U.S. FOOD & DRUG ADMINISTRATION

Liquid Chromatography-Electrospray Ionization-High Resolution Mass Spectrometry (LC-ESI-HRMS) Method for the Determination of Nitrosamine Impurities in Metformin Drug Substance and Drug Product

Background: Metformin is a prescription drug used to control high blood sugar in patients with type 2 diabetes. NDMA (N-nitroso-dimethylamine) has been classified as a Group 2A compound, thereby defining it as "probably carcinogenic to humans." FDA has set daily acceptable intake limits on NDMA in pharmaceuticals of 96 nanograms daily (immediate release (IR) dose is 0.038ppm based on 2550 mg maximum daily dose (MDD); extended release (ER) dose is 0.048 ppm based on 2000 mg MDD).

FDA's Office of Testing and Research has screened for NDMA in metformin drug substance and drug product in samples of selected drugs obtained commercially or directly through the manufacturers. A primary LC-HRMS screen for metformin is in place and posted here. Positive NDMA results can be confirmed with this orthogonal method, LC-ESI-HRMS.

Conclusions:
An LC-ESI-HRMS method was developed and validated in conformance with ICH Q2(R1) for the detection and quantitation of eight nitrosamine impurities, including N-nitroso-dimethylamine (NDMA), N-nitroso-diethylamine (NDEA), N-ethyl-N-nitroso-2-propanamine (NEIPA), N-nitroso-*di*isopropylamine (NDIPA), N-nitroso-*di*-n-propylamine (NDPA), N-nitroso-methylphenylamine (NMPA), N-nitroso-*di*-n-butylamine (NDBA) and N-nitroso-N-methyl-4-aminobutyric acid (NMBA) in metformin drug substance and drug product. The limit of detection (LOD), limit of quantitation (LOQ) and range of the method are summarized below:

	NDMA	**NDEA**	**NEIPA**	**NDIPA**	**NDPA**	**NMPA**	**NDBA**	**NMBA**
LOD (ng/mL)	0.5	0.2	0.3	0.1	0.1	0.1	0.1	0.2
(ppm)	0.005	0.002	0.003	0.001	0.001	0.001	0.001	0.002
LOQ (ng/mL)	1.0	2.0	2.0	2.0	0.5	0.5	0.5	0.5
(ppm)	0.01	0.02	0.02	0.02	0.005	0.005	0.005	0.005
Range (ng/mL)	1.0 – 10	2.0 – 10	2.0 – 10	2.0 – 10	0.5 – 10	0.5 – 10	0.5 – 10	0.5 – 10
(ppm)	0.01 – 0.1	0.02 – 0.1	0.02 – 0.1	0.02 – 0.1	0.005 – 0.1	0.005 – 0.1	0.005 – 0.1	0.005 – 0.1

Liquid Chromatography- Electrospray Ionization-High Resolution Mass Spectrometry (LC-ESI-HRMS) Method for the Determination of Nitrosamine Impurities in Metformin Drug Substance and Drug Product

Purpose

Development of an orthogonal LC-HRMS method to quantitate the following eight nitrosamine impurities in metformin drug substance or drug product: N-nitroso-dimethylamine (NDMA), N-nitroso-diethylamine (NDEA), N-ethyl-N-nitroso-2-propanamine (NEIPA), N-nitroso-*di*isopropylamine (NDIPA), N-nitroso-*di*-n-propylamine (NDPA), N-nitroso-methylphenylamine (NMPA), N-nitroso-*di*-n-butylamine (NDBA) and N-nitroso-N-methyl-4-aminobutyric acid (NMBA).

Principle

The eight nitrosamine impurities (NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, and NMBA) are separated from each other and from metformin by reverse phase chromatography and are detected by a high-resolution and high-mass accuracy (HRAM) mass spectrometer. A high sensitivity of detection is achieved by monitoring the accurate *m/z* values of the protonated or deprotonated impurity ions or their fragments. Quantitation is performed by comparing the peak area of an impurity in extracted ion chromatograms of samples to its standard in an external calibration standard solution containing the reference standards for the eight impurities.

