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Determination of the Dissociation Constants of Urocanic Acid Isomers in Aqueous Solutions

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Summary. (*E*)- and (*Z*)-Urocanic acids are endogenous chemicals in the normal mammalian skin. The first and the second thermodynamic dissociation constants (pK_{a1} and pK_{a2}) of urocanic acid isomers were determined using UV spectrophotometry in aqueous solutions. The values with standard deviation ($pK_{a1} = 3.43 \pm 0.12$ and $pK_{a2} = 5.80 \pm 0.04$) and ($pK_{a1} = 2.7 \pm 0.3$ and $pK_{a2} = 6.65 \pm 0.04$) were obtained to (*E*)- and (*Z*)-urocanic acids, respectively. The second dissociations were studied also by potentiometric titration in aqueous sodium chloride solutions up to the isotonic salt concentration ($0.154\,\mathrm{mol\,dm^{-3}}$), and the second thermodynamic dissociation constants as well as activity parameters for both isomers were determined at temperature 25°C and for (*E*)-urocanic acid also at 37°C. The obtained pK_{a2} values were close to those found by UV spectrophotometry. The equations for the calculation of the second stoichiometric dissociation constants of urocanic acid isomers on molality and molarity scale in aqueous sodium chloride solutions were derived. The obtained pK_{a1} and pK_{a2} values for (*Z*)-urocanic acid appear to be essentially lower than some previously reported values in literature.

Keywords. Acidity; Natural products; Potentiometric titration; Protonation; UV-Vis spectroscopy.

Introduction

The acidic *pH* of the skin surface is highly significant in skin barrier homeostasis and natural defence against pathogenic micro-organisms. Alteration of the skin surface *pH* related to skin diseases has been known since the 1950s. In atopic dermatitis the normal skin *pH* is elevated both on affected and uninvolved skin sites [1–3]. Dryness, itching, and eczematous skin symptoms correlate positively with skin *pH*, as reviewed by *Rippke et al.* [4].

One of the essential constituents participating in the *pH* homeostasis of the healthy mammalian skin is urocanic acid. The (*E*)-urocanic acid ((*E*)-3-(1*H*-imidazol-4-yl)prop-2-enoic acid, **1**), synthesized enzymatically from the amino acid histidine, is photoisomerised to (*Z*)-urocanic acid ((*Z*)-3-(1*H*-imidazol-4-yl)prop-2-enoic acid, **2**) upon UV irradiation. It has been well documented, both *in vitro* and *in vivo*, that **2** is able to produce immunosuppression [5].

The dissociation constants especially for **2** have not been studied much. The first and second thermodynamic dissociation constants (pK_{a1} and pK_{a2}) for **1** and **2** have been determined by *Roberts et al.* [6] from combined data at 25 and 40°C by ^{15}N NMR method. *Mehler* and *Tabor* [7] have determined the dissociation constants of **1** using UV spectrophotometry and *Halle et al.* [8] have used ^{1}H NMR measurements in their determinations for **1**. In addition *Zimmerman et al.* [9] have determined pK_{a2} values of **1** and **2** and *Cloninger* and *Frey* [10] pK_{a2} value of **1** in their study.

We determined the first and second dissociation constants for (*E*)- and (*Z*)-urocanic acid by using both UV spectrophotometry and potentiometric

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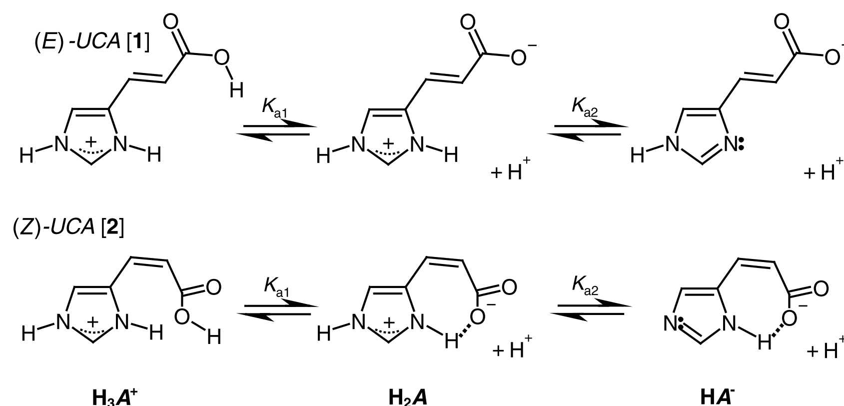

Fig. 1. The dissociation of urocanic acid isomers

titration, because at least for **2** these values were not well known. Because the urocanic acid isomers have two dissociation groups in the near-biological *pH* range, interference of the carboxylate and imidazolyl groups, which lie about 3 *pH* units apart, is inevitable when determining their dissociation constants. In the first stage, the carboxylic acid proton is delivered and a zwitter-ion (or neutral species $= H_2A$) is formed. In the second stage, the proton from the imidazolyl ring is delivered and an anion ($= HA^-$) is formed. The two dissociation processes are presented in Fig. 1 for the isomers.

The determination of the dissociation constants by spectrophotometry is possible if the substance of interest absorbs in the UV to visible wavelength region and when its absorbance characteristics differs between the molecular (electroneutral) and ionised forms. *Albert* and *Serjeant* [11] have presented the technique which was used here to determine the first and second thermodynamic dissociation constants for **1** and **2**.

The second dissociation constants of urocanic acid isomers were here also studied by potentiometric titration. This method has recently been successively used to determine the stoichiometric (*e.g.*, molality or molarity scale) and thermodynamic dissociation constants for some carboxylic and amino acids at 25°C, see, *e.g.*, Ref. [12]. The (*E*)-urocanic acid titrations were made also at 37°C (near human body temperature). The stoichiometric dissociation constant is dependent on the temperature and the composition of the solution, and this dependence is shown for the second stoichiometric dissociation constant of urocanic acid isomers.

Results and Discussions

UV Spectrophotometry

The absorbances of the urocanic acid solutions were measured at the 230–340 nm range. Pure (*E*)-urocanic acid (**1**) species H_3A^+ and HA^- were found to dominate at about *pH* 1 and *pH* 7–8, respectively, showing little change in absorbance in response to small *pH* changes. The species H_2A of **1** was located at *pH* 4.4. The greatest arithmetic difference in the spectra of H_3A^+ or HA^- species and H_2A species were calculated to be at 281.8 and 286.6 nm, respectively (Fig. 2), the chosen analytical wavelengths.

The thermodynamic dissociation constants (pK_{a1} and pK_{a2}) were calculated from the measured absorbances of the 50 μM **1** solutions as a function of *pH* at the analytical wavelengths. An approximate pK_{a1} or pK_{a2} were calculated from Eq. (1):

$$pK_a = pH + \lg \frac{A_I - A}{A - A_M} \qquad (1)$$

where A is the absorbance of the partly ionised form at the measured *pH*, A_I of the ionised form H_3A^+ (for pK_{a1}) or HA^- (for pK_{a2}), and A_M of the monoprotonated (zwitterionic or neutral) form H_2A. An antilogarithm (10^{pK_a}) was taken from those values, and the final values of pK_{a1} and pK_{a2} were calculated from the averaged antilogarithms. The detailed results are shown in Tables 1 and 2. For **1**, the pK_{a1} and the standard deviation is 3.43 ± 0.12, and respectively the pK_{a2} and the standard deviation is 5.80 ± 0.04.

