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Secondary electrospray ionization proceeds via gas-phase chemical ionization

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Our main goal was to gain further insights into the mechanism by which gas-phase analytes are ionized by interaction with plumes of electrospray solvents. We exposed target vapors to electrosprays of either water or deuterated water and mass analyzed them. Regardless of the solvent used, the analytes were detected in protonated form. In contrast, when the ionization chamber was humidified with deuterated water, the target vapors were detected in deuterated form. These observations suggest that either there is no interaction between analytes and electrospray charged droplets, or if there is any, a subsequent gas-phase ion–molecule reaction governs the process. Implications in practical examples such as breath analysis are discussed.

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Introduction

The concept of “ambient ionization-mass spectrometry” essentially involves the exciting possibility of direct analysis with minimal sample preparation.^{1,2} Well before a plethora of acronyms describing ambient ionization methods emerged during the second half of the 2000's, a number of pioneering papers describing similar concepts were presented. We feel that this pioneering work should be acknowledged in this special issue. For example, back in the 70's and early 80's Sciex's Trace Atmospheric Gas Analyzer (TAGA) was used to investigate human breath metabolites in real-time^{3–6} and for environmental monitoring.⁷ In these cases, chemical ionization coupled to atmospheric pressure ionization-mass spectrometry was at the core of such impressive early examples of the potential of what we call today ambient mass spectrometry.

Similarly, during the early days of electrospray ionization development, John B. Fenn and co-workers noted the remarkable ability of electrosprays to ionize not only analytes in the liquid-phase,⁸ but also pre-existing species in the gas-phase.^{9–11} Similar observations on the potential of electrospray to ionize vapors were noted contemporarily by other groups.^{12–14} Eventually, this technique was referred to as Secondary Electrospray Ionization (SESI) by Hill and co-workers to describe the use of a pure solvent electrospray to efficiently ionize gas-phase analytes.^{15,16} Ever since, SESI has been used to detect trace vapors in a wide range of applications by different groups.^{17–24}

While it is generally accepted that SESI coupled to modern atmospheric pressure mass spectrometry is a sensitive method to detect gases at trace concentrations in real-time, the lack of full understanding of the SESI mechanism prevents making rational choices for the optimal parameters. In addition, with the advent of several ambient mass spectrometry techniques with overlapping features, fundamental aspects of such techniques have been blurred or confused.²⁵ Our goal in this study was to further previous work on the mechanism by which strictly vapor species are ionized in contact with electrospray plumes of pure solvents.^{26,27}

Methods

SESI-MS

A commercially available SESI source (SEADM, Spain) was used in all experiments.²⁸ It was interfaced with an Orbitrap mass spectrometer (Thermo) and on one occasion (data shown in Fig. 5) with a TripleTOF 5600+ (Sciex).

The SESI was operated in the nanoliter range (~ 100 nL min^{-1}). We flushed the electrospray region with compressed air at a flow rate of 1 L min^{-1} . Compressed air contained around 5% relative humidity. Between the mass flow controller (Bronkhorst) and the ionization chamber, we placed a three-neck flask with rubber stoppers. Different liquids were injected into the flask to seed the carrier air with their corresponding vapors. Injected ambient vapors included water (H_2O), deuterated water (D_2O), ethanol (EtOH) and deuterated ethanol (EtOD). Relative humidity within the flask was recorded with a sensor (Alborn Almemo 2590A).

Breath analysis

SESI-MS breath analysis was accomplished by exhaling through the sampling tube of the ion source at a constant exhalation pressure of 10 mbar, as measured by a digital manometer.

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The study was approved by the local ethical committee (EK 2012-N-49) and the subject gave written informed consent to participate.

