# Characterization of the colouring molecules in the Mexican Indigo plant *(Indigofera suffruticosa Mill.)* by Ultraviolet-Visible spectrophotometry, Infrared and Protonic Nuclear Magnetic Resonance ($^1$HNMR)

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## Introduction

One of the most famous natural colourings used by the great civilizations of the world is the indigo, it has been obtained from vegetal and animal sources (Clark *et al.*, 1993; Galian and Gilles, 1993). In Mesoamerica, the Maya culture utilized it to elaborate the "azul maya". At the present, we know that is a nanoestructure in wich composition is involved a clay from the Yucatan peninsula and the dyeing principe from the Mexican Indigo plant (Tourbe, 1996; Yacaman *et al.*, 1996). The possible derivations from the dyeing indigotiers can be obtained from, at least, a dozen of vegetable families like: *Leguminosae, Brassicaceae, Polygonaceae, Acanthaceae, Orchidaceae, Apocynaceae* and *Asclepiadaceae* (Cardon and Chatenet, 1990). Around 50 species from these families has been studied; however, in these days, the most utilized and studied by the investigators are: *Isatis tinctoria* L. (Woad or Pastel), *Polygonum tintorium* Ait. (weed of dyers) and a group of leguminouses species called *indigotiers*. This group of species belong to the genre *Indigofera* and grow in different tropical climates around the world (Cannon *et al.*, 1994). In Asia "the genuine indigo plant" is the indian *Indigofera tinctoria* L. (Vavilov, N. I., 1992). In Africa, the *Indigofera arrecta* Hoschst is from Ethiopia (Cannon *et al.*, 1994). In America, there is one kind wich grow up from Mexico to South America, *Indigofera suffruticosa* Mill. It has different names according to the place and the languague. For example, the name *Xiquilite* or *jiquilite* was used by the mexicas; *Ch'oh* by the mayas, and *Yaga-Cohui pichacha* or *Nocuana-cohui* by the zapotecas (Moziño, S. J. M., 1994). Since the colonial period, the *Xiquilite* (ink weed), take the name *Añil*. The *añil* have one specie and two subspecies wich grow from central Mexico to Guatemala, El Salvador and Peru. The two subspecies are: *Indigofera suffruticosa* Mill., ssp. s*uffruticosa* and *Indigofera suffruticosa* Mill., ssp. *Guatemalensis* (Kort and Thigisse, 1984).

## The chemistry of the indigo in plants

In all the indigo producer plants doesn't exist, in their vegetable parts, some substances that have coloring properties. However, after a meticulous extraction, gradually the singular blue color of the indigo appears in the reactional medium. This is possible because of the presence of colorless glycoside precursors in the cells (especially in leafs), which, in specific conditions, evolve to indigo (Vilarem, G. 1999). It exist three indigo precursors that are product of the different indigotier species: Isatan B, Isatan C and Indican. (Epstein, E. *et al.*, 1967; Zhi-Quiang, X. and Meinhart, H. Z., 1992; Maugard, T. *et al.*, 2001).

Because of the characteristics of the indigo extraction, it is believe that the indican [1] is the primary precursor of añil, but there are no results that probe it. In general, when indigo precursors are submitted to hydrolysis, when the glucose is separated, the indoxyl [2] is liberated. It is highly reactional and, in presence of oxygen, forms the indigo or indigotin [3] joined to other similar molecule. When the indoxyl suffers an oxidation forms isatin [4]. If this specie joins to indoxyl, the reaction produces the indirubin [5]. This last compound is a structural isomer of the indigo, but it has a red color (Stoker, G. K., *et al.* 1998). When the indican is precursor, the enzyme indican-*β*-glucosidase realizes the hydrolysis. The enzyme and its substrate are separated in different compartments inside the cell. In *Polygonum tinctoreum* Ait., some evidences show that the indican is localized in the cellular vacuole, and the *β*-glucosidase is concentrated in the mesophyll cells at the stroma of the chloroplasts (Minami, Y., *et al.*, 1997).

According to the biosynthesis route of the indigo in some species, it has proposed a possible way of the indigo production in añil. The present work tries to confirm that the final products are the indigo and indirubin, as happens in other indigotier plants.

