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Spectrophotometric Study of the Reaction between Tryptophan and Ferroin at Different Forms

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THE INDICATOR ferroin III had been prepared by titration of Ferroin II against permanganate in sulfuric acid medium till blue color appeared of λ_{max} = 590 nm. The effect of time on the stability of blue ferroin (III) at λ_{max} = 590 nm was studied and it was found to completely change into red ferroin (II) of λ_{max} = 515 nm within 3 hr. Therefore, in this study the redox reaction between tryptophan (Trp) and ferroin indicator in its oxidized form (ferroin III) had been carefully studied; aiming to use both forms of this indicator in spectrophotometric micro-determination of Trp in pure form and in embryo synthetic and actual media. It had been found that; at λ_{max} = 590 nm the reaction between Trp and ferroin III involved oxidation of Trp into Trp^{-} and the indicator spontaneously reduced to ferroin II. Tracing the reaction between two forms of ferroin indicator after adding variable [Trp] = 10^{-3} M under proper selected conditions leads to the construction of two linear calibration curves, descending at λ_{max}= 590 nm and ascending at λ_{max} = 515 nm. This means that the reaction at λ_{max} = 590 nm is a redox reaction and at λ_{max} = 515 nm may be ion-pair formation $[Trp]^{-}[ferroin]^{++}$. The concentration ranges of the two calibration graph are found to be 2.05997−12.3594 and 41.058−164.2 µg mL^{-1}, respectively and the linearity was satisfactory (r= 0.9991and r = 0.999, respectively). The methods are successfully applied to a synthetic mixture containing some components of the human embryos' culture medium without interference and the results are satisfactory. This encouraged us to apply these procedures to the actual culture medium which yield a percent recovery of Trp in this medium of 99.51−101.5 and 100.2-100.3 % at λ=515 and 590 nm, respectively and with SD = 0.003464−0.01963 and RSD = 0.1640-0.3679 and Sandell sensitivity of S (µg cm^{-2}) = $7.424x10^{-7}$. These values refer to the accuracy and precision of the applied spectrophotometric procedures in embryo media analyses. The importance of this research work stems from; it is the first time to find cheap, simple and rapid spectrophotometric methods for Trp analyses that present in synthetic and actual embryo medium constituents. These methods can be used as alternative for expensive sophisticated tools such as HPLC, HNMR and mass famously used in literature.

Keywords: Ferroin indicator, Redox reaction, Ion-pair formation, Microdetermination of Trp and Analyses of embryo culture medium.

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Tryptophan is one of the culture medium components employed for clinical IVF to support development of human embryo *in vitro*. Studies on the embryos of several mammalian species such as mouse, hamster, sheep and cows, have all demonstrated that the inclusion of specific amino acids in the culture medium enhances embryo development to the blastocyst stage[1-11]. A number of the quantitative techniques that can monitor the uptake of specific nutrients by the embryo from the surrounding medium and detect the secretion of specific metabolites and factors are now being optimized to measure such changes in culture media[12]. The assessment tool must fulfil a number of criteria so that they can be applicable in IVF clinics. To be applied in clinical IVF, tools must have the ability to measure change without damaging the embryo, the ability to measure changes quickly and the ability to measure changes consistently and accurately.

Experimental

Materials and reagents

All chemicals were of the highest purity available. They included tryptophan (Trp) provided by WINLAB, UK; glucose provided by El Nasr Pharmaceutical Chemicals Co., Egypt and Sulfuric acid was supplied from Analytical Rasayan. Glycine (Gly) and potassium permanganate were purchased from ADWIC. Potassium chloride and sodium pyruvate were supplied from MERCK, Germany. Sodium bicarbonate was provided by El Gomhouria Co. Egypt.

The reagent used was 1, 10- phenanthroline iron (II) sulfate complex (ferroin) from FLUKA.

Solutions

2.017×10^{-4} M solution of Trp was prepared by dissolving 0.0103 g in 250 ml distilled water; $NaHCO_3$ was added as 0.0042 g/250 ml.

5×10^{-4} M ferroin (II) indicator solution was prepared by the appropriate dilution of conc. 0.025 M original solution by distilled water. Ferroin (III) oxidant indicator was freshly prepared, in each experiment, by titration of Ferroin (II), in 0.3 N sulfuric acid medium, against standard 5.247×10^{-4} M permanganate.

Potassium permanganate was prepared by dissolving 0.0207g in 250 ml distilled water. 2.036 x10-3M sodium pyruvate (C3H3NaO3, 110.044 g/mol.) and1.0028x10-2 M Gly (NH_2CH_2COOH, Molar mass: 75.0666 g/mol., C2H5NO2) were prepared by dissolving the accurately weighed amount in an accurate volume of distilled water to get the required concentration. Diluted solutions were prepared by accurate dilution from the stock solution to get the desired concentrations. Solutions were protected from light by keeping them in dark colored quick fit bottles during the whole work. 3.024 N sulfuric has been prepared by dilution from conc. acid (36 N). 1.003×10^{-1} M stock solution of sodium bicarbonate and 1.002×10^{-1} M stock solution of KCl were prepared by dissolving 2.106 g and 1.868 g of each respectively in 250 ml of distilled water.

Equipment

The spectrophotometric measurements were carried out using the automatic optizen pop spectrometer (Mecasys Co., Ltd/ made in Korea) in the wavelength range region from 200−800 nm and quartz cell of 1cm optical length was used. Small volumes were taken using automatic pipettes (Accupipet, USA) (0.01-1.0 ml). Weights measurement was performed by using Radwag wagi Elektroniczne Sensitive analytical balance 0.0001 g, Model: AS 220/C/1. Water used in the research was obtained by distillation from all glass equipment. All glassware used were washed carefully with distilled water and dried in the oven before use.

Methods

Preparation of FERR (III) oxidant indicator

Ferroin (III) oxidant indicator (FERR III) had been freshly obtained by direct titration of 0.025M Ferroin (II) (FERR II) in 0.3 N sulfuric acid medium against standard potassium permanganate. Freshly prepared FERR (III) was used during all experimental parts.

Selection of the suitable wavelengths (λmax) of FERR (II) and FERR (III) at different conditions

To obtain 2.5 x 10^{-3} M FERR (III) complex oxidant form, 1 ml of 2.5 x 10^{-2}M FERR (II) indicator was transferred to a 10 ml volume measuring flask followed by addition of 1 ml of 3.024 N H_2SO_4. This mixture had been carefully titrated against $KMnO_4$ (1.215 x10^{-3} M) until the formation of a blue color is reached at end point and complete the volume with distilled water. The absorption spectrum of FERR (III) was scanned against water in the wavelength region 400-765 nm. From the data obtained ,λ_{max} of ferroin in the oxidized form was determined and found to be 590 nm. The FERR (III) solution stability was affected by time and temperature.

