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Identification of Indigo Dye (*Indigofera tinctoria*) and Its Degradation Products by Separation and Spectroscopic Techniques on Historic Documents and Textile Fibers

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ABSTRACT

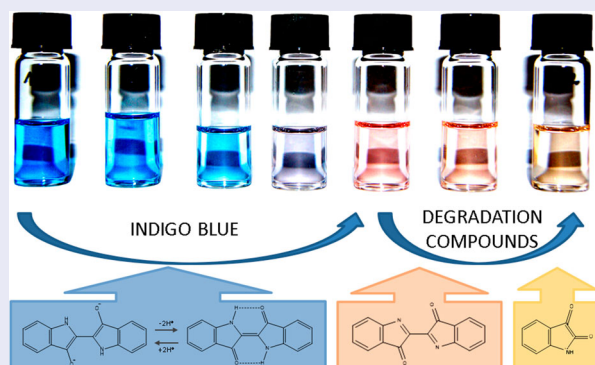
Understanding the use of colorant materials throughout history is now the objective of multiple studies centered on the identification and characterization of these materials. The main objective of this study is to increase knowledge of the degradation pathways of indigo blue dye (*Indigofera tinctoria*) and the detection of degradation products. This will enable the identification of the dye on historical objects, even when it is present in a degraded state. To this end, different analytical techniques were used for the identification of indigo blue and its degradation products. Capillary electrophoresis with diode array detection (CE-DAD) and fluorescence spectroscopy were used for characterization and detection of the colorant and its degradation products caused by the aging process. Nuclear magnetic resonance (NMR) spectroscopy was used to confirm the presence of degradation products. The evaluation of color variations (lightness, chroma, and tone) in indigo blue on paper samples was made with colorimetric study, while the changes in indigo solutions were evaluated with UV-visible spectrophotometry. Additionally, changes in the alkalinity and acidity of the colorant were made with pH measurements. Lastly, the main agents responsible for the modifications that resulted in the production of degradation products of indigo blue were also identified. These methods were applied to historic samples from the Collection of Drawings, Maps, and Plans (sixteenth to nineteenth centuries) and to the thread used in the binding of a notarial record book (*Registro Notarial de Torres*) (fifteenth century) held by the Royal Chancery Archive in Granada (Spain). This paper provides deeper insights into the materials used in artworks and helps in the identification of indigo blue despite the degradation caused by external agents or by its natural aging process.

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Introduction

Because of its nature, indigo blue can be considered both a pigment and a dye as it is an insoluble organic compound derived from plants whose microscopic particles can be deposited on the support's fibers without the use of a mordant. Traditionally, indigo blue has been extracted from several plants, mostly of genus *Indigofera*, which has around 300 species (Püntener and Schlesinger 2000a; Teanglum, Teanglum, and Saithong 2012). The main component

of indigo is indigotin ($C_{16}H_{10}O_2N_2$), with indirubin or indigo red, indigo brown or isindigo, gelatinous substances, nitrogenous materials and mineral salts, silicate, calcium, potassium, magnesium, iron, and other minor constituents also identified in the colorant (Degani, Riedo, and Chiantore 2015). In the solid state, indigo is bright deep blue and is soluble in nitrobenzene, pyridine, acetic acid, sulfuric acid, dimethylformamide, and dimethyl sulfoxide (Shirata 1996; Otlwska et al. 2018).

Indigo dye results from a complex process of extraction and treatment of the plant leaves. The precursor found in the leaves is the glucoside indican, which is naturally bonded to the glucose molecule. For adequate extraction, the plants are harvested at dawn and immediately placed in large vats with alkaline water during 24 h for maceration. After this, the mixture is energetically stirred for hours to allow the air to oxidize it and release the dying compounds. The mixture is left to settle until the following day when the water is drained. The resulting product is a deep blue paste that is formed into balls or bars that, once completely dried, can be stored until use (Roquero and Córdoba 1981; Kumar 2012).

This fermentation process of plant leaves allows the transformation of indican, colorless and soluble in aqueous media, into glucose and indoxyl, which exists in the leuco- and keto- tautomeric forms (Seixas de Melo, Moura, and Melo 2004; Sousa et al. 2008). When oxidized, two indoxyl molecules join with oxygen to form indigotin or indigo blue (insoluble in water) (Blackburn, Bechtold, and John 2009). This oxidation process also results in the formation of indirubin, a red colorant that when mixed with indigo blue gives a characteristic violet tone which helps distinguish it from indigo extracted from other plants and from synthetic indigo (Balfaur 2001) (Figure 1).

There is evidence that indigo was used as a textile colorant as early as 6000 BCE (Kramell et al. 2017), and for many years it was the only blue colorant available (Clementi et al. 2009; Sanz et al. 2010; Gulmini et al. 2013). In cultural heritage, indigo blue is commonly found in ceramics and mural paintings (Merwin, Morris, and Morris 1931; Cabrera 1969; Tagle et al. 1990; Pawlak et al. 2006; Vázquez, Domenech, and Domenech 2011; Domenech-Carbó et al. 2014) as

well as in several painting techniques such as oil, watercolor, and tempera painting (Mawatari et al. 2001; Doménech 2006; Karapanagiotis et al. 2009). Indigo has also been used to dye paper, wood, cork, or any type of natural fiber (López-Montes et al. 2007; Buti et al. 2018).

The identification of indigo in artworks may be complicated by its complex composition, changes related to its condition today, or its natural aging process (Archive of Royal Chancery 2012; González 2014). A review of the available literature has identified numerous studies on the identification, application, and behavior of natural organic colorants from animal or plant origin due to the interest of the cosmetic, pharmaceutical, and food industry (Püntener and Schlesinger 2000b; Chigurupati et al. 2002; García-Falcón and Simal-Gándara 2005). Studies on the identification of colorants used as paint layers or textile dyes in artworks have also been identified (Miliani, Romani, and Favaro 1998; Degani et al. 2009; Buti et al. 2014; Marras 2014; Poulin 2018), most of them focusing on identification and characterization of materials.

In recent years, there has been a growing interest in the evaluation of the damage caused by external agents or by the natural aging of materials arising from the need to know the behavior of these materials for their better preservation. The first studies on the effects of environmental factors on natural colorants (cochineal, brazilwood, saffron, Indian yellow, indigo, and others) (López-Montes et al. 2008; Villmann and Weickhardt 2018) were published in the last decades, as well as studies on accelerated aging of colorants used in graphic documents (cochineal, saffron, indigo, and madder) and the resulting tonal variations (López-Montes et al. 2006; Kampasakali and Varella 2008).

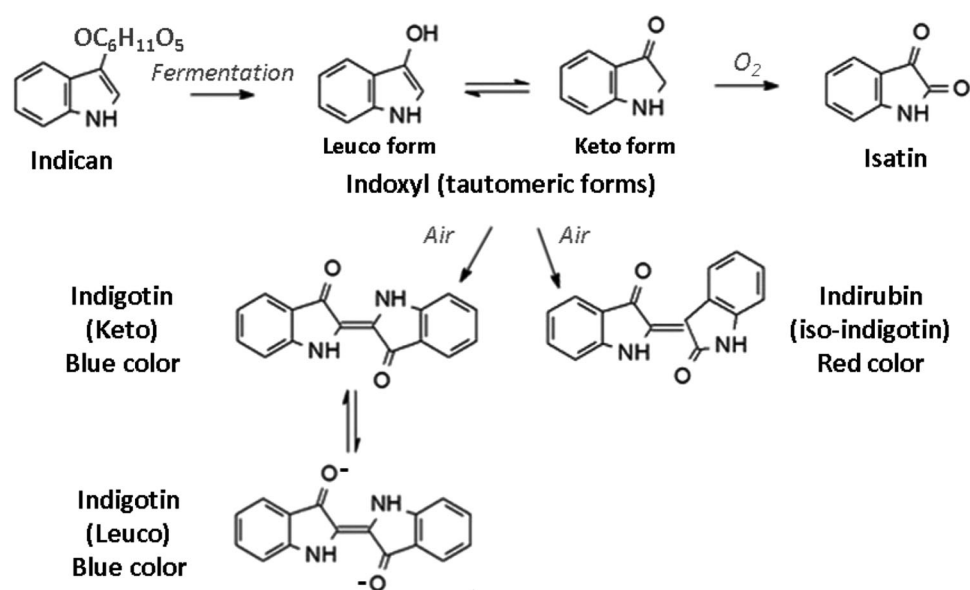


Figure 1. Molecules involved in the extraction process of blue indigo.

