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Effect of temperature control on the metabolite content in exhaled breath condensate

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Abstract

The non-invasive, quick, and safe collection of exhaled breath condensate makes it a candidate as a diagnostic matrix in personalized health monitoring devices. The lack of standardization in collection methods and sample analysis is a persistent limitation preventing its practical use. The collection method and hardware design are recognized to significantly affect the metabolomic content of EBC samples, but this has not been systematically studied.

Here, we completed a series of experiments to determine the sole effect of collection temperature on the metabolomic content of EBC. Temperature is a likely parameter that can be controlled to standardize among different devices. The study considered six temperature levels covering two physical phases of the sample; liquid and solid. The use of a single device in our study allowed keeping saliva filtering and collector surface effects as constant parameters and the temperature as a controlled variable; the physiological differences were minimized by averaging samples from a group of volunteers and a period of time. After EBC collection, we used an organic solvent rinse to collect the non-water-soluble compounds from the condenser surface. This additional matrix enhanced metabolites recovery, was less dependent on temperature changes, and may possibly serve as an additional pointer to standardize EBC sampling methodologies. The collected EBC samples were analyzed with a set of mass spectrometry methods to provide an overview of the compounds and their concentrations present at each temperature level.

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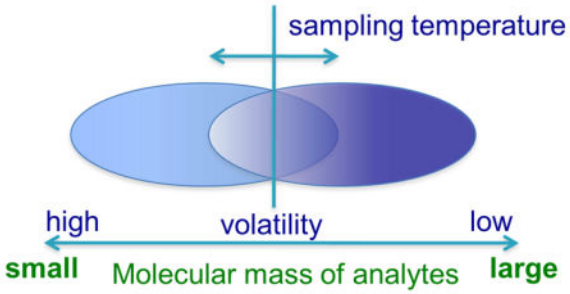
The total number of volatile and polar non-volatile compounds slightly increased in each physical phase as the collection temperature was lowered to minimum, 0 °C for liquid and −30, −56 °C for solid. The low-polarity non-volatile compounds showed a weak dependence on the collection temperature. The metabolomic content of EBC samples may not be solely dependent on temperature but may be influenced by other phenomena such as greater sample dilution due to condensation from the ambient air at colder temperatures, or due adhesion properties of the collector surface and occurring chemical reactions. The relative importance of other design parameters such as condenser coating versus temperature needs further investigation.

Graphical Abstract



Keywords

Exhaled Breath Condensate (EBC); breath metabolomics; collection temperature control; analytical methods

1. Introduction

The non-invasive, quick, and safe collection of exhaled breath condensate (EBC) makes it a candidate as a diagnostic matrix for use in personalized health monitoring devices. Single-use, sterile EBC collection device components can be mass produced cheaply and used safely in non-medical settings. The recent advances in microfluidic lab-on-a-chip analysis and cloud-based data analysis algorithms may make prescreening of a number of diseases possible in short time period at a small fraction of the current cost.

EBC is a complex matrix which has been shown to have a chemical composition resembling that of the extracellular lung fluid [1–5]. This biological sample is rich with a wide variety of compounds including: non-volatile biomolecules aerosolized from the airway lining fluid and water-soluble volatile compounds, proteins, lipids, antibodies, and carbohydrates. In total, in humans, EBC contains up to 2000 different compounds [6–8]. EBC analysis may not precisely measure solute concentrations in native airway fluid; however, if concentrations of certain compounds differ enough between a healthy and diseased state, EBC analysis can be a potential diagnostic tool [7]. Some individual compounds or a set of compounds can be reflective of diseased state and are called biomarkers. Currently, nitric oxide (NO) [9–12], hydrogen peroxide (H_2O_2) [13–16], and acetone, measured from breath condensate, are the three most studied and used compounds for diagnostic biomarkers of inflammatory responses in the respiratory system. Concentrations of lipids can also be

measured from EBC, including fatty acids, steroids, eicosanoids, and their subclasses, such as prostaglandins or isoprostanes [17, 18]. For instance, 8-isoprostane detected in EBC, is considered a biomarker of oxidative stress and antioxidant deficiency, showed differences between healthy smokers and patients with COPD [19]. Plasma lysozyme was found to be significantly higher in patients with adult respiratory distress syndrome (ARDS) as compared to healthy patients [20]. Other low-volatility compounds such as enzymes have been considered as effective biomarkers of illness diagnosis. The pH of EBC can also serve as a simple but robust biomarker of various lung diseases [21–23]. The compounds, including volatile organic compounds (VOCs) present in EBC are not limited to the respiratory system but may originate from blood borne biogenic compounds, and can be representative metabolites of a wide range of systemic processes [24, 25]. Patients with and without lung cancer, regardless of the cancer stage, were discriminated using 22 VOCs including alkanes, alkane derivatives, and benzene derivatives [26]. A combination of eight VOCs was sufficient to discriminate between asthmatic and healthy children [27].

EBC analysis has some current limitations; the persistent problem is the lack of standardization in the collection methods, the collection devices, and the sample analysis [28–30]. The collection procedure and hardware design are known to significantly affect the metabolomic content of the EBC sample [31, 32]. A number of parameters were examined: effect of sampling duration, breathing pattern, collected fraction of the exhaled breath (alveolar end tidal versus total expired volume), collection device material, condensation temperature, contamination from saliva, sample transfer, and storage [33–35]. The design and performance of commercially available EBC samplers such as the Rtube™ (Respiratory Research, Inc., Austin, TX, USA), ECoScreen® (Erich Jaeger GmbH, Hoechberg, Germany), and TurboDECCS (MEDIVAC, Parma, Italy) were compared to answer some of the questions about sample collection procedure and device choice [36–38].

In our previous work [38], we compared the performance of an engineered EBC collection device with that of RTube™ and TurboDECCS®. Though the three devices equilibrated in the volume of collected EBC sample, the EBC samples differed in the metabolomic content. The engineered device collected EBC samples that contained less saliva but higher number of compounds. There were some design differences for that; different collection temperatures, different surface materials, and different saliva filtering mechanism.

The engineered device had a PTFE housing (duct) and a glass condenser surface cooled by dry ice pellets; it warmed up from −56 °C to − 30°C in a 10 minute sampling period. RTube™ and TurboDECCS® had polypropylene condenser surfaces and significantly differed in the condensation temperature and its stability. RTube™ warmed up from −56 °C to 0 °C during a 10 minute sampling period and TurboDECCS® warmed up from −7 °C to 6 °C during the first minute of breath sampling [38]. The surface properties of the collector surface are also known to have an effect on the recovery of the metabolomic content of EBC.

Rosias et al. [35, 37] studied the effect of the condenser surface coatings on measurement of biomarkers in EBC. Five condenser coatings (silicone, glass, aluminum, polypropylene, and Teflon) were compared using the ECoScreen® device. Adhesive properties of different condenser coatings influenced the eicosanoids and proteins measurements in EBC. Silicone

and glass coatings were shown to be more efficient for measurement of 8-isoprostane or albumin in EBC. The relative importance of hardware parameters, e.g. temperature level versus surface material, and their effects on the content of EBC samples needs to be quantified with a rigorous set-up where both tested parameters are highly controlled.

The contamination with saliva needs to be minimized because oral microbiome contributes wide variety of metabolites that may obscure biomarkers originating in the lungs [39]. The level of saliva contamination in collected EBC samples was different; the engineered device had the least level of saliva contamination.

The previous studies that used different devices are informative and give some common points for estimation but lack to define the relative importance of design parameters because the compared devices differ in surface material temperature, and saliva filtering [32, 36, 38].

While all design parameters (saliva trap, material choice, breath flow, chamber design, and heat transfer) have their effect on the metabolomic content of EBC, here we investigate the significance of the condensation temperature while keeping other parameters constant. A custom EBC sampling device was used [38]. A refrigeration-based cooling system with an accurate temperature control was constructed for this experimental investigation. The EBC samples were collected at incremental temperatures, between 5 °C to −56 °C, from one group of volunteers, with the one device, and with the same procedure. The volatile fraction of the EBC samples was analyzed with a gas chromatography coupled to mass spectroscopy (GC-MS) method and the non-volatile fraction with high performance liquid chromatography-mass spectrometry (HPLC-MS) methods. Knowing some guidelines for the choice of an optimal temperature for EBC collection will be very useful for engineering future portable platforms for EBC analysis. From engineering point of view, the temperature level may directly determine the power requirements. From diagnostic point of view, it may affect the metabolomic content of EBC samples.

