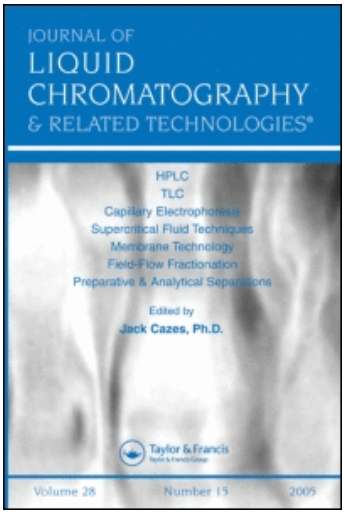

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Identification of the Coloring Constituents of Four Natural Indigoid Dyes

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To cite this Article Karapanagiotis, Ioannis , de Villemereuil, Violaine , Magiatis, Prokopios , Polychronopoulos, Panagiotis , Vougogiannopoulou, Konstantina and Skaltsounis, Alexios-Leandros(2006) 'Identification of the Coloring Constituents of Four Natural Indigoid Dyes', Journal of Liquid Chromatography & Related Technologies, 29: 10, 1491 — 1502

To link to this Article: DOI: 10.1080/10826070600674935

URL: http://dx.doi.org/10.1080/10826070600674935

Journal of Liquid Chromatography & Related Technologies®, 29: 1491–1502, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600674935

Identification of the Coloring Constituents of Four Natural Indigoid Dyes

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Abstract: A high performance liquid chromatography (HPLC) method combined with spectrophotometric UV-Vis detection is developed for the separation and identification of seven indigoid coloring compounds: indigotin, which is commercially available, and indirubin, 6-bromoindigotin, 6′-bromoindirubin, 6-bromoindirubin, 6,6′-dibromoindigotin, and 6,6′-dibromoindirubin, which are synthesized to be used as reference compounds in the HPLC analysis. The chromatographic method is employed for the identification of the blue/purple coloring compounds in samples extracted from four mollusks, which have been used for the production of Tyrian Purple since antiquity: *Hexaplex trunculus*, *Murex brandaris*, *Nucella lapillus*, and *Thais haemastoma*. The composition of the analyzed samples, with respect to the reference materials, is compared and discussed. All seven indigoids are identified in *Hexaplex trunculus* and some of them are identified in the other three purple extracts.

Keywords: HPLC, Dye, Tyrian Purple, Indigo, Art analysis

INTRODUCTION

Tyrian Purple is one of the oldest known organic pigments found in art objects of cultural heritage. Chemical studies have identified Tryian Purple on wall

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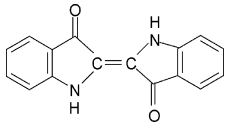
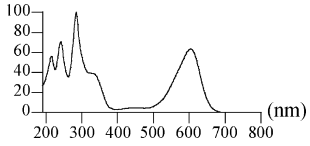
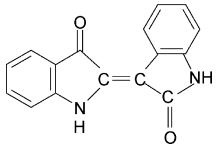
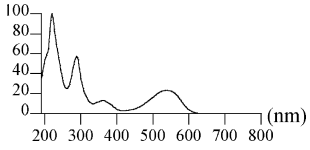
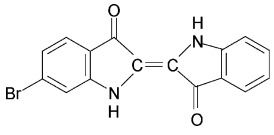
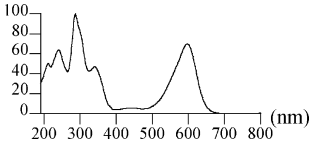
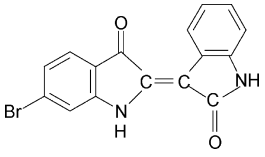
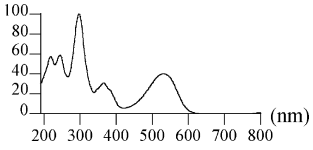
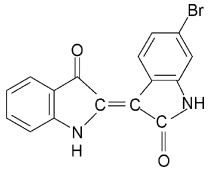
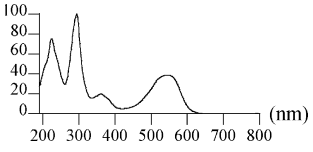
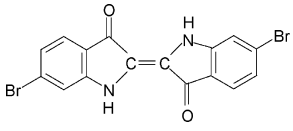
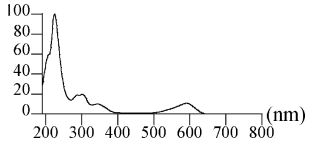
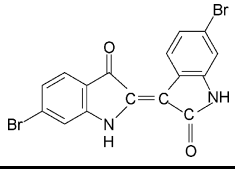
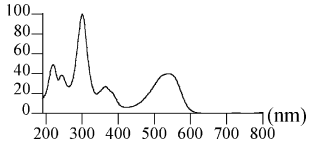
paintings of the 17th century BC in southern Greece.[1,2] Historical sources indicate that Tyrian Purple has been extensively used by the Achaeminian dynasty (559-330 BC, Persia).[1] After Persia's conquest by Alexander the Great (331 BC), the precious dye became well known in the northern part of Greece, and particularly in the area of Macedonia, according to the archaeological treasures found in 4th-century BC royal tombs in Vergina, Greece.[1] The use of the purple dye is reported in Roman and Byzantine sources; finally its massive use in Europe had been brought to a definite end in the 15th century AC.

