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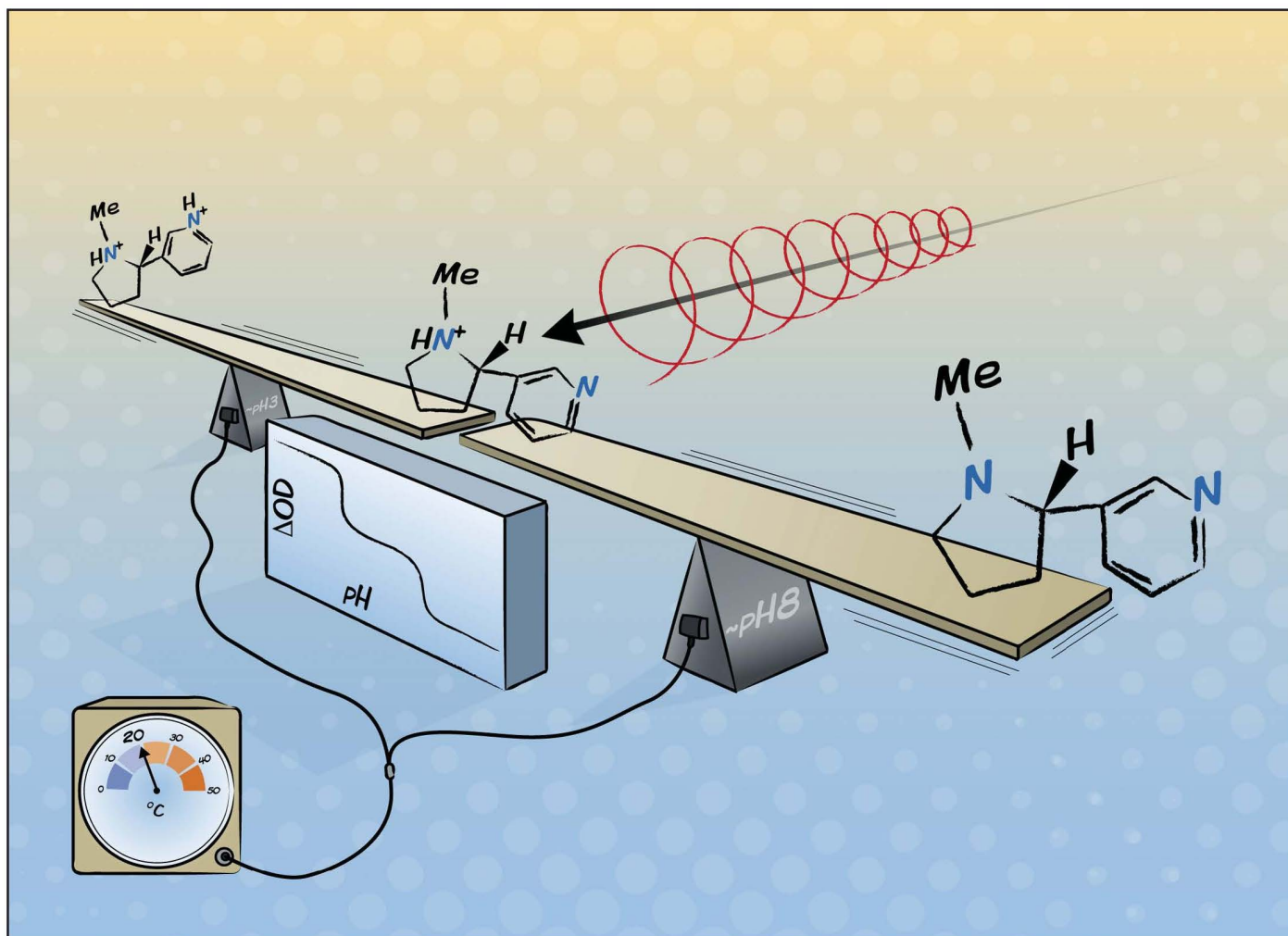
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Showcasing research into the spectroscopic properties of nicotine by Dr Peter Clayton and colleagues from British American Tobacco, Southampton, UK, and Applied Photophysics Limited, Leatherhead, UK.

Spectroscopic investigations into the acid–base properties of nicotine at different temperatures

Electronic circular dichroism spectrometric titration methodology was used to determine the  $pK_a$  values of (S)-(-)-nicotine. We believe this is the first report of the use of chiroptical spectroscopy to characterise the acid–base properties of a small bio-active molecule, and the technique could become the method of choice for defining these attributes in similar molecules.