2. Material and methods

2.1. EBC sampler and experimental hardware

A custom built EBC sampling device [38] was used in this study with a modification in the cooling method. Figure 1 shows the main components of the device. Its design employs a number of engineering solutions to make it optimal for collection of EBC samples [38]. Chemically inert materials (PTFE and glass) were used for parts that are in contact with the biological sample. An engineered mass-momentum-based flow filter was used to reduce sample contamination with saliva droplets carried with the breath flow from the mouth cavity. Resistance pressure drop and heat transfer were evaluated to provide comfort and to ensure that the condensation temperature was stable during the sampling period. The device was equipped with a pair of one-way valves that route the inhaled and exhaled air in such a way that only exhaled breath flowed through the device condenser chamber. The outlet valve, installed at the top end of the condenser tube, prevented the condensation from the ambient vapor. To evaluate and to have a reference point, the performance of the engineered device was compared to that of two commercial instruments, RTube™ and TurboDECCS®, in terms of sample volume, working temperature and its stability, level of saliva content in

EBC samples, and the number of detected metabolites in EBC [38]. The device demonstrated superior performance in terms of metabolite richness compared to the other devices and thus was chosen for use in this experiment.

In this study, the EBC collection element of the described engineered device [38] was used with a refrigeration cycle-based cooling system equipped with an accurate temperature control (Figure 2a). The cooling system consisted of a thermal mass, refrigerant coil and line, a compressor, and an Arduino®-based temperature control and user interface. An aluminum thermal mass (280 mm long, 50 mm OD, 25.3 mm ID) had a machined helix groove on the outer side (groove depth and diameter, 7 mm) that accommodated a copper tube (6.25 mm OD) for refrigerant circulation. For better thermal contact, the groove was partially filled with thermal paste (Cooler Master, IceFusion, RG-ICFN-200G-B1) before the copper line was woven into the helix groove. The copper line was secured with small bolts that were screwed into the aluminum mass. Two *k*-type thermocouples were placed with the thermal paste into small pockets drilled in the aluminum thermal mass at one-third and two-thirds of its length. The thermocouples and the copper line were wrapped with electrical insulation tape and placed into a polycarbonate tube (290 mm long, 101.6 mm OD, 91.9 mm ID). The hollow radial space (20.95 mm) was filled with thermal insulation foam (Dow, Product # 227112). The thermal mass was intended to buffer rapid temperature changes during breath sampling and when the refrigerant pump cycled through ON/OFF operational cycles. The refrigerant compressor (General Electric, # FCM5SHBWW; 1.35 Amp, 115 V, design pressure: high side 290 psig, low side 88 psig) was installed in a table-top housing box and connected to the copper helix line with a pair of flexible rubber Kevlar reinforced hoses, 914 mm long (McMaster, Part # 1874K11). The system was charged with refrigerant gas (R134a). Based on system performance, no refrigerant leak was noticed after 98 hours of operation and one year of storage.

An Arduino® microcontroller (ProTrinket, Adafruit, #: 2000) with an LCD display (16×2 LCD-RGB; Adafruit, #: 784), three push buttons (RadioShack, Product #: 2750609), and three LEDs were used for user interface and for temperature control (Figure 2b). Once the system was started, the controller entered the set-up mode where a user could input the desired temperature level, from 20 °C down to −30 °C at a half degree increment; temperature overshoot margin – number of degrees to go below the desired temperature before turning off the compressor; and the minimum time period for compressor pressure stabilization – the time period to equilibrate the pressure difference between the vacuum side and the pressure side in order to be able to start pumping after it was stopped. The temperature was measured with a thermocouple amplifier (MAX31855, Adafruit) from one of the two embedded thermocouples. The spare thermocouple was to be used in case the first got damaged or for measuring the average temperature between the two ends of the thermal mass, by connecting the thermocouples in parallel. The controller was powered with a power supply (9 V, 1 Amp; I.T.E., Model #: GEO151UB-9013). The solid state relay (Crydom®, CKRD2420) was used to power on/off the mechanical pump. The controller was packed in a small box panel on a side of the cooling sleeve (Figure 2b). As a safety precaution, the 9 volts power supply and all high power electrical connections were made inside the table-top pump housing and a 4 wire cable with was run along the flexible hoses to the user interface on a side of the hand-held collector element. Two wires were used for power supply to the

controller and the other two wires were used for relay control to operate the mechanical pump.

To calibrate the temperature settings, a custom Arduino® data logger was used for surface temperature measurements from eight thermocouples attached to the inner surface of the condenser tube (experimental details [38]).

Supplemental material S1 provides more details on the device design and fabrication.

2.2 Samples collection and aliquots for analysis

Figure 3 shows the flowchart of the EBC collection and sample distribution for analysis in this study per one of the temperatures; other temperatures were processed in the same manner. The EBC samples were collected from a group of six healthy volunteers (3 males and 3 females; matched from the three age groups of approximately 20, 30, and 40 years). All participants were in good health and had no history of smoking. The EBC sampling time was 12 minutes; the number of breaths and the expired volume were not measured. No nose clip was worn during EBC sampling. Volunteers were asked to keep normal tidal breathing with straight body position (straight back, ninety degrees chin). The volunteers restrained from food consumption three hours before EBC collection procedure and rinsed their mouths with water prior to breathing into the EBC collection device in order to reduce the effect of food related confounders. All other experimental set up parameters were kept constant for all volunteers and all collection temperatures. EBC sampling from human volunteers was performed under UC Davis IRB protocol 63701-3.

Six temperature levels (5, 0, −10, −20, −30, and −56 °C), common for commercial devices or used in previous studies, were tested in this study. Temperatures down to −30 °C were achieved with the refrigerant-based cooling system. Temperature of −56 °C was achieved with charging the device cooling chamber with dry ice pellets [38]. The same EBC collection device was reused by all volunteers. All parts of the device and sample retrieval press were thoroughly cleaned before and after each use. The cleaning protocol included three rinses: deionized (DI) water rinse, followed by 70% ethanol disinfectant rinse, followed by DI water rinse and drying. A new mouthpiece (Instrumentation Industries, Inc., part #: BE 120-22D) was installed for every sampling procedure. The cleaned device was assembled, the temperature controller was set at the appropriate temperature level, and the system was allowed to stabilize for three ON/OFF operational cycles to ensure the temperature stability during EBC collection. In case of dry ice cooling, the assembled clean device was charged with dry ice pellets and allowed 5 minutes for temperature stabilization. Both ends of the condenser tube were closed with PTFE caps to prevent vapor condensation from the ambient air during temperature stabilization period. After EBC collection, a sample was quickly retrieved into a vial (Sigma-Aldrich, part #: SU860099 SUPELCO), cap sealed (Sigma-Aldrich, part #: SU860101 SUPELCO), weighted for mass, and stored in a −80 °C freezer. The mass of the collected EBC sample was evaluated by subtracting the mass of the empty vial from the mass of the vial with the sample in it.

In order to better correlate the dependence of EBC sample volume on the collection temperature, the physiological differences of volunteers were noted [33]. Prior to EBC

sampling, the tidal expired volume per breath cycle and tidal peak expiratory flow were measured from each volunteer with a spirometer (Spirolab II; SDI Diagnostics, Easton, MA) on three different days.

A total of 36 EBC samples were collected from six volunteers at the six different temperatures in a randomized order during a four months period. Each volunteer breathed into the device at a set temperature for 12 minutes. The required volume sufficient for the planned analysis was 3 mL per one EBC sample (one volunteer, one temperature). This volume accounted for collection of extra sample in case the analysis needs to be repeated. It took two or sometimes three EBC collections (12 minutes each, different days) from a volunteer to collect this volume. In this case, the EBC samples (from the same volunteer and at the same temperature) were combined in one vial.

