Parent	•Accurate Mass of the Parent
•Isotopic Pattern	•Isotopic Abundance Pattern
	•Accurate Mass of Fragments
	•Library Search

Introduction

Types of Screening Applications Using Full MS Scan/MS^2

Method Screens for hundreds of compounds	Method Screens for a restricted number of compounds
•Ability to set up screening for hundreds of targeted and non-target compounds •Refer back to the data anytime without reruns to investigate samples for both target and non-target compounds •Calibrators and QCs do not have all the compounds present at once	•Inclusion list has only targeted compounds to perform MS^2 •Calibrators and QCs have all the compounds present at once •MS^2 is collected for all the compounds present in calibrators and QCs •All calibrators and QCs have to pass screening criteria.

Introduction

Reasons for choosing a targeted Screening method

- Require to have a certified reference standard for screening
- Demonstrate linearity
- QCs and calibrators need to be included in the analytical run
- Results are not reported below LLOQ
- Screening and Quantitation can be done with one single injection – Calibrators and QCs are required for both

Materials and Methods

Calibrators and quality control (QC) samples were prepared in Surine™ using certified reference standards from Cerilliant™

Sample Preparation Procedure

75µL of sample were diluted with 300 µL a master mix solution containing IMCSzyme®, and 47 internal standards, one for each of the analytes. Samples were incubated at 65°C for 60 minutes, and centrifuged at 4000 rpm for 7 min before analysis.

Materials and Methods

LC Parameters

Instrument:	UPLC
Analytical Column:	Agilent Poroshell™ 120 SB-C18 2.1 x 50mm 2.7µm
Gradient:	A: 0.1 % Formic Acid acid in LCMS grade water B: 0.1 % Formic Acid in Methanol

Time (min)	A (%)	B(%)
0.50	95	5
1.50	75	25
4.60	40	60
4.61	5	95
5.00	5	95
5.01	95	5

Flow Rate:	0.50 mL/min
End time:	5.5 min
Column Temp:	30 °C
Injection volume:	7.5 µL
Autosampler Temp:	5 °C

Materials and Methods

Mass Spectrometer

Source Parameters

Mode: HESI+

Sheat gas flow rate: 65

Aux gas flow rate: 20

Sweep gas flow rate: 2

Spray voltage: 3.5 kV

Capillary Temp: 300 °C
Aux gas heater temp: 420 °C
S-lens RF Level: 55

Full MS

Scan Range: 130-480 m/z
Resolution: 17 500, 35 000, 70 000

MS/MS

First fixed mass: 50 m/z
Resolution: 17 500

ddMS^2

Top 2, 3, 4, 5 (targeted)
First fixed mass: 50 m/z
Resolution: 17 500

Results

ANALYTE	Internal Standard	Analyte Calibration Range	ANALYTE	Internal Standard	Analyte Calibration Range
2-Hydroxyethylflurazepam	2-Hydroxyethylflurazepam-D4	20-1000	Methadone	Methadone-D3	20-1000
6-Acetylmorphine	6-Acetylmorphine-D3	20-1000	Methamphetamine	Methamphetamine-D5	100-5000
7-Aminoclonazepam	7-Aminoclonazepam-D4	20-1000	Morphine	Morphine-D3	20-2000
α-Hydroxyalprazolam	α-Hydroxyalprazolam-D5	20-1000	Norbuprenorphine	Norbuprenorphine-D3	20-1000
α-Hydroxymidazolam	α-Hydroxymidazolam-D4	20-1000	Norcodeine	Norcodeine-D3	20-1000
α-Hydroxytriazolam	α-Hydroxytriazolam-D4	20-1000	Nordiazepam	Nordiazepam-D5	20-1000
Alprazolam	Alprazolam-D5	20-1000	Norfentanyl	Norfentanyl-D5	2-100
Amphetamine	Amphetamine-D5	100-5000	Norhydrocodone	Norhydrocodone-D3	20-1000
Benzoylecgonine	Benzoylecgonine-D3	20-1000	Normeperidine	Normeperidine-D4	20-1000
Buprenorphine	Buprenorphine-D4	20-1000	Noroxycodone	Noroxycodone-D3	20-1000
Carisoprodol	Carisoprodol-D7	20-1000	Norpropoxyphene	Norpropoxyphene-D5	40-2000
Cocaethylene	Cocaethylene-D3	20-1000	O-Desmethyltramadol	O-Desmethyltramadol-D6	20-1000
Codeine	Codeine-D6	20-2000	Oxazepam	Oxazepam-D5	20-1000
EDDP	EDDP-D3	20-1000	Oxycodone	Oxycodone-D3	20-2000
Fentanyl	Fentanyl-D5	2-100	Oxymorphone	Oxymorphone-D3	20-2000
Gabapentin	Gabapentin-D10	20-1000	PCP	PCP-D5	20-1000
Hydrocodone	Hydrocodone-D3	20-2000	Pentazocine	Pentazocine-13C3	20-1000
Hydromorphone	Hydromorphone-D3	20-2000	Phentermine	Phentermine-D5	100-5000
Lorazepam	Lorazepam-D4	20-1000	Pregabalin	Pregabalin-D6	20-1000
MDA	MDA-D5	100-5000	Ritalinic acid	Ritalinic acid-D10	20-1000
MDEA	MDEA-D5	100-5000	Tapentadol	Tapentadol-D3	20-1000
MDMA	MDMA-D5	100-5000	Temazepam	Temazepam-D5	20-1000
Meperidine	Meperidine-D4	20-1000	Tramadol	Tramadol-13C, D3	20-1000
Meprobamate	Meprobamate-D7	20-1000			

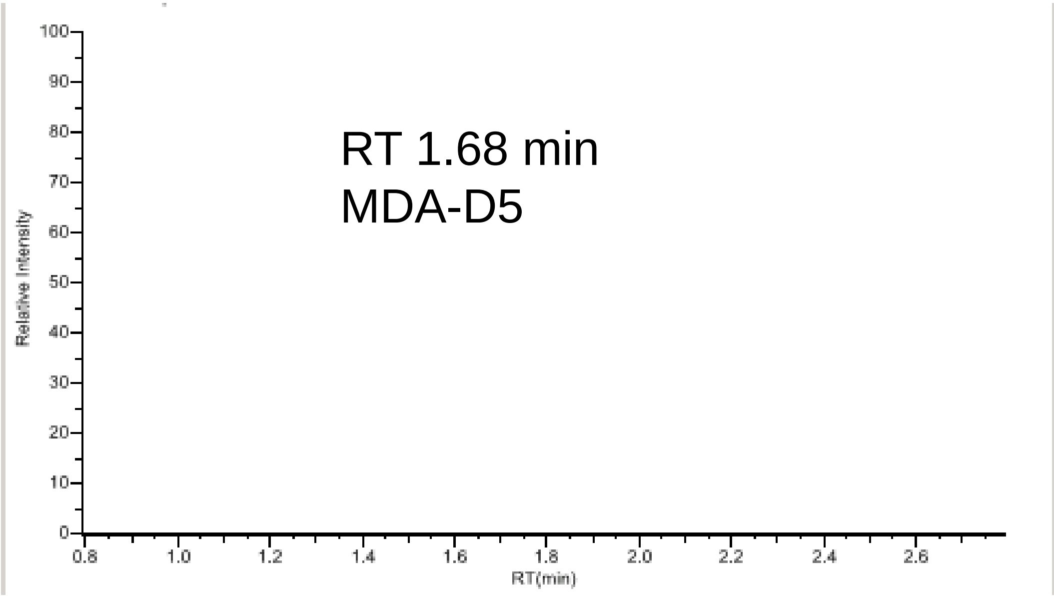
Results

Analyte identity criteria:

- Accurate Mass of the Parent: Full-scan extracted ion chromatograms (XIC) ±5 ppm
- Retention Time
- Isotopic Abundance Pattern
- At least two expected fragments (±10ppm) from the MS/MS scans
- Library Search: Custom MS/MS library built in-house using MS/MS spectra derived from calibrators

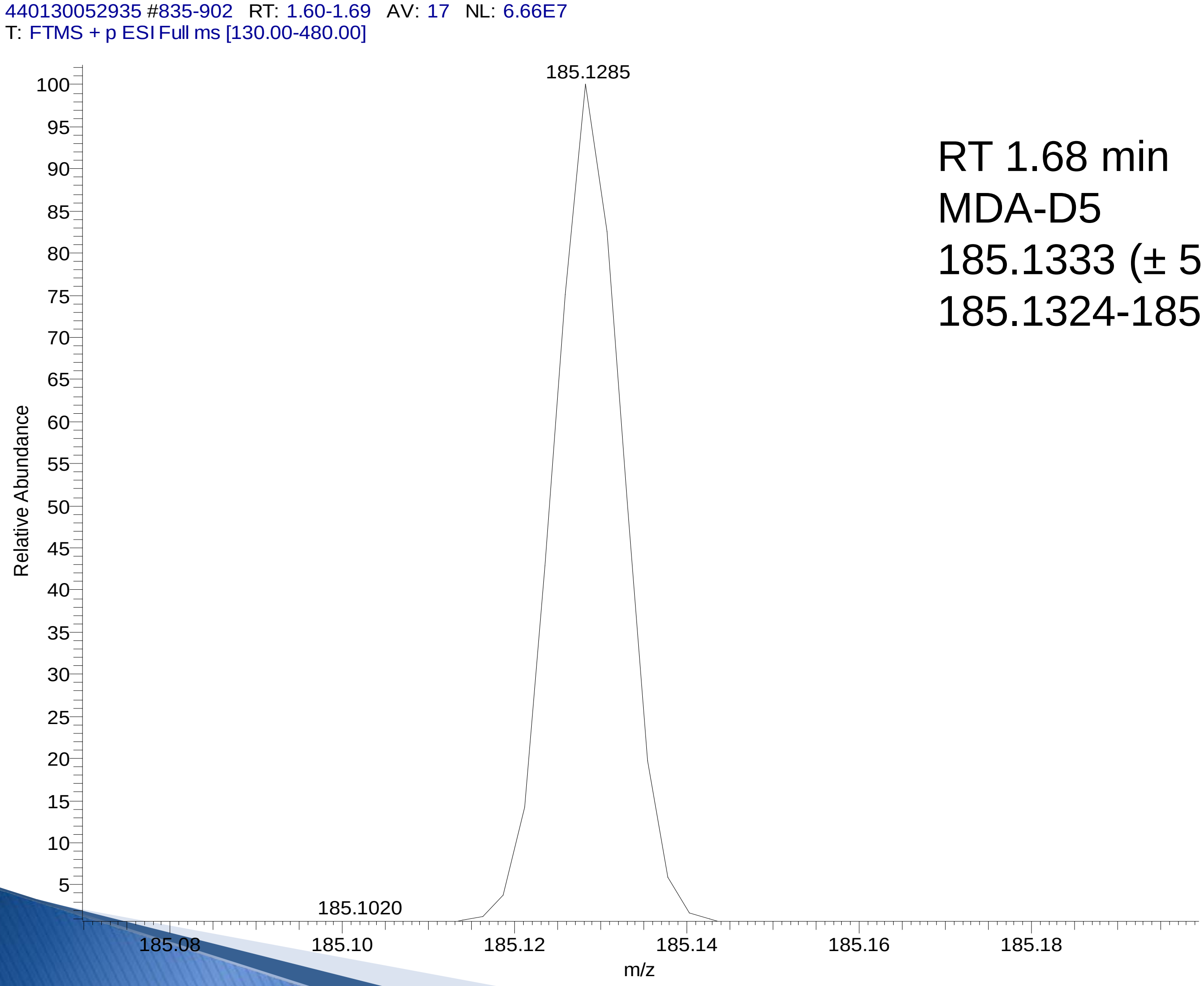
Results- MD 17500 Resolution

Internal Standard MDA-D5 is not detected in this donor sample even though it was added.



