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## SIMPLE SPECTROPHOTOMETRIC DETERMINATION OF INDIGO IN NATURAL SAMPLES

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**Abstract:** A simple and rapid spectrophotometric method for the determination of indigo is presented. This method is based on the formation of the N,N'-diacetyl derivative of indigo, which allows for a more stable and soluble product suitable for analysis. The method follows Beer's law in the concentration range of 1–20  $\mu\text{g mL}^{-1}$  at a maximum absorption of 614 nm. The developed technique has been successfully applied to the quantification of indigo in natural samples, demonstrating high sensitivity, reproducibility, and accuracy. Compared to traditional methods such as HPLC and UV-Vis, this approach offers a more straightforward and cost-effective alternative while maintaining high analytical performance. Additionally, the proposed method avoids issues related to the low solubility of indigo in various solvents, providing a more efficient derivatization process that enhances measurement accuracy. A comparative study with existing methodologies highlights the advantages of the N,N'-diacetyl derivative approach, particularly in terms of sample preparation, stability, and signal reliability. The results obtained confirm that this method can be reliably used for routine analysis of indigo in different natural matrices. Furthermore, statistical validation of the method ensures its applicability in industrial and research settings.

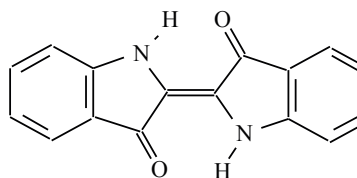
**Keywords:** Indigo, spectrophotometric analysis, natural dyes, analytical chemistry.

## INTRODUCTION

In recent years, there has been a significant resurgence of interest in natural dyes due to ecological concerns and stricter regulations regarding textile effluents. Additionally, there is increasing demand among artists and artisans interested in historical dyeing techniques. Historically, most natural dyes were obtained from wild plants, with only a few, such as indigo, woad, madder, and cochineal, being extensively cultivated. The development of

synthetic dyes, beginning with synthetic indigo by A. Bayer in 1878, significantly reduced reliance on natural dye sources. By 1914, only 4% of the indigo produced worldwide was extracted from plants.

The chemical indican, that is present in more than a hundred different plant species, produces the blue dye indigo. Indigo cake is produced in a centuries old fermentation process of indigo generating species.



## INDIGO STRUCTURE

Agricultural projects are currently expanding all over the world to produce natural dyes and one of them is indigo, the increasing importance of vegetable dyes can be gauged from the steep rise in prices of natural dyestuffs in the markets, prices have increased by five to eight times in the last year for some of them, therefore, is important to develop simple, fast and reliable methods for the analysis of natural dyes. Indigo and indirubin, an isomer of natural indigo, can be determined using UV-Vis spectrophotometry<sup>1–9</sup>, chromatography methods<sup>10–14</sup>, NMR<sup>15</sup>, electroanalytical methods<sup>16</sup>, and other spectroscopic techniques<sup>17–22</sup>.

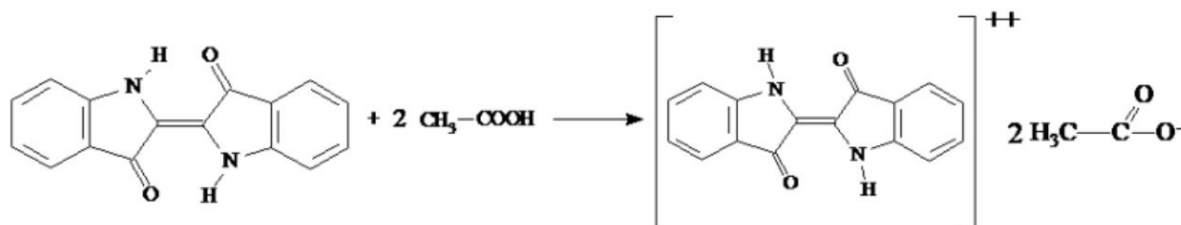
An evaluation of methods for the determination of indigo was made by Gutierrez et al<sup>4</sup>, they to point out that: colorimetry of the water-soluble leuco form is unsuitable because of colour instability. Turbidimetric measurement of the oxidized form is too dependent on particle size to be useful. Colorimetric measurement of a chloroform extract is possible, but difficulties arise due to low solubility of indigo in chloroform. More suitable is a method based on colorimetric measurement of the oxidized form at 625 nm in hot aq. 50 % pyridine at pH 9 to 10; reproducibility is 2 to 4%. A