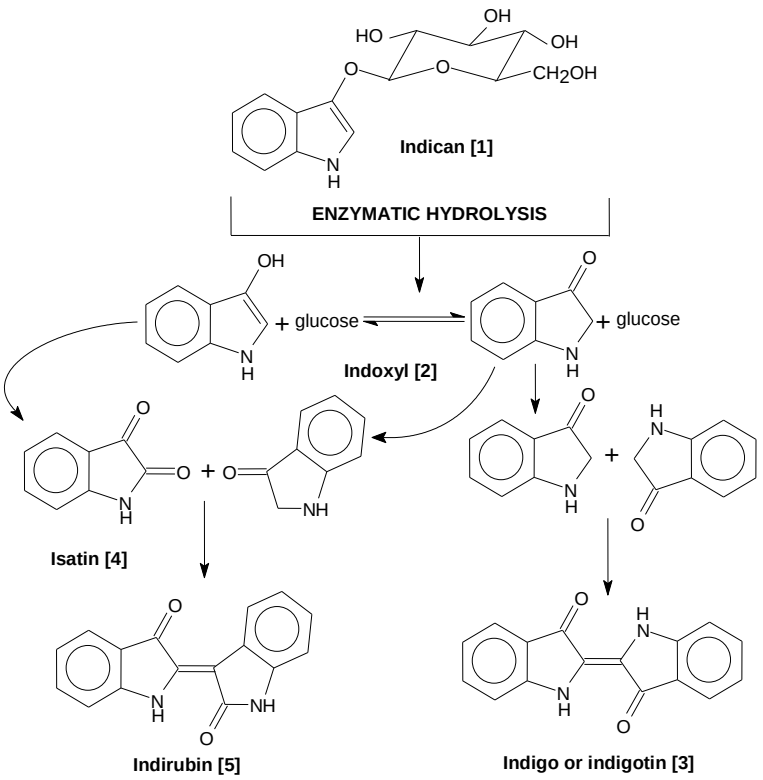


**Figure 1. Possible biosynthetic way of the coloring compounds of añil.**

At present, in many countries, mainly in Europe, the studies about indigo extraction and analysis methods have been intensified. In England, some investigation works are focus in optimize the extraction conditions, the obtaining of a solid extract with high colorant concentration, the analysis of the indigo precursors and the coloring itself (Stoker, G. K., Cooke, T. D., and Hill, J. D., 1998; Gilbert, K. G., *et al.*, 2004; García, M. P., and John, P., 2004). In Germany, the investigators have been searched the quantification of the precursors in *Isatis tinctoria* L. (Oberthür, C. *et al.*, 2004a; Oberthür, C. *et al.*, 2004b). In France, the works of investigation have permitted the commercial production of the indigo based on leafs of *Isatis tinctoria* L. (Matadamas O., E., 2002).

The Mexican production of the indigo is realized by farmer organizations in the regions of the Istmo Oaxaqueño and the Huacana, in the Michoacan state. The production of this natural coloring in Mexico rise to 400 Kg per year, and it is commercialized mainly in regional markets, but sometimes it has been exported, a no significant proportion, to France and Germany.

The product obtained from the extraction of añil leafs by the producers is a solid with the form of tiny stones that posses an intense blue color. When we study these solid extracts, the major part of the weight corresponds to no dyeing material, which means that those extracts have a variable concentration of coloring molecules with values from 4 to 40% and the rest are impurities (Matadamas O., E. J., 2001, Matadamas O., E. J., 2002, Matadamas O., E. J., 2006a and Matadamas O., E. J., 2006b).

The main purpose of this study was to characterize, by a chemical method, the coloring molecules of the blue solid extracts (BSE) obtained from the añil leafs using instrumental techniques of Ultraviolet-Visible spectrophotometry, infrared and protonic nuclear magnetic resonance. This procedure was useful to determinate, precisely, these compounds; in this way, it is possible to establish and improvement the industrial processes of application, besides to count with quality specifications to its sell.

## Materials and methods

### Vegetal material

The leafs from the extraction were obtained from the crop of añil "San Lorenzo", in the central valleys of Oaxaca, identified and preserved by the author. The ideal leaves were selected from plants that haven't blossom at the crop time.

### Extraction

A solid-liquid extraction was realized with 30°C water using as solvent, with 15 hours of maceration. After that, a blue precipitate was separated by filtration and was dried 12 hours at 55°C in a stove. The blue solid extract (BSE) was brought to Agro Industrial Chemistry Laboratory (LCA) of Superior National School of Engineers in Chemical and Technological Arts of Toulouse, France (ENSIACET) to do its characterization.

