



## EVALUATION OF POTENTIAL BREATH BIOMARKERS FOR ACTIVE SMOKING: ASSESSMENT OF SMOKING HABITS

Journal:	<i>Analytical and Bioanalytical Chemistry</i>
Manuscript ID:	ABC-01761-2009.R1
Type of Paper:	Original Paper
Date Submitted by the Author:	
Complete List of Authors:	Alonso, Monica; University of Girona, Chemistry Castellanos, Mar; Dr Josep Trueta University Hospital, Neurology Sanchez, Juan; University of Girona, Chemistry
Keywords:	Air / Gases, Clinical / Biomedical analysis, Drug monitoring / Drug screening, Sampling, Thermal methods



Analytical and Bioanalytical Chemistry  
Springer

Girona, 29<sup>th</sup> January 2010

Dear Sir,

I enclose the reviewed version of the manuscript entitled "*Evaluation of Potential Breath Biomarkers for Active Smoking: Assessment of Smoking Habits*" in order that it be considered for publication in *Analytical and Bioanalytical Chemistry*.

In this study we statistically demonstrate the effectiveness of 2,5-dimethylfuran as specific breath biomarker of smoking status (a large population group of 204 volunteers has been analyzed). This compound has been able to confirm smoking status in more than 99.5% of samples, without being affected by smoking habits. The main advantage of our results is the possibility to use only the qualitative determination of 2,5-dimethylfuran to demonstrate the smoking status of a person, including very light smokers (only some cigarettes per week). The application of a new "in-house" micro-trap specially developed to determine VOCs at pptv levels in gaseous samples has allowed us to detect this compound at very low levels, some orders of magnitude below other breath studies. This has permitted us to detect this compound in all smoker breath samples, whereas other studies were only able to detect its presence after short exposure due to their higher limits of detection.

Our results open new perspectives in breath analysis as, for the first time, we demonstrate that breath can be effectively used for screening of toxic substances consumption (e.g., tobacco in this case). When specific exogenous contaminants are found, a simple qualitative analysis can confirm the consumption.

After introducing the very constructive comments and suggestions made by the reviewers, we considerer that the manuscript has improved significantly its scientific level. Moreover, the changes added has resulted in a more readable and comprehensible manuscript.

I look forward to hearing from you.

Yours sincerely,

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7 Juan M. Sánchez, PhD

8 University of Girona  
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# EVALUATION OF POTENTIAL BREATH BIOMARKERS FOR ACTIVE SMOKING: ASSESSMENT OF SMOKING HABITS

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## ABSTRACT

Different compounds have been reported as biomarkers of smoking habit, but, to date, there is no an appropriate biomarker for tobacco-related exposure because the proposed chemicals seem to be nonspecific or they are only appropriate for short-term exposure. Moreover, conventional sampling methodologies require an invasive method because blood or urine samples are required. The use of a micro-trap system coupled to GC-MS analysis has been found to be very effective for the non-invasive analysis of volatile organic compounds in breath samples. The levels of benzene, 2,5-dimethylfuran, toluene, *o*-xylene, and *m-p*-xylene have been analyzed in breath samples obtained from 204 volunteers (100 smokers, 104 non-smokers; 147 female, 57 male; ages 16 to 53 years). 2,5-dimethylfuran was always below the limit of detection (0.005 ppbv) in the non-smoker population and always detected in smokers independently of the smoking habits. Benzene was only an effective biomarker for medium and heavy smokers, and its level was affected by smoking habits. Regarding the levels of xylenes and toluene they were only different in heavy smokers and after short-term exposure. The results obtained suggest that 2,5-dimethylfuran is a specific breath biomarker of smoking status independently of the smoking habits (e.g., short- and long-term exposure, light and heavy consumption) and so, this compound might be useful as a biomarker of smoking exposure.

*Keywords: breath biomarkers; smoking; 2,5-dimethylfuran; benzene*

## Introduction

Cigarette smoke has been described as “a complex mixture of chemicals produced by the burning of tobacco and its additives” [1] or “a complex aerosol that includes a gas vapor phase and a particulate phase” [2]. The gaseous phase consists mainly of nitrogen and oxygen, the constituents of air. Several combustion products such as carbon monoxide (CO), carbon dioxide and nitric oxide are exclusively found in the gaseous phase. Compounds that are important due to their known toxicity or carcinogenic properties, such as 1,3-butadiene, formaldehyde, acetaldehyde, acrolein, benzene, hydrogen cyanide, and nicotine, are also found in this phase. Some other compounds, such as phenol and cresols, are partitioned between the particulate and the gaseous phases [3].

From a clinical point of view, there is a need to find suitable biomarkers of smoking status because some patients do not admit to be smokers and recognize to smoke a smaller number of cigarettes than real. Moreover, as smoking is a well recognized risk factor for some pathologies, including vascular and lung diseases, it is important to have a marker of smoking habit that might be used to determine the real risk of developing such a diseases. Different compounds have been proposed as possible biomarkers of exposure (“biomarkers of exposure are chemicals found in the body providing evidence of environmental exposure to that chemical or to a precursor chemical” [4]) to tobacco smoke, and several studies have tried to find a correlation between the compound chosen as a biomarker and proof of the habit of smoking [5-10]. The analysis of these compounds has been performed in different matrices including urine, blood, hair, saliva, and exhaled air. Although urine and blood are well-established matrices for the analysis of smoking related compounds, there is a delay in obtaining the results of the sampling and, in the case of blood, the test is invasive. Breath sampling presents the advantage that is non-invasive and have not the discomfort associated with the sampling of blood and urine [11-15]. Moreover, breath sample analysis is simpler since the matrix is less complex than blood or urine, no work-up is required and it can be applied to a wide range of compounds [15].

