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2013 J. Breath Res. 7 026005

(http://iopscience.iop.org/1752-7163/7/2/026005)

IOP Publishing Journal of Breath Research

J. Breath Res. **7** (2013) 026005 (8pp) doi:10.1088/1752-7155/7/2/026005

Atmospheric pressure chemical ionization mass spectrometry of pyridine and isoprene: potential breath exposure and disease biomarkers

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Received 14 November 2012
Accepted for publication 14 March 2013
Published 12 April 2013
Online at stacks.iop.org/JBR/7/026005

Abstract

Volatile organic compounds (VOCs) in exhaled human breath can serve as potential disease-specific and exposure biomarkers and therefore can reveal information about a subject's health and environment. Pyridine, a VOC marker for exposure to tobacco smoke, and isoprene, a liver disease biomarker, were studied using atmospheric pressure chemical ionization mass spectrometry (APCI-MS). While both molecules could be detected in low-ppb levels, interactions of the ionized analytes with their neutral forms and ambient air led to unusual ion/molecule chemistry. The result was a highly dynamic system and a nonlinear response to changes in analyte concentration. Increased presence of ambient water was found to greatly enhance the detection limit of pyridine and only slightly decrease that of isoprene. APCI-MS is shown to be a promising analytical tool in breath analysis with good detection limits, but its application requires a better understanding of the ion/molecule chemistry that may affect VOC quantification from a chemically complex system such as human breath.

1. Introduction

Recent developments in the analytical methods of human breath analysis have revealed a continuously increasing list of chemicals that are found as traces in exhaled air and can be used to diagnose chronic diseases, airway infections and other changes from normal health [1–3]. An increased level of these biomarkers is associated with a permanent defect or a temporary imbalance in normal biochemical processes and the gas exchange physiology in the human body [1–3]. Isoprene is a byproduct of cholesterol metabolism and is one the most abundant volatile organic compounds (VOCs) in breath. Elevated breath isoprene has been linked to cholesterologenesis-related disorders [4, 5]. Breath isoprene concentrations are highly variable in both healthy and unhealthy populations. In normal subjects, isoprene values vary from 50 to more than 1000 ppb [4, 5]. Pyridine is a marker for exposure to tobacco smoke and thus is an environmental and occupational health marker [6]. It has been detected in healthy breath [7, 8] and is thought to be a product of collagen breakdown. Elevated pyridine levels have been found in subjects with periodontal disease. Unfortunately, pyridine is the least studied compound due to its toxic nature in its pure form and there are still no reported absolute quantitative measurements [9].

Several analytical approaches have been applied in recent years to identify and measure the levels of these biomarkers, including GC, GC/LC-MS, IMS, halogen detectors, EBC wet scrubbing fluorescence, IR and others [10–21]. The most applied tool in the area of breath research is mass spectrometry and the common ionization source used in MS breath analysis is electrospray ionization (ESI) in which exhaled breath condensate (EBC) [22] is sprayed back to the gaseous phase while compounds are ionized. The disadvantage of

