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Characterization of a portable method for the collection of exhaled breath condensate and subsequent analysis of metal content†‡

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Using exhaled breath condensate (EBC) as a biological media for analysis of biomarkers of exposure may facilitate the understanding of inhalation exposures. In this study, we present method validation for the collection of EBC and analysis of metals in EBC. The collection method was designed for use in a small scale longitudinal study with the goal of improving reproducibility while maintaining economic feasibility. We incorporated the use of an Rtube with additional components as an assembly, and trained subjects to breathe into the apparatus. EBC was collected from 8 healthy adult subjects with no known elevated exposures to Mn, Cr, Ni, and Cd repeatedly (10 times) within 7 days and analyzed for these metals *via* ICP-MS. Method detection limits were obtained by mimicking the process of EBC collection with ultrapure water, and resulted in 46–62% of samples falling in a range less than the method detection limit. EBC metal concentrations were found to be statistically significantly associated ($p < 0.05$) with room temperature and relative humidity during collection, as well as with the gender of the subject. The geometric mean EBC metal concentrations in our unexposed subjects were 0.57 μg Mn per L, 0.25 μg Cr per L, 0.87 μg Ni per L, and 0.14 μg Cd per L. The overall standard deviation was greater than the mean estimate, and the major source in EBC metals concentrations was due to fluctuations in subjects' measurements over time rather than to the differences between separate subjects. These results suggest that measurement and control of EBC collection and analytical parameters are critical to the interpretation of EBC metals measurements. In particular, rigorous estimation of method detection limits of metals in EBC provides a more thorough evaluation of accuracy.

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In this study, a method was developed and evaluated for collection and measurement of metals in exhaled breath condensate. Adult participants were monitored for typical biomarker concentrations (*i.e.* no exposure conditions were induced). The work presented here aims to provide guidance to future investigations of biomonitoring of metals by offering a thorough method description of a standardized protocol for sample collection. In addition, the characterization of the method is presented with supporting data such as a storage test, description of field blank collection and estimation of method detection limit, and statistical analysis.

1 Introduction

Estimation of inhaled dose from occupational and environmental exposures is typically challenged by variations in aerosol generation rate and by unique time–activity patterns of the exposed individual. The inhaled dose represents the pollutant quantity that has been deposited, absorbed or distributed in the body.[1] Measurement of respiratory media, in contrast to blood or urine, can be especially useful for understanding inhalation exposures if this is the route of concern.

Biological media that are commonly collected for sampling the respiratory tract include induced sputum, bronchoalveolar lavage, and exhaled breath condensate (EBC). Among these, the collection of EBC is attractive because it is non-invasive and

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samples can be collected repeatedly within a short time.[2] Research on the application of biomarkers in EBC has increased in the past decade; however, many questions remain about what EBC represents physiologically. EBC is primarily composed of water vapor (>99%), but a small fraction of EBC is derived from respiratory droplets that can contain either volatile or non-volatile components that are exhaled from within the respiratory tract.[2] Although the exact mechanism is unknown, one theory is that the respiratory droplets are released with the turbulence of respiration, while a newer theory is that respiratory fluid that lines the lower bronchioles is released from folds in the bronchiolar surface as the surface contracts during respiration.[3] The basic method for collecting EBC samples involves a subject breathing into a chilled tube equipped with a one-way valve. Vapor exhaled from the lungs condenses onto the side walls of the tube and is collected into a container. There is currently no standardized protocol for either collection of EBC or measurement of biomarkers in EBC, although the American Thoracic Society and European Respiratory Society (ERS) published guidelines for EBC collection and measurement in 2005,[2] and in 2010 the ERS published a monograph devoted to exhaled biomarkers (volume 49). These documents acknowledge that the conditions specified for collection and measurement may be highly dependent on the biomarker of interest and the population being studied.

The EcoScreen, EcoScreen Turbo (formerly available through Cardinal Health, Hoechberg, Germany), and EcoScreen 2 (FILT Lungen-und Thorax Diagnostik GmbH, Berlin, Germany) and the Rtube (Respiratory Research Inc., Charlottesville, VA, USA) are commercially available devices for EBC collection that have been used in most EBC studies. The condensing surface is polytetrafluoroethylene in the EcoScreen, polyethylene in the EcoScreen Turbo, and polypropylene in the Rtube. Research groups have also designed their own devices consisting of different materials.[4–7] The condenser coating can affect biomarker recovery.[8] A benefit of the EcoScreen and the EcoScreen Turbo is that they both contain refrigeration units to cool the EBC at a fixed temperature. In contrast, the Rtube uses an aluminum sleeve that surrounds the collection tube and is chilled prior to use in order to condense the EBC, but the temperature is not controlled during EBC collection. The EcoScreen and EcoScreen Turbo are more expensive than the Rtube and less portable, making the use of Rtubes more feasible for a small-scale field study.

