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Short communication

Electrospray ionization of volatiles in breath

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Abstract

Recent work by Zenobi and colleagues [H. Chen, A. Wortmann, W. Zhang, R. Zenobi, Angew. Chem. Int. Ed. 46 (2007) 580] reports that human breath charged by contact with an electrospray (ES) cloud yields many mass peaks of species such as urea, glucose, and other ions, some with molecular weights above 1000 Da. All these species are presumed to be involatile, and to originate from breath aerosols by so-called extractive electrospray ionization EESI [H. Chen, A. Venter, R.G. Cooks, Chem. Commun. (2006) 2042]. However, prior work by Fenn and colleagues [C.M. Whitehouse, F. Levin, C.K. Meng, J.B. Fenn, Proceedings of the 34th ASMS Conference on Mass Spectrometry and Allied Topics, Denver, 1986 p. 507; S. Fuerstenau, P. Kiselev, J.B. Fenn, Proceedings of the 47th ASMS Conference on Mass Spectrometry, 1999, Dallas, TX, 1999] and by Hill and colleagues [C. Wu, W.F. Siems, H.H. Hill Jr., Anal. Chem. 72 (2000) 396] have reported the ability of electrospray drops to ionize a variety of low vapor pressure substances directly from the gas phase, without an apparent need for the vapor to be brought into the charging ES in aerosol form. The Ph.D. Thesis of Martínez-Lozano [P. Martínez-Lozano Sinués, Ph.D. Thesis, Department of Thermal and Fluid Engineering, University Carlos III of Madrid; April 5, 2006 (in Spanish); http://hdl.handle.net/10016/655] had also previously argued that the numerous human breath species observed via a similar ES ionization approach were in fact ionized directly from the vapor. Here, we observe that passage of the breath stream through a submicron filter does not eliminate the majority of the breath vapors seen in the absence of the filter. We conclude that direct vapor charging is the leading mechanism in breath ionization by electrospray drops, though aerosol ionization may also play a role.

Keywords: Breath analysis; Electrospray; EESI; Volatile species

1. Introduction

In their early studies on electrospray ionization mass spectrometry (ESMS), Fenn and colleagues [3a,b] noted the extraordinary ability of an electrospray cloud to produce ions from trace vapor species accidentally present in their system. They then introduced controlled amounts of low vapor pressure species in the conventionally clean counter-flow gas of their ESMS instrument, and were able to sense vapors present at partial pressures in the range of parts per billion (ppb) and below. Initially [3a] they concluded that the vapors were formed by charge exchange from gas phase ions produced by the electrospray drops. However, they later [3b] argued that the drops were a more effective agent than gas phase ions for capturing and ionizing volatiles, a possibility previously noted by Hill and colleagues [4].

In view of the high sensitivities found in these pioneering studies for vapors, and given the large gains subsequently achieved in ESMS sensitivity, it appeared to us that ES charging coupled to a modern atmospheric pressure ionization (API) MS system ought to be able to achieve sensitivities in the parts per trillion (ppt) range. We tested this hypothesis by passing breath vapors through an electrospray cloud facing the entry region of Sciex's API-365 triple quadrupole mass spectrometer, used in the single MS mode. Acidified water (formic acid 0.1 M) was electrosprayed in positive mode from a capillary tip placed at a distance of several mm upstream from the conical curtain gas outlet of the MS (Fig. 1). This electrospray chamber was completely enclosed, except for two orifices 4 mm in diameter, one for entry of the sample flow into the chamber, another to exhaust the sample flow and the counter-flow drying gas. The vapors to be analyzed were carried in a stream of air or CO_2 flowing con-

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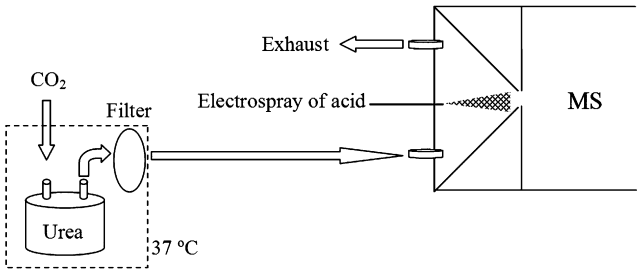

