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High mass resolution breath analysis using secondary electrospray ionization mass spectrometry assisted by an ion funnel

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In this study, we used secondary electrospray ionization mass spectrometry assisted by an ion funnel (IF) operating at ambient pressure to find compounds in the mass range of 100–500 m/z in online breath fingerprinting experiments. In low-resolution experiments conducted on an ion trap instrument, we found that pyridine is present in breath of individuals long after drinking coffee. In high-resolution experiments conducted on a Fourier transform ion cyclotron resonance, we found more than 30 compounds in the mass range of 100–500 m/z in analogous online breath experiments. More than a third of these compounds have molecular weights above 200 Daltons and have not been mentioned in previous studies. In low-resolution experiments as well as experiments without the IF, these compounds could not be detected. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: secondary electrospray ionization; ion funnel; breath analysis; online mass; spectrometry; FTICR

Introduction

Hardly any of the common techniques used for getting information on the general health condition of patients are completely noninvasive, fast or convenient, and safe for the patient. Quite the opposite is true for breath analysis, an approach that has been known for centuries. In 1950, the first publication on the level of blood alcohol based on breath analysis was published.[1] However, it took another 20 years until Linus Pauling showed in his work that breath condensate collected and sampled offline and analyzed with gas chromatography-mass spectrometry (GC–MS) contained more than 250 different substances.[2] Many different techniques emerged in the wake of this publication, which is usually regarded as the 'birth' of modern breath analysis. When analyzing breath, the techniques can be divided into two main categories: one can either collect and concentrate breath (offline sampling), or measure it directly and in real time (online sampling). Among the offline sampling techniques, exhaled breath condensate (EBC)[3–7] is probably the most widespread today. To process the data and characterize as many signals as possible in EBC, headspace solid-phase microextraction combined with GC-MS proved to be very useful[8]. Online sampling of breath is relatively simple as long as substances with high abundance in breath such as ethanol are investigated. As soon as the concentrations are lower than parts per million per volume, the sensitivity of the detection device becomes the bottleneck for the analysis. Being forced to quasi-simultaneously measuring a wide range of compounds additionally complicates the development of suitable devices.

During the last two decades, several MS-based techniques have been developed that deal with this challenge. They focus on volatile organic compounds with molecular weights usually not exceeding 200 Daltons (Da) and all belong to the category of ambient MS techniques. The most widely used technique is ion mobility[9] with its main areas of application being the detection of drugs and explosives in airports and in military operations. Other techniques that find regular applications are proton transfer reaction MS developed by Hansel *et al.*,[10] selected ion flow tube MS developed by Smith and Špan l,[11,12] as well as electrospray ionization MS (ESI-MS)[13]-based techniques such as secondary ESI (SESI)[14–17] and desorption ESI.[18,19] Here, we show the detection of compounds with molecular weights of up to 500 Da. Online techniques capable of monitoring trace amounts of compounds in breath in real time have a broad range of commercial applications. Since the amount of volatile compounds excreted via breath corresponds to the concentration in blood,[20] successful online breath sampling would, e.g. allow real-time, *in-vivo* observations of the pharmacokinetics of drugs, monitoring of anesthetics during surgery or fast screening of athletes for doping compounds prior to sport events. Additionally, and in comparison to time-consuming offline experiments, metastable compounds that degrade within minutes could be investigated in online breath analysis experiments.

In this study, we combined a breath with a secondary ESI (SESI) interface that is assisted by an ion funnel (IF) to find compounds with molecular weights above 200 Da in breath in real time. While our first approach by using a LCQ-Deca as mass spectrometer showed that the peak resolution was insufficient, we could find a lot of compounds that are exclusively present in breath when using a linear trap quadropole-Fourier transform ion cyclotron resonance (LTQ-FTICR) mass spectrometer. The high resolution even allowed us to unambiguously determine the sum formulae of 32 compounds with molecular weights of up to 500 Da that have so far never been detected in online experiments.

