Laboratory Procedure Manual

Analyte: **Perchlorate, Nitrate and Iodide**

Matrix: **Water**

Method: **Ion Chromatography with Tandem Mass Spectrometry (IC-MS/MS)**

Method No: **VOC-WP8-1.00**

Revised: **01/14/2009**

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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:

File Name	Variable Name	SAS Label
WPIN_D	LBXUP8	Perchlorate, water (ng/mL)
	LBXWIO	Iodide, water (ng/mL)
	LBXWNO	Nitrate, water (ng/mL)

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Perchlorate and nitrate are polyatomic anions that can disrupt thyroid function by competitively inhibiting iodide uptake at the sodium-iodide symporter (NIS).[1,2] Pharmacological doses of NIS-inhibitors or iodine deficiency can significantly reduce iodide uptake. Sufficient inhibition of iodide uptake can lead to decreased thyroid hormone production, and chronically impaired thyroid function can lead to hypothyroidism [3,4] and impaired neurodevelopment in infants [5]. Linkage between health effects and environmental exposure to NIS inhibitors requires improved exposure assessment. By assessing exposure to these three toxicologically-related analytes (perchlorate, nitrate and iodide) in one assay, the relative impact of each chemical on thyroid function can be estimated and thus provide useful information for assessing the potential association between exposure and health effects.

Nitrate is commonly found in physiological fluids resulting from both exogenous and endogenous sources including a variety of foods (green leafy vegetables, milk) and drinking water. Perchlorate exposure is widespread in the U.S. [6] Perchlorate has been associated with decreased thyroid function in females with urinary iodine < 100µg/L [7], indicating the need to assess exposure to perchlorate, other iodide uptake inhibitors and iodide.

b. Test Principle

This method is a quantitative procedure for the measurement of perchlorate, nitrate and iodide in tap water using ion chromatography coupled with electrospray tandem mass spectrometry. Chromatographic separation is achieved using an IonPac AS20 column with sodium hydroxide as the eluant. 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) of unknowns with known standard concentrations yields individual analyte concentrations.

2. Safety Precautions

a. Reagent toxicity or carcinogenicity

Perchlorate and other NIS inhibitors can reversibly inhibit thyroid function at doses of µg per kg body weight per day. Therefore avoid intake of perchlorate (oral or inhalational). Additionally, some perchlorate salts (e.g. ammonium perchlorate) are strong oxidizers. Take special care to prevent contact of solid ammonium perchlorate salt with combustible or oxidizable material, since this constitutes an extreme fire and explosion hazard. However, aqueous solutions of perchlorate do not present a fire or explosion hazard. Perchlorate solutions can irritate skin and

mucous membranes, and thus avoid dermal exposure. Observe Universal Precautions (wear gloves, lab coat, and safety glasses) while handling solutions. Place disposable supplies (pipette tips, autosampler tubes, gloves, etc.) in a biohazard autoclave bag. Keep autoclave bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with a surface disinfectant/decontaminant, when work is finished.

b. Radioactive hazards

None.

c. Microbiological hazards

None.

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 only be performed by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Take precautions when working in these areas.

e. Protective equipment

Follow standard safety precautions when performing this procedure, including using 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

Formal training in the use of the ion chromatograph and mass spectrometer is necessary. Users are required to read the operation manuals and should demonstrate safe techniques in performing the method.

g. Personal hygiene

Follow Universal Precautions. Take care when handling chemicals. Routinely use gloves and wash hands properly. 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

1) Dispose of waste materials in compliance with laboratory, federal, state, and local regulations.

2) Dispose of solvents and reagents in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood.

3) Place all disposable items that come in direct contact with the biological specimens in a biohazard autoclave bag that is kept in an appropriate container until sealed and autoclaved.

4) Immediately place unshielded needles, pipette tips and disposable syringes into a sharps container and autoclave when this container becomes full.

5) Wipe down all surfaces with a surface disinfectant/decontaminant when work is finished.

6) Wash any non-disposable glassware or equipment that comes in contact with biological samples with bleach solution before reuse or disposal.

7) Wash, recycle, or dispose of any other non-disposable glassware in an appropriate manner.

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

3. Computerization; Data-System Management

a. Software and knowledge requirements

This method has been validated using the Dionex IC system controlled by PeakNet Software coupled with an Applied Biosystems Inc. mass spectrometer run with Analyst 1.4 software. Results are exported from Analyst software to Microsoft Excel files and entered into the ATLIS relational 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

Enter information pertaining to particular specimens into the database either manually or transfer electronically. Transfer the result file electronically into the database. Use no personal identifiers; reference all samples to a blind-coded sample identifier.

c. Data maintenance

Check all samples and analytical data prior to being entered into the ATLIS database for transcription errors and overall validity. Routinely back up the database locally onto a computer hard drive and CDs through the standard practices of the NCEH network. Contact the local area network manager for emergency assistance.

d. Information security

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

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

a. Special instructions

None.

b. Sample collection

1) Collect water samples in pre-screened borosilicate glass vials, polystyrene cryo tube vials or polypropylene (PP) centrifuge tubes.

