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Molecular identification of bio-fluids in gas phase using infrared spectroscopy

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Bio-fluids are the source of a large number of metabolites. Identification and quantification of them can be an efficient step for understanding the internal chemistry of the body as well as for developing objective diagnostics of diseases. Several techniques have been developed so far; however, their metabolite identification and/or quantification are not reliable enough for acceptance by clinicians. As another promising step in this direction, we push infrared spectroscopy of bio-fluids in gas phase. Here we discuss features of breath and urine headspace realized with Fourier transform infrared spectroscopy. Molecular identification procedures based on component analysis of gas samples are proposed. In this paper, we show that aggregate data from different bio-fluids in gas phase can strengthen the diagnostics of the body state and disease.   © 2020 Optical Society of America

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1. INTRODUCTION

Each and every living organism undergoes thousands of biochemical reactions to maintain its life. The corresponding chemical compounds circulating in the body can be considered as messengers carrying information about its state [1]. Those compounds are called metabolites and in general are restricted to small organic molecules [2]. Metabolites become released from the body via urine, feces, sweat, saliva, exhaled air, etc. Hundreds of metabolites have been reported so far [3,4]. There is hope that the above-mentioned bio-fluids can be used for analysis of different body states including diseases [5]. Unfortunately, slow progress in this direction shows that still there is a lack of robust detection techniques capable of simultaneous identification of a high number of molecules and their accurate quantification [6,7]. We therefore push further the analysis of biological samples for molecular identification using vibrational spectroscopy. Vibrational spectroscopy is well established as a powerful and widely used tool for studying molecular structure and dynamics in the fields of chemistry and physics [8,9]. It uses molecular bond vibrations as a probe to identify the composition of the molecule and its 3D structure [10,11]. In recent years, development of ultrafast lasers has made it possible to observe the vibrational motion in real time [12]. This unique feature facilitates revealing chemical and biological processes even on a femtosecond (fs) timescale [13]. Therefore, it is natural to expect that vibrational spectroscopy can be a powerful tool for identification of metabolites in bio-fluids [14,15].

Vibrational spectroscopy uses infrared light to excite molecular bonds and measure absorption of light during their vibrations. Each chemical bond has a unique vibrational energy [16,17]. Therefore, each molecule has a set of unique spectral features in the infrared part of the spectrum. This set of spectral features is called a fingerprint of the molecule [18]. The molecular fingerprint is used to identify molecules from a mixture of molecules in bio-fluid [19,20]. However, infrared spectra in liquid phase in general yield broad low structured spectral features, thus making molecular identification a difficult or even impossible task. On the other hand, infrared spectra in gas phase yield narrow spectral features of molecules, thus allowing their identification to be much easier than liquid phase [21,22]. In this study, we demonstrate and illustrate a technique that we developed to analyze bio-fluids in gas phase.

The main advantage of the gas phase biological samples analysis over the tissue or liquid phase biological samples is that water can be removed from the sample without changing the molecular constitution. A highly efficient water removal technique was developed in our group and reported recently [23]. In combination with the water removal technique, Fourier transform infrared (FTIR) spectroscopy has already proved its potential for breath analysis [6,24]. In this paper, we show how

FTIR spectroscopy is used for analysis of gas-phase biological samples including breath and urine headspace.

2. MATERIALS AND METHODS

A schematic of the experimental procedure aimed at revealing molecular fingerprints is depicted in Fig. 1. The experimental setup consists of three major units, namely, (1) a sample collector, (2) a water condenser, and (3) an infrared FTIR spectrometer.

Before the sample collection, the complete system is evacuated by vacuum pump to remove any trace of contamination. Breath samples collected with Tedlar bags are transferred to the empty sample collector by releasing the valve. In the case of bio-fluid in liquid phase, it is collected in a specially designed well-sealed sample container in such a way that only part of the container is filled with bio-fluid in liquid phase. The sample is kept a sufficient time in the container to allow volatile compounds in bio-fluid to escape it via sublimation creating headspace, with their further collection. Finally, by releasing the valve, a headspace sample goes to the empty sample collector. Unlike breath, the headspace sample collection aimed at further spectroscopic analysis still has no established standard operating procedure (SOP).

