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Determination of aldehydes in exhaled breath of patients with lung cancer by means of on-fiber-derivatisation SPME–GC/MS☆

Diana Poli[a,b,*], Matteo Goldoni[a,b], Massimo Corradi[a], Olga Acampa[a,b], Paolo Carbognani[c], Eveline Internullo[c], Angelo Casalini[d], Antonio Mutti[a]

[a] Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, via Gramsci 14, 43100 Parma, Italy
[b] ISPESL Research Centre at the University of Parma, University of Parma, Italy
[c] Unit of Thoracic Surgery, University of Parma, Italy
[d] Pulmonary and Thoracic Endoscopy Unit, Azienda Ospedaliera di Parma, Parma, Italy

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ABSTRACT

A number of volatile organic compounds (VOCs) have been identified and used in preliminary clinical studies of the early diagnosis of lung cancer. The aim of this study was to evaluate the potential of aldehydes (known biomarkers of oxidative stress) in the diagnosis of patients with non-small cell lung cancer (NSCLC). We used an on-fiber-derivatisation SPME sampling technique coupled with GC/MS analysis to measure straight aldehydes C3–C9 in exhaled breath. Linearity was established over two orders of magnitude (range: 3.3–333.3 $\times 10^{-12}$ M); the LOD and LOQ of all the aldehydes were respectively 1×10^{-12} M and 3×10^{-12} M. Accuracy was within 93% and precision calculated as % RSD was 7.2–15.1%. Aldehyde stability in a Bio-VOC® tube stored at +4 °C was 10–17 h, but this became >10 days using a specific fiber storage device. Finally, exhaled aldehydes were measured in 38 asymptomatic non-smokers (controls) and 40 NSCLC patients. The levels of all of the aldehydes were increased in the NSCLC patients without any significant effect of smoking habits and little effect of age. The good discriminant power of the aldehyde pattern (90%) was confirmed by multivariate analysis. These results show that straight aldehydes may be promising biomarkers associated with NSCLC, and increase the sensitivity and specificity of previously identified VOC patterns.

1. Introduction

Lung cancer is the leading cause of cancer death in the world. Its prognosis is still poor because of the lack of valid approaches to its early detection, which means that it is frequently diagnosed at an advanced stage when treatment is less effective.

The high 5-year mortality rate of more than 80% is also related to the frequency of metastases at the time of diagnosis, frequent recurrence after surgery, and poor responsiveness to chemotherapy [1,2]. Moreover, despite the increased survival of patients undergoing the resection of stage I lung cancer, overall mortality remains unchanged or may even increase [3].

Nevertheless, its pre-invasive phase may last for years and this provides a window of opportunity for an early molecular diagnosis. There is therefore considerable interest in developing non-invasive methods of detecting early lung cancer which is mainly concentrated on determining the compounds arising from lipid peroxidation. Lipid peroxidation is one of the basic mechanisms of inflammatory processes, and has been implicated in the pathogenesis of many lung diseases including chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis, interstitial lung disease and also lung cancer [1,4–8].

Breath analysis is a promising non-invasive approach that allows the identification of the inflammatory and oxidative stress biomarkers involved in the pathogenesis of various respiratory conditions [1,4,5,9–13]. Various classes of volatile organic compounds (VOCs) can be measured in exhaled breath, including the saturated hydrocarbons and oxygen-containing substances formed during the fatty acid lipid peroxidation of cell membranes [2,12,14], and published data show that the patterns of VOCs in exhaled breath can distinguish patients with and without lung cancer [9,11,13,15–18].

In a previous study [19], we found that a pattern of selected VOCs (aliphatic and aromatic hydrocarbons) distinguished a group of NSCLC patients from healthy controls, asymptomatic smokers

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* Corresponding author at: Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, via Gramsci 14, 43100 Parma, Italy. Tel.: +39 0521 033079; fax: +39 0521 033076.
E-mail address: diana.poli@unipr.it (D. Poli).

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and COPD patients with a sensitivity of 72.2% and a specificity of 93.6%. In the light of this promising result, it was decided to seek other substances in exhaled breath that may be related to lung disease with the aim of identifying a more sensitive and specific pattern that could be used for the early detection of lung cancer by excluding substances that could also be related to other diseases.

As a wide variety of carbonyl compounds are generated as secondary oxidation products, we chose to study straight volatile aldehydes as biomarkers of tissue damage [14,20,21]. In particular, saturated aldehydes such as hexanal, heptanal and nonanal are formed by the peroxidation of ω3 and ω6 fatty acids (PUFAs), the basic components of cell membrane phospholipids [22–24]. Furthermore, volatile aldehydes are poorly soluble in blood and are therefore excreted into the breath within minutes of their formation in tissues [2,4].

As most of these substances have exhaled breath concentrations in the 10^{-12} M (pM) and 10^{-9} M (nM) range [9,11,15,19,25], a pre-concentration step is necessary. Solid-phase micro-extraction (SPME) has been successfully used to extract a series of volatile compounds from human breath, including such as aliphatic and aromatic hydrocarbons [19,26–30] and also carbonyl compounds by means of on-fiber derivatisation [28,31,32]. However, not all breath-borne volatile compounds can be directly extracted because of their physical properties and the characteristics of the available fiber coatings.

The aim of this study was to use the SPME on-fiber derivatisation sampling technique together with gas chromatography–mass spectrometry (GC–MS) analysis in order to detect straight aldehydes in the last portion of human breath (alveolar air) that enters the gas exchange region of the lung. Preliminary experiments were used to address methodological issues, optimise the SPME parameters and validate the method. We also studied the stability of aldehydes after on-fiber derivatisation (and consequently storage time) using a specific new device. Finally, the method was used to quantify selected aldehydes in the exhaled breath of patients with non-small cell lung cancer (NSCLC) and a control group of healthy non-smokers.

