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An improved HPLC method coupled to PCA for the identification of Tyrian purple in archaeological and historical samples

Ioannis Karapanagiotis [a,*], Dimitrios Mantzouris [b], Chris Cooksey [c], Mohammad S. Mubarak [d], Panagiotis Tsiamyrtzis [e]

[a] University Ecclesiastical Academy of Thessaloniki, Department of Management and Conservation of Ecclesiastical Cultural Heritage Objects, Thessaloniki 54250, Greece
[b] Ormylia Foundation, Art Diagnosis Center, Ormylia 63071, Greece
[c] 59 Swiss Avenue Watford WD18 7LL, UK
[d] The University of Jordan, Department of Chemistry, Amman 11942, Jordan
[e] Athens University of Economics and Business, Department of Statistics, Athens 10434, Greece

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ABSTRACT

The efficiencies of dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), and pyridine to treat and solubilise Tyrian purple are compared using high performance liquid chromatography (HPLC). For the comparative study, samples of *Hexaplex trunculus*, collected from the area of Carthage, are treated with the three solvents and the following compounds are monitored with HPLC: indigotin, indirubin, 6′-bromoindirubin, 6-bromoindirubin, 6-bromoindigotin, 6,6′-dibromoindigotin and 6,6′-dibromoindirubin. HPLC identifications are achieved as these compounds were synthesized in pure forms and characterized using ^{1}H NMR, elemental analyses and IR spectroscopy. It is shown that pyridine results in poor yields compared to the quantities solubilised using DMF or DMSO. However, the relative composition of the purple dye is not affected by the solvent used for sample treatment. DMSO resulted in improved HPLC signals (peak heights) over DMF and is therefore selected for further studies. The effects of treatment temperature and time are investigated, suggesting that the best conditions correspond to 80 °C and 15 min.

The improved method (treatment with DMSO at 80 °C for 15 min) is used to treat more molluscan samples which are then subjected to HPLC analysis. The results are investigated in the light of previously collected (published and unpublished) analytical data. In particular, principal component analysis (PCA) is applied, to investigate if it is possible to achieve a distinction between the three Mediterranean molluscan species (*H. trunculus* L., *Bolinus brandaris* L. and *Stramonita haemastoma*), using all the HPLC quantitative results reported up until now by various researchers. The PCA plot shows that *B. brandaris* and *S. haemastoma* species are not separated and *H. trunculus* samples are slightly separated from the other two species.

The above findings are used to investigate the possible biological origin of Tyrian purple detected in historical -initially studied using X-ray fluorescence spectroscopy- and archaeological samples. Furthermore, the improved method, devised herein, resulted in the identification of monobromoindirubins in the DMSO extracts of the archaeological samples, which were not detected in previous studies where DMF was used to treat the same significant samples.

1. Introduction

Among the various dyes used in objects of the cultural heritage, Tyrian purple, known also as royal or true purple, is the most prestigious, being mentioned in various sources including for instance the Mycenaean Linear B clay tablets (Knossos X 976, 13th century BC) and the Bible [1]. The dye is obtained from the hypobranchial glands of molluscs (Muricidae family) that are spread all over the world. In the Mediterranean Sea, three species are found [1,2]: *Hexaplex trunculus* L. (*Murex trunculus*), *Bolinus brandaris* L. (*Murex brandaris*) and *Stramonita haemastoma* (*Thais haemastoma*).

The impressive hue of true purple is generated from colouring compounds, including indigotin (IND) and its isomer indirubin (INR) which are also contained in the indigoid dyes of plant origin, such as those from *Indigofera* spp. and *Isatis tinctoria* [3]. The marker compound of Tyrian purple is, however, 6,6′-dibromoindigotin (DBI), which was first identified by Friedländer in 1909 after processing the hypobranchial glands of *M. brandaris* [4]. Analytical capabilities were enhanced by the application of high performance liquid chromatography (HPLC) [5]. The early HPLC study on *M. trunculus* samples, by Wouters and Verhecken (1991) [5], resulted in the identification of

* Corresponding author at: University Ecclesiastical Academy of Thessaloniki, Department of Management and Conservation of Ecclesiastical Cultural Heritage Objects, N. Plastira 65, Thessaloniki 54250, Greece. Tel.: +30 2310 397730; fax: +30 2310 300360.
E-mail address: y.karapanagiotis@aeath.gr (I. Karapanagiotis).