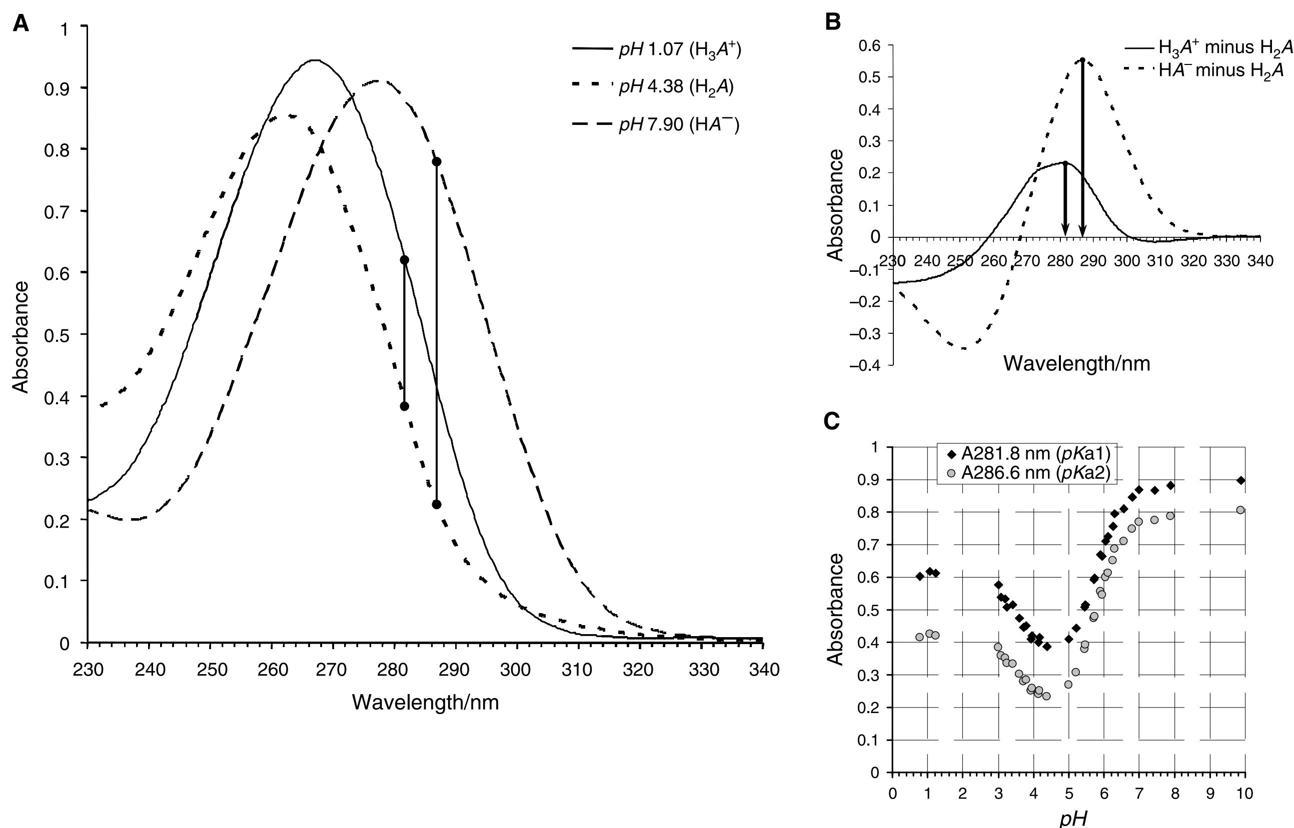

Fig. 2. Determination of the first and second dissociation constants for (*E*)-urocanic acid **1**. Graph A: absorption spectra of 50 μM **1** at *pH* representing the H_3A^+, H_2A, and HA^- species. The greatest difference between the H_3A^+ or HA^- species and the H_2A species is shown by vertical lines. Graph B: subtraction of the curves yields two curves with maximum differences at 281.8 and 286.6 nm. These wavelengths were chosen as the analytical wavelengths. Graph C: measured absorbances of 50 μM **1** as a function of *pH* at the analytical wavelengths. The pK_{a1} and pK_{a2} were calculated from the absorbances according to Eq. (1) and are shown in Tables 1 and 2

Table 1. Calculation of the first thermodynamic dissociation constant of (*E*)-urocanic acid from UV-spectrophotometric data

pH	A(281.8 nm)	$\lg\frac{A_I - A}{A - A_M}$	pK_{a1} [a]	$10^{pK_{a1}}$	pK_{a1}
1.07	0.61798 (= A_I)				
3.00	0.57577 (= A)	0.65263	3.65263	4494.0	
3.08	0.53876 (= A)	0.28495	3.36495	2317.1	
3.20	0.53351 (= A)	0.24188	3.44188	2766.2	
3.25	0.50861 (= A)	0.04934	3.29934	1992.3	
3.41	0.51438 (= A)	0.09287	3.50287	3183.2	
3.61	0.47476 (= A)	−0.20818	3.40182	2522.4	
3.72	0.44577 (= A)	−0.46016	3.25984	1819.0	
3.78	0.45256 (= A)	−0.39590	3.38410	2421.6	
4.38	0.38608 (= A_M)				
		Mean		2689.5	3.43 [b]
		Standard deviation			0.12

[a] Calculated from Eq. (1)
[b] Calculated from the mean of antilogarithms ($10^{pK_{a1}}$)

Table 2. Calculation of the second thermodynamic dissociation constant of (*E*)-urocanic acid from UV-spectrophotometric data

pH	A(286.6 nm)	$\lg\frac{A_I - A}{A - A_M}$	pK_{a2} [a]	$10^{pK_{a2}}$	pK_{a2}
4.38	0.23342 (= A_M)				
5.47	0.39162 (= A)	0.39782	5.86782	737597.1	
5.72	0.4735 (= A)	0.11590	5.83590	685323.2	
5.91	0.55527 (= A)	−0.14265	5.76735	585258.2	
6.11	0.61364 (= A)	−0.34106	5.76894	587406.8	
6.26	0.65237 (= A)	−0.49299	5.76701	584806.1	
6.56	0.71017 (= A)	−0.79270	5.76730	585189.7	
7.90	0.78701 (= A_I)				
		Mean		627596.9	5.80 [b]
		Standard deviation			0.04

[a] Calculated from Eq. (1)
[b] Calculated from the mean of antilogarithms ($10^{pK_{a2}}$)

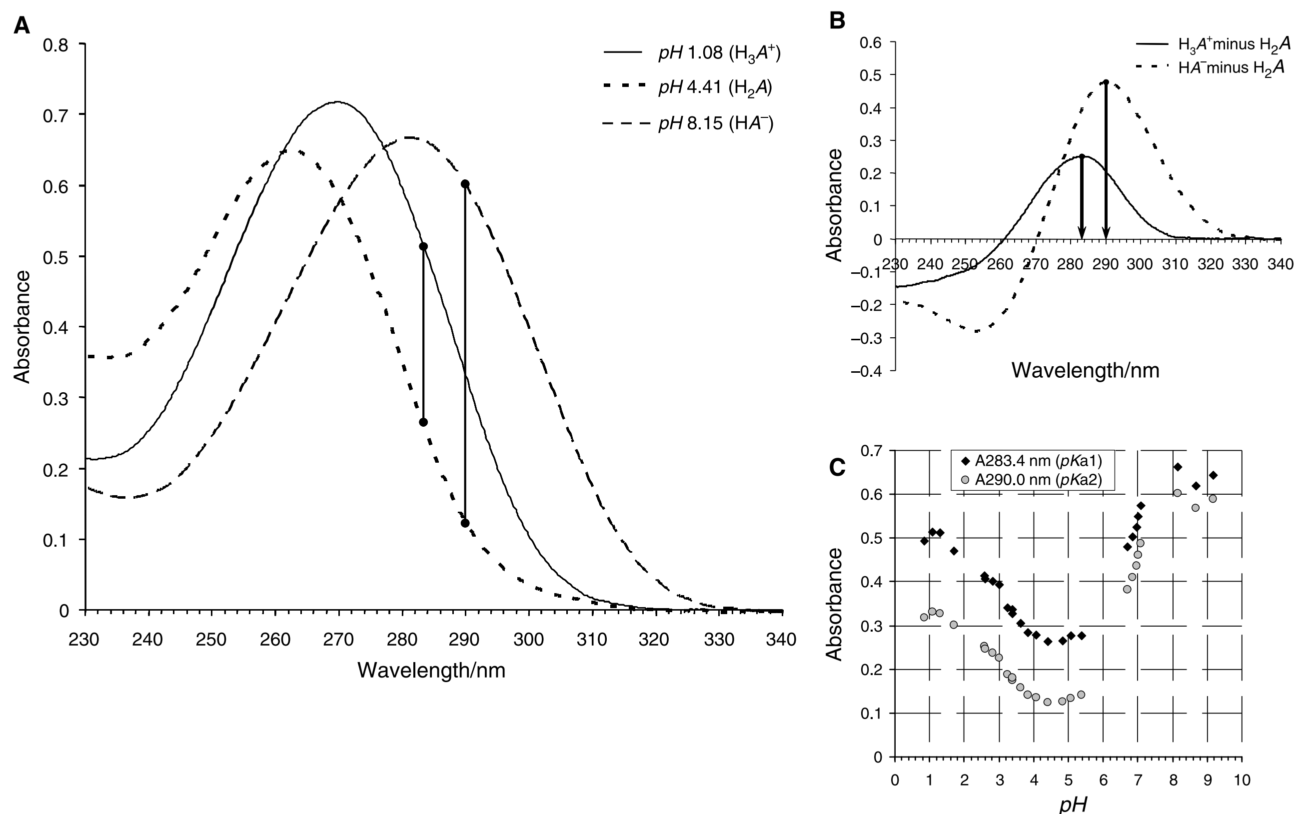

Fig. 3. Determination of the first and second dissociation constants for (*Z*)-urocanic acid **2**. Graph A: absorption spectra of 50 μM **2** at *pH* representing the H_3A^+, H_2A, and HA^- species. The greatest difference between the H_3A^+ or HA^- species and H_2A species form is shown by vertical lines. Graph B: subtraction of the curves yields two curves with maximum differences at 283.4 and 290 nm. These wavelengths were chosen as the analytical wavelengths. Graph C: measured absorbances of 50 μM **2** as a function of *pH* at the analytical wavelengths. The pK_{a1} and pK_{a2} were calculated from the absorbances according to Eq. (1) and are shown in Tables 3 and 4

The analyses with (*Z*)-urocanic acid (**2**) were conducted in an analogous manner to those with **1**. The pure H_3A^+ and HA^- species of **2** were found at around *pH* 1.1–1.3 and *pH* 8.1–9.2, respectively, whereas H_2A species was dominating at *pH* 4.4–4.8. The corresponding analytical wavelengths were located at 283.4 and 290 nm (Fig. 3).