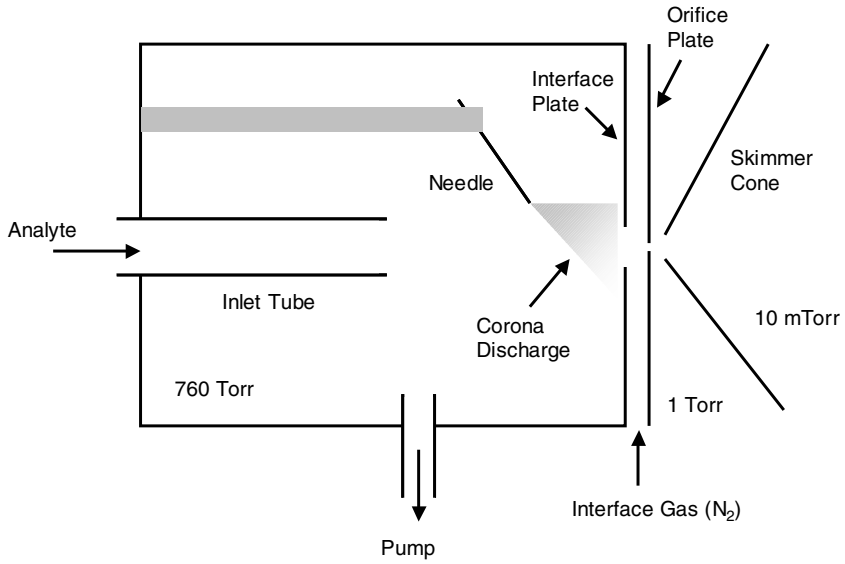

Figure 1. Basic schematic of the modified APCI source employed for gas analysis.

EBC-ESI-MS is that it does not provide real time, or 'online', analysis. The major drawback of online breath analysis is the air and breath moisture that affects instrument sensitivity and ionization dynamics. GC-MS bundles are the most common instruments in breath research and there have been very few attempts to analyze VOC directly in a mass spectrometer without any pre-separation steps [23]. Atmospheric pressure chemical ionization (APCI) has been the most useful ionization source to study online VOC sampling [24] and has shown some promising preliminary results in breath research [25].

In APCI-MS, H_2O molecules in air (also N_2 and O_2) pass through a corona needle inside the source, where electrons from neutral molecules are stripped off by several thousands of volts of positive potential generated by the corona needle, thus generating H_2O^+, N_2^+ and O_2^+ ions. These positive ions interact with neutral H_2O molecules and eventually transform to hydronium cluster ions, $H_3O^+(H_2O)_n$ with various values of n [28]. These protonated water clusters can then react with an analyte via bimolecular proton transfer reactions to produce a protonated analyte [25–28] (Reaction 1):

$$H^+(H_2O)_n + A \rightarrow AH^+ + (H_2O)_n. \quad (1)$$

The success and extent of such proton transfer will depend on several key factors and parameters such as the proton affinity (PA) of the analyte, humidity and therefore the amount of H_3O^+ in the carrier gas [27, 29], neutral analyte pre-ionization clustering and other neutral chemistry depending on analyte concentration. Such dynamic parameters and possible side-reaction chemistry must be recognized in any APCI-MS VOC analysis.

In 2006, Turner *et al* used a SIFT-MS with NO^+ ionization to detect and quantify breath isoprene by analyzing only an ionized isoprene monomer at 69 Th [30]. In 2008, Kushch *et al* measured breath isoprene with PTR (proton transfer reaction)-MS taking into account details of possible fragmentation reactions which may occur during quantification [31]. However, the previous analytical studies of breath isoprene using APCI or PTR MS generally give very little to no consideration to the factors mentioned above which could compromise the integrity of quantification. In this work, we study the APCI-specific ion/molecule chemistry of pyridine and isoprene that should be the basis of the next step in the calibration and measurement of VOCs in breath or a breath-like matrix.

2. Materials and methods

The instrument employed in this project was an AB Sciex API 2000 triple quadrupole mass spectrometer. The APCI source was redesigned by replacing the original sample introduction inlet with a 1-inch diameter glass tube which directed analyte gas into the corona discharge needle (shown in figure 1). Each experiment was performed with fixed ionization and MS parameters: a curtain gas flow of approx 2 L min^{-1} of nitrogen, a nebulizer current of 4 μA and a declustering potential of 20 V.

Isoprene and pyridine were obtained from Sigma with a Chemical Purity (C.P.) grade (99.6%). Before entering the APCI source VOCs were diluted by injecting liquid compounds or their vapors using a syringe pump. Pyridine and isoprene were injected as a liquid dissolved in isooctane in different% v/v because of their toxicity in high concentrations. Diluted VOCs were then carried into the APCI source using a suction pump connected to the source drawing air from the syringe at 90 L h^{-1} as shown in figure 2. Mass spectra were taken at gradually increasing concentrations after which a peak area of the ion of interest was background subtracted and used as a signal intensity unit to generate a calibration curve. The calibration range was different for each compound and targeted both healthy and unhealthy levels of that compound in human breath. Collision-induced dissociation (CID) was used to fragment and identify ions of interest. The collision energy for CID was varied between 35 and 65 V to produce the optimum fragmentation with the nitrogen collision gas (approx. 1 mTorr).