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## Spectroscopic investigations into the acid–base properties of nicotine at different temperatures

Cite this: *Anal. Methods*, 2013, 5, 81Peter M. Clayton,<sup>\*a</sup> Carl A. Vas,<sup>a</sup> Tam T. T. Bui,<sup>b</sup> Alex F. Drake<sup>b</sup> and Kevin McAdam<sup>a</sup>

Electronic circular dichroism (ECD) spectrometric titration methodology was used to determine the acid ionisation constants ( $pK_a$ ) of (*S*)-(–)-nicotine. Thermostatically controlled instrumentation allowed measurements to be conducted between 20 and 40 °C and the changes in  $pK_a$  with temperature were characterised. The methodology was robust, precise and could be utilised for other chiral chromogenic compounds. We believe this article is the first report of the use of chiroptical spectroscopy to characterise the acid–base properties of a small bio-active molecule, and the technique could become the method of choice for defining these attributes in similar molecules. For (*S*)-(–)-nicotine the two  $pK_a$  values were found to be 2.96 and 8.07 at 20 °C, 2.85 and 7.89 at 25 °C, 2.84 and 7.83 at 30 °C and 2.74 and 7.57 at 40 °C. Using the van't Hoff relationship the  $pK_a$  of the pyrrolidyl ionisation at 37 °C was calculated as 7.65. Changes in the ionisation status of nicotine with temperature are of interest to investigators studying the attributes of nicotine derived from the human use of oral tobacco products. The reversibility of UV and ECD spectra of nicotine with temperature between 10 and 87 °C was demonstrated for both enantiomers. Additionally there was good evidence that at pH 9.5 nicotine underwent conformational change, and the transition mid-point occurred at 49.7 and 49.4 °C for (*S*) and (*R*)-nicotine respectively.

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## Introduction

(*S*)-(–)-Nicotine [CAS 54-11-5], shown in Fig. 1, is the dominant alkaloid present in tobacco and is considerably more biologically potent than the *R* enantiomer [CAS 25162-00-9].<sup>1,2</sup> (*R*)-(+)-Nicotine is almost entirely absent from tobacco<sup>3</sup> and in high temperature in open tubular pyrolysis experiments pure nicotine shows remarkable stereochemical stability.<sup>4</sup> However in tobacco smoke the amount of (*R*)-(+)-nicotine has been quantified at 2–3% of the total amount of nicotine present.<sup>5,6</sup> Nicotine has two ionisable moieties, which are individually centred on the nitrogen atoms of the pyridine and the pyrrolidine heterocyclic rings (Fig. 1); nicotine undergoes acid–base equilibria as shown in Scheme 1.

In aqueous solution the heterocyclic pyrrolidyl moiety is considerably more basic than the aromatic pyridyl group and in the monoprotonated state the positive charge resides on the former moiety.<sup>7,8</sup> The acid ionisation constant for the two ionisable moieties of nicotine in aqueous solution is a fundamental property that will influence its physicochemical and biological properties.

In an acidic aqueous environment nicotine exists predominantly as the di-protonated cation with charge on both of the ionisable moieties. At neutral or mildly acidic pH values, nicotine will be present principally as the mono-protonated cation with a positive charge centred on the pyrrolidine nitrogen atom, while under alkaline conditions nicotine will adopt a non-protonated molecular state. These equilibria are depicted in Scheme 1.

The equilibria may be described by the acid dissociation constant,  $K_a$ , or, because of the many orders of magnitude spanned by  $K_a$  values, more conveniently by the acid ionisation constant, termed the  $pK_a$ . The  $pK_a$  is defined as the negative

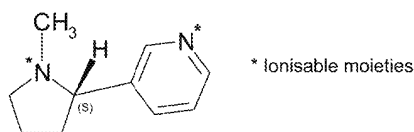
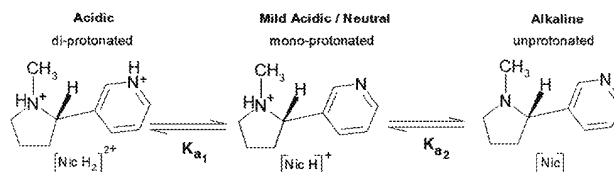


Fig. 1 Molecular structure of (*S*)-nicotine with its ionisable moieties.

<sup>a</sup>British American Tobacco, Group R&D, Regents Park Road, Southampton, SO15 8TL, UK. E-mail: peter\_clayton@bat.com

<sup>b</sup>Applied Photophysics Limited, 21 Mole Business Park, Leatherhead, KT22 7AG, UK



Scheme 1 (*S*)-Nicotine in aqueous solution.

logarithm of the acid dissociation constant, depicted in Eqn (1) and (2) for nicotine.

$$\text{p}K_{\text{a}1} = -\log_{10}K_{\text{a}1} \quad (1)$$

$$\text{p}K_{\text{a}2} = -\log_{10}K_{\text{a}2} \quad (2)$$

Eqn (1) and (2). Acid ionisation constants of nicotine with reference to Scheme 1.

Under ideal conditions, the determination of  $\text{p}K_{\text{a}}$  values for nicotine allows calculation of the proportion of neutral and cationic species present at any particular pH (and temperature and low ionic strength) as governed by the Henderson–Hasselbalch equation, Eqn (3) and (4). However in practise there can be complex relationships between the acid ionisation constant, temperature and ionic strength.

$$\text{pH} = \text{p}K_{\text{a}1} + \log[\text{NicH}]^+ / [\text{NicH}_2]^{2+} \quad (3)$$

$$\text{pH} = \text{p}K_{\text{a}2} + \log[\text{Nic}] / [\text{NicH}]^+ \quad (4)$$

Eqn (3) and (4). Henderson–Hasselbalch equation with reference to Scheme 1.

