Laboratory Procedure Manual

Analyte: **Volatile Organic Compounds (VOCs) Metabolites**

Matrix: **Urine**

Method: **Ultra Performance Liquid Chromatography with Electro Spray Tandem Mass Spectrometry [UPLC ESI/MSMS]**

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 Name	SAS Label
UVOC_I & UVOCS_I	URX1DC	N-acel-S-(1,2-dichlorovinl)-L-cys(ng/mL)
	URX2DC	N-Acel-S-(2,2-Dichlorvinyl)-L-cys(ng/mL)
	URX2MH	2-Methylhippuric acid (ng/mL)
	URX34M	3-methipurc acd & 4-methipurc acd(ng/mL)
	URXAAM	N-Ace-S-(2-carbamoylethyl)-L-cys(ng/mL)
	URXAMC	N-Ace-S-(N-methlcarbamoyl)-L-cys(ng/mL)
	URXATC	2-amnothiazolne-4-carbxylic acid(ng/mL)
	URXBMA	N-Acetyl-S-(benzyl)-L-cysteine(ng/mL)
	URXBPM	N-Acetyl-S-(n-propyl)-L-cysteine(ng/mL)
	URXCEM	N-Acetyl-S-(2-Carbxyethyl)-L-Cys(ng/mL)
	URXCYHA	N-Acetyl-S-(1-cyano-2-hydroxyethyl)-L-cysteine
	URXCYM	N-acetyl-S-(2-cyanoethyl)-L-cyst(ng/mL)
	URXDHB	N-Ace-S- (3,4-Dihydxybutl)-L-Cys(ng/mL)
	URXDPM	N-Ace-S-(dimethylphenyl)-L-Cys(ng/mL)
	URXGAM	N-ac-S-(2-carbmo-2-hydxel)-L-cys(ng/mL)
	URXHEM	N-Ace-S-(2-Hydroxyethyl)-L-cys(ng/mL)
	URXHP2	N-Ace-S-(2-hydroxypropyl)-L-cys(ng/mL)
	URXHPM	N-Ace-S-(3-Hydroxypropyl)-L-Cys(ng/mL)
	URXIPM1	N-Acetyl-S-(2-hydroxy-3-methyl-3-butenyl)-L-cysteine + N-Acetyl-S-(2-hydroxy-2-methyl-3-butenyl)-L-cysteine(ng/mL)
	URXIPM3	N-Acetyl-S-(4-hydroxy-2-methyl-2-butenyl)-L-cysteine
	URXPMM	N-A-S-(3-hydrxprpl-1-metl)-L-cys(ng/mL)
	URXMAD	Mandelic acid(ng/mL)
	URXMB1	N-A-S-(1-HydrxMet)-2-Prpn)-L-Cys(ng/mL)
	URXMB2	N-Ac-S-(2-Hydrxy-3-butnyl)-L-Cys(ng/mL)
	URXMB3	N-ace-S-(phenl-2-hydxyetl)-L-cys(ng/mL)
	URXPHE	N-ace-S-(phenl-2-hydxyetl)-L-cys(ng/mL)
	URXPHG	Phenylglyoxylic acid(ng/mL)
	URXPMA	N-Acetyl-S-(phenyl)-L-cysteine(ng/mL)
	URXTCV	N-Acetyl-S-(trichlorovinyl)-L-cys(ng/mL)
	URXTTC	2-thoxothazlidne-4-carbxylic acid(ng/mL)

1. Clinical Relevance and Summary of Test Principle

A. Clinical relevance

Volatile organic compounds (VOCs) are ubiquitous in the environment, originating from many different natural and anthropogenic sources. Human exposure to VOCs occurs through inhalation, ingestion, and dermal contact [1]. VOCs are present in virtually all homes and workplaces. Long-term exposure to certain VOCs may increase the risk for leukemia [2], bladder cancer [3], birth defects [4], and neurocognitive impairment [5]. In the United States, tobacco smoke is the major non-occupational source of exposure to a number of harmful VOCs. Tobacco smoke contains over 8000 chemicals, including a number of carcinogenic and toxic VOCs (e.g., benzene, vinyl chloride, ethylene oxide, 1,3-butadiene, and acrolein) [6-8]. Regardless of exposure source, high levels of toxic VOCs is an area of significant public health concern [9]. Monitoring urinary metabolites of VOCs provides complimentary data to measuring VOCs in exhaled breath or blood, and a longer time window during which biomarkers are elevated following cessation of exposure to VOCs. The non-invasive sampling of urine, longer physiological half-lives of mercapturic acids, and relatively high degree of specificity make urinary mercapturic acids useful biomarkers of exposure to VOCs. Mercapturic acids are formed primarily through the metabolism of VOCs via the glutathione pathway. VOCs and/or their metabolites can react with glutathione (GSH), and undergo further metabolism to form mercapturic acids. These metabolites are then removed from the blood by the kidneys and excreted into urine.

Table 1 shows the urinary VOC metabolites monitored using the current method. We also list the parent compound(s) from which these metabolites can be formed. Acrolein is present in various cooked foods and in the environment. It is formed from carbohydrates, vegetable oils, animal fats, and amino acids during heating of foods, and by combustion of petroleum fuels and biodiesel. Smoking tobacco products is typically the largest source of acrolein exposure [10]. Acrolein induces necrotic and apoptotic cell death in humans. Acrylamide is used for the production of polymers, formulation of cosmetics and body care products, and in textile industry. Acrylamide is also a constituent of normal diet. Acrylamide is formed during the heating of carbohydrate rich food (e.g., French fries, potato chips). It is also a component of cigarette smoke [11]. The acrylamide metabolite, glycidamide, is a putative mutagen and most directly related to acrylamide's carcinogenicity. Acrylonitrile is widely used in the manufacture of plastics, acrylic fibers, and synthetic rubber and is considered as a probable human carcinogen [12]. Benzene is a group 1 carcinogen [13]. It is found in crude oil, gasoline, and tobacco smoke. 1,3-Butadiene is mainly used for production of synthetic rubber alone or as a copolymer with styrene. Environmental sources of 1,3-butadiene are automobile exhaust, exhaust from heating, and cigarette smoke [14]. 1,3-Butadiene is characterized as being carcinogenic to humans by inhalation. Carbon disulfide exposure can affect cardiovascular and nervous systems [15]. A major source of exposure to crotonaldehyde is mainstream and sidestream tobacco smoke [16]. It also occurs naturally in food and is formed during combustion of organic materials. A recent study reported that crotonaldehyde exposure induces oxidative stress and apoptosis in human bronchial epithelial cells [17]. There are

multiple sources of exposure to cyanide other than tobacco smoke (e.g., cyanide from food and from amino acid catabolism) [18]. *N*,*N*-Dimethylformamide (DMF) is a solvent that is used in the production of electronic compounds, pharmaceutical products, and textile coatings, and in the manufacture of synthetic leather, polyurethane, and polyacrylonitrile fibers [19]. Ethylene oxide, which is an intermediate used in the production of ethylene glycol and other oxide derivatives, has been associated with leukemia [20]. Propylene oxide, which is used in industry as a chemical intermediate in the production of propylene glycols and glycol ethers, has been classified as a probable human carcinogen (group 2B) by the IARC [21]. Styrene is one of the most important chemicals used worldwide to manufacture plastics, synthetic rubber, and resins, and it is also an environmental contaminant present in food, tobacco, and engine exhaust. The IARC classified styrene as possibly carcinogenic to humans [22]. Xylenes and toluene are widely used in industry as organic solvents, ingredients of thinners, and in the synthesis of other chemicals [23]. Acute toluene exposure can cause disorientation, euphoria, exhilaration, and tinnitus [24]. Vinyl chloride exposure can cause angiosarcoma [25]. Isoprene, the 2-methyl analog of 1,3-butadiene, has been classified as possibly carcinogenic to humans (group 2B) by IARC. It is mainly used in synthetic rubber production. Tobacco smoke also imposes significant isoprene exposure in humans [29]. Except for perchloroethylene (PERC; also known as tetrachloroethene), 1-bromopropane, and trichloroethene (TCE) all other parent compounds in Table 1 are constituents of tobacco smoke. PERC and 1-bromopropane are widely used as dry cleaning and metal degreasing solvents. PERC is a hazardous air pollutant, a common contaminant detected at superfund waste sites, and is a surface and ground water pollutant [26]. Over 400 million pounds of PERC are produced annually in the United States. 1-Bromopropane is reported to cause reproductive toxicity in male rats and neurotoxicity in both rats and humans [27]. TCE is an important industrial chemical widely used because of its favorable solvent characteristics, chemical stability, and relatively low acute toxicity. However, the studies show that the mutagenic and nephrotoxic metabolite formed in human trichloroethene metabolism could be a risk of nephrocarcinogenesis associated with trichloroethene exposure [28].

Urinary VOC metabolite biomonitoring data will provide useful baseline information about VOC exposures in the US population.

Table 1. VOC metabolites and their parent compounds

Parent Compound	VOC Metabolite	Code
Acrolein	*N*-Acetyl-S- (2-carboxyethyl)-L-cysteine	CEMA
	N-Acetyl-S- (3-hydroxypropyl)-L-cysteine	HPMA
Acrylamide	*N*-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA
	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	*N*-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA
	N-Acetyl-S-(1-cyano-2-hydroxyethyl)-L-cysteine	CYHA
Acrylonitrile, vinyl chloride, ethylene oxide	*N*-Acetyl-S- (2-hydroxyethyl)-L-cysteine	HEMA
Benzene	*N*-Acetyl-S-(phenyl)-L-cysteine	PMA
1-Bromopropane	*N*-Acetyl-S-(*n*-propyl)-L-cysteine	BPMA
1,3-Butadiene	*N*-Acetyl-S- (3,4-dihydroxybutyl)-L-cysteine	DHBM
	N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHB1
	N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHB2
	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHB3
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid	TTCA
Crotonaldehyde	*N*-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMM
Cyanide	2-Aminothiazoline-4-carboxylic acid	ATCA
N, N- Dimethylformamide	*N*-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCA
Ethylbenzene, styrene	Phenylglyoxylic acid	PHGA
Isoprene	*N*-Acetyl-S-(2-hydroxy-3-methyl-3-buten-1-yl)-L-Cysteine + *N*-Acetyl-S-[1-(hydroxymethyl)-2-methyl-2-propen-1-yl)-L-Cysteine	IPM1
	N-Acetyl-S-(4-hydroxy-2-methyl-2-buten-1-yl)-L-Cysteine	IPM3
Propylene oxide	*N*-Acetyl-S-(2-hydroxypropyl)-L-cysteine	HPM2
Styrene	*N*-Acetyl-S-(1-phenyl-2-hydroxyethyl-L-cysteine + *N*-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	PHEM
	Mandelic acid	MADA
Tetrachloroethylene	*N*-Acetyl-S-(trichlorovinyl)-L-cysteine	TCVM
Toluene	*N*-Acetyl-S-(benzyl)-L-cysteine	BMA
Trichloroethylene	*N*-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1DCV
	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2DCV
Xylene	*N*-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + *N*-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + *N*-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	DPMA
	2-Methylhippuric acid	2MHA
	3-Methylhippuric acid + 4-Methylhippuric acid	34MH

B. Test principle

This method is a quantitative procedure for the measurement of VOC metabolites in human urine using ultra performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS) [30]. Currently, chromatographic separation is achieved by using a C18 reversed phase column with 15 mM ammonium acetate and acetonitrile as the mobile phases. The choice of column and mobile phases should be such that it ensures adequate baseline separation among the metabolites and minimizes any background interferences. The eluent from the column is ionized using an electrospray interface to generate and transmit negative ions into the mass spectrometer. Comparison of relative response factors (ratio of native analyte to stable isotope labeled internal standard) with known standard concentrations yields individual analyte concentrations.

2. Safety Precautions

A. Reagent toxicity or carcinogenicity

The chemical, physical, and toxicological properties of most of the VOC metabolites have not been thoroughly investigated. Contact of VOC metabolites with strong oxidizing agents should be avoided as this could generate toxic fumes of carbon monoxide, carbon dioxide, nitrogen oxides, and sulfur oxides. However, aqueous solutions of VOC metabolites do not present a fire or explosion hazard. These compounds may cause respiratory tract, skin, and eye irritation. Gloves, lab coat, and safety glasses must be worn while preparing solutions and handling human urine. Disposable plastics (pipette tips, autosampler tubes, gloves, etc.), glass, and paper that come in contact with urine are placed in a biohazard autoclave bag. These bags are kept in appropriate containers until sealed and autoclaved. All work surfaces are wiped down with 70% ethanol solution when work is finished.

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

Special precautions must be followed 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, it is to be quickly washed with lots of water.**

B. Radioactive hazards

None

C. Microbiological hazards

Follow Universal Precautions. Because of the possibility of exposure to various microbiological hazards, appropriate measures are to be taken to avoid any direct contact

with the urine specimen. Gloves, lab coats, and safety glasses must be worn while handling all human urine products. A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues.

D. Mechanical hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. The manufacturer's information regarding safe operation of the equipment need to be read and followed by the laboratorians. Direct contact with the mechanical and electronic components of the mass spectrometer must be avoided unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair are performed only by qualified technicians. The autosampler and the mass spectrometer contain a number of areas that are hot enough to cause burns. Precautions are to be taken when working in these areas.

E. Protective equipment

Standard safety precautions are followed when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood.

F. Training

Users are required to demonstrate safe and proper techniques in performing the method and to generate data with acceptable accuracy and precision based on their calibration curves, QCs, and PTs.

G. Personal hygiene

Follow Universal Precautions. Care has to be taken when handling chemicals or any biological specimen. Routine use of gloves, personal protective equipment, and proper hand washing must be practiced. The laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures are to be consulted for details related to specific activities, reagents, or agents.

H. Disposal of waste

Waste materials must be disposed in compliance with laboratory, federal, state, and local regulations. Solvents and reagents are disposed in an appropriate container clearly marked for waste products and are temporarily stored in a chemical fume hood. All disposable items that come in direct contact with the biological specimens are placed in a biohazard autoclave bag that is kept in appropriate container until sealed and autoclaved. Used unshielded needles, glass Pasteur pipettes, and disposable syringes are immediately placed into a sharps container and autoclaved when this container becomes full. All surfaces are wiped down with 70% ethanol solution (or equivalent) when work is finished.

3. Computerization; Data-System Management

A. Software and knowledge requirements

Different software packages (e.g., Analyst, MultiQuant) are used to control the UPLC system and the mass spectrometer during data acquisition and to analyze chromatograms after the run. Final reportable results are exported to the ATLIS database. Knowledge and expertise of these software packages (or their equivalent) are required to utilize and maintain the data management structure.

B. Sample information

Information pertaining to particular specimens is entered into the database either manually or electronically.

C. Data maintenance

All samples and analytical data are checked prior to being entered into the ATLIS database for transcription errors and overall validity. The data is routinely backed up locally onto a computer hard drive and in 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.

4. Specimen Collection, Storing and Handling Procedures; Criteria for Specimen Rejection

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

(2) The matrix type is urine.

(3) A total sample volume of 0.25-0.5 mL is required to allow for repeated analysis. An aliquot of at least 50 µl is needed for typical analysis.

(4) Acceptable containers include polystyrene cryovial tubes or polypropylene (PP) centrifuge tubes. Sterile collectors should be used for specimen acquisition.

(5) The criteria for unacceptable specimen is any suspected contamination due to

improper collection procedures or collection devices. In all cases, a second urine specimen should be requested.

(6) Specimen characteristics that may compromise test results are as indicated above including contamination of urine by contact with dust, dirt, etc. from improper handling.

(7) Detailed instructions for urine collection and processing are outlined in the DLS policies and procedures manual. Collection, transport, and special requirements are discussed. In general, urine specimens should be transported and stored chilled or frozen at -20ºC. Once received, the samples can be frozen at -70ºC until time for analysis. Portions of the sample that remain after analytical aliquots are refrozen at -20 or -70ºC. Freeze-thawing of samples more than five times are to be avoided.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable to this assay

6. Preparation of Reagents, Calibration (Standards), Controls, and all other Materials; Equipment and Instrumentation

A. Reagents and sources

Reagents that were used during the development, validation, and application of this method are listed in **Table 2** along with their suggested sources. All chemicals and solvents are used without further purification**.**

Table 2. Reagents and sources

Reagent	Code	Source
Solvents		
Acetonitrile (Optima LCMS grade)		Fisher Scientific, Fairlawn, NJ
Ammonium Acetate		Sigma Chemicals, St. Louis, MO
Methanol (Optima LCMS grade)		Fisher Scientific, NJ
Isopropyl alcohol (Optima LCMS grade)		Fisher Scientific, NJ
Water (HPLC grade)		Fisher Scientific, Fairlawn, NJ
Native Calibration and Control Materials		
N-Acetyl-S-(benzyl)-L-cysteine	BMA	Battelle Research, Columbus, Ohio
N-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA	C/D/N Isotopes Inc, Quebec, Canada
N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-carboxyethyl)-L-Cysteine	CEMA	Cambridge Isotopes, Andover, MA
N-acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1DCV	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2DCV	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3,4-dihydroxybutyl)-L-Cysteine	DHBM	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine	2,4-DPMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine	2,5-DPMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	3,4-DPMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-hydroxyethyl)-L-cysteine	HEMA	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	HPM2	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	HPMA	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHB1	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHB2	Kalexsyn Inc., Kalamazoo, MI
N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine)	MHB3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3-hydroxypropyl-1 methyl)-L-cysteine	HPMM	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCA	Sigma Chemicals, St. Louis, MO
N-Acetyl-S-(phenyl)-L-cysteine	PMA	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(1-phenyl-2-hydroxyethyl-L-cysteine	PHEM1	Toronto Research Chemicals,
N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	PHEM2	Toronto, Canada
N-Acetyl-S-(*n*-propyl)-L-cysteine	BPMA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(trichlorovinyl)-L-cysteine	TCVM	Battelle Research Institute, Columbus, Ohio
2-Aminothiazoline-4-carboxylic acid	ATCA	Chem-Impex International Inc., Woodale, IL
Mandelic acid	MADA	Sigma Chemicals, St. Louis, MO
2-Methylhippuric acid	2MHA	Sigma Chemicals, St. Louis, MO
3-Methylhippuric acid	3MHA	Sigma Chemicals, St. Louis, MO
4-Methylhippuric acid	4MHA	Sigma Chemicals, St. Louis, MO
Phenylglyoxylic acid	PHGA	Sigma Chemicals, St. Louis, MO
2-Thioxothiazolidine-4-carboxylic acid	TTCA	Sigma Chemicals, St. Louis, MO
N-Acetyl-S-(1-cyano-2-hydroxyethyl)-L-Cysteine	CYHA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-hydroxy-3-methyl-3-buten-1-yl)-L-Cysteine + *N*-Acetyl-S-[1-(hydroxymethyl)-2-methyl-2-propen-1-yl)-L-Cysteine	IPM1	Toronto Research Chemicals, Toronto, Canada

N-Acetyl-S-(4-hydroxy-2-methyl-2-buten-1-yl)-L-Cysteine	IPM3	Toronto Research Chemicals, Toronto, Canada
Isotopically Labeled Internal Standards		
N-Acetyl-S-(benzyl-$^{13}C_6$)-L-cysteine	BMA-$^{13}C_6$	Battelle Research Institute, Columbus, Ohio
N-Acetyl-S-(2-carbamoylethyl-D_4)-L-cysteine	AAMA- D_4	C/D/N Isotopes Inc, Quebec, Canada
N-Acetyl-D_3-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-carboxyethyl-$^{13}C_3$)-L-cysteine	CEMA-$^{13}C_3$	Cambridge Isotopes, Andover, MA
N-acetyl-D_3-S-(2-cyanoethyl)-L-cysteine	CYMA- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-^{13}C- D_3-S-(1,2-dichlorovinyl)-L-cysteine	1DCV-^{13}C- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-^{13}C- D_3-S-(2,2-dichlorovinyl)-L-cysteine	2DCV-^{13}C- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3,4-dihydroxybutyl-$^{13}C_4$)-L-cysteine	DHBM-$^{13}C_4$	Cambridge Isotopes, Andover, MA
N-Acetyl-D_3-S-(2,4-dimethylphenyl)-L-cysteine	2,4-DPMA- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-D_3-S-(2,5-dimethylphenyl)-L-cysteine	2,5-DPMA- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-D_3-S-(3,4-dimethylphenyl)-L-cysteine	3,4-DPMA- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-hydroxyethyl-D_4)-L-cysteine	HEMA- D_4	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(2-hydroxypropyl-D_3)-L-cysteine	HPM2- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(3-hydroxypropyl-D_6)-L-cysteine	HPMA- D_6	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(1-hydroxymethyl-2-propenyl-D_6)-L-cysteine	MHB1- D_6	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine-$^{13}C_3$-^{15}N	MHB2$^{13}C_3$-^{15}N	Kalexsyn Inc., Kalamazoo, MI
N-Acetyl-D_3-(4-hydroxy-2-buten-1-yl)-L-cysteine)	MHB3- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-D_3-S-(3-hydroxypropyl-1 methyl)-L-cysteine	HPMM- D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine -$^{13}C_3$-^{15}N	AMCA-$^{13}C_3$-^{15}N	Kalexsyn Inc., Kalamazoo, MI
N-Acetyl-S-(phenyl-$^{13}C_6$)-L-cysteine	PMA-$^{13}C_6$	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(1-phenyl-$^{13}C_6$-2-hydroxyethyl-L-cysteine	PHEM1-$^{13}C_6$	Toronto Research Chemicals,
N-Acetyl-S-(2-phenyl-$^{13}C_6$-2-hydroxyethyl)-L-cysteine	PHEM2-$^{13}C_6$	Toronto, Canada
N-Acetyl-S-(*n*-propyl-D_7)-L-cysteine	BPMA-D_7	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(trichlorovinyl-$^{13}C_2$)-L-cysteine	TCVM-$^{13}C_2$	Battelle Research Institute, Columbus, Ohio
2-Aminothiazoline-D_3-4-carboxylic acid	ATCA-D_3	Dr. Bill Draper's Lab, CDPH, CA
Mandelic-2,3,4,5,6-D_5 acid	MADA-D_5	C/D/N Isotopes Inc, Quebec, Canada
2-Methylhippuric-D_7 acid	2MHA-D_7	C/D/N Isotopes Inc, Quebec, Canada
3-Methylhippuric-D_7 acid	3MHA-D_7	C/D/N Isotopes Inc, Quebec, Canada
4-Methylhippuric-D_7 acid	4MHA-D_7	C/D/N Isotopes Inc, Quebec, Canada
Phenylglyoxylic-D_5 acid	PHGA-D_5	C/D/N Isotopes Inc, Quebec, Canada
2-Thioxothiazolidine-$^{13}C_3$-4-carboxylic acid	TTCA-$^{13}C_3$	Cambridge Isotopes, Andover, MA
N-Acetyl-S-(1-cyano-2-hydroxyethyl)-L-Cysteine-D_3	CYHA-D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(2-hydroxy-3-methyl-3-buten-1-yl)-L-Cysteine-D_3 + *N*-Acetyl-S-[1-(hydroxymethyl)-2-methyl-2-propen-1-yl)-L-Cysteine-D_3	IPM1-D_3	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-(4-hydroxy-2-methyl-2-buten-1-yl)-L-Cysteine	IPM3-D_3	Toronto Research Chemicals, Toronto, Canada

B. Solvents

HPLC grade water and LCMS grade solvents (e.g., acetone, methanol, isopropyl alcohol) are used to prepare mobile phases. Every run contains a water sample with 15 mM ammonium acetate, referred to as a double blank, to monitor the quality of the mobile phase and to detect any contamination.

