Chapter 5 Results and Discussion

5.1 pK_a Determinations

5.1.1 Method evaluation

Figure 6 shows 40 measured and fitted titration results from one of the twelve-pK_a solving exercises used to determine the thermodynamic pK_as and standardised absorptivity factors of fluorescein. The close correspondence between the fitted and measured values in Figure 6 demonstrates the precision of this approach. The combined pK_a and absorptivity results for the buffer solutions and salt solution tests at two different temperatures are listed in Table 4; again the high R^2 values confirm that the curve-fitting method was effective.

Albert and Serjeant (1984) suggested that a scatter value was the most useful indication of pK_a variance, and recommended a guideline value of less than 0.06 as an indication of an acceptable pK_a determination. Using this guideline pK_{a2} and pK_{a3} are acceptable values at 0.044 and 0.046 respectively, while pK_{a1} is not an acceptable value at 0.100 (see Table 4).

The pK_{a1} scatter value is high for two reasons. The first reason is that the absorptivity factors for the cation and neutral species are low compared to the dianion species at a wavelength of 492nm . This reduces the impact of a change in the pK_{a1} value such that a number of different pK_{a1} values can produce similarly good fits using the spreadsheet algorithm. The second reason is that the test titrations started at pH 1.9, close to the pK_{a1} of 2.22, 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 a lower pH because the ionic strength of the buffered solution increases during the titration and would approach 0.1 towards the end of the test. Ionic strengths above 0.1 were avoided because these would exceed the validity limit of the activity correction formulas used in the solving process (Section 3.4).

The high fluorescein pK_{a1} scatter value was not due to the buffer pK_a temperature correction approximation (Equation 23 described in Section 3.5). It was found that Equation 23 did