Fig. 1. Experimental set-up used to detect urea vapors.

tinuously through the ES chamber. There, they interacted with either the ES drops or the solvated protons produced by charged drop evaporation, and some were ionized, driven by the electric field against the counter-flow gas, and sucked into the atmospheric pressure inlet pinhole of the MS. Sensitivities in the 4 ppt range were demonstrated in calibration studies by injecting and completely evaporating metered flows of dilute trioctyl amine solution in the charging region.

Our initial results have been reported in [5], whose main figure. 6.9 (comparing breath and blank spectra for two subjects) is reproduced here as Fig. 2a. For clarity, we include here the breath of only one subject. Fig. 2b includes the full mass range,

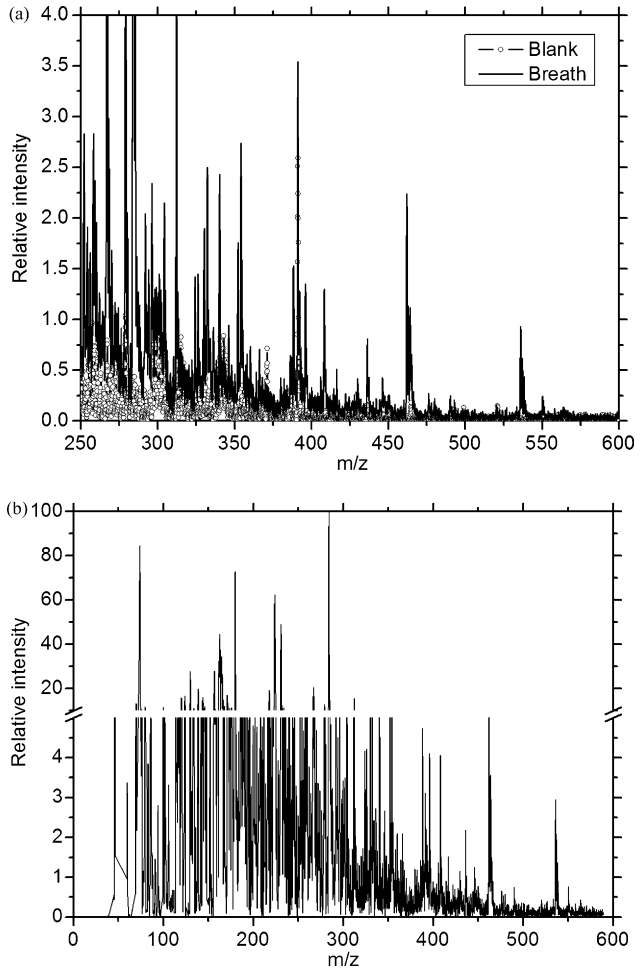

Fig. 2. ESI spectra from one subject's breath, from [5]. (a) (Top), breath and blank (clean CO_2). (b) (Bottom), breath spectra after subtracting the blank.

and shows breath spectra after subtracting the blank (clean CO_2). Prior to charging, the breath flow passes through a cold trap kept at 0 °C, to avoid water condensation in the Teflon line or the charging region. Downstream of the cold trap, the Teflon line is heated up to 60 °C. Note also the break at the *y*-axis between 5 and 10%. Fig. 2a and b shows a large number of characteristic breath ions with molecular weights going up to 600 Da, often with intensities several orders of magnitude above the clean CO_2 background.

