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Chapter 5

HPLC-PDA analysis of brominated indirubinoid, indigoid, and isatinoid dyes

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A reverse-phase HPLC-PDA method has been formulated for the identification of isatinoid, indigoid and indirubinoid dyes, and applied to their detection in Tyrian Purple pigments from *Murex trunculus* sea snails. These pigments are spontaneously produced from indoxyl precursors in excised hypobranchial glands of various mollusca. The linear gradient elution method consists of an aqueous acidic methanolic system and separates ten brominated and unbrominated indole and biindole dione colorants in under thirty minutes. This is the first time that all of these components were analyzed by a single elution method, which would allow for the multicomponental chemical fingerprinting of modern and archaeological molluskan pigments.

Keywords*:* HPLC, indigo, indirubin, isatin, purple

INTRODUCTION

The Purples and Violets of Phoenicia and of the Bible, the most royal and sacred of all ancient textile dyes, were produced from certain species of Levantine mollusks. The colors fashioned from these sea snails were known as Tyrian or Royal Purple and Biblical Blue. These Mediterranean *Muricidae* marine creatures, whose common familial approbation is *Murex,* were in use for the industrial biochemical production of the purple pigment from as early as perhaps 4,000 years ago (Stieglitz, 1994; Reese, 2000; Koren, 2005). Along the Mediterranean coasts of what are now Israel and Lebanon, only three *Muricidae* species have been associated with purple dyeing in antiquity (Spanier and Karmon, 1987). These species include *Murex trunculus* (also known as *Hexaplex trunculus, Phyllonotus trunculus,* and as *Trunculariopsis trunculus*), *Murex brandaris* (also *Bolinus brandaris*), and *Purpura haemastoma* (also *Thais haemastoma* and *Stramonita haemastoma*).

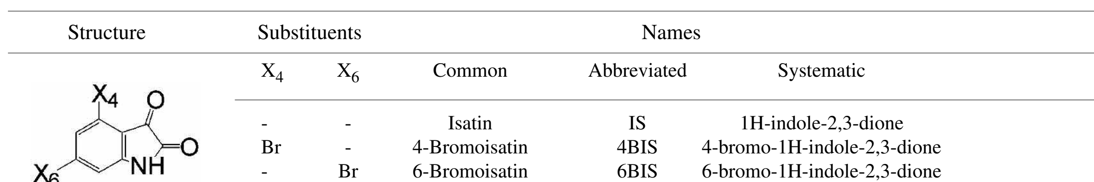
The chemistry of the dye and its production from the precursors – or chromogens – contained in the hypobranchial glandular fluid of the living animal, though not completely understood, has been studied (Fouquet, 1970; Fouquet and Bielig, 1971; Baker, 1974) and reviewed (Cooksey, 2001a; Cardon, 2003). These colorless precursors to the final pigment consist of substituted and unsubstituted indoxyl sulfates as well as their brominated and unbrominated complements (Fouquet, 1970; Fouquet and Bielig, 1971; Baker, 1974). The enzyme purpurase required for the hydrolysis of these indoxyls is also present in that gland, though not in the same compartment as the precursors (Naegel and Cooksey, 2002). Upon puncturing the gland, or when the animal expires, the enzyme comes into contact with these precursors and hydrolyzes them. These hydrolyzates then undergo a series of spontaneous oxidative and photochemical reactions to form the final purple pigment, which was analyzed and found to consist of, in part, indigoid and indirubinoid dye components (Koren, 1995).

