Struct Chem
DOI 10.1007/s11224-017-0932-0

ORIGINAL RESEARCH

The effect of light and dye composition on the color of dyeings with indigo, 6-bromoindigo, and 6,6′-dibromoindigo, components of Tyrian purple

Keith Ramig[1] · **Aygul Islamova**[1] · **John Scalise**[1] · **Sasan Karimi**[2] · **Olga Lavinda**[3] · **Christopher Cooksey**[4] · **Athina Vasileiadou**[5] · **Ioannis Karapanagiotis**[5]

Received: 4 January 2017 / Accepted: 17 February 2017

Abstract Quantitative HPLC and colorimetry are used to study color variations in dyeings with indigo, 6-bromoindigo, and 6,6′-dibromoindigo, the main components of the historic dye Tyrian purple. For the first time, visible light is identified conclusively as a cause of debromination of the leuco form of 6-bromoindigo. A dyeing run using 6-bromoindigo alone is found to yield a dyed fabric containing large amounts of indigo, when the vat is exposed to visible light. The extent of debromination is dependent upon the pH of the dye bath and also the source of the visible light. This information allowed development of a dyeing procedure which is demonstrated to give consistent colors through two passes. Quantitative HPLC analysis of extracts from the dyed fabrics indicates that the leuco form of 6-bromoindigo vs. the leuco forms of indigo and 6,6′-dibromoindigo has the strongest affinity for wool fabric. This is postulated to be due to attractive electrostatic interactions between the leuco form of 6-bromoindigo and wool.

Keywords Indigo · 6-Bromoindigo · 6,6′-Dibromoindigo · Dyeing · Tyrian purple · HPLC · Colorimetry

This article is dedicated to our colleague and friend Professor Lou Massa, in honor of his 75th birthday. Lou kindly introduced this project to three of us (KR, OL, and SK) and collaborated in much of the previous work on it.

Electronic supplementary material The online version of this article (doi:10.1007/s11224-017-0932-0) contains supplementary material, which is available to authorized users.

✉ Keith Ramig
keith.ramig@baruch.cuny.edu

✉ Ioannis Karapanagiotis
y.karapanagiotis@aeath.gr

[1] Department of Natural Sciences, Baruch College of the City University of New York, Box A0506, 1 Bernard Baruch Way, New York, NY 10010, USA

[2] Department of Chemistry, Queensborough Community College of the City University of New York, 222-05 56th Ave., Bayside, New York, NY 11364, USA

[3] Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, 180 Varick Street, Room 641, New York, NY 10014, USA

[4] 59 Swiss Avenue, Watford, Hertfordshire, England WD18 7LL, UK

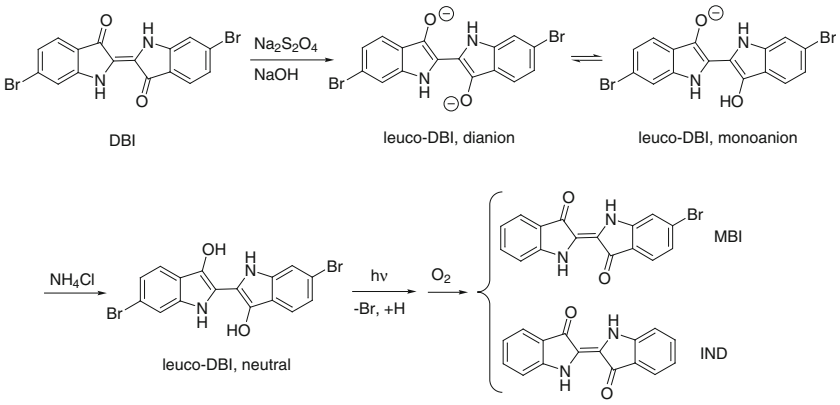
[5] University Ecclesiastical Academy of Thessaloniki, 54250 Thessaloniki, Greece

Introduction

In the course of our recent studies [1, 2] of dyeing with the indigoids indigo (IND), 6-bromoindigo (MBI), and 6,6′-dibromoindigo (DBI), we became interested in color variations caused by types of illumination during the vatting procedure, and by altering the proportions of the three indigoids. Dyeing with indigoids is complicated by their limited water solubility. The colored form of the dye must be chemically reduced in the dye vat to give the water-soluble leuco (colorless) form (Scheme 1). In the lab, this is done with sodium dithionite at high pH, resulting in the dianionic form alone, or a mixture of the dianionic and monoanionic forms of the leuco-indigoid [3]. Then, excess ammonium chloride is added to adjust the pH to around neutral, because many fabrics cannot tolerate high pH levels. The leuco species is then in its neutral form [3]. Finally, the fabric is dipped in the solution, removed, and subjected to oxygen in the air, which causes oxidation back to the colored form.

It is well-known that sunlight can cause debromination of the leuco forms of brominated indigoids, resulting in indigo-like blue dyeings [4, 5]. This photodebromination was used to