This result appeared to represent an important step forward in the field of breath analysis, previously dominated by far less sensitive GC–MS methods [6], where no species with a mass exceeding 290 Da had ever been detected, and where background intensities were in all cases rather close to corresponding breath values. Ref. [5] then focused on the potential of the technique for medical diagnosis. It never occurred to us initially that such peaks might have originated not from volatile species, but from aerosols also contained in breath. However, in a private discussion with Prof. Fenn, he noted that possibility, particularly in view of the recent discovery by Cooks and colleagues of the DESI mechanism [2b]. Although breath aerosol has been reported in certain instances not involving violent episodes such as coughing and sneezing, we did not follow up on that suggestion for a variety of reasons. First, breath aerosol would tend to contain involatile species of high molecular weight, while our data showed a gradual decrease of signal intensity with ion mass. The same trend is clear in the spectra from [1]. Second, our observed spectra were fairly steady, while the signal from an aerosol would tend to be less stable and repeatable than that sampled by the lung from the blood stream. Finally, we had carried out sensitivity studies with vapors of moderately volatile trioctyl amine, and found sensitivities in the range of ppt, at which it would be reasonable to expect sensing species with molecular weights above 300 Da well below their equilibrium vapor pressure. However, the suggestion of a possible aerosol origin of these heavy breath vapors has been recently reformulated in an entirely independent investigation on electrospray charging of breath [1]. The new study is of great interest for a number of reasons, including among others the observations of ions at masses up to 1200 Da, the initial exploration of the effect of diet on the composition of breath vapors, and the discovery of urea in the breath of fasting individuals.

The present note is an attempt to resolve the question of whether most of the many observed breath ions originate from volatiles in the blood (as assumed in [5]), or from aerosol accompanying the exhaled gas (as assumed in [1]). The difference is important in metabolic and medical diagnosis studies, because the lung achieves equilibrium between the gas and the liquid, offering a remarkable window on the composition of blood. Blood can of course be analyzed directly, but not continuously and almost instantaneously, nor without a considerable level of invasiveness. Furthermore, the chemical complexity of blood is such that the natural separation of its volatile and involatile components taking place in the lung offers a welcome simplifying opportunity for its study. Furthermore, if it were true that blood species with molecular weights beyond 1000 Da could be continuously and non-invasively monitored, this would enable a rather

direct probing of complex processes far more biologically (and diagnostically) relevant than previously possible from species with masses below 290 Da. On the other hand, if the large mass ions observed in breath are indeed involatile, and their presence responds, not to a simple liquid–vapor equilibrium, but to a surely far more complex process of aerosolization, the apparent promise of this breath analysis technique would be greatly diminished.

2. ES charging of urea, D-glucose and other vapors in breath

2.1. Urea

In view of the discovery of urea in the breath of fasting individuals [1], a first issue to be resolved is if breath urea can be sensed in vapor form, or if, as presumed in [1], it is involatile and can only be sensed in aerosol form. In preliminary experiments we placed some small grains of urea at the bottom of a glass flask held at room temperature, and introduced the flask in the path of the sample gas, so that the gas flow achieved urea vapor concentrations below room temperature saturation. These tests showed a large peak of protonated urea ($\sim 4 \times 10^5$ ion/s), without significant reduction of the signal observed by the filter. This point is in qualitative agreement with reported data on the room temperature vapor pressure of urea (1.2×10^{-5} mmHg at 25 °C [7]), obtained by extrapolation from higher temperatures at which such measurements were viable with previously existing techniques. Note however the need to limit the gas flow rate through the flask, since, above a certain flow, some of the crystals were aerosolized and contributed to the signal. It is also important to use a non-absorbing filter. For instance, MSA's filter #711561 removed the urea signal entirely, while a PTFE membrane filter with a polypropylene holder (Whatman Vacu-Guard^{TM}, 99.99% particle retention in air for particles $\geq$0.1 μm) did not retain any appreciable fraction.

In view of these encouraging results, and given that urea is one of the most abundant metabolites in human blood ($\sim$0.4 g/l, rising to 2.3 g/l in uremic patients; see Table 1 in [8]), we looked for the presence of urea in the breath of a healthy individual following 24 h of rigorous fasting. In order to reduce interference from a contaminant of the same mass present in the background, we performed collision-induced dissociation (CID), at 61.1 Da and monitored the fragment peak intensity at 44 Da (protonated urea-NH_3). As shown in Fig. 3a, this fragment dominates the spectrum. Fig. 3b displays the total ion current (TIC) versus time for this fragment, where the pattern seen corresponds to periods with or without breathing. In the latter case, the baseline is from clean CO_2. There is no measurable reduction of the signal when the breath is filtered. Accordingly, we conclude that the urea peak observed with this technique in fasting individuals originates predominantly from vapors and not from an aerosol.

