FluAnisot

A Simulated Experiment in Fluorescence Anisotropy Measurement

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THEORY

Fluorescence Anisotropy

If a molecule is excited using plane-polarized light only the component of the incident excitation vector that lies along the transition moment in the molecule is absorbed. If one considers a collection of such molecules, each with its transition moment randomly oriented, then each molecule will absorb a different component of the excitation vector. Moreover, if the molecules are fluorescent and one imagines them to be stationary during the fluorescence process then it is clear that the fluorescence emission will be depolarized to some extent relative to the excitation by virtue of the random orientation of the molecules. In reality molecules are not stationary but are rapidly moving and rotating during the fluorescence process. This has the effect of producing a greater extent of depolarization than what would be observed in the absence of molecular motion. In the *FluAnisot* software a schematic representation of the fluorescence depolarization phenomenon is presented both pictorially and in the form of a vector diagram[1].

If one were to conduct an experiment using a vertically polarized excitation source then both vertical and horizontal components of the emission would be observed. This will be due to both the random orientation of transition moments and the rotation of the molecules during the fluorescence lifetime. This phenomenon is referred to as fluorescence "polarization", "depolarization" or "anisotropy" the last term referring to the fact that the behavior depends on the direction in which it is observed.

Fluorescence anisotropy experiments thus provide information about rotational dynamics of molecules in solution. This can be applied to studying the rotational motion of proteins and synthetic polymers as well as probing the structures of bilayers and lipids. As long ago as the early 1920's Perrin[2,3] performed the original exploratory work in this field. Since then the technique of fluorescence anisotropy has been used among other things in clinical studies including fetal and neonatal lung maturity[4–6] as well as pregnancies complicated by hypertension[7]. Furthermore, the more sophisticated time-resolved anisotropy measurements (TRAMS) have been used in the early detection of Alzheimer beta-amyloid peptide aggregation[8].

More recently, steady-state measurements of fluorescence anisotropy have been widely adopted in the field of high-throughput screening in drug discovery[9], for studying biomolecular interactions[10] as well as performing various clinical immunoassays[11]. Fluorescence anisotropy has also been applied in modern imaging techniques[12].

Experimental Set-Up

To perform a fluorescence anisotropy experiment a fluorescent sample is irradiated using plane-polarized light. This is achieved by passing the excitation beam through a polarizing filter. The fluorescence emission is also passed through a polarizing filter so that the emission intensity can be recorded in both the direction that is parallel to the direction of the excitation plane ($I_{\parallel}$) and the direction that is perpendicular to the excitation plane ($I_{\perp}$).

Fluorescence anisotropy experiments can be performed using either a fluorescence polarimeter or a conventional spectrofluorimeter that has been equipped with polarizing filters on its excitation and emission monochromators. The advantage of using a polarimeter is that it has two photodetectors positioned either side of the sample and at right angles to the excitation beam axis thus enabling the simultaneous recording of both the parallel and perpendicular components of the fluorescence emission. In the case of a polarimeter the fluorescence emission is usually isolated from scattered excitation light using appropriate cut-off filters.

A xenon lamp can be used in both polarimeters and fluorimeters as a UV-visible excitation light source. Sometime a combination of a deuterium lamp that produces UV light along with a halogen lamp that produces visible light is used in modern spectrophotometers and spectrofluorimeters to enable excitation wavelengths to be selected from anywhere in the UV and visible regions of the electromagnetic spectrum. Excitation wavelengths that lie in the far blue region of the visible spectrum or in the UV region are common for many fluorimetric experiments.

In a fluorescence depolarization experiment, light from the excitation source is passed through a monochromator set to an appropriate excitation wavelength (usually the absorption maximum of the fluorophore) and the excitation beam emerging from the monochromator is focused and passed through a polarizing filter before it reaches the sample. The sample is typically contained in a 1 cm path length fluorescence cell mounted in a temperature-controlled holder. The fluorescence emission is passed through another polarizing filter and detected at right angles to the excitation beam by a photodetection device such as a photomultiplier tube (PMT).

The Perrin Equation

Much of the theory associated with the Perrin equation has been presented elsewhere[1,13]. A summary of the key aspects is presented herein.

The depolarization of fluorescence emitted by macromolecular spherical rotors undergoing rotational diffusion is described by the Perrin equation[13]:

$$1/A = (1/A_0)(1 + \tau_f/\theta) \quad (1)$$

where A is the measured anisotropy, A_0 is the limiting (or fundamental) anisotropy (i.e. the anisotropy in the absence of fluorophore rotation), τ_f is the fluorescence lifetime of the fluorophore and θ is the rotational correlation time. The above form of the Perrim equation can be obtained from first principles based on diffusional steps[13] and the anisotropy, A, can be calculated from the experimentally measured quantities $I_{||}$ and $I_{\perp}$ as follows:

$$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) \quad (2)$$

If fluorescence depolarization experiments are performed using a conventional fluorimeter, then a correction must be made for the systematic variations in transmission of $I_{||}$ and $I_{\perp}$ through the detection system. In these cases the anisotropy is calculated using equation (3):

$$A = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (3)$$

The parameters I_{VV} and I_{VH} in equation (3) are the fluorescence intensities determined using vertically polarized excitation light and monitoring vertically and horizontally polarized emission respectively. The instrumental correction factor, G, is equal to I_{HV}/I_{HH} where I_{HV} and I_{HH} are the respective vertically and horizontally polarized fluorescence emission intensities observed using horizontally polarized excitation light[1,13].

The Stokes-Einstein equation enables the rotational correlation time to be evaluated for a spherically shaped moiety whose molar volume is V in a solution of viscosity η and at a temperature T as follows:

$$\theta = V\eta/(RT) \quad (4)$$

where R is the ideal gas constant. From equations (1) and (4), the following equation can be derived:

$$1/A = (1/A_0)[1 + \tau_f RT/(V\eta)] \quad (5)$$

Thus the experimental study of fluorescence anisotropy can provide information on the size of the fluorescing moiety through the determination of its molar volume. The interaction of macromolecules such as proteins with other macromolecular species in immunoassays, for example, can be detected by the change in the fluorescence anisotropy signal that occurs when an interaction takes place in solution to produce a rotating entity that is of a larger size than its two interacting constituents.

Limiting Emission Anisotropy

Another quantity that can be calculated from polarized fluorescence intensity measurements is, P, the degree of polarization[1,13]:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (6)$$

The relationship between the degree of polarization, P, and the angle, α, between the absorption and emission transition moments of a fluorophore is:

$$P = (3\cos^2\alpha - 1)/(\cos^2\alpha + 3) \quad (7)$$

Consider the hypothetical case where there is no molecular rotation during the excited-state lifetime of the fluorophore. If the angle between the absorption and the emission transition moments is zero, then $P_0 = 0.5$ (i.e. $\alpha = 0$, in equation (7)) and thus $I_{\parallel} = 3I_{\perp}$ (from equation (6)). Thus from equation (2) the *limiting* emission anisotropy, A_0, is therefore equal to 0.4 in this case.

In a previous publication[1] the concept of the limiting fluorescence anisotropy was explored along with some simple computer code that can be used to demonstrate the concept. The *FluAnisot* software contains an animation that illustrates further the limiting fluorescence anisotropy calculated using a Monte Carlo approach.

