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Development of Methods for the Determination of pK_a Values

Jetse Reijenga, Arno van Hoof, Antonie van Loon and Bram Teunissen

Department of Chemical Engineering and Chemistry, Eindhoven University of Technology, Eindhoven, The Netherlands.
Corresponding author email: j.c.reijenga@tue.nl

Abstract: The acid dissociation constant (pK_a) is among the most frequently used physicochemical parameters, and its determination is of interest to a wide range of research fields. We present a brief introduction on the conceptual development of pK_a as a physical parameter and its relationship to the concept of the pH of a solution. This is followed by a general summary of the historical development and current state of the techniques of pK_a determination and an attempt to develop insight into future developments. Fourteen methods of determining the acid dissociation constant are placed in context and are critically evaluated to make a fair comparison and to determine their applications in modern chemistry. Additionally, we have studied these techniques in light of present trends in science and technology and attempt to determine how these trends might affect future developments in the field.

Keywords: review, history, dissociation constant, pK_a , pH

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Introduction

History

The centennial of the concept and of the quantitative measurement of pH was celebrated not long ago.^{1–3} By now, accurate and precise determination of pH seems to have few secrets left. The related concept of the acid dissociation constant (pK_a) as a substance property is recognized as being among the most commonly used parameters in modern-day chemistry. Both pH and pK_a are essential for understanding the behavior of chemical substances in everyday life. This realization came gradually and under different names. The first notion of acids comes from ancient Greece, where people noticed that some substances tasted sour. This is also where the word “acid” comes from; it is derived from the Greek word “oxein” which in Latin is “acere,” meaning “to make sour.” It was also noted that acids and bases could color certain substances.

The first description of an equilibrium constant came from Guldberg and Waage in 1864 in their “law of mass action.” With the help of van’t Hoff’s work on osmotic pressures, Ostwald formulated his “dilution law” for solutions. Measurements of osmotic pressures and conductivity of solutions gave insight into the degree of dissociation.⁴

It was in 1907 that Henderson published a paper first describing the relation between the hydrogen ion $[H^+]$ and the composition of a buffer.⁵ In 1909, Sørensen⁶ proposed the more convenient pH and pK_a terms as the negative logarithm of $[H^+]$ and the equilibrium constant, K , respectively. Although Henderson defined K in terms of a concentration ratio in 1908, it was not until 1916 that Hasselbalch⁷ proposed their now famous equation (1), which remains the most commonly used equation to calculate pK_a values: the Henderson-Hasselbalch equation. It relates pH and pK_a to the equilibrium concentrations of dissociated acid $[A^-]$ and non-dissociated acid $[HA]$ respectively:

$$pH = pK_a + \log ([A^-]/[HA]) \quad (1)$$

In many experimental methods to determine pK_a values, a certain parameter is measured as a function of pH. This results in a characteristic sigmoid curve (Fig. 1) from which the pK_a may be determined by locating the inflection point. Generally speaking, for acidic components X ranges from a bulk property of a solution of

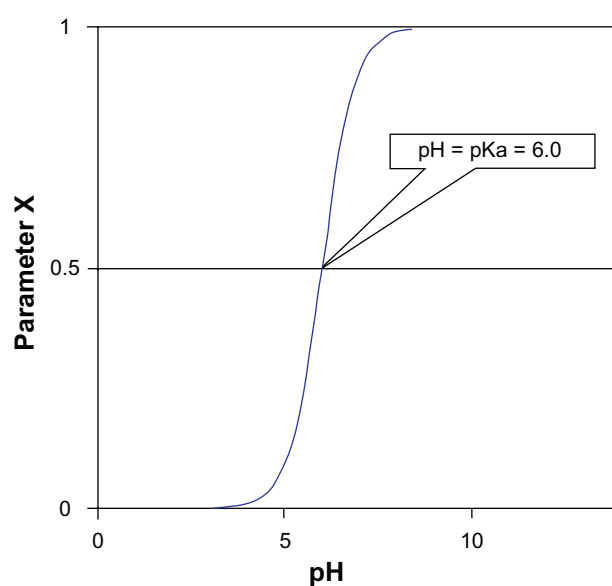


Figure 1. A classic example of a sigmoidal curve created by plotting a measured quantity versus pH. The inflection point corresponds to pK_a .

only non-dissociated acid to the situation where only dissociated acid is present. A more specific example is represented if parameter X denotes the degree of dissociation α ranging between 0 and 1, the inflection point will be at $\alpha = 0.5$, where pH equals pK_a . The degree of dissociation α for acids is defined as:

$$\alpha = [A^-]/([HA] + [A^-]) \quad (2)$$

Combining Equations 1 and 2 leads to (for anions):

$$\text{Log} (\alpha/(1 - \alpha)) = pH - pK_a \quad (3)$$

However, for concentrated solutions or pK_a 's near the extremes of the pH scale, it has been shown that there are significant differences between the predicted and actual concentration of the hydrogen ion.⁸

Since the Henderson-Hasselbalch equation only gives accurate results for dilute acids in aqueous solutions, another formula for the quantification of acid strength was developed by Hammett.⁹ Instead of measuring the concentration of the species present in solution, Hammett described the strength of an acid as the relation between the hydrogen ion activity (a) and the activity coefficients (f) of the various species in solution. However, difficulty associated with accurate determination of the parameters in this model has kept it from being as widely used as the Henderson-Hasselbalch equation.

Influences on pK_a

In spite of being universally referred to as a constant, the dissociation constant pK_a is not in fact truly constant; it depends on temperature (T), ionic strength (I), and the solvent dielectric constant (ϵ).

The temperature dependence of the pK_a values is sometimes fitted to a van't Hoff type equation:

$$d \ln K_a / dT = \Delta H / RT^2 \quad (4)$$

Here ΔH is the enthalpy change of dissociation and R is the gas constant. The value of ΔH is often negative. In the case where ΔH is independent of temperature, plotting pK_a versus $1/T$ will result in a linear plot. This is commonly used to interpolate to other temperatures.^{10,11} Extrapolation of experimental data is, of course, not advised. We can understand this by realizing that ΔH can be re-written as:

$$\Delta H = \Delta G + T\Delta S \quad (5)$$

Here ΔG is the Gibbs free energy change and ΔS is the entropy change. The linear relation mentioned only holds if $\Delta H \gg T\Delta S$. Moreover in some cases ΔS also depends on temperature, resulting in even more non-linear dependence of pK_a on $1/T$. An example of the pK_a temperature dependency is shown in Figure 2.

Measured pK_a values also depend on the ionic strength of the solution under investigation. The ionic strength is defined as the sum of concentrations (c) of all ionic species, corrected for their charge number (z):

$$I = \frac{1}{2} \sum z^2 \cdot c \quad (6)$$

At extreme values of the pH scale, the contribution of H^+ or OH^- should also be included. Activity coefficients (γ) of different ionic species in solution strongly depend on ionic strength (Debye-Hückel theory). Because pK_a depends on the activity coefficients, the ionic strength will also influence pK_a , especially at higher charge numbers (z). An example of this dependency is shown in Figure 3.