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Table 1
Identification of Tyrian purple in historical and archaeological objects from the Eastern Mediterranean (Greece, Turkey, Syria, Lebanon, Israel and Egypt) using chemical methods.

Date	Location	Object/Samples	Technique	Reference
17th c. BC (or earlier)	Akrotiri, Thera, Greece	Lump of pigment and wall paintings	XRF, HPLC-DAD, LC-MS-APCI, Raman, FTIR	18–24
17th c. BC (or earlier)	Raos, Thera, Greece	Wall painting	HPLC-DAD, Raman, FTIR	23,24
17th c. BC (or earlier)	Trianda, Rhodes Greece	Pigment	HPLC-DAD, Raman, FTIR	23,24
Late Bronze age	Royal tomb complex within the palace Qatna, Tell Mishrife, Syria	Several sediment samples originated from decayed fabrics; fossilised woollen textiles	FTICRMS, HPLC-DAD, NMR	25
13th c. BC	Sarepta (Sarafand), Lebanon	Pottery sherds with purple deposits	PIXE, ESCA, FTIR, MCT	26
11th c. BC	Tel Keisan, Israel	Vessel	MCT	27
9th/8th c. BC	Tel Shiqmona, Israel	Sherds	IR	28
7th c. BC	Tel Kabri, Isarel	Pigmented potsherd	HPLC-DAD	11
6th-2nd c. BC	Cave of Koroneia, central Greece	Astragalos made of sheep knucklebone	ESEM-EDX	29
486/485 BC	Bible Lands Museum, Jerusalem, Israel	Outer surface of Darius I stone jar	HPLC-DAD	30
4th c. BC	J. Paul Getty Museum	Greek marble vessel (accession nos. 88.AA.140.1-36)	n.a.	31
336 BC	Tomb of Phillip II, Vergina, Macedonia, Greece	Casket fabric	FTIR, MCT	3
last quarter of the 4th c. BC.	Tomb III at Agios Athanasios, Macedonia, Greece	Wall painting	XRF, HPLC-DAD	32,33
first half of the 3rd c. BC	The Tomb of the palmettes, Mieza, Macedonia, Greece	Wall paintings	HPLC-DAD	33
ca. 300 BC	Chania, Crete, Greece	Hellenistic figurines	FTIR, XRF	34
1st c. BC	Masada, Israel	Fabric excavated at the western Herodonian palace	HPLC-DAD	35
75 BC and 30 BC	Enkomi, Cyprus	Two textiles	TLC, UV/Vis	36
1st – 3rd c. AC	Eastern Desert of Egypt	Several textiles	HPLC-DAD	37, 38
3rd c. AC	Egypt	Egyptian mantle	HPLC-DAD	8
3rd c. AC	Palmyre, Syria	Textiles from tombs	MCT	39, 40
3rd c. AC	Egypt	Two textiles	MCT	41, 42
3rd/4th c. AC.	Egypt - collection of the University Museum of Archaeology and Anthropology, Philadelphia	Fragment of Coptic textile	MS	43
5th c. AC	Upper Egypt	Textile probably from a cemetery	UV/Vis	44
5th/6th c. AC & 8th/9th c. AC	Egypt	Two textiles of Belgian private collections	HPLC-DAD	45
7th–12th c. AC	Byzantium & Byzantium/Syria	Four relic wrappings	n.a.	3

ESCA: electron spectroscopic chemical analysis; ESEM-EDX: environmental scanning electron microscopy coupled with X-ray microanalysis; FTIR: Fourier transform infrared spectroscopy; FTICRMS: Fourier transform ion cyclotron resonance mass spectrometry; HPLC-DAD: high performance liquid chromatography-diode array detector; IR: infrared spectroscopy; LC-APCI-MS: liquid chromatography with atmospheric pressure chemical ionization-mass spectrometry; MS: mass spectrometry; MCT: microchemical tests; NMR: nuclear magnetic resonance spectroscopy; PIXE: proton-induced X-ray emission spectroscopy; Raman: Raman spectroscopy; TLC: thin layer chromatography; UV/Vis: ultraviolet/visible spectroscopy; XRF: X-ray fluorescence; n.a.: not available.

