Chapter 4 Materials and Methods

4.1 pK_a Determination

4.1.1 Overview

This experiment determines values for the three pK_as and four absorptivity factors of fluorescein by fitting the pH/absorbance profile of a dilute fluorescein solution to a known ionic model. The titration starts with an acidified, buffered fluorescein solution and adds small quantities of a strong potassium hydroxide solution. The pH is monitored and an absorbance sample is collected after each of these additions. These pH and absorbance data are curve-fitted to the known ionic forms (Zanker and Peter, 1958) according to the simplified ionic model tested by Klonis and Sawyer (1996). The curve-fitting procedure relies on standard spreadsheet functions and simultaneously determines values for both the pK_as and absorptivity factors.

The new pK_a determination method described here is different from the simplified spectrophotometric method of Clark and Cunliffe (1973) in that the quantity of test compound is measured allowing precise absorbance characteristics to be determined. Although the new method also does not measure the titrant volume, it is calculated using standard equilibrium chemistry equations. The same equilibrium chemistry equations are used to compensate for the buffer and salt solution activity effects.

4.1.2 Chemicals used

Chemical quality was certified to meet American Chemical Society specifications and supplied by either Fluka or J.T. 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.

4.1.3 Instruments used

Absorbance readings were made using a Turner Model 350 spectrophotometer at the fluorescein, high-pH absorbance maximum wavelength, which was found to be 492 nm on

this instrument. To prevent accidental adjustment during the measurement period the wavelength selector knob was removed once the wavelength maximum had been set. The samples were collected and analysed in new, polished, 13 × 100 mm borosilicate glass tubes.

pH readings were taken using a Corning Model pH-30 meter. 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.

