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Published version

LEESE, Elizabeth, MORTON, Jackie, GARDINER, Philip and CAROLAN, Vikki (2016). The simultaneous detection of trivalent & hexavalent chromium in exhaled breath condensate: A feasibility study comparing workers and controls. International Journal of Hygiene and Environmental Health, 220 (2), 415-423.

International Journal of Hygiene and Environmental Health 220 (2017) 415–423

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

The simultaneous detection of trivalent & hexavalent chromium in exhaled breath condensate: A feasibility study comparing workers and controls

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ARTICLE INFO

Article history:
Received 24 August 2016
Received in revised form 7 November 2016
Accepted 1 December 2016

Keywords:
Chromium
Hexavalent
Trivalent
Exhaled breath condensate
μLC-ICP-MS
Occupational exposure

ABSTRACT

The analytical method outlined in this feasibility study has been used to show that trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)) can be detected and measured in exhaled breath condensate (EBC) samples. EBC samples and urine samples were collected from a cohort of 58 workers occupationally exposed to hexavalent chromium compounds and 22 unexposed volunteers (control group). Levels of Cr(III) and Cr(VI) were determined in EBC samples and total chromium levels were determined in urine samples. Pre and post working week samples for both EBC and urine were collected in tandem. Total chromium in urine samples was analysed by inductively coupled plasma mass spectrometry (ICP-MS). Analysis of Cr(III) and Cr(VI) in EBC samples used a hyphenated micro liquid chromatography (μLC) system coupled to an ICP-MS. Separation was achieved using an anion exchange micro-sized column. The results showed that the occupationally exposed workers had significantly higher levels of Cr(III) and Cr(VI) in their EBC samples than the control group, as well as higher levels of total chromium in their urine samples. However, for the exposed workers no significant difference was found between pre and post working week EBC samples for either Cr(III) or Cr(VI). This study has established that Cr(III) and Cr(VI) can simultaneously be detected and measured in 'real' EBC samples and will help in understanding inhalation exposure.

1. Introduction

In Great Britain (GB), many activities/occupations can involve the use of chemicals considered hazardous to worker health. It is important that exposure to chemicals which are classified as carcinogens, mutagens and sensitisers be reduced to as low as reasonably practicable as stated in legislation (Health & Safety Executive (HSE), 2013a). Biological monitoring (the measurement of a chemical or its breakdown products in a biological sample) is one approach for assessing occupational exposure to a chemical in the workplace.

Hexavalent chromium (Cr(VI)), which includes chromates, dichromates and chromic acid is one such chemical that is classified as both a sensitising agent and a carcinogen (Agency for toxic substances and disease registry (ATSDR), 2012; International agency for research on cancer (IARC), 2016)). Exposure to Cr(VI) compounds in the workplace can be through inhalation directly into the lungs from breathing in dust, fumes or mists; dermal absorption through the skin by contact with chromium solutions or solids; or by ingestion as a result of hand to mouth contamination from handling food, smoking or biting nails when hands are contaminated with chromium dust or solutions. Exposure to Cr(VI) compounds can cause respiratory irritations such as nosebleeds, ulcers and holes in the nasal septum, inflammatory respiratory problems, skin irritation and rashes from allergic dermatitis, upset stomachs, kidney and liver damage along with lung and nasal cancer (ATSDR, 2012; HSE, 2013b; Rakhunde et al., 2012). The types of industry and commercial processes where occupational expo-

Abbreviations: EBC, exhaled breath condensate; Cr(III), trivalent chromium; Cr(VI), hexavalent chromium; ICP-MS, inductively coupled plasma mass spectrometry; μLC, micro liquid chromatography; COSHH, control of substances hazardous to health; ATSDR, agency for toxic substances and disease registry; IARC, international agency for research on cancer; HSE, Health & Safety Executive; RBC, red blood cells; HSL, Health and Safety Laboratory; BMGV, biological monitoring guidance value.

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http://dx.doi.org/10.1016/j.ijheh.2016.12.003

sure to Cr(VI) compounds might occur are chromate production, production of chromate pigments and dyes, electroplating and anodising, the production of stainless steel and other chromium alloys, the cutting, finishing and welding of chromium alloys and stainless steel, leather tanning and the production and spraying of paints used in the aeronautic and maritime industries (Genovese et al., 2015; HSE, 2013b; Rakhunde et al., 2012).

Exposure to Cr(VI) compounds leads to accumulation in various tissues such as the lungs, liver and kidneys (Genovese et al., 2015), with glutathione and ascorbate being responsible for reduction of Cr(VI) to trivalent chromium (Cr(III)) within cells with the aid of cysteine (De Flora et al., 1989; Paustenbach et al., 2003). Cr(III) is an essential element as it is needed for the regulation and metabolism of glucose, sugars and fats (Genovese et al., 2015) and thus is considered non-toxic. This difference in toxicity between the two chromium species is largely related to their permeability into cells. Although Cr(III) is more reactive than Cr(VI) (e.g. binding to DNA), Cr(III) is unable to permeate cell membranes, Cr(VI) however is easily taken up by cells; where it undergoes sequential reduction from Cr(V) to Cr(IV) and finally Cr(III) (De Flora et al., 1989; Hoet, 2005). Although exposure to Cr(VI) can be measured in red blood cells (RBC) determined as Cr(III), reduction of Cr(VI) to Cr(III) primarily occurs in plasma, reducing the uptake of Cr(VI) into RBC (Hoet, 2005). For the purposes of biological monitoring for exposure assessment, a urine sample is widely accepted, largely due to it being less invasive and easier to collect than a blood sample. All detectable chromium in a urine sample will be in the form of Cr(III) (De Flora, 2000) and although this is not an ideal approach to Cr(VI) exposure assessment, it is a practical one and widely used. A background unexposed reference range of <2.9 μmol/mol creatinine (Morton et al., 2014) has been established by HSE's, Health and Safety Laboratory, and a Biological Monitoring Guidance Value (BMGV) of 10 μmol/mol creatinine has been determined, based on a Health & Safety Executive (HSE) study where the 90th percentile was adopted from workplaces deemed to have good control (HSE, 2011). Urinary chromium levels can reflect both past and recent exposure, with daily accumulation through the working week of the occupationally exposed (Hoet, 2005). Studies have suggested the excretion of chromium in urine after exposure by inhalation as a two or three stage process (Hoet, 2005; Petersen et al., 2000), with elimination half-lives of 7 h, 15–30 days and 3–5 years (ATSDR, 2012; Hoet, 2005).

