



Facilitated Diffusion of Acetonitrile Revealed by Quantitative Breath Analysis Using Extractive Electrospray Ionization Mass Spectrometry

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By using silver cations (Ag^+) as the ionic reagent in reactive extractive electrospray ionization mass spectrometry (EESI-MS), the concentrations of acetonitrile in exhaled breath samples from the volunteers including active smokers, passive smokers, and non-smokers were quantitatively measured *in vivo*, without any sample pretreatment. A limit of detection (LOD) and relative standard deviation (RSD) were 0.16 ng/L and 3.5% ($n = 8$), respectively, for the acetonitrile signals in MS/MS experiments. Interestingly, the concentrations of acetonitrile in human breath continuously increased for 1–4 hours after the smoker finished smoking and then slowly decreased to the background level in 7 days. The experimental data of a large number of (> 165) samples indicated that the inhaled acetonitrile is excreted most likely by facilitated diffusion, instead of simple diffusion reported previously for other volatile compounds.

The analysis of human breath is of increasing importance due to the potential applications in clinical diagnosis, therapy monitoring, and pharmacokinetics studies^{1,2}. The breath test (e.g., respiration rate, respiration intensity) has been performed for the purpose of diagnosing diseases such as typhoid and kidney deficiency in Asian countries for more than two thousand years³. The instrumental analysis of breath was introduced by Linus Pauling⁴. With advanced gas-liquid partition chromatography, several hundred volatile organic compounds (VOCs) have been found in exhaled breath samples⁴. The exhaled breath also carries a lot of aerosols containing non-volatile compounds such as proteins⁵. Previous studies^{6,7} have shown that breath analysis serves as a chemical window to probe human metabolism. Fast and sensitive breath analysis is one of the most attractive research topics in the field of non-invasive clinical diagnosis. Several methods including gas chromatography/flame ionization^{8,9}, gas chromatography/mass spectrometry^{10,11}, gas chromatography/ion mobility spectrometry¹², proton transfer reaction/mass spectrometry^{13–16}, selected ion flow tube/mass spectrometry¹⁷, chemical sensors¹⁸, colorimetric analysis¹⁹, and micro-plasma⁷ have been developed for the analysis of exhaled breath samples. Among the abovementioned techniques, mass spectrometry is a preferred method for breath analysis, due to its premier sensitivity and the powerful capability for molecular structure elucidation. However, multiple-step sample pretreatment is usually required prior to MS analysis, especially a conventional ionization technique (e.g., electron impact (EI), chemical ionization (CI)) is used to create the analyte ions for MS analysis. In this case, the matrix containing nitrogen, oxygen, and water in the exhaled breath sample must be carefully separated to ensure successful detection of the analytes with high sensitivity and/or high mass resolution. Alternatively, extractive electrospray ionization mass spectrometry (EESI-MS), initially developed for the direct analysis of gas^{20,21}, liquid^{22–25}, aerosol^{26,27}, and viscous samples^{28,29}, has been demonstrated as a useful tool for the sensitive and *in vivo* analysis of breath samples²¹. Because the living subject is isolated from any high voltage or charged particles, EESI provides a MS tool to ensure the safety of living subjects under investigation. Nevertheless, *in vivo* detection of acetonitrile in breath samples remains a challenge since acetonitrile is difficult to be protonated due to its low proton affinity (779 kJ/mol).

Acetonitrile has been considered as one of the markers for lung cancer³⁰ and identified as a significant breath marker of smoking^{13,17,31}. Recently, increased concentrations of acetonitrile were also reported in the exhaled breath from drug addicts³². Therefore, the determination of acetonitrile in breath is important for disease diagnosis, clinic monitoring, and testing of drug abuse. It has been reported that high quality mass spectra were



obtained by reactive EESI-MS for sensitive detection of sulfur (S)-contained compounds in breath²⁰, because sulfur-contained compounds are soft Lewis bases and silver cations are soft Lewis acids, and the soft acids react fast to form strong bonds with soft bases as elucidated in the *Hard and Soft (Lewis) Acid and Base Theory*^{33,34}. Since the bonding energy of acetonitrile to Ag^+ is substantially high (39.4 kcal/mol, at 298 K)³⁵, in this work, silver cations produced by electrospray ionization (ESI) were used as the primary ionic reagents to be utilized in ion/molecule reactions with acetonitrile during the EESI process, resulting in a novel method for the direct, quantitative, and *in vivo* detection of acetonitrile in breath samples. Surprisingly, the data obtained from all the exhaled samples from volunteers including active smokers, passive smokers, and non-smokers showed that the acetonitrile levels increased significantly within 1–4 hours after they finished smoking. This positively supports that the acetonitrile was excreted through expiration by facilitated diffusion instead of simple diffusion.