Several cigarette smoke compounds have been analyzed in exhaled breath as potential biomarkers of cigarette smoking. Since no metabolic activation occurs, the most widely analyzed biomarker in breath has been CO [16]. Alveolar CO

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3 concentrations measured prior to and after every successive cigarette indicate that  
4 CO is accumulated in the human body with repeated smoking [17]. However,  
5 there is not a good correlation between reductions in CO concentration and the  
6 reduction in smoking [18], furthermore, CO can come from sources other than  
7 smoking, limiting the usefulness of CO as a biomarker of smoking exposure.  
8 Different volatile organic compounds (VOC's) have also been proposed as  
9 smoking breath biomarkers. Benzene, which is a known carcinogen, is the most  
10 common VOC analyzed in exhaled breath as a potential biomarker of cigarette  
11 smoking. Benzene levels detected in exhaled breath of people who had recently  
12 smoked were found to be ten times greater than in non-smokers [19]. This  
13 compound has proved to be a good biomarker to indicate recent use of tobacco  
14 [20,21]. However, it has two main limitations. Firstly, benzene concentration in  
15 breath is heavily time-dependent and falls extremely rapidly reaching background  
16 or pre-exposure levels in relatively short times and, furthermore, large variability  
17 has been reported in different studies [20,22,23]. Secondly, the fact that benzene  
18 is also present in the exhaled breath of non-smokers and there are many different  
19 sources of benzene in the environment, such as cars and other pollutants, makes it a  
20 poor biomarker for the gas phase, or as an indicator of passive smoking [22].  
21 1,3-butadiene has also been proposed as a breath smoking biomarker [22] but the  
22 same disadvantage of a fast return to pre-exposure levels has been found. In fact,  
23 Sanchez and Sacks [24] found that this compound is eliminated from the  
24 respiratory tract in less than 30 minutes after smoking.  
25 In recent years, 2,5-dimethylfuran has been postulated as a strong indicator of  
26 smoking status [22,24-27] given that this compound is not normally present at  
27 detectable levels in the breath of non-smokers. Ashley et al. confirmed the  
28 discrimination power of this compound in blood samples [28]. Unfortunately, the  
29 sample size used in each study (ranging from 10 to 31 samples) makes it difficult  
30 to establish statistically significant correlations for biomedical considerations. The  
31 purpose of this study was to evaluate the behavior of different potential smoking  
32 biomarkers in breath samples and to asses smoking habits.  
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## Materials and Methods

### Breath Collection

Breath analyses were conducted in 204 healthy adult volunteers, who were randomly asked to participate in the study. Fifty-seven were men (27.9 %) and 147 (72.1 %) women, and the mean age was 24.6 years (range 16-53). All participants were either employees or students of the University of Girona. Analyses were carried out between April 15<sup>th</sup>, 2008 and November 13<sup>th</sup>, 2008. Before taking breath samples, participants were informed regarding the nature of the test and the aims, and were asked to fill in a form with supplementary information (see Supplementary Materials). For the purpose of this study and to normalize answers to the question “are you a smoker?”, a person was considered to be a smoker when he/she admitted to a smoking habit of at least one cigarette/day. When a person smoked more occasionally, the answer of “non-smoker” was required and the participant was requested to specify the extent of his/her tobacco use in the “Remarks” section.

Given that the main objective of this study was to evaluate the possibility of finding a biomarker compound in breath samples that would allow a real determination of smoking status of an individual independently of the extent of their habit, no requirements related to the time after smoking or food and drink ingestion were made prior to breath sampling.

The participants were asked to take a deep breath and then exhale into a Tedlar gas-sampling bag (SKC Inc, Eighty Four, PA, USA). Approximately 1.0 L of breath sample was obtained for each participant and it was analyzed immediately after collection (less than 2 minutes after) to avoid the loss of analytes in the bags. For each sample, 775 cm<sup>3</sup> of breath were required for the chromatographic analysis (i.e. breath samples were moved through the microtrap over the course of 25 minutes at a fixed flow rate of 31 cm<sup>3</sup>·min<sup>-1</sup>). Each sampling bag was cleaned with purified nitrogen several times before to collect a breath sample. In order to determine background effects due to contaminants from the walls of the bags, the last portion of nitrogen used in the cleaning process was analyzed in the same conditions as breath samples. Using this procedure, no detectable background levels of any of the target compounds were found in the Tedlar bags. Moreover, to

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3 avoid contamination of the bags from breath samples, each bag was used for a  
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5 maximum of five breath samples.  
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7 It is known that significant changes in the concentrations of specific molecules in  
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9 breath take place as a result of variations in ventilation parameters during  
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11 sampling, and hyperventilation may occur when a breath sample is collected from  
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13 spontaneously breathing subjects [29]. However, this study was not aimed at  
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15 determining the amounts of compounds from metabolic sources but rather at  
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17 developing a reliable and quantitative method with low detection limits that  
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19 allows the determination of adequate breath biomarkers in order to establish  
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21 smoking status. For this reason, spontaneously breathing subjects were used. In  
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23 order to reduce dead-space air contamination, the first 2-3 s of exhalation were not  
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25 collected in the sampling bag. Indoor air samples at the different locations where  
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27 breath samples were obtained were also collected for background determination;  
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29 2,5-dimethylfuran was not detected in any indoor sample and benzene levels were  
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31 always at equivalent or slightly larger levels than those found in non-smoker  
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33 samples.  
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### Breath Analysis

Different VOCs were selected for evaluation as possible smoking biomarkers. Benzene was evaluated given that it is the VOC which has been most frequently proposed as a smoking biomarker in the literature. Toluene and xylenes were chosen because VOC emissions by cigarette smoke are usually dominated by benzene and these compounds [30]. 2,5-dimethylfuran was included as recent studies have showed its strong correlation with smoking status [22,24-28]. 1,3-butadiene was not evaluated as it has been found that the elimination of this compound from the respiratory tract is fast and cannot be detected at the limits of detection levels of the instrumental method used in this study after 30 minutes of smoking [24].

For the analysis of breath samples, an “in-house” capillary thermal desorption device connected to a gas chromatograph (Focus GC, Thermo Scientific, Waltham, MA, USA) with mass spectrometry detection (DSQ II, Thermo Scientific) was used. The microtrap used in this study was specifically developed for the analysis of VOCs in breath samples at ppbv-pptv levels [27]. The device uses a 80-mm-long, 1.35-mm-i.d. tube made of a Ni-Co alloy (Inconel 600, Accu-

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3 Tube Corp., Englewood, CO, USA) The tube contains a graded ensemble of three  
4 commercial adsorbent materials. Each bed contains  $2.5 \pm 0.2$  mg of Carboxen 1000  
5 and Carbopack X (Supelco, Bellefonte, PA, USA) and  $5.5 \pm 0.2$  mg of Carbopack  
6 B (Supelco). The packed tube was preconditioned by heating at  $300^\circ\text{C}$  for 4 h with  
7 a continuous flow of nitrogen ( $200 \text{ mL}\cdot\text{min}^{-1}$ ). To avoid memory effects, sampling  
8 and desorption flow were in opposite directions. Adsorption of VOCs from  
9 breath samples was done at  $22 \pm 1^\circ\text{C}$ . A fast heating pulse (1 s) at  $\sim 300^\circ\text{C}$  was  
10 enough to obtain quantitative thermal desorption of all retained compounds and  
11 send them as a sharp injection plug to the chromatographic column. Component  
12 separation was achieved by the use of a 30 m length of nonpolar 5% phenyl 95%  
13 dimethylpolysiloxane column with 0.25 mm ID and 0.25  $\mu\text{m}$  film thickness (ZB-  
14 5ms, Zebron, Phenomenex, Torrance, CA). The oven temperature program was as  
15 follows:  $40^\circ\text{C}$  held for 2 min, then ramped at  $10^\circ\text{C}/\text{min}$  to  $270^\circ\text{C}$  and held for 2  
16 min. The MS analyses were carried out in full-scan mode, with scan range 40-200  
17 amu. Electron impact ionization was applied at 70 eV. Helium carrier gas was  
18 used, with a constant inlet pressure of 31 kPa, after purification for water vapor,  
19 hydrocarbons, and oxygen. The acquisition of chromatographic data was  
20 performed by means of Xcalibur software (v. 1.4, Thermo Electron). Table 1  
21 shows the list of the target compounds and details of the GC-MS analysis. The  
22 analytical equipment developed allowed the total analysis of each breath sample  
23 to be performed in less than 45 minutes.