References

1. Bigger, S. W., Craig, R. A., Ghiggino K. P. and Scheirs, J., *J. Chem. Educ.*, 70, A234-239 (1993).
2. Perrin, F. J., *J. Phys.*, 7, 390 (1926).
3. Perrin, F. J., *Phys. Radium*, VII.7, 1 (1936).
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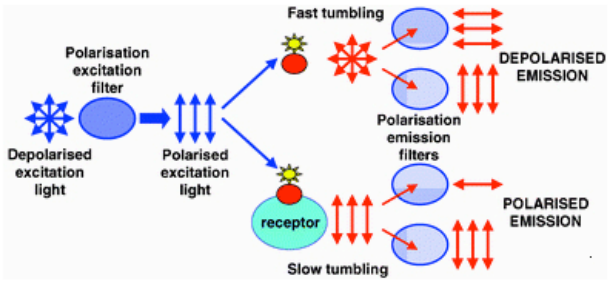
5. Henderson, C. E., Gewolb, I. H., Deutsch, J. and Cavalieri, R. L., *J. Perinatol.*, 13, 428-32 (1993).
6. Liu, K. Z., Shaw, R. A., Dembinski, T. C., Reid, G. J., Ying, S. L., Mantsch, H. H., *Am. J. Obstet. Gynecol.*, 183, 181-7 (2000).
7. Barkai, G., Reichman, B., Kokia, E., Segal, P., Lusky, A., Goldman, B. and Mashiach, S., *J. Perinat. Med.*, 22, 107 (1994).

8. Allsop, D., Swanson, L., Moore, S., Davies, Y., York, A., El-Agnaf, O. M. and Soutar, I., *Biochem. Biophys. Res. Commun.*, 285, 58-63 (2001).
9. Owicki, J. C., *J. Biomol. Screen*., 5, 297-306 (2000).
10. Huang X. and Aulabaugh A., *Methods Mol. Biol*., 565, 127-43 (2009).
11. Smith D. S. and Eremin S. A., *Anal. Bioanal. Chem*., 391, 1499-507 (2008).
12. Roberti M. J., Jovin, T. M. and Jares-Erijman, E., *PLoS ONE* 6(8): e23338. doi:10.1371/journal.pone.0023338 (2011).
13 Lakowicz, J. R., Principles of Fluorescence Spectroscopy, Vol. 1, 3rd edn., Springer, 2006, pp. 367-370.

Diagrams and Information Screens

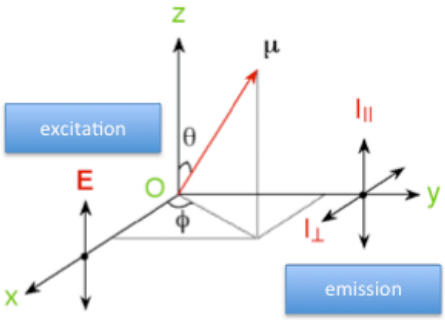
Fluorescence Depolarization



If polarized light is used to excite a group of randomly oriented fluorophores, most of the excited molecules will be those that are oriented within a particular range of angles relative to the applied polarization. If they do not move, the emitted light will also be polarized within a particular range angles to the applied light. If rotation occurs during the excited state lifetime then the fluorescence will be depolarized to a greater extent.

Source:
http://www.hi-techsci.com/public/backend/uploads/page/supporting/63/fluorescence-polarisation.gif

Vector Diagram



This vector diagram represents schematically the fluorescence depolarization phenomenon. The sample is located at the origin. The excitation beam, ***E***, is directed along ***Ox*** and is polarized in the ***z*** direction. The transition moment in the sample molecule is $\boldsymbol{\mu}$ and has the orientation (θ, Φ). The intensity of fluorescence emission (along ***Oy***) polarized parallel to the ***z*** axis is $I_{||}$ and is proportional to the square of the component of $\boldsymbol{\mu}$ in the ***z*** direction:

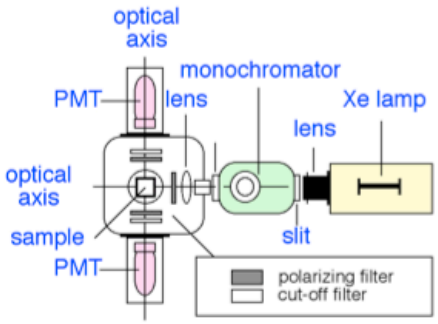
$$I_{||} \equiv (\mu_z)^2 = |\boldsymbol{\mu}|^2 \cos^2\theta$$

where the symbol "≡" means "equivalent to". Similarly, the intensity of fluorescence emission polarized parallel to the ***x*** axis is $I_{\perp}$ and is given by:

$$I_{\perp} \equiv (\mu_x)^2 = |\boldsymbol{\mu}|^2 (\sin^2\theta)(\cos^2\Phi)$$

Source:
Bigger, S. W., Craig, R. A., Ghiggino K. P. and Scheirs, J., *J. Chem. Educ.*, **70**, A234-239 (1993).

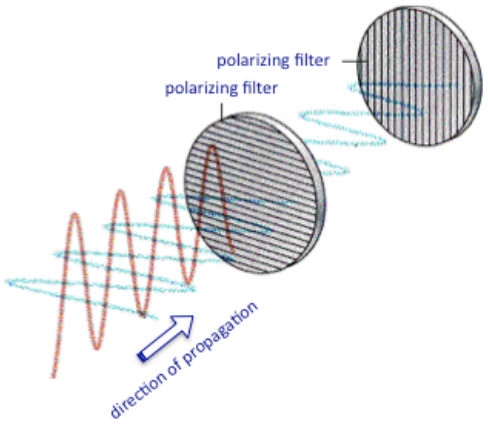
Fluorescence Polarimeter



A fluorescence polarization apparatus can be used to conduct fluorescence polarization experiments. Light from a suitable source is directed onto the slit of a high throughput monochromator that is set to the appropriate excitation wavelength. The excitation beam emerging from the monochromator is focused and passed through a polarizing filter before it reaches the sample. The sample is contained in a standard 1-cm path length fluorescence cell that is mounted in a temperature-controlled holder. The fluorescence emission is detected at right angles to the excitation axis by two photomultiplier tubes (PMTs). Each PMT has a polarizing filter and a cut-off filter in front of it and the use of two PMTs enables the convenient simultaneous recording of the parallel and perpendicular components of the fluorescence emission. The cut-off filters isolate the fluorescence emission from scattered excitation light.

Source:
Bigger, S. W., Craig, R. A., Ghiggino K. P. and Scheirs, J., *J. Chem. Educ.*, **70**, A234-239 (1993).

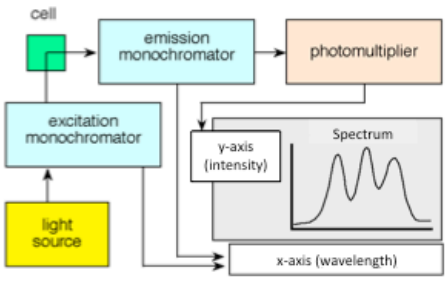
Polarizing Filters



If an electromagnetic wave is propagated in a given direction, the electric field vector, ***E***, is always perpendicular to the direction of propagation and the magnetic field vector, ***B***, is perpendicular to both. An un-polarized light beam consists of waves that move in a given direction and have their electric vectors pointing in random orientations with respect to the axis of propagation. If, say, a horizontal filter is placed before the beam then only those waves that have horizontal components of their of their electric vectors will pass, resulting in a beam comprised only of electric vectors oscillating in the horizontal plane. This would be referred to as a "horizontally polarized" beam. If a second, vertically oriented, filter were to be placed in the emerging bean (see diagram) then clearly no light would be passed by the second filter.