After each EBC sampling, the inside surface of the condenser tube was rinsed with 1 mL of 200 proof HPLC grade ethyl alcohol (Sigma-Aldrich, Product #: 459828). This solvent was used to rinse the non-aqueous EBC fraction residue from the collection tube and stored in a sealed vial for further analysis. A total of 36 EtOH surface rinse samples were collected. Water wash blanks were collected from a cleaned device each week. The cleaned condenser tube was rinsed 2 mL of DI water. The condenser tube wash outs were used to monitor the compounds adsorption and accumulation on the surface of the condenser tube.

After all individual EBC samples were collected, an average EBC sample per each temperature (10 mL) was prepared by aliquoting 1.67 mL from each individual EBC sample. This step was intended to buffer the individual differences of volunteers and to represent an EBC sample collected at a specific temperature. The “averaged” sample was then aliquoted into separate replicates for GC/MS, LC/MS, and LC/MS/MS analyses. The EtOH samples were aliquoted, 0.67 mL from each rinse sample, and “averaged” per each temperature (4 mL) in a similar manner. Figure 3 shows the schematics for one of the temperatures.

2.3 Metabolomic content analysis

To obtain a wide coverage of the metabolic content of EBC, different analytical techniques were applied. The volatile fraction was studied with GC-MS. The non-volatile fraction was studied with two complementary analytical platforms based on reversed phase (RP) and hydrophilic interaction (HILIC) LC-MS. Three technical replicates were processed for each of the tested temperatures. Water-based EBC samples and device blanks were analyzed with GC-MS and LC-MS techniques; ethanol samples were analyzed with LC-MS techniques only (Figure 3).

GC–MS analysis was done with Varian 3800 GC with a 4000 Ion Trap MS (Varian, Walnut Creek, CA) equipped with electron ionization (EI) source and a semi-polar VF-5ms column (5% phenol/95% PDMS, Agilent J&W). Before the analysis, 1 mL aliquots of each EBC sample were thawed and placed on a chiller plate in cap-sealed vials. The volatile compounds were extracted directly from the liquid in the vial using a polyacrylate (PA) solid phase microextraction tip (SPME) (Supelco, Bellefonte, PA) for 30 min at room temperature with constant agitation. After that, the samples were injected in the GC-MS system using the conditions described in our previous work [38]. Empty vials, DI water blanks, and aqueous

D8 naphthalene quality controls were analyzed alternately in a single batch with all the samples.

HPLC-MS analyses were performed with an Agilent 1290 series high-performance liquid chromatography system coupled with an Agilent 6230 time-of-flight (TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA). Prior the analysis, 0.5 mL aliquots of each EBC and ethanol samples were placed in vials and lyophilized directly. The dried extract was reconstituted in 70 μL of 9:1 (v/v) HPLC grade acetonitrile: water solvent with sonication. All the samples were stored at −20 °C in capped LC vials before LC-MS analyses with both platforms, HILIC-LC and RP-LC. The instrumental conditions were also previously described [38]. An Agilent Jet Stream (AJS) electrospray ionization (ESI) source was used in positive mode (+) and acquiring a mass range between 50 and 1700 (m/z). Quality controls were analyzed with every batch to monitor the system stability and data quality, together with the method blanks prepared with water, acetonitrile and the reconstitution solvent. All samples were housed in an autosampler maintained at 4 °C.

For the HILIC chromatography (HILIC-LC), 20 μL of each sample were injected in a Waters Acquity UPLC BEH Amide column (130A, 1.7μm, 2.1 × 100 mm) ((Waters, Milford, MA) held at 30 °C. A gradient of mobile phase was applied using water (A) and 90% acetonitrile in water (B), both at pH 5 with an ammonium acetate and acetic acid buffer. The HILIC quality control consisted in a Waters 1806006963 HILIC QC (Waters, Milford, MA) and a custom-made QC [40]. For the reverse phase analysis (RP-LC), 20 μL of each sample were injected in a Poroshell 120 EC-C18 column (2.7μm, 3.0 mm × 50 mm) (Agilent, Wilmington, DE) held at 30 °C. Mobile phases consisted in 60% acetonitrile in water (A) and 10% acetonitrile in isopropanol (B), both at containing 10 mM of ammonium formate and formic acid. The RP quality control was Waters 6963 RP QC (Waters, Milford, MA).

Additional analyses were carried out using tandem LC-MS/MS with an Agilent 1260 HPLC system coupled to an Agilent 6530 Q-TOF MS (Agilent Technologies, Santa Clara, CA) and the same techniques, HILIC and RP. The chromatographic conditions were described in our previous work [38]. Electrospray ionization in positive (ESI+) and negative (ESI-) mode was applied to detect a mass range between 60 and 1200 (m/z). All the tested temperatures were grouped into one EBC sample and another ethanol rinse sample. The liquid samples were lyophilized and reconstituted in the same way as described previously. 3μl of resuspended sample was injected for analysis together with an internal standard composed by CUDA (12-(((cyclohexylamino)carbonyl)amino) dodecanoic acid) in 9:1 v/v methanol:toluene.

2.4 Data analysis and compounds identification

After data acquisition, data processing procedures were applied in order to identify molecular features with a significant shift between the considered temperatures. First, the raw GC-MS data were visualized using MS Data Review software v6.6 (Varian), and then transferred into an Automated Mass Spectral Deconvolution and Identification System (AMDIS) environment to perform peaks deconvolution. The obtained peaks were filtered and aligned with Mass Profiler Professional (MPP) 13.1 software using an alignment window of 0.3 min and match factor of 0.5 (as described previously [38]).

For the LC-MS chromatograms, the raw data were first visualized with the Agilent Mass Hunter Qualitative Analysis B.05.00SP1 software. After that, the data were processed with the Batch Recursive Feature Extraction option in the Agilent MassHunter Profinder (version B 08.00) software. This step involved a simultaneous peak deconvolution and alignment, calculating the abundance value for each molecular feature as the sum of intensities of corresponding isotopic and adduct peaks. Data filtering and alignment were done using a minimum absolute abundance of molecular features 1000 counts, mass alignment window of 40 ppm and 25 mDa and retention time alignment window of 0.5 min.

For each sample, a compiled peak table was obtained with the corresponding intensities for each molecular feature. Molecular features were described by the aligned retention time and averaged molecular mass calculated after the feature extraction. The dataset was evaluated and filtered to remove low-abundant spurious peaks and inconsistent mass spectral features. There were three criteria to consider a detected peak as representative of a metabolite: repetitive detection in technical replicates, exceeding the minimum abundance threshold, and its presence/absence in the blanks. A detected peak was considered as representative of a metabolite only if it satisfied the minimum ion abundance threshold, 5000 a.u. for GC-MS and 3000 a.u. for LC-MS and was detected in at least two out of three technical replicates. The third criterion was the peak absence or presence in the blanks. A peak that satisfied the first two requirements and was not present in the blanks was considered as a representative of a metabolite. A peak that satisfied the first two requirements but was also present in the blanks was considered a representative of a metabolite only if its ratio of abundance (sample/blank) was higher than 10. The resulting peak tables were then analyzed for statistically significant differences between the temperatures. HILIC and RP methods were treated similarly but independently of each other.

The total number of peaks was counted per each temperature, representing the total number of metabolites detected with each analytical method. There was a number of common metabolites detected at all tested temperatures and unique metabolites specific per each temperature level. The ‘total unique peaks’ included the sum of ‘unique present’ and ‘unique absent’ peaks at the corresponding temperature (see example in Supplemental S2). A ‘unique present’ peak could be described as unique at certain temperature if this peak was absent (or present at significantly lower abundance) in all other temperatures. A ‘unique absent’ peak (or present at significantly lower abundance) at a certain temperature could also be described as unique at that temperature if it was present at all other temperatures. To detect these unique peaks with statistical differences a folder exchange and Student’s t-test between groups (each temperature *vs.* all the others) were performed. Peaks or metabolites that satisfied a cut-off value of $p<0.05$ and a fold change (FC) value of 2 were selected per each temperature.

Chemical identification of the metabolites was carried out using MS/MS fragmentation patterns matching with METLIN mass spectral libraries with 3 mTh window. For the compounds that were not identified by MS/MS, a tentative identification based on LC-MS exact mass was performed with HMBD, METLIN and LIPIDMaps databases. In order to define correct tentative annotation in each case, best match was selected manually.

