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Selecting and identifying gas-phase protonation isomers of nicotineH⁺ using combined laser, ion mobility and mass spectrometry techniques

Samuel Marlton

University of Wollongong, sjpm073@uowmail.edu.au

Benjamin McKinnon

University of Wollongong, bm125@uowmail.edu.au

Boris Ucur

University of Wollongong, bu749@uowmail.edu.au

Alan T. Maccarone

University of Wollongong, alanmac@uow.edu.au

William Donald

University of New South Wales, w.donald@unsw.edu.au

See next page for additional authors

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Abstract

The detection and assignment of protonation isomers, termed protomers, of gas-phase ions remains a challenge in mass spectrometry. With the emergence of ion-mobility techniques combined with tuneable-laser photodissociation spectroscopy, new experimental combinations are possible to now meet this challenge. In this paper, the differences in fragmentation and electronic spectroscopy of singly protonated (S)-nicotine (nicH⁺) ions are analysed using action spectroscopy in the ultraviolet region and field asymmetric ion mobility spectrometry (FAIMS). Experiments are supported by quantum chemical calculations (DFT, TD-DFT and CC2) of both spectroscopic and thermochemical properties. Electrospray ionisation (ESI) of (S)-nicotine from different solvents leads to different populations of two nicH⁺ protomers corresponding to protonation on the pyridine nitrogen and pyrrolidine nitrogen, respectively. FAIMS gives partial resolution of these protomers and enables characteristic product ions to be identified for each isomer as verified directly by analysis of product-ion specific action spectroscopy. It is shown that while characteristic, these product ions are not exclusive to each protomer. Calculations of vertical electronic transitions assist in rationalising the photodissociation action spectra. The integration of photodissociation action spectroscopy with FAIMS-mass spectrometry is anticipated to be a useful approach for separating and assigning protonation isomers of many other small molecular ions.

Keywords

identifying, gas-phase, nicotineh⁺, protonation, combined, selecting, laser, ion, isomers, mobility, mass, spectrometry, techniques

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Selecting and identifying gas-phase protonation isomers of nicotineH⁺ using combined laser, ion mobility and mass spectrometry techniques

Samuel J. P. Marlton,¹ Benjamin I. McKinnon,¹ Boris Ucur,¹ William A. Donald,² Stephen J. Blanksby,³ Adam J. Trevitt.^{1}*

¹ School of Chemistry, University of Wollongong, Wollongong, AUSTRALIA

² School of Chemistry, University of New South Wales, Sydney, AUSTRALIA

³ Central Analytical Research Facility, Institute for Future Environments, Queensland University of Technology, Brisbane, AUSTRALIA

*Corresponding author: adamt@uow.edu.au

1 Abstract

The detection and assignment of protonation isomers, or protomers, of gas-phase ions remains a challenge in mass spectrometry. With the emergence of ion-mobility techniques combined with tuneable-laser photodissociation spectroscopy, new experimental combinations are possible to now meet this challenge. In this paper, the differences in fragmentation and electronic spectroscopy of singly protonated (*S*)-nicotine (nicH^+) ions are analysed using action spectroscopy in the ultraviolet region and field asymmetric ion mobility spectrometry (FAIMS). Experiments are supported by quantum chemical calculations (DFT, TD-DFT and CC2) of both spectroscopic and thermochemical properties. ESI of (*S*)-nicotine from different solvents leads to different populations of two nicH^+ protomers corresponding to protonation on the pyridine nitrogen and pyrrolidine nitrogen, respectively. FAIMS gives partial resolution of these protomers and enables characteristic product ions to be identified for each isomer as verified directly by analysis of product-ion specific action spectroscopy. It is shown that while characteristic, these product ions are not exclusive to each protomer. Calculations of vertical electronic transitions assist in rationalising the photodissociation action spectra. The integration of photodissociation action spectroscopy with FAIMS-mass spectrometry is anticipated to be a useful approach for separating and assigning protonation isomers of many other small molecular ions.

2 Introduction

Detection and assignment of protonation site isomers (protomers) generated by electrospray ionisation (ESI) is a challenge for mass spectrometry. Not only are protomers isomers—and therefore unable to be mass-resolved by mass spectrometry—changes in ESI conditions are observed to affect relative protomer populations.¹⁻¹⁴ This can be an analytical problem when protomers dissociate to different product ions under activation, a phenomenon that can confound assignment by comparison to reference spectra. Differences in the photodissociation of protomers have been used to assign protomer populations,¹⁰⁻²⁶ although it is difficult to predict how the electronic spectroscopy and photodissociation will be affected by differences in protonation site. Hence, analysing the photodissociation of selected protomers, can provide insights into the effects of protonation on excited quantum states of ions while progressing towards the use of photo-dissociation as a method to disambiguate and assign protomers.

Previous studies have taken advantage of the differences in photoactivity of protomers and made assignments by comparing theoretical transition simulations to experimental results obtained by UV photodissociation action spectroscopy,^{13-17, 24-25} or IRMPD.^{10-11, 18-23} Dessent and co-workers have demonstrated the potential for moderate-resolution UV action spectra, coupled with TD-DFT analysis, to detect and assign protomers of nicotinamide and *p*-aminobenzoic acid.^{12, 26} These studies reported different action spectrum profiles of O-protonated or N-protonated isomers. To further explore this technique, we investigate two N-protonated protomers of (*S*)-nicotine, in which the pyridine nitrogen or the pyrrolidine nitrogen atoms are protonated.