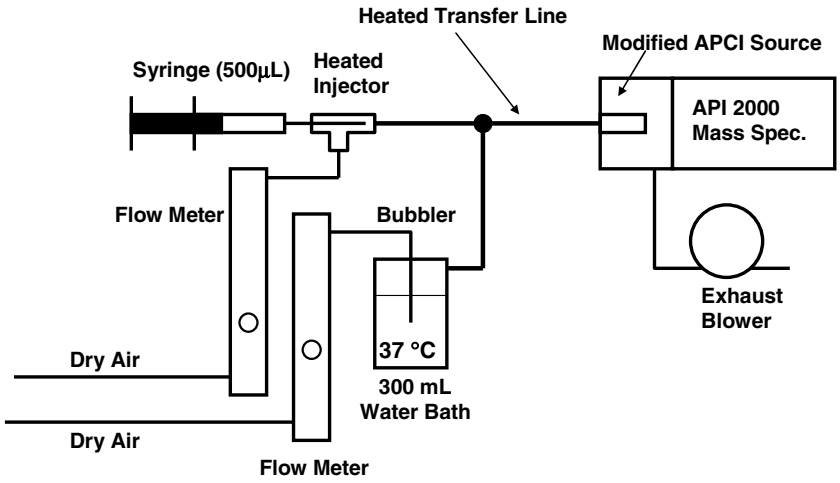

Figure 2. Final VOC dilution set-up designed for partial humidification of the airflow. VOC is injected using a syringe into a heated injector to ensure efficient vaporization. The syringe flow rate is controlled using a syringe pump.

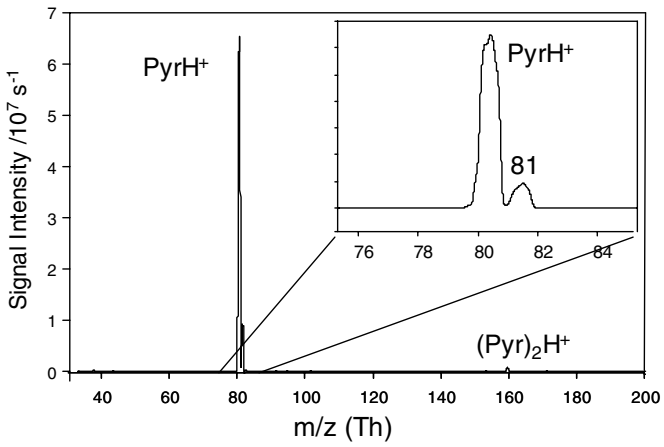

Figure 3. APCI mass spectrum of 200 ppb of pyridine in room air. Region close to the protonated pyridine has been enhanced for clarity.

Humid air was produced by incorporating a stainless steel bubbler into an airflow system as show in figure 2. Humidity was not measured. We created 100% relative humidity air by bubbling through water and then mixing with dry air in a known proportion. The bubbler was continuously heated to 37 °C in a water bath. The air was split through independent flow controllers, one calibrated from 5 to 20 L min^{-1} passing the dry air to the heating region prior to injection port. The other, calibrated from 0 to 5 L min^{-1}, was connected to the bubbler and then rejoined with the main airflow. The control of sample humidity without affecting the analyte concentration is achieved by varying the two flows while keeping the sum constant at 15 L min^{-1}.

3. Results and discussion

3.1. Pyridine

APCI positive ion mass spectrum of pyridine in room air shows ions at 80, 81 and 159 Th (figure 3). Those at 80 and 159 Th represent the protonated monomer and dimer of pyridine, respectively, whereas the ion at 81 Th is a product of pyridine ion/molecule chemistry in the APCI source. While the monomer peak appears at the detection limit, the dimer appears only at concentrations above 20 ppb. The ion at 81 Th was initially found to have a much lower intensity than protonated pyridine (5%), but increases to almost 50% at a pyridine concentration of 200 ppb. Figure 4 shows the individual signal response curves and the cumulative response of 80, 81 and 159 Th as the pyridine air concentration increases. After 40 ppb, the signal response of the protonated pyridine ion (80 Th) flattens out and begins to decrease while the sum of all three ions remains constant. The spatial charge limits the increase in overall intensity resulting in a decreasing intensity of protonated pyridine due to dimer formation (159 Th) and formation of 81 Th.

