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Biomonitoring method for the analysis of chromium and cobalt in human whole blood using inductively coupled plasma - kinetic energy discrimination - mass spectrometry (ICP-KED-MS)

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Abstract

The Centers for Disease Control and Prevention developed a biomonitoring method to rapidly and accurately quantify chromium and cobalt in human whole blood by ICP-MS. Many metal-on-metal hip implants which contain significant amounts of chromium and cobalt are susceptible to metal degradation. This method is used to gather population data about chromium and cobalt exposure of the U.S. population that does not include people that have metal-on-metal hip implants so that reference value can be established for a baseline level in blood. We evaluated parameters such as; helium gas flow rate, choice and composition of the diluent solution for sample preparation, and sample rinse time to determine the optimal conditions for analysis. The limits of detection for chromium and cobalt in blood were determined to be 0.41 and 0.06 μg/L, respectively. Method precision, accuracy, and recovery for this method were determined using quality control material created in-house and historical proficiency testing samples.

We conducted experiments to determine if quantitative changes in the method parameters affect the results obtained by changing four parameters while analyzing human whole blood spiked with National Institute of Standard and Technology traceable materials: the dilution factor used during sample preparation, sample rinse time, diluent composition, and kinetic energy discrimination gas flow rate. The results at the increased and decreased levels for each parameter were statistically compared to the results obtained at the optimized parameters. We assessed the degree of reproducibility obtained under a variety of conditions and evaluated the method's robustness by analyzing the same set of proficiency testing samples by different analysts, on different instruments, with different reagents, and on different days.

The short-term stability of chromium and cobalt in human blood samples stored at room temperature was monitored over a time period of 64 hours by diluting and analyzing samples at different time intervals. The stability of chromium and cobalt post-dilution was also evaluated over a period of 48 hours and at two storage temperatures (room temperature and refrigerated at 4°C).

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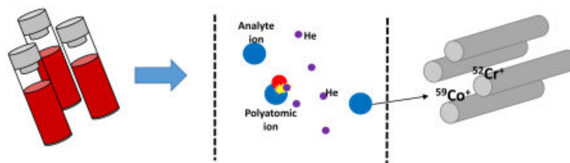
The results obtained during the stability studies showed that chromium and cobalt are stable in human blood for a period of 64 hours.

Graphical Abstract

An ICP-MS method to measure total chromium and cobalt in whole blood is validated and described.



Introduction

Chromium (Cr) is a naturally occurring element whose nutritional bioavailability and toxicity depends on its oxidation state. Trivalent chromium is considered an essential nutrient, while hexavalent chromium is a human carcinogen and a commonly encountered occupational hazard for humans.[1, 2] Cobalt (Co) is considered essential because it is part of the B12 vitamin which is important for human brain function, nervous center function, and cell metabolism.[3, 4] While it is essential at certain lower levels, exposures to high levels of cobalt can affect the heart and/or lungs. Elevated exposures in animals have been shown to affect the liver and kidneys. Research performed by the International Agency for Research on Cancer, in which direct contact with cobalt occurred, led the Agency for Toxic Substances and Disease Registry (ATSDR) to list cobalt as a possible carcinogen to animals.[3] Chromium and cobalt play important roles clinically in cases where metal-on-metal (MoM) hip implants fail and release these metals into the bloodstream. It is not known if the health effects of these two metals seen in animals will also be seen in humans. This uncertainty causes additional concerns for the health and safety of individuals with MoM hip implants that have failed and begun to degrade.

Once degradation of a MoM hip implant occurs, elevated levels of chromium and cobalt are seen in the bloodstream. In some instances, the increase can be up to ten times the levels seen in individuals without MoM implants.[5] Inflammation, carcinogenic and teratogenic effects, and allergic reactions have been linked to the accumulation of these metals in the body.[6] One step in gaining information about the effects of higher chromium and cobalt concentrations in the bloodstream resulting from failed MoM implants is determining the typical levels of these metals in the affected population. Biomonitoring data for these metals are needed for use as a baseline comparison. The reference values (Table 1) provided in the literature for these metals in whole blood are based on smaller scale studies, which typically target a small population for a short period of time within a narrow geographic area.[4, 7-10] As a result, the analytical results obtained are not reflective of typical values for the U.S. population as a whole. One avenue for gaining the required biomonitoring data is through the analysis of specimens collected in the National Health and Nutrition Examination Survey (NHANES). NHANES is used to assess the health and nutritional status of the United States

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population. Biomonitoring data about environmental exposures to numerous chemicals are obtained through this survey and made publicly available in the year following the end of each two-year NHANES cycle. The MoM implant degradation issues prompted the incorporation of chromium and cobalt data collection into the 2015–2016 NHANES survey cycle. With this a baseline of the US population exposure values for cobalt and chromium are established through the analysis of NHANES specimens by our laboratory. Researchers can then begin to more accurately correlate certain adverse health effects with certain levels of exposure.

Before sample analysis collection could begin, our laboratory had to develop an analytical method that could accurately quantify these two metals in whole blood. CDC laboratories have previously developed and published work of human whole blood analysis by ICP-MS to measure other elements[11-13]. We used the method developed by Jones *et al.*[13] in our laboratory for the selection of the sample preparation procedure. The sample preparation in alkaline dilutions is very straightforward and allows for direct sample analysis without any extra step like centrifugation. Previous methods of analyzing chromium in whole blood included the usage of atomic absorption spectrometry (AAS) or sector field inductively coupled plasma mass spectrometry (ICP-MS).[14] However, both of these techniques have their limitations and drawbacks. AAS does not provide the detection limits needed to analyze these metals at the trace levels needed for biomonitoring purposes, and it does not allow for the measurement of multiple analytes simultaneously.[10] When it comes to the detection of low level trace and toxic elemental analysis, ICP-MS is the method of choice given its sensitivity, multi-elemental analysis, high sample throughput and the ability to efficiently reduce and/or remove interferences by collision or reaction cells. Sector field ICP-MS is not a preferred technique to handle the sample throughput demands of a biomonitoring method because of the complexity and cost of the instrumentation.

The analysis of chromium and cobalt in blood matrices by quadrupole ICP-MS can be problematic due to polyatomic interferences. The interferences come from a number of different sources such as the sample matrix itself, the matrix of the reagents used for analysis, or the argon plasma[15]. Elimination or reduction of argon-based polyatomic interferences can take place in the collision cell by Kinetic Energy Discrimination (KED) using helium gas as the collision gas or in a reaction cell by reaction with a reactive gas like ammonia.[16] These options are available for most commercial ICP-MS. The collision cell is pressurized with helium gas which collides with the ions. Such collisions will occur more frequently with larger polyatomic ions than smaller atomic analyte ions, hence, lowering the kinetic energy of the polyatomic ions relative to the atomic ions. The cell is maintained at a voltage more negative than the analyzing quadrupole creating the KED. The faster moving analyte ions are able to overcome it while the larger polyatomic ions are not. As a result, the analyte ions are separated from the interference ions.

