Spectrophotometric determination of pKₐ values for fluorescein using activity coefficient corrections

SA Smith* and WA Pretorius
1 Worldthree, 735 SE High Street, Pullman, WA, 99163-2320, USA
2 Water Utilisation Division, University of Pretoria, Pretoria, South Africa

Abstract

The absorbance of the organic water tracer compound fluorescein is known to be pH dependent but differences between the reported pKₐ values make it difficult to predict these absorbance changes. A new pKₐ determination method, which incorporated activity corrections, was used to calculate the pKₐ values of fluorescein. Several published pKₐ 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 pKₐ values to account for this pH response. This paper describes a technique that was developed to measure the three pKₛ 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 pKₛ values so important.

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

Apart from the pKₛ 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 × 10⁵ with other published values of 7.4 × 10⁴ (Larsen and Johansson, 1989), 8.4 × 10⁴ (Hammond, 1979), 8.9 × 10⁴ (Delori et al., 1978, and Melhado et al., 1982) and 1.6 × 10⁷ M⁻¹cm⁻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 pKₛ determination method.

A number of different methods have been reported (Albert and Serjeant, 1984) for pKₛ determinations, which involve monitoring the response of the test compound throughout a titration. A simple and rapid pKₛ 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 pKₛ determination methods use mathematical techniques to simultaneously solve for the pKₛ 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 pKₛ 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
solution method, and includes activity and temperature corrections similar to those recommended by Albert and Serjeant (1984).

Theory

A three-pKₐ 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 pKₐ 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:

\[
\begin{align*}
H₃Flu^+ & \leftrightarrow H^+ + H₂Flu \\
H₂Flu & \leftrightarrow H^+ + HFlu^- \\
HFlu^- & \leftrightarrow H^+ + Flu²⁻
\end{align*}
\]

Relative equilibrium concentrations are denoted by the ionisation constant Kᵢ 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.

\[
\begin{align*}
K_{a1} &= \frac{[H^+][H₂Flu]}{[H₃Flu^+]} & (4) \\
K_{a2} &= \frac{[H^+][HFlu^-]}{[H₂Flu]} & (5) \\
K_{a3} &= \frac{[H^+][Flu²⁻]}{[HFlu^-]} & (6)
\end{align*}
\]

A fluorescein mass balance gives:

\[
[\text{Flu}_{\text{total}}] = [H₃Flu^+] + [H₂Flu] + [HFlu^-] + [Flu²⁻] & (7)
\]

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

\[
\begin{align*}
[H₃Flu^+] &= \frac{[\text{Flu}_{\text{total}}]}{1 + \frac{K_{a1}}{[H^+]^2} + \frac{K_{a1}K_{a2}}{[H^+]^3}} & (8) \\
[H₂Flu] &= \frac{[\text{Flu}_{\text{total}}]}{\frac{[H^+]}{K_{a1}} + 1 + \frac{K_{a2}}{[H^+]}} & (9) \\
[HFlu^-] &= \frac{[\text{Flu}_{\text{total}}]}{\frac{[H^+]}{K_{a1}K_{a2}} + \frac{[H^+]}{K_{a2}} + 1 + \frac{K_{a3}}{[H^+]}} & (10) \\
[Flu²⁻] &= \frac{[\text{Flu}_{\text{total}}]}{\frac{[H^+]}{K_{a1}K_{a2}K_{a3}} + \frac{[H^+]²}{(K_{a2}K_{a3})} + \frac{[H^+]}{K_{a3}} + 1} & (11)
\end{align*}
\]

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 Kᵢ 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:

\[
\text{Absorbance} = εbc
\]

Figure 1
Six ionic forms of fluorescein after Zanker and Peter (1958)
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 (F₁, F₂, F₃, and F₄). The total absorbance is the sum of the absorbencies of the different fluorescein species, such that:

\[ \text{Total absorbance} = F_1[H\text{Flu}] + F_2[H_2\text{Flu}] + F_3[H\text{Flu}] + F_4[\text{Flu}^\text{2-}] \]

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

\[ \text{Total absorbance} = F_i(\text{Eq.8}) + F_i(\text{Eq.9}) + F_i(\text{Eq.10}) + F_i(\text{Eq.11}) \]

If the total fluorescein, the pH and total absorbance are known there are seven unknowns: Ka₁, Ka₂, Ka₃, F₁, F₂, F₃ and F₄. 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:

\[ \log \gamma = -0.5Z^2 \frac{\mu}{1 + \sqrt{\mu}} \]

The ionic strength (µ) being:

\[ \mu = 0.5 \sum_i C_i Z_i^2 \]

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:

\[ \gamma_i = 10^{-0.5 \frac{0.5Z^2}{(1 + \sqrt{\mu})}} \]

And the activity coefficient formula for dionic species was:

\[ \gamma_i = 10^{-2 \frac{0.5Z^2}{(1 + \sqrt{\mu})}} \]

As pH meters measure the proton activity, pH readings are already activity corrected. This means that the mixed Kaᵢ 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 Kᵢ. Equations 4, 5 and 6 can be expanded to include this activity correction.

\[ K_{aT} = \frac{[H^+][H\text{Flu}]/[\gamma_i[H\text{Flu}^\text{1+}]]}{[H^+][H\text{Flu}]/[\gamma_i[H\text{Flu}^\text{2-}]]} = \frac{[H^+][H\text{Flu}]/[\gamma_i[H\text{Flu}]]}{[H^+][H\text{Flu}]/[\gamma_i[H\text{Flu}^\text{2-}]]} \]

Where the curved brackets denote activity, the square brackets denote molarity, and the activity coefficient of the uncharged species (H_Flu) 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 pKᵢ.

