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ANALYSIS OF HUMAN EXHALED BREATH IN A POPULATION OF YOUNG VOLUNTEERS

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Abstract - Analysis of volatile organic compounds (VOCs) in human breath can provide information about the current physiological state of an individual, such as clinical conditions and exposure to exogenous pollutants. The blood-borne VOCs present in exhaled breath offer the possibility of exploring physiological and pathological processes in a noninvasive way. However, the field of exhaled breath analysis is still in its infancy. We undertook this study in order to define inter-individual variation and common compounds in breath VOCs of 48 young human volunteers. Alveolar breath samples were analyzed by automated thermal desorption, gas chromatography with flame ionization detector (FID) and electron capture detector (ECD) using SUPELCO standards with 66 compounds. Predominant compounds in the alveolar breath of analyzed subjects are ethylbenzene, 1-ethyl-4-methylbenzene, 1,2,4-trimethylbenzene and 1,3,5-trimethylbenzene (over 50% of the subjects). Isopropyl alcohol, propylene, acetone, ethanol were found as well. We detected substituted compounds in exhaled breath.

*Key words***:** alveolar breath; volatile organic compounds; exhaled breath condensate; gas chromatography

INTRODUCTION

Our exhaled breath is a complex combination of thousands of molecules that constitute a breath print that carries information about us (similar to a fingerprint), and certain information about our state of health (similar to blood and urine) (Martinez-Lozano Sinues et al., 2013). One can reasonably argue that the history of using breath as a biomarker is as old as medicine itself. Hippocrates described *fetor oris* and *fetor hepaticus* in his treatise on breath aroma and disease (Hakkert, 1979). Lavoisier and Laplace in 1784 showed that respiration consumes oxygen and eliminates carbon dioxide, Nebelthau in the mid 1800s showed that diabetics exhale breath acetone, and Anstie in 1874 isolated ethanol from breath (which is the basis of breath alcohol testing today) (Dweik et al., 2008; Duveen et al., 1955). A major breakthrough in the scientific study of breath started in the 1970s when Linus Pauling demonstrated that there is more to exhaled breath than the classic gasses of nitrogen, oxygen, carbon dioxide and water vapor. Based on gas liquid partition chromatography Linus Pauling reported the presence of 250 substances in exhaled breath (Pauling et al., 1971). With modern mass spectrometry and gas chromatography

(GC-MC) instruments, we can indentify more than 1 000 unique substances in exhaled breath (Martinez-Lozano Sinues et al., 2013). These substances include elemental gases like nitric oxide and carbon monoxide and a multitude of volatile organic compounds. Exhaled breath contains aerosolized droplets as well, called exhaled breath condensate that have non-volatile compounds like proteins in them (Horvath et al, 2005; Borrill et al. 2008).

With recent advances in technology, anything in the blood that is potentially volatile or has volatile metabolites can be measured in exhaled breath. This includes substances that are produced as a part of our normal (or disease-related) metabolism. Since we are constantly inhaling air from our environment, exhaled air can also reflect our environmental exposure (Akmstrand et al, 2009; Dubowsky et al., 2007; Jacobs et al., 2010; Brunekreef et al. 2002). Furthermore, our breath contains volatile compounds produced by our internal environment – the bacteria in our gut and mouth.

The field of breath analysis is rapidly evolving as the new frontier in medical testing for disease states in the lungs and beyond (Amann A et al, 2007). Breath analysis is now used to diagnose and monitor asthma, various cancers like, such as lung cancer (Hakim et al., 2012), breast cancer (Phillips et al., 2010), colorectal cancer (Altomare et al.,2013), liver cirrhosis (Dadamio et al., 2012), heart transplant rejection (Cikasch Jr. et al, 2012), oxidative stress (Amman et al., 2007; Phillips et al., 2003), kidney damage (Pagonas et al., 2012). Breath analysis has the potential to offer a relatively rapid and noninvasive method for detecting a variety of diseases, including diabetes (Buszewski et al., 2007; Righettoni et al., 2012; Hubbard, 1920). Breath analysis has potential to be applied in fields beyond medicine including environmental monitoring, security and others.