3. Results and discussion

3.1 Condenser surface temperature optimization

The device was calibrated for temperature accuracy. Figure 4a shows measured temperature values with no forced air flow through the condenser tube; both ends of the tube were open to the ambient air (20 °C, 40% RH). Eight thermocouples were distributed, in a helix arrangement, on the inner side of the glass tube along its length. The significantly higher reading from TC8 is due to its position at the end of the tube. We suspect that it had a poor thermal contact with the cold condenser surface and it was exposed to the ambient air. Close agreement between measurements from the other seven thermocouples proved uniform temperature distribution in the condenser element. Temperature achieved a stable state after first three cycles; the thermal mass cooled down and temperature oscillations faded. At a stable state, the temperature value stayed below the set point temperature; the magnitude of oscillations corresponded to the set value of temperature overshoot margin. The actual temperature closely corresponded to the set temperature value when there was no air flow.

With the breath flow through the device, the temperature rose above the set value due to convective and latent heat transfer from the warm breath. Figure 4b shows the temperature calibration curve that accommodates this temperature raise. The set temperature value was decreased at increments, the system was allowed to stabilize with no breath flow for three cycles, then a volunteer (M30) breathed into the device at his/her comfort tidal rate for 10 minutes, the measured temperature values were recorded. The measured value (avg. from eight thermocouples) was higher than the set temperature value. This difference increased at colder temperatures. A calibration curve was built with linear fit between the set and the actual temperature values. Another two volunteers (F20 and M20) repeated the experiment with refined temperature settings to confirm the temperature difference between the set and the actual values. The measured values were very close to the set levels. The calibration curve was refined based on measured values from three volunteers (Figure 4b). There was no temperature rise (drift) during 10 minutes of sampling; the temperature stayed at the set level.

With the breath flow through the condenser, the cooling system could not achieve a stable temperature below −30 °C. Temperature colder than −30 °C was achieved with a cooling sleeve filled with dry ice pellets. The initial temperature was −56 °C, the temperature rose to a stable level below 30 °C during a 10 minute long EBC collection [38].

EBC samples collected during these temperature optimization trials were discarded.

3.2 Effect of temperature and breath flow rate on collected EBC volume

Figure 5a shows the average expired volume per breath cycle and peak flow for each volunteer at tidal breathing and the EBC collection rate averaged across all the temperatures per each volunteer. Gressner et al. [33] found that expired volume was the primary factor determining the volume of collected EBC sample at a set temperature (−20 °C). Other lung function parameters (TLC, VC, RV, FEV1) and physiology (age, body weight) did not correlate significantly with the collected EBC volume. About 0.95 mL of EBC was collected from every 100 L of expired breath, i.e. about 40 % of total expired water content in breath.

Our data cannot reflect this dependence because the breath flow measurements do not show the total expired volume per EBC collection but rather represent an average physiological tidal breath rate of each volunteer sampled at three random days during a four months period. In our study, there was also an uncounted loss of EBC sample during its retrieval from the condenser tube; some of the sample was left on the condenser wall. However, based on our experimental observations, we agree with Gressner et al. [33] findings on EBC volume dependence on expired volume and duration of the collection time. We would also hypothesize that hydration level might have an effect. Volunteer (F20) liked keeping hydrated and had the highest EBC collection volume rate out of all volunteers.

Figure 5b shows the dependence of EBC collection rate on the surface temperature of the collection device. The values are averaged from 12 collections, two from each volunteer, at each temperature. The surface temperature of the condenser element determines the physical phase of the sample. EBC samples collected at 5 °C were liquid. The samples collected at 0 °C were slush-like mixtures of water and ice. The samples collected at lower temperature were white powdery snowflake-like crystals growing from the surface of the tube towards its center. The ice crystals were fluffy, highly porous, and could partially be blown out of the tube with a strong exhalation maneuver.

The higher rate of EBC collection at 5 °C and subsequent mass increase at lower temperature levels in solid phase can be explained with thermodynamics, heat transfer, and energy barrier principles.

Liquid phase collection requires lower amount of heat exchange; the vapor cools down, changes phase from gas to liquid, and the condensate cools down to the surface temperature of the condenser. In solid phase (ice) collection, the amount of released heat is increased by additional amount of heat that the condensed liquid needs to cool down to its freezing temperature, undergo phase transition from liquid to solid, and then cool down to the surface temperature of the condenser (freezer).

The nucleation energy barrier on a clean glass surface (hydrophilic) is low and a film wise condensation occurs rapidly while energy barrier for a solid crystal formation on porous icy surface is significantly higher and requires more heat transfer for accumulation of the same mass [41]. Also, the heat transfer through a thin layer of liquid condensate is higher than through a layer of highly porous ice flakes which also diminish the rate of sample collection. There is also a significant difference in the amount of sample lost on the collector surface during sample retrieval step. Retrieval of the sample from the condenser tube into a storage vial is easier and more efficient for liquid phase, all the sample can be dripped into a vial. A solid sample is retrieved with a push plunger pole and some solid ice is left on the surface of the collector tube [38].

The increase in the EBC collection rate at lower temperatures (same phase, ice) is in agreement with the thermodynamic principles and the previous experimental works [5, 38, 42]. Lower collection rate at the coldest temperature (−56 °C) can be explained with the formation of a layer of porous frozen sample on the surface of the condenser during the first minute that suppresses efficient heat transfer and diminishes further condensation rate.

The error bars in Figure 5 show ±0.5 STD.

3.3 Metabolomic content of the EBC

Collected EBC samples were analyzed with mass spectrometric-based techniques to determine the metabolomic content at each temperature of condensation. Device-rinsed with ethanol (EtOH) samples were also assessed. Volatile metabolites were studied in EBC samples using the previously described GC-MS methodology [40]. Non-volatile metabolites were studied from EBC and EtOH samples using RP and HILIC LC-MS protocols to determine non-polar compounds and more polar/hydrophilic compounds, respectively. As previously described, the number of peaks detected with each technique resembles the number of metabolites. Table 1 shows the number of total and unique metabolites determined in EBC and EtOH samples with each temperature and each analytical method. Unique metabolites are expressed as the total unique peaks. Specific present- and absent-unique peaks are also listed. These numbers are divided between completely present/absent peaks and peaks with higher and lower abundances at certain temperatures in supplemental Table S3.

Figure 6 compares the effectiveness of the analytical methods to detect and distinguish the chemical compounds from EBC samples. The dark bars in Figure 6 show the total number of metabolites detected with each technique from all the temperatures. The blue bars in Figure 6 show the total sum of unique peaks distinguished with each technique from all the temperatures. The lowest number of compounds (total and unique) was detected in the EBC fraction analyzed with GC-MS, which contained the volatile compounds (VOCs). Although volatiles are usually present in EBC samples in higher concentrations than non-volatiles, the number of these molecules is limited, being often easily transformed to non-volatile derivatives [21]. As a consequence, EBC samples were richer in composition, containing non-volatile compounds that are detected by LC-MS. Hydrophilic platform (HILIC LC-MS) allowed the detection of more non-volatiles (~ 900) than RP (~ 500). This is because the EBC fraction is mainly formed by water vapor and water-soluble biomolecules (e.g. acids, proteins) [32]. Similarly, samples obtained after the subsequent device rinse with a low-polar solvent (EtOH) were richer in less polar compounds (e.g. lipids and arachidonic acid metabolites) that were efficiently detected by RP LC-MS [43]. EtOH fraction contained a considerable higher number of peaks than the EBC fraction, with more than 3000 compounds detected by RP and around 1500 by HILIC. The device-cleaning EtOH fractions are not usually collected in breath studies; however, these samples may contain information about the residual compounds retained on the collector surface and not included in the EBC fraction due to their interaction with the condenser material.

Figure 7 presents the differences in the number of detected compounds per temperature. The physical phase of the sample (liquid or ice) has a great influence on the collected fraction of the volatile compounds detected with GC-MS and the non-volatile compounds detected with HILIC LC-MS in it, but less so for RP LC-MS. In general, the number of compounds detected with each type of chromatography increases as the collection temperature gets colder (Fig. 7a) but the increase in the total number of compounds does not correlate with the number of unique compounds detected at the specific temperatures (Fig. 7b). The

metabolomic content of collected EBC sample may not be solely dependent on temperature but be influenced by other phenomena such as solubility at different temperatures, increased sample dilution due to condensation from the ambient air at colder temperatures, or due to occurring chemical reactions on the surface of the collector. The observed increase in the number of detected compounds in each phase at the corresponding lowest temperature, 0 °C for liquid and −56 °C for solid, might be due decrease in degradation or other chemical alterations of the compounds in the sample [21]. The captured fraction of the low-polarity compounds detected with RP LC-MS in EBC and ethanol rinse show a weaker dependence on the sample phase. A tentative annotation of the molecular formula (MF) or metabolites detected by GC-MS and LC-MS is provided in Supplemental material S4.