(*S*)-Nicotine is the bioactive agonist in tobacco²⁷ and it is suggested that the bioactive form of (*S*)-nicotine is singly protonated on the pyrrolidine nitrogen. This bioactivity arises as the cationic quaternary amino nitrogen is chemically similar to the cationic quaternary amino nitrogen of acetylcholine.²⁸⁻²⁹ Its bioactivity manifests when pyrrolidine protonated (*S*)-nicotine binds to the neuronal acetylcholine receptors in the brain.²⁸⁻³⁰ At physiological pH, (*S*)-nicotine is singly protonated. As an interesting aside, cigarettes and electronic cigarettes³¹ vaporise nicotine using different mechanisms and El Hellani *et al.* suggest that analytical studies do not adequately consider whether nicotine is sampled as a neutral species as a singly- or doubly- protonated ion.³²

Theoretical studies have predicted that both protomers of (*S*)-nicotine are likely present in the gas phase^{28, 33-34} and this is supported by experiment.^{21, 28, 35} Product ions arising from collision induced dissociation (CID) have been explained to arise from either nicH⁺ protonated on the pyridine nitrogen (PYRO-nicH⁺) or pyrrolidine nitrogen (PYRI-nicH⁺).³⁵ What is unclear from CID studies however, is whether both protomers are present in the gas phase or whether tautomerisation proceeds dissociation upon CID. An IRMPD spectrum of gas phase nicH⁺ provided experimental evidence for the presence of PYRI-nicH⁺, however, the presence or absence of PYRO-nicH⁺ could not be confirmed.²¹ By comparing experimental gas-phase basicity measurements to theoretical predictions, it was estimated that protomers formed in approximately a 2:1 ratio of PYRO-nicH⁺:PYRI-nicH⁺ from gas phase proton-transfer equilibrium between a (*S*)-nicotine and several reference compounds.²⁸ Because of the differences between formation of nicH⁺ through proton-transfer and formation by ESI, it is difficult to predict whether the 2:1 ratio will translate to ESI and how solvent conditions might modulate protomer populations in the gas phase.

Several theoretical studies are reported on neutral and protonated (*S*)-nicotine. These include the calculation of optimised ground-state structures and energies for isolated nicH^+ ,^{21, 28, 33-34} and solution-phase nicH^+ .^{28, 33} Calculations uniformly predict the presence of both protomers of nicH^+ in the gas-phase.^{21, 28, 33-34} Although the electronic transitions of neutral (*S*)-nicotine have also been calculated at high levels of theory,³⁶ an analysis of the gas-phase electronic transitions of nicH^+ is absent from the literature.

The photo-stability and absorption properties of neutral (*S*)-nicotine have been subjected to several studies over several decades, including IR,³⁷ electron-induced dissociation³⁸ and UV absorption spectra of solution phase (*S*)-nicotine in various protonated forms.³⁹⁻⁴¹ To our knowledge, there are no reported UV photodissociation studies of singly protonated (*S*)-nicotine in the literature.

For various ions, relative protomer populations have been obtained by comparing relative ion signal after separation using ion mobility mass spectrometry (IMS).^{2, 7, 9, 11, 42-45} The preponderance of these studies have employed classical drift-IMS (or closely related traveling-wave variants) that separate ions based on different collision-cross sections in a uniform electric field. In contrast, field asymmetric waveform ion mobility spectrometry (FAIMS) differs from drift-IMS in that, while drift-IMS typically separates ions depending on their ion mobility in the direction of ion flow, FAIMS separates species depending on both their high- and low-field ion mobility in fields applied orthogonal to the direction of ion transmission. FAIMS has proven to be a powerful tool for separating and analysing protomer populations at atmospheric pressure prior to detection by mass spectrometry.^{1, 4, 6, 8, 46} Relative-energy calculations are typically coupled with FAIMS analysis to rationalise experimental results. However, it has been shown that theoretical results cannot reliably

predict the relative protomer populations generated by ESI. For example, protomer populations can be shifted by changing ESI conditions¹⁻¹⁴ and less stable isomers can be kinetically trapped by the ESI process.^{4, 6, 9, 47-48} Furthermore, the relative energies of protomers can depend strongly on different levels of theory and basis sets, which can result in the prediction of different lowest energy protomers using different computational approaches.^{14, 49} Here, we report the integration of FAIMS, ion-trap mass spectrometry and UVPD action spectroscopy to detect and assign the protonation isomers of protonated nicotine generated from ESI.

3 Experimental

3.1 Materials

(*S*)-Nicotine sample of ($\geq 99\%$ purity), acetonitrile, methanol and formic acid of LC/MS grade were purchased from Sigma Aldrich and used without further purification. Samples 1 μM (*S*)-nicotine were prepared in either methanol or acetonitrile with 1% (v/v) formic acid.