The analytical methods that have been applied to the study of some of the components of that pigment and of related substances have included 3D fluorescence spectrometry (Shimoyama and Noda, 1994), NMR (nuclear magnetic resonance) spectrometry (Clark and Cooksey, 1997; Clark and Cooksey, 1999;

Voss, 2000; Voss and Schramm, 2000; Cooksey, 2001a; Cooksey, 2001b; Cooksey and Withnall, 2001), MS (mass spectrometry) (McGovern and Michel, 1990; McGovern *et al.*, 1990; Clark and Cooksey, 1997; Voss and Schramm, 2000; Benkendorff *et al.*, 2001; Cooksey, 2001a; Cooksey, 2001b; Cooksey and Withnall, 2001; Andreotti *et al.*, 2004; Papanastasiou, 2005), Raman spectrometry (Clark and Cooksey, 1999; Cooksey, 2001a; Cooksey, 2001b), IR (infrared) spectrometry (McGovern and Michel, 1985; Clark and Cooksey, 1997; Clark and Cooksey, 1999; Cooksey, 2001a; Cooksey, 2001b), PIXE (particle induced x-ray emission) and ESCA (electron spectroscopy for chemical analysis) (McGovern and Michel, 1984; McGovern and Michel, 1985), Visible spectrophotometry (Saltzman, 1978; Saltzman, 1986; Daniels, 1989; Saltzman, 1992; Koren, 1993; Cooksey, 2001b), TLC (thin-layer chromatography) (Cooksey and Withnall, 2001; Hiyoshi and Fujise, 1992), GC (gas chromatography) (Benkendorff *et al.*, 2001), and HPLC (high-performance liquid chromatography) (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1994a; Koren, 1995; Clark and Cooksey, 1997; Cooksey, 2001a; Cooksey and Withnall, 2001; Withnall *et al.*, 2003). As far as multicomponent analyses of the purple pigment are considered, it is clear that a chromatographic analysis, which involves a "separation" of information, is considerably more effective than a spectrometric method alone, which involves an "overlap" of information. It has been found that for the analysis of UV- and Visible-absorbing dyes, the HPLC method is undoubtedly the most powerful "workhorse" for such dye analyses.

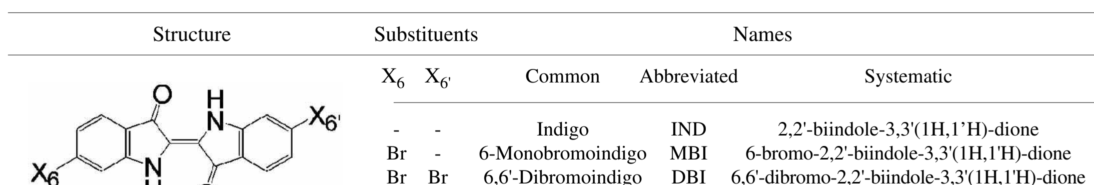
Previous instrumental methods of analysis of that marine colorant have primarily focused on a few of the components that constitute the purple dyestuff, referred to as C.I. Natural Vat Dye in the Colour Index (SDC, 1971a). Foremost of these components is the main – if not always the major – dye, namely 6,6'-dibromoindigo, often abbreviated as DBI (Table 1), with the Chemical Constitution Number of C.I. 75800 (SDC, 1971a). This dye is the ultimate "trademark" or "biochem-marker" of a true-purple dyeing, one that is

(a) Isatinoids



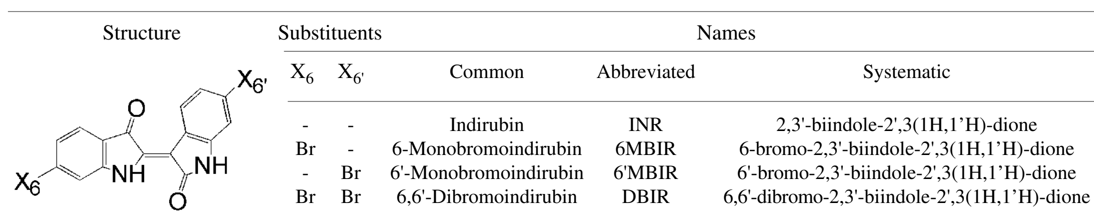
Structure	Substituents		Names		
	X_4	X_6	Common	Abbreviated	Systematic
	-	-	Isatin	IS	1H-indole-2,3-dione
	Br	-	4-Bromoisatin	4BIS	4-bromo-1H-indole-2,3-dione
	-	Br	6-Bromoisatin	6BIS	6-bromo-1H-indole-2,3-dione