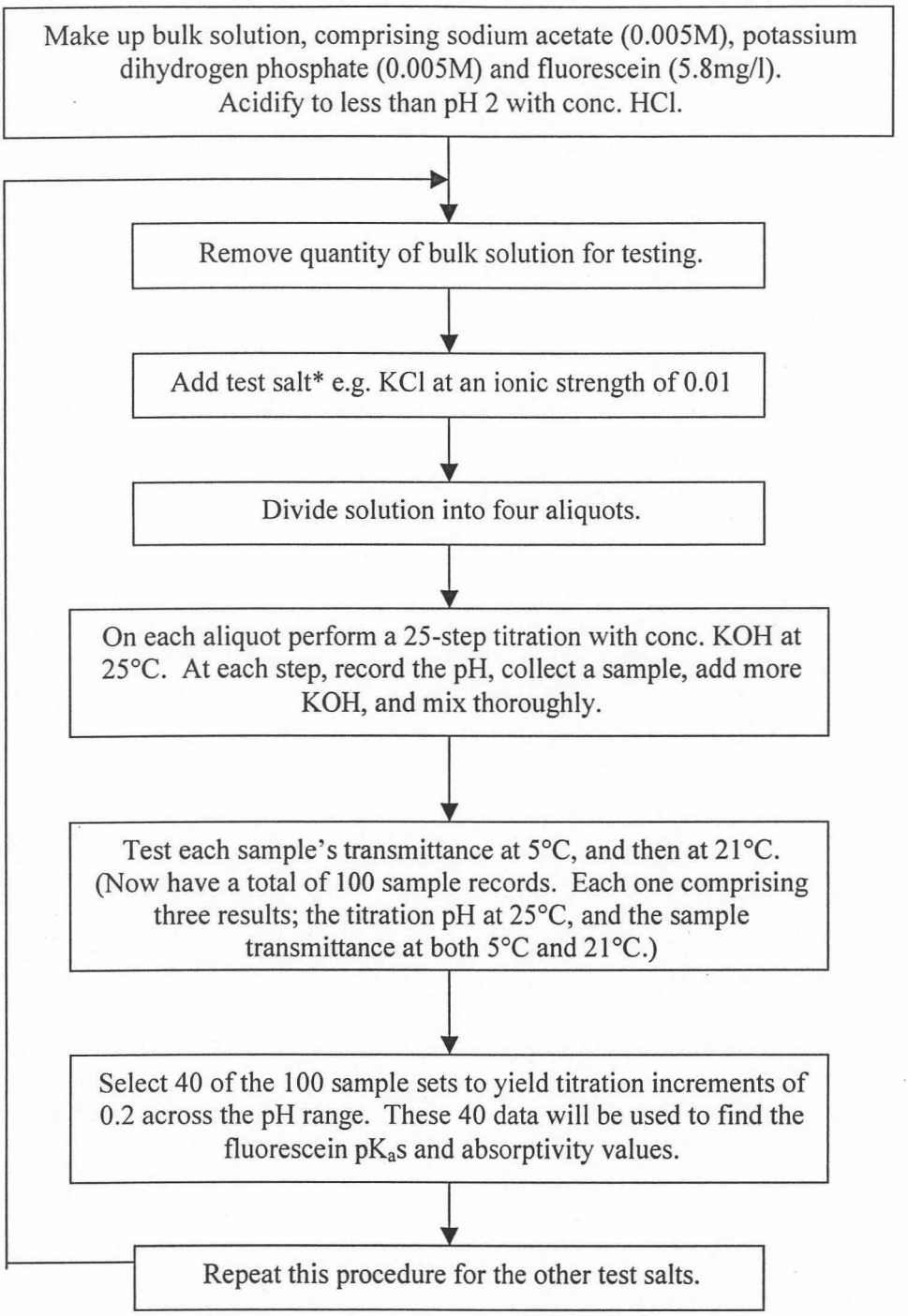
4.1.4 Test method

A flow diagram of this method is shown in Figure 3. A buffered bulk solution was used as the stock solution for all test solutions. This bulk solution comprised 0.005 M sodium acetate (NaAc), 0.005 M KH_2PO_4 and 5.8mg/l fluorescein, acidified to less than pH 2 with concentrated HCl.

In addition to measuring the fluorescein pK_a and absorptivity factors in buffer solution, the influence of five different salts was also tested at two different 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 0.01 and 0.05. A total of twelve solutions were tested. This total included two batches of buffer stock.

Normally, salts solutions would not be necessary to measure the pK_a of a compound. KCl, KNO_3, K_2SO_4, NaCl, and Na_2SO_4 salts were included in this experiment to test the influence of different ionic strengths and this range of salts was tested so as to discriminate between ionic strength effects and the influence of particular cations and anions.

Each test solution was divided into four aliquots and each aliquot was titrated separately. Separate titrations were necessary because the precise amounts of concentrated KOH added in the titration process were difficult to control and a single titration experiment might occasionally yield data with a large pH increment. Large pH increments must be avoided because they reduce the precision of the pK_a determination. Forty sets of measurements were selected from these combined data to give a series with small pH increments throughout the



* Test salts were KCl, KNO_3, K_2SO_4, NaCl, and Na_2SO_4 added at ionic strengths of 0.01 and 0.05. Bulk solution was also tested without salt addition.

Figure 3 Flow diagram for pK_a determination experiment

test range. This approach is similar to that recommended by Albert and Serjeant (1984), i.e. of at least seven data points having 0.2 pH increments around the expected pK_a.

Titrations were made by adding small quantities of a concentrated (>5 M) KOH solution to the test solution, stirring the solution, taking the pH, and collecting a sample for later absorbance testing. Twenty-five samples were collected from each titration. Titrations were performed in a temperature-controlled room at 25°C. Samples were bagged and stored in the dark at 4°C until absorbance testing was performed.

The titration samples were absorbance tested at two temperatures. Transmittance rather than absorbance readings were recorded as these could be read more precisely. These transmittance readings were then converted to the equivalent absorbance. The first readings were taken with the spectrophotometer placed in a cold room at 5°C. The instrument maximum and a sample blank were tested between every measurement to confirm stable instrument response under these conditions. These measurements were then repeated at room temperature (21°C). Titration and transmittance readings were entered onto a computer spreadsheet that solved for the pK_a and absorptivity factor values of fluorescein.

4.1.5 Spreadsheet calculation method

Standard spreadsheet functions were used throughout. The process is described in detail in Appendix B and examples of parts of the calculation are given in Appendices C and D.

