## 2.9 Absorbance principles

A number of studies have made the assumption that fluorescein obeys the Beer-Lambert law, (Adelman and Oster, 1956, Delori *et al.*, 1978, and Diehl, 1989) however if this assumption is not correct some of their observations, such as the absorptivity values may need to be adjusted.

The Beer-Lambert law states that when a beam of light passes through a solution containing a light-absorbing analyte, the relationship between the analyte concentration and the absorption is described by:

$$A = \log\left(\frac{P_o}{P}\right) = \varepsilon bc$$

Where A is the absorbance, $P_0$ the light beam radiant power before attenuation, P the attenuated radiant power, ε the molar absorptivity, b the path length through the absorbing medium and c the analyte concentration. The Beer-Lambert law can be split into two parts with Beer's law stating that there is a linear relationship between absorbance and concentration at a fixed path length, and Lambert's law stating that there is a linear relationship between absorbance and path length at a fixed concentration (Braude *et al.*, 1950). As this investigation uses a fixed path length only Beer's law will be discussed.

In practice there are frequently deviations from the direct proportionality of Beer's law but these deviations are well known spectrophotometry phenomena, and can be categorised as; real limitations to Beer's law, instrumental deviations and chemical deviations (Skoog *et al.*, 1992).

### 2.9.1 Real limitations to Beer's law

The real limitations to Beer's law concern its application to concentrated solutions when it should only be applied to dilute solutions: When solutions contain high concentrations of ions (> 0.01 M) these charged species affect the charge distribution on adjacent analyte molecules, which in turn can change the light absorbing character of the molecule. In the case of a dilute absorber in the presence of a non-absorbing electrolyte, the direct proportionality between absorbance and concentration of the absorber can also be disturbed by the electrolyte concentration (Skoog *et al.*, 1992).

### 2.9.2 Chemical deviations

Chemical deviations are the apparent deviations from Beer's law that occur when an analyte changes in the presence of the solvent to form compounds that have a different light absorbing character from the parent species. The ionisation reactions of acidic or basic indicators are examples of this behaviour. As the indicator concentration increases so does its influence on the pH, and when the pH changes so do the proportions of the different ionised species which in turn changes the absorptivity of the solution (Skoog *et al.*, 1992).

### 2.9.3 Instrumental deviations

There are two main types of instrumental deviations, those due to the presence of polychromatic radiation, and those due to the presence of stray-light. Polychromatic deviations occur when more than one wavelength of light is present and the radiant power of these wavelengths is absorbed in different proportions by the analyte. The difference between the molar absorptivities of the compound at the different wavelengths determines the size of this Beer's law deviation. However, if the analyte has a consistent absorptivity within the polychromatic range, this instrumental error will not be appreciable (Skoog *et al.*, 1992).

Light scattering inside the detection area of the spectrophotometer causes stray-light deviations from Beer's law. The resulting absorbance deviation increases with increasing absorbance because the stray-light represents an increasingly significant part of the signal that reaches the detector. Such instrumental deviations from Beer's law always cause underestimates of the analyte concentration (Meehan, 1981 as quoted by Skoog *et al.*, 1992).

## 2.10 Fluorescence principles

The fundamental difference between absorbance and fluorescence lies in the way an excited molecule loses its energy after it has been irradiated. Absorbed radiant energy normally dissipates quickly through a variety of nonluminescent pathways, but fluorescent molecules have configurations that stabilize the excited state and this longer excitement period increases the probability that a proportion of the absorbed energy will be re-emitted in the form of a detectable light signal. As some of the absorbed energy is lost during the conversion from absorbed radiation to emitted radiation, the wavelength of the emitted light is longer than that of the absorbed light (Skoog *et al.*, 1992).

As the fluorescence signal is radiated in all directions the fluorescence detector can be placed at right angles to the irradiation source and sample. Using this arrangement it is possible to provide greater radiant excitation power without an equivalent increase in detector signal because the beam passing through the sample does not fall directly on the detector. This allows an increase in detection sensitivity because the fluorescence signal is more dependent on the concentration of the analyte and less dependent on the transmitted radiant power. Furthermore, the wavelength difference between the absorbed and emitted wavelengths makes it possible to use filters or monochromators, to select only those wavelengths that yield the most specific response, and completely exclude the exciting wavelength.