Because the acid-base equilibrium occurs in solution, the solvent composition can also influence the pK_a values (Fig. 4). Measuring pH of mixtures of organic solvents and water is in itself far from straightforward, and are beyond the scope of the present contribution. When regarding an acid dissociation reaction, three thermodynamic steps are considered: (1) the dissolution of the acid from the solvent into the gas phase, (2) the dissociation of the acid into the ions, and (3) the solution step of the ions into the solvent. In the first and the last step, the solvent is involved. When considering the influence of the solvent, the difference between the solvation energies of

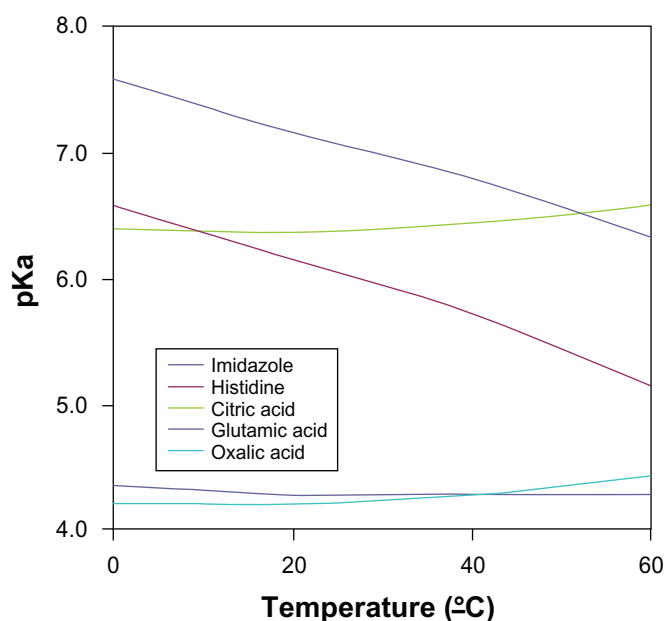


Figure 2. Various pK_a values as function of temperature. Each component has an own dependency. Values in water at infinite dilution, data from Everaerts et al.¹²

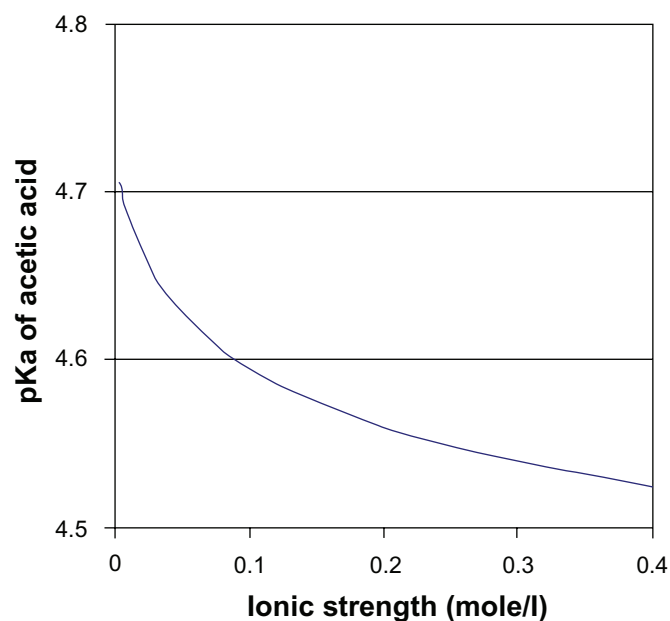


Figure 3. The dependency of the pK_a of acetic acid on the ionic strength, at 18 °C in water. Data from Cohn et al.¹³

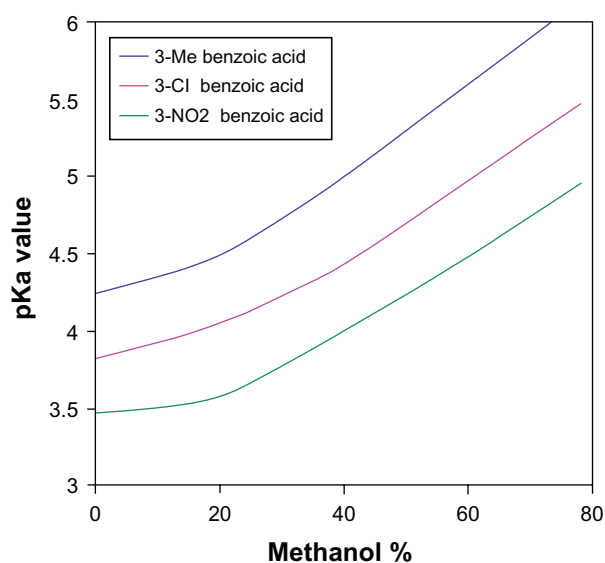


Figure 4. The dependency of pK_a of different Benzoic-acids on the solvent composition, methanol in water at 25 °C and $I = 0.02$ M. Data from Sarmini and Kenndler.¹⁴

the acid and the dissociated acid influences the final pK_a value. Hence the pH range of one solvent may differ from that of another solvent.

It must be stressed that when performing pK_a measurements, all parameters mentioned will have to be kept constant in order to produce a meaningful result.

It is often overlooked that this is also the case with pH measurements. Before use, pH meters should be calibrated under the same conditions of temperature, ionic strength, and solvent. Reporting pK_a values in literature also require that exact conditions of temperature, ionic strength, and solvent be stated. If these details are omitted, it cannot be assumed they were measured in water, at room temperature, and extrapolated to infinite dilution.

Many of the techniques mentioned in the subsequent section measure solutions in which not only the analyte is present, but also various other components for buffering. One has to be sure that there is no ionic

or other interaction between the analyte and these other components.

Complications and overview

Depending on the sample and matrix under investigation, the choice of technique can be a difficult one, even for the case of monovalent ions, to which this paper is limited to. For multivalent components, matters are more complicated as the pK_a differences are smaller. This is because all methods, except perhaps for nuclear magnetic resonance (NMR), require curve fitting in addition to the normal calculation procedure for the respective technique. Investigating both acidic and basic pK_a of amphoteric compounds also requires curve fitting in a much broader range. For such compounds, especially peptides and proteins, the isoelectric point is often relevant for identification purposes but beyond the scope of this paper.

Figure 5 displays an overview of the first time these techniques were used for this purpose.

Techniques

Potentiometry

The simplicity and low cost of potentiometric titration has made it one of the most commonly used methods for pK_a determination. In a potentiometric titration, a known volume of reagent is added stepwise to a solution of analyte. The change in potential (E) upon reaction is consequently measured with the use of two electrodes, an indicator, and a reference electrode. These are often integrated in what is now commonly called a combined pH electrode.

Plotting the potential versus volume subsequently gives rise to a sigmoid curve, where the inflection point gives the potential at equilibrium. With the use of standards with known pH, this potential can be linearly converted into a pH, equaling pK_a . Sigel et al have, however, correctly noted that there are multiple

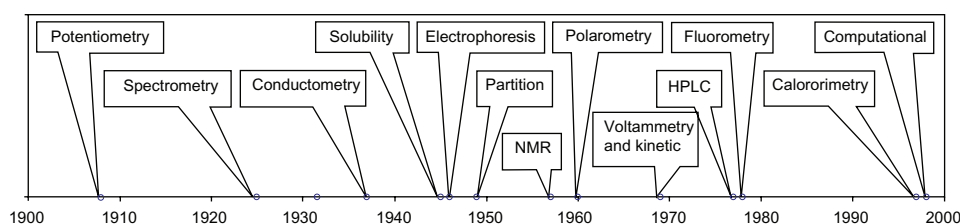


Figure 5. Timeline of the first notion of the various techniques to determine pK_a (dissociation constant, acid strength).



acid strength-models in use, each giving a different H⁺ concentration for a given pH.¹⁵

Increasing understanding of electrochemical processes in the late 19th century gave rise to the first potentiometer. This consisted of a platinum working electrode and a reference electrode and used a H₂/H⁺ equilibrium to determine its standard reduction potential, which is by definition 0 V. The first description of the use of a setup to determine equilibrium constants was made by Denham in 1908.¹⁶

It was not long until the cumbersome hydrogen electrode was replaced by the familiar glass electrode. Completely automated and self-adjusting pH-measuring equipment by Keeler is known from as early as 1928.¹⁷ Because of difficulty in obtaining reliable results, the first working pH-meter was only constructed in the 1930's.¹⁸

Although the glass electrode has become widely used, it has been proven that at higher temperatures or more extreme pH's the results deviate from theoretical predictions. The "alkaline-error" is certainly the most widely known. Because temperature influences not only the measurement but also the pK_a itself, it is of vital importance to conduct the titration at constant temperature. A good review about the various errors of the electrode itself is given by Gardiner¹⁹ while Bennett²⁰ discusses the various ways to determine pK_a from the measured potential.