2.2. D-Glucose

D-Glucose is one of the most abundant metabolites in blood ($\sim$0.9 g/l). It was also detected in breath and positively identified

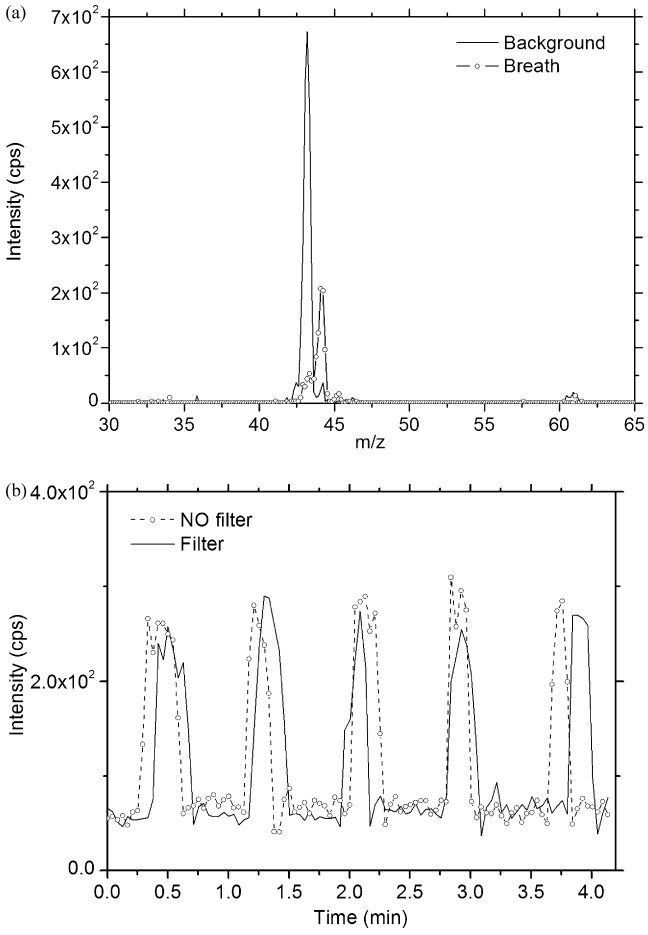

Fig. 3. Analysis of urea vapors in breath, with the first quadrupole fixed at 61.1 Da. (a) (Top), fragmentation spectrum showing the presence of a background peak with a characteristic fragment at 43 Da, mixed with breath urea, with a characteristic fragment at 44 Da (protonated urea-NH_3). (b) (Bottom), total urea ion current spectrum at 44 Da, from filtered and unfiltered breath.

by MS–MS in [1]. Accordingly, we did a preliminary examination of whether D-glucose vapors could be sensed by our method. However, no visible signal for protonated glucose was detected in breath. The result was also negative under the surely much higher vapor concentrations resulting when the sample gas was passed through a flask containing crystals of D-glucose. These experiments showed the difficulty involved in sensing glucose vapors, but were not sufficiently systematic to warrant a conclusion on the impossibility to do so. Note in particular the well-known difficulty of protonation of glucose in conventional ES–MS, which has been previously addressed by cationization with Li, Na, and Cs, or by chloride attachment in negative mode [9]. Pending future improvements in the charging technique for volatile glucose, our work so far provides no basis to doubt the prior assumption about the aerosol origin of breath glucose observed in [1].