CID studies of all three ions showed a great similarity between the CID of protonated pyridine (80 Th) and the CID of the 81 Th ion with a nearly identical fragmentation pattern and most fragment ions shifted by 1 Th. The CID of the protonated pyridine dimer (159 Th) did not show any evidence for 81 Th. The similarity in the fragmentation of protonated pyridine and the 81 Th ion suggests two possible candidates for the latter: $C_4H_5N_2^+$, protonated pyradizine, and $C_5H_7N^+$, a hydrogenated pyridine. Both of these could result from an endothermic reaction of two pyridine molecules in the presence of H_3O^+ as a source of activation energy [31]. Isotopic labeling experiments were performed to test which of the two structures is present in our experiments. Initially, D_2O was introduced as an APCI reagent to produce D_3O^+. If $C_4H_5N_2^+$ is protonated pyradizine, there is no opportunity for deuterium to be incorporated into the ring and the presence of D_2O would result in the formation of $C_4H_4N_2D^+$ (one D incorporation). However, if the $C_5H_7N^+$ is formed, independent of the exact chemical structure, a deuterium atom will be incorporated via H/D exchange in the CH_2 location during the formation producing the $C_5H_5D_2N^+$ ion (83 Th, two-D incorporation). Such an event would result in an 83 Th peak of significant intensity, which is not evident in the pyridine-D_2O spectrum (the slightly visible 83 Th peak is a result of isotopic abundances of the 81 and

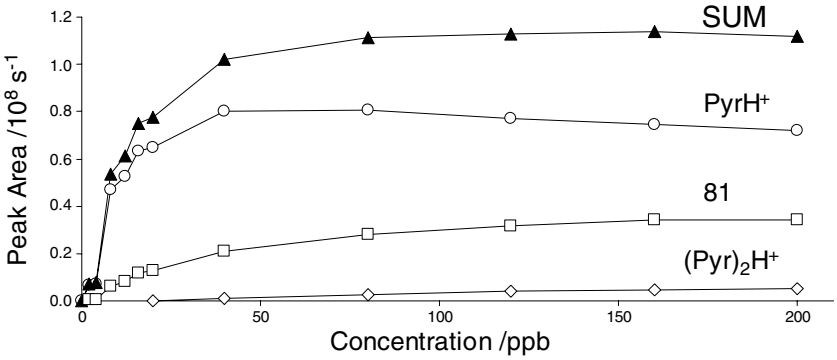

Figure 4. Signal response for the three ions observed in the chemical ionization of pyridine and their cumulative intensity (SUM) as measured by the area under the peak. Increase in the 81 Th ($PyrH^+$) to 80 Th ratio suggests some degree of neutral chemistry converting pyridine to some other compound that appears as 81 Th ion.

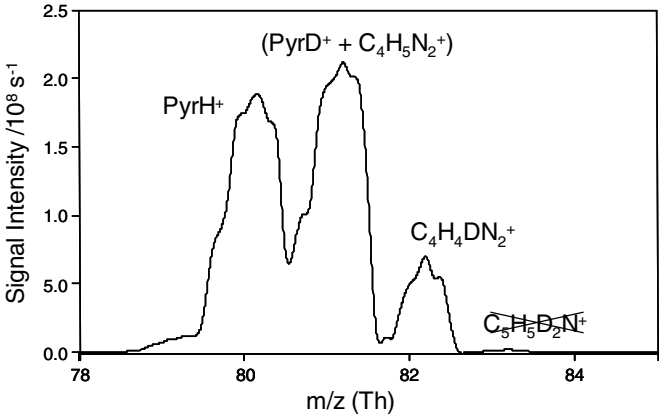

Figure 5. APCI-mass spectrum of pyridine with D_2O as the APCI reagent. The presence of background H_2O results in incomplete H/D exchange. The intensity of the 83 Th ion is consistent with the isotopic pattern of the 82 ion and it shows no presence of the $C_5H_5D_2N^+$ ion.

