Title

Human breath metabolomics using an optimized non-invasive exhaled breath condensate sampler

Journal

Journal of Breath Research, 11(1)

ISSN

1752-7155

Authors

Zamuruyev, Konstantin O

Aksenov, Alexander A

Pasamontes, Alberto

et al.

Publication Date

2017

DOI

10.1088/1752-7163/11/1/016001

Peer reviewed

HHS Public Access

Author manuscript

J Breath Res. Author manuscript; available in PMC 2017 December 22.

Published in final edited form as:

J Breath Res. ; 11(1): 016001. doi:10.1088/1752-7163/11/1/016001.

Human breath metabolomics using an optimized noninvasive exhaled breath condensate sampler

Konstantin O. Zamuruyev[a], **Alexander A. Aksenov**[a], **Alberto Pasamontes**[a], **Joshua F. Brown**[a], **Dayna R. Pettit**[a], **Soraya Foutouhi**[b], **Bart C. Weimer**[b], **Michael Schivo**[c,d], **Nicholas J. Kenyon**[c,d], **Jean-Pierre Delplanque**[a], and **Cristina E. Davis**[a,*]

[a]Department of Mechanical and Aerospace Engineering, One Shields Avenue, University of California, Davis, Davis, California 95616, U.S.A

[b]School of Veterinary Medicine, 1089 Veterinary Medicine Drive, University of California, Davis, Davis, California 95616, U.S.A

[c]Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of California, Davis, Sacramento, CA 95617, USA

[d]Center for Comparative Respiratory Biology and Medicine, University of California, Davis, Davis, CA 95616, USA

Abstract

Exhaled breath condensate (EBC) analysis is a developing field with tremendous promise to advance personalized, non-invasive health diagnostics as new analytical instrumentation platforms and detection methods are developed. Multiple commercially-available and researcher-built experimental samplers are reported in the literature. However, there is very limited information available to determine an effective breath sampling approach, especially regarding the dependence of breath sample metabolomic content on the collection device design and sampling methodology. This lack of an optimal standard procedure results in a range of reported results that are sometimes contradictory. Here, we present a design of a portable human EBC sampler optimized for collection and preservation of the rich metabolomic content of breath. The performance of the engineered device is compared to two commercially available breath collection devices: the RTube™ and TurboDECCS. A number of design and performance parameters are considered, including: condenser temperature stability during sampling, collection efficiency, condenser material choice, and saliva contamination in the collected breath samples. The significance of the biological content of breath samples, collected with each device, is evaluated with a set of mass spectrometry methods and was the primary factor for evaluating device performance. The design includes an adjustable mass-size threshold for aerodynamic filtering of saliva droplets from the breath flow. Engineering an inexpensive device that allows efficient collection of metalomic-rich breath samples is intended to aid further advancement in the field of breath analysis for non-invasive health diagnostic. EBC sampling from human volunteers was performed under UC Davis IRB protocol 63701-3 (09/30/2014-07/07/2017).

*Correspondence: cedavis@ucdavis.edu.

Keywords

exhaled breath condensate (EBC); volatile organic compounds (VOCs); non-volatile organic compounds (non-VOCs); breath analysis; metabolomics; portable non-invasive health diagnostic

1. Introduction

Exhaled breath condensate (EBC) analysis is a developing field with tremendous promise to advance personalized, non-invasive health diagnostic as new optimized analytical instrumentation platforms and detection methods are developed [1, 2]. Exhaled breath contains potentially valuable metabolomic content due to gas exchange with blood at the pulmonary alveolar membrane interface [3]. Recent studies showed that some biomarkers in exhaled breath [4] and exhaled breath condensate [5] highly correlate to traditional biomarkers in other biological fluids such as blood and urine that routinely aid health diagnostics. Due to the completely noninvasive nature of breath analysis, it offers a safe method for health assessment of children with inflammatory diseases such as asthma [6], patients with acute cardiovascular or pulmonary diseases, and for unresponsive patients who receive respiratory ventilator assistance [7]. Breath analysis also may find applications in pharmokinetics [8] and exposome monitoring [9]. Respiratory and gastrointestinal diseases [10] often significantly change the entire pattern and relative abundances of volatile organic compounds (VOCs) in exhaled breath. A number of recent case studies demonstrate the feasibility of respiratory diseases diagnostic with breath analysis. Kramer et al. [11] and Shestivka et al. [12] developed the method and could identify VOCs *in vitro* for rapid breath analysis in patients with cystic fibrosis using gas chromatography mass spectrometry (GC/MS) platforms. Purkhart et al. [13] were able to detect chronic intestinal mycobacteria infection using differential mobility spectrometry. Eng et al. [14] used a combination of five VOCs to distinguish children with chronic liver disease from healthy control subjects. Oncological diseases produce distinct changes in VOCs profile of exhaled breath that can be used for rapid noninvasive early-stage cancer diagnostic. Kumar et al. [15] developed mass spectrometric analysis of exhaled breath for the identification of VOC biomarkers in esophageal and gastric cancer. Capuano et al. [16] demonstrated differentiation of lung cancer based on a breath signature.

Although recent case studies demonstrate the applicability of breath analysis for health diagnostics, this field is still relatively limited for practical applications. One of the persistent problems is lack of sampling standardization. A number of parameters that affect composition of exhaled breath were elucidated earlier, including: EBC sampling period, breathing pattern (tidal versus forced capacity), collected fraction of the exhaled breath (alveolar end tidal versus total expired volume), collection device material and design, condensation temperature, passage of exhaled droplets and saliva trapping, sample transfer and storage [17]. These parameters still require optimization for the sample collection procedure [17, 18]. There is a great potential in optimized hardware and methods because they can help establish standard procedures and allow data comparison from different case studies. The ability to draw conclusions from multiple studies may help the field move

forward and understand the complicated metabolomic pathways to identify disease-specific biomarkers.

The EBC collection procedure and hardware design may significantly affect the metabolomic content of the sample. The design of commercially available EBC samplers may still be far from optimal for efficient collection of metabolomic content in EBC samples. Therefore, the performance of commercially available EBC collection devices 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.

Soyer et al. [19] compared pH and concentrations of lipid mediators and proteins in EBC samples collected with two devices: RTube™ and ECoScreen®. In addition to larger condensate volumes, EBC samples collected with ECoScreen® contained significantly higher concentrations of cysteinyl leukotrienes and eotaxins that allow detection of proteins and lipid mediators with greater sensitivity. There are a number of reasons for higher metabolomic content of EBC because the designs of the two compared devices are different in several aspects. The ECoScreen® has an active cooling mechanism that keeps the temperature of the condenser surface at a constant value below −15 °C during breath sampling period (10 min). The RTube™ uses a passive cooling from a thermal mass that is cooled in a freezer prior to breath sampling. The temperature of the RTube™ condenser surface is not stable. It rises significantly, from −20 °C to about 20 °C, during a typical breath sampling period of 10 min [19, 20]. The designs of the condensation chambers of the two devices are also different. The ECoScreen® has a closed chamber while the RTube™ chamber is open to ambient air. A closed chamber design minimizes condensation and contamination from the environment.

Rosias et al. [21, 22] studied the effect of condenser surface coatings on measurement of biomarkers in EBC. Five condenser coatings (silicone, glass, aluminum, polypropylene, and Teflon) were compared using the ECoScreen® device. The detected levels of 8-isoprostane and albumin were compared using EBC samples from 28 healthy volunteers. Adhesive properties of different condenser coatings influenced 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. Further guidelines on the choice of condenser coating to measure specific biomarkers are needed to standardize EBC collection.

The passage of aerosolized microdroplets and filtering of saliva droplets from the breath flow is also an important design factor. Exhaled breath is a complex mixture of gasses that contains nitrogen, oxygen, CO_2, inert gases, hundreds of VOCs (acetone, isoprene, methanol, ethanol) of exogenous and endogenous origin in trace concentrations along with water vapor and aerosolized droplets [23] and non-volatile compounds contained in microdroplets. The origin of the droplets exhaled with breath determines their metabolomic content. Therefore, the size and the origin of the microdoplets in exhaled breath at different breathing regimes and humidity have been studied [24–26]. Sub-micron size droplets may originate at the alveolar membrane and contain airway lining fluid (ALF). Slightly larger size droplets may be carried with turbulent flow from bronchial surfaces. Large droplets may

originate in the mouth cavity and introduce sample contamination and dilution with saliva and therefore should be avoided [25, 27]. Partial physical trapping of small microdroplets on the condenser element surface and condensation of vapors from exhaled breath may be an optimal way to collect exhaled breath condensate. EBC contains volatile, non-volatile, and water soluble compounds that can correlate to ALF sample. Non-volatile and water soluble compounds are more easily trapped in EBC. These compounds range from small inorganic ions (anions and cations), to organic compounds such as urea, organic acids, amino acids, and large molecular compounds such as peptides, proteins, and surfactants [28]. Concentrations of very volatile compounds in EBC are lower due to their high volatility, but multiple other compounds including cytokines can be detected [29]. Exhaled breath and breath condensate contain different volatile and nonvolatile fractions that provide complementary but different chemical information, and therefore each phase should be analyzed with specifically suitable methodology. In comparison to exhaled breath gas [30, 31], EBC contains more stable metabolomic content and allows easier sample manipulation, transfer, and storage [28].

For these reasons, direct comparison of metabolomic content collected with different commercial devices might not be possible because these devices often have different condensation temperatures, chamber designs, condenser coatings, and saliva filtering mechanisms. Different designs of breath collection devices offer specific advantages, but also have certain limitations. To address this need, we present an optimized design of a portable human breath sampler (HBS) that takes into account condenser chamber design to reduce contamination from the ambient conditions, condenser temperature and its stability to preserve the physical phase of the sample, hardware material choice for metabolomic content preservation, and aerodynamic trap for saliva droplets filtering from the breath flow. Figure 1 summarizes the key design parameters and evaluation criterion considered in this study. The main criterion for evaluation of device performance is the metabolomic content in EBC samples collected with each device from the same group of healthy volunteers. The significance of the biological content of the EBC samples is evaluated with multiple types of mass spectrometers. We present results and describe the methodology developed for the analysis of the metabolomic content of EBC samples. The performance of the HBS engineered device is compared to two currently commercially available EBC collection devices, RTube™ and TurboDECCS®. The three devices are compared in terms of the condenser surface temperature during sampling period, sample physical phase, condensation efficiency (sample mass/time), and saliva content in collected EBC samples. These findings may help correlate EBC sampler design parameters to the metabolomic content of collected samples and lead to development of optimal devices for standardized studies in human breath metabolomics.