RT 1.68 min
MDA-D5

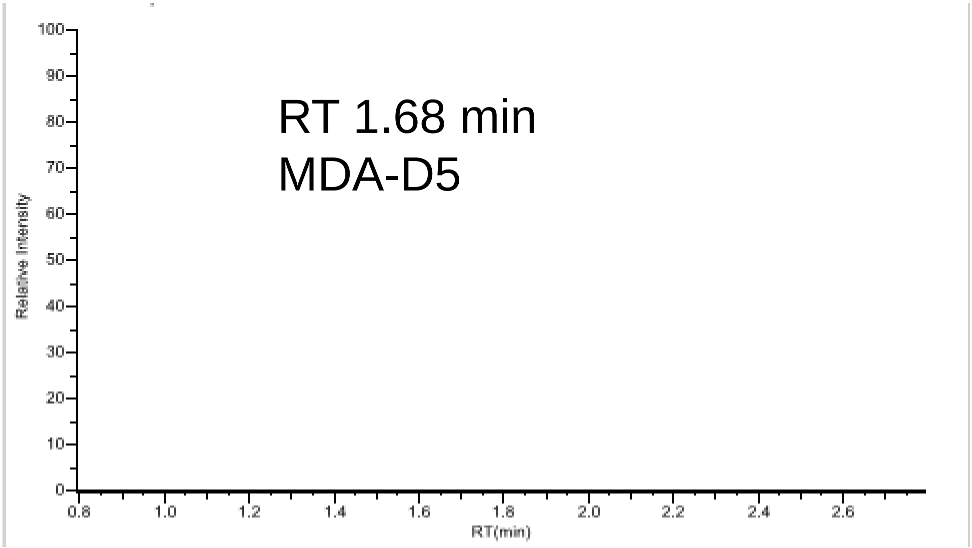
Results- MD 17500 Resolution



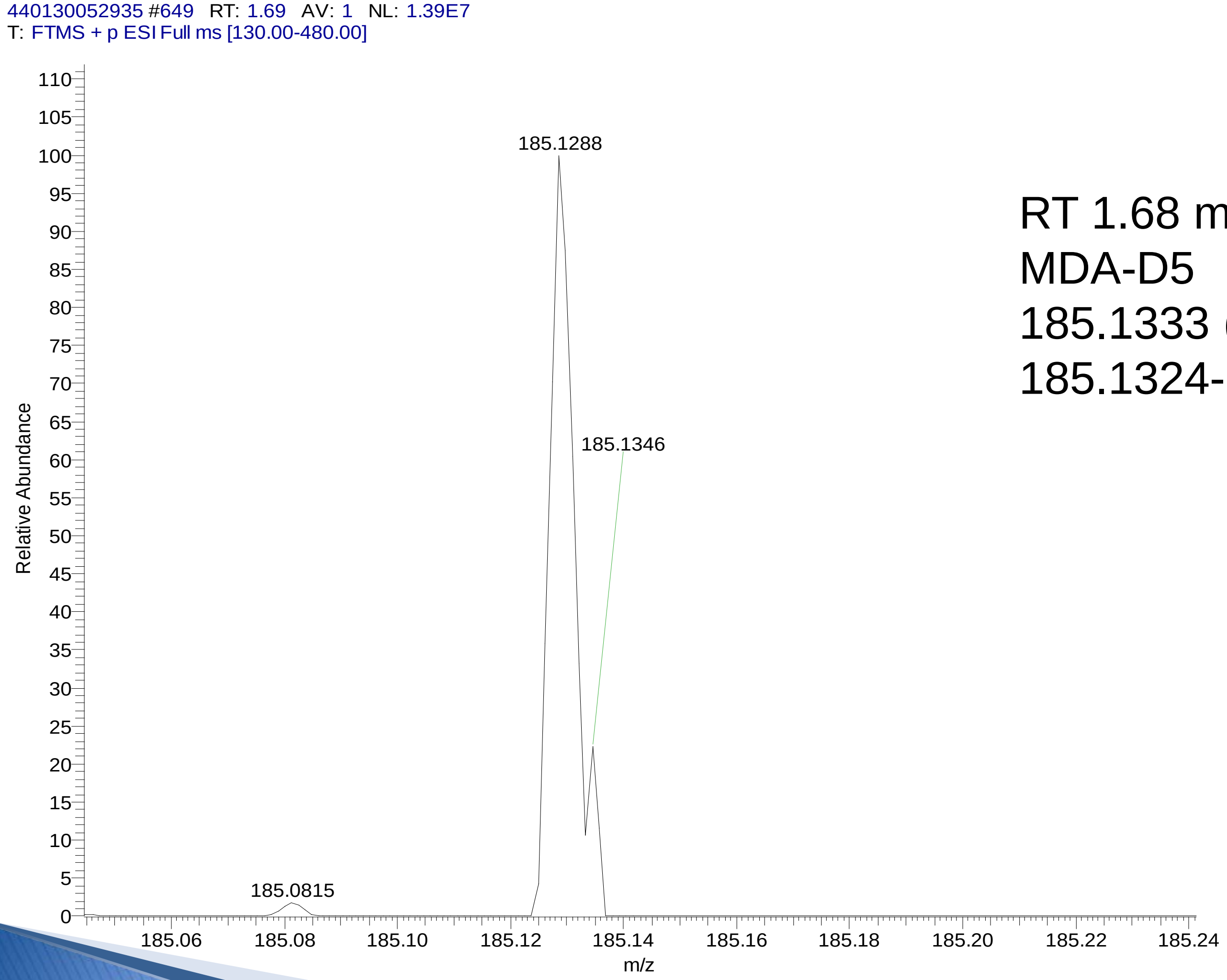
RT 1.68 min
MDA-D5
185.1333 (± 5 ppm window)
185.1324-185.1342

Results- MD 35000 Resolution

Internal Standard MDA-D5 is not detected in this donor sample even though it was added.

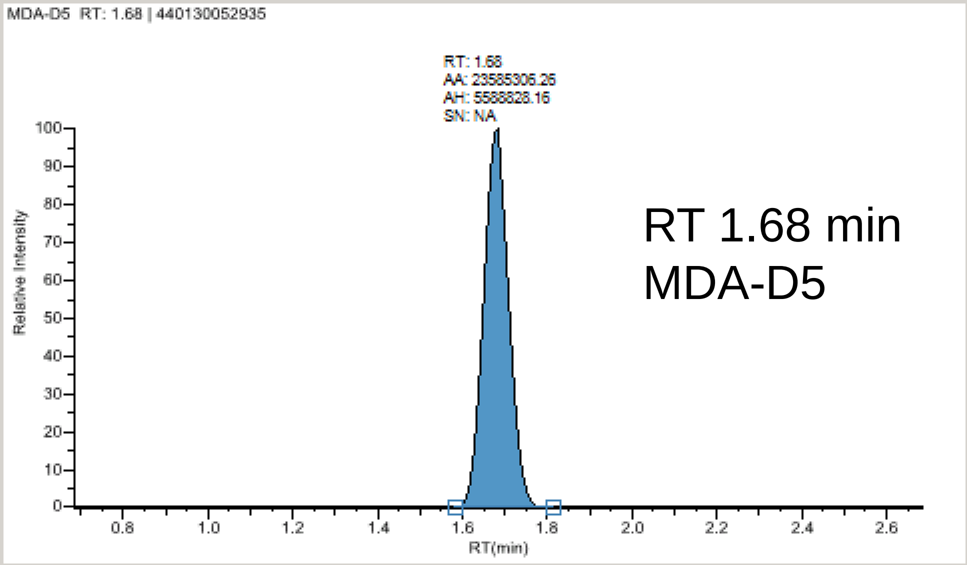


Results- MD 35000 Resolution



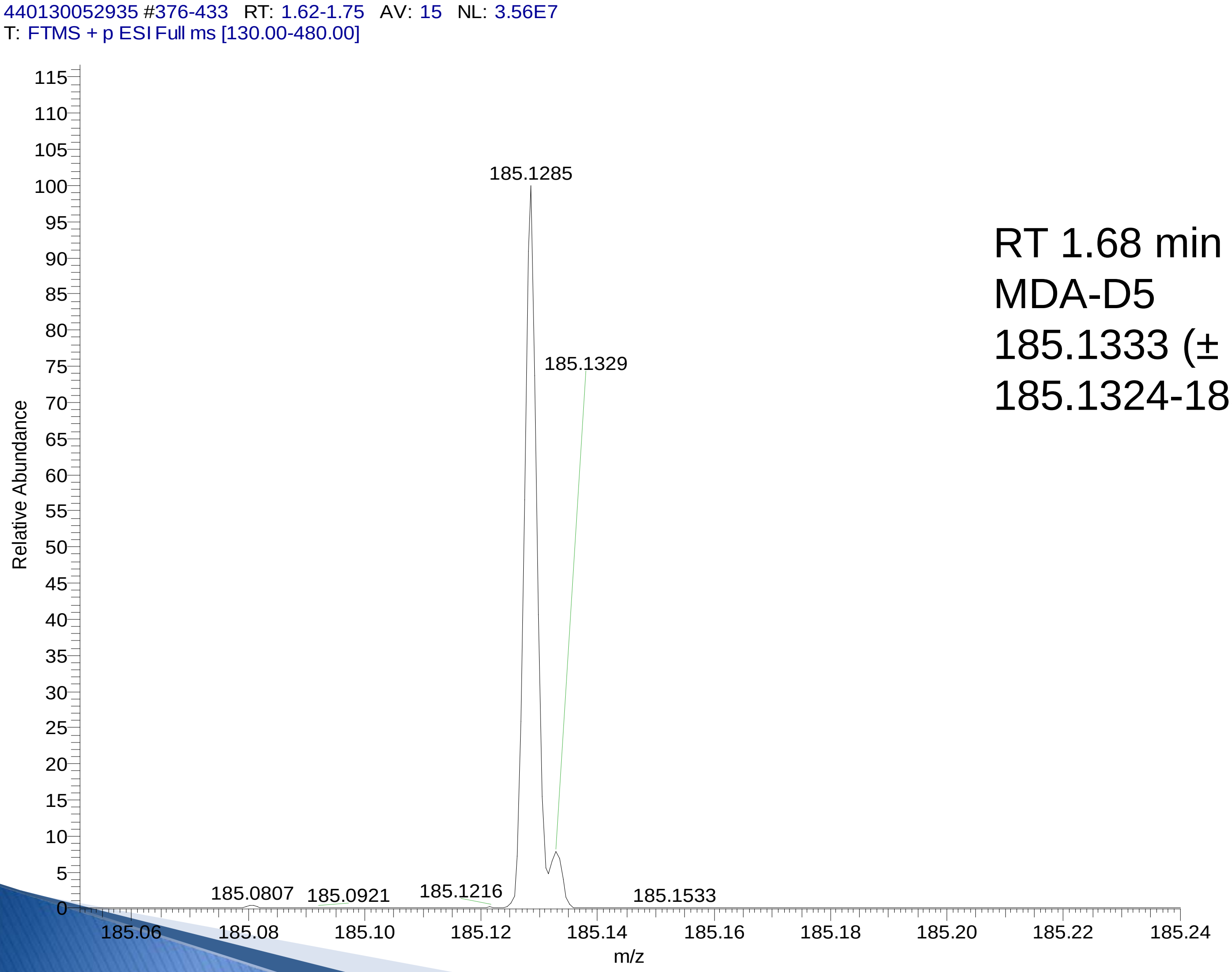
RT 1.68 min
MDA-D5
185.1333 (± 5 ppm window)
185.1324-185.1342