Traditional potentiometric titrations have a lower concentration limit of about 10⁻⁴ M, however various methods were developed to extend this range. Another practical complication is the pK_a-measurement of substances with a low water-solubility. One example is extrapolation of measurements in solvent mixtures.²¹ These require, however, a relatively high co-solvent concentration, and are inherently inaccurate and time consuming. An alternative approach using surfactants is proposed²² which avoids tedious extrapolating while giving the same level of accuracy (± 0.2 pK_a value).

A precise determination of the pH from the titration slope has also been difficult in the earlier period of its use. However, over the years various software programs have become available to minimize most of the previous mentioned errors. Potentiometric titration requires a relatively large amount of sample compared with separation methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

Completely automated potentiometric pH meters for a wide range of applications and with complicated calibration software are widely available. Because of this and the simplicity and relatively low costs associated with the potentiometric pH-meter, it will probably remain in use in the foreseeable future. This of course only holds for those analytes that are available in sufficient quantity and purity.

Conductometry

The determination of acid dissociation constants by conductometry relies on the assumption that strong electrolytes are completely dissociated at all concentrations, while weak electrolytes only attain complete dissociation at infinite dilution. A measurement of the conductivity of a sample yields a value that is the sum of the independent contributions of all ions present in the solution:

$$\Lambda = \sum \lambda_i \quad (7)$$

Here, Λ represents the equivalent conductance and λ_i represents the specific conductivity contribution of ionic species *i*. The equivalent conductance is dependent on the number of ions in solution, and reaches its limiting value Λ^0 at infinite dilution, where, by definition, activity coefficients are unity.

Λ is a measurable quantity and Λ^0 can be obtained from extrapolation in a plot of Λ versus the square root of the analytical concentration as shown in Figure 6. It can also be seen from Figure 6, however, that this linear

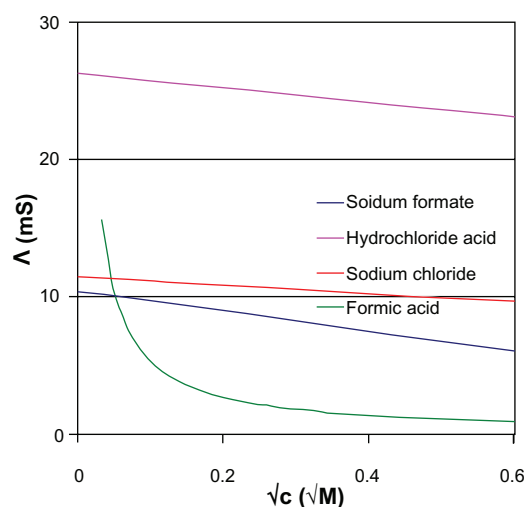


Figure 6. Plots of Λ versus \sqrt{c} required for pK_a determination of formic acid at room temperature in water. Data from Saxton and Darken²⁴ and Landolt-Börnstein et al.²⁵



extrapolation does not hold for weak acids (or bases). This is because for these electrolytes, the assumption that all ions are independent of their counter ions does not hold as these species are not fully dissociated.

Though the limiting conductance of a weak electrolyte cannot be directly extrapolated, their value can still be obtained quite readily. Even for weak electrolytes, the Kohlrausch law²³ of the independent migration of ions holds. This law can be stated as meaning that at infinite dilution, each ion makes a specific contribution to the conductivity regardless of the ions associated with it. It is not possible to measure the limiting conductance of ions directly, but Λ^0 for a salt may be expressed as a linear combination of its ionic components.

Expressing the limiting conductance of a salt in terms of ionic contributions is useful as it allows the calculation of the limiting conductance of a weak electrolyte. In order to do this, the limiting conductance of other relevant salts of a strong electrolyte is extrapolated. For a weak acid (formic acid) relevant plots are shown in Figure 6. One would obtain the ionic contributions for the limiting conductance of the acid from the salt of its conjugate base and from hydrochloric acid, subtracting the value for sodium chloride to eliminate the ionic contributions of the sodium and chlorine counter ions in the reference compounds. This yields the sum shown in Equation 8:

$$\Lambda_{\text{HA}}^0 = \Lambda_{\text{NaA}}^0 + \Lambda_{\text{HCl}}^0 - \Lambda_{\text{NaCl}}^0 \quad (8)$$

Once the limiting conductance is known, the degree of dissociation (α) for a weak electrolyte is given by $\alpha = \Lambda/\Lambda^0$. An apparent dissociation constant K' can be obtained directly from the degree of dissociation using Ostwald's dilution law by expressing it in terms of a concentration quotient, as shown in Equation 9.²⁶ In order to obtain the true thermodynamic acid dissociation constant K_a , one has to correct for the activity of the ions:

$$K' = \Lambda^2 \cdot c / (\Lambda^0 \cdot (\Lambda^0 - \Lambda)) \quad (9)$$

Equation 9 yields the dissociation constant for a given analytical concentration (c) and a series of measurements would yield the dissociation constant as a function of ionic strength. It is worth noting that this determination does not require knowledge of the pH of the solution, making it easily applicable

to non-aqueous systems where such a measurement of pH would be impracticable. It also means that the method, unlike many others which express their measured quantity as a function of pH, is not constrained by the precision of the pH electrode.

The conductometric method offers a relatively fast and reliable method of determining the $\text{p}K_a$ and is also capable of attaining a high degree of precision with $\text{p}K_a$ deviations as small as ± 0.01 – 0.03 units.²⁷ The main downside of the method is that conductometric measurements are nonspecific, ie, different ions cannot be measured separately. This requires working with pure compounds.

Foundational work that enabled the development of the conductometric method was undertaken by Friedrich Kohlrausch. Among the key ideas he developed was the use of alternating current to prevent electrolysis during conductivity measurements. His work examining a variety of electrolyte solutions led to the law of independent migration of ions that is ascribed to him. Building on this early work, and further contributions by Ostwald and Arrhenius, the method reached maturity during the late 1930s, and the accuracy of the method approached that of modern methods. The incorporation of Debye-Hückel activity coefficients enabled the calculation of true thermodynamic $\text{p}K_a$ values, and the calculation of these coefficients for a range of ions was published during this period.²⁸ In contrast to the earlier period, which focused mainly on obtaining thermodynamic dissociation constants, experiments performed after this period were additionally concerned with correlating this data with the structure of the organic acids being studied.²⁹

Work on the conductometric method came to a virtual standstill after the outbreak of World War II, and further research was practically abandoned after the end of the war. Renewed activity would not come until the 1970s, when two new conductance equations were published. These equations enabled the study of asymmetrical electrolytes, and even mixtures of electrolytes.^{30–33}

Since then, it has been remarked that the spectacular interest for the method that existed during its early period has waned with time, leaving the subject a somewhat unfashionable topic of research. With the developments made over its history, however, the method has achieved a high degree of precision.^{26,34,35}

Voltammetry

In voltammetry, a changing potential is applied over a sample solution and the resulting current is measured. When the potential reaches the reduction potential of the analyte this will give rise to an increase in current, followed by a decrease due to depletion of the molecule. In the case of cyclic voltammetry, for example, this will lead to results much like those shown in Figure 7.

