Determination of the Isoelectric Point of Ampholytes

Theoretical background: P.W. Atkins: *Physical Chemistry*.
Type of practice: Pairwise.
Aim of practice: This experiment shows those *p*H-metric techniques which can be used to determine the isoelectric point of amino acid and proteins.

1 Introduction

Amphoprotic substances (or shortly ampholytes) can either donate or accept a proton in aqueous solution, thus acting either as an acid or a base depending on the actual *p*H. The common feature of the ampholytes that they have at least two (but usually more) hydrogen ion acceptor groups. Commonly, one of them is a carboxyl group while the other one is an amino group. Typical ampholytes are the amino acids, the oligopeptides and the proteins.

The *p*H value belonging to the isoelectric point of an ampholyte ($p\mathrm{H_i}$) is a practically important parameter to characterize the substance. By definition, the aqueous solution of an ampholyte is in the isoelectric point if exactly the same amount of hydrogen ion is dissociated (usually from carboxyl groups) as the amount of hydrogen ion captured by other (usually amino) groups. In this state, the ampholyte is neutral electrically, it has neither positive nor negative charge.[1] If the *p*H in an ampholyte solution is changed by adding either an acid, a base or a buffer then the average degree of protonation (and also the average charge) is also changed. The different isoelectric points of the oligopeptides and proteins are used to separate their mixtures. A mixture of ampholytes is put on a gel-plate providing *p*H-gradient, and the plate is placed in an electric field which forces the charged particles to move. If an ampholyte reaches that part of the gel where the *p*H value equals to the $p\mathrm{H_i}$ of the ampholyte, this substance does not have any charge, consequently, it does not move further. This method of electroforesis is called isoelectric focusing.

During the practice, a $p\mathrm{H_i}$ value is determined by two different methods for one of the most simple amino acid having two acceptors.

2 Calculation of $p\mathrm{H_i}$ from the dissociation constants

The ampholytes with two hydrogen ion acceptor groups (HA in the followings) take part in two equilibrium processes. If the ampholyte has both a carboxyl and an amino groups then the next two deprotonation reactions characterize it in equilibrium:

$$\mathrm{H_2A^+ \rightleftharpoons HA + H^+} \qquad \mathrm{K_1 = \frac{[HA]\cdot[H^+]}{[H_2A^+]}} \quad \text{and} \tag{1}$$

$$\mathrm{HA \rightleftharpoons A^- + H^+} \qquad \mathrm{K_2 = \frac{[A^-]\cdot[H^+]}{[HA]}}, \tag{2}$$

where $\mathrm{K_1}$ is the dissociation constant of the protonated ampholyte, and $\mathrm{K_2}$ is the dissociation constant of the ampholyte. It follows from the definition of the isoelectric point (given in the introduction) that the $[\mathrm{H_2A^+}] = [\mathrm{A^-}]$ relation is valid in this point. If the $\mathrm{K_1 \cdot K_2}$ expression is simplified by this equation then the hydrogen ion concentration of the isoelectric point, ($[\mathrm{H^+}]_\mathrm{i}$) (and consequently $p\mathrm{H_i}$) can be given:

$$\mathrm{K_1 \cdot K_2 = \frac{[HA]\cdot[A^-]\cdot[H^+]^2}{[H_2A^+]\cdot[HA]} = [H^+]_i^2 \rightarrow [H^+]_i = \sqrt{K_1 \cdot K_2}}, \quad p\mathrm{H_i} = \frac{\mathrm{pK_1 + pK_2}}{2}. \tag{3}$$

[1] Really, both the protonated and the deprotonated forms exist in the solution, but the sum of their charges equals to zero.