The use of EBC as a medium for biomarkers of pulmonary exposure to metals, specifically manganese, has been of specific interest to our research team. Unlike biochemical biomarkers that can undergo degradation throughout the collection and analysis,[9] elemental analyses of metals in EBC are chemically stable biomarkers and present no concern of breakdown, since they are measured in elemental form. Conversely, contamination is a major area of concern during the collection and storage of EBC containing metals. This is a particular challenge for abundant metals, such as iron and manganese, where leaching of the metals from equipment surfaces used to collect or store the media can contaminate samples. Polytetrafluoroethylene products are generally recommended to reduce metal contamination, but their high expense often prohibits widespread use. Plastics are considered a good alternative, but there can be significant differences between plastics as well (*e.g.*, high density polyethylene has been found to contain lower levels of trace metal contamination than low density polyethylene[10]).

Few studies have investigated metals in EBC. In occupationally exposed groups, cobalt, copper, chromium, nickel, antimony, lead, and tungsten in EBC have been investigated. While pre- *vs.* post-shift concentrations have been shown to be significantly different for some metals, not all studies have demonstrated a significant correlation between elevated particle exposures and elevated post-shift measurements of EBC metals.[11–14] Other studies of metals in EBC have focused on a comparison of healthy subjects *vs.* those with respiratory illness or disease. In a study of subjects with COPD *vs.* those with normal spirometry results, Mutti *et al.* found that of 33 metals analyzed in EBC samples, only lead, cadmium, nickel, aluminum, copper, selenium, iron, and manganese were detected in greater than 50% of subjects, and there was no statistically significant difference in the concentrations between these subject groups.[15] Dodig and Vlasic measured magnesium, calcium and iron, and found statistically significant differences in iron and the ratio of magnesium to calcium between healthy subjects and subjects with asthma.[16,17]

These occupational and clinical studies have pioneered demonstration of the capability to detect metals in EBC and have provided insight into successful collection and analytical methods. Still, taking the next step of comparing the results between studies is challenged by variation in the method of collection and the approach for quality control.

The goals of the current study were to assemble and validate an EBC collection method that incorporated several quality control features that would allow for the standardized collection of EBC samples, and to establish an analytical method for measuring specific metals of interest: chromium (Cr), manganese (Mn), nickel (Ni) and cadmium (Cd), in EBC under conditions controlled for contamination. The collection and analytical methods discussed in this work were evaluated in samples collected from unexposed subjects in a longitudinal study to assess the bioavailability of Mn associated with inhalation exposure to welding fume. Measured metal concentrations in EBC were assessed for association with two key categories of factors: measured respiratory parameters of subjects generating the EBC and parameters of EBC collection.

2 Materials and methods

The EBC method development study reported was part of a longitudinal study approved by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board (FWA00000287). Informed consent was obtained from each subject prior to enrollment in the study.

EBC collection device

The Rtube was selected as the instrument of EBC collection to be adapted for our research application. The Rtube consists of a

polypropylene condensing tube, a silicon one-way valve, a t-connector with a closed bottom which acts as a saliva trap, and an attached mouthpiece. An aluminum sleeve that is chilled to the desired temperature prior to use cools the condensing tube.

Optimization of EBC collection for our study was accomplished through a combination of modifications made to the collection device and the establishment of a protocol to train subjects to breathe consistently during repeated sample collection periods. A spirometer designed to measure and record ventilation during tidal breathing (EcoVent, JAEGER, Höchberg Germany) was attached to the air intake end of the Rtube. In addition to the spirometer, a visual incentive spirometer (VIS) was attached to the breathing train to help subjects normalize the volume of air during each inspiration. A HEPA filter was attached to the inlet of the breathing train to reduce exposure to airborne particles during EBC collection. An extra t-connector and flex-tubing were used to connect the EcoVent, VIS, and HEPA filter in series with the Rtube, which together we term "the modified Rtube EBC collection (MREC) system" (Fig. 1).

The aluminum sleeve was stored on water ice until needed for sample collection. Immediately before sample collection the sleeve was inserted over the polypropylene collection tube and the temperature inside the collection tube measured. Sample collection commenced when the temperature of the tube was 0–5 °C. The room temperature and relative humidity were also recorded.