A typical voltammetry setup usually consists of 3 electrodes: reference, working, and auxiliary electrode. The working and auxiliary electrodes act as the anode and cathode, respectively, while the reference electrode acts as a fixed point to measure the applied voltage. Modern-day voltammetric methods can be extremely sensitive; with specific techniques it is possible to accurately measure very low concentrations.^{36,37}

The earliest use of voltammetry was the use of a dripping mercury electrode in 1922 by Jaroslav Heyrovsky to develop the first polarograph.³⁸ As early as 1941, voltammetric methods were deployed to determine local pH at the electrode surface.³⁹ However the use of voltammetry to specifically determine bulk pK_a values came into use in the 1960s.⁴⁰

When the pK_a of a substance is being determined voltammetrically, one could in principle measure the electrochemical response of the molecule itself.⁴¹ However, accurate knowledge of the electrochemical behavior of the sample substance is required and

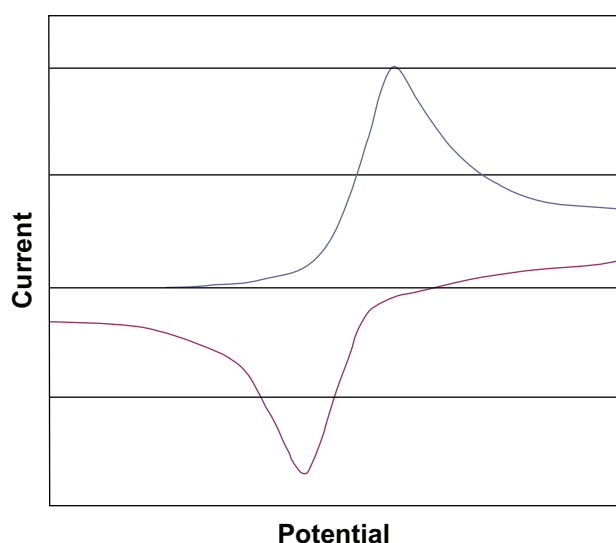


Figure 7. Example of a cyclic voltammetric curve. The upper line represents an increase in potential, the lower line the following decrease.

therefore a reference with known characteristics is usually added. The shift of the peaks of the reference upon addition of the acid is then used to determine the pK_a value.^{42,43} Care has to be taken since the reference will influence the ionic strength of the solution. Additionally, its concentration should be in the same order as that of the analyte to give optimal sensitivity.

More recently, advanced techniques have been developed such as those measuring the surface pK_a of self-assembled monolayers.⁴³ Voltammetry has not been used extensively for the measurement of pK_a values. The reason for this is the need for an electro-active molecule which is soluble in a conductive solvent. Also, it is often necessary to recalibrate the apparatus when different samples are used. Besides these “operational” drawbacks, polarography also requires the use of mercury electrodes.

On the other hand, voltammetry has some advantages. It is of particular use in measurements of pK_a values in less polar solvents, something which is often difficult to do accurately with the use of techniques such as potentiometric titration.⁴¹ When optimized, it is usually not very time-consuming and relatively cheap in comparison to other techniques such as NMR, separation methods, and spectrometry.

Another advantage over other techniques is that it is able to conduct a quantitative measurement of different oxidation states.⁴⁴

Calorimetry

All calorimetric methods work by the same principle: a physical or chemical process takes place in a sample and the amount of heat evolved is measured. For the measurement of pK_a values, a technique called Isothermal Titration Calorimetry (ITC) has been used. Here, a regular acid-base titration is carried out inside the calorimeter while the energy needed to keep the temperature constant is measured. It is also one of the oldest analytical techniques. The first recorded model was made by Lavoisier and Laplace in 1783.⁴⁵

In recent years, the ITC-method has been used to measure the dissociation constants of peptides and the influence of binding on the specific ionizable groups. This method also calculates the pK_a indirectly from a measured enthalpy change ΔH .⁴⁶

A related technique was developed which measures the pK_a directly: Isothermal Titration

Microcalorimetry (ITM). Here the reagent for the ionizable groups is added in equivalent amounts and at once. The resulting heat released upon reaction is measured in buffer solutions with different pH values (Fig. 8). By plotting the minima or maxima versus pH, a sigmoid curve is obtained from which the pK_a can be determined from the inflection point.⁴⁷ Uncertainties are in the range of 0.05–0.15 pK_a units depending on the calorimetric technique.⁴⁷

Nuclear magnetic resonance

Before NMR was used to determine acid dissociation constants, the technique was already applied to determine the site of deprotonation of an acid, or the site of protonation of a base. In these cases, the temperature was decreased to such extent, that the acid-base equilibrium was slow on the NMR time-scale, so that two separately peaks would be observed for HA and A^- .

In 1957, Grunwald et al⁴⁸ used NMR to determine the pK_a of mono-, di- and trimethyl-amine, thus determining the chemical shift of the triplet from the protons in the CH_3 group(s) as a function of pH. A linear correlation was found between the chemical shift and the acid-base ratio. Experiments could be carried out in water by using a reference measurement. A sigmoid curve is obtained from which the pK_a was calculated.⁴⁸

Pioneering work was performed by Lee et al,⁴⁹ who determined the pK_a values of a wide range of functional groups with good concurrence towards

existing literature. Based on his work, further knowledge of the pH-chemical shift relation was described and published.⁴⁹

When the pH dependent acid-base equilibrium is fast on the NMR time scale, a mean chemical shift of the HA and A^- groups can be measured. Because the equilibrium is pH dependent, the chemical shift will also change with the pH level. For this situation, the pK_a can be written as Equation 10:

$$pK_a = pH + \log [(\delta_A - \delta_{obs})/(\delta_A - \delta_{HA})] \quad (10)$$

The pK_a value can be determined by plotting the δ -component shown above versus the pH. This yields a familiar sigmoid curve where the pK_a is located at the inflection point.

Instead of performing a continuous titration, a known amount of a strong acid or base is added to different samples (constant volume titration), from which the pH is consequently calculated. This however neglects the influences of the target molecule on the pH and the risk of a systematic error is added.⁵⁰

The main advantage of the NMR technique is that it is possible to measure mixtures, even when impurities are present, because mole fractions are observed instead of the total acid concentration, in contrast to potentiometric titrations. When observing the chemical shift of one characteristic group, no other groups are involved, so even the pK_a values of diprotic acids with pK_a values close together can be observed separately, provided that the chemical shifts do not overlap. These individual constants are referred to as “microscopic pK_a values” and their determination is one of the most promising applications of this technique. Also, performing NMR with 2H , ^{13}C , ^{31}P or other nuclei with an electromagnetic moment can be used to measure the pK_a .

With this characterization method, even more complex molecules, such as enzymes can be fully characterized. So, the pK_a of individual acid sites on complex molecules can be determined, making this a promising technique for further development. This was discovered by Rabenstein and Sayer in 1976 who determined microscopic dissociation constants for polyprotic acids by means of curve fitting.⁵¹

The main errors for the NMR method are found to be caused by imprecision of the chemical shift and the pH level, which is calculated and not measured.

