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Complete List of Authors:	Capone, Simonetta; Istituto per la Microelettronica e Microsistemi Sezione di Lecce Tufariello, Maria; Consiglio Nazionale delle Ricerche Istituto di Scienze delle Produzioni Alimentari Unita Organizzativa di Supporto di Lecce Forleo, Angiola; Istituto per la Microelettronica e Microsistemi Sezione di Lecce Longo, Valentina; Istituto per la Microelettronica e Microsistemi Sezione di Lecce Giampetruzzi, Lucia; Istituto per la Microelettronica e Microsistemi Consiglio Nazionale delle Ricerche Sezione di Lecce Radogna, Antonio; Istituto per la Microelettronica e Microsistemi Sezione di Lecce Casino, Flavio; Istituto per la Microelettronica e Microsistemi Sezione di Lecce Siciliano , Pietro ; Istituto per la Microelettronica e Microsistemi Sezione di Lecce
Keywords:	SPME-GC/MS, breath analysis, exhaled VOCs, smoking biomarkers

Chromatographic analysis of VOC patterns in exhaled breath from smokers and nonsmokers

Simonetta Capone[1,*], Maria Tufariello[2], Angiola Forleo[1], Valentina Longo[1], Lucia Giampetruzzi[1], Antonio Vincenzo Radogna[1], Flavio Casino[1], Pietro Siciliano[1]

[1] National Research Council - Institute for Microelectronics and Microsystem (C.N.R.-I.M.M.), Lecce, Italy

[2] National Research Council - Institute of Sciences of Food Production (C.N.R.-I.S.P.A.), Lecce, Italy

* **E-mail**: simonetta.capone@cnr.it, phone: +39-0832-422507

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ABSTRACT

Cigarette smoking harms nearly every organ of the body and causes many diseases. The analysis of exhaled breath for Volatile Organic Compounds (VOCs) can provide fundamental information on active smoking and insight into the health damage that smoke is creating. Various exhaled (VOCs) have been reported as typical of smoking habit and recent tobacco consumption, but to date, no eligible biomarkers have been identified. Aiming to identify such potential biomarkers, in this pilot study we analysed the chemical patterns of exhaled breath from 26 volunteers divided in nonsmokers and smokers sampled at different periods of withdrawal from smoking. SPME-GC/MS method were applied.

Many breath VOCs were identified and quantified in very low concentrations, but only a few (toluene, pyridine, pyrrole, benzene, 2-butanone, 2-pentanone, 1-methyldecylamine) were found to be statistically significant variables. Probit prediction model based on statistical relevant VOCs-patterns (instead of individual VOCs) showed that the assessment of smoking status is heavily time dependent; it's possible recognise with high specificity and sensitivity smokers after a short-term exposure to tobacco (i.e. after 1 hour of smoking abstinence), whereas smokers after a long-term exposure to tobacco (i.e. after a night out of smoking) are more like nonsmokers.

1. Introduction

Smoking is the inhalation of tobacco smoke containing over 4000 chemical compounds including toxic and carcinogenic constituents (Rodgman et al. 2013; Hecht, 2011). The harmful effects of tobacco smoking both for active and passive smokers are well known; active smoking is the main risk factor and independent predictor of short survival in lung cancer, and involuntary exposure to environmental tobacco smoke, i.e. passive smoking, has been extensively investigated with respect to its potential health effects (U.S. Dept. of Health and Human Services, 2014; Hakim et al., 2012; Hecht, 2011). Many more Volatile Organic Compounds (VOCs) can be found in the exhaled breath of smoking and passive smoking persons than in breath samples from nonsmokers. The origin of these VOCs is mainly exogenous due to the inhalation of tobacco smoke, but they are also produced in the body as result of a metabolic defence response of the body to the irritant effects of smoking and of the oxidative stress processes induced by cigarette constituents (endogenous origin) (Rodgman et al., 2013; Talhout et al., 2011; Piadé et al., 2013). In particular, cigarette smoking influences the levels of nitric oxide (NO) and carbon monoxide (CO) in breath, during smoking; NO concentration decreases and CO increases, whereas after just 1 hour quit smoking, the NO and CO levels will usually return to pre-exposure values (Nadif et al. 2010; Kendrick 2013). Also many other exhaled VOCs, as alcohols, aldehydes, ketons, sulphur compounds, hydrocarbons

and nicotine-derived products, may be present in higher concentrations in the exhaled breath of smokers as compared to nonsmokers (Phillips, 2004).

The effects of smoking are cumulative over time and quietly a health status threshold can be overcome. The mechanisms of disease induction by tobacco smoke have well studied (U.S. Dept. of Health and Human Services, 2014). Tobacco smoke produces fine particulate matter that deposit on alveoli. Since nicotine creates addiction, it leads to prolonged exposure to tobacco smoke. In addition, smokers may increase the depth of inhalation and hold the smoke in their lungs longer to increase nicotine uptake. When smokers inhale smoke, each cigarette puff delivers a mixture of carcinogens and toxicants. These exogenous inhaled VOCs pass the alveolar epithelial barrier and enter the bloodstream by reaching cells/tissues of any organ in the body; endogenous VOCs produced by cellular metabolism return to the alveolar volumes via the blood vessels and are exhaled (Hakim, 2012).

As result of all these biochemical pathways, VOCs in alveolar air need time to decay to background or smoking pre-exposure levels (ranging from a few minutes to hours), so that the VOC pattern in exhaled breath of smokers may result altered compared to nonsmokers. A too short time elapsed from the last smoked cigarette hinders the recover to normal condition. The inhaled VOCs are retained in the alveolar volumes and trigger the above mentioned cumulative effects increasing the risk of a lot of smoking-related diseases (cancer, inflammatory processes, pulmonary emphysema, etc.). The health risk of tobacco smoking increases as the daily cigarette consumption increases (U.S. Dept. of Health and Human Services, 2014; Alonso, 2010).

In the last years, the analysis of exhaled VOCs is playing an increasingly prominent role in medical field, particularly in disease diagnostic. The complex pattern of hundreds of VOCs in the exhaled breath is a “snapshot” of the various biochemical pathways that the volatile compounds follow inside the body; it’s frequently referred as “breathprint” since it’s indicative of the health of a subject. The great expectation is that breath analysis may become a powerful non-invasive tool in

clinical practise for disease diagnostics and metabolic status monitoring (Pereira et al., 2014; Lourenço et al. 2014).