The measurement of the  $\text{p}K_{\text{a}}$  values of nicotine has been the subject of a number of investigations and the subject was examined by Jackson<sup>9</sup> and more recently by Banyasz.<sup>10</sup> Jackson evaluated that, with the use of potentiometric methodology,  $\text{p}K_{\text{a}1} = 3.22$  and  $\text{p}K_{\text{a}2} = 8.11$  (20 °C, ionic strength not defined).<sup>9</sup> In 1980, also using potentiometric titration, Gonzalez *et al.*<sup>12</sup> measured the acid ionisation constant of nicotine at a range of temperatures and ionic strengths;  $\text{p}K_{\text{a}2}$  was determined as 8.06 (20 °C, no supporting electrolyte). Finally Seeman *et al.*<sup>13</sup> in 1999 based calculations on the relative amounts of unprotonated and monoprotonated nicotine species on values of  $\text{p}K_{\text{a}1}$  and  $\text{p}K_{\text{a}2}$  equal to 3.1 and 8.0 respectively.

Latterly, spectroscopic methodologies have been employed to determine the  $\text{p}K_{\text{a}}$  values of acids and bases<sup>14,15</sup> and the approach offers some distinction advantages. The titration was carried out in unbuffered, low concentration solution so the effect of ionic strength on the measurement was minimal. The low concentration, in the micromolar range, means that the activity coefficient approaches unity. At high ionic strength – *i.e.* where there are significant concentrations of buffer or analyte ions, the  $\text{p}K_{\text{a}}$  value will be affected as predicted by the Debye–Hückel relationship. The titration is not affected by interference from dissolved carbon dioxide, and the technique requires only small amounts of sample, typically less than 500 µg. However, the method is only suitable for pure chromogenic compounds and requires the reliable measurement of pH. The spectroscopic methodology completed here utilised chiroptical spectroscopy in the UV region; the potential of chiroptical techniques was cited in a recent publication which reported on the Raman spectra of tobacco alkaloids<sup>8</sup> and included an examination of Raman optical activity of (*S*)-nicotine. To our best understanding this is the first utilisation of chiroptical spectroscopic methodology as a probe into the acid–base properties of a pharmacologically active molecule, although the technique was previously highlighted in an educational context.<sup>16</sup>

Understanding the properties of nicotine is of great interest not only from a fundamental standpoint, but establishing the behaviour of nicotine within the context of tobacco products. Methods for measuring the amounts and proportions of the different forms of nicotine in tobacco has been highlighted as an area of interest from a public health perspective.<sup>17</sup> Prior to the analysis of individual compounds in complex mixtures such as tobacco or cigarette smoke, it is important to thoroughly establish the properties of the individual compound in isolation and characterise the capability of the analytical method for the analyte of interest. In his comprehensive review published 1999 Banyasz<sup>10</sup> stated ‘though the value of  $\text{p}K_{\text{a}2}$  may be stated with some confidence, systematic re-determination of ionisation constants with proper controls would be highly desirable.’ This article is a contribution towards that end – a spectroscopic methodology based on circular dichroism spectroscopy is utilised in the determination of the two acid ionisation constants of nicotine across a range of temperatures between 20 and 40 °C.

## Experimental

### Chemicals

(*S*)-(–)-Nicotine free base, ERM®-AC802b, was purchased from LGC standards (Teddington, UK), declared total purity ( $\text{m m}^{-1}$ ): 99.65% (gravimetrically determined).

ANALYSIS OF (*S*)-NICOTINE SOLUTION IN-HOUSE. The enantiomeric purity of (*S*)-(–)-nicotine was determined by GC-FID using a dimethylated cyclodextrin GC column,<sup>11</sup> the kind gift of Prof. V. Schurig, University of Tübingen; the details of the chromatographic method will be published elsewhere. A small amount of hexane solvent was added to a stock aqueous solution ( $0.25 \text{ mg mL}^{-1}$ ) of (*S*)-nicotine used in ECD measurements followed by the addition of sodium carbonate. The enantiomeric purity of (*S*)-nicotine in the hexane layer was approximately 99.0%, and the chemical purity was approximately 99.6%.

(*R*)-(+)-Nicotine was purchased from Toronto Research Chemical (Toronto, Canada), declared chemical purity: 97% (NMR), quoted specific rotation: ( $+117^\circ \text{ dm}^{-1} \text{ cm}^3 \text{ g}^{-1}$ , 589 nm).

### Materials and instrumentation

Simultaneous ECD and UV absorption spectra were measured on a Chirascan-plus spectrometer (Applied Photophysics Ltd, Leatherhead, UK). The spectrometer was equipped with a peltier temperature controller for precise temperature control of the sample cell. The peltier device was based around a TC125 unit (Quantum North West Inc., WA, USA) and featured an in-cell temperature probe. Outside the spectrometer, the pH of solutions were measured using a Corning pH 105 pH meter equipped with a ThermoRussel K series electrode (Russell Mainstream Supply Ltd, Fife, UK). The solution was maintained at the defined temperature using a water bath with a digital thermometer. The temperature of the solution was also measured directly to ensure it attained the temperature of the water bath.