So pH_i can be calculated if the acidic dissociation constants have already been determined. If there are at least three orders of magnitude difference between the dissociation constants[2] then the (de)protonation processes do not overlap each other, consequently, the values of their dissociation constants can be determined independently based on the next considerations:

1. If the solution of an ampholyte also contains a strong acid then the concentration of the A^- can be neglected, only the (de)protonation process belonging to K_1 determines the pH of the solution. In the investigated solution, let c_{HA} and c_s be the concentrations of the ampholyte and the added strong acid, respectively. By omitting the A^-, – and also neglecting the auto-dissociation of the water – the following approximating formulas will substantially be valid:

$$c_{HA} = [H_2A^+] + [HA] \quad \text{and} \quad c_s = [H^+] + [H_2A^+] \,.$$

   Expressing $[HA]$ and $[H_2A^+]$, and substituting them into equation (1), the value of K_1 can be calculated if $[H^+]$ is known:

$$\boxed{K_1 = \frac{[H^+] \cdot (c_{HA} - c_s + [H^+])}{c_s - [H^+]}}. \tag{4}$$

2. Similar considerations can be applied to calculate the value of K_2. If the solution of an ampholyte also contains strong base then the concentration of the H_2A^+ form can be neglected. Denote c_{HA} and c_b the analytical concentrations of the ampholyte and the added strong base, respectively! The following approximating equations can be written from the mass-conservation:

$$c_{HA} = [HA] + [A^-] \quad \text{and} \quad c_b = [OH^-] + [A^-] \,.$$

   Expressing $[A^-]$ and $[HA]$, substituting them into equation (2) together with the $[OH^-] = K_v / [H^+]$ relation (taking the auto-dissociation of water into account where K_v is the ionic product of water), the value of K_2 can be calculated in the following way:

$$\boxed{K_2} = \frac{K_v \cdot (c_b - [OH^-])}{[OH^-] \cdot (c_{HA} - c_b + [OH^-])} \boxed{= \frac{[H^+] \cdot ([H^+] \cdot c_b - K_v)}{[H^+] \cdot (c_{HA} - c_b) + K_v}}. \tag{5}$$

   The values of c_{HA}, c_s and c_b (occurring in equations (4) and (5)) can be calculated from the mass measurements and / or from the extent of dilutions. Moreover, $[H^+]$ in each solution can be approximated by applying the primary measured pH values, so the above equations are applicable to calculate the values of K_1 and K_2 dissociation constants.

There are, however, two practical disadvantages of this method:

– Ampholytes occurring in biological systems have more hydrogen ion acceptor groups. The value of pH_i can still be calculated from the dissociation constants but more complicated mathematics is needed.[3]

– Several precisely measured data are necessary to get a punctual value for the dissociation constants.The majority of the ampholytes are difficult to handle experimentally (they cannot be cleaned easily, they are usually hygroscopic substances, etc.), therefore the measuring precision required by the calculation of the dissociation constants cannot be reached.

Because of these limitations, other methods were also worked out. These methods do not require the precise values of the dissociation constants. One (and probably the most important) of them is the Michaelis method.

[2]This condition is almost always valid if one carboxyl and one amino group can be found in the ampholyte.

[3]For example, ampholytes having three hydrogen ion acceptor groups and the composition of H_2A, the $[H^+]_i^3 = K_1 \cdot K_2 \cdot ([H^+]_i + 2 \cdot K_3)$ third-degree polynomial equation should be solved instead of a simple square root calculation required by (3).

3 Determination of $p\mathrm{H_i}$ by the Michaelis method

To understand this method, it should be thought over what is happening if an ampholyte is dissolved in pure water. In equilibrium, a portion of the carboxyl groups is deprotonated. A part of this amount of produced hydrogen ion protonates some amino groups while the rest remains free providing the *p*H of the solution. At appropriately large ampholyte concentration, almost all hydrogen ion deprotonated from a carboxyl group reconnected to an amino group, only a tiny portion of it remains free. It also means that the sum of the electric charges of the dissolved ampholyte particles is very close to the electric neutrality so *the pH value of the isoelectric point practically equals to that pH value which is valid in a concentrated aqueous solution of the ampholyte*.

