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Determinants of variability of protein content, volume and pH of exhaled breath condensate

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Summary
Collection of exhaled breath condensate (EBC) is a simple and noninvasive method to obtain information on the respiratory system. Different mediators can be determined in EBC. However, determinants of variability are not well described.
The aim of this study was to evaluate variability of pH, volume and protein concentration of EBC between individuals and between sampling times. Therefore, EBC was collected from 20 healthy volunteers on two different days.
Median pH for all samples, measured 5 min after collection without deaeration, was 6.17. Median volume was 1.70 ml and median total protein concentration was 1.02 μg/ml. Coefficients of variation were 5.17%, 21.84% and 37.93%, respectively. No intra- or interday variability could be found, except for the first collection time. Between individuals, significant differences were observed for all three mediators. Age, height and gender can explain part of this variation.
In conclusion, no significant difference between sampling times on the same day or on different days was obtained for pH, volume and total protein concentration, provided that subjects are experienced in collecting EBC.

Introduction

Exhaled breath condensate (EBC) is a biological fluid that can be collected by tidal breathing. Collection of EBC is a simple and completely noninvasive method and as such it is applicable to study airway status from fragile groups in the population such as children and the elderly and for repeated use in longitudinal studies. It is believed that EBC contains molecules that reflect the physiological state of the lung.[1] There is a growing interest in this area and an increasing amount of researchers study the presence of low amounts of molecules in EBC samples. Compounds that have already been identified in EBC, include hydrogen peroxide, isoprostanes, leukotrienes and some specific proteins.[2–5] The presence of these molecules can be influenced by oxidative stress or inflammation of the airways.

EBC is more attractive to study airway status than for example bronchoalveolar lavage because of its noninvasive

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character. However, much has to be done before this method can be used routinely in clinical studies or in biomonitoring. Information on determinants of variability of mediators in EBC is scarce. It was the aim of our study to measure intra-individual variability of pH, volume and total protein concentration of EBC collected during different days and at different samplings times a day. This knowledge should be taken into account in case of study design and for interpretation of results.

Methods

Subjects and study design

Twenty-one volunteers (9 men, 12 women) aged 14–54 years, were recruited for this study. For 15 of the volunteers, it was the first time to collect EBC samples. From each volunteer, 6 EBC samples were collected: five on the first day (at sampling times 9.00, 11.00, 13.00, 15.00 and 17.00 h) and one on the second day (at sampling time 11.00 h). These samples were taken to evaluate inter- and intraday repeatability. All subjects completed a short questionnaire about general health, gender, age, height, body weight, smoking habits and environment (proximity of industry and heavy road traffic). These variables were used to evaluate variability between subjects. All volunteers lived in areas without heavy road traffic or industry in the direct environment and this variable was not further evaluated. One volunteer was excluded from the study based on her self-reported health status.

Methods/procedures

Collection of EBC and pH measurement

Exhaled breath condensate was collected using a RTube (Respiratory Research, Inc). The aluminium sleeve was stored for at least 30 min in a home freezer (−18 °C) before collection. Subjects were asked to breath tidally through the mouthpiece for 15 min. No food was taken 1 h before collection. Volume of the condensate was measured immediately using a calibrated 1 ml pipette. The condensate was transferred into a 1.5 ml microfuge tube (LoBind Tube, Eppendorf). pH was measured with a biotrode (Hamilton) exactly 5 min after collection (without deaeration), after which samples were frozen immediately at −18 °C. Transport to the laboratory occurred on dry ice and samples were stored at −80 °C until further analysis.

Measurement of total protein concentration

Total protein concentration was measured with the NanoOrange Protein Quantitation Kit (Molecular Probes) in a fluorescence spectrophotometer with cuvettes (LS55, Perkin Elmer), according to the instructions of the manufacturer of the kit. Because very low protein concentrations were expected, 400 μl aliquots were lyophilised and analysed in triplicate. Positive control samples of 2 μg/ml bovine serum albumin (BSA) were included in the analysis.

Saliva contamination

All samples were checked for saliva contamination. Amylase was measured with the Infinity™ Amylase Liquid Stable Reagent kit (Thermo). Samples were measured in triplicate and saliva samples were measured as positive control samples.

