A sampler prototype for the simultaneous collection of exhaled air and breath condensate

T. Lomonaco, P. Salvo, *Member, IEEE,* S. Ghimenti, D. Biagini, S. Antoni, F. G. Bellagambi, F. Di Francesco and R. Fuoco

***Abstract*— Exhaled air and breath condensate contain a large number of health biomarkers, such as volatile and semi-volatile organic compounds, proteins and lipids. Nowadays, the collection of breath samples is carried out by commercial or lab-made sampling systems that collect only one type of sample (e.g. gaseous or condensate phase), thus limiting the diagnostic capability of breath tests. This work presents a portable prototype optimized for the simultaneous collection of gaseous exhaled breath and exhaled breath condensate within five minutes. The system is fully portable, requires no power supply and has a total weight of about 1 Kg. An illustrative determination of ethanol, isoprene, acetone, isopropyl alcohol, 1-propanol, 2-butanone, 2-pentanone, toluene and xylenes in breath, and cortisol and 8-iso-prostaglandin $F_{2\alpha}$ in breath condensate is discussed.**

I. Introduction

In the last decades, modern medicine has developed a significant interest for the unobtrusively collectable human body specimens [1-6]. Several studies highlighted the potential of breath analysis as an innovative diagnostic tool since the chemical composition of breath is related to the occurrence of diseases [7]. The main reason of this interest lies in the lower invasiveness and risks of breath collection procedures as well as in the possibility of a real time monitoring of physiological processes occurring in human body. In addition to nitrogen, oxygen and water vapor, breath contains a large number of exogenous (e.g. isopropyl alcohol [8, 9]) and endogenous (e.g. acetone and isoprene) volatile organic compounds (VOCs) besides a variable quantity of micro-droplets exhaled during respiration [10]. These droplets are produced in the alveolar membrane and contain less volatile compounds (e.g. isoprostanes and cytokines), which are dissolved in the airway lining fluid and can provide valuable information about the patient's health status [11-13]. These analytes can be collected by condensing the micro-droplets using a suitable cooling collection device [14]. A growing number of evidences suggest the diagnostic utility of breath biomarkers, often complementary or even alternative to those of blood and urine. For example, acetone is potentially useful for monitoring patients suffering from diabetes [15] and heart failure [16, 17], hydrocarbons for monitoring abnormal lipid peroxidation [18], isoprostanes are key biomarkers for investigating oxidative stress [12], and hydrogen peroxide for monitoring airway inflammation [19].

P. Salvo is with the Institute of Clinical Physiology, CNR, Via Moruzzi 1, 56127, Pisa, Italy (Corresponding author, Tel: +39 050 3152703, e-mail: pietro.salvo@gmail.com).

T. Lomonaco, S. Ghimenti, D. Biagini, S. Antoni, F. G. Bellagambi, F. Di Francesco and R. Fuoco are with the Department of Chemistry and Industrial Chemistry, University of Pisa, Italy (email: tommaso.lomonaco@unipi.it).

Nowadays, several approaches are available for collecting and analyzing exhaled breath (EB), depending on the markers of interest. VOCs are determined by sampling the gaseous phase [8, 9, 20] whereas non-volatile compounds are measured in exhaled breath condensate (EBC) [14]. However, common samplers only allow either EB or EBC to be collected, which excludes the possibility to obtain a comprehensive overview of breath biomarkers.

This study proposes a portable breath sampler separately collecting both EB and EBC. The prototype was preliminary tested by collecting samples from healthy volunteers, and ethanol, isoprene, acetone, isopropyl alcohol, 1-propanol, 2-butanone, 2-pentanone, toluene and xylenes, and cortisol and 8-iso-prostaglandin $F_{2\alpha}$ were determined in gaseous and condensate phase, respectively.