Published online: 03 March 2017



Scheme 1 Reduction of DBI to its ionic leuco forms, protonation to give the neutral form, photodebromination, and oxidation back to the colorful forms of MBI and IND

confirm that ancient textiles were, indeed, really dyed with shellfish purple. In the 1930s, Rudolf Pfister analyzed a Coptic textile dating from the second or third century AD, which is a fragment of a purple medallion decorated with four-leafed motifs in gold thread and is now in the *Österreichisches Museum für angewandte Kunst* in Vienna. He used chemical methods: reduction of the dye with dithionite followed by exposure of the solution to light and oxidation to give a blue-violet color. He concluded that *C'est. donc bien la pourpre véritable; il s'agit d'un tissu particulièrement riche puisque les fils d'or, également, sont très rare en Égypte* [It is, therefore, the true purple; it is a particularly rich fabric since gold threads are equally very rare in Egypt.] [6]. A more recent investigation has shown that the major colorant is MBI [7]. There are other examples from antiquity where MBI is the major component [8]. This technique to identify genuine shellfish purple was still in use in the 1980s when it was used on a thirteenth century BC pottery sherd [9]. Cooksey and Sinclair [10] have reported that in a study of DBI dyeing of wool, incandescent light does not cause a detectable level of debromination, as judged by TLC. It has long been assumed that ultraviolet light is needed to cause debromination of leuco-indigoids [11]. On the other hand, some of us [1] have noted that if leuco-MBI is exposed to standard fluorescent lighting for an overnight period, the dyeings produced are indistinguishable from IND dyeings. Another study [12] gives anecdotal evidence that even the colored oxidized forms of brominated indigoids are sensitive to light. It has also been reported [13] that if the dye vat is protected from sunlight by red cellophane, debromination of leuco-DBI is lessened dramatically. Whether the light-sensitive species is the neutral leuco-bromoindigo, the monoanion or the dianion, or some of all three, is not known. It has also not been fully established whether visible light can cause debromination.

DBI was first isolated from a *Murex* shell fish and identified as the colorant in Tyrian purple by Friedländer over 100 years ago [14]. Later, he also obtained from a different species a more blue uncharacterized indigoid which was assumed to be indigo [15]. Much later the minor bluer component was identified by HPLC as MBI [16]. The shellfish pigment, often known as Tyrian purple, has been found to contain a mixture of brominated and unbrominated indigoids and indirubinoids, the composition of which can vary according to the species of mollusk, region where they are collected, and other factors [17–23]. When the pure indigoids are used to dye wool, IND gives a nearly pure blue color, and MBI produces a nearly true purple shade (i.e. a roughly even mixture of red and blue), while DBI yields a reddish purple shade [2]. However, if the dye vat containing either MBI or DBI is very dilute, the dyeings can become blue [24]. The dye in a natural Tyrian purple dyeing can consist of mainly DBI, or DBI can be mixed with large amounts of MBI and IND, and a wool dyeing will still show a reddish hue [25, 26]. In a mixed indigoid dyeing, the question arises of whether the proportion of leuco-indigoids in the vat is the same as the proportion of the colored forms on the fabric. It has been suggested that leuco-DBI has a stronger affinity than leuco-IND for wool, since the uptake of leuco-DBI in the first pass appears to be greater than that of leuco-IND [25]. This apparently led to an IND-rich second pass that was more blue. However, extraction of the dye from wool and HPLC analysis of the dye components subsequently revealed that MBI may have been a major component in the dyeing [26], so it is just as likely that it is leuco-MBI that has a great affinity for wool, and which ultimately yielded the reddish hue. Whether the main dye component is MBI or DBI, the bluing of the second-pass dyeing could also be explained by an aggregation effect upon dilution of the vat, which makes late-pass dyeings appear bluer than earlier ones [24]. The differing affinity of leuco-indigoids for wool could be established unequivocally by comparing the HPLC profiles of a dyeing where the initial composition of dyes in the vat is controlled. If there is a differential uptake of leuco-indigoids by fabrics, then the proportions of indigoids initially produced from the mollusk may not be indicative of the relative amounts on the dyed fabric. An alternative explanation for the differing colors in multipass dyeings is chemical reduction of the bromo compounds in modern dye baths, but not in antiquity, by dithionite, a powerful reducer in the dye vat.

For years, the identity of the reductant of shellfish purple pigment was in doubt. In the first century AD, Pliny suggested that use of a lead or tin metal bath was required for a successful dyeing. In modern times, it was conjectured that lead or tin could be the reductant. These and other potential reducing agents were investigated [27], including methanethiol, sulfur compounds in wool, and glucose from grape juice. But none was very convincing. The problem was resolved by a retired engineer, John Edmonds (1931–2009), who had studied the centuries-old woad vat in which IND is reduced to leuco-IND by bacteria, specifically *Clostridium isatidis* [28]. Other species of bacteria, apart from *Clostridium*, will do the reduction, e.g., *Alkalibacterium* [29]. Edmonds demonstrated that DBI, derived from mollusk sources, could be reduced in an exactly analogous way using just mollusk flesh after 4 days at pH 9 and 50 °C [30]. Given this vital data, others were able to successfully repeat this biochemical reduction [25, 31].

We report here the effect of visible light on the debromination of leuco-MBI, in both its dianionic and neutral forms. The knowledge gained allowed development of a procedure which gave consistent colors through two passes of a dyeing run using wool fabric. This procedure was used in a comparative study of the amounts of dye in IND, MBI, and DBI dyeings, and dyeings produced from equimolar mixtures of IND and MBI, and MBI and DBI. The dyeings were characterized by CIELAB colorimetric data, which was then correlated with HPLC data from extracts of the wool dyeings. Conclusions about differing affinities of the leuco-indigoids for wool were then drawn.

