Secondary electrospray ionization–mass spectrometry: Breath study on a control group

Article *in* Journal of Breath Research · March 2011

DOI: 10.1088/1752-7155/5/1/016002 · Source: PubMed

CITATIONS
51

READS
117

4 authors, including:

Pablo M-L Sinues
University Children's Hospital Basel, University of Basel
78 PUBLICATIONS **789** CITATIONS
SEE PROFILE

Simone Cristoni
ISB srl
60 PUBLICATIONS **668** CITATIONS
SEE PROFILE

Secondary electrospray ionization-mass spectrometry: breath study on a control group

2011 J. Breath Res. 5 016002

(http://iopscience.iop.org/1752-7163/5/1/016002)

J. Breath Res. **5** (2011) 016002 (10pp) doi:10.1088/1752-7155/5/1/016002

Secondary electrospray ionization-mass spectrometry: breath study on a control group

P Martínez-Lozano[1,3], L Zingaro[2], A Finiguerra[2] and S Cristoni[1,2]

[1] National Research Council-Institute for Biomedical Technologies (CNR-ITB), Via Fratelli Cervi 93, 20090 Segrate (MI), Italy
[2] ISB Ion Source & Biotechnologies, via Fantoli 16/15, Milan, Italy

E-mail: pablo.mlsinues@gmail.com

Received 23 April 2010
Accepted for publication 19 October 2010
Published 11 January 2011
Online at stacks.iop.org/JBR/5/016002

Abstract

A series of fatty acids among other compounds have recently been detected in breath in real time by secondary electrospray ionization mass spectrometry (SESI-MS) (Martínez-Lozano P and Fernández de la Mora J 2008 *Anal. Chem.* **80** 8210). Our main aim in this work was to (1) quantify their abundance in breath calibrating the system with standard vapors and (2) extend the study to a control group for several days, both under fasting conditions and after sucrose intake. For the quantitative study, we fed our system with controlled amounts (∼140–1440 ppt) of fatty acid vapors (i.e. propanoic, butanoic, pentanoic and hexanoic acids). As a result, we found sensitivities ranging between 1 and 2.2 cps/ppt. Estimated concentrations of these particular acids in the breath of a fasting subject were in the order of 100 ppt. These values were in reasonable agreement with those expected from reported typical plasma concentrations and Henry constants. A second set of experiments on three fasting individuals before and after ingesting 15 g of sucrose showed that the concentration of propionic and butanoic acids increased rapidly in breath for two subjects. This response was attributed to bacterial activity in mouth and pharynx. In contrast, a third subject showed no response to the administration of sucrose. In addition, we performed a survey among six fasting subjects comparing nasal and mouth exhalations during 11 days, 4 months apart. The signal intensity was comparable for mouth and nose breath. This observation, in conjunction with the quantitative study, suggests that these compounds are mostly systemic when measured under fasting conditions. We finally used the *NIST MS search* algorithm to evaluate the possibility of recognizing a breathing subject based on his/her breath signature. The global recognition score was 63% (41 out of 65), while the probability by chance alone was 6×10^{-17}. This indicates that (i) there are statistically recognizable differences in individual breath patterns and (ii) the breath pattern for a given subject is relatively stable in time. This is consistent with previous NMR-based studies indicating the existence of stable individual metabolic phenotypes.

1. Introduction

Breath analysis has elicited considerable research efforts because it is non-invasive and carries metabolic information released during blood–air exchange. However, there are important pitfalls that have precluded its acceptance as a routine diagnostic approach. These difficulties and the different approaches taken to overcome them have recently been reviewed [1], providing an overview of the state of the art since the times of Lavoisier. One of the main difficulties is associated with the low concentrations of the most volatile organic compounds (VOCs) in breath. As VOCs are present at levels of parts per billion (ppb) to parts per trillion (ppt), a

[3] Author to whom any correspondence should be addressed.

preconcentration step is usually necessary. This manipulation is usually time consuming and may in some cases compromise the sample. Pioneering studies using atmospheric pressure chemical ionization (APCI) demonstrated the possibility of tracing gases in breath (e.g. ammonia and acetone) in real time [2, 3]. More recently, two well-established techniques have also overcome this problem: proton transfer reaction mass spectrometry (PTR-MS) [4] and selected ion flow tube mass spectrometry (SIFT-MS) [5]. In both techniques, the gas sample is mixed with precursor ions at low pressure leading to the detection of several compounds in real time. Their limits of detection are in the range of ppt for PTR-MS and ppb for SIFT-MS, and they are therefore suitable for the analysis of VOCs in breath. Recently, PTR has been combined with a time-of-flight instrument, enabling in this way the detection of several isobaric species in breath [6]. Among the compounds detected in [6] were acetic, propanoic and butyric acids, which is in good agreement with our measurements on a quadrupole time-of-flight (Q-TOF) instrument [7]. An alternative approach to ionize trace gases prior to mass spectrometric analysis was pointed out by Fenn and co-workers [8–10]. They noted that gas phase molecules were efficiently ionized in contact with an electrospray (ES) cloud at atmospheric pressure, leading to mass spectra akin to that of a sample ionized from the liquid phase. It is important to note that ESI is a 'gentle' ionization technique where usually none or little fragmentation is observed. This fact greatly simplifies mass spectral interpretation. This peculiar way of ionizing vapors has considerable intrinsic interest, and has also been studied by Hill and colleagues [11] in conjunction with ion mobility spectrometry, dubbing it 'secondary electrospray ionization' or SESI [12]. Eluents exiting a GC column have also been successfully ionized by SESI and mass analyzed [13]. Chen *et al* used this same approach to monitor changes in breath metabolites (e.g. urea) upon food intake [14]. However, their interpretation was that the observed low-volatility compounds could only be transported in an aerosol form. A subsequent study showed evidence that urea and the vast majority of the compounds observed when breath is mixed with an ES plume come as vapors [15]. Although the ionization mechanism is still unclear (i.e. dissolution of the neutral molecule in the droplet and re-emission of the ion, gas phase ion–molecule reaction or both) [16], independent quantitative experiments have proven its high sensitivity. Almost a decade ago, Kiselev and Fenn reported detections of 10 ppt of caffeine and 20 ppt of explosive RDX [17]. Recent studies combining SESI with several commercial atmospheric pressure mass spectrometers (API-MS) have measured limits of detection at sub-ppt levels [18, 19]. This approach is therefore sensitive enough to detect VOCs in minute concentrations in breath, in real time, and can be virtually applied to any commercial atmospheric pressure ionization MS instrument. We have actually explored its applicability for the breath analysis of one subject in positive ion mode using a triple quadrupole [15] and in negative ion mode with a Q-TOF instrument [7], and the applicability has also been more recently explored by Thomas and co-workers combining thermal desorption and ion mobility mass spectrometry [20]. Here we extend the study in negative ion mode with an ion trap MS, and we include quantitative estimations of concentrations in breath with a Q-TOF instrument.