2.3. Other breath peaks

The breath experiments of [5] have been repeated with and without the PTFE filter (Fig. 4), showing essentially identical spectra. Only three peaks show a clear reduction upon pass-

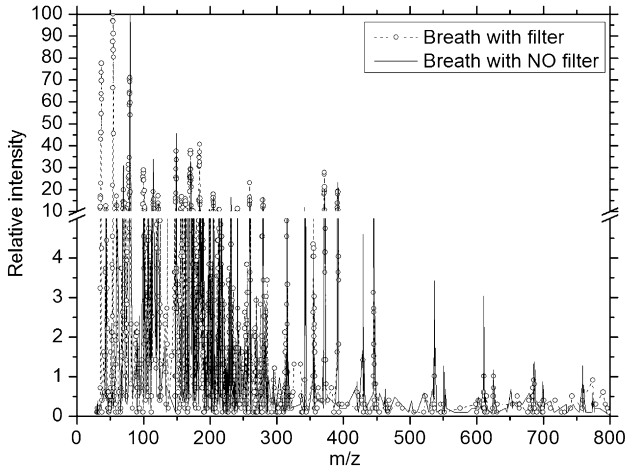

Fig. 4. Mass spectrum of a subject's breath, either blowing directly to the MS, or going first through a filter. Even the highest molecular weight peaks survive through the filter.

ing through the filter, but the variation observed even in these cases is comparable to that due to other incompletely controlled experimental parameters, such as the flow rate of breath. Note the break at the *y*-axis of the spectrum of Fig. 4, used to show more clearly breath peaks at masses approaching 800 Da. As in Fig. 2b, the blank of pure CO_2 has been subtracted. Of particular interest is the fact that the high mass compounds survive through the filter, and are therefore associated to vapors rather than aerosol. In order to better illustrate this important point, we targeted the peak appearing at 445 Da in an experiment similar to the one described in Fig. 3b. In this case, fragmentation was not necessary due to the rather low background signal. Fig. 5 shows that there is no difference in peak intensity with and without filter.

Most of the breath peaks observed remain unassigned, though a few have been identified provisionally by CID. A dominant peak appearing at 80 Da is from protonated pyridine, whose published vapor pressure is as high as 20 mmHg at 25 °C. Some other breath compounds identified in protonated form are: acetone (59 Da); 1-pyrroline (70 Da); 1-methyl-2-pyrrolidone (100 Da); and diphenylamine (170 Da).

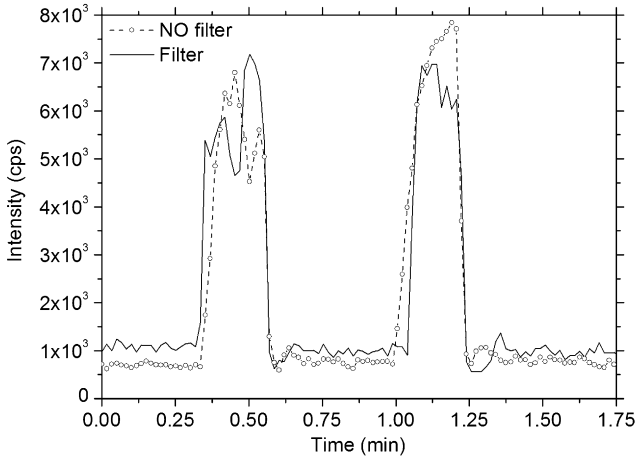

Fig. 5. Total ion current spectrum at 445 Da, breathing with and without filter in the line.

2.4. Effect of vapor contaminants in background air

We have shown that most of the breath peaks sensed originate in the vapor phase. However, it is well known that exhaled breath consists mostly of room air inhaled (containing many volatile contaminants), with additional vapors of human origin. In order to further facilitate the interpretation of our breath spectra, we have performed additional experiments where the *blank* consists of a mixture of 2 l/min of CO_2 mixed with 4 l/min of the same room air breathed by the subject, while the *sample breath* simply substitutes the 4 l/min of room air by the same flow rate of exhaled air. The CO_2 flow is fixed by a flowmeter upstream of the system, while the sum of 4 + 2 l/min is fixed downstream by a second flowmeter and a pump. In order to leave these flows and the system pressure exactly fixed when shifting from sample to blank, the subject simply exhales into the surroundings of the air sampling tube, so that his breath is sucked into the tube exactly at atmospheric pressure. No filter is used in these experiments, where we electrospray a formic acid solution 0.1 M in water/methanol (50/50 v/v). We then take mass spectra sequentially for the blank and the sample.