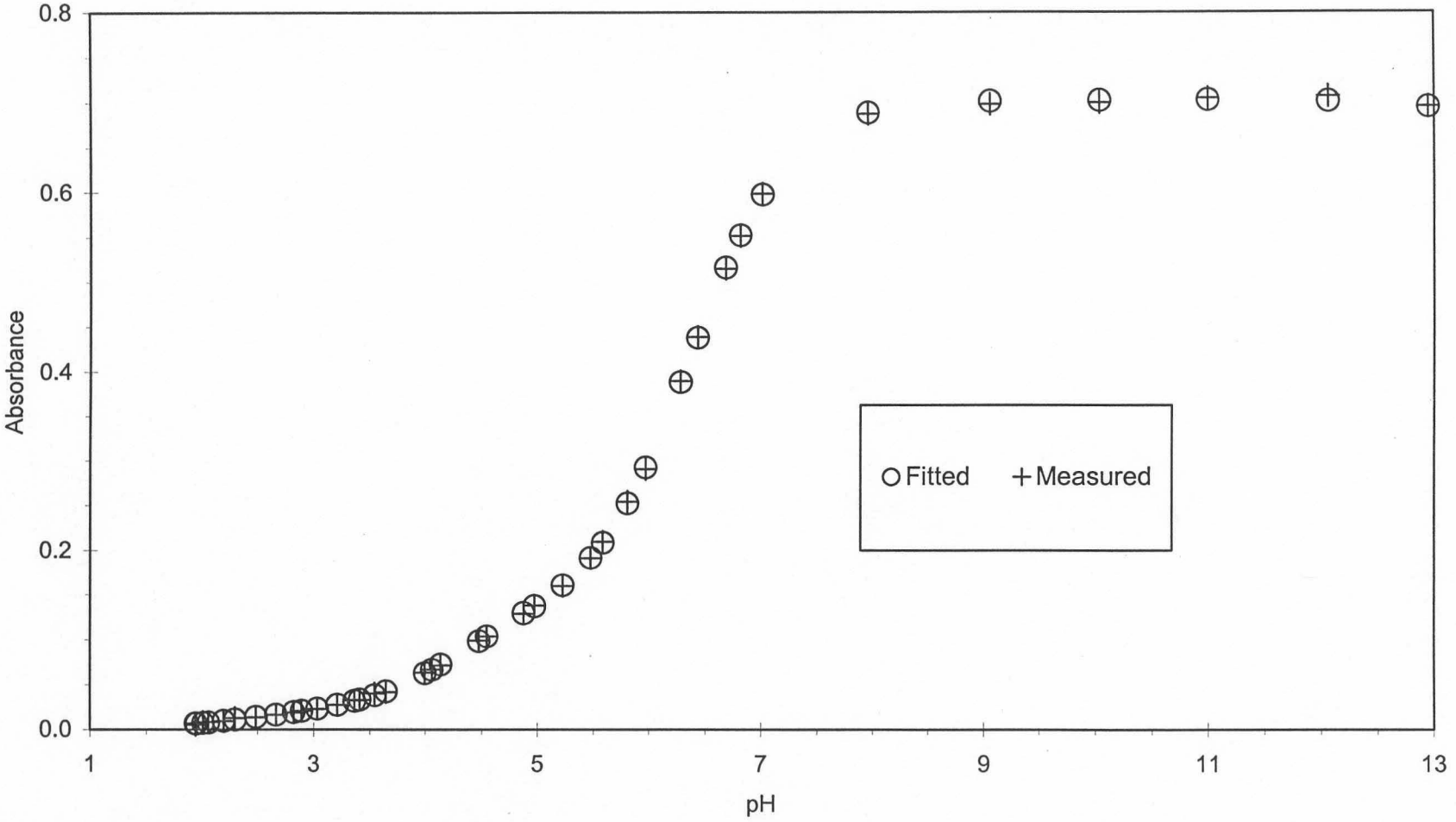

Figure 6 An example of fluorescein absorbance results showing close agreement between measured and fitted absorbance values. This titration used KOH on a buffered KNO_3 (0.05M) solution.

5°C Results - pKas corrected for ionic strength

Solution	Buffer 1	KCl (0.01)	KCl (0.05)	KNO_3 (0.01)	KNO_3 (0.05)	K_2SO_4 (0.01)	K_2SO_4 (0.05)	Buffer 2
pK_{a1}	2.25	2.36	2.40	2.42	2.33	2.25	2.23	2.37
pK_{a2}	4.33	4.35	4.35	4.35	4.34	4.31	4.33	4.30
pK_{a3}	6.77	6.76	6.81	6.77	6.82	6.78	6.83	6.78
Factor 1	18.75	18.75	18.75	18.75	18.75	18.75	18.75	18.75
Factor 2	1509	1661	1924	1821	1611	1611	1442	1629
Factor 3	10125	10255	10662	10639	10447	10458	10507	10440
Factor 4	49322	49282	49393	49390	49360	49353	49420	50094
Ionic Str.	0.024	0.034	0.074	0.034	0.074	0.034	0.074	0.022
R^2	0.99981	0.99984	0.99986	0.99986	0.99988	0.99985	0.99989	0.99993

Solution	NaCl (0.01)	NaCl (0.05)	Na_2SO_4 (0.01)	Na_2SO_4 (0.05)	Summary	Average	Scatter	Ionic Correl
pK_{a1}	2.36	2.38	2.37	2.34	pKa1	2.33	0.100	-0.008
pK_{a2}	4.31	4.32	4.33	4.34	pKa2	4.33	0.031	-0.366
pK_{a3}	6.78	6.81	6.79	6.83	pKa3	6.79	0.041	-0.900
Factor 1	18.75	18.75	18.75	18.75	Factor 1	18.75		-
Factor 2	1675	1852	1692	1576	Factor 2	1667		0.133
Factor 3	10600	10743	10647	10796	Factor 3	10526		0.505
Factor 4	49936	50058	50240	49967	Factor 4	49651		-0.086
Ionic Str.	0.032	0.072	0.032	0.072				
R^2	0.99992	0.99989	0.99992	0.99989	R^2	0.99988		

21°C Results - pKas corrected for ionic strength

Solution	Buffer 1	KCl (0.01)	KCl (0.05)	KNO_3 (0.01)	KNO_3 (0.05)	K_2SO_4 (0.01)	K_2SO_4 (0.05)	Buffer 2
pK_{a1}	2.17	2.26	2.27	2.30	2.20	2.19	2.19	2.27
pK_{a2}	4.36	4.37	4.35	4.36	4.35	4.34	4.32	4.33
pK_{a3}	6.70	6.68	6.73	6.67	6.71	6.68	6.75	6.69
Factor 1	18.75	18.75	18.75	18.75	18.75	18.75	18.75	18.75
Factor 2	1490	1608	1531	1572	1413	1445	1480	1485
Factor 3	9609	9581	9542	9471	9361	9458	9402	9627
Factor 4	48133	48358	48046	47989	48225	47938	48253	48610
Ionic Str.	0.024	0.034	0.074	0.034	0.074	0.034	0.074	0.022
R^2	0.99981	0.99990	0.99968	0.99975	0.99997	0.99975	0.99977	0.99979