In this paper, we discuss the biomonitoring method that we developed which uses quadrupole ICP-MS to rapidly and accurately quantify Co and Cr in human whole blood samples while eliminating the potential interferences that exist with the analysis of samples of this nature. The method is designed to support both small and large biomonitoring studies as well as epidemiological studies of possible exposures of public health significance. To

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ensure the accuracy of the measurements reported, our laboratory also focuses on lot screening of the devices used in the sample collection, storage, and preparation processes to ensure that background levels of the analytes, particularly chromium, are not present in the items used for sample collection, storage, preparation or analysis of the samples for the NHANES program. We validated our method by looking at accuracy, precision, recovery, robustness, ruggedness, short-term stability and other parameters by using proficiency testing specimens obtained from the Centre de Toxicologie du Quebec (CTQ), historical proficiency specimens obtained for the New York Department of Health's (NYDOH) proficiency testing program, in-house quality control (QC) and Seronorm reference material.

Experimental

Reagents

We prepared all reagents using 18 MΩ·cm water from AQUA Solutions® water purification systems (Jasper, GA). Triton® X-100 (Sigma Aldrich, St. Louis, MO), tetramethylammonium hydroxide (TMAH, 25% in H_2O, Alfa Aesar), ethanol (Pharmco Products Inc., Brookfield, CT), and ammonium pyrrolidine dithiocarbamate (APDC, Fisher Scientific) for the diluent/carrier and rinse solutions. We used ethanol, nitric acid (HNO_3, environmental grade, GFS Chemicals, Columbus, OH) and double distilled hydrochloric acid (HCl, 30–35%, GFS Chemicals Inc.) to investigate potential interferences. We purchased “Base blood,” human whole blood with EDTA, for matrix matching of calibration curve from Tennessee Blood Services. We used single element stock standards from various sources (Inorganic Ventures, Christiansburg, VA, NIST Standards, Gaithersburg, MD, SPEX CertiPrep, Metuchen, NJ, and High-Purity Standards, Charleston, SC) for the preparation of intermediate calibration standards in 2% v/v HNO_3 and 1% v/v HCl. All calibration standards are traceable to the National Institute for Standards and Technology (NIST, Gaithersburg, MD). Gallium (Ga) and scandium (Sc) are used as internal standards to allow for the correction of instrument noise and drift as well as sample-to-sample matrix differences. These elements were selected based on the fact that Sc and Ga are close to the mass range of the target analytes and that their concentration in whole blood is negligible or non-existent.[4] We prepared the internal standard intermediate for the Ga and Sc mixture in 1% v/v HNO_3. Sample diluent and carrier solutions consisted of 20 μg/L Ga, 20 μg/L Sc, 0.01% APDC, 0.4% (v/v) TMAH, 1% ethanol, and 0.05% Triton X-100. The rinse solution consisted of 0.01% APDC, 0.4% (v/v) TMAH, 1% ethanol, and 0.05% Triton X-100 as described previously.[13]

Calibrators and Sample Preparation

We prepared the Cr and Co spiked intermediate calibrations standards (S1-S8) with Cr and Co concentrations of 0.5, 1.5, 5.0, 7.5, 15, 45, 75, 100 μg/L in 2% HNO_3/1%HCl using the 1000 μg/mL stock analytical standards purchased from the vendor. From these solutions, two sets of matrix-matched working calibrators (S0-S5) and (S0, S5-S8) are prepared using a Hamilton Diluter by dispensing 0.25 mL of the intermediate standard, 0.25 mL of base blood, and 4.5 mL of diluent. Calibrator S0 is used as the blank for all working calibrators. A reagent blank is prepared using DI water instead of whole blood for patient samples, quality control and reference materials. We screened for Cr and Co the whole blood

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purchased from Tennessee Blood Services before using it as base blood to make sure that it didn't contain Cr and Co. More specific information for the calibration curve can be found in the Calibration, Limits of Detection and Carryover section under Results and Discussion. The unknown blood specimens, in-house quality control (QC), and PT samples were prepared by mixing 0.250 mL of the unknown blood specimen, 0.250 mL of DI H_2O, and 4.5 mL of diluent. All prepared aliquots are a 20× dilution of blood.

Quality Control (QC) and Reference Materials

We used four levels of blood-based bench QC materials with the concentrations of two levels (named low and high) falling within the boundaries of the regular calibration curve and the concentrations of the other two levels falling within the boundaries of the extended calibration curve. The bench QC materials were analyzed at the beginning of each analytical run and again at the end. Our laboratory used modified Westgard rules as detailed in Caudill *et al.*[17] to determine the acceptance or rejection of each analytical run from a quality control perspective. We established the bench quality control limits by characterization of data from 52 analytical runs over a three month time span. We assessed method accuracy by preparing two samples that were spiked with NIST standard reference materials (SRM) 3112a (Chromium Standard Solution) and SRM 3113 (Cobalt Standard Solution) so that the resulting concentrations were 3 μg/L and 11 μg/L for Cr and Co, respectively. In addition, we used reference materials from the Québec Multielement External Quality Assessment Scheme (QMEQAS), Institut National de Santé Publique Québec, and Seronorm™ Trace Element Whole Blood (SERO AS, Norway) during method development and validation.

Instrumentation

We used a Thermo® iCAP™ Qa (upgraded to an iCAP Qc) ICP-MS with collision cell (QCell™) (Thermo Fisher Scientific) equipped with a peristaltic pump, a PFA-ST MicroFlow Nebulizer, a peltier-cooled cyclonic spray chamber, a 2.0 mm quartz injector, platinum sampler cone, platinum skimmer cone, and skimmer cone insert 3.5 mm for all experiments. The instrument parameters are listed in Table 2. We used a SC-4DQ FAST autosampler (Elemental Scientific Inc., Omaha, NE) for the uptake of diluted blood samples for analysis and to control the FAST sample introduction timing. See Table S1 in the supplemental information (SI) for detailed information on the FAST method. Our laboratory used > 99.999% argon (Specialty Gases Southeast, Atlanta, GA) for the plasma and nebulizer gases. We operated the collision cell in KED mode using helium (99.999% grade, Airgas South) gas. We analyzed base blood samples for 20 minutes prior to all experiments and sample analysis to stabilize the collision cell. We prepared final dilutions (i.e. working calibrators, QC, and unknown samples) for analysis using a Hamilton Microlab 625 Advanced Dual Syringe Diluter (Hamilton Company, Reno, NV) equipped with a 10 mL dispensing syringe and a 500 μL sampling syringe.

Statistical Analyses

A mixed effect of the analysis of variance (ANOVA) was used to examine ruggedness. More specifically, the effect of small variations in these specific parameters: ethanol, KED gas flow, rinse time, TMAH, and sample dilution. We assumed that there were random effects from the vial (triplicates or quadruplicate from the same vial). Least square means from the

variations of the parameters were compared with the standard condition, and a p-value <0.05 denoted a statistically significant difference.

We examined the short-term stability of Cr and Co in blood by analyzing QC materials at different conditions: room temperature, 48-hour test, refrigeration, and freeze-thaw cycles. Equal means under different conditions were tested using one-way mixed effect ANOVA models. We assumed that there were random effects from time or cycle (set as the category variable) and also from vial (duplicates or triplicates from the same vial). The scenarios with a p-value <0.05 had a statistically significant difference among the conditions that we tested. For the scenarios with a significant p-value, further statistical comparisons were performed.

