



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:

Tobacco and Volatiles Branch
Division of Laboratory Sciences
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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_H & UVOCS_H	URX1DC	N-ace-S-(1,2-dichlorovinyl)-L-cys(ng/mL)
	URX2DC	N-Ace-S-(2,2-Dichlorovinyl)-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-methylcarbamoyl)-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)
	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)
	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 resources. 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 of 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 mainly 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 ubiquitously present in cooked food 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 of 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 (eg. French fries, potato chips). It is also a component of cigarette smoke (11). The acrylamide metabolite, glycidamide, is considered to be the putative mutagen and most directly related to acrylamide's carcinogenicity. Acrylonitrile is widely used in the manufacture of plastics, acrylic fibers, and synthetic rubber is considered as a probable human carcinogen (12). Benzene is a group 1 carcinogen (13). 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 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, textile coatings and in the manufacture of synthetic leather, polyurethane and polyacrylonitrile fibres (19). Ethylene oxide, which is an intermediate used in the production of ethylene glycol and other oxide derivatives could cause 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 human (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 provoke disorientation, euphoria, exhilaration, and tinnitus (24). Vinyl chloride exposure can cause angiosarcoma (25). 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 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. But 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 exposure in the US population.

Table 1. VOC metabolites and their parent compounds.

Parent Compound	VOC Metabolite	Common Name
Acrolein	<i>N</i> -Acetyl-S- (2-carboxyethyl)-L-cysteine	CEMA
	<i>N</i> -Acetyl-S- (3-hydroxypropyl)-L-cysteine	3HPMA
Acrylamide	<i>N</i> -Acetyl-S-(2-carbamoyl-ethyl)-L-cysteine	AAMA
	<i>N</i> -Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA
Acrylonitrile	<i>N</i> -Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA
Acrylonitrile, vinyl chloride, ethylene oxide	<i>N</i> -Acetyl-S- (2-hydroxyethyl)-L-cysteine	HEMA
Benzene	<i>N</i> -Acetyl-S-(phenyl)-L-cysteine	PMA
1-Bromopropane	<i>N</i> -Acetyl-S-(<i>n</i> -propyl)-L-cysteine	BPMA
1,3-Butadiene	<i>N</i> -Acetyl-S- (3,4-dihydroxybutyl)-L-cysteine	DHBMA
	<i>N</i> -Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	MHBMA1
	<i>N</i> -Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	MHBMA2
	<i>N</i> -Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA3
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid	TTCA
Crotonaldehyde	<i>N</i> -Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMMA
Cyanide	2-Aminothiazoline-4-carboxylic acid	ATCA
<i>N, N</i> - Dimethylformamide	<i>N</i> -Acetyl-S-(<i>N</i> -methylcarbamoyl)-L-cysteine	AMCC
Ethylbenzene, styrene	Phenylglyoxylic acid	PGA
Propylene oxide	<i>N</i> -Acetyl-S-(2-hydroxypropyl)-L-cysteine	2HPMA
Styrene	<i>N</i> -Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine + <i>N</i> -Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	PHEMA
	Mandelic acid	MA
Tetrachloroethylene	<i>N</i> -Acetyl-S-(trichlorovinyl)-L-cysteine	TCVMA
Toluene	<i>N</i> -Acetyl-S-(benzyl)-L-cysteine	BMA
Trichloroethylene	<i>N</i> -Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	1,2DCVMA
	<i>N</i> -Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	2,2DCVMA
Xylene	<i>N</i> -Acetyl-S-(2,4-dimethylphenyl)-L-cysteine + <i>N</i> -Acetyl-S-(2,5-dimethylphenyl)-L-cysteine + <i>N</i> -Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	DPMA
	2-Methylhippuric acid	2MHA
	3-Methylhippuric acid + 4-Methylhippuric acid	3MHA + 4MHA

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 electro spray tandem mass spectrometry (UPLC-ESI/MSMS) (29). Chromatographic separation is achieved using an Acquity UPLC[®] HSS T3 (Part no. 186003540, 1.8 μ m x 2.1 mm x 150 mm, Waters Inc.) column with 15 mM ammonium acetate and acetonitrile as the mobile phases. The eluant 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. Take care to prevent contact of VOC metabolites with strong oxidizing agents 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. Wear gloves, lab coat, and safety glasses while preparing solutions and handling human urine. Place disposable plastic, glass, and 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. Wipe down all work surfaces with 70% ethanol solution when work is finished.

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 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 should 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. 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, mechanical and electronic maintenance and repair should be performed only by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Precautions should be used 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, QCs and PTs.

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

Dispose of waste materials in compliance with laboratory, Federal, State, and Local regulations. 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 the Waters UPLC system coupled to the Sciex mass spectrometer both controlled and run by Analyst 1.51 software. Results are converted to Microsoft Excel files and entered into the ATLAS 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

Information pertaining to particular specimens is entered into the database either manually or electronically transferred. The result file is transferred electronically into the database.

c. Data maintenance

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

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

- a. No special instructions such as fasting or special diets are required.
- b. The matrix type is urine.
- c. An aliquot of 50 μ l is needed per assay. A volume of 0.25 -0.5 mL is required to allow for repeated analysis.
- d. Acceptable containers include polystyrene cryo tube vials or polypropylene (PP) centrifuge tubes (e.g.). Sterile collectors should be used for specimen acquisition.
- e. The criteria for unacceptable specimen with suspected contamination due to improper collection procedures or collection devices. In all cases, a second urine specimen should be requested.
- f. Specimen characteristics that may compromise test results are as indicated above including contamination of urine by contact with dust, dirt, etc. from improper handling.
- g. 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. In general, urine specimens should be transported and stored chilled or frozen at -20°C. Once received, the samples can be frozen at -70°C until time for

analysis. Portions of the sample which remain after analytical aliquots are withdrawn and should be refrozen at $\leq -20^{\circ}\text{C}$. Avoid freeze-thawing of samples more than five times.

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

Not applicable to this procedure

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

a. Reagent sources

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

Table 2. Reagents and sources.

Reagent	
Acetonitrile (Optima LCMS grade)	Fisher Scientific, Fairlawn, NJ
Ammonium Acetate	Sigma Chemicals, St. Louis, MO
Dimethylsulfoxide (HPLC grade)	Fisher Scientific, Fairlawn, NJ
Methanol (Optima LCMS grade)	Fisher Scientific, NJ
Iso-propyl alcohol (Optima LCMS grade)	Fisher Scientific, NJ
Negative PPG ($3 \times 10^{-5}\text{M}$) P/N 4405235	ABI Sciex, Foster City, CA
Water (HPLC grade)	Fisher Scientific, Fairlawn, NJ
<i>N</i> -Acetyl-S-(benzyl)-L-cysteine	Battelle Research, Columbus, Ohio
<i>N</i> -Acetyl-S-(benzyl- $^{13}\text{C}_6$)-L-cysteine	Battelle Research Institute, Columbus, Ohio
<i>N</i> -Acetyl-S-(2-carbamoylethyl)-L-cysteine	C/D/N Isotopes Inc, Quebec, Canada
<i>N</i> -Acetyl-S-(2-carbamoylethyl- D_4)-L-cysteine	C/D/N Isotopes Inc, Quebec, Canada
<i>N</i> -Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada
<i>N</i> -Acetyl- D_3 -S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada
<i>N</i> -Acetyl-S-(2-carboxyethyl)-L-Cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(2-carboxyethyl- $^{13}\text{C}_3$)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -acetyl-S-(2-cyanoethyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada
<i>N</i> -acetyl- D_3 -S-(2-cyanoethyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada
<i>N</i> -Acetyl-S-(1,2-dichlorovinyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada
<i>N</i> -Acetyl- ^{13}C - D_3 -S-(1,2-dichlorovinyl)-L-cysteine	Toronto Research Chemicals, Toronto, Canada

<i>N</i> -Acetyl-S-(2,2-dichlorovinyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl- ¹³ C- D ₃ -S-(2,2-dichlorovinyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(3,4-dihydroxybutyl)-L-Cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(3,4-dihydroxybutyl- ¹³ C ₄)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(2,4-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-D ₃ -S-(2,4-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(2,5-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-D ₃ -S-(2,5-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(3,4-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-D ₃ -S-(3,4-dimethylphenyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(2-hydroxyethyl)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(2-hydroxyethyl-D ₄)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(2-hydroxypropyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(2-hydroxypropyl-D ₃)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(3-hydroxypropyl)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(3-hydroxypropyl-D ₆)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(1-hydroxymethyl-2-propenyl-D ₆)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine	Kallexsyn Inc., Kalamazoo, MI
<i>N</i> -Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine- ¹³ C ₃ - ¹⁵ N	Kallexsyn Inc., Kalamazoo, MI
<i>N</i> -Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-D ₃ -(4-hydroxy-2-buten-1-yl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(3-hydroxypropyl-1 methyl)-L-cysteine dicyclohexylammonium salt	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-D ₃ -S-(3-hydroxypropyl-1 methyl)-L-cysteine dicyclohexylammonium salt	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(<i>N</i> -methylcarbamoyl)-L-cysteine	Sigma Chemicals, St. Louis, MO
<i>N</i> -Acetyl-S-(<i>N</i> -methylcarbamoyl)-L-cysteine - ¹³ C ₃ - ¹⁵ N	Kallexsyn Inc., Kalamazoo, MI
<i>N</i> -Acetyl-S-(phenyl)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(phenyl- ¹³ C ₆)-L-cysteine	Cambridge Isotopes, Andover, MA
<i>N</i> -Acetyl-S-(1-phenyl-2-hydroxyethyl-L-cysteine +	Toronto Research Chemicals,
<i>N</i> -Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine	Toronto, Canada
<i>N</i> -Acetyl-S-(1-phenyl- ¹³ C ₆ -2-hydroxyethyl-L-cysteine +	Toronto Research Chemicals,
<i>N</i> -Acetyl-S-(2-phenyl- ¹³ C ₆ -2-hydroxyethyl)-L-cysteine	Toronto, Canada
<i>N</i> -Acetyl-S-(<i>n</i> -propyl)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(<i>n</i> -propyl-D ₇)-L-cysteine	Toronto Research Chemicals, Toronto, Ca
<i>N</i> -Acetyl-S-(trichlorovinyl)-L-cysteine	Battelle Research Institute, Columbus, OH
<i>N</i> -Acetyl-S-(trichlorovinyl- ¹³ C ₂)-L-cysteine	Battelle Research Institute, Columbus, OH
2-Aminothiazoline-4-carboxylic acid	Chem-Impex International Inc., Woodale,
2-Aminothiazoline-D ₃ -4-carboxylic acid	Dr. Bill Draper's Lab, CDPH, CA

Mandelic acid	Sigma Chemicals, St. Louis, MO
Mandelic-2,3,4,5,6-D ₅ acid	C/D/N Isotopes Inc, Quebec, Canada
2-Methylhippuric acid	Sigma Chemicals, St. Louis, MO
2-Methylhippuric-D ₇ acid	C/D/N Isotopes Inc, Quebec, Canada
3-Methylhippuric acid	Sigma Chemicals, St. Louis, MO
3-Methylhippuric-D ₇ acid	C/D/N Isotopes Inc, Quebec, Canada
4-Methylhippuric acid	Sigma Chemicals, St. Louis, MO
4-Methylhippuric-D ₇ acid	C/D/N Isotopes Inc, Quebec, Canada
Phenylglyoxylic acid	Sigma Chemicals, St. Louis, MO
Phenylglyoxylic-D ₅ acid	C/D/N Isotopes Inc, Quebec, Canada
2-Thioxothiazolidine-4-carboxylic acid	Sigma Chemicals, St. Louis, MO
2-Thioxothiazolidine- ¹³ C ₃ -4-carboxylic acid	Cambridge Isotopes, Andover, MA

^aSigma – NMR/GCMS/LCMS; C/D/N Isotopes Inc. – NMR; TRC – MS; Cambridge Isotopes – GCMS; Kalexsyn Inc – NMR/MS; Battelle – NMR.

^bSigma – NMR/GC/HPLC; C/D/N Isotopes Inc. – HPLC; TRC – NMR, TLC or HPLC; Cambridge Isotopes – NMR; Kalexsyn Inc – HPLC/NMR; Battelle – NMR/HPLC; Chem Impex - HPLC

b. Reagent preparation

1) 15mM Ammonium acetate

Prepare the 15 mM ammonium acetate, which is used as Solvent A (mobile phase of UPLC), to prepare working calibration standards, and to dilute urine and quality control (QC) samples, by dissolving 2.31 g of ammonium acetate in 2 L of HPLC-grade water and mixing thoroughly in a 2 L reservoir bottle. Vacuum filter the prepared solution (0.45 micron) and check the pH (pH ~ 6.8).

c. Standards solutions preparation

1) Stock solutions and dilutions

a) VOC metabolite stock and the working mixed standard solutions

Prepare the VOC metabolite stock solutions individually by weighing VOC metabolite neat compounds (10 - 20 mg) using a pre-calibrated analytical balance and diluting each in 10 mL of solvent (**Table 3**). From stock, prepare the mixed standard working solution in water (final volume 50 mL) as shown in **Table 4a and Table 4b**. Aliquot (1 mL) working mixed standard into 2-mL glass vials and store them in -20°C until use. Once the vial is thawed and under use, stores it at 4°C.

Table 3. Solvent used to prepare initial stock solution.

Analyte	Solvent used to Prepare Initial Stock
AAMA; AAMA-D ₄	water
AMCC; AMCC- 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
1,2DCVMA; 1,2DCVMA- ¹³ C-D ₃	methanol
2,2DCVMA; 2,2DCVMA- ¹³ C-D ₃	methanol
DHBMA; DHBMA- 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
2HPMA; HPMA-D ₃	water
HPMMA; HPMMA-D ₃	water
MA; MA-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)
MHBMA1; MHBMA1-D ₆	water
MHBMA2; MHBMA2- ¹³ C ₃ - ¹⁵ N	water
MHBMA3; MHBMA3-D ₃	methanol:water (1:1)
PGA; PGA-D ₅	water
PHEMA; PHEMA- ¹³ C ₆	methanol
PMA; PMA- ¹³ C ₆	water
TCVMA; TCVMA- ¹³ C ₂	methanol
TTCA; TTCA- ¹³ C ₃	water

Table 4a. Typical individual stock standards and working mixed standard solution (to prepare STD1 to STD9).

Analyte STD	Stock A µg/mL	µl Vol. from Stock A	Working Mix A ng/mL
AAMA	1085	5	54
AMCC	2770	6.5	180
ATCA	1115	40	446
BMA	1100	2	22
BPMA	1535	2.5	38
CEMA	100	300	300
CYMA	1580	1.2	19
1,2DCVMA	1574	30	472
2,2DCVMA	1620	12	194
DHBMA	1000	20	200
2,4DPMA	10	37.8	4
2,5DPMA	13.3	28.4	4
3,4DPMA	12.6	30	4
GAMA	1181	25	295
HEMA	304	6.2	19
HPMA	1296	50	648
2HPMA	1321	10	132
HPMMA	1520	10	152
MA	1001	60	601
2MHA	1004	15.5	156
3MHA	1006	15.4	155
4MHA	1012	15.3	155
MHBMA1	456	5	23
MHBMA2	480	5	24
MHBMA3	1100	2.5	28
PHEMA	1000	2.5	25
PGA	1007	50	504
PMA	276	6.3	17
TCVMA	1510	5	60.4
TTCA	1340	12.5	168

Table 4b. Typical concentrations used for individual stock standards and working mixed standard solution (to prepare STD10).

STD	Stock A $\mu\text{g/mL}$	μl Vol. from Stock A	Working Mix B ng/mL
AAMA	1085	6.25	678
AMCC	2770	6.25	1731
BMA	1100	2.5	275
BPMA	1535	2.5	384
CYMA	1580	5	790
DHBMA	1000	25	2500
HEMA	304	5	152
HPMA	1296	62.5	8100
2HPMA	1321	50	6605
HPMMA	1520	40	6080
2-MHA	1004	17.15	1722
3-MHA	1006	17.1	1720
4-MHA	1012	17	1720
MHBMA3	1100	2.5	275
PGA	1007	50	5035
TTCA	1340	12.5	1675

b) Labeled internal standard and the working mixed internal standard solutions

Labeled stock standard solutions are prepared individually by weighing labeled neat compound (10 - 20 mg) using a pre-calibrated analytical balance and dissolving it in 10 mL of suitable solvent (**Table 3**). The stock internal standards are stored in -20°C . The mixed internal standard working solution is prepared as shown in **Table 5**. Aliquots (5 mL) of mixed internal standard in glass vials are stored in the refrigerator. An aliquot of 25 μl from the mixed internal standard solution is added to each sample.

Table 5. Individual labeled internal standard stock and mixed internal standard working solutions.

Labeled Internal Standard	Stock A µg/mL	Mixed Internal Standard Working Solution (final vol. 25 mL)		Final Conc. in 0.5 mL Sample ng/mL
		µl from Stock A	Final Conc. ng/mL	
AAMA-D ₄	1115	25	1115	56
AMCC- ¹³ C ₃ - ¹⁵ N	1000	25	1000	50
ATCA-D ₃	1080	125	5400	270
BMA- ¹³ C ₆	1370	2.5	137	7
BPMA-D ₇	1271	25	1271	64
CEMA- ¹³ C ₃	156	150	936	47
CYMA-D ₃	1000	25	1000	50
1,2DCVMA- ¹³ C-D ₃	2508	12.5	1254	63
2,2DCVMA- ¹³ C-D ₃	2446	5	489	24
DHBMA- ¹³ C ₄	1000	15	600	30
2,4DPMA-D ₃	10	26	10	0.52
2,5DPMA-D ₃	13	20	10	0.52
3,4DPMA-D ₃	10.3	25.2	10	0.52
GAMA-D ₃	1204	25	1204	60
HEMA-D ₄	308	32.5	400	20
HPMA-D ₆	100.8	25	101	5
2HPMA-D ₃	1216	5	243	12
HPMMA-D ₃	1000	25	1000	50
MA-D ₅	1000	50	2000	100
2MHA-D ₇	1005	50	2010	101
3MHA-D ₇	1005	25	1005	50
4MHA-D ₇	1005	25	1005	50
MHBMA1-D ₆	796	8.25	263	13
MHBMA2- ¹³ C ₃ - ¹⁵ N	1100	6.25	275	14
MHBMA3-D ₃	1000	7	280	14
PHEMA- ¹³ C ₆	1000	25	1000	50
PGA-D ₅	1020	25	1020	51
PMA- ¹³ C ₆	840	4.5	151	8
TCVMA- ¹³ C ₂	1260	25	1260	63
TTCA- ¹³ C ₃	1260	25	1260	63

d. Preparation of quality control material**1) Quality control pools**

QC materials were prepared spiking a known amount of mixed VOC metabolite standards solution into 100 mL urine to achieve the target concentration. Two urine pools were prepared with levels within the linear range established, QC low and QC high. Aliquot 150 µl into separate cryovials (for QC low and QC high) and store them at -20°C until use.

2) Proficiency testing samples

Proficiency testing materials are prepared from the original VOC metabolite stock standards. Four levels of PT standards are prepared diluting the stock A in HPLC grade water. Aliquots are stored in cryovials, at -20°C until use. Proficiency testing samples are run twice a year as well, and following any major maintenance on the instrumentation.

e. Other materials

- Acquity UPLC® HSS T3 1.8µm x 2.1 mm x 150 mm column [Part # 186003540 Waters Inc., Milford, MA]
- Inline Filter assembly [Part # 205000343, Waters Inc., Milford, MA]
- Reservoir bottles and caps [1 L and 2 L, Agilent, Santa Clara, CA]
- PEEK tubing [Agilent, Santa Clara, CA]
- Dionex 1.5 mL clear autosampler vials, septa and caps [Fisher Scientific, NJ]
- Agilent 2 mL amber autosampler vials, septa and caps [Fisher Scientific, NJ]
- Nalgene cryovials [Fisher Scientific, Fairlawn, NJ]
- VWR brand mini vortexer [VWR, West Chester, PA]
- Raining electronic pipettes and tips [10, 20, 100, 200, 1000-µl; Rainin Inc., CA]
- Eppendorf Repeater Plus pipette [Brinkman Instruments Inc., Westbury, NY]
- Volumetric flasks [5, 10, 25, 50-µl, Fisher Scientific, Fairlawn, NJ]
- Pasteur pipettes and bulbs [Fisher Scientific, Fairlawn, NJ]
- Glass containers with caps, beakers, Wheaton 125 mL bottles [Fisher Scientific, NJ]
- Wilmad Lab glass filter Apparatus [Cat. No. 50805333, Fisher Scientific, NJ]
- Mixed Cellulose Ester Nitrocellulose Millipore Filters D=47mm, Pore size=0.45 µm [Cat. No. 09-719-2E, Fisher Scientific, Fairlawn, NJ].

f. Instrumentation

Analyses were conducted with a Waters Acquity UPLC system equipped with a binary solvent manager, sample manager, a degasser and a movable column compartment, with 15 mM ammonium acetate (Solvent A)/acetonitrile (Solvent B) as mobile phase. The separation was performed using an Acquity UPLC® HSS T3 (1.8 µm x 2.1 mm x 150 mm) column. AB Sciex Triple Quad 5500 Mass Spectrometer (Applied Biosystems, Foster City, CA) with an electrospray interface was used for the detection of urinary VOC metabolites.

