Attachment 11

Laboratory Standard Operating Procedures

"Biomonitoring of Urban Anglers in Milwaukee's Area of Concern"
Biomonitoring of Great Lakes Populations Program III

Division of Laboratory Sciences

Laboratory Protocol

Analytes: **Cadmium, Lead, Manganese, Mercury, and Selenium**

Matrix: whole blood

Method: blood multi-element analysis by ICP-DRC-MS

Method code: DLS 3016.8-05

Branch: Inorganic and Radiation Analytical Toxicology

Prepared By: Deanna M. Jones, PhD[1] (author's name) Deanna M Jones (signature) 12/9/15 (date)

(author's name) (signature) (date)

Supervisor: Jeffery M Jarrett, MS (supervisor's name) (signature) 12/9/15 (date)

Branch Chief: Robert L Jones, PhD (Branch Chief) (signature) 3/2/16 (date)

Date current version of method first used in lab: 03/23/2016 (Date)

Director's Signature Block:

Reviewed: James J Pirkle (Signature) 3/21/16 (Date)

Procedure Change Log

Procedure: Blood multi-element analysis by ICP-DRC-MS

DLS Method Code: 3016.8-05

Date	Changes Made	By	Rev'd By (Initials)	Date Rev'd
4/1/2011	1UB and 2UB for Mn changed from 15 to 25 ug/L and from 30 to 50 ug/L, respectively.	JHJ8	JHJ8	4/1/2011
4/1/2011	Limit Rep Delta for Mn changed from 1.0 to 2.0.	JHJ8	JHJ8	4/1/2011
7/28/2011	Clarified matrix of internal standard intermediate from "dilute HNO3" to "1% v/v HNO_3".	JHJ8	JHJ8	7/28/2011
8/9/2011	Changed BMN 1UB (25 ug/L to 20 ug/L) and 2UB (50ug/L to 35 ug/L). Supporting references added.	JHJ8	JHJ8	8/9/2011
10/7/2011	Added comment to CV standard tables regarding use of gravimetric preparation.	JHJ8	JHJ8	10/7/2011
3/20/2012	Sample Diluent Preparation: Triton X-100 percentage correction (typo)	nap1	JHJ8	3/20/2012
3/20/2012	DRC Stability Test Preparation: alternate preparation procedure using the intermediate working calibrators	nap1	JHJ8	3/20/2012
3/20/2012	Preparation of Samples for Analysis: changed the Blood Blank name from "BldBlkChk" to "WB Blank" and WB Blank2"	nap1	JHJ8	3/20/2012
3/20/2012	Contaminated Blanks: added clarification on procedure to follow in the event of contaminated blanks	nap1	JHJ8	3/20/2012
3/20/2012	Linear Calibration Curves: clarification on dropping points	nap1	JHJ8	3/20/2012
3/20/2012	Appendix B, Table 1: Added description for method file names Method Parameters: updated sample flush times and sample wash times Autosampler Locations: Aq Blank location	nap1	JHJ8	3/20/2012
3/20/2012	Appendix B, Table 3: Clarification of stock standard preparation	nap1	JHJ8	3/20/2012
3/20/2012	Appendix B, Table 10: Typical sample/batch window: changed autosampler location to reflect current positions	nap1	JHJ8	3/20/2012
3/20/2012	Updated screenshots in Appendix B, Figures 1e, 1f, and 2d	nap1	JHJ8	3/20/2012
3/20/2012	Created Appendix C for "help sheets"	nap1	JHJ8	3/20/2012

3/20/2012	Method Procedures: Types of Quality Control: Removed reference to blind QC	nap1	JHJ8	3/20/2012
5/03/2012	Sample Diluent Preparation: Changed concentration of TMAH from 0.25% to 0.4%	nap1	JHJ8	5/03/2012
5/10/2012	Added Appendix A, Experiment 6: Validated extra dilutions up to 20x. Updated Reportable Range and Table 6 (descriptions of sample preparation).	EMU2	JHJ8	5/10/2012
9/10/2012	Sample Rinse Preparation: Changed concentration of TMAH from 0.25% to 0.4%	nap1	JHJ8	9/10/2012
1/22/2013	Extended calibration range S0-S8 adding a third bench QC level. Changed to weighted linear regression and dual detector mode.	JHJ8	KLC7	1/22/2013
1/22/2013	Clarified and updated handling elevated concentrations, Tables 8 – 11, Sections 7 – 11 and references. Added Figures 1 and 4.	JHJ8	KLC7	1/22/2013
1/22/2013	Added description of solutions for DRC and dual detector optimizations.	JHJ8	KLC7	1/22/2013
1/22/2013	Updated reference range Tables	JHJ8	KLC7	1/22/2013
1/22/2013	Added detail of potential MoO_2 interference on ^{130}Te	JHJ8	KLC7	1/22/2013
1/22/2013	Updated action levels	JHJ8	KLC7	1/22/2013
3/20/2013	Updated evaluating calibration curves language	nap1	JHJ8	3/20/2013
4/16/2013	Updated help sheets re: calibration std prep	nap1	JHJ8	4/16/2013
5/15/2013	Replaced references to urine with references to blood in Table of Figures, Section 7.c.ii, and Section 12. Updated reference from "Section 10a" to "Section 11a" in Section 10a. References to Tables 5, 10, and 11 updated.	JHJ8	Klc7	5/20/2013
9/15/2014	Clarified method details (esp. references to urine methods and solutions preparations). Bldblkchk to be made with S0 instead of water.	JHJ8	Klc7	9/15/2014
12/08/2015	Changed method name from Blood Metals Panel 3 (BMP3) by ICP-DRC-MS to Blood multi-element analysis by ICP-DRC-MS	JJ	KLC	12/9/2015
12/08/2015	Updated Title page to new DLS template	JJ	KLC	12/9/2015
12/08/2015	Updated Section 3 to specify not to freeze blood in blood collection tubes (esp. glass)	JJ	KLC	12/9/2015
12/08/2015	Clarified comments, updated examples, corrected typos: Increased use of active voice (eliminated 'may' and 'shall'). Clarified comments in Tables 8 and 9. Renamed second "Figure 2g" to "Figure 2h". Correct table references in Section 10.	JJ	KLC	12/9/2015

12/08/2015	Minor equipment updates: References to Digiflex pipette changed to Hamilton Microlab 625 benchtop automatic pipette and updated Table 8 volumes. Updated regulator part numbers for methane and oxygen compressed gases.	JJ	KLC	12/9/2015
12/08/2015	Updated instructions related to very elevated results. Set criteria to confirm proper washout after an elevated sample to ± 3SD limits of low bench QC wash check (Section 8.b.iv). Set criteria to confirm samples potentially affected by insufficient washout to ±10% or ±3SD of the low bench QC, whichever is greater (Section 8.b.vii.2.a). Updated extended wash details in Table 1. Added highest validated washout concentrations to Table 9. Updated Figure 4 (Flow Chart for handling an elevated result).	JJ	KLC	12/9/2015
12/08/2015	Added 2011-2012 NHANES reference level data to Table 10 and replaced statement about blood lead >10 µg/dL with statement about 5 µg/dL reference level.	JJ	KLC	12/9/2015
12/08/2015	Updated record retention in section Section 9.c to match DLS policy (3 years to 2 years).	JJ	KLC	12/9/2015
03/02/2016	Left justified text. Updated references. Removed "(esp. glass)" regarding do not freeze blood in blood tubes. Referenced highest calibrator and max extra dilution tables in reportable range section. Updated description of disinfectant. Changed "working calibration standard" to "working calibrator" throughout. Updated references to high purity water.	JJ	RLJ	03/02/2016

Laboratory Procedure Manual

Analytes: **Cadmium, Lead, Manganese, Mercury, and Selenium**

Matrix: **Whole Blood**

Method: **blood multi-element analysis by ICP-DRC-MS**

Method No: DLS 3016.8-05

As performed by: Inorganic and Radiation Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

Contact: Jeffery M. Jarrett, MS
Phone: 770-488-7906
Fax: 770-488-4097
Email: JJarrett@cdc.gov

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

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.

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1. Clinical relevance & summary of test principle

a. Clinical relevance:

Metals ions affect human health in various ways. Some metals (i.e. lead, cadmium, and mercury) show only deleterious effects on human health. Some (i.e. selenium and manganese) play an essential role in the human biological system if within certain concentration ranges, while negative health implications are observed when concentrations in biological systems are in deficit or excess. Determination of a person's level of environmental exposure to chemicals through direct measurement of the substances or their metabolites in human specimens such as blood is called biomonitoring. Biomonitoring reduces the uncertainty of determining levels of exposure over making these determinations through calculations of estimated dose based on analysis of environmental samples and assumptions about exposure pathways[1]. Biomonitoring measurements are the most health-relevant assessments of exposure because they indicate the amount of the chemical that actually gets into people from all environmental sources (e.g., air, soil, water, dust, or food) combined, rather than the amount that gets into them. The laboratory method described here is a multi-element technique for monitoring the concentrations of cadmium (Cd), lead (Pb), manganese (Mn), mercury (Hg), and selenium (Se) in whole human blood for the purpose of biomonitoring.

There is no known biological role of mercury in the human body. The main sources of mercury intake in humans are fish, dental amalgams, and occupational exposures[2]. The main organs affected by mercury are the brain and the kidneys. Exposure of childbearing-aged women is of particular concern because of the potential adverse neurologic effects of Hg in fetuses. The health effects of mercury are diverse and depend on the form of mercury encountered and the severity and length of exposure. The general population is be exposed to three forms of mercury: elemental, inorganic, and organic (predominantly methyl). However, this method tests only for the total amount of mercury in the blood without regard to chemical form. In the general population, total blood mercury is due mostly to the dietary intake of organic forms which are formed through microbial action from inorganic mercury that has deposited in aquatic environments and bioaccumulated through the food chain (especially into large predatory fish)[3]. Exposure to inorganic or elemental mercury (e.g. dental amalgams or occupational exposures) is particularly reflected in urine excretion rather than blood. Psychic and emotional disturbances are the initial signs of chronic intoxication by elemental mercury vapors or salts. Those exposed are at increased risk for parasthesia, neuralgias, renal disease, digestive disturbances, and ocular lesions [4]. Massive exposure over a longer period of time results in violent muscular spasms, hallucinations, delirium, and death[5]. Except for methylmercury exposures, blood is considered useful if samples are taken within a few days of exposure. This is because most forms of mercury in the blood decrease by one-half every three days if exposure has been stopped. Thus,

mercury levels in the blood provide more useful information after recent exposures than after long-term exposures. Several months after an exposure, mercury levels in the blood and urine are much lower. Blood mercury reference ranges for the U.S. population are listed in Table 10 in Appendix B.

There is no known biological role of lead in the human body. Lead, a naturally occurring metal, has had many different commercial uses from which a person can be exposed either in the occupational / manufacturing process or by the manufactured products such as paint (paint chips, or dust and soil contaminated from deteriorating paint), solder or pipes (only now in older homes), gasoline (now outlawed for all but specialized applications), glazes on pottery, hobby uses (e.g. stained glass), commercial products (e.g. batteries, lead-containing jewelry), home remedy medicines containing lead compounds and non-Western cosmetics. Soil contains lead naturally, or from man-made uses of lead such as paint (near older homes), gasoline (near roadways), mining, manufacturing, and disposal. The main target for lead toxicity is the nervous system, both in adults and children. The developing biological systems of children are most sensitive to the effects of Pb, where effects are being recognized even at blood lead levels <5 μg/dL [6-10]. Acute, elevated lead exposure is associated with anorexia, dyspepsia, and constipation followed by diffuse paroxysmal abdominal pain. When lead exposure is high, particularly in children, the person is at increased risk for encephalopathy [11]. The alkyl lead species are highly toxic to the central nervous system[12]. The primary screening method for lead exposure is blood lead, which primarily reflects recent exposures (excretory half-life in blood is approximately 30 days)[13]. Lead in blood is primarily (99%) in the red blood cells. Blood lead reference ranges for the U.S. population are listed in Table 10 in Appendix B. The CDC now uses a reference level of 5 μg/dL to identify children with blood lead levels that are much higher than most children's levels. This new level is based on the U.S. population of children ages 1-5 years who are in the highest 2.5% of children when tested for lead in their blood. This reference value is based on the 97.5th percentile of the National Health and Nutrition Examination Survey (NHANES)'s blood lead distribution in children. CDC will update the reference value every four years using the two most recent NHANES surveys [14].

There is no known biological role of cadmium in the human body. The predominant commercial use of cadmium is in battery manufacturing. Other uses include pigment production, coatings and plating, plastic stabilizers, and nonferrous alloys. Since 2001, U.S. cadmium use has declined in response to environmental concerns. In the United States, for nonsmokers the primary source of cadmium exposure is from the food supply. People who regularly consume shellfish and organ meats will have higher exposures. In general, leafy vegetables such as lettuce and spinach, potatoes and grains, peanuts, soybeans, and sunflower seeds contain high levels of cadmium due to bioaccumulation from the soil. Tobacco leaves accumulate high levels of cadmium from the soil, and smoking is the primary non-occupational source of

cadmium exposure for smokers. Generally, the critical organ for Cd is the kidney. Kidney dysfunction is one of the most characteristic signs of exposure to Cd. Workers in an environment with high exposure levels have developed proteinuria, renal glucosuria, aminoaciduria, hypercalciuria, phosphaturia, and polyuria. Chronic obstructive lung disease of varying degrees of severities is frequently seen in Cd workers. Concentration of cadmium in blood of healthy unexposed adults are in the range 0.1 – 4 μg/L[15]. Newborn babies are practically free of Cd[16]. Exposure to high concentration of fumes appearing from heated cadmium metal or compounds has led to acute poisoning and in some cases to the death of workers[11]. Principal symptoms reported were respiratory distress due to chemical pneumonitis and edema. It has been estimated that 8 hrs. exposure to 5 g Cd/m^3 will be lethal[11]. Ingestion of high amounts of Cd puts a person at increased risk to a rapid onset with severe nausea, vomiting, and abdominal pain. Cadmium levels in blood, urine, feces, liver, kidney, hair, and other tissues have been used as biological indicators of exposure to cadmium. Blood cadmium levels are principally indicative of recent exposure(s) to cadmium rather than whole-body burdens [17-20]. Urine cadmium levels primarily reflect total body burden of cadmium, although urine levels do respond somewhat to recent exposure[21]. Blood cadmium reference ranges for the U.S. population are listed in Table 10 in Appendix B.

Manganese (Mn) is a trace element essential to humans and is associated with the formation of connective and bony tissue, growth and reproductive functions and with carbohydrate and lipid metabolism [22]. Manganese is also a known neurotoxin but little information exists about levels of manganese that cause toxicity. Symptoms of manganese toxicity are similar to Parkinson's Disease and can also include disorientation, memory impairment, anxiety and compulsive behavior [23]. There is much concern for the levels of manganese in humans whom are occupationally exposed to it [24-30]. Recently, there are growing concerns over exposure due to contamination of drinking water with manganese [31-33] and as a result of methylcyclopentadienyl mangangese tricarbonyl (MMT) used as an anti-knocking additive in gasoline [34-40]. Populations suffering from iron deficiencies are at an increased risk to manganese toxicity because iron deficiency can result in an accumulation of manganese in the central nervous system [37]. To fully understand the essentiality and toxicity of manganese, further investigations are needed regarding the levels of manganese in biological matrices. Group average levels in blood appear to be related to manganese body burden, while average urinary excretion levels appear to be most indicative of recent exposures [41]. On an individual basis the correlation between the level of workplace exposure and the levels in blood or urine has always been found to be a reliable predictor of exposure [25, 41-43]. Manganese in blood or urine are useful in detecting groups with above-average current exposure, but measurements of manganese in these body fluids in individuals are sometimes be related to exposure dose after the exposure has ceased. In addition to individual variability, another factor that limits the usefulness of measuring manganese in blood, urine, or feces as a measure of excess manganese

exposure is the relatively rapid rate of manganese clearance from the body. Excess manganese in blood is rapidly removed by the liver and excreted into the bile, with very little excretion in urine [44, 45]. Thus, levels of manganese in blood or urine are not expected to be the most sensitive indicators of exposure [46]. Typical blood manganese concentrations in humans which have been reported in the literature are listed in Table 11 of Appendix B.

Selenium is an essential element that is required to maintain good health but both selenium deficiency and excessive levels of selenium are associated with several disorders[47, 48]. Selenium is a naturally occurring mineral element that is distributed widely in nature in most rocks and soils. Most processed selenium is used in the electronics industry, but it is also used: as a nutritional supplement; in the glass industry; as a component of pigments in plastics, paints, enamels, inks, and rubber; in the preparation of pharmaceuticals; as a nutritional feed additive for poultry and livestock; in pesticide formulations; in rubber production; as an ingredient in antidandruff shampoos; and as a constituent of fungicides. Radioactive selenium is used in diagnostic medicine. In the body, selenium is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. The antioxidant properties of selenoproteins help prevent cellular damage from free radicals. Free radicals are natural by-products of oxygen metabolism that increase risk of chronic diseases such as cancer and heart disease[48, 49]. Other selenoproteins help regulate thyroid function and play a role in the immune system [50-53]. Human selenium deficiency is rare in the U.S. but is seen in other countries where soil concentration of selenium is low[54]. There is evidence that selenium deficiency increases the risk of a form of heart disease, hypothyroidism, and a weakened immune system[55, 56]. There is also evidence that selenium deficiency does not usually cause illness by itself. Rather, it can make the body more susceptible to illnesses caused by other nutritional, biochemical or infectious stresses[57]. Symptoms of very high exposure to selenium, a condition called selenosis, include gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor, fatigue, irritability, and mild nerve damage[47]. Selenium can be detected in the blood, feces, urine, hair, and nails of exposed individuals, however, field studies have used primarily blood or urine levels to indicate the degree of selenium exposure[47]. Typical blood selenium concentrations in humans which have been reported in the literature are listed in Table 11 of Appendix B.

The laboratory method presented here can be used to achieve rapid and accurate quantification of five elements of toxicological and nutritional interest including cadmium (Cd), lead (Pb), mercury (Hg), manganese (Mn) and selenium (Se) in whole human blood. Use this method to screen blood when people are suspected to be acutely exposed to these elements or to evaluate chronic environmental or other non-occupational exposure.

b. Test principle:
This method directly measures the Cd, Mn, Hg, Pb, and Se content of whole blood specimens using mass spectrometry after a simple dilution sample preparation step.

During the sample dilution step, a small volume of whole blood is extracted from a larger whole blood patient specimen after the entire specimen is mixed (vortexed) to create a uniform distribution of cellular components. This mixing step is important because some metals (e.g. Pb) are known to be associated mostly with the red blood cells in the specimen and a uniform distribution of this cellular material must be produced before a small volume extracted from the larger specimen will accurately reflect the average metal concentration of all fractions of the larger specimen. Coagulation is the process in which blood forms solid clots from its cellular components. If steps are not taken to prevent this process from occurring, i.e. addition of anti-coagulant reagents such as EDTA in the blood collection tube prior to blood collection, blood will immediately begin to form clots once leaving the body and entering the tube. These clots prevent the uniform distribution of cellular material in the blood specimen even after rigorous mixing, making a representative sub-sample of the larger specimen unattainable. It is important that prior to or during sample preparation the analyst identify any sample having clots or micro-clots (small clots). Clotted samples are not analyzed by this method due to the inhomogeneity concerns (i.e. all results for the sample are processed as "not reportable").

Dilution of the blood in the sample preparation step prior to analysis is a simple dilution of 1 part sample + 1 part water + 48 parts diluent. The effects of the chemicals in the diluent are to release metals bound to red blood cells making them available for ionization, reduce ionization suppression by the biological matrix, prevent clogging of the sample introduction system pathways by undissolved biological solids, and allow introduction of internal standards to be utilized in the analysis step. Tetramethylammonium hydroxide (TMAH, 0.4% v/v) and Triton X-100® (0.05%) in the sample diluent solubilizes blood components. Triton X-100® also helps prevent biological deposits on internal surfaces of the instrument's sample introduction system and reduce collection of air bubbles in sample transport tubing. Ammonium pyrrolidine dithiocarbamate (APDC) in the sample diluent (0.01%) aids in solubilizing metals released from the biological matrix. Ethyl alcohol in the sample diluent (1%) aids solubility of blood components and aids in aerosol generation by reduction of the surface tension of the solution. The internal standards, rhodium, iridium, and tellurium, are at a constant concentration in all blanks, calibrators, QC, and samples. Monitoring the instrument signal ratio of a metal to its internal standard allows correction for instrument noise and drift, and sample-to-sample matrix differences.

Liquid samples are introduced into the mass spectrometer through the inductively coupled plasma (ICP) ionization source. The liquid diluted blood sample is forced through a nebulizer which converts the bulk liquid into small droplets in an

argon aerosol. The smaller droplets from the aerosol are selectively passed through the spray chamber by a flowing argon stream into the ICP. By coupling radio-frequency power into flowing argon, plasma is created in which the predominant species are positive argon ions and electrons and has a temperature of 6000-8000 K. The small aerosol droplets pass through a region of the plasma and the thermal energy vaporizes the liquid droplets, atomizes the molecules of the sample and then ionizes the atoms. The ions, along with the argon, enter the mass spectrometer through an interface that separates the ICP (at atmospheric pressure, ~760 torr) from the mass spectrometer (operating at a pressure of 10^{-5} torr). The ions first pass through a focusing region, then the dynamic reaction cell (DRC), the quadrupole mass filter, and finally are selectively counted in rapid sequence at the detector allowing individual isotopes of an element to be determined.

Generally, the DRC operates in one of two modes. In 'vented' (or 'standard') mode the cell is not pressurized and ions pass through the cell to the quadrupole mass filter unaffected. In 'DRC' mode, the cell is pressurized with a gas for the purpose of causing collisions and/or reactions between the fill gas and the incoming ions. In general, collisions or reactions with the incoming ions selectively occur to either eliminate an interfering ion, change the ion of interest to a new mass, which is free from interference, or collisions between ions in the beam and the DRC gas can focus the ion beam to the middle of the cell and increase the ion signal. In this method, the instrument is operated in DRC mode when analyzing for manganese, mercury, and selenium. For selenium, the DRC is pressurized with methane gas (CH_4, 99.999%) which reduces the signal from $^{40}Ar_2^+$ while allowing the $^{80}Se^+$ ions to pass relatively unaffected through the DRC on toward the analytical quadrupole and detector. Manganese and mercury are both measured when the DRC is pressurized with oxygen gas (O_2, 99.999%). They are analyzed at the same flow rate of oxygen to the DRC cell to avoid lengthening analysis time due to pause delays that would be necessary if different gas flows were used for the two analytes. The oxygen reduces the ion signal from several interfering ions ($^{37}Cl^{18}O^+$, $^{40}Ar^{15}N^+$, $^{38}Ar^{16}O^1H^+$, $^{54}Fe^1H^+$) while allowing the Mn^+ ion stream to pass relatively unaffected through the DRC on toward the analytical quadrupole and detector. In the case of mercury, collisional focusing of the mercury ions occurs, increasing the observed mercury signal at the detector by approximately a factor of two (2x).

Once ions pass through the DRC cell and electrically selected for passage through the analytical quadrupole, electrical signals resulting from the ions striking the discrete dynode detector are processed into digital information that is used to indicate the intensity of the ions. The intensity of ions detected while aspirating an unknown sample is correlated to an elemental concentration through comparison of the analyte: internal standard signal ratio with that obtained when aspirating calibration standards. This method was originally

based on the method by Lutz et al [58]. The DRC portions of the method are based on work published by Tanner et al. [59, 60].

2) Limitations of Method; Interfering Substances and Conditions

a. Interferences addressed by this method

i. Reduction of argon dimer ($^{40}Ar^{2+}$) interference on selenium ($^{80}Se^{+}$) using ICP-DRC-MS: $^{40}Ar^{2+}$ is a polyatomic ion formed in the plasma as a result of a reaction between the plasma gas (Ar) and itself. The dynamic reaction cell of the ICP-MS is used to reduce ion signals from polyatomic ions via ion-molecule reaction chemistry [60, 61]. In the reaction cell, methane (CH_4) molecules react with $^{40}Ar^{2+}$ ions through a charge transfer reaction. The products of the reaction are $^{40}Ar^{+}$ (ion at a different mass) and ^{40}Ar (neutral). The background ion signal at m/z 80 is reduced by six orders of magnitude because of this reaction.

ii. Reduction of argon nitride ($^{40}Ar^{15}N^{+}$), argon hydroxide ($^{38}Ar^{16}O^{1}H^{+}$) interference on manganese (^{55}Mn) using ICP-DRC-MS: $^{40}Ar^{15}N^{+}$ and $^{38}Ar^{16}O^{1}H^{+}$ are polyatomic ions formed in the plasma as a result of reactions between the plasma gas (Ar) and atmospheric gases (N_2, O_2) or the solvent (H_2O). The dynamic reaction cell of the ICP-MS is used to reduce ion signals from polyatomic ions via ion-molecule reaction chemistry [60, 61]. In the reaction cell, oxygen molecules react with $^{40}Ar^{15}N^{+}$ and $^{38}Ar^{16}O^{1}H^{+}$ ions through either charge transfer reactions or oxygen transfer reactions. The products of the reactions are either neutral molecules and are not detected (charge transfer), or a new ion with higher mass (oxygen transfer). In either case, attenuation of the background ion signal at m/z 55 occurs.

iii. Reduction of $^{37}Cl^{18}O^{+}$, $^{39}K^{16}O^{+}$, $^{54}Fe^{1}H^{+}$ interferences on manganese (^{55}Mn) using ICP-DRC-MS: $^{37}Cl^{18}O^{+}$, $^{39}K^{16}O^{+}$, $^{54}Fe^{1}H^{+}$ are polyatomic ions created in the plasma as a result of reactions between elements present in the blood matrix (Cl, K, and Fe) and the solvent (H_2O). Due to the high concentrations of Cl, K, and Fe in the blood matrix the resulting ion signals of $^{37}Cl^{18}O^{+}$, $^{39}K^{16}O^{+}$, and $^{54}Fe^{1}H^{+}$ interfere with the measurement of $^{55}Mn^{+}$ at m/z 55. The dynamic reaction cell of the ICP-MS is used to reduce ion signals from polyatomic ions via ion-molecule reaction chemistry [60, 61]. In the reaction cell, oxygen molecules react with $^{37}Cl^{18}O^{+}$, $^{39}K^{16}O^{+}$, $^{54}Fe^{1}H^{+}$ ions through either charge transfer reactions or oxygen transfer reactions. The products of the reactions are either neutral molecules and are not detected (charge transfer), or a new ions with higher mass (oxygen transfer). In either case, attenuation of the background ion signal at m/z 55 occurs.

b. Limitations of method (interferences remaining in method)

i. MoO_2 interference on ^{130}Te: Molybdenum will combine with oxygen in the DRC conditions used in this method for Hg analysis to form a polyatomic ion, $^{98}Mo^{16}O_2^+$, which interferes with the measurement of the internal standard $^{130}Te^+$. Increased signal at m/z 130 (due to measuring both $^{130}Te^+$ and $^{98}Mo^{16}O_2^+$) results in an erroneously low net intensity for Hg (net intensity = measured intensity for analyte isotope / measured intensity for internal standard isotope). If this interference occurs during the measurement of the calibration standards (i.e. a multi-element calibration stock standard includes high levels of Mo) it can result in a positive bias for observed mercury concentrations as a consequence of a nonlinear calibration curve having an artificially low slope. If this interference occurs during the measurement of an unknown sample, the reduced net intensity observed can result in reporting an erroneously low Hg result. This interference has been verified to be of concern (>5% effect negative bias) at blood molybdenum concentrations greater than 15 ug/L. However, typical levels of molybdenum in whole blood (0.2 – 4.6 ug/L [62, 63]) are below this. Also, levels of molybdenum in whole blood after acute exposures have been observed to be ≤15 μg/L [62]. Molybdenum concentrations below 5 μg/mL in stock calibration standard solutions do not produce an observable interference.

3) Procedures for collecting, storing, and handling specimens; criteria for specimen rejection; specimen accountability and tracking

a. Procedures for collecting, storing, and handling specimens: Specimen handling conditions, special requirements, and procedures for collection and transport are discussed in the Division of Laboratory Science's (DLS) Policies and Procedures Manual [64]. In general,

i. No fasting or special diets are required before collection of blood

ii. Specimen type – whole blood

iii. Optimal amount of specimen is 1^+ mL. Request a minimum volume of 0.25 mL. Volume for one analytical measurement is 0.05 mL.

iv. Verify sample collection devices and containers are free of significant contamination ("pre-screened") before use.

v. Draw the blood through a stainless steel needle into a pre-screened vacutainer.

vi. Do not freeze blood in blood collection tubes due to risk the tubes cracking. Transfer to plastic, pre-screened cryovials before freezing.

vii. Once received, store blood collection tubes at refrigerated temperatures (2–8 °C). Transfer to plastic, pre-screened cryovials before freezing. Specimen stability has been demonstrated for over 1 year at ≤ -20 °C.

b. Criteria for specimen rejection: The criteria for an unacceptable specimen include:

i. Contamination: Improper collection procedures, collection devices, or sample handling can contaminate the blood through contact with dust, dirt, etc. Manganese is present in the general environment, found often in combination with iron, and is present in many alloys (especially stainless steel).

ii. Low Volume: Request a minimum volume of 0.25 mL. Volume for one analytical measurement is 0.05 mL.

In all cases, request a second blood specimen.

c. Transfer or referral of specimens; procedures for specimen accountability and tracking: Location, status, and final disposition of the specimens will be tracked at least by paper document in the "Study Folder" (created before analysts receive the samples). Apart from this specimen tracking form, this folder will also contain the paper print outs of results from analysis of the specimens. Maintain records for a minimum of 3 years. Use only numerical identifiers for samples within the laboratory (e.g., case ID numbers) in order to safeguard confidentiality. Access to personal identifiers for samples will be limited to the medical supervisor or project coordinator (e.g. non-CDC personnel).

4) Safety precautions

a. General safety

i. Observe all safety regulations as detailed in the Laboratory Safety Manual and the Chemical Hygiene Plan. Participate in training regarding blood-borne pathogens prior to performing this method.

ii. Observe Universal Precautions when working with blood.

iii. Wear appropriate gloves, lab coat, and safety glasses while handling all solutions.

iv. Take special care when handling and dispensing bases and concentrated acids. Use additional personal protective equipment which protects face, neck, and front of body. **If TMAH or concentrated hydrochloric acid comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 minutes.**

v. Use secondary containment for containers holding biological or corrosive liquids.

vi. The use of the foot pedal on the benchtop automatic pipette is recommended because it reduces analyst contact with work surfaces that have been in contact with blood and also keeps the analyst's hands free to hold the specimen cups and autosampler tubes and to wipe off the tip of benchtop automatic pipette.

vii. There are many potential hazards on an operating ICP-MS instrument including ultraviolet radiation, high voltages, radio-frequency radiation, and high temperatures. This information is detailed in the ICP-MS System Safety Manual.

viii. Transport and store compressed gas cylinders with proper securing harnesses. For compressed oxygen gas, use regulators which are oil-free and are equipped with a flash arrestor.

ix. Wipe down all work surfaces at the end of the day with disinfectant. Disinfectant may be either daily remake of diluted bleach (1 part household bleach containing 5.25% sodium hypochlorite + 9 parts water) or an equivalent disinfectant

b. Waste disposal:

i. Autoclaving: All diluted biological specimens, original biological specimens being disposed, or consumables which come into contact with biological specimens (even diluted or aerosolized). Use sharps containers or special autoclave pans for broken glass / quartz or items which puncture autoclave bags (e.g. pipette tips).

ii. Other liquid waste

1. Waste discarded down sink: Only non-corrosive liquid waste (EPA defines as pH >2 and pH<12.5, 40CFR §261.22) from the ICP-MS instrument can be discarded at the sink. Flush the sink with copious amounts of water.

2. Waste to be picked up by CDC hazardous waste program: Submit request for hazardous waste removal of all other liquid waste generated in the CDC laboratory for this method.

5) Instrument & material sources

a. Sources for ICP-MS instrumentation

i. ICP-MS: Inductively Coupled Plasma Mass Spectrometer with Dynamic Reaction Cell Technology (ELAN® DRC II) (PerkinElmer Norwalk, CT, www.perkinelmer.com).

ii. Recirculating chiller / heat exchanger for ICP-MS: Refrigerated chiller (PolyScience 6105PE) or heat exchanger (PolyScience 3370) (PerkinElmer Norwalk, CT, www.perkinelmer.com).

iii. Autosampler: ESI SC4-DX autosampler (Elemental Scientific Inc., Omaha, NE) or equivalent.

iv. Computer: Computer controller provided or recommended by ICP-MS manufacturer is recommended to ensure proper communication between computer and ICP-MS. Recommend 1-2 Gb RAM and secondary internal hard disk for nightly backups (if network backups are not possible).

v. FAST sample introduction system (optional): Standard peristaltic pump on ICP-MS replaced by DXi-FAST micro-peristaltic pump / FAST actuator and valve combination unit. Like part # DXI-54-P4-F6. If DXi-FAST upgrade on ICP-MS is not used, a separate FAST actuator (built-in option on ESI SC4-DX autosampler or stand-alone FAST actuator) will be necessary to complete the FAST sample introduction system.

b. Sources for ICP-MS parts & consumables

NOTE: The minimum number of spares recommended before reordering (if owning one instrument) are listed as "# *Spares* = X amount" in the descriptions below.

i. Adapter, PEEK: Securely connects 1.6mm O.D. PFA tubing to 0.03" I.D. peristaltic tubing. Composed of three PEEK parts.

1. Female nut for 1.6mm O.D. (1/16") tubing. Like part P-420 (Upchurch Scientific, Oak Harbor, WA, www.upchurch.com).
2. PEEK ferrule. Like part P-260x (10pk SuperFlangeless ferrule, Upchurch Scientific, Oak Harbor, WA, www.upchurch.com).
3. Conical Adapter Body. Like part P-692 (Upchurch Scientific, Oak Harbor, WA, www.upchurch.com).

ii. Bottles (for rinse solution): Four liter screw-cap polypropylene container with built-in luer connections (2) designed for use with FAST sample introduction

system (like catalog# SC-0305-1, Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).

iii. Carboy and cap assembly for waste collection: 10-15 L, polypropylene wide-mouth carboy (100 mm neck size) with handles and no spigot (Like part #7BE-25126, Lab Safety Supply, Janesville, WI, www.lss.com) with cap assembly like part # N0690271 (PerkinElmer, Norwalk, CT, www.perkinelmer.com) with tubing connections built into the cap for addition of liquid waste.

iv. Coolant, for polyscience chiller or heat exchanger: Only PerkinElmer part # WE01-6558 (PerkinElmer Norwalk, CT, www.perkinelmer.com) is approved for use by PerkinElmer. *# Spares = 6.*

v. Cones: Platinum or Nickel cones have been used and tested to be comparable in performance from either PerkinElmer or Spectron. Platinum cones are more expensive, but will last longer, can be refurbished (often for free by the manufacturer), and will frequently yield higher sensitivity.

1. Sampler (nickel/platinum): PerkinElmer part # WE021140 / WE027802 (PerkinElmer Norwalk, CT, www.perkinelmer.com). *# Spares = 4.*
2. Skimmer (nickel / platinum): PerkinElmer part # WE021137 / WE027803 (PerkinElmer Norwalk, CT, www.perkinelmer.com). *# Spares = 4.*

vi. Connector (for tubing): Use to connect 1/8" I.D. PVC tubing to 0.125" I.D peristaltic pump tubing. Use part # 3140715 (PerkinElmer Norwalk, CT, www.perkinelmer.com) or equivalent. *# Spares = 4.*

vii. Detector, electron multiplier: Like part # N8125001 (PerkinElmer Norwalk, CT, www.perkinelmer.com). *# Spares = 1.*

viii. FAST accessories

1. Valve: CTFE High-flow valve head for SC-FAST (uses ¼-28 fittings). Like part # SC-0599-1010 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).
2. Stator: CTFE Stator for 6 port SC-FAST high flow valve (¼-28 fittings). Like part # SC-0599-1010-01 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).
3. Rotor: Composite rotor for 6 port SC-FAST high flow valve (¼-28 fittings). Like part # SC-0599-1010-05 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).
4. Sample Loop: 1 mL Teflon, white connector-nuts for high flow valve head(¼-28 fittings). Like part # SC-0315-10 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).
5. Probe, Autosampler: Teflon, carbon fiber support, 0.8mm i.d., blue marker, 1/4-28 fittings. Like part number SC-5037-3751 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). *# Spares = 2.*

6. Probe, Carrier Solution: Teflon, carbon fiber support, 0.5mm i.d., orange marker, 1/4-28 fittings. Like part number SC-5037-3501 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). *# Spares = 2.*
7. Tubing, FAST vacuum: Vacuum line for SC-FAST high flow valve, connects to port #6, black nut for connection to valve head, natural brown color nut on other end for connection to SC autosampler vacuum port. Like part # SC-0321 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).
8. Tubing, connects nebulizer to valve: See "Nebulizer, PolyPro-ST micro flow"

ix. Hose, for connection to chiller: Push on hose. I.D. = ½", O.D. = ¾". Use part # PB-8 (per inch, Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. Do not normally need spare hose (unless moving instrument into a new location).

x. Hose, for exhaust of ICP-MS: Available as part of ICP-MS installation kit from Perkin Elmer (PerkinElmer Norwalk, CT, www.perkinelmer.com). Available direct from manufacturer as part # S-LP-10 air connector (Thermaflex, Abbeville, SC, www.thermaflex.net), or equivalent. *# Spares = 10 feet of 4" diameter and 10 feet of 6" diameter hose.*

xi. Injector, quartz with ball joint: I.D. = 2.0 mm. PerkinElmer part # WE023948 (PerkinElmer Norwalk, CT, www.perkinelmer.com). Available direct from manufacturer as part # 400-30 (Precision Glass Blowing, Centennial, CO, www.precisionglassblowing.com) or from various distributors. *# Spares = 2.*

xii. Ion lens: PerkinElmer part # WE018034 (PerkinElmer Norwalk, CT, www.perkinelmer.com). # Spares = 3.

xiii. Nebulizer: PolyPro-ST micro flow polypropylene nebulizer with external 1/4-28 threaded connector for liquid delivery, low pressure version or equivalent. Like part # ES-4040-7010 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). *# Spares = 1.* Different nebulizers are acceptable, however, the nebulizer gas flow rate, sample flush time, read delay time, loop fill time, loop size, blood sample dilution preparation volume, and sample-to-sample carry-over must be evaluated and optimized.

1. Gas connection:
    a. Teflon tubing: 4mm o.d., 2.4mm i.d. Teflon tubing (like part # ES-2502, Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). *# Spares = 1.*
    b. Adapter kit: Plastic adapters to connect Teflon tubing (2.4 mm i.d) to ¼" male Swagelok (compression) port on ICP-DRC-MS. Parts can be obtained as components in a "gas fittings kit for microflow nebulizer", kit like part # ES-2501-1000 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). *# Spares = 1.*

2. Liquid connection: Connects nebulizer to port #3 of high flow FAST valve head with green, 1/4- 28 fitting. Like part # SC-0317-0250 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). # *Spares = 2.*

xiv. Nut: (for flanged connections of 1.59mm (1/16") o.d. PFA tubing) Flanged, for 1/16" o.d. tubing, 1/4-28 threads. Use part # P-406x (pkg. of 10, Upchurch Scientific, Oak Harbor, WA, www.upchurch.com) or equivalent. Use a Teflon-coated Viton o-ring with this nut instead of the stainless steel washer that comes with part # P-406x*). # Spares = 10.*

xv. Nut and ferrule set, 1/8" Swagelok: Such as part # SS-200-NFSET (stainless steel) or part # B-200-NFSET (brass) (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. For part numbers listed here a quantity of 1 means 1 nut, 1 front ferrule, and 1 back ferrule. *Spares = 20.*

xvi. Nut and ferrule set, 1/4" Swagelok: Such as part # SS-400-NFSET (stainless steel) or part # B-400-NFSET (brass) (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. For part numbers listed here a quantity of 1 means 1 nut, 1 front ferrule, and 1 back ferrule. *Spares = 20.*

xvii. Oil for roughing pumps:

1. Welch Directorr Gold: For roughing pumps. Available direct from manufacturer as part # 8995G-15 (1 gallon, Welch Rietschle Thomas, Skokie, IL, www.welchvacuum.com), or equivalent. *# Spares = 4.*
2. Fomblin Y14/5 fluid: PerkinElmer part # N8122265 (1 kg bottle, PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares =1 per instrument.*

xviii. O-ring: (for sampler cone) PerkinElmer part # N8120511 (pkg. of 5, PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares = 20 o-rings.*

xix. O-ring: (for skimmer cone) PerkinElmer part # N8120512 (pkg. of 5, PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares = 20 o-rings.*

xx. O-ring: (for flanged connections of 1.59mm (1/16") o.d. PFA tubing) Teflon-coated Viton o-ring, i.d. = 1/16", thickness = 1/16", o.d. = 3/16". Such as part # V75-003 (O-rings West, Seattle, WA, www.oringswest.com) or equivalent. *# Spares = 20.*

xxi. O-ring: (for injector support).

1. Internal o-rings: ID = ¼", OD = 3/8", thickness = 1/16". Need 2 o-rings per injector support setup. PerkinElmer part # N8122008 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent (such as part # V75-010, O-rings West, Seattle, WA, www.oringswest.com). *# Spares = 20.*
2. External o-rings: ID = 3/8", OD = 1/2", thickness = 1/16". Need 2 o-rings for each injector support setup. PerkinElmer part # N8122009 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent (such as

part # V75-012, O-rings West, Seattle, WA, www.oringswest.com). # *Spares = 20.*

xxii. O-ring (for inside nebulizer port on standard PerkinElmer cyclonic quartz spray chamber for the ELAN): Such as part # 120-56 (Precision Glass Blowing, Centennial, CO, www.precisionglassblowing.com). Additional o-rings can sometimes be obtained free of charge or at reduced price when acquired while purchasing spray chambers. # *Spares = 20.*

xxiii. O-ring: (for inside of bayonet torch mount): Part # WE017284 (PerkinElmer, Shelton, CT, www.perkinelmer.com). Do not substitute. The PerkinElmer o-ring is specially metal impregnated to minimize RF leakage though the torch mount. # *Spares = 2.*

xxiv. Photon stop: PerkinElmer part # WE018278 (PerkinElmer, Shelton, CT, www.perkinelmer.com). # *Spares = 1.*

xxv. Plugs, quick change for roughing pump oil: These plugs will only work on the Varian roughing pumps which come standard on ELAN DRC II ICPMS instruments. These plugs will not fit the Leybold pumps which come standard on the ELAN DRC Plus instruments. Part # W1011013 (PerkinElmer, Shelton, CT, www.perkinelmer.com). No spares typically needed.

xxvi. Probes

1. for ESI autosampler: Teflon, carbon fiber support, 0.8 mm i.d., blue marker, 1/4-28 fittings. Like part number SC-5037-3751 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). # *Spares = 2.*
2. for carrier solution of FAST sample introduction system: Teflon, carbon fiber support, 0.5mm i.d., orange marker, 1/4-28 fittings. Like part number SC-5037-3501 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com). # *Spares = 2.*

xxvii. RF coil: PerkinElmer part # WE02-1816 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. # *Spares = 2.*

xxviii. Spray chamber, quartz concentric: PerkinElmer part # WE025221 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. Available direct from manufacturer as part # 400-20 (Precision Glass Blowing, Centennial, CO, www.precisionglassblowing.com) or from various distributors. # *Spares = 2.*

xxix. Torch, quartz: PerkinElmer part # N812-2006 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. # *New Spares = 2.*

xxx. Tubing and adapter, for SC autosampler rinse station drain: Tygon tubing and adapter to attach to back of SC autosampler for draining rinse station waste (like part # SC-0303-002, Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).

xxxi. Tubing and adapters, for SC autosampler rinse station filling: Teflon tubing and adapters (to attach to back of SC autosampler for filling rinse stations and

to attach to rinse containers). Like part # SC-0302-0500, Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).

xxxii. Tubing and nut, for FAST carrier solution: 0.5 mm i.d. Teflon tubing (orange marker) with red ¼-28 male nut. Connects to high flow FAST valve head, port #2. Like part # SC-0316-0500 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).

xxxiii. Tubing, FAST vacuum: Vacuum line for SC-FAST high flow valve, connects to port #6, black nut for connection to valve head, natural brown color nut on other end for connection to SC autosampler vacuum port. Like part # SC-0321 (Elemental Scientific Inc., Omaha, NE., www.elementalscientific.com).

xxxiv. Tubing, main argon delivery to instrument: I.D. = 1/8", O.D. = ¼". Like part # C-06500-02 (pkg. of 100ft, polypropylene, Fisher Scientific International, Hampton, NH, www.fishersci.com) or equivalent. *# Spares = 50 ft.*

xxxv. Tubing, PFA: I.D. = 0.5 mm, O.D. = 1.59 mm (1/16"). Used to transfer liquidbetween rinse solution jug and peristaltic pump tubing

The Perfluoroalkoxy (PFA) copolymer is a form of Teflon®. Like part # 1548 (20ft length, Upchurch Scientific, Oak Harbor, WA, www.upchurch.com) or equivalent.*# Spares = 20ft.*

xxxvi. Tubing, peristaltic, 0.03" i.d. (carrier solution for ESI autosampler): *use either*

1. Standard PVC, 2-stop (black / black) peristaltic pump tubing, i.d. = 0.03". PerkinElmer part # 09908587 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares = 6 packs of 12 tubes.*
2. Standard PVC, 3-stop (black/ black/black) peristaltic pump tubing, i.d. 0.76 mm. Spectron part # SC0056 (Spectron, Ventura, CA, www.spectronus.com) or equivalent. #Spares = 6 packs of 12 tubes. *Use this type of tubing with ESI DXi micro-peristaltic pump.*

xxxvii. Tubing, peristaltic, 0.125" i.d. (spray chamber drain): *use either*

1. Standard PVC, 2-stop (black / white) peristaltic pump tubing, i.d. = 0.125" or equivalent. PerkinElmer part # N812-2012 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares = 6 packs of 12 tubes.*
2. Standard Santoprene, 3-stop (grey/ grey/ grey) peristaltic pump tubing, i.d. 1.30 mm. Spectron part # SC0311 (Spectron, Ventura, CA, www.spectronus.com) or equivalent. #Spares = 6 packs of 12 tubes. *Use this type of tubing with ESI DXi micro-peristaltic pump.*

xxxviii. Tubing, PVC, i.d. = 1/8", o.d. = 3/16". Used to transfer liquid

1. between spray chamber waste port and peristaltic pump
2. between peristaltic pump and liquid waste jug

Like part # 14-169-7A (pkg. of 50 ft, Fisher Scientific International, Hampton, NH, www.fishersci.com) or equivalent. *# Spares = 20ft.*

xxxix. Tubing, Stainless Steel, o.d. = 1/8", wall thickness = 0.028": Used to connect gas cylinders to NexIONUCT gas ports. Like part # SS-T2-S-028-20 (20ft, Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. *Spares = 20 ft.*

xl. Tubing, Teflon, corrugated, ¼" o.d.: Connects to the auxiliary and plasma gas side-arms of the torch. Part # WE015903 (PerkinElmer, Shelton, CT, www.perkinelmer.com) or equivalent. *# Spares = 2.*

xli. Tubing, vinyl (argon delivery to nebulizer): Vinyl Tubing, 1/8" ID x 1/4" OD. Like part # EW-06405-02 (Cole Parmer, Vernon Hills, Illinois, www.coleparmer.com) or equivalent. *# Spares = 10 ft.*

xlii. Union elbow, PTFE ¼" Swagelok (ELAN bayonet mount): Connects argon tubing to torch auxiliary gas sidearm on bayonet mount NEXION ICP-MS instruments. Like part # T-400-9 (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. *Spares = 2.*

xliii. Union tee, PTFE, ¼" Swagelok (ELAN bayonet mount): Connects argon tubing to torch plasma gas sidearm and holds igniter inside torch sidearm on bayonet mount NEXION ICP-MS instruments. Like part # T-400-3 (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. *Spares = 2.*

c. \Sources for ICP-MS maintenance equipment & supplies

i. Anemometer: Like digital wind-vane anemome*ter (Model* 840032, SPER Scientific LTD., Scottsdale, AZ, www.sperscientific.com) or equivalent. Use to verify adequate exhaust ventilation for ICP-MS (check with hoses fully disconnected).

ii. Pan, for changing roughing pump oil: Like part # 53216 (United States Plastics Corporation, Lima, OH, www.usplastic.com) or equivalent.

iii. Container, to hold acid baths for glassware: Polypropylene or polyethylene containers with lids (must be large enough for torch, injector, or spray chamber submersion). Available from laboratory or home kitchen supply companies.

iv. Cotton swabs: Any vendor. For cleaning of cones and glassware.

v. Cutter (for 1/8" o.d. metal tubing): Terry tool with 3 replacement wheels. Like part # TT-1008 (Chrom Tech, Inc., Saint Paul, MN, www.chromtech.com) or equivalent.

vi. Getter regeneration Kit: Part # WE023257 (PerkinElmer, Shelton, CT, www.perkinelmer.com). Use this as needed (at least annually) to clean the getter in the pathway of channel A DRC gas.

vii. Magnifying glass: Any 10x + pocket loupe for inspection of cones and other ICP-MS parts. Plastic body is preferred for non-corrosion characteristics. Like part # 5BC-42813 (Lab Safety Supply, Janesville, WI, www.labsafety.com).

viii. Ultrasonic bath: Like ULTRAsonik™ Benchtop Cleaners (NEYTECH, Bloomfield, CT, www.neytech.com) or equivalent.

d. Sources for general laboratory equipment and consumables

i. Bar code scanner: Like Code Reader 2.0 (Code Corporation, Draper, UT, www.codecorp.com) or equivalent. For scanning sample IDs during analysis setup. Any bar code scanner capable of reading Code 128 encoding at a 3 mil label density can be substituted.

ii. Carboy (for preparation of blood quality control pool and waste jug for ICPMS sample introduction system): Polypropylene 10-L carboy (like catalog # 02-960-20C, Fisher Scientific, Pittsburgh, PA, www.fischersci.com) or equivalent. Carboys with spouts are not advised due to potential for leaking.

iii. Containers for diluent and rinse solution: Two liter Teflon™ containers (like catalog# 02-923-30E, Fisher Scientific, Pittsburgh, PA., www.fishersci.com, or equivalent) and 4L polypropylene jugs (like catalog# 02-960-10A, Fisher Scientific, Pittsburgh, PA, www.fishersci.com, or equivalent) have both been used. Acid rinse before use.

iv. Gloves: Powder-free, low particulate nitrile (like Best CleaN-DEX™ 100% nitrile gloves, any vendor).

v. Paper towels: For general lab use, any low-lint paper wipes such as KIMWIPES®EX-L Delicate Task Wipers or KAYDRY®EX-L Delicate Task Wipers (Kimberly-Clark Professional, Atlanta, GA, www.kcprofessional.com). For sensitive applications in cleanrooms, use a wipe designed for cleanrooms such as the Econowipe or Wetwipe (Liberty, East Berlin, CT, www.liberty-ind.com).

vi. Pipette, benchtop automatic (for preparation of blood dilutions to be analyzed): Like the Microlab 625 advanced dual syringe diluter (Hamilton, Reno, NV, http://www.hamilton.com/) equipped with a 5.0 mL left syringe, a 250 µL right syringe, a 12 gauge Concorde CT probe dispense tip, the Microlab cable management system and a foot pedal. Alternatives are acceptable, including the Micromedic Digiflex™ (Titertek, Huntsville, AL, http://www.titertek.com/) equipped with 10.0-mL dispensing syringe, 200 µL sampling syringe, 0.75-mm tip, and foot pedal.

vii. Pipettes (for preparation of intermediate stock working standards & other reagents): Like Brinkmann Research Pro Electronic pipettes (Brinkmann Instruments, Inc., Westbury, NY, http://www.brinkmann.com/home/). 5-100 µL (catalog #4860 000.070), 20-300 µL (catalog #4860 000.089), 50-1000 µL (catalog #4860 000.097), 100-5000 µL (catalog #4860 000.100). Note: pipette catalog numbers are without individual chargers. Can purchase individual chargers (pipette catalog numbers will differ) or a charging stand that will hold four pipettes (catalog #4860 000.860). When purchasing pipette tips (epTips), purchase one or more boxes, then "reloads" for those boxes after that: 5-100

μL (box catalog # 22 49 133-4, reload catalog # 22 49 153-9), 20-300 μL (box catalog # 22 49 134-2, reload catalog # 22 49 154-7), 50-1000 μL (box catalog # 22 49 135-1, reload catalog # 22 49 155-5), 100-5000 μL (box catalog # 22 49 138-5, reload catalog # 22 49 198-9, bulk bag catalog # 22 49 208-0). Equivalent pipettes and tips can be substituted.

viii. Tubes for sample analysis (for autosampler): Like polypropylene 15-mL conical tubes, BD Falcon model #352097 (Becton Dickinson Labware, FranklinLakes, NJ, www.bd.com) or equivalent. Clear plastics tend to have lowest trace metal contamination. Blue colored caps have also been used successfully for this method.

ix. Tubes for storage of intermediate working stock standards: Like polypropylene 50-mL conical tubes, BD Falcon model #352098 (Becton Dickinson Labware, FranklinLakes, NJ, www.bd.com) or equivalent. For use in storage of intermediate working stock standards. Clear plastics tend to have lowest trace metal contamination. Blue colored caps have also been used successfully for this method.

x. Vortexer: Like MV-1 Mini Vortexer (VWR, West Chester, PA, www.vwr.com). Used for vortexing blood specimens before removing an aliquot for analysis. Equivalent item can be substituted.

e. Sources of chemicals, gases, and regulators

i. Acid, hydrochloric acid: Veritas™ double-distilled grade, 30–35% (GFS Chemicals Inc. Columbus, OH, www.gfschemicals.com) or equivalent. This is referred to as "concentrated" hydrochloric acid in this method write-up. For use in preparation of intermediate working stock standards.

ii. Acid, nitric acid: Veritas™ double-distilled grade, 68-70% (GFS Chemicals Inc. Columbus, OH, www.gfschemicals.com). For use in cleaning any bottles, vials, tubes, and flasks. This is referred to as "concentrated" nitric acid in this method write-up.

iii. Blood, whole (human or bovine): Bags of human blood can be purchased from various sources such as American Red Cross (http://www.redcross.org) or Tennessee Blood services (Memphis, TN, http://tennesseebloodservices.com/). Request that human blood be screened for infectious diseases such as Hepatitis B and HIV. Source for bovine blood includes the Wisconsin State Laboratory of Hygiene (WSLH, Madison, WI, http://www.slh.wisc.edu).

iv. Ethanol (EtOH): USP dehydrated 200 proof (Pharmco Products, Inc.) or equivalent.

v. Ammonium pyrrolidine dithiocarbamate, laboratory grade (Fisher Scientific, Fairlawn, NJ) or equivalent.

vi. Argon gas (for plasma & nebulizer) and regulator: High purity argon (>99.999% purity, Specialty Gases Southeast, Atlanta, GA, www.sgsgas.com) for torch and nebulizer. Minimum tank source is a dewar of liquid argon (180-250 L). Bulk tank (1500⁺L is preferred).