There is an overlap between the absorbance spectrum and emission spectra of fluorescein (Heller *et al.*, 1974). The result is that as the analyte concentration increases, in addition to the excitation signal, the compound also absorbs an increasing proportion of the emitted signal. This causes a deviation from the linear relationship between fluorescence and concentration and this self-quenching or inner-filter effect becomes significant at an absorbance above 0.05 (Skoog *et al.*, 1992). Some researchers have corrected for this problem by choosing excitation wavelengths that yield optical densities lower than 0.03 (Martin and Lindqvist, 1975) while others minimised this inner-filter error by keeping the total absorbance less than 0.06 (Sjöback *et al.*, 1995).

## 2.11 Fluorescein determinations using absorbance

Apart from the three Beer's law deviations noted in Sections 2.9.1 to 2.9.3, there is a fourth Beer's law deviation characteristic of fluorescent compounds. This is where the fluorescent emission reaches the instrument detector and thus reduces the absorbance signal (Gibson and Keegan, 1938). There is a further complication in that at higher sample concentrations this fluorescent emission is reabsorbed by the sample and this deviation becomes appreciable above 1.0 absorbance unit (Braude *et al.*, 1950). This reabsorption of fluorescence is also important in calorimetric measurements at fluorescein concentrations of $10^{-4}$ M (Seybold *et al.*, 1969) but different instrument geometries are expected to respond differently to these potential complications (Umberger and LaMer, 1945).

Imamura (1958) reported an error of 1.6% due to the absorbance/fluorescence interaction but did not see any deviations from Beer's Law due to fluorescence re-adsorption in the

fluorescein concentration range of 0 to $10^{-5}$ M (about 3.8 mg/l). Lindquist (1960) also tested the absorbance response of fluorescein at concentrations from up to $10^{-5}$ M at pH 1, 3.3 and 5.5, and concluded that Beer's law was valid for the three ionic forms present under these conditions, i.e. the cation, neutral form and anion. He avoided other Beer's law deviations by using buffers and dilute solutions. It should be noted however that Lindqvist's spectrophotometer was an unusual design in that it employed double monochromators on the light beam between the sample and the detector and that this design might be expected to be less susceptible to polychromatic deviations and stray-light problems. More importantly, there would only be low levels of the fluorescein dianion even at pH 5.5 so the problems caused by this highly fluorescent species would not be significant.

Similar fluorescent complications have been noted for Lambert's law (Moran and Stonehill, 1957) but as the sample path length is consistent throughout this investigation this deviation will not be an issue here. The guidelines to ensure accuracy are to avoid high absorbance readings, work in the absorbance range of 0.3 to 0.7, and to test Beer's law wherever possible (Braude *et al.*, 1950).

## 2.12 Molar absorptivity value of fluorescein

Table 2 shows a number of the molar absorptivities that have been reported for fluorescein and these are listed chronologically to show that there is no approaching consensus. This lack of agreement has been ascribed to a variety of factors including, the use of impure materials (Lindqvist, 1960, and Seybold *et al.*, 1969), neglecting to specify the concentrations and pH of the test solutions (Orndorff, Gibbs and Shapiro, 1928), or differences in instrument geometry (Umberger and LaMer, 1945). Purity problems are not always an issue however because Heller *et al.* (1974) noted similar absorptivity results for the fluorescein as purchased, as for samples prepared using two different purification methods and while the moisture content of fluorescein will affect its apparent purity this does not appear to be a problem as most researchers describe the precautions taken to avoid this complication (Boets *et al.*, 1992 and Sjöback *et al.*, 1995).