Effect of time on FERR (III) spectra

The effect of time on absorbance of freshly prepared 2.5 x 10^{3-}M FERR (III) oxidant solution in 1 ml 3.024 N sulfuric acid; in 10 ml measuring flask was measured at different time intervals ranged from 0 to 180 min. The absorbance values obtained in the wavelength range 400-700 nm were measured. The absorbance values obtained were plotted against time to check stability of FERR (III) indicator at this time interval. It was found that FERR (III) changed completely to FERR (II) in sulfuric acid medium after 3 hr at room temperature; it had λ_{max} = 515 nm .

Spectrophotometric study of the redox reaction between tryptophan (Trp) and ferroin indicator (FERR) in its different forms (FERR II and FERR III)

a. Stoichiometry of the reaction between ferroin (III) and Trp: The stoichiometric study of the reaction between ferroin (III) and Trp had been done by molar ratio method (mrm); in which different volumes (0.5-5.5 ml) of (1.01199 x 10^{-3}M) Trp were added to 2.5 ml of (1x10^{-3} M (FERR) III (in sulfuric acid medium in 10 ml measuring flask .The absorbance values were measured at λ_{max} = 590 nm and at normal temperature within 10-15 min. These values were plotted against [Trp] / [FERRIII] ratio from which 1:1 ratio had been detected .

b. Effect of variable concentration of Trp on spectra of FERR (III) reaction product at λmax = 590 nm: To 1.0 ml of ($2.5x10^{-2}$ M) of freshly prepared FERR (III) in 10 ml measuring flask; 0.2 to 0.8 ml of 1.0052 x 10^{-2} M Trp were added in 1 ml 3.024 N sulfuric acid medium. The absorbance values were measured at λ_{max}= 590 nm at normal temperature within 10−15 min. These absorbance values were plotted against [Trp] and found to be linear descending relation. This may be related to reduction of FERR (III) to FERR (II) and oxidation of Trp. This linear descending calibration curve can be used in Microdetermination of Trp via redox reaction with FERR (III) at λ_{max}= 590 nm and normal temperature.

c. Effect of time on reaction between FERR (III) and Trp : In 10 ml volume measuring flask, 1 ml of ($1x10^{-3}$ M) FERR (III) and 10. ml ($1.0047x10^{-3}$ M) of Trp were mixed in in 1 ml 3.024 N sulfuric acid medium and absorbance values were measured at different time intervals to 135 min at normal temperature. From obtained data it appears at λ_{max} =515 nm. This means that; the reaction product changes its nature from blue form (λ_{max} = 590 nm) into brown form (λ_{max} = 515 nm).

d. Validity of beer's law of reaction between standard Trp in its oxidized form and ferroin (II) reduced form at λmax = 515 nm: Different volumes (0.5−3.5 ml) of Trp ($2.0173x10^{-4}$ M) were added to 4 ml of FERR III ($5x10^{-4}$ M) in 1 ml 3.024 N sulfuric acid medium in 10 ml capacity measuring flask. The absorbance values of these mixtures were measured after 30 min at λ_{max}=515 nm against water as a blank. These values were plotted against [Trp] to give ascending calibration curve that can be used in micro-determination of Trp in its oxidized form via reaction with FERR (II) obtained reduced form.

The obtained calibration curves of standard Trp on reaction with both FERR (III) at λ_{max} = 590 nm and with FERR (II) at λ_{max} = 515 nm can be used in micro-determination of Trp in synthetic and actual embryo culture medium.

Microdetermination of Trp in synthetic and actual embryo culture medium at λ_{max} =590 and 515 nm

Before going to determine Trp in embryo's culture medium interference of other constituents should be studied, at both λ_{max} = 590 and 515 nm. Synthetic mixture components that were analyzed at λ_{max} = 590 are given in Table 1 and that analyzed at λ_{max} = 515 nm is given in Table 2.

a. Interference study of embryo's culture medium constituents on the accuracy of the proposed procedure at λ_{max} =590 nm: Different volumes (0.2−2.0 ml) of synthetic embryo medium mixture (Table 7) were added to 1.0 ml of FERR III ($2.5x10^{-2}$ M) in 1.0 ml 3.024 N sulfuric acid medium and the volume was completed to 10 ml by distilled water and absorbance was measured within 10 min at λ_{max} = 590 nm and at normal temperature. The concentration of Trp each mixture was calculated from previously constructed calibration curve at λ_{max} = 590 nm and at normal temperature. The data obtained was used to check the

effect of the other constituents of embryo medium on accuracy for determination of Trp in this medium via redox reaction with FERR (III).

TABLE 1. The composition of the synthetic mixture used for interference study at λ_{max} = 590 nm.

Component	Weight taken (g/250 ml)
Trp	0.2203
$NaHCO_3$	0.0894
Gly	0.0757
Sodium pyruvate	0.1131
glucose	0.1820
KCl	0.0833

b. Interference study of embryo's culture medium constituents on the accuracy of the proposed procedure at $= {}_{max}\lambda$ *515 nm:* Different volumes (0.5-3.5 ml) of synthetic embryo medium mixture (Table 2) were added to 4 ml of FERR III ($5x10^{-4}$ M) in 1.0 ml 3.024 N sulfuric acid medium and the volume was completed to 10 ml by distilled water and absorbance was measured within 30 min at λ_{max} = 515 nm and at normal temperature. The concentration of Trp each mixture was calculated from previously constructed calibration curve at λ_{max} = 515 nm and at normal temperature. The data obtained was used to check the effect of the other constituents of embryo medium on accuracy for determination of Trp in this medium via interaction with FERR (II).

TABLE 2. The composition of the synthetic mixture used for interference study at λ_{max} = 515 nm.

Component	Weight taken (g/ 250 ml)
Trp	0.0109
$NaHCO_3$	0.0046
Gly	0.0056
Sodium pyruvate	0.0059
glucose	0.0931
KCl	0.0037

Microdetermination of Trp in the actual embryo culture medium using $2.5x10^{-2}$*M ferroin at* λ_{max}*=590 nm*

Different volumes of the human embryo's culture media (1.5−3 ml) were added to1.0 ml of FERR III ($2.5x10^{-2}$ M) and 1.0 ml 3.024 N sulfuric acid medium and the mixture was completed with distilled water to 10 ml capacity in measuring flask. This procedure was repeated five times. The absorbance values were measured at λ_{max} = 590 nm against distilled water as a blank. The concentration of Trp in this actual medium had been calculated from previously constructed calibration curve under the same conditions.

