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SPECTROSCOPIC STUDY OF DIETARY EFFECTS ON VOLATILE BREATH BIOMARKERS

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Abstract. The objective of the present research was to monitor the response of the organism to different food habits (mixed, vegetarian, raw vegan and Dukan) using a CO_2 laser based photoacoustic spectroscopy technique (LPAS), a fast and precise method capable of detecting breath ethylene and ammonia biomarkers at low concentrations. Ethylene is a breath biomarker of oxidative stress and ammonia a breath biomarker of protein metabolism. Oxidative stress caused by a disturbance between free radicals and antioxidant defense remains as the major factor associated to the pathophysiology of many dysfunctions whereas ammonia originate in the amino acids catabolism and elevated breath ammonia can be associated with different diseases. The results reveal that the ethylene and ammonia breath biomarkers can be considered as a measure of wellness state in the involved volunteers. Implications for future research are also discussed.

Key words: photoacoustic spectroscopy, breath analysis, ethylene, ammonia, oxidative stress, protein metabolism.

1. INTRODUCTION

Laser photoacoustic spectroscopy (LPAS) [1] has emerged over the last decade as a very powerful investigation technique, capable of measuring trace gas concentrations at ppmV (parts per million by volume), or even sub-ppbV (parts per billion by volume) level. LPAS is a specific technique that differs from conventional optical absorption spectroscopy methods based on the Beer-Lambert law which states the exponential attenuation of the transmitted intensity (Fourier-transform infrared spectroscopy [2], cavity ring-down spectroscopy [3], intracavity spectroscopy [4]). Photoacoustic spectroscopy is a calorimetric method, in which the optical energy absorbed in a gaseous species is directly measured through the heating produced in the medium. The conversion from optical energy to heat is induced by molecular absorption of photons at proper wavelength and subsequent non-radiative relaxation of the excited state (collisional relaxation). The small local temperature variation in the sample is associated to a pressure variation. When the deposited optical energy is modulated, a periodic heating is produced, thus generating a modulation of the sample pressure. This results in an acoustic wave, which can be detected using a sensitive miniature microphone.

LPAS provide several unique advantages, notably the multicomponent capability, high sensitivity and selectivity, wide dynamic range, immunity to electromagnetic interferences, convenient real time data analysis, operational simplicity, relative portability, relatively low cost per unit, easy calibration, and generally no need for sample preparation. Photoacoustic (PA) detection provides not only high sensitivity but also the necessary selectivity for analyzing multicomponent mixtures by the use of line-tunable IR lasers, *e.g.*, CO or CO_2 lasers.

LPAS is a widely recognised method that is applied in many different applications: nondestructive evaluation of materials, environmental analysis, agricultural, biological, and medical applications, investigation of physical processes (phase transitions, heat and mass transfer, kinetic studies), and many others.

Breath analysis for human diagnostics: Human breath contains over 1,000 volatile organic compounds (VOCs) that are linked to various physiological conditions as they represent the products of metabolism in human body. In other words, the human breath can be considered as an information source of the health status of the body because some of the volatile compounds detected in human breath can be directly correlated to specific diseases [5–16]. Exhaled breath is a mixture of more than a thousand molecules, some of which are present at parts per billion (ppb) or even parts per trillion (ppt) concentration levels and [5–8]. These molecules provide a unique breath profile of the health condition and have endogenous and exogenous origins. The sources of endogenous molecules are normal and abnormal physiological processes, whereas the sources of exogenous molecules are: inspiratory air, ingested food and beverages, or any exogenous molecule that has entered the body by other routes (*e.g.* dermal absorption) [6, 7]. A healthy diet gives to the body the nutrients it needs to perform physically, maintain wellness, and fight disease, but food is also the body's main source of energy [11–13]. Unbalanced life styles, characterized by a diet rich in fat and calories, alcohol drinking and tobacco smoking, and low intake of vegetable, fruits and fibers as well as sedentary style has been associated with chronic diseases: obesity, cancers, dyslipedemia, diabetes, hypertension cardiovascular, and hypertension [14–16].

