Application Note Booklet

2710FP001

Spectrofluorometer

CONTENTS

Fluorescence depolarization measurement for Liposome

Fluorescence polarization and depolarization

The molecule is excited ($S_0 \rightarrow S_1$) by the absorption of light from ground state to transition state and stabilized in the most stable vibrational state by its molecular relaxation process, and then it returns again ($S_1 \rightarrow S_0$) to the ground state by radiating fluorescence light.In the process of $S_0 \rightarrow S_1$ or, $S_1 \rightarrow S_0$, there is a directionality (transition moment), determined by molecular structure, that is closely related to polarization direction of excitation and fluorescence light.

(1)Absorption process: With exciting by linearly polarized light, probability of excitation of each molecule is expressed as below.

$P(a) = P_0 \cos^2 a$ (a: Angle between polarization direction and transition moment, P_0: proportional constant)

When vector of electric field of excitation light and transition moment are in parallel condition, theprobability of excitation will become maximum.

(2) Radiation process: Fluorescence in the process of $S_1 \rightarrow S_0$ has polarizing direction in conformity with the direction of transition moment. Fluorescence light from the molecule excited by polarized light has polarization property depending on the polarizing direction of excitation light. Moreover, such polarization property of the fluorescence light will be affected also by the rotation of the molecule due to Brownian motion, etc. until the fluorescence is radiated. In other words, when fluorescence is radiated before the rotation of its molecule, fluorescence light will be strongly polarized towards direction of the polarization of excitation light, but on the other hand, when fluorescence is radiated after rotation of its molecule in completely random direction, the fluorescence light will be no longer polarized. This is called “depolarization”.

Measurement method and measured example for fluorescence depolarization

In order to measure the fluorescence depolarization by FP-8500 spectrofluorometer, automatic polarization measurement unit and its software are needed.

By setting the Excitation (E_x) side polarizer in the vertical position(V), both fluorescence intensities(I_{VV} and I_{VH}) are measured by setting the fluorescence(E_m) side polarizer in the horizontal position(H) and V. In order to correct the sensitivity difference of the Em side detector against polarization, the spectrum is measured by setting the Ex side polarizer in H position and setting the E_m side polarizer in V and H position to obtain instrumental function, $G(=I_{HV}/I_{HH})$, and the correction is applied multiplying I_{VH} by G factor. Accordingly, each parameter is expressed by the following formula.

Total fluorescence intensity (F): $I_{VV} + 2G \cdot I_{VH}$

Fluorescence anisotropy (r): $\dfrac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}}$

Polarization degree (P): $\dfrac{I_{VV} - G \cdot I_{VH}}{I_{VV} + G \cdot I_{VH}}$

As an example, measurement was made using 0.22 mg/L rhodamine B in glycerin solution.

[Measurement conditions]

Automatic polarization measurement unit:	Water-cooled Peltier thermostatted automatic polarization measurement unit.
Measurement mode:	Fluorescence
Excitation wavelength:	550 nm, Emission
Wavelength:	600 nm
Excitation bandwidth:	5 nm
Emission bandwidth:	5 nm
Response:	0.1 sec
Measurement temperature:	25 ºC

Table 1 Fluorescence polarization of 0.22 mg/L rhodamine B in glycerin solution

No.	I_{VV}	I_{VH}	G	F	P	r
1	244.1098	30.737	2.833	418.2683	0.4741	0.3754
2	244.3634	30.7426	2.833	418.553	0.4745	0.3757
3	243.714	30.6777	2.833	417.5363	0.4743	0.3755
4	243.3629	30.6519	2.833	417.0391	0.474	0.3753
5	242.9584	30.5799	2.833	416.2262	0.4743	0.3756
Ave.	243.7017	30.6778	2.833	417.5246	0.4742	0.3755
S.D.	0.5639	0.067	0	0.9398	0.0002	0.0002
C.V.	0.2314	0.2184	0	0.2251	0.0353	0.0419

Fluorescence polarization measurement of Liposome

Fluorescence polarization depends on the time from excitation of the molecule to its radiation and on how long polarization property of the initially excited molecule is kept without some movement of the molecule. Therefore, in other words, by measuring the degree of fluorescence polarization, it can be known how difficult the molecule can move. The factors related to molecule movement are as follows.

(1) Molecular size (The larger molecule is harder to move.)
(2) Viscosity of environment where molecule exists (In higher viscous condition, molecule is harder to move.)
(3) Strength and degrees of freedom of molecule in bondage.

The above factors are taken into consideration mainly for measurement of biological samples, and especially are utilized for analysis of the size and conformation change for biological polymer and the local conformation. Here is a result of polarization degree measured under the condition that diphenyl hexatriene (DPH) is added to liposome (lipid bilayer) that is generated by dispersion of the phosphatide in water and the temperature is changed from 25 ºC to 55 ºC.

[Measurement conditions]

Automatic polarization measurement unit:	Water-cooled Peltier thermostatted automatic polarization measurement unit.
Excitation wavelength:	357 nm
Emission wavelength:	430 nm
Excitation bandwidth:	3 nm
Emission bandwidth :	3 nm
Response:	2 sec
Data import interval:	0.1 ºC
Temperature gradient:	20 ºC /hour
Phosphatide:	130 µM
DPH:	0.6 µM

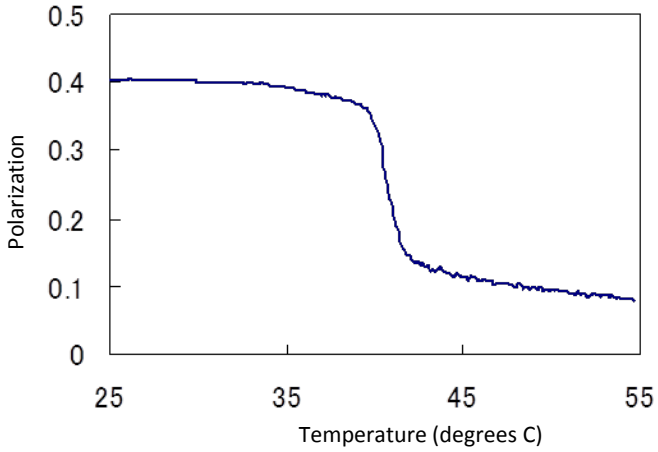


Figure 1 Temperature gradient measurement for fluorescence polarization of DPH added to liposome

DPH is held in oriented form between lipid bilayer and is restricted to move at a low temperature, but when membrane drastically flickers by the phase transition of lipid bilayer at 40 ºC, the movement of DPH is activated, and depolarization is clearly observed.

Measuring fluorescence anisotropy spectrum of Rhodamine B

Measuring fluorescence anisotropy is performed using polarizers on both excitation(Ex) side and emission(Em) side. It is known that Rhodamine B has wavelength dispersion of fluorescence anisotropy depending on the excitation wavelength. Therefore the Rhodamine B was measured to obtain wavelength dispersion of fluorescence anisotropy and degree of polarization.

[Measurement principle]

With Ex side polarizer in the vertical(V) position, each excitation spectrum is measured under Em side polarizer in the vertical(V) and horizontal(H) positions. These fluorescence intensities of the excitation spectra are defined as I_V and I_H.

In order to correct the difference of sensitivity for polarization on Em side detector, the spectra are measured with Ex side polarizer in the H and Em side polarizer in the V and H positions, and the ratio is multiplied by I_H to obtain I_H(corrected). Then calculated fluorescence anisotropy(r) is obtained using the following equation.

[Measurement condition]

Instrument:	FP-8500
Polarizer:	FDP-837 polarizer
Measurement mode:	Ex spectrum
Ex side bandwidth:	2.5 nm
Em side bandwidth:	2.5 nm
Response:	0.5 sec
Sensitivity:	Medium
Measurement wavelength range:	350-580 nm
Data acquisition interval:	0.2 nm
Em wavelength:	625.0 nm
Wavelength scan speed:	200 nm/min

FDP-837

[Measurement procedure]

1. Set Ex side polarizer in the V position and set Em side polarizer also in the V position. Using FP-8500 spectrofluorometer, measure Ex spectrum of ethylene glycol solution (0.588 mg/L). Then the fluorescence intensity of the spectrum is defined as I_V.
2. Set Ex side polarizer in the H position and set Em side polarizer in the V position, and measure spectrum in the same way like item 1.
3. Set Ex side polarizer in the H position and set Em side polarizer in the H position, and measure spectrum in the same way like item 1.
4. Set Ex side polarizer in the V position and set Em side polarizer in the H position, and measure spectrum in the same way like item 1.
5. Calculate the ratio of the above item 2.and 3. results, and then approximate average value is determined to be $ratio_{avg} = 3.0$.
6. Multiply the spectrum of the above item 4. with the $ratio_{avg} = 3.0$, to determine this fluorescence intensity as I_H(corrected).
7. Calculate the fluorescence anisotropy(r) using I_V and I_H(corrected).

[Measurement result]

The absorption band that has peak wavelength around 560 nm shows large constant fluorescence anisotropy(r). On the other hand, r around 430nm, 360 nm show negative peaks, and shows small positive peak around 380 nm. These fluorescence anisotropy variation suggests that different electronic transition bands are overlapped.

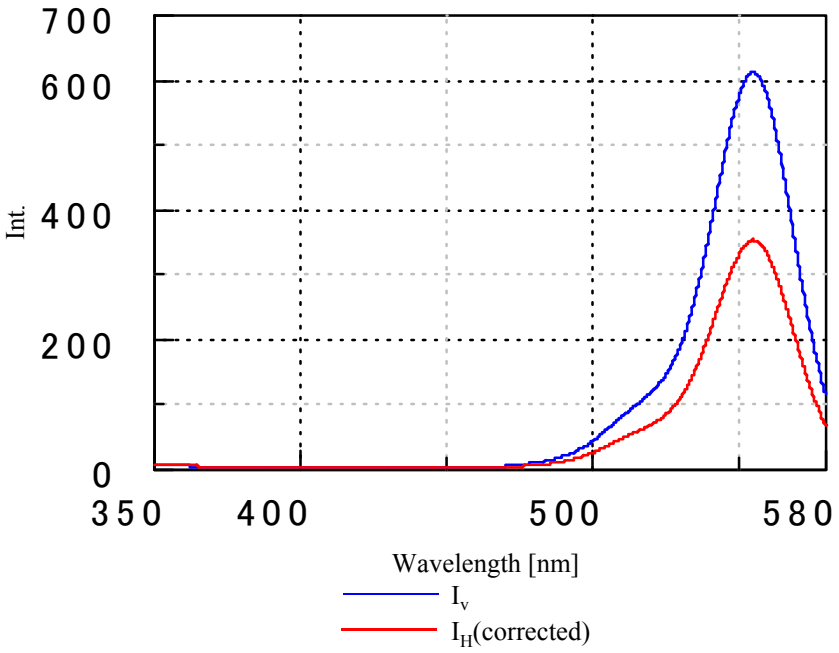


Fig. 1 Ex spectra of I_V and I_H(corrected)

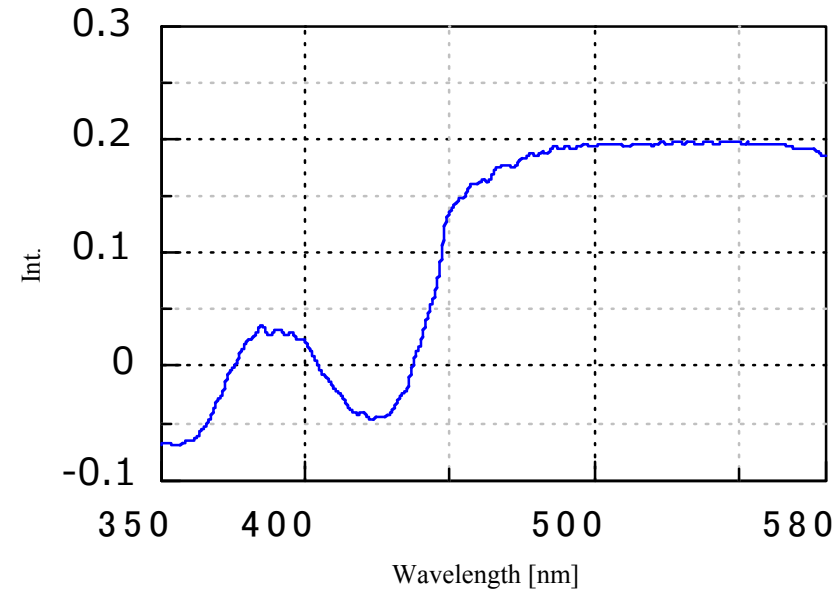


Fig. 2 Ex spectrum of fluorescence anisotropy

Fluorescence Measurement of Heat-Denatured Lysozyme

Experiments for the denaturation of proteins are generally measured by Circular Dichroism. However, proteins contain aromatic amino acids (AAA) and will fluoresce when excited with UV light. During heat denaturation of proteins, the secondary structure of the protein will change and the aromatic amino acid residues will change slightly which can be detected by fluorescence.