A single rectangular strain-free fused silica far UV cell, path length of 10 mm, was used for all measurements. ECD and UV absorption spectra were recorded at each determined pH between 300 and 200 nm (0.5 nm wavelength step size, 1.0 s time-per-point, and the spectral bandwidth was 1 nm). All spectra were baseline corrected with respect to a water spectrum measured at 20 °C.

### Procedure

An aqueous solution of (*S*)-(-)-nicotine was prepared using deionised water (18.2 MΩ cm) to give a solution concentration of 30.6 μg mL<sup>-1</sup> (189 μM). The pH value was 8.68 at 20 °C, and the ionic strength of the initial solution was estimated to be about 1 × 10<sup>-4</sup> mol dm<sup>-3</sup>, based on 50% ionisation of nicotine at pH 8. Approximately 50 mL of solution was prepared, 6 mL was aliquotted into small glass vials. These solutions were stored at ambient temperature for less than 6 hours and then subsequently at 4 °C until required. No changes in the UV or ECD spectra of these solutions were observed during the 5 days it took to complete these measurements.

The initial pH titration of (*S*)-(-)-nicotine was determined at 20 °C. In a small glass vial 6 mL of (*S*)-(-)-nicotine solution was thermostated at 20 °C. Small aliquots (~2 μL) of aqueous NaOH solutions (between 0.1 M and 10 M) were added; when the pH had changed significantly and stabilised the pH was recorded and an aliquot (~1.8 mL) of the solution was placed in the silica cell using a glass Pasteur pipette. The UV cell was positioned inside the Chirascan spectrometer where it was re-equilibrated to the same measurement temperature with the aid of the TC125 unit. UV absorbance and ECD spectra were simultaneously obtained following which the aliquot was returned to the glass vial where, after the addition of further alkali, the measurement cycle was repeated. In this way a series of 3 spectra of increasing alkalinity were obtained at pH values from 9.05 to 10.15. Subsequently small volumes of aqueous HCl (between 0.1 M and 10 M) were added to this solution and a series of 31 spectra of increasing acidity were obtained between pH 8.48 and 2.08.

Further titrations were conducted at 25, 30 and 40 °C each time using a fresh vial of 30.6 μg mL<sup>-1</sup> (*S*)-(-)-nicotine solution. Each temperature series was completed on consecutive days. Thermostating the solution at 25 °C 31 spectra were obtained between pH 9.85 and 1.79, at 30 °C 26 spectra were obtained between pH 10.13 and 1.95, and at 40 °C 30 spectra were obtained between pH 9.80 and 1.94.

The combined amount of acid and alkaline added to a sample aliquot were less than 2% of the initial volume, therefore volume changes were disregarded.

The Levenberg–Marquardt curve fitting algorithm was used to construct the titration curve.

## Results and discussion

Fig. 2 shows the UV and ECD spectra of (*S*)-(-)-nicotine between 200 and 300 nm at 20, 25, 30 and 40 °C as a function of pH.

A universal isosbestic point at 239 nm encompassing all pHs is a feature of the UV absorption spectra, and two partial isosbestic points featuring only the di-protonated and mono-

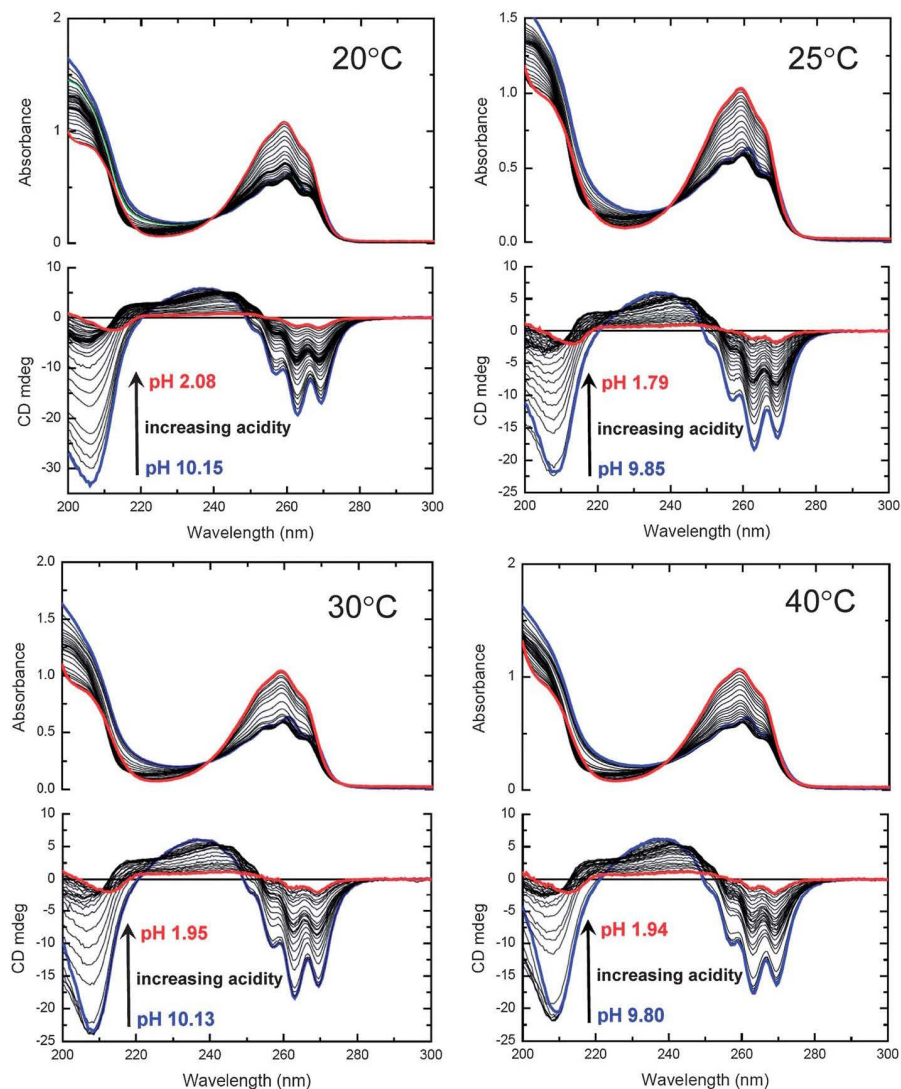
protonated species are seen at 219 and 211 nm. No universal isodichroic wavelength is observable for nicotine but partial ones are seen at 211 nm, incorporating the di-protonated and mono-protonated species, and at 226 and 242 nm, incorporating the mono-protonated and non-protonated species. These isosbestic and isodichroic wavelengths are independent of temperature between 20 and 40 °C. The occurrence of isosbestic and isodichroic points suggests an equilibrium between two species, either between di-protonated and mono-protonated, or mono-protonated and non-protonated nicotine. The universal isosbestic point at 239 nm is observed because: (1) between pH 2 and 6 di-protonated and mono-protonated species are in equilibrium, and, (2) between pH 6 and 10 the absorbance spectrum of nicotine remains unaltered.

