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SPONSOR REPORT

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Rapid quantification of 12 nitrosamines in metformin using triple quadrupole GC-MS/MS with Advanced Electron Ionization (AEI)

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Keywords: Nitrosamines, pharmaceutical, N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), GC-MS/MS, TSQ 9610 mass spectrometer

Goal: The aim of this application note is to demonstrate the suitability of the Thermo Scientific™ TSQ™ 9000 triple quadrupole mass spectrometer coupled to the Thermo Scientific™ TRACE™ 1310 gas chromatograph for the analysis of 12 nitrosamines in drug substance. The linearity of the new Thermo Scientific™ TSQ™ 9610 triple quadrupole mass spectrometer NeverVent AEI equipped with the Thermo Scientific™ XLXR™ detector was also tested for nitrosamines.

INTRODUCTION

Nitrosamines have become compounds of concern in pharmaceutical products after N-nitrosodimethylamine (NDMA) was first reported in valsartan in June 2018.[1] Subsequently, additional nitrosamines have been detected in various sartans as well as ranitidine containing products. Nitrosamines detected include N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), and N-nitroso-N-methyl-4-aminobutyric acid (NMBA).

The presence of nitrosamines in these products is concerning because they are classified as Class 1 impurities or mutagenic carcinogens by the ICH M7 (R1) guidelines[2] and as probable carcinogens by the International Agency for Cancer Research (IARC).[3]

By using triple quadrupole mass spectrometry, additional selectivity and lower detection limits can be achieved compared to single quadrupole systems. This additional selectivity can help to prevent false positives by utilizing the transition between pre-cursor and product ion as well as the ion ratios between different transitions and can improve method detection limits by decreasing the background noise, therefore increasing signal-to-noise ratios.

In this study, a method for the simultaneous analysis of 12 different nitrosamines was developed and tested for linearity, sensitivity, precision, and accuracy. An automated liquid-liquid extraction workflow with GC injection was implemented through the use of a robotic autosampler. This allows extraction volumes to be scaled down and extends unattended operation.

EXPERIMENTAL

Instrument and method setup

For this analysis, a TRACE 1310 GC was coupled to a TSQ 9000 MS and a Thermo Scientific™ TriPlus™ RSH autosampler. The TriPlus RSH autosampler allows for unattended switching between liquid injection and headspace analysis as well as providing the possibility of performing automated sample preparations when configured with dedicated sample handling tools such as the vortex mixer and the centrifuge. For the data shown in this application note, the liquid injection technique was used. Full instrument conditions are shown in Appendix 1. A second experiment was performed with the TSQ 9610 NeverVent AEI and the XLXR detector to assess the extended dynamic range using the same instrument conditions.

Standards and samples preparation

Calibration standards containing 12 nitrosamines and two labeled internal standards were acquired from Fisher Scientific, Sigma-Aldrich, and LGC. These are listed in Table 1 along with the CAS numbers and acronyms used.

For the calculation of method detection limits (MDLs) and method LOQs, serially diluted matrix-matched standards spiked at 0.25, 0.5, 1.0 and 2.0 ng/g were used. For the sample preparation, 100 mg of metformin was weighed into amber high recovery vials (P/N C4000-LV2W) and the extraction proceeded as follows in Figure 1. The total sample preparation time was 6 minutes.

Table 1. List of nitrosamine compounds analyzed, acronyms, and CAS numbers

Analyte name	Acronym	CAS number
N-Nitrosodimethylamine	NDMA	62-75-9
N-Nitrosomethylethylamine	NMEA	10595-95-6
N-Nitrosodiethylamine	NDEA	55-18-5
N-Nitrosodiisopropylamine	NDIPA	601-77-4
N-Nitroso-N-methyl-N-phenylamine	NMPA	614-00-6
N-Nitroso-di-n-propylamine	NDPA	621-64-7
N-Nitroso-N-ethyl-N-phenylamine	NEPhA	612-64-6
4-Nitrosomorpholine	NMOR	59-89-2
N-Nitrosopyrrolidine	NPYR	930-55-2
N-Nitrosopiperidine	NPIP	100-75-4
N-Nitroso-di-N-butylamine	NDBA	924-16-3
N-Nitroso-diphenylamine	NDPhA	86-30-6
N-Nitrosodimethylamine ($^{13}C_2$;D_6)	NDMA ($^{13}C_2D_6$)	2483824-56-0
N-Nitrosodipropylamine-D_{14}	NDPA (D_{14})	93951-96-3

