

A Sensitive Electrogenerated Chemiluminescence Assay For Determination of Melanin in Natural and Biological Samples

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A rapid, simple and highly sensitive flow-injection (FI) electrogenerated chemiluminescence (ECL) method was developed for the determination of melanin, a biological pigment present in the skin, eyes, hair, scales and it is responsible for color of them. The proposed method was based on the ECL reaction of $\text{Ru}(\text{bpy})_3^{2+}$ with melanin in an acidic medium. The different experimental parameters affecting the ECL intensity were carefully studied and incorporated into the procedure. The method permits the determination of $0.01 - 2.0 \mu\text{g mL}^{-1}$ and $3.0 - 35 \mu\text{g mL}^{-1}$ of melanin with correlation coefficients (r) > 0.9999 . The lower limit of detection (LOD) is $0.0005 \mu\text{g mL}^{-1}$ ($S/N = 3$) and the lower limit of quantitation (LOQ) is $0.01 \mu\text{g mL}^{-1}$. The proposed method was successfully applied to the determination of melanin in *Sepia Officinalis* and natural samples.

Keywords: Flow-injection electrogenerated chemiluminescence; Melanin; Tris(2,2'-bipyridyl) ruthenium(II); Natural samples; Biological samples

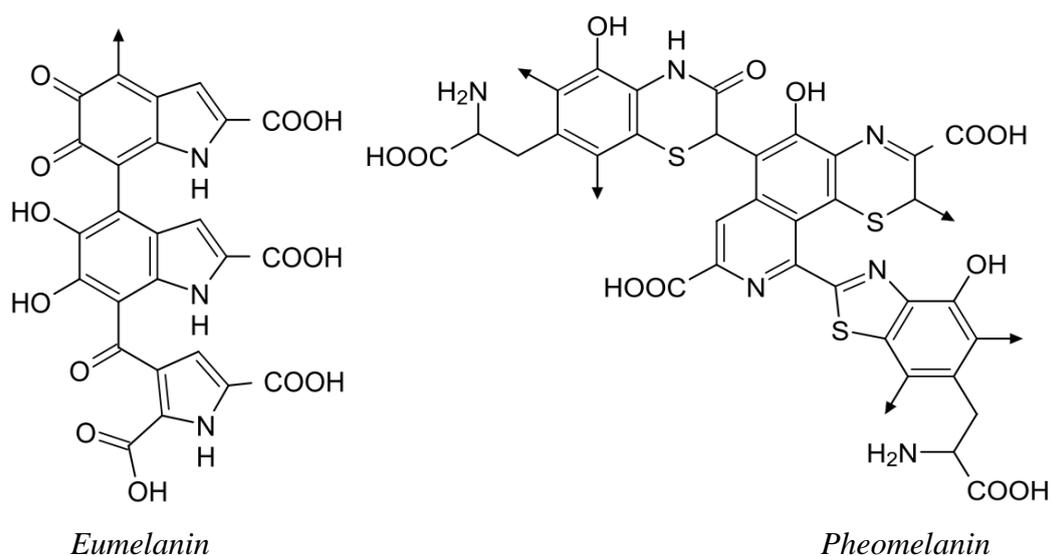
1. INTRODUCTION

Melanin is a biological pigment present in the skin, eyes, hair, scales and in the internal structures such as brain and inner ear [1,2], and it is responsible for color of them. Melanin is an irregular light-absorbing polymer containing indoles and other intermediate products derived from the oxidation of tyrosine. Melanin is widely dispersed in the animal and plant kingdoms [3], and it is an important key to human survival.

Melanin is synthesized in melanocytes, specialized skin cells located in the basal layer of the epidermis in mammals and birds which produce two chemically distinct types of melanin, black to dark-brown eumelanin and yellow to reddish-brown pheomelanin [4]. Most natural melanins are mixtures of eumelanin and pheomelanin [5], both eumelanin and pheomelanin are derived from the

common precursor dopaquinone (DQ) formed by oxidation of the common amino acid L-tyrosine by tyrosinase. The type of melanin and the structure, size, quantity, and distribution not only determine skin color, hair, and eyes in animals, but also play an important role in photoprotection, melanin protects skin against UV-induced damages acting as a sunscreen, free radical scavenger, and antioxidant, whereas pheomelanin is believed to be carcinogenic after UV radiation [6,7]. Melanin can play many different roles in microorganisms because of its unique physico-chemical properties [8].