Results- MD 70000 Resolution



RT 1.68 min
MDA-D5

Results- MD 70000 Resolution

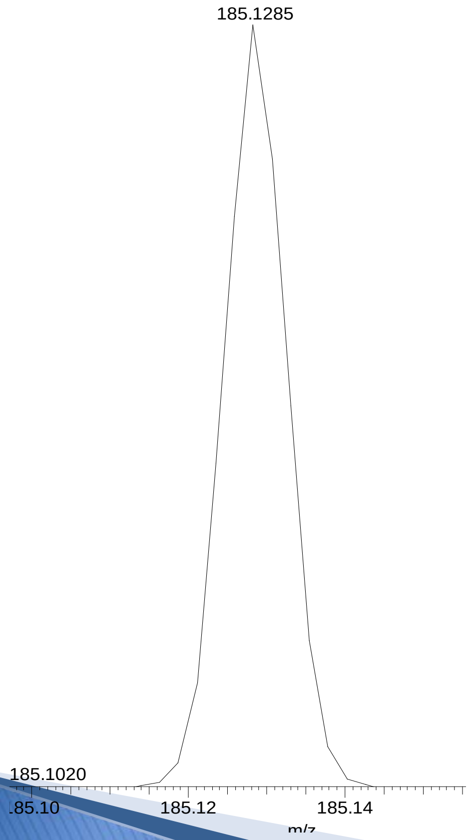


RT 1.68 min
MDA-D5
185.1333 (± 5 ppm window)
185.1324-185.1342

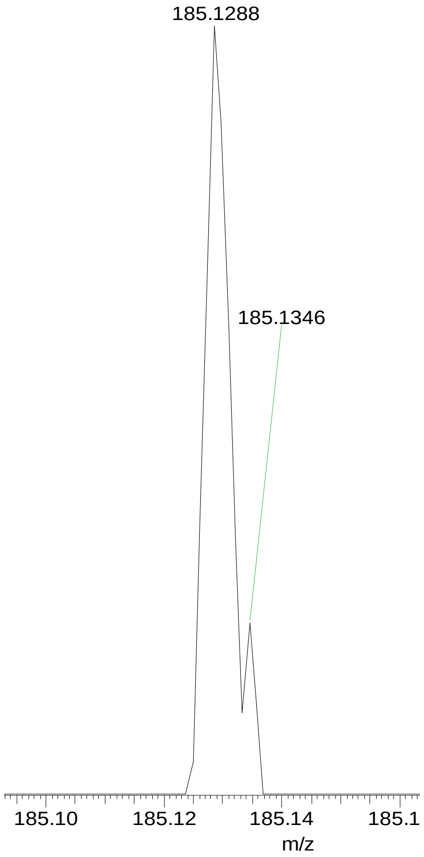
Results- MD All resolutions

MDA-D5 185.1333 (± 5 ppm window) 185.1324-185.1342

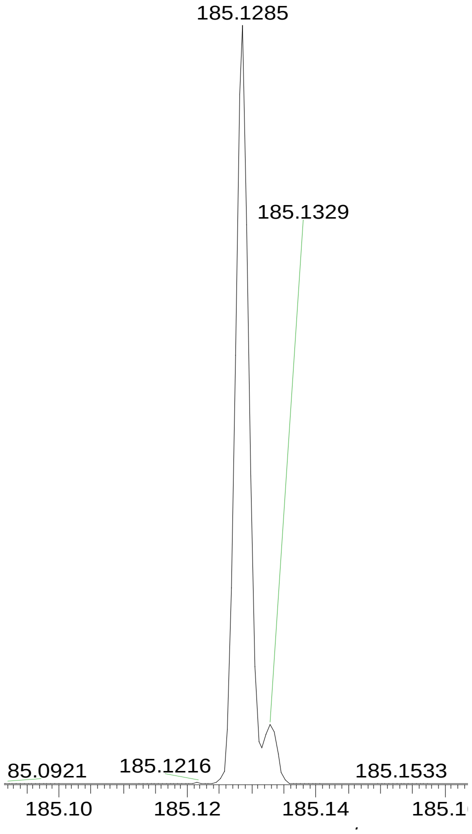
17 500



35 000

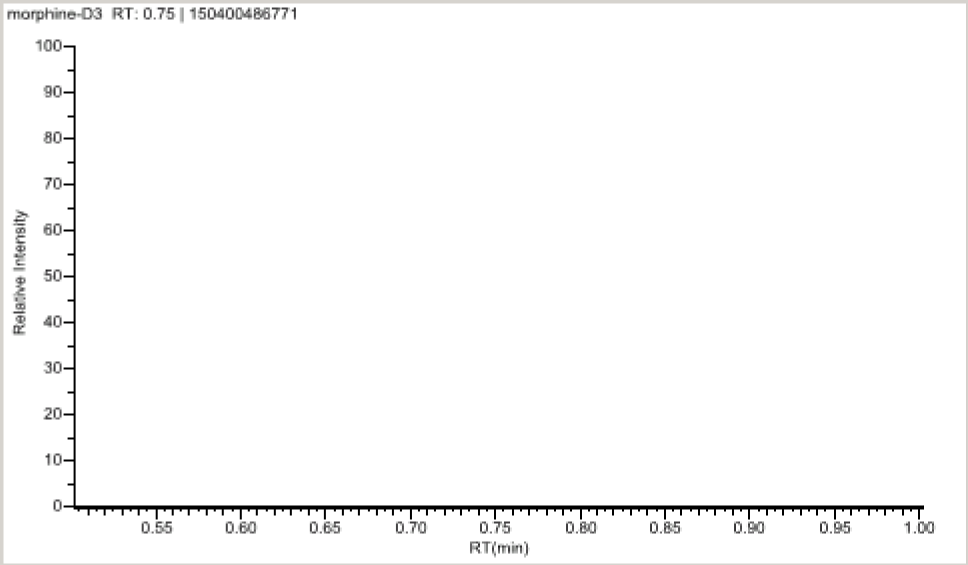


70 000



Results- MD 17500

Internal Standard Moprhine-D3 is not detected in this donor sample even though it was added.