## 24 Chemicals

25 All reagents were reagent grade (purity 99.0% or better) purchased from Sigma-  
26 Aldrich (Steinheim, Germany). Stocks were prepared in 10 L Tedlar gas-sampling  
27 bags filled with nitrogen (nitrogen 5.0 -99.9990 purity- purified for hydrocarbons,  
28 oxygen and water vapor with specific trap filters) by injecting 1 to 2  $\mu\text{L}$  of  
29 individual components. Calibration standards were prepared by taking a fixed  
30 volume of the stock gas with a gas tight syringe and diluting to 10 L with purified  
31 nitrogen in a Tedlar bag. Stocks and standards were freshly prepared for each  
32 calibration. The stability of the target compounds in the Tedlar bags was  
33 evaluated for the period used for calibration purposes (up to 3 hours): benzene,  
34 toluene and xylenes did not show any loss in this period, 2,5-dimethylfuran  
35 displayed losses lower than 5% after 3 hours. The “in-house” thermal desorption  
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unit control was provided by a 12-bit A/D board (PCI-1710HG, Advantech, Taiwan).

## Data Analysis

Statistical analysis was performed using SPSS for Windows Version 15.0. For calculations of statistical significance, two-sided testing was used and  $P < 0.05$  was considered as significant. A Kolmogorov-Smirnov test was used to study the distribution of the compounds evaluated in the samples. The results indicate that the chosen analytes do not follow a normal distribution and so the Mann-Whitney test was used to compare the values found between smokers and non-smokers. The Spearman correlation was used to determine the correlations between the levels of compounds and the daily intake of cigarettes and the time since smoking the last cigarette.

## Results and Discussion

The breath of a healthy non-smoker may contain more than 200 different VOC's [31]. Unfortunately, the majority of VOC's from tobacco smoke are also normally found in non-smokers breath, which makes it difficult to find suitable breath biomarkers for smoking status. It has been reported that all evaluated compounds in this study have exogenous origin [32]. Taking into account all references cited in the present study, 2,5-dimethylfuran is the only compound in this study that has not been reported in non-smokers breath.

Figure 1 shows typical chromatograms obtained in the analysis of breath samples from a smoker and a non-smoker. The target compounds were identified using qualifier ions and retention times. Scan detection was used in all analyses but the evaluation of these chromatograms were not adequate for complete breath analysis due to the large variation in the levels of each compound in these samples (i.e., acetone at ppmv level and other VOCs at ppbv to pptv levels). Monitoring of extracted ion chromatograms was used during data analysis to determine the target compounds.

The evaluation of indoor air (inspiratory concentrations) in different common spaces of the science faculty (those places where breath samples were usually obtained) showed that indoor levels (n=12) did not show significant differences from non-smoker breath levels for toluene, benzene and 2,5-dimethylfuran (the

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3 last has never been detected in indoor air samples). Xylenes, however, showed  
4 slightly higher concentrations in indoor air than in non-smoker samples and did  
5 not show significant differences with smoker breath levels. These first results  
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7 suggest that xylenes might not be appropriate compounds to assess the smoking  
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9 habits.

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11 Among the 204 participants in the study, 100 (49%) were smokers (72, 72%, were  
12 women and 28, 28%, men) and 104 (51%) were non-smokers (75, 72%, were  
13 women and 29, 28%, men). The concentrations of the evaluated compounds in  
14 smokers and non-smokers are shown in Table 2 and Figure 2. **These results show**  
15 **that there were significant differences in the levels of the five compounds**  
16 **evaluated between the whole population of smokers and non-smokers (Table 2).**

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18 The evaluation by gender, however, did not show any significant difference  
19 between the levels detected in male and female smokers for any of the evaluated  
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21 compounds.  
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### 24 **Effect of daily consumption of cigarettes**

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26 **The evaluation of the data supplied by the smokers population shows that** 44  
27 volunteers (44%) smoked less than 10 cigarettes/day, 19 (19%) claimed to smoke  
28 approximately 10 cigarettes/day, 27 (27%) smoked between 10 and 20  
29 cigarettes/day, and 10 (10%) smoked more than 20 cigarettes/day. There were  
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31 three people (3%) who indicated that they normally smoke at least 1 cigarette/day  
32 but the time that had passed since their last cigarette was more than 24 hours  
33 when the sample was obtained.  
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37 The effects of the daily intake of cigarettes was evaluated and a positive, although  
38 weak, significant correlation was found between the daily **number** of cigarettes  
39 smoked and the concentrations detected (Table 3). The correlations obtained gave  
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41 the best results for benzene and 2,5-dimethylfuran.  
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45 When analyzed by sex, it is interesting to note that concentrations of xylenes in  
46 men did not show any significant correlation with the parameter evaluated. In the  
47 case of women, correlations were significant for all compounds ( $r=0.314$  for *o*-  
48 xylene,  $r=0.246$  for *m-,p*-xylene,  $r=0.338$  for toluene,  $r=0.407$  for benzene, and  
49  $r=0.413$  for 2,5-dimethylfuran).  
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53 When subgroup analysis was performed and the smokers population is reduced to  
54 only light smokers ( $\leq 7$  cigarettes/day, 33 smokers), the differences were only  
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3 significant for 2,5-dimethylfuran and benzene. The results indicate a lack of  
4 specificity of xylenes and toluene as adequate smoking biomarkers, and it may be  
5 that these compounds are only useful for heavy smokers. Benzene gave better  
6 results and seems to be an effective breath smoking biomarker for light smokers.  
7 2,5-dimethylfuran was the most suitable compound to test as there were  
8 significant differences between smokers and non-smokers in all cases  
9 independently of the subgroup evaluated.