Reagent

- Reference standards for NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, and NMBA
- Formic acid, LC/MS grade (Fisher A117-50 or equivalent)
- Methanol, LC/MS grade (Fisher A456-4 or equivalent)
- Water, LC/MS grade or equivalent

Equipment

- HPLC or UHPLC system equipped with temperature-controlled autosampler and column compartment
- Q Exactive™ hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific)
- HPLC column: Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å, 150 x 3.0 mm (Part No. 00F-4622-Y0)
- Analytical Balance
- Vortex Mixer
- 15 mL glass centrifuge tubes
- Wrist action shaker
- 0.22 µm PVDF syringe filters
- Centrifuge
- HPLC vials

Mobile phase preparation

- Mobile phase A (0.1% formic acid in water): mix formic acid and water at a volume ratio of 1:1000

- Mobile phase B (0.1% formic acid in methanol): mix formic acid and methanol at a volume ratio of 1:1000

Diluent and Blank: Methanol

Mixed Stock Standard preparation
Prepare a mixed stock standard solution in methanol with the following concentrations.

Nitrosamine	Conc. (ng/mL)
NDMA	100
NDEA	100
NEIPA	100
NDIPA	100
NDPA	100
NMPA	100
NDBA	100
NMBA	100

Standard Preparation (3.0 ng/mL)
Transfer a 0.75 mL aliquot volume of the mixed stock standard into a 25 mL volumetric flask and dilute to volume with methanol. Prepare fresh daily.

Drug substance sample preparation
Accurately weigh 400 mg of drug substance into a 15 mL glass centrifuge tube. Add 4.0 mL of methanol and mix the solution using a vortex mixer. Shake the sample for 40 minutes using a mechanical wrist action shaker.

After extraction, centrifuge the sample for 15 minutes at 4500 rpm. Filter the supernatant using a 0.22 µm PVDF syringe filter, discard the first 1 mL and transfer the filtered sample into an hplc vial for LC/MS analysis.

Drug product sample preparation
Crush the appropriate number of tablet(s) to obtain a target concentration of 100 mg/mL of API in methanol, and transfer into a 15 mL glass centrifuge tube. Add the appropriate volume of methanol and mix for about a minute using a vortex mixer. Shake the sample for 40 minutes using a mechanical wrist action shaker.

After extraction, centrifuge the sample for 15 minutes at 4500 rpm. Filter the supernate using a 0.22 µm PVDF syringe filter, discard the first 1 mL and transfer the filtered sample into an hplc vial for LC/MS analysis.

Chromatographic Conditions

HPLC Column	Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å, 150 x 3.0 mm (Part No. 00F-4622-Y0)		
Column Temp.	40 °C		
Flow Rate	0.4 mL/min		
Mobile Phase A	0.1% formic acid in water		
Mobile Phase B	0.1% formic acid in methanol		
Gradient	Time (min)	A%	B%
	0	95	5
	3.0	95	5
	5.0	90	10
	6.0	40	60
	10.0	40	60
	13.0	20	80
	13.1	0	100
	15.0	0	100
	15.1	95	5
	18.0	95	5
Injection Volume	3 µL		
Autosampler Temp.	21 °C (Room Temperature)		
Needle Wash	80:20, Methanol:Water with 0.1% Formic Acid		

Mass spectrometer conditions

- Instrument
  Q Exactive™ mass spectrometer (ThermoFisher) or Q Exactive™ HF-X mass spectrometer (ThermoFisher)

- ESI Source Settings (apply to both negative and positive modes)
  Note: Ion source parameters can be adjusted to achieve the desired sensitivity.

