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Analysis of expired air of fasting male monks at Mount Athos

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Abstract

Expired air chemical analysis is investigated as a search and locate method for the early detection of entrapped people under the ruins of collapsed buildings after an earthquake. Fasting individuals were examined as a group that simulates the medical status of some of such victims. Exhaled air from seven fasting male monks (after 63 h) was analysed using thermal desorption–gas chromatography–mass spectrometry (TD–GC–MS) analysis. Over 150 volatile organic compounds (VOCs) were identified and the 43 most frequent are presented. Acetone showed by far the highest "positive alveolar gradient". Other compounds included phenol, di-limonene, 2-pentanone, isoprene and acetaldehyde. Quantitative results showed a 30-fold increase of acetone concentration (5.8 ppmv) compared to control measurements of a volunteer. Breath acetone was also identified through a portable gas chromatography-ion mobility spectrometer showing possible, under certain conditions, effectiveness of the method in the field.

Keywords: Expired air; VOCs; Fasting; Breath; Earthquake; TD–GC–MS; Ion mobility spectrometry

1. Introduction

Human breath is a bulk matrix consisting of nitrogen, oxygen, carbon dioxide, water vapor, inert gases and some trace components; volatile organic compounds (VOCs). This fraction is produced in the range of nmol/l–pmol/l (ppbv–pptv). Endogenous VOCs have gained scientific interest as diagnostic screening tools for monitoring metabolic and biochemical processes in the body [1,2]. The pulmonary alveolar membrane of the lung separates alveolar air from blood in the capillaries. VOCs can diffuse across the alveolar membrane from blood into the air and vice versa [3]. VOCs in exhaled air originate from biochemical processes within the human body, nutrition and the environment. When blood borne, their route depends on Henry's constant; molecular weight and hydrophobicity [4]. Currently, a common use of breath analysis is that of alcohol determination. Measurement of ethanol in breath through portable analyzers (alcoholometers) is based on its direct relation to the concentration in blood. A breath-to-blood ratio of 1:2100 is used when alcoholometers are calibrated to reflect blood alcohol concentrations [5]. Among several other useful clinical applications, breath VOCs are utilized in the detection of lung cancer and the prediction of heart and lung transplant rejections [6–8].

The purpose of our continued work is to investigate the use of breath analysis for the early location of entrapped people (i.e. in earthquakes). Earthquakes might cause massive destruction with many human casualties without warning. There is an urgent need for early location, medical support and rescue of entrapped people as the survival rate dramatically decreases in time [9]. Survivors are often trapped in voids of ruins, usually dehydrated and starved. VOCs, along with inorganic gases, can be piled up and an aerial odour (plume) may be created trailing in the direction of air currents. Expired air VOCs, along with volatiles of other biological fluids (blood, urine and sweat), might give indications of human life [10] or loss [11]. To study expired air under similar situations one needs to find volunteers for providing breath samples. However, identification of a group of volunteers undergoing starvation for 72 h (crucial time for search and rescue operations) for experimentation purposes may be difficult to find.

The source and physiological significance of most of VOCs in expired air is still unknown. However, among substances produced endogenously, acetone is considered the most abundant compound in human breath and has attracted scientific attention. Acetone is mainly formed by decarboxylation of acetoacetate and dehydrogenation of isopropanol [12]. It is produced by lipolysis, absorbed into the blood stream and is

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mostly excreted in expired air. As shown by Lord et al. [13], the concentration of acetone in breath changes during the day depending on the amount of carbohydrates received in the diet. Acetone is markedly influenced by the period of fasting [14], starvation or even type of diet [15]. Moreover, elevated acetone concentrations are produced in patients with uncontrolled diabetes mellitus [16]. However, acetone as a product of intermediary metabolism might vary widely between and within subjects [17,18]. Despite the obvious importance and interest in the analysis of expired air of fasting people, few authors have published complete trace component analysis in such conditions.