3.2 Cylindrical FAIMS-Mass Spectrometry

Field asymmetric waveform ion mobility spectrometry (FAIMS) experiments coupled with mass spectrometry were performed using a linear ion-trap mass spectrometer (LTQ XL Thermo Fischer Scientific) with a cylindrical geometry FAIMS attachment (Thermo Fischer Scientific). The FAIMS electrodes are positioned between the ESI source and the inlet transfer capillary of the mass spectrometer. The FAIMS carrier gas was 50 % N_2 and 50 % He at a total flow rate of 3.5 L/min. The dispersion voltage was set to -5000 V. The compensation voltage (CV) was scanned from -30 to -7 V at a step size of 0.3 V. The outer

electrode temperature was 90 °C and the inner electrode temperature was 70 °C. The ESI spray voltage was set to 6 kV. The underlying process of ion separation by FAIMS has been described in detail elsewhere.⁵⁰⁻⁵¹ Briefly, a gas flow carries ions through two electrodes across which an asymmetric voltage alternating between high and low voltage of opposite polarity is applied. Due to their differences in high-field and low-field ion mobility, ions are separated on laterally displaced trajectories. By scanning a DC compensation voltage (CV) across the electrodes, these displacements can be selectively corrected allowing each trajectory, in turn, to be directed towards the inlet to the mass spectrometer for subsequent detection. With the ion-trap mass spectrometer set to detect a mass-to-charge (m/z) population of interest, a CV scan can provide details of isomer populations—protonation isomers are targeted in this study—by resolving subsequent populations in the CV spectrum. It is noted that this FAIMS geometry is designed to enhanced ion transmission efficiency, compared to planar-FAIMS (see below), but with some sacrificed resolution.⁵²⁻⁵⁴

3.3 Planar-FAIMS-Mass Spectrometry

Additional FAIMS experiments were performed on a commercial triple-quadrupole mass spectrometer (SCIEX 5500) equipped with a pair of planar FAIMS electrodes (SCIEX SelexION); herein referred to as planar-FAIMS. Planar FAIMS geometries tend to offer better mobility resolution at the expense of transmission.⁵²⁻⁵⁴ The conditions were as follows: the separation voltage (SV) was set to 4500 V, the compensation voltage (CV) was scanned from -3 to 20 V at a step size of 0.1 V. The DMS temperature was set at 150 °C, DMS offset was set at a value of -3 V, nitrogen was used as a transport gas. IonSpray Voltage was set to 5500 V, the source temperature was 32 °C the curtain gas pressure was set to 20 psi, nebulizing gas pressure of 15 psi, source gas 2 was set to zero, declustering potential was set

to 150 V and nitrogen was used as a collision gas with the collision energy parameter set to 2. Although there is no laser photodissociation capabilities on this platform so only CID activation is reported, planar-FAIMS instruments have higher resolution and peak capacity than cylindrical-FAIMS.

3.4 Photodissociation Action Spectroscopy

The photo-dissociation action spectroscopy equipment and methods used here have been described in detail elsewhere and will only be outlined briefly.⁵⁵ Experiments were performed on a linear quadrupole ion trap mass spectrometer (Thermo Fisher Scientific LTQ XL), that has been modified to permit photodissociation experiments as described by Julian and co-workers.⁵⁶ This instrument is coupled to a tuneable laser system comprising a Nd:YAG (Spectra-Physics QuantaRay INDI) laser generating third harmonic (355 nm) light that pumps a mid-band optical parametric oscillator (OPO) (GWU-Lasertechnik FlexiScan) with a linewidth of $\sim 7\text{ cm}^{-1}$. Second harmonic generation (SHG) of the visible light OPO output allows access to 305-213 nm photons. Laser powers typically ranged from 0.5 mJ to 1.5 mJ per pulse within this region, recorded with a power meter (Gentec UP17P-6SW5).

To obtain a photodissociation action spectrum, m/z -selected ions are subjected to a single laser pulse which is directed through the ion trap via a quartz window mounted on the back of the mass spectrometer vacuum housing. This laser pulse overlaps the trapped ion population with very good efficiency.⁵⁷ After one laser pulse, the ions are scanned out of the ion trap to record a mass spectrum. The photofragment signal is reported as photofragment intensity divided by the total ion count of the mass spectrum. The photofragment signal is recorded

over a range of wavelength values (305-213 nm) at a set step size (1 nm), each step taking approximately ninety PD mass spectra, *i.e.*, 90 laser shots at one per mass spectrum. The process of scanning the laser wavelength and controlling the mass spectrometry is controlled using a custom LABVIEW program (National Instruments) developed in-house. All action spectra were power normalised to the laser power spectrum. For power dependence experiments, a power attenuator (STANDA 10MVAA) was installed in the beam path of a 266 nm set wavelength Nd:YAG laser (Continuum, MINILITE) and the linear response to power at this wavelength is included in the supporting information (Figure S7).

