Laboratory Procedure Manual

Analyte: **Trihalomethanes/MTBE/Nitromethane**

Matrix: **Whole Blood**

Method: **Solid Phase Microextraction with GC Separation/High Resolution MS**

Method No: **2101.01**

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As performed by:

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

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
VOCMWB_G & VOCMWS_G	LBXVBF	Blood Bromoform (pg/mL)
	LBXVBM	Blood Bromodichloromethane (pg/mL)
	LBXVCF	Blood Chloroform (pg/mL)
	LBXVCM	Blood Dibromochloromethane (pg/mL)
	LBXVME	Blood MTBE (pg/mL)
	LBXVNM	Blood Nitromethane (pg/mL)

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

The prevalence of disinfection by-products in drinking water supplies has raised concerns about possible adverse health effects from chronic exposure to these potentially carcinogenic compounds. To support studies exploring the relation between exposure to trihalomethanes (THMs), nitromethane (NM: biomarker for halonitromethanes), methyl *tert*-butyl ether (MTBE) and adverse health effects, an automated analytical method was developed using capillary gas chromatography (GC) and high-resolution mass spectrometry (MS) with selected ion mass detection and isotope-dilution techniques. This method quantifies trace levels of THMs (chloroform, bromodichloromethane, dibromochloromethane, and bromoform), nitromethane, and methyl *tert*-butyl ether (MTBE) in human blood (1,2).

b. Test Principle

THMs, NM, and MTBE are measured in whole blood by solid phase microextraction/gas chromatography/isotope dilution mass spectrometry (SPME/GC/isotope dilution MS) based on the method described by Bonin, et al. (1). The analytes are in equilibrium between the whole blood matrix and the headspace above the sample. A solid-phase microextraction (SPME) fiber is inserted into the headspace and the volatile organic compounds (VOCs) partition into the phase on the outside of the fiber shaft. The SPME fiber is then inserted into the heated GC inlet where the VOCs thermally desorb into the carrier gas stream. Extracted VOCs are focused at the head of the GC column using a cryogenically cooled trap (cryo-trap). Analytes are separated on a DB-624 capillary column and quantified using multiple ion detection (MID) mass spectrometry (high resolution at 10000 mass resolution using a Finnigan MAT 95XP mass spectrometer). Comparison of relative response factors with known standard concentrations yields individual analyte concentrations. The method is applicable to the determination of five VOCs in 3-mL samples of whole blood with detection limits in the low parts per trillion (pptr) range. Since non-occupationally exposed individuals have blood VOC concentrations in this range, the method is applicable for determining these quantities and investigating cases of low-level exposure to VOCs. This method proved adequate for measuring the THMs, NM, and MTBE in most blood samples tested from a diverse U.S. reference population (1).

2. Safety Precautions

a. Reagent toxicity or carcinogenicity

ALL OF THE COMPOUNDS USED IN THIS STUDY ARE HAZARDOUS CHEMICALS! Use a high draft fume hood and lower all the sashes to recommended operating height when working with neat (undiluted) materials or highly concentrated solutions since a number of these compounds are toxic. Wear nitrile gloves when

handling hazardous chemicals to prevent absorption through the skin. Some of the compounds used in this study are known or suspected carcinogens, mutagens and/or teratogens.

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 blood specimens. 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 gas chromatograph or 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 auto-sampler 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

Standard safety precautions should be followed 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

Formal training in the use of the gas chromatograph and mass spectrometer is necessary. Users are required to read the operation manuals and should demonstrate safe techniques in performing the method. Users should be trained in use of all other potentially hazardous equipment, including centrifuges, chemical fume hoods, etc.

g. Personal hygiene

Follow Universal Precautions. Care should be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing should be practiced. 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 wastes

Waste materials must be disposed of in compliance with CDC laboratory, federal, state, and local regulations. Solvents and reagents should always be disposed in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. Disposable plastic, glass, and paper (pipette tips, vacutainers, gloves, etc.) that contact blood are to be placed in a biohazard autoclave bag. The biohazard autoclave bags should be kept in appropriate containers until sealed and autoclaved. Wipe down all surfaces with fresh 70% ethanol solution when work is finished. Disposable needles used to remove blood from syringes should be placed immediately into a sharps container and autoclaved when the sharps container becomes full. All syringes and other non-disposable glassware that contact blood should be decontaminated with a freshly prepared bleach solution (a 10% dilution of commercial sodium hypochlorite (bleach) or equivalent) before re-use or disposal. Commercial sodium hypochlorite solutions contain significant amounts of chloroform and bromodichloromethane that can contaminate samples; routine disinfection with bleach should therefore be isolated from preparatory areas and VOC blood samples.

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/DLS guidelines for disposal of hazardous waste.

All syringes and other non-disposable glassware that contact blood should be decontaminated with a freshly prepared bleach solution (a 10% dilution of commercial sodium hypochlorite (bleach) or equivalent) before re-use or disposal.

3. Computerization; Data-System Management

a. Software and knowledge requirements

This method has been validated using the solid phase microextraction technique coupled with a gas chromatography and MAT 95 XP mass spectrometer run with the Thermo Finnigan Xcalibur 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

Information pertaining to particular specimens is entered into the database either manually or electronically transferred. The result file is transferred electronically into the database. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier.

c. Data maintenance

Integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data, storing of data in multiple computer systems, and redundantly archiving data. Original data files contain traceable header information (date, run number, sample type and sample identification) and are stored in duplicate on two separate recordable compact disks. Once the compact disk (CD) is filled and verified for integrity, both copies are permanently archived. Thus, two copies of all data are available to be accessed at any time. One CD is stored in an office adjacent to the laboratory and the other is stored in a separate facility. Data is transferred through a CD to the shared network drive along with relevant meta-data (including peak integrations, calibration curves, blanks, and isotope corrections). Processed results files are transferred electronically into the local area network (LAN) and stored in a shared directory. Data is loaded into the Microsoft Access database system using an automated data import module. The data files are backed up onto CD and tape media.

Routine backup procedures include: 1) weekly backup of hard disks; 2) daily backup of Access database files; 3) weekly backup of database files onto CD. A separate, backup tape database is stored off site. Either the supervisor or the local area network manager should be contacted for emergency assistance

d. Information security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of 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

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

b. Sample collection

Isopropyl alcohol, which may be used to disinfect the venipuncture site, can contaminate the collected sample and cause nonspecific interferences in the analytical measurement process. Isopropyl alcohol contamination can be easily prevented by swabbing the venipuncture site with a dry gauze bandage and allowing the site to dry for 5 to 10 sec after wiping with isopropyl alcohol.

The specimen type is whole blood collected in specially prepared, gray-top glass vacutainers containing potassium oxalate and sodium fluoride (2). Blood collection tubes (vacutainers) obtained from commercial sources commonly contain VOC contaminants that can mask the levels of VOC analytes originally in the blood at the time of sample collection, and thus prevent accurate exposure assessment. Vacutainers are obtained commercially and specially modified by laboratory staff (DLS VOC laboratory) to remove measurable levels of most VOCs present (see VOC Standard Operating Procedures [SOP] Manual - "Vacutainer Cleanup 1.0"). The SOP is based on our previously published research into VOC contamination from blood collection tubes (2). It is absolutely imperative that these specially treated vacutainers be used for all VOC blood collection to insure sample validity. The vacutainer tubes are supplied by DLS Lab staff for all VOC studies.

c. Sample handling

The Centers for Disease Control and Prevention (CDC)-prepared vacutainers contain milligram quantities of potassium oxalate and sodium fluoride. Potassium oxalate and sodium fluoride function to inhibit the metabolism of the analytes and prevent coagulation. Metabolic inhibition increases sample shelf life by minimizing metabolic impact on blood VOC levels during storage. Potassium oxalate and sodium fluoride do not prevent the clotting of blood as well as many other anticoagulants. Thus after collection, the samples must be mixed thoroughly to allow complete distribution of the anticoagulant. If a blood mixer is available, then samples should be placed on the mixer for at least 3 min. If a mixer is not available, then the blood can be mixed by hand by inverting the tube approximately 30 times. Since VOCs are highly volatile, care must be taken to insure that samples are kept at refrigerator temperatures, 4^{o}C, during storage and shipment. All samples should be placed on wet ice or into a refrigerator within 30 min of sample collection. In addition, samples should be shipped with enough wet ice or equivalent cooling material to insure that the samples will remain cool (but not frozen) throughout the shipment process. Samples should be shipped to insure that the samples will arrive at the CDC on normal business days to

insure the proper processing of the samples upon arrival. Samples should not be frozen or stored at freezer temperatures at any time during sample collection and shipment. Samples should be shipped within 1 to 2 days of collection so that the samples can be analyzed within 2 to 3 weeks of collection. For further detail please refer to the VOC SOP Manual - "VOC Blood Sample Collection 1.0". The analytes measured by this method have demonstrated stability in specimens stored for at least 10 weeks at refrigerated temperatures, 4–10 °C.

d. Sample quantity

The optimal amount of specimen required for analysis is 10 mL; the minimum amount is 3 mL.

e. Unacceptable specimens

The criteria for unacceptable specimen are a low volume (< 3 mL), failure to maintain sample temperature between 0 °C and 10 °C, suspected contamination, use of an untreated vacutainer, and significant clotting of the specimen. Clotting can occur due to the failure to properly mix the sample as described above.

Failure to obtain adequate sample volume is obvious when the samples are received. Visual inspection of the vacutainer reveals if the estimated blood volume is less than the required 3 mL. Maintenance of temperature during shipment is verified by examining the shipment temperature upon receipt. Clotting is indicated by failure of the sample to flow when the vacutainer tube is inverted. A description of reasons for each rejected sample is recorded in the Access database as the samples are logged into the lab.

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

1) <u>Solvents</u>

Solvents used during the development, validation, and application of this method are listed below.

Purge and Trap grade methanol is required for all dilutions of native standards and labeled analogs. Other grades of methanol typically contain unacceptable levels of THM contamination. Purge and trap grade methanol is available from Burdick and Jackson (Muskegon, MI).

HPLC grade water of acceptable purity has been acquired from Baker-Mallinckrodt. Variability in the contaminant levels in this product requires the testing of product lots. Once an acceptable lot has been found, a 1-year supply of water is purchased to insure an adequate supply. The purchased water is further processed by helium sparging and distillation to reduce VOCs before use. Directions for this procedure are given below under Section 6.d, and are based on previously published techniques for removing residual VOCs from reagent water (3).

2) Calibration and Control Materials

Material used for preparation of calibration standards, labeled internal standards, and Quality Control materials are listed in Table 1 below. All chemicals are used without further purification unless otherwise noted, and final concentrations of prepared materials are calculated using the purity specified with the raw material lot. Materials procured from other sources should meet or exceed these listed requirements.

Table 1: Reagents for Calibration and Control Materials

Compound	Formula	Acceptable Grade	Safety	Source
Chloroform	$CHCl_3$	99%	a, b	f
Bromodichloromethane	$CHCl_2Br$	98%	a, b	f
Dibromochloromethane	$CHClBr_2$	98%	e	f
Bromoform	$CHBr_3$	99%	a, c	f
tert-Butyl Methyl Ether	$(CH_3)_3COCH_3$	99%	d, e	g
Nitromethane	CH_3NO_2	99%	d	f
Chloroform-^{13}C	$^{13}CHCl_3$	99%	a, b	h
Bromodichloromethane-^{13}C	$^{13}CHCl_2Br$	99%	a, b	i
Dibromochloromethane-^{13}C	$^{13}CHClBr_2$	92%	e	i
Bromoform-^{13}C	$^{13}CHBr_3$	99%	a, c	h
tert-Butyl Methyl Ether-$^{13}C_3$	$(^{13}CH_3)_3COCH_3$	99%	d, e	j
Nitromethane-^{13}C	$^{13}CH_3NO_2$	99%	d	f

Key: a - Cancer suspect agent
b - Toxic
c - Lachrymator
d - Flammable liquid

e - Irritant
f - Aldrich Chem. (Milwaukee, WI)
g - Chem Service (West Chester, PA)
h - Cambridge Isotope Laboratories (Woburn, MA) - available commercially

b. Preparation of glassware

All glassware used in this study is carefully cleaned to be certain to remove possible nonspecific VOC contamination. To remove possible analytical interferences, rinse glassware (volumetric flasks, ampoules, and storage bottles) with reagent-grade methanol, and heat at 150°C in a vacuum oven with an independent vacuum source for at least 8 hrs. to remove adsorbed VOCs. The independent vacuum source is necessary to prevent cross-contamination from other laboratory operations. There is the risk of changing the calibration of volumetric glassware by heating, but the error resulting from this is small compared to other sources of error in the VOC method. Cool the glassware to room temperature under vacuum and restore pressure using nitrogen (UHP grade). Remove treated glassware from the oven and store sealed with Teflon-lined caps until used in standard preparation.

c. Preparation of vacutainers

Vacutainers containing potassium oxalate/sodium fluoride are specially treated to remove VOC contamination resulting chiefly from the rubber stopper. The individual vacutainers are disassembled and the glass tubes and rubber stoppers are both heated as described in the VOC SOP Manual - "Vacutainer Cleanup 1.0". The vacutainers are reassembled, the vacuum restored and the vacutainers are sterilized using a Cobalt-60 radiation source. This process produces a sampling container with substantially less contamination.

d. Preparation of Blank water

1) Apparatus

Distillation of the raw water is accomplished using a Fuchs continuous reflux apparatus which has been modified to run with helium stripping during the distillation process.

