Laboratory Procedure Manual

Analyte: **Aromatic Diamines**

Matrix: **Urine**

Method: **UPLC-APCI-MS/MS**

Method No: **2120**

As performed by:

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Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

DATA FILE NAME	VARIABLE	ANALYTE
UADM_I	URX4TDA	2,4-Diaminotoluene (4TDA) (ng/mL)
	URX6TDA	2,6-Diaminotoluene (6TDA) (ng/mL)
	URX4MDA	4,4'-Diaminodiphenylmethane (4MDA) (ng/mL)
	URX5NDA	1,5-Diaminonaphthalene (5NDA) (ng/mL)
	URXPPDA	p-Phenylenediamine (PPDA) (ng/mL)

1. CLINICAL RELEVANCE AND SUMMARY OF TEST PRINCIPLE

a) Clinical Relevance

Urinary aromatic diamines can be formed from the metabolism of diisocyanates, which are widely used in the polymer industry, particularly in the production of polyurethane-based consumer products (e.g., foam cushions, mattresses, pillows, and car seats), elastomers, coating, and adhesives.[1] Diisocyanates may be released into the environment through industrial waste, and volatilization and degradation of consumer products. Exposure to diisocyanates can occur through dermal contact, ingestion, and inhalation. The most commonly used aromatic diisocyanates include 4,4'-methylenediphenyldiisocyanate (MDI), 2,4-toluenediisocyanate (4TDI), and 2,6-toluenediisocyanate (6TDI). Others include, but are not limited to, 1,5-naphthalenediisocyanate (NDI) and *p*-phenylenediisocyanate (PPDI). All diisocyanates are classified as skin and respiratory sensitizers.[1, 2] Exposure to these chemicals can be lethal when inhaled at high concentrations by sensitized subjects, can elicit hypersensitivity pneumonitis and accelerated lung function loss, and is considered one of the most frequently reported causes of occupational asthma.[1, 3, 4] In addition, the *in vivo* hydrolyzed product of diisocyanates (i.e., corresponding diamines such as 4,4'-methylenedianiline (4MDA), 2,4 toluenediamine (4TDA), 2,6-toluenediamine (6TDA), 1,5-naphthalenediamine (5NDA), and *p*-phenylenediamine (PPDA)) have been reported as hepatotoxic and carcinogenic in human and animal models.[2, 5-7]

b) Test Principle

This method is a quantitative procedure for the measurement of urinary aromatic diamines using ultra performance liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry (UPLC-APCI-MS/MS). Prior to chromatographic separation, urine samples are hydrolyzed in 0.6 M hydrochloric acid at 80 °C for 4 hours. Hydrolyzed samples are passed through solid phase extraction (SPE) sorbents. Chromatographic separation is achieved using a reversed phase column with 5 mM ammonium acetate buffer at pH 9.2 (mobile phase A) and 5/95% 100 mM ammonium acetate buffer/acetonitrile (mobile phase B). The eluent from the column is ionized using an APCI interface to generate and transmit positive ions into the mass spectrometer.

2. SAFETY PRECAUTIONS

a) Reagent Toxicity or Carcinogenicity

Most of the amines used in this procedure are categorized as toxic following acute exposures. These compounds may also cause respiratory tract, skin and eye irritation. In animal models, aromatic diamines have shown genotoxic and hepatocarcinogenic effects.[2, 6] Laboratory analysts must wear gloves, lab coat, and safety glasses during sample preparation and handling of urine samples. Place disposable plastic, glass, paper, pipette

tips, autosampler tubes, gloves, etc. that contact urine in a biohazard autoclave bag. Keep these bags in appropriate containers until sealed and autoclaved.

Observe Universal Precautions. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/EHLS guidelines for disposal of hazardous waste.

Follow special precautions while handling acetonitrile. Acetonitrile is a flammable liquid and a mucous membrane, skin and eye irritant. **If acetonitrile comes in contact with any part of the body, quickly wash with water.**

b) Radioactive Hazards

This method does not use radioactive materials and is not associated with radioactive hazards

c) Microbiological Hazards

This assay uses human urine samples. Universal precautions must be followed to minimize biological hazards. Analysts working directly with the specimens must use proper technique and avoid direct contact with the samples. Gloves, lab coats and safety glasses must be worn while handling the samples. A Hepatitis *B* vaccination series is recommended for all laboratory analysts who may get exposed to human fluids and tissues.

d) Mechanical Hazards

There are minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the mass spectrometer unless all power to the instrument is off. Generally, only qualified technicians should perform mechanical and electronic maintenance and repair. The UPLC and the mass spectrometer contain a number of areas that are hot enough to cause burns. High voltages are found in certain areas of mass spectrometers. Care must be taken when working in these areas.

e) Protective Equipment

Follow standard safety precautions when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

f) Training

Users are required to demonstrate safe and proper techniques in performing the method, and generate data with acceptable accuracy and precision based on their calibration curves, quality control (QC) and proficiency testing (PT) samples. Educational and specific training information are maintained for all laboratory analysts certified to work on this method.

g) Personal Hygiene

Follow universal precautions. Take care when handling chemicals or any biological specimen. Practice routine use of gloves and proper hand washing. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

h) Disposal of Waste

All laboratory waste disposal must be in compliance with DLS policy. Dispose of solvents and reagents in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. Place all disposable items that come in direct contact with the biological specimens in a biohazard autoclave bag that is kept in appropriate containers until sealed and autoclaved. Immediately place unshielded needles, pipette tips and disposable syringes into a sharps container and autoclave when this container becomes full. Wipe down all surfaces with 70% ethanol solution or equivalent when work is finished.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a) Software and Knowledge Requirements

This method has been validated using Waters UPLC system coupled to the AB Sciex 5500 triple quadrupole mass spectrometer both controlled by Analyst software. Data analysis is performed using MultiQuant software and the quantitation reports are stored as .qsession and ASCII files and entered into the STARLIMS database. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b) Sample Information

Typically, an analytical run contains up to 96 samples. Corresponding batch files contain information such as run ID, sample ID, sample file number, date of analysis, analyst's initials, injection volume, standard and internal standard lot, and special notes and observations for each run. Information pertaining to particular specimens is entered into the batch file either manually or electronically.

c) Data Maintenance

All analytical data sets are checked prior to being entered into the STARLIMS database for transcription errors and overall validity. The database is routinely backed up locally onto a computer hard drive and through the standard practices of the NCEH network. The local area network manager should be contacted for emergency assistance.

d) Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of the results is protected by use of blind coded ID numbers only (no clinical specimen are labeled with personal identifiers).

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a) No special instructions such as fasting or special diets are required.

b) The sample type is urine.

c) An aliquot of 250 µL is needed per assay. A volume of 0.5–1.0 mL is required to allow for repeated analysis. If the sample volume is below 500 µL, a second urine specimen should be requested.

d) Acceptable containers include sterile polystyrene cryovials or polypropylene centrifuge tubes.

e) Specimen suspected of being contaminated due to improper collection procedures or collection devices are considered unacceptable.

f) Specimen handling conditions are outlined in the Division protocol for urine collection and handling (copies available in Branch, Laboratory and Special Activities specimen handling offices). Collection, transport, and special requirements are discussed. Urine specimens should be transported and stored chilled or frozen at -20 ºC. Once received, the samples can be frozen at -70 ºC until time of analysis. Portions of the sample that remain after analytical aliquots are withdrawn should be refrozen at ≤ -20 ºC.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable to this procedure

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a) Reagent Sources

Reagents used during the development, validation and application of this method and their sources are listed in **Table 1**. All chemical reagents are used without further purification**.**

Table 1. Reagents and sources

Reagent	Source	Catalog Number
Acetonitrile (Optima LCMS grade or similar)	Fisher Scientific, Fairlawn, NJ	A955
Methanol (Optima LCMS grade or similar)	Fisher Scientific, Fairlawn, NJ	A456
Isopropyl alcohol (LCMS chromasolv or similar)	Sigma Aldrich, St. Louis, MO	34965
Ammonium hydroxide (LCMS grade or similar)	Sigma Aldrich, St. Louis, MO	44273
6N Hydrochloric acid solution	Sigma Aldrich, St. Louis, MO	72033
0.1N Hydrochloric acid solution	Sigma Aldrich, St. Louis, MO	318965
Ammonium acetate (LCMS Ultra grade or similar)	Sigma Aldrich, St. Louis, MO	14267
1M Sodium hydroxide solution	Sigma Aldrich, St. Louis, MO	71463
Water (HPLC grade or similar)	Fisher Scientific, Fairlawn, NJ	14-650-357

b) Reagent Preparation

1) Mobile phase A: 5 mM ammonium acetate buffer solution (pH 9.2)

Prepare 100 mL of 100 mM master stock ammonium acetate buffer at pH 9.2 by mixing 0.7708 g of ammonium acetate and 400 µL ammonium hydroxide solution (~25% v/v) in water. The 5 mM ammonium acetate buffer is prepared by diluting master stock buffer 20 times in water (i.e., 25 mL of 100mM ammonium acetate in 475 mL water). The master stock buffer solution should be stored at 5 ± 3 ^{o}C for no more than one week. Mobile phase A should be replaced before each run.

2) Mobile phase B: 95/5% (v/v) acetonitrile/100 mM ammonium acetate buffer

Prepare 95/5% (v/v) acetonitrile/100 mM ammonium acetate buffer mixture by diluting master ammonium acetate buffer solution 20 times in acetonitrile (i.e., 12.5 mL of 100mM ammonium acetate in 237.5 mL acetonitrile). Mobile phase B should be replaced before each run.