Statistical analysis

All data are expressed as median values and corresponding interquartile range. The overall coefficient of variation (CV) and CV for measurements at different days, different sampling times per day and different individuals are given. Univariate main effect ANOVA ($P<0.05$) was used to analyse interday and intraday variability and variability between subjects for each variable of interest (volume, pH and protein content). If $P<0.05$, a Scheffé test was performed as post-hoc test to assess significant differences among the variables. A Bland–Altman plot was generated to compare each variable between samples collected at the two different days.[6] A paired Student t-test ($P<0.05$) was used to compare collections from similar time points (11.00 h) at two different days. Based on the information from the questionnaires; gender, age, smoking habit, weight, height and body mass index (BMI) of the subjects were statistically analysed. Correlation between the variables was evaluated in a correlation matrix. Influences of these variables were evaluated in a multiple regression analysis ($P<0.05$). To assess possible linear relationships between the explanatory variables in the multiple regression analysis (in which case the estimation of regression coefficients becomes unreliable), variance inflation factors (VIFs) were calculated. Based on these VIFs, the original variable 'weight' has been eliminated from the regression for introducing collinearity especially with regard to the explanatory variables 'height' and 'BMI'. Elimination of the weight variable reduced the amount of collinearity between the explanatory variables to an acceptable degree. Scatter plots show the influence of the individual variables on volume and total protein concentration. All statistics were performed with Statistica 7.

Results

Subjects

Volume, pH and total protein concentration could be determined for all samples. Characteristics of the subjects are given in Table 1.

Table 1 Characteristics of the subjects.

	Males	Females
Subjects (n)	9	11
Age (yrs)	26 (14–54)	25 (22–50)
Weight (kg)	78 (59–104)	67 (58–82)
Height (m)	1.87 (1.70–1.99)	1.69 (1.63–1.79)
Smokers	1/9	1/11

Characteristics of the volunteers, based on the completed questionnaires. For age, weight and height, the median (range) is given.

Variability

Volume

Median volume of EBC from samples collected during 15 min of collection was 1.70 ml with an interquartile range of 1.40–1.87. Median values and ranges at different sampling times are shown in Fig. 1a. The overall CV was 21.84%. CV between sampling days (interday variability) was 11.10% (range: 0–22.8%). Between sampling times/day (intraday variability), CV was 13.99% (range: 1.54–25.69%) and between subjects it was 31.18% (range: 28.79–35.83%). No significant interday variability was observed comparing sampling time 11.00 h with a paired Student *t*-test ($P = 0.163$) and as shown by a Bland–Altman plot (Fig. 2a). Results of ANOVA analysis showed significant intra-day variability in EBC volume (Table 2). As shown by the