If the ampholyte is dissolved in a buffer solution instead of pure water then the *p*H value of the buffer changes. Only one case is an exception when the *p*H of the buffer equals to the *p*H of the isoelectric point of the ampholyte (i.e., $\Delta p\mathrm{H} = p\mathrm{H}_{\text{puffer+amfolit}} - p\mathrm{H}_{\text{puffer}} = 0$ in an ideal case). Based on these considerations, the essence of the Michaelis method is the following:

1. A series of buffer solutions with different composition is to be prepared, and their *p*H values have to be measured.
2. The same buffer solutions are to be prepared but these solutions also contain dissolved ampholyte with the same concentration. If the ampholyte is dissolved in such a buffer solution that $p\mathrm{H}_{\text{buffer}} < p\mathrm{H_i}$ then the protonation of amino groups is dominant and thus a *p*H greater than $p\mathrm{H}_{\text{buffer}}$ will be measured. The change of *p*H of the original buffer solution upon dissolving the ampholyte is less if its original *p*H was closer to $p\mathrm{H_i}$. If $p\mathrm{H}_{\text{buffer}} = p\mathrm{H_i}$, the *p*H of the original buffer does not change upon ampholyte dissolution. Finally, if the ampholyte is dissolved in such buffer solution that $p\mathrm{H}_{\text{buffer}} > p\mathrm{H_i}$ then the deprotonation of carboxyl groups is dominant and thus a *p*H smaller than $p\mathrm{H}_{\text{buffer}}$ will be measured. Consequently, if $\Delta p\mathrm{H} = p\mathrm{H}_{\text{buffer+ampholyte}} - p\mathrm{H}_{\text{buffer}}$ is plotted as a function of $p\mathrm{H}_{\text{buffer}}$, a curve is obtained the inflection point of which is located at $p\mathrm{H_i}$.[4]

4 Students's measurements

Four *p*H-metric titration have to be carried out during the practice. The aim of the first two is to determine the values of the dissociation constants in order to calculate the *p*H of the isoelectric point based on equation (3):

1. At first, the solution of the ampholyte is titrated with hydrochloric acid solution. Based on equation (4), the value of $\mathrm{K_1}$ can be calculated in every point of the titration.
2. During the second titration, the ampholyte solution is titrated with NaOH solution. The value of $\mathrm{K_2}$ can be calculated in each point of this titration based on equation (5).

The purpose of the last two titrations is to determine the $p\mathrm{H_i}$ value using the Michaelis method:

1. In the third titration, a citric acid solution is titrated with a basic salt solution (the instructor determines which salt is to be used for this purpose). In this way, each titration point is a buffer with different measurable *p*H value.
2. The fourth titration is almost the same as the third one but both the titrant and the citric acid solution contain dissolved ampholyte in the same concentration. It is very important for this titration that the titration volumes must be exactly the same as they were during the third titration.

[4] In an ideal case, $\Delta p\mathrm{H} = 0$ at the inflection point of the curve. However, the solutions to be used during the practice are not ideal. Therefore, not $[\mathrm{H^+}]$ but the activity of hydrogen ions can be determined from the measured *p*H. Although we do not take this difference into account when applying equation (4) and (5), this assumption (together with loose *p*H measurements) can lead to serious problems during Michaelis evaluation method (where $\Delta p\mathrm{H} = 0$ is sought).

Table 1: The necessary volumes and the concentrations in the solutions used for the titrations and is to prepared by dilution of appropriate stock solutions.

	titrand	titrant
titration 1	$100\,\mathrm{cm}^3$ solution **A**: $\mathrm{c(ampholyte)} = \mathrm{T_{amf}}$	$50\,\mathrm{cm}^3$ solution **B**: $\mathrm{c(HCl)} = \mathrm{T_{amf}}/2$
titration 2		$50\,\mathrm{cm}^3$ solution **C**: $\mathrm{c(NaOH)} = \mathrm{T_{amf}}$
titration 3	$50\,\mathrm{cm}^3$ solution **D**: $\mathrm{c(citric\ acid)} = \mathrm{T_{cit}}$	$50\,\mathrm{cm}^3$ solution **E**: $\mathrm{c(Na_3PO_4)} = 3{,}0 \cdot \mathrm{T_{cit}}$ or $\mathrm{c(Na_4EDTA)} = 3{,}5 \cdot \mathrm{T_{cit}}$ or $\mathrm{c(Na_2B_4O_7)} = 1{,}8 \cdot \mathrm{T_{cit}}$
titration 4	$50\,\mathrm{cm}^3$ solution **F**: $\mathrm{c(ampholyte)} = \mathrm{T_{amf}}$, and $\mathrm{c(citric\ acid)} = \mathrm{T_{cit}}$	$50\,\mathrm{cm}^3$ solution **G**: $\mathrm{c(ampholyte)} = \mathrm{T_{amf}}$, and $\mathrm{c(Na_3PO_4)} = 3{,}0 \cdot \mathrm{T_{cit}}$ or $\mathrm{c(Na_4EDTA)} = 3{,}5 \cdot \mathrm{T_{cit}}$ or $\mathrm{c(Na_2B_4O_7)} = 1{,}8 \cdot \mathrm{T_{cit}}$