2. Materials and methods

2.1. EBC sampler hardware

Figure 2 shows the design of the human EBC sampler modeled in computer assisted design (CAD) software. The outer casing of the device is constructed from polycarbonate tubing (101.6 mm OD × 91.9 mm ID × 360 mm L) insulated with polyethylene foam pipe

insulation (12.7 mm thick), the inner condenser tube center guidings are machined from 6.35 mm thick PVC. The housing is hermetic; the bottom plate is sealed with silicone and the condenser tube orifice is sealed with fluorosilicone rubber o-ring. A borosilicate glass tube with 25.4 mm OD × 17.4 mm ID × 300 mm L (Wale Apparatus Co., Inc., Hellertown, PA, USA) is used as a condenser surface. The hollow space between the glass condenser tube and insulated housing (radial distance ~35 mm) is filled with cooling material, e.g. dry ice pellets. The insulated housing is closed on the top with a polyethylene foam cap to prevent dry ice evaporation into the ambient air. The total weight of the apparatus charged with dry ice coolant is about 3.7 kilograms. The breath sampling apparatus was fabricated in the Engineering Fabrication Laboratory at UC Davis.

The condenser airway chamber is equipped with a pair of one way valves. The exhale silicone flap valve is installed at the outlet of the condenser tube. The inhale membrane valve is installed in the PTFE housing in proximity to the mouthpiece in front of the saliva trap. The pair of asynchronous valves is designed to promote unidirectional breath flow and keep the condenser chamber closed for condensation from the ambient air. During operation, the valves allow only exhaled breath to pass through the condenser chamber, which prevents individuals from exposure to extreme temperature differences in inhaled air. The valves provide a sufficient, pressure drop free, flow during inspiration-expiration maneuvers. Chemically inert materials were used for parts that are in contact with breath to reduce any chemical absorbance and carry over effect among users [21, 22]. The device can be sanitized and reused; the only disposable part is a personal plastic mouthpiece adapter (Part umber BE 120-22D, Instrumentation Industries, Inc., Bethel Park, PA).

Close attention was given to the flow aerodynamic in the device to achieve minimum pressure resistance, efficient condensation rate, and enhance mechanism for filtering saliva droplets from the exhaled breath flow. The saliva trap section of the device, shown in Figure 2b, and circular inset of Figure 2a, has a designed flow path for passage of droplets with mass-diameter smaller than the set threshold. Any droplet above the threshold should be eliminated from the breath flow and collected in the saliva trap reservoir. The threshold size of the droplets is adjusted with the position of the vertical notch. No physical filtering element (membrane) is used in the sampler that can harm the biological content by eliminating useful compounds from the exhaled breath.

Efficient and quick sample transfer with no physical phase change from frozen ice into liquid is crucial for metabolomic content preservation [17]. To retrieve the collected sample from the condenser tube into a vial, the dry ice pellets are poured out from the insulated housing, and condenser tube is placed into the sample retrieval press (Figure 2c). The frozen EBC sample is removed from the inner lumen side of the glass tube with a PTFE plunger. The two sharp notches on the front end of the plunger, similar to an end mill tool, clear the ice out with a rotational movement. There are two PTFE gaskets installed in the middle part of the plunger body that are slightly larger than the its diameter. The two gaskets create a ridge that scribes the remaining frozen sample from the inner wall of the condenser tube.

2.2. Breath flow, heat exchange, and saliva filtering

Flow regime, pressure resistance, heat transfer, and condensation rate are estimated with analytical and numerical solutions. The pressure drop (Δp) in the condenser tube is estimated with Darcy friction factor and known fluid properties [32].

$$\Delta p = \rho g \left(\frac{f L v^2}{2dg} + \Delta z \right) \quad \text{Eq. 1}$$

where ρ is the fluid density, g is the acceleration of gravity, f is the Darcy friction factor, v is the flow velocity, and d is the tube inner diameter. Change due to gravity, Δz, is equal to the glass tube length, L, since the device operates in the vertical position.

The temperature at the outlet of the device (T_o), is evaluated with the average convection heat transfer coefficient, $\overline{h}$, assuming constant surface temperature, T_s, and negligible pressure gradient to treat the fluid as incompressible.

$$\frac{T_s - T_o}{T_s - T_i} = \exp\left(-\frac{P L \overline{h}}{\dot{m} C_p} \right) \quad \text{Eq. 2}$$

where, T_i is the inlet temperature, P is the tube inner perimeter $\dot{m}$, is the mass flow rate, and C_p is the specific heat. Corresponding fluid properties at the outlet of the device are evaluated at this estimated temperature, T_o. Constant surface temperature assumption is validated with the estimation of the heat that can be conducted through the glass tube wall to the heat absorbed from the fluid in condensation, freezing, and cooling. Under the most conservative conditions, at minimum temperature difference and highest breath flow rate, the possible amount of heat conducted through the glass wall exceeds the amount of heat released by warm breath.

The rate of heat transfer, $\dot{Q}$, between the moist breath stream and the chilled surface is evaluated with mass and energy rate balances. The mass of the air is preserved, while water vapor partially condenses which is resembled in the change of relative humidity. Assuming steady state and saturated fluid properties at the inlet and outlet temperatures, one can evaluate the condensation rate from Eq. 3.

$$\dot{Q}_{cv} = \dot{m}_a [h_{a_o} - h_{a_i} + \omega_o h_{g_o} - \omega_i h_{g_i} + (\omega_i - \omega_o) h_{f_o}] \quad \text{Eq. 3}$$

where, $\dot{m}_a$ is mass flow rate of dry air, h_a is the specific enthalpy of dry air, h_g is the specific enthalpy of water vapor, and h_f is the specific enthalpy of the condensate, ω is the humidity ratio at the inlet and outlet, respectively.

The analytical solution results for pressure resistance and heat exchange in the device were confirmed with a numerical simulation of non-isothermal flow using COMSOL®. The geometrical scale of the model was 1:1 corresponding to the actual dimensions of the device.

The inlet flow rate corresponded to an average tidal breathing rate (12–20 breaths/minute, tidal volume 0.5 L, exhaled in 1 second) [33, 34], the fluid properties at the inlet were approximated with those of saturated air mixture at body core temperature, the surface temperature of the condenser corresponded to the experimentally measured value (−30 °C). The numerical solution was performed in three steps. First, velocity and pressure distribution in the device were evaluated assuming steady state flow. Heat exchange between breath and condenser surface was evaluated with two models: heat transfer in fluids based on the previously estimated fluid velocity field; and with simulation of non-isothermal fluid flow in the device. The droplets flight paths inside the saliva filter were estimated with a particle tracing application in COMSOL® with assumption that droplets were at thermal equilibrium with the carrier fluid and underwent no phase change (no evaporation or condensation) in flight. The saliva trap shape and dimensions (notches lengths and positions) were chosen based on the results of the numerical solution.

2.3. EBC samples collection and aliquots for analysis

The performance of the engineered device was compared to two currently commercially available EBC collection devices, Rtube™ (Respiratory Research, Inc., Austin, TX, USA), and TurboDECCS (MEDIVAC, Parma, Italy). The main criterion for evaluation of device performance was the number of metabolites in EBC samples collected with each device from the same group of healthy volunteers (Figure 3). Collection of EBC was performed with three devices from a group of healthy volunteers (3 male and 3 female, matched from three age groups). All participants were in good health and had no history of smoking. The sampling time was time controlled, 10 min for all volunteers and devices; the number of breaths was not counted. No nose clip was worn during collection. Volunteers were asked to keep normal tidal breathing with straight body position (straight back, ninety degrees chin). Volunteers restrained from food consumption three hours before EBC collection procedure and rinsed their mouth with water prior to breathing into an EBC collection device to reduce the effect of food-related confounders. All other experimental set up parameters were held constant for all volunteers and devices. Individual EBC samples collected with each device were aliquoted into a composite sample to reduce effects of the physiological differences. The metabolomic content of composite sample from each device was analyzed with a number of methods. EBC sampling from human volunteers was performed under UC Davis IRB protocol 63701-3 (09/30/2014-07/07/2017).

The HBS device was reused by all volunteers. All parts of the engineered device and sample retrieval press were thoroughly cleaned before 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. New mouthpiece was installed for every sampling procedure. The device was assembled, charged with dry ice pellets, and allowed to stabilize for 5 min. Before normal breathing, volunteers first performed an exhalation maneuver into the tube to displace the volume of the cold air trapped in the device chamber during assembly and temperature stabilization period. The frozen condensate was retrieved from the condenser tube with sample retrieval press into a clean borosilicate glass vial (Sigma-Aldrich, SU860099 SUPELCO) and immediately sealed with a stainless steel threaded cap with PTFE fluorosilicone rubber septum (Sigma-Aldrich, SU860101 SUPELCO). Rapid

sample retrieval (within 1–2 min after collection) allowed avoiding sample phase change (thawing). Figure 4 shows HBS device in use as a volunteer is breathing into the device, and then an operator transfers frozen EBC sample into a storage vial using the plunger, retrieval press, and a spatula. The EBC sample loaded into a vial is preserved in its original frozen state.

New disposable RTube™ and TurboDECCS devices were used for each volunteer and each EBC collection. For RTube™, the aluminum thermal mass sleeve was kept at −80 °C overnight before sampling. The insulating cover was rapidly put on the cooled sleeve and the device assembled for sampling within 1 min. The slush-like EBC sample was retrieved into a clean borosilicate glass vial with the RTube™ sample plunger and immediately sealed with stainless steel threaded cap containing PTFE fluorosilicone rubber septum. For TurboDECCS, the device was assembled and allowed to equilibrate for 40 min to achieve a stable state temperature prior to EBC sampling at −10 °C (default minimum setting for the device). For this device, the EBC sample was always in liquid form and was transferred into a clean borosilicate glass vial with a pipet. The vial was immediately sealed after sample transfer. The mass of each glass vial (with cap, septum, and label) was measured before and after each sampling. The condensation efficiency of each device was evaluated based on the average mass of EBC collected from the same group of volunteers during the set period of time.

2.4. Condenser surface temperature measurements

The three devices were compared in terms of the condenser surface temperature during a 10 min EBC sampling period. Eight k-type thermocouples, distributed at equal distances were attached on the condenser surface of each device with 4 mm square pads of foam tape. The use of foam tape helped to reduce the thermocouple exposure to the warm exhaled breath flow and measure condenser surface temperature more accurately. An Arduino-based data logger recorded and saved temperature values every 2 seconds (assembled from: KTA-259K Thermocouple Multiplexer Shield from Ocean Controls, Seaford, Australia; Arduino Uno microcontroller board; and DEV-09530 microSD serial data logger from Sparkfun Electronics™). The EBC samples collected during temperature profile measurements were discarded.