1) LC parameters

The separation conditions were optimized (**Table 6**) to obtain good resolution among VOC metabolites.

Table 6. Chromatography parameters for the UPLC.

Parameter	Details
Column	Acquity UPLC [®] HSS T3 1.8 μm x 2.1 mm x 150 mm column. Operating temp: 40°C
Mobile Phase	15 mM Ammonium acetate (Solvent A); Acetonitrile (Solvent B)
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 25% Optima LCMS grade acetonitrile
Gradient :	
Time, flow, Solvent A : Solvent B	initial, 250 $\mu\text{l}/\text{min}$, 97% : 3% 2 min, 250 $\mu\text{l}/\text{min}$, 95% : 5% 3 min, 300 $\mu\text{l}/\text{min}$, 90% : 10% 5 min, 300 $\mu\text{l}/\text{min}$, 70% : 30% 6.5 min, 300 $\mu\text{l}/\text{min}$, 60% : 40% 7 min, 300 $\mu\text{l}/\text{min}$, 85% : 15% 7.5 min, 300 $\mu\text{l}/\text{min}$, 90% : 10% 8 min, 300 $\mu\text{l}/\text{min}$, 97% : 3% 9 min, 300 $\mu\text{l}/\text{min}$, 97% : 3%

3) Mass spectrometer parameters

Each one of the following parameters was optimized for the ions of interest. These parameters should be re-optimized when transferring the method to another instrument. The mass spectrometer was operated under scheduled MRM (SMRM –Scheduled Multiple Reaction Monitoring) mode. The transitions of interest are presented in **Table 7** and the typical mass spectrometer parameters used are presented in **Table 8** and **Table 9**.

Table 7. Scheduled 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
AMCC	219/162	220/163	AMCC- 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	204/75	204/84	BPMA-D ₇	211/82
CEMA	234/162	234/105	CEMA- ¹³ C ₃	237/162
CYMA	215/86	215/162	CYMA-D ₃	218/165
1,2DCVMA	257/127	257/128	1,2DCVMA- ¹³ C-D ₃	261/127
2,2DCVMA	257/127	257/128	2,2DCVMA- ¹³ C-D ₃	261/127
DHBMA	250/121	250/75	DHBMA- 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
2HPMA	220/91	221/91	2HPMA-D ₃	223/91
HPMMA	234/105	235/105	HPMMA-D ₃	237/105
MA	151/107	152/108	MA-D ₅	156/112
2MHA	192/148	192/91	2MHA-D ₇	199/155
3MHA + 4MHA	192/148	192/91	3MHA-D ₇ + 4MHA-D ₇	199/155
MHBMA1	232/103	233/103	MHBMA1-D ₆	238/109
MHBMA2	232/103	233/103	MHBMA2- ¹³ C ₃ - ¹⁵ N	236/103
MHBMA3	232/103	233/103	MHBMA3-D ₃	235/103
PGA	149/77	149/105	PGA-D ₅	154/82
PHEMA	282/153	282/123	PHEMA- ¹³ C ₆	288/159
PMA	238/109	239/110	PMA- ¹³ C ₆	244/115
TCVMA	290/161	290/35	TCVMA- ¹³ C ₂	294/165
TTCA	162/58	162/33	TTCA- ¹³ C ₃	165/58

^aQuantitation ion. ^bConfirmation ion.

Note: Monitored same transitions for 1,2DCVMA and 2,2DCVMA, 2MHA and 3MHA+4MHA, MHBMA1, MHBMA2, MHBMA3 in scheduled MRM as the ions eluted at different retention times.

Table 8. Typical mass spectrometer settings.

Parameter	Settings
Scan type	SMRM
Polarity	Negative
Ion Source	Turbo Spray
Temp	650 °C
IS	-4000 V
CAD	7 psi
CUR	45 psi
GSI	55 psi
GS2	65 psi
Probe Y distance (mm)	2

Table 9. Typical mass spectrometer parameters for each analyte transition.

Analyte	DP	EP	CE	CXP	Analyte	DP	EP	CE	CXP
AAMA (quan)	-65	-9	-18	-9	HPMA (quan)	-100	-10	-18	-9
AAMA (con)	-65	-9	-46	-9	HPMA (con)	-100	-10	-32	-7
AAMA-D ₄	-80	-10	-20	-9	HPMA-D ₆	-60	-9	-18	-7
AMCC (quan)	-50	-8	-10	-5	2HPMA (quan)	-170	-8	-18	-9
AMCC (con)	-50	-9	-12	-9	2HPMA (con)	-100	-10	-20	-9
AMCC- ¹³ C ₃ - ¹⁵ N	-65	-9	-12	-9	2HPMA-D ₃	-65	-8	-20	-7
ATCA (quan)	-45	-8	-14	-7	HPMMA (quan)	-60	-8	-18	-7
ATCA (con)	-45	-8	-46	-7	HPMMA (con)	-100	-10	-18	-11
ATCA-D ₃	-30	-10	-16	-1	HPMMA-D ₃	-55	-10	-22	-9
BMA (quan)	-40	-10	-20	-9	MA (quan)	-55	-8	-12	-3
BMA (con)	-55	-10	-22	-11	MA (con)	-55	-8	-20	-11
BMA- ¹³ C ₆	-60	-9	-16	-13	MA-D ₅	-35	-8	-14	-7
BPMA (quan)	-60	-9	-18	-9	2-MHA (quan)	-55	-9	-16	-19
BPMA (con)	-60	-9	-14	-7	2-MHA (con)	-55	-9	-20	-7
BPMA-D ₇	-50	-9	-20	-7	2-MHA-D ₇	-65	-8	-16	-11
CEMA (quan)	-45	-10	-14	-9	3MHA + 4MHA (quan)	-70	-8	-16	-17
CEMA (con)	-45	-10	-20	-9	3MHA + 4MHA (con)	-70	-8	-22	-11
CEMA- ¹³ C ₃	-25	-11	-14	-13	3MHA-D ₇ + 4MHA-D ₇	-55	-9	-18	-5
CYMA (quan)	-45	-10	-16	-5	MHBMA1 (quan)	-55	-7	-14	-17
CYMA (con)	-45	-10	-10	-7	MHBMA1 (con)	-40	-7	-18	-3
CYMA-D ₃	-45	-9	-12	-15	MHBMA1-D ₆	-70	-8	-18	-11
1,2DCVMA (quan)	-50	-8	-8	-13	MHBMA2 (quan)	-35	-10	-16	-11
1,2DCVMA (con)	-50	-8	-12	-9	MHBMA2 (con)	-45	-8	-18	-7
1,2DCVMA- ¹³ C-D ₃	-55	-7	-10	-7	MHBMA2- ¹³ C ₃ - ¹⁵ N	-50	-8	-18	-13
2,2DCVMA (quan)	-40	-10	-12	-15	MHBMA3 (quan)	-80	-9	-18	-7
2,2DCVMA (con)	-50	-10	-14	-11	MHBMA3 (con)	-60	-10	-20	-7
2,2DCVMA- ¹³ C-D ₃	-35	-10	-14	-9	MHBMA3-D ₃	-40	-9	-20	-3
DHBMA (quan)	-70	-9	-20	-7	PGA (quan)	-30	-8	-14	-19
DHBMA (con)	-70	-9	-32	-7	PGA (con)	-30	-8	-10	-7
DHBMA- ¹³ C ₄	-55	-9	-18	-9	PGA-D ₅	-30	-9	-16	-11
DPMA (quan)	-40	-9	-30	-11	PHEMA (quan)	-60	-8	-18	-17
DPMA (con)	-20	-9	-28	-9	PHEMA (con)	-60	-8	-34	-15
DPMA-D ₃	-70	-10	-28	-3	PHEMA- ¹³ C ₆	-60	-10	-18	-11
GAMA (quan)	-50	-9	-20	-9	PMA (quan)	-25	-8	-22	-9
GAMA (con)	-50	-9	-16	-9	PMA (con)	-80	-8	-22	-5
GAMA-D ₃	-50	-9	-24	-13	PMA- ¹³ C ₆	-65	-10	-18	-9
HEMA (quan)	-50	-7	-16	-3	TCVMA (quan)	-50	-10	-14	-11
HEMA (con)	-50	-7	-32	-3	TCVMA (con)	-50	-10	-48	-17
HEMA-D ₄	-60	-7	-18	-9	TCVMA- ¹³ C ₂	-40	-9	-12	-11
TTCA (quan)	-35	-9	-14	-7					
TTCA- ¹³ C ₃	-35	-9	-36	-17					
TTCA (con)	-45	-9	-14	-25					

7. Calibration and Calibration Verification

Preparation of calibration standards are done according to **Table 10**. There are nine plus one calibrators.

a. Creation of curve

1) Calibration data

- a) Prepare fresh calibrators for each set of unknown analyses.
- b) Analyze each set of unknowns to form the calibration curve for that set of samples.
- c) Generate a linear calibration curve with nine standards using the ratio of the peak area of the analyte to the labeled internal standard.

2) Calculation of curve statistics

Determine the slope, intercept and R-squared value for the nine-point calibration curve using a 1/x-weighted linear regression in Analyst 1.51 software.

3) Evaluation of curve statistics

Evaluate the calibration curve statistics to ensure that the R-squared value of the curve is equal to or greater than 0.990, and that the linearity of the standard curve extends over the entire standard range. If the calculated value of one calibrator deviates by greater than 20% from the actual value then that one calibrator can be excluded.

4) Calibration verification

Calibration is verified by analyzing a full set of calibrators with every batch of unknown samples. In addition, an external standard blind to the analyst is analyzed at least once every 6 months and whenever the instrument is non-operational due to repairs or maintenance. This external standard blind result must agree with certified or accepted values within the 95% confidence and range intervals.

b. Validation of aqueous calibrators for urinary measurements

Prepare duplicates of 15 different analyte concentrations distributed across the calibration range by diluting stock solutions with either 15 mM ammonium acetate or urine (diluted 1:10 with water). Analyze these solutions as unknowns and calculate analyte concentrations based on calibrators prepared in 15 mM ammonium acetate. Construct an XY plot of theoretical vs. observed amount, and draw a best fit line. The slopes of these best fit lines for aqueous and urine-based plots are shown in Appendix B, and are not statistically different ($p > 0.05$).

Therefore we recommend preparing calibrators in 15 mM ammonium acetate for this method.

c. Use of the calibration curve

The unknowns are assayed at 1:10 dilution. The formal LODs for urine are 10 times the analytical LODs for the assay. Only the data above or at the LOD are reported. The highest point of the calibration is above the expected range of results. The remaining points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns fall.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of a blank, 9 plus one calibration standards, 2 low level QCs, 2 high level QCs and up to 81 unknown urine samples.

a. Sample preparation

1) Preliminary sample preparation steps

- a) Allow frozen urine samples, quality control materials, and calibration standards to reach ambient temperature.
- b) Mix samples thoroughly by vortexing.
- c) Set up and label a series of 1.5-mL autosampler vials corresponding to the number of blanks, standards, QCs and samples to be analyzed.

2) Preparation of standards (1-10)

- a) Using a 1000- μ L and a 100- μ L pipettor add 15 mM ammonium acetate (as given in **Table 10**) into the appropriately-marked autosampler vials.
- b) Using micro pipettors transfer aliquots of appropriate working mixed standard solution (as given in **Table 10**) into the marked autosampler vials.
- c) Using a 100- μ L pipettor add 25 μ L working mixed internal standard to vials to make the final volume to 0.5 mL.
- d) Cap the vial and mix thoroughly for approximately 20 sec using a vortex mixer.

Table 10. Typical calibration standard preparation.

Working Mix Standard	A									B
Calibrator ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10
Mixed std μL	1	2	5	10	25	50	100	200	400	80
Mixed IS μL	25	25	25	25	25	25	25	25	25	25
15 mM Ammonium Acetate μL	474	473	475	465	450	425	375	275	75	395

Note: STD1 – STD10 are named as SSMMYY01 – SSMMYY10. MMY Y represents the two digit month followed by the last two digit of the year.

3) Preparation of the blank

- a) Using a 1000- μL pipettor transfer 475 μL of 15 mM ammonium acetate into the appropriately marked autosampler vial.
- b) Using a 100- μL pipettor add 25 μL of the working mixed internal standard solution to make a final volume of 0.5 mL.
- c) Cap the vial and mix for approximately 20 sec using a vortex mixer.

4) Preparation of the low quality control sample

- a) Using a 100- μL pipettor transfer 50 μL of the QC Low stock solution into the appropriately marked autosampler vial.
- b) Using a 100- μL pipettor add 25 μL of the working mixed internal standard solution.
- c) Using a 1000- μL pipettor add 425 μL of the 15 mM ammonium acetate to make a final volume of 0.5 mL.
- d) Cap the vial and mix thoroughly for approximately 20 sec using a vortex mixer.

5) Preparation of the high quality control sample

- a) Using a 100- μL pipettor transfer 50 μL of the QC High stock solution into the appropriately marked autosampler vial.
- b) Using a 100- μL pipettor add 25 μL of the working mixed internal standard solution.
- c) Using a 1000- μL pipettor add 425 μL of the 15 mM ammonium acetate to make a final volume of 0.5 mL.

- d) Cap the vial and mix thoroughly for approximately 20 sec using a vortex mixer.
- 6) Preparation of the unknown specimens
- a) Mix the unknown by vortexing for a few minutes.
 - b) Using a 100- μ L pipettor aliquot 50 μ L of unknown into the autosampler vial.
 - c) Using a 100- μ L pipettor add 25 μ L of the working mixed internal standard solution.
 - d) Using a 1000- μ L pipettor add 425 μ L of 15 mM ammonium acetate to make a final volume of 0.5 mL.
 - e) Cap the vial and mix thoroughly for approximately 20 sec using a vortex mixer.

b. Instrument and software setup for the LC-MS/MS

1) Preliminary system setup

- a) Tuning and calibration of the mass spectrometer
 - i. Set the y-distance of the probe to 6 mm and infuse the PPG negative solution at a flow rate of 10 μ L/min.
 - ii. Using **Manual Tuning**, load the PPG negative calibration file. In the tuning window make sure that the mass spectrometer is showing peaks for each ion in the calibration file. This is to make sure that the tuning solution is constantly flowing into the mass spectrometer.
 - iii. Once checked, perform a manual optimization with calibration upon success.
 - iv. Make sure that the following specified parameters are met. For peak width, the resolution is set to 0.8 ± 0.05 mass units and sensitivity is met using the ion 933 m/z with an intensity of **4.0×10^7 minimum (at Q1)** and **4×10^7 (at Q3)** (combined intensity of 50 milliscans).
 - v. Check the tune and mass calibration of the instrument weekly.
- b) LC system setup
 - i. Fill the mobile phase bottles (**Solvent A** bottle with 15 mM ammonium acetate, **Solvent B** bottle with acetonitrile, Weak wash bottle with HPLC grade water, Strong wash bottle with a mixture of 25% HPLC grade water + 25% Optima LCMS grade methanol + 25% Optima LCMS grade isopropyl alcohol + 25% Optima LCMC grade acetonitrile).
 - ii. Replace the filter inside the guard column when needed.

- iii. Operate the system purge before every run (go to Acquity Console, click Acquity UPLC System → go to “Control” and then click “Start up system” and press “start” (10 min, 7 cycles). This will prime the tubing, injector, wash the needle and seals.
- iv. After the purge, let mobile phase run through the column for 10 min at a flow rate of 0.25 mL/min.

c) Mass spec set up

- i. Clean the curtain plate (both sides) with DI water first and then with methanol before every run.

d) Performance evaluation

- i. Allow the system to equilibrate with the method to be run (both MS and LC).
- ii. To check the performance of the system, inject a standard three times to ensure equilibration of the system.
- iii. Examine the peak to ensure an acceptable signal-to-noise ratio ($S/N > 10$ for the lowest standard).
- iv. Once these limits are met the system is ready to start a run.

2) Final setup and operation

a) Create the run sequence

- i. Create the run sequence in ATLAS. The .csv file generated provides sequence information of the blanks, standards, QCs, and unknowns to be analyzed.

b) Assign the acquisition and quantitation methods

- i. Import the .csv file obtained from ATLAS into Analyst.
- ii. Select the acquisition method (*Immddy.dam*; where *mmddy* is the most recent date that the method was changed and / or saved) and the quantitation method (*Immddy.qmf*; where *mmddy* is the most recent date that the method was changed and / or saved).
- iii. The letter “I” before the methods name (*mmyy*) correspond to the first letter of the instrument name (S for Shorty).
- iv. Ensure that the icons on the right corner of the window are green both for LC and MS, indicating that the system has equilibrated and is ready to start.

c) Submit and start batch in Analyst

- i. Open and submit the **Equilibration** batch as well as the batch of the unknowns to be analyzed.
- ii. Press the “Start Sample” icon on top of the window to start the run.
- iii. The system will immediately start by turning green on the first sequence to run.

3) System shutdown

After the end of an analytical run flush the system with 50%:50% (Solvent A: Solvent B) to eliminate any salt residue and matrix accumulation (Shutdown.dam). After flushing, the system shuts down the LC instrument as well as the MS.

c. **Processing of data**

- 1) Using the quantitation capabilities of the Analyst Software, create a result file from raw data files and quantify the unknowns. The peaks are automatically integrated using the quantitation method created for the analysis.
- 2) Visually review the integration of each peak and manually correct when needed.
- 3) Generate a calibration curve from the calibrators; QCs, unknowns and blanks are quantified against the calibration curve.
- 4) Save the reviewed data files in a report file and export as a text file.
- 5) Open the text file in the Excel file mcrSCIEX.xls available on the Q drive and run the file through the macro “mcrFormatResults”.
- 6) Save the data as an Excel file and import it into the ATLAS VOC Metabolite database for further evaluation, including the QC evaluation described in Section 10.b.2.

9. **Reportable Range of Results**

a. **Linearity limits**

The reportable range of results for all the analytes using this method are given in **Table 11**. The urine LODs are 10 times the analytical LODs for the assay (samples are diluted at 1:10). 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 reassayed by diluting it in 15 mM ammonium acetate.

b. **Limit of detection**

The analytical limit of detection was calculated as described by Taylor (30). We extrapolated to the standard deviation at zero concentration based on the standard deviations of repeated measures of the lowest four calibrators. The intercept of the least squares fit of this line equals S_0 ; $3S_0$ equals the limit of detection (LOD).

c. Accuracy

The accuracy of the assay was established by analyzing Proficiency Testing (PT) samples blind to the analyst and by spiking urine at three different levels of VOC metabolites. As no certified reference materials are commercially available, the PT samples (4 dilutions) were prepared from the original stock solution, and the accuracy of the method is obtained by comparing the concentration calculated from analyzing the samples to the theoretical concentration. The results of PT measurements are given in **Table 12**, and **Table 13** presents the spike recovery data

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 over a one month period (**Table 14**).

Table 11. Typical calibration range and urine LODs.

Analyte STD	Calibration Range ng/mL	r	Urine LOD ng/mL
AAMA	0.11 - 109	0.999	2.20
AMCC	0.36 - 277	0.999	6.26
ATCA	0.89 - 357	0.999	15.0
BMA	0.04 - 44	0.996	0.500
BPMA	0.08 - 61	0.997	1.20
CEMA	0.60 - 240	0.999	6.96
CYMA	0.04 - 126	0.999	0.500
1,2DCVMA	0.94 - 378	0.993	12.6
2,2DCVMA	0.39 - 156	0.997	4.70
DHBMA	0.40 - 400	0.998	5.25
DPMA	0.02 - 9.00	0.998	0.500
GAMA	0.59 - 236	0.999	9.40
HEMA	0.04 - 24	0.999	0.791
HPMA	1.3 - 1296	0.999	13.0
2HPMA	0.26 - 1057	0.999	5.30
HPMMA	0.3 - 973	0.999	2.00
MA	1.2 - 480	0.999	12.0
2-MHA	0.31 - 276	0.998	5.00
3-MHA + 4-MHA	0.62 - 275	0.997	8.00
MHBMA1	0.05 - 18	0.999	0.700
MHBMA2	0.05 - 19	0.999	0.700
MHBMA3	0.06 - 44	0.999	0.600
PHEMA	0.05 - 20	0.998	0.700
PGA	1.0 - 806	0.999	12.0
PMA	0.03 - 13.8	0.996	0.600
TCVMA	0.15 - 60	0.997	3.00
TTCA	0.34 - 268	0.999	11.2

e. Analytical specificity

LC-MS/MS is a highly selective analytical method for quantifying the target analytes in complex aqueous matrices. Reverse phase liquid chromatography reproducibly resolves the target analytes, even in the most concentrated urine samples. The analyte peaks elute in well defined regions of the chromatogram with no visible interferences and very low background. 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 are determined by comparing the responses of the quantitation ion and the confirmation ion transitions over the standard and QC samples. The average value of this ratio $\pm 20\%$ is used to confirm the analyte determined in unknown samples.

Table 12. Typical method accuracy and precision (proficiency testing).

