Microchemical Journal 181 (2022) 107689

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

# Influence of pH measurement inaccuracy on the values of acidity constant determined on the basis of electrophoretic and thermophoretic data

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ARTICLE INFO

*Keywords:*
Acid-base equilibrium
Acid dissociation constant
Capillary electrophoresis
pH measurement
Microscale thermophoresis
Thermodynamics

ABSTRACT

The accuracy of the acid dissociation constant ($pK_a$) determined by experimental techniques depends on the potential errors in the pH measurement. This dependence is obvious, but, according to common practice, this effect is either ignored or treated in a greatly simplified manner when discussing the credibility of obtained $pK_a$ values. This paper investigates the influence of incorrect pH measurement on the $pK_a$ values obtained by capillary electrophoresis (CE) and microscale thermophoresis (MST). A simple self-control procedure has been proposed to control and reliably predict the corresponding total uncertainty of the acidity constant. The significance of the pH measurement error was also investigated in relation to the thermodynamic analysis, which requires determining the thermal dependency of $pK_a$ values. The obtained results clearly indicate that the investigated effect should not be ignored, and the actual accuracy of methods using electrophoretic separation may be worse than commonly assumed. It also points at the need to develop alternative methods that do not require measuring pH, such as a known internal standard-based approach. Besides pH-related effects, other sources of inaccuracy of $pK_a$ constant should also be considered.

## 1. Introduction

The acid-base dissociation constant written in logarithmic form ($pK_a$) is the basic physicochemical parameter characterizing chemical compounds. It determines the state of a molecule at a given pH. Since the neutral and ionized forms differ in properties such as water solubility, hydrophobicity, affinity for supramolecular interactions, etc., knowledge of the $pK_a$ value is crucial for predicting the properties of biological compounds, including drugs [1]. Accurate determination of the $pK_a$ value is therefore of great importance in pharmacology, molecular biology and also analytical chemistry, because the mechanism of many analytical techniques is based on the ionization state and corresponding properties.

The values of the $pK_a$ constant can be studied by many different experimental techniques [2]. The capillary electrophoresis (CE) technique is particularly useful, valued mainly for its accuracy, extremely low consumption of the sample and reagents, automation, as well as the possibility of simultaneous analysis of different compounds and complex mixtures, considering the fact that it is a separation technique characterized by high resolution [3,4]. The classic approach is to determine the relationship between the electrophoretic mobility of the analyte, which directly depends on the degree of ionization (Eq.1), and the pH value of the buffer being the separation medium [3]. The model of the nonlinear Boltzmann function (Eq.2) is fitted to the obtained dependence. The inflection point of obtained sigmoidal curve points the pH value at which half of the pool of analyte molecules is ionized, i.e. the $pK_a$ value. In this method, the mobility is measured in several buffers that differ in pH, but show the same ionic strength:

$$\mu_{ep} = \frac{L_{tot}\, L_{eff}}{U_{nom}} \bullet \left( \frac{1}{t_{tot}} - \frac{1}{t_{eof}} \right) \quad (1)$$

where $\mu_{ep}$ is the electrophoretic mobility, $L_{tot}$ and $L_{eff}$ are the total and effective capillary lengths (m), $U_{nom}$ is the nominal (programmed) separation voltage (V); $t_{tot}$ is the total (observed) migration time of analyte (s); while $t_{eof}$ is the time measured for the neutral marker of electroosmotic flow (EOF) (s).

The Boltzmann sigmoid in the case of acidic groups is described as:

*Abbreviations:* CE, capillary electrophoresis; FITC, fluorescein isothiocyanate; IS-CE, internal standard-capillary electrophoresis; MST, microscale thermophoresis; OVM, one-value method; TVM, two values method.

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https://doi.org/10.1016/j.microc.2022.107689
Received 14 April 2022; Received in revised form 9 June 2022; Accepted 10 June 2022
Available online 11 June 2022

$$\mu_{eff} = \left[\frac{\mu_{A^-} \bullet 10^{-pK_a}}{10^{-pK_a} + 10^{-pH}}\right] \quad (2)$$

where $\mu_{eff}$ is electrophoretic mobility at a given pH, and $\mu_{A^-}$ is mobility of the ionic form.

Another approach is the Internal Standard Capillary Electrophoresis (IS-CE) method developed by the group of Rosés and coworkers [5–11]. Briefly, this method assumes measurement of the electrophoretic mobility of an analyte and a standard with a known p$K_a$ value only in two buffers, corresponding to partial and total ionization, respectively (Eqs.3 and 4). Most importantly, it is not necessary to know the pH value of the buffers used, but the p$K_a$ value of the standard should be determined with high accuracy and should not significantly deviate from the determined p$K_a$ value of the analyte, for a monoprotic acid:

$$pK_a = pK_{aIS} + logQ - logQ_{IS} \quad (3)$$

given that,

$$Q = \frac{\mu_A - \mu_{eff}}{\mu_{eff}} \quad (4)$$

where $pK_{aIS}$ is the known value of the acidity constant of the reference compound, $\mu_A$ is the effective electrophoretic mobility corresponding to the totally deprotonated state and $\mu_{eff}$ is the effective electrophoretic mobility measured when both compounds are supposed to be partially ionized. To enable accurate analysis, the analyte compound and the internal standard compound should be injected together from the same vial. The value of Q is calculated for the analyte, whereas the value of $Q_{IS}$ is calculated for the internal standard.

