

# Simultaneous quantitation of nine nitrosamines using a highly sensitive and LC-HRAM mass spectrometry method in multiple drug products

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Keywords: Nitrosamine quantitation, drug products, high resolution, Orbitrap, single method, sartans, metformin, impurity analysis, GTI

## Goal

The aim of this study was to evaluate and report the quantitative performance of the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer in combination with liquid chromatography for the simultaneous determination and estimation of genotoxic nitrosamine impurities in sartan and metformin drugs (Active Pharmaceutical Ingredient (API) and tablets), employing the U.S. Food and Drug Administration (U.S. FDA) recommended methodology with minor improvisations.

## Introduction

The sartan group of drugs includes widely used anti-hypertensives known for the treatment of human high blood pressure, heart failure, kidney failure associated with diabetes, and chronic kidney diseases. The major



drugs are losartan, valsartan, irbesartan, azilsartan, and olmesartan. Metformin is a biguanides drug product and the first-line medication for the treatment of Type II diabetes. Controlling high blood sugar helps prevent kidney damage, blindness, nerve problems, and loss of limbs. Nitrosamines (Figure 1) are common in water and foods, including cured and grilled meats, dairy products, and vegetables. Everyone is exposed to some level of nitrosamines; however, the presence of nitrosamines in drug products has led to regulatory authorities issuing guidance to ensure the levels are kept within safe limits.<sup>1</sup> Nitrosamine impurities are formed during the production

of sartans that contain a specific ring structure known as a tetrazole ring under certain conditions and when certain solvents, reagents, and other raw materials are used (Figure 2).<sup>2</sup> These impurities are classified as probable carcinogens (i.e., potentially genotoxic impurities).

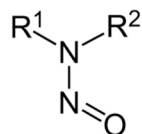


Figure 1. Nitrosamine structure

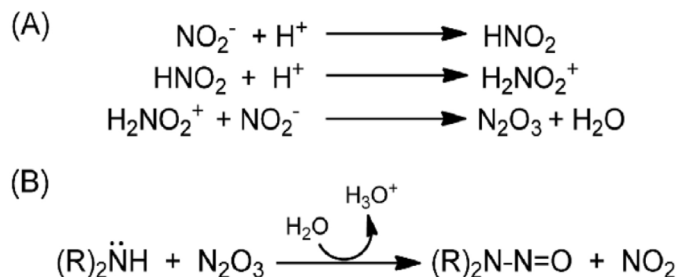


Figure 2. Production of nitrosamine (modified from Rostkowska *et al.*). (A) Formation of a nitrous anhydride from a nitrite (B) nitrosation from a nitrous anhydride and an amine.

A dominant valsartan API supplier in China reported the detection of *N*-nitroso-dimethylamine (NDMA) in their product in July 2018. Investigations carried out by the European Medicines Agency (EMA) and the United States Food and Drug Administration (U.S. FDA) showed that NDMA may cause cancer, and thus recall procedures for valsartan drugs were started. Furthermore, the U.S. FDA found an additional unexpected genotoxic impurity, *N*-nitroso-diethylamine (NDEA), in three batches of the recalled valsartan drugs on September 13, 2018, affecting more than half of the United States' supply of the drug. Since then, additional nitrosamines impurities were detected in other drugs belonging to the sartans family. In September 2019, the U.S. FDA announced that preliminary tests also found low levels of NDMA in ranitidine products. Because of this, large pharmaceutical companies announced recalls of generic ranitidine products. The U.S. FDA is imposing limits on the maximum daily exposure to nitrosamines in different drugs in the range of sub ppm levels in the final product,<sup>3-5</sup> but those levels are expected to decrease to non-detectable levels as soon as the manufacturing process is modified to avoid any nitrosamine formation.

The U.S. FDA has developed and published different methodologies to detect these nitrosamine impurities in ranitidine, sartans and metformin using gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC-MS), and liquid chromatography-high-resolution mass spectrometry (LC-HRMS).<sup>4-7</sup> A recent method was published on identification of eight impurities in metformin using an LC-HRMS method.<sup>3</sup>

Here, we present a robust and highly sensitive liquid chromatography-high-resolution accurate mass (LC-HRAM) Orbitrap parallel reaction monitoring (PRM) and targeted single ion monitoring (t-SIM) method for simultaneous quantitation of nine *N*-nitrosamine impurities as per U.S. FDA guidelines in supporting pharma testing laboratories. The nine impurities employed in the current study are *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), *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA) and *N*-nitroso-*N*-methylethylamine (NMEA).

## Experimental

### Reagents and laboratory equipment

- Water (H<sub>2</sub>O), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 7732-18-5)
- Methanol (MeOH), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 67-56-1)
- Acetonitrile (MeCN), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 75-05-8)
- Formic acid, Fisher Chemical™ Optima™ LC/MS solvent (CAS: 64-18-6)
- Metformin tablets/API
- Valsartan tablets/API
- Nitrosamine Impurities Standard from Clean Chem (Details provided in Table 1)
- Analytical balance
- Vortex mixer
- 15 mL glass centrifuge tubes
- Wrist action shaker
- 0.22 μm PVDF syringe filters
- Refrigerated centrifuge

**Table 1. Nitrosamine impurity information**

Abbreviation	Chemical name	Chemical formula	Monoisotopic mass	CAS no.
NDMA	<i>N</i> -nitroso-dimethylamine	C <sub>2</sub> H <sub>6</sub> N <sub>2</sub> O	74.048012	62-75-9
NMEA	<i>N</i> -nitroso-methyl ethylamine	C <sub>3</sub> H <sub>8</sub> N <sub>2</sub> O	88.06311	10595-95-6
NMBA	<i>N</i> -nitroso- <i>N</i> -methyl-4-aminobutyric acid	C <sub>5</sub> H <sub>12</sub> N <sub>2</sub> O	116.094963	61445-55-4
NDEA	<i>N</i> -nitroso-diethylamine	C <sub>4</sub> H <sub>10</sub> N <sub>2</sub> O	102.079315	55-18-5
NEIPA	<i>N</i> -ethyl- <i>n</i> -nitroso-2-propanamine	C <sub>5</sub> H <sub>12</sub> N <sub>2</sub> O	116.094963	25413-61-0
NDIPA	<i>N</i> -nitroso-diisopropylamine	C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O	130.110611	601-77-4
NDPA	<i>N</i> -nitroso-di- <i>n</i> -propylamine	C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O	130.110611	621-64-7
NMPA	<i>N</i> -nitroso-methylphenylamine	C <sub>7</sub> H <sub>9</sub> N <sub>2</sub> O	136.06311	614-00-6

### Sample preparation

Each nitrosamine impurity's certified reference material was used to prepare the stock solution by dissolving 10 mg in 10 mL methanol. Furthermore, a 100 ng/mL intermediate working standard solution was prepared in methanol through a serial dilution approach. The intermediate 100 ng/mL was further diluted to prepare the linearity solution(s) in the range of 0.1–20 ng/mL. Solutions for recovery studies were prepared in similar fashion.