(b) Indigoids



Structure	Substituents		Names		
	X_6	$X_{6'}$	Common	Abbreviated	Systematic
	-	-	Indigo	IND	2,2'-biindole-3,3'(1H,1'H)-dione
	Br	-	6-Monobromoindigo	MBI	6-bromo-2,2'-biindole-3,3'(1H,1'H)-dione
	Br	Br	6,6'-Dibromoindigo	DBI	6,6'-dibromo-2,2'-biindole-3,3'(1H,1'H)-dione

(c) Indirubinoids



Structure	Substituents		Names		
	X_6	$X_{6'}$	Common	Abbreviated	Systematic
	-	-	Indirubin	INR	2,3'-biindole-2',3(1H,1'H)-dione
	Br	-	6-Monobromoindirubin	6MBIR	6-bromo-2,3'-biindole-2',3(1H,1'H)-dione
	-	Br	6'-Monobromoindirubin	6'MBIR	6'-bromo-2,3'-biindole-2',3(1H,1'H)-dione
	Br	Br	6,6'-Dibromoindirubin	DBIR	6,6'-dibromo-2,3'-biindole-2',3(1H,1'H)-dione

Table 1. Molecular structures and common, abbreviated, and systematic names of the dyes: (a) isatinoids, (b) indigoids, and (c) indirubinoids

obtained only from purple-producing sea snails and not from a combination of vegetal dyestuffs, as only a marine source produces this dye. Two other components that have been investigated to which Colour Index constitution numbers have been given are indigo (also known as indigotin) and its isomer indirubin. Though natural indigo, C.I. 75780 (SDC, 1971a), can be present in a molluskan pigment, it was typically produced as a dye from a flora source, C.I. Natural Blue 1 (SDC, 1971b), such as from the leaves of *Indigofera tinctoria*, indigo-producing plants, and from woad, *Isatis tinctoria* L. Indirubin (CI 75790) - the red shade of indigo - is often naturally found together with the indigo dye in these vegetal dyestuffs (SDC, 1971a). Synthetically produced indigo (C.I. Vat Blue 1) is given the designation C.I. 73000 (SDC, 1971c).

DBI and some of the other components, such as IND, INR, MBI, and DBIR (Table 1), have been included in HPLC analyses (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1994a; Koren, 1995), and HPLC results have also been reported for a few of the components without publication of the chromatographic method or conditions (Clark and Cooksey, 1997; Cooksey, 2001a). Another study (Cooksey and Withnall, 2001), which used a method that was previously applied to the analysis of indigoidal dyes (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1994a; Koren, 1995), was also applied to study some of these components. However, recent analyses of the purple pigment have clearly indicated that other colorants, such as isatin, are also present in purple *Murex trunculus* pigments (Koren, 2001). Therefore a single HPLC elution method is needed to detect all the important colorants that constitute purple molluskan pigments from various zoological and geographical sources.

This article presents a new HPLC method with photo-diode array (PDA) detection for the identification of ten dyes consisting of brominated and unbrominated isatinoids, indigoids, and indirubinoids, which may be present in modern and archaeological purple pigments produced from *Murex* and related species. This analytical scheme would thus be able to qualitatively and quantitatively "chemically fingerprint" modern pigments produced from various purple-producing marine species collected at different geographical locations around the world. In addition, this method would aid in determining the zoological and geographical provenance of the sea snails used in producing the ancient royal and sacred pigments found on archaeological potsherds from ancient dyeing vats and on historic textile dyeings.

EXPERIMENTAL

The standard dyes, dye extraction method, and chromatographic system used in this study are described below.