Many VOCs are present in exhaled breath, which can reflect the current physiological state of an individual (Martinez-Lozano Sinues et al., 2013). Changed levels and composition of VOCs in diseased patients can provide insight into abnormal metabolism. Several VOCs can be directly linked to cholesterol metabolism in the body, with isoprene as the signature molecule (Phillips et al., 1997; Kalapos, 2003). Oxidative stress is a term referring to the generation of reactive oxygen species (ROS) that catalyze the breakdown of polyunsaturated lipids into lipids with a carbon radical. The carbon radical then becomes a lipid peroxyl radical by combining with molecular oxygen, which can lead to the breakdown of other polyunsaturated lipids. This type of oxidative stress has been shown to occur in heart transplant rejection, acute myocardial infarction and several respiratory diseases. Acetone is produced in the liver after degradation of acetyl coenzyme A. When the body uses fat for energy rather than glucose (during dieting, fasting or starvation), acetone production increases. Endogenous reduction of acetone leads to the formation of 2-propanol, which is a VOC that can be measured in the exhaled breath at concentrations lower than acetone (Kalapos, 2003; Deng et al., 2004; Nelson et al., 1998). Benzene and acetonitrile are two VOCs that are present in elevated concentrations in individuals exposed to tobacco smoke. Typical VOCs present in human breath are isoprene, methanol, acetone and 2-propanol. Acetonitrile, furan and 2-methylfuran have been found in smokers, limonene is of exogenous origin, hydrogen is from bacteria in the gut of persons suffering from fructose malabsorption while methane, ethane and pentane are lipid peroxidation products (Nelson et al., 1998; Phillips et al., 1999). Possible sources of cancer VOCs have been reported. Metabolic disorders or pathological processes can produce new VOCs or change the ratio between VOCs produced in the body. It has been proposed that cytochrome p450 enzymes are overactivated in lung and breast cancer (Phillips et al., 2007; Hakim et al., 2012).

Alveolar breath is a distinctive gas whose chemical composition differs markedly from inspired air. Volatile organic compounds are either subtracted from inspired air or added to alveolar breath as products of metabolism (Phillips et al., 1999). Alveolar breath samples in this study were collected using a commercial device (Bio-VOC sampler, Markes International Limited, UK) for exhaled air developed by

the UK Health and Safety laboratory. The principle of the method relies in the relationship between volatile chemical concentrations in the blood and air in the lungs, termed end-tidal air. The concentration of volatile chemicals in alveolar air responds rapidly to changes in the end-tidal air. An adult exhaling deeply typically breathes out over 4 L of air. Only the last 100 ml of this air from alveolar portion of the lungs is retained by the Bio-VOC sampler. The method is sensitive with a coefficient of variation between 5% and 15% for collection and analysis of breath samples (Handerson et al., 2002). Each volunteer was asked to exhale through a cardboard mouthpiece into the Bio-VOC sampler (Markes International, 2000). Alveolar breath was transferred to a sorbent trap. VOCs in the sorbent traps were analyzed by automated thermal desorption, gas chromatography with flame ionization detector (FID) and electron capture detector (ECD).

In this paper, volatile organic compounds found in exhaled breath of 48 young (between 19 and 33 years) volunteers are presented. The TD/GC/FID/ECD method was developed for the analysis of volatiles in breath. We analyzed variations in the composition and quantities of VOCs in the breath of normal young humans using gas chromatography combined with a flame ionization detector (FID) and electron capture detector (ECD). All of them had fasted from the previous midnight in order to minimize any potential confounding effects of recent meals. The study participants refrained from smoking, eating, teeth brushing and chewing gum for at least 2 h prior to the analysis. Subjects generally sat for approximately 15 min prior to collection of breath and air in order to allow time for equilibration between the VOCs in room air and blood. All volunteers were asked to fill in a questionnaire describing their current smoking status, drinking status and status of health, based on self-declaration.

