

THERMOTRANSFORMATION FROM INDICAN TO INDICAN ESTER B

ELÍAS-Jaime, MATADAMAS-ORTIZ

Autonomous University of Chapingo. Agroresources and Secondary Metabolites Laboratory.
Km. 38.5 Mexico-Texcoco Highway. C.P. 56230, Chapingo, State of México. MEXICO.
emata993@hotmail.com.

Abstract

Indican is the indigo precursor present in the leaves of Indigofera suffruticosa. This is a glycoside containing an indolic aglycone joined to the glucose. The indigo traditional extraction in Mexico is by enzymatic action of a β -glucosidase that attacks the indican at ambient temperature. However, when this precursor is subject to a temperature of about 70°C, undergoes a transformation into a form ester which we call indican B. This behavior of the indican allowed developing an experimental extraction method that produces a higher yield than the enzymatic method. This new method is by alkaline hydrolysis of indican B. This new form of precursor has not yet been characterized by our part and could be isatan B. This last is the majority precursor of Woad (Isatis tinctoria) whose extraction method also involves an alkaline hydrolysis. Leaves woad contain various precursors such as indican, isatan B or isatan C. It would not be strange that leaves of Indigofera species also contain isatan B. A hypothesis suggests that all indigo plants have a single precursor that is the indican which is affected by oxidative processes generated by thermal stress and transformed to isatan B.

Introduction

The extraction of natural indigo from plants has been an ancient practice in various parts of the world. Nowadays, this one is done traditionally in several countries as India, Japan, Vietnam, El Salvador, Guatemala and Mexico, and with modern methods in some European countries such as France, England and Germany (Vilarem, 1999; Vilarem, 2005; Stoker, *et al.*, 1998a; Stoker, *et al.*, 1998b., Oberthür, *et al.*, 2004). The need to optimize the yields and quality dye has led to the study of the evolution of indigoids dyes from their precursors (Maier *et al.*, 1989; Zhi-Qiang and Meinhart, 1992,. Maugard, *et al.*, 2001; Chanayath, *et al.*, 2002;. Gilbert, *et al.*, 2004; Garcia-Macias and John, 2004; Oberthür, *et al.*, 2004). The traditional extraction methods and those which have recently been generated primarily taking into account the indigo plant species to extract. Thus, the elaboration of "cocañas" was the predominant indigo extraction method in Europe, particularly in France, which consisted of crushing fresh leaves of *Isatis tinctoria* L. (Woad) and composting in order to encourage fermentation and evolution of precursors to indigo (Hurry, 1930; Caster, 1998; Kokubun, *et al.*, 1998). The final product of this method was a dehydrated organic material rich in coloring matter (*agranate*). The product was submitted to rehydration process then the juice was reduced in vats and carried to dyeing of fibers. Such method was almost exclusively for woad. In India and in America, the indigo extraction method, which nowadays is in use consists of

collocating fresh leaves of *Indigofera* genus in water and force out the reaction medium of the precursors and enzymes that attack them. After several hours of soaking and removing the leaves, the aqueous extract is stirred for several hours to induce hydrolysis of the precursor by the action of β -glycosidase and to induce formation the dye matter. The final product is a blue solid extract (BSE), also called *bluestone*. In the nineties decade of last century in France and England the production of natural indigo was relaunched from woad leaves and extraction methods were generated (Stoker *et al.*, 1998 ; Vilarem, 2005). In essence this is the same method used for *Indigofera* species, but with the difference that hydrolysis is chemical in nature. More clearly, there is the need to add an alkali to achieve hydrolysis of the major precursor. The enzymatic or chemical hydrolysis is the starting point for the formation of dyes indigoids. Although recent studies on the nature of indigo precursors in plants and its evolution to indigo or their derivatives, this aspects are not yet sufficiently clear. In addition, the dynamic and transformations of these precursors during the extraction are not studied in detail, and their results seem to support the idea that there are different precursors between indigotic species. Thus, the literature reports that the leaves of *Isatis tinctoria* (woad) contain **isatan B**, **isatan C** and **indican** (some authors also argue that there is **isatan A**); while the leaves of various genus of indigo plants including *Indigofera* (with at least 50 species around the world), only contains the indican precursor (Hoogerwerff and Meulen, 1900; Beijerinck, 1900, Epstein *et al.*, 1967; Maugard, *et al.*, 2001; Oberthür, *et al.*, 2004). The chemical characterization of indigo precursors reported in these studies reveals that isatan A, isatan B and isatan C are structures glycosides with ester linkage, while the link in indican precursor is of ether type. These research results agree in practice with the indigo extraction methods for different species. Therefore, leads us to associate these methods with an enzymatic hydrolysis extraction to plants belonging to the genus *Indigofera* and the rest of indigo plants with exception of woad associated with alkaline hydrolysis.

However, when we try to implement processes to optimize the extraction conditions, in order to increase yields and purity of the extracts, the precursors do not seem to behave as reported in the literature. We have observed that it is possible to perform an alkaline extraction in *Indigoferas* and that the indican is hydrolyzed by the action of a base after a heat treatment as with the ester precursors.

In this work we report the observations the precursor indican behavior from leaves of Mexican indigo plant (*Indigofera suffruticosa* Mill.) during its extraction, specifically its thermal transformation to an ester precursor which has allowed an alkaline extraction of indigo.

The indigo precursors in plants

Indigo precursors are chemically from heteroside nature whose constitution involves an indole nucleus attached to a glucose molecule. These compounds are found in at least 200 species in various genres of botanical families such as, Brassicaceae, Acanthaceae, Orchidaceae, Polygonaceae and Leguminosae. The most commonly used species for the indigo extraction are *Isatis tinctoria* L., *Polygonum tinctorium* Ait., and several species of the genus *Indigofera*, being the best known, *Indigofera tinctoria* L. (indigo plant in India), *Indigofera arrecta* (African indigo plant) and *Indigofera suffruticosa* Mill., (*Añil* or Mexican indigo plant) (Cannon, *et al.*, 1994; Cardon, 2007).