Reagents and dyes

The extracting solvent, dimethyl sulfoxide (DMSO), was spectrophotometric grade and supplied by Mallinckrodt (Paris, Kentucky, USA). The HPLC-grade eluents consisted of methanol and water, both supplied by J. T. Baker (Deventer, Holland), and 85% ortho-phosphoric acid (Fluka, Buchs, Switzerland). The concentrated acid was diluted with HPLC-grade water to provide a 5% w/v solution with a pH of 1.50 at 25°C.

The standard dyes were synthesized and kindly provided by Dr. Chris Cooksey (University College London, UK), except for indigo, which was obtained as "indigo rein" from BASF (Germany).

HPLC

The ambient-temperature reverse-phase all-Waters (Milford, MA, USA) chromatographic system consisted of a 600E Controller pump and a 996 PDA detector, controlled by Millennium-32 software. The stationary phase consisted of a 3.0 x 150 mm C_{18} Symmetry column (Part No. WAT054200) with 5 µm and 100 Å particle and pore diameters, respectively. The linear gradient elution method, which consisted of the ternary mobile phase system of water, methanol, and 5% w/v H_3PO_4 (pH = 1.50 at 25ºC), with a flow rate of 0.8 mL/min, is shown in Table 2. A 20-µL sample loop was used.

Extraction and sample preparation

Each dye was dissolved with dimethyl sulfoxide (DMSO) and processed under subdued lighting conditions as brominated indigoids can undergo photo-debromination. The sample was heated for 5 minutes at 100ºC, allowed to cool to room temperature for 15 min, and then filtered in a 0.45-µm micro-spin polypropylene centrifuge tube with nylon filter (Alltech, Part No. 2490).

For the quantification of this method for indigo, standard concentrations between 0.1 and 100 µg/mL were prepared and immediately analyzed.

Time (min)	Methanol (%)	Water (%)	5% H_3PO_4, pH=1.50 (%)
0 → 3	30 → 75	60 → 15	10
3 → 20	75	15	10
20 → 21	75 → 100	15 → 0	10 → 0
21 → 30	100	0	0

Table 2. Linear gradient elution method for the HPLC analysis of isatinoid, indigoid and indirubinoid dyes (flow rate of 0.8 mL/min).

RESULTS AND DISCUSSION

Chromatographic separation and retention properties

The mobile phase utilized in this study has been widely used for the liquid chromatographic analyses of natural dyes (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1994a; Koren, 1995; Cooksey and Withnall, 2001).

A typical chromatogram of a DMSO-solution containing all of the ten standard dyes is shown in Figure 1. The average (unadjusted) relative retention times for these components, RRT = $t_R/t_{R,indigo}$, together with their respective standard deviations (SD) and % relative standard deviations (RSD) are presented in Table 3.

The ultraviolet-visible spectra of these dyes, as produced by the photodiode array detector of the chromatograph, are presented in Figures 2–4.

The chromatographic method developed in this work shows clear group separations based on molecular structure (polarity) and size. The three relatively small indole diones – the isatinoids - are the first to elute between about 3-7 minutes, with the small polar isatin eluting first, but retained in the column for about 3 minutes, far away from the solvent front. The less polar (and heavier) isomeric bromoisatins are further upfield, and clearly separated from each other. The bromoisatin substituted in the 4-position is not a component of a molluskan purple pigment, but has been used in this study to show that the developed method shows sufficient resolution to separate it from the isomeric component substituted in the 6-position. The latter is a more likely candidate to be found in the final pigment as it is an intermediate in the photo-oxidative pathway leading to the purple product (Fouquet, 1970; Fouquet and Bielig, 1971). Previous indigoidal studies did not address the chromatographibility of the isatinoids

The second eluting group of dyes, between about 10–13 minutes, consists of the simple biindole diones (indigo and indirubin) and the singly brominated indigo, respectively. Of this group, indirubin, the asymmetric isomer of indigo, is the middle eluting component.