Oxidative stress is generated by a chemical reaction of oxidative substances (*e.g.*, free radicals) which damage the cell wall, proteins, and genetic material and can be directly attributed to biochemical events surrounding lipid peroxidation [17]. Lipid peroxidation is the free-radical-induced oxidative degradation of polyunsaturated fatty acids, where biomembranes and cells are thereby disrupted, and causing cell damage and cell death [17]. Many of the organs dysfunction because of the critical care required as a result of oxidative stress. The oxidative stress can be measured in blood, urine and breath. A number of studies of oxidative stress show significant biomarkers present in breath, which can give quantitative and qualitative results of the status in oxidative stress. Ethylene biomarkers in breath has been associated with oxidant stress [6, 7, 17–19]. The normal physiological range for human breath ethylene is in the region of 0.02 ppb3 – 46 ppb [18] (parts per billion).

Ammonia (NH_3) plays a significant role in the human body and is considered to be an important biomarker [20–25]. Ammonia is present in all body fluids, mainly as ammonium ion (NH+4) but also in the form of NH3. The biomarker is usually monitored as NH+4 concentration in blood or, noninvasively, in urine, saliva, sweat or breath. A high ammonia concentration has been linked to liver and kidney function and to the effects of exercise, bacterial activity, and halitosis [20–23].

Ammonia metabolism: When food is ingested, a fine balance of nutritional absorption and toxin removal takes place. The body must be specific about how amino acids are processed, or nitrogenous compound concentrations could prove fatal. Initially, the stomach, lumen and intestines break down food into amino acids, nucleotide bases, and other nitrogenous compounds which diffuse into the blood [20–23]. These excess nitrogenous compounds are then absorbed from the blood into the liver. The liver converts them into less toxic soluble forms which can be safely removed in relatively low volumes of water. The urea cycle [23], as it applies to humans, is the pathway upon which amino acids are effectively broken down. As the liver finishes processing, the urea is excreted into the bloodstream among excess ammonia and is absorbed by the kidneys. Kidneys serve the purpose of filtering the blood urea and excess ammonia out of the body in the form of urine, but ammonia in too high concentrations it becomes toxic to the human body. Thus, for a healthy person, blood ammonia is tightly regulated via the urea cycle, with excess ammonia being converted to urea and excreted through urine. The level of ammonia in human breath has been measured as being between 50 ppb (where 1 ppb of ammonia in human breath is approximately 0.67 $\mu g\ m^{-3}$) and 2000 ppb and is dependent on a range of factors including the health status of the patient, the route of sampling (nasal or oral), contribution from oral bacteria, as well as diet, pharmaceutical use and levels of metabolic activity [20, 21]. Lifestyle factors such as: alcohol, smoking, stress, physic activity or diet is important factors in ammonia level into the body [6, 7, 20–22]. Encourage liver detoxification and support kidney filtration by alkalizing the diet (water, lemon juice, miso soup, fruits and vegetables, sprouts, grains, fish and vegetable broths) is diet tips to reduce ammonia. Depending on the factors involved (social, cultural, economic, physiological, or psychological), is a diet is adopted or a food life style.

The aim of the study was to analyzed human breath samples from healthy volunteers with different food diets (mixed, raw vegan diet for ten days, vegetarian and Dukan diet) by measuring the ethylene and ammonia concentration as biomarkers of oxidative stress and protein metabolism using the LPAS method.

2. MATERIALS AND METHOD

2.1. METHOD

Laser Photoacoustic Spectroscopy method (LPAS) is a very powerful investigation technique which is capable of measuring trace gas concentration at sub ppb level [19, 26–31].

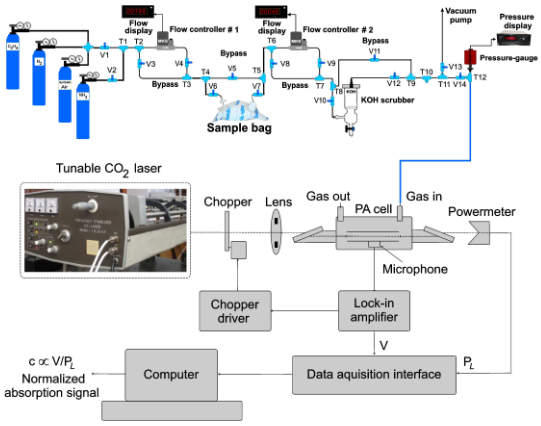

Fig. 1 – The LPAS system scheme.