Different analytical platforms can be used for analyse the breath VOC pattern. Spectrometric technologies (GC-MS as gold standard, SIFT-MS, DMS, PTR-MS/PTR-ToFMS, FAIMS) are sophisticated instrumental tools offering a lot of advantages in terms of selectivity and sensitivity, but they are inexpensive, time consuming and cannot be easily miniaturized (Mathew et al. 2015; Rattray et al. 2014, Garcia et al. 2014). Instead e-noses are portable analytical platforms, that provides a potential, relatively cheap and easy devices for breath analysis (Garcia et al. 2014; Bikov et al. 2015).

Most of the publications on breath analysis deals with the detection of endogenous volatile biomarkers that could be linked to specific diseases as consequence of the arising of relevant pathogenic processes. On the contrary, a smaller number of works considers breath analysis as challenging method to detect inhalational exposure to toxic or noxious vapours (Jarẽno-Esteban et al. 2013; Cheng et al. 2009; Witt et al. 2011; Capone et al. 2011). It's interesting to study how smoking influences the exhaled VOC pattern in a healthy population and testing whether breath analysis can be used to distinguish between smokers and nonsmokers.

In this work, we used breath analysis for the assessment of active smoking habit and recent use of tobacco. From a clinical point of view, prediction of smoking habit and smoking abstinence is indispensable because some patients do not admit to being regular smokers and claim to smoke a smaller number of cigarettes than they actually smoked. Different exhaled VOCs have been proposed as biomarkers of smoking status (carbon monoxide, 2,5-dimethylfuran, benzene, toluene, xylene, 1,3-butadiene, acetonitrile) (Kendrick et al., 2013; Sandberg et al., 2011; Al-Sheyab et al., 2015; Alonso et al., 2010; Buszewski et al., 2009; Gordon et al., 2002; Lirk et al., 2004; Crespo et al., 2012; Jarẽno-Esteban et al., 2013), but the correlation of a single compound to smoking habit is complex and dependent on many other factors (e.g. other exogenous sources different from

cigarettes, comorbidity, dependence of compound concentration from time since smoking, large inter- and intra- individuals variability). In general, identifying which exhaled VOCs are biomarkers of a specific pathology is more complex than formerly believed, because the relation between a biomarker and a specific disease is multi-fold. Indeed, an exhaled VOC can be biomarker of several diseases, and one particular disease can be characterized by different VOC biomarkers. A specific VOC pattern, instead of individual VOCs, is probably the biomarker that most realistically represents a specific morbidity (Pereira et al.,2014; Lourenço et al., 2014).

In particular, in relation with possible smoking biomarkers, exhaled carbon monoxide is a simple marker that can be measured by commercial end-user devices for the assessment of smoking cessation, but the correlation the correlation between decreasing CO levels in exhaled breath and reducing tobacco consumption is not so good. What limits the usefulness of CO as a biomarker of smoking habit is that it can also come from sources different from smoking. In addition, the actual level of CO (8 ppm or less) indicating that a patient continues to smoke is controversial. To date, no reliable cut point has been established to allow differentiation between smokers and nonsmokers (Kendrick et al., 2013; Sandberg et al., 2011; Al-Sheyab et al., 2015).

According to what reported in literature, toluene and xylenes may be already present in indoor air, and they have been found in the breath of light smokers and nonsmokers; they show a heavy time-dependence after smoking and a large variability from subject to subject, so that they could be smoking markers only for heavy smokers and for recent use of tobacco (Alonso et al., 2010). Benzene, which is carcinogenic, is more significant since it's frequently identified in higher concentrations in the breath samples of smokers compared to non-smokers. For this reason, it has been proposed as potential marker of smoking, but it also may come from other sources of air pollution (traffic, combustion process). Moreover, during smoking benzene rapidly increases in exhaled breath of smokers and after smoking it declines to values similar found in nonsmokers (within about 1 hour); it may be used only for detect recent use of tobacco (Buszewski et al., 2009; Alonso, 2010).

Cigarette smoke causes also an exposure to 1,3-butadiene, that is in B2 group like a probable human carcinogen with a unit risk factor 30 times higher than benzene. 1,3-butadiene shows short residence times (less than 30 min after smoking) in the body, so that also this compound cannot be useful as effective marker for smoking habit (Hecht, 2011; Gordon et al., 2002).

Increased levels of acetonitrile, a constituent of cigarette smoke, were found in the breath of smokers, but the studies that validate its use as marker of active smoking behaviour are still few (Phillips,2004; Buszewski et al., 2009; Lirk et al., 2004; Crespo et al., 2012).

Other authors studied the presence of aldehydes and carboxylic acids in the exhaled breath of healthy population (nonsmokers, ex-smokers and smokers): differently to previously mentioned VOCs, the metabolic origin of these compounds are known to derive from oxidative stress processes of cells. They found that nonanal in exhaled breath is associated to the status of being a smoker or ex-smoker, independently of the age, gender and amount of tobacco smoked (Jarẽno-Esteban et al., 2013).

This work was intended to contribute to the discussion: 1) which is the medical significance of VOCs in exhaled air?, 2) what are the differences between the exhaled breath of smokers and nonsmokers?, 3) which VOCs can be smoking biomarkers?

Here, solid-phase microextraction (SPME) and gas chromatography coupled with mass spectrometry (GC/MS) was used for the analysis of volatiles in breath. We identified and quantified the VOCs present in the alveolar breath samples of groups of nonsmokers and subgroups of smokers sampled at different periods of withdrawal from smoking (after 1 hour abstinence and 1 night out of smoking), and we used the patterns of VOCs concentrations to perform statistical analysis (Mann-Whitney test, predictive Probit model) in order to asses smoking habit.

