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## Assessment of ISNAG fluorimeter (Total fluorescence measurements at $+90^\circ$ & $-90^\circ$ using four solar cell on each side for 100mm distance at 2mm path length) with well-known fluorescent molecules via CFIA

Nagam S. Turkie<sup>\*1</sup>, Hayder Q. Munshid<sup>2</sup>

<sup>1</sup>Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq.

<sup>2</sup>Department of Pharmacy, Al-esraa University College, Baghdad, Iraq.

### Abstract

Two well-known fluorescent molecules: fluorescein sodium salt (FSS) and 2,7-dichloro fluorescein (DCF) were tried to prove the efficiency, trustability and repeatability of ISNAG fluorimeter by using discrete and continuous flow injection analysis modes. A linear range of 0.002-1 mmol/L for FSS and 0.003-0.7 mmol/L was for DCF, with LOD 0.0018 mmol/L and 0.002 mmol/L for FSS and DCF respectively, were obtained for discrete mode of analysis. While the continuous mode gave a linear range of 0.002-0.7 mmol/L and 0.003-0.5 mmol/L for FSS and DCF respectively, the LOD were 0.0016mmol/L and 0.0018 mmol/L for FSS and DCF respectively. The results were compared with classical method at variable  $\lambda_{ex}$  for both fluorescent molecules at 95% confidence level. The comparison data shows that ISNAG fluorimeter is the choice with excellent extended detection and a wider applicability.

**Keywords:** Fluorescein sodium salt, 2, 7-Dichlorofluorescein, Flow injection analysis, Fluorescence.

تقييم مقياس الفلورة ISNAG (القياس للفلورة الكلية على  $+90^\circ$  و  $-90^\circ$  باستخدام اربعة خلايا شمسية على كل جانب لمسافة 100mm وبمسار تشيع 2mm) مع جزيئات الفلورة المعروفة عن طريق تقنية الحقن الجرياني المستمر

نغم شاكر تركي<sup>1\*</sup> ، حيدر قيس منشيد<sup>2</sup>

<sup>1</sup> قسم الكيمياء، كلية العلوم، جامعة بغداد، بغداد، العراق.

<sup>2</sup> قسم الصيدلة، كلية الاسراء الجامعة، بغداد، العراق.

### الخلاصة

تم استخدام جزيئين متفلورة معروفة: ملح فلوريسين الصوديوم (FSS) و 2,7-ثنائي كلوريد الفلوريسين (DCF) لإثبات كفاءة وموثوقية وتكرار مقياس الفلورة ISNAG باستخدام تقنية تحليل الحقن الجرياني المنفصلة والمستمرة؛ وتم الحصول على مدى منحنى المعايرة 0.002-1 ملي مول/لتر لملاح فلوريسين الصوديوم و 0.003-0.7 ملي مول/لتر لجزيئة 2,7-ثنائي كلوريد الفلوريسين، مع حد الكشف 0.0018 ملي مول/لتر و 0.002 ملي مول/لتر لملاح فلوريسين الصوديوم و جزيئة 2,7-ثنائي كلوريد الفلوريسين على التوالي، لطريقة التحليل المنفصل. في حين أعطت الطريقة المستمرة مدى خطي من 0.002-0.7 مليمول/لتر و

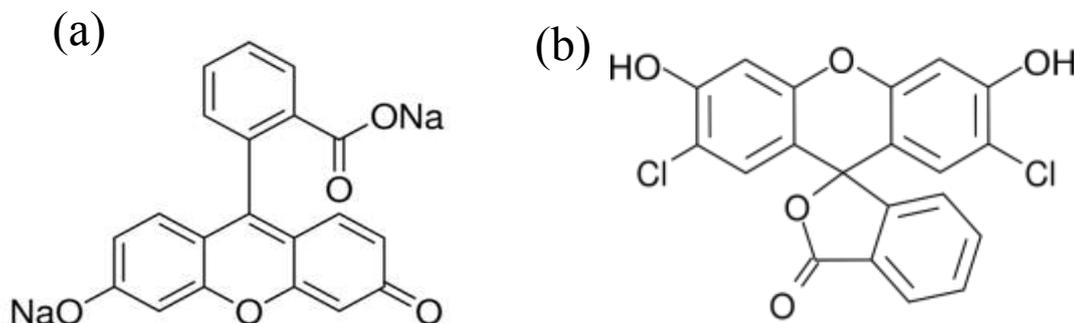
\*Email: Nagamturkey2013@gmail.com

0.003-0.5 ملي مول/لتر لملاح فلوريسين الصوديوم و جزيئة 7,2-ثنائي كلوريد الفلوريسين على التوالي، وكان حد الكشف 0.0016ملي مول/لتر و 0.0018 ملي مول/لتر لملاح فلوريسين الصوديوم و جزيئة 7,2-ثنائي كلوريد الفلوريسين على التوالي. تم مقارنة النتائج مع الطريقة التقليدية باستخدام  $\lambda_{ex}$  مختلفة لكلا الجزيئين المنفلورتين وبحدود ثقة 95%. تظهر نتائج المقارنة ان مقياس الفلورة ISNAG هو الاختيار الامثل ولمدى كشف ممتاز وتطبيق اوسع.