Results and discussion

Deuterated spray solvent

Two (simplified) ionization scenarios are plausible in SESI: (i) the neutral species dissolve into the charged electro-spray droplets to eventually be re-ejected in ionized form into the gas-phase; (ii) electro-spray droplets eject primary reactant ions that undergo an ion-molecule reaction with neutral species. Obviously, both mechanisms lead to very different scenarios to optimize the ionization efficiency. For the former mechanism, solubility of the analyte on the charging spray solvent would be crucial to maximize ionization efficiency. In the latter mechanistic scenario, analyte detection would be ultimately governed by thermochemistry.

In order to determine which of the two potential scenarios apply in SESI we exposed neutral vapors to electro-sprays of water and deuterated water. The exchange of hydrogen/deuterium has provided very valuable mechanistic insights since the initial developments of chemical ionization/mass-spectrometry.^{29–32} We hypothesized that, if the neutral vapors interact with the electro-spray charged droplets to be re-emitted as gas-phase ions, they should be detected as $[M + D]^+$ ions.

Typical SESI-MS spectra has a rich chemical background in the range 50–500 Da.^{33,34} For example, volatile polydimethylcyclosiloxanes in ambient laboratory air are well-known interfering species in mass spectrometry.^{35,36} Similarly, phthalates have also been reported to off-gas from vacuum o-rings.³⁷ Thus, we took advantage of the presence of these ubiquitous gas-phase contaminants to gain insights into how nano-electrosprays of solvent eventually lead to the ionization of such species. Fig. 1 shows the mass spectra resulting of flushing the electro-spray chamber with compressed air and using as spray solvent either 0.15% formic acid in H_2O (a) or 0.15% deuterated acetic acid (*i.e.* CD_3-COOD) in D_2O (b). The

resulting spectra are identical, including the known polysiloxanes and phthalates volatile species. Thus, regardless of the use of protonated or deuterated solvents, all species were detected in protonated form. This suggests that, even if the gas-phase species interact with the droplets to acquire a deuterium, eventually they exchange the deuterium with a proton in the gas-phase. In this case, with ambient water vapor, as the carrier gas contained $\sim 5\%$ of relative humidity.

Doping carrier gas with deuterated vapors

The data shown in Fig. 1b strongly suggest that a gas-phase proton reaction takes place in SESI. To further confirm this hypothesis, we humidified the carrier air with either water or deuterated water. As a result of humidifying with deuterated water, the entire chemical background mass spectrum shifted by 1 Da. Fig. 2 shows a zoomed view of the mass spectrum in the region of one representative example of a phthalate ($C_{16}H_{22}O_4$) and a polysiloxane ($C_{12}H_{36}Si_6O_6$) with H_2O -humidified air (bottom) and D_2O -humidified air (top). The fact that these species deuterate when the air is seeded with deuterated water vapor, but not when deuterated water is used in the spray solvent, strongly indicates that gas-phase ion chemistry plays a critical role in SESI, if not the only one.

Further insight was gained by seeding the carrier gas with ethanol instead of water. Ethanol has a greater gas-phase proton affinity than water (776.4 vs. 691 kJ mol⁻¹). Thus, it would be expected that, if SESI is governed by thermochemistry of the water and ethanol proton transfer reaction, it would be detected in the presence of the former but not the latter. Fig. 3a and b illustrate the behavior of another representative compound (benzothiazole; C_7H_5NS).³⁸ Fig. 3a shows the $[M + D]^+ / [M + H]^+$ ratio before and after humidifying with D_2O . Clearly, at the time point of D_2O injection, the protonated species decays, while simultaneously the deuterated adduct rises. Fig. 3b shows the result of a similar experiment, but instead of D_2O , we seeded the SESI chamber with vapors of EtOD. Likewise, the deuterated