Table 3. Calculation of the first thermodynamic dissociation constant of (*Z*)-urocanic acid from UV-spectrophotometric data

pH	*A*(283.4 nm)	$\lg\frac{A_I - A}{A - A_M}$	pK_{a1} [a]	$10^{pK_{a1}}$	pK_{a1}
1.08	0.51443 (= A_I)				
2.58	0.41353 (= *A*)	−0.17497	2.40503	254.114	
2.61	0.40651 (= *A*)	−0.12508	2.48492	305.436	
2.82	0.39998 (= *A*)	−0.07940	2.74060	550.297	
3.02	0.39412 (= *A*)	−0.03879	2.98121	957.659	
4.41	0.26257 (= A_M)				
		Mean		516.877	2.71[b]
		Standard deviation			0.27

[a] Calculated from Eq. (1)
[b] Calculated from the mean of antilogarithms ($10^{pK_{a1}}$)

Table 4. Calculation of the second thermodynamic dissociation constant of (*Z*)-urocanic acid from UV-spectrophotometric data

pH	*A*(290.0 nm)	$\lg\frac{A_I - A}{A - A_M}$	pK_{a2} [a]	$10^{pK_{a2}}$	pK_{a2}
4.41	0.12472 (= A_M)				
6.71	0.38239 (= *A*)	−0.06989	6.64011	4366298	
6.86	0.40937 (= *A*)	−0.17013	6.68987	4896337	
6.96	0.43682 (= *A*)	−0.27697	6.68303	4819836	
7.02	0.46138 (= *A*)	−0.37989	6.64011	4366301	
7.08	0.48679 (= *A*)	−0.49821	6.58179	3817614	
8.15	0.60176 (= A_I)				
		Mean		4453277	6.65[b]
		Standard deviation			0.04

[a] Calculated from Eq. (1)
[b] Calculated from the mean of antilogarithms ($10^{pK_{a2}}$)

The pK_{a1} and pK_{a2} values and their standard deviations calculated for **2** are 2.7 ± 0.3 and 6.65 ± 0.04, respectively. The detailed results are shown in Tables 3 and 4.

Thermodynamic dissociation constant values for **1** obtained in literature ($pK_{a1} = 3.5$ and $pK_{a2} = 5.8$ [7], $pK_{a1} = 3.58$ and $pK_{a2} = 5.94$ [8], $pK_{a2} = 5.89$ [9], $pK_{a2} = 5.92$ [10]) are quite close to the values that we obtained ($pK_{a1} = 3.43$ and $pK_{a2} = 5.80$). For **2**, we obtained the values $pK_{a1} = 2.71$ and $pK_{a2} = 6.65$. The pK_{a2} value 6.78 for **2** has been determined in Ref. [9]. *Roberts et al.* [6] have reported pK_a values 4.0 and 6.1 for **1** and 3.3 and 7.0 for **2** by ^{15}N NMR method. Both of these values are quite different comparing to those mentioned above. We cannot fully explain the reason for the difference, but it has been noted elsewhere that the effect of extra-molecular environmental factors especially on ^{15}N shifts can be large in the ^{15}N NMR method [13].

During the measurement of the UV spectra, photoisomerization of **1** and **2** could generate analysis error. It is known that photoisomerization of uroca-

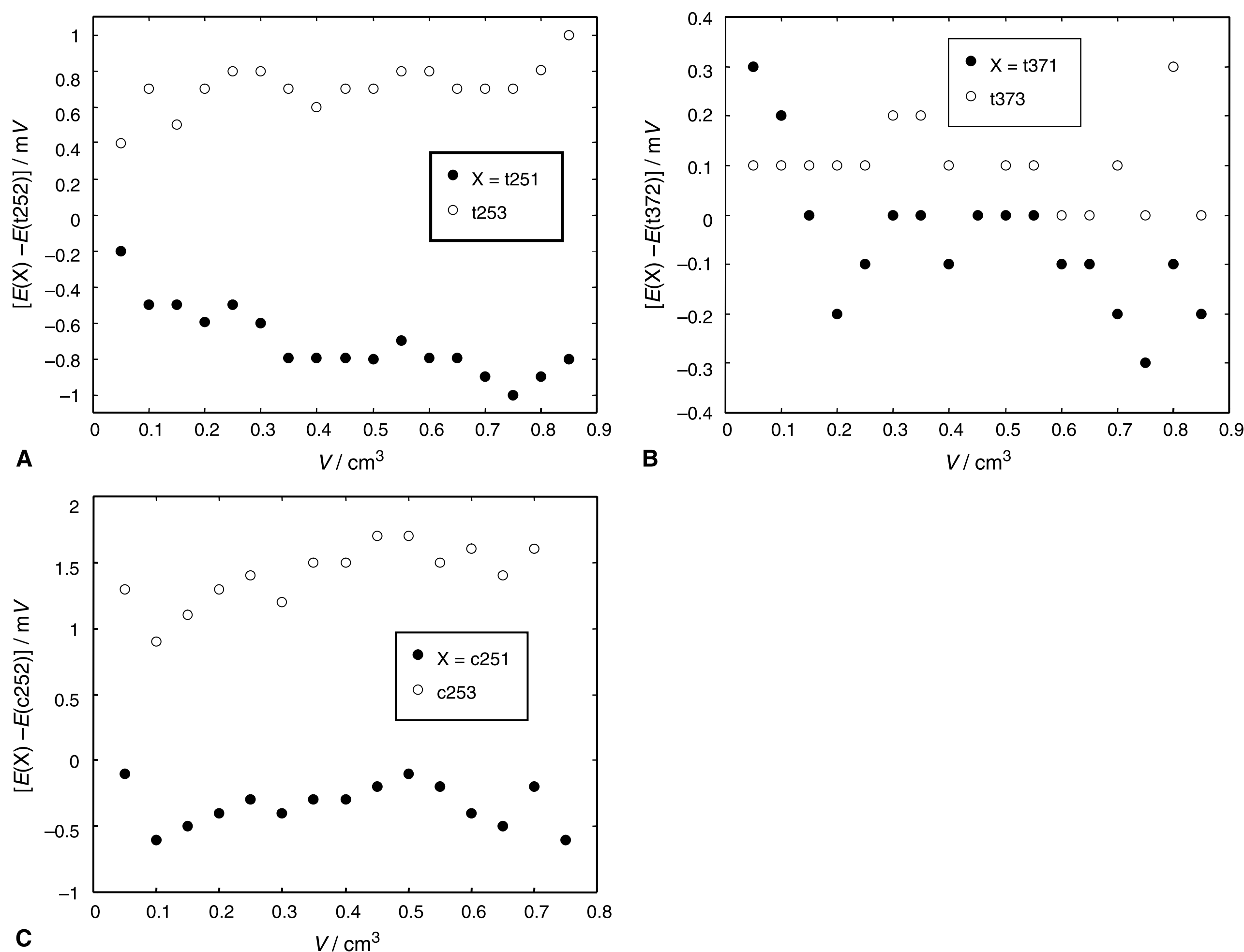

Fig. 4. The data obtained from the potentiometric titrations of **1** at 25°C (graph A), of **1** at 37°C (graph B), and of **2** at 25°C (graph C). For each titration is presented the difference of cell potential differences (cpds) measured in the titration and in a reference titration as a function of the base volume added. For **1** at 25°C solutions, the reference titration is the one where $I_c = 0.10\,M$ (t252), for **1** at 37°C solutions the one where $I_c = 0.10\,M$ (t372), and for **2** at 25°C solutions the one where $I_c = 0.10\,M$ (c252). The following cpds were measured for these reference titrations of **1**: $V = 0.05\,cm^3$, E(t252) = 127.7 mV, E(t372) = 138.8 mV; 0.1, 120.0, 132.0; 0.15, 113.0, 125.5; 0.2, 106.5, 119.5; 0.25, 100.4, 113.8; 0.3, 94.9, 108.3; 0.35, 89.8, 103.1; 0.4, 84.7, 98.3; 0.45, 79.8, 93.4; 0.5, 74.9, 88.6; 0.55, 69.9, 83.7; 0.6, 64.8, 78.7; 0.65, 59.5, 73.4; 0.7, 53.9, 67.7; 0.75, 47.5, 61.5; 0.8, 40.3, 54.1; 0.85, 31.5, 45.7. The following cpds were measured for these reference titrations of **2**: $V = 0.05\,cm^3$, E(c252) = 89.7 mV; 0.1, 74.3; 0.15, 63.3; 0.2, 54.5; 0.25, 47.2; 0.3, 41.0; 0.35, 35.1; 0.4, 29.7; 0.45, 24.4; 0.5, 19.3; 0.55, 14.4; 0.6, 9.3; 0.65, 4.1; 0.7, −1.8; 0.75, −7.9

nic acid is the most efficient at about 313 nm [14] and that appreciable absorption at this wavelength is observed in alkaline solutions only (Figs. 2 and 3). Paradoxically, the photoisomerization reaction is not the most efficient in alkaline but at the acidic *pH* 5.5 [15]. The reaction would not markedly affect the absorbances in this case, because **1** and **2** absorb rather similarly, as confirmed by ground state absorption measurements and flash photolysis techniques [15]. Considering these aspects, the short measuring time (about 20 s), and relatively low radiation intensity of a spectrophotometer, it is justified to assume that the possible photoisomerisation error due to exposure of the samples to UV irradiation during the measurement is not significant and can be neglected.