3) Elution: 75/20/5% (v/v/v) methanol/isopropyl alcohol/ammonium hydroxide solution

   Prepare a volumetric mixture of 150 mL methanol, 40 mL isopropyl alcohol, and 10 mL ammonium hydroxide solution (~25% v/v). Elution solvent should be prepared fresh for each run

4) Strong wash: 80/20% (v/v) acetonitrile/water

   Prepare a volumetric mixture of 800 mL acetonitrile and 200 mL water in a 1 liter solvent bottle. Strong wash should be replaced every month.

5) Seal/weak wash: 95/5% (v/v) water/acetonitrile

   Prepare volumetric mixture of 950 mL water and 50 mL acetonitrile in a 1 liter solvent bottle. Seal/weak wash should be replaced every month.

c) Calibration and Quality Control Materials

Compounds used for preparation of calibration standards and quality control materials are purchased from companies meeting guidelines of International Organization of Standards Guide 34. **Table 2a** shows the list of compounds and the recommended concentration of master stock solutions for each standards obtained. Other concentrations may be used when applicable. All master stocks are stored in -70 ºC freezer prior to use. Master stock solution is diluted 10 times to prepare working stock solution **(Table 2b)** and are stored at -70 ºC prior to use. Working stock solutions are further diluted 10 times to prepare calibration solutions **(Table 2c)** and are stored at -70 ºC prior to use.

Table 2a. Concentration of master stock standards supplied in acetonitrile

Analyte	CAS	Concentration Level (ng/mL)						
		1	2	3	4	5	6	7
p-Phenylenediamine	106-50-3	5.0	15.8	50	158	500	1580	5000
1,5-Napthalenediamine	2243-62-1	5.0	15.8	50	158	500	1580	5000
4,4'-Diphenylmethanediamine	101-77-9	1.0	3.16	10	31.6	100	316	1000
2,4-Toluenediamine	95-80-7	5.0	15.8	50	158	500	1580	5000
2,6-Toluenediamine	823-40-5	5.0	15.8	50	158	500	1580	5000

Table 2b. Concentration of working stock standards in acetonitrile

Analyte	CAS	Concentration Level (ng/mL)						
		1	2	3	4	5	6	7
p-Phenylenediamine	106-50-3	0.5	1.58	5.0	15.8	50	158	500
1,5-Napthalenediamine	2243-62-1	0.5	1.58	5.0	15.8	50	158	500
4,4'-Diphenylmethanediamine	101-77-9	0.1	0.32	1.0	3.2	10	32	100
2,4-Toluenediamine	95-80-7	0.5	1.58	5.0	15.8	50	158	500
2,6-Toluenediamine	823-40-5	0.5	1.58	5.0	15.8	50	158	500

Table 2c. Final concentration of calibration standards prepared in NH_4OAc buffer

Analyte	CAS	Concentration Level (ng/mL)						
		1	2	3	4	5	6	7
p-Phenylenediamine	106-50-3	0.05	0.16	0.5	1.58	5.0	15.8	50.0
1,5-Napthalenediamine	2243-62-1	0.05	0.16	0.5	1.58	5.0	15.8	50.0
4,4'-Diphenylmethanediamine	101-77-9	0.01	0.032	0.1	0.32	1.0	3.2	10.0
2,4-Toluenediamine	95-80-7	0.05	0.16	0.5	1.58	5.0	15.8	50.0
2,6-Toluenediamine	823-40-5	0.05	0.16	0.5	1.58	5.0	15.8	50.0

d) Internal Standards

Stable isotope labeled internal standards used in this method are listed in **Table 3**. Other isotopic analogs may be used when there is availability or cost limitation as long as the internal standard is stable, and there are no chromatographic or mass spectral interferences. Internal standard compounds of at least 98% pure can be used without further purification. All internal standard solutions are stored at -70 ºC prior to use.

Table 3. List of stable isotope labeled internal standards and sources

Internal Standards	Sources	Suggested final concentration (ng/mL)
p-Phenylenediamine-[$^{13}C_6$]	Isosciences, LLC	2.85
1,5-Napthalenediamine-[$^{15}N_2$]	Isosciences, LLC	5.40
4,4'-Diphenylmethanediamine-[$^{15}N_2$ ^{13}C]	Isosciences, LLC	0.56
2,4-Toluenediamine-[$^{13}C_6$]	Toronto Research Center	3.02
2,6-Toluenediamine-[$^{13}C_6$]	Toronto Research Center	1.50

e) Proficiency Testing Materials

PT materials are prepared by in-house PT program administered by the branch QC officer or statistician. PT samples are run at least twice a year. PT samples are run following any major maintenance on instrumentation as well.

f) Instrumentation and Other Equipments

- Ultra-high pressure liquid chromatography system (i.e., Waters Acquity UPLC®)
- Triple quadrupole mass spectrometer
- Autosampler
- Automated solid phase extraction system such as Biotage Extrahera
- Biotage SPE dry or similar system
- Dry block heater
- Vortex mixer
- Desktop computer

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

All calibration standards are prepared in initial gradient of the UPLC method. Matrix validation experiments are performed to verify that the slopes of the calibration curves resulting from the calibrators mixed in the calibration solution are comparable to those mixed in urine. These results validate the use of non-urine based calibrators for quantifying aromatic diamines in urine samples.

a) Instrument Response Calibration

A set of seven calibration solutions is analyzed twice, bracketing unknowns and QC materials in an analytical run. These calibration results are combined and used for the quantification of analytes in all samples and QC materials from that batch. Calibration curves are constructed for each analyte from the peak response ratio of standards to internal standards as the seven different concentration levels. The slope (response factor) and intercept are determined by linear least squares of 1/x weighted data. Calibration curve statistics are evaluated for each analyte to ensure that the coefficient of determination (R^2) value of the curve is equal to or greater than 0.98. The highest calibrator on the calibration curve is above the expected range of results for non-occupationally exposed subjects and the lowest calibrator is near or below the measurable detection limit. A calibrator can only be omitted from a calibration curve only if there is a known cause specific to that one sample.

b) Calibration Verification

Calibration is verified by analyzing a full set of calibrators with every batch of unknown samples as outlined in the previous paragraph. Absolute accuracy is verified by using PT samples at least twice per year.

8. PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

An analytical run consists of solvent blanks, QC blanks, calibration standards, low level QC, high level QC and urine samples.

a) Sample preparation

1) Thaw urine samples at room temperature and vortex mix for at least 10 minutes to homogenize the sample
2) Transfer 250 µL urine into a sample plate. QC samples are similarly thawed and aliquoted. For QC blank use HPLC grade water.
3) Add 50 µL internal standards mix and 100 µL 6 N HCl solution.
4) Add 600 µL HPLC grade water to make final volume of 1 mL in each well and place cap mat.
5) Mix thoroughly and allow samples to hydrolyze in a heating block at 80 ºC for 4 hours.
6) After hydrolysis, samples are removed from heating block and allowed to cool down to room temperature (15 ± 5 minutes).
7) Adjust pH of a solution to 1.0 ± 0.1 by adding 500 µL of 1 M NaOH.
8) pH adjusted samples are allowed to pass through SPE sorbent. An offline automated SPE is performed using systems such as Biotage Extrahera or Hamilton STARLET. SPE steps are as follows:
    a) Condition with 1 mL methanol
    b) Equilibrate with 1 mL water
    c) Load entire samples
    d) Wash with 1 mL of 0.1 N HCl solution
    e) Wash with 2 mL methanol
    f) Dry for at least 3 minutes
    g) Elute with 500 µL 75/20/5% (v/v/v) methanol/isopropyl alcohol/ammonium hydroxide mixture
    h) Repeat steps (f) and (g) once
9) Evaporate eluent to dryness in a SPE dry system operated at 60 ºC and under nitrogen
10) Reconstitute analytes into 90/10% (v/v) mobile phase A and B by adding 250 µL solvent mix followed by vigorous agitation for at least 10 minutes in a vortex mixer

11) Transfer the samples into LC autosampler and perform UPLC-MS/MS analysis

b) UPLC-MS/MS Analysis

A good quality chromatographic peak resolution is required for achieving reliable quantitative UPLC-MS/MS data. Peak resolution is defined as a degree of separation of two chromatographic peaks (or analytes):

$$\text{Peak Resolution } (R_s) = 2*(t_{R2} - t_{R1})/1.7*(w_{1/2,\,1} + w_{1/2,\,2}) \quad (1)$$

Where, t_{R1} and t_{R2} are retention time and $w_{1/2,\,1}$ and $w_{1/2,\,2}$ are full width at half maximum of peak 1 and peak 2, respectively. Theoretically, peak resolution of 1.25 or higher is expected for all quantitative peaks in the method and is determined based on the peak resolution of low level quality control (QL) samples. If peak resolution is less than 1.25 (i.e., visually indistinguishable) for any analyte in QL samples adjust gradient flow, column temperature, or other possible chromatographic parameters to obtain desired peak resolution. Suggested chromatographic parameters are shown in **Table 4**.

Table 4. Suggested UPLC parameters

Parameter	Details
Column	ACE EXCEL superC18 Ultra-inert 2.1 mm × 100 mm, 1.7 µm or similar column
Column temperature	35 °C
Sample manager temperature	5 °C
Mobile phase	Solvent A - 5 mM ammonium acetate buffer at pH 9.2 Solvent B - 95/5% (v/v) acetonitrile/100 mM ammonium acetate buffer
Seal wash	95/5% (v/v) water/acetonitrile
Strong wash (needle wash/sample manager purge)	80/20% (v/v) acetonitrile/water
Injection volume	5 µL
Gradient elution schedule (Time; flow rate; solvents A%:B% (v/v))	Initial; 500 µL/min; 86:14 1.0 min; 500 µL/min; 70:30 2.0 min; 500 µL/min; 10:90 2.5 min; 500 µL/min; 86:14 4.0 min; 500 µL/min; 86:14

Suggested MS parameters and compound-specific parameters are shown in **Tables 5a and 5b**.