Melanin is unique biopolymer, a macromolecule with undefined structure and molecular weight. Eumelanin is a very heterogeneous polymer consisting of different oxidative states of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, and pyrrole units derived from their peroxidative cleavage [9]. Pheomelanin is derived from the sulfur containing cysteinyl-dopa (CD), and again is thought to be heterogeneous macromolecule. Eumelanin and Pheomelanin are having the following structures:



Melanin has many biological functions, including coloring the skin and hairs or feathers, strengthening plant cell walls and insect cuticle, absorbing light ultraviolet (UV) and visible spectrum (VIS), and so providing photoreceptor shielding, thermoregulation, photoprotection and camouflage, and it is a powerful cation chelator and may act as a free radical sink. Melanin is used commercially as a component of photoprotective creams, although mainly for its free radical scavenging rather than its light absorption properties. The pigment is also a potential target for anti-melanoma therapy [3].

Few analytical methods have been reported for the determination of melanin in pure synthetic form, in natural samples and in biological samples. Most of the reported methods are spectroscopic and chromatographic methods.

Ito et al.[10] described a spectrophotometric method for assaying eumelanin in tissue samples. Ozeki et al. [11] used a spectrophotometric method for quantified detection of eumelanin and pheomelanin. Photoacoustic spectroscopy was used for the determination of melanin in human hair [12] and an electron energy loss spectroscopy (EELS) method was used for determination of eumelanin and pheomelanin in melanosomes [13]. The chromatographic methods used for melanin

determination include: high performance liquid chromatography with electrochemical detection (HPLC/ECD) [14-20], high performance liquid chromatography with UV detection (HPLC/UV) [21-23], high performance liquid chromatography with fluorimetric detection [24] and capillary electrophoresis (CE) [25,26]. The objective of this study is to develop a new analytical procedure for the determination of melanin in pure synthetic form, in natural samples and in biological samples. The investigation includes the usefulness of electrogenerated chemiluminescence technique. Also, it includes a study of experimental parameters that affect the selectivity, accuracy and precision of the developed procedure.

Electrogenerated chemiluminescence (ECL) is a powerful detection technique for analytical determinations because of its very low detection limit, its speed of analysis and its wide linear working range that can be achieved using relatively simple instrumentation [27].

One of the most interesting CL reactions involves the oxidation of tris(2,2'-bipyridyl) ruthenium(II), or $\text{Ru}(\text{bpy})_3^{2+}$, to $\text{Ru}(\text{bpy})_3^{3+}$, which is then reduced by an analyte species with a subsequent emission of light [28]. However, $\text{Ru}(\text{bpy})_3^{2+}$ ECL is observed when $\text{Ru}(\text{bpy})_3^{2+}$ reacts with $\text{Ru}(\text{bpy})_3^+$ and yields an excited state $\text{Ru}(\text{bpy})_3^{2+*}$. ECL emission can also be obtained when a variety of oxidants and reductants react with the reduced or oxidised form of $\text{Ru}(\text{bpy})_3^{2+}$. Either the reductant or the oxidant can be treated as an analyte [29]. This study describes the development of simple flow-injection electrogenerated chemiluminescence (FI-ECL) method for the determination of melanin. The method was based on the ECL generated by the reaction of melanin with acidified $\text{Ru}(\text{bpy})_3^{2+}$ after electrolysis.

To the best of our knowledge, no chemiluminescence method has been yet described for the determination of melanin in pure synthetic form, in natural samples and in biological samples. The present work describes a novel and highly sensitive method for the determination of melanin, using flow injection analysis (FIA) with electrogenerated chemiluminescence (ECL). The ECL method is based on the reaction between tris (2,2'-bipyridyl) ruthenium (II), $[\text{Ru}(\text{bpy})_3^{2+}]$ and melanin as a chemical reductant.

2. EXPERIMENTAL

2.1. Materials and Reagents

All the chemicals used were of Analytical Reagent grade and distilled water was used throughout. Synthetic pure sample of melanin was kindly supplied by (SIGMA Chemical Co., USA). An aqueous solution of 1×10^{-2} M Tris(2,2'-bipyridyl) dichlororuthenium(II) hexahydrate, $[\text{Ru}(\text{bpy})_3^{2+}]$ (ALDRICH, USA), was prepared in 0.05 M sulfuric acid (BHD Ltd., UK). Sodium hydroxide (BDH, UK), 5 M aqueous solution was used. Sepia Officinalis samples were supplied from (SIGMA Chemical Co., USA.). Natural samples of melanin were commercially available.

2.2. Instrumentation

The ECL measurements were made with a flow–injection cell and electrode control circuits built in-house. Potentials were applied to the electrodes using a two identical platinum electrodes potentiostat.