Analyte	PT	Theoretical (ng/mL)	Calculated Mean (ng/mL)	\pm SD ^a (ng/mL)	% Accuracy ^b
AAMA	PT1	0.27	0.30	0.00	111%
	PT2	1.36	1.29	0.01	95%
	PT3	8.68	8.73	0.13	101%
	PT4	21.7	20.80	0.28	96%
AMCC	PT1	0.55	0.65	0.01	118%
	PT2	2.77	2.80	0.23	101%
	PT3	13.9	13.90	0.71	100%
	PT4	55.4	59.7	2.62	108%
ATCA	PT1	1.84	2.13	0.03	116%
	PT2	7.81	9.12	0.16	117%
	PT3	39.0	39.4	1.48	101%
	PT4	112	119	0.71	106%
BMA	PT1	0.08	0.07	0.00	88%
	PT2	0.28	0.23	0.02	82%
	PT3	2.75	2.59	0.24	94%
	PT4	7.70	6.82	0.25	89%
BPMA	PT1	0.12	0.13	0.01	108%
	PT2	0.61	0.63	0.02	103%
	PT3	3.07	3.19	0.01	104%
	PT4	12.3	10.8	0.21	88%
CEMA	PT1	1.00	1.12	0.07	112%
	PT2	5.00	4.65	0.29	93%
	PT3	40.0	38.9	3.32	97%
	PT4	120	119	2.12	99%
CYMA	PT1	0.08	0.08	0.00	100%
	PT2	0.47	0.53	0.04	113%
	PT3	2.37	2.40	0.28	101%
	PT4	6.32	6.07	0.05	96%
1,2DCVMA	PT1	1.89	1.83	0.11	97%
	PT2	6.30	7.48	1.25	119%
	PT3	31.5	32.9	1.70	105%
	PT4	126	149	12.0	118%
2,2DCVMA	PT1	0.81	0.81	0.05	100%
	PT2	3.24	3.85	0.10	119%
	PT3	16.2	19.0	0.71	117%
	PT4	64.8	75.2	4.10	116%
DHBMA	PT1	0.80	0.84	0.17	105%
	PT2	2.50	2.45	0.03	98%
	PT3	25.0	24.5	0.14	98%
	PT4	80.0	73.9	2.76	92%
DPMA	PT1	0.07	0.08	0.01	114%
	PT2	0.33	0.35	0.01	106%
	PT3	2.40	2.25	0.13	94%
	PT4	4.53	4.58	0.13	101%

GAMA	PT1	0.94	1.10	0.15	117%
	PT2	4.72	4.74	0.08	100%
	PT3	47.2	46.7	0.28	99%
	PT4	94.5	97.3	2.4	103%
HEMA	PT1	0.08	0.09	0.00	113%
	PT2	0.38	0.42	0.05	111%
	PT3	3.80	4.24	0.07	112%
	PT4	6.08	7.11	0.05	117%
HPMA	PT1	2.59	3.1	0.23	120%
	PT2	6.48	7.12	0.18	110%
	PT3	77.8	72.9	1.27	94%
	PT4	259	245	4.95	94%
2HPMA	PT1	0.92	0.93	0.20	101%
	PT2	6.61	6.74	0.69	102%
	PT3	26.4	28.9	0.85	109%
	PT4	79.3	86.5	1.98	109%
HPMMA	PT1	0.61	0.63	0.01	103%
	PT2	1.52	1.48	0.06	97%
	PT3	12.2	12.5	0.42	103%
	PT4	38.0	36.5	0.42	96%
MA	PT1	2.40	2.08	0.08	87%
	PT2	7.01	7.85	0.76	112%
	PT3	40.0	46.7	0.57	117%
	PT4	200	234	3.54	117%
2MHA	PT1	0.65	0.58	0.01	89%
	PT2	2.51	2.42	0.14	96%
	PT3	10.0	9.67	0.61	96%
	PT4	50.2	53.1	0.99	106%
3MHA + 4MHA	PT1	0.81	0.85	0.07	105%
	PT2	4.04	4.25	0.15	105%
	PT3	20.2	21.1	1.06	104%
	PT4	101	115	0.71	114%
MHBMA1	PT1	0.08	0.08	0.01	100%
	PT2	0.41	0.38	0.00	93%
	PT3	2.74	2.60	0.02	95%
	PT4	7.30	7.20	0.02	99%
MHBMA2	PT1	0.08	0.07	0.00	88%
	PT2	0.41	0.42	0.00	102%
	PT3	2.88	3.02	0.04	105%
	PT4	7.68	8.02	0.22	104%
MHBMA3	PT1	0.11	0.10	0.00	91%
	PT2	0.94	0.90	0.02	96%
	PT3	6.60	5.53	0.05	84%
	PT4	17.6	14.9	0.07	84%
PGA	PT1	1.21	1.44	0.06	119%
	PT2	7.05	7.25	0.34	103%
	PT3	40.3	40.7	1.13	101%
	PT4	201	212	0.00	105%

PHEMA	PT1	0.07	0.06	0.02	86%
	PT2	0.35	0.34	0.01	97%
	PT3	1.50	1.40	0.01	93%
	PT4	8.00	6.72	0.10	84%
PMA	PT1	0.07	0.08	0.00	114%
	PT2	0.28	0.31	0.01	111%
	PT3	2.76	2.52	0.03	91%
	PT4	5.52	5.62	0.30	102%
TCVMA	PT1	0.30	0.31	0.04	103%
	PT2	1.51	1.60	0.05	106%
	PT3	7.55	7.90	0.74	105%
	PT4	22.7	24	0.14	106%
TTCA	PT1	0.67	0.64	0.11	96%
	PT2	2.01	1.75	0.02	87%
	PT3	20.1	19.9	0.71	99%
	PT4	53.6	47.45	0.64	89%

^aStandard deviation.

^bPercentage difference between theoretical and calculated amount.

Table 13. Typical spike recovery data – urine spiked at three different levels of VOC metabolites.

Analyte	Spiked Conc. in Urine ng/mL			Mean Spike Recovery (%) ± SD ^a ng/mL		
	Level I	Level II	Level III	Level I	Level II	Level III
AAMA	21.7	109	217	98 ± 11	103 ± 6	104 ± 7
AMCC	27.7	139	277	100 ± 10	100 ± 9	99 ± 6
ATCA	66.9	335	669	94 ± 8	97 ± 8	101 ± 10
BMA	3.96	19.8	40	96 ± 10	95 ± 13	99 ± 14
BPMA	30.7	154	307	91 ± 7	91 ± 5	102 ± 5
CEMA	60.0	300	600	98 ± 11	103 ± 11	95 ± 2
CYMA	3.16	15.8	32	99 ± 4	97 ± 3	99 ± 9
1DCVMA	63.0	315	630	96 ± 7	97 ± 6	95 ± 5
2DCVMA	32.4	162	324	93 ± 9	95 ± 7	99 ± 7
DHBMA	40.0	200	400	98 ± 6	106 ± 9	101 ± 4
DPMA	1.28	65.0	130	94 ± 3	97 ± 3	97 ± 2
GAMA	47.2	236	472	97 ± 12	103 ± 7	105 ± 5
HEMA	3.04	15.2	30	93 ± 13	96 ± 7	98 ± 5
HPMA	130	648	1296	96 ± 4	108 ± 4	107 ± 7
2HPMA	66.1	165	661	84 ± 5	83 ± 2	86 ± 3
HPMMA	15.2	76	152	96 ± 13	96 ± 12	100 ± 8
MA	100	501	1001	95 ± 11	98 ± 8	94 ± 8
2MHA	25	126	251	98 ± 8	91 ± 2	88 ± 6
3MHA+4MHA	101	503	1000	101 ± 4	101 ± 9	108 ± 7
MHBMA1	6.84	34.2	68	89 ± 11	94 ± 4	85 ± 2
MHBMA2	7.2	36	72	102 ± 5	105 ± 4	105 ± 4
MHBMA3	55.0	138	550	87 ± 1	86 ± 1	86 ± 3
PGA	101	504	1007	104 ± 7	105 ± 4	105 ± 4
PHEMA	20	100	200	99 ± 7	101 ± 9	101 ± 6
PMA	2.76	13.8	28	97 ± 9	98 ± 6	100 ± 7
TTCA	40.2	201	402	98 ± 7	102 ± 3	99 ± 9
TCVMA	15.1	75.5	151	93 ± 7	105 ± 4	97 ± 5

^aStandard deviation.

Table 14. Typical QC characterization statistics for VOC metabolites.

Analyte ID	QC ID	Mean ng/mL	± SD ^a ng/mL	CV ^b	LCL3 ^c ng/mL	LCL2 ^d ng/mL	UCL2 ^d ng/mL	UCL3 ^c ng/mL
AAMA	QH0811	183.02	9.16	5.01%	153.51	163.41	202.64	212.53
AAMA	QL0811	33.46	1.66	4.96%	28.12	29.91	37.01	38.8
AMCA	QH0811	494.14	23.17	4.69%	419.53	444.56	543.73	568.75
AMCA	QL0811	46.07	1.98	4.30%	39.69	41.83	50.31	52.45
ATCA	QH0811	1,175.48	49.14	4.18%	1,017.24	1,070.32	1,280.64	1,333.71
ATCA	QL0811	72.34	4.8	6.63%	56.89	62.07	82.61	87.79
BMA	QH0811	83.9	5.35	6.37%	66.68	72.46	95.35	101.13
BMA	QL0811	14.48	0.92	6.32%	11.54	12.52	16.44	17.43
BPMA	QH0811	94.19	4.08	4.33%	81.04	85.45	102.93	107.34
BPMA	QL0811	9.34	0.72	7.71%	7.02	7.8	10.88	11.66
CEMA	QH0811	1,115.00	110.37	9.90%	759.62	878.82	1,351.18	1,470.38
CEMA	QL0811	123.11	9.76	7.92%	91.7	102.23	143.99	154.52
CYMA	QH0811	66.4	2.99	4.50%	56.77	60	72.8	76.03
CYMA	QL0811	10.34	0.77	7.47%	7.85	8.69	11.99	12.82
1DCVMA	QH0811	1,006.67	96.57	9.59%	695.7	800	1,213.33	1,317.63
1DCVMA	QL0811	115.8	7.88	6.81%	90.42	98.93	132.66	141.17
2DCVMA	QH0811	578.17	49.48	8.56%	418.85	472.29	684.05	737.48
2DCVMA	QL0811	62.19	4.52	7.28%	47.62	52.5	71.87	76.76
DHBMA	QH0811	813.48	60.25	7.41%	619.47	684.54	942.41	1,007.48
DHBMA	QL0811	136.6	9.13	6.68%	107.19	117.06	156.13	166
DPMA	QH0811	40.17	1.55	3.85%	35.19	36.86	43.48	45.16
DPMA	QL0811	4.57	0.21	4.52%	3.91	4.13	5.02	5.24
GAMA	QH0811	970.07	44.16	4.55%	827.88	875.57	1,064.57	1,112.27
GAMA	QL0811	123.52	6.2	5.02%	103.56	110.26	136.79	143.49
HEMA	QH0811	130.94	5.75	4.39%	112.43	118.64	143.24	149.45
HEMA	QL0811	25.6	2.18	8.50%	18.59	20.94	30.25	32.6
HPMA	QH0811	1,491.19	110.52	7.41%	1,135.31	1,254.68	1,727.70	1,847.07
HPMA	QL0811	116.68	8.26	7.08%	90.07	99	134.36	143.28
2HPMA	QH0811	941.36	61.78	6.56%	742.41	809.14	1,073.58	1,140.30
2HPMA	QL0811	67.47	5.72	8.47%	49.07	55.24	79.7	85.87
HPMMA	QH0811	569.62	16.89	2.96%	515.25	533.48	605.76	623.99
HPMMA	QL0811	126.1	4.42	3.50%	111.87	116.64	135.55	140.32
MA	QH0811	2,112.86	94.85	4.49%	1,807.44	1,909.88	2,315.84	2,418.27
MA	QL0811	248.62	13.63	5.48%	204.75	219.46	277.78	292.49
2MHA	QH0811	569.17	29.47	5.18%	474.28	506.11	632.23	664.05
2MHA	QL0811	66.42	2.74	4.13%	57.6	60.56	72.29	75.25
3MHA + 4MHA	QH0811	1,119.55	46.41	4.15%	970.1	1,020.23	1,218.87	1,268.99
3MHA + 4MHA	QL0811	179.17	7.87	4.39%	153.82	162.32	196.01	204.51
MHBMA1	QH0811	72.5	2.23	3.07%	65.33	67.73	77.27	79.68
MHBMA1	QL0811	8.56	0.25	2.96%	7.74	8.02	9.1	9.37
MHBMA2	QH0811	79.8	2.72	3.41%	71.03	73.97	85.63	88.57

MHBMA2	QL0811	9.83	0.43	4.37%	8.45	8.91	10.75	11.22
MHBMA3	QH0811	182.62	6.57	3.60%	161.46	168.56	196.68	203.78
MHBMA3	QL0811	24.04	1.28	5.34%	19.91	21.3	26.79	28.17
PHEMA	QH0811	80.98	3.95	4.88%	68.26	72.53	89.43	93.7
PHEMA	QL0811	21.82	1.24	5.69%	17.82	19.16	24.48	25.82
PGA	QH0811	1,720.95	72.83	4.23%	1,486.44	1,565.10	1,876.81	1,955.46
PGA	QL0811	164.45	6.08	3.70%	144.87	151.44	177.47	184.04
PMA	QH0811	55.46	4.26	7.67%	41.76	46.35	64.56	69.16
PMA	QL0811	11.02	0.63	5.71%	8.99	9.67	12.36	13.04
TCVMA	QH0811	309.36	17.56	5.68%	252.8	271.77	346.94	365.91
TCVMA	QL0811	61.3	2.72	4.44%	52.53	55.47	67.12	70.06
TTCA	QH0811	524.31	25.67	4.90%	441.65	469.38	579.24	606.97
TTCA	QL0811	56.14	4.05	7.22%	43.09	47.47	64.81	69.19

^aStandard deviation. ^bCoefficient of variation. ^cMean \pm 3 σ (confidence limits: Lower - LCL3; Upper - UCL3).

^dMean \pm 2 σ (Lower: LCL2; Upper: UCL2), σ = standard deviation.

10. Quality Assessment and Proficiency Testing

a. Quality assessment

Quality assessment procedures follow standard practices (31). Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as 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 be linear with an R^2 value of at least 0.990. The results from the analysis of QC materials obtained using these calibration curves are compared with acceptance criteria given below to assure precision of the analysis.

b. Quality control procedures

1) Establishing QC limits

Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Two different pools of quality control material are used. Different variables are included in the characterization analyses (e.g. different analysts, columns, reagents) 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. Limits are based on statistical calculation accounting for 2 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 (31):

- a) If both the low and the high QC results are within the 2σ (σ – standard deviation) limits, then accept the run.
- b) If one of two QC results is outside the 2σ limits, then apply the rules below and reject the run if any condition is met.
 - i. **$1_{3\sigma}$** – Average of both low QC OR average of both high QC is outside of a 3σ limit.
 - ii. **$2_{2\sigma}$** – Average of both low QC AND average of both high QC is outside of 2σ limit on the same side of the mean.
 - iii. **$R_{4\sigma}$ sequential** – Average of both low QC AND average of both high QC is outside of 2σ limit on opposite sides of the mean.
 - iv. **10_x sequential** – The previous 9 average QC results (for the previous 9 runs) were on the same side of the mean.

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 were prepared from the original stock, diluted in water, and blind-coded by the in-house PT Coordinator. The samples are analyzed blind and the results evaluated by the in-house PT coordinator.

2) Frequency of PT

Four samples of unknown PT concentrations are analyzed 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; the analysis passes proficiency testing if $\geq 80\%$ of the results deviate $\leq 20\%$ 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 until the method 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 3σ limits for mean or range it fails the QC criteria described in section 10.b.2, then the following steps are taken.

- 1) If a particular calibration standard is obviously in error, remake a new dilution of that calibration standard (see section 8.a.2), reanalyze it, and reprocess the sample analyses using this new result as part of the calibration curve.
- 2) Prepare a fresh dilution of the failing QC material (working QC standard) (see sections 8.a.4 & 5) and re-analyze it.
- 3) Prepare calibration standards using a freshly prepared working mixed standard solution, and re-analyze the entire calibration curve using the freshly prepared calibration standards.

If these three 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. Due to excellent chromatographic and mass spectrometric resolution, we have not found any substances that have similar chromatographic and mass spectrometric characteristics. We evaluated 75 human urine samples and found no evidence of interferences with our quantitation transitions. In a small subset of samples (~5%), chromatography is distorted by urinary component(s); this problem is resolved by further diluting the sample and re-analyzing it.

13. Reference Ranges (Normal Values)

Reference ranges for smokers and non-smokers are presented in **Table 15**.

Table 15. 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	(32)
AMCC	5.0	38.9-498 µg/L 47.3- 449 µg/g creatinine	122-1453 µg/L 196-1153 µg/g creatinine	(32)
ATCA	25	85 ± 47	233 ± 237	(33)
BMA	0.02	2.4-81.4 µg/g creatinine	1.7-31.2 µg/g creatinine	(34)
CEMA	0.15	ND-94 µg/L ND-158 µg/g creatinine	29-1240 µg/L ND-744 µg/g creatinine	(35)
CYMA	0.50	<1.0-21.3 µg/L	2.0-1382 µg/L	(36)
DHBMA	0.14	ND-329 µg/L ND-582 µg/g creatinine	113-1830 µg/L 166-1092 µg/g creatinine	(35)
HEMA	0.03	ND-1.44 µg/L ND-1.05 µg/g creatinine	ND-20.8 µg/L ND-16 µg/g creatinine	(35)
HPMA	0.20	ND-128 µg/L ND-245 µg/g creatinine	80.9-4030 µg/L 75-3678 µg/g creatinine	(35)
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	(36)
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	(35)

Some of the mercapturic acids are formed endogenously in human body. Most of the non-mercapturic metabolites are non-specific and could also be formed from multiple sources. AMCC and DHBMA could be endogenous (37, 38). AMCC can be formed in two ways. The first possibility is the dietary intake of isothiocyanates, especially methyl isothiocyanate, which is a component of wine and cruciferous vegetables (such as cabbage, turnips and cress). The other possibility is the physiological formation of AMCC *via* carbamoylation and methylation. DHBMA may be formed from endogenous butadiene-diol.

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 urinary VOC metabolites has not been established. Therefore there are no critical call values for VOC metabolites at this time. But the biological exposure indices (BEI) reported by ACGIH (39) for some of the VOC metabolites in this method are given in (Table 16) as the maximum values allowable in urine samples collected from workers.

Table 16. Biological exposure indices.

VOC Metabolite	BEI	Parent Compound
AMCC	40 mg/L	<i>N, N</i> dimethylformamide
DHBMA	2.5 mg/L	1,3-butadiene
2MHA+3MHA+4MHA	1.5 g/g creatinine	<i>o</i> -, <i>m</i> -, <i>p</i> - xylenes
MA + PGA	400 mg/g creatinine	styrene
PMA	25 µg/g creatinine	benzene
TTCA	5 mg/g creatinine	carbon disulfide

15. Specimen Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. If the measurement is delayed until the next day, freeze the prepped samples at $-20 \pm 5^{\circ}\text{C}$.

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

Alternate validated methods have not been evaluated for measuring VOC metabolites in urine. If the analytical system fails, freeze the samples at -20°C until the analytical system is restored to functionality.

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

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

Once the validity of the data is established by the QC/QA system outlined above, these results are verified by a DLS statistician, and the data reported in both hard copy and electronic copy. This data, a cover letter, and a table of method specifications and reference range values will 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 $-20 \pm 5^{\circ}\text{C}$ 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.