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Experimental section

All experiments in this publication were conducted on two commercial ion trap mass spectrometers (LCQ-Deca and LTQ-FTICR Ultra, both from Thermo Finnigan, San Jose, USA). Breath was introduced online and the neutral, gaseous molecules contained in breath were ionized with a SESI interface that was incorporated into an ambient pressure IF system to enhance the overall sensitivity. Detailed information on both SESI interface as well as the setup and construction of the IF can be found in the literature.[21,22]

An overview of the breath interface used in this study is shown in Fig. 1. Breath is introduced into the system via a disposable non-return valve mouth piece (#1; ACE series, ACE GmbH, Freilassing, Germany) to prevent saliva from entering the breath interface and through an aluminum breath inlet (#2; id 9 mm, od 12 mm). Within the breath interface, the gaseous sample is split; one part leaves the interface through an outlet (#4; id 2 mm, od 12 mm), the other part is transferred towards the charging sprays (#11) through a stainless steel capillary (#5; id 1mm, od 1.3 mm) that is held in place by a Swagelok union (#6) and an aluminum adapter (#7) to connect the breath interface with the charging spray unit. A stainless steel plug (#3) allows for easy access to the breath interface for cleaning (#8; aluminum, id 9 mm, od 15 mm). The charging spray unit (#10) was heated to 75°C (less than boiling point of the solvent mixture used) with three heating cartridges (#9; Probag Wärmetechnik AG, Niederbuchsiten, Switzerland) to prevent sample molecules from condensing. Heat transfer through the aluminum adapter (#7) resulted in warming of the breath interface to roughly 40°C.

The charging spray solvent consisted of a MeOH:H_2O mixture in a 1:1 ratio acidified with 1% acetic acid, and three indentical sprays were infused at 0.05 ml/min each. A voltage of 3 kV was applied. The transfer capillaries on both the LCQ and the LTQ-FTICR were held at 250°C. All other parameters were optimized for maximum ion yield. Data acquisition was controlled by the Xcalibur 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were collected in scanning mode in the m/z range of 50–500 Th for LCQ experiments and 100–500 for LTQ-FTICR experiments.

Caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione) and MRFA (Met-Arg-Phe-Ala), a component of the ProteoMass™ LTQ/FT-Hybrid ESI Pos. mode CalMIX, as well as nicotine (3-(1-methyl-2-pyrrolidinyl) pyridine), were obtained from Sigma Aldrich (Buchs, SG, Switzerland). Norfentayl, morphine, cocaine, naloxone, fentanyl and sulfentanil were all obtained from Lipomed AG (Arlesheim, Switzerland). All compounds mentioned above were used for mass calibration of the LTQ-FTICR. Methanol and acetic acid were obtained from Acros Organics (Geel, Belgium). Nanopure water with a resistivity of >18.1 MΩ·cm was obtained from a NANOpure water purification system (Barnstead, IA, USA).

Results and discussion

In a first set of experiments and to prove that our breath interface is working, we performed a series of experiments with the LCQ as mass analyzer. Three individuals delivered their breath into the breath interface by exhaling for roughly 20–30 s per exhalation. In a typical experiment, an individual would exhale five to six times with 1.5 min breaks between consecutive exhalations, to allow for equilibrated background prior to the next exhalation. 'Background experiments' means sampling air that was humidified by blowing it through a gas wash bottle filled with water. Both in breath and background experiments, roughly 500 ml/min of volume was sampled, of which roughly 250 ml/min entered the SESI interface. In all experiments, the total ion current (TIC) measured rose during periods of exhalation and fell in between. This phenomenon has been described previously.[23] Figure 2 shows the intensity of m/z = 80 for nine different experiments that were conducted in triplicate. The experiments represent breath samples of individuals A, B and C, and breath samples after consuming different foods or drinks: (1) A; (2) B; (3) C; (4) B after eating an orange; (5) A after eating a chewing gum; (6) B after eating a peppermint candy; (7) B after drinking lime juice; (8) A after drinking coffee; (9) B after drinking coffee. The inset shows the peak shape of m/z = 80 during experiment (8) with a mass resolution of R = 150 at full width at half maximum, the resolution obtained with the LCQ in full scanning mode. Background experiments are not shown as no signal (S/N < 100 cps) could be detected at m/z = 80. Experiments prior to which coffee was consumed (8 and 9) show almost an order of magnitude higher intensity. The main compound with a $[M+H]^+$ of m/z = 80 is pyridine. This is in accordance with gas-chromatography-based publications that found high amounts of pyridine in coffee.[24–27] In addition to the m/z value shown, more than 20 other m/z values were found with differing intensities among the experiments. Principal component analysis that was performed on those selected m/z values allowed for the discrimination of experiments and therefore individuals from each other (data not shown).