Subject training

Before collecting EBC, subjects underwent a training session to assist in establishing a consistent breathing pattern throughout the sample collection period. Subjects were instructed to breathe tidally through the MREC system for a few minutes by sealing their lips on the mouthpiece. They were advised that salivary build-up may occur, and that they should stop to swallow saliva as needed. Once subjects felt comfortable breathing through the MREC system, they were asked to breathe tidally through the system for 2 minutes, and the resulting tidal volume measured by the Ecovent. Each subject's tidal volume was multiplied by 1.5, which corresponded to a slightly deeper than normal breath, and this was set as the target volume for subjects to visually monitor on the VIS. Subjects were provided a nose clip and practiced breathing at a rate of 10 breaths per minute with coaching and by counting while watching the VIS to achieve the target volume. Subjects were informed that if they needed to make adjustments to the rate or target volume for comfort, their focus should be on maintaining consistent breathing.

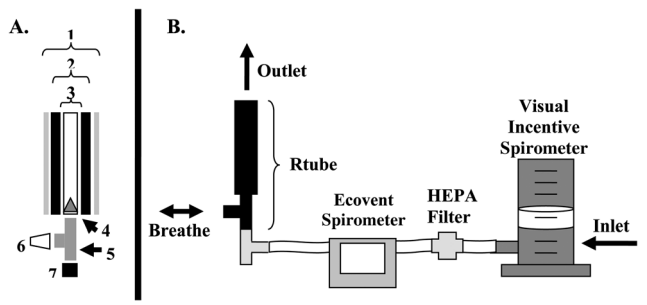

Fig. 1 Modified Rtube EBC Collection (MREC) apparatus. Left (A): Rtube components: (1) insulator, (2) aluminum sleeve, (3) condensing tube, (4) one-way valve, (5) t-connector, (6) mouthpiece and (7) saliva trap. Right (B): the MREC system.

Collection of EBC

EBC collection occurred over a 5-minute period with a nose clip. (Five minutes of EBC collection was selected instead of 10 minutes, as recommended by the ATS/ERS, because this was found to be less burdensome in our study requiring repeat measurements within a relatively short time period.) Although EBC is generated by exhalation, the MREC system measures the volume of inhalation; these were presumed to be equivalent in this closed system. If a subject had initiated an inhalation at the end of the 5 min sample collection period, they were instructed to complete that exhalation through the MREC system even if it passed the time period.

EBC metal analysis

Sample preparation protocol development and quality control. To evaluate the potential for instrument contamination, measures were made of metals in the condensing components of the MREC (the condensing tube, one-way valve, and silicon ring). Background measurements to establish metal concentrations in EBC samples resulting from surface contamination were assessed by mimicking the presence of EBC within the condensing tube. Condensing tube background samples were collected by rinsing the sides of the condensing tube with the one-way valve in place with 1 mL of ultra-pure water (Millipore, Billerica, MA). After 5 min, the sample water was removed, and 300 μL of the sample was digested in 1.5% HNO_3 (Optima grade, Fisher Scientific, Fairlawn, NJ) at a 1 : 4 dilution level prior to analysis.

Background concentrations of Mn were tested to determine the appropriate cleaning method for the Rtube condensing components. Mn concentrations were not significantly different in new compared to used and cleaned Rtube condensing components when used Rtube condensing components were soaked in 10% HNO_3 for 18 hours after use. (The mean Mn concentration in new components was 0.95 μg L^{-1} and in used components was 0.76 μg L^{-1}, *t*-test: $p = 0.48$ (ESI Table S1‡).) Soaking used condensing components in 10% NH_3OH (ACS grade, JT Baker, Phillipsburg, NJ) *vs.* 10% HNO_3 (ACS grade, Acros Organics, Geel, Belgium) significantly reduced Mn background levels (Mn concentration 0.21 *vs.* 1.0 respectively, *t*-test: $p = 0.0002$). Based on this assessment, the components of the Rtube were cleaned between uses by disinfecting with bleach and then by soaking in 10% NH_3OH (ACS grade) for a minimum of 5 days.

To avoid the potential of infection in subjects, the mouthpieces and t-connectors were not reused. Additional mouthpieces and t-connectors equivalent to those in the Rtube system

were purchased separately and bleached prior to use (Instrumentation Industries, Bethel Park, PA).

Control for background contamination. Field blanks were collected periodically during the course of the sample collection in both groups of subjects, such that at least 10% blanks were collected per group of subjects and there were 3 field blank samples for each subject's sample set ($n = 10$). In all cases, the Rtube condensing tube of the field blank was cleaned following the same procedure as the Rtube condensing tubes of the EBC samples it represented.

Field blanks were prepared by placing an Rtube condensing tube inside a chilled Rtube aluminum sleeve in open air during EBC collection in the same room. The field blank tube was transported in the same manner and at the same time as tubes containing collected EBC and then processed in the same way as background check samples with purified water. The liquid obtained from field blanks was then prepared and stored simultaneously and similarly to EBC samples.