indigotin, indirubin, 6,6′-dibromoindigotin and, for the first time, 6-bromoindigotin (MBI). The presence of the latter in dyes prepared from *M. trunculus* was thus first clearly proved using HPLC [5], although in 1990 McGovern et al. reported that monobromoindigotin could be contained in the purple dye *Purpura patula pansa* as the result of photodebromination of synthetic *leuco*-6,6′-dibromoindigotin followed by oxidation [6]. The same group identified 6-bromoindigotin in dyes from *M. trunculus* in 1992 [7]. A brominated indirubin, identified later as being 6,6′-dibromoindirubin (DBIR), by Koren, was first detected using HPLC in *M. brandaris* and *T. haemastoma* dyes in 1992 by Wouters [8] using samples prepared previously by Verhecken [9]. Some years later, Koren reported the identification of 6,6′-dibromoindirubin in *M. trunculus* [10,11]. Monobromoindirubins such as 6-bromoindirubin (6MBIR) and 6′-bromoindirubin (6′MBIR), which had been speculated as possible components of Tyrian purple for several years [10–12], were isolated from *M. trunculus* and identified using NMR by Meijer et al. in 2003 [13]. The two monobromoindirubins were detected in *M. trunculus* extracts, along with the previously mentioned indigoids and indirubinoids, using HPLC by our group in 2006 [14]. In the same year, similar HPLC results for *M. trunculus* were published by Koren who furthermore reported the HPLC identifications of isatinoids such as isatin and 6-bromoisatin [15]. Recently, Nowik et al. (2011) were able to detect in trace four new analogues of brominated and unbrominated indirubins in *M. trunculus* extracts [16]. It was suggested that the newly identified compounds could be cis-INR, cis-6MBIR, cis-6'MBIR and cis-DBIR [16]. Further investigations showed that the new compounds were in fact indirubin-3'-monoimines [17].

Despite the historical importance of Tyrian purple, its chemical identification in historical/archaeological samples is rare. This is evidenced in Table 1 that serves two purposes. First, the table is a brief review of previously reported chemical identifications of Tyrian purple in ancient objects from the Eastern Mediterranean Sea [3,11,18–45], an area where the royal purple played a significant role for centuries. Second, Table 1 summarizes the methods used for the identification of Tyrian purple in objects of the cultural heritage. Among the chemical methods included in Table 1, HPLC is the most informative as it can provide (semi-)quantitative data for the composition of the purple dye contained in a historical/archaeological sample. This is useful for several reasons including, for instance, the comparison of the purple pigments/dyes used in various objects with different provenance. Furthermore, the (semi-)quantitative data provide indications regarding the exact biological origin of Tyrian purple used in an ancient object. Within this spirit, the relative compositions, measured as HPLC peak areas, of purple pigments on painted cultural heritage objects were used to identify the exact molluscan species i.e. the raw source of the purple pigments [30,46]. These identifications were based on a criterion which takes into consideration the relative content only of brominated indigotins (MBI and DBI), disregarding thus the other colouring components of the purple dye [30]. In the present study, we follow a more general approach, as described next.

First, we enrich the available data for the relative compositions of the Mediterranean purple molluscan species. For this reason, HPLC analyses of new molluscan samples, collected in the Mediterranean, are carried out. Second, the results are investigated through an integrated approach, in light of previously published and unpublished analytical data that were collected after personal communications. Principal component analysis (PCA) is applied, for the first time, to investigate if this mathematical tool is effective to achieve the distinction of the three Mediterranean molluscan species, using all the HPLC

quantitative results reported up until now by various researchers. PCA has been applied only twice on historical dyes, to distinguish cochineal (*Dactylopius* species, *Porphyrophora hamelii*, and *Porphyrophora polonica*) [47] and dragon's blood (*Dracaena* and *Daemonorops* trees) [48] species, with quite satisfactory results. The distinction of the three Mediterranean molluscan species, *H. trunculus* L., *B. brandaris* L. and *S. haemastoma*, should not be easy because numerous variables (e.g. geographical location, age and sex of the snails, preparation conditions of the purple pigment, analytical method employed to record the composition etc.) might affect the reported composition of the purple dye [30,37].