Solution	NaCl (0.01)	NaCl (0.05)	Na_2SO_4 (0.01)	Na_2SO_4 (0.05)	Summary	Average	Scatter	Ionic Correl
pK_{a1}	2.32	2.22	2.34	2.22	pKa1	2.24	0.095	0.335
pK_{a2}	4.36	4.30	4.37	4.33	pKa2	4.34	0.044	0.539
pK_{a3}	6.69	6.70	6.70	6.73	pKa3	6.70	0.046	-0.763
Factor 1	18.75	18.75	18.75	18.75	Factor 1	18.75		-
Factor 2	1567	1444	1623	1442	Factor 2	1508		-0.515
Factor 3	9714	9448	9754	9607	Factor 3	9548		-0.578
Factor 4	48969	48482	48682	48773	Factor 4	48372		-0.082
Ionic Str.	0.032	0.072	0.032	0.072				
R^2	0.99991	0.99984	0.99982	0.99989	R^2	0.99982		

Table 4 Combined results of pK_a and absorptivity factor determinations.

introduce a small error in some cases, i.e. it underestimated the first dissociation constant of phosphate by 5.0% at low temperatures, however when the fluorescein pK_a calculation process was repeated with more accurate buffer dissociation constants (Lange's Handbook, 1992) the fluorescein pK_{a1} scatter value did not improve.

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 at the absorbance maximum wavelength of the cation rather than the dianion species, and using a lower starting pH combined with a lower buffer strength. This was not considered justified here as most of the experiments were performed at a pH above pK_{a2} where the influence of an imprecise pK_{a1} is negligible.

5.1.2 Factors affecting pK_as

Inspection of the pK_as listed in Table 4 shows that for the cations and anions tested, the ion type has little influence on the pK_a. In addition, Table 4 shows that while activity effects have been accounted for, there is still a correlation between pK_{a3} and the ionic strength (last columns of Table 4). The critical correlation value for 12 data pairs using a two-tailed test is 0.708 at the 1% probability level. The pK_{a3} and ionic strength correlation at –0.900 (5°C Results on Table 4) and –0.763 (21°C Results) is thus very significant even though it is numerically small. This suggests that the scatter value for pK_{a3} might be further reduced if the activity correction formula is refined.

The consistent pK_{a3} values suggest that once the activity effects are taken into account it makes no difference whether the predominant solution cation is sodium or potassium, or whether the predominant solution anion is chloride, nitrate or sulphate.

5.1.3 Thermodynamic pK_as of fluorescein

The summary in Table 4 shows both pK_a measurement temperatures and using these temperatures it is possible to calculate an activity corrected pK_a standardised to 25°C (the thermodynamic pK_a) using Equations 23 and 24, and these results are shown in Table 5.

Table 5 Calculated thermodynamic pK_as of fluorescein

Measurement temperature (°C)	4.7	21.2	Calculated Standard Enthalpy (J/Mol)	Thermodynamic pK_a
Average pK_{a1}	2.33	2.24	8742	2.22
Average pK_{a2}	4.33	4.34	-1501	4.35
Average pK_{a3}	6.79	6.70	8894	6.68

These thermodynamic pK_a values can be compared to a selection of the published pK_as and these are repeated in Table 6. This selection of pK_as only includes the most recent publications of researchers who used the three-pK_a model in aqueous systems.

Table 6 Reworked pK_a comparisons

Published Value			After reworking			Correction applied	Reference
pK_{a1}	pK_{a2}	pK_{a3}	pK_{a1}	pK_{a2}	pK_{a3}		
			2.22	4.35	6.68	-	This study
2.2	4.4	6.7	2.19	4.36	6.66	Pre-rounding	Lindqvist, 1960
2.19	4.24	6.36	2.07	4.36	6.72	Activity	Diehl & Markusewski, 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

Table 6 shows that the difference between Lindqvist's (1960) pK_as and the pK_as found in this study are the result of rounding because the pre-rounded values can be calculated from the published data and they are close to those of this study.