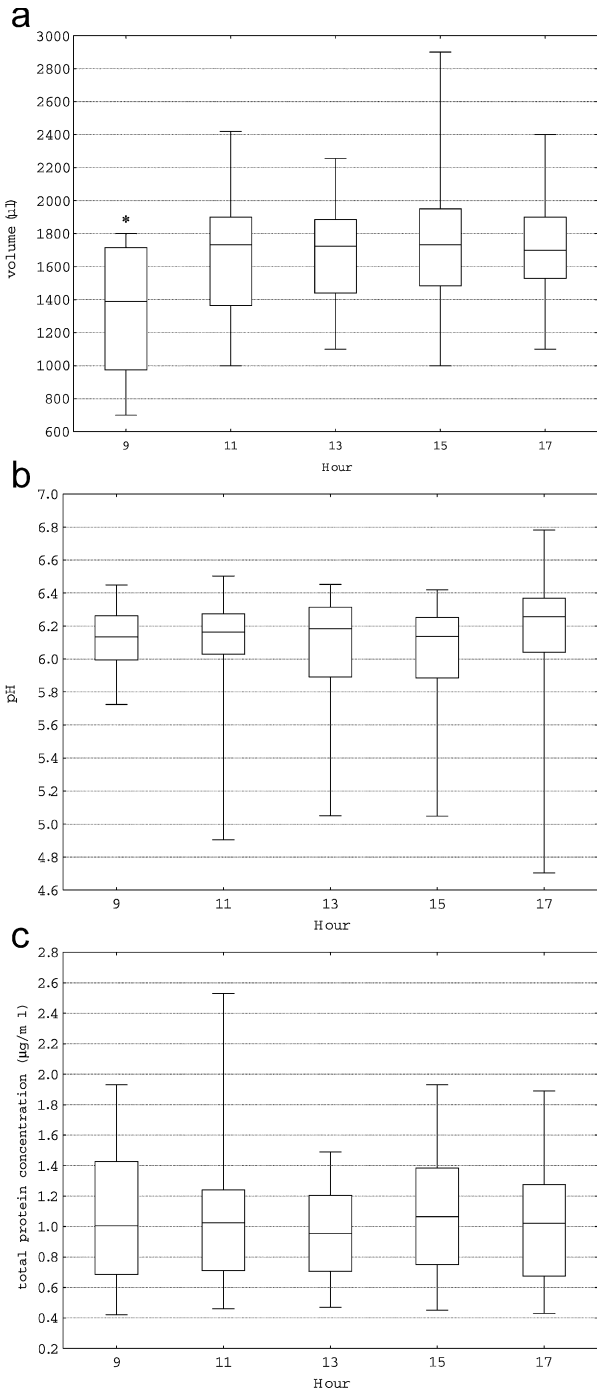

Figure 1 Variability during the day. Box–Whisker plot showing median value of EBC samples collected at the five different sampling times in all 20 individuals and the corresponding interquartile range and minimum/maximum value for: (a) volume, (b) pH and (c) total protein concentration.

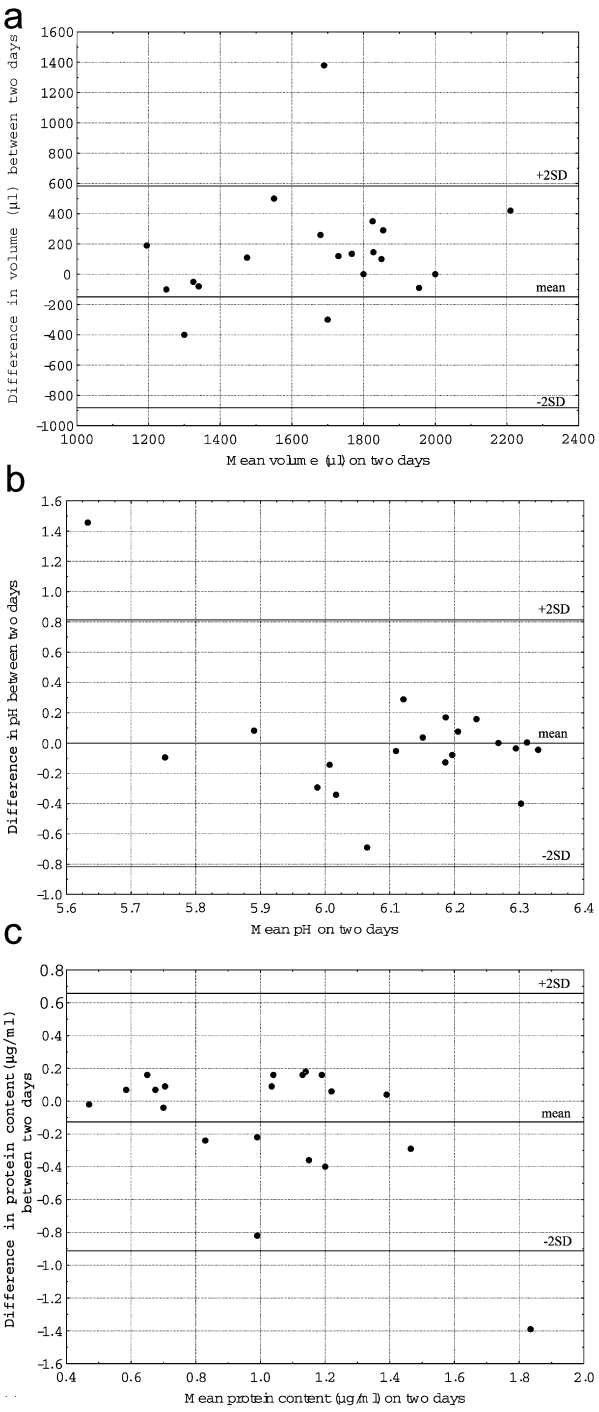

Figure 2 Bland–Altman plot for comparison of: (a) volume, (b) pH and (c) total protein concentration of EBC samples collected at two different days (collection time 11.00 h).

