Application Note

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Quantitative Analysis of Nitrosamine Impurities in Synthetic Oligonucleotides

Using the Agilent 6495D triple quadrupole LC/MS system

Suitable for Agilent
1290 Infinity III LC

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Abstract

Nitrosamines are genotoxic impurities of significant concern in pharmaceuticals due to their carcinogenic potential. Regulatory agencies require rigorous risk assessment and control for all drug products, including synthetic oligonucleotides. This application note presents a sensitive and robust LC/MS/MS method for the quantification of eight nitrosamine impurities in antisense oligonucleotide (ASO) samples. The method achieved ppt-level detection with excellent calibration linearity, high accuracy, good precision, high sensitivity for detection and quantification, good recovery, and outstanding reproducibility. These results demonstrate the reliability and suitability of this method for nitrosamine analysis in oligonucleotide-based therapeutics.

Introduction

The discovery of N-nitrosodimethylamine (NDMA) in certain drug products in 2018 prompted global regulatory agencies, including the FDA and EMA, to mandate nitrosamine risk assessments across all pharmaceutical classes. Nitrosamines are genotoxic impurities of significant concern due to their high carcinogenic potential, requiring stringent control to ng/day levels. While chemically synthesized oligonucleotides—such as ASOs, small interfering RNAs (siRNAs), and aptamers—contain primary aromatic amines in their nucleobases, these groups typically act as scavengers rather than forming stable nitrosamines. Consequently, the intrinsic risk of nitrosamine formation from oligonucleotide APIs is considered low.[1]

However, potential contamination from raw materials, reagents, or process conditions necessitates a robust risk assessment and control strategy to ensure patient safety and regulatory compliance. FDA[2], EMA[3], and ICH[4] require nitrosamine risk assessment and control for all drug products, including synthetic oligonucleotides. Manufacturers must document risk evaluation, confirmatory testing, and mitigation strategies.

In this application note, a quantitative analysis of eight nitrosamine compounds was carried out on the Agilent 6495D triple quadrupole LC/MS (LC/TQ) system coupled with the Agilent 1290 Infinity II LC system and Agilent atmospheric pressure chemical ionization (APCI) source. The results demonstrated ppt-level detection of the nitrosamine targets in an ASO sample.

Experimental

Sample preparation

A 21-mer phosphorothioate ASO with the sequence 5'-ACAUAUUCCCUGAUGAGGUdTdT-3' was prepared at a final concentration of 1 mg/mL by dissolving in a 10% methanol/water (v/v) solution.

Agilent nitrosamine standards (US-113N-1) were spiked into the above oligonucleotide solution at concentration ranging from 0.05 to 25 ng/mL.

Quality control (QC) samples were prepared in four technical replicates by spiking the oligonucleotide solution with nitrosamine standards at concentrations of 0.2 ng/mL low QC (LQC), 2 ng/mL middle QC (MQC), and 20 ng/mL high QC (HQC).

Injections were carried out in triplicates for calibration standards and duplicates for QCs.

Instrumentation

For separation, the Agilent 1290 Infinity II bio LC system was used, including:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent InfinityLab bio-inert Quick Connect heat exchanger

Samples were analyzed on the Agilent 6495D LC/TQ equipped with the Agilent APCI source.

Software

The following software was used in this study:

- Agilent MassHunter acquisition software (LC/TQ), version 12.2
- Agilent MassHunter Quantative Analysis software, version 12.1

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for LC and MS. Table 3 contains compound specific MRM settings.

Table 1. LC parameters.

Parameter	Value
Instrument	Agilent 1290 Infinity II LC system
Column	Agilent InfinityLab Poroshell 120 column EC-C18, 3.0 × 150 mm, 2.7 µm (p/n 693975-302)
Thermostat	8 °C
Solvent A	0.1% formic acid in H_2O
Solvent B	0.1% formic acid in methanol
Flow Rate	0.5 mL/min
Gradient	Time %B 0.0 5 3.5 5 7.0 45 9.0 60 11.0 60 15.0 65 16.0 90 16.1 5
Post-Time	4 min
Injection Volume	20 µL, needle wash flush port for 10 seconds with 50% acetonitrile/H_2O (v/v)
Column Temperature	40 °C

Table 2. MS data acquisition parameters.

