Analytical Methods

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Rapid In Vivo Fingerprinting of Nonvolatile Compounds in Breath by Extractive Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry**

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Quantitative analysis of trace constituents in exhaled gas can provide useful insights into biochemical processes[1–7] in the body, thus revealing information about metabolic dynamics and providing, theoretically, a scientific base for biomarker research[4,8] or clinical diagnoses.[2,8,9] Breath, however, is rarely used practically for diagnostic purposes in clinical medicine because of analytical difficulties.[2,8] Numerous attempts have been made for fast breath analysis by using methods that include proton transfer reaction mass spectrometry (PTR-MS),[10–12] selected ion flow tube (SIFT)[5–7] mass spectrometry, and exhaled breath condensate (EBC) analysis,[3,13,14] the latter being based on chromatographic separation.[11,15–18] However, these methods require tedious sample collection[3,19–21] and sample pretreatment procedures.[8,10,16,22,23] PTR-MS[10] and SIFT-MS[5,7] have been used for direct breath analysis, but require specially designed instruments that are not widely available. To date, only low-molecular-weight compounds (up to ca. 100 Da),[5–7] almost exclusively volatile species, have been detected in breath. With the exception of SIFT-MS, measurements are also compromised by the high water content in breath.[2,3,8] Actually, breath is a type of aerosol, and, in addition to volatile constituents, contains a vast variety of nonvolatile compounds dissolved in the microdroplets.[24,25]

We herein report an extractive electrospray ionization (EESI)[26] quadrupole time-of-flight mass spectrometry (QTOF-MS) method that has been established without modification of a commercial ESI interface (shown schematically in Figure 1) for the rapid in vivo fingerprinting of human breath. It presents a direct way to probe the dynamics of body metabolism and a simple, experimentally convenient method for the fast clinical diagnosis of oral malodors, based on

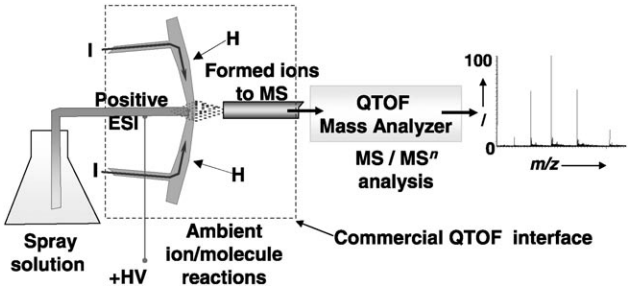

Figure 1. Schematic illustration of the concept and setup of EESI-QTOF-MS. I: desolvation gas inlet, used for sampling of breath; H: heating region in the desolvation gas outlet; HV = high voltage; QTOF = quadrupole time-of-flight; breath compounds are ionized in the region between the ESI spray and the breath stream. Note the intrinsic flexibility of EESI allows ambient ion/molecule reactions, including—but not limited to—protonation and cationization.