2. Experimental details

This work is divided into two main experimental sections: (i) quantification of fatty acids (FAs) in breath and (ii) a qualitative study of breath patterns for a group of volunteers, fasting and after ingesting sucrose. For the quantitative analysis, we have used the same experimental setup as that described previously for the detection of explosives and is depicted schematically in figure 1(*a*). Briefly, we replaced the original ionization source of a Q-TOF (QStar from Sciex, Concord, ON, Canada) instrument by a stainless steel chamber, which held a home-built nanospray source and two 1/4″ tubes. One of the tubes was connected by Teflon tubing to a vapor generator located 50 cm upstream. The vapor generator consisted simply of another nanospray source ('seeding ES'), which delivered known concentrations of analyte gas phase ions by controlling the ES flow rate, analyte liquid concentration and carrier gas flow rate. Thus, a set of solutions of propanoic (C3), butanoic (C4), pentanoic (C5) and hexanoic (C6) acid standards in methanol were prepared at different concentrations: 0.25, 0.5, 1 and 2.5 mM. These solutions were electrosprayed ('seeding ES') at typical flow rates of 130 nL min^{-1}. The acid vapors were diluted in 5.5 L min^{-1} of CO_2 and drawn downstream through a heated (100 °C) Teflon tube. The ions neutralize along their path toward the ionization chamber, arriving in this way as neutral gas-phase molecules when they come in contact with the 'charging ES' cloud. The gas carrying small concentrations of standards is exhausted through a second tube to the atmosphere. The charging ES buffer consisted of 0.1% NH_4OH in 1:1 $MeOH/H_2O$ (v/v) infused at 70 nL min^{-1}. It was located coaxially at approximately 1.5 cm from the mass spectrometer sampling orifice.

For the comparison survey with volunteers, we carried out our measurements with an ion trap mass spectrometer (HCT Ion Trap Bruker Daltonics, Bremen, Germany). Its commercial ES chamber features two windows allowing the monitoring of the ES performance by eye. In order to mix the breath samples with the charged ES droplets, we simply exchanged the windows by a couple of 1/4″ tubes (figure 1(*b*)). A third port at the bottom of the chamber, meant to collect the liquid from the ES, was capped with a plastic centrifuge tube in order to keep the pressure constant within the chamber.

Note also that the commercial ES source is located orthogonally to the MS sampling orifice to prevent the introduction of droplets into the MS vacuum system when the ES is operated in the microflow range. In order to run the ES at nanoflows, we exchanged the original metal capillary with a silica one (25 μm i.d.), which could be moved freely in the radial direction. The ES buffer, which consisted of 0.1% NH_4OH in 1:1 $MeOH/H_2O$ (v/v), was pulled with a syringe and delivered at ∼100 nL min^{-1}. The syringe metal needle was grounded and the MS capillary voltage was set at 3.8 kV to generate the ES.

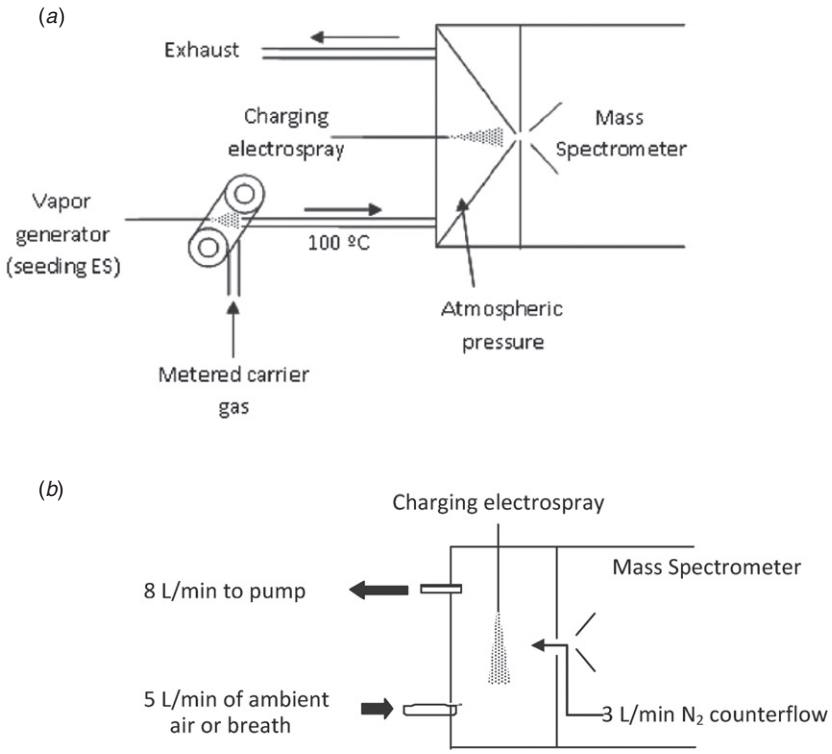

Figure 1. (*a*) Schematic of the experimental set-up to calibrate the system quantitatively. Known concentrations of vapors are released with the 'seeding' electrospray source, transported with a carrier gas to the ionization chamber at atmospheric pressure and mass analyzed with a Q-TOF instrument. (*b*) Experimental set-up used for the breath analysis experiments using an ion trap mass spectrometer.

One of the 1/4″ tubes was connected to a pump, which continuously sampled 8 L min^{-1} metered with a rotameter. This flow consisted of 3 L min^{-1} of N_2 (i.e. mass spectrometer counterflow) and 5 L min^{-1} of ambient air, sampled through the second disposable Teflon tube. The latter was meant to breathe directly into the ionization chamber. Note that since a pump is sucking constantly, the method requires minimal effort. However, in the case where nasal exhalations were required, the system may probably not be suitable for participants with reduced lung function. The local ethics committee (ITB-CNR) approved the protocol of this study.