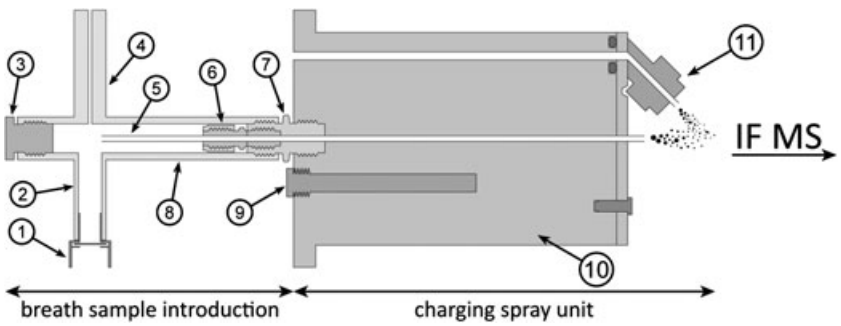

Figure 1. The breath interface: (1) mouth piece with non-return valve; (2) aluminum breath inlet (id 9 mm, od 12 mm); (3) stainless steel plug to allow for easy cleaning of breath interface; (4) aluminum breath outlet (id 2 mm, od 12 mm); (5) stainless steel capillary (id 1 mm, od 1.3 mm); (6) Swagelok union to mount (5); (7) aluminum adapter to connect breath interface with charging spray unit; (8) aluminum breath sampling tube (id 9 mm, od 15 mm); (9) heating cartridge; (10) heated charging spray unit; (11) charging sprays.

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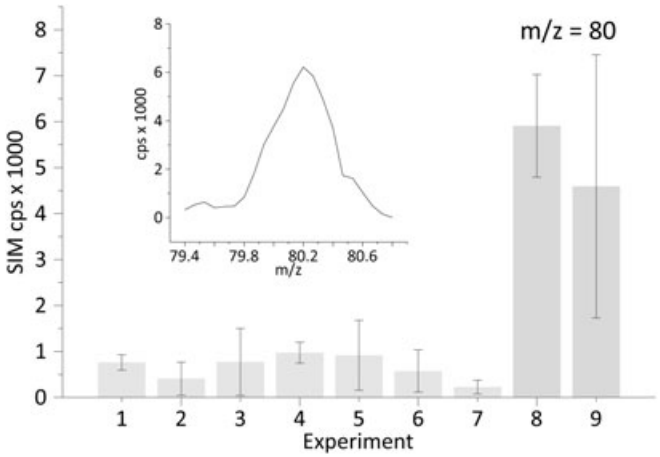

Figure 2. Single ion monitoring of m/z = 80, i.e. protonated pyridine on the LCQ-Deca. The experiments represent breath samples of individuals A, B and C and breath samples after consuming different foods or drinks: (1) A; (2) B; (3) C; (4) B after eating an orange; (5) A after eating a chewing gum; (6) B after eating a peppermint candy; (7) B after drinking lime juice; (8) A after drinking coffee; (9) B after drinking coffee. The inset shows the peak shape m/z = 80 during experiment (8).

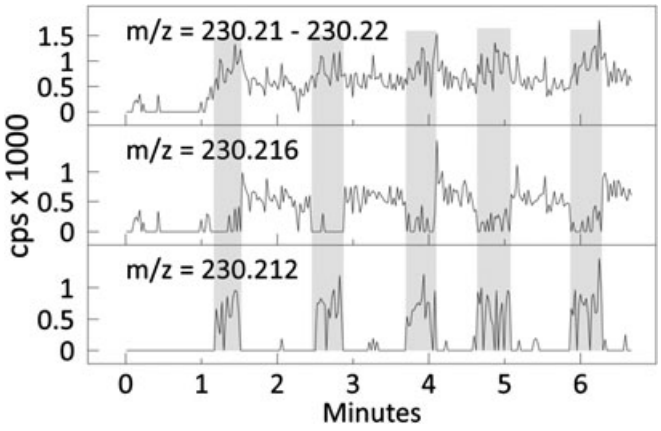

Figure 4. Single ion monitoring of m/z = 230 on the LTQ-FTICR with a resolution of 100 000. The spectra show the measured ion current in the mass range displayed. The grey shading is for visual help to show the time intervals during which breath was delivered into the system. A resolution of less than 25 000 would not reveal the signal from the background (top). Note that only with appropriate resolution, the compound with m/z = 230.212 (bottom) can be distinguished from background signals (middle).

