

A NEW APPROACH TO IDENTIFICATION OF BIOMARKERS FOR EARLY CANCER STAGE DETECTION

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Abstract: Gas chromatography and mass spectrometry (GC/MS) was applied for determination of concentrations of volatile organic compounds present in human breath samples. The technique allows for rapid determination of compounds in human air, at the level of parts per billion. It showed linear correlations ranging from 0.83-234.05 ppb, limit detection in the range of 0.31-0.75 ppb and precision, expressed as RSD, was less than 10.00%. Moreover, trained dogs are able to discriminate breath samples of patients with diagnosed cancer disease. We found a positive correlation between dog indications and the content of ethyl acetate and 2-pentanone in breath ($r=0.85$ and $r=0.97$, respectively).

Key words: volatile organic compounds, breath analysis, gas chromatography-mass spectrometry, canine olfaction

1. Introduction

Lung cancer belongs to most often of malignant tumors being a main cause of death both men and women in industrialized countries. The predominant factor of lung cancer is active and passive smoking because cigarette smoke contains about 200 substances with influence carcinogenic and mutagenic. Other common initiator cause of lung cancer belongs to exposure to radon, cadmium, arsenic, beryllium, asbestos (MCWILLIAMS *et al.*, 2002; BAKER, 2006). Lung cancer is classified into two broad groups: small cell lung carcinoma (SCLC) (20-25% frequency of occurrence) and non-small cell lung carcinoma (NSCLC) (70-75%). The latter category includes adenocarcinomas (25-30%), squamous cell (30-35%) and large cell carcinomas (10-15%). This classification takes into account histological type of lung cancer as well as facilitates method of treatment and prognosis of disease (COLLINS *et al.*, 2007; Yao *et al.*, 2010). In the recent years scientific interest in search of non-invasive techniques, painless and agreeable for patients which would facilitate diagnosis of early-stage of lung cancer without necessarily using invasive medical routine has been increased. Exemplary denouement could be analysis of breath which has numerous advantages in comparison with traditional diagnostic methods (PRADO, *et al.*, 2005; LIBARDONI, *et al.*, 2006). The determination of volatile organic compounds (VOCs) in exhaled breath air can provide valuable information about the condition of human health. Presence of VOC in breath air at the trace levels makes breath analysis difficult,

therefore, as the sampling technique, solid phase microextraction (SPME) (YU, *et al.*, 2005) and thermal desorption (TD) (HRYNIUK, *et al.*, 2009) are mainly applied. After sampling of VOCs, identification of the components is performed most often by using: gas chromatography-mass spectrometry (GC/MS) (DENG, *et al.*, 2004), selected ion flow tube-mass spectrometry (SIFT-MS) (SPANEL, *et al.*, 1996), proton transfer reaction-mass spectrometry (PTR-MS) (WARNEKE, *et al.*, 1996). An innovative, unconventional method is applied the dog smell to detect changes in carcinoma. In dogs and other macrosmatic animal species (e.g. rats, pigs) that are characterized by a well developed sense of smell, the area of olfactory epithelium in the nasal cavity is much larger, compared to in microsmatic species, including humans (Fig. 1, BUSZEWSKI *et al.*, 2012). Dogs could be used for the detection of different kinds of neoplastic diseases, e.g. melanoma, lung, breast, prostate, or ovarian cancer (HORVATH, *et al.*, 2008; PICKEL, *et al.*, 2004; WILLIS, *et al.*, 2004; MCCULLOCH, *et al.*, 2006). Sniffer dogs' employment has some advantages when compared to contemporary analytical methods for the identification of VOCs such as chromatography or mass spectrometry (GC/MS). In this research work, experiments which involved using trained dogs in order to detect odour markers of lung cancer in breath samples are presented. Additionally, the results obtained by dogs recognition are compared with those obtained by the GC/MS.

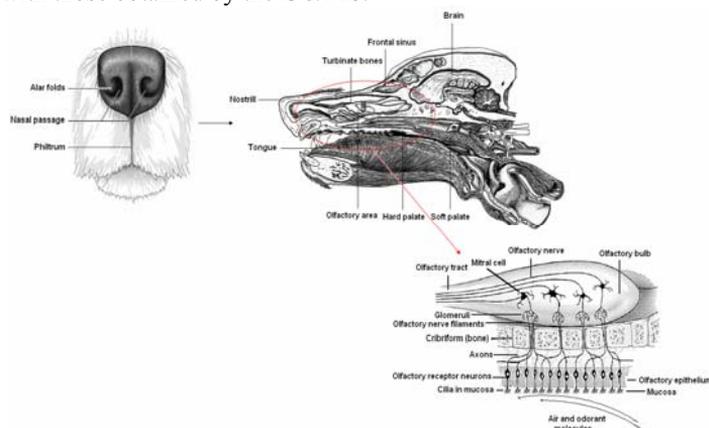


Fig. 1. Canine sense of smell (BUSZEWSKI *et al.*, 2012).