Source:
http://light.physics.auth.gr/enc/polarization_en.html

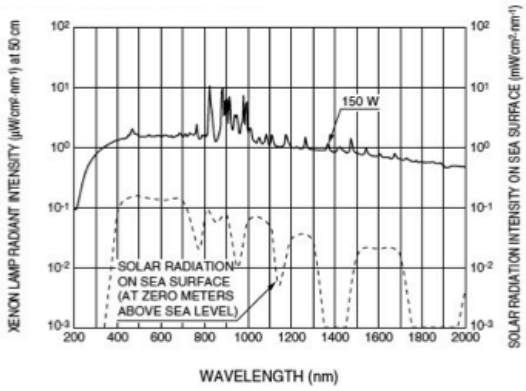
Spectrofluorimeter



The fluorescence emission of a sample can be spectrally dispersed using an instrument known as a "spectrofluorimeter" or simply "fluorimeter". In a fluorimeter the fluorescence is collected and detected at right angles to the direction of the excitation beam. Fluorimetric analysis is usually much more sensitive than colourimetric analysis. This is because it is easier to measure low light intensities at right angles to the excitation beam than small differences in the intensity of a transmitted beam. Although some fluorescence spectra are broad and featureless others contain much more fine structure and can be used to identify compounds due to their distinctive shape and peak maxima. The picture (left) shows a typical laboratory fluorimeter. The diagram (right) is a schematic representation of the functional elements of a fluorimeter.

Sources:
http://chemlab.truman.edu/CHEM322manual/perkinelmerls5b.htm
http://chemwiki.ucdavis.edu/Physical_Chemistry/Spectroscopy/Electronic_Spectroscopy/Electronic_Spectroscopy%3A_Application

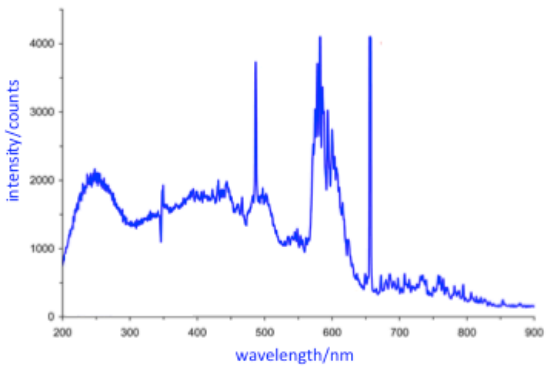
Xenon Lamp



A xenon arc lamp is a specialized gas discharge lamp that produces light by passing an electric current through ionized xenon gas at high pressure. The spectrum is close to that of natural sunlight (see above spectrum) and so xenon lamps are commonly used in accelerated exposure experiments that test the light-fastness of plastics and textiles. Small xenon lamps can be found in laboratory spectrophotometers and produce wavelengths in the visible region and some of ultraviolet region of the electromagnetic spectrum.

Sources:
http://jp.hamamatsu.com/products/light-source/pd024/index_en.html
http://en.wikipedia.org/wiki/Xenon_arc_lamp

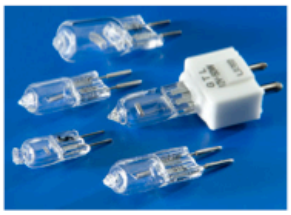
Deuterium Lamp

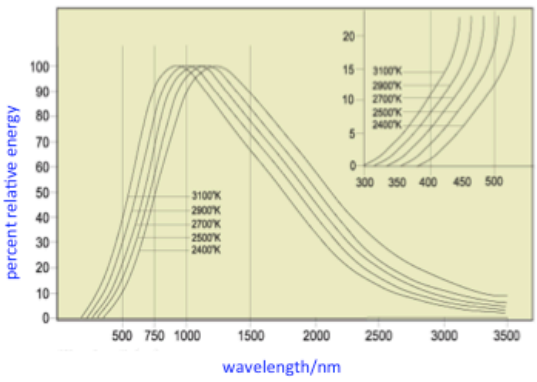


A deuterium arc lamp is a low-pressure gas discharge lamp that produces a continuous spectrum in the ultraviolet region. As such, it is often used in spectroscopy. The lamp emits wavelengths from 112 to 900 nm with its continuous spectrum considered to be from 180 to 370 nm (molecular deuterium continuum). The above spectrum shows also the characteristic hydrogen Balmer lines (486 and 656 nm) and the Fulcher band emission between *ca*. 560 to 640 nm.

Sources:
http://www.innovations-report.com/html/reports/energy_engineering/lamp_specialist_heraeus_noblelight_unveils_d_2_170664.html
http://en.wikipedia.org/wiki/File:Deuterium_lamp_1.png

Halogen Lamp

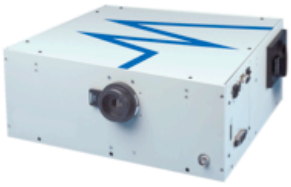


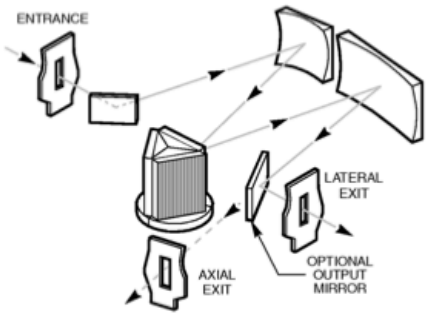


A halogen lamp (also known as a tungsten halogen lamp) is an incandescent lamp that comprises a tungsten filament contained within an inert gas and a small amount of a halogen such as iodine or bromine. The inclusion of the halogen increases the lifetime of the filament by re-depositing on the filament tungsten that would otherwise be deposited on the inside surface of the bulb. This process also prevents darkening of the bulb. Halogen lamps have higher luminous efficiencies compared with other gas-filled counterparts and because of their smaller size can be advantageously used in optical systems such as spectrophotometers, etc.

Sources:
http://www.intl-lighttech.com/applications/light-source-apps/halogen-lamps/halogen-lamps-index
http://en.wikipedia.org/wiki/Halogen_lamp

Monochromators

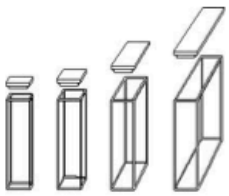


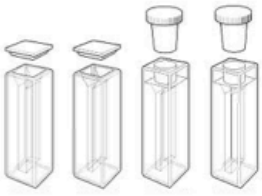


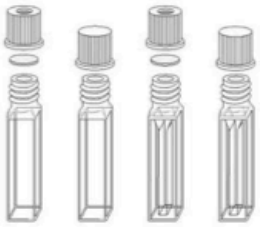
A monochromator is a device that selects a single wavelength or wavelength band from light that is incident on its entrance slit and transmits it through an exit slit. The incident light beam is collimated onto a diffraction grating. The relative motion of the grating with respect to the entrance and exit slit optics enables different wavelengths to be selected and as such a spectrum can be scanned. A common type of mono-chromator is the Czerny-Turner type depicted in the schematic diagram (above right) and can be used in the optics of a spectrophotometer. The picture (above left) shows a stand-alone monochromator that can be mounted, say, on an optical bench.

Sources:
http://search.newport.com/?x2=sku&q2=74126
http://gratings.newport.com/library/handbook/chapter6.asp

Fluorescence Cells



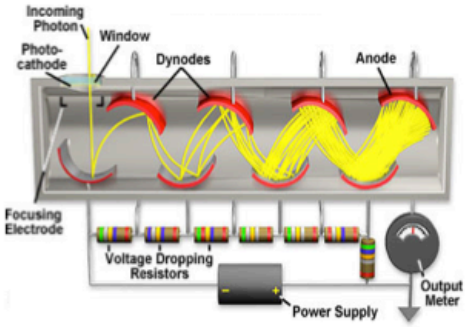




The picture and diagrams give an indication of the wide range of available fluorescence cells ranging from the common 1-cm square based cell that has four optical glass or quarts walls and an inert stopper to other types that have different optical path lengths and stopper systems.