1. Regulator for argon (at dewar): Stainless steel, single stage, specially cleaned regulator with 3000 psig max inlet, 0–200 outlet pressure range, CGA 580 cylinder connector, and needle valve shutoff on delivery side terminating in a ¼" Swagelok connector. Part number "KPRCGRF415A2/AG10-AR1" (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. *# Spares = 1.*
2. Regulator for argon (between bulk tank and PerkinElmer filter regulator): Single Stage 316SS Regulator, with 0-300 psi Inlet Gauge, 0-200 psi Outlet Gauge, Outlet Spring Range, 0-250 psi, ¼" Swagelok Inlet Connection, ¼ turn Shut off Valve on Outlet with ¼" Swagelok Connection and Teflon Seals. Part number KPR1GRF412A20000-AR1 (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com) or equivalent. *# Spares = 1.*
3. Regulator for argon (filter regulator on back of ICP-MS): Argon regulator filter kit. Catalog number N812-0508 (PerkinElmer, Shelton, CT, www.perkinelmer.com).

vii. Disinfectant, for work surfaces: Daily remake of diluted bleach (1 part household bleach containing 5.25% sodium hypochlorite + 9 parts water), or an equivalent disinfectant.

viii. Methane: Methane (Research Grade 5.0, 99.99% purity), for DRC channel A. Typically purchased in cylinder size 200 (part # ME R200, Airgas South, Atlanta, GA, www.airgas.com).

1. Regulator for methane: Stainless steel, two stage, specially cleaned regulator with 3000 psig max inlet, 0-25 outlet pressure range, CGA 350 cylinder connector, and needle valve shutoff on delivery side terminating in a ¼" Swagelok connector. Like part number KCYADPF412A2AD10 (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com), or equivalent. *# Spares = 1.*
2. Flash Arrestor: Like part # 6104a (Matheson Tri Gas, Montgomeryville, PA, www.mathesontrigas.com) or equivalent.

ix. Oxygen: Oxygen ("Research Grade Research Grade 5.0", 99.9999% purity) for DRC channel B. Like part # OX R33A (Airgas South, Atlanta, GA, www.airgas.com).

1. Regulator for oxygen: Stainless steel, two stage regulator for use with high purity oxygen (cleaned to be free of all oils). Maximum inlet pressure 3600-5000 psi. Inlet gauge pressure 0-5000 psi (no oil in gauge). Maximum delivery pressure 50–100 psi with a 0-30 psi outlet gauge (no oil in gauge). CGA 540 cylinder connector on inlet side and an angle pattern (90 degree) stainless steel needle valve on the delivery side terminating in

a 1/8" stainless steel Swagelok connector. Like part # GEORG/KCYCFR/ORS2/540 (Georgia Valve and Fitting, Atlanta, GA, www.swagelok.com), or equivalent.

2. Flash arrestor: Like part # 6104A (Matheson Tri Gas, Montgomeryville, PA, www.mathesontrigas.com), or equivalent. # *Spares = 1.*

x. Standard, iridium: Like 1,000 µg/mL, item #CGIR1-1 (Inorganic Ventures, Christiansburg, VA http://www.inorganicventures.com). Used as an internal standard in diluent. Standard must be traceable to the National Institute for Standards and Technology.

xi. Standard, multi-element stock calibration standard: Item number SM-2107-042 (High Purity Standards, Charleston, SC, http://www.hps.net/). Standard must be traceable to the National Institute for Standards and Technology.

xii. Standard, rhodium: Like 1,000 mg/L, item # PLRH3-2Y. (SPEX Industries, Inc., Edison, NJ, www.spexcsp.com). Used as an internal standard in diluent. Standard must be traceable to the National Institute for Standards and Technology.

xiii. Standard, single element stock standards for preparation of calibrators and blood quality control pools: National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs): 3108 (Cd), 3132 (Mn), 3128 (Pb), 3133 (Hg), 3149 (Se). (Gaithersburg, MD, www.nist.gov). Standard must be traceable to the National Institute for Standards and Technology.

xiv. Standard, tellurium: Like 1,000 mg/L, item #CGTE1-1 (Inorganic Ventures, Christiansburg, VA http://www.inorganicventures.com).Used as an internal standard in diluent. Standard must be traceable to the National Institute for Standards and Technology.

xv. Tetramethylammonium hydroxide, 25% w/w, or equivalent (AlfaAesar, 30 Bond St., Ward Hill, MA 01835).

xvi. Triton X-100™ surfactant: Like "Baker Analyzed" TritonX-100™ (J.T. Baker Chemical Co., www.jtbaker.com).

6) Preparation of reagents and materials

a. Internal standard intermediate mixture:

i. Purpose: Preparation of single intermediate solution containing all internal standards simplifies the addition of the internal standard(s) into the final diluent solution. This solution can be purchased rather than prepared.

ii. Preparation: To prepare 50 mL of 20 mg/L Rh, Ir, Te in 1% v/v HNO_3:

1. If not previously dedicated to this purpose, acid wash a 50 mL volumetric flask (PP, PMP, or Teflon™). For example, with 1% (v/v) HNO_3 and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate.

2. Partially fill the 50 mL volumetric flask with 1% v/v HNO_3 (approximately 25-40 mL).
3. Add 1 mL of 1,000 µg/mL Rh standard, 1 mL of 1,000 µg/mL Ir standard, and 1 mL of 1,000 µg/mL Te standard. If initial Rh, Ir, or Te standard concentration is different, adjust volume proportionally.
4. Fill to mark (50 mL) with 1% v/v HNO_3 and mix thoroughly.
5. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

b. Intermediate Triton X-100® solution:

i. Purpose: To ease daily preparation of the diluent and rinse solutions by first preparing an intermediate Triton X-100® solution.

ii. Preparation: To prepare 1 L of 20% Triton x-100®

1. If not previously dedicated to this purpose, acid wash a 200 mL volumetric flask (PP, PMP, or Teflon™). For example, with 1% (v/v) HNO_3 and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate.
2. Add 200 mL of Triton X-100® to the 1L container that is partially filled with ≥18 Mohm·cm water.
3. Fill to 1 L with ≥18 Mohm·cm water and mix until the Triton X-100® has completely dissolved into solution (overnight). A magnetic stirring plate can be used to assist mixing by adding an acid-washed Teflon®coated stirring bar to the bottle.
4. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

c. Sample diluent and carrier

i. Purpose: This solution will be used in the preparation of all samples and calibrators during the dilution process prior to analysis. Make all samples, standards, blanks, QC, etc. . . in a run from the same diluent solution so that the concentration of the internal standards will be the same among all calibrators and samples in the run. When using a flow-injection component in the sample introduction system (i.e. the Elemental Scientific SC4-FAST autosampler), use the same solution for the the 'carrier' and sample diluent. The diluent is an aqueous solution of 5 µg/L internal standard mixture (Rh, Ir, Te), in 0.4% v/v tetramethyl ammonia hydroxide (TMAH), 1% ethyl alcohol, 0.01% APDC, and 0.05% v/v Triton X-100®. Larger volumes of these solutions can be prepared by adjusting component volumes proportionally.

ii. Preparation: To prepare 2L of 5 µg/L Rh, Ir and Te, 0.01% APDC in 0.4% v/v TMAH, 1% ethanol, and 0.05% v/v Triton X-100:

1. If not previously dedicated to this purpose, acid wash a 2L container (PP, PMP, or Teflon™). For example, with 1% (v/v) HNO_3 and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate.
2. Partially fill the 2L container with ≥18 Mohm·cm water.
3. Add 0.2 g of APDC , 8 mL of 25% v/v TMAH, 20 mL of ethanol, and 5 mL of 20% Triton X-100®.
4. Dilute to volume (2L) with ≥18 Mohm·cm water.
5. Spike 500 µL of 20 mg/L Rh, Ir, Te to the final diluent.
6. Invert bottle a few times to insure thorough mixing. Allow to sit for several hours or overnight before using.
7. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

d. ICP-MS rinse solution

i. Purpose: The rinse solution used in this method is an aqueous solution of 0.01% APDC in 0.4% v/v TMAH, 1% ethanol, and 0.05% v/v Triton X-100. This solution will be pumped through the autosampler rinse station, probe, and sample loop between sample analyses to prevent carry-over of analytes from one sample measurement to the next.

ii. Preparation: To Prepare 4 L of 0.01% APDC in 0.4% v/v TMAH, 1% ethanol, and 0.05% v/v Triton X-100:

1. If not previously dedicated to this purpose, acid wash a 4L container (PP, PMP, or Teflon™). For example, with 1% v/v HNO_3 and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate.
2. Partially fill the 4 L bottle with ≥18 Mohm·cm water (approximately 2-3 L). Use of volumetric flask is not required.
3. Add 0.4 g of APDC
4. Add 16 mL of TMAH
5. Add 40 mL of ethyl alcohol,
6. Add 10mL of 20% Triton X-100®, (See Section 6.b for details on preparation)
7. Fill to 4 L using ≥18 Mohm·cm water.
8. Store at room temperature and prepare as needed. To prepare volumes other than specified here, add proportionally larger or smaller volumes of the solution constituents.

9. Invert bottle a few times to ensure thorough mixing. Allow to sit for several hours or overnight before using.
10. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

e. Standards, calibrators, base blood and QC

i. Multi-element stock calibration standards

1. Purpose: This multi-element stock standard will be used to prepare the intermediate working calibration standards.
2. Purchase & Storage:
   a. Purchasing from vendors: Whether purchased or prepared in-house, the starting materials must be NIST-traceable. Matrix and concentrations of Pb, Cd, Hg, Mn and Se are listed in Table 3 of Appendix B.
   b. Storage: Store at room temperature and label appropriately. Expiration is as defined by the manufacturer or 1 year from date of opening, whichever comes first.

ii. Diluent for intermediate calibration standard preparations:

1. Purpose: This diluent is used to dilute stock and intermediate stock calibration standards, not to prepare working calibrators or blood samples for analysis.
2. Preparation: To prepare 2L of 3% v/v HCl:
   a. If not previously dedicated to this purpose, acid wash a 2L container (PP, PMP, or Teflon™). For example, with 3% HCl and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate.
   b. In the 2 L flask, add 1-1.5L ≥18 Mohm·cm water.
   c. Add 60 mL high purity concentrated HCl.
   d. Fill to the mark and mix thoroughly.
   e. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

iii. Multi-element intermediate stock calibration standard

1. Purpose: This multi-element intermediate stock standard will be used to prepare the intermediate working calibration standards.
2. Preparation: To prepare 3% v/v HCl solutions containing Cd, Pb, Hg, Se, and Mn with concentrations listed in Table 4 of Appendix B:
   a. Acid-rinse one 100 mL, PP (or PMP) volumetric flask. For example, with 3% HCl and ≥18 Mohm·cm water (at least 3 times each) and verify

cleanliness through analysis of rinsate. Mark flask according to intended use. Dedicate to purpose.

b. Partially fill (50-75% full) the 100 mL flask with the 3% (v/v) HCl diluent prepared in Section 6.e.ii.

c. Using the volume listed in Table 4 of Appendix B, pipette the appropriate volume of the multi-element stock calibration standard solution into the volumetric flask. Dilute to the volumetric mark with the 3% HCl (v/v) diluent using a pipette for the final drops. Mix each solution thoroughly. Final concentrations are listed in Table 4 of Appendix B.

d. Once mixed, transfer to acid-cleaned, labeled, 50 mL containers (PP, PMP, or Teflon™) for storage.

e. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

iv. Intermediate working calibration standards

1. Purpose: Used each day of analysis to prepare the final working calibrators that will be placed on the autosampler.

2. Preparation: To prepare 3% v/v HCl solutions containing Cd, Pb, Hg, Se, and Mn with concentrations listed in Table 3 of Appendix B:

a. Acid-rinse eight 100 mL, PP (or PMP) volumetric flasks and one 2 L PP (or PMP) volumetric flasks. For example, with 3% HCl and ≥18 Mohm·cm water (at least 3 times each) and verify cleanliness through analysis of rinsate. Mark each flask according to intended use. Dedicate to purpose.

b. Fill each 100 mL flask 50-75% with the 3% (v/v) HCl diluent prepared in Section 6.e.ii.

c. Using the volumes listed in Table 5 of Appendix B, pipette the appropriate volume of the multi-element intermediate stock calibration standard solutions into each of the volumetric flasks. Dilute each to the volumetric mark with the 3% HCl diluent using a pipette for the final drops. Mix each solution thoroughly. Final concentrations are listed in Table 5 of Appendix B.

d. Once mixed, transfer to acid-cleaned, labeled, 50 mL containers (PP, PMP, or Teflon™) for storage.

e. Store at room temperature and label appropriately. Expiration is 1 year from date of preparation.

f. Pour aliquots of each standard into clean 15mL polypropylene tubes and label for daily use.

v. Working calibrators

1. Purpose: The working calibrators will be analyzed in each run to provide a signal-to-concentration response curve for each analyte in the method. The concentration of the analyte of interest in a patient blood sample dilution is determined by comparing the observed signal ratio (element/internal standard) from the dilution of the patient blood sample to the signal ratio response curve from the working calibrators.
2. Content: Dilutions (1:50) of the corresponding eight intermediate working calibration standards with base blood and sample diluent.
3. Preparation: Mix with base blood and diluent (Section 6.c) using a benchtop automatic pipette to make 1:50 dilutions of the corresponding eight intermediate working calibration standards immediately prior to analysis (see Table 8 of Appendix B).

vi. Base blood

1. Purpose: This blood pool material will be mixed with the intermediate working calibrators just prior to analysis to matrix-match the calibration curve to the blood matrix of the unknown samples.
2. Preparation: To prepare a mixture of multiple blood sources collected from anonymous donors to approximate an average blood matrix:
   a. Purchase several bags of whole blood.
   b. Screen each individual bag of blood for concentration of analytes of interest. See Table 2 in Appendix B for minimum acceptable values
   c. Once screened, mix the acceptable blood together in a larger container (i.e. acid washed polypropylene (PP), polymethylpentene (PMP), or Teflon™) and stir for 30+ minutes on a large stir plate (acid wash large Teflon™ stir bar before use).
   d. Store long-term as smaller portions for daily use (e.g. 2 mL cryovials) according the same storing and handling criteria described in Section 3.

vii. Internal quality control materials ("bench" QC)

1. Purpose: Internal (or "bench") quality control (QC) materials are used to evaluate the accuracy and precision of the analysis process, and to determine if the analytical system is "in control" (is producing results that are acceptably accurate and precise). They are included in the beginning and at the end of each analytical run.
2. Preparation: To prepare pooled animal or human blood at low-normal and high-normal concentrations:

   Both purchased or in-house prepared quality control materials are suitable for this purpose if volumes, concentrations meet method requirements and

any spikes of elemental levels are traceable to the National Institute for Standards and Technology (NIST).

3. Screening blood: Screen bags of blood for analyte of interest concentration before mixing together to make 2 separate base blood pools (for preparing the low and high bench QC materials). Samples can be screened individually
    a. Keep blood refrigerated whenever possible to minimize microbial growth.
    b. Because this is only a quick screen of the analyte of interest concentration, the number of replicates in the blood method can be reduced to one in order to reduce analysis time.
    c. Select blood for the low bench QC pool which has analyte concentrations in the low-normal population range. Select blood for the high and elevated bench QC pools which has analyte concentrations less than some pre-selected target concentration values in the high normal population range. See Table 2 in Appendix B for recommended concentration ranges.
4. Combining collected blood: The goal is for combining samples is to approach an 'average' matrix for each pool.
    a. Graduate four acid-washed 10 L carboys (PP or PMP) in 0.5 L increments (two will be used for decanting into).
    b. Combine collected blood samples into two separate acid-washed 10 L carboys (PP or PMP), according to their concentrations, for the low bench and high bench QC pools.
    c. Mix each blood pool using carboy stirrers and large stir plates. Keep blood refrigerated whenever possible.
5. Spiking of blood
    a. Analyze three samples of each blood pool. Record these results for future recovery calculations.
    b. Use these results to determine target analyte concentrations possible for the pools
    c. Calculate the volume of single element standards needed to spike each pool to the desired concentrations. See Table 2 in Appendix B for recommended concentration ranges.
    d. While stirring the pools on large stir plates, spike each pool with calculated volumes of single element standards (all spiking standards used must be traceable to NIST).
    e. Continue to stir pools overnight after spiking, then reanalyze.

f. Repeat steps 4 and 5 until all analytes reach target concentrations keeping track of the total volume of spiking solution added to each blood pool.

6. Dispensing and storage of blood

a. Container types: Dispense blood into lot screened containers (i.e. – 2 mL polypropylene tubes). If possible, prepare tubes of QC which have only enough volume for one typical run + 1 repeat analysis. This allows for one vial of QC to be used per day of analysis, reducing chances of contamination of QC materials due to multi-day use.

b. Labels: Place labels on vials after dispensing and capping if the vials are originally bagged separately from the caps. This minimizes the chance for contamination during the process. Include at least the name of QC pool (text and bar code), date of preparation, and a vial number on the labels.

c. Dispensing: Dispensing can be accomplished most easily using a benchtop automatic pipette in continuous cycling dispense mode. Dispense the pools in a clean environment (i.e. a class 100 cleanroom area or hood).

1. Allow blood to reach room temperature before dispensing (to prevent temperature gradients possibly causing concentration gradients across the large number of vials being dispensed and to prevent condensation problems during labeling of vials).

2. Replace the tubing attached to the dispensing syringe (left when looking at front of the benchtop automatic pipette) with a length of clean Teflon™ tubing long enough to reach into the bottom of the 10 L carboy while it is sitting on the stir plate.

3. Check cleanliness of the benchtop automatic pipette before use by analyzing 1-2% (v/v) HNO_3 which has been flushed through the benchtop automatic pipette with a portion of the same solution which has not been through the benchtop automatic pipette.

4. Approximately one hour before dispensing begins,

a. With the large stir plate close to the left side of the benchtop automatic pipette, begin stirring the blood pool to be dispensed.

b. Also during this time, flush the benchtop automatic pipette with blood from the pool to be dispensed. Place the ends of the tubing attached to both the sample and dispensing syringes into the carboy of blood so that blood won't be used up during this process. Be sure to secure both ends

of tubing in the carboy with Parafilm so they will not come out during the flushing process.

5. After dispensing the blood into the vials, cap the vials and label them. Placing labels on vials after capping minimizes the chance for contamination during the process.

d. Homogeneity test: Check homogeneity of analyte concentrations in pool aliquots.

e. Storage: Store long-term as smaller portions for daily use (e.g. 2 mL cryovials) according the same storing and handling criteria described in Section 3.

f. Optimization solutions

i. DRC optimization:

1. Purpose: For periodic testing of the DRC cell parameters. Procedure requires at a minimum a blank (i), an analyte solution (ii), a blank with interference (iii), and an analyte and interference containing solution (iv). For Se, only the blank (i), an analyte solution (ii) are needed because the interference on Se is plasma based.

2. Content:

Diluent in this section refers to sample diluent (5 μg/L internal standard mixture (Rh, Ir, Te), 0.4% v/v tetramethyl ammonia hydroxide (TMAH), 1% ethyl alcohol, 0.01% APDC, and 0.05% v/v Triton X-100® as described in Section 6c.

a. Solutions for testing elimination of $^{54}Fe^{1}H$ interference on ^{55}Mn:

i. Base blood in diluent (1 + 49)
ii. Base blood in diluent (1 + 49) + 4.5 μg/L Mn
iii. Base blood in diluent (1 + 49) + 500 μg/L Fe
iv. Base blood in diluent (1 + 49) + 4.5 μg/L Mn + 500 μg/L Fe

b. Solutions for testing elimination of $^{40}Ar_2$ interference on ^{80}Se:

i. Base blood in diluent (1 + 49)
ii. Base blood in diluent (1 + 49) + 90 μg/L Se

3. Preparation & storage: Prepare different volumes, if needed, by adding proportionally larger or smaller volumes of solution constituents. Interference concentrations can be prepared higher as needed by adjusting the volume of this spike. Keep interference spike volume small (<0.3 mL) using a high concentration stock solution (i.e. 1000 mg/mL). Analyte concentrations can be made higher if needed for sensitivity reasons by preparing a higher concentration calibrator.

a. Solutions for testing elimination of $^{54}Fe^{1}H$ interference on ^{55}Mn:
   i. Base blood in diluent (1 + 49)
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 0 as described in Table 6 (multiply volumes by 5).
   ii. Base blood in diluent (1 + 49) + 4.5 µg/L Mn
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 2 as described in Table 6 (multiply volumes by 5).
   iii. Base blood in diluent (1 + 49) + 500 µg/L Fe
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 0 as described in Table 6 (multiply volumes by 5).
      2. Add 0.025 mL of 1000 mg/mL Fe.
   iv. Base blood in diluent (1 + 49) + 4.5 µg/L Mn + 500 µg/L Fe
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 2 as described in Table 6 (multiply volumes by 5).
      2. Add 0.025 mL of 1000 mg/mL Fe.

b. Solutions for testing elimination of $^{40}Ar_2$ interference on ^{80}Se:
   i. Base blood in diluent (1 + 49)
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 0 as described in Table 6 (multiply volumes by 5).
   ii. Base blood in diluent (1 + 49) + 90 µg/L Se
      1. In a 50 mL lot screened or acid-washed polypropylene tube, prepare a 50 mL portion of working calibrator 2 as described in Table 6 (multiply volumes by 5).

c. Store at room temperature and prepare as needed.
d. Label appropriately (see Section 6.f.i.2), "Store at room temperature", preparation date, expiration date one year from preparation date, and preparer's initials.

ii. Dual detector calibration:

1. Purpose: Use as necessary to perform the dual detector calibration.
2. Content: Aqueous dilutions of single element stock standard solutions in 2% (v/v) nitric acid. Current solution in use contains: Pb with a final concentration of 200 ug/L.

3. Preparation & storage: Prepare different volumes, if needed, by adding proportionally larger or smaller volumes of solution constituents.
   a. To prepare a total of 50 mL: In a 50 mL lot screened polypropylene tubes, spike in 0.01 mL of 1000 mg/mL single element stock solution for each element desired in the final solution.
   b. Dilute to the 50 mL mark with 2% (v/v) nitric acid.
   c. Store at room temperature and prepare as needed.
   d. Label appropriately, e.g. "200 ug/L Pb in 2% (v/v) HNO3", "Store at room temperature", preparation date, expiration date one year from preparation date, and preparer's initials.

7) Analytical instrumentation setup

(see Section 5 for details on hardware used, including sources)

a. Instrumentation and equipment setup:

i. Configuration for liquid handling

1. FAST valve setup: See Appendix B, Figure 1 for diagram and Section 5.b "FAST / ESI SC4-DX autosampler accessories" for source information.
   a. Port 1: sample loop (white nut).
   b. Port 2: 0.5 mm ID probe (red nut) for carrier solution.
   c. Port 3: nebulizer line (green nut) for transfer of liquid to nebulizer.
   d. Port 4: sample loop (white nut).
   e. Port 5: 0.8 mm ID probe (blue nut) for diluted samples.
   f. Port 6: vacuum line (black nut).

2. Carrier solution uptake: Use peristaltic pump to control uptake flow rate of carrier solution to the SC-FAST valve. Use of a 'peristaltic to Teflon tubing adapter' for prevents damage to small i.d. tubing when making connections (see consumables descriptions in Section 5.b).

3. Spray chamber waste removal

   Use of a 'peristaltic to Teflon tubing adapter' for prevents damage to small i.d. tubing when making connections (see consumables descriptions in Section 5.b).

   a. Between spray chamber and peristaltic tubing:
      i. Spray chambers with threaded connection: Use vendor-supplied threaded connector on base of chamber, connecting tubing directly to peristaltic pump tubing through a PEEK adapter or directly.

ii. Spray chambers without threaded connection: Use of specialized push-on connectors available from various vendors (like UFT-075 from Glass Expansion, Pocasset, MA) are preferred for safety reasons to direct connection of PVC tubing (e.g. 1/8" i.d. x ¼" o.d.).

b. Between peristaltic pump tubing and waste container: Connect 1/8" i.d. x ¼" o.d. PVC tubing to the white / black peristaltic pump tubing using a tubing connector (PerkinElmer item # B3140715). Place the free end of the PVC tubing through the lid of the waste jug (be sure it is secure). Place waste container in a deep secondary containment tray in case of overflow.

4. Rinse solution for autosampler:

a. Rinse solution jug: Leave one of the caps on the top of the rinse jug loose to allow air venting into the jug as liquid is removed. Otherwise the jug will collapse on itself as the liquid is removed and a vacuum is created inside. Use secondary containment tray.

b. Rinse solution uptake to autosampler rinse station: Use tubing of different lengths and inner diameters between the rinse solution container and the autosampler rinse station to control uptake rate of rinse solution. These can be obtained from the autosampler manufacturer, their distributors, or custom built in the lab. Optimize these factors along with fill time in the software so that waste of rinse solution is minimized and rinse station does not go empty.

c. Autosampler rinse station waste removal: Gravity drain of waste to the waste container is sufficient. Use minimum drain tubing to make this connection. If this tube is too long, the rinse station will not drain properly.

ii. Gas delivery and regulation

1. ICP-MS modifications:

a. Plastic tubing between mass flow controllers and dynamic reaction cell have been replaced with stainless steel. Stainless steel tubing is preferred between the reaction gas cylinder / regulator and the back of the ICP-MS instrument.

b. A second mass flow controller will be needed (channel B) that does not send the DRC gas through a 'getter'.

2. Argon gas: Used for various ICP-MS functions including plasma and nebulizer.

a. Regulator for argon source (if a dewar): Set delivery pressure of this regulator at least 10 psi higher than the delivery pressure of the step-

down regulator to allow for pressure drop across tubing that stretches to the instrument.

b. Step down regulator (if source of argon is a bulk tank): Place this single stage regulator in the lab so that incoming argon pressure can be monitored and adjusted. Set delivery pressure to 10 psig above the delivery pressure of the filter regulator on the ICP-MS.

c. Filter regulator at ICP-MS: Single stage "argon regulator filter kit" supplied with the ICP-DRC-MS. Set the delivery pressure depending on the instrument setup:

i. ELAN with a 0-60psi gauge on the filter regulator: 52±1 psi when plasma is running (need 0-150 psi regulator if using a PolyPro or PFA nebulizer made by Elemental Scientific Inc).

ii. ELAN with a 0-150psi gauge on the filter regulator: 90-100 psi when plasma is running.

3. Methane (99.99%) gas: Used for dynamic reaction cell interference removal from selenium isotopes.
   a. Connect to DRC channel A
   b. Set the delivery pressure of regulator to 5-7 psig when gas is flowing. See section 5.e for part numbers and details.

4. Oxygen (99.999^{+}%) gas: Used for dynamic reaction cell interference removal from manganese isotopes.
   a. Connect to DRC channel B.
   b. Set the delivery pressure of regulator to 5-7 psig when gas is flowing. See Section 5.e for part numbers and details.
   c. Use a brass flash arrestor on outlet side of regulator. See Section 5.e for part numbers and details.

iii. Chiller / heat exchanger: If using refrigerated chiller, set temperature control to approximately 18 °C.

b. Instrument and method parameters: See Tables and Figures in Appendix B for a complete listing of the instrument and method parameters and software screen shots.

8) The run: quality, execution, evaluation, and reporting

a. Bench QC, reference materials and calibration verification:

i. Bench "QC": Analysis of bench QC permits assessment of methodological imprecision, determination of whether the analytical system is 'in control' during the run, and assessment of time-associated trends. Before QC materials can be used in the QC process, they must be characterized by at least twenty (20) analytical runs to determine appropriate QC parameters.

Bench QC pool analyte concentrations in this method span the analyte concentration range of the calibrators including "low-normal" ('Low QC'), "high-normal" ('High QC'), and "above-normal" ('Elevated QC') concentrations.

In each analytical run the analyst will test each of the three bench QC samples two times, subjecting them to the complete analytical process. Bench QC pool samples are analyzed first in the run after the calibration standards but before any patient samples are analyzed. This permits making judgments on calibration linearity and blank levels prior to analysis of patient samples. The second analysis of the bench QC pools is done after analysis of all patient samples in the run (typically 40-50 patient samples total when analyzing for all elements in the method) to ensure analytical performance has not degraded across the time of the run. If more patient samples are analyzed on the same calibration curve after the second run of the bench QC, all bench QC must be reanalyzed before and after the additional samples. For example, the schemes shown in Table 6 in Appendix B are both acceptable ways to analyze multiple consecutive "runs".

ii. Reference materials: Use standard reference materials (SRM) from the National Institute of Standards and Technology (NIST) (i.e. SRM 955c Levels 1-4) to verify method accuracy. Use previously characterized samples from proficiency testing program or commercially-produced reference materials when NIST SRMs are unavailable.

iii. Calibration verification: The test system is calibrated as part of each analytical run with NIST-traceable calibration standards. These calibrators, along with the QCs and blanks, are used to verify that the test system is performing properly.

b. Perform, evaluate and report a run

i. Starting the equipment for a run

1. Power on the computer, printer, and autosampler, and instrument computer controller.
2. Peristaltic pump: Set proper tension on peristaltic pump tubing.
3. Software: Start software for the ICP-MS and autosampler control.
4. Daily pre-ignition maintenance checks: Perform and document daily maintenance checks (e.g., Ar supply pressure, interface components cleanliness and positioning, interface pump oil condition, vacuum pressure, etc.).
5. Place probe in adequate volume of carrier or rinse solution: If using an ESI FAST, manually place carrier probe into carrier solution. If not, send the autosampler probe to a rinse solution (e.g. autosampler rinse station).
6. Start the plasma

7. Start the peristaltic pump: Start the pump running slowly, making sure that the rotational direction is correct for the way the tubing is set up.
8. Warm-up time: Allow warm-up time suggested by the manufacturer for the ICP-MS (e.g. RF generator) after igniting the plasma. There will be another warm-up time (or "stability time") for the DRC later in this procedure.
9. Daily performance check: Perform and document a daily performance check and any optimizations necessary.

   Save new parameters to the "default.tun" and "default.dac" files.
10. DRC stability time: Best analyte-to-internal standard ratio stability is typically observed after 1-1.5 hours of analysis of diluted blood samples using the DRC mode method (~15 measurements of the 5 element panel can be made in 1 hour). Prepare 50mL+ of a calibration standard (e.g. standard 2) to be analyzed repeatedly before the beginning of the run to achieve a stable analyte-to-internal standard ratio. Time to reach stability is instrument-specific and learned from performance of runs. See Table 7 in Appendix B for example of setup in the Samples / Batch window and Table 8 in Appendix B for details of making a working standard.
11. Readying the instrument for quick-start analysis: Leave the plasma running to eliminate the need for an initial instrument warm-up period and / or a DRC stabilization period as long as appropriate planning is made for sufficient solution supply and waste collection. Analysis of conditioning samples (diluted blood matrix) can also be scheduled to occur at roughly a predetermined time. Accomplish this by setting up multiple sample analyses with extended rinse times (e.g. one 5 element analysis with a 1500s rinse time will take approximately 30 minutes to complete). Initial samples would be non-matrix, while final samples would be diluted matrix for conditioning. If running a DRC-only method during these scheduled analyses, the ICP-MS will remain in DRC-mode for approximately 45 minutes without depressurizing the cell.
12. Software setup for analysis:
    a. Workspace (files & folders): Verify & set up the correct files and data directories for your analysis (See Table 1 in Appendix B for defaults).
    b. Samples / batch window: Update the software to reflect the current sample set. Use a bar code scanner to input data whenever possible. See Table 1 in Appendix B for times and speeds.

        1. Blood vs. aqueous method files:
            a. The difference: There are two method files for this one method (see Table 1 in Appendix B). It is necessary to use both to accomplish each run because the current PerkinElmer software will not allow for more than one blank

per method file. The ONLY DIFFERENCE between these two files is on the Sampling tab where one lists the autosampler positions of the blood blank and blood-based calibrators (the "bldblk" method file) and the other lists the autosampler position of the aqueous blank (the "aqblk" method file).

b. Use: The ONLY TIME when it matters which of these files is used is when the measurement action *includes* "Run blank" or "Run standards". When the measurement action is only 'run sample', it does not matter whether the "bldblk" or "aqblk" method file is used. Analysts typically follow the pattern below, however, for the sake of consistency and as a reminder of which blank must be used for which type of sample. See Table 7 in Appendix B.

   i. The *"bldblk" method file:* Use to analyze the initial blood blank (blank for the calibration curve), the blood calibrators, and the blood blank checks at the very beginning of the run. The blood blank method defines the autosampler location of the blood blank and the blood calibration standards.

   ii. The "*aqblk*" method file must be used to analyze all QC materials and patient samples. The aqueous blank method defines the aqueous blank in autosampler location.

ii. Preparation of samples for analysis (See Table 6 in Appendix B)

1. Thaw blood samples; allow them to reach ambient temperature.
2. Prepare the following solutions into pre-labeled containers using the benchtop automatic pipette or other volumetric sample transfer device. See Table 8 in Appendix B for a summary.

   a. *Aqueous Blank*: Prepare a minimum of two aqueous blanks. One will be the actual aqueous blank and the other will be a backup ("Aqueous Blank Check") in case the original aqueous blank is unusable.

   b. *Calibrators*: Prepare the working calibrators (S0-S8). Prepare S0 in triplicate. One of these S0 preparations will be the zero calibrator (blood blank) for the calibrators; the other two will be analyzed twice after the last calibrator to collect run blank data that can be used in calculating method limit of detection (LOD).

   c. *Patient & QC Samples*: Before taking an aliquot for analysis, homogenize the sample thoroughly.

   After preparation, mix and cover. Place prepared dilutions on the autosampler of the ICP-MS in the order corresponding to the sequence setup in the ICP-MS software.

Room temperature is acceptable for the original samples for the work day.

NOTE: ***Samples must be analyzed within 24 hours of preparation to obtain valid results for selenium***. The method has been validated to produce valid results for other Pb, Cd, Hg, and Mn even 48 hrs after sample preparation. See critical parameter test results in Appendix A for details.

iii. Start the analysis using the ICP-MS software.

iv. Monitor the analysis in real-time as much as possible. If necessary, leave the run to complete itself unattended as long as appropriate planning is made for either overnight operation or Auto Stop (see below).

Monitor the analysis for the following:

1. *Verify proper operation of the instrument* (proper loop filling, sample reaching nebulizer in correct timing, autosampler arm moving properly, etc . . .).
2. Verify that background signal from instrument and reagents are low. Helpful checks when diagnosing high background problems include:
   a. Water to be used in Aq Blank Checks and dilutions.
   b. Diluent before and after being flushed through the benchtop automatic pipette.

      If contamination is observed from the pipette, flush the pipette with ≥500 mL of nitric acid solution (≤ 5% v/v HNO_3) and retest.

   c. Comparison with other instruments.
3. *Verify analyte / internal standard ratio stability*

   The net intensity (analyte / internal standard ratio) of the measurements made while stabilizing the DRC can be evaluated to determine the readiness of the system to begin analysis. Continual trending in this ratio indicates that unwanted instrument drift will occur within the run.
4. *Verify calibration curves* meet R^2 requirements (minimum of 0.98, typically 0.99 to 1.000).
5. *Verify bench QC results are within the acceptable limits.*

   If an analyte result for the beginning QC material(s) falls outside of the ± 3SD limits, then the following steps are recommended:

   a. Evaluate the blank results.

   b. Evaluate the reproducibility of the 3 replicates within the measurements.

   c. Evaluate the consistency of the internal standard across the measurements (esp. the calibrators).

d. Evaluate calibration curves. If a particular calibration standard is obviously in error, it can be re-analyzed as a sample (old or new dilution) and incorporated into the curve through data reprocessing as a calibrator. As a last resort, a single calibration point per analyte between or including S2 and S7 can be removed from the curve (Do not drop S0, S1 or S8). Follow up problems with calibration standards with appropriate corrective actions (e.g. re-preparation of intermediate working standards or troubleshooting instrument parameters).

e. Prepare a fresh dilution of the failing QC material (same vial) and reanalyze it to see if the QC dilution was not properly made.

f. Prepare a fresh dilution of the failing QC material (unused vial) and analyze it to see if the QC vial had become compromised.

g. Prepare and analyze new working calibrators.

h. Test a different preparation of intermediate working calibration standards.

If these steps do not result in correction of the out-of-control values for QC materials, consult the supervisor for other appropriate corrective actions.

6. *Verify good precision among replicates* of each measurement.

7. *Verify consistent measured intensities of the internal standards.*

   Some sample-to-sample variations are to be expected, however, intensities drifting continuously in one direction resulting in failing results for ending QC indicate the instrument needs additional pre-conditioning before the run or environmental conditions are changing too much around the instrument.

8. *Verify elevated patient results.*

   Refer to Figure 4 in Appendix B for flowchart.

   a. Confirming an elevated concentration: Repeat for confirmation any sample having a concentration greater than the 1UB threshold. See Table 9 in Appendix B.

   b. Dilution of a sample to within the calibration range: Repeat in duplicate with extra dilution any sample having a concentration greater than the highest calibration standard to bring the observed result within the concentration range of the calibrators. See Table 7 in Appendix B for validated extra dilutions.

   c. Confirming proper washout after an elevated sample: When monitoring the analysis in real-time, if a sample concentration is greater than the highest concentration validated for washout (see Table 9 of Appendix B), do the following to verify that the run is still in control for low concentration samples before proceeding with analysis.

      i. Stop run following elevated sample

ii. Verify that the run is still in control for lower concentration samples before proceeding with analysis. Analyze 2 blood blank checks followed by a low bench QC washout check. If the low bench QC wash check is not in control (within ± 3SD limits), repeat these 3 check samples until washout is verified before proceeding with analysis.

Example:
3016 BldBlkChk Wash1
3016 BldBlkChk Wash2
LBXXXXX Wash

iii. If the run is not verified in-control for low concentration samples before the next samples are analyzed, see Section 8.b.vii.2. for directions.

v. Overnight operation or using auto stop: The run may be left to complete itself unattended as long as appropriate planning is made (e.g. sufficient solution supply and waste collection). Turn on the AutoStop feature of the ICP-MS software. Delay the shutdown at least 10 minutes (use peristaltic pump speed approximately that of the method wash) to rinse the sample introduction system of blood matrix before turning off the plasma. It will be necessary to replace the sample peristaltic pump tubing the next day since it will have been clamped shut overnight. Enable "Auto Start / Stop" is on the "AutoStop" tab of the Instrument window.

vi. Records of results: Run results will be documented after each run in both electronic and paper form.

1. Electronic records: Transfer data electronically to the laboratory information system. When keyboard entry must be used, proofread transcribed data after entry.

   a. Export data from the ICP-MS software using "original conditions" or files and folders used during the analysis. Use descriptive report filenames (e.g. 2005-0714a_group55.txt). In the ICP-MS software under "Report Format" (METHOD window, REPORT tab) choose the "Use Separator" option, and under the "File Write" Section choose "Append."

   b. Move the generated .TXT data file to the appropriate subdirectory on the network drive where exported data are stored prior to import to the laboratory information management system.

   c. Import the instrument file into the laboratory information system with appropriate documentation (e.g. instrument ID, analyst, calibration standards lot number, and run or sample specific comments).

2. Paper records: Printed run sheets must be documented with

   i. Analyst initials

ii. Instrument ID

iii. Date of analysis and run # for the day

vii. Analyst evaluation of run results:

1. Bench quality control: After completing a run, and importing the results into the laboratory information system, evaluate the run bench QC according to laboratory QC rules. The QC limits are based on the average and standard deviation of the beginning and ending analyses of each of the bench QC pools, so it will not be possible to know if the run is in control until statistically reviewed.

   a. Rules for bench quality control evaluation: The following are the CDC DLS QC rules for three QC pools per run with two or more QC results per pool.

      i. If all three QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.

      ii. If one of the three QC run means is outside a $2S_m$ limit - reject run if:

         1. Extreme Outlier – Run mean is beyond the characterization mean ± $4S_m$
         2. 3S Rule - Run mean is outside a $3S_m$ limit
         3. 2S Rule – Two or more of the run means are outside the same $2S_m$ limit
         4. 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

      iii. If one of the QC individual results is outside a $2S_i$ limit - reject run if:

         1. Extreme Outlier – One individual result is beyond the characterization mean ± $4S_m$
         2. R 4S Rule – 2 or more of the within-run ranges in the same run exceed $4S_w$ (i.e., 95% range limit)

            Note: Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only.

      Abbreviations:

      S_i = Standard deviation of individual results.

      S_m = Standard deviation of the run means.

      S_w = Within-run standard deviation.

   b. Implications of QC failures: If the DLS SAS program declares the run "out of control" for any analyte, use the following to determine the implications on usability of the data from the run.

i. For 1 or 2 analytes: ONLY the analytes which were "out of control" are invalid for reporting from the run.

ii. For 3 or more analytes: All results, regardless of analyte, are invalid for reporting from the run.

2. Patient results:

a. Elevated concentrations: Refer to Figure 5 in Appendix B for flowchart.

i. Boundaries requiring confirmatory measurement:

1. Results greater than the first (1UB) or second (2UB) upper boundaries.

The concentrations assigned to 1UB and 2UB for an element is determined by study protocol but default concentrations are in Table 9 in Appendix B.

a. Results greater than the first upper boundary (1UB): Confirm by repeat analysis of a new sample preparation concentrations observed greater than the "first upper boundary" (defined in the laboratory database as the "1UB"). Report the first analytically valid result, as long as the confirmation is within 10%. Continue repeat analysis until a concentration can be confirmed.

b. Analyst reporting of elevated results: Report any patient results confirmed to be greater than the second upper boundary (2UB) as an "elevated result".

2. Results greater than highest calibrator: Samples that exceed the high calibrator must be prepared with minimum extra dilution in duplicate to bring the observed result within the calibration range (≤ S8). Report the first analytically valid result (i.e. the first one within the calibration range), as long as the confirmation is within 10%. Continue repeat analysis until a concentration can be confirmed.

ii. Concentrations requiring verification of washout: Following a result greater than the highest concentrations validated for washout (see Table 9 of Appendix B) do the following:

1. If the run was determined to be in-control for low concentration samples before the next samples were analyzed, no further action is required.

2. If the run was not determined to be in-control for low concentration samples before the next samples were analyzed confirm by re-analysis the results for the 2 samples immediately following the elevated sample. Report the results if they confirm the initial results within ±10% or ±3SD of the low bench QC, whichever is greater.

b. Unacceptable reproducibility: If the range of the three replicate readings (maximum replicate concentration value - minimum replicate concentration value) for a single sample analysis is greater than the range maximum criteria listed in Table 9 in Appendix B ***and*** the range of the three replicate readings is greater than 10% of the observed concentration, do not use the measurement for reporting. Repeat the analysis of the sample.

viii. Submitting final work for review: All analyses must undergo quality control and quality assurance review. After appropriately documenting the run in the laboratory information system (e.g. sample and run QC, and run and sample comments), inform the first level reviewer of the completed work and submit any printed documentation.

9) Routine equipment maintenance and data backups

Maintenance activities will be documented in the instrument logbook.

a. Equipment maintenance: Analysts are expected to regularly evaluate the need for, and when necessary perform, cleaning, replacement, or re-positioning of components in ICP-MS the sample introduction system, interface, ion optics region, and equipment required resources (e.g. autosampler, exhaust, compressed gases, and coolant). Frequency of equipment maintenance will be dependent on instrument throughput.

b. Parameter optimizations: Analysts are expected to optimize instrument parameters.

i. Dual detector calibration: Perform dual detector calibration regularly for any element exceeding 1,000,000 cps for calibration standard 8. This is typically only Pb. Dual detector calibration solution is described in Section 6.f.ii. Frequency of dual detector calibration is typically monthly when throughput requires multiple analytical runs per week, or as needed for optimized linearity.

ii. DRC optimizations: DRC conditions (cell gas flow rate and RPq value) can be verified by analyzing the DRC optimization solutions (see Section 6.f.i) as needed to ensure proper reduction of potential ICP-MS interferences.

c. Data backup: Data on the instrument computer will be backed up via two backup routines. Files used and produced by the ICP-MS in analyzing samples will be backed up and kept a minimum of two years after analysis.

i. Daily backups to secondary hard drive: Program automatic backups of the relevant computer files to occur each night onto a secondary hard drive to prevent loss of data from failure of primary hard drive.

ii. Weekly backup: Backup relevant computer files weekly either to secondary hard drive which is remote to the laboratory or to removable media which will be placed remote to the laboratory for retrieval in the case of catastrophic data loss elsewhere.

10)Reporting thresholds

a. Reportable range: Blood elemental concentrations are reportable in the range between the method LOD and the highest calibrator (see 'calibrator concentrations' in Table 1) times the maximum validated extra dilution (see Table 8). Above the highest concentration verified, extra dilutions are made of the blood sample to bring it within the reportable range.

b. Reference ranges (normal values): In this method the 95% reference ranges (see Appendix B, Table 10) for these elements in blood fall within the range of the calibrators.

c. Action levels: Report concentrations observed greater than the "second upper boundary" (defined in the laboratory database as the "2UB") to the QC reviewer as an "elevated result". The concentration assigned to the 2UB for an element is determined by study protocol but default concentrations are listed in Table 9 in Appendix B. The protocol for supervisors reporting elevated results to medical personnel is defined according to the study protocol. But typically,

   i. Lead: Levels of lead in blood of children ages 1-5 are considered elevated above 5 μg/dL and chelation treatment is recommended at blood lead levels ≥45 μg/dL[65]. The Occupational Safety and Health Administration regulations use a blood lead level of 40 μg/dL as cause for written notification and a medical exam, and a blood lead level of 60 μg/dL as cause for medical removal from exposure[66].

   ii. Cadmium: Levels of concern for cadmium in blood is >5 μg/L[67, 68].

   iii. Mercury: The American Conference of Governmental Industrial Hygienists has a biological exposure index (BEI) of 15 μg/L for inorganic mercury in blood (end of shift at end of work week)[68].

   iv. Manganese: Insufficient data to establish an action level.

   v. Selenium: >500 μg/L [69, 70]

11) Method Calculations

a. Method limit of detection (LODs): The method detection limits for elements in blood specimens are defined as 3 times s_0, where s_0 is the estimate of the standard deviation at zero analyte concentration. S_0 is taken as the y-intercept of a linear or 2nd order polynomial regression of standard deviation versus concentration (4 concentration levels of the analytes in blood each measured 60 times across at least a 2-month timeframe). Method LODs are re-evaluated periodically.

b. Method limit of quantitation (LOQ): The Division of Laboratory Sciences does not currently utilize limits of quantitation in regards to reporting limits [71].

c. QC Limits: Quality control limits are calculated based on concentration results obtained in at least 20 separate runs. It is preferable to perform separate analyses on separate days and using multiple calibrator lot numbers,

instruments, and analysts to best mimic real-life variability. The statistical calculations are performed using the SAS program developed for the Division of Laboratory Sciences (DLS_QC_compute_char_stats.sas).

12) Alternate methods for performing test and storing specimens if test system fails:

If the analytical system fails, the analysis may be setup on other ICP-MS instruments in the laboratory. If no other instrument is available, store the specimens at ~4 °C until the analytical system can be restored to functionality. If interruption longer than 4 weeks in anticipated, then store blood specimens at ≤ -20 °C.

Appendix A: Critical parameter test results

Critical parameter test #1: Testing scenario of something preventing a set of prepared samples from being analyzed immediately.

Test details:

Day 1: Prepared a set of dilutions (calibrators, blanks, reference material, fake samples) for analysis in triplicate. Analyzed set 1 immediately (normal practice). Cap sets 2 and 3 and leave at room temperature for later analysis.

Day 2: Prepared run set 4 and analyzed it sequentially with run set 2

Day 3: Prepared run set 5 and analyzed it sequentially with run set 3

Table 1. Ruggedness testing results: Evaluating the significance of time from preparation to analysis on sample stability. Test performed 12/6-8/10 by Deanna Jones. Results are the average of the beginning and ending QC results for each analytical run.

ID	Time, prep to analysis	Hg (µg/L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
LB08707 WB2 I	*target mean and 3SD range*	0.585 0.318 – 0.852	2.12 1.99 – 2.25	0.488 0.353 – 0.623	7.98 6.38 – 9.59	
	0 hr	0.418	2.03	0.399	6.09	
	24 hr (fresh)	0.504 (0.522)	1.99 (2.18)	0.419 (0.47)	7.06 (7.88)	
	48 hr	0.396 (0.418)	2.04 (2.03)	0.509 (0.40)	7.82 (6.09)	
HB08708 WB2 I	*target mean and 3SD range*	6.19 5.74 – 6.63	10.1 9.73 – 10.4	3.14 2.84 – 3.44	14.9 12.8 – 17.1	
	0 hr	5.86	10.0	3.03	12.5	
	24 hr	5.46 (5.7)	9.5 (10.7)	2.85 (3.17)	13.6 (14.7)	
	48 hr	2.64 (5.9)	9.2 (10.0)	2.79 (3.03)	13.5 (12.5)	
QMEQAS 07B-03*	*target mean and 3SD range*					228 206 – 251
	0 hr					192
	24 hr					202 (217)
	48 hr					56 (192)
QMEQAS 10B-06*	*target mean and 2SD range*					239 215 – 253
	0 hr					212
	24 hr					221 (238)
	48 hr					62 (212)

*samples purchase from Le centre de toxicology du Quebec (Quebec, Canada)

Conclusion: Samples which have been diluted 1+1+48 for analysis up to one (1) day previously can still be analyzed.

Appendix A: Critical parameter test results (continued)

Critical parameter test #2: This test evaluated the significance of the RF Power setting of the ICP when analyzing blood samples for whole blood metals.

Test details:
1. Prepare a set of dilutions (calibrators, blanks, reference material, dummy samples) for analysis in triplicate (three separate sets of tubes).
2. Analyze them in three separate runs on the same day, same instrument.
3. Change the RF Power across the runs
4. Allow 15 minutes equilibration time between runs for RF Power to stabilize

Table 2. Ruggedness testing results: Evaluating the significance of RF Power setting on sample stability. Test performed on December 6 and December 10, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	RF power (W)	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
LB08707_W B2	*target mean and 2SD range*	0.585 0.407 – 0.763	2.12 2.03 – 2.21	0.488 0.398 – 0.578	7.98 6.91 – 9.05	
	1150 W	0.517	2.09	0.432	7.35	
	1450 W (default)	0.512	2.03	0.369	6.76	
	1600 W	0.529	2.02	0.418	7.17	
HB08708_W B2	*target mean and 2SD range*	6.19 5.89 – 6.48	10.1 9.84 – 10.3	3.14 2.94 – 3.34	14.9 13.5 – 16.4	
	1150 W	5.90	10.0	2.93	13.7	
	1450 W (default)	6.23	10.2	2.90	12.8	
	1600 W	5.99	10.1	3.07	13.3	
QMEQAS08 B-02*	*target mean and 2SD range*					293 273 - 313
	1150 W					269
	1450 W (default)					288
	1600 W					314
QMEQAS08 B-08*	*target mean and 2SD range*					165 154 - 176
	1150 W					179
	1450 W (default)					147
	1600 W					146

*samples purchase from Le centre de toxicology du Quebec (Quebec, Canada)

Conclusion: Results are not compromised by changes in RF power within the range of 1150W to 1600W.

Appendix A: Critical parameter test results (continued)

Critical parameter test #3: This test evaluated the significance of the dynamic reaction cell gas flow rate of the reaction gas (oxygen and methane) while analyzing blood samples for elements analyzed in DRC mode (Hg, Mn, and Se). The cell gas flow rate for Mn and Hg is oxygen (O_2) and the per method setting is 1.2 mL/min. The cell gas flow rate for Se is methane (CH_4) and the per method setting is 0.84 mL/min.

Test details:
1. Prepare a set of dilutions (calibrators, blanks, reference material, dummy samples) for analysis in triplicate (three separate sets of tubes).
2. Analyze them in three separate runs on the same day using the same instrument.
3. Change the cell gas flow rate.