2.5. Saliva contamination measurement

The amount of saliva contamination was evaluated by measuring activity of the α-amylase enzyme in EBC samples collected with each device. α-amylase is a calcium metalloenzyme that breaks starch down to sugar. In humans, amylases are produced by salivary glands and pancreas, but are absent in any of the lung-borne fluids. Therefore, the activity of the amylase is a convenient way to estimate contamination of the EBC sample with saliva. The measurements of amylase activity in EBC samples collected with the engineered device and the RTube™ and TurboDECCS were done with the amylase activity colorimetric assay K2225-100 (ApexBio, Houston, TX, USA).

The engineered device was tested with regards to two modes of saliva filtering: enhanced and basic (with and without the vertical saliva filtering notch installed in the saliva trap

cavity). For the HBS device, the effect of saliva filtering was confirmed by measuring amylase activity in both EBC sample from the condenser tube and in the filtered content from the saliva trap reservoir. The assay was conducted per manufacturer instructions as follows. The EBC samples from each device and saliva trap content from the engineered device were tested directly without sample pretreatment. 50 μl of each sample in triplicates was spotted onto a 96-well plate and mixed with 50 μl of assay buffer and 50 μl amylase substrate mix. Three amylase positive controls, three DI water blanks, and duplicates of nitrophenol standard mix at 0, 4, 8, 12, 16, 20 nmol/well were spotted on the same 96-well plate. Samples were mixed with reaction mix of 50 μl of assay buffer and 50 μl amylase substrate, appropriately. The 96-well plate was loaded into the plate reader and the absorbance was measured immediately (T_0) at 405 nm and then in 10 min intervals for 60 min while reactions incubated at 25 °C.

The amylase activity in each sample was calculated by using the following equation:

$$Amylse\ activity = \frac{B}{T_x V} * sample\ dilution\ factor$$; where amylase activity (in mU/mL, the U is the unit of amylase - the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmol of nitrophenol per min at pH 7.20 at 25 °C); B is the estimated amount of nitrophenol generated by amylase between T_0 and T_x (the value of B is estimated from the built nitrophenol standard curve); T is the time between T_0 and T_x (in min); V is the sample volume added to the reaction well (in ml). Sample dilution factor was 1 since all of the samples were used directly.

2.6. Metabolomic content analysis

The metabolomic content of the collected EBC samples was evaluated using gas chromatography mass spectrometry (GC/MS) for the volatile fraction, and the liquid chromatography mass spectrometry (LC/MS) for the non-volatile fraction.

The GC/MS analysis was carried out for three technical replicates of composite EBC sample per each tested device (see Figure 3). 1 mL aliquots of the EBC samples were stored in cap-sealed borosilicate vials in −80 °C freezer. Before analysis, the samples were allowed to thaw on 3 °C chilled tray of GC/MS instrument; the vials were kept cap-sealed. The polyacrylate (PA) solid-phase microextraction (SPME) (Supelco, Bellefonte, PA) tip was inserted by an autosampler into the liquid EBC, and the sample was agitated for 30 min at room temperature. The samples were analyzed with Varian 3800 GC (VF-5ms 5% phenol/95% PDMS column, Varian, Walnut Creek, CA) and a 4000 Ion Trap MS (Varian) equipped with Electron Ionization source (EI) instrument. DI water blanks (Evoqua, Denver, CO, USA), empty vial blanks and quality controls of aqueous D8 naphthalene solution were interspersed with the samples and randomized. GC/MS analysis was carried out as a single batch. The details of GC/MS analysis protocol were described [35].

For LC/MS analysis, three technical replicates of composite EBC sample, 0.5 mL each, from each device were lyophilized directly in the vial and then re-dissolved in 70 μL HPLC grade acetonitrile:water 9:1 v/v with sonication and 20 μL of sample was injected for analyses by hydrophilic interaction liquid chromatography (HILIC) and reversed-phase chromatography (RP). Chromatography was performed on an Agilent 1290 binary high-performance liquid

chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA). The HILIC and RP analyses were performed in turn, with the solvent lines flushed for 10 minutes in between. Before the analysis, the lyophilized samples were stored at −20 °C in capped LC vials with spring inserts (Agilent, Santa Clara, CA). During analysis the samples were housed in an autosampler maintained at 4 °C. The details of LC/MS HILIC and LC/MS RP analysis protocols were described previously[35, 36].

For the HILIC analysis, Waters ACQUITY UPLC BEH Amide 130 Å, 1.7 μm, 2.1 mm × 100 mm column (Waters, Milford, MA), held at 30 °C was employed. Mobile phase A consisted of ultrapure water, mobile phase B was 90% acetonitrile in water with ammonium acetate and acetic acid added to obtain pH 5. Exact phase, composition ratios, and ramp times are described elsewhere [35]. The mass range was set to 50–1700 Thomson (m/z). An internal standard of $TFANH_4$ and purine was infused along with the sample. The blanks of milliQ water used for sample preparation, the AcN solvent and mobile phase B were run along with the samples. Quality controls were also run with the samples: the Waters 186006963 HILIC QC (Waters, Milford, MA) a custom-made QC was used to verify instrument performance: carnitine, lysine, adenylputricine, aminocapricoic acid, ornithine, tigonelline, alaninol, acetylcarnitine, 1-(2 pyramidyl)piperazine, methoxychalcone, cholecalciferol, 13-docosenamide and oleamide, all at 5 μM aqueous solution.

For the RP analysis, Waters Acquity CSH C18 1.7 μm, UHPLC (2.1 × 100 mm) (Milford, MA USA) column was used; held at 30 °C during analysis. Mobile phase A consisted of 60% acetonitrile in ultrapure water, mobile phase B was 10% acetonitrile in isopropanol with formic acid and ammonium formate added to make the final concentration of each mobile phase 10 mM for both formic acid and ammonium formate. Exact phase, composition ratios, and ramp times are described elsewhere [35]. The mass range was 50–1700 Thomson (m/z). The standard of $TFANH_4$ and purine was infused along with the sample. The Waters 6963 RP QC (Waters, Milford, MA) standard was run with each sample batch. The blanks of milliQ water used for sample preparation, the AcN solvent and mobile phase A were run along with the samples.

Additional analysis of three technical replicates of composite EBC sample from each device was carried out using tandem mass spectrometry, for each HILIC and RP chromatography modes. For this mode of analysis, the samples were lyophilized directly in the vial and then re-dissolved as described above, and 3 μL of re-suspended sample was injected for analysis. The CUDA (12 [[(cyclohexylamino)carbonyl]amino]-dodecano ic acid) in methanol:toluene, 9:1 v/v internal standard was used for quality control. Sample was housed in an autosampler maintained at 4 °C; the chromatography was performed using Waters ACQUITY UPLC BEH Amide 130 Å, 1.7 μm, 2.1 mm × 100 mm column (Waters, Milford, MA), held at 40 °C during analysis. Mobile phase A consisted of ultrapure water with 10 mM ammonium formate + 0.125% formic acid, pH 3. Mobile phase B was 95:5 v/v acetonitrile:ultrapure water with 10 mM ammonium formate + 0.125% formic acid, pH 3. LC eluent was analyzed with an Agilent 6530 Q-TOF MS (Agilent Technologies, Santa Clara, CA) in positive and negative ionization modes. The mass range was set to 60–1200 Thomson (m/z). The electrospray ionization (ESI) capillary voltage was set at +4.5 kV for ESI (+). The fragmentation was carried out with collision energy of +45 eV for ESI (+). Untargeted

analysis of molecular data was carried out using data-independent acquisition (DIA) as described previously [37]. Device blanks of a milliQ water rinse of clean device inner surface that comes into contact with the sample were included along with the collected breath sample.

For the reverse phase tandem MS analysis, the Agilent 1260 HPLC system with the reverse phase UHPLC Waters Acquity CSH C18 1.7 μm, (2.1 × 100 mm) (Milford, MA USA) column was used. The sample was dried, and then re-suspended in 100 μL 9:1 methanol:toluene. 3 μL of re-suspended sample was injected for analysis. The CUDA in methanol:toluene, 9:1 v/v internal standard was used for quality control and to assess reproducibility. The samples were separated on the column held at 65 °C during analysis. Mobile phase A consisted of 60% acetonitrile in water. Mobile phase B was 10% acetonitrile in isopropanol. Formic acid and ammonium formate were added to make the final concentration of each mobile phase 10 mM for both formic acid and ammonium formate. Samples were analyzed with MS using an Agilent 6530 Q-TOF mass spectrometer in positive and Agilent 6550 Q-TOF mass spectrometer in negative ionization modes. The device blanks, milliQ water rinse of the clean device inner surface that comes into contact with the sample were included along the collected EBC samples.

2.7 Metabolomic data analysis

The GC/MS data for the three devices were analyzed as described earlier [35, 36]. The data were visualized using the MS Data Review software v. 6.6 (Varian). Deconvolution was carried out using Automated Mass Spectral Deconvolution and Identification System (AMDIS) software v. 2.64 with medium resolution and high sensitivity and shape requirements. Any peaks that appeared in any of the appropriate blanks (blank vials, DI water, clean SPMEs, quality controls) or compounds that are known external contaminants such as siloxanes (column, septa bleed) were excluded. Each peak was considered reproducible if it was present in at least 2 out of 3 technical replicates for each collection device.

The LC/MS raw data files were first processed with the “Find By Molecular” feature in Agilent’s Mass Hunter Qualitative Analysis B.05.00SP1 software in order to deconvolve each peak with the “Match factor” parameters set at 50. The peaks were aligned using Mass Profiler Professional 13.1 software. The alignment window was set at 1 min and the match factor is 0.3. The minimum quality score was set at 0.25.

For the LC/MS/MS analysis, the HILIC data were processed, collated and constrained into Agilent’s MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the NIST14/Metlin/MassBank libraries to identify metabolites with manual confirmation of adduct ions and spectral scoring accuracy. For the RP analysis, raw data were processed in an untargeted (qualitative) manner by Agilent’s software MassHunter Qual to find peaks in all chromatograms. The peaks were then collated and constrained into a MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the LipidBlast library to identify lipids with manual confirmation of adduct ions and spectral scoring accuracy.

Three criteria were used to obtain the global peak tables for LC/MS and LC/MS/MS analyses: peak abundance, peak sample/blank ratio, and peak reproducibility. All low-abundance spurious peaks, below the threshold of 1000 a.u., were removed from sample peaks. Then, each peak was examined and considered as a metabolite if it was not present in the blanks or if its ratio of peak abundance in sample versus abundance in blank was greater than 10 times. Also, similarly to GC/MS analysis, a peak was considered consistent if it appeared at least in 2 out of 3 technical replicates in each analysis. During data processing, HILIC and RP methods were treated similarly but independently of each other.