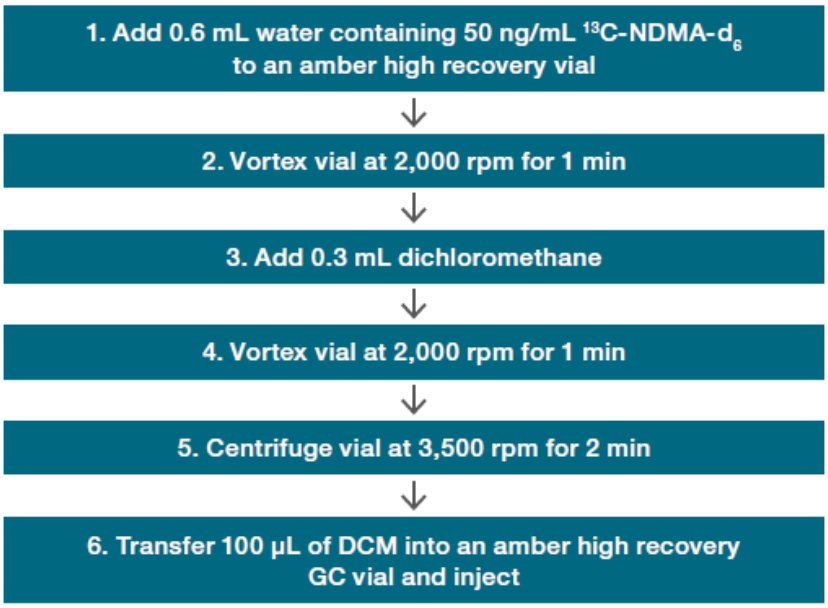


Figure 1. Workflow showing the sample extraction preparation procedure.

Data acquisition, processing, and reporting

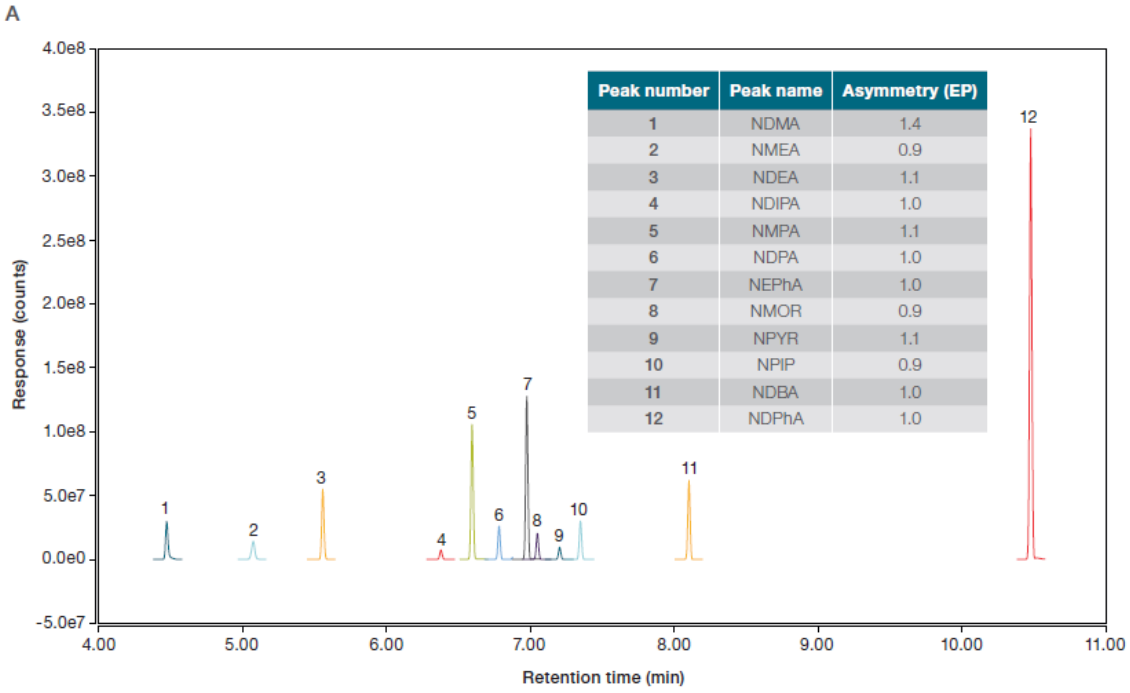
Data were acquired, processed, and reported using Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS). With the ever-evolving emphasis on data integrity, data security, and compliance, it is of vital importance that the CDS provides comprehensive preventative and detection technical controls 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 separation and linearity were assessed using solvent standards. Sensitivity, as method detection limit, precision, and accuracy were evaluated using extracted, spiked samples following the procedure described in the experimental section.

Chromatography

Using the Thermo Scientific™ TraceGOLD™ TG-1701 MS column, all compounds were separated in under 11 minutes with peak asymmetry values <1.5 obtained for all compounds, meeting the requirements of the European and US Pharmacopoeia, Figures 2A and 2B.



Peak number	Peak name	Asymmetry (EP)
1	NDMA	1.4
2	NMEA	0.9
3	NDEA	1.1
4	NDIPA	1.0
5	NMPA	1.1
6	NDPA	1.0
7	NEPhA	1.0
8	NMOR	0.9
9	NPYR	1.1
10	NPIP	0.9
11	NDBA	1.0
12	NDPhA	1.0

Figure 2A. Extracted ion chromatograms (EIC) of the quantitation ions for the 12 nitrosamine analytes in a 100 ng/mL standard with an inset table showing the peak asymmetry factors.