II. Materials And Methods

A. Sampler prototype for the simultaneous collection of exhaled breath and exhaled breath condensate

A schematic of the sampler prototype is shown in Fig. 1. It included a mouthpiece (Spectra 2000, Italy), fast mainstream CO_2 and flow sensors (Capnostat 5, Respironics, USA) with a response time lower than 60 ms, a non-rebreathing two-way three-port valve (Hans Rudolph Inc, USA), a series of 3 polyethylene T-piece connectors (Intersurgical, Italy) equipped with a luer-lock port, a sterile In-Stopper (Braun, Italy), a polypropylene conical tube (Eppendorf, Italy) with a capacity of 50 mL and a non-return valve. Capnostat 5 was equipped with RS-232 interface to communicate with a Mercury module (Respironics, Philips), which measured the airflow and pressure by a pneumotachometer. Exhaled airflow, pressure, volume and pCO_2 values were transmitted from the Mercury module to a computer in real time and were saved in a log file [21]. The conical tube cap was modified to allow the connection between the T-piece connectors and the non-return valve. A cylindrical 3C type Dewar flask (KGW Isotherm, Italy) was filled with a mixture of dry ice powder and sodium chloride (1:3 weight ratio) to let the breath condenser reach a temperature of -10 °C. The Dewar was closed with a lab-made Teflon cap, in which a Pt100 thermocouple (Tersil, Italy) was inserted to monitor temperature during collection. All connections were sealed with fluoroelastomer rubber O-rings to avoid the contamination of the breath sample from the ambient contaminants [8]. All the parts in contact with breath were made of chemically inert materials (e.g. polypropylene and polyethylene) in order to minimize the adsorption of biomarkers on collection tubes, and were suitably sanitized using an autoclave (121 °C per 15 min) for reuse. The mouthpiece and conical tube were disposable.

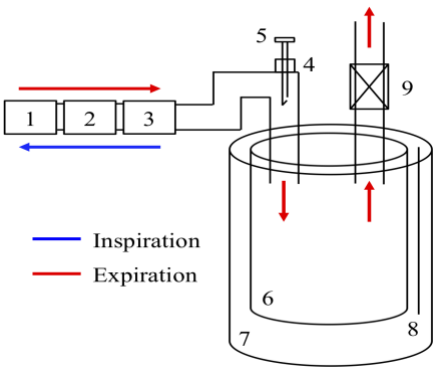

Figure 1. Schematic diagram of the sampler prototype. 1) mouthpiece, 2) capnostat 5 CO2/flow sensors, 3) non-rebreathing two-way three-port valve, 4) sterile In-Stopper, 5) needle trap device packed with 3 cm of Tenax GR (60/80 mesh), 6) polypropylene conical tube, 7) Dewar flask, 8) Pt100 thermocouple and 9) non-return valve

The system is fully portable, requires no power supply, has a size (height × width × length) of 30 × 10 × 20 cm and has a total weight of about 1 Kg. The estimated price is lower than 50 €. The first version of the sampler prototype is shown in Fig. 2.

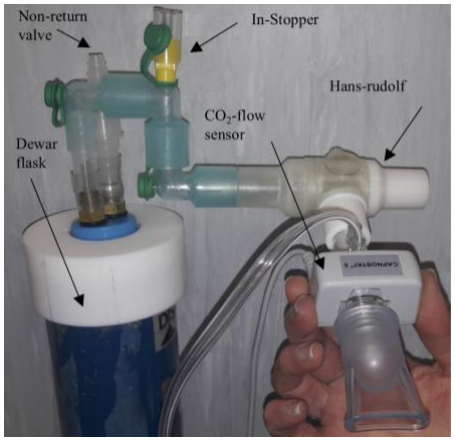

Figure 2. The breath sampler prototype.