Three solvents have been previously suggested and used to treat samples containing Tyrian purple: dimethyl sulfoxide (DMSO) [16,30,49], N,N-dimethylformamide (DMF) [11,14,50] and pyridine [8]. Another goal of the study is to investigate if the solvent, selected for sample treatment, affects the HPLC compositional data of Tyrian purple. This is important to elucidate if the type of the solvent is a source of the large variety of the compositional data reported for purple snails of the same species. Furthermore, a brief comparison of the efficiencies of DMSO, DMF and pyridine to treat and solubilise true purple is carried out. Then, extraction with DMSO is optimised by investigating the effects of treatment temperature and time on dyestuff extraction and solubilisation.

The results of the aforementioned studies are used to discuss the possible biological origin of Tyrian purple detected in historical and archaeological samples.

Finally, another task is included in the present work: prior to the chromatographic studies, components of Tyrian are synthesized in pure form and used as standards for identification purposes.

2. Experimental

2.1. Chemicals and standards

Seven compounds were used as standards for identification purposes in the HPLC studies. Indigotin (IND) was purchased from Fluka. Indirubin (INR), 6′-bromoindirubin (6′MBIR), 6-bromoindirubin (6MBIR), 6-bromoindigotin (MBI), 6,6′-dibromoindigotin (DBI) and 6,6′-dibromoindirubin (DBIR), were synthesized according to the procedures described in paragraph 2.2. Important chemicals used for synthesis were as follows: 6-bromoindole (Beta Pharma), 3-acetoxyindole (Acros Organics), 6-bromoisatin (Wako) and isatin (Aldrich).

The following HPLC-grade solvents were used for sample preparation, prior to HPLC: dimethyl sulfoxide (DMSO, Sigma-Aldrich), N, N-dimethylformamide (DMF, Lab-Scan) and pyridine (Sigma-Aldrich).

HPLC was operated using type I reagent-grade water with resistivity up to 18.3 MΩ/cm and organic content less than 5 ppb, produced by a Barnstead EASYpure water purification system, HPLC-grade acetonitrile (CH_3CN, J. T. Baker) and trifluoroacetic acid (TFA, Riedel-de Haën) of 99% purity.

2.2. Synthesis and characterization of standards

Compounds, which are either the target compounds, such as INR, 6MBIR, 6′MBIR, DBIR, MBI and DBI and intermediates, were synthesized according to the schemes shown in Fig. 1.

In particular, 3-acetoxy-6-bromoindole(**3**) was prepared in pure form according to the procedure published by Tanoue et al. [51] which involved addition of iodine (2.6 g, 10.2 mmol) and an aqueous solution of potassium iodide (1.70 g, 10.2 mmol) to a solution of the commercially available 6-bromoindole (**1**) (2.0 g, 10.2 mmol) and sodium hydroxide (0.41 g, 10.2 mmol) in methanol (100 mL). After the mixture was stirred at room temperature for 3 h, water was added, the precipitate was collected, washed with water and dried to afford 6-bromo-3-iodoindole (**2**) which was used in the following step without further purification. Silver acetate (3.41 g, 20.4 mmol) was added to a solution of 6-bromo-3-iodoindole, prepared in the previous step, in acetic acid (80 mL). After stirring for 1 h at 90 °C, the mixture was cooled to room temperature and filtered. The filtrate was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel using $CHCl_3$ as an eluent to afford 3-acetoxy-6-bromoindole (**3**). Melting point and NMR spectral data were in agreement with previously published data [51].