The purple dye can be obtained from various types of mollusks. The most common, which can be found in coastal areas of the European continent are: *Hexaplex trunculus (Murex trunculus)*, *Murex brandaris (Bolinus brandaris)*, *Thais haemastoma (Stramonita haemastoma)*, and *Nucella lapillus (Purpura lapillus)*.[1] The latter is present on the coasts of the Atlantic while the other three can be also found in the Mediterranean.[1] The main coloring matter of Tyrian Purple is 6,6′-dibromoindigotin and depending on the exact mollusk type other indigoid constituents, summarized in Table 1, can be contained. The identification of these compounds has been done primarily by high performance liquid chromatography (HPLC) with spectrophotometric detection (UV-Vis).[3–8] HPLC-UV-Vis has been employed in an effort to distinguish the various mollusk species on the basis of the relative quantitative composition of indigoid constituents, with relative success.[3–8] In these reports, the following compounds were identified and used as a basis to distinguish the different types of mollusks: indigotin, indirubin, 6-bromoindigotin, 6,6′-dibromoindigotin, and 6,6′-dibromoindirubin. The results are summarized by Cooksey et al.[8] Substantial deviations on the reported composition of the same mollusk, suggest that this cannot be used as a robust criterion to distinguish the various mollusk species. More meticulous methodologies, which will take into consideration several other parameters, such as the geographical origin and the gender of the mollusks, are required for the development of an effective strategy which will be able to distinguish the shells. More elaborate analytical methods comprising mass spectrometric (MS) detection have been rarely employed to investigate, primarily, the precursors of the coloring matters.[2,9–12] In a recent study, two more indigois were isolated from *Hexaplex trunculus*:[13] 6-bromoindirubin and 6′-bromoindirubin, along with the well known 6,6′-dibromoindirubin and indirubin. It is noteworthy, that in the same study, the indirubin derivatives of *Hexaplex trunculus* were found to possess very interesting biological activity as protein kinase inhibitors.

The goal of this study is to compare, for the first time, the four main mollusk species found in the coastal areas of Europe, using a single HPLC-UV-Vis method. So far, comparisons were made using results from different investigations which apparently employed different methods.[3–8] The approach consisted of three steps as follows. First, compounds of Table 1 (reference materials) were synthesized, except indigotin, which is

Table 1. Structures and UV-Vis absorption spectra of the investigated reference materials

a/a	Name	Structure	UV-Vis absorption spectrum
1	Indigotin (m/z = 261)		
2	Indirubin (m/z = 261)		
3	6-Bromo indigotin (m/z = 340)		
4	6′-Bromo indirubin (m/z = 340)		
5	6-Bromo indirubin (m/z = 340)		
6	6,6′-dibromo indigotin (m/z = 419)		
7	6,6′-dibromo indirubin (m/z = 419)		

commercially available. Then, an HPLC method was developed to achieve sufficient separation of the reference materials and to allow their UV-Vis spectrophotometric detection. The results were supported by preliminary investigations performed using LC-MS coupled to APCI ionization mode. Finally, dyestuff extracts were produced and analyzed by HPLC-UV-Vis, thus identifying their coloring constituents.