## Introduction

Fluorescein sodium salt (FSS); is a common dye used in fluorescent spectroscopy which is also known as uranine, it has the empirical formula of  $C_{20}H_{12}O_5Na_2$  Figure-1(a), it is freely soluble in water and alcohol [1]. It was reported that FSS solutions are unstable when heated due to the photochemical instability, which has a major pKa of 6.4 [2]. While, 2, 7-Dichlorofluorescein (DCF); is a fluorescein derivative belongs to the xanthene dyes group with an empirical formula of  $C_{20}H_{10}Cl_2O_5$  Figure-1(b). It is insoluble in water, soluble in ethanol and slightly soluble in ether and methanol. DCF has a pKa of 4.46 and give a weak green fluorescence at pH 4.0 to intense green fluorescence at pH 6.0 [3]. Both fluorescent molecules have a wide range of applications and usage as fluorescent indicator for the indirect detection of ions[4, 5], organic compounds[6-8], drugs[9, 10], biological molecules [11, 12]and a wide range of medical applications[13, 14]. These molecules were also used in quenching of continuous flow injection analysis (CFIA) [15-18].

In this study these two fluorescent molecules were used for the assessment of ISNAG fluorimeter. A homemade ISNAG fluorimeter with low-pressure mercury lamp; the Hg-lamp will supply two specific lambdas 184.9 & 253.7 nm (as it is regarded in candescent lamp (low pressure))[19], the emitted fluorescence was measured at  $\pm 90^\circ$  via  $2 \times 4$  solar cell (410-1150 nm spectral range). Which will detect the visible length region and any of the reflected if any reach the solar cell as being beyond its response efficacy.



**Figure 1**-Chemical structures of fluorescent molecules. (a) Fluorescein sodium salt; (b) 2, 7-Dichlorofluorescein.

## Chemicals and Apparatus

### Reagents and chemicals

All chemicals were used of analytical-reagent and distilled water was use to prepare all the solutions. A standard solutions of 1mmol/L of FSS and DCF, molecular weight 376.275 and 401.195 g/mole respectively, were prepared by dissolving 0.1811 g and 0.2006 g in 500 mL respectively. A pH range of 2.2–8.0 buffers were prepared according to McIlvaine citric acid–phosphate buffer systems [20]. A series of sodium hydroxide solutions were prepared from the dilution of standardized stock solution (0.1 mol/L) with distilled water.

### Apparatus

A homemade ISNAG fluorimeter (described above) was used with 4-channels peristaltic pump (Ismatec, Switzerland) and Six-port medium pressure injection valve (I D E X corporation, USA) with sample loop (1 mm i.d. Teflon, variable length). Potentiometric recorder to estimate the output signals (Siemens, Germany (1- 5 V)). Spectrofluorometer (RF-1501, shimadzu, Japan) was also used for classical spectrofluorometric methods.

### Methodology

A single line manifold system Figure-2 was tried via the use of distilled water as a carrier stream and injected volume of the fluorescent solution at 176  $\mu\text{L}$  of 0.1 mmol/L at 2.2 mL/min flow rate for FSS and 250  $\mu\text{L}$  of 0.1 mmol/L at 2.75 mL/min for DCF using 2 mm i.d. of silicone special tubing in the 4-channels Ismatic peristaltic pump. Figure-3 (a, b) show a response-time profile for six repeated successive measurements of the fluorescein sodium salt and 2, 7-dichlorofluorescein respectively. In order to study the optimum parameters by making all variables constant and varying one at a time i.e. fixed variable optimization. To optimized the chemicals involved in the reaction of variable; three modes were used; alkaline medium in addition to distilled water, disodium hydrogen orthophosphate – citric acid buffer as a carrier stream, sodium hydroxide solution as a carrier stream and direct sample preparation using (D.W, selected-pH, and variable concentrations of NaOH). The variation of physical parameters including flow rate, sample volume, purge time and effect of temperature were also studied using optimum parameters achieved in previous sections. Two sets of calibration graph were constructed based on mode of measurements (Discrete mode; a set of 0.001-1 mmol/L were prepared using 250  $\mu\text{L}$  sample injected on a carrier stream line (0.05 mol/L NaOH for FSS and distilled water for DCF), and continuous mode; the carrier stream in this case is the fluorescence molecule while distilled water is injected on as a sample loop) using 250 $\mu\text{L}$  as an injected sample volume with an open valve mode and flow rate of 2.75 mL/min for FSS and 2.2 mL/min for DCF. The classical methods also studied using direct fluorescence at variable  $\lambda_{\text{ex}}$  for both fluorescent molecules.

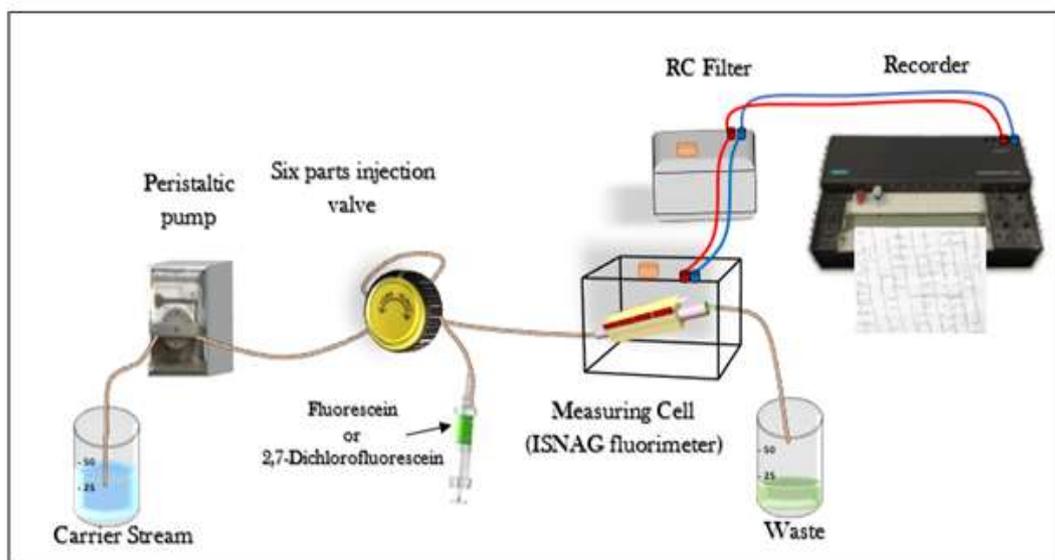


Figure 2- One-line manifold system design using FSS and DCF as an injected sample.