(1) Calibration and control materials

Currently used calibration and quality control materials including native compounds and isotopically labeled internal standards are at least 95% pure. Generally, isotopic purity of internal standards are at least 97%. Isotopically labeled compounds are checked for any spectral overlap with corresponding native analogs before use. Each run contains a blank sample (internal standard and 15 mM ammonium acetate) to monitor any changes in quality.

C. Reagent preparation

(1) 15mM ammonium acetate

15 mM ammonium acetate is used as Solvent A (mobile phase of UPLC), to prepare working calibration standards, and to dilute urine and quality control (QC) samples. To prepare the solution, ammonium acetate is dissolved in HPLC-grade water and is filtered through a glass filtration system. The pH of the solution needs to be checked, which should be between 6.5 and 7.

D. Standards solutions preparation

(1) Native analytical standards

a. Individual primary stock solutions

i. The primary stock solutions are prepared by dissolving the neat compounds individually in appropriate solvents (**Table 3**). For hygroscopic compounds, special procedures, such as drying the neat material in a desiccator before use, are to be taken. The prepared stocks are stored at -20ºC for future use.

Table 3. Solvent used to prepare initial stock solution

Analyte	Solvent used to Prepare Initial Stock
AAMA; AAMA-D_4	water
AMCA; AMCA-$^{13}C_3$-^{15}N	water
ATCA; ATCA-D_3	water
BMA; BMA-$^{13}C_6$	water
BPMA; BPMA-D_7	methanol:water (1:1)
CEMA; CEMA-$^{13}C_3$	water
CYMA; CYMA-D_3	water
1DCV; 1DCV-^{13}C-D_3	methanol
2DCV; 2DCV-^{13}C-D_3	methanol
DHBM; DHBM-$^{13}C_4$	water
2,5 DPMA; 2,5 DPMA-D_3	methanol
3,4 DPMA; 3,4 DPMA-D_3	methanol
2,4DPMA; 2,4 DPMA-D_3	methanol
GAMA; GAMA-D_3	water
HEMA; HEMA-D_4	water
HPMA; HPMA-D_6	water
HPM2; HPM2-D_3	water
HPMM; HPMM-D_3	water
MADA; MADA-D_5	methanol:water (1:1)
2MHA; 2MHA-D_7	methanol:water (1:1)
3MHA; 3MHA-D_7	methanol:water (1:1)
4MHA; 4MHA-D_7	methanol:water (1:1)
MHB1; MHB1-D_6	water
MHB2; MHB2-$^{13}C_3$-^{15}N	water
MHB3; MHB3-D_3	methanol:water (1:1)
PHGA; PHGA-D_5	water
PHEM; PHEM-$^{13}C_6$	methanol
PMA; PMA-$^{13}C_6$	water
TCVM; TCVM-$^{13}C_2$	methanol
TTCA; TTCA-$^{13}C_3$	water
CYHA; CYHA-D_3	methanol:water (1:1)
IPM1; IPM1-D_3	DMSO:methanol (1:1); methanol
IPM3; IPM3-D_3	methanol:water (1:1)

ii. Mixed intermediate stock solutions

Intermediate stock solutions are prepared for at least five levels and are 10 times higher than the corresponding working standards. A sample composition is given in **Table 4**. To prepare each level, the appropriate volume of each analyte is pipetted from the individual

primary stock solutions into a volumetric flask and the mixture is diluted with HPLC-grade water to attain the required final concentration. The solutions are aliquoted in cryovial tubes and are stored at -70ºC. Each set is thawed once and the remaining solution is discarded after use.

Table 4. A sample composition of mixed intermediate stock solutions (ng/mL)

Analyte	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
CYMA	0.50	0.75	1.58	5.00	15.8	50.0	158	500	1581
HPMM	3.04	4.56	9.61	30.40	96.1	304.0	961	3040	9613
MHB3	0.55	0.83	1.74	5.50	17.4	55.0	174	550	1739
HPM2	2.64	3.96	8.35	26.42	83.5	264.2	835	2642	8355
3MHA	3.10	4.65	9.80	31.00	98.0	310.0	980	3100	
4MHA	3.10	4.65	9.80	31.00	98.0	310.0	980	3100	
AAMA	1.10	1.65	3.48	11.00	34.8	110.0	348	1100	
BMA	0.44	0.66	1.39	4.40	13.9	44.0	139	440	
HPMA	12.96	19.44	40.98	129.60	409.8	1296.0	4098	12960	
DHBM	4.00	6.00	12.65	40.00	126.5	400.0	1265	4000	
2MHA	3.10	4.65	9.80	31.00	98.0	310.0	980	3100	
AMCA	3.60	5.40	11.38	36.00	113.8	360.0	1138	3600	
BPMA	0.77	1.15	2.43	7.68	24.3	76.8	243	768	
PHGA	10.07	15.11	31.84	100.70	318.4	1007.0	3184		
IPM1	1.43	2.14	4.51	14.27	45	143	451		
CEMA	6.00	9.00	18.97	60.00	189.7	600.0	1897		
GAMA	5.91	8.86	18.67	59.05	186.7	590.5	1867		
HEMA	0.38	0.57	1.19	3.77	11.9	37.7	119		
MADA	12.00	18.00	37.95	120.00	379.5	1200.0	3795		
ATCA	8.90	13.35	28.14	89.00	281.4	890.0	2814		
PHEM	0.50	0.75	1.58	5.00	15.8	50.0	158		
PMA	0.35	0.52	1.09	3.45	10.9	34.5	109		
2DCV	3.90	5.85	12.33	39.00	123.3	390.0	1233		
TCVM	1.51	2.27	4.78	15.10	47.8	151.0	478		
24DPMA	0.076	0.114	0.240	0.760	2.40	7.60	24.03		
25DPMA	0.076	0.114	0.240	0.760	2.40	7.60	24.03		
34DPMA	0.076	0.114	0.240	0.760	2.40	7.60	24.03		
MHB1	0.46	0.68	1.44	4.56	14.4	45.6	144		
MHB2	0.48	0.72	1.52	4.80	15.2	48.0	152		
1DCV	6.30	9.44	19.91	62.96	199.1	629.6	1991		
TTCA	11.17	16.75	35.31	111.67	353	1117	3531		
IPM3	0.53	0.80	1.69	5.33	16.9	53.3	169	533	1685
CYHA	2.60	3.90	8.22	26.00	82	260	822	2600	

iii. Working mixed standard solutions

Each level of intermediate stock is diluted 10 times with 15 mM ammonium acetate solution to prepare the corresponding working standard level. The preparation of the working standard solutions should follow certain criteria: (a) concentration at each level should

be separated from the next level by a maximum factor of √10, (b) the lowest concentration is to be equal to or less than the LOD, and (c) the highest standard should ideally cover the 99th percentile of the expected population level, whenever that information is available.

b. Isotopically labeled internal standard solutions

i. Individual primary stock solutions

The primary internal standard (IS) stock solutions are prepared by dissolving the neat compounds individually in appropriate solvents (**Table 3**). For hygroscopic compounds, special procedures, such as drying in a desiccator before use, are to be taken. The prepared stocks are stored at -20ºC for future use.

ii. Mixed intermediate stock solutions

The concentrations of internal standards (IS) in intermediate stock should be 20 times higher than that in the working solution. The appropriate volume of each IS is pipetted from the individual primary stock solutions into a volumetric flask and the mixture is diluted with HPLC-grade water to attain the required final concentration. These solutions are aliquoted in cryovial tubes and are stored at -70ºC. Each vial is thawed once and the remaining solution is discarded after use.

iii. Working mixed internal standard solutions

The intermediate stock is diluted 20 times with 15 mM ammonium acetate solution to prepare the working internal standard (IS). The final concentration of each IS should be between standard (native analyte) level 3-5 and yield an absolute peak intensity of at least 75,000 counts.

(2) Preparation of quality control material

a. Quality control pools

Quality Control (QC) materials are prepared at two concentration levels, QL (QC low) and QH (QC high), in urine. QL should be between standard levels 3 and 5, and QH between 5 and 7. The urine matrix can have high backgrounds for certain analytes, in those cases amount of analyte to be spiked should be adjusted to meet the target concentration. Aliquots of QL and QH are stored separately in cryovials at -70°C until use. Each vial is thawed once and the remaining solution is discarded after use. At least 20 separate QC samples are analyzed using different sample runs and instruments to characterize the QCs and to determine the mean values and

coefficient of variation (CV) for individual analytes.

b. Proficiency testing samples

Proficiency testing materials at four (analyte concentration) levels are prepared from the individual primary stock solutions in a manner similar to the mixed intermediate stocks. Aliquots are stored in cryovials, at -70°C until use. Proficiency testing samples are run at least two times a year. A proficiency testing coordinator, independent from the sample analysis team, blind-codes the PT stock vials and verifies accuracy of quantified results of four PT samples at each of the four concentration levels and one sample at any of the four different levels.

E. Instrumentation and operation

(1) Liquid chromatography (LC)

Chromatographic separation of the analytes is achieved with a UPLC system (e.g., Waters Acquity) fitted with a reversed phase C18 column (e.g., Acquity UPLC® HSS T3). A guard column is mounted upstream to protect the analytical column from impurities. The column and the sample manager are set at optimum temperatures, for example, 40°C and 25°C respectively. The needle and loop volumes are characterized monthly and after any repair or maintenance to ensure proper sample loading to the column.

The mobile phase consists of 15 mM ammonium acetate (Solvent A) and acetonitrile (Solvent B). The separation conditions are optimized to obtain good resolution among VOC metabolites, a representative example is given in **Table 5**. Before each run, the column is equilibrated with the initial mobile phase composition for at least 10 column volumes. After each sample injection, the needle is first cleaned with a strong wash and subsequently with a weak wash (**Table 5)**. At the end of each run, the column is washed with an aqueous solution (e.g., A:B = 97:3) followed by 100% acetonitrile and is stored in acetonitrile.

Table 5. Chromatography parameters for the UPLC

Parameter	Details
Weak Wash	HPLC grade water
Strong Wash	25% HPLC grade water 25% Optima LCMS grade Acetonitrile 25% Optima LCMS grade methanol 25% Optima LCMS grade isopropyl alcohol
Gradient : Time, flow, Solvent A : Solvent B	 initial, 250 µl/min, 97% : 3% 2 min, 250 µl/min, 95%: 5% 3 min, 300 µl/min, 90%:10% 5 min, 300 µl/min, 70%: 30% 6.5min, 300 µl/min, 60%:40% 7 min, 300 µl/min, 85%:15% 7.5 min, 300 µl/min, 90%:10% 8 min, 300 µl/min, 97%:3% 9 min, 300 µl/min, 97%:3%

(2) Mass spectrometer (MS)

A triple quadrupole mass spectrometer (e.g., AB Sciex Triple Quad 5500) with an electrospray ion source is used for the detection of urinary VOC metabolites. The mass spectrometer is operated under Scheduled Multiple Reaction Monitoring (SMRM) mode. The instrument parameters are optimized to obtain the maximum signal intensity, dynamic range, and signal to noise (S/N) ratio. Compounds (native analytes and internal standards) are optimized individually to select transitions and associated mass spectrometric parameters (e.g., declustering potential, collision energy, etc.) for maximum selectivity and signal intensity. These parameters should be re-optimized when transferring the method to a new instrument. **Table 6** lists suggested transitions for the VOC metabolites measured by this method.

Table 6. Example of MRM transitions for VOC metabolites

Analyte	Transition		Internal Standard	Transition
	Quan. Ion[a]	Conf. ion[b]		
AAMA	233/104	233/58	AAMA-D_4	237/108
AMCA	219/162	220/163 219/84	AMCA-^{15}N-$^{13}C_3$	223/166
ATCA	145/67	145/58	ATCA-D_3	148/70
BMA	252/123	253/124	BMA-$^{13}C_6$	258/84
BPMA	204/84	204/75	BPMA-D_7	211/82
CEMA	234/162	234/105	CEMA-$^{13}C_3$	237/162
CYHA	231/84	231/102	CYHA-D_3	234/102
CYMA	215/86	215/162	CYMA-D_3	218/165
1DCV	256/127	258/129	1DCV-^{13}C-D_3	260/127
2DCV	257/127	256/127	2DCV-^{13}C-D_3	261/127
DHBM	250/121	250/75	DHBM-$^{13}C_4$	254/125
DPMA	266/137	267/138	DPMA-D_3	269/137
GAMA	249/120	249/128	GAMA-D_3	252/120
HEMA	206/77	206/75	HEMA-D_4	210/81
HPMA	220/91	220/89	HPMA-D_6	226/97
HPM2	220/91	221/91	HPM2-D_3	223/91
HPMM	234/105	235/105	HPMM-D_3	237/105
IPM1	246/117	246/87	IPM1-D_3	249/117
IPM3	246/117	246/87	IPM3-D_3	249/87
MADA	151/107	151/77	MADA-D_5	156/112
2MHA	192/148	192/91	2MHA-D_7	199/155
34MH	192/148	192/91	34MH-D_7	199/155
MHB1	232/103	233/103 232/73	MHB1-D_6	238/109
MHB2	232/103	233/103 232/128	MHB2-$^{13}C_3$-^{15}N	236/103
MHB3	232/103	233/103 232/85	MHB3-D_3	235/103
PHGA	149/77	149/105	PHGA-D_5	154/82
PHEM	282/153	282/123 282/128	PHEM-$13C_6$	288/159
PMA	238/109	239/110	PMA-$^{13}C_6$	244/115
TCVM	290/161	290/35	TCVMA-$^{13}C_2$	296/167
TTCA	162/58	162/33	TTCA-$^{13}C_3$	165/58

[a]Quantitation ion. [b]Confirmation ion.

Note: Analytes with same SMRM transitions (e.g., MHB1, MHB2, & MHB3) elute at different retention times.

Mass spectrometers are tuned once a month and after any repair or performance maintenance. The curtain plate is cleaned before each run to remove any deposition from previous runs. The performance of the instrument is also checked before every scheduled run by injecting a low standard (e.g., std 2) three times and by calculating the S/N ratio, which should be at least 10. Additionally, the overall intensity and resolution between peaks are also evaluated.

(3) Robotic liquid handling system

All calibration standards, QCs, and urine samples are aliquoted, prepared, and mixed by a robotic liquid handling system such as Hamilton Microlab Star. **Table 7** exemplifies a sample preparation protocol. Routine maintenance is done weekly to check for any leakage and calibration error.

Table 7. An example of a sample preparation protocol using robotic liquid handler

Sample	Vol. of sample (µL)	Vol. of IS (µL)	Vol. of 15 mM ammonium acetate (µL)
Double Blank	0	0	500
Blank	0	25	475
Calibration standard	50	25	425
Quality Control	50	25	425
Urine	50	25	425
Proficiency Testing	50	25	425

7. Calibration and calibration verification

Different urine samples contain varying background levels of VOC metabolites and hence urine cannot be used as a reliable matrix to prepare calibration standards. Instead, 15 mM ammonium acetate solution is used for this purpose. Matrix validation experiments were performed to verify that the calibration curves in urine and in ammonium acetate had the same slope (Appendix, Table B1) [30].

A. Calibration curve

At least one set of calibrators is used for the quantitation of analytes in all urine samples from a batch. The calibration curve for each analyte is constructed from the response ratio, which is the area ratio of the unlabeled analyte to its corresponding internal standard. The slope and intercept of curves are determined by least squares regression of 1/x weighted data. Calibration curves should be composed of at least five standard levels that span the range of all detectable unknown samples, and should achieve an R-squared coefficient of at least 0.98.

B. Calibration verification

Calibration accuracy is tested with each run by analysis of blank (15 mM ammonium

acetate and IS) and quality control samples. A full set of calibrators is analyzed with each batch of urine samples. Absolute accuracy is verified by proficiency testing at least twice a year.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

A. Sample preparation

An analytical run consists of double blank (15 mM ammonium acetate), blank (15 mM ammonium acetate + internal standard), calibration standards, low level QC, high level QC, and unknown urine samples. Prior to analysis, all samples including urine, standards, and QCs are equilibrated at room temperature and vortexed for at least 3 seconds. A robotic liquid handling system prepares the samples following the protocol as demonstrated in **Table 7**. Briefly, urine samples and QCs are diluted 10 times with 15 mM ammonium acetate. Each sample is immediately spiked with the internal standard solution and mixed properly.

B. Data analysis

Unknown samples are quantified by the ratio of the analyte peak area to the internal standard peak area. Use of internal standard compensates for analyte-dependent selectivity biases, such as matrix effects associated with the ionization process, and confirms the presence of a native target when there is any shift in chromatographic retention time. Urine and QC sample concentrations are multiplied by the appropriate dilution factor.

C. Data processing

(1) Peak integration

Each peak is visually inspected and peak integration is corrected if the software erroneously integrates a peak. For each analyte, the confirmation ion signal is quantified above a certain concentration threshold.

(2) Excluding calibrators

A particular calibrator is only excluded if it significantly affects (>10%) the detectable results and the cause behind the anomaly is identified. Scenarios that might only affect a single standard include no or low addition of analyte or internal standard and missed injection because of instrument failure. However, the highest standard level can be excluded if the calibration curve is nonlinear over this region because all QCs fall below standard level 7. In that case, analysis of unknown samples, which exceed the calibration range, are diluted and repeated.

(3) Excluding sample data

Absolute internal standard response is evaluated for consistency among the standards, blanks, QCs, and urine samples. Sample data is excluded if low or excess IS is added to the urine sample, which is identified by the absolute IS response that varies by more than a factor of 2 as compared to similar sample types. Poorly resolved co-eluents can cause an unusually high internal standard response, which also warrants elimination of the sample.

9. Reportable range of results

A. Reportable limits

Only the data above or at LOD is reported. The upper reportable limit corresponds to the concentration of the highest standard. If the analyte level exceeds the upper calibration range, the sample is repeated by diluting it 10-100 times as required falls within the std curve with 15 mM ammonium acetate.

B. Limit of detection

The analytical limit of detection was calculated as described by the DLS policies and procedure manual.

C. Accuracy

The accuracy of the assay is established by blind analysis of Proficiency Testing (PT) samples and whenever necessary, by spike recovery experiment in which urine is spiked at three different concentration levels. The accuracy is obtained by comparing the calculated concentration to the theoretical concentration; the maximum allowed deviation is ±25%.

D. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficient of variation (CV) of the method was determined based on 20 independent analyses of the QC samples.

E. Analytical specificity

LC-MS/MS is a highly selective analytical method for quantifying the target analytes in complex aqueous matrices. Reversed phase liquid chromatography reproducibly resolves the target analytes, even in the most concentrated urine samples. Analytical specificity is established by comparing the retention times of an analyte relative to its internal standard. Tandem mass spectrometry provides a further degree of selectivity, by filtering out all ions except a specific transition of precursor-to-product ions for each analyte. Additionally, qualifier ratios, the area ratios of quantitation ion to the confirmation ion, are determined for the standards and QC samples. The average value of this ratio should be within ± 25%.