Experimental

Emission (E_m) spectra of lysozyme using an excitation (E_x) of 280 nm are measured while controlling the temperature, to examine the relation between temperature and the fluorescence spectrum. The model FP-8500 spectrofluorometer and an ETC-815 Peltier thermostatted cell holder are used for the measurements in this experiment, using the instrument parameters outlined below.

Parameters:

Measurement points: 16	Temperature range: 15 to 90 ºC	Temperature interval: 5 ºC
E_x bandwidth: 2.5 nm	E_m bandwidth: 5 nm	E_x wavelength: 280.0 nm
Response: 0.5 sec	Gain: Sensitivity	Wavelength range: 290 to 450 nm

A buffered aqueous solution of 0.1 mg/mL of lysozyme was used as the sample, the measurement performed by stirring the sample with a magnetic stirrer to ensure even sample temperatures in the cell. A temperature ramping rate of 1˚C/min was controlled by the Peltier cell holder. Spectra are measured within 60 seconds after reaching the individual set temperature points.

Results

The graph below (Figure 1) demonstrates the change of intensity vs. temperature at 340 nm. Lyzosyme is known to denature at a temperature of 70˚C; however the graph of fluorescence vs. temperature shows only a decrease in intensity as the temperature increases. However, the E_m Spectra from 40 to 90 degrees plotted using a contour view demonstrates a transition of the spectra at 70 degrees (Figure 2).

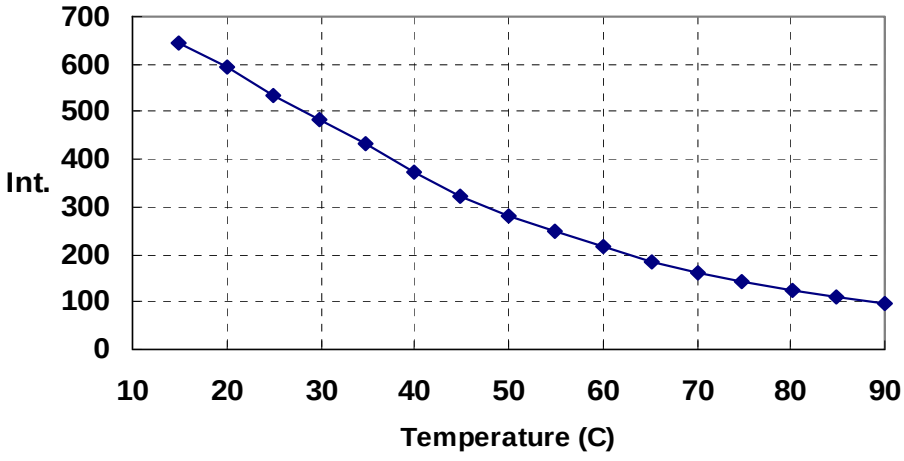


Figure 1: Temperature Dependence of Lysozyme aqueous solution at EX 280 nm, EM 340 nm

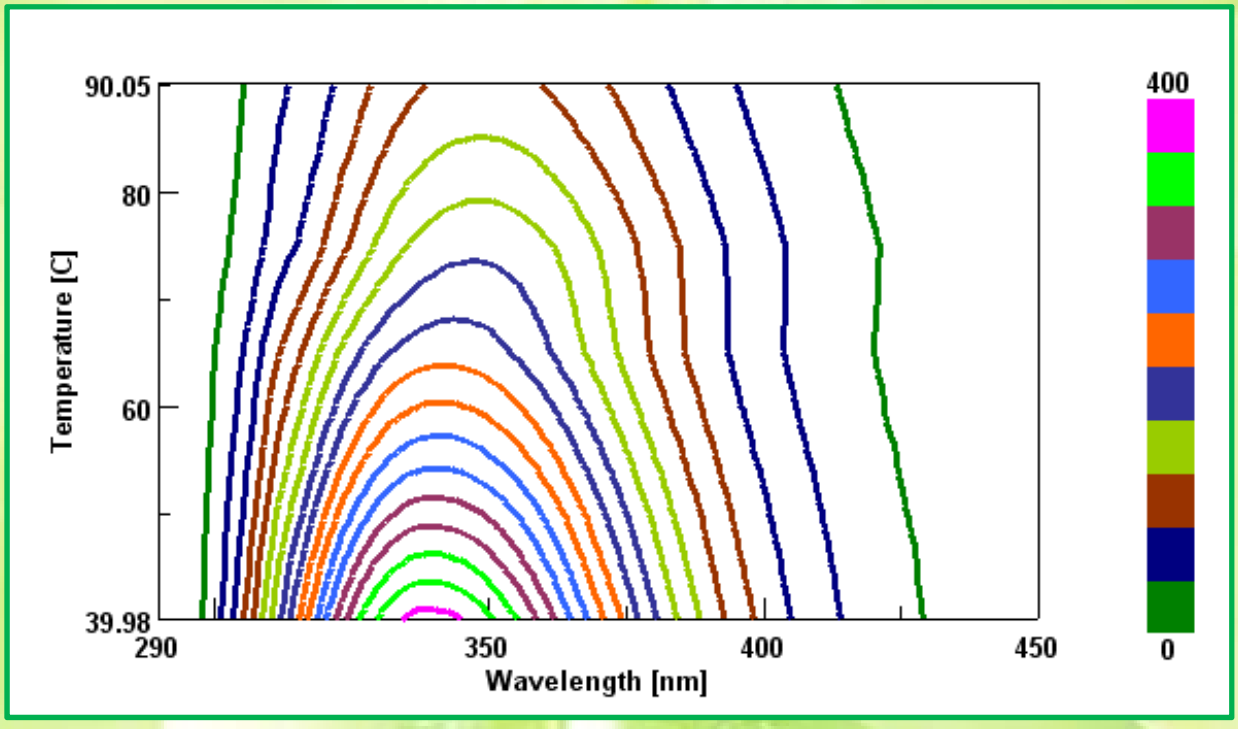


Figure 2: Contour view of EM spectra from 40 to 90 degrees.

The E_m spectra at room temperature have an E_m maximum at 340 nm with a corresponding maximum at 348 nm for 90 degrees. A plot of the intensity ratios for the two wavelengths versus temperature results in a graph that demonstrates a heat-denaturation at 70 ºC (Figure 3), in agreement with literature values.

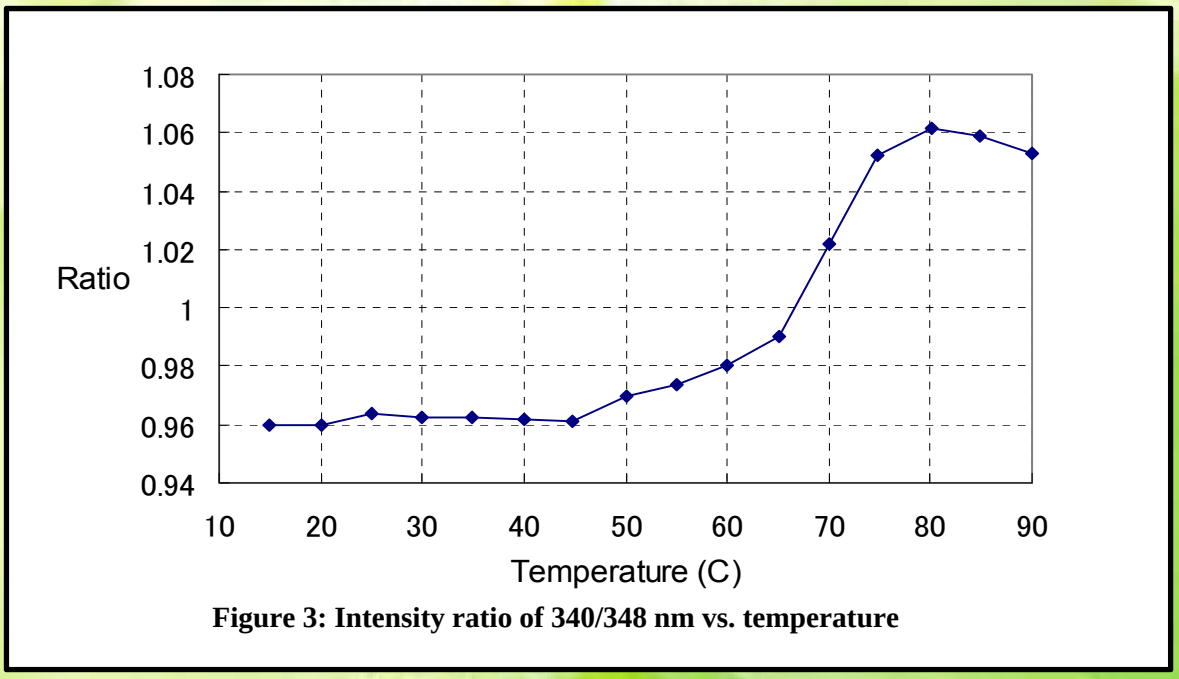


Figure 3: Intensity ratio of 340/348 nm vs. temperature

Diffuse Reflectance Measurement of Fluorescent Powder

Fluorescent powder has been evaluated by its fluorescence spectrum, excitation spectrum, external/internal quantum efficiency and luminescent color. In addition, diffuse reflectance spectrum is now required for obtaining the findings related to absorption spectrum. However, in the diffuse reflectance measurement by using of spectrophotometer with integrating sphere, since the scattered light and fluorescence from sample cannot be separated, only sum of scattered light and fluorescence light will be obtained as the result of reflectance measurement. Therefore, such artifact must be corrected to obtain the true reflectance.

In this application, by using sodium salicylate, a typical fluorescence powder as a sample, the procedure to eliminate the fluorescence component from such reflectance artifact will be demonstrated.
In order to reduce the reflectance artifact by fluorescence, firstly, the high cut filter is placed is mounted inside of main unit so that scattered light caused from irradiation light can be transmitted through the filter and, the fluorescence can be cut by the filter. Secondly, in the single beam mode, spectra of standard white plate and sample were measured the filter. Thirdly, the same single beam spectra were measured with using the filter.
The transmittance of the filter is shown as the ratio between two spectra of standard white plate measured with filter and without filter a) of Fig. 1). The single beam spectra of sodium salicylate measured with filter and without filter are shown in b) of Fig. 1. The filter can transmit the 50% of light at 420 nm, and can cut the light of wavelength shorter than 420 nm and so it is evident that the transmittance in the range shorter than 420 nm was significantly reduced when measured with filter. Also, the spectrum of sodium salicylate measured without filter indicates that most of the signal intensity shorter than 370 nm was from fluorescence.