2. Experimental

2.1. Chemicals and reagents

Propanal (>96.0%), *n*-butanal (>99.0%), *n*-pentanal (97%), *n*-hexanal (98.0%), *n*-heptanal (95.0%), *n*-octanal (99.0%), *n*-nonanal (95.0%), 2-methylpentanale (98.0%) used as the internal standard (IS), chloroform (>99.0%), and O-2,2,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA, 98%) were obtained from Sigma–Aldrich (Milan, Italy).

The standard aldehyde solutions were prepared in chloroform.

2.2. Calibration standards

The gaseous standards were directly prepared in a Teflon® bulb (Bio-VOC® sampler, Markers International Ltd., Rhondda Cynon Taff, UK) filled with purified helium, 1 μl of standard aldehyde solutions, 1 μl of IS, and 6 μl of deionised water. The standard aldehyde solutions (0.5×10^{-6} M, 10^{-6} M, 0.5×10^{-5} M, 10^{-5} M, 0.5×10^{-4} M) were prepared in chloroform.

The gaseous standards were stabilized at room temperature for almost 1 h before analysis.

2.3. Breath collection

The breath samples were collected as previously described [19]. Briefly, the subjects were asked to perform a single slow vital capacity breath in a one Bio-VOC® tube in order to trap the last 150 ml of exhaled breath.

After adding 1 μl of 10^{-5} M IS solution, the Bio-VOC® tubes were stored at 4 °C and analysed within 2 h.

Before being reused, the tubes were thoroughly cleaned by means of flushing with nitrogen.

2.4. Solid-phase micro-extraction on-fiber derivatisation

The aldehydes were extracted using a 65 μm PDMS/DVB fiber purchased from Supelco (Bellefonte, BA, USA). New fibers were conditioned for about 30 min at 250 °C under a stream of hydrogen in the GC injection port.

Before each use, the fiber was cleaned in the GC injection port for 1 min at 280 °C in order to release any contaminants, and then exposed to the headspace of a PTFE-capped 4 ml amber vial containing 1 ml of PFBHA 17 mg/ml aqueous solution for 10 min at room temperature under stirring conditions. After this loading phase, the fiber was placed directly in the Bio-VOC® breath sampler for 45 min at room temperature (the on-fiber derivatisation phase) and then thermically desorbed in the GC injection port at 280 °C.

The reaction of derivatisation of aldehydes with PFBHA reagent gives two PFBHA-oxime isomers (*cis*- and *trans*-isomers) for each aldehyde [32].

2.4.1. SPME parameter optimisation and validation

A series of experiments were carried out in order to ensure a reliable exhaled air sampling procedure. Gaseous standards were prepared directly in Bio-VOC® bulb filled with helium, 1 μL of aldehyde standard solution, 1 μL of I.S. (10^{-5} M) and 6 μL of deionised water. Standards were stabilized at room temperature for almost 1 h before analysis.

The SPME parameters were optimised by studying the loading time of the PFHBA (2, 5, 10 and 15 min) and by sampling for different times (5, 10, 30, 45 and 60 min) Bio-VOC® tubes containing 66.6×10^{-12} M of aldehyde gaseous concentrations (1 μl of 10^{-5} M standard solution).

Aldehyde stability in gas phase in a Bio-VOC® sampler at +4 °C was evaluated at 66.6×10^{-12} M aldehyde concentrations (1 μl of 10^{-5} M standard solution) by analysing the sample after 1, 3, 9, 17, 32 and 70 h. After optimising the SPME parameters, all of the analyses were made under equilibrium conditions using loading and sampling times of respectively 10 and 45 min.

Finally, the method was validated by studying its linear dynamic range (range: $3.3–333.3 \times 10^{-12}$ M, with five samples analysed in duplicate), limit of detection (LOD, calculated as a signal-to-noise ratio [S/N] of 3), limit of quantitation (LOQ), accuracy, and its intra- and inter-day analytical precision measured on standard samples (66.6×10^{-12} M aldehyde concentrations) and calculated as the relative standard deviation (RSD%).

2.5. Gas chromatography–mass spectrometry

The analyses were made using a Hewlett Packard HP 6890 gas chromatograph coupled with a HP 5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA), with the aldehydes being separated on a HP-5MS column (Agilent Technologies, 30 m × 0.25 mm ID; 0.50 μm film) using hydrogen as the carrier gas (flow rate 1 ml/min). The GC conditions were 8 °C/min from 100 °C to 150 °C, and then 30 °C/min until 250 °C and hold for 1 min. The chromatographic run was completed in 10.58 min.

A quantitative analysis was performed in selected ion monitoring (SIM) mode by acquiring the signal of the mass fragment m/z 181 (dwell time 400 ms). Each aldehyde was identified by the ion 181, the PFB-oxime characteristic fragment ion, and confirmed by comparison of the retention time with that of pure standard.

Table 1
Characteristics of study subjects.

	NSCLC (n = 40)	Controls (n = 38)
Age (years)	67.9 ± 9.9	49.3 ± 15.2
Gender (M/F)	(28/12)	(17/21)
Smokers/ex-smokers/non-smokers	21/12/7	0/10/28
Ex-smokers for (years ± SD)	14.1 ± 13.6	16 ± 11.8
Pack/years		
Smokers	57.0 ± 28.9	3.6 ± 3.4
Ex-smokers	46.6 ± 28.02	31.8 ± 20.13

Mean values ± SD.