Indirubin(**6**) was prepared following previously published routes [52,53] that involve addition of anhydrous sodium carbonate under nitrogen atmosphere (2.20 g, 20.0 mmol) to a stirred solution of the commercially available 3-acetoxyindole (**4**) (1.75 g, 10.0 mmol) and isatin(**5**) (1.55 g, 10.5 mmol) in methanol (50 mL). After 12 h of stirring, the precipitate was filtered, washed with aqueous methanol (1:1, 25 mL), then with methanol (30 mL), and allowed to dry to afford indirubin as a deep purple solid. NMR spectral data were in agreement with previously published data [53]. For instance, ^{1}H-NMR (300 MHz, DMSO): δ (ppm) 10.96 (brs, 2H, NH), 8.73 (d, $J = 7.3$ Hz, 1H), 7.63 (dd, $J = 8.0$, 0.5 Hz, 1H), 7.54 (td, $J = 8.2$, 0.6 Hz, 1H), 7.38 (d, $J = 8.0$ Hz, 1H) 7.22 (td, $J = 7.8$, 1.2 Hz, 1H), 6.98 (t, $J = 7.9$ Hz, 2H), 6.87 (d, $J = 7.8$ Hz, 1H).

6-Bromoindirubin(**8**) was prepared from the reaction of the commercially available 6-bromoisatin(**7**) and 3-acetoxyindole(**4**) by a procedure analogous to that of indirubin. 6′-Bromoindirubin(**9**) was prepared from 3-acetoxy-6-bromoindole(**3**) and isatin(**5**) using the procedure employed for the synthesis of indirubin. 6,6′-Dibromoindirubin(**10**) was synthesized using the same general procedure employed for the synthesis of the other indirubins by reacting 3-acetoxy-6-bromoindole (**3**) and 6-bromoisatin (**7**). NMR data of the produced brominated indirubins were in perfect agreement with previously published data [53].

6-Bromoindigotin(**12**) was prepared by heating a mixture of 6-bromoisatin(**7**) (5 g, 22.0 mmol) and PCl_5(5 g, 24.0 mmol) in chlorobenzene(150 mL) under nitrogen at around 100 °C for 4 h to give a 6-bromo-2-chloroindolone (**11**) that was not isolated but, after being cooled, was treated with 3-acetoxyindole (**4**) (3.58 g, 21 mmol) [51]. Then the reaction mixture was allowed to stand overnight to afford a dark blue solid which was washed with ethanol and recrystallized from ethyl benzoate. IR spectral results were in agreement with previously published data [54]. Anal.Calcd for $C_{16}H_9BrN_2O_2$ (341.16 g/mol): C 56.33, H 2.66, N 8.21, found: C56.18, H 2.64, N 8.13.

Finally, 6,6′-dibromoindigotin (**13**) was synthesized according to the previously published procedure [54] which involves addition of aqueous 1 M NaOH solution (400 mL) to a solution of 3-acetoxy-6-bromoindole (**3**) (2.15 g, 8.44 mmol) in ethanol (200 mL). After the mixture was stirred at room temperature for 2 h, water was added and the precipitate was collected by filtration. The product was washed thoroughly with water and ethanol and dried to afford the desired compound. Elemental analysis and IR spectral were in agreement with previously published data [54] and are also in agreement with the suggested structure. Anal.Calcd for $C_{16}H_8Br_2N_2O_2$ (420.05 g/mol): C 45.75, H 1.92, N 6.67, found: C45.67, H 1.90, N 6.63.

2.3. Molluscan (H. trunculus) samples

Four samples of *H. trunculus* pigment were provided by Nouira Mohammed Ghassen (Tunis, Tunisia) and prepared as described next.

Sample T1 (Carthaginian): a sample, described as Carthaginian purple, was prepared from *H. trunculus* (collected from Carthage, Tunisia) by crushing the shells and exposing them to air and sunlight. Water was added and the dark coloured pigment separated from the shell fragments and mollusc flesh.

Sample T2 (Croatian): the second sample was prepared from *H. trunculus* molluscs, collected in the coastline of Croatia. Molluscs were allowed to dry out and die when they exude purple mucus. The mucus was collected and dried.