The stock solution chemical composition was calculated first by determining how much HCl was needed to adjust the buffer solution to the measured acidified pH. Once the buffer solution composition was known along with the temperature corrected pK_as of NaAc and KH_2PO_4, these values were used as a starting point. An iterative routine adjusted the HCl addition (see Appendix C), determined the proton concentration at which the charges balanced and then compared the calculated pH (the activity corrected proton concentration at zero net charge) with the measured pH. The spreadsheet "Solver" function repeated this process until the difference between the calculated and measured pH was minimised.

The quantity of KOH added during titration was determined by a method similar to that used to calculate the HCl addition. The test salt quantity (where appropriate) was added to the

calculated stock solution composition and these data were combined with the relevant temperature-corrected pK_as to calculate the KOH needed to adjust the calculated pH to the titration pH. This process was repeated for each of the 40-titration samples so that the concentration of every solution ion, apart from the fluorescein species, was known for each sample.

The absorbance measurement temperature was used in conjunction with the calculated sample composition to determine the precise solution pH for the conditions under which the absorbance measurement was made. The exact pH and solution composition were then combined with the seven-fluorescein variables (three pK_as and four absorbance factors) according to Equations 8, 9, 10, 11, and 13, to determine an absorbance value for each of the 40-titration samples (see Appendix D). After compensating for the dilution effect of adding the KOH solution, the 40 calculated absorbance values were compared to the observed values and the seven variables were adjusted until the best least-squares fit was found for the complete titration.

The average pK_a values were calculated by taking the negative log of the average K_a values as opposed to calculating the average of the pK_a values. The three pK_as were converted to thermodynamic pK_as by multiplication with the appropriate activity correction factor and standardising this activity corrected pK_a at 25°C. Albert and Serjeant (1984) used the negative log of the maximum difference between any one measurement and the average K_a value to evaluate the pK_a variance and called this the scatter value, however the scatter value reported here is based on the pK_a results from twelve separate tests rather than a set of seven pK_a measurements within a single test.

Two correlation tests were performed using standard spreadsheet functions. The first quantified the correlation between the test solution ionic strength and the calculated pK_a, while a second quantified the correlation between the ionic strength and different ionic species absorbance factors.

4.2 Beer's law tests

This experiment measured the fluorescein absorbance and concentration relationship at different pH to determine whether there were any significant deviations from Beer's law. A bulk buffer solution (0.005 M NaAc and 0.005 M KH_2PO_4) was split into three quantities and the pH of these was adjusted to <pH 2, pH 5.25, and >pH 8.5 with concentrated HCl or KOH as appropriate. Each of these solutions was used to perform gravimetric serial dilutions with a dilute fluorescein solution, while monitoring the pH and changes in absorbance.

The Beer's law relationship was evaluated by calculating the square of the Pearson product moment correlation coefficient (R^2) between the fluorescein concentration and absorbance for each different pH. The product moment correlation coefficient (R) formula is a standard spreadsheet function and is shown below as Equation 28.

$$R = \frac{n(\sum XY) - (\sum X)(\sum Y)}{\sqrt{\left(n\sum X^2 - (\sum X)^2\right)\left(n\sum Y^2 - (\sum Y)^2\right)}} \tag{28}$$

Where n is the number of data pairs in a data array, and X and Y represent data points from pairs of observations from this array.

A stray-light determination was also included in this experiment. This involved fitting all of the absorbance measurements to Equation 29 (Skoog *et al.*, 1992)

$$Abs_{Fitted} = \log\left(\frac{1 + Stray}{10^{-Fc} + Stray}\right) \tag{29}$$

Where Abs_{Fitted} is the calculated absorbance value, F is a factor that incorporates both molar absorptivity and sample path length terms and is calculated using the pK_as and absorbance factors from Experiment 1, and c is the molar fluorescein concentration. The stray-light (Stray) percentage was adjusted until a minimum least-squares fit was found between the experimental and fitted absorbance values. This fit was evaluated using the R^2 determination.