The required number of tablet(s) were powdered using pestle and mortar and reconstituted in LC-MS grade 100% methanol to obtain a target concentration of 100 mg/mL of API. Samples were subjected to mechanical shaking for about 45 minutes. Extracted sample solution(s) were centrifuged for 15 minutes at 4,500 rpm and filtered through 0.22 µm PVDF syringe membrane filter. These filtered sample solutions were injected to the LC-HR Orbitrap MS for further analysis.

### LC-MS conditions

A Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid-Orbitrap mass spectrometer was used for the data acquisition. UHPLC configurations and parameters are listed in Table 2. The mass spectrometer configuration and acquisition scan parameters for the nitrosamine impurities are listed in Tables 3 and 4. Briefly, we have employed the combination of parallel reaction monitoring (PRM) and targeted selected ion monitoring (t-SIM) methods at positive and negative polarities. Both the PRM and t-SIM methods were found to offer excellent sensitivity and selectivity for all nine nitrosamine impurities in both the drug samples and drug products.

**Table 2. Liquid chromatography configuration and parameters**

Parameter	Value																																																																												
UHPLC	Vanquish Flex Quaternary UHPLC system with modules: Vanquish Horizon/Flex System Base (VF-S01-A-02) Vanquish Diode Array Detector (VH-D10-A) Vanquish Split Sampler FT (VF-A10-A) Vanquish Quaternary Pump F (VF-P20-A) Vanquish Column Compartment H (VH-C10-A)																																																																												
Analytical column	2.6 µm Biphenyl 100 Å, 150 × 3.0 mm																																																																												
Mobile phase	A - 0.1% Formic acid in water (Degassed) B - 0.1% Formic acid in methanol:acetonitrile (80:20) (Degassed) C - 0.1% Formic acid in acetonitrile (Degassed)																																																																												
Flow rate (mL/min)	0.400																																																																												
Gradient	<table border="1"> <thead> <tr> <th>Time</th> <th>%A</th> <th>%B</th> <th>%C</th> </tr> </thead> <tbody> <tr><td>00.0</td><td>95</td><td>05</td><td>00</td></tr> <tr><td>01.0</td><td>95</td><td>05</td><td>00</td></tr> <tr><td>04.0</td><td>65</td><td>35</td><td>00</td></tr> <tr><td>04.9</td><td>65</td><td>35</td><td>00</td></tr> <tr><td>05.0</td><td>50</td><td>50</td><td>00</td></tr> <tr><td>06.5</td><td>50</td><td>50</td><td>00</td></tr> <tr><td>07.5</td><td>35</td><td>65</td><td>00</td></tr> <tr><td>08.5</td><td>35</td><td>65</td><td>00</td></tr> <tr><td>09.5</td><td>30</td><td>70</td><td>00</td></tr> <tr><td>12.5</td><td>30</td><td>70</td><td>00</td></tr> <tr><td>13.0</td><td>00</td><td>100</td><td>00</td></tr> <tr><td>15.0</td><td>00</td><td>100</td><td>00</td></tr> <tr><td>15.5</td><td>00</td><td>05</td><td>95</td></tr> <tr><td>19.5</td><td>00</td><td>05</td><td>95</td></tr> <tr><td>20.0</td><td>20</td><td>80</td><td>00</td></tr> <tr><td>2.0</td><td>20</td><td>80</td><td>00</td></tr> <tr><td>22.5</td><td>95</td><td>05</td><td>00</td></tr> <tr><td>28.0</td><td>95</td><td>05</td><td>00</td></tr> </tbody> </table>	Time	%A	%B	%C	00.0	95	05	00	01.0	95	05	00	04.0	65	35	00	04.9	65	35	00	05.0	50	50	00	06.5	50	50	00	07.5	35	65	00	08.5	35	65	00	09.5	30	70	00	12.5	30	70	00	13.0	00	100	00	15.0	00	100	00	15.5	00	05	95	19.5	00	05	95	20.0	20	80	00	2.0	20	80	00	22.5	95	05	00	28.0	95	05	00
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Column temp. (°C)	40, Forced-air mode																																																																												
Needle wash	95:05 Acetonitrile:water with 0.1% formic acid																																																																												
Injection volume (µL)	3.0																																																																												
Run time (min)	28.0																																																																												

**Table 3. Q Exactive Plus Hybrid-Orbitrap mass spectrometer configuration and parameters**

MS ion source parameter	Value
Ion source type	HESI-II
Spray voltage (V)	3,500 (Pos) and 2,500 (Neg)
Sheath gas (arb)	65
Auxiliary gas (arb)	25
Sweep gas (arb)	0
Ion transfer tube temp. (°C)	250
Vaporizer temp. (°C)	450
S-lens (V)	55

### Data analysis

All the nitrosamine standards and drug samples were analyzed using the UHPLC and HRAM Orbitrap MS method described in Tables 2, 3, 4, and 5. Data analysis has been performed using Thermo Scientific™ Chromeleon™ 7.2.10 CDS software, which is a globally accepted gold standard for acquiring and analyzing LC-MS and HRMS datasets as per compliance requirements.

### Results and discussion

Representative chromatograms of nitrosamine impurities and drug matrices are shown in Figures 3–8.

#### Linearity

The linearity plot employing calibrant concentrations ranging from 0.5 to 20 ng/mL as mentioned in Table 6 is shown in Figure 9. R<sup>2</sup> values were greater than 0.99 for all the impurity standard(s), displaying linear responses throughout the concentration range. Figure 9 shows the representative linearity curve with the equation and R<sup>2</sup> value for each of the impurity standards. Tables 7 and 8 show reproducibility and recovery results for the method.