The alternatives to IS-CE are the two-values method (TVM) and one-value method (OVM), which show some similarity to it [12–14]. As in IS-CE, the electrophoretic mobility corresponding to partial and complete ionization of the analyte is used, with the difference that it is not necessary to use a standard; instead, it is necessary to measure the pH of the buffer in which partial ionization is observed. In the case of TVM, the mobility of the fully ionized form is measured experimentally, while in OVM it is theoretically predicted on the basis of a previously developed model linking mobility and molecular weight. For monoprotic acids, it is expressed as:

$$pK_a = pH + log\frac{\mu_A - \mu_{eff}}{\mu_{eff}} \quad (5)$$

where $\mu_{eff}$ is electrophoretic mobility – it needs to be measured in the partially ionized state of a molecule at known pH, and $\mu_{A^-}$ is mobility of the ionic form.

Other CE-based methods of acidity analysis embrace spectrophotometric approaches, in which electrophoretic separation is used to purify the analyte and supply it to the detector site, where the spectral properties corresponding to the ionization state are measured [3,15,16].

An interesting example of a modern technique that was used for the first time to determine p$K_a$ values just a few months ago is a microscale thermophoresis (MST) [17]. MST is a quite new and rapidly developing tool mainly used for the analysis of intermolecular affinity [18–22]. It uses the effect of changing the concentration of an analyte due to increase in temperature, known as thermophoresis, thermal diffusion or the Soret effect [23–26]. In practice, the measurement of the change in fluorescence is used, which occurs after the generation of a temperature gradient of 5–10 K in the microscopic volume of the capillary containing the analyte solution. After about half a minute, the equilibrium state is reached, usually characterized by a decrease in fluorescence intensity by several to several dozen percent. This is described by a parameter called normalized fluorescence (Eq.6):

$$F_{norm} = \frac{F_{hot}}{F_{cold}} \quad (6)$$

where $F_{norm}$ is the normalized fluorescence, $F_{hot}$ is the intensity of fluorescence measured during or after forming the microscopic temperature gradient by an IR laser, and $F_{cold}$ is the intensity of fluorescence measured before heating.

Affinity analysis with MST is possible because the achieved $F_{norm}$ value depends on parameters such as molecule size, charge, conformation, and structure of the hydration shell. It has recently been proved that the relationship between $F_{norm}$ and the pH value described by a model analogous to the electrophoretic method (Eq.2) can be used for the determination of p$K_a$ with high accuracy [17]:

$$F_{norm} = \frac{A - B}{1 + e^{(pH - pK_a)/0.45}} + B \quad (7)$$

where A is the $F_{norm}$ standing for the non-ionized state asymptote, B is the $F_{norm}$ standing for the totally ionized state asymptote, 0.45 determines the slope of the linear fragment of sigmoidal curve.

The CE and MST techniques presented above are only selected examples of acidity analysis methods. In the vast majority of cases (the exception is the aforementioned IS-CE method), the key factor for the accurate determination of p$K_a$ is the trueness of pH values characterizing solutions in which the parameters adequate for the given techniques are measured. It is worth noting that the uncertainties of the p$K_a$ values determined experimentally usually do not take into account the error of pH measurement, they are often limited to the repeatability expressed by the standard deviation (SD) or the error in determining the model fit parameter, i.e. the inflection point, which is often misunderstood as the actual p$K_a$ error. The aim of this study was to investigate the real influence of the potential inaccuracy of pH measurement on the obtained p$K_a$ values, and to compare this effect with other potential sources of inaccuracy. For this purpose, two previously described methods were selected: classical method of p$K_a$ determination by means of CE based on a nonlinear model, and the MST method. We used experimental data obtained in the past [12,17], and conducted systematic pH measurements in many buffers with a different ionic composition using six different pH meters.

## 2. Materials and methods

### 2.1. Measurements of pH

#### 2.1.1. Chemicals

Sodium acetate trihydrate, disodium hydrogen phosphate, acetic acid, sodium dihydrogen phosphate and disodium tetraborate decahydrate were supplied by Sigma Aldrich (USA, MO, St. Louis). Orthophosphoric acid was purchased from Merck (Germany, Dramstadt), sodium hydroxide solution from Avantor Performance Materials (Poland, Gliwice), while potassium chloride from Chempur (Poland, Piekary Śląskie). Certified pH standards (calibrators) solutions of pH: 4.00 ± 0.01, 7.00 ± 0.01, 10.00 ± 0.01 at 25 °C, were obtained from Mettler Toledo GmbH (Switzerland, Greifensee). All solution were prepared with deionized water (MilliQ, Merck-Millipore Billerica, USA, 111 MA).

