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NOVEL SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF PHYCOCYANIN IN SPIRULINA: APPLICATION TO ASSAY METHOD VALIDATION

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Abstract:

A reliable spectrophotometric method has been developed for quantitative analysis of Phycocyanin in capsule formulations by developing a suitable extraction scheme for complete extraction of phycocyanin from its biomass with the help of central composite design and then the phycocyanin thus obtained was treated with diazotised sulphanilic acid as an active coupling species. The red-colored species generated on an ice-cold condition has shown absorption maximum at about 490 nm. The method was validated in that its linearity was excellent within the concentration range of 100-500 µg/mL with correlation coefficient 0.9998. The intra-day and inter-day precision were found as less than 2.0 % RSD and the recovery studies proved its accuracy as the % recovery is of 99.97-100.36. Commercially available capsule formulation was assayed and the mean % assay was found as 99.87%.

KEYWORDS: *Spirulina,* ***Phycocyanin****, sulphanilic acid, diazotised, Validation*

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1. INTRODUCTION:

Phycocyanin (PC) is a nutraceutical compound with biological activity isolated and/ or purified from seaweeds. PC has shown anti-inflammatory, antiplatelet, anti-cancer, nephroprotective and hepatoprotective properties that may be explained, at least in part, by its antioxidant activity. PC is a protein from the phycobiliprotein (PBP) family characterized by its intense blue color. It is a peripheral accessory light-harvesting complex called phycobilisome (PBS), which is assembled on the surface of the thylakoid membrane. Its main function is to transfer the excitation energy to the center reaction where the maximum wavelength of absorption is near to 620 nm (Benedetti et al., 2006; De Marsac & Cohen-Bazire, 1977). PC is one of the main pigments of Mexican algae Spirulina, which is used as a dietary supplement due to its high content of protein, vitamins, minerals and essential fatty acids. This pigment is found in cyanobacteria and eukaryotic algae such as Rhodophyta and Cryptomonads. PC is classified into three types, C-PC (obtained from cyanobacteria), R-PC (obtained from red algae) and R-PCII (obtained from *Synechococcus* species). In the Spirulina algae, PC provides its characteristic green– blue color. Furthermore, PBPs are the most abundant proteins in many cyanobacteria and algae.

A thorough literature survey has been conducted and found many aspects regarding the extraction and purification of phycocyanin from various species by different techniques including Study of Physical-Chemical Properties of C-Phycocyanin Isolated from an Acido-Thermophilic Eukaryote, Cyanidium caldarium[1], Isolation and Characterization of Phycocyanins from the Blue-Green Alga *Spirulina platensis[2]*, Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine [4], Experimental Design as a Tool for Optimization of C-Phycocyanin Purification by Precipitation from *Spirulina platensis*[5], C-Phycocyanin extraction from *Spirulina platensis* wet biomass [6], Extraction and purification of C-phycocyanin from dry *Spirulina* powder and evaluating its antioxidant, anticoagulation and prevention of DNA damage activity[7], Extraction and purification of C-phycocyanin from Spirulina platensis (CCC540)[8], Optimization of protein extraction from *Spirulina platensis* to generate a potential co-product and a biofuel feedstock with reduced nitrogen content [9], Isolation, characterization and antioxidative activity of C-phycocyanin from Limnothrix sp. strain 37-2-1[10], a method to obtain C-phycocyanin of high purity [11], fractionation and purification of the phycobiliproteins from Spirulina platensis[12], comparison of Different extraction methods for phycocyanin Extraction and Yield from *Spirulina platensis[13]*, separation of phycocyanin from Spirulina platensis using ion exchange chromatography[14], and Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques[15]. **F**rom the literature survey, it was observed that several works have been done on spirulina but they were not meant for estimation of phycocyanin in either biomass products or pharmaceutical dosage forms. This facet prompts us to develop a simple but reliable analytical method which would able to determine the phycocyanin content of the spirulina in different strains with good accuracy and precision. Hence the aim of the work is to develop a visible spectrophotometric method specific for estimation of phycocyanin in commercially available capsule dosage forms and validate the method in terms of specificity, linearity, accuracy and precision.

2. MATERIALS AND METHODS:

2.1 Chemicals

All the chemicals and reagents used were of analytical grade and procured from different manufacturers. Analytically pure phycocyanin (99.98 %) was from TCI chemicals, sulphanilic acid, sodium nitrite, sodium chloride, hydrochloric acid (S.D. fine chemicals limited, Mumbai), and ethanol from Changshu yangyuan chemicals, China. Double distilled water was prepared in Vignan pharmacy college on daily basis for preparation of reagents. Commercial formulations of Spirulina capsules were purchased from local pharmacy and the strength is of 500 mg per capsule.