The MS detection performance for all impurities is attained using the Q Exactive Plus Hybrid Quadrupole-Orbitrap MS instrument. In this study, we have demonstrated the specificity and sensitivity of the instrument to detect ultralow levels of nitrosamine contents with very good signal to noise (S/N) and peak shape.

**Table 4. MS acquisition scan parameters**

Impurity number and name	1. NDMA	2. NMBA	3. NMEA	4. NDEA	5. NEIPA	6. NDIPA	7. NDPA	8. NMPA	9. NDBA
Scan type	PRM	t-SIM	PRM	t-SIM	PRM	t-SIM	t-SIM	t-SIM	PRM
Polarity	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
<i>m/z</i> isolated	75.0553	145.0619	89.0704	103.0866	117.1022	131.1179	131.1179	137.0709	159.1492
NCE	80	NA	15	NA	10	NA	NA	NA	50
Isolation width ( <i>m/z</i> )	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Micro scans	3	3	3	3	3	3	3	3	3
Resolution	70,000	70,000	35,000	70,000	35,000	70,000	70,000	70,000	35,000
AGC target	2e5	1e6	2e5	1e6	2e5	1e6	1e6	1e6	2e5
Max. IT	100	100	100	100	100	100	100	100	100

**Table 5. Extraction parameters**

	NDMA	NMBA	NMEA	NDEA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Acquisition mode	PRM	t-SIM	PRM	t-SIM	PRM	t-SIM	t-SIM	t-SIM	PRM
<i>m/z</i> to extract	75.0553	145.0619	61.0396	103.0866	75.0553	131.1179	131.1179	137.0709	103.0872, 159.1492
RT (min)	3.6	6.52	6.43	8.27	9.43	10.49	10.87	11.32	12.82

Tolerance used for *m/z* extraction is 15 ppm (as per FDA protocol).

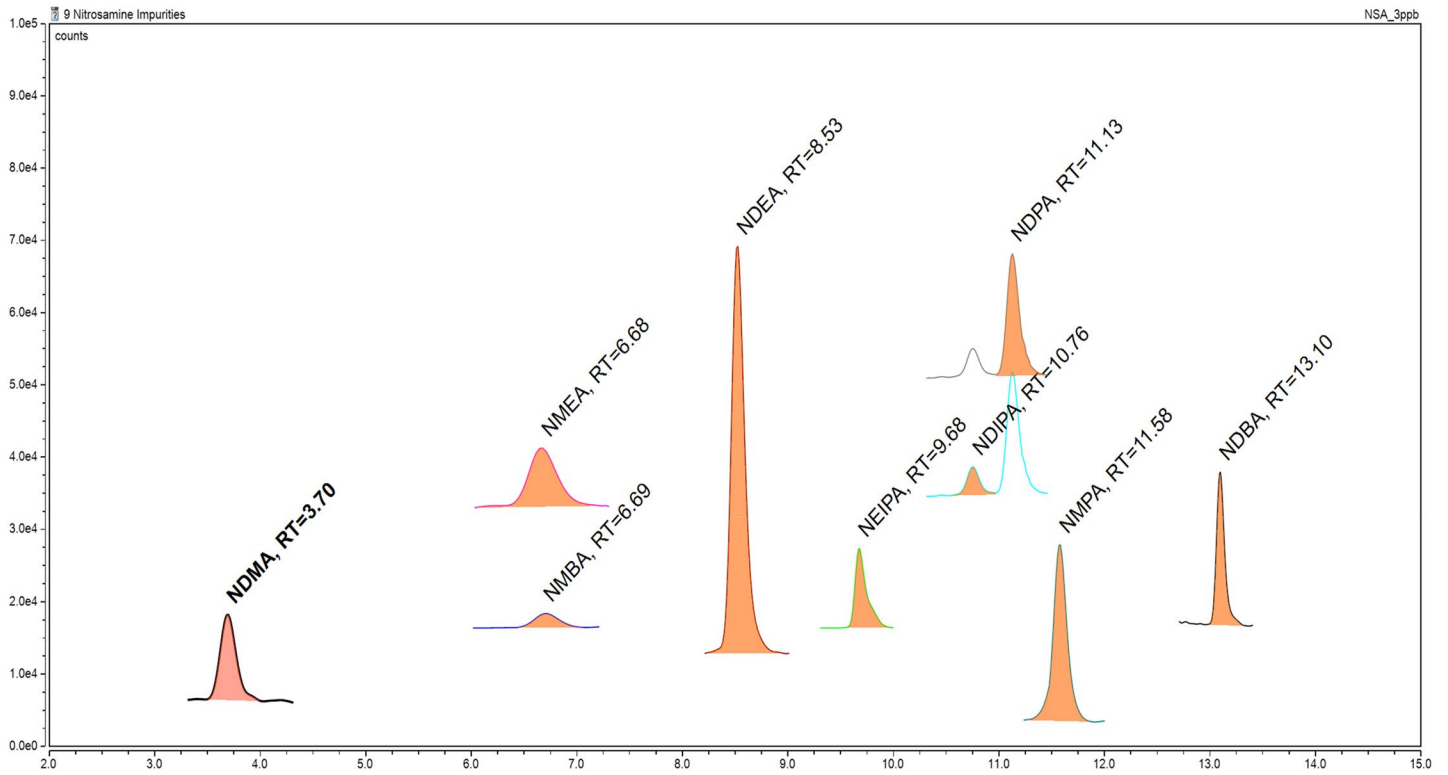


Figure 3. Extracted ion chromatograms (EICs) for all nine impurities in a single window