Table 5a. Suggested MS parameters (compound nonspecific)

Parameter	Settings
Scan type	Scheduled MRM
Polarity	Positive
Ionization	APCI
Heater Temperature	550 °C
Nebulizer current	2 µA
CAD	7 psi
CUR	30 psi
GS1	45 psi

Table 5b. Suggested compound specific MS parameters

Analytes	Ion-transitions *(m/z)*		DP	EP	CE	CXP
	Quantitation	Confirmation				
PPDA	109.1→92	-	80	10	20	8
	-	109.1→65	80	10	28	8
PPDA-$^{13}C_6$	115.1→98		80	8	21	8
5NDA	159.1→143.1	-	40	9	26	6
	-	159.1→142.1	40	9	26	6
5NDA-$^{15}N_2$	161.1→144.1		40	10	27	6
4MDA	199.2→106	-	40	9	30	10
	-	199.2→182.1	40	9	25	10
4MDA-$^{15}N_2$ ^{13}C	202.2→108		40	9	30	10
4TDA	123.1→108		70	5	24	6
	-	123.1→106	70	5	20	6
4TDA-$^{13}C_6$	129.1→112		70	8	24	6
6TDA	123.1→108	-	70	5	20	6
	-	123.1→106	70	8	24	6
6TDA-$^{13}C_6$	129.1→112		70	8	24	6

DP=declustering potential; EP=Entrance potential; CE=Collision energy; CXP=cell exit potential

c) Data Processing

1) Peak Integration

Chromatograms are processed individually after the corresponding samples are run. Each target peak is confirmed by retention time and compound specific MRM transition. Peaks are integrated using multianalyte data processing software (aka integrator) such as MultiQuant. Each peak is visually inspected and peak integration is corrected if the integrator erroneously integrates a peak. The integration approach for all samples in an analytical run is kept consistent for a specific target analyte.

2) Excluding calibrators

Calibrator data is excluded only if it can be determined that the calibrator data is biased independently of the samples in the analytical batch. Scenarios that might only affect a single calibrator are rare, however may be due to improper amount of internal standard addition and/or contaminated autosampler vials. Higher calibrators (calibrations solutions 6 and/or 7) can be excluded if the calibration curve is nonlinear over this range and all QCs and unknowns fall between calibrators 1 and 5.

3) Excluding sample data

Sample data is excluded if there is no or very low internal standard signal. Absolute response from internal standards is evaluated for consistency among the standards, QC blank, QCs, and unknowns. An unusually high internal standard level can occur if the internal standard is added twice. A low or no response is observed if internal standard is not added.

d) Formal Quality Control Material Evaluation

Following data analysis and import of data into a database, quality control sample results are formally evaluated by an independent quality control officer. The QC samples analyzed in an analytical run are evaluated against the QC characterized means and standard deviation limits determined by the QC officer. The QC samples are evaluated using modified Westgard rules as specified by DLS SAS program.[8, 9] Any failure of QC rules for an analyte disqualifies the corresponding data for that analyte for that specific run. Once the source of QC problem is identified, the samples are subsequently reanalyzed with new QC samples.

e) Additional Quality Assurance Data Evaluation

In addition to QC samples, other quality parameters such as quantitation/confirmation ion ratios, QC blank samples, and adequate internal standard response, are evaluated for acceptable precision and accuracy.

9. REPORTABLE RANGE OF RESULTS

a) Reportable Limits

Sample results above the LOD and that pass sample and batch QC evaluation are marked as reportable, otherwise they are marked as not reportable. The upper reportable limit corresponds to the concentration of the highest linear standard. If the analyte level exceeds the upper calibration range, the sample is diluted and reanalyzed.

b) Limit of Detection

The analytical limits of detection are based on the method described in the DLS Policies and Procedures Manual, equivalent to the recommendations of the Clinical and Laboratory Standards Institute and include Type II error.

c) Accuracy

The absolute accuracy is evaluated by blind analysis of independently prepared and certified PT materials as described in section 10c. Absolute accuracy may also be verified using spiked urine samples. The percent accuracy must fall between 100 ± 25%. Relative accuracy is evaluated upon comparison of characterized QC mean values with those obtained on each run. Error in relative accuracy should not exceed the precision of the characterized QC samples as defined in the DLS procedures, which is based on standard practices.[8]

d) Precision

Method precision is reflected in the variance of quality control samples analyzed over time and is described in section 10b. Precision of the characterized QC samples, as defined in the DLS policies and procedures manual, is based on standard practices.[8]

e) Analytical Specificity

Specificity of this method is conferred through the hyphenation of two established analytical approaches. The analytical specificity in liquid chromatography is defined by the retention time in a chromatogram, while tandem mass spectrometry adds another dimension in analytical specificity by monitoring ion-transitions specific to an analyte. Further assurance of peak identity is provided by estimating appropriate ion ratios between quantitation and confirmation ion-transitions and monitoring the presence of co-eluting interferences in QC blanks.

f) Ruggedness Testing

Ruggedness testing was performed to evaluate the potential variables that affect analytical results. The variables that are found to have a substantial influence on the final analytical results include formulation errors through the addition of internal standard to all samples, buffer pH, pH adjustment prior to SPE, reconstitution volume, and eluent evaporation time. These variables were altered to examine their influence on the analytical results and were optimized for higher sensitivity and accuracy. Ruggedness testing results for all 5 analytes are shown in **Tables 6a – 6e.**

Table 6a. Ruggedness data for PPDA

Five tested parameters	Final method level or choice	Result for analyte (ng/mL)	Lower level	Result for analyte at lower level (ng/mL)	Higher level	Result for analyte at higher level (ng/mL)
pH adjustment prior to SPE	pH 1.0	3.07	pH 0.8	3.00	pH 2.0	3.15
Solvent volume for reconstitution	250 µL	3.07	200 µL	3.02	300 µL	3.14
Mobile phase buffer pH	pH 9.2	3.07	pH 9.0	3.05	pH 9.5	3.15
Eluent evaporation time	10 min	3.07	5 min	3.08	15 min	3.18
Volume of internal standard added to spiked urine	50 µL	3.07	45 µL	2.99	55 µL	3.10

Table 6b. Ruggedness data for 4MDA

Five tested parameters	Final method level or choice	Result for analyte (ng/mL)	Lower level	Result for analyte at lower level (ng/mL)	Higher Level	Result for analyte at higher level (ng/mL)
pH adjustment prior to SPE	pH 1.0	0.59	pH 0.8	0.62	pH 2.0	0.65
Solvent volume for reconstitution	250 µL	0.59	200 µL	0.60	300 µL	0.61
Mobile phase buffer pH	pH 9.2	0.59	pH 9.0	0.59	pH 9.5	0.65
Eluent evaporation time	10 min	0.59	5 min	0.61	15 min	0.60
Volume of internal standard added to spiked urine	50 µL	0.59	45 µL	0.55	55 µL	0.62

Table 6c. Ruggedness data for 5NDA

Five tested parameters	Final method level or choice	Result for analyte (ng/mL)	Lower level	Result for analyte at lower level (ng/mL)	Higher Level	Result for analyte at higher level (ng/mL)
pH adjustment prior to SPE	pH 1.0	0.51	pH 0.8	0.48	pH 2.0	0.52
Solvent volume for reconstitution	250 µL	0.51	200 µL	0.51	300 µL	0.48
Mobile phase buffer pH	pH 9.2	0.51	pH 9.0	0.48	pH 9.5	0.53
Eluent evaporation time	10 min	0.51	5 min	0.51	15 min	0.50
Volume of internal standard added to spiked urine	50 µL	0.51	45 µL	0.60	55 µL	0.46

Table 6d. Ruggedness data for 4TDA

Five tested parameters	Final method level or choice	Result for analyte (ng/mL)	Lower level	Result for analyte at lower level (ng/mL)	Higher Level	Result for analyte at higher level (ng/mL)
pH adjustment prior to SPE	pH 1.0	2.68	pH 0.8	2.60	pH 2.0	2.65
Solvent volume for reconstitution	250 µL	2.68	200 µL	2.63	300 µL	2.66
Mobile phase buffer pH	pH 9.2	2.68	pH 9.0	2.72	pH 9.5	2.75
Eluent evaporation time	10 min	2.68	5 min	2.54	15 min	2.60
Volume of internal standard added to spiked urine	50 µL	2.68	45 µL	2.75	55 µL	2.60

Table 6e. Ruggedness data for 6TDA

Five tested parameters	Final method level or choice	Result for analyte (ng/mL)	Lower level	Result for analyte at lower level (ng/mL)	Higher Level	Result for analyte at higher level (ng/mL)
pH adjustment prior to SPE	pH 1.0	2.9	pH 0.8	3.00	pH 2.0	2.78
Solvent volume for reconstitution	250 µL	2.9	200 µL	2.87	300 µL	2.75
Mobile phase buffer pH	pH 9.2	2.9	pH 9.0	2.99	pH 9.5	2.90
Eluent evaporation time	10 min	2.9	5 min	2.86	15 min	2.76
Volume of internal standard added to spiked urine	50 µL	2.9	45 µL	2.96	55 µL	2.85

10. QUALITY ASSESSMENT AND PROFICIENCY TESTING

a) Quality Assessment

Quality assessment procedures follow standard practices.[8, 9] Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are included in each run sequence. At least three quality assessment sample types are analyzed in each analytical run that include a QC blank and QCs at two different concentrations. In addition, solvent blanks are prepared to monitor carry over effect.

b) Quality Control Procedures

1) Establishing QC limits

Precision is evaluated using the QC sample results. Up to two different pools of quality control material are used—typically, one at a low and the other at a high concentration. Expected precision ranges for the QC samples are established for a new QC batch using modified Westgard rules as specified by DLS SAS program.[8, 9] Different variables are included in the characterization analyses (e.g. different analysts, days, batches, and columns) to capture realistic assay variation over time. The mean, standard deviation, coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy on a daily basis.

