Spectrophotometric determination of pK_a values for fluorescein using activity coefficient corrections

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Abstract

The absorbance of the organic water tracer compound fluorescein is known to be pH dependent but differences between the reported pK_a values make it difficult to predict these absorbance changes. A new pK_a determination method, which incorporated activity corrections, was used to calculate the pK_a values of fluorescein. Several published pK_a values were re-evaluated and were in agreement once activity corrections were applied.

Introduction

Fluorescent and coloured organic tracers are used in a wide variety of water investigations such as evaluating the mixing regime of treatment systems (Levenspiel, 1972), characterising the performance of stabilisation ponds (Shilton et al., 2000), or calculating the parameters used in activated sludge modelling (Makinia and Wells, 2000). A number of tracers are pH-sensitive and this can affect the spectrophotometric and transport behaviour of the tracer (Behrens, 1986). Should precise tracer data interpretation be required it is vital to have accurate pK_a values to account for this pH response. This paper describes a technique that was developed to measure the three pK_a values and four absorptivity factors of fluorescein.

The most rudimentary ionic model that accounts for the pH response of fluorescein (Klonis and Sawyer, 1996) uses the six different ionic species proposed by Zanker and Peter (1958). These include a cation, monoanion and dianion ionic species and a lactone, zwitterion and quinonoid neutral species. While each ionic species has its own characteristic absorbance spectrum, the strongest absorptivity is associated with the dianion and there are substantial differences in the species absorptivities at the dianion analytical wavelength of 490 nm. Compared to the dianion absorptivity at 490 nm, the monoanion has only 19%, the neutral species only 3%, and the cation species only 0.04% of the absorptivity (Diehl, 1989, and Klonis and Sawyer, 1996) and it is these large relative absorbance differences that make the consequences of inaccurate pK_a values so important.

Unfortunately, it is difficult to predict exactly when these ionic changes occur because there is little agreement between the published fluorescein pK_a values. A wide variety of pK_a values have been reported and these range from a three pK_a ionic model using values of 2.25, 4.23 and 6.31 (Klonis and Sawyer, 1996) to a single pK_a value of 5.1 (Kasnavia et al., 1999). While the differences between some of the reported pK_a values appear to be small, they have a large impact on the apparent fluorescein concentration. For instance, if the Klonis and Sawyer (1996) pK_a values and absorptivities are used as reference values but fluorescein actually has the pK_a values of 2.2, 4.4 and 6.7 (Lindqvist, 1960) then at a pH of 6.1 only 70% of the expected fluorescein would be detected.

Apart from the pK_a value differences, questions have also been raised about the fluorescein absorptivity values by Boets et al. (1992) who compared their absorptivity value of 8.7×10^4 with other published values of 7.4×10^4 (Larsen and Johansson, 1989), 8.4×10^4 (Hammond, 1979), 8.9×10^4 (Delori et al., 1978, and Melhado et al., 1982) and 1.6×10^5 $M^{-1}cm^{-1}$ (Grotte et al., 1985). These inconsistent values may be the result of using an absorbance test to quantify a highly fluorescent compound (Braude et al., 1950), and as the magnitude of this error depends on the type of analytical instrument (Gibson and Keegan, 1938) it will be important to determine values specific for the spectrophotometer used in each study.

Additionally, some fluorescent compounds are influenced by the nature of the cations present (Smart and Laidlaw, 1977) so it will be important to eliminate any such effects from a pK_a determination method.

A number of different methods have been reported (Albert and Serjeant, 1984) for pK_a determinations, which involve monitoring the response of the test compound throughout a titration. A simple and rapid pK_a determination method (Clark and Cunliffe, 1973) may be adequate for some applications, however this technique does not incorporate activity corrections and the prescribed test buffers have concentrations of greater than 0.08M, which will yield ionic strengths greater than the 0.01M limit above which activity corrections are recommended (Albert and Serjeant, 1984). More recent pK_a determination methods use mathematical techniques to simultaneously solve for the pK_a values, however these methods either do not correct for activity effects (Klonis and Sawyer, 1996) or do not account for the activity complications caused by the test buffer (Sjöback et al., 1995).

The ideal pK_a determination method would have the accuracy of the Albert and Serjeant (1984) techniques, the simplicity of the Clark and Cunliffe (1973) method, and the minimal equipment requirements of the mathematical approaches (Klonis and Sawyer, 1996, and Sjöback et al., 1995), but must also include activity and temperature corrections. Thus, the proposed method gathers data in a manner similar to that of Clark and Cunliffe (1973) and eliminates the equipment required for precise temperature control. In addition, the method processes the data using a simultaneous