The negative ECD band (and the absorbance) between 250 and 280 nm arises from the  $\pi$ - $\pi_1^*$  transition of the pyridyl group, and it exhibits vibronic structure resulting from simultaneous changes involving both electronic and vibrational energies of the aromatic moiety.<sup>18</sup> This ECD transition is perturbed by the ionisation state of the two ionisable moieties in nicotine and so can be used to determine the values of both p*K*<sub>a1</sub> and p*K*<sub>a2</sub>. Changes in UV spectra of the pyridyl  $\pi$ - $\pi_1^*$  transition between pH 6.23 and pH 10.15 (resulting from the ionisation of the pyrrolidyl group) however are minimal, hence UV absorption is not a good spectrometric probe for monitoring changes associated with the ionisation of the pyrrolidyl moiety. This phenomenon is illustrated in Fig. 2 by the observation that in the UV absorbance spectra, the most alkaline (blue line) spectra is overlaid by other alkaline spectra. The ECD signal at 263 nm on the other hand is sensitive to changes in both ionisable moieties; here spectra around both p*K*<sub>a</sub> values show systematic progression. Titration curves were constructed using the ECD spectra (Fig. 2) with 263 nm as the monitoring wavelength at 20, 25, 30 and 40 °C and are shown in Fig. 3.

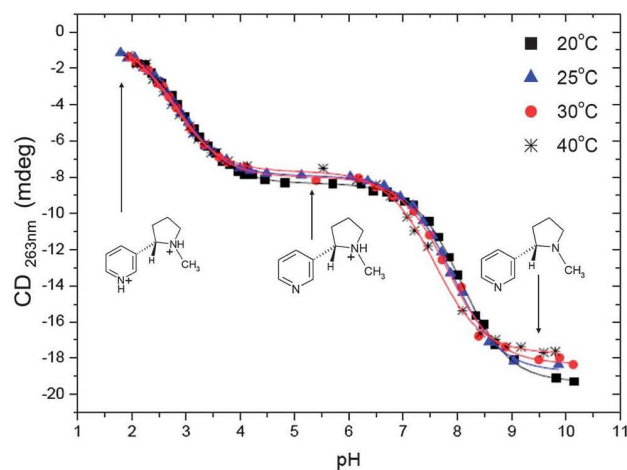
The Levenberg–Marquardt method for nonlinear least squares curve-fitting was employed on the data points of the ECD titration curve to obtain an appropriate regression (goodness of fit). The inflection points of the fitted line afforded the two p*K*<sub>a</sub> values at 20, 25, 30 and 40 °C detailed on Fig. 3 and the analytical precision of the measurements made at individual temperatures are also shown. The p*K*<sub>a</sub> values derived from these titration curves are shown tabulated in Table 1.

As described above the monitoring wavelength used in the titration curve was standardised on 263 nm which allowed the calculation of both p*K*<sub>a1</sub> and p*K*<sub>a2</sub>. Another possibility is to use the ECD band at 206 nm, a transition which is thought to be associated with the pyrrolidine lone pair electrons.<sup>18</sup> However, the wavelength of 206 nm was precluded as the monitoring wavelength for p*K*<sub>a1</sub> because although the ECD at this wavelength is overwhelmingly susceptible to the pyrrolidyl ionisation it is insensitive to the pyridyl ionisation, see Fig. 4. This wavelength was used in the calculation of p*K*<sub>a2</sub> (only at 20 °C) as a check to compare with the value calculated at 263 nm and is shown in Table 2.