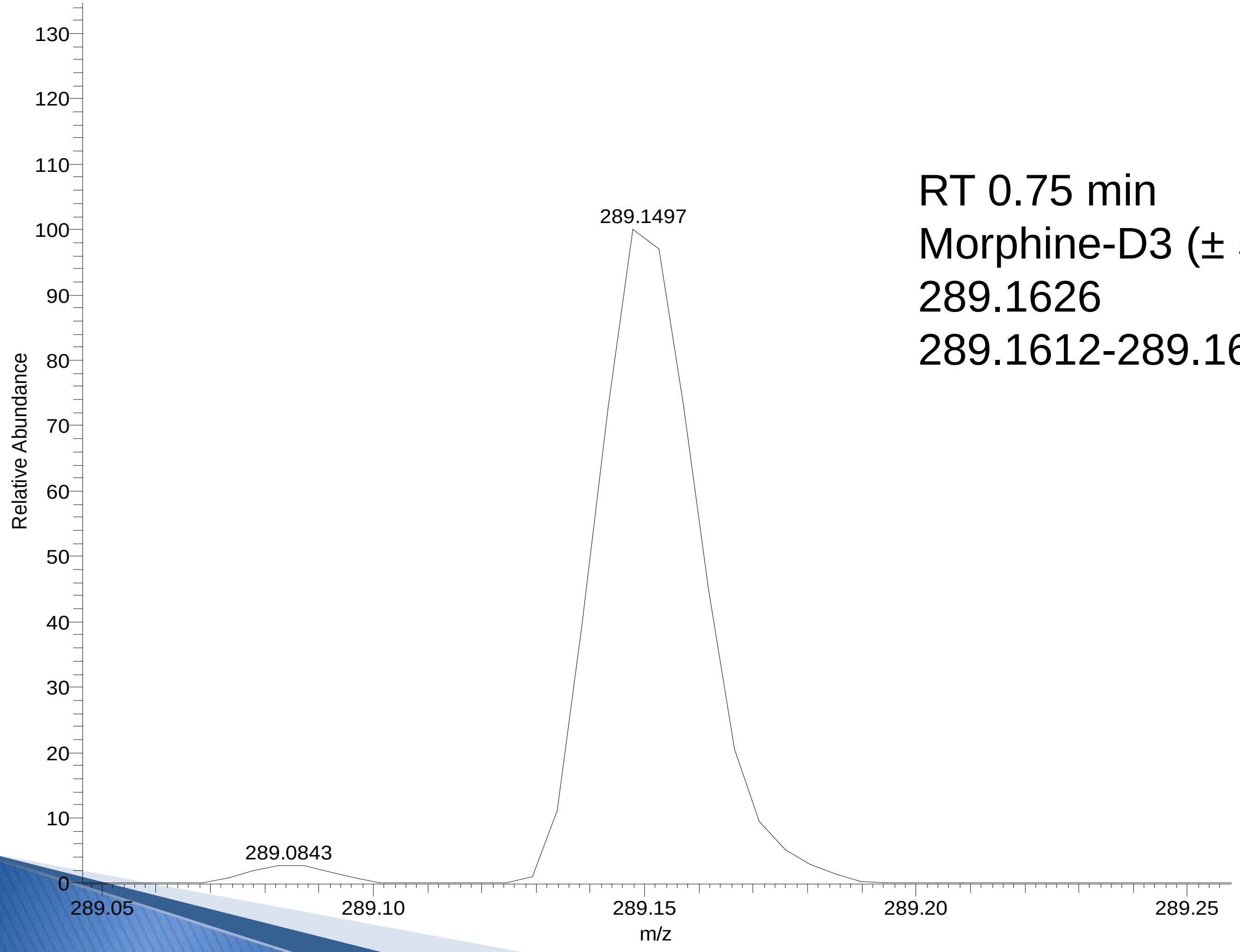


Positive for morphine

RT 0.72 min
Morphine-D3

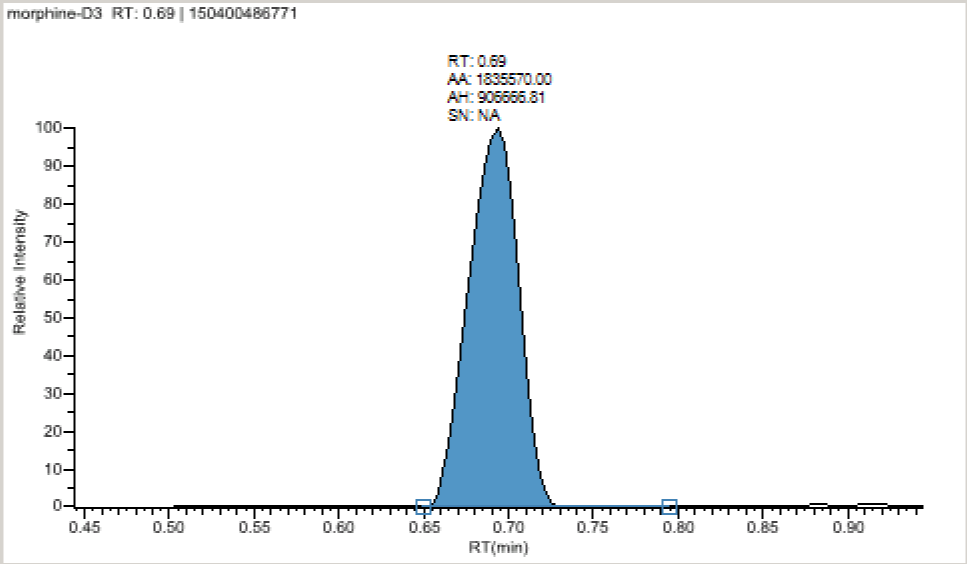
Results- MD 17500

150400486771 #136-280 RT: 0.68-0.86 AV: 72 NL: 1.71E6
T: FTMS + p ESI Full ms [130.00-480.00]



RT 0.75 min
Morphine-D3 (± 5 ppm window)
289.1626
289.1612-289.1640

Results- MD 35000

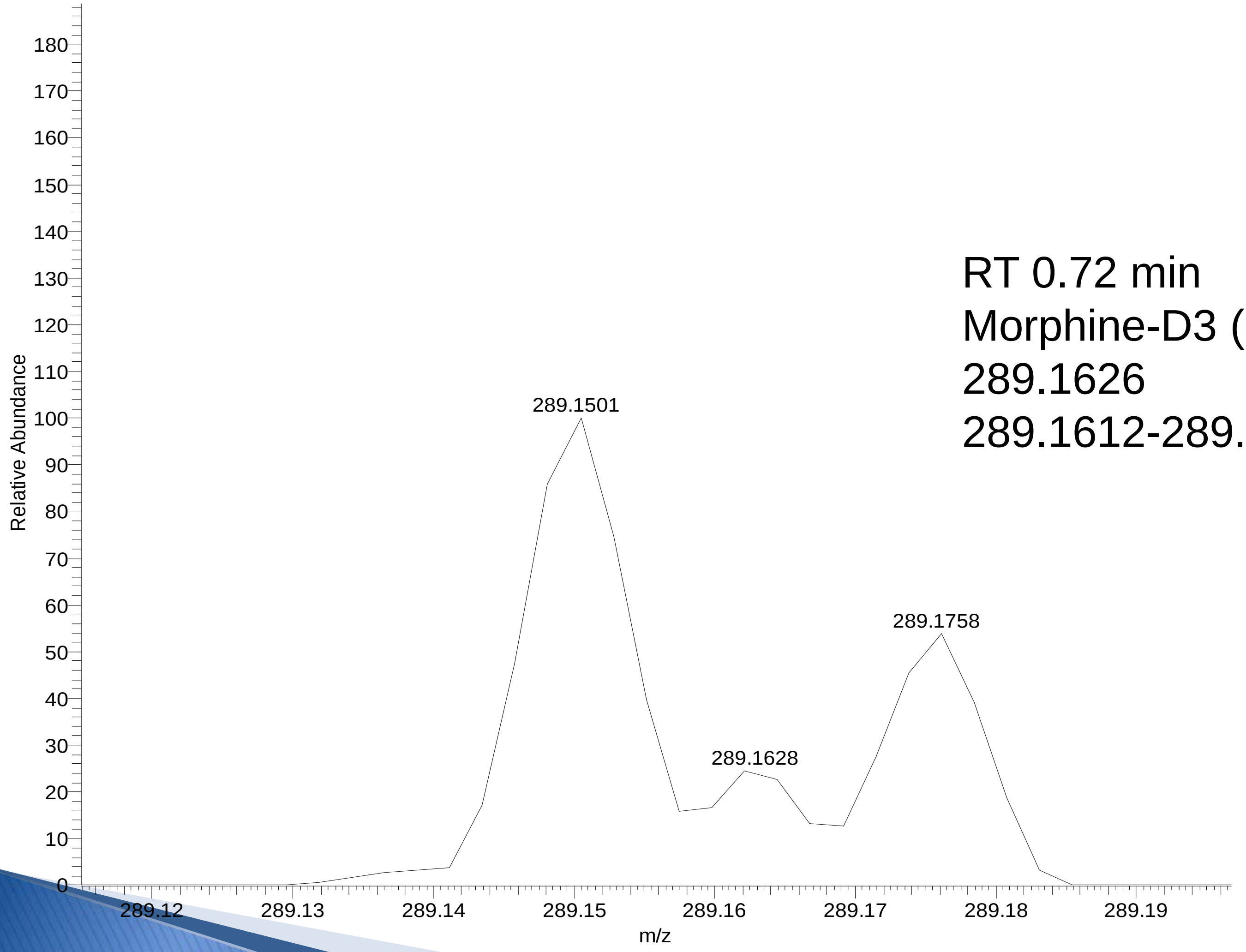


Positive for morphine

RT 0.72 min
Morphine-D3

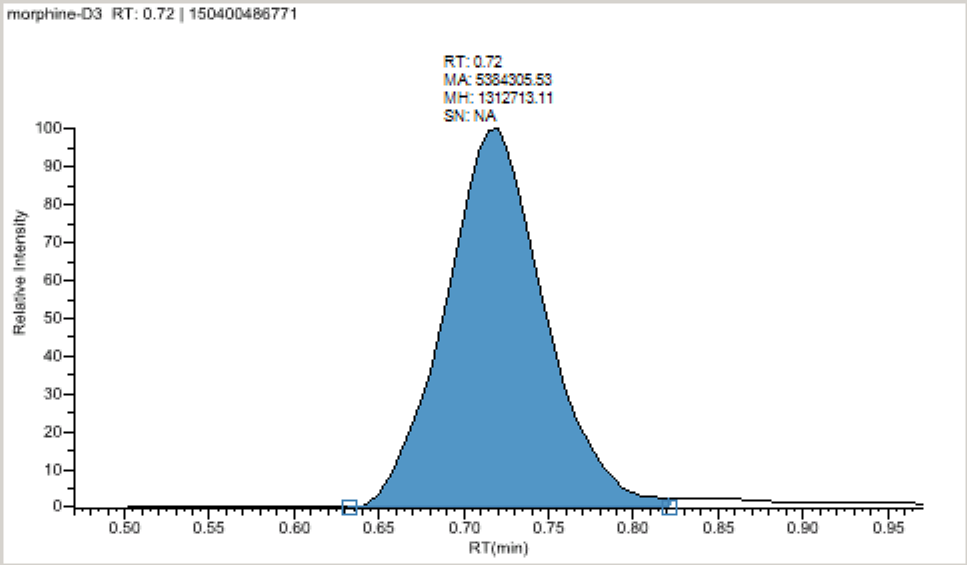
Results- MD 35000

150400486771 #69-113 RT: 0.66-0.74 AV: 23 NL: 1.62E6
T: FTMS + p ESI Full ms [130.00-480.00]



