Quantitative Analysis of indigo and indirubin in solid extracts of Woad (*Isatis tinctoria* L.) by Thin Layer Chromatography /Densitometry

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1 Introduction

Indigo and their derivatives are coloring molecules of industrial use and they can be obtained of natural sources (plants, animals or microorganisms) as synthesized of fossil materials [1,2,3]. In Europe, during the 16th century a plant utilized for the indigo extraction was the woad (*Isatis tinctoria* L.) and reason of an important industry that unchained a strong trade among the Southwest of France with Spain to the South and with England to the North. Toulouse became the nucleus of this industry based on the woad cultivation, the dye extraction and its sale [4,5]. With the chemical identification of the indigo at the end of the 19th century it was also achieved their synthesis to great scale. The indigo synthetic production displaced almost completely that of natural origin [6].
In the last decade the use of dyestuffs of natural origin has reached importance again in the entire world. In 1994 M. Lambert established the first company in France of extraction of the blue pigment of the woad leaves and the production of the diverse commodities ("Bleu de Lectoure") [7]. The production passed of some pounds in 1995 to around 0.4 tons in 2001, as a result of repeated extraction attempts at industrial level under the direction of the Laboratoire de Chimie Agro-Industrielle of the Ecole Nationale Supérieure des Ingénieurs en Arts Chimiques et Technologiques of Toulouse France.

The indigo extraction of the woad leaves implies the obtaining of the colorless precursors of indolic type by means of a liquid-solid extraction using like solvent the water [8,9]. These precursors are glucosides possessing a high likeness with the water and they go out the vegetable material with relative easiness after a period of maceration [10]. The major precursor in woad is the **Isatan B** (**Indoxyl-5-ketogluconate** (**1**)) that is constituted of a nucleus indolic united by a bond of type ester to a molecule of glucose. This bond is unstable in presence of an alkali [11,12].
Once extracted the isatan B, this is hydrolyzed with the addition of an alkali to the liquid extract, and free **indoxyl** (**2**) is released. Two indoxyl molecules combine to produce an **indigo** o indigotina (**3**) molecule in presence of the oxygen of the air. In conditions of high temperature and an oxygen-rich environment the indoxyl is oxidized forming **isatin** (**4**). The condensation of indoxyl with isatin produces **indirubin** (**5**) which is an isomer that imparts a red color. The indirubin production is determined by the extraction conditions as pH or temperature [13].

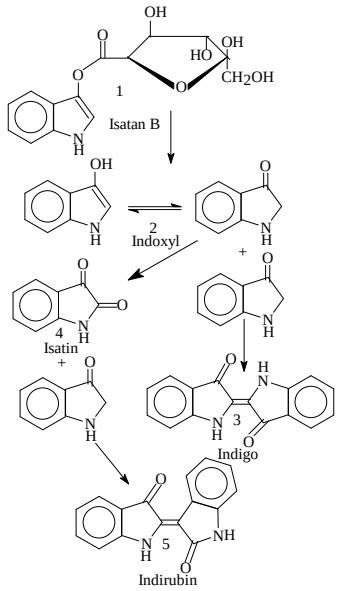

Figure 1. Formation of indigo and indirubin in *Isatis tinctoria* L.[14,15].

These coloring compounds in woad leaves are not extracted in pure form, but rather a solid extract of blue-reddish color. This appearance is obtained that is the result of the indigo and indirubin concentrations. Consequently, the blue solid extract is a powder with variable content of coloring matters.
The yield in extract solid and their indigo and indirubin concentrations in the commercial extractions are dependent of multiple factors like they are: the agronomic handling of the plants, the climatic conditions, the leaves harvest time and the condition extractions. Thus, the solid extracts cannot vary alone in each extraction time with different ages of leaves, but also in different extractions with leaves of the same age. The quality control of the solid extracts then demands a method of quantitative analysis.
The analysis by spectrophotometry with absorption of Ultraviolet-Visible revealed that the pigment woad spectra are so next that the maximum band absorption in the visible region of indigo eclipses that the indirubin when they were analyzed in separate form using

standards the two compounds. When the blue solid extract was analyzed containing the two pigments, a spectrum it was obtained with a maximum absorption in the visible region that embraces the two bands corresponding to the coloring molecules (Figure 2).

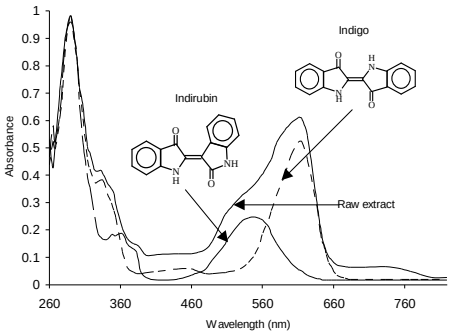

Figure 2.UV/Visible spectroscopy of pigments and raw extract in *N,N*-dimethylformamide.