### 16 Effect of time since smoking

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19 The evaluation of the data reported for the smoking population showed that 23  
20 smokers (23%) gave the breath sample after less than 5 minutes of smoking, 19  
21 (19%) between 5 and 30 minutes after smoking, 16 (16%) between 30 minutes  
22 and 1 hour, 22 (22%) between 1 and 4 hours, 14 (14%) between 4 and 15 hours, 3  
23 (3%) between 15 and 24 hours, and 3 (3%) more than 24 hours after their last  
24 cigarette. A negative significant correlation was found between the time since the  
25 last cigarette and concentrations detected (Table 3). As in the case of cigarette  
26 consumption, the correlations obtained gave the best results for benzene and  
27 2,5-dimethylfuran.

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29 When analyzed by sex, xylenes in men did not show any significant correlation  
30 and, in the case of women, correlations were significant for all compounds  
31 ( $r=-0.339$  for *o*-xylene,  $r=-0.457$  for *m*-,*p*-xylene,  $r=-0.521$  for toluene,  $r=-0.695$   
32 for benzene, and  $r=-0.751$  for 2,5-dimethylfuran).

33  
34 These results show that levels of VOC contaminants present in the breath of  
35 smokers are affected by smoking habits. Note that information about smoking  
36 habits was obtained from questionnaire answers and, although these were  
37 anonymous, they may not always correspond to the exact conditions.

38  
39 Different studies have evaluated the effect of time since smoking on the levels of  
40 benzene in breath samples [20-23], and conclude that benzene concentration in  
41 breath is heavily time-dependent and falls extremely rapidly immediately after  
42 each puff is taken. Moreover, the time required for benzene concentrations to  
43 return to pre-exposure levels varied largely from one study to another. Gordon et  
44 al. [22] reported a time of approximately 15 minutes whereas Jordan et al. [23]  
45 found that a time of around 1 h was needed. Jo and Pack [20] evaluated two  
46 kinetic models and found a mean half-life of 38.9 min with the one-compartment  
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3 model, and 11 min for the first half-life and 235 min for the second half-life with  
4 the two-compartment model.

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7 The results obtained in the present study also showed this fast return to pre-  
8 exposure levels in breath content and it took place for the five compounds  
9 evaluated (Figure 3). The evaluation of the expiration decay curves showed that  
10 the evaluated compounds returned to pre-exposure levels in smokers in a range  
11 from 45 minutes to two hours for all samples evaluated, after which levels were  
12 equivalent to those in non-smokers. Moreover, there was large variability in the  
13 time needed to return to pre-exposure levels for each individual. It is necessary to  
14 note that all volunteers who agreed to participate in this test (n=3) had a daily  
15 consumption of cigarettes of less than 5 per day. Therefore, the results obtained  
16 cannot be used to determine that subjects with a large cigarette consumption need  
17 less than 2 hours to return to pre-exposure levels.

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20 Evaluation of the decay curves shows that there are certain differences in the  
21 behavior of the different VOCs. The curves obtained for benzene, toluene, and  
22 2,5-dimethylfuran showed the same tendency for all individuals evaluated (n=3).  
23 There was an immediate rapid fall in the levels of these compounds in the breath  
24 and thereafter a more gradual decrease. Xylene curves, however, showed different  
25 trends and there were large variations in their behavior from one subject to  
26 another. The differences in the concentration for xylenes between smokers and  
27 non-smokers were the smallest (<2 times greater in smokers), which led to a fast  
28 return to pre-exposure levels. Moreover, in some samples there was no decay in  
29 the post-exposure curves as the pre-exposure levels were at the same range as  
30 after smoking. This is explained by the fact that, as seen before, xylene  
31 concentrations detected in light smokers are frequently equivalent to those levels  
32 found in non-smokers. In these conditions, there is not a fast decay in xylene  
33 content as takes place with benzene, toluene and 2,5-dimethylfuran.

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36 Jo and Pack [20] indicated that the variability in concentration decay could be  
37 explained by the difference in the metabolism of the subjects. Our results also  
38 indicate that daily intake and background levels remaining in the respiratory  
39 system from earlier smoking have a considerable effect on the variability in the  
40 VOC content observed. The decay curves obtained for benzene and toluene  
41 showed that a time of between one and two hours was required to return to pre-  
42 exposure levels for the subjects evaluated (light smokers). Those people typically  
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3 smoke one cigarette every two to three hours and so levels found before smoking  
4 a cigarette could be considered background levels. However, heavy smokers tend  
5 to smoke one cigarette every 20-30 minutes, at time which is not sufficient for a  
6 drop to background levels to be reached.  
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10 **The decay in the content found with the time since smoking makes necessary to**  
11 **compare the VOCs content in smokers and non-smokers at different time intervals**  
12 **after smoking. As showed previously, there was a significant difference between**  
13 **the values obtained for smokers and non-smokers was obtained for the five**  
14 **compounds tested when the whole population of smokers was used and no time**  
15 **subgroups were taken into account (Table 4,  $\geq 0$  min). Concentrations found for**  
16 **smokers and non-smokers were not significantly different in the case of xylenes**  
17 **and toluene when the subgroup of smokers used in the analysis only considered**  
18 **those people that gave the breath sample 45 minutes or more after smoking (43**  
19 **smokers). Benzene showed a larger time interval and it gave significant**  
20 **differences between smokers and non-smokers until approximately 12 hours of**  
21 **smoking. 2,5-dimethylfuran showed significant differences between the two**  
22 **groups in all the time intervals evaluated. This compound gave significant**  
23 **differences between smokers and non-smokers after more than 24 hours without**  
24 **smoking.**  
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### 38 **Combined effects**

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40 The wide variation found in the levels of benzene, toluene and xylenes between  
41 smokers with different consumption habits together with the fact that these  
42 compounds are also detected in the breath of non-smokers suggests that a large  
43 number of false negatives might be obtained with light smokers.  
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48 In the specific case of xylenes and toluene another drawback is that they are only  
49 effective for recent exposure conditions. In the samples evaluated, these  
50 compounds lost their ability to adequately determine smoking status  
51 approximately 45 minutes after smoking (Table 4, Figure 2d). Furthermore, it was  
52 found that the test was less effective for subjects who smoked fewer cigarettes  
53 (<30 minutes for individuals that smoked <10 cigarettes/day, no differences at any  
54 time when intake was reduced to <7 cigarettes/day).  
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60 Benzene, on the other hand, gave better time intervals and it could be used as an  
effective breath biomarker for some hours after smoking even in conditions of

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3 reduced cigarette consumption. Unfortunately, however, benzene is also present in  
4 the breath of healthy non-smokers and the significance of this compound is  
5 reduced in light smoking conditions and after 1-2 hours of smoking.