3. Results

3.1. EBC sampler and hardware design optimization

The purpose of this work is to design an optimal EBC sampler while using simple design solutions. In this work, we considered the following three design parameters: condenser temperature and its stability during sampling period [20]; removal of saliva droplets from the breath flow to reduce EBC sample contamination with compounds originating in the mouth cavity [25, 27]; and use of chemically inert materials for the parts that are in contact with breath sample [21, 22]. Each of these parameters may have a significant effect on the metabolomic content of EBC sample and requires a detailed study. We evaluated the effects of design optimization with the analysis of the metabolomic content of EBC samples in comparison to two commercially available devices. The metabolomic contents of volatile and non-volatile fractions of EBC samples were analyzed with a set of GC/MS and LC/MS methods, respectively. A set of chemometric tools was applied to identify metabolites in each analysis type.

3.2. Condenser temperature, sample phase and volume

Figure 5a shows the experimental setup for measuring surface temperature of the condensing elements of each device, particularly the HBS device. The thermocouples distributed in the condenser chambers were able to resolve the spatial and time temperature profiles for each type of device. For the RTube™ and HBS devices, the condenser area near the inlet is exposed to a stream of warm exhaled breath and is maintained approximately 3–5 °C warmer than the rest of the condenser surface. The rate of temperature data acquisition was fast enough to show oscillatory temperature readings that resembled tidal breathing pattern. Original temperature data is provided (Supplemental Table 1).

Figure 5b shows averaged condenser surface temperature, from 8 thermocouples and three volunteers, for each device during a 10 min EBC collection. Each device was given time to achieve stable temperature before EBC sampling began. The TurboDECCS instrument was operated for 40 min prior to EBC collection to achieve its minimum temperature (set at −10 °C, actual temperature −7 °C). The RTube™ aluminum sleeve was kept in a −80 °C freezer overnight before use. The device was assembled and ready for sampling within 1 min after the thermal mass was removed from the freezer. No additional stabilization period was allowed because the device warmed up quickly. The HBS device equilibrated within 5 min, after filling the chamber with dry ice pellets, to achieve a stable condenser surface temperature of −56 °C. The temperature plots are aligned in time to allow for visual direct

comparison. Figure 5c shows the net mass of EBC retrieved for storage/analysis, after 10 minute sampling period with each device; averaged from 6 subjects; the error bars (±1 standard deviation) highlight differences among subjects. The net mass of EBC obtained for analysis does not necessary represent device condensation efficiency per surface area of the condenser element (0.0164 m^2 for HBS, 0.0157 m^2 for RTube™, 0.098 m^2 for TurboDECCS) because a fraction of sample is lost during sample retrieval from the device into a storage container.

3.3. Flow optimization and aerodynamic saliva droplets filtering

Low airflow resistance and high condensation rates are two desired engineering parameters for a breath sampler. The back pressure is a significant problem for breath sampling apparatuses because deviation from normal respiration patterns affects the metabolomic content of breath [17]. Human respiratory devices are rated at or below 100 Pa pressure drop [38] for the average adult humans. Approximating average tidal respiratory rate of 20 breaths/min with tidal volume of 0.5 L exhaled in 1 second [34] as a constant flow rate of 0.5 L/s, we can estimate pressure and temperature drop in the device. Figure 6 (a, b) shows numerical solution results of non-isothermal laminar flow (Re = 1864) in the HBS device using COMSOL® software. Both types of solutions, numerical and analytical, closely agree on the pressure resistance and temperature drop in the device. The corresponding pressure resistance (ΔP) inside the device is 38.1 Pa estimated with the numerical solution and 31.5 Pa estimated with the analytical solution (Eq. 1). The estimated temperature of the fluid at the outlet (To) is 25.1 °C estimated with the numerical solution and 24.4 °C estimated with the analytical solution (Eq. 2).

Theoretical condensation rate estimated from the mass and energy balance (Eq. 3), based on the difference of saturated fluid properties at the inlet temperature (36.6 °C) and estimated fluid temperature at the outlet (25 °C), is 10.5 mg/s. Hence, the total theoretical condensed sample volume is 2.1 g assuming that condensation occurs only during exhalation maneuvers; 200 seconds (10 min sampling, 20 breaths/min, flow rate 0.5 L/s during exhalation). The condensation process was not numerically estimated in COMSOL®. Figure 6 (c, d) shows the results of the numerical solution for the passage of microdroplets through the saliva trap. The size of the microdroplets filtered from the breath flow in the saliva trap was estimated with the particle tracking mode of the COMSOL® software. Exhaled droplets with diameter less than 20 μm are predicted to pass with the breath flow (Figure 6c) and droplets with diameter greater than 20 μm deviate from the flow and are retained in the trap (Figure 6d). Although a more computationally accurate solution would resolve more details about the design of the saliva filter, this solution justified the design concept. The ability of the designed filter to reduce the amount of saliva was confirmed with the experimental measurement of amylase level present in the collected EBC samples. The beneficial side of this design is that it allows selective filtering of microdroplets; captures heavy saliva droplets but allows passing small droplets that originate in the deep lungs and contain meaningful metabolites for health diagnostic.

The HBS device can operate in basic and enhanced saliva filtering modes. In the basic saliva filtering mode, there is no vertical notch installed in the saliva trap. All exhaled

microdroplets pass to the condenser; only saliva liquid is directly removed from the breath flow and collected in the saliva trap reservoir. In the enhanced saliva filtering mode, there is a vertical notch installed in the saliva trap that makes the breath flow circulate through four orthogonal turns. The enhanced saliva filtering mode further reduces the amount of saliva in the EBC sample. Figure 7 shows experimental results of average α-amylase activity level (mU/mL) in EBC samples collected with each device. Error bars show ±1 standard deviation from the average value based on measurements from 6 volunteers. For the HBS device, the α-amylase activity was measured in EBC samples and the liquid fraction captured (retained) in the saliva trap reservoir. The saliva contamination in EBC samples was the lowest for the HBS device when used in enhanced saliva filtering mode (Figure 7a). Figure 7b demonstrates the effect of the saliva filtering method in the HBS device by comparing basic and enhanced modes (single measurement). The performance of HBS sampler, in basic saliva filtering mode, is comparable to that of RTube™ sampler. In basic mode, the saliva is filtered with a 90-degree turn in the flow, similar to RTube™, that may remove some of the heaviest droplets that are more likely to originate in the oral cavity. The EBC samples collected with the TurboDECCS device contain significantly more saliva. Supplemental Table 2 provides the data for OD measurements on the tested EBC samples.

3.4. Metabolomic content of the EBC

The metabolomic content of the EBC samples was assessed using mass spectrometry for each different device: the HBS in basic saliva filtering mode, the RTube™, and the TurboDECCS. The volatile EBC fraction was measured using GC/MS protocols, and the non-volatile fraction was measured using both polar (HILIC) and non-polar (RP) HPLC/MS analysis. The number of unique peaks resembles the number of metabolites covered only with the particular device (Figure 8). The number of common peaks resembles the number of metabolites covered with the particular device but also present in one of the other two or in both other devices. The number of total peaks resembles the total number of metabolites covered with each device per analytical method. Table 1 provides exact number of metabolites detected in samples collected with each device and analysis method. The global peak tables for LC/MS and LC/MS/MS analyses that include peak abundance, peak sample/blank ratio, and peak reproducibility are provided (Supplemental Table 3).

4. Discussion

The physical phase of EBC samples differ for the three compared devices. The TurboDECCS instrument operates at about 5 °C and collects a liquid EBC sample. The RTube™ starts condensation at −54 °C and warms up to 0 °C over a 10 min sampling period which limits its collection time and sample volume; it collects a slush-like EBC sample that completely melts into liquid during transfer into a storage container. The HBS device operates at significantly lower temperatures with the condenser rising from −56 °C to −30 °C during tidal breath sampling, and the temperature stabilizes at that level for longer sampling periods. The breath vapor condensate and exhaled aerosol microdroplets are frozen into a snow white powder on the condenser surface; the inner space of the condenser tube gets filled with sample at longer sampling period (40–50 minutes, sample mass 4.5 g).

Provided that inlet conditions are the same, the condensation efficiency of the device depends on the condenser chamber design, its temperature, and size. The greater mass of EBC sample was collected with TurboDECCS device, in spite of the highest temperature and smallest surface area of the condenser, is probably due to its chamber design. In TurboDECCS device, the condenser chamber is sealed from ambient air, with inlet and outlet positioned so that they enhance turbulence in the flow such that exhaled breath circulates several times before leaving the chamber. Whereas in RTube™ and HBS devices, the exhaled breath flows through the condenser chamber. Some part of the sample was lost during sample transfer from the device into a storage container. The liquid EBC sample collected with TurboDECCS was less prone to that loss because it was all pipetted into a vial.

The experimental value (Figure 5c) is much smaller than theoretical for a number of reasons. Sampling was performed time based (10 min), number of total exhalation cycles and total exhaled volume were not controlled. Also, the theoretical estimation does not include sample loss that occurred during sample transfer from condenser surface into a vial. The estimation does not take into account the time changing nature of exhalant flow. It is a multi-phase process with unsteady flow (vapor cooling, phase change from vapor to liquid, liquid cooling, phase change from liquid to solid, and solid cooling). Nevertheless the proposed solutions gave some predictions for the experimental results.

The primary design advantage of the HBS device is the captured fraction of the metabolomic content from exhaled breath condensate. The physical phase of the EBC sample (solid versus liquid) and the fraction of the captured VOCs are dependent on the condensation temperature and its stability during all collection steps [20, 39]. The hardware design is made for quick sample transfer to avoid sample contamination and physical phase change. A thick glass tube (4 mm wall) acts as a thermal mass and keeps the EBC sample frozen during the sample retrieval procedure, which is usually completed in 1–2 min. The collected EBC samples were transferred into storage vials in their original solid phase (Figure 4).

The aerodynamic saliva filtering in the saliva trap allows not only elimination of the apparently large saliva droplets from the breath flow but also selective mass filtering of the microdroplets with masses above the set mass threshold. The saliva trap design is based on the ability of a particle (droplet) to trace the flow. The behavior of a particle suspended in a fluid flow is characterized with the Stokes number defined as the ratio of the characteristic time of a particle (time required to change particle flight direction) to a characteristic time of the flow (time required for fluid flow to change its direction) around an obstacle. A small droplet (low Stokes number) follows the fluid flow closely because its momentum is easily affected by the change in fluid velocity (particle momentum changes as fast as the fluid flow). A heavy particle has a great momentum and requires a longer time period to change its momentum in response to the change in the flow velocity field. Provided that droplets with different masses are propagating in the same fluid flow, they all have the same time period for their momentum change to follow the fluid flow around an obstacle but require different amounts of time for that change to occur. Thus, large droplets fail to follow the fluid flow around an obstacle (because they need more time to change their momentum than it takes the fluid) and will rather follow their own (close to straight) path that will eliminate

them from propagating the fluid flow around an obstacle. This ability of a droplet to follow the fluid flow is not solely determined by its mass and size but also by the flow regime (turbulent or laminar) and fluid properties (density, viscosity). This dependence is described by Reynolds number and drag coefficient.