A solvent delay of 2.5 min was set to protect the filament from PFBHA vapor.

2.6. Study population

Forty patients with clinical stage I or II non-small cell lung cancer (NSCLC) were enrolled at the Thoracic Surgery Section of the University Hospital of Parma before undergoing lung resection with curative intent. All of the patients were staged by means of computerized tomography (CT) scans of the chest and abdomen; brain CT, abdominal ultrasound or bone scintigraphy were performed as indicated. None of the patients had received any form of anticancer therapy before surgery.

The control group consisted of 38 healthy asymptomatic subjects, none of whom reported a medical history of malignancy or clinically significant pulmonary disease.

Informed consent was obtained from each subject at the time of enrolment. The study was approved by the Ethics Committee of the University of Parma and conducted in conformity with the Declaration of Helsinki.

The characteristics of the study population are shown in Table 1.

Tobacco smoke exposure was evaluated on the basis of self-reported current smoking status. The number of cigarettes smoked per day and the numbers of years of smoking were recorded. The subjects who had stopped smoking at least 1 year before recruitment were defined ex-smokers.

The diagnosis of NSCLC was confirmed by a histological examination: there were 23 adenocarcinomas (ADCs), 13 squamous cell carcinomas (SCCs) and 1 large cell carcinoma. Although all of the patients were clinically staged I or II preoperatively, final pathology revealed stage I in 29 cases (18 stage IA, 11 stage IB), stage II in 6 (IIB), and stage IIIA in 5.

2.7. Statistical analysis

The aldehyde levels in the exhaled breath of a group of 10 healthy subjects and environmental air were compared using a paired sample t test because of the normality of the differences between variables.

Three groups of subjects were distinguished: group 0: 38 healthy controls; group 1: 19 ex- or non-smoker NSCLC patients; and group 2: 21 currently smoking NSCLC patients. Given the log-normal distribution of all of the clinical variables, the data are recorded as geometric mean (GM) (geometric standard deviation [GSD]) and median values (interquartile range).

One-way ANOVA followed by Tukey's post hoc test was used on the logarithm of the variables to test the univariate between-group differences; to test the effect of age, the analysis was repeated using the age as covariate (one-way ANCOVA). Multivariate analysis was performed by means of discriminant analysis of the logarithms, testing the significance of Wilks' Lambda of the two resulting factors and reporting the structure matrix (e.g. the matrix with the weight of single variables and factors) and the cross-validated classification of the subjects (leave-one-out method). The Factor2 score was plotted against the Factor1 score, identifying the position of the centroids of the three subject groups.

A p value of 0.05 was always considered significant. All of the statistical analyses were made using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The diagnosis of lung cancer at an earlier stage may improve survival, and breath may be a source of information regarding the neoplastic process in the airways [1,33]. Furthermore, the non-invasive and safe nature of exhaled breath sampling makes this approach useful in clinical practice.

The growing interest in discovering breath markers of the oxidative stress associated with lung cancer led us to consider straight aldehydes as secondary products of lipid peroxidation. As the lungs are directly exposed to higher oxygen concentrations than other organs, they can be more susceptible to the oxidative stress significantly involved in the pathogenesis of many respiratory diseases [34,35], and published data indicate that lung cancer is also characterised by an increase in oxidative stress [1,9,11,36].

Little is known about the behaviour of straight aldehydes in human breath, and only a few of them have been correlated with lung cancer [9,27,37,38], but promising results have been reported concerning aldehyde levels in other biological matrices of lung cancer patients, such as urine [39], blood/serum [37,38,40] and exhaled breath condensate (EBC) [21].

Aldehydes, particularly malondialdehyde, have been measured in EBC (the liquid phase of exhaled breath), in healthy subjects, asthmatics, and COPD patients [21,41,42]. Breath can be analysed in the gaseous phase (as exhaled breath) or the liquid phase, with the choice of the matrix depending on the physical–chemical properties of the target compounds, particularly their volatility and water solubility.

Breath analysis requires sophisticated and expensive equipment and excellent skills, because the target compounds arising from the lipid peroxidation pathway are only found in traces (e.g. 10^{-12} M or 10^{-9} M): a pre-concentration step is therefore crucial. The introduction of SPME has simplified the analysis of human breath and it has already been used to quantify various classes of VOCs [26–29,31,32]. In addition to the known advantages of adsorption, SPME is not affected by samples with a high water content, which makes it particularly suitable for breath analysis. The water content of breath can be critical because a high level of humidity may affect the pre-concentration step and GC–MS analysis of individual compounds.

However, SPME requires the rigorous preliminary optimisation of the parameters involved and careful validation is necessary especially in the case of trace compounds. Not all of the volatile compounds in breath can be easily extracted, which means that the choice of an SPME fiber that allows complete adsorption as well as easy desorption is crucial and, in many cases, derivatisation is necessary.

SPME on-fiber derivatisation using PFHBA as a reagent has been previously used to extract aldehydes from biological [32,37,40] and environmental samples [43,44]. In the light of these interesting results, especially the ability to detect trace amounts, we modified these methods to allow the determination of aldehyde levels in breath collected using commercially available Bio-VOC® tubes [19,26], which is a rapid non-invasive method of sampling alveolar air of the respiratory tract by excluding the air of the dead space that can contaminate or dilute the sample.