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8 2,5-dimethylfuran was detected in all smokers **breath samples** and it was only  
9 detected in 12 non-smokers **samples** (12% of the non-smoking population and  
10 5.9% of the total population). An exhaustive study of these **12** samples showed  
11 that four subjects indicated in the survey that they smoked less than one cigarette  
12 a day but smoked during weekends or on special occasions (**i.e., social smokers**):  
13 **two of them** claimed that they had smoked their last cigarette two days earlier;  
14 another, three days earlier; and the last one, five days earlier. When the  
15 concentrations of these subjects were introduced in the group of smokers for the  
16 calculations, the levels detected for 2,5-dimethylfuran still showed significant  
17 differences with non-smokers, and all other compounds, including benzene,  
18 showed no significant differences. Seven more subjects considered themselves to  
19 be passive smokers as they lived with people who smoked and said that they had  
20 been in contact with tobacco smoke in the previous hour before the sample was  
21 obtained. 2,5-dimethylfuran was detected in the sample of only one subject who  
22 claimed to be neither an active nor a passive smoker.

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35 The best results in all conditions evaluated (**i.e., short and long exposure, light and**  
36 **heavy cigarette consumption**) were obtained with 2,5-dimethylfuran (Figure 2a).  
37 Previous studies [22,24-27,32] have indicated that this compound has a high  
38 discrimination power as smoking breath biomarker. As can be seen in Table 4,  
39 this compound yielded significant differences in all conditions evaluated and  
40 maintained its significant difference between smokers and non-smokers during the  
41 whole time interval evaluated. Moreover, this compound is able to detect smoking  
42 status more than 24 hours after smoking for people with a very light smoking  
43 habit.

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The American National Research Council identifies different criteria for the  
establishment of an effective environmental tobacco smoke tracer. The first is  
“uniqueness” [33], a **criterion** which is also strongly recommended for an  
adequate smoking breath biomarker. 2,5-dimethylfuran has the advantage of not  
being an endogenous substance and that it can only be introduced in the  
respiratory system from exogenous sources: smoking is practically the only  
realistic source of this contamination. Moreover, recent studies have shown that

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3 2,5-dimethylfuran emissions do not differ by cigarette type [30]. The results  
4 obtained in the present study show that this compound yields significant  
5 differences between smokers and non-smokers in all conditions evaluated (e.g.,  
6 short- and long-exposure, light and heavy consumption). Furthermore, 2,5-  
7 dimethylfuran maintains its ability to differentiate between smokers and non-  
8 smokers even in the case of people who only smoke very occasionally, a situation  
9 in which benzene levels are unable to determine the true smoking status.  
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### 17 **Effect of smoking controlled substances**

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19 A large proportion of the volunteers participating in the study were students and  
20 many of them referred to smoking controlled substances. Thirty-five subjects  
21 admitted to smoking cannabis. Of these, 28 were also cigarette smokers and gave  
22 positive results in the analysis of 2,5-dimethylfuran. Seven subjects claimed only  
23 to smoke pure cannabis, without mixing this with tobacco. Six of these seven  
24 people gave negative results in the analysis of 2,5-dimethylfuran whereas the  
25 seventh person was a passive smoker who had had recent contact with tobacco  
26 smoke.  
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33 **These results indicate that** other drugs commonly related with smoking did not  
34 yield false positive results and confirm that 2,5-dimethylfuran is a specific breath  
35 biomarker for tobacco consumption. This compound has also demonstrated its  
36 effectiveness in detecting passive smoking when direct contact with the smoke  
37 has been produced just before a sample is analyzed. This makes 2,5-dimethylfuran  
38 a very promising biomarker as it is only necessary to detect its presence in order  
39 to be able to establish smoking status.  
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### 47 **Conclusions**

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49 Here, we showed that toluene and xylenes can only be used as potential breath  
50 biomarkers of smoking exposure for those smokers with a large daily  
51 consumption of cigarettes ( $\geq 10$  cigarettes/day) as well as recent exposure (less  
52 than 45 minutes after smoking). Benzene gave better results and could be used  
53 successfully as a biomarker with lower cigarette consumption (at least 1  
54 cigarette/day) and functions for a longer period after smoking (12-13 hours). The  
55 best results, however, were obtained with 2,5-dimethylfuran. This compound can  
56 be used as a breath biomarker of the smoking status **also for social smokers (a**  
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subgroup that is never considered as smokers due to their low cigarette consumption: 3-4 cigarettes/week) and is still effective long after the exposure (more than 24 hours after smoking). Moreover, 2,5-dimethylfuran is the only compound that is able to detect heavy passive smoking.

The results obtained in this study suggest that further attempts to evaluate whether there exist some correlation with the food ingestion or cigarette type smoked would be fruitful. Moreover, studies about second hand smoke exposure are needed to determine the exposure conditions required to detect 2,5-dimethylfuran in passive smokers.

## Acknowledgements

We gratefully acknowledge the financial support of the University of Girona (project # 003798, R+D grants within the framework of IdIBGi 2006) and MICINN (project # CTM2008-06847-C02-02/TECNO). M. Alonso acknowledges the Spanish Ministry of Education for her research grant (AP2008-01628). We would like to thank the staff and students who have participated in the study.

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4 **Figure 1.** GC-MS chromatograms of exhaled breath from a smoker (a) and a healthy non-smoker  
5 person (b). Chromatograms were **extracted** at  $m/z=78, 91$  and  $96$  to facilitate the view of the target  
6 compounds. Y-axis scale was the same for both chromatograms.  
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9 1: benzene; 2: 2,5-dimethylfuran; 3: toluene; 4: *m-, p*-xylene; 5: *o*-xylene.

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11 **Other compounds identified in the chromatograms:** a: acetone; b: alkane (C5); c: dichloromethane;  
12 d: alkane (C6); e: ethylbenzene; f:  $\alpha$ -pinene; g: phenol; h: trimethylbenzene; i: limonene; j:  
13 eucalyptol.  
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18 **Figure 2.** Box plots of data obtained for (a) 2,5-dimethylfuran, (b) benzene, (c) toluene, and (d)  
19 toluene when the subgroup of analysis for smokers only took into account those people that had  
20 smoked at least 120 minutes before. **The bottom and top of the box are 25<sup>th</sup> and 75<sup>th</sup> percentiles,**  
21 **the line inside the box is the median (50<sup>th</sup> percentile) and the whiskers indicate the lowest and**  
22 **highest data within 1.5 inter-quartile range.**  
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27 **Figure 3.** Normalized decay curves obtained for benzene, 2,5-dimethylfuran, toluene, and  
28 *o*-xylene as measured in the breath of three volunteers after smoking. Breath values were  
29 normalized to the compound level detected just after smoking a cigarette. Vertical bars show the  
30 standard deviation obtained for the three breath samples evaluated.  
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**Table 1.** Target compounds in chromatographic elution order, and their quantifier and qualifier ions. Method detection limits (MDL) are indicated for the analysis of 775 cm<sup>3</sup> of sample.