The indican precursor

Until the mid-19th century it was believed that there was only one colorless precursor present in all indigo plants, which evolved to indigo after hydrolysis and oxidation.

In 1855, Schunck isolated and described a compound considered to be the indigo precursor of *Isatis tinctoria*, he called indican and proposed a structural formula for this compound as $C_{26} H_{31} NO_{17}$ (Schunck, 1855; Hoogewerff and Meulen, 1900; Epstein, *et al.*, 1967). Although Schunck made attempts to cultivate *Indigofera* in England, his efforts were unsuccessful, so he did not have a comparison point of his precursor with that from other plants (Farrar, 1977). However, Shunck supposed the precursor that he had isolated from the woad leaves was different to other species such as *Indigofera* sp., and *Polygonum tinctorium*, basing their doubts in the fact that the precursor isolated from woad was unstable and easily hydrolyzed for bases and acids, while the "indican" from the other species was very crystalline and very stable in the presence of bases and acids (Shunck, 1855; Perkin and Bloxan, 1907). Anyway and despite of reactivity difference, precursors from different species received the same name indican.

Marchlewski, however, took with certain discretion the formula proposed by Schunck and in 1898 proposed his own formula, based on the hypothesis that it could be an indoxyl glycoside, with a $C_{14} H_{17} NO_6$ structure by illustrating the linkage between the indole group and glucose. Nevertheless, Marchlewski apparently did not have a vegetative material available to test experimentally the accuracy of his hypothetical formula, although it proved eventually he was right (Hoogewerff and Meulen, 1900).

In 1900, Hoogewerff and Meulen worked with vegetative material of *Polygonum tinctorium* and *Indigofera leptostachya* and isolated a compound which they identified as indoxyl- β -D-glucoside with the structural formula $C_{14} H_{17} NO_6$.

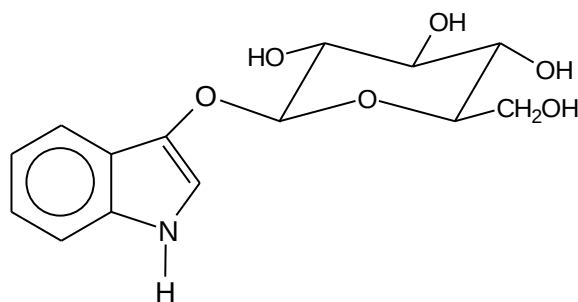


Figure 1. Indican or indoxyl- β -D-glucoside

These authors also stated that they did not observe differences between the indican of *Indigofera leptostachya* and that from *Polygonum tinctorium*, so that they concluded it was the same molecule.

Indican and indoxyl plants

Beijerinck in 1900, also presented the results of their research related to indigo precursors of *Isatis tinctoria*, *Polygonum tinctorium* and *Indigofera leptostachya* and tried to demonstrate for the first time the possible existence of two different precursors. For this author, *Polygonum tinctorium* and *Indigofera leptostachya* are plants containing indican (indoxyl- β -D-glucoside), whereas *Isatis tinctoria* is an "indoxyl plant." The Beijerinck argument was that extracts from *Isatis* only produce indigo when they are treated with alkali, while the indoxyl- β -D-glucoside is stable even in presence of strong bases and is hydrolyzable by a glycosidase enzyme present in the same plant leaves. Moreover, by incubating a crude extract of glycosidase enzyme of *Indigofera* or *Polygonum* with aqueous extracts of *Isatis* any coloring substance was not obtained. Finally this author concludes that the indican is not present in *Isatis tinctoria* and possibly there is a substance that liberates indoxyl and named "isatan". Beijerinck's demonstration was so convincing, that since the publication of his work is generally accepted that there are two precursors; the indican for *Indigofera* and *Polygonum tinctorium* and isatan for *Isatis tinctoria*.

The isatan B precursor.

Epstein *et al.*, (1967), reported to have isolated the indigo precursor from *Isatis tinctoria* that Beijerinck had predicted and called isatan. Beijerinck characterized this substance as indoxyl-5-ketogluconate and gave the name isatan B. What Beijerinck did not take into account was that the name isatan had already been allocated to an isolated molecule by Laurent in 1842 whose structure was suggested until 1916 by Lefèvre and was synthesized by Hansen eight years later as 3-hydroxyl-3,3'-bioxindole. Therefore the Epstein team decided to name his precursor as isatan B in order to differentiate from Laurent compound.

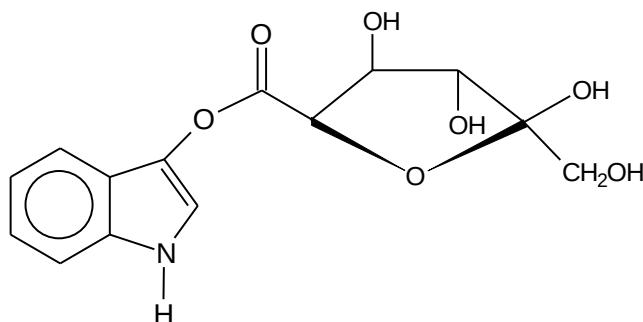


Figure 2. Indoxyl-5-ketogluconate or isatan B

In order to determine the origin of isatan B, Schraudolf in 1968 put etiolated seedlings of *Isatis tinctoria* in a rich medium radioactive compounds of [2-¹⁴C] indole and D, L-[2-¹⁴C]-tryptophan and noted that the indole isotope joined to the isatan B. He concluded that indole gives rise to isatan B. Years later Zhi-Qiang and Meinhart (1992) confirmed this.

Strobel and Gröger (1989) reported that both indican and isatan B are present in *Isatis* species, the majority being isatan B in leaves and indican in the roots.