3. Results and discussion

3.1. Quantification of FA in breath

One of the challenges in the field of analysis of trace gas metabolites in exhaled breath is to be able to correlate their concentrations to the corresponding plasma levels. To address this point, O'Hara *et al* [21] have measured simultaneously blood and breath concentrations for isoprene and acetone. They found *in vivo* arterial blood/breath average ratios of 580 for acetone and 0.47 for isoprene. Notably, these values were comparable to those previously measured for *in vitro* blood/air partition coefficients. Henry's law describes the partitioning between the liquid and vapor phases in equilibrium and is usually measured in water/air rather than in blood/air. Nonetheless, reported partitioning coefficient values covering several orders of magnitude have been found to be reasonably similar in water/air and blood/air (see table 2 in [22]). As noted in [21], using Henry's constants to estimate breath concentrations may be an oversimplification given the high complexity of the lung exchange system. However, in the absence of literature values, in this study we will use water/air Henry's constants to estimate the expected concentration of a series of FAs in breath. As an example, based on Henry's constants of acetone (30 M atm^{-1} [23]) and isoprene (1.3×10^{-2} M atm^{-1} [24]) and the arterial blood concentrations found in [21] (acetone 26 μmol L^{-1}; isoprene 6.8 nmol L^{-1}), we can estimate their abundance in breath to be 867 ppb (i.e. 867×10^{-9} atm) for acetone and 523 ppb (285–846) for isoprene. These values are comparable to those actually measured in breath: 1090 ppb for acetone and 376 ppb for isoprene. Thus, it follows that blood concentrations and Henry constants can provide a somewhat rough estimate of the order of magnitude we should expect in breath, for at least these two systemic compounds.

In an attempt to estimate the concentration of FAs in breath semiquantitatively in [7], we based our calculation on a calibration exercise performed on the same Q-TOF instrument toward explosive vapors [18]. Because the SESI ionization mechanism is still unclear, the sensitivity measurements need to be based on calibration standards. As described in the experimental section, we delivered known concentrations of FA vapors into the SESI source and measured the system's response. All the acids are detected in deprotonated form $[M\text{-}H]^-$. Figure 2(*a*) shows the single ion monitoring (SIM) trace at m/z 73.043–73.050 (propanoate, C3), 87.060–87.067 (butanoate, C4), 101.080–101.087 (pentanoate, C5) and 115.100–115.107 (hexanoate, C6). It displays four clear

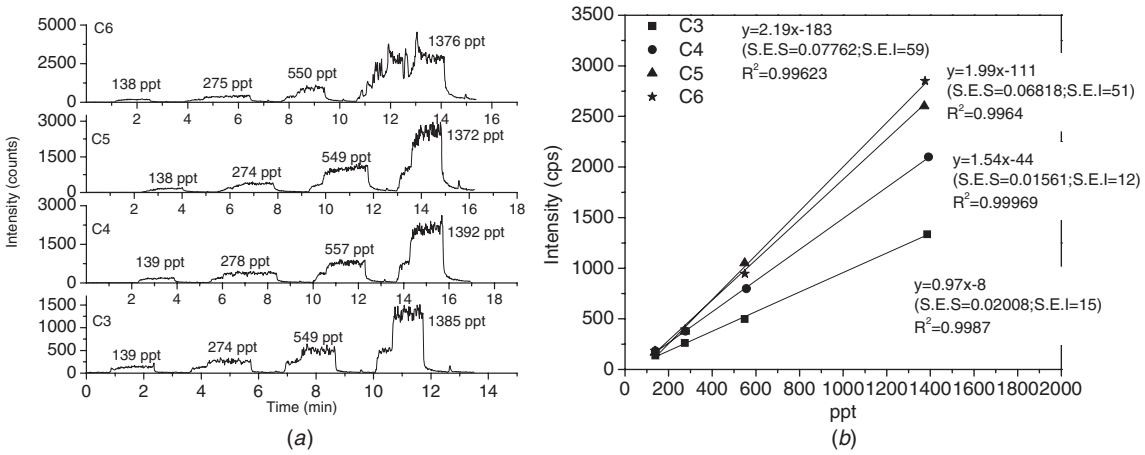

Figure 2. (*a*) Total ion current for deprotonated propanoic (C3), butanoic (C4), pentanoic (C5) and hexanoic (C6) acids at different vapor concentrations. (*b*) Signal versus vapor concentration. S.E.S: standard error slope; S.E.I: standard error intercept.

Table 1. Compilation of literature data used to calculate expected breath concentrations and measured concentrations.

Compound	Henry constant (M atm^{-1})	Blood concentration (M)	Calculated gas-phase concentration (ppt)	Measured gas-phase concentration (ppt)
Propanoic acid	5.7×10^3 [42]; 6.2×10^3 [43]	9.1×10^{-7} (0–2.1×10^{-6}) [44]	158 (0–368); 145 (0–339)	184
Butyric acid	4.7×10^3 [42]	1×10^{-6} (0.3×10^{-7}–1.5×10^{-6}) [44]	213 (64–319)	106
Isobutyric acid	1.1×10^3 [42]; 5.7×10^3 [43]	2.3×10^{-6} (0.7×10^{-7}–4.4×10^{-6}) [44]	2091 (636–4000); 404 (123–772)	
Valeric acid	2.2×10^3 [42]	6×10^{-7} (3×10^{-7}–1.2×10^{-6}) [44]	273 (136–545)	119
Isovaleric acid	1.2×10^3 [42]	1.6×10^{-6} (0.3×10^{-7}–2.7×10^{-6}) [44]	1333 (250–2250)	
Hexanoic acid	1.4×10^3 [42]	8×10^{-7} (0–1.6×10^{-6}) [44]	571 (0–1143)	138

steps as a result of switching on and off the seeding ES, delivering in each step different concentrations of FA (∼140–1400 ppt). Plotting the response versus vapor concentration we obtain the corresponding calibration curves for each compound (figure 2(*b*)), showing a linear response, and an increasing sensibility with increasing FA length. A similar correlation between sensitivity and mass has been found for explosives vapors. This has been suggested to be explained by the increased polarizability of the heavier vapor molecules, which tends to increase the ionization cross section [25]. Because the sampling period, or dwell time, used during the experiments was 1 s, the typical sensitivity values for these particular compounds were of the order of 1–2 cps/ppt. Thus, based on the sensitivity found for each compound, we can now estimate the exhaled concentration of these particular FAs in the breath of a fasting subject (see for example figure 2 in [7]), being in the order of ∼100 ppt. Table 1 lists these values along with those expected based on Henry constants and reported blood concentrations. Note that we have included isomeric species for butyric (C4) and valeric (C5) acids, which would obviously appear at the same m/z, and the corresponding intensity would be approximately the sum of both. For propanoic and hexanoic acids, the agreement between expected and measured concentrations is within the expected boundaries, whereas for C4 (butyric + isobutyric) and C5 (valeric + isovaleric), the measured values are underestimated. Note however that both Henry constants and blood concentrations may differ notably from reference to reference. Also, the dynamic range for a given compound may vary widely for a given control group (e.g. propanoic acid). These values should therefore be taken as rough estimates, and being conservative, we should expect them in the range 0.1–1 ppb. It should therefore be noted that only simultaneous measurements of plasma and breath FA concentrations could establish to what extent this method is suitable to probe systemic FAs in breath.

3.2. Monitoring breath after sucrose ingestion

Concerning the origin of acetic, propanoic and butanoic acids, these are the major end products of bacterial-gut fermentation [26]. In this regard, it is well known that there exists a significant interplay between bacterial and mammalian metabolism [27]. However, short-chain FAs are also produced by mouth bacteria [28, 29], further complicating the determination of their origin.