Results and discussion

Effect of light on debromination of leuco-MBI

A short study was made of the effectiveness of light-protection measures and what kind of light source will cause debromination of the various forms of leuco-MBI. The dye vat during leuco formation and the developing dyeings are normally covered with aluminum foil to prevent possible debromination by stray light. We have found that the vat containing leuco-MBI (after NH_4Cl addition), while protected from light in this fashion, undergoes very little debromination, as judged by HPLC (Table 1). A three-pass dyeing run was simulated by heating the vat at reflux for 1 h with no fabric present, while either covering the vat or exposing it to light. Then, the vat was opened to air, causing oxidation back to the colored form of the dye, which was isolated by suction filtration. This protocol was used to determine the debrominative effect of ambient fluorescent light on both the leuco-MBI neutral form and dianion, and of incandescent light on the leuco-MBI dianion (see "Dyeing procedures" section). The amounts of dyes were estimated from the relative area percentages of the peaks arising from the three indigoids, assuming similar detector responses. The analyses were done in triplicate, and the standard deviations (SD) were low. The data show that, with both the vat and the developing dyeings protected from light, there is very little debromination. However, when the leuco form in either its neutral or dianionic forms is subjected to ambient fluorescent lighting, an appreciable level of debromination to give leuco-IND occurs. Even a low-wattage incandescent desk lamp, if put near the uncovered reaction flask, can cause a large amount of debromination of the dianionic form of leuco-DBI.

Table 1 The effects of covering the leuco-MBI dye vat and of exposing the vat to fluorescent or incandescent light

	HPLC area %					
Conditions	DBI	(SD)	MBI	(SD)	IND	(SD)
(Starting)[a]	5.0		92.5		2.0	
Covered, neut[b]	3.6	(0.1)	90.8	(0.6)	5.6	(0.6)
Fluor, neut[c]	2.7	(0.2)	75.5	(2.1)	21.8	(2.2)
Fluor, dian[d]	2.7	(0.1)	70.9	(0.8)	26.4	(0.9)
Incan, dian[e]	1.9	(0.2)	48.3	(0.5)	49.8	(0.6)

[a] Purity (HPLC area %) of starting MBI [32]

[b] Vat covered with aluminum foil; NH_4Cl present

[c] Vat exposed to ambient fluorescent light; NH_4Cl present

[d] Vat exposed to ambient fluorescent light; no NH_4Cl present

[e] Vat exposed to direct incandescent light; no NH_4Cl present

MBI dyeing: exposure of the vat to incandescent light

Then, a dye vat containing leuco-MBI was again exposed to incandescent light, under the conditions of "Dyeing procedures" section (corresponding to the last row of Table 1). This time, a two-pass wool dyeing run was carried out after the exposure, with the intention of extracting the dye from the fabric and quantifying by HPLC [33]. CIELAB values of the dyeings were also collected, to correlate color with amounts of MBI and IND on the fabric (Table 2). Since the three indigoids vary appreciably in their molecular masses, and it is the ratio of the numbers of dye molecules of each kind on the fabric which is likely to determine color in a mixed dyeing, the masses of IND and MBI found by quantitative HPLC were converted to micromoles. This allowed a more meaningful correlation between color and amounts of the two dyes. The first pass of the incandescent-light-exposed dyeing run showed nearly 80% IND, on a molar basis. Not surprisingly, this gave a fairly dark, nearly pure blue dyeing, as judged by the CIELAB values (see the Online Resource file for additional data). The second pass contained predominantly IND as well and gave a lighter blue dyeing, starting to head towards green.

Table 2 Quantitative HPLC data of extracts from wool produced by an incandescent-light-exposed two-pass MBI dyeing run and colorimetric data from the fabric

		Dye amt. (mg/g wool)			CIELAB values		
Pass		mg	(SD)	μmol	*L**	*a**	*b**
1st	IND	1.0	(0.02)	3.8	38	0.2	−20
	MBI	0.3	(0.02)	0.9			
2nd	IND	0.75	(0.005)	2.9	47	−2.0	−18
	MBI	0.4	(0.001)	1			

Comparing the IND/MBI ratio in the vat (last row of Table 1) to the ratio of the two on the fabric after the first pass in Table 2, it would appear that leuco-IND has a stronger affinity for wool. The incandescent-exposure data of Table 1, when converted to moles of IND and MBI, gives a ratio of 1.4:1, in terms of actual numbers of molecules. On the fabric, the IND/MBI ratio is 3.8:1, appearing to indicate that leuco-IND was preferentially adsorbed by the fabric. But, the data of Table 1 may only give a rough idea of mass ratios, as the three indigoids may have differing detector responses. Indeed, the HPLC chromatogram of the extracts from the first-pass dyeing (corresponding to the first row of Table 2) shows MBI with a greater peak area than IND (Fig. 1). Also, reproducibility of the effect of exposure of the vat to incandescent light was not determined. So, possible differing affinity of the leuco forms of the indigoids for wool remained an open question, to be answered with further experiments.

IND, MBI, and DBI dyeings: affinity of leuco forms for wool

With the effect of light on debromination of the leuco form established, attention was turned to development of a dyeing procedure which would give consistent colors, the ultimate goal being to determine which leuco form of the dye has the strongest affinity for wool. We have noted [1, 2] that the procedure of "Dyeing procedures" section when performed at 50 °C gave widely varying colors in the dyeings, especially for DBI. The procedure performed at this temperature was used to generate indigoid dyeings used in an earlier comparative study [32]. We report here that a dyeing procedure where the vat is heated at reflux [10] while protecting from light gives reproducible dyed colors through two passes. The third-pass dyeings for the brominated indigoids were often highly mottled in appearance and thus did not give consistent colors. Figure 2 shows the results of an average dyeing for IND, MBI, and DBI (all three at the same molar amount in the vat), and two 1:1 mixtures which will be discussed later. Noteworthy are the progressive increase in redness on proceeding from IND to DBI first passes, the lack of change in hue of indigo dyeings upon dilution, and the marked bluing of MBI dyeings resulting from dilution of the vat. The bluing of brominated indigoid dyeings upon dilution has been ascribed to inadvertent debromination [32], but now that we have established here that very little debromination occurs, the explanation [24] based on changes in size of dye aggregates is more likely.