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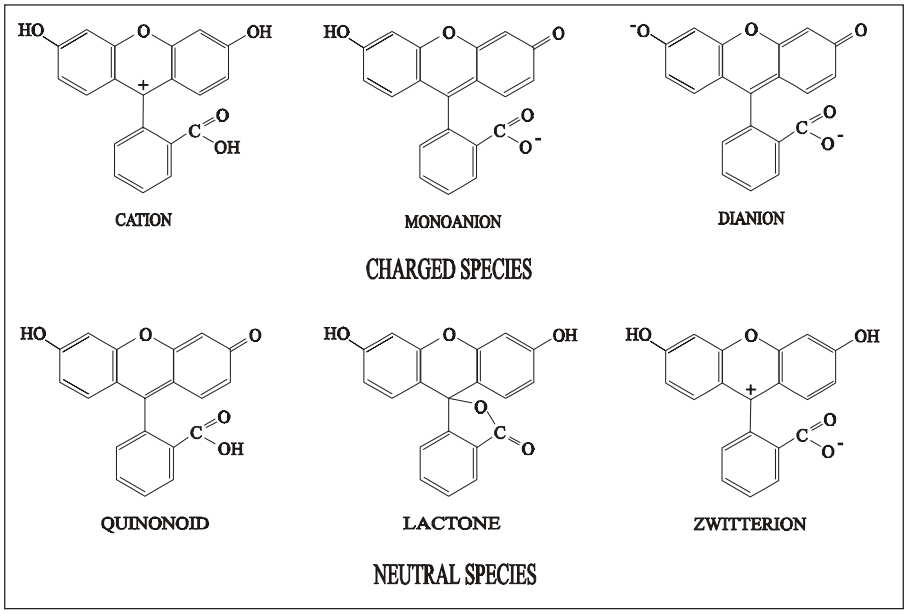

Figure 1
Six ionic forms of fluorescein after Zanker and Peter (1958)

solution method, and includes activity and temperature corrections similar to those recommended by Albert and Serjeant (1984).

Theory

A three-pK_a fluorescein model (model A, Klonis and Sawyer, 1996) was used in this study. This model uses the six ionic fluorescein species shown in Fig. 1 but treats the lactone, zwitterion and quinonoid neutral species as a single group.

The method presented here calculates the pK_a values and absorptivity factors of fluorescein by fitting the pH/absorbance profile of a dilute fluorescein solution to the known ionic model. The titration starts with an acidified, buffered fluorescein solution and adds small quantities of a strong alkali solution. The pH was monitored and an absorbance sample was collected after each of these additions. These pH and absorbance data were then curve-fitted using standard spreadsheet functions.

The approach is based on the following assumptions:

- Fluorescein behaves according to the laws of equilibrium chemistry.
- The different ionic species have characteristic absorptivities and obey the Beer-Lambert law within the test concentration range.
- The presence of small amounts of fluorescein has a negligible effect on the buffer solution pH.

Equilibrium expressions

Equilibrium expressions were used to develop a concentration formula for each ionic species. The three ionic reactions of fluorescein are listed below, with "Flu" denoting the deprotonated fluorescein molecule:

$$H_3Flu^+ \leftrightarrow H^+ + H_2Flu \quad (1)$$
$$H_2Flu \leftrightarrow H^+ + HFlu^- \quad (2)$$
$$HFlu^- \leftrightarrow H^+ + Flu^{2-} \quad (3)$$

Relative equilibrium concentrations are denoted by the ionisation constant K_a, with the numerical subscript indicating the ionic reaction and the square brackets denote molar concentrations (Eqs. (4), (5) and (6)). As the use of molar concentrations is only appropriate for dilute solutions activity corrections are included later.

$$K_{a1} = [H^+][H_2Flu]/[H_3Flu^+] \quad (4)$$
$$K_{a2} = [H^+][HFlu^-]/[H_2Flu] \quad (5)$$
$$K_{a3} = [H^+][Flu^{2-}]/[HFlu^-] \quad (6)$$

A fluorescein mass balance gives:

$$[Flu_{Total}] = [H_3Flu^+] + [H_2Flu] + [HFlu^-] + [Flu^{2-}] \quad (7)$$

Rearrangement of Eqs. (4), (5) and (6) followed by their substitution into Eq. 7 produces four equations. These are:

$$[H_3Flu^+] = [Flu_{Total}]/(1 + K_{a1}/[H^+] + (K_{a1}K_{a2})/[H^+]^2 + K_{a1}K_{a2}K_{a3}/[H^+]^3) \quad (8)$$
$$[H_2Flu] = [Flu_{Total}]/([H^+]/K_{a1} + 1 + K_{a2}/[H^+] + K_{a2}K_{a3}/[H^+]^2) \quad (9)$$
$$[HFlu^-] = [Flu_{Total}]/([H^+]^2/(K_{a1}K_{a2}) + [H^+]/K_{a2} + 1 + K_{a3}/[H^+]) \quad (10)$$
$$[HFlu^{2-}] = [Flu_{Total}]/([H^+]^3/(K_{a1}K_{a2}K_{a3}) + [H^+]^2/(K_{a2}K_{a3}) + [H^+]/K_{a3} + 1) \quad (11)$$

These last four equations ((8) to (11)), allow the precise concentration of each species to be calculated as long as the total fluorescein concentration, pH and individual K_a values are known.