2. Experimental

2.1 Subjects

Breath analyses were conducted in 26 healthy adult volunteers (10 nonsmokers and 16 smokers) recruited within the university campus in Lecce, Italy. All smokers aren't heavy smokers (<10 cigarettes/day). The smokers individuals were asked to fill three bags in three different times: a) in the morning, fast, 1 night out of smoking and before smoking the first cigarette of the day (1° bag) ("blank" smokers), b) after 1 hour abstinence of smoking the 1st cigarette of the day (2° bag), c) after 1 hour of abstinence of smoking the nth- cigarette of the day (3° bag). The collected bags from subgroups of smokers were labelled as Fi_jbag (i=1,…,16, j=1,2,3). Nonsmokers individuals were asked to fill one bag in the morning, fast; the codes for nonsmokers bags were NFi, i=1,…,10. We collected total 58 bags from smokers and nonsmokers individuals.

The study was designed to assess whether the group of all smokers (Fi_jbag, i=1,…16, j=1,2,3), can be distinguished from the group of nonsmokers (NFi, i=1,…,10). The sub-group of "blank" smokers (Fi_1bag, i=1,…,16) was also compared with the nonsmokers group (NFi, i=1,…,10), in order to evaluate if the smoking habit in regular smokers can be detected in "blank" conditions, i.e. after the longest time of abstinence of smoking in a day, i.e. 1 night out of smoking. Finally, the sub-group of smokers after the 1st and the nth- smoked cigarette of the day (Fi_2bag + Fi_3bag, i=1,…16, 1 h smoking abstinence) was compared with the subgroup of "blank" smokers (Fi_1bag, i=1,…,16, 1 night out of smoking) in order to assess the recent use of tobacco for a regular smoker. A scheme of the study design and groups definition was shown in figure.

2.2 Breath sampling

We sampled breath of the volunteers by a commercial breath sampling system (QUINTRON, USA). The system consists in a special 3-way valve and two bags, one (discard bag, 250 ml) discards the expired air contained in the first respiratory airways (dead-volume) and the other (collecting bag, 750 ml) collects the only alveolar air (end tidal). When the discard bag is full, a

membrane-valve opens automatically and the collecting bag fills with the alveolar air. A 1-way stopcock (into which a SPME septum was fitted) was inserted into the Luer port on the collecting bag. The volunteer was asked for doing a single deep exhalation through the mouthpiece of the breath sampling system.

2.3 Analytical procedure

The GC-MS analysis was performed on Agilent 5973 mass spectrometer (MSD) coupled with 6890N series gas chromatograph (Agilent Technologies) with a split-splitless injector. The injector temperature was 250 °C. A DB-WAX capillary column (60 m, 0.25 mm I.D., 0.25 μm thickness) was used. The oven temperature program was as follows: initial 40 °C held for 5 min, ramped at 3 °C/min to 140 °C held for 10 min, next ramped at 10 °C/min to 230 °C and held for 3 min. Electron impact ionization was applied at 70 eV. The MS analyses were carried out in full-scan mode with a scan range 30-500 amu at 3.2 scans/s. The identification of the volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST 98, P > 80%). A manual SPME holder with a SPME fiber assembly Carboxen/Polydimethylsiloxane (CAR/PDMS) from Supelco (black fiber, Supelco, Bellefonte, USA) was used. Internal standard (I.S. bromobenzene, 23 ppbv) was added by a suitable multiple-steps dilution into the collecting bag after breath sample collection in order to apply semiquantitative analysis method to evaluate the concentrations of the identified volatile compounds. Since the breath exhaled at the body temperature is highly humid, it inevitably condenses on the colder inner walls of the collecting bag; it was necessary to slightly heat the bag in order to return the condensate breath to the vapour phase. This was realized by firmly placing the collecting bag onto a by a heating plate at 38°C, during the exposure of the SPME fiber for 30 min.

2.4 Statistical analysis

Statistical analysis was performed using STATISTICA software. For descriptive statistics, two-sided testing and median with interquartile (IQR) range were used. Non parametric Mann-Whitney test was preferred in alternative to *t*-test for independent samples. By Mann-Whitney test, statistical significance of differences between the two compared groups (Figure 1) was assessed in terms of most significant variables (VOCs), and a $p<0.05$ was considered significant. Further a Probit model was used as non-linear regression model for binary responses; the method is more suitable to breath metabolomics data that typically show nonlinear patterns. This model describes the relationship between one or more continuous independent variable(s) to dichotomous (binary) variables, here corresponding to breath VOCs and binary categorical variables (i.e. the two compared groups) respectively. In particular, we retained all and only the statistical relevant variables highlighted by Mann-Whitney test, and we evaluated how this VOC pattern can predict a correct class membership.

3. Results and discussion

Eighty-three different compounds were identified in the collected breath samples. Their concentration was calculated by normalizing picks areas to internal standard area (I.S.) and referring to its concentration (Table 1). They belong to different classes of compounds: alcohols, aldehydes, ketons and hydrocarbons. The breath patterns of the group of the nonsmokers and the subgroup of "blank" smokers sampled in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day, were found less rich in VOCs than breath pattern of the subgroup of smokers after 1 h from smoking the 1st and the nth cigarette of the day. At a first qualitative analysis of data distribution in the sample population, we observed an increased occurrence of heterocyclic aromatic organic compound and alkanes hydrocarbons in the group of smokers. Given that the main objective of this study was to evaluate which VOCs can allow a real determination of smoking habit and/or smoking event, we performed Mann-Whitney test on data set

by groups. By applying Mann-Whitney test the most informative features regarding the statistical differences between the two groups under analysis were filtered.

First, we consider the whole group of smokers regardless of whether they had smoked recently or not (*analysis 1*). In *analysis 1,* the complete group of the smokers sampled three times in a day Fi_jbag (i=1,…,16; j=1 in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day; j=2 smokers after 1 h from the 1st smoked cigarette of the day; j=3 smokers after 1 h from the nth smoked cigarette of the day) was compared to the group of nonsmokers NFi (i=1,…,10). By Mann-Whitney test, the VOCs found to be variables with statistical relevance ($p<0.05$) in analysis 1 were: a) toluene, b) pyridine, c) pyrrole. Descriptive statistic results (median, 25th-75th percentiles, minimum/maximum) for these compounds were respectively shown in Figure 2a, 2b, 2c for nonsmokers and smokers in order to easily compare the differences between the two groups. A trend in higher occurrence and higher concentrations in the complete group of smokers were registered for these VOCs in our sample population. The presence of toluene, pyridine and pyrrole in human breath was confirmed by literature (Alonso et al., 2010; Schmidt et al., 2015; Pennazza et al., 2010; Ji et al., 2002; Mansoor et al., 2014; Kapishon et al., 2013; Bazemore et al., 2006; Marco et al., 2015; Filipiak et al., 2014; Hanai et al. 2012; Talty et al., 2013). Moreover, in next analysis (*analysis 2*), the sub-group of the "blank" smokers Fi_1bag (sampled in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day) was compared with the group of nonsmokers NFi; by this comparison the Mann-Whitney test didn't show any VOCs really statistically significant. We can mention compound nonane, 2,3-dimethyl ($p=0.093$), an alkane found more in smokers than in nonsmokers (Figure 2d).