2) Quality control evaluation
After the completion of an analytical run, the calculated results from the analyses of QC samples are compared to the established QC limits to determine if the run is "in control". The quality control rules apply to the average of the beginning and ending analyses of each of the QC pools. The quality control results are evaluated using modified Westgard rules as specified by DLS SAS program.[8, 9] If a QC result is declared "out of control", the results for all patient samples analyzed during that run are invalid for reporting.

c) Proficiency Testing

Certified standard reference materials (SRM) from the National Institute of Standards & Technology (NIST) are the first choice for independent validation of method accuracy; however, NIST does not have SRM for urinary diamines. Therefore, the PT scheme for this method is administered by an in-house proficiency testing coordinator. Aqueous proficiency testing materials were prepared and blind-coded by an in-house PT Coordinator. The samples are analyzed blind and the results evaluated by the in-house PT coordinator.

1) Frequency of PT

Five of four different levels of PTs are analyzed at least twice a year using the same method as for unknown samples.

2) Documentation of proficiency testing results

Analytical PT results are reviewed by the analyst and laboratory supervisor and submitted to the in-house PT Coordinator electronically. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if $\geq$ 80% of the results deviate $\leq$ 25% from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens are not analyzed or analyte results are not reported until the method successfully passes proficiency testing.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTMES FAIL TO MEET ACCEPATABLE CRITERIA

a) Internal Reference Area Counts

Internal standards are used to compensate for sample loss such as those caused by matrix effects. Since sample matrices vary for the standards, QCs and among urine samples, the differences in internal standard response can vary up to a factor of 10. If the intensity drop for analytes is on the order of a factor of 10 relative to the median response among the other samples of similar matrices, the sample might have suffered poor recovery during SPE or analyte loss during other sample handling steps. The cause of this decrease in response should be investigated, determined, and resolved.

b) Analyte in Blank Material Only

When an unprecedented amount of analyte is observed in the blank samples, but not in other samples, there could be a possible contamination of the blank. The source of the contamination should be determined to prevent biasing of sample results.

c) Analyte in all Samples

When an unprecedented amount of analyte is observed in all samples for a particular day, there is likely a continual source of contamination. Steps should be taken to identify and eliminate the source of contamination. Contamination specific to analytes could come from new lots of chemical reagents, SPE cartridges, sample collection tubes, and other sample processing materials.

d) QC Sample Outside the Confidence Limits

If results for a QC sample fails the QC criteria described in section 10b(2), no data for that analyte from that particular batch is reported. The cause for the QC failure is to be investigated, isolated, and solved. No analytical results are reported for runs that are not in statistical control. Note that in all cases, the supervisor should be consulted for the appropriate corrective actions.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

This method uses isotope dilution UPLC-MS/MS, widely regarded as the definitive method for the measurement of toxicants in human body fluids. Alteration of this method might result in major biases. Care should be taken to produce non-contaminated standard, quality control, and blank samples. The quantification range and LODs are to be determined as described in section 9.

13. REFERENCE RANGES (NORMAL VALUES)

Reference ranges for these compounds are not available.

14. CRITICAL CALL RESULTS ("PANIC" VALUES)

There are no critical call values for aromatic diamines at this time.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach ambient temperature prior to sample preparation; however, biological samples (unknowns and QCs) are to be racked into a chilled tray (5 ± 3 ºC) while awaiting analysis. If the measurement is delayed to the next day, samples must be frozen at -70 ºC.

16. ALTERNATE METHODS FOR PERFORMING TEST AND STORING SPECIMENS IF TEST SYSTEM FAILS

Alternate methods have not been evaluated or validated for measuring urinary aromatic diamines; however, based on the stability and freeze-thaw cycle experiments performed, if the analytical system fails, samples can be frozen at -70 ºC or less for at least a week before they are analyzed.

17. TEST-RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Analytical results are reportable once the validity of the data is established by the division's QC/QA policies and procedures, and are verified by a DLS statistician. One hardcopy and one electronic copy (ASCII format) of the data will be generated. This data, a cover letter, and a table of method specifications will be routed through the appropriate channels for approval (i.e., supervisor, branch chief, division director). After approval the report will be sent to the contact person who requested the analyses.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

If greater than 500 µL of sample remains after analysis, this material should be returned to storage at -20 °C or less in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

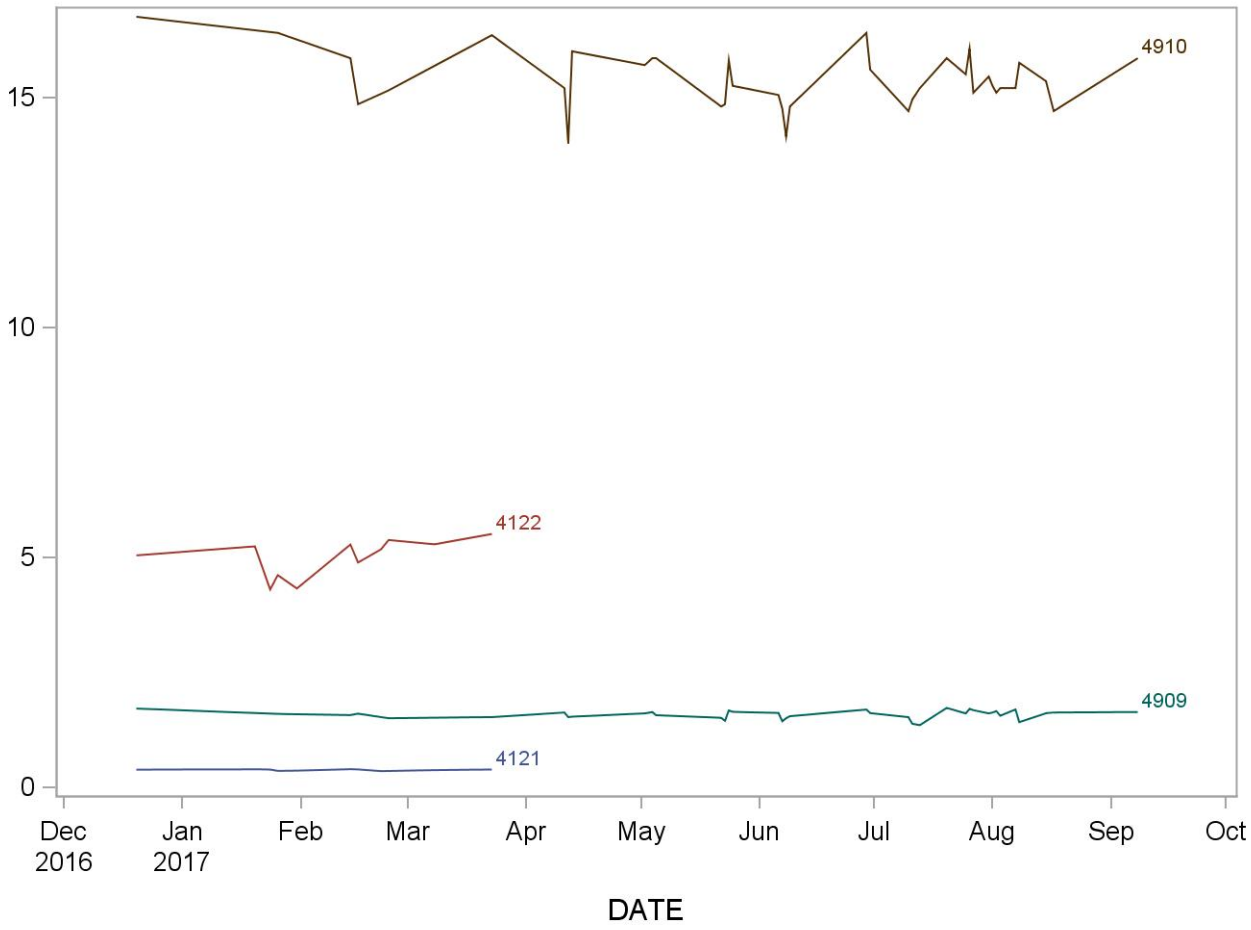
Standard record keeping (e.g., sample ID, database, notebooks, and data files) is used for tracking specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer.

19. SUMMARY STATISTICS AND GRAPHS

See the following pages.