Sources:
http://www.made-in-china.com/showroom/minyotec/product-detailNbixMvmThyhQ/China-Fluorometer-Cell-With-Stopper-TYPE-13-.html
http://shop.spectrecology.com/Quartz-cells-for-Fluorescence_c33.htm
http://www.optiglass.com/ukhome/d_cells/d_cells_f/T003.html
https://wbphotonics.com/index.php/starna-cells/fluorometer-cells.html

Photomultiplier Tubes



In a photomultiplier tube (PMT) the absorption of a photon results in the emission of an electron from a photoactive material (photocathode). The emitted electrons are amplified by electrodes known as metal channel dynodes and electrons are collected by the anode at the end of the chain. The flux of incident photons (intensity) thus produces a proportional electric current over a large range of incident photon fluxes. Photomultiplier tubes produce a signal in the absence of incident light due to a small amount of thermal emission of electrons from the photocathode (dark current) and other sources that create electronic noise. The spectral sensitivity of a PMT depends on the chemical composition of its photocathode. Gallium-arsenide materials are typically used for photocathodes and provide good sensitivity in the range of 300 to 800 nm.

Sources:
http://learn.hamamatsu.com/articles/photomultipliers.html
http://www.hofstragroup.com/product/rca-6342-photomultiplier-tube-2-10-stage-s-11/

EXPERIMENT

Hydrodynamic Molar Volume of a Globular Protein

Introduction

Some fluorescent "probe" molecules can bind directly to macromolecules causing a dramatic change to the emission characteristics of the probe. This behavior can be used to determine the conformational and structural properties of the macromolecule. One example of a probe molecule as such is 8-anilino-1-naphthalene sulfonic acid or "ANS" that exhibits a relatively strong fluorescence (λ_{max}fl = 460 nm) when it is attached to a protein molecule such as bovine serum albumin (BSA) in buffered solution, pH = 7.2. Indeed, the ANS species is almost non-fluorescent in H_2O but becomes highly fluorescent when non-covalently bound to BSA on mixing.

In the Theory section of the *FluAnisot* software one form of the Perrin equation is given as:

$$1/A = (1/A_0)[1 + \tau_f RT/(V\eta)] \qquad (1)$$

where A is the fluorescence anisotropy, A_0 is the limiting fluorescence anisotropy, τ_f is the fluorescence lifetime of the fluorophore, R is the ideal gas constant, T is the absolute temperature, V is the molar volume of the rotating moiety (macromolecule in this case) and η is the viscosity of the solution.

The value of A can be calculated from experimental measurements of the fluorescence intensity at 460 nm that is observed using different combinations of the relative orientations of the polarizing filters:

$$A = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \qquad (2)$$

where I_{VV} and I_{VH} are the fluorescence intensities determined using vertically polarized excitation light and monitoring vertically and horizontally polarized emission respectively. The instrumental correction factor, G, is equal to I_{HV}/I_{HH} where I_{HV} and I_{HH} are the respective vertically and horizontally polarized fluorescence emission intensities observed using horizontally polarized excitation light.

This experiment simulates the use of a conventional fluorimeter equipped with polarizing filters in its excitation and emission optical axes to determine the molar volume of BSA that has been "tagged" with ANS (i.e. a BSA-ANS conjugate) in a buffered aqueous solution. It is assumed that [ANS] << [BSA] so that no multiple binding sites are produced and that any ANS not bound to BSA does not interfere with the anisotropy measurements.

Two experimental techniques are explored to determine the molar volume: (i) the temperature variation method and (ii) the viscosity variation method.

Temperature Variation Method

This method involves varying the temperature and assuming that the variation of the solution viscosity with temperature is equal to the variation of the viscosity of water with temperature. Such an assumption is valid if a dilute aqueous solution of BSA is used. The method also assumes there is no thermal degradation of the BSA, or indeed the BSA-ANS conjugate, as the temperature is increased, particularly at the higher temperatures used. In reality, any such degradation would presumably be reflected by non-linearity in the ultimate plot of the processed experimental data.

The temperature variation method involves calculating the fluorescence anisotropy from intensity data that were collected at different temperatures over a range of, say, 20 to 60°C.

Procedure

(1) Click the *Flu Anisotropy* tab and access the simulator.

(2) Adjust the temperature to 20°C using the slider control. Notice that the value of the viscosity of water at this temperature automatically appears in the viscosity edit field.

(3) Select the desired orientation of the polarizing filters by clicking on the square control tabs. The default setting (i.e. excitation = V; emission = V) can be used to make the first reading.

(4) Click the *Start* button to run the simulator and observe the reading of the emission intensity. For the default settings of the polarizing filters this will be displayed as "I_{VV}". [Note: The first subscript denotes the orientation of the excitation polarizing filter; the second denotes the orientation of the emission polarizing filter.]

(5) Notice that when the simulator is running there is some minor fluctuation in the reading. This is usually observed in a real instrument due to the inherent "noise". When the reading appears to be relatively stable record it by clicking the *Capture* button.

(6) When the *Capture* button is clicked, a copy of the recorded data appears in the window at the bottom left-hand corner of the screen. The captured data are simultaneously copied to the *Lab Book*.

(7) Click the *Stop* button to halt the simulator. You can now view the recorded data in the *Lab Book* if you wish by clicking on the *Lab Book* tab. You can return to the simulator by clicking on the *Flu Anisotropy* tab. [Note: Changes to the settings of the simulator and access to the *Lab Book* and other functions of the program can only be achieved if the simulator has been stopped.]

(8) Change the orientation of the emission polarizing filter to horizontal (i.e. emission = H). Leave the excitation polarizing filter set to its vertical orientation (i.e. excitation = V).

(9) Start the simulator and record the emission intensity "I_{VH}".

(10) Stop the simulator and using a similar procedure to that above record the emission intensities "I_{HV}" and "I_{HH}". [Note: After completing this step you should have recorded a total of four readings: I_{VV}, I_{VH}, I_{HV} and I_{HH}. It does not matter in which order these reading are made, so long as all combinations of the relative orientations of the excitation and emission polarizing filters have been used.]

(11) Once the four readings have been recorded at 20°C repeat the above procedure to similarly record I_{VV}, I_{VH}, I_{HV} and I_{HH} readings, each at a temperature of 30, 40, 50 and 60°C. [Note: Temperature increments of 5°C could be used instead if time permits. The temperature range could also be increased slightly if required.]

(12) When all data are recorded enter the *Lab Book* by clicking on its tab. You may edit the data in the results field in the usual way by "clicking, wiping, cutting and pasting", etc. Data editing may be necessary during the experiment if, say, a reading was erroneously recorded when the simulator was not quite stable, or an incorrect setting was inadvertently used, etc. [Note: All data in the results edit field can be deleted by clicking the *Clear* button.]

(13) When you are satisfied that you have collected all necessary data for analysis click the *Clipboard* button. This will copy the data into the clipboard.

(14) Import the data in the clipboard into a suitable spreadsheet program for processing and plotting.

(15) Perform the "Viscosity Variation" experimental method found immediately below and/or proceed to the Calculations and Questions section.

Note: An advanced procedure that enables an error analysis to be performed involves recording multiple readings for each setting of the instrument at, say, 10 s intervals. A sample analysis illustrating this technique where four measurements have been made at each setting can be accessed in the *Lab Book* section. The analysis shows that the mean $1/A$ values are significantly different at each instrumental setting.

Viscosity Variation Method

This method involves keeping the temperature constant, at say 20°C, and varying the viscosity of the solution by the addition of an inert viscosity modifying agent such as sucrose. If this experiment were to be performed in a laboratory the viscosity of each test solution would need to be determined in a separate experiment. If the latter measurements were to be made, say, using a capillary viscometer, then this could be quite time-consuming. Nonetheless, the viscosity variation method has the advantage in that it does not invoke any assumptions regarding the stability of the BSA-ANS conjugate, providing the temperature is set at ambient (*cf*. temperature variation method above).

For the purposes of the current simulated experiment it will be assumed that a series of solutions each containing a sufficiently low concentration of the BSA-ANS conjugate and a certain quantity of an inert viscosity modifier (sucrose) has been prepared and equilibrated at 20°C in the instrument. Furthermore, it will be assumed that the viscosity of a given BSA-ANS/sucrose solution is equal to the viscosity of an aqueous sucrose solution containing the same concentration of sucrose alone. This assumption is reasonable if a sufficiently low concentration of BSA-ANS conjugate is used.