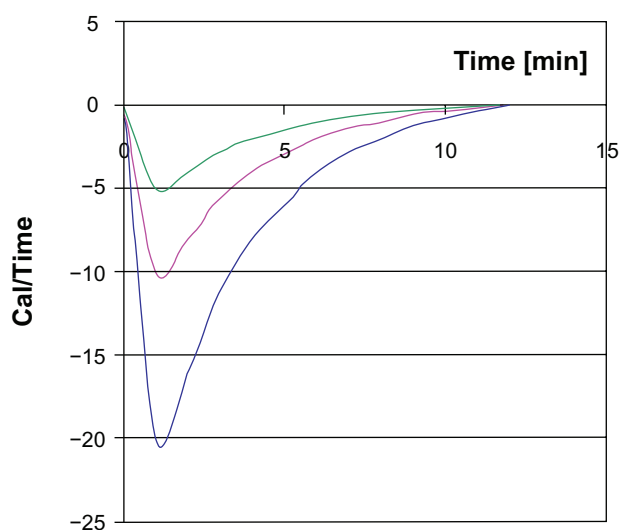


Figure 8. Curves created after a typical direct measurement of pK_a with the use of calorimetry. The amplitude of the minimum at different pH values is proportional to the degree of dissociation.

The pK_a can be measured precisely up to ± 0.05 log units.⁵²

For all NMR measurements, a reference compound is required to acquire a ‘lock’ for the apparatus; this process is called shimming. Because an internal reference can interfere with the acid-base equilibrium, an external reference is recommended, either with an extra measurement or a second co-axial sample tube.

Control of temperature in NMR is often not a problem, but when performing NMR the fact that energy dissipation can occur must be taken to account; locally, at the vibrating nucleus of interest the temperature can rise.

Glaser et al presented the first fully-automated NMR apparatus for measuring the pK_a .⁵² This method also has good concurrence with available data in literature. With this automation and stronger magnets, the NMR technique is a promising technique to measure multiple enzymes at specific (de-)protonation sites within a reasonable time frame. For less complex acids and bases with only one protonation site, however, this technique is rather expensive.

Electrophoresis

In electrophoresis, charged species are separated under the influence of an electric field, migrating with a velocity proportional to their size-to-charge ratio. The ratio of the linear velocity v_i and field strength E is defined as the electrophoretic mobility m_i :

$$m_i = v_i/E \quad (11)$$

The relation with previously discussed conductometric methods and electrophoretic ones is important: in the latter we measure individual mobilities m_i (Equation 11) whereas in the former we measure the sum of mobilities of all ions together (Equation 7). They are interrelated on the basis of individual ions using the Faraday constant F :

$$m_i = \lambda_i/F \quad (12)$$

The use of electrophoresis for the determination of the pK_a value depends on the differing mobilities of the protonated and deprotonated forms of the analyte. As the two forms exist in fast equilibrium, a net mobility is measured that can be related to the degree of dissociation (α) of the analyte. When the degree of dis-

sociation for acids is expressed in terms of mobility, one obtains the relation shown in Equation 13:

$$m_{\text{eff}} = (1 - \alpha) \cdot m_0 + \alpha \cdot m_d \quad (13)$$

Here, m_{eff} is the effective mobility, m_d is the mobility of the fully dissociated species, and m_0 the mobility of the non-dissociated species (which equals zero). For bases, a similar equation is derived. A sigmoid curve is obtained by plotting m_{eff} vs. pH, with the inflection point of $\alpha = 0.5$ at $\text{pH} = pK_a$.

Model equations can be derived for weak acids and bases with any number of ionizable centers by rewriting Equation 13. These models for species with up to three ionizable centers were summarized in a 2004 review article by Poole.⁵² The pK_a may be determined by numerically fitting the resulting relations to a plot of mobility versus pH through nonlinear regression, as shown in Figure 9 for the case of 2-aminopyridine.⁵⁴

The shape of these curves depends only upon m_d and the position depends only upon pK_a , therefore this regression directly yields the pK_a value(s) of interest. Remarkably, few data points are needed to provide an accurate fit for these models, and a precision of ± 0.03 – 0.08 can be achieved depending on the analyte, placing the method roughly on par with potentiometry in terms of precision.⁵⁵

The method has a number of key advantages in comparison to more traditional alternatives.

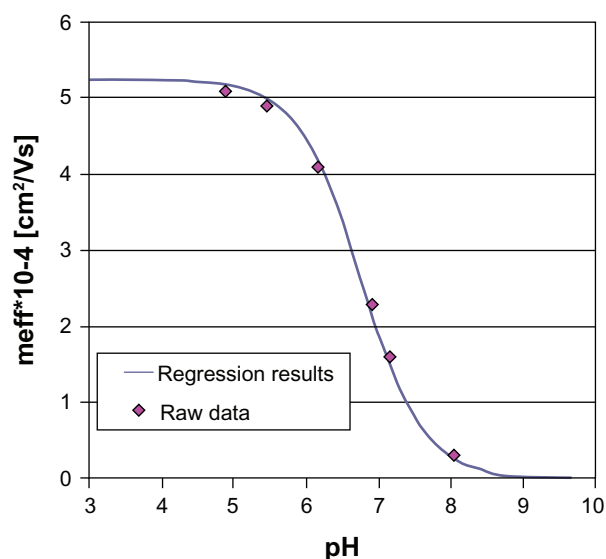


Figure 9. Curve fitted to data for electrophoretic mobility of 2-aminopyridine as a function of pH, data from Poole et al.⁵⁴



Firstly, only a very small amount of the sample is required for a measurement, on the order of microlitres in terms of volume, and with detection limits in the 10^{-6} M range. This allows for the processing of poorly soluble species without much difficulty. Since electrophoresis is a separation technique, impure samples can be readily processed and as the molecules are measured directly, exact knowledge of sample concentrations is not required. In addition, commercially available equipment is capable of automatic operation without requiring modification, allowing large numbers of measurements to be conducted at speed. This makes the method quite suitable for screening applications.⁵⁶ A potential problem of the method is the requirement of preparing buffer solutions for each pH to be measured, since the buffer compounds must be carefully chosen to avoid undesirable interactions between buffer and analyte.

The relationship between the electrophoretic mobility of an acid or base and the pH of the background electrolyte was already considered when Consden and Martin⁵⁶ published their paper on ionophoresis in 1946, which discussed the separation of two analytes based on a difference in their pK_a values. This relation was explicitly applied to the estimation of acid dissociation constants by Waldron in the 1950s⁵⁷ and by Kiso et al in the 1960s⁵⁸ using a paper strip as a supporting medium. However, despite the known advantage of requiring only minute amounts of sample, both methods were found to be impractical in application. Improvements over the next decades would eventually lead to the introduction of the fused silica capillaries.

By the end of the 1980s, isotachopheresis was considered a viable method for the determination of dissociation constants. While the method showed reduced precision in comparison to potentiometric or conductometric methods, this was offset by its faster measurement times and lower sample requirements.⁵⁹ However, determining pK_a values by isotachopheresis was not without disadvantages which are discussed in detail by Beckers et al.⁶⁰ It was remarked that the calculations required are quite laborious as the zones formed during the separation all have different parameters (pH, ionic strength, and even temperature). To avoid the complexities and limitations of isotachopheresis, it was proposed that the simpler method of capillary zone electrophoresis be used

specifically for the determination of mobilities and pK_a values. The potential sensitivity of the method had already been demonstrated⁶¹ and further refinements of the technique would be made over the next few years. A general methodology for the determination of pK_a values by CE was proposed in 1992, and further refined by the addition of terms concerning the rate of electroosmotic flow and a method of handling potential discontinuities between buffer electrolytes.^{62,63} An important step was made by Ishihama⁶⁴ when he studied multivalent compounds and derived relationships between pK_a and zone mobility that were more easily applicable than the earlier equation proposed by Kiso.⁵⁸

Developments over the next decade would include further examinations of the pK_a equations, comparing different regression techniques, and better experimental methods.⁶⁵ Improvements included using charged polymer coatings of the capillary wall to influence the rate, influencing the electroosmotic flow to enable measurements at a lower pH,⁶⁶ and the use of pressure applied over the capillary to shorten measurement times for screening purposes.⁶⁷ Recent work includes efforts undertaken by Fuguet et al⁶⁸⁻⁷⁰ to improve the internal standard method first proposed by Gluck and Cleveland.⁶⁵

High performance liquid chromatography

The first observation that the time of elution can be changed by adjusting the pH level was made by Singhal.⁷¹ By changing the pH, the performance of the HPLC was optimized, in this case ion-exchange chromatography.