The main route of exposure to hexavalent chromium is by inhalation (De Flora, 2000; Hoet, 2005) so a suitable biological matrix to represent exposure by inhalation to hexavalent chromium compounds might be exhaled breath condensate (EBC). After an inhalation exposure of chromium into the lungs, it is partially removed by mucociliary clearance, but some is known to remain (Hoet, 2005). The exact bioavailability of respirable chromium is not known, however animal studies have shown that 53–85% of respirable Cr(VI) is cleared from the lungs by absorption into the blood stream or by mucociliary clearance in comparison to only 5–30% of respirable Cr(III), with the rest remaining in the lungs (ATSDR, 2012). The collection and measurement of EBC is a promising technique primarily due to its non-invasiveness and the lack of interfering solutes found in other biological matrices. EBC is the collection of condensate from cooled exhaled breath during regular tidal breathing. This collected exhaled air contains mostly water vapour but also droplets of fluid from the respiratory tract (Corradi and Mutti, 2005), meaning EBC measurements will reflect markers and molecules found in the mouth, tracheobronchial system and the alveoli (Kharitonov and Barnes, 2001). EBC is reported to contain both volatile substances in the gaseous phase along with low volatile and non-volatile substances in the form of droplets originating from the epithelial lining fluid, as aerosol particles in EBC (Rosias, 2012). The non-volatile substances will include salts, proteins, lipids and environmental and/or occupational contaminants (Corradi and Mutti, 2005; Goldoni et al., 2013; Kharitonov and Barnes, 2001).

There are only a small number of studies concerning the relationship between EBC measurements and occupational exposure, where detectable levels of metals in EBC have been reported such as; for cobalt and tungsten (Broding et al., 2009; Goldoni et al., 2004), beryllium (Hulo et al., 2016), manganese (Hulo et al., 2014), lead (Felix et al., 2015) and iron, nickel, aluminium and chromium (Caglieri et al., 2006; Goldoni et al., 2010; Goldoni et al., 2006; Gube et al., 2010; Hoffmeyer et al., 2011). To date there are only two published studies where Cr(VI) was measured in EBC samples of workers (Goldoni et al., 2010; Goldoni et al., 2006), however both used a method combining solvent extraction with complexation with diphenylcarbazide and measurement performed by atomic absorption spectrometry. There are no publications measuring Cr(VI) by inductively coupled plasma – mass spectrometry (ICP-MS) and there are no publications reporting the measurement of Cr(III) in EBC samples of workers or background populations.

We have previously validated and published our novel analytical methodology in which the two chromium species were determined in EBC samples (Leese et al., 2016). The objective of this feasibility study was to determine if both Cr(III) and Cr(VI) could be detected and measured in 'real' EBC samples. Having collected samples the aim was to determine whether there was a difference between the levels of Cr(III) and Cr(VI) found in EBC samples between a control group not occupationally exposed to chromium compounds and an occupational group who are potentially occupationally exposed to Cr(VI) compounds by inhalation. In addition, any difference in chromium EBC levels would be investigated between pre and post working week samples from the occupationally exposed volunteers.

2. Experimental

2.1. Study group

The occupationally exposed group (n = 58) consisted of 53 males and 5 females, all were over the age of 18 years, with a mean age of 45 years. Individuals volunteered in response to an email sent to companies who had previously sent urine samples to the Health & Safety Laboratory for chromium analysis as part of a biological monitoring program. The occupationally exposed workers were categorised into three types of workers and termed Cr(VI) Workers, Non-Cr(VI) Workers and Other Workers;

Cr(VI) Workers – Individuals who work directly with chromium compounds (e.g. Cr(VI) platers, jiggers and blenders).

Non-Cr(VI) Workers – Individuals who did not work directly with chromium compounds, but worked alongside colleagues working directly with chromium (e.g. nickel, silver or copper electroplaters, maintenance).

Other Workers – Individuals who work neither with chromium nor alongside chromium workers (e.g. inspectors, administration staff, and managers).

Table 1 shows all the types of occupations detailed by the occupationally exposed workers and in which of the three worker categories they were placed in this study.

The volunteers in the control group (n = 22) consisted of 16 males and 6 females, who were not occupationally exposed to Cr(VI) compounds. All were over the age of 18 years, with a mean age of 39 years. Individuals volunteered in response to an email request outlining the study, sent to staff at the Health & Safety Laboratory.

The study was approved by the NHS Research Ethics Committee London – Camden & Islington, REC number 14/LO/1273. All partici-

Table 1
Types of occupational or workplace role listed on the questionnaire by all individual volunteers potentially exposed to occupational Cr(VI) compounds.

Cr(VI) Workers
Chrome electroplater
Grinder
Plasma cutter
Blenders (chemical production of powders, liquids or solids)
Anodiser
Jigger
Passivater
Polisher
Non-Cr(VI) Workers (not working directly with chromium)
General area duties
Silver electroplating
Nickel electroplater & electroless-plater
Copper plater
Zinc plater
Tin plater
Lab analyst
Other Workers
Production Team Leader
Inspector
Manager
Office worker
Supervisor

pating volunteers gave informed consent, and were provided with a participant information sheet. Volunteer information was collected via a questionnaire. Information collected included: gender, age and smoking status, and for the occupationally exposed group only; tasks and duties undertaken since providing the pre working week sample. Because this is a feasibility study only, information on length of employment status and instances of work related ill health were not obtained.

For the occupationally exposed workers, workplace site visits were conducted by the authors to collect both a urine and EBC sample from each volunteer on a Monday morning at the start of shift (pre working week) and again on a Thursday afternoon (post working week).

For the control group, urine samples and EBC samples were collected onsite at the Health and Safety Laboratory. Urine and EBC samples were collected from each control volunteer at the beginning of the week and again at the end of the working week as was done for the occupationally exposed group.

2.2. Instrumentation

All sample analysis was undertaken using inductively coupled plasma – mass spectrometry (ICP-MS). Both total chromium in urine and chromium species Cr(III) and Cr(VI) in EBC samples were determined using an XSERIES 2 ICP-MS (Thermo Fisher Scientific, Hemel Hempstead, UK) monitoring ^{52}Cr. For both methods the ICP-MS was operated in collision cell (CCT) mode using 7% hydrogen in helium, RF power 1400 W, using Ni Xt skimmer and sample cones. The ICP-MS conditions were optimised daily.