Zhia-Qiang and Meinhart (1992) isolated the indican of *Isatis tinctoria*, *Polygonum tinctorium*, *Baphicanthus cusia* and *Calanthe veratrifolia*. According to the knowledge so far it established that only *Isatis tinctoria* contains two precursors and all other indigotic plants contain only indican.

Kokubun et al., (1998) quantified indican and isatan B in *Isatis tinctoria* leaves and they concluded that in young leaves approximately 24% of the dry weight was represented by the precursors in an approximate relation of 3:1 (isatan B / indican). These authors also found that adding a base to crude extracts of isatan B and to reach a pH value of 10.7, there was an immediate hydrolysis and indigo was formed. They remarked that this instability in alkaline medium is consistent with the ester nature of isatan B. Meanwhile the indican was resistant to hydrolysis even in pH values of 13.1.

Maugard et al., (2001) reported a new indigo precursor in *Isatis tinctoria*, which he called **isatan C**. This precursor was tentatively identified as dioxindole ester with a molecular weight of 395.0 and a possible molecular formula C₂₀H₁₃O₈N. Like isatan B, isatan C was shown to be unstable in alkaline medium to hydrolyze quickly, which was assigned an ester nature.

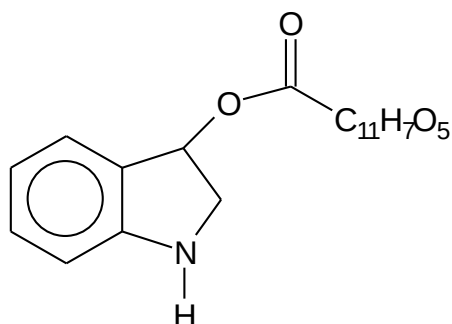


Figure 3. Isatan C or dioxindole ester.

This author concludes that the *Isatis tinctoria* leaves harvested in June produced a significant amount of isatan C which promotes the production of red pigments such as indirubin and isoindirubin. It is the first time reported that there may be specific precursors for the formation of a dye molecule in particular.

At the University of Jena, Germany, Oberthür *et al.*, (2004) extracted and studied precursors with higher polarity than the indican and isatan B in *Isatis tinctoria* leaves. He identified a new precursor which he called isatan A (1H-indole-3-6'-O-(carboxyacetyl)-β-D-ribohex-3'ulopiranoside). Furthermore, he rectified the isatan B structure; instead of indoxyl-5-ketogluconate was found to be 1H-indol-3-yl-β-D-ribohex-3-ulopiranoside.

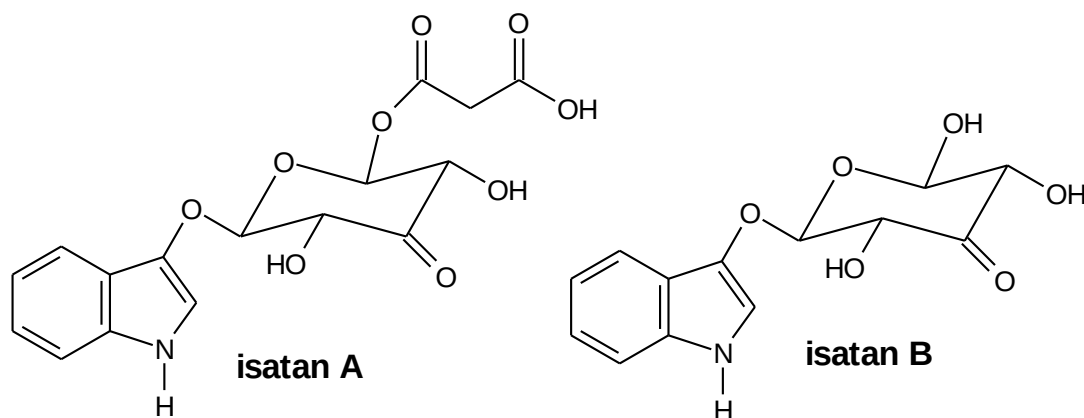


Figure 4. The isatan A and Oberthür's isatan B new structure.

In these works, isatan A proved to be the major precursor and in the same case that isatan B produced indigo under a treatment with bases or acids. Moreover it arise the hypothesis that indican can become in isatan B through an oxidoreductase enzyme.

Chemical model of indigo production in plants.

With the knowledge generated to date it has established a chemical model that aim to explain the precursor's evolution to dyes. It starts with the formation of precursors from indole which undergo hydrolysis during extraction in order to liberate indoxyl and glucose. This hydrolysis is carried out for the case of ester precursors (isatan A, isatan B and isatan C) by the alkaline pathway in leaves of *Isatis* species, while indican hydrolysis (in *Indigofera* sp. *Polygonum tinctorium* and other plants) is effected enzymatically.

Minami *et al.*, (1996) isolated a β -glucosidase enzyme of leaves of *Polygonum tinctorium* that hydrolyze to indican but also he found that it attacks other β -glucosides. This enzyme showed high activity in a pH range of 5,5 to 7,5, and it dramatically decreased to a pH of 5.0, it indicates that this catalyst has different properties because the majority of glucosidases that its pH range is 4,0 to 5,5 . The thermostability of this enzyme were also determined and found to be in a temperature of 37°C for a time of 25 minutes or in 0°C for one hour, its activity decreased by 50%. At a temperature of 60°C for 5 minutes its activity completely disappeared. Furthermore, some divalent cations such as Cu^{2+} , Ag^+ , Hg^{2+} , Co^{2+} , Ni^{2+} , Fe^{2+} , Zn^{2+} and Cd^{2+} inhibited the enzyme activity of *P. tinctorium* as in the majority of β -glucosidases.

According to the above it is possible to establish that the extraction methods are determined by the hydrolysis type of the precursors in each of the indigo-producing species. The extraction method for woad is by alkaline via and for other species is performed enzymatically. This differentiation lies in the chemical characteristics of the precursors.