Sample storage. A storage test was conducted by measuring metal concentrations over time in multiple aliquots from a pooled EBC sample (Fig. 2). Stored aliquots were processed simultaneously and in the same manner as study samples, and then stored at −20 °C until the specified analysis time. Three aliquots were analyzed in each time point. For Cr, no statistically significant change was detected at 11 days of storage. A significant increase in metal concentration, presumably due to leaching from the storage containers, was detected at 11 days for Mn, Ni and Cd. The increase in Mn concentration at 11 days was considered negligible because the mean magnitude of this change in concentration was low (8% increase). However, for Ni the mean increase was 16%, and for Cd it was 77%. All four metals demonstrated statistically significant increases in concentration after 29 days ($p < 0.05$). Based on these results, all samples were analyzed as quickly as possible, which was always within 9 days of collection.

Sample analysis. Samples were analyzed for metal content *via* inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, Agilent Technologies, Newark, DE). All samples were run against a 4–7 point calibration curve, depending on the concentration range of the samples. Calibration blanks and standards were analyzed after every ten samples to check for instrument drift. Internal standard (ISTD) elements were selected based on both their chemical similarity to the measured analytes and low expected contamination and background levels. Analyte concentrations were adjusted with ISTD concentrations.

Since no standard reference material (SRM) for metals in EBC was available and EBC consists of >99% water, a NIST standard for metals in water was used to establish metal recoveries in each analytical batch ("Trace Elements in Water", 1643e, NIST, Gaithersburg, MD). For each SRM measurement, 300 μL of SRM was added directly to a polystyrene tube and then processed by digesting in the same digest solution and at the same dilution level as EBC samples.

EBC sample collection

For this study, each subject provided 10 EBC samples during a total of 10 visits within a 5 day period; from 1 to 3 samples were collected on each day of participation. All study visits were conducted within a research institution (laboratory and office space) and samples were processed in a single laboratory. Following EBC collection, the condensing tubes were stored on ice or in a refrigerator (4 °C) until processed. The collected volume of EBC was measured using a calibrated pipette. For each EBC analysis, 300 μL of EBC was diluted 1 : 5 parts in 1.5%

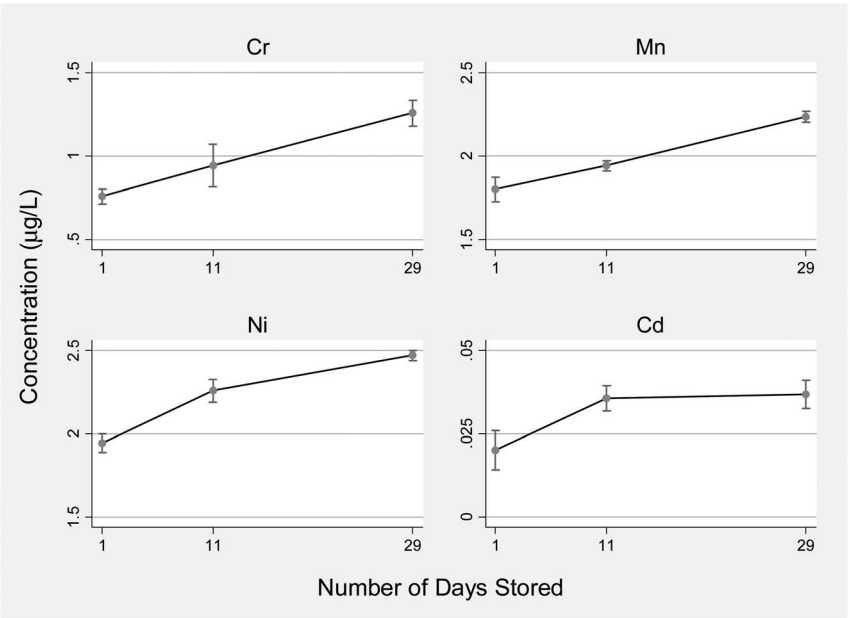

Fig. 2 EBC metals storage stability for Cr, Mn, Ni, and Cd. At each point $n = 3$, error bars = SD.

Optima grade HNO_3 (Fisher Scientific, Fairlawn, NJ) containing an internal standard mixture (Agilent, Santa Clara, CA) in an acid-washed 15 mL polystyrene centrifuge tube (red caps, Sarstedt, Nümbrecht, Germany). Replicate samples were prepared and analyzed when there was a sufficient volume of individual EBC volume available. The samples, field blanks, and SRM samples were processed on the same day as EBC collection and then stored at −20 °C until analysis.

Statistical analysis

Data processing, including determination of blank subtraction factors, method detection limits (MDL), and SRM correction factors, was performed in Excel (Microsoft Office, 2003). All statistical analysis was performed in STATA (Intercooled STATA 8, College Station, TX).