**Table 2 Molar absorptivity values reported for fluorescein**

| Year | Absorptivity ($M^{-1} cm^{-1}$) | Reference |
|---|---|---|
| 1928 | $7.62 \times 10^4$ | Orndorff *et al.* |
| 1931 | $7.82 \times 10^4$ | Lewschin, as quoted by Umberger and LaMer (1945) |
| 1945 | $7.85 \times 10^4$ | Umberger and LaMer |
| 1956 | $5.46 \times 10^4$ | Adelman and Oster |
| 1958 | $7.80 \times 10^4$ | Imamura |
| 1960 | $8.8 \times 10^4$ | Lindqvist |
| 1969 | $8.79 \times 10^4$ | Seybold *et al.* |
| 1974 | $8.932 \times 10^4$ | Heller *et al.* |
| 1978 | $8.9 \times 10^4$ | Delori *et al.* |
| 1979 | $8.4 \times 10^4$ | Hammond |
| 1982 | $8.9125 \times 10^4$ | Melhado *et al.* |
| 1985 | $1.6 \times 10^5$ | Grotte *et al.* |
| 1989 | $7.79 \times 10^4$ | Diehl |
| 1989 | $7.4 \times 10^4$ | Larsen and Johansson |
| 1992 | $8.70 \times 10^4$ | Boets *et al.* |
| 1995 | $7.69 \times 10^4$ | Sjöback *et al.* |
| 1996 | $8.7692 \times 10^4$ | Klonis and Sawyer |

The molar absorptivity values listed in Table 2 were determined at analytical wavelengths between 490 and 495nm because this is the absorbance maximum of the dianion fluorescein species, which has the highest absorptivity value of the four ionic species. However it should be noted that the monoanion, neutral and cation species also have high absorptivity values (Klonis and Sawyer, 1996) and the potential presence of these ionic species obviously makes it essential that reported absorptivity values use the same analytical wavelength.

Questions about the accuracy of reported absorptivities continue to be raised. Boets *et al.* (1992) were especially concerned about this because standardised fluorescein solutions are used to calibrate ophthalmic fluorometers, but as fluorescein is strongly fluorescent it is reasonable to assume that it is also subject to the complications noted by Gibson and Keegan (1938) and Braude *et al.* (1950).

## 2.13 The $pK_a$ values of fluorescein

As the absorbance and fluorescent character of fluorescein is pH dependent it is vital to know the exact pH at which these changes occur so that the behaviour of the compound can be predicted. Unfortunately there is little agreement between the published $pK_a$ values of fluorescein and this is seen in Table 3. The values have been listed chronologically to show that there is no approaching consensus.

There appear to be four main reasons for the $pK_a$ discrepancies noted in Table 3 and these can be categorized as the absence of activity corrections, a reliance on fluorescent measurements, the use of non-aqueous solvents, and differences in terminology.

**Table 3 Published values for fluorescein $pK_a$s**

| Year | Reported $pK_a$s | | | Researchers |
|---|---|---|---|---|
| | $pK_{a1}$ | $pK_{a2}$ | $pK_{a3}$ | |
| 1958 | 1.95 | 5.05 | 7.00 | Zanker and Peter |
| 1960 | 2.2 | 4.4 | 6.7 | Lindqvist |
| 1967 | 2.25 | 5.1 | 7.1 | Vig as quoted by Bannerjee and Vig (1971) |
| 1971 | 2.0 | 4.75 | 7.0 | Bannerjee and Vig |
| 1983 | ( | 6.7 | ) | Wolfbeis *et al.* |
| 1985 | 2.13 | 4.44 | * | Diehl and Markuszewski. |
| 1985 | ( | 6.4 | ) | Grotte *et al.* |
| 1986 | * | * | 6.36 | Diehl *et al.* |
| 1987 | 2.18 | 4.40 | 6.36 | Diehl and Horchak-Morris |
| 1989 | 2.19 | 4.24 | 6.33 | Diehl |
| 1989 | 2.19 | 4.24 | 6.36 | Diehl and Markuszewski. |
| 1995 | 2.08 | 4.31 | 6.43 | Sjöback *et al.* |
| 1996 | 2.25 | 4.23 | 6.31 | Klonis and Sawyer |
| 1997 | ( | 5.6/5.7 | ) | Kasnavia |
| 1999 | ( | 5.1 | ) | Kasnavia, *et al.* |