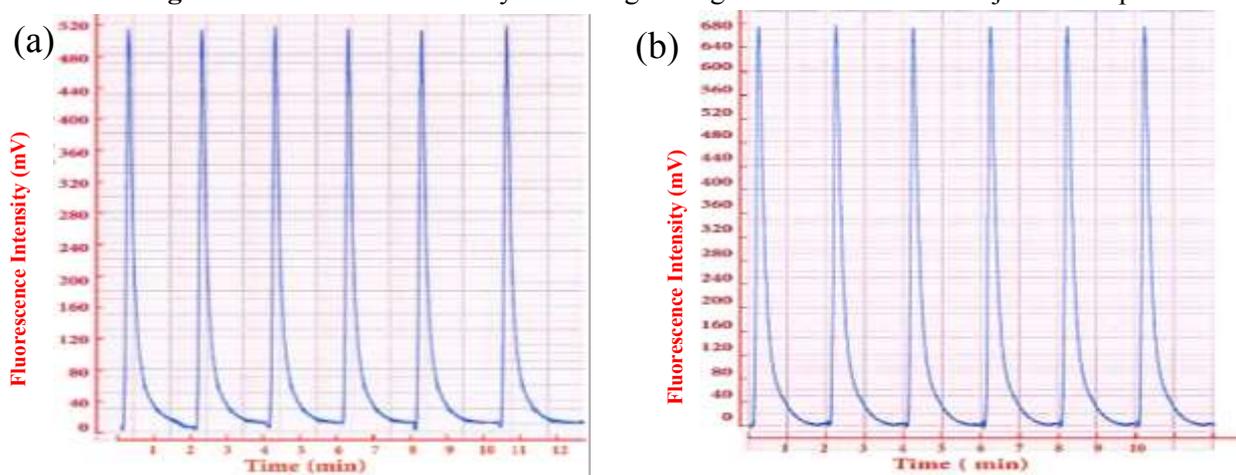


Figure 3- Response profile for the fluorescence intensity, (a) Fluorescein sodium salt; (b) 2, 7-Dichlorofluorescein.

## Results and Discussion

### Study of the optimum parameters

The chemical and physical parameters for the flow injection manifold system which shown in Figure-2 were investigated, in order to obtain optimum conditions for studying the fluorescence of FSS and DCF, it was noticed that when using variable hydrogen ion concentrations as a carrier stream, via disodium hydrogen orthophosphate – citric acid buffers, at high pH values the emission intensity of fluorescent molecules increase up to pH value of 7; then followed by a stable just about constant level of fluorescent emission of FSS. While using DCF; fluorescence emission increases starting at pH=6 followed by a slight variation in emission Figure-4. Also both molecules show an intersection at pH 3.5, which means that at this pH value no significant difference can be noticed in measurements using both tried fluorescent molecules. While the use of sodium hydroxide solution as a carrier the responses obtained show good acceptable repeatable measurements Figure-5. Based on these studies; constant concentration of FSS and DCF (0.1mmol/L of each) were prepared in different media (D.W, selected-pH, and variable concentrations of NaOH) and D.W used as a carrier stream. It can be noticed from the obtained results (Table 1) that the  $\sigma_{n-1} = 5.99$  and  $RSD\% = 1.24$  is very small; therefore, it can be regarded as there is no significant difference using either D.W or any alkaline medium tried in the above mentioned experiments. So, it was decided that 0.05 mol/L NaOH concentration as a carrier stream was the most suitable medium for have a maximum fluorescence intensity, which might be attributed to the increase number of resonance species that are associated with the basic forms of the molecules. While for DCF, it was noticed that a decrease in the fluorescence intensity with variable NaOH concentrations, which might be due to the external conversion effect by which a molecule passes to a lower energy electronic state without emission of radiation. Therefore, pH = 6 was chosen as the optimum medium that used in all subsequent experiments for DCF.

