Evaluation and Validation of GC-MS/MS Method EEA 521.1 adopting U.S. EPA ATP Protocol for Determination of N-Nitrosamines in Drinking Water

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1. Introduction

N-Nitrosamines (aka nitrosoamines) is a general term used to designate a large group of compounds having the generic chemical structure R_2N-N=O. Nitroso compounds are formed in drinking water as byproducts of the disinfection process, when nitrate or other nitrogen-containing compound in water react with chlorine or chloramine[1]. The presence of N-nitrosamines in drinking water present a health concern due to their carcinogenicity. In 2005 the US EPA published method EPA 521 for the quantification of seven N-nitrosamines in drinking water however, the method did not include N-nitrosomorpholine (NMOR) which is of current interest due to its presence in recycled water[2].

In 2018, method EEA-521.1 which includes all seven analytes from method EPA 521 as well as NMOR was developed by Eurofins Eaton Analytical and Agilent Technologies, Inc[3]. To accurately quantify NMOR, the new method employed new instrumentation, an Agilent Technologies Inc. triple quadrupole GC-MS/MS with a high-efficiency ion source system. While EPA 521 uses GC-MS/MS with large volume injection with chemical ionization (CI) using either ion trap or triple quadrupole mass spectrometry, method EEA-521.1 analyzes a small volume of sample, uses electron ionization (EI), and can only be performed with a triple quadrupole mass spectrometer. Sample preparation procedure using solid phase extraction (SPE) in both methods is identical.

In the current method development study, method EEA-521.1 was validated on a ThermoFisher Scientific TSQ8000 Evo triple quadrupole mass spectrometer with ExtractaBrite ion source and Trace 1310 gas chromatograph by the Chemistry Unit (CU) of the Drinking Water and Radiation Laboratory (DWRL). As requested by the Division of Drinking Water (consultation No. CU20200129001**)** the validation study followed the validation and quality criteria given in US EPA publication, Protocol for the Evaluation of Alternate Test Procedures for Organic and Inorganic Analytes in Drinking Water[4]. The target N-nitrosamines were N-Nitrosodimethylamine (NDMA), N-Nitrosomethylethylamine (NMEA), N-Nitrosodiethylamine (NDEA), N-Nitrosodi-n-propylamine (NDPA), N-Nitrosomorpholine (NMOR), N-Nitrosopyrollidine (NPYR), N-Nitrosopiperidine (NPIP), and N-Nitrosodi-n-butylamine (NDBA). The method's applicability was further tested by performing an intra-laboratory

[1] https://www.sciencedirect.com/topics/neuroscience/nitrosamines

[2] US EPA. Method 521 Determination of nitrosamines in drinking water by solid phase extraction and capillary column gas chromatography with large volume injection and chemical ionization tandem mass spectrometry (MS/MS); Version 1.0; EPA/600/R-05/054.

[3] Agilent Technologies application note. EEA-Agilent Method 521.1 Nitrosamines analysis in drinking water using GC/MS/MS-meeting equivalence to EPA method 521.

[4] US EPA. Protocol for the evaluation of alternate test procedures for organic and inorganic analytes in drinking water, Feb 2015; EPA 815-R-15-007.

validation on an Agilent Technologies Inc. 7000 instrument with an Extractor ion source, and the method performance was compared to those from the TSQ8000 Evo. In addition, an inter-laboratory study was performed by collaborating with ThermoFisher Scientific on a TSQ9000 Evo instrument with advanced electron ionization (AEI) ion source to compare instrument sensitivity with that of the TSQ8000 Evo and to further test the applicability of the method.

2. Experimental

2.1 Equipment and supplies

2.1.1 GC-MS/MS

For a detailed description of the instrument specifics see **Table 1**.

2.1.2 Equipment for sample preparation

For sample extraction a 24-port HyperSep™ Glass Block Vacuum manifold (ThermoFisher Scientific) was connected to the laboratory vacuum system.

2.2 Standards and reagents

All solvents used were demonstrated to contain minimal levels of analyte interferences. Aliquots of dichloromethane and methanol were tested and confirmed to have less than the instrument detection levels of the target analytes.

2.2.1 Solvents and reagents

Solvents:

- Dichloromethane (DCM) (Honeywell GC299-4, purity ≥ 99.9%).
- Methanol, Honeywell, Burdick and Jackson, LC-MS Grade Mfr. Model #: GC230-4
- Methanol, Honeywell, Burdick and Jackson, Mfr. Model #: LC230-1, purity ≥ 99.9% by GC analysis
  Note: the above methanol solvents were used interchangeably throughout this validation.
- Reagent water (obtained in-lab from the Barnstead GenPure Pro by ThermoFisher supplied with de-ionized water equipped with an Ultrapure Polisher® resin and UV treatment).

Gases:

- Helium (Airgas, 99.999%) – used as the GC carrier gas
- Argon (Purity Plus Specialty Gases, 99.999%) – used as the collision gas in the TSQ8000 Evo GC-MS/MS
- Nitrogen (Airgas, 99.995%) – used for extract concentration and used as the collision gas in the Agilent7000 GC-MS/MS

Reagents:

- Sodium thiosulfate (Fisher S445-500, purity ≥ 99.5%) – dechlorinating agent
- Pre-packed coconut charcoal cartridges (Restek 26032 ≤100 mesh, 2 g, 6 mL) – for solid phase extraction.
- Pre-packed sodium sulfate drying cartridges (Biotage 802-0250-M) – for drying dichloromethane extract.
- Nitrosamines analyte standard stock solution (Agilent US-113N-1) – containing NDMA, NMEA, NDEA, NDPA, NMOR, NPYR, NPIP, NDBA, and NDPhA in dichloromethane (DCM), 2000 µg/mL of each analyte
- Internal standard stock solution: NDPA-D14 (1000 µg/mL, AccuStandard M-521-IS)
- Surrogate standard stock solution: NDMA-D6 (1000 µg/mL, AccuStandard M-521-SS)

2.2.2 Calibration standards

Primary dilution standards (PDSs): PDSs were prepared in both DCM and in reagent water. PDSs prepared in DCM were used for spiking calibration standards while PDSs prepared in reagent water were used for spiking water samples. For the preparation of reagent water PDSs, the amount of the standard stock solution spiked to water could be no more than 1% (v/v) for the DCM-based standard stock solution in order to be miscible in water at room temperature.

The surrogate PDS (S-PDS) was prepared, in both water and DCM at a concentration of 0.2 µg/mL for each analyte.

The internal standard PDS (ISTD-PDS) was prepared in DCM at a concentration of 0.2 µg/mL. The ISTD-PDS is added after the completion of sample extraction procedure and evaporation. The final extract containing the target analytes are in DCM.