Measures of association. We investigated the association between EBC volume/metals and collection/physiological characteristics. The EBC metal concentration can be reported either as μg L^{-1} of EBC collected (as reported throughout this paper), or by incorporating the EBC volume collected and the air volume respired, it can be reported as ng L^{-1} air respired. Hence we also explored the association between EBC metals concentrations measured as μg L^{-1} EBC and ng L^{-1} air exhaled.

For these analyses, in order to account for autocorrelation of measurements, only metal concentrations that were above the MDL were included. The associations were estimated using a mixed models approach that accounts for repeated measures obtained from each subject ("xtreg" command in STATA). The level of association was estimated using a regression coefficient with an associated *p*-value indicating level of statistical significance. The measured EBC metal concentration from the first visit of subjects (baseline concentration) was found to be lognormally distributed across subjects for all four metals, hence for all EBC metal concentrations presented the regressions were performed with log transformed data.

Measures of means and variability. One goal of this study was to estimate concentrations of metals in EBC of unexposed subject and to get an idea of the main sources of variability in these measurements. When determining the mean concentration of each EBC metal of this data that was highly skewed and had a high percentage of concentrations below the MDL (or left censored), the left censored concentrations were replaced with half of the MDL.[18]

To estimate the variability of the metals concentrations within and between subjects, a likelihood function which accounts for autocorrelation present in repeat measures was used ("xtintreg" in STATA). In the analysis of the variability of the EBC metals concentration, we used the non-transformed concentrations above the MDL, rather than the log of EBC metals concentrations.

3 Results

EBC collection characteristics

In total, 79 EBC samples (*n*) from 8 subjects (*N*) were collected and available for this assessment. Collection characteristics are detailed in Table 1. The mean room temperature and relative humidity during EBC collection were 23 °C and 36%, respectively. The mean start temperature of the Rtube condensing tube was 4 °C. The mean total volume respired throughout the 5 minute period of collection was 53.0 L (overall SD = 10.6 L), and the mean EBC volume collected was 0.540 mL (overall SD = 0.099 mL).

Table 1 EBC collection characteristics on 79 EBC samples from 8 subjects

Parameter	Mean	Min	Max	SD[a]
Room temp (°C)	23	19	26	1.7
Relative humidity (%)	36	14	51	10
Start collect temp (°C)	4.2	0	9.5	2.8
Diff collect temp (°C)	11.8	7	17	2.0
EBC volume (mL)	0.540	0.300	0.750	0.099
Respired volume (L)	53.1	33.2	72.0	10.6

[a] Overall standard deviation.

EBC metal analysis

Quality control. The EBC samples were analyzed in two batches. Samples from multiple subjects were analyzed in each batch, with 50 EBC samples analyzed in the first batch and 29 EBC samples in the second batch. Both analytical batches had a unique set of field blanks that were collected at the same time as the subject samples. The sample concentrations were blank-corrected by subtracting the mean field blank concentration corresponding to the analytical batch. The MDL was calculated as 3 times the standard deviation of the concentrations in the field blank samples. The MDL's of the two analytical batches were: 0.47 and 0.92 μg L^{-1} for Mn, 0.12 and 1.27 μg L^{-1} for Cr, 0.54 and 1.97 μg L^{-1} for Ni, and 0.28 and 0.06 μg L^{-1} for Cd (Table 2). Applying the batch-specific MDL's to each sample resulted in a total of 46% of Mn, 62% of Cr, 59% of Ni, and 61% of Cd sample concentrations falling below the MDL. For EBC samples with replicate measurements (39% of samples), the precision of the sample estimate was measured as the difference between measurements divided by the mean measurement. The mean precision of EBC metals concentrations were: 0.42, 3.37, 1.03, and 1.04 μg L^{-1} for Mn, Cr, Ni and Cd, respectively.

The SRM recoveries in the two batches were 108% and 105% for Mn, 109% and 105% for Cr, 105% and 100% for Ni, and 105% and 106% for Cd (Table 2). Since all of the SRM recoveries were within 10% of the expected value, no correction factors were applied to the EBC samples.

Association of collection parameters to EBC metal concentrations

Collection of EBC volume was found to be significantly associated ($p < 0.05$) with lower ambient relative humidity during collection, lower condensation tube temperature before and after EBC collection, larger volume respired during collection, and gender (larger EBC volumes were collected from males) (Table 3). There was no significant association between EBC

Table 2 Analytical measures of quality control for EBC metal concentrations (μg L^{-1})

	Batch 1 ($N = 50$)				Batch 2 ($N = 29$)			
	Field blank	MDL[a]		SRM[b]	Field blank	MDL		SRM
Metal	Avg	Estimate	% <MDL	% Recovery	Avg	Estimate	% <MDL	% Recovery
Mn	0.27	0.47	44	108	0.42	0.92	48	105
Cr	0.02	0.12	54	109	0.78	1.27	76	105
Ni	0.35	0.54	62	105	1.05	1.97	55	100
Cd	0.02	0.28	64	105	0.03	0.06	55	106

[a] Method detection limit. [b] Standard reference material.

volume collected and age of subjects or the room temperature during collection.