2.2.1. EBC samples – chromium speciation

Speciation analysis was performed using a hyphenated μLC system with the XSERIES2 ICP-MS. The separation of Cr(III) and Cr(VI) was achieved using a 5 cm anion exchange column, (Dionex AG7 4 mm × 50 mm i.d., 10 μm, Thermo Fisher Scientific, Hemel Hempstead, UK). The delivery of both sample and mobile phase onto the column was accomplished using an ESI OneFAST system (Elemental Scientific, Warrington, UK), with the ICP-MS peristaltic pump controlling a constant flow rate of 1 mL/min, with a six port switching value and 500 μL sample loop. The operating conditions of the μLC-ICP-MS were: nebuliser gas flow rate 0.86–0.96 L/min using a PFA-ST nebuliser (Elemental Scientific, Warrington, UK), collision gas flow rate 3.4–3.7 mL/min, dwell time of 100 ms for ^{52}Cr. The specific conditions of the μLC-ICP-MS method used in this study and the optimised conditions for the collection, stability and analysis of Cr(III) and Cr(VI) in EBC have been reported elsewhere (Leese et al., 2016).

2.2.2. Urine samples – total chromium

Analysis was performed on the XSERIES2 ICP-MS using a Burgener Miramist (Burgener Research Inc., Ontario, Canada) nebuliser. Operating conditions were: nebuliser gas flow rate 0.81–0.96 L/min, collision gas flow rate 3.5 mL/min, dwell times were 100 ms for ^{52}Cr and 20 ms for ^{72}Ge (internal standard), with 100 sweeps per replicate and three replicates per sample.

2.3. Reagents

Potassium dichromate ($K_2Cr_2O_7$) (analytical grade), chromium(III) chloride ($CrCl_3$)(laboratory grade) and EDTA (diaminoethanetetracetic acid) were obtained from Fisher Scientific (Loughborough, UK). SpA ammonia solution (NH_3) and UpA Nitric Acid were obtained from Romil (Cambridge, UK). BDH ICP-MS single standards of 1000 mg/L chromium and germanium were obtained from VWR International (Lutterworth, UK).

All solutions were prepared fresh each day of analysis using ultrapure deionised water (18.2 MΩhm cm) from a Millipore system (Merck Millipore, Billerica, MA, USA).

2.4. Sample collection

All urine samples were collected in 25 mL urine collection bottles (Sterilin Ltd, Newport, UK). EBC samples were collected using a TURBO-DECCS (Transportable Unit for Research on Biomarkers Obtained from Disposable Exhaled Condensate Collection Systems) by ItalChill (Parma, Italy). The TURBO-DECCS technique is as previously described (Leese et al., 2016). Immediately after the collection of each EBC sample an aliquot of EBC was diluted 10 fold with the 0.5 mM EDTA (pH was adjusted to pH 8 using 10% v/v aqueous ammonia solution) solution onsite by the authors. Urine and EBC samples were returned to the laboratory on the day of collection by the authors. Urine samples had creatinine content analysed upon arrival by the Jaffe alkaline picrate method (Cocker et al., 2011) on a Pentra 400 spectrophotometer (Horiba UK Ltd, Northampton, UK). Creatinine (a breakdown product of creatine phosphate in muscle, which is usually produced at a constant rate by the body) allows a urine sample to be normalised for hydration. Both urine samples and EBC samples were refrigerated at between 2 and 8 °C until analysis.

2.5. Sample preparation and analysis

2.5.1. EBC samples – chromium speciation

A single mobile phase was prepared in water by adding 4% v/v ammonia solution and 3.2% v/v ultrapure nitric acid pH was adjusted to between pH 1.8–2 using the ammonia solution.

Exhaled breath condensate samples were allowed to reach room temperature and mixed on a roller mixer before analysis. The samples were analysed in duplicate. For the calibration standards, stock solutions of 1000 mg/L of Cr(III) and Cr(VI) were prepared in water. Using these 1000 mg/L stock solutions a 100 μg/L mixed species working solution was prepared in water. Using the 100 μg/L mixed species working solution, standards of 0.01, 0.02, 0.05, 0.1, 0.5, 1 and 2 μg/L were prepared in the 0.5 mM EDTA solution, giving a linear calibration range up to 20 μg/L. Samples above the linear calibration range were diluted further with the 0.5 mM EDTA solution where necessary

A commercially available proficiency testing material (PTM) was obtained from Sigma Aldrich (Dorset, UK) for Cr(VI) in drinking water (sample 1045/PE1453, Lot LRAA1427). This PTM was within date with a certified range. Whilst the manufacturer's instructions suggest the PTM is diluted 100 fold in water, for the purpose of this study the PTM was diluted 1000 fold in water to more accurately reflect the levels of total chromium and Cr(VI) in EBC samples previously reported (Caglieri et al., 2006; Goldoni et al., 2010).

It was also necessary to collect a large volume of EBC from a volunteer not occupationally exposed to Cr(VI) to create an EBC blank sample and spike to use as an in-house quality control material. Three equal volumes of "blank" EBC were spiked to create three in-house controls: 2 μg/L Cr(III), 2 μg/L Cr(VI) and a mixed 2 μg/L Cr(III) and Cr(VI) EBC sample.

Both the 1000 fold dilution of the PTM and the 2 μg/L in-house spiked EBC samples were diluted 10 fold with 0.5 mM EDTA solution for analysis and refrigerated at between 2 and 8 °C for up to 6 weeks between analyses, as per the storage stability study undertaken by Leese et al. (2016). The PTM and the spiked EBC samples were analysed at the beginning and the end of the analytical run as well as every 10 samples.

2.5.2. Urine samples – total chromium

For total chromium analysis, a single standard stock solution of 1 mg/L chromium was prepared in water from the 1000 mg/L single standard. Using the 1 mg/L stock solution, standards of 0.5, 2.5, 5, 10 and 20 μg/L were prepared in water, giving a linear calibration up to 80 μg/L.

Urine samples were allowed to reach room temperature and mixed on a roller mixer before analysis. Samples were diluted 20 fold and calibration standards 5 fold with a 1% v/v nitric acid solution containing 10 μg/L Ge internal standard, following the Health & Safety Laboratory UKAS accredited method for total chromium in urine. The certified reference materials (CRMs) were Lyphocheck urine metals control Level 1 (Lot 69161 & 69171) (Bio-RAD Laboratories, Hemel Hempstead, UK) and Clinchek urine control for trace elements Level 2 (Lot 122 & 432) (Recipe, Munich, Germany) and were analysed like the urine samples at the beginning and the end of each analytical run and after every 20 samples. In addition, a 2 μg/L standard was analysed at the beginning and end of each analytical run as well as every 10 samples to monitor drift within the analysis.