Toluene is an aromatic hydrocarbon widely used as an industrial feedstock and as a solvent, and it's one of the many compounds found in cigarettes (Hecht, 2011; Rodgman et al., 2013; Talhout et al., 2011). It's thus commonly identified in breath samples independently of the source (Schmidt et al., 2015; Pennazza et al., 2010). Toluene is classified as an intoxicative inhalants, but although less

dangerous than benzene, under the Guidelines for Carcinogen Risk Assessments (US. EPA, 2005), the EPA considers that there is an inadequate information to assess the carcinogenic potential of toluene. We identified toluene in greater amount in the smokers breath (Figure 2a).

Pyridine is a basic heterocyclic organic compound used as a precursor to agrochemicals and pharmaceuticals; it's also an important solvent and reagent as well as a cigarette constituent (Hecht, 2011; Rodgman et al., 2013). It's known it causes damage to cells (Ji et al., 2002; Mansoor et al., 2014). Pyridine was also proposed as a VOC marker for exposure to tobacco smoke (Kapishon et al., 2013); other work identified pyridines and pyrazines as compounds responsible for the typical malodour of smokers breath, being likely generated during cigar pyrolysis by cleavage of nicotine or by Maillard reaction (Bazemore et al., 2006).

Pyrrole is another heterocyclic aromatic organic compound typically listed as a chemical constituent in cigarettes and cigarette smoke (Hecht, 2011;Rodgman et al., 2013; Talhout et al., 2011; Piadé et al., 2013; Marco et al., 2015). It has been detected both in the exhaled breath of lung cancer patients and in the headspace of cancer cells *in vitro* (Filipiak et al., 2014; Hanai et al., 2012). Abnormal concentrations of pyrrole are also related to a less known *Pyroluria*, or *pyrrole disorder*, a metabolic condition with many physical and psychological symptoms, suffered by 10% of the population (Talty et al., 2013), it's caused by stress that depletes the body of certain vitamins (mainly vitamin B6) and minerals (mainly magnesium and zinc) before they are able to be absorbed.

By considering the VOC pattern with statistical relevance ($p<0.05$) for analysis 1 (toluene, pyridine, pyrrole), we performed a Probit Regression by comparing the nonsmokers with the complete group of smokers. The predictive results with a good significance level p ($p=0.0043$) are shown in Table 2. The classification test has an high sensitivity (100%) but a low specificity (20%) due to the high number of false positives. The sensitivity measures the percentage of positives that are correctly identified as such (i.e. here the smokers who are correctly identified as such), whereas

the specificity measures the percentage of negatives nonsmokers that are correctly identified as such (i.e. here the nonsmokers who are correctly identified as such). The test is even null specificity if we consider the "blank" smokers compared to the nonsmokers, whereas the sensitivity is optimum (100%) (analysis 2, table 3). In both data analysis, the high number of false positives may be influenced by the small sample size of this pilot study, and of course a more extended experiment on larger population could better show the small relations between nonsmokers and "blank" smokers. However, we can argue that in our sample population, all the smokers were light smokers; the breath patterns of nonsmokers and subset group of "blank" smokers, are really quite similar, hence the classification test, that forces them to belong to two different classes in analysis 1, gives unavoidably a misclassification for nonsmokers. Prediction model based on statistical relevant VOCs-patterns showed that assessment of smoking status is heavily time dependent. Since the level of exhaled VOCs decays with time since smoking, the discrimination between nonsmokers and smokers depend on the time lag between the smoking event and the breath sample collection. A breath sampling after the longest period since smoking for a regular smoker in a day (i.e. as after 1 night out of smoking) ensures the recovery to pre-exposure or "non-smoker" condition; the "blank" smokers of our sample population are thus realistically more like non-smokers. Our highly sensitive test can thus be deemed effective only at ruling out a smoking habit when negative.

Later, we focused on how breath pattern is altered by cigarette smoking, aiming in finding VOCs markers of smoking event. At this aim we analysed data distribution within the group of smokers, by comparing the sub-group of the smokers after 1 h from the 1st and the nth- smoked cigarette of the day (Fi_2bag + Fi_3bag) with the sub-group of "blank" smokers i.e. sampled in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day (Fi_1bag) (*analysis 3*). First, Mann-Whitney test filtered the most informative features regarding the statistical differences between the two groups of smokers after 1 h from last smoked cigarette and "blank" smokers. More statistical relevant variables ($p<0.05$) compared to *analyses 1* and *2* were found: a) toluene, b) pyridine, c) pyrrole, d) benzene, e) 2-butanone, f) 2-pentanone, g) 1-methyldecylamine.

Descriptive statistic results (median, 25^{th}-75^{th} percentiles, minimum/maximum) for these compounds were respectively shown in Figure 4a,b,c,d,e,f,g. It can be easily observed that the smoking event altered the breath pattern by increasing the levels of the identified VOCs with statistical relevance. In addition to toluene, pyridine and pyrrole, another aromatic hydrocarbon, i.e. benzene known to be a cigarette smoking product and to have carcinogenic effects on health, was found in higher concentrations in the exhaled breath of smokers after smoking. Benzene is generally considered a marker of lung cancer (Alonso et al., 2010; Buszewski et al., 2009; Gordon et al., 2002; Filipiak et al., 2014). More interesting is the role of 2-butanone and 2-pentanone, that are two important ketones whose origin could be both exogenous and endogenous. Ketone bodies are physiologically produced when the body uses fat instead of glucose for energy (Buszewski et al., 2007). It was observed variation of ketones levels as a function of tumor growth in *in-vitro* cancer cells studies suggesting that ketogenetic pathways may be involved in lung cancer (Hakim et al., 2012, Kischkel et al., 2010). Several recent studies reported their increased characteristic presence in the breath of Río et al., 2015; Fu et al., 2014).