2) Lot screen sample collection containers to ensure the absence of any analyte contamination.

3) Use sterile collectors for sample acquisition.

c. Sample handling

Sample handling conditions are outlined in the DLS protocol for water collection and handling (copies available in the laboratory and specimen handling offices). Collection, transport, and special requirements are discussed in the division protocol.

1) Transport and store water samples at 4 ± 3^{o}C.

2) Once received, store water samples at 4±3°C (in the refrigerator) until time for analysis.

d. Sample quantity

The minimum amount of sample required for analysis is 0.50 mL, with the optimal amount being 2 mL.

e. Unacceptable specimens

1) Reject samples if suspected of contamination due to improper collection procedures or devices. Specimen characteristics that may compromise test results include contamination of water by contact with dust, dirt, etc. from improper handling.

2) Record on the sample transfer sheet a description of reasons for each rejected sample such as low sample volume, leaking or damaged container.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibration Materials, Control Materials, and all Other Materials; Equipment and Instrumentation

a. Reagents and sources

Reagents and sources used during the development, validation, and application of this method are listed in Table 1. All chemicals and solvents are used without further purification. Reagents procured from other sources should meet or exceed these listed requirements.

Table 1. Reagents and Sources.

Reagent	Grade	Source *
Sodium Perchlorate	98%	Sigma Aldrich, St. Louis, MO
Ammonium Perchlorate	99.999%	Sigma Aldrich, St. Louis, MO
Perchlorate 1000µg/mL	Certified Solution	Accustandard, New Haven, CT
Nitrate 1000µg/mL	Certified Solution	Accustandard, New Haven, CT
Sodium Iodide 1000µg/mL	Certified Solution	Spex Certiprep (Metuchen, NJ)
Labeled Sodium Perchlorate ($^{18}O_4$)	98%	Isotec, Miamisburg, OH
Labeled Potassium Nitrate (^{15}N)	99%	Cambridge Isotope Lab, Andover, MA
3-chlorobenzene sulfonic acid		Texas Tech University
Deionized Water	18 MOhm-cm	Aqua Solutions water Purification System, Japer, GA

[#]3-Chlorobenzene was used as the internal standard for NHANES 2005-2007 tap water iodide quantitation.

b. Preparation of Calibration Materials

1) Stock Solutions and dilutions

a) *Stock Solution*

Stock solutions are prepared by dilution of certified solutions (1000 µg/mL) for each of the analytes into deionized (DI) water to give target concentrations of 100, 10, and 1 µg/L depending on the current needs. For example, prepare these stock solutions in volumetric flasks by diluting 10 mL into 100 mL total volume (100 µg/L), 1 mL into 100 mL total volume (10 µg/L), or 1 mL into 1000 mL total volume (1 µg/L). These stock solutions are used to prepare the working standard solutions as shown in Tables 2-4.

b) *Internal Standard Solution*

1. Labeled Perchlorate

i. Weigh approximately 2.5 mg of ^{18}O-labeled sodium perchlorate, transfer to a 25-mL volumetric flask and take to volume with DI water to produce an approximate 100-ppm concentrated stock solution.
ii. Dilute the initial stock solution approximately 1:20 (1.25 mL of a 100-ppm stock into a 25-mL volumetric flask diluted with DI water) to produce a final concentration of 5 ppm.

2. Labeled Nitrate ($^{15}NO3$)

Weigh approximately 25 mg of $^{15}NO_3$, transfer to a 25-mL volumetric flask and dilute to volume with DI water to produce an approximate 1000 ppm solution.

3. 3-Chlorobenzene sulfonic acid (internal standard for iodide)

3-Chlorobenzene sulfonic acid (100 ng/µL) was provided by Dr. P. K. Dasgupta, Texas Tech University.

4. Internal Standard Solution Mix

i. Prepare 100 m of the working labeled internal standard solution every 2 weeks by adding 800 µl of 5 ng/µl labeled perchlorate, 10,000 µL of 1000 ng/µl labeled nitrate, and 1000 µL of 100 ng/µL 3-chlorobenzene sulfonic acid into a 1-L glass bottle.
ii. Dilute this solution to 1000 mL with DI water.

iii. The concentration of the working solution for Cl18O4, 15NO3 and 3-chlorobenzene sulfonic acid is approximately 0.004, 1.3, and 0.1 ng/µL, respectively, from which 500 µL is added to the sample.

2) Working Standard Solutions

Prepare working standard solutions by aliquoting known amounts of each analyte from previously prepared stock solutions (6.b.1) and diluting to final volume with DI water in a volumetric flask. Standard solutions (1-9) are prepared as presented in Tables 2-4, which specifies stock solution to use, volume to aliquot and final volume for each standard.