2.2 Instrumentation

An ultrasonic cell crusher noise isolating chamber from *ORCHID Scientifics* SJIA 500 W ultrasonic homogenizer was used for extraction of phycocyanin from spirulina biomass. A Elico SL 210 double beam UV-Visible spectrophotometer with 1 cm matched cuvettes was used. The instrument was calibrated before the study. A *Elico* CI 59 digital pH meter was used for all pH adjustments after calibrating the instrument. *Remi* centrifuge capable of producing a 9000g rpm was used.

2.3 Phycocyanin standard solution

Accurately weighed 100 mg of phycocyanin into a cleaned 100 ml volumetric flask containing double distilled water. volume was made with water and the resulting solution was filtered through a Whatman paper. Carefully transferred 5ml of filtrate into

another volumetric flask and the volume was made up to the mark to get the working standard solution of 100-500 µg/mL.

2.4 Sodium chloride solution, 10 % w/v
10 g of sodium chloride was dissolved in a 100 ml container to get the solution and clarified through Whatman paper.

2.5 Sodium nitrite solution, 4.5 % w/v
Accurately, 4.5g of sodium nitrite was dissolved in a 100 ml container to get the solution and clarified through Whatman paper.

2.6 Dilute sodium carbonate solution
10 g of anhydrous sodium carbonate was dissolved in a 100 ml container to get the solution and clarified through Whatman paper.

2.7 Sulphanilic acid solution diazotized
Dissolved with warming, about 0.9 g sulphanilic acid in 9 mL of concentrated hydrochloric acid and diluted to 100 mL with water. The solution was allowed to cool. 10 mL of this solution was added to a flask containing 10 mL of sodium nitrite solution (4.5 % w/v) on an ice-cold bath with occasional mixing. The mixture was kept on ice bath for about 15 min. 20 mL of dilute sodium carbonate was added to this solution prior to develop colour.

2.8 Construction of calibration curve
To a series of 10 mL volumetric flasks, carefully transferred about 1.0, 2.0, 3.0, 4.0, and 5.0 mL standard drug solution, equalized the volume and added 5.0 mL of diazotized sulphanilic acid in an ice-cold bath to develop color. The resulting solution was kept aside for about 20 min and the volume was adjusted to 10 mL with distilled water. The absorbance of each calibration standard was recorded on a spectrophotometer against its reagent blank at about 490 nm. A graph was plotted by taking concentration of standard phycocyanin on x-axis and absorbance on y-axis.

2.9 Estimation of Phycocyanin in capsules
The contents of randomly selected 20 capsule were weighed and average weight of one capsule content was determined. capsule content about 1.0 g was transferred to a beaker containing double distilled water, 10 ml of sodium chloride (10 % w/v) was added with continuous stirring and the volume was adjusted to about 100 mL with distilled water. The resulting solution was kept under ultrasonic homogenizer and applied the ultrasound frequency pulses in a short period of time by several cycles. Then the solution was kept under ultra bath Sonicator for about 45 min at room temperature. The solution was filtered through a cotton wool, centrifuged at 3000 rpm for about 45 min and the clear supernatant was diluted to get the working sample solution. Few mL of the sample was treated in the same manner as to that of calibration curve and the concentration of phycocyanin was computed from calibration curve.

3. RESULTS AND DISCUSSION:

3.1 Extraction of phycocyanin
Though several methods of extraction were reported in the literature, a simple and reliable extraction method was followed after optimizing the procedures developed through Design of experiments using central composite design (CCD). About 14 experiments were conducted to know the best yield of phycocyanin and each experiment was different in terms of the following variables (TABLE 1). Among the experiments that we have tried, D2, D3, D5, D9, D10, D13, D17, D19 and D20 extractions gave very low yield of phycocyanin as seen in their corresponding absorption spectra (figure 1, 4, 5, and 6), in that there was a clear indication of lack of characteristic absorption maximum. Hence those experiments were considered unsuccessful. Extraction of phycocyanin from spirulina with the aid of citrate buffer and calcium chloride was also tried and found unsuitable for this work (figure 7). Extraction design D7 and D14 experiments have given satisfactory results in terms of phycocyanin content, as seen in their spectra (figure 2 and 3) and therefore these experiments were used for phycocyanin estimation procedures.