The consistency of the dyeing procedure is illustrated by three identical two-pass dyeing runs using an equimolar mixture of MBI and DBI (Table 3). (The full sets of colorimetric data for the other dyeings shown in Fig. 2 are in the Online Resource file.) The CIELAB values from the fabric are given from dyeing runs done in triplicate. The standard deviations within a run are the result of at least ten measurements on various parts of the fabric. These low standard deviations indicate an evenly colored fabric. The color differences (ΔE_{ab}*) between runs are insignificant. We have found that the colors of two dyed fabric samples are indistinguishable from each other if the ΔE_{ab}* value is less than 4. The third passes of the brominated indigoid dyeings tend to be highly mottled in appearance, and thus the colorimetric data show a high amount of variability.

The first-pass MBI dyeing of Fig. 2 looks darker and richer in color than the corresponding IND and DBI dyeings. But, does this mean that wool has the greatest affinity for leuco-MBI? We present here HPLC data from extracts of dyeings

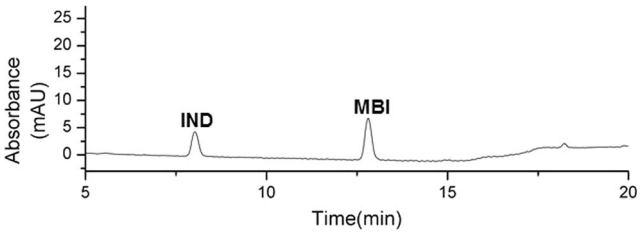


Fig. 1 HPLC chromatogram of extracts from the first-pass dyeing of Table 2

Fig. 2 Composite photograph of dyeings from five three-pass dyeing runs: (1st–5th columns) DBI, 1:1 DBI:MBI, MBI, 1:1 MBI:IND, IND; top-row squares are first-pass dyeings

Table 3 Colorimetric data of fabric from three identical two-pass equimolar MBI/DBI mixed dyeing runs

Pass	Value	Run 1	SD	Run 2	SD	Run 3	SD	Avg.	SD	ΔE_{ab}*	
1st	L*	33.8	1.1	36.4	0.7	34.8	0.5	35.0	1.3	2.7	(Run 1, run 2)
	a*	25.3	0.3	25.5	0.1	28.2	0.1	26.3	1.6	3.4	(Run 2, run 3)
	b*	−22.9	0.4	−23.4	0.5	−24.6	0.3	−23.6	0.9	3.5	(Run 1, run 3)
2nd	L*	51.3	1.1	49.1	0.5	48.9	0.4	49.8	1.3	2.2	(Run 1, run 2)
	a*	21.6	0.1	21.4	0.1	23.2	0.1	22.1	1.0	2.1	(Run 2, run 3)
	b*	−22.0	0.6	−22.3	0.3	−23.4	0.2	−22.6	0.7	3.2	(Run 1, run 3)

that unequivocally establish that leuco-MBI has the strongest affinity for wool. Table 4 shows quantitative HPLC data of extracts from the three one-component dyeings of Fig. 2. Also shown are the colorimetric data for each pass. After one pass, only one third of the original amount of MBI remains in the vat. The vats of the other two still retain most of the leuco-dyes after one pass. Also, after two passes, the MBI vat is nearly completely exhausted, while the other vats are less than half so. The colorimetric data bear out what is seen by the eye: The MBI dyeing is darkest and highly saturated in color, through both passes.

Mixed indigoid dyeings: competition experiments

The strong affinity of leuco-MBI for wool was confirmed by direct competition experiments. Two two-pass mixed dyeing runs using equimolar starting dye ratios (18 μmol each dye/g of wool) were performed, one with IND mixed with MBI, and another with DBI mixed with MBI. Table 5 shows the colorimetric data for the four dyed fabric samples and quantitative HPLC data for extracts from each. The first-pass data clearly show that, when equal numbers of each dye's leuco form are present in the vat, the uptake of leuco-MBI is preferred over both leuco-IND and leuco-DBI. Colorimetrically, the dyeings behave as a hybrid: While there is no clear trend in lightness differences between the hybrid and pure dyeings, the redness and blueness values of the hybrids are between their respective pure-dyeing values (see Table 4). This can be discerned qualitatively from Fig. 2.

Table 4 Amounts of dye extracted from the fabric of three two-pass one-component wool dyeing runs and colorimetric data from the fabric

Dyeing		Amt.[a]	(SD)	% exh.[b]	CIELAB values		
					L*	a*	b*
IND	Max.[c]	9.3					
	First pass	2.5	(0.01)	27%[d]	37	−2.2	−20
	Second pass	1.8	(0.01)	46%[e]	41	−2.5	−21
MBI	Max.[c]	12					
	First pass	7.9	(0.1)	66%[d]	28	27	−27
	Second pass	3.7	(0.25)	97%[e]	39	25	−28
DBI	Max.[c]	15					
	First pass	5.0	(0.05)	33%[d]	39	28	−21
	Second pass	1.9	(0.03)	46%[e]	54	22	−19

[a] mg dye/g wool

[b] % exhaustion: amount of dye on fabric compared to amount of leuco-dye in the vat before dyeing

[c] Maximum amount of dye on fabric if all of the leuco form had been absorbed in one pass

[d] Amount on fabric after 1st pass compared to max. amount

[e] Total amount absorbed after two passes compared to max. amount

Conclusions

The strong affinity of leuco-MBI for wool may be due to its polarity. Leuco-MBI is the only one of the three leuco-indigoids that has a molecular dipole. Theoretical calculations using Gaussian09 [34] at HF/6-31G** level of theory predict a molecular dipole moment of 2.76 debye for leuco-MBI, while the molecular dipole moments for both leuco-IND and leuco-DBI are zero. Thus, leuco-MBI may be expected to be more strongly attracted to the highly polar surface of wool fibers. That the concentrations of leuco-indigoids in the dye bath may be different than what is seen on the dyed fabric indicates that caution is needed in interpreting data from dyed-wool extracts.