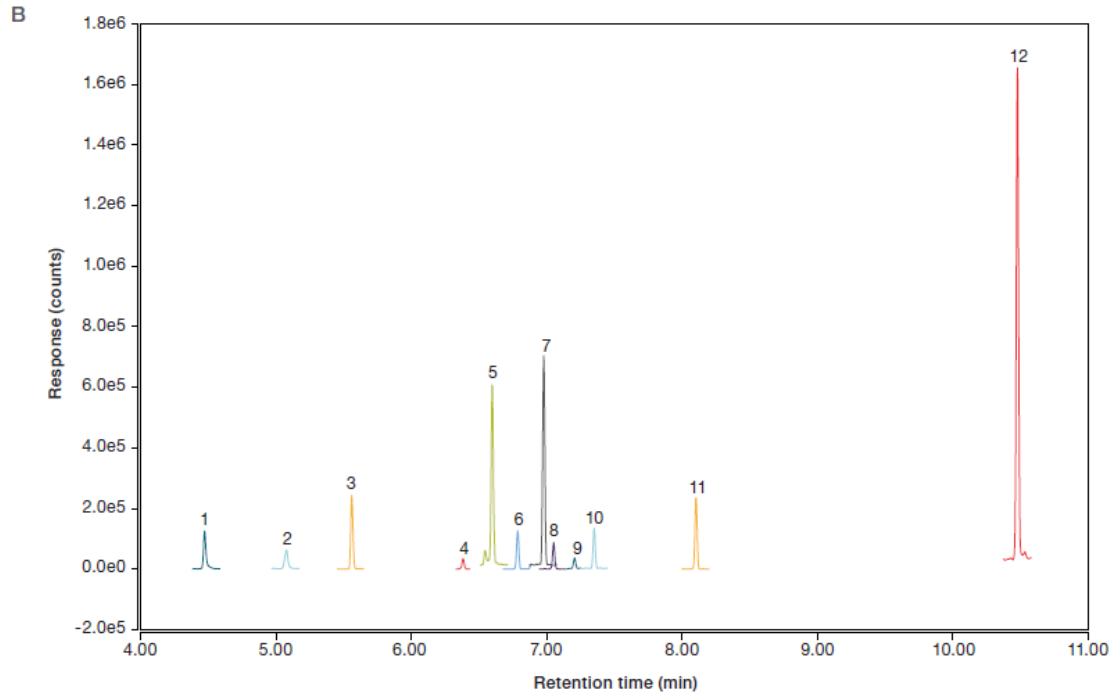


Figure 2B. Extracted ion chromatograms (EIC) of the quantitation ions for the 12 nitrosamine analytes in a metformin sample spiked with nitrosamines at 2.5 ng/g or 5 ng/g.

Linearity

Linearity was assessed using triplicate solvents standards and the linear range was determined. Examples of the calibration curves generated are shown in Figure 3, with a zoomed in area for NDMA at 0.05 ng/mL to 2 ng/mL level shown in Figure 4.