Results

Reactive EESI-MS spectra of acetonitrile. All the experiments carried out on human subjects including active smokers, passive smokers, and non-smokers have passed the assessment by the Ethics Committee in the East China Institute of Technology. All the exhaled breath was directly introduced into the EESI source through disposable mouthpieces to eliminate any carry over effect. In the EESI-MS spectra collected using pure methanol solvent without silver nitrate as the cationization reagent, no signal corresponding to acetonitrile was detected; this is because acetonitrile is a compound with relative low proton affinity (779 kJ/mol), which makes it difficult to be protonated using either ESI or EESI technique. Fig. 1 shows a typical EESI-MS spectrum recorded for the headspace analysis of the acetonitrile solution (1 ppb) with silver cations as the primary ionic reagents in the ESI channel. The signals at m/z 107, 109 corresponded to the ions of $^{107}\text{Ag}^+$, $^{109}\text{Ag}^+$, respectively. The signals at m/z 148, 150 were designated to the adducts of $[\text{}^{107}\text{Ag} + \text{CH}_3\text{CN}]^+$, $[\text{}^{109}\text{Ag} + \text{CH}_3\text{CN}]^+$, respectively. These assignments were confirmed by the fragments (insets of Fig. 1) created by the collision induced dissociation (CID) experiments. During the CID processes, the fragmental ions of m/z 107, 109 ($^{107}\text{Ag}^+$, $^{109}\text{Ag}^+$) were observed by the neutral loss of ACN. The peaks at m/z 166/168 correspond to the adduct ions $[\text{}^{107}\text{Ag} + \text{CH}_3\text{CN} + \text{H}_2\text{O}]^+$, and $[\text{}^{109}\text{Ag} + \text{CH}_3\text{CN} + \text{H}_2\text{O}]^+$. The signals at m/z 189/191, which show up only at high concentration of acetonitrile, correspond to the adduct ions $[\text{}^{107}\text{Ag} + 2\text{CH}_3\text{CN}]^+$ and $[\text{}^{109}\text{Ag} + 2\text{CH}_3\text{CN}]^+$. The structures of these ions were confirmed by using CID experiments (see Supplementary

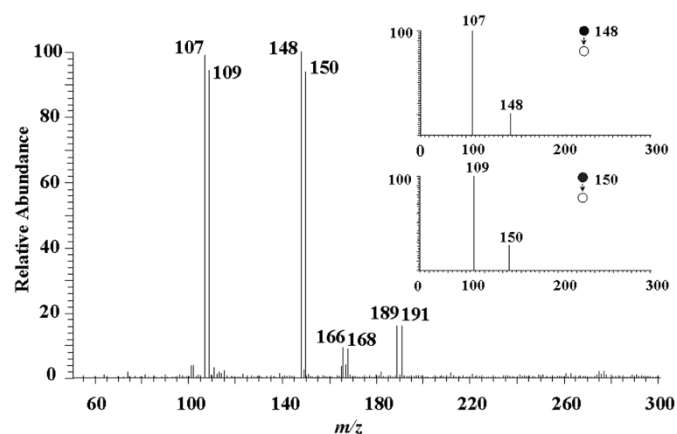


Figure 1 | The MS spectrum of acetonitrile recorded using a standard acetonitrile solution by reactive EESI-MS; the insets show the CID spectra of m/z 148, 150.