ESI Source

Sheath Gas Flow Rate	55 arbitrary units
Aux Gas Flow Rate	15 arbitrary units
Sweep Gas Flow Rate	0 units
Spray Voltage	3.5 kV
Capillary Temp.	400 °C
S-Lens	55 (applied to Q Exactive™)
Aux Gas Heater Temp.	350 °C

- Scan Settings
  Note:
  1) The scan start–end time should be adjusted for the user's HPLC system since the retention times of the impurities may vary between different HPLC systems
  2) The divert valve can be used to divert the eluent to waste when a scan is not performed.

Impurity	NDMA	NMBA	NMBA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Scan Type	PRM	SIM	SIM	PRM	SIM	SIM	SIM	PRM
Polarity	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Scan Start - End (min)	3.0 – 6.0	7.5 – 8.5	8.5 – 9.3	9.0 – 10.0	9.9 – 11.2	9.9 – 11.2	11.0 – 12.0	13.5 – 14.5
m/z Isolated for PRM	75.0553	N/A	N/A	117.1022	N/A	N/A	N/A	159.1492
(N) CE	80	N/A	N/A	10	N/A	N/A	N/A	50
Isolation Window	1.5 m/z	1.5 m/z	1.5 m/z	1.5 m/z	1.5 m/z	1.5 m/z	1.5 m/z	1.5 m/z
Microscans	3	3	3	3	3	3	3	3
Resolution	35,000	70,000	70,000	35,000	70,000	70,000	70,000	35,000
AGC target	2e5	1e6	1e6	2e5	1e6	1e6	1e6	2e5
Max. IT	100 ms	100 ms	100 ms	100 ms	100 ms	100 ms	100 ms	100 ms

Injection Order

- Inject Blank (use diluent) at least once at the beginning of a sequence
- Inject Standard solution for six consecutive times before the injection of the first sample
- Inject Standard solution once every six injections of samples and at the end of a sequence.
- Example:

Order	Solution	No. of Injections
1	Blank	2
2	Standard	6
3	Blank	1
4	Sample 1	1
5	Sample 2	1
6	Sample 3	1
7	Sample 4	1
8	Sample 5	1
9	Sample 6	1
10	Standard	1
…	…	…

System Suitability

- The % RSD of the peak area for each nitrosamine impurity for the first six injections of standard solution should be no more than 10%.

- The cumulative % RSD of the peak area for each nitrosamine impurity should be no more than 15%. (cumulative % RSD of the peak area is calculated by combining the initial six replicate injections of the standard solution and each subsequent bracketing standard)

Data Processing

- Peak areas in the extracted ion chromatograms (EIC) with a *m/z* tolerance of 15 ppm are used for quantitation. The *m/z* values to be extracted are listed below:

Impurity	**NDMA**	**NMBA**	**NDEA**	**NEIPA**	**NDIPA**	**NDPA**	**NMPA**	**NDBA**
m/z to be extracted	75.0553	145.0619	103.0866	75.0553	131.1179	131.1179	137.0709	57.0704, 103.0872, 159.1492
Ret. Time (min)	4.34	8.01	8.79	9.46	10.39	10.82	11.39	14.17

- The retention time difference of any impurity in the analyzed samples should not be more than 2% of the retention time of the corresponding standard in the standard solution.

Calculation

Drug Substance:

$$\text{Nitrosamine impurity (ppm)} = \frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1\times 10^6\ ng} \times \frac{V}{W} \times 10^6$$

Where: Nitrosamine impurity refers to NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, or NMBA
A_{spl} = Area of the nitrosamine impurity peak in the sample solution
As = Average area (n = 6) of the nitrosamine impurity peak from the first six consecutive injections of the standard solution
C_s = Concentration of the nitrosamine impurity in the standard solution (3.0 ng/mL)
W = Weight of drug substance (mg)
V = Volume of the diluent in the sample solution (mL)

Drug Product:

$$\text{Nitrosamine impurity (ppm)} = \frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1\times 10^6\ ng} \times \frac{1}{100\ mg/mL} \times 10^6$$

Where: Nitrosamine impurity refers to NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, or NMBA
A_{spl} = Area of the nitrosamine impurity peak in the sample solution
As = Average area (n = 6) of the nitrosamine impurity peak from the first six consecutive injections of the standard solution
C_s = Concentration of the nitrosamine impurity in the standard solution (3.0 ng/mL)