2) Procedure

(a) Water distillation

Fill the 3000-mL 2-neck flask with approximately 2500 mL of HPLC grade water. Add 10 micro-porous boiling chips to the flask and seal the device. Adjust the helium flow to produce an active flow through the sparger. Allow the helium to bubble through the raw water for approximately 17 hr. After 17 hr, turn on the heating mantle to bring the water to a boil. Allow the water to reflux for approximately 4 hr. At the end of the reflux period, begin collecting the finished blank water. Dispense the finished water into 100-mL

glass stoppered Pyrex bottles and cap immediately as described in Section 6.b. If more blank water is needed, allow the storage head to refill and repeat the process.

(b) Water storage

The blank water is either used directly from the glass stoppered Pyrex bottles or stored in 2, 5, and 20-mL flame sealable Pyrex ampoules. Water is aliquoted for storage by transfer from the Pyrex bottles using a Portapet Pipetter equipped with a 10-mL long tip serum pipette. A torch (natural gas and oxygen fuel) is used to melt the ampoule neck to produce a gas-tight seal. A tight seal is checked by tapping the sealed end on a hard surface and looking for a water leak. The sealed ampoules are stored in the dark at room temperature.

e. Preparation of native analytical standards

1) Procedure for handling neat compounds

All analytes are purchased as neat liquids in flame sealed ampoules. After opening the ampoule the remaining (unused) material is discarded. A few of the most expensive analytes (custom synthesis products) are aliquoted into an individual amber borosilicate glass container and flame sealed for future use. After transferring the compounds, flame seal the ampoule and store in an explosion-proof freezer. Cover all containers with aluminum foil to reduce light exposure to all neat compounds. Store neat standards in a separate location from blood samples, blanks and quality control materials.

2) Procedure for filling and sealing glass ampoules

Aliquot about 0.2 mL of the neat standard into a liquid nitrogen cooled 1-mL borosilicate glass vacule. Place the vacule in a metal rack that has been cooled with liquid nitrogen. Always wear cryo-gloves and a face shield when handling liquid nitrogen. If there are a substantial number of vacules to be flame sealed (over 25), then the metal rack should be continuously cooled in an insulated container that is filled with liquid nitrogen. The cooling of the vacule will require approximately 1 min. If the vacule gets too cold and builds up frozen condensate, then oxygen and/or nitrogen will dissolve in the liquid and can cause the vacule to shatter or develop leaks that can compromise the standard. Remove the vacule from the cooled metal rack. Use a glass capillary and zero headspace micro-dispensor to transfer the liquid. Before using, rinse the pipette by initially filling with the neat standard and expelling to waste. (NOTE: There may not be enough neat standard to perform this rinse step). Make sure the liquid is placed in the bottom of the vacule and is not adhering to the neck. Otherwise, during the sealing procedure, ignition of the liquid will produce a loud pop and could shatter the vacule. Use a torch that is fueled with ultra high purity grade methane and oxygen to seal the vacule by melting and pulling the molten

neck to affect a seal. Dark filter protective eyewear is required during flame sealing to shield eyes from the arc of the flame. Allow the sealed vacule to come to room temperature in a separate storage rack. In a chemical fume hood, invert the vial and gently tap the sealed end on a lab wipe-lined hard surface. If the vacule does not leak, the vacule is ready to label and store. If a leak does occur, do not attempt to reseal the vacule. Dispose of the vacule and make a new one. Repeat the above steps until at least 12 sealed vacules are made. Place the sealed vacules in an appropriate holder and store in a freezer at -70 °C.

3) Transfer of liquids used in making standards

Positive displacement pipettes are used for all transfer of liquids in the µL range. Transfers in the 5-µL to 30-µL range use a pipette with 0.1-µL increments. Transfers in the 31-µL to 100-µL range use a pipette with 0.2-µL increments. Transfers in the 101-µL to 250-µL range use a pipette with 1-µL increments. Volumetric flasks, 25 mL class A, are used to make all standards. Standard concentrations are based on the gravimetric measure of mass transferred to the volumetric flask. Standards are prepared in methanol (purge and trap grade).

4) Balance calibration

Before sample weighing procedures for each standard set, the balance must be calibrated and checked for accuracy. With an empty pan, select the 160 g/0.1 mg weighing range. Tare and then wait for stability. Press the CAL button; the display will show 'C' and the busy symbol. When 'CC' appears, press the CAL button again, completing the calibration stage. Check the balance accuracy by weighing the 50 g weight. Record weight check results in the “Sartorius Analytical Balance Maintenance Log book”.

5) Final concentrations of the standards

Standards are prepared in helium-sparged, distilled water at levels that are determine accurately, at the approximate concentration levels listed in Table 2 below. Preparation steps are listed in Sections 6.e.6 & 7 below.

Table 2: THM/MTBE calibration standards.

Analyte	Concentration in final 25 mL water standard (pptr)							
	7.204	**6.204**	**5.204**	**4.204**	**3.204**	**2.204**	**1.204**	**0.204**
Chloroform	490	196	78.4	39.2	19.6	5.88	1.96	0.78
Bromodichloromethane	390	156	62.4	31.2	15.6	4.68	1.56	0.62
Dibromochloromethane	389	155	62.2	31.1	15.5	4.66	1.55	0.62
Bromoform	357	143	57.1	28.6	14.3	4.29	1.43	0.57
Methyl tert-Butyl Ether	497	199	79.6	39.8	19.9	5.97	1.99	0.80
Nitromethane	7205	2882	1153	576	288	86.5	28.8	8.65

6) Stock solutions and concentrated standards

Add about 20 mL purge and trap grade methanol to eleven 25-mL volumetric flasks. Label these flasks 1 through 11. (Keep all flasks sealed when not directly adding standard materials.)

Weigh the flask labeled “1" accurately to 0.001 g. Set aside flasks labeled 2 through 11. Sequentially add the following neat compounds listed in Table 3 below to the flask labeled “1" and determine the total weight, to 0.001 g, between each addition. Keep the lid tightly screwed onto the flask unless volume is being added, minimizing the loss of methanol and/or analyte due to evaporation.

Table 3: Formulation of stock solution 1.

Analyte	Volume (µL)	Density (g/mL)	Expected Wt. (mg)
Chloroform	53	1.48	76.6
Bromodichloromethane	30	1.98	60.9
Dibromochloromethane	25	2.45	60.7
Bromoform	20	2.89	55.8
Methyl tert-Butyl Ether	110	0.74	77.7
Nitromethane	1000	1.136	1136

Once all of the neat standards are added to the flask labeled “1", fill the flask to the 25 mL mark with purge and trap grade methanol. Invert flask “1” 5 times and sonicate for 3 min to insure complete mixing. Stock solution S1 is now prepared and ready to be further diluted for the preparation of standards.

Use the flask labeled “2” to dilute 500 µL of stock solution S1 in 25 mL of purge and trap grade methanol. Invert flask “2” 5 times and sonicate for 3 min to insure complete mixing. Stock solution S2 is now prepared and ready to be further diluted for the preparation of standards.

Use the flask labeled “3” to dilute 500 µL of stock solution S2 in 25 mL of purge and trap grade methanol. Invert flask “3” 5 times and sonicate for 3 min to insure complete mixing. Stock solution S3 is now prepared and ready to be further diluted for the preparation of standards.

Prepare working standards 0 - 7 by preparing dilutions of stock solutions S2 and S3 as listed in Table 4 below.

Table 4: Formulation of calibration standards.

Standard number	Stock Solution Used	Volume Added (µL)	Final Volume (mL)
0	S3	20	25
1	S3	50	25
2	S3	150	25

3	S3	500	25
4	S2	20	25
5	S2	40	25
6	S2	100	25
7	S2	250	25

Mix all solutions by sonicating for 3 min. Label the ampoules with the standard level (0 - 7) and a standard batch identifier. For example "2.204005", where “2" identifies the standard made (as given in column 1 above) and “204" indicates that this is the batch number of standards produced. Dispense the standard solutions into glass ampoules and flame seal as described above in Section 6.e.2. Make at least 100 sealed ampoules of each standard, 0 - 7. Label each ampoule with a sequential 3 digit number indicative of the preparation order of that aliquot and sealed (e.g. 005 is the fifth aliquot prepared for the above standard 2. After leak checking the flame sealed ampoules as described in Section 6.e.2, place the ampoules in a 10 x 10 grid box and store in a freezer at approximately -70 °C.

7) Daily standard

Daily standards are made by taking particular concentrations of the above ampoules and diluting in helium sparged/distilled water. For example, standard 5.204003 is created by taking 40 µL from ampoule 5.204003 and diluting in 25 mL of helium-sparged, distilled water.

f. Preparation of labeled analog solutions

1) Procedure for handling neat compounds

The majority of the labeled compounds contain ^{13}C and are shipped in ampoules or thick walled glass containers of various sizes. It is advisable to cool these containers in ice before opening in order to minimize pressurization and volatilization problems.

2) Procedure for storing labeled analog solutions

Aliquot the appropriate amount of primary or secondary analog solution into a 1-mL flame-sealable borosilicate glass ampoule. Use a glass Pasteur pipette to transfer the solution. Before using the pipette, rinse by initially filling the pipette with the solution and expelling to waste. Make sure the solution is placed in the bottom of the ampoule and is not adhering to the neck of the ampoule. Otherwise, during the sealing procedure, the ignition of the methanol will produce a loud pop and could shatter the ampoule. Place the ampoule in a Dewar containing liquid nitrogen until the solution is cooled, but not frozen. This will require approximately 5 sec. If the ampoule remains in the liquid nitrogen for too long, then oxygen and/or nitrogen will dissolve in the liquid and can cause the

ampoule to shatter or develop leaks that can compromise the standard. Remove the ampoule from the Dewar. Use a propane/oxygen torch to seal the ampoule by pulling the molten neck to create a gas-tight seal. Allow the sealed ampoule to come to room temperature then invert the vial and tap the sealed end on a hard surface. If the seal does not leak, the ampoule is ready to store. If a leak does occur, do not attempt to reseal the ampoule. Dispose of it and make a new one.

3) Final concentrations of the labeled solutions

Labeled standards are prepared at the approximate concentrations listed in Table 5 below. Preparation steps are listed in Sections 7.e.4, 5, & 6 below.

Table 5: THM/MTBE/NM labeled internal standards.

Analyte	Concentration (pptr)
Chloroform	30
Bromodichloromethane	10
Dibromochloromethane	10
Bromoform	25
Methyl tert-Butyl Ether	60
Nitromethane	460

4) Primary analog stock solutions

Primary analog stock solutions are made by initial dilution of the neat compound into 25 mL of purge and trap grade methanol. This provides a consistent source of these compounds for further dilutions. For each analog add approximately 20 mL purge and trap grade methanol to a 25-mL volumetric flask. Keep the flask sealed when not directly adding standards. Label the flask and dilute the compounds according to the scheme described in Table 6 below:

Table 6: THM/MTBE/NM labeled internal stock solutions.

Standard	Compound	Volume or Weight - µL(g)	Approximate Concentration (ppm)
L12	^{13}C-Chloroform	25 (0.0370)	1480
L18	^{13}C-Bromodichloromethane	10 (0.0198)	792
L19	^{13}C-Chlorodibromomethane	10 (0.0245)	980
L19	^{13}C-Bromoform	25 (0.0723)	2890
L38	$^{13}C_3$ Methyl tert-Butyl Ether	100 (0.0740)	2960
LNM	^{13}C-Nitromethane	55 (0.0539)	2156

Fill the flasks to the 25-mL mark with purge and trap grade methanol. Seal approximately 0.75 mL of these solutions in ampoules as described above. Repeat these steps until as many sealed ampoules (at least 20) as possible are

made. Label and place the sealed ampoules in an appropriate holder and store in a freezer at approximately -70 °C.