Oxidation of $\text{Ru}(\text{bpy})_3^{2+}$ occurs at the surface of one of the platinum electrodes and reduction of $\text{Ru}(\text{bpy})_3^{2+}$ occurs at the surface of the other platinum electrode. A voltage suitable period of time allowed for oxidation of $\text{Ru}(\text{bpy})_3^{2+}$ to $\text{Ru}(\text{bpy})_3^{3+}$. A FI-ECL analyzer which features two basic components, detector housing and a flow-through system which allows mixing of the sample with $\text{Ru}(\text{bpy})_3^{3+}$ solution and then combination just before the detector. The flow cell was backed by a mirror for maximum light collection and a sensitive photomultiplier tube (PMT) (Thorn EMI, 9789 QB) for measurement of the emitted light intensity.

The PMT was operated at 1100 V, provided by a stable high voltage power supply (Thorn EMI, Model PM 28BN). No wavelength selection was involved. A 1-channel peristaltic pump (Gilson MiniPuls 3MP4) was used to deliver the reagent.

A solenoid activated rotary valve (Rheodyne 5020) was used to inject the sample solution through a carrier stream of 1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ in 0.05 M H_2SO_4 . Polytrifluoroethylene tubing (PTFE), (0.06 mm i.d.) was used throughout the remainder of the manifold and a strip-chart recorder (REC111, Amersham Pharmacia Biotech, REC111) was fed to the PMT and the peak heights were measured manually. Other instruments used were: analytical balance (Denver Instrument, U.S.A) and a stirrer (Snijders, 34531).

2.3. Standard Solution

A stock solution containing 0.01 g in 100 mL ($100 \mu\text{g mL}^{-1}$) synthetic pure sample of melanin was prepared in 2 mL of 1 M sodium hydroxide (NaOH) and left for one day for complete dissolution. This solution was stable when kept in the refrigerator for one week.

2.4. Procedures

2.4.1. General Procedure

The ECL reagent (1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ in 0.05 M H_2SO_4) solution (a carrier) was feed by pump at a flow rate of $3.7 \mu\text{L min}^{-1}$. $\text{Ru}(\text{bpy})_3^{2+}$ was oxidized to $\text{Ru}(\text{bpy})_3^{3+}$ using platinum electrodes and a suitable voltage 0.8V during 80 min.

Working standard solutions of melanin were prepared from the stock solution in the ranges (0.01 – 2.0 and 3.0 – 35 $\mu\text{g mL}^{-1}$). A 250 μL portion of each sample concentration was injected into a stream of reagent solution and the resulting peak heights were measured. Calibration graphs were prepared by plotting the ECL intensities (mV) against the sample concentrations. Alternatively, the corresponding regression equations were calculated.

2.4.2. Application to the Analysis of Melanin in Biological Samples (*Sepia Officinalis*)

An accurately weighed amount of the powder of *Sepia Officinalis* equivalent to 0.01g of sample, was transferred into a 100 mL volumetric flask. 2 mL of 1 M NaOH was added to the flask and left for one day for complete dissolution and then completed to the mark with distilled water, then proceeded as described under general procedure . The nominal content was calculated either from the previously plotted calibration graphs or using the corresponding regression equations.

2.4.3. Application to the Analysis of Melanin in Natural samples (*Nigella sativa* and *Helianthus annuus*)

An accurately weighed amount of powdered samples of *Nigella sativa* (black seeds) or *Helianthus annuus* (sunflower seeds) equivalent to 1g, were transferred into two different flasks. Melanin was extracted by adding 10 mL of 5 M NaOH to each flask and the flasks were shaken for 1 min and left for one day until the contents dissolved. The mixture of each flask was filtered in a separate conical flask by using a filter paper, then 5 mL of 2 M NaOH were added to each flask for two times. Each solution was transferred into a 100 mL volumetric flask and volume was completed with distilled water, and then analyzed according to the general procedure described above. A blank experiment was carried out adopting the above procedure. The nominal contents of melanin were determined either from the previously plotted calibration graphs or using the corresponding regression equations.

3. RESULTS AND DISCUSSION

The ECL signal for the blank samples of $\text{Ru}(\text{bpy})_3^{2+}$ solution increased gradually with increasing the oxidation time of the reagent up to 80 min after which the signal became stable. The optimum applied voltage was measured at the maximum oxidation peak, which gave the best response.

A weak ECL signal (recorded as a base-line) was appeared when the reagent solution was flowed in the FIA-ECL system. This signal was notable increased when melanin was injected, and gave a peak which height increased proportionally with the melanin concentration.