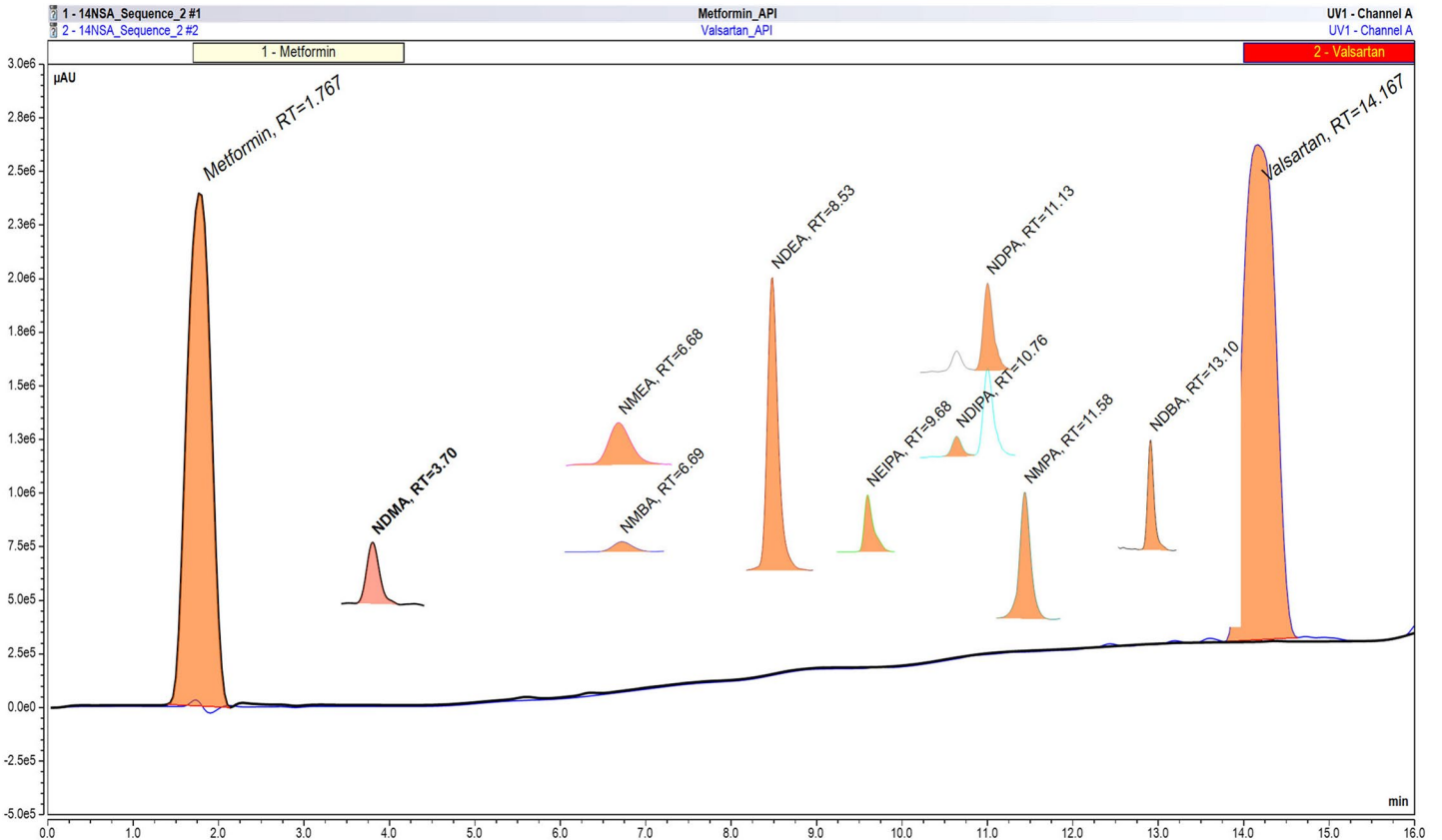


Figure 4. Overlay view of MS and UV chromatogram(s) of nitrosamine impurities with sartan and metformin