MATERIALS AND METHODS

Exhaled breaths of forty-eight subjects (between 19 and 33 years) were analyzed. None of the volunteers complained of discomfort during donation of the breath sample. Alveolar breath samples were collected using a commercial device (Bio-VOC sampler, Markes International Limited, UK). Each subject had to keep exhaling until the lungs were emptied, thereby capturing the end-tidal air. Once the breath had been collected in the Bio-VOC sampler, a screw-in plunger was used to steadily discharge the sample into a concentrating (sorbent) trap, a sampling tube. Alveolar breath was transferred immediately from the sampler to the sorbent tube containing 200 mg TenaxTA and 200 mg Unicarb (carbonized molecular sieve) (Markes International Limited). The sampling tube was then capped and was stable for transportation and subsequent analysis by gas chromatography. Samples were analyzed using TD/GC/FID/ECD (Agilent 7890) associated with the thermal desorber (Unity MARKES 1). Separation of the components was performed on a capillary column DB-624, 60 m in length. TD/GC/FID/ECD was previously calibrated with 66 compounds contained in SUPELCO standard Cat. No. 41973-U balanced in nitrogen. Compounds present in each sample were quantified according to the prepared standard curve.

RESULTS AND DISCUSSION

Human subjects were volunteers recruited from faculty colleagues and young faculty staff. There were 48 subjects studied, 16 males and 32 females, aged between 19 and 33 years. The subjects remained in the same room for at least 15 min before breath collection so that equilibrium between the lung and ambient air was created.

Human subjects' etiology and study design

The average age of the subjects was 25.6 years. The research was approved by the Faculty Review Board.

Among the subjects there were 2 persons who have hypertension, none had diabetes, and 4 had high values of lipids in the blood. Forty-two subjects had normal values of blood pressure, levels of sugar and lipids in blood, 29 were females and 13 males. Six of the subjects had a chronic problem (high values of lipids in blood, hypertension) or more than one con-

Table 1. Characteristics of the study population

Group etiology	Number	Female/male
Healthy controls	43	29/13
Hypertension	2	1/1
Diabetes	0	0/0
Smokers	11	10/1
Drinkers	32	20/12
Fats in blood	4	3/1

Table 2. Variation in the number of volatile organic compounds identified

Breath VOC	Range of concentrations (per 1lof alveolar breath in μg)	Number of subjects
Propylene	5.72×10^{-3}	1
1,3-Butadiene		
Ethanol	1.16×10^{-2}-6.83×10^{-4}	11
Acetone	1.12×10^{-2}-9.90×10^{-3}	19
Carbon disulfide	4.46-8.71×10^{-4}	5
Isopropyl alcohol	9.92×10^{-3}-5.68×10^{-4}	3
Tert-butyl methyl ether	11.21×10^{-1}-6.84×10^{-2}	11
n-Hexane	1.15×10^{-1}-5.12×10^{-3}	10
Vinyl acetate	2.73×10^{-1}-6.20×10^{-3}	3
2-butanone	2.06×10^{-1}-9.18×10^{-3}	9
Ethyl acetate	1.06×10^{-1}-7.51×10^{-3}	6
Tetrahydrofuran	8.73×10^{-2}-8.62×10^{-4}	6
Ciklohexane	4.85×10^{-2}-1.43×10^{-3}	6
Benzene	1.04×10^{-2}-6.37×10^{-4}	12
Heptane	1.29×10^{-2}-8.43×10^{-4}	14
1,4-Dioxane	4.40×10^{-1}-9.83×10^{-3}	22
Methyl isobutyl ketone	11.18×10^{-3}-2.65×10^{-3}	5
Toluene	1.81×10^{-4}-2.19×10^{-4}	2
n-octane	1.22×10^{-2}-4.90×10^{-4}	16
Methyl n-Butyl ketone	1.41×10^{-3}-1.80×10^{-4}	9
Ethylbenzene	3.66×10^{-2}-8.40×10^{-5}	9
m-Xylene	1.48×10^{-2}-1.48×10^{-2}	27
Nonane	91.21-9.24×10^{-1}	19
p-Xylene	8.42×10^{-1}-5.94×10^{-4}	19
o-Xylene	1.02×10^{-1}-9.87×10^{-2}	7
Styrene	1.00×10^{-3}-7.94×10^{-3}	13
n-Decane	1.76×10^{-2}-8.19×10^{-4}	22
1-Ethyl-4-methylbenzene	1.04×10^{-3}-6.00×10^{-4}	27
1,2,4-Trimethylbenzene	1.36×10^{-2}-9.83×10^{-4}	37
1,3,5-Trimethylbenzene	1.02×10^{-2}-8.74×10^{-4}	43