Also demonstrated here for the first time unequivocally is the high sensitivity of leuco-MBI (and by a highly probable extension, leuco-DBI as well) towards light in the visible region of the spectrum. The light-sensitivity is slightly dependent on the pH of the dye bath: The leuco dianion appears to be more prone to debromination than the protonated form.

Ancient dyers knew that exposure of the Tyrian purple dye vat to sunlight would cause a bluer dyeing. For over 70 years since the chemical basis of this effect was discovered, it had been assumed that ultraviolet light was the sole culprit that caused debromination. We conclude here that the leuco form of MBI, either at a high or neutral pH level, is extraordinarily sensitive to debromination by visible light.

Table 5 Amounts of dye molecules on the fabric of two equimolar mixed dyeings and colorimetric data

Dyeing	Pass	Dye amounts (SD) (μmol/g wool)					CIELAB values		
		IND		MBI		MBI/IND	L^*	a^*	b^*
1:1 IND/MBI	1st	6.9	(0.01)	8.8	(0.03)	1.3	31	11	−24
	2nd	5.0	(0.03)	4.1	(0.04)	0.82	41	5.7	−25
		DBI		MBI		MBI/DBI			
1:1 MBI/DBI	1st	1.9	(0.02)	2.6	(0.04)	1.4	35	26	−24
	2nd	1.5	(0.005)	2.2	(0.005)	1.5	50	22	−23

Materials and methods

Chemicals and materials

HPLC-grade dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA) (assay >99.0%), tetrahydrofuran (≥99.9%, inhibitor-free), and sodium hydrosulfite (sodium dithionite; technical grade, 85%) were obtained from Sigma-Aldrich (USA). HPLC-grade acetonitrile and water were obtained from ChemLab (Belgium). Wool fabric was obtained from Akn Fabrics Inc. (New York City, USA).

Indigo was purchased from TCI (Japan) (>98% purity) or Sigma-Aldrich (95% purity). DBI was synthesized by the procedure of Tanoue et al. [35], and MBI was prepared by the procedure of Clark and Cooksey [24]. Purity was established by NMR and TLC analysis of the *N*,*N*′-bis(trifluoroacetyl) derivatives [24, 36] and by HPLC [32].

Instrumentation and analysis methods

Colorimetric analysis

CIELAB data were collected with a Konica Minolta Color Reader CR-10 tristimulus colorimeter, with a 10-mm/8-mm aperture/viewing area, 8° illumination angle with CIE standard illuminant D_{65}, and CIE 10° viewing angle with diffuse viewing. All samples were backed with an opaque white background. Averages were taken of at least 10 measurements from different spots on the fabrics. In the indigo dyeings, approximately 90% of the area of the fabric was of an even color for all three passes, while approximately 75% of the area of the fabric was of an even color in the first and second passes of brominated indigoid dyeings. Measurements were taken from evenly colored areas of all dyeings. The third passes of MBI and DBI dyeings were highly mottled in appearance, and therefore the third-pass data were not useable.

In the CIELAB color space [37], a color is characterized by three attributes: the lightness, L^*, the hue, which is given by two values, a^* and b^*, and the saturation, which is indicated by the absolute values of a^* and b^*. The L^* value varies from 0 (black) to 100 (white), while the a^* value is positive for redness and negative for greenness, and the b^* value is positive for yellowness and negative for blueness. High absolute values of a^* and b^* signify a highly saturated or vivid color, while low absolute values of these mean a color towards gray. The ΔE_{ab}^* value [37] is a measure of the difference between two colors and is the Euclidean distance between two points in the three-dimensional color space. The formula for ΔE_{ab}^* is the square root of the sum of the squares of the changes in L^*, a^*, and b^* values. We have found that a color change in these samples is just noticeable for ΔE_{ab}^* values around 4.

HPLC analysis

Instrumentation The HPLC-DAD system (Ultimate 3000, Dionex) consisted of a LPG-3000 quaternary HPLC pump with a vacuum degasser, a WPS-3000SL autosampler, a column compartment TCC-3000SD, and a UV–Vis Diode Array Detector (DAD-3000). Analyses were carried out by injecting 20 μL into an Alltima HP C18 Grace column (250 mm × 3 mm i.d., 5 μm particle size; Alltech Associates Inc., USA) at a stable temperature of 35 °C. Data acquisition was carried out via the Chromeleon software, version 6.80 (Dionex).

HPLC method and sample preparation The original version of the applied chromatographic method [38] was recently improved [22] and validated [33]. Two solvent reservoirs, containing (A) 0.1% (*v*/*v*) TFA in H_2O and (B) 0.1% (*v*/*v*) TFA in CH_3CN, were used for chromatographic separation, under a gradient which is described in detail elsewhere [37]. The monitoring wavelength of the detector was 288 nm.

Prior to HPLC, the samples were solubilized in DMSO by heating at 80 °C for 15 min, followed by centrifugation at 3000 rpm for 3 min. The injected supernatants were clear.

Analysis of samples from "Effect of light on debromination of leuco-MBI" section HPLC analyses were performed in triplicate, i.e., three subsamples were generated from each of the four given samples and analyzed separately. For each sample, an amount of around 200 μg was dissolved in 3 mL of hot DMSO and analyzed as described above. The

injected supernatants were clear. Integrated areas were measured at a detector wavelength of 288 nm.