At the beginning of the practice, the instructor determines *(1)* which ampholyte is to be investigated, *(2)* the concentration of the ampholyte ($\mathrm{T_{amf}}$) both in the titrant and the titrand solutions (between $0.01 - 0.03\,\mathrm{M}$), *(3)* the concentration of citric acid in the solutions to be titrated ($\mathrm{T_{cit}}$) for the Michaelis method (between $0.001 - 0.003\,\mathrm{M}$), *(4)* the basic salt the solution of which is to be used as titrant (it can be either $\mathrm{Na_3PO_4}$, $\mathrm{Na_4EDTA}$ or $\mathrm{Na_2B_4O_7}$) and *(5)* the volume differences in the titrations belonging to the Michaelis method (between $0.3 - 1.0\,\mathrm{cm}^3$). If the instructor does not give different instructions then *(1)* the ampholyte is the glycine, *(2)* $\mathrm{T_{amf}} = 0.02\,\mathrm{M}$, *(3)* $\mathrm{T_{cit}} = 0.002\,\mathrm{M}$, *(4)* the basic salt is the $\mathrm{Na_2B_4O_7}$ and *(5)* $\Delta\mathrm{V} = 0.8\,\mathrm{cm}^3$ during the third and fourth titration.

Three stock solutions must be prepared at first by weighting the calculated mass of the appropriate solid material:[5]

- $100\,\mathrm{cm}^3$ 0.1 M stock solution of the ampholyte,
- $50\,\mathrm{cm}^3$ 0.01 M stock solution of the citric acid and
- $50\,\mathrm{cm}^3$ stock solution of the basic salt from one of the following possibilities: 0.03 M $\mathrm{Na_3PO_4}$, 0.035 M $\mathrm{Na_4EDTA}$ or 0.018 M $\mathrm{Na_2B_4O_7}$.

With this, the solutions used for the titrations are to be prepared (they are denoted as solutions **A**, **B**, ..., **F** and **G**). Their volumes and compositions are given in Table 1. *For the preparation of the solutions given in the table, the previously prepared there stock solutions should be used. Additionally, a 0.1 M HCl and a 0.1 M NaOH stock solution are provided for the students.* Before any experimental work, all necessary calculations about the dilutions must be done in the laboratory notebook and the instructor should check them.

The precision of the *p*H measurements primarily influences the final results. Therefore, before the titrations, the *p*H-meters should be calibrated using two buffers. If there are more buffer solutions then the buffers with *p*H$\approx$4 and *p*H$\approx$10 are worth to be chosen or the buffers having the nearest *p*H. The calibration standards must be at room temperature. Furthermore, the calibration must be checked by measuring the *p*H of the standards once the calibration is fully executed. If the measured *p*H differs from the nominal values more than $0.01 - 0.02$, the calibration procedure must be repeated.

[5]The crystal water content of the solid material is also to be taken into account during the necessary calculations!

During the first titration, 20.0 cm^3 of solution **A** is to be titrated with solution **B**. The titration volume must be increased by 1 cm^3 portions until the final volume of 15 cm^3, and the titration volume – $p\mathrm{H}_{\mathrm{measured}}$ data pairs must be recorded. The second titration is almost the same but the titrant is the solution **C** (instead of **B**). The instructor may change the difference between the subsequent titration volumes as well as the final volume of the titrant. In the third titration, 20.0 cm^3 of solution **D** must be titrated with solution **E** until 20 cm^3 consumption of the titrant has been reached. The difference between the subsequent titration points is ΔV. The fourth titration is very similar to the third one but solution **F** must be titrated with solution **G**. During the last titration, *it is very important that the volumes of the titration points must be exactly the same as they are in the third titration!* The instructor may change the value of ΔV, moreover, the last 15 % of the titration curve (consumption of 17 – 20 cm^3) is worth to measure with smaller ΔV in cases of Na_3PO_4 and Na_4EDTA.