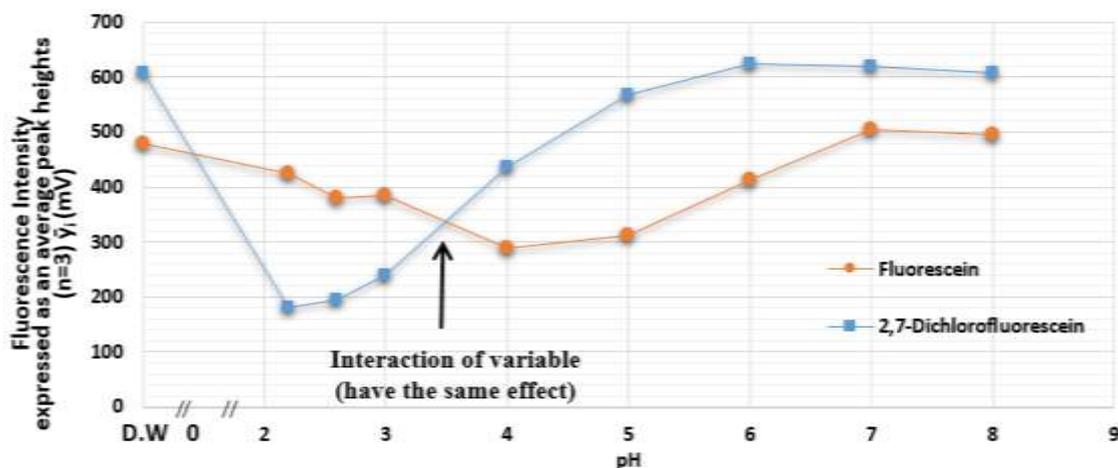


Figure 4-Variation of fluorescence intensity at different pH value.

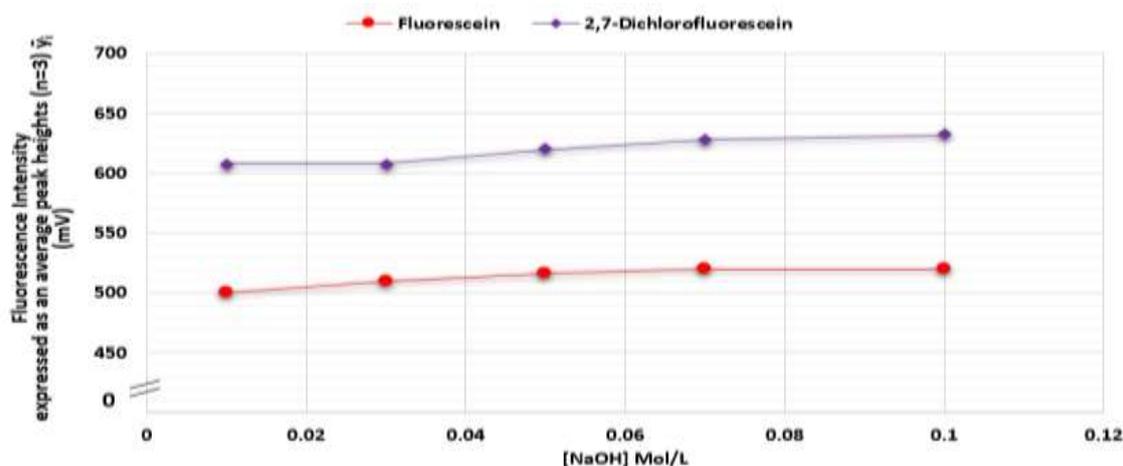
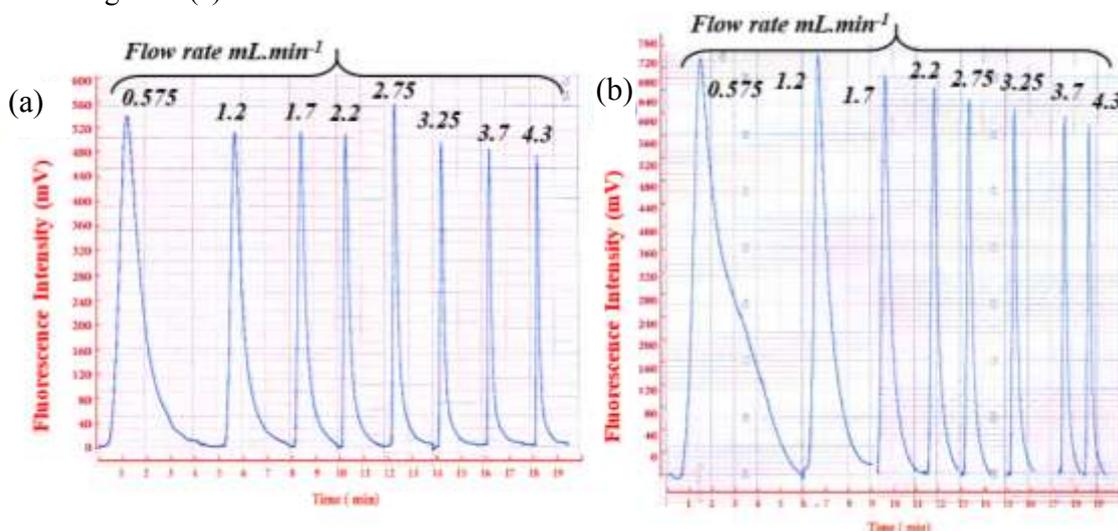


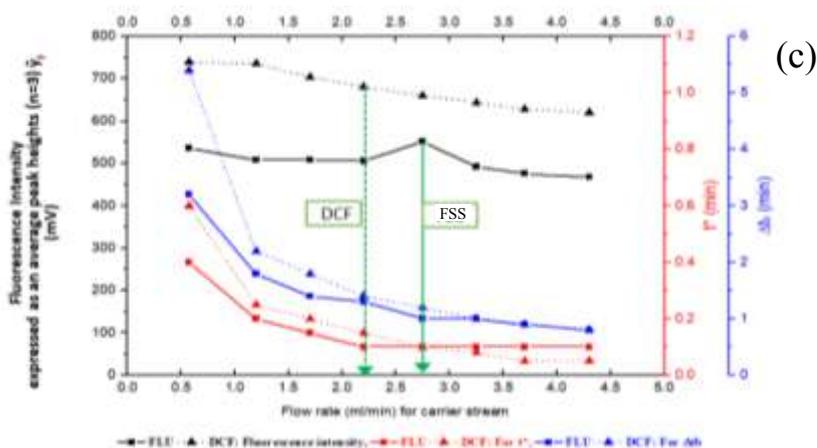
Figure 5- Variation of NaOH concentration that effect on fluorescence of FSS and DCF using 176µL (0.1mmol/L) of FSS and 250µL (0.1mmol/L) for DCF.