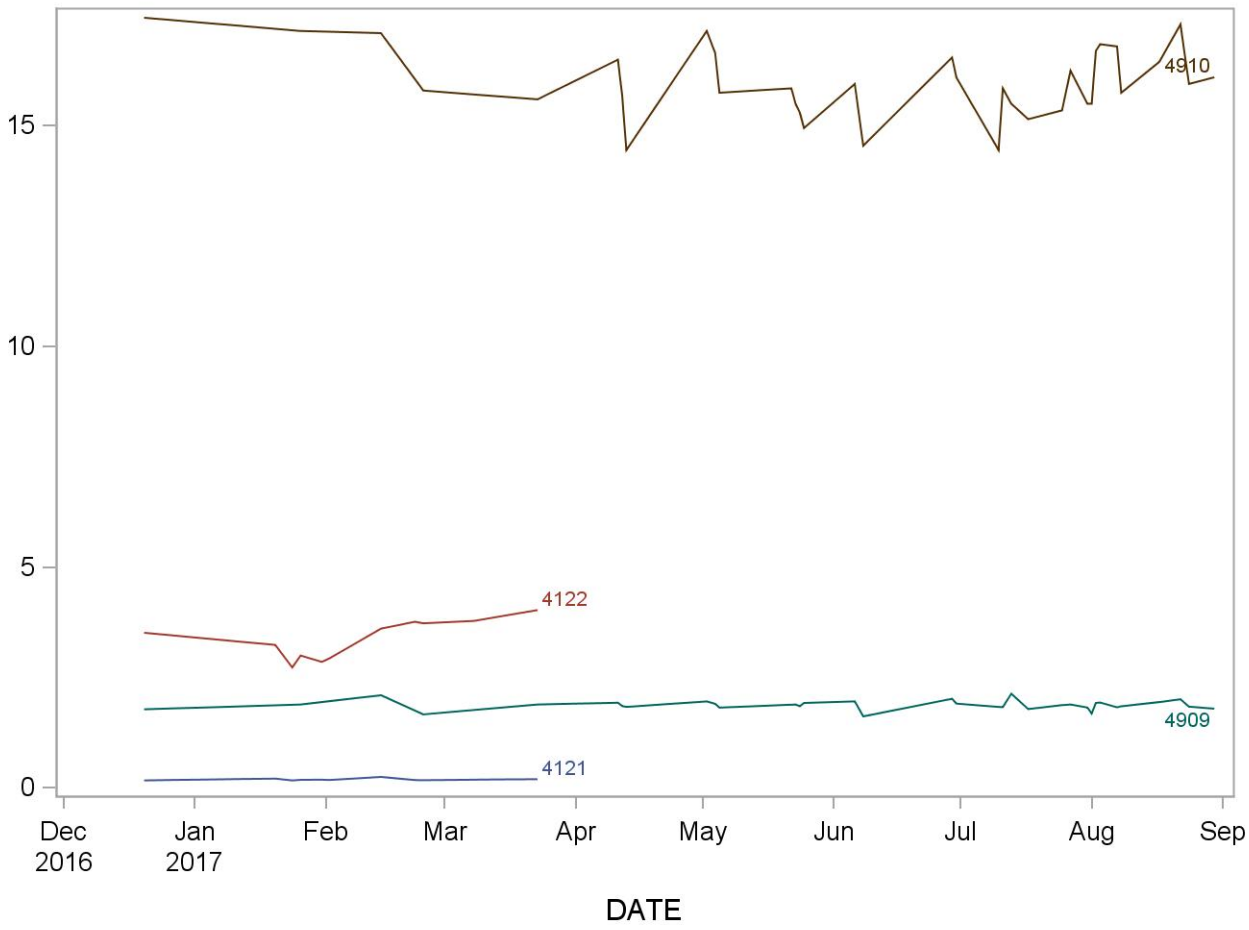
2015-2016 Summary Statistics and QC Chart for 1,5-Diaminonaphthalene (5NDA) (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4122	11	20DEC16	23MAR17	5.0045	0.4187	8.4
4910	38	20DEC16	08SEP17	15.3842	0.6143	4.0
4121	11	20DEC16	23MAR17	0.3794	0.0159	4.2
4909	38	20DEC16	08SEP17	1.5847	0.0918	5.8



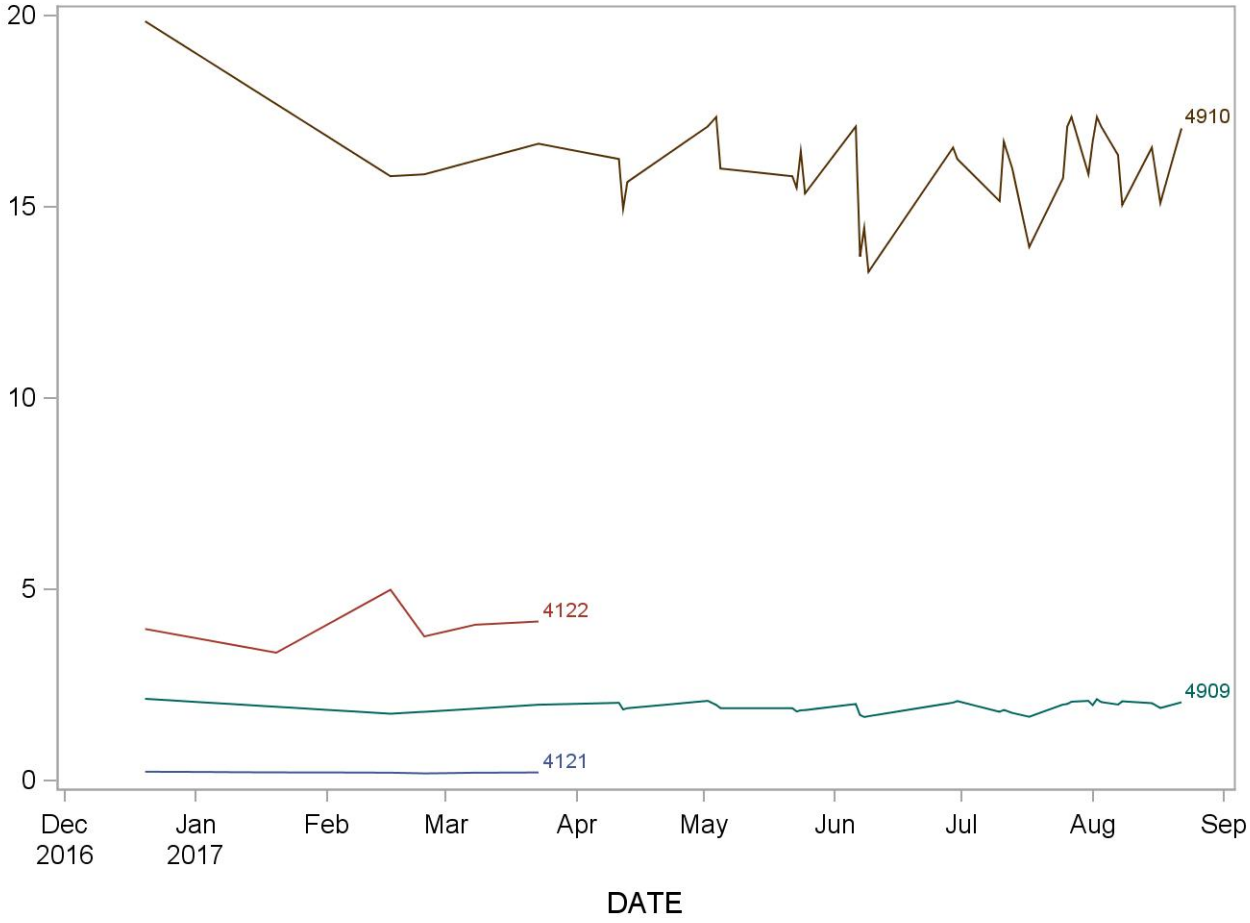
2015-2016 Summary Statistics and QC Chart for 2,4-Diaminotoluene (4TDA) (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4122	11	20DEC16	23MAR17	3.3773	0.4449	13.2
4910	35	20DEC16	30AUG17	15.9857	0.8019	5.0
4121	11	20DEC16	23MAR17	0.1839	0.0232	12.6
4909	35	20DEC16	30AUG17	1.8713	0.1053	5.6



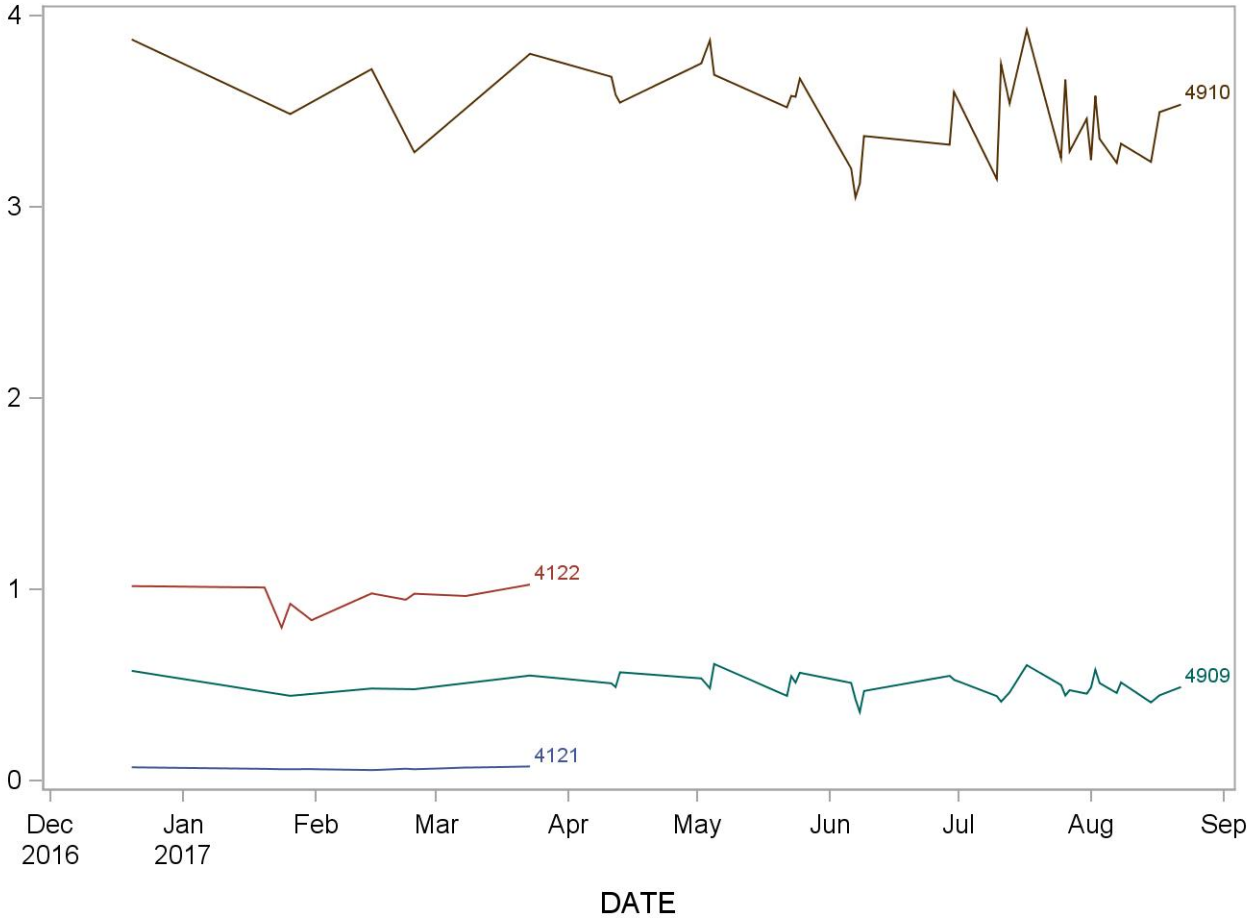
2015-2016 Summary Statistics and QC Chart for 2,6-Diaminotoluene (6TDA) (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4122	6	20DEC16	23MAR17	4.0508	0.5439	13.4
4910	36	20DEC16	22AUG17	16.0833	1.2206	7.6
4121	6	20DEC16	23MAR17	0.2117	0.0144	6.8
4909	36	20DEC16	22AUG17	1.9289	0.1377	7.1