Fig. S1 online). Similarly, in the analysis of a breath sample from an active smoker (adult male), trace amount of acetonitrile in breath was detected in the EESI-MS spectrum after addition of silver cations (Fig. 2), showing the characteristic isotopic peaks of $[\text{Ag} + \text{CH}_3\text{CN}]^+$. As shown in the insets of Fig. 2, the product ion spectra of the selected ions (m/z 148, 150) yielded the ionic Ag^+ isotopes by the loss of neutral CH_3CN . These data confirmed that acetonitrile in the exhaled breath sample was sensitively detected using the EESI technique. Several other peaks in Fig. 2 correspond to the ionized other species in the breath sample. The absolute intensities of Ag^+ in Fig. 1 and Fig. 2 are both about 1×10^4 cps. For the quantitative analysis of acetonitrile, the selected reactive monitoring operation mode was used, therefore the other peaks will not affect the quantitative data.

The establishment of calibration curve. For several applications such as clinical diagnosis and biomarker discovery, it is required to perform quantitative analysis of acetonitrile in human breath. According to the Henry's law, there is a linear relationship between the concentrations of analytes in the solution headspace (c_g) and that in the liquid phase (c_l) as discussed in details elsewhere²¹. The calibration curve of acetonitrile was established according to the procedure developed based on the Henry's coefficient of acetonitrile (detailed in SI Text). The characteristic fragment derived from the precursor ions (m/z 148) was used for quantitative measurements. A good relative standard deviation (RSD) of 3.5% for 8 measurements was obtained (as listed in Supplementary Table S1), providing a linear dynamic range from 0.747 ng/L to 747 ng/L with a linearity coefficient of 0.992, as shown in Fig. 3. The limit of detection (LOD, $\text{S/N} = 3$) was determined to be 0.16 ng/L by gradually decreasing the acetonitrile concentration until the signal/noise reached 3 in selected reaction monitoring operation mode.

Quantification of acetonitrile in the exhaled breath samples of active smokers. Samples donated from 15 human subjects including 4 non-smokers, 4 smokers smoking about 5 cigarettes per day, 3 smokers smoking about 10 cigarettes per day, 2 smokers smoking about 15 cigarettes per day, and 2 smokers smoking about 20 cigarettes per day, have been analyzed as soon as they consumed a cigarette (type A). The plot of breath acetonitrile concentrations as a function of the daily cigarette consumption is shown in Fig. 4. Clearly, Fig. 4 shows a positive correlation between the breath acetonitrile concentrations and the smoking amounts per day. This is because of the accumulation of acetonitrile in heavy smokers.

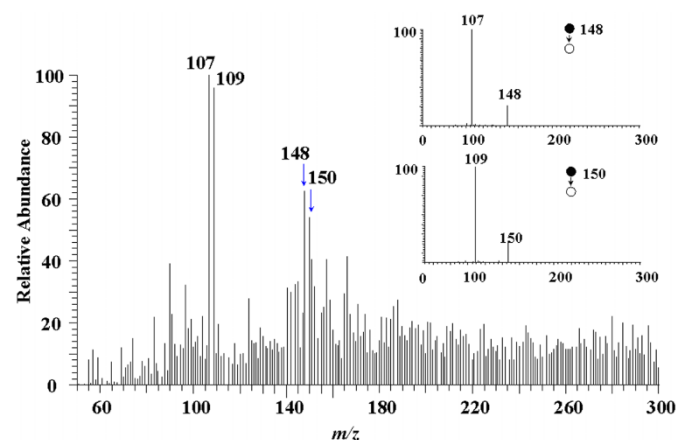


Figure 2 | The MS spectrum of an exhaled breath sample by reactive EESI-MS; the insets show the CID spectra of m/z 148, 150.

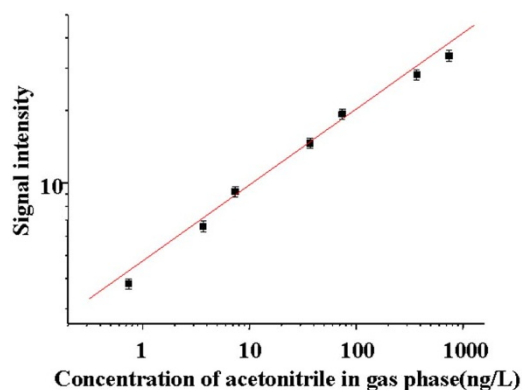


Figure 3 | Calibration curve of acetonitrile obtained using EESI-LTQ-MS/MS. The signal at m/z 107 was used for plotting this calibration curve.