### Separation and obtaining of pure fractions

The separation of coloring fractions from the remaining compounds was realized by chromatography in an open column (40 mm of diameter). The system of elution determined by preliminary essays of thin layer chromatography was integrated by chloroform and a gradient of ethyl acetate in proportions: 5, 10, 15 and 20%. This solvents system permitted to obtain two fractions, one blue and one red. The fractions were concentrated and the crystals were obtained for their analysis.

### Thin Layer Chromatography

To keep the purity of the coloring fractions, before to be submitted to the analytic techniques, we proceeded to do a control using the thin layer chromatography (TLC). Some silica badges and a mobile phase composed with chloroform and methanol (90/10) were utilized, and clear bands with frontal relations ($R_f$) of 0.54 and 0.65 from the red and the blue fraction, respectively, were obtained.

### Ultraviolet-Visible spectrophotometric analysis

One sample of 15 mg of BSE and samples of the red and blue extractions, each one with 5 mg, were dissolved in dimethylformamide (DMF) and passed by the spectrophotometer (VECTRA ES/12 Hewlett Packard – 8452 A) to obtain the maximum absorptions and the ultraviolet and visible spectrum.

### Infrared Spectroscopy

50 mg of solid samples from the blue and red fractions were taken to elaborate pills with KBr and were passed by the infrared equipment Perkin Elmer, Nicolet 210 with transformed of Fourier (IR-FT).

### Nuclear Magnetic Resonance Spectroscopy

The purified samples were dissolved in DMSO- $d_6$ and then they were brought to the Broker ARX 400 MHz equipment of the Polytechnic National Institute of Toulouse, France.

## Results and discussion

### Ultraviolet and visible absorption spectrophotometry

The absorption of the three analyzed samples (the BSE and the purified blue and red fractions) was almost equal in the ultraviolet region (250 to 295 nm). There were two absorption bands at 255 and 290 nm. These bands were the most visible in all the spectrums and increase their absorbance if the concentration of the samples rises. In the visible region, the BSE and blue fraction had a maximum absorption at 610 nm wavelength. The red fraction presented a maximum band of absorption at 542 nm. When we passed by the spectrophotometer some samples of indigo and synthesis indirubin, we found the same bands of absorption. The spectrums are shown in figure 2.

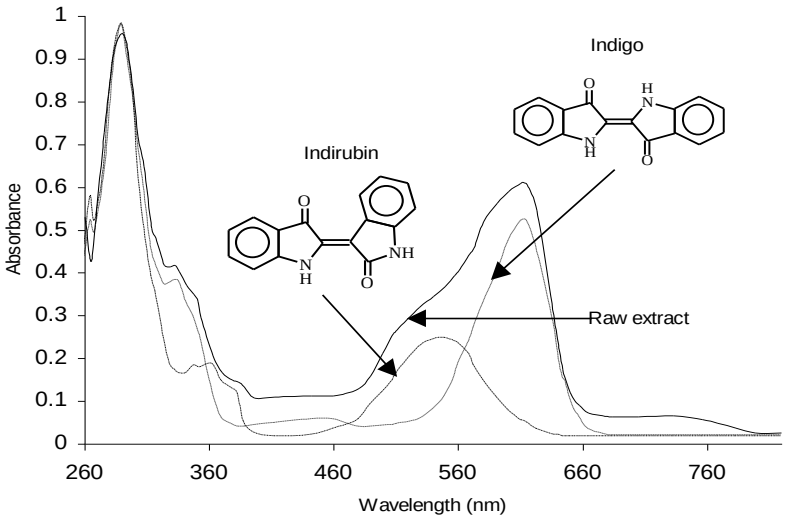


**Figure 2. Spectrums from the blue solid extract and the red and blue fractions.**

The absorption bands that appear between 250 and 295 nm in ultraviolet region are consequence of the electronic orbital transitions $n \rightarrow \pi^*$, $n \rightarrow \sigma^*$ and $\pi \rightarrow \pi^*$ from the C=O group of the indolic nucleus and the double conjugated liaisons of the blue and red fractions.