3. Results

3.1. Analytical method & validation

3.1.1. EBC samples – chromium speciation

The method described in this study is a sensitive and robust method for the determination of Cr(III) and Cr(VI) in EBC samples. The chromatograms in Fig. 1 show that this novel speciation method can reproducibly separate Cr(III) and Cr(VI) in both aqueous standards and an EBC matrix within 2 min 20 s (200,000 ms).

Based upon the results of twenty two analyses of the determination of chromium species in EBC samples, the limit of detection (LOD) and limit of quantification (LOQ) have been established for both Cr(III) and Cr(VI). The LOD were calculated as three times the standard deviation of the mean of the blank; these were 0.007 μg/L and 0.002 μg/L for Cr(III) and Cr(VI) respectively. The LOQ were calculated as ten times the background equivalent concentration (BEC); these were 0.067 μg/L and 0.008 μg/L for Cr(III) and Cr(VI) respectively. The results of the three in-house spiked EBC samples shown in Table 2 gave an average spiked recovery of 105% for the EBC sample spiked only with 2 μg/L Cr(III); 98% for the EBC sample spiked only with 2 μg/L Cr(VI), along with 99% and 95% for the EBC sample spiked with both 2 μg/L Cr(III) and Cr(VI) respectively. In each analysis the PTM for Cr(VI) in water gave consistently accurate results which were all within the acceptance range. As shown in Table 2, no conversion of chromium species was observed during analysis with the Cr(III) concentrations less than the LOQ when measuring the PTM and EBC in-house sample spiked only with 2 μg/L Cr(VI) (no Cr(III) should be present). The Cr(VI) concentration was also less than the LOQ when measuring the EBC in-house sample spiked only with 2 μg/L Cr(III) (no Cr(VI) present).

3.1.2. Urine samples – total chromium

The determination of total chromium in urine is an established UKAS accredited method at the Health and Safety Laboratory. The LOD is 1 nmol/L (0.05 μg/L) (uncorrected for creatinine). Both urine CRMs, Biorad Level 1 and Clinchek level 2 are certified for total chromium in urine; all gave good results within the target range, this is shown in Table 2.

3.2. Study results

Of the 22 volunteers (16 males, 6 females) in the control group only 1 (male) was a smoker, the remaining 21 volunteers were non-smokers. Of the 58 volunteers (53 males, 5 females) in the occupationally exposed worker group, 33 were non-smokers (3 female, 29 male) and 25 were smokers (1 female, 24 male). Of these 58 workers, 35 were classified as Cr(VI) Workers, 12 as Non-Cr(VI) Workers and 11 as Other Workers. Of the 35 Cr(VI) Workers only 1 volunteer was female, similarly of the 12 Non-Cr(VI) Workers only 1 volunteer was female. Of the 11 Other Workers 3 volunteers were female and 8 were male.

The EBC results for controls and workers are shown in Fig. 2 and presented in Table 3. The results for the EBC analysis from the control group showed that 84% and 91% were below the LOQ for Cr(III) and Cr(VI) respectively, compared with only 5% and 3% for the occupationally exposed workers.

Fig. 2 and Table 3 show that the median values for Cr(III) in EBC from the control group are below the LOQ. Furthermore all Cr(III) found in EBC results from a Non-Cr(VI) worker or Other Worker were above the LOQ. The median concentration of Cr(III) in EBC samples for both pre and post working week of all 58 occupationally exposed workers is very similar, with a median value of 0.59 μg/L for pre working week and 0.54 μg/L for post working week. The Cr(VI) Workers also showed similar median concentrations of Cr(III) in EBC of 0.58 μg/L and 0.51 μg/L for pre and post working week samples respectively. The Other Workers had the highest median concentrations of Cr(III) in EBC of 0.70 and 0.66 μg/L for pre and post working week samples. The Non-Cr(VI) Workers gave the lowest median concentrations of pre and post working week samples for Cr(III) in EBC of 0.38 and 0.54 μg/L respectively. The maximum Cr(III) concentration measured was 11.02 μg/L and was from a post working week EBC sample of a Cr(VI) Worker, compared to the maximum Cr(III) in EBC from a Non-Cr(VI) Worker and an Other Worker both resulting from a pre working week sample, of 9.1 μg/L and 2.97 μg/L respectively.

Fig. 2 and Table 3 show that the median values for Cr(VI) in EBC from the control group are below the LOQ. As with Cr(III), there were no Cr(VI) in EBC results from a Non-Cr(VI) worker or Other Worker less than the LOQ. The results of Cr(VI) in EBC samples for all 58 occupationally exposed workers showed a median concentration increase from 0.58 to 0.72 μg/L of pre to post working week samples. An increase in Cr(VI) EBC levels was observed between the pre and post working samples for both median (0.72–0.91 μg/L) and 90th percentile concentrations (2.81–4.56 μg/L) for the Cr(VI) Workers. This trend was also observed in the Non-Cr(VI) Workers to a lesser extent with a pre working week median concentration of 0.29 μg/L increasing to a post working week median concentration

