Designation: D8456 – 22

Standard Test Method for Determination of Nitrosamines in Water by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)[1]

This standard is issued under the fixed designation D8456; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the liquid chromatography tandem mass spectrometry (LC-MS/MS) detection and quantitation of N-nitrosamines after direct injection. It has been validated for groundwater, surface water, wastewater influents, and wastewater effluents. This test method is not limited to these aqueous matrices; however, the applicability of this test method to other aqueous matrices must be demonstrated.

1.2 This test method is applicable to nitrosamines that can be chromatographed and detected using a mass spectrometry procedure. Table 1 lists the compounds that have been validated for this test method. This test method is not limited to the compounds listed in Table 1; however, the applicability of the test method to other compounds must be demonstrated.

1.3 Analyte concentrations from 0.05 µg/L up to approximately 5 µg/L may be determined without dilution of the sample. Analytes with insufficient sensitivity will not be detected, but they can be measured with acceptable accuracy and precision when present in sufficient amounts. In addition, newer instruments, or instruments of improved sensitivity may be used to lower detection limits.

1.4 Analytes that are not separated chromatographically, but that have different mass spectra and noninterfering quantitation ions, can be identified and measured in the same calibration mixture or water sample. Analytes that have very similar product ions cannot be individually identified and measured in the same calibration mixture or water sample unless they have different retention times.

1.5 It is the responsibility of the user to ensure the validity of this test method for untested matrices.

1.6 This test method is restricted to use by or under the supervision of analysts experienced in the use of a liquid chromatograph with tandem mass spectrometry (LC-MS/MS).

1.7 Depending on data usage, you may modify this test method but limit to modifications that improve performance while still meeting method quality acceptance criteria. Shortening the chromatographic run simply to save time is not allowed. Use Practice E2935 or similar statistical tests to confirm that modifications produce equivalent results on non-interfering samples. In addition, use Guide E2857 or equivalent statistics to re-validate the modified test.

1.8 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.9 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.10 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water
D3370 Practices for Sampling Water from Flowing Process Streams
D4448 Guide for Sampling Ground-Water Monitoring Wells
D6089 Guide for Documenting a Groundwater Sampling Event
D6538 Guide for Sampling Wastewater With Automatic Samplers
D6759 Practice for Sampling Liquids Using Grab and Discrete Depth Samplers
E2857 Guide for Validating Analytical Methods

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.06 on Methods for Analysis for Organic Substances in Water.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

TABLE 1 Analytes Validated for this Method

Sr. No.	Name	Abbrevations	CAS No
1	N-Nitroso dimethylamine	NDMA	62-75-9
2	N-Nitroso Morpholine	NMOR	59-89-2
3	N-Nitroso-N-methyl-4-aminobutyric Acid	NMBA	61445-55-4
4	N-Nitroso pyrrolidine	NPYR	930-55-2
5	N-Nitroso ethylmethylamine	NMEA	10595-95-6
6	N-Nitroso diethylamine	NDEA	55-18-5
7	N-Nitroso piperidine	NPIP	100-75-4
8	N-Nitroso isopropylethylamine	NEIPA	16339-04-1
9	N-Nitroso diisopropylamine	NDIPA	601-77-4
10	N-Nitroso di n-Propyl amine	NDPA	621-64-7
11	N-Nitroso dibutylamine	NDBA	924-16-3
12	N-Nitrosmethylphenylamine	NMPA	614-00-6
13	N-Nitroso diphenylamine	NDPhA	86-30-6
14* ISTD	N-Nitroso dimethylamine-D6	NDMA-D6	17829-05-9
15* ISTD	N-Nitroso diethylamine-D10	NDEA-D10	1219794-54-3

E2935 Practice for Evaluating Equivalence of Two Testing Processes

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *atmospheric pressure chemical ionization (APCI), n*—an ionization method used in mass spectrometry which uses a gas-phase ion-molecule reaction at atmospheric pressure coupled with high-performance liquid chromatography (HPLC).

3.2.1.1 *Discussion*—APCI is a soft ionization method similar to chemical ionizationwhere primary ions are produced on a solvent spray. The main usage of APCI is for mid-polar and relatively less polar thermally stable compounds with molecular weight less than 1500 Da.