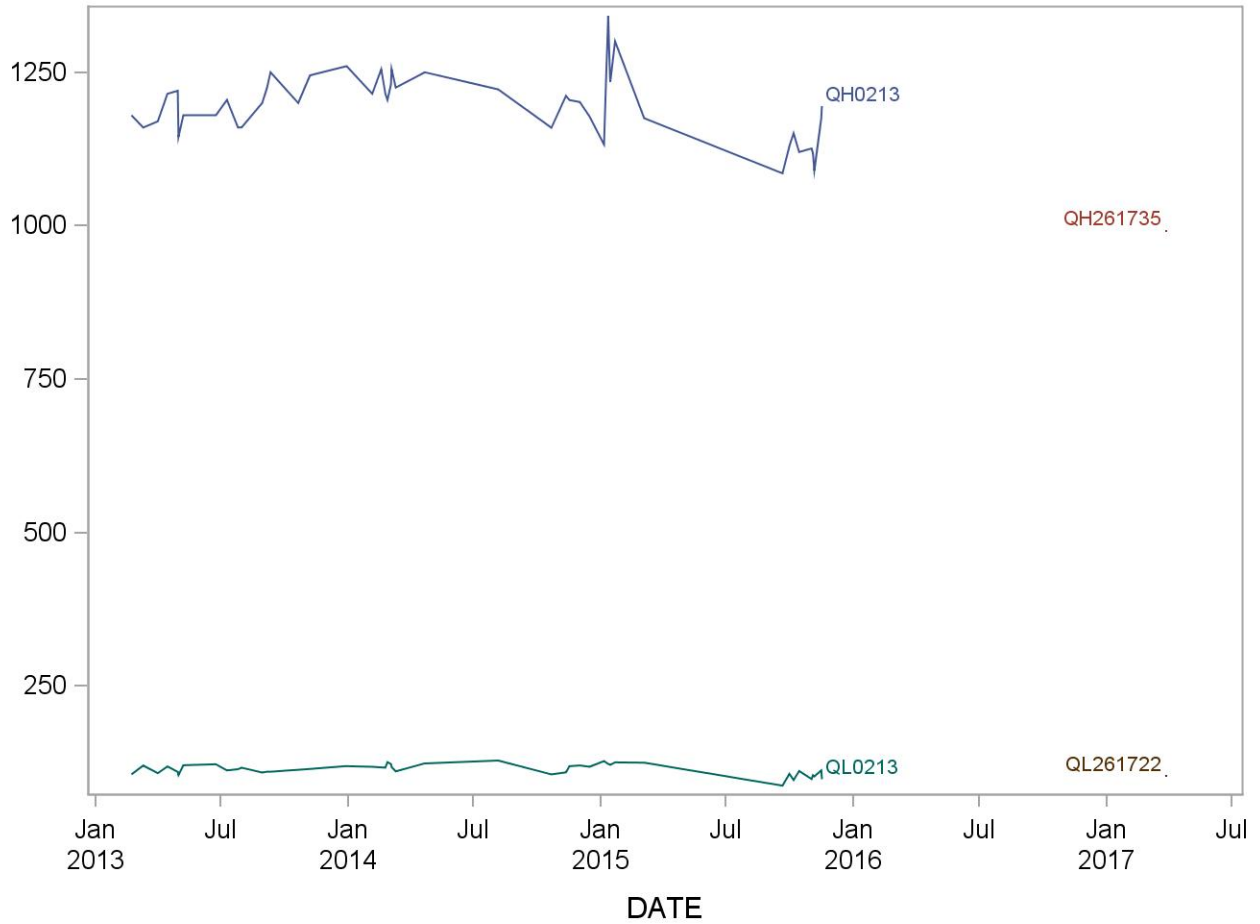
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers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

19. Summary Statistics and QC Graphs

See next pages.

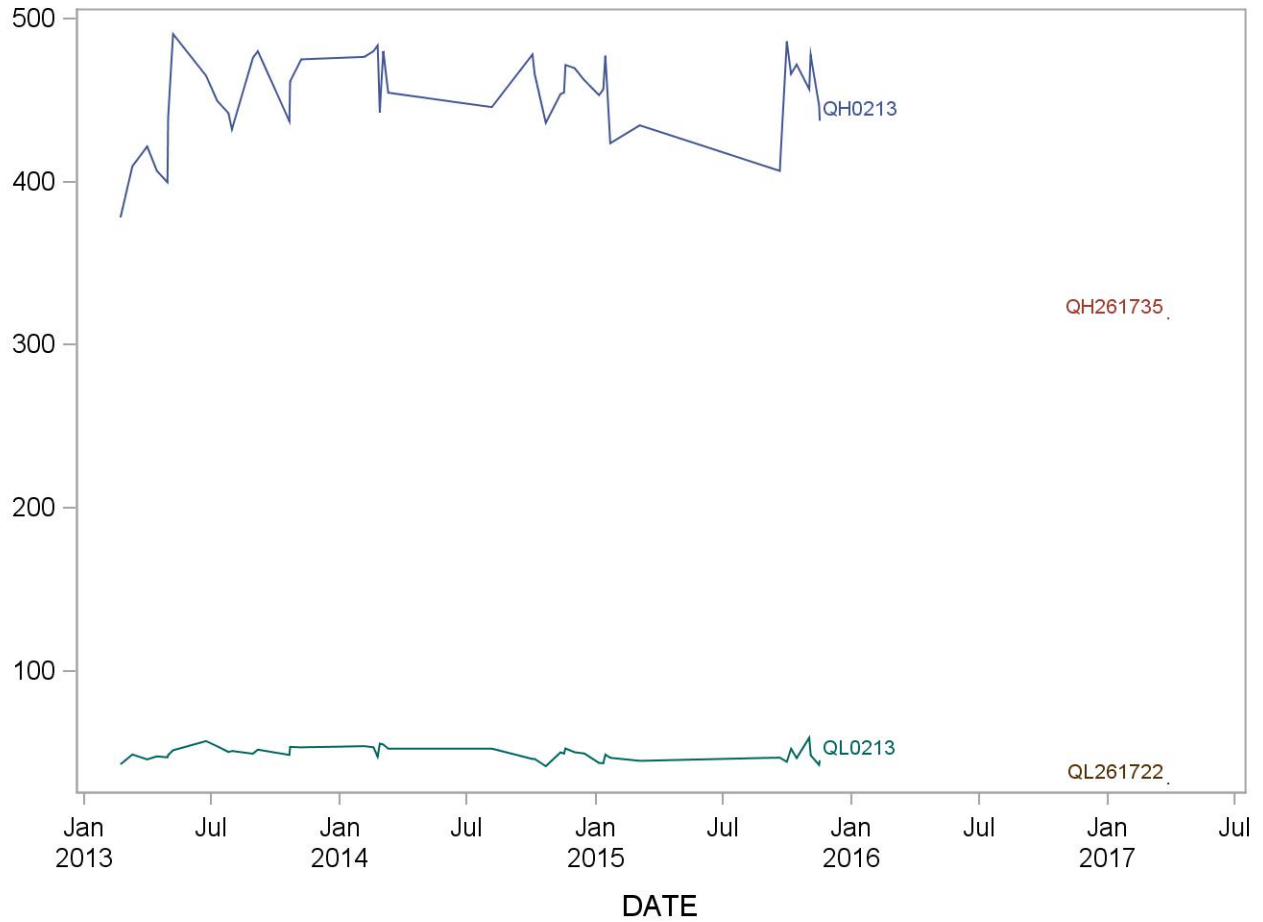
2013-2014 Summary Statistics and QC Chart for 2-aminothiazolne-4-carboxylic acid(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	45	22FEB13	17NOV15	1194.60	52.18	4.4
QL0213	45	22FEB13	17NOV15	114.09	9.05	7.9



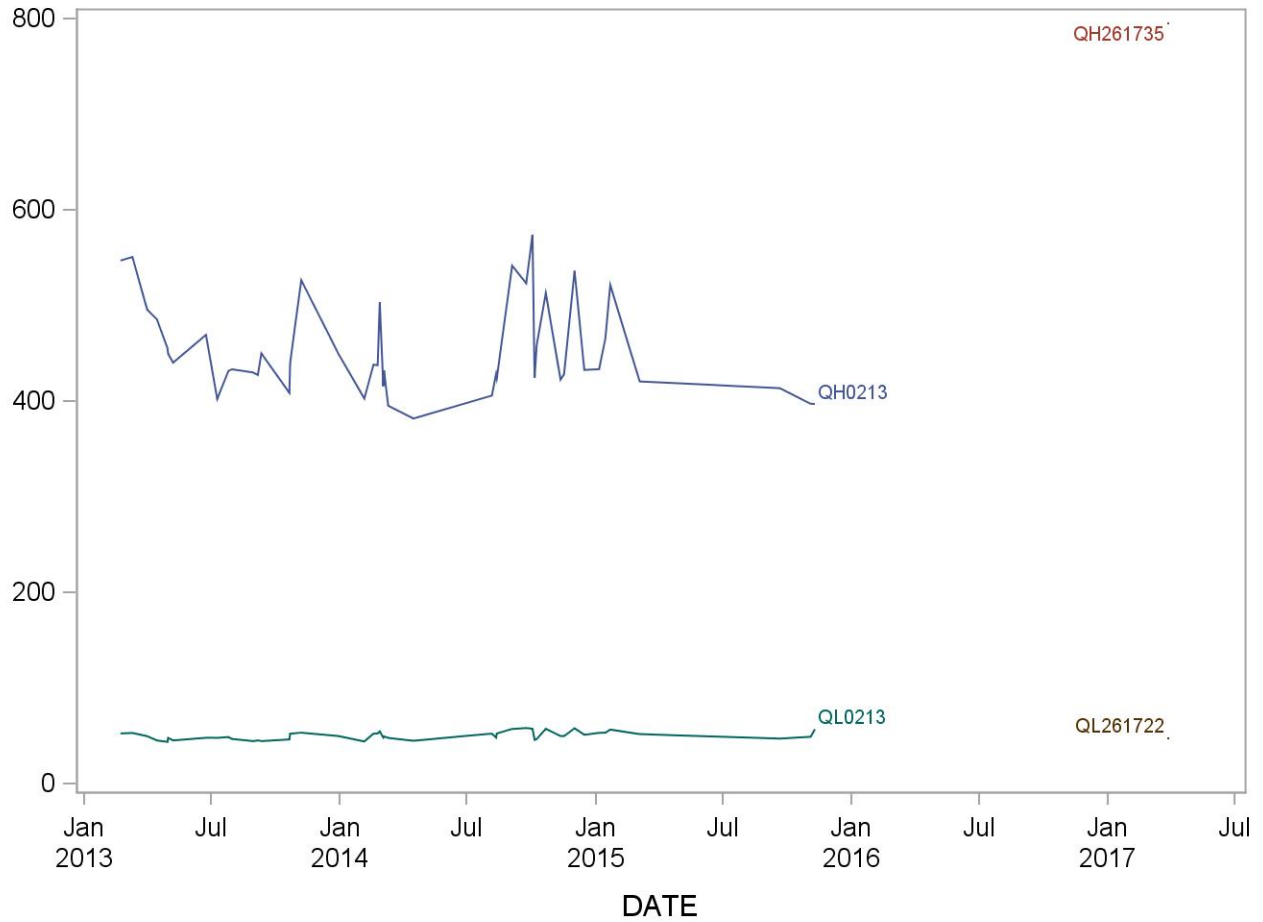
2013-2014 Summary Statistics and QC Chart for 2-methylhippuric acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	44	22FEB13	17NOV15	452.518	26.097	5.8
QL0213	44	22FEB13	17NOV15	49.023	4.069	8.3



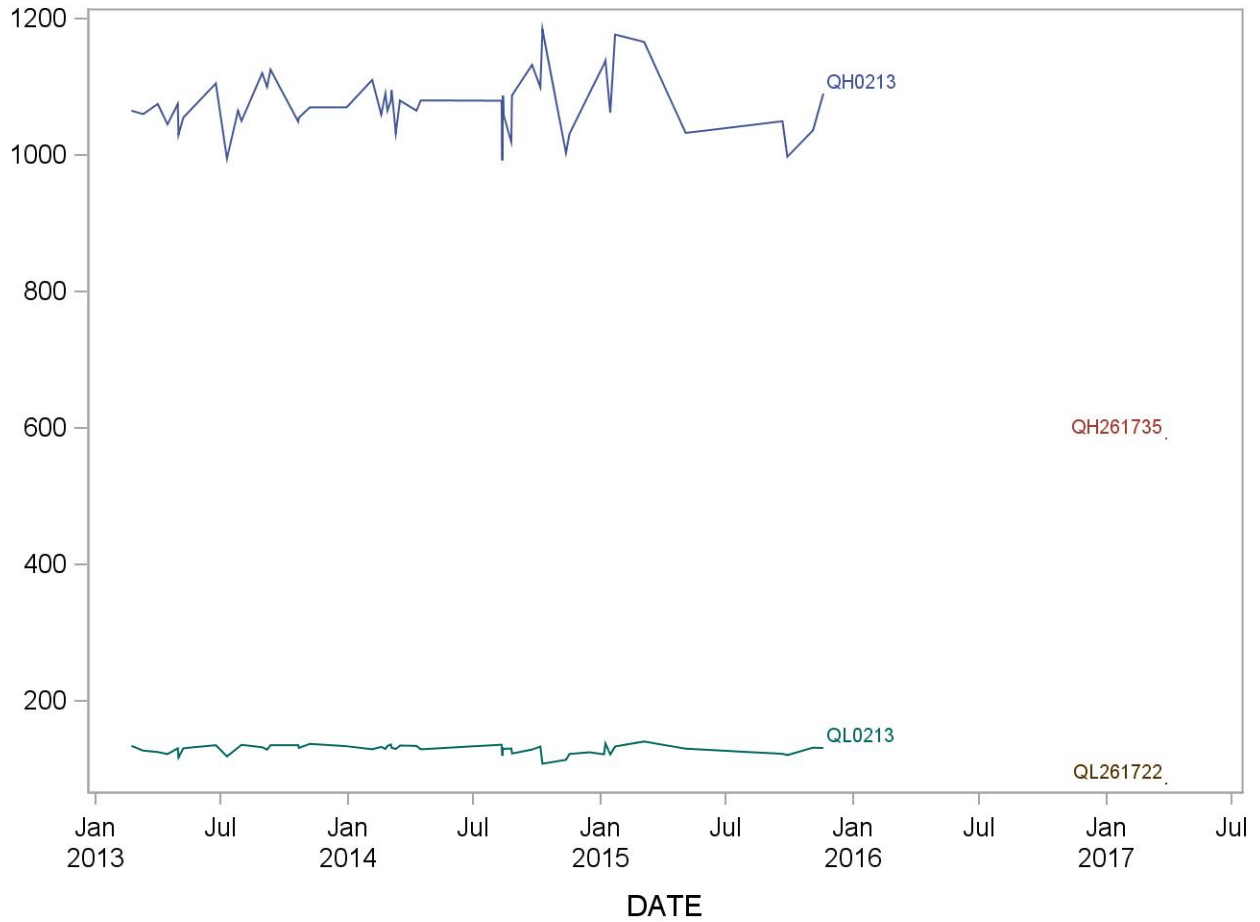
2013-2014 Summary Statistics and QC Chart for 2-thoxothazlidne-4-carboxylic acid(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	47	22FEB13	10NOV15	452.810	48.659	10.7
QL0213	47	22FEB13	10NOV15	50.204	4.174	8.3



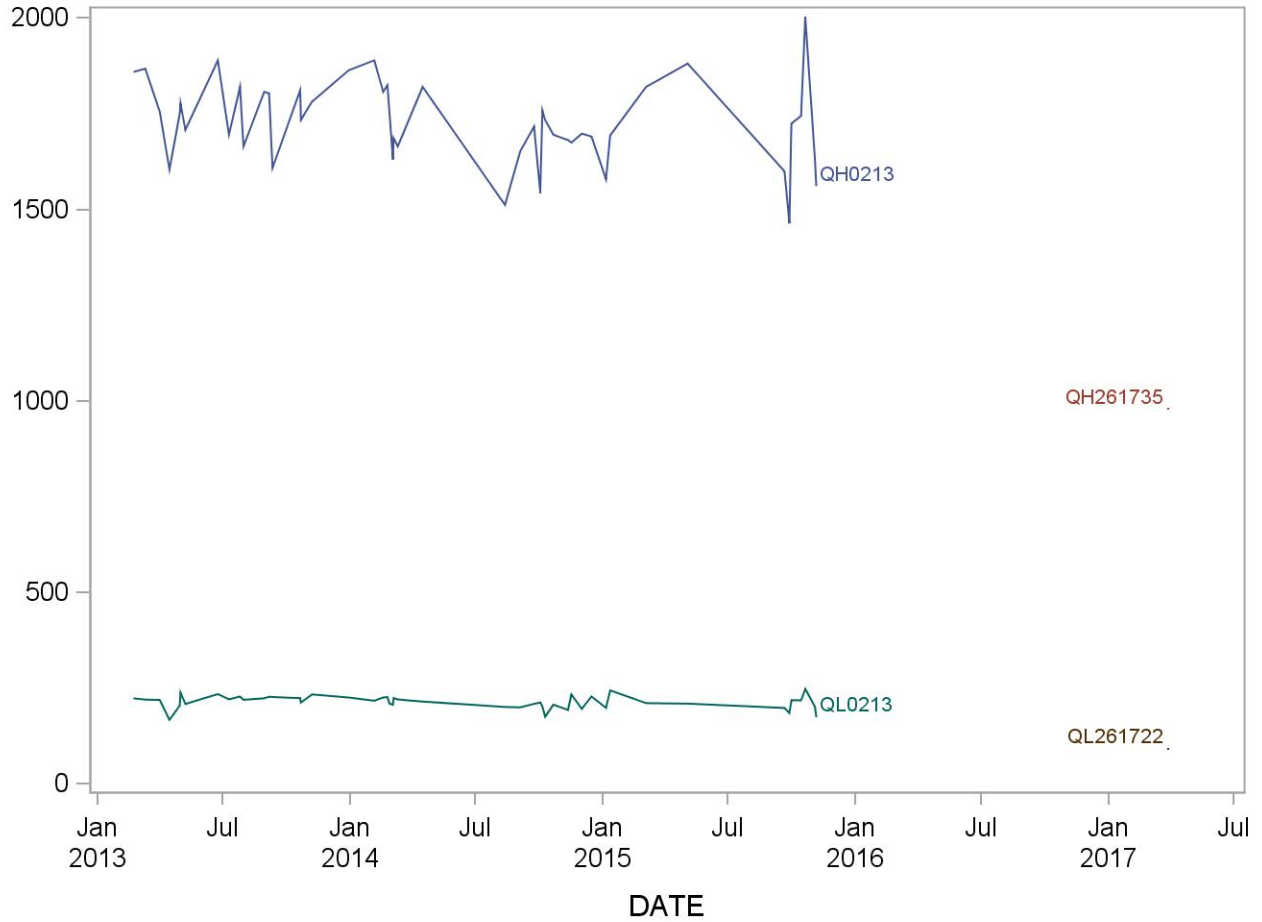
2013-2014 Summary Statistics and QC Chart for 3-methipurc acd & 4-methipurc acd(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	19NOV15	1074.223	43.379	4.0
QL0213	50	22FEB13	19NOV15	129.394	6.637	5.1



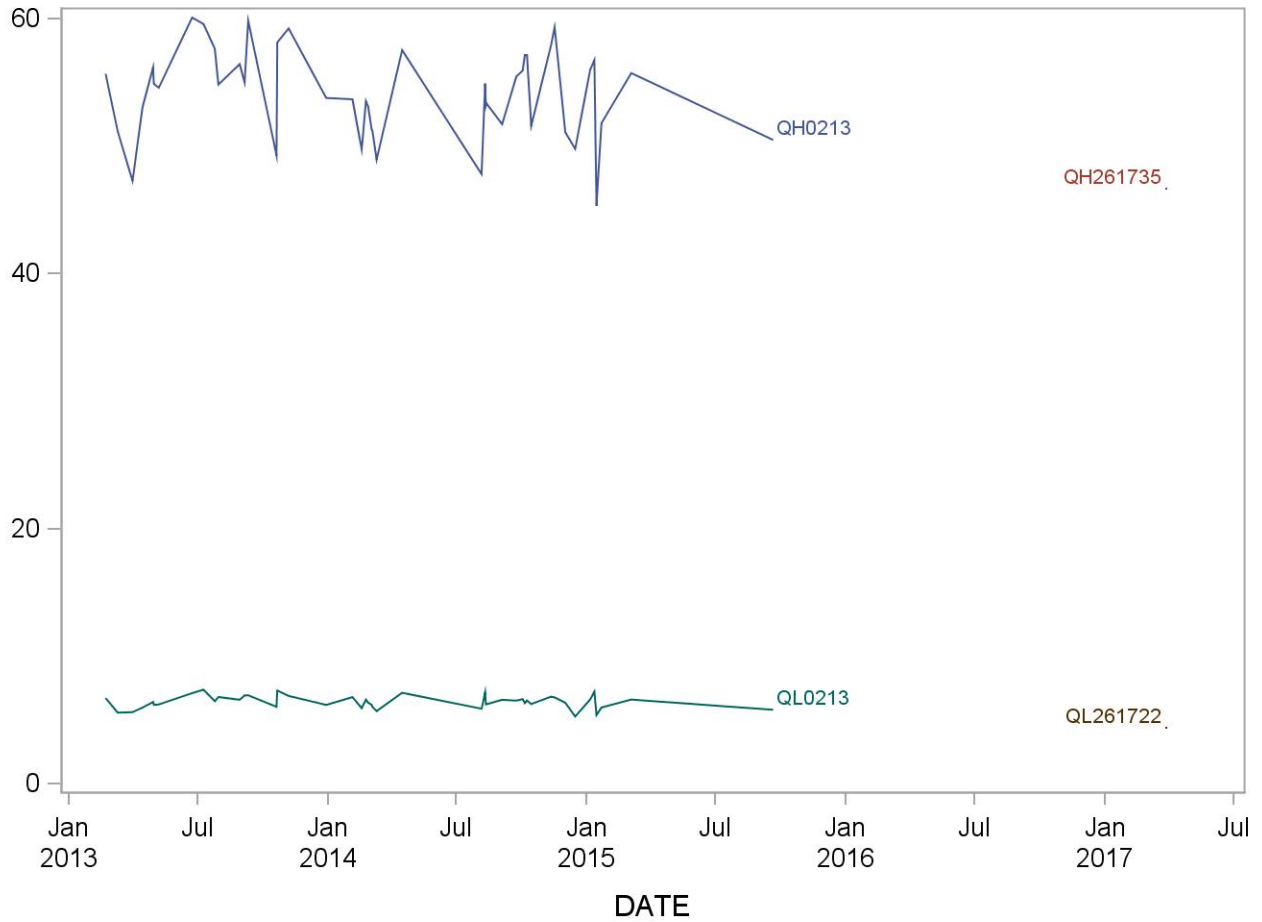
2013-2014 Summary Statistics and QC Chart for Mandelic acid(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	48	22FEB13	06NOV15	1727.59	109.96	6.4
QL0213	48	22FEB13	06NOV15	213.41	17.27	8.1