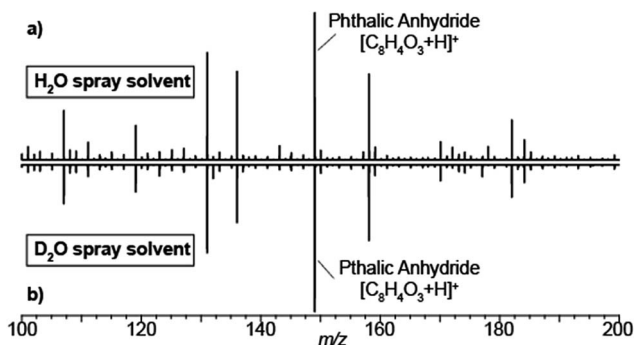


Fig. 1 SESI mass spectra are independent of whether electro-spray solvent is water or deuterated water. SESI chemical noise mass spectra with different electro-spray solutions: (a) 0.15% formic acid in H_2O ; (b) 0.15% deuterated acetic in D_2O . In both cases, the analytes were detected in protonated form. For reference, the dominating peak of phthalic anhydride is labelled.

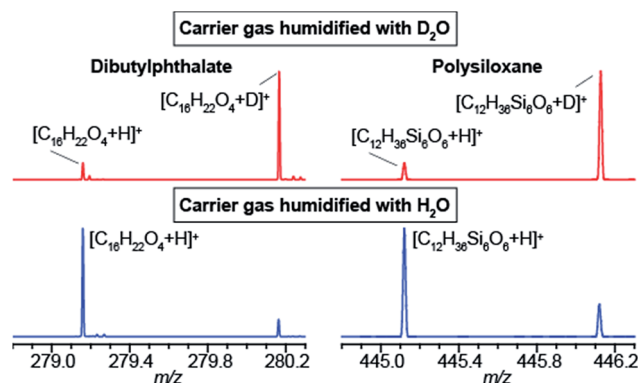


Fig. 2 Humidifying the carrier air with deuterated water leads to deuterated ions in SESI-MS. Electro-spray solvent was 0.15% formic acid in H_2O . Mass spectra of dibutylphthalate (left) and a polysiloxane (right). As expected, humidifying with H_2O leads to protonated species (*i.e.* $[M + H]^+$; bottom) and humidifying with D_2O leads to deuterated species (*i.e.* $[M + D]^+$; top).



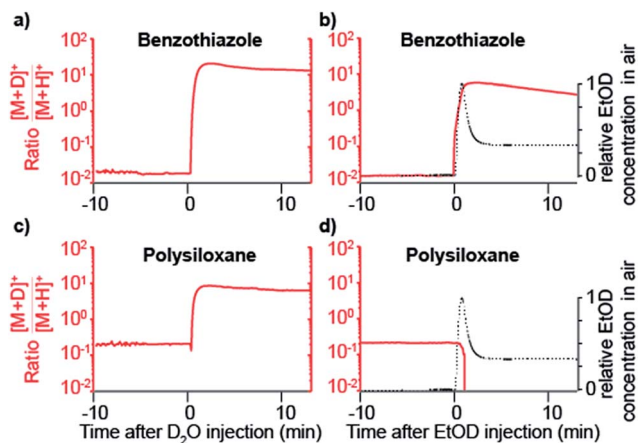


Fig. 3 Gas-phase proton affinity of seeding vapors have an effect in SESI analyte detection. Benzothiazole deuterates after adding vapors of D_2O (a) and EtOD (b) in the carrier gas. In contrast, the deuterated form of the polysiloxane was detected with D_2O (c), but depleted upon exposure to EtOD vapors (d). Relative concentrations of EtOD in the carrier gas are shown for reference.

adduct rose sharply in parallel with the relative EtOD concentration in air, suggesting a direct correlation between EtOD levels and $[C_7H_4NS + D]^+$ signal intensity. In contrast, the polysiloxane experienced the same response upon exposure to D_2O vapors (Fig. 3c), but the signal dropped abruptly to 0 after introducing EtOD (Fig. 3d). Hence, Fig. 3c and d suggests that the polysiloxane has a gas-phase proton affinity greater than water, but lower than ethanol.