Table 1: Central composite design for extraction of phycocyanin

Run	Biomass weight (g)	Extraction Time (min)	Salt concentration (% w/v)
1	1	300	20
2	2	172.5	15
3	2	172.5	15
4	2	172.5	6.59
5	2	172.5	15
6	2	41.92	15
7	1	45	10
8	3.68	172.5	15
9	2	172.5	15
10	2	172.5	15
11	2	386.92	15
12	3	300	10
13	2	172.5	15
14	1	45	20
15	3	45	10
16	1	300	10
17	3	45	20
18	3	300	20
19	0.318	172.5	15
20	2	172.5	23.4

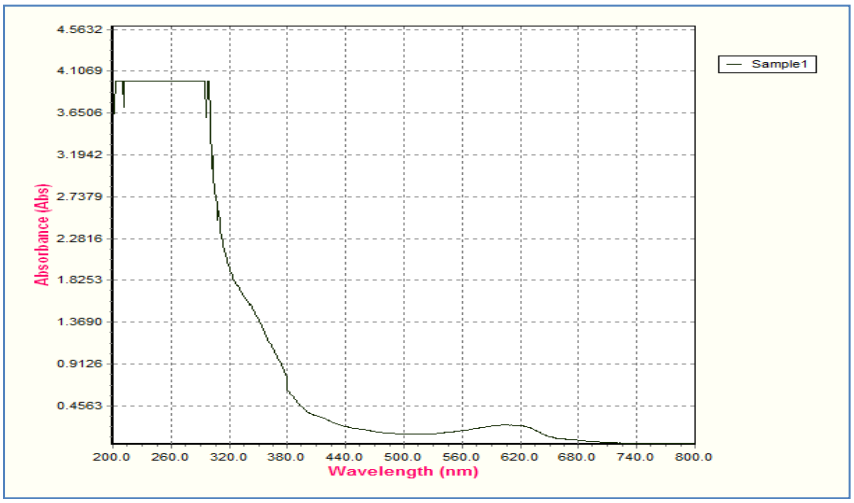

Figure 1:Absorption spectrum of phycocyanin from D2, D3, D5, D9, D10, and D13 extractions