Evaluating the dependence of the metabolomic content of EBC on collection temperature, one should note that the considered temperature range covers two physical phases and includes two different experimental set-ups. The physical phase of the sample, liquid or solid, has its effect on the EBC content and thus must be taken into account. The −56 °C temperature level was not possible to be achieved with the refrigerant-based cooling system and an alternative set-up using dry-ice pellets was used. This difference must be noted too.

Although we did not compare the difference in concentration of compounds at different temperatures, it is implied with their presence or absence. The compounds were counted as present only if they satisfied the minimum value for abundance threshold and peak/blank ratio in at least two out of three samples, as described in the methods section. Thus the increase in the number of volatile (GC-MS) and polar non-volatile (HILIC LC-MS) compounds with decrease in collection temperature in each phase implies the increase in their concentration at colder temperatures.

Considering the number of unique volatile compounds detected at each temperature level with GC-MS method, we can note that for temperatures from +5 to −30°C only 10–30 metabolites were considered unique from 150–200 total peaks that were determined per each temperature, representing less than 20% variability. This means that most of the metabolites were present at more than one temperature and showed no significant differences between these temperatures. However, the number of both, total and unique, detected compounds raised to more than 30% at −56 °C, 95 unique from almost 300 total compounds. From these unique metabolites, most of them (91) were present or present at statistically higher abundance at −56 °C than at other temperatures, while only 4 metabolites were not detected or present at significantly lower abundances. This rapid increase in the number of unique compounds makes us suspect that it resulted from the use of a different set-up rather than the colder temperature. The overall increase in the number of detected compounds is consistent with the trend observed in this experiment; the concentration of compounds increases in each physical phase as the collection temperature decreases.

A similar behavior was also observed for total and unique number of polar non-volatile compounds detected with LC-MS HILIC method. A high number (~ 720 peaks) of total detected peaks was observed at +5 and 0°C. This number diminished at −10°C (646 peaks) and then, the metabolic profile was enhanced reaching almost 800 compounds at the lowest temperatures (−30 and −56°C).

Comparison of unique compounds for temperature from +5 to −30 °C did not show any significant difference, representing only 15–20% of unique signals. There was a significant difference in the number of compounds considered unique at −56 °C. The number of metabolites considered exclusive by presence at −56°C was around 90% (146 of 164), while at −30 °C it was around 20% (24 of 117).

From these unique compounds, some endogenous metabolites (and molecular formulas) have been tentatively identified at −56°C in Table S (Supplemental Material S4). Peptides or amino acids such as serine, valine or proline betaine were only detected at the lowest temperature. Although this study was carried out with healthy individuals with no pulmonary diseases, related compounds like 3-nitrotyrosine, tyrosine, hydroxyproline and proline known as markers of inflammation in airway diseases (e.g. nitrosactive stress) [44] were present in collected EBC samples. Other compounds detected in the EBC were 3-dehydroteasterone, triethylamine, coniferyl acetate, acids like nonynoic, tridecynoic or docosanedioic acid, and cinnamic derivatives which span some endogenous and exogenous compounds in human breath.

The hydrophobic non-volatile compounds detected in EBC with RP LC-MS method were less susceptible to temperature changes; an average total number of peaks (~ 450) was detected per each temperature. For temperature range from +5 to −30 °C, there was also no statistically significant difference in the number of unique compounds. However, the number of unique peaks detected in EBC samples collected at −56°C was considerably higher. At −56 °C, 50% of the total peaks (215 of 435) were considered unique; 30% of the unique peaks (64/215) were specific by presence, other metabolites were considered unique by absence in comparison to other temperatures. This is the case of some acids like butyric/isobutiric, hydroxy-hexanoic or cinnamic acid or possible ketones like hydroxybutan-2-one. The solubility of hydrophobic compounds is not affected by the changes in temperature, as these compounds are insoluble at any temperature and thus are captured equally well.

Device rinse with ethanol after EBC collection presented a similar behavior regarding the total number of compounds detected with HILIC and RP methods. A higher number of metabolites (more than 2500) was detected with RP, while only ~1200 metabolites were detected with HILIC. All EtOH samples were collected at room temperature after corresponding EBC collection. The organic solvent was in liquid state, so, the temperature differences should be resembled only due to the prior EBC collection. HILIC and RP profiles of the total number of peaks (Fig. 5, 7a) showed similar trends in the temperature effect between EBC and EtOH collection. For example, the number of metabolites was much lower at −10°C and increased to a maximum at −56°C for HILIC, and was lower at −20°C and increased to a maximum at −30/−56°C for RP. The same considerations apply for ethanol rinse as for neat EBC.

Note that a high number of total metabolites were detected in liquid phase (at +5 and 0°C) with both techniques. Regarding the unique peaks (Fig. 7b), at high temperature of EBC collection (+5°C), organic wash fractions presented an elevated number of unique peaks (20% of the total number of peaks). From these, more than the 70% were unique metabolites present only at that temperature (194 of 240 for HILIC and 346 of 462 for RP). For instance,

metabolites like eicosanoic acid or terpene derivatives (10b,14b-dihydroxytaxa-4(20),11-dien-5alpha-yl acetate, 3a,7a-dihydroxy-5b-cholestan-26-oic acid or 3a,7a,12a-trihydroxy-5b-cholestan-26-al). Similar to the total number of peaks, the number of unique peaks was lower at 0°C but then increased to the maximum value at −56°C, except for unique compounds at −10°C determined with HILIC. In this particular case (−10°C), 70% from the total detected peaks (742) were considered unique (516), 11 compounds specific by presence at that temperature and 505 compounds specific by absence. However, at −56°C and +5°C only the 20% of the total detected peaks (1200) were considered unique (240), with more than 70% of the unique peaks specifically described as present for both, HILIC and RP.

4. Conclusions

This work contributes to the prior knowledge in the field of EBC analysis and helps to standardize the collection protocol that will determine the design of an optimal EBC sampling hardware in terms of operating temperature. Here, we considered the sole effect of collection temperature that might be a viable primary parameter for the purpose of standardization among different devices [5, 32, 42, 45, 46]. The experimental results corroborate the findings of previous studies about effects of collection temperature on EBC volume and content [33]. Goldoni et al. [5] considered the effect of the condensation temperature on selected exhaled breath parameters at temperatures between −10°C and 5°C using a TurboDECCS device. The concentration of hydrogen peroxide and malondialdehyde, and condensate conductivity increased with increase in the condenser temperature. Loyola et al. [45] studied the effect of the condensation temperature on the concentration of acetone in EBC with a regular, passively-cooled, and a custom thermoelectric cooling setup made for RTube™. A colder starting temperature resulted in increased concentration of acetone in EBC from 140±20 k-counts at 0 °C to 310±20 k-counts at −50 °C. We agree with the trend of conclusions from the previous studies, but note that the actual temperature of the collector s needs to be verified and reported when a device is in use because it can significantly deviate from the set temperature level [36, 38].

We completed a controlled experiment to determine the effect of collection temperature on the metabolomic content of EBC. The use of a single device allowed keeping saliva filtering and collector surface effects as constant parameters and the temperature as a controlled variable; the physiological differences were minimized by averaging samples from a group of volunteers and a period of time. We note that the temperature of −56 °C is the only experimental data point that may be affected due to using a different set-up, dry ice pellets instead of an actively cooled thermal mass. This difference must be noted when making further conclusions about the effect of temperature on EBC content. Due to the wide scope and versatility of untargeted metabolomics, we just considered the effect of temperature on relative concentrations and our ability to detect compounds in EBC with a set of mass spectrometric methods in two different physical phases and temperature levels in each phase. This effort shows a “big picture” and helps determine the engineering design for efficient EBC sampling hardware.