Potentiometric Titration

The second thermodynamic dissociation constants of **1** and **2** were also determined using potentiometric titration method that has been used in our previous studies, see, *e.g*., Ref. [16]. In this method the stoichiometric dissociation constants (here the second stoichiometric dissociation constant values on molality scale, K_{m2} values) were individually determined from the titration data. Then these observed stoichiometric dissociation constants were used to determine the thermodynamic dissociation constant and activity parameters of ions. Finally, the experimental potentiometric titration data were tested with the determined thermodynamic dissociation constant and activity parameters.

The titration data of (*E*)-urocanic acid at 25°C are shown in graph A of Fig. 4, at 37°C in graph B of Fig. 4, and (*Z*)-urocanic acid at 25°C in graph C of this figure. It was observed during the study that the second stoichiometric dissociation constant on the molality scale, K_{m2}, values of **1** or **2** could not be estimated from the titration data before the first stoichiometric dissociation constant, K_{m1}, is known. The chosen K_{m1} value affects the value obtained for K_{m2} in the calculation procedure of the titration data (shown below). The value $K_{m1} = 10^{-pK_{a1}} = 3.72 \times 10^{-4}$, where $pK_{a1} = 3.43$, was used for **1**, and the value $K_{m1} = 10^{-pK_{a1}} = 19.5 \times 10^{-4}$, where $pK_{a1} = 2.71$, for **2**. These values were obtained from the UV-spectrophotometric results of this study.

In the *Hückel* method, the following equation is generally used for the activity coefficient (γ) of a univalent ion *i* on the molality scale.

$$\ln \gamma_i = \frac{-\alpha\sqrt{I_m}}{1 + B_i\sqrt{I_m}} + b_{i,MCl}(I_m/m^o) \qquad (2)$$

where $m^o = 1\,\mathrm{mol\,kg^{-1}}$, I_m is the ionic strength on the molality scale and α is the *Debye-Hückel* parameter equal to 1.1744 $(\mathrm{mol\,kg^{-1}})^{-1/2}$ at 25°C and 1.2001 $(\mathrm{mol\,kg^{-1}})^{-1/2}$ at 37°C [17]. B_i and $b_{i,MCl}$ are parameters that are dependent on ion *i*, and $b_{i,MCl}$ is also dependent on the salt present in the system. The parameter values previously determined for this equation and used in the present calculations are given in Table 5.

Table 5. Ion parameters for the *Hückel* Eq. (2), at 25 and 37°C and results of the regression analysis obtained by Eq. (24) from the titration data shown in

Parameter	H^+	Cl^-	HA^- for **1**	HA^- for **2**
K_{a2}(25°C)[a]			1.35×10^{-6}	2.04×10^{-7}
pK_{a2}(25°C)[b]			5.870 ± 0.011	6.69 ± 0.02
K_{a2}(37°C)[c]			1.83×10^{-6}	
pK_{a2}(37°C)[b]			5.737 ± 0.008	
$B(\mathrm{mol\,kg^{-1}})^{-1/2}$	1.25[d]	1.25[d]	2.2[e]	2.2[e]
b_{NaCl}	0.238[d]	0.238[d]		
q_{NaCl}(25°C, obsd)[f]			0.5 ± 0.2	0.8 ± 0.3
q_{NaCl}(37°C, obsd)[f]			1.0 ± 0.2	

[a] Determined second thermodynamic dissociation constant of (*E*)- or (*Z*)-urocanic acid at 25°C
[b] $pK_{a2} = -\lg K_{a2}$, the standard deviation is also given
[c] Determined second thermodynamic dissociation constant of (*E*)-urocanic acid at 37°C
[d] Determined from *Harned* cell data (Ref. [18])
[e] Determined for glycine species from *Harned* cell data (Ref. [19]). The same value is used here for anion of (*E*)- and (*Z*)-urocanic acid
[f] Determined in this study from the titration data ($q = b_{HA} - b_{H2A}$) and the standard deviation is also given

The thermodynamic value of the second dissociation constant (K_{a2}) of **1** or **2** is given by

$$K_{a2} = \frac{a_H a_{HA}}{a_{H_2A}} = \left(\frac{\gamma_H \gamma_{HA}}{\gamma_{H_2A}}\right)\left(\frac{m_H m_{HA}}{m_{H_2A} m^o}\right) = \left(\frac{\gamma_H \gamma_{HA}}{\gamma_{H_2A}}\right) K_{m2} \quad (3)$$

where a is the activity, m the molality, and K_{m2} the second stoichiometric dissociation constant of **1** or **2** on the molality scale. For simplicity, the ion charge superscripts are omitted from equations, *e.g.*, $H^+ = H$ and $HA^- = HA$. It is assumed that the activity coefficient of the neutral species H_2A follows the *Kirkwood* equation [20, 21] for zwitterions, *i.e.*,

$$\ln \gamma_{H_2A} = b_{H_2A} I_m / m^o \quad (4)$$

The following equation can be derived from Eqs. (2)–(4) for K_{m2} of **1** or **2** in aqueous NaCl (generally MCl) solutions:

$$\ln K_{m2,MCl} = \ln K_{a2} + \frac{\alpha\sqrt{I_m}}{1 + B_H\sqrt{I_m}} + \frac{\alpha\sqrt{I_m}}{1 + B_{HA}\sqrt{I_m}} - (b_{H,MCl} + q_{MCl})(I_m/m^o) \quad (5)$$

where $q_{MCl} = b_{HA,MCl} - b_{H_2A,MCl}$. In the present study, the values of $B_H = 1.25$ $(\text{mol kg}^{-1})^{-1/2}$, $B_{HA} = 2.2$ $(\text{mol kg}^{-1})^{-1/2}$ (the used value is the same as the value that has been determined for glycine in Ref. [19]), and $b_{H,NaCl} = 0.238$ were used in this equation (see Table 5), and the values of the second thermodynamic dissociation constant K_{a2} and parameter q_{NaCl} were determined from the new potentiometric titration data.

The thermodynamic quantity for the ionic product of water ($K_{a,w}$) is defined by equation

$$K_{a,w} = \frac{\gamma_H \gamma_{OH} m_H m_{OH}}{a_w (m^o)^2} = \frac{g_w m_H m_{OH}}{(m^o)^2} \quad (6)$$

where OH refers to hydroxide ions, w refers to water, the quantity g_w is defined by $g_w = \gamma_H \gamma_{OH} / a_w$. *Harned* and *Hamer* [22] have determined a value of 1.008×10^{-14} for $K_{a,w}$ at 25°C, and a value of 2.41×10^{-14} can be used at 37°C. In a previous study [23], the following equation was determined from the *Harned* cell data [24] for $\ln g_w$ in NaCl solutions, containing only one parameter depending on the salt, *i.e.*, parameter b_w:

$$\ln g_w = \ln\left(\frac{\gamma_H \gamma_{OH}}{a_w}\right) = -\frac{2\alpha\sqrt{I_m}}{1 + B_H\sqrt{I_m}} + b_w\left(\frac{I_m}{m^o}\right). \quad (7)$$

For NaCl solutions at 25°C, the value of parameter b_w is 0.412, and Eq. (7) is valid up to an ionic strength of about $3\,\text{mol kg}^{-1}$ [23]. In this study, the same values of B_H and b_w were used at 25 and at 37°C.

The theoretical equation for the molality of hydrogen ions can be derived for the titration data from the first stoichiometric dissociation constant equation ($K_{m1} = m_H m_{H_2A} / (m_{H_3A} m^o)$), the second stoichiometric dissociation constant equation ($K_{m2} = m_H m_{HA} / (m_{H_2A} m^o)$), the electroneutrality equation ($m_{H_3A} + m_H + m_b = m_{HA} + m_{OH}$), the mass balance equation ($m_t = m_{H_3A} + m_{H_2A} + m_{HA}$), and thermodynamic quantity for the ionic product of water, Eq. (6). m_b in the third equation is the molality of base NaOH, in the solution titrated, and $m_b = c_b V / w_1$ where c_b and V are the molarity and volume of base solution added in the titration, respectively, and w_1 is the mass of water in the solution titrated. m_t in the fourth equation is the total molality of **1** or **2** in the solution titrated and $m_t = n_t / w_1$ where n_t is the amount of this substance. From these five equations the following equation, Eq. (8), was obtained:

$$\begin{aligned} & m_H{}^4 + (K_{m1} m^o + m_b + m_t) m_H{}^3 \\ & + (K_{m1} m_b m^o - K_{a,w} (m^o)^2 / g_w \\ & + K_{m1} K_{m2} (m^o)^2) m_H{}^2 \\ & + (K_{m1} K_{m2} m_b (m^o)^2 - K_{m1} K_{m2} m_t (m^o)^2 \\ & - K_{m1} K_{a,w} (m^o)^3 / g_w) m_H \\ & - K_{m1} K_{m2} K_{a,w} (m^o)^4 / g_w = 0 \end{aligned} \quad (8)$$

In the present calculations, m_H had to be determined by this equation for each titration. It was determined numerically by the *Newton-Raphson* method [25].

