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Development of a method for metabolomic analysis of human exhaled breath condensate by gas chromatography–mass spectrometry in high resolution mode

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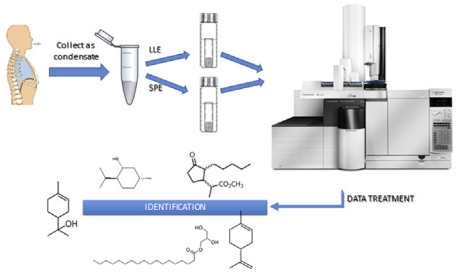
HIGHLIGHTS

- A study for sample preparation of breath for metabolomics analysis was developed.
- Liquid–liquid extraction with hexane was a suited approach in metabolomics studies.
- 51 metabolites were identified in breath using in source fragmentation information.

GRAPHICAL ABSTRACT



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ABSTRACT

Exhaled breath condensate (EBC) is a promising biofluid scarcely used in clinical analysis despite its non-invasive sampling. The main limitation in the analysis of EBC is the lack of standardized protocols to support validation studies. The aim of the present study was to develop an analytical method for analysis of human EBC by GC–TOF/MS in high resolution mode. Thus, sample preparation strategies as liquid–liquid extraction and solid-phase extraction were compared in terms of extraction coverage. Liquid–liquid extraction resulted to be the most suited sample preparation approach providing an average extraction efficiency of 77% for all compounds in a single extraction. Different normalization approaches were also compared to determine which strategy could be successfully used to obtain a normalized profile with the least variability among replicates of the same sample. Normalization to the total useful mass spectrometry signal (MSTUS) proved to be the most suited strategy for the analysis of EBC from healthy individuals (n = 50) reporting a within-day variability below 7% for the 51 identified compounds and a suited data distribution in terms of percentage of metabolites passing the Skewness and Kurtosis test for normality distribution. The composition of EBC was clearly dominated by the presence of fatty acids and derivatives such as methyl esters and amides, and volatile prenol lipids. Therefore, EBC offers the profile of both volatile and non-volatile components as compared to other similar biofluids such as exhaled breath vapor, which only provides the volatile profile. This human biofluid could be an

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alternative to others such as serum/plasma, urine or sputum to find potential markers with high value for subsequent development of screening models.

1. Introduction

The most common biofluids in metabolomics analysis applied to clinical and nutritional studies are blood (serum or plasma) and urine. Nowadays, alternative biofluids such as sweat, tears, saliva or exhaled breath, with a less complex composition, are gaining popularity as they can be easily obtained in a non-invasive manner [1,2]. Among these biofluids, exhaled breath is one of the most accessible samples as it can be obtained without adverse effects, even in children and patients with serious respiratory diseases [3,4]. There are two types of exhaled breath samples, depending on the sampling protocol: exhaled breath vapor (EBV) and exhaled breath condensate (EBC). The former is obtained by collecting the breathing in a suited bag or a similar device. The main fraction of EBV (>99%) comprises nitrogen, oxygen, CO_2, water vapor, and other inert gases, and the remaining fraction is formed by a mixture of volatile organic compounds (VOCs) present at concentration ranges from few μg mL^{-1} to pg mL^{-1} [5,6]. The levels of VOCs such as aldehydes [7,8] and alkane derivatives as 4-methyldecane [9,10] in clinical samples such as urine or EBV have been associated to cancers, lung cancer among them [11]. This type of cancer has been linked to predictive models based on canine detection, ascribed to the presence of VOCs [12].

On the other hand, EBC is collected by cooling the exhaled breath that condensates to give a liquid solution composed by soluble exhaled gases and metabolites of the extracellular lining fluid. Despite the main component of EBC is water, hundreds of different components at trace concentrations can be found in this sample [13–16], ranging from small inorganic ions through large organic molecules to peptides, proteins, surfactants, macromolecules and VOCs [17]. Therefore, EBC is characterized by a more varied composition than EBV.

Concerning the analysis of EBC and EBV, the main difference lies in the sample preparation strategy selected as a function of the physical state of each sample. Solid-phase microextraction (SPME) seems to be a suited technique for treatment of EBV after collection in Tedlar bags and prior to GC–MS analysis of VOCs [18–20]. One other option is the use of sorbents such as activated carbon to retain sample components, which are further desorbed in the injection unit of GC–MS equipment [21]. On the other hand, the liquid nature of EBC increases the number of alternatives to analyze this sample. Thus, liquid–liquid extraction (LLE) by taking advantage of the polarity of the extractant could be a fast alternative to separate compounds from the aqueous matrix before GC–MS characterization. One other competing alternative could be solid-phase extraction (SPE) that could be used to concentrate the EBC components and remove matrix interferents.

Several target strategies have been used in metabolomics to determine compounds of clinical relevance in EBC samples [17] such as those involved in the NO-oxidation pathway [22], lipid peroxidation products such as isoprostanes and leukotrienes, hydrogen peroxide and cytokines. Concerning untargeted analysis, different platforms have been proposed to seek for markers of different diseases, especially lung diseases. A recent study by Laurentiis et al. has proposed NMR to discriminate between patients with smoking related diseases and healthy individuals by some EBC components [23]. The number of patients selected for this study was particularly low and external validation was carried out. Despite the wide variety of methods in the literature to identify or determine metabolites in EBC, a generic characterization of the EBC sample has not so far been carried out, possibly owing to the methodological limitations [17], as the absence of normalization protocols. In fact, the variability observed in GC–MS results obtained from targeted and non-targeted metabolomics analysis of EBC was highly dependent on the strategy for normalization. With these premises, the aim of the present study was to develop a protocol for EBC analysis, with special emphasis on sample preparation. For this purpose, LLE and SPE were evaluated for sample preparation by using different extractants and eluting solutions, respectively. The resulting approach was used for tentative profiling of EBC samples from healthy individuals. A normalization study has also been carried out by testing four strategies: the total useful MS signal (MSTUS), the internal standard (IS) response and the combination of the IS response with the collected EBC volume or the expired EBC volume.

2. Experimental

2.1. Reagents

Cyclohexane, hexane, dichloromethane, acetonitrile and ethyl acetate *Trace*SELECT® grade from Sigma–Aldrich (St. Louis, USA) were the organic solvents for sample preparation. Deionized water, 18 MΩ cm, was from a Millipore Milli-Q water purification system (Bedford, USA). A standard mixture containing ten linear alkanes from C10 to C40 designed for performance tests in GC from Sigma–Aldrich was used to establish the retention index (RI) calibration model. Triphenylphospate, also from Sigma–Aldrich, was used as internal standard (IS).

2.2. Instruments and apparatus

An ECOScreen2 device (FILT Thorax-und Lungen Diagnostik GmbH, Berlin, Germany) was the EBC collector. A centrifugal SPE procedure was carried out by a Mixtasel centrifuge (Selecta, Barcelona, Spain), and homogenization of the extracts by an MS2 Minishaker Vortex (IKA, Germany).

An Agilent 7890A Series GC system coupled to an Agilent 7200 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with an electron impact (EI) ionization source (Santa Clara, CA, USA) was used. The analytical sample was monitored in high resolution mode.

2.3. Cohort selected for the study

All experiments were carried out in accordance with ethical principles of human medical research (World Medical Association, Helsinki Declaration, 2004). The ethical review board of Reina Sofía University Hospital (Córdoba, Spain) approved and supervised the clinical study (Project "Development of methods for early cancer detection, December 29, 2011"). The EBC samples were obtained from 50 healthy volunteers early in the morning before breakfast. The individuals of the cohort, aged between 40 and 80, were recruited in the Respiratory Medicine Department. The samples