4. Summary of Test Method

4.1 Internal standard is added to a known volume of sample. The sample is injected into LC-MS/MS system operated with an APCI source. Alternatively, extract an isotopically labelled analog of each analyte (isotope dilution), if available, and correct for recovery.

4.2 Compounds eluting from the LC column, are identified by comparing their MRM transition and retention times to reference standards. MRM transitions and retention times for analytes are obtained by the measurement of calibration standards under the same conditions used for the samples. The concentration of each identified component is measured by relating the Selected Reaction Monitoring (SRM) response of the SRM produced by that compound to the SRM transition response produced by a compound that is used as an internal standard. Surrogate analytes, whose concentrations are known in every sample, are measured with the same internal standard calibration procedure. Qualitative identity is made by measuring the relative abundance ratios of Multiple Reaction Monitoring (MRM) from the same compound and comparing it to the relevant standard. Quantitative analysis is performed by using the authentic standard(s) to produce a response factor or calibration curve. The calibration data, along with the sample volume extracted and the final sample extract volume, is used to determine the concentration of a target compound in the sample extract.

5. Significance and Use

5.1 Nitrosamines are a class of nitrogen-containing compounds with a known occurrence in wastewater. They may also be created in deionization and are a potential contaminant in water for reuse. The World Health Organization has issued a guideline for NDMA in drinking water at 0.1 µg/L. NDMA may occur in chlorinated effluents and other wastewaters. Other methods for nitrosamines employ solid phase extraction, which may not be applicable to wastewaters that contain particulate matter or a high organic load. This method analyses nitrosamines directly using LC-MS/MS.

6. Interferences

6.1 Interferences are largely eliminated by use of tandem MSMS technology. Reagents should also be checked for the presence of contaminants. Subtracting blank values from sample results is not permitted.

6.2 Interfering contamination may occur when a sample containing low concentrations is analyzed immediately after a sample containing higher concentrations. After analysis of a sample containing high concentrations of semi-volatile organic compounds, one or more solvent blanks should be analyzed to check for cross contamination. After analyzing a highly contaminated sample, it may be necessary to wash out the system by injection of blanks.

6.3 Nitrosamines may be present in trace amounts in rubber products. Repeat injections from auto-sampler vials with polytetrafluoroethylene coated rubber septa may introduce method analytes into the sample.

7. Apparatus

7.1 *Tandem Liquid Chromatography/Mass Spectrometer/Data System (LC-MS/MS):*

7.1.1 *Liquid Chromatography System*—A complete LC system is required to analyze samples, this includes a sample injection system, a solvent pumping system capable of mixing

solvents, a sample compartment capable of maintaining required temperature and a temperature-controlled column compartment.

7.1.2 *Liquid Chromatography Column*—Any column that meets the performance specifications of this test method may be used. Separations of the calibration mixture must be equivalent or better than those described in this test method. As examples, the following columns have been found to be suitable:

7.1.3 *Column 1*—C18 150 mm × 4.6 mm, 5 microns.

7.1.4 *LC-MS/MS Interface*—Any interface that meets the requirements of the method may be used.

7.1.5 *Tandem Mass Spectrometer System*—A MS/MS system capable of multiple reaction monitoring (MRM) analysis or any system that can perform at the requirements in this test method shall be used. The mass spectrometer should be capable of operating in APCI positive mode and acquiring up to 4 MRM per target with dwell times long enough for 10 or more data points during the elution of each LC peak.

7.1.6 An interfaced data system is required to acquire, store, reduce, and output mass spectral data. The computer software shall have the capability of processing stored LC-MS/MS data by recognizing a LC peak within any given retention time window, comparing the MRM transition from the LC peak with spectral data in a user-created database, and generating a list of tentatively identified compounds with their retention times. The software must allow integration of the ion abundance of any specific ion between specified time or scan number limits. The software must also allow calculation of response factors or construction of a second order regression calibration curve, calculation of response factor statistics, and calculation of concentrations of analytes using either the calibration curve or the equation in 12.6.4.