2. Materials and methods

2.1 Materials and reagents

The GC-TOF/MS analysis was performed on gas chromatograph 7890 A (Agilent, Waldbronn, Germany) coupled with spectrometer TruTOF (Leco, St. Joseph, MI, USA) equipped with CP-Porabond-Q (Varian Inc., Middelburg, The Netherlands) 25 m × 0.25 m × 3 μm column. Oven temperature program was as follows: initial 40 °C held for 2 min, then ramped at 10 °C/min to 140 °C and the ramped at 5 °C/min to 270 °C and held for 5 min. The temperature of the split-splitless injector was 200 °C.

Acquisition was performed at mass range m/z 30 – 300, acquisition rate 30 spectra/sec. Spectra were collected at electron ionization (EI) 70 eV, both ion source and line transfer temperatures were set to 200 °C. The acquisition of chromatographic data was performed by means of Chroma TOF software (Leco). Carboxen/Polydimethylsiloxane coated fibre (Supelco) was used for the SPME method. All the chromatographic standards (aldehydes, alcohols, hydrocarbons and ketones) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2 Breath collection

Breath samples were collected from 44 healthy volunteers and 29 patients with lung cancer (including 18 people with SCLC and 11 with NSCLC and volunteers). Breath samples were collected in Department of Lung Diseases, Collegium Medicum, Toruń. The study was approved by the local ethic commission. In each patient's case, a questionnaire on cancer and its stage was filled. Data such as age, sex, other diseases, prescribed drugs, smoking habits, and information about previously consumed meal were also collected.

The alveolar breath samples were collected by means of a CO₂ controlled sampler (Department of Anesthesiology and General Intensive Care, Innsbruck Medical University, Innsbruck, Austria) in a 1 L Tedlar bags. Before collection of breath, all bags were cleaned by flushing with argon gas and then filled with argon and heated at 60 °C for 12 h to remove any contaminations. Afterwards, a 200 ml sample was transferred into second bag. The SPME fibre was introduced into the bag through the septum and exposed to a sample. After 15 min. of extraction, the SPME fibre was desorbed in the hot GC injector port for 2 min at 200 °C. Ambient air samples were taken for blank measurement.

2.3 Standard preparation

A gaseous standard was prepared by injecting 1 or 3 µL of liquid compounds into 1 L glass bulb. The liquid was then evaporated. Next, the mixture was subsequently diluted in Tedlar bags filled with nitrogen to obtain concentration in the range 0.5 – 200 ppb.

2.4 Experiments with the use of sniffing dogs

In tests were used male dogs (German shepherd mix) that successfully underwent a three-phase training in the scent line-up were used for the lung cancer detection. The dogs were 20-22 months old.

The experimental procedure and keeping conditions for the dogs were approved by the Local Ethical Commission for Animal Experimentation in Warsaw. For testing with the use of dogs, breath odour samples were collected from the same donors and at the same time as for the GC-MS analysis. The breath samples for the canine training and detection were taken by exhaling 2-3 times through disposable polypropylene sampling tubes (Defencetek, Pretoria, South Africa) 15 cm long and 3 cm in diameter.

In order to test the samples, the removable insertions were taken out of the tubes and put into sterile polypropylene boxes covered with hole-punched lids to prevent a direct contact of a dog's nose with the insertion, salivation, etc. One breath odour sample taken from patients with lung cancer was placed in a line-up together with four samples from healthy volunteers (controls). The dogs were taught to indicate the lung cancer sample by the sitting-down response in front of the sample. The trials with dogs were repeated approximately 30 times on different days. The positions of odour samples in the line-up were randomly changed for every trial. In order to prevent suggesting answers to the dogs during detection, the experimenter was invisible to the dog, and the dog handler was not aware of the cancer sample position in the line-up.

3. Results and discussion

3.1 Validation of the method

The relative standard deviation (RSD) was in the range from 3.3 % to 9.5 % for hydrocarbons, alcohols, aldehydes, ketones and aromatic compounds. A calibration curve was linear for aliphatic hydrocarbons (pentane, hexane, 2-methylpentane, 3-methylpentane) in the range 0.9-150.0 ppb, for alcohols (1-propanol, 2-propanol) 1.6 - 163.5 ppb, for aldehydes (butanal, propanal, 2-methylpropanal) 1.3 - 170.4 ppb, for ketones (acetone, 2-butanone, 2-pentanone) 1.3 - 166.4 ppb, for aromatic compounds (benzene, toluene, ethylbenzene, o-xylene) 1.00 - 165.61 ppb. Regression coefficient values were high reaching at least 0.991. The lowest LOD values obtained for hydrocarbons and aromatic compounds varied from 0.31 to 0.49 ppb.