10. Quality Assessment and Proficiency Testing

A. Quality assessment

Quality assessment procedures follow standard practices [32]. Daily experimental checks are made on the stability of the analytical system. Blanks, standards, and QC materials are added to each run sequence. A blank is analyzed at the beginning of each run to check the system for possible contamination. Relative retention times are examined for the internal standard to ensure the choice of the correct chromatographic peak. A calibration curve is developed for the batch using a complete set of calibration standards. The calibration curve must have a coefficient of determination, R^2-value of at least 0.98. The results from the analysis of QC materials obtained using these calibration curves are compared using the acceptance criteria given below to assure precision of the analysis.

B. Quality control procedures

(1) Establishing QC limits

Two different pools of quality control material are used, one at a low and the other at a high concentration. Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Different variables are included in the characterization analyses (e.g., different analysts, columns, instruments, etc.) to capture realistic assay variation over time. One instrument characterizes no more than two samples from one pool per day. 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 and examined. Quality control limits are used to document assay precision and accuracy on a daily basis. Limits are based on statistical calculation accounting for two QCs analyzed in each analytical run.

(2) Quality control evaluation

After the completion of a run, the calculated results from the analysis of quality control samples are compared to the established quality control 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 according to the DLS policies and procedures manual. 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

(1) Scope of PT

The proficiency testing (PT) scheme for this method is administered by an in-house Proficiency Testing Coordinator. Aqueous proficiency testing materials are prepared from the primary stock solutions, diluted in water, and blind-coded by the in-house PT Coordinator. The samples are analyzed and the results are

evaluated by the in-house PT coordinator.

(2) Frequency of PT

Four samples of unknown PT concentrations are analyzed at least twice a year using the same method described for unknown samples.

(3) Documentation of PT

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; if the value falls between 75% and 125% of the expected value, then the analysis passes the proficiency test. A summary report of the PT evaluation is maintained by the laboratory quality control officer. If the assay fails proficiency testing, then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Analyte data for unknown specimens may only be reported if that analyte successfully passes proficiency testing.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If an analyte result for a quality control material falls outside of the acceptable range, then it fails the QC criteria, and following steps should be taken.

(1) Calibration Standards: If R-squared value is less than 0.98 for the fitted curve, then the individual calibration standards are evaluated for any obvious error (e.g., missed IS or analyte or injection, improper peak integration, etc.). If not, then a new calibration set (working standard) is prepared and acquisition and analysis of the entire batch, including QCs & unknown samples, is repeated.

(2) Quality control material: If the QC material is the suspected cause of the error, then a fresh QC sample is prepared and analyzed.

(3) Internal standard response: If no missed IS aliquoting or missed injection is detected, then the absolute IS response should be compared to an earlier run. If the observed change exceeds 25%, then a new IS working solution is prepared and the run is repeated.

(4) Contamination: Blank (internal standard and ammonium acetate) and double blank (ammonium acetate only) samples should be investigated for any contamination, e.g., presence of a ghost co-eluent peak or high background of unlabeled analyte in blank. The mobile phase is to be prepared fresh and the LC system need to be cleaned prior to any measurement.

(5) Intermediate stock solution: Occasionally the composition of the intermediate stock solution for native analytes could be erroneous. In that case, new

intermediate stock solutions followed by the working standards should be prepared and used for further measurements.

If these steps do not result in correction of the "out of control" values for QC materials, the supervisor should be consulted for other appropriate corrective actions. Analytical results are not reported for runs that are out of statistical control.

12. Limitations of Method, Interfering Substances and Conditions

The described method is highly selective. Because of excellent chromatographic and mass spectrometric resolution, we typically do not find other interfering substances that have similar chromatographic and mass spectrometric characteristics. However, in some urine samples, chromatography can be distorted by unknown co-eluents; usually, this problem is resolved by further diluting the sample and re-analyzing it. In those situations, where a co-eluent cannot be resolved from the target analyte, the data is not reported.

13. Reference Ranges (Normal Values)

Reference ranges for smokers and non-smokers are presented in **Table 8**.

Table 8. VOC metabolites in urine collected from non-smokers and smokers.

Analyte	Analytical Limit of Detection (LOD)	Range		Ref.
		Non-smokers	Smoker	
AAMA	2.5	12.7-171 µg/L 9.8-171 µg/g creatinine	30.3-447 µg/L 35.1-401 µg/g creatinine	[33]
AMCC	5.0	38.9-498 µg/L 47.3- 449 µg/g creatinine	122-1453 µg/L 196-1153 µg/g creatinine	[33]
ATCA	25	85 ± 47	233 ± 237	[34]
BMA	0.02	2.4-81.4 µg/g creatinine	1.7-31.2 µg/g creatinine	[35]
CEMA	0.15	ND-94 µg/L ND-158 µg/g creatinine	29-1240 µg/L ND-744 µg/g creatinine	[36]
CYMA	0.50	<1.0-21.3 µg/L	2.0-1382 µg/L	[37]
DHBMA	0.14	ND-329 µg/L ND-582 µg/g creatinine	113-1830 µg/L 166-1092 µg/g creatinine	[36]
HEMA	0.03	ND-1.44 µg/L ND-1.05 µg/g creatinine	ND-20.8 µg/L ND-16 µg/g creatinine	[36]
HPMA	0.20	ND-128 µg/L ND-245 µg/g creatinine	80.9-4030 µg/L 75-3678 µg/g creatinine	[36]
2HPMA	5	<5-49.3 µg/L <5-73.6 µg/g creatinine	<5- 252 µg/L <5-206 µg/g creatinine	(32)
HPMMA	28	192-1740 µg/24hr	815-5457 µg/24hr	[16]
MHBMA	1.0	<2.0-2.5 µg/L	<2.0-17.5 µg/L	[37]
PMA	0.01	ND-0.26 µg/L ND-0.45 µg/g creatinine	ND-37.7 µg/L ND-18.4 µg/g creatinine	[36]

14. Critical Call Results ("Panic Values")

Mercapturic acids are specific biomarkers of VOC exposure. High levels of urinary VOC metabolites could indicate excessive exposure to VOCs. However, the stoichiometric relationship of VOCs and many of the urinary VOC metabolites has not been established. Therefore, there are no critical call values for VOC metabolites at this time. The biological exposure indices (BEI) reported by ACGIH [38] for some of the VOC metabolites in this method are given in (**Table 9**) as the maximum values allowable in urine samples collected from workers.

Table 9. Biological exposure indices.

VOC Metabolite	BEI	Parent Compound
AMCC	40 mg/L	*N*, *N*-dimethylformamide
DHBMA	2.5 mg/L	1,3-butadiene
2MHA+3MHA+4MHA	1.5 g/g creatinine	*o*-, *m*-, *p*- xylenes
MADA + PHGA	400 mg/g creatinine	styrene
trans, trans-Muconic acid	500 µg/g creatinine	benzene
PMA	25 µg/g creatinine	benzene
TTCA	5 mg/g creatinine	carbon disulfide

15. Specimen Storage and Handling during Testing

Specimens must be stored at ≤ -20°C until analysis; however, they may be kept at ambient temperature during analysis.

16. Alternate Methods for Performing Test or Storing Specimens if Test System Fails

Alternate methods have not been evaluated for measuring VOC metabolites in urine.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)

Results are reported to three significant figures based on assay sensitivity calculations. Study subject data is reported in both concentration units (ng/mL) and as adjusted values based on creatinine excretion (µg/g creatinine).

Once the validity of the data is established by the QC/QA system outlined above, results are verified by a DLS statistician, and the data is reported in both hard and electronic forms. This data, a cover letter, and a table of method specifications and reference range values will then be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). After approval at the division level, 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 0.25 mL of sample remains following successful completion of analysis, this material should be returned to storage at ≤ -20°C in case further analysis is required. These samples should be retained until valid results have been obtained, reported, and sufficient

time has passed for review of the results.

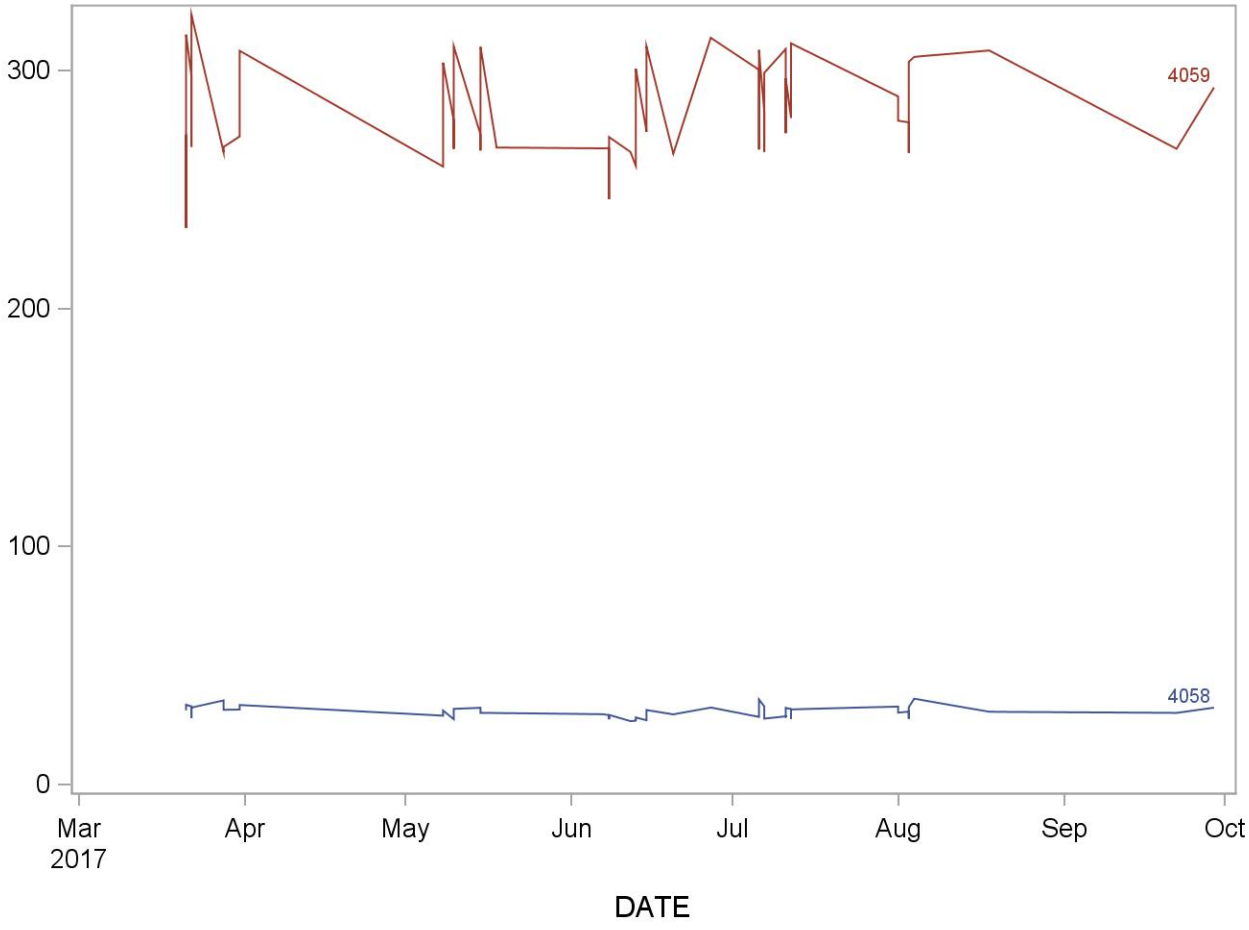
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Records are maintained for 3 years, including related QA/QC data. Additionally, duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal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

19. Summary Statistics and QC Graphs

See next pages.

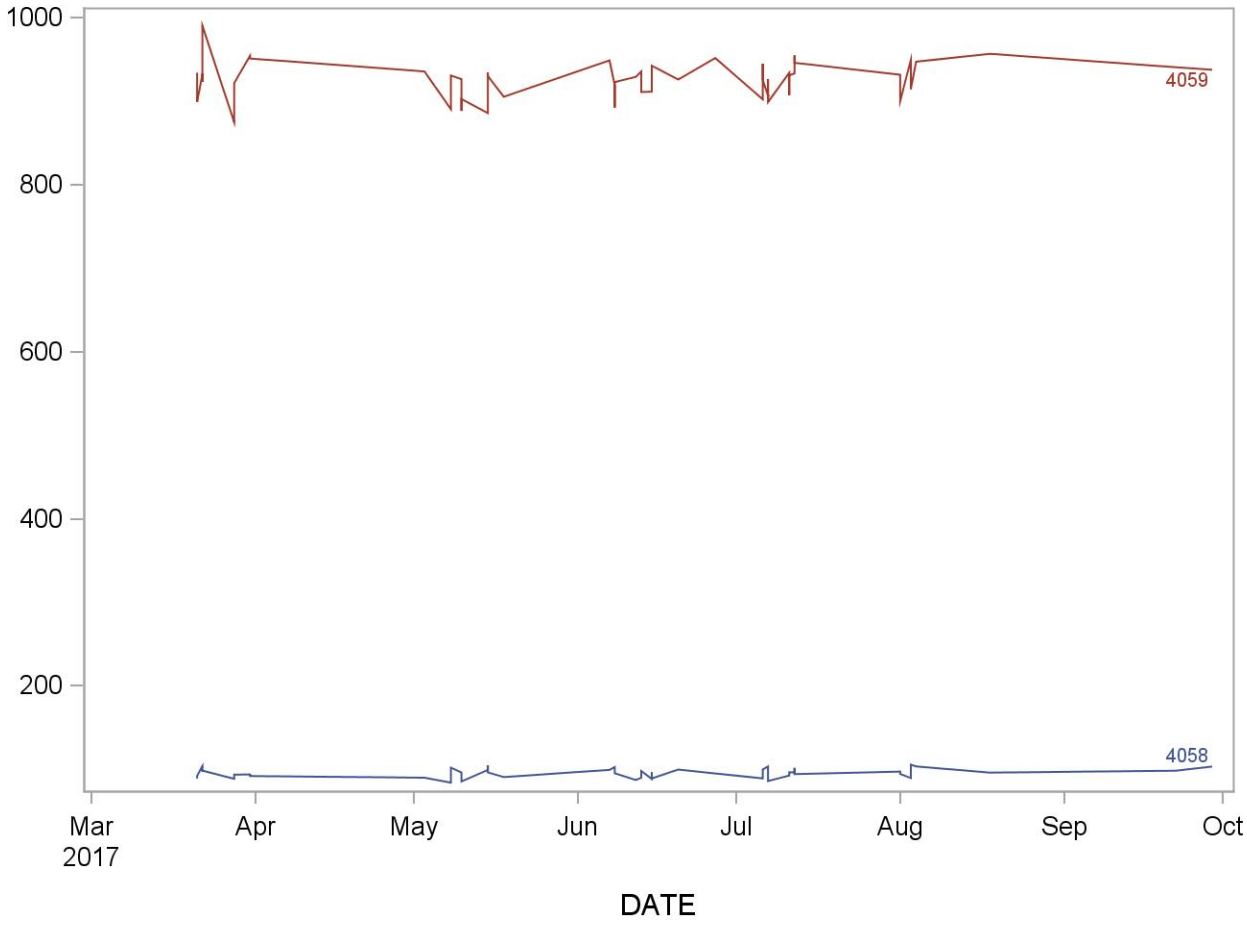
2015-2016 Summary Statistics and QC Chart for 2-Methylhippuric acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	283.6324	20.4771	7.2
4058	52	21MAR17	29SEP17	30.5316	2.3079	7.6



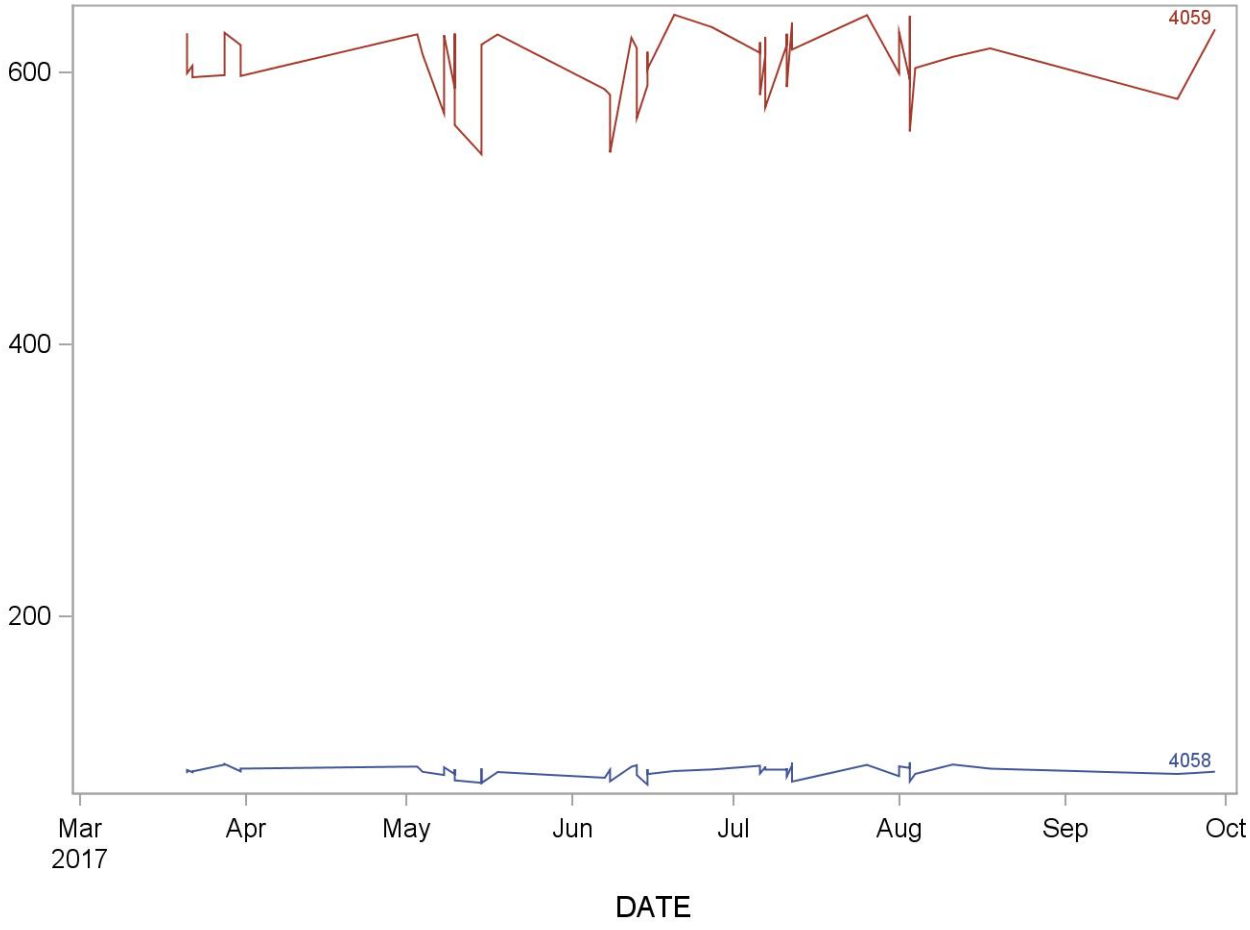
2015-2016 Summary Statistics and QC Chart for 2-amnothiazolne-4-carbxylic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	925.8491	21.9489	2.4
4058	52	21MAR17	29SEP17	95.3850	5.4707	5.7