Despite this, only few studies have focused on indigo blue and its degradation (Grosjean, Whitmore, and Cass 1988; Novotná et al. 2003; Witkos, Lech, and Jarosz 2015). There are no studies about indigo degradation on paper. Also, there are very limited studies on the use of capillary electrophoresis for indigo degradation studies (López-Montes et al. 2007). Therefore, the main objective of the present work is to evaluate the physical and chemical changes that indigo blue undergoes on paper support by using processes that simulate its natural aging. This will allow the identification of this colorant in historic objects on paper despite the degradation it may have undergone.

In this study, indigo blue in solution and as a simulated watercolor on paper was exposed to an accelerated aging process. The evaluation of alkalinity changes was made with pH measurements; color changes were evaluated with colorimetry and UV-vis spectrophotometry; and capillary electrophoresis with diode array detection (CE-DAD), fluorescence spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy were used for the evaluation of composition changes.

Lastly, samples from the Collection of Drawings, Maps, and Plans and from the blue colored sewing thread used in the binding of a notarial record book (*Registro Notarial de Torres*) were analyzed to determine the presence of indigo blue and its degradation products. The collection of graphic documents (sixteenth to nineteenth centuries) and the record book (found in 1504 in the municipality of Torres in Jaen, Spain, dating between 1382 and 1400) are held by the Royal Chancery Archives in Granada (Spain) (Archive of the Royal Chancery of Granada 2012).

Materials and methods

Instrumentation and software

All pH measurements were made with a pH meter (220 V; 0.1 A; 50 Hz) from Crison Instruments S.A. combined with a glass plane electrode HI1413S/50 from HANNA Instruments, which provide measurements from the surface pH of samples.

For the colorimetric analysis, a portable Konica Minolta CM-2600d spectrophotometer (measurement area: 8 mm \varnothing ; wavelength range: 360–740 nm; wavelength interval: 10 nm) and the CM-S9w SpectraMagic software v. 3.61, also from Konica Minolta, were used.

An Agilent 8453E (Waldbronn, Germany) UV-Vis spectrophotometer was used to record the absorbance spectra of indigo and data were analyzed using the Agilent ChemStation software package.

A Shimadzu RF-150 spectrofluorophotometer and Shimadzu PC-1501 Personal Fluorescence Software v. 1.2 were used for the spectrofluorometric analysis.

CE-DAD separation was made with an Agilent HP3D instrument equipped with diode-array detector,

thermostated column cartridge, high voltage built-in power supply, and autosampler. Separations were carried out in fused-silica capillaries (72 cm \times 75 μ m id) with 56 cm inlet-to-detector distance. An Agilent 8453E UV-vis spectrophotometer was used to record the absorbance spectra of indigo. ChemStation v. A.0901 software package was used for acquisition and processing of electropherograms.

NMR analysis was made with a 600 MHz NMR spectrometer (4 channels) VARIAN DIRECT DRIVE, equipped with: triple resonance probe ^1H $\{^{13}\text{C}, ^{15}\text{N}\}$ indirect detection with 5 mm XYZ gradients, quadruple resonance probe ^1H $\{^{13}\text{C}, ^{15}\text{N}, ^{31}\text{P}\}$ indirect detection with 5 mm XYZ gradients, triple resonance crossover $\{^1\text{H}, ^{19}\text{F}\}$ $\{^{13}\text{C}, ^{15}\text{N}\}$, temperature regulator without liquid nitrogen and automatic tuning system on the ^1H channel.

Accelerated aging tests were made in a SOLARBOX 3000eRH chamber equipped with xenon lamps and indoor filter (S208/S408). Parameter control inside the chamber was recorded using the XEN 32 Report Level software.

An ultrasonic bath from Selecta (Barcelona, Spain) was also used for the extraction and dissolution of dyes.

Reagents

A bar of compact indigo blue (IND) paste of Indian origin and obtained from *Indigofera tinctoria* plants and gum arabic (GA) powder from *Acacia senegal* were purchased from Kremer Pigmente (Aichstetten, Germany). Hemp fiber paper from *Cannabis sativa* was from a batch of handmade Chinese paper, with no colorants or glue.

Reference standards of indigotin (INDt), indirubin (INR), and isatin (IS) were provided by Sigma Aldrich (St. Louis, MO, USA).

The solvents methanol, ethanol, acetonitrile (ACN), 1-propanol, 2-propanol, boric acid, and acetic acid (17.5 M) and deuterated dimethyl sulfoxide (DMSO) (all HPLC-hypergradient grade) were obtained from Merck (Darmstadt, Germany).

Sodium tetraborate decahydrate was provided by Sigma Aldrich (St. Louis, MO, USA), sodium dodecyl sulphate (SDS) by Sigma-Aldrich Chemie (Steinheim, Germany), and sodium hydroxide by Panreac (Barcelona, Spain).

All reagents were analytical grade, unless stated otherwise. Water was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA).

Sample collection and treatment

Reference compounds

Indigo blue was extracted from the bar of *Indigofera tinctoria* paste using acetic acid in an ultrasonic bath

for 45 min. The extract was filtered through a 0.2 μm nylon filter, evaporated to dryness and redissolved in 0.1 M SDS.

Standard solutions of synthetic indigo (INDt) were prepared at an initial concentration of 0.2 g/L in acetic acid and a similar extraction procedure of natural indigo was applied.

Standard solutions of indirubin (INR) and isatin (IS) were prepared in 0.1 M SDS at 0.2 g/L.

Gum arabic (GA) was prepared in distilled water (1:6) and left to hydrate for 24 h. After this period, it was dissolved in a double boiler (Castillo-Valdivia et al. 2014).

Samples of indigo blue in solution

For the accelerated aging tests, 0.003 g of indigo blue was prepared in glass vials with 10 mL acetic acid (D-IND samples). Dissolution was made in an ultrasonic bath for 45 min. For the identification of blue indigo components (INDt, INR, and IS) all the reference standards and spiked samples were previously analyzed.

Acetic acid was selected as a solvent among other possible ones such as pyridine, sulfuric acid, or nitrobenzene since it accelerates the degradation of the dye facilitating the appearance of degradation products and does not pose a risk in handling (López-Montes 2006; Qian et al. 2005).

Samples of indigo blue in paper

Layers of indigo blue were applied to the hemp paper without the use of a binder (P-IND), with binder (P-INDAG), and paper blanks (P-BL). To do this, distilled water was added dropwise to 1 g of thin IND powder in a mortar at room temperature and mixed to obtain a homogeneous paste. This paste was left to stand for a few hours to allow the larger particles to settle at the bottom. The preparation of P-INDAG samples required the addition of the previously made GA solution. For both P-IND and P-INDAG, the color layer was applied twice and in opposite directions to ensure complete coverage of the paper.

For the analysis of these samples the colorant was removed from the support by placing a 1.5 cm^2 paper sample into a vial with 4.5 mL acetic acid that was placed in an ultrasonic bath for 45 min. The solution of acetic acid with the dye was dried using a stream of nitrogen at 60°C and redissolved in 0.1 M SDS for further analysis by capillary electrophoresis. SDS was selected to facilitate micellar separation.

Historical samples

Sample collection and treatment. Collection of drawings, plans, and maps. A paint brush dampened with 0.1 M SDS solution (Blanc et al. 2007) was used for removing the samples off the different documents. For analysis, the brush was subsequently dipped into a vial with SDS. Samples were dried and dissolved in 0.3 mL acetic acid in an ultrasonic bath for 45 min.