* Not determined

### 2.13.1 Activity corrections

Wolfbeis *et al.* (1983) measured the $pK_a$s of a number of fluorescent compounds and also reported detailed results for the fluorescent tracer 1-hydroxy-pyrene-3,6,8-trisulphonate (pyranine). They noted that their result was dependent on the buffer ionic strength and this suggests that their method does not correct for activities. A number of other studies also do not report correcting for activity effects (Diehl and Markuszewski, 1985, Diehl, 1989, Klonis and Sawyer, 1996, and Diehl and Horchak-Morris, 1987) and this suggests that the use of activity corrections is not standard practice. This is contrary to the recommendation that activity corrections be used for all but the most dilute solutions (Albert and Serjeant, 1984).

### 2.13.2 Reliance on fluorescence measurements

Grotte *et al.* (1985) used a fluorescence $pK_a$ determination method and identified the steepest gradient region of the fluorescence response as the $pK_a$. Wolfbeis *et al.* (1983) also relied on a fluorescence based $pK_a$ determination method. As most of the fluorescent response is associated with the dianion species (Martin and Lindqvist, 1975) this sort of test should only identify the $pK_{a3}$ value. The similarities between the single $pK_a$s of Wolfbeis *et al.* (1983) and Grotte *et al.* (1985) and the $pK_{a3}$ values of other studies appears to support this conclusion.

### 2.13.3 Non-aqueous $pK_a$ determinations

Lindqvist (1960) questioned the applicability of the Zanker and Peter (1958) $pK_a$ figures as they used dioxane solutions that have different solvent character and ionic strength. This observation does appear to be justified because the Zanker and Peter (1958) $pK_a$s are different from most of the other three-$pK_a$ values.

### 2.13.4 Terminology differences

Kasnavia *et al.* (1999) refer to the work of Kasnavia (1997) and report a single $pK_a$ for fluorescein determined using a potentiometric method. This value appears to be a mistake however because while Kasnavia (1997) did calculate a single $pK_a$, it was reported as both 5.6 and 5.7. The idea that a polyprotic molecule can have a single $pK_a$ stems from the $pK_a$ definition expressed by Kasnavia *et al.* (1999); that the $pK_a$ of a molecule is the pH where

half of its functional groups are neutralised and half are ionic. This is different from the traditional definition, where each ionisable group has its own ionisation constant.

### 2.13.5 The impact of $pK_a$ values

Thermodynamic $pK_a$s of a given compound should be consistent because, by definition, these $pK_a$s are standardised for temperature and activity effects. Table 3 shows that the differences between the Lindqvist (1960), Diehl and Markuszewski (1989), Sjöback *et al.* (1995) and Klonis and Sawyer (1996) $pK_a$ figures are apparently small but these small differences can have a large impact on the apparent fluorescein recovery. This is shown in Figure 2 where the Klonis and Sawyer (1996) $pK_a$ values are assumed correct and the apparent fluorescein concentration is calculated using the $pK_a$s of other studies. Thus if fluorescein was expected to behave according the Klonis and Sawyer (1996) $pK_a$s but actually has the $pK_a$s reported by Lindqvist (1960) then only 70% of the fluorescein would be detected at pH 6.1 at the absorbance wavelength of 490nm. This sort of discrepancy has obvious implications for the apparent conservative nature of a tracer and highlights the importance of accurate $pK_a$ values.

## 2.14 $pK_a$ determination methods

The precautions required for accurate $pK_a$ measurements are not normally described in detail so the laboratory manual of Albert and Serjeant (1984) was used extensively in this investigation. These researchers provide detailed information about the steps that must be taken to reduce errors, and also discuss the limitations of different approaches. They recommend that potentiometric determinations be made wherever possible, mainly because of its speed and accuracy.