Instrument	Agilent 6495D LC/TQ
Source	Agilent APCI source
Polarity	Positive
Gas Temperature	290 °C
Gas Flow	11 L/min
Nebulizer	23 psi
APCI Vaporizer Temperature	350 °C
Gas Flow	12 L/min
Capillary Voltage	1,000 V
Corona Current	4 µA
Scan Type	dMRM
Detector Gain Factor (+)	10

Table 3. Detailed MRM settings and compound information for the Agilent 6495D LC/TQ. The quantifier ions are bolded.

Compound Full Name	Compound Abbreviated Name	Precursor *m/z*	Product *m/z*	CE (V)	iFunnel Mode	CAV (V)	Polarity	Retention Time (min)
N-Nitrosodimethylamine	NDMA	75	43	16	Fragile	3	+	2.50
	NDMA	75	58	10	Fragile	3	+	2.50
N-Nitrosomorpholine	NMOR	117	45	17	Fragile	3	+	3.93
	NMOR	117	87	9	Fragile	3	+	3.93
N-Nitrosomethylethylamine	NMEA	89	61	10	Fragile	3	+	5.32
	NMEA	89	43	10	Fragile	3	+	5.32
N-Nitrosompyrrolidine	NPYR	101	55	15	Fragile	3	+	5.80
	NPYR	101	41	30	Fragile	3	+	5.80
N-Nitrosodiethylamine	NDEA	103	75	8	Fragile	3	+	7.83
	NDEA	103	47	16	Fragile	3	+	7.83
N-Nitrosopiperidine	NPIP	115	41	22	Fragile	3	+	8.26
	NPIP	115	69	14	Fragile	3	+	8.26
N-Nitrosodi-n-propylamine	NDPA	131	43	10	Fragile	3	+	10.53
	NDPA	131	89	6	Fragile	3	+	10.53
N-Nitrosodi-n-butylamine	NDBA	159	57	10	Fragile	3	+	14.00
	NDBA	159	41	20	Fragile	3	+	14.00

Results and discussion

The calibration curve of the eight nitrosamine compounds was established with concentrations ranging from 0.05 to 25 ng/mL in the ASO matrix. Excellent chromatographic separation and peak shapes were achieved for all analytes, as shown in Figure 1. The NPIP peak baseline was impacted by matrix interference due to coelution with the ASO sample. To reduce matrix effects and improve method performance, a divert valve can be programmed to direct the matrix peak to waste at its elution time window.

Excellent calibration linearity with $R^2 > 0.99$ (Figure 2) was achieved for all the eight analytes within 0.05 to 25 ng/mL concentration range, except NPIP (0.25 to 25 ng/mL) and NDBA (0.1 to 25 ng/mL). Accuracy was consistently within the range of 90 to 120% at all tested levels (Table 4). High analytical sensitivity was achieved, enabling quantification of all the targeted analytes down to ppt level. Table 4 summarizes limits of detection (LODs) and limit of quantification (LOQs) for each nitrosamine. LOD values were determined using software-driven automated based on eight LQC samples. The LOQ values corresponded to the lowest calibration level.

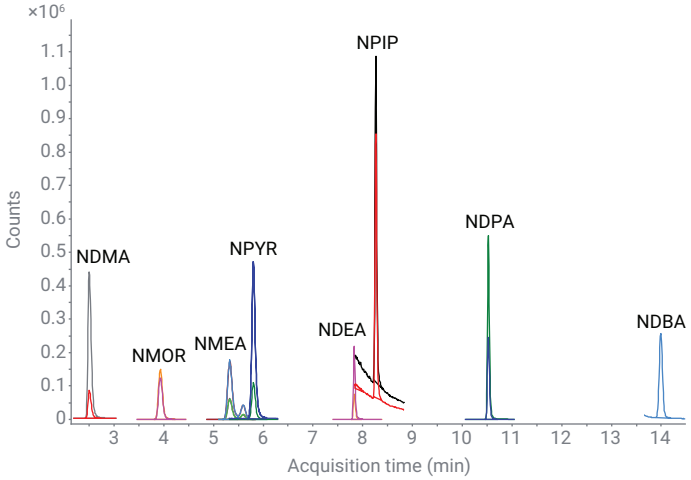

Figure 1. MRM chromatogram of eight nitrosamines in ASO matrix at 5 ng/mL.