After total dissolution, the samples were dried and redissolved in 0.1 M SDS. Due to the limited amounts of samples, they were only centrifuged, not filtered, to prevent undissolved particles from passing to the capillary.

Sample collection and treatment. Registro Notarial de Torres. The sewing thread used in the binding of the record book was placed in a vial with 2 mL of 17.5 M acetic acid and maintained in an ultrasonic bath for 45 min. The thread was withdrawn from the vial. The solution of acetic acid with the dye was introduced into a quartz cuvette for analysis by fluorescence spectroscopy. After this, the solution was filtered by a 0.2 μm nylon filter, evaporated using a stream of nitrogen at 60°C to dryness, and redissolved in 2 mL of 0.1 M SDS for further analysis by capillary electrophoresis.

Accelerated aging tests

D-IND, P-IND, and P-INDAG samples were subjected to four aging conditions: (a) Reference samples (REF) were kept in a compartment in the laboratory at 25°C, 30% relative humidity (RH) and no irradiance; (b) Darkness samples (DARK) were placed in the aging chamber exposed to 80°C, 65% RH and no irradiance (covered with foil); (c) Light samples (LIGHT) were placed in the aging chamber exposed to 550 W/m^2 intensity light, 80°C and 65% RH; (d) Open-air samples (OUT) were exposed to open-air, without UV filter (UV radiation index provided by the National Meteorological Agency 10/11). For each group, triplicates were obtained from all the samples, as well as a blank sample. The conditions selected for the aging chamber comply with the ISO 5630-3:1996 and ISO 11341:2004 standards (80°C, 65% RH, and 550 W/m^2 irradiances with indoor filter). Total duration of the tests carried out in the aging chamber was 144 h with sampling at 0, 24, 48, 72, and 144 h.

pH measurements

pH measurements were made in P-IND and P-INDAG samples and in blank paper (P-BL sample), in compliance with the TAPPI 5529-1988 standard.

Colorimetric analysis

The colorimetric analysis was performed in P-IND and P-INDAG samples in compliance with the following measurement conditions: CIE 1964 standard colorimetric observer; CIE 1967 standard illuminant D65; measurement/illumination area: SAV: 3 mm; measurement mode: 3 automatic measurements x 3 manual measurements in 3 points of each sample (estimation of the mean); specular component excluded (SCE); UV component illumination (100% UV), and CIELAB color

space (CIE 1976 $L^*a^*b^*$, CIE 1976 L^*C^*h). The total color differences [CIELAB (ΔE_{ab}^* , 10)] between testing samples and the corresponding reference standard were calculated. These differences can also be expressed as differences in lightness (ΔL_{10}^*), chroma ($\Delta C_{ab,10}^*$), and hue ($\Delta H_{ab,10}^*$), represented in the CIELAB coordinates (L_{10}^* , a_{10}^* and b_{10}^*) (Collado-Montero et al. 2018).

Spectrophotometric analysis

The spectrophotometric analysis of P-IND and P-INDAG samples and of sample solutions was performed before and after each aging procedure. The absorption spectra of samples were recorded in a 250–950 nm range.

Fluorescence spectroscopy analysis

Fluorescence spectroscopy analysis was performed only in sample solutions by placing the adequate volume of each sample in a quartz cell. The fluorescence emission and excitation spectra were obtained at 300, 310, and 347 nm scanning in a range 250–900 nm. After the analysis, the sample solution was placed again in the vial to resume the aging process.

Capillary electrophoresis analysis

CE-DAD analysis was made in P-IND and P-INDAG samples and in sample solutions. New capillaries were preconditioned by rinsing with 1 M NaOH for 20 min at 60°C, followed by a 5 min rinse with deionized water and 20 min with buffer. For subsequent analyses, the capillary was rinsed with 0.1 M NaOH for 5 min at 25°C, followed by a 3 min rinse with deionized water to ensure good repeatability. The capillary was equilibrated with the running buffer (40 mM tetraborate pH 9.25) for 5 min before each sample injection (López-Montes 2015).

Samples were injected hydrodynamically into the anodic end of the capillary with a pressure mode of 50 mbar for 13 s. Electrophoretic separation was performed at 25 kV for 25 min, resulting in a current of about 117 μ A. The temperature of the capillary was constant at 25°C. After each analysis, the capillary was rinsed for 3 min with deionized water.

All solutions, buffers, and samples were filtered through a 0.20 μ m membrane filter. The running buffer was changed after three runs. UV detection was carried out by monitoring at 252 nm. DAD was used over the range 190–600 nm for the spectral data.

Nuclear magnetic resonance spectroscopy study

The NMR study was made based on results found using other analytical methods. NMR analysis was completed for only the LIGHT samples at time intervals 0–6, 12, and 24 h. The sample solutions used in the aging procedure were dried first and subsequently redissolved in

2 mL deuterated DMSO, applying hot air to improve dissolution.

Results and discussion

Results of pH study

pH values of paper samples before and after each aging interval are shown in Table 1.

Samples showed slightly acidic values (6.0–6.3) before starting the aging procedures. After aging, the pH of the P-BL samples was more stable in all conditions compared to P-IND and P-INDAG samples. The samples that exhibit a greater variation in pH (maximum permitted error 0.5) are P-INDAG in all conditions, due to the influence of the binder which, being formed by sugars, presumably acidifies over time. This shows us the great stability of the dye.

Results of colorimetric study

P-BL samples did not show significant variations under any of the aging conditions compared to the variations found in colored paper samples.

Lightness, CIELAB (L_{10}^*), was the parameter that showed more variation compared to the reference standard (P-IND 0 h) for colored samples with and without binder in all aging conditions. This parameter showed a progressive increase related to the number of aging hours, with more variation found in samples without the GA binder because it acts as a color protector. LIGHT and OUT samples were those showing more CIELAB lightness after the aging procedures (Figure 2 (a)). We can indicate that it is the light that brings brightness to the dye.

The values of a_{10}^* , b_{10}^* coordinates showed a progressive increase of tone related to increased aging hours, with variations of colored samples from blue to green occurring in all the conditions tested (REF, DARK, LIGHT, and OUT) and regardless of the presence of binder. Higher color differences were found in LIGHT

Table 1. pH values of P-IND (indigo paper samples), P-INDAG (indigo with gum arabic samples), and P-BL (blank paper samples) for every aging condition and for every aging interval.

	Aging (hours)	REF	DARK	LIGHT	OUT
P-BL	0 h	6	6	6	6
	24 h	5.9	5.9	5.9	5.8
	48 h	5.8	5.9	5.7	5.7
	72 h	5.8	5.8	5.6	5.7
	144 h	5.7	5.8	5.6	5.6
P-IND	0 h	6.3	6.3	6.3	6.3
	24 h	6.2	5.9	5.8	5.8
	48 h	6	6.1	5.8	5.7
	72 h	5.9	6	5.6	5.7
	144 h	5.8	5.8	5.5	5.5
P-INDAG	0 h	6.1	6.1	6.1	6.1
	24 h	5.6	5.3	5.5	5.7
	48 h	5.4	5.4	5.3	5.6
	72 h	5.3	5.2	5.3	5.3
	144 h	5.3	5.2	5.1	5.2