As a result of precursor's hydrolysis, depending on the specie, an indoxyl is liberated. When two indoxyl molecules spontaneously condense forms a molecule of indigo or indigotin. The indoxyl can also be oxidized to become in isatin and then it reacted with an indoxyl molecule and producing an indirubin molecule. Isatan C, meanwhile a once hydrolyzed is oxidized to dioxindole and later to an isatin that by joining with the indoxyl finally form indirubin as well.

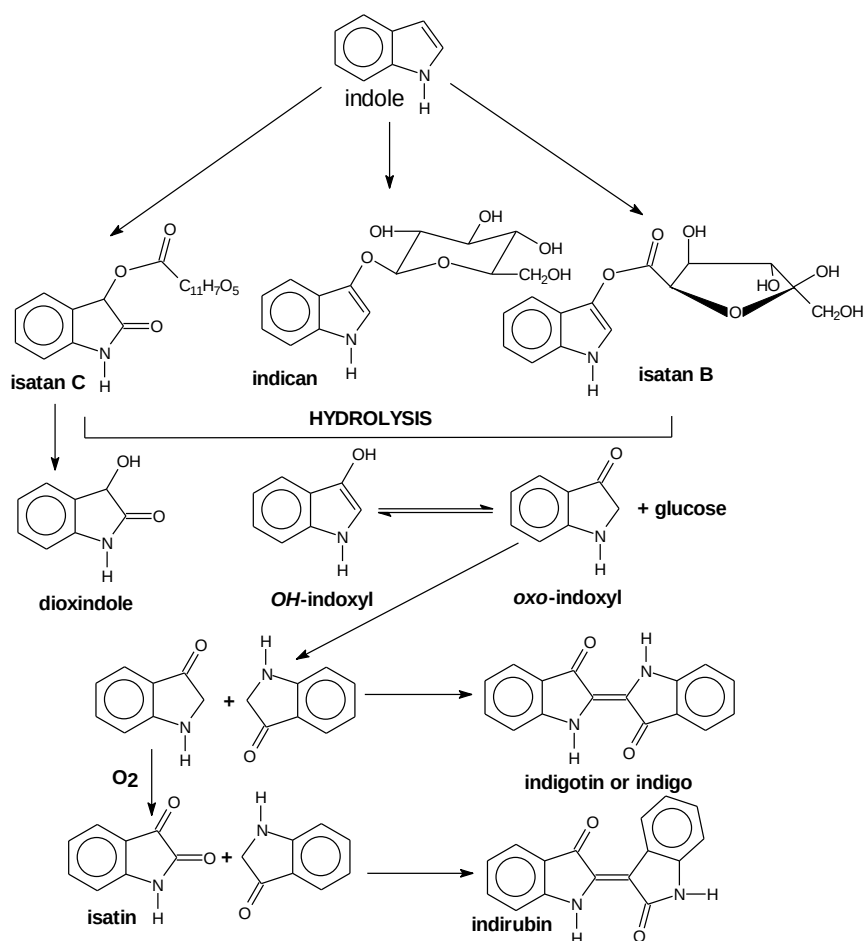


Figure 5. Chemical model of dye molecules formation. (Kokubun et al, 1998. Stoker et al, 1998a;. Stoker et al, 1998b. Maugard et al, 2001.).

Indigotin and indirubin are structural isomers having different physical and chemical properties.

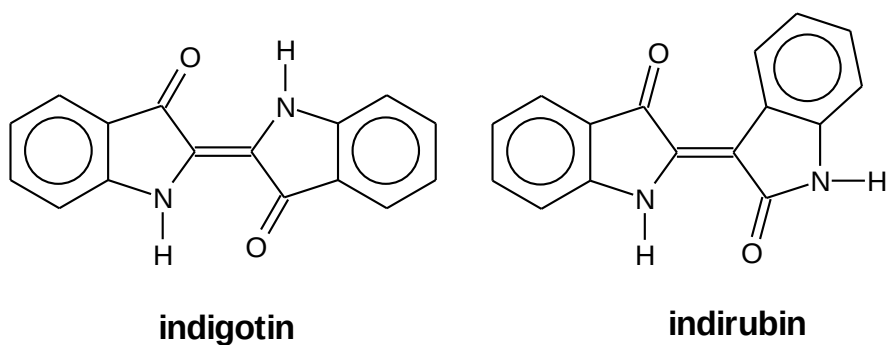


Figure 6. Main dye molecules that evolve from the indigo precursors in plants.

From fundamental knowledge that so far we have, it is accepted that the indican precursor is resistant to basic hydrolysis (this has been achieved only in laboratory conditions at very high temperatures and a strongly basic pH) and liberation of indoxyl only be achieved by effect of β -glucosidase enzyme, causing the extraction is a long process and resulting indigo having varying concentrations of dye molecules.

Observations on the indican behavior of *Indigofera suffruticosa*.

The traditional method for extracting indigo from Mexican indigo plant (*Añil*) is based on enzymatic hydrolysis. So far there are no reports on possible chemical hydrolysis in this specie. However, we have observed that if the extraction solvent with the leaves are heated to a temperature of about 70°C, the resulting extract do not react to oxygenation and it does not form indigo, as with the aqueous extracts product of a maceration at room temperature (enzymatic hydrolysis). By raising the pH to values of 9.0-10.0 by the addition of a base, occurs immediately indigo formation, as it were a hydrolysis of precursor esters (isatan A, isatan B and isatan C). This has led us to set the hypothesis that the ether indican undergoes a thermal transformation and becomes an ester precursor, which we have called indican B.