The current design of the saliva trap proves the concept and needs further optimization. The optimal size of the droplets that are allowed to pass into the breath condensing region in the HBS device needs to be determined experimentally by considering metabolomic content of exhaled microdroplets versus their size. Although the correlation of microdoplets mass and diameter to their origin was studied [24–26], their metabolomic content and biomarker use was not fully assessed. While some microdrolets may introduce sample contamination with saliva and dilution, others may originate from lungs and contain metabolites of significant value for a particular disease diagnostic. In the HBS device, the mass threshold for passage of exhaled microdroplets can be adjusted with the position of the vertical saliva trap notch. This feature can be beneficial for further optimization of a standardized EBC sampling procedure.

For the volatile fraction of the EBC samples, the RTube™ and HBS device showed similar performance with largely overlapping metabolomic content, and both were superior to the TurboDECCS device. The overlap in the number and types of detected breath metabolites is likely due to similarities in the design of the HBS and RTube™ devices, although certain differences were observed. For example, carbonyls such as 6-methyl-5-hepten-2-one and nonanal were consistently more abundant in HBS samples. These minor differences are presumed to result predominantly from the use of different materials (glass in HBS and plastic in RTube™), although differences in collection temperature and its stability may also play a role. Both the number and abundances of most EBC metabolites were consistently lower in samples collected with the TurboDECCS device. This is presumed to result from several factors: temperature of condensation collection, length of tubing in the device, and the physical state of the EBC sample. Unlike in the other two devices, the collected EBC remains in liquid form throughout the duration of sampling due to a higher collection temperature (Figure 5b). This is presumed to result in the loss of some volatile metabolites during the collection and sample transfer steps.

For the non-volatile fraction, the HBS device demonstrated significantly higher coverage of metabolomic content than either the RTube™ or TurboDECCS devices. Although a significant degree of metabolite content overlap is still observed, the design differences between the devices clearly distinguish their performance. Some of the differences and the enhancement of the metabolomic content coverage for the HBS device are presumed to stem from the use of glass material in the condenser tube. Loss of breath metabolites due to retention on plastic surface has been reported in the literature, and more non-polar compounds are prone to be retained. For example, oxylipin content in EBC sample is greatly reduced when EBC comes in contact with a plastic surface [40]. A significant improvement in capture of polar compounds with the HBS device was observed. The number of metabolites observed in the HILIC chromatography mode was significantly greater than in the other two devices (Figure 8). One potential reason for this is better saliva filtering from the exhaled breath flow and more efficient condensation of metabolites in the exhaled vapor.

This could also be due, in part, to a more consistent collection temperature and better nucleation of the frozen condensate material onto the glass surface. Another potential reason is diminished contamination of the EBC sample with saliva due to a more efficient droplet filtering mechanism.

5. Conclusions

This manuscript considers the differences between two commercial and one novel exhaled breath condensate samplers for the purpose of developing an optimal breath sampling methodology. The condenser surface temperature profiles and saliva level in the collected EBC samples were compared, along with the metabolomic content measured from their samples. The number and abundance of unique breath metabolites sampled using each device varies, and the metabolomic content and types of molecule classes vary by device as well. It appears the HBS device allows for enhanced retention of broad-spectrum metabolomic content; however, those metabolites are still of unknown diagnostic value today. Future work may allow for device designs that are tailored for specific clinical applications.

The novel HBS device design allows EBC collection with a number of methods: full facial mask, mouthpiece, or installation in respiratory ventilator pipeline. The choice of the method may affect the metabolomic content of the collected sample and may require optimization for particular type of biomarkers [17, 41, 42]. Future improvements could be achieved by integrating the current EBC sampling device with a spirometer [43] or capnography [44] devices. Breath sampling based on total exhaled breath volume may reduce physiological and breathing pattern differences and be a more consistent approach than time based sampling. Sampling end-tidal, alveolar, fraction of exhaled breath may further increase the concentration of biomarkers in collected EBC sample. Another possible improvement of the device could be modifying the surface material of the condenser region. Finally, the aerodynamic in-line filtering of exhaled breath droplets during EBC collection may be a substantial aid to enhance targeted metabolomic content, and might be more fully explored in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by: NIH award U01 EB0220003-01 [CED, NJK, MS, JPD], the NIH National Center for Advancing Translational Sciences (NCATS) through grant UL1 TR000002 [CED, NJK, JPD], and NIH award 1P30ES023513-01A1 [CED, NJK]. Partial support was also provided by: the Office of Naval Research (ONR) grant N-00014-13-1-0580 [CED, BCW], NIH award 1K23HL127185-01A1 [MS], and The Hartwell Foundation [CED, NJK]. Student support was provided by NIH award T32 HL07013 [KOZ] and NIH award P42ES004699 [KOZ]. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies.

References

1. Amann A, Smith D. Volatile Biomarkers Non-Invasive Diagnosis in Physiology and Medicine Foreword. Volatile Biomarkers: Non-Invasive Diagnosis in Physiology and Medicine. 2013:Xxvii–Xxix.
2. Buljubasic F, Buchbauer G. The scent of human diseases: a review on specific volatile organic compounds as diagnostic biomarkers. Flavour and Fragrance Journal. 2015; 30(1):5–25.
3. Phillips M. Breath Tests in Medicine. Scientific American. 1992; 267(1):74–79. [PubMed: 1502511]
4. Amann A, et al. The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. Journal of Breath Research. 2014; 8(3)
5. Stiegel MA, et al. Analysis of inflammatory cytokines in human blood, breath condensate, and urine using a multiplex immunoassay platform. Biomarkers. 2015; 20(1):35–46. [PubMed: 25495125]
6. Baraldi E, et al. Safety and success of exhaled breath condensate collection in asthma. Archives of Disease in Childhood. 2003; 88(4):358–360. [PubMed: 12651772]
7. Schnabel R, et al. Analysis of volatile organic compounds in exhaled breath to diagnose ventilator-associated pneumonia. Scientific Reports. 2015; 5
8. Beauchamp J. Inhaled today, not gone tomorrow: pharmacokinetics and environmental exposure of volatiles in exhaled breath. J Breath Res. 2011; 5(3):037103. [PubMed: 21654021]
9. Spanel P, Dryahina K, Smith D. A quantitative study of the influence of inhaled compounds on their concentrations in exhaled breath. Journal of Breath Research. 2013; 7(1)
10. Markar SR, et al. Exhaled Breath Analysis for the Diagnosis and Assessment of Endoluminal Gastrointestinal Diseases. Journal of Clinical Gastroenterology. 2015; 49(1):1–8. [PubMed: 25319742]
11. Kramer R, et al. A rapid method for breath analysis in cystic fibrosis patients. European Journal of Clinical Microbiology & Infectious Diseases. 2015; 34(4):745–751. [PubMed: 25431363]
12. Shestivska V, et al. Quantitative analysis of volatile metabolites released in vitro by bacteria of the genus Stenotrophomonas for identification of breath biomarkers of respiratory infection in cystic fibrosis. Journal of Breath Research. 2015; 9(2)
13. Purkhart R, et al. Chronic intestinal Mycobacteria infection: discrimination via VOC analysis in exhaled breath and headspace of feces using differential ion mobility spectrometry. Journal of Breath Research. 2011; 5(2)
14. Eng K, et al. Analysis of breath volatile organic compounds in children with chronic liver disease compared to healthy controls. Journal of Breath Research. 2015; 9(2)
15. Kumar S, et al. Mass Spectrometric Analysis of Exhaled Breath for the Identification of Volatile Organic Compound Biomarkers in Esophageal and Gastric Adenocarcinoma. Annals of Surgery. 2015; 262(6):981–990. [PubMed: 25575255]
16. Capuano R, et al. The lung cancer breath signature: a comparative analysis of exhaled breath and air sampled from inside the lungs. Scientific Reports. 2015; 5
17. Horvath I, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal. 2005; 26(3):523–548. [PubMed: 16135737]
18. de Lema JB, et al. Exhaled breath condensate: Standardized collection of samples from healthy volunteers. Archivos De Bronconeumologia. 2005; 41(10):584–586. [PubMed: 16266673]
19. Soyer OU, et al. Comparison of two methods for exhaled breath condensate collection. Allergy. 2006; 61(8):1016–8. [PubMed: 16867057]
20. Loyola BR, et al. Temperature changes in exhaled breath condensate collection devices affect observed acetone concentrations. Journal of Breath Research. 2008; 2(3)
21. Rosias PP, et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. European Respiratory Journal. 2006; 28(5):1036–1041. [PubMed: 16870660]
22. Rosias PP, et al. Biomarker reproducibility in exhaled breath condensate collected with different condensers. European Respiratory Journal. 2008; 31(5):934–942. [PubMed: 18184682]
23. Phillips M, et al. Variation in volatile organic compounds in the breath of normal humans. Journal of Chromatography B. 1999; 729(1–2):75–88.