Report

- Report the nitrosamine impurity content in ppm with three significant figures if the value is ≥ LOD
- Report ‘not detected’ if no nitrosamine impurity is detected or the value is < LOD

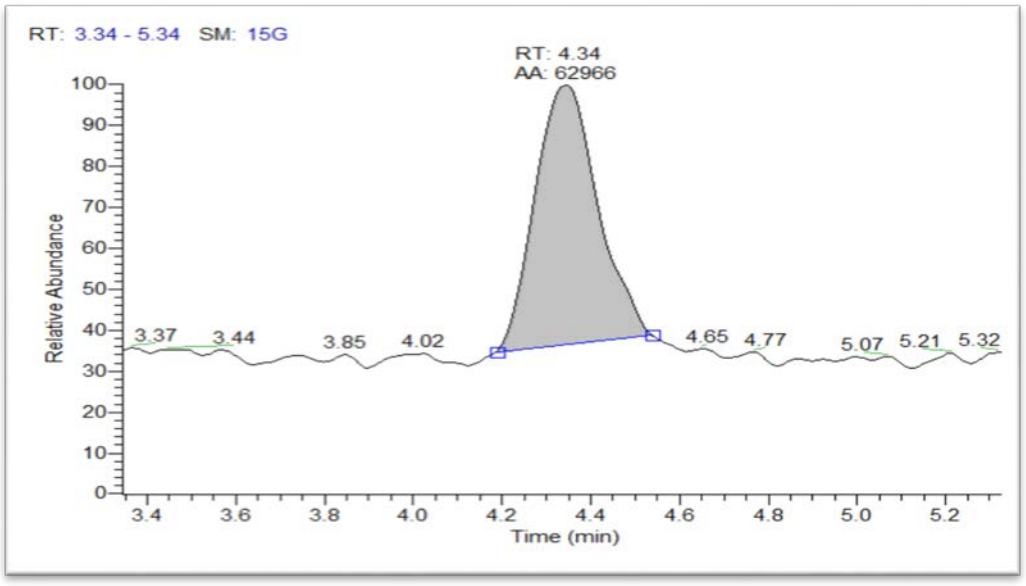
Reference

1. *FY20-058-DPA-S:* Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Method for the Determination of NDMA Metformin Drug Substance and Drug Product.

Example Chromatograms

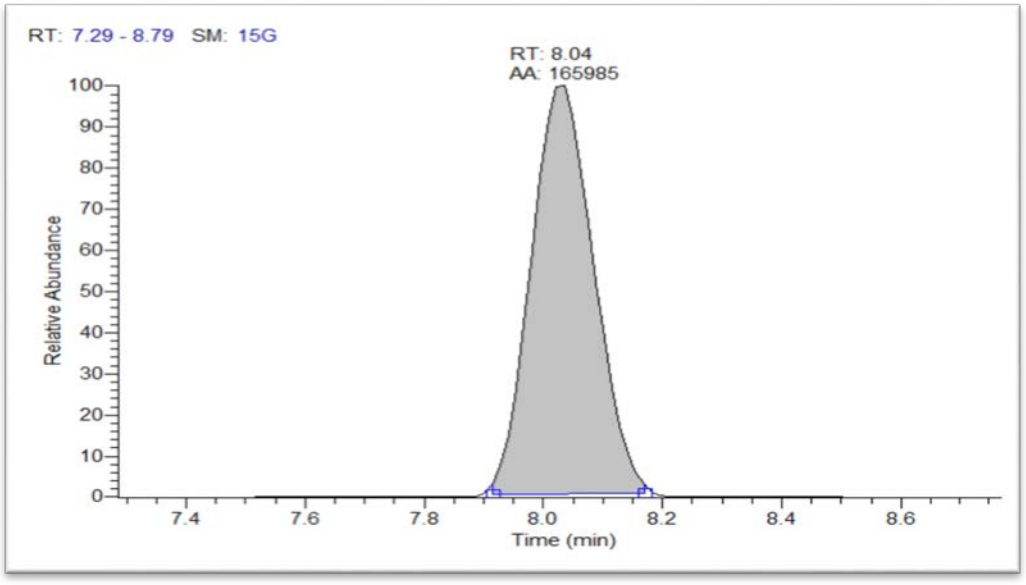
NDMA (3.0 ng/mL Standard)