**Table 1-** Effect of different medium on fluorescence intensity expressed as an average peak heights (n=3) at constant concentration of (0.1 mmol/L) for both molecules

Type of medium	Fluorescence Intensity expressed as an average peak heights (n=3) $\bar{y}_i$ (mV)		RSD%		Confidence interval of the average response (at 95% confidence level) $\bar{y}_i \pm t_{0.05/2, n-1} \sigma_{n-1} / \sqrt{n}$	
	FSS	DCF	FSS	DCF	FSS	DCF
D.W	478	628	0.17	0.11	478 ± 2.06	628 ± 1.71
pH 7	490	660	0.16	0.14	490 ± 1.91	660 ± 2.29
pH 6	492	596	0.20	0.14	492 ± 2.39	596 ± 2.06
pH 8	474	614	0.17	0.20	474 ± 1.96	614 ± 3.01
[NaOH] (Mol/L)	0.005	480	0.21	0.21	480 ± 2.53	618 ± 3.28
	0.01	482	0.20	0.20	482 ± 2.43	622 ± 3.08
	0.03	482	0.14	0.17	482 ± 1.71	618 ± 2.61
	0.05	480	0.21	0.22	480 ± 2.53	620 ± 3.33
	0.1	480	0.21	0.22	480 ± 2.53	620 ± 3.33

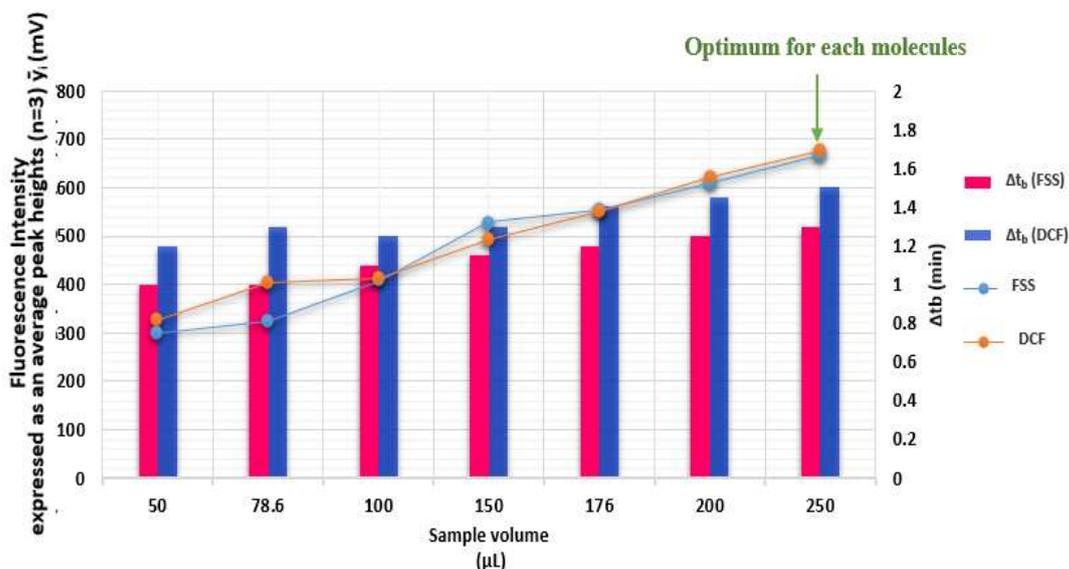
While the physical variables study was carried out for the determination of preferred flow rate within the range of 0.58 to 4.3 mL/min using 0.1 mmol/L of FSS (176 $\mu$ L) with NaOH 0.05 mol/L as carrier stream and 0.1 mmol/L of DCF (250 $\mu$ L) prepared in pH 6 solution (12.63 mL (0.2mol/L) Na<sub>2</sub>HPO<sub>4</sub> + 7.37 mL (0.1mol/L) citric acid). Figure-6(a, b) show that at low flow rate there was an increase in diffusion and dispersion which causes to enlargement of sample segment of the fluorescent species which in turn to increase of peak base width ( $\Delta_{tb}$ ) and distorted profile, while at higher flow rate (i.e. > 0.58 mL/min) a slightly decreased of fluorescence intensity followed by; there was an increase height up to 2.75 mL/min for FSS and 2.2 mL/min for DCF and then decrease of fluorescence emission (i.e. > 2.75 and 2.2 mL/min for FSS and DCF respectively), due to departure of fluorescent species from measuring cell at a short time. So the best flow rate for fluorescence measurement, sharp maxima and minimize the consumption of solutions was 2.75 and 2.2 for FSS and DCF respectively as shown in Figure-6 (c).





**Figure 6-** Effect of flow rate on the fluorescence intensity. (a) Response profile for FSS; (b) Response profile for DCF; (c) Relationship between flow rate with F.I.,  $t$  and  $\Delta t_b$ .