The remaining published pK_as values shown in Table 6 failed to correct for activity. Diehl and Markuszewski (1989) report that their measurements were all made at an ionic strength

of 0.1, therefore if Equations 19, 20 and 21 are used the activity corrected pK_{a2} and pK_{a3} results are close to the thermodynamic pK_as 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_as in the presence of 0.05M NaCl and 0.005M phosphate buffer at a pH of 6.14, as 2.09, 4.30 and 6.41 (different from their thermodynamic pK_a values shown in Table 6). When these values are activity corrected using Equations 19, 20 and 21, the pK_{a2} and pK_{a3} values are again close to the thermodynamic pK_as 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_as by reanalysing data from Diehl and Horchak-Morris (1987) who performed their experiments at an ionic strength of 0.10. Once activity corrections are applied, the pK_a values are close to the thermodynamic pK_as found in this study (Table 6).

The similarities between the thermodynamic pK_as of this study and the reworked values of the other publications (Table 6) show that it is the absence of activity corrections that causes most of the discrepancies between the published pK_as of the three-pK_a models of fluorescein.

5.1.4 Absorptivity factors of fluorescein

The absorptivity factors determined in this study and shown in Table 7 are different from standard molar absorptivity values in two ways: The first difference is that these absorptivity factors incorporate both the molar absorptivity value and the sample path length. The second difference is that the sample vessels used in the spectrophotometer were tubes with a diameter of 1.1 cm, when they should ideally have been flat-sided cuvettes with a path length of 1.0 cm. While calibrating using identical sample tubes will have eliminated most of the tube effects the uncertainty about the combined effects of the sample tube shape and the path length suggests that these absorptivities be interpreted as relative rather than absolute values.

Table 7 Comparison of absorptivity values

	Reported absorptivity			Ratio of this study's values with	
Fluorescein Ionic Species	Ref. 1	Ref. 2	This study	Ref. 1	Ref. 2
Cation	20.8	34	18.75	0.90	0.55
Neutral	2280	2695	1496	0.66	0.56
Monoanion	15300	16425	9387	0.61	0.57
Dianion	78900	87692	48299	0.61	0.55

Ref. 1 is Diehl (1989) and Ref. 2 is Klonis and Sawyer (1996)

The importance of calculating absorptivity factors for each tracer, spectrophotometer and sample vessel combination can be seen by comparing the temperature standardized absorptivity factors of this study with those of the two studies shown in Table 7. These data show that if the Klonis and Sawyer (1996) absorptivity factors had been adopted the fluorescein concentrations would appear to be 45% lower than expected.

The fluorescein cation absorptivity was fixed at 18.75 for the reasons given earlier in this section but the consistency in the absorptivity ratios of the neutral, monoanion and dianion species suggest that the spectrophotometer used in this investigation is functioning properly but has a different response from the tracer and spectrophotometer combinations used in the Diehl (1989) and Klonis and Sawyer (1996) investigations.

According to the fluorescein 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 shown in Table 7. While this purity may appear to be low, this is the grade of material that might typically be used in a tracer test 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.

5.1.5 Effect of temperature on fluorescein absorptivities

Feuerstein and Selleck (1963) reported a single temperature coefficient for fluorescein that was based on fluorescence measurements, i.e. –0.0036 per degree Celsius. They did not describe the temperature coefficients of the different ionic forms probably because most of the fluorescein fluorescent response is associated with the dianion species.

The temperature coefficients calculated in Table 8 show that the absorbance temperature coefficient of each ion is different (the cation was not calculated because its value had been fixed at 18.75). The Factor 4 dianion absorbance temperature coefficient is nine times smaller than the fluorescence temperature coefficient reported by Feuerstein and Selleck (1963) and this suggests that temperature corrections are probably not essential for absorbance measurements made at a high pH where the dianion predominates.

Table 8 Temperature effect on fluorescein absorptivity factors

Temperature (°C)	4.6	21.2	Temperature Coefficient	Absorptivity at 25°C and 492nm
Ionic species	Calculated absorptivity			
Factor 1 – Cation	18.75*	18.75*	Not calculated	18.75
Factor 2 – Neutral	1560	1508	-0.0020	1496
Factor 3 – Monoanion	10281	9548	-0.0045	9387
Factor 4 – Dianion	48689	48372	-0.0004	48299

* Fixed at 18.75 to reduce pK_{a1} variability

5.1.6 pK_a Determination summary

This study has shown that the published pK_a data do agree once activity corrections are made and the pK_a determination method developed for this investigation is precise, and has the advantage of automatically compensating for activity effects. Additionally, this experiment shows that while absorptivity constants need to be established for each fluorescein batch and spectrophotometer/sample vessel combination, absorbance temperature corrections are probably not essential for high pH samples that are close to room temperature.