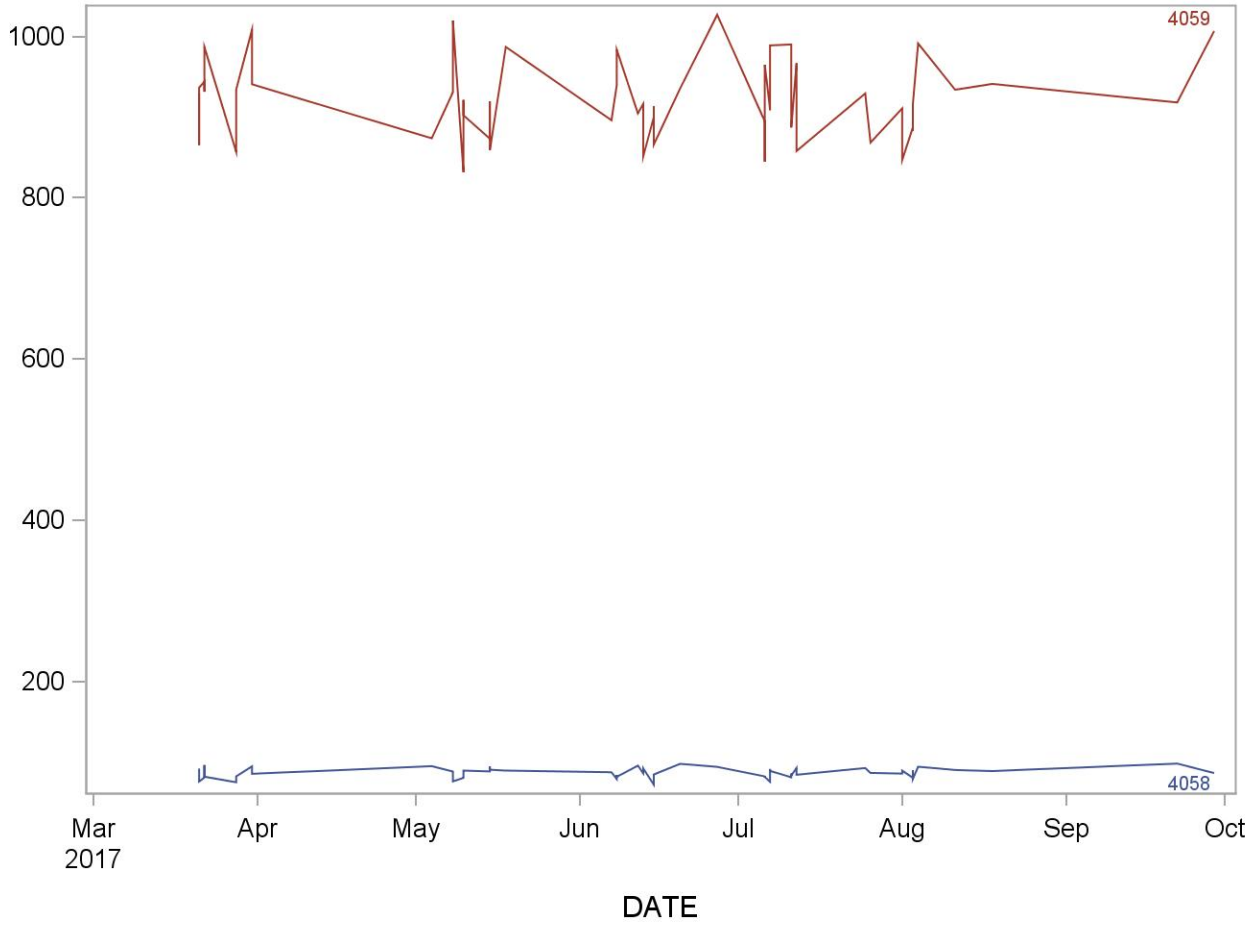
2015-2016 Summary Statistics and QC Chart for 3-methipurc acd & 4-methipurc acd (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	55	21MAR17	29SEP17	605.3888	25.8537	4.3
4058	55	21MAR17	29SEP17	86.0040	4.0589	4.7



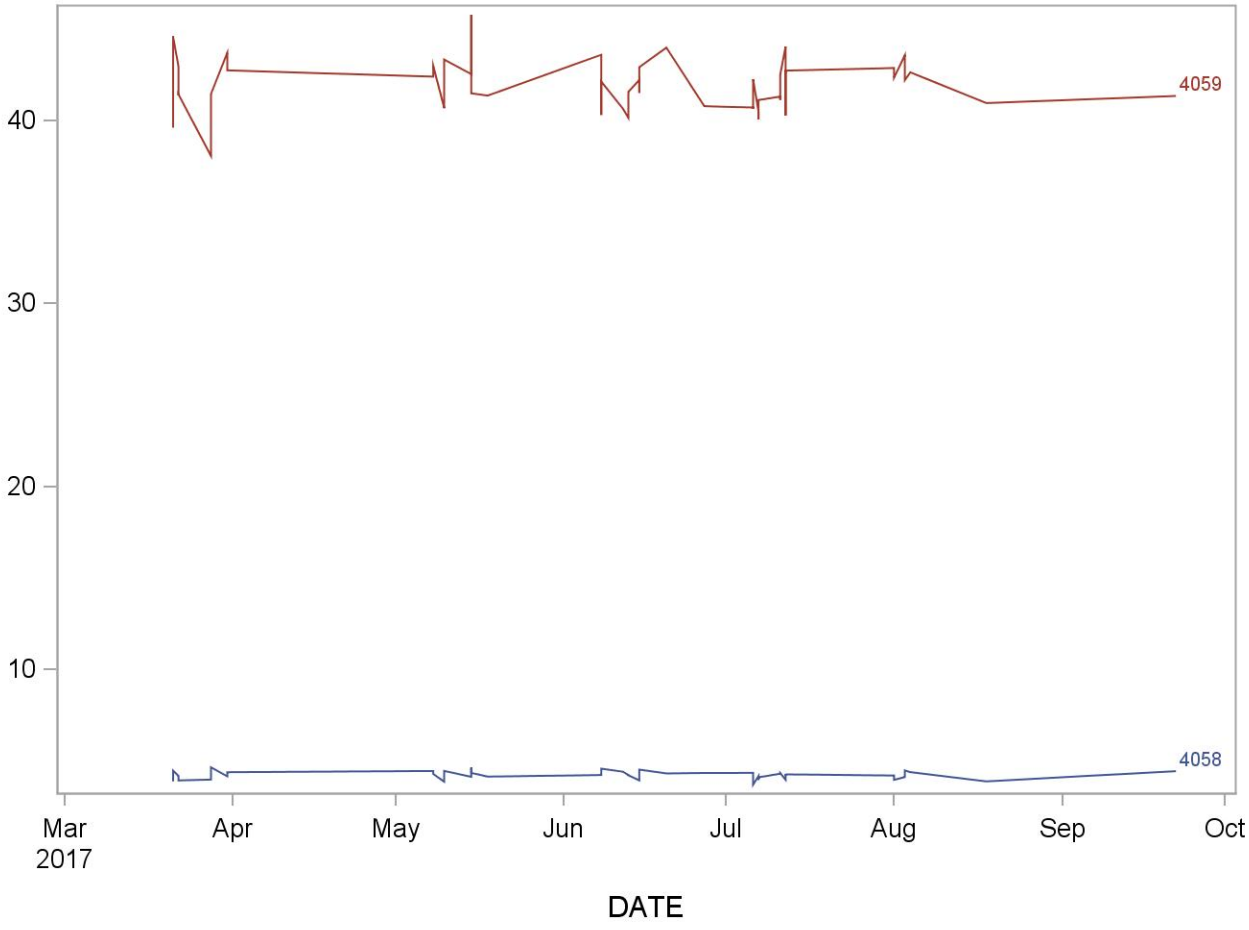
2015-2016 Summary Statistics and QC Chart for Mandelic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	55	21MAR17	29SEP17	921.5885	48.5951	5.3
4058	55	21MAR17	29SEP17	86.5985	6.3813	7.4



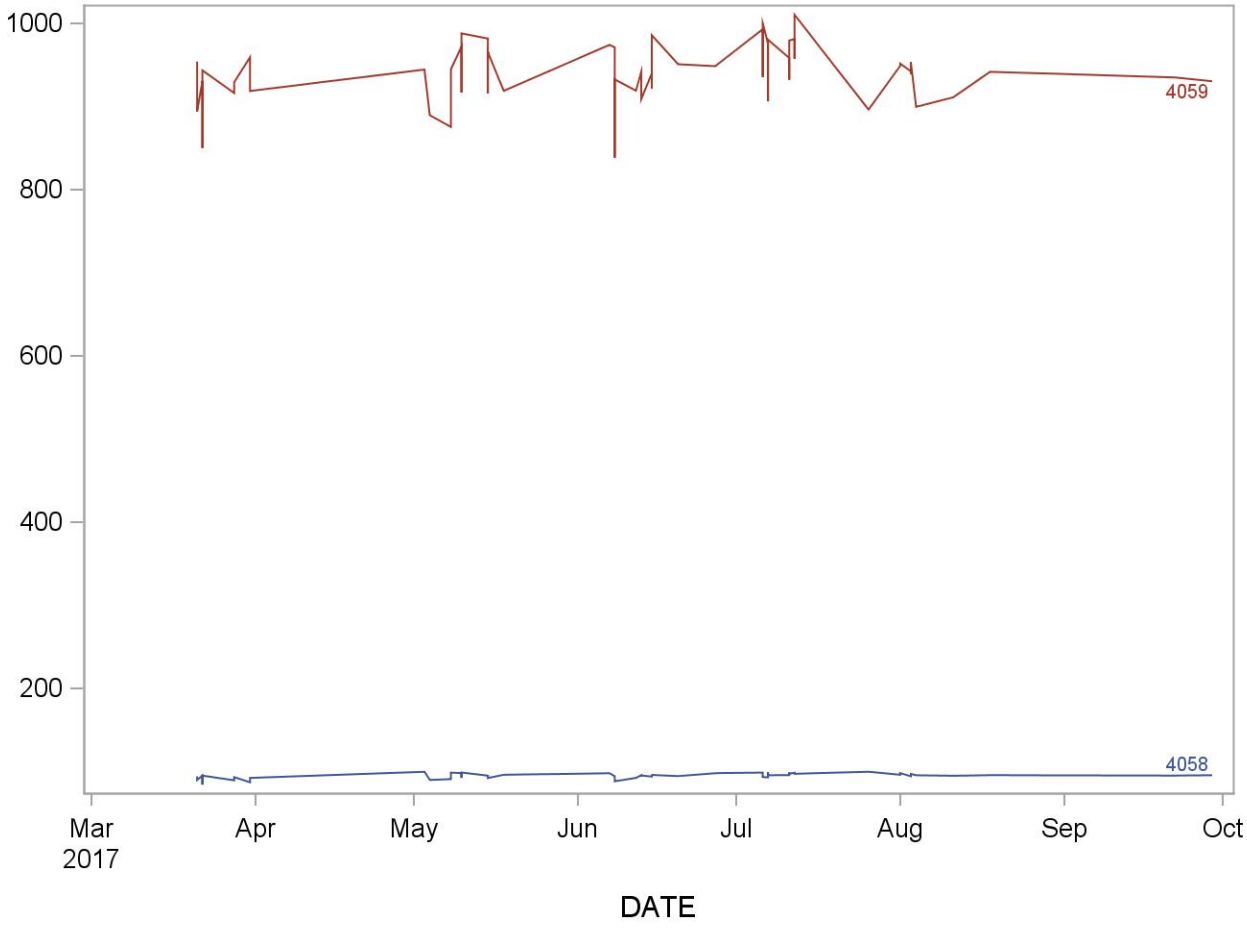
2015-2016 Summary Statistics and QC Chart for N-A-S-(1-HydrxMet)-2-Prpn)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	50	21MAR17	22SEP17	41.8872	1.4185	3.4
4058	50	21MAR17	22SEP17	4.1963	0.2138	5.1



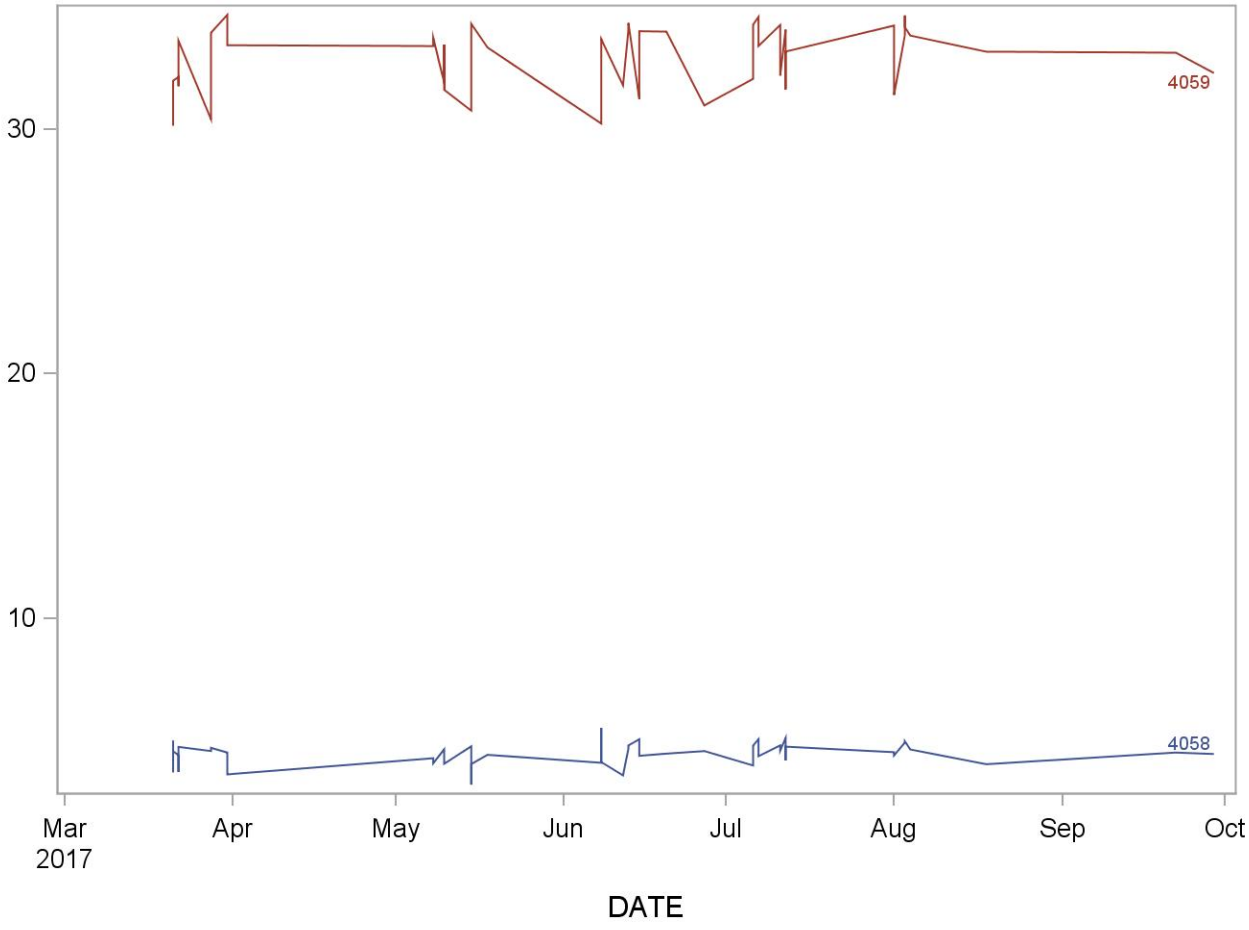
2015-2016 Summary Statistics and QC Chart for N-A-S-(3-hydrxprpl-1-metl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	55	21MAR17	29SEP17	940.5580	35.1452	3.7
4058	55	21MAR17	29SEP17	95.4547	3.3010	3.5



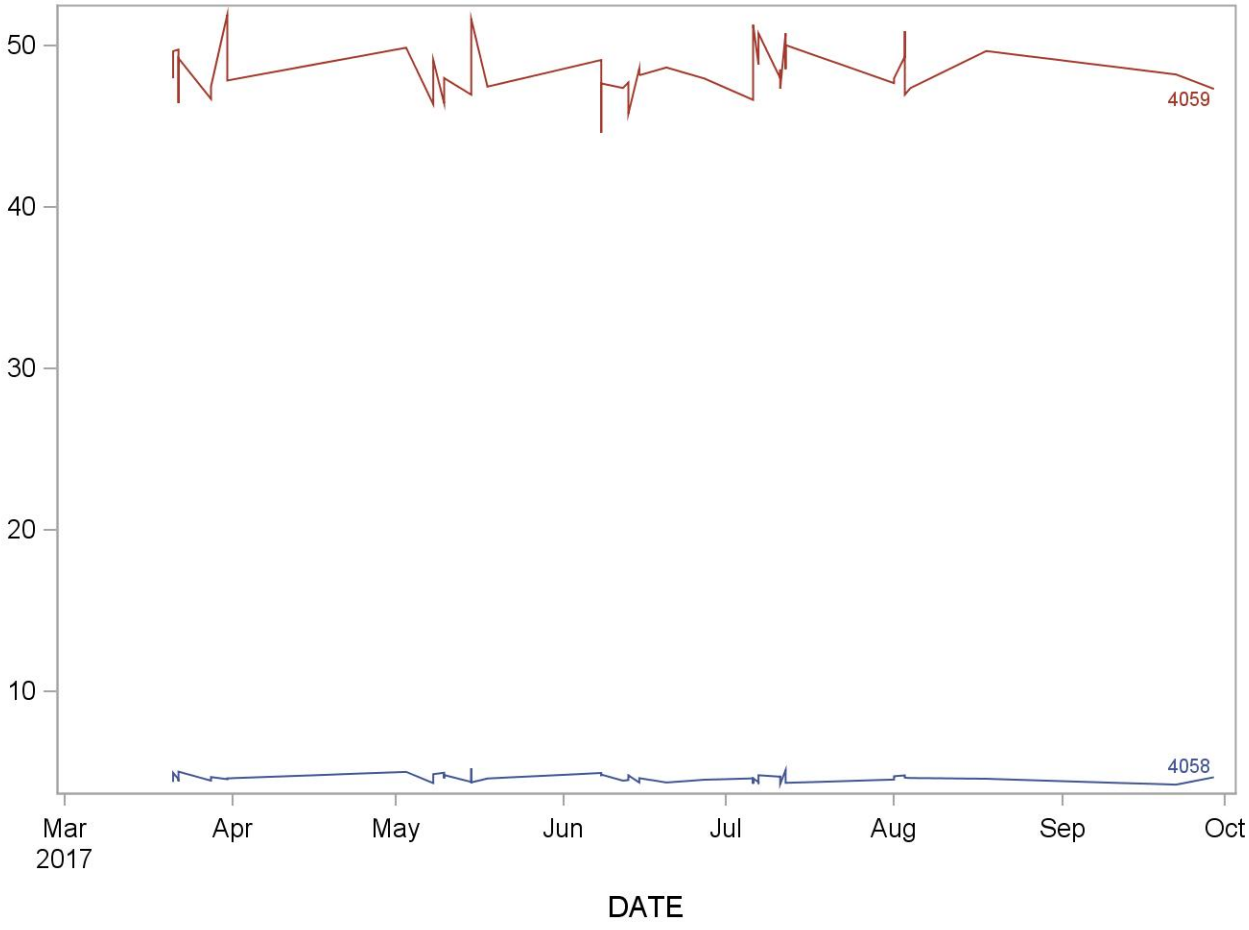
2015-2016 Summary Statistics and QC Chart for N-A-S-(4-hydrxy-2butnyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	51	21MAR17	29SEP17	32.9833	1.2776	3.9
4058	51	21MAR17	29SEP17	4.4594	0.4440	10.0



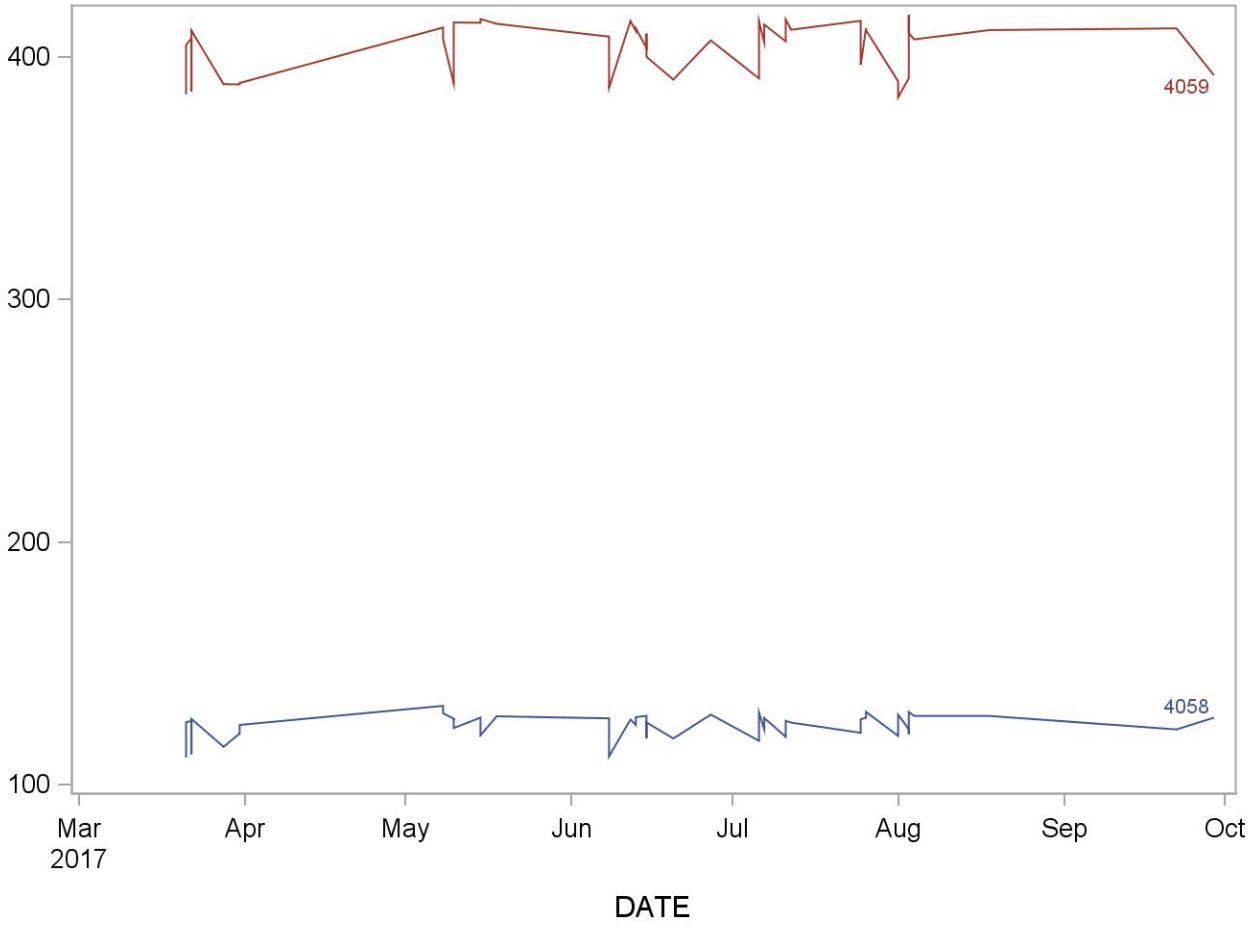
2015-2016 Summary Statistics and QC Chart for N-Ac-S-(2-Hydrxy-3-butnyl)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	51	21MAR17	29SEP17	48.3573	1.5312	3.2
4058	51	21MAR17	29SEP17	4.6114	0.2413	5.2