Comparing mouth breath with nose breath is a common approach to differentiate between systemic and oral borne volatiles [30, 31]. In order to shed light on this issue, we compared the mouth and nasal breath of an overnight fasting individual and monitored the evolution of the breath FAs after

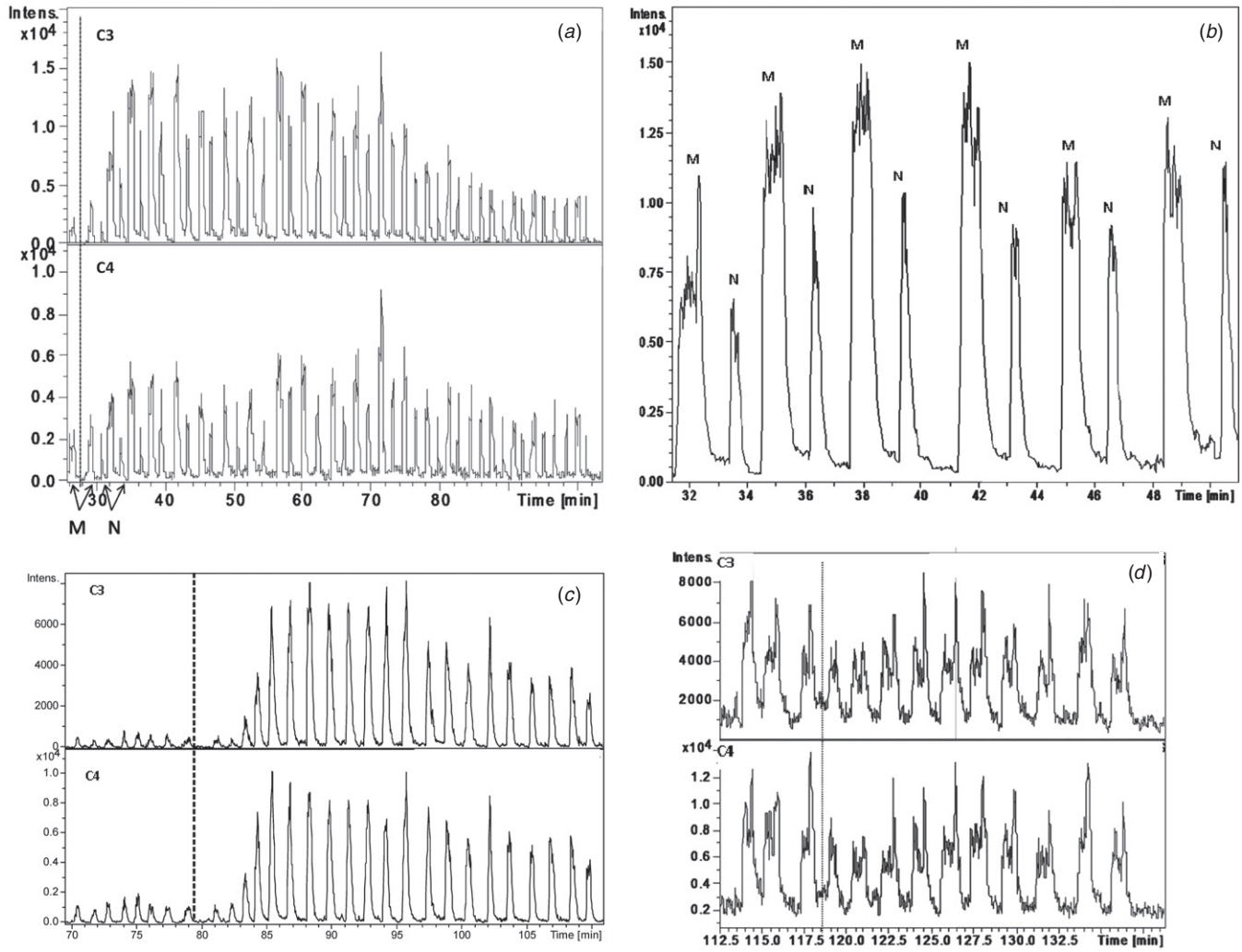

Figure 3. (*a*) Monitoring in real time of the concentration changes of propanoic (C3) and butanoic (C4) acids in mouth after ingesting 15 g of sucrose. The dashed line indicates the moment at which the sugar was administered. 'M' and 'N' stand for mouth and nasal exhalations, respectively. The subject breathed alternatively through the mouth and the nose. (*b*) Magnification of the period where a first maximum of propanoic acid signal intensity is observed. (*c*) Propanoic (C3) and butanoic (C4) acid changes in mouth exhalations for a second participant after ingestion of sucrose. (*d*) Mouth exhalations for a third fasting subject who, in contrast with the other participants, showed no response to sugar intake.

ingestion of 15 g of sucrose dissolved in 100 mL of water. The subject was asked to breathe alternatively through the mouth and through the nose. Note that two different sampling tubes were used to preserve the independence of the results between mouth and nose exhalations. Figure 3(*a*) shows the SIM trace for deprotonated propanoic (m/z 73) and butanoic (m/z 87) acids for one volunteer. The different periodic steps observed above the baseline are obtained as a result of a breath exhalation. The first step observed was recorded breathing through the mouth, showing a clear response at about 1000 and 2000 au of intensity for propionic and butanoic acids, respectively, under fasting conditions. The dashed line indicates the moment at which the subject ingested the sucrose. At this moment, he was asked to breathe alternately through his mouth and nose. The signal evolution for propanoic and butanoic acids is very similar, where the intensity increases after sugar intake to reach a first local maximum at minute 38–42 of the chromatogram (i.e. 10–14 min after sugar intake). The signal then gradually drops (up to min ∼50–54), then increases (min ∼ 56), and then drops (min ∼64) and increases (min ∼72) again. Lastly, C3 gradually tails off, while C4 drops until min ∼92, to finally increase once again. Note that the same pattern is observed for both nasal and mouth exhalations. However, the mouth exhalations show a higher intensity compared to nose exhalations, clearly indicating that at least a fraction of the detected acids originates in the mouth. Figure 3(*b*) magnifies the chromatogram of propanoic acid in the period just after sucrose intake. It can be clearly seen how the nasal exhalations follow the same trend as the mouth breath, although its intensity is approximately 2/3 of that of the mouth. Only at the end of the chromatogram (from min 90 onwards), do the signal intensities level off and match each other. Because there is nonetheless a clear response in the nasal breath, one may think that either there is a systemic production of propionic and butanoic acids that are released into the blood stream as a consequence of the sucrose ingestion,