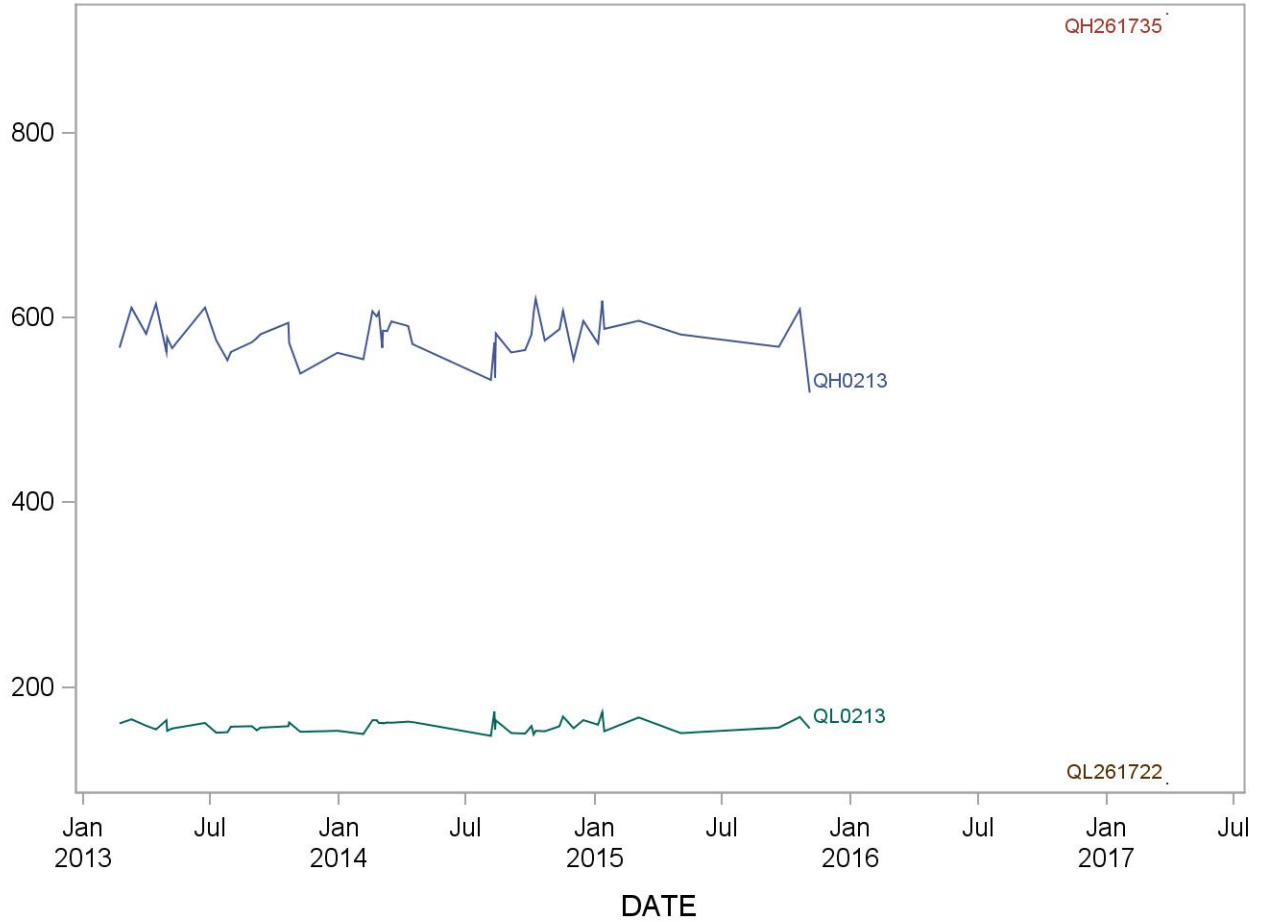
2013-2014 Summary Statistics and QC Chart for N-A-S-(1-hydrxmet)-2-Prpn)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	46	22FEB13	21SEP15	54.0592	3.5835	6.6
QL0213	46	22FEB13	21SEP15	6.4089	0.5098	8.0



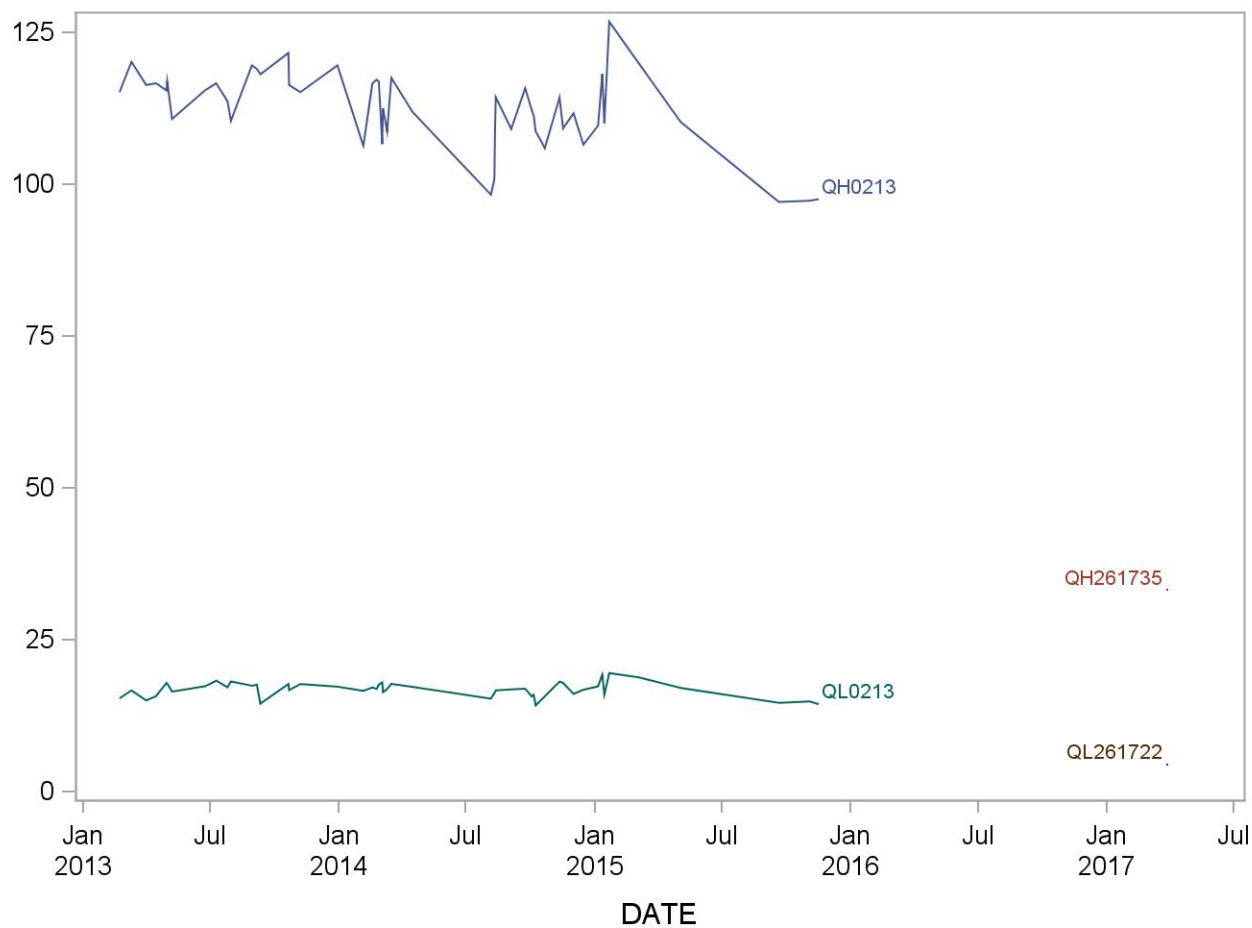
2013-2014 Summary Statistics and QC Chart for N-A-S-(3-hydrxprpl-1-metl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	04NOV15	579.815	22.866	3.9
QL0213	50	22FEB13	04NOV15	157.966	6.305	4.0



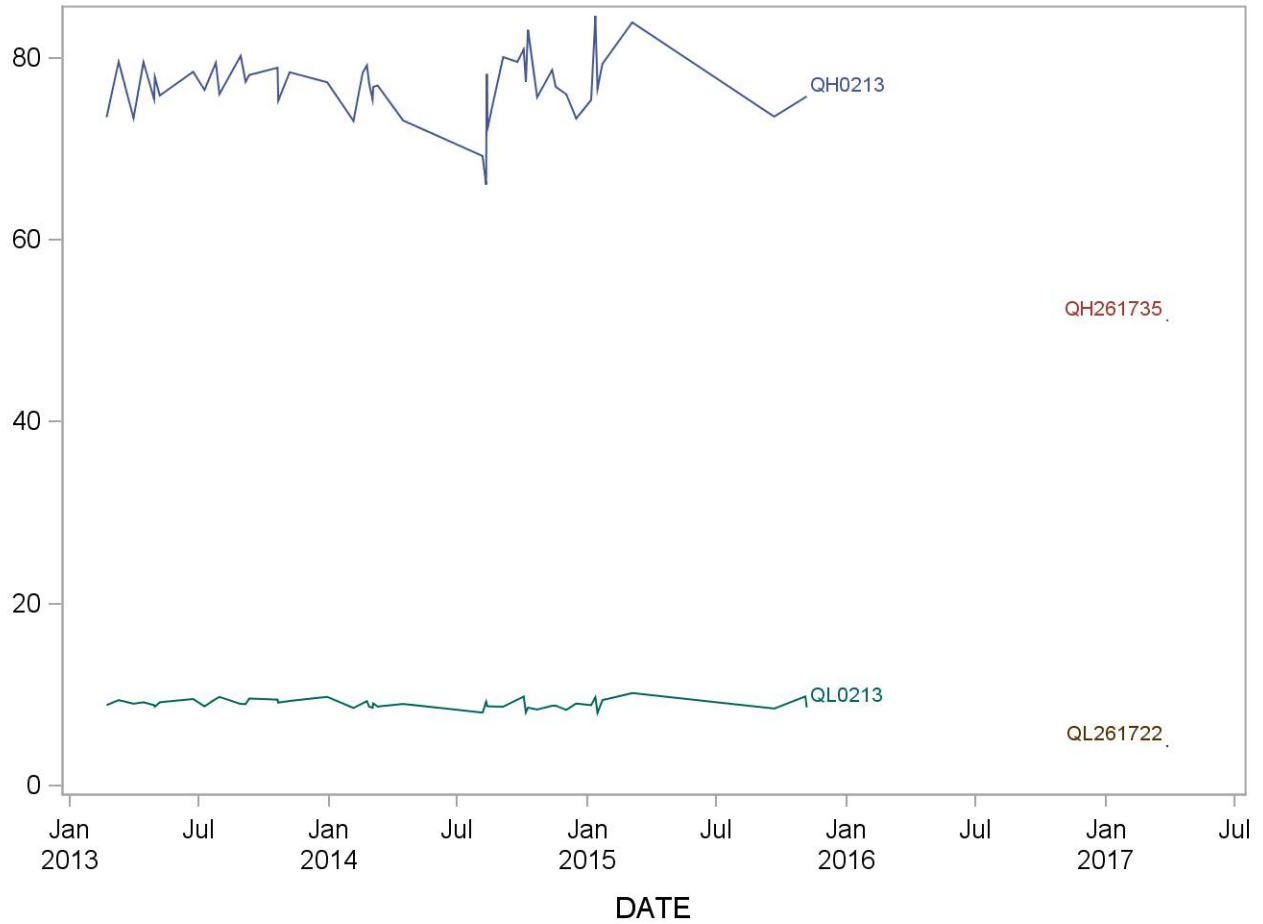
2013-2014 Summary Statistics and QC Chart for N-A-S-(4-hydrxy-2butn-l-yl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	17NOV15	112.5351	6.5417	5.8
QL0213	50	22FEB13	17NOV15	16.8054	1.2262	7.3



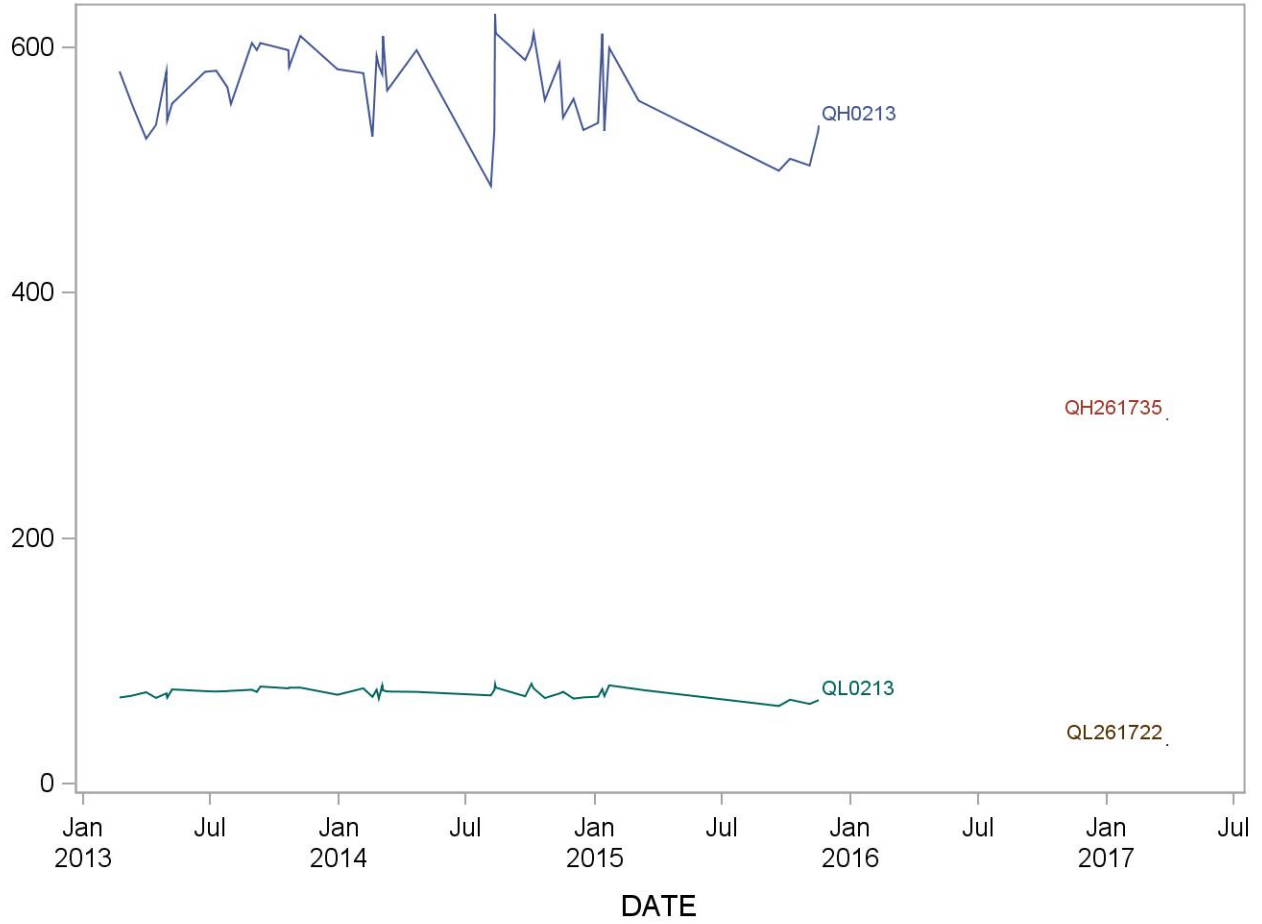
2013-2014 Summary Statistics and QC Chart for N-Ac-S-(2-hydroxy-3-butnyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	48	22FEB13	06NOV15	76.9302	3.3687	4.4
QL0213	48	22FEB13	06NOV15	9.0036	0.4988	5.5



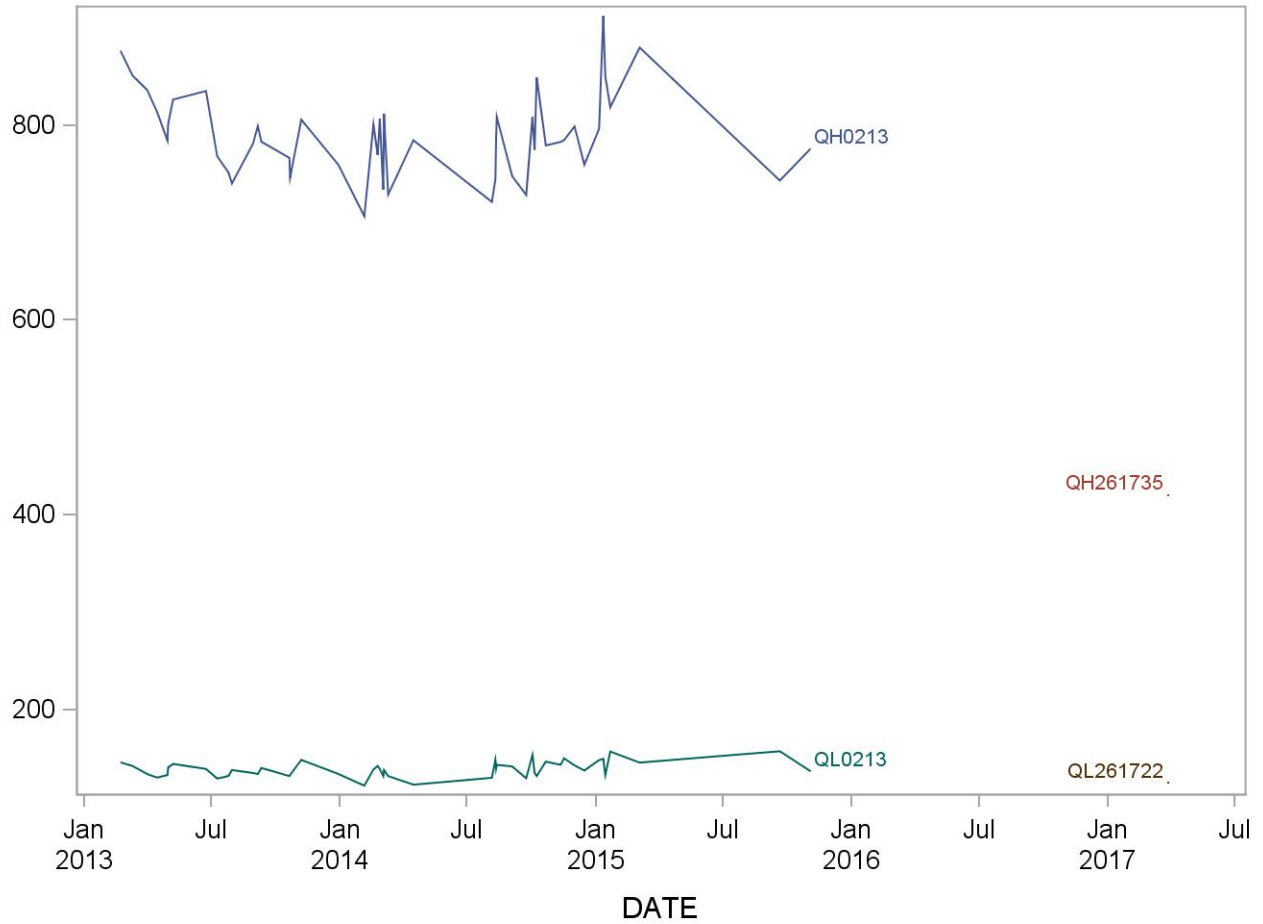
2013-2014 Summary Statistics and QC Chart for N-ac-S-(2-carbmo-2-hydxel)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	48	22FEB13	17NOV15	567.179	34.357	6.1
QL0213	48	22FEB13	17NOV15	73.953	4.191	5.7