Note: table containing the viscosity of aqueous sucrose solutions as a function of sucrose concentration at 20°C is given at the end of these notes.

Procedure

(1) Click the *Flu Anisotropy* tab and access the simulator. If during this session a previous experiment has been run using the simulator without quitting the software and re-opening it, click the *Lab Book* tab and erase all data from the data edit field by clicking the *Clear* button. Return to the simulator.

(2) Adjust the temperature to 20°C using the slider control. [Notice that the value of the viscosity of water at this temperature automatically appears in the viscosity edit field. This setting can be used to obtain data for a solution that contains no sucrose.]

(3) Use the information in the accompanying table to determine the viscosity of BSA-ANS/sucrose solutions containing: 0, 10, 20, 30 and 40% (w/w) sucrose. Assume the concentration of BSA-ANS conjugate is low enough so that these solutions can be treated as aqueous solutions of pure sucrose. [Note: If time permits you may wish to collect data covering a wider viscosity range or at increments of 5% (w/w) sucrose.]

(4) Following the procedure given in the previous (temperature variation method) experiment record I_{VV}, I_{VH}, I_{HV} and I_{HH} readings at 20°C for each the BSA-ANS solution. Important: For each run

you will need to ensure that the viscosity is set to the appropriate value by typing that value into the viscosity edit field before running the simulator.

(5) When all runs are complete ensure that all necessary data appear in the *Lab Book*. Edit the data in the *Lab Book* to remove any readings that you know have resulted from an erroneous procedure.

(6) Click the *Clipboard* button and import the data into a suitable spreadsheet program for processing and plotting.

(7) Proceed to the Calculations and Questions section.

Calculations and Questions

(1) Values of viscosity in this experiment are given and entered into the simulator in units of centipoise (cP). If the units of the ideal gas constant, R, are taken to be those that are commonly used (i.e. $R = 8.314$ J mol^{-1} K^{-1}) then this will require the units of viscosity to be kg m^{-1} s^{-1} for use in the calculations. Thus determine the conversion factor from cP to kg m^{-1} s^{-1}.

(2) If the value of $R = 8.314$ J mol^{-1} K^{-1} is used in the calculations, state the resultant units of V, the molar volume. What are the units of A? Show that the units of A are consistent with the units of the "$\tau_f RT/(V\eta)$" term in the form of the Perrin equation used in this work.

(3) Clearly explain how a plot of $1/A$ versus T/η can be used to determine the molar volume of a globular protein such as BSA. Include in your answer all necessary equations and define all symbols that are used.

(4) Use your data obtained in either experiment above to plot a graph of $1/A$ versus T/η. From the values of the gradient and vertical axis intercept of the plot calculate the molar volume of the BSA-ANS conjugate given the fluorescence lifetime $\tau_f = 15.6$ ns. You may assume that this value was obtained from a separate time-resolved fluorescence decay experiment.

Note: Perhaps the most convenient way of performing this task is to use the programming capabilities of your spreadsheet software to perform the simple, but repetitive, arithmetic needed to calculate the required quantities. The program can also be used to plot the graph and assess its linearity. Examples of typical results that can be obtained using the simulator along with a suggested guide to setting out the calculations using a spreadsheet are given in the *Lab Book* section.

(5) Observe the plot obtained in Question (4) and comment on its linearity. Hence comment on the validity of any assumptions used in the experimental procedure.

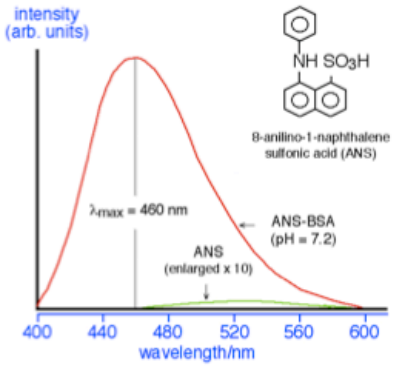
(6) Note the value of the vertical axis intercept of your plot and comment on how close this value lies to the theoretically expected value.

(7) Compare the value/s of the molar volume of BSA you obtained by the fluorescence anisotropy simulator with the value of 0.104 m^3 mol^{-1} that is obtained from X-ray scattering experiments. Comment on any differences.

(8) Briefly discuss the advantages and disadvantages of the each of the two methods used in this experiment to study the molecular dynamics of a molecular probe-globular protein conjugate.

Diagrams and Information Screens

Fluorescence Behavior of ANS



The fluorescence probe 8-anilino-1-naphthalene sulfonic acid (ANS, $\lambda_{max}(fl)$ = 460 nm) can attach to a protein molecule such as bovine serum albumin (BSA) in a buffered aqueous solution at pH = 7.2. If a dilute aqueous solution is used (0.40 g L^{-1} BSA in the presence of 8 x 10^{-6} M ANS) then the viscosity of the solution is approximately equal to that of water. Although the ANS probe is virtually non-fluorescent in water it becomes highly fluorescent when it is non-covalently bound to the BSA on mixing (see fluorescence spectra). Thus any ANS that is unbound to the protein should not affect experimental results in the laboratory.

Source:
Bigger, S. W., Craig, R. A., Ghiggino K. P. and Scheirs, J., *J. Chem. Educ.*, **70**, A234-239 (1993).

Composition, Viscosity and Density of Sucrose Solutions at 20°C

Sucrose Concentration % w/w	Sucrose Concentration g L^{-1}	Relative Viscosity η_{soln}/η_{water}	Density ρ g mL^{-1}
0	0	1.00	0.998
5	50.9	1.144	1.018
10	103.8	1.333	1.038
15	158.9	1.589	1.059
20	216.2	1.941	1.081
25	275.9	2.442	1.104
30	338.1	3.181	1.127
35	402.9	4.314	1.151
40	470.6	6.150	1.176
45	541.1	9.360	1.203
50	614.8	15.40	1.230
55	691.6	28.02	1.258
60	771.9	58.37	1.286
65	855.6	146.9	1.316
70	943.0	480.6	1.347
75	1034	2323	1.379

Source:
http://lclane.net/text/sucrose.html

Answers to Questions

(1) $1\text{P} = 1\text{ g cm}^{-1}\text{ s}^{-1}$

$\therefore\ 1\text{cP} = 0.01\text{ g cm}^{-1}\text{ s}^{-1}$

$= [0.01\text{ g cm}^{-1}\text{ s}^{-1}] \times [100\text{ cm m}^{-1}] \times [10^{-3}\text{ kg g}^{-1}]$

$= 1 \times 10^{-3}\text{ kg m}^{-1}\text{ s}^{-1}$

(2) The anisotropy, A, is calculated from the ratio of intensity values in accordance with, say, equation (2) above. Thus the quantity will be dimensionless. For the form of Perrin equation shown in equation (1) to be dimensionally correct the "$\tau_f RT/(V\eta)$" term must be dimensionless. Whence, considering units:

$\tau_f RT/(V\eta) \equiv \{[\text{s}] \times [\text{J mol}^{-1}\text{ K}^{-1}] \times [\text{K}]\} / \{[\text{m}^3\text{ mol}^{-1}] \times [\text{kg m}^{-1}\text{ s}^{-1}]\}$

$\equiv \{[\text{s}] \times [\text{N m mol}^{-1}\text{ K}^{-1}] \times [\text{K}]\} / \{[\text{m}^3\text{ mol}^{-1}] \times [\text{kg m}^{-1}\text{ s}^{-1}]\}$

$\equiv \{[\text{s}] \times [\text{kg m s}^{-2}\text{ m mol}^{-1}\text{ K}^{-1}] \times [\text{K}]\} / \{[\text{m}^3\text{ mol}^{-1}] \times [\text{kg m}^{-1}\text{ s}^{-1}]\}$

$= 1$

(3) Recall the following equation relating the reciprocal of the anisotropy, A, to the ratio T/η:

$$1/A = (1/A_0)[1 + \tau_f RT/(V\eta)]$$

where A_0 is the limiting fluorescence anisotropy, τ_f is the fluorescence lifetime of the fluorophore, R is the ideal gas constant, T is the absolute temperature, V is the molar volume of the dye-polymer conjugate and η is the viscosity of the solution. The equation can be re-written as follows:

$$1/A = (1/A_0) + (\tau_f R/A_0 V) \times (T/\eta)$$

The latter way of expressing this equation makes it clear that a plot of $1/A$ versus T/η should produce a straight line with intercept equal to $1/A_0$ and gradient equal to $\tau_f R/A_0 V$. The molar volume, V, can thus be determined from the gradient of the plot if τ_f is known because R is known and A_0 can be determined from the vertical axis intercept of the plot.