B. *Collection protocol*

All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki and a written informed consent was obtained from all volunteers prior to enrollment. Samples were collected from three nominally healthy subjects in resting conditions. Before collection, the subject breathed normally (12-15 breaths per minute) through a sterile mouthpiece for 1 min to evaluate the CO_2 partial pressure (pCO_2) threshold (i.e. 80% of the end-tidal pCO_2). After this period, the sampler prototype was fully assembled, charged with cooling bath mixture and allowed to stabilize the temperature for 5 min. Then, the subject started to breathe through the sampler prototype and twenty-five milliliters of breath sample were transferred at 15 mL/min in a 6 cm long needle trap device (NTD), packed with 3 cm of Tenax GR (60/80 mesh), by an automatic sampling device (PAS Technology, Germany). This device opened/closed an electronic valve (delay <50 ms) based on real-time pCO_2 values measured during each respiratory cycle (the valve opened after exceeding the pCO_2 threshold value). After completing sample collection, both ends of NTD were immediately sealed with Teflon caps and stored at room conditions (25 ± 2 °C and RH 50 ± 5%) until analysis. In the case of EBC, a reliable sampler should guarantee: i) a reduced contamination from saliva, ii) a limited back pressure and iii) a stable condenser temperature.

A first set of experiments was carried out to evaluate a sampler geometry capable to remove saliva droplets from the breath flow without reducing the amount of smaller lungs particles. It is well know that an effective removal of these droplets can be easily obtained by including one or more bends in the system [22]. In this way, droplets with a diameter higher than 20 µm, which cannot follow the air flow around an obstacle, are removed, whereas the smaller particles bypass the bends and reach the condenser chamber [22]. The mass of each polypropylene conical tube was measured before and after sampling procedure to determine the amount of EBC collected. The sample was recovered by centrifuging the conical tube for 1 min at 5000 rpm and then stored in polypropylene tubes until analysis at -80 °C.

C. *Sample analysis*

Volatile organic compounds in breath gaseous phase were analyzed using a slightly modified analytical procedure described elsewhere [23].

Cortisol and 8-iso-prostaglandin $F_{2\alpha}$ in breath condensate were determined in an aliquot (500 µL) of EBC sample extracted with 2 mL of ethyl acetate. The resulting mixture was vortex-mixed for 30 s and then centrifuged at 5000 rpm for 5 min at room temperature. The upper organic phase was transferred to a screw top V-Vial and evaporated to dryness under a gentle stream of nitrogen at 25 °C. The residue was dissolved in 30 µL of H_2O + 0.1 % formic acid and then analyzed by an Agilent 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer equipped with a Jet Stream electrospray (ESI) ionization source (Agilent Technologies, Santa Clara, USA). Cortisol and 8-iso-prostaglandin $F_{2\alpha}$ were separated using a Polaris C18-A (100 × 4.6 mm, 3 µm) column at 25 °C and combination of A (H_2O + 0.1 % formic acid) and B (acetonitrile + 0.1 % formic acid). The optimized elution gradient resulted the following: 30% of B held for 0.5 min and then increased to 50% in 5.5 min, increased to 80% in 0.5 min and held for 3.5 min, followed by column equilibration time in 3 min. The Agilent 1290 high performance well-plate auto-sampler was set at a temperature of 4 °C and 10 µL were injected in the system. The Agilent 6495 Triple Quadrupole operated in multiple reaction monitoring (MRM) with unit mass resolution. Nitrogen was used as source (purity 99.5%) and collision (purity 99.999%) gas. For all analytes, the ESI operation conditions were: drying gas temperature 240 °C, drying gas flow 18 L/min, nebulizer gas pressure 35 psi, sheath gas temperature 360 °C, sheath gas flow 12 L/min, capillary voltage 3000 V and nozzle 0 V. The fragmentor voltage was fixed at 380 V and high and low pressure funnel voltages were set at 120 and 160 V for all mass transitions, respectively.

The contamination of breath condensate with saliva was evaluated by measuring the activity of the α-amylase enzyme as described elsewhere [24].

III. Results And Discussion

Table I reports the α-amylase enzymatic activity measured in saliva and EBC samples collected from three volunteers, and in a water blank solution for a better evaluation of the contamination level.