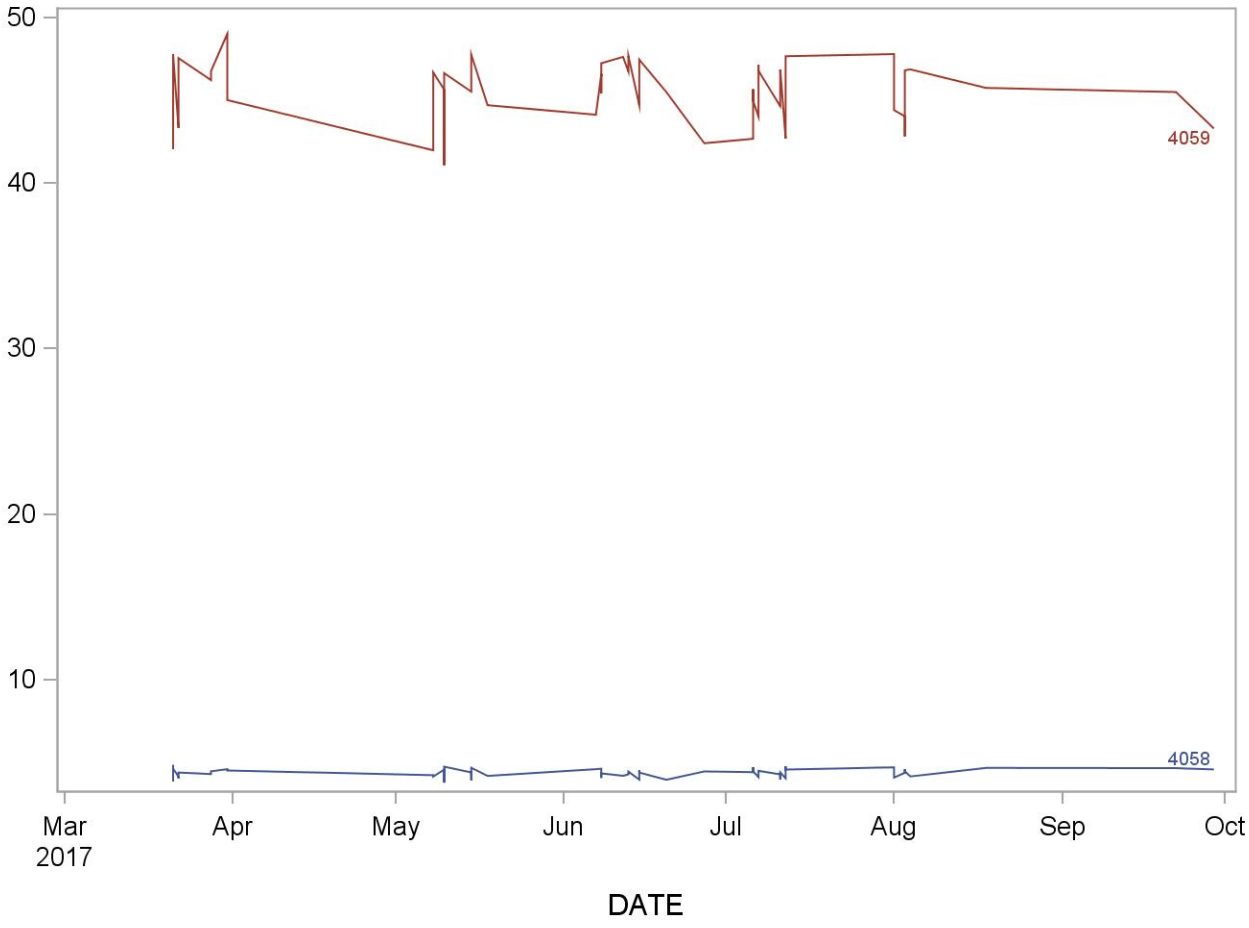
2015-2016 Summary Statistics and QC Chart for N-Ace-S- (3,4-Dihidxybutl)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	47	21MAR17	29SEP17	403.8993	10.4839	2.6
4058	47	21MAR17	29SEP17	124.4656	4.9808	4.0



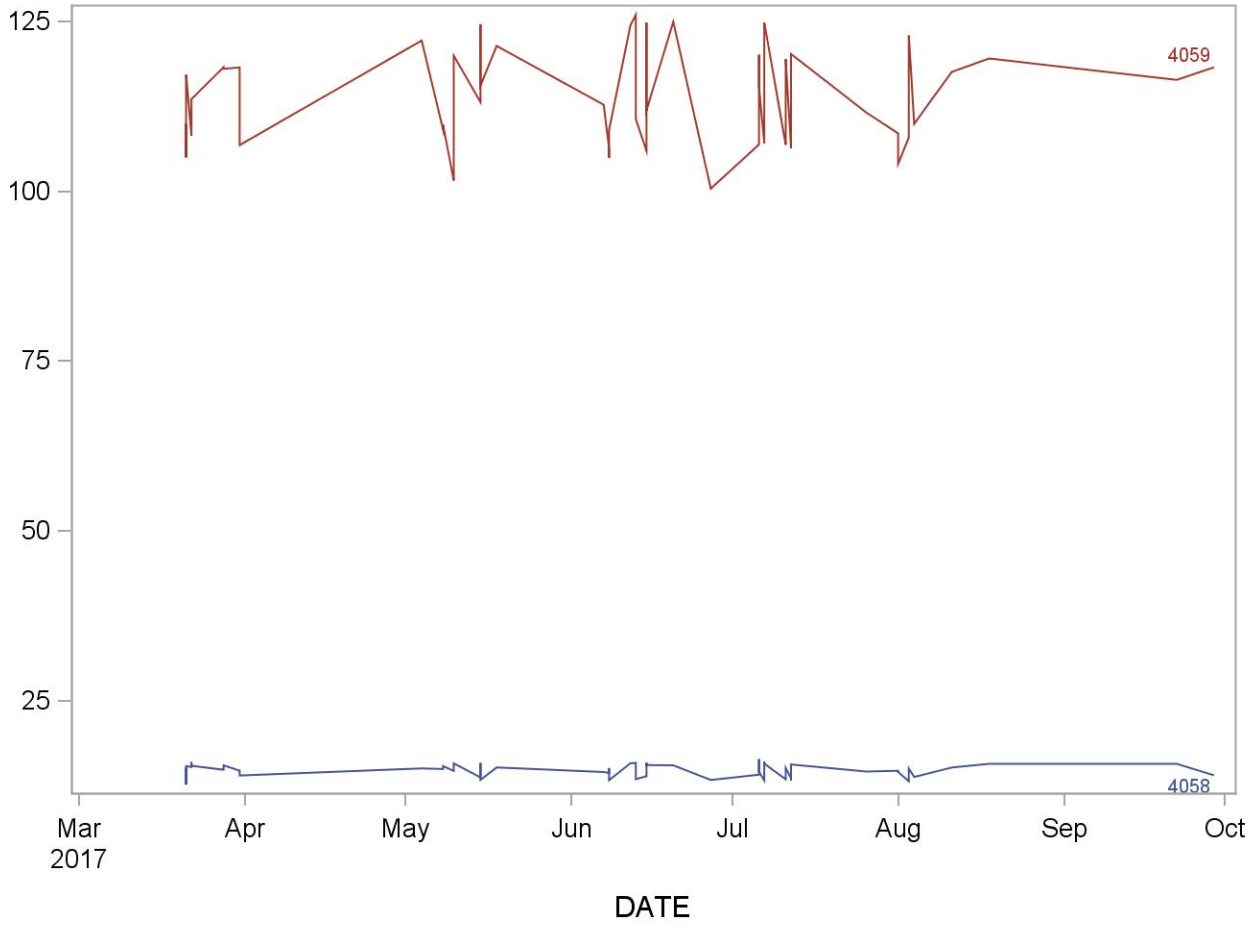
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(2-Hydroxyethyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	45.4824	1.8695	4.1
4058	52	21MAR17	29SEP17	4.3501	0.2653	6.1



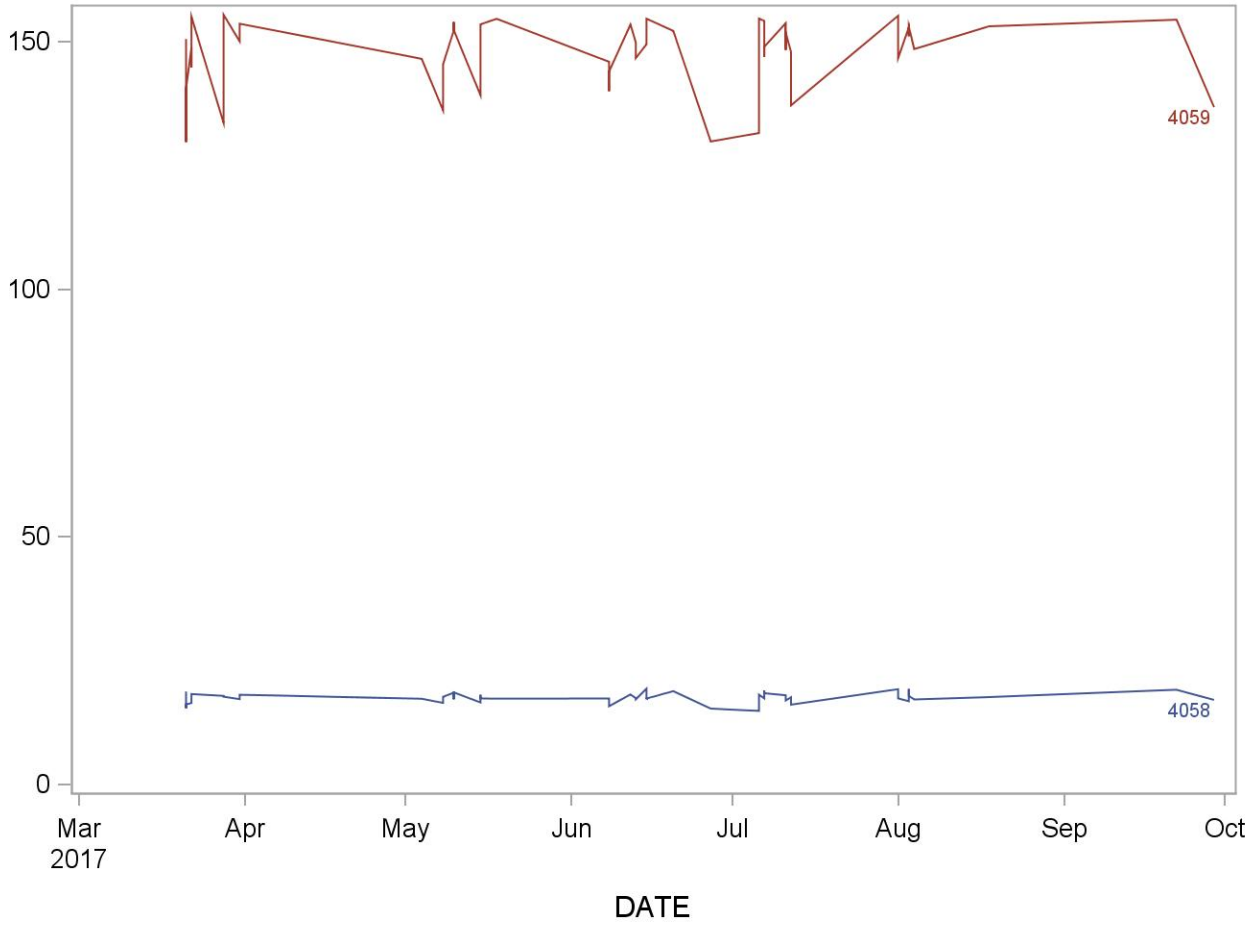
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(2-carbamoylethyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	55	21MAR17	29SEP17	113.8830	6.7709	5.9
4058	55	21MAR17	29SEP17	14.7597	0.9303	6.3



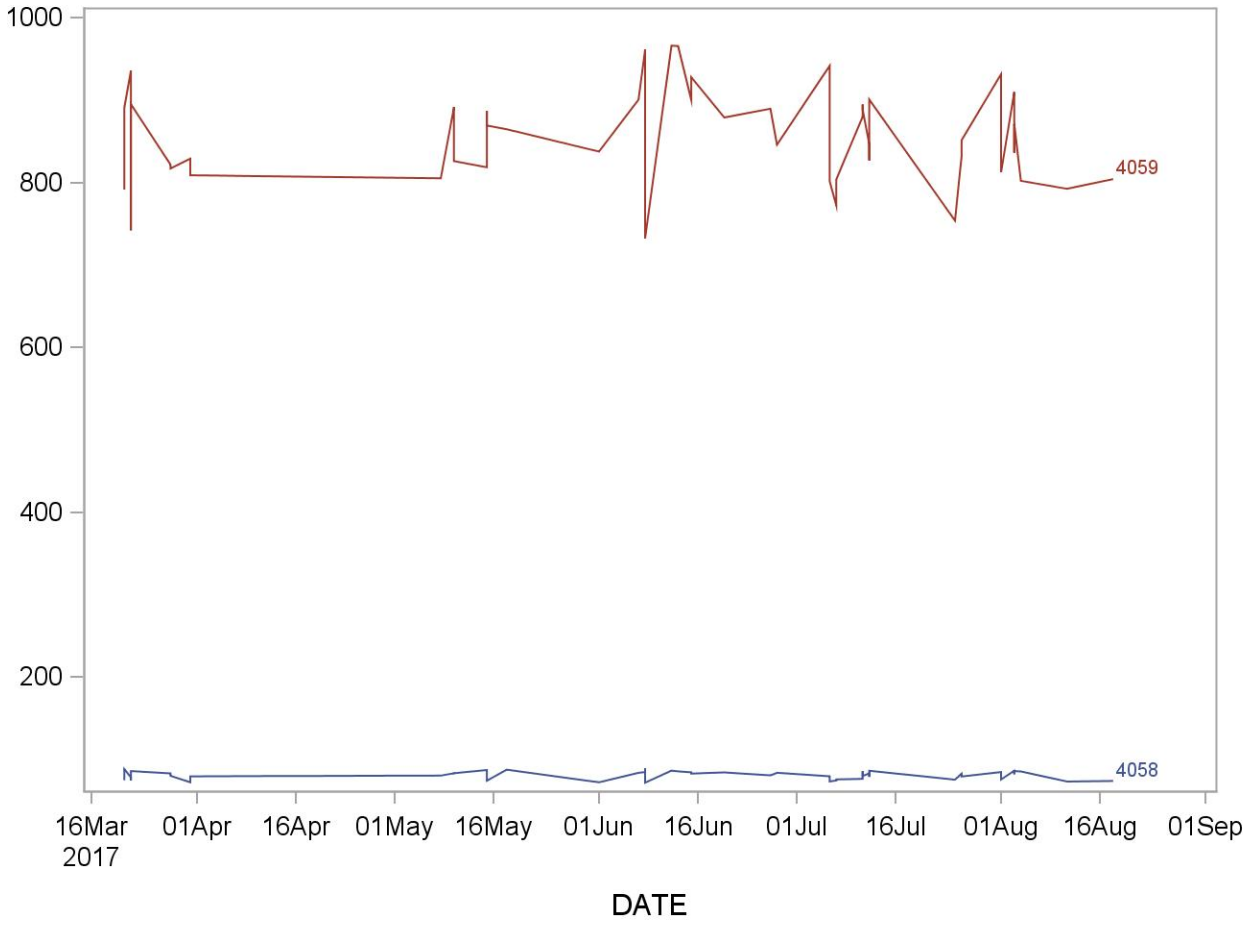
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(2-hydroxypropyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	147.7941	7.0321	4.8
4058	52	21MAR17	29SEP17	17.5329	1.0124	5.8



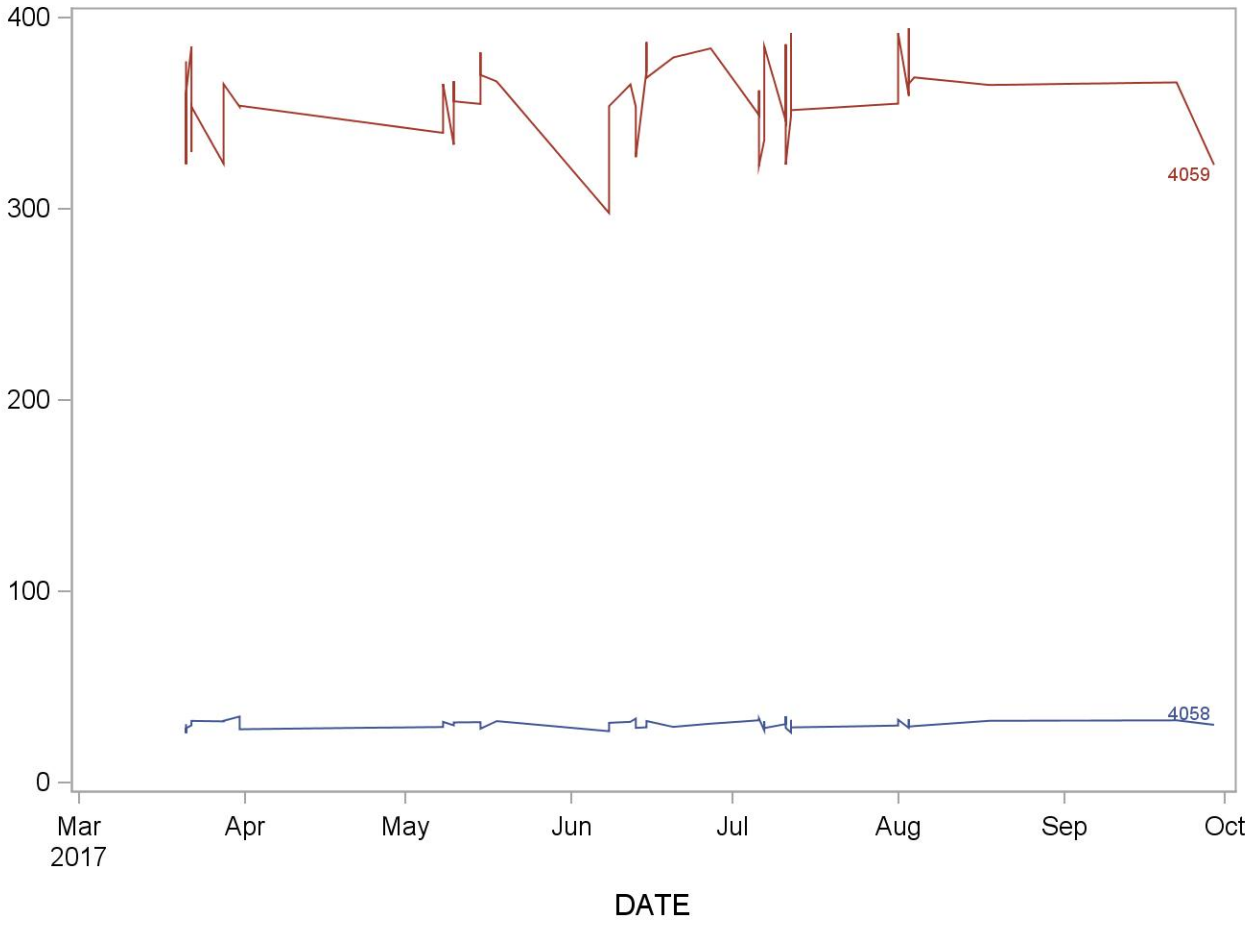
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(3-Hydroxypropyl)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	50	21MAR17	18AUG17	855.4327	57.0618	6.7
4058	50	21MAR17	18AUG17	81.0230	4.9594	6.1