Maximum CL intensity was obtained when the sample was injected into a stream of acidic 1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ and mixed prior to the detector . Various chemical and instrumental parameters affecting the ECL intensity were investigated and optimized for the determination of the studied samples.

3.1. Configuration Designs

A schematic diagram of a FIA-ECL system is shown in Fig 1. The flow injection configuration used for the determination of melanin, was so designed to provide different reaction conditions for magnifying the ECL signal generated by the reaction.

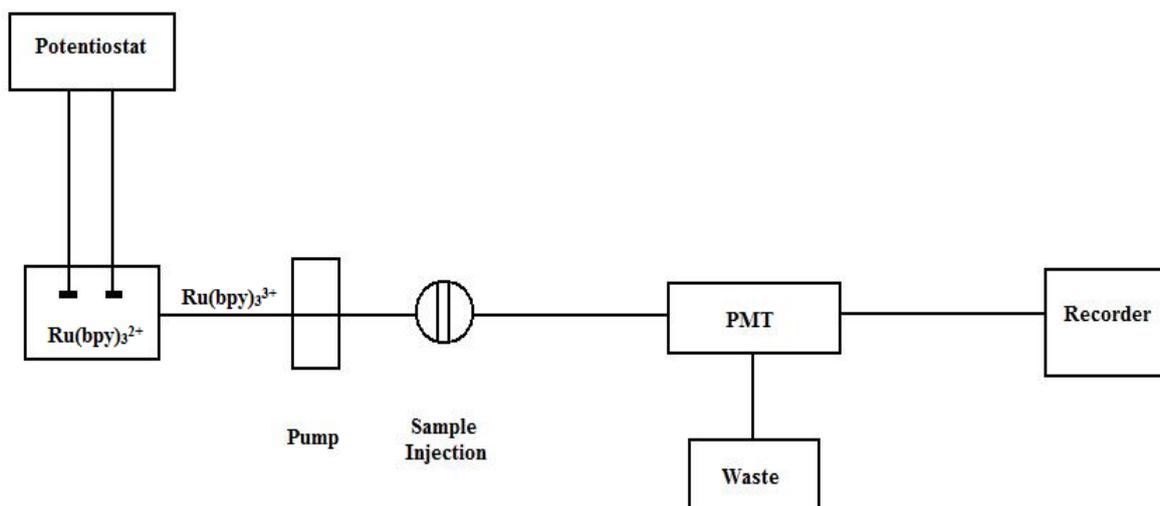


Figure 1. Schematic diagram of the FIA-ECL system for ECL determination of melanin.

ECL intensity signal was obtained when the sample was injected and mixed into a stream of Ru(bpy)₃²⁺ solution prior to the detector. A voltage was applied to the platinum working electrodes in the solution of Ru(bpy)₃²⁺ to initiate the ECL reaction. A weak ECL radiation emitted from this reaction is continuously recorded as the base line. When the sample solution is injected into the reagent stream, the intensity is enhanced in proportional to the concentration of melanin.

3.2. Optimization of Experimental Variables

The effect of experimental conditions on the ECL intensity was studied. A series of experiments were conducted to establish the optimum analytical variables. The parameters optimized included the best oxidation potential of Ru(bpy)₃²⁺, the oxidation time of Ru(bpy)₃²⁺ which depends on platinum electrode surface when a potential is applied, the reagents concentrations and some physical variables, including the flow rate, the sample volume and reaction coil length to PMT where in each time changing one variable in every turn and keeping the others at their optimum values.

3.2.1. Effect of the Oxidation Potential of Ru(bpy)₃²⁺

The applied potential for oxidation of Ru(bpy)₃²⁺ was investigated, the highest ECL emission was obtained at 0.8 V. This potential was used for oxidation of Ru(bpy)₃²⁺ to Ru(bpy)₃³⁺ solution for melanin determination.

3.2.2. Effect of the Time of Oxidation of Ru(bpy)₃²⁺

The highest intensity of the emitted red light was investigated following the General procedure (section 2.4.1) and measuring the ECL intensity every 10 min. It was found that the highest intensity

was obtained after 80 min as the oxidation of $\text{Ru}(\text{bpy})_3^{2+}$ to $\text{Ru}(\text{bpy})_3^{3+}$ was completed, after which the intensity was seemed to be constant with increasing time.