Calibration standards (CAL): A PDS of target analytes was prepared in DCM at a concentration of 20 µg/mL (PDS). Two secondary dilution standards (SDSs) were prepared in DCM at 0.2 µg/mL (SDS-1) and 0.02 µg/mL (SDS-2) by serial dilution. Calibration standards were prepared in final volume of 1 mL of DCM as follows and as shown in **Table 2**: 100 µL of ISTD-PDS and 50 µL of S-PDS were added to the appropriate volume of SDS-1 or SDS-2 for each CAL accordingly, and DCM was added to a final volume of 1 mL. Each CAL concentration of the target analytes in 1 mL DCM extract (ppb) are given in the table.

Similarly, PDS, SDS-1 and SDS-2 in reagent water were prepared at 20 µg/mL, 0.2 µg/mL and 0.02 µg/mL, respectively, by serial dilution. These mixtures are used to spike reagent water for extraction.

2.3 Sample Preparation

A 500-mL of reagent water sample was treated with 40-50 mg of sodium thiosulfate for dechlorination and spiked with 50 µL of S-PDS, and varying amounts of the target analyte PDS. The sample containing the analytes and surrogate was extracted via solid phase extraction (SPE) using a pre-packed coconut charcoal cartridge on a 24-port block vacuum manifold. Prior to extraction, the cartridge was conditioned with DCM, MeOH, and reagent water following the procedure outlined in method EEA 521.1. During the extraction of the water sample, minimal low vacuum (~2 psi) was applied. At this low vacuum (~2 psi) extraction flowrate was approximately 3 mL/min. The cartridge was dried by applying a high vacuum (~10 psi) for 10 minutes to remove all remaining water and was eluted immediately with DCM. The eluate was passed through two drying columns to remove any remaining water; each drying column contained 2.5 g of sodium sulfate and had been pre-conditioned with DCM. After passing through the cartridge and drying column, the DCM eluate was collected in a 15-mL conical glass centrifuge tube. The elution step, through the cartridge and drying column, was repeated until a total volume of 14 mL DCM eluate was collected.

The sample extracted in DCM was capped, wrapped in Parafilm, and stored in a -20 ºC freezer until the sample concentration step was completed (usually the next day). The sample concentration step was performed using a gentle stream of nitrogen to evaporate the solvent to a final volume of approximately 0.5-0.85 mL. The concentrated extract was transferred from the 15-mL centrifuge tube to a 1 mL volumetric flask, by rinsing the tube with a small amount of DCM to ensure quantitative transfer. A 100 µL of IS- PDS was spiked to each extract and the volume was adjusted with DCM to a final volume of 1 mL. This final 1-mL solution is referred to as the DCM extract in this report.

The 1 mL DCM extracts were stored until analysis in 2-mL amber glass vials (Sun-Sri 200 514) at -20 ºC with caps wrapped in Parafilm. Aliquots of the extract were analyzed in amber GC autosampler vials with 100- 200 µL inserts. All DCM extract samples were analyzed within the14-day holding time for the extract.

2.4 Sample analysis by GC-MS/MS

The single-lab validation was performed on a Thermo TSQ8000 Evo mass spectrometer (MS) interfaced with a Thermo GC Trace 1310. The MS was operated in electron ionization mode (EI) and tandem mass spectrometry (MS/MS). For the identification and quantitation of the analytes the product ions listed in **Table 3** were used. Specifically, a multiple reaction monitoring method (MRM) was developed, in which specific ion transitions per analyte were selected and collision energy for each optimized. For each analyte the transition used for quantitation was based on; 1) signal intensity, 2) peak shape and S/N ratio, and 3) absence of interferences in extracted blanks. Retention times with 1-minute windows were established for each analyte with

the GC oven temperature program and column as shown in **Table 1**. Ten scans across each GC peak were collected.

3. Validation Study

The water samples are spiked and prepared at ng/L or parts per trillion (ppt) levels. The sample preparation concentrates the water sample to a ug/L or parts per billion (ppb) level and achieves a 500-fold concentration factor. Hence, the instrument detection levels are in the unit of ppb. For example, a 500 mL of a water sample is extracted and concentrated to a final volume of 1 mL (in DCM solvent). An 8 ppt water sample corresponds to a 4 ppb concentration level on the calibration.

The terminology used in this document corresponds to those used in the ATP requirements such as Minimum Reporting Level, Method Blank, Dilution Factors.

3.1 Calibration

The calibration curve is the range of concentrations where the instrument signals are directly proportional to the concentration of the analyte in the sample. The concentration of the analytes is calculated using an internal standard; the single ISTD (NDPA-D14) is added to samples after extraction prior to instrument analysis.

The calibration curves had a minimum of 8 points and up to a maximum of 10 points per analyte. The best fit line was established where the correlation coefficients were higher or equal to R^2>0.99 and at least one calibration standard was at <RL. The calibration curves were established following these acceptance criteria: For each CAL standard except for the lowest one, the quantitative and confirming transition of each analyte should have a signal to noise (S/N) $\geq$ 3 and a recovery of 70 – 130% to the true value. For the lowest CAL standard recovery levels were accepted at 50 – 150% of the true value. The instrument calibration curve ranges for each of the target analytes are given in **Table 4** which varied at the low end from 0.1 ppb (NDEA) to 1 ppb (NPIP), while the high end was at 100 ppb for all analytes.

3.2 Accuracy

Accuracy studies are used to evaluate whether systematic errors (method bias) are associated with the use of the method within a single day and between different days. Method bias is estimated by analyzing spiked reagent water at three levels, at the extremes of the quantitation range and at regulatory levels (if any) and calculating the recoveries to the true value. The ATP acceptance criteria for accuracy is for concentrations > 2xMRL at ± 30% and for concentrations $\leq$ 2MRL at ± 50% (Section 3.7).

Accuracy of the method was evaluated intra-day and inter-day at three concentrations, 1 ppt, 10 ppt and 100 ppt for each target analyte in spiked water samples. The intra-day study was

conducted by extracting and measuring replicate samples in a single day at 1 ppt (N=3), 10 ppt (N=6), and 100 ppt (N=3). The inter-day bias study was conducted over three or four different days where replicate extractions and analyses were at 1 ppt (N=8), 10 ppt (N=10), and 100 ppt (N=8). Results of the accuracy study is given in **Table 5**. Data show average recoveries at all concentrations to be within the acceptance criteria.

3.3 Precision

Precision studies are used to evaluate random (indeterminate) errors associated with the method within a single day and between different days. Precision is evaluated similar to accuracy, by analyzing spiked reagent water near or at the extremes of the quantitation range and at regulatory levels (if any) and calculating either the dispersion based on standard deviation or the relative standard deviation (RSD) of the measurements. In this method the acceptance criteria for precision were set at RSD ± 30%.