EXPERIMENTAL

Reference Materials

Indigo (indigotin) was purchased from Fluka (Sigma-Aldrich, Germany). 6-Bromoindigotin and 6,6′-dibromoindigotin were synthesized according to previously described methods.[14] Indirubin, 6-bromoindirubin, 6′-bromoindirubin, and 6,6′-dibromoindirubin were synthesized by the dimerization of isatin or 6-bromoisatin with 3-acetoxyindol or 6-bromo-3-acetoxyindol.[15] The synthesized reference materials were identical with those previously isolated from the extract of *Hexaplex trunculus*.[13] Figure 1 shows the general synthetic route, followed for the production of the reference materials.

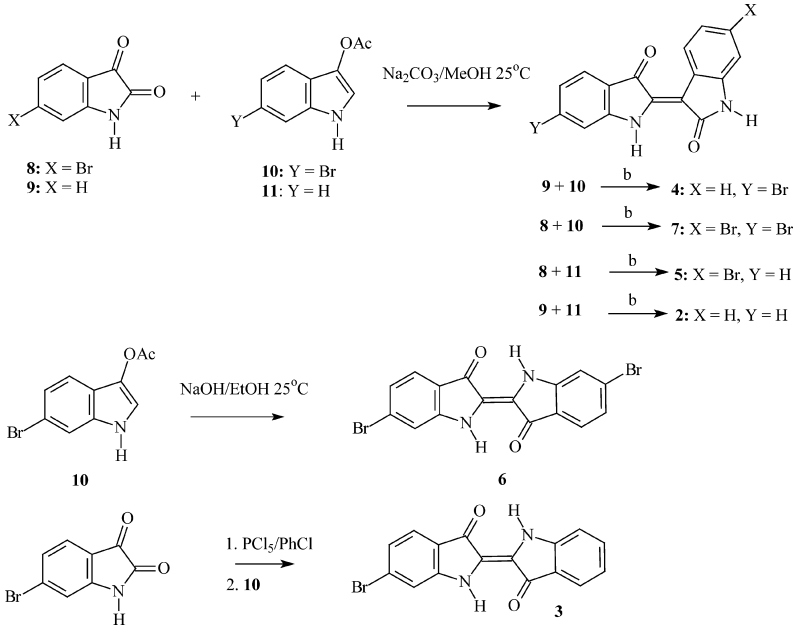


Figure 1. Synthetic scheme used for the production of the reference materials.

Dyestuff Extracts

Animal Material

The marine mollusks, *Hexaplex trunculus*, *Murex brandaris*, and *Thais haemastoma* were collected by diving in the Saronikos gulf near the Salamina island (Greece). *Nucella lapillus* was collected by hand in shallow waters in Roscoff, Bretagne (France). Voucher specimens are deposited in the collection of the Goulandris Natural History Museum.

Extraction Procedure

After removal of the shell of the fresh mollusks, the hypobranchial glands (50 pieces from each mollusk) were separated from the body, placed on paper sheets, and exposed to direct sunlight for 6 h. The colorless glands rapidly changed to green and then to red-blue color. Then, the paper with the glands was cut in small pieces and extracted for 1 h with DMF (50 mL), at 80°C under magnetic stirring. The final solution was filtered and the solvent was removed under reduced pressure (using a high vacuum pump) at 40°C, to afford the vividly colored extracts.

Solvents

N,N-Dimethylformamide (DMF) was purchased from Carlo Erba (Italy) and trifluoroacetic acid from Merck (Germany). Type I reagent grade water, with resistivity up to 18.3 MΩ/cm and organic content <5 ppb, was produced by passing deionized water through Barnstead EASY pure RF water purification system (Fisher Scientific, UK), and was used for buffer solution preparation. HPLC grade acetonitrile was provided by Riedel-deHaan (Sigma-Aldrich, Germany) for liquid chromatography. All HPLC solvents were filtered through a 0.2 μm filter prior to use.