Concentrations of Mn, Cr, Ni, and Cd in EBC were significantly associated with higher room temperature and relative humidity ($p < 0.05$). The condensation tube temperatures measured either before or after EBC collection were not significantly associated with any of the EBC metal concentrations. Larger volume respired during collection was significantly associated with an increase of EBC concentration of Mn and Cd, and marginally associated with Cr ($p = 0.06$) and Ni ($p = 0.07$). Larger EBC volume collected was significantly associated with higher EBC concentration of Mn but not Cr, Ni, or Cd.

Among the subject characteristics, the age of subjects was not associated with the concentrations of the EBC metals. However, gender was significantly associated with EBC concentrations of all four metals, with females exhaling significantly higher EBC metals concentrations than males (Table 3).

We compared the association between EBC metal concentration measured as μg L^{-1} of EBC collected and as ng L^{-1} air respired. In comparing these two units of EBC metal concentrations, the coefficient of the EBC metal concentration for the four metals ranged from 18.8 to 21.3 (μg L^{-1} EBC) per (ng L^{-1} air respired). The overlapping confidence intervals of the regression of EBC measured as μg L^{-1} EBC and ng L^{-1} air respired among Mn, Cr, Ni and Cd suggest that the relationship is not different for the 4 different metals (ESI Table S2‡).

We also estimated mean EBC metals concentrations for healthy unexposed adults (Table 4) along with the sources of variability in these measurements. The geometric means and geometric standard deviations (GSD) of the uncensored concentrations were: 0.58 μg L^{-1} (GSD 2.42) for Mn, 0.18 μg L^{-1} (GSD 4.40) for Cr, 0.80 μg L^{-1} (GSD 4.62) for Ni, and 0.11 μg L^{-1}

Table 4 EBC metal concentrations (μg L^{-1}) and EBC volume (mL). ($N = 8$, $n = 79$)

Metal	Geometric mean	Var_{inter}[a]	Var_{intra}[b]	$Var_{overall}$[c]	CV[d]
Mn	0.57	0.19	0.87	0.45	0.0085
Cr	0.25	<0.0001	1.36	0.61	0.0099
Ni	0.87	1.87	6.64	15.39	0.0497
Cd	0.14	0.09	0.48	0.08	0.0036

[a] Variation of concentrations between subjects (inter-subject). [b] Variation of concentrations within subjects (intra-subject). [c] Variation of concentrations overall. [d] Coefficient of variation (SD/n).

Table 3 Estimated associations between EBC volume (mL) and metal concentrations (μg L^{-1}), and subject and collection parameters. Bolded estimates indicate there is a statistically significant association between the EBC volume or EBC metal and the subject or collection parameter ($p < 0.05$). ($N = 8$)

	EBC volume ($n = 79$)		EBC Mn ($n = 43$)		EBC Cr ($n = 28$)		EBC Ni ($n = 32$)		EBC Cd ($n = 31$)	
Parameter	Coefficient[a]	p-Value	Coefficient	p-Value	Coefficient	p-Value	Coefficient	p-Value	Coefficient	p-Value
Collection parameters										
Room temp (°C)	−0.008	0.212	0.68	**<0.001**	0.33	**<0.001**	0.37	**<0.001**	**2.5**	**<0.001**
Relative humidity (%)	**−0.004**	**<0.001**	0.92	**<0.001**	0.84	**0.002**	0.86	**<0.001**	**1.1**	**<0.001**
Condense start temp (°C)	**−0.011**	**<0.001**	0.91	0.183	1.00	0.980	1.06	0.666	1.0	0.827
Condense end temp (°C)	**−0.010**	**0.001**	0.88	0.212	1.06	0.700	1.13	0.627	1.3	0.159
Diff condense temp (°C)	**0.014**	**<0.001**	1.04	0.698	1.03	0.849	0.96	0.830	1.1	0.429
Volume respired (L)	**0.006**	**<0.001**	1.05	**0.002**	1.10	0.060	1.10	0.066	**0.9**	**0.011**
EBC volume (mL)	—	—	955	**0.003**	384	0.265	29	0.526	0.005	0.192
Subject characteristics										
Age (year)	0.002	0.515	0.96	0.284	1.00	0.972	0.95	0.668	1.0	0.837
Gender (female *vs.* male)	**−0.107**	**0.049**	**0.28**	**<0.001**	**0.01**	**<0.001**	**0.04**	**<0.001**	**13.8**	**0.001**

[a] Mixed model regression coefficient.