Absorbance equations

The assumption that each ionic species has a characteristic absorptivity and obeys the Beer-Lambert law is fundamental to quantitative absorbance spectrophotometry but needs to be confirmed wherever possible (Braude et al., 1950). For the concentrations at which the Beer-Lambert law applies:

$$\text{Absorbance} = \varepsilon bc \quad (12)$$

where:

ε is the molar absorptivity constant at the analytical wavelength
b is the path length of the light beam through the sample
c is the fluorescein species concentration.

As the same spectrophotometer and wavelength were used throughout the determination the molar absorptivity constant and instrument path length were combined to produce a factor for each of the four ionic species (F_1, F_2, F_3 and F_4). The total absorbance is the sum of the absorbencies of the different fluorescein species, such that:

$$\text{Total absorbance} = F_1[H_3Flu^+] + F_2[H_2Flu] + F_3[HFlu^-] + F_4[Flu^{2-}] \quad (13)$$

Or where Eq.# symbolises the right-hand side of equation #…

$$\text{Total absorbance} = F_1(\text{Eq.8}) + F_2(\text{Eq.9}) + F_3(\text{Eq.10}) + F_4(\text{Eq.11}) \quad (14)$$

If the total fluorescein, the pH and total absorbance are known there are seven unknowns: K_{a1}, K_{a2}, K_{a3}, F_1, F_2, F_3 and F_4. During the solving process, these seven values were adjusted until the calculated total absorbance matched the measured total absorbance.

Activity corrections

In this method, activity corrections were based on an approximation in which the "mean ionic diameter" parameter of the extended Debye-Hückel formula is set to 3.0Å (Guggenheim and Schindler, 1934). This approximation is useful for mixtures of electrolytes up to ionic strengths of 0.1M (Guggenheim, 1935) therefore buffer strengths were designed to yield ionic strengths lower than 0.1M throughout the titration.

The Guggenheim and Schindler (1934) approximation describes the relationship between the activity coefficient (γ) of a particular ion, and the solution ionic strength (μ) as:

$$\log\gamma = -0.5Z^2\frac{\sqrt{\mu}}{1+\sqrt{\mu}} \quad (15)$$

The ionic strength (μ) being:

$$\mu = 0.5\sum_i C_i Z_i^2 \quad (16)$$

where:

C is the concentration of a particular species
Z is the charge of the species and
i is the number of different species present.

The activity correction was made by multiplying the species concentration by its activity coefficient. For monoionic species the activity coefficient formula simplified to:

$$\gamma_1 = 10^{\left(-0.5\frac{\sqrt{\mu}}{(1+\sqrt{\mu})}\right)} \quad (17)$$

And the activity coefficient formula for diionic species was:

$$\gamma_2 = 10^{\left(-2\frac{\sqrt{\mu}}{(1+\sqrt{\mu})}\right)} \quad (18)$$

As pH meters measure the proton *activity*, pH readings are already activity corrected. This means that the *mixed* K_a' found after using the proton activity in combination with uncorrected molarity data needed to be activity corrected (and standardised to 25°C) to find the K_a. Equations 4, 5 and 6 can be expanded to include this activity correction.

$$K_{a1} = \{H^+\}[H_2Flu]/(\gamma_1[H_3Flu^+]) = \{H^+\}[H_2Flu]/[H_3Flu^+]\times 1/\gamma_1 = K_{a1}'\times 1/\gamma_1 \quad (19)$$

$$K_{a2} = \{H^+\}(\gamma_1[HFlu^-])/[H_2Flu] = \{H^+\}[HFlu^-]/[H_2Flu]\times\gamma_1 = K_{a2}'\times\gamma_1 \quad (20)$$

$$K_{a3} = \{H^+\}(\gamma_2[Flu^{2-}])/(\gamma_1[HFlu^-]) = \{H^+\}[Flu^{2-}]/[HFlu^-]\times\gamma_2/\gamma_1 = K_{a3}'\times\gamma_2/\gamma_1 \quad (21)$$

Where the curved brackets denote activity, the square brackets denote molarity, and the activity coefficient of the uncharged species (H_2Flu) is unity and is not shown. These last three equations ((19) to (21)) are used to correct the *mixed* ionisation constant for activity effects and this value must then be standardised to 25°C to find the pK_a.

pK_a temperature corrections

The K_a of solutes can be temperature dependent and are normally standardised to 25°C. Equation (22) was used to compensate for changes in the ionisation constants due to temperature changes. This is the integrated form of the Gibbs-Helmholtz equation where the Gibbs free energy equation is $\Delta G° = -RT\ln(K)$, and the enthalpy is assumed constant (Sawyer et al., 1994).

$$\ln\left(\frac{K_{aT2}}{K_{aT1}}\right) = \frac{-\Delta H^0}{R}\left(\frac{T_1 - T_2}{T_1T_2}\right) \quad (22)$$

Equation (22) was rearranged to give:

$$K_{aT2} = K_{aT1}e^{\left(\frac{-\Delta H^0}{R}\left(\frac{T_1-T_2}{T_1T_2}\right)\right)} \quad (23)$$

where:

H^0 is the standard enthalpy of reaction
R is the gas constant
K_{aT1} is the ionisation constant at 298.15 Kelvin (T_1)
K_{aT2} is the apparent ionisation constant at the experimental temperature T_2

While this formula is recognised to be an approximation (Sawyer et al., 1994) it does offer a degree of temperature compensation for the relatively small changes within the test temperature range.