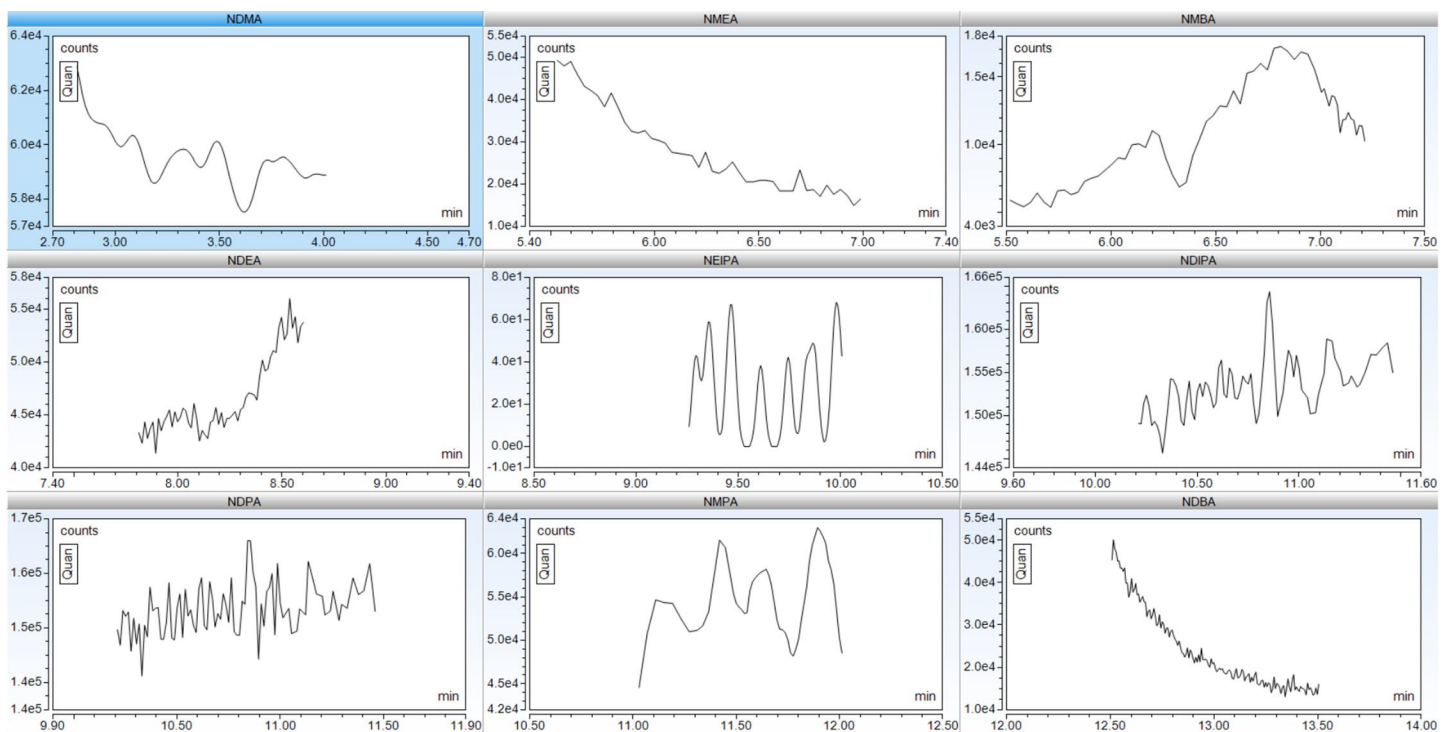


Figure 5. EICs for solvent blank injections

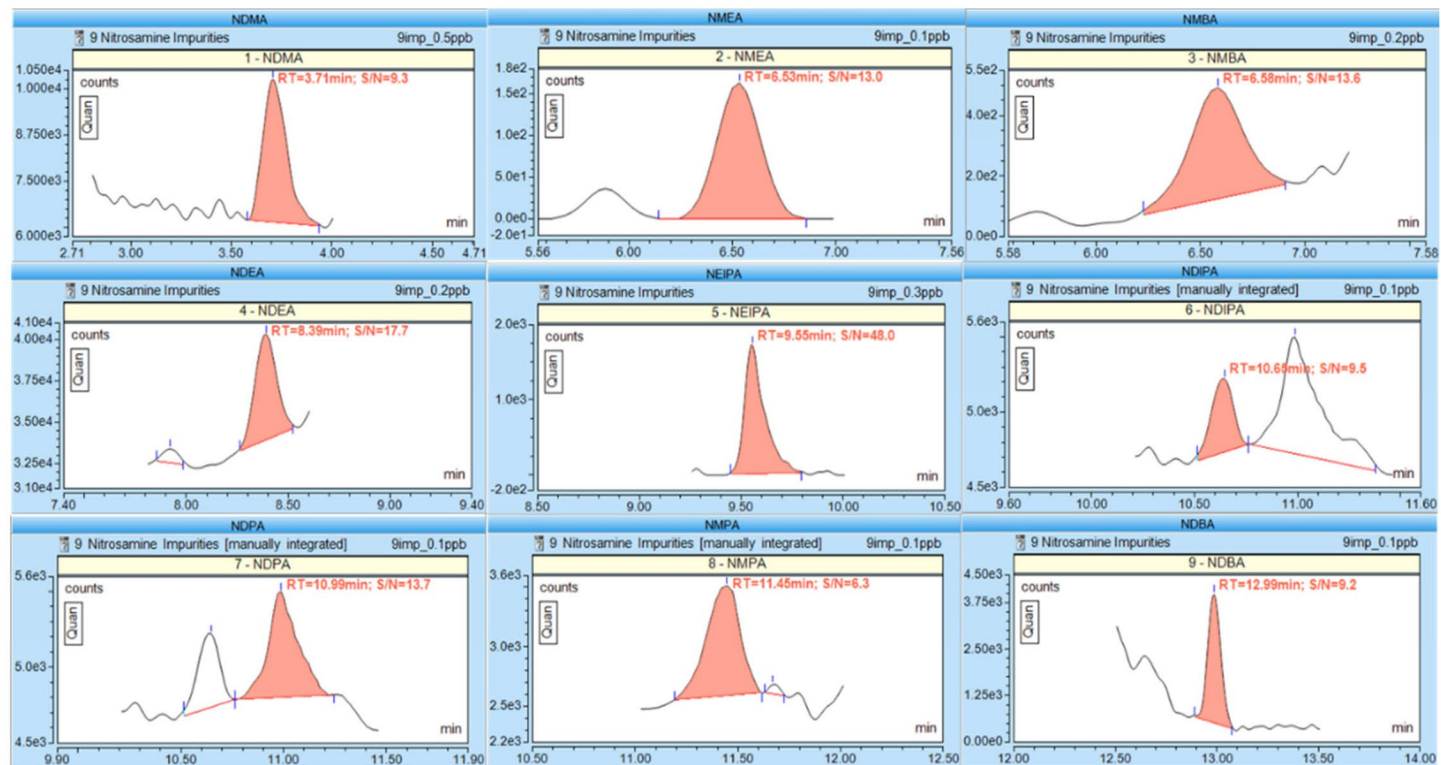


Figure 6. EICs for nitrosamine standard injection at respective LOD levels (NDMA: 1 ppb; NMEA, NMBA, NDPA, NMPA, NDBA: 0.5 ppb; NDEA, NEIPA, NDIPA: 2 ppb)