#### 2.1.2. pH meters

The following pH meters were used: Mettler Toledo Expert Pro-ISM - reference pH meter, selected due to the smallest uncertainty of measurements declared by the manufacturer in the instrument specification (Switzerland, Greifensee), Mettler Toledo Micro-Pro-ISM - pH meter I (Switzerland, Greifensee), Beckman Coulter PHi 510 - pH meter II (USA, CA, Brea), Elmetron CP-401; 2607/12 - pH meter III (Poland, Zabrze), Elmetron CP-501; 0502/06 - pH meter IV (Poland, Zabrze), Elmetron CP-501; 0541/06 - pH meter V (Poland, Zabrze). They are shown in Fig. 1.

#### 2.1.3. Procedure

The solutions were prepared according to procedure proposed by P. Nowak et al. [15], according to the recipes presented in Table 1. Their ionic strength was 100 mM.

The composition of pH standards was not specified by their


**Fig. 1.** The pH meters used in the experiment.

manufacturers; the presented volumes refer to the total volume of 50 mL assuming filling up with deionized water.

At the beginning the calibration process was conducted using pH standards (calibrators I-III). Afterwards the pH value of the previously prepared solutions was measured. Then, the pH value of standards (calibrators) was measured. All measurements were done in triplicate. Between the measurements, the electrode was rinsed with deionized water. The room temperature of 22 ± 1 °C was constant throughout the whole experiment.

### 2.2. Influence of the pH measurement-related error on $pK_a$ values

The electrophoretic and thermophoretic data collected during our previous research were used to analyze the pH-related effects [12,17], as described in detail below.

Electrophoretic data.

Five model compounds were selected, all of them represent coumarin derivatives family and their acidity ($pK_a$) differs quite significantly: 4-hydroxycoumarin (4.16), 10-hydroxywarfarin (5.94), 3-hydroxycoumarin (6.95), 7-hydroxycoumarin (8.01) and 6-hydroxycoumarin (9.10) [12]. Based on the $pK_a$ values and the electrophoretic mobility values corresponding to the totally ionized forms, available in the work [12], the mobility-pH relationship models were simulated using the OriginPro program, for all these compounds (Eq.2). From the obtained models, the 14 values of electrophoretic mobility were read. They corresponded exactly to the pH values measured earlier for 14 model electrolyte solutions using a reference pH-meter (Mettler Toledo) - characterized by the lowest uncertainty of pH measurement declared by the manufacturer from all tested pH meters. Then, five new models were fitted for each compound, using the same 14 electrophoretic mobility values previously read, but different pH values that were measured in the same solutions with different pH meters. The inflection point of the obtained sigmoid curves indicated the $pK_a$ value. By comparing the $pK_a$ values obtained with different data sets corresponding to different pH meters, it was possible to investigate the discrepancy in $pK_a$ values resulting only from the pH measurement, excluding the influence of other effects related to the CE technique.

#### 2.2.1. Thermophoretic data

An analogous procedure was used to investigate the potential influence of the pH measurement error on the $pK_a$ values obtained by the MST technique. For this purpose, the $pK_a$ values determined for fluorescein isothiocyanate (FITC) in our previous work were used [17], corresponding to four different temperatures (22, 27, 32, 37 °C). Then, similarly as in the case of electrophoretic data, non-linear models describing the dependance of $F_{norm}$ on pH were obtained (Eq.7). Afterwards, the $pK_a$ values corresponding to various pH meters were determined. The difference was the use of $F_{norm}$ values only for the pH range $> 6$, because the specificity of the MST method for FITC excludes the pH range $< 6$ as ineffective (it is related to the dissociation of another acidic group, which has an opposite impact on the thermophoretic effect [17]). Afterwards, the obtained $pK_a$ values, adequate for various temperatures, were used for a thermodynamic analysis based on the Van't Hoff model:

**Table 1**
Composition of all solutions used in the study, including those prepared in the laboratory and the purchased certified pH standards used for pH meter calibration.

| Solution | Buffer composition [mL] | |
|---|---|---|
| I | $H_3PO_4$ (100 mM) | $NaH_2PO_4$ (100 mM) |
| | 18.52 | 4.80 |
| II | certified pH standard (calibrant) I | |
| III | $CH_3COOH$ (500 mM) | $CH_3COONa{\cdot}3H_2O$ (500 mM) |
| | 14.16 | 10.00 |
| IV | $CH_3COOH$ (500 mM) | $CH_3COONa{\cdot}3H_2O$ (500 mM) |
| | 2.52 | 10.00 |
| V | $NaH_2PO_4$ (100 mM) | $Na_2HPO_4$ (100 mM) |
| | 3.59 | 4.70 |
| VI | $NaH_2PO_4$ (100 mM) | $Na_2HPO_4$ (100 mM) |
| | 1.01 | 13.29 |
| VII | certified pH standard (calibrant) II | |
| VIII | $NaH_2PO_4$ (100 mM) | $Na_2HPO_4$ (100 mM) |
| | 0.12 | 16.25 |
| IX | $Na_2B_4O_7{\cdot}10H_2O$ (50 mM) | NaOH (1 M) |
| | 39.77 | 1.02 |
| X | certified pH standard (calibrant) III | |
| XI | $Na_2B_4O_7{\cdot}10H_2O$ (50 mM) | NaOH (1 M) |
| | 28.67 | 2.13 |
| XII | $Na_2B_4O_7{\cdot}10H_2O$ (50 mM) | NaOH (1 M) |
| | 25.05 | 2.50 |
| XIII | NaOH (1 M) | KCl (1 M) |
| | 0.64 | 4.36 |
| XIV | NaOH (1 M) | KCl (1 M) |
| | 4.55 | 0.45 |