A water condenser is a closed metal chamber containing a 12 m long, spiral copper tube, through which a gas sample is moved from the sample collector to a multipass gas cell attached to a FTIR spectrometer. The chamber is filled with a special liquid operating at temperatures between $-95°C$ and $+45°C$. Before the gas sample is allowed to pass through the water condenser, the liquid is cooled to $-60°C$ by the refrigerated circulator. Then the gas sample is allowed to pass through the spiral tube with a precisely controlled flow rate of 3 ml/s. A large amount of water vapor is removed from the gas sample when it passes through the cold copper tube. A reduction of approximately a factor of 2500 is achieved for the breath sample at $-60°C$. Finally, the water suppressed gas sample is transferred to the multipass gas cell, attached to the FTIR spectrometer. After each experiment, the copper tube is cleaned by heating with the heat circulator and vacuum pump. A detailed description of
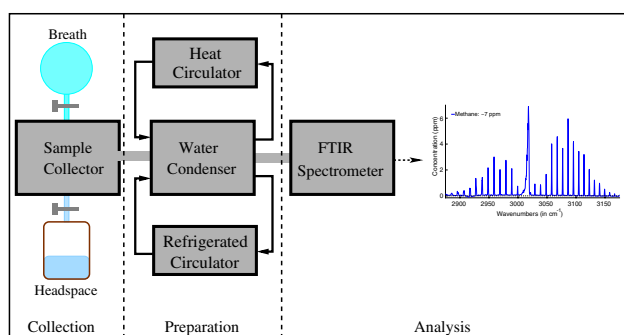

Fig. 1. Schematic of the experimental scheme, with three main parts: (1) collection—a collector, where breath or headspace of liquid bio-fluids is collected; (2) preparation—a water suppressed sample is prepared when gas-phase bio-fluids are passing through "the water condenser"; (3) analysis—a water suppressed gas sample is collected to the multipass gas cell and measured with a FTIR spectrometer.

the setup and its working principle was reported in a separate article [23].

Finally, the water suppressed gas sample is collected in the 4 m multipass cell, which is placed inside the Bruker Vortex70 FTIR spectrometer. Spectral data are collected by a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector in a range from $500\ cm^{-1}$ to $4000\ cm^{-1}$. Data are analyzed by component analysis, which uses known molecular fingerprints for fitting the observed spectral features.

Two experiments were performed: one that included a single-case aimed at comparison of absorption spectra of breath and urine headspace, and another aimed to find out statistical differences in breath between healthy volunteers and patients with cerebral palsy (CP). Breath and urine samples were collected from a healthy volunteer in our working group. Twelve persons with cerebral palsy (23–52 years), recruited from a day care center for persons with disabilities, and 12 healthy adults (25–77 years), recruited among the personnel working at Ludwig Maximilian University (LMU), participated in this study. For the participants with CP, the experimental procedures were approved by the ethics committee of the Faculty of Medicine of the Technical University of Munich, and for the healthy participants, from the ethics committee of LMU. Participation in the study was voluntary, and all subjects, or in some cases, their legal protectors, gave their written informed consent before the sample collection. Each breath sample was collected in a single-use 1 liter Tedlar bag via multiple exhalations of normal breathing [6].

3. RESULTS AND DISCUSSION

It is a common practice in medical studies that collected data are analyzed statistically. Most commonly used statistical methods are unsupervised ones such as principal component analysis (PCA) often combined with analysis of variance (ANOVA), as well as supervised ones such as random forest, etc. For these methods, experimental data are analyzed blindly, i.e., no certain spectral features can be attributed to the result. Hence, in some cases, the method of choice cannot distinguish between real data variations and experimental noise or measurement errors, thus leading to misinterpretation of the data. We proposed and successfully tested the component analysis, where each spectral component is matched with a known molecular spectral feature (reference fingerprint). The reference spectra were collected from PNNL, HITRAN, or NIST databases [25,26]. However, in many cases, when fine structure features are required to distinguish between several molecular candidates, digital spectra have been collected experimentally from the same spectrometer by applying the same spectral resolution used for the breath spectra [7]. Only in the case of matching was a spectral component considered for further analysis. The peak strength of the identified spectral feature (i.e., absorbance) can be transferred into the corresponding concentration and used then to quantify the state of the body. A detailed analysis of the body state made with such a procedure was reported in a separate article [6].