RT 0.72 min
Morphine-D3 (± 5 ppm window)
289.1626
289.1612-289.1640

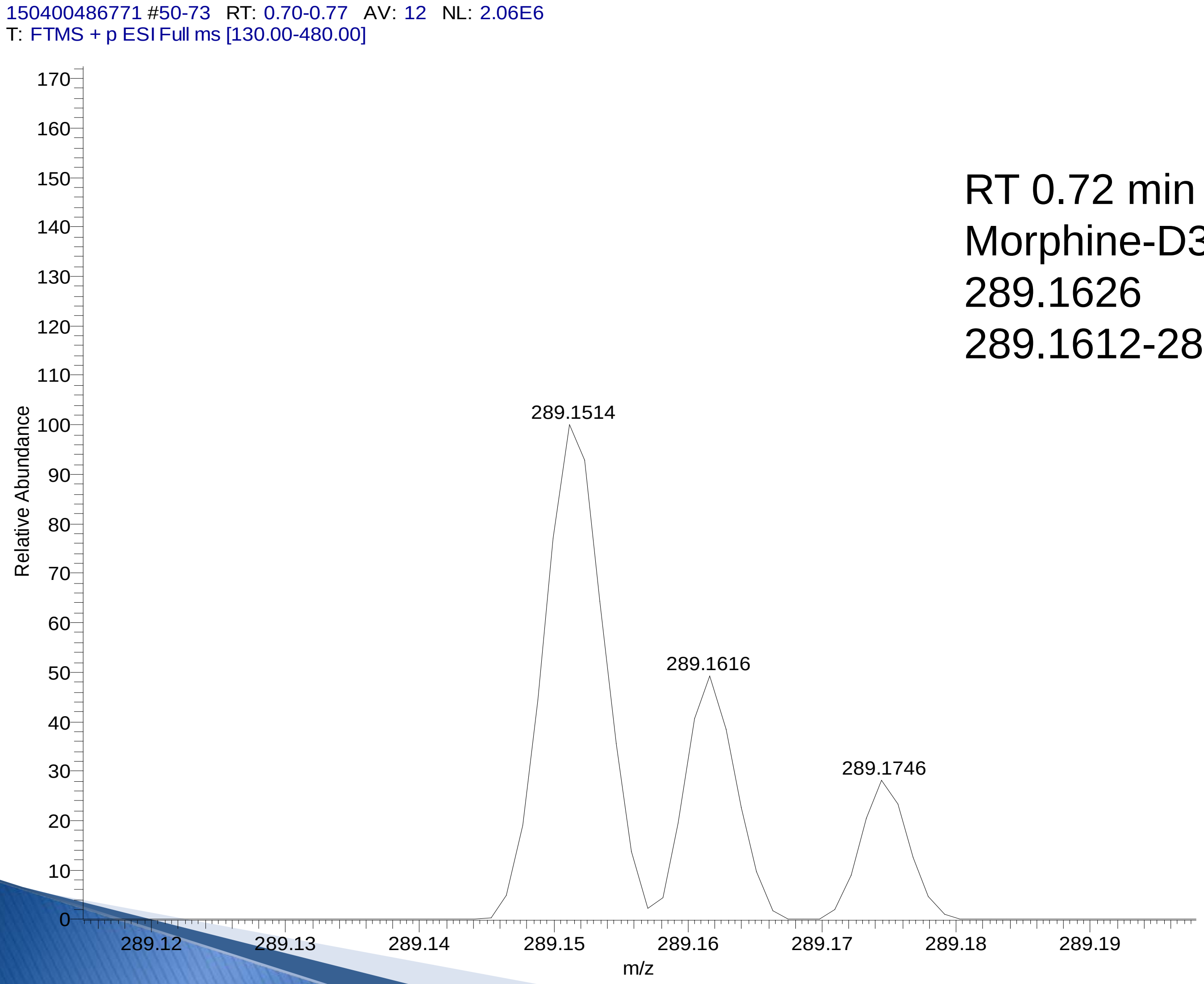
Results- MD 70000



Positive for morphine

RT 0.72 min
Morphine-D3

Results- MD 70000

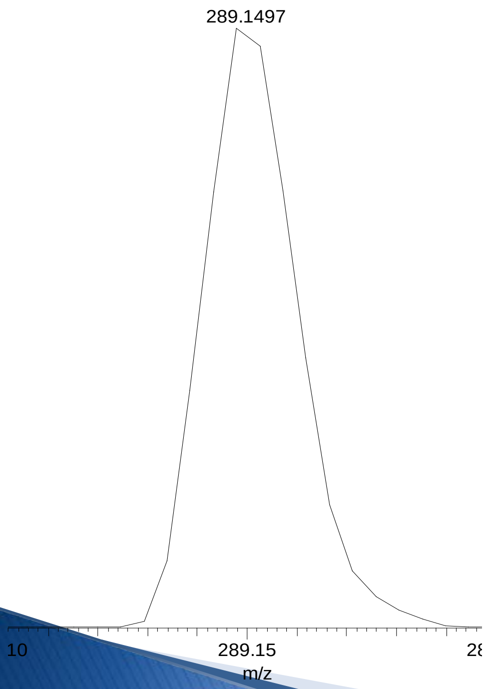


RT 0.72 min
Morphine-D3 (± 5 ppm window)
289.1626
289.1612-289.1640

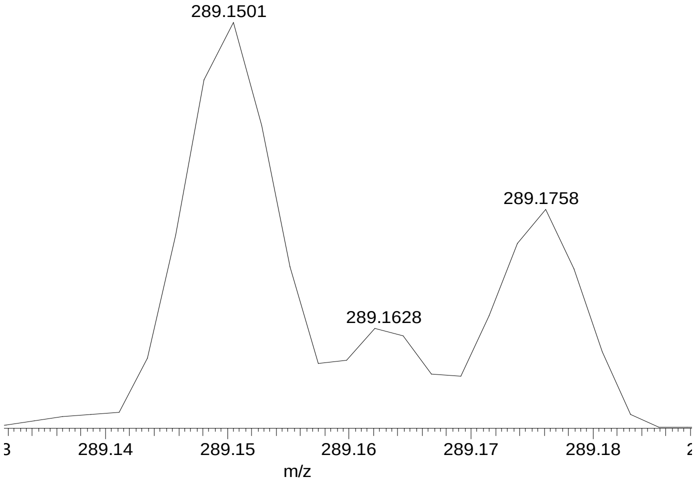
Results- MD All Resolutions

Morphine-D3 (± 5 ppm window) 289.1626 (289.1612-289.1640)

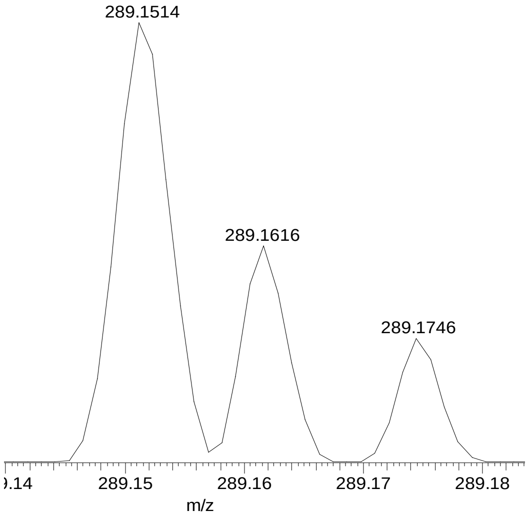
17 500



35 000



70 000



Results- MD MS/MS vs ddMS^2

- **Full MS OR MS/MS** : Mode where full MS spectrum scans and MS^2 scans are collected . Compounds included in the inclusion list have a RT time window where MS^2 scans are collected.

- **Full MS/ddMS^2** : Mode where full MS spectrum scans and MS^2 scans are collected. MS/MS is performed by the software on the top ions observed in the MS spectrum. The ions are automatically selected on the basis of their signal intensity and/or scheduling time.

- DIA proved to be superior to ddMS^2 to collect consistently at least one MS^2 scan for all the analytes at the LLOQ level.

Results

Validation criteria:

- Quantitation performed using the analyte monoisotopic mass extracted from full-scan ion chromatograms (XIC)

- Specificity/Selectivity

- Linearity ($R^2 \geq 0.99$)

- Repeatability (RSD $\leq$20%)

- Recovery and Accuracy Standards and QCs (RSD $\leq$20%)

- Stability

- Matrix interferences (%RSD $\leq$15% for the internal standard-normalized matrix factor)

- Interferences from OTC drugs, blood contamination (5% v/v).

Results Validation

Precision and Accuracy				
QC	Accuracy (% Dev)		Precision (% RSD)	
	Intra (n=6)	Inter (n=18)	Intra (n=6)	Inter (n=18)
LLOQ	≤18.8	≤ 9.7	≤ 10.5	≤ 13.4
Low	≤ 17.8	≤ 6.7	≤ 5.7	≤ 13.9
Mid	≤ 6.6	≤ 3.2	≤ 7.4	≤ 7.1
High	≤ 12.9	≤ 8.3	≤ 5.4	≤ 7.6
Correlation Coefficient (r^2)	≥ 0.997			

Results Validation

Stability		
Stability	Condition	Accuracy (% Dev)
Freeze / Thaw	3 Cycles, -20°C	≤ 9.8
Room Temperature	24 hrs	≤ 15.7
Autosampler Stability	1 week, 5°C	≤ 12.0
Long – Term Storage Stability	4 week, 5°C	≤ 17.7

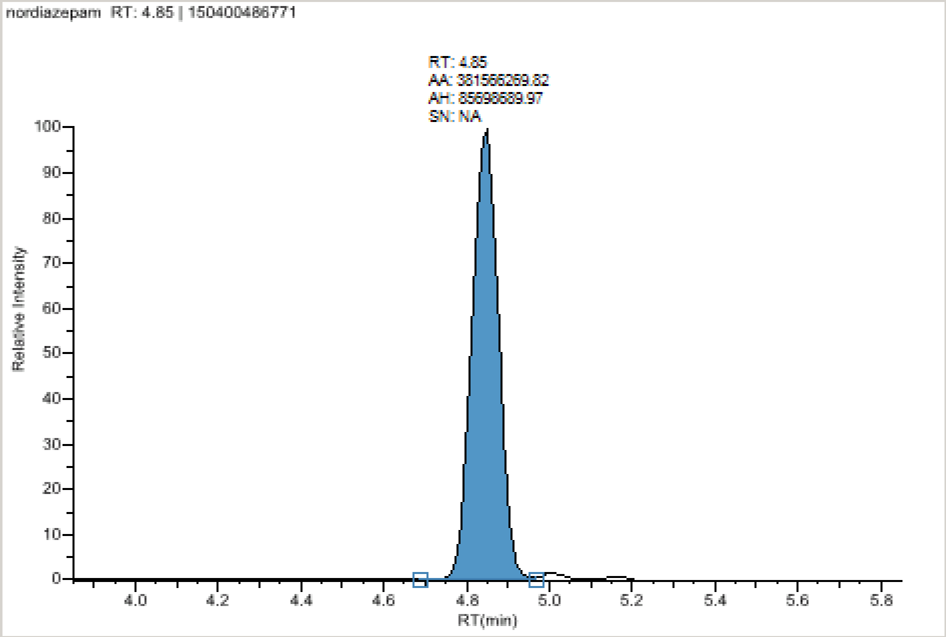
Results Validation

- The internal standard-normalized matrix factor for nine independent matrix sources was ≤ 14.0%RSD for all the analytes.

- Assessments of specificity, carryover, impact from blood contamination (5% v/v), and OTC drugs were also performed, and found to be acceptable.

Results: Screening criteria example

Nordiazepam Retention Time and Parent Accurate Mass

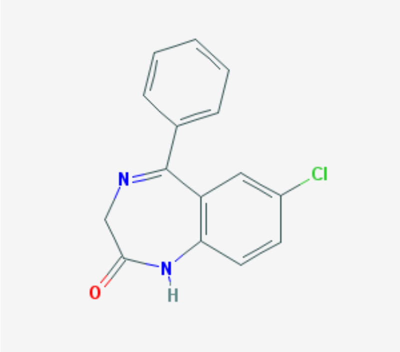


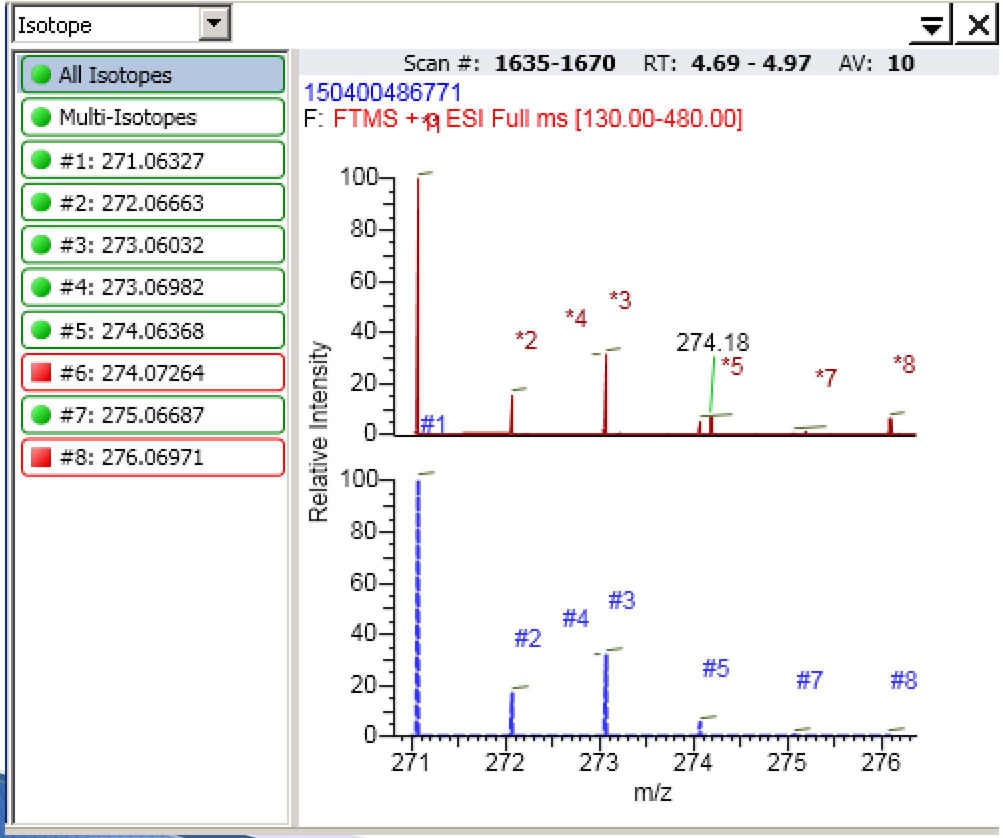
Expected RT	Actual RT	RT Delta
4.83	4.82	0.02