For the determination of K_{m2} of **1** or **2** from the titration data, two glass electrode parameters, in addition to this dissociation constant, were simultaneously estimated from each titration data set. In general, the following equation is valid for the cell potential differences (= cpds) measured on a glass electrode cell:

$$E = E^o + k\left(\frac{RT}{F}\right) \ln a_H \quad (9)$$

where a_H is the activity of protons, k is a glass electrode parameter, and E^o is another glass electrode parameter. The latter potential parameter includes

the contributions of the reference electrode, liquid junction, standard-glass electrode, and asymmetry potentials; see, *e.g.*, Ref. [26]. It is assumed in all present titrations that this term remained constant during the titration. Eq. (9) can also be presented in the form:

$$E = E^{o} + \frac{kRT}{F}\ln\gamma_{H} + \frac{kRT}{F}\ln\left(\frac{m_{H}}{m^{o}}\right) = E_{0} + \frac{kRT}{F}\ln\left(\frac{m_{H}}{m^{o}}\right) \quad (10)$$

where $E_0 = E^{o} + (kRT/F)\ln\gamma_H$ is also a glass electrode parameter that is constant during each titration at a constant ionic strength; see Eq. (2). Parameters E_0 and k must be estimated from the titration data.

K_{m2} and glass electrode parameter E^{o} were calculated for each **1** or **2** data set presented in Fig. 4 containing N points by the following equations (Eqs. (20) and (21) are used iteratively, *Newton-Raphson* method):

$$\sum(E_{i} - E_{pred,i}) = 0 \quad (11)$$

$$\ln(h_{i}/m^{o}) = \frac{(E_{i} - E_{0})F}{kRT} \quad (12)$$

$$m_{H_3A} = \frac{h_{i}(n_{t}/w_{1,i}) - h_{i}^{2} - h_{i}m_{b,i} + K_{a,w}(m^{o})^{2}/g_{w}}{K_{m1}m^{o} + 2h_{i}} \quad (13)$$

$$m_{H_2A} = \frac{K_{m1}m_{H_3A}m^{o}}{h_{i}} \quad (14)$$

$$m_{HA} = n_{t}/w_{1,i} - m_{H_3A} - m_{H_2A} \quad (15)$$

$$K_{m2,i} = \frac{h_{i}m_{HA}}{m_{H_2A}m^{o}} \quad (16)$$

$$K_{m2} = \frac{\sum K_{m2,i}}{N} \quad (17)$$

$$\begin{aligned} f(h_{i}) = {} & h_{i}^{4} + (K_{m1}m^{o} + m_{b,i} + n_{t}/w_{1,i})h_{i}^{3} \\ & + (K_{m1}K_{m2}(m^{o})^{2} + K_{m1}m_{b,i}m^{o} \\ & - K_{a,w}(m^{o})^{2}/g_{w})h_{i}^{2} + (K_{m1}K_{m2}m_{b,i}(m^{o})^{2} \\ & - K_{m1}K_{m2}(n_{t}/w_{1,i})(m^{o})^{2} \\ & - K_{m1}K_{a,w}(m^{o})^{3}/g_{w})h_{i} \\ & - K_{m1}K_{m2}K_{a,w}(m^{o})^{4}/g_{w} \end{aligned} \quad (18)$$

$$m_{H,j}(j = 1) = h_{i} - \frac{f'(h_{i})}{f(h_{i})} \quad (19)$$

$$\begin{aligned} f(m_{H,i}(j)) = {} & (m_{H,i}(j))^{4} + (K_{m1}m^{o} + m_{b,i} + n_{t}/w_{1,i}) \\ & \times (m_{H,i}(j))^{3} + (K_{m1}K_{m2}(m^{o})^{2} \\ & + K_{m1}m_{b,i}m^{o} - K_{a,w}(m^{o})^{2}/g_{w})(m_{H,i}(j))^{2} \\ & + (K_{m1}K_{m2}m_{b,i}(m^{o})^{2} \\ & - K_{m1}K_{m2}(n_{t}/w_{1,i})(m^{o})^{2} \\ & - K_{m1}K_{a,w}(m^{o})^{3}/g_{w})(m_{H,i}(j)) \\ & - K_{m1}K_{m2}K_{a,w}(m^{o})^{4}/g_{w} \end{aligned} \quad (20)$$

$$m_{H,i}(j) = m_{H,i}(j-1) - \frac{f'(m_{H,i}(j-1))}{f(m_{H,i}(j-1))} \quad (21)$$

$$E_{pred,i} = E_{0} + k\left(\frac{RT}{F}\right)\ln\left(\frac{m_{H,i}}{m^{o}}\right) \quad (22)$$

where h_i is the experimental molality of H^{+} ions in point i. In Eqs. (19)–(21), j is the number of rounds in the iterative process. Additionally, the glass electrode parameter k was optimized by setting the following square sum, $S(E)$, at a minimum:

$$S(E) = \sum_{i=1}^{N}(E_{i} - E_{predicted,i})^{2} \quad (23)$$

Table 6. Results calculated from titrations of (*E*)-urocanic acid (see Fig. 4) in aqueous NaCl solutions at 25°C

Symbol	t251	t252	t253
I_c (mol dm^{-3}) at 20°C	0.07	0.10	0.154
I_m (mol kg^{-1})	0.070215	0.10036	0.15471
10^6 K_{m2} (obsd)[a]	1.95	2.05	2.08
10^6 K_{m2} (recd)[b]	1.97	2.04	2.10
k[c]	0.986	0.984	0.982
E_0 (mV)[d]	407.53	406.79	406.11

[a] The second stoichiometric dissociation constant of (*E*)-urocanic acid determined from titration data

[b] The second stoichiometric dissociation constant of (*E*)-urocanic acid calculated by Eq. (5) with recommended activity parameters and K_{a2} value shown in Table 5

[c] The value of glass electrode parameter k used in the calculation

[d] The value of glass electrode parameter E_0 used in the calculation of the cpd errors for Fig. 5. It was determined by requiring that the sum of errors in the data set is zero [*i.e.*, by Eqs. (18) and (19) and then iteratively by Eqs. (20) and (21) for $m_{H,i}$ and also by Eqs. (25) and (26)]

This parameter had to be adjusted in the NaCl titrations (probably because of the selectivity problems of a glass electrode in aqueous NaCl solutions). For the ideal case (the glass electrode cell has theoretical *Nernst* slope), $k = 1$. It was observed here that the deviation from unity at temperature 37°C is larger than at 25°C. In our earlier studies at 25°C [27–30], the parameter k also differed from unity in aqueous NaCl solutions. The results from the calculations of the titration data of **1** are shown in Table 6 for temperature 25°C and in Table 7 for temperature 37°C. Correspondingly, the results from the calculations of the titration data of **2** at 25°C are shown in Table 8.

Table 7. Results calculated from titrations of (*E*)-urocanic acid (see Fig. 4) in aqueous NaCl solutions at 37°C

Symbol	t371	t372	t373
I_c (mol dm^{-3}) at 20°C	0.07	0.10	0.154
I_m (mol kg^{-1})	0.070215	0.10036	0.15471
10^6 K_{m2} (obsd)[a]	2.60	2.69	2.68
10^6 K_{m2} (recd)[b]	2.60	2.66	2.67
k[c]	0.945	0.944	0.944
E_0 (mV)[d]	414.10	413.37	413.33

[a] The second stoichiometric dissociation constant of (*E*)-urocanic acid determined from titration data
[b] The second stoichiometric dissociation constant of (*E*)-urocanic acid calculated by Eq. (5) with recommended activity parameters and K_{a2} value shown in Table 5
[c] The value of glass electrode parameter k used in the calculation
[d] The value of glass electrode parameter E_0 used in the calculation of the cpd errors for Fig. 5. It was determined by requiring that the sum of errors in the data set is zero [*i.e.*, by Eqs. (18) and (19) and then iteratively by Eqs. (20) and (21) for $m_{H,i}$ and also by Eqs. (25) and (26)]

Table 8. Results calculated from titrations of (*Z*)-urocanic acid (see Fig. 4) in aqueous NaCl solutions at 25°C

Symbol	c251	c252	c253
I_c (mol dm^{-3}) at 20°C	0.07	0.10	0.154
I_m (mol kg^{-1})	0.070215	0.10036	0.15471
10^7 K_{m2} (obsd)[a]	2.88	3.02	3.00
10^7 K_{m2} (recd)[b]	2.92	2.99	3.03
k[c]	0.986	0.987	0.982
E_0 (mV)[d]	400.49	400.61	399.77

