ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

A Nobel Chemiluminescence and Fluorescence Energy Transfer Three Inlets Cell for the Determination of Hydrogen Peroxide via Continuous Flow Injection Analysis

Nagam Shakir Turkie Al-Awadi

Chemistry Department-College of Science University of Baghdad-Baghdad/Iraq.

Abstract

The study presents a sensitive, fast and easy assay method that was developed for determination of hydrogen peroxide via continuous mode of FIA i.e. new noble chemiluminescence and fluorescence cell with specially designed unit cell of three inlets and one outlet to measure a total luminescence which include the chemiluminescence generated and the fluorescence that was created by the in situ radiation of the released chemiluminescence light using fluorescence molecule which gives an emission spectrum at 530 nm The method is based on the use of fluorescent molecule as an acceptor fluorophore where by it is irradiated internally and instantly when the generation of luminol chemiluminescence light is used as internal source for irradiation of fluorescein molecule to give fluorescence light , this reaction and analysis occur in a specially homemade designed cell. Experimental parameters that lead to optimum concentrations of all chemicals and physicals variables .A sample volume of 75 µL was used throughout the whole work. It was found that the linear working range was between 20-500 µmol. L^{-1}, with correlation coefficient of 0.9969 and limit of detection L.O.D. (S/N =3) 1.275ng/sample by using step wise dilution of the minimum concentration that was achieved by the calibration graph. Repeatability of less than 0.25% for ten successive injections of 250 µmol.L^{-1} & 400 µmol .L^{-1} of hydrogen peroxide .The method was applied successively in determination of hydrogen peroxide in some pharmaceutical disinfectants.

Keywords: *Chemiluminescence, Flow injection analysis, Hydrogen peroxide, In situ fluorescence.*

Introduction

Chemiluminescence (CL) is defined as the production of electromagnetic radiation (ultraviolet, visible or infrared) observed when a chemical reaction yields an electronically excited intermediate or product, which either luminesces or donates its energy to another molecule, which then luminesces. If radiation is emitted by energy –transfer, the process is normally called chemi-excitation; likewise when the chemiluminogenic reaction is enzymatic and/or occurs within a organism living, the phenomenon is named bioluminescence (BL) [1, 2].

In general, a CL reaction can be generated by two basic mechanisms. In a direct reaction, two reagents, usually a substrate and an oxidant in presence of some co-factor, react to form a product, then some fraction of the product will be formed in an electronically excited state which can subsequently relax to the ground state with emission of a photon [3, 4].On the contrary, indirect or sensitized CL is based on a process of transference of energy of the excited species to a fluorophore. This process makes it possible for those molecules that are unable to be directly involved in CL reactions to transfer their excess of energy to a fluorophore that in turn is excited, releasing to its ground state with photon emission [5, 6]. The oxidation of luminol (3-aminophthalhydrazide) in alkaline medium is one of the most efficient CL reactions. Luminol undergoes CL reactions with a range of oxidants.

The oxidants including hydrogen peroxide, oxygen, potassium permanganate, ferricyanide, tetravalent cerium ion, lead dioxide and oxygen-free radicals such as super oxide anion ($O_2^{-\cdot}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide (NO) were often used in this CL system. Some transition metals in their highest oxidation states, such as tetravalent nickel, can be stabilized by

chelating with suitable polydentate ligand [7]. There are a variety of methods concerning the determination of trace levels of hydrogen peroxide, the most common more sensitive methods based on the turbid metric (T_{180^o})[8] fluorimetric –flow injection system [9], chemiluminescence methods under optimum conditions [10, 14] based on the oxidation of luminol by hydrogen peroxide in the presence of Co(II) as a catalyst. The use of the total luminescence was first used by Shakir [15, 17] using flow inection analysis.

The principle involved was to make use of chemiluminescence light generated by luminol molecule oxidation with hydrogen peroxide in the presence of Co (II) ion as a catalyst which gave a band extended from 365 to 570 nm, while feeding the system by fluorescence molecule, the insitu fluorescence from fluorophore molecule such as fluorescein, rhodamine -6G and rhodamine- B was generated for determination of hydrogen peroxide [18- 20].

In this work hydrogen peroxide at various concentration will be presented though a known reaction pattern of chemiluminescence using a new noble chemiluminescence and fluorescence cell[21] with specially designed unit cell of three inlets enter cell body at a tangential angle one for the donner molecule, the second for the acceptor fluorophore molecules and the carrier stream line. Using a powerful signal amplification that can measure 10^{-12} A. Noise free signal S/N was easily handled. A RCA2055 PMT was used to measure a repeated and reproducible response. The design is Patented [21]. The designed cell can measure chemiluminescence (CL) and fluorescence energy transfer (FRET) for continuous flow injection analysis.

Experimental

Chemicals

All chemicals were used of analytical-reagent grade while distilled water was used to prepare the solutions. A stock solution (1mmol.L^{-1}) of Luminol solution (5-amino phthalylhydrazide) $C_8H_7N_3O_2$ (177.16 g.mol^{-1},BDH) was prepared by dissolving 0.17716g in 1L of 0.1mol.L^{-1} solution of sodium carbonate Na_2CO_3(105.97 g.mol^{-1} , BDH), prepared by dissolving 5.2985g in 500 ml distilled water. A standard solution of 1000 µg.ml^{-1} Cobalt (II) ion as $Co(NO_3)_2.6H_2O$ (291.03 g.mol^{-1}, BDH) was prepared by dissolving 4.9383g in 1L distilled water. A stock solution of hydrogen peroxide H_2O_2 (100 mmol.L^{-1}) was prepared by pipetting 19.44 ml of hydrogen peroxide (35%, 1.01 g.mol^{-1}, BDH.) and complete the volume with distilled water to 2L volumetric flask. Hydrogen peroxide molarity was fixed in sulfuric acid medium (1:1) with potassium permanganate solution $KMnO_4$ (0.1 mol. L^{-1}) (158.03g.mol^{-1}, Hopkin&William) was prepared by dissolving 7.9015g in 500 ml of distilled water.

This solution was standardized previously against Sodium oxalate solution $Na_2C_2O_4$ 0.1 mol.L^{-1} (134.0g.mol^{-1}, BDH) prepared by dissolving 3.35g in 250 ml distilled water. a stock solution (1mmol.L^{-1}) of fluorescein sodium salt $C_{20}H_{10}O_5Na_2$(376.3g.mol^{-1}, BDH) prepared by dissolving 0.3763 g in 0.1mol.L^{-1} solution of sodium carbonate Na_2CO_3 and complete the volume to 1L by the same solution .