**pKᵢ temperature corrections**

The Kᵢ 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).

\[ \ln \left( \frac{K_{aT_2}}{K_{aT_1}} \right) = -\frac{\Delta H}{R} \left( \frac{T_1 - T_2}{T_1 T_2} \right) \]

Equation (22) was rearranged to give:

\[ K_{aT_2} = K_{aT_1} e^{\frac{-\Delta H}{R} \left( \frac{T_1 - T_2}{T_1 T_2} \right)} \]

where:
- H° is the standard enthalpy of reaction 
- R is the gas constant 
- Kᵢ₁ is the ionisation constant at 298.15 Kelvin (T₁) 
- Kᵢ₂ is the apparent ionisation constant at the experimental temperature T₂

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.

\[ \Delta H^0 = -R \left( \frac{T_1 - T_2}{T_1 T_2} \right) \ln \left( \frac{K_{aT_2}}{K_{aT_1}} \right) \]

**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).

\[ F_i = F_i e^{(T_2 - T_1)} \]

Available on website http://www.wrc.org.za

ISSN 0378-4738 = Water SA Vol. 28 No. 4 October 2002 397
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:

\[
F_T = \frac{F_{T_1}}{e^{n(T_s - T)}} = \frac{F_{T_2}}{e^{n(T_s - T)}},
\]

where:
- \( F_{T_1} \) and \( F_{T_2} \) are the absorbance factors at temperatures \( T_1 \) and \( T_2 \).

Solving for \( n \) gives:

\[
\ln\left(\frac{F_{T_2}}{F_{T_1}}\right) = \frac{n(T_2 - T_1)}{T_1 - T_2},
\]

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.

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 KH₂PO₄ and 5.8 mg/G6C fluorescein, acidified to less than pH 2 with concentrated HCl) was used to measure the fluorescein pKₐ and absorptivity factors. The influence of five salts was also tested at two concentrations. The salts tested were KCl, KNO₃, K₂SO₄, NaCl and Na₂SO₄, 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 pKₐ 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
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 pKₐ 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 pKₐ 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 pKₐ values were then corrected for activity using the ionic strength present at the pKₐ pH. The enthalpy of reaction for each fluorescein ionisation constant was determined using the apparent pKₐ values at two different temperatures (Eq. (24)) to calculate the equivalent pKₐ 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 pKₐ scatter value was found by calculating the negative log of the maximum difference between the individual Kₐ measurements and the average Kₐ value.

The χ² 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 χ² value to that found during the pKₐ-solving exercise.

Figure 3
Flow diagram for fluorescein pKₐ calculation procedure
was the most useful indication of $pK_a$ variance, and suggested a
gives precise results.

4.3×10$^{-2}$ for the pH measurements this parameter fitting process
centrations above 20 mg/G6C
was a negligible deviation from Beer's law at a fluorescein
values (Table 1) while $pK_{a1}$ is an unacceptable value. The $pK_{a1}$
typical $pK_a$-solving trial and the close correspondence between
limits based on the
and 0.99997. The combined $pK_a$ results for the buffer solution and
values for the curve fitting were high and ranged between 0.99967
these values demonstrates the precision of this approach. The $R^2$
error of the estimate was 5.4×10$^{-3}$ for the absorbance values, and
$pK_a$ 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 0.99997
and 0.99997. The combined $pK_a$ results for the buffer solution and
different salt tests are listed in Table 1. Table 1 also shows $pK_a$
limits based on the $\chi^2$ value of the absorbance measurements.
Variations in the value of the $pK_a$ parameter have less impact on
the $\chi^2$ statistic than $pK_{a1}$ and $pK_{a2}$. However, as the standard
error of the estimate was $5.4\times10^{-4}$ for the absorbance values, and
$4.3\times10^{-3}$ 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 $pK_{a1}$ 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 absorptivity 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 $pK_a$ 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
close 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,
when 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.