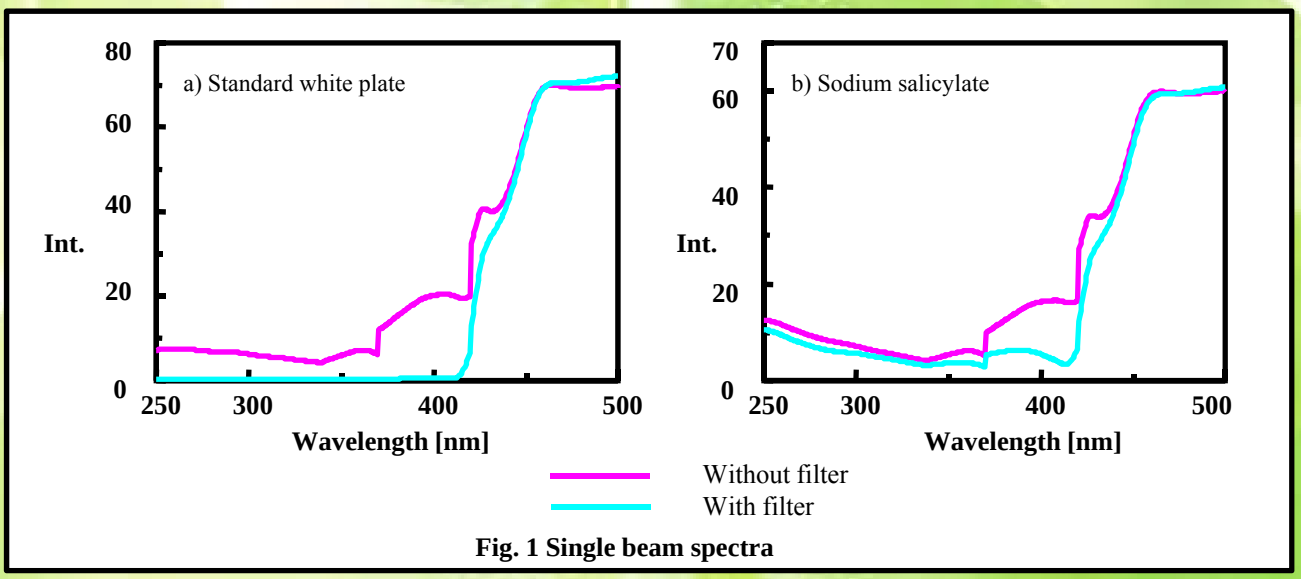


Fig. 1 Single beam spectra

The fluorescence spectra of sodium salicylate measured with or without the filter are shown in Fig. 2. The peak measured with filter (light blue in color) was observed as fluorescence and the peak without filter (pink in color), as total fluorescence and then the ratio of peak area was calculated, which is a factor to obtain the total fluorescence from fluorescence. The calculated ratio was 1.0556. By multiplying the factor 1.0556 with the single beam spectrum of sodium salicylate measured with filter (light blue in color; Fig.1, b), the total fluorescence was obtained. Then, the true diffuse reflectance component was obtained by the subtracting the total fluorescence from single beam spectrum of sodium salicylate measured without filter (pink in color; Fig. 1, b).

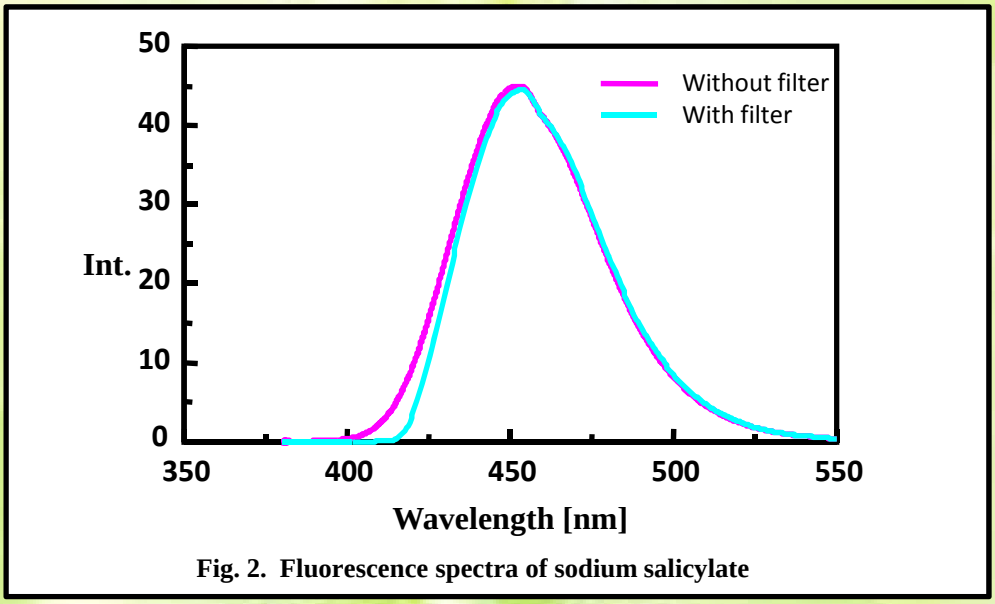


Fig. 2. Fluorescence spectra of sodium salicylate

The true diffuse reflectance component of sodium salicylate is shown in Fig. 3 together with the diffusive reflectance component of the standard white plate obtained in the same procedure. Since such component of standard white plate can be considered as 100% Line, the diffuse reflectance spectrum of sodium salicylate (Fig. 4) was obtained by the ratio of two spectra in Fig. 3.

NOTE:
Cut-off wavelength of the filter is 410 nm. Therefore, this correction procedure can be applied only to the wavelength range shorter than 410 nm.

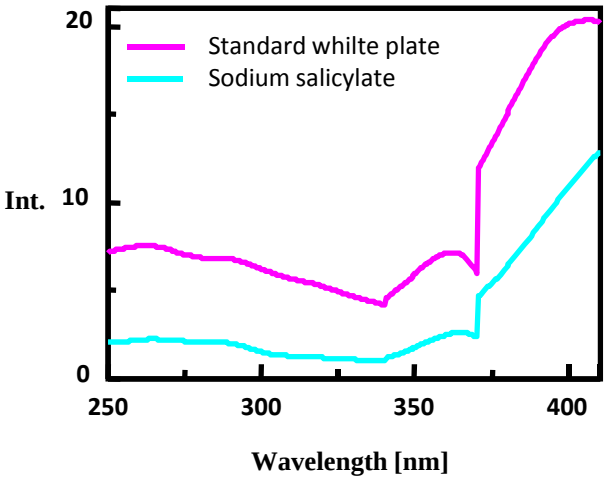


Fig. 3. Diffuse reflectance compnent.

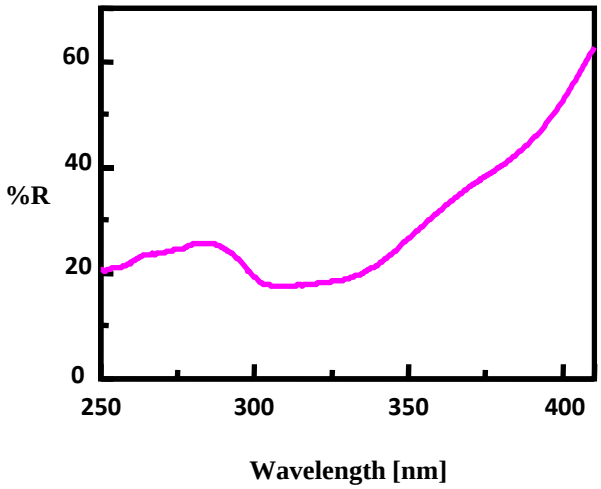


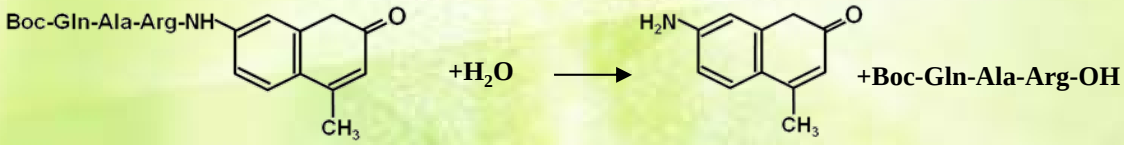
Fig. 4. Diffuse reflectance spectrum of sodium salicylate

Activity measurement of trypsin using a fluorescence peptide substrate

Introduction

Hydrolysis reaction is caused by making protease act on METHYLCOUMARIN-AMIDE (MCA) of peptide substrate, and 7-AMIDO-4-METHYLCOUMARIN (AMC) isolated.

This isolated AMC, fluorescence becomes the maximum by wavelength 440 nm. Protease activity can be measured using fluorescence spectrophotometer. We introduce the example which performed activity measurement of trypsin using a fluorescence peptide MCA substrate.



Boc-Gln-Ala-Arg-MCA　　Isolation AMC

Fig. 1 The hydrolysis reaction of MCA by protease

Keywords: Kinetics, Enzyme activity, Lineweaver-Burk, Michaelis-Menten

Measurement system

FP-8300	Spectrofluorometer
STR-812	Water thermostatted cell holder with stirrer
CSP-829	Sample compartment lid with syringe port
CTU-100	Mini Circulation Bath*1)
VWKN-772	Kinetics Analysis Program

*1) The temperature of a circulation bath is set as 37 degrees by all the measurement.

Samples

Intensity standardization sample: 50 µmol/L AMC solution

Enzyme solution: 10 nmol/L Trypsin bovine pancreas typeVIII, 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1.0 mmol/L $CaCl_2$, 0.1 mg/mL BSA

Substrate solution: Boc-Gln-Ala-Arg-MCA solution

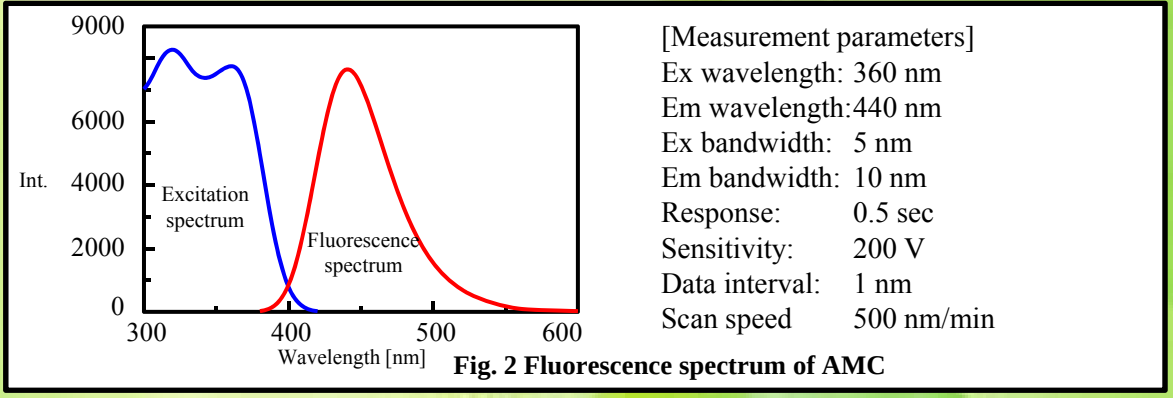
(The concentration after mixture is adjusted to 0.5, 1, 2.5, 5, 10, 20, 40 µmol/L)

Concentration for adjustment. [µmol/L]	240	120	60	30	15	6	3
The last concentration. [µmol/L]	40	20	10	5	2.5	1	0.5

Measurements

(1) Fluorescence-spectrum measurement of AMC

Excitation and the Fluorescence spectrum of 50 µmol/L AMC were measured (Fig. 2). It turns out that the fluorescence maximum wavelength from this result is 440 nm.



[Measurement parameters]
Ex wavelength: 360 nm
Em wavelength:440 nm
Ex bandwidth: 5 nm
Em bandwidth: 10 nm
Response: 0.5 sec
Sensitivity: 200 V
Data interval: 1 nm
Scan speed 500 nm/min

Fig. 2 Fluorescence spectrum of AMC

(2) The vertical axis is changed into concentration from fluorescence intensity

Intensity standardization was performed in order to change the vertical axis into the numerical value equivalent to concentration. 50 µmol/L AMC solution of 0.5 mL is dropped at enzyme solution 2.5 mL. Fluorescence intensity of last concentration of 8.333 µmol/L AMC solution was set to 8333 µmol/L AMC solution.