A variety of sensitive instruments and techniques are employed in expired air analysis. The most frequent include TD–GC–MS [19], solid phase mictroextraction (SPME) [20], proton transfer reaction-mass spectrometry (PTR-MS) [21], selected ion flow tube-mass spectrometry (SIFT-MS) [22], laser spectroscopy [23] and ion mobility spectrometry (IMS) [24]. Preconcentration on solid sorbent tubes is a widely applicable technique providing semi-quantitative results. On the other hand, IMS is a highly selective detector allowing direct but less specific detection.

The aim of the present study was the preliminary determination of VOCs found in exhaled air of fasting male individuals. For the needs of this work, a selected group of fasting monks volunteered and provided samples of expired air and made this work possible. As part of the religious tradition, all the monasteries located in Mount Athos (Chalkidiki, Greece) abstain from food and water for 3 days at the onset of Easter fast in order to cleanse body and spirit. The 3-day fast begins on a Monday, called “Clean Monday”, and ends on Wednesday. During this severe fasting period the monks only exert their religious obligations in the monastery and refrain from hard work and travel. One of the monasteries which follows this tradition for years is the Great and Holy Monastery of Vatopaidi; it stands on the coastline of a gulf in the center of the north-eastern side of the Athos peninsula, next to the Aegean sea.

After the identification of fasting breath VOCs, a classification of compounds according to “alveolar gradient” (abundance in breath minus abundance in air) for the most common substances was performed. Substances showing positive alveolar gradient (rate of synthesis greater than the rate of clearance) and negative alveolar gradient are presented and discussed. Furthermore, the effectiveness of a real time sensitive instrument such as ion mobility spectrometer for the determination of breath VOCs during fasting was investigated.

2. Experimental

2.1. Human subjects

The sample was highly homogeneous as the volunteer monks follow a common daily program and a specific diet. Although the sample is small, it could be considered rare. Seven non-smoking, healthy, white male monks (mean age 32 years, range 24–37 years) of the Great and Holy Monastery of Vatopaidi (mount Athos), willingly participated in the experiment that took place in March 2004. None had diabetes mellitus or nutritional abnormalities and none took regular medication. They all participated in the 3-day abstinence of food and water. The last meal was served at 7.00 p.m. on Sunday comprising of fish, salad and a glass of wine. Fasting ended on Wednesday at 11.00 a.m. The first meal was a special hot fruit soup called “housafi” consisting of plums, figs, grapes, oranges, etc. One volunteer was monitored at 3, 48 and 63 h with double samples. The other six monks were sampled at the end of the fasting period (63 h).

2.2. Sample collection

Breath sampling was carried out using 5-l tedlar bags (Alltech). Prior use, the bags were cleaned three times by using pure Ar. The subjects were asked to blow into the bag with moderate effort, while inspiring from the nose. The air was immediately transferred to the sorbent tubes with the use of a portable pump at a constant flow of 200 ml min^{-1}. Meanwhile, blank air samples were also taken from the sampling location. Three layer sorbent tubes (6 mm i.d., 115 mm length, 60/80 mesh) consisting of 300 mg Carpopack C, 200 mg Carbograph and 125 mg Carbosieve S-III (Supelco, USA) were employed. The tubes were preconditioned for 2 h, at 300 °C, with constant flow of He at 150 ml min^{-1}. They were then sealed by both Swagelok fitting and PTFE ferrules and stored at 4 °C. Prior sampling, they were spiked with 1 μl of a methanolic standard solution (50 mmol l^{-1}) of chlorobenzene-d5 serving as internal standard (IT-Chem, Greece).

2.3. Equipment and procedures

2.3.1. Sorbent tube desorption

Sorbent tubes were thermally desorbed to an HP 5890/5972 GC/MS system using an in-house-made thermal desorption unit which stands on top of the gas chromatograph. The system has been tested and evaluated for its performance with prior use. Detection limit for hexane was found to be lower than 10 ng when the system worked as an injector. System linearity exhibited an r^2 value of 0.986 for calibration curves at nanogram to a low microgram region. Reproducibility experiments showed R.S.D. less than 5%. Desorption flow of He was set at 30 ml min^{-1} while the temperature was kept constant at 200 °C. Desorption and refocusing duration was 20 min in order to maximize recovery. The cryo trap capillary was a 22 cm part of a 0.53 mm i.d., AT-Q, Q-PLOT column (Alltech Associates). A 20-s heating pulse (220 °C) has proved to be adequate for flush desorption of trapped analytes in the GC column. Cryogen used was liquid nitrogen.