Precision was evaluated intra-day and inter-day, at three concentrations, 1 ppt, 10 ppt and 100 ppt for each target analyte in water samples. The intra-day precision study was conducted by extracting and analyzing a total of 12 replicate samples in a single day at 1 ppt (N=3), 10 ppt (N=6), and 100 ppt (N=3). The inter-day precision study was conducted over three or four different days where a total of 26 replicate samples were extracted and analyzed at 1 ppt (N=8), 10 ppt (N=10), and 100 ppt (N=8). Results of the precision study are given in **Table 6**. Both intra- and inter-day precision for all analytes were within the acceptable criteria and in general RSD was <10%.

3.4 Upper limit of quantitation

Linearity of the calibration curve was evaluated up to 1000 ppt concentration in water sample. A low range calibration curve with a maximum concentration of 200 ppt and a high range calibration curve with a maximum of 1000 ppt were generated. The acceptable criteria for all the calibration points were the same as those mentioned in section 3.1 above. The R^2 was calculated using a linear, 1/A-weighted curve fit from the lowest to highest end of the calibration curve, and the results are tabulated in **Table 7**. For all analytes R^2>0.99 with that for NMOR R^2=0.999.

3.5 Dilution factors

Dilution factors or concentration factors are used to relate the calibration curve to the quantitation range. Sample dilution can affect method performance including accuracy, precision and reporting limits. When validating a method one of the requirements is to demonstrate that sample dilution does not affect the final analytical result. For this study the water samples were diluted prior to extraction and the diluted samples followed the entire procedure (i.e. extraction, concentration, measurement).

Five water samples at 1000 ppt were prepared. One of the 1000 ppt water samples was diluted by a factor of 2 to prepare two 500 ppt water samples while the second 1000 ppt water sample was diluted by a factor of 10 to prepare two 100 ppt water samples. After the dilution, S-PDS was added to each sample, i.e. 1000 ppt (N=3), 500 ppt (N=2), and 100 ppt (N=2). Samples at all three concentrations were extracted and analyzed following the method. Dilution effect was evaluated by calculating the percent recoveries. Results of the study is given in **Table 8**.

Sample dilution (x2, x10) did not affect the method performance as demonstrated from the comparable analyte recoveries obtained at all three dilution experiments. Cartridge breakthrough was not observed. This study also shows that 2 g charcoal substrate used for SPE can retain up to 1000 ppt of all eight target nitrosamines from a 500 mL water sample.

3.6 Method detection limit and Reporting limit

The Method Detection Limit (MDL) was established using spiked reagent water samples following the procedure described in 40 CFR 136, Appendix B. MDL values for each target analyte was determined by spiking reagent water at 4 ppt. Eight replicate samples were prepared and analyzed on four different days. Since sample spike level was above the CAL-1 level, all required QC criteria equal to an LFB sample were applied and met. The experimentally determined MDL values for each analyte are given in **Table 9**.

The MDL value was multiplied by 5 to calculate the Reporting Level (RL) for each analyte per our laboratory reporting policy. **Table 9** shows the MDL and RL values for the target analytes.

3.7 Minimum reporting level

The Minimum Reporting Level (MRL) is the minimum concentration of the target analyte that can be quantified and reported following the entire sample analysis, including sample extraction. MRL is an experimentally confirmed value (with at least 7 spiked replicate samples extracted and analyzed on at least three different days). The MRL is recommended to be no lower than the concentration of the lowest calibration standard.

The MRL is confirmed by measuring replicate spiked samples at a target concentration, calculating the mean recovery and standard deviation, and determining statistically whether the prediction interval of results (PIR, an interval of recoveries in which 99% of future samples analyzed are expected to fall) is between 50-150% of the spiked amount. When PIR is 50-150% of the spiked amount, the MRL is confirmed at the target concentration; if not, a sample spiked at a higher concentration is tested to attempt confirmation. In addition, MRL confirmation samples must meet the QC acceptance criteria that are applied to all samples, specifically the percent recovery of each analyte be 50-150% (the 70-130% recovery acceptance limits apply to samples measured where concentrations are higher than the MRL).

MRL values for the analytes were determined from either eight replicates spiked at 1 ppt analyzed on three different days, eight replicates spiked at 4 ppt analyzed on four different days, or from seven replicates spiked at 8 ppt analyzed on three different days. Results of the MRL values for all target analytes are given in **Table 10**. For the analyte NDPA, the MRL confirmation failed at all three levels tested (i.e. the lower PIR limit for each was <50%) and therefore MRL value could not be determined. It is the intention to spike at a higher level and repeat the study for NDPA in future.

In this method RLs (Section 3.6) were found to be above or equal to the MRL values for all the target analytes except for NDPA, NPIP and NDBA. For these analytes the measured MRL values were above the statistically calculated RLs, therefore an adjustment to the RL values was necessary. The adjusted RL value was found to be 8 ppt for NDBA and NPIP, but for NDPA an MRL is pending since the 10 ppt spiked samples failed the required criteria (recovery and PIR limits).

3.8 Ruggedness

Ruggedness is the ability of an analytical method to remain unaffected by small variations in the method parameters and influential environmental factors. During method validation work, different factors can be tested to evaluate ruggedness including the use of different solvents, instruments, analysts, and consumables lot numbers. In this validation study we investigated four factors (but others might need to be investigated in the future):

3.8.1 Extract volume during concentration: During the initial extract concentration step when the sample is evaporated with nitrogen, it is critical to keep the final volume at $\geq$0.5 mL, as specified in the method. When extracts were concentrated to less than 0.5 mL, e.g. due to longer evaporation time or higher nitrogen flow, analyte losses were observed. Especially for the most volatile compounds a loss of up to 80% was observed.

3.8.2 SPE cartridges lot numbers: two different lot numbers of cartridges were tested for this method. The target analyte recoveries for water samples spiked at 10 ppt were slightly better from one lot number over the other (N=3 for each lot number). Overall, the relative percent difference between the two lot numbers ranged within 0 – 7% depending on the analyte.

3.8.3 Temperature of reagents: Primary dilution standards (PDSs) in DCM are prepared and stored at -20 ^{o}C. It is crucial to allow the PDSs to warm to room temperature prior to analysis. When the ISTD-PDS was not allowed to fully warm/thaw, dispensing the normal volume of ISTD-PDS into samples yielded results with higher levels of ISTD compared to samples where the ISTD-PDS was warmed to room temperature prior to use. PDSs' temperature can cause failure of the QC criteria.