(3) Enzyme activity measurement

Substrate solution 0.5 mL of each concentration was dropped at enzyme solution 2.5 mL, and time course measurement of the fluorescence intensity of the isolation AMC was performed to it. A result is shown in Fig. 3.

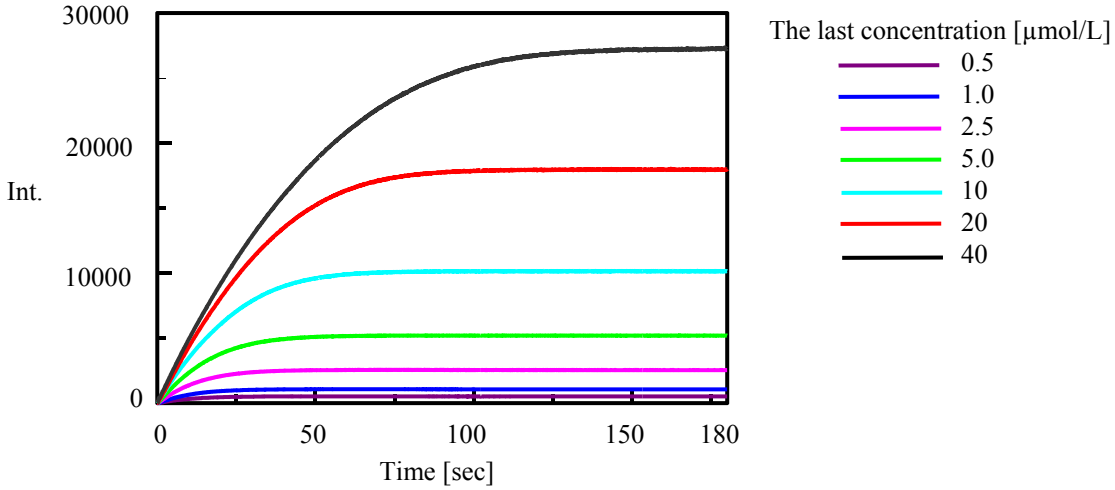


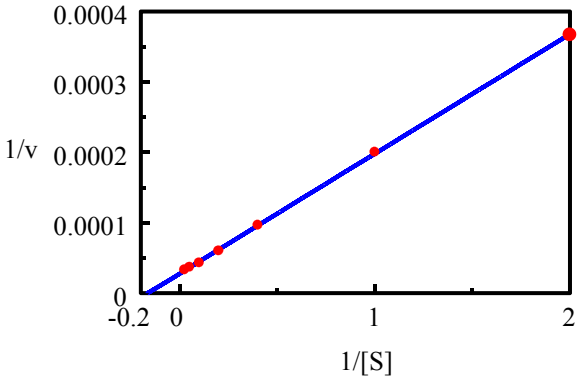
Fig. 3 The pursuit result of an enzyme reaction process

[Measurement parameters]

Ex wavelength:	360 nm	Em wavelength:	440 nm
Ex bandwidth:	5 nm	Ex bandwidth:	10 nm
Data interval:	0.1 sec	Response:	0.1 sec
Scan speed:	200 V		

Analysis

[Kinetics Analysis] In quest of each initial velocity, the Lineweaver-Burk plot was performed using the program from inclination of the time variation data of each substrate concentration (Fig. 4). Km=5.99 and V*max*=35270 nmol/L.min^{-1} were obtained from this result.



[Calculation result]

Calculation method:	Lineweaver-Burk Plot
V*max*:	35270
Km:	5.99

1/v = 0.000170 x 1/[S] + 0.0000284

Fig. 4 Lineweaver-Burk plot

Acid Unfolding of Horse Cytochrome C Measured with a Fluorescence Stopped-Flow System

The fluorescence characteristics of the tryptophan residue in proteins will vary depending on the structures surrounding the residue. This characteristic of cytochrome C is derived from the tryptophan in the residue position 59. The natural state of this tryptophan residue is so close to the Heme iron residue that the fluorescence is quenched by nonradiative energy transfer to the Heme iron. When Cytochrome C is denatured by an acid, the distance between the tryptophan and Heme iron changes and the fluorescence intensity enlarges. This application note introduces the measurement example of the change in fluorescence intensity by the acid denaturation of Cytochrome C as measured by the JASCO stopped-flow measurement system.

Measurement/Analysis Systems

FP-8500 Spectrofluorometer
SFS-852 Stopped-Flow system (Cell length: 10 mm)
[Stopped-Flow Measurement] program
[Reaction Rate Calculation] program

Stopped-Flow System

Cell Holder and Cell

Syringe configuration

S1 : 10 mL, 0.5 mg/mL Cytochrome C
S2 : 10 mL, 0.1N sulfuric acid

Measurement Parameters

Ex bandwidth	5 nm
Em bandwidth	5 nm
Response	2 sec
Sensitivity	Manual
Measurement range	0 - 5000 msec
Measurement interval	5 msec
Ex wavelength	280 nm
Em wavelength	340 nm

No. of accumulations	4
Flow time	35 msec
Mixing ratio	S1:S2 = 1:1
Flow volume	S1: 100µL S2: 100µL
Start data acquisition	35ms before flow time ends

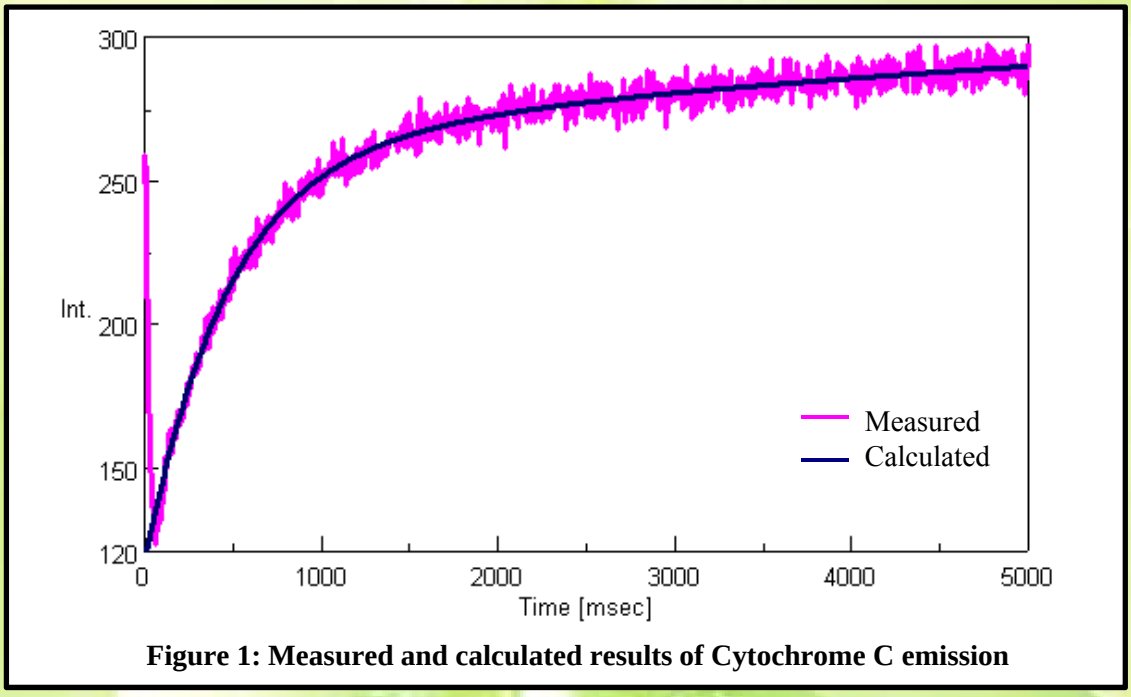


Figure 1: Measured and calculated results of Cytochrome C emission

Figure 1 illustrates the measured and calculated results of the Cytochrome C emission during the stopped-flow experiment.

The measured data shows an extreme change in the fluorescence intensity corresponding to the acid denaturation of Cytochrome C. The JASCO stopped-flow system enables data acquisition before the syringe movement is completed to ensure that the early stage of the reaction data before and after the flow time ends can be acquired.

The reaction rate was calculated with the [Reaction Rate Calculation] program. The calculated range was 35 to 5000 msec and a 2-step reaction mechanism was applied for the calculation. The calculated results show an excellent fit to the experimental data.

Calculation range:	35 to 5000 msec
Reaction rate formula:	Y(t) = -142.667 * exp(-t / 432.854) + -47.7112 * exp(-t / 3611.11)
Step 1 time constant:	432.854 msec
Step 1 rate constant:	0.00231025 $msec^{-1}$
Step 2 time constant:	3611.11 msec
Step 2 rate constant:	0.000276923 $msec^{-1}$

Quantitative analysis of λDNA using SAF-850 One drop accessory

Introduction

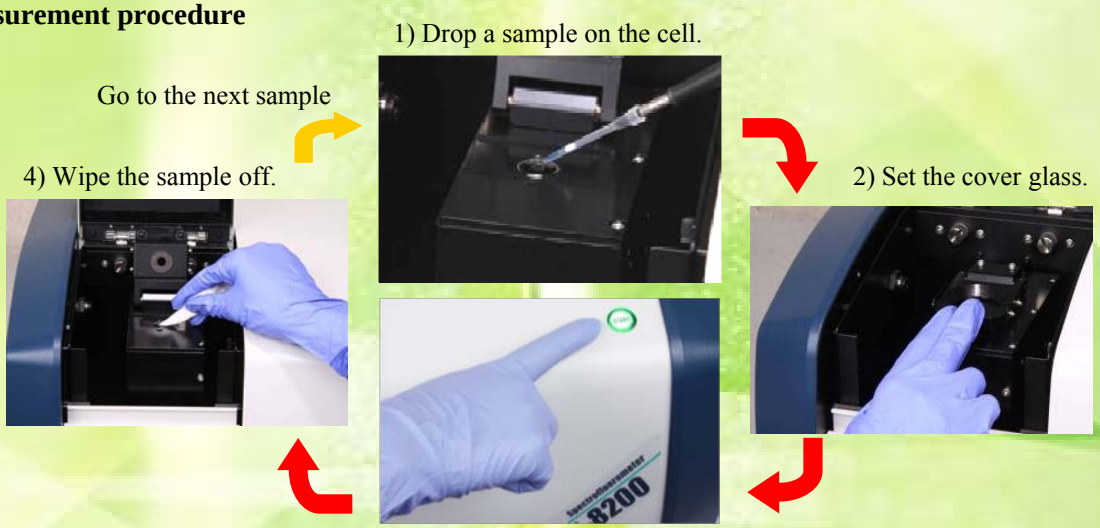
SAF-850 One Drop accessory is a special accessory, enabling fluorescence measurement by just dropping 5 µL of the sample on a cell and then covering it with another glass. This simple way of measurement is the most suitable and effective for quantitative analysis of multiple samples such as fluorescently-labeled DNA and various kinds of fluorescent dye. In this application data, the measurement result of λDNA labeled with PicoGreen is shown.

Keywords: One Drop, nucleic acid, reproducibility, linearity

Measurement system

FP-8200	Spectrofluorometer
SAF-850	One Drop accessory

Measurement procedure



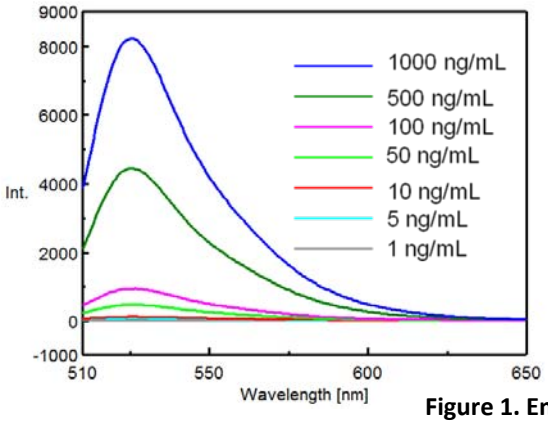
Samples

· λDNA labeled with PicoGreen®: 0, 1, 5, 10, 50, 100, 500, 1000 ng/mL

Results

1) Spectra

The emission spectra of sample with each concentration with Ex. Wavelength of 480 nm are shown in Figure 1. The result indicates that the peak wavelength is 523 nm.