Results and Discussion

Specificity

As previously mentioned, the analysis of Cr and Co in blood matrices by ICP-MS can be problematic due to polyatomic interferences. The interferences come from a number of different sources such as the sample matrix, the matrix of the reagents used for analysis, and/or the argon plasma.[15] Since the interferences are the same mass as the analyte being measured, proper interference removal is necessary to ensure analytical specificity, meaning that only the analyte of interest is being quantified. To assess the specificity of chromium and cobalt in this method, we examined the potential interferences that could be present during ICP-MS analysis. To determine if the potential interferences were significant, we determined background equivalent concentrations (BEC) for Cr and Co during both standard mode (no interference removal used) and KED mode (used helium gas for interference removal) analysis by preparing spiked solutions with concentrations based on the maximum levels of the potential interference expected in human whole blood.[4] The elimination or reduction of argon-based polyatomic interferences takes place in the collision cell by KED using helium gas as the collision gas. For Cr at m/z 52 in standard mode, significant levels of interference from chlorine (Cl), oxygen (O), hydrogen (H), nitrogen (N), argon (Ar), carbon (C), sulfur (S) and vanadium (V) in these species $^{35}Cl^{16}O^{1}H^{+}$, $^{40}Ar^{12}C^{+}$, $^{37}Cl^{15}N^{+}$, $^{34}S^{18}O^{+}$, $^{36}S^{16}O^{+}$, $^{35}Cl^{17}O^{+}$,$^{1}H^{51}V^{+}$ are expected.[18] When our laboratory simulated the component concentrations of the blood matrix by spiking solutions with sulfur (500,000 μg/L), no interference was observed for Cr at m/z 52 from $^{34}S^{18}O^{+}$ or $^{36}S^{16}O^{+}$ polyatomic interferences. For the other interferences, we observed a level of interference in the standard mode. This was observed in the BEC values obtained from spiked solutions of Cl (1% HCl), calcium (Ca) (200,000 μg/L), and V (100 μg/L). In the case of Co at mass-to-charge ratio (m/z) 59, interference from calcium, oxygen, hydrogen, magnesium (Mg), chlorine, nitrogen, scandium (Sc), nickel (Ni), argon and sodium (Na) in these species $^{43}Ca^{16}O^{+}$, $^{42}Ca^{16}O^{1}H^{+}$, $^{24}Mg^{35}Cl^{+}$, $^{14}N^{45}Sc^{+}$, $^{1}H^{58}Ni^{+}$, $^{36}Ar^{23}Na^{+}$, and/or $^{40}Ar^{18}O^{1}H^{+}$ is expected.[18] No interferences were observed from $^{24}Mg^{35}Cl^{+}$, $^{14}N^{45}Sc^{+}$, or $^{1}H^{58}Ni^{+}$ when solutions were spiked with Mg (50,000 μg/L), Cl (1% HCl), Sc (100 μg/L) and Ni (100 μg/L). The BEC obtained for Ca and Na in standard mode showed some degree of interference. Overall, for some of the elements, BEC values were obtained in the standard mode; therefore, KED mode using helium at a flow rate of 5.0 mL/min was used for the

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removal of these interferences. Initially, the helium gas flow rate used during the KED mode was optimized by the ICP-MS software for ^{51}ClO interference. In addition it was observed in the literature that the He gas for the collision cell fell between 4.0-5.0 mL/min[9, 14, 19, 20]. After the instrument optimization, the He gas flow rate was further evaluated for Cr and Co by looking at their intensities while in matrix solutions at flow rates between 4.0-6.0 mL/min. It was determined that 5.0 mL/min was the best flow rate for the removal of the interferences. The data in table 3 substantiates the idea that the use of the collision cell significantly reduces the level of interferences present in human blood coming from Cl, S, V, and Ca for Cr at m/z 52 and from Ca and Na for Co at m/z 59. These results show that KED mode is necessary for ICP-MS analysis of Cr and Co in blood with this analytical method.

Calibration, Limits of Detection and Carryover

After optimization of the operating parameters, our laboratory determined the analytical response characteristics for chromium and cobalt. We used the reference ranges in Table 1 to determine the analyte concentrations to be covered by our method during the method development process. For this reason, we determined that two separate calibration curves would be used for sample analysis. The regular calibration (0.5, 1.5, 5.0, 7.5, 15 μg/L) covers analyte concentrations up to 15 μg/L. We anticipated that the majority of the samples analyzed would most likely fall below 15 μg/L based on the reference ranges found in the literature (Table 1). The extended calibration (15, 45, 75, 100 μg/L) covers samples with analyte concentrations above our normal threshold of 15 μg/L but below 100 μg/L. With both calibration ranges, linear responses and 0.9950 coefficients of correlation (R^2 values) were obtained for Cr and Co response function.

Limit of detection (LOD) determinations were based on the analysis of four concentration levels (matrix blank and first three calibrators) from 60 analytical runs over three months. For this method, the matrix blank was used to satisfy the criterion of having a level below the LOD while the first three calibrators of the standard calibration curve were used for the other three levels. The method used to calculate the LODs is based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI)[21] and take both Type I and Type II errors into consideration. For linear relationships the following equation is used to calculate the LOD: $conc_{LOD} = [mean_b + 1.645*(S_b + B)] / (1-1.645*A)$ where $mean_b$ is the mean of the blank, S_b is the standard deviation of the blank, B is the y-intercept, and A is the slope. For quadratic relationships ($y=Ax^2 + Bx+C$), the following equation is used to calculate the LOD: $conc_{LOD} = [-b \pm sqrt\ (b^2 -4ac)] / 2a$ where $a=1.645*A$, $b= (1.645*B)-1$, and $c= mean_b + 1.645*(S_b+C)$. With both linear and quadratic relationships, the resulting calculated LOD has a 5% false negative rate. A linear relationship was the best fit with this analytical method, and the limits of detection for chromium and cobalt in whole blood were determined to be 0.41 μg/L and 0.06 μg/L, respectively.

We evaluated analyte carryover multiple days by alternating the analysis of blood samples (the highest calibrator in extended curve) containing approximately 100 μg/L of each analyte and aqueous blanks over the period of ~3.5 hours (analysis time of 2 minutes per sample with 98 samples analyzed). The intensities and resulting concentrations of both analytes in the 100 μg/L sample were stable over the full 3.5 hour run. (Figure S1a-b). The data for the

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aqueous blanks showed a spike in Co and Cr intensities at sample 33 bringing the calculated Co concentration of that sample to 0.74 μg/L which was above the cobalt limit of detection (LOD) of 0.06 μg/L. The Cr concentration remained below LOD of 0.41 μg/L (Figure S1c-d). Our laboratory decided to limit the number of patient samples in an analytical run to 30 samples, thereby ending the analytical run before the point where an intensity spike would potentially be observed. For the next run a new calibration curve and QC material needs to be prepared.

Precision, Accuracy, and Recovery

To ensure the method's short-term and long-term reproducibility, we evaluated CTQ proficiency samples and in-house CDC quality control (QC) samples. The short term precision was evaluated by analyzing 19 CTQ proficiency testing samples in one analytical run. All of the results for chromium and cobalt were within the allowable ranges established by the PT program as summarized in Table 4. Several samples had recoveries slightly outside of the 90–110% range; however, average recoveries for chromium and cobalt were 99 ± 9% and 101 ± 12%, respectively. We determined the analyte concentrations in the in-house QC samples by characterizing “low” and “high” blood quality control pools spiked with these analytes. Table 5 summarizes the calculated results for the QC samples over 50 analytical runs for low and high QC samples and over 30 runs for both elevated QC levels. For chromium and cobalt, the among-run precision for the low (Co 1.66 μg/L and Cr 2.58 μg/L) and high (Co 8.89 μg/L and Cr 12.3 μg/L) QC are <13% and <7% relative standard deviation (RSD), respectively, over 52 analytical runs over a time period of 2 months.