The standard enthalpy of reaction for each buffer compound was calculated using the CODATA (Cox et al., 1989) values as quoted in the CRC Handbook (1992). Equation (24) was used to determine the standard enthalpy of reaction for fluorescein but used the ionisation data produced by this investigation.

$$\Delta H^0 = -R\left(\frac{T_1T_2}{T_1 - T_2}\right)\ln\left(\frac{K_{aT2}}{K_{aT1}}\right) \quad (24)$$

Absorptivity temperature corrections

Molar absorptivity values were temperature standardised in this investigation using an analogous formula to that used to compensate for fluorescence intensity (Feuerstein and Selleck, 1963).

$$F_T = F_s e^{n(T-T_s)} \quad (25)$$

where:

F_T is the absorptivity factor at the test temperature
F_s is the absorptivity factor at the standard temperature of 0°C
T_s is the standard temperature
T is the test temperature
n is the temperature coefficient.

Absorbance factor data were collected at two different temperatures and the temperature coefficient was calculated by rewriting Eq. (25) as:

$$F_s = \frac{F_{T1}}{e^{nT_1}} = \frac{F_{T2}}{e^{nT_2}} \qquad (26)$$

where:

F_{T1} and F_{T2} are the absorbance factors at temperatures T_1 and T_2.

Solving for n gives:

$$n = \frac{\ln(\frac{F_{T2}}{F_{T1}})}{(T_2 - T_1)} \qquad (27)$$

Once the temperature coefficient was known for each ionic species, standardised absorptivity factors were calculated by substitution into Eq. (25).

Materials and method

Chemical quality was certified to meet ACS (American Chemical Society) specifications and supplied by either Fluka or JT Baker. Distilled water was used throughout. Standard grade Fluka fluorescein was used without further purification. The fluorescein moisture content was measured by calculating the mass lost after overnight drying at 105°C under vacuum. This moisture correction was applied to all fluorescein mass measurements.

Absorbance readings were made using a Turner Model 350 spectrophotometer at the fluorescein, high-pH absorbance maximum, which was 492 nm on this instrument. Samples were collected and analysed in new, polished, 13 × 100 mm borosilicate glass tubes.

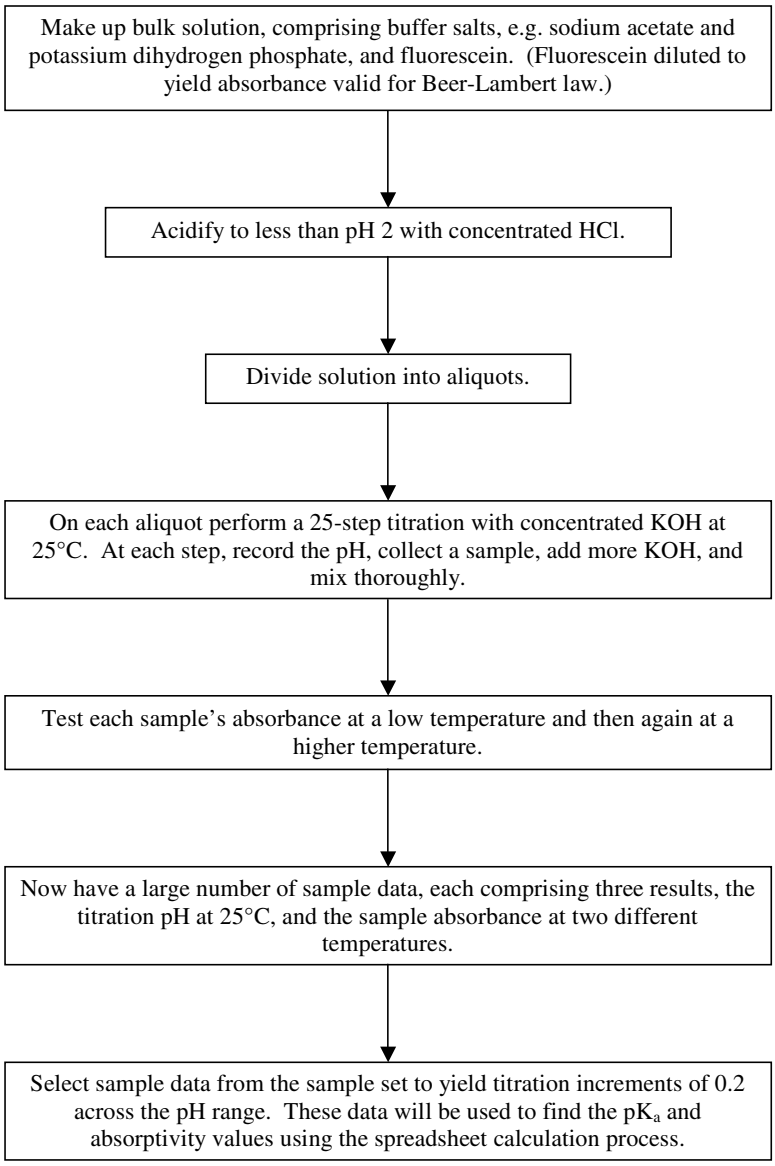

Figure 2
Flow diagram for pK_a determination titration

A Corning Model pH-30 meter was used and pH calibrations were performed before, during, and after each test session. Calibrations were performed in a water bath at 25°C (the titration temperature) at pH 4.00 and pH 7.00 with calibration solutions prepared using "pHydrion" buffer capsules supplied by Micro Essential Laboratories.