5) Secondary analog stock solutions

Label and fill a 50-mL volumetric flask with approximately 45 mL of purge and trap grade methanol. Add the following solutions (L12: 37.5 µL; L18: 22.5 µL; L19: 18.75 µL; L24: 15 µL; L38: 37.5 µL; LNM: 400 µL) to give solution L. Fill to the mark with purge and trap grade methanol and mix the solution by sonicating for 3 min. Seal approximately 150 µL of these solutions in ampoules as described above in Section 6.f.2. Repeat these steps until as many sealed ampoules as possible are made (at least 50). Label and place the sealed ampoules in an appropriate holder and store in a freezer at approximately -70 °C.

6) Working analog stock solutions

Prepare the working stock solution for a given 2-week period of analysis by adding 50 µL from solution L to a 25-mL flask containing approximately 25 mL purge and trap grade methanol. This solution can be reused for 2 weeks if it is sealed thoroughly and stored at approximately -20 °C. To achieve the desired concentrations of internal standard, pipette 40 µL of the working analog stock solution into each sample (blood, QC, water blank or aqueous standard).

g. Preparation of Quality Control materials

QC Materials are prepared with the final concentrations (pptr) given in Table 7 below. These concentrations allow for the loss of some compounds by volatilization during the mixing step.

Table 7: Approximate concentrations of THMs, MTBE, and NM for low and high QC pools

Analyte	Low QC (pptr)	High QC (pptr)
Chloroform	24	90
Bromodichloromethane	8	25
Dibromochloromethane	7	22
Bromoform	9	26
tert-Butyl Methyl Ether	26	120
Nitromethane	800	1500

1) Pretreatment of serum

Screen stock serum samples for inordinate levels of ethanol and VOCs before further processing. Much of the VOC contamination can be removed by incubating the serum with PDMS septa. Add approximately 100 VOC-free PDMS septa (Supelco) to 500 ml serum bottle and stored in refrigerator at

approximately 4°C for 48 hr. Pour the pre-filtered calf serum from the serum bottle into a three-neck, 2000-mL round bottom flask.

2) Spiking serum for QC preparation

(a) Spiking serum if PDMS septa are used to lower the VOC levels

Spike the relatively VOC free serum by adding the appropriate amount of each spiking solution into serum to yield each QC material. After all the spiking solutions have been added, stopper the flask and swirl the contents for approximately 2 min to mix. Place the stoppered flask on cold packs for approximately 30 min. About approximately every 15 min, swirl the serum for approximately 1 min. Continue this procedure until the QC material has been aliquoted and sealed into glass ampoules.

(b) Spiking serum if used as received without PDMS septa treatment

Fetal bovine serum can be obtained with low enough levels of MTBE and the measured THMs for use without resorting to PDMS septa treatment. In this case, before spiking, cool the serum in a flask using cold packs instead of ice water to prevent the introduction of chloroform from the city water supply. About approximately every 15 min, swirl the serum for approximately 1 min. Continue this procedure until the QC material has been aliquoted and sealed into glass ampoules.

3) Preparation of stock solutions Q2 and Q1 for low QC

Add approximately 20 mL purge & trap grade methanol to a 25-mL volumetric flask. Keep the flask sealed when not directly adding standards. Add the following neat compounds: bromodichloromethane, 30 µL; bromoform, 20 µL; chloroform, 50 µL; dibromochloromethane, 25 µL; tert-butyl methyl ether, 500 µL; Nitromethane, 500 µL. Fill the flask to the 25 mL mark with purge & trap grade methanol to give stock solution Q2.

Dilute 200 µL of solution Q2 in 25 mL of purge & trap grade methanol to give solution Q1.

Dilute 200 µL of solution Q1 in 25 mL of purge & trap grade methanol to give spiking solution A.

Table 8: Calculations of approximate concentrations of spiking solution A for low QC

Analyte	Density (g/ml)	Volume (µL)	Exp wt (g)	Soln Q2 (10^6 pg/ml)	Soln Q1 (10^6 pg/ml)	Spike Soln A (10^6 pg/ml)
Bromodichloromethane	1.98	30	0.059	2400	19	0.15
Bromoform	2.89	20	0.058	2300	18	0.14
Chloroform	1.48	50	0.074	3000	24	0.19
Dibromochloromethane	2.45	25	0.061	2400	19	0.15
MTBE	0.74	500	0.370	15000	120	0.96
Nitromethane*	1.136	1000	1.136	22516	450	4.5

*For nitromethane: Q2 solution was prepared in 25ml and spike Q1 solution made from 500 µL of Q2 in 25 ml methanol. Spike solution A was made from 250 µL of Q1 in 25 ml methanol.

4) Preparation of stock solutions Q4 and Q3 for high QC

Add approximately 20 mL purge & trap grade methanol to a 25-mL volumetric flask. Keep the flask sealed when not directly adding standards. Add the following neat compounds: bromodichloromethane, 30 µL; bromoform, 20 µL; chloroform, 150 µL; dibromochloromethane, 25 µL; methyl tert-butyl ether, 500 µL. Fill the flask to the 25 mL mark with purge & trap grade methanol to give stock solution Q4.

Dilute 200 µL of solution Q4 in 25 mL of purge & trap grade methanol to give solution Q3.

Dilute 200 µL of solution Q3 in 25 mL of purge & trap grade methanol to give spiking solution B.

Table 9: Calculation of approximate concentrations of spiking solution B for high QC

Analyte	Density (g/ml)	Volume (µL)	Exp wt (g)	Soln Q4 (10^6 pg/ml)	Soln Q3 (10^6 pg/ml)	Spiking Solution B (10^6 pg/ml)
Bromodichloromethane	1.98	30	0.059	2400	19	0.15
Bromoform	2.89	20	0.058	2300	18	0.14
Chloroform	1.48	150	0.222	8880	71	0.57
Dibromochloromethane	2.45	25	0.061	2400	19	0.15
MTBE	0.74	500	0.370	15000	120	0.96
Nitromethane	1.136	500	1.136	22300		44.6

*For nitromethane: Q4 solution was prepared in 25ml and spike solution B made from 50 µL of Q4 in 25 ml methanol.

5) Preparation of Low QC Material

Add approximately 40 µL of spiking solution A to 1500 ml of fetal bovine serum. Keep the flask sealed when not directly adding standards. Also add 50 µL of spike solution A of nitromethane to 1500 ml of fetal bovine serum.

6) Preparation of High QC Material

Add approximately 200 µL of working solution B to 1500 ml of fetal bovine serum. Keep the flask sealed when not directly adding standards. Also add 20 µL of spike solution B of nitromethane to 1500 ml of fetal bovine serum.

Table 10: Calculated concentrations of analytes for low and high QC

Analyte	Calculated concentration of QL206 (pg/ml)	Calculated concentration of QH206 (pg/ml)
Bromodichloromethane	4.0	20
Bromoform	3.7	19
Chloroform	5.0	76
Dibromochloromethane	4.0	20
MTBE	25.6	128
Nitromethane	800	1500

h. Proficiency Testing (PT) Materials

Proficiency Testing materials are prepared from neat compounds in a manner similar to standard preparation. Several levels are prepared for all analytes to cover the calibration range. The PT materials are sealed in ampoules, blind coded and stored at approximately -70 °C until use. PT testing is performed twice a year and following any major maintenance of the instrumentation. Proficiency testing samples are blind coded for analysis; results are evaluated by an external quality control officer.

i. Storage of standard solutions

Except while in use, all standard stock solutions, labeled analog stock solutions, and quality control materials are stored at approximately -70 °C. The stock solutions can be stored for up to 2 weeks at approximately 4 °C. Once ampoules containing stock solutions have been opened, they must be used immediately. The working analog stock solution may be preserved and used over the next 2 weeks if it is carefully sealed and stored at approximately 4 °C within 8 hr of initial preparation. All stock solutions are labeled to include a reference to the preparation procedure, batch and date.

j. Supplies

Supplies used during the development, validation, and application of this method are listed below. Supplies procured from other sources should meet or exceed these listed requirements.

(a) Disposable Pasteur pipettes, (Kimble Glass, Inc., Marietta, GA) or equivalent
(b) Pipette bulbs (Fisher Scientific, Suwanee, GA) or equivalent
(c) Clear pre-scored ampoules; 2-mL, 5-mL, and 20-mL (Wheaton Scientific, Millville, NJ) or equivalent
(d) Clear glass vacuoles, 1-mL and 10-mL (Wheaton Scientific, Millville, NJ) or equivalent
(e) Fisher brand heavy glass desiccator, 250-mm I.D., with cover (Fisher Scientific, Suwanee, GA) or equivalent
(f) Graduated 10-mL glass pipette (Fisher Scientific, Suwanee, GA) or equivalent
(g) Portapet pipette, 10-mL volume (Fisher Scientific, Suwanee, GA) or equivalent
(h) High Vacuum grease, Dow Corning (Fisher Scientific, Suwanee, GA) or equivalent
(i) Stainless steel desiccator (Boekel scientific, Philadelphia, PA) or equivalent
(j) Activated charcoal (Fisher Scientific, Suwanee, GA) or equivalent
(k) Molecular sieve (EM Science, Lab Depot, Alpharetta, GA) or equivalent
(l) Ultra-high purity helium gas, 99.999% (Airgas South, Chamblee, GA) or equivalent
(m) Nalgene high-density polyethylene Dewar flask (Fisher Scientific, Suwanee, GA) or equivalent
(n) VWR positive displacement Micro-pipettors, 40-µL, 50-µL, 250-µL, (Lab Depot, Alpharetta, GA) or equivalent
(o) VWR replacement glass capillaries, 40-µL, 50-µL, 250-µL, (Lab Depot, Alpharetta, GA) or equivalent
(p) SMI positive displacement Micro-pipettors, 20-100 µL, 50-250 µL, (Lab Depot, Alpharetta, GA) or equivalent
(q) SMI replacement glass capillaries, 20-100 µL, 50-250 µL, (Lab Depot, Alpharetta, GA) or equivalent
(r) Pyrex volumetric flasks with screw caps, 25-mL (Fisher Scientific, Suwanee, GA) or equivalent
(s) Wheaton caps, No. 415-18 (LabSource, Inc., Chicago, IL) or equivalent
(t) Nitrile gloves, non-powdered (LabSource, Inc., Chicago, IL) or equivalent
(u) Ultrasonic cleaner with heater and timer, Model B-221 (Thomas Scientific, Swedesboro, NJ) or equivalent
(v) Liquid nitrogen, 160-L, 22-psi (Airgas South, Chamblee, GA) or equivalent
(w) Heavy-duty, micro polypropylene test tube rack, stackable, for 11-mm diameter tubes (Cole-Palmer Instruments Co., Chicago, IL) or equivalent
(x) SPME Vials, 10-mL (MicroLiter, Suwanee, GA) or equivalent
(y) Septa, Teflon Red/White silicone, Level IV (MicroLiter, Suwanee, GA) or equivalent
(z) Stainless steel washers, metric size 10-cm (Hillman Fastener Company, Cincinnati, OH) or equivalent
(aa) Hand-operated Crimper (Wheaton Scientific, Millville, NJ) or equivalent
(bb) Open Center seals 20-mm (SUPELCO, Bellefonte, PA) or equivalent
(cc) Oxygen, 99.99%, 200-300 cu. ft. (Airgas South, Chamblee, GA) or equivalent

(dd) Vacutainer brand sterile blood collection tubes, 10-mL draw, 16 X 100, potassium oxalate, sodium fluoride (Becton-Dickinson Vacutainer Systems, Rutherford, NJ) or equivalent
(ee) Gastight Teflon luer-lock tip syringe, 5-mL (Hamilton Company, Minneapolis, MN) or equivalent
(ff) Sharps container (Pro Tec US Clinical Products, INC., Richardson, TX) or equivalent
(gg) Fisher hematology mixer (Fisher Scientific, Suwanee, GA) or equivalent
(hh) Orbital shaker (Fisher Scientific, Suwanee, GA) or equivalent
(ii) Sodium hypochlorite (James Austin Co., Mars, PA) or equivalent
(jj) Flow tube, 150-mm, for helium 0 to 100 cc/min (Alltech Associates, Inc., Deerfield, IL) or equivalent
(kk) Adapter 1/8" to 1/8" MPT (Alltech Associates, Inc., Deerfield, IL) or equivalent
(ll) DB-624 Capillary Column, 0.20-mm I.D., 25-m, 1.12-µm film thickness (J&W Scientific, Folsom, CA) or equivalent
(mm) Standard Printer paper (local office supply) or equivalent
(nn) HP LaserJet 1200 and 5Si microfine toner cartridges (Government Technology Services, Inc., Atlanta, GA) or equivalent

k. Equipment

Equipment used during the development, validation, and application of this method are listed below. Equipment procured from other sources should meet or exceed these listed requirements.