Table 2. Perchlorate Calibration Standards

Standard ID	Stock Solution		Final Solution	
	Concentration ng/µL (ppm)	Volume (µL)	Concentration µg/L (ppb)	Total Volume (mL)
SS*mmyy*01	1.00	12.5	0.05	25
SS*mmyy*02	1.00	25	0.10	25
SS*mmyy*03	1.00	33	0.33	10
SS*mmyy*04	1.00	100	1.0	10
SS*mmyy*05	10	33	3.3	10
SS*mmyy*06	10	100	10	10
SS*mmyy*07	10	330	33	10
SS*mmyy*08	100	75	75	10
SS*mmyy*09	100	100	100	10

* *mmyy* represents the month and year of standard preparation.

Table 3. Nitrate Calibration Standards

Standard ID	Stock Solution		Final Solution	
	Stock Solution µg/mL (ppm)	Volume (µL)	Concentration µg/L (ppb)	Total Volume (mL)
SS*mmyy*01	10	2500	100	25
SS*mmyy*02	100	1250	500	25
SS*mmyy*03	100	1000	1000	10
SS*mmyy*04	1000	500	5000	10
SS*mmyy*05	1000	1000	10000	10
SS*mmyy*06	1000	2500	25000	10
SS*mmyy*07	1000	5000	50000	10

SS*mmyy*08	1000	7500	75000	10
SS*mmyy*09	1000	X[a]	100000	X[a]

[a]Nitrate in standard mix solution 9 is added separately when preparing calibration curve. See procedure below in section 8.2

Table 4. Iodide Calibration Standards

Standard ID	Stock Solution		Final Solution	
	Stock Solution ng/µL (ppm)	Volume (µL)	Concentration µg/L (ppb)	Total Volume (mL)
SS*mmyy*01	1.0	25	0.1	25
SS*mmyy*02	1.0	82.5	0.33	25
SS*mmyy*03	1.0	100	1	10
SS*mmyy*04	10	33	3.3	10
SS*mmyy*05	10	100	10	10
SS*mmyy*06	10	330	33	10
SS*mmyy*07	100	75	75	10
SS*mmyy*08	100	100	100	10
SS*mmyy*09	CERT STOCK	50	500	10

* *mmyy* represents the month and year of standard preparation.

Store aliquots of these solutions in 1.5-mL vials at -20±5°C until use. After the vial is used, store it at 4±3°C.

c. Preparation of Control Materials

1) Quality Control materials

a) Prepare separately two quality control (QC) levels (low QC and high QC) in 18 MOhm-cm aqua water.
b) Fortify each pool with the different analytes to achieve levels within the linear range of the method: a low QC with target levels of 5, 25, and 5000 µg/L for perchlorate, iodide and nitrate, respectively; and a high QC with 75, 250, and 60,000 µg/L for perchlorate, iodide, and nitrate, respectively.
c) After fortifying the water to reach target concentrations, store the QC solutions overnight at 4±3°C for equilibration.
d) After overnight equilibration let QC solutions reach room temperature and aliquot into 1.2-mL labeled cryo vials.
e) Store the vials at -20±5°C until use.

2) Proficiency Testing materials

a) Prepare proficiency testing (PT) materials from certified 1000 µg/L reference solutions for each of the analytes (AccuStandard, New Haven, CT).

b) Four target concentrations covering the linear range for each analyte are selected.

c) Dilute to final concentration with water in a 25-mL volumetric flask.

d) Blind-code aliquots and store in cryo-vials at -20±5°C until use.

e) Analyze PT samples twice a year as well as following any major maintenance on the instrumentation

f) Proficiency testing samples are blind coded for analysis; results are evaluated by an external quality control officer.

Note: Proficiency Testing materials are prepared by the team lead and blind to the analyst. Consult with the team lead when additional PT materials need to be prepared.

d. Other materials and supplies

Materials / supplies and sources used during the development, validation, and application of this method are listed below. Materials/supplies procured from other sources should meet or exceed these specifications. All materials that have direct contact with sample matrix were lot-screened to verify no perchlorate contamination.

Nalgene 1.8-mL cryo-vials (Fisher Scientific, Fairlawn, NJ).

Eppendorf Repeater Plus Pipette (Brinkmann Instruments Inc., Westbury, NY).

Rainin Electronic Pipettes (100, 250, and 1000-µL; Rainin, California)

Pasteur pipettes and bulbs (Kimble Glass, Inc., Vineland, NJ).

VWR Brand Mini vortexer (The Lab Depot, Alpharetta, GA).