To confirm that gas-phase thermochemistry ultimately dictates which analytes are detectable by SESI, we chose 2,2,2-trifluoroethanol as target analyte vapor. This particular compound has gas-phase proton affinity of $700.2 \text{ kJ mol}^{-1}$,³⁹ which is in between that of water and ethanol. As expected, this compound was detected as deuterated ion upon doping the carrier gas with D_2O , but not when EtOD was used.

Fig. 4a shows how the $[M + D]^+ / [M + H]^+$ ratio rises sharply immediately after injecting D_2O . For reference, relative humidity levels, as measured with the sensor, are overlaid. When the same experiment is performed but instead of D_2O , EtOD is injected in the carrier gas, 2,2,2-trifluoroethanol is no longer detected (Fig. 4b).

We have just shown compelling evidence indicating that SESI leads to protonated species, even if the spray solvent is deuterated, as long as some water vapor is present in the carrier gas of the sample (in our case $\sim 5\%$ relative humidity). This directly leads to a gas-phase chemical ionization scenario, at least in the final step of the process. Our hypothesis to explain this observation is that, initially, $[(D_2O)_n + D]^+$ clusters are ejected from the electrospray droplets by ion evaporation.^{40–44} These primary ions rapidly exchange deuterium by protons with ambient water, which is in excess in ambient air, leading to eventually dominating $[(H_2O)_n + H]^+$ clusters. These water clusters would then undergo a proton transfer reaction with neutral vapor species, provided the reaction is thermodynamically favorable. Of course, kinetically-controlled reactions are

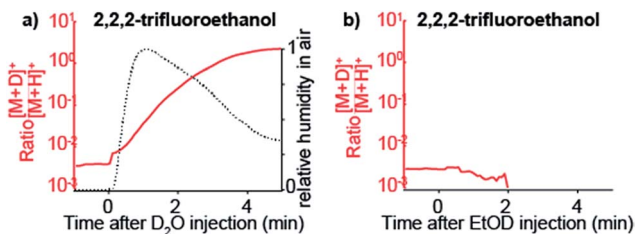


Fig. 4 SESI selectivity towards target vapors is governed by the proton affinity of the dopant seeded in the carrier gas. Response of 2,2,2-trifluoroethanol vapors in the presence of D_2O (a) and EtOD (b) in the carrier gas. When D_2O is introduced in the system the protonated ion declines in favor of the deuterated species (a). In contrast, when EtOD is introduced in excess in the carrier gas the signal of $[C_2H_3F_3O + H]^+$ drops quickly to zero, but the $[C_2H_3F_3O + D]^+$ ion is not formed (b). This is explained by the fact that the gas-phase proton affinity of 2,2,2-trifluoroethanol is greater than water, but smaller than ethanol.

also conceivable for slow proton transfer, which indeed is known to be the case for many reactions in chemical ionization.^{44–46} For example, under our particular experimental conditions in Fig. 1b, we formed the electrospray by infusing D_2O at $\sim 100 \text{ nL min}^{-1}$. If we assume that all D_2O is immediately vaporized, and considering that the ambient air flushing the SESI chamber at 1 L min^{-1} contained 5% relative humidity, the expected ratio H_2O/D_2O in the ionization chamber is in the order of 10. This excess of H_2O would then shift the equilibrium of reactant ions from $[(D_2O)_n + D]^+$ towards $[(H_2O)_n + H]^+$. This hypothesis is supported by the data shown in Fig. 5. It shows a SESI mass spectrum using water–formic acid as electrospray solvent and the carrier gas bathing the SESI chamber humidified with a 50/50 mixture of H_2O and D_2O . It shows two binomial distributions for the water trimer and tetramer with all possible combinations of hydrogen and deuterium atoms.