The complex biindole diones consisting of singly-substituted indirubinoids and doubly substituted indigo, are the third group to elute, between about 18.5–20.5 minutes. The dibromoindigo component is sandwiched between the two monobromoindirubins, with the 6’-substituted isomer eluting last. The use of an acidic elution system was found to maximize the

Dye	Run-to-run (*n* = 7)			Run-to-run (*n* = 6)			Week-to-week (*n* = 13)		
	AVG	SD	%RSD	AVG	SD	%RSD	AVG	SD	%RSD
IS	0.3022	0.0017	0.57	0.3049	0.0008	0.25	0.3034	0.0019	0.63
4BIS	0.6629	0.0011	0.17	0.6631	0.0003	0.04	0.6630	0.0008	0.12
6BIS	0.7187	0.0004	0.06	0.7176	0.0006	0.09	0.7182	0.0008	0.11
IND[a]	1	0	0	1	0	0	1	0	0
INR	1.1901	0.0017	0.15	1.1951	0.0019	0.16	1.1924	0.0031	0.26
MBI	1.2925	0.0024	0.19	1.3000	0.0030	0.23	1.2959	0.0047	0.36
6MBIR	1.7894	0.0102	0.57	1.8209	0.0093	0.51	1.8039	0.0188	1.04
DBI	1.8962	0.0097	0.51	1.9312	0.0124	0.64	1.9124	0.0210	1.10
6'MBIR	1.9640	0.0119	0.61	2.0065	0.0153	0.76	1.9836	0.0256	1.29
DBIR	2.6635	0.0053	0.20	2.6925	0.0031	0.11	2.6769	0.0156	0.58

[a]Average respective t_R values (in minutes) for IND were: 10.338, 10.200, 10.274.

Table 3. Average relative retention times (RRT), standard deviations (SD), and % relative standard deviations (RSD) of the eluted dye components.