The titration procedure is shown as a flow diagram in Fig. 2. A stock solution (5mM sodium acetate (NaAc), 5mM KH_2PO_4 and 5.8 mg/ℓ fluorescein, acidified to less than pH 2 with concentrated HCl) was used to measure the fluorescein pK_a and absorptivity factors. The influence of five salts was also tested at two concentrations. The salts tested were KCl, KNO_3, K_2SO_4, NaCl and Na_2SO_4, and were added to the stock buffer solution at ionic strengths of 0.01M and 0.05M, therefore, in total 12 solutions were tested, including two batches of buffer solution.

Separate aliquot titrations were necessary (Fig. 2) because the small amounts of concentrated alkali solution added in the titration process were difficult to control and sometimes produced data with a large pH increment, which would have reduced the precision of the pK_a determination. However, as the aliquot solutions were identical, the titration data from each test were combined to produce a single data set and forty sets of measurements were selected from the combined data to yield a data series with small (≈ 0.2) pH increments throughout the test range. Titrations were made by adding small quantities of concentrated (>5 M) KOH solution to the test solution, stirring the solution, taking the pH, and collecting a sample for later absorbance testing. Samples were bagged and stored in the dark at 4°C until absorbance testing was performed. It was not necessary to measure volumes during the titration process because molar concentrations were calculated directly using the buffer concentration, pH and absorbance readings.

Titrations were performed at the pH meter calibration temperature (25°C) to ensure maximum accuracy but the absorbance measurements were made at different temperatures because the calculation method incorporated temperature corrections. The absorbance of the titration samples was measured at 5°C and 21°C. The absorbance maximum and a sample blank were tested between every 5°C measurement to confirm stable instrument response at