Extracted ion chromatogram of m/z 75.0553 from PRM scan of m/z 75.0553 at a mass accuracy of 15 ppm



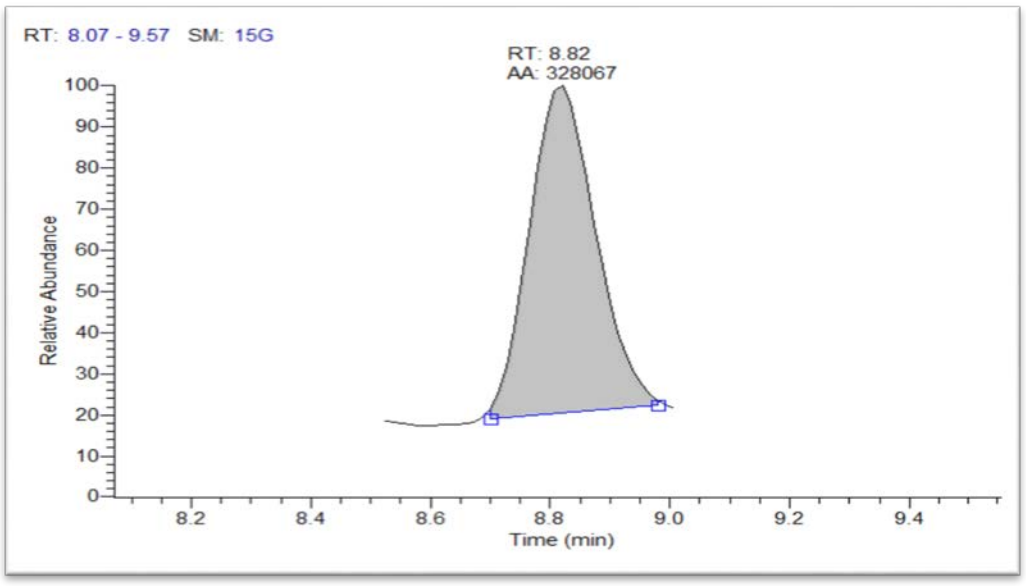
NMBA (3.0 ng/mL Standard)

Extracted ion chromatogram of m/z 145.0619 from Targeted-SIM scan of m/z 144.3 to 145.8 at a mass accuracy of 15 ppm