(GSD 4.71) for Cd. The estimated geometric means with censored values replaced with half of the detection limit for the four metals considered were: 0.57 μg L^{-1} for Mn, 0.25 μg L^{-1} for Cr, 0.87 μg L^{-1} for Ni, and 0.14 μg L^{-1} for Cd. For all these metals the overall standard deviation was greater than the mean estimate, and the variance within is larger than the variance between subjects. The majority of the variability in EBC metals concentrations is due to fluctuation in subjects' measurements over time rather than to the differences between subjects (Table 4).

4 Discussion

We developed a series of quality control measures that would allow for the robust characterization of metal content in samples of EBC. These measures included training subjects to breathe consistently during sample collection, additions to the Rtube assembly to allow for the quantitative assessment of volume of air respired by each subject, measurement of numerous collection parameters, and evaluation of all sources of contamination related to the components of the Rtube. Rosias suggests three categories of methodologic issues to be important to consider in EBC collection and biomarker measurement: pre-condenser conditions (subject and environment), condenser conditions (device used for collection), and post-condenser conditions (preservation and analysis).[19] The results of this work are evaluated in this framework.

Pre-condenser conditions

Our finding that the standard deviation of EBC concentrations for all metals tested was greater than the means and that the variation in sample concentrations was primarily driven by within-subject, rather than between-subject variance suggests that a high degree of fluctuation in EBC metal concentrations within individuals may be typical in healthy adults. The mean estimates here should be interpreted cautiously due to the high percentage of values less than the MDL and uncensored values that are highly skewed (as indicated by the geometric standard deviation),[18] yet they provide relative estimates. Mutti *et al.* present scatterplots of EBC concentrations from unexposed subjects that indicate median values of approximately 0.2 μg L^{-1} for Mn, 1 μg L^{-1} for Ni, and <0.01 μg L^{-1} for Cd (visually estimated from figure),[15] which are all much lower than the values we observed. Goldoni *et al.* also measured a lower mean concentration for EBC Cr of 0.18 μg L^{-1} (range <0.05 to 0.43 μg L^{-1}) in unexposed subjects with lung cancer.[20] One potential reason for higher concentrations measured in this current study may be a difference in the condensers used, as both Mutti and Goldoni used the TURBO-DECCS at −5 °C to collect EBC, while the temperature of collection was higher in this study with the use of the Rtube (and an increase of temperature is associated with an increase in EBC metals concentrations). It is likely that other collection parameter differences beyond temperature of collection also contribute to the observed differences.

The relationship between volume respired during sample collection *vs.* the volume of EBC sample collected was 106 L air respired/1 mL of EBC collected (95% CI: 97, 114). Using the EcoScreen, Gessner obtained similar results of 105 and 98 L air respired/mL EBC in healthy volunteers and COPD patients respectively.[21] This consistent finding that air volume respired and EBC volume collected are highly correlated suggests that a conversion factor can be applied to EBC metals concentrations to reported units as either μg L^{-1} of EBC or ng L^{-1} air respired (Table 4). Measuring EBC concentration as μg L^{-1} of EBC is much easier, as reporting EBC concentrations as ng L^{-1} air respired requires measurement of both the total volume of EBC and the total volume respired, while reporting concentrations as μg L^{-1} of EBC does not require either measurement.

Several pre-condenser conditions were found to influence EBC metal concentrations. Increases in room temperature, relative humidity, and air volume respired during EBC collection are either significantly or marginally associated with EBC concentrations of all four metals. The EBC volume collected was associated with EBC Mn concentrations, but not the other measured EBC metals concentrations. Effros has raised a concern that EBC volumes change the dilution level of the biomarker within the water vapor, and thus the measurement of the EBC biomarker would be inconsistent if ventilation patterns are altered.[22] While an affect from dilution has not appeared to be evident in measurement of nitrate, protein concentrations, pH, cysteinyl leukotrienes in EBC,[23,24] it may be an issue for Mn in EBC.

Gender and age also appeared to be important determinant of EBC metal concentrations. In contrast to the results reported by Liu and Thomas,[4] in our study an association between gender and EBC volume collected was found, where males generated a greater volume of EBC than females. This is consistent with the observation that males respired greater volumes during EBC collection than females in this study (female mean total volume during 5 minutes: 48.3 L, male mean total volume: 64.1 L). A gender difference was also measured in the EBC metals concentrations, with higher concentrations of all four metals measured in females. Also in contrast to findings by Liu and Thomas, an association between age and EBC volume was found in the present study. However, EBC metals concentrations were not associated with age. It is not clear why there is disagreement in results of EBC volume of the present study *vs.* those of Liu and Thomas; this could be attributed to subject differences such as where they live (and what air they inhale), and it may be attributed to differences in collection and breathing patterns (subjects breathed tidally in the other study). Because the small number of subjects investigated, observations about physiological characteristics of the subjects are inconclusive but may serve as indication for further research.