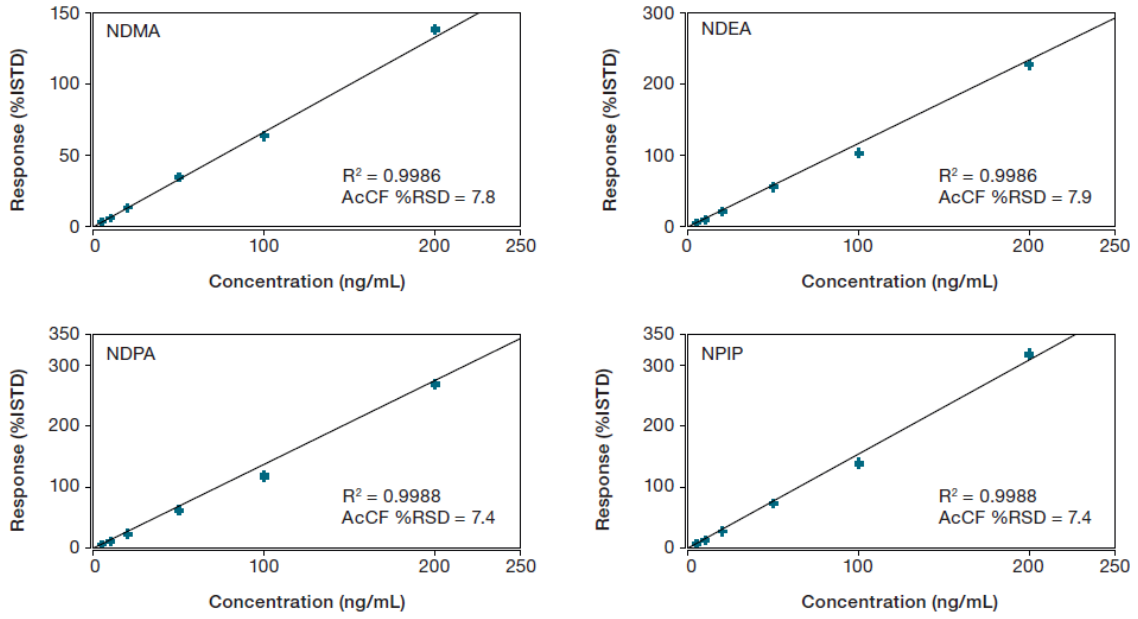


Figure 3. Example of linearity for four nitrosamine compounds (NDMA, NDEA, NDPA and NPIP), annotated with R^2 and AvCF %RSD (residual) values.

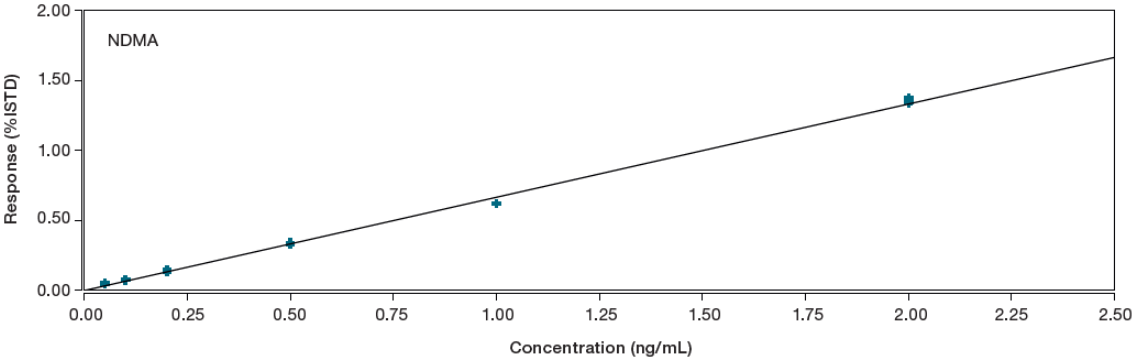


Figure 4. Zoomed in calibration plot for NDMA from 0.05 ng/mL to 2 ng/mL.

Within the stated range, all compounds showed excellent linear responses with coefficient of determination (R^2) values >0.995 and average calibration factor (AvCF) %RSDs <14. The results for all 12 nitrosamines are shown in Table 2.

Table 2. Table showing the calibration range, retention time AvCF %RSD, and R^2 values for the 12 nitrosamine analytes

Peak name	Range (ng/mL)	Ret. time (min)	AvCF %RSD	R^2
NDMA	0.05–2,000	4.47	7.8	0.9986
NMEA	0.025–1,000	5.07	9.7	0.9979
NDEA	0.05–2,000	5.56	7.9	0.9986
NDIPA	0.05–1,000	6.38	9.1	0.9981
NMPA	0.025–1,000	6.59	13.6	0.9955

Table 2. Table showing the calibration range, retention time AvCF %RSD, and R^2 values for the 12 nitrosamine analytes (continued)

Peak name	Range (ng/mL)	Ret. time (min)	AvCF %RSD	R^2
NDPA	0.05–2,000	6.78	7.4	0.9988
NEPhA	0.025–1,000	6.98	10.5	0.9974
NMOR	0.025–1,000	7.05	8.9	0.9983
NPYR	0.1–2,000	7.20	7.2	0.9987
NPIP	0.05–2,000	7.35	7.4	0.9988
NDBA	0.05–2,000	8.10	8.3	0.9985
NDPhA	0.05–250	10.47	4.2	0.9996

Sensitivity

To assess the MDLs, n=12 replicate injections of the lowest spiked standard with a peak area %RSD of <15 were used. The MDL for each compound 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. The results are summarized in Figure 5 with MDLs values ranging from 35 to 184 fg on column o.c. with a mean of 94 fg o.c. (which corresponds to 0.05 to 0.28 ng/g, with a mean result of 0.14 ng/g). These results significantly exceed the FDA regulatory limit of 30 ng/g.