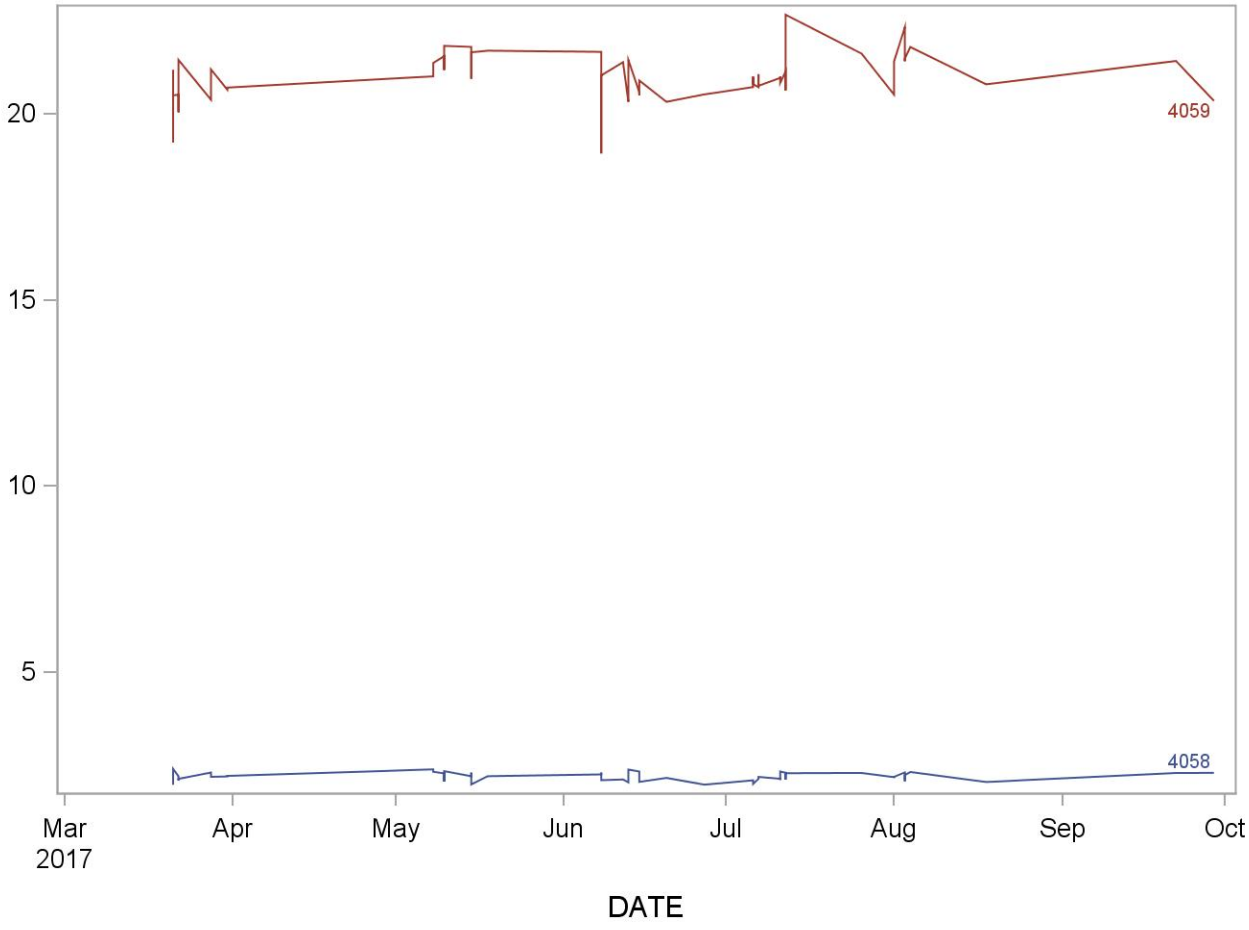
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(N-methlcarbamoyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	50	21MAR17	29SEP17	358.2349	21.8639	6.1
4058	50	21MAR17	29SEP17	30.6426	2.1190	6.9



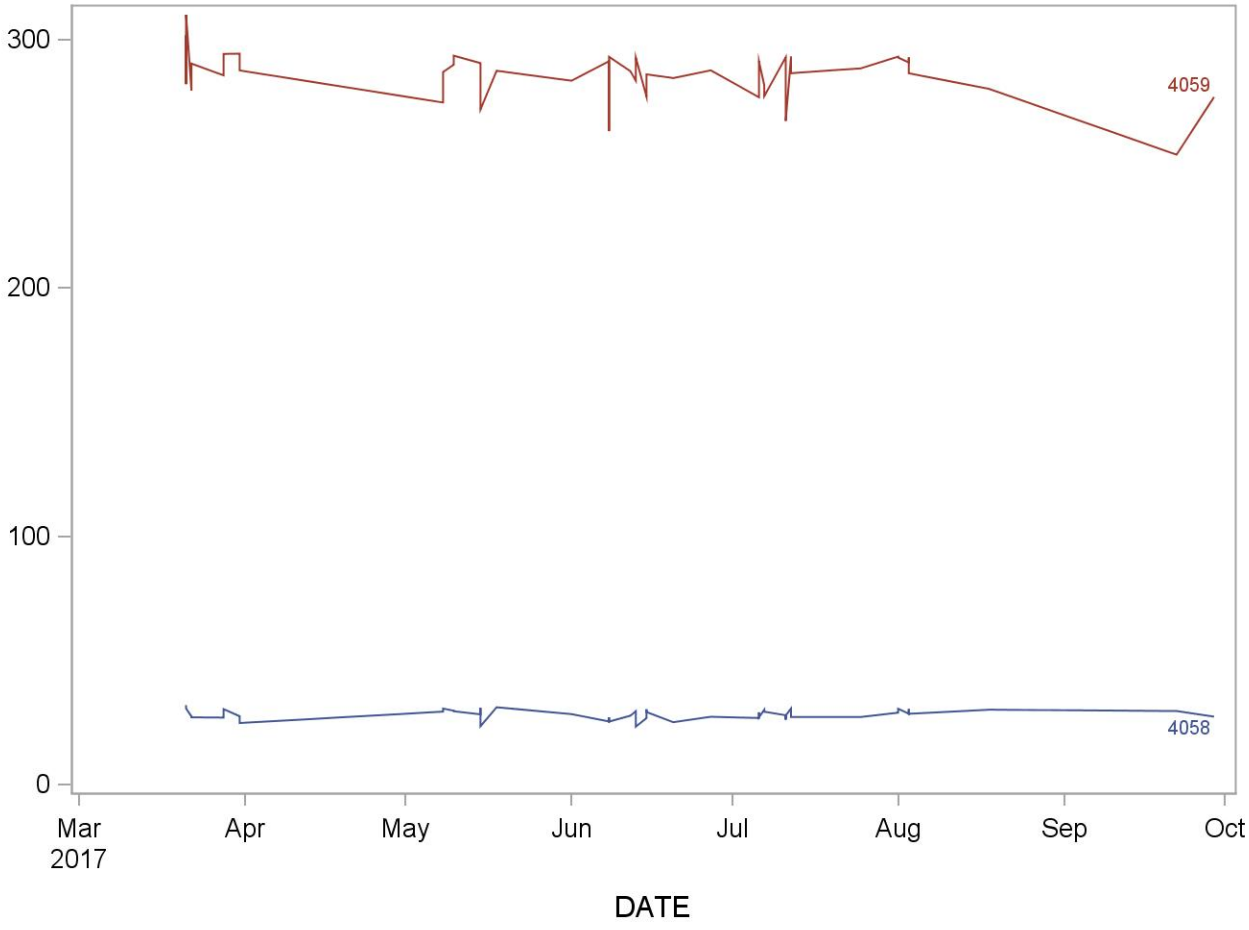
2015-2016 Summary Statistics and QC Chart for N-Ace-S-(dimethylphenyl)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	21.0088	0.6604	3.1
4058	52	21MAR17	29SEP17	2.1807	0.1174	5.4



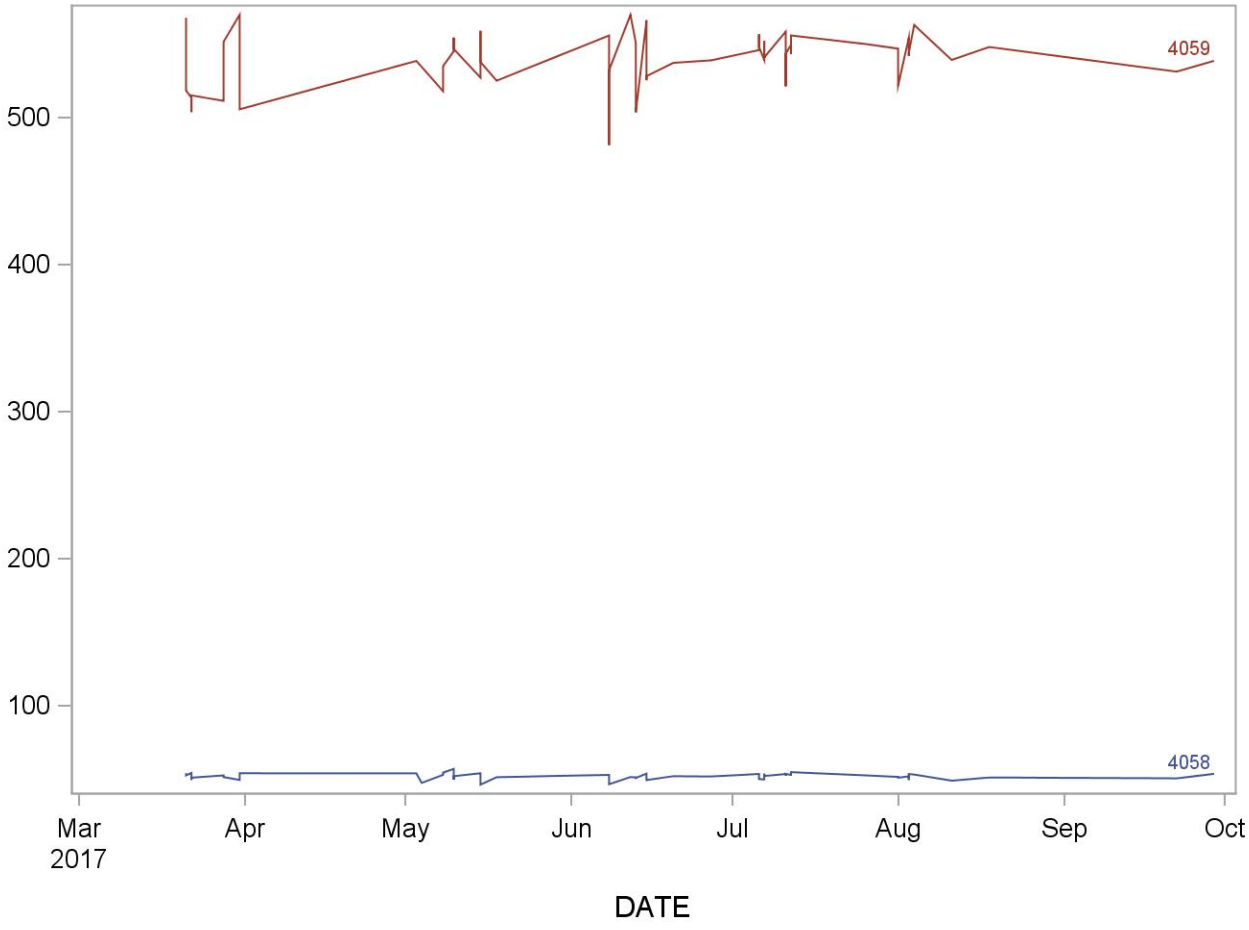
2015-2016 Summary Statistics and QC Chart for N-Acel-S-(2,2-Dichlorvinyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	48	21MAR17	29SEP17	285.5682	9.5894	3.4
4058	48	21MAR17	29SEP17	28.3537	2.0343	7.2



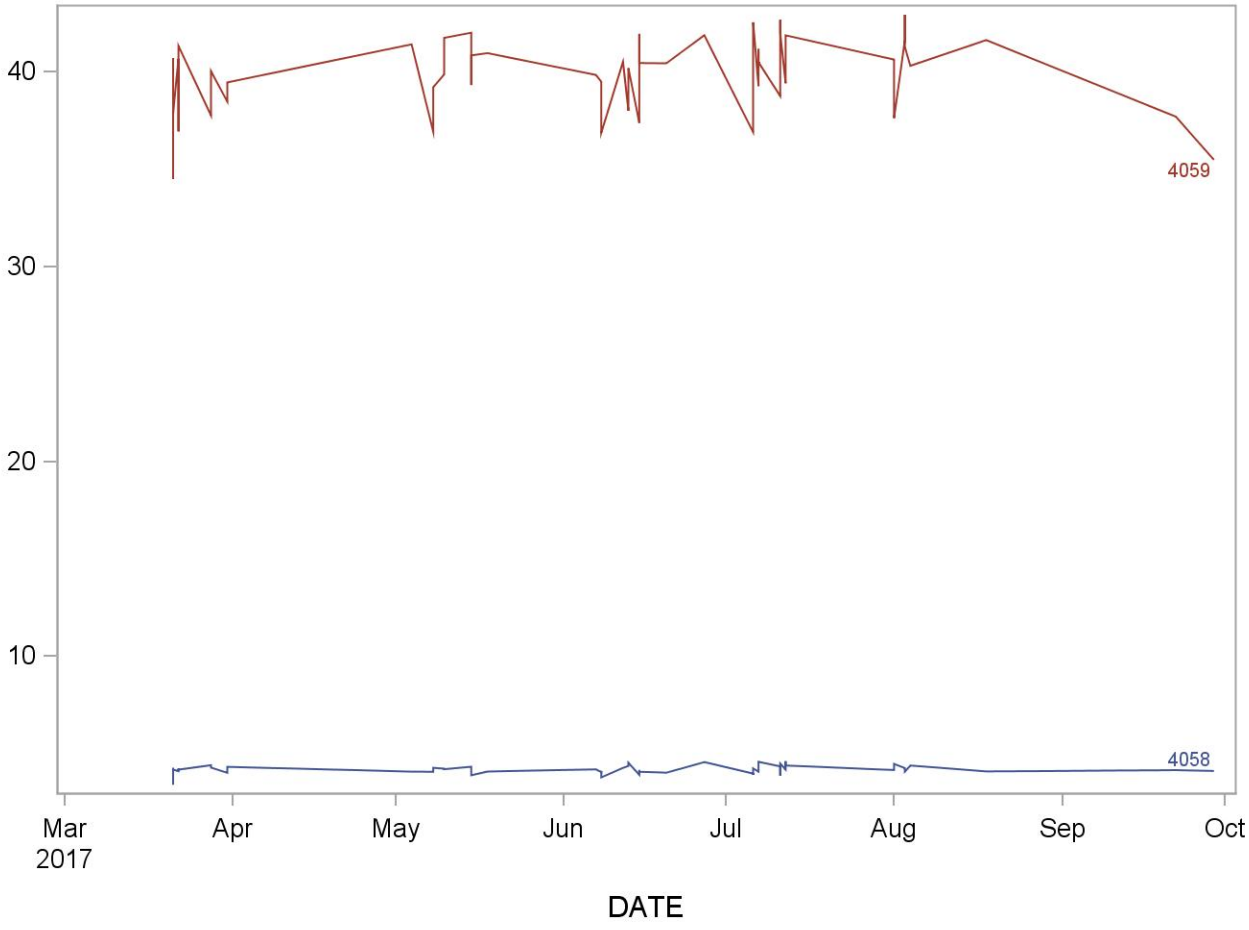
2015-2016 Summary Statistics and QC Chart for N-Acetyl-S-(2-Carbxyethyl)-L-Cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	54	21MAR17	29SEP17	538.7243	18.6875	3.5
4058	54	21MAR17	29SEP17	52.1666	2.0821	4.0



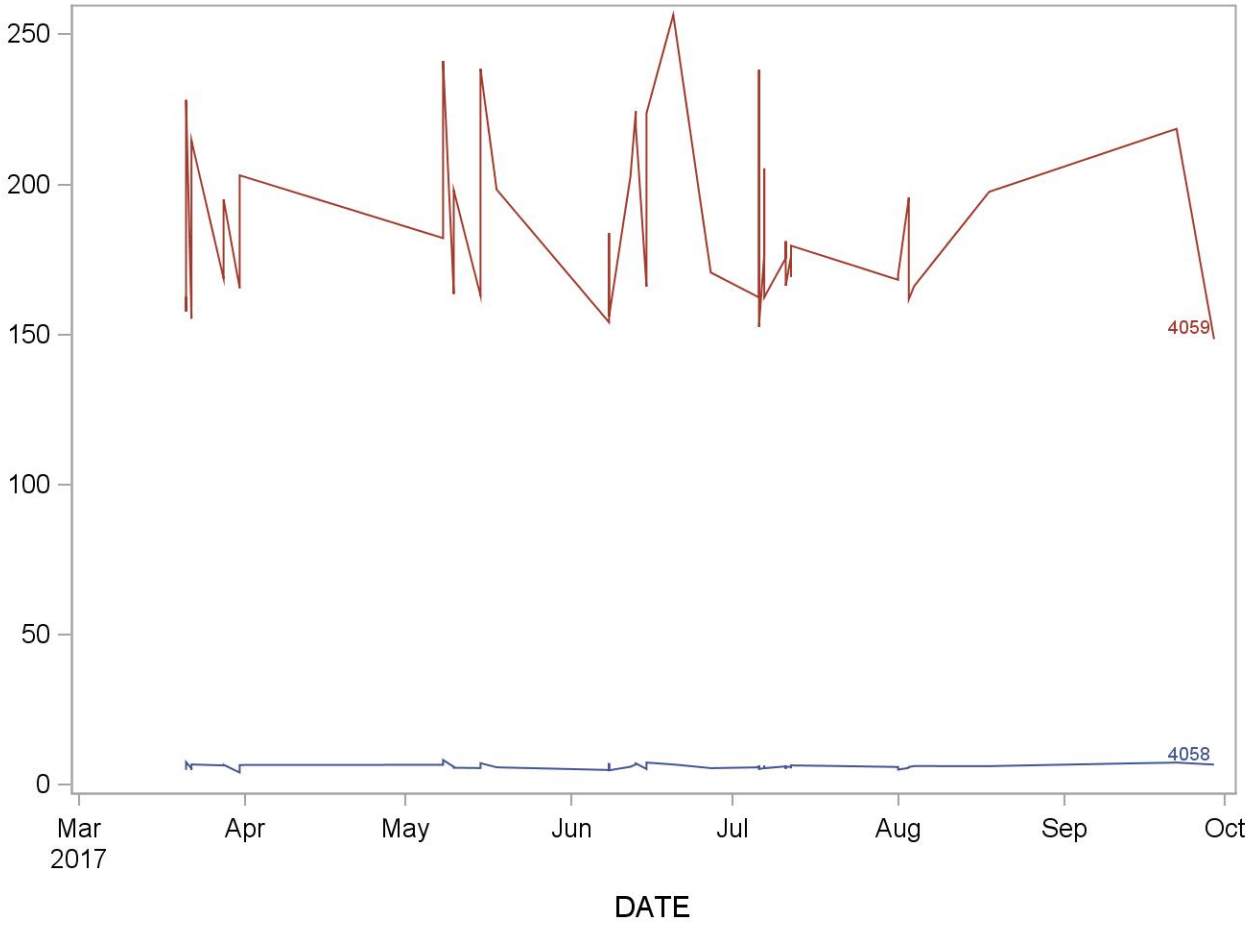
2015-2016 Summary Statistics and QC Chart for N-Acetyl-S-(benzyl)-L-cysteine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	53	21MAR17	29SEP17	39.8311	1.9314	4.8
4058	53	21MAR17	29SEP17	4.1765	0.2162	5.2



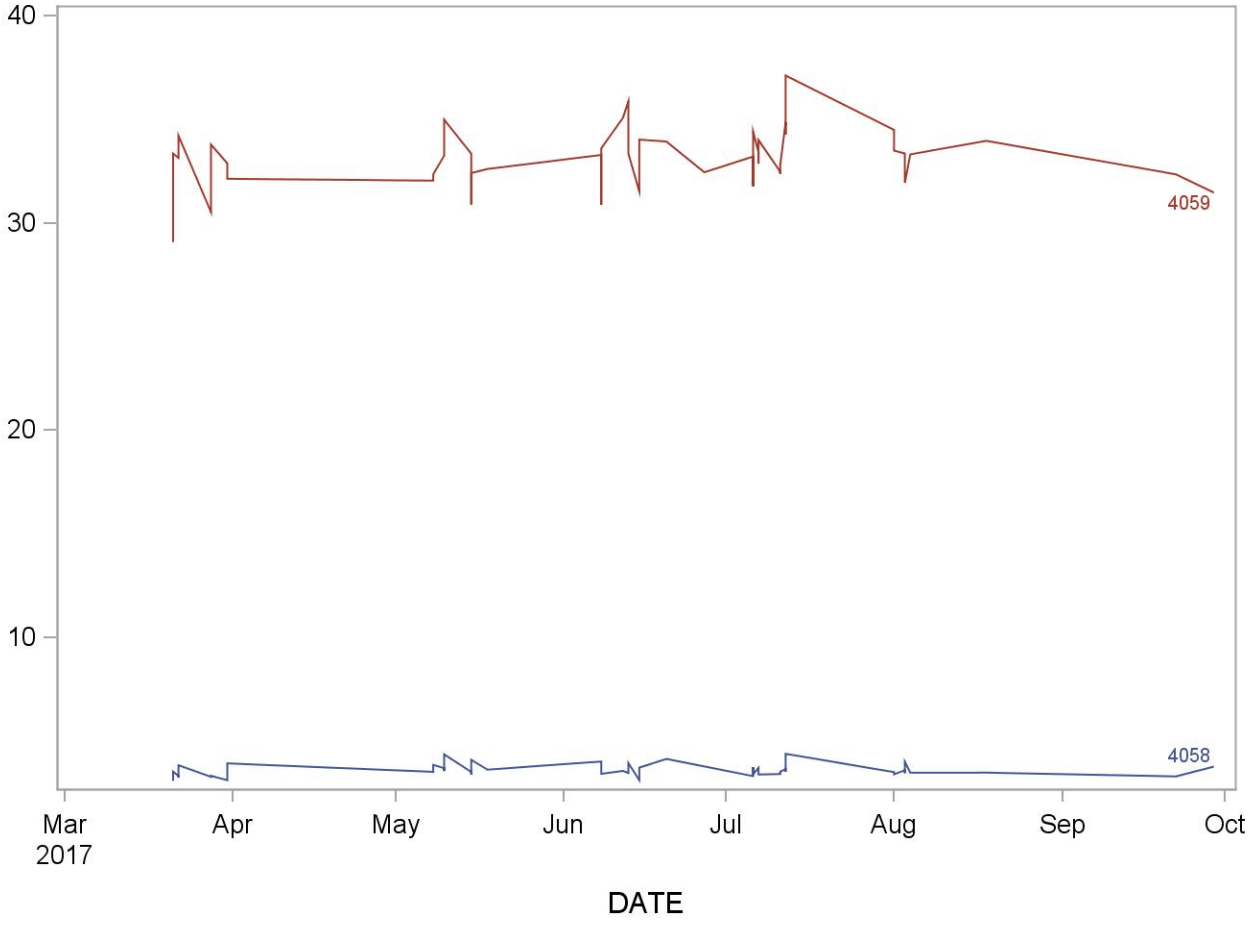
2015-2016 Summary Statistics and QC Chart for N-Acetyl-S-(n-propyl)-L-cysteine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	50	21MAR17	29SEP17	186.3295	27.6583	14.8
4058	50	21MAR17	29SEP17	6.0485	0.8389	13.9