We can conclude that the temperature of the EBC collector surface does not alter the metabolomic content of EBC but has some effect on the abundances of compounds, and therefore our ability to detect them. Higher magnitudes can be detected at colder temperature in each physical phase. The concentrations amounts of volatile compounds are more readily affected by colder temperatures. The effect of physical phase of the EBC sample, liquid versus solid, are not known but no significant differences were observed in this study.

We also would like to note that the use of an organic solvent rinse to collect non-water-soluble compounds from the condenser surface after EBC collection [40, 47] enhances metabolites recovery, is less dependent on temperature changes, and may possibly serve as an additional pointer to standardization of EBC sampling methodologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at **xxx**

- We investigate the effect of collection temperature on the metabolomic content of exhaled breath condensate over a range of temperature and two physical phases.
- Collection temperature and corresponding physical phase have an influence on the concentrations of the volatile compounds detected with GC-MS and the non-volatile compounds detected with HILIC LC-MS in it, but less so for RP LC-MS.
- Detection of specific type of compounds may be more efficient at a specific temperature.
- The importance of collection temperature relative to other design parameters such as saliva filtering and surface coatings need to be further investigated.

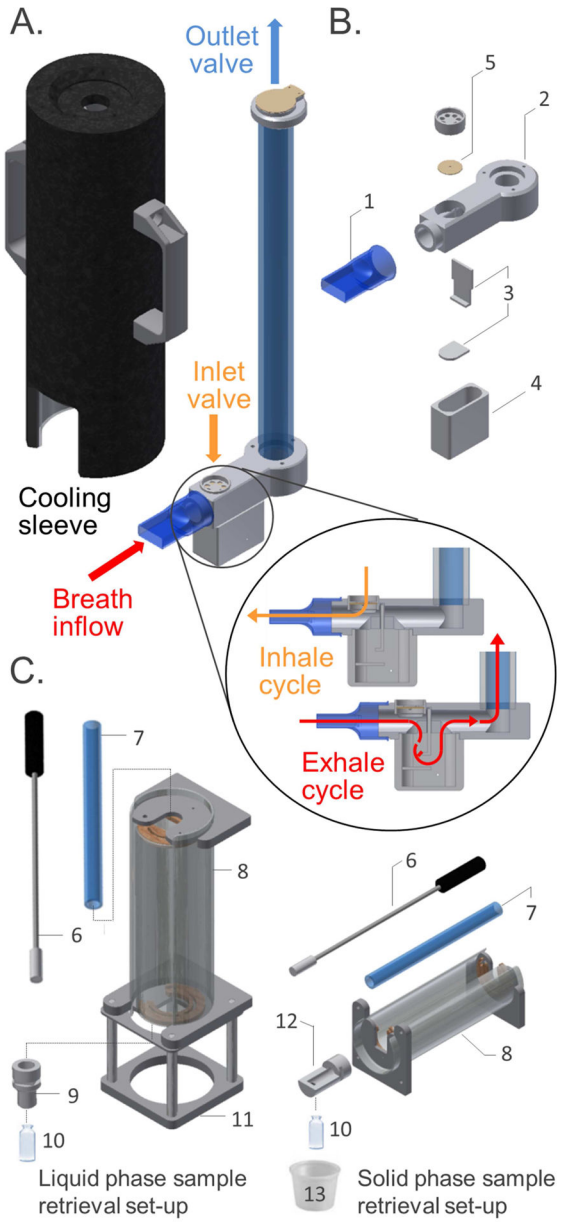

Figure 1.

(A) CAD design of the EBC sampler. Main parts: saliva trap assembly, condenser tube, insulated cooling sleeve. Inset circle shows the breath flow diagram for inhale and exhale cycles. (B) Saliva trap components: 1. mouthpiece, 2. saliva trap housing, 3. trap notch, 4. saliva reservoir, 5. inhale valve. (C) Sample retrieval press components (for liquid phase sample collection): 6. stainless steel sample retrieval plunger with PTFE head, 7. condenser glass tube, 8. retrieval press housing, 9. threaded vial connector, 10. glass vial, 11. vertical stand with suction cups; (for solid phase sample collection): vertical vial holder (part 9) is exchanged for horizontal vial holder (part 12), vertical stand (part 11) is removed, ice bowl (part 13) is added to keep the vial cold.

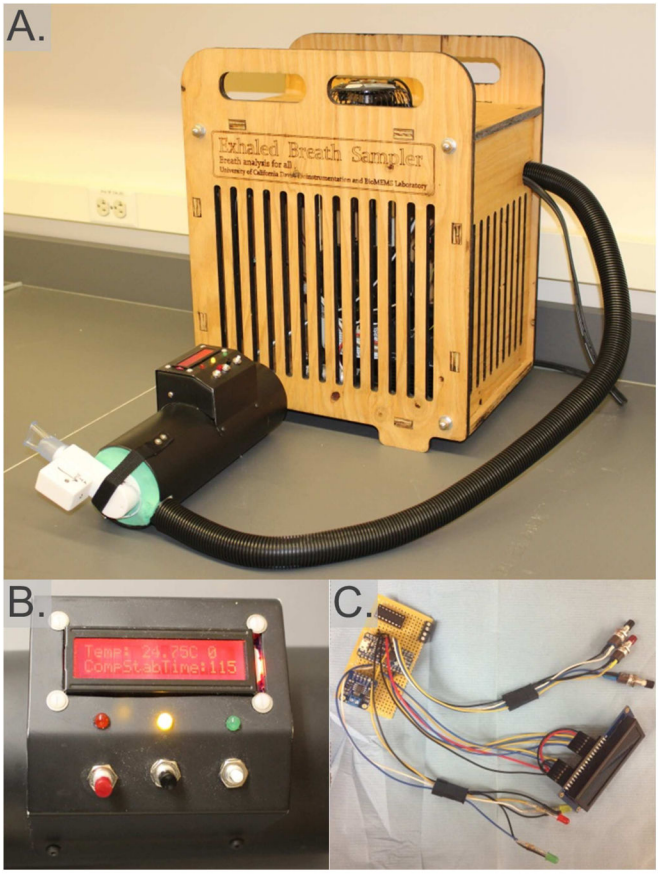

Figure 2.

(A) EBC sampler with a cooling system. Condenser tube and saliva trap assembly is inserted into the insulated cooling sleeve. The system consist of an insulated actively cooled thermal mass, refrigerant line and a pump. (B) An Arduino®-based user interface and temperature control system. (C) Microcontroller board with periphery.

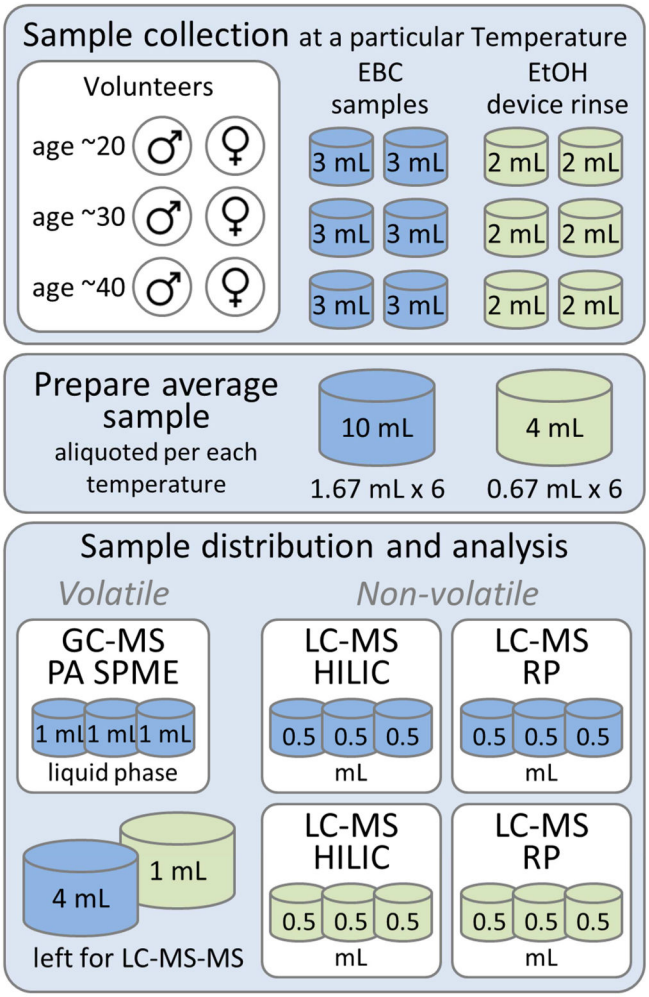

Figure 3.