The presence of a significant quantity of an unknown impurity in the fluorescein rules out a potentiometric pK_a determination because the potentiometric method uses higher concentrations of test material (Albert and Serjeant, 1984) and the impurity would introduce uncertainties because its ionic effects would be unpredictable. Under these circumstances a spectrophotometric pK_a determination method appears justified because the highly diluted impurity would have negligible impact on the titration chemistry and any absorbance interference would either be automatically incorporated into the calculated absorptivity values, or produce unacceptably large scatter values in the pK_a solving process.

Apart from including activity and temperature corrections this new pK_a determination method is a significant departure from that of Clark and Cunliffe (1973) in that the pH of the titration step can be measured in a water bath at 25°C and the absorbance measurements can be performed later on samples taken from this titration. The new method calculates the pH at the time of absorbance measurement by using the absorbance measurement temperature and known chemistry of the buffer solution and eliminates the uncertainties of pH meter temperature correction. This uncouples the titration and absorbance measurement processes and places the emphasis on accurate temperature measurement rather than accurate temperature control.

5.2 Beer's Law Tests

The absorbance of a series of gravimetric fluorescein dilutions was measured to test the assumption that fluorescein conforms to Beer's law. The data are listed in Appendix E and are plotted in Figure 7. Figure 7 shows a linear relationship between fluorescein concentration and absorbance at absorbance readings of less than 0.75 and that these values correlate well with Beer's law. The R^2 values for Beer's law in this region is 0.9945, 0.9988 and 0.9999 for solutions with pH<2, pH 5.2 and pH>8 respectively, but there is a pronounced deviation from Beer's law linearity as the absorbance increases. This deviation is most noticeable in the tests where the pH is 5.2 or higher. The deviation is not simply an absorbance related phenomenon as the pH 5.2 data in the 20 mg/l range deviate more from linearity than the data of pH>8 at the same absorbance. It is also not simply a concentration related phenomenon because there is little deviation for the pH<2 data.

The upper absorbance limit of 0.75 is of the same order as the validity limit reported by Lindqvist (1960) of 0.88 (88000ε at 10^{-5}M) but is lower than that reported by Adelman and Oster (1956) of 5.46 (54600ε at 10^{-4}M) and Diehl (1989) of 1.9 (78900ε at 2.407×10^{-5}M).

The "Fit" values in Figure 7 are the values that would be produced by 1.16% stray-light in combination with Beer's law. The R^2 values for a 1.16% stray-light fit across the whole range of absorbance readings were 0.9915, 0.9991 and 0.9968 for the pH<2, pH 5.2 and pH>8 solutions respectively. The stray-light fit also shows some deviation and it seems that the complex interaction between fluorescence and absorbance phenomena described in Chapter 2 may be playing a role. If fluorescence is playing a role its influence is expected to increase in direct proportion to the fluorescein concentration until the self-quenching effect becomes dominant. This behaviour would be a special case of stray-light deviation, with the stray-light being proportional to the fluorescence and its quenching rather than consistent instrument geometry.

The Beer's law relationship can be seen more clearly in Figure 8 where the absorbance measurements are plotted against the Beer's law predictions on Log-Log axes so that the lower range values can be distinguished. These Beer's law values have been pH corrected