Sample Preparation

The analysis of H_2O_2 in three different of pharmaceutical preparation (Baghdad company, Al-Amire company and Al- Areje company) have 25% , 20% and 7% concentration respectively to evaluate the newly developed methodology for estimating hydrogen peroxide. After standardization with $KMnO_4$ solution, the concentration of those samples obtained as 20% (5.88 mol .L^{-1}, 15 % (4.41 mol .L^{-1}) and 5 % (1, 47 mol .L^{-1}) respectively, these different on concentration may be bad storage or closures is not tight for these samples. and a series of solutions were prepared for standard addition curve.

Apparatus

The flow systems consist of three parts Figure No. 1:

Pumping system: variable speeds peristaltic pump3- channels (Switzerland- an Ismatic type ISM796) to pump reagents separately .Each in a silicone rubber tube of 2mm I.D.

Manifold system : Include a rotary 6-port injection valve (Teflon) (Rheodyne, U.S.A.) with sample loop of 1mm i.d. Teflon, variable length, and a new design for chemiluminescence and fluorescence cell [21] (Figure No. 2) that can be used for on-line or flow injection analysis for fast chemiluminescence reaction was established.

The total volume of this new designed all is 163µL.

Three inlets are available two of them is to create the chemiluminescence in the luminol-H_2O_2-Co (II) system that give a band spectrum of 365-425(max.)-then fades at wavelength greater than 600nm. While the third inlet is used to introduce an acceptor fluorophore e;g. in this case the most suitable fluorophore is fluorescein sodium salt (Uranyl sodium salt with a stock shift of ≈ 100nm i.e; absorbing most of the blue spectrum and emitting the golden-green colour emission of fluorescein .The design of the three inlet two of them are at same level while the third one is at a lower level in the cell. The three inlets are arranged to create a tangential cyclone movements of the inlet solutions leaving the cell at lower wider tube (outlet) diameter to insure no delay will be established at the outlet of the cell therefore a wider outlet tube diameter was constructed within the specially designed flow chemiluminescence cell.

The front face of the cell is sealed by silicone rubber O-ring covered by a mirror to enhance strong then the light emitted from either chemiluminescence or/and fluorescence created by insitu entra irradiation by chemiluminescence reaction. A nanosecond PMT is used to detect any released light and recorded via 10^{-12} A .Linear Amplifier-Y (Z)-t (d) potentiometric recorder.

Electronic measuring system consist of photomultiplier tube PMT (covers UV-VIS Region) enclosed with the chemiluminescence cell by a black leather in order to reduce the background interferences. DC voltage power supply (0-1.6 KV) type (JOBIN YVON-France). Dual detector (United Detector Technology, U.S.A.) capable of measuring pA –nA level. The read out of the system composed of y (z) – t (d) potentiometric recorder (1-500 mV or 1-500 Volt) (KOMPENSO GRAPH C-1032) SIEMENS (Germany).

General Procedure

Figure No. 1 shows a schematic diagram of the manifold system that was used for the determination of hydrogen peroxide. In which 50 µL of hydrogen peroxide is injected through the sample loop (1mm i.d., Teflon, length = 6.4 cm) into a stream of Co (II) ion. Three lines system were used. Line 1 was used for 0.7 $mmol.L^{-1}$ luminol in $0.1mol.L^{-1}$ solution of Na_2CO_3 at 2 $ml.min^{-1}$.Line 2 was used for Co(II) ion (15 $\mu g.ml^{-1}$) as a carrier stream at 1.5 $ml.min^{-1}$ and third line was for fluorescein sodium salt ($0.05mmol.L^{-1}$) at 2 $ml.min^{-1}$.

Total emission was recorded for three successive samples (each sample 75 µL). Peak height was used during this work due to the symmetric peak obtained. The mechanism of Luminal oxidation by hydrogen peroxide in presence of Co (ll) ion as catalyst to generate chemiluminescence light ($\lambda_{emission}$ = 425 nm) and is shown in scheme 1 [22, 23] This was regarded as the light source that provide the UV-Light necessary as an internal source to irradiated fluorescein molecule which was used as an acceptor fluorophore to generate insitu fluorescence ($\lambda_{emission}$ = 520 nm), this method will measure both lights in the UV- VIS regions.

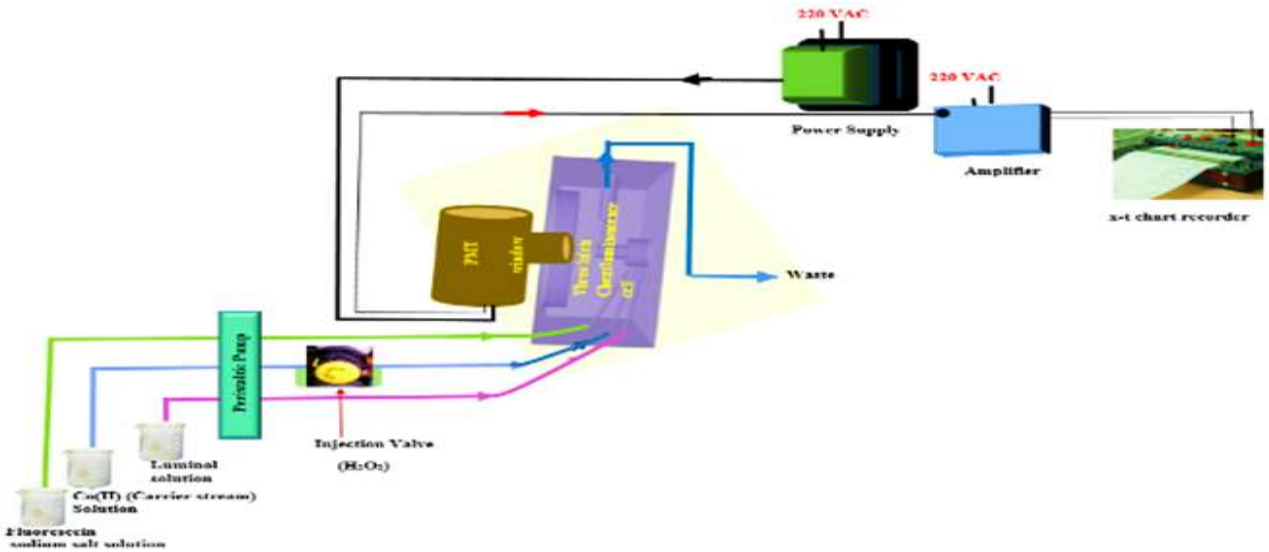
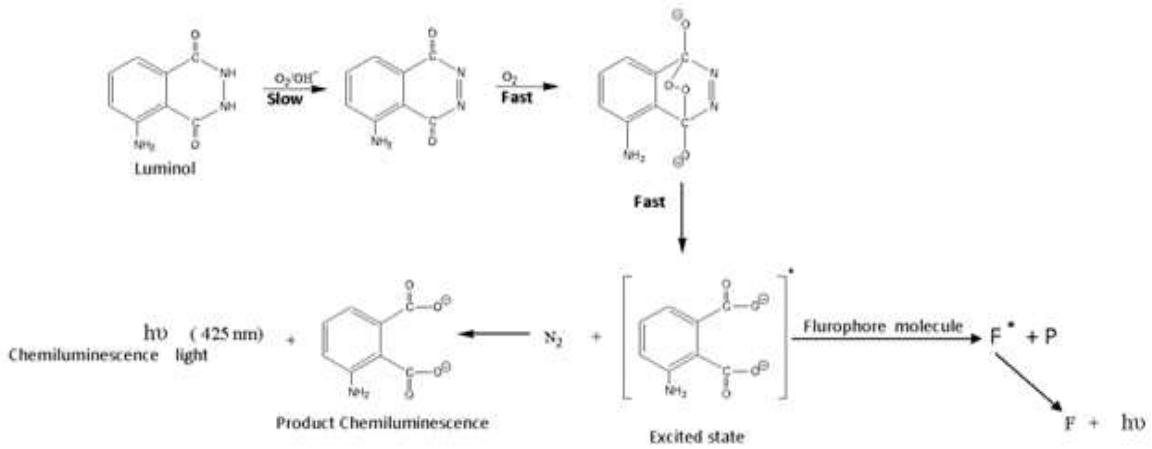