3.8.4 Type of reagent water: Several sources of reagent water were tested, and results indicated the importance of using ultrapure reagent water that has undergone UV treatment (resistivity of 18.2 MΩ.cm, TOC < 10 ppb and bacterial count <10 CFU/ml). Water that was passed through ion-exchange resin without being subsequently UV-treated was found to contain high levels of NDMA (between 15-20 ppt). In contrast, water from an in-lab purification system that uses UV treatment was found to contain minimal levels of each analyte. A commercial source ultrapure water, Alfa Aesar (PN: 22934, Ultrapure, HPLC grade) contained similar levels of background analyte to the in-lab UV-purified water and was also acceptable for use.

3.9 Method blank

A method blank (MB) is reagent water taken through the entire experimental procedure and analyzed in the same manner as a drinking water sample. Method blanks are used to assess any contamination or interference caused by reagents or by any part of the measurement procedure in the laboratory environment, including instrumentation.

The ATP acceptance criterion for MBs is for the determined concentration of the target analytes be <1/3 MRL value (where MRL is defined in Section 3.7). **Table 11** shows results for ten MB extracts measured on four different days. For each target analyte the ten measurements were averaged, and the standard deviation determined. All MB values were less than 1/3 the MRL for each analyte.

3.10 Initial demonstration of capability

The initial demonstration of capability (IDC) for a method is performed to demonstrate the accuracy and precision of the method by preparing, extracting, and analyzing four to seven replicate LFB samples.

The IDC study was performed by spiking six water samples at 10 ppt with each target analyte, extracting the samples, and analyzing them on a single day. Each IDC sample was processed and analyzed as an unknown sample. The percent recovery for each analyte was calculated, averaged and the relative standard deviation determined. Results of the IDC study is shown in **Table 12**. The required acceptance criteria for an IDC study are similar to those of any sample analyzed with this methodology. Specifically, the requirement is to achieve a 70 – 130% recovery from the true value for all analytes with an RSD< 20%. As shown in **Table 12** the recoveries ranged between 85 – 98% with RSD values between 4 – 8%.

3.11 Internal standard and surrogate recoveries

The internal standard (ISTD) and surrogate recoveries are used to assess the method for each sample that is analyzed. The ISTD used was deuterated-NDPA (NDPA-D14) and the surrogate was deuterated-NDMA (NDMA-D6).

The peak area of the ISTD was monitored for each sample injection. The ISTD peak area for all sample measurements was monitored to ensure there is no deviation from the ISTD peak area of the most recent continuous calibration check sample (CCC) with acceptable levels between 70-130%; and be ≥50% from the average ISTD area measured for the current calibration curve. The average area counts for the ISTD from 50 samples measured over five different days was found to be 4276 ± 1146 counts.

The surrogate concentration was found to be within 70-130% of true value of the spiked amount of NDMA-D6, which was 20 ppt in water samples. The average surrogate recovery from 44 samples measured over six days was 85% ± 6%.

3.12 Sample extract stability

The method allows a holding time of 14-days for the DCM extract at -20 ^{o}C temperature. We studied the stability of the extract at 14 days, 21 days and 28 days after extraction.

Spiked reagent water samples at two concentrations, 4 ppt and 8 ppt, were taken through the extraction process and the DCM extracts were stored in amber glass vials at -20 ^{o}C until analysis. At each concentration six replicate water samples were prepared, and extractions were performed on two different days (N=3 at each concentration). An aliquot of each DCM extract was analyzed between 2-5 days of extraction, which is within the holding time (<14-days), followed by aliquots measured at 14-days, 21-days, and 28-days after extraction. The percent recoveries of the target analytes were calculated to evaluate the stability of the extracts. The stability study results are shown in **Graph 1** (4 ppt) and **Graph 2** (8 ppt) indicate that the extracts were stable up to 28-days when stored at -20 ^{o}C protected from light.

3.13 Sample storage stability

Sample holding time per method EPA 521 is 14 days after sample collection when stored at <6 ^{o}C. In this method validation study sample storage stability was not further evaluated.

4. Intra-Laboratory Validation Study

An intra-laboratory validation study of the method EEE 521.1 was performed by collaborating with the Biomonitoring Unit (BU) of DWRL on an Agilent Technologies Inc. 7890A/7000 instrument. For this study, split DCM extracts of the spiked study samples (prepared by the CU) were provided to the BU for analysis. These extracts were analyzed on the Agilent7000 instrument simultaneously within the same week as those analyzed on the TSQ8000 Evo instrument. The BU instrument was used to validate the method for calibration, upper limit of quantitation, accuracy, precision, and sensitivity (MDL).

4.1 Equipment

Refer to **Table 1** for a detailed comparison of the instrument parameters and analysis conditions between the Agilent7000 instrument and TSQ8000 Evo instrument.

4.2 Calibration

Calibration curves for the target analytes were established on the Agilent7000 instrument following the criteria specified in section 3.1 above. **Table 13** shows the comparison of the calibration curves for the two instruments. The lowest calibration standard for all the target analytes except NPIP were higher on the Agilent7000 (0.5 ppb) compared to the TSQ8000 Evo (0.1 – 0.25 ppb). For NPIP the Agilent7000 instrument was able to reach 0.5 ppb while TSQ8000 Evo was higher at 1 ppb.

4.3 Accuracy

Intra- and Inter-day accuracy for the intra-lab comparison was determined on both instrumentations and the percent recoveries were compared (**Tables 14A and 14B**). Eight reagent water samples were spiked at 4 ppt and analyzed over four different days and seven reagent water samples spiked at 8 ppt were analyzed over three different days. Results indicate excellent comparison between the two instruments for seven target analytes, with recoveries ranging between 66% - 106% for both systems.

4.4 Precision

Intra-day and Inter-day precision was measured on both instruments and the relative standard deviation of replicate measurements for each target analyte was calculated. Precision was calculated using the data from the intra-day and inter-day accuracy study and the results (%RSD) are shown in **Tables 15A and 15B**. Overall, precision on both instruments compared well with the Agilent7000 instrument having slightly better precision on average than the TSQ8000 Evo instrument: for example, with the 4 ppt water samples, the average inter-day RSD for all analytes was 8.1% on the TSQ8000 Evo and 5.8% on the Agilent7000. Differences in RSDs on the two instruments affect the MDL values as well, as shown in Section 4.5.

4.5 Method detection limit

Water samples spiked at 4 ppt were used to determine the MDL values for each target analyte on both systems. Eight replicate samples were analyzed on four different days. **Table 16** provides the comparison of MDL values for each target analyte between the two instruments. Overall, the MDL values are comparable on the TSQ8000 Evo and Agilent7000 with the latter being slightly better than the former. The lower MDL values on the Agilent7000 can be explained by two factors that affected precision. First, the measurements were performed during a shorter time span on the Agilent7000 than on the TSQ8000 thus, the precision was higher in the former. Second, several analytes in the samples measured on the Agilent7000 were shown to have lower intra-day variability (Section 4.4).