Table 3. Ruggedness testing results: Evaluating the significance of dynamic reaction cell gas flow rate on sample stability. Test performed on December 6, 2010 and January 4, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	cell gas flow rate	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
LB08707_WB2	*target mean and 2SD range*	0.585 0.407 – 0.763	2.12 2.03 – 2.21	0.488 0.398 – 0.578	7.98 6.91 – 9.05	See Table 4
	0.96 mL/min O_2; 0.7 mL/min CH_4	0.457	2.10	0.471	8.49	
	1.2 mL/min O_2; 0.84 mL/min CH_4	0.479	2.10	0.438	8.15	
	1.44 mL/min O_2; 1.0 mL/min CH_4	0.555	2.11	0.457	8.12	
HB08708_WB2	*Target Mean and 2SD Range*	6.19 5.89 – 6.48	10.1 9.84 – 10.3	3.14 2.94 – 3.34	14.9 13.5 – 16.4	
	0.96 mL/min O_2; 0.7 mL/min CH_4	4.71	10.0	3.19	14.4	
	1.2 mL/min O_2; 0.84 mL/min CH_4	5.45	10.1	2.92	15.2	
	1.44 mL/min O_2; 1.0 mL/min CH_4	5.34	10.3	3.04	14.6	

Conclusion: Accuracy of Mn and Hg results are not compromised by changes in cell gas flow rate within the range tested (0.96 – 1.44 mL/min).

Appendix A: Critical Parameter Test Results (Continued)

Table 4. Ruggedness testing results: Evaluating the significance of dynamic reaction cell gas flow rate on sample stability. Test performed on December 6, 2010 and January 4, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	cell gas flow rate	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
QMEQAS07B-09*	target mean and 2SD range	See Table 3, Appendix A				157 146 - 168
	0.96 mL/min O_2; 0.7 mL/min CH_4					187
	1.2 mL/min O_2; 0.84 mL/min CH_4					186
	1.44 mL/min O_2; 1.0 mL/min CH_4					191
QMEQAS08B-02*	target mean and 2SD range					293 273 - 313
	0.96 mL/min O_2; 0.7 mL/min CH_4					328
	1.2 mL/min O_2; 0.84 mL/min CH_4					334
	1.44 mL/min O_2; 1.0 mL/min CH_4					339

*samples purchase from Le centre de toxicology du Quebec (Quebec, Canada)

Conclusion: Accuracy of Se results are not compromised by changes in cell gas flow rate within the range tested (0.7 – 1.0 mL/min).

Appendix A: Critical parameter test results (continued)

Critical parameter test #4: This test evaluated the significance of the RPq value while analyzing blood samples for Se, Mn and Hg. The RPq value setting per method for Mn and Hg is 0.6, and for Se it is 0.65. The reduced and elevated RPq values for Mn and Hg are 0.48 and 0.72, respectively. The reduced and elevated RPq values for Se are 0.52 and 0.78, respectively. The results are presented in Tables 5 and 6.

Test details:
1. Prepare a set of dilutions (calibrators, blanks, reference material, fake samples) for analysis in triplicate (three separate sets of tubes).
2. Analyze them in three separate runs on the same day, using the same instrument.
3. Change the RPq value.

Table 5. Ruggedness testing results: Evaluating the significance of RPq value on sample stability. Test performed on December 21, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	RPq	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
LB08707_WB2	*Target Mean and 2SD Range*	0.585 0.407 – 0.763	2.12 2.03 – 2.21	0.488 0.398 – 0.578	7.98 6.91 – 9.05	See Table 6
	0.48 Mn and Hg; 0.52 Se	0.455	1.97	0.361	7.86	
	0.6 Mn and Hg; 0.7 Se	0.418	2.03	0.399	6.09	
	0.72 Mn and Hg; 0.78 Se	0.402	2.07	0.402	7.99	
HB08708_WB2	*Target Mean and 2SD Range*	6.19 5.89 – 6.48	10.1 9.84 – 10.3	3.14 2.94 – 3.34	14.9 13.5 – 16.4	
	0.48 Mn and Hg; 0.52 Se	5.54	9.4	2.79	14.4	
	0.6 Mn and Hg; 0.7 Se	5.86	10.0	3.03	12.5	
	0.72 Mn and Hg; 0.78 Se	5.53	9.7	2.88	14.9	

Conclusion: Accuracy of Mn and Hg results are not compromised by changes in RPq settings within the range tested (0.48 – 0.72).

Appendix A: Critical Parameter Test Results (Continued)

Table 6. Ruggedness testing results: Evaluating the significance of RPq value on sample stability. Test performed on December 21, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	RPq	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
QMEQAS07B-09*	*target mean and 2SD range*	See Table 5, Appendix A				293 273 – 313
	0.48 Mn and Hg; 0.52 Se					262
	0.6 Mn and Hg; 0.7 Se					250
	0.72 Mn and Hg; 0.78 Se					277
QMEQAS08B-02*	*target mean and 2SD range*					361 337 - 385
	0.48 Mn and Hg; 0.52 Se					347
	0.6 Mn and Hg; 0.7 Se					349
	0.72 Mn and Hg; 0.78 Se					364

*samples purchase from Le centre de toxicology du Quebec (Quebec, Canada)

Conclusion: Accuracy of Se results are not compromised by changes in RPq settings within the range tested (0.52 – 0.78 for Se).

Appendix A: Critical parameter test results (continued)

Critical parameter test #5: This test evaluated the significance of the Axial Field Voltage (AFT) while analyzing blood samples for whole blood metals. The Axial Field Voltage may vary on each instrument. The Axial Field Voltage was increased and decreased by 20%. The results are presented in Table 7.

Test details:
1. Prepare a set of dilutions (calibrators, blanks, reference materials, fake samples) for analysis in triplicate (three separate sets of tubes).
2. Analyze them in three separate runs on the same day, same instrument.
3. Change the AFV value +/- 100 V.

Table 7. Ruggedness testing results: Evaluating the significance of Axial Field Voltage on sample stability. Test performed on December 20, 2010 by Deanna Jones. Results below are the average of the beginning and ending QC results for each analytical run.

ID	axial field voltage	Hg (µg /L)	Pb (µg /dL)	Cd (µg /L)	Mn (µg /L)	Se (µg /L)
LB08707_ WB2	Target Mean and 2SD Range	0.585 0.407 – 0.763	2.12 2.03 – 2.21	0.488 0.398 – 0.578	7.98 6.91 – 9.05	
	(optimized - 100V)	0.511	2.00	40.415	7.77	
	(optimized)	0.461	2.04	0.394	6.36	
	(optimized + 100V)	0.414	2.01	0.376	6.95	
HB08708_ WB2	Target Mean and 2SD Range	6.19 5.89 – 6.48	10.1 9.84 – 10.3	3.14 2.94 – 3.34	14.9 13.5 – 16.4	
	(optimized - 100V)	5.50	9.8	2.91	14.3	
	(optimized)	5.62	9.8	2.84	12.0	
	(optimized + 100V)	5.75	10.1	2.99	12.8	
QMEQAS 07B-09*	Target Mean and 2SD Range					157 146 – 168
	(optimized - 100V)					139
	(optimized)					147
	(optimized + 100V)					138
QMEQAS0 9B-08*	Target Mean and 2SD Range					548 511 - 585
	(optimized - 100V)					501
	(optimized)					556
	(optimized + 100V)					532

*samples purchase from Le centre de toxicology du Quebec (Quebec, Canada)

Conclusion: Accuracy of Mn, Hg and Se results are not compromised by changes in AFV settings within the range tested (optimized setting +/- 100V).

Appendix A: Critical parameter test results (continued)

Parameter test #6: Evaluate the impact on observed concentration if an extra dilution is performed on the sample relative to the calibration standards.

Test details:

1. A large blood sample was spiked to elevated concentrations, and mixed well. The spiked sample was then prepared for analysis at various extra dilution levels and the observed results compared to results obtained with no extra dilution performed.

normalized concentration ± 1RSD

Dilution level	Mn	Hg	Se	Cd	Pb
No Extra (N=8)	1.00	1.00	1.00	1.00	1.00
2x dilution (N=8)	1.00 ± 0.01	1.03 ± 0.05	1.02 ± 0.03	1.00 ± 0.01	1.01 ± 0.01
5x dilution (N=6)	1.01 ± 0.01	1.06 ± 0.06	1.01 ± 0.02	1.01 ± 0.01	1.02 ± 0.01
10x dilution (N=8)	1.01 ± 0.03	1.04 ± 0.06	1.04 ± 0.06	1.00 ± 0.02	1.02 ± 0.02
20x dilution (N=8)	1.02 ± 0.04	1.09 ± 0.05	1.06 ± 0.08	1.01 ± 0.03	1.02 ± 0.02

Conclusion: Results show that all analytes of the method (Pb, Cd, Hg, Mn, and Se) can be analyzed at up to a 20x extra dilution without significant effect (> ± 10% error) to the observed concentration.

Appendix B

Table 1. Instrument and method parameters.	
Instrument: PerkinElmer ELAN DRC II ICP-MS ESI SC4 autosampler with (optional) PC3 Peltier cooled spray chamber	
Optimization window parameters	
RF power	1450 W
Plasma Gas Flow (Ar)	15 L/min
Auxiliary Gas Flow (Ar)	1.2 L/min
Nebulizer Gas Flow (Ar)	~0.90 – 1.0 L/min (optimized as needed for sensitivity)
Ion Lens Voltage(s)	AutoLens (optimized as needed for sensitivity)
AFV, QRO, CRO, CPV, Discriminator Threshold	Optimized per instrument by service engineer, or advanced user.
Parameters of x-y alignment, nebulizer gas flow, AutoLens voltages, mass calibration, dual detector calibration and detector voltages are optimized regularly. Optimization file name = default.dac.	
Configurations window parameters	
cell gas changes pause times	Pressurize Delay (From Standard to DRC mode) = 60 Exhaust Delay (From DRC to Standard mode) = 30 Flow Delay (Gas changes while in DRC mode) = 30 Channel Delay (Gas channel change in DRC mode) = 30
File names & directories	
method file names	*calibration curve (programmed for blood blank)* CDC_DLS3016_bldblk.mth *For QC & patient sample analysis (programmed for aqueous blank)* CDC_DLS3016_aqblk.mth
dataset	Create a new dataset subfolder each day. Name as "2011-0820" for all work done on August 20, 2011
sample file	Create for each day's work
report file name	*For sample results printouts* cdc_quant comprehensive.rop *For calibration curve information* CDC_Quant Comprehensive (calib curve info).rop
tuning	Default.tun
optimization	Default.dac
calibration	N/A
polyatomic	elan.ply
report options template (transferring results to the database)	CDC_Database Output.rop *Report Format Options: select only "Use Separator"* *File Write Option: Append* *Report File name: make descriptive including date (e.g. 2005-0311b_DRC2A_HM-0364.txt)*

Table 1. Instrument and method parameters.	
Method Parameters	
Method Parameters: Timing Page (see Figures 1a, 2a and 2d in Appendix B)	
sweeps/reading	30
readings/replicate	1
replicates	3
enable qc checking	On
isotopes monitored and internal standard associations (exact mass)	use ^{103}Rh, ^{130}Te, ^{193}Ir as internal standards ^{103}Rh (102.905): ^{55}Mn (54.93805) ^{130}Te(129.907): ^{202}Hg (201.971), ^{80}Se(79.9165) 193Ir(192.963):^{208}Pb(207.977), ^{114}Cd(113.904)
dwell times	100 ms for ^{55}Mn,^{202}Hg, ^{80}Se, ^{208}Pb, and ^{114}Cd 50 ms for ^{130}Te,^{103}Rh, and ^{193}Ir
scan mode	Peak Hopping for all isotopes (1 MCA channel)
DRC channel A gas flow rate	99.999% methane (5-7 psig delivery pressure) typically 0.84 L/min (0.7 – 1.0) * *optimized per instrument, and periodically verified
DRC channel B gas flow rate	99.99% oxygen (5-7 psig delivery pressure) typically 1.2 L/min (0.96 – 1.44) * *optimized per instrument, and periodically verified
RPa	0 for all isotopes
RPq	Typically* 0.6 (0.48 – 0.72) for ^{103}Rh,^{55}Mn,^{130}Te, and ^{202}Hg. 0.65 (0.52 – 0.78) for ^{130}Te and ^{80}Se. 0.25 for ^{193}Ir, ^{208}Pb, and ^{114}Cd Use the same RPQ for each analyte and its IS. (* Optimize per instrument, and periodically verified)
Method parameters: processing page (see Figures 1b in Appendix B)	
detector mode	Dual
process spectral peak	N/A
autolens	On
isotope ratio mode	Off
enable short settling time	Off
blank subtraction	After internal standard
measurement units	cps
process signal profile	N/A
Method parameters: equations page (see Figure 1c in Appendix B)	
equations	+Hg 200 -0.027250 * Sn118 +Pb 206 +Pb 207

Table 1. Instrument and method parameters.	
Method parameters: calibration page (see Figures 1d in Appendix B)	
calibration type	external std.
curve type	weighted linear
sample units	"µg/L" or "ppb"
calibrator concentrations (µg/L)	Mn (µg /L): 1.5, 4.5, 10.5, 15, 30, 75, 225, 600 Cd and Hg (µg /L): 0.5, 1.5, 3.5, 5, 10, 25, 75, 200 Pb (µg /dL): 1, 3, 7, 10, 20, 50, 150, 400 Se (µg /L): 30, 90, 210, 300, 600, 1500, 4500, 12000
Method parameters: sampling page (see Figures 1e and 1f in Appendix B)	
"peristaltic pump under computer control"	On
autosampler tray port sampling device	***If using ESI autosampler*** Autosampler Type: AS-93plus Tray Name: esi.try Sampling Device: None If using other autosampler, refer to user guide.
sample flush	default is 4s at 1.5 rpm (~160 uL/min, ESI DXi peristaltic pump, FAST sample introduction system) Time can be optimized as needed to adequately fill the FAST loop. Time and rpm can be optimized as needed to using a different style peristaltic pump (maintaining approximate liquid flow rate). As a matter of lab practice, set this time to equal the loop fill time in the ESI FAST program. As long as the combined time of sample flush + read delay is equal to the time required for signal to reach stability, analytical measurement will be good.
read delay	60s at 1.5 rpm (~160 uL/min, ESI DXi peristaltic pump, FAST sample introduction system) Time can be optimized as needed to reach signal stability before beginning analysis. Time and rpm can be optimized as needed to using a different style peristaltic pump (maintaining approximate liquid flow rate). As a matter of lab practice, set this time equal to the total time required for the signal to reach stability minus the loop fill time. As long as the combined time of sample flush + read delay is equal to the time required for signal to reach stability, analytical measurement will be good.
wash	30s at 1.5 rpm (~160 uL/min, ESI DXi peristaltic pump, FAST sample introduction system) Time can be optimized to allow for changes in FAST loop rinsing (must be greater than total time of steps in FAST program after the initial "on rinse" command). Time and rpm can be optimized as needed to using a different style peristaltic pump (maintaining approximate liquid flow rate).

Table 1. Instrument and method parameters.

extra wash (via ICP-MS software QC checking)	For sample concentrations greater than these, setup the ICP-MS software's 'QC checking' feature to "Wash for X and continue" **Analyte** **Concentration** **Extra Rinse Time** Cd 200 µg/L 200s Hg 200 µg/L 200s Mn 600 µg/L 200s Pb 400 µg/dL 200s Se 1200 µg/L 200s
autosampler locations of blanks and standards	*For calibration curve (points to blood blank)* CDC_DLS3016_bldblk.mth Calibration Stds 0 – 8 in autosampler positions 105 – 113 by default, but can be customized. *For QC & patient sample analysis (points to aqueous blank)* CDC_DLS3016_aqblk.mth Aqueous Blank in autosampler position 117 by default, but can be customized.
FAST parameters: See Figures 4a through 4h in Appendix B for details	
configuration file	default.sc (saved at C:\Program Files\ESI\ESI-SC\)
FAST program	cdc_dls3016_5element_loop1ml_scfast.txt
Potential Emergency Response Modifications:	
mercury:	Analyze mercury in standard mode with tellurium as the internal standard. Set dwell time to 100ms, DRC gas flow to 0, and RPq to 0.25.
Non-FAST sample introduction system:	If the FAST sample introduction system is not available on any instruments, the method can still be implemented, but these changes will need to be made in the ELAN (and ESI software if present). Peristaltic pump speeds are for DXi pump; adjust accordingly if another pump is installed. • sample flush: Default is ~30s at -16 rpm. Set so that solution reaches nebulizer. • read delay: Default is 45s at -5 rpm. Set for best reproducibility of replicate measured intensities. • wash: Default is 60s at -11rpm. Set to prevent significant carry-over from one sample to the next. • If using ESI autosampler without FAST, disable FAST in the ESI software before running analysis.

Appendix B (continued)

Table 2. Suggested concentrations for base blood	
analyte (units)	**suggested concentration**
Cd (µg/L)	<0.5
Hg (µg/L)	<0.5
Mn (µg/L)	< 8
Pb (µg/dL)	<2
Se (µg/L)	<200

Table 3. Stock calibration standard concentrations	
Analyte	**Stock calibration concentration (mg/L)** High Purity Standards Item # SM-2107-042 10% v/v HCl
Cd	50
Hg	50
Mn	150
Pb	1000
Se	3000

Appendix B (continued)

Table 4. Preparation of intermediate stock calibration standard	
volume of flask (mL)	100
volume of spike of stock standard solution	2
	concentrations (mg /L)
Cd	1
Hg	1
Mn	3
Pb (mg /dL)	20
Se	60

Table 5. Preparation of intermediate working standards								
Standard #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
volume of flask (mL)	100	100	100	100	100	100	100	100
volume spike of stock std. (mL)						0.05	0.15	0.4
volume spike of int. stock Std. (mL)	0.05	0.15	0.35	0.50	1.00			
concentrations (µg /L) *								
Cd	0.5	1.5	3.5	5	10	25	75	200
Hg	0.5	1.5	3.5	5	10	25	75	200
Mn	1.5	4.5	10.5	15	30	75	225	600
Pb (µg /dL)	1	3	7	10	20	50	150	400
Se	30	90	210	300	600	1500	4500	12000

* These same concentrations are entered in the ICP-MS software's calibration page to describe the concentrations of the working calibrators (preparations analyzed during a run). This eliminates the need to multiply ICP-MS observed results by a dilution factor except for the case of extra dilutions (see Table 8).

Appendix B (continued)

Table 6. Acceptable ways to perform two consecutive analytical runs, bracketing with bench quality control samples.

setup 1	setup 2
Run #1 calibration standards low bench QC high bench QC elevated bench QC patient samples low bench QC high bench QC elevated bench QC *Run #2* low bench QC high bench QC elevated bench QC patient samples low bench QC high bench QC elevated bench QC	*Run #1* calibration standards low bench QC high bench QC elevated bench QC patient samples low bench QC high bench QC elevated bench QC *Run #2* *calibration standards* low bench QC high bench QC elevated bench QC patient samples low bench QC high bench QC elevated bench QC

Appendix B (continued)

Table 7. A typical SAMPLE/BATCH window.

AS Location*	Sample ID	Measurements Action	Method
233	DRCstability1	Run sample	...DLS3016_bldblk.mth
233	DRCstability2	Run sample	...DLS3016_bldblk.mth
233	DRCstability3	Run sample	...DLS3016_bldblk.mth
233	DRCstability4	Run sample	...DLS3016_bldblk.mth
Continue DRC stability samples . . .			
233	DRCstability9	Run sample	...DLS3016_bldblk.mth
233	DRCstability10	Run sample	...DLS3016_bldblk.mth
114	3016 BldBlkChk1	Run blank, standards, and sample **	...DLS3016_bldblk.mth
115	3016 BldBlkChk2	Run sample	...DLS3016_bldblk.mth
116	3016 AQBLK	Run blank and sample ¥	...DLS3016_aqblk.mth
125	L Bench QC	Run sample	...DLS3016_aqblk.mth
126	H Bench QC	Run sample	...DLS3016_aqblk.mth
127	E Bench QC	Run sample	...DLS3016_aqblk.mth
137	Sample 1	Run sample	...DLS3016_aqblk.mth
138	Sample 2	Run sample	...DLS3016_aqblk.mth
125	L Bench QC	Run sample	...DLS3016_aqblk.mth
126	H Bench QC	Run sample	...DLS3016_aqblk.mth
127	E Bench QC	Run sample	...DLS3016_aqblk.mth

* The exact autosampler positions of QCs and patient samples do not have to be those shown above. QC samples do not have to be run in the order of low, then high, then elevated.

** When executing this row, the ELAN will first analyze the standard 0 (blood blank) at AS position 105, then standards 1-8 at autosampler positions 106-113, then the "3016 BldBlkChk1" sample at A/S position 114. The sampling information about AS positions 105-113 are stored in the "bldblk" method file.

¥ When executing this row, the ELAN will first analyze the aqueous blank at AS position 117, then the "Aq blank " at AS position 103. The sampling information about AS positions 117 is stored in the "aqblk" method file.

Appendix B (continued)

Table 8. Preparation of samples, working calibrators, and QC materials for analysis *

If a different total volume is prepared, adjust the volumes for each component proportionally.

* These directions are written with the expectation of a 5,000 µL syringe on the left side and a 250 µL syringe on the right side of the benchtop automatic pipette.

Description	Water (µL)	Base Blood (µL)	AQ Intermediate Working Standard (µL)	Patient or QC blood sample (µL)	Diluent (µL)**
Working Calibrators (S0-S8) and Bldblkchk (S0)	-	50 x 1	50 x 1	-	2,400 (1,200 x 2)
AQ Blank	100 x 1	-	-	-	2,400 (1,200 x 2)
Patient blood or Blood-Based QC	50 x 1	-	-	50 x 1	2,400 (1,200 x 2)
Patient Blood *2x Extra Dilution* [H]	150 x 1	-	-	50 x 1	4,800 (2,400 x 2)
Patient Blood *5x Extra Dilution* [H]	450 (225 x 2)			50 x 1	12,000 (4,000 x 3)
Patient Blood *10x Extra Dilution* [H]	950 (190 x 5)			50 x 1	24,000 (4,000 x 6)
Patient Blood *20x Extra Dilution* [H]	1950 (195 x 10)			50 x 1	48,000 (4,000 x 12)

** By splitting the dispense step of diluent into two or more portions, liquids pulled up into the right pipette tip are flushed out more completely. For example, when preparing a working calibrator, do the preparation in two steps: in step 1, dispense 2400 µL diluent + 50 µL; in step 2, dispense 2400 µL diluent + 50 µL base blood to prepare a 2.5 mL total volume dilution.

[H] Extra dilution is performed on urine samples whose concentration is greater than the highest calibrator listed in the 'calibrator concentrations' section of Table 1 in the Appendix B.

Maximum extra dilution (see Appendix A, ruggedness test #6 for details)
20x for Cd, Hg, Mn, Pb, and Se

Any extra level of dilution up to 20x (see Appendix A, Experiment 6) can be prepared as long as the 4.8:5 ratio of diluent to total dilution volume is maintained. Use of the lowest possible dilution level is preferred to minimize differences between the calibrators and the samples (i.e. 2x dilution is preferred over 10x if 2x is sufficient to dilute analyte into the documented linearity range).

Appendix B (continued)

Table 9. Boundary concentrations for whole blood concentrations

analyte (units)	1st upper boundary ("1UB") *	2nd upper boundary ("2UB") **	range maximum ("Lim Rep Delta") †	Highest Concentration Validated for Washout
Mn (μg/L)	20	35	2.0	600
Pb (μg/dL)	5.0	5.0	1.0	400
Cd (μg/L)	5.0	5.0	1.0	200
Hg (μg/L)	10.0	10.0	1.0	200
Se (μg/L)	400	400	20	12,000

* Typically, the 1st upper boundary (1UB) is the 99th percentile of non-weighted concentration results from the NHANES 1999-2000 subset groups, a concentration significant to public health, or a concentration defined by study protocol. The default 1UB concentrations are listed in this table.

** The 2nd upper boundary (2UB) may be 2x the 1UB, a concentration significant to public health, or defined by study protocol.

† Range maximum (Lim Rep Delta) is the allowed limit to the range of the three replicate readings for a single sample analysis.

Appendix B (continued)

Table 10. Reference ranges for blood concentrations [72].

analyte (units)	survey years	geometric mean	50th	75th	90th	95th	N
Cd (µg/L)	07-08	0.315	0.270	0.500	1.00	1.52	8266
	09-10	0.302	0.260	0.480	0.960	1.40	8793
	11-12	0.279	0.250	0.460	0.960	1.50	7920
Hg (µg/L)	07-08	0.769	0.740	1.48	2.95	4.64	8266
	09-10	0.863	0.790	1.68	3.43	5.13	8793
	11-12	0.703	0.640	1.38	2.87	4.40	7920
Pb (µg/dL)	07-08	1.27	1.22	1.90	2.80	3.70	8266
	09-10	1.12	1.07	1.70	2.58	3.34	8793
	11-12	0.973	0.930	1.52	2.38	3.16	7920
Mn (µg/L)	11-12	9.35	9.22	11.5	14.4	16.7	7920
Se (µg/L)	11-12	190	190	206	223	236	7920

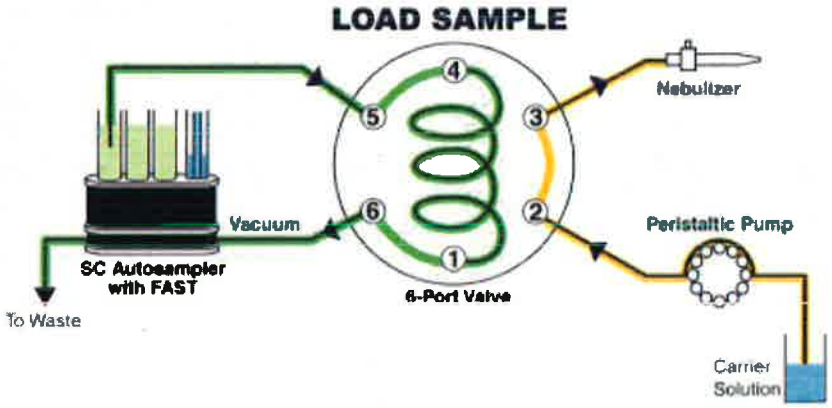
Table 11. Reference concentrations from published literature for blood Mn and Se.

analyte (units)	published concentrations
Se (µg/L)	157 – 265 µg/L [73]
Mn (µg /L)†	Non-exposed 4 – 14 (µg /L) [46]
	Exposed workers (adults) 3.2 – 101 µg /L [28]
	Children receiving long term parenteral nutrition 33.8 – 101 µg /L [74]
	Ohio adults (N=49) residing near a refinery (possible Mn emission): Mean (range) 9.4 (4.2-21.7) µg/L [30]
	Mexican infants Age 1, mean (SD) = 24.3 (4.5) µg/L, median = 23.7 µg/L, N=270 Age 2, mean (SD) = 21.1 (6.2) µg/L, median = 20.3 µg/L, N=430 [75]
	Japanese women (N = 1420) GM 13.2 µg/L overall, Range of median (max) across 8 regions 12.0-14.3 (25.0-33.4) µg/L [76]
	South African children, ages 8-10 years old (n = 49) Mean (SD) 8.48 (2.45) µg/L, range 4.58-18.20 µg/L. [34]

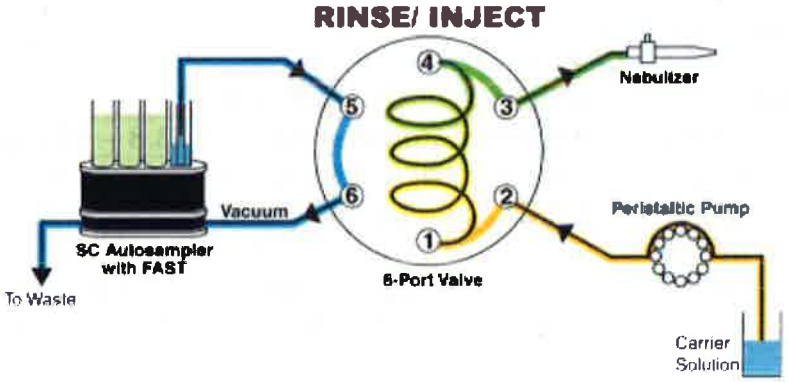
Appendix B (continued)

Figure 1. Configuration of tubing and devices for liquid handling using FAST sample introduction.

Below shows the correct connections to the 6-port FAST valve. The two diagrams show the differences in liquid flow directions when the valve changes from "Load" to "Inject" This change is internal to the valve. The shift of the valve cannot be seen, but it can be heard, and felt (with hand on the valve). The light indicators on the actuator body also indicate the valve position.



Teflon vacuum pump loads sample into loop while carrier solution is nebulized

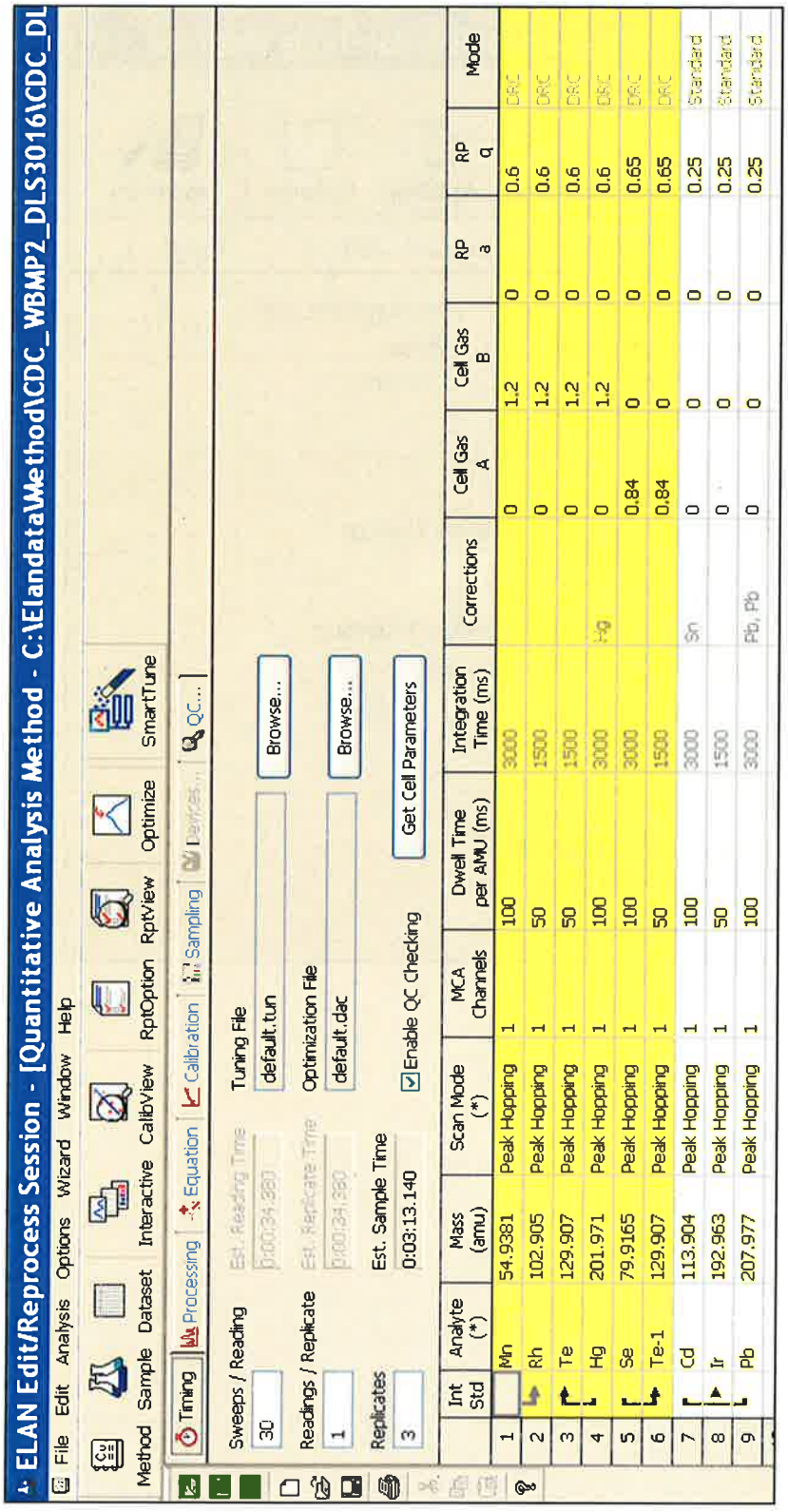


Carrier solution pushes sample into nebulizer at the same time sample line is rinsed

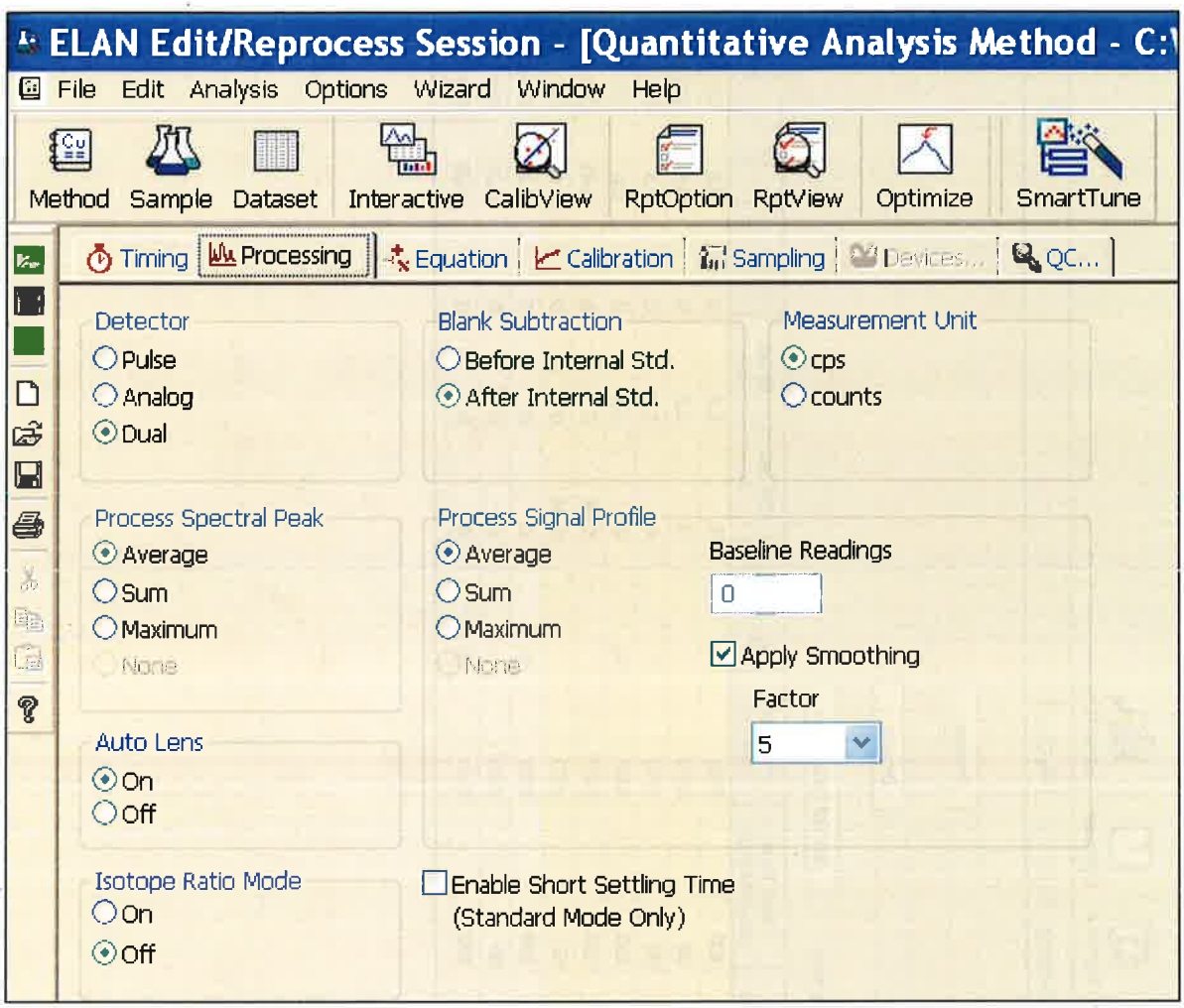
The connections to the valve are color-coded (see Section 7.a.i).

Enable the FAST program in the ESI software before running the method, but optimizations can be done in either FAST or non-FAST mode.

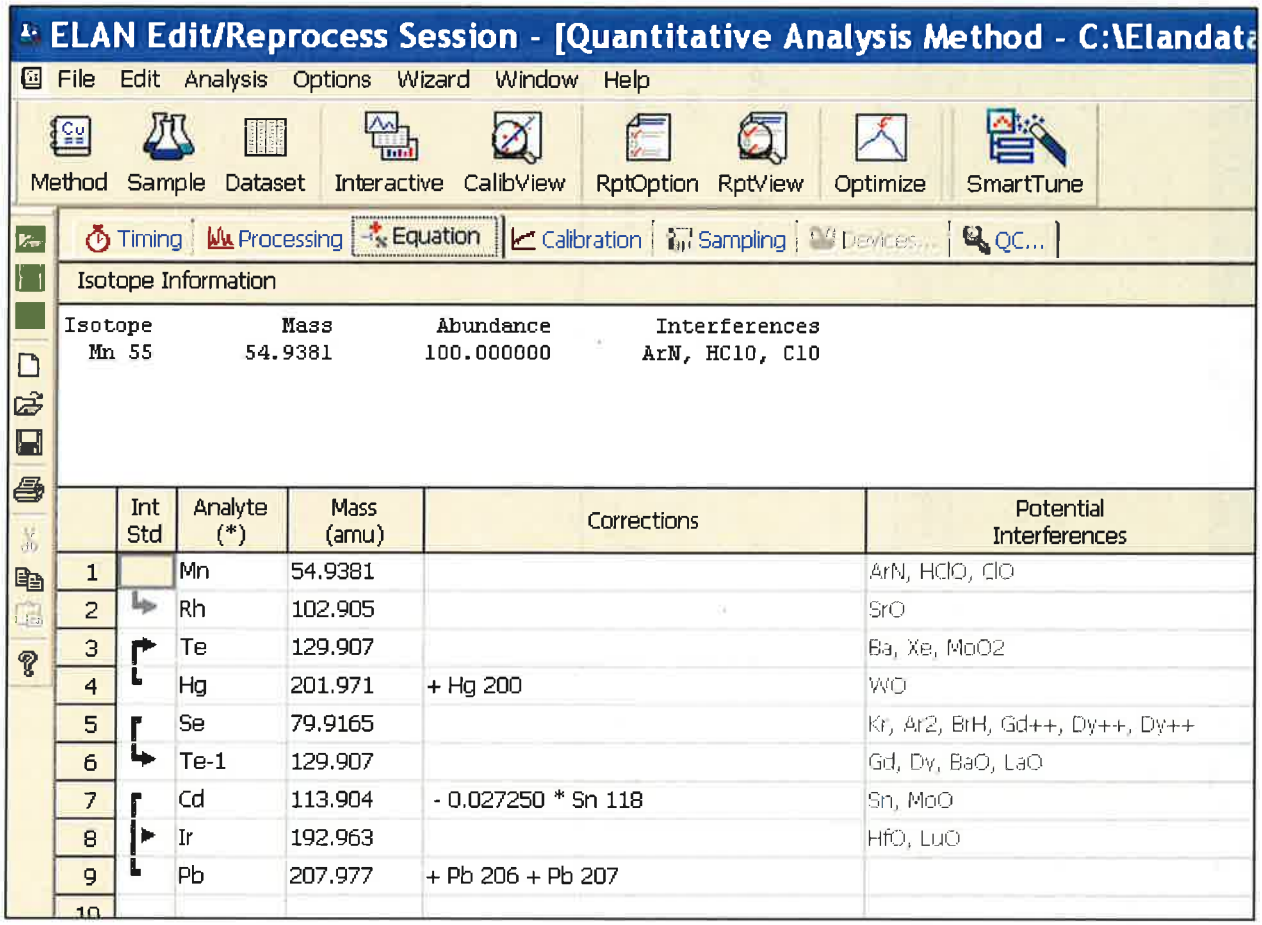
Appendix B (continued)
Figure 2a. ELAN ICP-MS method screen shots (timing page).



Appendix B (continued)
Figure 2b. ELAN ICP-MS method screen shots (processing page).

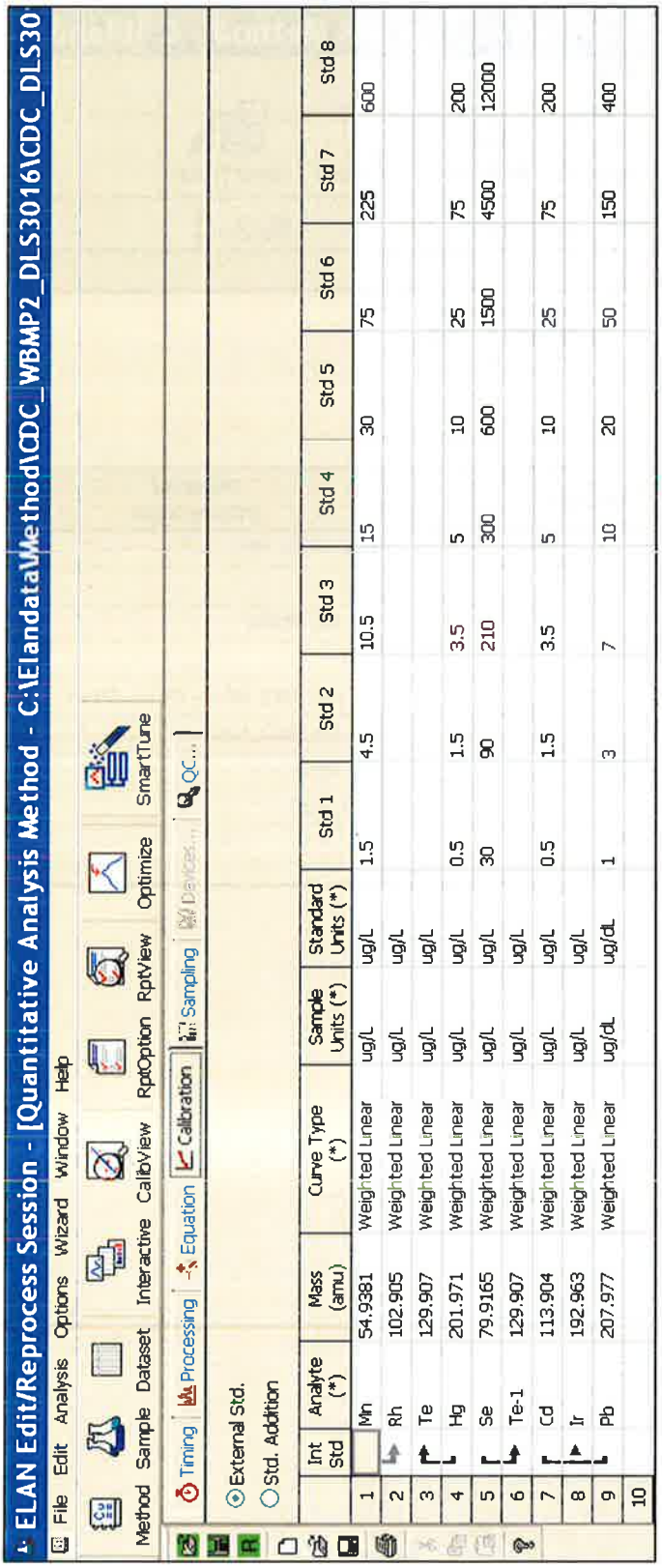


Appendix B (continued)
Figure 2c. ELAN ICP-MS method screen shots (equation page).



	Int Std	Analyte (*)	Mass (amu)	Corrections	Potential Interferences
1		Mn	54.9381		ArN, HClO, ClO
2		Rh	102.905		SrO
3		Te	129.907		Ba, Xe, MoO2
4		Hg	201.971	+ Hg 200	WO
5		Se	79.9165		Kr, Ar2, BrH, Gd++, Dy++, Dy++
6		Te-1	129.907		Gd, Dy, BaO, LaO
7		Cd	113.904	- 0.027250 * Sn 118	Sn, MoO
8		Ir	192.963		HfO, LuO
9		Pb	207.977	+ Pb 206 + Pb 207	
10					

Appendix B (continued)
Figure 2d. ELAN ICP-MS method screen shots (calibration page).



ELAN Edit/Reprocess Session - [Quantitative Analysis Method - C:\Elandata\Method\CDC_WBMP2_DLS3016\CDC_DLS30

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Method Sample Dataset Interactive CalibView RptOption RptView Optimize SmartTune

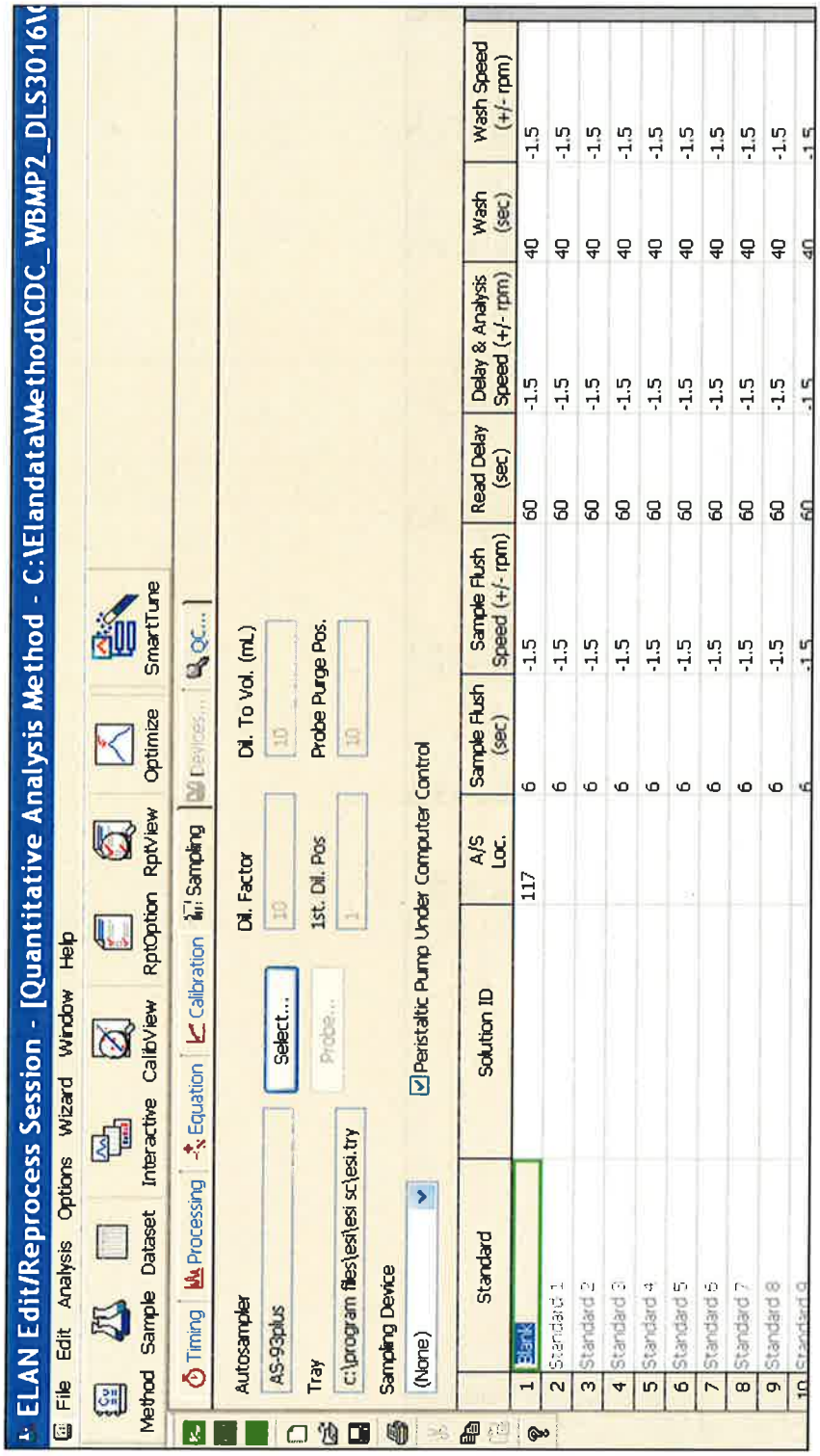
Timing Processing Equation Calibration Sampling Devices... QC...

External Std.
Std. Addition

	Int Std	Analyte (*)	Mass (amu)	Curve Type (*)	Sample Units (*)	Standard Units (*)	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
1		Mn	54.9381	Weighted Linear	ug/L	ug/L	1.5	4.5	10.5	15	30	75	225	600
2		Rh	102.905	Weighted Linear	ug/L	ug/L								
3		Te	129.907	Weighted Linear	ug/L	ug/L								
4		Hg	201.971	Weighted Linear	ug/L	ug/L	0.5	1.5	3.5	5	10	25	75	200
5		Se	79.9165	Weighted Linear	ug/L	ug/L	30	90	210	300	600	1500	4500	12000
6		Te-1	129.907	Weighted Linear	ug/L	ug/L								
7		Cd	113.904	Weighted Linear	ug/L	ug/L	0.5	1.5	3.5	5	10	25	75	200
8		Ir	192.963	Weighted Linear	ug/L	ug/L								
9		Pb	207.977	Weighted Linear	ug/dL	ug/dL	1	3	7	10	20	50	150	400
10														

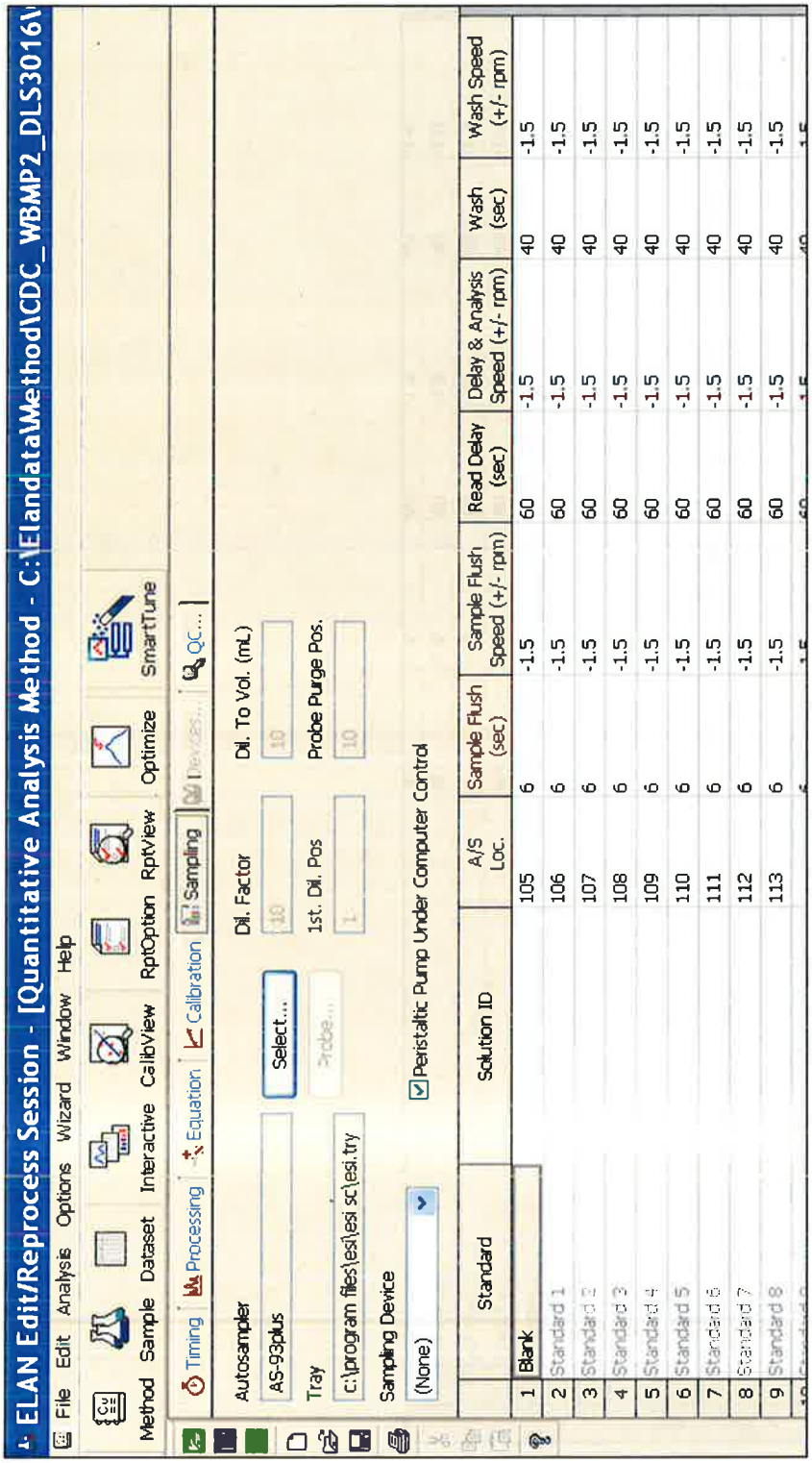
Appendix B (continued)

Figure 2e. ELAN ICP-MS method screen shots (sampling page, AqBlank method).