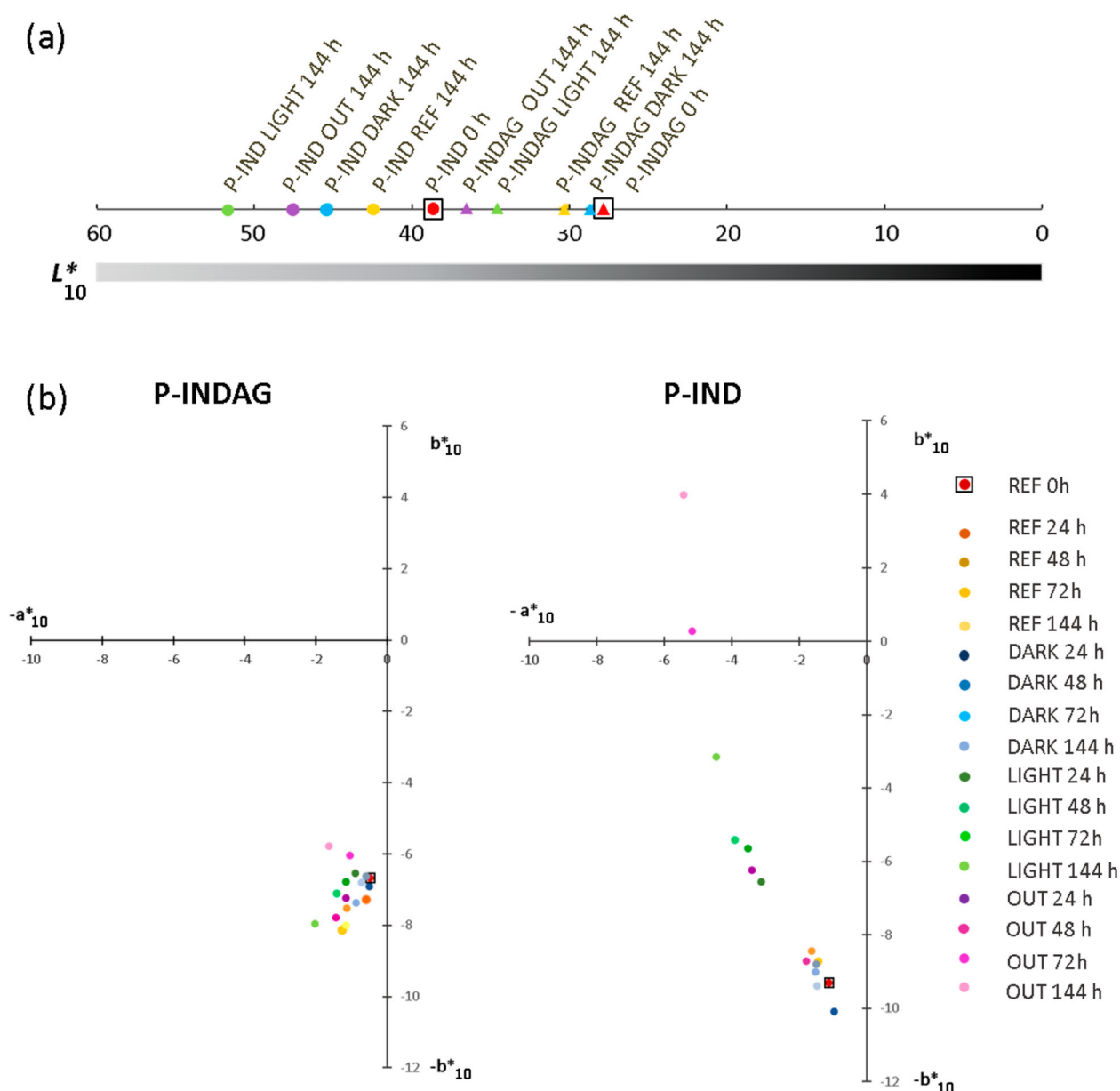


Figure 2. (a) Values of lightness CIELAB L^*_{10} coordinates: 0 h samples (highlighted symbol) and 144 h samples in all conditions (• P-IND samples without binder; ▲ P-INDAG samples with binder); (b) chroma and hue (a^*_{10} , b^*_{10}) coordinates for P-IND (indigo paper samples) and P-INDAG (indigo with gum arabic) after the aging procedure for all the conditions tested.

and OUT samples, with higher differences ($\Delta E^*_{ab,10}$) in indigo colored samples without binder in contrast with colored samples with binder, which were more stable in all the conditions tested (Figure 2(b)).

A clear relationship is observed between the aging produced by exposure to light (LIGHT and OUT samples) and the alteration of the samples towards values greater than b^* . This modification of the colorimetric coordinates of bluish tonalities to greenish tonalities may be due to the degradation of the indigo molecule, of a characteristic blue color, to its degradation product, isatin, of a characteristic yellow color. These results indicate that light is the main cause of degradation for indigo blue.

The accentuation of this gradient in the samples without binder is also evident, which would indicate that GA confers protection to indigo molecules in

terms of resistance to light exposure. There is evidence in the literature that supports that gum arabic is used to varnish to protect and improve the optical and mechanical properties of paper-based artifacts (Young 1999; Vetter 2019).

Results of spectrophotometric study

Indigo blue in solution has three characteristic absorption bands at 286, 348, and 623 nm. After the aging procedure, the spectrophotometric analysis of indigo sample solutions revealed that REF and DARK samples maintained their characteristic absorbance values. LIGHT and OUT samples turned yellow after 24 h and their absorbance spectra showed new absorption bands at 250 nm, 307 nm, and 412 nm, with the loss of the initial absorption bands at 286,

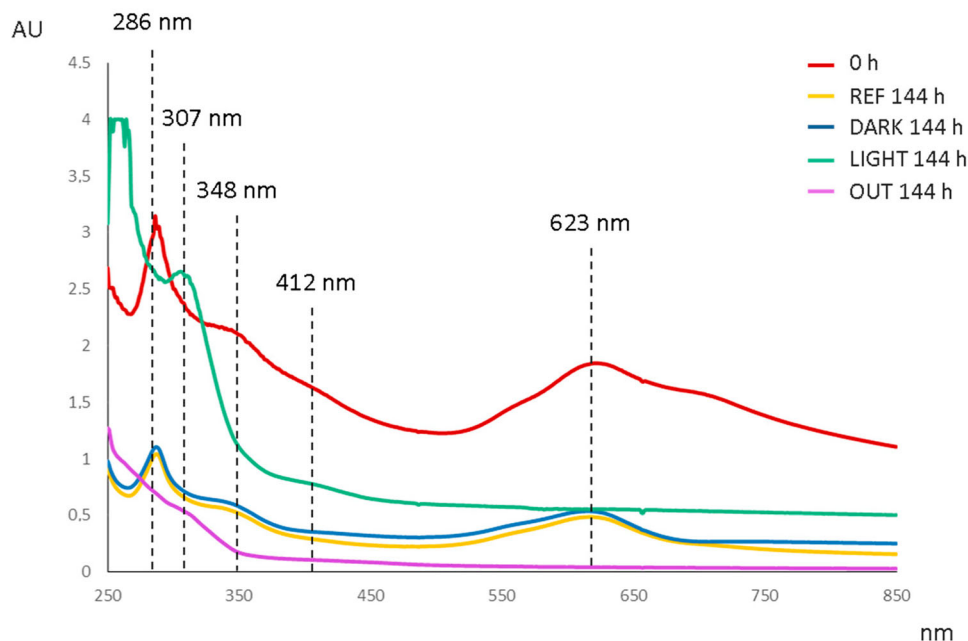


Figure 3. Absorption spectra of D-IND sample (indigo blue solution in acetic acid) for all the tested conditions after 144 h aging.

348, and 623 nm after 144 h aging, as indicated by Novotna et al. (2006) (Figure 3).

This conversion is related to the changes registered in the colorimetric study of the paper samples, but it is not comparable since for the dye on paper support the time necessary for the degradation compounds to form is different. The spectrophotometric analysis of paper samples during 144 h revealed no significant changes in the absorbance of the different samples.

Considering the results of the spectrophotometric study, a new series of aging tests was made to observe the gradual sequence of degradation of indigo blue solution during the first aging interval

(24 h). To this end, a new series of sample solutions of indigo were prepared and subjected to the LIGHT aging condition as this condition results in more changes and in a more controlled manner (OUT condition caused a rapid colorant degradation). The aging intervals were 0, 1, 2, 3, 4, 5, 6, and 12 h.