The FTIR spectrum of breath of a healthy volunteer is presented in the top panel of Fig. 2. Only water and CO_2 absorption peaks can be observed. To note, CO_2 is produced in many biological processes in the body and therefore is not
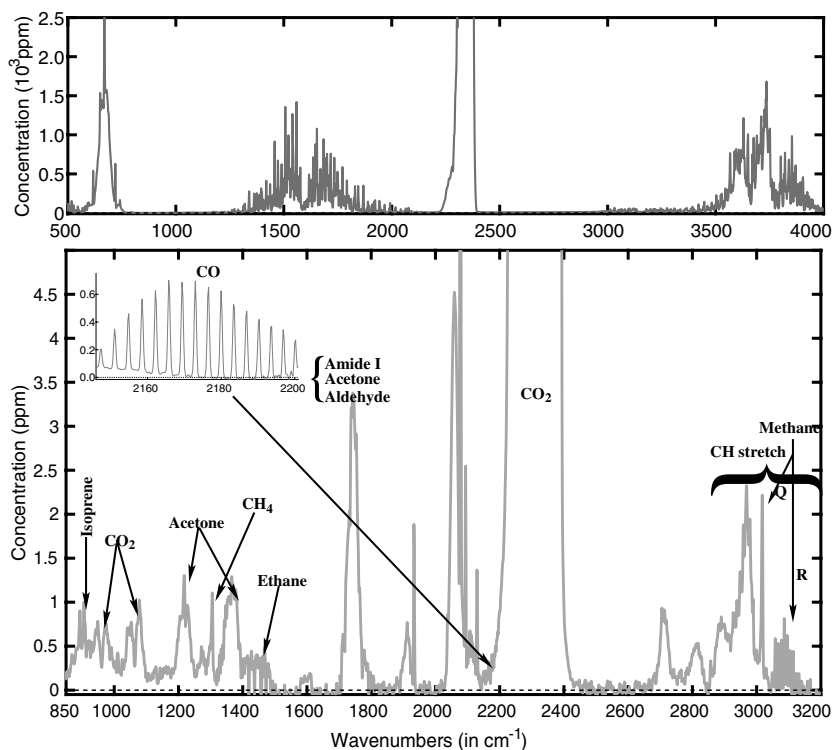
Fig. 2.   Top: FTIR spectra of a breath sample of a healthy volunteer without water suppression. Bottom: water suppressed breath spectrum of the same healthy volunteer. The y axis in the top panel is 10^3 times larger than that in the bottom panel. Inset: zoomed carbon monoxide absorption spectrum. A few fingerprints are identified and labeled.

specific enough for detailed analysis of the body state. All other spectral signatures of volatile organic compounds (VOCs) are buried mostly under the water absorption. The typical water suppressed breath spectrum of the same healthy volunteer is presented in the bottom panel of Fig. 2. A large amount of water suppression (a factor of 2500) makes the spectrum cleaner, especially in the range from 1100 to 2000 cm^{-1} and 3000 to 4000 cm^{-1}. We expect that other than water and CO_2, small (not more than 120 atomic units) VOCs can be specific for certain diseases, leading thus to distinct statistical spectral differences between the groups under study. The main task of the analysis of biological samples in gas phase is to identify those particular VOCs and to find their origin and a transportation scheme in the body. In this paper, we discuss only the identification of VOCs.

Now, let us discuss the most promising spectral regions with distinct features that we observed. The spectral range from 2800 to 3200 cm^{-1} is populated mostly with C–H stretch vibrational absorption bonds [27,28]. The C–H bond is the characteristics of biological molecules; therefore, all of them contribute to this spectral region, resulting in a congested spectral structure. It means that, practically, it is difficult to identify individual molecules with their fingerprints in this region. However, a distinguished spectral feature observed around 3100 cm^{-1} was identified as the **R** branch of methane. A very strong **Q** branch of methane is observed around 3020 cm^{-1}; however, the **P** branch is buried under broad C–H absorption spectra of other biological molecules and still cannot be revealed.

Many prominent spectral features are observed in the spectral range between 900 and 1800 cm^{-1}. The features are relatively less congested, making the identification of the corresponding molecules easier [7]. For example, a double-peak structure around 900 cm^{-1} is a unique feature of isoprene produced in the human body via cholesterol biosynthesis [29,30]. The peak at 1220 cm^{-1} is a clear signature of acetone, identified unambiguously. However, the peak at 1375 cm^{-1}, which is also a fingerprint of the acetone molecule, is a bit higher in absorbance strength than it should be in relation to other peaks. Therefore, this spectral feature must be a product of several molecules. Acetaldehyde also yields an absorption peak there, thus contributing to the observed peak at 1375 cm^{-1} [7]. Very strong absorption is observed around 1750 cm^{-1} due the C=O bond of acetone, aldehyde, and some small molecule with the amide band.