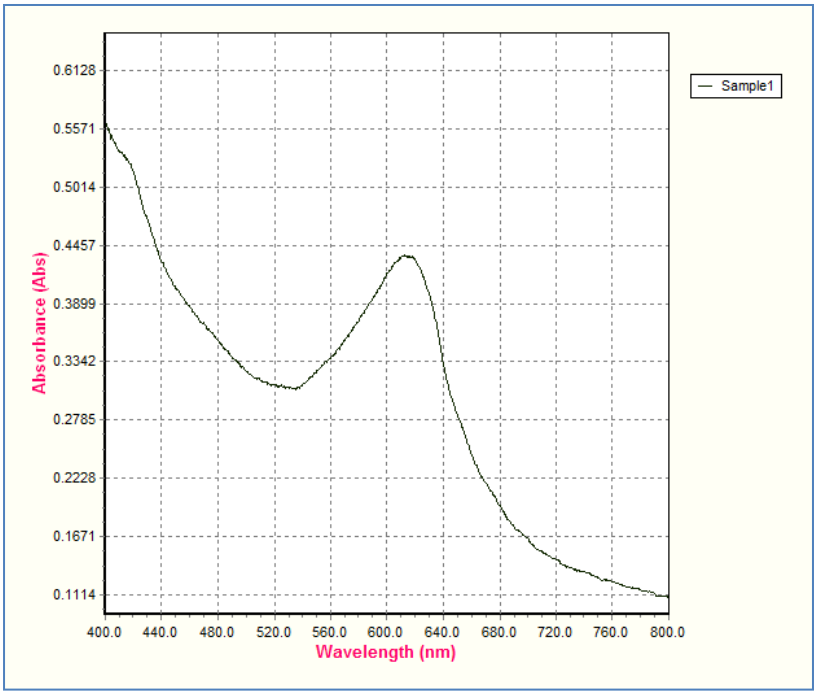

Figure 2: Absorption spectrum of phycocyanin from D7 extraction

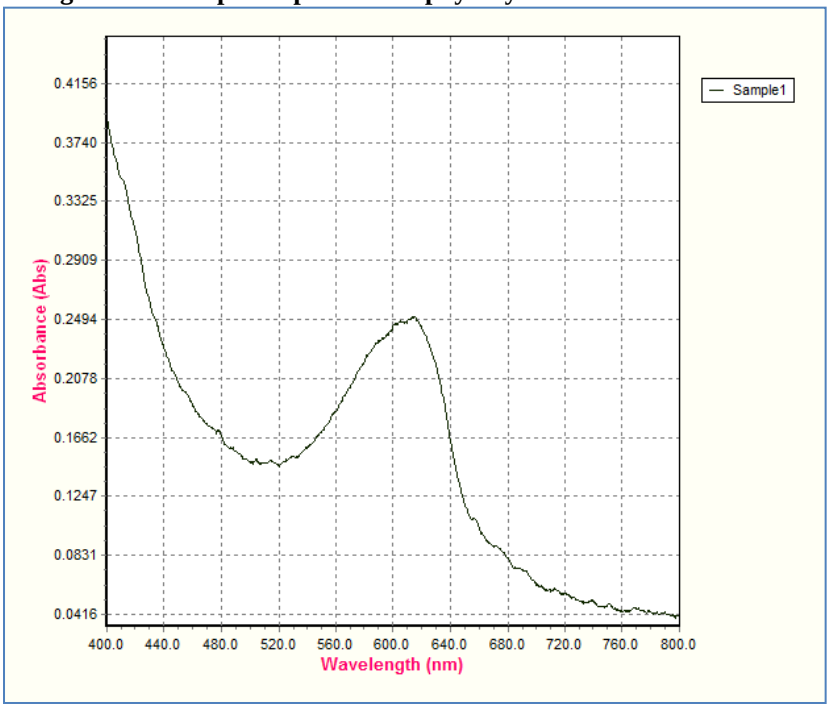

Figure 3:Absorption spectrum of phycocyanin from D14 extraction

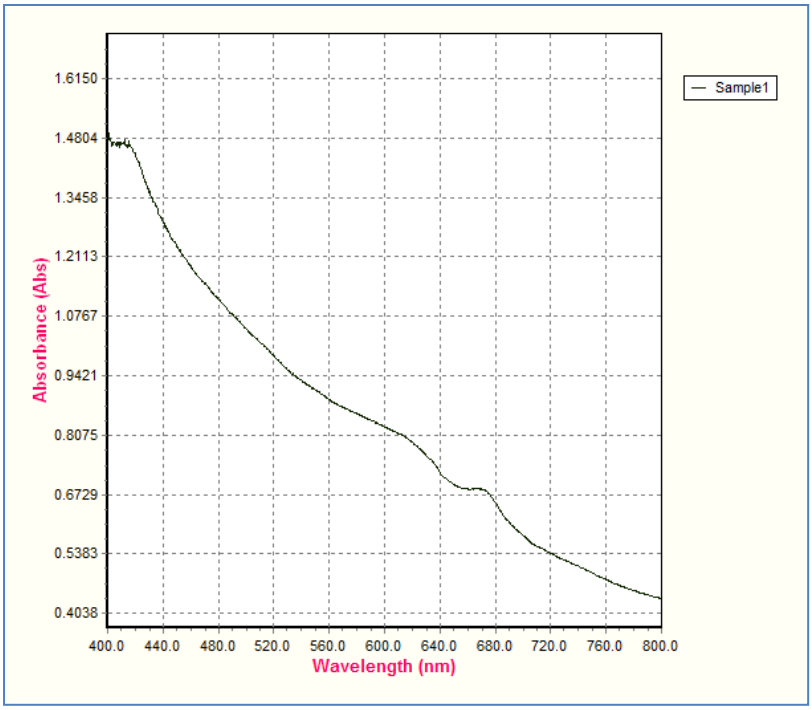

Figure 4:Absorption spectrum of phycocyanin from D17 extraction

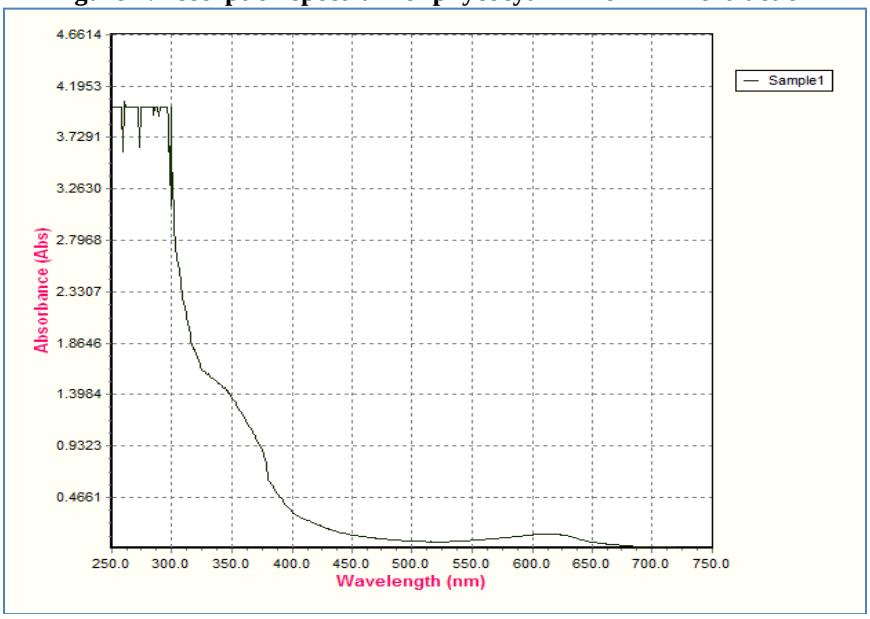

Figure 5: Absorption spectrum of phycocyanin from D19 extraction

Figure 6: Absorption spectrum of phycocyanin from D20 extraction

Figure 7: Absorption spectrum of phycocyanin by citrate buffer and Calcium chloride

3.2 Optimum conditions for color development:

In developing the proposed method, the nature of solvent was selected based on the solubility of the absorbing substance and by the absorption behavior of the solvent at the analytical wavelength. Water was selected as an ideal solvent for the entire analysis due to less absorbance at the wavelength selected for assay and it provides no chemical interference. Assay for phycocyanin in capsules by direct spectrophotometric method is subjected to interference from the excipients and its related phycobiliproteins that may present as a result of oxidation or reduction or decomposition during storage. Thus, an indirect spectrophotometric method has been developed to estimate phycocyanin by diazotised sulphanilic acid as an active coupling

species. The current research work utilizes diazotised sulphanilic acid in an ice-cold condition as a chromogenic reagent due to its specific reaction with phycocyanin and stability of the colour for spectral measurements and it was also observed that the color is stable for more than 3 hours (figure 8). The phycocyanin upon reaction with diazotized sulphanilic acid, gives a blood red colored coupling species that has formed as a result of specific reaction involved between the analyte and the reagent as proposed by the following scheme (figure 9). The absorption spectrum of phycocyanin standard treated with reagent was presented in Figure 11-12 and the wavelength selected for spectral measurements is at 490 nm.