In addition to hexanal, heptanal and nonanal, which are formed by the peroxidation of ω3 and ω6 fatty acids [22–24], we investigated all of the other straight aldehydes and thus covered the

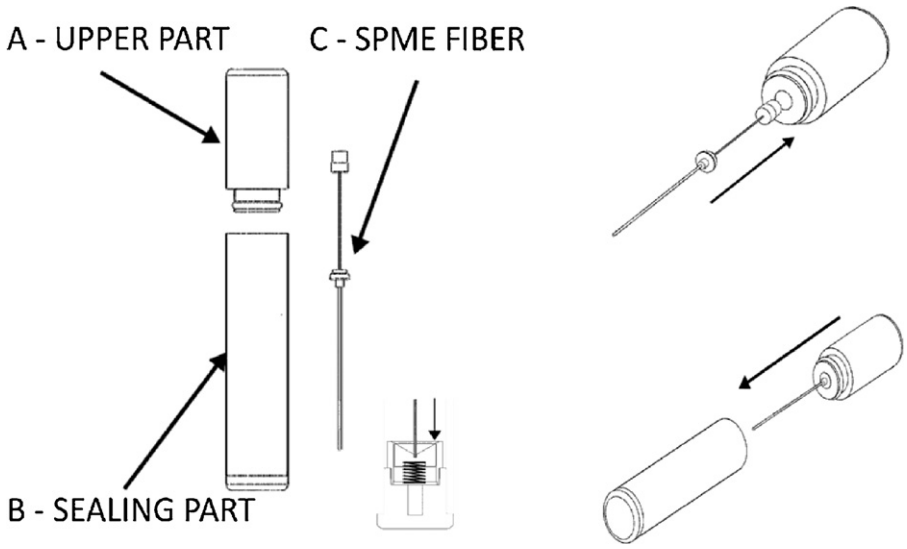

Fig. 1. SPME storage device. The tube consists of an upper part (A) and a sealing part (B). The fiber (C) must be screwed into the upper part (A) of the device and then inserted into the lower part (B) of the tube, which is closed by a PTFE cap and spring.

entire range from propanal (C3) to nonanal (C9). Formaldehyde and acetaldehyde were excluded because of their presence in the indoor and outdoor environments [45,46] and as combustion products of tobacco smoke [47,48], particularly considering that many patients with lung cancer are ex- or current smokers. Furthermore, breath acetaldehyde levels are closely related to the metabolism of ethanol [49–51].

The experiments in the first part of this study addressed methodological issues, defined standard operating procedures, and validate analytical methods relating to aldehyde collection and analysis.

Preliminary experiments studied the fiber loading time of PFBHA (2, 5, 10 and 15 min) by exposing the fiber to the headspace of a 17 mg/ml PFBHA water solution. The detection of unmodified PFBHA adsorbed confirmed that the optimum loading time (corresponding to the maximum PFBHA adsorbed by the fiber) was equal to 10 min corresponding to the achievement of equilibrium condition in the loading phase. In fact, no increase in PFBHA adsorption was observed after a longer time.

After loading, the SPME fiber was directly inserted into the Bio-VOC® sampler and the time profile of fiber exposure necessary to ensure complete aldehyde extraction and on-fiber derivatisation was performed. Different extraction times were evaluated (5, 10, 30, 450 and 60 min) by sampling Bio-VOC® tubes containing 66.6×10^{-12} M of aldehyde gaseous concentrations. The equilibration time (extraction plus on-fiber derivatisation) had been reached after 45 min for all aldehydes (data not shown) since a further increase in extraction time did not increase the amount of aldehyde extracted by the fiber.

Another crucial step was to assess the stability of gas phase aldehyde in a Bio-VOC® sampler. Bio-VOC® tubes containing 66.6×10^{-12} M of aldehyde gaseous concentrations were stored at +4 °C for various times (1, 3, 9, 17, 32, and 70 h) and thus analysed. The results showed that straight aldehydes from C3 to C6 were stable at +4 °C for up to 17 h (±20% SD compared with mean values), whereas octanal (C8) and nonanal (C9) depletion started after 10 h (Fig. 2). These findings underlined the fact that, in order to obtain reliable results, the analyses should be made within half a day, thus restricting the method's field of application of the method.

In an attempt to overcome this drawback, we tested the new and commercially available SPME storage device (Chromline srl Prato, Italy), a hollow tube made of delrin, a polyoxymethylene polymer, that can house the SPME fiber after the sampling procedure and minimises the release of the previously adsorbed substances. It is equipped with a sealing system that consists of a PTFE septum with a spring mechanism that pushes the PTFE onto the SPME needle surface as shown in Fig. 1.

After sampling Bio-VOC® tubes containing 66.6×10^{-12} M of aldehyde gaseous concentrations, the fiber was inserted into the device and stored for various times (3, 9, 17, 32, 120 and 240 h). The results showed that all of the aldehydes were stable throughout the investigated range (Fig. 2), and clearly indicated that the fiber can be stored for at least 10 days without any significant loss of its aldehyde content (±20% SD compared with mean values). This makes it possible to extend the application of the method because the breath collection and extraction steps can be separated by GC–MS analysis. Furthermore, the chemical and thermal resistance of the lightweight material of which it is made (from −20 °C to +100 °C) means that the tube can be easily shipped.

After optimising the SPME parameters and assessing aldehyde stability, the method was validated by studying its linear dynamic range, limit of detection (LOD, calculated as a signal-to-noise ratio [S/N] of 3), limit of quantitation (LOQ), accuracy, and intra- and inter-day analytical precision on standard samples calculated as the relative standard deviation (RSD%). Accuracy was determined by means of a recovery study on the basis of the found and added concentrations [52].