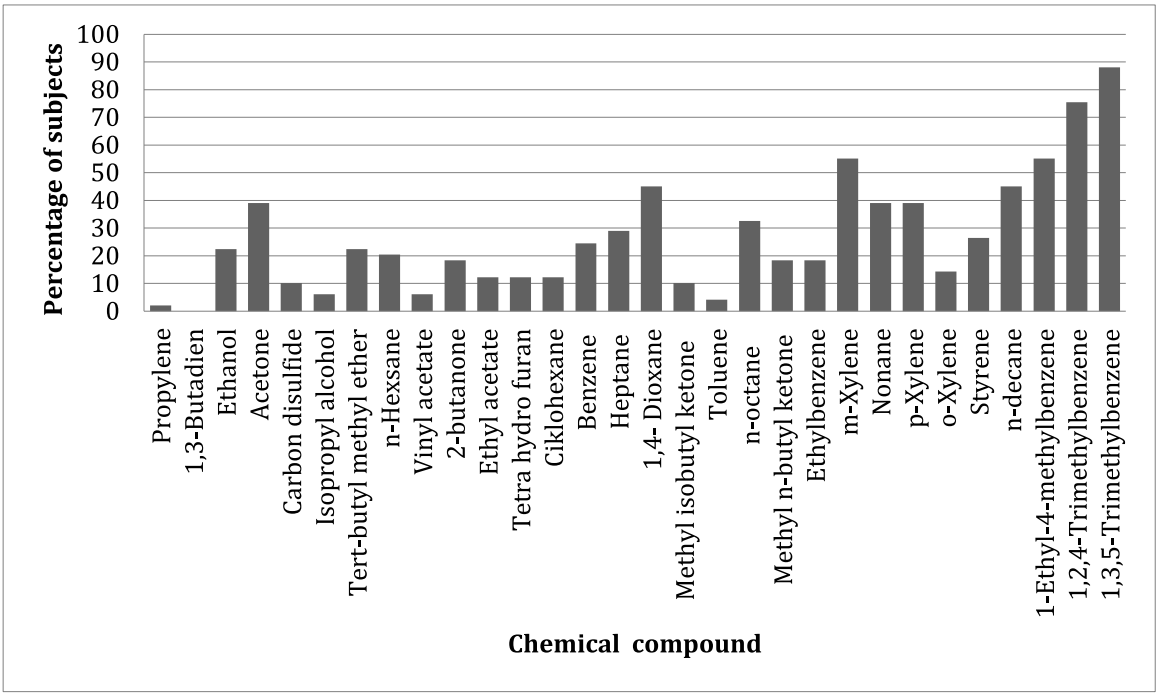

Fig. 1. The frequency of detection of compounds in the analyzed population

dition at the same time. More than two-thirds of the subjects (67%) consumed alcohol to some extent. Six (3 females and 3 males) consumed alcoholic drinks two or three times a week, 16 consumed alcohol two or three times a month (9 females and 5 males), 10 rarely consumed alcoholic beverages (5 females and 5 males). Eleven subjects smoked, 10 females and 1 male. The extent to which the subjects consumed cigarettes varied from 20 cigarettes (1 male) to 2 cigarettes per day (1 female); 3 females consumed 10 cigarettes per day. Seven persons practiced some form of recreation (athletics, fitness), 2 females and 5 males. Four persons consumed some type of prescribed medication (for migraine, anemia, depression and asthma). These conditions were in normal values at the moment of sampling.