Quantification of acetonitrile in the exhaled breath samples of passive smokers. The concentrations of acetonitrile in the breath samples from passive smokers were also detected using EESI-MS. The experiments were carried out in a clean room of about 32 m³. No furniture but 4 wood chairs for 4 people was kept inside the room. 3 non-smokers sat concentrically around the smoker, with radial intervals of 0.5 m, 1.0 m, and 2.0 m between the smoker and non-smoker 1, non-smoker 2, and non-smoker 3, respectively. The door was closed and no air flow inside the room was detected during the experiments. The first cigarette was lit when the non-smoker volunteers went into the room. The exhaled breath samples were quickly analyzed when the cigarette ran out. The door remained closed during the measurements, and no intentional air ventilation was performed. The next cigarette was lit when the volunteers came back to the room from the laboratory after measurements. This procedure was repeated 5 times to complete all the measurements. As shown in Fig. 5, the acetonitrile levels in the breath samples from passive smokers increased as more cigarettes were smoked by the active smoker. After the first cigarette was consumed, the increase of the breath acetonitrile levels were calculated with subtraction of the individual original acetonitrile concentrations before smoking and the calculation details are shown in Supplementary Table S2. The final results of the 3 non-smokers were ordered in the sequence: non-smoker 1 (12.9 ng/L) > non-smoker 2 (12.4 ng/L) > non-smoker 3 (11.7 ng/L). The corresponding uncertainties of the final results were 0.6, 0.5, and 0.4, respectively. The experiment was repeated and the same tendency was obtained as listed in Supplementary Table S3. A reasonable explanation was that under the experimental conditions the non-smoker 1 consumed much more smoke than the non-

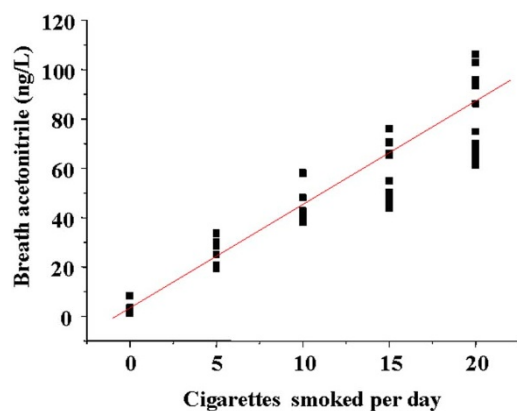


Figure 4 | Breath acetonitrile concentrations as a function of the daily smoking amounts (type A). Each point designates a single measurement. Each volunteer was measured 6 times.

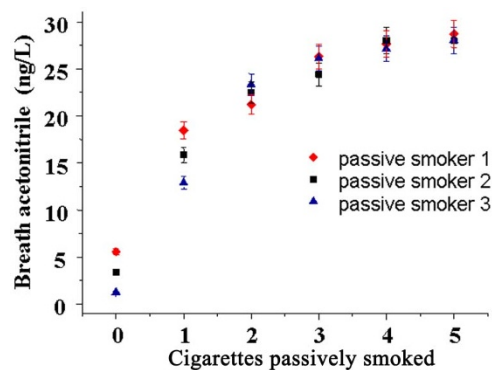


Figure 5 | Breath acetonitrile concentrations as a function of the passively smoked amounts.

smoker 3 because non-smoker 1 sat the most closely to the smoker. The difference of the breath acetonitrile levels among the 3 non-smokers was gradually blurred when more cigarettes were consumed by the smoker, and the breath acetonitrile reached the same level when 5 cigarettes were consumed. This was because all the 3 non-smokers consumed similar amounts of smoke since the concentration of smoke eventually was evenly distributed in the whole room. The total time for the non-smokers stayed with the smoker was about 25 min, during which 5 cigarettes were completely smoked by the active smoker. The final concentrations of acetonitrile in the breath samples from passive smokers ranged between 26–30 ng/L. Note that the breath acetonitrile concentration was measured to be 20–30 ng/L for the light active smokers (5 cigarettes per day) after consuming 1 cigarette (Fig. 4). This indicates that, from the acetonitrile level point of view, it is still harmful to the passive smoker even they are 2 m away from the active smoker. Staying in a closed space (~32 m³), where people are smoking heavily, for about 25 min is equivalent to actively smoking more than 1 cigarette. These findings provide quantitative data to evaluate the side effects of passive smoking, and the data at least partially support that smoking in public buildings such as restaurants and airports should be avoided.