Figure No.1: schematic diagram of the continuous flow injection analysis system with noble chemiluminescence and fluorescence energy transfer three inlet cell that used for determination of H2O2

Figure No.2: A specially designed flow chemiluminescence and fluorescence cell consists of three inlets are arranged two of them are at same level while the third one is at a lower level in the cell



Scheme 1: Proposed mechanism of Luminol –H2O2- Co (II) ion CL- system and insitu fluorescence in the presence of fluorescein molecule

Results and Discussion

Optimization of Variables

Chemical as well as physical parameters were studied by employing the flow injection manifold that was shown in Figure No. 1, in order to obtain the optimum parameters for the conditions of the total luminescence reaction. The reason for this was to avoid any losses in luminescence (chemiluminescence & fluorescence) due to pre-mix step of H_2O_2 with either Co (II) ion or luminol and fluorescein molecule.

Effect of Co (II) ion Concentration on Total Luminescence

Using 0.5mmol.L^{-1} luminol and variable concentration of Co (II) ion as a catalyst and a carrier stream in the manifold design from 5-25 µg.ml^{-1} and fluorescein sodium salt (0.05mmol.L^{-1}) in the third line with a constant concentration of Hydrogen peroxide (0.3mmol.L^{-1}) was used as an oxidant for Luminol to generate the CL-emission as a (sample loop (50µl) , using open valve mode**)** as an internally source to irradiated fluorescein molecule which was used as an acceptor fluorophore to generate insitu fluorescence.

The results are shown in figure No. 3 and Table No. 1, that shows the response profile of emission versus concentration, where clear indication that 15 µg.ml^{-1} of Co(II) ion is the most suitable concentration for maximum total luminescence.

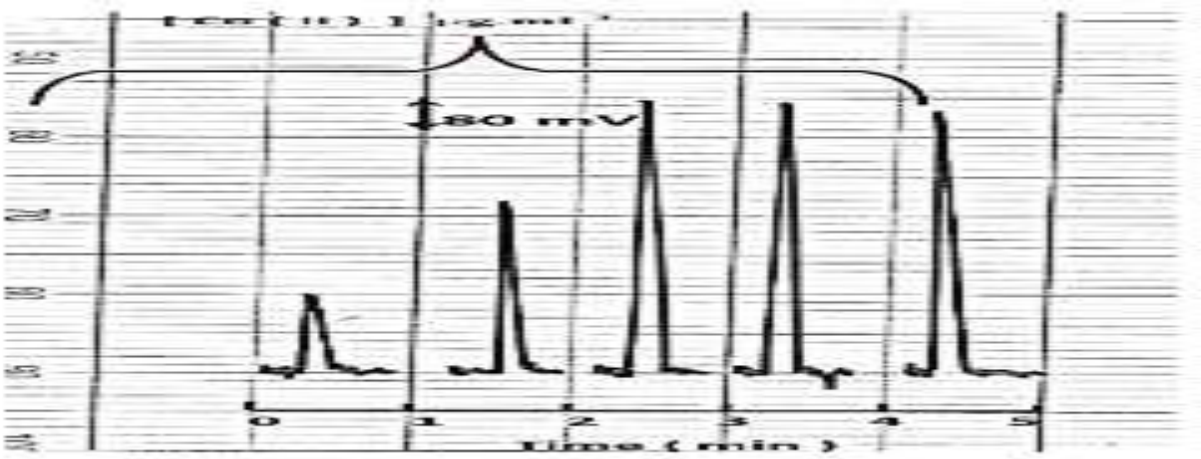


Figure No. 3: variation of total luminescence response- time peak profile versus concentration of Co (II) ion

Table No.1: Variation of the Co (II) ion concentration ontotal Luminescence Using: Luminol (0.5 mmol.L^{-1}) Hydrogen peroxide (0.3mmol.L^{-1}) – Co(II) –fluorescein sodium salt (0.05mmol.L^{-1}) system , with sample volume 50µL at 1.3 , 1.9 , 1.9 ml min^{-1} for Co(II) , Luminol ,& fluorescein respectively

[Co(II)] µg.ml^{-1}	Total luminescence emission expressed as an average peak heights (n=3) ȳi (mV) and confidence interval of the average response (at 95% confidence level) $\bar{y}_i$ (mV)$\pm t_{0.05/2,\ n-1}\ \sigma_{n-1}/\sqrt{n}$
5	190 ± 2.23
10	428 ± 2.45
15	685 ± 2.03
20	680 ± 3.98
25	675 ± 4.56

Effect of Either Luminal and Fluorescein Sodium Salt Concentration on Total Luminescence

Using variable concentration of either luminol (0.1, 0.2 0.5 ,0.7, 1, 5) mmol.L^{-1} in 0.1 mol/ L solution of Na_2CO_3 and fluorescein sodium salt (5, 10, 20, 40, 50, 100, 200) µmol .L^{-1} were prepared in this study, with a constant concentration of Hydrogen peroxide (0.3 mmol.L^{-1}) and optimum concentration of Co(II) ion. A study was carried out to optimize the concentration of luminol and fluorescein that will be used for the rest of this work. Figure No. 4 shows the profile of total Luminescence emission versus concentrations. It indicate that at high concentration of luminol lead to a constant emission therefore 0.7 mmol.L^{-1} most suitable as a light source to provide the UV- light necessary for fluorescence emission and due to unnecessary consumption of high reagent concentration even though that minimum concentration of fluorescein was 40 µmol .L^{-1} is the best to obtain suitable sensitivity .Any increase in fluorescein sodium salt concentration causes a deformed response and decrease of response height, this might be attributed to the self-quenching, self-absorption, and deactivation of electronic excited states that can be occur either by internal or external conversion .In addition due to the saturation of electronic system i.e. a constant saturated signals obtained (Figure No. 5). All of these effects are illustrated by the data in Table No. 2.