5. Inter-Laboratory Comparison

The method EEA-521.1 was developed and validated by Eurofins using the Agilent7010B instrument system which incorporates a much more sensitive source for ionization that of high efficiency than both the TSQ8000 Evo and Agilent7000 instruments used in the current study. The comparable Thermo system to the Agilent7010B is the TSQ9000 with an advanced electron ionization (AEI) ion source. In order to evaluate the performance of EEA-521.1 method on a TSQ9000 and to assess detection sensitivity compared to the TSQ8000 Evo, DWRL collaborated with Thermo Scientific in New Jersey to perform an inter-laboratory comparison study.

All eight target analytes were included in the study. The required samples were prepared and extracted by DWRL, stored at -20 ^{0}C and shipped to the Thermo laboratory by overnight delivery. The new DCM extracts were analyzed by both laboratories during the same week. Three parameters were compared, (1) calibration curve range, (2) instrument sensitivity using a method blank sample, and (3) accuracy and method sensitivity. As expected, the TSQ9000 demonstrated better signal, about 25x higher ion count with its AEI source compared to TSQ8000 Evo ExtractaBrite source when analyzing the same quantity of calibration compound.

5.1 Calibration

Calibration curves were established by analyzing 14 calibration standards (0.05 – 100 ppb). The acceptable criteria applied to evaluate each calibration point were the same as those followed in Section 3.1. The TSQ9000 showed a wider calibration curve range starting at 0.05 ppb for all target analytes compared to TSQ8000 Evo which had the lowest calibration sample between 0.1 – 1 ppb (**Table 17**). ThermoFisher laboratory indicated that the TSQ9000 had demonstrated good signal/noise ratio (S/N $\geq$ 3 for both quant and qual ions) at an even lower concentration, that of 0.025 ppb for all analytes.

5.2 Instrument sensitivity using method blanks

A method blank (MB) sample was processed and run on the two instruments (**Table 18**). The TSQ8000 Evo detected the presence of two target analytes (NDMA and NDBA) while the TSQ9000 was able to detect background interferences from four analytes (NDMA, NDEA, NMOR, NDBA) in the same MB extract. Similar ion ratio and signal/noise >3 criteria were implemented in both instruments. While the amounts of the interferences detected in the MB on the TSQ8000 Evo is not significant enough (<1/3 MRL) to be a concern during sample analysis, for the TSQ9000 having lower levels of quantitation the MB interference amounts are significant, and a background subtraction was found to be necessary for sample quantitation.

5.3 Accuracy and method sensitivity

To assess the sensitivity of the method on the two instruments, we compared the accuracy of samples spiked at four different concentrations. Spiked reagent water at four low concentrations of 0.1 ppt, 0.5 ppt, 1 ppt, and 4 ppt were prepared in duplicate and taken through the extraction procedure. The acceptance criteria implemented for recovery were the same as in section 3.2. As shown in **Table 19** the TSQ9000 performed significantly better at spike levels < 1 ppt in comparison to TSQ8000 Evo. More precisely, at 0.5 ppt the TSQ 9000 presented acceptable recoveries for all analytes in duplicate samples and at 0.1 ppt three of the analytes were acceptable.

6. Conclusion

Method EEA-521.1 has been evaluated and validated adopting the US. EPA ATP protocol and requirements for use on a ThermoFisher Scientific TSQ8000 Evo triple quadrupole mass spectrometer with an ExtractaBrite ion source. An intra-laboratory validation study with split extract samples demonstrated the applicability of the method on an Agilent Technologies 7000 instrument with an Extractor ion source. Following the guidelines from US EPA ATP validation criteria, the method was shown to be selective, precise, linear and accurate for the target analytes tested and showed excellent correlation of parameters achievable between the two instruments. However, during the MRL determinations DWRLB was unable to achieve ultralow MRL levels with TSQ 8000 instrument. Therefore, DWRLB has optimized the solid-phase extraction flow rate and the final extraction volume to enhance sensitivity (Section 2.3). In addition, DWRLB has experienced high background interference (nitroso amines and unknown compounds) in sample blanks. DWRLB found that different types of reagent water had varying amounts of background contamination and as such, laboratory had to switch from the original water source to a different water source to have sufficiently low background levels (Section 3.8.4).

The RL values, which is 5xMDL according to laboratory reporting policy, ranged from 1.6 ppt to 6 ppt in water samples measured by both instruments, while the MRL values determined only on

the TSQ8000 Evo ranged from 1 ppt and 8 ppt in water samples. For the analyte NDPA, the MRL confirmation criteria failed at all three levels tested (1 ppt, 4 ppt, 8 ppt) and therefore MRL failed verification in these experiments (Table 10). The systems were sensitive to detect the presence of a trace amount (1 ppt) of only two N-nitrosamines in reagent water samples. Sample DCM extracts were found to be stable up to 28-days when preserved at -20 ^{o}C.

The inter-laboratory study included a comparison between TSQ8000 Evo and TSQ9000 (AEI ion source) for the detection of all eight target analytes. Results suggested TSQ9000 to have a higher sensitivity with acceptable accuracy at 0.5 ppt for all analytes. However, higher sensitivity made it possible to detect high background interference for four target analytes in method blanks; two more than those detected with the TSQ8000 Evo. Some background interference issues can be laboratory specific and those need to be solved by each individual laboratory. The TSQ9000 had a wider estimated measurement range (starting at 0.1 ppt in water sample) which is comparable to that of the Agilent 7010B used in method EEA-521.1. Best practice for determination of all 8 nitrosamines at ultralow concentrations (< 4 ppt) using EEA521.1 method in a laboratory should be: 1) using the highest sensitive instruments, 2) identifying the background contamination issues and finding solutions to overcome them before using the method for sample measurements.

7. Acknowledgments

DWRL would like to thank ThermoFisher Scientific laboratory in New Jersey for testing the DCM extracts for the inter-laboratory study.