Sample Preparation

Reference materials and dyestuff extracts were dissolved in DMF and heated at 80°C for about 15 min. After dissolution, samples were immediately subjected to HPLC analysis. Extracts were centrifuged prior to HPLC analysis.

Instrumentation

Liquid chromatography was carried out using Thermoquest (Manchester, UK) HPLC system which consisted of P4000 quaternary HPLC pump, SCM 3000

vacuum degasser, AS3000 auto sampler with column oven, Reodyne 7725i Injector with 20 μL sample loop, and Diode Array Detector UV 6000LP. Chromatographic separation was carried out on a XTerra C18 5 μm 3.0 × 250 mm HPLC column (Waters Co., USA) thermostatted at 40°C. Data were received and analyzed using an XcaliburTM (Thermoquest) data system. Preliminary investigations with LC-MS were performed using a Finningan AQA mass spectrometer (Thermoquest) coupled to a negative APCI ionization mode. The instrument was equipped with a single quadrupole mass filter, which was used to record ion signals acquired in the selected ion monitoring (SIM) mode with $m/z = 261$, 340, and 419. The APCI probe temperature was 350°C and corona and cone voltages were 4.5 kV and 30 V, respectively.

HPLC Analysis

Solutions of the reference materials and dyestuff extracts were analyzed using a gradient elution program, shown in Table 2. An analytical run was always followed by the injection of a blank sample, to ensure that no endogenous peaks or carryover effects that might interfere with the indigoid materials are detected.

RESULTS AND DISCUSSION

Figure 2 provides the HPLC-PDA chromatogram obtained for a mixture of six reference materials. The compounds of Table 1 are included in the analyzed mixture, except 6′-bromoindirubin (**4**). Sufficient separation of the investigated compounds are obtained, which allows their identification based on the reported retention times and absorption spectra, shown in Table 1. The separation of 6′-bromoindirubin (**4**) and 6-bromoindirubin (**5**) cannot be clearly shown in the time scale of Figure 2. Figure 3 focuses on a short

Table 2. Gradient elution program for HPLC. The flow rate was 0.4 mL/min

Time (min)	H_2O + 0.1% TFA (%)	CH_3CN + 0.1% TFA (%)
0	60	40
1	60	40
5	20	80
10	10	90
15	10	90
18	0	100

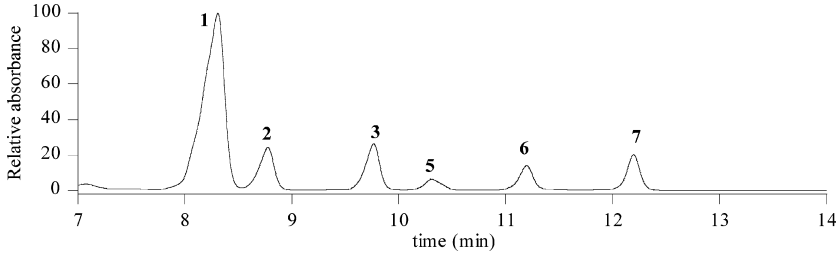


Figure 2. PDA chromatogram of a mixture containing six reference materials. Numbers correspond to the materials of Table 1 as follows: **1** for indigotin, **2** for indirubin, **3** for 6-bromoindigotin, **5** for 6-bromoindirubin, **6** for 6,6′-dibromoindigotin, and **7** for 6,6′-dibromoindirubin. Material **4** (6′-bromoindirubin) is not included in the mixture. PDA: 500–610 nm.

time scale of around 2 min in which the separation of the two materials is presented for a binary mixture. The absorption spectra of Table 1 suggest that "bluish" indigotins (compounds **1**, **3**, and **6**) appear to have a characteristic absorption maximum at a wavelength of around 600 nm, while "purple" indirubins (compounds **2**, **4**, **5**, and **7**) fall to a smaller characteristic wavelength (around 550 nm).