Analysis of samples from "MBI dyeing: exposure of the vat to incandescent light," "IND, MBI, and DBI dyeings: affinity of leuco forms for wool," and "Mixed indigoid dyeings: competition experiments" sections The standard additions method was adopted by fortifying aliquots of a pooled sample, which was derived by blending equal quantities (100 μg) of the investigated samples, subsequently dissolved in 1 mL of DMSO and heated according to the "sample preparation" procedure described above. Five 100-μL aliquots of the clear supernatants were spiked with 100 μL of standard multicomponent mixtures of the analytes (IND, MBI, DBI), in order to obtain final added concentrations of 0.1, 0.2, 0.3, 0.5, and 1.5 μg/mL. For the preparation of the blank sample, to a 100-μL aliquot of the pooled sample, 100 μL of DMSO was added. The blank and the fortified aliquots were measured in triplicate. Calibration curves were constructed by plotting the means of peak areas against added concentrations of the analytes.

Linearity and accuracy of HPLC method Linearity was evaluated by calibration using spiked samples in order to compensate for matrix effects (Table 6). Calibration curves were constructed by plotting the coloring compounds' peak areas against concentrations. Linear least squares regression was applied for the calculation of slopes (*b*), intercepts (*a*), and correlation coefficients (*r*). The limits of detection (LOD) and limits of quantification (LOQ) were calculated as $3{\cdot}3S_a/b$ and $10S_a/b$, respectively, where S_a is the standard deviation of the *y*-intercept and *b* the slope of the regression equations.

According to the results of Table 6, linearity was obeyed in the range 0.1–1.5 μg/mL with LOD and LOQ ranging between 0.03 to 0.04 μg/mL and 0.09 to 0.12 respectively, for the three detected compounds. The linearity, expressed as the correlation coefficient of the calibration curves, was higher than 0.999 in all cases. LODs and LOQs reported in Table 6 are in excellent agreement with a previously published report [33] where shellfish purple samples were quantified using the same HPLC-DAD method applied herein.

For the assessment of the accuracy of the method, the recovery of the analytes from spiked samples was calculated by means of the standard additions method calibration. The accuracy was determined with the percentage recovery using the spiked pooled samples, at three concentration levels of the analytes. The accuracy was calculated as the percentage of the [(mean measured − initial)/added] concentrations of the analytes. The experimental findings are summarized in Table 7. The recoveries were acceptable in all cases, ranging between 92 and 100%.

Dyeing procedures

Equimolar MBI/DBI dyeing A 250-mL three-neck round-bottom flask fitted with a water condenser was swept with N_2 and kept under a positive pressure of N_2 during the dyeing. Distilled water (100 mL) and THF (15 mL) were introduced, followed by NaOH (0.50 g, 13 mmol). The solution was brought to reflux (75–80 °C) with magnetic stirring, and $Na_2S_2O_4$ (0.50 g, 85% purity, 2.4 mmol) was added, followed immediately by DBI (10.5 mg, 0.0250 mmol) and MBI (8.5 mg, 0.025 mmol), both of which had been finely ground in an agate mortar. At this point, the window shades were drawn, and the flask was wrapped in aluminum foil. The fluorescent room lights were kept on. A yellow-green solution, containing a small amount of dark particles, was obtained after 15 min at reflux. Addition of NH_4Cl (2.0 g, 37 mmol) gave the protonated leuco form of the dye. Wool fabric (1.4 g), which had been soaked in dilute soap solution, was introduced. The stirring rate was reduced, and the solution was heated at reflux for 15 min. From this point until color had fully developed, the fabric was handled with gloves. The fabric was removed from the flask and exposed to air, underneath a shield of aluminum foil. After at least 30 min of air exposure, the fabric was rinsed in aqueous 1% aqueous HOAc solution and allowed to dry. Second and third passes were made in the same manner with additional fabric.

The equimolar IND/MBI dyeing run was done in the same fashion. The other dyeing runs using only one of the three indigoids were done by the same procedure, using 0.050 mmol of the dye.

Table 6 Linearity data, limits of detection (LOD), and limits of quantitation (LOQ) of the HPLC method

cpd	Regression equation $y = (a \pm SD_a) + (b \pm SD_b)x$	Correlation coefficient (*r*)	LOD(μg/mL)	LOQ (μg/mL)
IND	$y = (0.2739 \pm 0.0256) + (2.5635 \pm 0.0386)x$	0.9995	0.03	0.09
MBI	$y = (0.5881 \pm 0.0276) + (2.5092 \pm 0.0416)x$	0.9995	0.04	0.12
DBI	$y = (0.1722 \pm 0.0149) + (1.6486 \pm 0.0224)x$	0.9996	0.03	0.09

y and *x* values were measured in spiked samples

y peak area ($n = 3$), *x* concentration (μg/mL), *a* intercept, *b* slope, SD_a and SD_b standard deviations of intercept and slope, respectively

Table 7 Precision and accuracy of assay for the determination of purple components in spiked samples and standard deviations (SD)

cpd	Initial conc. (μg/mL)	Added conc. (μg/mL)	Mean measured conc. (SD)[a] (μg/mL)	Recovery[b] (%)	RSD (%)
IND	0.11	0.2	0.31 (0.018)	100	5.8
		0.5	0.58 (0.03)	94	5.2
		1.5	1.61 (0.017)	100	1.1
MBI	0.23	0.2	0.43 (0.004)	100	0.9
		0.5	0.69 (0.06)	92	8.7
		1.5	1.73 (0.027)	100	1.6
DBI	0.1	0.2	0.30 (0.01)	100	3.3
		0.5	0.59 (0.038)	98	6.4
		1.5	1.60 (0.01)	100	0.6

[a] Means of values calculated from the regression line equations ($n = 3$) on the same day

[b] Recovery(%) = [(mean measured conc. − initial conc.)/added conc.] × 100

Isolation of MBI from oxidation of the dye vat containing neutral leuco-MBI A dye vat of leuco-MBI monoanion was prepared as above, using 34 mg (0.10 mmol) of MBI and doubling the amounts of the other reagents and solvents. After addition of NH_4Cl (4.0 g, 74 mmol), the vat was heated at reflux while protecting from light for 1 h, to simulate a three-pass dyeing run. The vat was opened to the air, while protecting from light. After an overnight period of stirring completely in the dark, the blue fine precipitate which had formed was isolated by suction filtration, washing with distilled water. Isolated was 13 mg of fine blue powder.