The column used in this study was calibrated with 66 compounds. The system TD/GC/FID/ECD was also calibrated with 36 compounds substituted with Br or Cl contained in the calibration mixture, and compounds present in each sample were quantified according to the standard curve.

In earlier reports on breath samples of healthy persons, acetaldehyde, ethanol, acetone, 2-methyl-1-propene, carbon disulfide, isoprene, pentane, 2- and 3-methylpentane, hexane and toluene are usually identified. Compounds such as ethanol, acetone, isoprene, carbon disulfide, 2- and 3-methylpentane, benzene, methylcyclopentane, hexane and toluene were found in all breath samples (Buszewski et al., 2009; Phillips, 1999; Phillips et al., 1999). Ethanol, acetone, carbon disulfide, isoprene and hexane are endogenous and the remaining compounds are exogenous.

In Table 2 and Fig. 1 it can be seen that the most abundant volatile organic compounds in exhaled breath of young population were 1-ethyl-4-methylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, found in over 50% of subjects. Benzene was present in a lower number of subjects (24.5%), probably due its modification in the above-mentioned compounds. In previously published studies, benzene was present in the exhaled breath of smokers, non-smokers and passive smokers, but it has been

Table 3. Substituted compounds in breath

Chlorinated breath VOC	Range of concentrations (per 1l of alveolar breath in μg)	Number of subjects
Dichlorodifluoromethane	/	/
1,2-Dichlorotetrafluoroethane	/	/
Vinyl chloride	1.01×10^{-3}-1.27×10^{-6}	6
Bromomethane	1.57×10^{-2}-9.13×10^{-5}	10
Ethyl chloride	7.65053-7.21×10^{-2}	16
Trichlorofluoromethane	1.54254-7.98×10^{-3}	12
Ethylene 1.2-dichloro (trans)	1.44×10^{-1}-3.21×10^{-5}	14
1.1.2-Trichlorotrifluoroethane	7.11759-6.47×10^{-4}	13
Methyl chloride	1.78×10^{-1}-8.92×10^{-5}	11
Methylene chloride	1.05×10^{-1}-9.42×10^{-6}	10
Cis-1.2-Dichloroethylene	3.08803-5.47×10^{-4}	7
1.1-Dichloroethane	3.17×10^{-1}-8.20×10^{-4}	14
1.1-Dichloroethylene	1.13×10^{-1}-8.16×10^{-3}	13
Chloroform	1.95×10^{-3}-1.79×10^{-4}	4
1.1.1- Trichloroethane	1.75×10^{-4}-5.49×10^{-6}	5
Carbon tetrachloride	1.55×10^{-4}-8.49×10^{-6}	18
1.2-Dichloroethane	6.59×10^{-3}-9.48×10^{-5}	20
Trichloroethylene	1.10×10^{-2}-2.67×10^{-5}	27
1.2-Dichloropropane	1.94×10^{-4}	1
Bromodichloromethane	1.23×10^{-2}-9.79×10^{-3}	7
Cis-1.3-Dichlorpropane	1.32×10^{-2}-1.40×10^{-5}	6
Trans-1.3-Dichlorpropene	1.80×10^{-2}-8.17×10^{-5}	23
1.1.2-Trichloroethane	1.21×10^{-4}-4.97×10^{-5}	8
Tetrachlorethylene	1.19×10^{-1}-8.57×10^{-5}	17
cis-1.2-Dichloroethylene	1.01×10^{-4}-5.70×10^{-5}	6
Dibromochloromethane	1.33×10^{-2}-5.83×10^{-5}	14
1.2-Dibromoethane	1.87×10^{-2}-3.97×10^{-5}	42
Chlorobenzene	6.20×10^{-2}-9.64×10^{-5}	15
Tribromomethane	1.24×10^{-2}-4.79×10^{-4}	7
1.1.2.2-Tetrachloroethane	3.74×10^{-2}-8.06×10^{-4}	49
1.3-Dichlorobenzene	1.26×10^{-2}-8.67×10^{-5}	39
1.4-Dichlorobenzene	2.40778-6.76×10^{-7}	36
Benzyl chloride	2.26×10^{-3}-5.75×10^{-5}	11
1.2-Dichlorobenzene	3.51×10^{-1}-5.14×10^{-6}	43
Hexachloro-1-3-butadiene	2.59163-6.35×10^{-3}	44
1.2.4-Trichlorobenzene	2.53×10^{-2}-9.87×10^{-5}	37