However, the differences in intensities were not very distinct, and the low mass resolution gave rise to overlap of signals originating from breath and from chemical noise.

In a second set of experiments, we mounted the interface in front of a LTQ-FTICR. Individuals exhaled into the instrument after washing their mouth with water. The advantage of using a mass analyzer with high resolution becomes apparent in Fig. 3 and Fig. 4. Figure 3 shows normalized breath spectra recorded with the LCQ (dashed line) and the FTICR (solid line) the in the m/z range 176–184 Th. While for the LCQ experiment, no signal was significantly higher than the background in the whole mass range, sharp signals can be detected in the FTICR experiment. The high resolution allows for the determination of the chemical formulae of signals. For example the peak marked with an asterisk originates from a neutral compound (probably a glucono δ-lactone) with $C_6H_{10}O_6$ as chemical formula. Figure 4 shows five consecutive exhalations (grey) recorded over a period of 7 min.

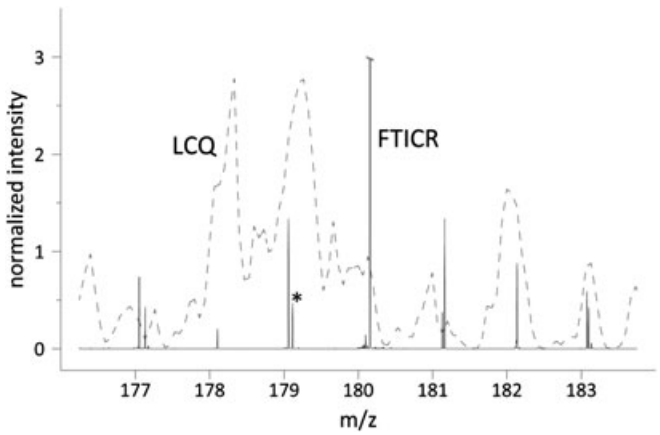

Figure 3. Normalized breath mass fingerprints recorded with the LCQ (dashed line) and the FTICR (solid line) in the m/z range 176–184 Th. For example, the signal marked with an asterisk can only be detected with high resolution and originates from a neutral compound (probably a glucono delta-lactone) with the chemical formula $C_6H_{10}O_6$.

The top spectra show the ion current in the mass range of 230.21–230.22, the middle and bottom selected ion currents recorded with a resolution of R = 100 000. It can easily be seen that the signal at m/z = 230.212 (bottom) is only present in breath and would have been hidden in the background at lower resolution. Figure 5 shows the TIC of humidified air, the TIC during a breath experiment and the single ion monitoring of m/z = 259.150 and m/z = 223.098 (top to bottom). Two typical observations made during our breath experiments can be seen here. First, the TIC gradually increases during each exhalation. Second, compounds that originate from breath follow one of two

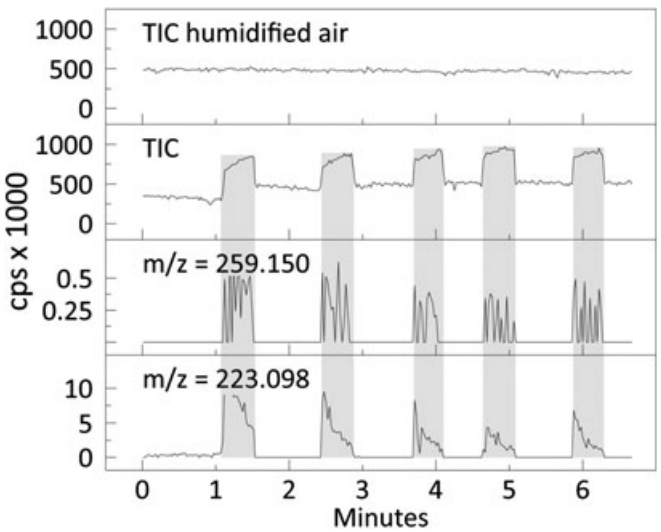

Figure 5. Mass spectra of humidified air (background), total ion current during breath experiments, m/z = 259.150, and m/z = 233.098 (from top to bottom). The grey shading is for visual help to show the time intervals during which breath was delivered into the system. Note that compounds in breath show two different behaviors: they either remain constant in intensity over one breath cycle and over time as shown with m/z = 259.150, or their intensity drops during single breath cycles and within a short time span as shown with m/z = 233.098.