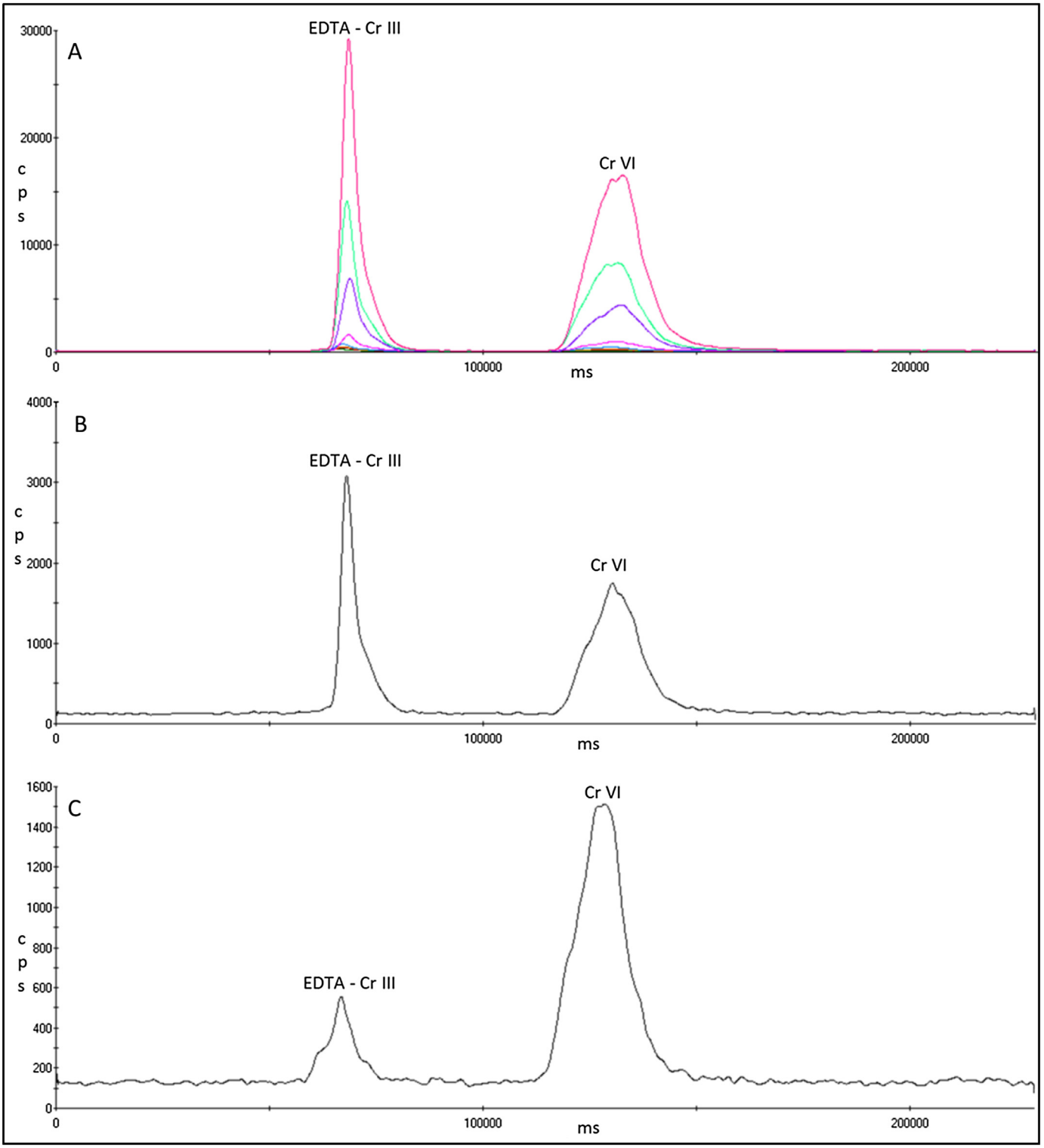

Fig. 1. Chromatograms showing full separation of EDTA-Cr(III) and Cr(VI) using an ESI OneFAST system coupled to a Dionex AG7 anion exchange column and ICP-MS using a nitric acid and ammonia mixed mobile phase. (A) Overlay of the individual standard calibration chromatograms of 0–2 μg/L calibration. (B) An in-house spiked EBC sample 2 μg/L mixed Cr(III) and Cr(VI). (C) An EBC sample from an occupationally exposed volunteer.

of 0.44 μg/L. A two-fold increase from 0.31 μg/L (pre working week) to 0.62 μg/L (post working week) was observed in the median EBC of the Other Workers. The Other Workers showed a higher median and 90th percentile for both the pre and post working week Cr(VI) in EBC samples than the Non-Cr(VI) Workers, but not the Cr(VI) Workers. The maximum Cr(VI) concentration in EBC of 27.3 μg/L was from the same Cr(VI) Worker's post working week EBC sample that contained the highest Cr(III) concentration in EBC. Similarly to Cr(III) in EBC the highest level of Cr(VI) in both Non-Cr(VI) Workers and Other Workers was from a pre working week sample, of 8.31 and 2.59 μg/L respectively.

Table 2
Results obtained for the two species of chromium in the in house spiked EBC samples and the PTM samples and the urinary chromium CRM.

	In-house spiked EBC (2 μg/L) Cr(III) (n = 58) (% recovery)	In-house spiked EBC (2 μg/L) Cr(VI) (n = 56) (% recovery)	In-house spiked EBC (2 μg/L) Cr(III) & Cr(VI) mix (n = 58) (% recovery)	PTM LRAA1427 (n = 57) Target range 2.28–3.28
Cr(III) EBC	2.09 ± 0.3 (105%)	<LOQ	1.98 ± 0.26 (99%)	<LOQ
Cr(VI) EBC	<LOQ	1.95 ± 0.16 (98%)	1.90 ± 0.26 (95%)	2.63 ± 0.17
	Biorad Level 1–69161 (n = 14) Target range 0.359–0.995 μg/L	Biorad Level 1–69171 (n = 35) Target range 0.575–2.53 μg/L	Clinchek Level 2–122 (n = 18) Target range 16.2–24.4 μg/L	Clinchek Level 2–432 (n = 31) Target range 15.9–23.8 μg/L
Urinary total chromium	0.82 ± 0.09	0.90 ± 0.16	18.9 ± 1.20	18.6 ± 0.75

All of the urinary total chromium results for the occupationally exposed workers were above the limit of quantification. As shown in Fig. 3 all the urine samples from the control group were below the HSL background unexposed reference range of <2.9 μmol/mol creatinine.

Analysis of the urine samples from the occupationally exposed workers showed; that 53% were below the background unexposed reference range, 31% were above the HSL UK background unexposed reference range but below the BMGV of 10 μmol/mol creatinine whilst 16% exceeded the BMGV. As shown in Table 3, the median values for all 58 workers of the occupationally exposed group showed a moderate increase in total chromium in urine from 1.9 to 3.3 μmol/mol creatinine for pre to post working week samples. However, the 90th percentile values showed over a 4-fold increase from 3.1 to 13.7 μmol/mol creatinine for pre to post working week samples. The increase of the median and 90th percentile from the pre working week samples to the post working samples was also observed for all three categories of worker, with a significant increase observed in the 90th percentile for both Cr(VI) Workers and Non-Cr(VI) Workers from pre to post working week samples. The highest urinary total chromium value was found in a post working week sample of a Non-Cr(VI) Worker, being 37.1 μmol/mol creatinine, compared to maximum value of 6.0 μmol/mol creatinine for an Other Worker. The highest urinary total chromium value for both pre and post working week from a Cr(VI) worker was 29.4 and 34.1 μmol/mol creatinine respectively and came from the same volunteer. However this volunteer was not responsible for the highest Cr(III) and Cr(VI) levels found in EBC.