Microdetermination of Trp in the actual embryo culture medium using 1x10^{-4} M ferroin at λ_{max}515 nm

Two different volumes of the human embryo's culture media were added to 4ml of FERR III (2.5x10^{-4} M) in 1.0 ml 3.024 N sulfuric acid medium and the mixture was completed with distilled water to 10 ml capacity in measuring flask. This mixture was repeated five replicates. The absorbance values were measured at λ_{max} = 515 nm within 30 min against distilled water as a blank. The concentration of Trp in this actual medium had been calculated from previously constructed calibration curve under the same conditions.

Results and Discussion

Selection of the suitable wavelength of ferroin indicator (λ_{max}) at different conditions

The absorption spectrum of 2.5x10^{-4} M ferroin (II) indicator solution which is red in color was scanned in the wavelength region 400-800 nm against distilled water as a blank. The data obtained are represented in Fig. 1. It is clear from these results that λmax of the indicator occurs at 510-515 nm (ε = 1.0184x10^{4} Lmol^{-1}cm^{-1}).

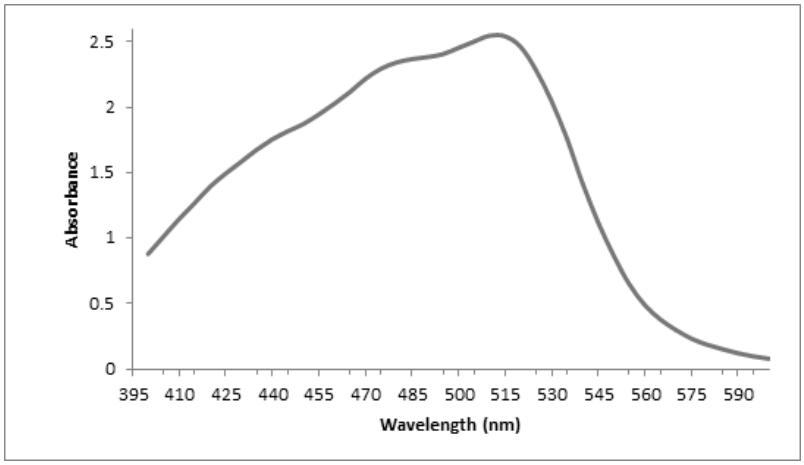


Fig. 1. Absorption spectrum of ferroin indicator solution.

The absorption spectrum of the oxidized form of ferroin (2.5x10^{-3}M blue colored solution of iron III complex) was scanned against distilled water as a blank in the wave length range 400–765 nm. The data are represented in Fig. 2. It is clear from these results that, the indicator exhibits λmax at 590 nm (ε=638.8 Lmol^{-1}cm^{-1}).

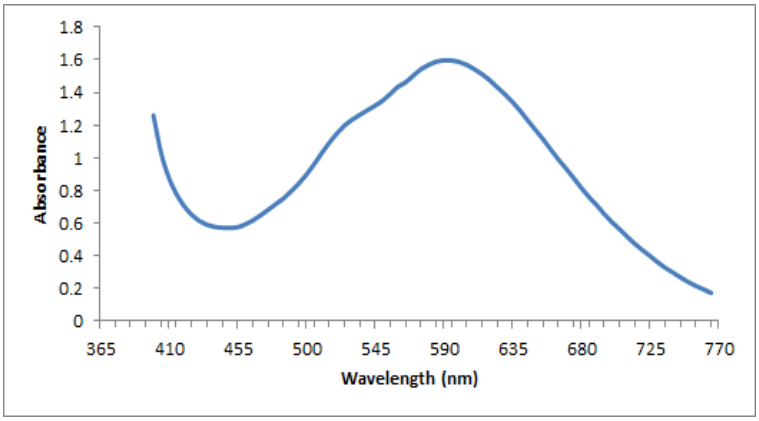


Fig.2. Absorption spectrum of ferroin solution (iron III sulfate complex) .

Study of the reaction between Trp and ferroin indicator in different forms

Effect of time on ferroin (III) spectra

The effect of time on the stability of the oxidized form of ferroin ($2.5x10^{-3}$ M blue solution) is studied carefully and the data obtained are shown in Table 3 and illustrated in Fig. 3. It is obvious from these results that, the absorbance of this indicator at λ_{max} =590 nm (of the blue color) decreases within 2 hr after which a constant absorbance is attained.

TABLE 3. Effect of time on the stability of the oxidized ferroin at λmax = 590 nm.

Absorbance	Time (min)
1.597	0
1.401	5
1.179	15
0.983	30
0.806	45
0.648	60
0.513	75
0.398	90
0.304	105
0.242	120
0.231	135
0.23	150
0.229	165
0.229	180

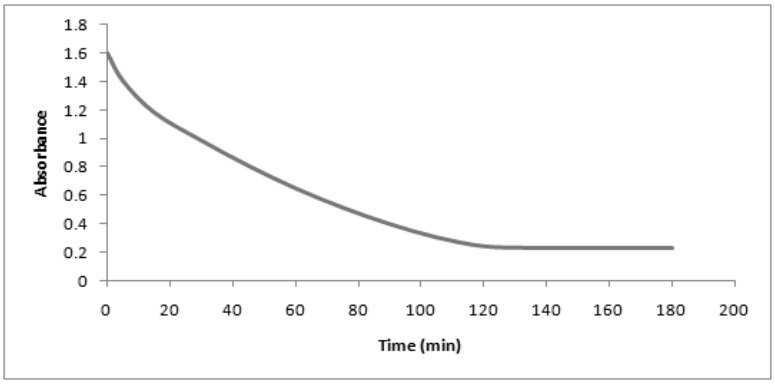


Fig. 3. Effect of time on the stability of the oxidized ferroin at λmax = 590 nm.

It is expected from these results that, ferroin (III) indicator is completely changed at a long time, more than 3 hr, into ferroin (II) form. This also means that ferroin (III) indicator obtained by reaction of ferroin (II) with permanganate in sulfuric acid medium is stable at normal temperature but is affected by time factor. To illustrate this conclusion; the absorption spectrum of the same solution after 10 folds dilution ($2.5x10^{-4}$ M) was scanned in the same wavelength region. The results obtained are shown in Fig.4. It gives a λmax = 515 nm with ε = $1.664x10^{3}$ $Lmol^{-1}cm^{1}$.