3.2 Exhaled air

All the compounds detected in breath samples were compared to ambient air samples, and only those compounds showing concentration values at least 10 % higher than those in ambient air. The concentration of pentane, which is regarded to be an oxidative stress marker, ranged from 6.8 to 14.3 ppb in the case of healthy people and from 0.7 to 17.5 ppb in the case of patients with cancer. Furan and its derivatives are considered to be smoking status markers. 1-propanol was observed in breath of patients with cancer at concentration values in the range 4.37 – 13.15. Moreover, 2-propanol was found in healthy and ill people's breath samples. Additionally, its concentrations in exhaled and ambient air were on a similar level.

The original data from VOCs measurement did not meet the normality assumption of parametric ANOVA and even log transformation did not managed to produce a normal distribution for some compounds. Therefore, the alternative nonparametric ANOVA (Kruskal–Wallis test) was used to verify the null hypothesis assuming that three studied groups (cancer patients, control smokers, and control of non-smokers) came from the same population. This measures the probability that a random observation from one group is the same as a random observation from another group. The value of the χ^2 test confirms the significance of an observation obtained in the Kruskal–Wallis test. For 12 substances the hypothesis of uniform concentrations of VOCs in patients' breath and in that of healthy controls could be rejected. For butanal,

2-butanone, ethyl acetate, ethyl benzene, 2-pentanone, 1-propanol, and 2-propanol the tendency of greater concentration in the breath of cancer patients than in controls was found to be significant at $P < 0.001$.

3.3 Dog experiments

Detection sensitivity and specificity were calculated by use of a yes/no response criterion toward each sniffed sample in the line-up, i.e. at 50 % probability of getting the correct response by chance. To evaluate differences in dogs' indications between cancer and control samples, and the results obtained from particular dogs, the χ^2 test was used. The dogs indicated correctly the pattern of breath samples from lung cancer patients with detection sensitivity and specificity of 82.2 % and 82.4 %, respectively. False positive indications toward healthy controls amounted to 17.8 % of trials. The differences between dogs' indications of cancer samples vs. controls were highly significant ($\chi^2 = 1056$, $P < 0.000$). There were significant differences between dogs in detection sensitivity ($\chi^2 = 25.17$, d.f = 1, $P < 0.001$) but no significant differences in detection specificity.

3.4 Correlation between a dog's indications and chemical analysis

The data concerning the dogs' indications were analysed. We tried to find the correlation between the VOCs in exhaled air and the dogs' indications. Two data sets, for control people ($N = 49$) and for patients ($N = 29$), contained the percentage of a dog's indications and the chosen compounds. For the patients group, positive Pearson's correlations between the dogs' indications and VOCs content in breath air exhibited significant positive or negative tendency, e.g. ethyl acetate and 2-pentanone positively correlated with the dog's indications positively ($r = 0.85$ and $r = 0.97$, respectively), whereas for acetonitrile, propanal, and 1-propanol, the contents were negatively correlated with the dog's indications ($r = -0.78$, $r = -0.87$ and $r = -0.98$ respectively).

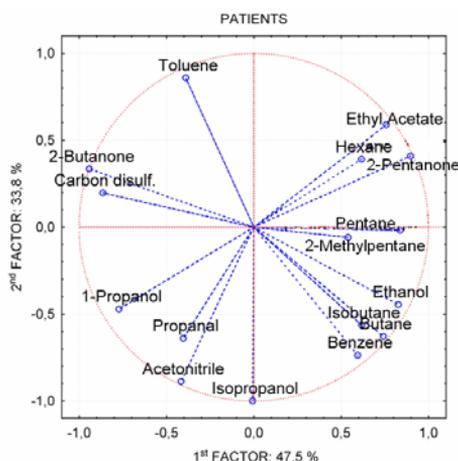


Fig. 2. Classification compounds by PCA.

Two multivariate methods, i.e. factor analysis (FA) and principal component analysis (PCA), were applied for data calculation. Data of each dimension were standardised within the individuals and, to obtain a meaningful structure of principal components, the number of factors has been finally limited to two. Factor analysis with varimax rotation was carried out as a first approach to the datasets. These two factors explained over 80% of the total variation. Factor 1 gave positive signs of loading coefficients for ethanol, isobutane, butane, isoprene, pentane, and benzene, whilst negative signs were apparent for carbon disulfide, 2-butanone and toluene. A principal component analysis (PCA) was also used for the classification to show the relations between VOCs and the dog's indications towards two components (Fig. 2).

4. Conclusions

Trained dogs are able to discriminate breath samples of patients with diagnosed cancer disease from those of healthy donors at a „better than by chance” rate. The canine method has the following advantages: dog training and testing is relatively simple and inexpensive in comparison to analytical equipment application (GC/MS) and relatively high and easily interpretable detection sensitivity and specificity in relation to pattern samples were ascertained.

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