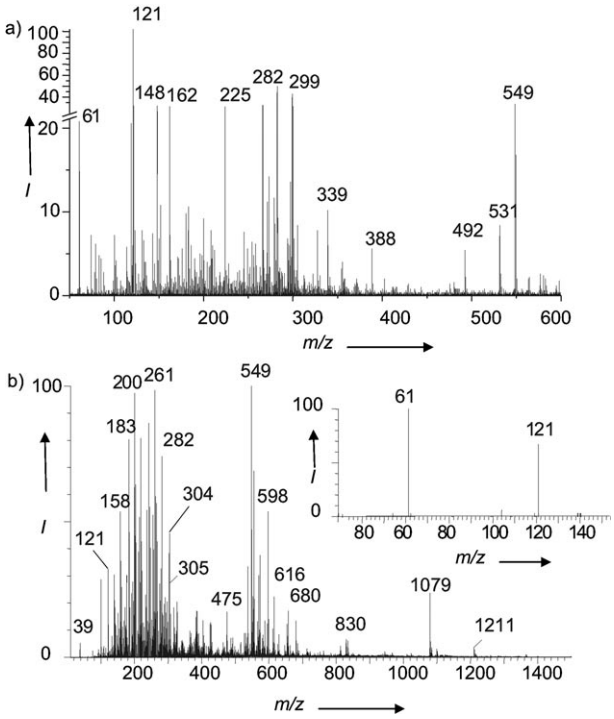

Figure 2. Breath fingerprints obtained under different conditions: a) after overnight fasting, a higher urea content (showing dominant proton-bound dimer *m*/*z* 121 and monomer *m*/*z* 61) was detected in the breath. Note that there is a break in the *y*-axis between 20 and 30%; b) urea at a lower level was also found in breath 2 h after consuming cheese, a high-protein diet. The inset shows the CID spectrum of the proton-bound urea dimer (*m*/*z* 121).

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fingerprinting of both volatile and nonvolatile trace components in breath without any sample pretreatment.

Initial experiments were performed to explore the relationship between the breath fingerprint and body metabolism. Figure 2a shows breath fingerprints of an overnight-fasted healthy Asian male. The mass spectrum is dominated by urea (protonated urea at *m*/*z* 61, proton-bound dimer at *m*/*z* 121) and metabolites (for example, glutaminic acid (*m*/*z* 148) and 2-amino-adipic acid (*m*/*z* 162)) generated in metabolic pathways of amino groups.[27] Note that urea and other metabolites might be released from the surfaces in the airways and the mouth in addition to the lung. Interestingly, urea was still dominant in the breath spectra even after drinking coffee or green tea (both unsweetened), but disappeared after a normal meal. The data provide good agreement with the well-known fact that the body utilizes fat and/or protein to compensate for the lack of carbohydrates. Urea is not usually abundant in the breath of healthy subjects; however, it is increasing significantly when the urea cycle and/or metabolic pathways of amino groups are more active than they usually are, for example, when there is not enough glucose or the level of amino acids is too high in the blood stream. The latter was confirmed by having a subject eat a high protein diet (low-fat cheese); the amount of urea (Figure 2b), however, was still much lower than that in the case where there was a lack of carbohydrates.