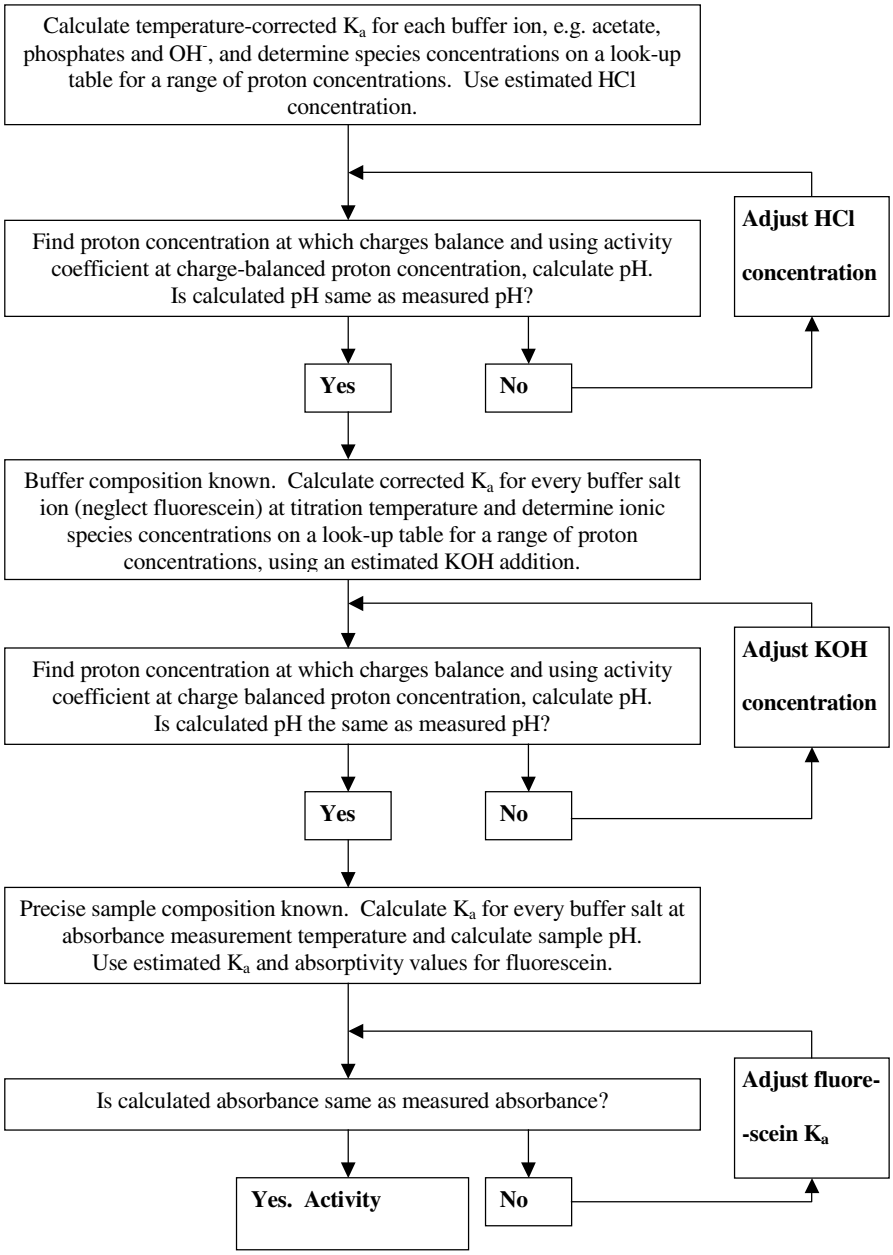

Figure 3
Flow diagram for fluorescein pK_a calculation procedure

this temperature. The titration and absorbance readings were then entered onto a computer spreadsheet that solved for the pK_a and absorptivity values of fluorescein.

Calculation procedure

The calculation procedure is shown as a flow diagram in Fig. 3. The chemical composition of the stock solution was calculated first by determining how much HCl was needed to adjust the buffer solution to the measured acidified pH. Each buffer ion species concentration was expressed in terms of the proton concentration, temperature-corrected ionisation constant and total salt concentration (similar to Eqs. (8) to (11)) and a look-up table was set up with a series of decreasing proton concentration values. The charge balance at each proton concentration was calculated on the look-up table using the buffer ion species concentrations and an estimated HCl addition, and a precise charge-balanced proton concentration was calculated. The activity coefficient was calculated at the charge-balanced ionic strength and this was used to calculate the pH. The spreadsheet "Solver" function was used to find the precise HCl addition concentration where the activity-corrected proton concentration was the same as the measured pH.

Once the chemical composition of the buffer was known a similar procedure was used to calculate the KOH added at each titration step using ionisation constants corrected for the titration temperature. Once the quantity of KOH was determined for each of the titration steps, the precise chemical composition of each titration sample was known. This information was used to calculate the pH at the temperature at which the absorbance was measured. At the end of these calculation steps the chemical composition, the ionic strength and the pH of the absorbance test solution were known as well as the KOH addition dilution effect.

Estimated test compound pK_a values were then used to calculate the different fluorescein ionic species concentrations at the calculated pH, and in conjunction with estimated absorptivity factors, a total absorbance value was predicted. The estimated pK_a values and absorptivity factors were then adjusted by the "Solver" function until the best least-squares fit was found between the predicted absorbance and the measured absorbance value for all of the titration samples. These *mixed* pK_a values were then corrected for activity using the ionic strength present at the pK_a pH.

The enthalpy of reaction for each fluorescein ionisation constant was determined using the apparent pK_a values at two different temperatures (Eq. (24)) to calculate the equivalent pK_a value at 25°C (Eq. (23)).

The standard error of the estimate was calculated for the absorbance values by comparing the measured values with those predicted using the calculated fluorescein parameters at the measured pH. A similar calculation was used to evaluate the standard error of the estimate for the pH measurements by comparing the measured pH with that predicted using the calculated fluorescein parameters at the measured absorbance. The pK_a scatter value was found by calculating the negative log of the maximum difference between the individual K_a measurements and the average K_a value.

The χ^2 statistic of the absorbance values was used as a measure of the confidence levels and model sensitivity. To test the model sensitivity, each model parameter was adjusted individually to determine what range of values produced an identical χ^2 value to that found during the pK_a-solving exercise.

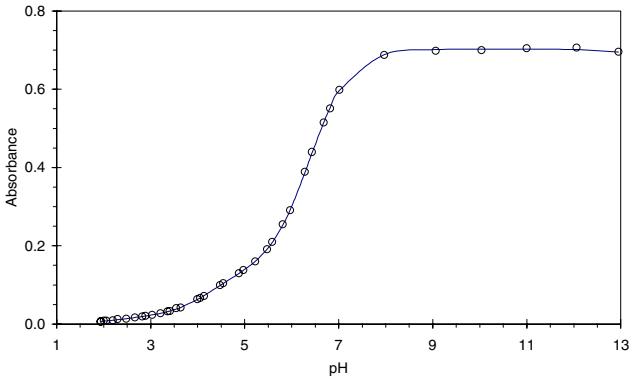

Figure 4
A typical example of fluorescein absorbance results showing close agreement between measured (o), and fitted (—) absorbance values. This titration used KOH on a buffered KNO_3 solution. Absorbance measurements were made at 21°C.

Results and discussion

pK_a determination evaluation

Fluorescein's Beer-Lambert law correlation was tested before performing the pK_a determination. These tests showed that there was a negligible deviation from Beer's law at a fluorescein concentration of 6 mg/ℓ, but that deviations became significant at concentrations above 20 mg/ℓ (data not shown). Therefore, the fluorescein concentration was kept at or below 6 mg/ℓ throughout the study.