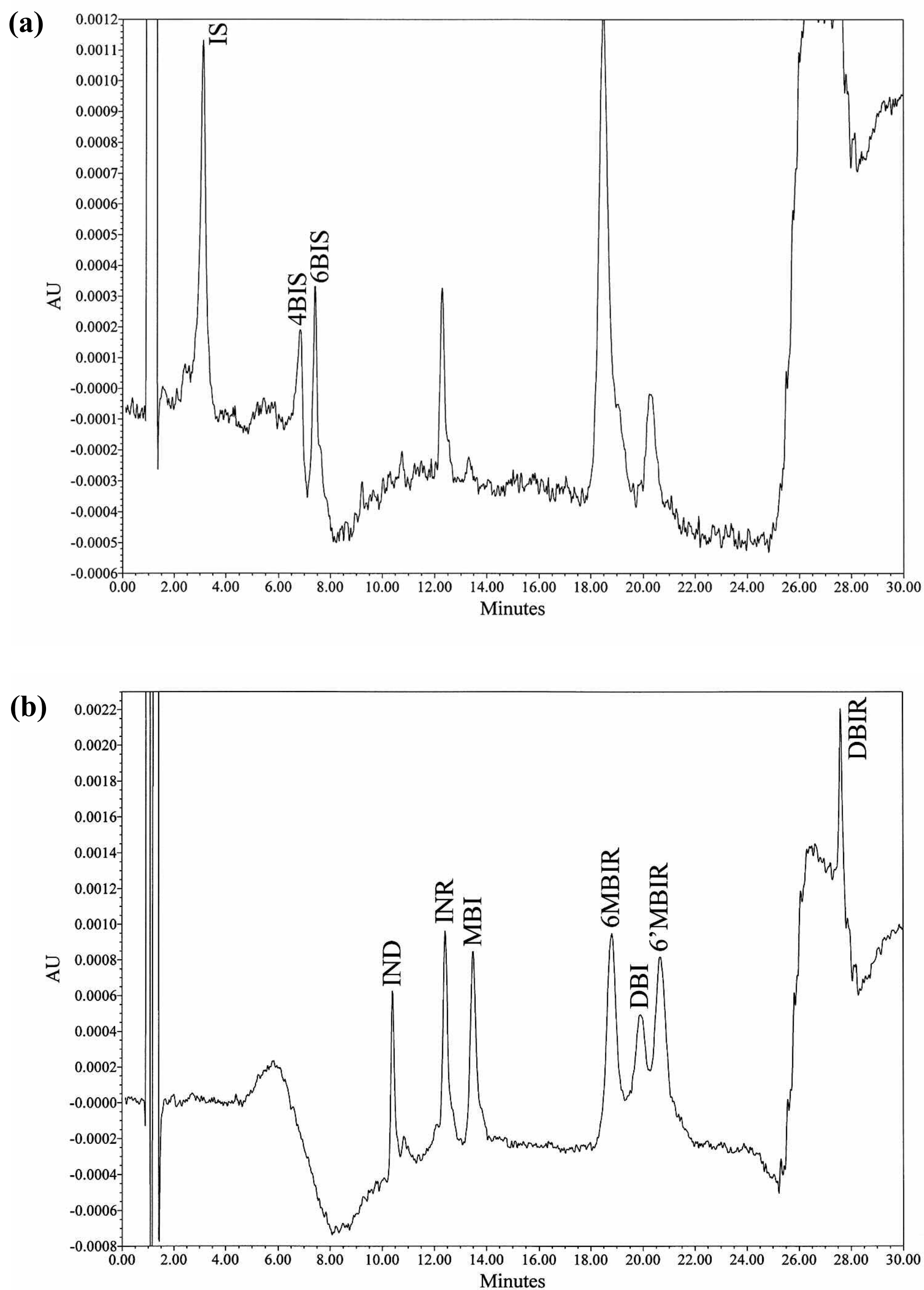


Figure 1. Chromatograms of a DMSO extract of the standard dyes: (a) isatin (IS), 4-bromoisatin (4BIS), and 6-bromoisatin (6BIS), visualized at 400 nm; (b) indigo (IND), indirubin (INR), 6-monobromoindigo (MBI), 6-monobromoindirubin (6MBIR), 6,6'-dibromoindigo (DBI), 6'-monobromoindirubin (6'MBIR), and 6,6'-dibromoindirubin (DBIR), visualized at 594 nm.

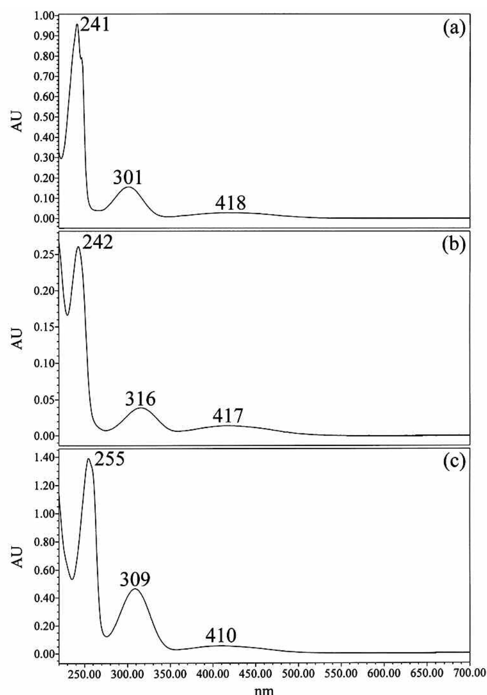


Figure 2. UV/Vis spectra of the isatinoids: (a) isatin, (b) 4-bromoisatin, and (c) 6-bromoisatin.