Increased acetonitrile levels found in the exhaled breath of smokers after smoking. Taking the advantages of EESI-MS for rapid and quantitative detection, the acetonitrile concentrations of breath samples from 4 volunteers (1 male, adult smoker; 1 female, adult smoker; 1 male, adult non-smoker; 1 female, adult non-smoker) were *in vivo* monitored (40 samples, 10 measurements for each volunteer) using the method reported here. The breath acetonitrile concentrations have been plotted as a function of the time after volunteers consumed a cigarette (type A). As shown in Fig. 6a, the concentrations of acetonitrile in the breath samples from smokers increased noticeably (17.8%–35.5%) to their highest levels within 4 hours and then decreased slowly down to the background levels within 1 week. Note that all the volunteers maintained their normal daily activities, but all of them did not consume any more cigarettes during the test (7 days).

The dynamic change of acetonitrile was measured in the previous study¹⁶, showing that the dynamics is identical to the post-24-hour data reported here. In the current study, however, it was surprisingly found that the breath acetonitrile concentration increased within the first 4 hours after smokers stopped smoking. Unfortunately, similar data of the breath acetonitrile levels were not available in the literature¹⁶.

To validate the increase of acetonitrile observed in breath after smoking, extra studies involving a large sample set (125 samples from 25 smokers, consuming 1–5 type B cigarettes) have been performed using the same technique reported here. The breath acetonitrile levels

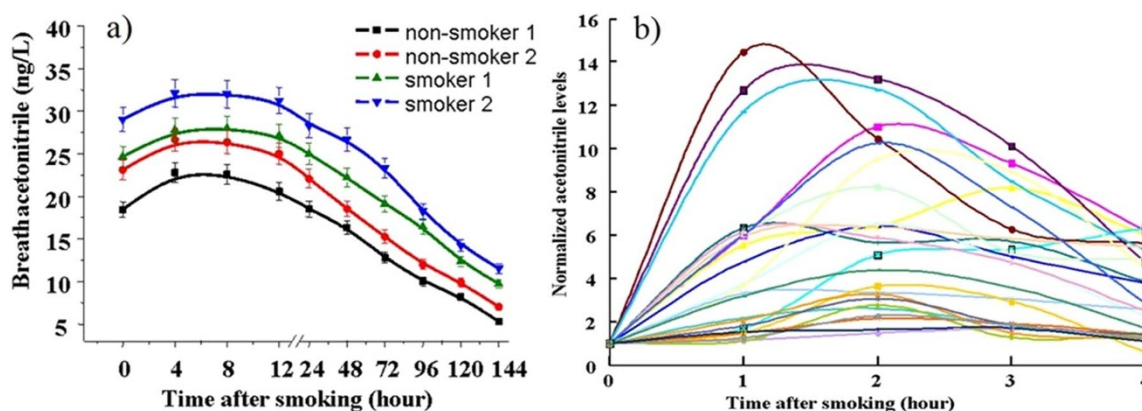


Figure 6 | Increasing acetonitrile levels found in the exhaled breath after smoking. a) Acetonitrile concentrations in the breath of 4 volunteers after they smoked type A cigarettes; the break is from 14–20 hours. b) Acetonitrile concentrations in the breath of 25 volunteers after they smoked type B cigarettes; the acetonitrile concentrations were normalized to the initial concentrations measured immediately after smoking.

ranged between 8–69 ng/L, some of which were much higher than those detected after smoking a type A cigarette. For better comparison, the concentrations of acetonitrile were normalized to the initial concentration measured immediately after smoking. As shown in Fig. 6b, the acetonitrile levels in breath samples increased from 1 to 1.47–14.5 (85–200 ng/L) in about 1–2 hours after stopping smoking; the breath acetonitrile levels gradually decreased to 0.5–6.3 in 4 hours. The time difference for acetonitrile concentrations to reach the maximum probably was due to the individual differences of volunteers. The dynamic response was repeated in various volunteers who were examined multiple times on different dates. The consistent results confirmed the responses of breath acetonitrile observed in Fig. 6b. The experiments were also repeated after two years and the same tendency for the alteration of acetonitrile levels was observed as shown in Supplementary Fig. S2. These data provided good evidences to support the long-term stability of the method, indicating good reproducibility of the observation.