3.2.3. Effect of Sulfuric Acid Concentration as a Solvent of $\text{Ru}(\text{bpy})_3^{2+}$

$\text{Ru}(\text{bpy})_3^{2+}$ is readily soluble in water and acids. Sulfuric acid was used to dissolve $\text{Ru}(\text{bpy})_3^{2+}$ and increase its oxidation potential. The effect of sulfuric acid concentration on the ECL emission for melanin determination was studied in the range 5×10^{-3} - 2 M. It was found that the ECL intensity increases with increasing concentrations of sulfuric acid and the highest ECL emission was obtained with 0.05 M sulfuric acid (Fig 2). Higher concentrations than 0.05 M sulfuric acid will cause incomplete mixing of reagent and analyte (viscosity increases) leading to incomplete reaction. Thus, this concentration was used in the preparation of the reagent solution for melanin determination.

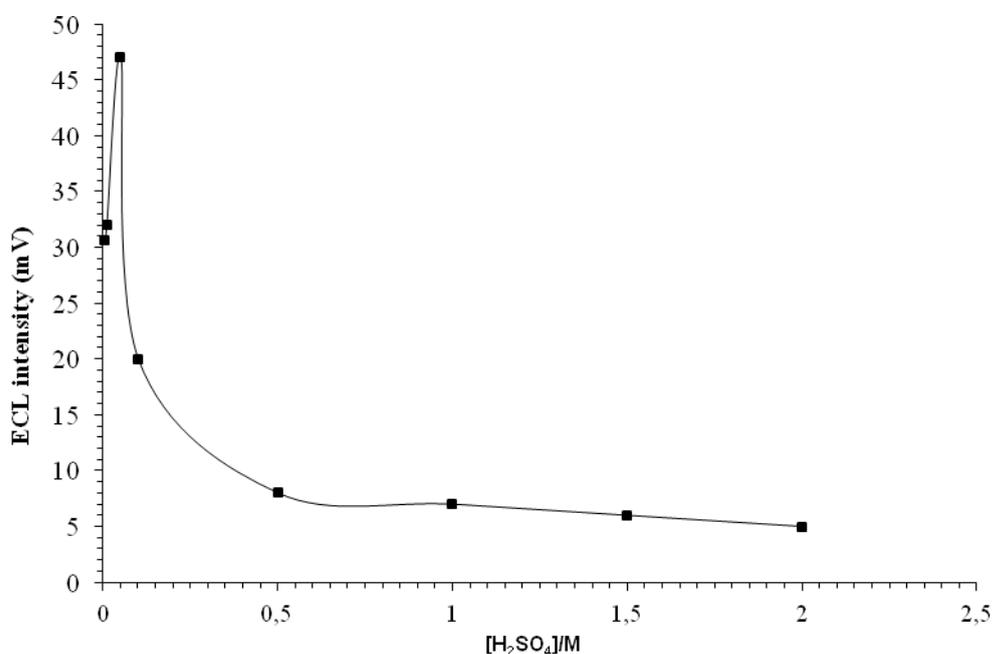


Figure 2. Effect of H_2SO_4 concentration as a solvent of $\text{Ru}(\text{bpy})_3^{2+}$ on the ECL intensity of melanin ($15 \mu\text{g mL}^{-1}$), $\text{Ru}(\text{bpy})_3^{2+} 1 \times 10^{-3}$ M, flow rate 3.7 mL min^{-1} and loop size $250 \mu\text{L}$.

3.2.4. Effect of $\text{Ru}(\text{bpy})_3^{2+}$ Concentration

The effect of $\text{Ru}(\text{bpy})_3^{2+}$ concentration on the ECL intensity of melanin was studied using different concentrations of $\text{Ru}(\text{bpy})_3^{2+}$ in the range 1×10^{-4} to 1×10^{-2} M prepared in 0.05 M sulfuric acid. It is obvious from Fig 3 that the ECL signal was increased with increasing of $\text{Ru}(\text{bpy})_3^{2+}$ concentration and the highest intensity was observed with 1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ after which the ECL

signal was decreased. Higher concentrations than 1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ will cause quenching of the ECL intensity and this may be due to self absorption of light.