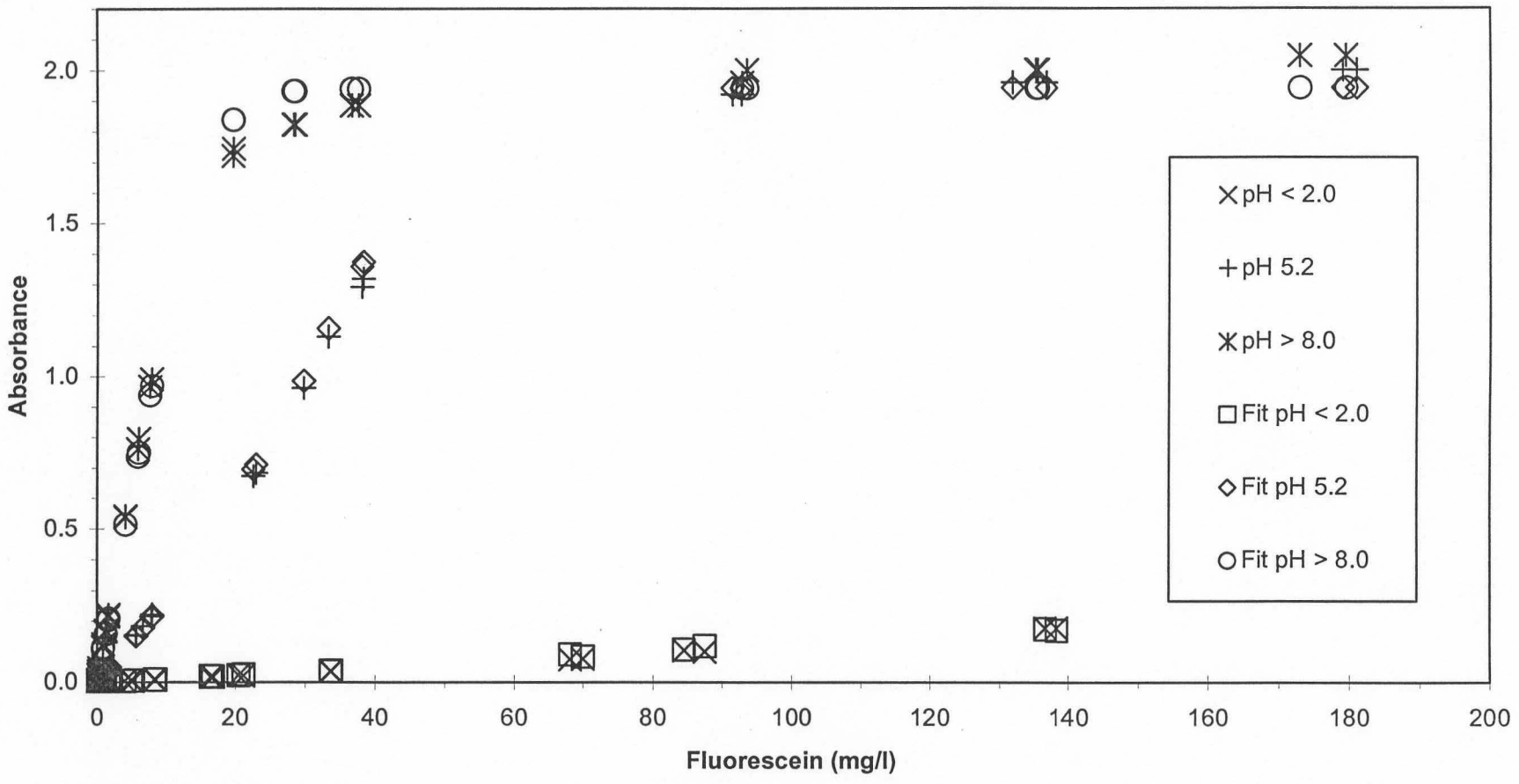

Figure 7 Beer's law test results overlaid with a fitted stray-light corrected formula