A variable sample volumes were injected using ranged sample loop (50-250  $\mu\text{L}$ ), while the other parameters were fixed for (0.1mmol/L) of both FSS; flow rate 2.75 mL/min; and DCF; flow rate 2.2 mL/min; in order to determine the optimum sample volume. It was noticed that an increased in sample segment (sample loop volume) for all fluorescent molecules leads to an increase of fluorescence at a wavelength that the solar cell is capable in detecting these emissions. On this basis 250  $\mu\text{L}$  Figure- 7 was chosen as the optimum volume for carrying out the rest of studies. The optimum sample loop was purged for variable injection time (2-25 sec) in addition to the open valve mode. Increased fluorescence emission was noticed as allowing without disturbing the flow of sample loop i.e.; continuous mode of open valve technique is the best to conduct this work at this stage as shown in Figure-8.



**Figure 7-** Variation of sample volume on fluorescence intensity and peak base width for each fluorescent molecules.

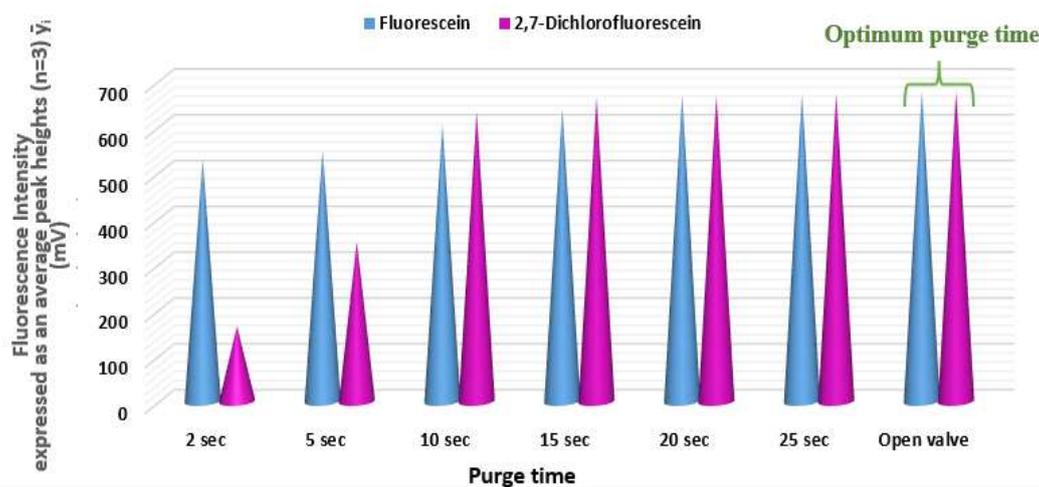


Figure 8-Effect of purge time on fluorescence intensity using FSS and DCF.

The final parameter in this study deals with the variation of temperature during the journey of the sample (fluorescent solution as a segment) from injection valve to the measuring cell, this study was carrying out using optimum open valve sample loop of 250  $\mu$ L for 0.1 mmol/L of FSS and DCF; flow rate 2.75 mL/min and 2.2 mL/min respectively. A sand bath was used to rise the temperature of the placed beaker in it with constant stirring. It should place in mind that UV-lamp (LP Hg Lamp) generate heat while it is on, but because of the design and material used to enclose the lamp; a brass metal covers most of the exposed area of the lamp as it works as heat sink. In addition, that a constant solution flow; the 100 mm length (flow cell) will keep an approximate steady ( $\sigma_{n-1} = 2$  & 2.153) with minimum of 27°C up to 33°C corresponding to the inlet feed temperature of 23°C up to 80°C ( $\sigma_{n-1} = 18.312$  & 17.758) for FSS and DCF respectively. As a final conclusion the variation of inlet feed temperature will cause a fluctuation in signal profile and distorted. On this fall study no high temperature (beyond room temperature) is required Figure-9.

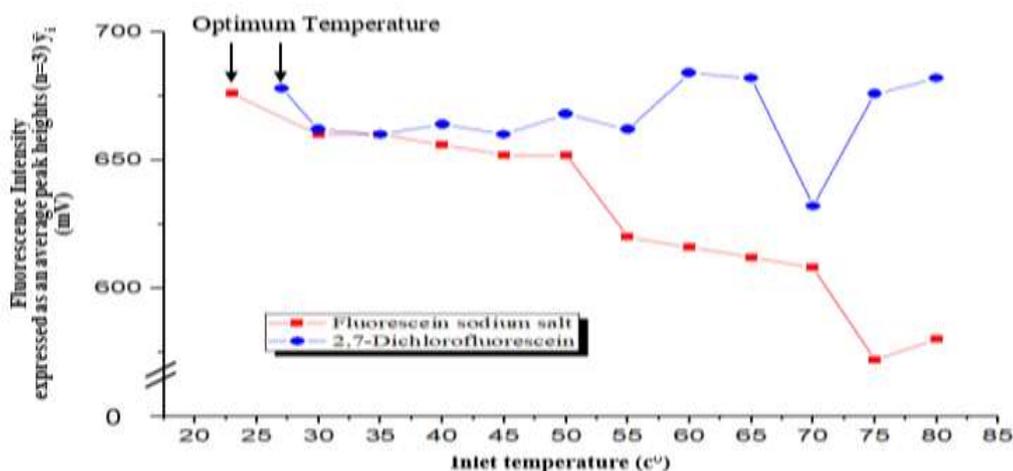


Figure 9- Effect of temperature on the fluorescence intensity of FSS and DCF.