4.3 Light versus heat degradation

This experiment compared the effect of light and heat by monitoring any absorbance changes in dilute fluorescein solutions and a flow diagram of this method is shown in Figure 4.

Fluorescein was added to a bulk buffer solution (0.005 M NaAc and 0.005 M KH_2PO_4) to give a fluorescein concentration of about 5 mg/l, and this solution was split into two halves. To test the impact of water chemistry, sodium sulphide (0.0029 M) was added to one half of the solution. After standing overnight in airtight bottles these two solutions were split again, with one half of each being adjusted to pH 11.2 and the other half to pH 5.2. These solutions were labelled A to D as shown in Figure 4. The four test solutions were pH tested, subdivided into three lots and poured into Ziploc® bags. These bags were sealed after removing any air bubbles.

During each experimental run two bags were kept in the dark; one at room temperature and the other in a water bath more than 60°C; while a third bag was placed in full sun. Samples were taken from the bags at intervals and tested for transmittance. The test was performed three times.

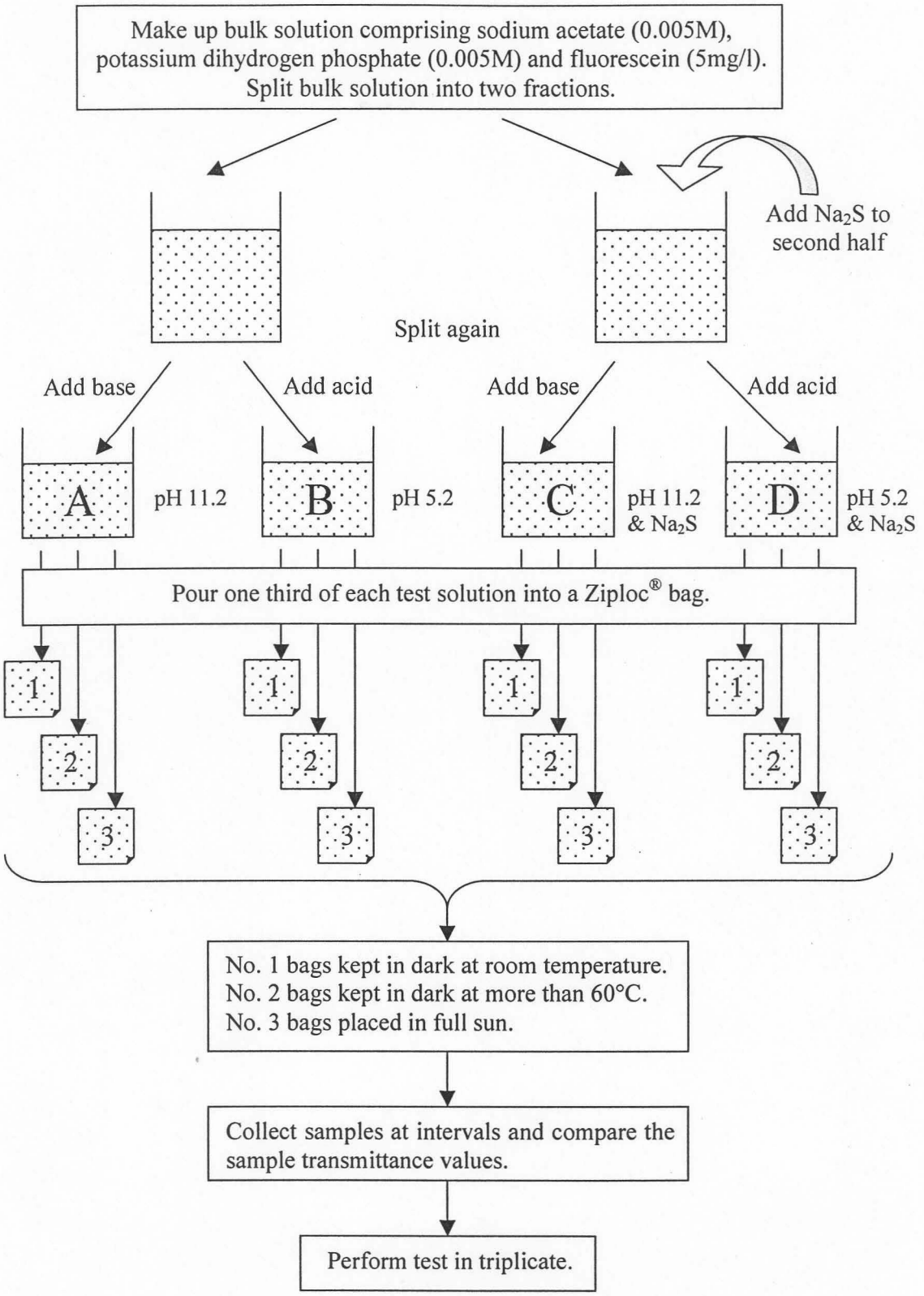


Figure 4 Flow diagram for photodegradation experiment

4.4 Elution tests

4.4.1 Overview

This experiment determined the amount of fluorescein recovered from a test column under different pH conditions using the fluorescein characteristics calculated in experiment 4.1 and also compared this to the amount detected after increasing the sample pH by adding solid KOH. The test apparatus comprised a constant pressure feed, connected to a glass column filled with gravel (see Figure 5). Test solution flowed through the gravel column into a tracer-sensor and then into a sample collector. The tracer-sensor was connected to a data-logging system to ensure regular measurements.

4.4.2 Chemicals used

In addition to the chemicals described in section 4.1.2 this experiment used pre-washed, non-epoxy coated, aquarium gravel comprising silicates and agates, with a particle size of about 5 mm. The gravel bulk-density and volume were measured before filling the column.

4.4.3 Instruments used

In addition to the instruments described in section 4.1.3 this experiment used an elution column, tracer sensor, datalogger and computer, arranged as shown in Figure 5. The elution column comprised a glass tube, 1.5 metres long and with an internal diameter of 37 mm, that was filled with gravel. An inverted funnel was placed over the topmost gravel to provide an even inflow distribution (see Figure 5). During tests the tracer was injected into the neck of this funnel.

The tracer-sensor was constructed specifically for this project. Two T-pieces were connected to a 12 cm tube, so that a light source and light detector faced each other at either end of the tube length (See Figure 5). Sample fluid entered the tube at one end, passed down the length of the tube and out of the sensor. A blue light emitting diode (LED) served as a light source, while a light-dependent resistor was used as the detector. The detector was connected to a digital multimeter, which communicated directly with a computer. This combined multimeter/computer arrangement served as a datalogger. As the LED was expected to produce a broad spectrum of wavelengths within the blue range it was calibrated using