8. Tables and Graphs

Table 1: GC-MS/MS Systems

Injection parameter	TRACE 1310/TSQ8000 Evo setpoint	Agilent 7890A GC/7000 setpoint	TRACE 1310/TSQ9000 setpoint
Temperature	240 °C	250 °C	240 °C
Injection volume	2 µL	2 µL	2 µL
Liner	Thermo splitless, single-tapered, 4 mm deactivated liner with quartz wool (P/N 453A1925)	Agilent splitless, double-tapered, 4 mm deactivated liner (P/N 5181-3315)	Restek splitless, single-tapered, 4 mm deactivated liner with CarboFrit (P/N 20799-209.5)
Mode	Splitless with surge	Splitless	Splitless with surge
Carrier gas	Helium	Helium	Helium
Column flow	1.3 mL/min	1.2 mL/min	1.3 mL/min
Septum purge flow	5.0 mL/min	3.0 mL/min	5.0 mL/min
Splitless time	1.01 min	0.8 min	1.00 min
Split flow	81.0 mL/min	100 mL/min	81.0 mL/min
Surge pressure	173.0 kPa	N/A	172.4 kPa
Surge duration	1.00 min	N/A	1.01 min

GC parameter	TRACE 1310/TSQ 8000 Evo	Agilent 7890A GC/7000	TRACE 1310/TSQ 9000
Column	Thermo TG-1701MS (P/N 26090-2960)	Thermo TG-1701MS (P/N 26090-2960)	Thermo TG-1701MS (P/N 26090-2230)
Column phase	14% Cyanopropylphenyl 86% dimethylpolysiloxane	14% Cyanopropylphenyl 86% dimethylpolysiloxane	14% Cyanopropylphenyl 86% dimethylpolysiloxane
Column dimensions	30 m length × 250 µm diameter, 1 µm film thickness	30 m length × 250 µm diameter, 1 µm film thickness	30 m length × 250 µm diameter, 0.5 µm film thickness
Oven temperature	Initial: 35 °C (hold 1 minute) Rate 1: Ramp 25 °C/min to 130 °C Rate 2: Ramp 20 °C/min to 250 °C (hold 2 minutes)	Initial: 35 °C (hold 1 minute) Rate 1: Ramp 25 °C/min to 130 °C Rate 2: Ramp 20 °C/min to 250 °C (hold 3 minutes)	Initial: 35 °C (hold 1 minute) Rate 1: Ramp 25 °C/min to 130 °C Rate 2: Ramp 20 °C/min to 250 °C (hold 2 minutes)
Equilibration time	0.5 minutes	3-5 minutes	0.5 minutes

MS parameter	TRACE 1310/TSQ 8000 Evo	Agilent 7890A GC/7000	TRACE 1310/TSQ 9000
Ion source	EI, Thermo ExtractaBrite	EI, Agilent Extractor	EI, Thermo AEI
Electron energy	70 eV	70 eV	50 eV
Source temperature	300 °C	230 °C	300 °C
Transfer line temperature	250 °C	280 °C	250 °C
Collision gas	Argon	Nitrogen	Argon
Gain	34.3	1.0	21.0

Table 2: Calibration Standards

Calibration Level	Volume of SDS-2 (0.02 ug/mL analyte in DCM) (µL)	Volume of SDS-1 (0.2 ug/mL analyte in DCM) (µL)	Analyte concentration in 1 mL DCM extract (ppb)
CAL 1	2.5		0.05
CAL 2	5		0.1
CAL 3	10		0.2
CAL 4	12.5		0.25
CAL 5	15		0.3
CAL 6	20		0.4
CAL 7	25		0.5
CAL 8	50		1
CAL 9		12.5	2.5
CAL 10		25	5
CAL 11		50	10
CAL 12		125	25
CAL 13		250	50
CAL 14		500	100

Table 3: Selected Transitions for the Instruments

SRM Transitions	TSQ8000 Evo Transitions				Agilent7000 Transitions					TSQ9000 Transitions				
	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	collision energy	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	collision energy	dwell time (ms)	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	collision energy	dwell time prioritization
NDMA-D6 quan		80	50	5		80	50	8	75		80	50	5	normal
NDMA-D6 qual 1	5.68	80	46	15	5.78	80	46	25	75	4.78	80	46	15	normal
NDMA-D6 qual 2		80	30	15							80	30	15	normal
NDMA quan		74	44	5		74	44	6	75		74	42	15	high
NDMA qual 1	5.7	74	42	15	5.79	74	42	22	75	4.79	74	44	5	high
NDMA qual 2											74	43	10	high
NMEA quan		88	71	5		88	71	23	100		88	71	5	high
NMEA qual 1	6.33	88	42	15	6.47	88	42	4	100	5.41	88	42	15	high
NMEA qual 2											88	43	5	high
NDEA quan		102	85	5		102	85	4	100		102	85	5	high
NDEA qual 1	7.01	102	44	10	7.01	102	44	12	100	5.9	102	44	10	high
NDEA qual 2											102	56	15	high
NDPA-D14 quan		78	46	10		144	126	10	50		78	46	10	normal
NDPA-D14 qual 1	8.21	110	78	5	8.01	144	50	20	50	7.1	110	78	5	normal
NDPA-D14 qual 2		144	126	5							144	126	5	normal
NDPA quan		130	113	5		130	43	10	100		130	113	5	high
NDPA qual 1	8.27	130	43	10	8.34	101	70	10	100	7.14	70	41	10	high
NDPA qual 2											130	43	10	high
NMOR quan		116	56	10		116	86	2	100		116	56	10	high
NMOR qual 1	8.61	86	56	5	8.65	116	56	15	100	7.41	116	86	5	high
NMOR qual 2														high
NPYR quan		100	55	5		100	55	7	100		100	55	5	high
NPYR qual 1	8.75	100	70	5	8.8	100	70	7	100	7.56	100	43	5	high
NPYR qual 2						100	43	10	100		100	70	5	high
NPIP quan		114	97	5		114	84	7	100		114	84	5	high
NPIP qual 1	8.91	114	84	5	9	114	55	25	100	7.71	114	97	5	high
NPIP qual 2											114	41	10	high
NDBA quan		116	99	5		158	141	10	75		116	99	5	high
NDBA qual 1	9.72	158	99	5	9.72	158	99	10	75	8.47	158	99	5	high
NDBA qual 2						116	99	10	75		158	141	5	high

Table 4: Instrument Calibration Curves for Target Analytes

Analyte	TSQ8000 Evo calibration curves in DCM extract (ppb)
NDMA	0.2 - 100
NMEA	0.2 - 100
NDEA	0.1 - 100
NDPA	0.2 - 100
NMOR	0.25 - 100
NPYR	0.25 - 100
NPIP	1 - 100
NDBA	0.25 - 100

Table 5: Accuracy Study

Analyte	Average Recovery of 1 ppt water sample for TSQ8000 Evo		Average Recovery of 10 ppt water sample for TSQ8000 Evo		Average Recovery of 100 ppt water sample for TSQ8000 Evo	
	Intra-day (N=3)	Inter-day (N=8)	Intra-day (N=6)	Inter-day (N=10)	Intra-day (N=3)	Inter-day (N=8)
NDMA	125%	114%	89%	86%	81%	82%
NMEA	74%	78%	86%	82%	79%	81%
NDEA	85%	82%	86%	81%	77%	79%
NDPA	70%	66%	85%	78%	76%	76%
NMOR	70%	84%	90%	88%	85%	87%
NPYR	140%	108%	93%	90%	92%	90%
NPIP	102%	96%	98%	96%	87%	87%
NDBA	110%	104%	87%	83%	82%	78%