1.5-mL Vial Kit with Split Septum (Dionex, Sunnyvale, Ca)

ASRS Ultra II, 2mm Suppresor (Dionex, Sunnyvale, Ca)

Ion Pac ® AS 20 Column (Dionex, Sunnyvale, Ca)

Nalgene Sterilization filter unit (Fisher Scientific, Fairlawn, NJ)

Envirocide Surface Disinfectant/ Decontaminant Cleaner

d. Instrumentation

Analyses were conducted with a Dionex ion chromatography system equipped with a GP50 gradient pump, AS50 autosampler, AS50 thermal compartment and a 2-mm anion self-regenerating suppressor (ASRS Ultra II) operated in the external water mode (Dionex Corp, Sunnyvale, CA). PeakNet 6 chromatography software was used for system control. The separation was performed using an IonPac AS20column (2

x 250mm, Dionex) with a 25-µL injection loop. An Applied Biosystems API4000 triple quadrupole mass spectrometer (Foster City, CA) with electrospray interface was used for the detection of perchlorate.

1) Ion chromatograph configuration

The ion chromatograph configuration is described in Table 6 below. The separation conditions were optimized to obtain resolution between perchlorate and other interferences present in water (e.g. sulfate).

Table 6. Ion Chromatograph Configuration

Parameter	Setting
Column type	AS20 (2 x 250 mm)
Column temperature	30°C
Eluant	50 mM sodium hydroxide
Flow	0.5 mL/min
Injection Loop Volume	25 µL
Suppressor	ASRS Ultra II

2) Mass spectrometer SRM configuration

The following parameters were 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 Multiple Reaction Monitoring (MRM) mode. The transitions of interest are presented in Table 7 and typical mass spectrometer parameters are presented in Tables 8 and 9.

Table 7. Perchlorate MRM Transitions

Analyte	MRM Transition
Perchlorate ClO_4^-	
Quantification	98.9 / 83.1
Confirmation	100.6 / 85.2
Labeled Perchlorate, $Cl^{18}O_4^-$	106.9 / 88.97
Iodide	127.0/127.0
3-Chlorobenzene sulfonic acid	191.0/79.9
Nitrate, NO_3	
Quantification	62.0 / 45.8
Confirmation	62.0 / 62.0
Labeled Nitrate, $^{15}NO_3$	63.0 / 47.1

[#]3-chlorobenzene was used as the internal standard for NHANES 2005-2007 tap water analysis for iodide. The MRM transition was 191.0/79.9

Table 8. Mass Spectrometer Configuration.

Parameter	Setting
Scan type	MRM
Polarity	Negative
Ion Source	Turbo Spray
Temperature	600°C
IS	-4000 V
CAD	12
CUR	10
GSI	45 psi
GS2	45 psi
Dwell Time	400 msec
Probe Y distance	2.0 mm

Table 9. Mass Spectrometer Parameters Characteristics for each Analyte

Analyte	DP	EP	CE	CXP
Perchlorate				
Quantification	-55	-10	-45	-1
Confirmation	-60	-10	-38	-3
Iodide	-60	-5	-65	-6
Nitrate				
Quantification	-40	-10	-40	-5
Confirmation	-40	-10	-35	-6

7. Calibration and Calibration Verification

a. Creation of curve

1) Calibration Data

i. Prepare fresh calibrators for each set of unknown analyses.
ii. Analyze each set of unknowns to form the calibration curve for that set of samples.
iii. 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 1.4 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 run. 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. Use of the calibration curve

The lowest point on the calibration curve is the lowest reportable level and the highest point 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 calibration standards, 2 low level QCs, 2 high level QCs and up to 80 unknown water samples.

a. Sample preparation

1) Preliminary sample preparation steps

c) Allow water samples, frozen quality control materials, and calibration standards to reach ambient temperature.

d) Mix samples thoroughly by inversion or vortexing.

e) 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 (1-8)

a) Using a 100-µL pipettor transfer 50 µL of the appropriate standard stock solution into the appropriately marked autosampler vial.

b) Using a 1000-µL pipettor add 450 µL of DI water.

c) Using a 1000-µL pipettor add 500 µL of the internal standard solution to make a final volume of 1 mL.

d) Cap the vial and mix thoroughly for a few seconds using a vortex mixer.

3) Preparation of standard 9

a) Using a 100-µL pipettor transfer 50 µL of standard mix 9 into the appropriately marked autosampler vial.

b) Using a 100-µL pipettor add 50 µL of the 1000 ppm nitrate certified stock solution.

c) Using a 1000-µL pipettor add 400 µL of DI water.

d) Using a 1000-µL pipettor add 500 µL of the internal standard solution to make a final volume of 1 mL.

4) Preparation of the blank

a) Using a 1000-µL pipettor transfer 500 µL of DI Water into the appropriately marked autosampler vial.

b) Using a 1000-µL pipettor add 500 µL of the internal standard solution to make a final volume of 1 mL.

c) Cap the vial and mix for a few seconds using a vortex mixer.

5) Preparation of the low Quality Control sample

a) Using a 1000-µL pipettor transfer 500 µL of the QC Low stock solution into the appropriately marked autosampler vial.

b) Using a 1000-µL pipettor add 500 µL of the internal standard solution to make a final volume of 1 mL.

c) Cap the vial and mix thoroughly for a few seconds using a vortex mixer.

