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Analytical methodologies for broad metabolite coverage of exhaled breath condensate

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ABSTRACT

Breath analysis has been gaining popularity as a non-invasive technique that is amenable to a broad range of medical uses. One of the persistent problems hampering the wide application of the breath analysis method is measurement variability of metabolite abundances stemming from differences in both sampling and analysis methodologies used in various studies. Mass spectrometry has been a method of choice for comprehensive metabolomic analysis. For the first time in the present study, we juxtapose the most commonly employed mass spectrometry-based analysis methodologies and directly compare the resultant coverages of detected compounds in exhaled breath condensate in order to guide methodology choices for exhaled breath condensate analysis studies.

Four methods were explored to broaden the range of measured compounds across both the volatile and non-volatile domain. Liquid phase sampling with polyacrylate Solid-Phase MicroExtraction fiber, liquid phase extraction with a polydimethylsiloxane patch, and headspace sampling using Carboxen/Polydimethylsiloxane Solid-Phase MicroExtraction (SPME) followed by gas chromatography mass spectrometry were tested for the analysis of volatile fraction. Hydrophilic interaction liquid chromatography and reversed-phase chromatography high performance liquid chromatography mass spectrometry were used for analysis of non-volatile fraction. We found that liquid phase breath condensate extraction was notably superior compared to headspace extraction and differences in employed sorbents manifested altered metabolite coverages. The most pronounced effect was substantially enhanced metabolite capture for larger, higher-boiling compounds using polyacrylate SPME liquid phase sampling. The analysis of the non-volatile fraction of breath condensate by hydrophilic and reverse phase high performance liquid chromatography mass spectrometry indicated orthogonal metabolite coverage by these chromatography modes.

We found that the metabolite coverage could be enhanced significantly with the use of organic solvent as a device rinse after breath sampling to collect the non-aqueous fraction as opposed to neat breath condensate sample. Here, we show the detected ranges of compounds in each case and provide a practical guide for methodology selection for optimal detection of specific compounds.

1. Introduction

Breath analysis has been garnering attention as a diagnostic methodology with the potential for broad-scale application [1]. Inherent non-invasive nature of breath sampling and ease of collection make exhaled breath a very attractive matrix [2–5]. In some cases, breath metabolites abundances can be used as a proxy for concentration of certain compounds in blood. The alveoli in the lungs contain a large number of capillary blood vessels in close proximity to the lung surface to facilitate oxygen uptake and carbon dioxide release. This creates an optimal condition for the release of various compounds including volatile organic compounds (VOCs) dissolved from the blood into the exhaled breath. Many of these chemicals may be biomarkers indicative of a specific disease. Indeed, several disorders such as influenza [6,7], diabetes [8–10], gastrointestinal diseases [11], and pneumonia [12] were reported to be potentially amenable to diagnosis or monitoring

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based on breath biomarkers. In addition, there was a major effort in identifying breath biomarkers of cancer [3,13–17]. However, further advances are likely necessary to achieve the full practical utility of that breath analysis methodology.

One perceived disadvantage of using breath for diagnostic purposes is that it contains significantly fewer compounds at trace concentrations [18–20] compared to other matrices such as blood plasma. Another critical impediment that has affected widespread adoption is the lack of standardization of breath collection and analysis. It is challenging to ensure reproducibility and comparability of breath collection and storage methodologies such as to minimize any variations in abundances of compounds of interest. In this regard, exhaled breath condensate (EBC) appears to be more advantageous as opposed to gaseous breath [21]. Although methodologies for gaseous sample storage exist, e.g. Tedlar® (polyvinyl fluoride) bags, it is challenging to ensure sample preservation [22,23]. Liquid EBC samples have an advantage that they can be stored for extended periods of time under cryogenic conditions without apparent loss of metabolite content [21,24].

For EBC sample analysis, mass spectrometry (MS) is the method of choice for comprehensive metabolic analysis, most often used in conjunction with gas and liquid chromatography (GC and LC, respectively). In addition to offering a tremendous volume of chemical information, MS offers incredible sensitivity down to attomolar levels [25], and selectivity appropriate for chemical species identification. Depending on circumstances, it is possible to elucidate a metabolomic profile of a sample for "untargeted" metabolomics approaches to seek new biomarkers that associate with a specific condition. It is also possible to specifically measure amounts of selected compounds for "targeted" metabolomics approaches where a biomarker is known *a priori*. Since MS is predominantly a laboratory-based methodology due to high cost and size of MS instrumentation, sample collection occurs prior to analysis at a separate location such as hospitals or other medical facilities in the majority of studies.