82 Th ions) (figure 5). The same experiment was repeated using deuterated pyridine analyte, C_5D_5N, to test the possibility that pyridine is the possible source of hydrogen. In this case the two candidates, $C_4D_5N_2{}^+$ (86 Th) and $C_5D_7N^+$ (88 Th), differ by 2 Th and only the 86 Th ion was observed.

A detection limit for pyridine of 200 pptv was estimated from the measured signal to background noise levels (in the single ms mode). Improvements may well be achieved by performing CID with MRM (multiple reactant monitoring) measurements and that increasing the size of the sampling orifice may also help. This is done, for example, in the recent API 3200 (AB Sciex) instrument that is 10-fold more sensitive than the API 2000 (AB Sciex) used in our studies.

3.2. Isoprene

Mass spectra of isoprene in sub- and low-ppb concentrations show the principal ion at 57 Th with smaller 69 Th and 71 Th ions. Higher isoprene concentrations produce a complex sequence of ions between 81 and 261 Th at 14 Th intervals, sometimes as an ion pair 2 Th apart (see figure 6). The 20 ions with the highest signal response, including 57, 69 and 71 Th, were monitored. Taking the spatially constrained ionization as the start of the reaction region and the orifice of the MS as the end of the reaction region, one could consider this region as a flow reactor so that experiments at various concentrations become similar to a flow-based kinetics experiment such as one might perform with a SIFT-MS [32–34]. The 57, 69 and 71 Th ions are the (primary) isoprene ions that appear first in the mass spectra and these are then consumed by further chemistry in the reaction region, giving rise to the higher mass species. The relative distribution of the three primary ions (57, 69 and 71 Th) does not change significantly with increased isoprene concentration from low- to mid-ppb levels. 57 Th is the dominant ion at all isoprene concentrations and, combined with the products of its subsequent chemistry, comprises approximately 60% of the total ion signal. Isoprene was studied at concentrations between 1 and 1000 ppb, with detection limits similar to those in pyridine, approx. 200 pptv.

While the 69 Th ion is clearly the protonated isoprene, the identity of the other two primary ions, 57 and 71 Th, is less obvious. The hydronium ion H_3O^+, aside from its ability to transfer a proton, also could react with isoprene to cleave one of its carbon bonds to form the butyl cation and formaldehyde (equation (2), $\Delta G^\circ = -10.5$ kcal mol^{-1}):

$$H_3O^+ + C_5H_8 \rightarrow C_4H_8H^+(57\ \text{Th}) + CH_2O. \qquad (2)$$

The proton affinity of formaldehyde is relatively low, 170 kcal mol^{-1} [35] and protonation of the C_4H_8 fragment is more favorable. One of the possible structures for the C_4H_8 fragment is isobutene with PA = 191 kcal mol^{-1} and other possible structures will have similar proton affinities due to the presence of a double bond in the molecule. Isoprene is known to undergo oxidation by ozone and other active oxygen species such as hydroxyl radicals and NO_3 [36–39]. Although none of those species mentioned above are major constituents in air, both room and zero air, it has been reported that ozone is generated by a corona discharge in atmospheric air at a normal humidity [40, 41]. Isoprene oxidation has two major products: methyl vinyl ketone (MVK) and methacrolein (MARC or MAC) [36–39], whose protonated ion corresponds to 71 Th, $C_4H_6OH^+$, but cannot be distinguished further. The highest mass ions correspond to isoprene clusters of $C_4H_8H^+$ (125, 193 and 261 Th), protonated isoprene $C_5H_8H^+$ (137, 205 and 243 Th) and MVK/MAC (139, 207 and 275 Th). A detailed overview is shown in figure 7.