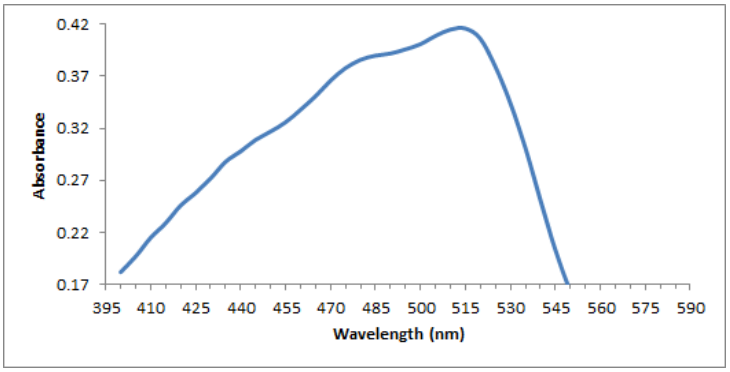


Fig. 4. Absorption spectrum of the oxidized ferroin solution after 3 hr from the beginning of the oxidation reaction.

From these data it is clear that, these results are matching with the previous data of absorption spectrum of $2.5x10^{-4}$ M ferroin (II) in the reduced form.

Selection of the suitable wavelength (λ_{max}) of reaction between Trp and ferroin (III) indicator in sulfuric acid medium

The absorption spectrum of ferroin (III)-Trp reaction was scanned in the wavelength range 400−570 nm (Fig. 5). It is clear from these data that, Trp instantaneously reduces ferroin (III) of blue color (λ_{max} = 590 nm) into ferroin (II) red color of λ_{max} = 515 nm.

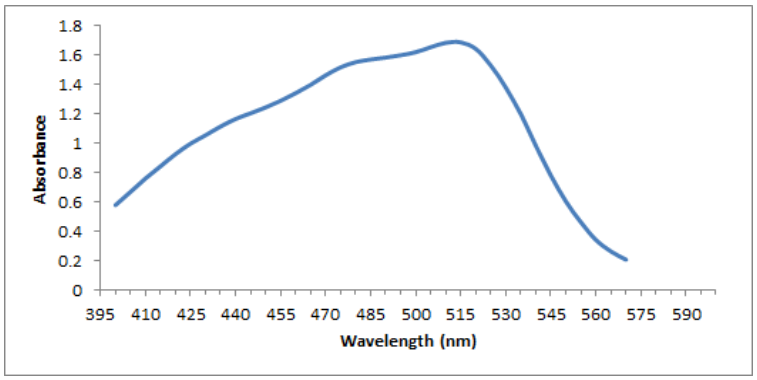
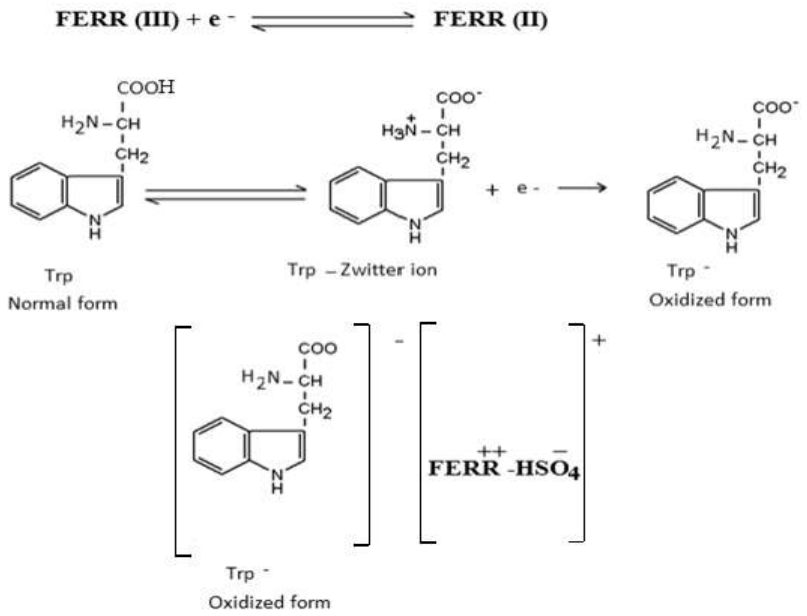


Fig. 5. Absorption spectrum of ferroin-Trp ion pair.

The value of λ_{max} of the reaction product is the same as that of ferroin (II) itself which means that the reaction proceeds via the reduction of ferroin (III) into its reduced form as given by the following proposed mechanism:



Scheme 1. Redox reaction of Trp-Ferroin (III).

The stoichiometric ratio of this reaction (1:1) as given by the proposed mechanism needs further study. It is also deduced from the above data that, there is another possible way of reaction between ferroin (II) reduced form and Trp in oxidized form; which needs more research study.

This mechanism is also confirmed by the decrease of ferroin (III) absorbance at λ_{max} = 590 nm with the increase of Trp concentration as a result of the proposed reduction process (Fig. 6).

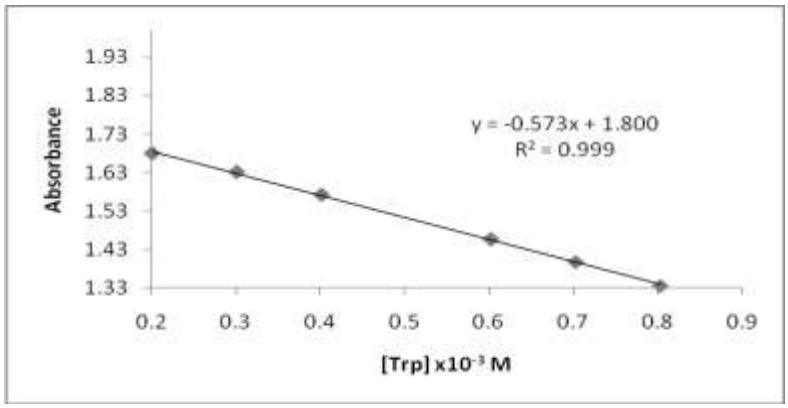


Fig. 6. Linear relation between absorbance –Trp concentration at λ_{max}= 590 nm.

Therefore, this descending calibration curve can be used for micro-determination of Trp in its pure form using ferroin (III) indicator. It was noticed that when these samples were measured at λ_{max} = 515 nm of ferroin (II); it gives ascending calibration curve. This means that, the reaction between ferroin (II) and Trp in oxidized form takes place by another mechanism; it needs further conformational studies. This conclusion is confirmed by direct reaction between ferroin (II) and Trp in its initial form. In this study, the absorbance of ferroin (II) and variable concentrations of Trp in its initial form; that measured at λ_{max} = 515nm which gives a straight line (Fig. 7).