[a] The second stoichiometric dissociation constant of (*Z*)-urocanic acid determined from titration data
[b] The second stoichiometric dissociation constant of (*Z*)-urocanic acid calculated by Eq. (5) with recommended activity parameters and K_{a2} value shown in Table 5
[c] The value of glass electrode parameter k used in the calculation
[d] The value of glass electrode parameter E_0 used in the calculation of the cpd errors for Fig. 5. It was determined by requiring that the sum of errors in the data set is zero [*i.e.*, by Eqs. (18) and (19) and then iteratively by Eqs. (20) and (21) for $m_{H,i}$ and also by Eqs. (25) and (26)]

The second thermodynamic dissociation constant and activity parameters for urocanic acid isomers were determined from the experimental K_{m2} values obtained from titration data. Three titrations at both temperatures 25 and 37°C were only made at low ionic strengths. Because the activity parameter B_{HA} cannot be estimated from the data, we have used here the value $B_{HA} = 2.2$ (mol kg^{-1})$^{-1/2}$. It was determined from accurate *Harned* cell data of glycine [19, 31], and this value has also been successively used for other amino acids [16]. The activity parameter $q_{NaCl} = b_{HA,NaCl} - b_{H_2A,NaCl}$ and the thermodynamic value of the dissociation constant K_{a2} were determined at temperatures 25°C and for **1** also at 37°C. The following equation, derived from Eq. (5), was used:

$$\ln K_{m2,NaCl} - \alpha\sqrt{I_m}\left(\frac{1}{1+B_H\sqrt{I_m}} + \frac{1}{1+B_{HA}\sqrt{I_m}}\right) = y = \ln K_{a2} - (b_{H,NaCl} + q_{NaCl})\left(\frac{I_m}{m^o}\right) \quad (24)$$

This equation shows that y is a linear function of (I_m/m^o). The thermodynamic dissociation constant K_{a2} can be obtained from the intercept of the line with the y-axis, and the slope of the straight line is $-(b_{H,NaCl} + q_{NaCl})$. At temperature 37°C, the same $b_{H,NaCl}$ value as obtained earlier at 25°C was used (Table 5). The results from the linear regression analysis are shown in Table 5. It can be seen that the pK_{a2} values obtained using potentiometric titration were close to pK_{a2} values obtained by UV spectrophotometry (see above).

The second thermodynamic dissociation constants of urocanic acid isomers and activity parameters recommended (shown in Table 5) were tested with the titration data presented in Fig. 4. Parameter E_0 for Eq. (10) was calculated for each data set by using Eqs. (18) and (19) and then iteratively Eqs. (20) and (21) for $m_{H,i}$ and also the following equations:

$$E_{0,i} = E_i - \left(\frac{kRT}{F}\right)\ln\left(\frac{m_{H,i}}{m^o}\right) \quad (25)$$

$$E_0 = \frac{\sum_{i=1}^{N} E_{0,i}}{N} \quad (26)$$

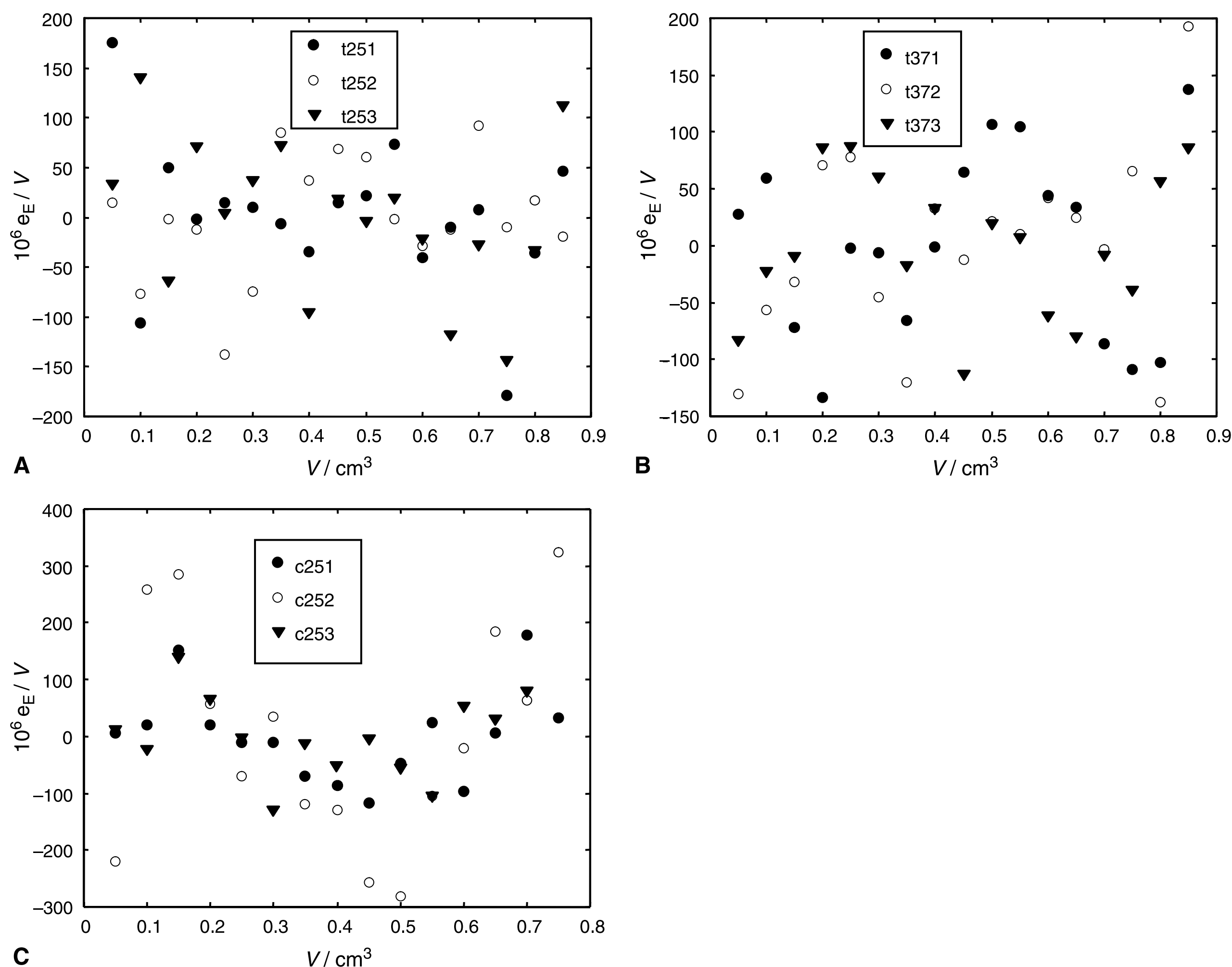

Fig. 5. The difference between the observed and predicted cpd values, e_E in Eq. (27), as a function of the titrant volume in the titrations of urocanic acid solutions with the base (NaOH) solution. Graph A shows the results of the (*E*)-urocanic acid titrations obtained at 25°C (Series t25), graph B shows (*E*)-urocanic acid titrations obtained at 37°C (Series t37), and graph C shows the results of the (*Z*)-urocanic acid titrations obtained at 25°C (Series c25). The predicted cpd was calculated by using Eqs. (5)–(8) and (10) using the second thermodynamic dissociation constant value at the studied temperature, the recommended ion parameters (shown in Table 5), and the glass electrode parameters k and E_0 shown in Tables 6–8. The symbols of the different sets are shown in the graphs

In these calculations, the values shown in Tables 6–8 were used for the other glass electrode parameter k. The resulting values for E_0 are included in these tables. The results of the tests are shown as error plots in graph A for **1** at temperature 25°C, in graph B for **1** at 37°C, and in graph C for **2** at 25°C of Fig. 5, where cpd error defined by

$$e_E = E(\text{observed}) - E(\text{predicted}) \qquad (27)$$

is presented for each data set as a function of the added base volume. Most of the error plots in these graphs appear random, and most of the errors in these plots are comparable to the resolution of the *pH* meter (0.1 mV). Therefore, the titration data of urocanic acid isomers support well the suggested second thermodynamic dissociation constants and the activity parameters used.

