APPLICATION NOTE 10753

A validated method for the rapid determination of fifteen nitrosamines in metformin drug substance

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Goal

The purpose of this study was to develop a validated workflow for the determination of fifteen nitrosamines in metformin drug substance and to assess the quantitative performance of the Thermo Scientific™ Orbitrap Exploris™ GC Mass Spectrometer.

Introduction

Nitrosamines are considered a matter of concern as the ICH M7 (R1)[1] guideline categorizes them as Class 1 impurities or mutagenic carcinogens or probable carcinogens by the International Agency for Cancer Research (IARC).[2] Nitrosamine impurities in pharmaceutical substances can originate from the reaction between nitrites or amines present as unintentional contaminants of raw materials, reagents, and solvents with a nitrosating agent (e.g., sodium nitrite) used during the production processes.[3] Since June 2018, several drugs have been recalled due to the presence of nitrosamine impurities in angiotensin II receptor blockers known as “sartans” and in the histamine antagonist/proton pump inhibitor ranitidine.[3]

The U.S. Food and Drug Administration (FDA) has indicated the presence of NDMA at unacceptable levels in several lots of the extended release (XR) formulation of metformin used in the treatment of diabetes.[1] A transition period of two years has been granted by the regulatory authorities to make changes in manufacturing processes to minimize nitrosamine impurities in finished pharmaceutical products.

The European network of Official Medicines Control Laboratories (OMCLs) has released several methods for the analysis of NDMA and NDEA in metformin drug substances and drug products.[2] Although the official method from the OMCL network specifies only two impurities, the FDA recently released a new guidance document with seven nitrosamine impurities that should be monitored in drug products.[2] In addition, this application note further expands the scope of nitrosamines to fifteen, including many of the GC amenable nitrosamines studied by the European Medicines Agency.[4-6]

There are a number of challenges with the use of unit mass resolution quadrupole mass spectrometery for nitrosamine analysis. These include the detection of false positives through (i) impurities of NDMA in N,N-dimethylformamide (DMF), (ii) challenging DMF separation from NDMA—even with triple quadrupole mass spectrometers the common precursor to product ion transitions do not provide sufficient selectivity, and (iii) data cannot be looked back on retrospectively to check for additional impurities when operating in tSIM and tSRM modes. To resolve DMF interferences, a minimum mass resolution of 15,000 FWHM @ *m/z* 200 is essential and without sacrificing sensitivity.

In this application note a validated analytical method using GC Orbitrap technology was developed for the selective quantification of fifteen nitrosamine impurities in metformin drug substance at ultra-trace levels.

Experimental

Sample preparation

Calibration standards containing fifteen nitrosamines at fifteen concentration levels (Appendix 1) and three (^{13}C labeled) internal standards (Appendix 2) were acquired from Fisher Scientific, Sigma-Aldrich, and LGC.

For the calculation of solvent standard instrument detection limits (IDLs) and limits of quantification (LOQ), DCM was spiked at 0.2, 0.4, 1.0, 2.5 and 5.0 ng/mL. In addition, method detection limits (MDLs) and method LOQs were determined using serially diluted matrix-matched standards spiked at 1.0, 2.0, 2.5, 5.0, 10, and 20 ng/mL. For the sample preparation, 100 mg of metformin was weighed into the amber high recovery vials and the extraction proceeded as follows in Figure 1. The total sample preparation time was 6 minutes.

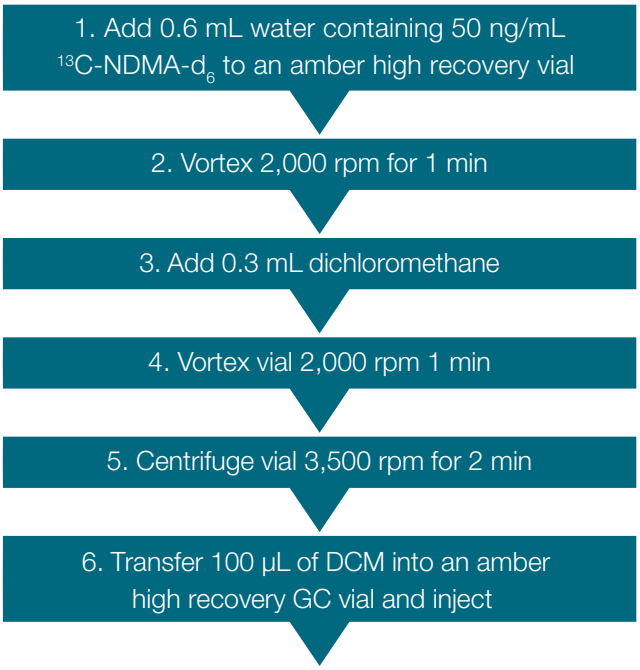


Figure 1. Extraction procedure for metformin drug substance

Validation

All tests were designed in accordance with ICH guidelines [ICH Topic Q 2 (R1)] used in FDA and worldwide GMP regulatory validation procedures.

Specificity

1,000 ng/mL mixed nitrosamine standard followed by injection solvent blanks was used to determine any carryover. In addition, three procedural blanks and six unspiked metformin extracts were also prepared to confirm that no nitrosamines were present.

Accuracy

For the determination of accuracy, three extractions were prepared at 1.0, 2.0, 10, and 20 ng/g *(w/w)* by spiking 100 mg of metformin prior to extraction. Further details of the workflow can be found in Figure 1. Recovery of 70–130% and recovery % RSD <20% was assessed at the lowest possible concentration for each nitrosamine.

Precision

For the determination of precision, <20% RSD for concentration was achieved for the lowest possible level.

Method and instrument robustness

The peak area and mass accuracy of internal standard spiked into a metformin extract was monitored over a period of two weeks. More than 100 sample extract injections were carried out without any maintenance of the inlet. However, mass calibration was performed daily, and injector septa were changed after ~100 injections.

GC-MS analysis

An Orbitrap Exploris GC mass spectrometer equipped with the Thermo Scientific™ ExtractaBrite™ electron ionization source was used for analysis. This configuration allows vent-free column changes and ionization source maintenance in <2 minutes representing a 98% time saving versus traditional venting approaches, which take up to 4 hours. Liquid injections of the sample extracts were performed using a Thermo Scientific™ TriPlus™ RSH series autosampler, and chromatographic separation was achieved by a Thermo Scientific™ TraceGOLD™ TG-1701MS 30 m × 0.25 mm i.d. × 0.50 µm film (P/N 26090-2230) capillary column. Additional details of instrument parameters are detailed in Appendixes 3 and 4. Consumable details are listed in Appendix 5.

Data processing

Data were acquired using full scan (FS) mode with tSIM, processed, and reported using Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS). With the ever-evolving emphasis on data integrity, data security, and compliance, it is of vital importance that the CDS provides comprehensive preventative and detection technical controls to enable analytical laboratories to meet modern regulatory requirements including FDA 21 CFR Part 11 and European Commission (EU) Annex 11.

Results and discussion

Chromatographic performance, sensitivity, and linearity were evaluated using solvent-based standards. Assessment of method performance (MDLs), method LOQs, selectivity, accuracy, precision, and long-term extraction repeatability (method and instrument stability) were performed using the method conditions described in the experimental section.

