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Identification and Quantification of Metabolites in Exhaled Breath in a Sample Population in México

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Abstract. We have identified a set of common metabolites present in breath samples from 27 Mexican individuals, classified as healthy. This has been achieved by analyzing breath samples by gas chromatography (GC) coupled with mass spectrometer (MS). For each compound, we did a semiquantitative analysis of the metabolites by measuring the area under the curve of each corresponding peak in the chromatogram.

With this methodology we have been able to define a baseline, i.e. the values and identity, of compound present in the GC spectra of the 27 breath samples. This baseline consists of 87 metabolic compounds (metabolites). It is expected this compounds will help to define a "normal" set of values; the departure from which may be indicative of a specific pathology. In particular, in the future, it is expected to contrast the value of the "normal" metabolites with those presented by a study groups composed of people diagnosed with prediabetes and type II diabetes. The ultimate goal will be, in the future, to develop a non-invasive tool to diagnose prediabetes. The baseline defined by this study is the first step towards achieving this goal.

1. INTRODUCTION

The analysis of metabolites present in the exhaled breath of humans is currently a very active topic of research, especially in Europe, Asia and U.S. The aim of these studies is to produce a non-invasive diagnostic method for several diseases. The method of breath analysis, also known as exhaled breath proof, is based on the fact that, within the human body, there exists a continuous generation of volatile compounds. These compounds, also known as metabolites, are partially emitted through the exhaled breath. These volatile compounds contain information regarding the metabolic and physiological conditions of a particular subject under study. [1-2]

This methodology has proven to be an important procedure in the evaluation of the health and monitoring of the development of a disease by analyzing trace amounts of molecular gas phase samples known to be associated with particular pathologies. [1]

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The breath analysis method can provide, in addition, a complementary tool for conventional medical diagnosis of a disease. This is because, with breath analysis it is possible to analyze hundreds of metabolites related to human metabolism, simultaneously, in real time (proton transfer reaction mass spectrometer PTR-MS), for example. [3]

In Mexico, one of the leading causes of death is the Type II Diabetes (T2D). Detecting this disease at early stages is of crucial importance, since diabetes and its side effects can be controlled with proper diet and exercise at early stages. The main motivation for the present study is to start the development of standardized procedures for sample collection, analysis and quantification of volatile biomarkers that may define "normality". This definition can then, in future studies, be contrasted with samples from individuals with specific pathologies non-invasive tools that may help out in the early diagnose of the disease. [4-6].

As a part of this long-term program, one of the biggest challenges of breath analysis is to relate unequivocally, biomarkers detected and quantified, with the metabolic pathways that occur as a result of the health condition of the patient. [7-8].

In relation of our specific aim to devote the present studies to T2D and prediabetes, it is of great importance to have a good definition of the baseline or normal values of metabolites of healthy subjects. Further studies should contrast this normal values with those presented by diabetic and pre-diabetic patients. This baseline values should be quantitative and have assigned to them the error bars associated to the intrinsic variations among individuals considered as healthy. Therefore, the main barrier to successful implementation of a breath analysis is the standardization and the definition of baselines of normalcy in a population [7-10].

The present work is the first step towards this desirable goal of quantification and standardization for breath analysis studies in Mexico. This study involves a population group of 27 metabolically healthy subjects.

2. MATERIALS AND METHODS

2.1 Healthy Volunteers Characterization

The physiological characterization of the metabolically healthy population whom participated in this study was held in the General Hospital of Mexico. The population was selected among a database of 1000 individuals and the criteria for health involved not only absence of diabetes, but of several other diseases, in order to avoid potential complications in the definition of our baseline as introduced by other pathologies.

To define the "healthy" population we carried out a series of hematological tests. These studies included: the curve of glucose tolerance, nominal values of glycosylated hemoglobin and the nominal values of fasting glucose. In addition to the aforementioned values we also considered body mass index, triglyceride and cholesterol levels. Of the selected volunteers, 27 were chosen classified as healthy using the normal values reported by the American Diabetes Association. [11]

2.2 Sample Collection and Preparation

Breath samples were taken at the General Hospital of Mexico at the pulmonary physiology department, under controlled parameters of temperature, barometric pressure and relative humidity. Tested patients were submitted to eight hours of fasting and an extensive examination of family history. Human breath consists of a mixture of breath from the alveoli and from the so called "dead space", that is the regions of the respiratory system that are not part of the exchange between the blood stream and the inhaled air.