As in the previous analysis, we didn't use the 7 identified statistical relevant VOCs as individual breath markers, whereas we used the 7-VOCs chemical patterns to assess if the recent tobacco consumption (1 h abstinence from smoking). At this aim Probit Regression was applied to VOCs data pattern and a prediction of class membership to the groups of "blank" smokers and smokers after smoking was get. In this case a good prediction ($p<0.01$) was obtained with both high sensitivity (84%) and specificity (100%) (Table 4). The high sensitivity means that it's possible to correctly detect smokers, who effectively smoked 1 h before sampling, as smokers after a smoking event. The full specificity means that it's possible to correctly identify smokers, who effectively abstained from smoking before testing. Such information are fundamental for physician to assess the degree of compliance with a smoking cessation programme.

4. Conclusions

In conclusion, chromatographic measurements showed that tobacco smoking undoubtedly causes a voluntary inhalation of hundreds of harmful substances into lungs as evidenced by the alteration of breath VOCs profile. Indeed, cigarette smoking directly affects the level of several VOCs in human breath, some of which are toxic and carcinogenic, increasing the risk of lung cancer and other tumour diseases. Smokers showed higher concentrations of toluene, pyridine and pyrrole, as compared to nonsmokers. As consequence of a smoking event also the level of other VOCs, as benzene, 2-butanone, 2-pentanone, 1-methyldecylamine, increases as compared to the reference breath profile of a "blank" smoker who really observed a longer time of abstinence from smoking (i.e. 1 night out of smoking). Our statistical approach to data analysis was based on filtering the statistical relevant variables (VOCs), that highlights the differences between two classes of samples, by non parametric Mann-Whitney test, and on using the breath patterns of statistically relevant VOCs to assess the membership to the two classes under analysis by non-linear Probit regression model.

The prediction results based on our dataset showed that the assessment of smoking status is heavily time dependent. Time is required for breath VOC to reach background or pre-exposure levels ranging from a few minutes to hours. A too short time elapsed from the last smoked cigarette leads to a perturbation of the alveolar air composition; the inhaled VOCs are retained in the alveolar volumes and trigger the smoking cumulative effects increasing the risk for smoking-related diseases. Probit prediction model based on statistical relevant VOCs-patterns showed that it's possible recognise with high specificity and sensitivity smokers after a short-term exposure to tobacco (i.e. after 1 hour of smoking abstinence), whereas smokers after a long-term exposure to tobacco (i.e. after a night out of smoking) are more like non-smokers.

However, the present authors are aware of the many pitfalls that a statistical analysis can lead (confounders, false positive or "voodoo" correlations, overfitting); they admit that, up to now, the

small sample size used in this pilot study doesn't allow to establish statistically significant correlations for biomedical considerations. Larger data set are needed in order to draw the conclusion that a given VOCs pattern is a biomarker for a smoking status and/or smoking event based on a reliable pattern recognition methodology. An extended study on a larger sample population of both light and heavy smokers will be considered as well as a more detailed study of the effect of time from smoking at different sampling times from smoking.

Declaration of Interests

The other authors declare that they have no conflicts of interest.

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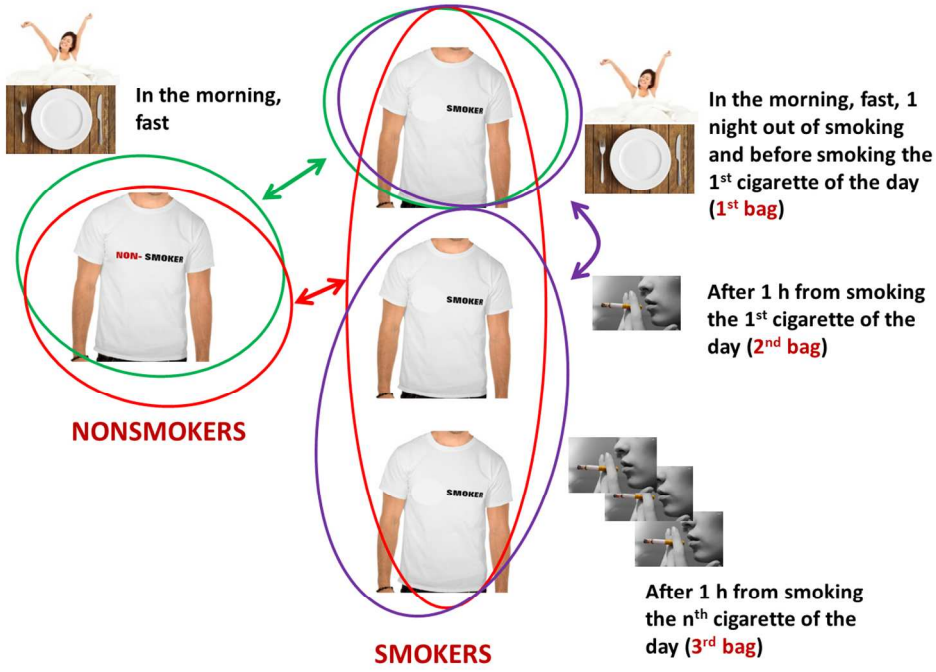
Table 1 Compounds identified in the exhaled breath of the sample population. Concentration ranges (min-max in ppbv) were reported for the groups of nonsmoker (NF), "blank" smokers (Fi_1bag) and smokers after 1 h from smoking last cigarette (Fi_2bag + Fi_3bag).