Table 6: Precision Study

Analyte	RSD at 1 ppt for TSQ8000 Evo		RSD at 10 ppt for TSQ8000 Evo		RSD at 100 ppt for TSQ8000 Evo	
	Intra-day (N=3)	Inter-day (N=8)	Intra-day (N=6)	Inter-day (N=10)	Intra-day (N=3)	Inter-day (N=8)
NDMA	3.6%	11%	5.6%	6.8%	5.4%	4.6%
NMEA	8.7%	7.9%	4.5%	8.0%	4.9%	6.3%
NDEA	9.2%	10%	5.0%	8.8%	5.1%	7.3%
NDPA	34%	24%	7.4%	14%	5.0%	8.6%
NMOR	47%	27%	8.8%	8.1%	5.7%	4.6%
NPYR	2.1%	32%	7.0%	6.7%	3.8%	3.2%
NPIP	15%	21%	7.6%	7.2%	2.6%	5.2%
NDBA	5.5%	11%	6.3%	8.7%	5.2%	6.5%

Table 7: Upper Limit of Quantitation

Analyte	Calibration up to 100 ppb TSQ8000 Evo		Calibration up to 500 ppb TSQ8000 Evo	
	Lowest acceptable cal standard (ppb)	R^2	Lowest acceptable cal standard (ppb)	R^2
NDMA	0.2	0.998	0.2	0.995
NMEA	0.05	0.998	0.05	0.996
NDEA	0.1	0.998	0.1	0.996
NDPA	0.4	0.997	0.4	0.995
NMOR	0.2	0.999	0.05	0.996
NPYR	0.3	0.998	0.3	0.993
NPIP	0.3	0.997	0.3	0.993
NDBA	0.2	0.995	0.1	0.996

Table 8: Effects of Water Sample Dilutions

Analyte	Recovery range for 1000 ppt water sample (N=3)	Recovery range for 1000 ppt water sample, diluted by factor of 2 (500 ppt, N=2)	Recovery range for 1000 ppt water sample, diluted by factor of 10 (100 ppt, N=2)
NDMA	81-82%	76-82%	78-84%
NMEA	79-81%	75-79%	75-80%
NDEA	77-79%	72-77%	71-77%
NDPA	76-79%	72-78%	73-78%
NMOR	87-90%	82-89%	80-89%
NPYR	87-91%	82-88%	83-87%
NPIP	82-85%	80-83%	77-82%
NDBA	76-80%	71-76%	71-73%

Table 9: MDL and RL Study

Analyte	MDL (ppt) (N=8) TSQ8000 Evo	RL (=MDLx5) (ppt)
NDMA	0.75	3.77
NMEA	0.32	1.59
NDEA	0.51	2.55
NDPA	0.76	3.82
NMOR	0.97	4.86
NPYR	0.99	4.94
NPIP	0.79	3.95
NDBA	1.19	5.95

Table 10: MRL Verification Study

Analyte	MRL (ppt) TSQ8000 Evo
NDMA	4
NMEA	1
NDEA	1
NDPA	ND
NMOR	4
NPYR	4
NPIP	8
NDBA	8

Table 11: Method Blank Study

Analyte	Average Method Blanks (ppt) (N=10) TSQ8000 Evo*	Standard deviation of Method Blanks (ppt) (N=10) TSQ8000 Evo
NDMA	0.27	0.07
NMEA	0.01	0.01
NDEA	0.04	0.02
NDPA	0.04	0.03
NMOR	0.05	0.05
NPYR	0.23	0.18
NPIP	0.10	0.19
NDBA	0.69	0.28
* values are <MDL		

Table 12: IDC and Method Bias

Analyte	Average Recovery (N=6) TSQ8000 Evo	Relative standard deviation (N=6) TSQ8000 Evo
NDMA-D6	90%	4.1%
NDMA	89%	5.6%
NMEA	86%	4.5%
NDEA	86%	5.0%
NDPA	85%	7.4%
NMOR	90%	8.8%
NPYR	93%	7.0%
NPIP	98%	7.6%
NDBA	87%	6.3%

Table 13: Calibration Curves Comparison between TSQ8000 Evo and Agilent7000

Analyte	TSQ8000 Evo calibration curves in DCM extract (ppb)	Agilent7000 calibration curves in DCM extract (ppb)
NDMA	0.2 - 100	0.5 - 100
NMEA	0.2 - 100	0.5 - 100
NDEA	0.1 - 100	0.5 - 100
NDPA	0.2 - 100	0.5 - 100
NMOR	0.25 - 100	0.5 - 100
NPYR	0.25 - 100	0.5 - 100
NPIP	1 - 100	0.5 - 100
NDBA	0.25 - 100	0.5 - 100

Table 14 A: Intra-day Accuracy Comparison between TSQ8000 Evo and Agilent7000

Analyte	4 ppt Intra-day Recovery (N=3)		8 ppt Intra-day Recovery (N=3)	
	TSQ8000 Evo	Agilent7000	TSQ8000 Evo	Agilent7000
NDMA	82%	83%	76%	69%
NMEA	74%	90%	71%	74%
NDEA	72%	91%	76%	75%
NDPA	73%	89%	67%	74%
NMOR	83%	93%	77%	75%
NPYR	82%	119%	74%	93%
NPIP	75%	95%	76%	79%
NDBA	71%	103%	76%	87%

Table 14 B: Inter-day Accuracy Comparison between TSQ8000 Evo and Agilent7000

Analyte	4 ppt Inter-day Recovery (N=8)		8 ppt Inter-day Recovery (N=7)	
	TSQ8000 Evo	Agilent7000	TSQ8000 Evo	Agilent7000
NDMA	85%	83%	77%	69%
NMEA	75%	86%	73%	74%
NDEA	76%	91%	74%	76%
NDPA	73%	87%	66%	75%
NMOR	87%	91%	78%	77%
NPYR	89%	114%	79%	96%
NPIP	76%	97%	80%	80%
NDBA	81%	106%	75%	89%