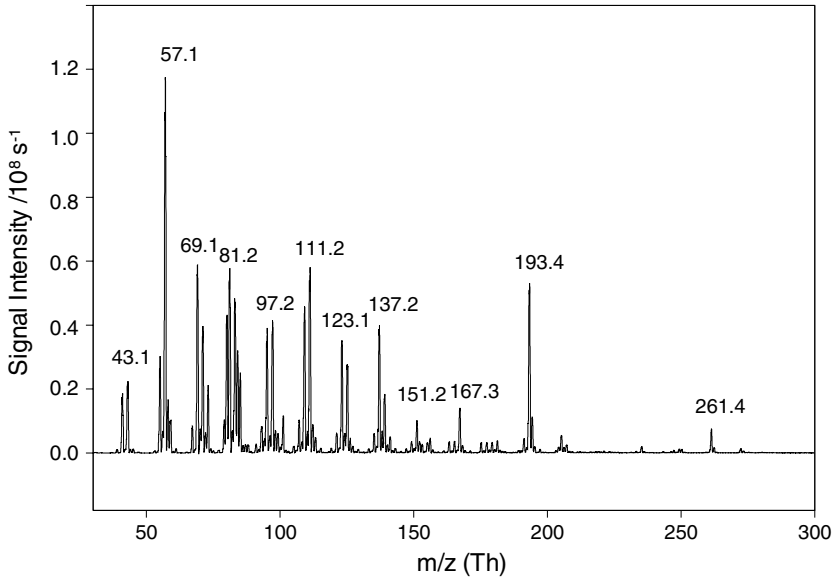

Figure 6. APCI-MS of 800 ppb isoprene showing the richness of the ion/molecule chemistry occurring in the source region of the instrument.

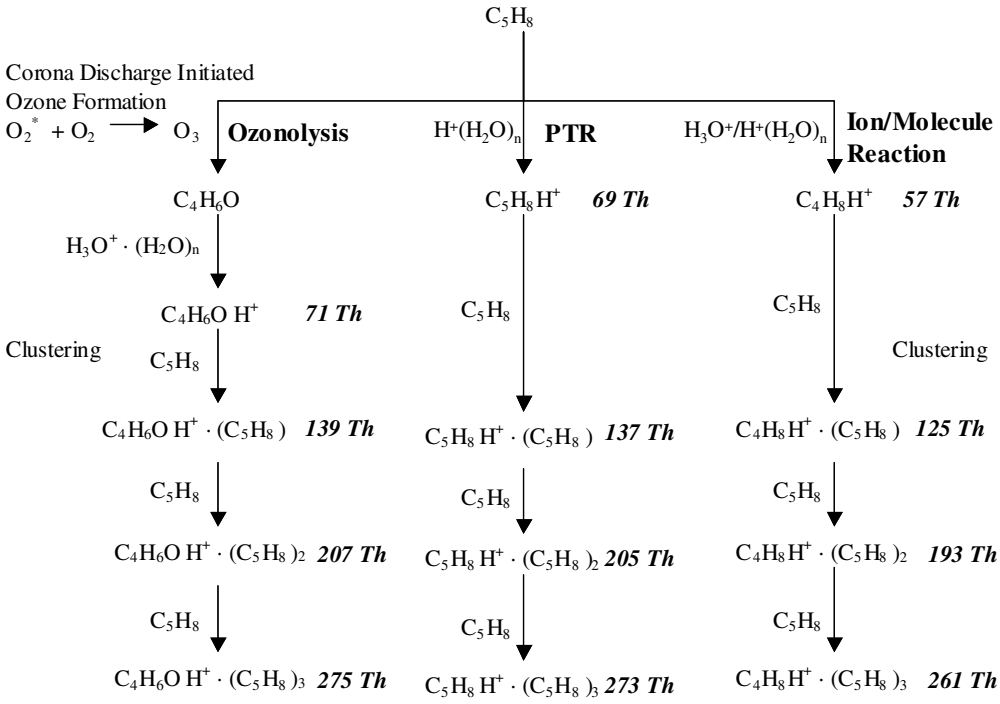

Figure 7. Overview of the isoprene ion/molecule reactions proposed to occur in the APCI ion source. Ozone is formed by oxygen excitation at the corona discharge needle.