#	Compound	Quantifier ion	Qualifier ions	MDL	
				(pptv)*	(ng·m <sup>-3</sup> )
1	benzene	78	77, 51, 52	5	16
2	2,5-dimethylfuran	96	95, 81, 53	5	20
3	toluene	91	92	5	19
4	<i>m</i> -, <i>p</i> -xylene	91	106, 105, 77	10	43
5	<i>o</i> -xylene	91	106, 105, 77	10	43

\* values determined at 25°C and 760 mm Hg.

**Table 2.** Median and quartiles [25 and 75%] for the analyte concentrations in the breath of smokers and non-smokers. Concentrations are expressed in ppbv.

Compound	Smokers (n=100)	Non-smokers (n=104)	<i>P</i> value
benzene	7.366 [2.831, 23.091]	0.927 [0.289, 1.295]	< 0.001
2,5-dimethylfuran	0.978 [0.253, 3.186]	0.000 [0.000, 0.000]*	< 0.001
toluene	2.798 [0.830, 6.047]	1.208 [0.451, 2.350]	< 0.001
<i>m-, p</i> -xylene	0.101 [0.063, 0.168]	0.068 [0.047, 0.095]	< 0.001
<i>o</i> -xylene	0.045 [0.037, 0.071]	0.037 [0.034, 0.042]	< 0.001

\* not detected (a value of 0 was used for statistical analysis).

**Table 3.** Spearman correlation coefficients for the compounds evaluated in smokers (n=100) and the daily intake of cigarettes, and the time since the last cigarette smoked.

<b>Compound</b>	Number of cigarettes smoked daily	Time since last cigarette
benzene	0.417 *	-0.675 *
2,5-dimethylfuran	0.417 *	-0.721 *
toluene	0.334 *	-0.502 *
<i>m-, p-xylene</i>	0.249 *	-0.439 *
<i>o-xylene</i>	0.251 *	-0.293 *

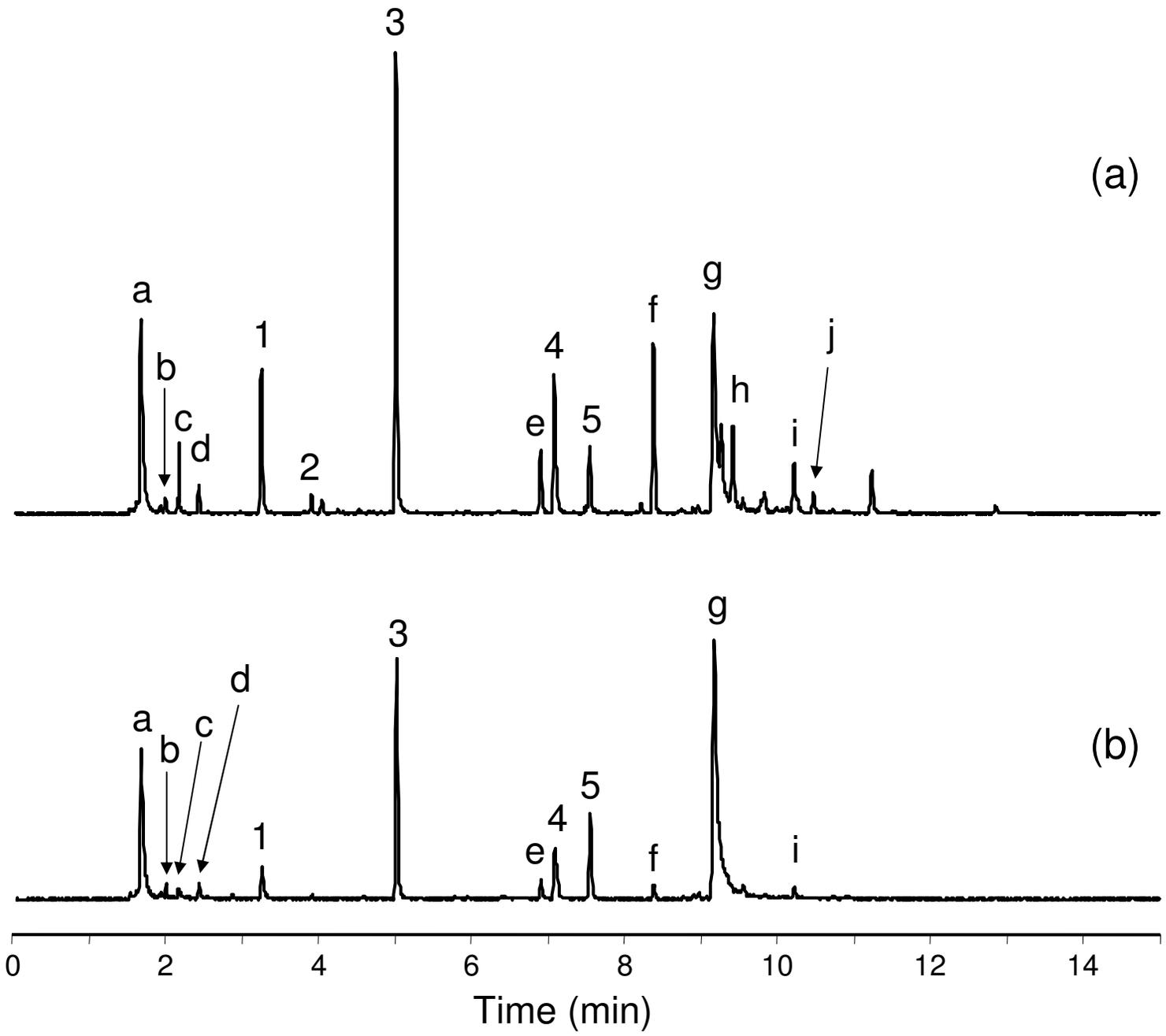
\* significant

**Table 4.** Median and quartiles [25 and 75%] for the analyte concentrations (ppbv) in the breath of smokers at different time intervals from the smoking of last cigarette.

	time since last cigarette			
	≥ 0 min	≥ 30 min	≥ 45 min	≥ 13 hours
# smokers	100	58	43	15
benzene	7.366 [2.831, 23.091] *	4.061 [1.941, 9.516] *	3.280 [1.507, 6.332] *	1.112 [0.667, 2.871]
2,5-dimethylfuran	0.978 [0.253, 3.186] *	0.448 [0.131, 1.074] *	0.248 [0.101, 0.733] *	0.093 [0.039, 0.169] *
toluene	2.798 [0.830, 6.047] *	1.546 [0.628, 4.029] *	1.312 [0.590, 3.058]	0.590 [0.310, 1.487]
<i>m-, p</i> -xylene	0.101 [0.063, 0.168] *	0.082 [0.058, 0.146] *	0.073 [0.057, 0.118]	0.062 [0.046, 0.143]
<i>o</i> -xylene	0.045 [0.037, 0.071] *	0.042 [0.035, 0.102] *	0.038 [0.035, 0.062]	0.036 [0.038, 0.047]