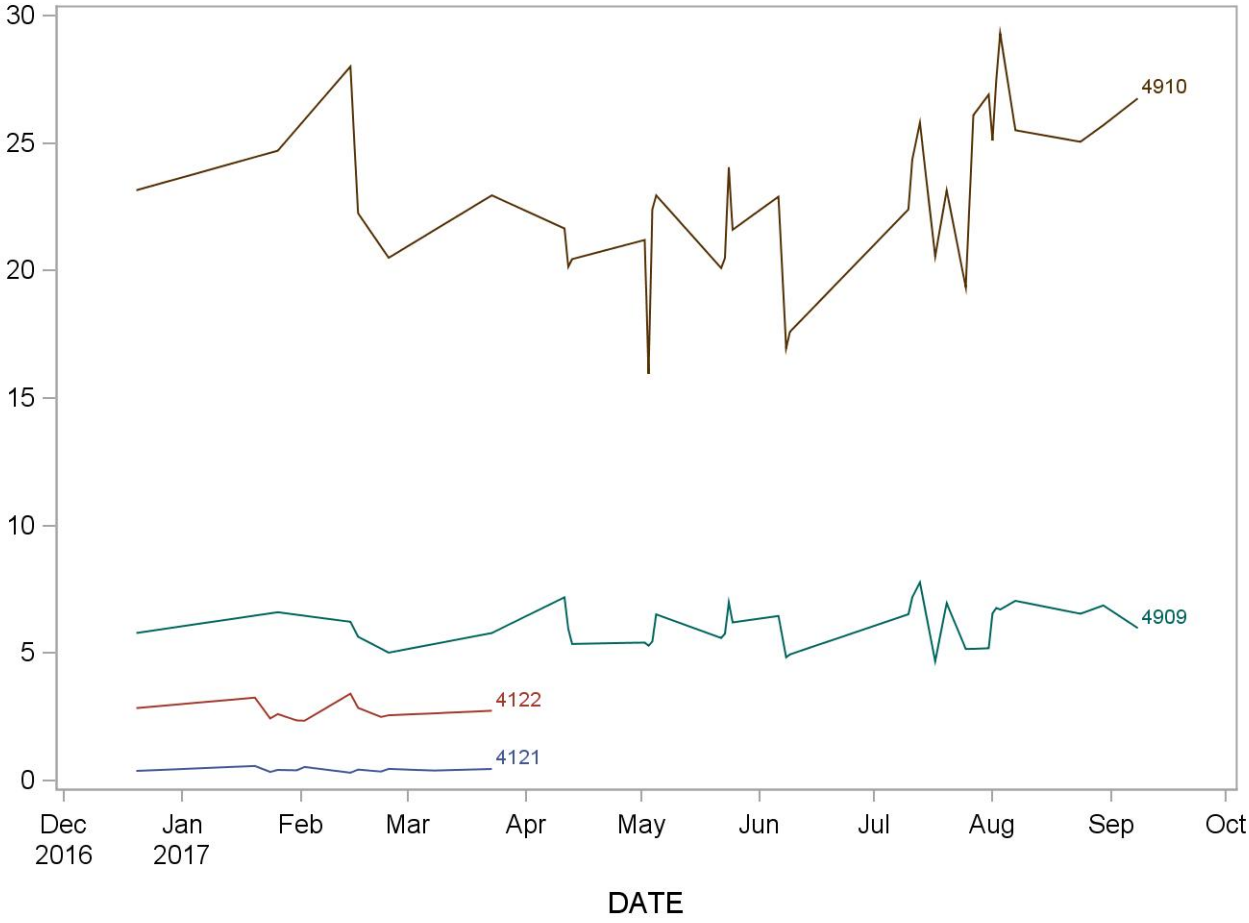
2015-2016 Summary Statistics and QC Chart for 4,4'-Diaminodiphenylmethane(4MDA)(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4122	10	20DEC16	23MAR17	0.9484	0.0753	7.9
4910	37	20DEC16	22AUG17	3.4953	0.2300	6.6
4121	10	20DEC16	23MAR17	0.0632	0.0058	9.2
4909	37	20DEC16	22AUG17	0.4949	0.0571	11.5



2015-2016 Summary Statistics and QC Chart for p-Phenylenediamine (PPDA) (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4122	13	20DEC16	23MAR17	2.7027	0.3231	12.0
4910	36	20DEC16	08SEP17	22.8889	3.1356	13.7
4121	13	20DEC16	23MAR17	0.4263	0.0742	17.4
4909	36	20DEC16	08SEP17	6.0340	0.8067	13.4



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APPENDIX A: Method Performance Documentation

Method performance documentation for this method including accuracy, precision, specificity, and stability is provided in Appendix A of this method documentation. **The signatures of the Branch Chief and Director of the Division of the Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**

Table A1. Accuracy using spike recovery

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Urinary Aromatic Diamines by UPLC-MS/MS
Method #: 2120
Matrix: Urine
Units: ng/mL
Analyte: **PPDA**

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Q170809	Q170810	Mean	Recovery (%)	Spike concentration	Measured concentration Q170809	Q170810	Mean	Recovery (%)		
Sample	1	0.00	1.66	1.25			0.00	2.05	4.18			95.8	2.40
	2		1.37	1.38	1.36			2.24	2.95	2.80			
	3		1.40	1.12				2.04	3.32				
Sample + Spike 1	1	2.06	3.24	3.02			2.06	3.58	5.78				
	2		3.61	3.43	3.32	95.2		4.19	4.57	4.83	98.7		
	3		3.36	3.29				5.32	5.55				
Sample + Spike 2	1	8.23	9.37	9.51			8.23	9.81	11.8				
	2		9.08	9.48	9.15	94.6		10.3	11.8	10.9	98.9		
	3		8.05	9.38				9.04	12.9				
Sample + Spike 3	1	16.5	16.2	16.9			16.5	17.4	18.1				
	2		17.8	16.5	16.8	93.5		16.5	20.4	18.2	93.9		
	3		16.6	16.5				17.2	19.9				

Analyte: **6TDA**

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Q170809	Q170810	Mean	Recovery (%)	Spike concentration	Measured concentration Q170809	Q170810	Mean	Recovery (%)		
Sample	1	0.00	0.656	0.529			0.00	0.453	0.483			104	7.83
	2		1.66	0.554	0.782			0.486	0.497	0.507			
	3		0.696	0.601				0.574	0.547				
Sample + Spike 1	1	2.06	2.31	2.43			2.06	2.17	2.59				
	2		2.28	2.52	2.37	115		2.09	2.34	2.31	112		
	3		2.10	2.56				2.17	2.47				
Sample + Spike 2	1	8.23	7.32	8.30			8.23	7.81	8.44				
	2		7.58	8.37	7.97	96.9		8.48	8.49	8.32	101		
	3		7.84	8.43				8.23	8.48				
Sample + Spike 3	1	16.5	14.9	16.6			16.5	16.9	15.9				
	2		14.8	16.8	15.8	95.9		17.4	17.0	16.9	103		
	3		15.3	16.3				17.5	16.6				

Analyte: **5NDA**

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Q170809	Sample 1 Measured concentration Q170810	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Q170809	Sample 2 Measured concentration Q170810	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0.00	0.497	1.25			0.00	0.498	0.469				
	2		0.485	1.38	0.870			0.504	0.468	0.501		95.4	3.0
	3		0.487	1.12				0.583	0.484				
Sample + Spike 1	1	2.06	2.32	3.02			2.06	2.25	2.42				
	2		2.51	3.43	2.80	93.7		2.43	2.45	2.35	89.9		
	3		2.24	3.29				2.22	2.36				
Sample + Spike 2	1	8.23	7.82	9.51			8.23	8.23	8.56				
	2		8.36	9.48	8.86	97.1		8.49	9.00	8.50	97.2		
	3		8.61	9.38				8.39	8.34				
Sample + Spike 3	1	16.5	17.0	16.9			16.5	16.4	16.8				
	2		17.0	16.5	16.8	96.6		16.9	16.8	16.6	97.6		
	3		16.6	16.5				15.5	17.0				