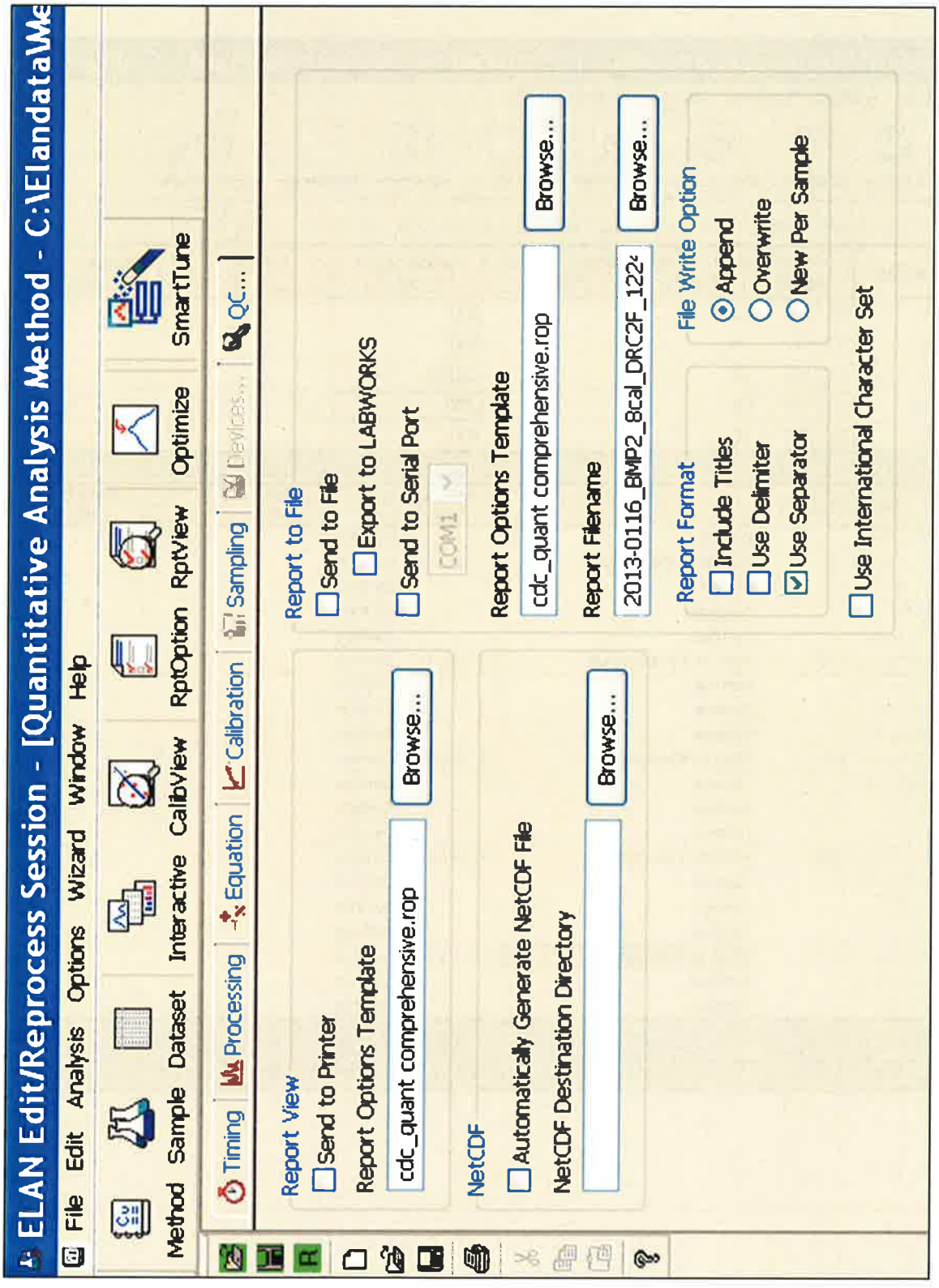


Appendix B (continued)

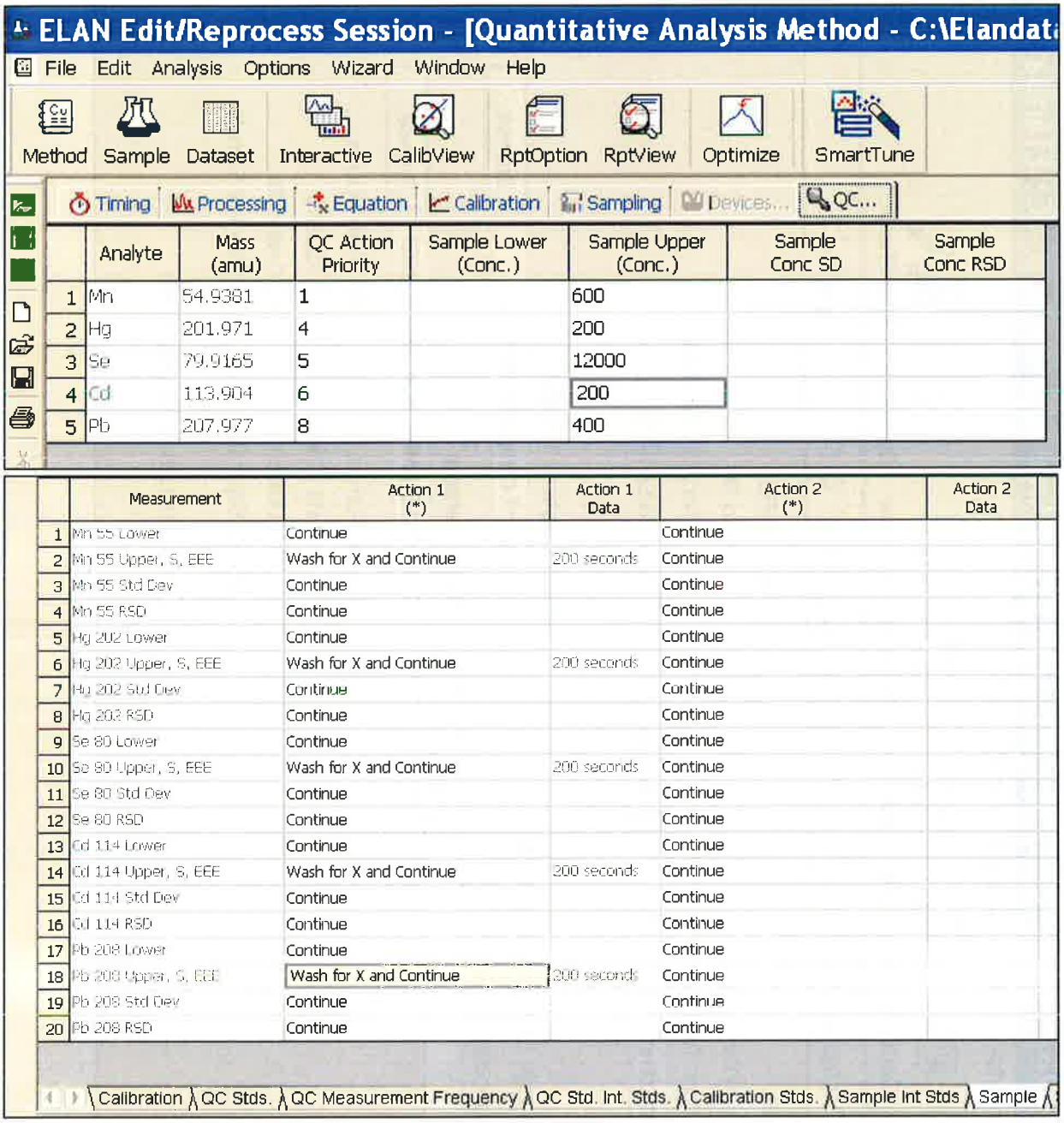
Figure 2f. ELAN ICP-MS method screen shots (sampling page, BldBlank method).



Appendix B (continued)
Figure 2g. ELAN ICP-MS method screen shots (report page).



Appendix B (continued)
Figure 2h. ELAN ICP-MS method screen shots (QC / Sample page).

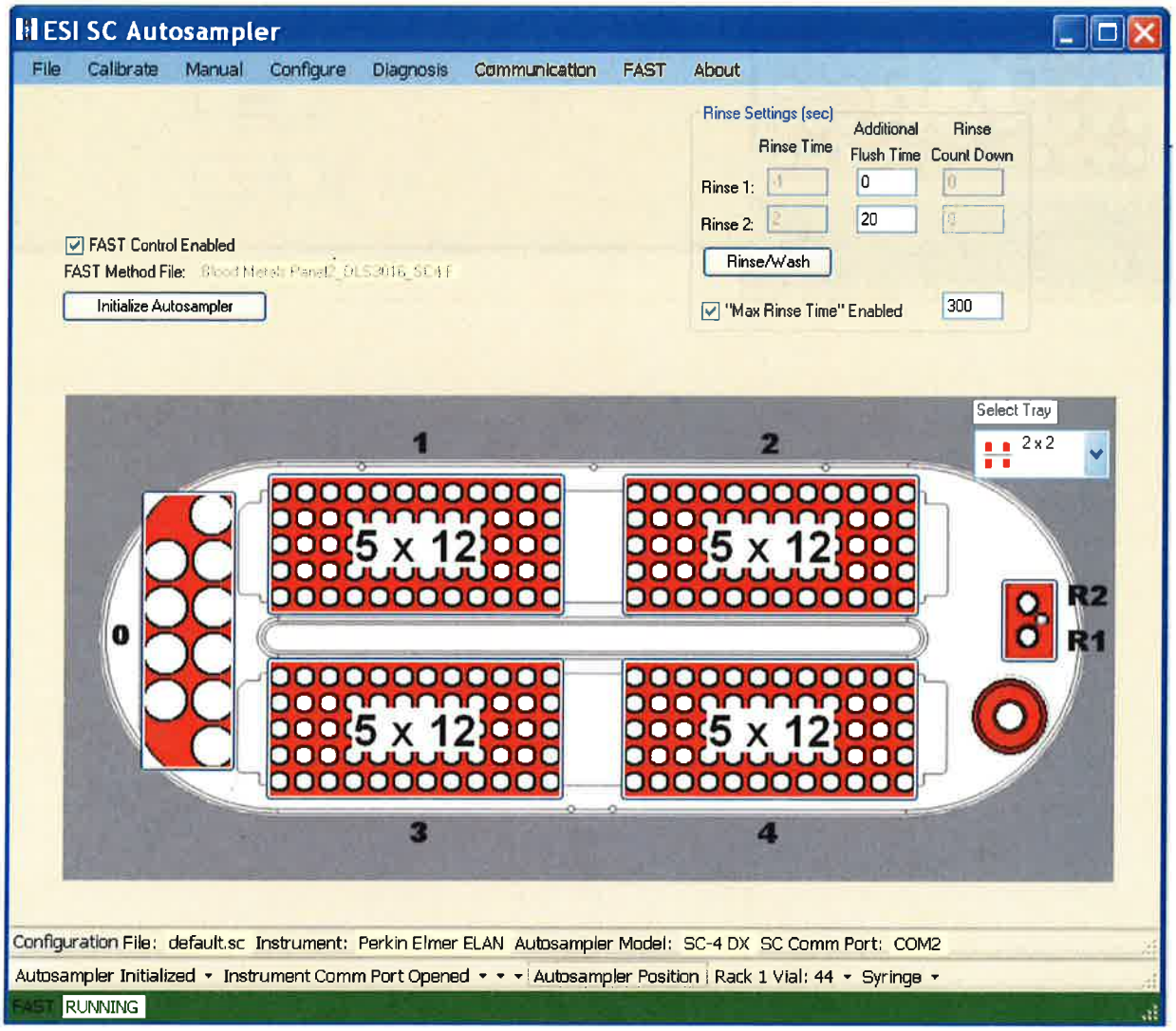


Appendix B (continued)

Figure 3a. ESI SC4 autosampler screen shots (main page). Additional flush times and "Max Rinse Time" are approximate. Optimize these for best reduction of elemental carry-over between samples. Tray types can be changed to allow for different volumes of diluted sample digests. 'FAST control' must be enabled before start of method, but does not need to be used in instrument optimization (pre-analysis) steps. Rinse and additional flush times for eliminating carry-over from one sample to the next while using the minimum amount of rinse solution.

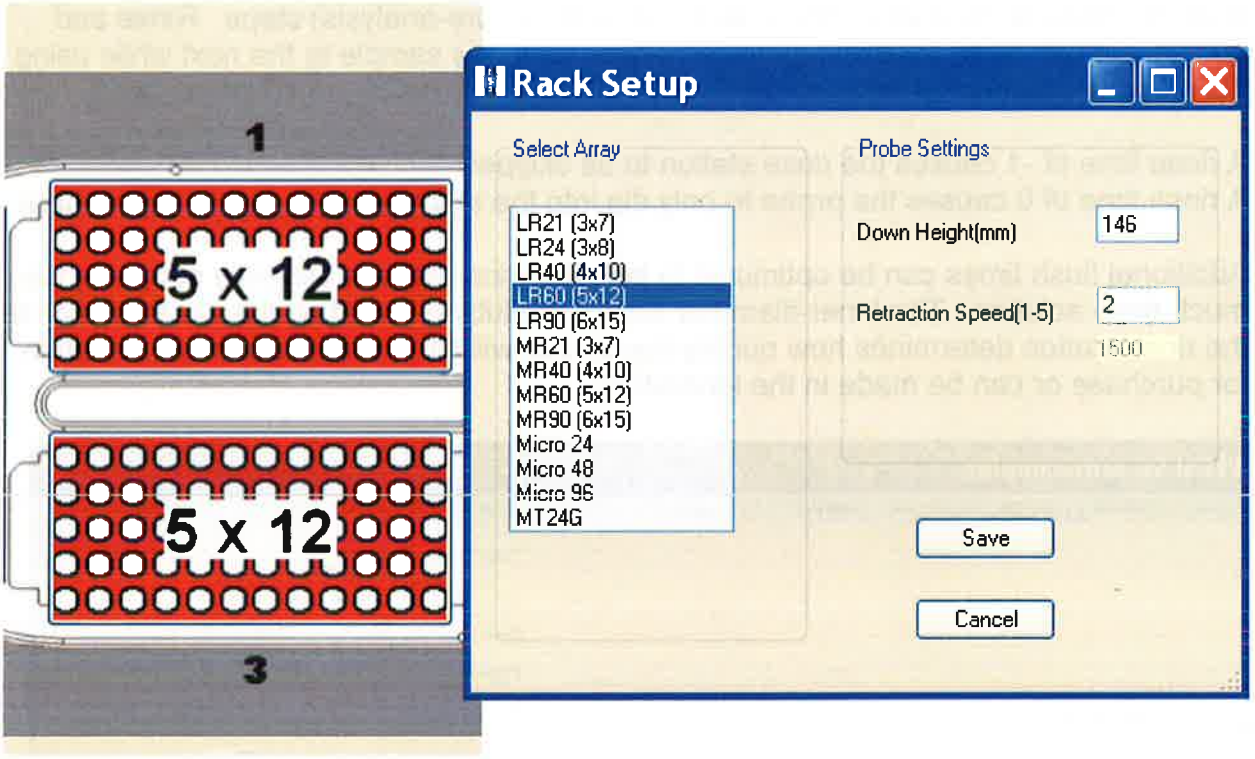
A rinse time of -1 causes the rinse station to be skipped.
A rinse time of 0 causes the probe to only dip into the station, but spends no time there.

Additional flush times can be optimized to keep the rinse station full while not using too much rinse solution. The inner diameter size of the tubing providing the rinse solution to the rinse station determines how quickly the station will fill. Various sizes are available for purchase or can be made in the laboratory.



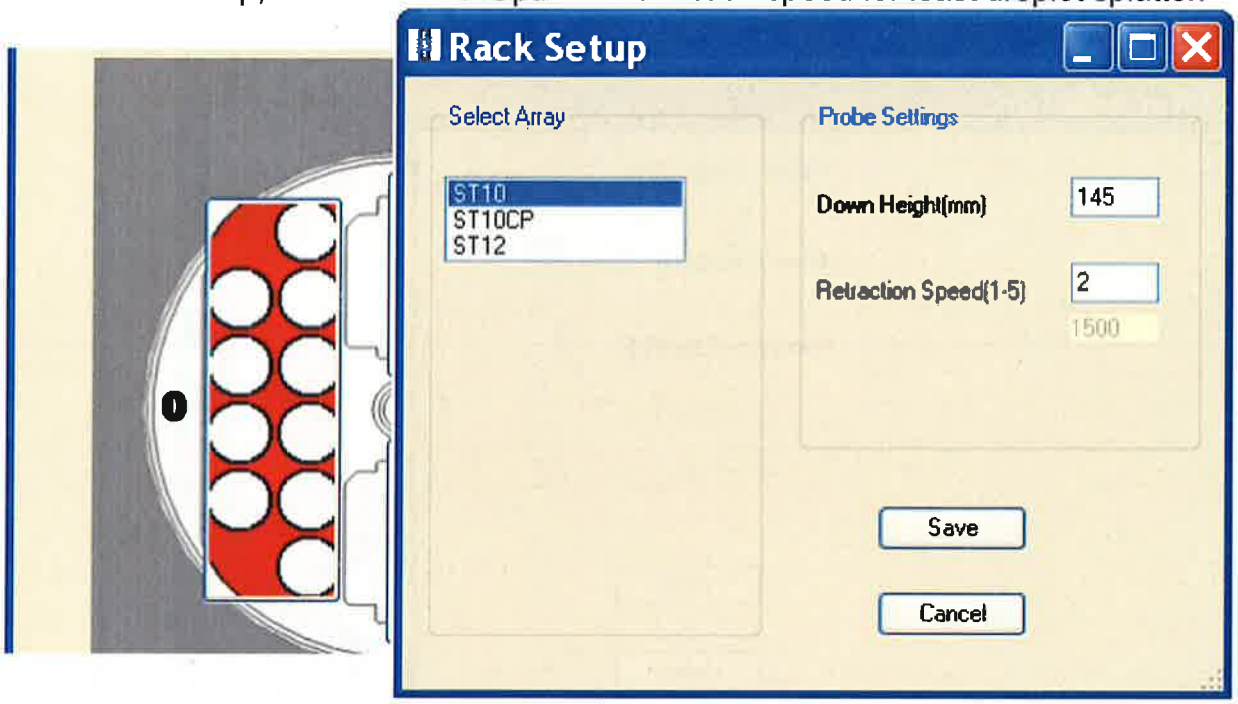
Appendix B (continued)

Figure 3b. ESI SC4 autosampler screen shots (5x12 rack setup window). Settings are approximate. To be sure the loop is filled, set the probe to go close to the bottom of the cup, but not touch. Optimize retraction speed for least droplet splatter.



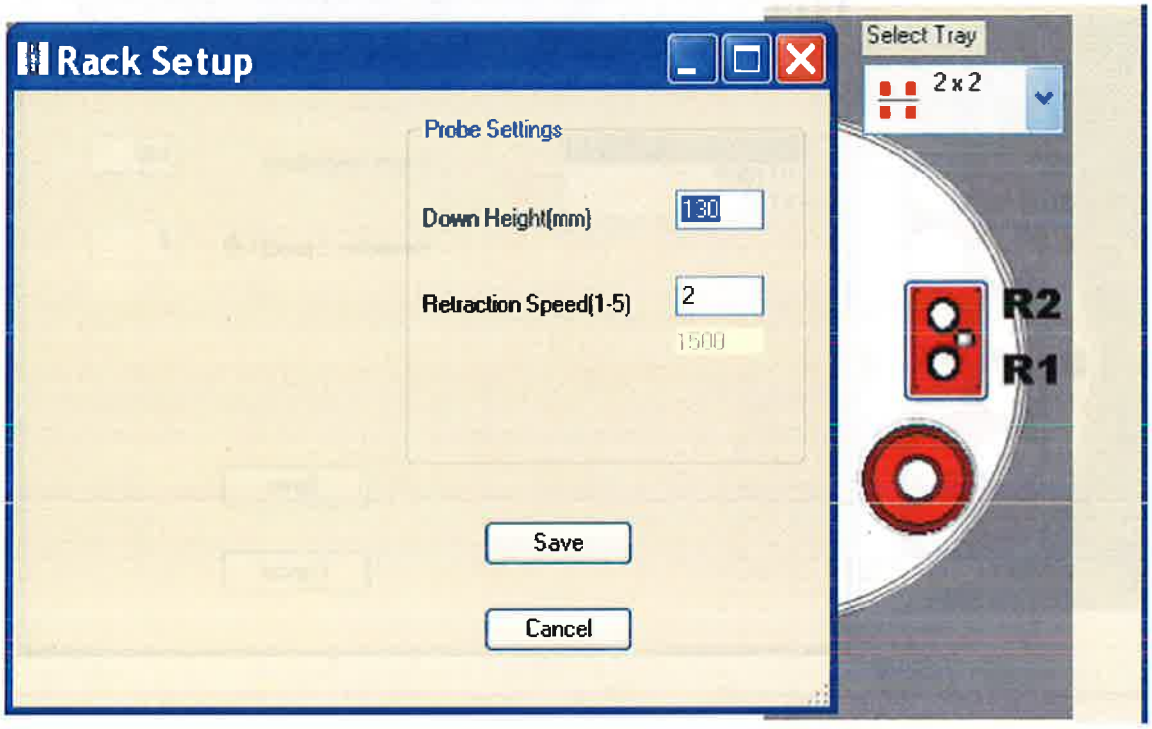
Appendix B (continued)

Figure 3c. ESI SC4 autosampler screen shots (50mL tube rack setup window). Settings are approximate. To be sure the loop is filled, set the probe to go close to the bottom of the cup, but not touch. Optimize retraction speed for least droplet splatter.



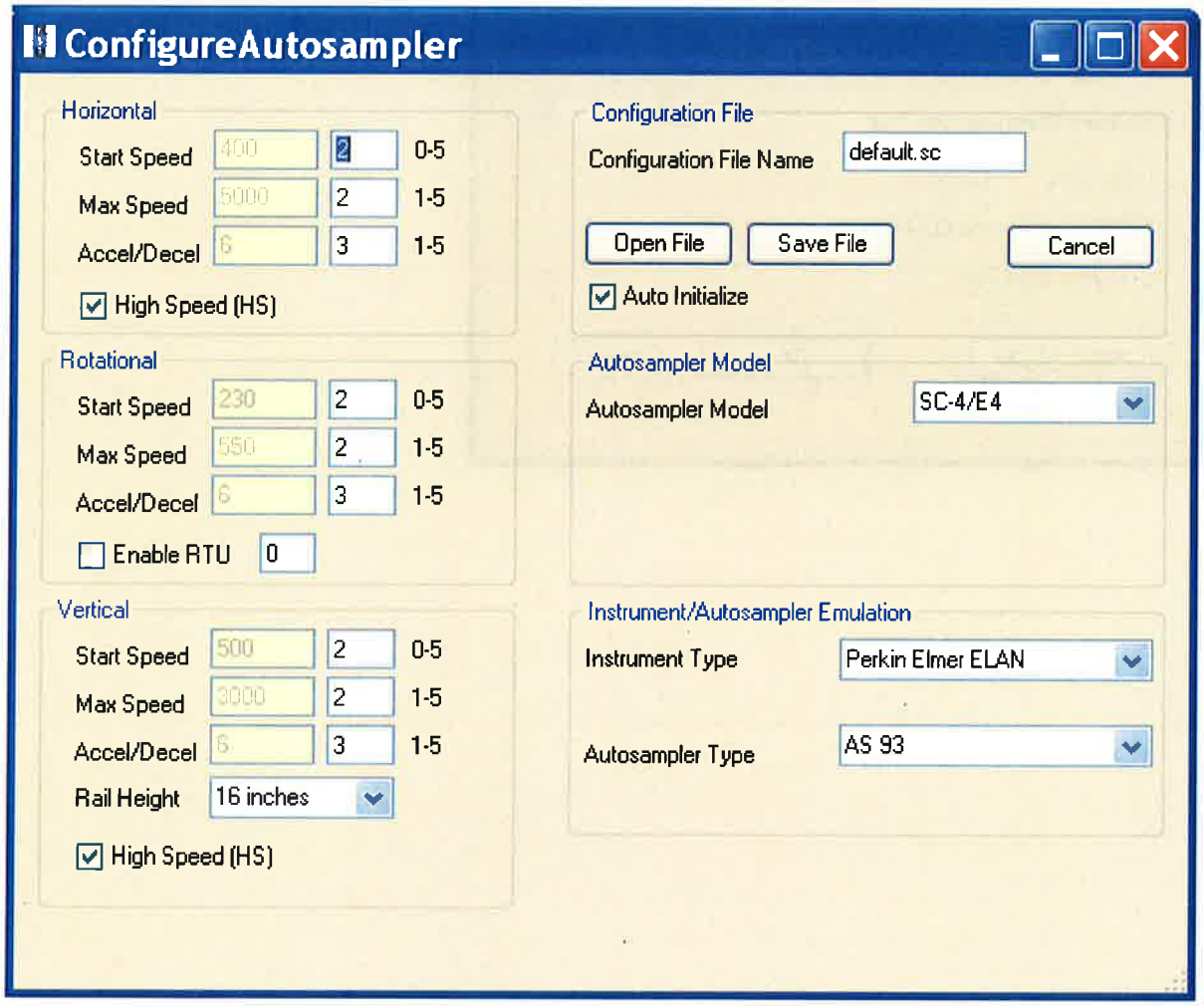
Appendix B (continued)

Figure 3d. ESI SC4 autosampler screen shots (rinse station rack setup window). Settings are approximate. Optimize down height for best probe cleaning, and retraction speed for least droplet splatter.



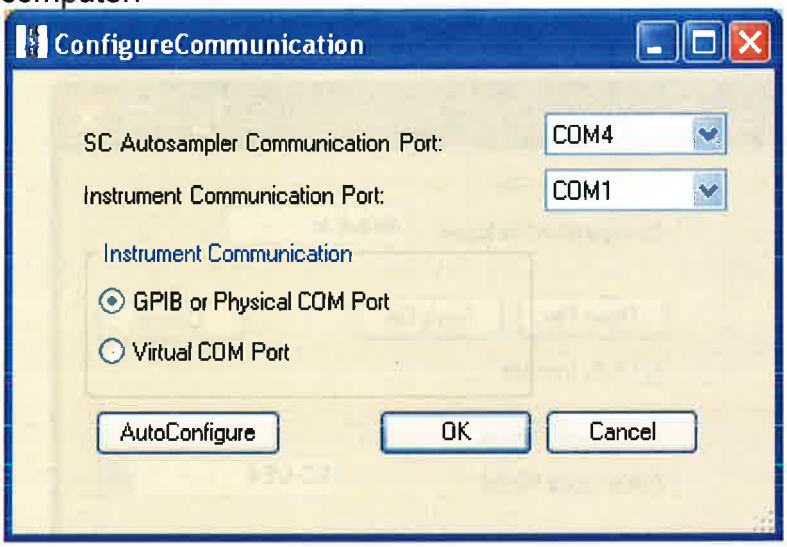
Appendix B (continued)

Figure 3e. ESI SC4 autosampler screen shots ("Configure" page). "High Speed" option is to only be used for 'High Speed' models of the SC4 (look for "HS" in serial number). Speeds and accel / decel values can be optimized per analyst preference and to minimize droplet splatter off of probe.



Appendix B (continued)

Figure 3f. ESI SC4 autosampler screen shots ("Communication" page). Communication ports will differ depending on available ports on instrument control computer.

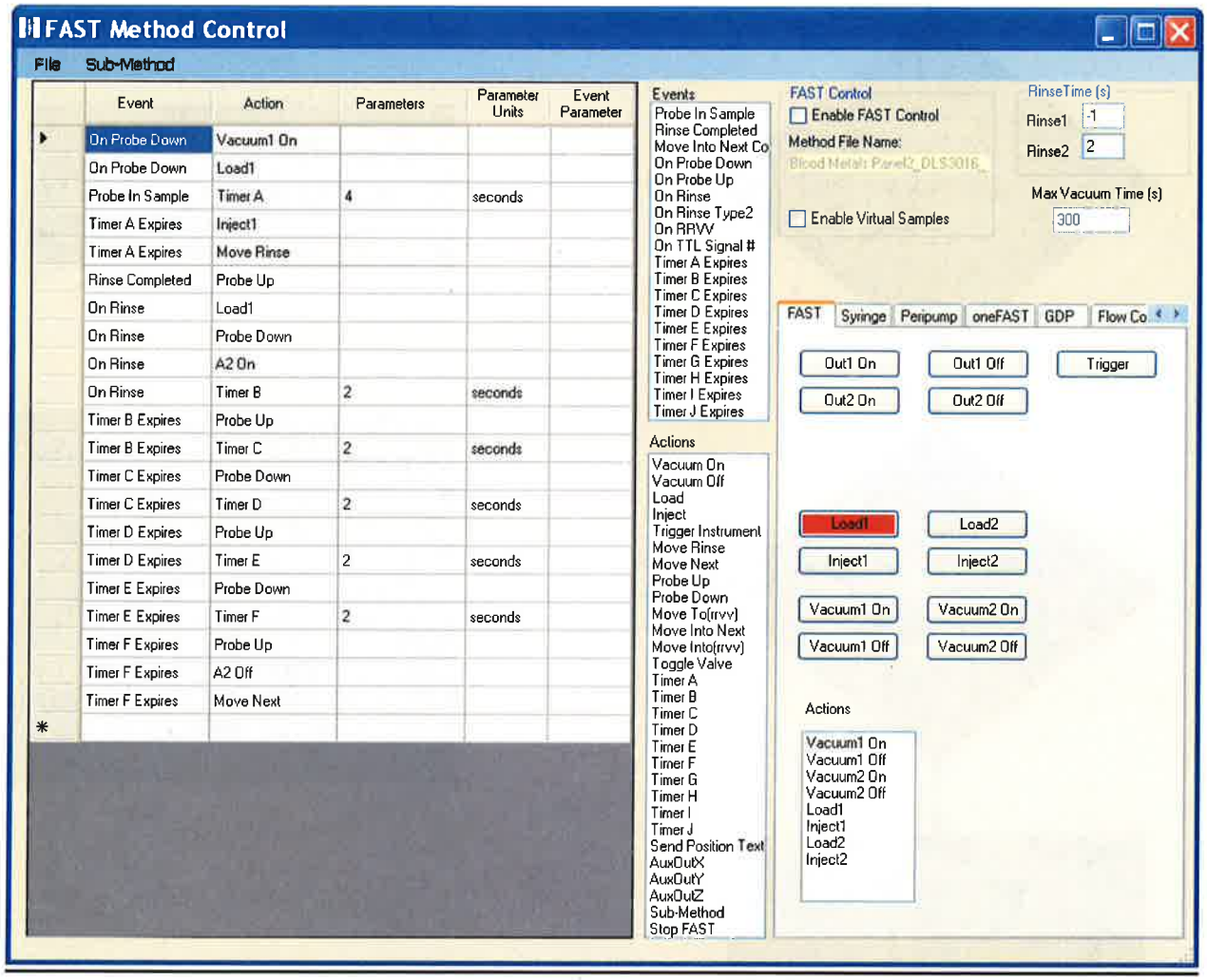


Appendix B (continued)

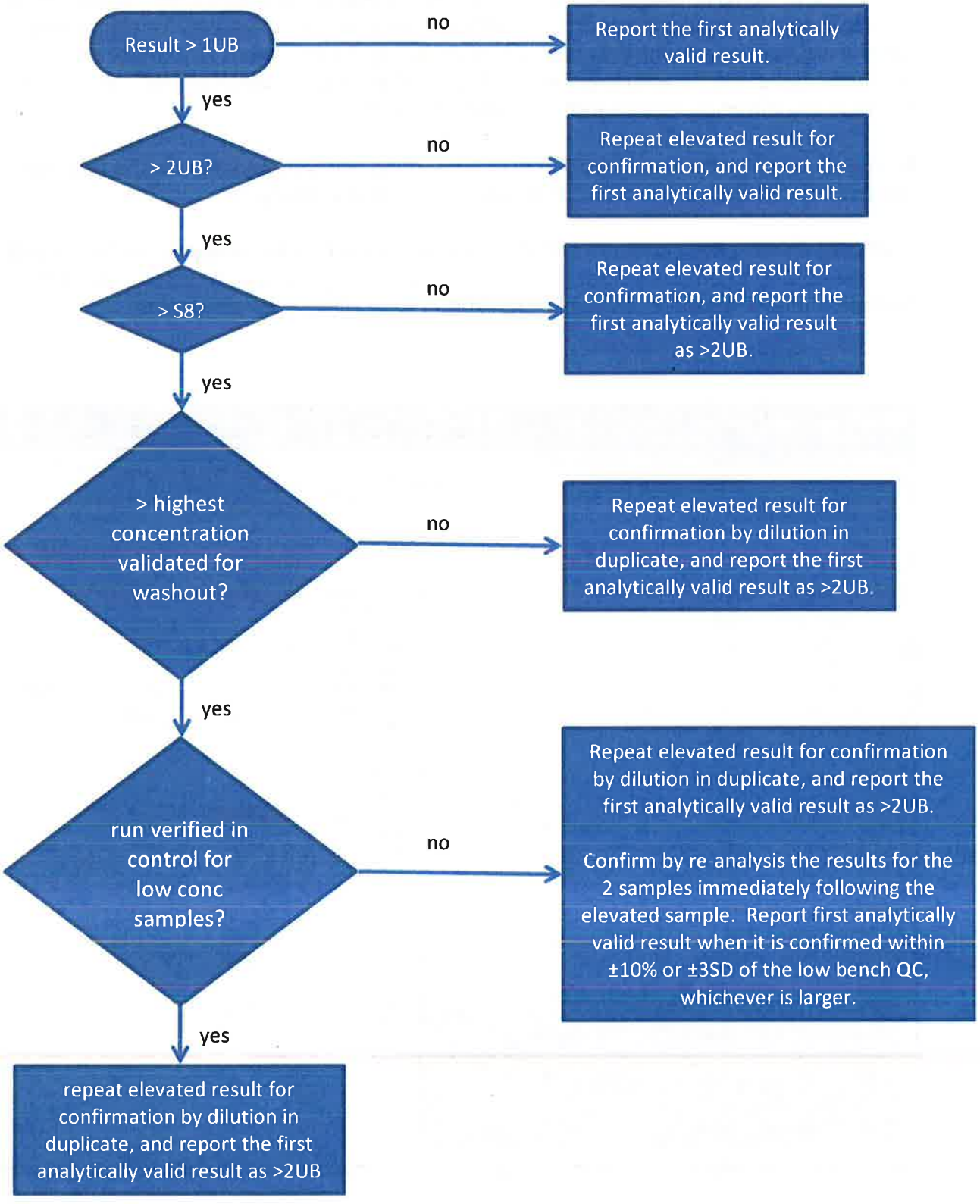
Figure 3g. ESI SC4 autosampler screen shots ("FAST" page). Timer A can be optimized to achieve proper filling of loop with diluted sample digestate. Timers B, C, D, E, and F control rinsing the loop after analysis and can be optimized for eliminating carry-over from one sample to the next while using the minimum amount of rinse solution. Save the file with the name "DLS 3016.8 FAST parameters.txt". It can be found in the directory C:\Program Files\ESI\ESI-SC\.

Manually clicking the "Load" button prior to starting analysis will ensure the position of the actuator is always the same at the beginning of the analysis.

Manually clicking the "Vacuum On" button prior to starting the analysis will help initial sample uptake to be consistent (the vacuum pump may be slow to start for the first sample if this is not done, possibly resulting in loop filling inconsistencies).



Appendix B (continued)
Figure 4. chart for handling an elevated result



Appendix C: Help Sheets

Reagent Preparation (page 1 of 3)

NOTE:
mg/L = ppm
ug/L = ppb
ug/mL = ppm

Rinse solution
(0.4% TMAH, 0.05% Triton X-100, 1% ethyl alcohol, 0.01% APDC)

1. Partially fill a 4 liter bottle with ≥18 Mohm·cm water.
2. Add 0.4 grams of APDC.
3. Add 16 mL of TMAH (Tetramethylammonium hydroxide, 25% w/w ((CH3)4NOH).
4. Add 40 mL of ethyl alcohol (C2H5OH, 200 proof)
5. Add 200 mL of 1% Triton X-100 (OR add 10mL of 20%Triton X-100).
6. Add enough ≥18 Mohm·cm water to bring to 4 liter mark.
7. Mix well by gently inverting several times.

Sample diluent
(0.4% TMAH, 0.01% APDC, 0.05% Triton X-100, 1% Ethanol, 5ppb Te, Rh, Ir)

1. Partially fill a 2 liter bottle with ≥18 Mohm·cm water.
2. Add 0.2 gram of APDC.
3. Add 8 mL of TMAH.
4. Add 20 mL of ethyl alcohol.
5. Add 500 uL of a 20 mg/L stock solution of Te, Rh, and Ir.
8. Add 100 mL of 1% Triton X-100 (OR, if using a 20% Triton X-100 solution, add 5mL)
9. Add enough ≥18 Mohm·cm water to bring to 2 liter mark.
10. Mix well by gently inverting several times.

0.5% HNO3
(Carrier solution for optimization)

1. Partially fill a 2 liter bottle with ≥18 Mohm·cm water.
2. Add 10 mL of conc. HNO_3.
3. Add enough ≥18 Mohm·cm water to bring to 2 liter mark.
4. Mix well by gently inverting several times.

Appendix C: Help Sheets (continued)

Reagent Preparation (page 2 of 3)

1% v/v HNO_3

1. Partially fill a 10 liter bottle with ≥18 Mohm·cm water.
2. Add 100 mL of conc. HNO_3.
3. Add enough ≥18 Mohm·cm water to bring to 10 liter mark.
4. Mix well by gently swirling several times.

5% v/v HNO_3

1. Partially fill a 2 liter bottle with ≥18 Mohm·cm water.
2. Add 100 mL of conc. HNO_3.
3. Add enough ≥18 Mohm·cm water to bring to 2 liter mark.
4. Mix well by gently inverting several times.

20% Triton X-100

1. Partially fill a 1 liter bottle with ≥18 Mohm·cm water.
2. Add 200 mL of Triton X-100.
3. Add enough ≥18 Mohm·cm water to bring to 1 liter mark.
4. Allow to dissolve overnight (or add a Teflon magnetic stirring bar and stir on stirrer until dissolved).
5. Mix well by gently inverting several times.

1% Triton X-100

1. Partially fill a 1 liter bottle with ≥18 Mohm·cm water.
2. Add 10 mL of Triton X-100.
3. Add enough ≥18 Mohm·cm water to bring to 1 liter mark.
4. Allow to dissolve overnight (or add a Teflon magnetic stirring bar and stir on stirrer until dissolved).
5. Mix well by gently inverting several times.

20 ppm Rh, Te and Ir internal standard solution

1. Partially fill an acid rinsed, 50 mL flask with 1% v/v HNO_3.
2. Add 1mL of Rh from 1000ppm stock standard.
3. Add 1mL of Te from 1000ppm stock standard.
4. Add 1mL of Ir from 1000ppm stock standard.
5. Add enough 1% v/v HNO_3 to fill to 50mL mark.
6. Mix well by gently inverting several times.
7. Pour the standard solution over into an appropriately labeled 50mL polypropylene tube.

Appendix C: Help Sheets (continued)

Reagent Preparation (page 3 of 3)

Daily solution (1ppb) in 2% v/v HNO_3

1. Partially fill a 1 liter volumetric flask with ≥18 Mohm·cm water.
2. Add 1mL of High Purity Standard: SM-2107-018 (or current lot #)
3. Add 20mL of concentrated HNO_3
4. Add enough ≥18 Mohm·cm water to bring to 1 liter mark.
5. Mix well by gently inverting several times.

Stability test solution (1 liter bulk prep)

1. Use a 1 liter bottle dedicated to stability test solution preparation
2. Add 960 mL of Sample Diluent
3. Add 20 mL of "junk" whole blood
4. Add 20 mL of Intermediate Working Calibration Standard (may use S1 or S2) OR add 1.5mL of Intermediate Stock Calibration Standard.
5. Mix well by gently inverting several times.
6. Store in the refrigerator (when not using).

Appendix C: Help Sheets (continued)

Standard Preparation (page 1 of 1)

(from single element stock standards)

Prepare 3% HCl v/v solution:

1. Partially fill a clean 2 liter bottle with ≥18 Mohm·cm water.
2. Using a clean 50 mL polypropylene tube to measure, add 60 mL of high purity concentrated HCl.
3. Add enough ≥18 Mohm·cm water to bring to 2 liter mark.
4. Gently invert to mix.

Prepare intermediate stock standard (see Table 4 in Appendix B):

1. Partially fill a 100 mL volumetric flask with 3% v/v HCl solution.
2. Label as: "HgPbCdMnSe Intermediate Stock Std"
3. Add 2 mL of HgPbCdMnSe multi-element stock solution.
4. Add enough 3% v/v HCl to bring to 100 mL mark.
5. Mix well by gently inverting several times.

Prepare intermediate working standards (see Table 5 in Appendix B):

1. Partially fill each of eight, 100 mL volumetric flasks with 3% v/v HCl solution.
2. Label as: Intermediate Working Std "S1", "S2", "S3" and "S4", "S5", "S6", "S7" and "S8".
3. For "S1 Intermediate Working Std": add 50 uL of the Intermediate Stock Std.
4. For "S2 Intermediate Working Std": add 150 uL of the Intermediate Stock Std.
5. For "S3 Intermediate Working Std": add 350 uL of the Intermediate Stock Std.
6. For "S4 Intermediate Working Std": add 500 uL of the Intermediate Stock Std.
7. For "S5 Intermediate Working Std": add 1mL of the Intermediate Stock Std.
8. For "S6 Intermediate Working Std": add 50 uL of the Multi-Element Stock Std.
9. For "S7 Intermediate Working Std": add 150 uL of the Multi-Element Stock Std.
10. For "S8 Intermediate Working Std": add 400 uL of the Multi-Element Stock Std.
11. Add enough 3% v/v HCl solution to bring to 100 mL mark.
12. Mix well by gently inverting several times.
13. These intermediate working standards may be poured over into clean 15 mL Falcon tubes for daily use (NOTE: "S0 Intermediate Working Std" is 3% HCl only).

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Division of Laboratory Sciences

Laboratory Protocol

Analytes:	**Polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), polychlorinated biphenyls (PCBs) and persistent pesticides.**
Matrix:	**Serum / Plasma**
Method:	**Liquid-Liquid Extraction (LLE) / Silica-sulfuric acid cleanup and Gas Chromatography/Isotope Dilution High Resolution Mass Spectrometry (GC/IDHRMS) Analysis**
Method code:	**6701.04**
Branch:	**Organic Analytical Toxicology Branch (OATB)**

Prepared By: Richard Jones (Author's name) _____ (Signature) 6/14/16 (Date)

_____ (Author's name) _____ (Signature) _____ (Date)

Supervisor: Andreas Sjodin (Supervisor's name) _____ (Signature) 6/17/16 (Date)

Branch Chief: Antonia Calafat (Branch Chief's name) _____ (Signature) 6/17/16 (Date)

Date current version of method first used in lab: _____ (Date)

Director's Signature Block:

Reviewed: _____ (Signature) 6/24/16 (Date)

Procedure Change Log

Procedure: POPs in serum/plasma DLS Method Code: 6701.04

Date	Changes Made	By	Rev'd By (Initials)	Date Rev'd

Laboratory Procedure Manual

Analyte: **Polybrominated diphenyl ethers (PBDEs), Polybrominated Biphenyls (PBBs), Polychlorinated biphenyls and Persistent Pesticides (PPs)**

Matrix: **Serum**

Method: **Isotope dilution High resolution Mass Spectrometry (IDHR-MS)**

Method No: **6701.04**

Revised: **June 17, 2016**

as performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:

Dr. Andreas Sjodin
Phone: 770-488-4711
Fax: 770-488-0142
Email: ASjodin@cdc.gov

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

Important Information for Users

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.

DLS Method Code: 6701.03

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1. Clinical Relevance and Summary of Test Principle

1.1. Clinical Relevance

Organohalogen compounds may be characterized as halogen substituted hydrocarbons, neutral and lipophillic organic compounds that are only very slowly degraded or transformed under environmental conditions. According to the United Nations Environmental Program, 12 polychlorinated compounds or compound groups have been defined as persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) [1]. The physicochemical properties of such man-made chemicals have led to their accumulation in fatty tissues of wildlife and humans. This behavior of POPs was basically unknown at the time of World War II, when the chemical industry developed these substances and made them available in increasing quantities. Organohalogen compounds were commercially produced for use in agricultural, industrial and/or household applications, while others were formed unintentionally during municipal waste incineration, in other combustion and thermal processes or as by-products in the chemical industry. For example, PCB products are industrial chemicals that were used as dielectric and heat-exchange fluids, as sealants and much more [2]. DDT was applied as a pesticide, in agriculture and household applications [3].

The environmental implications first of DDT and later of PCB were not realized until the 1960s, when DDT and also PCBs were detected at high concentrations (several hundred to a few thousand ppm) in wildlife from the Baltic Sea region [3;4]. These high concentrations of DDT; 2,2-bis(4-chlorophenyl)-1,1-dichloroethene (DDE), and PCB were later found to correlate with toxicological effects observed in e.g. white-tailed sea eagles [5] and seals living in the Baltic Sea region [6-8]. However, the list of organohalogen compounds present in the environment is long today, including chemicals such as toxaphene, polychlorinated paraffins (CPs), polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), polychlorinated naphthalenes (PCNs), bis(4-chlorophenyl) sulfone (BCPS) and numerous other pesticides and technically applied substances. This illustrates the research needs about environmental issues and persistent pollutants to hopefully avoid future problems similar to those caused by PCB and DDT including bioaccumulation and biomagnifications in fatty tissues.

Polybrominated diphenyl ethers (PBDEs) included in the group of chemicals known as Brominated Flame Retardants (BFRs), have been and are still heavily used as additive chemicals in polymers and textiles [9;10]. Hence humans may be exposed though food and/or though contact with flame retarded products [11-13]. Increasing PBDE levels have been observed in mothers' milk from Sweden [14] as well as in blood from Germany [15] and Norway [16]. The PBDE levels are in general lower than that of polychlorinated biphenyls (PCBs) in Europe [13;17]. However, the PBDE concentrations found in the North Americans are considerably higher compared to European subjects [11;13;17;18]. The PBDEs are dominated by 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) [11;13;17;18]. Decabromodiphenyl ether (BDE-

209) is reported both in the general population and in occupationally exposed persons showing the bioavailability of this high molecular weight compound [11;18;19]. While the lower and medium brominated diphenyl ethers are persistent BDE-209 has a fairly short half-life of approximately two weeks [19].
PBDEs have in pregnant mice been shown to cause neurodevelopmental disorders in the offspring, as measured by behavioral test systems [20;21]. Neurodevelopmental disorders in relation to exposure to PBDEs in humans has to date not been assessed, although, such investigations are currently ongoing

Polybrominated biphenyls (PBBs) are another type of chemicals that in the past has been used and applied for similar application areas as PBDEs [9;22]. No known commercial production of PBBs currently exists. HexaBB has in humans been shown to have a half-life of approximately 30 years [23].

1.2. Test Principle

The method described in this manual assesses human body burden of BFRs, specifically PBDEs and PBBs, as well as polychlorinated biphenyls (PCBs) and persistent pesticides (PPs) in serum and/or plasma. This is done by measuring the concentration in serum/plasma through the use of automated liquid/liquid extraction and subsequent sample clean-up. Final determination of target analytes is performed by isotope dilution gas chromatography high-resolution mass spectrometry GC/IDHRMS.

Concentrations of target analytes are reported on two different concentration bases, i.e., (i) fresh weight basis (i.e., pg/g serum) and (ii) lipid weight basis (i.e., ng/g lipid). Lipid adjusted concentration values are preferable because (i) organohalogen compounds are lipophillic and hence distribute in the body mainly according to the tissues lipid content. Lipid adjusted concentrations correlates with the adipose tissue concentrations of the chemical. Normalization according to lipid content further reduces variability since differences in individuals serum lipid concentrations are cancelled out.

The samples are extracted using LLE, employing an automated Liquid Handling instrument (Gilson 215 Liquid Handler®, Gilson, Inc.). Required sample pretreatment prior to extraction is performed on the Gilson 215 liquid handler, including automated addition of (*i*) internal standards, (*ii*) methanol with a manual vortexing step in-between each addition. Hydrochloric acid is added manually to denature proteins in the sample enabling efficient extraction of target compounds. During the extraction step the target analytes are transferred from a water medium to an organic solvent.

Sample cleanup, i.e., removal of co-extracted lipids, is obtained by elution (5% DCM in hexane; 10 mL) of the extract through a column containing from the top 0.25 g of silica and 1 g of silica/sulfuric acid (33% by weight). Serum lipids are during this procedure degraded in the sulfuric acid layer while cholesterol is removed in the top layer consisting of activated silica gel. Without the activated silica gel layer cholesterol

would eliminate water forming cholestene when coming in contact with the sulfuric acid. Cholestene is not removed in the silica gel/sulfuric acid layer and would then interfere in the final HR-MS analyses. The presence of cholestene causes an ion suppression in the region of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) and 2,2',4,4',6-pentabromodiphenyl ether (BDE-100).

The lipid removal is automated using the Rapid Trace® (Caliper Life Sciences). The samples are evaporated and transferred to GC vials. Evaporization is performed on the Caliper TurboVap using increased temperature and a stream of nitrogen to aid evaporization.

Serum concentrations are determined using gas chromatography isotope dilution high resolution mass spectrometry (GC/IDHRMS), which minimizes or eliminates many interferences associated with low-resolution measurement of organohalogen compounds. Splitless injection is used employing a short GC column (DB-5HT; 15 m length, 0.1 µm film thickness, 0.25 mm ID) enabling the determination of high molecular weight compounds such as decabromodiphenyl ether (BDE-209) having a molecular weight close to 1000 amu. Electron impact ionization (EI) is used. The two most abundant ions in the isotopic cluster (fragment or molecular ion) are monitored for the target analyte as well as for the 13C-labeled internal-surrogate standard. Quantification is made against a calibration curve covering the full concentration range of the target analytes. Serum PCB/PP concentration is also determined using GC/IDHRMS but using a longer column (DB-5MS, 30 m length, 0.25 µm film thickness, 0.25 mm ID).

2. Safety Precautions

2.1 Biohazards

Follow Universal Precautions. Wear appropriate gloves, lab coat, and protective eye glasses while handling human serum. Serum may be contaminated with pathogens such as hepatitis or HIV; hence all safety precautions must be followed as outlined in the laboratory hazardous chemicals exposure plan. Wear gloves, lab coat and glasses at all times, and conduct all work in fume hood or biological safety cabinets (BSCs).

Place disposable plastic, glass, and paper (e.g., pipette tips, autosampler tubes, and gloves) that come in contact with serum in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved. When work is finished, wipe down all work surfaces where serum was handled with a 10% (v/v) sodium hypochlorite solution or equivalent.

After an accident the CDC/ATSDR Incident Report must be filed according to hazardous exposure control plan by supervisor.

2.2. Chemical hazards

Acids and Bases: Exercise caution when handling and dispensing concentrated sulfuric acid, formic acid and nitric acid. Always remember to add acid to water. Acids and bases are capable of causing severe eye and skin damage. Wear powder-free gloves, a lab coat and safety glasses. If acids or bases come in contact with any part of the body, quickly wash the exposed area with copious quantities of water for at least 15 minutes. Use safety shower if exposed area is not limited to hands and/or arms. Use eye wash station in the event of eye exposure to acids and/or bases. In the event of an accident, lab colleagues will contact the clinic by phone or emergency medical response by dialing 9-911.

Solvents: Solvents may penetrate skin causing long-term adverse health effects. Exercise caution and always use gloves when handling solvents and other chemicals. In the event of spill on gloves immediately change to a new glove since solvents do penetrate many gloves with time.

After an accident the CDC/ATSDR Incident Report must be filed according to hazardous exposure control plan by supervisor.

2.3. Hazardous waste handling

Solvent waste: Collect solvent waste in waste bottles (empty solvent bottles may be used). Clearly write **WASTE** on bottles, and the solvent(s) the waste bottle contains. If possible, always keep different solvents separated in different waste bottles, since this will make the final disposal of the different solvent wastes easier. When a bottle is filled, arrange for waste pickup according the Chemical Hygiene Plan.

Serum waste: Dispose of serum waste originating as a waste fraction in the extraction step on the Gilson Liquid Handler by completing the forms as outlined by Chemical Hygiene Plan. Also attach a Memorandum stating that the contents of the bottle are a mixture of hydrochloric acid, water, and serum that is considered to be biologically inactivated by the acid present.

Solid wastes: Sort solid waste in three fractions and placed in metal boxes with lid according to below and Chemical Hygiene Plan:

- **Non-Biogenic Contaminated Reusable Glassware** (e.g. beakers, cylinders and other reusable glassware). When the container is filled, label and return to Glassware Services according to CDC protocol.

- **Broken glass** includes used Pasteur pipets contaminated with biogenic materials, or serum bottles and vials that are not reused. When this container is filled (*i*) add approximately 1 L water to container, (*ii*) place sticker with your name, room and building number on container, (*iii*) place autoclave tape over lid and down the side

of the box and (*iv*) bring the container to autoclave located in the loading dock, building 103.

- **Gloves and other plastic parts contaminated with biogenic material** - Place biohazard bag in metal container before placing any waste in container. When container is filled (*i*) add approximately 1 L water to container, (*ii*) place sticker with your name, room and building number on container, (*iii*) place autoclave tape over lid and down the side of the box, (*iv*) place autoclave sticker on container and (*v*) bring the container to DLS designated handling area.

3. Computerization; Data System Management

3.1. Data Entry and Transfer

Sample analysis results generated by this method are stored in SAS and/or Microsoft Excel™ software. The analytical results should include at least the analysis date; analytical run number, quality-control (QC) results for the run, results of specimen analysis by specimen identification (ID), and method identifier.