Table 15 A: Intra-day Precision Comparison between TSQ 000 Evo and Agilent7000

Analyte	4 ppt Intra-day RSD (N=3)		8 ppt Intra-day RSD (N=3)	
	TSQ8000 Evo	Agilent7000	TSQ8000 Evo	Agilent7000
NDMA	2.6%	1.9%	3.8%	5.7%
NMEA	1.9%	2.7%	3.6%	6.5%
NDEA	1.9%	2.6%	0.8%	2.0%
NDPA	7.1%	4.3%	2.8%	6.1%
NMOR	9.6%	4.0%	9.4%	6.4%
NPYR	6.4%	4.2%	3.0%	2.6%
NPIP	11.3%	3.3%	4.2%	3.1%
NDBA	4.2%	3.0%	1.6%	5.5%

Table 15 B: Inter-day Precision Comparison between TSQ8000 Evo and Agilent7000

Analyte	4 ppt Inter-day RSD (N=8)		8 ppt Inter-day RSD (N=7)	
	TSQ8000 Evo	Agilent7000	TSQ8000 Evo	Agilent7000
NDMA	7.4%	4.8%	4.0%	5.8%
NMEA	3.5%	6.0%	4.8%	4.7%
NDEA	5.6%	3.5%	6.2%	4.4%
NDPA	8.7%	4.0%	8.1%	7.1%
NMOR	9.3%	6.4%	7.2%	5.1%
NPYR	9.3%	5.6%	11%	3.7%
NPIP	8.7%	6.6%	7.9%	6.4%
NDBA	12%	9.4%	4.5%	5.1%

Table 16: MDL Comparison between TSQ8000 Evo and Agilent7000

Analyte	TSQ8000 Evo MDL (ppt) (N=8)	Agilent7000 MDL (ppt) (N=8)
NDMA	0.75	0.48
NMEA	0.32	0.62
NDEA	0.51	0.38
NDPA	0.76	0.42
NMOR	0.97	0.70
NPYR	0.99	0.76
NPIP	0.79	0.78
NDBA	1.2	1.2

Table 17: Inter-Laboratory Instrument Calibration Curves Comparison

Analyte	Instrument Calibration Curves in DCM extract (ppb)	
	TSQ8000	TSQ9000
NDMA	0.2 – 100	0.05 – 100
NMEA	0.2 – 100	0.05 – 100
NDEA	0.1 – 100	0.05 – 100
NDPA	0.2 – 100	0.05 – 100
NMOR	0.25 – 100	0.05 – 100
NPYR	0.25 – 100	0.05 – 100
NPIP	1 – 100	0.05 – 100
NDBA	0.25 – 100	0.05 – 100

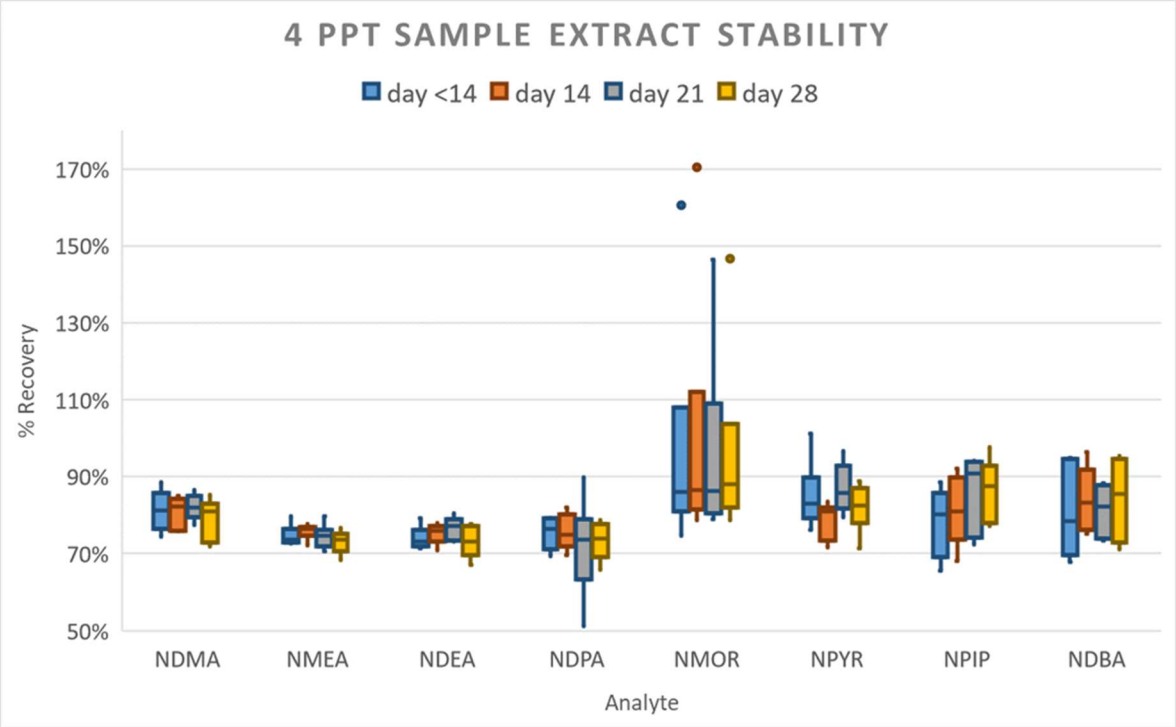
Table 18: Inter-Laboratory Comparison Instrument Sensitivity from Method Blank Samples

Analyte	TSQ8000 blank (ppt) (N=1)	TSQ9000 blank (ppt) (N=1)
NDMA	***0.656***	***0.396***
NMEA	ND	ND
NDEA	ND	***0.116***
NDPA	ND	ND
NMOR	ND	***0.352***
NPYR	ND	ND
NPIP	ND	ND
NDBA	***0.442***	***0.470***
*ND = Non Detect		

Table 19: Inter-Laboratory Extract Recovery Comparison

Analyte	0.1 ppt in water sample average recovery (N=2)		0.5 ppt in water sample average recovery (N=2)		1 ppt in water sample average recovery (N=2)		4 ppt in water sample average recovery (N=2)	
	TSQ8000	TSQ9000	TSQ8000	TSQ9000	TSQ8000	TSQ9000	TSQ8000	TSQ9000
NDMA	--	--	--	74%	53%	90%	78%	91%
NMEA	--	88%	--	92%	72%	97%	73%	98%
NDEA	--	98%	62%	102%	63%	113%	73%	111%
NDPA	--	97%	--	103%	--	117%	71%	122%
NMOR	--	--	--	100%	50%	107%	66%	111%
NPYR	--	--	--	71%	--	109%	67%	124%
NPIP	--	--	--	130%	77%	130%	73%	131%
NDBA	--	--	--	131%	63%	147%	70%	138%
Note: Entries denoted as "--" did not meet the acceptance criteria for recovery (<50%)								

Graph 1: Stability of DCM Extract from 4 ppt Water Sample



Graph 2: Stability of DCM Extract from 8 ppt Water Sample