Many solutions are prepared during the practice, therefore, the stock, the titrand and the titrant solutions can easily be mixed. Because of this fact, a precise plan should be written into the notebook for the preparation procedure of the solutions given in Table 1. Without this plan, it is very easy to prepare wrong solution(s). The titrations should be carried out in the thinnest and tallest 50 – 100 cm^3 beaker since the use of the combined glass electrode requires a minimum height of the investigated solution.

If the instructor does not state otherwise, one of the students performs the first two titrations with one of the pH-meters, while –in parallel– the other student uses the other instrument for the other two titrations.

5 Evaluation of measured data

– The weighted masses and the concentrations of the prepared solutions (i.e., the stock solutions and solutions given in Table 1) based on the weighted masses, and the temperature of the laboratory have to be summarized in the notebook.

– The value of K_1 has to be determined from the measured points of the first titration. The measured and calculated data should be arranged in the following table:

Table 2: Summary of the experimental results.

V_0= .... cm^3, M_r(ampholyte)= ....

V_B (cm^3)	$p\mathrm{H}_{\mathrm{measured}}$	$[H^+]$(M)	c_{HA}(M)	c_s(M)	K_1

In the table, V_B is the titration volume of solution **B**. To calculate the value of K_1, equation (4) should be used where the $[H^+]$, c_{HA} and c_s values can be calculated by the

$$\boxed{[H^+] = 10^{-p\mathrm{H}_{\mathrm{measured}}}}, \quad \boxed{c_{HA} = \frac{V_0 \cdot T_{amf}}{V_0 + V_B}} \quad \text{and} \quad \boxed{c_s = \frac{V_B \cdot T_{amf}/2}{V_0 + V_B}}$$

expressions (they are based on the definition of *p*H and the rules of dilution). After completing the above table, the average and the deviation of the calculated K_1 values should be given. The obviously wrong values (if there is any) must be omitted before the calculation.

– The value of K_2 has to be determined from the measured points of the second titration. The measured and calculated data should be arranged in the following table:

In the table, K_v is the ionic product of water at the temperature and ionic strength of the measurement and V_C is the titration volume of solution **C**. The related equation of the Appendix should be used

Table 3: Summary of the experimental results.

V_0= .... cm^3, K_v= ....

$V_C\,(cm^3)$	$p\mathrm{H}_{\mathrm{measured}}$	$[H^+](M)$	$c_{HA}(M)$	$c_b(M)$	K_2

to calculate K_v where the ionic strength equals to the concentration of the ampholyte ($I = T_{amf}$). To calculate K_2 equation (5) is used where c_{HA} and c_b can be given by the

$$\boxed{c_{HA} = \frac{V_0 \cdot T_{amf}}{V_0 + V_C}} \quad \text{and} \quad \boxed{c_b = \frac{V_C \cdot T_{amf}}{V_0 + V_C}}$$

expressions ($[H^+]$ must be given by its definition). The average and the deviation of K_2 should be given in the same way as it happened for K_1 earlier.

– According to the determined K_1, K_2 and the equation (3); the values and the deviations of $[H]_i$ and $p\mathrm{H}_i$ (i.e., the isoelectric point of the ampholyte) must be calculated. With the aid of Appendix, take the error spreading into account as well.

– The measured and calculated data of the third and fourth titrations must be summarized in the following table:

Table 4: Summary of the experimental results.

M_r(citric acid)= ...., M_r(basic salt)= ....