3.2. Routine Computer Hard-Drive Maintenance

Defragment the computer hard drive regularly by using software such as Norton Utilities™ to maximize computer performance and maintain data integrity for files on the hard drive.

3.3. Data Backup and Schedule of Back-ups

GC/IDHRMS: Instrument raw data files are mirrored through a local network connection with each HRMS instrument computer to a local share drive Network Path: \\192.168.210.3\volume_1) which is mirrored to a network share drive (Network Path: \\cdc.gov\project\NCEH_OATB_HRMS_Archive). Between the 10th and the 14th of each month all generated instrument raw data is copied into the folder X:\LONG_TERM_BACKUP_001 on the network share for compression into a monthly compressed ZIP-file. The creation of the ZIP-file is an automatic process that runs at mid-night on the 15th of each month. The created ZIP-file is called POPLab_YYYY-MM-DD where YYYY-MM-DD is a date time stamp. After completion of the monthly backup all instrument operators will be informed over email that the backup has been completed and any raw-files from the preceding month should be transferred to a local archive folder on the instrument computer. After completion of the monthly backup the compressed ZIP-file will be made available on the lab share in the folder Z:__Shared_Folders_01_BACKUP_GOING_BACK_6_MONTHS which is a synchronized folder between the lab and network share drives. The monthly backup ZIP-file will be made available on the Lab Share for at a minimum 6 months after which older backups are accessible on the Network Share drive in the folder X:\LONG_TERM_BACKUP_001\ZipFiles.

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

- No special instructions for fasting or special diets are required, although, preferably the sample has been drawn in the morning before breakfast (i.e. fasting).
- The specimen type is serum or plasma.
- Minimum preferred serum amount is 0.5grams and the minimum acceptable amount is 0.125 grams.
- Acceptable containers for storage are thick-walled glass vials with Teflon™-lined caps or cryovials or equivalent container. Rinse containers using the same procedure as for other glassware used in the current method (see section 6.1). Preferred container is a 10 mL Wheaton glass serum vial.
- The criteria for an unacceptable specimen are either a low volume (< 0.125 g) or suspected contamination due to improper collection procedures or collection devices. In all such cases, request a second serum specimen. The limit of detection for the minimum acceptable serum amount 0.125 to 2 g of serum is given in Table 1.
- Transport and ship frozen serum specimens on dry ice. Upon receipt, they must be kept frozen at ≤ -60 °C until time for analysis. Refreeze at ≤ -60 °C any portions of the sample that remain after analytical aliquots are withdrawn. Samples thawed and refrozen several times are not compromised.

Table 1. Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]	Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]
BFR	PBDE17	0.125	8.8	BFR	PBDE99	0.125	40
		0.25	4.4			0.25	20
		0.375	2.9			0.375	13
		0.5	2.2			0.5	10
		1	1.1			1	5
		2	0.55			2	2.5
BFR	PBDE28	0.125	14	BFR	PBDE100	0.125	21
		0.25	6.8			0.25	10
		0.375	4.5			0.375	6.9
		0.5	3.4			0.5	5.2
		1	1.7			1	2.6
		2	0.85			2	1.3
BFR	PBDE47	0.125	88	BFR	PBDE153	0.125	96
		0.25	44			0.25	48
		0.375	29			0.375	32
		0.5	22			0.5	24
		1	11			1	12
		2	5.5			2	6
BFR	PBDE66	0.125	29	BFR	PBDE154	0.125	22
		0.25	14			0.25	11
		0.375	9.6			0.375	7.2
		0.5	7.2			0.5	5.4
		1	3.6			1	2.7
		2	1.8			2	1.4
BFR	PBDE85	0.125	17	BFR	PBDE183	0.125	1000
		0.25	8.4			0.25	520
		0.375	5.6			0.375	350
		0.5	4.2			0.5	260
		1	2.1			1	130
		2	1.1			2	65

[a] Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on gennerated measurements during 2015 and 1st and 2nd quarter of 2016.

Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]	Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]
BFR	PBDE209	0.125	180	PCB	PCB99	0.125	56
		0.25	92			0.25	28
		0.375	61			0.375	19
		0.5	46			0.5	14
		1	23			1	7
		2	12			2	3.5
BFR	PBB153	0.125	11	PCB	PCB105	0.125	60
		0.25	5.6			0.25	30
		0.375	3.7			0.375	20
		0.5	2.8			0.5	15
		1	1.4			1	7.5
		2	0.7			2	3.8
PCB	PCB28	0.125	75	PCB	PCB114	0.125	19
		0.25	38			0.25	9.6
		0.375	25			0.375	6.4
		0.5	19			0.5	4.8
		1	9.4			1	2.4
		2	4.7			2	1.2
PCB	PCB66	0.125	68	PCB	PCB118	0.125	69
		0.25	34			0.25	34
		0.375	23			0.375	23
		0.5	17			0.5	17
		1	8.5			1	8.6
		2	4.3			2	4.3
PCB	PCB74	0.125	68	PCB	PCB138-158	0.125	120
		0.25	34			0.25	60
		0.375	23			0.375	40
		0.5	17			0.5	30
		1	8.5			1	15
		2	4.3			2	7.5

[a] Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on gennerated measurements during 2015 and 1st and 2nd quarter of 2016.

Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]	Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]
PCB	PCB146	0.125	51	PCB	PCB170	0.125	53
		0.25	26			0.25	26
		0.375	17			0.375	18
		0.5	13			0.5	13
		1	6.4			1	6.6
		2	3.2			2	3.3
PCB	PCB153	0.125	70	PCB	PCB172	0.125	14
		0.25	35			0.25	7.2
		0.375	23			0.375	4.8
		0.5	18			0.5	3.6
		1	8.8			1	1.8
		2	4.4			2	0.9
PCB	PCB156	0.125	62	PCB	PCB177	0.125	17
		0.25	31			0.25	8.4
		0.375	21			0.375	5.6
		0.5	15			0.5	4.2
		1	7.7			1	2.1
		2	3.9			2	1.1
PCB	PCB157	0.125	56	PCB	PCB178	0.125	51
		0.25	28			0.25	26
		0.375	19			0.375	17
		0.5	14			0.5	13
		1	7			1	6.4
		2	3.5			2	3.2
PCB	PCB167	0.125	54	PCB	PCB180	0.125	59
		0.25	27			0.25	30
		0.375	18			0.375	20
		0.5	14			0.5	15
		1	6.8			1	7.4
		2	3.4			2	3.7

[a] Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on gennerated measurements during 2015 and 1st and 2nd quarter of 2016.

Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]	Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]
PCB	PCB183	0.125	54	PCB	PCB199	0.125	54
		0.25	27			0.25	27
		0.375	18			0.375	18
		0.5	14			0.5	14
		1	6.8			1	6.8
		2	3.4			2	3.4
PCB	PCB187	0.125	54	PCB	PCB206	0.125	80
		0.25	27			0.25	40
		0.375	18			0.375	27
		0.5	13			0.5	20
		1	6.7			1	10
		2	3.4			2	5
PCB	PCB189	0.125	58	PCB	PCB209	0.125	52
		0.25	29			0.25	26
		0.375	19			0.375	17
		0.5	15			0.5	13
		1	7.3			1	6.5
		2	3.7			2	3.3
PCB	PCB194	0.125	55	PST	HCB	0.125	110
		0.25	28			0.25	56
		0.375	18			0.375	37
		0.5	14			0.5	28
		1	6.9			1	14
		2	3.5			2	7
PCB	PCB196-203	0.125	100	PST	B-HCCH	0.125	220
		0.25	52			0.25	110
		0.375	35			0.375	75
		0.5	26			0.5	56
		1	13			1	28
		2	6.5			2	14

[a] Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on gennerated measurements during 2015 and 1st and 2nd quarter of 2016.

Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]	Class	Analyte	Serum Weight (g)	Method LOD (pg/g serum) [a]
PST	G-HCCH	0.125	63	PST	PP-DDT	0.125	140
		0.25	32			0.25	68
		0.375	21			0.375	45
		0.5	16			0.5	34
		1	7.9			1	17
		2	4			2	8.5
PST	OXYCHLOR	0.125	58	PST	MIREX	0.125	60
		0.25	29			0.25	30
		0.375	19			0.375	20
		0.5	14			0.5	15
		1	7.2			1	7.5
		2	3.6			2	3.8
PST	T-NONA	0.125	71				
		0.25	36				
		0.375	24				
		0.5	18				
		1	8.9				
		2	4.5				
PST	PP-DDE	0.125	260				
		0.25	130				
		0.375	85				
		0.5	64				
		1	32				
		2	16				
PST	OP-DDT	0.125	48				
		0.25	24				
		0.375	16				
		0.5	12				
		1	6				
		2	3				

[a] Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on gennerated measurements during 2015 and 1st and 2nd quarter of 2016.

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

Not Applicable

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

6.1 Reagents and consumables

The method has been validated using the chemicals, solvents and consumables listed in Table 2 and 3. Other manufacturer's products of equivalent purity can be used after verification of chemicals purity.

Table 2. Solvents and chemicals used for development of current methodology, equivalent products from other manufacturer may be used with exception to the SPE sorbent.

Chemical/Solvent	Manufacturer	Grade
Acids		
Hydrochloric acid	Aldrich	37%
Sulfuric acid	Aldrich	95-97%
Solvents		
Dichloromethane	TEDIA	Pesticide
Dodecane	EM Science	min 99%
Hexane	TEDIA	Pesticide
Methyl *tert*-Butyl Ether (MTBE)	TEDIA	Pesticide
Methanol	TEDIA	Pesticide
n-Nonane	Sigma	99%
Water	TEDIA	Pesticide
SPE sorbents		
OASIS HLB®	Waters	n/a
Silica gel	Sigma	100-200 mesh

Table 3. Expendables used for development of current methodology, equivalent products from other manufacturer may be used.

Item	Manufacturer/Source
Glassware and caps	
Test tube 16 x 100 mm	Fisher Scientific
Septum for test tube	Fisher Scientific
Open top cap for test tube	Fisher Scientific
Borosilicate GlassPasteur pipette	Fisher Scientific
Boston Round (amber glass bottle)	Fisher Scientific
V-vial (3 mL) with septum-cap	Fisher Scientific
GC vials and caps	Fisher Scientific
Others	
Label printer (Brady TLS PClink)	Fisher Scientific
Magnetic stirrer (heavy duty, large)	Fisher Scientific
Pipette dispenser	VWR

6.1.1 Rinsing of Consumables Prior to Use

PBDEs and other brominated flame retardants are common indoor pollutants. Clean all glassware including new glassware according to following procedure to eliminate risk of sample contamination.

Culture tubes and other glassware: Rinse glassware first in dishwasher (Labconco, Steam Scrubber or equivalent dish washer). Place test tubes in racks and insert them in the dishwasher. Place detergent in reservoir in the door, and start the dishwasher using program **"Scientific".**

After completion of the program, transfer the glassware to the oven located next to the dishwasher. After a heat cycle of at least 12 hours at >200 °C, the glassware is ready to be used.

For satellite bottles such as glass tapered-stopper bottles intended for storing for small volume, everyday use in the BSC the normal large labels are not to be used because it would interfere with the proper procedure for re-cleaning them. Instead label by hand using a "Sharpie" pen and affix a small hazard pictogram sticker to the bottle or alternatively attach a sheet of paper on the fume hood/BSC were relevant chemicals are listed by name with appropriate pictogram.

Caps and septa: Rinse caps and septums for test tubes prior to use to remove contaminants. This is done by Soxhelet extraction for five hours using methanol as the extraction solvent. Alternatively, if the Soxhelet apparatus cannot be used it is also acceptable to sonicate the items in methanol (20 min x 3 times). After cleaning the items, allow them to dry on aluminum foil. After the caps are completely dry, place them in a large glass beaker or in plastic re-sealable bags (not in cardboard boxes) for safe storage until used.

Gas Chromatography Vials: Heat GC vials in an oven at >200 °C overnight prior to use. Store vials in a beaker covered with aluminum foil. The caps for GC vials are cleaned by Soxhelet extraction, using the same procedure as for caps and septum's.

Pasteur Pipets: Place glass Pasteur pipets in oven on aluminum foil and heat the oven to >200 °C overnight. After completing the heating cycle for at least 12 hours, the pipets are ready to be used.

6.1.2 Internal standards (IS)

The current method is validated for BFRs, PCBs, and acid stable persistent pesticides (PPs). Use three internal standard spiking solutions for quantification of the three compound classes included. Order these standards pre-made from Cambridge Isotope Laboratory (CIL). The PBDE standard contains 7.5 pg/µL of 10 different $^{13}C_{12}$-labeled PBDE and PBB congeners. The PCB standard contains 7.5 pg/µL of 21

different PCB congeners and the PP standard contains 11 ^{13}C-labeled PPs. CIL supplies the spiking standard, in 10-mL ampoules.

When opening a new ampoule transfer the standard to a Wheaton 3-mL vial. Label the vial with "BFR IS", "PCB IS" or "PP IS" using a computer-generated label. Note the weight of the container, and the date the ampoule was opened. (The weight is used to detect any potential evaporation of the standard during storage) One vial of each standard is consumed in each analytical run on the automated liquid handler. (See 8.3)

File the certificate of analysis from CIL for each internal standard solution in the SOP binder located in building 103, room 2103.

6.1.3 Recovery standard (RS)

Use one recovery standard for measurement of recovery. This standard contains 1234-$^{13}C_6$-TCDD (2.5 pg/µL), $^{13}C_{12}$-CB-208 (10.0pg/µL) and $^{13}C_{12}$-BDE-139 (10.0pg/µL) in hexane containing 10% nonane and 2% dodecane by volume. Add the standard (100µL) to the GC vial during initial liquid handling. Transfer and mix the final extracted and purified sample with the recovery standard at the end of the procedure. Nonane and dodecane is present in the standard to act as a "keeper" (solvent that will not evaporate or evaporate to a lesser degree during subsequent evaporation step) to reduce evaporation losses during the final evaporation step. (This recovery standard is ordered pre-made from CIL)

When opening a new ampoule the standard is transferred to a Wheaton 3-mL vial, and the vial is labeled using a computer-generated label. The weight of the container is noted as well as the date the ampoule was opened. The weight is used to detect any potential evaporation of the standard during storage. One vial of recovery standard is consumed in each analytical run on the automated liquid handler. See 8.3.

File the certificate of analysis from CIL for the recovery standard solution in the SOP binder located in building 103, room 2103.

6.1.4 GC/IDHRMS Calibration Standard (CS)

The calibration standards includes several calibration levels denoted CSX (X=1 through 10). This standard is prepared by CIL and delivered in ampoules.

When opening a new ampoule, aliquot the standard into GC vials (5-10uL in each vial). Label the vials BFRX, PCBX, and PSTX where X corresponds to the calibration point 1through 10 using a computer-generated label. Replace the standards used for calibration of the DFS after completion of every run.

6.2 Instrumentation

6.2.1 Gilson 215 liquid handler: Liquid handling is automated using the Gilson 215 Liquid handler, cf. Figure 1. Place the samples in the auto-mix to the far right in Figure 1. The probe (moving arm) picks up and dispenses reagents (internal standards, methanol and water) to the samples according to a predefined sequence with mixing in-between each type of addition.

Recovery of the internal standards, as a percentage, is an important quality measurement of the analytical run. In order to enable recovery measurements, in this automated procedure, recovery standard will be added to empty GC vials located in a rack at the far left in Figure 1. These GC vials will be stored capped until the last step of the sample preparation method in which the purified extract will be transferred to the GC vials and mixed with the recovery standard.

Figure 1. Gilson 215 Liquid Handler used for automated additions of internal surrogate standards and water to the serum samples with mixing by rotation in-between the additions. This equipment also adds recovery standard to GC vials.

6.2.2 Rapid Trace®, SPE work station: The Rapid Trace® SPE workstation (Caliper Life Sciences) (Figure 2) includes (A) syringe pump for drawing and dispensing solvents and sample (B) mixing chamber (not used in this method), (C) plunger, compressing SPE cartage and dispensing liquids through cartridge, (D) cannula used for drawing serum sample from test tube and (F) rack containing serum samples and collected fractions. The Rapid Trace® instrument processes the samples in sequence. Up to 10 samples

per module for unattended cleanup. Six modules are used for the default batch size of 30 samples, resulting in simultaneous processing of six samples at any one time.

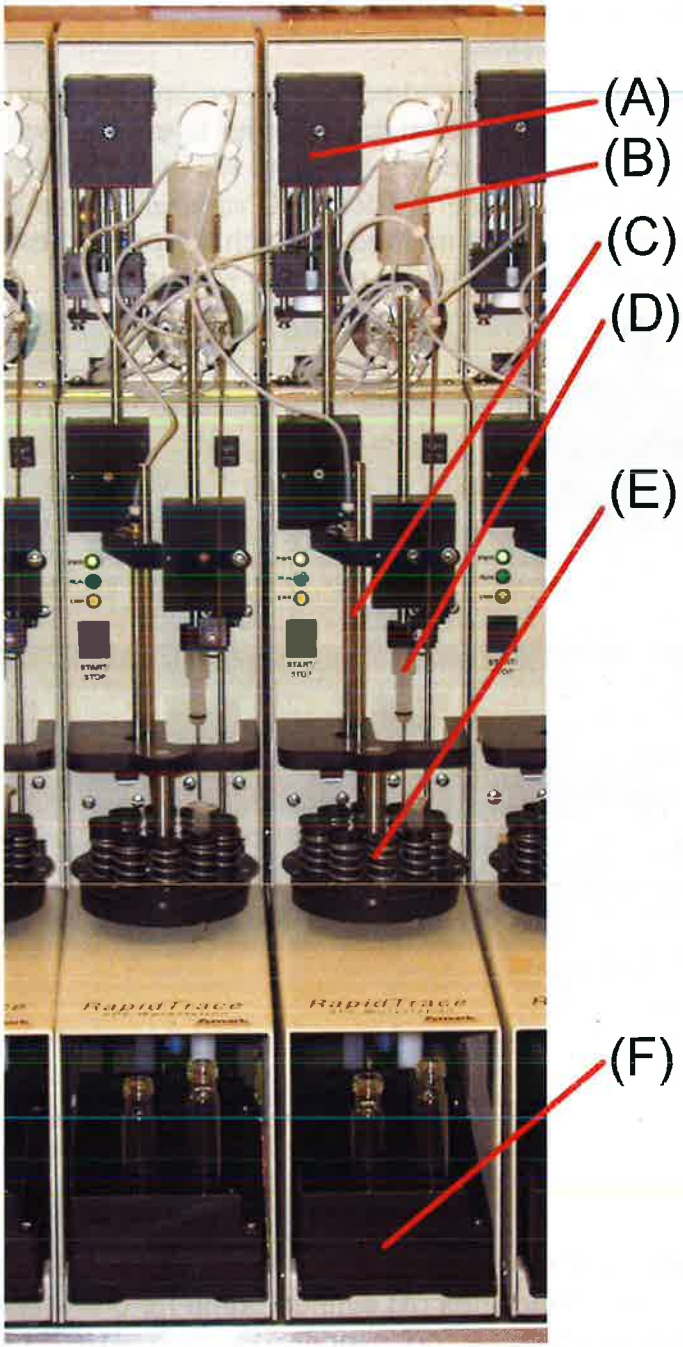

Figure 2. Rapid Trace modular SPE work station up to 10 modules controlled by one computer. Instrument includes (A) syringe pump, (B) mixing chamber [not used in current method], (C) plunger, (D) cannula and (F) sample and fraction collection rack.

6.3 Procedures for preparing quality control materials

The QC material for this assay is bovine serum in which the concentrations of the target analytes have been certified. One QC sample is analyzed in every set of 10 samples to ensure comparability and reliability between different sets of samples over time. In addition to the QC sample, a bovine blank is analyzed in every set of 10 samples. The method is designed to include several sets of 10 samples to be analyzed in parallel in one batch. (See Sample preparation below).

Specific predefined rules are applied in order to determine if the QC sample analyzed in one set is in agreement with previously analyzed QC samples. If the QC sample is found to be an outlier that set has to be reanalyzed. Example QC rules are below. All QC rules are checked by the DLS QC program available in StarLIMS.

(i) The QC determination must not deviate more than 3 times the standard deviation from the mean value of previous determinations of the same QC pool, and

(ii) No more than ten consecutive QC samples may fall either above or below the mean value of previous determinations of the same pool after one data point has fallen outside of +/- 2SD. If the QC sample fails any of these tests the set of unknown study samples must be reanalyzed.

For further details, see data handling section below.

Day 1: Rinse the vials (including caps in which the serum will be aliquoted) according to the procedure outlined in glassware rinsing procedures before use (see section 6.1.2. Label the vials with computer-generated labels.

This label should contain a unique name, constructed from the page number in the pool note book. For example SERUM:02:03 where 02 is the notebook number and 03 is the page number. State the date of the pool preparation on the label.

Thaw the serum by submerging the container in water (37 °C) until the serum is completely thawed. Pour the serum into a large beaker (4 L) containing a heavy-duty stir bar (45-mm length). Spike with native analytes to appropriate concentration level, e.g., 500 pg/mL, and stir solution overnight using a magnetic stirrer.

Day 2: While still stirring the solution, transfer serum in 6.0 mL aliquots to each of the vials. Cap the vials and place them in cardboard boxes (e.g., a lid for Xerox paper boxes) for simple freezer shelf organization. Place one identifying label on the edge of the cardboard box and place in freezer (-70 °C).

7. Calibration and Calibration Verification

7.1. Calibration of Mass Spectrometer

Calibrate and tune the Thermo DFS mass spectrometer using the appropriate calibration gas (either high boiling PFK (perfluorokerosene) for BFR analysis or FC43 for PCB/PP analysis) according to the instructions in the operator's manual.

Sensitivity Check prior to analytical run:

- ***BFRs:*** After tuning the instrument to 10,000 resolution, a greater than 10:1 signal to noise ratio for the native ions is required for an injected CS1 standard (0.2pg/uL) except PBDE209 which needs to meet a signal to noise ratio of 10:1 for the CS4 standard (5pg/uL).

- ***PCB/PST:*** After tuning the instrument to 10,000 resolution a 100:1 signal to noise ratio for the injection of 0.01pg/ul of 2378-tetrachloro-p-dibenzodioxin (TCDD) with a 2ul injection (20fg on-column).

Mass Spectrometer gain checks are performed when the multiplier is replaced or as needed. A Magnetic Calibration (MCAL) is performed during routine PMs and/or as needed. An Electric Calibration (ECAL) is performed during routine PMs and/or as needed.

7.2. Creation of Calibration Curve

A linear calibration curve, consisting of at least five CS standards with concentrations ranging from 0.5 to 500 pg/µL, is generated using the ratio of the peak area of the analyte to the labeled internal standard.

The R-squared value of the curve must be equal or greater than 0.995. Linearity of the standard curve must extend over the entire standard range.

The lowest point in the calibration curve is the lowest reportable level and the highest point is highest reportable value. The remainder of the points are equally distributed between the two extreme concentrations (on a log scale).

Generate a new calibration curve with every new set of samples to be analyzed, using the certified calibration standards from CIL. Before using a new batch of standards with the current method, verify that the new standards agree with in 20% of the old standard, this is accomplished by quantifying the new standard using the old standard. The certified value (pg/µl) of the new standard must be within 20% of the in-house quantified value (pg/µL). The tolerance of 20% between new and older standard is derived from the certificate of analysis giving a 10% tolerance of each standard released by CIL. Due to the fact that the response ratio between a native and ^{13}C-labeled internal standard is measured, a maximum deviation of 20% is used. This is accomplished by quantifying the new standard using the old standard. The certified value of the new must be within 20% of the in-house quantified value.

7.3. Calibration Verification

Calibration verification of the test system is done by the inclusion of quality control samples with a determined concentration in every run of unknown specimens and by the analysis of Proficiency Testing (PT) samples at least twice per year. See section 10 for further information on PT procedures.

7.4. Standard concentrations and target isotopic ratios

The specified concentration for analytical standards and target isotopic ratios for all measured analytes are given in the files MS_PARAM_SASEG_20YY-MM-DD.xlsx and STD_CONS_20YY-MM-DD.xlsx where YY-MM-DD is the creation date of the file. These files are located at the DLS share drive at the location:
\\cdc.gov\project\NCEH_OATB_SASEG_HRMS\LOOKUP_TABLES
Standard concentrations are also specified in the manufacturers COA included in the SOP binder.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

Formal training in the use of a high resolution mass spectrometer is necessary for all GC/HRMS operators. Users are required to read the operation manuals and must demonstrate safe techniques in performing the method. New operators must be evaluated after 6 months of initial training by the supervisor to certify that they are appropriately qualified to perform the assay.

Anyone involved in sample preparation must be trained in sample preparation equipment, chemical handling, and have basic chemistry laboratory skills. The training may be delegated to more experienced analyst.

8.1 Sending aliquot of serum for lipid determination

Serum lipid concentration in serum is determined in an aliquot of the sample (100 μl) using enzymatic methods by the Clinical Chemistry Branch (CCB). Aliquot 100 μl of each sample into polypropylene vials after mixing the thawed serum samples; use a new pipette tip for every sample to avoid cross contamination. Label vials for lipid weight determination with Study name, Study Number and notebook number. A lipid aliquot may have been drawn upon arrival of the samples to CDC and prior to the samples being sent to the POPs laboratory in which case no additional lipid aliquot needs to made prior to analysis.

If the available sample amount is low (<1mL of serum) then the entire sample may be sent to CCB for lipids measurements to minimize losses of serum during aliquoting. In this case the sample is returned to the POPs lab upon completion of the lipids measurements.

8.2. Thawing and weighing samples

Store samples in a –70 °C freezer before starting analysis. Samples are taken out from the freezer to thaw completely; this can be done the day before analysis and the samples placed in a refrigerator overnight. Thoroughly mix the samples by vortex. For each batch of 30 samples, complete a run sheet. On the run sheet, enter ALL requested information under heading "Contact Information", e.g., analyst's name or initials, the date and run number.

Print four complete sets of labels for the samples to be used during the cleanup procedure.

To ensure optimum performance of the balance (Ohaus Adventure) used for weighing serum samples, verify the balance calibration using NIST calibration weights spanning the range 1.000 g and 10.000 g before weighing each batch of samples and document recorded weights on the run sheet in the "Balance Calibration" section. Calibration weights are placed on the balance after taring, and the reading is recorded on the run sheet. The difference from true value may not exceed +/- 0.01 g. If this limit is exceeded, any problems must be resolved, such as cleaning the balance tray, recalibration of balance and/or calling for service of balance. After verifying the balance calibration, weigh serum samples into 16 x 100 mm test tubes with septum-equipped open-top screw caps. Record all sample weights on the run sheet.

8.3. Sample pretreatment, using Gilson 215 - Liquid handler

Procedure

A. Adjust the volume of the sample to 2mL if less serum was available for the measurement.
B. Place new internal standards vials containing the internal standards in the rack on the Gilson 215.
C. Begin the Gilson Spiking Application in Trilution LH. During the procedure all samples are fortified with the internal surrogate standards (Approximately 20 minutes).
D. After completion the Gilson spiking application is complete, the samples are removed from the Gilson and vortexed manually for at least 10 seconds each.
E. Next 0.5mL of 6M hydrochloric acid is added to each sample. All samples are then vortexed again for at least 10 seconds each.
F. To each sample, add 2.5mL methanol and vortex for at least 10 seconds each.

8.4. Liquid-Liquid Extraction, using the Gilson 215 Liquid Handler

The extraction procedure is automated using the Gilson 215 Liquid Handler®

The software controlling the Gilson Liquid Handler is called Trilution LH and a shortcut/icon is located on the desktop. After launching the software, the main menu is displayed (Figure 3). For setting up the software for extraction, first click on "Applications" button in the menu. In the Application Menu (Figure 4) select the application named "LLE Methanol Extraction – Neutral Fraction Only". Make sure that number of samples to be extracted is correct for each method in the application. Then click the "Run" button to begin the extraction procedure outlined below. After the first sample transfer step, the samples will be removed from the 818 AutoMix, vortexed, and centrifuged (3min, @2000rpm) to separate the organic/aqueous phases. Then, the samples are placed back in the 818 AutoMix and the Application proceeds with the second transfer of the organic phase.

Figure 3. Detail of the Trilution Main Menu. **A:** The Application Menu button.

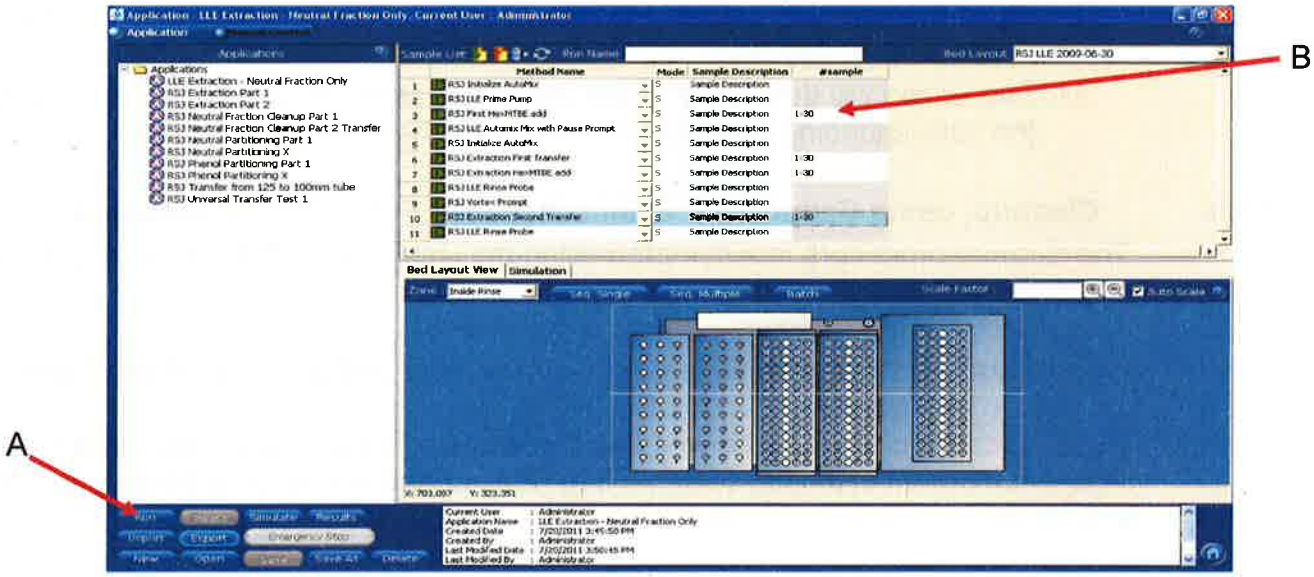

Figure 4. Detail of the Application Menu in Trilution LH. **A**: The Application Run Button. **B**: The column where the number of samples to be extracted is entered.

Check List - Extraction

A. Ensure that sufficient quantities of all solvents and reagents are present in containers under the Gilson 215 instrument and that all solvent lines are kept at the bottom of each container by an attached weight at the end of the solvent line.
B. If necessary, empty waste containers by replacing the container with an empty one.
C. Place the sample tubes in positions 1-30 in the rack in the 818 AutoMix.
D. Place empty 16x100mm tubes in positions 1-30 in the "Sample Extract" rack on the tray.
E. Select the application named "LLE Methanol Extraction – Neutral Fraction Only – Std Rinse Port". Click on the "Run" button.
F. The Gilson will add the hexane/MTBE solution to each sample and then mix the samples automatically by rotation via the 818 AutoMix for 10 minutes.
G. After mixing the Gilson will prompt the user to remove the samples and centrifuge them.
H. After centrifuging, the samples are placed back in the rack in the AutoMix and click on the "OK" button on the prompt window in the software.
I. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x100mm tube.
J. After transferring all samples, the Gilson will add more hexane/MTBE solution to each original sample tube. The Application will then pause and prompt the user to vortex the samples.
K. Remove the samples from the AutoMix and mix by vortexing for at least 10 seconds each.
L. Place the samples back in the rack in the AutoMix and click the "OK" button to continue the Application.
M. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x100mm tube. Then the Application will end.

8.5. Cleanup, using Caliper Life Sciences, Rapid Trace SPE workstation

The cleanup procedure is automated using the Rapid Trace® modular SPE system, cf. section 6.2.2).

Preparation of Silica gel / Silica gel:Sulfuric acid and packing of SPE cartridges

The SPE cartridges packed a with Silica and Silica:Sulfuric acid have a shelf life of 2 days when stored in plastic sealable bag (Ziploc) and hence must be prepared directly prior to use.

Procedure for preparation of cartridges

A. See section 6.1 for Manufacturer, grade and brand for all chemicals used

B. Activate silica gel in oven at >200 °C overnight
C. Using laboratory balance add 6.6 g Silica gel to 50-mL glass tube fitted with Teflon lined cap and add 3.3 g of concentrated sulfuric acid to the tube with. After adding the acid, vigorously shake mixture to break up large lumps. Standard laboratory Personal Protective Equipment must be used, such as lab coat, safety glasses and gloves. See section 2.2 for additional safety precautions when handling concentrated acids.
D. Allow the mixture to rotate overnight using rotating mixer. After overnight rotation confirm that no lumps are present in mixture.
E. Press frit to bottom of empty 3-mL SPE
F. Add 1.0 Silica/Sulfuric acid mixture to the cartridge, and place another frit on top
G. Add 0.25 g activated Silica gel (>200 °C overnight) and place another frit on top of the silica
H. Store packed cartridges in a reseal-able plastic bag in dessicator until just prior to use

Setting up the Equipment for Processing Samples (Cleanup)

The software controlling the workstation is launched by the RapidTrace™ Development Icon on the desk top. After launching the software the main menu is displayed (Figure 3). For setting up the software for cleanup click on "Setup Racks", the menu given in Figure 4 is displayed. Select the modules to be used in lower left corner in this menu and transfer method CL#1ONLY.spe to position "one". Transfer method CL2to10.spe to positions 3, 5, 7 and 9. Exit this menu by pressing "OK". Enter the "Run Monitor Menu" and launch the modules to be used for cleanup, cf. Figure 5.

Check List - Cleanup

A. Evaporate all unknowns and QC samples to dryness and blank samples to approximately 0.2-0.5mL by placing samples in the Caliper TurboVap evaporator and using the settings 50deg C water bath temperature and ~5psi.
B. Make certain that sufficient quantities of the 5% DCM in Hexane solution are present in the solvent bottle under the RapidTrace™ instrument and that all solvent lines are kept at the bottom of the container by an attached weight at the end of the solvent line.
C. If necessary, empty waste containers by replacing the container with an empty one.
D. Place extracts in racks (one rack per module) on the right hand side of the racks, and remove screw caps.
E. Place collection tubes on the left hand side of the racks.
F. Place racks in tray at the bottom of each module.

G. Assign method to each module by clicking "Setup racks" in the main menu of the RapidTrace™ software and placing method "CL#1ONLY.spe" as sample one for each module used and method "CL2to10.spe" for remaining positions.
H. Exit the setup racks menu by pressing OK.
I. Enter the Run Monitor Screen. Wait a few seconds after entering the Run Monitor Screen to allow the software time to detect all modules present. Press start on modules to be run.
J. Watch the instrument for a few minutes to ensure that all modules has been initiated and inspect the modules running during the initial purge to ensure that all solvents lines are connected properly.

8.6. Evaporization and transfer to final GC-vial

A. Conduct all in a fume hood or BSC or at the Caliper TurboVap evaporator.
B. Samples from cleanup step are evaporated to approximately 0.5 mL using the Caliper TurboVap evaporator and starting the evaporization with the following settings as a guide: 50deg C water bath temperature and ~5psi line pressure. ***It is essential that the samples are not evaporated to dryness at this step, since all volatile analytes would be lost.***
C. Transfer the sample to the GC vial that was spiked with recovery standard in section 8.3. **MAKE CERTAIN THAT THE SAMPLES ARE TRANSFERRED TO THE CORRECT VIAL !!!**
D. Rinse the sample test tube with ~0.5mL of hexane and transfer to the GC-vial
E. Evaporate samples until <10uL remains using the Caliper TurboVap evaporator. Start the evaporization with the following settings as a guide: ~5-10psi line pressure. Adjust the final volume to 10uL with nonane.
F. Complete any lab notes, and bring samples to HR-MS operator.

8.7 GC/IDHRMS analysis of BFRs

GC/IDHRMS analysis is performed on a DFS (ThermoFisher, Bremen, Germany) instrument. The chromatographic separations are carried out on an Trace 1300 gas chromatograph (GC) (ThermoFisher, Bremen, Germany) fitted with a Rxi 5HT [(15 m length, 0.25 mm I.D. and 0.10-µm film thickness); Restek, Bellfonte, PA] capillary column. Splitless injection is used with an injector temperature of 260°C, the oven is programmed to increase from 140 °C (1 min) to 320°C (0 min) with a ramp rate of 10 °C/min. The source temperature is 290°C in the electron impact mode using a filament bias of 45 eV. Refer to the MS_PARAM file for all monitored masses. Injections (2uL) are performed using the TriPlus RSH (ThermoFisher, Bremen, Germany) autosampler. All wash solutions should be changed at least weekly.

8.8 Final Preparation of GC Vials for PCB Analysis

A. After analysis for BFRs the samples are returned to the Controlled-Air Environment Clean Room. If necessary, reconstitute the samples with nonane to bring the volume back to 10uL.
B. Recap the samples.
C. Bring samples to HR-MS operator for PCB/PP analysis.

8.9 GC/IDHRMS analysis of PCBs/PPs

GC/IDHRMS analysis is performed on a Thermo DFS (ThermoFinnigan, Bremen, Germany) instrument. The chromatographic separations are carried out on an Trace 1300 gas chromatograph (GC) (ThermoFisher, Bremen, Germany) fitted with a Rxi-5sil MS [(30-m length, 0.25 mm I.D. and 0.10-µm film thickness); Restek, Bellfonte, PA] capillary column. Splitless injection is used with an injector temperature of 260°C, the oven is programmed to increase from 140 °C (1 min) to 320°C (0 min) with a ramp rate of 10 °C/min. The source temperature is 300°C in the electron impact mode using a filament bias of 40 eV. Refer to the MS_PARAM file for all monitored masses. Injections (2uL) are performed using the TriPlus RSH (ThermoFisher, Bremen, Germany) autosampler. All wash solutions should be changed at least weekly.

9. Reportable Range of Results

The linear range of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. However, samples with a concentration exceeding the highest reportable limit may be re-extracted using a smaller volume and re-analyzed, so that the result is in the reportable range or the extract may be diluted so that the native area counts are less than the corresponding area count for the highest calibration standard

a. Linearity Limits

Calibration standards are linear for all analytes through the range of concentrations evaluated. The linear range for all analytes except p,p'-DDE were 0.5 to 1000 pg/ul. Calibration curves for p,p'-DDE were extended to 6,000 pg/µL, due to higher concentrations in unknown specimens. Samples exceeding the calibration curve must be diluted or analyzed using a smaller volume of serum.

Certificate of analysis for all standards used are stated in the certificate of analysis as provided by the manufacturer, Cambridge Isotope Laboratory (CIL).

b. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficients of variance (CV) of the method are listed in Table 3 below.

Table 3. Mean Concentration and CV for QC samples (QC identifier SSP:01:08).

Analyte	Mean (pg/g fw)	CV	N	Analyte	Mean (pg/g fw)	CV	N
PBDE17	462.7	5.0	39	PCB138/158	839.0	2.7	36
PBDE28	455.7	3.9	39	PCB128	415.8	1.9	36
PBDE47	643.7	6.2	39	PCB167	401.6	1.9	36
PBDE66	448.4	13.9	39	PCB156	412.2	1.9	36
PBDE100	474.6	5.0	39	PCB157	417.3	1.7	36
PBDE99	486.0	4.7	39	PCB178	396.1	3	36
PBDE85	512.4	13.0	39	PCB187	396.3	4.4	36
BB153	425.0	5.2	29	PCB183	393.7	3.7	36
PBDE154	427.5	3.7	29	PCB177	399.1	3.2	36
PBDE153	470.3	3.6	29	PCB172	391.5	2.2	36
PBDE183	413.7	4.3	39	PCB180	429.3	1.7	36
PBDE203	409.0	16.4	39	PCB170	419.5	1.8	36
PBDE209	417.0	3.3	29	PCB189	392.1	2	36
PCB018	399.0	7	36	PCB199	393.2	1.5	36
PCB028	401.9	1.7	36	PCB196/203	753.9	2.3	36
PCB052	408.9	1.9	36	PCB195	414.0	11	36
PCB049	429.8	4.5	36	PCB194	383.0	2.8	36
PCB044	453.8	5.2	36	PCB206	365.4	3.5	36
PCB074	415.4	3.8	36	PCB209	341.2	2.3	36
PCB066	426.2	3.4	36	PCB114	.	.	0
PCB101	410.7	1.8	36	PCB123	.	.	0
PCB099	400.7	1.8	36	HCB	438.8	1.3	36
PCB087	426.0	3.4	36	BHCCH	209.1	3.2	36
PCB110	430.3	3.6	36	GHCCH	374.4	2.7	36
PCB118	426.5	1.9	36	OXYCHLOR	243.2	5.5	36
PCB105	418.1	2.1	36	TNONA	476.6	3.1	36
PCB151	399.6	6.7	36	PPDDE	1265.2	4	36
PCB149	378.8	8.8	36	OPDDT	345.6	4.5	36
PCB146	398.8	2.2	36	PPDDT	248.0	2.7	36
PCB153	443.9	2.4	36	MIREX	399.5	1.3	36

d. Analytical specificity

Isotope Dilution High Resolution Mass Spectrometry (ID-HRMS) coupled with gas chromatography is used for sample analysis. This instrumentation offers a high mass resolution (10,000 resolution) measurement which provides excellent specificity. In

addition, two ions are monitored for each native analyte and 13C-labeled internal standard. For each measurement, the ratio between these two ions is verified to be with +/- 26% from the theoretical isotope ratio. This provides additional confirmation of the identity of the target analyte.

In addition, the relative retention time of native compound divided with its ^{13}C-internal standard is verified for each measurement to eliminate the risk of mistakes during integration.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

In this method, a set of samples is defined as 24 unknown samples, prepared and analyzed together with 3 analytical blanks and 3 QC sample. Quality control limits are established by characterizing assay precision with repeated analyses of the QC pool.

For QA/QC purposes measurement of a target analyte in a set of samples is considered valid only after the QA/QC sample have fulfilled the following criteria as verified by the Division QC program available in StarLIMS:

(i) If all of the QC samples are within 2σ limits, then accept the run.

(ii) If one or more QC results is outside the 2σ limits, then apply the rules below and reject the run if any conditions are met.

- **Extreme outliner:** the result is outside the characterization mean by more than 4σ.

- **$1_{3\sigma}$**, Average of three QCs is outside of the 3σ limit.

- **$2_{2\sigma}$**, QC results from two consecutive runs are outside of 2σ limit on the same side of the mean.

- **$R_{4\sigma}$ sequential**, QC results from two consecutive runs are outside of 2σ limit on opposite sides of mean.

- **10_x sequential**, QC results from ten consecutive runs are on the same side of the mean.

If the QC result for an analyte is declared "out of control", then the results of that analyte for all samples analyzed during that run are considered invalid for reporting.

Further, every measurement of a set of samples must fulfill the following criteria to be considered a valid measurement:

(i) The ratio of the two ions monitored for every analyte and 13C-labelled internal standard, must not deviate more than 26% from the theoretical value.

(ii) The ratio of the retention time of the analyte over its corresponding 13C-labeled internal standard must be within the range 0.99 – 1.01. For analytes that do not have an identical 13C -labeled IS the ratio to the IS used may not deviate more than 1% from the average of the same ratio of the calibration standards analyzed in the same analytical run

(iii) The measured recovery of the IS must be within the range 10-150%.

b. Proficiency testing (PT): Currently the only established PT program for this assay is the Arctic Monitoring and Assessment program (AMAP) in which our lab participates. In this program 3 serum samples are received three to four times per year and analyzed with respect to PCB/PP/PBDEs. The program provides a report after each set of PT samples has been reported. In addition, our lab uses an in house PT program (as specified in the Division Policy and Procedures manual) where 5 blinded PT samples are measured twice per year.

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance a failure of the mass spectrometer or a pipetting error, correct the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure). After re-establishing calibration or quality control, resume analytical runs. Document the QC failures, review the cases with supervisor to determine source(s) of problem, and take measures to prevent re-occurrence of the same problem.

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. By using high resolution mass spectrometry, most interferences are eliminated. Due to the matrix used in this procedure, occasional unknown interfering substances have been encountered. If chromatographic interference with the internal standards occurs, reject that analysis. If repeat analysis still results in an interference with the internal standard, the results for that analyte are not reportable.

13. Reference Ranges (Normal Values)

Reference ranges have been reported for BFRs in the NHANES survey and are available at www.cdc.gov/exposurereport

14. Critical Call Results ("Panic Values")

It is unlikely that any result would be a "critical call", which would only be observed in acute poisonings. There are no established "critical call" values. Application of this method to NHANES studies will assist in determining levels of BFRs normally found in the US populations. Test results in this laboratory are reported in support of epidemiological studies, not clinical assessments. Data will help determine critical exposures.

15. Specimen Storage and Handling During Testing

Store serum samples in -70 °C freezer before and after analysis. Keep extracts at room temperature covered with aluminum foil for storage, due to documented UV-sensitivity of target analytes.

After analysis, keep GC vials in Styrofoam boxes for storage at room temperature until the final analytical data have been reported.

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

Alternate validated methods have not been evaluated for measuring BFRs in human serum. If the analytical system fails, refrigerate the samples (at 4 - 8 °C) until the analytical system is restored to functionality. If long-term interruption (greater that one day) is anticipated, then store serum specimens at <-40 °C.

The method is designed to run on a GC/IDHRMS instrument, and is not generally transferable to other instrumentation. If the system fails, store sample extracts at room temperature covered with aluminum foil until the analytical system is restored to functionality.

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

Study subject data is reported in both concentration units (pg/mL serum) and adjusted based on serum lipids (ng/g lipid).

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 report is created. These data and a cover letter will be routed through the appropriate channels for approval (i.e. supervisor and/or branch chief, DLS statistician, division director) as outlined in the DLS Policy and Procedure Manual. After approval at the division level, the report will be sent to the contact person or principal investigator who requested the analyses typically in an email.

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

If greater than 0.1 mL of sample remains following successful completion of analysis, this material must be returned to storage at <-40 °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 formats (e.g., database, notebooks, data files) are used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Specimens may be stored at the CDC specimen handling and storage facility (CASPIR).

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Division of Laboratory Sciences

Laboratory Protocol

Analytes: Nine monohydroxy-polycyclic aromatic hydrocarbons: 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 3-hydroxyfluorene, 1-hydroxyphenanthrene, 2- & 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 1-hydroxypyrene

Matrix: Urine

Method: Isotope Dilution Online Solid Phase Extraction- High Performance Liquid Chromatography-Tandem Mass Spectrometry (online SPE-HPLC-MS/MS)

Method code: 6705.02

Branch: OAT

Prepared By:

Yuesong Wang			1/5/2015
Author's name	Signature		Date
Lei Meng			1/5/2015
Author's name	Signature		Date
Supervisor: Xiaoyun Ye			1/6/2015
Supervisor's name	Signature		Date
Branch Chief: Antonia Calafat			1/6/15
Branch Chief's name	Signature		Date

Date current version of method first used in lab: _____
Date

Director's Signature Block:

Reviewed: _____ 1/7/15
Signature Date

Procedure Change Log

Procedure: **Nine monohydroxy-polycyclic aromatic hydrocarbons**
DLS Method Code: 6705.02

Date	Changes Made	By	Rev'd By (Initials)	Date Rev'd
12/29/2014	1. Changed the configurations, columns, and gradients for HPLC and SPE systems. 2. Prepared new pools of standards, QCs, and PTs. 3. Updated the QC ranges and LODs.	YW	XY	1/2/2015

Laboratory Procedure Manual

Analyte: **Nine monohydroxy-polycyclic promatic hydrocarbons: 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 3-hydroxyfluorene, 1-hydroxyphenanthrene, 2- & 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 1-hydroxypyrene**

Matrix: **Urine**

Method: **Isotope Dilution Online Solid Phase Extraction High Performance Liquid Chromatography/Tandem Mass Spectrometry (online SPE-HPLC-MS/MS)**

Method No: **6705.02**

As performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

Contact:

Antonia Calafat, Ph.D.
Phone: 770-488-7891
Email: ACalafat@cdc.gov

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

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.

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1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental contaminants formed during incomplete combustion processes. Many of them have been identified as suspected human carcinogens (1), but threshold levels for carcinogenicity have not been determined for most PAHs. Occupational exposure may occur through work involving diesel fuels and coal tars such as paving and roofing. Possible environmental exposures include smoking, diet, smog and forest fires (2). Because of potential widespread human exposure and potential risk to health, biomonitoring of PAHs is relevant for environmental public health (3,4). Upon exposure, PAHs are metabolized in humans; some of these metabolites are excreted in urine. Information on the concentration of metabolites of PAHs in people is important for understanding human exposure.

b. Test Principle

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection of several monohydroxylated metabolites of PAHs (OH-PAHs) in urine. The procedure involves enzymatic hydrolysis of glucuronidated/sulfated OH-PAH metabolites, centrifugation, dilution and analysis using isotope dilution online solid phase extraction (SPE) coupled with HPLC-ESI-MS/MS. Ion transitions specific to each analyte and carbon-13 labeled internal standards are monitored, and the abundances of each ion are measured. The analytes measured in this procedure are shown in Table 1.

Table 1. Analytes, their parent compounds, and their abbreviations.

No.	Metabolite/Analyte	Parent PAH	Abbreviation	Note
1	1-hydroxynaphthalene	Naphthalene	1-NAP	
2	2-hydroxynaphthalene		2-NAP	
3	2-hydroxyfluorene	Fluorene	2-FLU	
4	3-hydroxyfluorene		3-FLU	
5	1-hydroxyphenanthrene	Phenanthrene	1-PHE	2-, 3-PHE measured together
6	2-hydroxyphenanthrene		2-PHE	
7	3-hydroxyphenanthrene		3-PHE	
8	4-hydroxyphenanthrene		4-PHE	
9	1-hydroxypyrene	Pyrene	1-PYR	

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents needed to perform this procedure are toxic. Special care must be taken to avoid inhalation or dermal exposure to these reagents.

β-Glucuronidase is a known sensitizer. Prolonged or repeated exposure to the sensitizer may cause allergic reactions in certain sensitive individuals.

Note: Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at http://intranet.cdc.gov/CHATS/OHS/chem_search.asp or http://www.msdsxchange.com/english/index.cfm. Laboratory personnel are advised to review the MSDS before using chemicals and solvents.

b. Radioactive Hazards

There are no radioactive hazards associated with this procedure.

c. Microbiological Hazards

Although urine is generally regarded as less infectious than serum, the possibility of being exposed to various microbiological hazards exists. Appropriate measures must be taken to avoid any direct contact with the specimen. CDC recommends a Hepatitis B vaccination series and a baseline serum test for health care and laboratory workers who might be exposed to human fluids and tissues. Laboratory workers observe universal precautions to prevent any direct contact with the specimen. Also, laboratory personnel handling human fluids and tissues are required to take the "Bloodborne Pathogens Training" course and subsequent refresher courses offered at CDC to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratory analysts must read and follow the manufacturers' information regarding safe operation of 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 must only be performed by qualified technicians. Avoid contact with the heated surfaces of the mass spectrometer (e.g., interface). Multiple solvent bottles (1L) are located on and around the instruments. Numerous tubing lines are used to transfer solvents from storage bottles to the instrument and the waste bottles. Precautions must be used when working in these areas.

e. Protective Equipment

Standard safety precautions must 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 is necessary in the use of the Staccato® automated sampler, Agilent 1200 HPLC, AB Sciex 5500/6500 MS-MS, and online SPE system. Users are required to read the operation manuals and demonstrate safe techniques in performing the method. Laboratorians involved in sample preparation must be trained for all sample preparation equipment, chemical handling, and have basic chemistry laboratory skills.

g. Personal Hygiene

Follow Universal Precautions. Care must be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing must 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 laboratory, local, state, and federal regulations. Solvents and reagents must always be disposed of in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. Disposable items that come in direct contact with the biological specimens are to be placed in a biohazard autoclave bag that must be kept in appropriate containers until sealed and autoclaved. Needles, pipette tips and disposable syringes must be discarded into sharps containers and autoclaved. Contaminated surfaces should be disinfected with a freshly prepared bleach solution (e.g., ~0.5% available chlorine, or a 100 mL/L dilution of commercial sodium hypochlorite solution containing 5% available chloride). Any non-disposable glassware or equipment that comes in contact with biological samples must be washed with bleach solution before reuse or disposal. Any other non-disposable glassware must be washed and recycled or disposed of in an appropriate manner. To insure proper compliance with CDC requirements, laboratory personnel are required to take annual hazardous waste disposal training courses.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

Spiking of samples and hydrolysis of conjugates normally take place on a Staccato® Automated System controlled by the Perkin Elmer iLink and Maestro softwares. The SPE-HPLC-MS/MS system uses an Agilent 1200 series HPLC pump and AB Sciex 5500/6500 MS/MS, controlled by AB Sciex Analyst™ software, and an iChrom Symbiosis™ online SPE system, controlled by Sparklink® software. Analyte chromatographic peaks are integrated by MultiQuant™ or Analyst®. Results can be exported from Multiquant™ or Analyst®as text files which are subsequently processed using Excel, Access or SAS Enterprise Guide (SAS EG). Knowledge 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 (Access or STARLIMS) either manually or electronically using the files received from Sample Logistics. The result file is transferred electronically into the database. No personal identifiers are used, only coded sample identifiers.

c. Data Maintenance

All sample and analytical data are reviewed for overall validity. The database is routinely backed up locally through the standard practices of the CDC network. The local area network manager can be contacted for emergency assistance.