Analyte: **4MDA**

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Q170809	Sample 1 Measured concentration Q170810	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Q170809	Sample 2 Measured concentration Q170810	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0.00	0.439	0.399			0.00	0.749	0.752				
	2		0.496	0.462	0.459			0.783	0.700	0.761		86.5	3.39
	3		0.492	0.463				0.895	0.687				
Sample + Spike 1	1	0.410	0.659	0.857			0.410	1.09	1.08				
	2		0.851	0.924	0.820	88.0		1.12	1.02	1.09	80.2		
	3		0.763	0.863				1.11	1.12				
Sample + Spike 2	1	1.65	1.98	1.80			1.65	2.27	2.11				
	2		1.95	1.92	1.92	88.7		2.14	2.32	2.24	89.8		
	3		2.02	1.86				2.38	2.24				
Sample + Spike 3	1	3.29	3.24	3.10			3.29	3.77	3.33				
	2		3.31	3.34	3.30	86.4		3.37	3.79	3.59	86.0		
	3		3.48	3.33				3.68	3.59				

Table A2. Precision

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL
Analytes:	**PPDA, 6TDA, 4TDA, 5NDA, and 4MDA**

PPDA Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	6.04	6.29	6.16	1.55E-02	1.55E-02	7.59E+01
2	4.79	5.23	5.01	4.97E-02	4.97E-02	5.02E+01
3	5.76	5.82	5.79	7.02E-04	7.02E-04	6.70E+01
4	5.78	7.24	6.51	5.28E-01	5.28E-01	8.47E+01
5	5.60	6.53	6.06	2.16E-01	2.16E-01	7.35E+01
6	6.08	7.13	6.60	2.74E-01	2.74E-01	8.72E+01
7	6.94	8.39	7.67	5.23E-01	5.23E-01	1.18E+02
8	5.41	7.09	6.25	7.04E-01	7.04E-01	7.80E+01
9	5.64	6.09	5.86	5.04E-02	5.04E-02	6.87E+01
10	6.94	5.52	6.23	5.04E-01	5.04E-01	7.77E+01

Grand sum	124	**Grand mean**	6.21

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	5.73E+00	5.73E-01	7.57E-01	12.2
Between Run	8.27E+00	9.19E-01	4.16E-01	6.69
Total	1.40E+01		8.64E-01	**13.90**

PPDA Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	22.3	26.5	24.4	4.54E+00	4.54E+00	1.19E+03
2	20.3	19.9	20.1	4.24E-02	4.24E-02	8.10E+02
3	22.4	23.9	23.2	5.50E-01	5.50E-01	1.07E+03
4	25.4	28.4	26.9	2.18E+00	2.18E+00	1.45E+03
5	23.6	24.4	24.0	1.73E-01	1.73E-01	1.15E+03
6	23.9	25.5	24.7	6.42E-01	6.42E-01	1.22E+03
7	26.5	26.6	26.6	3.54E-03	3.54E-03	1.41E+03
8	23.4	23.8	23.6	2.91E-02	2.91E-02	1.11E+03
9	22.2	23.7	23.0	6.37E-01	6.37E-01	1.05E+03
10	25.2	30.8	28.0	7.90E+00	7.90E+00	1.57E+03

Grand sum	489	**Grand mean**	24.4

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	3.34E+01	3.34E+00	1.83E+00	7.48
Between Run	9.33E+01	1.04E+01	1.87E+00	7.67
Total	1.27E+02		2.62E+00	**10.7**

6TDA Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.92	1.91	1.91	6.25E-06	6.25E-06	7.32E+00
2	2.27	2.34	2.31	1.26E-03	1.26E-03	1.06E+01
3	1.99	2.29	2.14	2.28E-02	2.28E-02	9.18E+00
4	2.01	1.89	1.95	3.66E-03	3.66E-03	7.60E+00
5	1.98	1.86	1.92	3.42E-03	3.42E-03	7.38E+00
6	2.01	1.91	1.96	2.55E-03	2.55E-03	7.69E+00
7	1.97	2.04	2.00	1.48E-03	1.48E-03	8.04E+00
8	2.02	2.02	2.02	6.25E-06	6.25E-06	8.17E+00
9	1.75	1.89	1.82	4.56E-03	4.56E-03	6.61E+00
10	2.05	2.06	2.05	2.50E-05	2.50E-05	8.41E+00
Grand sum	40.2	**Grand mean**	2.01			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	7.95E-02	7.95E-03	8.92E-02	4.44
Between Run	3.36E-01	3.74E-02	1.21E-01	6.04
Total	4.16E-01		1.51E-01	**7.49**

6TDA Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	15.9	16.6	16.2	1.25E-01	1.25E-01	5.26E+02
2	18.5	18.4	18.5	2.35E-03	2.35E-03	6.81E+02
3	19.5	20.2	19.8	1.19E-01	1.19E-01	7.86E+02
4	16.7	16.9	16.8	1.19E-02	1.19E-02	5.64E+02
5	16.6	17.5	17.0	2.23E-01	2.23E-01	5.80E+02
6	16.7	16.2	16.4	7.54E-02	7.54E-02	5.40E+02
7	16.6	15.3	16.0	4.42E-01	4.42E-01	5.09E+02
8	16.8	17.2	17.0	2.92E-02	2.92E-02	5.77E+02
9	18.7	17.5	18.1	3.38E-01	3.38E-01	6.57E+02
10	17.0	17.0	17.0	2.50E-07	2.50E-07	5.81E+02
Grand sum	346	**Grand mean**	17.3			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.73E+00	2.73E-01	5.23E-01	3.02
Between Run	2.52E+01	2.80E+00	1.12E+00	6.50
Total	2.79E+01		1.24E+00	**7.17**

4TDA Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.89	1.93	1.91	4.20E-04	4.20E-04	7.27E+00
2	1.87	1.83	1.85	2.89E-04	2.89E-04	6.85E+00
3	1.85	1.70	1.78	5.33E-03	5.33E-03	6.32E+00
4	1.84	1.86	1.85	4.90E-05	4.90E-05	6.84E+00
5	1.83	1.80	1.82	2.10E-04	2.10E-04	6.61E+00
6	1.88	1.89	1.88	9.03E-05	9.02E-05	7.10E+00
7	1.92	1.82	1.87	2.26E-03	2.26E-03	6.98E+00
8	1.89	1.89	1.89	6.25E-06	6.25E-06	7.14E+00
9	1.81	1.83	1.82	5.63E-05	5.63E-05	6.63E+00
10	2.03	2.16	2.09	4.42E-03	4.42E-03	8.77E+00

Grand sum	37.5	**Grand mean**	1.88

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.63E-02	2.63E-03	5.12E-02	2.73
Between Run	1.33E-01	1.48E-02	7.80E-02	4.16
Total	1.59E-01		9.33E-02	**4.97**

4TDA Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	16.7	15.8	16.2	1.87E-01	1.87E-01	5.27E+02
2	16.3	16.8	16.6	7.56E-02	7.56E-02	5.49E+02
3	16.9	18.0	17.4	3.12E-01	3.12E-01	6.07E+02
4	17.3	17.1	17.2	1.85E-02	1.85E-02	5.92E+02
5	17.3	16.6	17.0	1.22E-01	1.22E-01	5.77E+02
6	17.5	16.8	17.1	1.27E-01	1.27E-01	5.88E+02
7	17.2	16.1	16.6	2.90E-01	2.90E-01	5.54E+02
8	17.2	17.0	17.1	3.54E-03	3.54E-03	5.84E+02
9	17.2	17.1	17.2	6.48E-03	6.48E-03	5.89E+02
10	17.2	17.0	17.1	9.90E-03	9.90E-03	5.85E+02

Grand sum	339	**Grand mean**	17.0

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.30E+00	2.30E-01	4.80E-01	2.83
Between Run	2.34E+00	2.60E-01	1.21E-01	0.72
Total	4.64E+00		4.95E-01	**2.92**

5NDA Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.68	1.60	1.64	1.52E-03	1.52E-03	5.36E+00
2	1.68	1.62	1.65	7.02E-04	7.02E-04	5.44E+00
3	1.76	1.67	1.72	1.98E-03	1.98E-03	5.89E+00
4	1.67	1.61	1.64	9.92E-04	9.92E-04	5.40E+00
5	1.72	1.82	1.77	2.86E-03	2.86E-03	6.26E+00
6	1.64	1.56	1.60	1.41E-03	1.41E-03	5.12E+00
7	1.67	1.58	1.63	1.85E-03	1.85E-03	5.29E+00
8	1.55	1.59	1.57	4.20E-04	4.20E-04	4.90E+00
9	1.56	1.57	1.56	7.22E-05	7.23E-05	4.89E+00
10	1.56	1.59	1.57	1.96E-04	1.96E-04	4.95E+00
Grand sum	32.7	**Grand mean**	1.63			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.40E-02	2.40E-03	4.90E-02	3.00
Between Run	7.98E-02	8.87E-03	5.69E-02	3.48
Total	1.04E-01		7.51E-02	**4.59**