(a) Distillation Equipment (Ace Glass, Inc., Louisville, KY)
  (i.) Twin connecting hose adapter
  (ii.) Column, vacuum jacketed
  (iii.) Condenser, Allihn
  (iv.) Head, Storage, 3000-mL
  (v.) Flask, two necks, 3000-mL
  (vi.) Mantle, 3-L
  (vii.) Powerstat, 0-140 volts
  (viii.) Teflon sleeves, 0.076-mm
  (ix.) Adapter, vacuum short stem, 14/20
  (x.) Teflon sleeves, 0.13-mm, 14/20
  (xi.) Bottle, single neck, 14/20 joint
(b) Squaroid vacuum oven, 2.3-cu. ft. (Fisher Scientific, Suwanee, GA)
(c) Direct-drive vacuum pump (Fisher Scientific, Suwanee, GA)
(d) Sartorius analytical balance (Fisher Scientific, Suwanee, GA)
(e) Bar code scanner (Wellch-Alleyn, Skaneateles Falls, NY)
(f) Datamax label matrix label printer (Computype, Inc., St. Paul, MN)
(g) Brady printer labels (Brady Worldwide, Milwaukee, IL)
(h) Ultra-low temperature freezer (Fisher Scientific, Suwanee, GA)
(i) Refrigerator (Fisher Scientific, Suwanee, GA)
(j) Standard laboratory freezer (Fisher Scientific, Suwanee, GA)

(k) QC Purging equipment (Ace Glass, Inc., Louisville, KY)
  (i.) Flask, three necks, vertical, 2000-mL, center neck 29/42, side necks, 24/40
  (ii.) Adapter, bushing, bottom inner 29/42 top outer 24/40
  (iii.) Stopper, full length 24/40
  (iv.) Stopper, medium length 14/20
  (v.) Adapter, Claisen, 24/40
  (vi.) Adapter, Distilling trap, 24/40
(l) Sterilized hood biological safety cabinet (Baker, Inc., Sanford, ME)
(m) Agilent 6890 Gas Chromatograph with sub ambient cooling (Agilent Technologies Co., Atlanta, GA)
(n) Finnigan MAT95XP mass spectrometer (Thermo Finnigan, Inc. Miami, FL)
(o) Hewlett Packard LaserJet 5Si and 1200 Printers (Hewlett-Packard Co., Atlanta, GA)
(p) Dell OPTIPLEX GX260 w/ 4.0 GB hard disk, 52 Mb RAM, flat screen monitor, and Pentium(IV) 2.4 GHz processor (Government Technology Services, Inc., Atlanta, GA)
(q) Microsoft Access based database (Government Technology Services, Inc., Atlanta, GA)
(r) PC SAS (Statistical Analysis System, Inc., Cary NC)

I. Instrumentation

1) MAT 95XP mass spectrometer (Thermo Finnigan) combined with a Combi-PAL auto-sampler (CTC Analytics) using SPME-fiber technology (Supelco) and with gas chromatographic separation (Agilent 6890 GC) using a 25-m DB-624 capillary column (J&W Scientific) with 1.12-µm film thickness and 200-µm internal diameter. The carrier gas is helium.

(a) Agilent 6890 GC Parameters

Table 11: Event Run Table

Time (min)	Switching Valve #	Set Point	Condition Initiated
0.00	8	ON	Cryo-Trap Cools to -100°C
0.01	7	ON	Closure Signal for MAT 95XP
0.05	7	OFF	Closure Signal for MAT 95XP
2.00	8	OFF	Cryo-Trap Heats to 220°C
20.00*	8	ON	Cryo-Trap Cools to -100°C

* Valve #8 must close (ON) before the end of GC run.
Event RUN Table (only exists in the GC microprocessor)

Injector type: Split/splitless inlet (back inlet)
- Injection mode: Splitless
- Purge flow: 70 mL/min
- Purge time: 2.00 min

- Inlet temperature: 250 °C
- Carrier gas: Helium
- Carrier gas control mode: Constant flow at 0.70 mL/min

Front Detector: 240 °C GC/MS transfer line

GC column: DB-624 (J&W Scientific)
- Length: 25 m
- Diameter: 200 µm
- Film thickness: 1.12 µm

GC Oven temperature program:
- Initial Temp: -1 °C for 3 min
- Ramp 1: 50 °C /min to 30 °C; hold at 30 °C for 1 min
- Ramp 2: 10 °C /min to 140 °C; no hold at 140 °C
- Ramp 3: 50 °C /min to 220 °C; hold at 220 °C for 3 min
- Equilibrium time: 0.05 min
- Run time: 20.22 min

Cryogenic cooling for GC Oven:
- Liquid nitrogen at 22 psi
- Quick cooling enabled
- Ambient temp: 25 °C
- Cryo timeout: 40 min
- Shutdown enabled

(b) Combi-PAL Auto-sampler (CTC Analytics) Parameters

PAL Method: B_VOC_03 (SPME Agitated & Heated)
- Carboxen-PDMS fiber 75 µm coating thickness
- Pre-incubation time: 10 sec
- Heated agitator temperature: 30 °C
- Agitator speed: 350 rpm
- Agitator on time: 5 sec
- Agitator off time: 2 sec
- Vial penetration: 22 mm
- Extraction time: 10 min
- Desorb to: GC Inj1
- Injection penetration: 50 mm
- Desorption time: 23:35 min
- GC run time: 20:24 min

Peltier Tray (CTC Analytics)

Peltier Tray uses solid state technology to cool the metal sample tray
- Temp set point: 15 °C
- Actual cooling range: 15-16 °C

(c) Cryo-Trap (Scientific Instrument Services, Inc)

Cryo-Trap used to trap volatiles on head of GC column by cryogenic cooling
- Temp set point: -100 °C
- Liquid nitrogen at 22 psi

Cryo-Trap desorbs trapped volatiles by ballistic heating
- Temp set point: 220 °C
- Electric Heater

The cooling and heating cycles of the Cryo-Trap are controlled by the Agilent 6890 GC through Switching Valve Driver #8 in the RUN Table.

(d) MAT 95XP (Finnigan MAT) Parameters

ICL Procedure: sleep.icl 5
Tune File: tune1
MCAL File: mcal1 (for small mass range)
Calibrate the EDAC (electric scan) using the ICL command:
.ecalib 131 169 5 (done once a week or after resetting the MAT 95XP)

Acquisition START is controlled by the Agilent 6890 GC through Switching Valve Driver #7 in the RUN Table.

Calibration and Lock Mixture: PFK (high boiling range) with n-butyl benzene
- Lock Masses used: 68.9947 (PFK for Time Segments 1 and 2), 118.9915 (PFK for Time Seqment 3), and 168.9882 (PFK for Time Segment 4).
- Cali Masses used: 91.0542 ($C_7H_7^+$ of n-butyl benzene for Time Segments 1 and 2), 130.9915 (PFK for Time Segment 3), and 180.9883 (PFK for Time Segment 4).
- Starting intensities for the Lock and Cali masses (at 10000 mass resolution [5% valley definition] at beginning of sample queue at the operational voltage of the electron multiplier): 130.9915 signal at 1 to 1.5 volts (used to set ion signals from the PFK) and 91.0542 signal at 4 to 5 volt (range for the Cali mass from n-butyl benzene). Neat PFK (about 2-2.5 µL) and neat n-butyl benzene (about 1 µL) are added by 10-µL syringes to heated reservoir of the Reference Gas Inlet and the needle valve of the reservoir is adjusted to deliver the above signal intensities. Over a 24-hr period the signals of the Lock and Cali masses will decrease slowly as the gaseous mixture pumps away.

Multiple Ion Detection (MID) Method Global Settings:

- MID file: voc_pfkpb_03.mid
- Mass resolution: 10000
- MID mode: Lock
- Offset: 20 µV
- Measure/Lock ratio: 1
- Width first lock: 0.10 m/z
- Acquisition mode: Centroid
- Sweep peak width: 3
- Electric jump time: 8 ms
- Magnetic jump time: 60 ms
- Electric range: 300%
- Number of time segments: 4
- Ionization mode: EI Positive
- Acquisition Time: 6 to 16.00 min

MID Segment Time Settings for MID file voc_pfkpb_03.mid (4 segments):

Table 12A: Segment 1 Time Settings.

Segment 1	**min**	**L/C**	**Mass *m/z***	**Gain**	**Int.**	**Time (ms)**	**Ion Type**
Start Time	6.00	Lock	68.9947	1	11	9	PFK CF_3^+
Measure Time	2:30		73.0648	1	1	102	MTBE Quant $C_4H_9O^+$
End Time	7.75		74.0681	1	1	102	MTBE Confirm[a] $^{13}CC_3H_9O^+$
Cycle Time 0.5 ms			75.0715	1	1	102	MTBE ISTD $^{13}C_2C_2H_9O^+$
			83.9528	1	1	102	$CH_2Cl_2^+$
		Cali	91.0542	1	11	9	n-Butyl Benzene $C_7H_7^+$

a There is a small variable contribution from the ion-radical $C_4H_{10}O^{+\bullet}$ (m/z 74.0726; not separable at 10000 resolution) produced during the ionization of MTBE in presence of water.

$CH_2Cl_2^+$ is Methylene Chloride used to stabilize the centroiding of the Cali mass.

Table 12B: Segment 2 Time Settings.

Segment 2	**min**	**L/C**	**Mass *m/z***	**Gain**	**Int.**	**Time (ms)**	**Ion Type**
Start Time	7:75	Lock	68.9947	1	15	9	PFK CF_3^+
Measure Time	0:45		60.0080	1	1	139	NM Confirm $CH_2NO_2^+$
End Time	8:30		61.0158	1	1	139	NM Quant $CH_3NO_2^+$
Cycle Time 0.5 ms			62.0192	1	1	139	NM ISTD $^{13}CH_3NO_2^+$
		Cali	91.0542	1	15	9	n-Butyl Benzene $C_7H_7^+$

Table 12C: Segment 3 Time Settings.

Segment 3	**min**	**L/C**	**Mass *m/z***	**Gain**	**Int.**	**Time (ms)**	**Ion Type**
Start Time	8:30	Lock	68.9947	1	15	9	PFK CF_3^+
Measure Time	3:29		82.9450	1	1	139	Chloroform Quant & BDCM Quant $CHCl_2^+$
End Time	12:00		83.9483	1	1	139	Chloroform ISTD & BDCM ISTD $^{13}CHCl_2^+$
Cycle Time 0.5 ms			84.9420	1	1	139	Chloroform Confirm & BDCM Confirm $CH^{37}ClCl^+$
		Cali	91.0542	1	15	9	n-Butyl Benzene $C_7H_7^+$

Table 12D: Segment 4 Time Settings.

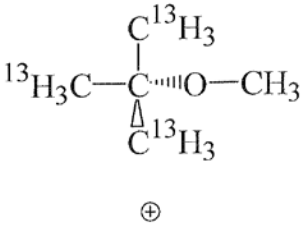
Segment 4	**min**	**L/C**	**Mass *m/z***	**Gain**	**Int.**	**Time (ms)**	**Ion Type**
Start Time	12:00	Lock	118.9915	1	15	9	PFK $C_2F_5^+$
Measure Time	1:40		126.8945	1	1	139	DBCM Confirm $CHClBr^+$
End Time	13:40		128.8919 mean	1	1	139	DBCM Quant $CH^{37}ClBr^+$ & $CHCl^{81}Br^+$
Cycle Time 0.5 ms			129.8953 mean	1	1	139	DBCM ISTD $^{13}CH^{37}ClBr^+$ & $^{13}CHCl^{81}Br^+$
		Cali	130.9915	1	15	9	PFK $C_3F_5^+$

Table 12E: Segment 5Time Settings.

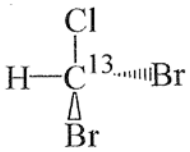
Segment 5	**min**	**L/C**	**Mass *m/z***	**Gain**	**Int.**	**Time (ms)**	**Ion Type**
Start Time	13:40	Lock	168.9883	1	15	9	PFK $C_3F_7^+$
Measure Time	2.20		172.8419	1	1	139	Bromoform Quant $CH^{81}BrBr^+$
End Time	16:00		173.8453	1	1	139	Bromoform ISTD $^{13}CH^{81}BrBr^+$
Cycle Time 0.5 ms			174.8399	1	1	139	Bromoform Confirm $CH^{81}Br_2^+$
		Cali	180.9883	1	15	9	PFK $C_4F_7^+$

(e) Carbon-13 analogs for MTBE, NM, and THMs
The structures of the carbon-13 analogs are listed in Figure 1 below:

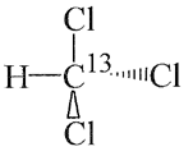
MTBE-ISTD



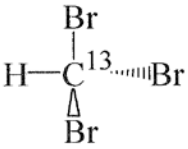
DBCM-ISTD



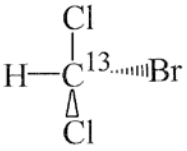
Chloroform-ISTD



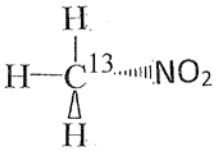
Bromoform-ISTD



BDCM-ISTD



Nitromethane-ISTD



(f) Typical retention time and mass for THMs and MTBE for level 3 water calibrator (Gretel run number G0312733) are listed in Tables 13A & B below.