TABLE I. ALPHA-AMYLASE ENZYMATIC ACTIVITY IN SALIVA, EBC AND WATER.

α-amylase enzyme activity (U/mL)[a]		
Saliva	***EBC***	***Water***
(580 ± 15) × 10_3	27 ± 2	25 ± 4

[a]Values are reported as mean ± standard deviation, n = 3.

The activity of α-amylase enzyme in EBC and water were lower than 0.01% of that of saliva, and their difference was not statistically significant (p >0.05). These data highlighted that the use of three T-piece connectors connected in series (Fig. 1) excluded any EBC sample contamination with saliva. Compared to other EBC sampler devices (e.g. ECoScreen and TURBO-DECCS), our prototype obtained the same results without including a saliva-trap, thus minimizing the cost of the system.

The use of the three T-piece connectors, commonly used in breathing systems and other respiratory management products, connected to a conical tube and a non-return valve produced a limited pressure drop that did not exceeded the back pressure of 100 Pa, which usually characterizes the common human respiratory devices [25]. Therefore, the prototype did not modified the normal inspiration-expiration maneuvers, limiting the possible variations of the breath composition due to the changes of breathing patterns [26]. In fact, during a 5-min self-paced breathing experiment (10 breaths per minute), real-time end-tidal pCO_2 fluctuated randomly within a 10%, highlighting the absence of hyperventilation effect. Regarding the condenser temperature, a slight increase (from -10 °C up to -7 °C) of the temperature was observed during a 5 min EBC collection. Longer times (i.e. 10, 15, and 20 minutes) entailed a constantly increase overtime of the temperature that reached almost zero degrees after 20 min (Fig. 3).

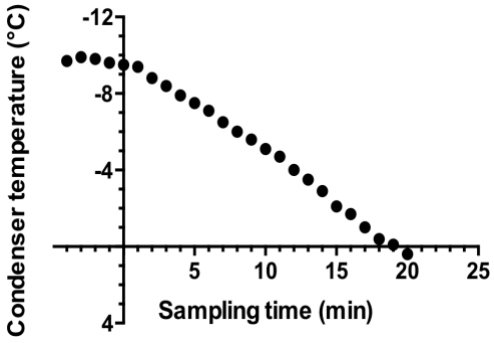

Figure 3. Condenser temperature trend over time during breath sampling at a self-paced breathing pattern at 10 breaths per minute. Before sampling (negative time in the figure), temperature stabilization period was 5 minutes.

A. Effect of temperature and collection time on the condensation efficiency

The condensation efficiency of the device depends on the design, size and temperature of the condenser chamber [22]. In addition, the amount of EBC is proportional to the lung ventilation, i.e. the volume of air exchanged from the lungs in one minute ($\dot{V}$, L/min), which is calculated by multiplying the tidal volume (V_T, L) for the respiratory rate (*RR*, breaths per minute). Fig. 4 shows the effect of sampling time (2, 5, 10, 15 and 20 min) and condenser temperature (-0.5, -4.5 and -9.5 °C) on the amount of EBC collected from three volunteers. The first set of experiments was carried out at -9.5 °C, whereas the second set using a sampling time of 10 min.

According to Lema *et al.* [27], the relationship between the volume of inhaled air (V_i, L) and the volume of collected EBC (V, mL) for each subject can be estimated using the follow equation:

$$V = 0.013 \times V_i + 0.255$$

During a self-breathing experiment performed at 10 breaths per minute, the mean volume (relative standard deviation) of inhaled air was 9 L (20%), 25 L (15%), 57 L (15%), 84 L (10%) and 100 L (10%) L at 2, 5, 10, 15 and 20 min, respectively. Thus, the theoretical EBC volume resulted: 350 mL (2 min), 570 mL (5 min), 990 mL (10 min), 1350 mL (15 min) and 1550 mL (20 min). The experimental and theoretical values were in good agreement, except for the EBC collected at 15 and 20 min that showed a statistically significant (p ≤0.05) variation of -30 and -40%, respectively. The lower amount of EBC collected at these times may be due to the warming up to zero degrees of the cooling chamber, as discussed before (Fig. 3).