Chromatography

All compounds were separated in <12 minutes. Using the TraceGOLD TG-1701 MS column, Gaussian peak shape was obtained for all compounds, including NDMA (particularly challenging due to its polarity) with an EP peak asymmetry value of 1.2, which meets EU and USP Pharmacopoeia criteria of <1.5. (Figure 2).

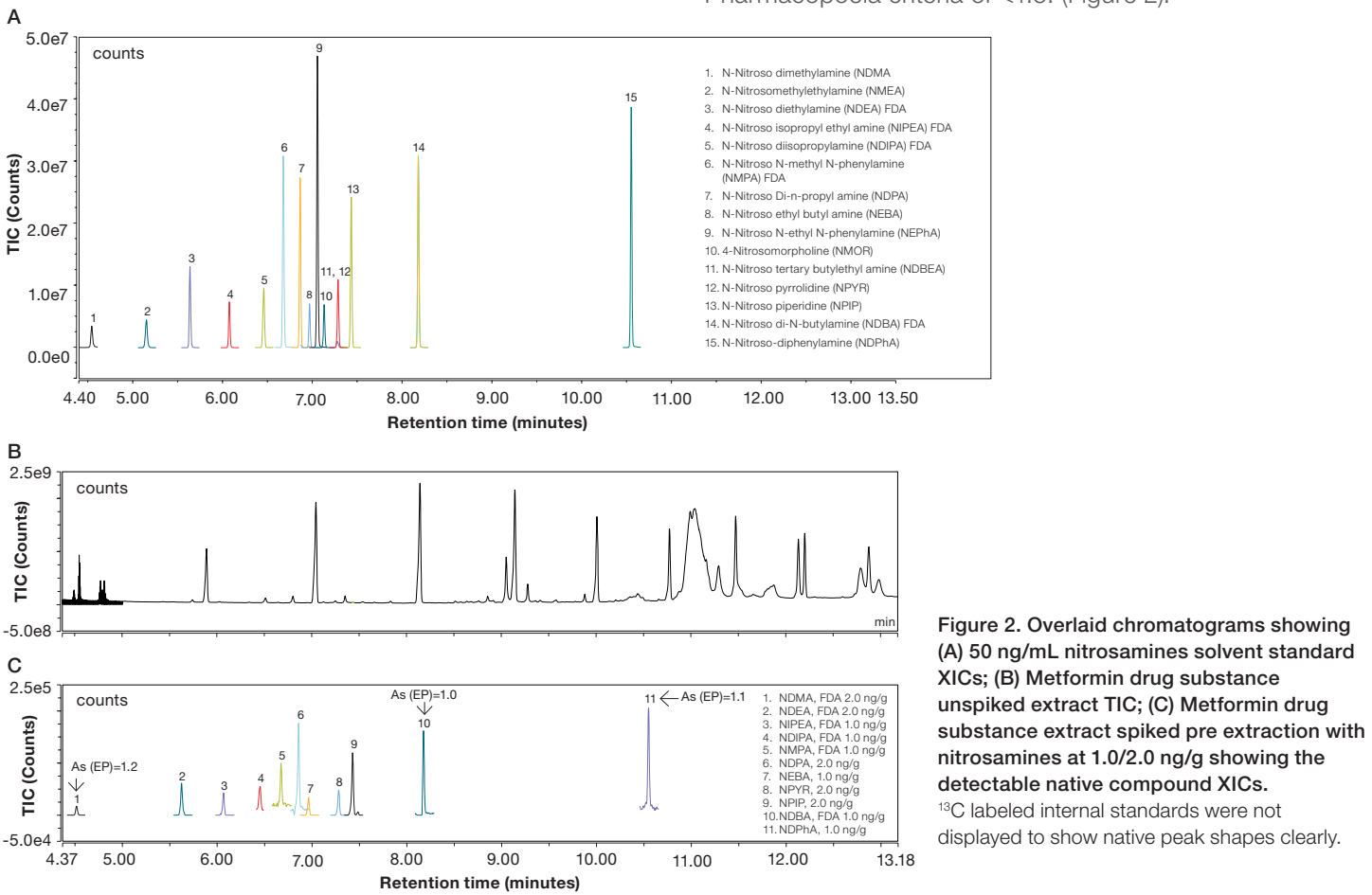


Figure 2. Overlaid chromatograms showing (A) 50 ng/mL nitrosamines solvent standard XICs; (B) Metformin drug substance unspiked extract TIC; (C) Metformin drug substance extract spiked pre extraction with nitrosamines at 1.0/2.0 ng/g showing the detectable native compound XICs. ^{13}C labeled internal standards were not displayed to show native peak shapes clearly.

Sensitivity: determination of IDL and LOQ

To assess the IDLs, n=13 replicate injections of the lowest serially diluted solvent standard (0.2, 0.4, 1.0, 2.5, and 5.0 ng/mL) with a peak area <15% RSD was used. The IDL was then calculated by considering the injected amount, peak area % RSD, and t-score of 2.681, corresponding to 12 (n-1) degrees of freedom at the 99% confidence interval (Figure 3). The calculated IDL values ranged from 42 to 388 fg on column (OC) (corresponding to 0.1–1.3 ng/g in sample) with a mean of 133 fg OC. The standard used to calculate each IDL was set as the LOQ, which varied from 0.2 to 1.2 pg OC (0.3–1.8 ng/g in metformin) (Appendix 6). These results easily meet the FDA regulatory requirements of 30 ppb (ng/g) total nitrosamines per day.

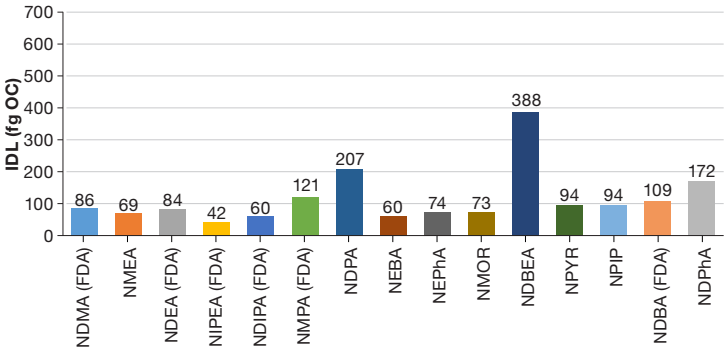


Figure 3. Individual IDLs (as detectable fg on column) for fifteen native nitrosamines calculated from n=13 replicate injections of the lowest concentrations of serially diluted solvent standards

In addition to this, for supplementary reading during method development, it was found that 30 eV provided a significant boost in sensitivity versus 70 eV. For most of the compounds analyzed, this was typically 3x peak area as highlighted by early, mid, and late eluting nitrosamines. (Appendix 7).

Linearity

Using solvent standards, the linear range was 0.2–2,000 ng/mL, equivalent to 0.6–6,000 ng/g in metformin drug substance. The calibration of each compound was performed using the linear average calibration factor function (AvCF) in Chromeleon CDS over three injections at each concentration level (Figure 4). All compounds show excellent linear responses with coefficients of determination $R^2 \geq 0.995$, and average response factor values (RF, % RSD) across the calibration range <8.5%. The R^2 values ranged from 0.9993 to 0.9999 with an average value of 0.9997 (Appendix 8).