The identified molecules mentioned above are present in the breath of a healthy volunteer with relatively high concentration, between a few hundred ppb (parts per billion) and a few ppm (parts per million). However, many more molecules are present in human breath at lower concentrations. In the current state, the detection limit of our gas analysis system is about 40 ppb [23]. Therefore, still there is a chance to identify more molecules with the setup shown in Fig. 1. With a proper zoom of the spectra, more molecules can be found on the pedestal of the above-mentioned peaks. For example, with a ×10 zoom, a distinct fine structure becomes visible (see inset in Fig. 2) at the pedestal of the left shoulder of the carbon dioxide peak around 2300 cm^{-1}. This fine structure is identified as the absorption
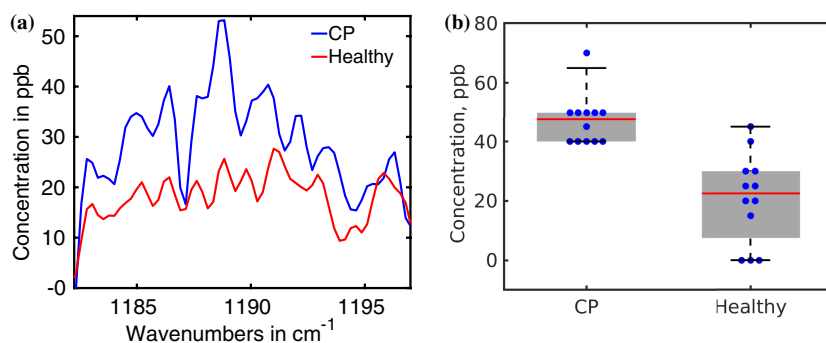
Fig. 3. (a) Average breath spectra of CP and healthy volunteers in the spectral range between 1180 and 1200 cm^{-1}. (b) Illustration of statistical component analysis of patients with CP and matched healthy individuals made in the spectral range of 1180 to 1200 cm^{-1}. A vertical scale shows the average concentrations of the metabolites, and the plot boxes show data deviations from the mean value for the two groups.

signature of carbon monoxide [31] produced in the human body due to catabolic processes, oxidative stress, and tissue injury [32]. Similarly, using a higher zoom and careful investigation, many more molecules can be identified [7]. So far, a few VOCs have been identified; however, many more revealed spectral features are on the way of their identification.

To illustrate how the measurement and identification steps can be realized in a real biomedical experiment, here we present the result of our pilot study that has been carried out for a group of patients with CP. For statistical comparison, the number of volunteers in the reference healthy group and their average age and gender ratio were comparable to those in the group of patients with CP. A distinguished spectral feature has been observed around 1189 cm^{-1}. The average spectra of healthy and CP persons are presented in Fig. 3(a). The result of the statistical analysis provided in the most sensitive spectral range of 1180 to 1200 cm^{-1} is shown in Fig. 3(b) [7,33]. The red lines in the gray boxes are average spectral strength at the spectral position 1189 cm^{-1} for CP and healthy volunteer groups. It is clear from the box plot that CP and healthy groups are clearly distinguishable. A conventional statistical analysis based on unsupervised PCA (PCA + ANOVA) demonstrated data clustering for the healthy and CP groups, with the p-value below 10^{-5}. A supervised analysis (support vector machine and random forest) resulted in approximately 91% accuracy of distinguishing the corresponding groups.

Breath is only one example of bio-fluid in gas phase. Another example is urine headspace, which can also be collected noninvasively [34]. The absorption spectrum of the urine headspace accompanied with water suppression was collected in the range of 500 to 4000 cm^{-1}. The spectrum is also populated with many characteristic molecular features, and a few of them are common molecular features observed in the breath sample. However, the common features in the two types of samples appear in very different relative and absolute concentrations. For comparison, both spectra are plotted in Fig. 4. The most striking difference is the CO$_2$ absorption peak, which saturates the detector in the case of the breath sample, but appears at a significantly lower level in the case of the urine headspace sample. Due to the significantly lower concentration of CO$_2$, a fingerprint of alcohol is revealed in the urine headspace spectra (see the left inset in Fig. 4). Similarly, carbon monoxide that

appears in breath at the pedestal of the left shoulder of the CO$_2$ peak at 2175 cm^{-1} can be observed in a clear spectral window in urine.