The steps of sampling, sample storage, and preparation are critical to ensure that a sample is compatible with the analysis mode of choice (GC/MS or LC/MS). The variations in sampling may introduce biases that obscure biomarker features of interest. For example, selecting an appropriate solid phase extraction (SPE) sorbent can allow for discrimination against abundant matrix compounds while enhancing sensitivity toward compounds of interest. Sorbent choices and elution conditions can induce tremendous effects on metabolite abundances in the breath sample [26]. In another example, derivatization of molecular species that can form hydrogen bonds (such as carbonic acids) is commonly used to promote volatility of these compounds to enhance their detection using GC/MS, but differences in reagent choice and reaction conditions may lead to large differences in resulting metabolite coverages.

EBC is a very low concentration aqueous solution of various organic and inorganic compounds that arise from both biogenic and extraneous sources [27]. Due to the trace nature of EBC biomarkers, we seek appropriate methodologies suitable for analysis of these types of samples. The volatile and semi-volatile fractions of EBC samples are most commonly analyzed using GC/MS in conjunction with Solid-Phase MicroExtraction (SPME) [28] or Stir Bar Sorptive Extraction (SBSE/Twister®) [29]. For the non-volatile fraction, hydrophilic interaction liquid chromatography (HILIC) and reversed-phase (RP) liquid chromatography (LC/MS) are used to analyze very polar and moderately polar/non-polar compounds, respectively [30].

The intent of the present study is to compare these commonly employed analytical methodologies directly for the first time, in order to compare and contrast the detected compounds distributions and outline a set of recommendations to guide the method selection for EBC analysis. In this study, we conducted GC/MS analysis of the volatile and semi-volatile fractions using SPME from liquid and headspace as well as extraction from liquid using polydimethylsiloxane (PDMS) sorbent as a low-cost alternative to SBSE/Twister® methodology. The non-volatile fraction of EBC was analyzed with HILIC and RP HPLC/MS chromatography modes. The residual non-aqueous fraction of EBC was collected from the condenser surface with an organic solvent and was analyzed with HILIC and RP HPLC/MS chromatography modes. To eliminate sample bias at the sampling step, an "averaged" EBC sample aliquoted from individual EBC samples collected from a group of six volunteers, spanning both genders and a range of ages, was used to represent a "typical" breath sample. The individual EBC samples were collected with a collection device that demonstrated metabolite capture [31]. A systematic metabolite survey is presented in this paper.

2. Materials and methods

2.1. EBC collection device

The engineering design and operational principle of the EBC collection device employed for the EBC collection was recently described elsewhere [31]. The device is portable, suitable for a wide-spread use similar to the RTube®, a common commercially available device for EBC collection: the exhaled breath is passed through a chilled tube where it is condensed and then physically removed. Unlike the RTube®, the employed device uses a glass condenser surface to enhance measurement of metabolites in exhaled breath condensate [32], a flow-controlled saliva filter to reduce sample dilution and contamination with saliva microdroplets originating in the mouth cavity [33–35], and an active thermal mass cooling mechanism to maintain low condenser temperature to avoid physical change of the sample and preserve greater concentrations of VOCs [36]. These design factors contribute to reduced variability in the metabolomic content caused by the collection device [31].

2.2. EBC sample collection

The device is comprised of a glass tube placed inside a plastic casing. The space between the glass tube and the outer casing is filled with dry ice pellets that serve as an active thermal mass to keep the condenser surface at low temperature (~ −30 °C). The ice-frozen exhalant is cleared from the condenser tube with the fitted plunger into a glass vial for storage. The physical phase of the sample is preserved in collection and sample transfer steps. The samples are stored in glass cap-sealed vials at −80 °C until further analysis. For a healthy subject of ~80 kg, the described device can typically collect 1.2 ± 0.3 g of ice-frozen EBC in 10 min [31].

A representative average EBC sample was aliquoted from individual samples collected from a group of six healthy adult volunteers representing three age groups of 20, 30, and 40 years old and two gender groups (3 males and 3 females). All participants were in good health, with no history of smoking. Volunteers restrained from consuming food for 2 h before sampling, and rinsed their mouths with drinking water before sampling. The clean collection device was assembled, charged with dry ice pellets, and allowed to sit for 5 min before sampling for the temperature to equilibrate within the device. Each participant sat in a relaxed, upright position during the collection. All participants were asked to breathe normally (tidal breathing) into the device for a 10 min period. After sampling, the EBC sample was transferred from the condenser tube into a clean borosilicate vial (Sigma-Aldrich, part #: SU860099 SUPELCO), cap sealed (Sigma-Aldrich, part #: SU860101 SUPELCO), and placed into a −80 °C freezer. This process was repeated until ~4 mL of EBC was collected from each participant. All parts of EBC collection 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 total of 24 EBC samples (~ 4 mL from each volunteer) were collected randomly during a 6 weeks period. Each volunteer performed 10 min sampling four times; the four EBC samples from the same