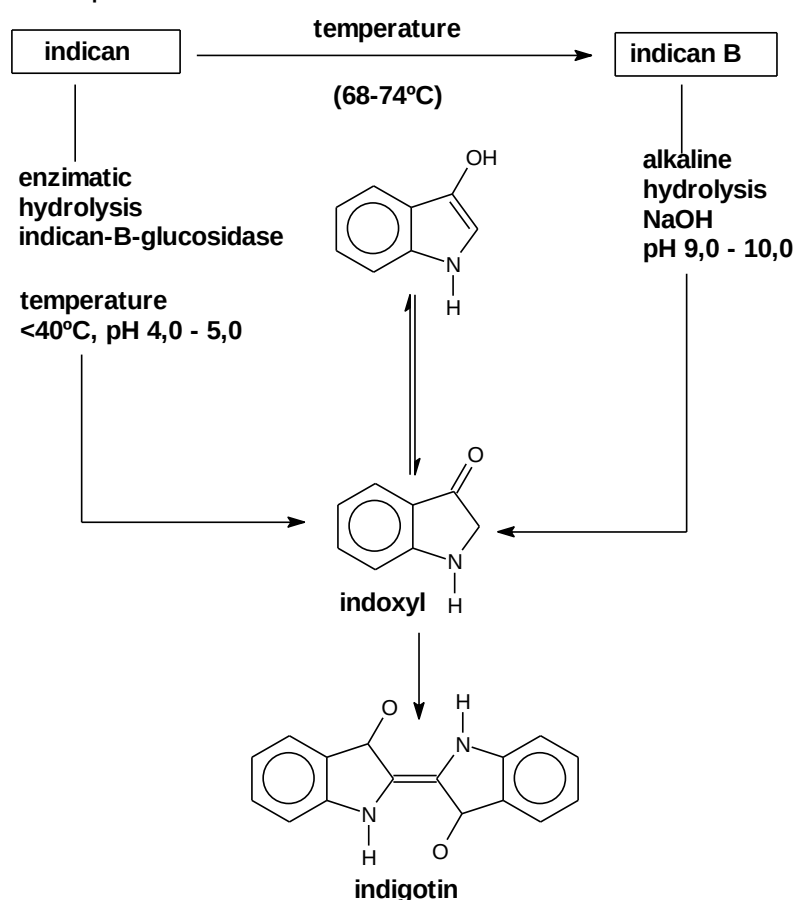


Figure 7. Indican behavior in high temperatures.

If leaves are not subjected during extraction at temperatures of about 70°C, the indigo extraction is only possible by the enzymatic via by the action of a β -glucosidase, but if the leaves are subjected to this temperature, the action of any enzyme is discarded for the reason that by increasing temperature a protein denaturation is raised.

At the same time we perform various tests to achieve an alkaline hydrolysis of a standard of indoxyl- β -D-glucoside (plant indican, SIGMA ALDRICH-Mexico), but we did not get it.

The indigo alkaline extraction was first reported by us and we obtained in higher yields than indigo traditional enzymatic extraction.

By instability and high reactivity of this transformed product it has not been chemically possible characterize it, but we try to see if really it was a chemical hydrolysis of the indican B. We hypothesized that if indeed it was an ester precursor, adding a base, the hydrolysis should release indoxyl. As the indoxyl condensation and isatin, according to literature, form indirubin, the formation of latter would be a proof of the indican transformation.

Materials and Methods

Vegetative Material

Leaves were collected from plants of *Indigofera suffruticosa* Mill., grown in the Agricultural Experimental Station "San Ignacio" at Autonomous University of Chapingo, Mexico. The plants were pruned on February with which the growth of new branches and leaves was promoted. The age of the leaves was 3.5 months and only healthy and integer leaflets were harvested.

Treatments

Seven treatments with three replicates were made where 50, 100, 200, 400, 600, 800 and 1000 g of fresh leaves were used. The leaves were pre-washed and placed into containers using a water/leaves relation of 0.5 liters per 100g of fresh leaves. Leaves with water were heated until to reach a temperature of approximately 70°C. In this point, the extract was cooled and the leaves were removed by filtration through a sieve. To indican B crude extract of each treatment was added an amount of 5 g of isatin, it stirred vigorously and the pH was adjusted to between 9.0 and 9.5. Upon stirring for 10 minutes the alkaline extract, it became an intense dark red color. The precipitate was decanted into a time of 24 hours and the supernatant was removed. The precipitate was washed repeatedly with distilled water until to get a supernatant with a neutral pH.

Liquid-liquid extraction

A liquid-liquid extraction of each treatment with dichloromethane was made until to deplete the red dye from the aqueous phase. The organic phase was brought to dryness under vacuum in a rotary evaporator. A red-brown product crystals was recovered which weighed and wherein a sample which was dissolved in ethyl acetate.

Thin Layer Chromatography (TLC)

In aluminum support plates of 10 x 5 cm of Chromatography with silica gel 60 F₂₅₄ (Merck, Mexico), the samples dissolved in ethyl acetate were deposited and were placed in a chromatography chamber with a mobile phase composed of acetone: ethyl acetate (50:50 v/v).

¹H Nuclear Magnetic Resonance (¹H NMR)

A standardized sample of red solid extract (RSE) was subjected to an analysis of proton nuclear magnetic resonance on a Bruker Avance spectrometer in 300 MHz. Sample was dissolved in DMSO-*d*₆ at a temperature of 27°C.

Results and Discussion

Red Solid Extract (RSE) yields

Graph 1 shows the yields of Red Solid Extract (RSE) resulting from the isatin and indoxyl condensation. The indoxyl was liberated by alkaline hydrolysis of indican B.