$$pK_a = \frac{\Delta H^\circ}{2.303RT} - \frac{\Delta S^\circ}{2.303R} \quad (8)$$

where R is the gas constant (8.3145 $J{\cdot}mol^{-1}{\cdot}K^{-1}$). Accordingly, the p$K_a$ values determined at various temperatures were plotted against the inverse absolute temperature (1/T) and fitted by the linear function. Subsequently, the enthalpic (ΔH°) and entropic (-TΔS°) terms were calculated from the slope and intercept, respectively. The temperature of 25 °C (298 K) was used to calculate the –TΔS° term.

On the basis of this model, the values of the enthalpy and entropy factors describing the deprotonation of FITC were determined in relation to the data obtained with the use of various pH meters. In consequence, it was possible to estimate the error and variability of these parameters related to the trueness of the pH value.

## 3. Results and discussion

### 3.1. Variability of pH

The pH values measured in all prepared electrolyte solutions using all six pH meters, including the reference pH meter and the others, are shown in Table 2. These values are the average of three consecutive measurements, separated by rinsing an electrode. The significant discrepancy in the pH value across the entire range of the tested solutions is noteworthy. The greatest discrepancies in relation to the reference pH meter were noted for the pH meter III, which gave values lowered by about 0.5 units in all solutions. This observation proves a significant systematic error that could result, for example, from microdamage of the electrode used. The span of the values measured for the remaining five pH meters as the difference between the maximum and minimum, ranged from 0.14 to 0.52, which proves the overall lack of consistency between tested devices. This discrepancy is greatest for the solution XIV expressing highest pH (about 13), which seems understandable considering that all pH meters were calibrated with solutions of pH not exceeding 10.

The direct precision, understood as the repeatability of pH measurements with the same pH meter, after rinsing the electrode and its reimmersion in the same solution, was expressed by the SD values (n = 3) presented in Table 3. Notably, SD values are low, in most cases they do not exceed 0.03. They are similar for all pH meters and show no explicit relationship with the acidity of the tested solutions. This indicates a little significance of accidental effects which could be eliminated by repeating the pH measurements several times in the same solution. In other words, the procedure consisting in a single pH measurement in a given solution does not introduce significant uncertainty, and considering the significant time-efficiency, it should be considered justified.

### 3.2. Influence on p$K_a$ values

The differences in p$K_a$ values obtained for five model compounds according to the procedure described in Section 2.2 are presented in Table 4. As expected, the greatest changes in p$K_a$ were recorded for the pH meter III, which provided pH values burdened with a significant error. Then, the pH meter IV was the worst, followed by pH meter I, pH meter V, and the best pH meter II. The differences in the p$K_a$ values in relation to the reference pH meter, though excluding the pH meter III, still range from + 0.11 to −0.26, which should be considered as significant discrepancies. In particular, it should be admitted that the accuracy of p$K_a$ determination by the classical CE method is generally considered to be very high, amounting to 0.10 or even less [3]. It turns out that the pH meter II is the only one characterized by a fully

**Table 2**
The pH values measured in 14 electrolyte solutions using the six pH-meters.

| Solution | Reference pH meter | pH meter I | pH meter II | pH meter III | pH meter IV | pH meter V | Median | Span* |
|---|---|---|---|---|---|---|---|---|
| I | 2.00 | 1.98 | 1.91 | 1.39 | 2.13 | 2.11 | 1.99 | 0.22 |
| II | 3.97 | 3.88 | 3.99 | 3.46 | 3.93 | 4.11 | 3.95 | 0.23 |
| III | 4.52 | 4.49 | 4.42 | 3.91 | 4.36 | 4.59 | 4.45 | 0.23 |
| IV | 5.27 | 5.21 | 5.21 | 4.67 | 5.10 | 5.32 | 5.21 | 0.22 |
| V | 7.17 | 7.09 | 7.05 | 6.52 | 6.93 | 7.11 | 7.07 | 0.25 |
| VI | 8.09 | 7.96 | 8.05 | 7.43 | 7.86 | 7.98 | 7.97 | 0.23 |
| VII | 7.01 | 6.93 | 7.02 | 6.42 | 6.88 | 7.02 | 6.97 | 0.14 |
| VIII | 8.90 | 8.68 | 8.74 | 8.24 | 8.63 | 8.75 | 8.71 | 0.27 |
| IX | 9.51 | 9.42 | 9.47 | 8.85 | 9.27 | 9.46 | 9.44 | 0.24 |
| X | 10.00 | 9.91 | 9.94 | 9.34 | 9.74 | 9.94 | 9.93 | 0.25 |
| XI | 10.06 | 9.98 | 9.99 | 9.46 | 9.82 | 9.98 | 9.98 | 0.24 |
| XII | 10.46 | 10.42 | 10.46 | 9.85 | 10.25 | 10.42 | 10.42 | 0.22 |
| XIII | 12.14 | 12.01 | 11.93 | 11.45 | 11.80 | 11.98 | 11.95 | 0.34 |
| XIV | 12.91 | 12.78 | 12.66 | 12.11 | 12.39 | 12.57 | 12.61 | 0.52 |