m/z (Expected)	m/z (Apex)	m/z (Delta(ppm))
271.0633	271.0628	-1.8255

Results: Screening criteria example

Nordiazepam Isotopic Abundance Pattern




Isotope

● All Isotopes
● Multi-Isotopes
● #1: 271.06327
● #2: 272.06663
● #3: 273.06032
● #4: 273.06982
● #5: 274.06368
🟥 #6: 274.07264
● #7: 275.06687
🟥 #8: 276.06971

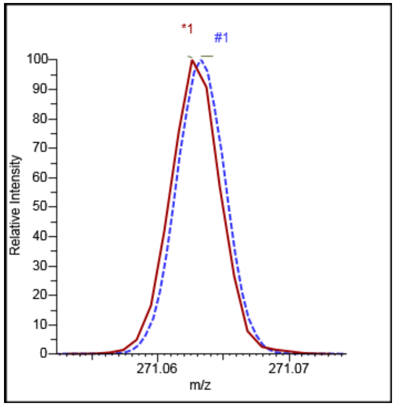
Scan #: **1635-1670** RT: **4.69 - 4.97** AV: **10**
150400486771
F: FTMS + p ESI Full ms [130.00-480.00]

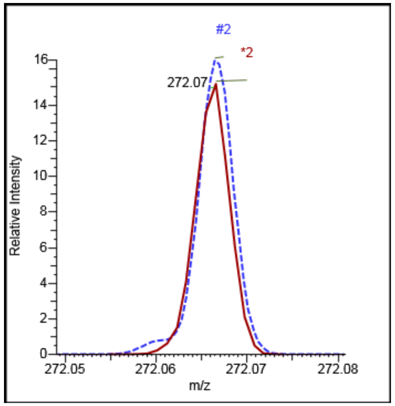
Measured

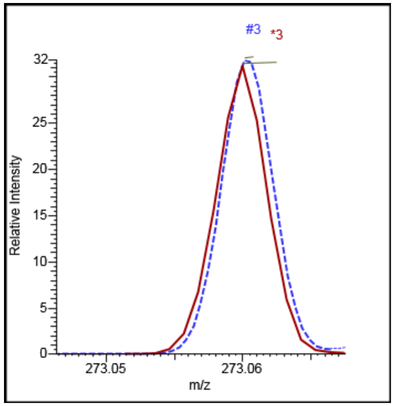
Theoretical

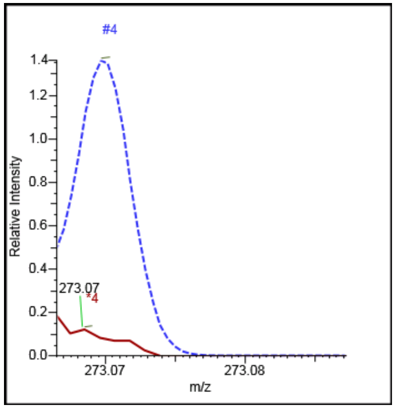
Results: Screening criteria example

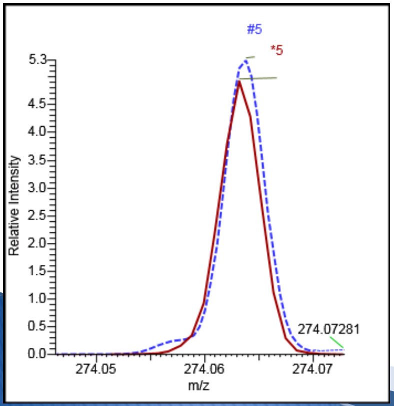
Nordiazepam Isotopic Abundance Pattern

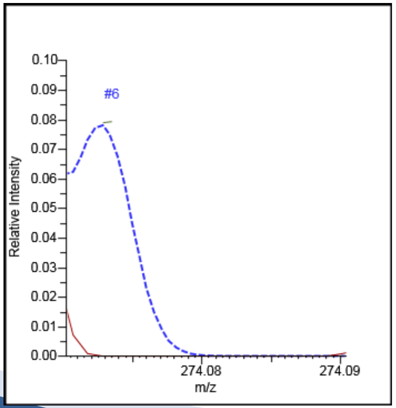


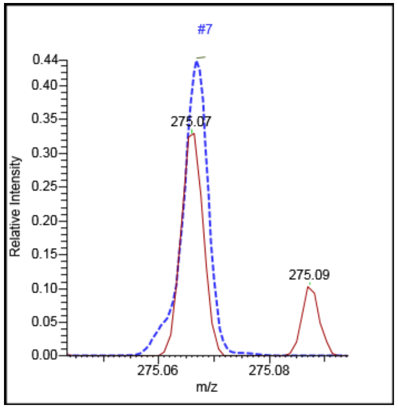


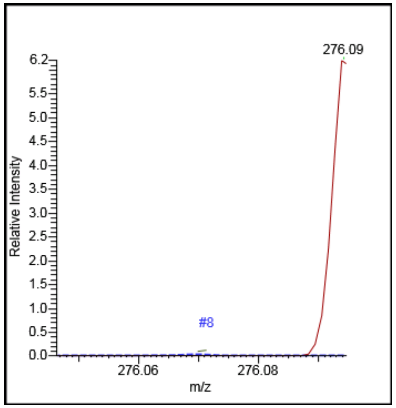






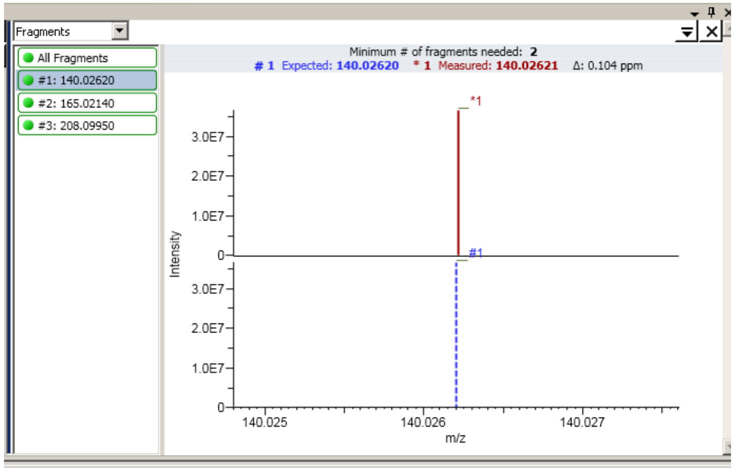






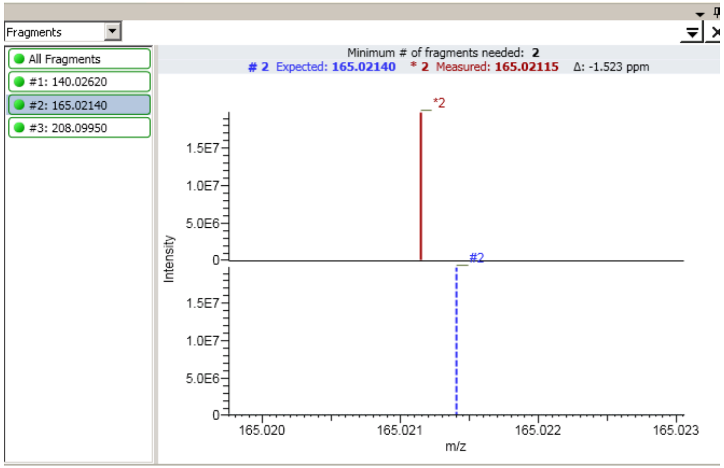
Results: Screening criteria example

Nordiazepam Fragments Accurate Mass



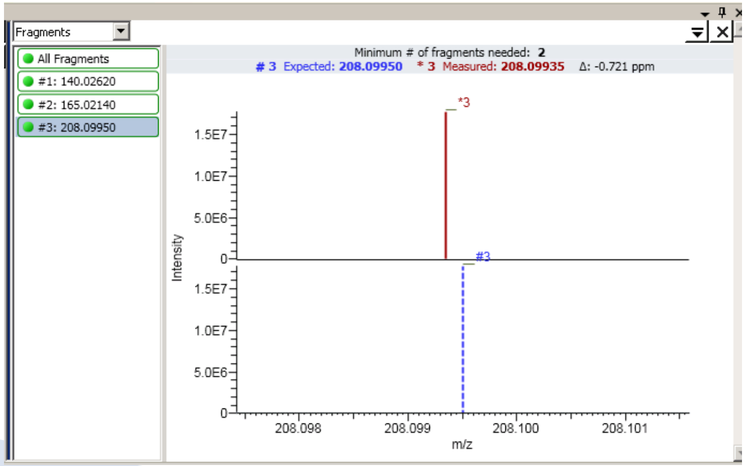
Minimum # of fragments needed: 2

1 Expected: 140.02620 * 1 Measured: 140.02621 Δ: 0.104 ppm



Minimum # of fragments needed: 2

2 Expected: 165.02140 * 2 Measured: 165.02115 Δ: -1.523 ppm

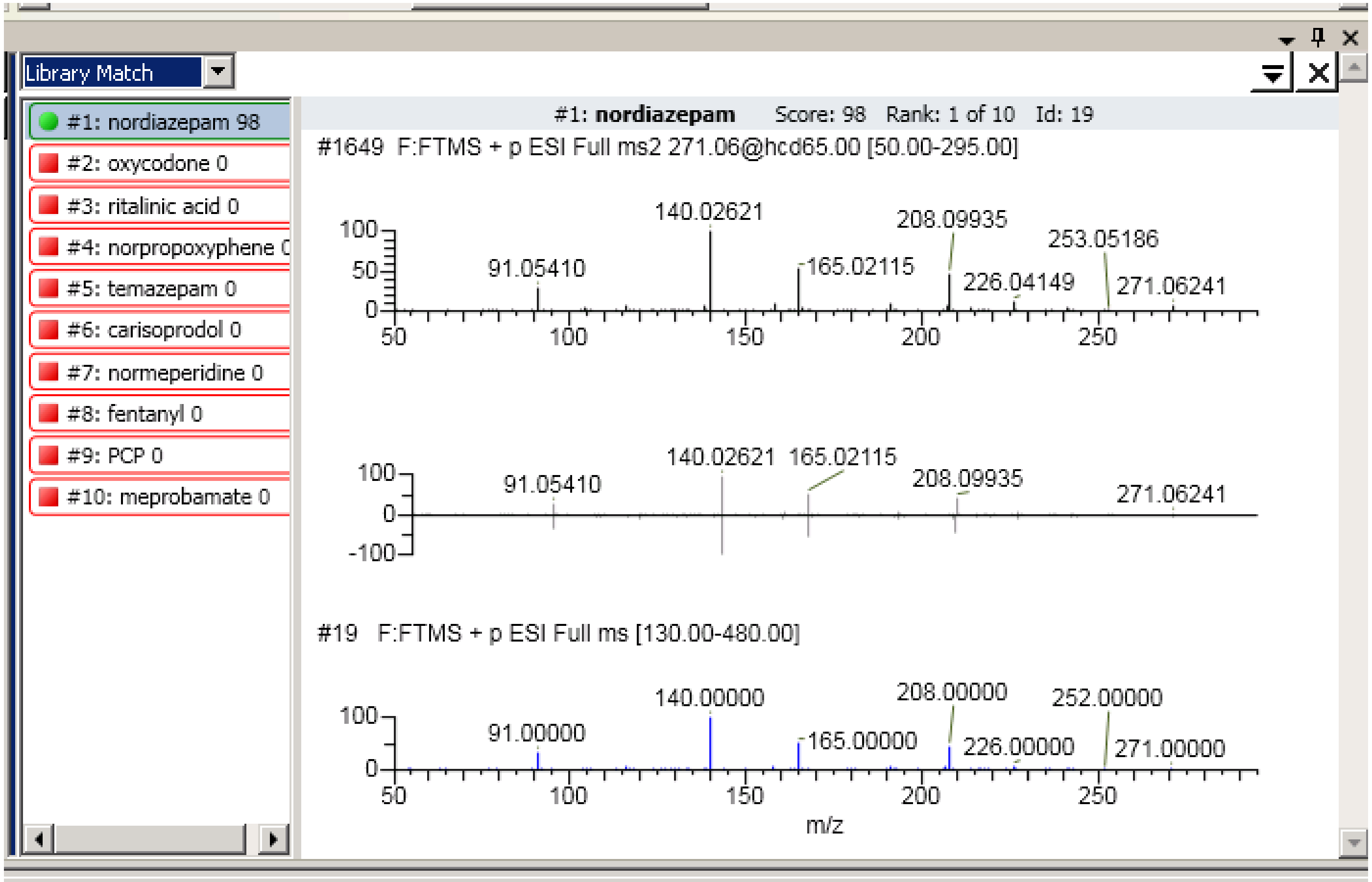


Minimum # of fragments needed: 2

3 Expected: 208.09950 * 3 Measured: 208.09935 Δ: -0.721 ppm