Scheffé test, EBC volume was significantly lower at the first sampling time compared to the other times ($P<0.05$). It has to be taken into account that for most individuals (fifteen), this was the first time to collect EBC. In the five subjects from which EBC was collected before, this difference was not observed. There were no significant differences between the other sampling times ($P = 0.133$). Volume of EBC samples differed significantly between subjects ($P<0.0001$).

pH

Median pH was 6.17 (interquartile range: 5.96–6.31). Fig. 1b shows median pH values and ranges at different sampling times. Overall CV for pH was 5.17. CV for interday variability was 2.79% (range: 0.04–8.04%), for intraday variability 3.63% (range: 1.34–8.23%) and between individuals it was 5.02% (range: 2.92–7.06%). Statistical analysis was performed on pH values after they were converted by inverse log transformation. Main effect ANOVA showed no significant differences between samples collected at different days (interday) or different sampling times a day (intraday) ($P = 0.494$ and 0.671, respectively; Table 2). Fig. 2b shows the Bland–Altman plot to compare pH values from samples collected on two different days. Between volunteers, pH values of samples were significantly different ($P = 0.0001$).

Total protein concentration

Protein concentration was measured in all samples. Median values for each sampling time with the corresponding range are shown in Fig. 1c. Median total protein concentration was 1.02 μg/ml with an interquartile range of 0.71–1.27 and an overall CV of 37.93%. Part of this variation resulted from the concentration procedure (lyophilisation) and from the protein analysis (CV for the three replicas: 18.32%). CV for interday variability was 13.87% (range: 2.03–58.57%), for intraday variability 14.55% (range: 4.12–33,77%) and between individuals it was 36.64% (range: 29.23–42.29%). No significant differences were present for samples collected at different days (interday) or at different sampling times per day (intraday) ($P = 0.057$ and 0.154, respectively; Table 2, Fig. 2c). Between individuals, total protein concentration of EBC differed significantly ($P<0.0001$).

Correlation of variables

Correlation between gender, age, smoking habit, height and BMI of the subjects and their contribution to the effect on EBC pH, volume and protein content was evaluated. It has to be taken into account that these values result from 20 individuals and larger populations are needed to confirm these results. In this population, a significant correlation between height and age ($P = 0.02$), height and gender ($P<0.001$), BMI and age ($P<0.001$) and BMI and smoking habit ($P<0.001$) is present.

Volume

As shown by multiple regression analysis (Table 2), gender and height significantly influence collected EBC volume. Age, smoking habit and BMI do not contribute significantly. Women and taller individuals collected larger EBC volumes. Overall significance of the multiple regression measured by the f-test amounts to $P<0.0001$. The regression accounts for 36% of the variance in volume of EBC. Trends in gender and height (single regression analysis) are shown in Fig. 3.

pH

No significant effect of gender, age, height, BMI or smoking behaviour could be found.

Total protein concentration

Age and height have a significant influence on total protein concentration, as shown by multiple regression analysis,

Table 2 Variability and correlation.

	Volume	pH	Total protein concentration
A. *Variability obtained by ANOVA*			
Interday	**0.033** (0.163)*	0.494 (0.472)*	0.057 (0.307)*
Intraday	<**0.0001**	0.671	0.154
Between subjects	<**0.0001**	**0.0001**	<**0.0001**
B. *Correlation of different variables in multiple regression analysis*			
Gender	<**0.0001**	0.343	0.071
Age	0.485	0.230	**0.003**
Smoker	0.115	0.670	0.491
Height	<**0.0001**	0.721	<**0.0001**
BMI	0.753	0.165	0.625
Combined†	<**0.0001**	0.445	<**0.0001**

A. Variability data are P-values obtained by univariate main effect ANOVA on all sampling times. B. Correlation data are P-values obtained by multiple regression analysis. Results from the first sampling time (9.00 h) were excluded from this multiple regression analysis because of a significant different volume (see also Fig. 1). Values in bold are significant differences ($P<0.05$). BMI = body mass index.

*Results from the paired Student t-test (P-value), to compare only collections from similar time points (11.00 h) at the two different days, are shown in brackets.

†The significance levels under the 'Combined' row heading give the significance levels using the f-test of the regression between the corresponding dependent variable with all explanatory variables.