Another striking difference can be seen for the isoprene absorption spectrum. Urine contains a significantly higher amount of isoprene than that in breath. Isoprene is slightly too big to easily escape the bloodstream through the alveolar membrane, but easily leaves the urine sample. Various lines of supportive evidence suggest that isoprene is related to cholesterol biosynthesis [30]. Therefore, its measurements could potentially be used for monitoring lipid disorders and could serve as an additional parameter to complement invasive tests for monitoring the efficacy of lipid-lowering therapy, pharmacological and dietary or lifestyle [35]. Therefore, in the case of lipid disorder diagnostics, the urine sample would be advantageous over the breath sample.

The spectral feature around 1800 cm^{-1} in the urine headspace spectrum is not observed in the breath spectrum. This peak is identified as the amide I band of anhydride amino acids [22]. Though we still do not attribute it to a certain amino acid, monitoring of the level of amino acids in general will allow researchers to say more about the body state. Another peak is observed around 3100 cm^{-1}. This peak is also not assigned to a molecule; however, definitely this is not methane, since there is no **Q** branch observed. On the contrary, methane is always observed in the breath sample. A possible explanation of that is based on the small molecular size of methane. Because of
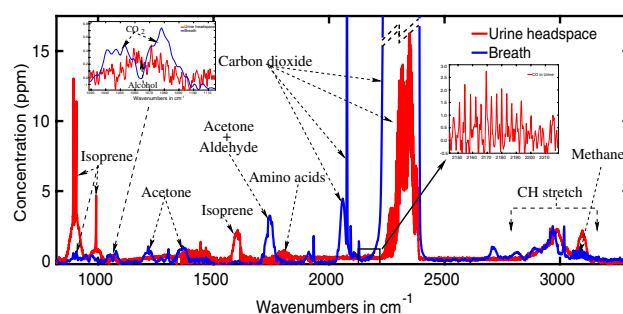

Fig. 4. Absorption spectrum of water suppressed urine headspace (red) plotted together with the breath absorption spectrum (blue) of a healthy volunteer. Fingerprints of alcohol and CO from urine headspace are presented in inset. A few fingerprints are labeled.

this, methane easily escapes the bloodstream via alveoli, and nothing remains to escape via the kidney. Similarly, several other small molecules such as acetone, aldehyde, etc., are observed in breath but absent in urine. From this short comparison, we see that in terms of available information, urine headspace is not a competitor of breath. Rather, considered together, they enrich the body diagnostics. Moreover, the infrared detection technique that we have developed for breath analysis is capable of handling both breath and urine samples simultaneously. Moreover, other bio-fluids such as saliva, blood, sweat, etc., can be analyzed via headspace sampling. To the best of our knowledge, the headspace of bio-fluids has not been analyzed so far via infrared spectroscopy.

4. CONCLUSION

In this paper, a spectroscopic analysis technique of bio-fluids has been presented. Several important issues of analysis of gas samples of biological fluids via FTIR spectroscopy have been pointed out and discussed. The spectroscopy was used to record the absorption spectra of the water suppressed urine headspace and breath samples. The molecular fingerprint-based spectral analysis was used to identify the VOCs in gas phase. We found that a significant number of volatile metabolites appears in the headspace of urine as well as in breath. Many of them are common for urine and breath, however, present in different absolute and relative concentrations. For example, isoprene is present for both samples, but in urine, it is present at a much higher concentration than in breath. On the contrary, breath contains a large amount of acetone in comparison to urine headspace. So far, only a few VOCs have been identified with infrared spectroscopy, and many more observed absorption peaks should be identified. We show that urine headspace analysis is rather complementary to breath analysis, together making the diagnostics via infrared spectroscopy more powerful. Since the experimental technique that we developed is suitable for extraction of headspace from any kind of liquid samples, the spectroscopy-based diagnostics can be enriched via blood, saliva, sweat, etc.

Disclosures. The authors declare that there are no conflicts of interest related to this paper.

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