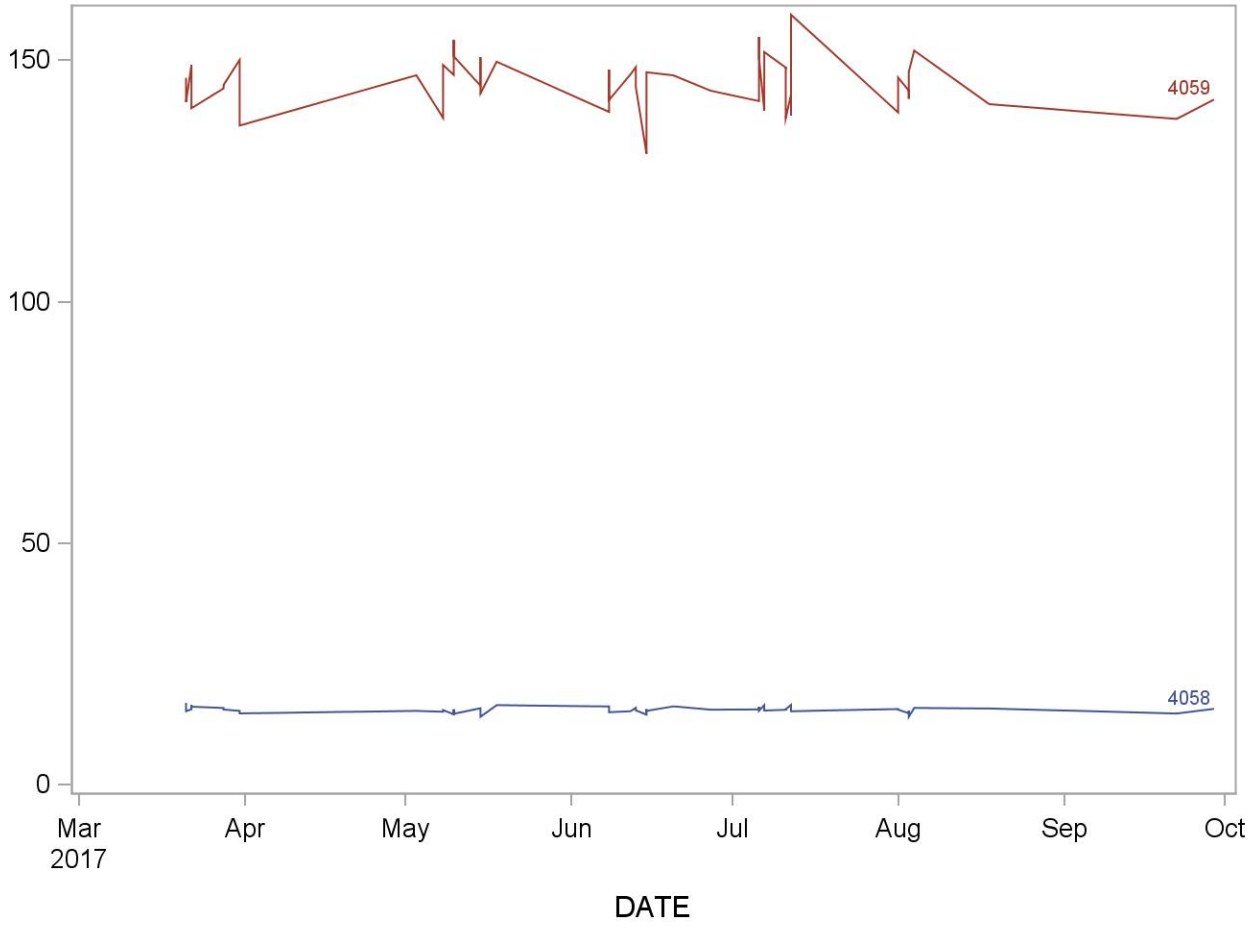
2015-2016 Summary Statistics and QC Chart for N-Acetyl-S-(phenyl)-L-cysteine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	51	21MAR17	29SEP17	33.0720	1.3643	4.1
4058	51	21MAR17	29SEP17	3.5914	0.2924	8.1



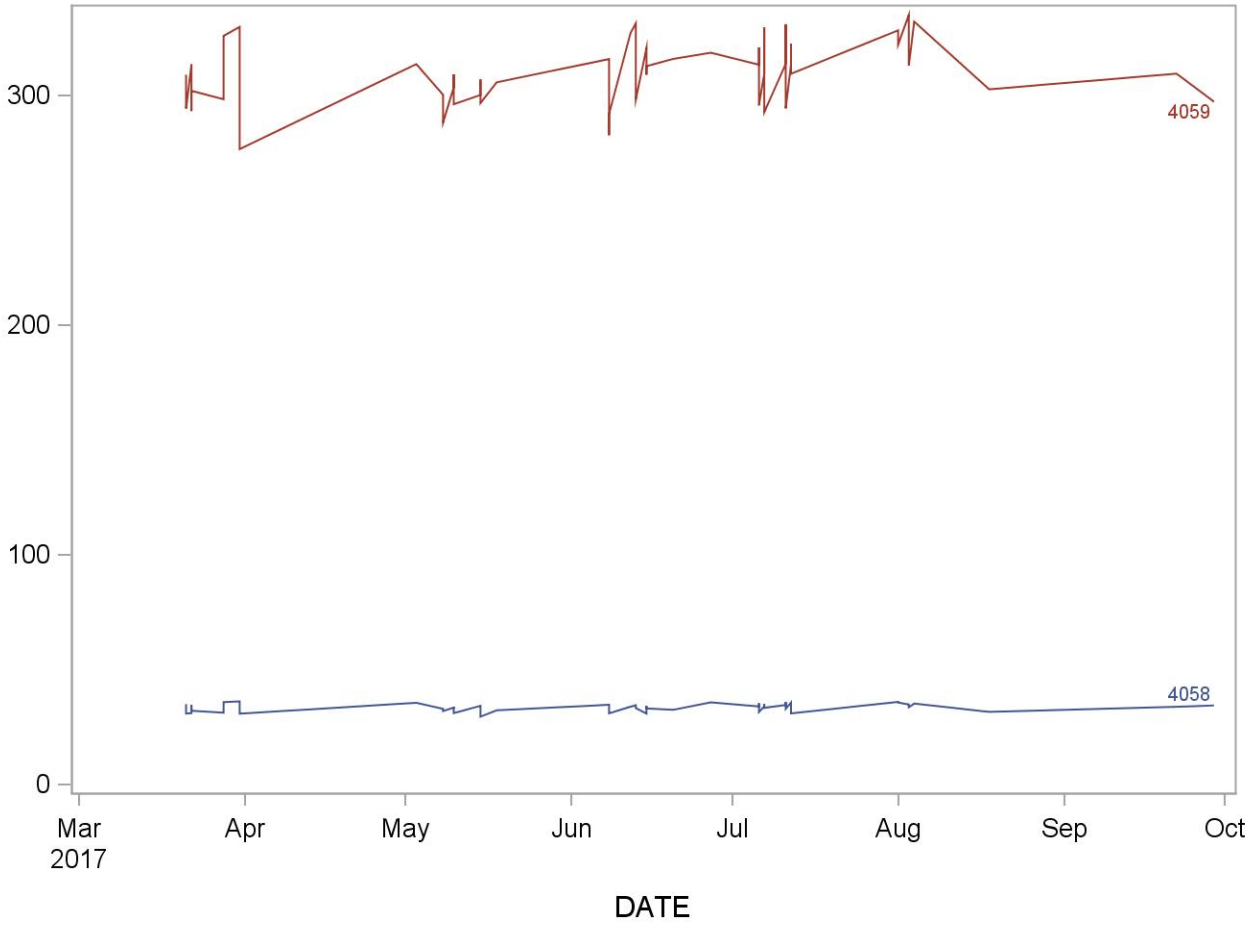
2015-2016 Summary Statistics and QC Chart for N-Acetyl-S-(trichlorovinyl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	51	21MAR17	29SEP17	145.3488	5.3336	3.7
4058	51	21MAR17	29SEP17	15.5261	0.6144	4.0



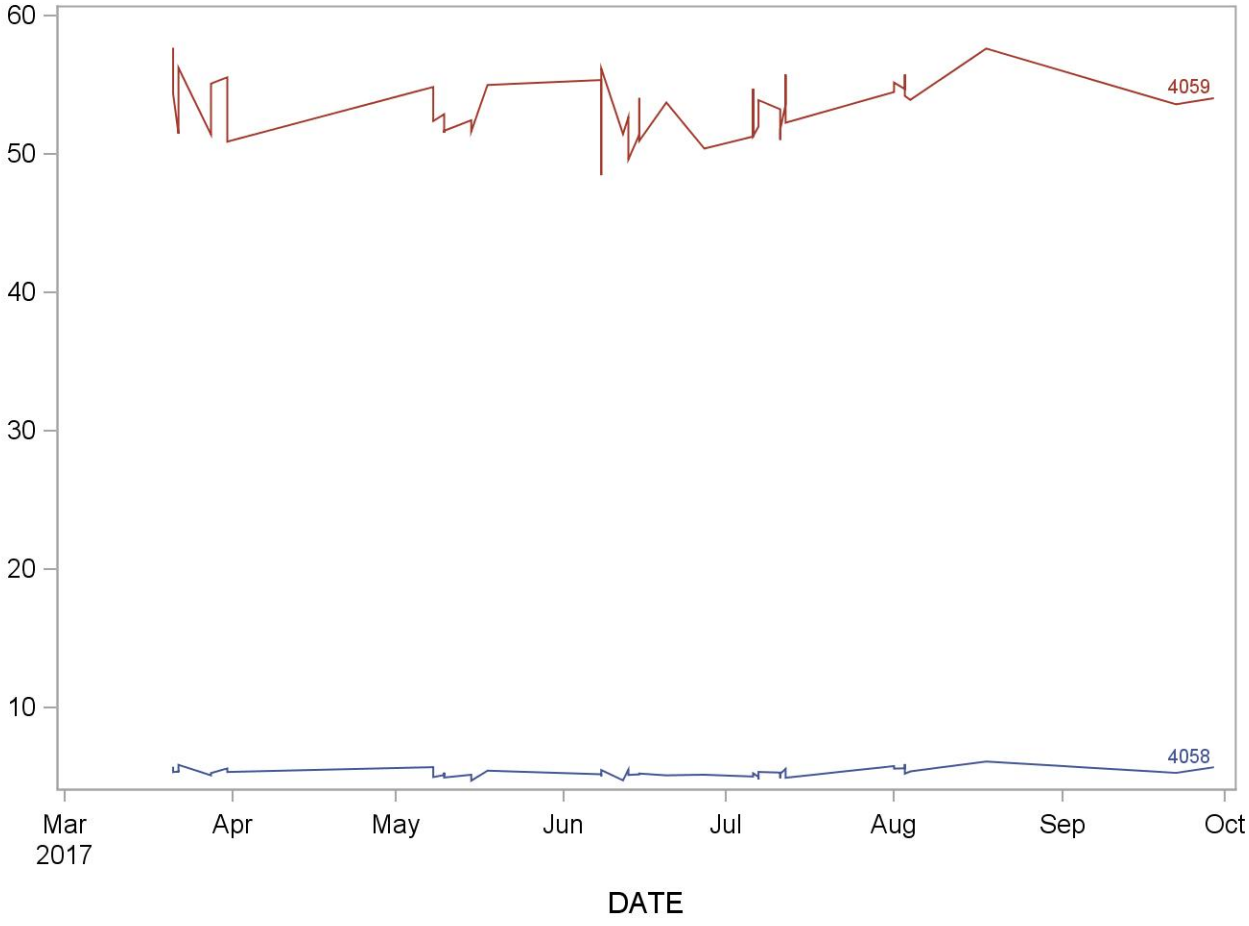
2015-2016 Summary Statistics and QC Chart for N-ac-S-(2-carbmo-2-hydxel)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	51	21MAR17	29SEP17	309.9590	14.0376	4.5
4058	51	21MAR17	29SEP17	33.6239	1.7344	5.2



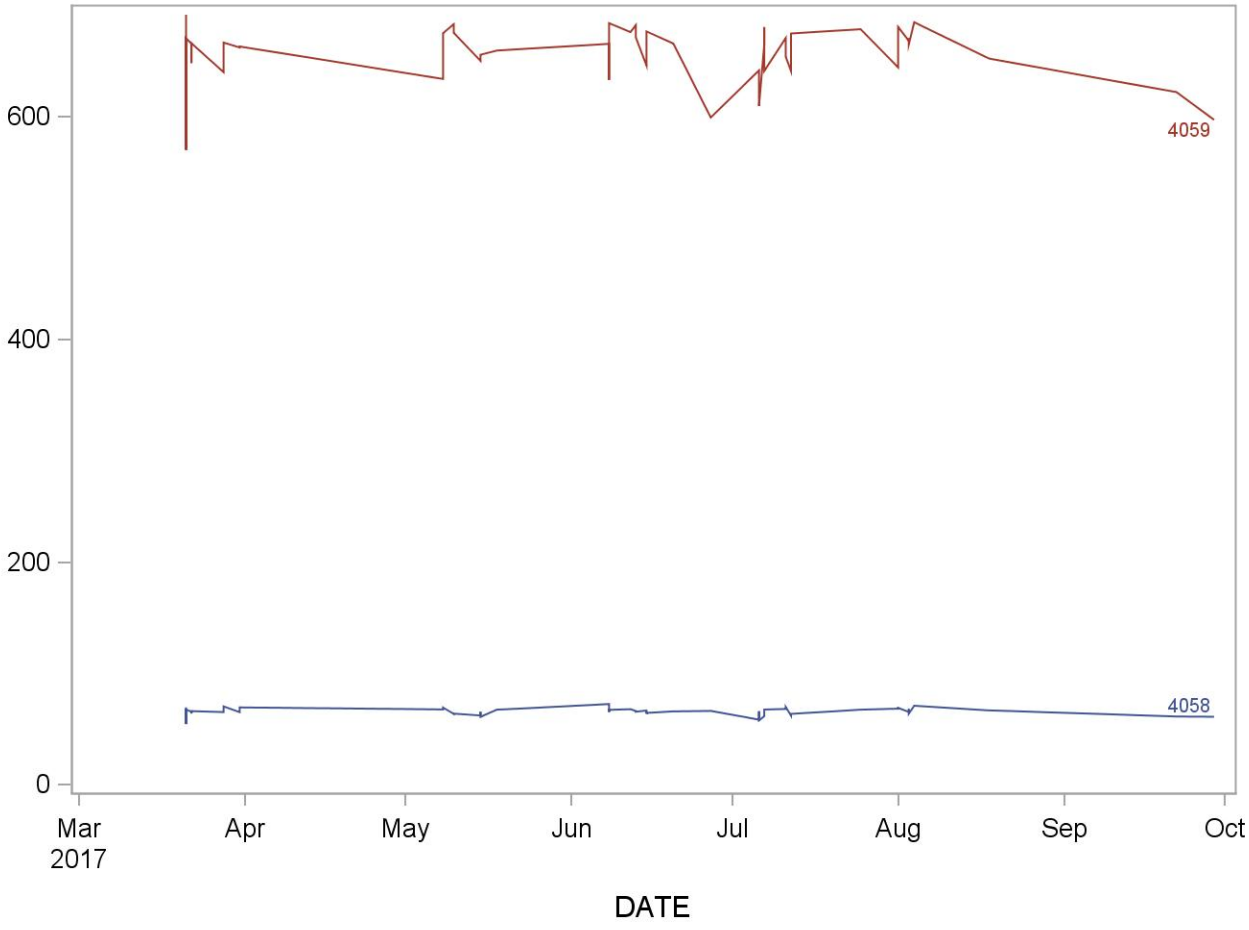
2015-2016 Summary Statistics and QC Chart for N-ace-S-(phenl-2-hydxyetl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	50	21MAR17	29SEP17	53.2106	2.0212	3.8
4058	50	21MAR17	29SEP17	5.2501	0.3059	5.8



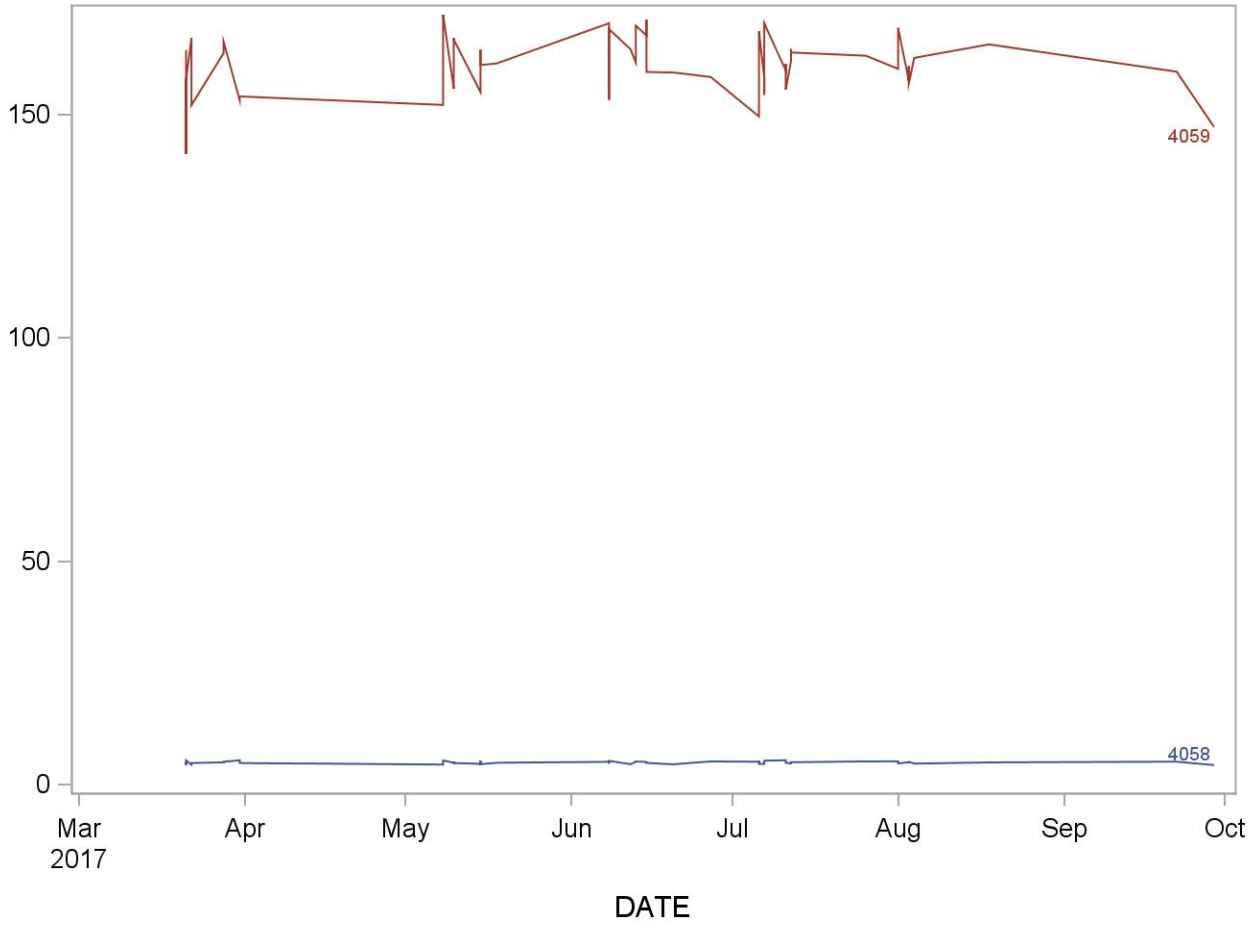
2015-2016 Summary Statistics and QC Chart for N-acel-S-(1,2-dichlorovinl)-L-cys (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	49	21MAR17	29SEP17	657.0476	25.0389	3.8
4058	49	21MAR17	29SEP17	65.5572	3.4800	5.3