### Calibration curve

Two sets of calibration graph were made based on mode of measurements; discrete mode; a linear range was 0.002-1 mmol/L for FSS while 0.003-0.7 mmol/L was for DCF. A narrower range in DCF is expected due to the presence two chlorine atom in the molecule that will affect the space occupation by the molecule causing less sensitive extended range even it is relatively small. The practical limits of detection (based on the gradual dilution for the minimum concentration) were 0.0018 mmol/L (169.324 ng/sample) and 0.002 mmol/L (200.598 ng/sample) for FSS and DCF respectively. Continuous mode; A linear range of 0.002-0.7 mmol/L and 0.003-0.5 mmol/L for FSS and DCF respectively were obtained, high concentration will be avoided at this stage due to different quenching

effects (might be e.g., self-quenching, internal conversion and external conversion). The limits of detection were 0.0016 mmol/L and 0.0018 mmol/L for FSS and DCF respectively. The results were tabulated in Table-3 using two modes and compare with classical methods at variable  $\lambda_{ex}$  for both fluorescent molecules at 95% confidence level.

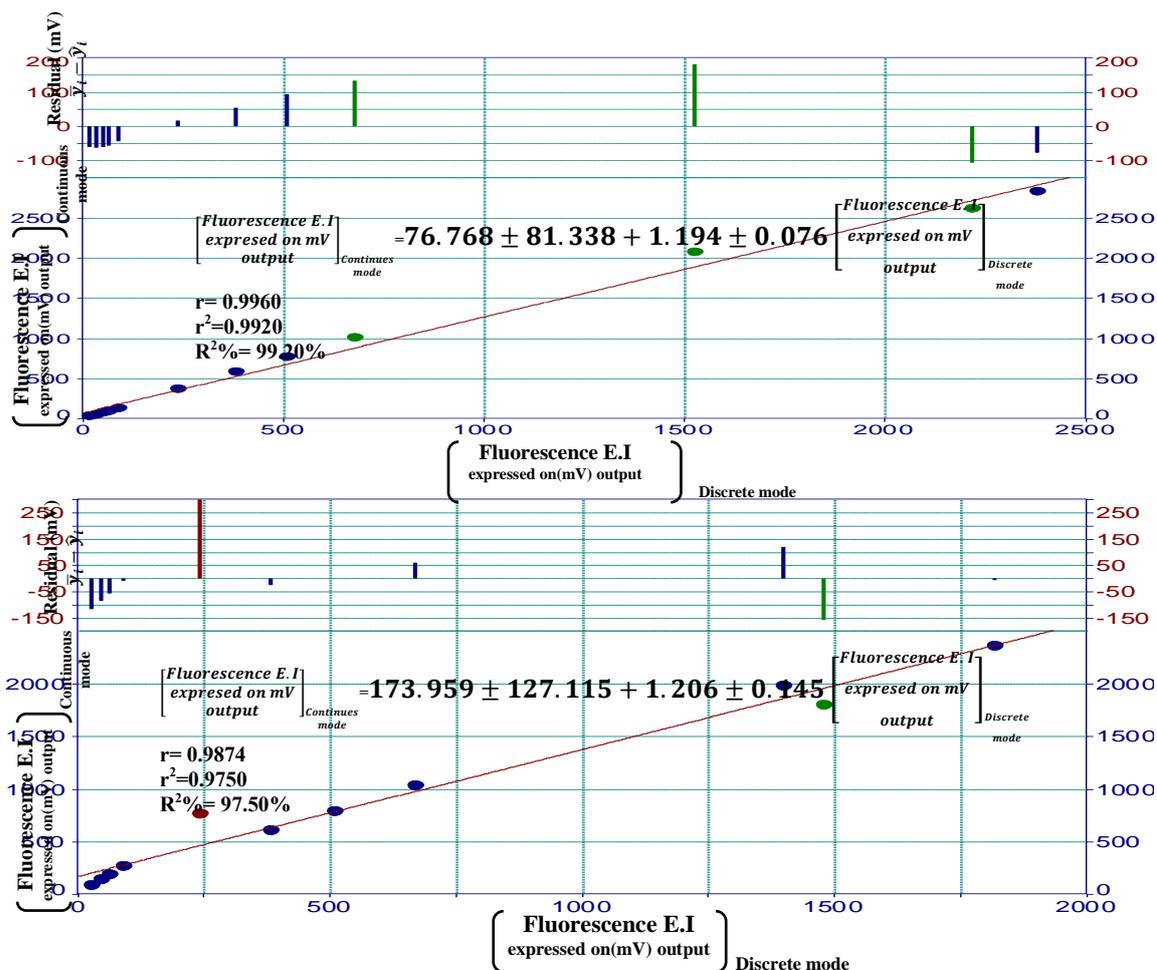
In order to decide the biasness of one mode of reaction pattern using single line manifold (discrete and or continuous) and based on the data in Table-3, a plot of responses of discrete as independent variable was plotted against responses of continuous mode for the same concentration (ranging from 0.002-0.7 mmol/L) for FSS. The graph plot Figure-10(a) shows the biasness toward the continuous mode; as the slope is  $> 45^\circ$  (it is  $55.61^\circ$ ). While DCF is the same way; i.e: biasness toward continuous mode Figure-10(b), the slope was  $> 45^\circ$  (it is  $56.13^\circ$ ). A comparison Table-2 between finally arrived ISNAG procedure emissions intensity regarding all data treatments parameters. It was recognized that due to different of scales of dependent and independent variables (i.e concerning the linear equation  $y = a + bx$ ).

It was realized that no comparison can be made due to different status of population, therefore a narrower range is obtained with spectrofluorometric while a wider range was the characteristic of ISNAG fluorimeter.