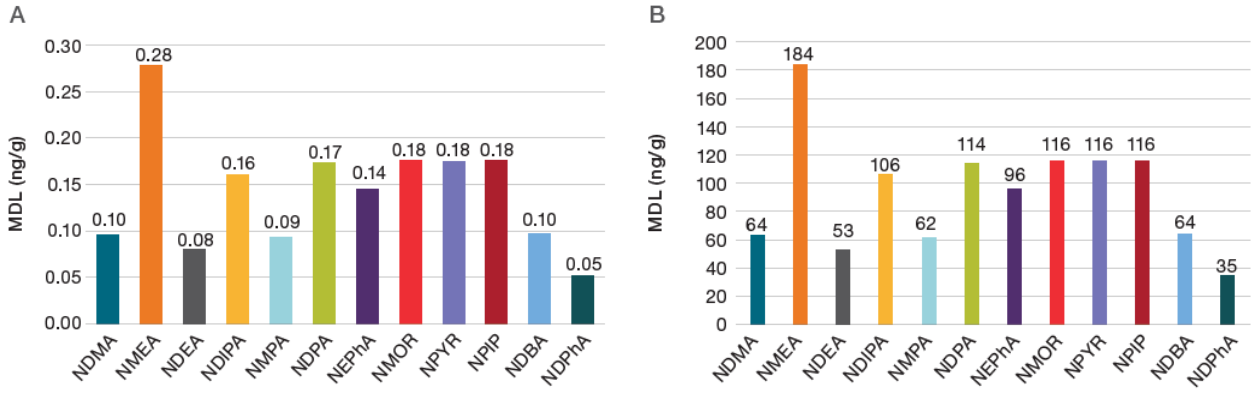


Figure 5. Charts showing the calculated MDL for the 12 nitrosamine analytes in ng/g (A) and fg oc (B).

Figure 6 shows examples of the extracted ion chromatograms (EIC)s for the three of the nitrosamines spiked at the level used to determine the MDL. Ion ratios obtained for qualifier transitions were within 80 to 120% of the expected ratio (as the mean ion ratio for the qualifier transitions across the calibration range).

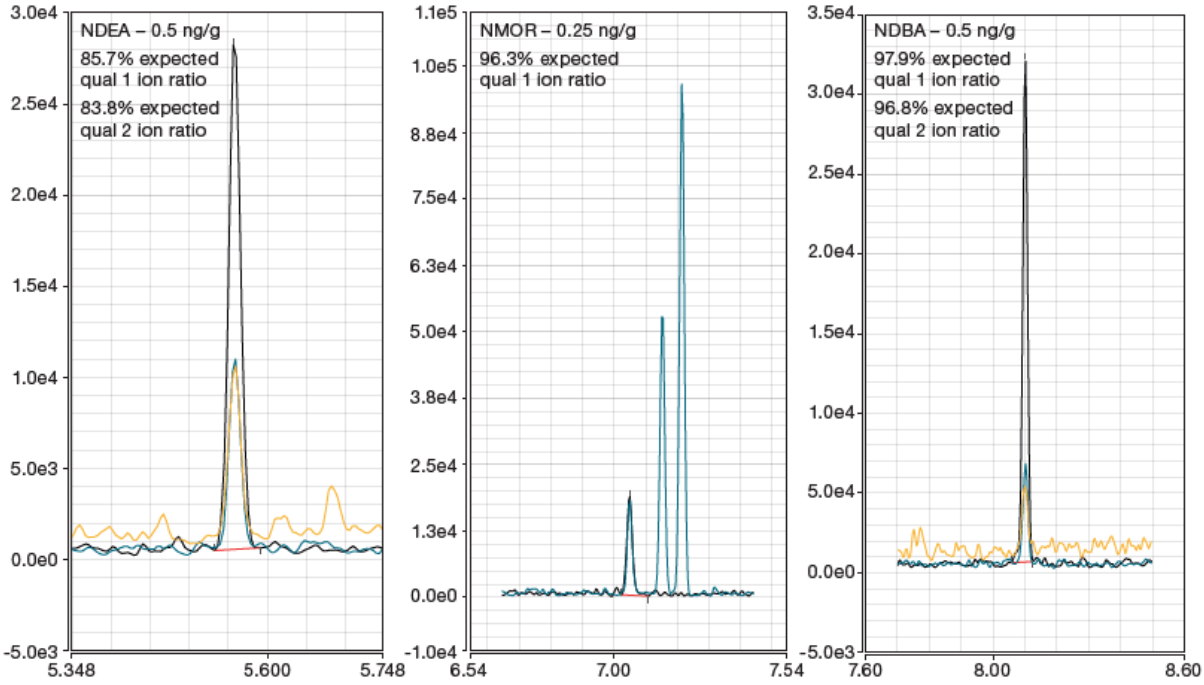


Figure 6. EICs for NDEA, NMOR, and NDBA for metformin sample spiked at MQL, annotated with % of expected ion ratio for the qualifier ions.

Precision and accuracy

For the determination of precision and accuracy, three extractions were prepared at 2.5 ng/g or 5 ng/g, depending on analyte, by spiking 100 mg of metformin prior to extraction and then following the extraction procedure in Figure 1. ICH Guidelines Q2 (R1) performance criteria were met, with mean recovery of 70–130% and RSD of <20% for all analytes, as shown in Figure 7.