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 (Wolffbeis et al., 1983), or a different
$pK_a$ definition is adopted (Kasnavia et al., 1999).
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 \(\chi^2\) value as that measured in the pKa-solving trials. A positive or negative bias of

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Ionic strength (M)</th>
<th>Tested @ 5°C</th>
<th>Tested @ 21°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (Batch 1)</td>
<td>0.024</td>
<td>2.26 4.36 6.81</td>
<td>2.17 4.36 6.71</td>
</tr>
<tr>
<td>Buffer (Batch 2)</td>
<td>0.022</td>
<td>2.38 4.34 6.82</td>
<td>2.27 4.33 6.69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.034</td>
<td>2.36 4.38 6.80</td>
<td>2.25 4.37 6.69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.074</td>
<td>2.40 4.38 6.86</td>
<td>2.27 4.35 6.73</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>0.034</td>
<td>2.43 4.39 6.81</td>
<td>2.30 4.36 6.68</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>0.074</td>
<td>2.34 4.37 6.86</td>
<td>2.19 4.35 6.71</td>
</tr>
<tr>
<td>K(_2)SO(_4)</td>
<td>0.034</td>
<td>2.26 4.35 6.83</td>
<td>2.18 4.34 6.68</td>
</tr>
<tr>
<td>K(_2)SO(_4)</td>
<td>0.074</td>
<td>2.24 4.37 6.88</td>
<td>2.19 4.33 6.75</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.032</td>
<td>2.36 4.35 6.82</td>
<td>2.32 4.36 6.70</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.072</td>
<td>2.39 4.36 6.85</td>
<td>2.22 4.30 6.71</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>0.032</td>
<td>2.38 4.36 6.83</td>
<td>2.33 4.37 6.70</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>0.072</td>
<td>2.35 4.37 6.87</td>
<td>2.22 4.33 6.73</td>
</tr>
<tr>
<td>Average</td>
<td>2.34 4.36 6.84</td>
<td>2.24 4.35 6.71</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1
Activity corrected pK\(_a\) results

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Ionic strength (M)</th>
<th>Tested @ 5°C</th>
<th>Tested @ 21°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (Batch 1)</td>
<td>0.024</td>
<td>2.26 4.36 6.81</td>
<td>2.17 4.36 6.71</td>
</tr>
<tr>
<td>Buffer (Batch 2)</td>
<td>0.022</td>
<td>2.38 4.34 6.82</td>
<td>2.27 4.33 6.69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.034</td>
<td>2.36 4.38 6.80</td>
<td>2.25 4.37 6.69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.074</td>
<td>2.40 4.38 6.86</td>
<td>2.27 4.35 6.73</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>0.034</td>
<td>2.43 4.39 6.81</td>
<td>2.30 4.36 6.68</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>0.074</td>
<td>2.34 4.37 6.86</td>
<td>2.19 4.35 6.71</td>
</tr>
<tr>
<td>K(_2)SO(_4)</td>
<td>0.034</td>
<td>2.26 4.35 6.83</td>
<td>2.18 4.34 6.68</td>
</tr>
<tr>
<td>K(_2)SO(_4)</td>
<td>0.074</td>
<td>2.24 4.37 6.88</td>
<td>2.19 4.33 6.75</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.032</td>
<td>2.36 4.35 6.82</td>
<td>2.32 4.36 6.70</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.072</td>
<td>2.39 4.36 6.85</td>
<td>2.22 4.30 6.71</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>0.032</td>
<td>2.38 4.36 6.83</td>
<td>2.33 4.37 6.70</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>0.072</td>
<td>2.35 4.37 6.87</td>
<td>2.22 4.33 6.73</td>
</tr>
<tr>
<td>Average</td>
<td>2.34 4.36 6.84</td>
<td>2.24 4.35 6.71</td>
<td></td>
</tr>
</tbody>
</table>

pK\(_a\) limits using \(\chi^2\) value

<table>
<thead>
<tr>
<th>Published value</th>
<th>After reworking</th>
<th>Correction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK(_{a1})</td>
<td>pK(_{a2})</td>
<td>pK(_{a3})</td>
<td>pK(_{a1})</td>
</tr>
<tr>
<td>2.2</td>
<td>4.4</td>
<td>6.7</td>
<td>2.19</td>
</tr>
<tr>
<td>2.08</td>
<td>4.31</td>
<td>6.31</td>
<td>2.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Published value</th>
<th>After reworking</th>
<th>Correction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK(_{a1})</td>
<td>pK(_{a2})</td>
<td>pK(_{a3})</td>
<td>pK(_{a1})</td>
</tr>
<tr>
<td>2.22</td>
<td>4.34</td>
<td>6.68</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE 2
Reworked pK\(_a\) comparison

4.0 \times 10^{-2} \text{ pH units}, 9^\circ \text{C} or 1.7 \times 10^{-3} \text{ absorbance units produced}

2M ionic strength bias at an ionic strength of 7.5 \times 10^{-2} \text{M to yield a strength bias required at an ionic strength of 2.5 \times 10^{-2} \text{M, and a 2} \times 10^{-2} \text{M ionic strength bias at an ionic strength of 7.5 \times 10^{-2} \text{M to yield a similar } \chi^2 \text{ 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.

**Acknowledgements**

We thank Kelly Brayton for helpful discussion and critical review of the manuscript and Tex Hunter for providing the Turner 350 spectrophotometer. This work is in partial fulfilment of an M.Sc. degree (Smith, 2001).

**References**