EBC collection and distribution for chemical analysis from a group of human volunteers at one of the temperatures. EBC collections and analysis at other temperatures were done from the same group of volunteers and with the same methodology.

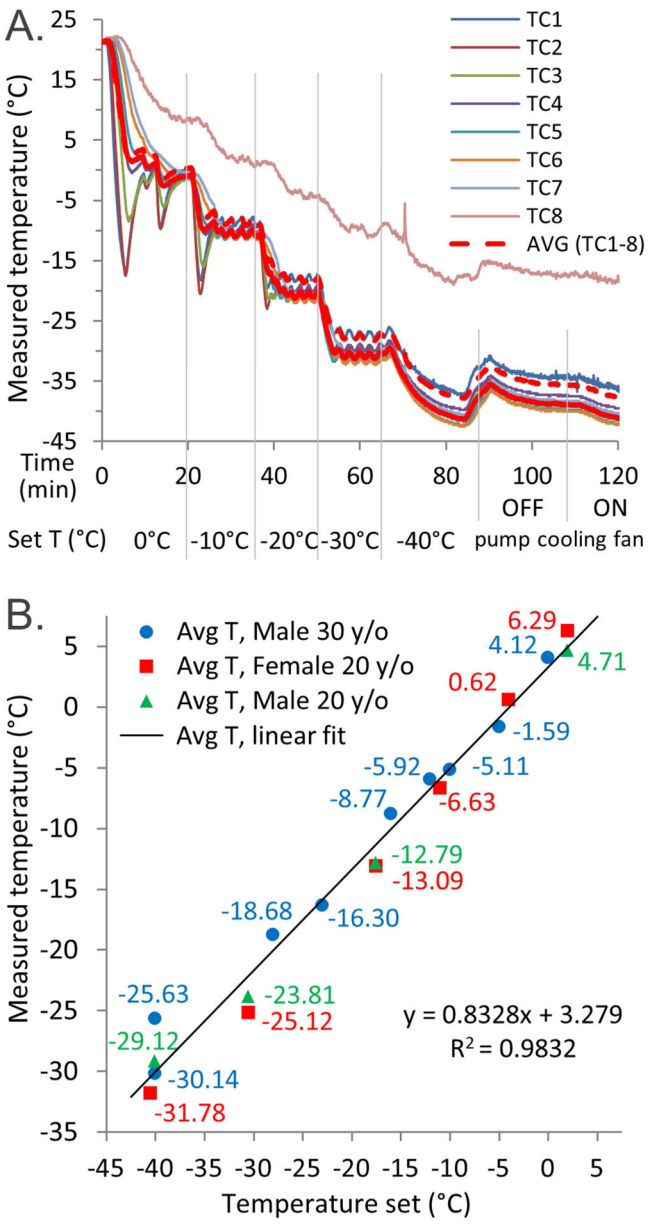

Figure 4.

Temperature calibration; measured temperature vs. set temperature. (A) No breath flow, static ambient air. The effect of pump cooling fan is observed only at the lower temperature limit. (B) Breath flow, 3 volunteers. The temperature raised above the set value due to warm exhaled breath and its physical change in the condenser.

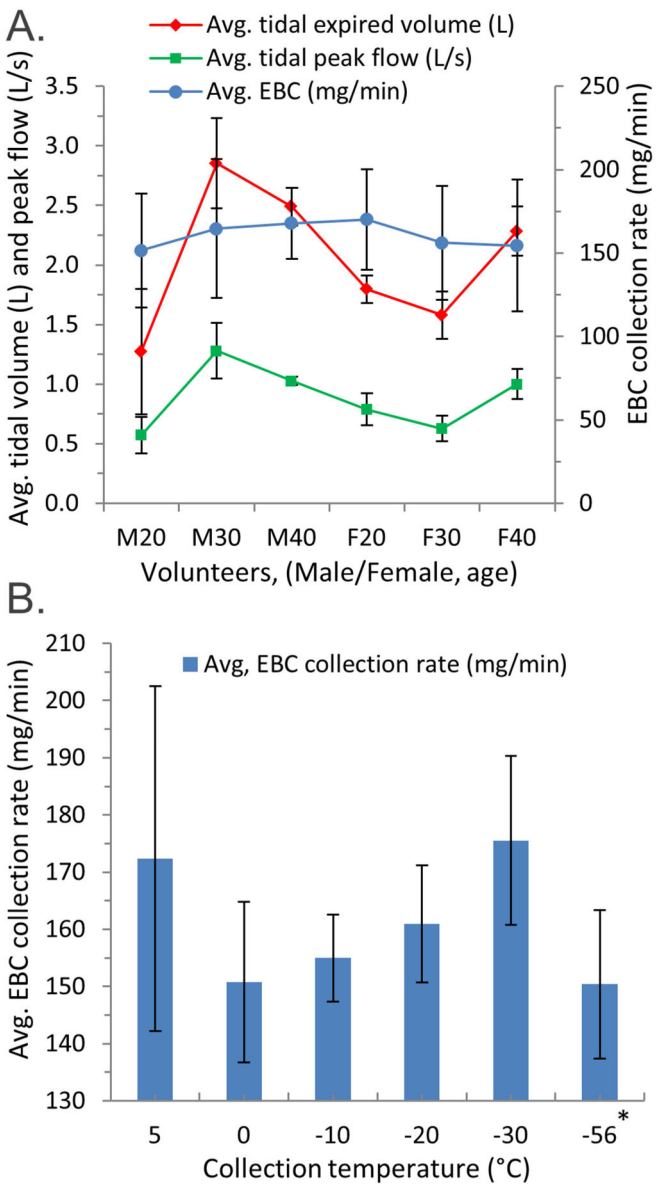

Figure 5.

EBC collection rate (mg/min). (A) Effect of breath flow rate. Tidal peak flow rate (PEF) and forced vital capacity (FVC) for each volunteer based on three measurements. Average EBC collection rate per volunteer, combined for all temperatures. (B) Average EBC collection rate per temperature, combined from 12 collections per each temperature from 6 volunteers. Error bars show ± 1 STD.