We conclude that this analysis technique is not useful for the indigo and indirubin determination due the interaction among the two compounds and to its maximum absorption so near, that which difficult the indirubin quantification that is generally in weak concentration that the indigo in the solid extracts. Consequently, the quantitative analysis method in this case requires of a previous pigments separation before to be analyzed by spectroscopy.
In this paper we report the results of the analysis of the solid extracts of *Isatis tinctoria* L., by Thin Layer Chromatography/Densitometry, that consist of two phases: the separation by chromatography and plates evaluation by densitometry.

2 Experimental

2.1 Solid extracts

The woad solid extracts were obtained from essays to industrial level carried out at Castelnaudary, France in the summer 2000, and which were of different drying treatments: (1) drying to air to full sun; (2) drying in an electric dryer, and (3) drying to air under protection of the solar rays. We took samples of 1 g of each one of the three extracts and they were analyzed by the method proposal.

2.2 Spectrophotometry UV-Vis

The spectrophotometric analyses of the solid extract, indigo and indirubin standards were run in a spectrophotometer type Vectra ES/12 (Hewlett Packard 8452 A) using like solvent *N,N*-dimethylformamide (Fluka, France) in an spectrum of 260 a 800 nm.

2.3 Standards

Synthetic indigo (Sigma, France) with more than 99% of purity and indirubin prepared in the laboratory according to the procedure of Glen A. Russell [16]. 1.76 g of isatin (Fluka, France) and 2.00 g of indoxyl acetate (Fluka, France) were made react in presence of carbonate of sodium. After filtration, the residual was washed twice with methanol and several times with demineralized water until neutral reaction of the filtrate. A dark crystal violet product was obtained. Finally, this product was washed with acetone and the filtrate was concentrated *in vacuo* giving a pure product, which was verified by thin layer chromatography being obtained a *Rf* =0.44 (hexane /ethyl acetate 3:2 v/v).

2.4 Thin Layer Chromatography

Separation optimal conditions of pigments were determined like the first part of the method.

2.4.1 Choice of the solvent for pigments

A solvent was selected for the indigo, indirubin and the solid extract. As a result of several essays it was determined that the *N,N*-dimethylformamide is the best solvent. Indigo presents good solubility in concentrations among 0.020 a 0.050 g/L in chloroform, ethyl acetate and acetone.

2.4.2 The Stationary phase

Silica gel glass plates 60 F_{254} de 20x20 cm (Merck, France) were selected as stationary phase for the method. It is obtained a better concentration and resolution of spots with the use of HPTLC plates.

2.4.3 The mobile phase

The elution system that produced the best results in pigments separation was a mixture of toluene/ethyl acetate (3:2 v/v). The following systems of benzene/acetonitrile (2:1 v/v) and hexane/ethyl acetate (3:2 v/v) were presented satisfactory.

2.4.4 Preparation of the samples

10 mg were weighed of each one of the samples of solid extracts for the analysis of the indigo and they were diluted in 25 mL of *N,N*-Dimethylformamide and 100 mg in 20 mL for the analysis of the indirubin. It was necessary to spend the samples to the ultrasound for three minutes to assure an appropriate dilution.

2.4.5 Preparation the solution standards

Two standard solutions series of each pigment were prepared with the purpose of their application on the TLC plates beside of the samples. A solution pattern was made of the indigo as the indirubin and after successive dilutions in *N,N*-dimethylformamide were obtained the standard solutions with the following concentrations:

0.04, 0.08, 0.12 and 0.16 g/L for the indigo and 0.03, 0.06, 0.09 y 0.13 g/L for the indirubin.

2.4.6 Application of the standards and the samples

With this method it is necessary to apply on TLC plates a series of standard solutions of the pigment to analyze beside the samples. The band applications were made with a semiautomatic applicator Desaga AS 30. The samples and standards were injected inside the applicator by means of a syringe of a capacity of 25 μL. This applicator was programmed to make the deposits of a length of 5 mm, with a distance among them of 15 mm of center to center. The volume of each band it varied from 4 to 10 μL.

2.4.7 Handling of the plates after making the application the spots

It is important to dry the plates with the deposits and to protect them the most possible of the light and to avoid the pigments degradation. For this purpose we covers the plates with aluminum paper.

2.4.8 Preparation of the chromatographic chamber

In a chromatographic glass chamber was put the mobile phase and a paper filter was placed to saturate the chamber atmosphere with the elution system in order that to obtain a good development.

2.4.9 Development of plates

It is allowed that the solvent front to migrate among 10 to 15 cm from the border of the plate being obtained a good separation of the pigments. Later, the plate is taken up of the chamber, dries it off and evaluate it to the densitometer.

2.5 Evaluation the plates to densitometer

A quantitative evaluation the plates was possible by photodensitometry after elution. This consists on the second part of the analysis. The method allows to analyze a single pigment of several samples at the same time, since the software of the apparatus it integrates and it elaborates the calibration curve starting from the series of deposits of the standards, which serves as reference to calculate the concentration of the samples. The scanning the plates was made to a wavelength of 610 nm for the indigo and to 548 nm for the indirubin. Three deposits of each sample were made. The evaluation of plates was made in a photodensitometer CD 60 Desaga-Heidelberg.