Table 13A: GC retention times for the analytes and labeled analogs:

Analyte Native	RT Native (min)	ISTD Label	RT ISTD (min)	Code ANALYTE
tert-butyl methylether	7.49	^{13}C3	7.48	VME
Nitromethane	8.09	^{13}C1	8.09	NM
Chloroform	9.02	^{13}C1	9.01	VCF
Bromodichloromethane	11.00	^{13}C1	10.99	VBM
Dibromochloromethane	12.95	^{13}C1	12.94	VCM
Bromoform	14.85	^{13}C1	14.84	VBF

Table 13B: Ion masses used for analysis:

Analyte Native	RT Native (min)	Mass 1[a]	Mass 2[a]	RT ISTD (min)	Mass ISTD
Tert butyl methylether	7.49	73	74	7.48	75
Nitromethane	8.09	61	60	8.09	62
Chloroform	9.02	83	85	9.01	84
Bromodichloromethane	11.00	83	85	10.99	84
Dibromochloromethane	12.95	129	127	12.94	130
Bromoform	14.85	173	175	14.84	174

a Nominal masses used for brevity.

7. Calibration and Calibration Verification

All calibration standards are created in water because it proved impractical to consistently reduce the background VOC levels in serum or whole blood below detectable levels. Matrix spike experiments established that calibration curves in whole blood and water have the same slope. This validates the use of water-based calibrators for quantifying VOCs in whole blood.

a. Creation of curve

1) Data Collection

A full set of 8 calibrators is analyzed with each batch of data and used for the quantification of analytes in all samples from that batch. The calibration curves are constructed for each analyte from the relative response factors for each of the 8 calibrators.

2) Calculation of curve statistics

The slope, intercept and R-squared value for the eight point calibration curves are generated using a 1/x-weighted linear regression using the ThermoFinnigan Xcalibur Quan Browser software.

3) Evaluation of curve statistics

The R-squared values for each analyte calibration curve must in all cases be greater than 0.95. In more than 90% of the cases the R-squared values must be greater than 0.995. Linearity of standard curves should extend over the entire standard range, three orders of magnitude. Intercepts calculated from the least squares fit of the data should not be significantly different from 0. If the y-intercept is significantly different from 0, then the source of this bias must be established and corrected. Possible sources include incorrect ion ratios, contamination of water used to dilute standards, and contamination of analog spiking solution.

b. Use of curve

The lowest point on the calibration curve is at or below the measurable detection limits and the highest point is above the expected range of results. The remaining points in this curve are distributed between these two points with more calibrators in the concentration range at which more unknowns are determined. The calibration curve spans three orders of magnitude. The values of these standards are given above in Table 2.

c. Calibration verification

Calibration is performed as part of each analytical run and a calibration curve is constructed from the eight calibration standards. Additional verification is conducted by quantitating Quality Control samples of known value against the calibration curve and statistically comparing the calculated results to known values.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

a. Morning startup procedure

1) Data System Startup

The data system PC may need to be reset.

(a) Turn off the data system PC by using the START menu and shut down.

(b) After the system has restarted, log into Windows 2000 by entering the user id and password.

Double-click the PAL icon to reset the PAL auto injector. (This must be done before initiating the Xcalibur software).

2) Mass Spectrometer Setup

First double-click the Xcalibur Ion from desktop of the PC to connect the PC with mass spectrometer (MAT 95XP). If the MAT 95XP is connected to the PC, then the Xcalibur road map and tune parameters window will be appear. If not, press the red reset button on the left rear section of the MAT 95XP. Once the connection is established, the Xcalibur road map and tune parameter windows will appear; then the MAT 95XP will be ready for tuning.

3) Mass Spectrometer (MAT 95XP) Tuning

The tuning of the MAT 95XP is usually done once each day as follows.

(a) In the tune window, type **.run 0.8 1.6** and press enter. Then this will be set the filament current to 0.8 mA and multiplier gain to 1.6 kV. Add enough Lock mass calibrant (low boiling PFK) and Cali mass calibrant (n butyl benzene) to achieve 1-1.5 V for mass 131 and 4-5 V for mass 91.
(b) Select mass 131 and obtain 10,000 resolution by changing the entrance and exit slit widths and also optimizing the beam rotation and focus quad. Select Autotune from the tune window. Wait for the tune to complete and print a report. This report is saved with archived raw chromatographic data.
(c) Run **.ecalib 131 169 5** and press enter in the peak window to calibrate the electric scans between masses 131 and 169.
(d) Run a short program of MID to ensure that the masses will lock properly during the run by selecting the **voc_pfkpb_03_0** mid program in the MID window.
(e) Change the MID program to the proper MID for the study. (e.g. For 2003 NHANES study, the MID program is voc_pfkpb_03).

b. Analysis of samples

Samples are transferred to the 10-mL SPME vials via 5-mL luerlock gas-tight syringes.

1) Sample Handling – Water Solutions

(a) Attach a 3-inch needle to the syringe, Rinse the syringe and needle once with methanol (purge and trap grade) and then 2 times with water (Baker, HPLC grade).

(b) Load non-biological samples to be examined. Withdraw 1 mL from the 25-mL volumetric flask or the ampoule containing the sample. Expel this to waste. Withdraw 3 mL of the sample. Weigh and record empty weight of

preassembled 10-mL SPME vial (using a Supelco open center seal, a steel washer, and a MicroLiter PTFE /Silicone faced septum) on the ATLIS work sheet. After removing the seal, gently push the contents of the syringe into the SPME vial. Discharge 40 µL of the internal standard solution into the aliquoted sample using the fixed 40-µL Micropipettor. Place the used disposable glass micropipettes in a sharps container. Crimp and seal the SPME vial using a hand crimper. Reweigh the assembled SPME vial and record the weight on the ATLIS work sheet. The net weight is the weight of solution used.

2) Sample Handling – Biological Samples

(a) Perform the wash cycles using a 3-inch needle with the syringe, as described for the water solutions. Before expelling the "final rinse" water, remove the 3-inch needle and attach a 1.5-inch 18-gauge needle. Expel the water. **For blood samples**, puncture the vacutainer septum and withdraw the 3-mL sample. **For serum samples**, snap the neck of the glass ampoule and use two syringes to remove two 3-mL samples (one used for quality control evaluation and the other for ongoing quality control characterization).

(b) Depending on the preference of the analyst, the needle can be either retained or removed. Transfer the contents of the syringe to a weighed, preassembled 10-mL SPME vial. Discharge 40 µL internal standard solution into the aliquoted sample in the SPME vial using the fixed 40-µL Micro-pipettor. Crimp and seal the SPME vial using a hand crimper. Reweigh the assembled SPME vial and record the weight on the ATLIS work sheet. The net weight is the weight of the blood or serum sample. Place the used disposable glass micropipettes in a sharps container. Remove the needle from the syringe using a pair of hemostats and place it in a sharps container. Place the syringe in a beaker containing water for later decontamination.

c. Sample sequence set up

1) From the Xcalibur road map select the sequence that has been developed to access a sample queue. Usually an existing file can be recalled and modified.

2) Type the sample type (e.g. Blank, QC, Std bracket, or unknown).

3) For each sample, fill in the sample comments, sample ID, sample weight, dilution factor, file name (based of the Julian date), path (for data), instrument method, process method, vial position, levels of standard or QC, and laboratory (initials of analyst).

4) For NHANES study instrument method is c:\Xcalibur\methods\voc_trap_03 and processing method is c:\Xcalibur\methods\THM_range_03.

5) Once the sequence set up is complete, samples can be run by choosing **Actions menu** and **run this sample or sequence.**

6) The last sample to be run should be the **shutdown** procedure.

Note: Make sure the Dewar contains adequate liquid nitrogen and that the liquid access valve is open.

d. Data acquisition and initial analysis

The first "sample" is a fiber cleaning procedure, and the second one is the blank water sample (Bench QC). The calculated ion ratios (native to internal standard area per analyte) are compared with historical ratios to check for possible contamination.

1) If the blank water passes the contamination check, run either the low or high QC serum sample and calculate the ion ratios (native to internal standard area per analyte). Using these ratios, ensure that the sample meets the QC Criteria described in section 10.b.2. If the QC sample passes, all the unknowns will be run and the second aliquot of the same QC will be run at the end of the sequence.

e. Data Analysis

1) Delete the fiber cleaning and shutdown entries from the sequence and save the file.

2) Go to the Qual Browser from the sequence menu.

3) Open the sequence file for the run (the run number is set according to the Julian date and year (IYYJJJ, where I is an alphabetic instrument identifier, YY is the 2 digit year identifier, and JJJ is the Julian date).

4) For each analyte in turn, examine the chromatographic peaks to verify that the automated peak detection routine has correctly identified the peak for both the native compound and the internal standard.

5) Create the Quan file by returning to the run sequence menu and clicking on the **Batch Reprocess** button on the Tool Bar. This processes the run queue using the method THM_range_03.

6) Recall the processed file by going to the Quan browser and opening the correct sequence file (the one that was just batch processed). The Quan browser will

display the calibration curve and all the data parameters including the concentrations that were calculated using the calibration curve.

7) Inspect the integration of all peaks and the baseline for all analytes; reintegrate manually where needed.

8) Inspect the calibration curve in at the low concentration end and perform the correction to maximize the correlation coefficient. Due to the limited mass difference between native and labeled compounds, the native analytes have some response at the mass used for the labeled isotope. Likewise, the labeled isotope often contributes some response at the mass being used for analyte quantitation. In these cases, corrections must be made for the contribution of the labeled analyte to the native ion and vice versa. In order to properly determine the relative response factors between the analyte and analog, these effects must be taken into account. Xcalibur Quan Browser allows for these adjustments using the “Isotope%” function under “Calibration Settings”.

9) Save the Quan Browser file using the nomenclature IYYJJJ.XQN.

10) Create the Excel data summary sheet using the same menu **“import short Excel report”** and save as the same run number format.

11) Save all the data in the folder on the CD for a particular run number.

12) Make a duplicate copy of the same data on a different CD.

f. Transfer Data to the Q: Drive

1) Transfer the data and other files associated with the run to the appropriate instrument/study folder (e.g. Q:\VOC\DBPBLD\Gretel\).

2) Copy the XQN file and raw files to C:\Xcalibur\Data.

3) Inspect the data and calibration curve, Colby corrections, create the long Excel report and save (IYYJJJ.xls; ok to overwrite the short report name).

4) Copy the long Excel report to the Q folder for this data set.

g. Transferring Data to the ATLIS Data Base

1) Log onto the ATLIS data base from your PC.

2) Import the Long Excel sheet for the particular data set by selecting the proper instrument, analyst, Assay, Study reference number and Run number.

3) Inspect the data and change the dates for all runs so that they all are the same date (if your run goes over two days) and transfer the data by selecting transfer button. This is to allow for the proper logic in evaluating which unknown results correspond to a particular QC sample.

4) Finally, review the run by using the review command to make sure data are transferred.

h. Evaluation of QA/QC Data

Two QC materials are included in each analytical run (one blank water and one enriched pool of serum either low or high). Acceptability of results for that entire analytical run is dependent upon the agreement of the results from these QC materials with established ranges.

For QC processing, examine each QC result and compare to the statistical data from the characterization runs (described in Section 10.b). If a QC result is “out-of-control,” the cause of the failure must be determined and corrected. No results from the associated batch may be reported.

The following additional conditions will also necessitate elimination of results:

Low label (ion-2) counts
Possible contamination
Outside of standard curve range
Unconfirmed result
Ion Ratio out of limits
Saturated signals

i. Vacutainer Processing

The butyl rubber stopper in blood collection tubes (e.g. Vacutainers) can contain significant quantities of bromoform and other volatile analytes. The vacutainer stoppers are heated under vacuum as described by Cardinali, et al (2) to remove bromoform and other volatile analytes from these blood collection tubes. Each lot of treated vacutainers is screened as previously described (2) to confirm that any residual contamination is reduced to levels below the lowest reportable value.