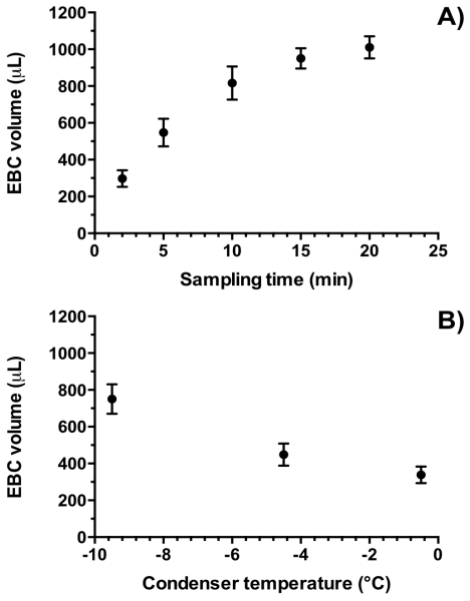

Figure 4. Effect of sampling time (A) and condenser temperature (B) on the amount of EBC collected. Experiments were performed at a self-paced breathing pattern of 10 breaths per minute. Error bars correspond to the standard deviation of 3 replicates.

B. Metabolic profile of exhaled breath collected from one nominally healthy volunteer

The sampler prototype was preliminary tested by collecting breath from one healthy volunteer for 5 min. EBC was obtained by cooling breath at -10 °C. Table II reports the respiratory data measured during a self-paced breathing pattern at 9 breaths per minute. The major volatile

components of exhaled breath were ethanol (20 ppbv), isoprene (370 ppbv), acetone (220 ppbv) and isopropyl alcohol (12 ppbv). Other analytes, such as 1-propanol, 2-butanone, 2-pentanone, toluene and xylenes ranged from 0.1 up to 1 ppbv. These VOCs are related to both metabolic pathways (e.g. acetone and isoprene [7]) and environmental contamination (e.g. isopropyl alcohols, ethanol, toluene and xylenes) [8].

TABLE II. RESPIRATORY DATA COLLECTED FROM A VOLUNTEER DURING A SELF-PACED BREATHING PATTERN AT 9 BREATHS PER MINUTE.

Respiratory rate (breaths per min)	End-tidal pCO_2 (mmHg)[a]	Tidal volume (mL)[a]	Airflow (L/min)[a]
9	45 ± 3	750 ± 40	10 ± 2

a. Values are reported as mean ± standard deviation, n = 46 breathes.

In the EBC sample, cortisol concentration was about 30 ppt, whereas the concentration of 8-iso-prostaglandin $F_{2\alpha}$ was below the limit of detection (10 ppt). Breath collection was also performed at three respiratory rates (10, 30 and 50 breaths per minute) with the assistance of an audible digital metronome (Real Metronome). An increase of respiratory rate provoked a clear decrease of tidal volume (i.e. 750 ± 40 mL at 10 breaths per minute, 540 ± 60 at 30 breaths per minute and 310 ± 70 at 50 breaths per minute), and at least one order of magnitude decrease of the isoprene concentration. This behavior was due to an increased ventilation of dead-space, which induced a reduction of compounds with a limited water-solubility as isoprene. The phenomenon was less marked (two fold decrease) for compounds with a high affinity for water (e.g. acetone), which can be released from the wet surfaces of the upper airways mucosa acting as a reservoir. In the same way, a slight decrease of cortisol (-20%) was also observed.

IV. CONCLUSION

In this work, we developed an innovative sampler prototype for the simultaneous collection of exhaled breath and exhaled breath condensate that allows a comprehensive evaluation of a panel of biomarkers potentially useful for screening and monitoring several diseases. The small dimension, low weight, and the possibility to use it without a power supply make the device easily portable and useful to sample patients who are unable to move.

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