more precise but time-consuming method depends on evaporation of a sample to dryness, sulphonation by heating with concentrated of sulfuric acid and colorimetric measurement at 605 nm of the resulting sulphonated indigo in water; reproductibility of this method is 0.3 to 2 %. This method is usually utilized for the determination of the indigo.

Despite these advances, to our knowledge, there is currently no officially accepted method for the determination of indigo. In this

study, we propose a new spectrophotometric method based on the formation of an N,N'-diacetyl derivative, which offers improved solubility and analytical performance.

In this work, a new spectrophotometric method for the determination of indigo based on the formation of N, N'-diacetyl derivative is proposed. The method is applied to the determination of indigo dye in natural (technical) samples.



## EXPERIMENTAL

### APPARATUS

A Spectronic 3000 Diode Array Milton Roy spectrophotometer with 0.35 nm resolution, coupled to a 486 PC was used. An User Data version 2.01 Milton Roy software was used for the acquisition, storage and manipulation of spectral data.

### REAGENTS AND MATERIALS

Indigo obtained from Aldrich (22,929-6, Milwaukee, WI) was used. Standard solutions of indigo were prepared in a 50 ml calibrated flask by dissolving the appropriate amount in 25 ml glacial acetic acid, which was then sonicated for 1 minute, boiled for another minute, and allowed the solution to cool down to room temperature before diluting to the mark with glacial acetic acid. All other chemicals were of analytical reagent grade.

### GENERAL PROCEDURE

A suitable volume of standard solution was transferred into a 10 ml standard flask to which 5 ml chloroform were added and glacial acetic acid to complete the volume. The absorption spectra of the solution was recorded from 400 to 700 nm against a glacial acetic acid-chloroform blank and the absorbance of the samples were measured at a wavelength of 614 nm. The content of indigo was determined by using the appropriate calibration graph.

### TECHNICAL GRADE SAMPLES

About 3 mg of technical grade sample were accurately weighed and suspended in 25 ml acetic acid, sonicated and boiled for 1 minute, allowed to cool to room temperature and diluted to 50 ml in a standard flask. Suitable aliquots of this solution were taken and processed as above.

## RESULTS AND DISCUSSION

One of the problems of the analysis of indigo is its low solubility in almost all possible solvents, thus making difficult the development of analytical methods. The currently used method is based on the formation of the corresponding disulfonic acid derivative through the treatment of indigo with concentrated sulfuric acid under heating, yielding a new derivative which is soluble in water. Unfortunately under this conditions is difficult to ensure that only disulfonated product has been formed. The proposed method is based on the formation of N,N'-diacetyl derivative<sup>23-26</sup> throughout the treatment of indigo with glacial acetic acid under the above stated conditions. This reaction ensure the formation of only one derivative which is more soluble in organic solvents than indigo. As mentioned in the introduction colorimetric measurement of indigo in a chloroform extract is possible but difficult due to low solubility of indigo in chloroform. Formation of N,N'-diacetyl derivative let the complet solubilization of indigo in chloroform.

### VARIABLE OPTIMIZATION

Absorption spectra of N,N'-diacetyl indigo derivative in chloroform are shown in figure 1. This product is more soluble than indigo in several solvents but specially in chloroform. Process variables were optimized to obtain maximum color development and stability. The influence of solvent on the analytical signal was studied with methylene chloride, chloroform and carbon tetrachloride. The results are summarized in table 1. Optimum response on the intensity of the absorption spectrum and stability was obtained with a 1:1 glacial acetic acid:chloroform mixture. The acetylation reaction was completed by heating to boil for 1 minute. Sample preparation is fast, but 10 minutes of standing time is necessary to ensure reproducible results. Color is stable for least 60 minutes.