Results: Screening criteria example

Nordiazepam Library Match



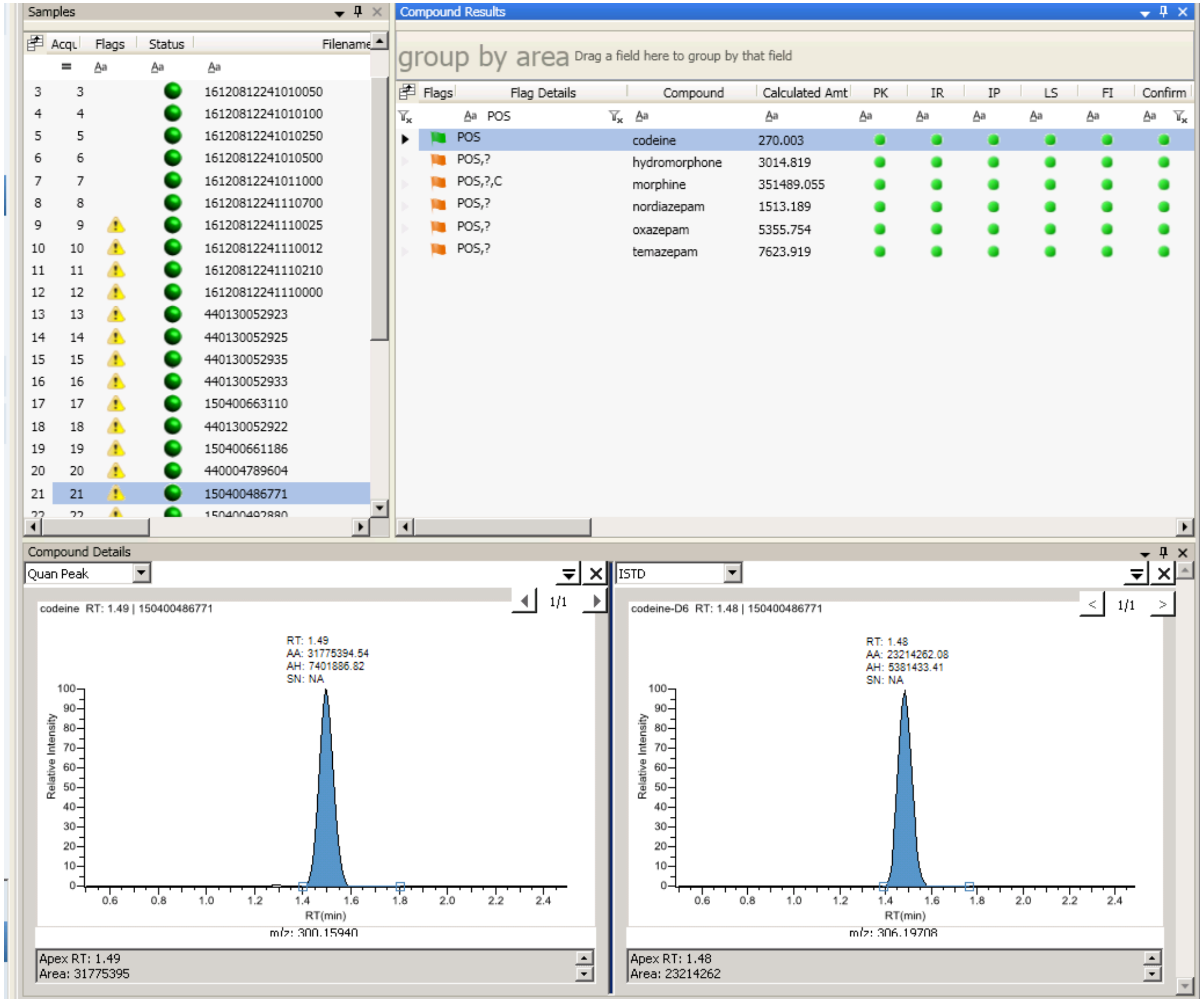
- ● #1: nordiazepam 98
- 🟥 #2: oxycodone 0
- 🟥 #3: ritalinic acid 0
- 🟥 #4: norpropoxyphene
- 🟥 #5: temazepam 0
- 🟥 #6: carisoprodol 0
- 🟥 #7: normeperidine 0
- 🟥 #8: fentanyl 0
- 🟥 #9: PCP 0
- 🟥 #10: meprobamate 0

#1: **nordiazepam** Score: 98 Rank: 1 of 10 Id: 19

#1649 F:FTMS + p ESI Full ms2 271.06@hcd65.00 [50.00-295.00]

#19 F:FTMS + p ESI Full ms [130.00-480.00]

Results



Samples

	Acqu	Flags	Status	Filename
3	3		●	16120812241010050
4	4		●	16120812241010100
5	5		●	16120812241010250
6	6		●	16120812241010500
7	7		●	16120812241011000
8	8		●	16120812241110700
9	9	⚠	●	16120812241110025
10	10	⚠	●	16120812241110012
11	11	⚠	●	16120812241110210
12	12	⚠	●	16120812241110000
13	13	⚠	●	440130052923
14	14	⚠	●	440130052925
15	15	⚠	●	440130052935
16	16	⚠	●	440130052933
17	17	⚠	●	150400663110
18	18	⚠	●	440130052922
19	19	⚠	●	150400661186
20	20	⚠	●	440004789604
21	21	⚠	●	150400486771
22	22	⚠	●	150400492880

Compound Results

group by area Drag a field here to group by that field

Flags	Flag Details	Compound	Calculated Amt	PK	IR	IP	LS	FI	Confirm
	POS								
🟩	POS	codeine	270.003	●	●	●	●	●	●
🟧	POS,?	hydromorphone	3014.819	●	●	●	●	●	●
🟧	POS,?,C	morphine	351489.055	●	●	●	●	●	●
🟧	POS,?	nordiazepam	1513.189	●	●	●	●	●	●
🟧	POS,?	oxazepam	5355.754	●	●	●	●	●	●
🟧	POS,?	temazepam	7623.919	●	●	●	●	●	●