As seen in Fig. 6, the simplification resulting in the mass spectrum formed by simple subtraction of the sample and blank spectra is striking. This drastic reduction of complexity is highly reproducible because the new procedure preserves faithfully the charging probability of the electrospray charger in the sample and the blank. However, essentially all the peaks shown in Fig. 6 are also present in the background spectrum. Most of them are in higher concentrations in room air than in breath, producing negative peaks not shown in Fig. 6 (the so-called "negative alveolar gradient"). These negative peaks tend to be associated to synthetic contaminants. Positive peaks tend to be metabolites, which are also present in the room atmosphere, but are more

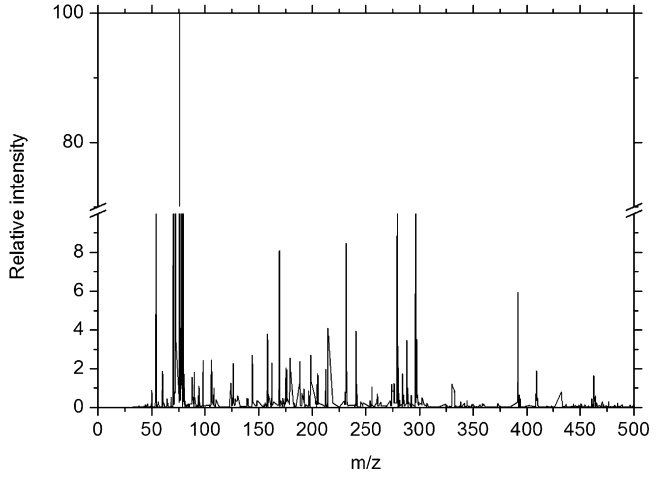

Fig. 6. Breath mass spectrum obtained after subtracting ambient air background in a protocol (discussed in Section 2.4) which preserves exactly the charging probability of the breath and the background.

concentrated in breath. All the positive peaks shown in Fig. 6 are reproducibly above the background. Note in particular that the heaviest compound clearly detected now is at 462 Da, which has a background intensity of 6500 cps and a breath intensity of 10,000 cps. We are in much debt to Dr. Bruce Thomson (Sciex) for his identification of several high mass peaks of Fig. 4 as common laboratory contaminants [11].

Among the peaks appearing both in Figs. 6 and 4 we have identified the following by MS-MS: 1-pyrroline, from the urea cycle and the metabolism of amino groups; and pyridine, which plays a role in the thiamine metabolism. Some other compounds identified in the simplified new spectra are 1-aminopropan-2-ol (76 Da), from the porphyrin metabolism; cysteamine (78 Da), from the taurine and hypotaurine metabolism; 4-aminobutanal (88 Da), from the urea cycle and the metabolism of amino groups and beta-alanine.

In conclusion, (i) ES ionization of volatiles is efficient enough to permit the detection of a large number of ions from products carried by breath; (ii) the source of most of these ions penetrates through chemically inert submicron filters and must therefore be the vapor itself in the gas phase; (iii) Because the surface area available for transfer of vapors from the body into the breathed gas flow is dominated by the lung, many of these vapors must originate primarily in the lung, from volatiles dissolved in the blood stream; (iv) urea in the breath of fasting individuals is positively detected in vapor form by our technique; (v) protonated glucose is not detected in either breath or in a stream saturated at room temperature with glucose vapors, though more work remains to be done with charging mechanisms more appropriate for glucose; (vi) while all major breath ions observed here survive the filter without significant dilution, other minor species could possibly have been suppressed by the filter. Breath aerosol is in fact known to contain ionic substances of essentially null volatility, which have been detected [10a,b], and could not possibly come in vapor form; (vii) a simple background subtraction protocol permits the clean distinction between the many volatiles present in ambient air (normally inhaled and exhaled by the subject) from the much smaller number of vapors strictly generated in the lung.

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