3.3. Statistical results

General statistical analysis was performed using Prism Graphpad Version 6 for Windows. Due to the skewness and distribution from normality of the data, all the data was log transformed. The log of the data is normally distributed enabling parametric statistical analysis. Results below the LOQ were replaced with half the LOQ. Paired *t*-tests showed there was a significant difference for total chromium levels in urine between pre and post working week samples for all the occupationally exposed workers ($p = 0.03$). However there was no significant difference between pre and post working week EBC samples for Cr(III) ($p = 0.63$) or Cr(VI) ($p = 0.13$). Unpaired *t*-tests found there was a strong significant difference between workers and controls for total chromium in urine ($p < 0.0001$), as well as Cr(III) in EBC samples ($p < 0.0001$) and Cr(VI) in EBC samples ($p < 0.0001$). As demonstrated in Fig. 4, no correlation was found when comparing post working week levels of total chromium in urine with Cr(III) in EBC ($r^2 = 0.02$, $p = 0.35$), or post working week levels of total chromium in urine samples with the sum of Cr(III) and Cr(VI) in EBC samples ($r^2 = 0.04$, $p = 0.12$). However, a weak moderate correlation was observed when comparing post working week levels of total chromium in urine with Cr(VI) in EBC samples ($r^2 = 0.11$, $p = 0.01$).

Statistical analysis was not performed for gender differences because of the small proportion (9%) of females forming the occupationally exposed group in this study. There was also no statistical significant difference observed between smokers and non-smokers or between workers who wore facemasks/respiratory protective equipment (RPE) and those who did not.

4. Discussion

Reported here are the determined chromium speciation results of Cr(III) and Cr(VI) concentrations in EBC and total chromium concentrations in urine from pre and post working week samples collected from 58 occupationally exposed workers and 22 controls not occupationally exposed to chromium.

The LOD and LOQ reported in this novel chromium speciation study are very low, thus making this a suitable method for the

Table 3
Statistical summary of the mean, median and 90th percentile concentrations of pre working week (Pre W-Wk) and post working week (Post W-Wk) samples for Cr(III) and Cr(VI) in EBC in μg/L and total chromium in urine in μmol/mol creatinine for both controls and workers.

		Cr(III) in EBC μg/L		Cr(VI) in EBC μg/L		Total Cr in Urine μmol/mol creatinine	
		Pre W-Wk	Post W-Wk	Pre W-Wk	Post W-Wk	Pre W-Wk	Post W-Wk
All Workers:	Median	0.59	0.54	0.58	0.72	1.9	3.3
n = 58	P90	2.43	4.21	2.01	2.45	3.1	13.7
	Range	0.19–9.1	0.14–11.03	0.04–9.69	0.01–27.3	0.5–29.4	0.6–37.1
Cr(VI) Workers:	Median	0.58	0.51	0.72	0.91	3.4	4.7
n = 35	P90	2.15	2.98	2.81	4.56	14.8	20.4
	Range	<LOQ-3.38	<LOQ-11.02	<LOQ-9.69	<LOQ-27.3	0.6–29.4	1.1–34.1
Non Cr(VI)	Median	0.38	0.54	0.29	0.44	1.5	2.1
Workers	P90	0.86	1.41	0.76	1.71	3.7	23.7
n = 12	Range	0.19–9.1	0.20–4.56	0.04–8.31	0.06–2.45	0.6–8.6	0.6–37.1
Other Workers	Median	0.70	0.66	0.31	0.62	1.2	1.5
n = 11	P90	2.2	2.05	2.01	1.84	1.6	2.7
	Range	0.27–2.97	0.14–2.21	0.03–2.59	0.01–2.20	0.5–5.1	0.8–6.0
Controls	Median	<LOQ	<LOQ	<LOQ	<LOQ	0.4	0.4
n = 22	P90	0.09	0.09	0.01	<LOQ	0.6	1.1
	Range	<LOQ–0.27	<LOQ–4.97	<LOQ–0.07	<LOQ-0.12	<LOQ-1.3	<LOQ-1.4

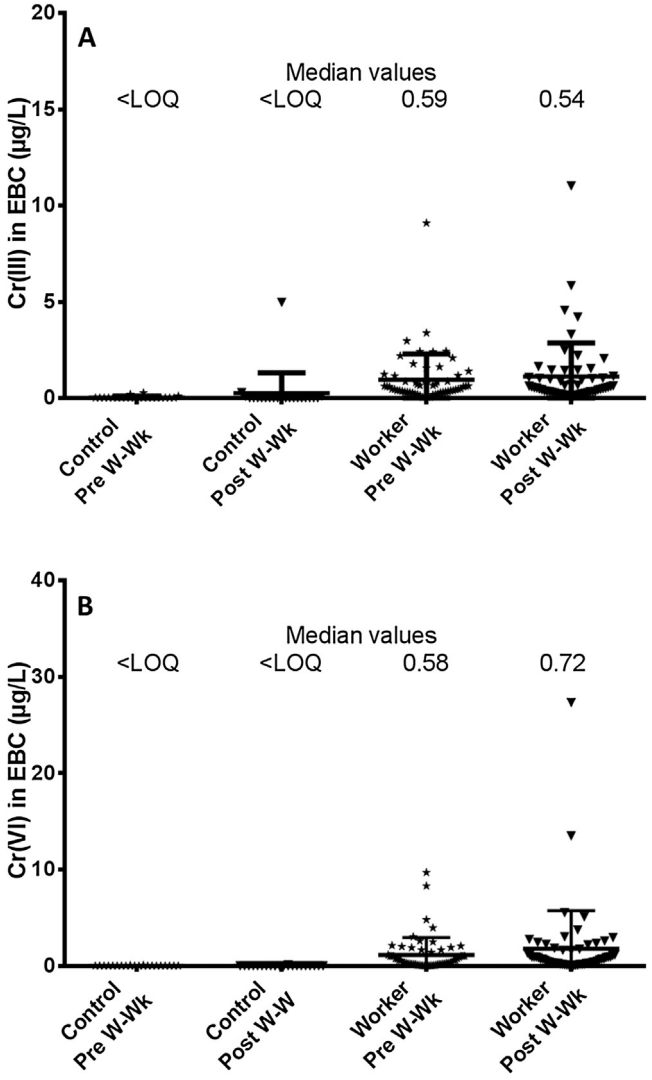

Fig. 2. Scatter plots with median concentrations (µg/L) from pre working week (Pre W-Wk) to post working week (Post W-Wk) samples for (A) Cr(III) in EBC and (B) Cr(VI) in EBC for both occupationally exposed volunteers and the unexposed control group.