3.5 Computational Details

All calculations were performed with the Gaussian16 program package,⁵⁸ with the exception of CC2 calculations which were performed with the TURBOMOLE (V7.2) package⁵⁹ on the Australian National Computational Infrastructure. Density functional theory (DFT) and time dependant density functional theory (TD-DFT) calculations utilised several methods and the aug-cc-pVDZ basis set.⁶⁰⁻⁶¹ Conformational minima of (*S*)-nicotine were located using M06-2X⁶² by optimising the *cis* and *trans* forms of both protomers then deforming and scanning the pyrrolidine dihedral labelled ν_2 shown in Figure S1. The χ dihedral angle of each of the minima was subsequently scanned and optimised. Conformers found with M06-2X were re-optimised using CAM-B3LYP⁶³ and ω B97X-D⁶⁴ and these optimised structures were used to calculate the vertical electronic transition energies. Further analysis with the CC2 method utilised the cc-pVDZ basis set.⁶⁰⁻⁶¹ The lowest energy conformer for each protomer (as calculated with M06-2X) was re-optimised using MP2.⁶⁵⁻⁶⁹ These geometries were used to calculate vertical excitation energies with the CC2 method⁷⁰ which employ the resolution-of-the-identity approximation.⁷¹

Figure 1: Optimised structures for the lowest energy conformers of the two protomers of nicH⁺. Their relative energies are given in kcal/mol as calculated using M06-2X/aug-cc-pVDZ.

4 Results and Discussion

4.1 Calculations

Optimised structures for the electronic (S_0) ground-state nicH⁺ were first located using the M06-2X method and subsequently optimised using both CAM-B3LYP and ω B97X-D with the aug-cc-pVDZ basis set in all cases. Results indicate that both the pyrrolidine nitrogen and pyridine nitrogen are energetically plausible protonation sites for singly protonated (S)-nicotine (see Table S1 and S2) as the minimum structure and conformation in each case differ by only 0.5 kcal/mol, in favour of pyridine-protonation (Figure 1), which is in accord with the 0.45 kcal/mol calculated by Yoshida *et al.* using MP2/6-31G(d,p).³⁴ Previous theoretical studies using B3LYP calculated proton affinity differences and free energy differences of less than 3 kcal/mol between protomers.^{28, 33}

It is well known that proton affinity is an oversimplified predictor for ESI generated gas-phase protomer populations, and this is well evidenced by reports showing that protomer populations can vary drastically with ESI and source conditions.^{3, 12-14, 25-26} Furthermore, cases are reported that show predictions with different computational methods can scramble the order of proton affinities, thus making it tricky to confidently predict the most stable gas-phase protomer.^{13-14, 49} Therefore, for mass spectrometry, experimental tools that detect and assign protonation isomers are vital for validation of computational methods.

As is discussed later, the computational predictions of two optically-active electronic transitions for PYRO-nicH⁺ and PYRI-nicH⁺ aid in the assignment of the action spectra. Conformational variations do not affect the assignments as the transition energies remain essentially consistent over several conformation scans (see Figure S3). It is therefore sufficient here to consider the global minimum conformation of each protomer in the analysis of photodissociation action spectra.

4.2 Photodissociation and Collision-Induced Dissociation

The PD and CID mass spectra of nicH⁺ (m/z 163), without FAIMS selection, are shown in Figure 2. Several of these product ions have been assigned to be characteristic of a particular protomer based on mechanistic arguments which are summarised below.³⁵ As will be explained in more detail, the characteristic product ions targeted in this study are m/z 84 and m/z 80 for the PYRI-nicH⁺ protomer and m/z 132 and m/z 130 for the PYRO-nicH⁺ protomer. For these product mass spectra in Figure 2, the same product ions are detected for both CID and PD methods, but since PD provides a more uniform distribution of abundance across

these product ions, in particular by enhancing key product ions, only PD product spectra used herein (unless otherwise noted).

Figure 2: (top) The CID and (bottom) PD ($\lambda=266$ nm) product mass spectra of nicH^+ (m/z 163). Spectra are normalised to the total ion count.

The links between individual product ions of nicH^+ to precursor protomers have been explored by previous groups.³⁵ Product ions m/z 84 and m/z 80 have been assigned to the PYRI- nicH^+ precursor ion product ions at and m/z 117, m/z 130 and m/z 132 product ions

have been assigned to the PYRO-nicH⁺ precursor ion. The product ion at m/z 106 has been assigned to the PYRO-nicH⁺ precursor ion, but as will be shown here, it appears to be formed from both protomers. Although some mechanistic explanation does exist, a comprehensive computational analysis would be useful to better explore the mechanism leading to each product ion.

Variations in relative protomer populations will result in changes in the relative populations of characteristic product ions and studies have documented that protomer population can be altered by changing the ESI solvent.^{1, 10-12} In this context, Figure 3A plots PD (266 nm) mass spectra of nicH⁺ acquired from either methanol or acetonitrile as the ESI solvent. In Figure 3B, the difference mass spectrum of the two mass spectra in Figure 3A is shown, in which positive peaks are enhanced in the case of methanol as the ESI solvent and negative peaks are enhanced with acetonitrile. The m/z 84 and m/z 80 product ions—which are the purported characteristic products for the PYRI-nicH⁺ protomer—are favoured in methanol relative to acetonitrile while the opposite is true for the m/z 132 and 130 product ions purportedly characteristic products of PYRO-nicH⁺. Thus, the PYRI-nicH⁺ protomer appears to be significantly enhanced using methanol as an ESI solvent and PYRO-nicH⁺ is enhanced with acetonitrile. There remains a question whether these pairs of characteristic product ions are exclusive to each protomer. To explore this, one would need to isolate pure populations of each protomer within the ion trap, for subsequent activation and analysis, and thus complete separation of these isomers is desired.