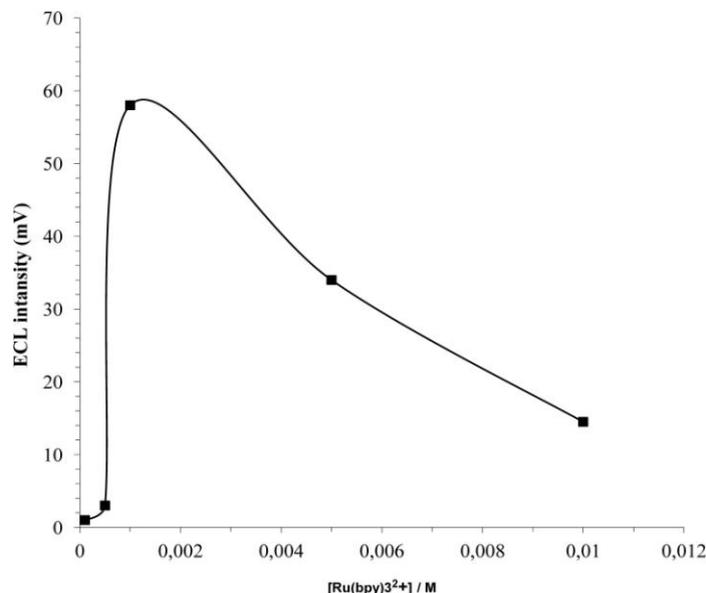


Figure 3. Effect of $\text{Ru}(\text{bpy})_3^{2+}$ concentration on the ECL intensity of melanin ($15 \mu\text{g mL}^{-1}$), flow rate 3.7 mL min^{-1} and loop size $250 \mu\text{L}$.

3.2.5. Effect of Flow Rate

Once the concentrations of reagents were optimized, the effect of the flow rate was studied. The flow rate is an essential parameter, therefore its variation has a great influence on the intensity of the light emitted. The effect of the flow rate was studied keeping all other parameters constant. The solution of reagent (1×10^{-3} M $\text{Ru}(\text{bpy})_3^{2+}$ in $0.05 \text{ M H}_2\text{SO}_4$) was introduced into the manifold at different flow rates. It is found that as we increase the flow rate the emission intensity increased up to 3.7 mL min^{-1} (the pump speed is 20 rpm) after which it starts to decrease. This flow rate is sufficiently high flow rate and is used to enable the excited product to reach the photomultiplier detector in a minimum time, hence achieving maximum collection of the emitted light. So, 3.7 mL min^{-1} was chosen as the optimum flow rate without much consumption of reagent.

3.2.6. Effect of Sample Volume

The variation of the ECL emission with the injected sample volume was studied in the range $10 - 600 \mu\text{L}$ by changing the length of the sample loop connected to the injection valve. It was found that the ECL intensity of melanin was increased sharply with the increase of the sample volume between $25 - 250 \mu\text{L}$. Above $250 \mu\text{L}$ ($250 - 600 \mu\text{L}$), there was a slight increase in the ECL intensity which was considered almost constant. Therefore, $250 \mu\text{L}$ was chosen as the optimum sample volume for a complete reaction without much consumption of analyte.

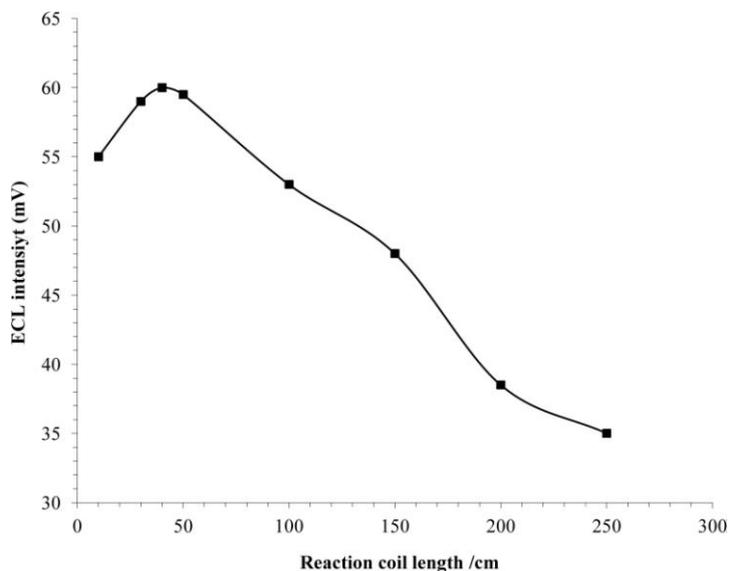


Figure 4. Effect of the reaction coil length on the ECL intensity of melanin ($15 \mu\text{g mL}^{-1}$), $\text{Ru}(\text{bpy})_3^{2+}$ $1 \times 10^{-3} \text{ M}$, flow rate 3.7 mL min^{-1} and loop size $250 \mu\text{L}$.

3.2.7. Effect of Reaction Coil Length to PMT

The effect of reaction coil length was studied in the range 10–250 cm. Fig. 4 shows the effect of reaction coil length on the ECL intensity. Here again it is clear that when the reaction occurs it produces light away from the photomultiplier tube if the reaction coil used is longer than 50 cm. Therefore, 40 cm length were chosen to enable the excited product to reach the detector in a minimum time and hence achieving maximum collection of the emitted light.