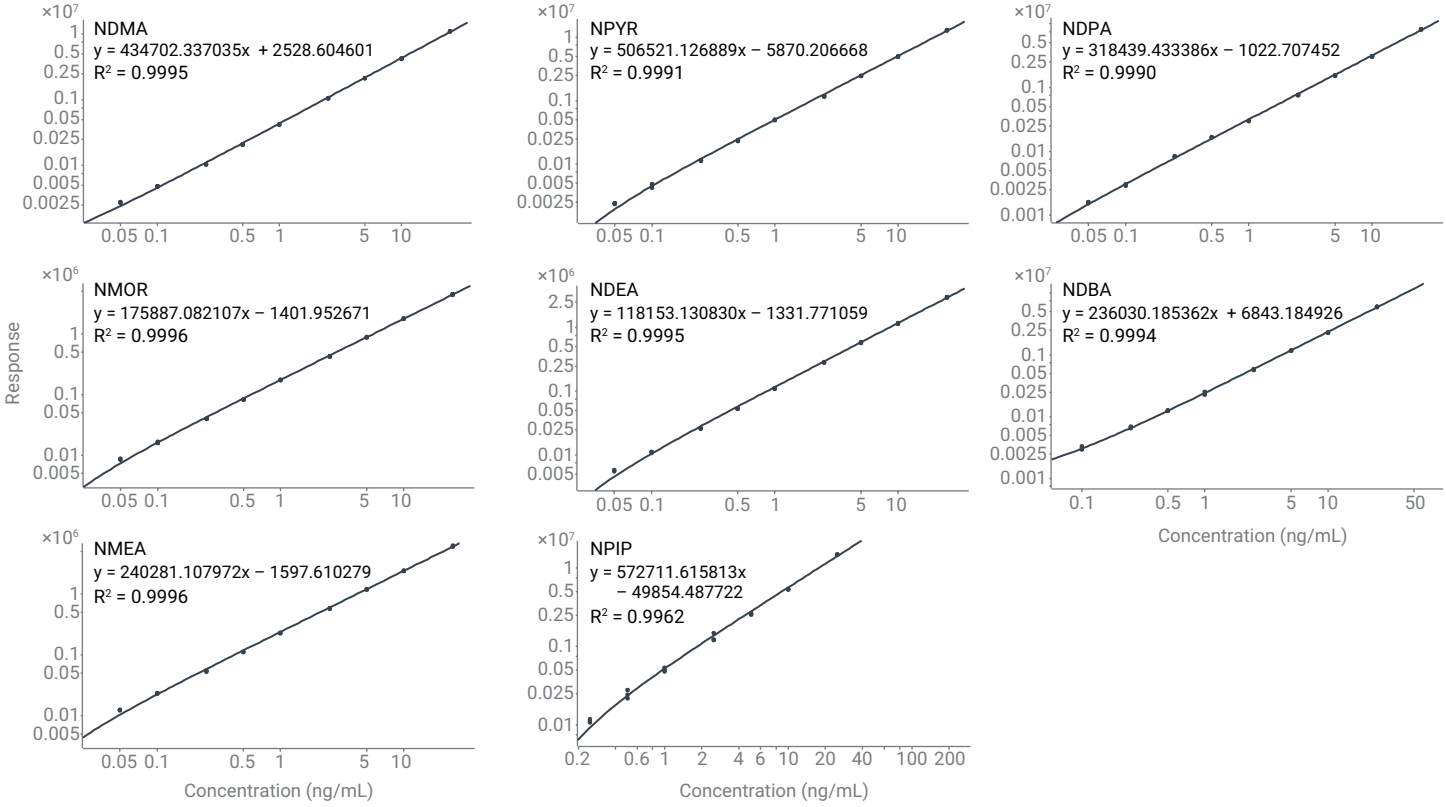

Figure 2. Calibration curves for the eight nitrosamine compounds.

Table 4. Targeted eight nitrosamine screening results in the ASO sample.