(4) Analyses of typical data sets obtained in each part of the experiment, along with a more extensive set of data that was recorded to undertake a comprehensive error analysis can be accessed in the

Lab Book section of the *FluAnisot* software. Reproductions of these screens appear below (see Results and Error Analyses section).

(5) The linearity of the plots shown in the Results and Error Analyses section is evidenced by the respective regression coefficients and thus validates the assumptions made in deriving and applying the analytical equation used to find the molar volume. These assumptions are mentioned in the preamble to the experiments as well as in the theory section and include: (i) the assumed spherical geometry of the rotating entity in solution, (ii) no multiple binding sites of the ANS, (iii) sufficiently low concentrations of dye-polymer conjugate such that solutions can be treated as being either pure water or sucrose solutions only and (iv) no thermal degradation occurs at the higher temperatures used in the "temperature variation" method.

(6) From the vertical axis intercepts and the error analyses conducted in the next section the following values of A_0 have been calculated along with the 95% confidence intervals:

$A_0 = 0.39 \pm 0.03$ (temperature variation method)
$A_0 = 0.40 \pm 0.01$ (viscosity variation method)

In each case the confidence interval contains the theoretical value of A_0 which is 0.40.

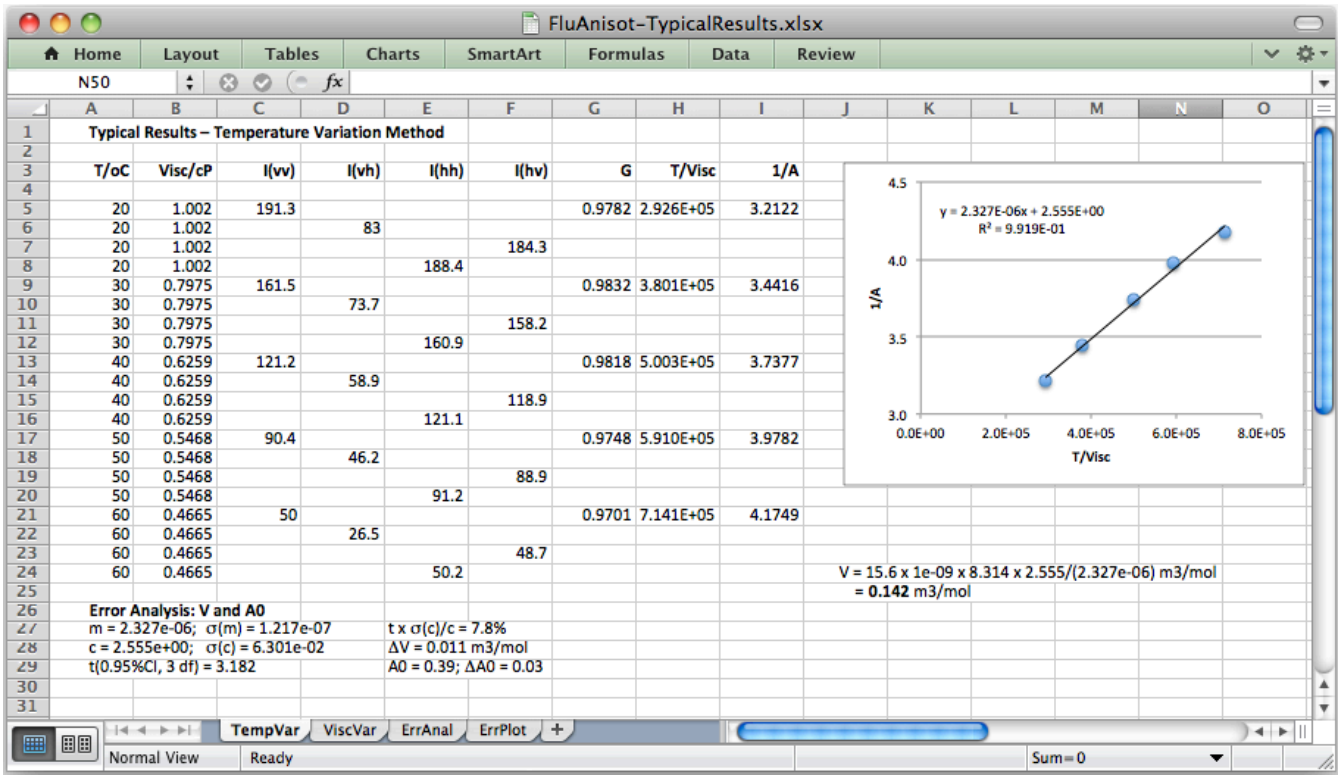
(7) Values of the molar volume of BSA obtained in the experiments are as follows:

$V = 0.142 \pm 0.011$ m^3 mol^{-1} (temperature variation method)
$V = 0.139 \pm 0.002$ m^3 mol^{-1} (viscosity variation method)

These values are significantly greater than the value obtained by X-ray scattering, 0.104 m^3 mol^{-1}. The latter result is has been obtained in the solid state and is therefore expected to be less than the hydrodynamic volume obtained in solution. In solution the protein is expected to be solvated and more swollen than in the solid state. The hydrodynamic volume may also include a sheath of water molecules on the outer surface of the spherical rotor that are dragged about during its rotation and may contribute to the observed volume.

(8) In the laboratory, the temperature variation method is quicker to perform than the viscosity variation method. However, the temperature variation method may present problems if the polymer under investigation is not thermally stable and thus decomposes at the higher temperatures that may be required to collect a wide enough range of data for analysis. The viscosity variation method, on the other hand, does not suffer from this potential problem but it will require much more time to complete because the viscosity of each solution must be measured in a separate experiment.