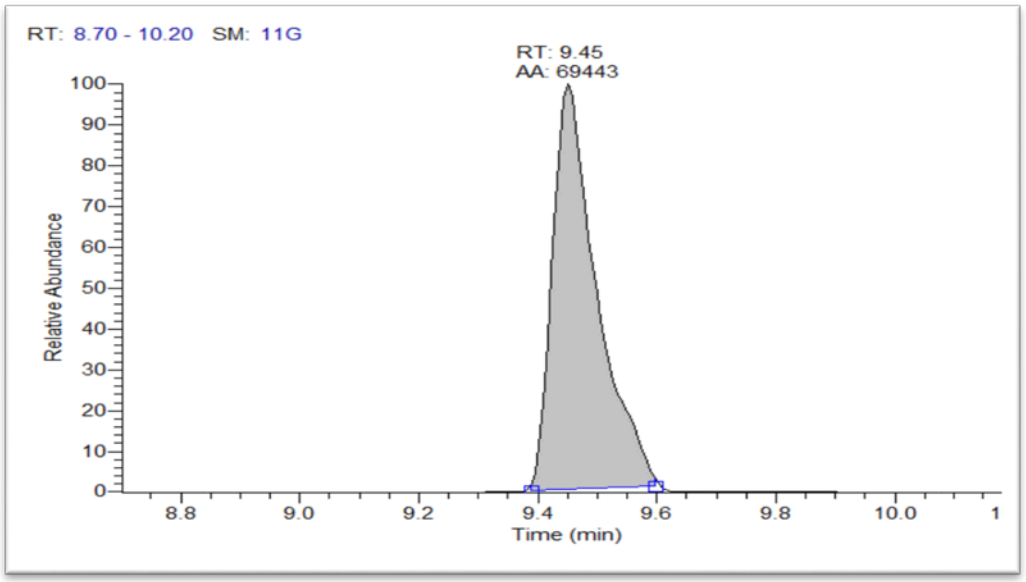


NDEA (3.0 ng/mL Standard)

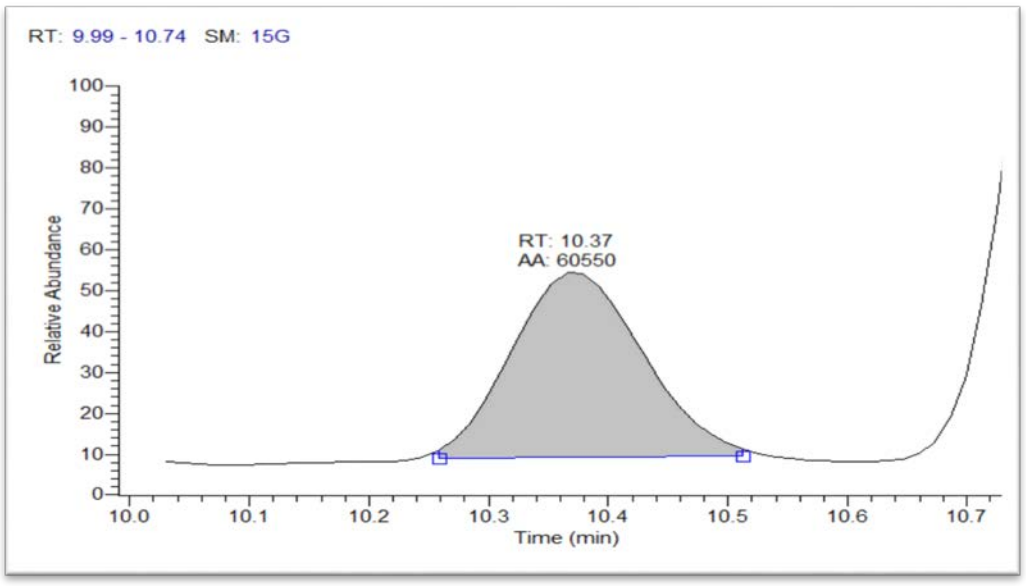
Extracted ion chromatogram of m/z 103.0866 from Targeted-SIM scan of m/z 103.0866 at a mass accuracy of 15 ppm