Table No.2: Variation of either luminol and fluorescein sodium salt concentration on total Luminescence:*Using: Luminol* - Hydrogen peroxide (0.3mmol.L^{-1}) – Co(II) 15 µg.ml^{-1} – fluorescein sodium salt system , *with sample volume 50µL at 1.3 , 1.9 , 1.9 ml min^{-1} for Co(II) , Luminol ,& fluorescein respectively.*

[Luminol] mmol.L^{-1}	Total luminescence emission expressed as an average peak heights (n=3) ȳi (mV) and confidence interval of the average response (at 95% confidence level) $\bar{y}_i$ (mV)$\pm t_{0.05/2,\ n-1}\ \sigma_{n-1}/\sqrt{n}$	[Fluorescein sodium salt] µmol .L^{-1}	Total luminescence emission expressed as an average peak heights (n=3) ȳi (mV) and confidence interval of the average response (at 95% confidence level) $\bar{y}_i$ (mV)$\pm t_{0.05/2,\ n-1}\ \sigma_{n-1}/\sqrt{n}$
0.1	210 ± 2.35	5	398 ± 3.68
0.2	350 ± 3.56	10	596 ± 4. 32
0.5	685 ± 3.79	20	638 ± 4.98
0.7	776 ± 3.08	40	899 ± 2.24
1	770 ± 4.13	50	778± 3.23
5	765 ± 4.39	100	710 ± 4.67
		200	638 ± 4.45

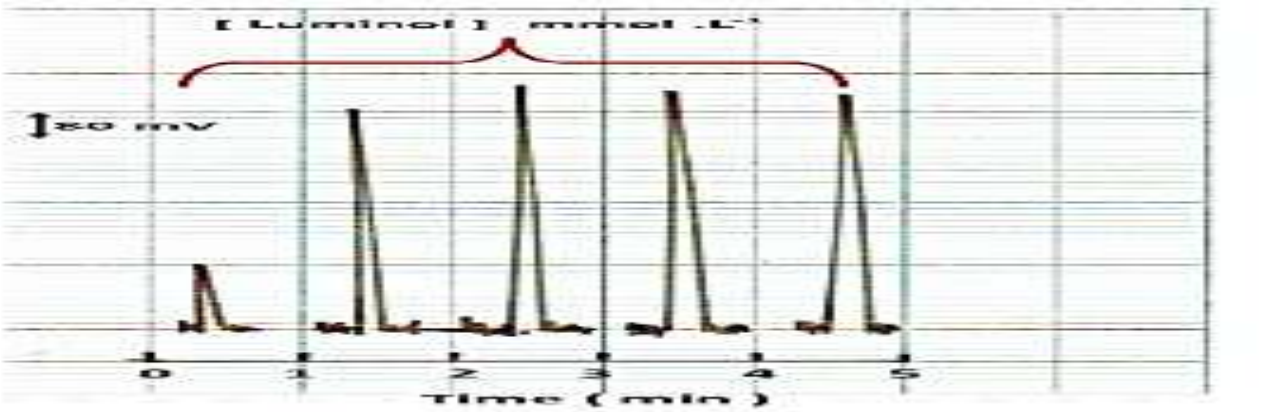


Figure No. 4: Variation of total Luminescence response versus Luminol concentration for some of the used concentration

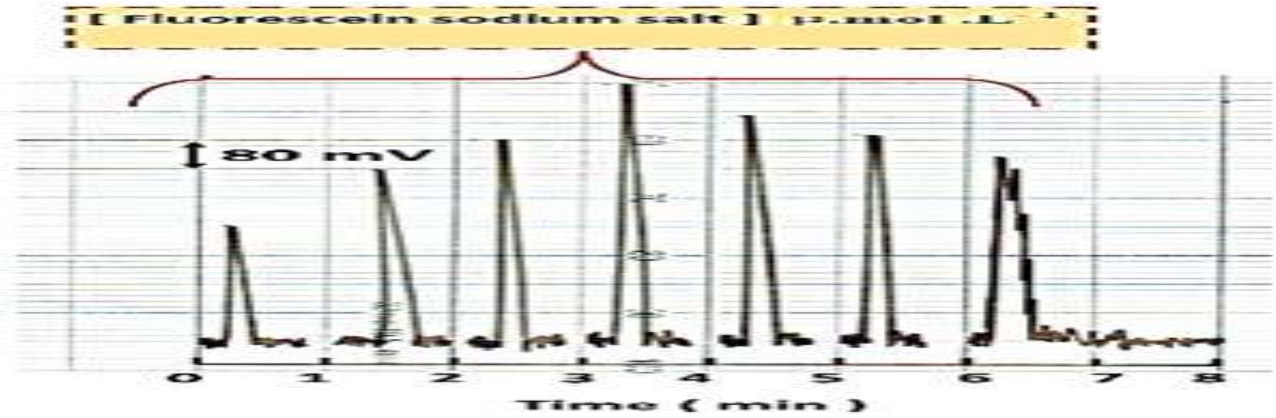


Figure No. 5: Variation of total Luminescence response versus Fluorescein sodium salt concentration

Physical Variable

Effect of Flow Rate & Sample Volume

The experimental parameters for maximum total Luminescence intensity (Chemiluminescence plus in situ fluorescence by the Chemiluminescence irradiation) (determined above) were used .Flow rates of 0.4 – 3.0 ml.min^{-1} for Co (II) ion as a carrier stream, 0.7 – 3.5 ml.min^{-1} for the luminol line and 0.9 – 3.5 ml.min^{-1} for fluorescein sodium salt line. In addition to variable sample volume (50, 75, 100) µl with open valve technique.Figure No. 6 shows the effect of flow rate on the response- time peak profile.