This study revealed the gradual shifting from the characteristic spectrum of blue indigo to the spectrum of degradation product after 5 h aging. This degradation product could be dehydroindigo or isatin because its absorbance spectra are similar, as indicated by Domenech, Domenech, et al. (2013). A decrease in absorption of 98% was observed after 5 h aging at 623 nm (Figure 4).

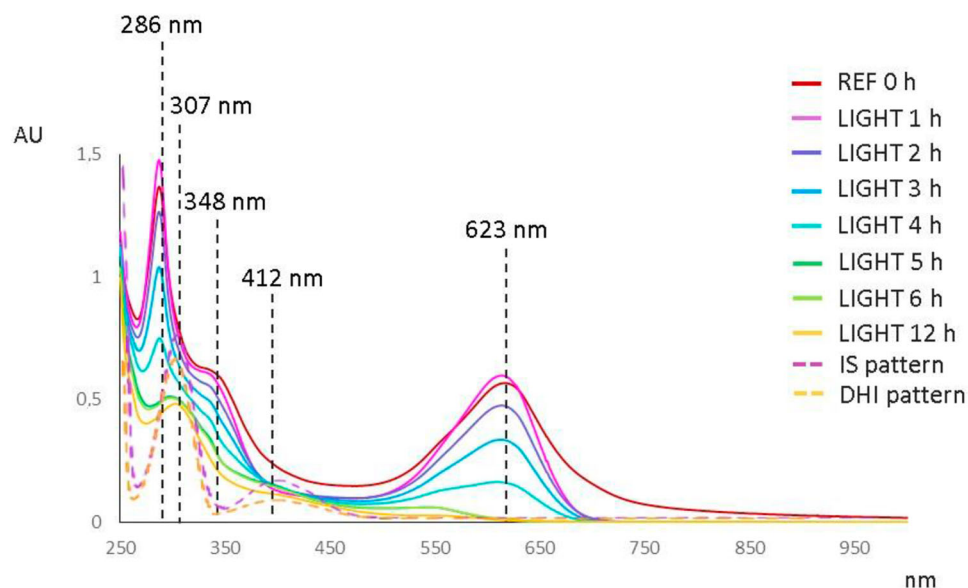


Figure 4. Absorption spectra of D-IND samples (solution of blue indigo in acetic acid) in LIGHT conditions (aging in chamber with light) at 1–6 h and at 12 h aging and absorption spectra of IS and DHI pattern.

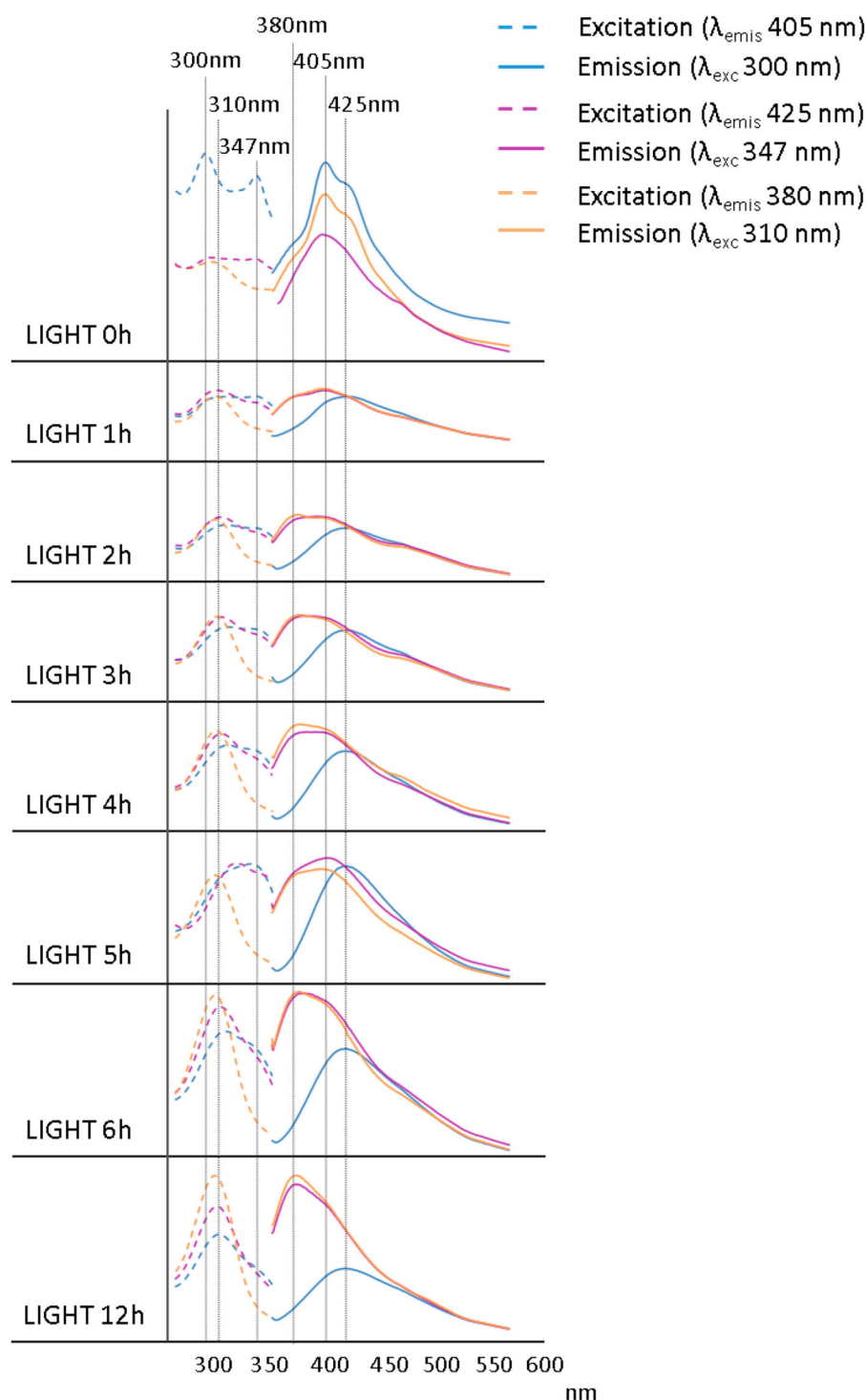


Figure 5. Excitation and emission spectra of D-IND samples (indigo blue in acetic acid) at each hour from 1 to 6 h and at 12 h of the aging process in LIGHT conditions (aging in chamber with light).

Results of fluorescence spectroscopy study

A fluorescence spectroscopy study was also performed during the first 12 h of aging (each hour from 0 to 6 h and at 12 h) in LIGHT conditions to the indigo blue in solution (Figure 5). The solution of indigo blue at 0 h showed two excitation peaks with maximum wavelengths at 300 and 347 nm, one emission peak with maximum wavelength at 405 nm and a secondary emission peak at 425 nm.

After the first hour of aging, the excitation peak at 300 nm and the emission peak at 405 nm disappear.

After two hours of aging, the excitation peak at 310 nm and the emission peak at 380 nm appear. After six hours of aging, the excitation peak at 347 nm disappears and the excitation peak at 310 nm and the emission peak at 380 nm are most noticeable. These maximums continue to increase during aging until 144 h (data not shown).

From the observation of these results we suggest that initially we have the two-tautomer species of the indigo, leuco and keto (Figure 1). When the degradation of the indigo begins, the leuco form is degraded first (at 1 h)



The fluorescence spectroscopy studies performed in REF aging conditions do not change. However, in DARK and OUT aging conditions the changes are the same as in LIGHT conditions but slower. In OUT aging conditions the maximum excitation at 347 nm and the emission at 425 nm appear at 24 h, and the

These results show us that the conversion from the leuco form to the keto form and from the keto form to its oxidized form, dehydroindigo, occur due to the influence of temperature, humidity, and especially light, as shown by the fact that in REF samples this conversion does not occur and in OUT samples the fluorescence intensity at $\lambda_{\text{ex}} = 310 \text{ nm}$ and $\lambda_{\text{em}} = 380 \text{ nm}$ decreases with the time due to polychromatic sunlight with UV component (Abdel-Ghani et al. 2012).