Figure 8: Reaction conditions for obtaining stable color

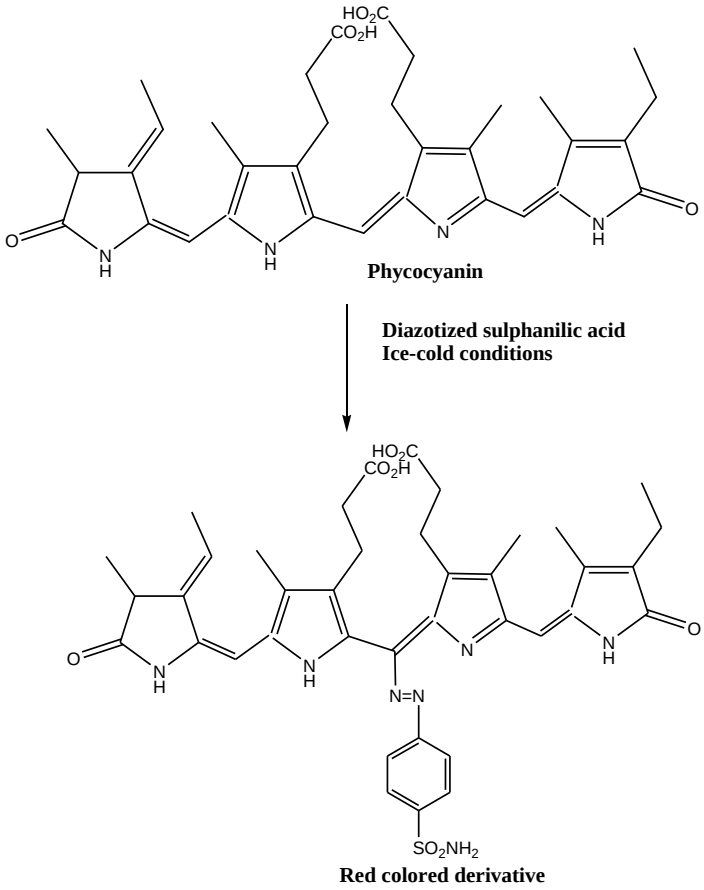

Figure 9:Proposed reaction scheme for the method

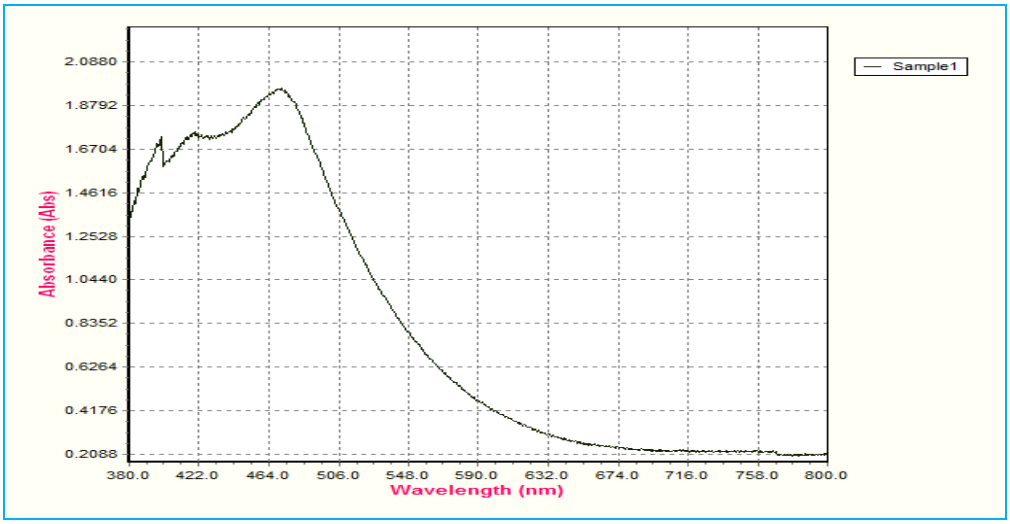

Figure 11: Absorption spectrum of phycocyanin treated with 3mL D14 solution and 2 mL reagent