From every point of the titrations presented in Fig. 4, an experimental value of K_{m2} can also be calculated from Eqs. (12)–(16) by using the glass electrode parameter values shown in Tables 6–8. These values are compared with the recommended values in the three graphs of Fig. 6. Graph A in this figure shows the results of **1** at 25°C, graph B the

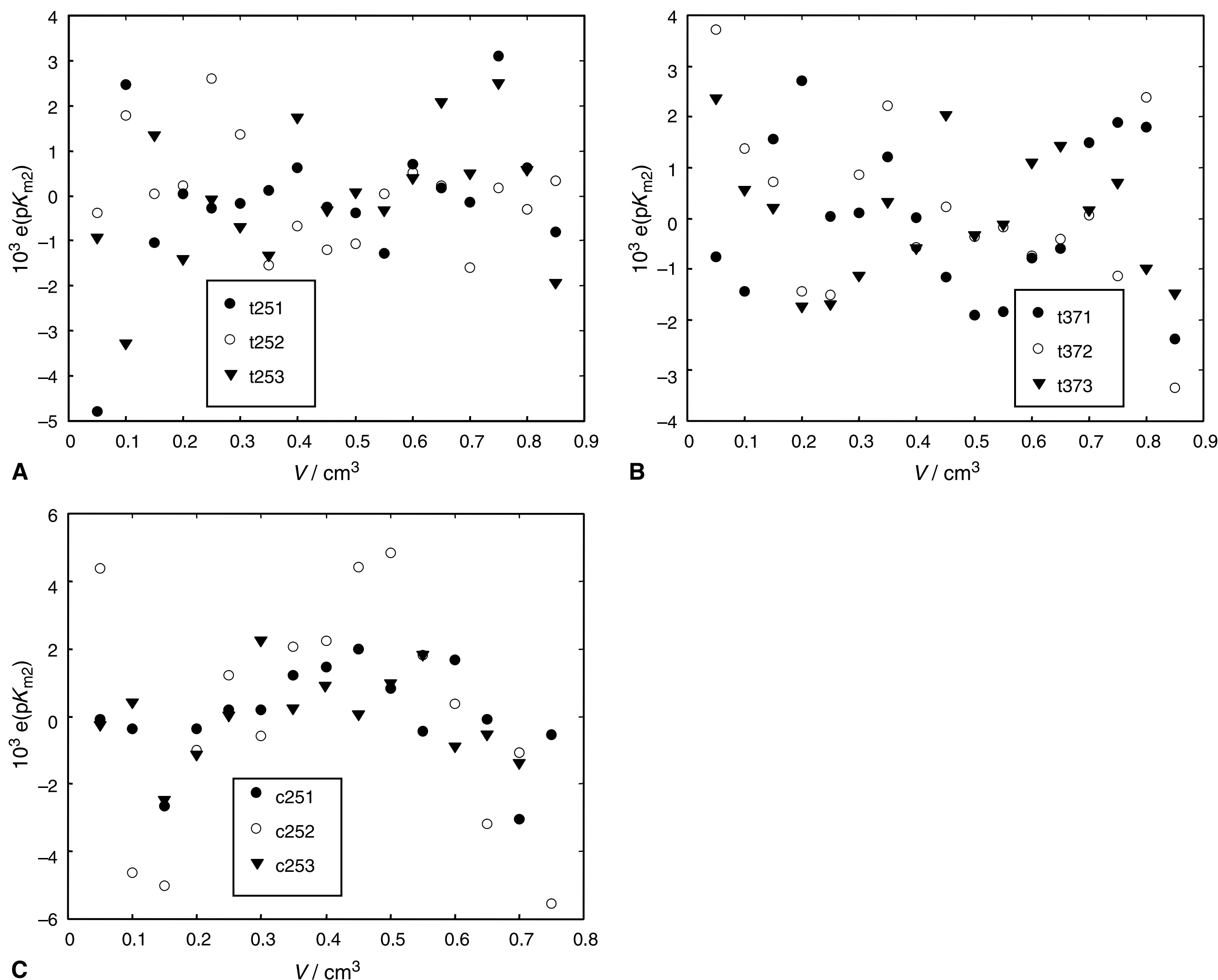

Fig. 6. The difference, $e(pK_{m2})$ in Eq. (28), between the observed pK_{m2} values and those predicted by the recommended *Hückel* method as a function of the titrant volume in the titrations of urocanic acid solutions with a base (NaOH) solution. Graph A shows the results of (*E*)-urocanic acid titrations obtained at 25°C (Series t25), graph B shows the results of (*E*)-urocanic acid titrations obtained at 37°C (Series t37) and graph C shows the results of (*Z*)-urocanic acid titrations obtained at 25°C (Series c25). The observed K_{m2} was calculated from the titration points by Eqs. (6), (7), (12)–(16) with the glass electrode parameters shown in Tables 6–8. The symbols of the different sets are shown in the graphs

results of **1** at 37°C and graph C the results of **2** at 25°C. In these graphs, the $pK_{m2}(= -\lg(K_{m2}))$ errors, defined by

$$e(pK_{m2}) = pK_{m2}(\text{observed}) - pK_{m2}(\text{predicted}) \tag{28}$$

are presented as a function of the added base volume. All pK_{m2} errors in Fig. 6 are within the range ± 0.006 and they form a random pattern. The maximum error can also be compared with the conventional pK_m error of 0.06 suggested by *Albert* and *Serjeant* [11].

We have performed potentiometric titration in aqueous urocanic acid solutions were the salt (NaCl) concentration has been much larger than urocanic acid concentration, so the ionic strength of the solution is practically the same as the salt concentration. *Harned* and *Owen* [32] have given an equation for some aqueous salt solutions to convert their molarity to molality or *vice versa*. This equation for aqueous NaCl solution has also been presented in Eq. (29).

$$r = \frac{c_{\text{NaCl}} m^{\text{o}}}{m_{\text{NaCl}} c^{\text{o}}} = \rho_{\text{H}_2\text{O}}/(\text{kg dm}^{-3}) - A\frac{m_{\text{NaCl}}}{m^{\text{o}}} \tag{29}$$

In this equation r is the molarity – molality ratio, c_{NaCl} is the molarity of the aqueous NaCl solution, m_{NaCl} is the molality of the aqueous NaCl solution, ρ_{H_2O} is the density of water at the measurement temperature (*e.g.*, $\rho_{H_2O} = 0.9970\,\mathrm{kg\,dm^{-3}}$ at 25°C and $\rho_{H_2O} = 0.9933\,\mathrm{kg\,dm^{-3}}$ at 37°C), and A is the constant value depending on the salt and temperature [32]. At 25°C, the value $A = 0.0183$ and, at 37°C, the value $A = 0.0188$ can be used.

Second stoichiometric dissociation constants are shown in Tables 9–11 at some ionic strengths on concentration scale, I_c. The I_m and r values have been calculated by using Eq. (29). The K_{m2} values for urocanic acid isomers have been calculated by using Eq. (5) with the K_{a2} values and the activity parameters shown in Table 5. The second stoichiometric dissociation constant on molarity scale, K_{c2}, values are also shown, calculated by using Eq. (30).

$$K_{c2} = K_{m2} r \qquad (30)$$

K_{m2} and K_{c2} values of **1** at aqueous NaCl solutions at 25 and 37°C are shown in Tables 9 and 10, respectively. The corresponding constant values for **2** at 25°C are shown in Table 11. It can be seen from these tables that pK_{c2} values of both urocanic acid isomers decrease by 0.1 to 0.2 units when salt molality increases from infinite dilute to isotonic salt concentration.

Table 9. The second stoichiometric dissociation constant on molarity scale (K_{c2}) of (*E*)-urocanic acid (**1**) as a function of the ionic strength on concentration scale (I_c) in aqueous NaCl solution at 25°C. The concentration of NaCl is much larger than the concentration of **1**

I_c (mol dm^{-3})	I_m (mol kg^{-1})	r[a]	$10^6\ K_{m2}$	$10^6\ K_{c2}$	$pK_{c2} = -\lg K_{c2}$
0	0	1	1.35	1.35	5.87
0.01	0.01003	0.9968	1.64	1.63	5.79
0.05	0.05020	0.9961	1.91	1.90	5.72
0.10	0.1005	0.9952	2.04	2.03	5.69
0.154	0.1549	0.9942	2.10	2.09	5.68

[a] The r is the molarity – molality ratio of salt (NaCl), see Eq. (29)

Table 10. The second stoichiometric dissociation constant on molarity scale (K_{c2}) of (*E*)-urocanic acid (**1**) as a function of the ionic strength on concentration scale (I_c) in aqueous NaCl solution at 37°C. The concentration of NaCl is much larger than the concentration of **1**

I_c (mol dm^{-3})	I_m (mol kg^{-1})	r[a]	$10^6\ K_{m2}$	$10^6\ K_{c2}$	$pK_{c2} = -\lg K_{c2}$
0	0	1	1.83	1.83	5.74
0.01	0.01007	0.9931	2.22	2.20	5.66
0.05	0.05039	0.9924	2.54	2.52	5.60
0.10	0.1009	0.9914	2.66	2.63	5.58
0.154	0.1555	0.9904	2.67	2.64	5.58

[a] The r is the molarity – molality ratio of salt (NaCl), see Eq. (29)

Table 11. The second stoichiometric dissociation constant on molarity scale (K_{c2}) of (*Z*)-urocanic acid (**2**) as a function of the ionic strength on concentration scale (I_c) in aqueous NaCl solution at 25°C. The concentration of NaCl is much larger than the concentration of **2**

I_c (mol dm^{-3})	I_m (mol kg^{-1})	r[a]	$10^7\ K_{m2}$	$10^7\ K_{c2}$	$pK_{c2} = -\lg K_{c2}$
0	0	1	2.04	2.04	6.69
0.01	0.01003	0.9968	2.47	2.46	6.61
0.05	0.05020	0.9961	2.84	2.83	6.55
0.10	0.1005	0.9952	2.99	2.97	6.53
0.154	0.1549	0.9942	3.03	3.02	6.52