D-IND sample in REF 0 h condition showed a peak at t_m 17.14 min whose absorption spectrum corresponds to the spectrum obtained in the spectrophotometric

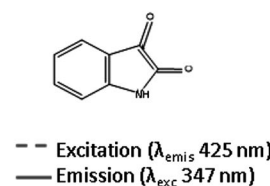


Figure 7. Spectra, molecules, and excitation-emission wavelengths proposals of the degradation products formed during artificial aging of D-IND samples (indigo blue in acetic acid).

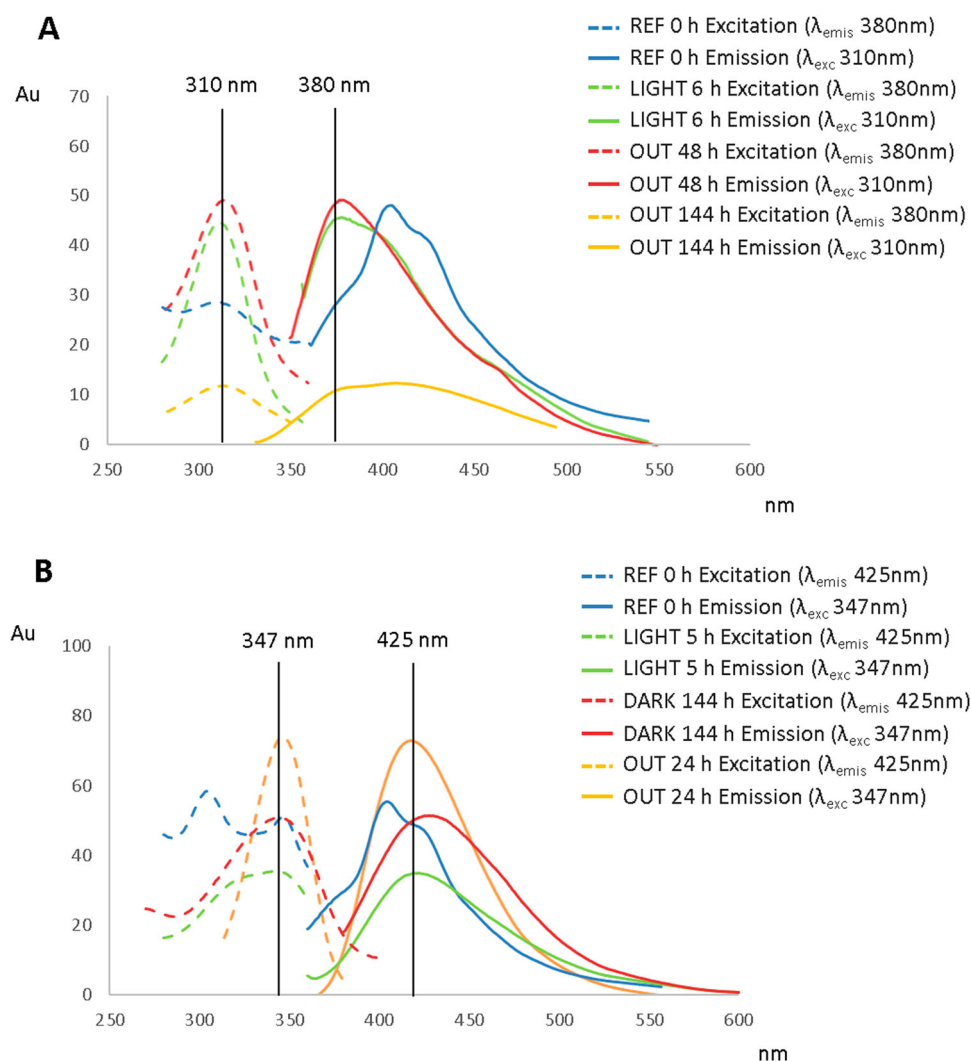


Figure 8. Excitation and emission spectra of DHI in REF, LIGHT, and OUT aging conditions (A) and excitation and emission spectra of IND in REF, LIGHT, DARK, and OUT aging conditions (B).

studies for the same sample, which allowed the identification of indigo (Figure 9(A)). In addition, the sample was spiked with the reference standard of indigotin to confirm this result.

After 6 h aging (LIGHT samples), the UV-vis and fluorescence analyses revealed the conversion into a degradation product that could correspond to dehydroindigo or isatin. The CE-DAD study of the same sample revealed a new peak at t_m 17.83 min with an absorption spectrum different to the one obtained for sample 0 h, which corresponds to the UV-vis spectrum recorded for dehydroindigo and isatin (Figure 9(B)). It is ruled out that it is indirubin because the electropherogram of a reference standard was registered and its migration time is 23.57 min (Figure 9(C)).

Dehydroindigo and isatin have different t_m . To confirm the presence of Isatin, a study of a reference standard of isatin by CE-DAD was made. It revealed a characteristic peak with t_m 8.027 min (Figure 9(D)).

After 12 h aging (LIGHT samples), the isatin and dehydroindigo were identified, confirming the coexistence of both substances in the degradation of the indigo (Figure 9(E)).

In P-IND and P-INDAG samples the three compounds could be observed simultaneously during the aging process in all conditions (Figure 10) except in P-INDAG samples under REF and DARK conditions, where there is only indigo.

The CE-DAD study of the sample solutions and paper samples allowed the separation and identification of indigo, dehydroindigo, and isatin.

Results of nuclear magnetic resonance spectroscopy study

NMR studies confirmed the transformation of indigo into isatin (Figure 11). The NMR spectrum of indigo blue includes a doublet, triplet, doublet, and triplet that can be identified during the first 12 h aging. At 6 h aging, the last doublet allowed the identification of the degradation product isatin, which was perfectly identified by comparison with the NMR spectrum of isatin reference standard (triplet, doublet, triplet, and doublet).

A similar NMR spectrum was observed at 12 h but showing a different configuration in the second