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

a. Special Instructions

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

b. Sample Collection

Urine specimens are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible, and preferably transferred to specimen vials within 24 hours of collection. If possible, a minimum of 2 milliliters of urine is collected and poured into vials (e.g., polypropylene, glass) with screw-cap tops. The specimens should be labeled, frozen at or below -20 ºC, and stored on dry ice for shipping. Special care must be taken to protect vials from breakage

during shipment. At CDC, samples are kept frozen, preferably at -70 ºC, until and after analysis.

c. Sample Handling

Specimen handling conditions are outlined in the Division of Laboratory Sciences (DLS) protocol for urine collection and handling (e.g., copies available in branch, laboratory). In general, urine specimens should be transported and stored frozen. Once received, they should be frozen, preferably at -70 °C, until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn must be refrozen as soon as possible after use.

d. Sample Quantity

The regular sample size for analysis is 0.1 mL; the minimum amount of specimen generally required for a regular analysis is 0.05 mL.

e. Unacceptable Specimens

Specimens must be frozen when delivered to the lab. The minimum volume generally required for a single analysis is 0.1 mL. If either of these criteria is violated, the specimen may be rejected. Specimens can also be rejected if suspected of contamination due to improper collection procedures or devices. Specimen characteristics that may compromise test results include contamination of urine from improper handling. Samples with visible microbiological growth (e.g., mold, bacteria) might be inadequate for analysis. In case of rejected specimens, we would request a second specimen if possible. A description of reasons for rejecting a sample must be recorded on the sample transfer sheet (e.g., 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

See Table 2.

Table 2. Reagents and suggested manufacturers

Reagent	Suggested Manufacturers*
Water (HPLC grade), acetonitrile, methanol	ThermoFisher Scientific, Inc., Waltham, MA
β–glucuronidase/arylsulfatase (H-1, powder enzyme), glacial acetic acid, sodium acetate, ascorbic acid, formic acid	Sigma-Aldrich Chemical, St. Louis, MO
$^{13}C_6$ 1-NAP, $^{13}C_6$ 2-NAP, $^{13}C_6$ 2-FLU, $^{13}C_6$ 3-FLU, $^{13}C_6$ 1-PHE, $^{13}C_6$ 2-PHE , $^{13}C_6$ 3-PHE, , $^{13}C_6$ 4-PHE,$^{13}C_6$ 1-PYR	Cambridge Isotope Laboratories, Andover, MA
1-NAP, 2-NAP, 1-PYR	Sigma-Aldrich Chemical, St. Louis, MO
2-FLU, 3-FLU, 1-PHE, 2-PHE, 3-PHE, 4-PHE	Cambridge Isotope Laboratories, Andover, MA

* Products from other manufacturers with similar purity or specifications may be used.

b. Preparation of Reagents

1) Sodium Acetate Buffer Solution (~1 mol/L, pH 5.5±0.2)

Weigh sodium acetate and record in logbook. Transfer contents to a clean glass bottle and add necessary volume of de-ionized water (DI H_2O) to make a 1 mol/L solution. An example solution is 10.25 g sodium acetate diluted with 125 mL of DI water. Stir on a stir plate until the solid completely dissolves. Measure pH and record the value in logbook. Adjust the pH to 5.5 with glacial acetic acid; record final volume in logbook.

2) β-glucuronidase/arylsulfatase enzyme/Buffer solution (~10 g/L)

Weigh the needed amount of β-glucuronidase/arylsulfatase (H-1, powder enzyme) into a glass vial to have a final concentration of ~10 g/L. Add 1 mL of the sodium acetate buffer solution (1 mol/L, pH 5.5) for each 0.01 g of enzyme and cap the vial (e.g. 0.40 g enzyme to 40 mL buffer). Place vial on a rotating mixer at ~40 rpm until the enzyme is completely dissolved. Store unused enzyme/buffer solution refrigerated for up to one week.

3) Ascorbic Acid Solution (~12.5 g/L)

Weigh L-ascorbic acid into a glass vial or test tube. Add 80 μL of deionized water for each milligram of ascorbic acid (e.g. 14 mg ascorbic acid to 11.2 mL water) and cap the vial. Place vial on a rotating mixer at 40 rpm until the solute is completely dissolved. Store unused ascorbic acid solution at room temperature for up to one week.

4) Synthetic Urine

For a 1L final volume, add the following chemicals in order, then fill to 1L with D.I. water. Store the solution in the refrigerator or freezer until use.

- 500 mL Water
- 3.8 g Potassium Chloride
- 8.5 g Sodium Chloride
- 24.5 g Urea
- 1.03 g Magnesium Sulfate ($MgSO_4.7H_2O$)
- 1.03 g Citric Acid
- 0.34 g Ascorbic Acid
- 1.18 g Potassium Phosphate, dibasic
- 1.4 g Creatinine
- 0.64 g Sodium Hydroxide (add slowly)
- 0.47 g Sodium Bicarbonate
- 0.28 mL Sulfuric Acid (concentrated.)

c. Preparation of Calibration Materials

Standard preparation is based on gravimetric and volumetric determination. Actual calculated concentrations based on weight are used in all data calculation and processing, and the actual preparation and final concentrations may slightly deviate from the normal procedure or target concentrations.

1) Individual Standards and Mixed Working Standards

Nine individual monohydroxylated PAH standard solutions were prepared by dissolving weighted amounts of target analytes in ethanol. The mixed working standards containing nine analytes were prepared from serial dilution of the individual stock solutions in 40 % (v/v) ethanol.

2) Calibration Standards

The calibration standards were prepared from serial dilution of the working standards in 40 % (v/v) ethanol. The calibration standards were aliquot into glass vials, capped and kept frozen until use.

3) Working Standard Solution of ^{13}C-labeled Standard Mix and Internal Spiking Solution (ISS)

Combine individual ^{13}C-labeled internal standard (I.S.) stock solutions (90 ng/µL) to generate the working internal standard solution (WSI). Homogenize the mixture.

Transfer 1 mL of WSI into a 500-mL volumetric flask. Dilute the solution with HPLC grade water to prepare internal spiking solution (ISS). Aliquot into amber 4-mL standard vials, cap and keep frozen until use.

d. Preparation of Quality Control Materials

1) Quality Control (QC) Materials

Prepare quality control materials by spiking a known amount of native standard mixture (in acetonitrile) into 2000 mL of an anonymous filtered urine pool. Homogenize the QC solutions for at least 3 h. Aliquot into 4-mL vials and store at -70 °C until use.

2) Proficiency Testing Material (PT)

Prepare proficiency testing materials by spiking 100 mL of an anonymous urine pool (filtered) with a known amount of working standard solution to achieve the target concentration (preferably different from those of the QCs). Prepare at least three urine pools at levels within the linear range of the method.
After spiking the urine pool with a known amount of working standard solution, homogenize the PT solutions overnight for equilibration. Then, aliquot the PT solutions into 16 x 100 mm test tubes (2 mL in each tube). PT samples are then randomized by an external PT administrator, labeled by external lab technicians, and stored at -70 °C until use.

e. Other Equipment, Materials, and Supplies

Materials / supplies and sources, or their equivalent, used during the development, validation, and application of this method are listed below.

- Incubator ovens (Fisher Scientific, INHECO)
- pH meter (Corning)
- Microbalance (Mettler-Toledo)
- Rotary suspension mixer (Glas-Col)
- Stirring/heating plates (Corning)
- Miscellaneous glassware (Pyrex, Kimax, Wheaton, Corning)
- Repeater Plus Pipette (Eppendorf)
- Electronic and Manual Pipettes (Rainin)
- Maxi-mix Vortex mixer (Barnstead International)
- Amber screw top vials of various volumes (Supelco, Inc)
- 96-Well plates (Axygen, Eppendorf)
- SBS Format Reservoirs (Seahorse Bioscience)
- Sample Tubes (Fluidx)

- Oasis WAX On-Line SPE Cartridges for Symbiosis and Prospekt 2 Systems (Merck KGaA, Darmstadt, Germany)
- Chromolith HighResolution RP-18 endcapped HPLC Column 100×4.6 mm (Merck KGaA, Darmstadt, Germany)
- Chromolith HighResolution RP-18 endcapped Guard Column 5×4.6 mm (Merck KGaA, Darmstadt, Germany)

f. Instrumentation

The sample preparation procedure can be fully automated on a Staccato® Automated System (Perkin Elmer Co.) with the following components:

- Sciclone G3/G3T
- Fluidx CESD-24PRO decapper
- Fluidx XTR-96-Cryo 2D barcode reader
- Turntable/1D barcode reader
- Hettich Rotanta 460 centrifuge
- ThermoScientific ALPS3000 sealer
- IVD Inheco Incubator Shaker DWP (4)
- Mitsubishi robotic arm

The analyses are performed on an iChrom Symbiosis online SPE system, coupled with an Agilent 1260 HPLC and AB Sciex 5500 or 6500 MS operated under negative electrospray ionization mode.

1) Online SPE

The SPE tubing and the valve switching system is used in concurrent SPE/HPLC mode controlled by the SparkLink software. The method uses both left and right cartridge clamps, the four switching valves, the high pressure dispenser, and the autosampler. The left clamp, the left clamp valve (LCV), and left integrated Stream Switching (ISS1) are used for SPE clean-up while the right clamp, the right clamp valve (RCV) and right integrated Stream Switching (ISS2) are used for the HPLC elution.

The SPE run of each sample starts with the conditioning of an Oasis WAX online cartridge with HPLC-grade acetonitrile (4 mL) and 0.1% formic acid (2 mL). Afterward, 50-500 µL of the sample (~ 50 µL of urine in 500 µL sample solution) is injected into the 1 mL sample loop and loaded onto the SPE column using 1.5 mL 0.1% formic acid. Next, the SPE column is washed with 1.5 mL Acetonitrile/Methanol/Water (1/1/2, v/v/v). The duration of the SPE procedure (including injection time) is approximately 11 min. Before starting the clean-up of the next sample, the cartridge containing the extracted analytes is transferred by a robotic gripper from the left clamp into the right clamp. While

the right clamp is used for analyte elution and HPLC-MS/MS acquisition, the left clamp could be used for the clean-up of the next sample.

2) HPLC Configuration

After online SPE, the extract is loaded onto a Chromolith HighResolution RP-18 endcapped Guard Column (5×4.6 mm) with 350 µL methanol at a flow rate of 100 µL/min. The HPLC gradient (Table 4) starts with 1% methanol (with 0.1 mM ammonium fluoride) as mobile phase B at 500 µL/min for the first 3.5 minutes to focus the analytes onto the guard column. After the first 3.5 minutes, the eluent from the guard column is connected to the waste. Afterwards, the valve switches and the eluent from the guard column connects to a Chromolith column.

Column and guard column:

- Chromolith HighResolution RP-18 endcapped HPLC Column 100×4.6 mm (Merck KGaA, Darmstadt, Germany)
- Chromolith HighResolution RP-18 endcapped Guard Column 5×4.6 mm (Merck KGaA, Darmstadt, Germany)

HPLC Mobile Phase:

- Mobile Phase A: 0.1 mM ammonium fluoride in Water
- Mobile Phase B: 0.1 mM ammonium fluoride in Methanol

Table 4: HPLC Gradient.

Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0.0	500	99.0	1.0
3.5	500	99.0	1.0
3.9	500	40.0	60.0
4.3	500	40.0	60.0
5.0	800	38.0	62.0
18.0	800	32.0	68.0
19.5	800	30.0	70.0
20.0	1000	15.0	85.0
21.0	1000	15.0	85.0
22.0	1000	10.0	90.0
24.0	1000	5.0	95.0
24.5	1000	5.0	95.0
24.6	1000	99.0	1.0
27.0	1000	99.0	1.0

3) Tandem Mass Spectrometer (MS/MS) Configuration
Detection of the target analytes is conducted on the AB Sciex tandem mass spectrometer in the negative electrospray ionization mode (Table 5).

Table 5. Representative Mass spectrometric parameters*

	Precursor ion (m/z)	Product ion (m/z)	Dwell Time (ms)	DP (volts)	CE (volts)
2-NAP	143	115	25	-120	-34
2-NAP2**	143	41	25	-260	-34
2-NAP-IS	149	115	25	-120	-34
1-NAP	143	115	25	-120	-34
1-NAP2**	143	41	25	-260	-34
1-NAP-IS	149	115	25	-120	-34
3-FLU	181	180	25	-100	-26
3-FLU-IS	187	186	25	-100	-26
2-FLU	181	180	25	-100	-34
2-FLU-IS	187	186	25	-100	-34
2-3PHE***	193	165	25	-150	-41
2-3PHE-IS***	199	171	25	-150	-41
1-PHE	193	165	25	-100	-38
1-PHE-IS	197	168	25	-100	-38
4-PHE	193	165	25	-100	-38
4-PHE-IS	197	168	25	-100	-38
1-PYR	217	189	25	-100	-45
1-PYR-IS	223	195	25	-100	-45

* Actual parameters are optimized for different instruments.
** 1-NAP2 and 2-NAP2 results may be used when concentrations are ~20-200 ng/mL.
*** 2-PHE and 3-PHE are measured together (2-3PHE).

7. Calibration and Calibration Verification

a. Tuning and Calibration of Mass Spectrometer

The AB Sciex MS/MS is calibrated and tuned at least once per year using a polypropylene glycol (PPG) solution according to the instructions contained in the operator's manual.

b. Creation of Calibration Curve

1) Calibration data

At least five calibration standards are analyzed with every analytical run. Calibration curves are generated by plotting the analyte area ratios (i.e., analyte area/internal standard area) against the native analyte concentrations through linear regression analysis with 1/X weight.

2) Evaluation of Curve Statistics

The R-squared value of the curve must be ≥0.95. Linearity of the standard curve must extend over the entire calibration range. Samples with concentrations of a given analyte exceeding the highest point in the calibration curve are reanalyzed using less urine volume.

c. Calibration Verification

1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.

2) All calibration verification runs and results shall be appropriately documented.

3) According to the updated Clinical Laboratory Improvement Amendments (CLIA) regulations from 2003 (http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/downloads/6065bk.pdf), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.

4) All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

a. Sample Preparation

Sample preparation can be performed manually or on a Staccato® Automated System.

1) Enzymatic Hydrolysis

- Allow calibrators (CS), quality control samples (QCs), synthetic urine, and urine samples to thaw and reach room temperature.
- Aliquot 100 μL of specimens into a vial or plate well.
- Add 20 μL of ascorbic acid solution (12.5 mg/mL) to each vial or well.
- Add 50 μL of ISS.
- Add 50 μL of 1M sodium acetate buffer (pH 5.5) containing β-glucuronidase/arylsulfatase enzyme from *Helix pomatia* (10mg enzyme/1mL buffer) to all aliquots and mix well.
- Cap or seal all samples and incubate overnight (~18 hours) at ~37°C.

2) Centrifugation and sample transfer

After overnight enzymatic hydrolysis, add at least 175 μL methanol (total volume = 220+175 μL) to all samples and mix well to precipitate the enzyme. Cap or seal all samples and centrifuge for ~15 minutes at 3000 – 5000 rpm. Transfer 200 μL of the supernatant layer to a new sample container containing 350 μL of deionized H_2O and mix well. Transfer the rest of the supernatant layer (around 150 μL) to a second new sample container containing 350 μL of deionized H_2O, mix well and store it as backup. The backup container may be used for LC-MS analysis, and will be discarded after a month.

b. Instrument and software setup for the online SPE-HPLC-MS/MS

1) Preliminary System Setup and Performance Check

The ABSciex 5500/6500 TripleQuad mass spectrometer is calibrated and tuned periodically using positive and negative polypropylene glycol (PPG) solution provided by the manufacturer. The instrument sensitivity is checked before each analytical run by injecting the instrument test solution.

2) Runsheet and Batch Setup

A typical analytical run consists of 11 calibration standards, 3 blanks, 2 QCLs, 2 QCHs and 78 study samples.

Create a runsheet using a MS Excel runsheet template. In the **Excel** runsheet, two batch files, one for the Analyst software and one for the Sparklink software, are generated for each analytical run. The two batch files are saved as text files (.txt or .csv) to be imported to the Analyst software and Sparklink software, respectively.

3) Online SPE-HPLC-MS/MS Analysis Procedure

- Check the basic instrument functions and settings according to the manufacturer's instructions.
- Check the instrument performance by running an instrument test sample.
- In the **SparkLink** software, create a new runtable and import the Sparklink batch text or excel file created from the batch runsheet. The runtable will be automatically populated with the method, sample name, vial position, injection volume and SPE cartridge position. Make sure the correct vial position and SPE cartridge are used. Click the "start" icon on the top of the runtable.
- In the **Analyst** software, create a new batch file, then import the Analytst batch text file and the batch table will be populated with sample name, sample ID, and data file name. Make sure the correct acquisition method and quantitation method are selected. Go to the quantitation tab, and make sure all the levels of the calibration curve are filled in. Go to submit tab. Highlight the rows of samples to run and click "submit" on the top right corner. All samples on the Queue Manager should be in "waiting."
- Click "Start" on the runtable in **SparkLink** and the online SPE will start with the first sample. Then submit the batch in Analyst and click "run." Once the sample clean-up finishes, the cartridge in the left clamp will be moved by the gripper to the right clamp for elution, and the mass spectrometer will promptly start the data collection.

c. Processing of Data

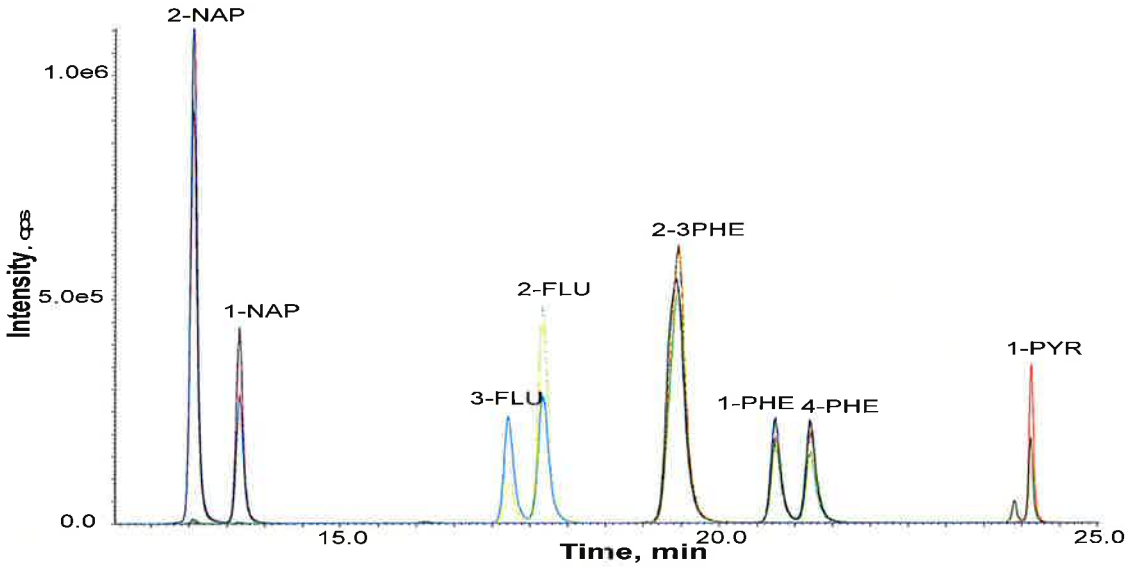

Figure 1. An example chromatogram of the standard (level 6).

An example HPLC chromatogram is given in Figure 1. Process the data using the Analyst, MultiQuant or Indigo software.

After peak integration is reviewed, go to the calibration curve page. Review the calibration curve for each analyte and confirm that $R^2>0.95$.

Export the results table as a text file (.txt), and place a copy of this result text file on the CDC shared network drive. Run the DLS SAS program to check the batch QCs.

d. Replacement and Periodic Maintenance of Key Components

The instrumentation used is serviced according to the manufacturers' guidance included in the instruments manuals or based on the recommendations of experienced analysts/operators after following appropriate procedures to determine that the instruments perform adequately for the intended purposes of the method.

9. Reportable Range of Results

The linear range of the standard calibration curve and the method limit of detection (LOD) determine the highest and lowest reportable concentrations for the target analytes. However, urine samples with analyte concentrations exceeding the highest reportable limit may be re-extracted using a smaller volume (e.g., 50 μL or 2 fold dilution) and re-analyzed so that the result is in the reportable range. Generally, samples with extremely high values can be diluted up to 100 times.

a. Linearity Limits

Calibration curves constructed with the analytical standards are linear for all analytes through the range of concentrations evaluated (Table 6). The linear range is up to 200 ng/mL for 1-NAP and 2-NAP (using their secondary ions) and up to 25 ng/mL for the remaining analytes. Urine samples whose OH-PAH concentrations exceed these ranges must be diluted and re-analyzed using a smaller sample size (up to 100 times dilution).

b. Limit of Detection

The limit of detection (LOD) for each analyte is presented in Table 6.

Table 6. Limits of detection (LOD) and linearity limits.

Analyte	LOD (ng/mL)	Upper linearity limit (ng/mL)
1-NAP*	0.06	200
2-NAP*	0.09	200
2-FLU	0.008	25
3-FLU	0.008	10
1-PHE	0.009	10
2-3PHE**	0.01	20
4-PHE	0.007	10
1-PYR	0.07	10

* Upper limits for 1-NAP and 2-NAP are achieved by using the first transition using the ABSciex 6500 and the secondary ion using the ABSciex 5500, "1-NAP2" and "2-NAP2."
** 2-PHE and 3-PHE are measured together.

c. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The mean concentrations and coefficients of variation (CV) of QC samples are listed in Table 7. These QC samples were analyzed over 6 weeks using two online SPE-HPLC-/MS/MS instruments.

Table 7. Mean, standard deviation, and CV for QC samples. The parameters are QC pool specific.

Analyte	QCL				QCH			
	Mean (ng/mL)	Between Day SD (ng/mL)	Within day SD (ng/mL)	CV%	Mean (ng/mL)	Between Day SD (ng/mL)	Within day SD (ng/mL)	CV%
1-NAP	1.17	0.09	0.05	6.9%	7.65	0.62	0.46	6.9%
2-NAP	1.53	0.14	0.08	8.0%	8.84	0.65	0.41	6.6%
2-FLU	0.26	0.02	0.01	8.6%	1.12	0.10	0.07	8.0%
3-FLU	0.29	0.02	0.01	6.5%	1.20	0.08	0.06	5.2%
1-PHE	0.18	0.03	0.01	13.7%	0.70	0.09	0.05	12.2%
2-3PHE	0.39	0.05	0.02	11.2%	1.47	0.15	0.09	9.3%
4-PHE	0.14	0.02	0.01	16.7%	0.55	0.07	0.05	12.1%
1-PYR	0.37	0.05	0.03	12.8%	0.73	0.11	0.08	13.8%

d. Analytical Specificity

The analyte peaks are located in well-defined regions of the chromatogram with no visible interferences and low background.

e. Accuracy

The accuracy of this method was evaluated by analyzing two NIST Standard Reference Materials (SRMs) and by comparing the results obtained to their certified concentrations for the 9 OH-PAHs (Table 8). The smoker SRM 3672 and the non-smoker SRM 3673 were analyzed 4 times in 2 different runs. Averages of the measured concentrations are given in Table 8.

Table 8. Measured concentrations (ng/mL) using this method in comparison to the certified concentrations in two NIST SRMs (5)

Analyte	SRM 3672 Smoker urine			SRM 3673 non-Smoker urine		
	This method	NIST Certified	Accuracy	This method	NIST Certified	Accuracy
2-NAP	8.182	8.730	93.7%	1.438	1.345	106.9%
1-NAP	36.853	34.400	107.1%	216.064	211.000	102.4%
3-FLU	0.414	0.428	96.8%	0.044	0.039	112.0%
2-FLU	0.720	0.870	82.8%	0.090	0.107	83.7%
2-3PHE	0.201	0.209	96.1%	0.055	0.053	103.0%
1-PHE	0.115	0.136	84.3%	0.048	0.049	98.4%
4-PHE	0.046	0.049	94.2%	0.021	0.010	213.9%*
1-PYR	0.177	0.173	102.2%	0.051	0.030	169.7%*

*: <3LOD.

The accuracy of the method was further assessed by repeated analyses (n=7) of three spiking concentrations in synthetic urine (Table 9).

Table 9. Accuracy

Analyte	Level -1		Level -2		Level -3	
	Spiked (ng/mL)	Accuracy	Spiked (ng/mL)	Accuracy	Spiked (ng/mL)	Accuracy
2-NAP	0.708	112.8%	3.845	99.7%	19.226	99.7%
1-NAP	0.705	113.3%	3.826	95.3%	19.130	98.7%
3-FLU	0.168	107.2%	0.915	98.6%	4.573	98.1%
2-FLU	0.176	107.4%	0.956	99.3%	4.778	100.3%
2-3PHE	0.357	113.1%	1.937	99.4%	9.684	101.9%
1-PHE	0.175	109.8%	0.951	99.4%	4.753	101.2%
4-PHE	0.173	108.5%	0.939	98.3%	4.697	98.2%
1-PYR	0.179	105.5%	0.972	93.6%	4.860	101.8%

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Daily experimental checks are made on the stability of the analytical system. Two QCLs and two QCHs are prepared and placed randomly on each run. The concentrations of the two QCH and two QCL are averaged to obtain one measurement of QCH and QCL for each batch.

b. Quality Control Procedures

1) Individual Sample Quality Checks

Each individual sample will be subjected to a number of quality checks:

- Auto integrations must be reviewed and integrated manually if needed
- The relative retention time (RRT) of each analyte, if detectable, in relation to its respective IS must be within 0.995 – 1.005 (e.g. 0.12 min difference between the native and labeled 1-PYR). Check integration if the RRT falls out of the range. If a peak is present with its retention time out of the limit in

relation to its IS retention time, an analytical interference may prevent the correct measurement of the target analyte, and the result for that analyte is coded as non-reportable (NR).

2) Establishing QC limits

Quality control limits are established by characterizing assay precision with repeated analyses of the QC pools. Different variables are included in the analysis (e.g. multiple analysts and instruments) to capture realistic assay variation over time. The mean, standard deviation (within day and between day), 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. QC characterization statistics for OH-PAH analytes are listed in Table 7. Characterization statistics are pool specific.

3) Quality Control Evaluation

After the completion of a run, the quality control concentrations are evaluated to determine if the run is "in control." The quality control rules apply to the average of the two replicates of each of the QC pools. The quality control results are evaluated according to a multi-rule QC check (6), and standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control.

Two QC pools *per run* with two or more QC results *per pool*

A) If both QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.

B) If 1 of the 2 QC run means is outside a $2S_m$ limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- $4S_m$
b) 3S Rule - Run mean is outside a $3S_m$ limit
c) 2S Rule - Both run means are outside the same $2S_m$ limit
d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

C) If one of the 4 QC individual results is outside a $2S_i$ limit - reject run if:

a) Extreme Outlier – One individual result is beyond the characterization mean +/- $4S_i$

b) R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
S_m = Standard deviation of the run means (the limits are shown on the chart).
S_w = Within-run standard deviation (the limits are not shown on the chart).

If the QC result for an analyte is declared "out of control", the results of that analyte for all study samples analyzed during that run are invalid for reporting.

c. Proficiency Testing (PT)

The in-house proficiency testing (PT) scheme for this method is administered by an in-house PT coordinator. PT samples are prepared in-house by spiking a known amount of standard into a well characterized urine pool and blind-coded by an in-house PT coordinator. PT samples are analyzed twice a year using the same method described for unknown samples.

In addition to the in-house PT program, a minimum of once per year, we also analyze two reference urine samples fortified with 1-NAP, 2-NAP and 1-PYR (as of 2015) as part of the German External Quality Assessment Scheme (G-EQUAS). G-EQUAS is organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). The program, evaluation, and certification are based on the guidelines of the German Federal Medical Council (http://www.g-equas.de/).

All proficiency results shall be appropriately documented. If the assay fails proficiency testing, then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error.

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, failure of the mass spectrometer or a pipetting error, the problem is immediately corrected. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration standards (in the case of

calibration failure). After re-establishing calibration or quality control, resume analytical runs. Document the QC failures, review the situation with the supervisor and/or his/her designee to determine source(s) of problem, and take measures to prevent re-occurrence of the same problem.

12. Limitations of Method, Interfering Substances and Conditions

This is an isotope dilution mass spectrometry method, widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. By using tandem mass spectrometry, most analytical interferences are eliminated. However, unknown endogenous substances may interfere with the chromatographic separation of certain analytes and/or suppress instrument sensitivity, especially when the urine samples are non-fasting or may also contain many other potential substances in addition to the target analytes (e.g., smokers' urine). To overcome inadequate chromatography or analytical sensitivity, dilute the sample and re-inject ("instrument rerun"). If the instrument rerun results are not acceptable, repeat the sample preparation by using up to 100 times dilution. If the diluted analysis still results in an interference that cannot be separated chromatographically or severe suppression of the target analyte signal and/or its IS, the result for that analyte is coded as not reportable (NR).

13. Reference Ranges (Normal Values)

Reference range values for the OH-PAH metabolites, established based on the National Health and Nutrition Examination Survey (NHANES), can be found at http://www.cdc.gov/exposurereport.

14. Critical Call Results ("Panic Values")

Insufficient data exist to correlate urinary OH-PAH concentrations with serious health effects in humans. Therefore, no established "critical call" values exist. Test results in this laboratory are reported in support of epidemiological studies, not for clinical assessments.

15. Specimen Storage and Handling During Testing

Urine specimens may reach and maintain ambient temperature during analysis. The urine extracts are stored in a sample collection plate (when prepared using the Staccato automated method) at -70 °C after analysis. CDC's unpublished data suggest that these extracts are stable for at least one month.

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

A GC-MS/MS method is available on site if necessary for measuring these PAH metabolites in urine (3). Furthermore, aliquoting and spiking of the urine can also be done manually if the Staccato automated system fails. Upon system failures, urine extracts can be refrigerated for up to a week until the analytical system is restored to functionality.

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

Study subject data can be reported both in concentration units (e.g., µg/L, ng/mL, ng/L, pg/mL) and/or adjusted based on creatinine excretion (e.g., µg/g creatinine).

a. The data from each analytical run are initially processed and reviewed by the laboratory supervisor, his/her designee or Quality Control officer to check sample Quality Control parameters (e.g., recovery, relative retention time, blank levels, calibration curve). The supervisor and/or his/her designee can provide feedback to the analyst and request confirmation of the data as needed.
b. The Quality Control officer reviews each analytical run, identifies the quality control samples within each analytical run, and determines whether the analytical run is performed under acceptable control conditions.
c. One of the Division statisticians reviews and approves the quality control charts pertinent to the results being reported.
d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a memorandum to the Branch Chief, and a letter reporting the analytical results to the person(s) who requested the analyses to be signed by the Division Director.
e. The data are sent (generally electronically by e-mail) to the person(s) who made the initial request.

Final hard copies of correspondence are maintained in the office of the Branch Chief and/or his/her designee and/or with the quality control officer.

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

Following successful completion of analysis, the urine must be returned to storage at -70 °C in case reanalysis is required. Urine samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results. Residual urine may be returned to the study PI or properly discarded upon completion of the project.

Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Specimens can be transferred or referred to other DLS Branch laboratories or, if required, to other laboratories. Transfer is normally carried out through the DLS Samples Logistic Group. Specimens may also be stored at CDC specimen handling and storage facility (CASPIR).

19. References

1. IARC. 2010, vol 92 IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Non-heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures. http://monographs.iarc.fr/ENG/Monographs/vol92/mono92.pdf.

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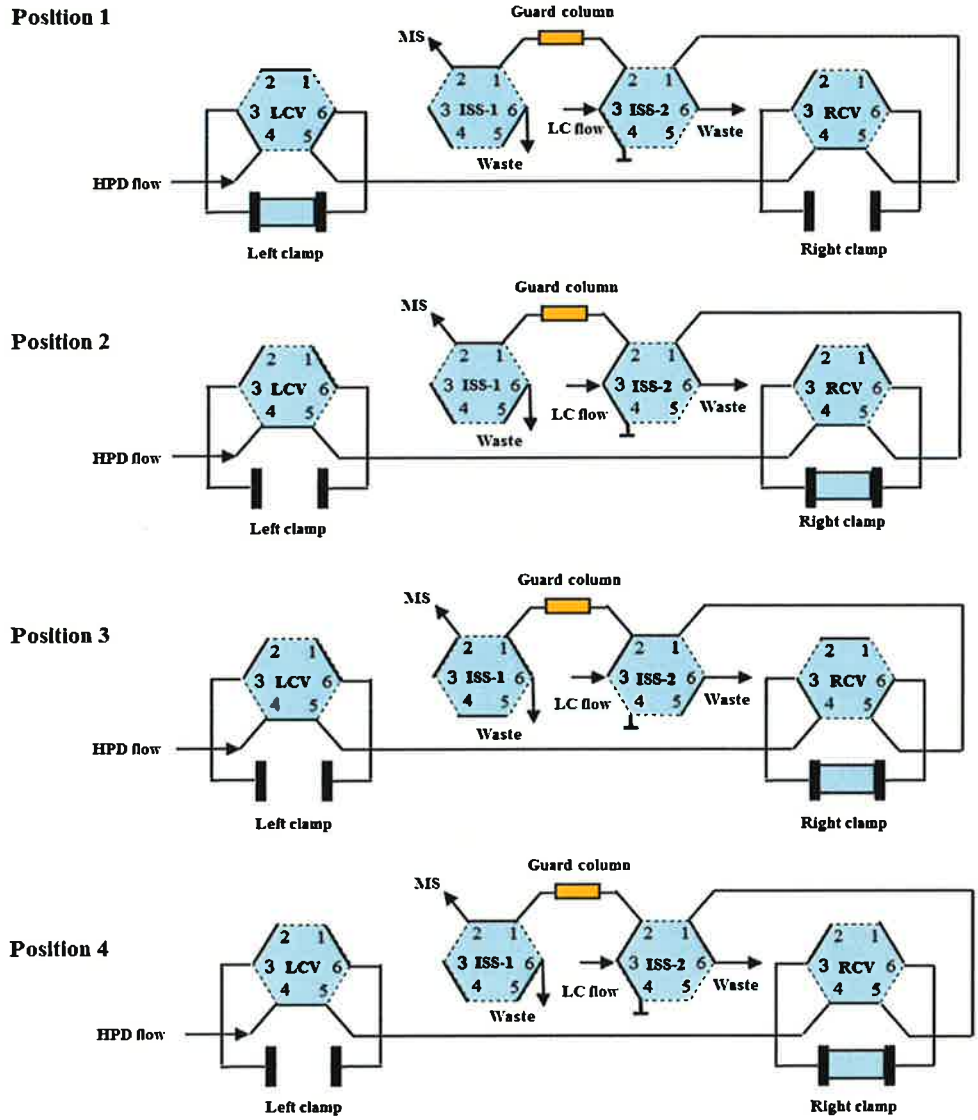
Appendix A – SPE tubing and valve switching system



Position 1: The Cartridge is activated at the left clamp with 4 mL acetonitrile and equilibrated with 2 mL 0.1% formic acid in water. Sample solution (50-500 µL) is injected with 1.5 mL 0.1% formic acid in water, followed by wash with 1.5 mL acetonitrile/methanol/water (25/25/50, v/v/v) (the actual wash solution is ~0.4 mL by counting the volume of sample loops).

Position 2: The cartridge is moved to the right clamp. Methanol (2 mL) is used to clean the loop.

Position 3: Methanol (350 µL) is used to elute out the analytes from the cartridge to the guard column (pre-column). The HPLC flow passes the guard column at the same time to focus the analytes onto the guard column.

Position 4: Methanol (2 mL) is used to clean the tubing system.

Appendix B – Ruggedness Testing

Procedure

Ruggedness testing was conducted to evaluate 5 parameters: enzyme amount, buffer strength, buffer pH value, deconjugation time and deconjugation temperature. For each parameter, 3 or 5 levels were tested, including 1 or 2 lower level(s), 1 or 2 higher level(s), and the method level. An anonymous urine pool was used in the ruggedness experiments. Samples were run in triplicate. Reported below are results on the major detectable OH-PAH analytes.

Results in Tables

Enzyme amount (mg/sample)		2-NAP	1-NAP	3-FLU	2-FLU	2-3PHE	1-PHE	4-PHE	1-PYR
		average concentration (ng/mL)							
Lower level1	5mg	38.39	32.66	1.77	3.06	3.29	1.70	0.39	1.29
Lower level2	9mg	37.56	33.01	1.76	3.06	3.23	1.68	0.40	1.27
Method	10mg	37.91	33.09	1.76	3.09	3.24	1.70	0.48	1.27
Higher level1	11mg	39.54	33.91	1.77	3.06	3.28	1.70	0.49	1.29
Higher level2	20mg	38.38	33.50	1.79	3.06	3.28	1.69	0.49	1.28
		standard deviation							
Lower level 1	5mg	0.43	0.02	0.00	0.02	0.03	0.01	0.01	0.01
Lower level 2	9mg	0.25	0.10	0.03	0.02	0.01	0.01	0.01	0.01
Method	10mg	0.18	0.65	0.01	0.04	0.03	0.00	0.00	0.01
Higher level1	11mg	0.28	1.12	0.03	0.03	0.04	0.02	0.01	0.02
Higher level2	20mg	0.31	0.31	0.01	0.02	0.01	0.01	0.00	0.03

Buffer strength (M)		2-NAP	1-NAP	3-FLU	2-FLU	2-3PHE	1-PHE	4-PHE	1-PYR
		average concentration (ng/mL)							
Lower level1	0.5M	35.59	31.60	1.73	3.09	3.21	1.73	0.49	1.22
Lower level2	0.9M	36.07	31.71	1.75	3.03	3.23	1.71	0.45	1.23
Method	1M	35.63	31.29	1.75	3.07	3.22	1.69	0.44	1.23
Higher level1	1.1M	35.52	31.31	1.75	3.04	3.21	1.71	0.37	1.25
Higher level2	1.5M	36.01	32.17	1.75	3.07	3.20	1.75	0.37	1.23
		standard deviation							
Lower level1	0.5M	0.53	0.51	0.03	0.04	0.04	0.04	0.04	0.02
Lower level2	0.9M	1.43	1.70	0.02	0.02	0.05	0.06	0.03	0.02
Method	1M	0.58	0.54	0.02	0.05	0.07	0.03	0.02	0.02
Higher level1	1.1M	0.87	0.81	0.03	0.04	0.05	0.01	0.01	0.01

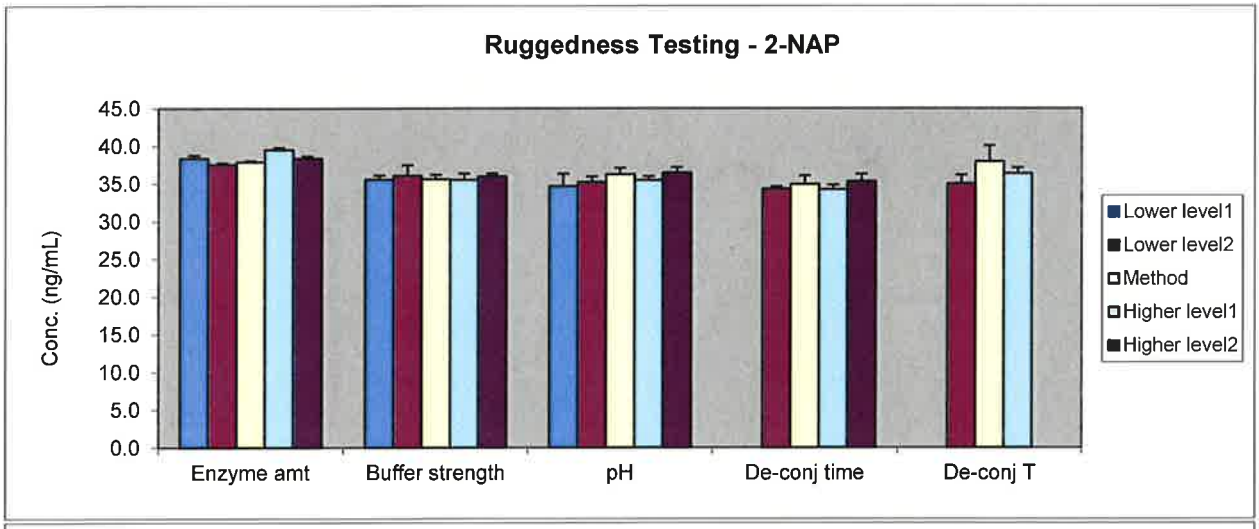
Higher level2	1.5M	0.35	1.10	0.02	0.08	0.06	0.04	0.02	0.02

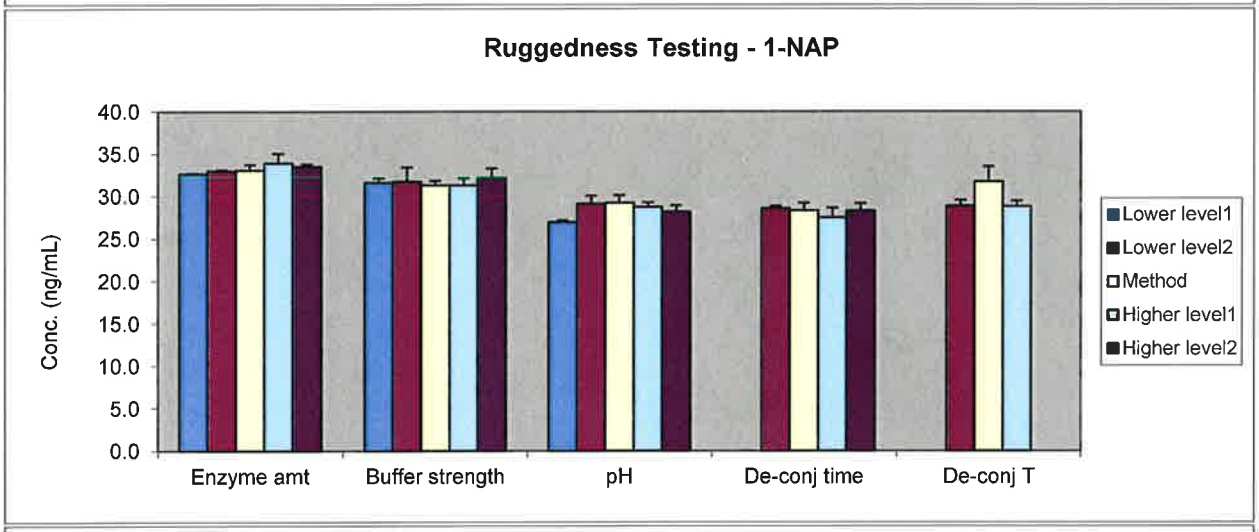
Buffer pH		2-NAP	1-NAP	3-FLU	2-FLU	2-3PHE	1-PHE	4-PHE	1-PYR
		average concentration (ng/mL)							
Lower level1	pH4.5	34.66	26.97	1.77	2.94	3.25	1.70	0.44	1.19
Lower level2	pH5.3	35.25	29.12	1.75	3.06	3.20	1.67	0.40	1.29
Method	pH5.5	36.27	29.22	1.75	3.10	3.26	1.70	0.47	1.26
Higher level1	pH5.7	35.47	28.72	1.73	3.02	3.22	1.63	0.44	1.21
Higher level2	pH6.5	36.46	28.17	1.79	3.11	3.27	1.63	0.44	1.24
		standard deviation							
Lower level1	pH4.5	1.67	0.18	0.12	0.01	0.02	0.02	0.02	0.03
Lower level2	pH5.3	0.72	0.92	0.02	0.03	0.05	0.02	0.05	0.02
Method	pH5.5	0.83	0.91	0.01	0.05	0.04	0.03	0.00	0.04
Higher level1	pH5.7	0.51	0.55	0.04	0.08	0.08	0.03	0.01	0.01
Higher level2	pH6.5	0.73	0.76	0.07	0.08	0.05	0.05	0.03	0.03

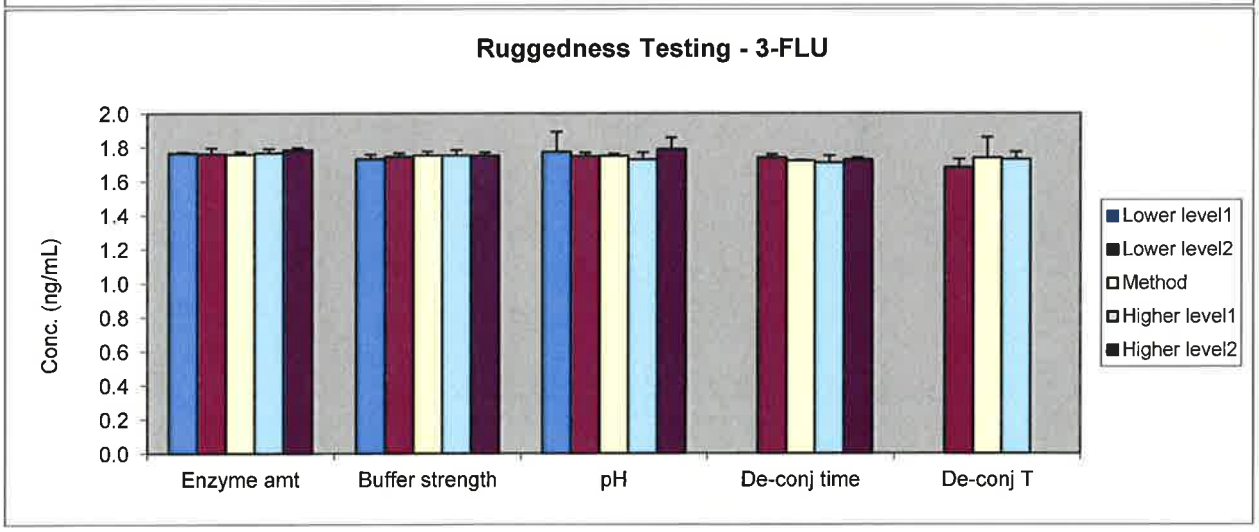
De-conjugation time (hours)		2-NAP	1-NAP	3-FLU	2-FLU	2-3PHE	1-PHE	4-PHE	1-PYR
		average concentration (ng/mL)							
Lower level2	17 hr	34.33	28.59	1.74	2.98	3.00	1.63	0.43	1.23
Method	18 hr	34.94	28.29	1.72	2.95	3.02	1.60	0.41	1.21
Higher level1	19 hr	34.27	27.46	1.71	2.95	2.99	1.59	0.41	1.20
Higher level2	24 hr	35.31	28.28	1.73	2.98	3.06	1.63	0.33	1.21
		standard deviation							
Lower level2	17 hr	0.33	0.26	0.02	0.04	0.04	0.02	0.01	0.04
Method	18 hr	1.16	0.92	0.00	0.01	0.03	0.01	0.01	0.00
Higher level1	19 hr	0.58	1.15	0.04	0.09	0.06	0.02	0.02	0.03
Higher level2	24 hr	0.98	0.86	0.01	0.03	0.05	0.03	0.00	0.01

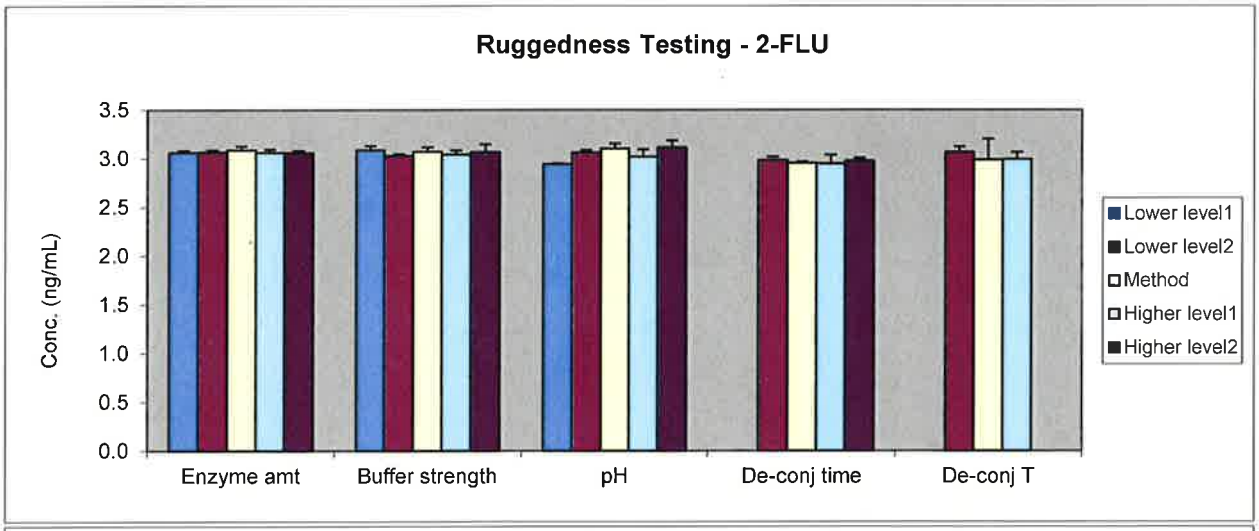
Hydrolysis temperature (°C)		2-NAP	1-NAP	3-FLU	2-FLU	2-3PHE	1-PHE	4-PHE	1-PYR
		average concentration (ng/mL)							
Lower Level	32°C	35.03	28.84	1.68	3.07	3.11	1.64	0.49	1.22
Method	37°C	37.94	31.71	1.74	2.98	3.00	1.57	0.42	1.20
Higher Level	42°C	36.39	28.79	1.73	2.99	3.15	1.60	0.45	1.14
		standard deviation							
Lower Level	32°C	1.13	0.69	0.05	0.06	0.04	0.01	0.01	0.03
Method	37°C	2.14	1.77	0.12	0.21	0.18	0.11	0.03	0.07
Higher Level	42°C	0.73	0.65	0.04	0.07	0.07	0.04	0.02	0.03