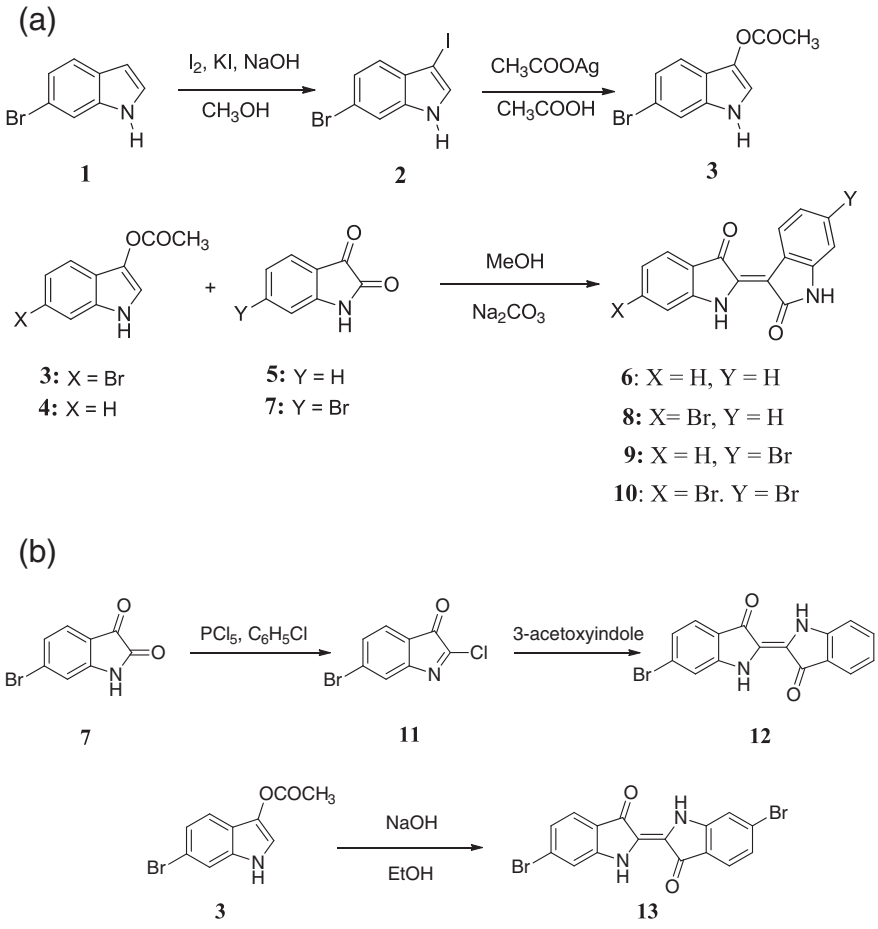

Fig. 1. Synthesis of (a) indirubin and brominated indirubins and (b) brominated indigotins. Structures of the target compounds used for identification purposes are as follows: INR (**6**), 6MBIR (**8**), 6′MBIR (**9**), DBIR (**10**), MBI (**12**) and DBI (**13**).

Sample T3 (Tunisian-red): the Tunisian-red (*H. trunculus*) sample was prepared by removing the hypobranchial glands in the dark and after the pigment was produced, separating it as in T1.

Sample T4 (Tunisian-blue): the Tunisian-blue (*H. trunculus*) sample was obtained from the product from T3 by heating a suspension of it in the dark. When the boiling temperature was reached, the colour changed to blue. The purpose of doing the conversion of mollusc to pigment in the dark was to try to reduce or eliminate the photodebromination of any intermediate leuco-bromoindigos that would lead, after oxidation, to a more bluish product.

The first sample, named as Carthaginian purple, was used for extensive studies, to investigate the effects of hot DMSO, DMF and pyridine on the HPLC compositional data of the purple dye, according to the procedures described in paragraph 2.4. The other three molluscan samples were treated only with hot DMSO (80 °C for 15 min), which corresponds to the finally selected treatment method.

2.4. Procedures for the selection of solvent and conditions for sample treatment

The effects of three solvents (DMSO, DMF and pyridine) on the HPLC compositional data of Tyrian purple were investigated on a comparative basis, according to the following. Carthaginian sample (2.5 mg) was diluted in 500 μl of solvent (DMSO, DMF and pyridine). The solution was heated at 80 °C for 15 min and then centrifuged for 1 min at 4000 rpm. The upper, clear liquid phase was subjected immediately to HPLC analysis.