Linearity was established over two orders of magnitude (range: $3.3–333.3 \times 10^{-12}$ M) in five samples analysed in duplicate); LOD and LOQ were respectively 1×10^{-12} M and 3×10^{-12} M for all aldehydes; accuracy was within 93%; and precision was in the range of 7.2–15.1% for all intra- and inter-day standard determinations, as reported in Table 2. The wide linear range, the good accuracy and precision of the method and LODs in the range of 10^{-2} M levels, make this method appropriate to monitor aldehyde levels in human breath even from healthy asymptomatic subjects.

The study was designed to measure aldehydes as biomarkers related to lung cancer in order to extend the pattern of previously selected VOCs [19] as a potential means of early diagnosis. In addition to their endogenous origin associated with lung cancer, exhaled compounds may arise from exogenous sources and can provide a retrospective indication of past exposure and the accumulation of environmental and smoke related pollutants. Therefore, our preliminary experiments were aimed at evaluat-

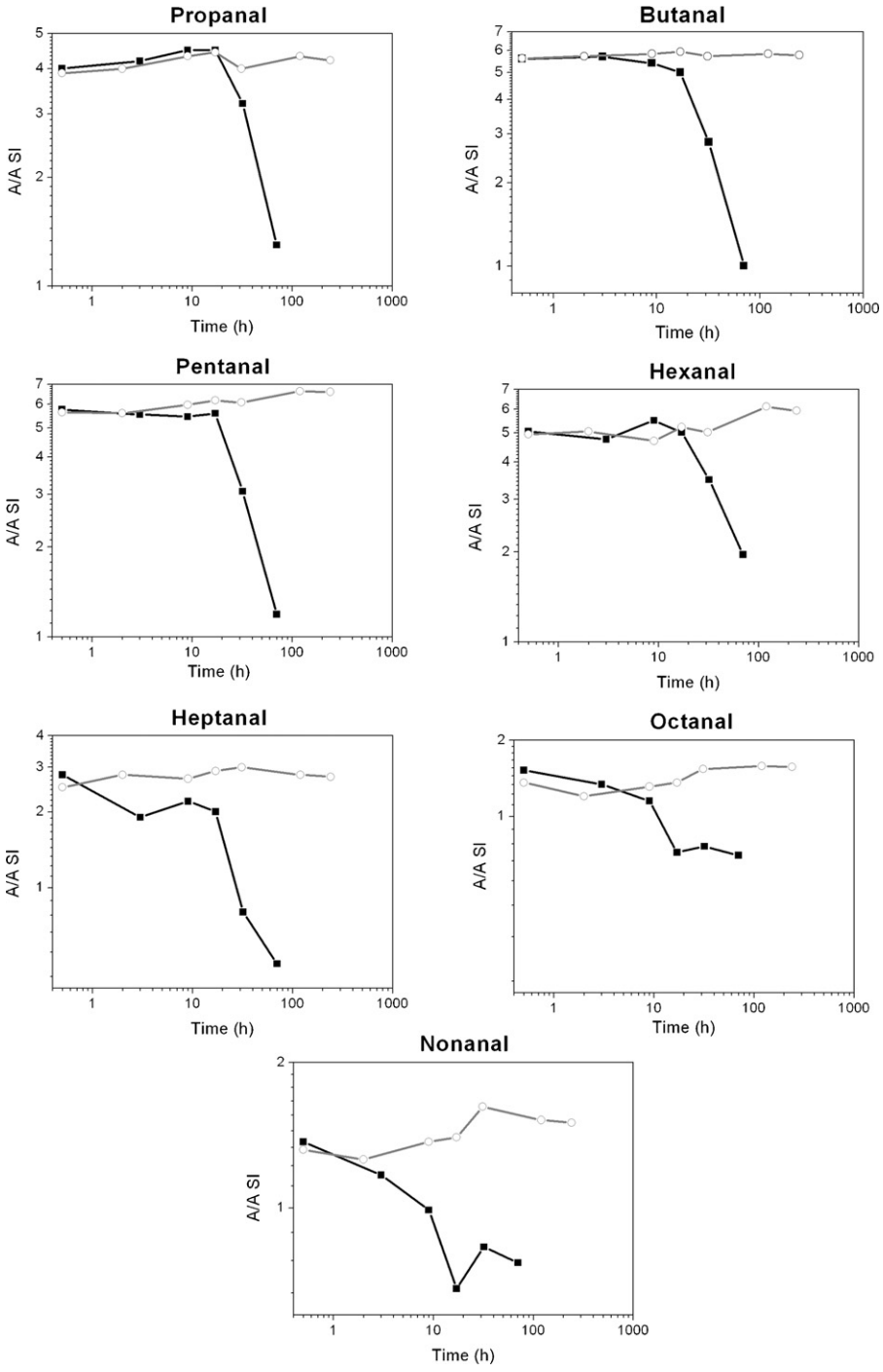

Fig. 2. Aldehyde storage stability in Bio-VOC® sampler at +4 °C (■) and on SPME fiber in SPME storage device (○).

ing environmental contamination by analysing breath samples of 10 subjects and the corresponding air inside the rooms in which the volunteers stayed for at least 2 h before breath collection. The results (Fig. 3) show that all aldehyde concentrations were statistically higher in breath than in environmental air.