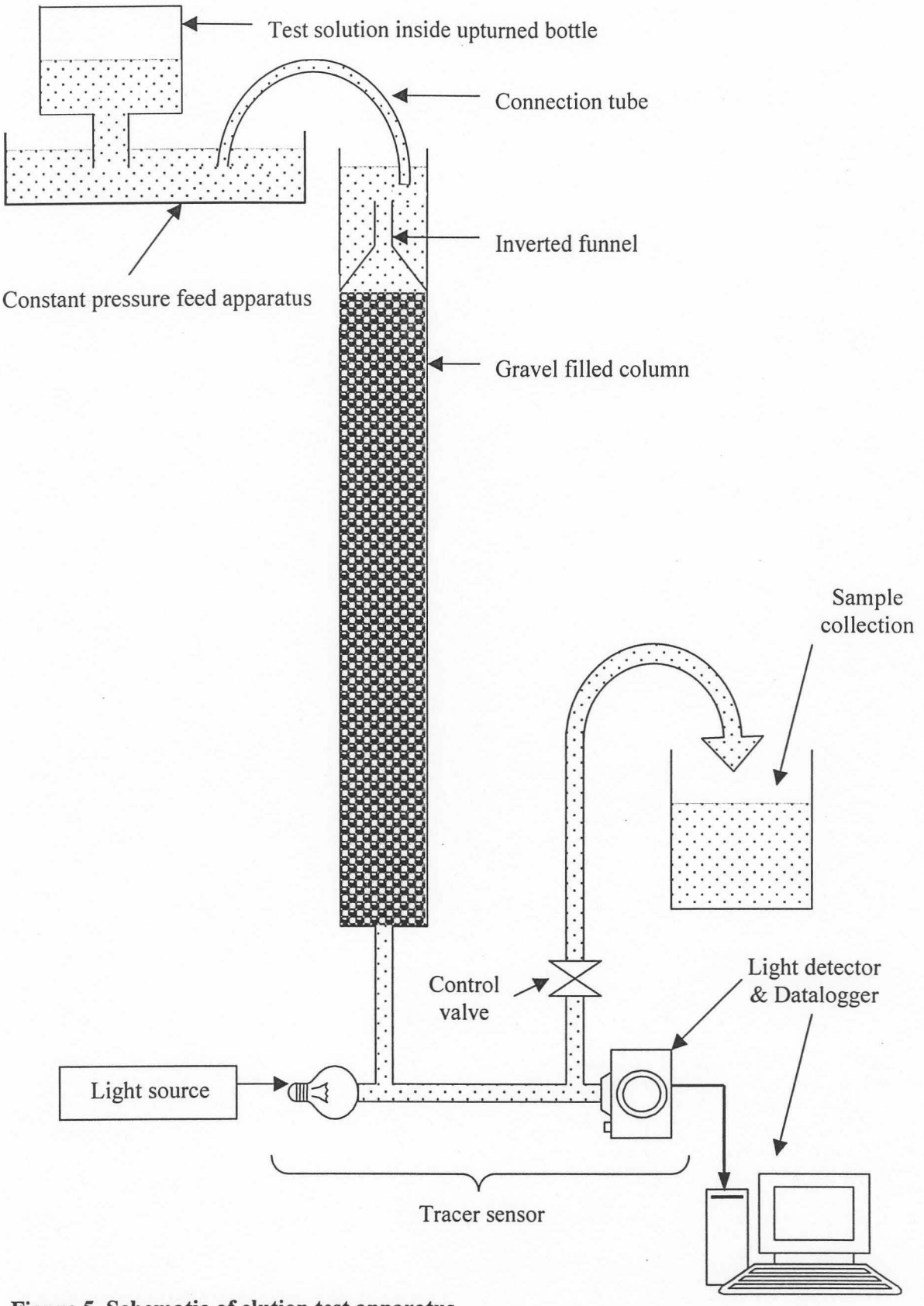


Figure 5 Schematic of elution test apparatus

solutions at a similar pH to the test solutions. These sensor readings were compared and correlated with the spectrophotometer.

4.4.4 Test method

Quantities of buffered (0.005 M NaAc and 0.005 M KH_2PO_4) test solutions were prepared and pH adjusted, using solid KOH or concentrated HCl, to pH 8, pH 7, pH 6, and pH 5. Test solution was placed in the feed apparatus and allowed to purge the column at the start of each run. During this time the flow was adjusted to about 25 ml/min. The datalogger was started during the column-purging period and used to establish a background reading.

A filled, preweighed syringe was used to deliver tracer solution into the top of the column and the injection time was recorded. The syringe mass difference was recorded as the tracer mass.

In addition to the sensor/datalogger information, 100 ml elutant samples were collected in preweighed containers for flow rate, pH and absorbance tests. Solution temperature was monitored regularly throughout the run. The test was stopped when the sensor reading approached its pre-test levels. After the test run was complete the elutant samples were retested using the tracer sensor and then measured using the spectrophotometer. After each absorbance reading was taken, a small quantity of solid KOH was added to the sample and it was retested at the higher pH.

If gravel-bed bubbles were noticed these were removed before the test run by draining the column completely, flushing the column with carbon dioxide, refilling the column with water, and then purging the column with fresh water.

4.4.5 Calibration method

The tracer sensor was built specifically for this project. While this experimental sensor allowed much more frequent sampling of the elutant and automatic datalogging (every five seconds) it also introduced a number of other variables, and as the sensor rather than the spectrophotometer readings served as the primary data source for this experiment, the sensor needed to be calibrated. This calibration process is described in Appendix H.