3.3. Validation

3.3.1. Linearity and Range

The calibration graph for determination of melanin by the proposed method was constructed by plotting the ECL intensity versus the concentration of melanin. The graph was found to be rectilinear over the concentration ranges cited in Table 1.

Statistical analysis [30] of the data gave high value of the correlation Coefficient (r) of the regression equations and small values of standard deviations of intercept (δ_a) and standard deviations of slope (δ_b) as shown in Table 1. These data proved the linearity of the calibration graphs.

3.3.2. Accuracy and Precision

To prove the accuracy of the proposed method, the % error was determined over the concentration range $0.01 - 35 \mu\text{g mL}^{-1}$. The % errors were $-1.13 - 0.89\%$ (Table 2).

Table 1. Analytical performance data for the FIA-ECL determination of melanin.

Parameter	Short-range	Long-range
Linear range ($\mu\text{g mL}^{-1}$)	0.01 – 2.0	3.0 – 35
Regression equation, $I^a = a + b C$	$I = 1.714 + 18.496 C$	$I = 50.99 + 4.085C C$
Correlation coefficient (r)	0.99997 ^b	0.99996 ^c
δ_a : standard deviation of intercept	0.065	0.303
δ_b : standard deviation of slope	0.069	0.014
LOD ^d ($\mu\text{g mL}^{-1}$)	0.0005	
LOQ ($\mu\text{g mL}^{-1}$)	0.01	

^a Intensity (mV).^b 6 data points.^c 8 data points^d S/N = 3.**Table 2.** Analysis of melanin in pure samples by the proposed FIA-ECL method.

Concentration taken ($\mu\text{g mL}^{-1}$)	Concentration found ($\mu\text{g mL}^{-1}$)	% Error ^a	% Found ^b
			Proposed method
0.01	0.01	0.67	100.6
0.1	0.10	0.89	100.8
0.5	0.50	0.41	100.4
1.0	0.98	-1.13	98.8
2.0	2.00	0.25	100.2
10	10.04	0.41	100.4
25	24.97	-0.10	99.8
35	35.01	0.03	100.0
Mean \pm S.D.			100.1 \pm 0.62

^a Calculated as $[(\text{measured value} - \text{true value})/\text{true value} \times 100]$.^b Each result is the average of the three separate determinations

Intraday and interday precisions were assessed using three concentrations with three replicates of each concentration. The relative standard deviations (% RSD) were found to be very small indicating reasonable repeatability and high precision of the proposed method (Table 4).

3.3.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD was determined practically by establishing the minimum level at which the analyte can reliably be detected (signal-to-noise ratio is 3:1). LOQ is the lowest amount of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions; the results are shown in Table 1.

Table 3. Precision data for the determination of melanin by the proposed FIA-ECL method.

Parameter		Melanin concentration ($\mu\text{g mL}^{-1}$)					
		Short-range			Long-range		
		0.1	0.5	1	10	20	30
%Found		100.3	100.5	99.4	100.6	99.2	100.4
		99.2	99.3	100.4	99.9	99.4	100.5
		100.6	99.8	98.5	100.1	99.0	100.2
Mean		100.0	99.8	99.4	100.2	99.2	100.3
S.D.		0.73	0.60	0.95	0.36	0.2	0.15
% RSD		0.73	0.60	0.95	0.35	0.20	0.14
%Found		100.1	99.6	98.9	100.4	100.3	100.8
		101.2	100.4	99.5	99.1	99.7	100.0
		100.4	99.8	100.0	99.4	99.5	99.5
Mean		100.5	99.9	99.4	99.6	99.8	100.1
S.D.		0.56	0.41	0.55	0.68	0.41	0.65
% RSD		0.55	0.41	0.55	0.68	0.41	0.64

N.B. Each result is the average of three separate experiments.

3.3.4. Ruggedness

To examine the ruggedness of the procedure, the intraday and interday precisions were evaluated as shown in Table 3. The precision of the proposed method was fairly high, as indicated by the low values of % RSD.

3.4. Applications

3.4.1. Analysis of Biological samples

In order to evaluate the analytical usefulness of the proposed ECL method, melanin was determined in its biological sources. The proposed method was applied to samples of *Sepia Officinalis*.

The recoveries of different concentrations of melanin were based on the average of three replicate determinations, the nominal content of melanin in *Sepia Officinalis* was determined using the corresponding regression equation of pure form. (Table 4).

