Application Note

FP-0002

Upgrade of simple coumarin analysis system to high sensitivity one

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.

1. 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.

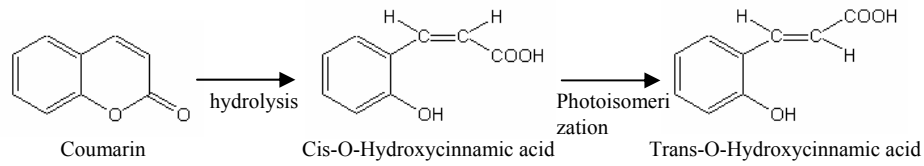

Fig. 1 Hydrolysis and photoisomerization of coumarin

2. Measurement system

Filters on both EX and EM sides are used to reduce scattering light, enabling the analyzing system to assure high sensitivity measurement.

- FP-6300 Spectrofluorometer
- Test tube holder for coumarin measurement
- U330 filter (EX side), WG305 filter (EM side) *1)

3. 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

4. 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.

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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

5. 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.

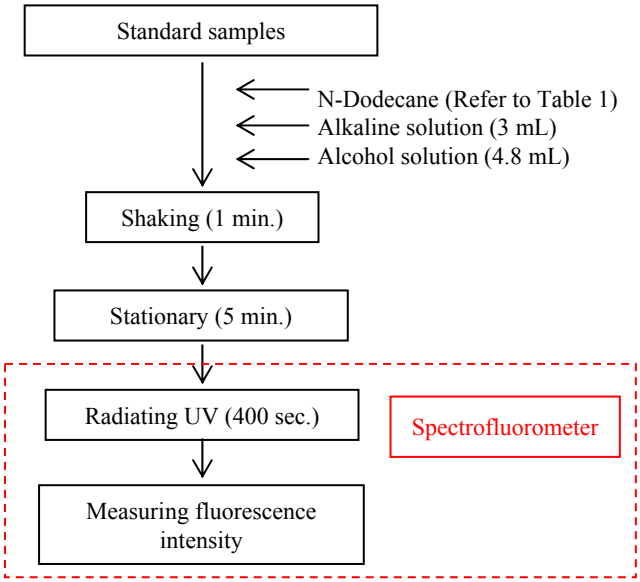

Fig. 2 Flow chart of analysis procedure

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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 EX 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.

6. 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.

7. 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.

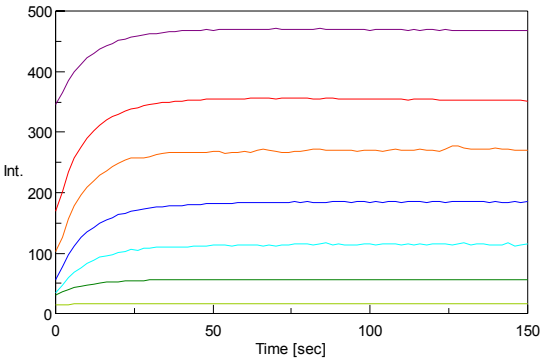

Fig. 3 Photoisomerization situation

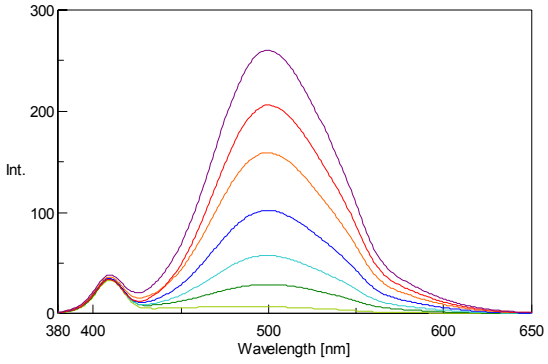

Fig. 4 Spectra after finishing photoisomerization

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.

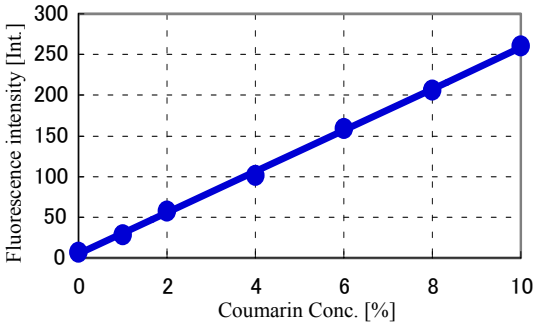

Fig. 5 Calibration curve

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.

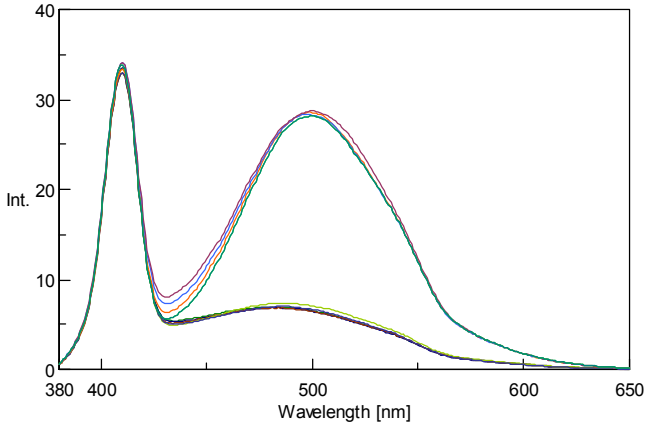

Fig. 6 Spectra of conc. 0 and 1 % coumarin solution(5 spectra each)

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