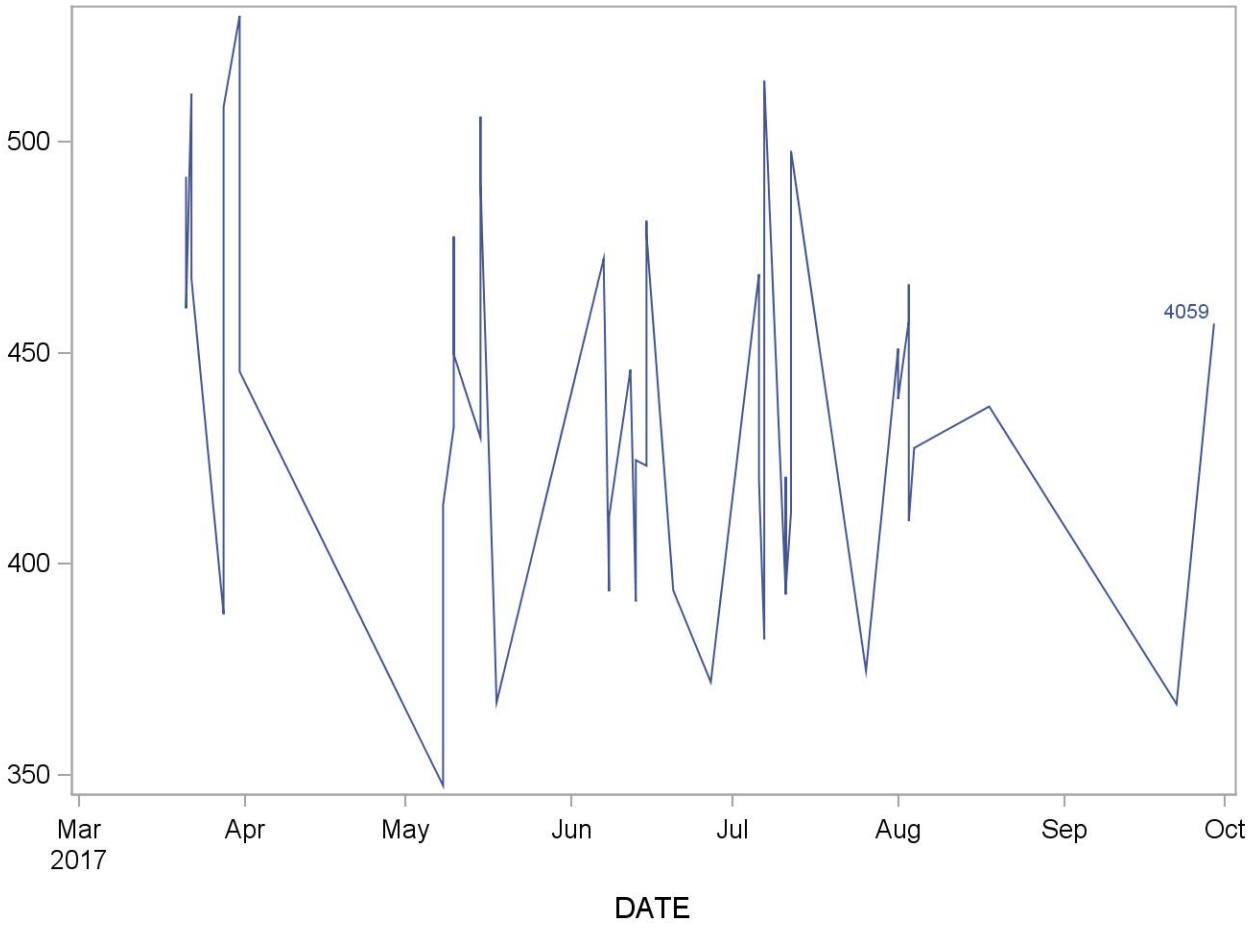
2015-2016 Summary Statistics and QC Chart for N-acetyl-S-(2-cyanoethyl)-L-cyst (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	52	21MAR17	29SEP17	161.2306	6.6340	4.1
4058	52	21MAR17	29SEP17	4.9769	0.2976	6.0



2015-2016 Summary Statistics and QC Chart for Phenylglyoxylic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
4059	53	21MAR17	29SEP17	439.5343	44.7295	10.2



APPENDIX A

A. Ruggedness testing

To evaluate the ruggedness of the method the following parameters were assessed through independent experiments:

(1) Urine samples were run at three different column temperatures (25°C, 35°C and 40°C):

No statistically significant difference between data for any analyte at 25°C, 35°C, and 40°C was observed when assessing column temperature. For GAMA, better chromatography (the peak shape) was observed at 40°C.

(2) Methanol as organic phase (Solvent B):

When we used methanol as Solvent B, we observed an interference co-eluting with MHB2. Using acetonitrile as the organic phase separated MHB2 from the interference.

(3) Freeze-thaw cycles:

Spiked urine samples kept at -20°C were freeze-thawed for ten times. Freeze thaw affected TTCA levels in urine. Do not freeze-thaw more than 5 times.

(4) Stability at 4°C and -20°C:

Samples stored at 4°C and -20°C for a week showed no statistically significant difference among data for any analyte. For long-term storage, store samples at -70°C.

(5) Samples run at 1:5, 1:10 and 1:20 dilutions:

Samples were prepared at 1:5, 1:10 and 1: 20 dilutions and were analyzed for all the analytes. The percentage difference among final estimates was < 10%.

(6) Samples run at different pH values:

Spiked urine samples were adjusted to different pH values and analyte concentrations were measured. All analytes were stable within the pH range from 2-11 (Table A1).

Table A1: Effect of pH on urinary metabolite concentrations

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
2	CEMA	223.95	219.77	-2%
2	ATCA	291.69	300.73	3%
2	GAMA	182.03	207.45	14%
2	AAMA	50.80	53.58	5%
2	HEMA	11.82	10.15	-14%
2	DHBM	157.56	168.45	7%
2	AMCA	125.58	112.79	-10%
2	TTCA	355.88	387.49	9%
2	HPMA	488.78	448.66	-8%
2	HPM2	90.85	106.88	18%
2	MADA	365.70	377.98	3%
2	CYMA	17.33	18.36	6%
2	MHB1	14.84	15.36	4%
2	MHB2	15.95	15.70	-2%
2	MHB3	19.37	22.18	14%
2	HPMM	136.56	131.83	-3%
2	PHGA	368.26	352.52	-4%
2	2MHA	125.69	116.28	-7%
2	BPMA	21.17	22.97	9%
2	34MH	105.12	106.36	1%
2	PHEM	16.78	14.52	-13%
2	1DCV	235.23	197.89	-16%
2	PMA	12.39	12.27	-1%
2	2DCV	116.94	133.87	14%
2	BMA	16.71	18.06	8%
2	TCVM	58.70	49.30	-16%
2	DPMA	2.41	2.61	8%
2	IPM1	40.94	42.61	4%
2	CYHA	88.63	89.81	1%
2	IPM3	17.08	20.28	19%
3	CEMA	223.95	230.59	3%
3	ATCA	291.69	307.95	6%
3	GAMA	182.03	220.03	21%
3	AAMA	50.80	58.07	14%
3	HEMA	11.82	13.42	14%
3	DHBM	157.56	168.42	7%
3	AMCA	125.58	120.68	-4%
3	TTCA	355.88	391.20	10%
3	HPMA	488.78	480.16	-2%
3	HPM2	90.85	87.44	-4%
3	MADA	365.70	397.78	9%
3	CYMA	17.33	20.40	18%
3	MHB1	14.84	15.36	4%
3	MHB2	15.95	16.83	5%
3	MHB3	19.37	16.64	-14%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
3	HPMM	136.56	134.05	-2%
3	PHGA	368.26	371.34	1%
3	2MHA	125.69	122.95	-2%
3	BPMA	21.17	22.69	7%
3	34MH	105.12	107.47	2%
3	PHEM	16.78	15.58	-7%
3	1DCV	235.23	198.12	-16%
3	PMA	12.39	11.51	-7%
3	2DCV	116.94	131.81	13%
3	BMA	16.71	17.44	4%
3	TCVM	58.70	54.97	-6%
3	DPMA	2.41	2.10	-13%
3	IPM1	40.94	39.22	-4%
3	CYHA	88.63	82.80	-7%
3	IPM3	17.08	19.13	12%
4	CEMA	223.95	227.65	2%
4	ATCA	291.69	286.64	-2%
4	GAMA	182.03	192.95	6%
4	AAMA	50.80	53.68	6%
4	HEMA	11.82	11.74	-1%
4	DHBM	157.56	161.28	2%
4	AMCA	125.58	127.33	1%
4	TTCA	355.88	366.90	3%
4	HPMA	488.78	536.26	10%
4	HPM2	90.85	94.02	3%
4	MADA	365.70	370.62	1%
4	CYMA	17.33	16.40	-5%
4	MHB1	14.84	18.34	24%
4	MHB2	15.95	14.45	-9%
4	MHB3	19.37	17.61	-9%
4	HPMM	136.56	133.18	-2%
4	PHGA	368.26	354.84	-4%
4	2MHA	125.69	128.76	2%
4	BPMA	21.17	22.44	6%
4	34MH	105.12	103.80	-1%
4	PHEM	16.78	14.81	-12%
4	1DCV	235.23	221.76	-6%
4	PMA	12.39	10.91	-12%
4	2DCV	116.94	134.14	15%
4	BMA	16.71	19.56	17%
4	TCVM	58.70	52.53	-11%
4	DPMA	2.41	2.54	5%
4	IPM1	40.94	44.22	8%
4	CYHA	88.63	99.58	12%
4	IPM3	17.08	19.55	14%
5	CEMA	223.95	205.16	-8%
5	ATCA	291.69	274.52	-6%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
5	GAMA	182.03	194.48	7%
5	AAMA	50.80	44.83	-12%
5	HEMA	11.82	10.99	-7%
5	DHBM	157.56	155.80	-1%
5	AMCA	125.58	128.50	2%
5	TTCA	355.88	372.35	5%
5	HPMA	488.78	437.26	-11%
5	HPM2	90.85	94.19	4%
5	MADA	365.70	374.83	2%
5	CYMA	17.33	17.41	0%
5	MHB1	14.84	16.50	11%
5	MHB2	15.95	14.39	-10%
5	MHB3	19.37	18.61	-4%
5	HPMM	136.56	121.27	-11%
5	PHGA	368.26	345.49	-6%
5	2MHA	125.69	114.29	-9%
5	BPMA	21.17	22.58	7%
5	34MH	105.12	102.02	-3%
5	PHEM	16.78	16.15	-4%
5	1DCV	235.23	194.62	-17%
5	PMA	12.39	10.94	-12%
5	2DCV	116.94	132.18	13%
5	BMA	16.71	18.37	10%
5	TCVM	58.70	46.03	-22%
5	DPMA	2.41	2.34	-3%
5	IPM1	40.94	38.64	-6%
5	CYHA	88.63	95.40	8%
5	IPM3	17.08	17.02	0%
6	CEMA	223.95	206.78	-8%
6	ATCA	291.69	282.28	-3%
6	GAMA	182.03	188.66	4%
6	AAMA	50.80	51.21	1%
6	HEMA	11.82	11.06	-6%
6	DHBM	157.56	158.73	1%
6	AMCA	125.58	119.40	-5%
6	TTCA	355.88	343.18	-4%
6	HPMA	488.78	465.91	-5%
6	HPM2	90.85	79.93	-12%
6	MADA	365.70	352.12	-4%
6	CYMA	17.33	17.82	3%
6	MHB1	14.84	14.80	0%
6	MHB2	15.95	15.24	-4%
6	MHB3	19.37	17.65	-9%
6	HPMM	136.56	127.44	-7%
6	PHGA	368.26	344.67	-6%
6	2MHA	125.69	117.61	-6%
6	BPMA	21.17	20.30	-4%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
6	34MH	105.12	104.17	-1%
6	PHEM	16.78	15.09	-10%
6	1DCV	235.23	195.87	-17%
6	PMA	12.39	13.63	10%
6	2DCV	116.94	117.06	0%
6	BMA	16.71	20.11	20%
6	TCVM	58.70	50.72	-14%
6	DPMA	2.41	2.43	1%
6	IPM1	40.94	39.35	-4%
6	CYHA	88.63	77.53	-13%
6	IPM3	17.08	19.89	16%
7	CEMA	223.95	208.71	-7%
7	ATCA	291.69	294.32	1%
7	GAMA	182.03	167.40	-8%
7	AAMA	50.80	47.67	-6%
7	HEMA	11.82	12.97	10%
7	DHBM	157.56	141.00	-11%
7	AMCA	125.58	130.01	4%
7	TTCA	355.88	355.25	0%
7	HPMA	488.78	403.29	-17%
7	HPM2	90.85	93.09	2%
7	MADA	365.70	339.19	-7%
7	CYMA	17.33	17.74	2%
7	MHB1	14.84	16.59	12%
7	MHB2	15.95	15.90	0%
7	MHB3	19.37	19.00	-2%
7	HPMM	136.56	110.33	-19%
7	PHGA	368.26	339.88	-8%
7	2MHA	125.69	112.43	-11%
7	BPMA	21.17	21.97	4%
7	34MH	105.12	101.98	-3%
7	PHEM	16.78	17.50	4%
7	1DCV	235.23	213.00	-9%
7	PMA	12.39	12.43	0%
7	2DCV	116.94	120.20	3%
7	BMA	16.71	18.20	9%
7	TCVM	58.70	52.30	-11%
7	DPMA	2.41	2.81	17%
7	IPM1	40.94	39.95	-2%
7	CYHA	88.63	93.64	6%
7	IPM3	17.08	19.59	15%
8	CEMA	223.95	212.62	-5%
8	ATCA	291.69	318.47	9%
8	GAMA	182.03	192.92	6%
8	AAMA	50.80	49.10	-3%
8	HEMA	11.82	12.12	3%
8	DHBM	157.56	146.62	-7%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
8	AMCA	125.58	130.64	4%
8	TTCA	355.88	360.97	1%
8	HPMA	488.78	444.95	-9%
8	HPM2	90.85	82.88	-9%
8	MADA	365.70	387.61	6%
8	CYMA	17.33	16.17	-7%
8	MHB1	14.84	18.45	24%
8	MHB2	15.95	14.47	-9%
8	MHB3	19.37	18.68	-4%
8	HPMM	136.56	118.43	-13%
8	PHGA	368.26	352.75	-4%
8	2MHA	125.69	114.11	-9%
8	BPMA	21.17	22.57	7%
8	34MH	105.12	102.31	-3%
8	PHEM	16.78	17.42	4%
8	1DCV	235.23	201.52	-14%
8	PMA	12.39	12.43	0%
8	2DCV	116.94	112.84	-4%
8	BMA	16.71	19.52	17%
8	TCVM	58.70	50.08	-15%
8	DPMA	2.41	2.37	-2%
8	IPM1	40.94	41.80	2%
8	CYHA	88.63	86.57	-2%
8	IPM3	17.08	18.84	10%
9	CEMA	223.95	212.58	-5%
9	ATCA	291.69	299.61	3%
9	GAMA	182.03	201.87	11%
9	AAMA	50.80	46.93	-8%
9	HEMA	11.82	12.09	2%
9	DHBM	157.56	153.24	-3%
9	AMCA	125.58	98.41	-22%
9	TTCA	355.88	345.07	-3%
9	HPMA	488.78	501.72	3%
9	HPM2	90.85	85.72	-6%
9	MADA	365.70	400.78	10%
9	CYMA	17.33	18.89	9%
9	MHB1	14.84	16.59	12%
9	MHB2	15.95	17.43	9%
9	MHB3	19.37	16.56	-15%
9	HPMM	136.56	122.98	-10%
9	PHGA	368.26	366.67	0%
9	2MHA	125.69	119.87	-5%
9	BPMA	21.17	24.52	16%
9	34MH	105.12	102.54	-2%
9	PHEM	16.78	15.34	-9%
9	1DCV	235.23	205.40	-13%
9	PMA	12.39	11.27	-9%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
9	2DCV	116.94	111.87	-4%
9	BMA	16.71	15.63	-6%
9	TCVM	58.70	55.61	-5%
9	DPMA	2.41	2.40	-1%
9	IPM1	40.94	42.56	4%
9	CYHA	88.63	91.47	3%
9	IPM3	17.08	19.15	12%
10	CEMA	223.95	209.07	-7%
10	ATCA	291.69	301.94	4%
10	GAMA	182.03	185.12	2%
10	AAMA	50.80	43.03	-15%
10	HEMA	11.82	11.04	-7%
10	DHBM	157.56	146.21	-7%
10	AMCA	125.58	101.21	-19%
10	TTCA	355.88	330.45	-7%
10	HPMA	488.78	521.40	7%
10	HPM2	90.85	86.73	-5%
10	MADA	365.70	354.00	-3%
10	CYMA	17.33	18.61	7%
10	MHB1	14.84	14.98	1%
10	MHB2	15.95	14.50	-9%
10	MHB3	19.37	19.37	0%
10	HPMM	136.56	114.17	-16%
10	PHGA	368.26	321.19	-13%
10	2MHA	125.69	118.43	-6%
10	BPMA	21.17	25.19	19%
10	34MH	105.12	104.14	-1%
10	PHEM	16.78	16.31	-3%
10	1DCV	235.23	198.81	-15%
10	PMA	12.39	11.73	-5%
10	2DCV	116.94	129.39	11%
10	BMA	16.71	17.45	4%
10	TCVM	58.70	49.38	-16%
10	DPMA	2.41	2.52	4%
10	IPM1	40.94	40.59	-1%
10	CYHA	88.63	88.91	0%
10	IPM3	17.08	17.68	4%
11	CEMA	223.95	217.72	-3%
11	ATCA	291.69	300.91	3%
11	GAMA	182.03	185.76	2%
11	AAMA	50.80	39.76	-22%
11	HEMA	11.82	12.65	7%
11	DHBM	157.56	143.28	-9%
11	AMCA	125.58	102.62	-18%
11	TTCA	355.88	323.33	-9%
11	HPMA	488.78	440.49	-10%
11	HPM2	90.85	91.36	1%

pH	Analyte	Target Concentration (ng/mL)	Measured Concentration (ng/mL)	% Difference
11	MADA	365.70	347.19	-5%
11	CYMA	17.33	16.22	-6%
11	MHB1	14.84	14.99	1%
11	MHB2	15.95	14.70	-8%
11	MHB3	19.37	17.25	-11%
11	HPMM	136.56	122.82	-10%
11	PHGA	368.26	323.43	-12%
11	2MHA	125.69	108.07	-14%
11	BPMA	21.17	22.68	7%
11	34MH	105.12	93.93	-11%
11	PHEM	16.78	15.45	-8%
11	1DCV	235.23	225.07	-4%
11	PMA	12.39	10.09	-19%
11	2DCV	116.94	132.68	13%
11	BMA	16.71	18.62	11%
11	TCVM	58.70	49.41	-16%
11	DPMA	2.41	2.47	2%
11	IPM1	40.94	38.39	-6%
11	CYHA	88.63	73.34	-17%
11	IPM3	17.08	19.00	11%

APPENDIX B

Table B1. Typical slopes of matrix based (urine) and solvent based (15 mM ammonium acetate) concentration plots.

		Slope	
Analyte	P-value	Urine matrix	15 mM Ammonium acetate matrix
AAMA	0.81	0.9242	0.9262
AMCA	0.87	0.9623	0.9626
ATCA	0.97	1.0047	1.0048
BMA	0.86	1.0111	1.0103
BPMA	0.94	0.9737	0.9737
CEMA	0.9	0.9725	0.9723
CYMA	0.99	0.9992	0.9993
1DCV	0.99	0.9866	0.9868
2DCV	0.99	1.0233	1.0239
DHBM	0.56	0.9529	0.9530
DPMA	0.99	1.0040	1.0041
GAMA	0.98	1.0110	1.0110
HEMA	0.99	1.1831	1.1843
HPMA	0.94	1.0149	1.0153
HPM2	0.87	0.9638	0.9640
HPMM	0.47	0.9662	0.9660
MADA	0.96	0.9999	1.0022
2MHA	0.99	0.9646	0.9655
34MH	0.83	0.9904	0.9906
MHB1	0.96	0.9881	0.9883
MHB2	0.99	1.0131	1.0131
MHB3	0.97	1.2050	1.2040
PHGA	0.92	0.9930	0.9929
PHEM	0.99	0.9839	0.9837
PMA	0.99	0.9925	0.9915
TCVM	0.99	0.9873	0.9877
TTCA	0.98	0.9404	0.9412

[a]Probability (two-tailed) for urine based estimates and solvent based estimates for 15 matching concentrations.

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