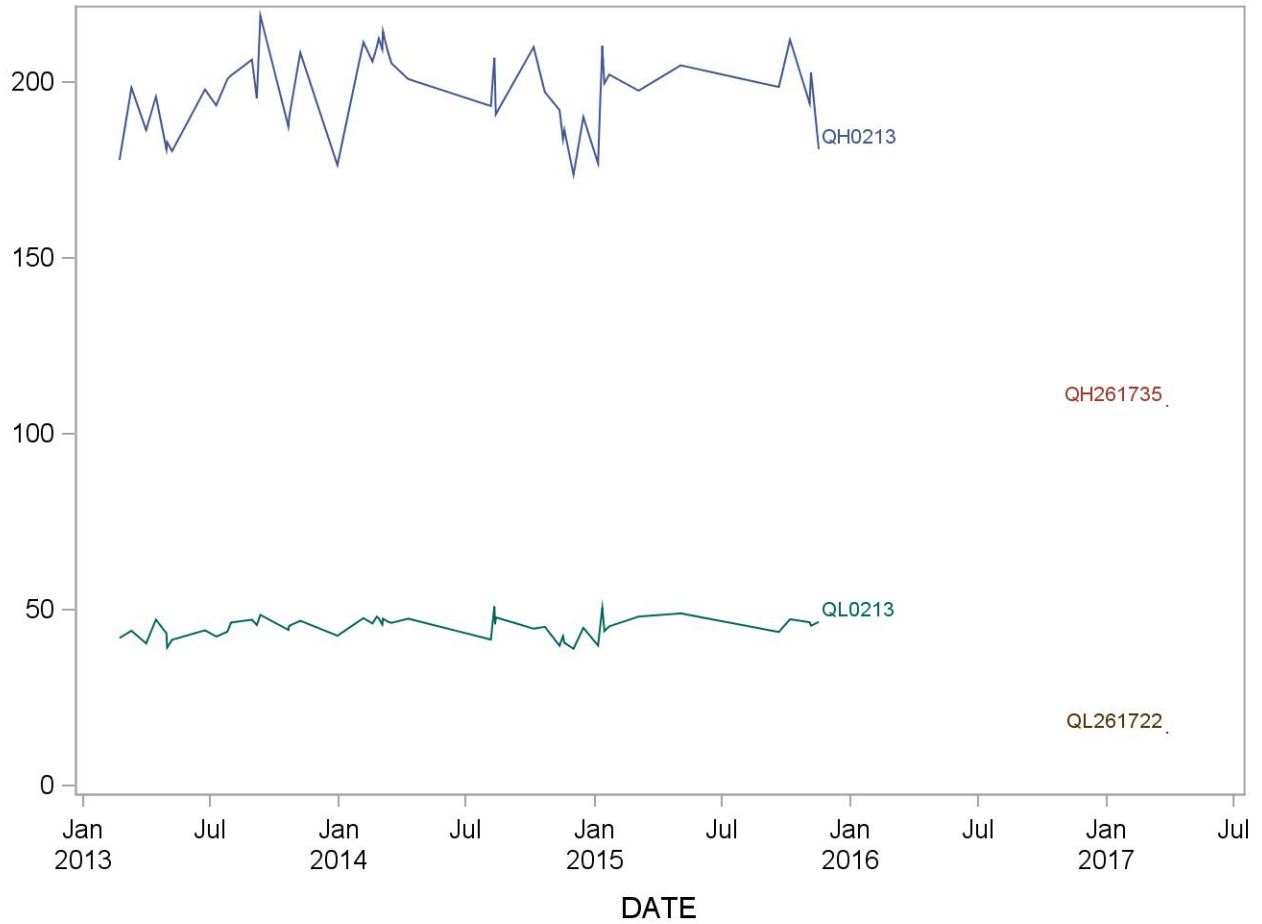
2013-2014 Summary Statistics and QC Chart for N-ace-S- (3,4-Dihidxybutl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	47	22FEB13	04NOV15	789.387	43.907	5.6
QL0213	47	22FEB13	04NOV15	139.233	8.079	5.8



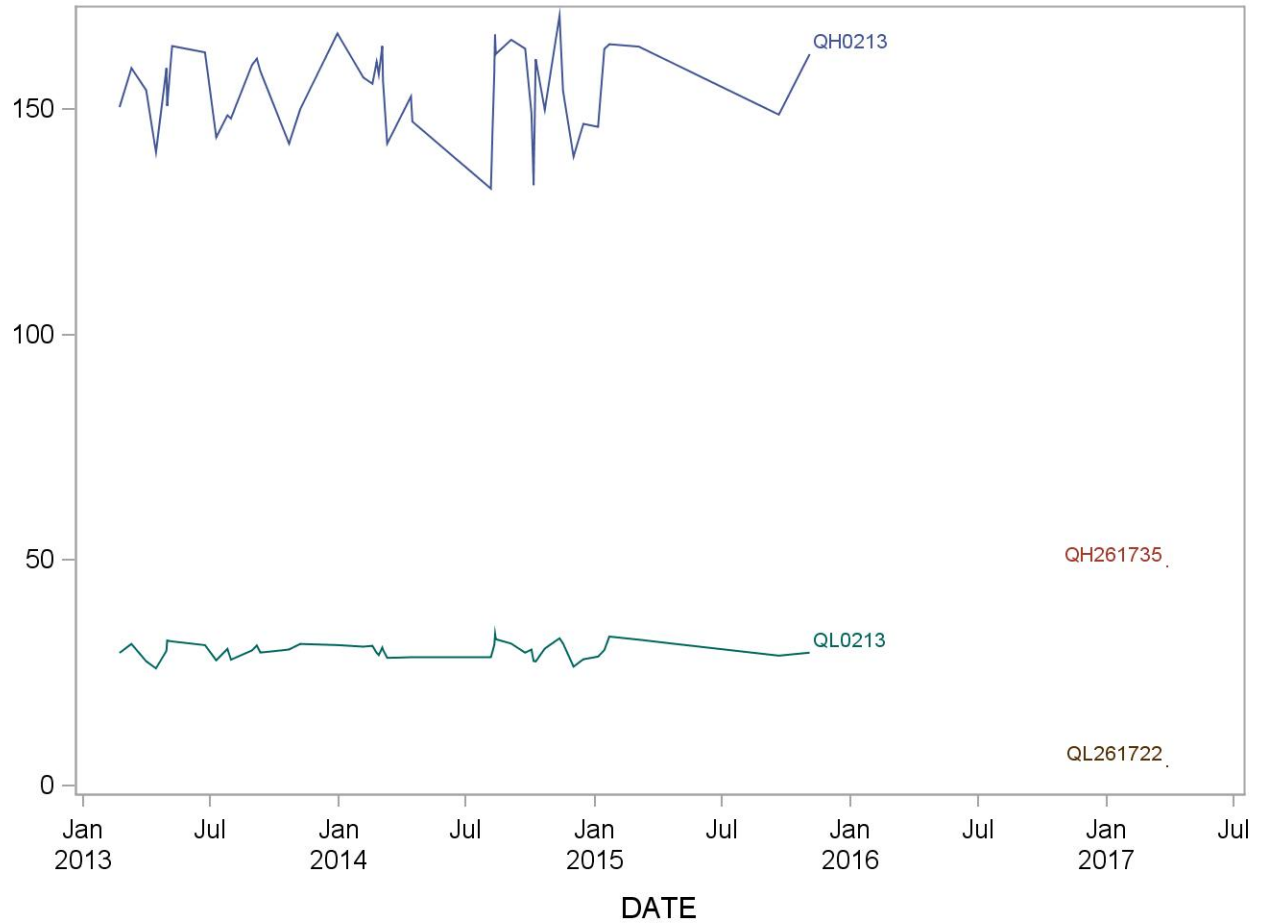
2013-2014 Summary Statistics and QC Chart for N-ace-S-(2-carbamoylethyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	49	22FEB13	17NOV15	197.294	11.504	5.8
QL0213	49	22FEB13	17NOV15	45.063	2.910	6.5



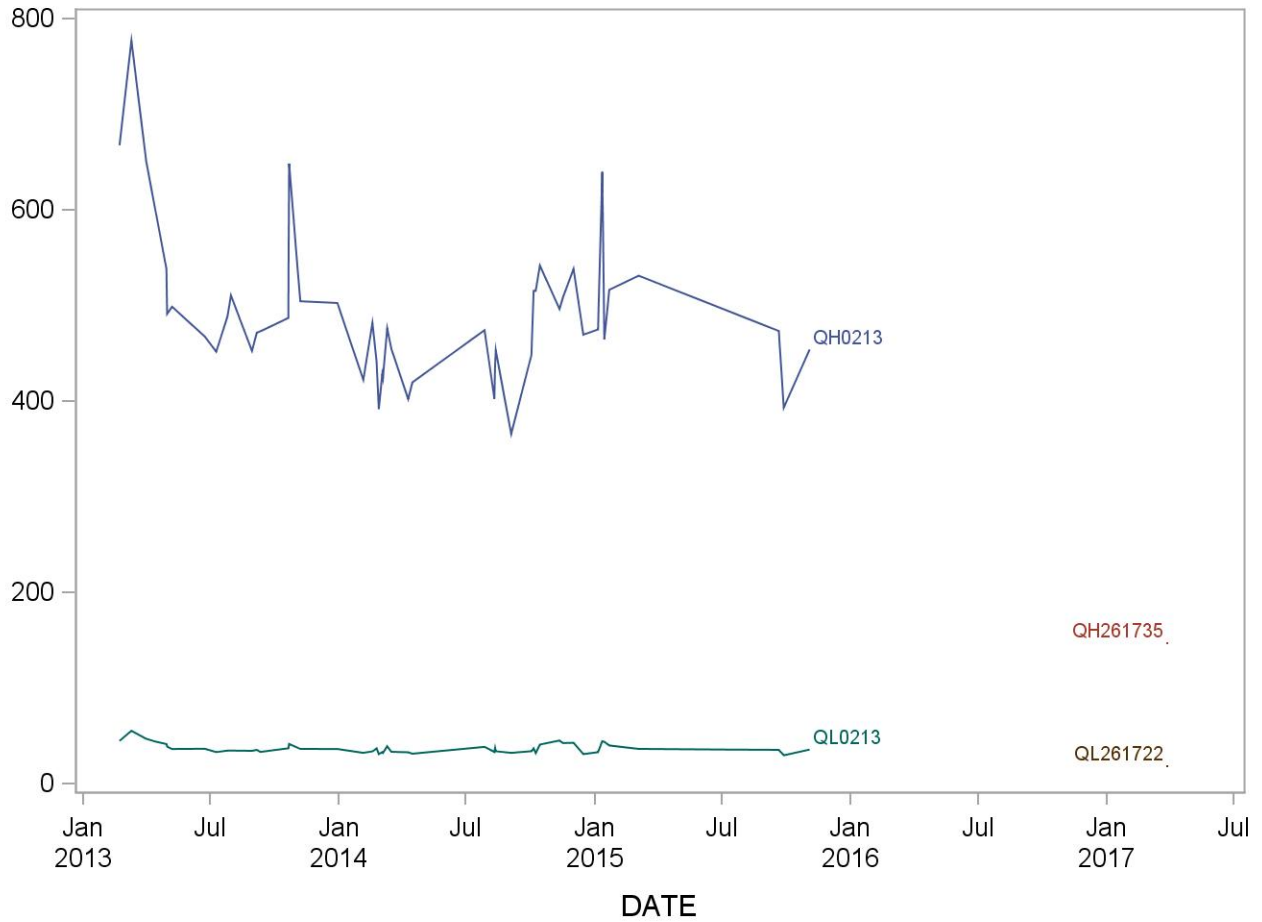
2013-2014 Summary Statistics and QC Chart for N-ace-S-(2-hydroxyethyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	46	22FEB13	04NOV15	154.5647	9.1899	5.9
QL0213	46	22FEB13	04NOV15	29.9591	1.7724	5.9



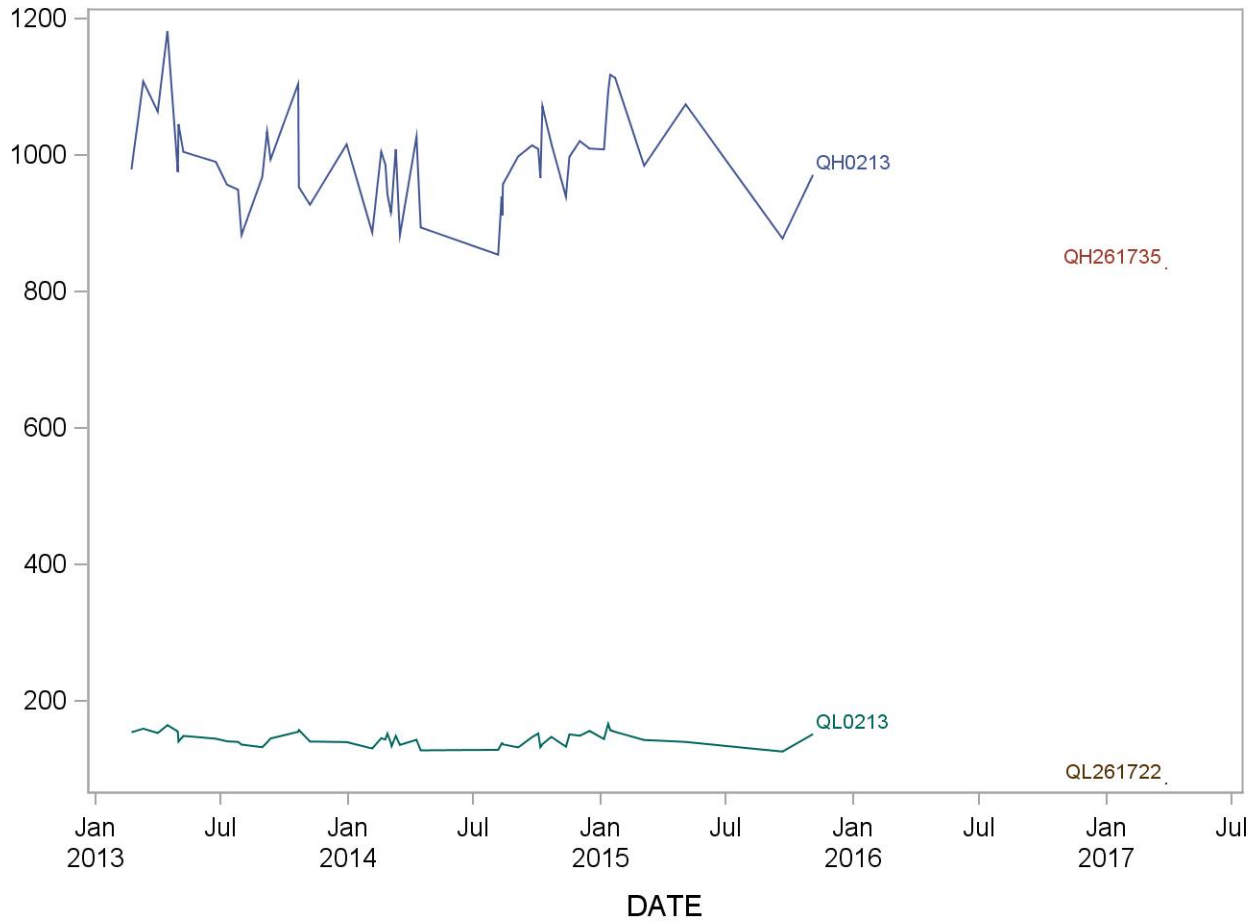
2013-2014 Summary Statistics and QC Chart for N-ace-S-(2-hydroxypropyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	49	22FEB13	04NOV15	492.481	78.395	15.9
QL0213	49	22FEB13	04NOV15	36.997	5.141	13.9



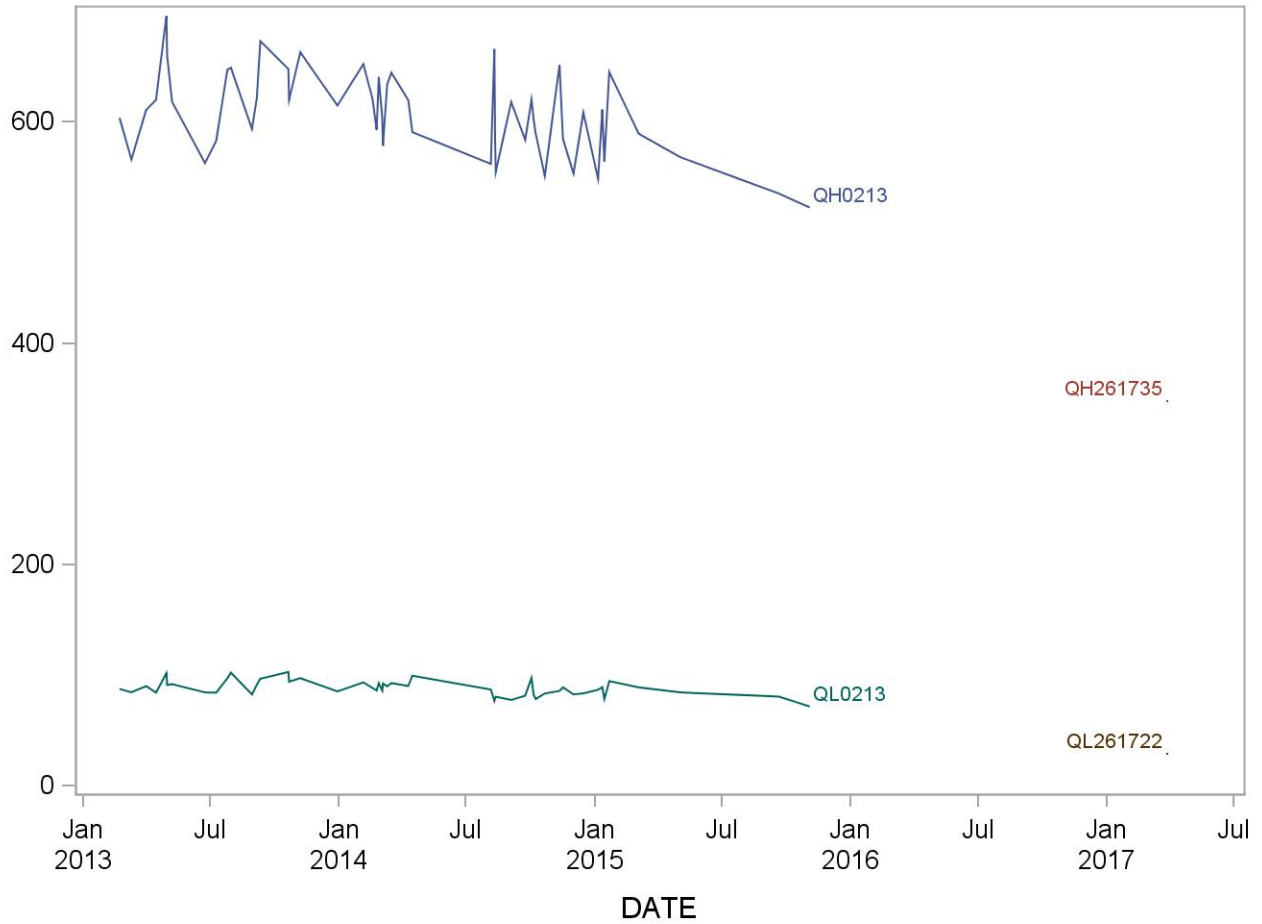
2013-2014 Summary Statistics and QC Chart for N-ace-S-(3-hydroxypropyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	04NOV15	990.69	70.71	7.1
QL0213	50	22FEB13	04NOV15	143.29	9.79	6.8



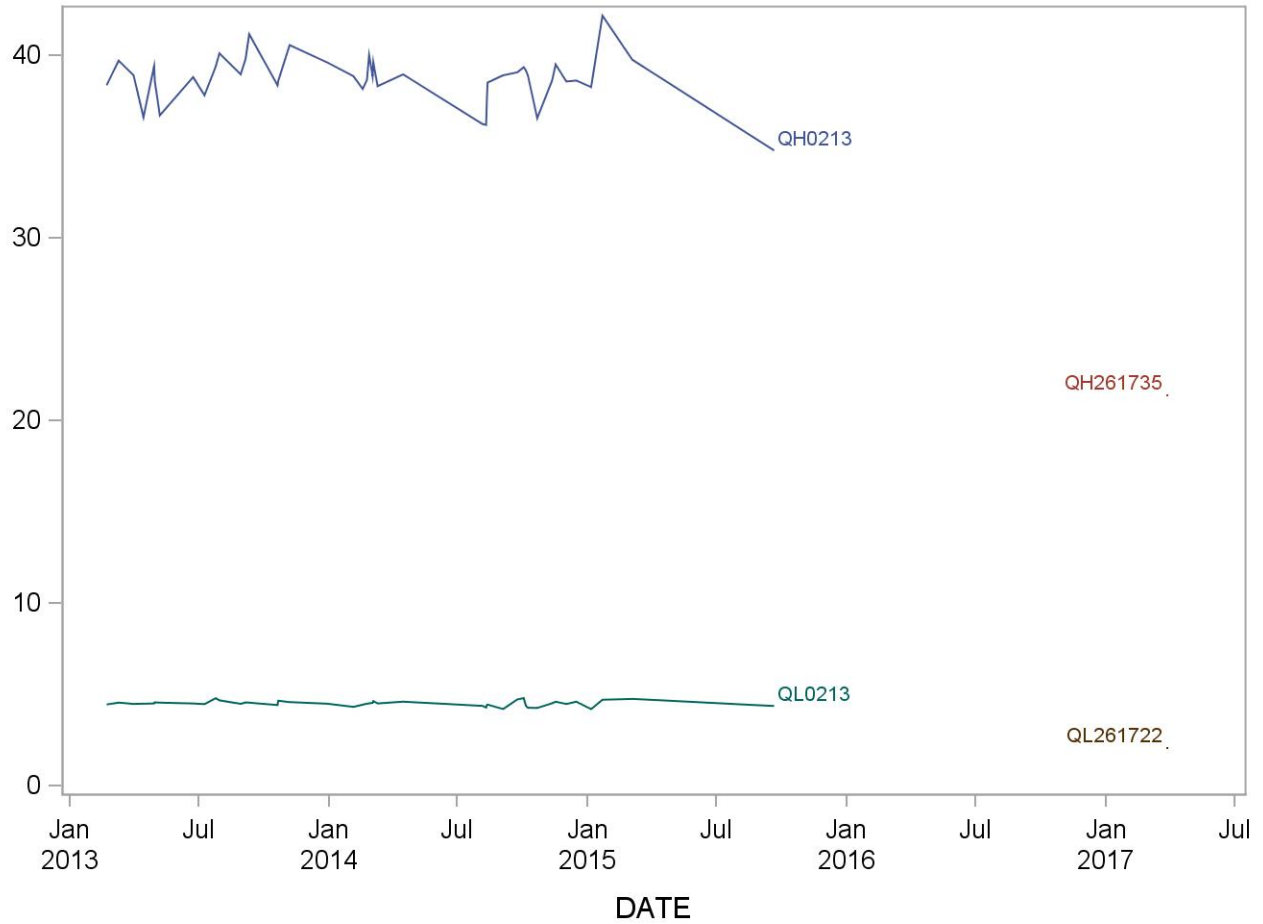
2013-2014 Summary Statistics and QC Chart for N-ace-S-(N-methylcarbamoyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	04NOV15	606.815	39.009	6.4
QL0213	50	22FEB13	04NOV15	87.912	7.071	8.0