NOTE 1—Instead of a nitrogen generator, an ultra-pure nitrogen cylinder (Purity: >99.9) was employed. It was observed the considerable reduction in baseline especially for NDMA (MRM: 75→43) transition. Liquid nitrogen can also be used as an alternative to a nitrogen generator to minimize a high NDMA baseline.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, providing it is pure enough to be used without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type I or Type II.

8.3 *Nebulizing gas*—Nitrogen (99.9 %).

8.4 *Standard Solutions, Stock*—Standards that are to be used for calibration shall be Certified Reference Materials (CRMs), where available. If a CRM is not available, then the standards shall be Reference Materials (RMs). If a CRM and/or an RM is not available, the standard shall be manufactured under the requirements of ISO Guide 34.

8.5 *Standard Solutions, secondary dilution standards*—Use stock standard solutions to prepare secondary dilution standard solutions that contain the analytes in reagent water. The secondary solutions should be prepared at concentrations that can be easily diluted to calibration standards that will bracket the calibration range, such as 100 µg⁄L, 10 µg⁄L, and 1 µg⁄L.

8.6 *Standard Solutions, secondary dilution for the internal standards (ISTD)*—Prepare a dilution at 100 µg/L each. For isotope dilution, lower the concentration of each labelled standard to approximate the estimated analyte concentration of native analyte in the samples.

8.7 *Standard Solutions, Calibration Standards*—Prepare standards from 0.02 µg/L to 2 µg/L with an ISTD concentration of 2 µg/L to bracket the expected range of target analytes in the samples according to Table 2.

8.8 *Mobile Phase A* (0.1% formic acid)—Add 1 mL of formic acid to 500 mL of reagent water in a 1000 mL volumetric flask. Dilute to the mark with reagent water and mix.

8.9 *Mobile Phase B* (0.1% formic acid in methanol)—Add 1 mL of formic acid to 500 mL of methanol in a 1000 mL volumetric flask. Dilute to the mark with methanol and mix.

8.10 *Rinsing solvent* (water: methanol)—(1:1) v/v water and methanol.

8.11 *Syringe* (5 mL to 10 mL Luer lock) and 0.2 µm filters.

8.12 *Vials,* 5 mL plastic vial or test tube, nitrosamine-free.

9. Hazards

9.1 *Precaution*—The toxicity or carcinogenicity of chemicals used in this test method has not been precisely defined; each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each

TABLE 2 Example Preparation of Calibration Standards

Sr.No.	Concentration (µg/L)	Stock conc. (µg/L)	Vol. of stock (µL)	ISTD conc. (µg/L)	ISTD Vol. (µL)	Diluent (µL)	Final Vol. (µL)
1	0.05	1	150	100	60	2790	3000
2	0.10	1	300	100	60	2640	3000
3	0.20	10	60	100	60	2880	3000
4	0.50	10	150	100	60	2790	3000
5	1.00	10	300	100	60	2640	3000
6	2.00	100	60	100	60	2880	3000
7	5.00	100	150	100	60	2790	3000

laboratory is responsible for maintaining awareness of OSHA regulations regarding safe handling of chemicals used in this test method.

9.2 **Warning—**The compound analytes in this test method have been classified as known or suspected human or mammalian carcinogens. Pure standards and stock solutions should be handled in a hood or glovebox.

10. Sample Collection, Preservation, and Storage

10.1 *Sample Collection, Dechlorination, and Preservation:*

10.1.1 Collect all chlorinated samples in 40 mL amber glass bottles containing ~3 mg of sodium thiosulfate crystals. If collecting more sample, use more sodium thiosulfate as appropriate. Unchlorinated samples do not require sodium thiosulfate.

10.1.2 Collect non-chlorinated, non-tap water samples in accordance with Practices D3370, Guide D4448, Guide D6538, or Practice D6759. Document field activities according to Guide D6089.

10.1.3 The samples must be chilled to above freezing but not to exceed 6 °C on the day of collection and must be maintained at that temperature until analysis. Field samples that will not be received at the laboratory on the day of collection must be packaged for shipment with sufficient ice to ensure that they will be at ≤6 °C on arrival at the laboratory.