confirmed that its origin is exogenous. Ethanol, acetone and isopropyl alcohol, which are usually found in exhaled breath, were present in a lower percentage of the analyzed population. Ethanol and acetone were present in over 20% of the volunteers, whereas isopropyl alcohol was present in only 6.1%. A peak corresponding to 1,3-butadien was not found in the analyzed population, where propylene was found in only one person.

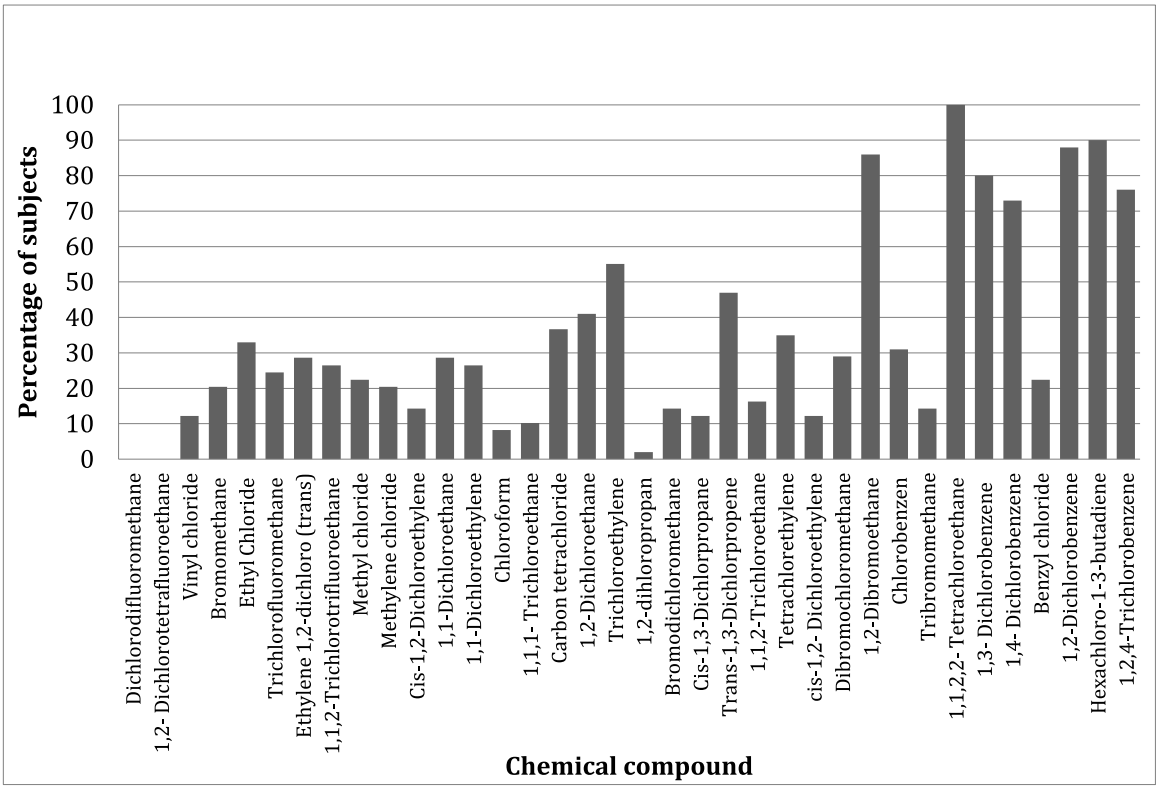

Fig. 2. The frequency of detection of substituted compounds in the analyzed population

Vinyl acetate, ethyl acetate and tetrahydrofuran were present in 3 and 6 persons, respectively, which suggests that this may be of exogenous origin. Toluene was found in two persons. There are reports that show a connection between furan, octane and decane in the breath of smokers and passive smokers. In our study, we could not find a significant connection between smoking and the existence of octane, decane or nonane, although all three compounds were present in over 30% of the analyzed subjects. Tetrahydrofuran was present in 6 samples which cannot be connected to smoking. However, further studies need to confirm these findings. In our analyzed samples, styrene, p-xylene, and o-xylene were present as well, but based on published data, we can conclude that their origin is exogenous (Buszewski et al., 2009).