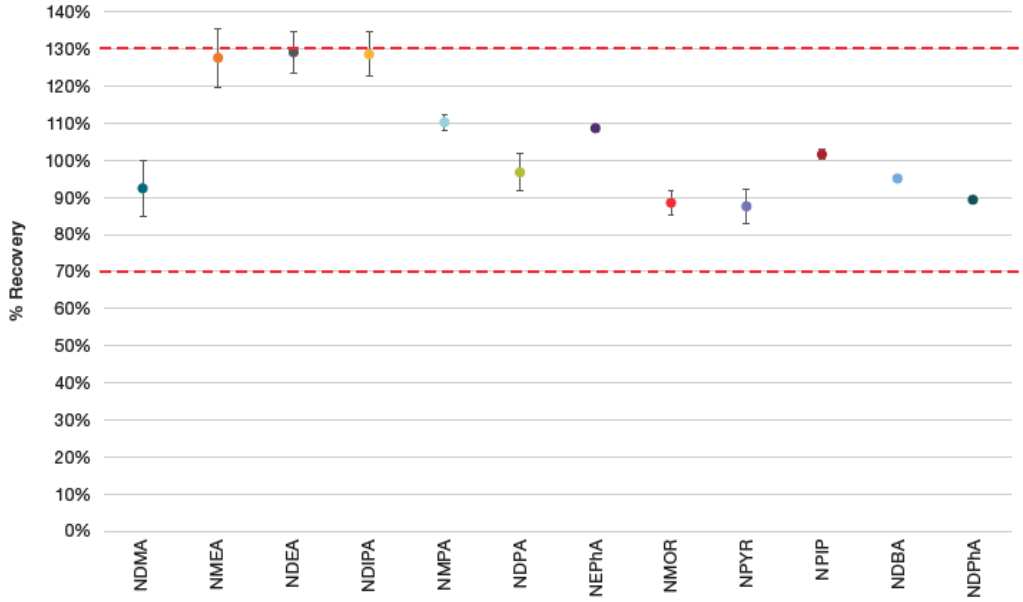


Figure 7. Mean accuracy (as % recovery) for triplicate analysis of metformin samples spiked at the tested level (NDMA, NDEA, NDPA, NPYR, NPIP, and NDBA at 5.0 ng/g and NMEA, NDIPA, NMPA, NEPhA, NMOR, and NDPhA at 2.5 ng/g), with standard error bars and a dashed line showing the allowed limits included.

Extended linearity using the TSQ 9610 NeverVent AEI

The TSQ 9610 NeverVent AEI offers the advantage of a removable AEI ion source, which allows for source maintenance, filament removal, and GC column changes without breaking instrument vacuum, thereby increasing lab productivity. The TSQ 9610 NeverVent AEI is equipped with the XLXR detector, which is an electron multiplier detector that offers extended dynamic range and detector lifetime.

To test the performance of the XLXR detector, the calibration range was extended by doubling the concentration of the highest level. Each concentration level was prepared in solvent standard (dichloromethane) and analyzed in triplicate (n=3). The calculated coefficients of determination (R^2) and the residual values (measured as %RSD of average response factors -AvCF %RSD) are reported in Table 3 with average values of 0.9991 and 6.5%, respectively, thus confirming a wider linear range can be easily achieved with the new electron multiplier. The full range (0.05–4,000 ng/mL) and the zoomed in calibration plots (B: 0.05–80 ng/mL and C: 0.05–2 ng/mL) for NDMA and NDPA are reported as an example in Figure 8.

The IDLs can be evaluated based on the precision of a measurement at low analyte levels in solvent based standards.[4] The IDL of target nitrosamines was assessed from n=12 consecutive injections of solvent standard spiked at 0.01 and 0.025 ng/mL and calculated by multiplying the Student's *t*-value for one-tailed distribution at 99% confidence (for n=12, *t*=2.718), the amount of analyte on-column (o.c.) and the relative standard deviation of the response. The calculated IDL ranged from 11 to 56 fg o.c. with average value of 27 fg o.c. as reported in Table 3.