3 Results and Discussion

3.3 Spectrophotometry UV-Vis

The indigo and indirubin standards in several solvents were analyzed to spectrophotometer. A effect displacement of the maximum absorption of the pigments was observed along the spectrum, probably by interaction of the solvent polarity and the molecules. These results are shown in the Table 1.

Table 1. Absorption bands of indigo and indirubin in several solvents.

Solvent	λ_{max} indigo (nm)	λ_{max} indirubin (nm)
DMSO	620	558
N,*N*-dimethylformamide	610	548
Chloroform	606	544
Ethyl acetate	598	538
Acetone	596	534

3.1 Thin Layer Chromatography (TLC)

The deposits were made at a distance of 18 mm of the inferior edge of the plate and leaving a space of 25 mm of each lateral border. The volume deposited was 4 μL to 10 μL and the distance among them of 15 mm. Each deposit had a length of 5 mm and in total 10 were made. Four of them corresponded to the standard series and six to two samples of the solid extracts with three deposits for sample. The semiautomatic applicator allow to deposit to a speed of 5 s μL^{-1}.
Three solvent systems produced good separation (Table 2).

Table 2. Elution systems and *Rf* values.

Solvent system	Rf indigo/indirubin	Δ(Rf)
Hexane/Ethyl acetate (3:2 v/v)	0.73 / 0.44	0.29
Benzene/Acetonitrile (2:1 v/v)	0.78 / 0.54	0.24
Toluene/Ethyl acetate (3:2 v/v)	0.61 / 0.47	0.14

The system hexane/ethyl acetate produced a good separation but the bands did not have suitable definition. The mixture of benzene/ethyl acetate gave good separation and definition of bands but the use of benzene is risky. Finally, we use the third system made up of toluene/ ethyl acetate.
The development of the plate took around ten minutes and it was allowed that the elution system to emigrate to 120 mm starting from the inferior border of the plate, that which is enough to obtain the separation of the pigments.

3.4 Densitometry

The developed plates were read to photodensitometer and a chromatogram was obtained that locates the bands of each pigment. At same time, the apparatus built a calibration curve starting from the standard solutions series and it integrated the results of the scanning to provide us the concentrations of each sample bands. A chromatogram type is shown in the Figure 3.

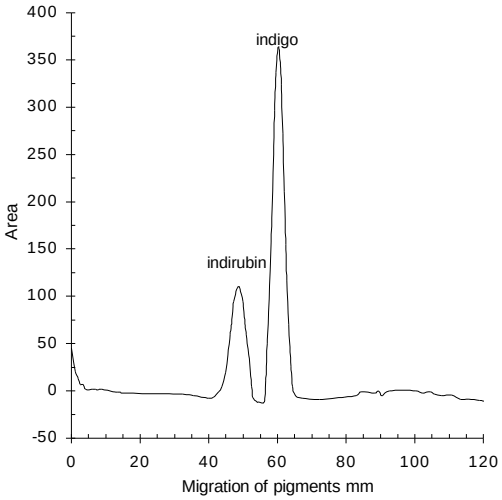

Figure 3. Chromatogram of the indigo and the indirubin of the woad solid extract separated by TLC provided by the photodensitometer.

The calibration curves of indigo and indirubin calculates by the photodensitometer are shown in the Figures 4 and 5

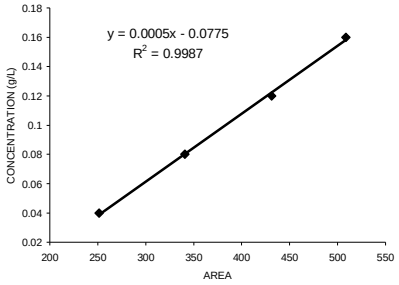

Figure 4. Calibration curve of the indigo .

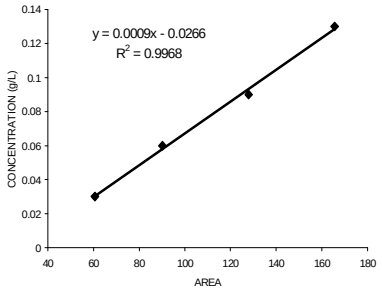

Figure 5. Calibration curve of the indirubin.

3.5 Content of pigments in solid extracts

To the concentration data of the pigments in the solid extracts were applied a variance analysis that indicated us the existence of significant differences among drying treatments, for what we proceeded to make a multiple Tukey (Table 3).

Table 3. Content of indigo and indirubin in solid extracts of woad with different drying treatments.

Extract	Indigo (%)	Indirubina (%)
Drying to air to full sun	3.95 ± 0.3 b	0.2116 ± 0.011a
Drying in a electric dryer	4.51 ± 0.16 b	0.1116 ± 0.008 c
Drying to air under protection of the sun	5.84 ± 0.2 a	0.1581 ± 0.005 b

Different letters among columns denote differences to α=0.05 for the Tukey test.

The solar rays and the heat cause a degradation of the indigo and a probable photorevertion from this pigment to indirubin. The solid extracts should dry off under the protection of the light.

4 Conclusion

Quantitative analysis of indigo and indirubin in solid extracts of woad by TLC/Densitometry it give reproducible results and it can also be used for the quality control in extractions at industrial level of these dyes.

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