DMSO was then selected to further study the effects of treatment temperature (40, 60, 80, 100 and 120 °C were investigated) and time (1, 5, 15 and 30 min were investigated) on the solubilisation of the Tyrian purple components.

HPLC analyses were carried out in triplicate and mean values were calculated. Dye yields were within ±8% of the mean.

2.5. Historical and archaeological samples

Two textile samples, indicated hereinafter as E1 and E2, were removed from a Byzanitne epitaphios (Benaki Museum, Greece) dated in the 14th c. AC. Samples were first studied with XRF and were subsequently subjected to HPLC analysis.

Small remaining quantities of five archaeological samples, which have been previously investigated [19,20,23,24] and are included in the first three rows of Table 1, are re-analysed herein. The samples constitute the earliest direct chemical evidence for the use of true purple in painted objects (wall paintings) as they are dated to the Late Bronze Age (~17th century BC or earlier). They were found in three different sites of the Aegean Sea: Akrotiri, Raos (both are located in the island of Thera) and Trianda (island of Rhodos). The samples are [23,24]: (i) a lump of purple pigment found in Complex D (Akrotiri, Ak1), (ii) a microsample taken from a purple paint detail on a wall painting fragment (Akrotiri, Ak2), (iii) a purple pigment found at the New Pillar Pit 53A (Akrotiri, Ak3), (iv) a microsample removed from a wall painting, excavated in the Site Raos (Ra) and (v) a purple pigment found in Trianda (Tri). Archaeological information about these significant samples is provided in detail elsewhere [24].

Table 2
Gradient elution program for HPLC. Flow rate: 0.5 mL min^{-1}.

Time (min)	H_2O + 0.1% TFA (%)	CH_3CN + 0.1% TFA (%)
0	50	50
0,5	50	50
12	40	60
12,5	40	60
14	30	70
17	15	85
18	15	85
21	0	100
22	0	100

The analyses described in the present report were carried out using improved HPLC (Table 2) and sample treatment (hot DMSO) methods, rather than as in the previous reports where hot DMF and a shorter HPLC method were used for sample treatment and analysis [23,24]. Furthermore, the results reported herein were collected using 288 nm as a monitoring wavelength, instead of 590 nm that was previously used for the analyses of Ak2, Ak3, Ra and Tri samples [23,24]. Because most of the quantitative data that is available in the literature for the Mediterranean molluscs have been reported at 288 nm, the use of this particular wavelength is necessary to investigate the possible biological source of the purple dye in archaeological samples. It is noted that only for sample Ak1, some unpublished HPLC compositional data collected at 288 nm was available [19]; but in this study, Ak1 had been treated with DMF [19] and not with DMSO, used herein.

Historical and archaeological and samples were treated with DMSO (80 °C for 15 min) and subjected to HPLC analysis.

2.6. Instrumentation

^{1}H-NMR spectra were recorded on a Bruker DPX-300 MHz spectrometer (Germany) with TMS as the internal standard. Chemical shifts were expressed in δ units. Elemental analyses were acquired with a Euro EA3000 CHNS-O Elemental Analyzer (Milan, Italy). Infrared spectra were obtained with the aid of a Nicolet Impact 400 FTIR-Spectrophotometer (Madison, WI, USA) from 4000 to 400 cm^{-1}.

The HPLC-DAD system (Thermoquest, Manchester, UK) consisted of a 4000 quaternary HPLC pump, a SCM 3000 vacuum degasser, an AS3000 auto sampler with column oven, a Rheodyne 7725i Injector with 20 μL sample loop and a Diode Array Detector UV 6000LP. Analyses were carried out with an Altima C18 (Alltech Associates Inc., USA) column (5 μm particle size, 250 mm × 3.0 mm) at a stable temperature of 35 °C. The monitoring wavelength was 288 nm.

The gradient elution program is summarized in Table 2. The use of TFA in the buffer solution to analyse Tyrian purple samples using HPLC-DAD was first introduced by our group [14,19,20] and was then adopted by others [55]. The gradient program presented in Table 2 is different from its original version [14,19,20], to improve the separation of monobromoindirubins, as shown in Fig. 2.