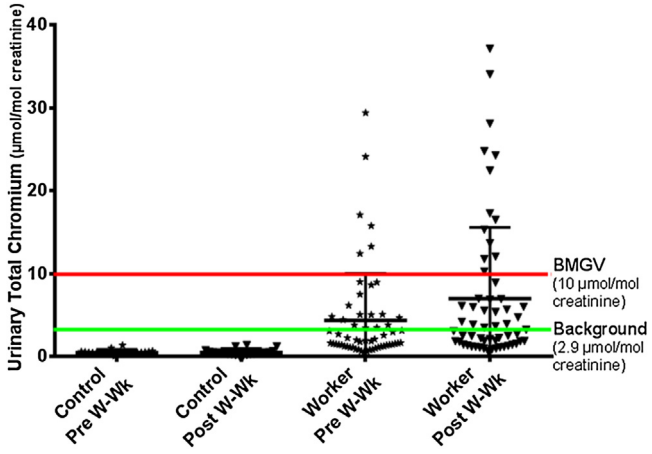

Fig. 3. Scatter plot showing the results of total chromium in urine (µmol/mol creatinine) for both the occupationally exposed workers and the control group for both pre working week (Pre W-Wk) and post working week (Post W-Wk) samples. Showing results in comparison to the background unexposed reference range and the BMGV.

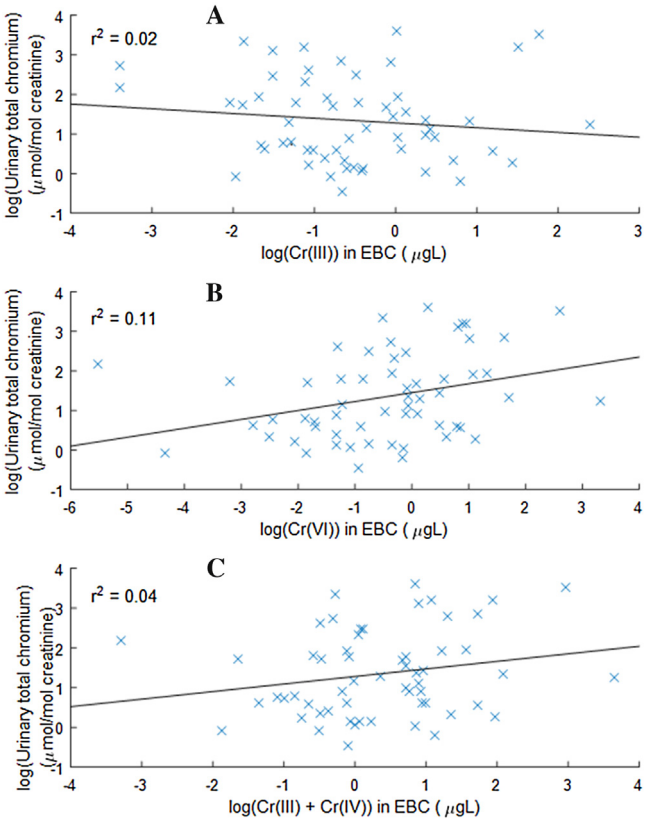

Fig. 4. Correlations of log transformed data between post working week total chromium in urine and; (A) Cr(III), (B)Cr(VI) and (C) the sum of Cr(III) and Cr(VI) in post working week EBC samples.

analysis of EBC where median concentration of Cr(III) and Cr(VI) were found to be less than 2 µg/L. No other studies have published actual Cr(III) results in EBC samples, only Cr(VI) and total chromium so there are no LOD/LOQ values to compare against. The Cr(VI) LOD and LOQ reported here is lower than other reported studies (Goldoni et al., 2010; Goldoni et al., 2006). However, the LOD and LOQ reported in this study are achieved using ICP-MS whilst the LOD of 0.2 µg/L reported by Goldoni et al. (2010, 2006) uses electrothermal atomic absorption spectrometry (ETAAS). Other publications reporting chromium in EBC were for total chromium only and the LODs reported were a limiting factor in their results (Gube et al., 2010; Hoffmeyer et al., 2011).

The background unexposed reference range established by the Health and Safety Laboratory, for total chromium in urine is <2.9 µmol/mol creatinine (Morton et al., 2014). For the urinary total chromium results for exposed workers, 47% exceeded this value, indicating that nearly half of the urine samples provided by the 58 workers showed some occupational exposure to Cr(VI) compounds. Furthermore, 16% were above the BMGV of 10 µmol/mol creatinine, indicating that 19 urine samples provided by the 58 workers showed significant exposure to Cr(VI) compounds. This exposure demonstrates that the occupationally exposed workers who took part in this study were a suitable cohort to successfully meet the objective of this study and determine if Cr(III) and Cr(VI) could be detected and measured in real EBC samples. As shown in Table 3, the 90th percentile of urinary total chromium for both Other Workers and the control group are within the background unexposed reference range, whereas the Cr(VI) Workers and Non-Cr(VI) Workers is twice the BMGV of 10 µmol/mol creatinine, indicating a strong association with chromium occupational exposure and urinary chromium measurements.

It is important to note that the study outlined here was a pilot feasibility study to ascertain if both Cr(III) and Cr(VI) could be determined in EBC samples from workers occupationally exposed to Cr(VI) compounds by inhalation. Consequently, no environmental monitoring such as air monitoring, surface wipes or hand wipe sampling was performed, nor was information taken regarding length of time since last potential exposure or length of time and levels of potential exposure from any of the occupationally exposed workers prior to providing a EBC or urine sample.

The statistical analysis found a lack of correlation between levels of total chromium in urine with Cr(III) in EBC and levels of total chromium in urine with the sum of Cr(VI) and Cr(III) in EBC, however a low moderate correlation was found between levels of total chromium in urine with Cr(VI) in EBC. This lack of correlation is in agreement with other studies where no correlation was found between total chromium in urine with Cr(VI) in EBC (Goldoni et al., 2006) or with total chromium in urine compared with total chromium in EBC (Caglieri et al., 2006; Goldoni et al., 2010). However, this may be expected considering urinary chromium levels will reflect all three routes of exposure (inhalation, dermal and ingestion); whereas EBC samples will reflect mainly inhalation exposure with some contribution from what is present in the mouth but not exposure by dermal absorption or ingestion. Interestingly, Goldoni et al. (2010) found there was a significant correlation between total chromium in post shift EBC samples and RBC samples only. This is not always the case, as better correlation of urine with EBC samples has been observed for other elements (Goldoni et al., 2004).