Results and Error Analyses



FluAnisot-TypicalResults.xlsx

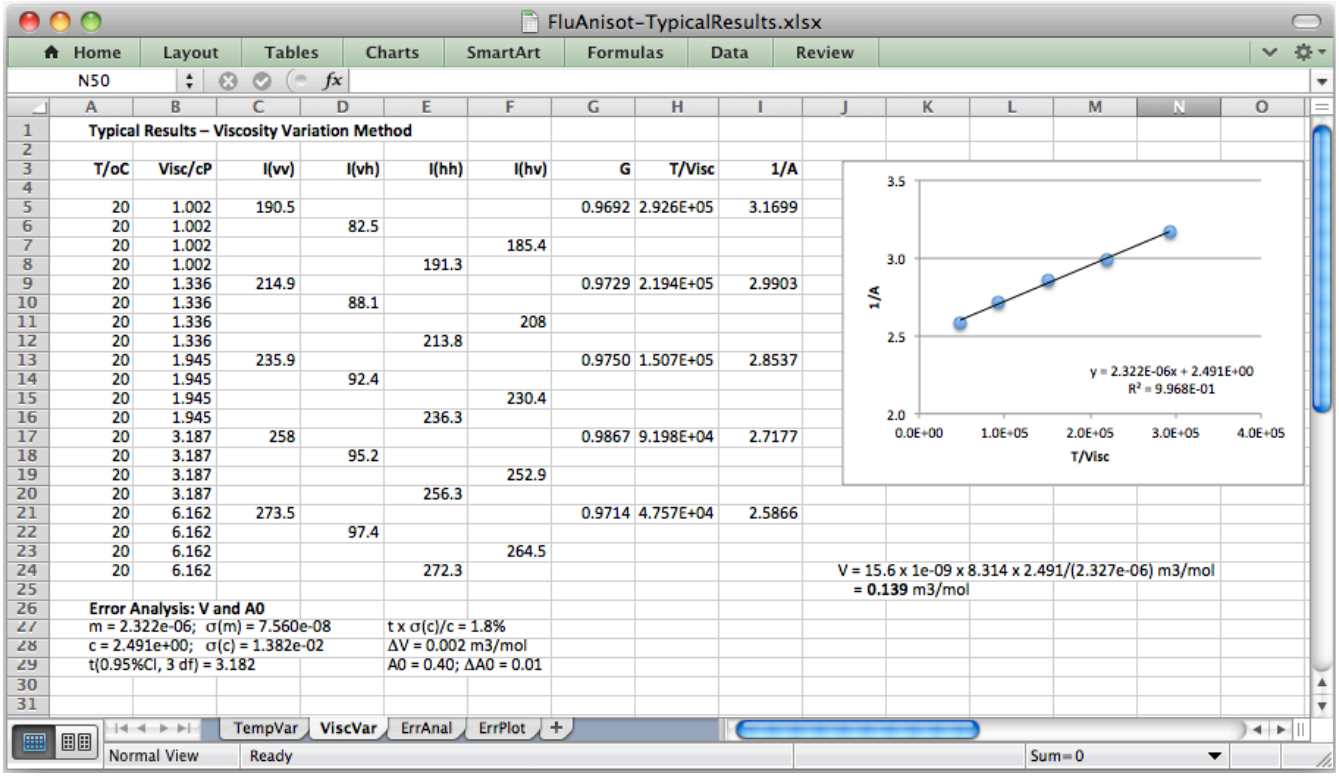
Typical Results – Temperature Variation Method

T/oC	Visc/cP	I(vv)	I(vh)	I(hh)	I(hv)	G	T/Visc	1/A
20	1.002	191.3				0.9782	2.926E+05	3.2122
20	1.002		83					
20	1.002				184.3			
20	1.002			188.4				
30	0.7975	161.5				0.9832	3.801E+05	3.4416
30	0.7975		73.7					
30	0.7975				158.2			
30	0.7975			160.9				
40	0.6259	121.2				0.9818	5.003E+05	3.7377
40	0.6259		58.9					
40	0.6259				118.9			
40	0.6259			121.1				
50	0.5468	90.4				0.9748	5.910E+05	3.9782
50	0.5468		46.2					
50	0.5468				88.9			
50	0.5468			91.2				
60	0.4665	50				0.9701	7.141E+05	4.1749
60	0.4665		26.5					
60	0.4665				48.7			
60	0.4665			50.2				

V = 15.6 x 1e-09 x 8.314 x 2.555/(2.327e-06) m3/mol
= **0.142** m3/mol

Error Analysis: V and A0

m = 2.327e-06; σ(m) = 1.217e-07 — t x σ(c)/c = 7.8%
c = 2.555e+00; σ(c) = 6.301e-02 — ΔV = 0.011 m3/mol
t(0.95%CI, 3 df) = 3.182 — A0 = 0.39; ΔA0 = 0.03



FluAnisot-TypicalResults.xlsx

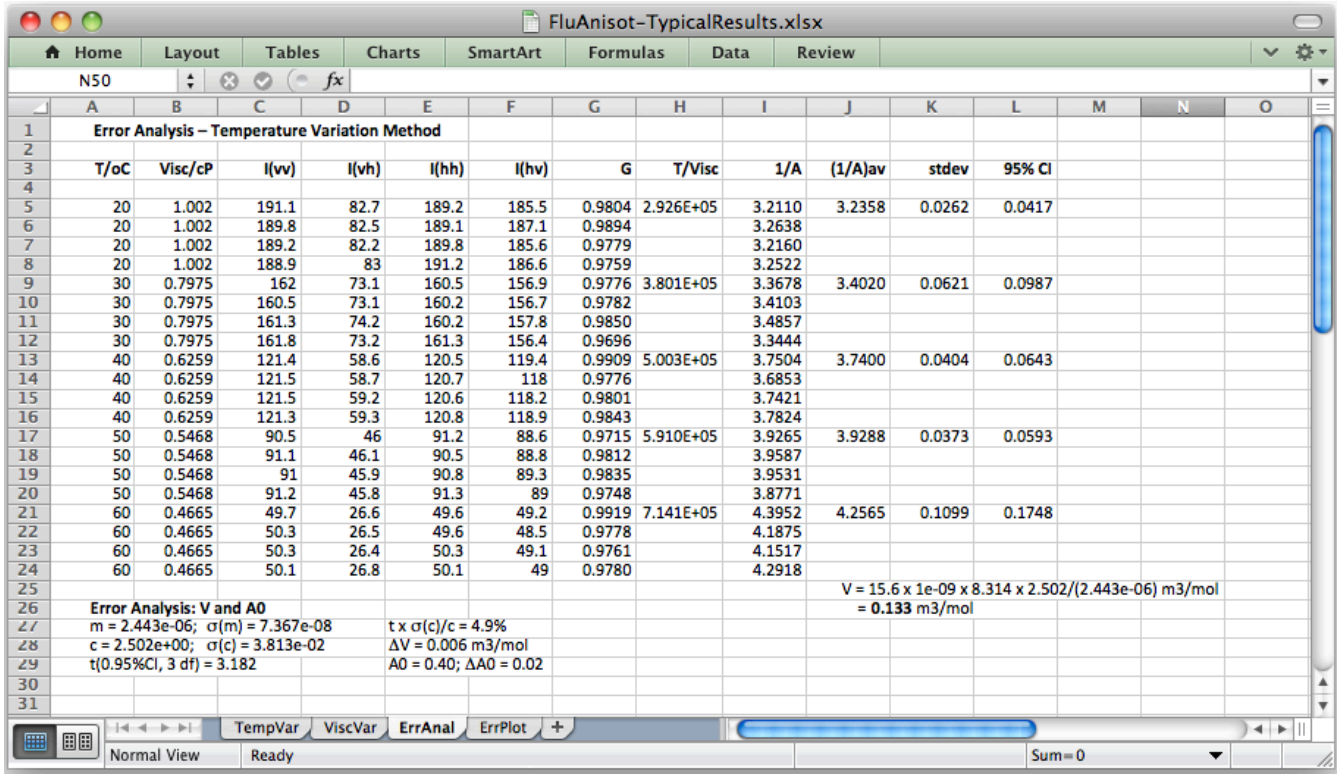
Typical Results – Viscosity Variation Method

T/oC	Visc/cP	I(vv)	I(vh)	I(hh)	I(hv)	G	T/Visc	1/A
20	1.002	190.5				0.9692	2.926E+05	3.1699
20	1.002		82.5					
20	1.002				185.4			
20	1.002			191.3				
20	1.336	214.9				0.9729	2.194E+05	2.9903
20	1.336		88.1					
20	1.336				208			
20	1.336			213.8				
20	1.945	235.9				0.9750	1.507E+05	2.8537
20	1.945		92.4					
20	1.945				230.4			
20	1.945			236.3				
20	3.187	258				0.9867	9.198E+04	2.7177
20	3.187		95.2					
20	3.187				252.9			
20	3.187			256.3				
20	6.162	273.5				0.9714	4.757E+04	2.5866
20	6.162		97.4					
20	6.162				264.5			
20	6.162			272.3				