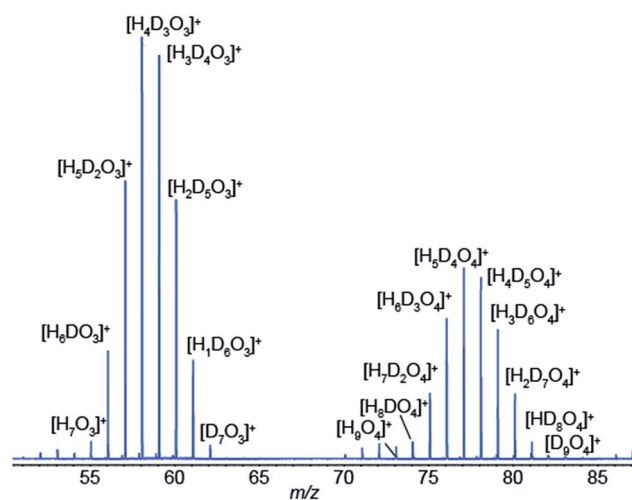


Fig. 5 SESI gas-phase reactant ions in the presence of humidity are water clusters. Example of an electrospray of 0.15% formic acid in water flushed with a 50/50 mixture of H_2O/D_2O vapors, leading to all possible $[H_xD_yO_3]^+$ ($x + y = 7$) combinations for the trimer (left-hand-side) and $[H_xD_yO_4]^+$ ($x + y = 9$) for the tetramer (right-hand-side).



Warnings about the importance of gas-phase ion chemistry in the standard electrospray process have been given in the literature.⁴⁷ For example, Enke and co-workers convincingly showed that analytes present in solution are very likely to be ultimately depleted in the gas phase by the presence of molecules that are stronger gas-phase bases, resulting in ion suppression.³⁹ Our data support this view, and extend it to SESI. Hence, we have advocated that one should be cautious when distinguishing the real-time analysis of gas- vs. condensed-phase analytes with sprays of pure solvent.²⁶ The main reason is that a number of studies have suggested that for samples delivered in aerosol form (*i.e.* extractive electrospray ionization), solubility of the analytes and interaction with the charging spray solvent is the limiting factor to be considered.^{48,49} We argue here that, in the case of vapor samples (*i.e.* secondary electrospray ionization), gas-phase ion chemistry considerations are ultimately governing the detection of such analytes. For this reason, we claim that water electrosprays operated in the cone-jet mode⁵⁰ are preferred in positive ion mode SESI over widely used H₂O/MeOH mixtures, as MeOH will probably deplete species detectable otherwise.

Breath analysis

The analysis of exhaled metabolites in breath is an attractive approach to monitor health status and therapeutic intervention.⁵¹ Selected ion flow tube mass spectrometry (SIFT-MS)⁵² and proton transfer reaction mass spectrometry (PTR-MS)⁵³ are two well established methods for real-time detection of volatiles in breath. One of the main strengths of SIFT-MS is its capability to provide absolute quantifications of trace gases in the parts-per-million to parts-per-billion range.⁵⁴ PTR-MS provides semi-quantitative information with limits of detection down to parts-per-trillion. Exhaling onto an electrospray plume in front of an atmospheric pressure ionization MS is another highly efficient way to perform such analyses. A wide range of species have been reported using this method; however chemical identification and absolute quantification of the species detected remain a challenge. Given that, low-volatility species such as urea have been detected using this approach,^{55,56} there has been some debate regarding whether the metabolites detected in breath following this approach come in aerosol form or simply the method is sensitive enough to detect low-volatility vapors.^{17,55,56} The debate is of importance because aerosols and volatiles have different physiological origins and therefore offer different diagnostic opportunities. Aerosols in breath are thought to be generated by the turbulence-induced aerosolization mechanism of the fluid film in the respiratory tract.⁵⁷ This leads to a high variability on particle size distribution and number depending on the inhalation and exhalation maneuvers⁵⁷ and hence difficulties in reproducibility. In contrast, the origin of gas-phase species is predominantly blood borne and therefore enable monitoring different metabolic processes.⁵⁸ We argue that breath analysis by SESI-MS detects volatile and non-volatile gas-phase species following that: (i) truly non-volatile macromolecules such as cytokines have been reported in exhaled breath condensate,⁵⁹ but are not detectable by SESI-