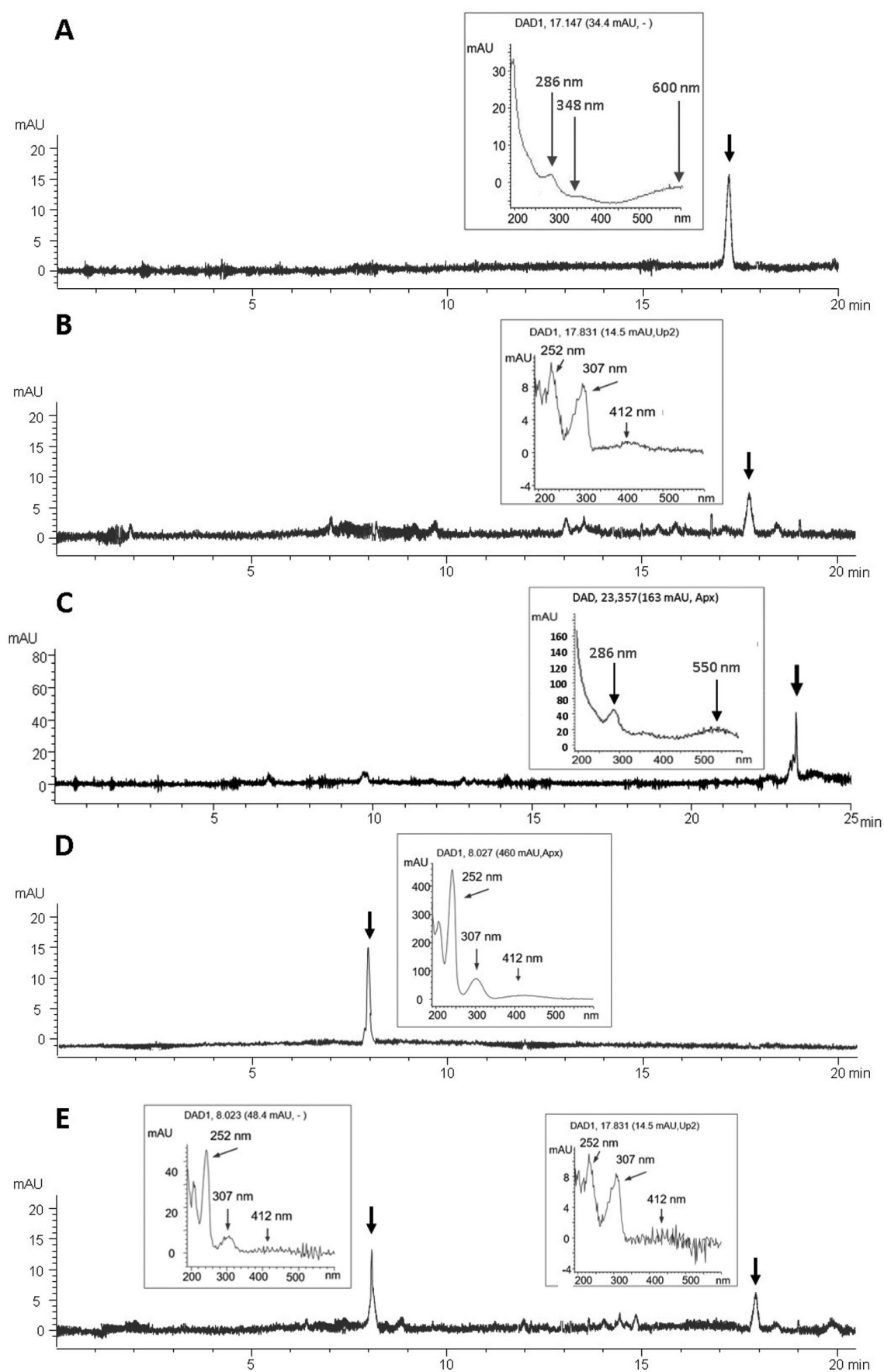


Figure 9. Electropherogram of D-IND samples (indigo blue in acetic acid) and its degradation products at different aging stages. **(A)** D-IND sample REF 0 h; **(B)** LIGHT 6 h; **(C)** indirubin reference standard; **(D)** isatin reference standard; **(E)** D-IND sample LIGHT 12 h.

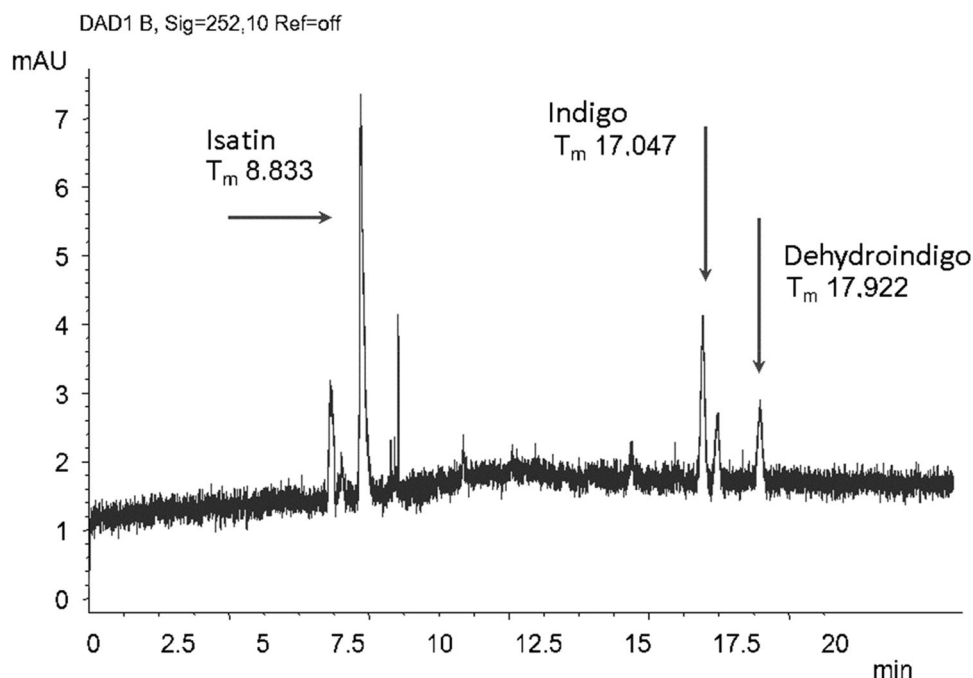


Figure 10. Electropherogram of P-IND sample (indigo blue in paper) in LIGHT 48 h conditions (aging in chamber with light).

doublet which may be indicative of the presence of dehydroindigo coexisting with isatin. Lastly, at 24 h aging the original compound had clearly disappeared from the sample and only isatin was identified.

Methodology for historical samples

Collection of Drawings, Maps, and Plans

A total of 64 maps of the Collection of Drawings, Maps, and Plans with blue, green, and earth

coloration, which could contain indigo, were analyzed using EC (Torres and Bonachera 2005). This dye and / or its degradation products were unequivocally found in only five of the 64 maps (Table 2). In some cases (particularly maps 69, 116, and 136), the use of indigo was identified only by the presence of its degradation products (DHI and IS) instead of being inferred by the color of the object. This fact allowed us to conclude that indigo had been the dye used and had suffered degradation.

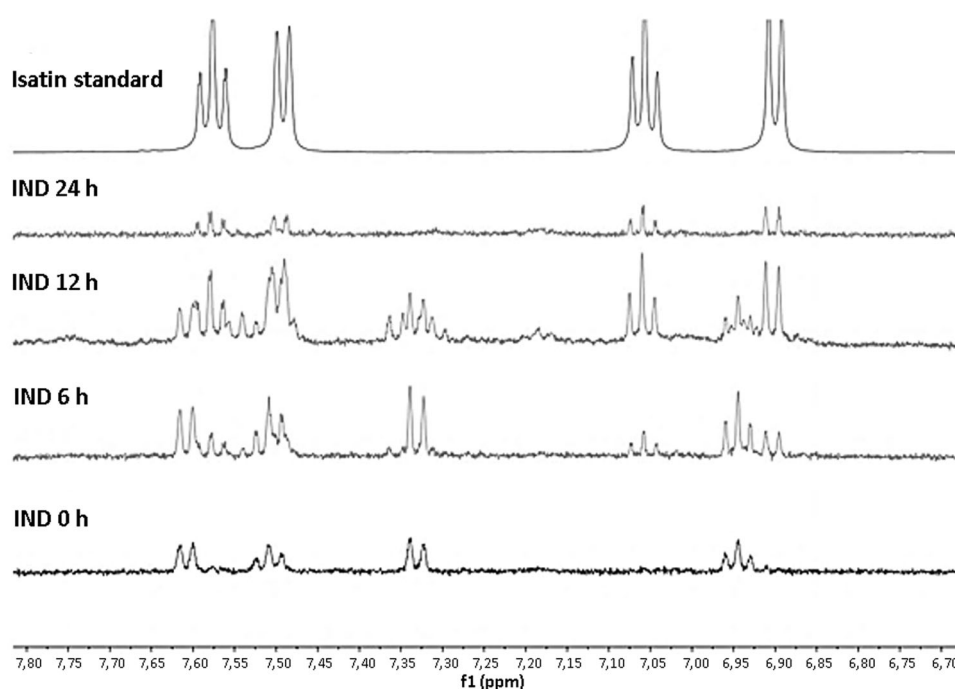
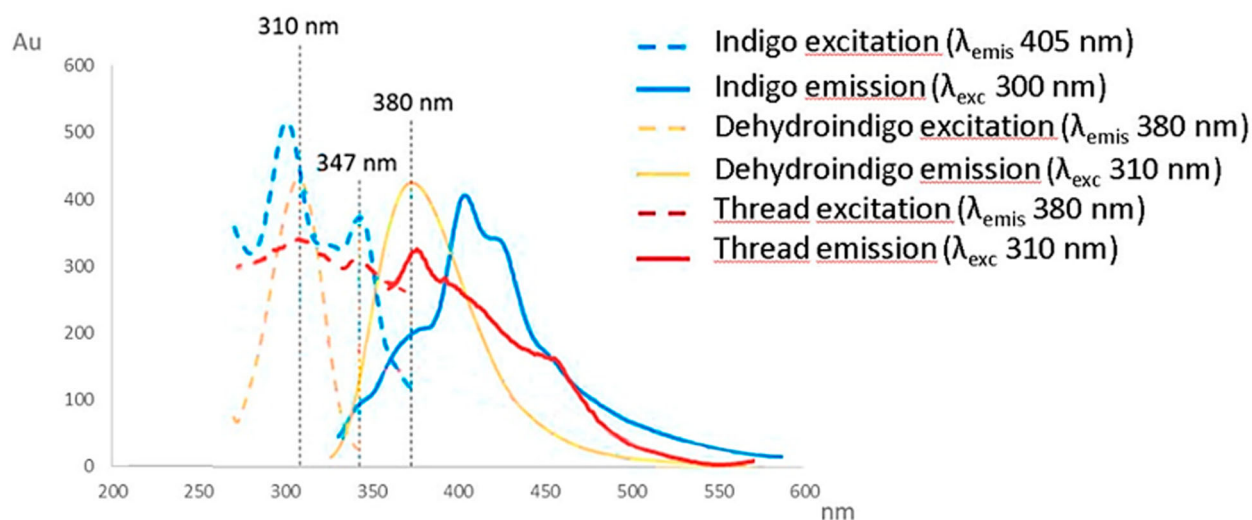