Figure 3: (A) The PD (266 nm) spectrum of nicH⁺ with methanol (blue line) and acetonitrile (green line) as the ESI solvent. (B) A difference spectrum (methanol – acetonitrile). (C) Bias calculated for the seven major photoproduct ions as calculated using Eq. (1).

To better quantify the link between product ions and protomers, bias values, as defined by Equation 1 were calculated.

$$\text{BIAS} = \frac{I_{\text{MeOH}} - I_{\text{ACN}}}{I_{\text{MeOH}} + I_{\text{ACN}}} \quad (1)$$

Bias values are used to quantify the proportional change of product ion populations between mass spectra taken under two different sets of conditions, as the difference in fragment intensity between two mass spectra (such as that plotted in Figure 3B) does not clearly communicate the relative magnitude of change in ion population. Bias as defined here by Eq. 1, where I_{ACN} is the signal of a fragment using acetonitrile as an ESI solvent and I_{MeOH} is the signal intensity from the same peak using methanol as the ESI solvent. Mass spectra are normalised to their total product ion count before respective I values are calculated. In this case, Bias values will therefore be 1 if the product ion is only detected with methanol as the ESI solvent and, at the other extreme, will be -1 if the product ion is only detected when acetonitrile is used. If a bias value of a fragment is 0 then the abundance of that ion is the same in both experiments. Bias values for the two ESI solvent spectra are plotted for the seven most abundant product ions in Figure 3C. The product ions with the strongest methanol bias are m/z 84 and m/z 80, which are characteristic of PYRI-nicH⁺. The product ions with the strongest acetonitrile bias are m/z 132, m/z 130 and m/z 117 and are characteristic of PYRO-nicH⁺. Despite the m/z 117 product ion having a low abundance compared to other products (Figure 3A), it has a relatively large acetonitrile bias (-0.2 ± 0.1), albeit with a large uncertainty. In contrast, product ions at m/z 106 and m/z 120 high signal intensity compared to other products yet are rather insensitive to ESI solvent with bias values of -0.0048 ± 0.0003 and 0.15 ± 0.03 , respectively. These findings suggest that this pair of product ions are likely common to both protomers.

4.3 Action Spectroscopy

To date, the assignment of characteristic product ions to respective protomers of nicH^+ emanates from mechanistic arguments in the literature.³⁵ While it is true that there is no major controversy surrounding these assignments, although we have reported above that m/z 106 responds as a common product ion, no direct evidence exists that supports these protomer assignments, thus, a direct measure of spectroscopic signatures would fulfil this requirement. Using UVPD action spectroscopy, the photoproduct yield of the m/z 132 product ion (green line) and the m/z 84 product ion (blue line) are shown in Figure 4. Acetonitrile is used as the ESI solvent in Figure 4A, whereas methanol was used in Figure 4B.

Figure 4: UVPD action spectra of nicH^+ generated by (A) ESI from acetonitrile with 1% formic acid and (B) methanol with 1% formic acid. The spectra follow the signal of the m/z 84 product ion (blue trace) and the m/z 132 product ion (green trace). Vertical bars represent vertical transition energies as calculated with CC2/cc-pVDZ based on geometries optimised with MP2/cc-pVDZ for PYRI- nicH^+ (blue bars) and PYRO- nicH^+ (green bars). Calculated transitions are all offset by -0.35 eV relative to their predicted values. Additional scans over the high energy portion of the spectra (*ca.* 5.5 eV) are appended to both (A) and (B.)

The UVPD trace following formation of the m/z 132 product ion (green trace in Figure 4) shows a strong transition centred at approximately 4.9 eV but there is a tailing-shoulder on the low-energy side of this main peak. This shoulder is distinct from the action spectrum following the m/z 84 fragment (blue trace in Figure 4), which reveals a strong band centred around 4.8 eV but essentially no signal between 4 and 4.4 eV. Also notable is a weak feature centred around 5.5 eV for the m/z 84 product-ion. Additional scans over this feature were performed and are added to both (A) and (B) in Figure 4 (blue circles). These scans confirm that this high-energy band is almost exclusive to the m/z 84 product ion. Two general features, (i) signal—or otherwise—between 4.0-4.4 eV and (ii) the high-energy feature centred at 5.5 eV, appear to discriminate between the two protomers. Comparing the two solvent cases (A and B in Figure 4), the relative abundance of the product ions changes but the general peak shape of the respective fragment ions remains the same.