RT (min)	Compound	Min-Max (ppbv)			RT (min)	Compound list	Min-Max (ppbv)		
		Non Smokers	**"Blank" Smokers** (1 night out of smoking)	**Smokers** (after 1h from smoking last cigarette)			**Non Smokers**	**"Blank" Smokers** (1 night out of smoking)	**Smokers** (after 1h from smoking last cigarette)
7,038	2-Methyl-1,3-Butadiene (isoprene)	0 ÷ 99,9	0 ÷ 403	0 ÷ 810	31,617	Pyridine, 3-methyl-	/	/	0 ÷ 5,9
7,052	1-Methyldecylamine	/	/	0 ÷ 591	33,471	Tetradecane, 5-methyl-	/	0 ÷ 2,5	/
8,005	1,3-Pentadiene	/	/	0 ÷ 359	34,640	2-Cyclopenten-1-one	/	/	0 ÷ 3,8
9,369	Acetone	0 ÷ 33,4	0 ÷ 69	0 ÷ 96	35,295	2-Ciclopenten-2-Methyl-1-One	/	/	0 ÷ 5,3
10,570	2-Methyl Furan	/	/	0 ÷ 9,8	35,449	Acetic acid 2-Ethylexyester	/	/	0 ÷ 5,1
11,654	2-Ethyl Butanale	/	/	0 ÷ 6,4	35,975	10-Methylnonadecane	/	0 ÷ 2,6	0 ÷ 5,9
11,720	2-Butanone	/	/	0 ÷ 51,4	35,981	Heptadecane, 8-methyl-	/	/	0 ÷ 4,5
11,780	Butanale	/	/	0 ÷ 21,9	36,090	Nonanal	/	0 ÷ 1,6	/
13,062	Ethanol	/	/	0 ÷ 74,5	36,800	Cyclohexane, (1,2-dimethylbutyl)-	/	0 ÷ 6,6	/
13,611	Benzene	/	/	0 ÷ 34,7	36,812	Cyclohexane, 1,2-diethyl-, cis-	0 ÷ 2,6	0 ÷ 3,5	0 ÷ 1,8
14,656	2-Pentanone	/	/	0 ÷ 30,9	36,820	Cyclohexane, 1-ethyl-1, 3-dimethyl-, trans-	0 ÷ 4,1	0 ÷ 2,0	0 ÷ 3,2
15,045	Pentanale	/	/	0 ÷ 66,6	37,288	2-Heptene, 4-methyl-,(E)-	0 ÷ 0,9	0 ÷ 2,8	0 ÷ 0,8
15,522	Cyclopentanol	/	/	0 ÷ 8,9	38,327	Benzene1,2,3triethyl-5methyl	0 ÷ 5,1	0 ÷ 10,8	0 ÷ 1,1
15,629	Decane	/	0 ÷ 10,8	0 ÷ 4,0	38,583	Hydrazine, 1,2-dimethyl-	/	/	0 ÷ 9,5
17,687	Toluene	0 ÷ 7,5	0 ÷ 12,9	0 ÷ 22,8	39,313	Benzenamine, 4-(trifluotomethyl)-	/	0 ÷ 1,5	/
19,632	Hexanal	/	/	0 ÷ 24,6	39,545	Nonane, 3-methyl-	/	0 ÷ 2,9	/
20,169	1,3-butanediamine	/	0 ÷ 1,5	/	39,553	Furfural	/	/	0 ÷ 2,4
22,169	1,2-Dimethyl-Benzene	/	/	0 ÷ 4,3	40,006	Decane, 5,6-dipropyl-	0 ÷ 1,5	0 ÷ 5,9	0 ÷ 7,7
22,205	Ethanone, 1-cyclopropyl-	/	/	0 ÷10,6	40,013	1,3-cyclopentamedione, 2-methyl-	0 ÷ 5,9	/	0 ÷ 9,1
23,031	1-Butanol	/	0 ÷ 18,3	0 ÷ 37,1	40,016	Cyclohexanone,5methyl-2-(1methylethyl)	/	0 ÷ 12,6	/
23,043	1H-Pyrrole, 1-methyl-	/	/	0 ÷ 7,4	40,618	1-hexanol	/	0 ÷ 8,6	/
23,710	3-Heptanone	/	/	0 ÷ 8,5	40,626	1-Hexanol, 2-ethyl-	0 ÷ 12,6	0 ÷ 26,6	0 ÷ 17,9
25,563	Pyridine	/	/	0 ÷ 19,9	40,846	Decane5,6dipropyl	/	0 ÷ 8,7	/
25,771	Dodecane	/	0 ÷ 1,9	/	40,853	Nonane, 2,3-dimethyl	/	0 ÷ 6,1	0 ÷ 7,0
26,046	Cyclooctanone	/	/	0 ÷ 4,8	40,857	dl-2-Ethylhexyl chloroformate	/	/	0 ÷ 1,6
26,312	Heptadecane	/	0 ÷ 16,4	/	42,233	Pyrrole	/	/	0 ÷ 17,5
26,963	Benzene, chloro-	0 ÷ 4,1	/	0 ÷ 3,8	48,942	Pentanoic acid	/	/	0 ÷ 9,0
27,474	Pyridine, 2-methyl-	/	/	0 ÷ 2,7	43,186	Propanoic acid	/	/	0 ÷ 3,4
28,150	1-Tridecene	/	0 ÷ 4,9	/	48,972	Butanoic acid	/	/	0 ÷ 12,9
28,664	1-Pentanol	/	/	0 ÷ 17,4	49,986	Butyrolactone	/	/	0 ÷ 10,5
28,723	Methyl-Ethyl-Benzene	/	0 ÷ 4,3	0 ÷ 4,5	50,052	Cyclohexane, 1-methyl-4-(1-methylethenyl)-cis-	/	0 ÷ 9,7	/
29,120	1,3,5,7-Cyclooctatetraene	/	0 ÷ 13,5	/	52,675	Benzene, 1,1'-(1-methyl-2-butynylidene)bis-	/	/	0 ÷ 12,6
29,154	Styrene	0 ÷ 8,9	0 ÷ 18,8	0 ÷ 12,7	52,707	Hydroxy Toluene Butilated	0 ÷ 102	0 ÷ 41,6	0 ÷ 25,5
29,924	Benzene, 1-methyl-2-(1-methylethyl)-	0 ÷ 14,2	0 ÷ 3,4	0 ÷ 14,5	55,945	Phenol, 2, 4-bis(1,1-dimethylethyl)	/	0 ÷ 7,6	0 ÷ 3,2
29,925	1-Methyl-4-(1-Methylethyl)	0 ÷ 21,9	0 ÷ 19,5	0 ÷ 32,2	56,049	Diethyl Phthalate	/	0 ÷ 8,6	/
29,954	Methyl-Benzene	/	0 ÷ 33,6	/	57,446	3-Hydroxy-4-methoxybenzoic acid	/	/	0 ÷ 17,9
30,237	Nonane,2,2,4,4,6,8,8-heptamethyl-	/	0 ÷ 7,6	0 ÷ 6,7	58,490	Propanoic acid, 2-methyl-, 3-hydroxy-2,4, 4-trimethylpentyl ester	/	0 ÷ 2,5	0 ÷ 7,9
30,256	Nonane	/	0 ÷ 7,2	0 ÷ 2,1	58,845	Benzoic acid, 4-hydroxy-3,5-dimethoxy-	0 ÷ 31,8	0 ÷ 11,8	0 ÷ 4,4
30,726	2-Octonal	/	/	0 ÷ 9,1	59,699	Butylated hydroxytoluene	0 ÷ 104	0 ÷ 176	0 ÷ 125