10.2 *Sample Storage:*

10.2.1 Store samples at above freezing to 6 °C until analysis.

10.2.2 Analyze all samples within 14 days of collection.

10.3 *Field Blanks and Duplicates:*

10.3.1 Field blank samples must be handled along with each sample set, which is composed of the reagent water collected from the same general sample site at approximately the same time. At the laboratory, fill field blank sample bottles with water, seal, and ship to the sampling site along with empty sample bottles. Using the same methodology as the samples taken, samplers will transfer the reagent water to a sample bottle at the site and then ship back to the laboratory with all the filled sample bottles. For maximum value, collect field blanks under the worst contamination conditions in the field.

10.3.2 Collect a field duplicate with each sampling event, or as defined by the project quality assurance plan.

11. Preparation of the LC-MS/MS

11.1 *LC Chromatograph Operating Conditions:*

11.1.1 Injections of all standards and samples are made at a 300 µL volume. Other injection volumes may be used to optimize conditions. Standards and samples shall be in aqueous, filtered samples. In the case of extreme concentration differences amongst samples, it is wise to analyze a blank after a concentrated sample and before a dilute sample to minimize carryover of analytes from injection to injection. A blank sample following a concentrated sample shall be lower than the MDL. The gradient conditions for liquid chromatography are shown in Table 3.

11.1.2 *Mixer volume*—40 µL.

TABLE 3 Example Analytical Conditions (may vary by manufacturer)

HPLC Column	C18 (150 mm × 4.6 mm, 5 micron)		
Column Temperature	40 °C		
Flow Rate	0.7 mL/min		
Mobile Phase A	0.1% formic acid in water		
Mobile Phase B	0.1% formic acid in methanol		
Gradient Program	Time (Min)	A%	B%
	0.01	85	15
	1.00	85	15
	3.50	30	70
	11.00	10	90
	11.10	85	15
	15.00	STOP	
Injection Volume	300 µL		
Autosampler Temp.	15 °C		

11.1.3 Rinse needle with a rinsing solvent volume of 1000 µL (8.8) for 2 s before and after each injection. Follow the instrument manufacturer's instructions.

11.1.4 *Diverter valve settings*—See Table 4.

11.2 *Mass Spectrometer Parameters:*

11.2.1 To acquire the maximum number of data points per MRM channel while maintaining adequate sensitivity, optimize the tune parameters according to the manufacturer's instructions. Each peak should have at least 10 scans per peak for adequate quantitation. MRM quantifier, and qualifier transitions are shown in Table 5. Mass spectrometer parameters used in the development of this method are listed in Table 6. See Fig. 1 for an example chromatogram.

12. Calibration and Standardization

12.1 The mass spectrometer shall be calibrated as in accordance with manufacturer's specifications before analysis. Analytical values satisfying test method criteria have been achieved using the following procedures.

12.2 To calibrate the instrument, analyze at least five calibration levels when using a linear calibration curve and six calibration levels when using a quadratic calibration curve. Weighted fits (1/x or $1/x^2$) are preferred. Avoid using unweighted curves since they tend to result in unacceptable error at the lower end of the curve.

12.3 Analyze a second source verification standard near the midpoint of the calibration range immediately after each calibration. Results shall be within ±30 % of the theoretical concentration. If a second source is not available, a second lot number from the same vendor may be used.

12.4 Inject each standard and obtain its chromatogram. An internal calibration technique is used to monitor the primary and confirmatory SRM transitions of each analyte. Calibration software conducts the quantitation of the target analytes the primary transition. The ratios of the primary/confirmatory transition area counts will vary depending on the individual tuning conditions. The quantifier/qualifier MRM transition area