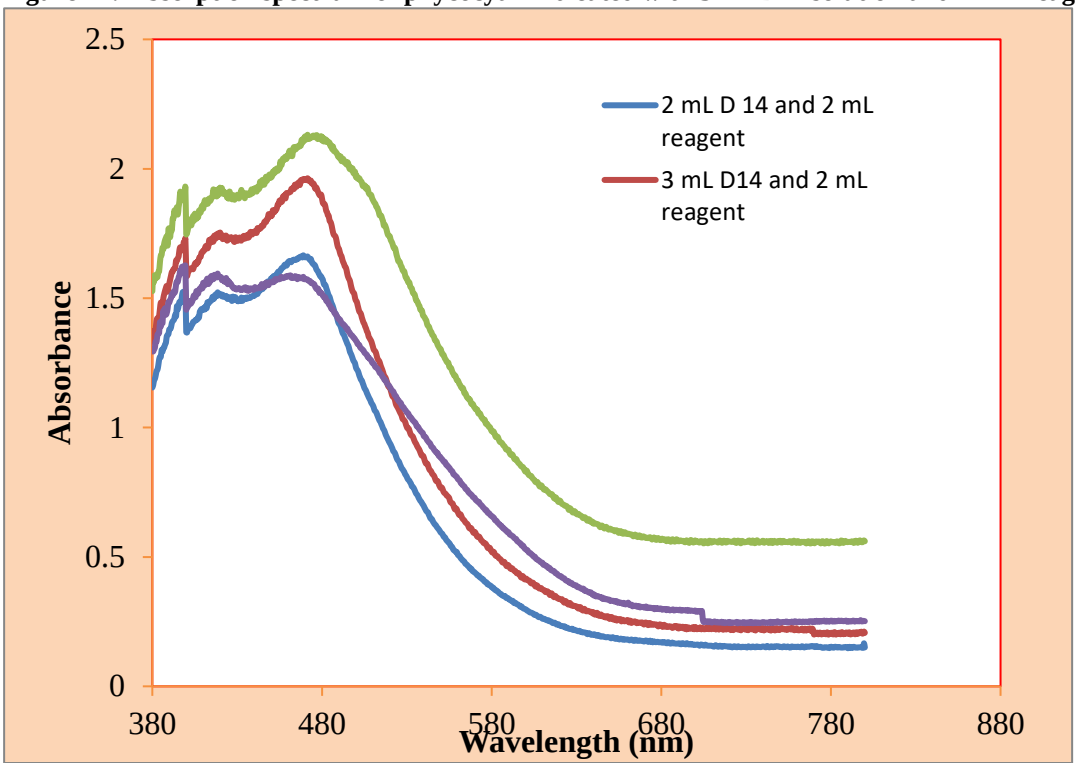

Figure 12: Absorption spectra of phycocyanin treated with sulphanilic acid diazotised in different occasions

3.3 Chemistry of reagents Diazotized-sulphanilic acid:

The process of formation of diazonium compound is called "diazotization" or "diazotization" or "diazotization". The reaction was first reported by peter Griess in 1858, which subsequently discovered several reactions of this new class of compounds. The most important method for the preparation of diazonium salt is treatment of aromatic amines such as aniline with nitrous acid. Usually the nitrous acid is generated in–situ (in the same flask) from sodium nitrite and mineral acid, in aqueous solution diazonium salts are unstable at temperature above +5 ^{0}C 'then $N^{\equiv}N^+$ group tends to be lost as N_2 (nitrogen gas). One can isolate diazonium salt tetrafluoroborate salts, which are stable at room temperature. Often, diazonium compounds are not isolated and once prepared, used immediately in further reactions. It is often preferred that the diazonium salt remains in solution, but they do tend to supersaturate.

3.4 Method validation

An integral part of analytical method development is validation. Once the method has been devised, it is necessary to evaluate under the conditions expected for real samples before being used for a specific purpose. The following parameters were evaluated.

a) Specificity:

The effect of wide range of other constituents and other additives usually present in the sample was investigated to know the specificity of the method and it shows no interference from other compounds. The common excipients such as lactose anhydrous, microcrystalline cellulose, crosscaramellose sodium and magnesium stearate have been added to the sample solution and analyzed. In fact many have no absorption at this UV maximum.

b) Linearity:

For linearity, aliquots of primary working standard solutions containing phycocyanin were diluted in a way such that the final concentrations are in the range of 15- 150 μg/mL. A calibration curve was plotted between concentration and Absorbance and statistical analysis of the calibration curve was performed. Method of least squares analysis was carried out for obtaining the slope, intercept and correlation coefficient values and the results of linearity characteristics were presented in TABLE 2. The proposed method was found linear in the concentration range of 100-500 µg/ml which states that the method was linear and the linearity curve was given in Figure 22. The correlation coefficient was found as 0.9998. Optical characteristics of the proposed method was presented in Table 3.

Table 2: Linearity of the method

S No	Concentration of Phycocyanin (µg/mL)	Absorbance at 490 nm
1	0	0
2	100	0.21
3	200	0.41
4	300	0.62
5	400	0.81
6	500	1.01