Figure 11. NMR spectrum of the isatin reference standard and spectra of D-IND samples in LIGHT conditions.

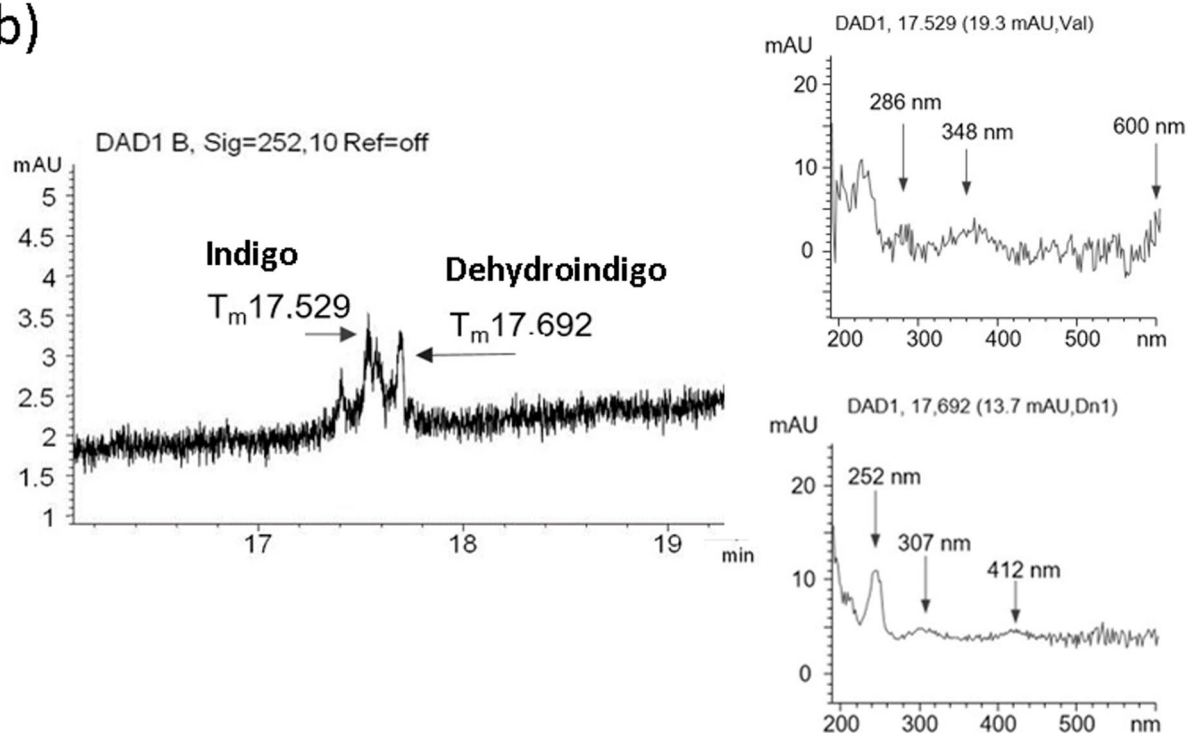
Table 2. Map samples analyzed with CE-DAD that revealed the presence of indigo blue and/or its degradation products.

Map No.	Date	Location	Sample color	Identification		
				IND	DHI	IS
69	1754	Almuñécar (Granada)	Earth	x	✓	✓
83	17??	Guadix (Granada)	Blue	✓	✓	✓
			Green			
87	17??	Monteagudo (Murcia)	Earth			
			Blue	✓	✓	✓
			Green			
116	1804	Alhaurín (Málaga)	Green	x	✓	✓
			Earth			
136	1784	Pechina (Almería)	Green	x	✓	✓

(a)



(b)

**Figure 12.** Fluorescence spectra and excitation-emission wavelengths of the thread compared with those of a sample of indigo blue prepared in laboratory (a); Electropherogram and UV-vis absorption spectra of the sewing thread (b).

Notarial record book (Registro Notarial de Torres)

The identification of indigo and its degradation status in the sewing thread from the *Registro Notarial de Torres* was made using fluorescence spectroscopy and CE-DAD and comparing the results with the results obtained from the aging tests. In fluorescence spectra, a maximum excitation at 347 nm is observed that can correspond to the indigo and a maximum excitation at 310 nm and a maximum emission at 380 nm corresponding to the dehydroindigo. These results are confirmed by observing the electropherogram where dehydroindigo and indigo are identified with migration times and absorbance spectra at the apex (Figure 12). Therefore, we suggest that the thread was dyed with indigo that is currently in the degradation phase.

Conclusions

The combination of the different analytical techniques employed in the present work has allowed us to explain and evaluate the degradation process of indigo blue. Our results revealed the transformation of the main component, indigo blue, caused by temperature, light (visible and ultraviolet), and humidity, both in solution and on a support, as well as the presence of the degradation products dehydroindigo and isatin.

The samples that showed more sensitivity to temperature were those in solution. A chemical change, confirmed by the electrophoretic and fluorescence studies, that resulted in the formation of dehydroindigo from indigo was detected.

Ultraviolet and visible light were the main causes responsible for the oxidation of indigo blue. The colorimetric study revealed an increase in sample lightness, which indicates photolysis of the molecular bonds. The fluorescence study revealed that sunlight was the main cause responsible for colorant degradation because of its more energetic radiation. Light in combination with high temperature resulted in faster degradation of the indigo molecule in comparison with degradation caused by UV radiation alone.

The effect of high RH on indigo degradation was limited, with no significant changes of the initial parameters. In addition, no significant changes were observed with RH in combination with the parameters that had an effect on degradation.

Degradation of indigo blue was decreased by the presence of GA and probably by the protection provided by the fibers of the support. The presence of GA slowed down the lightening process, contributed to stabilizing and reducing tone variations and preventing the colorant in the samples from degrading chemically.

The information obtained from the aging tests allowed us to demonstrate the presence of indigo blue in both the sewing thread from the *Registro Notarial de Torres* and in some of the documents of the Collection of Drawings, Maps, and Plans held in the Royal Chancery Archives of Granada as well as to describe the conservation status of this colorant.

Our results confirm that the degradation of indigo blue in both solid state and solution is an inevitable process; therefore, careful attention has to be paid to its environmental conditions in order to slow down the changes associated with degradation as they are permanent.

In addition, applying the different analysis techniques, we can identify the use of indigo blue in works of art despite its degradation caused by external agents or by its natural aging.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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