TABLE 4 Diverter Valve Settings

Time (min)	Divert Valve Position
0.0	To Waste
3.4	To Mass Spectrometer

TABLE 5 MRM Transitions

Name	Type	ISTD group	MRM (Quantifier)	MRM (Qualifier)
NDMA	Target	1	75 >43	75 >58
NMOR#	Target	2	TIC (117>87;117>45;117>28)	-
NMBA	Target	1	147>117	147>44
NPYR	Target	2	101>55	101>41
NMEA	Target	2	89>61	89>43
NDEA	Target	2	103>29	103>45
NPIP	Target	2	115>69	115>41
NEIPA	Target	2	117>75	117>27
NDIPA	Target	2	131>89	131>43
NDPA	Target	2	131>89	131>43
NDBA	Target	2	159>41	159>29
NMPA	Target	2	137>66	137>107
NDPhA	Target	2	170>93	170>65
NDMA-D6	ISTD	1	81>46	-
NDEA-D10	ISTD	2	113>34	-

TABLE 6 Ion Source Settings

Interface Parameters
Interface/Polarity : APCI/Positive
Nebulizing Gas Flow : 4.00 L/Min
Interface Temperature : 300 °C
DL Temperature : 180 °C
Heating Block Temperature : 200 °C
Drying Gas Flow : 5.00 L/min
CID gas : 180 kPa

ratio shall be within 35 % of the individual labs accepted primary/confirmatory MRM transition area ratio. The quantifier SRM transition of each analyte is used for quantitation and the qualifier MRM transition for confirmation. This gives added confirmation by isolating the parent ion, forming two product ions by means of fragmentation, and relating it to the retention time in the calibration standard.

12.5 Demonstration and documentation of acceptable initial calibration for compounds of interest is required before any samples are analyzed and is required intermittently throughout sample analysis as dictated by results of continuing calibration checks. After initial calibration is successful, a continuing calibration check is required at the beginning of each 12-h period during which analyses are performed. Additional periodic calibration checks are good laboratory practice. The criteria in this section were used for the method validation. Other criteria may be more appropriate in each situation depending on the data quality objectives.

12.6 *Performance Criteria for the Medium Calibration:*

12.6.1 *LC Performance*—Good column performance will produce symmetrical peaks with minimum tailing for most compounds. If peaks are broad, or sensitivity is poor, see 12.8.5 for some possible remedial actions.

12.6.2 *MS Sensitivity*—The LC-MS/MS peak identification software shall be able to recognize a LC peak in the appropriate retention time window for each of the compounds in the calibration solution and make correct tentative identifications. If any of the compounds are not recognized, system maintenance is required.

12.6.3 If all performance criteria are met, inject an aliquot of each of the other calibration standards using the same operating conditions.

12.6.4 Calculate a relative response factor (RRF) for each analyte for each calibration standard. Table 5 contains suggested quantitation ions for all compounds. If there is significant interference with a primary ion, then a secondary or alternative ion should be selected for quantitation. Experience gained from the method validation has shown that the use of these suggested ions and the suggested internal standards minimizes method interferences. The calculation of RRF is supported in acceptable data system software. The RRF is a unitless number, but units used to express quantities of analyte and internal standard must be equivalent.

$$RRF = \frac{(Ax)(Qis)}{(Ais)(Qx)} \quad (1)$$

where:

Ax = integrated abundance of the quantitation ion of the analyte,

Ais = integrated abundance of the quantitation ion of the internal standard,

Qx = quantity of analyte purged, ng or concentration units, and

Qis = quantity of internal standard purged, ng or concentration units.

12.6.5 For each analyte and surrogate, calculate the mean (M) RRF from the analyses of the calibration standards. Calculate the standard deviation (SD) and the relative standard deviation (RSD) from each mean: $RSD = 100(SD/M)$.

12.6.6 For the initial calibration to be acceptable, the following criteria must be met. These criteria verify the linearity of the calibration curve:

12.6.7 The RSD of the mean RRF of at least 90 % of the analytes and surrogates must be below 20 %.

12.6.8 For any analyte or surrogate with a RSD greater than 20 %, the RSD must be less than or equal to 30 %.

12.6.9 The RSD of a given analyte or surrogate must not exceed 20 % for more than three calibrations in a row.

12.6.10 If the acceptance criteria are not met, take action to improve performance and recalibrate.

12.7 As an alternative to calculating mean response factors and applying the RSD test, use the data system software or other proven software to generate a calibration curve. Residuals should agree within 20 %.