## INDIGO CALIBRATION GRAPH

Once the optimum conditions were established, a calibration graph for indigo was produced. Good linearity was obtained within a concentration interval of 1 to 20 mg×ml<sup>-1</sup>. The linear regression equation obtained is as follows:  $Abs = (0.0535 \pm 0.0007) C_{IND} + (0.0032 \pm 0.0013)$  ( $r = 0.9998$ ). The relative standard deviation (rsd) obtained from the analysis of 11 samples ( $p = 0.05$ ) with 10 mg ml<sup>-1</sup> of indigo was 1.67 %, with a relative error of 1.22 %. Detection limit (LD, 3s/ m)<sup>27</sup> and determination limit (LQ, 10s/m)<sup>27</sup> were 0.09 ppm and 0.32 ppm of indigo, respectively.

### APPLICATION

The proposed method for the analysis of indigo has been applied to the determination of this compound in technical samples and the results compared with the spectrophotometric method reported previously<sup>1</sup> These results were compared with those obtained by sulfonating method. The evaluation of method bias<sup>27</sup> (for each analyzed sample) was carried out using statistical tests ( $F$ - and  $t$ -tests,  $p < 0.05$ ) and no statistically significant difference was detected for the indigo determination ( $t_{theoretical} > t_{calculated}$  and  $F_{theoretical} > F_{calculated}$  in all the cases). The results obtained in the analysis of indigo are shown in Table 2. Also, the statistical studies for each sample are shown. The results of real samples analysis were in good agreement.

## CONCLUSIONS

The present method does not involve time consuming procedures as reported elsewhere. Furthermore, the present method is superior to earlier methods due to the simplicity of the sample preparation and is as sensitive as the former. Therefore, the present method is simple, sensitive, rapid and useful for routine analysis.