Condenser/post-condenser conditions

We established a procedure for measuring the method detection limit based on field blanks with the intent of imitating the complete method from sample collection through analysis: condensing tubes brought into the field during sampling, returned to the laboratory, with added water within the tubes of a similar volume to the EBC volumes collected. We applied the

MDL to establish the sensitivity of measurements, which resulted in 46–62% of EBC samples (depending on the metal) that were left-censored.

The MDL's estimated in this investigation were at least an order of magnitude greater than analytical limits of detection used to establish sensitivity reported by other investigators. Accounting for these differences with previous studies is difficult due to differences in the approach for generating detection limits or lack of detail about how the blanks were measured and calculated. It is common to report instrument detection limits rather than method detection limits, which are typically calculated as: 3 × SD of the concentration of the lowest analytical calibration standard, or 3 × SD of the concentration of the calibration check standard. Our reported sensitivity would have been as much as an order of magnitude lower if we had incorporated either type of instrumental detection limit (ESI Table S3‡).

The MDL is generally the most conservative measure and reflective of the overall sensitivity of the measurement, as it assesses both condenser and post-condenser conditions. Given the pervasive opportunity for contamination in trace metals analysis, the case could be made that method detection limits for EBC metals analysis are a better reflection of the true accuracy of the measurements than detection limits that are based on samples prepared as blanks or for the calibration curve in the laboratory, which only assesses post-condenser conditions.

While it is has not been standard to report MDL's, Hoffmeyer *et al.* presented supplemental data of a blank check for EBC metals analysis that was collected in a manner that is quite comparable to our approach.[25] In this test, 10 mL of ultra-pure water was incubated in a used EcoScreen for 10 minutes. Based on these concentrations, we estimate that the MDL using the above definition is 0.42 μg L^{-1} for Cr and 1.57 μg L^{-1} for Ni. (The concentrations for this estimation were kindly provided by F. Hoffmeyer *via* personal communication, 7/14/11). Although the incubation times are double the incubation time in the present experiment (5 *vs.* 10 minutes) and the collection devices used differ, these MDLs are similar to those estimated in our research.

Post-condenser conditions

The storage test of a pooled EBC sample is useful in evaluating the condenser and post-condenser conditions. This revealed that standard sample tubes leach metals over a relatively short time period in EBC samples (29 days in 1.5% HNO_3 at −20 °C). At shorter durations we found differences between metals in EBC in terms of the degree of contamination: within 11 days EBC Mn and Cr concentrations increased <10% in contrast with Ni and Cd, which had higher increases. Accordingly, the results of Ni and Cd should be interpreted cautiously. We are unaware of information about storage containers used for EBC prior to analysis and the duration between collection and analysis in other studies.

Numerous differences in condenser materials, sample preparation, storage containers, and analysis conditions could also quite possibly result in significant differences in measurement sensitivity among research groups. We suggest that reporting a detailed description of subject and collection parameters and attempting and improving QA/QC measurements could greatly facilitate the interpretation and improvement of EBC metals assessments.

5 Limitations

This was a small scale study was primarily designed to answer a series of focused questions about method performance under "real world" conditions. While our results of EBC metals concentrations in individuals help establish the magnitude of EBC metals concentrations in an unexposed population of healthy adults, further research will be required to provide better measures of the typical range in the general population. Another limitation of our study is the focus of the method optimization on Mn only. Variations and differences are to be expected if we were focusing on the other metals presented (Cr, Ni, and Cd).

6 Conclusions

The MREC system is highly portable, economical, and can be practical for small scale exposure studies. Our assessment of the EBC metals indicated that there can be multiple sources of metal contamination that are difficult to completely remove. This resulted in a relatively high method detection limits and a corresponding high percentage of measurements that were below the method limit of detection. While it is possible that high backgrounds of the metals of interest in this study are unique to the Rtube, it is likely that contamination is also an issue with other methods of EBC collection. Thus, it is clear that when using the Rtube for EBC sample collection and subsequent metals analysis, it is critical to account for all sources of contamination. The variability of the field blank measures may be reduced with the use of other materials for the condensing tube that may be more suitable for the collection of EBC for the purpose of metals analysis.

To our knowledge, this is the first research presenting typical concentrations and variability obtained from repeat measures of EBC Mn, Cd, Ni, and Cr in an unexposed population. Research of EBC metals is still in its early stages, and it is clear that disparate data are obtained from small changes in methods. Until further research has enabled the standardization of EBC metals analysis, we recommend that future groups continue to measure and report associations of EBC metals concentrations with sample collection and subject parameters.

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