[Measurement conditions]

Measurement mode:	Emission
Ex wavelength:	485 nm
Measurement range:	510-650 nm
Ex bandwidth:	10 nm
Em bandwidth:	10 nm
Scanning speed:	100 nm/min
Data interval:	0.5 nm
Response:	2 sec
Sensitivity:	700V

Figure 1. Emission spectra of λDNA labeled with PicoGreen®

2) Measurement reproducibility

Table 1 shows the result of 5 times measurements of the sample with each concentration.

Table 1. Measurement reproducibility

Conc. [ng/mL]	1	2	3	4	5	Ave	SD	CV(%)
0	53.3	52.4	55.9	53.1	55.2	54.0	1.49	2.8
1	63.6	68.1	65.9	66.5	65.1	65.8	1.68	2.6
5	110.3	106.7	105.1	104.0	110.0	107.2	2.86	2.7
10	157.6	155.5	156.1	153.0	151.7	154.8	2.39	1.5
50	447.1	465.3	460.2	455.8	469.0	459.5	8.56	1.9
100	865.9	856.9	848.3	850.6	853.9	855.1	6.86	0.8
500	3842.7	3831.0	3858.0	3828.9	3811.0	3834.3	17.42	0.5
1000	7766.2	7992.1	7925.3	7972.8	7805.7	7892.4	101.15	1.3

[Measurement conditions]

Measurement mode:	Emission		
Ex wavelength:	480 nm	Em wavelength:	523 nm
Ex bandwidth:	20 nm	Em bandwidth:	20 nm
Response:	1 sec	Sensitivity:	620 V

3) Linearity

The calibration curve was generated using the average value of the results in Table 1. Figure 2 shows the good linearity over a wide concentration range from 1ng/mL to 1000 ng/mL.

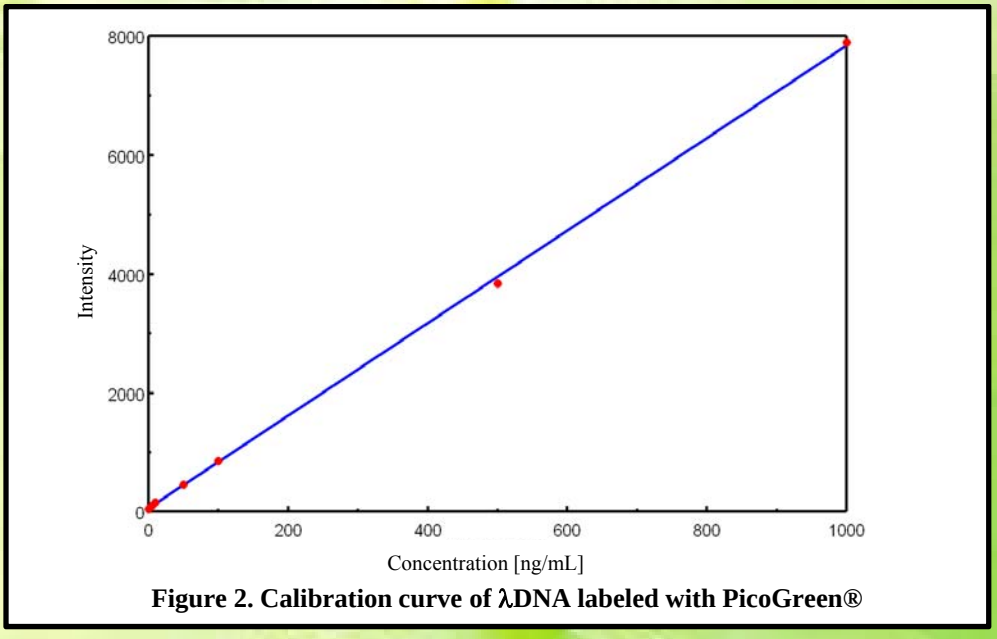


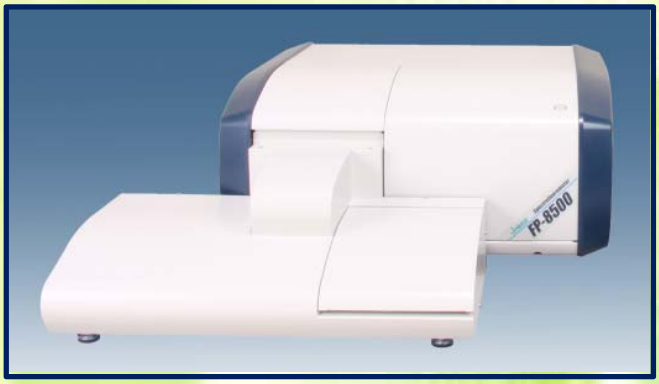
Figure 2. Calibration curve of λDNA labeled with PicoGreen®

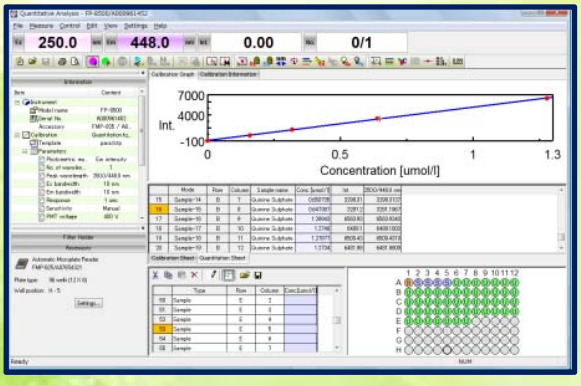
Calibration curve equation : Int.= 7.780 x Conc.+ 57.5211
Correlation coefficient : 0.9999
Standard error : 6.819

Fluorescence Measurements for Quinine Sulfate using Microplate Reader

Introduction

The FP-8000 series spectrofluorometer with the FMP-825 microplate reader allows multiple liquid samples to be automatically measured by moving the wells. Using this system, quantitative analysis, in addition to spectrum and fixed-wavelength measurements can be carried out.

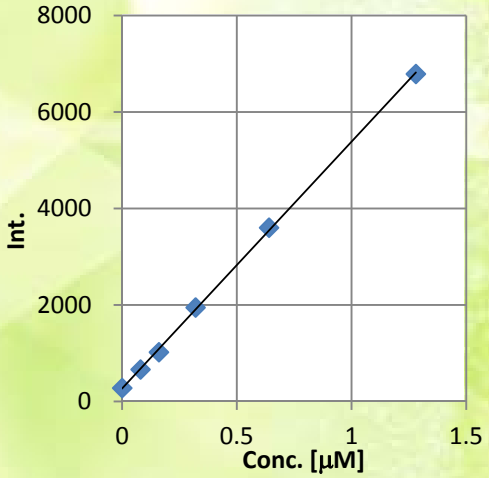




FP-8500 Spectrofluorometer
FMP-825 Microplate reader

[Quantitative Measurement] program

A calibration curve for quinine sulfate was created using a 96-well microplate reader. Based on the reproducibility for blank samples, the quantitation and detection limits were calculated.



Calibration curve for quinine sulfate

Calibration: Int=5117 x Conc.+270
Corr. Coeff.: 0.9998
Quantitation limit: 5 x 10^{-8}mol/

Measurement parameters

Sample volume: 300 mL
Ex wavelength: 250 nm
Em wavelength: 448 nm
Ex bandwidth: 10 nm
Em bandwidth : 10 nm
Speed: 30 mm/sec
Response: 50 msec
Gain: 400 V

System	Model	Product name	Code	Remarks
Instrument	FP-8500ST	Spectrofluorometer	6960-J001A	Model FP-8500DS is shipped with a PC.
Optional accessory	FMP-825	Microplate reader	6961-J025A	
Optional accessory		96-well Microplate	6961-H425A	50 plates/box, for fluorescence measurements, black, SBS standard
Optional accessory		384-well Microplate	0410-0127	

Absolute quantum yield measurement using FP-8000 series

Introduction

Fluorescence quantum yield is defined as the ratio of the number of photons emitted from sample as fluorescence to the number of photons in the excited light absorbed. Absolute method and relative method are known as measuring methods. Relative method is comparing the intensity of standard fluorescence with unknown sample to calculate quantum yield of the unknown sample. Therefore obtained results depend on the accuracy of standard sample's quantum yield value. On the other hand, quantum yield can be obtained directly by the absolute method, because the absolute method allows to detect all the fluorescence from the sample and integrates using integrating sphere, enabling more accurate quantum yield measurement.

In this experiment, some examples will be shown for the calculation of solution sample's quantum yield of which published values from literature are known by the absolute method.

Keyword: Quantum yield, Absolute method, Solution

<Measuring system>

FP-8500 Spectrofluorometer*1)
ILF-835 100mmφ Integrating sphere unit
1 mm pathlength solution cell
FWQE-880 Quantum yield calculation program

*1) Emission spectrum to which spectral correction is performed is required for quantum yield calculation. The spectra correction was performed using Rhodamine B on EX side ,and was also performed on EM side using standard white plate for synchronous spectrum (250-450 nm) and ESC-842 (450-700 nm).

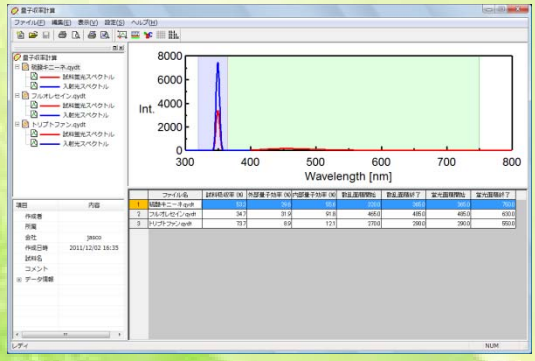


Fig.1 Quantum yield calculation program screen

<Samples>

-200 ppm Quinine sulfate (Solution: 1.0 N H_2SO_4)
-15 ppm Fluorescein (Solvent: 0.1 N $NaOH_{aq}$)
-200 mg/mL tryptophan (Solvent:Ultra pure water)

<Measuring method for absolute quantum yield>

1) Measuring incident light
   Confirm nothing is set on the sample cell holder in the integrating sphere, and measure spectrum of the incident light. Obtained peak area is defined as area from incident light, S_0 (equivalent number of photons in the incident light).
2) Measuring sample
   Set the sample on the sample holder, and measure scattering and emission spectra of the sample. Obtained excitation wavelength peak area is defined as area scattered from sample, S_1(equivalent number of photons which were not absorbed), and peak area in the emission wavelength range is defined as area emitted from sample, S_2.

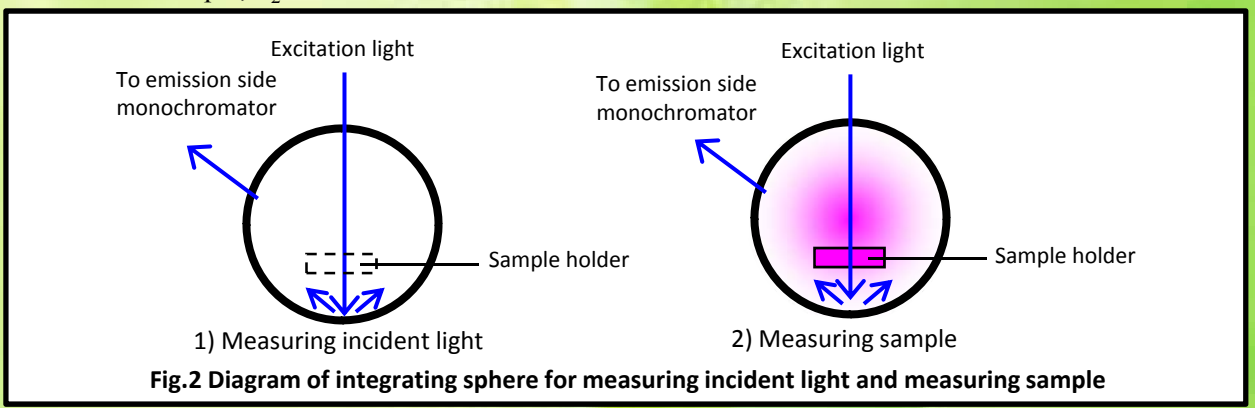


Fig.2 Diagram of integrating sphere for measuring incident light and measuring sample

3) Calculating quantum yield

Calculate in accordance with the following.