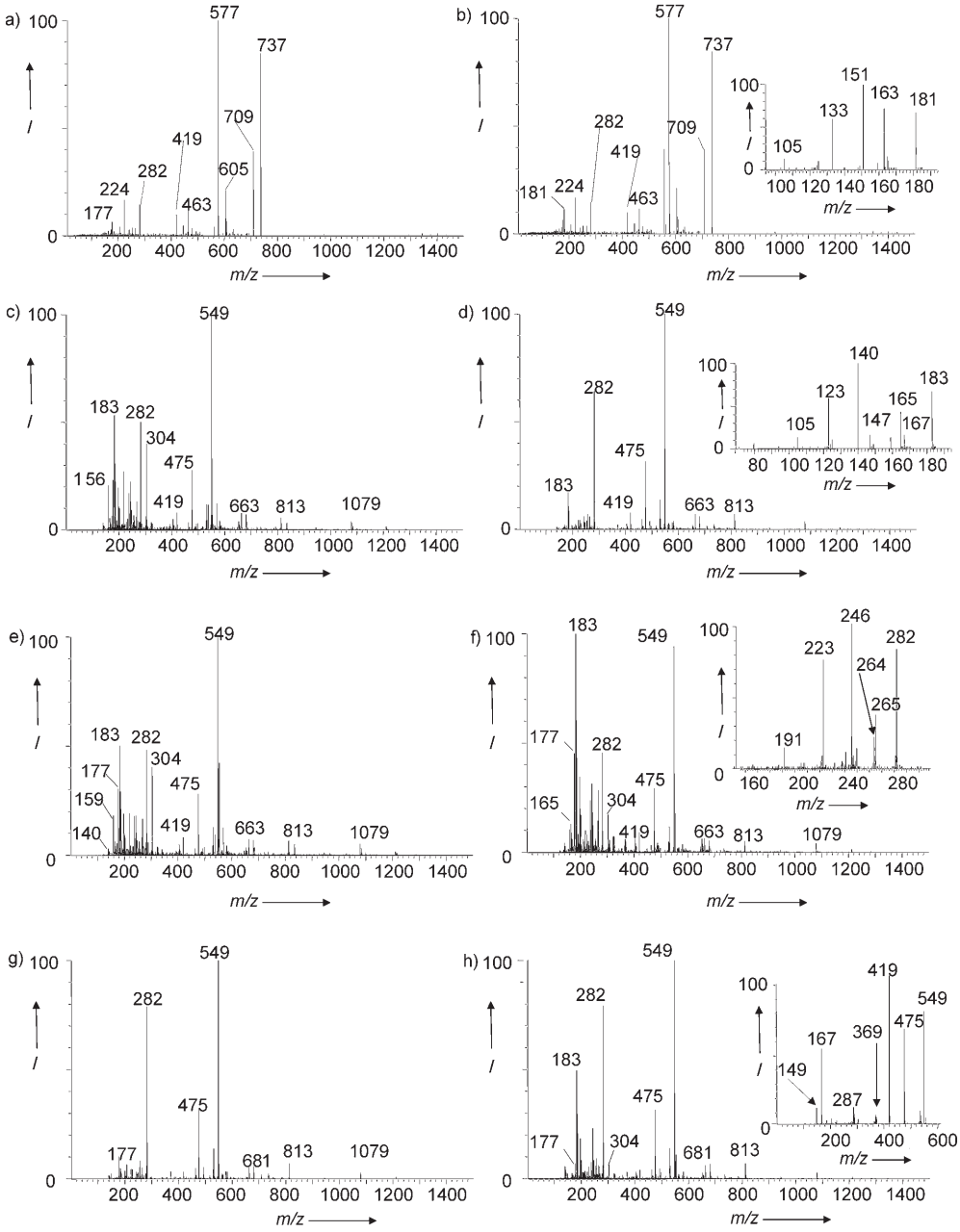

Figure 3. Breath fingerprints, obtained by EESI-QTOF-MS using water/HAc (5:1) as the spraying solution, of an individual Asian (left column) and European (right column) subject before and after drinking beer which reflect body metabolic dynamics. a,b) similar fingerprints before drinking beer; c,d) after one bottle (500 mL) of beer; e,f) after three bottles of beer; g,h) 50 min after finishing three bottles of beer. The insets in b, d, f, and h show fragmentation of the ions at *m*/*z* 181, 183, 282, and 549, respectively.

Figure 3 shows the mass spectral fingerprints of an individual adult male Asian (left column) and a European (right column) subject before and after drinking beer. In the positive-ion mode EESI-QTOF mass spectra, both individuals show a similar breath fingerprint before drinking any beer (Figure 3a,b). The signal at *m*/*z* 181 in the breath of the European subject (Figure 3b) was identified as glucose by collision-induced dissociation (CID)—in which it loses H_2O, HCHO, and (H_2O + HCHO) to yield fragments of *m*/*z* 163, 151, and 133, respectively (shown in the inset in Figure 3b). This signal reflects carbohydrates (for example, cane sugar) consumed a few hours prior to the analysis. Note that urea, amino acids, and glucose are typically nonvolatile compounds. Successful detection of them is possible because EESI tolerates the high moisture level in breath and requires no sample pretreatment, such that trace amounts of sugar present in the breath aerosol will be sampled without significant loss. SIFT[5–7] depends on reactions of species such as H_3O^+, NO^+, or O_2^+ with volatile analytes in the long reaction/flow tube, thereby resulting in mass spectra that often contain some high-intensity unassigned peaks. Ionic clusters are practically absent in EESI, with the exception of