Our laboratory evaluated accuracy and recovery by analyzing aliquots from two pools of human whole blood materials which were previously spiked with known amounts of chromium and cobalt. We spiked one pool so that the resulting concentration of each analyte was 3 μg/L and the other pool to have a resulting concentration of 11 μg/L for each analyte. NIST SRM 3112a (Chromium Standard Solution) and SRM 3113 (Cobalt Standard Solution) were used for the spiking. The samples were analyzed with different sets of calibrators (different lots from different vendors). Bench QC material, CTQ samples, and New York Department of Health (NYDOH) proficiency samples were also evaluated in terms of accuracy and recovery.

We generated correlation and bias plots containing acceptable results from the analysis of the various types of samples (NYDOH, CTQ and Seronorm) used them to evaluate accuracy and recovery for Cr and Co (Figure S2a-d). A slight, positive concentration bias was seen with cobalt, particularly with concentrations above 2 μg/L. With NYDOH proficiency testing materials, the positive bias was more pronounced in comparison to the bias in CTQ and Seronorm materials (Table S2-S3). The percent bias (% bias) seen with the NYDOH samples for Co range between 0 – 29 % bias and for Cr between 0 – 46 % bias (with the exception of an outlier sample at 100% bias). The correlation bias was also more pronounced with the Seronorm material than the CTQ material. A contributing factor to this bias is that unlike the CTQ samples which are made from human whole blood, the NYDOH materials are made from caprine (goat) blood and the Seronorm is made from lyophilized blood. NYDOH and CTQ results are based on the robust average obtained by employing

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algorithms described in ISO 13528:2005 guidelines from the results reported by all participating laboratories in the event. In our study, we use human whole blood for all of our analyses, but other laboratories most likely use caprine blood to make calibration and QC materials because of the difficulty in obtaining human blood. With all of the materials analyzed, our results were within the acceptability ranges specified by the vendors. A summary of the chromium and cobalt results for the spike solutions, QC samples, CTQ samples, PT samples, and reference materials is provided in Tables 6 and 7.

Ruggedness

We tested the capacity of our method to remain unaffected by small variations in specific parameters. Meaning, we wanted to determine how much the accuracy varies with changes to five method parameters likely to affect accuracy. This assessment is part of our method validation as stated in the CDC Division of Laboratory Sciences Policies and Procedures Manual. We tested five parameters through the analysis of high bench QC samples: the sample rinse time, the percentage of TMAH and ethanol in the diluent, and the KED gas flow rate. Tables S4–S8 show the analytical results obtained for the high bench QC that was analyzed during the assessments of the different parameters and the results for the statistical evaluations performed on this data. A mixed effect of ANOVA was used to check for a statistically significant difference between the two settings (standard value and lower or higher parameters). A p-value less than 0.05 indicates that there is a statistically significant difference between the two settings. Table 8 shows the p-values for the parameters examined.

With our analytical method, the dilution factor used for sample preparation is 20×. We evaluated dilution factors of 15× (25% lower than the typical dilution factor) and 25× (25% higher than the typical dilution). For both analytes, statistical evaluation of the results showed a statistical significance ($p < 0.05$) between samples prepared with the normal dilution factor and samples prepared with the smaller / higher dilution factors. When comparing the results obtained to the characterized QC results (target mean and ±2SD range), we found that the results using 15× and 25× dilutions were not within our QC acceptance limits; therefore, the statistical significance seen is valid. We concluded that the method does not provide acceptable results at dilution factors outside of the established level for this method.

The autosampler rinse time used in our established method is 33 seconds. Rinse times of 24 seconds (approximately 27% lower than the typical rinse time) and 39 seconds (approximately 18% higher than the typical rinse time) were evaluated. For both analytes, statistical evaluation of the results showed a statistical significance ($p < 0.05$) between the normal rinse time and the shorter rinse time. When comparing the least squares means to the characterized QC results (target mean and 2SD range), we found that the results using a 24-second rinse time were well within our QC acceptance limits. The presence of a statistical significance does not translate into scientific significance in this case. A statistically significant difference was not found between the normal rinse time and the longer rinse time. We concluded that our method is robust enough to withstand fluctuations in the rinse time.

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The percentage of TMAH in the diluent in the established method is 0.4%. We evaluated diluent TMAH percentages of 0.3% (25% lower than the typical TMAH percentage in the diluent) and 0.5% (25% higher than the typical TMAH percentage in the diluent). For both analytes, statistical evaluation of the results showed statistical significance ($p < 0.05$) between the normal and lower diluent TMAH percentages for Cr, the normal and lower TMAH percentages for Co, and the normal and higher TMAH percentages for Co. When comparing the least squares means to the characterized QC results (target mean and 2SD range), we found that the analytical results were well within our QC acceptance limits. In this test, the presence of a statistical significance does not translate into scientific significance. A statistically significant difference was not found between the results from the diluents with normal and higher TMAH percentages for Cr. We concluded that our method is robust enough to withstand fluctuations in the percentage of TMAH in the diluent.

Further, the percentage of ethanol in the diluent in the established method is 1%. We evaluated diluent ethanol percentages of 0.8% (20% lower than the typical ethanol percentage in the diluent) and 1.2% (20% higher than the typical ethanol percentage in the diluent). For both analytes, statistical evaluation of the results showed a statistical significance ($p < 0.05$) between the normal and increased ethanol amounts for Cr and Co. When comparing the least squares means to the characterized QC results (target mean and 2SD range), we found that the results were well within our characterized QC acceptance limits for both analytes. In this test, the presence of a statistical significance does not translate into scientific significance. A statistically significant difference was not found between results from the diluents with normal and lower ethanol percentages for either analyte. We concluded that the method is robust enough to withstand fluctuations in the percentage of ethanol in the diluent.

The KED gas flow used in the established method is 5 mL/minute (min). Our laboratory evaluated gas flows of 4 mL/min (20% lower than the typical gas flow) and 6 mL/min (20% higher than the typical gas flow). For both analytes, statistical evaluation of the results showed statistical significance ($p < 0.05$) between the normal gas flow rate and the higher gas flow rate. There was also a statistically significant difference between the normal gas flow rate and the lower gas flow rate for Cr but not Co. It is important to note the statistical significance does not necessary signify scientific importance. Since chromium least squares means of13.02, 12.25, and 12.08 μg/L for 4, 5, and 6 mL/min gas flow, respectively and cobalt least square means of 8.99 and 9.11 μg/L for 5 and 6 mL/min, respectively are well within our QC acceptance limits (Cr - 12.26 ± 1.2 μg/L and Co - 8.89 ± 0.94 μg/L, mean ± 2SD), we conclude that there is no definitive evidence that one flow rate is better than another in range of 4-6 mL/min. However, we chose 5 mL/min KED gas flow as the optimum condition for this parameter since Cr and Co concentrations came the closest to the QC target mean (which also correlates the closest with the actual amount of Cr and Co spiked into the QC pool). Additionally, it was determined that 5.0 mL/min was the best flow rate for the removal of the interferences (specificity section).

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Robustness

The degree of reproducibility obtained under a variety of conditions within the laboratory were assessed using the Pearson Correlation Coefficient, r. The method was evaluated by analyzing the same set of proficiency testing samples by different analysts, on different instruments, with different reagents (different calibrator lots), and on different days. The r for each analyte under each condition was calculated to be greater than 0.99 (values larger than 0.95 signify there is no difference between two variables), Table 9, meaning no statistically significant differences were found in Cr and Co concentrations from two different analysts, instruments, calibrators, and analysis time. We conclude that the developed method passes robustness testing and is stable.