3.4.2 Analysis of Natural Samples

The high sensitivity of the proposed method allowed the determination of melanin in natural samples, the extraction procedure for natural samples was performed by using NaOH as the suitable extraction solvent for melanin. The nominal content of melanin in *Nigella sativa* (Black seeds) or sunflower seeds were determined using the corresponding regression equation. Table 5 shows the results of determination of melanin in (Black seeds) or sunflower seeds coat.

The % melanin in *Nigella sativa* was found to be very close to that mentioned before by Hassib [31] which indicate that our proposed method is very accurate and precise.

Table 5. Determination of melanin in *Sepia Officinalis* by the proposed FIA-ECL method.

Preparations	Concentration taken ($\mu\text{g mL}^{-1}$)	% Found ^a
		Proposed method
<i>Sepia Officinalis</i> powder ^b	0.05	100.9
	0.25	99.1
	0.5	98.5
	1.0	99.6
	1.25	101.4
	2.5	99.7
Mean \pm S.D.		99.86 \pm 1.09

^a The average of three separate determinations, ^b Products of SIGMA Chemical Co., USA.

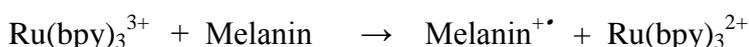
Table 6. Analysis of melanin in Natural samples by the proposed FIA-ECL method.

Natural Samples	% w/w ^a
	Proposed method
<i>Nigella sativa</i> (Black seeds)	2.60 % \pm 0.62
Sunflower seeds coat	0.24 % \pm 0.79
Sunflower seeds	2.3 % \pm 0.86

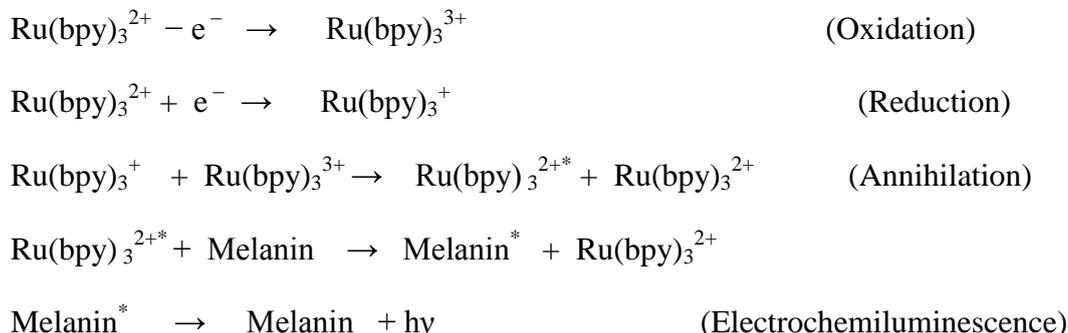
^a Each result is the average of five separate determinations.

3.5. ECL Mechanism

$\text{Ru}(\text{bpy})_3^{2+}$ has proven to be a very sensitive detection system for compounds which contain a secondary or tertiary aliphatic amine [32]. Melanin contains amino groups, thus the proposed reaction mechanism is presumably similar to that reported previously for amine determination utilizing its electrogenerated CL reaction with $\text{Ru}(\text{bpy})_3^{2+}$ [33,34]. $\text{Ru}(\text{bpy})_3^{2+}$ is oxidized by applying an appropriate voltage to the platinum electrodes. The amine group in melanin molecule is reduced to form an intermediate ion radical which reacts with $\text{Ru}(\text{bpy})_3^{2+}$ to form $\text{Ru}(\text{bpy})_3^+$. This reduces the $\text{Ru}(\text{bpy})_3^{3+}$ to the excited state that subsequently emits light. Therefore the possible ECL mechanism of melanin by analogy is shown below:



In other way, the oxidized and reduced forms can be electrochemically generated from $\text{Ru}(\text{bpy})_3^{2+}$ by applying a suitable voltage that's in first step. Second step, the excited melanin* is formed by collisions with $\text{Ru}(\text{bpy})_3^{2+*}$ that subsequently emit light. The proposed mechanism for the ECL reaction of melanin is shown below.



4. CONCLUSION

The proposed FIA-ECL based on $\text{Ru}(\text{bpy})_3^{2+}$ has proved to be a suitable sensitive analytical technique for the determination of melanin. Since $\text{Ru}(\text{bpy})_3^{2+}$ ECL emission is generated in situ in the reaction/detection cell with the original $\text{Ru}(\text{bpy})_3^{2+}$ species regenerated during the reaction, it does not need an external excitation light source and additional instrumentation to cut out scattered light for the excitation source, so sample dilution and band broadening are eliminated in a simple instrumental set-up. The procedure can be applied successfully for the determination of melanin in natural and biological samples.

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