4.4 Action Spectroscopy Compared to Calculations

Ground state DFT and MP2 as well as excited-state TD-DFT and CC2 calculations were performed to rationalise the differences in the UVPD action spectra of nicH^+ protomers. The CC2 and CAM-B3LYP predictions are listed in Table 1 (PYRO- nicH^+) and Table 2 (PYRI- nicH^+), which also includes the orbital and state analysis for CAM-B3LYP. Other TD-DFT (M06-2X and ω B97X-D) predictions are shown in Figure S4 and included in Tables S3 and S4 and these are generally in accord with the CAM-B3LYP results. Natural transition orbital (NTO) analysis was performed with CAM-B3LYP, the output of which is shown in Figure 5. We predominantly discuss the CC2 and CAM-B3LYP output here.

The vertical electronic excitation energies of PYRO- nicH^+ (green) and PYRI- nicH^+ (blue) calculated by the CC2 method, and shifted by -0.35 eV to align with the main features in the

experimental spectra, are plotted in Figure 4 along with the experimental spectra. The computational predictions were uniformly shifted by -0.35 eV to align with the main features in the experimental data. It was observed by Kulesza *et al.* that CC2 calculations accurately predict vertical transition energies, as compared with experimental PD action spectroscopy of cations, while TD-DFT overestimates these energies by approximately 0.5 eV.⁷² Although we note that it can be somewhat subjective to assign the “vertical transition energy” from an experimental spectrum, there is little doubt that CC2 offers significant improvement on transition energy predictions in many published studies of aromatic ions.^{13, 73} For the CAM-B3LYP method, comparisons are generally good with -0.5 eV shift applied to the predicted values. Many attempts to optimise excited states geometries, to obtain adiabatic (0-0) transition energies, were unsuccessful due to state mixing between the optical bright states and lower-lying dark states. It should be noted that different basis sets were utilised for the CC2 (cc-pVDZ) and TD-DFT (aug-cc-pVDZ) methods and warrants further investigation and comparison.

For the PYRO-nicH⁺ protomer, all computational methods predict a strong transition at around 5.23 - 5.52 eV, corresponding to the S₃←S₀ transition for all methods, and a relatively weaker transition at around 4.46 - 4.95 eV corresponding to the S₁←S₀ transition for the TD-DFT methods and S₂←S₀ in the case of CC2. Together these transitions manifest in the experimental action spectrum following the evolution of *m/z* 132 in the form of a major peak with a shoulder on the red-side as followed in the green trace in Figure 4, both A and B. A very strong transition is predicted at 6.45 eV (6.13 eV for CAM-B3LYP) but is not observed in the experimental spectra and is probably just outside of the experimental window when considering the aforementioned -0.35 eV and -0.5 eV shifts.

Table 1: Calculated vertical transition energies and key orbitals for the (*W*, *trans*, *anti*) PYRO-nicH⁺ using CC2/cc-pVDZ and CAM-B3LYP/aug-cc-pVDZ. H = HOMO and L = LUMO.

PYRO-nicH ⁺ State:	CC2 Transition Energies (eV)	CC2 Oscillator Strength (f)	CAM-B3LYP Transition Energies (eV)	CAM-B3LYP Oscillator Strength (f)	CAM-B3LYP Orbitals	CAM-B3LYP Contribution (%)
S ₁	4.87	0.00001	4.89	0.0036	L← H	60
					L+1← H	4
					L+2← H	30
					L+3← H	2
S ₂	4.94	0.0035	5.02	0.0001	L← H	3
					L+1← H	90
S ₃	5.23	0.021	5.52	0.04	L+1← H-2	26
					L← H-1	48
					L+2← H-1	21
S ₄	6.45	0.97	6.13	0.063	L← H-2	12
					L+2← H-2	5
					L+1← H-1	78
S ₅	7.23	0.70	6.74	0.0115	L← H	30
					L+2← H	47
					L+3← H	6
					L+5← H	7

For PYRI-nicH⁺, a strong transition is predicted around 5.15 - 5.19 eV which corresponds to the S₃←S₀ transition for all methods and this is assigned to the major peak in the *m/z* 84 action spectrum in Figure 4 (both A and B). The higher energy transition centred around 5.5 eV in Figure 4 is assigned to the S₄←S₀ for the CC2 method but assigned as the S₅←S₀ for the TD-DFT methods.

Table 2: Calculated vertical transition energies and key orbitals for (*S*, *trans*, *anti*) PYRI-nicH⁺ using CC2/cc-pVDZ and CAM-B3LYP/aug-cc-pVDZ. H = HOMO and L = LUMO.

PYRI-nicH ⁺ State:	CC2 Transition Energies (eV)	CC2 Oscillator Strength (f)	CAM-B3LYP Transition Energies (eV)	CAM-B3LYP Oscillator Strength (f)	CAM-B3LYP Orbitals	CAM-B3LYP Contribution (%)
S ₁	3.29	0.00006	3.27	0.0005	L ← H	98
S ₂	4.27	0.0006	4.31	0.0014	L+1 ← H	96
S ₃	5.15	0.11	5.19	0.13	L ← H-4 L+1 ← H-4 L ← H-1	4 3 87
S ₄	6.09	0.029	5.81	0.0004	L+2 ← H L+4 ← H	77 15
S ₅	6.62	0.0092	6.04	0.012	L ← H-5 L ← H-4 L ← H-1 L+1 ← H-1	9 40 4 39