Linearity

To evaluate the linearity of this method, we analyzed spiked blood samples with concentrations outside of the regular and extended calibration ranges. We analyzed three base blood samples spiked with 20, 50, and 100 μg/L of each analyte using the regular calibration curve. These samples were analyzed in triplicate on two different days with three different calibration lots. Average measured concentrations and recoveries are listed in Table S9. Figures 1 (a) and (b) display two linear curves. Curve (a) is a plot of the five regular calibration standards versus their relative ion signal (analyte of interest's intensity divided by the internal standard intensity), plus three spiked samples (20, 50, and 100 μ/L). When examining the two calibration lines, we can assess how linear the calibration is beyond the highest calibration point of 15 μg/L. For both analytes, the two curves have identical slopes and very close correlation coefficients (R^2) values which led us to conclude that the linearity continues beyond the top of the regular calibration range (15 μg/L up to 100 μg/L). It is important to note that average percent recoveries for Co at 20 and 100 μg/L were higher than 110%, Table S9.

To test the linearity of the method beyond the extended calibration range, we analyzed ten spiked base blood samples with concentrations in the range of 150 to 5000 μg/L (Table S10). We analyzed the samples in triplicate on two different days with two calibration lots with the exception of the 150 and 750 μg/L samples which were analyzed in triplicate with one calibration lot. Figures 1 (c) and (d) display two linear curves plus ten elevated samples (see concentrations in Table S10). For both analytes, the two curves (a) and (b) have identical slopes and R^2 values of approximately one. This led us to conclude that the linearity continued beyond the extended calibration range. However, average percent recoveries are higher than 110% for Co over 500 μg/L and for Cr over 1500 μg/L. Additionally the detector switches to the analog mode (higher than 1.5 million counts per second) at these two concentrations. Traditionally, we like to avoid analyzing samples in analog mode if calibration curves are analyzed in pulse detector mode[22]. Per the standard procedures of this method, samples above 100 μg/L would be diluted, and patient results would be reported from the dilution.

Range

The majority of the patient samples that our laboratory would receive for analysis would have concentrations towards the lower end of our calibration range [4, 7-10]. To determine how

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to treat samples with concentrations above 15 μg/L, we looked at two different concentration ranges: samples between 15 μg/L and 100 μg/L and samples above 100 μg/L. The first range is representative of patient samples with MoM implants, and the second range is for extreme cases with dangerous degradation of the MoM hip implants or total failure of the implants[7, 9, 23-30].

For samples between 15 μg/L and 100 μg/L, we tested the possibility of diluting elevated samples with base blood in order to bring them within our established calibration range. Two spiked samples were diluted with base blood: a 25 μg/L sample was diluted 2× and 5×, and a 100 μg/L sample was diluted 10× and 20× (Table S11). Samples were prepared on two different days with three different sets of calibration materials. The average recovery for 100 μg/L samples diluted 10× was approximately 120% for both Cr and Co; therefore, we decided to use an extended calibration curve instead of dilution for samples with concentrations between 15 μg/L and 100 μg/L.

To cover extreme cases where the concentration of one or both analytes can approach or extend into the parts per million range, we explored the analysis of samples with concentrations above 100 μg/L by evaluating dilution of specimens that fall into that category. Dilution was done with deionized (DI) water. We tested a number of sample concentrations and dilution factors were tested to determine the maximum concentration and dilution factor that could be used for this analytical method. The results are shown in Table S12 show that the average recovery for Cr up to 6000 μg/L (100× dilution) was acceptable (within 10% of the target). Co showed acceptable results up to 20,000 μg/L (200×) dilution. However, when this study was repeated, the results were inconsistent.

To investigate this further, we spiked seven elevated samples with standards used to make working calibrators and diluted them with base blood instead of DI water (Table S13). The samples were analyzed in triplicate with three different calibration curves on three different days. For samples with Cr and Co concentrations of 5500 μg/L, a 100× dilution gave recoveries that exceeded 110%. We subsequently spiked two samples (3000 and 5000 μg/L) with standards used to make the working calibrators and diluted these samples 50× with base blood (Table S14). These samples were analyzed in triplicate with four different calibration curves over two days. The recoveries were 102% and 99% for Cr and 100% and 97% for Co, respectively. Examining the data in its entirety, we set our clinically reportable range to 5000 μg/L. A 50× dilution is the maximum dilution that can be performed using base blood and an extended calibration curve.

Analyte Short-Term Stability

We examined the short-term stability of Cr and Co in blood by analyzing QC material at different storage conditions. First, we tested the stability of Cr and Co in undiluted low bench QC at room temperature for 64 hours. Next, we tested analyte stability by prolonging the time between sample preparation and sample analysis. The maximum anticipated delay time before analysis was 48 hours. We evaluated a number of time points at two different temperatures – ambient temperature and 4°C. The stability of the analytes in the matrix was also evaluated for two freeze-thaw cycles. The least squares means under different conditions were tested using one-way mixed effect ANOVA models (Table 10). We made the

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following assumptions: there are random effects resulting from time or cycle (set as the category variable) and random effects resulting from vial-to-vial variation or variation among replicates from the same vials. P-values of < 0.05 indicated a statistically significant difference among the conditions being compared. For scenarios with a significant p-value, further statistical comparisons were performed.

Room Temperature

We stored fifteen undiluted low bench QC samples at room temperature. At three different time points (24, 48 and 64 hrs.), we used five vials to make preparation samples for analysis in triplicate (15 samples to be analyzed at each time point). This experiment covered a 64-hour time period. For Cr, a statistically significant difference was not seen amongst the results ($p=0.7958$), Table 10. However, with Co, a p-value of <0.0001 (Table 10) indicated a statistically significant difference amongst the results at the different time points. As a further comparison, we compared the results at 48 hours with the results at 24 hours and the results at 64 hours with the results at 24 hours. Both revealed a statistically significant difference. When we compared the cobalt results obtained in this stability study to the characterized QC limits, although the majority of the Co results were above the characterized mean, none of the results were outside of +2SD of our characterized mean. Only one third of the results were outside of +1SD. We concluded that while a statistically significant difference did not exist in Cr results across time, a statistically significant difference existed for cobalt. The statistical significance seen with the cobalt results did not have scientific relevance because our recoveries were still within our statistical limits (±2SD) for our QC materials.

48-Hour Test

We diluted twenty-one low bench QC samples in triplicate. We stored the 63 diluted samples at room temperature and analyzed nine samples for each of the different time points over the 48-hour testing period. For both analytes, a statistically significant difference was not seen amongst the results; therefore, we concluded that leaving the digested samples at room temperature for up to two days has no effect on the recovery of the analytes.

Refrigeration

We diluted fifteen low bench QC samples in triplicate. We refrigerated the resulting 45 prepared samples at 4°C. We analyzed groups of 15 samples at each of the three time points over a 48-hour period. For both analytes, a statistically significant difference was seen amongst the results at the different time points. As a further comparison, we statistically compared the results at 24 hours with the results at 4 hours and the results at 48 hours with the results at 4 hours. In the case of Cr, a statistically significant difference was seen between the results at 24 hours and the results at 4 hours. With Co, a statistically significant difference was seen between the results at 48 hours and the results at 4 hours. With both analytes, the results were compared to the characterized QC data for this QC pool, and the results were within our laboratory's QC statistical limits of acceptability (±2SD); therefore, the statistical significance seen does not translate into scientific relevance. It was concluded that diluted samples can remain in the refrigerator for up to two days prior to analysis without adversely affecting the recovery of the analytes.