Figure 4 shows 40 measured and fitted titration results from a typical pK_a-solving trial and the close correspondence between these values demonstrates the precision of this approach. The R^2 values for the curve fitting were high and ranged between 0.99967 and 0.99997. The combined pK_a results for the buffer solution and five different salt tests are listed in Table 1. Table 1 also shows pK_a limits based on the χ^2 value of the absorbance measurements. Variations in the value of the pK_{a1} parameter have less impact on the χ^2 statistic than pK_{a2} and pK_{a3}. However, as the standard error of the estimate was 5.4×10^{-3} for the absorbance values, and 4.3×10^{-2} for the pH measurements this parameter fitting process gives precise results.

Albert and Serjeant (1984) recommended that the scatter value was the most useful indication of pK_a variance, and suggested a guideline value of less than 0.06 as an indication of an acceptable pK_a determination. Using this guideline pK_{a3} and pK_{a2} are acceptable values (Table 1) while pK_{a1} is an unacceptable value. The pK_{a1} scatter value is high for two reasons: The first is that the test titrations started at pH 1.9, close to the pK_{a1} value, which limited the spread of data-points that could be measured in this area. As a result, the solving method generated a number of equally acceptable solutions that satisfied the best-fit criteria. The titrations started at pH 1.9 rather than at a lower pH because the ionic strength of the buffered solution increases during the titration and would approach 0.1M towards the end of the test. Ionic strengths above 0.1M would exceed the validity limit of the activity correction formulas used in the solving process (Guggenheim, 1935). The second reason for the high pK_{a1} scatter value is that the absorptivity factors for the cation and neutral fluorescein species are low compared to the dianion species and this reduces the impact of a change in the pK_{a1} value.

The impact of the pK_{a1} variability was reduced by fixing the cation absorbance factor at 18.75. This value uses the cation:dianion absorptivity ratio of Klonis and Sawyer (1996) with the dianion absorptivity factor determined in this study. If a researcher is particularly interested in having a precise pK_{a1} value then the tests could be repeated using a lower starting pH combined with a lower buffer strength. This was not considered justified here as fluorescein is generally used at a pH above pK_{a2} where the influence of an imprecise pK_{a1} is negligible. Also, the best-fit solving process used in this study is intrinsically biased towards finding the most important influences on the pH/absorbance.

Influence of cations and anions

Inspection of the pK_a values listed in Table 1 shows that for the cations and anions tested, the ion type has little influence on the pK_a. Once the activity effects have been taken into account, it makes no difference whether the predominant cation is potassium or sodium, or whether the predominant anion is chloride, nitrate or sulphate.

pK_a comparison

The pK_a values found in this study were compared to other values in the literature and these comparisons are shown in Table 2. In some cases it is possible to rework these values, for instance Lindqvist (1960) rounded his pK_a data to the nearest significant figure but using his graphical technique his pre-rounded pK_a values can be recalculated. These are shown in Table 2 and are very similar to those calculated in this study.

The remaining published pK_a values shown in Table 2 failed to correct for activity. Diehl and Markuszewski (1989) report that their measurements were all made at an ionic strength of 0.1M, once activity corrections are used their pK_{a2} and pK_{a3} results are close to the pK_a values found in this study. Similarly, Sjöback et al. (1995) used a weak phosphate buffer in combination with a range of different salt concentrations, and while they did correct for the activity of the salts they did not include the buffer in their activity corrections. They report *mixed* pK_a values in the presence of 0.05 M NaCl and 0.005 M phosphate buffer at a pH of 6.14, as 2.09, 4.30 and 6.41 (different from their pK_a values shown in Table 2). When these values are activity-corrected, the pK_{a2} and pK_{a3} values are again close to the pK_a values found in this study. Likewise, Klonis and Sawyer (1996) used weak buffers and did not correct for activity effects in their own experiments. They calculated their pK_a values by reanalysing data from Diehl and Horchak-Morris (1987) who performed their experiments at an ionic strength of 0.1M. Once activity corrections are applied, the pK_a values are close to the pK_a values found in this study (Table 2).

The similarities between the pK_a values of this study and the reworked values of the other publications (Table 2) show that it is the absence of activity corrections that causes most of the discrepancies between the published pK_a values of the three-pK_a models of fluorescein. It was not possible to re-evaluate all of the reported pK_a values. Some researchers use solvent systems other than water (Zanker and Peter, 1958) so their pK_a values might be expected to be different. In other cases, the determination method is not described in sufficient detail to allow reworking of the data (Bannerjee and Vig, 1971), or the researcher is only interested in a single pK_a near physiological values and uses fluorescent rather than absorbance techniques (Wolfbeis et al., 1983), or a different pK_a definition is adopted (Kasnavia et al., 1999).