Whilst there is a lack of statistical significance between the pre and post working week samples for both Cr(III) and Cr(VI) in EBC, all the levels were higher than the controls. The data showed that 48% of the occupationally exposed group showed an increase of the concentrations of Cr(III) in EBC from the pre to post work week sample, and 59% showed an increase of the concentrations of Cr(VI) in EBC from the pre to post working week sample. However, chromium in lungs has shown to accumulate upon repeat inhalation (Hoet, 2005), something that is likely to happen in an occupational setting. In addition, reduction of Cr(VI) to Cr(III) by glutathione was found to be much slower than ascorbate leading to a longer residence of chromium in lungs (Petersen et al., 2000), meaning the higher levels of chromium in pre working week EBC samples may be the result of high exposure the previous week. It must also be noted that although both urine and EBC samples collected on the Monday morning were considered pre working week, the workers had already entered the work place. Although workers had been instructed not to start official work that could result in inhalation exposure (e.g. grinding or electroplating), they had started pre work preparation, meaning some exposure may have already occurred from chromium levels in the air of the workplace or from preparation procedures. This lack of correlation may also be related to the combination of a high proportion of low μg/L values for a sample size of only 58 occupationally exposed workers. In addition, the lack of information regarding exposure of the workers prior to providing the post working week EBC sample will be a contributing factor. No statistically significant difference was found between smokers and non-smokers for Cr(III) or Cr(VI) in EBC or total chromium in urine. Nor was there a statistical significant difference found between those individuals that wore any type of facemask/respiratory protective equipment (RPE) and those that did not. However, this outcome is not unexpected when tandem environmental analysis has not been performed and little information is known on length of time since last exposure or length of time the worker has experienced Cr(VI) exposure or the levels of exposure. This uncertainty, along with the fact these comparisons would reduce the dataset substantially, make it difficult to compare these more specific data groups and extrapolate any type of significance that would be statistically robust to investigate as to whether wearing RPE or smoker status affected observed levels of Cr(III) or Cr(VI) in EBC samples.

A similar study by Goldoni et al. (2006), determined Cr(VI) in EBC samples from 10 chromium electroplaters; they looked at overnight chromium decrease with EBC samples taken post shift and again the following morning pre shift (15 h since exposure). A decrease in Cr(VI) levels from overnight post to pre shift was observed, with concentrations of Cr(VI) found in EBC samples ranging from 0.1–2.9 μg/L for the post shift EBC samples and 0.1–2.1 μg/L for the pre shift EBC samples. However only 10 workers were sampled and seven of the 20 EBC samples collected were found to be below the LOD. The concentrations of Cr(VI) in EBC reported here in the current study were higher, with overall (n = 58) concentrations of Cr(VI) in workers ranging from 0.04–9.69 μg/L for the pre shift EBC samples and 0.01–27.35 μg/L for the post shift EBC samples. A later study by Goldoni et al. (2010) reported a similar study method but with tandem urine, red blood cell and plasma sampling alongside the EBC sample collection. The median values reported in this later study compare well with the results reported here in this study. For example, median total chromium in urine levels increased from 4 to 6 μmol/mol creatinine in Goldoni's study compared to a median increase of 1.9–3.3 μmol/mol creatinine for all workers and 3.4–4.7 μmol/mol creatinine for Cr(VI) Workers reported here in this study. Additionally, in Goldoni's study median values for Cr(VI) in EBC samples increased from 0.3 to 1.0 μg/L compared to median values increasing from 0.58 to 0.72 μg/L for all workers and 0.72–0.91 μg/L for Cr(VI) Workers reported here in this study. The studies by Goldoni et al. (2006, 2010) in addition to the work reported here indicate the need for a comprehensive EBC collection protocol to better understand dwell, half-life and excretion rates of chromium in EBC.

Also of interest is the two fold increase of 0.31 μg/L (pre working week) to 0.62 μg/L (post working week) median levels of Cr(VI) in EBC for the Other Workers. Although the median values are not higher than the Cr(VI) Workers, they are higher than the median values of the Non-Cr(VI) Workers, indicating inhalation exposure is not limited solely to workers operating directly in areas of the workplace where chromium processes occur. Chromium exposure of Other Workers is more evident when the median values for both Cr(III) and Cr(VI) in EBC are compared against the control group values, which were all below the LOQ, however there were only 11 volunteers in the Other Workers group. No other studies have determined chromium EBC measurements in this type of worker cohort for comparison.

In recent literature, there is reference to the need for a marker in EBC samples to standardise for dilution (Kuban and Foret, 2013; Rosias, 2012), in the same way creatinine is utilised to correct for concentration of urine. It is suggested that the droplets of fluid from the respiratory tract can be diluted by the water vapour found in EBC and will vary amongst individuals (Rosias, 2012). At present, there is no consensus on a suitable and accurate biomarker to correct for dilution, as smoking status, inflammatory conditions such as asthma or the inhaled contaminant itself have affected their usefulness in biomarkers studies (Dodig and Cepelak, 2013; Hoffmeyer et al., 2015). However Rosias (2012) and Reinhold and Knobloch (2010) have suggested EBC results be expressed per volume of EBC collected. Unfortunately, in this study neither approach was explored. However, it is something that must be taken into consideration in future EBC studies.

To enable the future of EBC as a biomarker for occupational exposure to chromium species there needs to be an understanding of the dwell time of chromium species detectable in an EBC sample after exposure has ceased. In addition to a better understanding of the relationship between levels of exposure and EBC measure-

ments is essential. The latter could be achieved with correlation to air measurements.

Previously known CRMs for either Cr(III) and/or Cr(VI) by NIST and IRMM have either been discontinued or withdrawn. For the future success of chromium speciation in EBC it is important that CRMs in either water or EBC are made available. In addition, the future of EBC as a biological matrix will rely on CRMs for other trace elements.

5. Conclusion

This is the first study to report the detection and measurement of Cr(III) and Cr(VI) in 'real' exhaled breath condensate (EBC) samples of volunteers. The analytical method outlined in this feasibility study has shown that both Cr(III) and Cr(VI) can be simultaneously detected and measured in EBC samples from a cohort of volunteers who are occupationally exposed to Cr(VI) compounds by inhalation. The occupationally exposed workers showed significantly higher levels of both Cr(III) and Cr(VI) than in the control group. However no statistically significant difference was found between pre and post working week samples for Cr(III) and Cr(VI) in EBC of the occupationally exposed workers. The speciation method demonstrates high sensitivity with low limits of detection and quantification. The next stage of this study will be to undertake site visits to perform air monitoring in tandem with EBC sample collection to further understand and correlate the relationship between levels of exposure and EBC results for chromium.

Funding

This publication and the work it describes were funded by the Health & Safety Executive (HSE). Its contents, including any opinions and/or conclusions expressed, are those of the authors alone and do not necessarily reflect HSE policy.

Acknowledgements

The authors would like to thank all the volunteers for participating in this study, along with independent metal finishing consultants John Torr and John Williams for all their knowledge and help during this project.

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