For breath analysis to be effective, it is desirable to maximize the amount of alveolar breath. The importance of taking alveolar sample lies in the fact that this sample is rich in metabolites present in the blood stream, and exchange in the alveoli due to Henry´s law, that relates the amount of gas dissolved in a liquid to the partial pressure. [12] For the present breath sample collection; a first sample of 300 ml, consisting of a mix of breath from dead space and alveolar breath, was collected using a Tedlar bag (SKC Inc., Eighty Four, PA, USA). This first

sample is discarded in order to get rid of the air from dead space. The rest of the exhalation consisting of alveolar breath was collected in a second bag, and further used for the chromatographic analysis of metabolites.

A Nafion tube coupled to Tedlar bag was used to remove the humidity from the sample breath.

Before collecting breath, all bags were sanitized to remove residual contaminants and marked to identify each sample. This was achieved by flushing the bags two times with dry air and nitrogen 5.0 gas (Praxair Technology Inc., MX). In each flushing, the bags were heated 20 min at 80 C° and emptied with a vacuum bomb. The cleanliness of the samples after this procedure was verified by carrying out gas chromatography of a sample of dry nitrogen from these bags, prior to their use for the sample collection process.

After the breath sampling procedure, the alveolar breath samples were pre-concentrated in sorbent cartridges (Carbotrap C, carbosive SIII and Carbopack C). To this purpose, a vacuum pump was connected to the sorbent cartridges and the contents of the Tedlar bag were adsorbed by the tube. To achieve this, a flow of 120 ml/min was used. After this procedure the cartridges, loaded with the samples, were transferred to the Center for Atmospheric Sciences, UNAM, MX. for analysis in GC / MS.

2.3 GC/MS Conditions

The GC/MS analysis was performed on a chromatograph Agilent Technologies brand mod. 7890A GC System. This gas chromatograph is coupled with mass spectrometry (MS) Agilent 5975C.

To introduce the sample contained in the cartridges, we used an automatic thermodesorber (ATD) built in house, with a desorption temperature of 320 °C by 6 min with a cryogenic trap at -20 °C.

A chromatography column of Dimethyl Polysiloxane 60 m × 0.25 mm × 1 um (phase 007-1 Quadrex Corp.) was programmed at an initial temperature of -20 °C for 2.3 min, then at 5 °C / min to reach 250 ° C, was used.

As carrier gas, helium of research grade and with a flow velocity of 1.0 ml min^{-1} was used.

The MS analyses were carried out in a full scan (scan range 35-200 amu) and at scan rate of 7.22 scan s^{-1}. An ionization energy of 70 eV was applied to the sample effusing from the column for mass analysis. This ionization region was held at temperature of 230 °C. The mass analysis was carried out with a quadrupole mass spectrometer held at 150 °C. These temperatures are maintained so as to avoid condensation of the sample in their respective walls of the ionization and the mass quadrupole region.

2.4 VOC´s Identification and Indirect Quantitative Analysis

The organic compounds present in the breath were identified and assigned by using four main libraries: the NIST library 2005 (Gatesburg, USA) that includes: nist_msms, nist_ri, and the software ChemStation that includes: mainlib and replib. The Match of each compound with the libraries was at least 95%.

In order to verify the accuracy of our assignments, some of the detected compounds were injected into the GC-MS. The retention time and the mass spectrum of the injected compounds were in agreement with the assignment.

In order to quantify, in a semi-qualitative way, we take the Area Under the Curve (AUC) of the peaks found for each compound in the chromatogram as a relative measure of its concentration. The AUC is used as a good enough estimate of the density of a given compound.