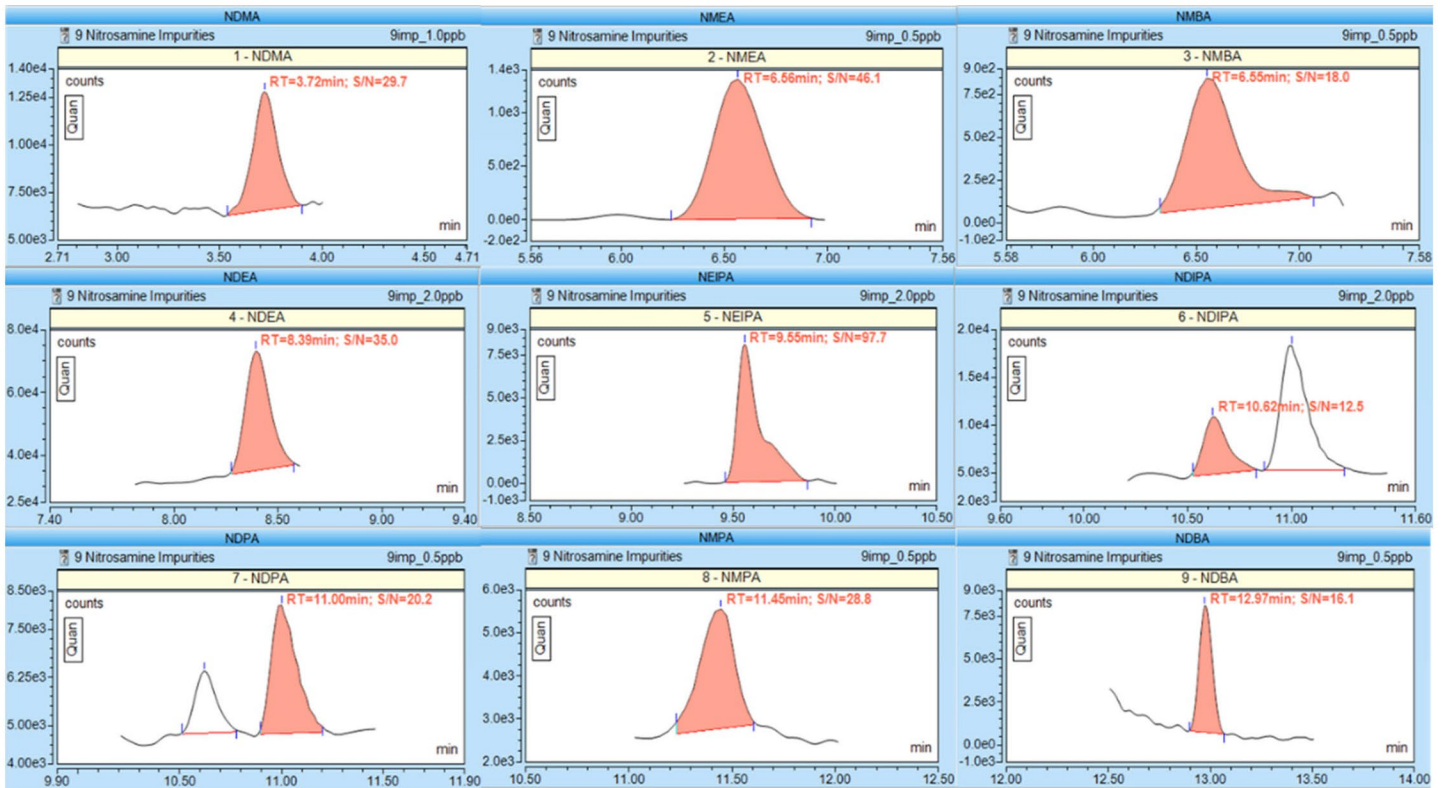


Figure 7. EICs for nitrosamine standard injection at respective LOQ levels (NDMA: 1 ppb; NMEA, NMBA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA: 0.5 ppb)

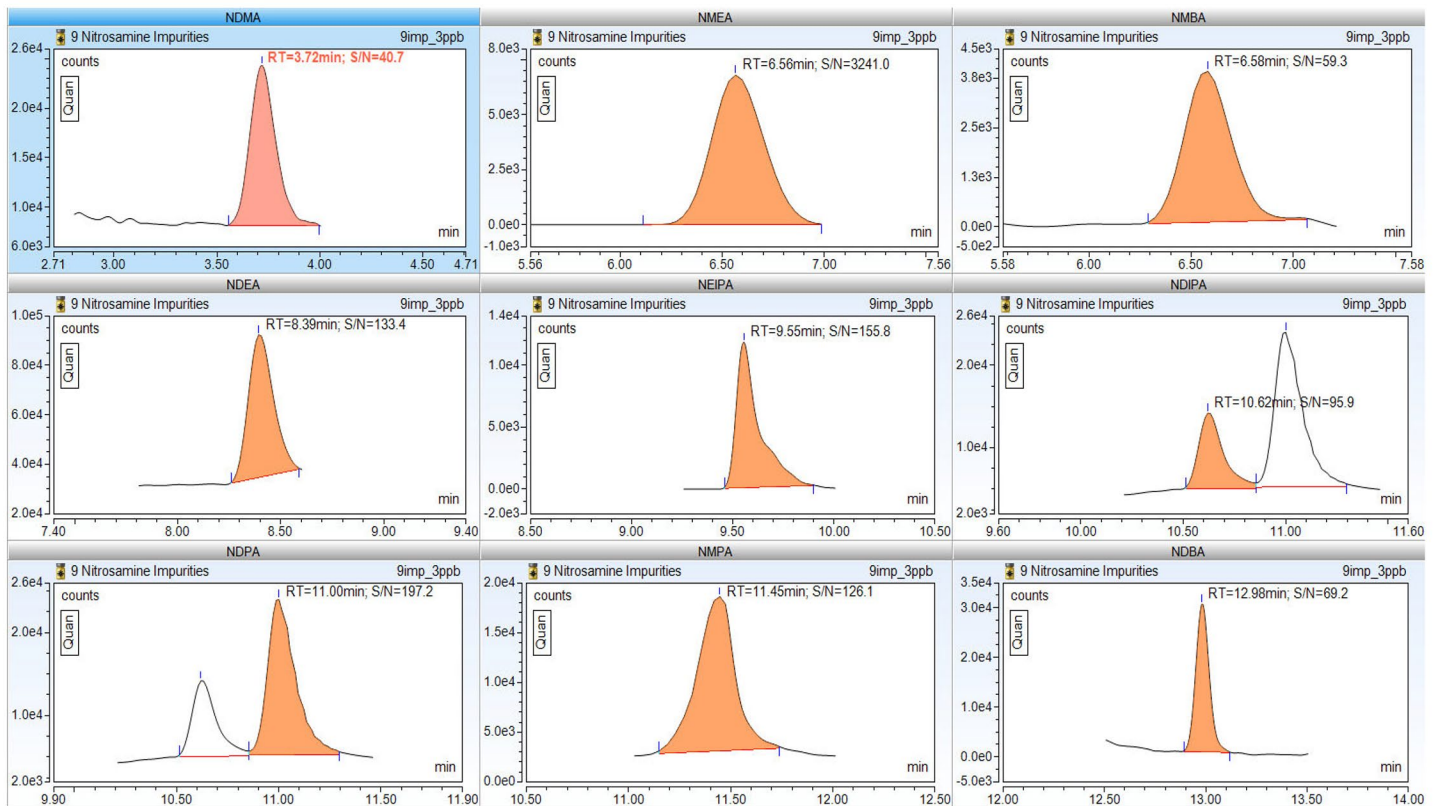


Figure 8. EICs for nitrosamine standard injections at 3 ng/mL (3 ppb)

Table 6. Quantitative performance of the nine impurities with excellent linearity and dynamic range