Aldehydes can exist as exogenous contaminants (above all products of combustion) [47] and so the exclusion of possible contamination from the room used for breath collection should be a primary endpoint. Pentanal and hexanal levels in the indoor air of built-up areas in which several combustions occur are at least one order of magnitude higher than those measured in the rooms used for this study, in which no combustion occurred (about 70×10^{-12} M for pentanal and $200–250 \times 10^{-12}$ M for hexanal) [53,54]. The levels of several aldehydes in environmental air collected in downtown Santiago, Chile, confirm the order of magnitude of the levels measured by us with the exception of butanal, whose levels varied depending on the day (120 ± 120 pM) [55]. These data confirm that environmental aldehydes can represent a possible source of contamination and underline the importance of assessing the room in which breath is collected.

Furthermore, the use of the Bio-VOC® device allows the collection of the last portion of exhaled breath (alveolar air), which should be minimally affected by environmental contamination and hence more representative of lung status. The concentrations of

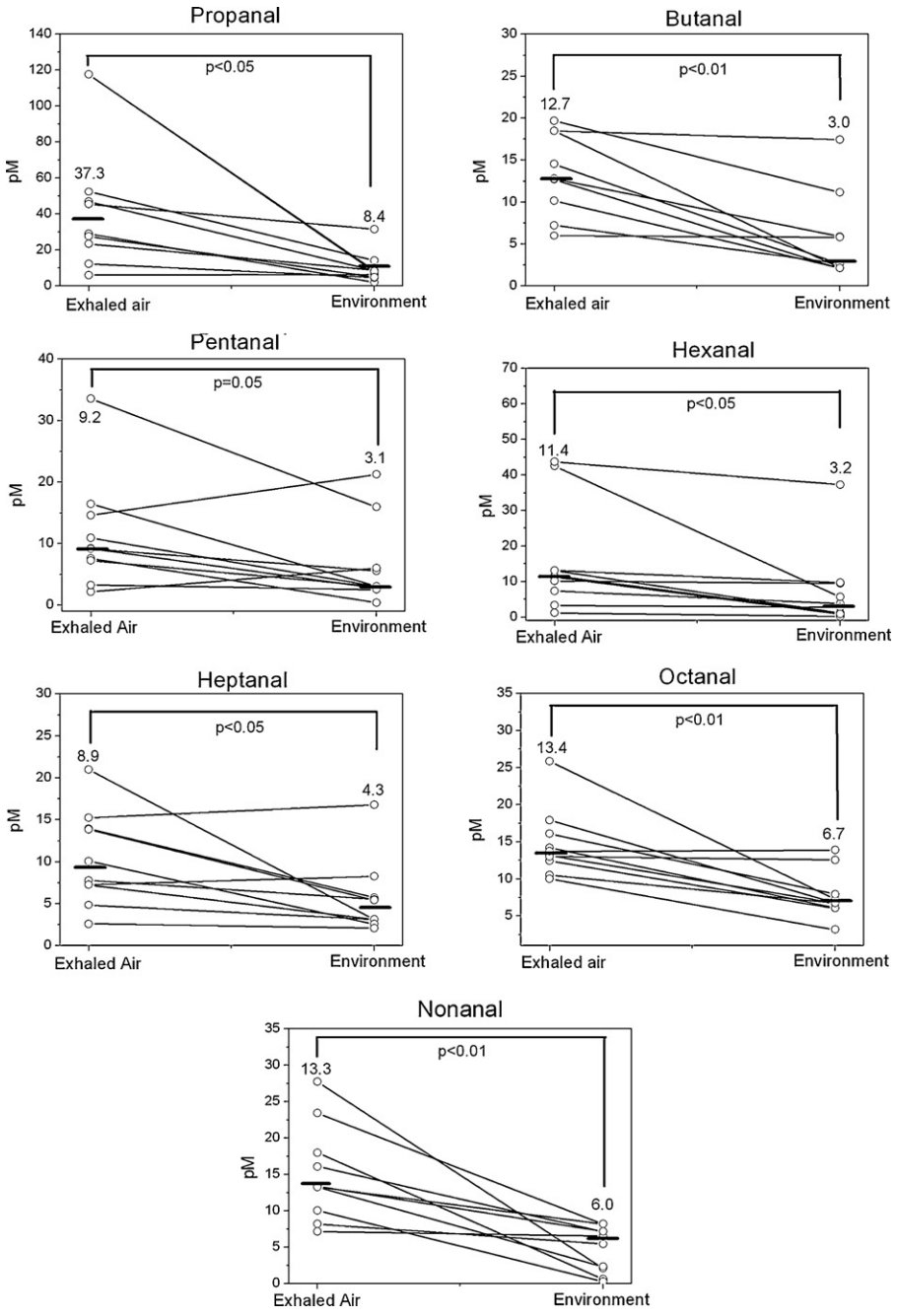

Fig. 3. Aldehyde levels measured in exhaled air and in the rooms (environment) where the asymptomatic volunteers remained for at least 2 h before breath collection.

endogenous VOCs in alveolar air should be higher than in mixed expiratory samples (alveolar and dead space air) because there is no dilution of the sample due to dead space air. The fact that the aldehyde levels in the exhaled breath of our healthy controls were always higher than those in the environmental samples demonstrates that increased breath aldehyde levels cannot be attributed to environmental contamination.

Another means of limiting environmental contamination is to calculate alveolar gradients from the levels measured in mixed expiratory samples by subtracting the levels measured in environment samples that can affect the VOC concentration in dead space air [11]. However, this method does not take into account the complexity of pulmonary adsorption and the exhalation of volatile substances, and the dilution and/or contamination effect of dead space air may vary and cannot be accurately quantified [5].