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Freeze-thaw Cycles

We removed ten low bench QC samples from the freezer, thawed them, prepared aliquots taken from the vials in duplicate, and returned the unused portions of the original 10 samples to the freezer. We analyzed the diluted samples. The following day, we removed the same ten original from the freezer, thawed them, and prepared aliquots taken from the vials in duplicate. We analyzed the diluted samples and statistically compared the results from both days. For Cr, a statistically significant difference was not seen amongst the results ($p=0.7122$). However, with Co, a p-value of <0.0001 indicated a statistically significant difference amongst the results from the two days. To further evaluate cobalt, we compared day one results against day two results, and a p-value of <0.0001 was obtained again. We compared the individual results from both days to the characterized QC mean for Co of 1.66 μg/L, and the results from this experiment ranged from 1.60 μg/L to 1.72 μg/L. All results were within one standard deviation of the mean; therefore, although a statistical significance is present, the difference has no scientific relevance.

Conclusion

We successfully developed a biomonitoring method to accurately and rapidly quantify Cr and Co in human whole blood by ICP-MS operated in KED mode. Because there is no whole blood reference material with certified target values for Cr and Co, the commercially available reference material lyophilized blood Seronorm Trace Elements Whole Blood, proficiency testing samples, and in-house QC were employed and characterized during the day-to-day method validation analysis. We determined that an analytical run will include 30 patient samples in addition to the blanks, calibrators, and quality control materials. We conducted experiments to determine ruggedness by quantitatively altering method parameters to determine if the variations affected the analytical results obtained. We concluded that only changes in the dilution factor during sample preparation had a significant impact on the results because these changes produced resulting QC concentrations outside of our QC acceptance ranges for both analytes. The same set of proficiency testing samples by different analysts, on different instruments, with different reagents, and on different days showed that the method is rugged. Statistical analysis, such as fixed effects linear model, step-down pairwise comparison, and equivalence test confirmed the short-term stability of Cr and Co in human whole blood.

This analytical method is used to support the acquisition of U.S. population data for chromium and cobalt in whole blood by analyzing specimens collected during the 2015–2016 National Health and Nutrition Examination Survey (NHANES) cycle. For chromium and cobalt in blood, NHANES targets exposure for the demographic group comprised of individuals over 40 years old which is the group most likely to have had a MoM implant. When this data is evaluated and made publicly available at the end of the cycle, researchers will be able to compare exposure data for the baseline population with data from individuals who have had an implant to determine if people with the implant have elevated levels of Co or Cr. Hence, researchers can then begin to more accurately correlate certain adverse health effects with certain levels of exposure to these two metals.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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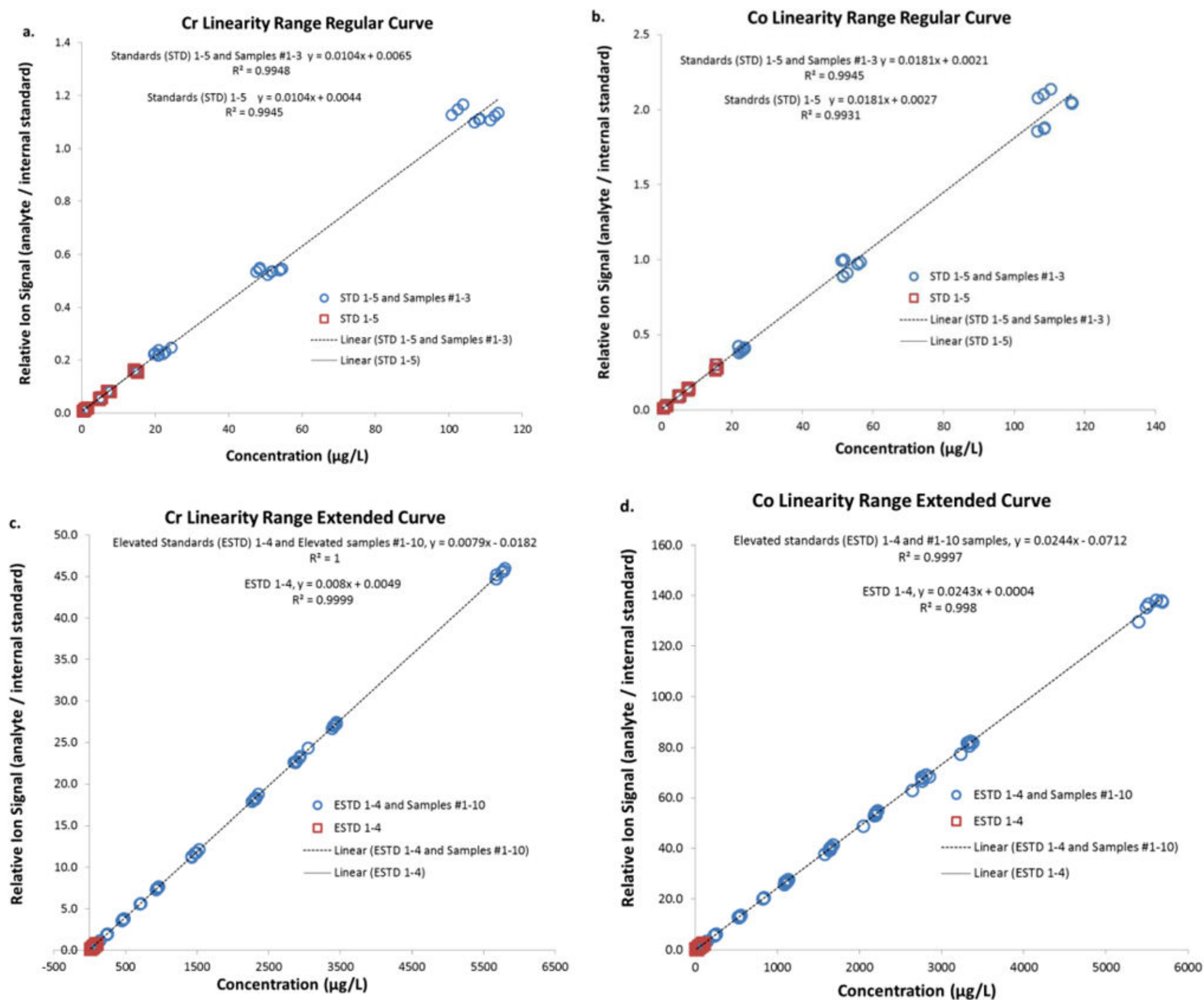

Figure 1.

(a) Regular calibration curve and linearity range for Cr, (b) regular calibration curve and linearity range for Co, (c) extended calibration curve and linearity range for Cr, (d) extended calibration curve and linearity range for Co.