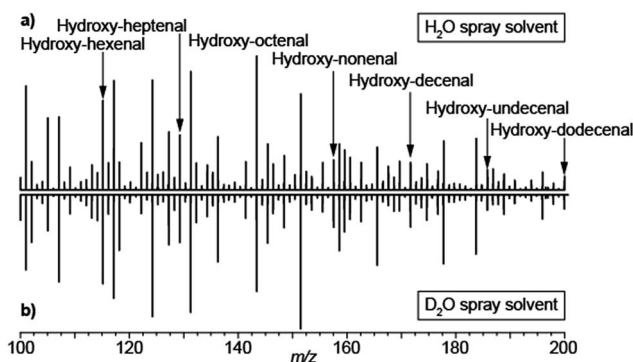


Fig. 6 Breath metabolites are ultimately protonated *via* gas-phase ion–molecule reactions in SESI: (a) breath mass spectrum using 0.15% formic acid in water; (b) breath mass spectrum using 0.15% acetic acid-d₄ in heavy water. Breath metabolites were detected in protonated form regardless of the solvent used to generate the electrospray. For reference, a series of hydroxy-alkenals previously identified by García-Gómez⁵³ are indicated.

MS; (ii) exhaling through a particle filter leads to essentially the same mass spectrum as without such a filter;⁵⁵ (iii) analytical²⁷ and numerical²⁸ models capture experimental observations with reasonable accuracy in systematic mechanistic studies²⁶ and (iv) we have shown how small amounts of injected drugs in mice can be detected in breath shortly afterwards and correlate with plasma levels.^{60,61} For example, ketamine and its main metabolites, which have molecular weights in the range of 250 Da and a relatively low estimated vapor pressure in the order of 8.5×10^{-5} mmHg at 25 °C (SPARC online calculator).⁶²

To further shed light on this mechanistic issue we performed SESI-MS breath analysis using H₂O and D₂O as spray solvents. Aerosolized samples have been shown to interact in the liquid-phase between charged electrospray droplets and neutral sample aerosols.⁴⁸ Hence we hypothesized that, if the detected compounds would come in aerosol form, they would necessarily pick up a deuterium from the D₂O charged droplets and a proton from H₂O sprays. Fig. 6 shows the breath mass spectrum of one subject exhaling onto a spray of 0.15% formic acid in water (a) and 0.15% acetic acid-d₄ in D₂O (b). In both cases, the spectra were identical, with the vast majority of the species detected in protonated form. This may also explain the noted sensitivity enhancement of SESI in the presence of water vapor in breath.^{55,64} Admittedly, these data cannot completely exclude the possibility of exhaled aerosols and vapors dissolving in the charged droplets, generation of $[M + D]^+$ gas-phase ions and subsequently exchanging H–D in the gas phase with ubiquitous water vapor. However, it clearly shows that, even if this is the case, gas-phase ion chemistry ultimately plays a critical role in the ionization of breath metabolites.

Conclusions

We conclude that (i) neutral vapors carried in an air stream (5% relative humidity) and exposed to an electrospray of deuterated water leads to protonated ions; (ii) inversely, neutral vapors carried in an air stream seeded with deuterated solvents (*e.g.*



D₂O), and exposed to an electrospray of water leads to deuterated ions; (iii) gas-phase proton affinity of the target analytes plays a crucial role in whether or not they are finally detected in SESI; (iv) breath metabolites are detected in protonated form regardless of whether the solvent used is H₂O or D₂O. These observations lead us to conclude that, in the final stage of SESI, gas-phase ion–molecule reactions govern the mechanism by which electrosprays ionize vapor species.

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