A 60 m SPB-624 capillary column with 1.4 μm stationary phase and an internal diameter of 0.25 mm (Supelco) was applied for high resolution chromatographic separation. Column head pressure of helium purge gas was set to 25 psi. GC program was selected as follows: 35 °C initial for 5 min, ramp of 4 °C min^{-1} up to 180 °C, hold for 20 min. Mass range was applied from 35 to 350 amu due to the expected detection of VOCs.

2.3.2. GC-IMS determination

The equipment used was a portable GC-IMS EVM II (Femtoscan, USA) with ^{63}Ni radioactive source (370 MBq). The limit of detection for vapor acetone was found below 100 ppbv. The temperatures of the instrument were set as follows: inlet temperature = 70 °C, GC oven = 70 °C, transfer temperature = 70 °C. The AVS (automated vapor sampling) pressure was at 100 Torr. The cycle time was set at 28 s and the sample time 10,000 ms. The GC column utilized was a 5 m length methyl silicone (DB-1), 0.25 μm film thickness and 500 μm (i.d.). The sample flow rate was set at 25 ml min^{-1}. For the determination of acetone using GC-IMS, the tedlar bag was connected to a teflon tube, which was adjusted in front of the inlet of the instrument. GC-IMS peaks were identified using the retention time and drift time of vapor standards in hexane. The vapor calibration standards were prepared as described in the literature [25].

2.3.3. Inorganic gases determination

CO_2 and CO measurements in expired air were also carried out in all volunteers. Thus, double samples of exhaled CO_2 and CO were collected in 2 l bags and analyzed by portable sensors (Anagas CD 98 plus, Environmental Instruments, England).

2.3.4. Data management

GC-MS chromatographic peaks have been initially identified with the help of Wiley 138 mass spectrum library and further enhanced by an in house-made substance database using the "Easy ID" tool of HP productivity Chemstation. External standard mixtures purchased from ITChem (Greece) and Sigma-Aldrich (UK) provided the data needed for each record in the database.

For each VOC identified in the chromatogram, the alveolar gradient was determined using the ratio of the area (A) of each compound identified compared to the value obtained from the blank sample, as detailed by Phillips et al. [8,17]:

$$\text{Alveolar gradient} = \frac{A_{\text{VOC in breath}}}{A_{\text{internal standard}}} - \frac{A_{\text{VOC in air}}}{A_{\text{internal standard}}}$$

Alveolar gradient is a correlate of molar concentration and has been used to estimate the relative abundance of each VOC in breath and air. It should be emphasized that this approach adopts the assumption of equilibrium between VOCs in the body and in room air. Further semi-quantitative results were calculated with the internal standard method presented elsewhere [10,11].

3. Results and discussion

A variety of VOCs were identified from the volunteers examined. More than 150 substances were detected. The 43 most frequent substances present in five of seven volunteers were further examined according to their positive or negative alveolar gradient. Table 1 presents the 29 most common VOCs in breath with positive alveolar gradient. Table 2 shows the remaining 14 VOCs with negative alveolar gradient. Fig. 1 demonstrates a typical TD–GC–MS chromatogram of a fasting individual. Fig. 2 shows an IMS spectrum of the same individual recorded by directing the content of the tedlar bag to the inlet of the GC-IMS. Acetone was the most abundant substance identified by both techniques.