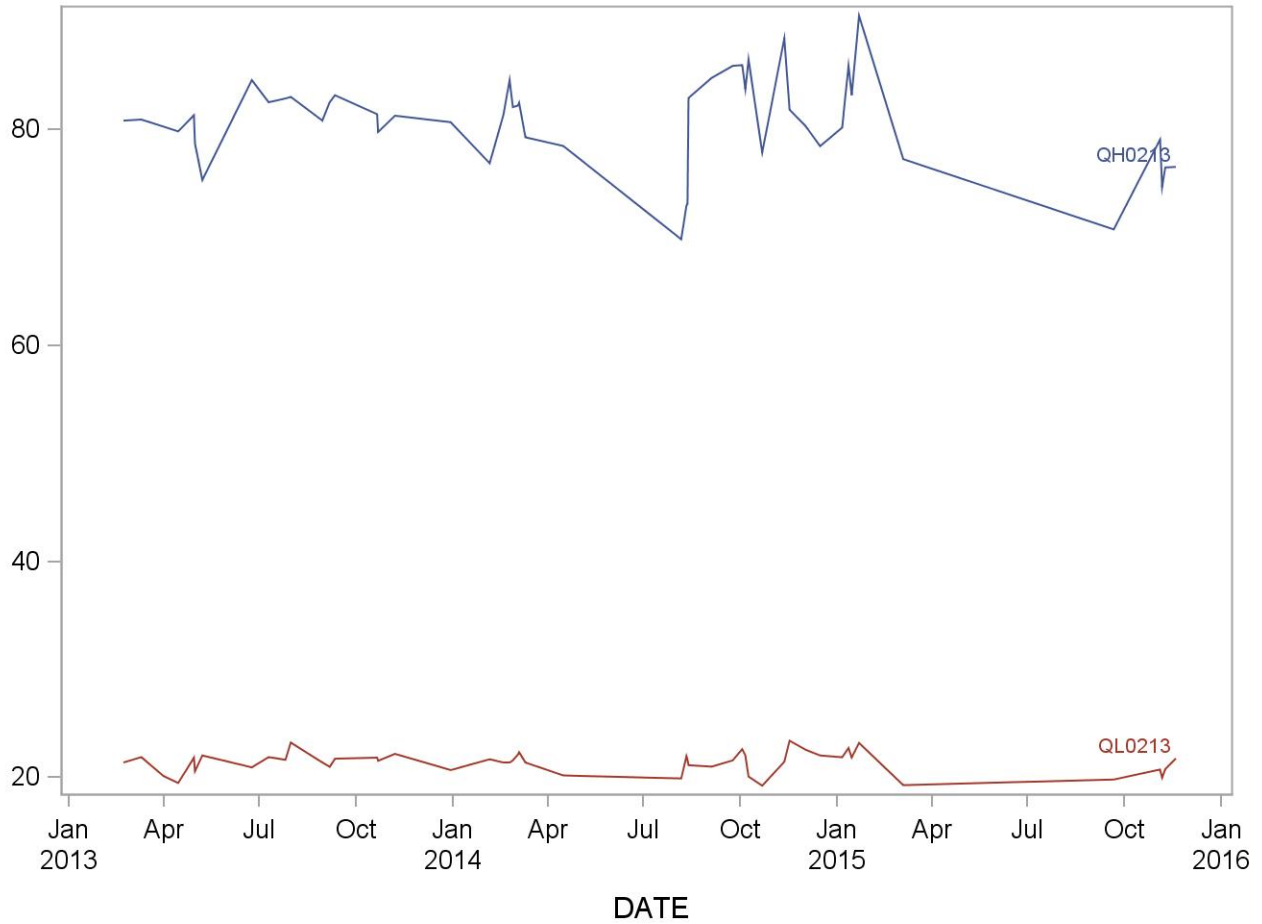
2013-2014 Summary Statistics and QC Chart for N-ace-S-(dimethylphenyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	44	22FEB13	21SEP15	38.7094	1.3356	3.5
QL0213	44	22FEB13	21SEP15	4.4995	0.1460	3.2



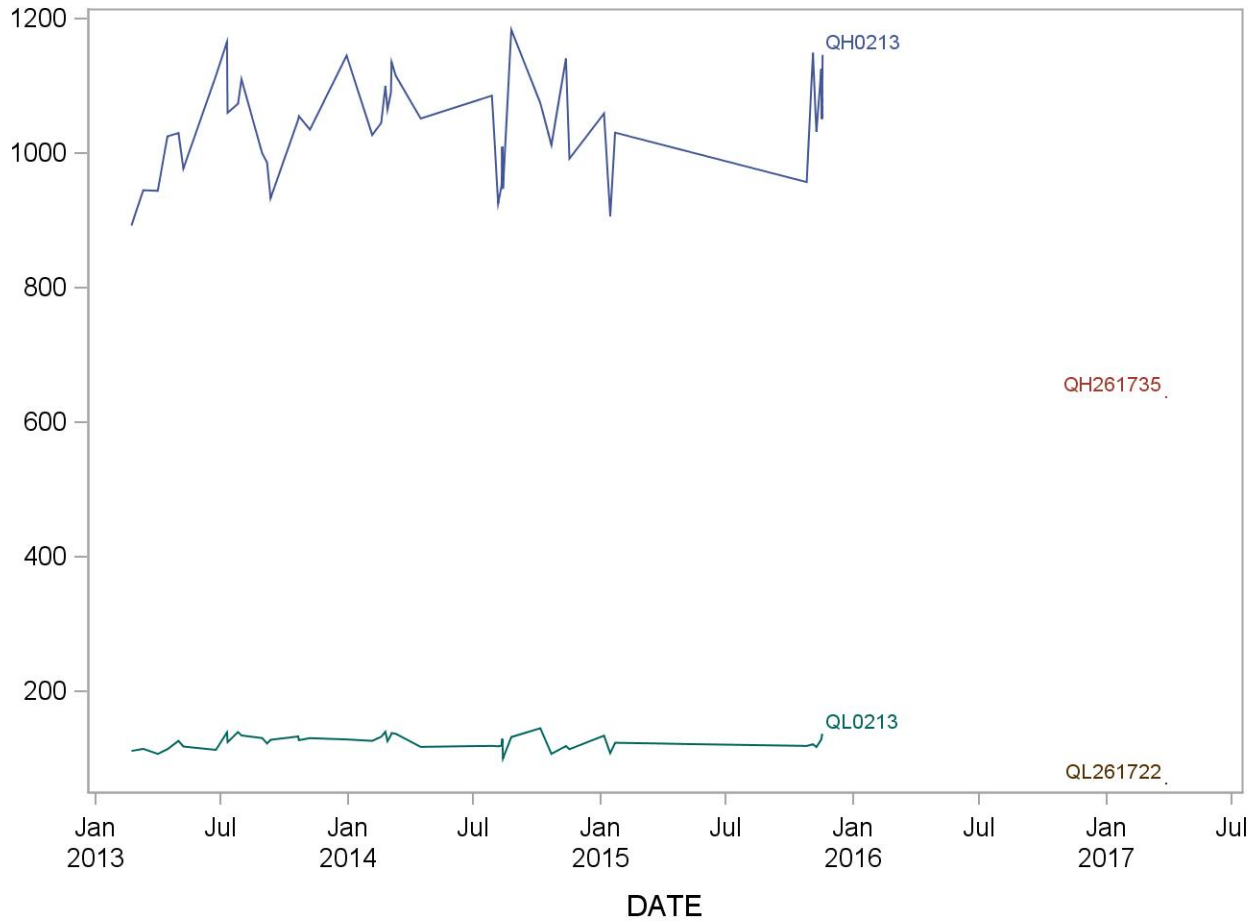
2013-2014 Summary Statistics and QC Chart for N-ace-S-(phenl-2-hydxyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	19NOV15	80.6631	4.1972	5.2
QL0213	50	22FEB13	19NOV15	21.3671	0.9857	4.6



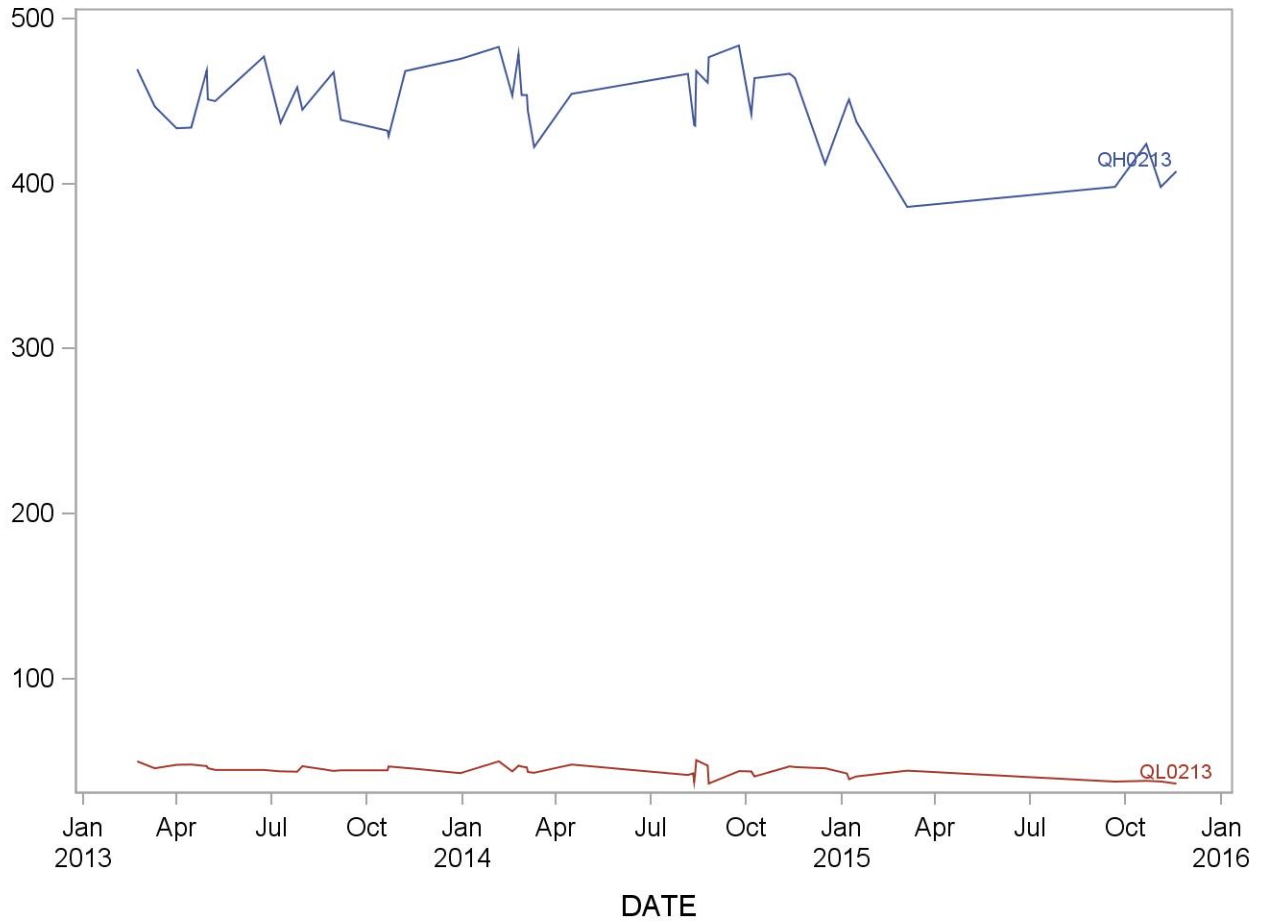
2013-2014 Summary Statistics and QC Chart for N-acel-S-(1,2-dichlorovinyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	45	22FEB13	18NOV15	1043.270	74.404	7.1
QL0213	45	22FEB13	18NOV15	125.449	10.404	8.3



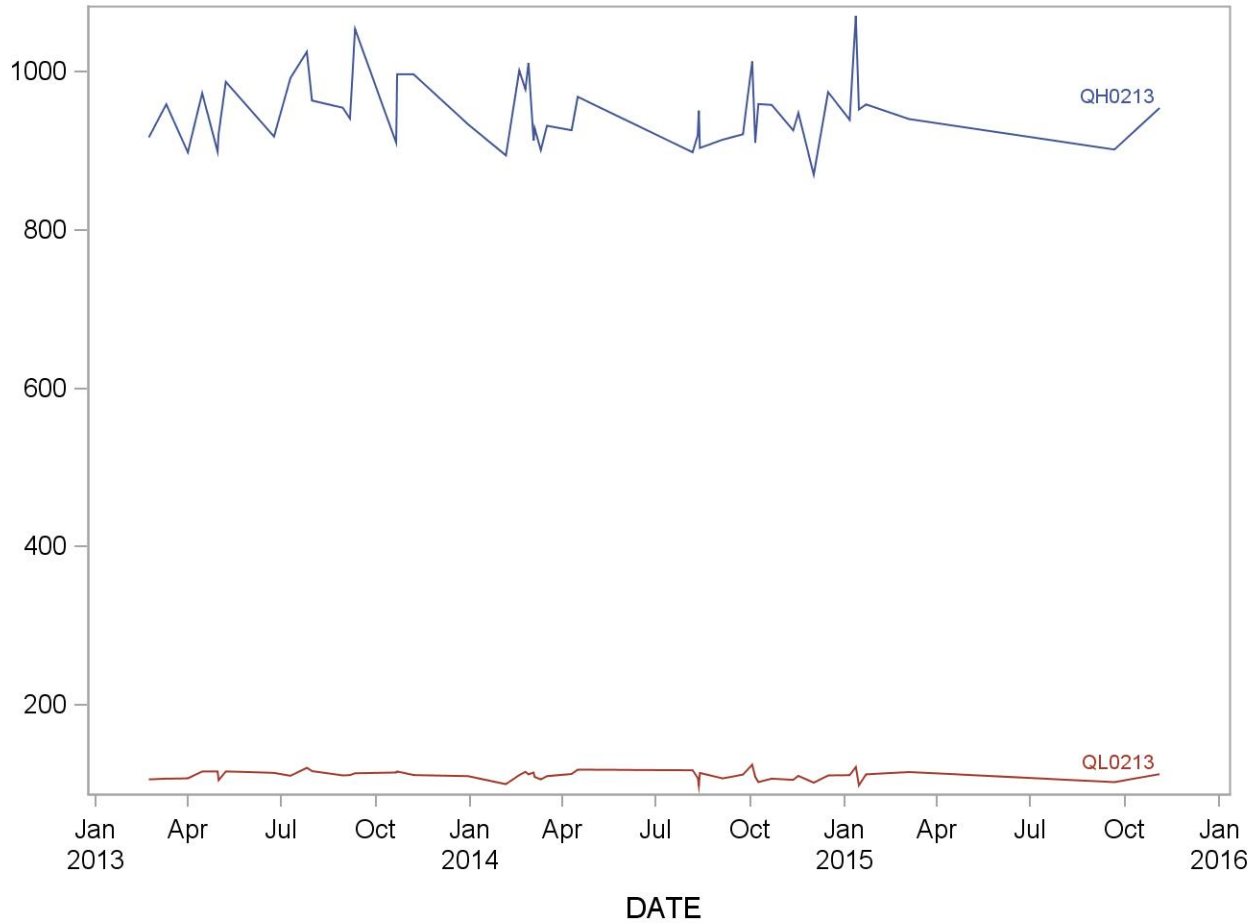
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(2,2-Dichlorovinyl)-L-cystine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	46	22FEB13	19NOV15	447.559	23.279	5.2
QL0213	46	22FEB13	19NOV15	44.079	3.597	8.2



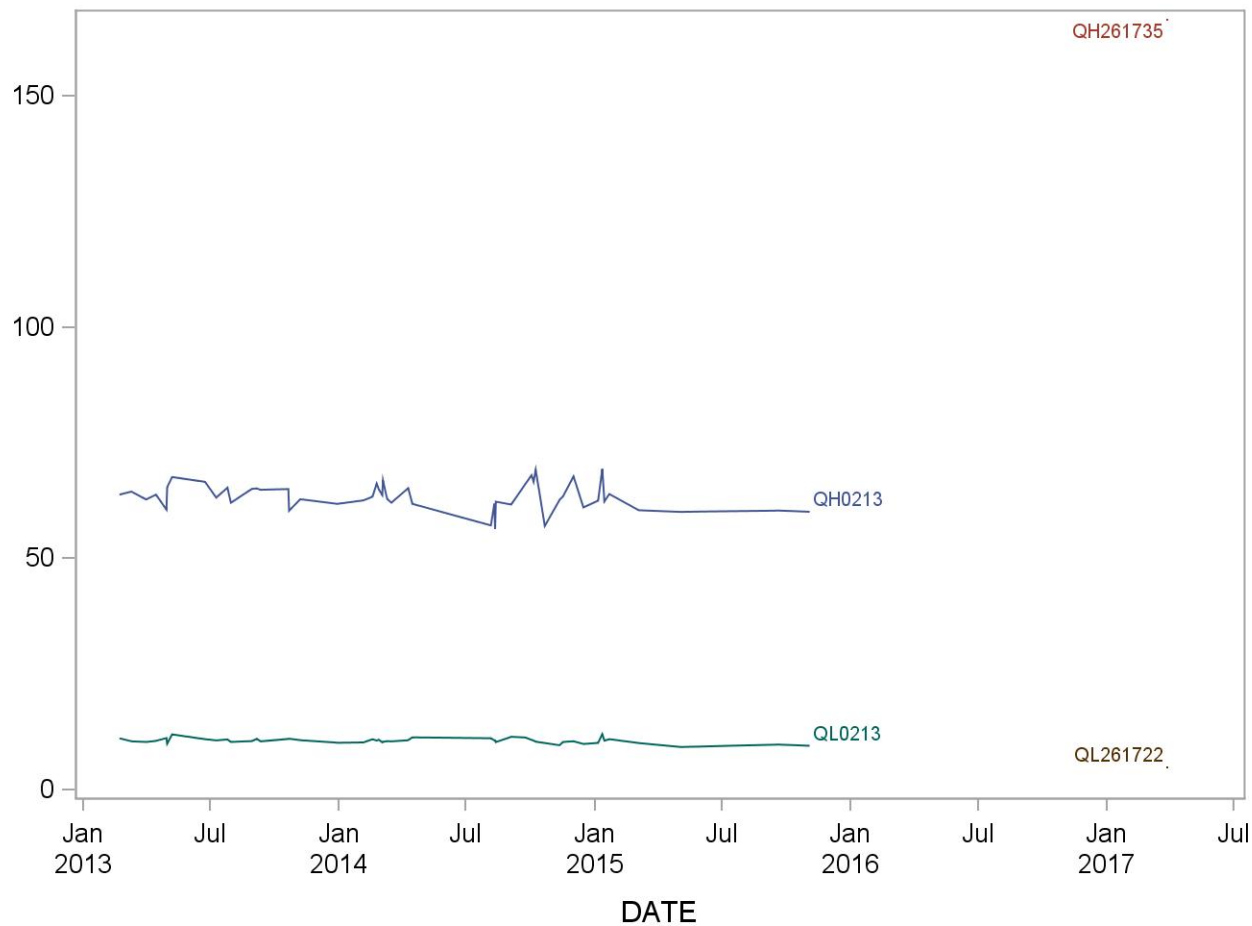
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(2-carboxyethyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	49	22FEB13	04NOV15	948.715	43.056	4.5
QL0213	49	22FEB13	04NOV15	110.525	5.776	5.2