The experimental system of the photoacoustic detection system is presented in Fig. 1. LPAS experimental setup used in the present work consists of a CO_2 laser, a photoacoustic (PA) cell where the gas sample is analyzed, a vacuum/gas handling system, and a detection unit. As a radiation source is used a CO_2 laser home-built, line-tunable between 9.2 and 10.8 μm on 57 different vibrational-rotational lines and frequency-stabilized, that emits continuous wave radiation with an output power of 2–5 W. The requirement for gases to be detected is that they should possess high absorption strength and a characteristic absorption pattern in the wavelength range of the CO_2 laser. Inside the PA cell, traces of gas can absorb the laser radiation and the absorbed energy is released into heat, which creates an increase in pressure inside a closed volume.

The laser beam is amplitude modulated by an optical chopper, focused by a ZnSe lens and introduced in the PA cell. The laser power used to excite the sample gas inside the PA cell is measured by a two channel powermeter. The acoustic waves produced in the PA cell are detected with four miniature microphones connected in series. The PA signal, proportional to the trace gas concentration is applied to a lock-in amplifier which detects and measures very small single frequency AC signals. The output signals of the lock-in amplifier and of the powermeter are then converted into digital signals and processed by a computer. A software program (TestPoint) for graphic and instrumentation permits to obtain and process the experimental results. The absolute trace gas concentrations are processed by the computer and the results are displayed on the screen. The gas handling system is an important part of the experimental set-up for the gas level concentration measurements (upper part of the general scheme of the photoacoustic detection system from Fig. 1), ensuring gas purity in the PA cell. The handling system can be used to introduce the sample gas in the PA cell at a controlled flow rate, to pump out the gas sample from the cell, and to monitor the total and partial pressures of gas mixtures.

An important parameter of the PA cell is the responsivity R [Vcm/W] which is defined as the amplitude of the electric signal provided by the microphones on the unity absorbed power of the molecules on the unity length:

$$R = C \cdot S_M. \tag{1}$$

Representation of this dependence is presented in Fig. 2, where for above measured average pressures $R \sim 300$ cmV/W and ~ 200 cmV/W, respectively.

The minimum detectable concentration which can be detected with the LPAS system is calculated with the following equation:

$$c_{\min} = \frac{V_N}{\alpha P_L C S_M}, \tag{2}$$

where $V = V_N$ is the voltage of the photoacoustic signal for a signal to noise ratio equal with 1 (SNR = 1), α [cm^{-1}·atm^{-1}] is the absorption coefficient for a given laser line, P_L [W] is the unmodulated peak value of the power laser, C [Pa·cm/W] is cell constant, and S_M [mV/Pa] is the total responsivity of the four microphones (S_M = 80 mV/Pa).

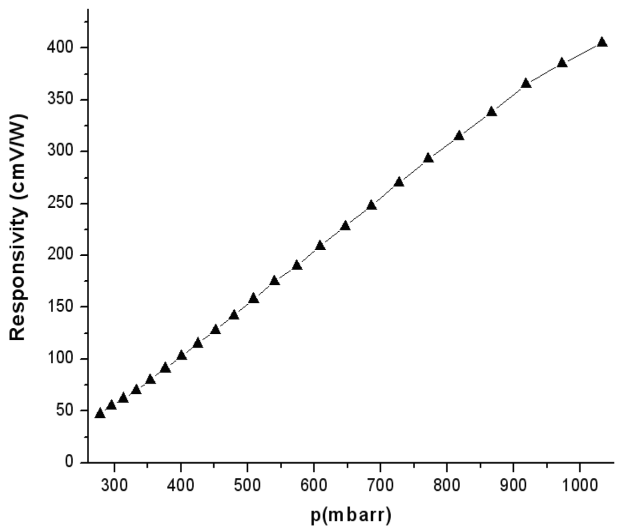

Fig. 2 – The responsivity of the PA cell against the pressure.

The absorption coefficients for ethylene and ammonia at different CO_2 laser wavelengths were measured previously (Dumitras *et al.* 2007, Dumitras *et al.* 2010) and the CO_2 laser was kept tuned at the 10P(14) line (10.53 μm), where ethylene had a strong absorption coefficient of 30.4 $cm^{-1}atm^{-1}$ and at 9R(30) laser line (9.22 μm), where ammonia had a strong absorption coefficient of 57 $cm^{-1}atm^{-1}$.