6) Preparation of the high Quality Control sample

a) Using a 1000-µL pipettor transfer 500 µL of the QC High stock solution into the appropriately marked autosampler vial.

b) Using a 1000-µL pipettor add 500 µL of the internal standard solution to make a final volume of 1 mL.

c) Cap the vial and mix thoroughly for a few seconds using a vortex mixer.

7) Preparation of the unknown specimens

a) Mix (either by vortexing or repetitive sample inversion) the unknown sample.

b) Using a 1000-µL pipettor aliquot 500 µL of unknown into the autosampler vial.

c) Using a 1000-µL add 500 µL of the internal standard solution to make a final volume of 1 mL.

d) Cap the vial and mix thoroughly for a few seconds using a vortex mixer.

Note: For the delivery of internal standard and DI water an Eppendorf Repeater Plus Pipette can be used.

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

1) Preliminary system setup

a) Tuning and calibration of the mass spectrometer

i. Set the y-distance of the probe to 6mm and infuse the PPG 3000 solution at a flow rate of 10 µL/min.
ii. Using **Manual Tuning,** load the ppg 3000 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 **Resolution Optimization** with **Calibration** upon success.
iv. Make sure that the following specified parameters are met. For peak width, the resolution is set to 0.60 ± 0.05 mass units and sensitivity is met using the ion 932 m/z with an intensity of **2.0×10^7 minimum** (combined intensity of 10 scans).
v. Check the tune and mass calibration of the instrument weekly.

b) IC system setup

i. Fill the mobile phase bottles with filtered and sonicated (5-min) fresh DI water.
ii. Ensure that the water reservoir for the suppressor is full.

iii. Load the program file mmddyy_INIS.pgm and start the pump.
iv. Allow the system to equilibrate for 1 hr prior to starting a run
v. Once the total conductivity in the system reaches a value less than 3 µSiemens, the system is ready.

c) Performance evaluation

i. Allow the system to equilibrate with the method to be run (both MS and IC).
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

In the PeakNet software of the IC system, create a sequence for the run using the wizard. Make sure that the appropriate number of samples is loaded and the appropriate program is selected (*mmddyy* – NIS.pgm; where *mmddyy* is the most recent date that the program was changed and/or saved).

b) Assign the acquisition and quantitation methods

i. Import the .csv file obtained from ATLIS into Analyst. The .csv file includes the sequence information of the standards, QCs, and unknowns to be analyzed.
ii. Select the acquisition method (*mmddyy*_INIS.dam; where *mmddyy* is the most recent date that the method was changed and / or saved) and the quantitation method (*mmddyy*_INIS.qmf; where *mmddyy* is the most recent date that the method was changed and / or saved).
iii. The letter "I" before the methods name (NIS) correspond to the first letter of the instruments name (J for joker and M for Mcdreamy).
iv. Ensure that the icons on the right corner of the window are green 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 instrument waits for a sync signal from the IC to start the acquisition.

f) Start the sequence in IC

i. Click **Batch** in the main menu and select edit.
ii. Once the window is open, select the sequence to be run starting with the equilibration sequence.
iii. Once the sequences are selected, press **Start,** making sure that the MS is ready to start.
iv. 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 DI water to eliminate any salt residue accumulation. After flushing the system shut down the IC instrument as well as the MS.

c. Processing of data

1) Once the run has finished, note the final pressure as well as conductivity in the instrument maintenance book.

2) Quantify all raw data files using the quantitation capabilities of the Analyst software. The peaks are automatically integrated using the quantitation method created for the analysis.

3) Visually review the integration of each peak and manually correct when needed.

4) Generate a calibration curve from the calibrators; QCs, unknowns and blanks are quantified against the calibration curve.

5) Save the reviewed data files in a report file and export as a text file.

6) Open the text file in the Excel file mcrSCIEXup8.xls available on the Q drive and run the file through the macro “mcrFormatResults”.

7) Save the data as an Excel file and import into the ATLIS Perchlorate 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 perchlorate using this method is 0.05 to 100 µg/L. The lower reportable limit corresponds to the lowest standard 0.05 µg/L which is greater than the detection limit for the method. The upper reportable limit corresponds to the concentration of the highest standard 100 µg/L. In the case of

nitrate and iodide the lowest reportable levels are 500, and 0.33 µg/L respectively. The upper calibration ranges are 100,000, and 500 µg/L. If the analyte level exceeds the upper calibration range, the sample is reassayed by diluting it 2-fold in water.