V = 15.6 x 1e-09 x 8.314 x 2.491/(2.327e-06) m3/mol
= **0.139** m3/mol

Error Analysis: V and A0

m = 2.322e-06; σ(m) = 7.560e-08 — t x σ(c)/c = 1.8%
c = 2.491e+00; σ(c) = 1.382e-02 — ΔV = 0.002 m3/mol
t(0.95%CI, 3 df) = 3.182 — A0 = 0.40; ΔA0 = 0.01



FluAnisot-TypicalResults.xlsx

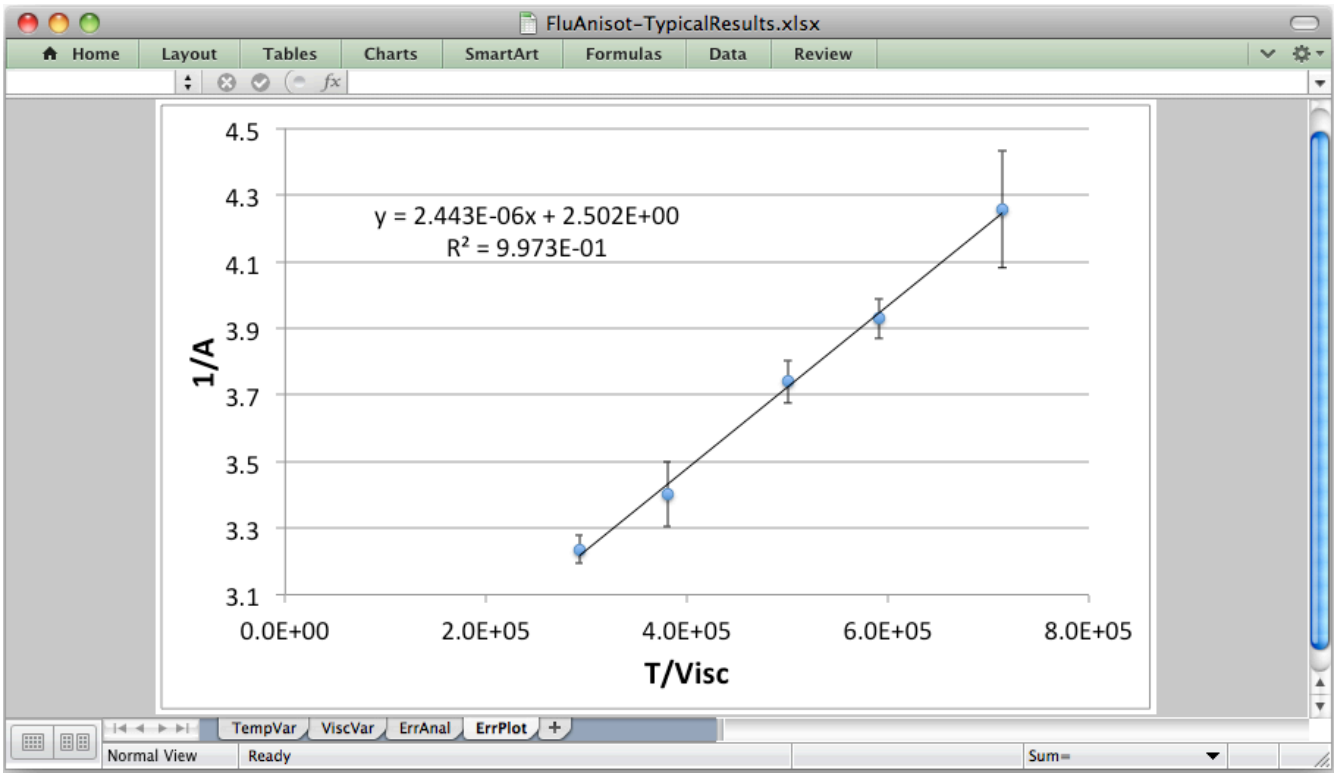
Home | Layout | Tables | Charts | SmartArt | Formulas | Data | Review

N50

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	**Error Analysis – Temperature Variation Method**														
2															
3	**T/oC**	**Visc/cP**	**I(vv)**	**I(vh)**	**I(hh)**	**I(hv)**	**G**	**T/Visc**	**1/A**	**(1/A)av**	**stdev**	**95% CI**			
4															
5	20	1.002	191.1	82.7	189.2	185.5	0.9804	2.926E+05	3.2110	3.2358	0.0262	0.0417			
6	20	1.002	189.8	82.5	189.1	187.1	0.9894		3.2638						
7	20	1.002	189.2	82.2	189.8	185.6	0.9779		3.2160						
8	20	1.002	188.9	83	191.2	186.6	0.9759		3.2522						
9	30	0.7975	162	73.1	160.5	156.9	0.9776	3.801E+05	3.3678	3.4020	0.0621	0.0987			
10	30	0.7975	160.5	73.1	160.2	156.7	0.9782		3.4103						
11	30	0.7975	161.3	74.2	160.2	157.8	0.9850		3.4857						
12	30	0.7975	161.8	73.2	161.3	156.4	0.9696		3.3444						
13	40	0.6259	121.4	58.6	120.5	119.4	0.9909	5.003E+05	3.7504	3.7400	0.0404	0.0643			
14	40	0.6259	121.5	58.7	120.7	118	0.9776		3.6853						
15	40	0.6259	121.5	59.2	120.6	118.2	0.9801		3.7421						
16	40	0.6259	121.3	59.3	120.8	118.9	0.9843		3.7824						
17	50	0.5468	90.5	46	91.2	88.6	0.9715	5.910E+05	3.9265	3.9288	0.0373	0.0593			
18	50	0.5468	91.1	46.1	90.5	88.8	0.9812		3.9587						
19	50	0.5468	91	45.9	90.8	89.3	0.9835		3.9531						
20	50	0.5468	91.2	45.8	91.3	89	0.9748		3.8771						
21	60	0.4665	49.7	26.6	49.6	49.2	0.9919	7.141E+05	4.3952	4.2565	0.1099	0.1748			
22	60	0.4665	50.3	26.5	49.6	48.5	0.9778		4.1875						
23	60	0.4665	50.3	26.4	50.3	49.1	0.9761		4.1517						
24	60	0.4665	50.1	26.8	50.1	49	0.9780		4.2918						
25										V = 15.6 x 1e-09 x 8.314 x 2.502/(2.443e-06) m3/mol					
26	**Error Analysis: V and A0**									= **0.133** m3/mol					
27	m = 2.443e-06; σ(m) = 7.367e-08				t x σ(c)/c = 4.9%										
28	c = 2.502e+00; σ(c) = 3.813e-02				ΔV = 0.006 m3/mol										
29	t(0.95%CI, 3 df) = 3.182				A0 = 0.40; ΔA0 = 0.02										
30															
31															

TempVar | ViscVar | ErrAnal | ErrPlot

Normal View | Ready | Sum=0



Systems

It is anticipated the software will successfully run on a wide range of Macintosh and PC systems. The following systems are examples of ones that have been successfully trialed:

Macintosh

- iMac 2.66 GHz Intel Core i5; Mac OS X 10.6.8 (Leopard); Fuji Xerox ApeosPort IV C5570 network printer
- Macbook 2.16GHz Intel Core 2 Duo; Mac OSX 10.7.2 (Lion); HP Deskjet F4400 Series and Apple PDF printer

PC Systems

- Macbook 2.1.6GHz Intel Core 2 Duo; Windows 7 Professional; HP Deskjet F4400 Series and Cute PDF Printer
- Windows XP SP2 Virtual Machine running on Macbook 2.16 GHz Intel Core 2 Duo; HP Deskjet F4400 Series and Cute PDF Printer
- Lenovo ESA4C406-86, Intel®Core™2 DuoCPU, E8400 @ 3.00GHz, 1.97GHz, 3.25 GB of RAM, Microsoft Windows XP Professional Version 2002 Service Pack 3; Xerox Phaser 6360 Printer, Model P6360DT