No.	Compound Name	Curve Fit	R^2	Calibration Range (ng/mL)	Accuracy (%)	LOD* (ng/mL)	LOQ# (ng/mL)	MQC Recovery (%)	MQC Recovery Intraday Repeatability (%) (n = 8)	MQC Recovery Interday Reproducibility (%) (n = 16)
1	NDMA	Linear, Ignore, 1/x	0.999	0.05–25	92–117	0.02	0.05	95–98	0.97	0.89
2	NMOR	Linear, Ignore, 1/x	0.999	0.05–25	93–115	0.01	0.05	98–101	0.89	1.16
3	NMEA	Linear, Ignore, 1/x	0.999	0.05–25	90–117	0.02	0.05	94–100	2.04	1.65
4	NPYR	Linear, Ignore, 1/x	0.999	0.05–25	92–119	0.02	0.05	94–98	1.20	1.55
5	NDEA	Linear, Ignore, 1/x	0.999	0.05–25	91–119	0.01	0.05	96–99	1.73	3.66
6	NPIP	Linear, Ignore, 1/x	0.996	0.25–25	91–116	0.10	0.25	91–96	1.81	3.73
7	NDPA	Linear, Ignore, 1/x	0.998	0.05–25	93–108	0.03	0.05	90–98	2.54	5.34
8	NDBA	Linear, Ignore, 1/x	0.999	0.10–25	94–117	0.07	0.10	90–100	3.75	7.10

* MassHunter Quantitative Analysis software-driven calculation of eight LQC results, except NPIP was based on MQCs as its LQC was below LOD.
# Lowest calibration level.

The MQC samples were used to evaluate method precision, recovery, repeatability, and robustness. As shown in Table 5, excellent retention time (RT) and peak area precision was achieved among replicated injections (n = 8), with RT %RSD < 0.17 and area %RSD < 3.71. Good recovery was attained within the range of 90 to 101% for all the compounds. Intraday repeatability was maintained below 4%, while interday reproducibility remained less than 8%. These results collectively confirmed the reliability and robustness of the method for nitrosamine quantification in the ASO sample.

As one of the most critical nitrosamines under regulatory surveillance, NDMA poses some analytical challenges. Due to its volatility and low molecular weight (74 Da), NDMA is prone to losses during sample handling. Chromatographically, it typically elutes early in reversed-phase separation, often coeluting with polar substances in the matrix, which can result in low recovery and ion suppression. These factors complicate accurate quantification and necessitate careful method optimization to ensure reliable detection at trace levels.

Table 5. RT and area precision data of MQC samples.

	NDMA		NMOR		NMEA		NPYR		NDEA		NPIP		NDPA		NDBA	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
MQCl	2.501	848918	3.934	345605	5.32	457452	5.802	954777	7.829	225973	8.259	1013642	10.526	595240	13.997	451455
MQCl	2.509	840101	3.935	349987	5.328	451746	5.802	965645	7.829	232926	8.259	1050028	10.526	597702	13.989	453842
MQC2	2.501	840404	3.926	349118	5.32	479856	5.802	974086	7.829	224225	8.259	1018063	10.526	577686	13.989	432706
MQC2	2.509	829319	3.935	353833	5.328	457272	5.802	985974	7.829	226684	8.259	1003340	10.526	588357	13.989	434307
MQC3	2.501	845020	3.935	347246	5.328	469514	5.794	989666	7.829	234628	8.259	1013283	10.526	606874	13.989	467299
MQC3	2.501	856434	3.926	344345	5.32	471871	5.794	963650	7.829	232953	8.259	1047266	10.526	628334	13.989	469804
MQC4	2.509	839527	3.926	345360	5.328	460099	5.794	972887	7.829	226334	8.259	995860	10.526	595732	13.997	456015
MQC4	2.501	849345	3.926	348810	5.328	469940	5.802	966740	7.829	231592	8.259	1017969	10.526	610396	13.989	481380
Average	2.50	843633.50	3.93	348038.00	5.33	464718.75	5.80	971678.13	7.83	229414.38	8.26	1019931.38	10.53	600040.13	13.99	455851.00
Std	0.00	8202.78	0.00	3082.20	0.00	9475.09	0.00	11625.57	0.00	4008.81	0.00	19268.01	0.00	15295.94	0.00	16906.02
RSD	0.17	0.97	0.12	0.89	0.08	2.04	0.07	1.20	0.00	1.75	0.00	1.89	0.00	2.55	0.03	3.71

Figure 3 presents the chromatograms of NDMA in a matrix blank and at the LOD level of 0.05 ng/mL. The results demonstrate that NDMA could be confidently quantified at 0.05 ng/mL in the ASO matrix, with a good signal-to-noise ratio (S/N) of 80 and a strong peak response. This indicates the method's suitability for NDMA quantification.