2.2. PROTOCOL FOR BREATH AMMONIA MEASUREMENTS

Patient breath samples were collected inside aluminized bags (0.75 liter aluminum-coated bags Quintron) equipped with valves that sealed them after filling. After an approximately normal inspiration (avoiding filling the lungs at maximum), the subject places the mouthpiece in his/her mouth, forming a tight seal around it with the lips. A normal expiration is then made through the mouth, in order to empty the lungs of as much air as required to provide the alveolar sample. The first portion of the expired air goes out, after which the valve (from the tee-piece) is opened, the remaining expired air being redirected into the collection bag. When a suitable sample is collected, the patient stops exhaling and removes the mouthpiece. The exhaled air sample is transferred to the PA cell at a controlled flow rate of 600 sccm [sccm – standard cubic centimeters per minute], and the total pressure of the gas in the PA cell was measured. To increase the accuracy of LPAS method for measurements of biomarkers in exhaled air of subjects, the sample gas is passed first through a trap filled with potassium hydroxide (KOH) pellets (with a volume larger than 100 cm^3) to remove the carbon dioxide and the water vapors before being collected into the PA cell and renewed after each measurement [26]. That measurements should not be altered by the molecules previously adsorbed on the PA cell wall and on the pathway, a cycle of N_2 washing was performed at the end of each sample. An average over several independent measurements at each line was used to improve the accuracy of the results.

2.3. SUBJECTS INVOLVED

Exhaled breath was collected from 10 healthy and non-smoking volunteers (4 males and 6 females with age between 30 and 36 years) with different food diets and different physical activity, no food supplements or pillows, and with a mean body index (BMI) of 22 ± 2.7. Form this group were 5 subjects with mixed diet and two from this kept the raw vegan diet for 10 days (ethylene and ammonia concentrations where measured at the end of the ten days of raw vegan diet and after three days return to the mixed diet), 3 vegetarians (from 8, 3 and 2 years) and 2 subjects with Dukan diet in phases 2 and 3. A detailed questionnaire was used to obtain baseline information (age, sex) and dietary habits (food preferences and avoidances, self-estimated amount of raw food, vegetarian, mixed or Dukan diet consumed, and duration of diets).

The mixed diet includes fruit, vegetables, grains, legumes, nuts, seeds and animal products. A vegetarian or vegan diet focuses on plants for food, which excludes all meat and animal products and no animal products. These include fruits, vegetables, dried beans and peas, grains, seeds and nuts. A raw food [16, 32] diet consists of unprocessed raw vegan foods that have not been heated above 46 degrees Celsius. A raw food diet includes: all raw fruits and vegetables, nuts and seeds, sprouts, roots, root vegetables and squashes, fresh herbs and raw spices, seaweeds, cold pressed oils, unprocessed olives, raw nut butters, raw nut "milks", fermented foods, dried fruits and vegetables, vinegars and foods cured in vinegar, unprocessed raw cacao (raw chocolate). "Raw foodists" believe that foods cooked above this temperature have lost their enzymes and thus a significant amount of their nutritional value and are harmful to the body, whereas uncooked foods provide living enzymes and proper nutrition. Dukan [33, 34] diet is a protein-based diet which consists in four phase: attack, cruise, consolidation, and stabilization. In the attack phase, dieters are allowed to eat as much as they want of 68 protein-rich foods. The cruise phase is designed to allow dieters to eat protein rich-foods with the addition of 28 specific vegetables (avoiding those starchy or fatty, *e.g.* beans, peas, lentils, potatoes or avocado). During the consolidation phase fruit, bread, cheese and starchy foods are reintroduced into diet. In the stabilization phase dieters can eat whatever they want by following a few rules: protein once a week, eating oat bran every day.

All samples were given in the laboratory between 09:00 and 11:00 in the morning and analyzed after 15 minutes to 3 hours. Informed consent was obtained from all individuals.

3. RESULTS

In this study, ethylene and ammonia concentrations from breath samples were measured at subjects with mixed, raw vegan, vegetarian and Dukan diets and the results were compared using CO_2 LPAS. The results for ethylene and ammonia biomarkers for each group are presented in Tabel 1.