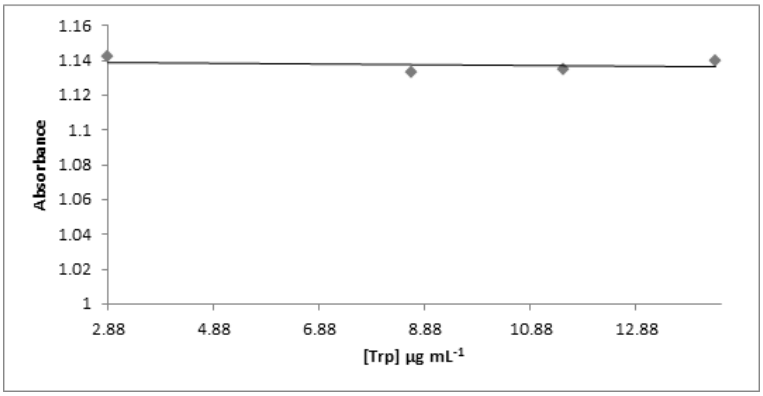


Fig. 7. The effect of variable [normal Trp] on the spectrum of ferroin (II)-Trp at λ_{max}= 515 nm.

These results means that, there is no direct reaction between ferroin (II) and Trp in its initial form; but the reaction actually goes through binding between this indicator in its reduced form and Trp in its oxidized form. Therefore, this reaction needs further study.

Stoichiometry of the reaction between ferroin (III) and Trp

The stoichiometric ratio of reduction of ferroin (III) to Trp was determined by using the molar ratio method. The data obtained are shown in and Fig. 8. These results indicate that a 1:1 [ferroin (III)]: [Trp] ratio is formed through the binding between the formed ferroin (II) and Trp oxidized form (Scheme 1).

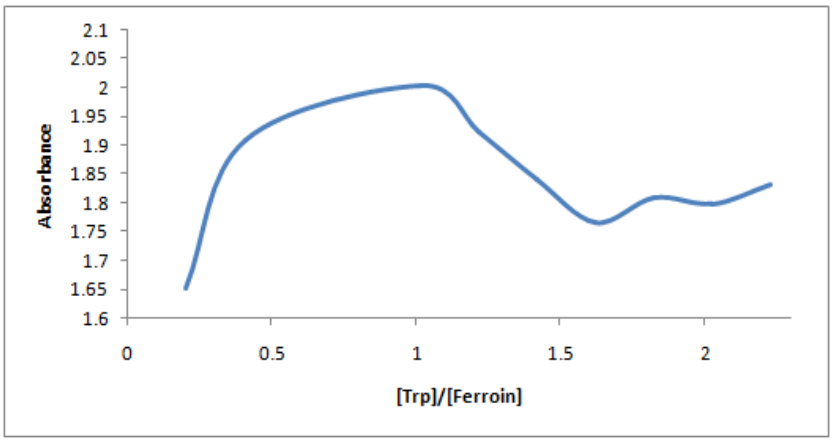


Fig. 8. Stoichiometric ratio of the reaction of ferroin (III) with Trp at λmax = 515 nm using molar ratio method.

During the follow of reaction between ferroin (II) and Trp in its oxidized form; it was noticed that, the red color of the reaction product fades with time. Therefore, it is important to study the effect of time on this product.

Effect of time on reaction between ferroin (II) and Trp in its oxidized form

The data obtained on studying the effect of time on reaction between ferroin (II) and Trp in its oxidized form are illustrated in Fig. 9. It is obvious from these data that the absorbance of the product decreases gradually with time until 135 min; where a constant minimum absorbance is attained. This can be explained by the conversion of Trp from oxidized form into normal form by time. This confirms the above conclusion that, no direct reaction between ferroin (II) and Trp in its initial form. This conclusion is also inferred by carrying out the complete run of $1x10^{-4}$ M of the same solution which shows no λ_{max}. This means that, there is no detection of the product between ferroin (II) and Trp in its initial form in the visible region.

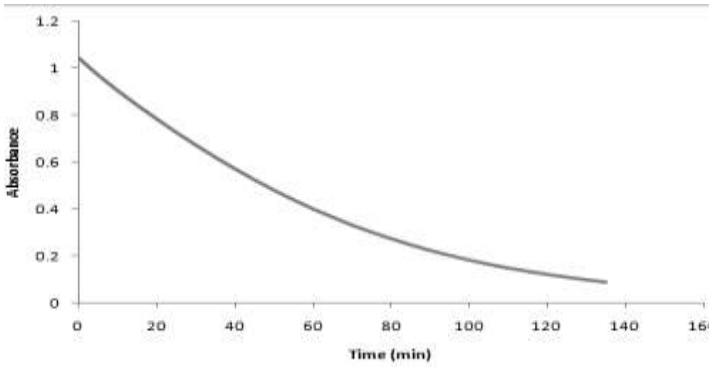


Fig. 9. Effect of time on the ion-pair formation between ferroin (III) and Trp at λ_{max}= 515 nm.

Therefore, we tried to measure the same solution in the UV region after dilution (Fig. 10).

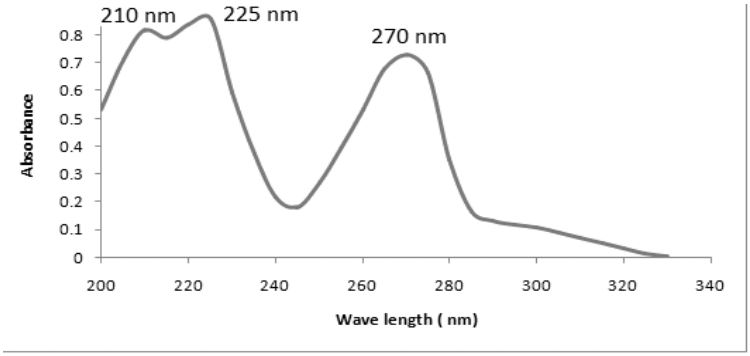


Fig. 10. The UV absorption spectrum of the ferroin (II)-Trp oxidized form product ($1x10^{-5}$ M) at 135 min.

To investigate if the product was dissociated forming the original ferroin reacted, absorption spectrum of ferroin of the same concentration was scanned in the same wave length region (Fig. 11).

It is clear from the above spectral data of ferroin (II) - Trp (Fig 10), ferroin (II) (Fig. 11) and Trp in its normal form (Fig.12) that, the only reaction proceeds between ferroin (III), ferroin (II) and Trp in its oxidized form. Also, the reaction between ferroin (II) and Trp in its normal form does not completely proceed. From the above detailed studies; it is possible to use ferroin (III) indicator in micro-determination of Trp in pure and in embryo culture medium, in two different cases. These cases are, the use of blue ferroin in sulfuric acid medium at λ_{max} = 590 and 515 nm.