It has been reported that acetonitrile detected in breath comes from the cigarette smoke³⁶. To demonstrate that the further increased acetonitrile after smoking was not a metabolite induced by the other chemicals in the cigarette smoke, deuterated acetonitrile (2–5 μ L) with comparable amounts as that of acetonitrile in the cigarette was spotted onto the top of cigarette. Then the experiments were carried out as described above. Deuterated acetonitrile and un-deuterated acetonitrile have almost identical chemical properties, and thus they must have very similar respiratory behaviors. If the exhaled acetonitrile had not originated from inhaled cigarette smoke (*i.e.*, it might be a metabolite induced by some other chemicals in the cigarette smoke), the time-course changes of the isotope labeled and the unlabeled acetonitrile should be different, because the deuterated acetonitrile was known to be only from inhalation of the smoke in this study. In our experiments, increased deuterated acetonitrile levels were also observed; and the experimental data showed that the deuterated acetonitrile in the breath had the same behavior as the un-deuterated acetonitrile (see Supplementary Fig. S3 and Fig. S4 online). This confirmed that the increased acetonitrile originates from cigarette smoke, not a metabolite induced by some specific chemicals in cigarette smoke.

Discussion

The metabolism of volatile toxic gases through aspiration is generally assisted by simple diffusion³⁷, during which a substance passes through a membrane driven by the diffusion force without the aid of any carrier protein. Therefore, in this process, the levels of inhaled substances in breath will decrease monotonically. For example, immediate and continuous decrease of nicotine levels in the breath after smoking was observed by EESI-MS²¹. However, the acetonitrile

levels increased after smoking, which demonstrated that acetonitrile was not excreted by a simple diffusion process, but likely by a facilitated diffusion through a specific carrier protein. This might be because acetonitrile has a high hydrophilicity and thus has a high solubility in the blood, which makes it difficult to transport from the blood into the alveolus, even under a positive concentration gradient. The exhaled acetonitrile concentration first increased after smoking, probably because a carrier protein was increased due to the increased stress of acetonitrile. Although the proteins responsible for the facilitated diffusion have not been identified, these findings are useful for further understanding the bio-transportation and detoxification mechanisms of the environmental contaminants.

A novel selective extractive electrospray ionization method has been developed for the quantitative and *in vivo* detection of acetonitrile in the exhaled breath. The acetonitrile concentrations in the exhaled breath samples from the active smokers with different cigarette consumptions, the passive smokers with different cigarette consumptions, and non-smokes have been quantitatively detected without any sample pretreatment. The time-course changes of acetonitrile have been followed using the technique reported here. Interestingly, our data demonstrated that the concentrations of acetonitrile in breath increased significantly before they dropped down after the smokers stopped smoking. The experimental data of many samples indicated that acetonitrile was transported by the process of facilitated diffusion instead of simple diffusion. Although the fundamental biological mechanism is still unknown, the analytical technique established in this study is particularly useful due to its capability to profile breath samples *in vivo*, with high throughput, high sensitivity, and high selectivity.

Methods

Instrumentation and working conditions. All the experiments were carried out using a Thermo Finnigan LTQ-XL mass spectrometer (San Jose, CA) coupled with a homemade EESI source. The EESI design and principle were described in details elsewhere^{20,23}. Briefly, the EESI source was constructed for the *in vivo* breath analysis, and it was composed of two channels as shown in Fig. 7. Channel 1 was used for the generation of the primary ions of Ag^+ by electrospraying silver nitrate in the methanol solution (10 $\mu\text{g}/\text{mL}$). The channel 1 was made from two capillaries. A long piece of thin capillary (ID 0.10 mm, OD 0.15 mm, Agilent Technologies Co., Ltd., USA) was used as an electrospray emitter, and a short piece of thick capillary (ID 0.25 mm, OD 0.40 mm, Agilent Technologies Co., Ltd., USA) was coaxially installed outside of the thin capillary to introduce the sheath gas for electrospray ionization. Channel 2 was made of a piece of Teflon tube (ID 3.0 mm, OD 3.5 mm, Qi Wei Co., LT), which was used for the breath sample introduction. A gas flow meter was used to monitor the breath flow. The breath flow rate exhaled by volunteers was maintained at about 800 mL/min. The distance (a) between the ESI spray emitter and the inlet of the mass spectrometer was 10 mm, and the distance (b) between the ESI spray emitter and the sample injector was 2 mm. The angle (α) formed between the electrospray emitter and the inlet of the mass spectrometer was 90°, and the angle (β) formed between the electrospray emitter and the sample injector was 120°. A high voltage of