TABLE 1
Activity corrected pK_a results

Test solution	Ionic strength (M)	Tested @ 5°C pK_{a1}	Tested @ 5°C pK_{a2}	Tested @ 5°C pK_{a3}	Tested @ 21°C pK_{a1}	Tested @ 21°C pK_{a2}	Tested @ 21°C pK_{a3}
Buffer (Batch 1)	0.024	2.26	4.36	6.81	2.17	4.36	6.71
Buffer (Batch 2)	0.022	2.38	4.34	6.82	2.27	4.33	6.69
KCl	0.034	2.36	4.38	6.80	2.25	4.37	6.69
KCl	0.074	2.40	4.38	6.86	2.27	4.35	6.73
KNO_3	0.034	2.43	4.39	6.81	2.30	4.36	6.68
KNO_3	0.074	2.34	4.37	6.86	2.19	4.35	6.71
K_2SO_4	0.034	2.26	4.35	6.83	2.18	4.34	6.68
K_2SO_4	0.074	2.24	4.37	6.88	2.19	4.33	6.75
NaCl	0.032	2.36	4.35	6.82	2.32	4.36	6.70
NaCl	0.072	2.39	4.36	6.85	2.22	4.30	6.71
Na_2SO_4	0.032	2.38	4.36	6.83	2.33	4.37	6.70
Na_2SO_4	0.072	2.35	4.37	6.87	2.22	4.33	6.73
Average		2.34	4.36	6.84	2.24	4.35	6.71
pK_a limits using χ^2 value		+0.14 -0.14	+0.09 -0.09	+0.05 -0.05	+0.16 -0.15	+0.10 -0.09	+0.05 -0.05
pK_a scatter value		0.10	0.03	0.04	0.09	0.04	0.05
Standard enthalpy (J/Mol)		9602	1702	12255			
pK_a values standardised@25°C		2.22	4.34	6.68			

TABLE 2
Reworked pK_a comparison

Published value pK_{a1}	Published value pK_{a2}	Published value pK_{a3}	After reworking pK_{a1}	After reworking pK_{a2}	After reworking pK_{a3}	Correction	Reference
			2.22	4.34	6.68	-	This study
2.2	4.4	6.7	2.19	4.36	6.66	Rounding	Lindqvist, 1960
2.19	4.24	6.36	2.07	4.36	6.72	Activity	Diehl & Markuszewski, 1989
2.08	4.31	6.43	2.00	4.39	6.69	Activity	Sjöback et al., 1995
2.25	4.23	6.31	2.13	4.35	6.67	Activity	Klonis & Sawyer, 1996

Absorptivity results

After adopting a cation absorptivity factor of 18.75 the 492 nm absorptivity factors for the neutral, monoanion and dianion fluorescein species were found to be 1476, 9393 and 48752 Mol^{-1} at 25°C. These values are only 62% and 55% of those reported by Diehl (1989) and Klonis and Sawyer (1996) respectively. However, according to the certificate of analysis the gravimetric purity of this fluorescein batch was only 53.8%. Thus, the 46.2% impurities adequately account for this investigation's absorptivity values being 45% lower than the Klonis and Sawyer (1996) values. While this purity may appear low, this is the grade of material that might typically be used in a tracer study, as it is more than ten times cheaper than the high purity grades. This highlights the need to establish absorptivity values specific for the fluorescein being used and shows that the purity concerns expressed by Lindqvist (1960) and Seybold et al. (1969) are well founded.

The absorptivity temperature coefficients for the different ionic species were -5.77×10^{-3} for the neutral species, -5.89×10^{-3} for the anion, and -1.60×10^{-3} for the dianion (the cation was not calculated because its value had been fixed at 18.75). The differences between the absorbance temperature coefficients for each ion suggest that fluorescein's absorbance/temperature response will not be simple. However, the dianion absorbance temperature coefficient is only half of the fluorescence temperature coefficient -3.6×10^{-3} (Feuerstein and Selleck, 1963), which suggests that temperature corrections are probably not essential for absorbance measurements made at a high pH where the dianion predominates.

Method evaluation

A 2% increase or decrease in the fluorescein concentration or sample dilution produced the same absorbance χ^2 value as that measured in the pK_a-solving trials. A positive or negative bias of

4.0×10^{-2} pH units, 9°C or 1.7×10^{-3} absorbance units produced similar differences. The solving method sensitivity to ionic strength variations was dependent on the ionic strength with a 1×10^{-2}M ionic strength bias required at an ionic strength of 2.5×10^{-2}M, and a 2×10^{-2}M ionic strength bias at an ionic strength of 7.5×10^{-2}M to yield a similar χ^2 value. This suggests that the solving method is more sensitive to absorbance, pH, concentration, and dilution bias and less sensitive to ionic strength and temperature variations.

Summary

The pK_a determination method used in this investigation can produce acceptable pK_a values and absorptivity factors for fluorescein, and while the published pK_a values of fluorescein do appear to differ, many of these values agree once activity corrections are applied. These corrected pK_a values are close to those found in this study: 2.22, 4.34 and 6.68.

Absorptivity values differ substantially between the various studies and should be determined for each fluorescein batch, spectrophotometer and sample cuvette combination.

The main advantage of this pK_a determination method is that it includes activity corrections, an important consideration because pK_a determination buffers have a marked influence on the pK_a measurements. This method also separates the titration and absorbance measurement processes, corrects for temperature changes and does not require sophisticated laboratory equipment.

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