In this study, chlorinated compounds (with the exception of dichlorodifluoromethane and 1,2-dihlorotetrafluoroetan) were detected in the exhaled breath of the studied population (Table 3, Fig, 2.) Almost all of the 36 listed compounds were present in the exhaled breath of some subjects. Some compounds, like 1,1,2,2-tetrahloroetan, 1,2-dibromoethane and hexaclor-1-3-butadiene, were found in more than 90% of the population. Compounds like trichloroethylene, trans-1,3-dichloropropene, 1,3-dichlorobenzene,1,4-dichlorobenzene and 1,2,4-trichlorobenzene are present in over half of the volunteers. To our knowledge, this is the first report that considers the existence of substituted volatile organic compounds in human breath that is not the result of previous exposure to these substituted hydrocarbons. Methylene chloride was previously reported in human breath, but the authors assumed that its origin was exogenous (Buszewski et al., 2009). This result requires further detailed investigation. A typical recorded chromatograms showing exhaled breath profile is shown in Fig. 3.

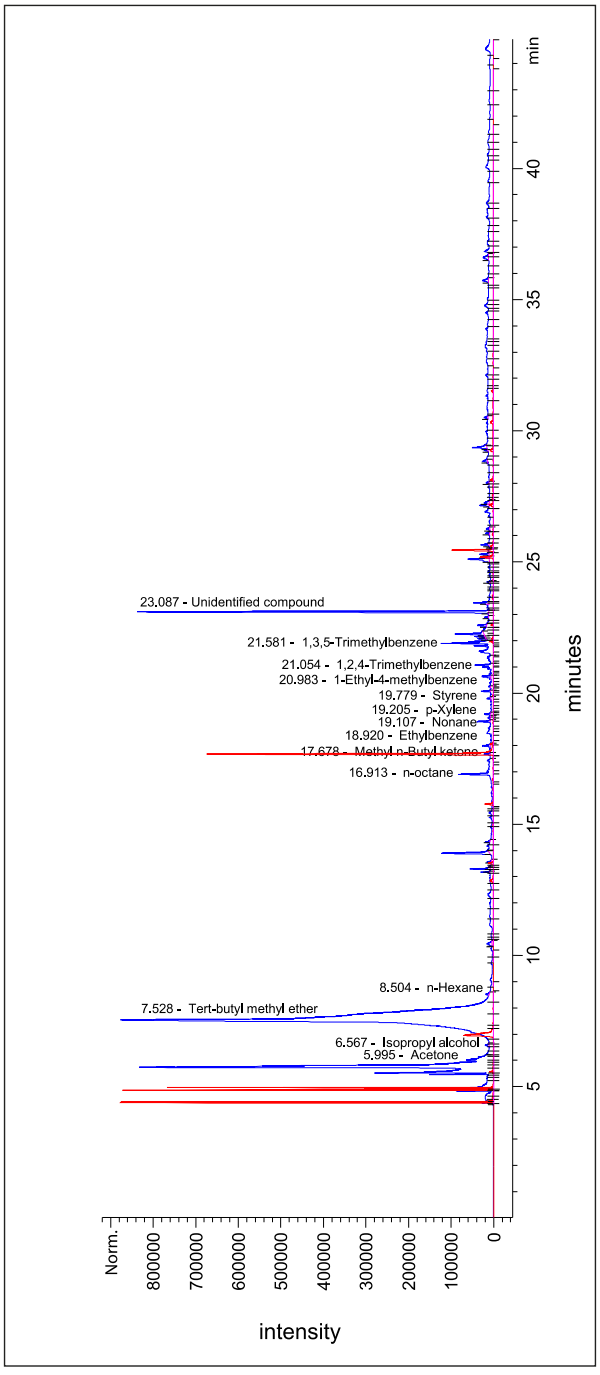

Fig. 3. Typical chromatogram of alveolarbreath sample (red line represents electron capture detector and blue line flame ionization detector)