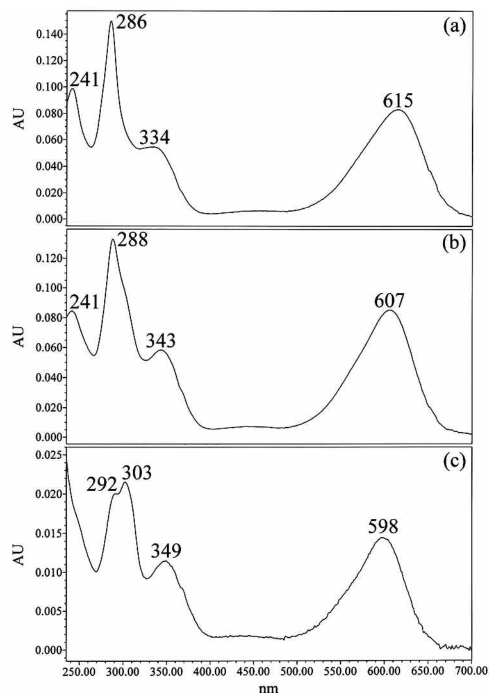


Figure 3. UV/Vis spectra of the indigoids: (a) indigo, (b) 6-monobromoindigo, and (c) 6,6'-dibromoindigo.

separation of the latter two nearly chromatographically equivalent dyes, DBI and 6'MBIR, to an average of about 0.75 minute. Though these latter two components elute close to each other, nevertheless, both of their respective peaks are still resolvable (Fig. 1). In addition, as their molecular weights are substantially different, additional mass spectrometric detection of these dyes would be helpful for future work. The method developed in this study produced a much better separation of these three "stubborn" dyes, 6MBIR, DBI, and 6'MBIR, which were separated by a total of about 2 minutes, compared with only 1 minute as previously reported (Clark and Cooksey, 1997).

The last eluting dye, the bulky doubly brominated indirubin (DBIR), is far "upfield" at about 27 minutes. In order to hasten its elution, the acidity of the mobile phase was reduced from 10% to 0% phosphoric acid (Table 2). In addition, to further shorten the retention time of DBIR, the mobile phase flow rate could also be increased from the time of elution of the previously eluting component, 6'MBIR.

The relative retention time, RRT, has been widely used as a characteristic retention quantifier in liquid chromatographic studies of natural dyes. In the current work, these RRT's have resulted in the highest degree of reproducibility – lowest %RSD values (Table 3) - of all the most common retention parameters. Two daily sets of analyses, a week apart, were performed and yielded same day run-to-run RRT values with %RSD values of 0.06–0.61% and 0.04–0.76% and week-to-week values of 0.11–1.29%.

Concentration quantification for indigo

Linear quantification of the concentrations was performed for indigo, the most common of these dyes, as the purities of the other components were not ascertained. All the dissolved indigoidal components are thermally and photochemically labile; therefore the analyses were immediately performed on freshly prepared solutions. The indigo dissolution procedure described in the sample preparation steps above simulates the process associated with actual analyses of dyes extracted from archaeological textiles (Koren, 1994b; Koren, 1999). Hence, the concentrations analyzed for linear behavior are those typically encountered in HPLC analyses of ancient textile dyeings.

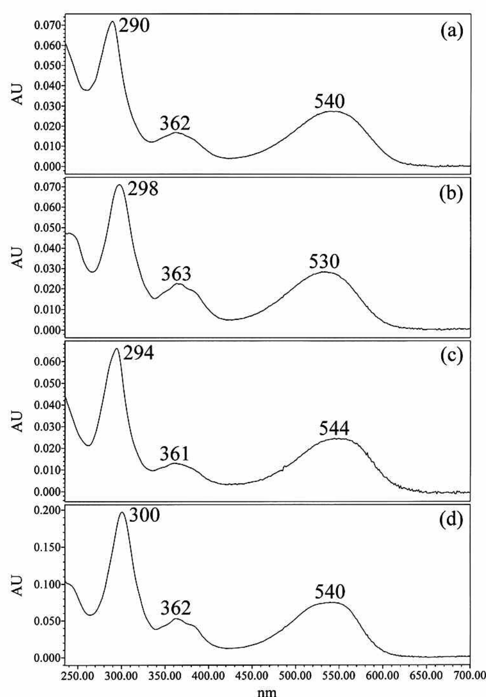


Figure 4. UV/Vis spectra of the indirubinoids: (a) indirubin, (b) 6-monobromoindirubin, (c) 6'-monobromoindirubin, and (d) 6,6'-dibromoindirubin.