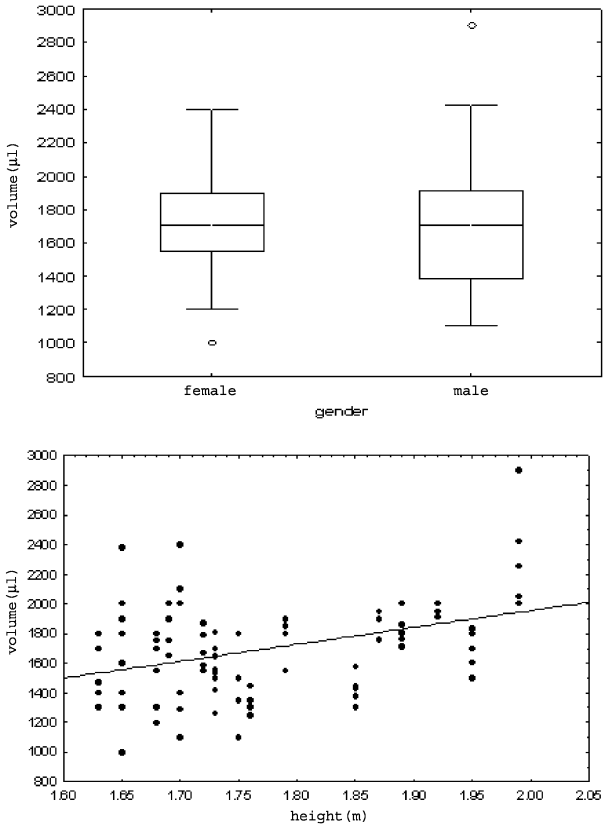

Figure 3 Trends in EBC volume in function of gender and height. All measurements of the 20 volunteers are shown, except the first collection time 9.00 h. Multiple regression analysis shows significant effects of gender and height on volume (see also Table 2). Single regression analysis shows no significant influence for gender ($P = 0.785$). Height has a significant effect on collected volume ($P = 0.0002$). The reduced explanatory power of gender in the single regression model is caused by second order interactions between gender, height and age.

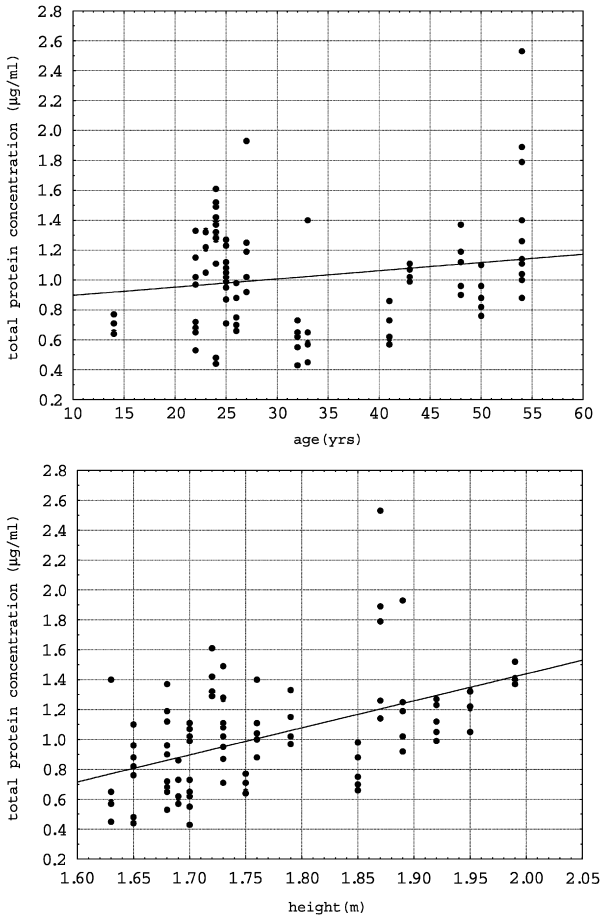

Figure 4 Trends in total protein concentration of EBC samples in function of age and height. All measurements of the 20 volunteers are shown, except the first collection time 9.00 h. By using single regression analysis to evaluate the effect on total protein concentration, $P = 0.083$ for age and $P < 0.0001$ for height.

(respectively, $P = 0.003$ and < 0.0001; Table 2). Older and taller subjects had a higher protein concentration in their EBC. Gender, smoking or BMI did not significantly influence protein concentration in EBC. Overall significance of the multiple regression measured by the f-test amounts to $P < 0.0001$. The regression accounts for 39% of the variance in protein concentration in EBC. Trends in age and height (single regression analysis) are shown in Fig. 4.