9. Reportable Range of Results

a. Reportable Limits

The reportable range of results for the analytes using this method is reported in Table14A. The lower reportable limit is either the detection limit or the lowest

standard, whichever is higher. The upper reportable limit is the highest linear standard.

Table 14A: Reportable range of results.

Analyte	Method LOD (pptr)	Lowest calibration standard (pptr)	Lower reportable limit (pptr)	Upper reportable limit (pptr)
MTBE	1.44	0.80	1.44	497
NM	60.0	8.65	60.0	7200
$CHCl_3$	2.11	0.78	2.11	490
BDCM	0.13	0.62	0.62	390
DBCM	0.40	0.62	0.62	388
$CHBr_3$	1.00	0.57	1.00	357

b. Limit of Detection

The limits of detection for these analytes are given in Table 14A above. These values were determined by calculating the standard deviation at each standard concentration following repeated measurements of the standards. These standard deviations were then plotted versus concentration (4). The y-intercept of the least squares fit of this line equals S_0, with 3 S_0 being the calculated detection limit (4). The detection limits are generally in the low pptr range.

c. Accuracy

No standard reference material is available for VOCs in blood. The accuracy basis for this method was established by analyzing independently prepared and characterized proficiency testing (PT) solutions as unknowns. The PT solutions were made from neat THMs and MTBE. The actual determined accuracy values for all analytes and PT levels are (based on analytical recoveries) ranging from 89-104% (Table 14B).

Table 14B: Accuracy results for all analytes for all PT levels

Analyte	PT20401		PT20402		PT20403		PT20404	
	Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)
MTBE	38	103.7	76	100.9	152	98.6	303	102.8
NM	424	105.2	849	103.7	1273	104.8	2122	102.2
$CHCl_3$	38	89.1	76	93.5	152	98.3	303	97.9
BDCM	30	97.9	61	97.7	122	97.4	243	99.9
DBCM	31	96.7	63	97.2	125	95.9	251	101.6
$CHBr_3$	30	92	59	89.6	118	90.3	237	101.4

d. Precision

The results of repeated measurements on spiked blood samples at four different concentrations are given in Table 15A below. Relative standard deviations are in

most cases less than 7%. As expected, most of the exceptions were found in the low spike samples. These standard deviation results are actually higher than would be encountered in typical blood determinations since they include variation in the blood both before and after spiking. Multiple measurements on spiked QC materials show somewhat lower standard deviation results, averaging 5% for all analytes (Table 15B).

Table 15A: Percent recovery and standard deviation for THMs, NM, and MTBE in blood

Analyte	Spiked Concentration (pptr)	Measured Concentration (pptr)	Recovery (%)	Relative Standard Deviation (%)
MTBE	8.9	9.2	103	3.2
	61.2	58.9	96	0.74
	160.1	144.1	90	2.2
	489.9	440.9	90	0.89
NM	1010	890	88	20
	2080	2030	98	7.3
	3080	3050	99	8.8
$CHCl_3$	8.8	4.4	50*	20
	60.1	48.1	80	1.0
	157.3	125.8	80	3.4
	481.1	370.4	77	2.3
BDCM	6.8	5.4	79	3.4
	46.4	40.4	87	1.5
	121.4	104.4	86	5.2
	371.5	315.8	85	3.5
DBCM	6.8	5.7	84	2.9
	46.6	42.4	91	3.0
	121.8	108.4	89	2.2
	372.7	339.2	91	4.1
$CHBr_3$	6.4	5.3	83	6.9
	44.2	38.9	88	2.8
	115.7	99.5	86	6.5
	353.9	304.4	86	3.8

Note: Chloroform blank level is 15 pptr for the blood used in this experiment. (*): Spike concentration for this sample is below the blank. Therefore recovery is lower in this spike sample for chloroform.

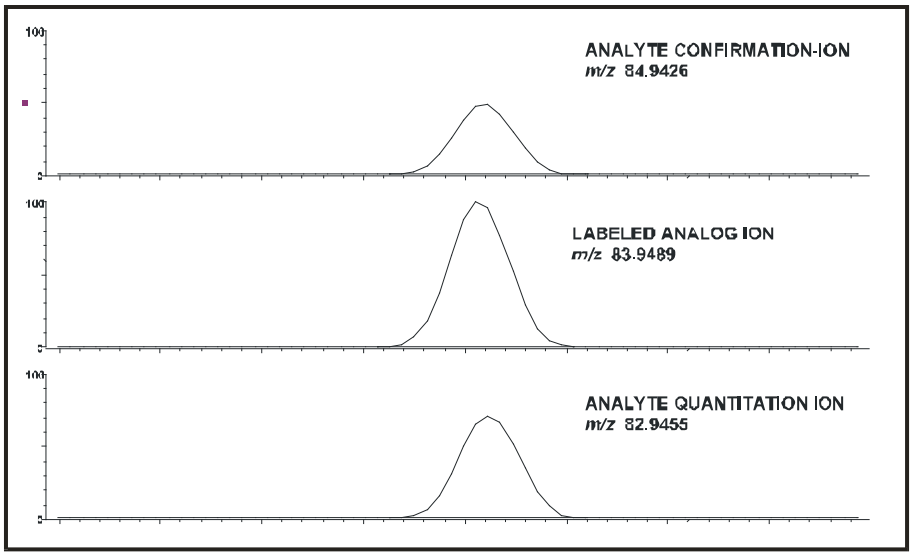
Table 15B: Percentage of recovery and standard deviation for THMs, NM, and MTBE in Quality Control Samples

Analyte	Spiked Concentration (pptr)	Measured Concentration (pptr)	Recovery (%)	Relative Standard Deviation (%)
MTBE	27.4	26.6	97	3.9
	123.7	118.6	96	3.6
NM	800	775	97	16
	1548	1557	99	9.3
$CHCl_3$	23.0	21.2	92	9.8
	92.2	89.4	97	4.6
BDCM	7.9	7.4	94	5.2
	24.9	25.7	103	6.0
DBCM	6.6	5.9	90	3.9
	22.5	21.2	94	4.9
$CHBr_3$	9.3	9.6	103	4.2
	26.2	27.8	106	5.2

e. Analytical Selectivity

Analytical selectivity is a measure of the extent to which a method can determine a particular compound in the matrix of interest without interference from matrix components. Because this method utilizes high resolution mass spectrometry (10,000 resolution) it is highly selective for the method analytes. This selectivity is shown in Figure 2, a typical extracted ion chromatogram of bromodichloromethane in human blood.

Figure 2: Extracted ion chromatogram of bromodichloromethane in human blood.



Method selectivity was further assessed by evaluation of the ratio of confirmation ion peak areas to quantitation ion peak areas.

f. Contamination

THMs and MTBE are ubiquitous in our environment. Therefore special analytical procedures are required for quantifying trace levels of these compounds. All glassware and consumables are specially treated to remove possible volatile interferences and contamination.

The water used for dilution of standards and as water blanks is an extremely critical potential source of interference. No commercial filtering or purification system was found which could consistently yield water with acceptably low levels of VOCs (< 1 pptr for most analytes). An acceptable commercial source of water has been identified, but this must be screened for acceptable lots. Under some circumstances even this source of water failed to yield acceptable levels of volatile organic compounds. In this case, the water is further purified by helium refluxing to yield blank water with acceptable levels of VOCs. To prevent further contamination from the laboratory air, water samples are sealed in glass ampoules. In all cases, typical blank water levels are below the detection limits given above.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Quality assurance and quality control procedures follow standard practices (4). Daily experimental checks are made on the stability of the analytical system, standards, blanks, and quality control materials which are added to each day's run sequence. Three QC samples (1 blank and 2 serum pools) are included in each day’s run. A water blank containing the internal standard is run at the beginning of each day to check for the presence of contamination. In addition, determination of label ion counts for this material is used to check daily method sensitivity. Relative retention times are examined for each analyte to ensure the choice of the correct chromatographic peak. All data entry errors are evaluated by the QC Officer or supervisor and corrected only after consultation with the analyst and positive identification of the correct information.

b. Quality Control Procedures

1) <u>Establishing QC limits</u>

Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Two different pools of serum quality control material are used, QC low and QC high. Different calibration materials are included in the analysis (e.g. different sets of standards and internal standards

and 20 different sets of QC low and high) 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 for MTBE, NM, and THMs are listed in Table 16.

Table 16. Quality Control Samples.

Analyte	QC material	CV	Mean - 3σ	Mean - 2σ	Mean	Mean + 2σ	Mean + 3σ
MTBE	QL206	5.54%	22.49	23.99	26.97	29.96	31.46
	QH206	4.99%	104.27	110.40	122.64	134.89	141.01
NM	QL208	16.3%	396.42	522.82	775.62	1028.42	1154.82
	QH208	9.3%	1123.18	1267.89	1557.31	1846.73	1991.44
$CHCl_3$	QL206	17.5%	10.98	15.02	23.11	31.20	35.25
	QH206	9.06%	66.92	75.25	91.89	108.54	116.86
BDCM	QL206	5.07%	6.61	7.02	7.81	8.60	9.00
	QH206	5.79%	20.18	21.60	24.42	27.26	28.67
DBCM	QL206	3.19%	5.89	6.09	6.51	6.92	7.13
	QH206	4.90%	19.06	20.16	22.35	24.54	25.63
$CHBr_3$	QL206	3.75%	8.13	8.48	9.16	9.85	10.20
	QH206	4.40%	22.37	23.05	25.77	28.04	29.18

2) Quality Control evaluation

After the completion of a run, the quality control limits are consulted to determine if the run is “in control”. The results of the analysis of the quality control samples measured at the beginning and the end of the analytical run are averaged and the quality control rules are applied to this average. The quality control results are evaluated according to modified Westgard (5, 6) rules:

Standard Shewhart QC charts are maintained for this internal QC specimen. A separate QC chart is to be maintained for each QC material used for this internal QC specimen. Standard criteria for run rejection based on statistical probabilities are used to declare a run either in control or out-of-control. These rules are:

Analytical run with 2 QC results**:**

1) If both QC results within 2s limits then accept the run
2) If one of two QC results is outside the 2s limits then apply rules below and reject if any condition is met:
   a) 1-3s – either of the two QC results is outside a 3s limit
   b) 2-2s – both QC results in current run are outside 2s limit (same side of mean)
   c) R4s sequential – the two QC results in current run are outside 2s limit on the opposite sides of the mean
   d) 10x sequential – previous 9 QC results were on same side of 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. The samples are analyzed 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 $\leq$ 25% from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens are not analyzed until the method successfully passes proficiency testing.

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

a. Internal reference area counts

If the labeled ion counts of the blank samples fall below 20% of the median of these values, this indicates that the instrumental sensitivity has fallen below acceptable limits. The following steps should be taken and the instrument sensitivity rechecked after each is performed. Once sensitivity has been reestablished and calibration verification checked, further steps are not necessary.

1) Check for an air leak in the system.

2) Remove and clean the mass spectrometer ion volume. Replace the filament and any ceramics which may be conducting.

3) Test the electron multiplier gain and replace if electron energy is 2.5 kV or higher.

4) Remove and clean the mass spectrometer outer source. Replace any ceramics which may be conducting.

b. Analyte in blank material

If an inordinately large amount of analyte is measured in the blank, but this is not seen in the remainder of the samples, this indicates a temporary contamination of the blank. The source of this incident should be investigated to prevent repeat occurrences but, no further action is required.

c. Analyte in all samples

If an inordinately large amount of analyte is present in all measurements for a particular day, either the labeled analog solution is contaminated or there is a continual source of contamination. The following steps should be taken until the contamination is removed.

1) Check the immediate area of the mass spectrometer and the laboratory where standards are made for use of the contaminating agent.

2) Discard the purge and trap grade methanol used for dilution of the internal standard. For further analyses use a new bottle of purge and trap grade methanol (or new lot of methanol).

3) Check all glassware which has been used.

d. QC sample outside of 99% confidence limits

If one or more of the quality control sample concentration results fall outside the 99% limits, one of the above is the most likely cause. Follow the steps outlined above to isolate and correct the problem.

In all cases the supervisor should be consulted for the appropriate corrective actions. No analytical results will be reported for runs not in statistical control. After corrective actions are carried out, calibration verification and quality control materials must be analyzed before proceeding with unknown analyses.

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope dilution mass spectrometry method, widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. Alteration of particular aspects of this method can result in major interferences. Care is required in order to produce non-contaminated blanks, vacutainers, and quality control

materials. The range of linearity and limits of detection are given above in Sections 9.a. and 9.b., respectively.