Partial debromination of neutral leuco-MBI with fluorescent light A dye vat of leuco-MBI monoanion was prepared as above, using 34 mg (0.10 mmol) of MBI and doubling the amounts of the other reagents and solvents. After the NH_4Cl was added, the vat was uncovered and exposed to ambient fluorescent light for 1 h, keeping the vat at reflux. The vat was opened to the air, while protecting from light. After an overnight period of stirring completely in the dark, the blue fine precipitate which had formed was isolated by suction filtration, washing with distilled water. Isolated was 15 mg of fine blue powder.

Partial debromination of leuco-MBI dianion with fluorescent light A dye vat of leuco-MBI dianion was prepared as above, using 34 mg (0.10 mmol) of MBI and doubling the amounts of the other reagents and solvents. After the 15-min reflux period and before the NH_4Cl was added, the vat was uncovered and exposed to ambient fluorescent light for 1 h, keeping the vat at reflux. NH_4Cl (4.0 g, 74 mmol) was added, and the vat was protected from light again. After another hour at reflux to simulate the time of a three-pass dyeing, the vat was allowed to cool to ambient temperature and opened to the air, while still protecting from light. After an overnight period of stirring completely in the dark, the blue fine precipitate which had formed was isolated by suction filtration, washing with distilled water. Isolated was 14 mg of fine blue powder.

Partial debromination of leuco-MBI dianion with incandescent light A dye vat of leuco-MBI dianion was prepared as above. After the 15-min reflux period and before the NH_4Cl was added, the vat was uncovered and exposed to a 50-W small desk lamp (approx. 6-in separation between lamp and vat) for 1 h, with all other lights off, keeping the vat at reflux. The vat immediately turned a deep red color upon exposure to the light. NH_4Cl (4.0 g, 74 mmol) was added, causing a color change to dark green, and then the vat was protected from light again. After another hour at reflux to simulate the time of a three-pass dyeing run, the vat was allowed to cool to ambient temperature and opened to the air, while still protecting from light. After an overnight period of stirring completely in the dark, the fine blue precipitate which had formed was isolated by suction filtration, washing with distilled water. Isolated was 15 mg of fine blue powder.

Acknowledgments Ms. Jo Kirby, formerly of The National Gallery, London, and Mr. Greg Rohaus of Konica Minolta are thanked for helpful discussions. Dr. Frank Jacob of Queensborough Community College is thanked for translating articles in Japanese. Akn Fabrics Inc. is thanked for supplying the wool fabric.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Lavinda O, Mironova I, Karimi S, Pozzi F, Samson J, Ajiki H, Massa L, Ramig K (2013) Singular thermochromic effects in dyeings with indigo, 6-bromoindigo, and 6,6'-dibromoindigo. Dyes Pigments 96:581–589