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Table 1

Reference Ranges for Chromium and Cobalt[4, 7-10]

Analyte	Information	Reference Ranges μg L^{-1}
Cr	Healthy adults in fasting state	0.7 to 28.0
Co	(N=64) 9 with no implants	0.224 to 0.565
	National Institute for Research and Safety	0.8
	(N=130) Germany	0.04-8
	French study healthy individuals (N=100)	0.04 – 0.64

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Table 2

Instrument Parameters for the Thermo ICAP-Qc

Plasma Power	1550 W
Cool Flow (Ar)	14 L min^{-1}
Auxiliary Flow (Ar)	0.8 L min^{-1}
Nebulizer Flow (Ar)	~ 0.95 L min^{-1}
KED Mode Gas Flow (He)	5 mL min^{-1}
Method Parameters	
Sweeps/Reading	20
Survey Run	1
Replicates	3
Dwell Times	200 ms for ^{52}Cr and ^{53}Cr, 250 ms for ^{50}Co 100 ms for ^{71}Ga and ^{45}Sc
Scan Mode	Peak Hopping
KED Mode Gas Flow (He)	5 mL min^{-1}
Peristaltic Pump	40 rpm
Sample Loop	2.0 mL
Sample Flush Time	4 s
Read Delay Time	15 s
Wash Time	30 s
Internal Standard	^{71}Ga and ^{45}Sc (20 μg L^{-1})
Calibration Type	External Simple Linear Matrix Matched
Calibration (μg L^{-1})	0.0, 0.5, 1.5, 5.0, 7.5, 15.0
Sample Preparation	20× dilution

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Table 3

Potential interferences and Background Equivalent Concentration (BEC) for Cr and Co in ICP-MS.[15]

		BEC ($\mu g\ L^{-1}$)	
Potential interferences	**Concentration Tested ($\mu g\ L^{-1}$)***	**Standard mode**	**KED Mode**
	Cobalt (^{59}Co)		
$^{43}Ca^{16}O^{+}$, $^{42}Ca^{16}O^{1}H^{+}$	200,000	0.43	0.07
$^{24}Mg^{35}Cl^{+}$	50,000 for Mg / 1% HCl for Cl	0/0	0/0
$^{36}Ar^{23}Na^{+}$	500,000	0.01	0
$^{14}N^{45}Sc^{+}$	5/100	0	0
$^{1}H^{58}Ni^{+}$	100	0	0
$^{40}Ar^{18}O^{1}H^{+}$, $^{40}Ar^{19}F^{+}$, $^{36}Ar^{23}Na^{+}$,	ICP gas	-	-
	Chromium (^{52}Cr)		
$^{35}Cl^{16}O^{1}H^{+}$, $^{37}Cl^{15}N^{+}$, $^{35}Cl^{17}O^{+}$	1% HCl	1.9	0.02
$^{40}Ar^{12}C^{+}$, $^{36}Ar^{16}O^{+}$, $^{38}Ar^{14}N^{+}$, $^{36}Ar^{15}N^{1}H^{+}$	ICP gas	-	-
$^{34}S^{18}O^{+}$, $^{36}S^{16}O^{+}$	500,000	0	0
$^{1}H^{51}V^{+}$	100	0.50	0
$^{40}Ca^{12}C$	200,000	0.16	0.6

*Concentration based on maximum levels expected in human whole blood.[4]

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Table 4

CTQ proficiency testing sample results for chromium and cobalt in a single analytical run

Sample Name	Chromium					Cobalt				
	CDC Value µg/L	Reference Value µg/L	Lower Limit µg/L	Upper Limit µg/L	Recovery %	CDC Value µg/L	Reference Value µg/L	Lower Limit µg/L	Upper Limit µg/L	Recovery %
QM-B-Q1102	1.74	1.44	0.30	2.58	121	3.47	3.01	2.33	3.70	115
QM-B-Q1105	2.40	2.78	1.42	4.13	87	0.34	0.43	0.14	0.72	80
QM-B-Q1108	2.41	2.69	1.39	4.00	90	0.95	0.91	0.55	1.27	105
QM-B-Q1201	4.70	4.40	2.86	5.93	107	1.13	1.07	0.68	1.45	106
QM-B-Q1204	2.31	2.40	1.14	3.66	96	5.92	5.13	4.13	6.14	115
QM-B-Q1207	3.92	3.46	2.06	4.86	113	0.62	0.63	0.31	0.95	97
QM-B-Q1301	2.81	3.01	1.67	4.35	93	0.86	0.86	0.51	1.22	99
QM-B-Q1302	2.34	2.71	1.40	4.01	86	0.37	0.42	0.13	0.71	87
QM-B-Q1307	2.50	2.70	1.42	3.99	92	2.53	2.34	1.76	2.92	108
QM-B-Q1308	4.28	4.38	2.88	5.88	98	0.37	0.45	0.16	0.75	81
QM-B-Q1313	2.49	2.61	1.33	3.88	96	1.64	1.46	1.01	1.90	112
QM-B-Q1314	2.33	2.53	1.26	3.81	92	5.50	4.82	3.87	5.77	114
QM-B-Q1403	2.98	3.26	1.89	4.62	92	4.07	3.65	2.87	4.42	112
QM-B-Q1404	5.49	5.36	3.70	7.02	103	0.67	0.69	0.36	1.02	98
QMEQAS09B-05	1.87	1.81	0.91	2.41	104	5.06	4.64	3.25	5.48	109
QMEQAS09B-08	7.42	7.50	6.00	8.50	99	0.52	0.65	0.33	0.93	80
QMEQAS10B-03	4.42	4.27	2.99	5.13	104	1.44	1.40	0.70	1.78	103
QMEQAS10B-06	1.76	1.60	0.80	2.14	110	8.07	7.40	5.92	8.68	109
QMEQAS10B-09	1.88	1.96	0.98	2.62	96	0.46	0.51	0.26	0.85	91
Average					**99**					**101**
STD dev					**9**					**12**

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Table 5

In-house CDC quality control pool results for chromium and cobalt

Analyte	QC sample	Target Value µg/L	Observed Mean µg/L	STDEV	%RSD	% Bias	% Recovery	N	Analytical Runs	% CV Among Runs	% CV Within Measure
Cobalt	Low QC	1.6	1.66	0.09	5.6	3.8%	104%	104	52	5.6%	3.1%
	High QC	8.6	8.89	0.5	5.3	3.4%	103%	104	52	5.3%	2.2%
	Elevated QC1	24.5	24.5	1.2	4.9	0.0%	100%	70	35	4.9%	2.0%
	Elevated QC2	64.1	63.8	4.3	6.7	-3.3%	100%	70	35	6.7%	2.3%
Chromium	Low QC	2.3	2.58	0.3	12.5	12.1%	112%	104	52	12.5%	9.6%
	High QC	11.9	12.3	0.6	4.9	3.0%	103%	104	52	4.9%	4.3%
	Elevated QC1	34.6	34.9	1.2	3.5	0.9%	101%	70	35	3.5%	3.6%
	Elevated QC2	74.6	72.8	2.7	3.6	-2.5%	98%	70	35	3.6%	3.1%