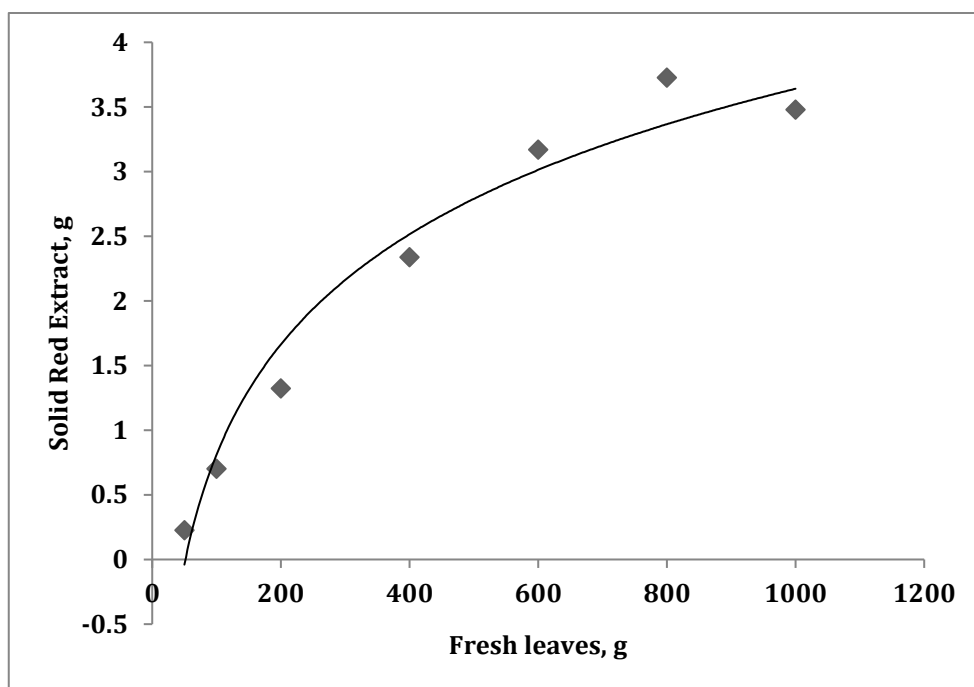


Figure 8. Red Solid Extract (RSE) yields.

According as plant material was increased also the amount of RSE increased, which indicates that the released amount of indoxyl as chemical hydrolysis product was condensed with a certain amount of isatin. The average production of RSE is 4,75 mg. g⁻¹ of fresh leaves.

Thin Layer Chromatography (TLC)

The RSE were controlled by TLC and just a red band appeared with an $R_f = 0,63$.

¹H Nuclear Magnetic Resonance

A RSE sample was subjected to analysis by ¹H Nuclear Magnetic Resonance Spectrometry, it gives the following spectrum:

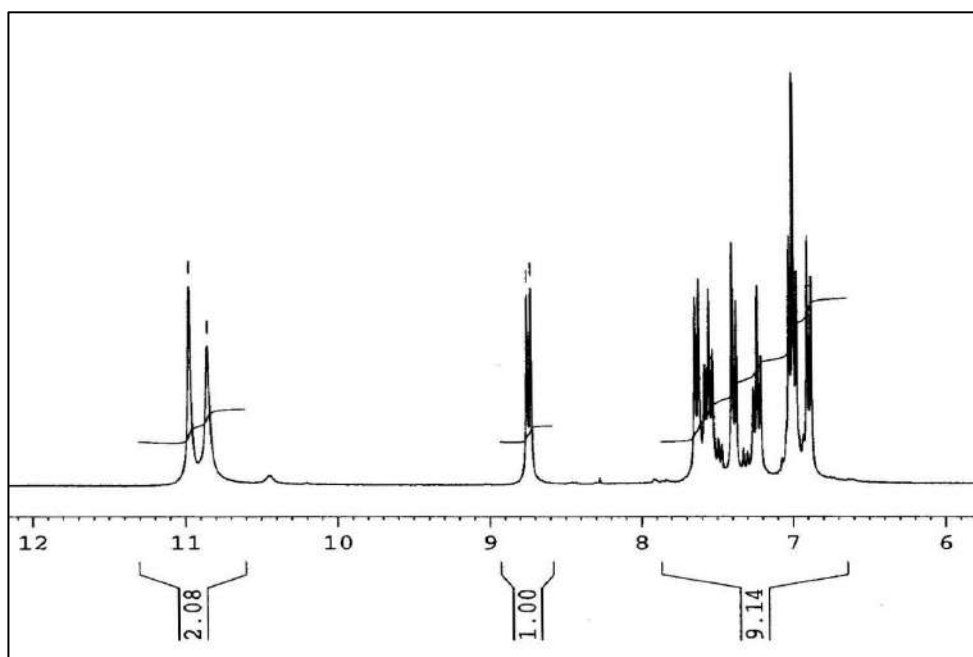


Figure 9. ^1H Nuclear Magnetic Resonance Spectrum of Red Solid Extract (RSE).

The identification of signals from chemical shifts corresponds to indirubin.

Indirubin: ^1H -RMN ($\text{DMSO-}d_6$), 27°C , 300 MHz, δ 6.89 (d, $J = 7.8$, CH'_4 and CH'_3), 7.00 (t, C_2H and CH_2), 7.24 (t, CH'_3 with CH'_2 and CH'_4), 7.39 (d, $J = 8.1$, CH_4 with CH_3), 7.56 (t, CH_3 with CH_2 and CH_4), 7.63 (d, $J = 7.5$, CH_1 with CH_2), 8.74 (d, $J = 7.8$, CH'_1 with CH'_2), 10.97 (s) and 10.85 (s) (NH' and NH).

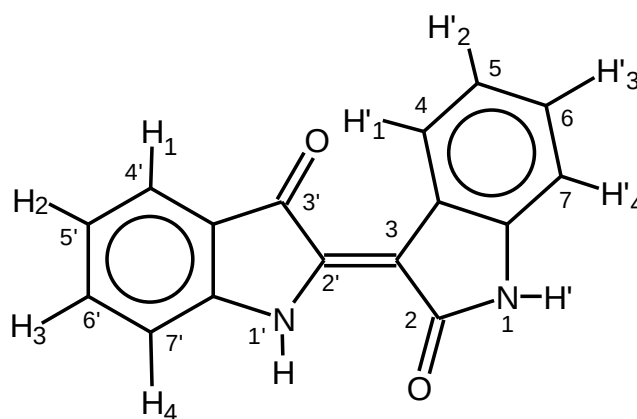


Figure 10. Red Solid Extract (RSE) in indirubin.