All pH values are the average from three consecutive measurements separated by the step of rinsing an electrode. (*) Span values are presented for five pH-meters, without pH meter III (large error excluding the feasibility of reliable pH measurement).

**Table 3**
Direct precision of pH measurements for the reference and tested pH meters (I-V), expressed as standard deviation (n = 3).

| Solution | Reference pH meter | pH meter I | pH meter II | pH meter III | pH meter IV | pH meter V | Mean |
|---|---|---|---|---|---|---|---|
| I | 0.026 | 0.012 | 0.021 | 0.010 | 0.012 | 0.006 | 0.014 |
| II | 0.038 | 0.020 | 0.051 | 0.006 | 0.000 | 0.010 | 0.021 |
| III | 0.010 | 0.010 | 0.010 | 0.006 | 0.000 | 0.021 | 0.009 |
| IV | 0.025 | 0.036 | 0.051 | 0.006 | 0.012 | 0.010 | 0.023 |
| V | 0.021 | 0.006 | 0.021 | 0.006 | 0.000 | 0.020 | 0.012 |
| VI | 0.010 | 0.076 | 0.056 | 0.021 | 0.010 | 0.010 | 0.030 |
| VII | 0.015 | 0.006 | 0.049 | 0.020 | 0.000 | 0.010 | 0.017 |
| VIII | 0.012 | 0.006 | 0.035 | 0.000 | 0.006 | 0.012 | 0.012 |
| IX | 0.012 | 0.000 | 0.012 | 0.006 | 0.006 | 0.000 | 0.006 |
| X | 0.015 | 0.012 | 0.012 | 0.010 | 0.010 | 0.010 | 0.011 |
| XI | 0.010 | 0.020 | 0.006 | 0.021 | 0.006 | 0.010 | 0.012 |
| XII | 0.015 | 0.017 | 0.020 | 0.006 | 0.010 | 0.006 | 0.012 |
| XIII | 0.000 | 0.006 | 0.067 | 0.006 | 0.006 | 0.026 | 0.018 |
| XIV | 0.026 | 0.010 | 0.045 | 0.006 | 0.006 | 0.023 | 0.019 |
| Mean | 0.017 | 0.017 | 0.032 | 0.009 | 0.006 | 0.012 | 0.016 |

**Table 4**
The differences in $pK_a$ values obtained between the particular pH-meters (I-V) and reference pH-meter, based on the same set of electrophoretic mobility data.

| Compound | pH meter I | pH meter II | pH meter III | pH meter IV | pH meter V |
|---|---|---|---|---|---|
| 4-hydroxycoumarin ($pK_a = 4.16$*) | −0.06 | −0.03 | −0.55 | −0.10 | 0.11 |
| 10-hydroxywarfarin ($pK_a = 5.94$*) | −0.07 | −0.02 | −0.61 | −0.18 | 0.03 |
| 3-hydroxycoumarin ($pK_a = 6.95$*) | −0.08 | −0.03 | −0.62 | −0.19 | −0.03 |
| 7-hydroxycoumarin ($pK_a = 8.01$*) | −0.13 | −0.01 | −0.66 | −0.23 | −0.10 |
| 6-dydroxycoumarin ($pK_a = 9.10$*) | −0.17 | 0.01 | −0.66 | −0.26 | −0.12 |

(*) – the values taken from [12].

**Table 5**
The values of thermodynamic functions describing the deprotonation of FITC, obtained with the reference and tested pH meters (I-V), expressed in the absolute values and percentages of reference data.

| | Reference pH meter | pH meter I | pH meter II | pH meter III | pH meter IV | pH meter V |
|---|---|---|---|---|---|---|
| ΔH° (kJ/mol) | 24.6 | 24.4 | 24.4 | 24.1 | 24.2 | 24.0 |
| -TΔS° (kJ/mol) | 15.5 | 15.2 | 15.5 | 12.4 | 14.8 | 15.8 |
| ΔH° (%) | 100.0 | 99.3 | 99.4 | 98.2 | 98.4 | 97.8 |
| -TΔS° (%) | 100.0 | 98.2 | 99.9 | 79.8 | 95.6 | 102.3 |

satisfactory agreement of the obtained $pK_a$ values with the reference pH meter (from + 0.01 to −0.03), hence, only in this case the assumed accuracy of the CE method seems maintained. For pH meters I, IV and V, the greatest $pK_a$ shifts were recorded for 6-hydroxycoumarin, i.e. the analyte showing the weakest acidity compared to the other compounds ($pK_a$ = 9.10). This is probably due to the intrinsic specificity of the potentiometric method and the aforementioned calibration procedure.