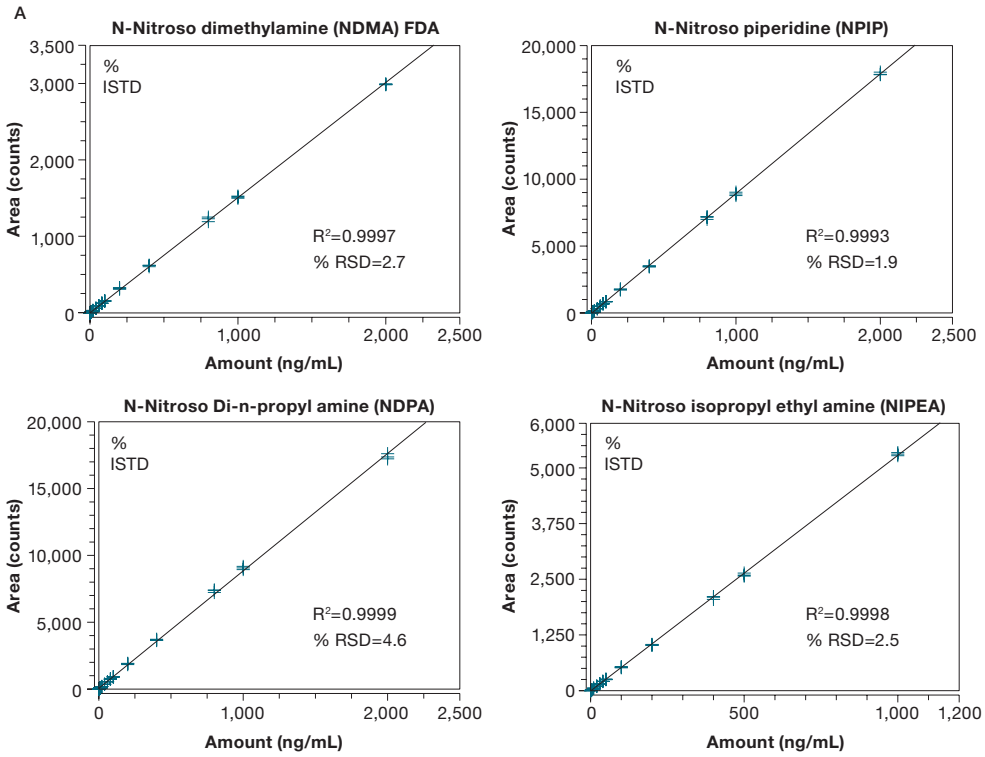


Figure 4. (A) Linearity of example nitrosamines as demonstrated using a solvent-based calibration curves ranging from 0.2 to 2,000 ng/mL (corresponding to 0.6–6,000 ng/g in metformin). Average calibration factor function (AvCF) was used in Chromeleon CDS, and three replicate injections at each concentration with internal standard adjustment was performed. Coefficient of determination (R^2) and average response factor values (RRF, % RSD) are displayed.

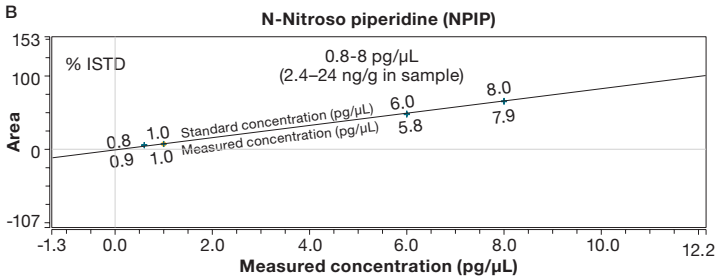


Figure 4 continued. (B) A magnified region of the calibration for NPIP ranging from 0.8 to 8 ng/mL (corresponding to 2.4–24 ng/g in metformin), showing excellent precision and accuracy for triplicate injections per point

Method detection limits (MDL) and method quantification limits (MQL)

To assess the MDLs, n=12 replicate injections of the lowest matrix matched serially diluted standard (1.0, 2.0, 2.5, 5.0, 10, 20 ng/mL) with a peak area % RSD of <15% was used. The MDL was then calculated by considering the injected amount, peak area % RSD, and t-score of 2.718, corresponding to 11 (n-1) degrees of freedom at the 99% confidence interval (Figure 5). The MDL values calculated ranged from 51 to 550 fg oc (corresponding to 0.2–1.8 ng/g in sample). The mean was 185 fg oc, which was comparable to the solvent standard IDLs. The standard used to calculate each MDL was set as the MQL, which varied from 0.4 to 2.0 pg OC (0.6–3.0 ng/g in metformin), Appendix 9. These results easily meet the FDA regulatory requirements of 30 ppb (ng/g).

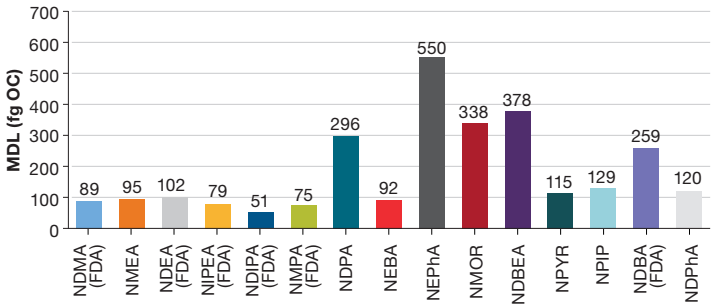


Figure 5. Individual MDLs (as detectable fg on column) for fifteen native nitrosamines calculated from n=12 replicate injections of the lowest serially diluted matrix matched standards

Specificity

A 2,000 ng/mL mixed nitrosamine DCM standard followed by a DCM blank were prepared and carryover in the blank was investigated (Figure 6). No carryover was detected in the DCM blank.

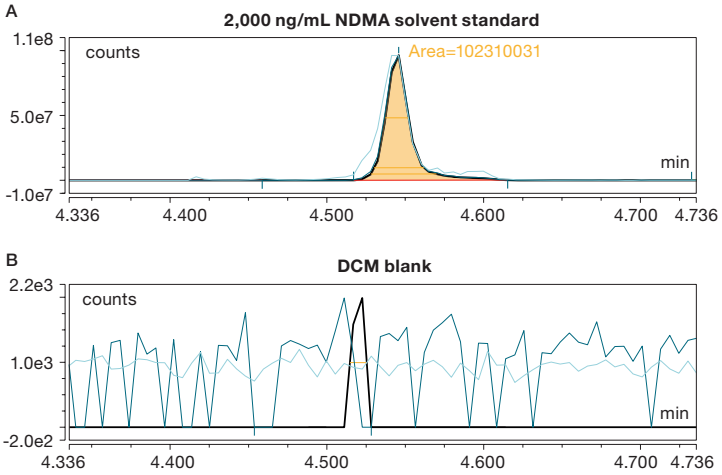


Figure 6. Overlaid XIC chromatograms of stacked quantification ion *m/z* = 74.04746, qualification ion 1 *m/z* = 42.03375 and qualification ion 2 *m/z* = 44.04940 for (A) 2,000 ng/mL NDMA solvent standard and (B) a consecutive DCM blank with no NDMA carryover.