Compound	LOD		LOD S/N	LOQ		LOQ S/N	R <sup>2</sup>	Weighting factor	Linearity range	
	ng/mL	ppm		ng/mL	ppm				ng/mL	ppm
NDMA	0.5	0.005	94	1.0	0.01	179	0.9993	1/x	0.5–20	0.005–0.2
NMEA	0.2	0.002	53	0.5	0.005	136	0.9990			
NMBA	0.2	0.002	37	0.5	0.005	52	0.9970			
NDEA	0.2	0.002	33	2.0	0.02	159	0.9995			
NEIPA	0.3	0.003	131	2.0	0.02	206	0.9988			
NDIPA	0.1	0.001	11	2.0	0.02	38	0.9988			
NDPA	0.1	0.001	41	0.5	0.005	86	0.9995			
NMPA	0.1	0.001	25	0.5	0.005	62	0.9992			
NDBA	0.1	0.001	114	0.5	0.005	148	0.9990			

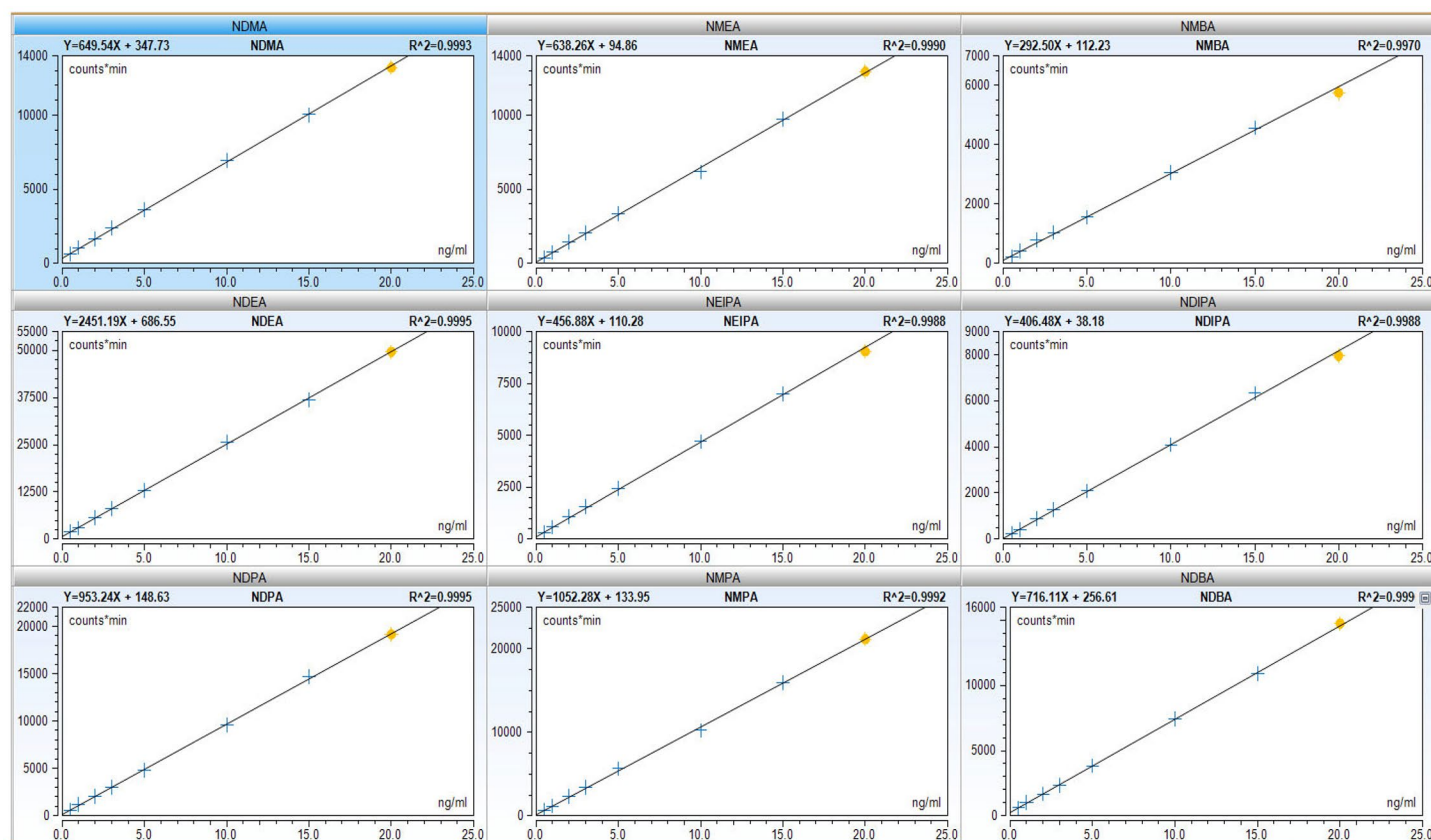


Figure 9. Linearity curves plot of respective nitrosamine impurity standards (0.5–20 ng/mL)



**Table 7. Reproducibility of the method at LOQ and standard level.** Reproducibility results were attained using six replicate injections of nitrosamine impurity mix at LOQ and standard level(s).

	Reproducibility results for all nine nitrosamine impurities								
	NDMA	NMEA	NMBA	NDEA	NEIPA	NDIPA	NDPA	NMPA	NDBA
%RSD at LOQ	5.9	6.5	6.8	4.4	2.4	4.8	3.8	4.5	2.9
%RSD at standard	2.8	2.0	3.6	2.5	2.7	4.6	5.4	4.1	0.4