Natural transition orbitals (NTO)⁷⁴ are a way of rationalising electronic transitions and thus NTOs calculated with CAM-B3LYP/aug-cc-pVDZ are presented in Figure 5 and can be compared with the results in Tables 1 and 2. Using this method it is evident that both protomers have an S₃←S₀ transition of $\pi^* \leftarrow \pi$ character and these transitions have the highest oscillator strength within this part of the UV spectrum but a major difference is the evidence for charge transfer in the S₃ ← S₀ for the PYRI-nicH⁺ protomer. The feature on the red edge of the PYRO-nicH⁺ action spectrum, assigned to the S₁←S₀ transition (S₂←S₀ for CC2), and has $\pi^* \leftarrow n$ character and by inspection is reminiscent of the $\pi^* \leftarrow n$ (S₁←S₀) for neutral pyridine. This transition is assigned to the shoulder observed *m/z* 132 product action spectra in Figure 4. Pyridine protonation, PYRI-nicH⁺, eliminates this $\pi^* \leftarrow n$ transition and therefore no analogous transition is present in this case.

The high-energy feature of PYRI-nicH⁺ is assigned to the S₅ ← S₀ transition (S₄ ← S₀) for CC2) has mostly $\pi^* \leftarrow \pi$ character associated with the pyridine ring but with some charge-transfer (CT), as shown in Figure 5. The presence of charge-transfer character in the PYRI-nicH⁺ case dramatically increases the oscillator strength relative to all other relevant transitions in this energy range (Table 2).

Figure 5: Calculated natural transition orbitals (NTOs) for the major transitions using CAM-B3LYP/aug-cc-pVDZ. The charging proton is circled in red.

It is plausible that charge-transfer accounts for the red-shift of the S₃ ← S₀ transition as the excited state π^* -system of PYRI-nicH⁺ can be stabilised relative to the ground state π system by increasing the localisation of the electrons around the charged site. Protonation on a heterocyclic nitrogen in a chromophore has been observed to red shift $\pi^* \leftarrow \pi$ transitions for protonated lumichrome²⁴ as well as other types of protonation on aromatic chromophores red shifted transition energies relative to their neutral (unprotonated) forms.^{13-14, 75} Neutral (S)-nicotine has a calculated S₃ ← S₀ vertical transition of $\pi^* \leftarrow \pi$ character at 5.42 eV with CAM-B3LYP/aug-cc-pVDZ³⁶ compared to 5.19 eV predicted of the PYRI-nicH⁺ protomer for the analogous transition and is closer to the S₃ ← S₀ transition of PYRO-nicH⁺ (5.52 eV).

When the pyridine ring is not protonated, as is the case of PYRO-nicH⁺, the chromophore has closer resemblance to that of neutral pyridine and hence the $\pi^* \leftarrow \pi$ transition more closely resembles neutral pyridine and higher in energy than the $\pi^* \leftarrow \pi$ transition of PYRI-nicH⁺.

The action spectra of all photofragment ions are shown in intensity image plots in Figure S5. Characteristic PYRO-nicH⁺ product ions, m/z 130 and m/z 132 are detected across a wider range of photon energies than m/z 80 and m/z 84, which is characteristic of PYRI-nicH⁺. A difference intensity plot comparing the two ESI solvents (acetonitrile subtracted by methanol plot) is shown in Figure S5C. Across all wavelengths, the characteristic product ions of PYRO-nicH⁺ are dominant when acetonitrile is used as the ESI solvent whereas characteristic product ions of PYRI-nicH⁺ dominate when methanol is used as an ESI solvent. The action spectra images reveal that the PYRO-nicH⁺ ion photodissociates across a wider wavelength range (*i.e.*, it has a wider band) than PYRI-nicH⁺. Secondly, the two protomers have characteristic photo-product branching ratios and hence when protomer populations shift in the different solvents, there is a change in relative fragment populations, however one needs to keep in mind the relative absorption and dissociation efficiencies of each protomer at each photon energy.

4.5 FAIMS

Figure 6A shows the FAIMS ‘ionogram’ of nicH^+ (m/z 163) obtained using methanol as the ESI solvent. There is a wide region of signal from -30 V to -18 V of relatively low signal intensity and a relatively high intensity region from -18 V to -8 V. Some separation of the two protomers by FAIMS is achieved by analysing the photodissociation spectrum of nicH^+ and tracking the formation of two protomer characteristic photoproducts as shown in Figure 6B. The blue trace from -18 V to -12 V is the m/z 84 photoproduct ion signal and has a maximum at -15 V. The green trace from -18 V to -8 V is the abundance of the m/z 132 photoproduct ions and has a maximum at -13 V. To probe the action spectra of these FAIMS selected nicH^+ ions to verify the separation of protomers, the changes in relative intensity of product ions at these compensation voltage values are plotted as action spectra profiles in Figures 6C and 6D. These data reveal a clear reversal of product ion abundance ratios between CV values of -16.5 V and -11.5 V, respectively. There is also a difference in the shape of the bands, with the m/z 132+130 action spectrum having a sharp point but a tailing low-energy shoulder. To better compare the band shapes in both cases, the bands are normalised to the same intensity value (Figure S6) revealing similarities between the two bands particularly in the distinctive region between 4.0-4.5 eV. To explain this point, inspection of Figure 6(D) and Figure S6(A) shows that at a FAIMS set voltage of -11.5V, where we expect a significant enhancement of the PYRO- nicH^+ protomer in the ion trap. The product-ion action spectrum of m/z 84 actually begins to show some resemblance of the the PYRO- nicH^+ protomer as is most noticeable in that 4.0-4.5 eV region. This is despite that the m/z 84 photoproduct is assigned to be largely characteristic of the PYRI- nicH^+ protomer (as shown in Figure 4). These results are direct evidence that under photoexcitation, there are