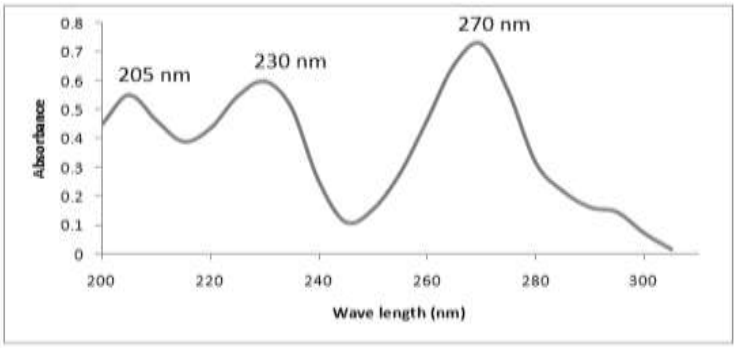


Fig. 11. Absorption spectrum of ferroin ($1x10^{-5}$ M).

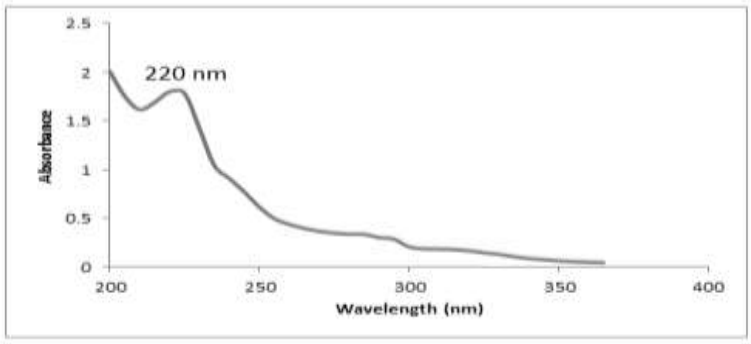


Fig. 12. Spectrum of $1.004x10^{-4}$ M Trp in 0.3024 N H_2SO_4 medium.

Microdetermination of Trp in pure and in embryo culture medium at λ_{max} = 590 and 515 nm

The calibration curve of standard Trp at λ_{max} = 590 nm

The reaction between ferroin (III) and Trp at λ_{max} =590 nm gives a descending calibration curve in the concentration range 41.058-164.2 µgmL^{-1} (Fig.13); which can be used for micro-determination of Trp in pure form (Table 4).

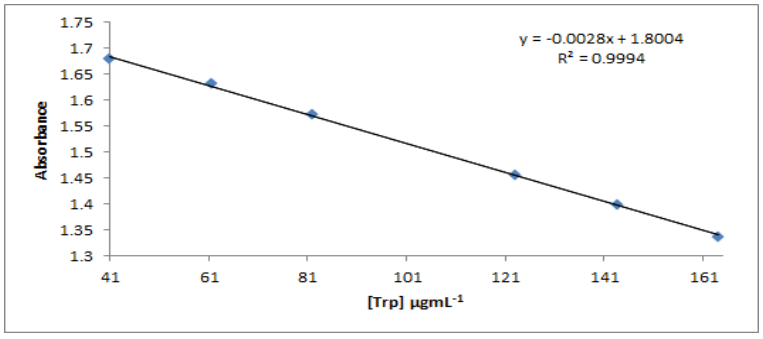


Fig.13. Calibration graph of the reaction between Trp and ferroin (III).

TABLE 4. Microdetermination of Trp using ferroin (III) at 590 nm.

Wt. taken μgmL^{-1}	Wt. found μgmL^{-1} (*)	% recovery (%)	SD	RSD (%)
41.058	41.71	101.6	0.1350	0.3226
61.59	60.15	97.66	0.0115	0.019
82.12	81.15	98.82	0.0874	0.1077
123.2	122.4	99.38	0.2517	0.2056
143.7	143.1	99.57	0.0577	0.0403
164.2	165.1	100.6	0.2517	0.1526

(*) are the means value of five replicates

These data refer to the high accuracy and precision of the applied procedure in determination of standard Trp by using ferroin (III) under proper conditions in the concentration range 41.058−164.2 μgmL^{-1}; which is confirmed by the values of % recovery = 97.66− 101.6, SD = 0.011547 − 0.2517 and RSD = 0.01526 – 0.3226 %.

Table 5 shows the different analytical parameters obtained such as slope, intercept, correlation coefficient, Sandell's sensitivity, molar absorptivity (ε), standard deviation, limit of quantification, limit of detection and relative standard deviation.

TABLE 5. Analytical parameters for the determination of Trp by the proposed method using ferroin indicator reagent λ_{max} = 590 nm.

Analytical parameter	Value
λmax (nm)	590
[Trp] μgmL^{-1}	41.058−164.2
ε ($Lmol^{-1}cm^{-1}$)	0.573 x 103
% Recovery (%)	97.66−101.6 %
SD	0.0115−0.2517
RSD (%)	0.0153−0.3226
LOD ($\mu g\ mL^{-1}$)	0.7775
LOQ ($\mu g\ mL^{-1}$)	2.356
Y = a X+ b, a a R^2	 -0.573 1.800 0.999
S ($\mu g\ cm^{-2}$)	$1.745x10^{-6}$

The small value of Sandell sensitivity indicates the high accuracy of the proposed method in the determination of the standard Trp using ferroin (III) and proper conditions at λ_{max} = 590 nm.

Validity of beer's law of reaction between standard Trp in its oxidized form and ferroin (II) at λ_{max} =515 nm

The reaction between standard Trp in its oxidized form and ferroin (II) at λ_{max}= 515 nm gives an ascending rectilinear line (Fig. 14) in the concentration range 2.05997 − 12.3598 µgmL^{-1} (Table 6). This ascending behavior may be related to the formation of a reaction product of a pronounced stability between ferroin (II) and Trp in its oxidized form which increases with the increase of Trp (Scheme 1).

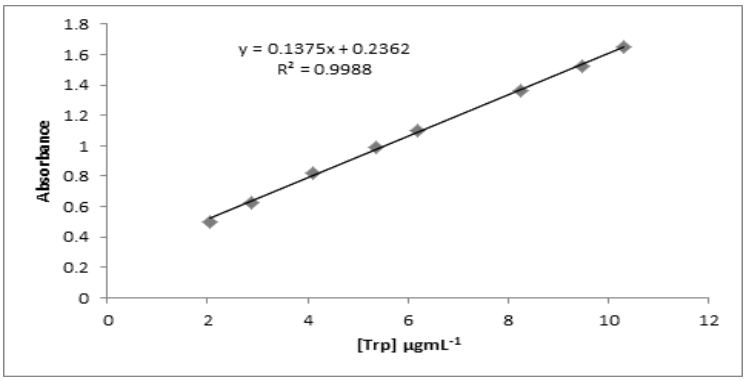


Fig.14. Calibration graph of the reaction between Trp in its oxidized form and Ferroin (II) at λ_{max} =515 nm.