\* Significant differences with concentration values detected in non-smokers (see Table 2 for concentration values in the breath of non-smokers)

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Figure 2

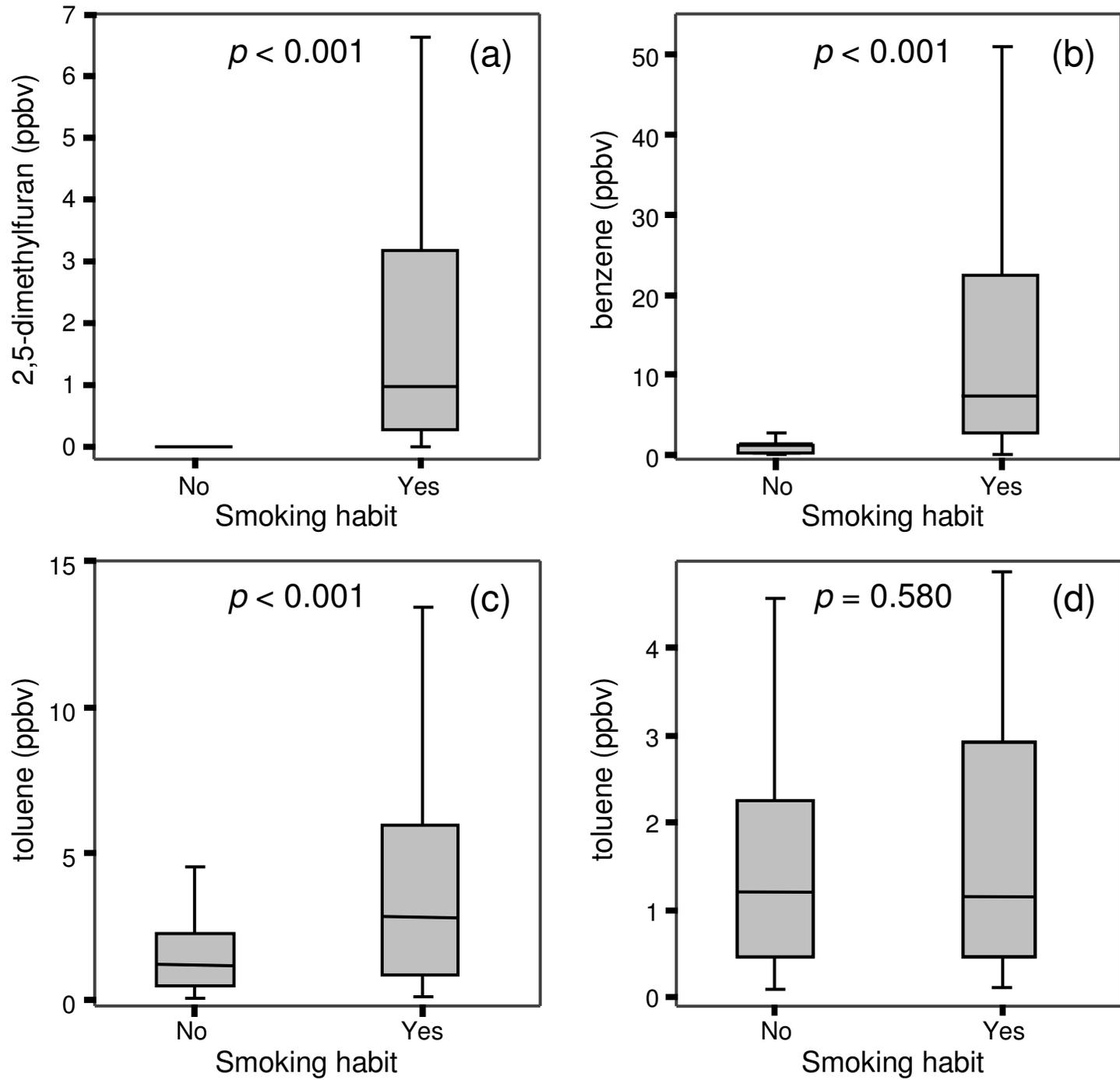
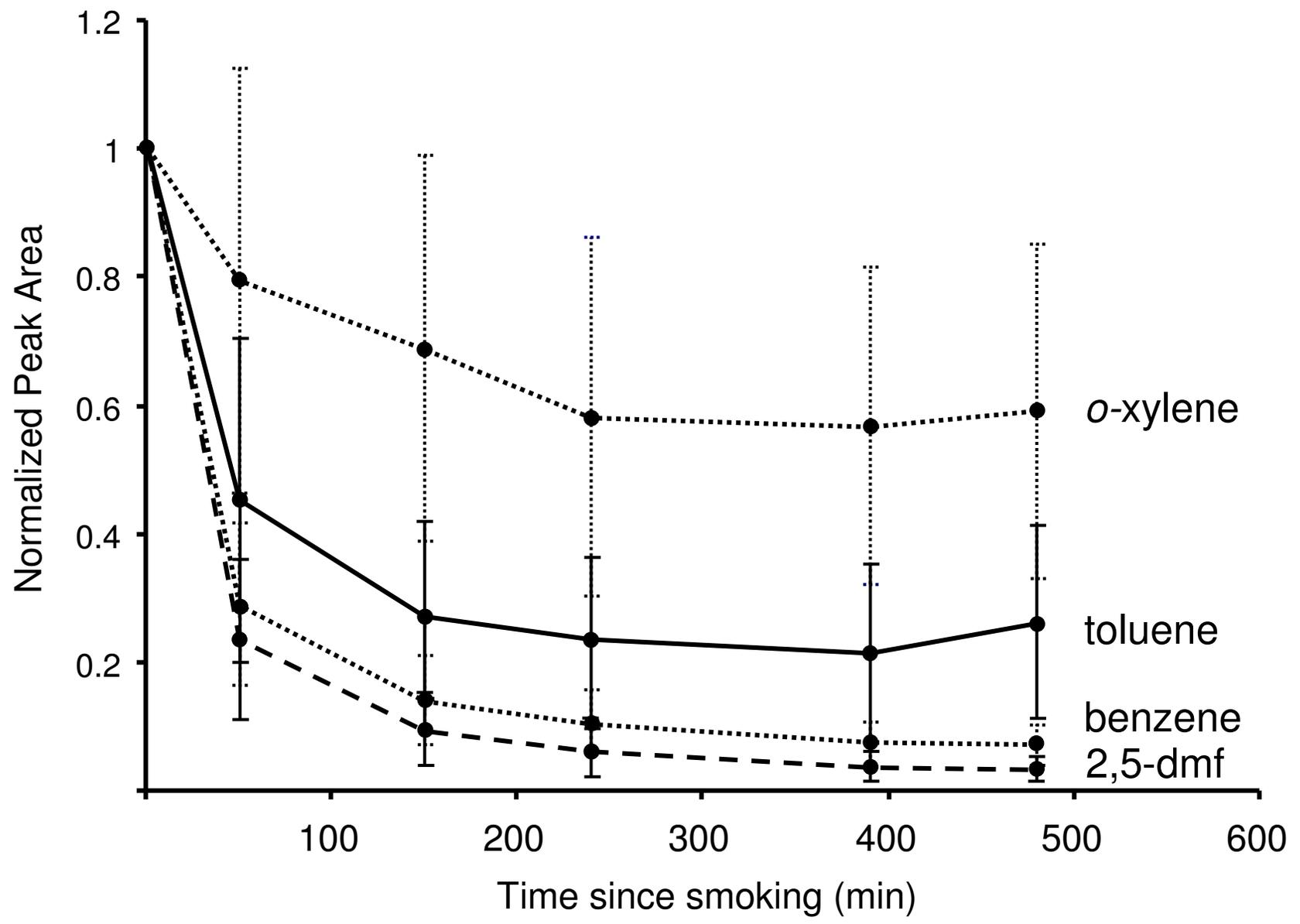
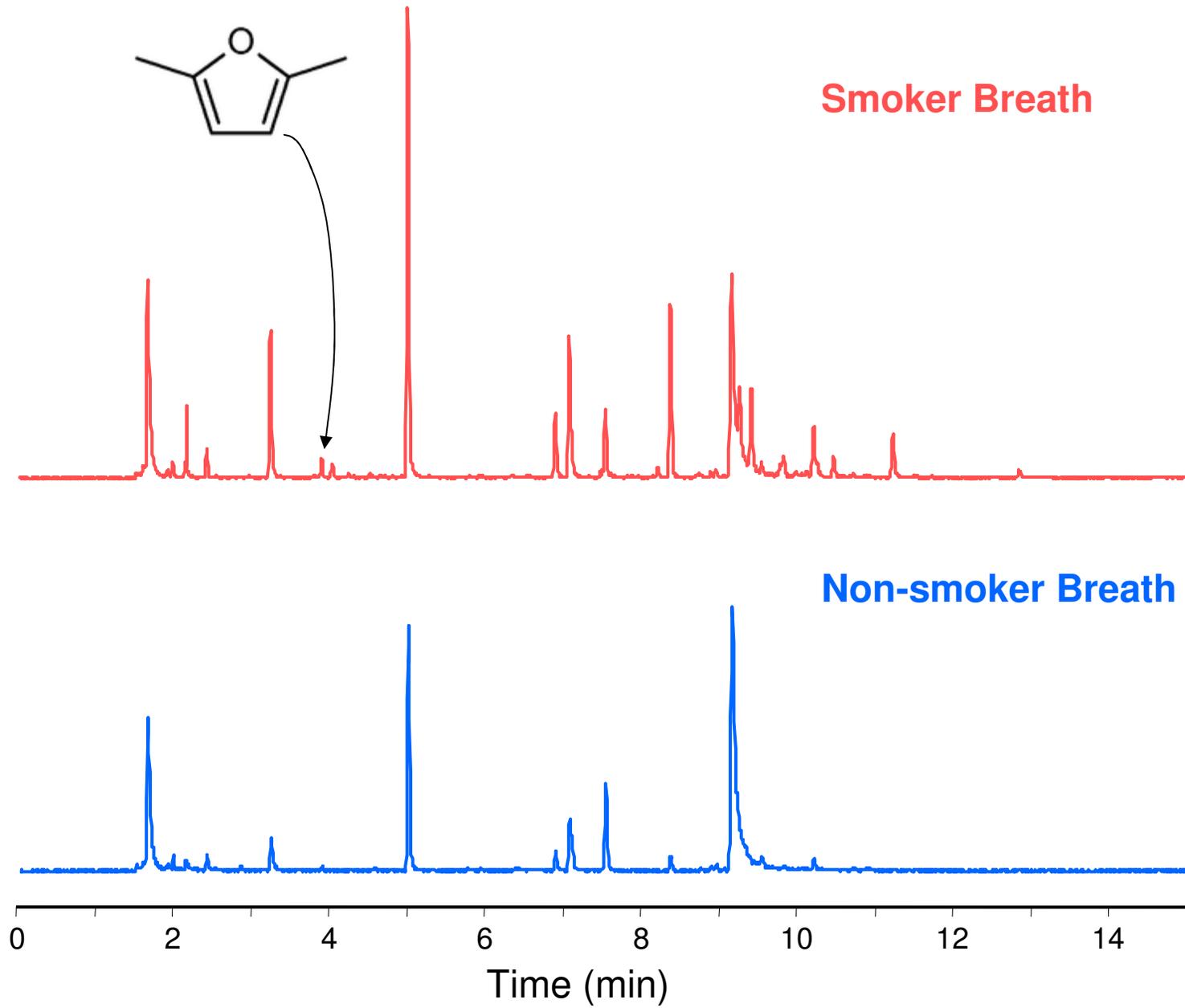


Figure 3



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**BREATH BIOMARKER ANALYSES IN SMOKERS**

## (QUESTIONNAIRE)

1. Age:
2. Sex:     Male      Female
3. Do you have any kind of pulmonary problem? No  Yes  If yes, please specify.
4. Are you a smoker? No  Yes  (if you answered "no", please go to question 8)
5. Approximately how many cigarettes do you smoke per day?
6. Which type of cigarettes do you smoke? (full flavor/black/light/cigars)
7. How long has it been since you smoked your last cigarette? \_\_\_\_ days, \_\_\_\_ hours, \_\_\_\_ minutes.
8. Have you consumed any drug in the last few days? No  Yes  Which type?
9. Have you consumed any food in the last hour? No  Yes  If yes, please specify.
10. Have you consumed any drink other than water during the last hour? No  Yes  Which?
11. Have you had a coffee in the last hour? No  Yes
12. Is there any history of lung cancer in your family? No  Yes
13. Do you consider yourself to be in regular contact with smokers? No  Yes  (if you have answered "no", there are no further questions.)
14. Are these smokers friends or family members?
15. How long do you consider yourself to be in contact with cigarette smoke during the day?

Sample code:

Sampling date:

Time of sampling: