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Determination of ionization constants of heterocyclic aromatic amines using capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) is a very convenient technique for the determination of ionization constants. The technique is rapid, precise, uses small quantities of solute, and the exact concentration of the compound is not needed. This work represents the first report on the ionization constants of eight heterocyclic aromatic amines: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, 2-amino-9H-pyrido[2,3-*b*]indole, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate, 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline, 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline and 9-H-pyrido[4,3-*b*]indole. The ionization constants determined using CZE were confirmed with independent determinations by ultraviolet spectrometry.
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1. Introduction

Heterocyclic aromatic amines (HAAs) are a class of mutagenic/carcinogenic substances found in cooked meat and fish. They have also been found in environmental samples such as airborne particles, rain water, soil and cigarette-smoke-polluted indoor air [1]. All of the known HAAs test positive in the Ames/*Salmonella* microsome-mediated mutagenicity test [2]. Many of them are known to induce tumors in liver, lung, breast, small and large intestines and other sites [3]. Kataoka [4] has published a review on the methods for the determination of mutagenic heterocyclic amines and their applications in environmental analysis. Several papers have appeared recently on the analysis of HAAs using liquid chromatography–mass spectrometry [5–8].

Knowledge of ionization constants is important in the prediction of migration order of solutes in capillary zone electrophoresis (CZE) [9] and in the understanding of certain chemical phenomena such as biological uptake, biological activity, biological transport [10], and the binding of these molecules to environmental matrices. Recently, CZE has been used to determine ionization constants [11–18]. Close agreement between ionization constants determined by CZE and literature values has been observed, usually within 0.07 units or less. CZE has the distinct advantage in that very small sample sizes can be used. In this work, ionization constants of eight HAAs have been determined using CZE, and these values were confirmed with ultraviolet (UV)

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spectroscopy. To the best of our knowledge, this is the first time ionization constants of these compounds have been reported.

2. Theoretical

2.1. Single protonation model for CZE

$$B + H^+ \leftrightharpoons BH^+ \quad (1)$$

When a base, B, is protonated, its net electrophoretic mobility (μ_e) is given by [11,17]

$$\mu_e = \alpha_{BH^+}\mu_{BH^+} \quad (2)$$

where α_{BH+} is the degree of dissociation of BH^+, and μ_{BH+} is the electrophoretic mobility of the fully protonated species. The thermodynamic dissociation constant (pK_a^T) has been discussed by others [14,18,19]. Non-linear regression analysis of an equation derived from Eq. (2), with α_{BH+} as a function of activity corrected $[H^+]$ and $K_{BH+,}$ gave the thermodynamic pK_a^T.

2.2. Double protonation model for CZE

$$B + H^+ \leftrightharpoons BH^+ \quad (3)$$

$$BH^+ + H^+ \leftrightharpoons BH_2^{2+} \quad (4)$$

The effective electrophoretic mobility, μ_d, of a diacidic base is given by [15,16,18],

$$\mu_d = \alpha_{BH^+}\mu_{BH^+} + \alpha_{BH_2^{2+}}\mu_{BH_2^{2+}} \quad (5)$$

where α is the degree of dissociation of the respective species, μ_{BH+} is the electrophoretic mobility of BH^+, and $\mu_{BH_2^{2+}}$ is the electrophoretic mobility of BH_2^{2+}. The terms for the first and second dissociation constants for a diacidic base are K_{a1} and K_{a2}. It can be shown that μ_d is a function of the terms in Eq. (6) [15,18].

$$\mu_d = \frac{K_{a2}[H^+]\mu_{BH^+} + [H^+]^2\mu_{BH_2^{2+}}}{K_{a1}K_{a2} + K_{a2}[H^+] + [H^+]^2} \quad (6)$$

Non-linear regression analysis of Eq. (6) using activity corrected $[H^+]$, yielded pK_{a1}^T and pK_{a2}^T

2.3. Single protonation model for spectroscopy

Non-linear regression analysis was performed using the following equation [19]:

$$pK_a = pH + \log\frac{A - A_M}{A_I - A} \quad (7)$$

where A is the observed absorbance, A_M is the absorbance of the molecular species and A_I is the absorbance of the ionized species. The activity correction was made in the same way as outlined above for the single protonation model with CZE.

2.4. Double protonation model for spectroscopy

Non-linear regression was carried out using the following equation [19]:

$$A = A_N(K_{a1}K_{a2}/G) + A_M(K_{a2}[H^+]/G) + A_D([H^+]^2/G) \quad (8)$$

where A is the observed absorbance, A_N is the absorbance of the neutral species, A_M is the absorbance of the monoprotonated species, A_D is the absorbance of the diprotonated species, and G is defined as

$$G = K_{a1}K_{a2} + K_{a2}[H^+] + [H^+]^2 \quad (9)$$

The parameters K_{a1} and K_{a2} were converted to pK_{a1}^T and pK_{a2}^T using the same activity correction terms outlined above for the double protonation model for CZE.

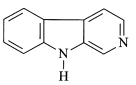
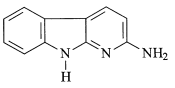
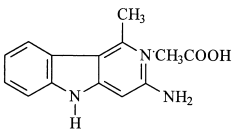
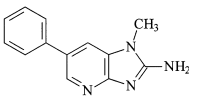
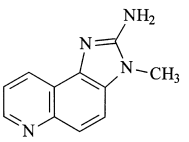
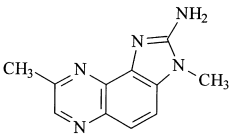
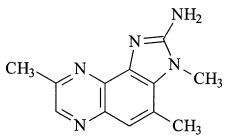
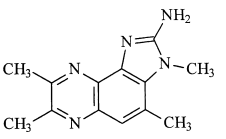
3. Experimental

3.1. Reagents

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate (Trp-P-2 acetate), 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*] quinoxaline (4,8-DiMeIQx), and 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*] quinoxaline (4,7,8-TriMeIQx) were pur-

chased from Toronto Research Chemicals (Ontario, Canada). 9-H-Pyrido[4,3-*b*]indole (norharman), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), tricine, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), (2-acetamido)-2-aminoethanesulfonic acid (ACES), 2-(*N*-morpholino)ethanesulfonic acid (MES), and formic acid were purchased from Aldrich (Milwaukee, WI, USA). Table 1 gives the structures of the compounds. Glacial acetic acid was purchased from Mallinckrodt (Phillipsburg, NJ, USA). Sodium hydroxide and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade water, HPLC-grade methanol, and citric acid were

Table 1
Structures of the eight HAAs

Compound	Structure	Abbreviation
9H-Pyrido[4,3-*b*]indole		Norharman
2-Amino-9H-pyrido[2,3-*b*]indole		AαC
3-Amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate		Trp-P-2 acetate
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine		PhIP
2-Amino-3-methyl-3H-imidazo[4,5-*f*]quinoline		IQ
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline		MeIQx
2-Amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline		4,8-DiMeIQx
2-Amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline		4,7,8-TriMeIQx

purchased from Fisher Scientific (Fair Lawn, NJ, USA).

3.2. Instrumentation

All separations were performed on a Beckman P/ACE 5000 Series Capillary Electrophoresis System with System Gold version 8.1 software (Beckman Instruments, Fullerton, CA, USA). The CZE separations were all done in the conventional operating mode with the cathode at the detector end of the capillary. An uncoated fused-silica CE column [57 cm (50 cm to detector)×75 μm I.D.×375 μm O.D.] obtained from Beckman Instruments was used. The temperature of the capillary was maintained at a fixed level by means of a liquid coolant in the capillary cartridge. All injections were done in the hydrodynamic mode and the solutes were monitored using UV detection at 214 nm. At the beginning of each day and whenever necessary the capillary was rinsed with 0.1 *M* NaOH for 30 min, water for 30 min, and running electrolyte for 30 min. Between runs the capillary was washed with 0.1 *M* NaOH for 2 min and equilibrated by flushing with the running electrolyte for 5 min.

All spectroscopy data were acquired on a Perkin-Elmer Lambda 9 UV–Vis–near IR spectrometer. GraphPad Prism version 2.01 software was used to perform non-linear regression analysis on the data.

3.3. Sample and buffer preparation and determination of pK_a values

Methanol stock solutions of 20 μg/ml of all eight HAAs were prepared and used for further dilutions as necessary. Methanol was used as a neutral marker to measure electroosmotic flow. The buffers listed in Table 2 were prepared fresh each day, and the pH was adjusted with 0.1 *M* NaOH or 0.1 *M* HCl. The ionic strengths of the buffers ranged from 0.005 to 0.02. For the acetic acid buffer the appropriate amount of 0.1 *M* NaOH was added to achieve the pH of the buffer. With the sodium formate buffer, the proper amount of 0.1 *M* HCl was added to obtain the pH of the buffer. Buffer pH was measured using an Orion Research Digital Ionalyzer/501 pH meter (Orion Research, Cambridge, MA, USA). The buffers were first filtered through a 0.2-μm filter and sonicated for 15 min to degas the buffers. The buffer series shown in Table 2 was used.

Table 2
Buffer series used for the CZE determination of pK_a values

Buffer	pH	pK_a	Concentration (m*M*)
TAPS	9.03	8.51	24
Tricine	8.58	8.26	23
Tricine	8.23	8.26	25
HEPES	7.82	7.66	21
HEPES	7.38	7.66	23
HEPES	7.03	7.66	25
ACES	6.78	6.91	24
ACES	6.50	6.91	25
MES	5.99	6.21	25
MES	5.56	6.21	30
Acetic acid[a]	4.97	4.76	11
Acetic acid	4.49	4.76	13
Sodium formate[b]	3.99	3.75	15
Sodium formate	3.57	3.75	14
Sodium formate	2.99	3.75	12

[a] A 0.1 *M* NaOH solution was added to acetic acid to obtain the pH of the buffer.
[b] A 0.1 *M* HCl solution was added to sodium formate to obtain the pH of the buffer.

A sample solution containing 4 μg/ml of a HAA and 20% methanol was injected for 3 s. Methanol acted as a neutral marker. For the separation, 20 kV was applied; the temperature was maintained at 25°C; and detection was at 214 nm. Individual samples of HAAs were run in triplicate and the average migration times were used in the calculation of electrophoretic mobilities. The electrophoretic mobilities were calculated using the following formula:

$$\mu_e = \frac{L_d L_t}{V} \cdot \left[\frac{1}{t_m} - \frac{1}{t_0} \right] \quad (10)$$

where μ_e is the electrophoretic mobility of the solute ($cm^2/V\ s$), L_d is the length of the capillary to the detector (50 cm), L_t is the total length of capillary (57 cm), V is the applied voltage (20 kV), t_m is the migration time of the analyte in s, and t_0 is the migration time of the neutral marker in s.

Samples of 1.8 μg/ml of a given HAA were used for each spectroscopic determination. The experimental procedure used was the one outlined in

Ref. [19], and non-linear regression analysis was performed on the data using either Eq. (7) or Eq. (8).

4. Results and Discussion

4.1. CZE determinations of pK_a

Electrophoretic mobilities were plotted against activity corrected pH of the buffer, and non-linear regression analysis was then performed on the data. Activity corrected pH values were employed because the buffers had different ionic strengths. The activity corrected pH values were calculated as described in Ref. [15]. Fig. 1 shows the best fits of the non-linear regression for PhIP, norharman, and TriMeIQx. DiMeIQx, MeIQx and AαC had similar plots with one break in the curve (data not shown). Similar plots for IQ and Trp-P-2 acetate gave two breaks, indicating two pK_a values for these compounds. Their plots are shown in Fig. 2.

4.2. Ultraviolet spectroscopic determination of pK_a values

The results of the non-linear regression analysis are shown in Fig. 3 for MeIQx. The other compounds that gave only one pK_a had similar shapes. The results for IQ are shown in Fig. 4. The shape of the curve is somewhat unusual for a titration curve but this is a result of the fact that the absorption spectra of the fully protonated and the neutral form of IQ are somewhat similar in the region of the analytical wavelength chosen (260 nm). The absorption spectrum of the monoprotonated form is, however, different from that of the fully protonated and the neutral forms. Titration curves similar to this shape have been reported before for *m*-aminobenzoic acid [20].

The spectroscopy data for Trp-P-2 acetate indicated only one pK_a, contrary to what the CZE data showed (Fig. 2). From Fig. 2, it is clear that the second break in the titration curve (lower pH) for

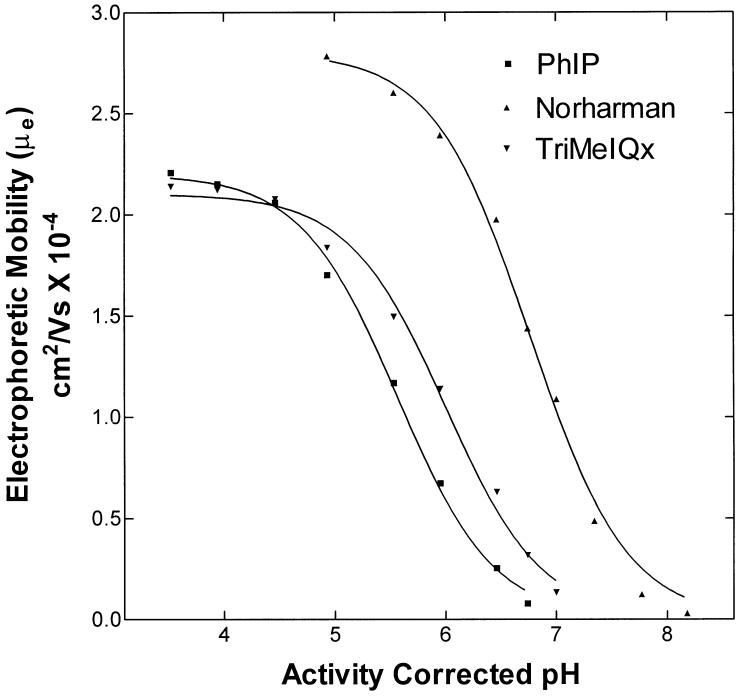


Fig. 1. Electrophoretic mobilities as a function of activity corrected pH for PhIP, norharman and TriMeIQx with best fits from non-linear regression analysis. Voltage: 20 kV, temperature: 25°C, and UV detection at 214 nm.

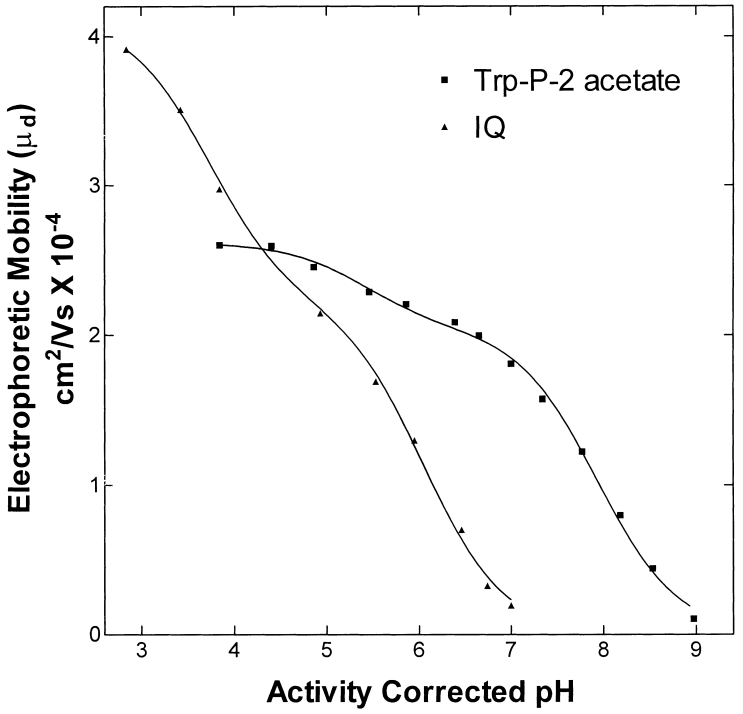


Fig. 2. Electrophoretic mobilities as a function of activity corrected pH for Trp-P-2 acetate and IQ with best fits from non-linear regression analysis. Voltage: 20 kV, temperature: 25°C, and UV detection at 214 nm.

Trp-P-2 acetate is weak. This suggests that the second pK_a for Trp-P-2 acetate obtained by CZE is, in fact, not real. A possible reason why Trp-P-2 acetate showed two breaks with the CZE data and only one break with spectroscopy is that the acetate salt was used, which could affect the mobility of the Trp-P-2 molecule and hence affect the shape of the μ_d vs. pH plot (Fig. 2). With spectroscopy, the acetate ion does not affect the absorbance of the Trp-P-2 molecule so only one break was seen.

Table 3 lists the pK_a values of the eight HAAs obtained with CZE and UV spectroscopy.

4.3. Correlation of structure with pK_a

Table 1 shows the structures of the eight HAAs. For several of the compounds, it is easy to see where protonation takes place. For norharman, the most basic nitrogen is the aromatic nitrogen in the six-membered ring. For Trp-P-2 acetate the fact that the acetate salt was used would not affect the pK_a to a large extent and the most basic site would be the aromatic ring nitrogen. For PhIP, the nitrogen in the 3 position of the imidazole ring would be the most basic because of the electron donating character of the amino substituent in the 2 position. Amino groups are known to increase the basic strength of the ring nitrogen atom [21,22]. IQ, which shows two pK_a values, would first protonate at the ring nitrogen atom in the 3 position of the imidazole ring, for the same reason given for PhIP. The second protonation would occur at the nitrogen in the quinoline ring. MeIQx, 4,8-DiMeIQx, and 4,7,8-TriMeIQx would also have the nitrogen atom in the 3 position of the imidazole ring as their most basic center. The reason why they, unlike IQ, show only one pK_a is that they have a quinoxaline system which has two nitrogen atoms in the same ring. The addition of a second ring

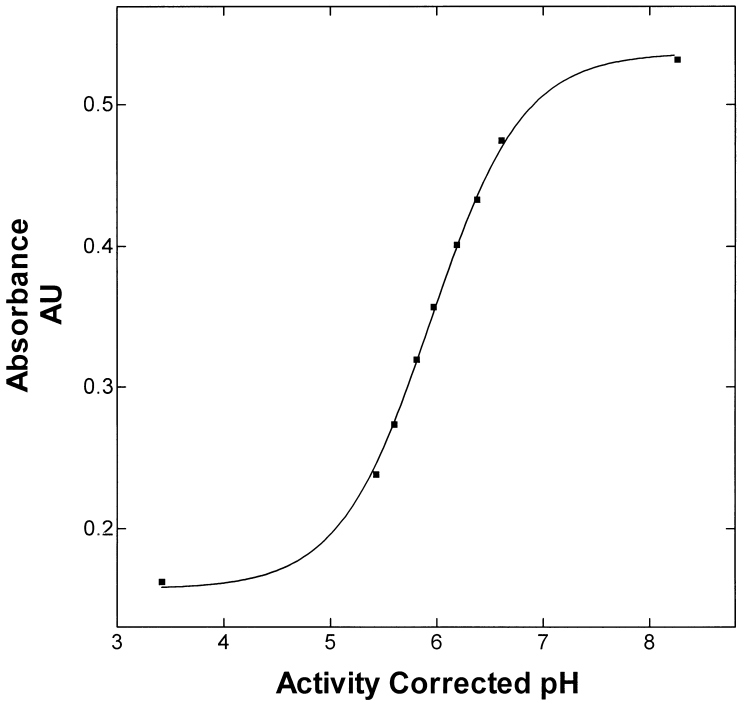


Fig. 3. Absorbance at 271 nm as a function of activity corrected pH for MeIQx with results of the non-linear regression analysis. Buffer: 0.01 *M* MES.

nitrogen atom is known to lower basic strength, because a doubly bound nitrogen atom (=N–) has the same base weakening effect as a nitro group [23]. Therefore, the second pK_a for these three compounds would be too weak to be determined under the conditions used in this study.

5. Conclusions

This is the first reported study on the determination of ionization constants of a very important class of heterocyclic aromatic amines. The pK_a values were obtained using two independent techniques. These pK_a values could help in explaining CZE migration characteristics of these compounds and aid in predicting migration order of similar compounds [24].

This study also demonstrates the usefulness of CZE in determining ionization constants. CZE determinations use very small volumes, typically nl amounts of sample, compared to much larger amounts used in potentiometry and in spectroscopy where a fresh amount of sample has to be used at each pH value. With CZE, since only the migration time is needed, the exact concentration of the compound need not be known. Automation of CZE instruments makes the technique very precise and accurate with the largest source of error lying in the measurement of buffer pH. The determination of ionization constants by CZE are rapid, and since the instrument is automated, the actual time spent by the analyst is even less than with UV spectroscopy.

Acknowledgements

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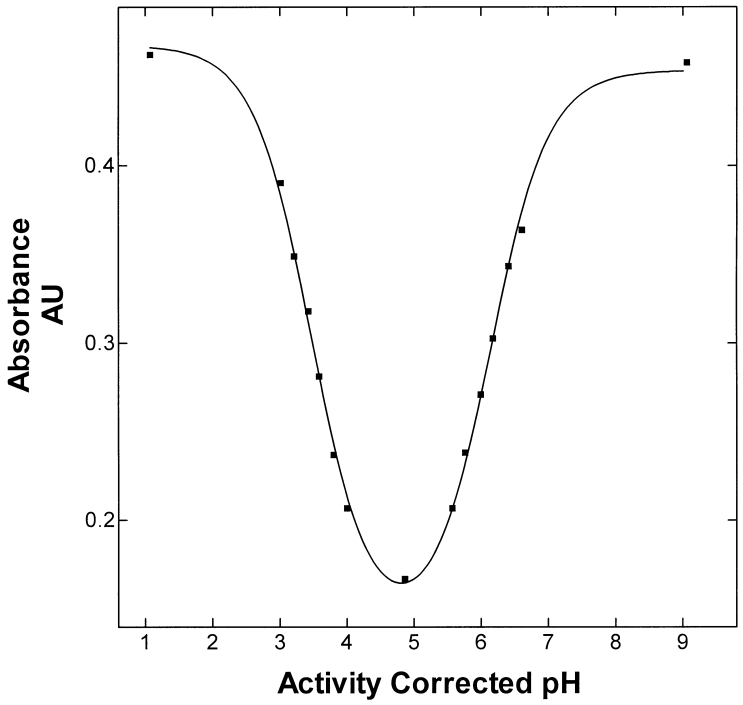

Fig. 4. Absorbance at 260 nm as a function of activity corrected pH for IQ with results of the non-linear regression analysis. Buffers: 0.01 *M* MES and 0.01 *M* formic acid.

Table 3
pK_a values of the eight HAAs obtained with CZE and UV spectroscopy

Compound	CZE		Spectroscopy	
	pK_a	95% CI[a]	pK_a	95% CI[a]
AαC	4.40	0.12	4.43	0.08
PhIP	5.56	0.07	5.65	0.13
MeIQx	5.95	0.09	5.94	0.05
DiMeIQx	5.86	0.12	5.85	0.13
TriMeIQx	6.00	0.10	6.02	0.13
Norharman	6.76	0.09	6.81	0.09
Trp-P-2 acetate	7.94 and 5.42	0.08 and 0.38	7.87	0.17
IQ	6.06 and 3.72	0.12 and 0.24	6.14 and 3.47	0.08 and 0.07

[a] 95% confidence interval.

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