5NDA Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	15.6	16.0	15.8	3.39E-02	3.39E-02	4.98E+02
2	15.3	15.7	15.5	4.12E-02	4.12E-02	4.79E+02
3	16.5	17.0	16.7	5.88E-02	5.88E-02	5.60E+02
4	16.2	16.4	16.3	6.16E-03	6.16E-03	5.30E+02
5	16.1	16.0	16.1	3.42E-03	3.42E-03	5.15E+02
6	17.0	15.8	16.4	3.59E-01	3.59E-01	5.35E+02
7	15.3	15.7	15.5	2.84E-02	2.84E-02	4.80E+02
8	15.8	15.7	15.7	1.68E-03	1.68E-03	4.94E+02
9	15.9	14.8	15.4	3.14E-01	3.14E-01	4.73E+02
10	15.7	16.0	15.8	2.42E-02	2.42E-02	5.01E+02
Grand sum	318	**Grand mean**	15.9			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.74E+00	1.74E-01	4.17E-01	2.62
Between Run	3.49E+00	3.88E-01	3.27E-01	2.05
Total	5.23E+00		5.30E-01	**3.33**

4MDA Quality material 1

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.596	0.495	0.546	2.55E-03	2.55E-03	5.95E-01
2	0.407	0.414	0.411	1.23E-05	1.23E-05	3.37E-01
3	0.519	0.634	0.577	3.31E-03	3.31E-03	6.65E-01
4	0.468	0.465	0.467	2.25E-06	2.25E-06	4.35E-01
5	0.481	0.489	0.485	1.60E-05	1.60E-05	4.70E-01
6	0.500	0.493	0.497	1.23E-05	1.23E-05	4.93E-01
7	0.501	0.466	0.484	3.06E-04	3.06E-04	4.68E-01
8	0.591	0.622	0.607	2.40E-04	2.40E-04	7.36E-01
9	0.541	0.497	0.519	4.84E-04	4.84E-04	5.39E-01
10	0.526	0.434	0.480	2.12E-03	2.12E-03	4.61E-01
Grand sum	10.1	**Grand mean**	0.507			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.81E-02	1.81E-03	4.25E-02	8.39
Between Run	5.84E-02	6.49E-03	4.84E-02	9.54
Total	7.65E-02		6.44E-02	**12.7**

4MDA Quality material 2

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	3.74	4.10	3.92	3.24E-02	3.24E-02	3.07E+01
2	3.81	3.85	3.83	2.89E-04	2.89E-04	2.93E+01
3	3.92	3.87	3.90	5.06E-04	5.06E-04	3.03E+01
4	3.90	3.50	3.70	4.12E-02	4.12E-02	2.74E+01
5	3.59	3.70	3.65	3.08E-03	3.08E-03	2.66E+01
6	3.50	3.58	3.54	1.44E-03	1.44E-03	2.50E+01
7	3.52	3.57	3.55	8.41E-04	8.41E-04	2.51E+01
8	3.68	3.68	3.68	2.25E-06	2.25E-06	2.71E+01
9	3.42	3.28	3.35	4.42E-03	4.42E-03	2.24E+01
10	4.08	4.12	4.10	4.00E-04	4.00E-04	3.36E+01
Grand sum	74.4	**Grand mean**	3.72			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.69E-01	1.69E-02	1.30E-01	3.50
Between Run	8.68E-01	9.65E-02	1.99E-01	5.36
Total	1.04E+00		2.38E-01	**6.40**

Table A3. Stability

Stability - fill in yellow shaded cells

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 2 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at room temperature for 1 day
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name: Urinary Aromatic Diamines by UPLC-MS/MS
Method #: 2120
Matrix: Urine
Units: ng/mL
Analyte: **PPDA**

Quality Material Lot #	**QM170927L**		**QM171121L**		**QM171128L**		**QM171121L**	
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**	**Initial measurement**	**Long-term stability**
Replicate 1	4.24	4.53	6.37	5.96	3.07	3.02	6.37	
Replicate 2	3.82	3.94	6.52	6.20	2.78	2.48	6.52	
Replicate 3	4.77	4.83	6.34	6.31	3.01	3.06	6.34	
Mean	4.28	4.43	6.41	6.2	2.95	2.852	6.41	#DIV/0!
% difference from initial measurement	--	**3.64**	--	**-3.97**	--	**-3.39**	--	**#DIV/0!**

Quality Material Lot #	**QM170927H**		**QM171121H**		**QM171128H**		**QM171121H**	
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**	**Initial measurement**	**Long-term stability**
Replicate 1	8.58	8.83	11.2	9.16	15.6	14.8	11.2	
Replicate 2	9.27	8.30	10.5	9.63	15.5	14.3	10.5	
Replicate 3	7.52	8.58	10.7	9.23	15.0	14.3	10.7	
Mean	8.46	8.57	10.8	9.3	15.4	14.5	10.8	#DIV/0!
% difference from initial measurement	--	**1.33**	--	**-13.4**	--	**-5.77**	--	**#DIV/0!**

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL
Analyte:	**6TDA**

Quality Material Lot #	QM170927L		QM171121L		QM171128L		QM171121L	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	5.30	4.56	5.70	5.81	2.16	2.11	5.70	
Replicate 2	5.37	4.90	6.18	5.58	2.28	2.35	6.18	
Replicate 3	5.59	5.30	5.86	6.00	2.17	2.10	5.86	
Mean	5.42	4.92	5.91	5.80	2.20	2.19	5.91	#DIV/0!
% difference from initial measurement	--	**-9.23**	--	**-9.49**	--	**-0.64**	--	**#DIV/0!**

Quality Material Lot #	QM170927H		QM171121H		QM171128H		QM171121H	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	9.74	9.68	10.7	10.1	13.3	12.3	10.7	
Replicate 2	10.7	9.93	11.3	10.4	11.8	10.7	11.3	
Replicate 3	10.6	10.3	11.5	10.5	11.4	12.0	11.5	
Mean	10.3	9.96	11.2	10.3	12.2	11.6	11.2	#DIV/0!
% difference from initial measurement	--	**-3.47**	--	**-4.43**	--	**-4.30**	--	**#DIV/0!**

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL
Analyte:	**4TDA**

Quality Material Lot #	QM170927L		QM171121L		QM171128L		QM171121L	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.81	4.74	6.22	5.70	2.68	2.64	6.22	
Replicate 2	5.05	4.87	6.49	5.75	2.77	2.66	6.49	
Replicate 3	5.08	5.13	6.43	5.99	2.68	2.77	6.43	
Mean	4.98	4.91	6.38	5.81	2.71	2.69	6.38	#DIV/0!
% difference from initial measurement	--	**-1.33**	--	**-9.33**	--	**-0.750**	--	**#DIV/0!**

Quality Material Lot #	QM170927H		QM171121H		QM171128H		QM171121H	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	9.66	9.44	12.3	11.0	16.1	15.1	12.3	
Replicate 2	10.0	9.37	12.0	10.9	14.5	13.7	12.0	
Replicate 3	9.86	9.31	11.7	11.1	14.3	14.6	11.7	
Mean	9.84	9.37	12.0	11.0	14.9	14.4	12.0	#DIV/0!
% difference from initial measurement	--	**-4.79**	--	**1.71**	--	**-3.35**	--	**#DIV/0!**

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL
Analyte:	**5NDA**

Quality Material Lot #	QM170927L		QM171121L		QM171128L		QM171121L	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.87	5.42	5.75	5.53	3.06	3.16	5.75	
Replicate 2	5.50	5.66	5.88	5.54	2.86	2.68	5.88	
Replicate 3	5.24	5.31	6.01	5.97	2.93	2.48	6.01	
Mean	5.20	5.46	5.88	5.7	2.95	2.8	5.88	#DIV/0!
% difference from initial measurement	--	**5.02**	--	**-11.3**	--	**-6.06**	--	**#DIV/0!**

Quality Material Lot #	QM170927H		QM171121H		QM171128H		QM171121H	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	10.1	10.6	11.3	10.2	17.1	17.1	11.3	
Replicate 2	10.3	10.9	11.8	10.8	18.2	16.4	11.8	
Replicate 3	9.89	10.9	11.4	10.8	16.0	16.8	11.4	
Mean	10.1	10.8	11.5	10.6	17.1	16.8	11.5	#DIV/0!
% difference from initial measurement	--	**6.90**	--	**-1.70**	--	**-1.88**	--	**#DIV/0!**

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL
Analyte:	**4MDA**

Quality Material Lot #	QM170927L		QM171121L		QM171128L		QM171121L	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.11	1.27	1.78	1.81	0.571	0.544	1.78	
Replicate 2	1.26	1.28	1.73	1.77	0.540	0.552	1.73	
Replicate 3	1.24	1.21	1.77	1.92	0.557	0.578	1.77	
Mean	1.20	1.25	1.76	1.8	0.56	0.6	1.76	#DIV/0!
% difference from initial measurement	--	**4.01**	--	**1.17**	--	**0.360**	--	**#DIV/0!**

Quality Material Lot #	QM170927H		QM171121H		QM171128H		QM171121H	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.21	2.22	2.76	2.88	3.30	3.26	2.76	
Replicate 2	2.19	2.15	2.91	2.98	3.06	3.42	2.91	
Replicate 3	2.13	2.24	2.96	3.13	3.22	3.18	2.96	
Mean	2.18	2.20	2.87	3.0	3.19	3.3	2.87	#DIV/0!
% difference from initial measurement	--	**1.19**	--	**1.16**	--	**2.86**	--	**#DIV/0!**

Table A4. LOD, Specificity, Fit for intended use

LOD, specificity and fit for intended use - fill in yellow shaded cells

Method name:	Urinary Aromatic Diamines by UPLC-MS/MS
Method #:	2120
Matrix:	Urine
Units:	ng/mL

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
PPDA	2.00	yes	yes
6TDA	0.640	yes	yes
4TDA	0.640	yes	yes
5NDA	1.00	yes	yes
4MDA	0.048	yes	yes