Compound Details

Quan Peak

codeine RT: 1.49 | 150400486771

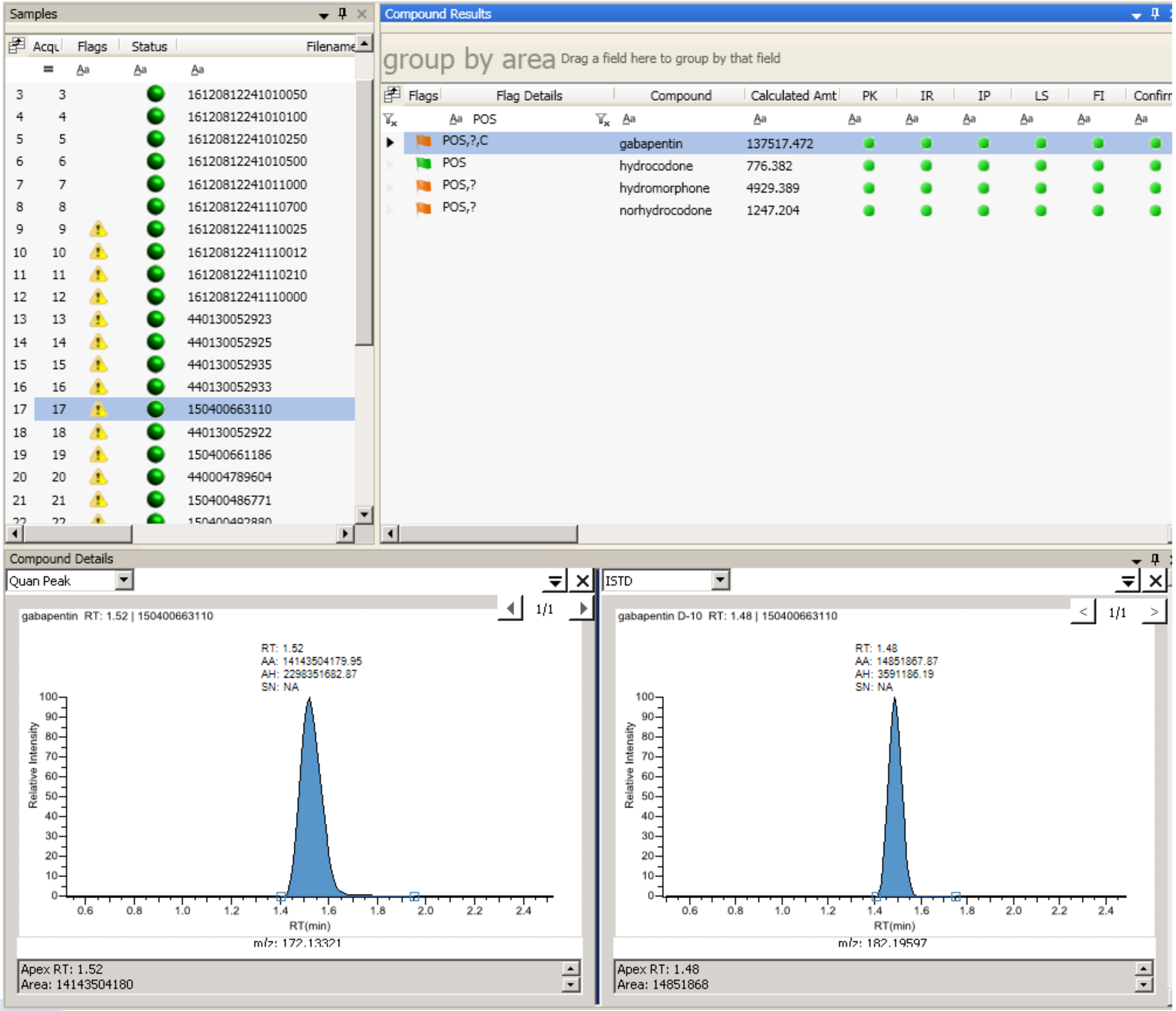
Apex RT: 1.49
Area: 31775395

ISTD

codeine-D6 RT: 1.48 | 150400486771

Apex RT: 1.48
Area: 23214262

Results



Samples

	Acqu	Flags	Status	Filename
3	3		●	16120812241010050
4	4		●	16120812241010100
5	5		●	16120812241010250
6	6		●	16120812241010500
7	7		●	16120812241011000
8	8		●	16120812241110700
9	9	⚠	●	16120812241110025
10	10	⚠	●	16120812241110012
11	11	⚠	●	16120812241110210
12	12	⚠	●	16120812241110000
13	13	⚠	●	440130052923
14	14	⚠	●	440130052925
15	15	⚠	●	440130052935
16	16	⚠	●	440130052933
17	17	⚠	●	150400663110
18	18	⚠	●	440130052922
19	19	⚠	●	150400661186
20	20	⚠	●	440004789604
21	21	⚠	●	150400486771

Compound Results

group by area Drag a field here to group by that field

Flags	Flag Details	Compound	Calculated Amt	PK	IR	IP	LS	FI	Confir
	POS								
🟧	POS,?,C	gabapentin	137517.472	●	●	●	●	●	●
🟩	POS	hydrocodone	776.382	●	●	●	●	●	●
🟧	POS,?	hydromorphone	4929.389	●	●	●	●	●	●
🟧	POS,?	norhydrocodone	1247.204	●	●	●	●	●	●

Compound Details

Quan Peak

gabapentin RT: 1.52 | 150400663110

RT: 1.52
AA: 14143504179.95
AH: 2298351682.87
SN: NA

m/z: 172.13321

Apex RT: 1.52
Area: 14143504180

ISTD

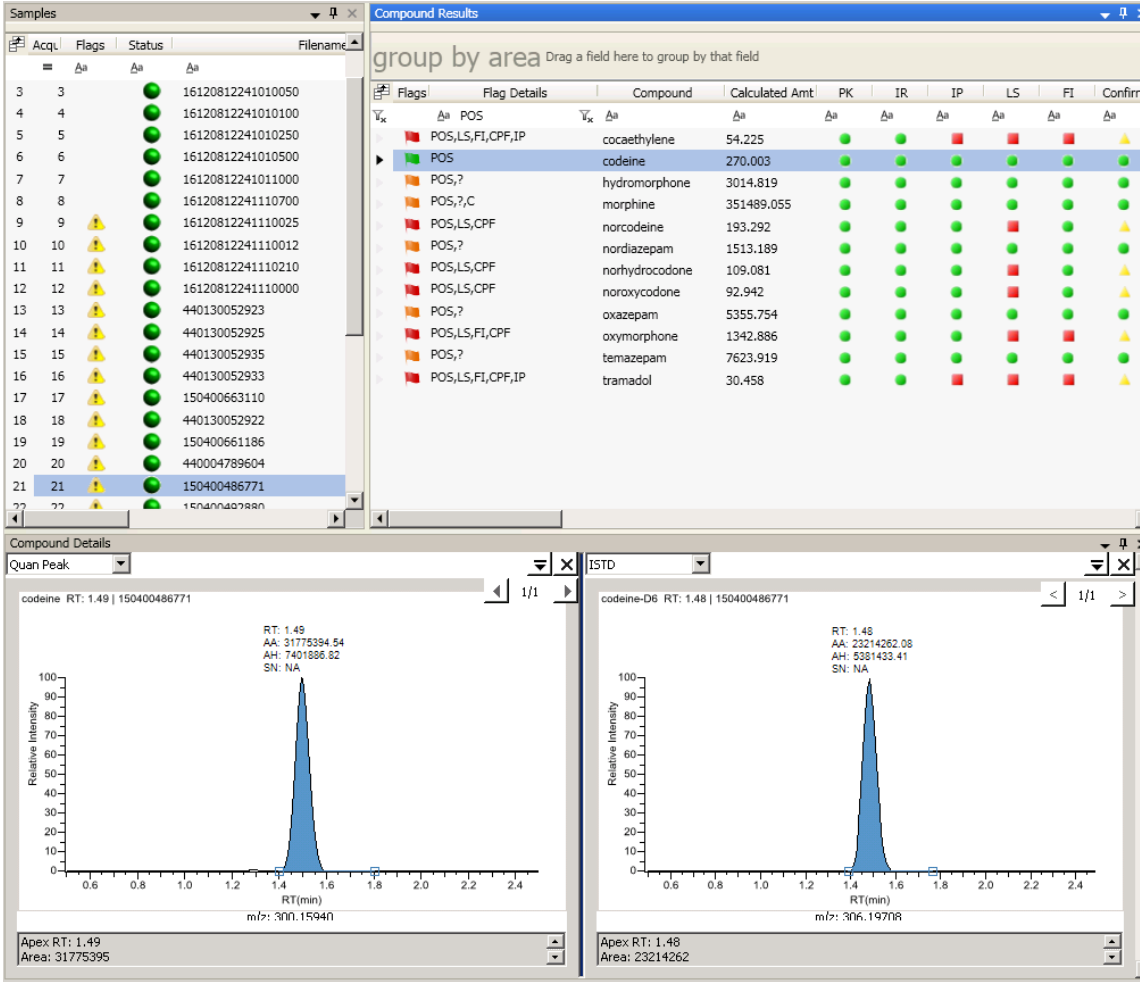
gabapentin D-10 RT: 1.48 | 150400663110

RT: 1.48
AA: 14851867.87
AH: 3591186.19
SN: NA

m/z: 182.19597

Apex RT: 1.48
Area: 14851868

Results



Samples

	Acqu	Flags	Status	Filename
3	3		●	16120812241010050
4	4		●	16120812241010100
5	5		●	16120812241010250
6	6		●	16120812241010500
7	7		●	16120812241011000
8	8		●	16120812241110700
9	9	⚠	●	16120812241110025
10	10	⚠	●	16120812241110012
11	11	⚠	●	16120812241110210
12	12	⚠	●	16120812241110000
13	13	⚠	●	440130052923
14	14	⚠	●	440130052925
15	15	⚠	●	440130052935
16	16	⚠	●	440130052933
17	17	⚠	●	150400663110
18	18	⚠	●	440130052922
19	19	⚠	●	150400661186
20	20	⚠	●	440004789604
21	21	⚠	●	150400486771

Compound Results

group by area Drag a field here to group by that field

Flags	Flag Details	Compound	Calculated Amt	PK	IR	IP	LS	FI	Confirr
	POS								
🚩	POS,LS,FI,CPF,IP	cocaethylene	54.225	●	●	🟥	🟥	🟥	▲
🚩	POS	codeine	270.003	●	●	●	●	●	●
🚩	POS,?	hydromorphone	3014.819	●	●	●	●	●	●
🚩	POS,?,C	morphine	351489.055	●	●	●	●	●	●
🚩	POS,LS,CPF	norcodeine	193.292	●	●	●	🟥	●	▲
🚩	POS,?	nordiazepam	1513.189	●	●	●	●	●	●
🚩	POS,LS,CPF	norhydrocodone	109.081	●	●	●	🟥	●	▲
🚩	POS,LS,CPF	noroxycodone	92.942	●	●	●	🟥	●	▲
🚩	POS,?	oxazepam	5355.754	●	●	●	●	●	●
🚩	POS,LS,FI,CPF	oxymorphone	1342.886	●	●	●	🟥	🟥	▲
🚩	POS,?	temazepam	7623.919	●	●	●	●	●	●
🚩	POS,LS,FI,CPF,IP	tramadol	30.458	●	●	🟥	🟥	🟥	▲

Compound Details

Quan Peak

codeine RT: 1.49 | 150400486771

Apex RT: 1.49
Area: 31775395

ISTD

codeine-D6 RT: 1.48 | 150400486771

Apex RT: 1.48
Area: 23214262

Conclusion

- Resolution value of 70 000 was necessary for the full scan data.

- MS/MS proved to be superior to ddMS^2 to collect consistently at least one MS^2 scan for all the analytes at the LLOQ level.

- The optimal conditions that yielded consistent hydrolysis values ≥80% for all the commercially available glucuronide standards were incubation at 65°C for one hour, with 5000 Fishman units/mL of enzyme concentration in the master mix solution. Codeine 6-glucuronide was the analyte that took longer to hydrolyze.

- By combining the high resolution capabilities of the Thermo Scientific™ Q Exactive™ MS with the high rate of hydrolysis of the IMCSzyme it was possible to decrease sample analysis cost while increasing accuracy and the number of analytes screened and quantified on a single injection.

Acknoledgments

Thermo Fisher Scientific:

- Marketing group
- Russ Constantineau
- Susan DiPietro