[a] The r is the molarity – molality ratio of salt (NaCl), see Eq. (29)

Urocanic Acid in the Skin

The results suggest that the pK_{a2} of **2** may be lower than the value 7.0 reported by some investigators [6]. This may have implications on the physico-chemical behaviour and biological mechanism of function of **2** in the skin. In addition to numerous scientific reports showing potent immunomodulating activities [5], a profound role for urocanic acid has been suggested in the maintenance of acidity in the *stratum corneum*. In the normal healthy skin, the homeostasis of **1** has a self-regulatory mechanism that responds to perturbations in skin *pH* [33]. In the diseased skin, this homeostasis may be disturbed, and reduced endogenous concentrations of total urocanic acid have been reported in atopic dermatitis [34–36] and psoriasis [36, 37]. When the epidermal *pH* tends to increase due to a skin disease [1–4], **1** and **2** may also act as buffer molecules according to their pK_{a2} values, resisting the *pH* change. The lower than previously reported pK_{a2} of **2** suggests that the buffering effect of **2** is more significant already at *pH* below 7. However, **2** exists in the skin normally in minor concentrations only [33], whereas its concentration is significantly elevated after UV irradiation of the skin. The dependence of the photoisomerisation reaction on protein interaction and temperature has been addressed in Ref. [38].

Experimental

For UV-spectrophotometric study, (*E*)-urocanic acid ((*E*)-3-(1*H*-imidazol-4-yl)prop-2-enoic acid, **1**) was purchased from Acros Organics (product 22896-0000; Geel, Belgium). (*Z*)-Urocanic acid ((*Z*)-3-(1*H*-imidazol-4-yl)prop-2-enoic acid, **2**) was manufactured from **1** by BioCis Pharma (Turku, Finland). The purity of the products was 99.75 and 99.95%, respectively, according to HPLC analysis with UV detection at 268 nm, and they were characterized also by IR spectrometry and melting point analysis. The identity of **2** was further confirmed by ^{1}H NMR and ^{13}C NMR spectrometry. The water content was 0.25% (loss on drying) for **1** and 0.09% (*Karl Fischer* method) for **2**.

1 and **2** were dissolved in ultrapure water (Quantum EX Ultrapure Organex Cartridge and Millipak 40 0.22 μm filter from Millipore) at 50 m*N* and then diluted 1:10 in ultrapure water. The 5 m*M* urocanic acid stock solutions were used in measurements by adding 10 mm^3 of the stock in 990 mm^3 of *Dulbecco*'s phosphate-buffered saline (D8537; Sigma-Aldrich), thus yielding a final analysis concentration of 50 μM. For the measurement of the corresponding control base line, a blank reference solution was made by mixing 10 mm^3 ultrapure water and 990 mm^3 of the phosphate-buffered saline. When measuring samples of *pH* below 2 or above 11, 0.01–1 *M* HCl or KOH was used instead of the buffer solution, respectively.

The urocanic acid isomer in its final 50 μM measurement concentration was transferred into a clean 50 ml plastic tube in a volume of 3–5 cm^3. The *pH* was measured with a *pH*/temperature combination electrode (SE-102 *pH*/Pt 1000 Electrode, Knick, Berlin, Germany) connected to Knick Calimatic *pH*-Meter 766. The *pH* was adjusted with small additions (0.5–20 mm^3 in volume) of 1 *M* HCl or KOH. Exactly identical additions of the same solutions were made in the corresponding blank reference solution without urocanic acid using the same volume. Thus, the volume, buffer constituents, and ionic strength were identical in the test and blank samples, the only difference being the presence of **1** or **2**. The *pH* was measured in the urocanic acid solutions only. The exact *pH* value of the solution was recorded when the reading was close to the desired value and stable (within ± 0.01 *pH* units) for at least 10 s under constant mixing. The electrode was calibrated immediately before the measurements using standard reference solutions. The measured temperature of the calibration and test solutions was in the range 23.0–24.8°C.

Immediately after *pH* adjustment, the test sample and the corresponding blank reference sample were transferred into two similar quartz cuvettes with 10.00 mm path length (Quartz SUPRASIL, Hellma Optik, Jena, Germany). The base line was measured in the blank reference sample, where after the absorption spectra of the urocanic acid test sample was measured against that base line. Each absorption spectrum of a test sample was obtained against an individual base line recorded from the corresponding blank reference sample. The measurements were performed with Jasco V-560 UV/VIS spectrophotometer (Jasco, Tokyo, Japan) through the 230–340 nm wavelength range using 0.2 nm bandwidth and data pitch. The measurement cuvette slot was equipped with a *Peltier*-type temperature control element that was preset to 23.5°C. The actual temperature of the slot was observed constantly during the measurement through the built-in thermometer display. The temperature was always within ± 0.2°C from the preset value during the measurements.

To obtain the spectra of the pure ionic species, the urocanic acid stock solutions were diluted in 0.01–1 *M* HCl or KOH, the *pH* were measured, and adjusted when necessary. A narrow *pH* range was searched both in the acidic and alkaline regions where the absorption spectrum showed a change of less than 1% [11]. These *pH* ranges represent the pure ionic urocanic acid species H_3A^+ and HA^- (Fig. 1). Approximately in the midpoint between these two *pH* regions, a third *pH* region was searched that represents the neutral H_2A species. Also here, the absorption spectrum should show minimal change within 0.3–0.5 *pH* units.

The absorption spectra of H_3A^+ and H_2A were overlaid and subtracted one from the other utilizing the software of the instrument manufacturer (Jasco). A single wavelength with the maximum difference between the two spectra was identified and appointed as the analytical wavelength. The absorbances of the two species recorded at the analytical wavelength represent the values for the parameters A_I and A_M, respectively (see Eq. (1)). These values were used to calculate the first thermodynamic dissociation constant K_{a1}

($pK_{a1} = -\lg K_{a1}$). The analytical wavelength was determined similarly for the second thermodynamic dissociation constant K_{a2} ($pK_{a2} = -\lg K_{a2}$) measurements by investigating the spectra for H_2A and HA^-.

A buffer solution was prepared in which urocanic acid is only in a partly ionised form. For pK_{a1}, this pH was assumed to be close to the midpoint of the pH values of the H_3A^+ and H_2A species, and, for pK_{a2}, this pH was assumed to be close to the midpoint of the pH values of the H_2A and HA^- species. The absorbances at the analytical wavelengths for pK_{a1} and pK_{a2} of urocanic acid isomers were measured.

The test solutions of urocanic acid isomers were adjusted to pH values equalling the approximate estimates of pK_a and up to ± 0.2 and ± 0.4 pH units from the approximate pK_a. The spectra of each of these solutions were measured and the corresponding pK_a values were calculated separately according to Eq. (1).

Potentiometric titrations of **1** and **2** with a base solution were carried out in aqueous NaCl solutions at 25 and 37°C. The same urocanic acid products were used than in UV spectrophotometry (see above). The NaCl concentrations of the measuring solutions were 0.07, 0.10, or 0.154 *M* (isotonic salt solution), while the concentrations of **1** and **2** were 0.00074 *M*. For measurements, two series of NaCl (pro analysi, Riedel-de Haën) solutions were prepared in RO-filtered water (Millipore) at 20°C, and the concentrations in these series were as follows: 0.0945, 0.135, and 0.2079 *M*. 0.0100 *M* solutions of **1** and **2**, and 0.100 *M* NaOH (Titrisol, Merck) were also prepared.

The solutions titrated were prepared by mixing 100.0 cm^3 NaCl solution, 25.00 cm^3 water and 10.00 cm^3 solution of **1** or **2** (see Ref. [39]). Because the titrations of both urocanic acid solutions were made in a near neutral milieu, the influence of carbon dioxide on the solutions was prevented by prior degassing with Ar for about 10 min. The gas stream was then stopped and the titration vessel was closed to form an Ar mattress over the titrated solution, because the density of Ar gas is higher than that of air.

Titrations were carried out by reading the cell potential difference (cpd) after increments of 0.050 cm^3 of the added base solution. The cpd was measured using a BlueLine 12 *pH* combination electrode and a CG841 *pH* meter (Schott Instruments, Mainz, Germany). The resolution of the meter was 0.1 mV. The titrant was added by a Dosimat device (Metrohm, Herisau, Switzerland). The measurements of **2** at 37°C could not be performed satisfactorily and were not included here. Standard buffer solutions (a reference value *pH* standard solution, 0.05 $mol\,kg^{-1}$ potassium hydrogen phthalate and a primary *pH* standard solution, 0.025 $mol\,kg^{-1}$ potassium dihydrogen phosphate $+0.025$ $mol\,kg^{-1}$ disodium hydrogen phosphate) [40] were used to check the stability of the measuring system between titrations. The *pH* meter reproduced usually the same reading within 0.2 mV in these buffer solution tests at 25°C. At 37°C, the deviations were about ± 1 mV.

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