In addition to these three procedural blanks, six unspiked metformin extracts were also prepared to confirm that no nitrosamines were present (Appendix 10). No nitrosamines were detected in the blank and as such the level is reported as a <LOQ based on method LOQ results in Appendix 7.

Accuracy

For the determination of accuracy, three extractions were prepared at 1.0, 2.0, 10, and 20 ng/g *(w/w)* by spiking 100 mg of metformin prior to extraction with nitrosamine spiking solutions. Further details of the extraction workflow can be found in Figure 1. Triplicate injections of each extract at the lowest possible level that satisfied ICH Guidelines Q 2 (R1) performance criteria (i) recovery of 70–130% and (ii) recovery % RSD <20% were observed as follows with an average recovery across the nitrosamines of 96% and all within 70–130% (Figure 7 and Appendix 11).

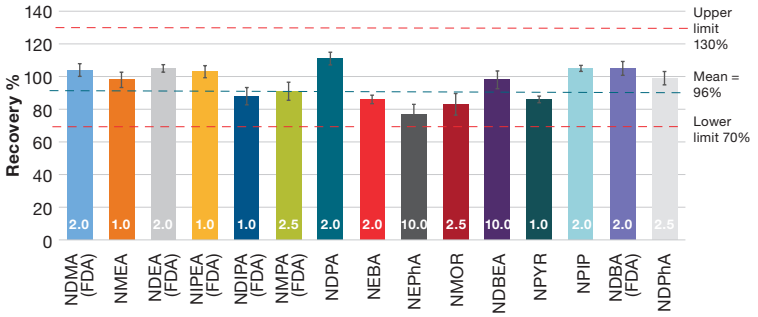


Figure 7. Mean (n=12 measurements) % recovery of nitrosamines spiked into metformin drug substance prior to extraction at various amounts with associated STDERROR bars and spiked amount at the bottom of each bar. Upper and lower % recovery tolerance (red) and mean recovery across all nitrosamines (blue) were added. *The 2.5 ng/g accuracy level was assessed by serially diluting the 10 ng/g extract with unspiked matrix.

Precision

For the determination of precision, the same nitrosamine extracts described previously in the accuracy section prepared in triplicate across four levels and injected in triplicate were used (Figure 8 and Appendix 12). All nitrosamines had peak amount % RSDs <20% and the mean % RSD was 8.3%.

Quantification of nitrosamines in metformin drug substance extracts

Chromatograms of metformin drug substance, which was extracted as described in the experimental section and spiked at the LOQ levels of nitrosamines that pass both accuracy and precison criteria, are shown (Figure 9). The quantitative performance of the method in terms of sensitivity and selectivity is highlighted below with examples of low-level quantification of the six GC-amenable FDA nitrosamines in terms of chromatographic peak shape and minimal matrix interferences, accuracy of the spiked amount % recovery, and ppm mass error.

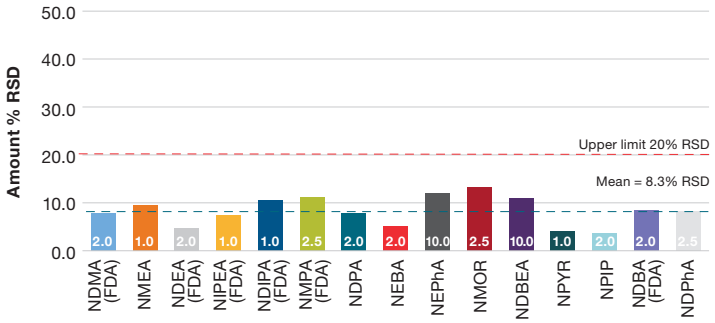
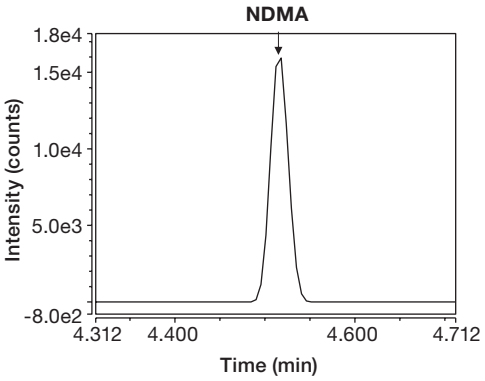
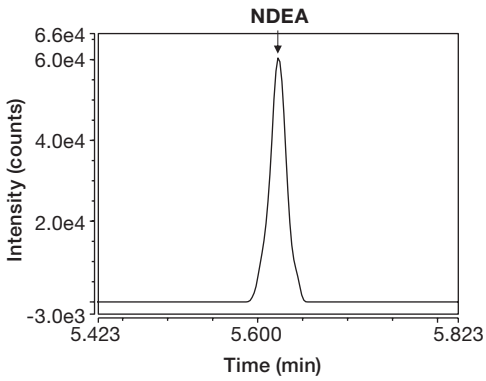


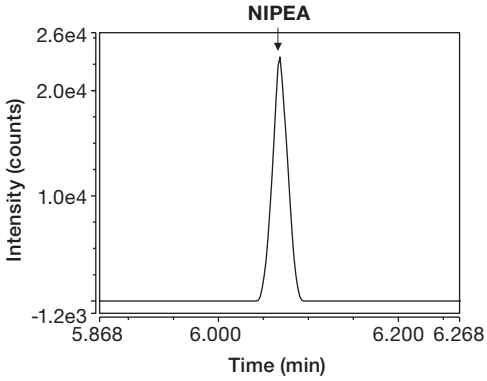
Figure 8. Repeatability of measurements as amount % RSD for target nitrosamines spiked into metformin drug substance at various amounts with spiked amount at the bottom of each bar. Upper amount % RSD tolerance line (red) and mean amount % RSD across all nitrosamines (blue) were added.