Table 1
Breath VOCs in male fasting individuals ranked by abundance (positive alveolar gradients)

VOCs	Mean alveolar gradient ($\times 10^{-2}$)	% Subjects
Acetone	505.96	100
Phenol	16.64	100
di-Limonene	15.34	100
2-Pentanone	1.49	100
Isoprene	0.89	100
Acetaldehyde	0.43	100
n-Octyl acetate	0.42	100
Dichloromethane	0.32	100
Octane	0.25	100
Hexane, 3-methyl	0.23	100
Hexane, 2-methyl	0.19	100
Heptane	0.14	100
2-β Pinene	0.14	90
Heptane, 2-methyl	0.14	90
Heptane, 4-methyl	0.14	90
Heptane, 3-methyl	0.13	80
Carbonic acid, dimethyl ester	0.10	100
Heptane, 2,4-dimethyl	0.10	90
Ethanone, 1-phenyl	0.10	100
Benzene, 1,2,3-trimethyl	0.09	100
Cyclohexane, methyl	0.08	80
Cyclohexanone	0.07	100
Benzene, (1-methylethenyl)	0.06	90
Toluene	0.04	100
Nonane	0.04	100
1-hexanol, 2-ethyl	0.04	100
2-butanone	0.03	100
Cyclohexane, 1,4-dimethyl	0.03	90
Benzene, 1,2-dimethyl	0.001	100

Acetone is produced by hepatocytes via decarboxylation of excess Acetyl-CoA and is an important physiological metabolic marker in breath. Ketone bodies are oxidized via the Krebs cycle in peripheral tissue. Fasting for 63 h led to a 30-fold increase of acetone content in expired air of the individual with successive measurements. Skrupskii reported a 15-fold increase for individuals after fasting for 24 h [26]. Taking into consideration

Table 2
Breath VOCs in male fasting individuals ranked by abundance (negative alveolar gradients)

VOCs	Mean alveolar gradient ($\times 10^{-2}$)	% Subjects
Ethanol	−2.22	80
Naphthalene	−1.47	100
Benzene, 1-ethenyl-4-ethyl	−0.73	100
Alpha-pinene	−0.44	100
Benzene, 1,3-dimethyl	−0.32	100
Benzene, 1-ethyl-4-methyl	−0.26	100
Nonanal	−0.23	100
Methane, chloro	−0.19	100
Hexanal	−0.12	90
Decanal	−0.11	100
Styrene	−0.09	100
Benzene, ethyl	−0.06	100
Decane	−0.02	100
Benzene	−0.003	100

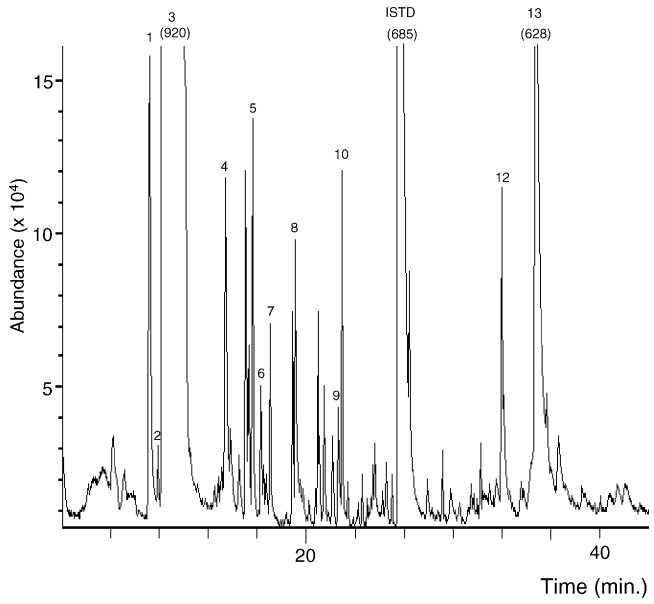

Fig. 1. A typical chromatogram (TD–GC–MS) of a fasting individual. The number in peaks indicate: (1) isoprene, (2) ethanol, (3) acetone, (4) 2-butanone, (5) hexane 3-methyl, (6) benzene, (7) heptane, (8) 2-pentanone, (9) toluene, (10) octane, (11) chlorobenzene d-5 (ISTD), (12) di-limonene, (13) phenol.

the variability between individuals and the analytical difficulties of the methodology used (high water content, possible adsorption in bags, appropriate selection of sorbent tubes, incomplete desorption, breakthrough, memory effects), there may be some loss during the analysis. Consequently, acetone concentration is expected to be even higher.