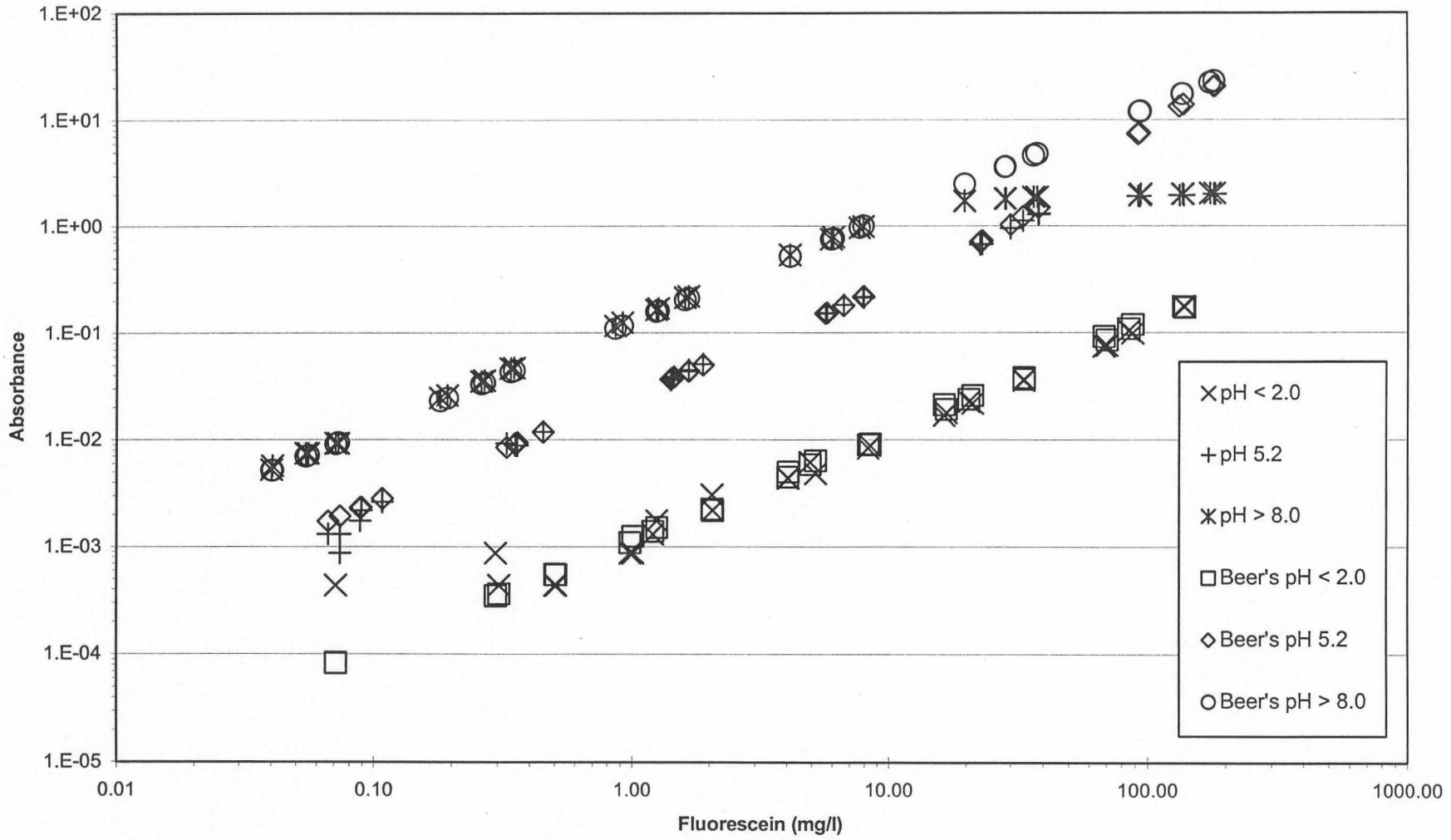

Figure 8 Absorbance results compared to the Beer's law prediction

but have no stray-light correction factor. While the three sets of absorbance data are parallel at lower absorbance levels the pH>8 and pH 5.2 absorbance measurements level out as they approach 2 absorbance units.

Figure 8 shows that the pH 5.2 absorbance values curve upwards slightly at the top end. This was because the test solutions containing high concentrations of fluorescein had a slightly higher pH than the others in the series and the data shown in Appendix E indicate that this pH deviation was only seen in the pH 5.2 trial. Figure 8 also shows that measurements at absorbance levels below 0.001 show considerable variation. This is because these measurements were made at transmittance levels greater than 99.7 % where the impact of small measurement inaccuracies is more pronounced.

There is an absorbance level where Beer's law is no longer valid, e.g. at an absorbance of 0.75 the difference between Beer's law and the stray-light fitted equation rises above 2%, however these Beer's law deviations at high absorbance levels have negligible impact on the pK_a determinations performed during this investigation because the pK_a determinations were performed at a fluorescein concentration of 5.8mg/l. As can be seen in Figure 8 a fluorescein concentration of 5.8mg/l is within the linear response region for this instrument but as an additional precaution these absorbance values were also corrected for the effects of stray-light. The same 0.75 absorbance limit is not expected to apply to other instruments or sample vessel combinations because different stray-light levels (Skoog *et al.*, 1992) and detector arrangements (Gibson and Keegan, 1938 and Umberger and LaMer, 1945) will determine each instrument's sensitivity to various absorbance and fluorescence phenomena.

The Beer's law deviation observed in this experiment provides further support for the precaution; that it is desirable to test the Beer-Lambert law wherever possible (Braude *et al.*, 1950).