Sample absorption[%]=$(S_0-S_1)/S_0$ x 100

External quantum yield[%]=S_2/S_0 x 100

Internal quantum yield[%]=$S_2/(S_0-S_1)$ x 100

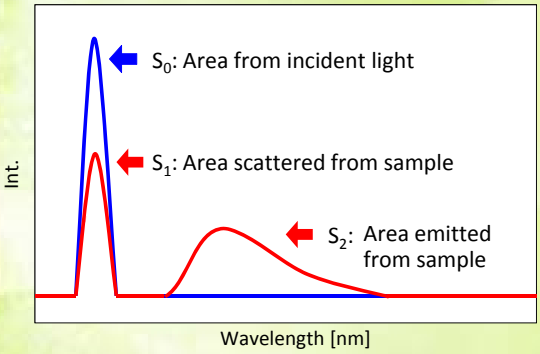
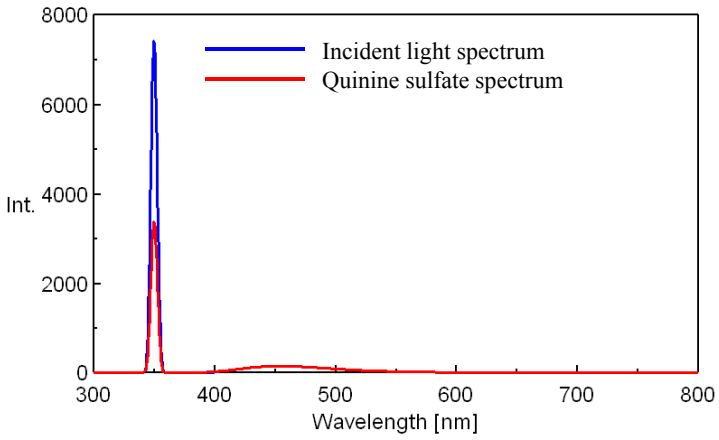


Fig. 3 Image of S_0, S_1, S_2

<Measurement results>

Sample spectra measurement results are shown in the Fig 4 ~ 6.

1. Quinine sulfate

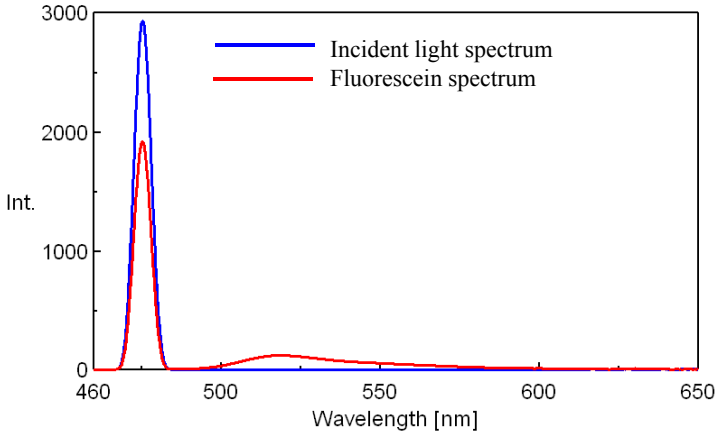


[Measurement condition]

Mode:	Emission
Ex bandwidth:	5 nm
Em bandwidth:	5 nm
Ex wavelength:	350.0 nm
Measurement range:	300 - 800 nm
Scan speed:	200 nm/min
Data interval:	0.1 nm
Response:	0.5 sec
PMT voltage:	350 V

Fig. 4 Emission spectrum of quinine sulfate

2. Fluorescein



[Measurement condition]

Mode:	Emission
Ex bandwidth:	5 nm
Em bandwidth:	5 nm
Ex wavelength:	475.0 nm
Measurement range:	460 - 650 nm
Scan speed:	200 nm/min
Data interval:	0.1 nm
Response:	0.5 sec
PMT voltage:	250 V

Fig. 5 Emission spectrum of fluorescein

3.Tryptophan

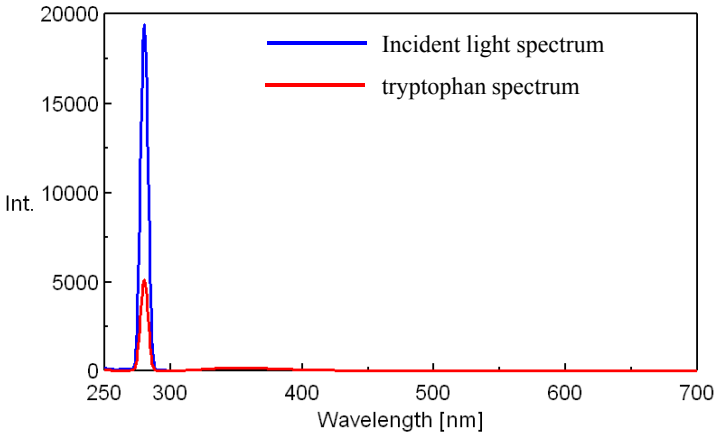


Fig.6 Emission spectrum of tryptophan

[Measurement condition]

Mode:	Emission
Ex bandwidth:	5 nm
Em bandwidth:	5 nm
Ex wavelength:	280.0 nm
Measurement range:	250 - 700 nm
Scan speed:	200 nm/min
Data interval:	0.1 nm
Response:	0.5 sec
PMT voltage:	400 V

<Analysis results>

Table 1 shows area from incident light (S_0), area scattered from sample (S_1), area emitted from sample (S_2) calculated by each of sample's spectra and wavelength range.

Table 1 Detail of quantum yield calculation

Sample name	Area from incident light [S_0]	Area scattered from sample [S_1]	Area emitted from sample [S_2]	Scattered WL range [nm]	Emitted WL range [nm]
Qunine sulfate	48267	22538	14304	320 - 365	365 - 750
Fluorescein	19174	12515	6116	465 - 485	485 - 630
Tryptophan	136135	35842	12101	270 - 290	290 - 550

Calculation results of quantum yield using the values on the Table 1 and equations on the 3) are shown in the Table 2. Obtained results are within the range of published values from literatures for any samples.

Table 2 Calculation results of quantum yield

Sample name	Sample absorbance	External quantum yield	Internal quantum yield	Internal quantum yield [published values]
Qunine sulfate	53.3%	29.6%	55.6%	50-57%*2)
Fluorescein	34.7%	31.9%	91.8%	85-92%*2)
Tryptophan	73.7%	8.9%	12.1%	12-14%*3)

*2) Literature: The Spectroscopical Society of Japan, (Japan Scientific Societies Press)
*3) Literature: Principles of fluorescence spectroscopy, Joseph R. Lakowicz, Springer

Relative quantum yield measurement using FP-8000 series

Introduction

Fluorescence quantum yield is defined as the ratio of the number of photons emitted from sample as fluorescence to the number of photons in the excited light absorbed. Absolute method and relative method are known measuring methods. Regarding the absolute method, it is necessary to detect all the fluorescence from the sample, requiring the integrating sphere, while relative method can calculate quantum yield of unknown sample by comparing the intensity of standard fluorescence with unknown sample, and accordingly the relative method is easier to get the results of quantum yield. In this experiment, an example will be shown for the calculation of quantum yield of Rhodamine B when fluorescein is used as the standard sample.

<Calculation method for relative quantum yield>

In order to calculate relative quantum yield, 1) Quantum yield of standard sample is required and in addition 2) Absorbance at excitation wavelength and 3) Area of emission spectrum with spectral correction are also required for the standard sample and the unknown sample respectively. Moreover, when the solvent of the standard sample is different from that of unknown sample, 4) Average refractive index value in the wavelength range to calculate the area of emission spectrum is required. When the standard or unknown samples for emission spectrum measurement are diluted, 5) Dilution ratio is required.

Table 1 Required parameters to calculate relative quantum yield

	Item	Samples	Expression
1)	Quantum yield	Standard	ϕ_{st}
2)	Absorbance at Ex Wavelength	Standard Unknown	A_{st} A_x
3)	Area of emission spectrum	Standard Unknown	F_{st} F_x
4)	Average refractive index value of solvent	Standard Unknown	n_{st} n_x
5)	Dilution ratio	Standard Unknown	D_{st} D_x

By using the parameters in the Table 1, relative quantum yield of unknown sample, f_x is shown by the following equation (1).

$$\Phi_x = \Phi_{st} \bullet \left(\frac{A_{st}}{A_x}\right) \bullet \left(\frac{F_x}{F_{st}}\right) \bullet \left(\frac{n_x^{\,2}}{n_{st}^{\,2}}\right) \bullet \left(\frac{D_x}{D_{st}}\right) \qquad (1)$$

In addition to [Absorbance measurement] program to measure 2)Absorbance at excitation wavelength and [Spectra measurement] program to get 3)Emission spectrum area, [Relative quantum yield calculation] program to calculate relative quantum yield of unknown sample based on equation (1) is installed as standard to the FP-8000 series. So everything from measuring to analyzing relative quantum yield can be performed only by using standard programs.

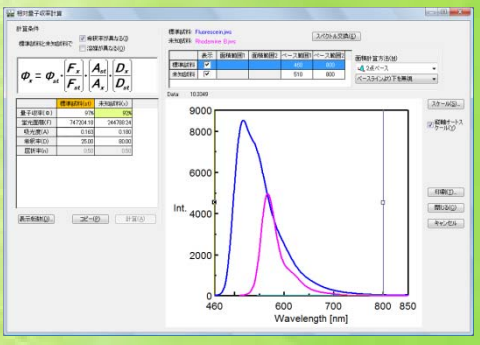


Fig. 1 [Relative quantum yield calculation] program screen

Keyword: Quantum yield, Relative method, and Solution

<Measuring System>

FP-8500 Spectrofluorometer*1)

FUV-803 Absorbance measurement cell block

*1) Emission spectrum with spectral correction is required for relative quantum yield calculation. The spectral correction was performed using Rhodamine B on Ex side, and ESC-842Calibrated WI light source on Em side for this measurement.

<Samples>

	Name	Concentration	Solvent
Standard	Fluorescein	For Absorbance:5400 µg/L For Emission:72 µg/L	Ethanol
Unknown	Rhodamine B	For Absorbance:7200 µg/L For Emission:36 µg/L	Ethanol

<Calculation procedure of parameters required to calculate relative quantum yield >

1) Quantum yield of standard sample

Published value of 0.97 from literature*2) was used for quantum yield of fluorescein.

*2) Literature: Kazuhiko Kinoshita and Koshin Mihashi, *Fluorescence measurements - Applications for Biochemical Sciences.* (The Spectroscopic Society of Japan, Measurement Method Series 3) Japan Scientific Societies Press, 1983.

2) Absorbance at Ex wavelength

Absorption spectra was measured using [Absorbance measurement] program and FUV-803 Absorbance measurement cell block. Results are shown in Fig. 2. From the results, it was confirmed that absorbance of the fluorescein at Ex wavelength,450 nm was 0.490 and the absorbance of the Rhodamine B at Ex wavelength 500 nm was 0.225.