the proton-bound urea dimer, which is easily identified by CID (shown in the inset of Figure 2b). Real-time total ion current (TIC) traces of various components in different analyses (see the Supporting Information) confirm that EESI offers good precision for real-time monitoring of breath. After the two subjects had drunk one bottle (500 mL, alcohol 5.5% (v/v)) of beer, the breath fingerprint changed immediately, and yielded patterns dominated by signals at *m*/*z* 549, 475, 282, and 183 for both subjects (Figure 3c, d). Compared to previous ESI mass spectra of beer,[28] the EESI mass spectra are quite different, and support the proposal that metabolites rather than the beer components in breath are detected in EESI-MS. The intensity ratio of signals (*m*/*z* 183:*m*/*z* 549) increased significantly in the breath of the European (Figure 2f) but deceased in the breath of the Asian subject (Figure 3e) after three bottles of beer. The signal at *m*/*z* 183 was nearly undetectable (Figure 3g) in the breath of the Asian subject, while it was still abundant in the breath of the European subject (Figure 3h) 50 minutes after drinking the beer. Fragmentation (seen in the insets in Figure 3d, f, h) of ions of *m*/*z* 183, 282, and 549 gave useful structure-related information (for example, there are -NH_2 and -OH groups in the compound at *m*/*z* 282). Although the structures have not yet been unambiguously identified by CID (MS^2), they could be elucidated with tandem MS studies using ion-trap mass analyzers.[26,29,30] However, the data clearly showed that metabolic dynamics of the body can be followed by direct fingerprinting of breath using EESI-MS.

It is well known that health risks for smokers are much higher than for nonsmokers. As an example, breath fingerprints of a female smoker were also investigated using EESI-QTOF-MS, and showed dynamic changes in the intensity ratio (*R*) of nicotine (*m*/*z* 163) to cotinine (one of its main metabolites, *m*/*z* 177) associated with smoking. The value of *R* was about 0.01:1 in the first spectrum obtained 12 h after finishing the last cigarette (see the Supporting Information); after smoking a cigarette, the *R* value rose to about 1:1 immediately (see the Supporting Information) and dropped down to about 0.15:1 in 80 minutes (see the Supporting Information). The decrease was mainly caused by the bioconversion of nicotine to cotinine.[31] About five hours later, the *R* value returned back to about 0.05:1 (see the Supporting Information), thus indicating the equilibrium in the metabolism of nicotine to cotinine after the smoking. The signals at *m*/*z* 549, 282, and 183 yielded the same fragmentation patterns as those in Figure 3, thus indicating they were the same compounds, and also showed interesting dynamic changes associated with biochemical activities in the body (for example, smoking). This finding supports the hypothesis that these compounds could be useful as potential biomarkers for studies of metabolism. Other previously identified biomarkers of smoking,[32–34] such as 2,3,5-trimethylpyridine, isoprostane, and arachidonic acid, were also found in the breath fingerprints (see the Supporting Information). Some small molecules such as acetonitrile were not detected in this experiment, because the instrument was not tuned optimally for detecting low-molecular-weight species. Other low-molecular-weight compounds in breath may have a relatively low proton affinity.

Similar to ESI, the sensitivity of EESI is compound-dependent. Pure nicotine vapor in nitrogen gas was introduced into the EESI source to determine the limit of detection (LOD). The LOD was found to be 0.86 pg (signal/noise = 3:1) using a one-minute average spectrum. No optimization was performed with the commercial QTOF interface, thus suggesting that the sensitivity could be further enhanced with a specially designed EESI source.[26]

Another area with a strong interest in breath analysis is in the assistance of the clinical diagnosis of numerous diseases linked to malodors. To simulate malodors, healthy volunteers ate garlic 30 minutes prior to breath analysis. Some polar sulfur-containing species (for example, allicin, *m*/*z* 163) were directly detected in the breath, along with numerous other signals in the spectrum (Figure 4a). However, the spectrum showed no signals corresponding to nonpolar sulfur-containing compounds. To address this problem, a highly sulfur-selective in situ silver cationization method,[35–39] in which an $AgNO_3$/water solution was used to generate the electrospray, was implemented in the EESI. In the positive ESI mode, this method generates silver cations, followed by selective ion/molecule (Ag^+/M) reactions occurring in the EESI source (see Figure 1) to facilitate detection of sulfur-containing molecules. By comparing the spectrum obtained with Ag^+/M reactions (Figure 4b) to the one without any Ag^+/M reactions (Figure 4a), it is clear that the spectrum features silver isotopes captured by different sulfur-containing species in the breath mixture. Unlike the HAc/water mixture, the $AgNO_3$/water spraying solution had a much lower propensity for protonation so that interferences caused by other species