The results of Figure 2 are confirmed by LS-MS-APCI, shown in Figure 4. The mass spectra received in the SIM mode provide additional evidence for the identification of the materials of interest, as the major recorded ions correspond to the molecular ions of the compounds (Table 1). In the present investigation, LC-MS was only used on a preliminary basis. The developed chromatographic method (Table 2) involves a strong acid containing mobile phase, which results in a signal suppression for mass spectrometry.[16] Preliminary measurements using standard solutions of indigotin (the only reference material which was available in large quantities)

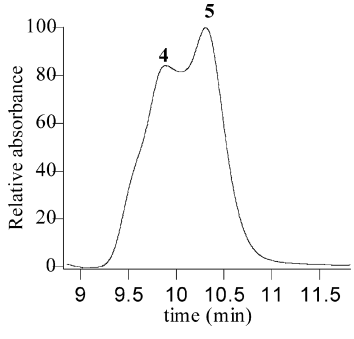


Figure 3. Chromatogram of a binary mixture containing two reference materials. Numbers correspond to the materials of Table 1 as follows: 4 for 6′-bromoindirubin and 5 for 6-bromoindirubin. PDA: 500–610 nm.

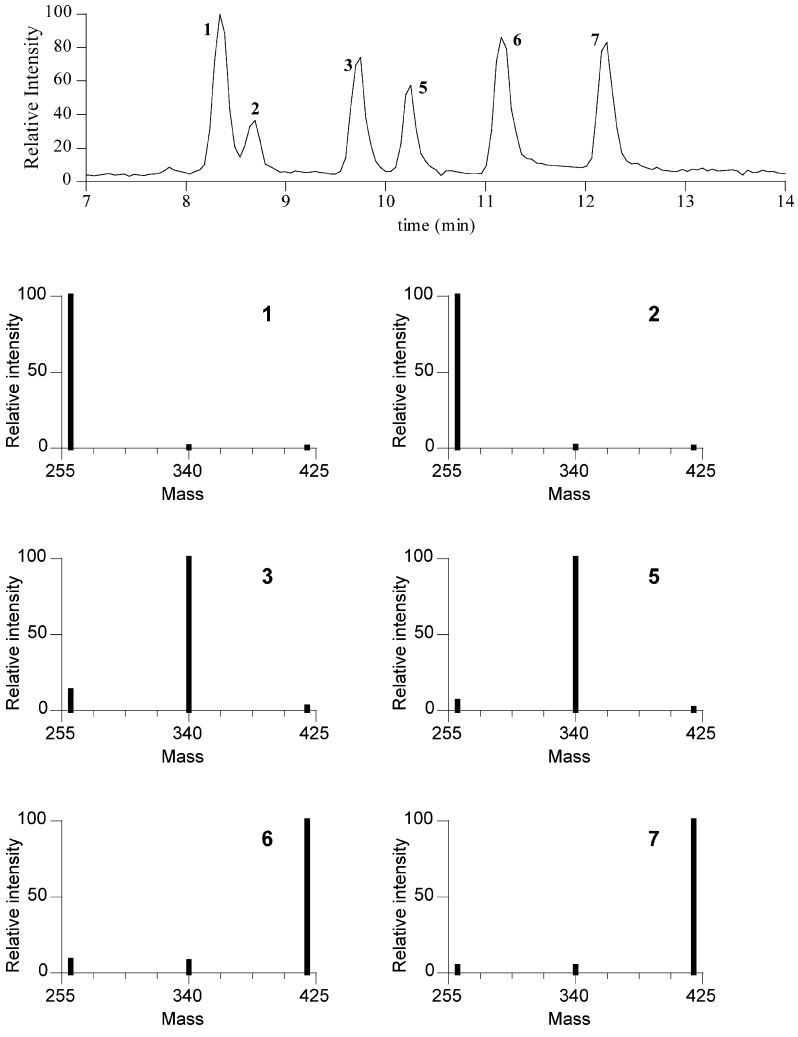


Figure 4. LC-MS-APCI chromatogram and mass spectra of a mixture containing six reference materials (similar to Figure 2). SIM mode.

showed that the detection limit of UV-Vis (0.02 μg/mL) was an order of magnitude superior than the corresponding limit of LC-MS (0.2 μg/mL). For the comparison, five standard solutions of indigotin were prepared (14–0.8 μg/mL) and analyzed in triplicate. The acquisition wavelength was 603 nm (characteristic for indigotin) and the acquisition ion mass was 261 (molecular ion of indigotin). We also note that TFA is an aggressive chemical and is usually not recommended for LC-MS. For these reasons, LC-MS was not further employed in the present study.