13. Reference Ranges (Normal Values)

Reference ranges for VOCs have been measured in a population of 1212 - 1355 persons selected from the National Health and Nutrition Examination Survey (NHANES) for the years 1999 - 2003. The sample is not representative of the U.S. population but it is designed to examine the influence of age, sex, race/ethnicity, urban/rural status and region of the country on VOC levels.

Detectable analyte levels of THMs and MTBE were found in blood samples from most of the people in this reference population. Statistical results are given below.

Table 17: Blood levels of THMs, NM, and MTBE in a reference range of the non-occupationally exposed U.S. population

Analyte	Detection Limit	N	5th Percentile	Median	95th Percentile
Bromoform	1.00	1341	<LOD	1.5	21
BDCM	0.62	1330	<LOD	2.0	12
Chloroform	2.11	1255	2.4	13	70
DBCM	0.62	1355	<LOD	0.77	8.7
MTBE	1.44	1212	<LOD	20	182
NM	60	8786	407.6	720.8	1592.0

14. Critical Call Results (“Panic” Values)

The health effects resulting from exposure to low levels of volatile organic compounds is currently unclear. The method described here is designed for the measurement of low level exposure to VOCs.

15. Specimen Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. If the measurement is delayed to the next day, samples should be refrigerated and not left at ambient temperature overnight. Most sample queues run for extended time periods of up to 24-hr duration. As a precaution biological samples (unknowns and QC) are racked into a chilled tray (15 $\pm$ 1 °C) while awaiting analysis.

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

The analysis of VOCs in whole blood at parts-per-trillion levels is an extremely complex measurement. There are no acceptable alternative methods for this analysis. If the analytical system fails, storage of unprepared and partially prepared specimens at 4 °C is recommended up to 24 hr.

a. Length of Time Samples may be Banked

Repeat measurements of samples stored at 4 °C indicate that whole blood VOC samples may be banked for at least 7 weeks. Since these are whole blood samples, longer storage results in samples which are harder to manipulate and produce additional analytical problems. Thus, even though analytical results may not change over this time, samples may be less amenable to analysis. Volatile organic compounds occur naturally in the body, and metabolism may alter the concentration of some of these analytes with storage.

b. Proper banking procedures

Whole blood samples for VOC measurement should be stored in the dark at 4-10 °C. This prevents blood cell rupture which would occur during freezing. In addition, freezing of blood can lead to breakage of vacutainers and loss of sample in some cases. Since VOCs are lost whenever the containers in which they are stored are opened, it is not appropriate to transfer the blood samples to another container which would be more resistant to breaking.

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

Results are generally reported to 2 significant digits. In addition, reports of reference range means and medians should also accompany all reports since these values are not available elsewhere.

The health effects resulting from exposure to low levels of volatile organic compounds is currently unclear. Therefore no critical call levels are set.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

If greater than 3 mL of sample remain after analysis, this material should be returned to storage at 4 °C.

Samples are tracked using a Microsoft Access-based relational database that tracks the sample location and shelf life. It is recommended that records be maintained for 3 years, including related QA/QC data, and that duplicate records be kept off-site in electronic format. All personal identifiers should be available only to the medical supervisor to maintain confidentiality.

Due to the complex nature of the analyses and the unique testing capabilities of this laboratory, it is not expected that specimens will be referred to other laboratories for testing. Should such a need arise, the laboratory supervisor will consult with local subject matter experts to establish an appropriate mechanism and work process.

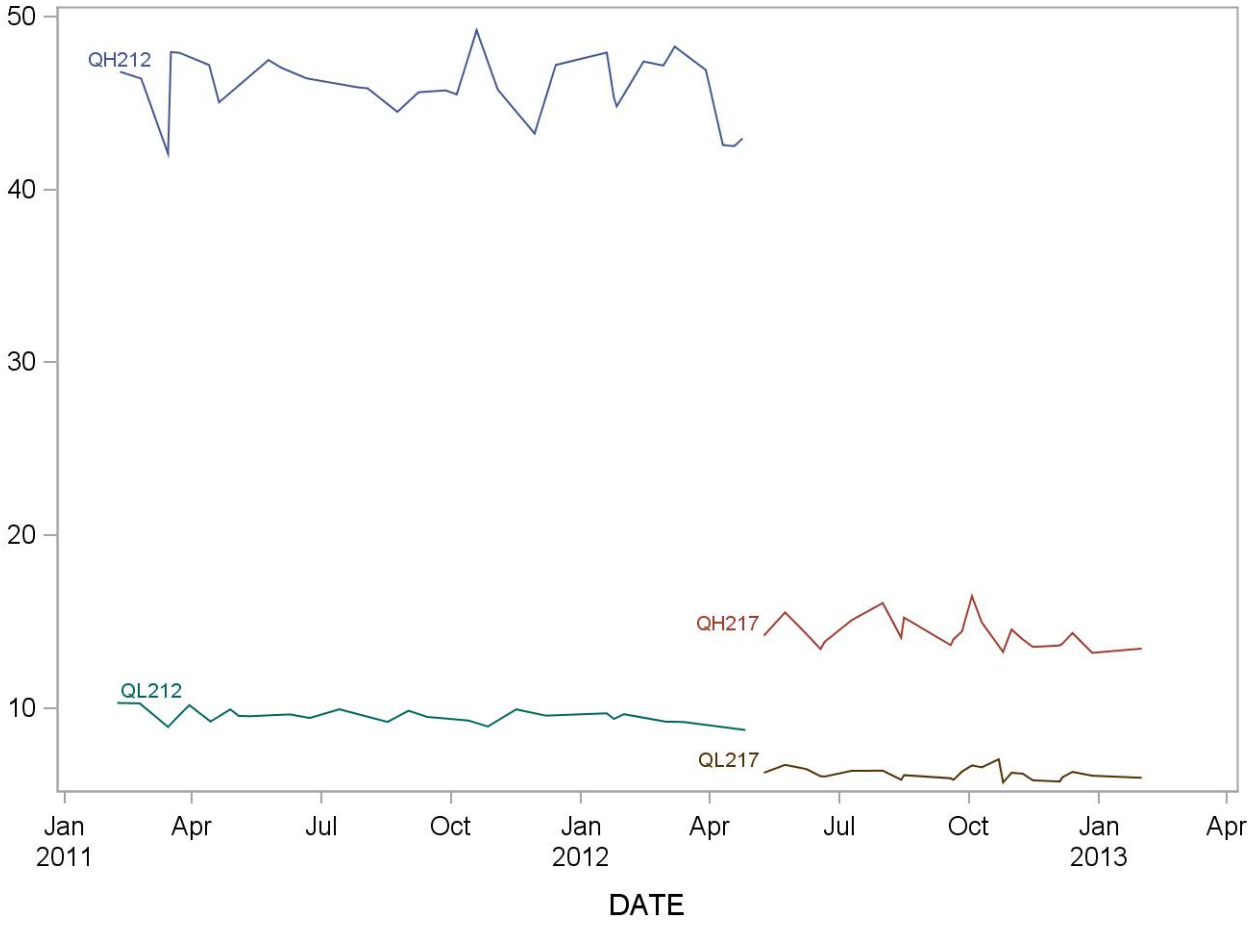
Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

19. SUMMARY STATISTICS and QC GRAPHS

See following pages.

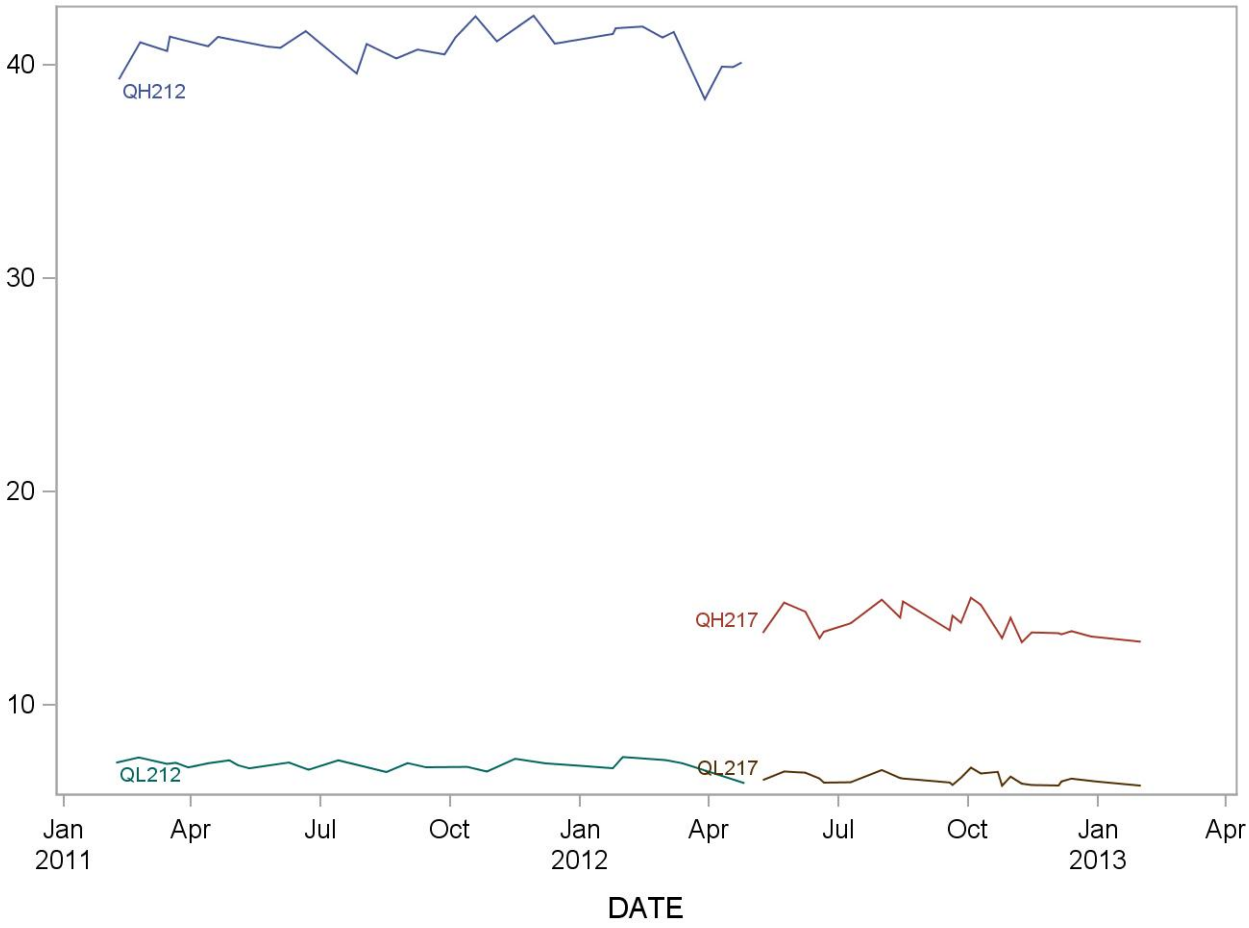
2011-2012 Summary Statistics and QC Chart for Blood Bromodichloromethane (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL212	25	07FEB11	26APR12	9.53705	0.41228	4.3
QH212	30	09FEB11	24APR12	45.95553	1.85531	4.0
QH217	23	09MAY12	31JAN13	14.30035	0.89338	6.2
QL217	24	09MAY12	31JAN13	6.20161	0.33571	5.4