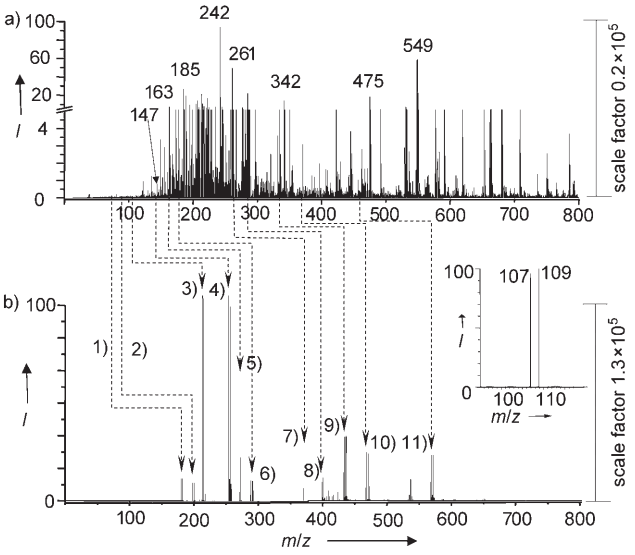

Figure 4. Highly selective detection of sulfur-containing species present in breath after eating garlic: a) breath fingerprint showing no nonpolar compounds in the absence of any Ag^+/molecule reactions; the inset shows the isotope signals of silver produced by electrospraying the $AgNO_3$ water solution; b) simplified spectrum showing adducts formed between silver isotopes and sulfur-containing molecules.
1) *m*/*z* 181, 183; 2) *m*/*z* 195, 197; 3) *m*/*z* 212, 214; 4) *m*/*z* 253, 255; 5) *m*/*z* 269, 271; 6) *m*/*z* 288, 290; 7) *m*/*z* 371, 373; 8) *m*/*z* 399, 401; 9) *m*/*z* 435, 437; 10) *m*/*z* 466, 468; 11) *m*/*z* 570, 572.

in the breath mixture were substantially reduced. Furthermore, the intensity was enhanced about 10 times with the Ag^+/M reactions. Some nonpolar sulfur-containing species such as methyl vinyl sulfide (M_r 74) and allyl methyl sulfide (M_r 88), which usually require EI for ionization,[17,40] were successfully detected as silver ion adducts at m/z 181 and 195, with their isotopes at m/z 183 and 197, respectively. This result shows that EESI is capable of detecting both nonvolatile and volatile species in breath, which is in agreement with previous studies.[26] Experimentally it was found that the CID mass spectra of protonated polar species differed from those of the silver-bound adducts (see the Supporting Information). If the cleavage of the entire sulfur-containing species, such as methyl vinyl sulfide, from the precursor silver ion adduct (m/z 181, 183) is exclusively observed (see the Supporting Information), it indicates that the silver ion is not strongly bound and that after cleavage of the $[Ag–M]^+$ bond, the positive charge will reside on the silver atom, but not on the sulfur-containing species. When multiple sulfur atoms are present in the sulfur-containing molecule, a relatively strong bond is formed, thus more fragments could be observed upon CID (see the Supporting Information).

In conclusion, EESI-QTOF-MS has been established for the first time with commercial instrumentation without modification of the hardware, and has been applied successfully for the rapid in vivo fingerprinting of breath without sample pretreatment. Metabolic dynamics are promptly reflected by breath fingerprints, thereby providing a rapid and convenient method for in vivo research into metabolism. This method predominantly detects nonvolatile compounds contained in breath condensates/aerosols. Selective ion/molecule reactions of silver cations with sulfur-containing molecules were also demonstrated for the first time for the analysis of breath, thus showing potential for application in clinical diagnosis.

Experimental Section

Experiments were carried out using a commercial QTOF-MS instrument (QTOF Ultima, Waters Micromass, Manchester, UK) without further modification. Breath was directly blown into the ESI source (maintained at 80 °C) through the desolvation gas transfer line with an outlet placed orthogonally to the ESI spray. Neutral analytes were subjected to numerous collisions with protons generated by spraying an acetic acid/water solution (1:5) such that EESI was established conveniently. An $AgNO_3$ water solution (10 ppm) was used instead of the HAc solution for the selective detection of sulfur-containing molecules in breath. CID was performed with 10–25 units of collision energy.

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