or the pharynx region allows air exchange between the mouth and nasal cavity, thereby allowing these acids formed on the back of the tongue to be carried up during a nasal exhalation. Given the fact that only 15 g were administered, this would hardly produce a short-chain FA pulse that would perturb the circulating concentration by a factor of 3–5 because only very small quantities of simple sugars are not absorbed from the small intestine in healthy people [26]. Moreover, absorbed butanoic and propanoic acids in the gut are efficiently cleared by the liver, and little appears in peripheral blood. Therefore, the explanation of a contribution of the bacteria on the back of the tongue in the nasal exhalations is more likely. The observed signal intensity modulations for C3 and C4 may most probably reflect the fermentation dynamics in the mouth and pharynx. To our knowledge, this is the first time that such an event is captured. A second participant was invited to repeat the exercise providing mouth exhalations. Figure 3(*c*) shows that the signal intensity is relatively stable under fasting conditions (left-hand side of the dashed line) and increases rapidly after sugar intake. Interestingly, we observed similar signal modulations as with the first participant, suggesting again a significant bacterial activity in the mouth–pharynx. Figure 3(*d*) shows the result of the same experiment on a third volunteer (mouth breath only). In contrast with the other two participants, no appreciable changes in propionic acid (or any other compound) were observed after sucrose ingestion. This suggests that the size and/or composition of his microbial mouth population is different. Overall, with these experiments we reinforce the notion that it is important to sample under fasting conditions to minimize interferences from bacterial by-products when one wishes to target systemic compounds. These experiments also indicate that in some cases, nasal exhalations may carry non-systemic compounds.

Regarding the potentiality of this technique in breath analysis, SESI-MS operated in the negative ion mode (i.e. detecting mostly FAs) seems to offer an attractive approach to investigate the bacterial activity in the mouth with high sensitivity and in real time. This may be relevant for instance to study the correlation between bacterial by-products and periodontal illnesses [29].

Intestinal flora also plays a major role in the metabolism of compounds ultimately excreted via breath. Indeed, hydrogen breath tests are a common practice to diagnose disorders associated with gut microflora such as irritable bowel syndrome and common food intolerances [32]. However, there are cases of people that do not exhale hydrogen. This is one of the reasons why, unfortunately, the sensitivity and specificity of hydrogen breath tests are inadequate for routine clinical use. Hence, complementary approaches are welcome to increase diagnostic accuracy [33]. Given that short-chain FAs are the major end products of gut bacterial fermentation, this technique appears to represent a complement to current breath tests used to diagnose these types of disorders.

Organic acidemias cause a build up of toxic organic acid intermediates due to the body's inability to breakdown certain amino acids and odd-chain organic acids. SESI-MS may well show potential in the monitoring of some types as for instance isovaleric and propionic acidemias.

Finally, the concentrations of short-chain FAs in blood have been found to be significantly elevated in patients with hepatic encephalopathy caused by cirrhosis (362 $\pm$ 83 μmol L^{-1}) compared with cirrhotic patients without encephalopathy (178 $\pm$ 57 μmol L^{-1}) and healthy individuals (60 $\pm$ 8 μmol L^{-1}) [34]. Actually, early breath analysis studies noted the presence of greater concentrations of volatile FAs in the breath of decompensated cirrhotic patients than in healthy subjects [35]. Therefore, this is an area in which this technique may also be ripe for analytical exploration.

3.3. Study on a control group: individual breath pattern recognition

One of the major issues in metabolic studies is the different sources of temporal variability (e.g. biochemical cycles, diet, etc) that ultimately change the metabolic fingerprint within a subject. It has been indicated that gut microbiome is largely responsible for interday variation of microfolora-related metabolites [36]. This greatly complicates the discovery of alterations of metabolic fingerprints due to the appearance/disappearance or concentration changes of metabolites that can provide early evidence of the onset of diseases. Fortunately, some recent nuclear magnetic resonance (NMR) metabolic studies on urine have shown that in spite of this 'temporal noise', there exists a stable metabolic fingerprint strongly individual specific, even in timescales as long as 3 years [36, 37]. The main purpose of this section was to investigate the temporal variability of individual breath-metabolic phenotypes.

Following the same approach as in the previous section, we performed breath analysis in six subjects (three males and three females) during 11 days, 4 months apart. All the volunteers were non-smokers, not subjected to any particular imposed treatment or diet and were in the same age range (28–35). After overnight fasting, they provided three nasal and three mouth exhalations, alternately. Two different Teflon sampling tubes for mouth and nasal exhalations for each subject were used. Some representative results of the experiments are given in figure 4. It displays the chromatograms of several FAs obtained for the six subjects. It shows the background level corresponding to the laboratory atmosphere (baseline) and the clear response observed for both mouth and nasal exhalations. The first, third and fifth steps are due to mouth exhalations, and the other three are associated with nasal exhalations. Some conclusions can be extracted from this figure. (i) The measurements are clearly repeatable for both nasal and mouth exhalations, indicating that control of the sampling flow rate is an efficient way to preserve ionization probabilities. (ii) There are modest, but clear differences in the intensities for the different subjects. (iii) Clearly, the intensities corresponding to mouth and nasal exhalations are comparable for a given individual, suggesting that under fasting conditions the observed FA may be systemic. We also found some differences between nasal and mouth exhalations for other compounds. For example, figure 4(*c*) includes the SIM trace for m/z 93. Its identity remains unknown, but it exhibits a similar response to that of ammonia in [31], where it