24. Papineni RS, Rosenthal FS. The size distribution of droplets in the exhaled breath of healthy human subjects. J Aerosol Med. 1997; 10(2):105–16. [PubMed: 10168531]
25. Morawska L, et al. Size distribution and sites of origin of droplets expelled from the human respiratory tract during expiratory activities. Journal of Aerosol Science. 2009; 40(3):256–269.
26. Holmgren H, et al. Relation Between Humidity and Size of Exhaled Particles. Journal of Aerosol Medicine and Pulmonary Drug Delivery. 2011; 24(5):253–260. [PubMed: 21671751]
27. Effros RM, et al. Exhaled Breath Condensates: Analyzing the Expiratory Plume. American Journal of Respiratory and Critical Care Medicine. 2012; 185(8):803–804. [PubMed: 22505753]
28. Kuban P, Foret F. Exhaled breath condensate: Determination of nonvolatile compounds and their potential for clinical diagnosis and monitoring. A review. Analytica Chimica Acta. 2013; 805:1–18. [PubMed: 24296139]
29. van Mastrigt E, de Jongste JC, Pijnenburg MW. The analysis of volatile organic compounds in exhaled breath and biomarkers in exhaled breath condensate in children - clinical tools or scientific toys? Clinical and Experimental Allergy. 2015; 45(7):1170–1188. [PubMed: 25394891]
30. Mochalski P, et al. Stability of selected volatile breath constituents in Tedlar, Kynar and Flexfilm sampling bags. Analyst. 2013; 138(5):1405–1418. [PubMed: 23323261]
31. Ghimenti S, et al. Comparison of sampling bags for the analysis of volatile organic compounds in breath. Journal of Breath Research. 2015; 9(4)
32. Colebrook CF. Turbulent flow in pipes, with particular reference to the transition region between the smooth and rough pipe laws. Journal of the ICE. 1939; 11(2):133–156.
33. Lindh WQ, et al. Delmar's Comprehensive Medical Assisting: Administrative and Clinical Competencies. Cengage Learning p. 2009:573.
34. Boron, WF., Boulpaep, EL. Medical Physiology: A Cellular and Molecular Approach. W.B. Saunders; 2003.
35. Zamuruyev KO, A AA, Baird M, Pasamontes A, Parry C, Foutouhi S, Venn-Watson S, Weimer BC, Delplanque J-P, Davis CE. Enhanced non-invasive respiratory sampling from bottlenose dolphins for breath metabolomics measurements. Journal of Breath Research. 2016
36. Aksenov AA, et al. Metabolite Content Profiling of Bottlenose Dolphin Exhaled Breath. Analytical Chemistry. 2014; 86(21):10616–10624. [PubMed: 25254551]
37. Tsugawa H, et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nature Methods. 2015; 12(6):523. + [PubMed: 25938372]
38. Smyth RJ, Chapman KR, Rebuck AS. Maximal Inspiratory and Expiratory Pressures in Adolescents-Normal Values. Chest. 1984; 86(4):568–572. [PubMed: 6478896]
39. Ahmadzai H, et al. Exhaled breath condensate: a comprehensive update. Clinical Chemistry and Laboratory Medicine. 2013; 51(7):1343–1361. [PubMed: 23420285]
40. Yang J, et al. Quantitative Profiling Method for Oxylipin Metabolome by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. Analytical Chemistry. 2009; 81(19):8085–8093. [PubMed: 19715299]
41. Vass G, et al. Comparison of nasal and oral inhalation during exhaled breath condensate collection. American Journal of Respiratory and Critical Care Medicine. 2003; 167(6):850–855. [PubMed: 12493648]
42. Maurer F, et al. Wash-out of ambient air contaminations for breath measurements. Journal of Breath Research. 2014; 8(2)
43. Kwan AM, et al. Personal Lung Function Monitoring Devices for Asthma Patients. Ieee Sensors Journal. 2015; 15(4):2238–2247.
44. Montpetit A, et al. Volumetric Capnography As A Candidate Exhaled Biomarker For Patients With Rib Fractures. American Journal of Respiratory and Critical Care Medicine. 2015; 191

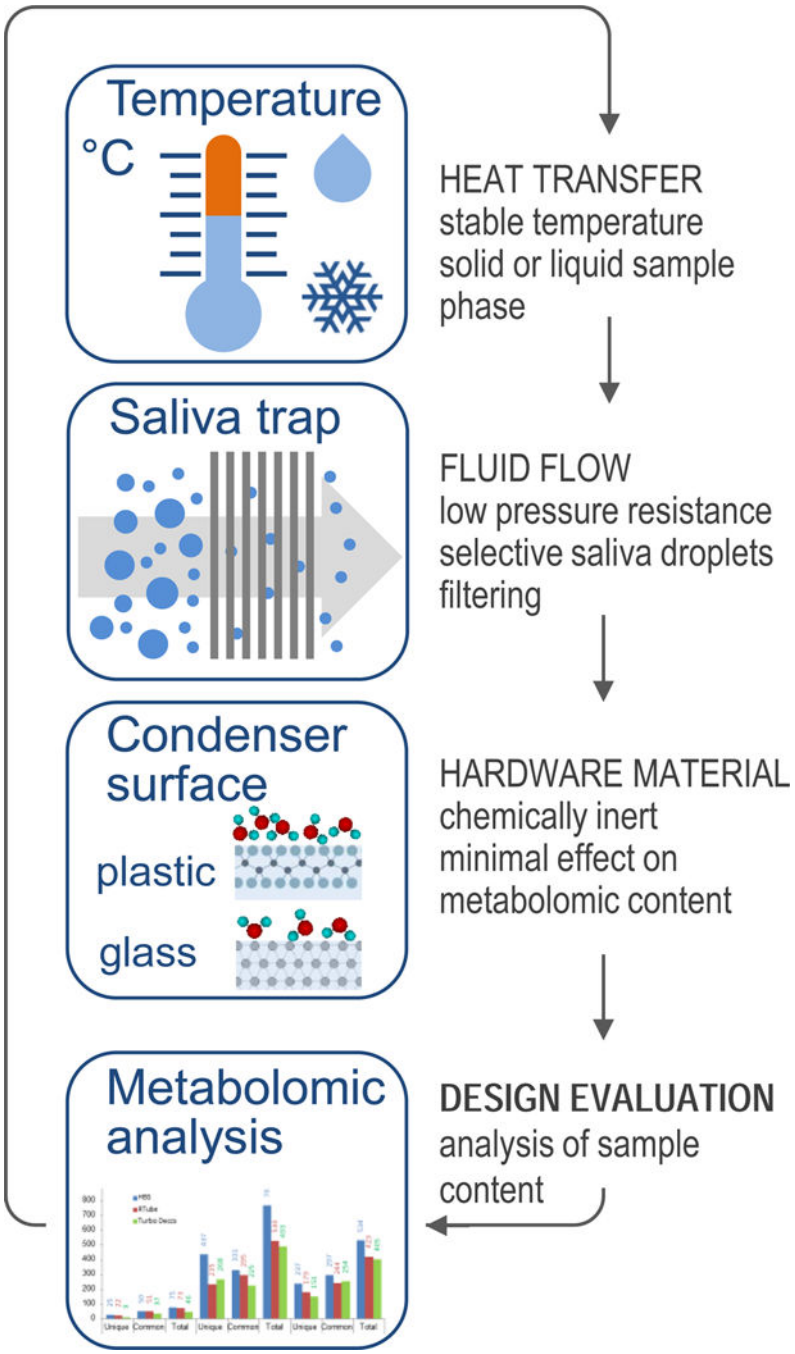

Figure 1.

Key parameters considered in the design of exhaled breath condensate sampler: temperature and its stability, saliva trap and microdroplets passage, choice of chemically inert materials. Performance of the device was evaluated with metabolomic analysis of EBC samples.

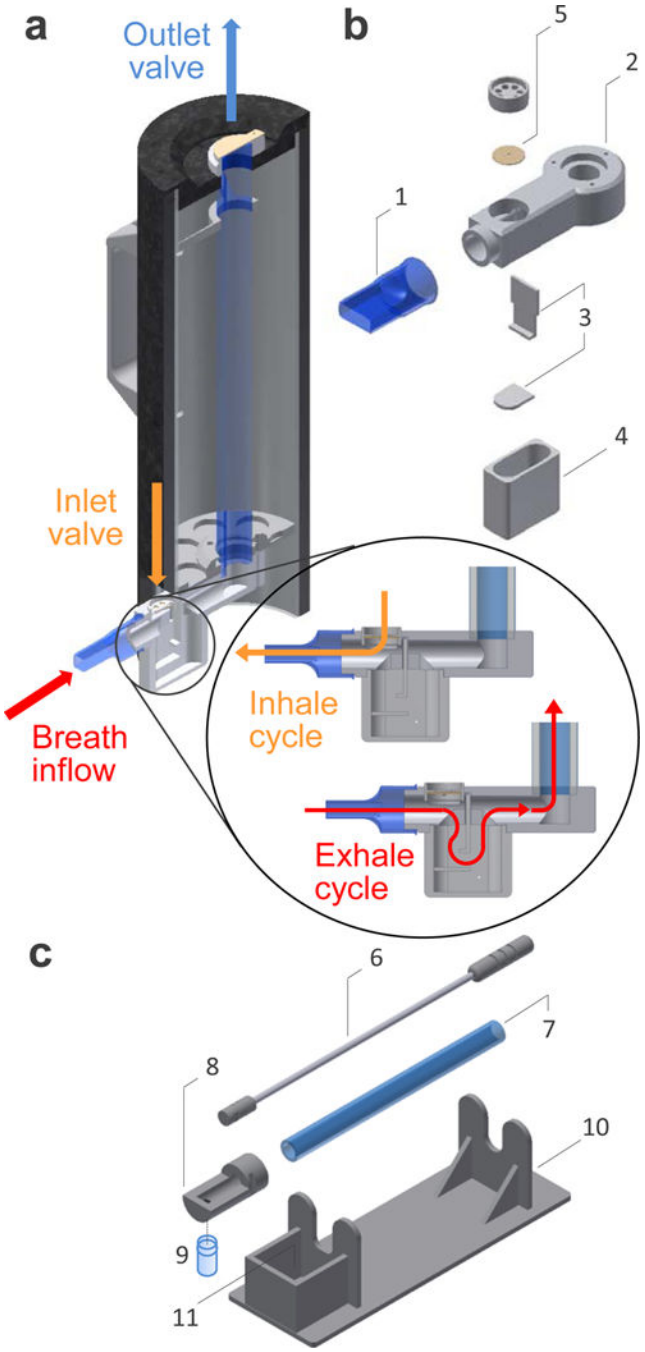

Figure 2.

(a) CAD model of the human breath sampler. Main parts: saliva trap assembly, condenser tube, insulated ice bath housing. Inset circle shows the breath flow diagram for inhale and exhale cycles. **(b)** Detailed view of saliva trap components: 1. mouthpiece, 2. saliva trap housing, 3. trap notch, 4. saliva reservoir, 5. inhale valve. **(c)** Sample extraction press components: 6. sample retrieval plunger with PTFE head, 7. condenser glass tube, 8. threaded vial connector, 9. 10 mL glass vial, 10. press base, 11. ice pocket for vial.