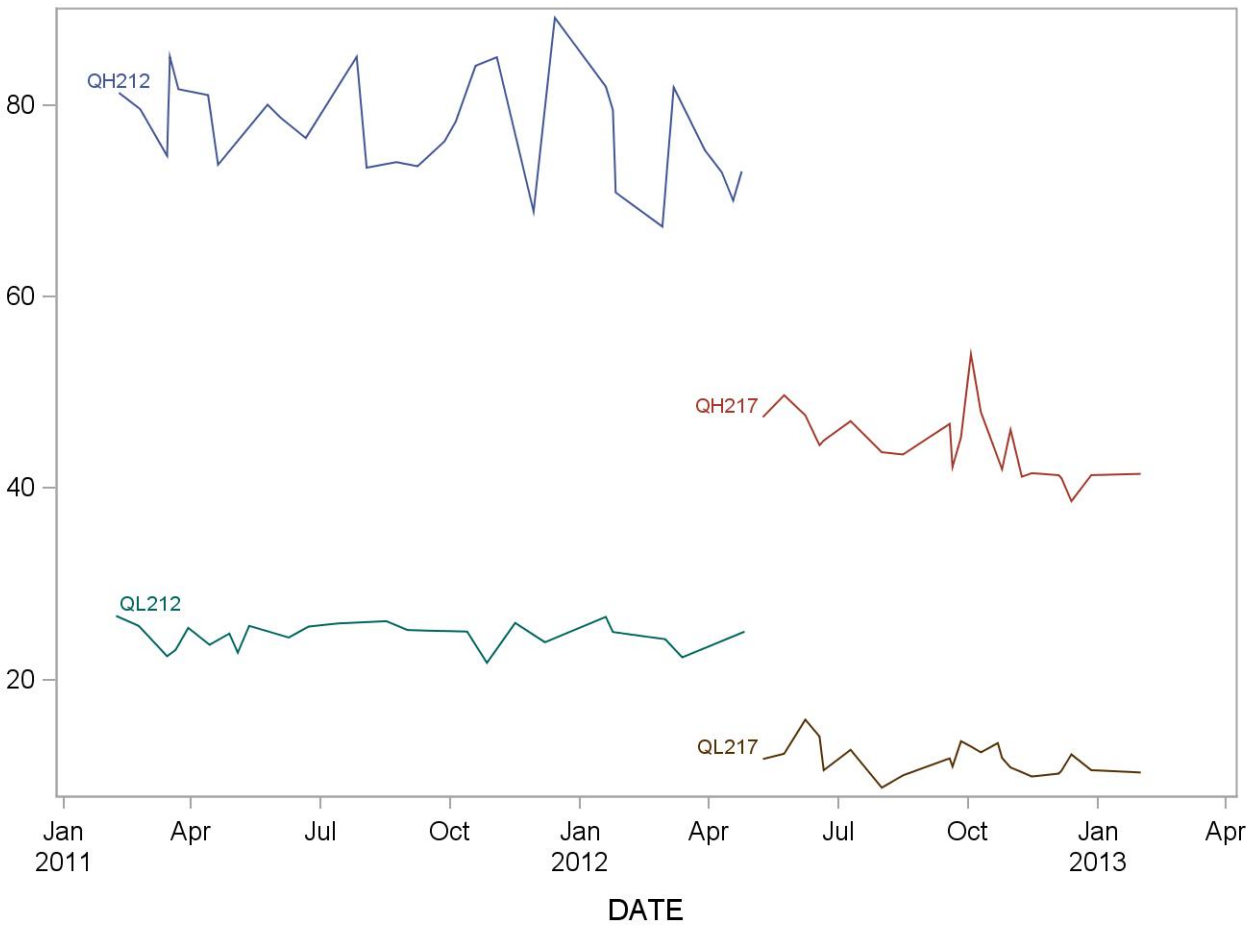
2011-2012 Summary Statistics and QC Chart for Blood Bromoform (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL212	24	07FEB11	26APR12	7.18500	0.26568	3.7
QH212	29	09FEB11	24APR12	40.83952	0.86587	2.1
QH217	23	09MAY12	31JAN13	13.81412	0.67697	4.9
QL217	24	09MAY12	31JAN13	6.52640	0.25210	3.9



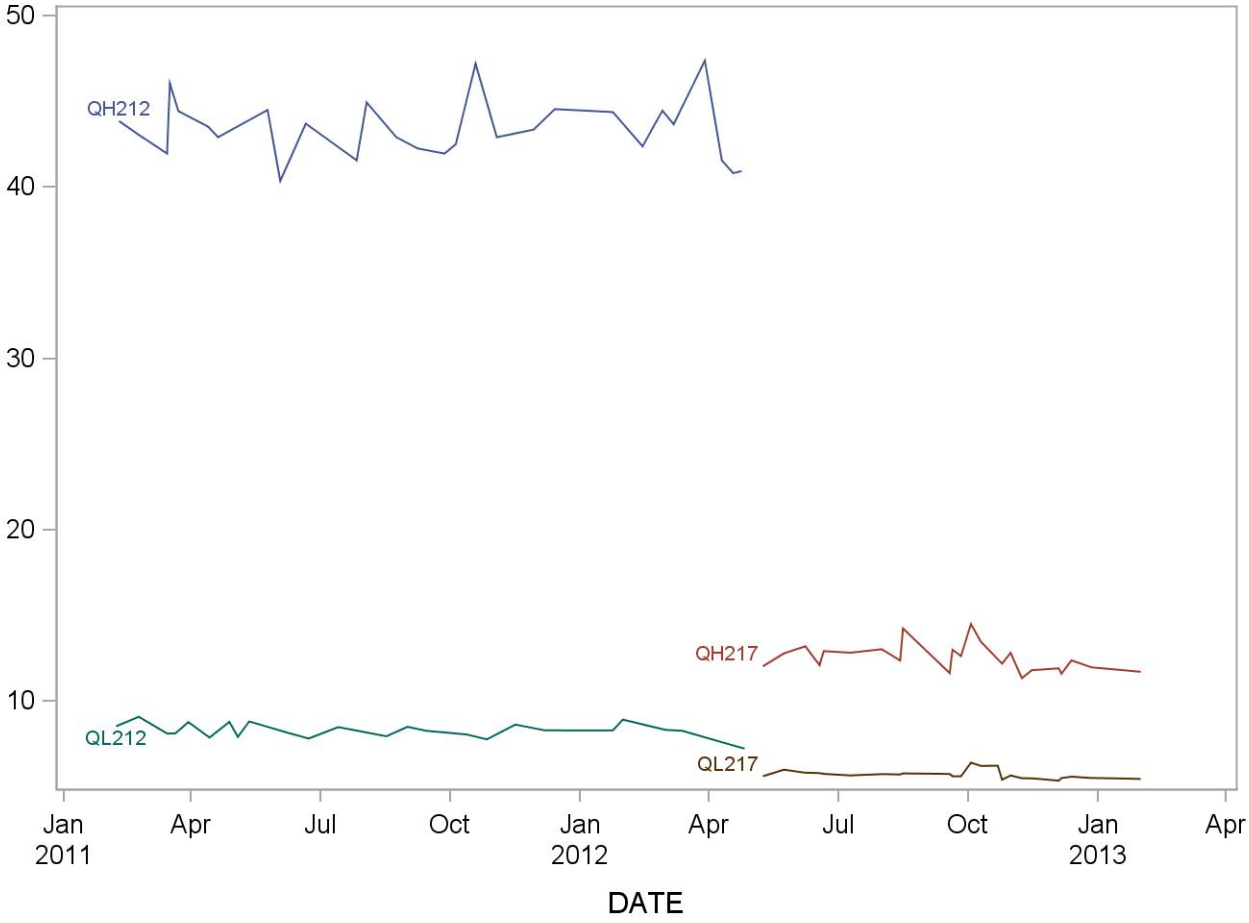
2011-2012 Summary Statistics and QC Chart for Blood Chloroform (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL212	24	07FEB11	26APR12	24.65643	1.37042	5.6
QH212	29	09FEB11	24APR12	77.68922	5.47147	7.0
QH217	22	09MAY12	31JAN13	44.50862	3.58660	8.1
QL217	23	09MAY12	31JAN13	11.61422	1.64019	14.1



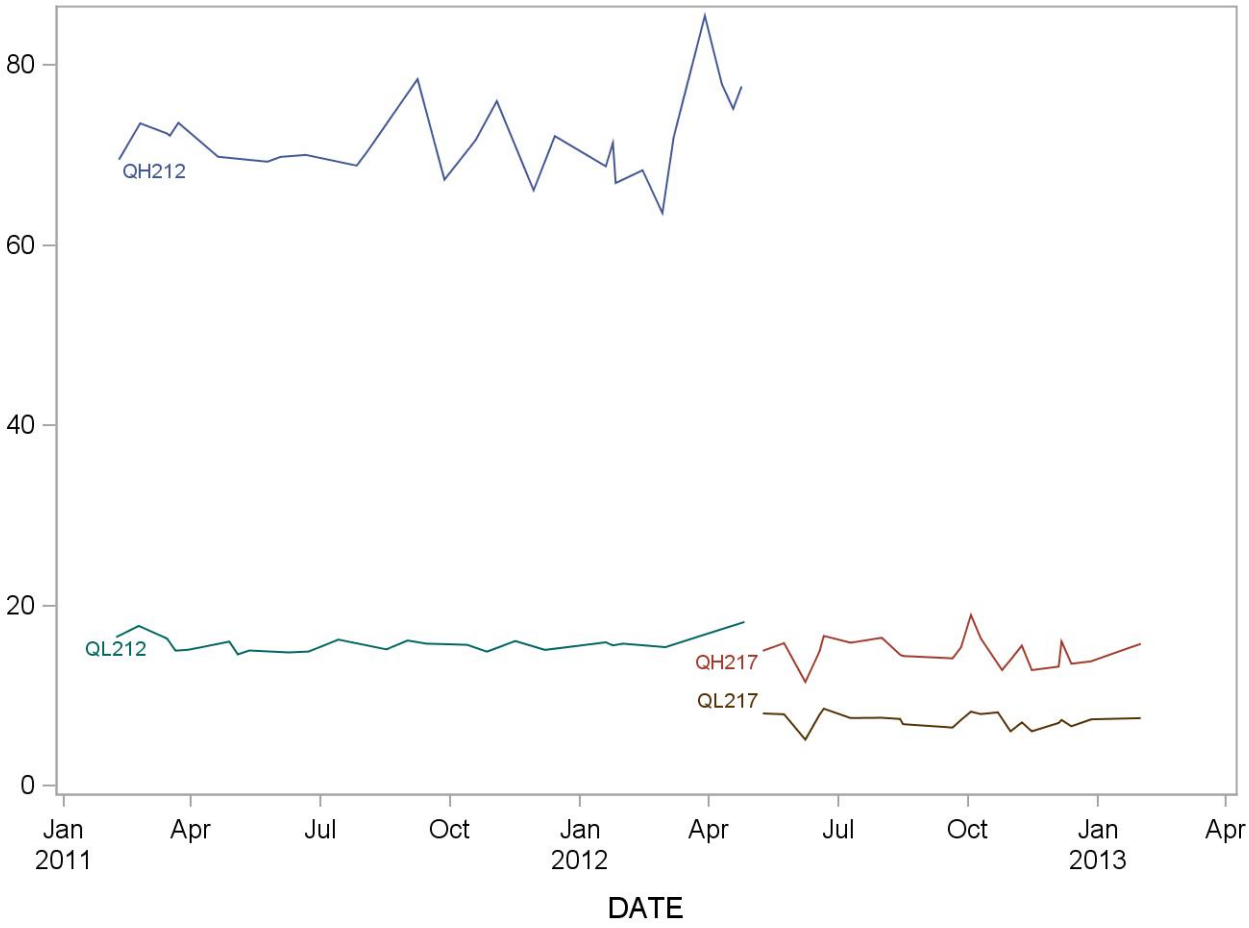
2011-2012 Summary Statistics and QC Chart for Blood Dibromochloromethane (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL212	24	07FEB11	26APR12	8.27899	0.42987	5.2
QH212	28	09FEB11	24APR12	43.34525	1.74970	4.0
QH217	23	09MAY12	31JAN13	12.53262	0.81354	6.5
QL217	24	09MAY12	31JAN13	5.70757	0.26513	4.6



2011-2012 Summary Statistics and QC Chart for Blood MTBE (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL212	23	07FEB11	26APR12	15.71677	0.89431	5.7
QH212	27	09FEB11	24APR12	71.75458	4.54711	6.3
QH217	22	09MAY12	31JAN13	14.87542	1.63087	11.0
QL217	22	09MAY12	31JAN13	7.25320	0.83126	11.5



References

1. Bonin, M.A., Silva, L.K.; Smith, M.M.; Blount, B. C. and Ashley, D.L., Measurement of Trihalomethanes and Methyl Tertiary-Butyl Ether in Whole Blood using Gas Chromatography and High Resolution Mass Spectroscopy, J. Analytical Toxicology, Vol. 29: 81-89 (2005).

2. Cardinali, F. L., McCraw, J. M., Ashley, D. L., Bonin, M. A., and Wooten, J.V. Treatment of vacutainers for the analysis of volatile organic compounds in human blood at the low parts-per-trillion level. *J. Chromatogr. Sci.* **33**: 557-560 (1995).

3. Cardinali, F. L., McCraw, J. M., Ashley, D. L., and Bonin, M. A. Production of blank water for the analysis of volatile organic compounds in human blood at the low parts-per-trillion level. *J. Chromatogr. Sci.* **32**: 41-44 (1994).

4. Taylor, J.K. Quality Assurance of Chemical Measurements, Lewis Publishers, Chelsea, MI, 1987.

5. Westgard, J. O.; Barry, P.L.; Hunt, M. R.; Groth, T.; Clin. Chem. 1981, 27, 493-501.

6. Caudill, S. P.; Schleicher, R. L.; Pirkle, J. L. Multi-rule quality control for the age-related eye disease study. *Stat. Med.* **2008,** *27* (20), 4094-4106.