After having evaluated environmental breath contamination, we applied the method to 40 NSCLC patients evaluated before surgery and 38 healthy non-smokers. Throughout the study, the environmental air in the room in which the patients stayed was analysed at least once a week, and the results confirmed the environmental values found in the previous experiments (±20% SD compared with median values) (data not shown).

Table 3A shows the exhaled breath aldehyde levels and Table 3B the results of the univariate analysis. Our findings are in line with those of Svensson et al. [32]. There were significant overall differences in the levels of all of the aldehydes: all but propanal (6/7) were significantly higher in the ex- and non-smoking NSCLC patients than in the controls, and 7/7 in the currently smoking NSCLC patients. The levels in the two patient groups were never significantly different. This finding is particularly relevant because

Table 2
Validation of the on-fiber-derivatisation SPME/GC–MS method for all straight aldehydes from propanal (C3) to nonanal (C9): linear range, correlation coefficients, limit of detection (LOD), limit of quantitation (LOQ), accuracy and intra- and inter-day precision.

	Straight aldehydes from C3 to C9 ($\times 10^{-12}$ M)
Linear range[a]	3.3–333.3
r^2	0.97–0.99
LODs[b]	1
LOQs	3
Accuracy[c]	97–93%
Precision (% RSD)[d]	
Intra-day	7.2–10.4%
Inter-day	9.5–15.1%

SPME fiber: 65 μm PDMS/DVB; loading phase: 10 min; extraction time: 45 min at room temperature.
[a] Calibration fitting: $y = bx$ ($n = 5$ samples analysed in duplicate) ± 95%CI; linear regression analysis using least-square method.
[b] Limit of detection (S/N = 3) calculated under *sim* conditions.
[c] Accuracy calculated on $n = 3$ samples.
[d] Intra- and inter-day precision ($n = 3$) calculated on standard samples.

smoking can modify the concentrations of the VOCs found in cigarette smoke as well as induce oxidative damage [4,19,26]. It is therefore important to evaluate smoking habit as a confounding factor.

However, our data showed that smoking habits did not influence aldehyde levels in the breath of NSCLC patients. One possible reason for this may be that the aldehyde produced as a result of the inflammatory processes associated with the development of the disease is higher than the acute aldehyde production induced by tobacco smoke exposure. Furthermore, aldehyde levels in cigarette smoke [47] do not seem to be so persistent as to contaminate exhaled breath and our NSCLC smokers stopped smoking at least 2 h before their breath was collected. The increased aldehyde levels in lung cancer patients are therefore probably due to the overall status of their airways, although this is currently being further investigated studies in a group of asymptomatic smokers.

Finally, the use of age as a covariate had only a marginal effect on significance in the case of all of the aldehydes except propanal, for which significance was lost ($p = 0.08$), which suggests that the age difference between the controls and NSCLC patients (67.9 ± 9.9 years *vs* 49.3 ± 15.2 years) was not sufficient to explain their different aldehyde profiles despite the well-known age-related increase in oxidative stress [10,56–58].

To confirm the univariate results, discriminant (multivariate) analysis was applied to all three subject groups and all of the aldehydes grouped together. Most of the variance was explained by the first factor of the discriminant analysis (94.8%, significance of Wilks' Lambda [chi-squared] $p < 0.01$), whereas the second factor explained only 5.2%, with a non-significant Wilks' Lambda ($p = 0.44$).

The structure matrix of the correlations between each variable and any discriminant function (Table 4A) shows that the weight of the variables was nonanal > hexanal > octanal > heptanal > butanal in the first factor, and pentanal > propanal in the second factor. In the overall classification of the subjects (leave-one-out classification, Table 4B), 35/38 controls were correctly classified (92.1%), but the two groups of NSCLC patients were not efficiently separated.

Table 3A
Exhaled aldehyde levels. GM [GSD] and median values (25–75th percentile).

	NSCLC	NSCLC		Controls
		Non-/ex-smokers	Smokers	
Propanal (pM)	53.6 [1.5] 52.4 (42.4–72.6)	47.9 [1.4] 49.8 (40.7–61.3)	59.3 [1.6] 66.3 (47.8–81.7)	30.6 [2.8] 24.4 (17.1–46.9)
Butanal (pM)	26.2 [1.8] 26.2 (18.7–41.0)	24.5 [1.7] 23.6 (17.9–33.8)	27.9 [2.0] 28.6 (19.1–46.1)	10.9 [2.4] 10.8 (6.9–18.6)
Pentanal (pM)	19.1 [2.4] 17.7 (12.7–42.6)	16.1 [2.4] 17.1 (12.8–22.3)	22.2 [2.5] 20.3 (12.1–49)	7.6 [2.7] 8.2 (4.4–14.7)
Hexanal (pM)	37.3 [1.9] 38.1 (26.6–57.7)	37.1 [1.6] 38.2 (26.7–54.6)	37.5 [2.2] 35.9 (20.9–66.7)	8.5 [2.7] 10.3 (7.0–13.8)
Heptanal (pM)	13.9 [1.8] 16.1 (9.3–21.3)	15.2 [1.6] 15.4 (10.4–21.3)	12.9 [2.1] 17.0 (8.4–24.0)	6.1 [2.0] 6.9 (3.8–10.1)
Octanal (pM)	23.0 [1.7] 23.6 (17.7–33.2)	25.7 [1.5] 26.9 (19.1–33.5)	20.8 [1.9] 22.4 (16.9–34.2)	10.0 [1.8] 11.6 (7.2–16.2)
Nonanal (pM)	44.0 [1.8] 48.2 (31.6–62.5)	50.9 [1.7] 51.7 (32.4–72.1)	38.5 [1.9] 36.5 (31.1–60.1)	12.7 [1.8] 13.3 (7.2–22.7)

Table 3B
Total significance of one-way ANOVA (first column) and ANCOVA using age as covariate (third column) in healthy controls (group 0), non- or ex-smoking NSCLC patients (group 1), currently smoking NSCLC patients (group 2). Tukey's post hoc test was used for multiple comparisons.