2. Ramig K, Lavinda O, Szalda DJ, Mironova I, Karimi S, Pozzi F, Shah N, Samson J, Ajiki H, Massa L, Mantzouris D, Karapanagiotis I, Cooksey C (2015) The nature of thermochromic effects in dyeings with indigo, 6-bromoindigo, and 6,6′-dibromoindigo, components of Tyrian purple. Dyes Pigments 117:37–48
3. Baig GA (2011) Indigo dyeing of polyester (PET) – pH effects. J Text I 102:87–92
4. Driessen LA (1944) Über eine charakteristische Reaktion des antiken Purpurs auf der Faser. Melliand Textilberichte 25:66
5. Van Alphen J (1944) Remarks on the action of light on several substances, most of them containing halogen, in particular several indigo dyes, in a reducing medium. Recl Trav Chim Pay-B 63:95–96
6. Pfister R (1937) Nouveaux textiles de Palmyre. Les Editions d'Art et d'Histoire, Paris, p. 12
7. Hofmann-de Keijzer R, van Bommel MR (2008) Dyestuff analysis of two textile fragments from late antiquity. Dyes in History and Archaeology 21:17–25
8. Bruni S, Guglielmi V, Pozzi F (2010) Surface-enhanced Raman spectroscopy (SERS) on silver colloids for the identification of ancient textile dyes: Tyrian purple and madder. J Raman Spectrosc 41:175–180
9. McGovern PE, Michel RH (1985) Royal purple dye: tracing the chemical origins of the industry. Anal Chem 57:1514A–1522A
10. Cooksey CJ, Sinclair RS (2005) Colour variations in Tyrian purple dyeing. Dyes in History and Archaeology 20:127–135
11. Voss G, Schramm W (2000) Selectively C-deuterated indigotins. Helv Chim Acta 83:2884–2892
12. Koren ZC (2008) A new HPLC-PDA method for the analysis of Tyrian purple components. Dyes in History and Archaeology 21: 26–35
13. Sawada T, Ishii H, Ichikawa H, Watanabe K, Aoki J, Ueda T (2011) Development of vat-dyeing method using 6,6'-dibromoindigo and prevention of photodebromination. Nippon Silk Gakkaishi (Journal of Silk Science and Technology of Japan) 19:15–21
14. Friedländer P (1909) Über den Farbstoff des antiken Purpurs aus *Murex brandaris*. Ber Dtsch Chem Ges 42:765–770
15. Friedländer P (1922) Über die Farbstoffe aus *Purpura aperta* und *Purpura lapillus*. Ber Dtsch Chem Ges 55:1655–1658
16. Wouters J (1992) A new method for the analysis of blue and purple dyes in textiles. Dyes in History and Archaeology 10:17–21
17. Cooksey CJ (2001) Tyrian purple: 6,6'-dibromoindigo and related compounds. Molecules 6:736–769
18. Cardon D (2007) Natural dyes: sources, tradition, technology and science. Archetype Publications, pp:551–606
19. Terada T (2008) Sea snail purple in contemporary Japanese embroidery. Textile Society of America 11th Biennial Symposium: Textiles as Cultural Expressions, Honolulu, Hawai'i, paper 138
20. Surowiec I, Nowik W, Moritz T (2012) Mass spectrometric identification of new minor indigoids in shellfish purple dye from *Hexaplex trunculus*. Dyes Pigments 94:363–369
21. Cooksey C (2013) Tyrian purple: the first four thousand years. Sci Prog 96:171–186
22. Karapanagiotis I, Mantzouris D, Cooksey C, Mubarak MS, Tsiamyrtzis P (2013) An improved HPLC method coupled to PCA for the identification of Tyrian purple in archaeological and historical samples. Microchem J 110:70–80
23. Mantzouris D, Karapanagiotis I (2014) Identification of indirubin and monobromoindirubins in *Murex brandaris*. Dyes Pigments 104:194–196
24. Clark RJH, Cooksey CJ (1999) Monobromoindigos: a new general synthesis, the characterization of all four isomers and an investigation into the purple colour of 6,6'-dibromoindigo. New J Chem 23: 323–328
25. Koren ZC (2005) The first optimal all-murex all-natural purple dyeing in the eastern Mediterranean in a millennium and a half. Dyes in History and Archaeology 20:136–149
26. Koren ZC (2006) HPLC-PDA analysis of brominated indirubinoid, indigoid, and isatinoid dyes. In: Meijer L, Guyard N, Skaltsounis L, Eisenbrand G (eds) Indirubin, the red shade of indigo. Life in Progress Editions, Roscoff, pp. 45–53
27. McGovern PE, Michel RH (1990) Royal Purple dye: the chemical reconstruction of the ancient Mediterranean industry. Accounts Chem Res 23:152–158
28. Padden AN, Dillon VM, Edmonds J, Collins MD, Alvarez N, John P (1999) An indigo-reducing moderate thermophile from a woad vat, *Clostridium isatidis* sp. nov. Int J Syst Evol Micr 49:1025–1031
29. Yumoto I, Hirota K, Nodasaka Y, Tokiwa Y, Nakajima K (2008) *Alkalibacterium indicireducens* sp. nov., an obligate alkaliphile that reduces indigo dye. Int J Syst Evol Micr 58:901–905
30. Edmonds J (2000) Tyrian or imperial purple dye, Historic dye series no. 7, ISBN 0 9534133 65, self-published
31. Kanold IB (2005) The purple fermentation vat: dyeing or painting parchment with *Murex trunculus*. Dyes in History and Archaeology 20:150–154
32. Koren ZC (2012) Chromatographic and colorimetric characterizations of brominated indigoid dyeings. Dyes Pigments 95:491–501
33. Vasileiadou A, Karapanagiotis I, Zotou A (2016) Determination of Tyrian purple by high performance liquid chromatography with diode array detection. J Chromatogr A 1448:67–72
34. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone V, Petersson GA, Nakatsuji H, Li X, Caricato M, Marenich AV, Bloino J, Janesko BG, Gomperts R, Mennucci B, Hratchian HP, Ortiz JV, Izmaylov AF, Sonnenberg JL, Williams-Young D, Ding F, Lipparini F, Egidi F, Goings J, Peng B, Petrone A, Henderson T, Ranasinghe D, Zakrzewski VG, Gao J, Rega N, Zheng G, Liang W, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Vreven T, Throssell K, Montgomery Jr JA, Peralta JE, Ogliaro F, Bearpark MJ, Heyd JJ, Brothers EN, Kudin KN, Staroverov VN, Keith TA, Kobayashi R, Normand J, Raghavachari K, Rendell AP, Burant JC, Iyengar SS, Tomasi J, Cossi M, Millam JM, Klene M, Adamo C, Cammi R, Ochterski JW, Martin RL, Morokuma K, Farkas O, Foresman JB, Fox DJ (2016) Gaussian 09, Revision E.01. Gaussian, Inc., Wallingford CT, USA
35. Tanoue Y, Terada A, Sakata K, Hashimoto M, Morishita SI, Hamada M, Kai N, Nagai T (2001) A facile synthesis of Tyrian purple based on a biosynthetic pathway. Fisheries Sci 67:726–729
36. Cooksey CJ (1995) Making Tyrian purple. Dyes in History and Archaeology 13:7–13
37. Schanda J (2007) CIE colorimetry. In: Schanda J (ed) Colorimetry understanding the CIE system. Wiley, Hoboken, pp. 58–64
38. Karapanagiotis I, de Villemereuil V, Magiatis P, Polychronopoulos P, Vougogiannopoulou K, Skaltsounis A-L (2006) Identification of the coloring constituents of four natural indigoid dyes. J Liq Chromatogr Rel Tech 29:1491–1502