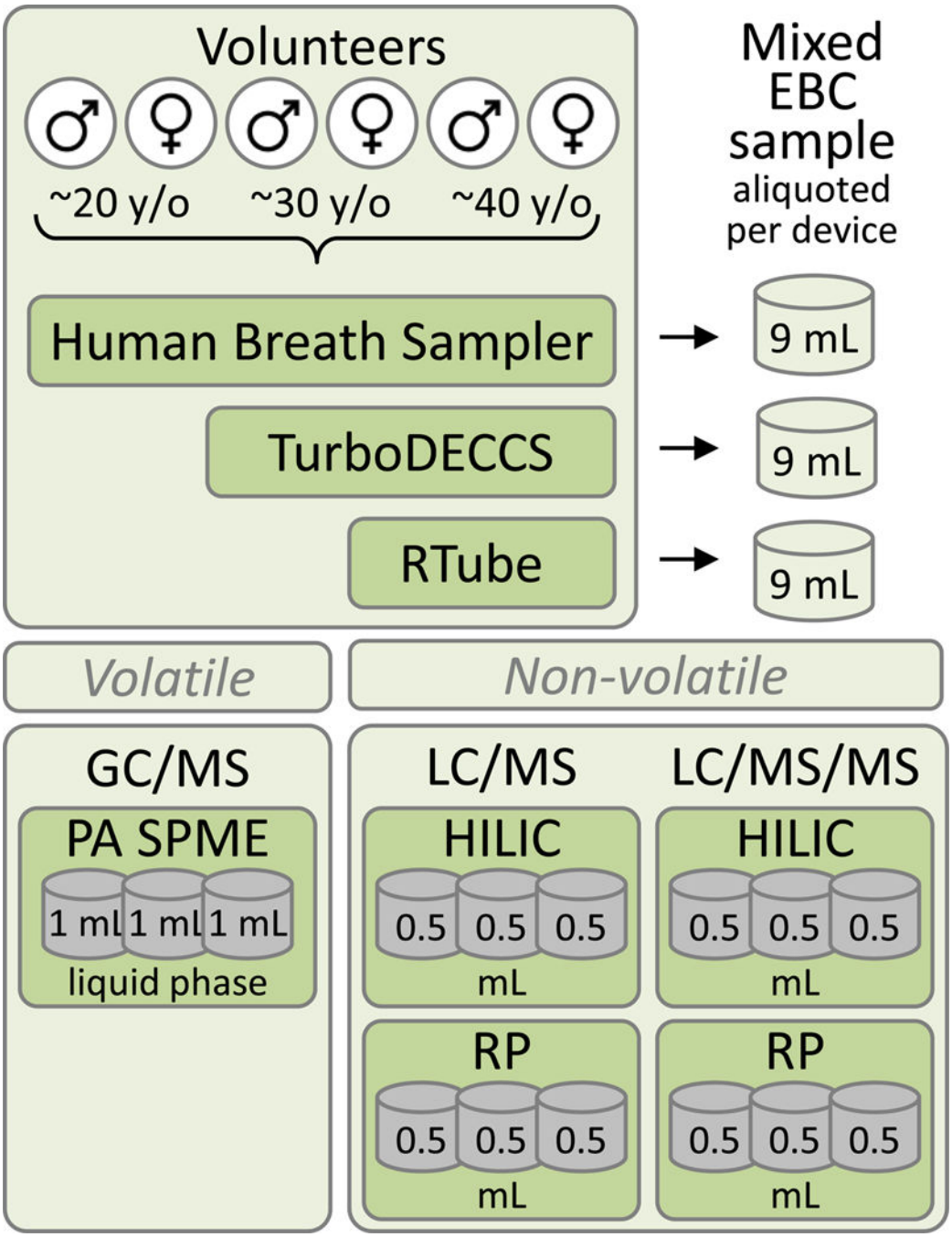

Figure 3.

EBC sampling from six healthy volunteers: 3 males, 3 females; three age groups: 20, 30, 40 years old. An averaged/mixed 9 mL sample (1.5 mL from each person) was aliquoted from individual samples collected with each device. The metabolomic content of the EBC sample was analyzed with GC/MS and LC/MS methods.

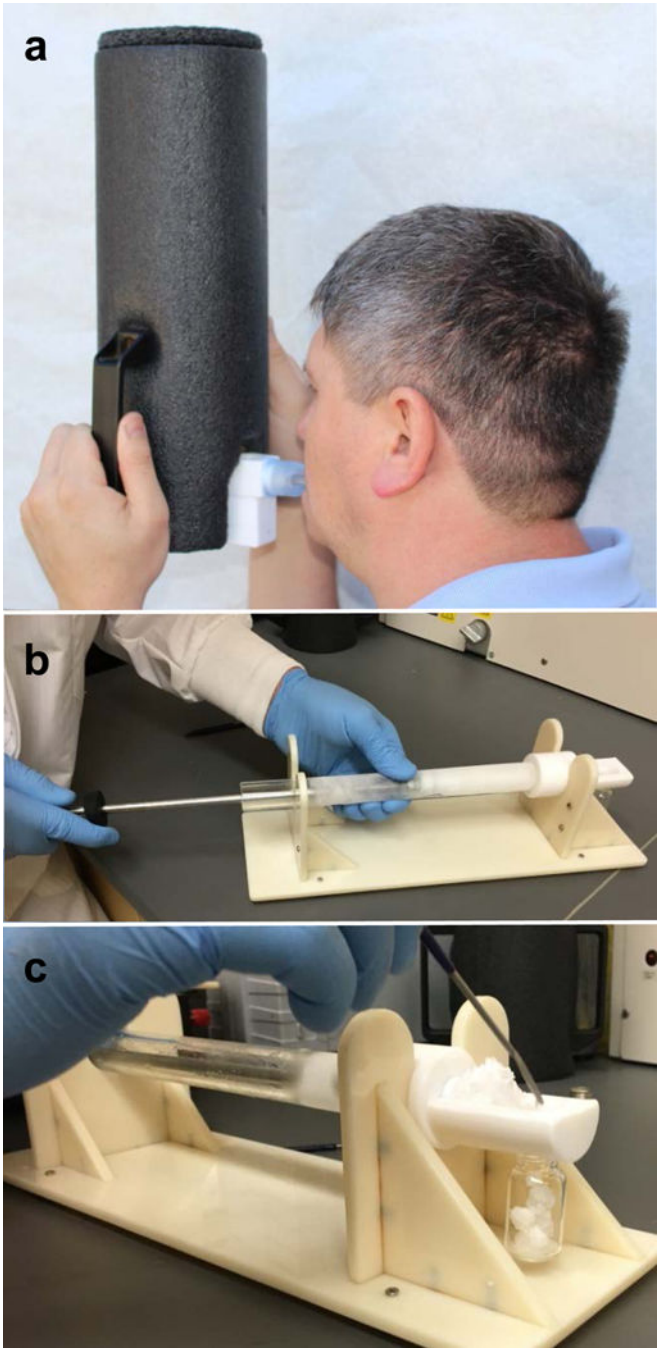

Figure 4.
HBS in use. **(a)** volunteer is breathing into the sampler. **(b, c)** Quick and efficient sample transfer for storage or analysis. Frozen EBC sample is cleared from the condenser tube with a PTFE plunger and loaded into a glass vial with spatula. The EBC sample is kept ice-frozen at all steps.

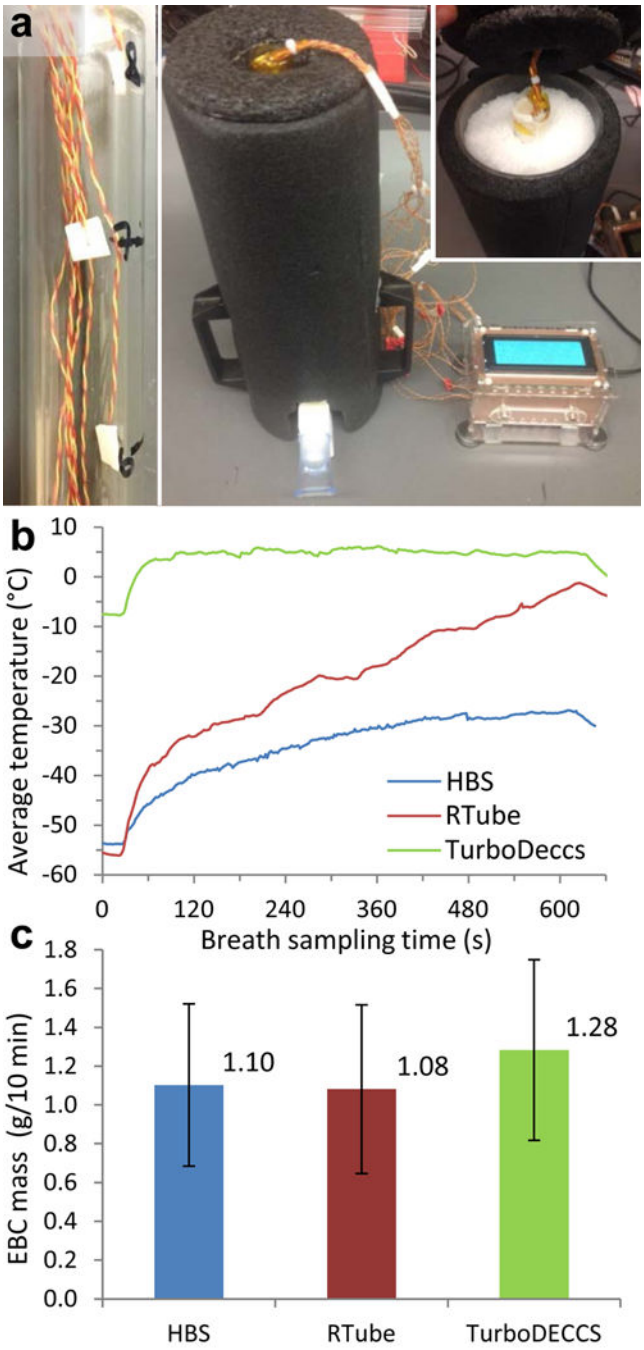

Figure 5.

(a) Eight thermocouples were attached on the condenser surface of each device. Here, HBS device is shown charged with dry ice for temperature recording with Arduino data logger. **(b)** Average condenser surface temperature (3 volunteers). Temperature stabilization period before sampling was 1 min for RTube, 5 min for HBS, and 40 min for TurboDECCS. The graphs are time-synchronized at the moment that breathing began (plotted at 30 s). **(c)** Average net mass of EBC sample retrieved for storage/analysis after sampling 10 minutes (4 volunteers).