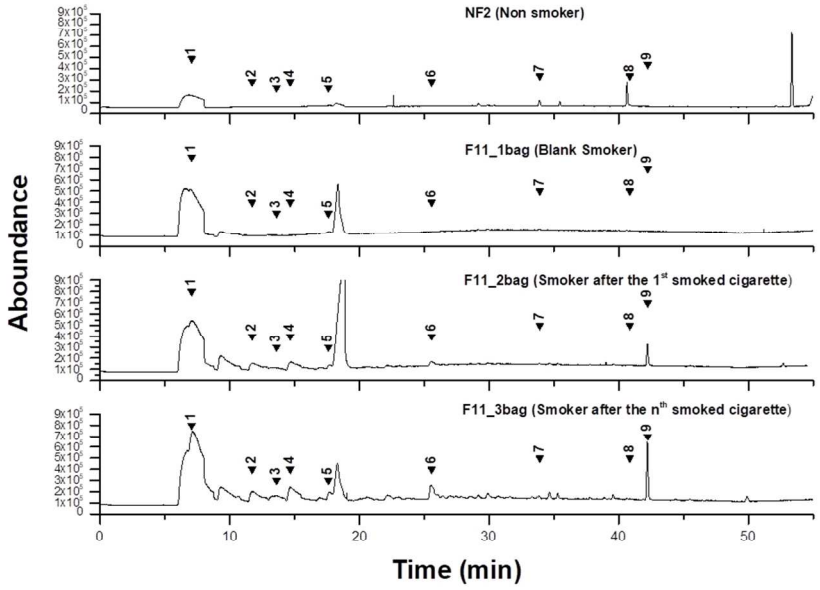
Table 2 Confusion matrix of the predictive Probit model (analysis 1, p=0.0043; analysis 2, p=0.0374; analysis 3, p=0.0000016)

	Predicted condition	**True condition**	
Analysis 1 (smoker vs nonsmoker) p=0.0043	Predicted condition positive (smoker) Predicted condition negative (nonsmoker) Accuracy (ACC)= (TP+TN)/Total population=86%	**True positive** (TP)=48 **False negative** (FN)=0 **Sensitivity** or True Positive Rate TPR=TP/(TP+FN)=100% **False negative rate** (FNR), Miss rate= FN/(FN+TP)=0%	**False positive** (FP)=8 **True negative** (TN)=2 **False positive rate** (FPR) or Fall-out =1-SPC= 80% **Specificity** (SPC) or True Negative Rate (TNR) TNR=TN/(TN+FP) =20%
Analysis 2 ("blank" smoker vs nonsmoker) p=0.0374	Predicted condition positive ("blank" smoker) Predicted condition negative (nonsmoker) Accuracy (ACC)= (TP+TN)/Total population=61%	**True positive** (TP)=16 **False negative** (FN)=0 **Sensitivity** or True Positive Rate TPR=TP/(TP+FN)=100% **False negative rate** (FNR), Miss rate= FN/(FN+TP)=0%	**False positive** (FP)=10 **True negative** (TN)=0 **False positive rate** (FPR) or Fall-out =1-SPC= 100% **Specificity** (SPC) or True Negative Rate (TNR) TNR=TN/(TN+FP) =0%
Analysis 3 (smoker after 1 h from smoking vs "blank" smoker) p=0.0000016	Predicted condition positive (smoker after 1 h from smoking last cigarette) Predicted condition negative ("blank" smoker) Accuracy (ACC)= (TP+TN)/Total population=89%	**True positive** (TP)=27 **False negative** (FN)=5 **Sensitivity** or True Positive Rate TPR=TP/(TP+FN)=84% **False negative rate** (FNR), Miss rate= FN/(FN+TP)=16%	**False positive** (FP)=0 **True negative** (TN)=16 **False positive rate** (FPR) or Fall-out= 1-SPC= 0% **Specificity** (SPC) or True Negative Rate (TNR) TNR=TN/(TN+FP) =100%