With the advance of reversed-phase HPLC in the late 1970s, advanced models were made relating the capacity factor (k) to the degree of dissociation (α). An excellent overview of the principle is presented by Horváth,⁷² who presented a generally applicable equation for acidic components:

$$k = (1 - \alpha) \cdot k_0 + \alpha \cdot k_{-1} \quad (14)$$

in which k_0 and k_{-1} are the capacity factors on the non-ionic and the ionic species respectively, where in reversed-phase HPLC $k_0 \gg k_{-1}$. Plotting k vs. pH gives a sigmoid curve with the inflection point of $\alpha = 0.5$ at $pH = pK_a$. The similarity of Equations 13 and 14 are immediately obvious.



A solid theory was formulated by Foley^{73,74} where the pH dependence of the capacity factor follows the dissociation curve. Again, some recommendations and optimizations were formulated to improve selectivity, retention, and efficiency.

As long as the analyte has a chromophore for detection, this method works fine, even with samples that are not 100% pure, as HPLC is a separation method. On the other hand, the full range of α from 0 to unity cannot always be utilized, because k_0 is often unacceptably large. Of course, the addition of organic modifiers decreases all k values but hugely complicates matters if we desire aqueous pK_a values.

Partition and distribution

The first investigation into the division of a substance between two immiscible solvents was carried out by Berthelot and Jungleich in 1872. They came to the important conclusion that the ratio of the concentration was a constant, e.g., it does not depend on the relative amounts of solvents used. Following up on this research, Nernst concluded in 1891 that the partition coefficient (P) was only a constant if a single substance was considered.⁷⁵

Later insight further revealed that for ionizable components, the partition coefficient depended on the pH of the aqueous phase. In the limiting case where the ionization is completely suppressed by pH (for bases, for example, at high pH) a distribution coefficient (D) can be defined mathematically (for bases):

$$D = [B]_o/[B]_w \quad (15)$$

$$P = [B]_o/([B]_w + [BH^+]_w) \quad (16)$$

Here B is the uncharged and BH⁺ the charged species, subscript o refers to the organic and subscript w, the water phase. Several derivations for pK_a from P and D are found in literature,^{76,77} but all of them result in roughly the same expression (for bases) as they combine Equations 1, 15, and 16:

$$pK_a = \log((P - D)/D) + pH \quad (17)$$

These are usually determined by adding a known amount of sample to an organic solvent/water mixture, followed by the measurement of the concentration in one of the phases.

There are two main techniques used to determine log P. One is the “shake-flask” technique⁷⁸ where an octanol/water mixture together with the sample is shaken in a separation funnel, after which the concentration in one or both of the phases is measured.

Another is the so called Filter probe⁷⁹ which was later improved to the “filter chamber technique.”⁸⁰ These are automated separating funnels connected to a pump which directs the layer of choice to an analytical device (usually a spectrophotometer) and back.

In some specific cases, the technique used to determine pK_a by P is largely unaffected by the choice of solvent.⁷⁷ Although it might seem that log P is a relatively simple quantity to determine, it has been proven that there are still many sources of errors,⁷⁸ one of which being the case of mutual miscibility of the two phases.

The use of partition coefficients to determine pK_a is not used extensively. Partition coefficients are however still very much in use in drug development as they give information about the uptake of a certain drug in various parts of the body.

A particularly illuminating example of this follows. In some surgeries, the body temperature and blood pH values deviate from normal values. This naturally affects the log P value and drug efficacy. Thurlkill et al⁸¹ reported an additional aspect for the case of Fentanyl, a local anesthetic. It was found that its pK_a significantly depends on temperature, which might lead to further complications in drug administration, in other words a temperature and blood gas dependent bioavailability.

Solubility

In 1945, Krebs⁸² described the relationship between pH, pK_a, and the solubility of sparingly soluble weak acids and bases, where a derivation of the Henderson-Hasselbalch equation was used to describe the behavior. A drawback of this method at that time was the solubility of the non-ionized analyte had to be known, which was not often the case.⁸²

This theory was further expanded by Zimmerman et al⁸³ who made mathematical derivations by which it was possible so determine the solubility of the neutral analyte and the pK_a. By these means, the solubility of the neutral compound was no longer required and the use of solubility data to determine the pK_a was much more applicable.⁸³



A derivation of the Henderson-Hasselbalch equation allows us to determine the pK_a from solubility data, the graphical representation is shown in Figure 10.

$$\log S = \log S_0 + \log (10^{-pK-pH} + 1) \quad (18)$$

Here S_0 is equal to the intrinsic solubility. When $pH \gg pK_a$ or $pH \ll pK_a$ assumptions can be made and linear $\log S/pH$ functions are obtained. By extrapolating these two functions and calculating the intercept, the pK_a can be calculated:

$$\log S = (\log S_0 - pK_a) + pH \quad (19)$$

In the later years, pK_a values for zwitterionic compounds were determined⁸⁵ and a solid method for the bi-functional bases and acids was formulated.⁸⁶ Avdeef⁸⁴ found a special way of determining the pK_a . Under certain conditions the pH does not change under addition of more titrant. This pH is known as the Gibbs pK_a .

Nowadays, the solubility data are used to determine pK_a values for a wide range of drugs, where the pK_a value is of great interest.⁸⁷ The power of this technique is that poorly soluble drug can be analyzed at very low concentrations, in the order of μM , and with a precision up to ± 0.5 in log units.⁸⁴ A drawback is that for most solubility determinations, a time frame of 1–24 hours is required where a measurement with spectroscopy only takes a few minutes.

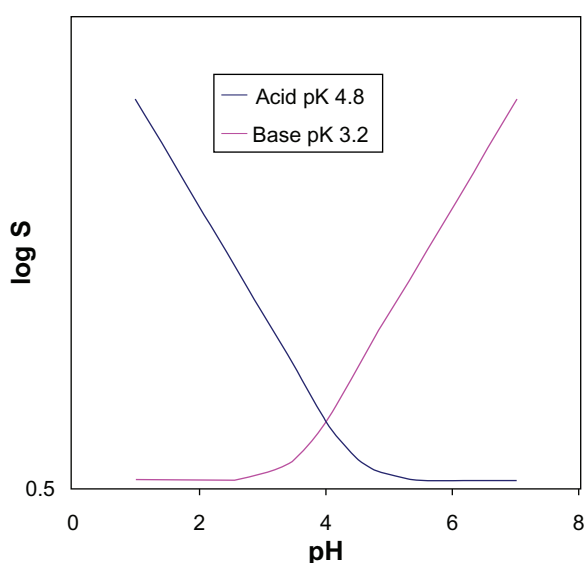


Figure 10. A plot of the pH dependence of the solubility of an acid with pK_a 4.8 and a base with pK_a 3.2.

Therefore, solubility measurements for pK_a determinations can be used for sparingly soluble compounds which have a chromophore near the ionization center.

UV/Vis spectrometry

Well before 1900, it was already known that a change in acidity could lead to color changes of natural substances. Spectrometry with visible light made it possible to measure pK_a values of acid/base indicators and this in turn was extended to the use of UV light to measure pK_a 's of other components.