It was noticed that at low flow rates, there were an increase in peak base width (Δt_b).This might be due to the dilution and dispersion due to diffusion which causes an irregular response. While at higher flow rate (> 1.3, 1.9 and 1.9 ml.min^{-1} for the carrier stream of Co (II) ion, luminol and fluorescein sodium salt respectively), although the effect of physical parameter (dilution and dispersion due to convection) was very crucial on the response profile; which that leading to regular response and very sharp maxima. Figure No. 6 shows that the best flow rate for the completion of the reaction of Hydrogen peroxide as an oxidant for Luminol to generate the CL-emission at the presence of Co (II) ion as a catalyst for irradiation of the photon to the fluorescein molecule was 1.5, 2, 2 ml.min^{-1} for the carrier stream of Co (II) ion, luminol and fluorescein sodium salt respectively to obtain a regular response, narrower Δt_b, and minimize the consumption of reactants solutions. The time required from the moment of departure of the sample from the injection valve to the newly specially designed flow chemiluminescence cell is 10 sec.

At the same time, It was noticed that an increase in sample volume led to an increase in the height of profile for total Luminescence without affecting on the response profile of obtained up to the sample volume 75µL. Above 75 µL, there were a broadening at the peak maxima and an increase in the base width (Δt_b) this is illustrated in Figure No. 6 which shows that the optimum volume was 75µL for better response profile and this might be explained to excess of fluorescence emission species , which in turn to stock shift i.e., toward lower frequencies or longer wavelength. All of these obtained data are illustrated in Table No. 3.

Table No.3: Variation of flow rate on total Luminescence: Using: Luminol (0.7 mmol.L^{-1}) - Hydrogen peroxide (0.3mmol.L^{-1}) – Co (II) (15 µg.ml^{-1}) – fluorescein sodium salt (40 µmol .L^{-1}) system, with open valve mode

Speed of peristaltic pump (indication approximate)	Flow rate (mL/min)			Total luminescence emission expressed as an average peak heights (n=3) ȳi (mV) and confidence interval of the average response (at 95% confidence level) $\bar{y}_i$ (mV)$\pm t_{0.05/2,\ n-1}\ \sigma_{n-1}/\sqrt{n}$		
	Luminol line1	Co(II) line2	*Fluorescein Line 3*	*Variable sample volume (µL)*		
				50	75	100
10	0.7	0.4	0.9	395	682	690
15	1.3	1.0	1.4	783	975	920
20	1.9	1.3	1.9	899	1050	1000
25	2	1.5	2.0	1090	1200	1220
30	2.5	2.0	2.4	1020	1100	1075
35	3.0	2.3	2.9	998	1050	1040
40	3.5	3.0	3.5	775	836	800

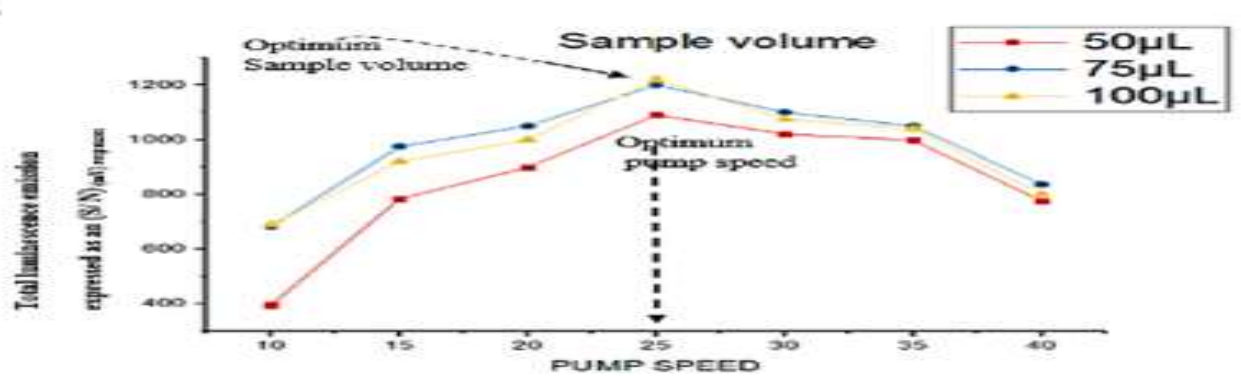


Figure No. 6: Effect of variation of the flow rate on total Luminescence expressed as an average peak heights (mV)

Scatter Plot of Total Luminescence versus Hydrogen Peroxide Concentration

A series of hydrogen peroxide solution having the concentrations of 0.01- 1mmol.L^{-1} .Using the optimum parameters that have been already established in previous section .Figure No. 7-A show the total luminescence vs. time profile for some of the used concentration. In addition to Figure No. 7-B show the total luminescence emission intensity versus hydrogen peroxide concentration that have a correlation coefficient of 0.9969 for the range of concentration ranging from 0.01- 0.5 mmol.L^{-1} or . 10 -500 µmol. L^{-1}.

In which a plot of the total luminescence emission intensity of solutions depends on concentration of the emitting species which in turn to depend on concentration of hydrogen peroxide So, at higher concentration (i. e, more than 0.5 mmol.L$^{-1)}$, the linearity of calibration graph is lost and total luminescence emission intensity in mV then lies below an extrapolation of the straight-line plot. This might be attributed of : two factors: self-quenching , self-absorption and the collisions between excited molecules lead to reabsorbed the energy of fluorescence by other fluorescent molecules .Linear regression equation with acceleration coefficient of determination (C.O.D. = r^2) which is equivalent to 0.9939 was obtained as follow :

Total Luminescence (average peak height (Ŷi) in (mV) = 28.983 ±17.234 + 3798.739 [H_2O_2] mmol.L^{-1} for n= 17 and confidence level 95%

Another mathematical treatment was conducted that an application of Savitzky-Golay [24] smoothed data filtering treatment was applied for each mathematically best fit linear regression equation [25-27]. The plot is shown in Figure No.8-A, B.