In normal individuals acetone breath concentration varies between 10–48.4 nmol l^{-1} (mean 23.2 nmol l^{-1}) [27] or 2.9–8.9 nmol l^{-1} [28]. Acetone in the control sample of a monk which was taken prior to the onset of fasting was indicatively found 8 nmol l^{-1}, which is in close agreement to the literature. On the other hand, Crote and Pawliszyn have reported increased concentrations of acetone (>50 nmol l^{-1}) in the breath of patients suffering from diabetes [20]. The indicative median value of 170 nmol l^{-1} (4.1 ppmv) identified from the monks examined is very close to the range 1.7–3.7 ppmv reported for diabetic breath [16]. In both situations the mechanism of acetone production is likely the result of lipid mobilization.

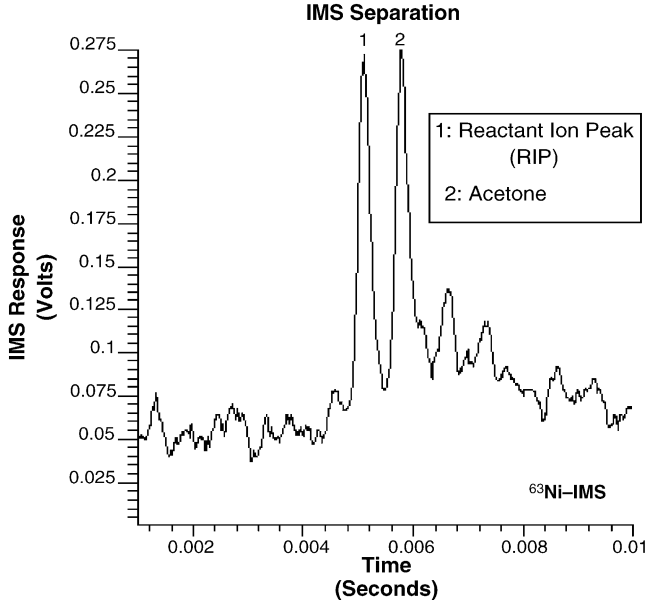

Fig. 2. Ion mobility spectrum of acetone in expired air of a fasting male.

It should be noted that during sample taking, the odour of acetone was detectable by smell in the expired air of the monks. The presence of ketone odour in their breath suggests that ketogenesis was taking place (production of ketone bodies: acetoacetate, β-hydroxybutyrate and acetone), resulting from fatty acid oxidation.

Acetone was also detected using GC-IMS analysis; it was identified using both GC retention time (5.32 s) and IMS drift time (5.2 ms). Acetone peak in IMS showed a clear separation from the reactant ion peak (RIP). RIP is formed by chemical ionization of the carrier gas in ambient temperature by ion mobility spectrometers equipped with radioactive ionization sources. It was observed that RIP peak was decreased when acetone was present due to charge transfer. The IMS spectrum was produced in positive polarity at room temperature and ambient pressure. It should be emphasized that the detection of acetone was further verified by the TD–GC–MS analysis of the duplicate sample.

For the same group of volunteers CO_2 and CO measurements in expired air were also carried out. No fluctuations of the CO_2 or CO were observed before and during the fasting procedure. CO was detected only in two volunteers (4 and 2 ppm, respectively); however, this is attributed to contaminants from the environment since similar values were also measured in the room coming probably from the burning wood at the fireplace. Furthermore, according to Middleton and Morice, a level of 6 ppm might be considered as a cut off point for outpatient non smoking individuals [29].

Broadly speaking the origin and distribution of VOCs in humans is mostly unknown. They may be produced in the body (endogenous), or may be absorbed or introduced as contaminants from the environment or foods. In the present study, in order to distinguish between endogenous substances and exogenous contaminants the calculation of the alveolar gradient was performed for each of the substances observed in expired air for over 71% of the volunteers. Thus, substances with positive alveolar gradient indicate that their rate of synthesis in the body is greater than their rate of clearance by metabolism or excretion.