A requirement for this UV/pH measurement is the presence of a chromophore close to the ionization site in the molecule. If this is fulfilled, then the spectra of the dissociated and the non-dissociated form can be expected to differ. In principle any wavelength can be used for the determination of pK , except at the isobestic point at which wavelength of both forms have the same molar absorptivity. The best choice however is a wavelength at which the molar absorptivities are as different as possible.

The method was further improved by measuring the absorption of two different wavelengths at a variable pH . The ratio in absorption at those two wavelengths is plotted against the pH . In this way, a sigmoid curve is obtained and the pK_a can be determined from the inflection point as normal. One of the wavelengths has to be assigned to the chromophore and the other wavelength should be invariant under change of pH (if this is possible). By using a 2nd wavelength as reference, change in total concentration will not affect the final result and activity/concentration issues and assumptions are bypassed.

This method was introduced by Holmes and Snyder in 1925⁸⁸ when the “decomposition” constant of a dye was measured. This was then further elaborated by Flexser et al⁸⁹ in 1935 by determining different ionization constants. In the 1960s, Wigler et al^{90,91} were the first to determine pK_a values of di-protic compounds.

Up to this point, the pK_a values calculated required prior knowledge of experimental data, such as the absorption coefficients of the neutral and ionized compound. By measuring over a whole wavelength range, Allen et al⁹² were able to determine the pK_a values without this prior knowledge. The measurements could also be done much faster. This method



showed good concurrence with previous single wavelength methods⁹³ and was later highly automated by Saurina et al.⁹⁴

Fluorometry

It can be argued that fluorometry is a specific form of spectrometry as any fluorescence is the result of light absorption, while the reverse is not the case. The use of fluorescence spectroscopy to determine pK_a values depends on the difference in the fluorescence spectrum between a free acid or base and its conjugated form. While fluorometry can potentially be more sensitive and selective than conventional spectrometry, it has the disadvantage of being only applicable to fluorescent analytes. Additionally, it is known that the pH dependence of fluorometry often does not agree with those obtained from spectrometry or other methods. The reason for this is that the former depends on excited state proton exchange as well as the ground-state equilibrium. This causes problems because the pK_a for the ground state and pK_a^* for the excited state can differ quite strongly, and the position of the inflection point in a fluorometric titration will depend on both values as well as the kinetics of the excited state proton transfer.⁹⁵ Another problem may occur when buffer components lead to pH-dependent fluorescence quenching.

In spite of these challenges, a successful determination of the ground-state pK_a values of sparingly soluble N-heterocyclic bases was accomplished by Rosenberg et al.⁹⁶ They made use of dilute solutions of a strong acid or base to perform a pH titration; strong buffers were not used in order to prevent reactions of the buffer ions with the highly reactive excited state forms of the aromatic analyte. By carefully selecting the excitation wavelength, they were able to obtain a titration curve in which the inflection point corresponded to the ground-state pK_a . The more precise of the two methods suggested involved choosing an excitation wavelength such that only the conjugate acid can absorb, meaning that no excited-state proton transfer can occur to the free base, in order for the fraction of conjugate acid to be measured directly. The bottom line is that the linear relationship between fluorescence intensity and the degree of dissociation is not as common as in absorption spectrometry.

Polarimetry

Determination of an acid dissociation constant by polarimetry involves the measurement of the optical rotation of plane polarized light by the sample solution as a function of pH. This method depends on the difference in optical rotation between the ionized and non-ionized forms of an analyte.

Only very few examples of polarimetry were found. A determination of the pK_a values of tartaric acid was performed by Katzin and Gulyas⁹⁷ using this method. They expressed the optical rotation of the sample as the sum of the optical rotation of the ionic fractions derived from the analyte.

When the fractions are expressed as a function of the acid dissociation constants and the solvated proton concentration, the pK_a values may be determined by curve fitting of a plot of the optical rotation against the pH, where small differences in pK_a inhibit accurate determination.

While polarimetry was shown to be a reasonably sensitive method to determine the pK_a values, the method has a number of significant drawbacks. Firstly, the method can only be applied to optically active analytes, and samples must be relatively pure enantiomers as the presence of the opposite enantiomers will cause a reduction in the sensitivity of the measurement with a corresponding drop in precision. Secondly, the determinations are carried out at high concentrations meaning that relatively large amounts of sample are required.

Kinetic method

The kinetic method of determining pK_a values depends on measuring the rate of reaction of a control reaction that is influenced by the pH of its reaction medium. While determinations based on reaction kinetics are rarely encountered in literature, Bunnett and Nudelman⁹⁸ demonstrated a method by which the principle might be applied systematically. Their method involved a reference reaction, the reaction of thiophenoxide ion with 2,4-dinitrofluorobenzene to form a conjugated double ring system, that could be easily monitored using absorption spectrometry.

The rate of this reaction is determined by the dissociation of thiophenol to yield the free ion, and this dissociation is repressed by an increase in the solvated proton concentration. When the dissociation constant of thiophenol is known, performing the reaction in a



buffering solution of the analyte allows for the calculation of the pK_a . The assumption is made that the solvated proton concentration, and thus the position of the thiophenol/thiophenoxide equilibrium, is entirely controlled by the unknown buffer, and that the reaction rate thus depends only on the buffer composition and the pK_a of the buffering acid.

The method as described seemingly has several advantages. Firstly, no exact knowledge of the pH is required since the solvated proton concentration is directly linked to the analytical composition of the buffering solution and it in turn is directly linked to the thiophenoxide concentration. A second advantage is that the pK_a can, in principle, be determined in a single measurement. In spite of this, the method still remains complicated and not generally applicable.

Computational method

Finally, we consider the method of computational chemistry. This method is unusual when compared to those previously described in that it is a mathematical method rather than an experimental one. Computation requires no sample and is in principle unrestrained in terms of physical conditions, but the accuracy of the values obtained depends entirely upon the model used to perform the calculations.

Since the science of computational chemistry began to take shape in the early 1970s with the development of efficient software for the calculation of molecular orbitals and the development of the first molecular mechanics methods, many different methods have been developed to computationally estimate molecular properties. Several of these methods have been evaluated for the determination of acid dissociation constants.

The most precise results available are generally obtained from *ab initio* calculations, meaning that these calculations tend to converge to an exact mathematical solution. However, since the equations used to describe the system are entirely derived from theoretical principles, any flaw in the model will still produce a result that may deviate from its physical value. For instance, considerable difficulties exist when attempting to calculate the pK_a value in a solvated or aqueous phase, since the problem of how to accurately take solvent-analyte interactions into account remains largely unsolved. An article by da Silva et al⁹⁹ published in 1999 compares several methodologies.

It compares the results obtained by their own method with those published by Schuurman et al¹⁰⁰ a year earlier. It is remarked that the methodology used in describing the molecules in the system and the computational methods used to determine the properties can drastically influence the outcome of the calculation, illustrating the difficulty in deriving *ab initio* systems. Another major downside of these calculations compared to other computational techniques is their enormous cost, making their application in any context where large numbers of molecules must be investigated impractical.

Semi-empirical quantum-mechanical (QM) methods are based on the same formalisms as the *ab initio* calculations, but make use of further approximations and obtain many parameters from experimental data. A good treatment of these methods was given by Tehan.¹⁰¹ The method works by taking information from a large database of known molecules and using this information to obtain approximate QM parameters for the calculation. While these methods are much faster than *ab initio* calculations, the results of the calculation are only reliable if the molecule being computed is similar enough to those stored in the database. More recently, there has been much work on the development of quantum structure-property relationship (QSAR) based methods.¹⁰² These may be considered a further development of the semi-empirical methods described above in the sense that empirical and *ab initio* data are correlated with the contribution of certain fragments within a molecule. This dataset is then used for further calculations. The most recent methods make use of Molecular Tree Structured Fingerprints to describe the chemical neighborhood of an ionizable center.¹⁰³

In terms of the accuracy of their predictions, computational methods suffer where multiple competing models exist, since the choice of parameters and starting assumptions can dramatically affect the outcome of the calculations. In addition, there are often significant deviations from literature values as shown in Figure 11.