The present work shows that breath analysis by TD/GC/FID/ECD (Agilent 7890) is able to identify VOCs that are associated with normal human breath. Separation of the components was performed on a 60 m capillary column DB-624. The earliest and still most widely used application of breath VOC analysis is the monitoring of blood alcohol concentration. Since ethanol is not normally present in ambient air, ethanol detected in the breath can be assumed to originate from the body. Only the most abundant breath VOCs, like ethanol, acetone and isoprene, were detected with assays of unconcentrated breath. However, many of the human breath VOCs can be detected in normal room air. Thus, researchers are faced with a dilemma: does VOC originate from within the body or is it an artifact due to contamination from environmental air? Some researchers have responded to this issue by ignoring the problem. Some authors provided the subject with VOC-free air to breathe prior to collection of breath sample. This is not possible to achieve in practice, because high purity air also contains VOCs in low concentrations. Some researchers measure background air and subtract from the VOCs observed in the exhaled breath.

In some studies, human subjects remain in the same room for at least 10 min before breath collection so that equilibrium can be created between lung and ambient air. We elected to follow the last option, and to calculate the concentrations of compounds in exhaled breath based on a calibration curve.

Benzene, toluene, styrene and 2,5-dimethylfuran are present. Styrene is considered exogenous. Sources of exhaled breath compounds need not be limited to human physiology. As stated previously, bacteria in the gut and mouth can often be sources of many volatile compounds in the breath (Buszewski et al., 2009). Hydrogen sulfide is a by-product of bacterial metabolism in the mouth and can be a sign of periodontal disease. Elevated levels of the compound have been observed in exhaled breath, and these levels can change significantly after a simple mouth rinse. In addition to VOCs produced within the body, exogenous sources of compounds need to be considered as well. Many compounds measured in exhaled breath occur after ingestion of certain foods and beverages (etha-

nol or nitrogen oxide) or inhalation of car exhaust gases and cigarette smoke. Benzene and acrylonitrile are two VOCs that are present in elevated concentrations in individuals exposed to tobacco smoke. The large majority of both compounds are conjugated by the cytochrome P450 enzymes in the liver and excreted in the urine; however, the rest is exhaled in the breath unchanged. The ability to monitor the smoking habits or exposure of subjects could provide evidence that lifestyle changes must occur to limit their exposure to cigarette smoke pollutants. Exogenous sources include exposure to cigarette smoke, alcohol, pollution and radiation.

According to our standard curve, nonane was present in highest concentrations in 19 individuals. Based on literature data, nonane has an exogenous origin (Buszewski et al., 2009). Propylene, ethanol and isopropyl alcohol were present as well in high concentrations in some subjects. The usual measured concentrations are in accordance with nanomolar or picomolar concentrations measured by other authors.

CONCLUSIONS

Breath samples of healthy young population of volunteers were analyzed. More than 50% of the subjects exhaled ethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene and 1-ethyl-4-methylbenzene, which is probably the result of internal modification of benzene which is assumed to be an exogenous pollutant. The usual exhaled volatile organic compounds such as isopropyl alcohol, propylene, acetone and ethanol were detected as well. The most common identified exogenous compounds, xylene isomers, styrene and furan derivatives, were found as well. In this preliminary investigation, we found 34 substituted compounds in lower or higher frequency of occurrence. Some of the compounds like 1,1,2,2-tetrachloroetane, 1,2-dibromoethane and hexachloro-1,3-butadiene, were found in more than 90% of the population. The presence of not previously detected substituted compounds in exhaled breath of humans requires further detailed investigation.

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