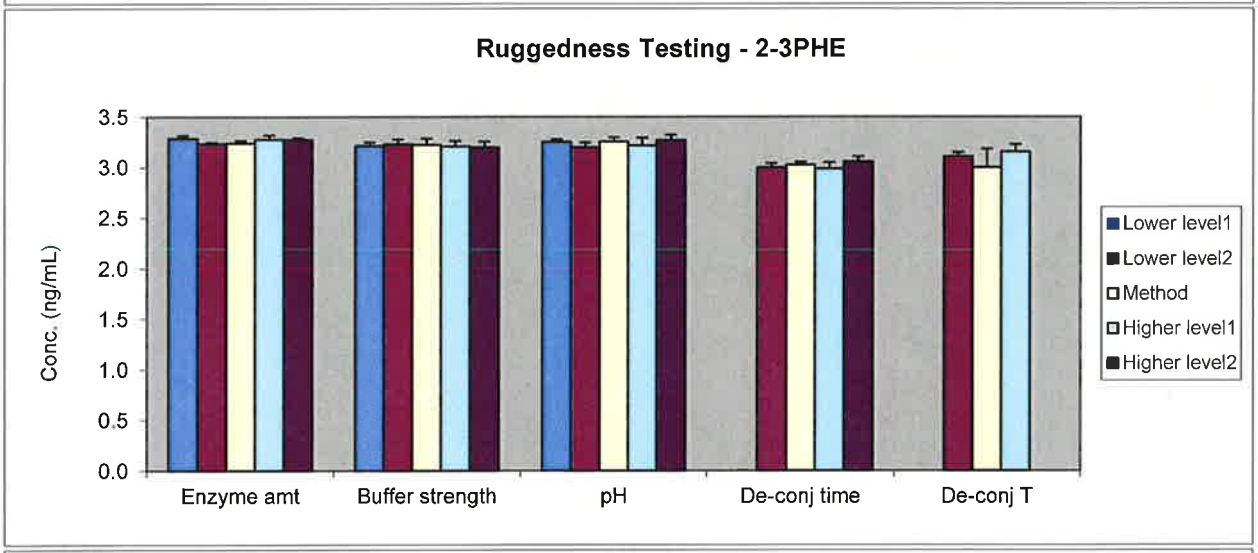
Results in Graphs

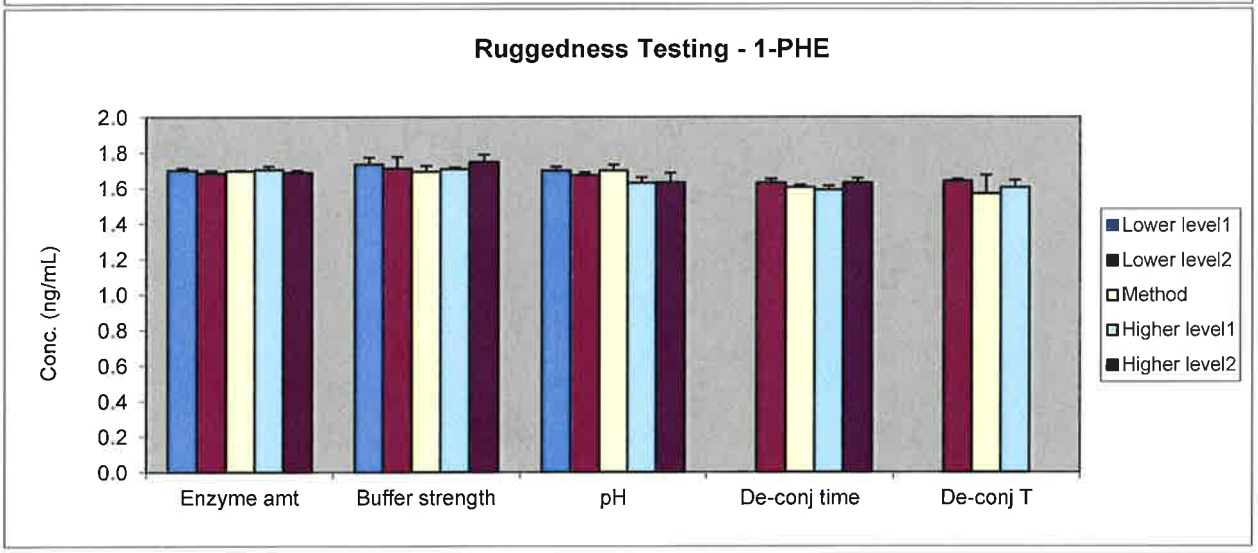


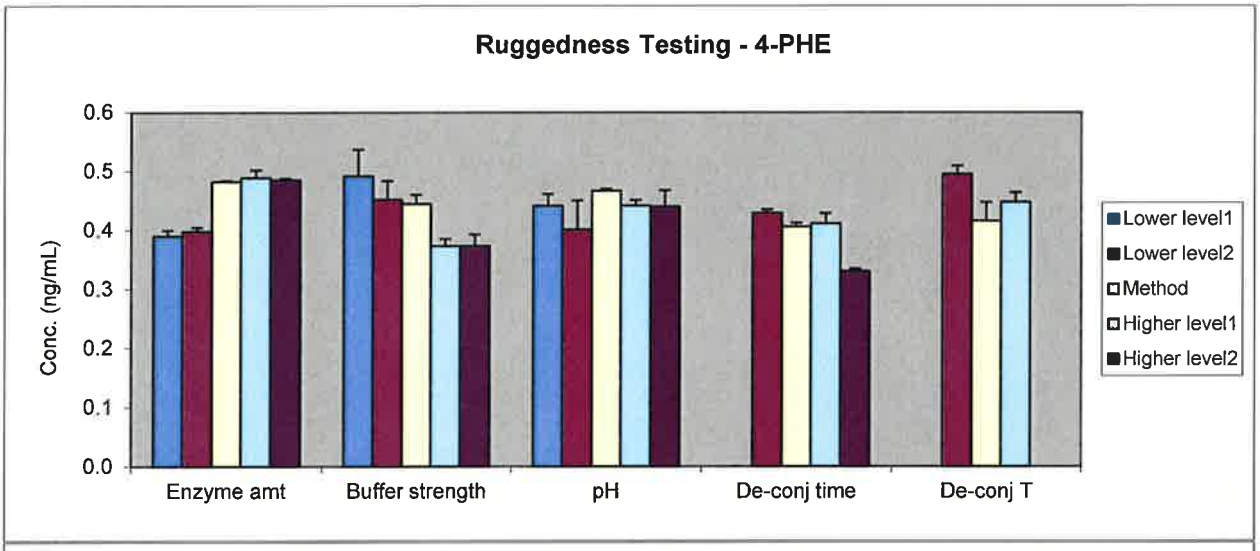


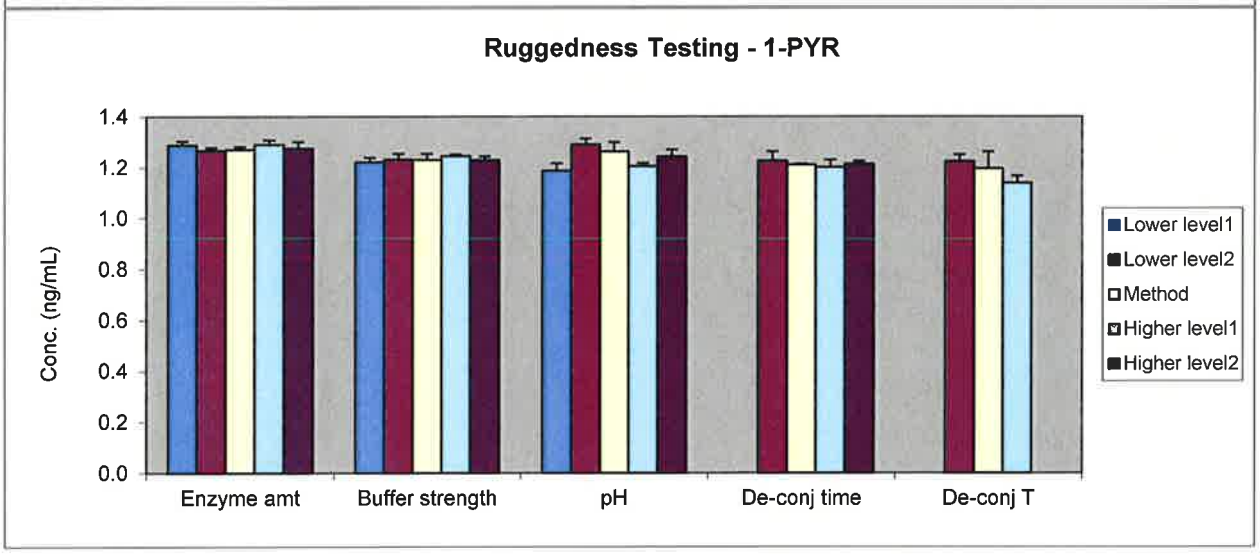



Ruggedness Testing - 2-FLU
Conc. (ng/mL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Lower level1
Lower level2
Method
Higher level1
Higher level2


Ruggedness Testing - 2-3PHE
Conc. (ng/mL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Lower level1
Lower level2
Method
Higher level1
Higher level2


Ruggedness Testing - 1-PHE
Conc. (ng/mL)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Lower level1
Lower level2
Method
Higher level1
Higher level2


Ruggedness Testing - 4-PHE
Conc. (ng/mL)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Lower level1
Lower level2
Method
Higher level1
Higher level2

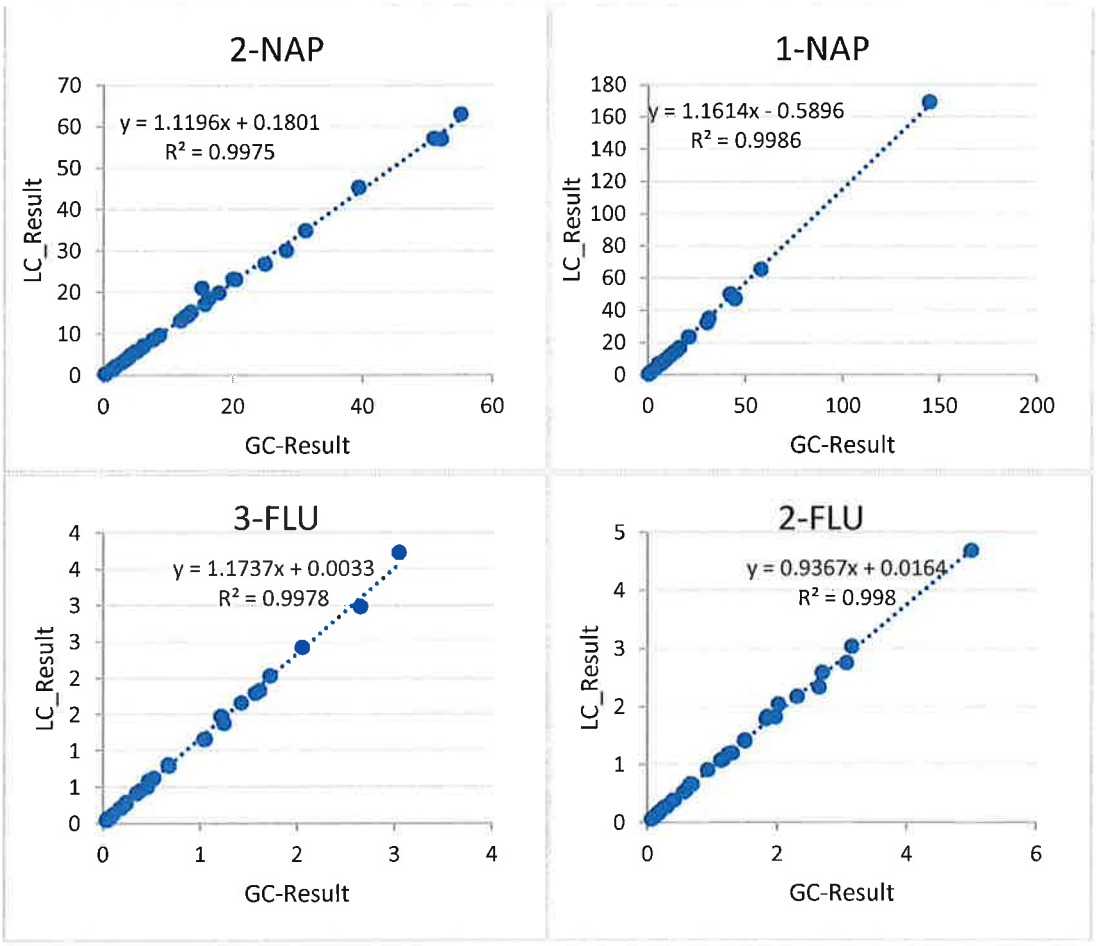

Ruggedness Testing - 1-PYR
Conc. (ng/mL)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Lower level1
Lower level2
Method
Higher level1
Higher level2

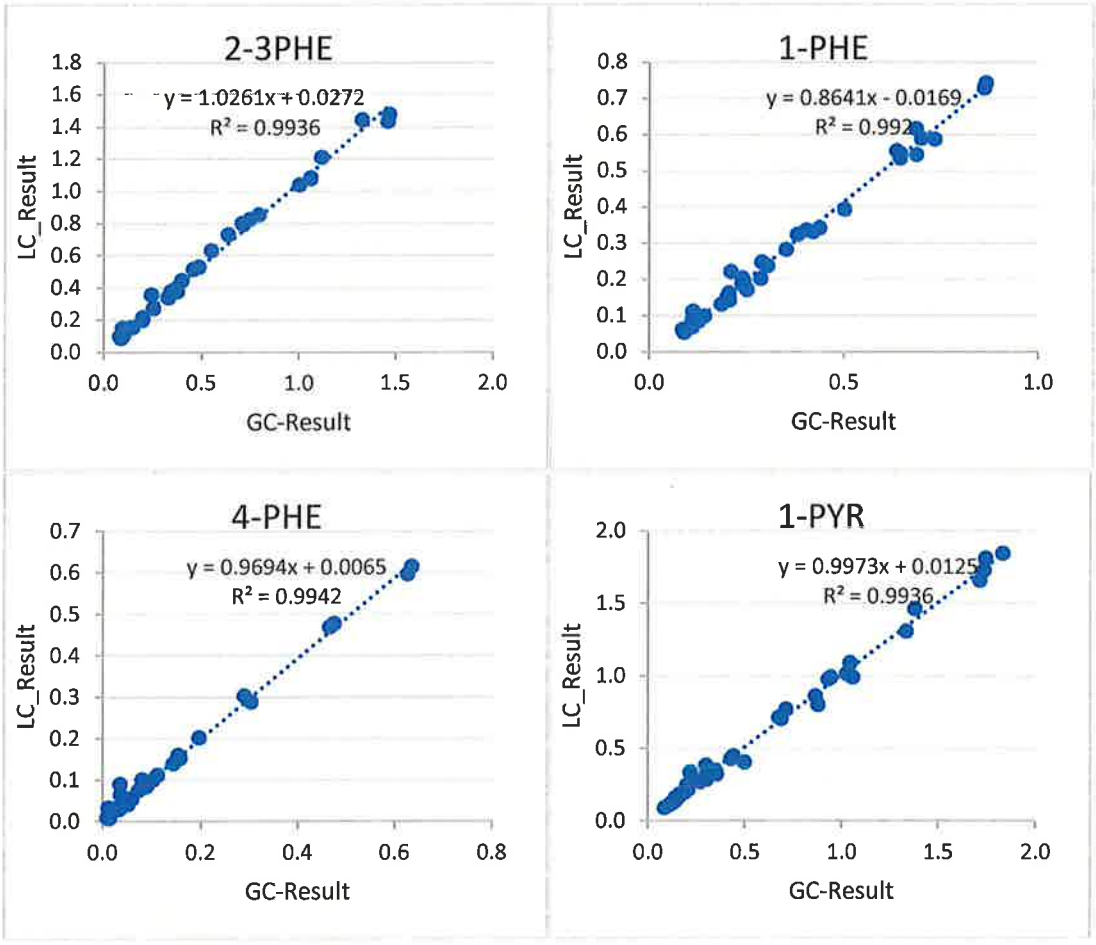
Appendix C – Method Comparison Results

Method comparison was performed by analyzing samples using the previous method by Agilent 7000 GC/MS/MS (Method #6703.04). The results are evaluated by both linear regression plots between the previous ("GC") and current ("LC") results, as well as Bland-Altman plot analysis (e.g., graphs of differences between the results from the two methods against the mean of the two results). A total of 38 samples were included in the linear regression analysis.

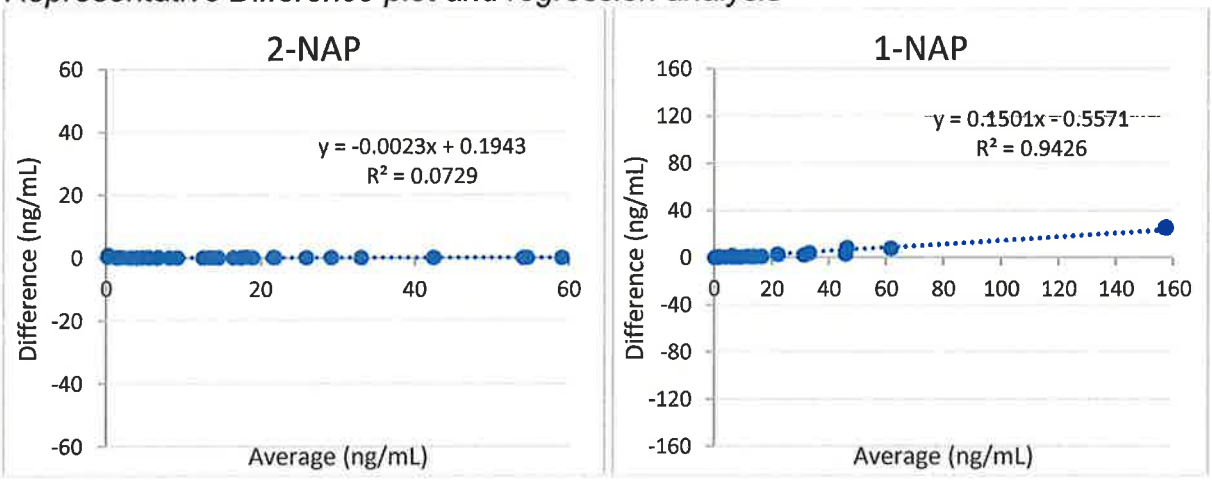
PLOTS

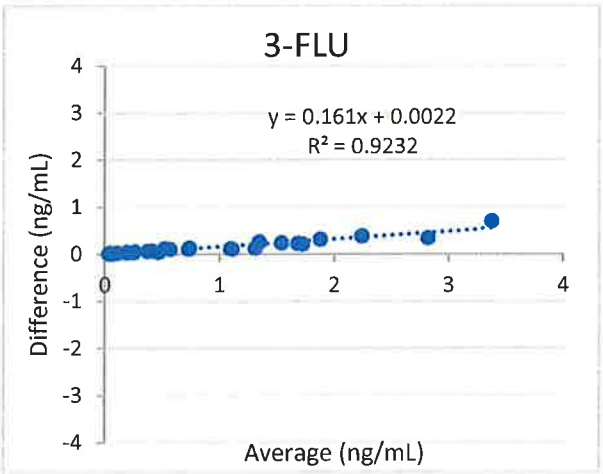
Representative Correlation plot and regression analysis

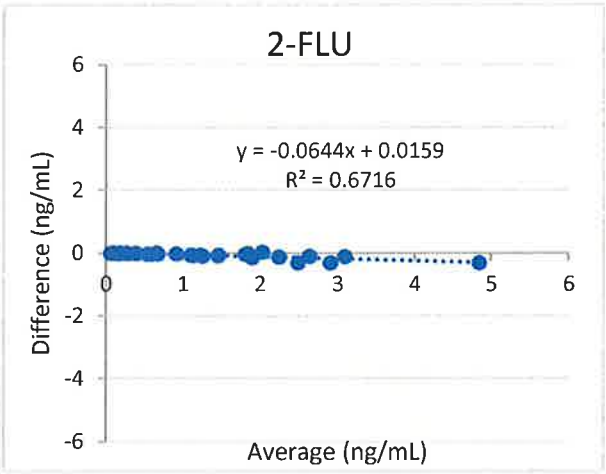


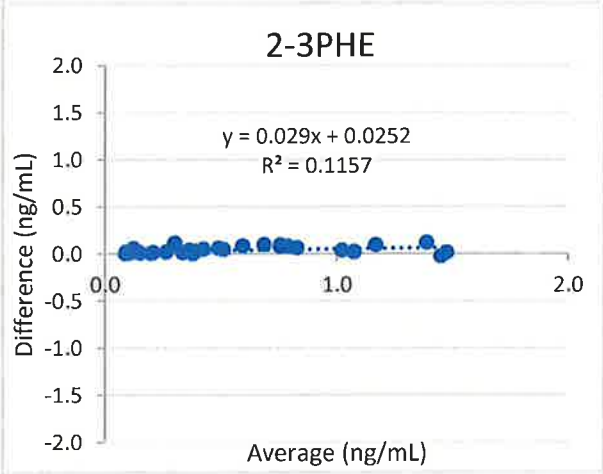


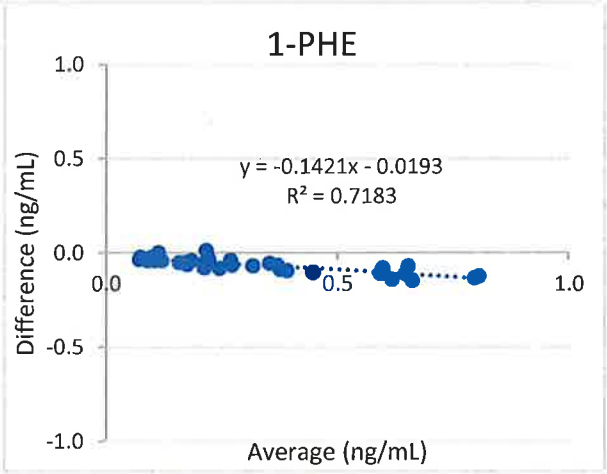
Representative Difference plot and regression analysis

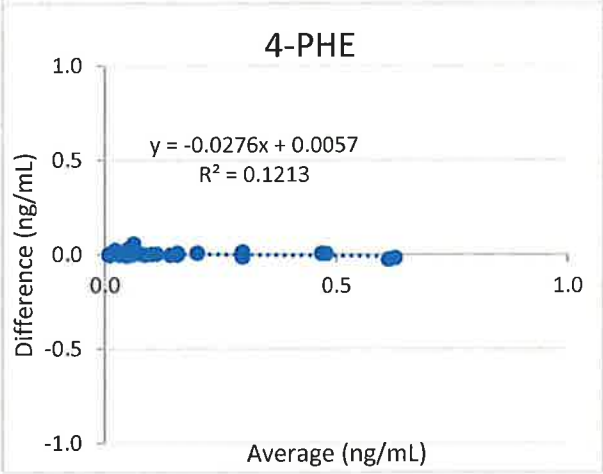


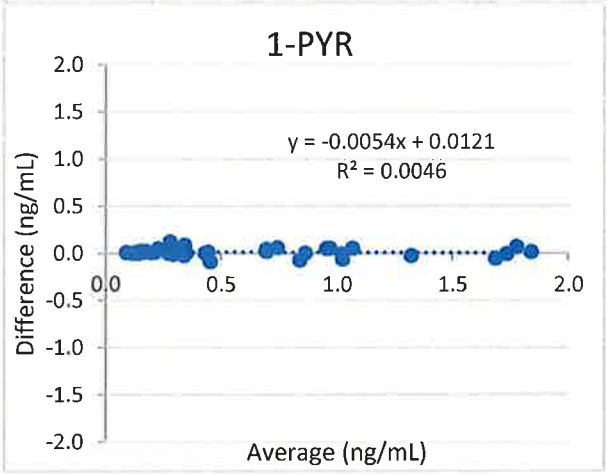

3-FLU
y = 0.161x + 0.0022
R² = 0.9232
Difference (ng/mL)
Average (ng/mL)


2-FLU
y = -0.0644x + 0.0159
R² = 0.6716
Difference (ng/mL)
Average (ng/mL)


2-3PHE
y = 0.029x + 0.0252
R² = 0.1157
Difference (ng/mL)
Average (ng/mL)


1-PHE
y = -0.1421x - 0.0193
R² = 0.7183
Difference (ng/mL)
Average (ng/mL)


4-PHE
y = -0.0276x + 0.0057
R² = 0.1213
Difference (ng/mL)
Average (ng/mL)


1-PYR
y = -0.0054x + 0.0121
R² = 0.0046
Difference (ng/mL)
Average (ng/mL)

Laboratory Procedure Manual

Analyte:	**Polyfluoroalkyl Substances: 2-(N-methyl-perfluorooctane sulfonamido) acetate, perfluorobutane sulfonate, perfluorohexane sulfonate, n-perfluorooctane sulfonate, sum of perfluoromethylheptane sulfonate isomers, sum of perfluorodimethylhexane sulfonate isomers, perfluoroheptanoate, n-perfluorooctanoate, sum of branched perfluorooctanoate isomers, perfluorononanoate, perfluorodecanoate, perfluoroundecanoate, and perfluorododecanoate**
Matrix:	**Serum**
Method:	**Online Solid Phase Extraction-High Performance Liquid Chromatography-Turbo Ion Spray-Tandem Mass Spectrometry (online SPE-HPLC-TIS-MS/MS)**
Method No:	**6304.06**

As performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

Contact:

Xiaoyun (Sherry) Ye, M.S.
Phone: 770.488.7502
Email: XYe@cdc.gov

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

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 (and SI units)
PFAS_H	LBXPFDE	Perfluorodecanoic acid (ng/mL)
	LBXPFHS	Perfluorohexane sulfonic acid (ng/mL)
	LBXMPAH	2-(N-methyl-PFOSA) acetic acid (ng/mL)
	LBXPFBS	Perfluorobutane sulfonic acid (ng/mL)
	LBXPFHP	Perfluoroheptanoic acid (ng/mL)
	LBXPFNA	Perfluorononanoic acid (ng/mL)
	LBXPFUA	Perfluoroundecanoic acid (ng/mL)
	LBXPFDO	Perfluorododecanoic acid (ng/mL)
SSPFAS_H	SSNPFOA	Linear perfluorooctanoate (ng/mL)
	SSBPFOA	Branched isomers of perfluorooctanoate (ng/mL)
	SSNPFOS	Linear perfluorooctane sulfonate (ng/mL)
	SSMPFOS	Monomethyl branched isomers of PFOS (ng/mL)

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Some per- and polyfluoroalkyl substances (PFASs), including perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), persist in humans and the environment and have been detected worldwide in wildlife [1]. Exposure to PFOS and PFOA in the general population is also widespread, although demographic, geographic, and temporal differences exist [2-14]. In animals, exposure to PFOS and PFOA is associated with adverse health effects [15-17] albeit at serum concentrations orders of magnitude higher than the concentrations observed in the general population [18,19]. PFOS was used in a wide variety of industrial and consumer products including protective coatings for carpets and apparel, paper coatings, insecticide formulations, and surfactants. In 2000, 3M, the sole manufacturer of PFOS in the United States and the principal manufacturer worldwide, announced that it was discontinuing its perfluorooctanyl chemistries, including PFOS. Shortly after, EPA also identified possible related concerns with respect to PFOA and fluorinated telomers. PFOA has been used primarily to produce its salts which are used in the production of fluoropolymers and fluoroelastomers. These polymers are used in many industrial and consumer products, including soil, stain, grease, and water resistant coatings on textiles and carpet; uses in the automotive, mechanical, aerospace, chemical, electrical, medical, and building/construction industries; personal care products; and non-stick coatings on cookware.

The electrochemical fluorination (ECF) manufacturing method used from the 1950s until the early 2000s to produce PFASs including PFOA, and PFOS and its precursors yielded branched and linear isomers. By contrast, another method, telomerization, produces almost exclusively linear compounds [20]. The structural isomer patterns of PFOA and PFOS in humans may be useful for understanding routes and sources of exposure [20].

b. Test Principle

Online solid phase extraction coupled to high performance liquid chromatography-turboionspray ionization-tandem mass spectrometry (online SPE-HPLC-TIS-MS/MS) is used for the quantitative detection of PFASs: 2-(N-methyl-perfluorooctane sulfonamido) acetate (Me-PFOSA-AcOH), perfluorobutane sulfonate (PFBuS), perfluorohexane sulfonate (PFHxS), n-perfluorooctane sulfonate (n-PFOS), sum of perfluoromethylheptane sulfonate isomers (Sm-PFOS, monomethyl branched isomers of PFOS), sum of perfluorodimethylhexane sulfonate isomers (Sm2-PFOS, dimethyl branched isomers of PFOS), perfluoroheptanoate (PFHpA), n-perfluorooctanoate (n-PFOA), sum of branched perfluorooctanoate isomers (Sb-PFOA, branched PFOA isomers), perfluorononanoate (PFNA), perfluorodecanoate (PFDeA), perfluoroundecanoate (PFUA), and perfluorododecanoate (PFDoA)[21]. Briefly, after dilution with formic acid, one aliquot of 50 µL of serum is injected into a commercial column switching system allowing for concentration of the analytes on solid-phase extraction column. Separation of the analytes from each other and from other serum components is achieved with high-performance liquid

chromatography. Detection and quantification are done using negative-ion TurboIonSpray ionization, a variant of electrospray ionization, tandem mass spectrometry. This method allows for rapid detection of these PFASs in human serum with limits of detection in the low parts per billion (ppb or ng/mL) range.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents used are toxic. Special care should be taken to: 1) Avoid contact with eyes and skin, 2) avoid use of the organic solvents in the vicinity of an open flame, and 3) use solvents only in well-ventilated areas.

Note: Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at www.ilpi.com/msds/index.html; some of them may be found in a binder in the laboratory. Laboratory personnel are advised to review the MSDS before using chemicals.

Care should be exercised in the handling of all chemical standards.

b. Radioactive Hazards

None.

c. Microbiological Hazards

The possibility of being exposed to various microbiological hazards exists. Appropriate measures (i.e., universal precautions) should be taken to avoid any direct contact with biological specimens (i.e., use gloves, laboratory coats, safety glasses, chemical or biological hoods). Any residual biological material should be appropriately discarded and prepared for autoclaving after analysis is completed. All disposable laboratory supplies must also be placed in an autoclave bag for disposal. The Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel who handles human fluids and tissues is required to take the “Bloodborne Pathogens Training” course offered at CDC to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should avoid any direct contact with the electronics of the mass spectrometer, unless all power to the instrument is off. Generally, only qualified technicians should perform the electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer (e.g., interface) should be avoided.

e. Protective Equipment

Standard safety protective equipment should be utilized when performing this procedure. This includes lab coat, safety glasses, durable gloves (e.g., nitrile or vinyl), and/or a chemical fume hood or biological safety cabinet.

f. Training

Training and experience in the use of a triple quadrupole mass spectrometer and the on-line SPE extractor should be obtained by anyone using this procedure. Operators are required to read the operation manuals or laboratory SOP. Formal training is not necessary; however, an experienced user should train all of the operators.

g. Personal Hygiene

Care should be taken in handling any biological specimen. Routine use of gloves and proper hand washing should be practiced. No food or drink is allowed in laboratory areas.

h. Disposal of Wastes

Solvents and reagents are disposed of in an appropriate container clearly marked for waste products and temporarily stored in one of the chemical fume hoods. Containers, glassware, etc., that come in direct contact with the specimen are either autoclaved or decontaminated with 10% bleach. Contaminated analytical glassware is treated with bleach, washed and reused; disposable labware is autoclaved before disposal. To insure proper compliance with CDC requirements, laboratory personnel are required to attend annual hazardous waste disposal courses.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

All samples are queued for analysis in a database created using Microsoft Access. Mass spectrometry data are collected and stored using the Analyst Software of the ABI 5500 and ABI 6500 Qtrap mass spectrometers. During sample preparation and analysis, samples are identified by their Sample Name and Sample ID. The Sample Name is used to identify each specimen and links the laboratory information with the demographic data recorded by the sample takers. The Sample ID is used to identify each specimen and links the laboratory information with the demographic data recorded by the sample takers. In case of repeated measurements, one specimen in the database may have more than one Sample Name, but only one Sample ID. All raw data files are processed using the Analyst software and are archived for future reference. The Analyst software selects the appropriate peak based on the precursor/product ion combination and chromatographic retention time and subsequently integrates the peak area. It also allows manual peak selection and area integration. The raw data (peak area, peak height, retention time, analyte name, MRM transition name) are exported to the Access database used for storage and retrieval of data. The Access database is stored on a network drive; it may also be backed up in additional archive locations. Statistical analysis of the data, programming, and reporting are performed using the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Knowledge and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b. Sample Information

Sample names and Sample IDs are entered into the Access database before sample preparation. If possible, for unknown samples, sample study IDs are read in by a barcode reader directly from the vials labels. Sample names for Standards, and Blanks (SBs, HSBs, QCBs) are entered manually. The Sample Log Sheet, containing Sample Names, Sample IDs, and sample study IDs is printed from the Access database and is used to record information during sample preparation. Sample Names, Sample IDs, and sample study IDs are exported as tab delimited text files from the Access database and imported into the Acquisition Batch table (*.dab) of the Analyst program on the mass spectrometer. After MS data collection and peak integration, data are saved as a tab delimited file and imported into the Access database. Further manipulation of the data, including QC evaluation and statistical analyses, are performed using SAS statistical software. After any additional calculations or corrections by the analyst are completed and the reviewing supervisor approves the final values for release, a comma-delimited file (SAS output) is generated.

c. Data Maintenance

Raw files are regularly backed up onto an external hard drive. Sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely backed up onto a computer hard drive and onto a network drive. Data from completed studies are saved on an external hard drive and/or a network drive. Additionally, paper copies of signed final report memos are scanned and saved as official government records.

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

a. Sample Collection and Storage

Follow recommended phlebotomy practices for the collection of blood and separation of blood serum. Preferably, a minimum of 0.5 mL of serum (plasma may also be used) should be placed in standard collection containers, refrigerated as soon as possible, and transferred to labeled containers for storage. Sera should be stored frozen preferably in polypropylene or polyethylene containers. Glass containers may be used if the specimens are to be analyzed for other environmental chemicals for which storage in plastic may be a problem. Teflon® coated materials should be avoided.

b. Sample Handling

In general, serum specimens should be shipped or transported cold (dry ice, ice or blue ice can be used). Special care must be taken in packing to protect vials from breakage during shipment.

Before analysis, samples are thawed, vortexed, aliquoted, and the residual specimen is refrozen and stored. The integrity of samples thawed and refrozen several times doesn’t appear to be compromised.

c. Criteria for Specimen Rejection

Specimens can be rejected if tubes/vials leaked, are broken, appear compromised or tampered with, or hold inadequate volume for analysis.

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

Not applicable for this procedure.

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

a. Reagents and Sources

Methanol (MeOH), acetonitrile, and water were HPLC grade purchased from Honeywell Burdick & Jackson (Muskegon, MI). Formic acid (99%) was purchased from EM Science (Gibbstown, NJ). Acetic acid (glacial) was purchased from J.T. Baker (Phillipsburg, NJ). The following PFASs were purchased form Wellington Laboratories (Guelph, ON, Canada): N-methylperfluoro-1-octanesulfonamidoacetic acid (Me-PFOSA-AcOH), sodium perfluoro-1-hexanesulfonate (PFHxS), potassium perfluoro 1-butanesulfonate (PFBuS), sodium perfluoro 1-octanesulfonate (n-PFOS), mixture of sodium perfluoro-5-methylheptane sulfonate (P5MHpS) and perfluoro-5-methylheptanoic acid (P5MHpA), mixture of sodium perfluoro-5,5-dimethylhexane sulfonate (P55DMHxS) and perfluoro-5,5-dimethylhexanoic acid (P55DMHxA), perfluoroheptanoic acid (PFHpA), ammonium perfluorooctanoate (n-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA). Perfluoro-n-[1,2,3,4,5-^{13}C]-heptanoic acid ($^{13}C_5$-PFHpA), perfluoro-n-[1,2,3,4-^{13}C]-octanoic acid ($^{13}C_4$-PFOA), perfluoro-n-[1,2,3,4,5-^{13}C]-nonanoic acid ($^{13}C_5$-PFNA), 2-Perfluorooctyl [1,2-^{13}C]-ethanoic acid ($^{13}C_2$-PFDeA), 2-perfluorooctyl [1,2-^{13}C]-undecanoic acid ($^{13}C_2$-PFUA), perfluoro-n-[1,2-^{13}C]-dodecanoic acid ($^{13}C_2$-PFDoA), N-methyl-d_3-perfluoro-1-octanesulfonamide acetic acid (D_3-Me-PFOSA-AcOH), and sodium perfluoro 1-hexane [$^{18}O_2$]-sulfonate ($^{18}O_2$-PFHxS), sodium perfluoro 1-[1,2,3,4-^{13}C]-octanesulfonate ($^{13}C_4$-PFOS) were purchased form Wellington Laboratories. All reagents were used without further purification. Other standards and reagents with similar specifications may be used.

b. Working Solutions

(1) HPLC Mobile Phase, 20mM Ammonium Acetate Buffer/acetonitrile (95:5), pH 4.

To prepare 20 mM Ammonium acetate buffer (pH4.0), dilute 1140 µL of concentrated acetic acid with approximately 800 mL water in a beaker. Adjust pH to 4±0.1 by adding drop-wise 1:10 ammonium hydroxide:water mixture. Transfer into a 1 L volumetric flask and fill up to volume with deionized water.

Mix 950 mL of ammonium acetate buffer with 50 mL of acetonitrile in a glass bottle. Prepare as needed and store at room temperature.

(2) HPLC Organic Mobile Phase, 100% HPLC acetonitrile

Refill as needed and store at room temperature.

(3) Organic solvent for SPE column regeneration, 100% Acetonitrile

Refill as needed and store at room temperature.

(4) Solid phase extraction (SPE) Acid Wash Solution, 0.1M formic acid

Dilute 3810 µL of 99% concentrated formic acid with water to 1000 mL in a volumetric cylinder. Prepare monthly and store at room temperature.

c. Standards Preparation

(1) Analytical Calibration Standards

The native standard stock solutions of all the analytes are prepared in methanol from the commercial solutions. The concentrations of the commercial solutions are: 50 µg/mL for Me-PFOSA-AcOH and n-PFOS; 2 µg/mL for PFHxS, PFBuS, n-PFOS, PFHpA, n-PFOA, PFNA, PFDeA, PFUA, and PFDoA; 1 µg/mL for P5MHpS and P55DMHxS; 1.96 µg/mL for P5MHpA and P55DMHxA. We used P5MHpS to quantify Sm-PFOS and P55DMHxS to quantify Sm2-PFOS. We used the combined response of the P5MHpA and P55DMHxA standards for the quantitation of Sb-PFOA. The PFOA isomers known to be included in Sb-PFOA are perfluoro-3-methylheptanoic acid, perfluoro-4-methyheptanoic acid, perfluoro-5-methyheptanoic acid, perfluoro-6-methyheptanoic acid, perfluoro-4,4-dimethylhexanoic acid, perfluoro-5,5-dimethylhexanoic acid, perfluoro-3,5-dimethylhexanoic acid, and perfluoro-4,5-dimethylhexanoic acid. Similarly, the PFOS isomers known to be included in Sm-PFOS are perfluoro-3-methylheptane sulfonate, perfluoro-4-methylheptane sulfonate, perfluoro-5-methylheptane sulfonate, perfluoro-6-methylheptane sulfonate. The PFOS isomers known to be included in Sm2-PFOS are perfluoro-4,4-dimethylhexane sulfonate, perfluoro-5,5-dimethylhexane sulfonate, perfluoro-4,5-dimethylhexane sulfonate, and perfluoro-3,5-dimethylhexane sulfonate.

The spiking standard solutions are prepared in MeOH from native standard stock solutions such as a 50-µL spike into 50 µL serum provides concentrations that cover the linear range of the method (Table 1). The spiking solutions are stored frozen in 1.0 mL aliquots in polypropylene cryogenic vials until use.

Table 1. Concentrations of standards #1-9 (in μg/mL)

Standard No	n-PFOS	Sm-PFOS (P5MHpS)	Sm2-PFOS (P55DMHxS)	PFHxS	PFBuS	Sb-PFOA (P5MHpA+ P55DMHxA)	All other analytes
Standard 1	0.015	0.01	0.005	0.005	0.004	0.0294	0.005
Standard 2	0.06	0.05	0.01	0.009	0.009	0.118	0.01
Standard 3	0.15	0.1	0.05	0.05	0.043	0.294	0.05
Standard 4	0.60	0.25	0.1	0.095	0.086	0.686	0.1
Standard 5	1.5	0.50	0.2	0.47	0.428	1.37	0.5
Standard 6	6.0	1.0	0.2	0.945	0.855	2.45	1.0
Standard 7	15.0	2.5	0.5	4.70	4.28	5.88	5.0
Standard 8	60.0	5.0	1.00	9.45	8.55	11.8	10.0
Standard 9	115.	10.0	2.50	18.9	17.1	24.5	20.0

(2) Internal Standard Spiking Solution

The internal standard spiking solution is prepared by dissolving appropriate amounts of $^{13}C_2$-PFHxA, $^{13}C_5$-PFHpA, $^{13}C_4$-PFOA, $^{13}C_4$-PFOS, $^{18}O_2$-PFHxS, $^{13}C_5$-PFNA, $^{13}C_2$-PFDeA, $^{13}C_2$-PFDoA, and D_3-Me-PFOSA-AcOH (4-6 ng/mL) in water/methanol (50/50). A 50 µL spike of this solution provides concentrations of 4-6 ng/mL in 50 µL serum. Spiking solutions are stored frozen in 2.0 mL aliquots in polypropylene cryogenic vials until use.

(3) Mass-Spec Operational Check Standard

The instrument test sample is prepared by spiking the reagent blank with all analytes to final concentrations of 0.3-0.5 ng /mL.

(4) In-house Proficiency Testing (PT) Standards

Appropriate aliquots of each stock standard are added to calf serum pools to produce 3 sets of in-house proficiency testing (PT) standards. The PT standards are mixed, aliquoted into polypropylene vials and frozen until needed. PT standards are characterized by at least 20 repeated analyses to determine the mean and standard deviation of the measurements.

d. Materials

1) HySphere C8-SE (7μM) cartridge (i-Chrome solutions, Plainsboro, NJ)
2) Chromolith® HighResolution RP-18e column (4.6 × 100 mm) (Merck KGaA, Germany).
3) Chromolith® HighResolution RP-18e Guard column (5 X 4.6 mm) (Merck KGaA, Germany).
4) Chromolith® HighResolution RP-18e column (4.6 × 25 mm) (Merck KGaA, Germany).
5) 750 µL polypropylene autosampler vials with polyethylene snap caps (National Scientific Company, Rockwood, TN).
6) Tip ejector variable volume micropipettes (Wheaton, Millville, NJ) and pipette tips (Rainin Instruments Co., Woburn, MA).

7) 5.0 mL and 2.0 mL polypropylene cryovials (National Scientific Company, Rockwood, TN).
8) Assorted glass and polypropylene labware.

e. Equipment

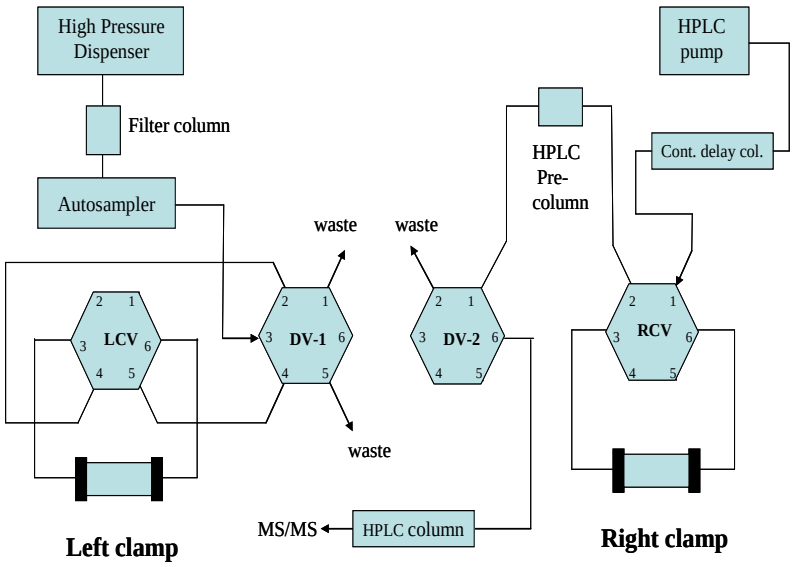
1) Symbiosys extractor equipped with an Alias autosampler run by the SparkLink software program (Spark Holland Inc. dba iChrom Solutions, Plainsboro, NJ).
2) Agilent 1200 binary pump and degasser (Agilent Technologies).
3) Applied Biosystems ABI 5500 or ABI 6500 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA).
4) Sartorius Genius Series ME models Electronic Analytical & Semi – microbalances (Sartorius AG, Goettingen, Germany).
5) Sartorius top – loading balance (Sartorius AG, Goettingen, Germany).
6) pH meter (AB 15 pH Meter, Fisher Scientific).
7) Vortex mixer (Type 16700, Barnstead International, Dubuque, Iowa).

f. Instrumentation

(1) Automated SPE

Tubing diagram for the Symbiosis column switching system used in concurrent SPE/HPLC mode. (LCV: left clamp valve; DV-1: divert valve 1; DV-2: divert valve 2; RCV: right clamp valve).

The method uses both left and right cartridge clamps, the four switching valves, and the high pressure dispenser. The left clamp, the left clamp valve (LCV), and left divert valve (DV-1) are used for SPE separation while the right clamp, the right clamp valve (RCV) and right divert valve (DV-2) are used for the HPLC elution. The SPE run of each sample starts with the conditioning of a HySphere C8-SE (7μM) cartridge with HPLC-grade acetonitrile (2 mL) and 0.1 M formic acid (2 mL). Afterward, 500 µL of the sample (containing 50 µL serum) injected into the 1 mL sample loop is loaded onto the SPE column using 2 mL 0.1 M formic acid with 1 mL/min flow rate. Next, the SPE column is washed with 2 mL 90% 0.1 M formic acid/10% Acetonitrile. The time of the SPE cleanup (including injection time) is 10 min long. Before starting the clean up of the next sample, the cartridge containing the extracted analytes is transferred by a robotic gripper from the left clamp into the right clamp. Therefore, while the right clamp is used for analyte elution and HPLC-MS/MS acquisition, the left clamp could be used for the clean up of the next sample. Once, the SPE column is in the right clamp, the right clamp valve remains in by-pass (1-2) position until the HPLC-MS/MS system becomes ready to begin acquisition.



The Symbiosis system is used in concurrent SPE/HPLC mode controlled by the SparkLink software (Table 2).

Table 2. Valve configurations used for concurrent SPE clean up and HPLC-MS/MS acquisition.

Steps[a]	Method	LCV	DV-1	DV-2	RCV	Time (min)
1	Move cartridge from left clamp to right clamp	6-1	1-2	6-1	1-2	0.1
2	Load new cartridge into left clamp	6-1	1-2	6-1	1-2	0.2
3	Send contact closer signal to HPLC-/MS/MS	6-1	1-2	6-1	1-2	0.1
4	Begin HPLC gradient elution by-pass HPLC column and MS/MS	6-1	1-2	1-2	6-1	3.0[b]
5	Condition left cartridge (2 mL acetonitrile, 2 mL/min)	1-2	1-2	6-1	6-1	1.2
6	Equilibrate left cartridge (2 mL 0.1 M formic acid, 2 mL/min)	1-2	1-2	6-1	6-1	1.2
7	Load 500 µL sample on left cartridge (2 mL, 0.1 M formic acid, 1 mL/min)	1-2	1-2	6-1	6-1	4.4
8	Forward wash left cartridge (2 mL 90% 0.1 M formic acid/10% acetonitrile, 1 mL/min)	1-2	1-2	6-1	6-1	1.2
9	Return right cartridge to tray	6-1	1-2	6-1	1-2	0.1

[a] The method used for the first sample included only steps 2 and 5-8. The method used for the acquisition of the last sample included only steps 1, 3, 4, and 9.
[b] For the acquisition of the last sample duration of step 4 was 13 min.

(2) HPLC configuration

At the beginning of the HPLC-MS/MS acquisition, the right clamp valve is turned into 6-1 position for the first 10 min of the HPLC gradient program to transfer the analytes from the SPE column to the HPLC column. At 10 min, the right clamp valve turns back to 1-2 position and the SPE column is returned to the cartridge tray while the HPLC gradient program continues. The HPLC pump is operated at a 1000 µL/min flow rate with 95% of 20 mM ammonium acetate (pH 4) and 5% of acetonitrile as mobile phase A and 100% acetonitrile as mobile phase B. The analytes are separated from each other and other extracted components on two Chromolith® HighResolution RP-18e columns (4.6 × 100 mm) preceeded by a Chromolith® HighResolution RP-18e (5 X4.6 mm) guard column and a Chromolith® HighResolution RP-18e (4.6 × 25 mm) column. To delay the elution of the PFAS contaminants leaching out

from Teflon parts of the HPLC pump, a 4.6 mm x 25 mm Chromolith® HighResolution RP-18e column is inserted between the HPLC pump and the right clamp valve. Because contaminants have to go through twice the column length, their peaks elute 1 min after the main analytes bands without interfering with the measured concentration.

Table 3. HPLC configuration

Parameters	Setting
Mobile Phase A	95% 20 mM ammonium acetate, pH = 4/5% acetonitrile
Mobile Phase B	100% acetonitrile
Flow rate	1000 μL/min

Table 4. Mobile phase gradient

Time (min)	0	1	2	8.5	8.51	12	12.1	13	13.2
Mobile phase B%	25	25	45	49	60	60	80	80	80
Flow rate (mL/min)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Time (min)	13.3	13.5	13.6	15.5	15.6	16.5	16.6	18	18.1
Mobile phase B%	80	95	95	95	95	95	25	25	25
Flow rate (mL/min)	1.5	1.5	1.8	1.8	2.0	2.0	1.5	1.5	1.0

(3) Mass Spectrometer Configuration

Detection of the target analytes is conducted on the ABI 5500 or ABI 6500 Qtrap mass spectrometer in the negative ion Turbo Ion Spray (TIS) mode. The TIS ionization source is a variant of the electrospray source and is used to convert liquid phase ions into gas phase ions. We use laboratory-grade air heated turbo ion spray gas (GS1=50 and GS2=50) gas. The heated turbo ion spray gas temperature is set at 400 °C. The curtain and collision gas (nitrogen) settings are as follows: collision (medium), curtain gas (CUR=30 [ABI 5500], CUR=45 [ABI 6500]). Ionization parameters and collision cell parameters are optimized individually for each analyte (**Table 5**). Unit resolution is used for both Q1 and Q3 quadrupoles. The dwell time is 50 msec for all compounds.

Table 5. Mass spectrometric parameters for measuring PFASs

	$(M-H)^-$ Precursor ion (m/z)	Product ion (m/z)	DP (volts)	CE (volts)
Me-PFOSA-AcOH	570	512	-45	-30
D_3-Me-PFOSA-AcOH(IS)	573	515	-45	-30
PFBuS-1	299	99	-70	-80
PFBuS-2[a]	299	80	-70	-85
PFHxS-1	399	99	-70	-80
PFHxS-2 [a]	399	80	-70	-85
PFHxS-$^{18}O_2$-1 (IS)[b]	403	103	-70	-80
PFHxS-$^{18}O_2$-2 (IS)[a]	403	84	-70	-85
n-PFOS-1 [a]	499	80	-70	-90
n-PFOS-2	499	99	-70	-80
PFOS-$^{13}C_4$-1 (IS)[c]	503	80	-70	-85
PFOS-$^{13}C_4$-2 (IS)	503	99	-70	-85
Sm-PFOS	499	80	-70	-90
PFHpA	363	319	-25	-13
PFHpA-$^{13}C_5$(IS)	368	323	-25	-13
n-PFOA	413	369	-27	-14
Sb-PFOA	413	369	-27	-14
PFOA-$^{13}C_4$ (IS)[d]	417	372	-30-	-15
PFNA	463	419	-30	-13
PFNA-$^{13}C_5$ (IS))	468	423	-30	-13
PFDeA	513	469	--30	-15
PFDeA-$^{13}C_2$ (IS)	515	470	-30	-15
PFUA	563	519	-30	-17
PFUA -$^{13}C_2$ (IS)	565	520	-30	-17
PFDoA	613	569	-30	-18
PFDoA-$^{13}C_2$ (IS)	615	570	-45	-15

[a] used only as confirmation ion
[b] PFHxS-$^{18}O_2$-1 was used as IS for PFBuS.
[c] PFOS-$^{13}C_4$ was used as IS for n-PFOS, Sm-PFOS, and Sm2-PFOS.
[d] PFOA-$^{13}C_4$ was used as IS for both n-PFOA and Sb-PFOA.

1. Calibration and Calibration-Verification Procedures

a. Calibration Curve

Nine-point calibration curves are normally constructed with each quantitative run from the analyte area ratios (i.e., analyte area/internal standard area) obtained from extracted standards in calf serum. A linear regression analysis (weighted by 1/x) of the area ratio versus standard concentration is performed. The area of the Q1 ion for each analyte is used for quantification (for PFOS we use Q2). Correlation coefficients are generally greater than 0.97. Samples with values exceeding the highest point in the calibration curve are reanalyzed using less serum.

b. Mass Spectrometer Calibration

The ABI 5500 or ABI 6500 Qtrap mass spectrometer is calibrated and tuned at least once per year using a polypropylene glycol (PPG) solution according to the instructions contained in the operator's manual. The instrument sensitivity is checked periodically by injecting the Instrument Test sample.

c. Calibration Verification

1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.

2) Calibration verification must be performed at least once every 6 months.

3) All calibration verification runs and results shall be appropriately documented.

4) According to the updated CLIA regulations from 2003 (http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/downloads/6065bk.pdf), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.

5) All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

d. Proficiency Testing (PT)

(1) Three pools of PT samples, which encompass the entire linear range of the method, are prepared in-house as described in the standard preparation section. Characterization of PT materials requires at least 20 separate determinations. Once the PT pools are characterized, the mean concentration and standard deviation of the PT materials are forwarded to a DLS representative (PT administrator) responsible for executing the PT program. These PT samples are blind-coded by the PT administrator and returned to the laboratory staff for storage.

(2) Proficiency testing should be performed a minimum of once per 6 months. When proficiency testing is required, the laboratory supervisor or his/her designee will notify the PT administrator, and the PT administrator will randomly select five PT materials for analysis. Following analysis, the results will be forwarded directly to the PT administrator for evaluation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the PT administrator. The PT administrator will notify the laboratory supervisor and/or his/her designee of the PT results (i.e., pass/fail).

(3) All proficiency results shall be appropriately documented.

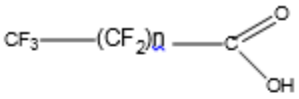
(4) In addition to the in-house PT program, since 2005 we have successfully participated in the international round-robin program organized by Intercal

(Sweden) and RIVO (The Netherlands) when it is conducted for human serum/plasma [22,23].

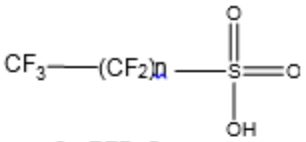
(5) Also, since 2006, at least once per year, we participate in the ongoing German External Quality Assessment Scheme (G-EQUAS) for PFOS and PFOA in serum, organized and managed by the Institute and Outpatient clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). The design, evaluation and certification of G-EQUAS are based on the guidelines of the German Federal Medical Council.

(6) Since 2011**,** three times a year we also participate in the ongoing Arctic Monitoring and Assessment Program (AMAP) Ring Test for several PFASs in human serum, conducted by the Institut National de Santé Publique du Québec (INSP) in Canada.

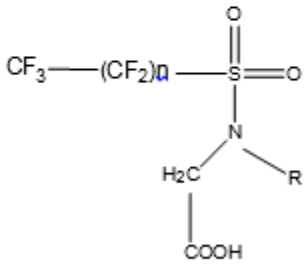
(7) Analytes nomenclature and structures



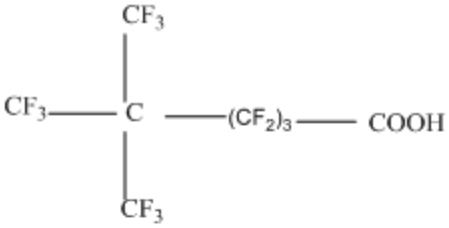
n=5 PFHpA
n=6 n-PFOA
n=7 PFNA
n=8 PFDeA
n=9 PFUA
n=10 PFDoA



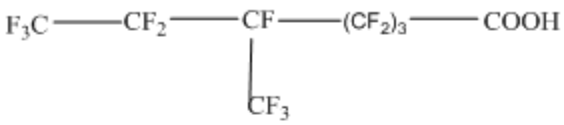
n=3 PFBuS
n=5 PFHxS
n=7 n-PFOS



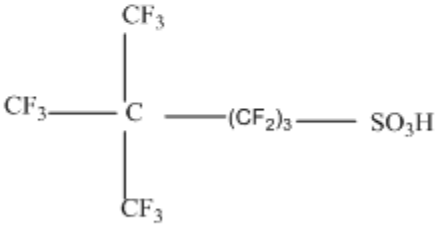
n=7, R=Me, Me-PFOSA-AcOH



perfluoro-5,5-dimethylhexanoic Acid



perfluoro-5-methylheptanoic Acid



perfluoro-5,5-dimethylhexane sulfonic acid

perfluoro-5-methylheptane sulfonic acid

8. Operating Procedures; Calculations; Interpretation of Results

a. Sample preparation

(1) Unknown, QC, Blank, and Standard Preparation

(a) Remove serum samples, working standard solutions and internal standard solution from the freezer, and let them thaw. Label polypropylene snap-cap autosampler vials with appropriate Sample Names. Aliquot 0.1 M formic acid (500 µL for QCBs; 450 µL for UNKs, QCs, serum blanks (SBs), and human serum blanks (HSBs); 400 µL for STDs) into appropriate vials.