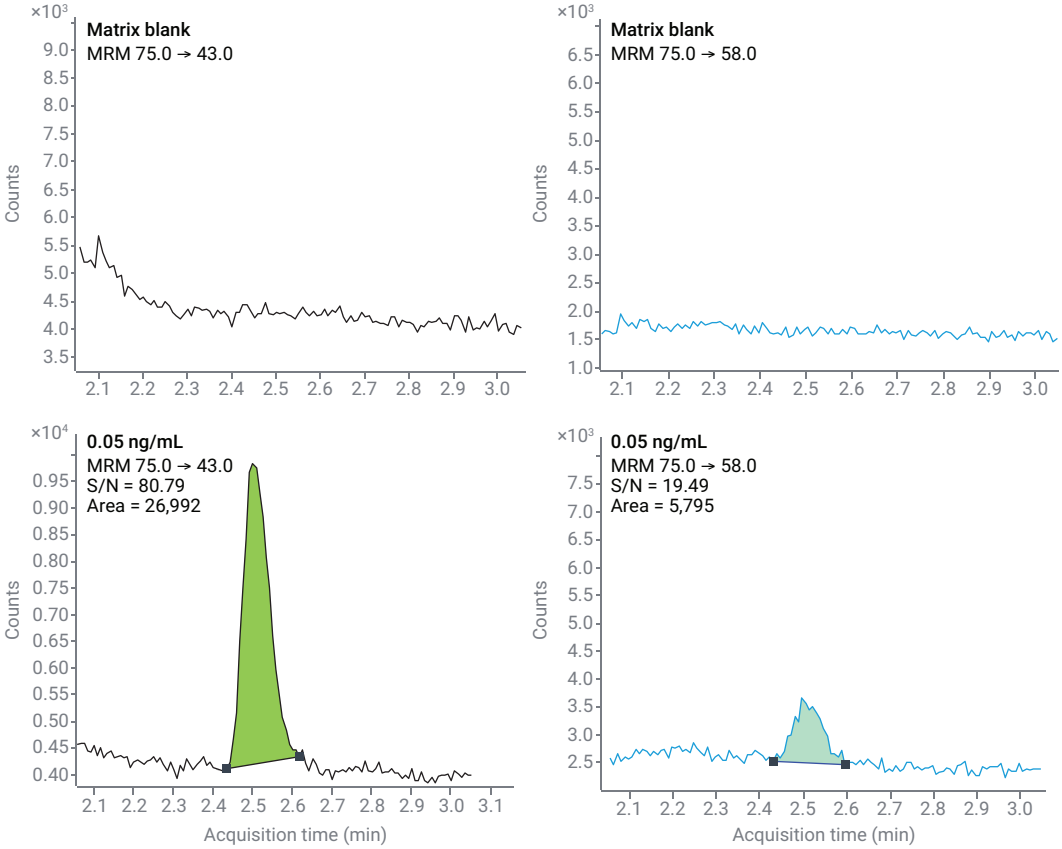

Figure 3. Representative chromatograms of NDMA quantifier and qualifier ions in ASO matrix at blank and LOQ levels.

Conclusion

This application note underscores the critical importance of mutagenic impurity analysis in oligonucleotide-based therapeutics, which is in alignment with regulatory requirements.

A sensitive and robust LC/MS/MS method was successfully developed on Agilent 6495D LC/TQ system. The method can quantify nitrosamine impurities in ASO sample down to ppt levels. The method demonstrated excellent performance across eight nitrosamines, with strong calibration linearity ($R^2 > 0.99$), high accuracy (80 to 120%), and outstanding retention time and area precision (%RSD < 0.17 and < 3.71, respectively). LOD and LOQ were below 0.10 and 0.25 ng/mL, respectively, confirming high sensitivity in complex matrices. Recovery and reproducibility results further validated the method's robustness. Overall, the findings confirm the method's reliability and suitability for routine nitrosamine analysis in oligonucleotide therapeutics, supporting regulatory compliance.

References

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