There was good agreement between the two calculated values for p*K*<sub>a2</sub>; 263 nm was preferred for the calculated data reported in this publication for the reasons given above and, in



**Fig. 2** UV and ECD spectra of (S)-nicotine at different acidities at 20, 25, 30 and 40 °C, 200–300 nm, pathlength 10 mm.



**Fig. 3** ECD titration curve of (S)-nicotine monitored at 263 nm.

**Table 1**  $pK_a$  values determined from ECD titrations at 20, 25, 35 and 40 °C

Temperature (°C)	$pK_{a1}$	$pK_{a2}$
20	$2.96 \pm 0.02$	$8.07 \pm 0.01$
25	$2.85 \pm 0.02$	$7.89 \pm 0.01$
30	$2.84 \pm 0.05$	$7.83 \pm 0.03$
40	$2.74 \pm 0.03$	$7.57 \pm 0.02$

general, higher wavelength measurements are preferred in the UV region.

#### van't Hoff graphs

van't Hoff relationships are shown in Fig. 5 for  $pK_{a1}$  and  $pK_{a2}$  by plotting  $pK_a$  values against reciprocal absolute temperature. The relationships appear linear over the temperature range examined.

Using the equations derived from the van't Hoff graphs, Fig. 5 it is possible to calculate the  $pK_a$  values at different

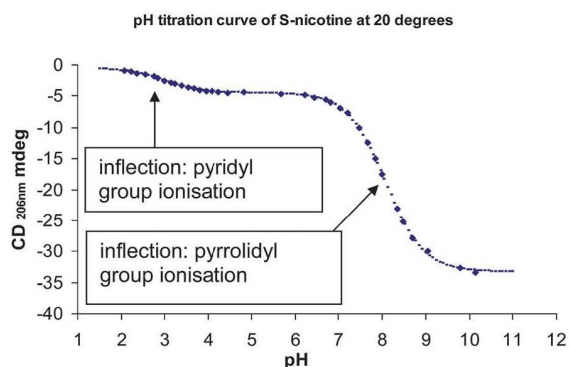


Fig. 4 ECD titration curve of (S)-nicotine at 20 °C monitored at 206 nm.

Table 2  $pK_{a2}$  values determined from ECD titrations at 206 and 263 nm

$pK_{a2}$ 20 °C, 206 nm	$pK_{a2}$ 20 °C, 263 nm
8.08	8.07 (from Table 1)

temperatures. The calculated values of  $pK_{a1}$  and  $pK_{a2}$  at 37 °C are shown in Table 3. The value of the ionisation constant of the pyrrolidyl group of nicotine is biologically significant at this temperature.

Additionally the standard enthalpy ( $\Delta H^0$ ) and entropy ( $\Delta S^0$ ) changes may be estimated from the gradient and y-intercepts of the van't Hoff graphs respectively shown in Fig. 5.

#### van't Hoff graph: di- and mono-protonated nicotine species, Fig. 5 (left)

$$\Delta H^0 = -17\,300 \text{ J mol}^{-1}$$

$$\Delta S^0 = -2.92 \text{ J mol}^{-1} \text{ K}^{-1}$$

The standard energy of protonation of pyridine, measured calorimetrically, is reported<sup>19</sup> as  $-20\,800 \text{ J mol}^{-1}$  which is consistent with the value estimated from this work. In the same publication the change in standard entropy is stated as  $+29.3 \text{ J mol}^{-1} \text{ K}^{-1}$ . This differs somewhat from the value estimated in this work, however van't Hoff graphs have large inherent errors in determination of intercepts and they are not a preferred method of finding  $\Delta S^0$ .

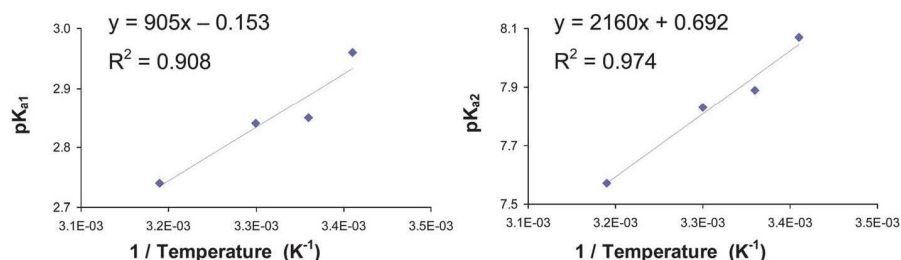


Fig. 5 van't Hoff graph relating to  $pK_{a1}$  (left) and  $pK_{a2}$  (right) ionisation.