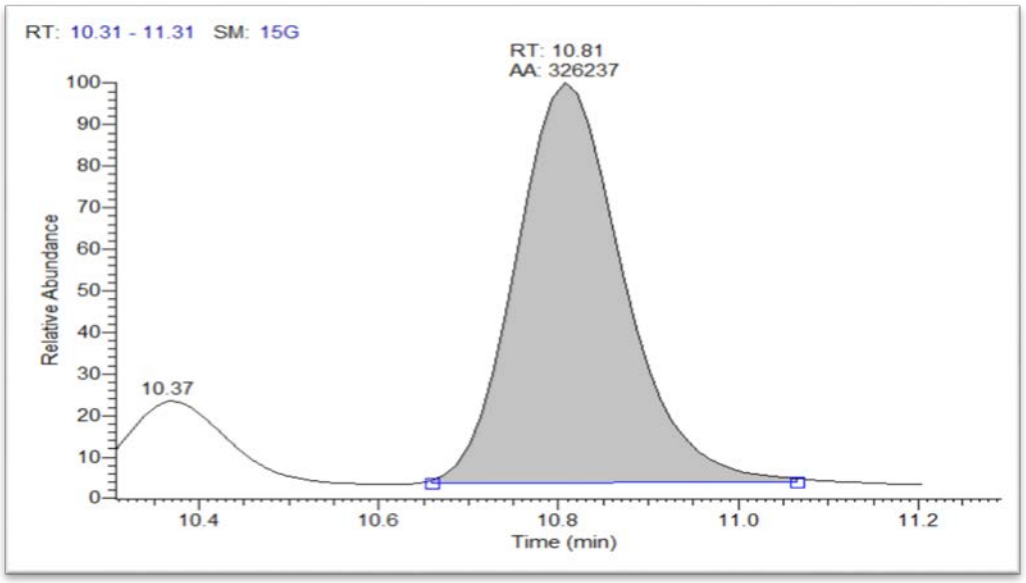
NEIPA (3.0 ng/mL Standard)
Extracted ion chromatogram of m/z 75.0553 from PRM scan of m/z 117.1022 at a mass accuracy of 15 ppm



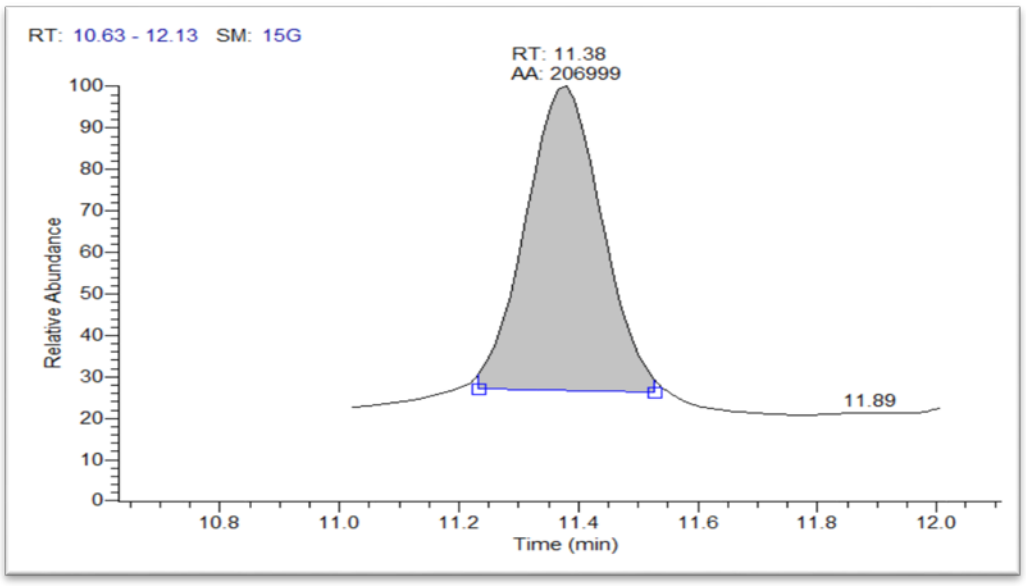
NDIPA (3.0 ng/mL Standard)
Extracted ion chromatogram of m/z 131.1179 from Targeted-SIM scan of m/z 130.4 - 131.9 at a mass accuracy of 15 ppm



NDPA (3.0 ng/mL Standard)
Extracted ion chromatogram of m/z 131.1179 from Targeted-SIM scan of m/z 130.4 - 131.9 at a mass accuracy of 15 ppm



NMPA (3.0 ng/mL Standard)
Extracted ion chromatogram of m/z 137.0709 from Targeted-SIM scan of m/z 136.3 - 137.8 at a mass accuracy of 15 ppm



NDBA (3.0 ng/mL Standard)

Extracted ion chromatogram of m/z 57.0704, 103.0872 and 159.1492 from PRM scan of m/z 159.1492 at a mass accuracy of 15 ppm