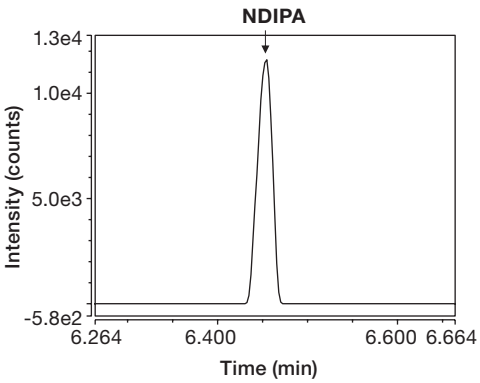
Spiked amount added	2.0 ng/g
Amount found in sample	1.9 ng/g
Recovery %	95%
Measured mass (*m/z*)	74.04750
Theoretical mass (*m/z*)	74.04746
Chemical formula	$C_2H_6N_2O$
Mass error (ppm)	-0.48



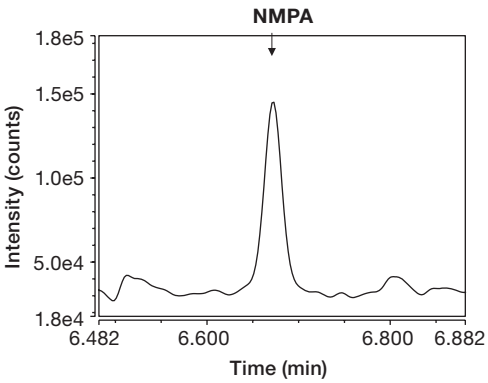
Spiked amount added	2.0 ng/g
Amount found in sample	2.1 ng/g
Recovery %	105%
Measured mass (*m/z*)	102.07877
Theoretical mass (*m/z*)	102.07876
Chemical formula	$C_4H_{10}N_2O$
Mass error (ppm)	-0.08



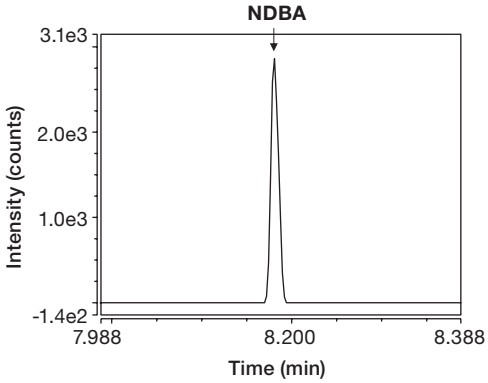
Spiked amount added	1.0 ng/g
Amount found in sample	1.0 ng/g
Recovery %	103%
Measured mass (*m/z*)	116.09441
Theoretical mass (*m/z*)	116.09441
Chemical formula	$C_5H_{12}N_2O$
Mass error (ppm)	-0.03



Spiked amount added	1.0 ng/g
Amount found in sample	0.9 ng/g
Recovery %	88%
Measured mass (*m/z*)	113.10731
Theoretical mass (*m/z*)	113.10732
Chemical formula	$C_6H_{13}N_2$
Mass error (ppm)	-0.04



Spiked amount added	2.5 ng/g
Amount found in sample	2.3 ng/g
Recovery %	91%
Measured mass (*m/z*)	106.06514
Theoretical mass (*m/z*)	106.06513
Chemical formula	C_7H_8N
Mass error (ppm)	-0.05



Spiked amount added	2.0 ng/g
Amount found in sample	2.1 ng/g
Recovery %	105%
Measured mass (*m/z*)	158.14120
Theoretical mass (*m/z*)	158.14136
Chemical formula	$C_8H_{18}N_2O$
Mass error (ppm)	0.99

Figure 9. Examples of FS XIC chromatograms of metformin spiked at the LOQ level pre-extraction looking at the six GC amenable FDA nitrosamines

In summary, the results obtained in these experiments demonstrate excellent accuracy in terms of the recoveries calculated versus a solvent calibration curve at the LOQ level, as well as close agreement between the theoretical exact mass and measured mass expressed as mass error in ppm. For all compounds, the ppm error was close to zero except for NDBA, which was 0.99 ppm. Minimal matrix interferences were observed with near zero baseline noise for all compounds even while operating in FS mode predominantly.

Long term extraction repeatability (method and instrument stability)

The internal standard peak area and mass accuracy were monitored over a period of two weeks in unspiked metformin extracted standards during the method development and validation phases of the study where >100 sample extracts were injected (Figure 10A and B). The peak area 6.9% RSD represents excellent system and method stability considering that these are separate extractions over a period of several weeks. No inlet maintenance or tuning was performed over this period, however MS calibration was performed daily taking approximately 30 seconds to complete within Chromeleon CDS, which is native with tuning.

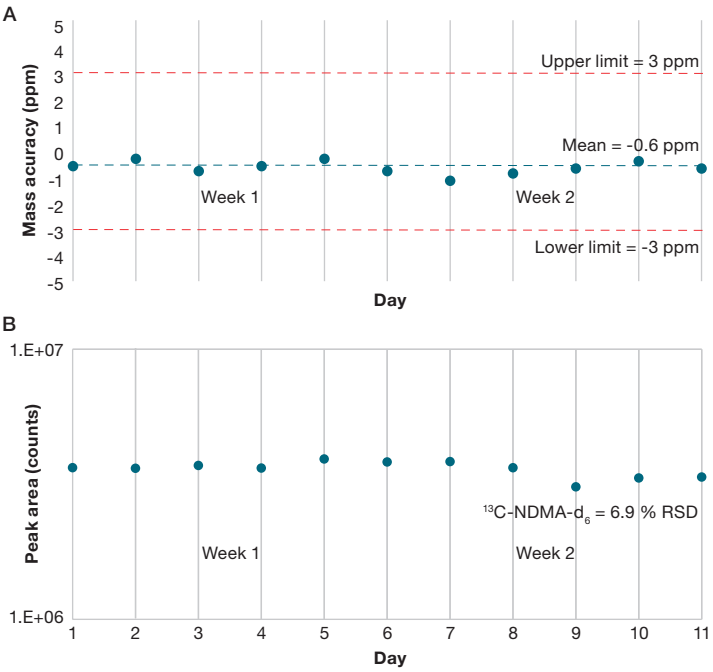


Figure 10. Example data showing (A) ^{13}C-NDMA-d$_6$ ppm mass error with upper limit 3 ppm (red line), lower limit 3 ppm (red line) and mean mass error (blue line), and (B) ^{13}C-NDMA-d$_6$ peak area repeatability plus mean peak area % RSD of unspiked metformin extracts over a period of two weeks during which over 100 matrix injections were performed.

Conclusions

The results of these experiments demonstrate:

- Rapid separation of fifteen nitrosamines in <12 minutes
- Excellent linearity over four orders of magnitude (0.2–2,000 ng/mL)
- Sub-ppb levels of sensitivity with MDLs ranging from 51 to 550 fg OC (corresponding to 0.2–1.8 ng/g in sample) and MQLs ranging from 0.6 to 3.0 ng/g in metformin drug substance, easily surpassing FDA regulatory limits of 30 ppb (ng/g)
- Precise and confident quantification as demonstrated through accuracy and precision of 70–130% recovery and amount % RSD <20% for triplicate extracted standards ranging from 1 to 20 ng/g
- Excellent method and instrument robustness was demonstrated over a period of 2 weeks with over 100 matrix injections with minimal inlet maintenance

Taken together, these results demonstrate that an excellent sample preparation procedure in combination with the Orbitrap Exploris GC provides an ideal solution for Pharma QC-Analytical-Science-Laboratories looking to improve productivity and deliver confident results.

References

1. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; "ICH Harmonised Guideline - Assessment And Control Of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, M7(R1)"; March 31, 2017. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m7r1-assessment-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit_en.pdf
2. International Agency for Research on Cancer; "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Smokeless Tobacco and Some Tobacco-specific N-Nitrosamines, volume 89, 200.
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4. https://www.edqm.eu/en/ad-hoc-projects-omcl-network#Methods%20for%20determination%20of%20nitrosamines%20in%20metformin
5. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf
6. 20 August 2019 EMA/351053/2019 rev 1: Temporary interim limits for NMBA, DIPNA and EIPNA impurities in sartan blood pressure medicines.