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Table 6

Summary of accuracy and recovery results for chromium

Sample Type	Sample ID	Target Value µg/L	CDC Overall AVG µg/L	CDC Overall SD	n (number of samples)	CDC Overall Bias	% Bias	CDC %CV	Recovery %
Spiked Samples and Bench QC	3 µg/L	3	2.98	0.29	15	-0.02	-1	9.8	101
	11 µg/L	11	11.17	0.82	16	0.17	2	7.4	98
	Low QC	2.3	2.58	0.37	104	0.28	12	14.2	89
	High QC	11.9	12.26	0.71	102	0.36	3	5.8	97
	Elevated QC1	34.6	34.9	1.5	68	0.3	1	4.3	99
	Elevated QC2	74.6	72.75	3.07	68	-1.85	-2	4.2	103
CTQ and Seronorm	QM-B-Q1102	1.44	1.39	0.36	12	-0.05	-3	25.8	104
	QM-B-Q1105	2.78	2.65	0.49	13	-0.13	-5	18.6	105
	QM-B-Q1108	2.69	2.54	0.38	13	-0.15	-6	15.0	106
	QM-B-Q1201	4.4	4.55	0.32	13	0.15	3	7.0	97
	QM-B-Q1204	2.4	2.42	0.51	13	0.02	1	21.1	99
	QM-B-Q1207	3.46	3.5	0.55	13	0.04	1	15.6	99
	QM-B-Q1301	3.01	3.11	0.31	13	0.1	3	10.1	97
	QM-B-Q1302	2.71	2.48	0.43	13	-0.23	-8	17.3	109
	QM-B-Q1307	2.7	2.67	0.34	13	-0.03	-1	12.6	101
	QM-B-Q1308	4.38	4.63	0.67	13	0.25	6	14.5	95
	QM-B-Q1313	2.61	2.63	0.39	13	0.02	1	14.9	99
	QM-B-Q1314	2.53	2.5	0.36	13	-0.03	-1	14.5	101
	QM-B-Q1403	3.26	3.3	0.48	13	0.04	1	14.7	99
	QM-B-Q1404	5.36	5.61	0.69	13	0.25	5	12.3	96
	QMEQAS09B-05	1.81	1.89	0.39	13	0.08	4	20.7	96
	QMEQAS09B-08	7.5	7.35	0.91	13	-0.15	-2	12.3	102
	QMEQAS10B-03	4.27	4.71	0.82	13	0.44	10	17.5	91
	QMEQAS10B-06	1.6	1.75	0.43	13	0.15	9	24.5	91
	QMEQAS10B-09	1.96	1.95	0.33	13	-0.01	-1	16.7	101
	Seronorm Level 1	0.86	0.69	0.2	14	-0.17	-20	29.9	125
	Seronorm Level 2	11.8	12.7	0.41	11	0.9	8	3.2	93

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Sample Type	Sample ID	Target Value µg/L	CDC Overall AVG µg/L	CDC Overall SD	n (number of samples)	CDC Overall Bias	% Bias	CDC %CV	Recovery %
	Seronorm Level 3	25.46	27.48	1.12	6	2.02	8	4.1	93

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Table 7

Summary of accuracy and recovery results for cobalt

Sample Type	Sample ID	TargetValue µg/L	CDC Overall AVG µg/L	CDC Overall SD	n (number of samples)	CDC Overall Bias	% Bias	CDC %CV	Recovery %
Spiked Samples and Bench QC	3 µg/L	3	3.04	0.12	17	0.04	1	3.9	99
	11 µg/L	11	10.9	0.43	16	-0.1	-1	3.9	101
	Low QC	1.6	1.66	0.1	102	0.06	4	6.0	96
	High QC	8.6	8.91	0.47	100	0.31	4	5.3	97
	Elevated QC1	24.5	24.51	1.25	68	0.01	0	5.1	100
	Elevated QC2	64.1	63.75	4.37	64	-0.35	-1	6.9	101
CTQ and Seronorm	QM-B-Q1102	3.01	3.3	0.28	13	0.29	10	8.5	91
	QM-B-Q1105	0.43	0.34	0.04	13	-0.09	-21	10.7	126
	QM-B-Q1108	0.91	0.96	0.07	13	0.05	5	7.5	95
	QM-B-Q1201	1.07	1.09	0.08	13	0.02	2	7.8	98
	QM-B-Q1204	5.13	5.71	0.39	13	0.58	11	6.9	90
	QM-B-Q1207	0.63	0.62	0.04	13	-0.01	-2	6.8	102
	QM-B-Q1301	0.86	0.89	0.06	13	0.03	3	7.2	97
	QM-B-Q1302	0.42	0.37	0.03	13	-0.05	-12	8.7	114
	QM-B-Q1307	2.34	2.53	0.17	13	0.19	8	6.8	92
	QM-B-Q1308	0.45	0.37	0.03	13	-0.08	-18	8.4	122
	QM-B-Q1313	1.46	1.61	0.12	13	0.15	10	7.8	91
	QM-B-Q1314	4.82	5.44	0.53	13	0.62	13	9.7	89
	QM-B-Q1403	3.65	4.08	0.28	13	0.43	12	6.9	89
	QM-B-Q1404	0.69	0.68	0.06	13	-0.01	-1	8.4	101
	QMEQAS09B-05	4.64	4.98	0.36	13	0.34	7	7.1	93
	QMEQAS09B-08	0.65	0.5	0.06	13	-0.15	-23	12.7	130
	QMEQAS10B-03	1.4	1.43	0.1	13	0.03	2	7.0	98
	QMEQAS10B-06	7.4	8.04	0.54	13	0.64	9	6.7	92
	QMEQAS10B-09	0.51	0.45	0.04	13	-0.06	-12	7.8	113
	Seronorm Level 1	0.16	0.19	0.02	14	0.03	19	10.8	84
	Seronorm Level 2	5.8	6.17	0.43	11	0.37	6	6.9	94

Sample Type	Sample ID	TargetValue μg/L	CDC Overall AVG μg/L	CDC Overall SD	n (number of samples)	CDC Overall Bias	% Bias	CDC %CV	Recovery %
	Seronorm Level 3	11.4	12.77	0.54	8	1.37	12	4.3	89

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Table 8

Statistical evaluation results of comparing five established method parameters to their lower/higher variations

	Ethanol %			KED gas flow (mL/min)			Rinse time (s)			TMAH %			Sample dilution		
	0.8	**1**	**1.2**	**4.0**	**5.0**	**6.0**	**24**	**33**	**39**	**0.3**	**0.4**	**0.5**	**15×**	**20×**	**25×**
Chromium															
Comparison (decreased vs. method norm)	p=0.7707			p<0.0001			p=0.0128			p=0.0002			p<0.0001		
Comparison (increased vs. method norm)			p<0.0001			p=0.008			p=0.0817			p=0.9767			p=0.001
Cobalt															
Comparison (decreased vs. method norm)	p=0.5079			p=0.9185			p=0.0441			p=0.0463			p<0.0001		
Comparison (increased vs. method norm)			p=0.0084			p<0.0001			p=0.5421			p=0.0069			p<0.0001

Note: Statistical Significance (p<0.05)

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Table 9

The Pearson Correlation Coefficient for Cr and Co for four analytical comparison parameters

	Pearson Coefficient r	
Comparison	**Chromium**	**Cobalt**
Different analysts	0.995	0.999
Different instruments	0.992	0.999
Different reagents	0.996	0.998
Different days	0.991	0.998

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Table 10

Statistical evaluation results of assessing the stability of spiked samples at different conditions

	Scenario	p-value	Comparison
Chromium	48 hours	0.3700	
	Room Temperature	0.7958	
	Refrigerated	0.0004	24 hr vs 4 hr: p-value =0.0001; 48 hr vs 4 hr: p-value =0.1793
	Freeze-thaw cycles	0.7122	
Cobalt	48 hours	0.8764	
	Room Temperature	<0.0001	48 hr vs 24 hr: p-value =0.0019; 64 hr vs 24 hr: p-value <0.0001
	Refrigerated	0.0033	24 hr vs 4 hr: p-value =0.0578; 48 hr vs 4 hr: p-value =0.0428
	Freeze-thaw cycles	<0.0001	Cycle 2 vs cycle 1: p-value <0.0001