### 3.3. Influence on thermodynamic parameters

Another issue in this experiment was to investigate the potential change in thermodynamic parameters values due to the use of different pH datasets. For this purpose, the procedure described in Section 2.2 was used, based on thermophoretic data. The related MST method recently allowed us to determine the deprotonation enthalpy and entropy values of FITC based on the Van't Hoff plot displaying excellent linearity [17]. The obtained absolute and relative values of these parameters for the individual pH meters are presented in Table 5. In general, the differences in the enthalpy values are small, not exceeding 3%, even for the pH meter III, which previously turned out to be highly inaccurate. The discrepancies in the case of entropy are bigger, but still, apart from the pH meter III, they do not exceed 5%. For the pH meter III, however, they exceed 20%. These results seem to be fully understandable. Enthalpy is calculated from the slope of the Van't Hoff plot, while entropy, from its intersection with the y axis. Enthalpy values can therefore remain unchanged, despite significant changes in the $pK_a$ values themselves, as long as their thermal dependency (1/temperature) remains constant. The fulfillment of this condition entails in turn the change of intercept, and thus the obtained value of the entropy factor. In conclusion, the thermodynamic analysis of the acid-base equilibrium is also susceptible to errors related to the accuracy of pH measurement, however, due to the inherent compensation of some effects, the expected changes in the values of thermodynamic factors, especially enthalpic one, may be relatively small. It is worth noting, however, that this experiment did not analyze the temperature-dependent variability of pH value, but only the effect of using different pH meters with a characteristic uncertainty of indications. The omission of the need to measure pH at altered temperature can be another source of uncertainty for both CE and MST. However, this problem is in our humble opinion too broad to be addressed in this article.

### 3.4. Other effects affecting the uncertainty of $pK_a$

In addition to analyzing the uncertainty of $pK_a$ values directly related to the error of the pH meter, to correctly estimate the total uncertainty of the method, it is important to know other sources of inaccuracy. One such potential source is the change in pH over time from the measurement of pH to the determination of the pH-dependent parameter, the migration time for CE (used to calculate electrophoretic mobility), and $F_{norm}$ for MST measurements. To find out the potential impact of these effects, two additional experiments were carried out. In the first one, the pH values in all previously used solutions were measured twice, immediately after preparation and stabilizing the pH for several hours, and then after a month of storage in a volume of about 50 mL at room temperature. In a second experiment, solutions of known pH were transferred to vials used in the CE technique in a volume of 1.4 mL, which were also stored at room temperature, however, not isolated from the ambient air hermetically. In this case, the pH values were measured after filling the vials and again after 4 and 22 h. In both experiments, the reference pH meter with the highest declared accuracy was used. In the

case of measurements in vials, a special microelectrode was used. Additionally, two types of vials were compared: glass - compatible with the Beckman MDQ instrument, and plastic - compatible with the Beckman PA800 plus instrument. The detailed results of these experiments are provided in Tables S1, S2, and Fig. S1 in the Electronic Supplementary Material (ESM).

Briefly, the estimated averaged pH change during storage of the sealed bulk solutions was 0.04 per month, while for the non-hermetic low volume CE vials, around 0.01 per hour for both vial types. These values seem to be of great importance for estimating the total uncertainty of the p$K_a$ values obtained with the CE methodology. In particular, a change in pH in vials sometimes seems unavoidable, as programmed and sequentially triggered electrophoretic analyzes often take several hours or even longer. One should also take into account that pH stability is inherently related to buffering capacity, which may vary significantly.

In the case of CE, the reliability of the p$K_a$ value is also determined by uncertainties in the determination of the electrophoretic mobility values, which may be associated with many different effects. These include uncertainty of migration time of the analyte and the EOF marker, inaccuracy related to undesirable interactions of the analyte and the EOF marker with the electrolyte components and inner wall surface, the divergence of the actual and assumed ionic strength of the electrolyte, as well as a number of phenomena related to the generation of Joule heat after the application of high voltage and insufficient temperature control: change in viscosity, local distortion of electric field strength, ramping of the applied voltage and change in ionization resulting from the increase in temperature [27]. The solution of this problem may be the use of known methods for correcting electrophoretic mobility values which, however, are often quite complicated and time-consuming. An alternative approach is to rationally consider these effects when estimating the total uncertainty of the p$K_a$ value.