Figure 5 provides the chromatograms obtained for *Hexaplex trunculus* (Figure 5a), *Murex brandaris* (Figure 5b), *Nucella lapillus* (Figure 5c), *and Thais haemastoma* (Figure 5d). Identified compounds are indicated with numbers which correspond to the serial numbers of Table 1. In several cases, magnifications of the peaks, which correspond to blue/purple coloring compounds are shown. The results obtained from Figure 5 are summarized in Table 3. All seven compounds of Table 1 are clearly identified in the *Hexaplex truculus* extract (Figure 5a). For the first time, 6′-bromoindirubin and 6-bromoindirubin (compound **4** and **5**, respectively) are clearly identified as major coloring components by HPLC. Previous analyses of *Hexaplex trunculus* identified five compounds as follows: indigotin, indirubin, 6-bromoindigotin, 6,6′-dibromoindigotin, and 6,6′-dibromoindirubin.[3–9] We note, that all these five coloring matters were not identified in a single

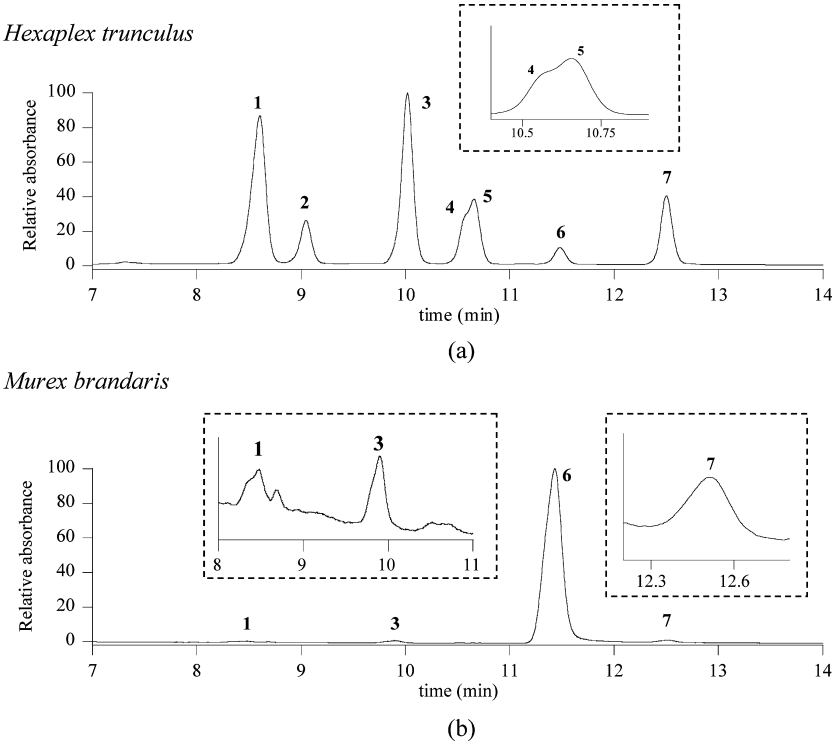
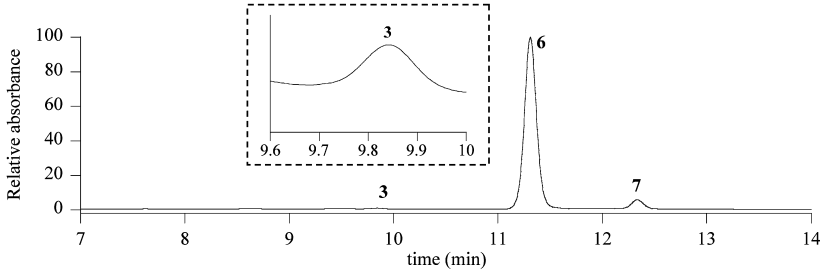


Figure 5. Chromatograms of (a) *Hexaplex trunculus*, (b) *Murex brandaris*, (c) *Nucella lapillus* and (d) *Thais haemastoma.* Identified compounds are indicated with numbers which correspond to the serial numbers of Table 1. In several cases, magnifications of the peaks which correspond to compounds of Table 1 are provided. PDA: 500–610 nm.