$V_E, V_G\,(cm^3)$	$p\mathrm{H}_{\mathrm{measured}}$ (pure buffer)	$p\mathrm{H}_{\mathrm{measured}}$ (buffer+ampholyte)	ΔpH

In the table, $\Delta p\mathrm{H} = p\mathrm{H}_{\mathrm{ampholyte+buffer}} - p\mathrm{H}_{\mathrm{buffer}}$. Plot ΔpH as a function of pH measured in the pure buffer solution. According to the Michaelis method, $p\mathrm{H}_i$ is given as the intersection of the horizontal axis and the *curve* fitted thorough the data. Because of the technical properties of the measurements (i.e., the measured signal is proportional to the activity of hydrogen ions and not to $[H^+]$) and of a loose calibration of the pH-meter, the closely flat part of the curve (which contains the inflection point as well) might systematically run above or below the x-axis. For this reason $p\mathrm{H}_i$ can be sought as the inflection point of the curve rather than $\Delta p\mathrm{H} = 0$ (nevertheless, this must be also done!). In this context, fit the data with a polynomial and determine its equation (see Appendix; Excel trendline and LINEST function, Origin, QtiPlot, etc.). If needed, skip points from the two ends of the data set, but fitting the middle flat part together with the smooth curvatures around it is necessary (and do not forget to include each data in the plot). Search for the inflection point of the fitted polynomial to determine $p\mathrm{H}_i$ (see Appendix).

– Compare the $p\mathrm{H}_i$ values obtained with the three different methods (i.e., applying dissociation constants, the original Michaelis evaluation method, and the determination of the inflection point of a polynomial) to literature data (add the source of the data); discuss the precision of the methods. Explain the observed differences.

Questions

1. What kind of substances are the ampholytes?
2. Describe shortly what is the isoelectric point of an ampholyte!
3. Give the essence of the isoelectric focusing!
4. Derive the equation which expresses the value of $p\mathrm{H_i}$ by the dissociation constants in case of an ampholyte with two hydrogen ion acceptor groups and with the composition of HA!
5. Derive the expression of the first dissociation constant of the ampholyte detailed in question 4!
6. Derive the expression of the second dissociation constant of the ampholyte detailed in question 4!
7. What are the limitation of the calculation of $p\mathrm{H_i}$ from the dissociation constants?
8. Describe the essence of the Michaelis method shortly!
9. What chemical considerations are necessary to use the Michaelis method?
10. We have to titrate an alanine solution ($c_{\mathrm{alanine}} = 0.032\,\mathrm{M}$) with a hydrochloric acid solution ($c_{\mathrm{HCl}} = c_{\mathrm{alanine}}/2\,\mathrm{M}$). How to prepare $50\,\mathrm{cm}^3$ of titrant solution from the available 0.12 M stock solution?
11. We have to titrate a citric acid solution ($c_{\mathrm{cit}} = 2 \times 10^{-3}\,\mathrm{M}$) during the practice. To do so, we require $50\,\mathrm{cm}^3$ titrant solution, which is 0.035 M for glycine and $c_{\mathrm{Na_3PO_4}} = 3 \times c_{\mathrm{cit}}\,\mathrm{M}$ for $\mathrm{Na_3PO_4}$. $50\,\mathrm{cm}^3$ of 0.2 M glycine and the same volume of 0.03 M $\mathrm{Na_3PO_4}$ stock solutions are available. How to prepare the titrant?
12. $20.0\,\mathrm{cm}^3$ 0.0300 M glycine solution is titrated with 0.01 M HCl solution. Calculate the total concentrations of the glycine and the strong acid when the titration volume is $7.00\,\mathrm{cm}^3$!
13. $20.0\,\mathrm{cm}^3$ 0.0300 M alanine solution is titrated with 0.02 M NaOH solution. Calculate the total concentrations of the alanine and the strong base when the titration volume is $17.00\,\mathrm{cm}^3$!
14. In a titration point, the analytical concentration of valine and added strong acid are 0.0175 M and 0.00637 M, respectively. What is the value of the first dissociation constant of the valine if the measured $p\mathrm{H} = 2.542$?
15. In a titration point, the analytical concentration of serine and added strong base are 0.0148 M and 0.00891 M, respectively. What is the value of the second dissociation constant of the serine if the measured $p\mathrm{H} = 9.229$ and the negative logarithm of the ionic product of water is $p\mathrm{K_v} = 13.78$?
16. The first and second dissociation constants of an ampholyte are 1.4×10^{-3} and 5.7×10^{-10}, respectively. Calculate the $[\mathrm{H}^+]_{\mathrm{i}}$ and $p\mathrm{H_i}$ values of the ampholyte!