A group of five subjects with mixed diet and from these, two kept the raw vegan diet for ten days. The ethylene and ammonia biomarkers were measured before the raw vegan diet, after the ten days of raw vegan diet and then after three days return to the mixed diet. Figure 3 shows the average concentration of breath ethylene and ammonia for subjects with mixed diet, raw vegan diet and after return to the mixed diet with an increase of ethylene concentration after the raw vegan diet and decrease of ammonia concentration after keeping the raw vegan diet. The ethylene and ammonia concentration return approximately to the previous value after three days return to the mixed diet.

Table 1

Average ethylene and ammonia concentrations from samples of human breath with different food diets: mixed, mixed at two subjects before raw vegan diet, after 10 days of raw vegan diet, after 3 days return to mixed diet, vegetarian and Dukan diet

Type of diet	No. of subjects	Ethylene concentration [ppb]	Ammonia concentrations [ppb]	Observations
Mixed (S1-S5)	5	40	1423	Moderate activity
Mixed (S1,S2)	2	52	1620	Moderate activity
Raw vegan (S1,S2)	2	945	1300	Moderate activity, after 10 days of raw vegan diet
Mixed (S1,S2)	2	56	1470	Moderate activity, after 3 days return to mixed diet
Vegetarian (S6-S8)	3	60	2633	Low and moderate activity
Dukan (S9,S10)	2	293	1830	Intense activity

Figure 4 shows the average concentrations of ethylene as oxidative stress indicator biomarker and ammonia as protein metabolism breath biomarker at mixed, vegetarians and those with Dukan diet. We see that the subjects with Dukan diet present a high level of breath ethylene and ammonia, but ammonia does not exceed the normal physiological range. The vegetarians present a normal level of ethylene but had a high level of breath ammonia.

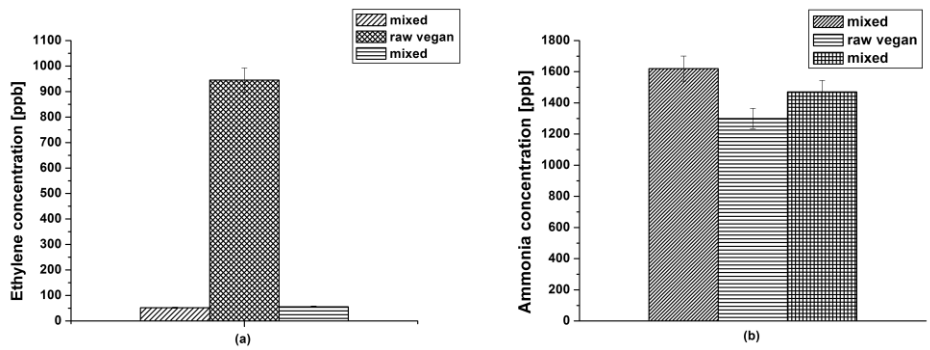

Fig. 3 – Breath biomarkers at the two subjects with mixed diet, before 10 days of raw vegan diet and after three days return to the mixed diet:
a) ethylene concentration;
b) ammonia concentration.

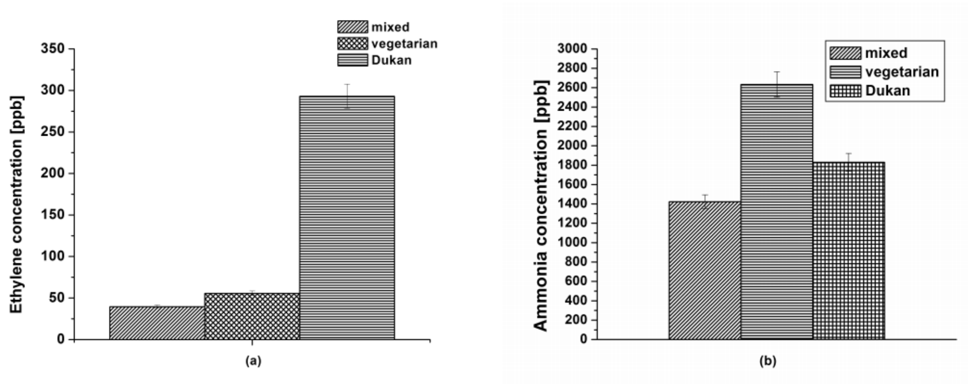

Fig. 4 – Breath biomarkers subjects with mixed diet, vegetarian and Dukan diet:
a) ethylene concentration;
b) ammonia concentration.