Despite these problems, there is still considerable interest in computational pK_a determination methods in such fields as drug discovery, where the method might be used to reduce uncertainty of the physical/chemical properties of (modifications of) molecules without even having to synthesize all of them. However, for the time being the method is purely of

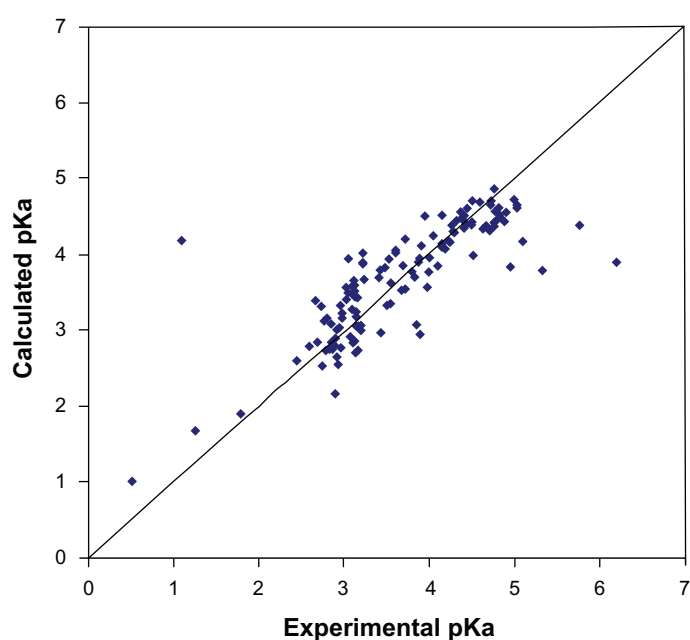


Figure 11. Plot of calculated versus experimentally observed pK_a for a set of 143 aliphatic carboxylic acids, data from Schüürmann et al.¹⁰¹

interest as an estimator, since the reliability of the results leaves much to be desired. It may be expected that the method will find more use in the future as the computational power available increases and better models are derived, but it shall be some time before computational methods are able to compete with actual measurement in terms of accuracy. There are several software packages available (ACD/labs was one of the first) and five of these are compared in a recent review by Manchester et al.¹⁰⁴

Comparison and Discussion

Comparing the incomparable is usually difficult and can only be done in a qualitative way as it depends on many factors, among which those specific for each analyte and experimental facilities available. This is even more so as the introduction and application of the different methods described in this paper cover many decades. As such, they also provide a fascinating historical cross-section of chemical experimentation. We are, however, able to make a tabulated comparison of the methods discussed, see Table 1.

The amount and concentration of analyte available are the most obvious factor for method selection. If only minute amounts in organic micro-synthesis are available for characterization, most of the methods cannot be used. Other sample restrictions include, for example, overlapping pK_a 's, very low solubility, lack of a chromophore, etc. If the available sample has impurities, only separation methods such as HPLC and particularly electrophoresis can be used successfully. Measuring in the extremes of the pH scale is sometimes impossible. Financial factors must also be taken into consideration. It is not fiscally responsible to invest in NMR equipment for the purpose of determining only a few pK_a values annually.

In regards to precision, for a very rough estimate (± 1 pK unit), the only thing needed is a software package,^{104,108} however experienced chemists are often able to estimate this from the structural formula. Additionally, if the pure analyte is available as an acid and as a salt of a strong base, a 1:1 solution of both

Table 1. Overview of the strong (++) and weak (—) points of the methods discussed. Precision based largely on Xie et al,⁴⁷ Manchester et al,¹⁰⁵ Barbosa et al,¹⁰⁶ and Beltrain et al.¹⁰⁷ T, I and ϵ denotes effects of Temperature, Ionic Strength and organic modifier, respectively.

	Amount/conc	Restrictions	pK_a range	Costs/time	Precision	T, I and ϵ
Potentiometry	—	+	—	++	+	+
Conductometry	—	++	—	+	+	—
Voltammetry	—	+	+	+	+	+
Calorimetry	—	++	++	+	+	+
NMR	—	—	++	—	++	—
Electrophoresis	++	+	+	+	++	++
HPLC	++	+	—	+	+	+
Solubility	—	—	+	—	+	—
Spectrometry	+	+	+	+	++	++
Fluorimetry	+	—	+	+	—	+
Polarimetry	+	—	+	+	+	+
Kinetic	—	—	—	—	+	+
Computational	++	—	++	—	—	—



will by definition have a pH equal to the pK_a within the range $4 < \text{pH} < 10$.

On the other hand, precisions claimed in literature generally refer only to specific analytes under investigation, and as we all know, are sometimes brightly colored. Three decimals is impossible and two decimals is considered very good. The last column in Table 1, in our view, is a most important one for reasons mentioned in section 1.2. In most cases, pK_a are measured under conditions imposed by the method of choice. These are not always the conditions relevant for the eventual application of the analyte. As already indicated in the Fentanyl example,⁸¹ physiologically relevant pK_a values of drugs inherent to their application are those in water at 37 °C and with an ionic strength of 0.9% NaCl (154 mM). Thermodynamic values at 25 °C and infinite dilution are irrelevant in this area. In a recent 700 page compilation¹⁰⁹ of aqueous pK_a values of drugs, only half are listed with details of temperature and ionic strength.

The most versatile method able to accurately measure under these conditions appears to be CE. The theoretical basis is straightforward and well understood and the experiments can use a high degree of automation.^{110–112} In the world of drug characterization and analysis, the method is well established.¹¹³ The pK range in which reliable values can be determined is expanding to very acidic regimes^{114,115} unavailable to other techniques.

Conclusion and Recommendations

Being able to actually measure under the conditions relevant for the application is a great advantage, but alas often impossible due to limitations of the technique used. Extrapolation to the desired conditions is often possible at the cost of accuracy and precision inherent to all extrapolations.

Depending on the sample purity, a choice can be made between a spectrometric method and a separation method. HPLC is time consuming in the case of small dissociation constants (α) whereas CE is faster and cheaper. In the absence of a chromophore for optical detection, CE has the additional advantage over chromatography in that it is possible to apply indirect detection.^{116,117}

Whichever method is used, matrix effects can always occur, especially ionic interaction between

analyte and buffer ions. These are checked with analyte behavior in two different buffers of same pH but different buffering constituents. Ionic strength dependence is checked by adding sodium chloride buffer made with the analyte under investigation. Temperature dependence is most easily checked by measuring a buffer of the analyte at different temperatures.

A laboratory where accurate pK_a determinations are essential would be wise to invest in equipment and experience of CE, complemented with spectrometry, pH, and conductivity meters. All of the former should be well thermostated.

Besides those, it would be advised to monitor future developments in pK_a estimation software, especially those programs that can be fed with experimental data for more accurate prediction (rather than estimation) of similar structures.

Author Contributions

Entire literature search and first draft versions of manuscript chapters: AHM van Loon, AJF van Hoof, AJP Teunissen. Jointly developed the final structure, illustrations and revised chapters of the paper: AHM van Loon, AJF van Hoof, AJP Teunissen, JC Reijenga. Made critical revisions and approved final version: JC Reijenga. All authors reviewed and approved of the final manuscript.

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