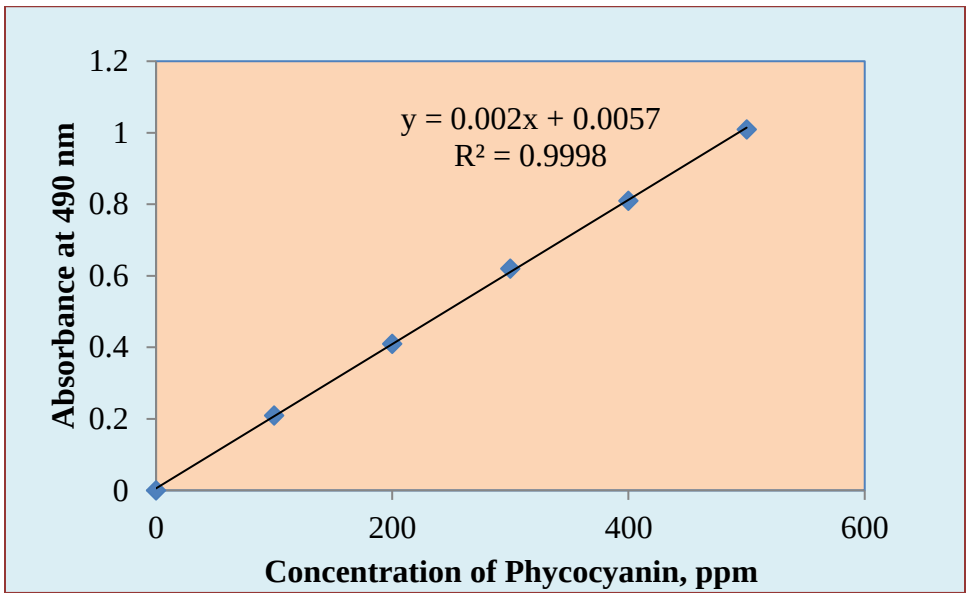

Figure 13: Beer's plot of phycocyanin treated with diazotized sulphanilic acid

Table 3: **Optical and Spectral characteristics of the method**

Parameters	Method
Detection wave length	By visible at 490 nm
Linearity range(mg/ml)	100-500 µg/ml
Regression equation	y = 0.002x + 0.0057
Slope(b)	0.002
Intercept(a)	0.0057
Standard deviation of slope(s_b)	0.0012
Standard deviation of intercept(s_a)	0.0016
Standard error of estimation (Se)	0.0005
Correlation coefficient($r^{2)}$	0.9998
% Relative standard deviation.	0.85
% Range of errors	Mean* ± 0.00241

**Average of six determinations*

c) Precision:

Precision was determined by intraday and inter-day study. Precision of the method was evaluated by carrying out the assay and analyzing corresponding responses 6 times on the same day and on different days for the sample solution. The precision of the method was ascertained from the absorbance obtained by determination of six replicates of capsule samples of phycocyanin. The percent relative standard deviation was found to be 5.2 parts per thousand (ppt) or 0.52 %.

d) Accuracy:

In order to ascertain the accuracy of the method recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredient by the proposed method. Accuracy studies were performed for phycocyanin at three different levels (25%, 50%, and 100%) and the mixtures were analyzed in triplicate by the proposed method. A known amount of standard phycocyanin at 25%, 50%, and 100% of sample (which was previously analyzed) was added and it was reanalyzed by the proposed method. And the percentage recovery was evaluated. A known amount of pure drug was then added to each of the previously analyzed formulation and the total amount of drug was once again determined by the proposed method. the mean % recovery of the drug when determined by spiking the same in a pre-analyzed sample was found between 99.66 ± 0.13 and 100.51 ± 0.29 and suggests that the proposed method has high degree of accuracy and can compete with the existing method. The results were presented in Table 4.

Table 4: Accuracy of the method

Formulation	Labelled Amount (mg)	% Recovery Found by reference method [14] ± S.D	% Recovery by proposed method*± S.D	Proposed Method		
				Amount found* (mg) ± S.D	t (Value)	F (Value)
Brand -I	500	100.9	100.36 ± 0.13	501.80 ± 0.009	0.672	1.249
Brand –II	500	100.1	99.76 ± 0.24	498.81 ±0.016	0.984	3.129
Brand -III	500	101.3	99.97 ± 0.60	499.86 ± 0.012	1.098	2.456
Brand –IV	500	101.2	100.10 ± 0.29	500.51 ± 0.08	1.564	1.439

**Mean of six determinations*

(e)Limit of detection and Limit of Quantitation

Limit of detection and limit of quantification were calculated using the following formula LOD = 3.3 (σ)/S and LOQ = 10 (σ)/S, where (σ) = standard deviation of response (peak area) and S = slope of the calibration curve. LOD and LOQ of the proposed method were found as 2.64 µg/mL and 8.713 µg/mL for the proposed method

f) Robustness:

The effect of small changes in certain variables on the developed method was determined. Robustness of the proposed methods was evaluated by making small changes that deliberately effect the method such as wavelength of spectral measurements ± 2 nm and it does not affected by these small alterations.

(g)system suitability

System suitability was ascertained by six replicate analyses of the drugs at concentrations of 200 μg/mL of phycocyanin. The percentage of RSD of the standard drug solutions in terms of their absorption maximum and absorbance.

CONCLUSION:

The proposed method is simple, accurate, precise, robust, and specific and has the ability to estimate, Phycocyanin including its sulphoxide in the capsules. Further short span of time for analysis reveals the time saving. The simplicity of the method allows for application in laboratory for routine quality check also it may be utilized for the determination of content uniformity and dissolution profiling of this product. Overall, the method provides solution for determination of Phycocyanin in the capsules with excellent selectivity, precision and accuracy in a cost effective manner.

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