It was previously reported that isoprene in gas phase undergoes ion/molecule polymerization and polymer-related fragmentation in an electron impact ionization source [42, 43]. The fragmentation by the electron impact is caused by a series of elimination reactions and bond formations within clusters of the isoprene cation with neutral isoprene molecules and is described as a cationic polymerization [42, 43], also producing a sequence of ions that again are 14 Th apart. Although the APCI-initiated polymerization of isoprene appears not to have been reported previously, the APCI source is known to provide additional activation energy for gas phase organic reactions that form new covalent bonds. All this suggests that the APCI-initiated polymerization of isoprene is initiated by all three ion–molecule cluster channels and therefore explains the series of ions (81, 83, 95, 97, 109, 111, 123, 151, 163, 167, 175, 121, 235, 249 Th and others) in the mass spectrum. The increasing complexity of the mass spectrum with a continuously increasing number of ions as the isoprene concentration increases suggests that there is additional clustering of the fragments with neutral isoprene and further polymerization and fragmentation reactions.

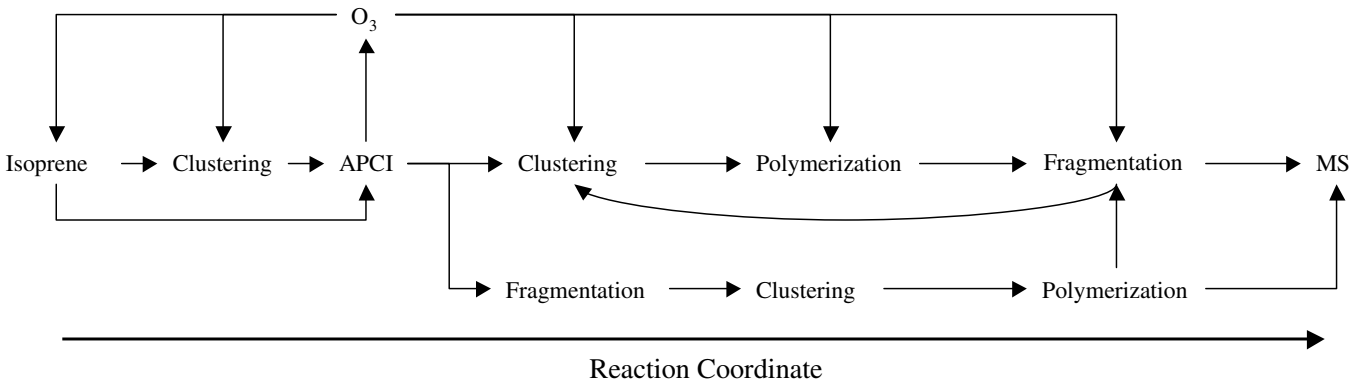

Figure 8. Network of chemical processes resulting from the APCI of isoprene proposed to be responsible for the observation of complex mass spectra.

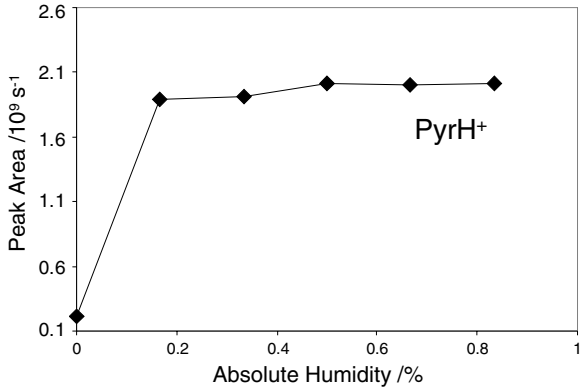

Figure 9. Measured signal response of protonated pyridine in APCI-MS as a function of absolute humidity. The increase in signal is dramatic al low absolute humidities, but the signal quickly plateaus. The pyridine concentration is 50 ppb.

The overall chemistry of isoprene proposed to occur in the APCI source is depicted in figure 8. As both isoprene monomers and neutral clusters enter the APCI source, they are ionized, oxidized and fragmented in the manner described above. These ion clusters and fragments undergo polymerization, clustering and fragmentation in a form of cyclic chain reactions that propagate until they exit the reaction region and enter the mass filter. Because the isoprene ozonation (oxidation) reaction is highly exothermic and the neutral isoprene is found in both clusters and fragments, it is likely that sequential and parallel reactions within the reaction region are also affected by ozonation.