Usually the colorant from about a 1-cm long woolen yarn (up to 1 mg) is extracted in at least 100 µL of solvent; for an utmost dye mass of 1% of the textile substrate, a maximum dye solution of about 100 µg/mL (400 µM in the case of indigo) is obtained.

The reproducibility of this method was tested by replicate injections of standard indigo solutions (Table 4). Seven different solutions with concentrations of between 0.1 µg/mL and 100 µg/mL were analyzed and at least three injections of each solution were performed. The run-to-run precisions of the 1-100 µg/mL solutions, as calculated by their respective relative standard deviations (RSD) of the integrated areas, varied from as low as 0.2% to 1.6%, and for the lowest concentration studied, 0.1 µg/mL, the uncertainty in establishing the baseline resulted in an RSD value of 4.5%.

The linear behavior was assessed for a total of 12 indigo concentrations of between 0.1 and 100 µg/mL, and the excellent response yielded an R-square regression value of 0.99991.

Concentrations (µg/mL)	No. of samples, *n*	%RSD
100	3	1.18
10	3	0.72
10	6	1.16
10	4	1.60
1	3	0.64
1	3	0.19
0.1	3	4.46
Regression coefficient R^2	12	0.99991

Table 4. Reproducibility and linearity of the chromatographic method for indigo.

With this method, the limit of detection (LOD) of indigo in a 20 µL injected sample, calculated based on three times the noise level, is about 50 pg.

APPLICATION TO *MUREX TRUNCULUS* SNAILS

Application of this HPLC method to the pigment and dye analyses of *trunculus* species has interestingly shown that “not all *Murex trunculus* species are created equal” (Koren, 2001). Some, but not all, of the species analyzed have shown the clear presence of a trace of isatin in the final purple pigment. No brominated isatin has yet been detected in these species. All of the *trunculus* pigments analyzed to date contain all three indigoids – IND, MBI, and DBI. However, while all of the purple pigments from these snails have shown the presence of indirubin (INR) and the doubly brominated complement (DBIR), some do not contain any of the brominated indirubins, while others consist of 6MBIR, and others also contain 6'MBIR.

HPLC chromatograms of a DMSO extract from wool that was completely naturally vat dyed with this purple pigment according to the procedure given by this author (Koren, 2001; Koren, 2005) show (Fig. 5) the presence of all three indigoids (IND, MBI, and DBI) as well as all four indirubinoids (INR, 6MBIR, 6'MBIR, and DBIR). This is the first time that these four indirubinoids were detected in purple woolen dyeings.

CONCLUSIONS

This work has shown that more indigoidal and indirubinoidal and related components than previously analyzed can be chromatographed by a single method, which yields quantifiable retention parame-

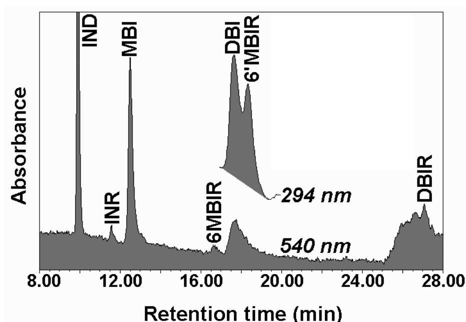


Figure 5. Chromatograms of a DMSO extract from wool naturally purple-dyed with a *Murex trunculus* pigment (wavelengths indicated are for visualization purposes).

ters, precision in concentration, and excellent linearity. This chromatographic method should therefore be used in future investigations involving the full multicomponent chemical characterization of modern and ancient molluskan purple pigments from various geographical regions and zoological species. This approach would thus assist in the geo-biochemical decipherment of the provenance of the purple dyestuff found on shard fragments from archaeological dye vats and on purple-dyed textiles prized by the royals and consecrated by the high priests of the ancients.

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