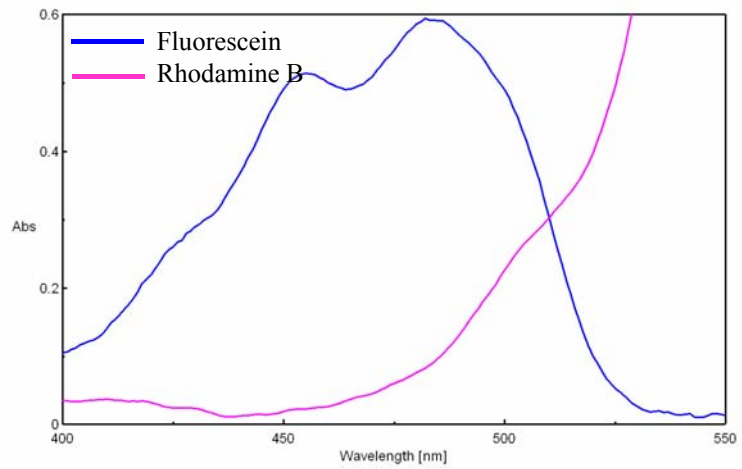


Fig. 2 Absorption spectra

Table 2 Absorbance at Ex wavelength

Standatrd (Fluorescein)	Unknown (Rhodamine B)
0.490	0.225

[Measurement conditions]

Ex bandwidth	2.5 nm	Em bandwidth	10 nm
Scan speed	200 nm/min	Data interval	1 nm
Response	0.5 sec	PMT voltage	230 V
Filter	Used		

Attenuator B was used.*3)

*3) The attenuator B is available as a standard of spectrofluorometer.

3) Area of emission spectrum

Emission spectra were measured using [Spectra measurement] program. In order to prevent from reabsorption of fluorescence, sample solution was diluted so that the absorbance of the sample solution becomes less than 0.02 for this measurement.(Details of dilution ratio will be described in the item 5).) Obtained emission spectra were shown in Fig. 3.

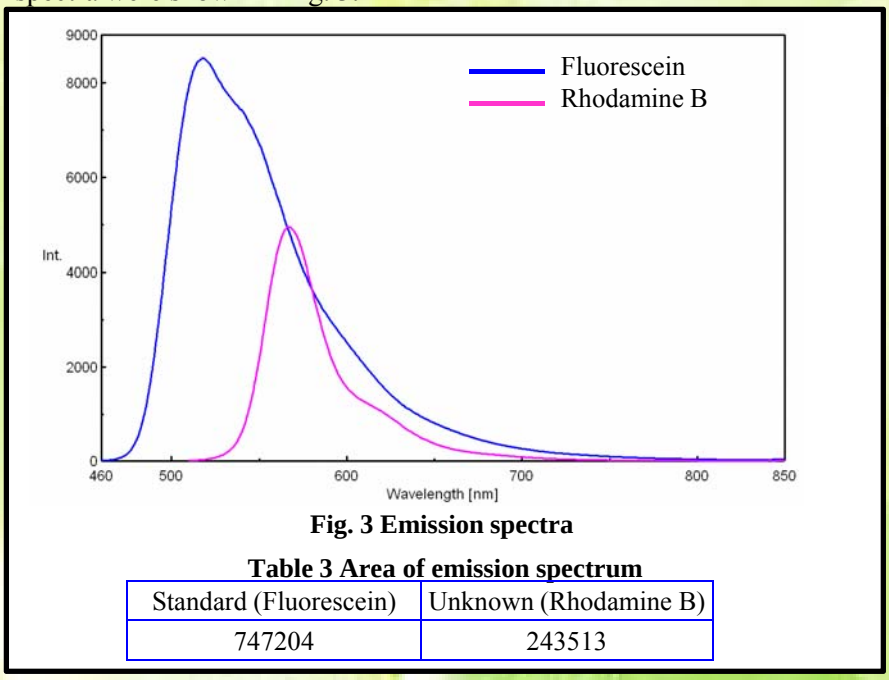


Fig. 3 Emission spectra

Table 3 Area of emission spectrum

Standard (Fluorescein)	Unknown (Rhodamine B)
747204	243513

[Measurement condition]

Ex bandwidth	5 nm	Em bandwidth	5 nm
Scan speed	200 nm/min	Data interval	0.5 nm
Response	0.5 sec	PMT voltage	430 V
Filter	Used	Spectral correction	ON

4) Average refractive index of solvent

As ethanol is used on both standard and unknown samples for this measurement, average refractive index of the solvent is not required. When the solvents used are different from each other, published value is used.

5)Dilution ratio

Comparing with absorbance measurement in the item 2), standard sample solution was diluted by 75 times and unknown sample, by 100 times in the item 3) emission spectra measurement.

Table 4 Dilution ratio

Standard (Fluorescein)	Unknown (Rhodamine B)
75	100

<Analysis results>

Quantum yield of Rhodamine B was calculated by applying parameters obtained by “calculation methods 1)~5) to get parameters which are needed to calculate relative quantum yield” to the equation (1). As a result, quantum yield of 92 % which is within the range of published value, [*3)] 69~97% was obtained.

Table 5 Quantum yield of rohdamine B

Published value	Measured value
69~97%	92%

Phosphorescence Spectrum Measurement for Quantum Efficiency

Introduction

Phosphorescence substances have attracted attention as luminescent material for organic EL device. Quantum efficiency of phosphorescence substances is required for developing such materials. Although the conventional integrating sphere measures sample spectrum at room temperature, phosphorescence is observed by cooling the sample to the temperature of liquefied nitrogen at 77K. JASCO developed a new dedicated system for calculating quantum efficiency from the measured phosphorescence spectra at 77K.

Phosphorescence Quantum Efficiency Measurement System

FP-8500	Spectrofluorometer
ILFC-847	100-mm Cooling Integrating Sphere
FWQE-880	Quantum yield calculation program
ESC-842	Calibrated light source (WI)
LPH-140	Phosphorescence measurement cell kit for liquid sample
Sample:	solid (6 to 7mm sq × 1.0 to 1.5mm tick)
	powder
	liquid

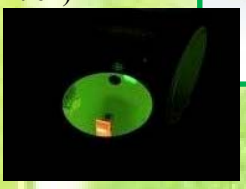


Figure 1: Phosphorescence Quantum Efficiency Measurement System

3. Measurement Procedure to Calculate Quantum Efficiency

Quantum efficiency is obtained by the ratio between "photon number absorbed by sample" and "photon number emitted by sample". To measure phosphorescence spectra with an integrating sphere for the calculation, first, place a Dewar vessel with coolant such as liquefied nitrogen in the sphere and measure a spectrum of incident light as illustrated in Fig. 2-a. The peak area appears in the Ex wavelength range of the spectrum, illustrated with blue in Fig. 3, indicates incident photon number S_0. Then, place a sample inside the vessel and measure a spectrum including scattering light of incident light and sample emission as illustrated in Fig. 2-b. The peak area appears in the Ex or Em wavelength range of the spectrum, illustrated with red in Fig. 3, indicates photon number unabsorbed by sample S_1 or emitted by sample S_2, respectively. The quantum efficiency is calculated from "photon number absorbed by sample = $S_0 - S_1$ and "photon number emitted by sample S_2":

$$\textbf{Quantum Efficiency} = \frac{\textbf{Photon number emitted by sample}}{\textbf{Photon number absorbed by sample}} = \frac{S_2}{S_0 - S_1}$$

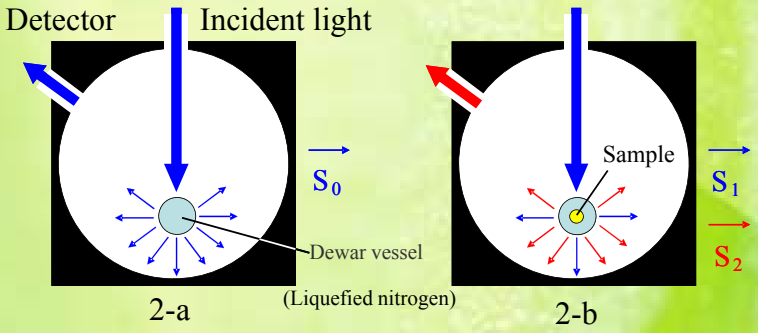


Figure 2 Sample position in the integrating sphere

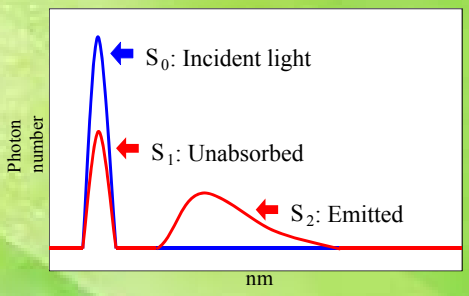


Figure 3 Model spectra

Fluorescence Quantum Efficiency of Quinine Sulfate

To confirm whether placing the Dewar vessel and coolant[*1)] in the sphere has any effect on the measurement results, quinine sulfate with well-known fluorescence quantum efficiency was measured by using this system. Fig. 4 illustrates the measured spectra and Table 1 shows the calculation results of quantum efficiency (f). The calculated fluorescence quantum efficiency was 0.56 that corresponds well with the literature-based value of 0.546[*2)]. From this calculation results, the effect of placing vessel inside the sphere can not be confirmed.

<Measurement Parameters>

Ex bandwidth	5 nm	Em bandwidth	5 nm	Response	0.5 sec
Ex wavelength	350.0 nm	Data interval	1 nm	Scan speed	1000 nm/min

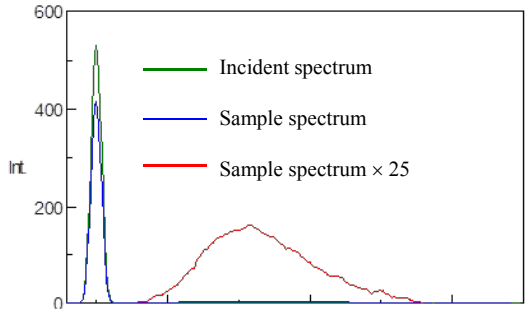


Figure 4 Spectra of Quinine Sulfate

	Int.
S_0	5377.3
S_1	4092
S_2	715.4
Φ [Measured]	0.56
Φ [Literature][*2)]	0.546

Table 1 Fluorescence quantum efficiency of quinine sulfate

[*1)] In this measurement, the Dewar vessel was filled with water instead of liquid nitrogen

[*2)] Melhuish, W.H., J.Phys.Chem. **65**, 229, 1961

Phosphorescence Quantum Efficiency of Benzophenone

Benzophenone was was measured as a representative phosphorescence substance. The sample was cooled by liquefied nitrogen. Fig. 5 illustrates the measured spectra and Table 2 shows the calculation results of quantum efficiency (f). The calculated phosphorescence quantum efficiency was 0.93 that corresponds well with the literature-based value of 0.9[*3)].

<Measurement Parameters>

Ex bandwidth	5 nm	Em bandwidth	5 nm	Response	0.5 sec
Ex wavelength	335.0 nm	Data interval	1 nm	Scan speed	1000 nm/min

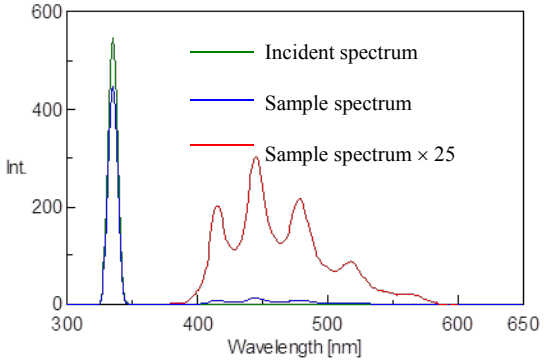


Figure 5 Spectra of Benzophenone

	Int.
S_0	4954.3
S_1	4074.0
S_2	819.3
Φ [Measured]	0.93
Φ [Literature][*3)]	0.9

Table 2: Phosphorescence quantum efficiency of Benzophenone

[*3)] The chemical society of Japan, Courses in Experimental Chemistry 3 basic physical chemistry, Maruzen ISBN: 4-621-07303-6

Upgrade of simple coumarin analysis system to high sensitivity one

Introduction

In order to prevent from producing illegal light diesel oil which contains kerosene or heavy oil, 1 ppm of coumarin is added in the related oils of the diesel (kerosene or A heavy oil) as discrimination label. The analysis procedures to determine mixing with the discrimination label and its mixing concentration are standardized by Advisory body in National Petroleum Dealers Association. Simple analysis using test tube and quantitative analysis using separating funnel are described in the instruction manual of the procedure. We have already introduced simple quantitative analysis system incorporating easy-to use simple analysis and accuracy of quantitative analysis with the preparation performed using test tube, detecting fluorescence intensity and judging concentration using spectrofluorometer.