It is assumed that the substances showing positive alveolar gradient (Table 1) are those that derive from one or different metabolic processes that are constantly in progress and may also have a cumulative behavior. Among them acetone showed significantly greater rates of synthesis than other substances, especially after 48 h of fasting. Other ketones identified were 2-pentanone and 2-butanone. Phenol was also among the substances with highest positive alveolar gradient. Phenol originates from foodstuffs and arises from metabolism of aromatic amino acids [30]. Terpenes determined with positive alveolar gradient include di-limonene and 2-β pinene. Di-limonene likely originates from the incense which is frequently used in

the several daily ceremonies in the monastery. A significant number of hydrocarbons (saturated, methylated, unsaturated) have also been identified. These might be correlated with lipid peroxidation as volatile hydrocarbons are considered in vitro and in vivo markers of oxidative degradation of polyunsaturated fatty acids. Isoprene is formed along the mevalonic pathway of cholesterol synthesis whereas acetaldehyde is probably produced by oxidation of endogenous ethanol [1]. However, it is of interest that pentane was not among them. An explanation might be probable coelution with the extremely high peak of acetone.

On the other hand, ethanol showed the highest negative alveolar gradient (Table 2). This is probably due to contaminants of room air. However, this is in agreement with the literature [19], although ethanol is considered among the endogenous volatile biomarkers. Moreover, a number of substituted benzene derivatives were found. Most of them appeared with a negative alveolar gradient as they are considered exogenous. Furthermore, it is of interest that two chlorinated substances were also measured (dichloromethane, chloromethane). Generally speaking they are considered exogenous; however, some of them (i.e. trichloroethane) were reported in the literature with a positive alveolar gradient [17].

Using analysis of expired air for locating entrapped people seems as a promising search and rescue method, especially if field analytical chemistry and technology is used. Effective urban search and rescue (US&R) operations are among the most promising means to reduce human casualties in earthquakes. The equipment of US&R teams is based on different principles, working either as active or passive systems. Search cameras might record picture, however size, cost and battery duration must be considered. Although electronic listening devices might cover large areas picking up faint noises and vibrations, their range for depth is limited and may have several artifactual interferences. Unconscious victims might be detected by canines which cover large areas in short time and gain access to voids. Disadvantages are their short work period and the great effort and cost to train them [31]. Ultra-sound detection is helpful with the main limitation of inability to survey areas inaccessible to the ultra-sound beam. Consequently, no single tool is sufficiently effective on its own to ensure that a complete search operation has been conducted. VOCs analysis of entrapped people in earthquakes could well be incorporated in audio-visual devices and fill in efficiently where other methods have limitations. The chemical method under study could be very helpful, especially when portable sensitive instruments are used on-line in the field.

However, tracing the dispersion of the odour plume of expired air in the ruins after an earthquake is a complicated task. VOCs are excreted in low concentrations and possible variation of values might appear. Furthermore, in a real situation VOCs distribution is affected by background influences such as dust, particles, household materials and wastes [32]. The physicochemical properties of the substances of interest should also not be overlooked (i.e. stability, absorption in particles, volatility, water solubility, degradation). Consequently, more research is needed in order to further improve the chemical method used in our study for the early location of individuals in distress.

4. Conclusions

Fasting male monks were sampled as a group of volunteers that resembles the medical status of victims trapped in the ruins of collapsed buildings after an earthquake. The TD–GC–MS analysis carried out in their expired air showed a prominent "positive alveolar gradient" for acetone, phenol, di-limonene, 2-pentanone, isoprene and acetaldehyde. This may imply that they accumulated endogenously during the 3-day fast. Acetone was further identified through a portable sensitive instrument (GC-IMS). The confirmation of the results in further studies might constitute this chemical method a useful complement applicable directly in the field after earthquake disasters.

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