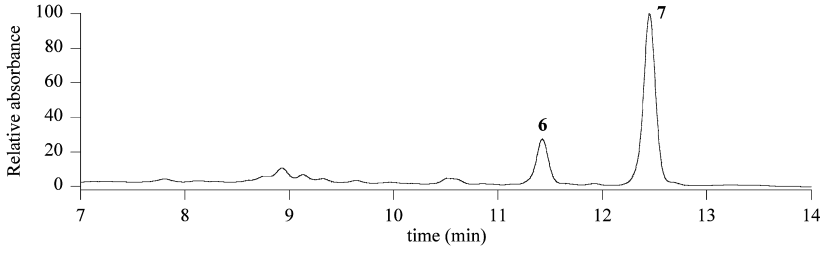
(*continued*)

Nucella Lapillus



(c)

Thais Haemastoma



(d)

Figure 5. Continued.

investigation, but they were scarcely reported in several studies.[3–9] In the present report, all five indigoids are identified along with compounds **4** and **5**. Figure 5b suggests that the following indigoid constituents are identified in *Murex brandaris*: indigotin, 6-bromoindigotin, 6,6′-dibromoindigotin,

Table 3. Summary of blue/purple compounds identified in Figure 4. Serial numbers correspond to Table 1, as follows: **1** for indigotin, **2** for indirubin, **3** for 6-bromoindigotin, **4** for 6′-bromoindirubin, **5** for 6-bromoindirubin, **6** for 6,6′-dibromoindigotin and **7** for 6,6′-dibromoindirubin

a/a	*Hexaplex trunculus*	*Murex brandaris*	*Nucella lapillus*	*Thais haemastoma*
1	X	+		
2	X			
3	X	X	X	
4	X			
5	X			
6	X	X	X	X
7	X	X	X	X

and 6,6′-dibromoindirubin. This result is in agreement with previously reported data with the exception of indigotin.[3,5,8] Traces of the latter were found to be present in *Murex brandaris* (Figure 5b) in contrast to previous investigations, which did not report indigotin as an ingredient of the dyestuff.[5] The results for *Nucella lapillus* agree with other investigations, which indicated that the main coloring constituents of this mollusk are 6-bromoindigotin, 6,6′-dibromoindigotin, and 6,6′-dibromoindirubin.[7] We note, however, that in another investigation, in addition to the three detected indigoids, a small quantity of indigotin was also detected in *Nucella lapillus*.[8] Finally, a relatively good agreement of Table 3 with previous reports has been obtained regarding the results of *Thais haemastoma*.[3,5,8] 6,6′-Dibromoindigotin and 6,6′-dibromoindirubin were clearly identified in the extract of this mollusk. We note, however, that traces of 6-bromoindigotin reported elsewhere[5] were not detected in our study.

SUMMARY

Synthetic routes for the production of indirubin, 6-bromoindigotin, 6′-bromoindirubin, 6-bromoindirubin, 6,6′-dibromoindigotin, and 6,6′-dibromoindirubin were developed to create a library of seven reference materials (indigotin was commercially available). HPLC combined with spectrophotometric UV-Vis detection was subsequently used to identify the organic colorants of samples extracted from four mollusks, which can be found in coastal areas of the European continent. In particular, samples extracted from *Hexaplex trunculus*, *Murex brandaris*, *Nucella lapillus*, and *Thais haemastoma* were analyzed. The investigated mollusks have been extensively used for the production of the purple dye (Tyrian Purple) since antiquity. The results are summarized in Table 3. Extract originated from *Hexaplex trunculus* is found to be the richest on the basis of the variety of indigoid compounds.

ACKNOWLEDGMENT

This work has been partially developed within the framework of MED-COLOUR-TECH project, which is supported by the European Commission, contract No: FP6-2003-INCO-MPC-2-015406.