Appendix

Appendix 1. Details of the fifteen native compounds analyzed, including CAS number and calibration range

Native standard	CAS number	Calibration range (ng/mL)
NDMA (FDA)	62-75-9	0.2–2,000
NMEA	10595-95-6	
NDEA (FDA)	55-18-5	
NIPEA (FDA)	16339-04-1	
NDIPA (FDA)	601-77-4	
NMPA (FDA)	614-00-6	
NDPA	621-64-7	
NEBA	4549-44-4	
NEPhA	612-64-6	
NMOR	59-89-2	
NDBEA	3398-69-4	
NPYR	930-55-2	
NPIP	100-75-4	
NDBA (FDA)	924-16-3	
NDPhA	86-30-6	

Appendix 3. GC and injector conditions

TRACE 1310 GC parameters	
Injection volume (µL)	2.0
Liner	Single gooseneck with glass wool Thermo Scientific™ LinerGOLD™ (P/N 453A1925-UI)
Inlet (˚C)	240
Inlet module and mode	SSL, Splitless with surge
Splitless time (min)	1.0
Split flow (mL/min)	80
Surge time (min)	1.01
Surge pressure (psi)	25
Septum purge flow (mL/min)	5.0
Carrier gas, flow rate (mL/min)	He, 1.3
Oven temperature program	
Temperature 1 (˚C)	40
Hold time (min)	1.0
Temperature 2 (˚C)	130
Rate (˚C/min)	25
Hold time (min)	0
Temperature 3 (˚C)	270
Rate (˚C/min)	20
Hold time (min)	2
Total GC run time (min)	13.6

Appendix 2. Details of the internal standards, including their concentration

Internal standard	Concentration (ng/mL)
^{13}C-NDMA-d_6	50
NPYR-d_8	50
NDPA-d_{14}	50

Appendix 4. MS parameters

Orbitrap Exploris GC EI GC-MS parameters	
Transfer line (˚C)	250
Ion source (ionization type)	ExtractaBrite (EI)
Ion source (˚C)	320
Electron energy (eV)	30
Emission current (µA)	50
Acquisition mode	Full scan (FS) + timed sim (t-SIM)
Mass range (*m/z*)	40–440
Time (min)	0–13.6
Mass resolution	60,000 (FWHM @ *m/z* 200, scan speed 7 Hz):
Lock masses (*m/z*)	73.0468, 133.01356, 207.03235, 225.04292, 281.05114, 299.06171, 355.06993
t-SIM masses (*m/z*)	74, 44, 42, 82
RT (min)	4.5
RT window (min)	1.0
Mass resolution	30,000 (FWHM @ *m/z* 200)
Number of MXP	5
Scan width (*m/z*)	4
t-SIM masses (*m/z*)	NDMA (74, 44, 42) ^{13}C-NMDA-d_6 (82)

Nitrosamines (RTs, *m/z*)

Mass (*m/z*)	Polarity	Start (min)	End (min)	Comment
74.04746	Positive	4.447	4.577	N= N-Nitroso dimethylamine (NDMA) FDA;F=$C_2H_6N_2O$;FDA
42.03375	Positive	4.447	4.577	N= N-Nitroso dimethylamine (NDMA) FDA;F=$C_2H_6N_2O$;FDA
44.0494	Positive	4.447	4.577	N= N-Nitroso dimethylamine (NDMA) FDA;F=$C_2H_6N_2O$;FDA
82.09183	Positive	4.460	4.580	N= ^{13}C-NDMA-D_6;F=[13]$C_2D_6N_2O$
71.06035	Positive	5.103	5.192	N= N-Nitrosomethylethylamine (NMEA);F=$C_3H_8N_2O$
88.06311	Positive	5.103	5.192	N= N-Nitrosomethylethylamine (NMEA);F=$C_3H_8N_2O$
42.03374	Positive	5.103	5.192	N= N-Nitrosomethylethylamine (NMEA);F=$C_3H_8N_2O$
102.07876	Positive	5.605	5.675	N= N-Nitroso diethylamine (NDEA) FDA;F=$C_4H_{10}N_2O$;FDA
85.07603	Positive	5.605	5.675	N= N-Nitroso diethylamine (NDEA) FDA;F=$C_4H_{10}N_2O$;FDA
42.03375	Positive	5.605	5.675	N= N-Nitroso diethylamine (NDEA) FDA;F=$C_4H_{10}N_2O$;FDA
99.09167	Positive	6.045	6.115	N= N-Nitroso isopropyl ethyl amine (NIPEA) FDA;F=$C_5H_{12}N_2O$;FDA
116.09441	Positive	6.045	6.115	N= N-Nitroso isopropyl ethyl amine (NIPEA) FDA;F=$C_5H_{12}N_2O$;FDA
70.06513	Positive	6.045	6.115	N= N-Nitroso isopropyl ethyl amine (NIPEA) FDA;F=$C_5H_{12}N_2O$;FDA
70.06511	Positive	6.429	6.499	N= N-Nitroso diisopropylamine (NDIPA) FDA;F=$C_6H_{14}N_2O$;FDA
113.10731	Positive	6.429	6.499	N= N-Nitroso diisopropylamine (NDIPA) FDA;F=$C_6H_{14}N_2O$;FDA
88.06311	Positive	6.429	6.499	N= N-Nitroso diisopropylamine (NDIPA) FDA;F=$C_6H_{14}N_2O$;FDA
106.06513	Positive	6.647	6.717	N= N-Nitroso N-methyl N-phenylamine (NMPA) FDA;F=$C_7H_8N_2O$;FDA
79.05422	Positive	6.647	6.717	N= N-Nitroso N-methyl N-phenylamine (NMPA) FDA;F=$C_7H_8N_2O$;FDA
77.03858	Positive	6.647	6.717	N= N-Nitroso N-methyl N-phenylamine (NMPA) FDA;F=$C_7H_8N_2O$;FDA
144.19794	Positive	6.782	6.852	N=13 NDPA D14;F=$C_{14}H_{14}N_2O$
70.06511	Positive	6.835	6.905	N= N-Nitroso Di-n-propyl amine (NDPA);F=$C_6H_{14}N_2O$
130.11006	Positive	6.835	6.905	N= N-Nitroso Di-n-propyl amine (NDPA);F=$C_6H_{14}N_2O$
88.06311	Positive	6.835	6.905	N= N-Nitroso Di-n-propyl amine (NDPA);F=$C_6H_{14}N_2O$
88.06311	Positive	6.939	7.009	N= N-Nitroso ethyl butyl amine (NEBA);F=$C_6H_{14}N_2O$
130.11006	Positive	6.939	7.009	N= N-Nitroso ethyl butyl amine (NEBA);F=$C_6H_{14}N_2O$
70.06513	Positive	6.939	7.009	N= N-Nitroso ethyl butyl amine (NEBA);F=$C_6H_{14}N_2O$
106.06513	Positive	7.027	7.097	N= N-Nitroso N-ethyl N-phenylamine (NEPhA);F=$C_8H_{10}N_2O$
77.03858	Positive	7.027	7.097	N= N-Nitroso N-ethyl N-phenylamine (NEPhA);F=$C_8H_{10}N_2O$
86.06005	Positive	7.1	7.17	N= 4-Nitrosomorpholine (NMOR);F=$C_4H_8N_2O_2$
116.05803	Positive	7.1	7.17	N= 4-Nitrosomorpholine (NMOR);F=$C_4H_8N_2O_2$
56.04941	Positive	7.1	7.17	N= 4-Nitrosomorpholine (NMOR);F=$C_4H_8N_2O_2$
108.11333	Positive	7.226	7.296	N= 13 NPYR D_8;F=$C_5H_{10}N_2O$
70.0651	Positive	7.252	7.322	N= N-Nitroso tertary butylethyl amine (NDBEA);F=$C_6H_{14}N_2O$
100.11208	Positive	7.252	7.322	N= N-Nitroso tertary butylethyl amine (NDBEA);F=$C_6H_{14}N_2O$
41.0385	Positive	7.252	7.322	N= N-Nitroso tertary butylethyl amine (NDBEA);F=$C_6H_{14}N_2O$
100.06311	Positive	7.257	7.327	N= N-Nitroso pyrrolidine (NPYR);F=$C_4H_8N_2O$
68.04947	Positive	7.257	7.327	N= N-Nitroso pyrrolidine (NPYR);F=$C_4H_8N_2O$
41.0385	Positive	7.257	7.327	N= N-Nitroso pyrrolidine (NPYR);F=$C_4H_8N_2O$
114.07876	Positive	7.404	7.474	N= N-Nitroso piperidine (NPIP);F=$C_5H_{10}N_2O$
97.07602	Positive	7.404	7.474	N= N-Nitroso piperidine (NPIP);F=$C_5H_{10}N_2O$
42.03375	Positive	7.404	7.474	N= N-Nitroso piperidine (NPIP);F=$C_5H_{10}N_2O$
84.08072	Positive	8.153	8.223	N= N-Nitroso di-N-butylamine (NDBA) FDA;F=$C_8H_{18}N_2O$;FDA
158.14136	Positive	8.153	8.223	N= N-Nitroso di-N-butylamine (NDBA) FDA;F=$C_8H_{18}N_2O$;FDA
99.0916	Positive	8.153	8.223	N= N-Nitroso di-N-butylamine (NDBA) FDA;F=$C_8H_{18}N_2O$;FDA
168.0808	Positive	10.523	10.593	N= N-Nitroso-diphenylamine (NDPhA);F=$C_{12}H_{10}N_2O$
168.08078	Positive	10.523	10.593	N= N-Nitroso-diphenylamine (NDPhA);F=$C_{12}H_{10}N_2O$
167.07303	Positive	10.523	10.593	N= N-Nitroso-diphenylamine (NDPhA);F=$C_{12}H_{10}N_2O$