The choice of a spectrophotometric $pK_a$ determination method in this study runs counter to the recommendation that spectrophotometric methods be used only when potentiometric determinations are not applicable (Albert and Serjeant, 1984). The main reason for selecting the spectrophotometric method here is that the fluorescein user is interested in the compound's photometric behaviour. Also, if water from the study system is used during the spectrophotometric $pK_a$ determination it might identify effects that would be difficult to

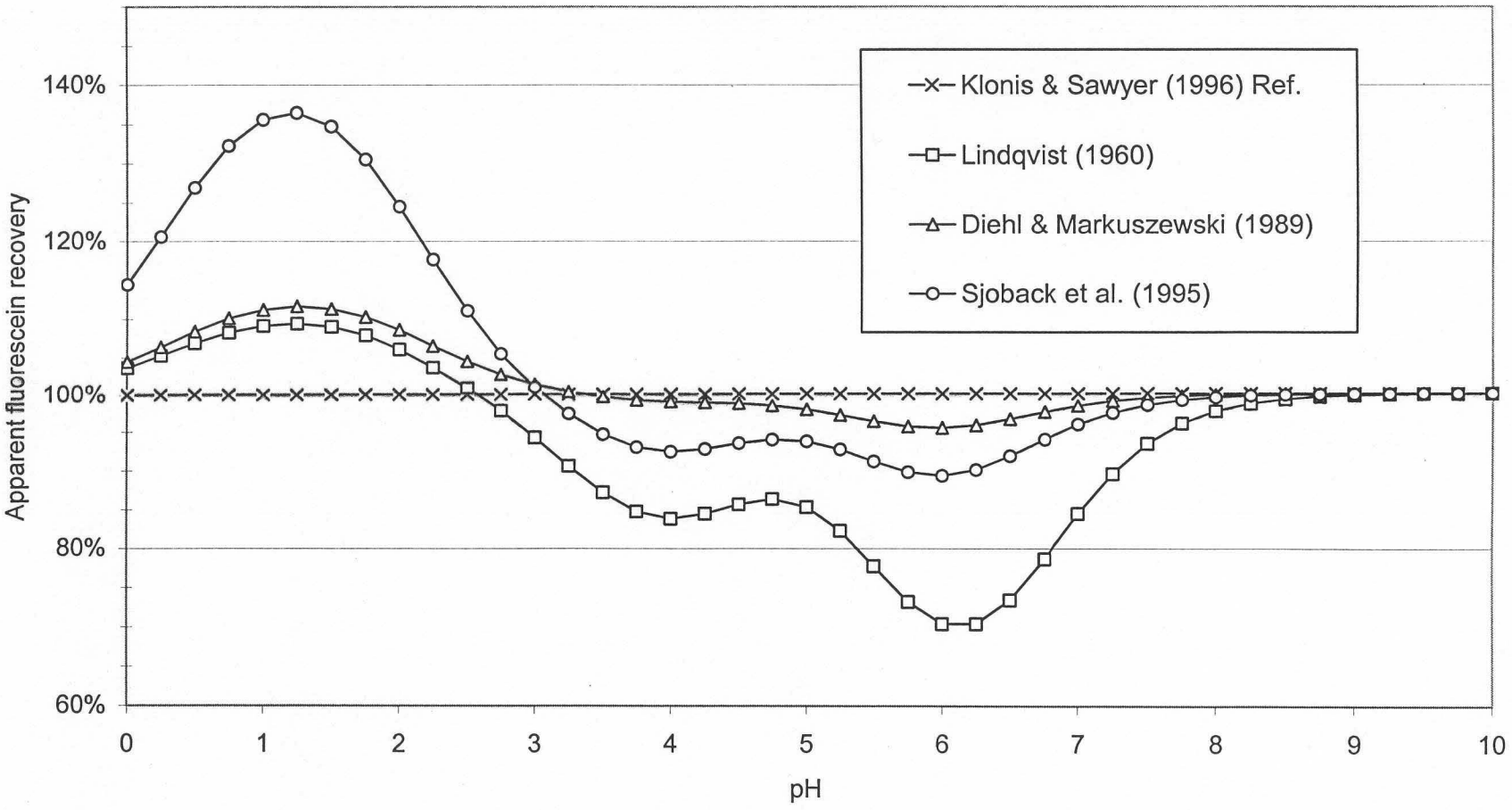


**Figure 2 The impact of different $pK_a$ values using Klonis and Sawyers' (1996) $pK_a$s as a reference.**

detect using the potentiometric method. Examples of this include:

- Chelating metals may be present that change the fluorescent signal in a manner similar to the equivalent quantity of base (Meinke and Scribner, 1967).
- Charge transfer systems may be present, e.g. bromide, iodide, thiocyanate and thiosulphate, that cause fluorescence quenching (Meinke and Scribner, 1967).
- Spectrophotometric $pK_a$ determination methods require test concentrations similar to those measured in the field, whereas the larger test concentrations required by the potentiometric method might mask the more subtle concentration dependent influences.
- If the spectrophotometer used for the $pK_a$ determination is the same instrument used for sample measurements then instrument problems may be anticipated and eliminated.

A common sense approach is required in the precautions taken to ensure accuracy and these precautions will be dictated by the intended application of the $pK_a$ value (Albert and Serjeant, 1984). They propose a scatter value of 0.06 as an indication of the precision of a series of $pK_a$ measurements. This scatter value is the logarithm of the difference between the average ionisation constant (the $K_a$ not the $pK_a$ value) and the reading that lies furthest from this average. They stress the correct calibration of the pH meter, as this is obviously crucial to all $pK_a$ determinations.

The rapid $pK_a$ approximation technique (Clark and Cunliffe, 1973) may be adequate for some applications (Albert and Serjeant, 1984). This method is a simplified spectrophotometric method that eliminates the need to weigh the test compound, make up volumetric solutions, or measure the volume of titrant. These are important simplifications that make it attractive for routine use, but this method does not incorporate activity corrections and although Clark and Cunliffe (1973) recommend using a buffer of low total ionic strength, their preferred buffers have concentrations greater than 0.08 M. This is larger than the 0.01 M maximum limit above which activity corrections are recommended (Albert and Serjeant, 1984). More recent fluorescein $pK_a$ determinations have used mathematical techniques to simultaneously solve for the $pK_a$s but 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 buffers (Sjöback *et al.*, 1995).

The ideal method would combine the precision of the Albert and Serjeant (1984) approach, the simplicity of the Clark and Cunliffe (1973) method and minimal equipment requirements of the mathematical approaches (Klonis and Sawyer, 1996, and Sjöback *et al.*, 1995) but must also include activity and temperature corrections.

## 2.15 Aims

This review has shown that a variety of molar absorptivities have been reported for fluorescein and that these differences are important. It has also shown that different $pK_a$ values have been reported and that these differences have a substantial impact on fluorescein measurements. This lack of agreement makes it difficult for the fluorescein user to select values appropriate for their circumstances. However, despite the differences there is agreement on the ionic forms of fluorescein and the nature of the ionic changes with pH. This foundation can be used to develop a method that will allow fluorescein users to calculate molar absorptivity values specifically for their own analytical instruments while simultaneously confirming the $pK_a$ values of fluorescein.

The aims of this study are:

1. To develop and test an alternative fluorescein $pK_a$ determination method. This method must be practical with a minimum of equipment, be reproducible, and be capable of calculating absorptivity values specific for the field analytical instrument and the fluorescein quality used in the investigation.
2. To determine the fluorescein concentration at which Beer's law deviation becomes significant for the spectrophotometer used in this study.
3. To compare the relative effects of light degradation and heat degradation, so that water researchers can take appropriate tracer preservation precautions.
4. To quantitatively test the recovery of fluorescein from a gravel-packed test column using the pre-determined $pK_a$ values and compare these results with an alternative recovery method.

The investigation focuses on the apparent non-conservative nature of fluorescein. This non-conserved fluorescein might more properly be called: undetected.