Table 1. The m/z found in breath shown with the proposed chemical formulas

Exact masses found in breath and proposed chemical formulas as $[M+H]^+$

m/z	Chemical formula	m/z	Chemical formula	m/z	Chemical formula
102.128	$C_6H_{16}N$	178.109	$C_8H_{12}N_5$	230.212	$C_{13}H_{28}NO_2$
117.103	$C_5H_{13}N_2O$	179.056	$C_6H_{11}O_6$	237.197	$C_{14}H_{25}N_2O$
128.144	$C_8H_{18}N$	181.134	$C_{10}H_{17}N_2O$	265.253	$C_{18}H_{33}O$
136.076	$C_8H_{10}NO$	181.170	$C_{11}H_{21}N_2$	302.139	$C_{17}H_{20}NO_4$
147.150	$C_7H_{19}N_2O$	183.077	$C_8H_{11}N_2O_3$	334.322	$C_{21}H_{40}N_3$
160.134	$C_8H_{18}NO_2$	193.134	$C_{11}H_{17}N_2O$	348.217	$C_{20}H_{30}NO_4$
163.124	$C_{10}H_{15}N_2$	197.154	$C_{12}H_{21}O_2$	358.213	$C_{20}H_{28}N_3O_3$
167.144	$C_{11}H_{19}O$	198.149	$C_{11}H_{20}NO_2$	389.342	$C_{26}H_{25}O_2$
172.134	$C_9H_{18}NO_2$	209.165	$C_{12}H_{21}N_2O$	392.280	$C_{23}H_{38}NO_4$
176.176	$C_8H_{22}N_3O$	215.212	$C_{12}H_{27}N_2O$	417.373	$C_{28}H_{49}O_2$
177.139	$C_{11}H_{17}N_2$				

different patterns: they either retain the same or a similar intensity over several consecutive exhalations (e.g. m/z = 259.150), or their intensity fades both within one exhalation as well as over consecutive exhalations (e.g. m/z = 223.098). Our interpretation is that the first behavior is typical for compounds that stem from the lung and reach a fast equilibrium between blood and breath concentration, while the second behavior relates to compounds that originate from the mouth cavity and are not as volatile. This would explain why in comparison to the 'steady' compounds the intensity of the 'fading' compounds drops so sharply after the first few seconds of every exhalation and does not recover between consecutive exhalations.

Table 1 shows 31 m/z values with exact masses and their elemental composition that could be found in breath experiments. None of these compounds could be detected in the low mass resolution experiments using the LCQ-Deca, as the resolution to distinguish signal from noise was not sufficiently high. Only FTICR measurements with a resolution between 12 500 and 100 000 allowed for unambiguous detection of these compounds. For a m/z value to be classified as present in breath, we defined that it had to be present in all experiments (>50), and that the m/z must not be present in any humidified air (background) experiment. As expected, most of the proposed compounds contain at least one nitrogen atom, which probably renders them to be more easily protonated. While some of the chemicals formulae found belong to low molecular weight and volatile compounds, a third have molecular weights of more than 200 Da. MS/MS confirmation of the signals found in breath was attempted, but not successful. In order to select a precursor m/z, the ion intensity was generally not sufficiently high to allow for MS/MS analysis. Nevertheless, our results show that this breath interface assisted by an IF is a powerful technique. To our knowledge, no other online mass spectrometric technique has detected components in breath with such high molecular weights.

Conclusions

A heated breath interface was built and coupled to an IF to allow for higher sensitivity. Experiments using a LCQ-Deca as mass analyzer showed that the interface works, but that higher resolution is required in order to detect more compounds in breath. By exchanging the LCQ-Deca with a LTQ-FTICR, more than 30 compounds unique to exhaled breath in the mass range of up to m/z = 500 could be detected. Running the experiments on this high-resolution mass analyzer additionally allowed for the determination of the elemental composition of more than 30 compounds.

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