Saliva contamination

No amylase activity was measured in the EBC of all volunteers, thus no saliva contamination could be detected in these samples.

Discussion

Because of its non-invasiveness, the study of EBC is a promising technique to study airway function and lung diseases. The numerous recent publications show an increase in interest on this subject. A task force for EBC was recently established by the American Thoracic Society/European Respiratory Society, with recommendations for further research.[7] Information on the role of different determinants of variability remains scarce, although this is very important for further study design and interpretation of results. Only for H_2O_2, a marker of oxidative stress that has already been studied successfully in EBC, circadian variation has been demonstrated.[8,9] A significant increase in H_2O_2 concentrations during the day was found both in COPD patients and in normal subjects. Mean H_2O_2 concentration did not change over 3 weeks. Recently, variation was studied for NO metabolites in EBC: a poor intra- and interday repeatability was found.[10] Knowledge of possible fluctuations during the day and variation in factors of interest, is very important.

In this study, variability in EBC pH, volume and total protein concentration were examined. The volume of EBC was significantly lower at the first collection point at 9.00 AM. This might be related to inexperience. For most volunteers (15/20), it was the first time to collect condensate and this difference was not seen in the five volunteers that collected EBC before. Except for the first

collection time, interday and intraday variability were not significantly different. Therefore it is acceptable to assume that the first experience with EBC collection of an individual significantly influences the EBC volume. Once familiar with the procedure, EBC volumes are constant for the same individual. Earlier data[11] showed a higher EBC volume in the afternoon, but this may possibly also be explained by the inexperience of the volunteers at the first sampling time because in that study, samples were only taken at three different times (8–9 AM, 12–2 PM and 5–6 PM).

pH values are comparable with pH measured in other studies where samples were not deaerated.[12,13] Acidification of EBC is a known characteristic of airway inflammation. Differences in pH were shown for patients with asthma and cystic fibrosis compared with healthy individuals.[12–14] In this study, not much variation was observed in pH of EBC samples collected at different days (interday), different sampling times/day (intraday) and different individuals, as shown by small coefficients of variability. This corresponds to earlier results for healthy individuals,[15] although more variability between COPD patients was observed.[16]

Total protein concentration ranged from 0.42 to 3.22 μg/ml, what is in agreement with earlier publications.[17,18] No significant trends could be observed for interday or intraday collection. This is in agreement with earlier results, where no difference in total protein concentration was reported in 12 healthy volunteers based on sampling time.[11] Despite the lower EBC volume collected during the first sampling time, no effect was seen in protein concentration.

Between subject variability in EBC volume was observed. Based on the questionnaires, the role of some explanatory factors such as gender, age, height, BMI, and smoking habit, could be evaluated. A significant trend was observed caused by gender and height: females and taller persons collected higher volumes EBC. Although statistical associations are described, no causal relationship can be determined. The study group is limited and results should be verified in a larger study population. Volume may be related to differences in lung volume, minute ventilation and total expired volume.[17,19] It is unclear if these factors were different in these twenty subjects because lung function was not measured in this study.

The EBC pH was not influenced significantly by gender, age, height, BMI or smoking habit. Further research is required to define the factors responsible for changes in pH of the EBC from an individual, but it was already suggested that gases and ions could be involved.[20]

Total protein concentration levels were significantly influenced by age and height: EBC of older and taller subjects contained higher protein concentrations. Garey et al.[18] found a higher protein concentration in the EBC from smokers. We were unable to find this, but only 2 of our volunteers were smokers, and they were asked not to smoke during 1 h before sample collection.

In conclusion; gender, age and height contributed significantly to the variation in volume and protein content of EBC in this study. They did not affect significantly the pH of EBC. Volume and total protein concentration were higher in taller subjects. Females were found to collect a higher volume EBC than males. Protein concentration was higher in EBC samples from older individuals. In this study, no significant differences in volume, pH and protein content were found for smokers compared to non-smokers. However, no conclusions can be drawn because only two of the twenty individuals were smokers. This study showed no significant differences between inter- and intraday variability in EBC pH, volume and total protein concentration, provided that individuals are experienced with the collection method. This means that sampling time will not influence these results and does not have to be taken into account for further study planning or comparing of results.

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