**Table 8a. Recovery results for valsartan drug matrix spiked at three different levels**

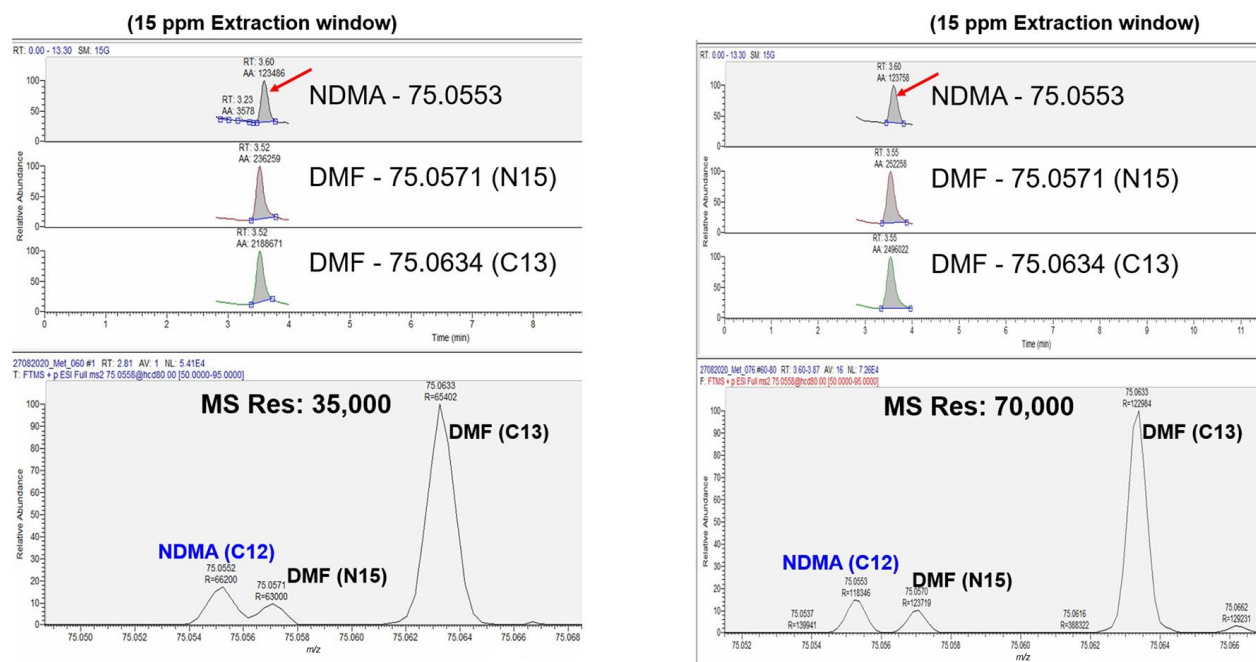
Impurity	Recovery results for all nine nitrosamine impurities								
	NDMA	NMEA	NMBA	NDEA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Impurity recovery at low concentration level									
Concentration	1.0 ng/mL								
% Recovery	87.3	97.3	90.1	95.6	83.1	93.8	101.1	95.9	99.1
Impurity recovery at mid concentration level									
Concentration	3.0 ng/mL								
% Recovery	92.0	84.2	94.2	90.6	87.9	89.3	88.7	95.8	96.1
Impurity recovery at high concentration level									
Concentration	20.0 ng/mL								
% Recovery	92.7	102.2	108.7	103.8	99.7	103.0	113.9	90.8	94.3

**Table 8b. Recovery results for metformin drug matrix spiked at three different levels**

Impurity	Recovery results for all nine nitrosamine impurities								
	NDMA	NMEA	NMBA	NDEA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Impurity recovery at low concentration level									
Concentration	1.0 ng/mL								
% Recovery	104.6	110.0	106.9	109.5	97.7	86.8	85.3	99.3	99.4
Impurity recovery at mid concentration level									
Concentration	3.0 ng/mL								
% Recovery	84.2	100.8	93.2	87.3	96.1	95.3	98.2	96.3	94.4
Impurity recovery at high concentration level									
Concentration	20.0 ng/mL								
% Recovery	93.5	108.8	101.2	93.7	102.3	101.7	100.3	100.2	95.3

During this study, we experimentally evidenced the importance of MS resolution in nitrosamine quantitation. The <sup>12</sup>C isotope of NDMA and <sup>15</sup>N isotope of DMF differ only by 0.0018 Da; therefore, one must employ high-resolution MS methods to selectively quantify the impurity or analyte of interest without compromising the sensitivity.

In Figure 10, we show that at a MS resolution setting of 60,000, the NDMA (<sup>12</sup>C) and DMF (<sup>15</sup>N) were not well separated, whereas at a resolution setting of 120,000, both isotopes were found to be baseline resolved. The extracted ion chromatogram (EIC) areas of NDMA also were found to be similar at both the resolution settings.



**Figure 10. Importance of high resolution in differentiating near isobaric interference(s) such as DMF from the analyte of interest (NDMA). 3 ppb NDMA and DMF spiked into metformin API (100 mg/mL)**

## Summary

Key outputs attained in the current study are as below:

- In this study using a Vanquish Flex Quaternary UHPLC system coupled with HRAM Orbitrap MS, we were able to confidently estimate the contents of nine nitrosamine impurities for two different matrices (valsartan and metformin) in a single method. Valsartan was observed to elute after 13 min and metformin before 3 min.
- By employing high resolution for NDMA acquisition (resolution set to 70,000), we were able to separate the interference of DMF isotopes from NDMA in the drug matrix samples (Figure 10).
- This method can be further modified to quantify much lower levels of the nitrosamines.
- By employing polarity switching, we were able to confidently separate two impurities that were chromatographically co-eluting (NMBA and NMEA) without increasing the gradient time.
- U.S. FDA prescribed LODs and LOQs were attained for all the impurities with better peak shape and S/N ratio.

- The % RSDs at LOQ and standard concentration levels of each nitrosamine impurity were found to be less than 10%.
- Recovery has been performed at three levels, including the 3.0 ng/mL (standard) level. The results were within permissible limits (80–120%).

## Conclusion

The Q Exactive Plus Orbitrap MS instrument can be employed to simultaneously identify and quantify nine nitrosamine impurities at very low concentrations as per regulatory requirements. Since Orbitrap MS has the capability to deliver high resolution and accurate mass while maintaining sensitivity, this can be confidently employed to resolve near isobaric interferences from the analytes of interest, and thereby can be routinely utilized for small molecule quantitation such as nitrosamines. In the current study, we have adopted a U.S. FDA prescribed LC-HRMS method with minor improvements so that we can attain better peak shape and peak response of nine impurities for two different drug matrices without the need for change in any of the LC and MS parameters. The method is highly sensitive, robust, and reproducible even at lower concentrations utilizing only 3  $\mu$ L injection volume. One can easily attain levels lower than the ones that are mentioned in this report by simply increasing the injection volumes to 9  $\mu$ L.

## References

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