Table 10. Method Detection Limits, Lowest Reportable Values and Calibration Ranges

Compound	Linear Range (µg/L)	R^2	Limit of Detection (µg/L)	Lowest Reportable Level (µg/L)
Nitrate	500 - 100000	0.9930	143	500
Perchlorate	0.05 - 100	0.9998	0.004	0.05
Iodide	0.33 - 500	0.9991	0.150	0.33

b. Limit of Detection

The limit of detection was determined (using Taylor's method[8]) by calculating the standard deviation at different standard concentrations following repeated measurements of the concentration standards in water. The standard deviations were then plotted versus concentration. The intercept of the least squares fit of this line equals S_0; $3S_0$ equals the limit of detection (LOD). Since the LOD is below the lowest standard, the lowest standard is used as the lowest reportable level.

c. Accuracy

The accuracy of the assay was established by analyzing certified perchlorate standards blind to the analyst (i.e. Proficiency Testing samples). The accuracy of the method was obtained by comparing the concentration calculated from analyzing the samples to the theoretical concentration. The results of these measurements are given in Table 11.

Table 11. Method Accuracy and Precision (Proficiency Testing)

Analyte	Sample	Average %CV [a]	Average Absolute % Diff [b]
Nitrate	1500 - 62000 µg/L	1.88	4.54
Perchlorate	0.19 - 72.0 µg/L	4.7	3.78
Iodide	3.0 - 76 µg/L	2.8	7.24

[a] Coefficient of Variation

[b] Average absolute value of % difference between theoretical and calculated amount

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 determined by analyzing 20 QC samples is listed in Table 12 below.

c. Analytical specificity

IC-MS/MS is the most selective analytical method in use for quantifying the target analytes in complex aqueous matrices. Ion chromatography produces reproducible chromatographic resolution of the target analytes, even in the most concentrated water 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 parent to daughter ion 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 that are found at levels above the limit of detection.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Quality assessment procedures follow standard practices[9]. Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are added to each day's run sequence. The QC blank is analyzed at the beginning of each run to check the system for possible contamination or in the spiking solutions and/or reagents. 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 a QC standards obtained using this calibration curve are compared with acceptance criteria given below to assure the proper operation 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. Typical QC characterization statistics are listed in Table 12. Limits are based on statistical calculation accounting for 2 QCs analyzed in each analytical run.

Table 12. NIS Quality Control Samples QC 0407

Analyte ID	QC ID	Count	Mean	σ	%CV	Mean - 3σ	Mean - 2σ	Mean + 2σ	Mean + 3σ
Iodide[#]	QH0407	20	252.8	5.51	2.2%	235.1	241.0	264.6	270.5
	QL0407	20	25.50	0.95	3.7%	22.44	23.47	27.53	28.56
Nitrate	QH0407	20	60543	1448	2.4%	55,881	57,444	63,641	65,204
	QL0407	20	5435	232.12	4.3%	4,688	4,938	5,932	6,182
Perchlorate	QH0407	20	75.80	0.66	0.9%	73.67	74.39	77.21	77.93
	QL0407	20	5.06	0.09	1.8%	4.77	4.87	5.25	5.35

σ = standard deviation, %CV = % coefficient of variation
[#]3-Chlorobenzene was used as the internal standard to quantify iodide in the above QC characterization

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 Westgard[9] rules:

a) If both the low and the high QC results are within the 2σ 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 purchased, 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

Five 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 fresh dilutions of the calibration standards (see section 8.a.1), and re-analyze the entire calibration curve using the freshly prepared 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. In less than 0.2% of water samples the presence of an unknown compound does distort perchlorate chromatography. Interference was observed with nitrate chromatography in about 18% of NHANES 2005-2007 tap water samples. The problem was resolved by diluting the sample 2-fold and re-analyzing it.

13. Reference Ranges (Normal Values)

Reference ranges for tap water perchlorate, nitrate and iodide are presented in Table 13, as derived from NHANES 2005-2007 data for study participants ages 6+.

Table 13. Perchlorate, nitrate and iodide levels in US tap water.

Analyte	Specimen	Units of measure	Analytical limit of detection (LOD)*	Reference range **
Perchlorate	Water	mg/L	0.1 mg /L	<LOD – 2.0 mg /L
Iodide	Water	mg/L	0.2 mg /L	0.57 – 37 mg /L
Nitrate	Water	mg/L	700 mg /L	<LOD – 16,100 mg /L

*Results near the limit of detection (LOD) are subject to greater uncertainty. LOD is calculated for the entire measurement system, not just the instrument.
The LOD = $3*S_0$ is the standard deviation of the concentrations derived from the measurement process as the concentration approaches zero.
** limited to the 5^{th} to the 95^{th} percentile intervals.

14. Critical Call Results ("Panic Values")

The health effects of chronic exposure to trace levels of perchlorate are unclear. Therefore a definitive panic value has not been established. The National Academy of Sciences has reviewed the toxicological literature for perchlorate, and recommended a reference dose of 0.0007 mg/Kg-day. This dose correlates to a tap water perchlorate level of 24.5 µg/L and would be flagged as a "high exposure level". Greer et al reported possible inhibition of thyroid hormones at a dose of 0.5 mg/Kg-day of perchlorate [10]. This dose correlates to a tap water perchlorate level of 17,500 µg/L, which would be set as the "Critical Call Value". The Environmental Protection Agency has set up a maximum contaminant level (MCL) of 10 ppm for nitrate (measured as nitrogen) in drinking water. Iodide is a required nutrient and thus does not have a critical call value.