REFERENCES

1. Hofenk de Graaff, J.H. *The Colourful Past: Origins, Chemistry and Identification of Natural Dyestuffs*; Abbeg-Stiftung: Riggisberg & Archetype Publications, Ltd: London, 2004; 264–273.

2. Karapanagiotis, I.; Sotiropoulou, S.; Chryssikopoulou, E.; Magiatis, P.; Andrikopoulos, K.S.; Chryssoulakis, Y. Investigation of Tyrian Purple occurring in historical wall paintings of Thera. Dyes History Archaeol. 2006, In press.
3. Cooksey, C.J. Tyrian Purple: 6,6′-Dibromoindigo and related compounds. Molecules **2001**, *6*, 736–769.
4. Wouters, J.; Verhecken, A. High-performance liquid chromatography of blue and purple indigoid natural dyes. J. Soc. Dyers Colour. **1991**, *107*, 266–269.
5. Wouters, J. A new method for the analysis of blue and purple dyes in textiles. Dyes History Archaeol. **1992**, *10*, 17–21.
6. Koren, Z.C. HPLC analysis of the natural scale insect, madder and indigoid dyes. J. Soc. Dyers Colour. **1994**, *110*, 273–277.
7. Cooksey, C.; Withnall, R. Chemical studies on *Nucella lapillus*. Dyes History Archaeol. **2001**, *1617*, 91–96.
8. Withnall, R.; Patel, D.; Cooksey, C.; Naegel, L. Chemical studies of the purple dye of *Purpura pansa*. Dyes History Archaeol **2003**, *19*, 109–117.
9. Andreotti, A.; Bonaduce, I.; Colombini, M.P.; Ribechini, E. Characterization of natural indigo and shellfish purple by mass spectrometric techniques. Rapid Commun. Mass Spectrom. **2004**, *18*, 1213–1220.
10. Szostek, B.; Orska-Gawrys, J.; Surowiec, I.; Trojanowicz, M. Investigation of natural dyes occurring in historical Coptic textiles by high-performance liquid chromatography with UV-Vis and mass spectrometric detection. J. Chromatogr. A **2003**, *1012*, 179–192.
11. Puchalska, M.; Poteć-Pawlak, K.; Zadrożna, I.; Hryszko, H.; Jarosz, M. Identification of indigoid dyes in natural organic pigments used in historical art objects by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry. J. Mass Spectrom. **2004**, *39*, 1441–1449.
12. Karapanagiotis, I. Identification of indigoid natural dyestuffs used in art objects by HPLC coupled to APCI mass spectrometry. Am. Lab. **2006**, *38*, 36–40.
13. Meijer, L.; Skaltsounis, A.L.; Magiatis, P.; Polychronopoulos, P.; Knockaert, M.; Leost, M.; Ryan, X.P.; Vonica, C.A.; Brivanlou, A.; Dajani, R.; Crovace, C.; Tarricone, C.; Musacchio, A.; Roe, S.M.; Pearl, L.; Greengard, P. GSK-3 selective inhibitors derived from Tyrian Purple indirubins. Chem. Biol. **2003**, *10*, 1255–1266.
14. Clark, R.J.H.; Cooksey, C.J. Monobromoindigos: A new general synthesis, the characterization of four isomers and an investigation into the purple color of 6,6′-dibromoindigo. New J. Chem. **1999**, 323–328.
15. Polychronopoulos, P.; Magiatis, P.; Skaltsounis, A.L.; Myrianthopoulos, V.; Mikros, E.; Tarricone, A.; Musacchio, A.; Roe, S.M.; Pearl, L.; Leost, M.; Greengard, P.; Meijer, L. Structural basis for the synthesis of indirubins as potent and selective inhibitors of glycogen synthase kinase-3 and cyclin-dependent kinases. J. Med. Chem. **2004**, *47*, 935–946.
16. Apffel, A.; Fischer, S.; Goldberg, G.; Goodley, P.C.; Kuhlmann, F.E. Enhanced sensitivity for peptide mapping with electrospray liquid chromatography-mass spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases. J. Chromatogr. A **1995**, *712*, 177–190.

Received December 31, 2005
Accepted February 3, 2006
Manuscript 6807