The obtained results show that thermal processing occurs of indican non hydrolyzable ether with bases at a compound that so far we have not identified and have called ester **indican B** that is readily hydrolyzed in an alkaline

medium. It is also demonstrated that liberation of indoxyl as hydrolysis product in the presence of isatin condenses with this one to form indirubin.

Indican quantification in leaves of Mexican indigo plant.

Indirectly, we have quantified the presence of indican in the leaves of *Indigofera suffruticosa*. Approximately 1,125 g of indican and 0,562 g of isatin are required to make 1 g of indirubin and liberate 0,687 g of glucose. We have obtained an average 4,75 g of indirubin per Kilogram of fresh leaves, so that we have approximately 5,3 mg.g⁻¹ of indican in fresh leaves.

Alkaline hydrolysis of indoxyl- β -D-glucoside.

We have performed several tests to try an alkaline solution hydrolysis of indican heating it and so far it has not been possible. This indicates that the temperature and base are not factors to achieve the hydrolysis and the liberation of indoxyl. Based on the above, we have proposed a hypothesis that could eventually explain the indican transformation in its ester form.

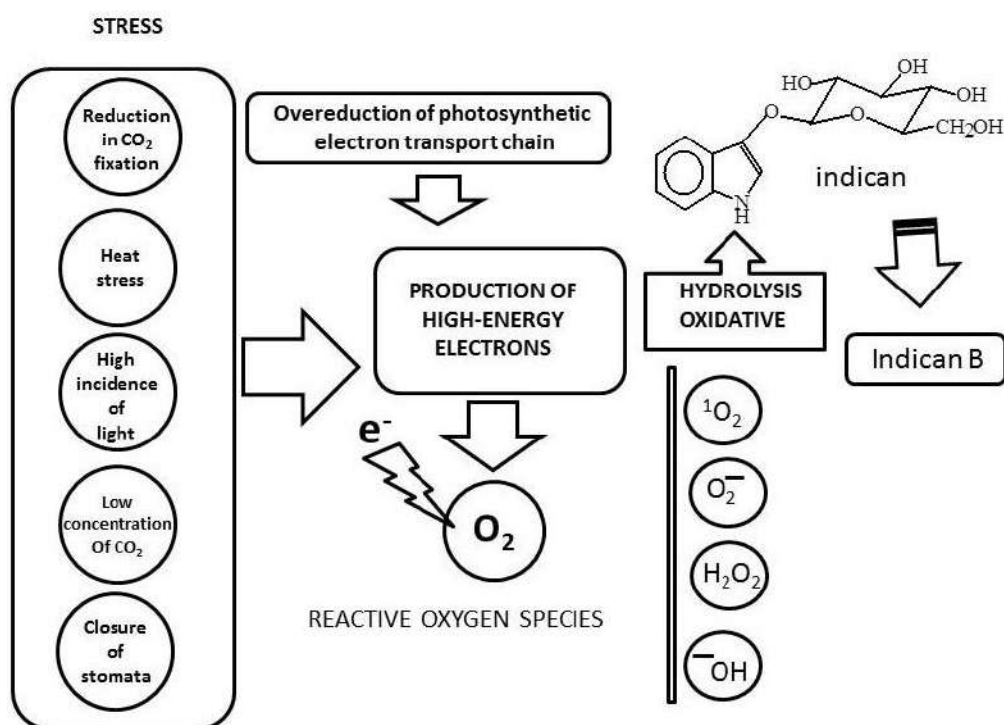


Figure 11. Oxidative model of indigo production in indigo plant leaves (*Indigofera suffruticosa* Mill.)

Oxidative theory of transformation from indican to indican B

Considering that the vegetative material which is subjected to extraction is alive and functional, it is possible to suggest that at the cellular level the oxidation reactions are carried out. The leaves when cut, stored, immersed in water and heated to certain temperatures are subjected under stress as a result of various factors including:

- Reduction in CO₂ fixation
- Low concentration of O₂
- Closure of stomata
- Disruption of photosynthesis
- High incidence of light
- Heat Stress
- Action of organic solvents

The electron transport chain is interrupted to give high energy free electrons that attack the oxygen producing *Reactive Oxygen Species (ROS)*, such as: ¹O₂, O₂⁻, H₂O₂ and ⁻OH. These species cause lipid peroxidation, the esterification of fatty acids and oxidative hydrolysis of indican possibly transforming it into a compound of the type of an **indole-3-carboxylic acid** that is highly sensitive to the presence of bases and free indoxyl.

If we do not add isatin to the reaction medium then the indigotin formation is produced (indigo alkaline production) to condense indoxyl molecules. If we add isatin to the reaction medium then the indoxyl liberated through hydrolysis of indican B is condensed with isatin to produce indirubin, as it has demonstrated in this work.

When the extraction is performed at temperatures below 30°C, a β -glucosidase enzyme hydrolyzes indican. The presence of this enzyme has been reported by Minami, *et al.*, (1996). Although also indican crude extracts can be attacked by microorganisms.

The sensitivity to stress and oxidative hydrolysis intensity varies among indigo plant species. *Indigofera* species are less sensitive that for instance in the case of *Isatis tinctoria*, since this oxidative mechanism is used to adapt to extreme temperatures and drought.

According to this theory it is possible to make the following statements:

- 1) All producing indigo plants contain only a single precursor that is the **indoxyl- β -D-glucoside** or indican, including *Isatis* species.

2) Isatan A, isatan B and isatan C, are products of the indican oxidative hydrolysis.