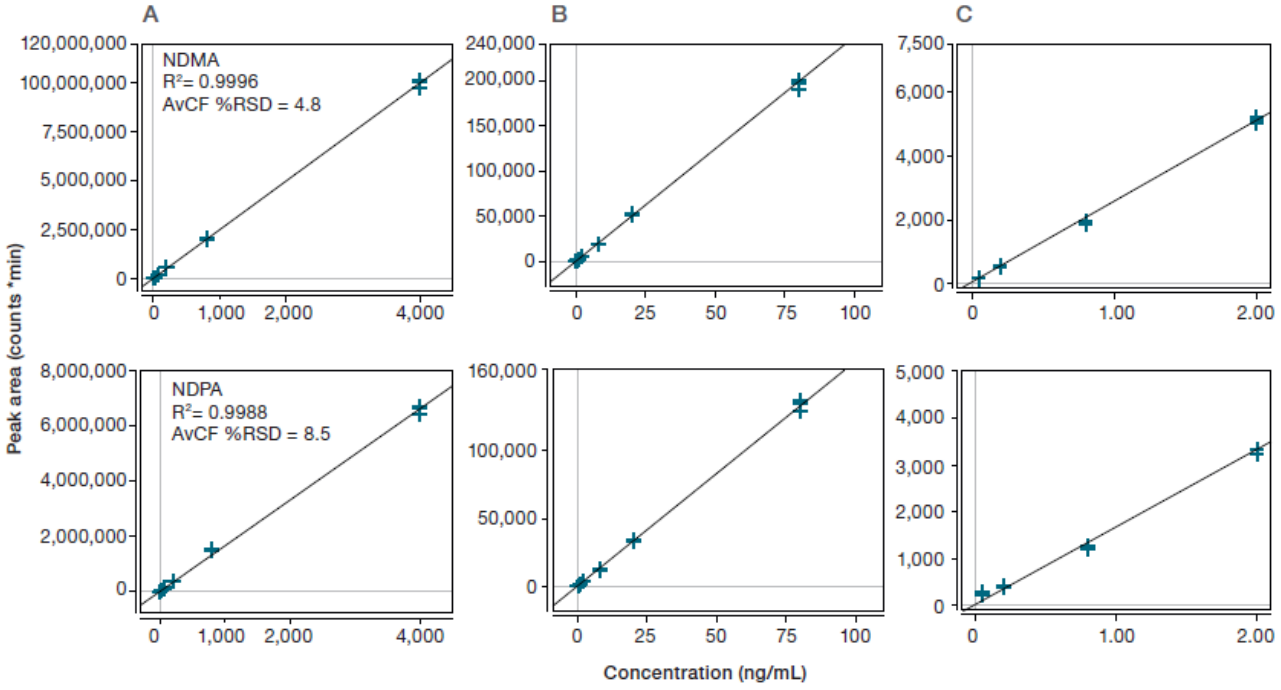


Figure 8. Examples of full range (0.05–4,000 ng/mL) and zoomed in calibration plots (B: 0.05–80 ng/mL and C: 0.05–2 ng/mL) for NDMA and NDPA obtained by injecting ten concentration levels. The R^2 as well as response factors relative standard deviations (AvCF %RSD) are annotated. Each calibration level was prepared and analyzed in triplicate.

Table 3. Calibration ranges, calculated coefficients of determination (R^2), average calibration factor (AvCF) %RSDs, and instrument detection limits (IDLs)

Peak name	Range (ng/mL)	Ret. time (min)	AvCF %RSD	R^2	Calculated IDL (fg OC)
NDMA	0.05–4,000	4.65	4.6	0.9996	40
NMEA	0.05–2,000	5.28	3.7	0.9997	13
NDEA	0.05–4,000	5.78	3.5	0.9998	17
NEIPA	0.025–2,000	6.24	7.2	0.9990	32
NDIPA	0.025–2,000	6.64	11.3	0.9976	56
NMPA	0.025–2,000	6.86	4.6	0.9996	17
NDPA	0.05–4,000	7.05	8.1	0.9988	53
NPYR	0.1–4,000	7.48	9.1	0.9985	15
NPIP	0.05–4,000	7.64	5.7	0.9995	16
NDBA	0.05–4,000	8.41	7.2	0.9991	11

CONCLUSIONS

The results of these experiments clearly demonstrate the suitability of the Thermo Scientific TRACE 1310 GC and Thermo Scientific TSQ 9000 and TSQ 9610 mass spectrometers with the Thermo Scientific TraceGOLD TG-1701MS column for the fast separation and analysis of nitrosamines in drug substance as demonstrated by:

- Rapid separation of 12 nitrosamine compounds in <11 minutes.
- Sub-ppb levels of sensitivity with MDLs ranging from 35 to 184 fg oc (corresponding to 0.05 to 0.18 ng/g) with MQLs ranging from 0.25–2 ng/g, comfortably surpassing the FDA regulatory limit of 30 ng/g.
- Precise and confident quantification as demonstrated through accuracy and precision of 70–130% recovery and amount %RSD <20 for triplicate extracted standards at 2.5 ng/g or 5 ng/g.
- Moreover, the XLXR electron multiplier detector of the TSQ 9610 triple quadrupole mass spectrometer allows for extended nitrosamine linearity of up to 4,000 ng/mL, between an on-column range of 100 ppb to 8 ppm. This linear range of over 5.5 orders of magnitude ensures accurate quantitation of these impurities at low and high levels.
- Chromeleon software tools allow for rapid implementation of this method into any lab. The eWorkflows can be downloaded and contain everything needed to run the analysis. SmartTune ensures that the instrument produces optimal results for every user. Chromeleon can be operated for CFR GMP compliance to meet regulatory needs.