Usual coumarin determination purpose is to analyze quantitatively more than a couple of percent of the related oils, while there is another analysis case which needs to analyze quantitatively to less than 1 percent. We would like to introduce a system to upgrade the above simple analysis system to high sensitivity system with improved detection limit and quantitation limit drastically.

Measurement principle

Coumarin is hydrolyzed in alkaline solution and becomes Cis-O-hydroxycinnamic acid. In addition, the Cis-O-hydroxycinnamic acid is isomerized by ultraviolet radiation and becomes Trans-O- hydroxycinnamic acid.The Trans-o-hydroxycinnamic acid radiates green fluorescence(Ex 360 nm, Em 500 nm). In this quantitative analysis procedure, this green fluorescence is detected.

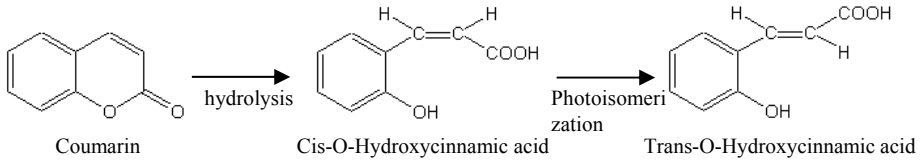


Fig. 1 Hydrolysis and photoisomerization of coumarin

Measurement system

CTS-855
FCQM-822

Tools to be used

- Round-bottom screw cutting test tube (18 mm outer diameter x 160 mm length)
- Stirrer bar (3 mm diameter x 10 mm length)
- Shaker

Preparation of reagents

1) Alkaline solution reagents
Dissolve 10 g of sodium hydroxide and 20 g of sodium nitrate into Millipore water, and prepare 100 mL solution. The alkaline solution is kept in polyethylene vessel.
2) Alcohol solution
Mix 40 mL of 1-butanol and 30 mL of ethanol in this proportions.

3) Undiluted coumarin solution [1000 ppm]
Dissolve 100 mg of the coumarin into aromatic solvent (such as n-propyl benzene).

4) Standard coumarin solution [0.1 ppm]
Dilute 100 µL of the undiluted coumarin solution using n-dodecane (1 ppm).
Take 100 µL of the 1 ppm coumarin solution, and dilute it using the n-dodecane and prepare 100 mL solution.

5)Standard sample
Mix each of solutions in accordance with the following ratio.

Table 1 Mixing ratio of standard solution

Conc. of additive [%]	Standard coumarin solution [0.1 ppm] (mL)	n-Dodecane(mL)	Alkaline solution (mL)	Alcohol solution (mL)
0%	0	4.2	3	4.8
1%	0.06	4.14	3	4.8
2%	0.12	4.08	3	4.8
4%	0.24	3.96	3	4.8
6%	0.36	3.84	3	4.8
8%	0.48	3.72	3	4.8
10%	0.96	3.24	3	4.8

Measurement procedure

Prepare test tubes containing standard samples which were prepared in the [4. Preparation of reagents 5) Standard sample]. Shake these test tubes to hydrolyzes coumarin in the test samples, and the coumarin is extracted in the alkaline solution. Then, perform photoisomerization reaction by radiating excitation light (360 nm) of spectrofluorometer on the alkaline solution, and detect fluorescence intensity at 500 nm and generate calibration curve.

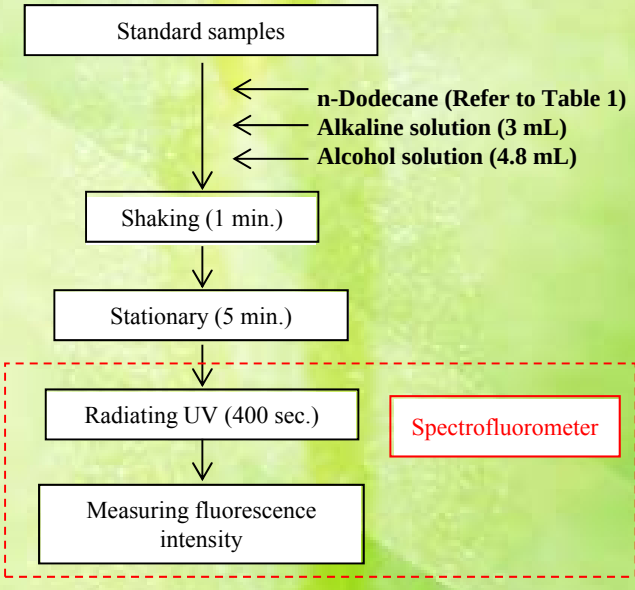


Fig. 2 Flow chart of analysis procedure

1) Put each of standard samples into test tubes *1) .

2) Add n-dodecane (refer to Table 1), 3 mL of alkaline solution and 4.8 mL of alcohol solution.

3) Put stoppers on the test tubes, and shake 1 minute using shaker to hydrolyze coumarin and extract to alkaline solution.

4) Keep stationary for 5 minutes after the shaking. By keeping stationary, the above extracted solutions are separated as lower layer of alkaline solution, middle layer of alcohol solution and upper layer of diesel oil.

5) After keeping stationary for 5 minutes, put a stirrer into test tube and set it to test tube holder for spectrofluorometer. Radiate UV light (360 nm) on the alkaline solution layer for 400 seconds with rotating the stirrer, for photoisomerization reaction. Stop rotating the stirrer and read fluorescence intensity with E_x 360 nm, EM 500 nm and generate calibration curve.

*1) Regarding low concentration coumarin measurement, it is necessary to wash thoroughly those test tool such as test tube.

Measurement condition

After monitoring the process of photoisimerization by using [Time course measurement] program, the spectra were measured using [Spectrum measurement] program and fluorescence intensity was detected at Em wavelength = 500 nm.

Time course measurement

Ex bandwidth *2)	20 nm
Em bandwidth	10 nm
Response	2 sec.
Sensitivity	High
Measurement range	0 - 400 sec.
Data acquisition interval	2 sec.
Ex wavelength	360 nm
Em wavelength	500 nm

Spectrum measurement

Ex bandwidth	10 nm
Em bandwidth	10 nm
Response	Fast
Sensitivity	High
Measurement range	380 - 650 nm
Data acquisition interval	1 nm
Ex wavelength	360 nm
Scan speed	1000 nm/min

*2) Ex bandwidth was set at 20 nm to perform photoisomerization effectively for the Time course measurement. Ex bandwidth was set at 10 nm for the Spectrum measurement in order to suppress reduction of fluorescence intensity due to photolysis.

Calibration curve

Time course measurement data and spectral measurement data of standard samples with additive materials concentration of 0 ~ 10 % are shown in the Fig. 3 and 4. From the Fig. 3, it is observed that photoisomerization finished in 150 seconds from starting UV light radiation.

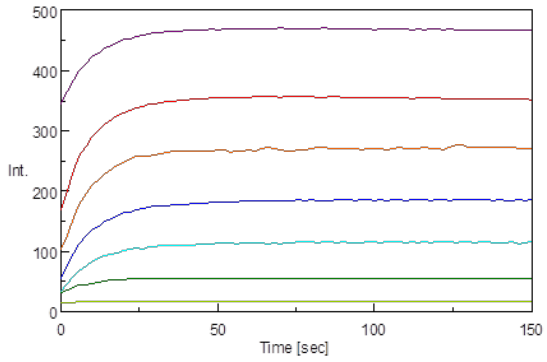


Fig. 3 Photoisomerization situation

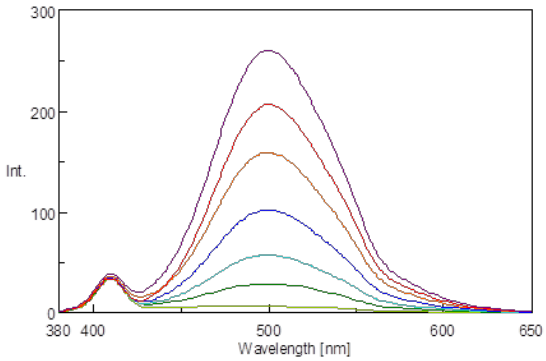


Fig. 4 Spectra after finishing photoisomerization

Calibration curve plotting fluorescence intensity at spectrum peak wavelength of 500 nm with additive material concentration is shown in Fig. 5.
0.9993 of correlation coefficient for the calibration curve was obtained, showing good linearity.

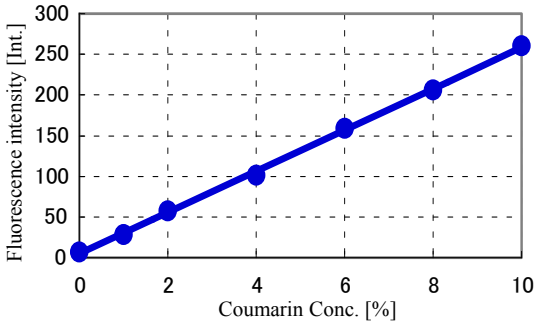


Fig. 5 Calibration curve

Table 2 Fluorescence intensity to additive concentration

Additive conc.[%]	Fluorescence intensity
0	6.7077
1	28.7548
2	57.3873
4	101.829
6	158.903
8	205.882
10	260.236

Calibration curve information
y = 25.367x + 4.7604
R2 = 0.9993

The measurement using 0% and 1 % concentration standard solution was repeated 5 times, and standard deviation for fluorescence intensity was 0.4357 and standard deviation for coumarin concentration was 0.0172. Considering such results, it is possible to perform analysis with 0.06% detection limit and 0.2% quantitation limit. *3)

*3) Detection limit was calculated by 3 sigma and quantitation limit was calculated by 10 sigma.

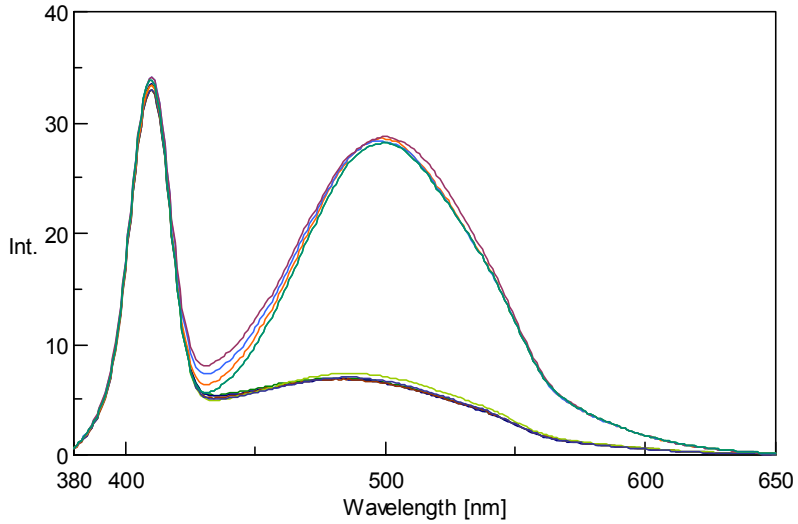


Fig. 6 Spectra of conc. 0 and 1 % coumarin solution(5 spectra each)