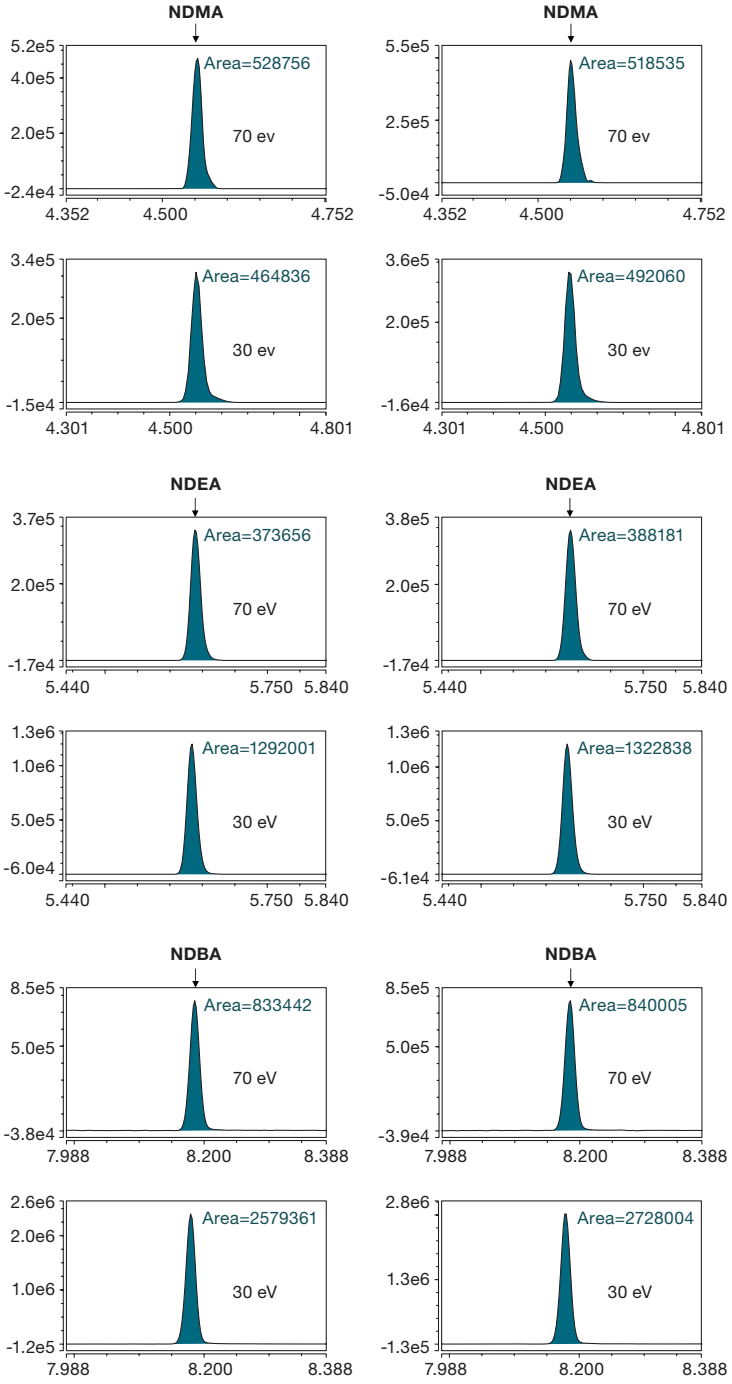
Appendix 5. Consumables

Consumable	Part of workflow	Part number
1.4 mL screw cap amber high recovery vials	Sample preparation: Drug extraction step	C4000-V2
9 mm magnetic caps with soft septa	Sample preparation: Drug extraction step + LC extracts vials	C5000-46M
9 mm screw cap amber fixed insert high recovery vials	Sample preparation: GC extract vials	C4000-LV2W
9 mm screw caps	Sample preparation: GC extract vials	9-SCK(B)-ST1
10 µL syringe for TriPlus RSH d7 tool, 57 mm needle	GC: Injection	365D0291
TraceGOLD TG-1701MS 30 m × 0.25 mm i.d. × 0.50 µm film column	GC: Column	26090-2230
Single gooseneck with glass wool LinerGOLD	GC: Liner	P/N: 453A1925-UI
BTO septa 11 mm	GC: Septa	31303233-BP
Gold inlet base seal	GC: Gold seal	290GA082
Spring loaded transfer line nut	MS: Transfer line nut	1R120434-0010

Appendix 6. LOQs were calculated using serially diluted solvent standards at 0.1, 0.2 0.3, 0.5, 0.6, 1.0, 3.0, 5.0 ng/mL. Thirteen (n=13) replicate injections of each of the diluted standards ranging between 0.1 and 5.0 ng/mL were performed (0.3–15 ng/g in sample). The criteria used to assess individual LOQs were (i) peak area repeatability of <15% RSD and (ii) ppm mass error <5 ppm.