3.3. Effects of humidity on pyridine and isoprene signal response

Pyridine ionization by APCI is greatly affected by humidity and this can be attributed to the formation of protonated water cluster ions and the high proton affinity of pyridine (222.0 kcal mol^{-1} [35]) which leads to proton transfer to pyridine even as the protonated water clusters themselves increase in size and in proton affinity. An increase from 0% to $\leqslant$0.17% in absolute humidity produced a ten-fold pyridine signal enhancement (see figure 9), but a further increase in absolute humidity shows no significant signal enhancement. These observations are in agreement with previous studies of APCI humidity effects on pyridine [44]. After the large initial increase at relatively low absolute humidity, the protonated pyridine signal response is independent of humidity. This behavior simplifies breath analysis measurements as the sample water concentration would not have to be controlled, provided the humidity is kept above 0.17%.

Water has a negative effect on the production of the three primary isoprene ions, reducing their absolute intensities. The relative intensity of $C_4H_8H^+$ is increased compared to that of oxidized and protonated isoprene. $C_4H_8H^+$ benefits from the increased concentration of one of its precursors, $H_3O^+/H^+(H_2O)_n$. On the other hand, the precursor for oxidized isoprene (71 Th), ozone, is suppressed by water, while formation of protonated isoprene is decreased due to the formation of larger protonated water clusters which have a higher proton affinity and so reduce the exothermicity of proton transfer. Previous studies have identified an analyte proton affinity of 200 kcal mol^{-1} as the boundary separating the relatively weak and strong responders to humidity in the APCI [43], consistent with the predominant observation of $H^+(H_2O)_2$ (37 Th, PA = 197 kcal mol^{-1} [35]) at low humidity and $H^+(H_2O)_3$ (55 Th, PA = 217 kcal mol^{-1} [33]) at high humidity. Isoprene (PA = 197.5 kcal mol^{-1}, [35]) is very sensitive to humidity since its proton affinity is equal to the deprotonation enthalpy of $H^+(H_2O)_2$, while pyridine (PA = 222.0 kcal mol^{-1} [35]) is insensitive since its proton affinity is above that of the deprotonation enthalpy of $H^+(H_2O)_3$.

4. Conclusions

Our experiments have demonstrated the flexibility of APCI-MS when applied to simulated breath analysis. The dynamic range for detection was seen to be large and different ionic species showed a good response over different analyte concentration ranges. The APCI chemistry of pyridine and isoprene showed unexpected complexity and neither could be reliably quantified by simple monitoring of the protonated analyte. The in-source ion/molecule chemistry of pyridine produced large amounts of protonated pyradizine, while isoprene produced a complex sequence of oxidation, polymerization and fragmentation products. The post-ionization chemistry of VOCs in APCI-MS was shown

to be a highly dynamic process at various concentrations and increases in analyte concentration produced significant secondary products that must also be monitored for reliable quantification.

While we did not perform breath analysis *per se,* we address the ion chemistry of two important components of breath and how it may impact the efficacy of breath analysis.

The detection limits for pyridine and isoprene (approx. 0.2 ppb) exceed the concentrations of isoprene measured in individuals (of the order of 100–1000 ppb [45, 46]) and the concentrations of pyridine found in ambient air (up to 5 ppb [47]). The installation of a bubbler as a humidifier inside the mixing system proved successful in achieving near-breath levels of humidity (4.4% from the bubbler compared to 5–6% from breath) with a good dynamic range. The final VOC mixing set up with the installed humidifier proved to be successful in producing trace VOC quantities with humidity levels similar to breath vapors. Breath sampling using this system would satisfy online-sampling, humidity and temperature requirements, which are critical to real-time breath analysis.

Acknowledgments

Financial support from the Natural Sciences and Engineering Research Council of Canada is greatly appreciated. As holder of a Canada Research Chair in Physical Chemistry, DKB thanks the contributions of the Canada Research Chair Program to this research.

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