TABLE 6. Microdetermination of Trp in its oxidized form via reaction with ferroin (II) at λ_{max} = 515 nm.

Weight taken of Trp (µgmL^{1})	Weight found of Trp (µgmL^{-1})	% recovery (%)	SD	RSD (%)
2.060	1.942	94.29	0.0088	0.5348
2.884	2.848	98.76	0.0092	0.3404
4.120	4.254	103.3	0.0115	0.2597
5.356	5.479	102.3	0.0055	0.0942
6.180	6.312	102.1	0.0060	0.0944
8.240	8.204	99.56	0.0161	0.2013
9.476	9.356	98.74	0.0050	0.0533
10.300	10.31	100.1	0.0076	0.0721
12.360	12.45	100.7	0.0058	0.0472

Table 7 shows the different analytical parameters obtained such as slope, intercept, correlation coefficient, Sandell's sensitivity, molar absorptivity (ε), standard deviation, limit of quantification, limit of detection and relative standard deviation .

TABLE 7. Analytical parameters for the determination of Trp by the proposed method using ferroin indicator reagent at λ_{max} = 515 nm.

Analytical parameter	**value**
λmax (nm)	515
[Trp] μgmL^{-1}	2.0600−12.36
ε (Lmol^{-1}cm^{-1})	0.2818 x 10^{5}
% Recovery	94.29−103.3 %
SD	0.0050−0.01612
RSD (%)	0.0472-0.5348
LOD (μg mL^{-1})	0.1041
LOQ (μg mL^{-1})	0.0315
S (μg cm^{-2})	3.549x10^{-8}

The small value of Sandell sensitivity (3.549x10^{-5} μg cm^{-2}) indicates the high accuracy of the proposed method in the determination of the Trp. Five replicated measurements is performed at different concentrations of Trp. The standard deviation (0.002449−0.007587) and relative standard deviation values (0.1398−0.8474%) indicate the high accuracy and precision of the proposed spectrophotometric method used for determination of Trp using ferroin (III) indicator at λ_{max} = 515 nm.

Before going to apply ferroin (III) indicator in micro-determination of Trp in actual embryo medium we have to check the accuracy of the proposed procedure on its application to analysis of synthetic embryo medium and possible interference due to the presence of other components.

Interference study of embryo's culture medium constituents on the accuracy of the proposed procedure for determination of Trp

1- First case at λ_{max}=590 nm: Before going to apply ferroin indicator in its forms (II and III) for micro-determination of Trp as an essential amino acid in actual embryo media; it was applied on a synthetic medium at two different wavelengths λ_{max}=590 and 515 nm, respectively. Table 6 represents the equimolar concentrations of the synthetic medium constituents such as, glucose, sodium pyruvate, Trp, Gly, sodium bicarbonate and potassium chloride; in order to test interference possibility of other constituents to Trp micro-determination at λ_{max}=590 nm under proper conditions .

TABLE 8. The composition and concentration of the equimolar synthetic mixture used for the interference study at λ_{max}=590 nm.

Component	**Concentration X10^{-3} M**
Trp	4.315
$NaHCO_3$	4.257
Gly	4.034
Sodium pyruvate	4.111
glucose	4.041
KCl	4.469

Therefore; it is possible to do the micro-determination of Trp in a synthetic mixture (Table 8) by measuring the absorbance of the product of its reaction with ferroin (III) at λ_{max}= 590 nm. The obtained results are shown in Table 9 using the calibration graph (Fig.15); which represents the effect of interfering constituents of the synthetic mixture on the accuracy of micro-determination on Trp using ferroin (III) indicator in this medium.

TABLE 9. Microdetermination of Trp in the synthetic mixture at λ_{max} =590 nm.

Wt. taken (μgmL^{-1})	Wt. found (μgmL^{-1})	% recovery (%)	SD	RSD (%)
17.63	16.76	95.07	0.0451	0.2683
35.25	37.44	106.2	0.2219	0.5879
44.06	42.91	97.39	0.1747	0.4076
70.5	71.05	100.8	0.0231	0.0325
158.6	158.5	99.9	0.2517	0.1587
176.3	177.2	100.5	0.6351	0.3592

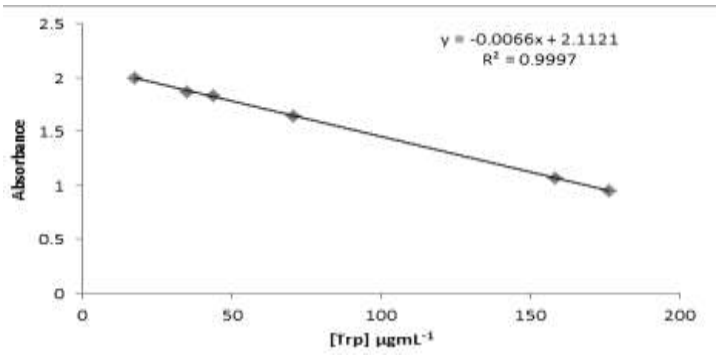


Fig. 15. Microdetermination of Trp in the synthetic mixture at λ_{max}=590 nm.

TABLE 10. Analytical parameters for microdetermination of Trp in the synthetic mixture at λ_{max}=590nm.

Analytical parameter	Value
[Trp] μgmL^{-1}	17.63-176.3
ε ($Lmol^{-1}cm^{-1}$)	$0.1347x10^4$
% Recovery	99.9−100.8
SD	0.0231−0.6351
RSD (%)	0.0325−0.5879
LOD ($\mu g\ mL^{-1}$)	1.103
LOQ ($\mu g\ mL^{-1}$)	47.15
S ($\mu g\ cm^{-2}$)	$7.424x10^{-7}$

It is clear from these data that, there is no pronounced interfering possibility from other constituents to Trp micro-determination using ferroin (III) at λ_{max}=590nm . This conclusion is confirmed by the calculated values of SD(0.20306-1.100) , RSD (0.4838-106360) (and recovery) % (95.07-106.2 %) of Trp determination in the given synthetic mixture. These data refer to the high accuracy and precision of the proposed procedure applied for determination of Trp using ferroin (III) indicator in synthetic mixture. This successful application encouraged to apply the proposed procedure for micro-determination of Trp in actual embryo media in presence of other constituents without possible interference at λ_{max}=590 nm.

2-Second case at λ_{max}=515 nm : Table 11 represents the equimolar concentrations of the synthetic medium constituents such as, glucose, sodium pyruvate, Trp, Gly, sodium bicarbonate and potassium chloride[14]; in order to test interference possibility of other constituents to Trp micro-determination at λ_{max}= 515 under proper conditions.

TABLE 11. The composition and concentration of the equimolar synthetic mixture used for the interference study at λ_{max}= 515 nm.