common photodissociation pathways from these two protomers. To quantify this, the absorption and dissociation cross-section values for both protomers are required, across this photon range, however these values are unknown.

Ultimately, the evidence suggests that bias values of 1 or -1 might not be possible for nicH^+ . Looking at the bias values for this FAIMS selection, PD product mass spectra for nicH^+ ions for FAIMS CV values of -11.5 V and -16.5 V are shown in Figure 7 A with bias values calculated and shown in Figure 7 B. The larger bias values for product ions of m/z 80, m/z 84, m/z 130, and m/z 132 achieved by FAIMS illustrates that, in this case, the protomer populations are shifted more dramatically than by changing ESI solvent. However, due to decreased ion signal, the FAIMS bias had more statistical uncertainty than the solvent bias but presumably this could be improved with increased acquisitions. Ultimately, the same characteristic product ions are present as discussed earlier, in accord with the above assignments.

Figure 6: (A) Cylindrical-FAIMS ionogram of m/z 163 nicH^+ . (B) Cylindrical-FAIMS ionogram of photoproducts (PD 266 nm) for m/z 132 (green line) and m/z 84 (blue line). (C) FAIMS-modulated action spectra of photoproducts assigned to PYRO- nicH^+ (m/z 130 + 132 in green) and PYRI- nicH^+ (m/z 80 + 84 in blue) acquired at FAIMS CV voltages of -16.5 V (B) and -11.5 V (D). The square and diamond symbols link the compensation voltage value used for the respective action scans to the position on the FAIMS ionogram.

Figure 7: (A) PD mass spectra of nicH^+ with FAIMS CV voltages -11.5 V (blue) and -16.5 V (green). (B) Bar graph showing the bias values of several photo-products to formation from nicH^+ with a CV voltage of -11.5 V and -16.5 V.

To further explore if complete separation of nicH^+ protomer is possible, experiments on a planar-FAIMS system were performed, however, only CID activation was available on this triple-quadrupole mass spectrometer. As shown in Figure 8, this planar-FAIMS system afforded almost complete separation of protomers by CV. Parking the CV at +4.5 V and +6.5 V and acquiring CID product spectra provided a significantly improved bias analysis, shown

in Figure 9, but characteristic peaks still fall short of +1 and -1. Action spectroscopy would be required here to unequivocally determine if these protomers give rise to exclusive product ions. Do the protomers isomerise before dissociation? Do they have some common product ions? The answers to these questions will depend on the activation method (*i.e.*, laser versus collision) and furthermore depend on the laser wavelength and the CID strategy (ion-trap resonant excitation versus beam-type collisions).

Figure 8: Planar-FAIMS multiple-reaction monitoring scans following the formation of the m/z 84 CID product ions (red) from and m/z 132 (blue) which correspond to fragmentation from different promoters of nicH^+ (m/z 163) comparing acetonitrile (dashed-line) and methanol (solid line) as the solvent.

Figure 9. (A) CID spectra of planar-FAIMS separated nicH^+ protomers in a SCIEX 5500 with SelexION attachment. The green spectrum originates from ions isolated at 4.5 CV and are assigned as pyrrolidine protonated nicH^+ . The inverted blue spectrum originates from ions isolated at 6.5 CV and are assigned as pyridine protonated nicH^+ . (B) Bar graph showing the bias of several CID product ions to formation from nicH^+ with a CV voltage of 4.5 V and 6.5 V.

5 Conclusions

Wavelength-dependent photodissociation and product ion branching ratios, which are observed in the PD mass spectra of nicH^+ , confirm both the presence of two nicH^+ protomers in the gas phase. These protomers have distinguishable photodissociation characteristics and

characteristic—although it appears not exclusive—product ions. Separation of nicH^+ protomers via FAIMS and analysis of separated species with PD mass spectrometry validated the assignments of the two nicH^+ protomers. Although both protomers appear to be formed in both solvents, relative protomer populations were shifted by changing ESI solvent (methanol and acetonitrile), with protonation being favoured on the pyridine nitrogen in methanol and at the on the pyrrolidine nitrogen in acetonitrile. Quantum chemical calculations supported the assignments of the protomers and their PD action spectra. It is anticipated that the integration of ion photodissociation action spectroscopy with the methods of ion mobility and mass spectrometry should be useful in the future for determining the impact of protonation sites on the photochemistry and photodissociation of many other protonation isomers.

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