Compound	Injected amount (pg OC)	Peak area % RSD	LOQ (pg OC)	LOQ metformin (ng/g)
NDMA (FDA)	0.4	8.0	0.4	0.6
NMEA	0.2	12.8	0.2	0.3
NDEA (FDA)	0.4	7.8	0.4	0.6
NIPEA (FDA)	0.2	7.9	0.2	0.3
NDIPA (FDA)	0.2	11.2	0.2	0.3
NMPA (FDA)	0.6	7.5	0.6	0.9
NDPA	1.2	6.4	1.2	1.8
NEBA	0.2	11.1	0.2	0.3
NEPhA	0.2	13.8	0.2	0.3
NMOR	0.6	4.6	0.6	0.9
NDBEA	1.0	14.5	1.0	1.5
NPYR	0.4	8.8	0.4	0.6
NPIP	0.4	8.7	0.4	0.6
NDBA (FDA)	0.4	10.2	0.4	0.6
NDPhA	0.6	10.7	0.6	0.9

Appendix 7. Peak area boost when comparing 70 eV and 30 eV for selected FDA mid early and late eluting nitrosamines (NDMA, NDEA, and NDBA) for duplicate injections of a 5 ng/mL solvent standard in DCM



Appendix 8. Calibration range 0.2–2,000 pg/µL, coefficient of determination (R^2) and residual average response factor (AVCF % RSD)

Compound	R^2	AVCF % RSD
NDMA (FDA)	0.9997	2.7
NMEA	0.9999	1.9
NDEA (FDA)	0.9996	5.8
NIPEA (FDA)	0.9999	2.5
NDIPA (FDA)	0.9998	3.2
NMPA (FDA)	0.9994	5.9
NDPA	0.9999	4.6
NEBA	0.9998	2.1
NEPhA	0.9997	3.2
NMOR	0.9999	2.6
NDBEA	0.9993	2.5
NPYR	0.9998	2.6
NPIP	0.9993	1.9
NDBA (FDA)	0.9997	8.5
NDPhA	0.9997	2.4
MIN	**0.9993**	**1.9**
MAX	**0.9999**	**8.5**
MEAN	**0.9997**	**3.5**

Appendix 9. Method LOQs were calculated using serially diluted matrix-matched standards at 1.0, 2.0, 2.5, 5.0, 10, and 20 ng/mL. Twelve (n=12) replicate injections of each of the diluted standards ranging between 1.0 and 20 ng/mL were performed (3–60 ng/g in sample). The criteria used to assess individual LOQs were peak area repeatability of <15% RSD and ppm mass error <5.

Compound	Injected amount (pg OC)	Peak area % RSD	LOQ (pg OC)	LOQ metformin (ng/g)
NDMA (FDA)	0.8	4.4	0.8	1.2
NMEA	0.5	7.0	0.5	0.8
NDEA (FDA)	0.8	5.0	0.8	1.2
NIPEA (FDA)	0.4	7.8	0.4	0.6
NDIPA (FDA)	0.4	5.0	0.4	0.6
NMPA (FDA)	0.4	7.4	0.4	0.6
NDPA	0.8	14.5	0.8	1.2
NEBA	0.4	9.0	0.4	0.6
NEPhA	2.0	10.1	2.0	3.0
NMOR	1.0	12.4	1.0	1.5
NDBEA	1.0	13.9	1.0	1.5
NPYR	0.8	5.7	0.8	1.2
NPIP	0.8	6.3	0.8	1.2
NDBA (FDA)	0.8	12.7	0.8	1.2
NDPhA	0.4	11.8	0.4	0.6

Appendix 10. Table showing the average detected nitrosamines in three procedural blanks and six unspiked metformin extracts. None were detected so a <LOQ was reported.

	Procedural blank	Unspiked metformin
Compound	Mean amount detected ng/g	Mean amount detected ng/g
NDMA (FDA)	<0.8	<0.8
NMEA	<0.5	<0.5
NDEA (FDA)	<0.8	<0.8
NIPEA (FDA)	<0.4	<0.4
NDIPA (FDA)	<0.4	<0.4
NMPA (FDA)	<0.4	<0.4
NDPA	<0.8	<0.8
NEBA	<0.4	<0.4
NEPhA	<2.0	<2.0
NMOR	<1.0	<1.0
NDBEA	<1.0	<1.0
NPYR	<0.8	<0.8
NPIP	<0.8	<0.8
NDBA (FDA)	<0.8	<0.8
NDPhA	<0.4	<0.4

Appendix 11. Accuracy table showing the spiked concentration, mean % recovery, and recovery % RSD for three replicate extractions of metformin drug substance, triplicate injections per extract

Compound	Spiked concentration (ng/g)	Mean recovery %	Recovery % RSD
NDMA (FDA)	2.0	104	7.8
NMEA	1.0	98	9.4
NDEA (FDA)	2.0	105	4.6
NIPEA (FDA)	1.0	103	7.4
NDIPA (FDA)	1.0	88	10.6
NMPA (FDA)	2.5	91	11.1
NDPA	2.0	111	7.8
NEBA	2.0	86	5.2
NEPhA	10.0	77	12
NMOR	2.5	83	13.2
NDBEA	10.0	98	10.9
NPYR	1.0	86	4.1
NPIP	2.0	105	3.7
NDBA (FDA)	2.0	105	8.5
NDPhA	2.5	99	8.2

Appendix 12. Precision table showing the spiked concentration, mean amount detected, amount % RSD, and STDEV for three replicate extractions of metformin drug substance, triplicate injections per extract

Compound	Spiked concentration (ng/g)	Mean amount detected (ng/g)	Amount % RSD	STDEV
NDMA (FDA)	2.0	2.1	7.8	0.1620
NMEA	1.0	2.0	9.4	0.0920
NDEA (FDA)	2.0	2.1	4.6	0.0960
NIPEA (FDA)	1.0	1.0	7.4	0.0770
NDIPA (FDA)	1.0	0.9	10.6	0.1768
NMPA (FDA)	2.5	2.3	11.1	0.2534
NDPA	2.0	2.2	7.8	0.1730
NEBA	2.0	1.7	5.2	0.0851
NEPhA	10.0	7.7	12	0.9300
NMOR	2.5	2.1	13.2	0.2602
NDBEA	10.0	9.8	10.9	1.0704
NPYR	1.0	0.9	4.1	0.1175
NPIP	2.0	2.1	3.7	0.0780
NDBA (FDA)	2.0	2.1	8.5	0.1780
NDPhA	2.5	2.5	8.2	0.20