Table 3  $pK_{a1}$  and  $pK_{a2}$  values of (S)-nicotine at 37 °C using equations in Fig. 5

$pK_{a1}$ 37 °C	$pK_{a2}$ 37 °C
2.77	7.65

#### van't Hoff graph – mono and non-protonated nicotine species, Fig. 5 (right)

$$\Delta H^0 = -41\,300 \text{ J mol}^{-1}$$

$$\Delta S^0 = +13.2 \text{ J mol}^{-1} \text{ K}^{-1}$$

The enthalpy of protonation for the pyrrolidine ring ( $pK_{a2}$ ) is substantially greater than that for the pyridine ring ( $pK_{a1}$ ). This is reflected in the van't Hoff graph where the gradient of  $pK_{a2}$  graph is steeper than the  $pK_{a1}$  graph. The reason for this divergence may be in part explained by steric hindrance at the pyrrolidine ring resulting from the presence of pyridine ring which will have the effect of shielding pyrrolidine lone pair electrons from the bulk solution.<sup>10</sup> At a higher temperature the molecule becomes more conformationally mobile, increasing the exposure of the pyrrolidine ring nitrogen atom to the bulk solution, and as a result  $pK_{a2}$  shows enhanced temperature dependence. The pyridine nitrogen atom is significantly less sterically hindered and has greater exposure to the bulk solution, hence  $pK_{a1}$  shows reduced temperature dependence compared to  $pK_{a2}$ .

#### Racemisation and reversibility of UV and ECD spectra of nicotine with temperature

Racemisation of nicotine would undermine the integrity of the experiment; however the molecule shows remarkable stereochemical stability<sup>4</sup> as regards to temperature. Another consideration is whether nicotine undergoes chemical decomposition across the temperature range studied which would result in changes in the ECD spectrum unrelated to acid–base equilibria. In order to verify that (S)-(–)-nicotine does not undergo decomposition; a thermal stability experiment was conducted. A solution of  $0.15 \text{ mg mL}^{-1}$  of (S)-(–)-nicotine was prepared in 5 mM sodium borate, pH 9.5. In order to minimise possible interference from trapped air bubbles, the solution was degassed by sonication for 1 minute.

The UV absorbance and ECD spectra of (*S*)-(-)-nicotine (pathlength 2 mm) were first recorded at 20 °C. Using the TC125 Peltier unit, spectra were then acquired at 10 °C, following which the sample was heated to 87 °C, cooled to 10 °C and then heated again to 20°. The temperature of the sample was measured directly with a thermocouple probe in the solution; UV and ECD spectra of (*S*)-(-)-nicotine at various temperatures are shown in Fig. 6. All UV and ECD spectra were water/buffer baseline corrected. As can be seen the nicotine spectra are unchanged following the heating-cooling cycle; hence, the effects of heat on (*S*)-(-)-nicotine are entirely reversible, suggesting the molecule is chemically unaltered.

### Temperature induced conformational change

A further consideration is whether nicotine undergoes temperature-induced conformational change independent of pH. Such a change would imply that variations in the ECD signal arise from not only acid-base equilibria, but temperature-induced conformational mobility might be a confounding outcome in the determination of the  $pK_a$  values. Fig. 6 shows that between 20 and 87 °C (*S*)-nicotine does undergo conformational change. A parallel measurement was conducted on (*R*)-nicotine which produced the same outcome except the signs of the Cotton effects were reversed, data not shown. The issue is at what temperature do changes in the conformation of nicotine commence. During the heating-cooling experiment, described above, simultaneous UV and ECD spectra [for both (*S*) and (*R*)-nicotine] were collected during the heating process

(10 → 87 °C) as described above. The same parameters were set for the cooling process (87 → 10 °C). The UV and ECD spectra were monitored between the extremes of temperature (2 °C step size). The ECD temperature profile and the three dimension plots (of ECD *versus* temperature *versus* wavelength) for (*S*)-nicotine is shown in Fig. 6.

From the temperature profile graphs of ECD at pH 9.5, shown in Fig. 6, (*S*)-nicotine remains conformationally stable up to around 40 °C, after which it exhibits change up to a temperature of about 60 °C, above which the ECD signal is again stable. Studies on the conformations that nicotine adopts in aqueous environments and the gas phase have been published.<sup>20,21</sup> The inflection point of the ECD temperature dependent change, calculated using the Levenberg–Marquardt method, was  $49.7 \pm 0.2$  °C and  $49.4 \pm 0.2$  °C for (*S*) and (*R*)-nicotine respectively. Conceptually this measurement for the two enantiomers should be identical; the slight divergence may be accounted for by differences in the impurity profiles of the isomers, or possibly by accumulated experimental errors.

The question arises, as to the nature of the conformational change that nicotine undergoes when heated from ambient to 90 °C. Nicotine is a fairly rigid molecule and the only available degree of freedom in the molecular structure is rotation about the C3–C2' axial carbon–carbon linking the two rings. This was confirmed recently by Baranska *et al.*<sup>8</sup> in their Raman spectroscopy study of nicotine; their results suggest that the energy difference between two *trans* conformations is  $\sim 2$  kJ mol<sup>-1</sup>, and that the conformers are interconverted by rotation about the C–C bond. (The calculations of Elmore and Dougherty<sup>20</sup> predict