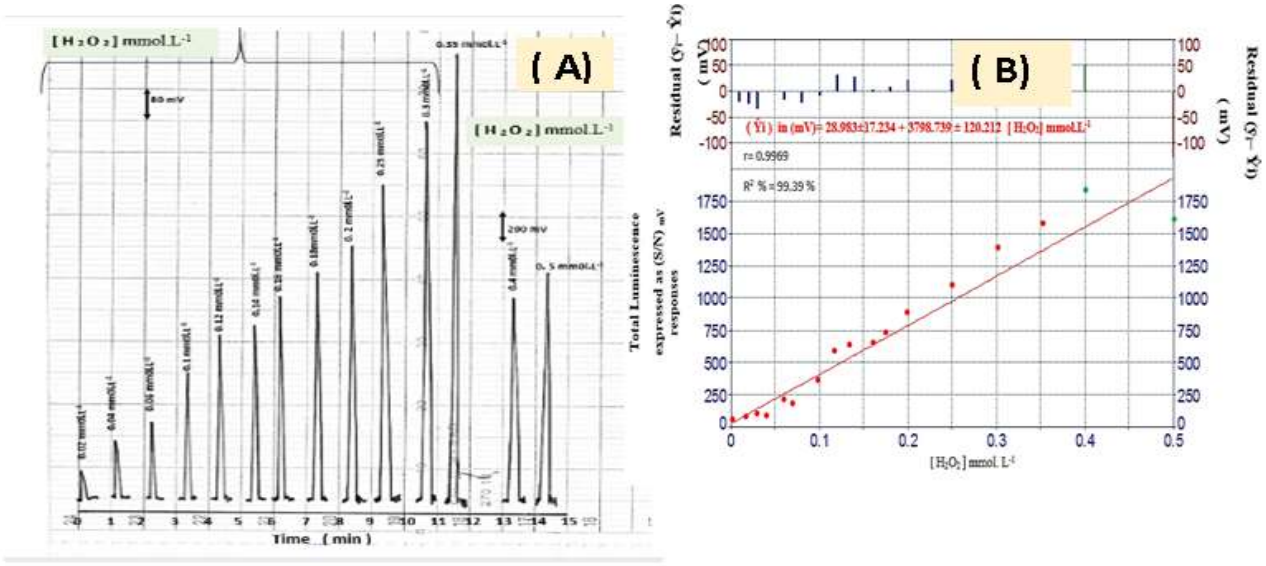


Figure No 7: A: variation of total Luminescence response versus H2O2 concentration B: Effect of H2O2 concentration on the total luminescence expressed as an average peak height in (mV) by linear equation, (residual (ȳi– Ŷi), ȳi: practical value, Ŷi: estimated value) Using 75µl, 15 µg.ml-1 Co (II) & [fluorescein] = 40 µmol .L-1

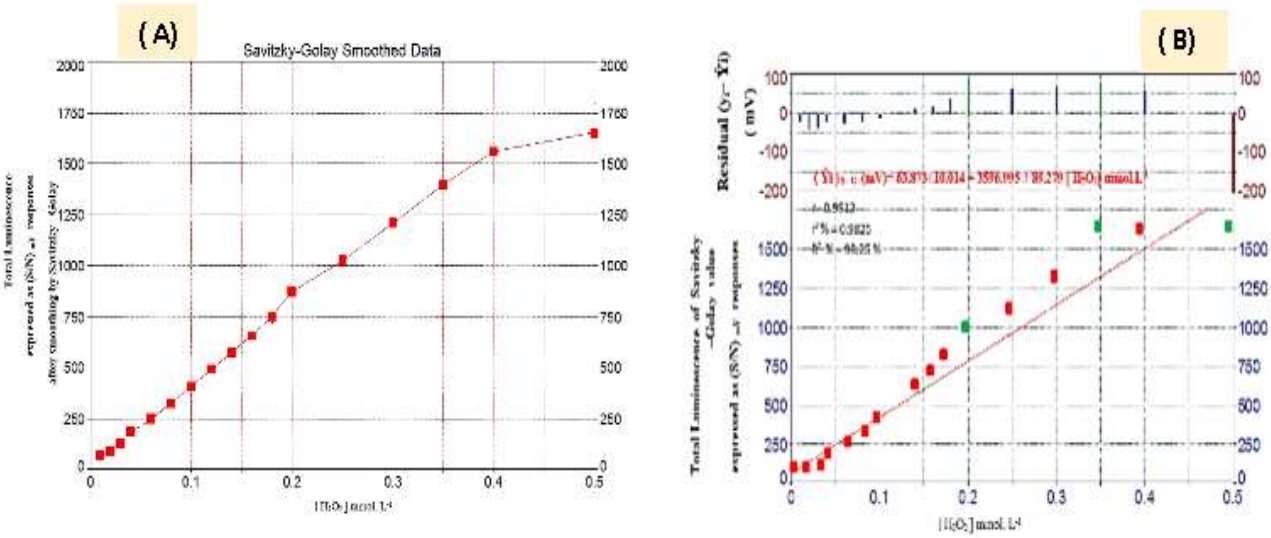


Figure No.8-A, B: Shows variation of Response vs. concentration of H2O2 expressed in mmol.L-1 according to Savitzky – GOLAY smoothening of obtained Lab. Data (A) .While in B: it show the linear regression of Savitzky – GOLAY smoothed data which gives 98.25% of explained data according to chosen equation

Limit of Detection (L.O.D)

Using successive dilution of lowest used concentration in the calibration graph 1.275 ng/ sample was the L.O.D of this used methodology

Repeatability

Repeatability of measurements was studied at two variable concentrations of H_2O_2 solutions at optimum parameters. The repeatability of 0.21% with σ_{n-1} of 2.105 mV obtained for ten successive measurements of 75 µL H_2O_2 of 250 µmol.L^{-1} and of 0.19% with σ_{n-1} of 2.986 mV obtained for ten successive measurements of 75 µL H_2O_2 of 400 µmol.L^{-1}, while figure No shows a kind of response-time profile for the used concentrations. All results tabulated in Table 4 and Figure No 9.

Table 4: Repeatability results of H_2O_2 at optimum parameters by CFIA-CL/fluorescence method

[H_2O_2] µmol.L^{-1}	Total luminescence response expressed as average peak heights (mV)	Standard deviation σ_{n-1}	RSD %	Confidence interval of the mean at 95% $\bar{y}_i \pm t_{0.05/2,\, n-1} \times \sigma_{n-1}/\sqrt{n}$ (n = 10)
250	1000	2.105	0.21	1000 ± 1.336
400	1600	2.986	0.19	1600 ± 1.967