Aldehyde	One-way ANOVA significance	Post hoc significant comparisons	One-way ANCOVA significance
Propanal	$p = 0.006$	0 *vs* 2, $p < 0.01$	$p = 0.08$
Butanal	$p < 0.001$	0 *vs* 1, $p < 0.01$ 0 *vs* 2, $p < 0.01$	$p < 0.001$
Pentanal	$p < 0.001$	0 *vs* 1, $p < 0.05$ 0 *vs* 2, $p < 0.01$	$p = 0.001$
Hexanal	$p < 0.001$	0 *vs* 1, $p < 0.01$ 0 *vs* 2, $p < 0.01$	$p < 0.001$
Heptanal	$p < 0.001$	0 *vs* 1, $p < 0.01$ 0 *vs* 2, $p < 0.01$	$p < 0.001$
Octanal	$p < 0.001$	0 *vs* 1, $p < 0.01$ 0 *vs* 2, $p < 0.01$	$p = 0.002$
Nonanal	$p < 0.001$	0 *vs* 1, $p < 0.01$ 0 *vs* 2, $p < 0.01$	$p < 0.001$

Table 4A
Structure matrix of discriminant analysis.

	Function	
	1	2
Nonanal	.866[a]	−.310
Hexanal	.725[a]	.252
Octanal	.598[a]	−.269
Heptanal	.538[a]	−.146
Butanal	.476[a]	.378
Pentanal	.394	.552[a]
Propanal	.289	.443[a]

[a] Largest absolute correlation between each variable and any discriminant function.

Table 4B
Cross-validated classification of subjects (only for cases in the analysis); each case was classified by the functions derived from all other cases. Group 0: healthy controls; group 1: non- or ex-smoking NSCLC patients; group 2: currently smoking NSCLC patients.

			Predicted group membership			
			Group 0	Group 1	Group 2	Total
Cross-validation	Count No.	Group 0	35	0	3	38
		Group 1	0	10	9	19
		Group 2	4	5	12	21
	Count %	Group 0	92.1	.0	7.9	100.0
		Group 1	.0	52.6	47.4	100.0
		Group 2	19.0	23.8	57.1	100.0

However, 36/40 NSCLC patients (90%) were correctly distinguished from controls. Plotting the Factor2 *vs* Factor1 scores (Fig. 4B) showed that the significant Factor1 mainly discriminated the controls and the two groups of NSCLC patients, whereas the non-significant Factor2 did not efficiently distinguish the two groups of NSCLC patients.

These results confirm that aldehydes may be promising biomarkers associated with NSCLC and that their combination with previously selected VOCs [19] could increase the sensitivity and specificity of the method.

Further ongoing studies include patients with other pulmonary diseases associated with inflammatory processes (e.g. COPD, asthma, cystic fibrosis, interstitial lung disease) and will thus clinically validate the strength of breath analysis in the early diagnosis of lung cancer.

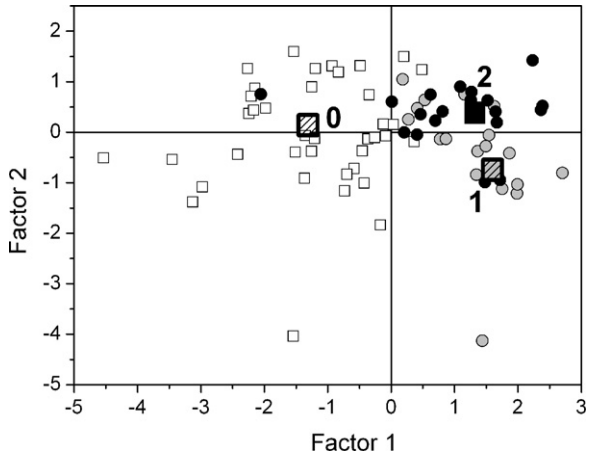

Fig. 4. Factor2 *vs* Factor1 scores for all of the study subjects. (□) Healthy controls (group 0); (◎) non- or ex-smoking NSCLC patients (group 1); (●) currently smoking NSCLC patients (group 2). The estimated centroids for the three groups are also shown.

4. Conclusions

In this study, we developed an on-fiber-derivatisation SPME/GC–MS method for detecting aldehydes in human breath. SPME offers many advantages over the conventional breath sampling methods by combining sample extraction, concentration and also improving aldehydes stability. However, preliminary experiments showed how critical the choice of the parameters affecting SPME process and their rigorous set-up can be, mainly in the case of gaseous substances at trace levels. Additionally, the broad linear dynamic range, the precision and the accuracy of the method and the low LODs all confirmed that on-fiber SPME/GC–MS is suitable and reliable to detect aldehydes in human breath.

The method was then applied to patients with early-stage NSCLC and a control group of asymptomatic subjects. Results demonstrated the good power of the pattern of selected aldehydes in distinguishing asymptomatic non-smokers and patients with early-stage NSCLC: 92.1% of controls *vs* 90% of NSCLC patients were correctly classified. Smoking habits or age had a limited influence on the results as confounding factors, thus confirming the promising role of breath analysis in the diagnosis of lung cancer.

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