3. RESULTS AND DISCUSSION

From the analysis of the metabolites present in the chromatograms from breath samples we observed that, in all of the samples 50 compounds were present in all individuals. The most abundant compounds out of this set were: isobutene, ethanol, acetone, isopropyl alcohol, isoprene, 2 methyl 2 propanol, hexane, 2 ethoxy ethanol, toluene, 2,2 dimethyl octane, 3,3 dimethyl octane, 2, 2, 6 trimethyl octane, clopidol and decane. In addition to these 50 compounds, other compounds appear that not appear in all the analyzed population. Both groups were 87 compounds. All these compounds are shown in figure 1 with percentages of appearance in the sample population which range from 60% to 100%. Although several other compounds were observed, these only appear in a fraction less than 60% of the total sample population, making them not representative, and are discarded for the purpose of defining the common metabolites present in a healthy sample population

The average age of volunteers ranged as follows: for women from 24 to 56 years (average age of 33 years), for men from 24 to 57 years (average age 38 years). All of the subjects were fasting for 8 h before breath sampling, and were not allowed to brush their teeth, other than these restrictions, no special instructions and diet was followed by healthy volunteers.

The 87 compounds detected and their relative abundance, are presented in figure 1.

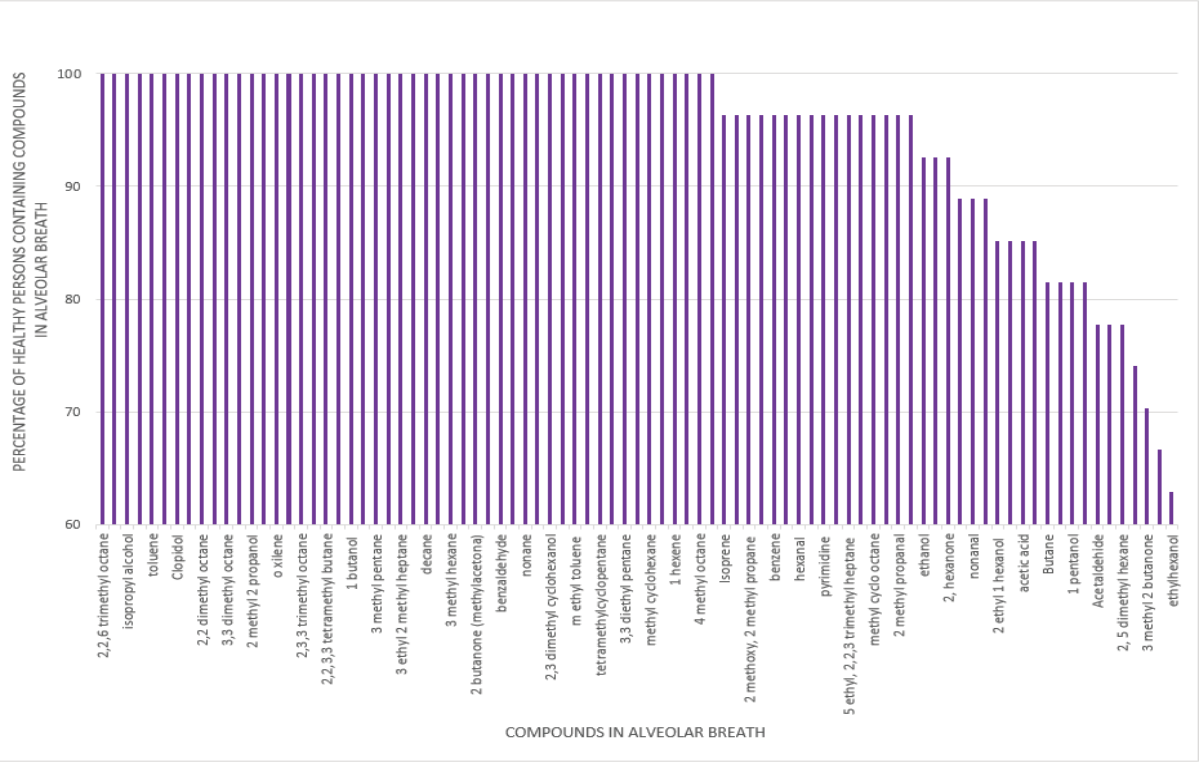

FIGURE 1. The distribution of appearance in the alveolar breath of healthy persons for 87 compounds