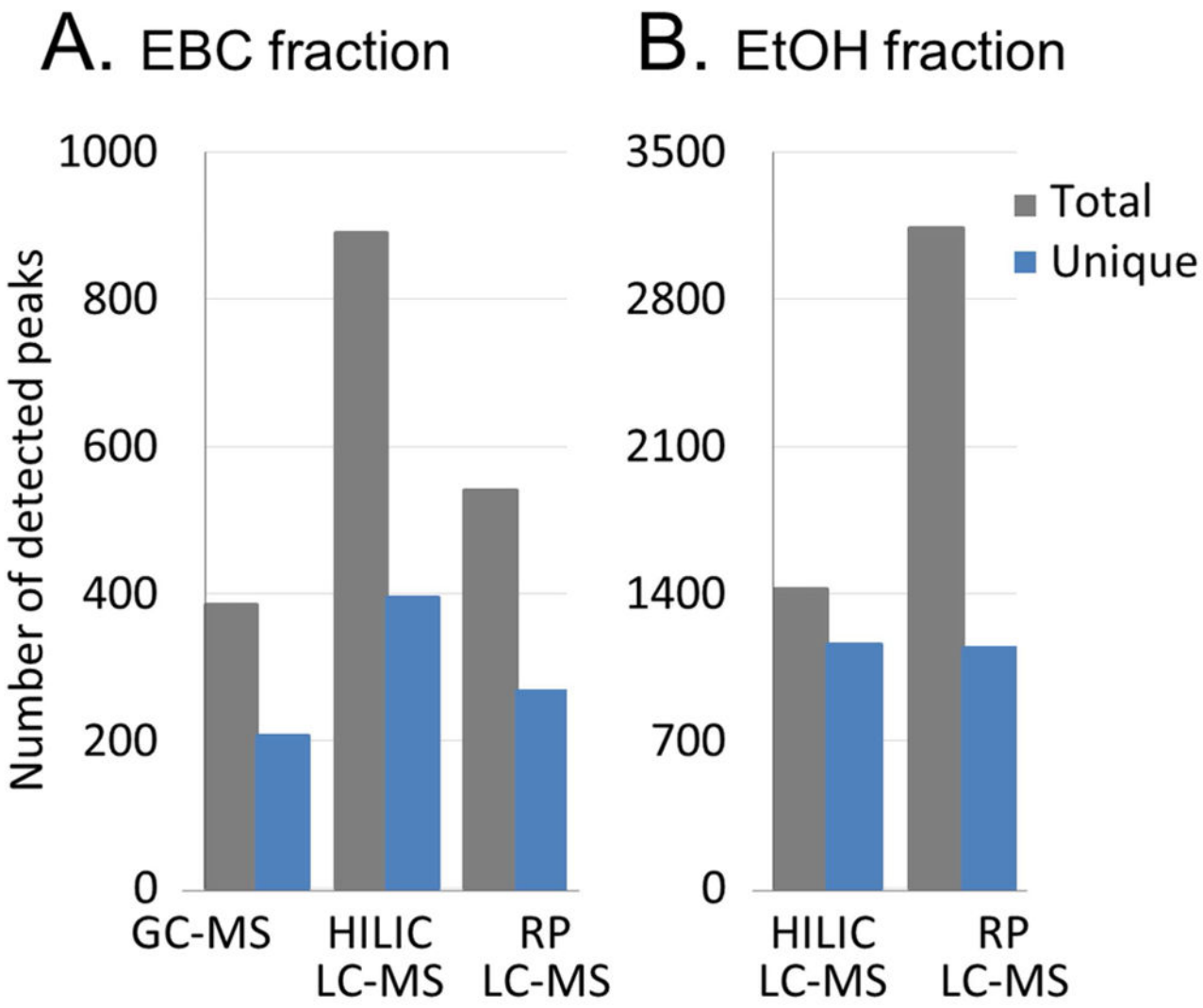

Figure 6.
Comparison of the total (grey) and unique (blue) number of peaks (metabolites) detected with the analytical methods: GC-MS, HILIC LC-MS and RP LC-MS, for all the temperatures together. (A) for the EBC fraction and (B) for the EtOH fraction.

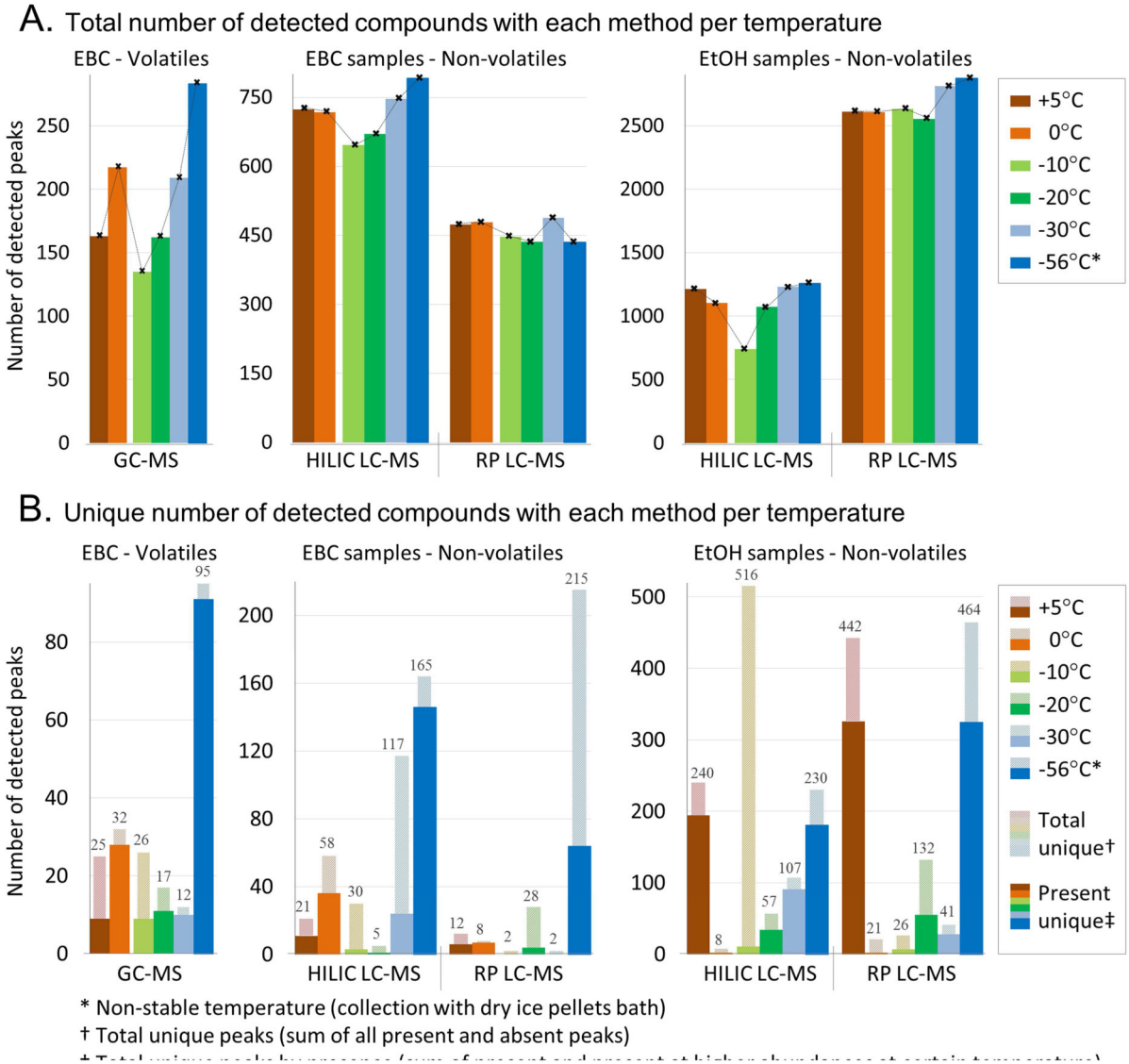

Figure 7.

Comparison of the total (A) and unique (B) number of peaks (metabolites) detected with the analytical methods: GC-MS, HILIC LC-MS and RP LC-MS for each temperature: +5°C (dark-red), 0°C (orange), −10°C (light-green), −20°C (green), −30°C (light-blue) and −56°C (blue).

Table 1

Number of total and unique peaks detected in EBC and EtOH fractions with all the temperatures studied and all the analytical techniques.

			Temperature (°C)						
Fraction	**Technique**	**# peaks**	**All**	**5**	**0**	**−10**	**−20**	**−30**	**−56***
EBC	GC-MS	Total	385	163	217	135	162	209	283
		Unique†	207	25	32	26	17	12	95
				(9/16)	(28/4)	(9/17)	(11/6)	(10/2)	(91/4)
EBC	HILIC	Total	890	724	718	646	671	747	791
	LC-MS	Unique†	395	21	58	30	5	117	164
				(11/10)	(36/22)	(3/27)	(1/4)	(24/93)	(146/18)
	RP	Total	541	473	479	447	436	488	435
	LC-MS	Unique†	267	12	8	2	28	2	215
				(6/6)	(7/1)	(0/2)	(4/24)	(0/2)	(64/151)
EtOH	HILIC	Total	1428	1215	1103	742	1073	1230	1259
	LC-MS	Unique†	1158	240	8	516	57	107	230
				(194/46)	(2/6)	(11/505)	(34/23)	(91/16)	(181/49)
	RP	Total	3140	2612	2607	2630	2553	2810	2871
	LC-MS	Unique†	1146	442	21	26	132	41	464
				(326/116)	(2/19)	(7/19)	(55/77)	(28/13)	(325/139)

*Non-stable temperature at −56°C (with dry ice) during the collection time period.

†Unique peaks expressed as the sum of present and absent peaks. Present peaks include peaks that satisfy the threshold and peak/blank abundance ratio requirements at certain temperature. Absent peaks include peaks that do not satisfy the threshold and peak/blank abundance ratio requirements at certain temperature (Suppl. Material S2).