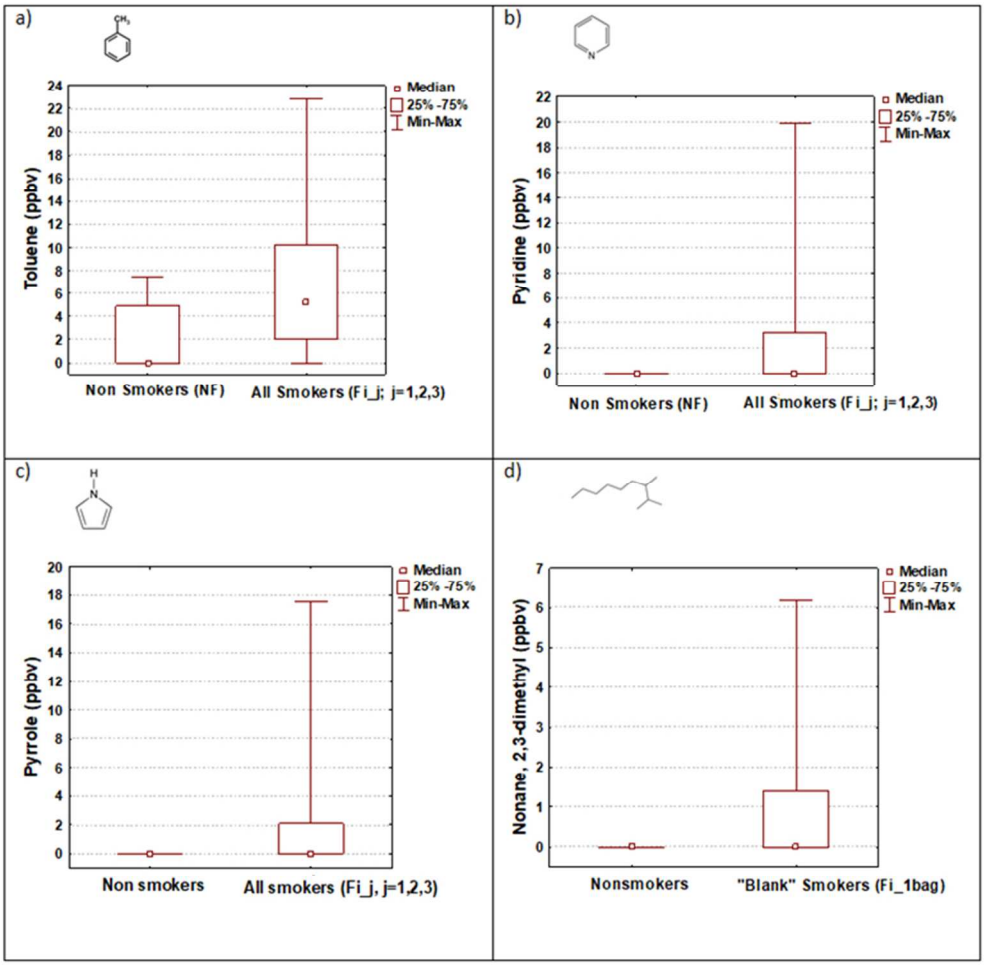
Scheme of the study design and definition of the groups. Analysis 1: the complete group of the smokers sampled three times in a day Fi_jbag (i=1,…,16; j=1 in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day; j=2 smokers after 1 h from the 1st smoked cigarette of the day; j=3 smokers after 1 h from the nth smoked cigarette of the day) compared to the group of nonsmokers NFi (i=1,…,10). Analysis 2: sub-group of the "blank" smokers Fi_1bag compared with the group of nonsmokers NFi. Analysis 3: sub-group of the smokers after 1 h from the 1st and the nth- smoked cigarette of the day (Fi_2bag + Fi_3bag) compared with the sub-group of "blank" smokers i.e. sampled in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day (Fi_1bag).

247x176mm (150 x 150 DPI)



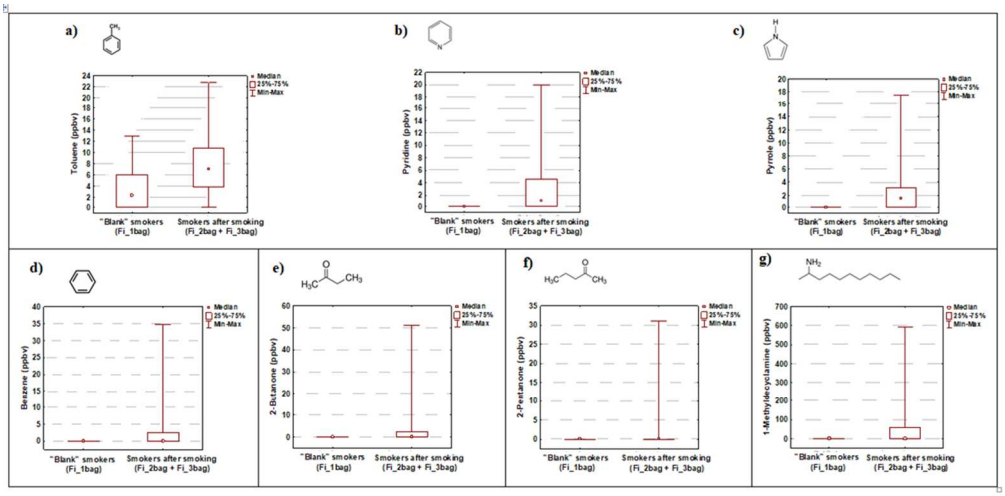
GC chromatograms of the exhaled air samples from a nonsmoker (NF2) and a smoker subject (F11), the last sampled in three different times in a day (F11_1bag: in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day; F11_2bag: after 1 h from smoking the 1st cigarette of the day); F11_3 bag: after 1 h from smoking the nth cigarette of the day. Significant VOCs: 1) 1-Methyldecylamine; 2) 2-Butanone; 3) Benzene; 4) 2-Pentanone; 5) Toluene; 6) Pyridine; 7) I.S.; 8) Nonane, 2,3-dimethyl; 9) Pyrrole.

196x140mm (150 x 150 DPI)



Box plot of exhaled significant VOCs. For analysis 1: a) toluene, b) pyridine, c) pyrrole concentrations (in ppbv) (Analysis 1: the complete group of the smokers sampled three times in a day Fi_jbag (i=1,…,16; j=1 in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day; j=2 smokers after 1 h from the 1st smoked cigarette of the day; j=3 smokers after 1 h from the nth smoked cigarette of the day) compared to the group of nonsmokers NFi (i=1,…,10)). For analysis 2: d) nonane,2,3dimethyl- from nonsmokers and "blank" smokers (Analysis 2: sub-group of the "blank" smokers Fi_1bag compared with the group of nonsmokers NFi). The bottom and top of the box are 25th and 75th percentile; the little square inside the box the median (50th percentile), and the whiskers indicate the lowest and the highest data.

138x135mm (150 x 150 DPI)



Box plot of exhaled significant VOCs. For analysis 3: a) toluene, b) pyridine, c) pyrrole, d) benzene, e) 2-butanone, f) 2-pentanone, g) 1-methyldecylamine concentrations (in ppbv) from blank smokers and smokers after cigarette smoking (Analysis 3: sub-group of the smokers after 1 h from the 1st and the nth-smoked cigarette of the day (Fi_2bag + Fi_3bag) compared with the sub-group of "blank" smokers i.e. sampled in the morning, fast, 1 night out of smoking and before smoking the 1st cigarette of the day (Fi_1bag). The bottom and top of the box are 25th and 75th percentile; the little square inside the box the median (50th percentile), and the whiskers indicate the lowest and the highest data.

208x102mm (150 x 150 DPI)