The three samples show different absorption bands in the visible region, this is explained by the production of transitions from the C-C and C=C molecular orbital electrons which are localized in the aromatic rings of the N-H and C=O liaison electrons. The links with N and O has $n$ no connectable electrons that suffer transitions $n \rightarrow \sigma^*$ (N-H) and $n \rightarrow \pi^*$ (C=O). In addition to the absorption produced by the transition of electrons $\pi \rightarrow \pi^*$ (C=O and C=C) and $\sigma \rightarrow \sigma^*$(C-C and C-H) produce a band in the visible region. The absorption band of the BSE

includes the absorptions of the blue and red fractions. The spectrum of the red fraction presents a hypsochromic shift of the absorption band, attributed to a minor formation of O-H electrostatic bridges. The consequence of this is that *n* electrons need more energy to produce a transition $n \rightarrow \pi^*$ from the C=O liaison. When two samples of indigo and synthesis indirubin are shifted, we can notice that their spectrums are equal to the blue and red fractions. With this technique it is possible to conclude that the blue fraction corresponds to the indigo or indigotin and the red fraction to the indirubin. This last compound is a structural isomer of indigo that have λ=542 nm as its maximum absorption band.

## Infrared Spectroscopy

It is notable that two medium intensity absorption bands appear at 3419.9 and 3265.0 $cm^{-1}$, at the 3500–3200 $cm^{-1}$ region in the infrared spectrum from the blue fraction. And, we can notice 6 bands with variable intensity at the 1600 and 1400 $cm^{-1}$ region. The relation between the absorption bands for the two fractions is shown in table 1.

**Table 1. Absorption bands in infrared.**

| Band | Bands localization ($cm^{-1}$) | |
|---|---|---|
| | Blue fraction | Red fraction |
| 1 | 3419.9 | 3345.9 |
| 2 | 3265.0 | 2929.6 |
| 3 | 1626.0 | 2853.1 |
| 4 | 1613.9 | 1742.2 |
| 5 | 1585.3 | 1663.7 |
| 6 | 1482.7 | 1621.0 |
| 7 | 1461.8 | 1462.4 |
| 8 | 1393.4 | ----- |

In the red fraction spectrum, we observe 7 absorption bands between 4000 and 1400 $cm^{-1}$. And in comparison, with blue fraction spectrum, two additional bands between 3000 and 2800 $cm^{-1}$ region at 2929.6 and 2853.1 $cm^{-1}$. In the blue fraction spectrum, one of the *v* medium intensity elongation bands (N-H) corresponds to N-H connected by molecular hydrogen (3265.0 $cm^{-1}$) and the other one (3419.9 $cm^{-1}$) corresponds to a free N-H. In the red fraction spectrum, the absorption band at 3345.9 $cm^{-1}$ is caused by free N-H vibrations. The position from one of the molecular halves explains the absorption bands at 2929.6 and 2853.1 $cm^{-1}$ in the indirubin or red fraction, the C-H groups tend to be less influenced by the O-H intermolecular and intramolecular connections than in the indigo molecule: these vibrations do not appear in the blue fraction spectrum. A strong absorption band (*s*) at 1626.0 $cm^{-1}$ in the blue fraction spectrum can explain a *v* elongation vibration (C=O) from the conjugated ketones. This vibration is attenuated by the strong conjugation with the aromatic ring and the C=C double connection that separates the indolic nucleus of the indigo molecule. The bands at 1613.9, 1585.3, 1482.7 and 1461.8 $cm^{-1}$ are attributed to lengthening vibrations from the *v* double connection (C=C). The band at 1393.4 $cm^{-1}$ is caused by an angular deformation vibration from $\delta$ (N=H). In the red fraction spectrum, besides the bands described, exist one at 1742.2 $cm^{-1}$, caused by *v* lengthening vibrations (C=O) from one of the ketones belong to a half of the indirubin molecule. The absorption band that appears at 1663.7 $cm^{-1}$ is attributed to lengthening oscillations from the *v* conjugated amide (C=O) and the absorption band at 1621.0 $cm^{-1}$ is caused by *v* amine vibrations (C=O). The more important functional groups from the indigo and the indirubin are represented by the absorption bands in their respective spectrums.