(b) Dispense 50 µL of internal standard into each polypropylene autosampler snap cap vials. In specific cases, the method can be performed using a smaller volume of matrix; the applied dilution factor must be noted appropriately.

(c) Add 50 µL of the appropriate native standard solution (S1-S9) into the polypropylene vials designated for standards.

(d) Aliquot 50 µL of UNKs, QCs, SBs and HSBs into the designated autosampler vials. For standards, aliquot 50 µL of blank serum. Analysis may also be conducted with a smaller amount of serum; in these circumstances, the volume used must be noted appropriately throughout the analytical procedure.

(e) Vortex all vials for at least 10 seconds to make sure all the internal standard and standard mixed into the sample.

(2) Automated SPE-HPLC-MS/MS Analysis Procedure

(a) Put the Alias into load position. Initialize the high pressure dispenser (HPD) and the automated cartridge exchanger (ACE) unit.

(b) Exchange the cartridge tray after every 500 samples.

(c) Purge the solvent lines on the HPLC binary pump and equilibrate the HPLC column.

(d) In the **SparkLink** software, go to RunTables and open and set up the batch table. For the first sample enter xx-method 1 which runs the injection and cleanup of the first sample. For the second and consecutive samples use xx-method 2 which initiates the HPLC/MS acquisition and runs the injection and cleanup of the next sample. For the last sample enter xx-method 3 which initiates the HPLC/MS acquisition of the last sample. For injection volume, enter 500 µL. Make sure the

right vial positions are entered and there is no sequential duplication of cartridge numbers. The sample names and sample IDs do not matter, since they will not be part of the acquired data.

(e) Go to **Excel**, open the text file containing the batch table created from Sample Login Table in the Microsoft Access database. This file should not require any editing. Save the table into the text file named import.txt into the Batch directory (overwrite). Remember to CLOSE THE FILE IN EXCEL!!!!!

(f) Go to **Analyst** and import the import.txt file (Sample pull down, go to gray header and click RMB, then Import From/File, select Alias autosampler). Make sure that the proper Acquisition Method and Quantitation Method are entered. Then, submit the batch (highlight and/or click Submit, go to View Queue, and click Start Sample). All samples on the Queue Manager should be in "waiting".

(g) Start the batch table in **SparkLink**. From this on everything should run automatically.

b. Analysis

(1) Check out the LC/MS interface

(a) If the instrument is in ready mode, wait until the interface cools down. When the interface is cool enough, take out the capillary from the MS interface. Rinse the capillary with MeOH, sonicate in MeOH for 20 min if necessary. Periodically, take off the interface housing, and wipe out the skimmer plate.

(b) Open the rough pump cabinet, check for oil leaks and unusual noise. Report anything unusual.

(2) Check out the LC system

After the column has been conditioned, click on the Equilibrate icon, select the current method, and let the system equilibrate for approximately 30 minutes. Run the Instrument Check sample by opening the batch file named Instrument_test.dab. Change the date in the Sample Name field. Make sure the proper Acquisition Method and Vial Position are entered, and submit the batch. The file should be saved into the Instrument_test.wiff file. Open the chromatogram and compare the intensities and peak shape to those obtained a day and a week before. If peaks appear distorted (tailing peaks, broad peaks, etc.) change the column and submit the Instrument Check sample again. If the absolute intensity is too low (peak intensity should not be <70% less intense than before) check with the laboratory supervisor or his/her designee.

(3) Check out the SPE system

a) In SparkLink, put the Triathlon autosampler into load position. Initialize the high pressure dispenser (HPD) and the automated cartridge exchanger (ACE) unit.

b) Exchange the cartridge tray as needed (generally after approximately 500 samples).

c) Make sure that the MS remote cable is connected ACE unit.

(4) Building batch files

(a) In the **SparkLink** software, go to RunTables and open and set up the batch table. For the first sample enter xx-method 1 which runs the injection and cleanup of the first sample. For the second and consecutive samples use xx-method 2 which initiates the HPLC/MS acquisition and runs the injection and cleanup of the next sample. For the last sample enter xx-method 3 which initiates the HPLC/MS acquisition of the last sample. For injection volume, enter 500 µL. Make sure that the proper vial positions are entered and there is no sequential duplication of cartridge numbers. The sample names and sample IDs do not matter, since they will not be part of the acquired data.

(b) In the Analyst software, open a new the subproject folder for each new run. The subproject should have the same YYYY-MMDD name as the unknowns it includes. Each subproject should have separate Acquisition Methods, Quantitation Methods, Batch, Data, and Results directories. Copy the latest Acquisition Method and Quantitation Method from the previous subfolder.

(c) From Excel, open the text file containing the batch table created from the Access database using Microsoft Access. This file should not require any editing. Save the table into the text file named import.txt into the Batch directory (overwrite). Remember to CLOSE THE FILE IN EXCEL!!!!! Go to Analyst, open a new batch table and import the import.txt file (Sample pull down, go to gray header and click RMB, then Import From/File, select Alias autosampler).

(d) Make sure that the proper Acquisition Method and Quantitation Method are entered. Although the vial positions entered in Analyst will not be used they should agree with the vial positions used on the Triathlon autosampler.

(5) Starting the SPE-HPLC-MS/MS run

a) Start the batch table in **SparkLink**.

b) Submit the batch table in Analyst (highlight and/or click Submit, go to View Queue, and click Start Sample). From this on everything should run automatically. After the SPE cleanup of the

first sample, each N+1 sample in the SparkLink batch table will correspond with sample N in the Analyst batch table.

c. Processing data

(1) Quantification

All raw data files are analyzed using the Quantitation Wizard application in the Analyst software, which allows both automatic and manual peak selection and area integration. The area values and retention times are exported into a tab delimited text file and imported into the Access database with the name YYYY-MMDD.txt.

(2) Importing Data into the Database

The tab-delimited file is read into the Access database. No prior editing is required.

(3) Statistical Analysis and Interpretation of Data

Data are exported from the Access database to a fixed ASCII text file and imported into SAS. SAS programs for standard curve generation, QC analysis, blank analysis, limit of detection determination, unknown calculations, and data distribution have been created and may be executed in SAS when this information is needed.

d. Replacement and periodic maintenance of key components

(1) ABI 5500 or ABI 6500 Qtrap Mass Spectrometer

Preventative maintenance is done by a qualified engineer at least once a year. In addition, to ensure proper performance of the system, a periodic maintenance of the system may be required.

(a) When a partial blockage of the vacuum is suspected, the orifice is probed with a syringe-cleaning wire.

(b) Cleaning of the spray shield and the entrance end of the heated capillary is performed weekly as described in the Sciex ABI 5500 or ABI 6500 Qtrap Hardware Manual. First, wash with a solution of water: methanol (1:1) and then, with 100% methanol. Wipe the area using flake free paper wipes.

(c) The pump oil is changed approximately every six months as part of the periodic maintenance of the system.

(2) Agilent 1200 HPLC

Preventative maintenance is done by a qualified engineer at least once a year. Additional maintenance may be necessary if there is a general decrease in instrument performance (see below). In general, performance maintenance procedures are performed after detecting a decrease in the system performance (sensitivity and/or S/N ratio) without any other apparent technical reasons.

(a) The HPLC column is replaced when analyte resolution decreases. Once the analyte peaks start tailing, the HPLC column should be replaced.

(b) If high pressure (>250 bar) error messages are observed, the purge valve frit, the guard column, analytical column frit, HPLC lines, needle seat, or injector components may need to be replaced. See also section 8b.

(c) Reestablishment of performance and calibration. Every time the system is disturbed for cleaning or maintenance, a mass spec operational check standard is analyzed to assess the HPLC and MS performance. For the mass spectrometer, a retune of the system may or may not be necessary. If the instrument does not pass this test, then the instrument is retuned using PPG as described previously.

(3) Spark system

Preventative maintenance is done by a qualified engineer at least once a year. Additional maintenance may be necessary if there is a general decrease in instrument performance.

If the SparkLink error “HPD 1 high pressure problem” occurs, check the SPE lines and HPD 6 port valve. The HPD valve stator and/or rotor may need to be replaced.

The instrumentation used is serviced according to the manufacturer’s guidance included in the instrument manuals or based on the recommendation of experienced analysts/operators after following appropriate procedures to determine that the instrument performs adequately for the intended purposes of the method.

9. Reportable Range of Results

The linear range of the standard calibration curves and the method limit of detection (LOD) determine the reportable range of results. The reportable range must be within the range of the calibration curves. However, samples with concentrations exceeding the highest reportable limit may be diluted, re-extracted, and reanalyzed so that the measured value will be within the range of the calibration.

If a sample needs more than 100 times dilution (which would require using less than 1 µL of specimen) the dilution can be performed in at least two steps. For example, first, at least 10 µL specimen is diluted up to 1 mL with water in a 2 mL Eppendorf tube (or equivalent), then a second dilution is performed by aliquoting the appropriate fraction of the dilute into an autosampler vial and adding 100 µL blank calf serum. With very concentrated specimens it may be difficult to estimate the dilution that is necessary, and the measured value may be higher than the highest calibration point even after the dilution.

Formula to calculate the dilution factor to be entered into the Analyst batch file:

$D= (1000 / V_{1st}) \times (200 / V_{2nd})$.

Formula to calculate the volume of specimen to be entered into the Access database:

$V= V_{1st} \times (V_{2nd} / 1000)$

Where V_{1st} is the volume of the aliquot taken from the original specimen and V_{2nd} is the volume of the dilute measured into the autosampler vial.

1) Analytical Sensitivity

The limits of detection (LOD) for each analyte are listed in **Table 6**.

2) Analytical Specificity

This is a highly selective method that requires that the PFASs 1) elute at a specific retention time; 2) have precursor ions with specific mass/charge ratios; 3) have specific product ions formed from the precursor ion with specific mass/charge ratios.

3) Linearity Limits

The calibration curve is linear for all analytes (generally $R^2>0.95$). The limit on the linearity is determined by the highest standard analyzed in the method. Due to the wide variation of PFASs levels in humans, we set our highest standard near the high end of the linear range (**Table 6**). Unknown samples whose concentrations exceed the highest standard concentration must be re-extracted using a smaller aliquot. The low end of the linear range is limited by the method LOD. Concentrations below the method LOD (or the concentration of the lowest standard in the calibration curve) are reported as non-detectable.

Table 6. Linear range (lowest – highest standard concentration) and LOD for each PFAS measured in serum.

Analyte	LOD	Linear range (ng/mL)
Me-PFOSA-AcOH	0.1	0.005-20
PFBuS	0.1	0.004-17.1
PFHxS	0.1	0.005-18.9
n-PFOS	0.1	0.015-115
Sm-PFOS	0.1	0.01-10
PFHpA	0.1	0.01-20
n-PFOA	0.1	0.01-20
Sb-PFOA	0.1	0.029-24.5
PFNA	0.1	0.01-20
PFDeA	0.1	0.01-20
PFUA	0.1	0.01-20
PFDoA	0.1	0.01-20

1) Accuracy

The accuracy of the method is determined by enriching serum samples with known concentrations of PFASs and comparing the calculated and expected concentrations. To examine their consistency over the range of levels encountered in serum, the measurements are taken at 3 different concentrations, namely using standards near 3*LOD, middle level (~1.0 ng/mL, except n-PFOS (6 ng/mL), Sm-PFOS and Sm2-PFOS (0.5 ng/mL), and Sb-PFOA (0.7 ng/mL)), and high level (~10.0 ng/mL, except n-PFOS (60 ng/mL), Sm-PFOS and Sm2-PFOS (1.0 ng/mL), and Sb-PFOA (2.5 ng/mL)). The accuracy is calculated from 5 independent measurements (Table 7).

Table 7. Spiked recoveries of extracted standards in serum

Analyte	Accuracy (%) at ~3*LOD/middle/high		
Me-PFOSA-AcOH	105±20	93±5	101±3
PFBuS	113±25	105±19	110±12
PFHxS	106±12	98±5	98±6
n-PFOS	110±14	99.7±6	97.0±2
Sm-PFOS	105±23	92±4	90±4
PFHpA	115±12	110±11	103±10
n-PFOA	92±14	105±5	101±2
Sb-PFOA	112±15	106±8	102±5
PFNA	95±13	103±7	102±3
PFDeA	103±13	96±6	98±4
PFUA	92±17	102±3	101±3
PFDoA	110±22	96±10	101±3

1) Precision

The precision of this method is reflected in the variance of two quality control (QC) pools over a period of three weeks. The coefficient of variation (CV) of repeated measurements of these QC pools, which reflects both inter and intra-day variations, is used to estimate precision (Table 8).

Table 8. Mean QC concentrations (ng/mL) and CV%

Analyte	VQC	CV%	QCH	CV%
Me-PFOSA-AcOH	0.4	14.3	6.5	10.5
PFBuS	0.4	18.5	6.7	15.7
PFHxS	0.4	9.3	6.3	7.0
n-PFOS	1.0	10.6	15.8	9.8
Sm-PFOS	0.4	10.5	2.3	10.3
PFHpA	0.5	6.5	6.3	9.4
n-PFOA	0.4	13.6	7.4	12.0
Sb-PFOA	0.8	15.5	6.5	11.0
PFNA	0.4	10.6	8.1	9.4
PFDeA	0.5	10.9	6.3	9.7
PFUA	0.5	12.5	6.4	9.7
PFDoA	0.5	20.0	6.4	9.1

10. Quality Control (QC) Procedures

a. Individual samples (i.e., standards, unknown samples, serum blanks, and quality control (QC) materials) QC procedures

1) For each analyte, the relative retention time (RT) (ratio of $RT_{analyte}$ and RT_{IS}) of standards, unknowns, and QCs should be checked. If the relative RT falls outside the range, check the integration to make sure the analyte or IS peak was properly picked up.

2) For each analyte, the IS area counts should meet minimum area count requirements. Low IS area counts suggest a) strong ion suppression from the matrix, or b) missing of IS. Depending on the findings, either re-extract the sample as usual or re-extract the sample after dilution.

3) For each analyte, the calculated concentration of the calf serum blanks (SB) should be less than three times the LOD. Using the current method, all standards, blanks and unknown samples are prepared following the same procedure, thus background blank values (reflected in the intercept of the calibration curve) are automatically subtracted from the concentrations of unknown samples. If background levels are above the threshold above, the reagents used for sample preparation and (or) mobile phases need to be checked for potential contamination.

4) For each analyte, if the concentration in an unknown sample is above the highest calibration standard, the sample needs to be re-extracted with a smaller volume of serum.

b. Quality control of the QC materials

1) QC Materials

The QC materials were prepared in bulk from calf serum (Gibco, Grand Island, NY). The target ranges for the pools were set to encompass the expected concentration ranges in human populations.

2) Preparation of QC Pools

The calf serum purchased was pooled and the QC pools were mixed uniformly, divided into four subpools and stored frozen. One subpool was used as a blank QC and to prepare the calibration standards, and the other three were enriched with PFASs as needed to afford very low concentration (VQC, ~0.3-1.0 ng/mL), low concentration (QCL, ~2.0 ng/mL) and high concentration (QCH, ~0.8-15.8 ng/mL) subpools. The QC pools were characterized to define the mean and the 95% and 99% control limits of PFASs concentrations by a minimum of 30 repeated measurements in a three week period. QC materials reextracted and analyzed after the initial characterization showed that the PFASs remained stable frozen for at least 3 months [21].

3) Characterization of QC Materials

For characterization, a minimum of 30 runs of QCL and QCH were measured over 1 month. In each run, one pair of QCL and QCH materials were analyzed and averaged. Using the pair average value from the 30 runs, the mean, and upper and lower 99% and 95% control limits were established.

QC samples are analyzed along with unknown samples to monitor for accuracy and precision throughout the analysis batch. Maximum 50 unknown samples are run with randomly placed 2 QCL, 2 QCH, and 2 reagent blank samples. The concentrations of the two QCL and two QCH in each batch are averaged to obtain one average measurement of QCL and QCH.

4) Final evaluation of Quality Control Results

Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control[24].

QC rules for: Analytical run with 1 QC pool per run (must also include a blank QC specimen)**:**

<u>One QC pool per run with one QC result per pool</u>
1) If QC run result is within 2Si limits, then accept the run.

2) If QC run result is outside a 2Si limit - reject run if:

a) Extreme Outlier – Run result is beyond the characterization mean +/- 4Si
b) 1 3S Rule - Run result is outside a 3Si limit
c) 2 2S Rule - Current and previous run results are outside the same 2Si limit
d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
e) R 4S Rule – The current and the previous run results differ by more than 4Si.
Note: Since runs have a single result per pool and only 1 pool, the R 4S rule is applied across runs only.

<u>One QC pool per run with two or more QC results per pool</u>
1) If QC run mean is within 2Sm limits and individual results are within 2Si limits, then accept the run.

2) If QC run mean is outside a 2Sm limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
b) 3S Rule - Run mean is outside a 3Sm limit
c) 2 2S Rule – Current and previous run means are outside the same 2Sm limit
d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

3) If one of the two QC individual results is outside a 2Si limit - reject run if:

a) R 4S Rule – Within-run range for the current run and the previous run exceeds 4Sw (i.e., 95% range limit)

Abbreviations:
S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
S_m = Standard deviation of the run means (the limits are shown on the chart).
S_w = Within-run standard deviation (the limits are not shown on the chart).

QC rules for: Analytical run with 2 QC pools per run:

Two QC pools per run with one QC result per pool

1) If both QC run results are within 2Si limits, then accept the run.

2) If 1 of the 2 QC run results is outside a 2Si limit - reject run if:

a) Extreme Outlier – Run result is beyond the characterization mean +/- 4Si
b) 3S Rule - Run result is outside a 3Si limit
c) 2S Rule - Both run results are outside the same 2Si limit
d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
e) R 4S Rule – Two consecutive standardized run results differ by more than 4Si. Note: Since runs have a single result per pool for 2 pools, comparison of results for the R 4S rule will be with the previous result within run or the last result of the previous run. Standardized results are used because different pools have different means.

Two QC pools per run with two or more QC results per pool

1) If both QC run means are within 2Sm limits and individual results are within 2Si limits, then accept the run.

2) If 1 of the 2 QC run means is outside a 2Sm limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
b) 3S Rule - Run mean is outside a 3Sm limit
c) 2S Rule - Both run means are outside the same 2Sm limit
d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

3) If one of the 4 QC individual results is outside a 2Si limit - reject run if:

a) R 4S Rule – Within-run ranges for all pools in the same run exceed 4Sw (i.e., 95% range limit). Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

QC rules for: Analytical run with 3 QC pools per run:

Three QC pools per run with one QC result per pool

1) If all 3 QC run results are within 2Si limits, then accept the run.

2) If 1 of the 3 QC run results is outside a 2Si limit - reject run if:

a) Extreme Outlier – Run result is beyond the characterization mean +/- 4Si
b) 3S Rule - Run result is outside a 3Si limit
c) 2S Rule - 2 or more of the 3 run results are outside the same 2Si limit
d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
e) R 4S Rule – Two consecutive standardized run results differ by more than 4Si. Note: Since runs have a single result per pool for 3 pools, comparison of results for the R 4S rule will be with the previous result within the current run or with the last result of the previous run. Standardized results are used because different pools have different means.

Three QC pools per run with two or more QC results per pool

1) If all 3 QC run means are within 2Sm limits and individual results are within 2Si limits, then accept the run.

2) If 1 of the 3 QC run means is outside a 2Sm limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
b) 3S Rule - Run mean is outside a 3Sm limit
c) 2S Rule - 2 or more of the 3 run means are outside the same 2Sm limit
d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

3) If one of the QC individual results is outside a 2Si limit - reject run if:

a) R 4S Rule - 2 or more of the within-run ranges in the same run exceed 4Sw (i.e., 95% range limit). Note: Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only.

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

If the QC systems or the calibrations failed to meet acceptable criteria, operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable (e.g., failure of the mass spectrometer or a pipetting error), the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure) are reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

12. Limitations of Method; Interfering Substances and Conditions

Occasionally, the concentration of the PFASs in serum may be higher than the highest standard in the calibration curves, and 0.1 mL of sample may be too much to use. This

is evident by the low recovery of the isotope-labeled standard after the SPE extraction. In this case, a smaller aliquot of serum can be used. Most likely, the LOD is not higher in this case because of the concentrated nature of the specimen.

13. Reference Ranges (Normal Values)

Results (http://www.cdc.gov/exposurereport) from the National Health and Nutrition Examination Survey (NHANES) can be used as reference ranges for the general US population [25].

14. Critical-Call Results ("Panic" Values)

Critical call values have not been established for any PFAS concentrations.

15. Specimen Storage and Handling During Testing

Specimens are stored in the laboratory frozen prior to analysis. Frozen samples are allowed to thaw completely at room temperature prior to the initiation of the analytical procedure.

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

Alternate procedures do not exist in-house for the measurement of PFASs. If the analytical system fails, storage of samples refrigerated is recommended until the system is operational again.

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

a. The Quality Control officer reviews each analytical run, identifies the quality control samples within each analytical run and determines whether the analytical run is performed under acceptable quality control conditions.

b. The data from analytical runs of unknowns are initially reviewed by the laboratory supervisor.

c. If the quality control data and results are acceptable the laboratory supervisor generates a memorandum to the Branch Chief reporting the results.

d. These data are then sent to the person(s) that made the initial request.

e. Final hard copies of correspondence are maintained in the office of the Branch Chief and with the quality control officer.

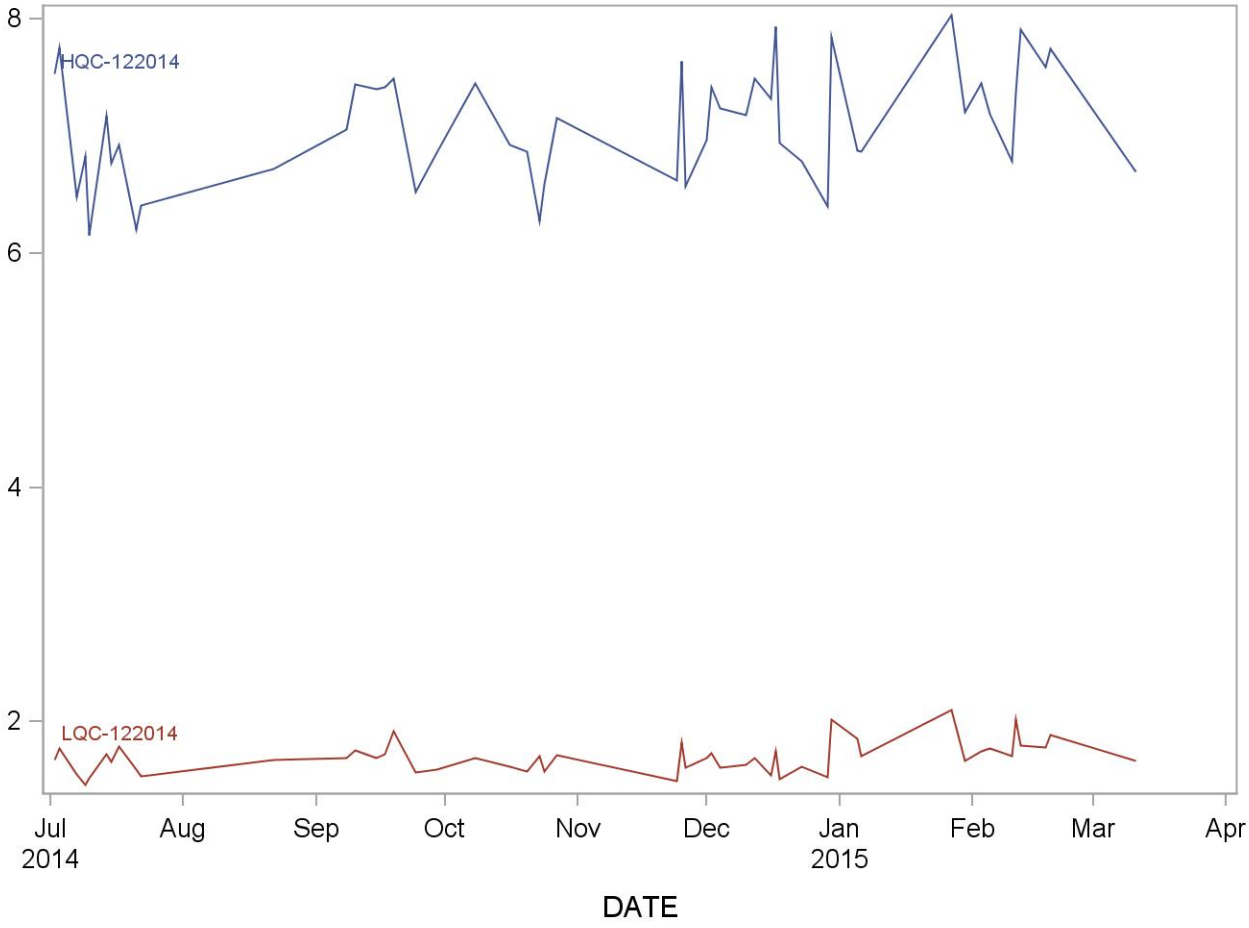
18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Standard record keeping systems (e.g., notebooks, sample logs, data files) should be employed to keep track of all specimens. One spreadsheet form with information for receiving/transferring specimens is kept in the laboratory. In this form, the samples received are logged in when received and when stored/transferred after analysis. For NHANES samples, the person receiving the specimens signs and dates the shipping manifests. The shipping manifests for NHANES and other samples are kept in a binder in the Laboratory.

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.

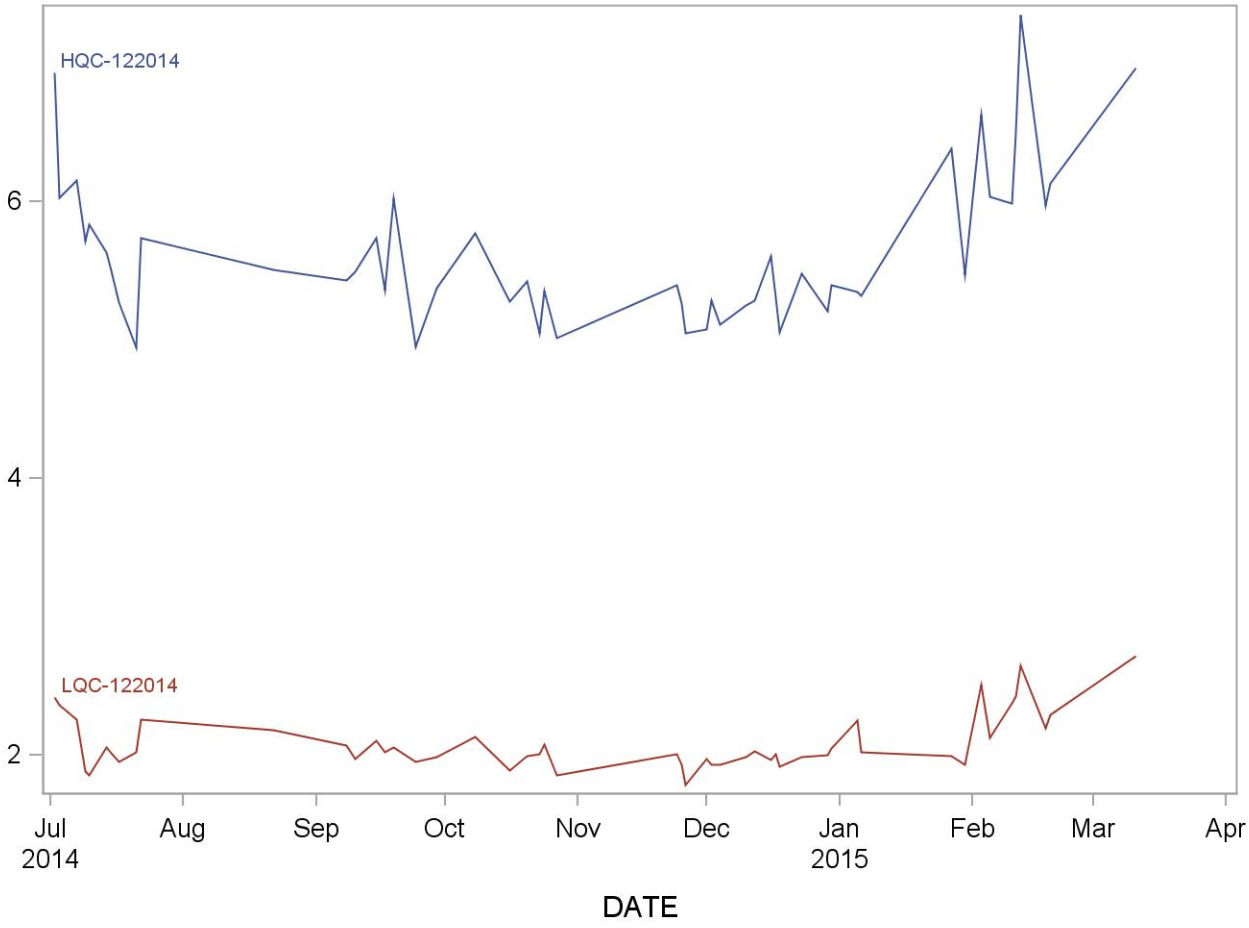
2013-2014 Summary Statistics and QC Chart for 2-(N-methyl-PFOSA) acetate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	7.09100	0.48089	6.8
LQC-122014	50	02JUL14	11MAR15	1.69470	0.13635	8.0



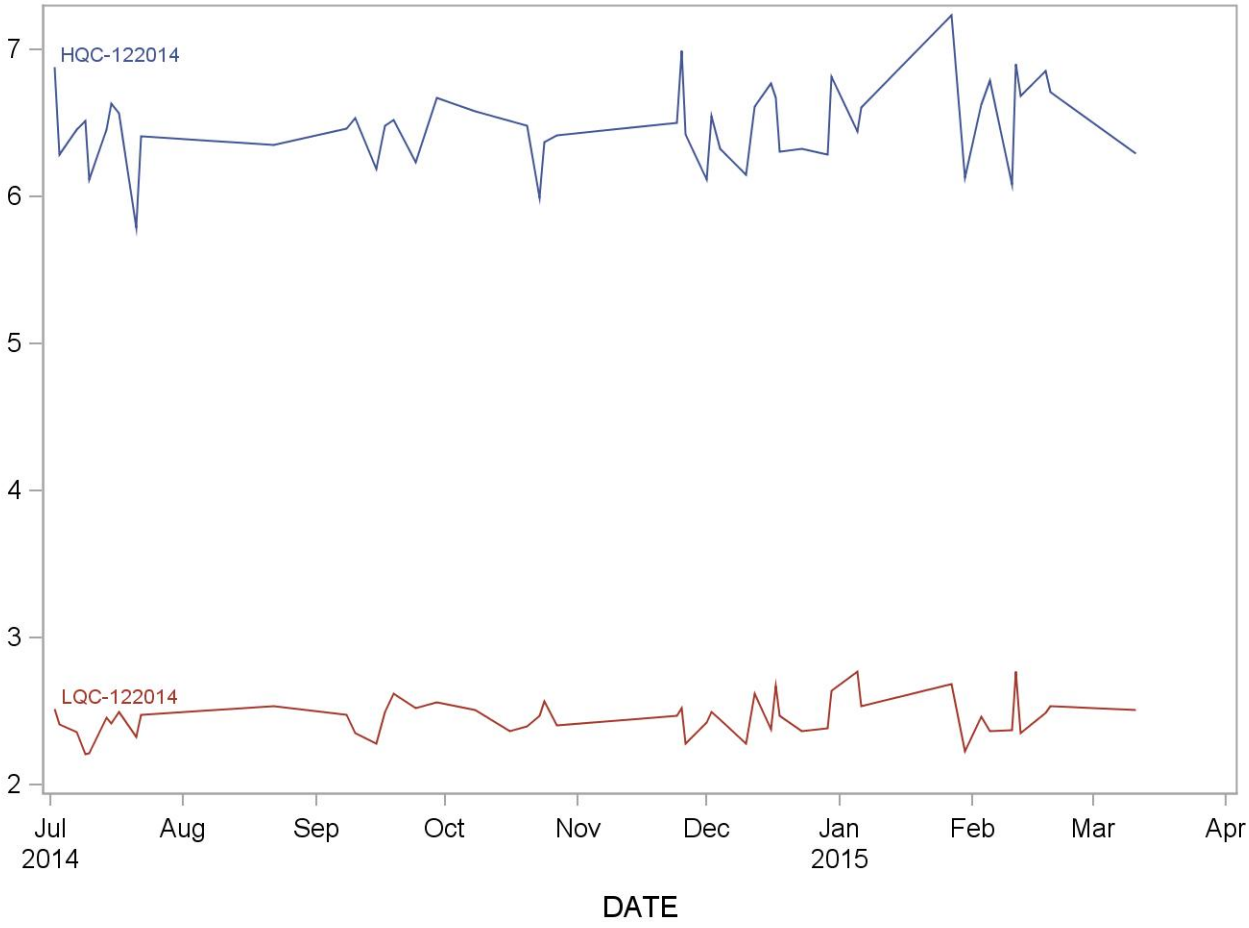
2013-2014 Summary Statistics and QC Chart for Perfluorobutane sulfonic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	5.629	0.548	9.7
LQC-122014	50	02JUL14	11MAR15	2.088	0.202	9.7



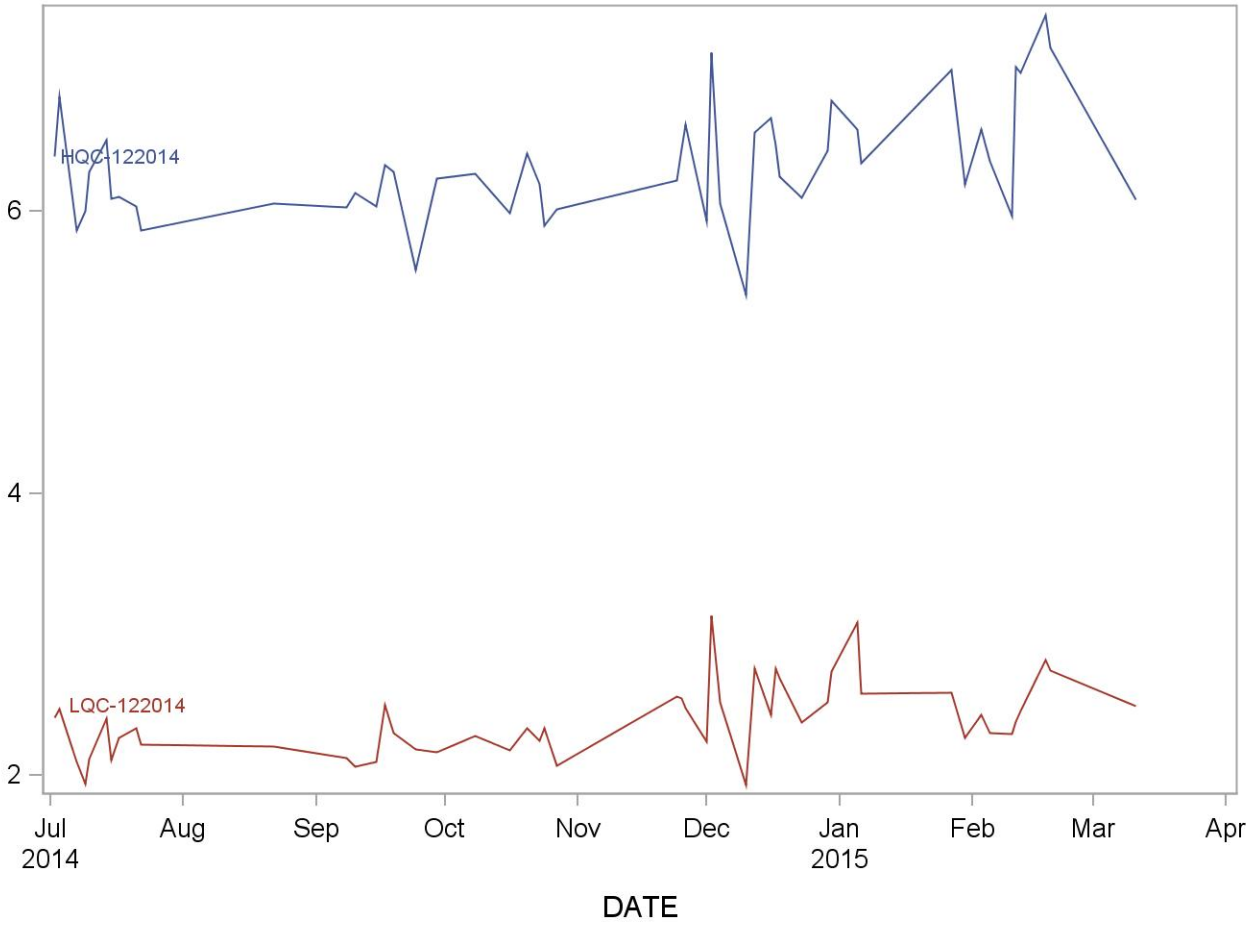
2013-2014 Summary Statistics and QC Chart for Perfluorodecanoic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.484	0.272	4.2
LQC-122014	50	02JUL14	11MAR15	2.461	0.129	5.3



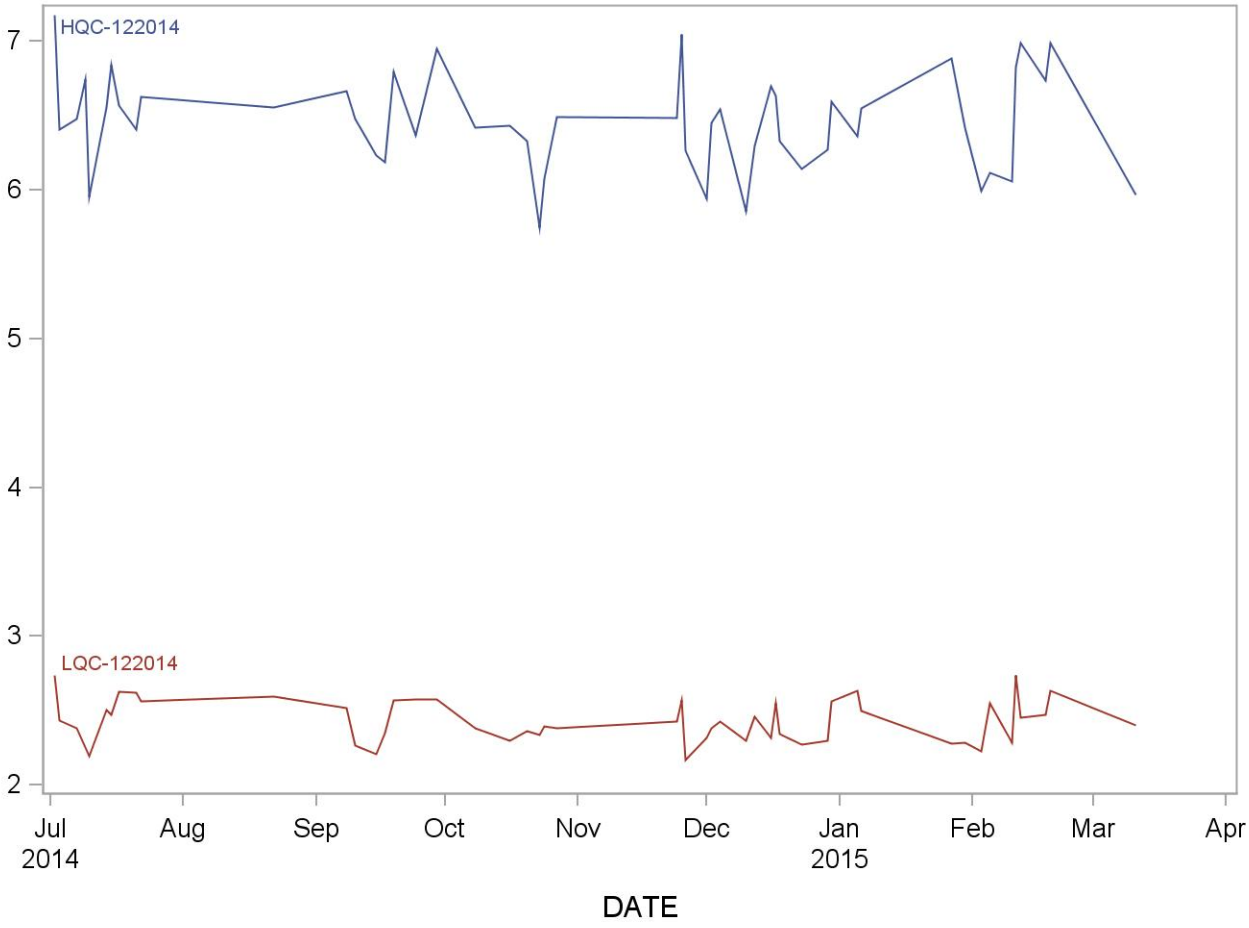
2013-2014 Summary Statistics and QC Chart for Perfluorododecanoic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.324	0.406	6.4
LQC-122014	50	02JUL14	11MAR15	2.390	0.263	11.0



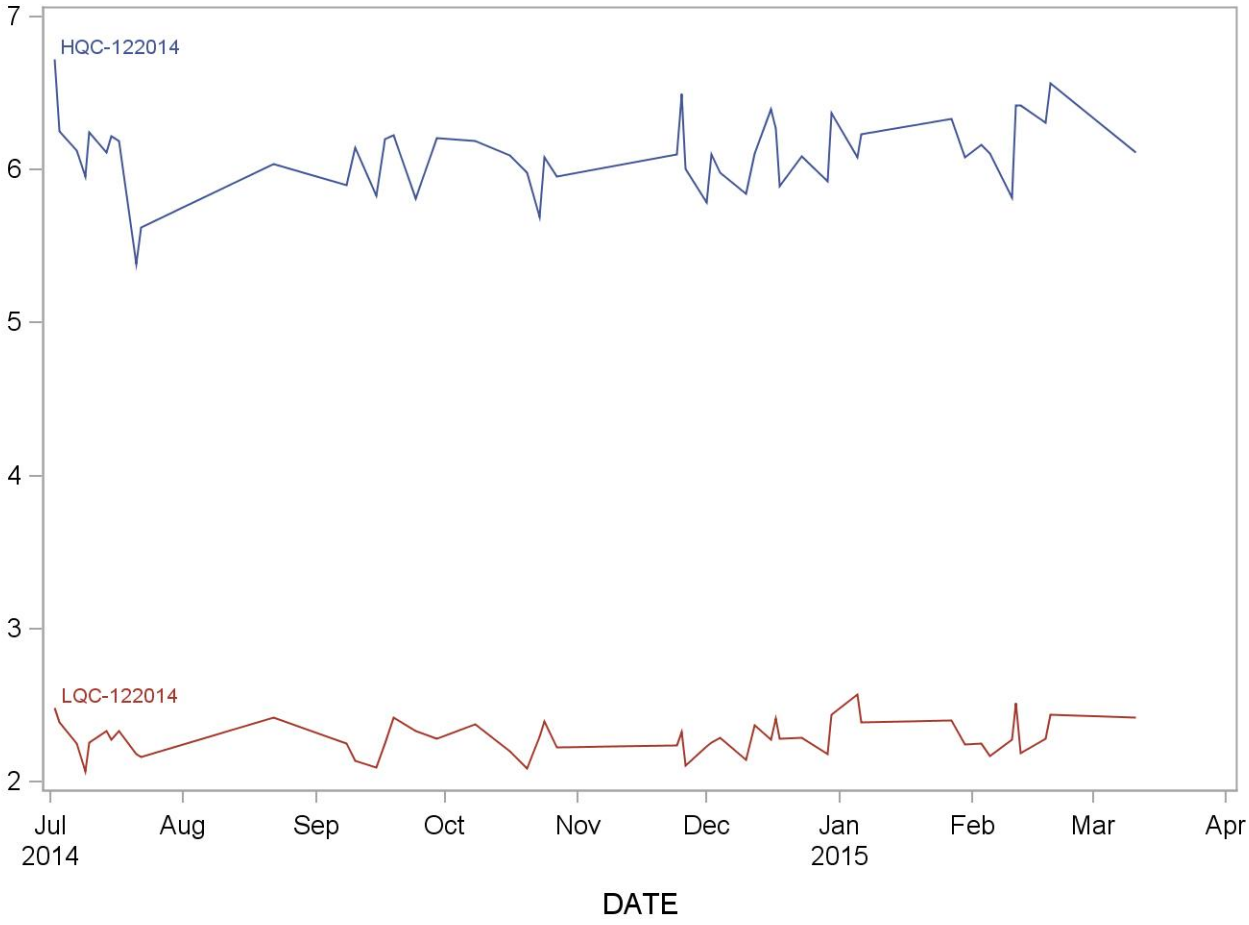
2013-2014 Summary Statistics and QC Chart for Perfluoroheptanoic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.458	0.328	5.1
LQC-122014	50	02JUL14	11MAR15	2.429	0.144	5.9



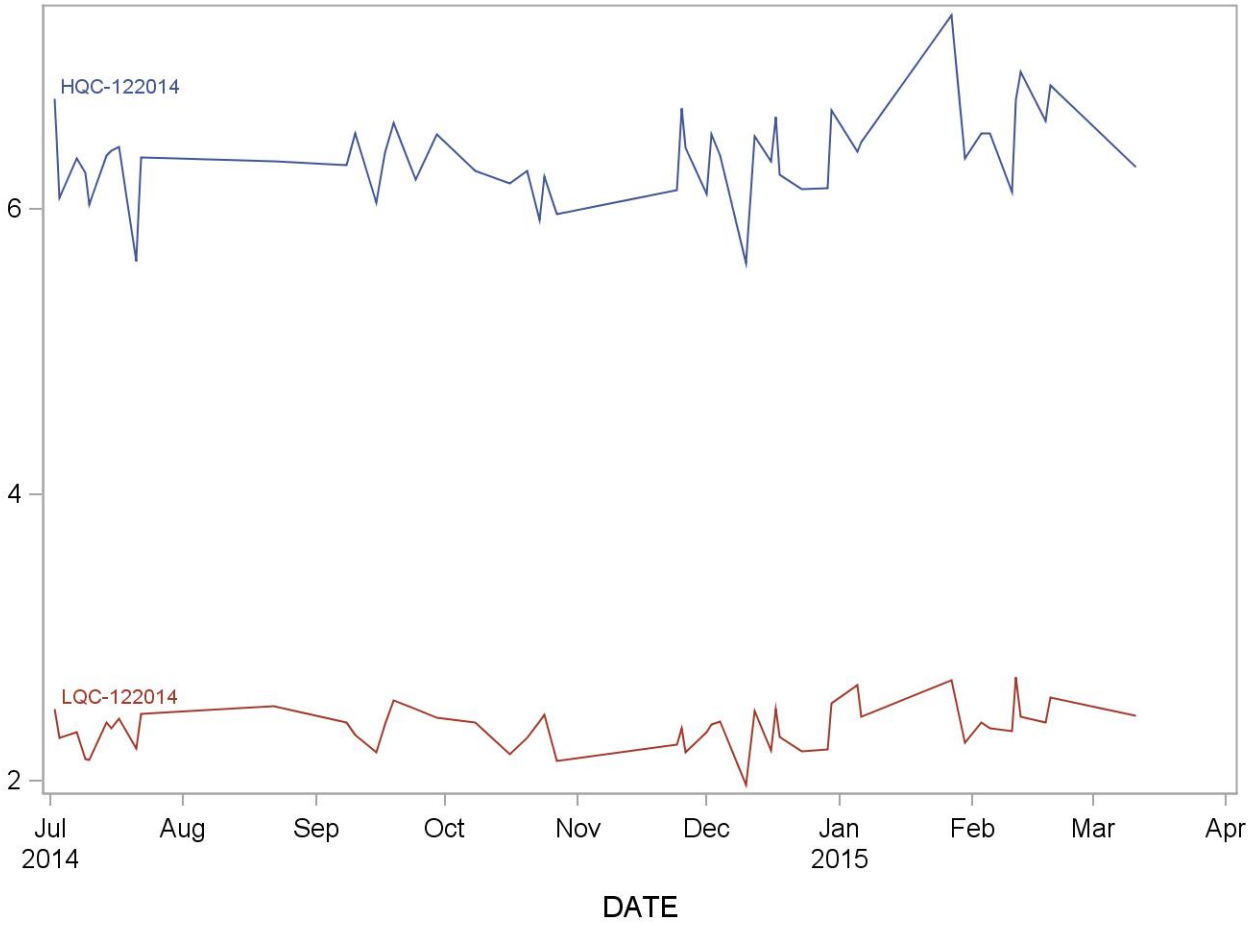
2013-2014 Summary Statistics and QC Chart for Perfluorohexane sulfonic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.105	0.243	4.0
LQC-122014	50	02JUL14	11MAR15	2.291	0.114	5.0



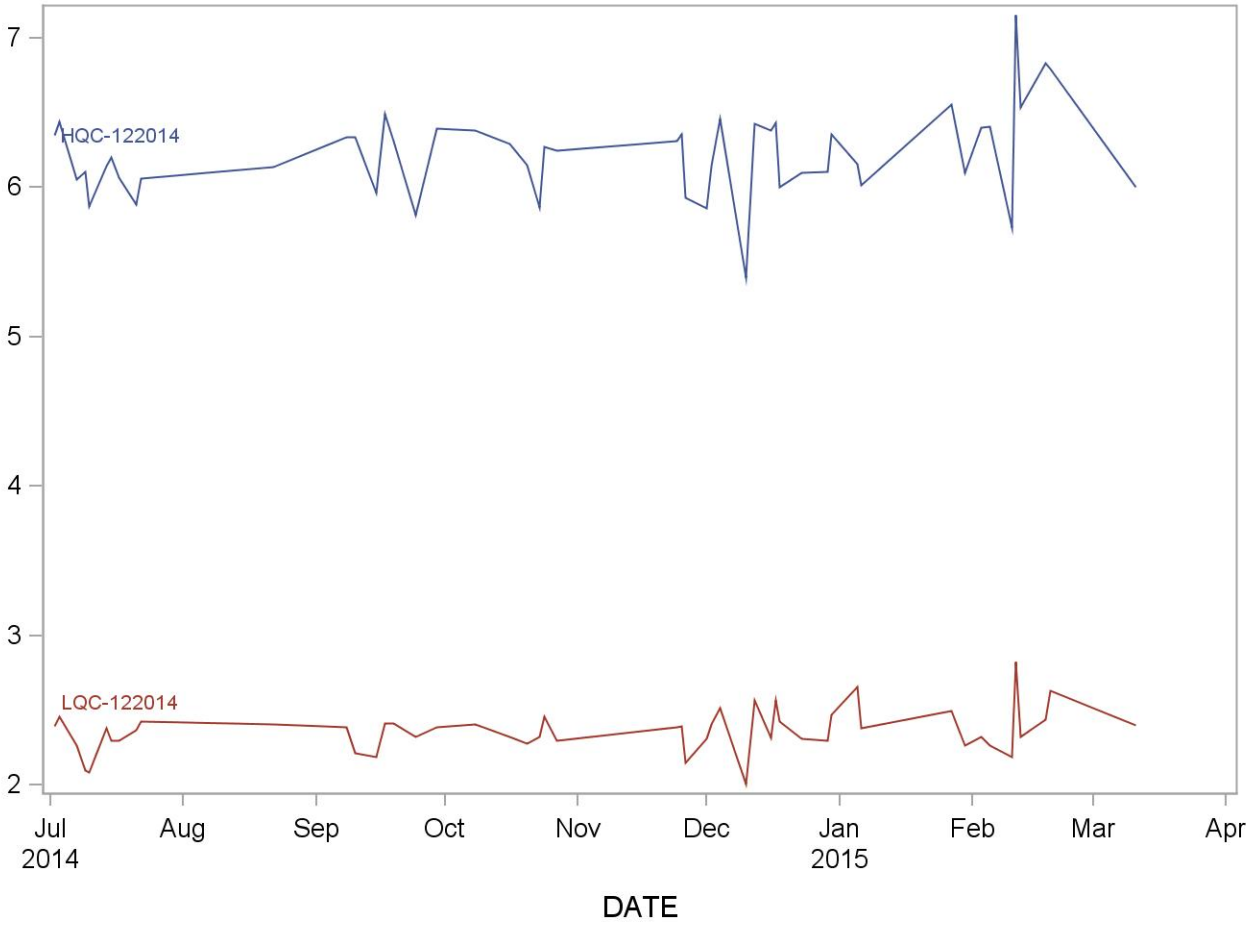
2013-2014 Summary Statistics and QC Chart for Perfluorononanoic acid (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.3701	0.3081	4.8
LQC-122014	50	02JUL14	11MAR15	2.3770	0.1517	6.4



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

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-122014	50	02JUL14	11MAR15	6.223	0.297	4.8
LQC-122014	50	02JUL14	11MAR15	2.364	0.145	6.1



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