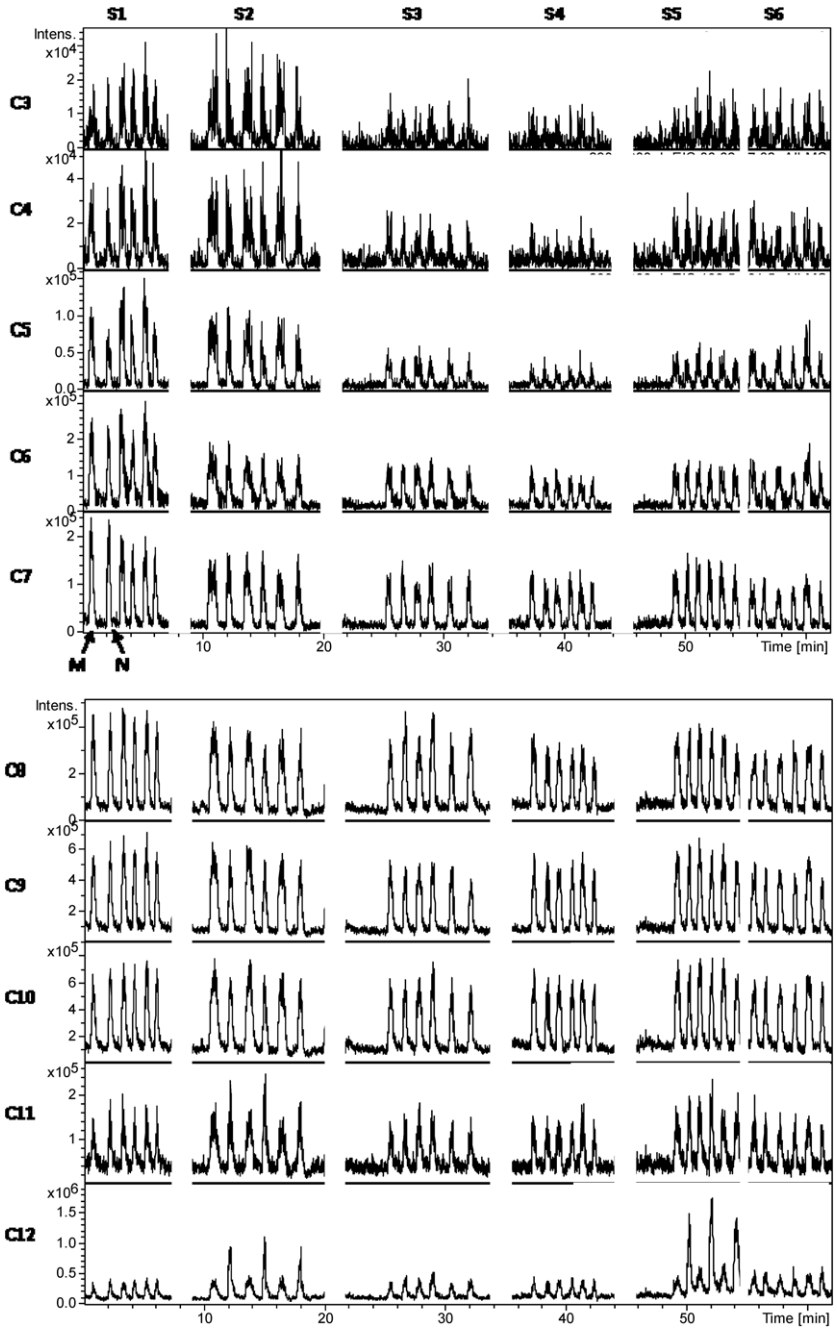

Figure 4. Monitoring of a series of FAs (C3–C18) for six fasting subjects (S1–S6) who exhaled three times through the mouth and three times through the nostril, alternately. For the series C3–C10, the response is comparable between mouth and nasal breath. For the acids above C11, the FAs emanating from the nostril skin clearly interfere, mostly for subjects S2 and S5. The ion at m/z 93 arises only in mouth exhalations—three steps each subject, except S4 who breathed twice—indicating that it is not systemic.

is present in the mouth but almost absent in nasal exhalations for all the subjects. This strongly indicates that this particular compound originates strictly in the oral cavity. For some other ions (>C11), the response is the opposite for subjects S2 and S5 (i.e. stronger nasal intensity), whereas for the other subjects the intensities are comparable. As noted previously [7], above C12 the signal enhancement may be largely affected by the humidity in breath. However, because the humidity levels for nasal and mouth exhalations for a given subject must be identical, the distinct response of S2 and S5 must be explained by other means. We have also reported the detection of a series of FAs emanating from the skin of the hand by SESI-MS [38]. The striking nasal response for these two subjects indicates that skin emanations interfere with nasal exhalations. FAs with chains of more than 12 carbons are well-known constituents of bacterial activity on skin. The fact that there are differences in the skin (nostril) FA profile among different subjects is consistent with a recent study [39], which revealed an impressive bacterial diversity both depending on the skin location (including nostril) and the subject. Therefore, this obvious difference indicates that the bacterial population in the nostrils of these two subjects is larger compared to the

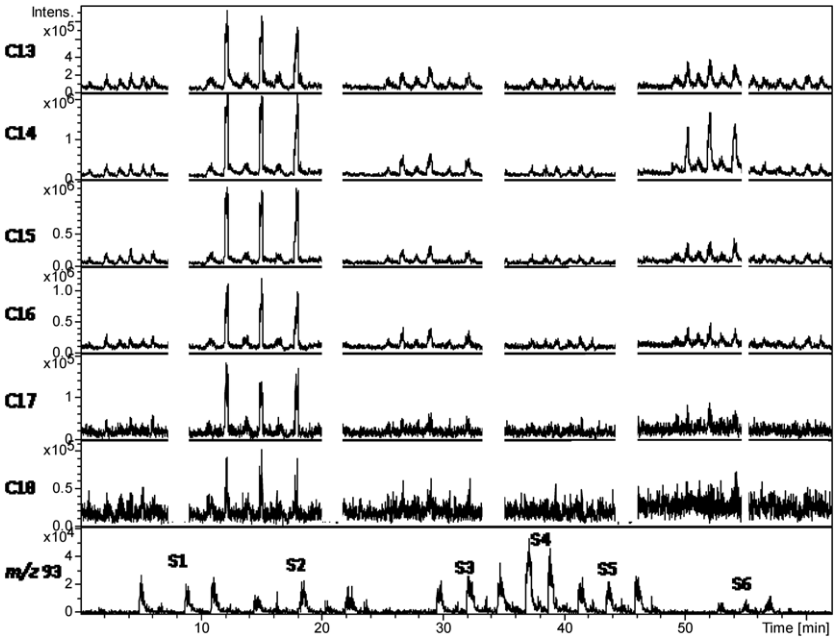

Figure 4. (Continued.)

other four subjects and/or the bacterial families populating these regions are to a large extent different in the other four subjects.

Upon visual inspection of the chromatograms, the breath FA profiles of different subjects are slightly different. The question we sought to address in this section is whether these individual differences are significant enough and if they were stable in time to be statistically discriminated. In other words, can a given breath signature be successfully attributed to the corresponding breathing subject from day to day? *NIST MS search* software is a widely used tool for the identification of unknown chemical compounds upon fragmentation via electron impact. It basically works as follows: the software contains a library including the fragmentation patterns of several standards. One then interrogates the library against the unknown fragmentation spectrum, and it provides a 'hit list' of potential candidates ranked from more similar to less similar. Any spectra to be searched must first appear in this list. The degree of similarity between the query and the hit list spectra is given by the so-called match factor, which is derived from the dot-product mass spectral search algorithm. A perfect match (i.e. identical spectra) results in a value of 999. Further details on the algorithm can be found elsewhere [40].