4. DISCUSSION

Several studies have shown that diet could influence the intensity of oxidative stress damage [35]. Oxidative stress is a physiological process that supposes an imbalance between free radical production and the ability of the body to neutralize the free radicals. Free radicals can contribute to a healthy response of the human body. Antioxidants are molecules that reduce the damage associated with oxidative stress [35, 36]. Many factors cause oxidative stress, some of which include: energy metabolism, detoxification, stress (physical, mental or emotional), poor dietary and lifestyle factors (high fat, high glycemic foods, smoking), environmental contaminations (air and water pollutants, radiation, soil contamination) [37]. At the subjects with mixed diet that kept for ten days the raw vegan diet, can be observed a high concentration of ethylene as a biomarker of oxidative stress presence in the body due to the stress on the body by switching from a diet that containing animal products to a strict diet like raw vegan diet. However, the ammonia concentration decreases after the ten days of raw vegan diet. Alkaline food (water, lemon juice, miso soup, fruits and vegetables, grains, fish) and reducing acidic forming foods (tea, coffee, alcohol, wheat sugar, red meats, refined and processed foods) are methods of diet tips to reduce ammonia by support the liver in its role of detoxifying and eliminating wastes from the body and improve kidney filtration and elimination. The decrease of ammonia concentration at these subjects is associated with liver detoxification.

The subjects with vegetarian diet present a normal concentration of ethylene in breath, but they present a normal level of ethylene in the breath. Ammonia is the byproduct of protein/ amino acid catabolism (break down) in the digestive process. Ammonia production may be elevated if there is an underlying factor such as

Helicobacter pylori, stress, amino acid deficiency or liver cirrhosis. In the case of vegetarian, the high concentration of ammonia is cause by a deficiency of essential amino acids. Ammonia is produced as a byproduct of amino acid/protein ingestion, however these amino acids are also required to detoxify toxins in the liver. A deficiency of these may result in insufficient detoxification pathways and therefore an accumulation of ammonia in breath.

Low or no consumption of vegetables and fruits in the diet leads to a decrease level of antioxidant, so it appears an imbalance between antioxidants and free radicals in the body. Due to the high protein consume and poor in vegetable or fruits, the subjects with Dukan diet, in phases 2 and 3 has a high level of ethylene in the breath. Also, the ammonia concentration is in high but does not exceed the normal physiological level of 2000 ppb. However, the ammonia concentration should be monitored at these subjects with Dukan diet over a long period or in different periods of the day, given that the breath samples were taken in the morning.

5. CONCLUSIONS

The purpose of this study was to measure ethylene and ammonia concentrations as breath biomarkers of oxidative stress and protein metabolism and to observe the body reaction to different food diets.

The ethylene and ammonia concentrations differ by adopted diet and also differ from one person to another; however the persons with mixed diet recorded the lowest concentrations of ethylene and ammonia.

The raw vegan diet adopted for a short period of time help liver detoxification, but also induces stress into the body, due to the transition from a diet with animal products to a diet based on raw vegetables and fruits.

The vegetarians (vegans) have a normal level of ethylene but present a high level of ammonia in the breath due to an amino acids deficiency.

The protein based diet, Dukan diet induce the presence of oxidative stress in the body. In this case, where the breath samples were taken in the morning this subjects have a high level of ammonia in the breath, but this level does not exceed the normal physiological range.

However, diet affect the concentrations of compounds in breath, and it will be important to better understand how these factor influence breath composition and breath analysis should probably take place after a complex monitorization.

Breath test is noninvasive, easily repeated, and does not have the discomfort or associated with blood tests. LPAS system is a fast and sensitive trace gas detector and will play an important role in exhaled breath analysis. Laser spectroscopy is a high-resolution technique capable of detecting traces of specific molecular species and the gas samples can be measured in real time without the need for sample treatment or preparation.

Implications for future research are also discussed, wanting to expand the research on a longer period of time and at different times of the day.

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