### 3.5. *Estimating the total uncertainty of p$K_a$*

It is obvious that in order to reduce the p$K_a$ error due to inaccurate pH measurement, the use of most accurate pH meter available is pivotal. However, even in such a case, there will be some significant uncertainty introduced, which should be verified each time. A simple self-monitoring procedure has been proposed for this purpose. Its essence is to properly calibrate the pH meters on fresh, certified standard solutions (calibrators), carry out the assumed pH measurements in working solutions, and then, immediately after completing these measurements, measure the pH value directly in the standard solutions previously used to calibrate the pH meter (mostly these will be measurements in buffers with pH 4.00, 7.00, and 10.00). We have found out a quite clear dependence between the average discrepancy of the pH values measured in the calibrators against the values declared by the manufacturer, and the resulting change in the p$K_a$ value.

Fig. 2 shows the data obtained for pH meters I-V. The mean absolute change in p$K_a$, averaged for 5 model compounds, resulting from the use of a different pH data set, was taken as y-value. The mean absolute deviation of the pH values measured in the calibrators from the declared values was, in turn, taken as x-value. This relationship is linear and could be described by the empirical formula: y = 1.08x + 0.03. This indicates that the expected p$K_a$ error related to the pH meter inaccuracy is approximately equal to the average pH measurement error revealed for the calibrators. This result is not surprising considering that the p$K_a$ value is read as the position of the inflection point of the electrophoretic mobility curve in relation to the pH axis. Therefore, the proposed self-control procedure can be considered an effective mean for reliable estimation of the p$K_a$ uncertainty related to the pH measurement.

The above considerations led us to propose a general formula for estimating the total uncertainty of the p$K_a$ values obtained by the classical CE method, resulting directly from the uncertainty of the pH value. A calculation method was proposed, quite often found in simplified

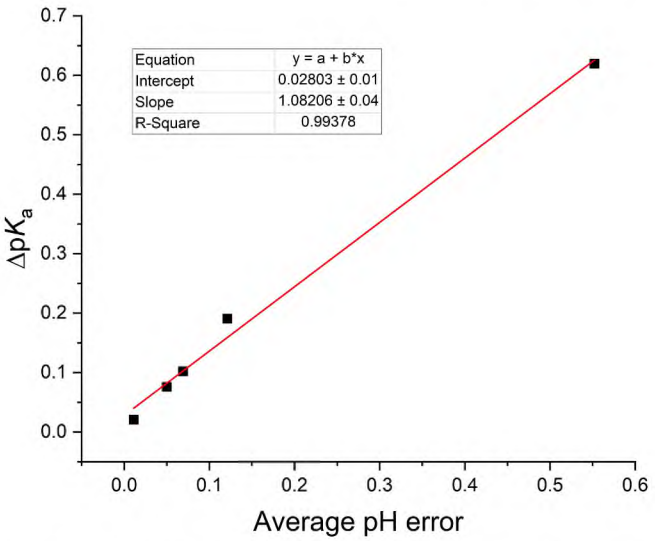


**Fig. 2.** The model relationship between the shift of p$K_a$ (caused by alteration of the input pH data) and average pH error measured in calibrant solutions (pH = 4.00 ± 0.01, 7.00 ± 0.01 and 10.00 ± 0.01).

estimations of the total uncertainty of parameters determined experimentally, as the root of the sum of squares related to the individual partial uncertainties:

$$u_{pK_{a(pH)}} = \sqrt{u_a^2 + u_b^2 + u_c^2 + u_d^2} \quad (9)$$

where: $u_{pKa(pH)}$ is the total uncertainty of the p$K_a$ related to pH; $u_a$ is the partial uncertainty related to the pH meter error, which can be estimated based on the self-control procedure described previously; $u_b$ is the partial uncertainty related to random effects, which can be expressed by SD obtained for consecutive pH measurements in the same solution; $u_c$ is the partial uncertainty of pH related to storing solutions after pH measurement in a hermetic vessel in a large volume (in order to ensure high accuracy of the method, it should be avoided and pH measurements should be performed immediately before electrophoretic measurements); and $u_d$ is the partial uncertainty of pH related to their storage in vials placed on a buffer tray inside the CE instrument. The values of the calculated total uncertainties for the tested pH meters and the total expanded uncertainties, along with the source data, are presented in Table 6.

As shown in Table 6, the estimated total uncertainties of p$K_a$ are large, even after assuming that the measurement of electrophoretic mobility takes place immediately after the pH measurement ($u_b$ and $u_c$ equal to zero). This can be seen especially from the expanded

**Table 6**
Partial and total uncertainties of p$K_a$ values related to pH, obtained for individual pH meters.

| | pH meter I | pH meter II | pH meter III | pH meter IV | pH meter V |
|---|---|---|---|---|---|
| $u_a$* | 0.102 | 0.021 | 0.620 | 0.191 | 0.076 |
| $u_b$** | 0.017 | 0.032 | 0.009 | 0.006 | 0.012 |
| $u_c$*** | – | – | – | – | – |
| $u_d$*** | – | – | – | – | – |
| $u_{pKa}$ | 0.104 | 0.039 | 0.620 | 0.191 | 0.077 |
| $U_{pKa}$ | 0.207 | 0.077 | 1.239 | 0.382 | 0.154 |

(*) – obtained as the average difference of the pH values obtained for three calibrant solutions from the declared true values; pH values were the average of three consecutive measurements; (**) obtained as the average SD of the pH values determined for a given pH meter in all tested solutions; (***) – not applicable, it was assumed that the solutions were used for electrophoretic separation immediately after pH measurement; $U_{pKa}$ – expanded total uncertainty (k = 2, confidence level of 95%).

uncertainty estimates, which correspond to an increased confidence level (up to 95%). They range from 0.08 for the most accurate pH meter II, 0.15 for the pH meter V, 0.21 for the pH meter I, to 0.38 for the pH meter IV, omitting the distinctly different pH meter III.