The spectrofluorometric gave a four peaks in FSS, three of them falls in the visible region while the DCF gave three strong bands one at UV region (290 nm) while the rest full in the blue and yellow orange region (445 nm (blue) & 580 nm (yellow orange)). The rest of data which are tabulated in Table-2 that includes  $r$  (correlation coefficient),  $r^2$  (coefficient of determination) and range; which indicate all together the wider applicability with excellent correlation and % R squared which can explain at excellent level for all data obtained using ISNAG fluorimeter; which was measure total fluorescent emission released via the irradiation of nearly specific  $\lambda$ . No monochromator was used to restrict the out coming emission when measured at certain specified  $\lambda$ . While ISNAG fluorimeter measure any visible emission. ISNAG fluorimeter is the choice with excellent extended detection and a wider applicability.

**Table 2-** Summary of calibration curve results for FSS and DCF at 95% confidence level.

Molecule	Type of method	Range of calibration curve (mmol/L)	Equation of calibration curve $\hat{y}_i = a \pm S_a t + b \pm S_b t[X]$ at confidence level 95%, n-2	r	$t_{tab} = t_{0.025, n-2}$	$t_{cal} = \frac{ r \sqrt{n-2}}{\sqrt{1-r^2}}$	
				$r^2$			R <sup>2</sup> %
Fluorescein sodium salt	Developed	0.002 – 1 (n=13)	$\hat{y}_i = 205.77 \pm 215.21 + 2951.04 \pm 570.72[X]$	0.9601	2.201 << 11.381		
				0.9217			
				92.17			
	Developed	0.002 – 0.7 (n=12)	$\hat{y}_i = 252.12 \pm 229.72 + 4330.07 \pm 863.91[X]$	0.9621	2.226 << 11.157		
				0.9256			
				92.56			
	Classical	$\lambda_{ex} = 232$ nm	$1 \times 10^{-5} - 0.004$ (n=9)	$\hat{y}_i = 47.38 \pm 35.99 + 213545.97 \pm 19440.46[X]$	0.9948	2.365 << 25.979	
					0.9897		
					98.97		
Classical	$\lambda_{ex} = 254$ nm	$1 \times 10^{-5} - 0.002$ (n=7)	$\hat{y}_i = 52.48 \pm 53.59 + 350740.60 \pm 58670.93[X]$	0.9895	2.571 << 15.370		
				0.9792			
				97.92			
Classical	$\lambda_{ex} = 481$ nm	$1 \times 10^{-5} - 1.5 \times 10^{-4}$ (n=6)	$\hat{y}_i = 261.02 \pm 85.04 + 4204780.00 \pm 873255.81[X]$	0.9889	2.776 << 13.367		
				0.9781			
				97.81			
2,7-Dichlorofluorescein	Developed	0.003 – 0.7 (n=12)	$\hat{y}_i = 172.96 \pm 144.69 + 3092.63 \pm 499.09[X]$	0.9747	2.226 << 13.793		
				0.9501			
				95.01			
	Developed	0.003 – 0.5 (n=11)	$\hat{y}_i = 342.61 \pm 215.32 + 4255.80 \pm 991.77[X]$	0.9554	2.262 << 9.706		
				0.9128			
				91.28			
	Classical	$\lambda_e = 243$ nm	$1 \times 10^{-9} - 1 \times 10^{-7}$ (n=6)	$\hat{y}_i = 31.08 \pm 50.18 + 5402.12 \times 10^6 \pm 906.27 \times 10^6[X]$	0.9927	2.776 << 16.547	
					0.9856		
					98.56		
Classical	$\lambda_{ex} = 254$ nm	$1 \times 10^{-9} - 2 \times 10^{-7}$ (n=7)	$\hat{y}_i = 49.14 \pm 52.91 + 3528.38 \times 10^6 \pm 579.28 \times 10^6[X]$	0.9899	2.571 << 15.659		
				0.9800			
				98.00			



$$Residual = \bar{y}_i(\text{practical value}) - \hat{y}_i(\text{estimated value})$$

**Figure 10-** Fluorescence E.I for discrete mode vs fluorescence E.I for continuous mode. A, Fluorescein sodium salt (FSS); B, 2,7-Dichloro fluorescein (DCF).

The reality and repeatability of the method were studied at a selected concentration of (0.1 mmol/L) using 250µL sample volume with open valve mode for both molecules. Repeat measurements for eight successive injections were measured and the obtained results are tabulated in Table-3.

**Table 3-** Repeatability results of 0.1 mmol/L of FSS and DCF using 250µL injected sample, an open valve mode for discrete mode.

Type of fluorescent compounds	Fluorescence intensity expressed as an average peak heights (n=8) $\bar{y}_i$ (mV)	RSD%	Confidence interval of the average response (at 95% confidence level) $\bar{y}_i \pm t_{0.05/2, n-1} \sigma_{n-1} / \sqrt{n}$
FSS	678	0.34	678 ± 1.940
DCF	679	0.29	679 ± 1.656

$$t_{0.025,7} = 2.365$$

## Conclusion

The assessment of ISNAG fluorimeter through this research work was applied using comparison between finally arrived ISNAG procedure with classical spectrofluorometric method. It was recognized that a narrower range is obtained with spectrofluorometric while a wider range was the characteristic of ISNAG fluorimeter, which indicate the wider applicability with excellent correlation and % R squared. ISNAG fluorimeter is the choice with excellent extended detection and a wider applicability.

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