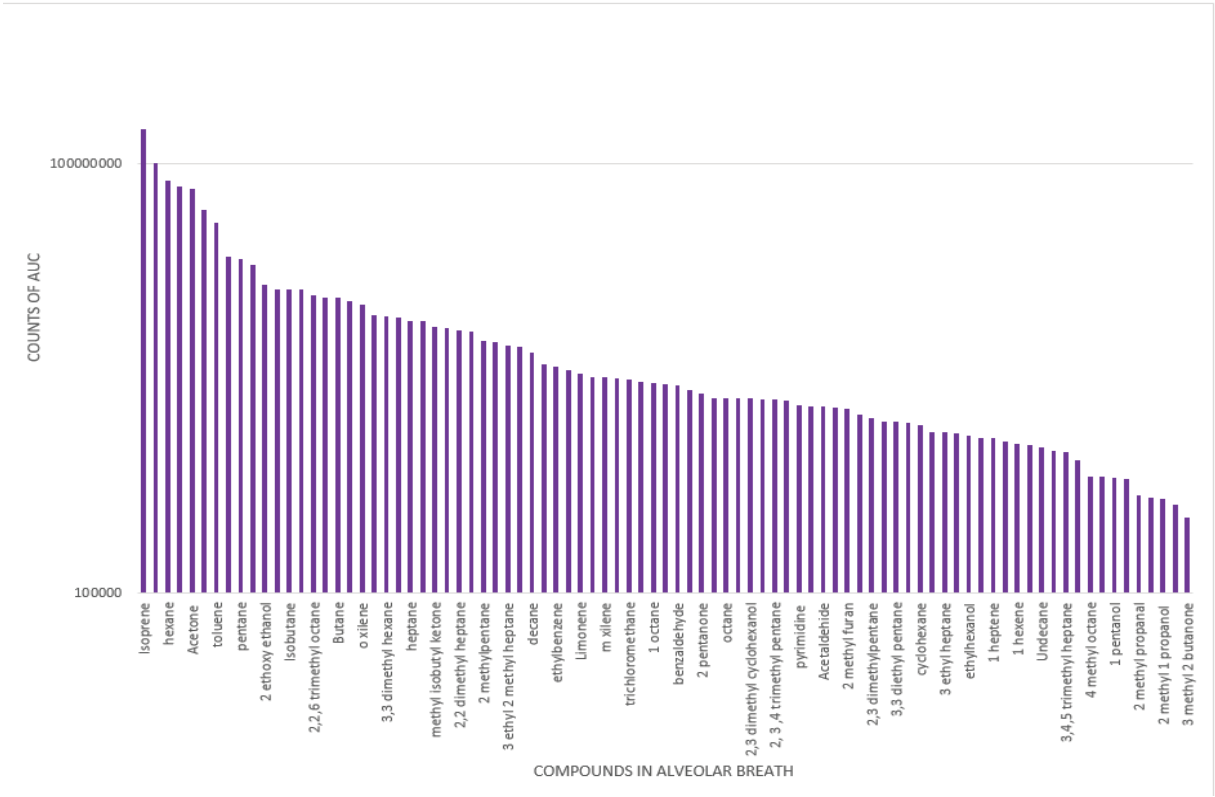

FIGURE 2. Common compounds detected in 60% of healthy subjects and their relative abundance

4. CONCLUSIONS

By measuring the AUC, we have been able to identify at least 87 biomarkers which are common in the samples of healthy individuals. 50 of these appear in 100% of the individuals tested and the other 37 compounds in 60% of the population. Identifying these common compounds and their relative concentrations is of great importance since it is possible, in principle to consider the definition of health from the values of these compounds, common to a healthy population of Mexicans. The deviation of the normal values of these compounds could potentially help out to develop diagnostic methods to identify a specific pathology.

The present work is in progress and the results obtained here are preliminary. In order to correctly define the baseline from common compounds in healthy individuals, the alveolar breath of healthy people should be quantified using standards for the volumetric density of the compounds, as well as the maximum variability of each compound, stemming from the intra and inter individual differences in metabolism. The value of the present study is that we have defined and developed a standardized sample acquisition and analysis protocol, and secondly we have identified the compounds which appear in all of the healthy individuals sampled. The future work planned is to provide further comparisons of healthy and non-healthy individuals and find out that the nominal values of the metabolites differ by several standard deviations, in a systematic fashion.

The results of this research, although semiquantitative in character, are encouraging. We plan in the near future to perform a calibration gas standards that connect the peak area of each compound absolute concentration, therefore approaching to our aims of developing a non-invasive technique with a more precise quantitative results.

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