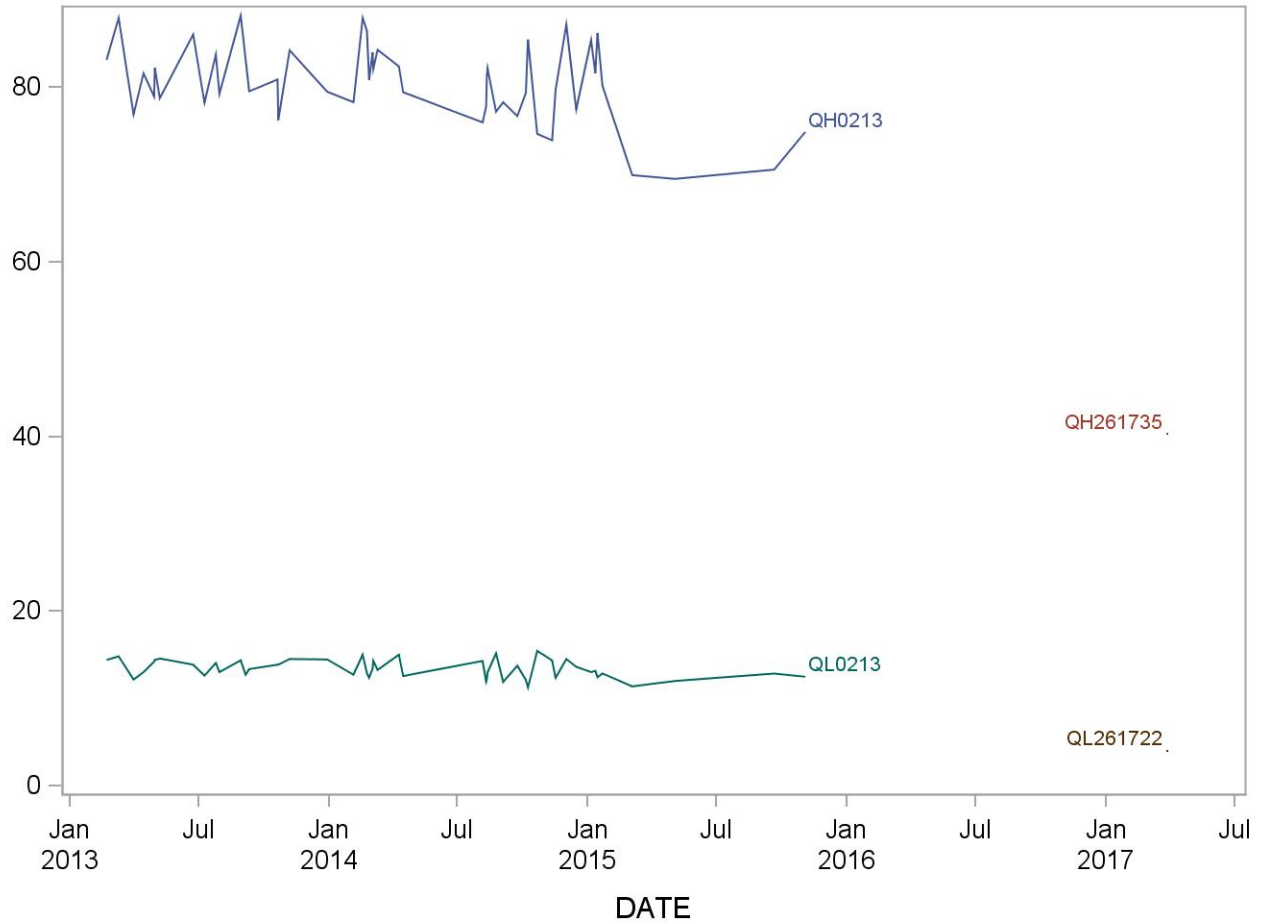
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(2-cyanoethyl)-L-cyst(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	50	22FEB13	04NOV15	63.3469	2.8933	4.6
QL0213	50	22FEB13	04NOV15	10.5683	0.5521	5.2



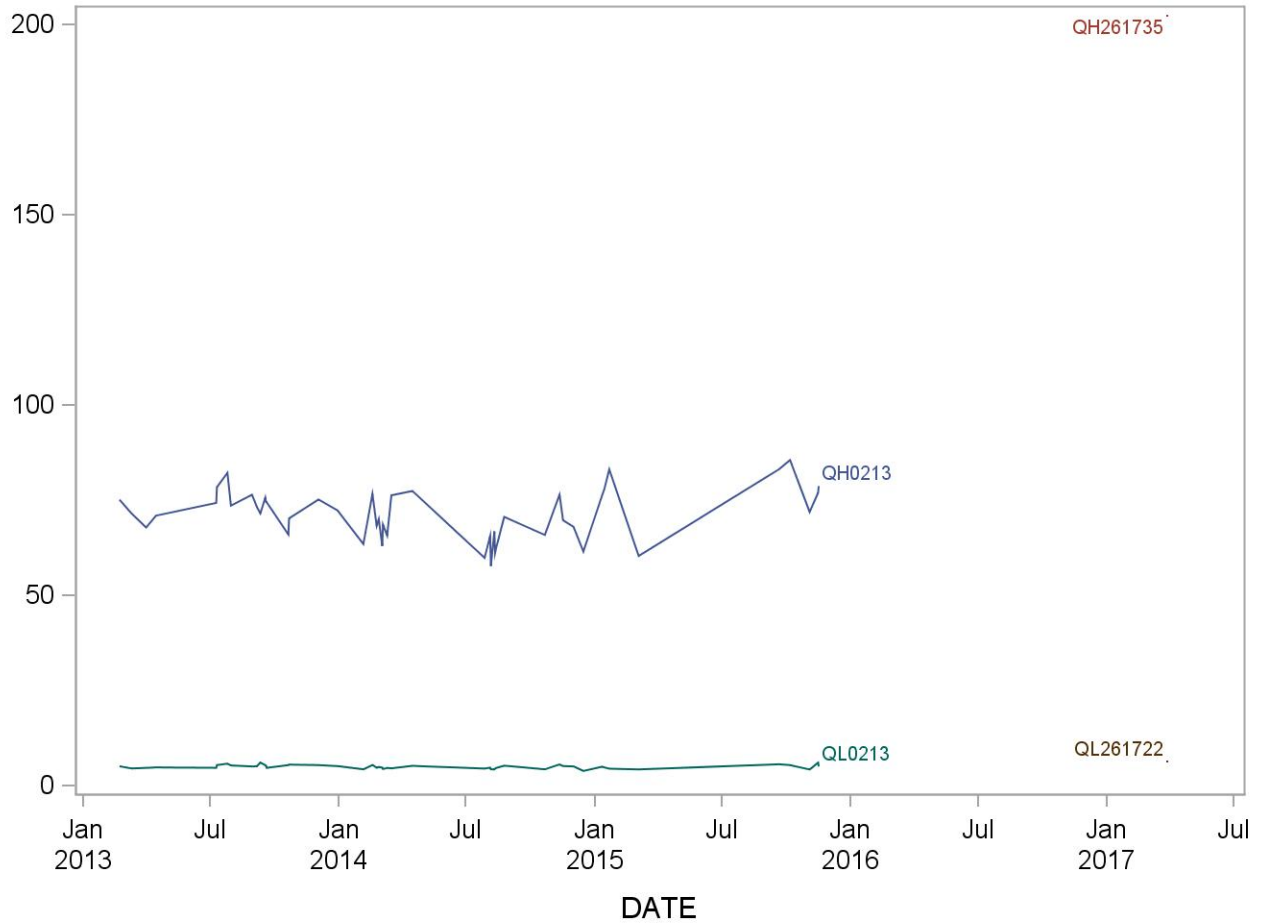
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(benzyl)-L-cysteine(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	49	22FEB13	04NOV15	80.3644	4.5694	5.7
QL0213	49	22FEB13	04NOV15	13.3716	1.0642	8.0



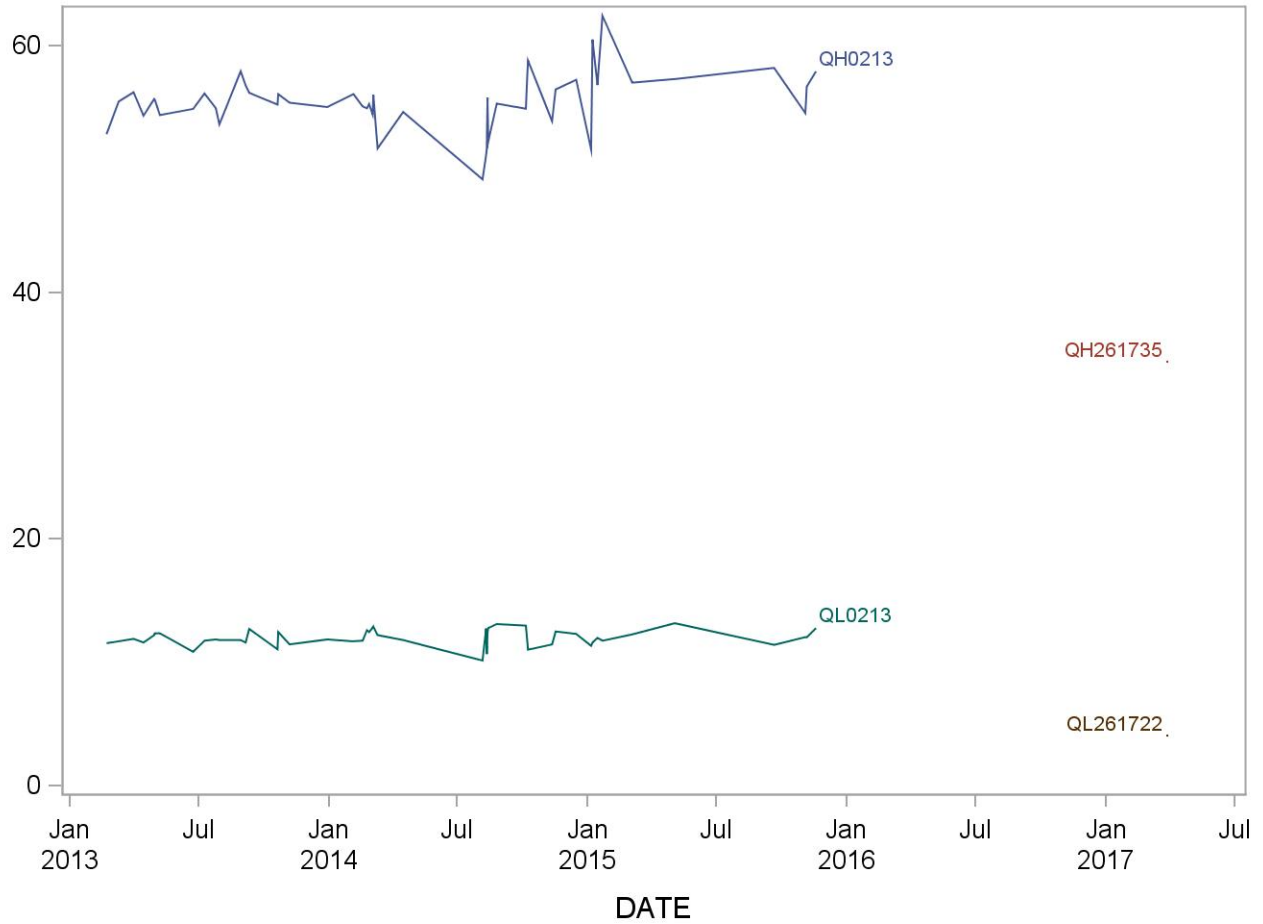
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(n-propyl)-L-cysteine(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	48	22FEB13	17NOV15	71.523	6.646	9.3
QL0213	48	22FEB13	17NOV15	4.943	0.504	10.2



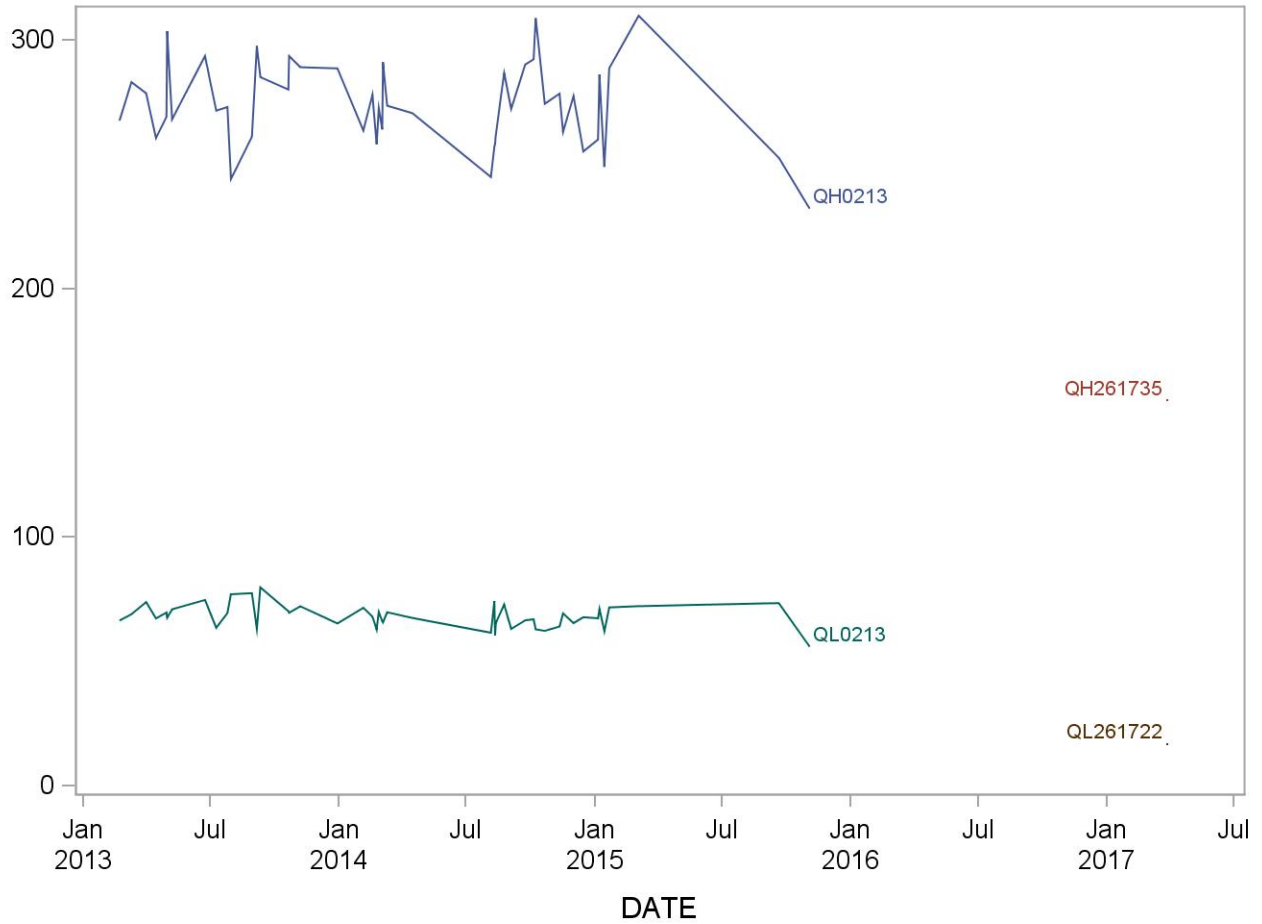
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(phenyl)-L-cysteine(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	47	22FEB13	19NOV15	55.3850	2.3489	4.2
QL0213	47	22FEB13	19NOV15	11.9881	0.6578	5.5



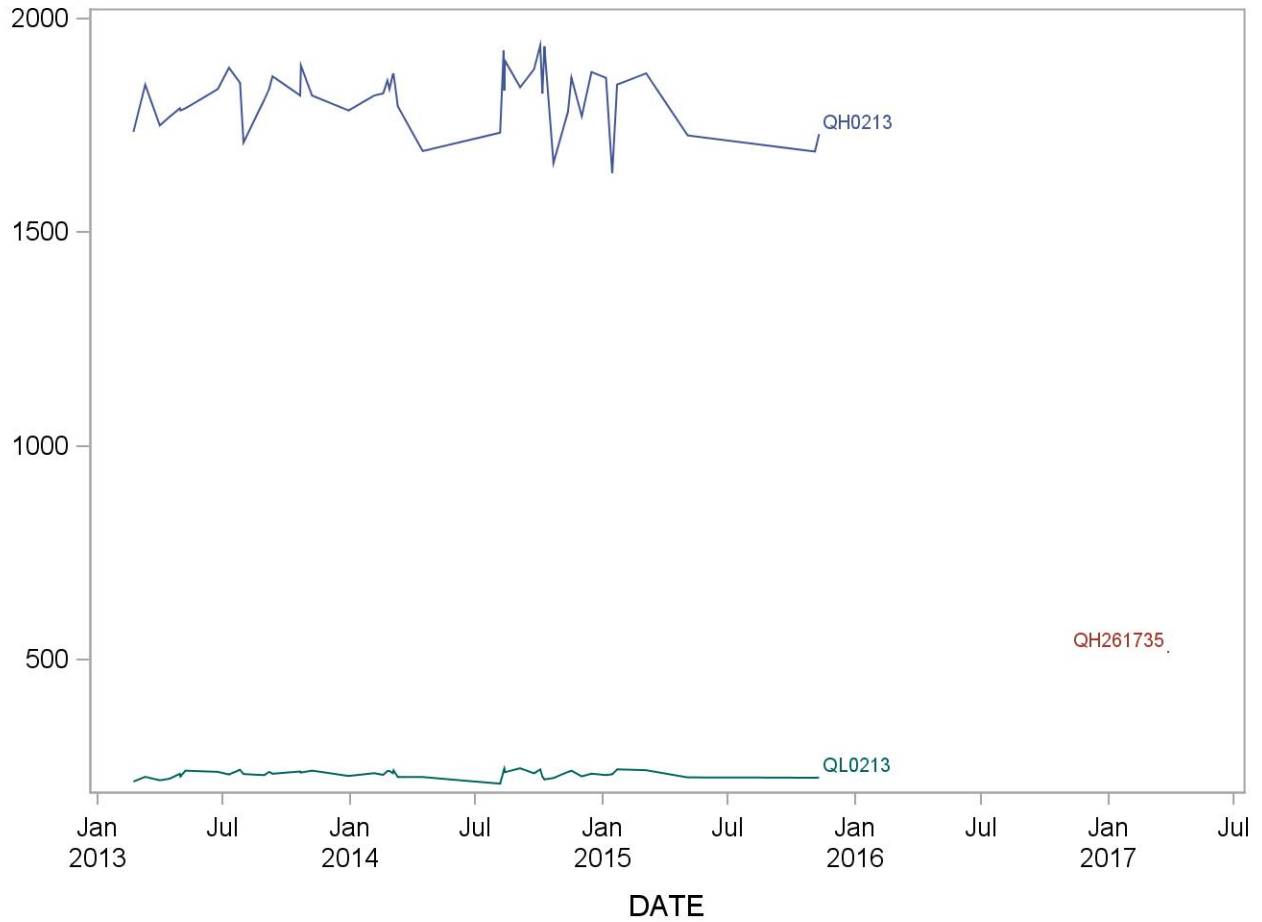
2013-2014 Summary Statistics and QC Chart for N-acetyl-S-(trichlorovinyl)-L-cys(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	47	22FEB13	04NOV15	273.935	17.260	6.3
QL0213	47	22FEB13	04NOV15	68.170	4.820	7.1



2013-2014 Summary Statistics and QC Chart for Phenylglyoxylic acid(ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH0213	47	22FEB13	10NOV15	1813.891	70.497	3.9
QL0213	47	22FEB13	10NOV15	232.886	8.317	3.6



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Use of trade names is for identification only and does not imply endorsement by the public Health Services or the U. S. Department of Health and Human Services.

APPENDIX A

Ruggedness testing

To assess the ruggedness of the accuracy of the method the following parameters were experimented:

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

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

ii. Methanol as organic phase (Solvent B):

When we used methanol as Solvent B, we observed an interference co-eluting with MHBMA2. Using acetonitrile as the organic phase separated MHBMA2 from the interference.

iii. 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.

iv. 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.

v. Samples run at 1:5, 1:10 and 1:20 dilutions:

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

vi. Micro-pipette performance test:

The micro-pipette used for the pipetting urine was tested for accuracy as follows.

Expt 1: without double-pumping, tip was not touched to vial surface when dispensing 100 µl urine.

Expt 2: without double-pumping, touched tip to vial surface when dispensing 100 µl urine.

Expt 3: with double-pumping, touched tip to vial surface when dispensing 100 µl urine.

The specific gravity of urine was measured using a pocket digital hand-held refractometer. The double pumping (Expt 3) and touching the tip to the vial surface while dispensing was found to give better accuracy for the pipette performance.

	Sample I	Sample II
	Mean ± STD	Mean ± STD
Expt 1	0.0969 ± 0.0005	0.0978 ± 0.0001
Expt 2	0.0971 ± 0.0005	0.0983 ± 0.0006
Expt 3	0.1001 ± 0.0006	0.1008 ± 0.0012

APPENDIX B

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

Analyte	P^a	Slope	
		Urine matrix	15 mM Ammonium acetate matrix
AAMA	0.81	0.9242	0.9262
AMCC	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
1,2 DCVMA	0.99	0.9866	0.9868
2,2 DCVMA	0.99	1.0233	1.0239
DHBMA	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
2HPMA	0.87	0.9638	0.9640
HPMMA	0.47	0.9662	0.9660
MA	0.96	0.9999	1.0022
2MHA	0.99	0.9646	0.9655
3MHA + 4MHA	0.83	0.9904	0.9906
MHBMA1	0.96	0.9881	0.9883
MHBMA2	0.99	1.0131	1.0131
MHBMA3	0.97	1.2050	1.2040
PGA	0.92	0.9930	0.9929
PHEMA	0.99	0.9839	0.9837
PMA	0.99	0.9925	0.9915
TCVMA	0.99	0.9873	0.9877
TTCA	0.98	0.9404	0.9412

^aProbability (two-tailed) for urine based estimates and solvent based estimates for 15 matching concentrations.