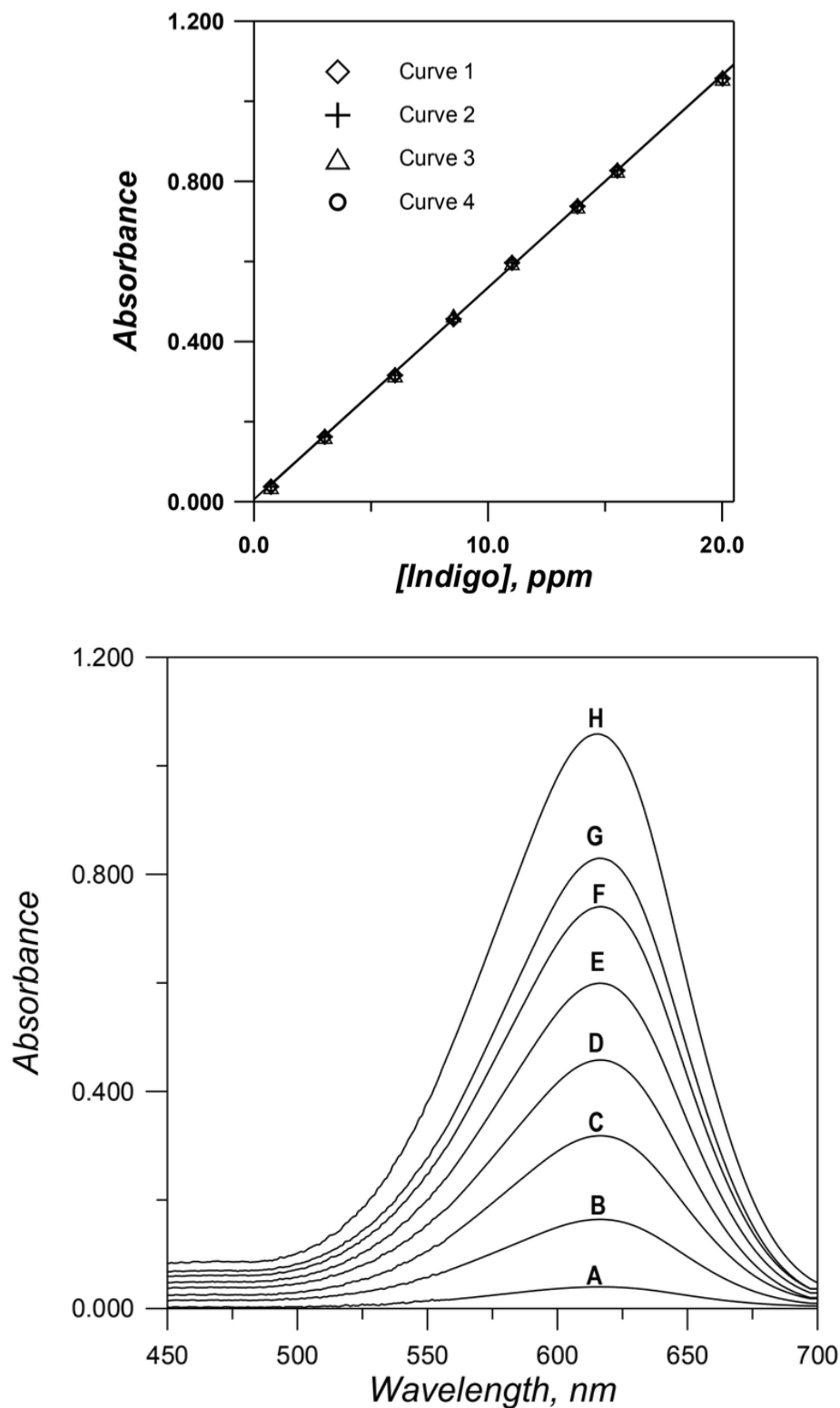


Figure 1.- Absorption spectra and calibration graphs of N,N'-diacetate indigo derivative. Indigo concentration, in (mg×ml<sup>-1</sup>) : A, 0.7; B, 3.0; C, 6.0; D, 8.5; E, 11.0; F, 13.8; G, 15.5; H, 20.0.

Solvent	Time, minutes			
	0	10	30	60
	Absorbance values			
*CH <sub>2</sub> Cl <sub>2</sub>	0.291	0.384	0.412	0.435
**CH <sub>2</sub> Cl <sub>2</sub>	0.181	0.223	0.236	0.253
*CHCl <sub>3</sub>	0.419	0.448	0.449	0.450
**CHCl <sub>3</sub>	0.189	0.226	0.432	0.246
*CCl <sub>4</sub>	0.109	0.143	0.185	0.197
Acetic acid,100%	0.158	0.161	0.160	0.163

\*solvent-acetic acid ratio, 1:1,

\*\*solvent-acetic acid ratio, 8:2

Table 1 Influence of the solvent on absorption spectra of N,N'-diacetyl indigo derivative,  $\lambda_{\max} = 614 \text{ nm}$ .  
Indigo concentration =  $9 \text{ mg} \times \text{ml}^{-1}$ .

Sample	Sulphonation Method* (%)	Proposed Method* (%)	$t_{\text{calculated}}$	$F_{\text{calculated}}$
1	$31.22 \pm 1.48$	$30.43 \pm 0.84$	1.10	3.10
2	$49.34 \pm 3.29$	$48.13 \pm 2.40$	0.70	1.88
3	$15.08 \pm 0.76$	$15.97 \pm 1.03$	1.65	1.85
4	$8.54 \pm 2.34$	$8.89 \pm 1.78$	0.28	1.74
5	$53.09 \pm 1.32$	$53.89 \pm 0.82$	0.50	2.59

\*n = 4, a = 0.05,

$t_{\text{theoretical}} = 2.365$ ,

$F_{\text{theoretical}} = 9.28$

Table 2. Assay of Indigo

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