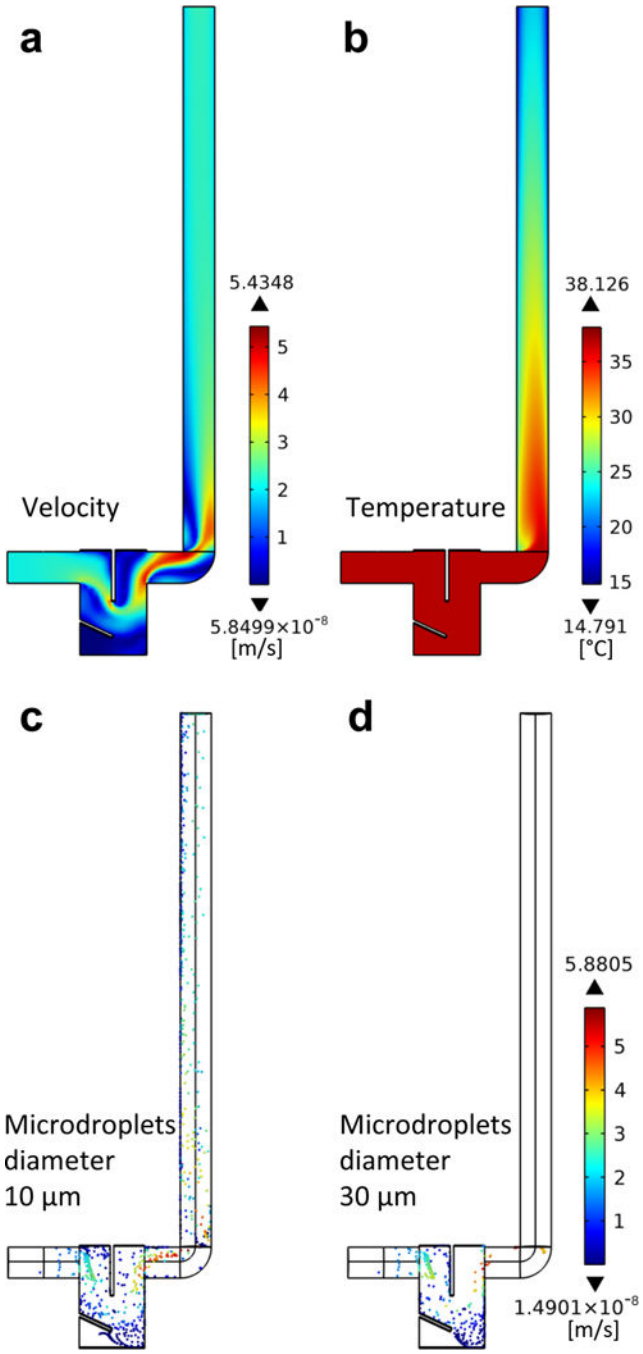

Figure 6.

Numerical solution results. Fluid flow simulation in COMSOL®. **(a)** Velocity profile. **(b)** Heat exchange and temperature of the breath flow. **(c, d)** Trajectories of particles with mass-diameter below (c) and above (d) the set mass threshold for aerodynamic filtering of saliva droplets from the breath flow. Droplets with smaller mass (probably originating in the lungs) pass into the condenser chamber but droplets with greater mass (saliva droplets originating the mouth cavity) are eliminated in the saliva trap.

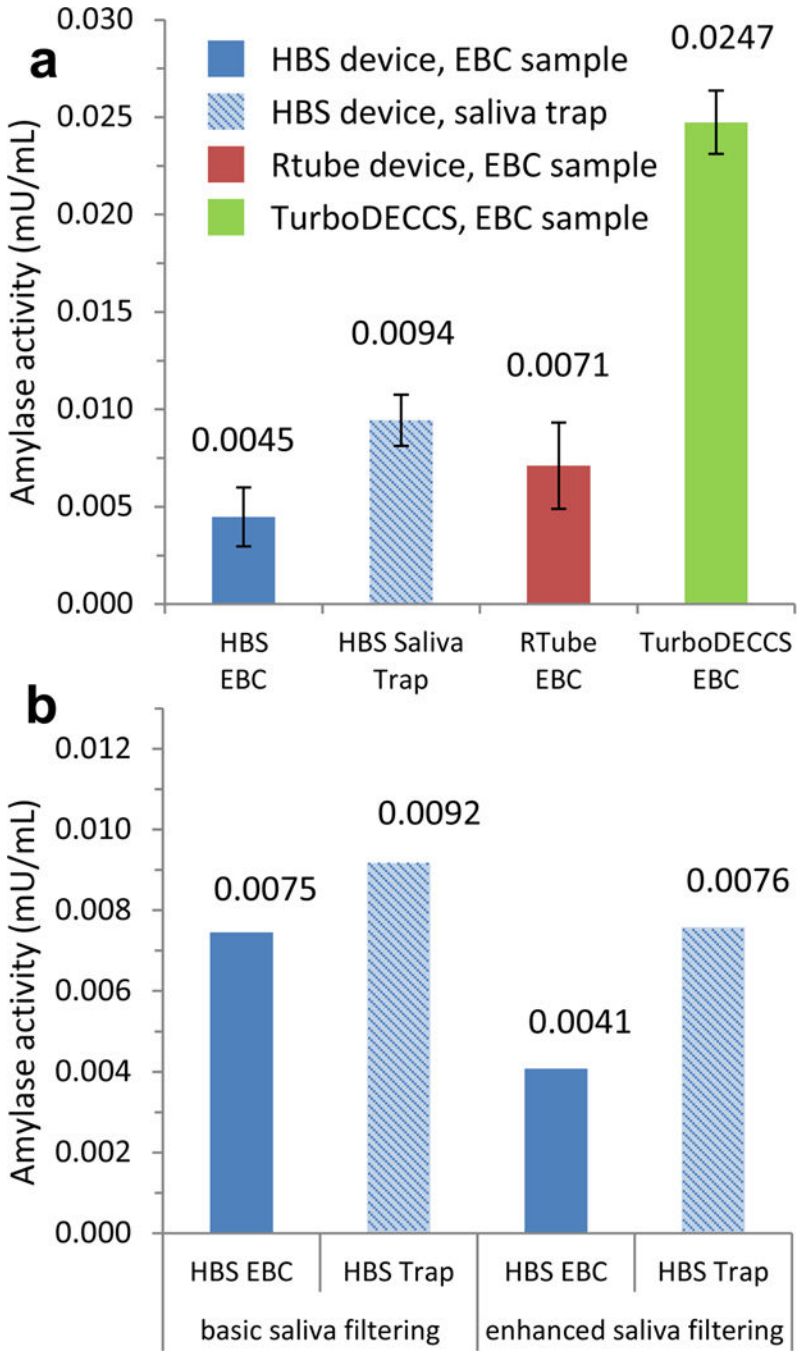

Figure 7.

Comparison of the saliva contamination in collected EBC sample based on amylase activity assay measurements. **(a)** Comparison of three EBC collection devices and content of HBS saliva trap. Averaged from three volunteers per each device. **(b)** Effect of the enhanced (with notch) versus basic (no notch) saliva filtering modes in HBS device. Single measurement.

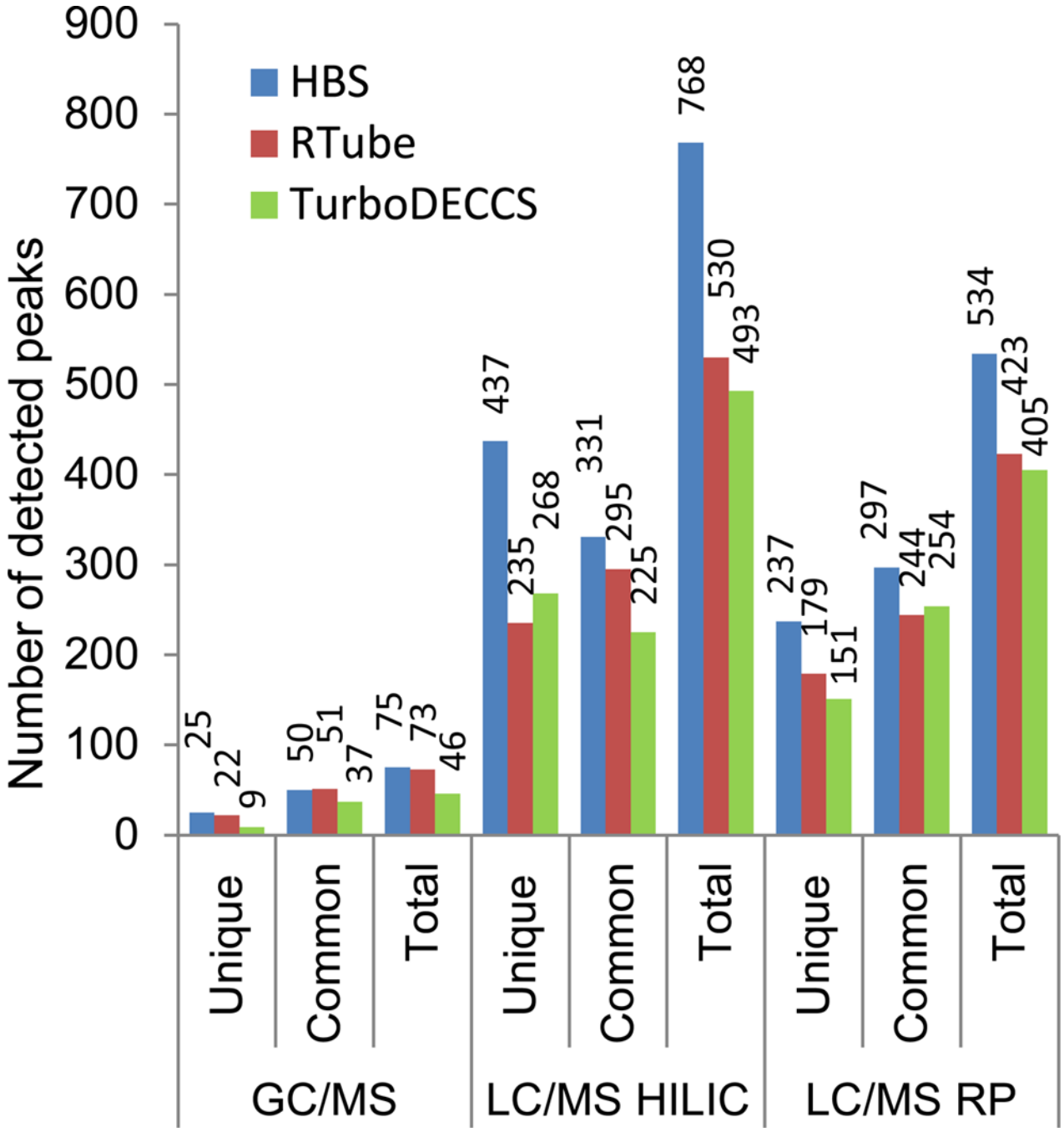

Figure 8.

Comparison of the number of metabolites detected with different methods: GC/MS analysis, LC/MS HILIC analysis, LC/MS RP analysis. The number of peaks per each device is confirmed from three replicates.

Table 1

Number of total and common peaks detected in EBC samples collected with three devices.

Peaks	Collection device	GC/MS	LC/MS HILIC	LC/MS RP
Unique peaks for device	HBS	25	437	237
	RTube	22	235	179
	TurboDECCS	9	268	151
Common for 2 devices	HBS/RTube	16	151	75
	HBS/TurboDECCS	2	81	85
	RTube/TurboDECCS	3	45	32
Common for 3 devices		32	99	137
Total	HBS	75	768	534
	RTube	73	530	423
	TurboDECCS	46	493	405