Component	Concentration X10^{-4} M
Trp	2.135
$NaHCO_3$	2.190
Gly	2.984
Sodium pyruvate	2.144
glucose	2.598
KCl	1.985

Therefore; the micro-determination of Trp in a synthetic mixture is possible by measuring the absorbance of the product of its reaction with ferroin (III) at λ_{max}=515 nm. The obtained results are shown in Table 12 using the calibration graph (Fig. 15); which represents the effect of interfering constituents of the synthetic mixture on the accuracy of micro-determination on Trp using ferroin (II) indicator in this medium.

TABLE 12. Microdetermination of Trp in the synthetic mixture at λ_{max}=515 nm.

Wt. taken of Trp µgmL^{-1}	Wt. found of Trp µgmL^{-1}(*)	% Recovery (%)	SD	RSD (%)
2.180	2.301	105.5	0.0116	0.5033
3.052	3.056	100.1	0.0205	0.6755
4.360	4.136	94.86	0.0285	0.6782
6.541	6.503	99.42	0.0043	0.0663
8.721	8.895	101.2	0.0035	0.0395
10.03	9.917	96.28	0.0226	0.2284
10.90	10.81	99.11	0.0569	0.5283
13.08	13.07	99.94	0.0693	0.5269

(*)Trp] are the means of five replicates.

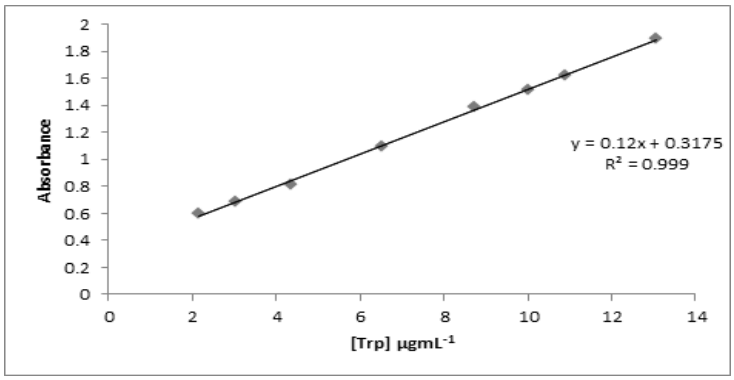


Fig.16. Calibration graph of Trp in the synthetic mixture using [ferroin III] = 2.0x10^{-4}M at λ_{max} =515 nm.

It is clear from these data that, there is no pronounced interfering possibility from other constituents to Trp micro-determination using ferroin (III) at λ_{max}=515nm. This conclusion is confirmed by the calculated values of SD (0.0035−0.0693), RSD (0.0395−0.6782) and recovery % (94.86−101.2 %) of Trp determination in the given synthetic mixture. These data refer to the high accuracy and precision of the proposed procedure applied for determination of Trp using ferroin (III) indicator in synthetic mixture. This successful application encouraged us to apply the proposed procedure for micro-determination of Trp in actual embryo media in presence of other constituents without possible interference at λ_{max}= 515 nm.

The comparison between data in Tables 13 and 14 refers to the successful application of ferroin indicator in both forms (II and III) in micro-determination of standard Trp in both normal and oxidized forms. Therefore, it is possible to apply this indicator in micro-determination of Trp as an essential amino acid in 0.5 and 1.0 ml embryo's culture media. The data obtained at λ_{max}= 515 nm are shown in Table 13.

TABLE 13. Microdetermination of Trp in the actual embryo culture medium using 1x10^{-4} M ferroin as a final concentration at λ_{max}= 515 nm.

Volume of medium taken (mL)	Absorbance Average (*)	Weight taken µgmL^{-1}	Weight found µgmL^{-1} (*)	% recovery (%)	SD	RSD (%)
0.5	0.554	2.124	2.114	99.51	0.0035	0.1640
1.0	0.952	5.214	5.290	101.5	0.0196	0.3679

(*) is average of five replicates

Wt. taken is calculated from the calibration curve of Trp microdetermination in the pure form at λ_{max}= 515 nm.

Wt. found is calculated from the calibration curve of Trp microdetermination in the synthetic mixture at λ_{max}= 515 nm.

TABLE 14. Microdetermination of Trp in the actual embryo culture medium using $2.5x10^{-3}$ M ferroin as a final concentration at λ_{max}= 590 nm.

No. of replicates	Absorbance	Weight taken μgmL^{-1}	Weight found μgmL^{-1}	% recovery (%)	SD	RSD (%)
1	1.893	33.15	33.23	100.2	0.1793	0.5414
2	1.875	32.83	32.91	100.2		
3	1.892	33.13	33.21	100.2		
4	1.854	32.47	32.55	100.3		
5	1.997	34.97	35.06	100.3		
6	1.862	32.61	32.69	100.3		

Volume of culture medium taken =1.0 ml

Conclusion

These data reveal that the applied method is simple, sensitive, precise and accurate. Also, the reagent utilized in the proposed methods is cheap, readily available and the procedures do not involve any critical reaction conditions or tedious sample preparation. Thus, this proposed spectrophotometric method can be successfully applied for the determination of Trp in the pure form and in embryos' culture medium mixture.

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دراسة طيفية للتفاعل بين تريبتوفان و فيروين فى صور مختلفه

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تم تحضير كاشف فيروين III بمعايرة فيروين Π مع برمنجنات الصوديوم فى وسط حمضى من حمض الكبريتيك حتى ظهور لون أزرق يمتص الطيف بدرجة قصوى عند طول موجى 590 نانومتر. تم دراسة تأثير الوقت على ثبات فيروين III الأزرق عند طول موجى 590 نانومتر ووجد أنه يتغير بالكامل الى فيروين Π الاحمر والذى يحدث له أقصى امتصاص للطيف عند 515 نانومتر وذلك بعد مرور 3 ساعات من تكوينه.

لذلك تمت هذه الدراسة للتفاعل بين تريبتوفان و فيروين فى صورته المؤكسدة بهدف استخدام الكاشف فى كلتى الصورتين للتقدير الدقيق لتريبتوفان فى صورته النقيه وفى وسط زراعة الأجنة البشرية الصناعى و الفعلى.

النتائج التى تم الحصول عليها فى هذه الدراسة تعكس دقة الطريقة المستخدمة فى تعيين تريبتوفان كأحد مكونات وسط زراعة الأجنة البشرية. وأهمية هذا البحث تنشأ من كونها المرة الأولى التى يتم فيها إيجاد طريقة سهلة و بسيطة وسريعة لتقدير تريبتوفان الموجود فى وسط زراعة الأجنة البشرية طيفيا ويمكن استخدام هذه الطريقة كبديل للطرق المعقدة والمكلفة المستخدمة من قبل.