## Nuclear magnetic resonance spectroscopy ($^1$HNMR)

In the blue fraction, the absorption bands from the protonic nuclear magnetic resonance spectrums are: (DMSO-d6) δ 6.96 (t, J = 7.5, C2H with CH1 y CH3), 7.34 (d, J = 8.0, CH4 and CH3), 7.52 (t, J = 8.2, CH3 with CH2 and CH4), 7.62 (d, J = 7.5, CH1 and CH2), 10.48 (s). And the absorption bands from the red fraction spectrum are: (DMSO-d6) δ 6.92 (d, J = 8.1, CH'4 and CH'3), 7.04 (t, C2H and CH'2), 7.27 (t, CH'3 with CH'2 and CH'4), 7.43 (d, J = 8.1, CH4 with CH3), 7.59 (t, CH3 with CH2 and CH4), 7.67 (d, J = 7.4, CH1 with CH2), 8.77 (d, J = 7.7, CH'1 with CH'2), 11.01 (s) and 10.88 (s) (NH' y NH).

The chemical displacements and the couplings are equals from one to another part of the C=C connection because the indigo molecule is symmetric in relation to the C=C double connection. Consequently, we can indicate just the results for the middle part of the molecule. The singlet at 10.48 ppm is the proton connected to the nitrogen (N-H), being the most displaced. The doublet at 7.62 ppm is caused by the coupling between the $H_1$ proton and $H_2$ proton, with a constant of coupling 7.5 Hz. The triplet at 7.52 is originated by the $H_3$ coupling with the $H_2$ and $H_4$ protons, with a constant of coupling 8.2 Hz. The doublet at 7.34 ppm is caused by the coupling between the $H_4$ proton and the $H_3$ proton, with a constant of coupling 8.0 Hz. The triplet at 6.96 ppm is caused by the coupling from $H_2$ proton with the $H_1$ and $H_3$, with a constant 7.5 Hz. The sequence, in which the signals appear in the spectrum, permits to deduce that there are some partial charges (δ+) in the carbons with $H_1$ and $H_3$, and some partial charges (δ-) in the carbons with $H_2$ and $H_4$. In consequence, the $H_1$ and $H_3$ will be more displaced, and $H_1$ will be more displaced than $H_3$ because it is very near to the carbonyl group. About to the least displaced, $H_2$ and $H_4$, their chemical displacements (6.96 y 7.34, respectively) are attributed to a multiplicity of signals: doublet to $H_4$ and triplet to $H_2$. The nuclear magnetic resonance spectrum from the red fraction presents three kinds of signals: 2 singlets at 11.01 and 10.88 ppm; 4 doublets at 8.77, 7.67, 7.43, and 6.92 ppm; and 3 triplets at 7.59, 7.27, and 7.04 ppm. The singlets are produced by the couplings between N-H' and N-H, respectively. The doublet at 8.77 ppm is caused by the coupling between $H'_1$ and $H'_2$; the doublet at 7.67 ppm is produced by the coupling between $H_1$ and $H_2$; the doublet at 7.43 ppm is caused by the coupling between $H_4$ and $H_3$ and the doublet at 6.92 ppm is the consequence from the coupling between $H'_4$ and $H'_3$. Finally, the interpretation of the triplets: the one at 7.59 ppm, is produced by the coupling between $H_3$, $H_2$ and $H_4$; the triplet at 7.27 is caused by the coupling between $H'_3$, $H'_2$ and $H'_4$; and the triplet at 7.04 ppm is produced by the resonance between $H_2$ and $H'_2$.

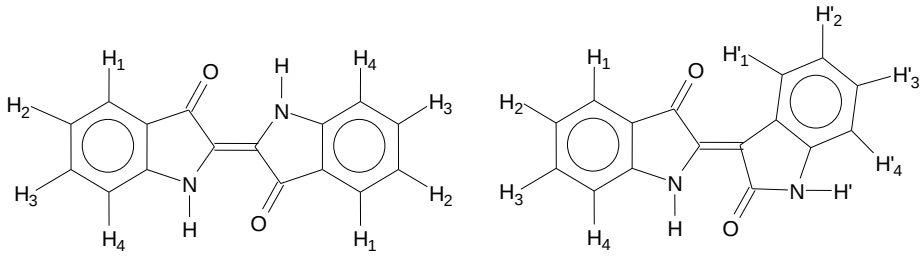


**Figure 3. Chemical structure from the indigo and the indirubin.**

## Conclusions

Based on the results obtained utilizing the thin layer chromatography, the Ultraviolet-Visible spectrophotometry, the infrared and the protonic nuclear magnetic resonance, it is possible to conclude that the blue solid extracts obtained from the añil leafs have two coloring molecules: indigo and indirubin. It doesn't exist other coloring materials as usually it has been said verbally.

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