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4. Thermo Fisher Scientific, Technical Note 10499: Practical Determination and Validation of Instrument Detection Limit of Thermo Scientific™ ISQ™ LT Single Quadrupole GC-MS. https://tools.thermofisher.com/content/sfs/brochures/TN-10494-GC-MS-ISQ-LT-Single-Quadrupole-TN10494-EN.pdf

Appendix 1 – Instrument parameters

TRACE 1310 GC parameters	
Injection volume	2 µL
Liner	Single gooseneck with glass wool Thermo Scientific™ LinerGOLD™ (P/N 453A1925-UI)
Inlet temperature	240 ˚C
Inlet module and mode	SSL, splitless with surge
Splitless time (min)	1
Split flow (mL/min)	80
Surge pressure (psi)	25
Carrier gas, flow rate (mL/min)	He, 1.3
Oven profile	40 ˚C (1 min hold), 25 ˚C/min to 130 ˚C, 20 ˚C/min to 270 ˚C (2 min hold)
Column	TraceGOLD TG-1701MS 30 m × 0.25 mm i.d. × 0.50 µm film (P/N 26090-2230)

TSQ 9000 MS parameters	
Transfer line temperature	270 ˚C
Ionization source	Advanced Electron Ionization (AEI)
Ion source temperature	340 ˚C
Electron energy	50 eV
Emission current	50 µA
Acquisition mode	Timed-SRM

Appendix 2

Name	RT (min)	Window (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Dwell time priority
NDMA-$^{13}CD_6$	4.44	1	82.1	32.1	15	Normal
NDMA-13CD_6	4.44	1	82.1	48.1	15	Normal
NDMA-13CD_6	4.44	1	82.1	52.2	5	Normal
NDMA	4.46	1	74.1	42.1	15	High

Appendix 2 (continued)

Name	RT (min)	Window (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Dwell time priority
NDMA	4.46	1	74.1	43.1	10	High
NDMA	4.46	1	74.1	44.1	5	High
NMEA	5.06	1	88.1	42.1	15	High
NMEA	5.06	1	88.1	43.1	5	High
NMEA	5.06	1	88.1	71.1	5	High
NDEA	5.55	1	102.1	44.1	10	High
NDEA	5.55	1	102.1	56.1	15	High
NDEA	5.55	1	102.1	85.1	5	High
N-Nitroso diisopropylamine (NDIPA)	6.38	1	130.1	41.1	10	High
N-Nitroso diisopropylamine (NDIPA)	6.38	1	130.1	42.1	10	High
N-Nitroso N-methyl N-phenylamine (NMPA)	6.61	1	77	50.1	30	High
N-Nitroso N-methyl N-phenylamine (NMPA)	6.61	1	77	51.1	10	High
N-Nitroso N-methyl N-phenylamine (NMPA)	6.61	1	106	51.1	30	High
N-Nitroso N-methyl N-phenylamine (NMPA)	6.61	1	106	77.1	10	High
NDPA-D14	6.73	1	78.1	46.1	10	Normal
NDPA-D14	6.73	1	110.1	78.2	5	Normal
NDPA-D14	6.73	1	144.2	126.3	5	Normal
NDPA	6.78	1	70.1	41.1	10	High
NDPA	6.78	1	130.1	43.1	10	High
NDPA	6.78	1	130.1	113.2	5	High
N-Nitroso N-ethyl N-phenylamine (NEPhA)	6.97	1	77	51.1	10	High
N-Nitroso N-ethyl N-phenylamine (NEPhA)	6.97	1	106	77.1	10	High
N-Nitroso N-ethyl N-phenylamine (NEPhA)	6.97	1	120	51.1	30	High
N-Nitroso N-ethyl N-phenylamine (NEPhA)	6.97	1	120	77.1	20	High
NMOR	7.04	1	116.1	56.1	10	High
NMOR	7.04	1	116.1	86.1	5	High
NPYR	7.20	1	100.1	43.1	5	High
NPYR	7.20	1	100.1	55.1	5	High
NPYR	7.20	1	100.1	70.1	5	High
NPYR-D_8	7.21	1	122.1	49.1	10	Normal

Appendix 2 (continued)

Name	RT (min)	Window (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Dwell time priority
NPYR-D_8	7.21	1	122.1	92.2	5	Normal
NPYR-D_8	7.21	1	122.1	105.1	5	Normal
NPIP	7.34	1	114.1	41.1	10	High
NPIP	7.34	1	114.1	84.2	5	High
NPIP	7.34	1	114.1	97.1	5	High
NDBA	8.10	1	116.1	99.2	5	High
NDBA	8.10	1	158.2	99.2	5	High
NDBA	8.10	1	158.2	141	5	High
N-Nitroso-diphenylamine (NDPhA)	10.48	1	167	166.1	10	High
N-Nitroso-diphenylamine (NDPhA)	10.48	1	168	167.1	10	High
N-Nitroso-diphenylamine (NDPhA)	10.48	1	169	168.1	10	High

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