$t_{0.05/2,\, 9}$= 2.262

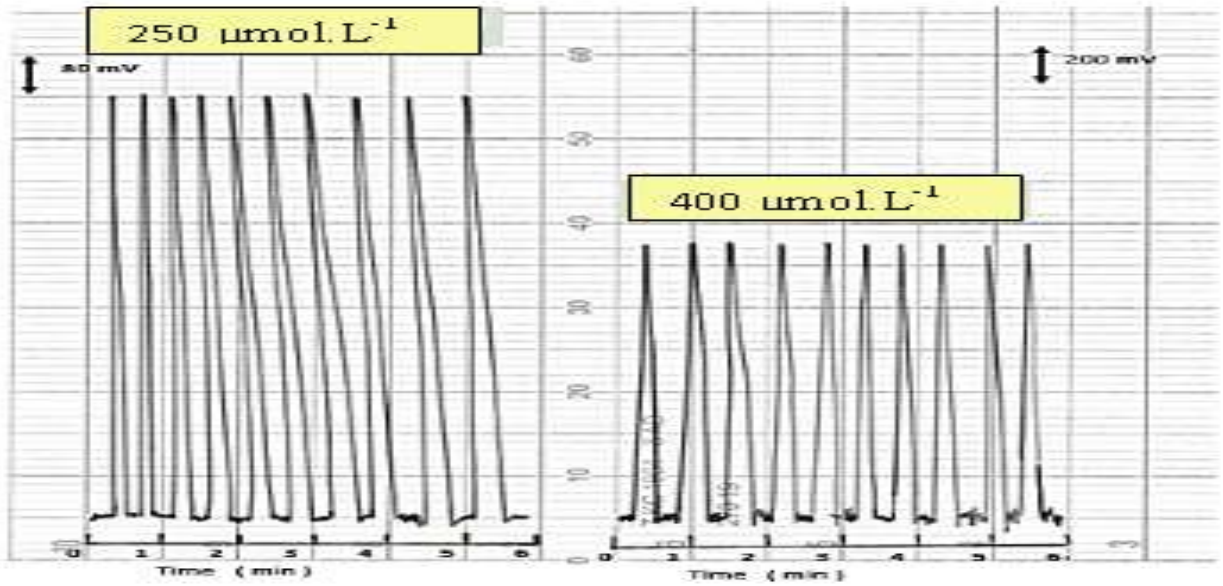


Figure No 9: Total luminescence response -time profile for ten successive repeatable measurements of H2O2; 250 µMol .L-1, 400 µMol .L-1 Using: Luminol(0.7 mmol.L-1) - Hydrogen peroxide – Co(II) (15 µg.ml-1) – fluorescein sodium salt (40 µmol .L-1) system

Analysis of Pharmaceutical Drugs

The established method was used for the determination of H_2O_2 in three different kind of H_2O_2 samples from three different well known manufactures (Baghdad company- 25% ,Al-Amire company- 20% and Al-areje company- 7%) using total luminescence of H_2O_2- Lu - Co (II) ion- Flu. System .And compared with measured the attenuation of incident light (turbidity) as well as reflection of light at two opposite positions and algebraic sum of them [8] by a homemade Ayah $4S_W$-3D-T_{180} - $2N_{90}$ - Solar -CFI Analyser [28] for Determination of Hydrogen Peroxide by Cr (III) - OH^- - H_2O_2 - Ba (II) system . The standard additions method was applied by preparing a series of solutions from each pharmaceutical drug via transferring 0.02 mL (50 $mmol.L^{-1}$) to five volumetric flask (50 ml) , followed by the addition of (0 , 0.02 , 0.03, 0.04, and 0.05 mL) from 50 $mmol.L^{-1}$ standard solution of H_2O_2 in order to have the concentration range from 0-0.05 $mmol.L^{-1}$ of each sample of drugs for the preparation of standard additions calibration plot. The measurements were conducted by both methods. Results were mathematically treated [25-27] for standard additions method and tabulated in Table.5 at confidence interval of 95 %. Paired t-test was used as shown in Table. 6 which show a comparison-treatment of data for the obtained results from both methods with neglecting the difference of origin .It was noticed that there were no significant differences between two methods as shown in column 7 at 95% for the determination of H_2O_2 in pharmaceutical drugs.

Table 5: Results of hydrogen peroxide determination in pharmaceutical drugs by by a homemade design [21] chemiluminescence cell / CFI -Total luminescence measurements method

No. of sample	Company, Country, Percentage%, $[H_2O_2]$ mol..L^{-1}	percentage% $[H_2O_2]$ mol L^{-1} After standardization	Volume draw (mL) to prepare 50 mmol L^{-1} H_2O_2 in 100 mL	Volume draws (mL) to prepare 0.02mmol. L^{-1} H_2O_2 in 50 mL	Total luminescence response expressed as average peak heights (mV)					Practical $[H_2O_2]$ µmol L^{-1} (n=3)	Practical $[H_2O_2]$ mol L^{-1} and percentage at origin sample
					Linear equation $\hat{Y}i$ (mV) = a ± ts_a +b ± ts_b $[H_2O_2]$ mmol/L at confidence level 95% , n – 2						
					Vol draw (mL)from 50mmol/L H_2O_2					In 50 ml	
					$[H_2O_2]$ mmol/L						
					0	0.02	0.03	0.04	0.05	In 100 ml	(Recovery %)
					0	0.02	0.03	0.04	0.05		
1	Baghdad company	20% 5.88		0.02	**63**	**130**	**150**	**179**	**210**	0.023	6.71 22.82 %

	(Iraq) (25%) 7.35 Mol L^{-1}		0.85		65.73± 4.62+3881.08± 110..45[X] r = 0.9976 , r^2 = 0.9951 ,R^2 % = 99.51%					57.03 ±2.321	(114.08 %)
2	Al-Amire company (Syria) (20%) 5.88 Mol L^{-1}	15% 4.41	1.13	0.02	**85**	**150**	**198**	**230**	**260**	0.0235	5.21 17.71% (118.09)
					84.41± 4. 58+3578.38± 124..47 [X] r = 0.9975 , r^2 = 0.9952 ,R^2 % = 99.52%					58.97	
3	Al- Areje company (Iraq) (7%) 2.06 Mol L^{-1}	5% 1.47	3.4	0.02	**50**	**100**	**130**	**150**	**180**	0.019	1.397 4.75% (95.00 %)
					49.73± 9. 12+2581.08± 313.45[X] r = 0.9989 , r^2 = 0.9979 ,R^2 % = 99.79%					47.50 ±3.454	

Ŷi (mV) =Estimated Total emission as an average peak height for (n=3), [H_2O_2] in mmol $.L^{-1}$, r = correlation coefficient (C.C), r^2: coefficient of determination (C.O.D) R^2% = % Capital R- square. $t_{ab} = t_{0.05/2,\ n-2}$ at 95% confidence level

Table.6: paired t –test results based on newly developed methodology (total luminescence) with classical method by Ayah 4SW-3D-T180 - 2N90 – Solar-CFI Analyser using standard additions method for determination of H2O2 in pharmaceutical drugs

Sample no.	Practical [H_2O_2] mol L^{-1} and percentage at origin sample		Xd	□ d	σ_{n-1}	$t_{cal} = \frac{□d\sqrt{n}}{\sigma_{n-1}}$ at 95%	*t_{tab} at 95%
	Total luminescence measurement	*Turbidity ($T_{(0-180)}$) measurement					
1	6.71 22.82 %	6.89 23.43 %	- 0.61	-0.375	0.399	|- 1.627| << 4.303	
2	5.21 17.71 %	5.81 19.75 %	-0.6				
3	1.397 4.75%	1.311 4.46 %	0.086				

$t_{cal} \ll t_{tab}$ (4.303) at 95% , * $t_{tab} = t_{0.05/2\ ,\ 2} = 4.303$

Conclusion

The present paper introduce an alternative high sensitivity flow method for ON – Line determination of H_2O_2 via total Luminescence i.e. chemiluminescence plus the in situ fluorescence by the Chemiluminescence irradiation of luminol G.B. - H_2O_2- Co (II) ion system. An improved linearity and detection limit compared with the available literatures cited in the introduction. Comparison of results obtained via the new three inlet chemiluminescence cell can be used as a new methodology in flow injection analysis with high trust ability and confidence.