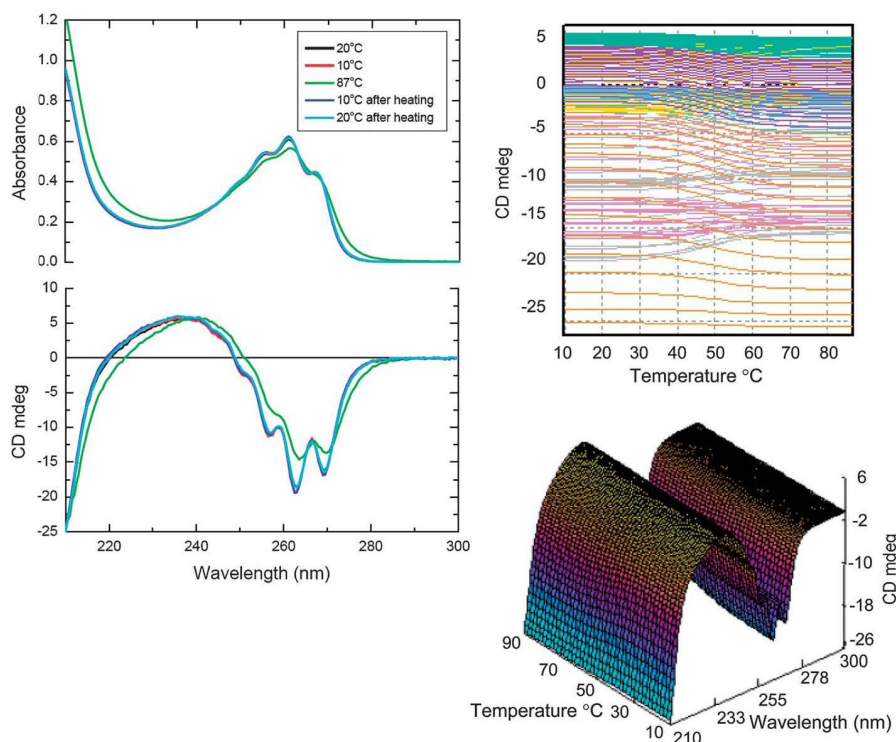


Fig. 6 UV and ECD of 0.15 mg mL<sup>-1</sup> (*S*)-nicotine in 5 mM sodium borate buffer (pH 9.5), 10–87 °C, 2 mm pathlength – see details above.

that the *N*-methyl *trans* species are the most stable conformers for all protonation states in gas and aqueous phases; they calculate that no more than 6% should be in a *cis* conformation even at physiological temperatures, although the energy difference between *trans* and *cis* conformations is reduced in aqueous solution). There is scope for *ab initio* calculations to indicate the lowest energy conformers adopted by nicotine, the results of these calculation this will feature in a subsequent publication.

## Conclusion

Spectroscopic methodology, in particular circular dichroism augmented with UV absorption, is a technique which may be used to characterise the acid–base properties of small molecules as illustrated in this article by the example of the bio-active compound (*S*)-nicotine. The two necessary conditions, in addition to chemical purity, are that the subject molecules need to be chiral and chromogenic. Enantiomeric purity is not an essential requirement albeit the amplitude of the Cotton effects will be progressively lessened as the enantiomeric purity declines. No doubt the methodology described here could find more general applicability in the determination of the acid–base properties of a number of chiral bio-active molecules, it is worth commenting that various bio-active molecules have a *pK<sub>a</sub>* close to physiological pH and therefore will exist in two ionised forms in biological solution. In these situations it is important to recognise that physiological temperature is supra-ambient and the proportions of the ionised entities will be affected by the increased temperature. It is important to take into consideration the elevated temperature prevalent *in vivo* systems compared to the temperature in the laboratory.

The two ionisation constants (*pK<sub>a</sub>*) of (*S*)-(–)-nicotine at 20, 25, 30 and 40 °C were spectrometrically determined using ECD methodology under thermostated control. The ECD of the  $\pi$ – $\pi^*$  transition associated with the band centred on 263 nm was sensitive to both ionisable moieties present in the nicotine molecule, unlike the UV absorption spectra. Measurements were performed in dilute, unbuffered solution and need not be corrected for ionic strength and the ingress of carbon dioxide. Ionisation constants at different temperatures are presented in Table 1 and these values are comparable with values reported in the scientific literature: *pK<sub>a1</sub>* and *pK<sub>a2</sub>* (20 °C) values were 2.96 and 8.07 compared to 3.22 and 8.11 reported previously using potentiometric method.<sup>10</sup> Using the van't Hoff relationship the ionisation constants at 37 °C were calculated as 7.65 and 2.77 for the pyrrolidyl (*pK<sub>a2</sub>*) and pyridyl (*pK<sub>a1</sub>*) moieties respectively.

The reversibility of UV and ECD spectra of nicotine with temperature was also demonstrated for both (*R*) and (*S*)-nicotine and the two enantiomers exhibited equal but opposite ECD spectra. Additionally there was good evidence from ECD spectra that, at pH 9.5, nicotine underwent a significant change in conformation. The mid-point temperature of the transition between the two conformers was determined as 49.7 °C and 49.4 °C for (*S*) and (*R*)-nicotine respectively. However between 20 and 40 °C the conformation as monitored by ECD across many wavelengths showed little variation.

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