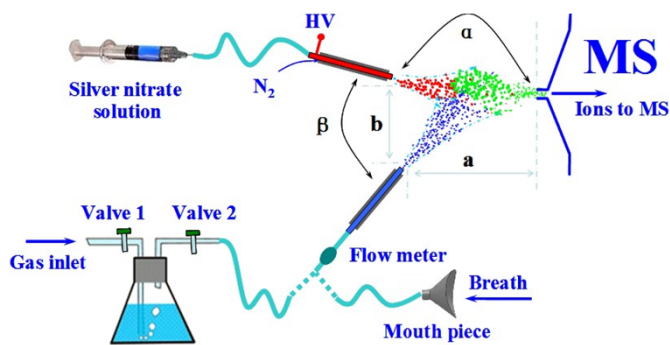


Figure 7 | Schematic diagram of the EESI-LTQ-MS for in vivo breath analysis (A flask was used when establishing the calibration curve, while breath sample directly passed through the Teflon gas line to the EESI source).

3.5 kV was applied to the ESI spray emitter to generate the primary ions (i.e., Ag^+) in the positive ion detection mode. The ESI solution (silver nitrate in methanol) was delivered to the electrospray emitter by a syringe pump at a flow rate of 2.0 $\mu\text{L}/\text{min}$. The analytes in exhaled breath were ionized directly without any sample pretreatment when the breath intersected the primary ion beam. The temperature of the inlet capillary was maintained at 150°C during all the experiments. The pressure of the sheath gas (N_2) was 1.4 MPa. The ion injection time was set to 100 ms for the LTQ-MS instrument. To perform CID experiments, ions of interest were isolated using an m/z window width of 1 unit. Helium was used as the collision gas and the CID energy was 25% with a duration time of 30 ms for tandem mass spectrometry experiments. The other parameters used in the experiments were the default values of the mass spectrometer. All the mass spectra were obtained using the Xcalibur software of the LTQ instrument without background subtraction. Naturally, a single breath exhalation last 15–25 s, depending on each individual of the volunteers. During the exhalation process, many MS scans (ca 500 ms for each scan) were completed since the EESI-MS instrument worked continuously. For better quantification, the mean value of 20 MS scans was used as one measurement result. The average of 6–8 measurements from the same volunteer was used as the final result for this subject. Single breath exhalation profiles are shown in Supplementary Fig. S5. A single sample analysis was completed within 3 minutes. The concentrations of acetonitrile in breath samples were calculated from the calibration curve.

Statement. The present study was approved by the Ethics Committee of the East China Institute of Technology and adhered to the tenets of the Declaration of Helsinki³⁸. Additionally, the benefits and risks of this study were clearly presented to the volunteers, and thereafter the written consent was obtained if they agreed to join the study.

Samples preparation. Commercial cigarettes (type A and B) used in the experiments were purchased from a local store without any pretreatment. As noted by the manufacturers, one cigarette A produces cigarette tar 13 mg, carbon monoxide 13 mg, and nicotine 1.3 mg; one cigarette B creates cigarette tar 13 mg, carbon monoxide 15 mg, and nicotine 1.31mg. All the male and female volunteers in the experiments were selected from healthy adults (age from 21–25). They maintained their daily lifestyle as usual during the experiment periods without any special care.

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Author contributions

H.C. designed the research; J.D., Y.Z., H.G., performed the research; M.L., N.X., S.P., H.L. analyzed data; M.L., H.C. wrote the manuscript and completed the revision.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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