3) When indican is hydrolyzed can produce both indigo and indirubin. Still need to know how the indoxyl is oxidized to form isatin.

Conclusions

Subjecting leaves of Mexican indigo plant at temperatures near 70°C, the indican precursor becomes an ester compound, which we have called indican B and to be hydrolyzed liberates indoxyl. If we add isatin, it condenses with indoxyl to form indirubin. This could be a quick and inexpensive method to produce indirubin in large-scale. The indican contents in leaves of Mexican indigo plant are about 5,3 mg.g⁻¹ of fresh leaves.

Literature cited.

- Beijerinck, M. K. 1899. The production of indigo from woad (*Isatis tinctoria*). *Nature* 61(1568):71.
- Beijerinck, M. K. 1900. On the formation of indigo from the Woad (*Isatis tinctoria*). In: KNAW, Proceedings, 2,1899-1900, Amsterdam, 1900, pp.120-129.
- Cannon, M., Cannon, J. and Dalby-Quenet, G. Dye plants. London. Ed. The Herbet Press Ltd. The Royal Botanic Gardens, Kew. U.K.
- Cardon, D. 2007. Natural Dyes- Sources, Tradition, Technology and Science. London : Archetype Publication. 430 pp.
- Caster, G. 1998. Les routes de Cocagne. Le siècle d'or du pastel 1450-1561. Ed. Privat. Toulouse, France. 223 pp.
- Chanayath, N., Lhieochaiphant, S. and Phutrakul, S. 2002. Pigment Extraction Techniques from the Leaves of *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. and Chemical Structure Analysis of Their Major Components. *CMU. Journal*. 1(2):149-160.
- Epstein, E., Nabors, M.W. and Stowe, B.B. 1967. Origin of indigo of woad. *Nature* 216(11):547-549.
- Farrar, W.V. 1977. Edward Schunck, F.R.S. A Pioneer Product Chemistry. *Notes and Records of the Royal Society of London*. 31:271-296.
- García-Macías, P. and John, P. 2004. Formation of Natural Indigo Derived from Woad (*Isatis tinctoria* L.) in Relation to Product Purity. *Journal of Agricultural and Food Chemistry*. 52:7891-7896.
- Gilbert, G.K., Maule, G.H., Rudolph, B., Lewis, M., Vandenburg, H., Sales, E., Tozzi, S. and Cooke, D. 2004. Quantitative Analysis of Indigo and Indigo Precursors in Leaves of *Isatis* spp. and *Polygonum tinctorium*. *Biotechnol. Prog.* 20:1289-1292.
- Hoogewerff, S. and Meulen, H. 1900. Contribution to the knowledge of indican. *Proc. Royal Netherlands Academy of Arts and Sciences* 2, 1899-1900:520-525.
- Hurry, J.B. 1930. The Woad plant and its dye. London, Oxford Univ. Press. U.K., 315 p.

- Kokubun, T., Edmonds, J. and Jhon, P. 1998. Indoxyl derivatives in Woad in relation to medieval indigo production. *Phytochemistry* 49(1):79-87.
- Maier, W., Schumann, B. and Gröger, D. 1990. Biosynthesis of indoxyl derivatives in *Isatis tinctoria* and *Olygonum tinctorium*. *Phytochemistry* 29(3):817-819.
- Maugard, T., Enaud, E., Choisy, P. and Legoy, D. 2001. Identification of an indigo precursor from leaves of *Isatis tinctoria* (Woad). *Phytochemistry* 58:897-904.
- Minami, Y., Kanafuji, T. and Miura, K. 1996. Purification and characterization of a β -Glucosidase from *Polygonum tinctorium*, Which catalyses preferentially the hydrolysis of indican. *Biosci. Biotech. Biochem.* 60(1):147-149.
- Oberthür, C., Schneider, B., Graf, H., and Hamburger, M. 2004. The Elusive Indigo Precursors in Woad (*Isatis tinctoria* L.)- Identification of the Major Indigo Precursor, Isatan A, and a Structure Revision of Isatan B. *Chemistry & Biodiversity* 1:174-182.
- Oberthür, C., Graf, H. and Hamburguer, M. 2004. The content of indigo precursors in *Isatis tinctoria* leaves- a comparative study of selected accessions and post-harvest treatments. *Phytochemistry*. 65:3261-3268.
- Perkin, A.G. and Bloxan, P.W. 1907. Indican. Part I. *Journal of the Chemical Society Transactions*. 91:1715-1728.
- Schraudolf, H. 1968. Untersuchung zur biogenese von Isatan B der indigovorstufe aus dem fäberwaid (*Isatis tinctoria* L.). *Z. Naturforsch.* 23b:572-573.
- Strobel, J. und Groger, D. 1989. Über das Vorkommen von Indigovorstufen in *Isatis*-Species. *Biochem. Physiol. Pflanzen*, 184:321-327.
- Stoker, K.G., D.T. Cooke and D. J. Hill. 1998a. Influence of light on natural indigo production from woad (*Isatis tinctoria*). *Plant Growth Regulation* 25:181-185.
- Stoker, K.G., D.T. Cooke and D. J. Hill. 1998b. An Improved Method for the Large-Scale Processing of Woad (*Isatis tinctoria*) for Possible Commercial Production of Woad Indigo. *J. Agric. Engng. Res* 71:315-320.
- Shunck, E. 1855. X. On the formation of indigo-blue. Part I. *Philosophical Magazine* Serie 4 15(97):73-95.
- Vilarem, G. 1999. La chimie du Pastel. *Espaces pour Demain* 61:16-17.
- Vilarem, 2005. *Isatis tinctoria*. In : *Les Travaux de l'Académie des Arts & des Sciences du Pastel*. No. 1 Juin 2005. France. pp 6-7.
- Zhia-Quiang, X. and Meinhart, H.Z. 1992. Biosynthesis of indigo precursors in higher plants. *Phytochemistry* 31(8):2695-2697.