15. Specimen Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. Perchlorate in water is stable at room temperature. If the measurement is delayed until the next day, refrigerate the samples at 4±3°C.

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

Alternate validated methods have not been evaluated for measuring perchlorate in water. If the analytical system fails, refrigerate the samples at 4±3°C until the analytical system is restored to functionality. If long-term interruption (greater than 4 weeks) is anticipated, store water specimens at –20±5°C.

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

Results are reported to two 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 1 mL of sample remains following successful completion of analysis, this material should be returned to storage at -20±5°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.

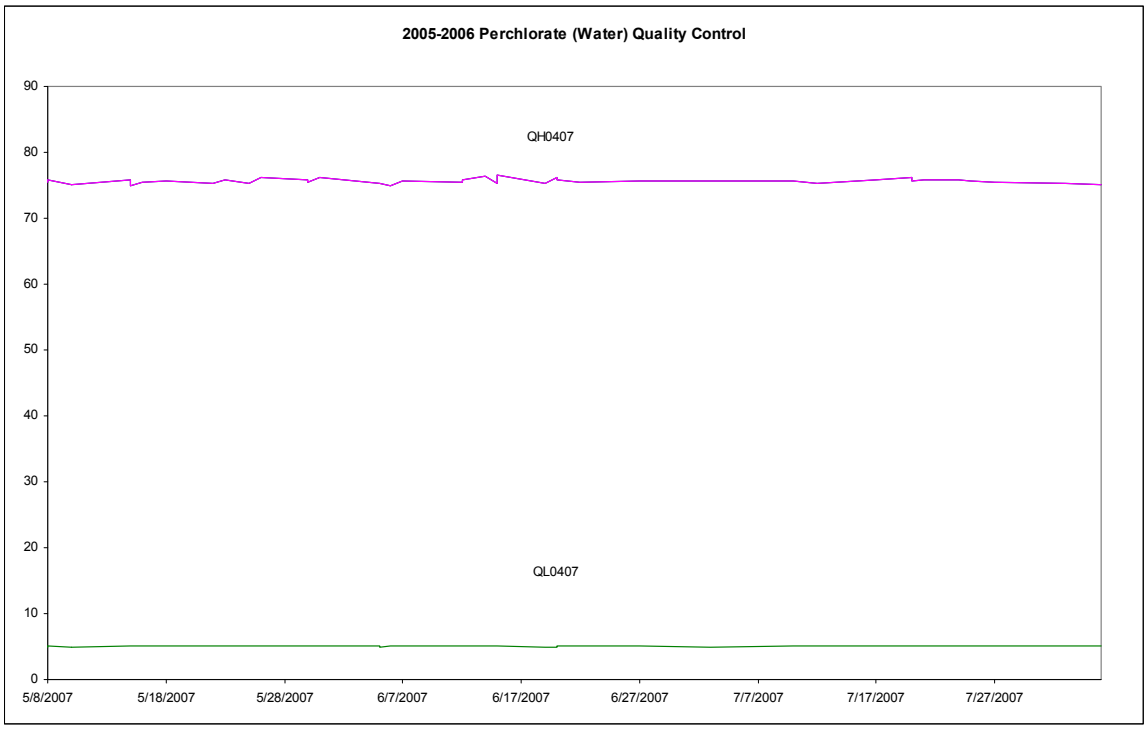
Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers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