We have reported the potentiality of the *NIST MS search* algorithm for the diagnosis of colorectal cancer [41]**,** revealing that the same principles used to identify a compound can also be applied to discriminate mass spectral differences between two groups (i.e. pattern recognition). We explore here its capabilities in this more challenging exercise, which is identifying six individuals based on their breath fingerprints. For this purpose, we created customized libraries containing the breath mass spectra from the six subjects. In particular, we averaged the three consecutive mouth-breath mass spectra for each subject and obtained in this way 65 mass spectra (11 days $\times$ 6 subjects; one subject missing one day). We transformed our files into text *.MSP format and created 11 libraries. Thus, for a given day (D), we compared this day against the other 10 days (e.g. D1 against D2–D11; D2 against D1 and D3–D11; D3 against D1, D2 and D4–D11, and so on). Library search options were set to 'Spectrum search type/similarity/simple'. For our purpose, we considered that the recognition of the breathing subject was successfully achieved if the first proposed candidate in the hit list was the same as the 'unknown'. Note that in two cases, the first candidate was correctly assigned, along with another candidate with the same 'match' and 'reverse match' factors. In this case, we considered that the assignment was performed correctly. In the case where any of the other five breathing subjects was suggested as first candidate, we considered the assignment as wrong. The results of this exercise are summarized in table 2. The individual recognition score ranged from 45% to 73% and globally, it correctly assigned the breath signature 41 out of 65 times (63%). Note that the probability that such an event occurs randomly is as low as 6×10^{-17} (given by a binomial distribution with success probability of 1/6). Therefore, even though the number of samples is limited, it provides a proof of principle. Also, these values are in fair agreement with learning curves from previous NMR studies (see figure 4(*a*) from reference), where the average probability of correct classification was about 75% for $n = 10$ spectra. Noteworthy is that typically, for a correct assignment, the match and reverse match factors are above 965, and also the other candidates appear at very close values. For example, in the case of subject 6–day 11 (S6–D11), the algorithm suggests the following first five candidates in decreasing order (match/reverse match values): 1 S6–D8 (967/967), 2 S6–D7 (965/965), 3 S6–D9 (965/965), 4 S4–D6 (965/965), 5 S6–D6 (963/963). This indicates that the profiles among the different subjects are similar. This is not surprising given that the six subjects were

Table 2. Results obtained during the breath pattern subject recognition exercise. In the rows are represented the subjects (S) and in the columns the days (D). •: the person was correctly identified; ○: the person was wrongly identified; ×: missing measurement.

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	Recognition score for each subject (%)
S1	○	•	○	•	•	○	•	•	•	•	•	73
S2	•	•	•	○	○	○	•	○	○	○	•	45
S3	•	•	○	•	○	○	•	○	•	•	○	55
S4	•	○	•	○	•	○	○	•	•	•	•	64
S5	•	○	•	•	•	○	×	•	•	•	○	70
S6	○	•	•	○	•	•	○	•	•	•	•	73

all healthy, from the same geographical region and within the same age range, and therefore no major differences may be expected. Globally, individual-specific differences detected by the NIST software are based on differences in peak ratios, rather than just the presence/absence of a given compound, in line with NMR urine studies [37]. In summary, together with that shown previously [36, 37], this study based on breath-MS reinforces the notion of the existence of stable individual metabolic phenotypes.

4. Conclusions

We have extended here our preliminary results in breath analysis using SESI-MS in negative ion mode. In particular, we have quantified a series of short-chain FAs (C3–C6) based on their standard vapors. The measured sensitivities were in the range of 1–2.2 cps/ppt, with increasing sensitivity as a function of chain length. We conclude that their concentration in the breath of a fasting individual is in the order of 100 ppt, which is in reasonable agreement with what it would be expected based on typical plasma concentrations and Henry constants (∼0.1–1 ppb). We have compared nasal and mouth exhalations for six subjects and found that the response for most FAs is comparable. These experimental observations reinforce the hypothesis that these compounds originate mostly as a result of blood–lung exchange, when sampled under fasting conditions. However, experiments on fasting individuals before and after drinking sucrose solutions showed that bacterial activity in the mouth may pose a serious interference in targeting these compounds.

We have finally attempted the identification of six breathing subjects based on their breath fingerprint, collecting multiple samples for about 4 months. *NIST MS search* software was employed as a pattern recognition tool. Globally, the interrogation exercise provided 41/65 (63%) successful recognitions. This indicates the existence of individual profiles constantly in time, which is in accordance with previous metabolic NMR-based studies.

Acknowledgments

PML is indebted to Prof. Juan Fernández de la Mora (Yale University) for his encouragement and guidance on the art of electrospray ionization. We are most grateful to SEADM (Boecillo, Spain) for providing access to their vapor generator–Q-TOF system. The ISB and SEADM teams are acknowledged for providing support during the experiments and Dr Cristina Canton for useful discussions. We thank Giancarlo Caramenti for his assistance in the modification of the ionization source. This research was supported by a Marie Curie Intra European Fellowship (PML) within the 7th European Community Framework Programme (PIEF-GA-2008-220511).

References

[1] Szulejko J E, McCulloch M, Jackson J, McKee D L, Walker J C and Solouki T 2010 Evidence for cancer biomarkers in exhaled breath *IEEE Sensors J.* **10** 185–210

[2] Lovett A M, Reid N M, Buckley J A, French J B and Cameron D M 1979 Real-time analysis of breath using an atmospheric pressure ionization mass spectrometer *Biomed. Mass Spectrom.* **6** 91–7

[3] Benoit F M, Davidson W R, Lovett A M, Nacson S and Ngo A 1983 Breath analysis by atmospheric pressure ionization mass spectrometry *Anal. Chem.* **55** 805–7

[4] Lindinger W, Hansel A and Jordan A 1998 On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research *Int. J. Mass Spectrom. Ion Process.* **173** 191–241

[5] Smith D and Španěl P 2005 Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis *Mass Spectrom. Rev.* **24** 661–700

[6] Herbig J, Müller M, Schallhart S and Titzmann T 2009 On-line breath analysis with PTR-TOF *J. Breath Res.* **3** 027004

[7] Martínez-Lozano P and Fernández de la Mora J 2008 Direct analysis of fatty acid vapors in breath by electrospray ionization and atmospheric pressure ionization mass spectrometry *Anal. Chem.* **80** 8210

[8] Whitehouse C M, Levin F, Meng C K and Fenn J B 1986 *34th ASMS Conf. on Mass Spectrom. and Allied Topics (Cincinnati, OH)* p 507

[9] Fuerstenau S 1994 Aggregation and fragmentation in an electrospray ion source *PhD Thesis* Yale University

[10] Fuerstenau S, Kiselev P and Fenn J B 1999 ESIMS in the analysis of trace species in gases *47th ASMS Conf. on Mass Spectrometry (Dallas, TX, June 1999)*

[11] Chen Y H, Hill H H and Wittmer D P 1994 Analytical merit of electrospray ion mobility spectrometry as a chromatographic detector *J. Microcolumn Separations* **6** 515

[12] Wu C, Siems W F and Hill H H 2000 Secondary electrospray ionization ion mobility spectrometry—mass spectrometry of illicit drugs *Anal. Chem.* **72** 396