References

1. WW Ward (1985) In “Chemiluminescence and Bioluminescence”, Dekker, (J.G. Burr. Editor), New York, 321-329.
2. FM McCapra, KD Perring (1985) In “Chemiluminescence and Biolumin”, Dekker, (J.G. Burr Editor), New York, 360.
3. AM Garcia-Campana, WRG Baeyen (2000) Principle and recent analytical applications of chemiluminescence, Luminescence Spectroscopy, Analysis, 28 (8): 686- 698.
4. V Ya Swyapintokh, ON Karpukhin, LM Postnikov, VF Tesepalov (1968) “Chemiluminescence Techniques in chemical Reaction”, consultants, Burean, New York.
5. D Rendell (1987) “Fluorescence and phosphorescence spectroscopy”, John Wiley, New York, 1.
6. K Nakashima, K Imai (1993) In “Molecular luminescence spectroscopy”, part 3, (S.G. Schulman, Ed.), New York: Wiley, chapter 1.
7. TJN Carter, LJ Kricka (1982) “Analytical application of chemilumin- escence”, Dekker, New York, 136.
8. N ST Al-Awadie, FK Ahmed (2015) Determination of hydrogen peroxide in some local pharmaceutical disinfectants by continuous flow injection analysis via turbidimetric (T_{180^o}) and scattered light effect at two opposite position ($2N_{90^o}$) using Ayah $4S_w$-3D-T_{180^o}-$2N_{90^o}$-Solar-CFI analyser, Iraqi journal of science, 56(1C): 577-592.
9. H Hwang, PK Dasgupta (1985) fluorimetric determination of trace hydrogen peroxide in water with FIA, Anal. Chim. Acta, 170: 347-352.

10. IMA Shakir, NST Al-Awadie (1997) New mode in semi automated on line analysis (Study and Application). M.Sc thesis, Baghdad University.
11. IMA Shakir, NST Al-Awadie (2005) New mode for the study of chemiluminescence reactions by tracking (distance or time) via multi detection points through optical fibers in a completely newly designed cell. Ph.D thesis, Baghdad University.
12. IMA Shakir, NS Turkie (2000) New mode of on- line automation analysis: chemiluminescence determination of hydrogen peroxide using continuous flow and chemiluminescence detection via generation of luminol chemiluminescence , Iraqi J. Sci., 41A (3): 29-47.
13. IMA Shakir, NST Al-Awadie (2018) Junction cell for ON- Line fast Chemiluminescence reaction and light energy transfer by optical fiber, Patent present to Central Organization for Standardization and Quality control-Baghdad- Iraq G01N2021/754, G01N21/76.
14. M Stigbrand, E Ponten, K Irgum (1994) 1, 1 oxalyldiimidazole as Chemiluminescence reagent in the determination of low hydrogen peroxide by FIA, Anal. Chem., 66(10): 1766-1770.
15. IMA Shakir, FH Zaineb (2015) Ultra trace chemi and total luminescence determination of selected metal ions via entrapment of chemiluminescent reagent in poly acrylic acid polymer. Ph.D, Baghdad University.
16. IMA Shakir, NM Yousif (1993) On-line ultra trace determination of Co(II) ion and hydrogen peroxide through simulated insitu fluorescence using fluorphor molecule individually or through chain energy transfer process, M.Sc thesis, Salahaddin university, 125.
17. IMA Shakir, FS Sirwan (1991) Chemiluminescence in fluorescence excitation in the visible region and its application in chemical analysis, M.Sc thesis, Salahaddin University.
18. IMA Shakir (2015) New mode of on-line automated : chemilumino-fluoro metric method for the determination of hydrogen peroxide by fluorescence energy transfer using poly acrylic acid gel beads, Iraqi journal of science, 56(2B):1289-1302.
19. IMA Shakir, NST Al-Awadie (1999) New mode of on-line determination of hydrogen peroxide by total luminescence utilizing the chemiluminescence energy released ftom 3-amino phthylhydrazide Oxidation as internal source for irradiation of Rhodamine -6G molecule, Iraqi journal of science, 40(2): 223-239.
20. IMA Shakir, IMA, NST Al-Awadie, NST (2001) New mode of on-line automation determination of hydrogen peroxide by total luminescence utilizing the chemiluminescence energy released from luminol oxidation as internal source for irradiation of Rhodamine -B molecule, Iraqi journal of science, 42A(2): 23-57.
21. IMA Shakir, NST Al-Awadie (2017) Chemiluminescence (CL) and FluoRescence Energy Transfer (FRET) cell for Continuous Flow Injection Analysis (CFIA). Patent present to Central Organization for Standardization and Quality control-Baghdad- Iraq, G01N27/ 76.
22. L Erdey (1972) In "Indicator", (E. Bishop, Editor), pergamon, Oxford, 714
23. TA Turney (1965) Oxidation mechanisms, Butterworth's & Co, London, 50.
24. Abraham Savitzky, Marcel JE Golay (1964) Smoothing and differentiation of data by simplified least square procedures, Anal. Chem., 36 (8): 1627-1639.
25. AG Bluman (1997) "Elementary Statistics", 3rd Ed., WCB/MC Graw-Hill, New York.
26. LR Jaisingh (2000) "Statistics for the Utterly Confused", 2nd Ed., McGraw-Hill, London.
27. D Brink (2010) "Essentials of Statistics", Ventus Publishing ASP, London.
28. IMA Shakir, NS Turkey (2014) "Ayah $4S_W$-3D-T_{180^o}-$2N_{90^o}$ -Solar cell - CFI Analyser", Patent present to Central Organization for Standardization and Quality control-Baghdad-Iraq, G01N21/ 00.