19. Summary Statistics and QC Graphs

A. Perchlorate in water

Summary Statistics for Perchlorate (Water) by Lot

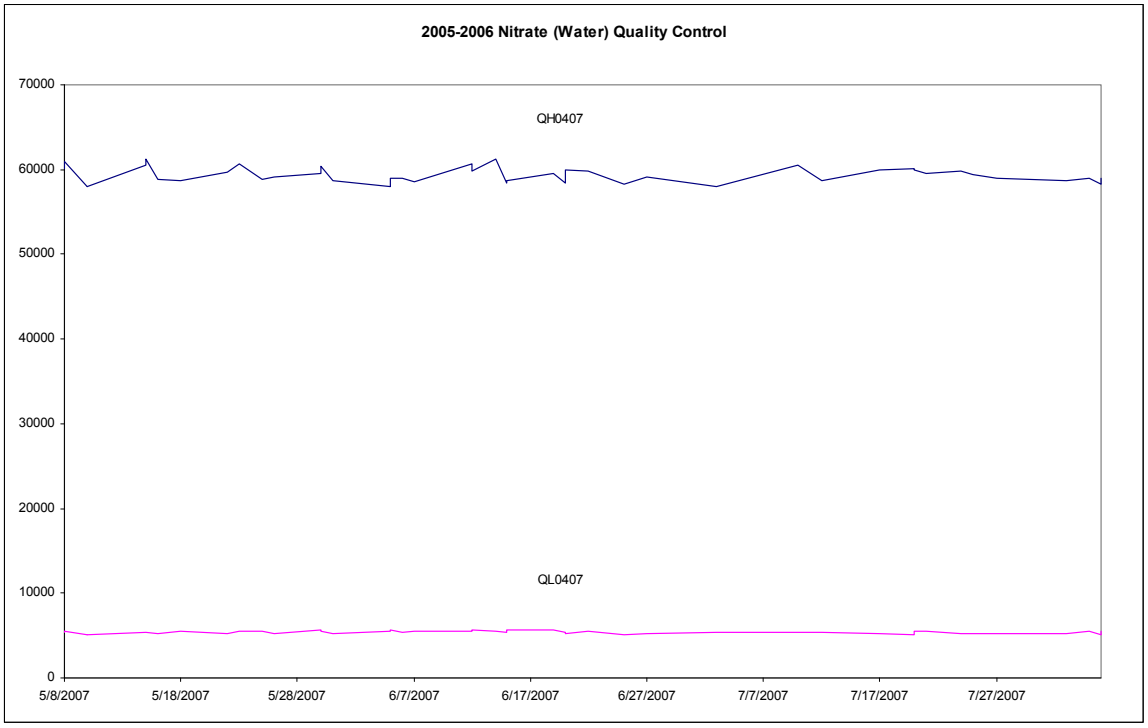
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL0407	40	5/8/2007	8/5/2007	5.040	0.038	0.7
QH0407	40	5/8/2007	8/5/2007	75.621	0.363	0.5



B. Nitrate in water

Summary Statistics for Nitrate (Water) by Lot

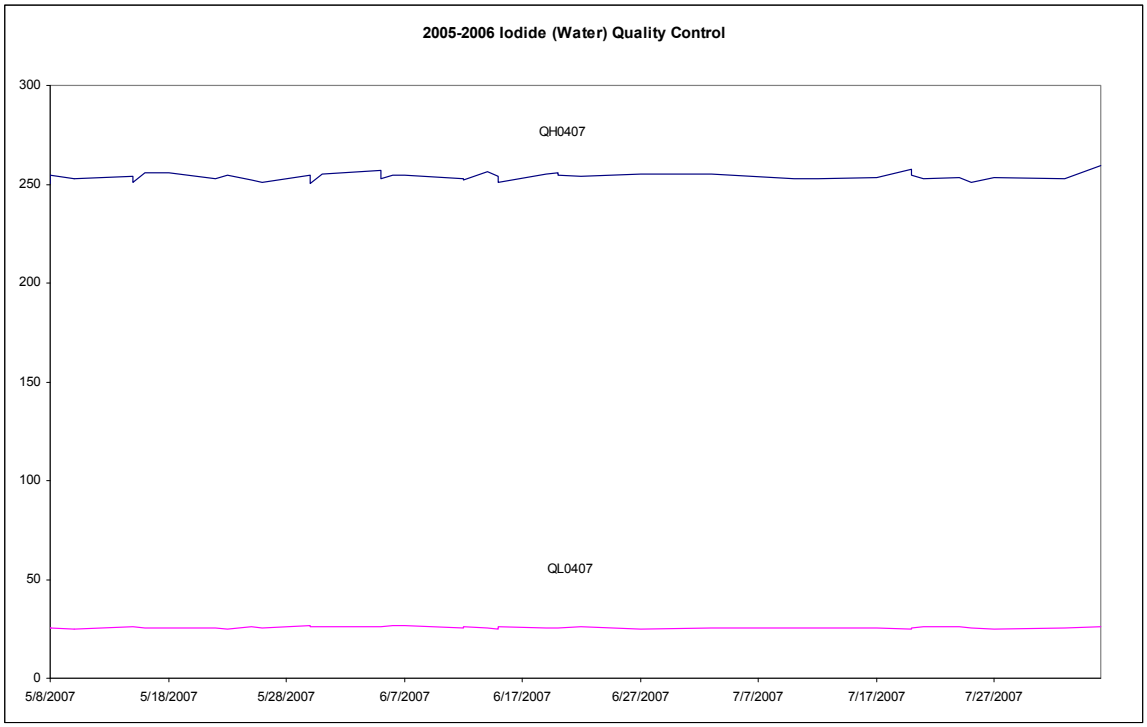
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL0407	43	5/8/2007	8/5/2007	5391.977	158.404	2.9
QH0407	43	5/8/2007	8/5/2007	59391.861	891.222	1.5



C Iodide in water

Summary Statistics for Iodide (Water) by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL0407	40	5/8/2007	8/5/2007	25.679	0.477	1.9
QH0407	40	5/8/2007	8/5/2007	253.825	1.992	0.8



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