[13] Lee C Y and Shiea J 1998 Gas chromatography connected to multiple channel electrospray ionization mass spectrometry for the detection of volatile organic compounds *Anal. Chem.* **70** 2757–61
[14] Chen H, Wortmann A, Zhang W and Zenobi R 2007 Rapid *in vivo* fingerprinting of nonvolatile compounds in breath by extractive electrospray ionization quadrupole time-of-flight mass spectrometry *Angew. Chem. Int. Ed.* **46** 580–3
[15] Martínez-Lozano P and Fernández de la Mora J 2007 Electrospray ionization of volatiles in breath *Int. J. Mass Spectrom.* **265** 68
[16] Fernández de la Mora J 2010 Ionization of vapor molecules by an electrospray cloud *Int. J. Mass Spectrom.* doi:10.1016/j.ijms.2010.09.009
[17] Kiselev P and Fenn J B 2001 ESIMS analysis of vapors at trace levels *Proc. 49th ASMS Conf. on Mass Spectrometry and Allied Topics (Chicago, IL, May 2001)*
[18] Martinez-Lozano P, Rus J, Fernández de la Mora G, Hernández M and Fernández de la Mora J 2009 Secondary electrospray ionization (SESI) of ambient vapors for explosive detection at concentrations below parts per trillion *J. Am. Soc. Mass Spectrom.* **20** 287
[19] Dillon L A, Stone V N, Croasdell L A, Fielden P R, Goddard N J and Thomas C L 2010 Optimisation of secondary electrospray ionisation (SESI) for the trace determination of gas-phase volatile organic compounds *Analyst* **135** 306–14
[20] Reynolds J C *et al* 2010 Detection of volatile organic compounds in breath using thermal desorption electrospray ionization-ion mobility-mass spectrometry *Anal. Chem.* **82** 2139–44
[21] O'Hara M E, Clutton-Brock T H, Green S and Mayhew C A 2009 Endogenous volatile organic compounds in breath and blood of healthy volunteers: examining breath analysis as a surrogate for blood measurements *J. Breath Res.* **3** 027005
[22] Anderson J C, Babb A L and Hlastala M P 2003 Modeling soluble gas exchange in the airways and alveoli *Ann. Biomed. Eng.* **31** 1402–22
[23] Staudinger J and Roberts P V 1996 A critical review of Henry's law constants for environmental applications *Crit. Rev. Environ. Sci. Technol.* **26** 205–97
[24] Yaws H C and Yang C L 1992 *Henry's Law Constant for Compound in Water* (Houston, TX: Company Gulf Publishing) pp 181–206
[25] Mesonero E, Sillero J A, Hernández M and Fernández de la Mora J 2009 Secondary electrospray ionization detection of explosive vapors below 0.02 ppt on a triple quadrupole with an atmospheric pressure source *57th ASMS Conf. on Mass Spectrometry and Allied Topics (Philadelphia, PA)* p 314
[26] Cummings J H 1983 Fermentation in the human large intestine: evidence and implications for health *Lancet* **321** 1206–9
[27] Li M *et al* 2008 Symbiotic gut microbes modulate human metabolic phenotypes *Proc. Natl Acad. Sci. USA* **105** 2117–22
[28] Gorbach S L, Mayhew J W, Bartlett J G, Thadepalli H and Onderdonk A B 1976 Rapid diagnosis of anaerobic infections by direct gas liquid chromatography of clinical specimens *J. Clin. Invest.* **57** 478–84
[29] Kurita-Ochiai T, Fukushima K and Ochiai K 1995 Volatile fatty acids, metabolic by-products of periodontopathic bacteria, inhibit lymphocyte proliferation and cytokine production *J. Dent. Res.* **74** 1367–73
[30] Tangerman A and Winkel E G 2007 Intra- and extra-oral halitosis: finding of a new form of extra-oral blood-borne halitosis caused by dimethyl sulphide *J. Clin. Periodontol.* **34** 748–55
[31] Wang T, Pysanenko A, Dryahina K, Španěl P and Smith D 2008 Analysis of breath, exhaled via the mouth and nose, and the air in the oral cavity *J. Breath Res.* **2** 037013
[32] Metz G, Drasar B S, Gassull M A, Jenkins D J and Blendis L M 1976 Breath-hydrogen test for small-intestinal bacterial colonisation *Lancet* **307** (7961) 668–9
[33] Saad R J and Chey W D 2007 Breath tests for gastrointestinal disease: the real deal or just a lot of hot air? *Gastroenterologica* **133** 1763–6
[34] Clausen M R, Mortensen P B and Bendtsen F 1991 Serum levels of short-chain fatty acids in cirrhosis and hepatic coma *Hepatology* **14** 1040–5
[35] Chen S, Mahadevan V and Zieve L 1970 Volatile fatty acids in the breath of patients with cirrhosis of the liver *J. Lab. Clin. Med.* **75** 622–7
[36] Bernini P, Bertini I, Luchinat C, Nepi S, Saccenti E, Schäfer H, Schütz B, Spraul M and Tenori L 2009 Individual human phenotypes in metabolic space and time *J. Proteome. Res.* **8** 4264–71
[37] Assfalg M, Bertini I, Colangiuli D, Luchinat C, Schäfer H, Schütz B and Spraul M 2008 Evidence of different metabolic phenotypes in humans *Proc. Natl Acad. Sci. USA* **105** 1420–4
[38] Martínez-Lozano P and Fernández de la Mora J 2009 On-line detection of human skin vapors *J. Am. Soc. Mass Spectrom.* **20** 1060–63
[39] Grice E A *et al* 2009 Topographical and temporal diversity of the human skin microbiome *Science* **324** 1190–2
[40] Stein S 1994 Estimating probabilities of correct identification from results of mass spectral library searches *J. Am. Soc. Mass Spectrom.* **5** 316–23
[41] Cristoni S *et al* 2009 MALDI-MS-NIST library approach for colorectal cancer diagnosis *Rapid Commun. Mass Spectrom.* **23** 2839–45
[42] Khan I, Brimblecombe P and Clegg S L 1995 Solubilities of pyruvic acid and the lower (C1-C6) carboxylic acids. Experimental determination of equilibrium vapour pressures above pure aqueous and salt solutions *J. Atmos. Chem.* **22** 285–302
[43] Servant J, Kouadio J, Cros B and Delmas R 1991 Carboxylic monoacids in the air of Mayombe Forest (Congo): role of the forest as a source or sink *J. Atmos. Chem.* **12** 367–80
[44] Lentner C and West Cadwell N J c1981–1992 *Geigy Scientific Tables* 8th edn (Basel, Switzerland: Medical education Div., Ciba-Geigy Corp.) pp 165–77