These values should be interpreted with caution. On the one hand, there is no doubt that the effects related to the inaccuracy of the pH measurement are significant, the resulting p$K_a$ inaccuracy may be even several times greater than the often assumed accuracy of the CE method as a whole. On the other hand, it is obvious that the principles of good laboratory practice should encourage the use of modern and proven pH meters, for which the error is as small as possible. In this respect, all pH meters except II could be considered to not comply with these guidelines. However, their use for the purposes of this experiment allowed one to illustrate the effects discussed here and to outline the scale of the potential problem.

Furthermore, as discussed above, the total uncertainty of the p$K_a$ values determined by the CE technique is not limited to the pH-dependent effects. It is worth mentioning that on the basis of our previous work, it can be concluded that the average p$K_a$ error related only to thermal effects is at least 0.05 [28], and it does not yet contain all sources of inaccuracy. Therefore, there is no doubt that the overall accuracy of the p$K_a$ determination method at the level of 0.10 or less, even in the most optimistic scenario, seems to be unachievable.

## 4. Conclusions

This work proves that the reliability of pH measurements should always be verified and its influence on p$K_a$ values obtained by CE, MST and other experimental techniques, should not be ignored. Even assuming the use of an accurate pH meter, the uncertainty associated with the pH measurement cannot be completely eliminated. It should be controlled and limited. A simple method is to validate the credibility of a pH meter by measuring pH in standard solutions previously used for calibration, immediately after finishing target measurements. According to the outcomes obtained herein, the mean pH error measured in this way roughly translates into the same numerical p$K_a$ error. Another sources of inaccuracy are the random effects which can be estimated by the SD values obtained for consecutive measurements in the same solution, separated by the electrode rinsing step. In addition, one must take into account the variability of pH over time, which may directly result from the actual buffer capacity of the electrolytes used. Accordingly, the measured mean pH change of the tested buffer solutions in the CE compatible vials, of about 0.01 pH unit per hour, is of interest and potential influence on the final p$K_a$ values as well.

To estimate the total uncertainty related to pH, we suggest using Eq.9 and expressing the total expanded uncertainty at the 95% significance level ($k = 2$), which may be more informative regarding the actual scale of the effects discussed: Nevertheless, the total uncertainty of methodology should also include other important effects: Joule heat, ionic strength, and the accuracy of the mobility determination procedure. A thorough analysis of these effects was not the aim of this work. However, it can be predicted that the actual accuracy of the classical approach to determining p$K_a$ by means of CE will be most likely in the range of 0.15–0.25 pH unit, not less. The use of simplified TVM and OVM methods based on only two electrophoretic mobility values does not eliminate the problem of pH measurement, thus these methods cannot be considered as alternatives in this respect. Here, however, the IS-CE method developed by Rosés and co-workers may be very helpful [5–11], as it does not require pH measurements at all, but requires meeting other conditions, e.g. finding a suitable standard with an exactly known p$K_a$ value. In some situations, the use of IS-CE may lead to more reliable data than the classical method.

The accuracy of the MST method, because of the general similarity of both methodologies, seems to be exposed to pH-dependent effects to an extent similar to CE. In the case of thermodynamic analysis, regardless of the choice of the experimental technique, some errors related to pH may be compensated. In particular, this may be the case when determining the enthalpy of deprotonation, which does not depend directly on the absolute p$K_a$ values but on their temperature change.

Noticeably, the approach presented in this article is consistent with the idea of green chemistry. The previously collected electrophoretic and thermophoretic data were reused to deliver new findings. They were accompanied by the indispensable measurements allowing to predict potential pH variations, without a need to use advanced instruments. Therefore, the number of required experiments, waste production and energy intake were appreciably reduced. It highlights a need for publishing comprehensive scientific data in the open-access format, taking into account their second potential utilization.

### *CRediT authorship contribution statement*

**Paweł Mateusz Nowak:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. **Iwona Biel:** Investigation, Data curation, Visualization, Writing – review & editing. **Gabriela Kózka:** Investigation, Data curation, Visualization, Writing – review & editing. **Maria Klag:** Investigation, Data curation, Visualization, Writing – review & editing. **Michał Woźniakiewicz:** Conceptualization, Methodology, Supervision, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### *Acknowledgments*

This research was funded by National Science Centre, Poland (P.M. N.; OPUS, 2020–2024, grant no. 2019/35/B/ST4/01022).

### *Data Availability Statement*

Raw data supporting the manuscript content are publicly available using the DOI link: https://doi.org/10.26106/cc71-jj37.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.107689.

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