Finally, a handheld X-ray fluorescence (XRF) spectrometer (Tracer IV-SD, Bruker) was used to carry out preliminary studies on the historical textile (E1 and E2) samples.

3. Results and discussion

3.1. Method development for sample treatment

Fig. 2 shows an HPLC chromatogram collected for the Carthaginian sample (T1) using the gradient elution program of Table 2. Good separation of the purple components is achieved, including the separation of monobromoindirubins (6′MBIR and 6MBIR), which was problematic using a previously devised gradient elution method [14]. Retention times increase with molecular size for both indigotins (IND, MBI, DBI) and indirubins (INR, 6MBIR, 6′MBIR, DBIR). Furthermore, indirubins

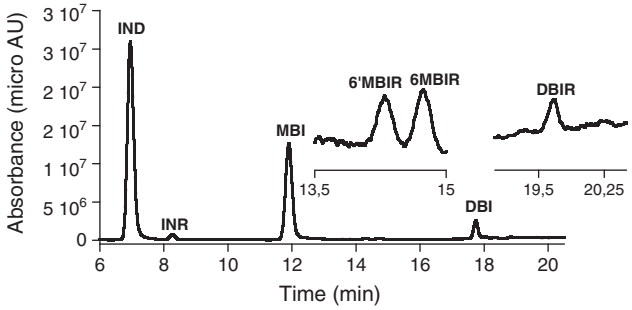

Fig. 2. HPLC chromatogram at 288 nm of Carthaginian sample (T1) analysed using the gradient elution program of Table 2. Good separation of the purple components is achieved.

are more retained than the corresponding indigotins because they have “free” NH and CO groups, whereas in the indigotins these groups are intramolecularly hydrogen bonded.

The five archaeological samples have been previously analyzed with HPLC after treatment with DMF [19,23,24]. In these previous investigations treatment temperature and time were set to 80 °C and 15 min, respectively. Using these conditions as a starting point, the efficiencies of DMSO, DMF and pyridine to solubilise Tyrian purple are compared in Fig. 3. The latter shows HPLC (288 nm) peak areas measurements of seven purple components detected in the Carthaginian sample. For each compound, the results are normalised to the highest value (taken as 100%) recorded for one of the three solvents. Fig. 3 suggests that the use of pyridine results in poor yields compared to the results obtained using DMF or DMSO, except for IND that was dissolved almost equally well in any of the three solvents. DMSO gave the best results for the brominated indirubins (6′MBIR, 6MBIR, DBIR). DMF gave probably somewhat better but clearly comparable results with DMSO for brominated indigotins (MBI, DBI). No difference in the yields of DMSO and DMF was recorded for INR.

It is important to note that despite the differences in the HPLC peak areas recorded for the seven purple components treated with the three solvents, the latter did not have any major effect on the relative composition of the Carthaginian sample, as shown in the results of Table 3. The relative integrated HPLC (288 nm) peak areas measured for the components of the Carthaginian sample treated with DMSO, DMF and pyridine are very similar. This is important, as it does suggest that the comparison of the relative compositions, reported by various researches who used different solvents to treat purple samples, is valid.

A further comparison of the efficiencies of DMSO and DMF, to treat purple samples, is carried out in Fig. 4. The latter shows two chromatograms obtained after treating Carthaginian samples with hot DMSO and DMF. Better signals (i.e. higher HPLC peaks) are achieved for the sample dissolved in DMSO. This is clearly observed for IND and its brominated derivatives which are contained in relatively high amounts. Similarly, treatment of the sample with DMSO yielded in slightly higher HPLC peaks (than DMF) for INR and its brominated derivatives, which are contained in the Carthaginian molluscs in small amounts. Interestingly, HPLC peaks recorded with DMSO are higher even for the brominated indigotins for which the use of DMF resulted in slightly larger peak areas, according to the results of Fig. 3. Apparently, the peak signal/height which is directly related to the signal-to-noise ratio (S/N) recorded by HPLC for each extracted colouring compound of interest, is extremely important for chromatographic protocols which are devised to be applied for archaeometric studies [56,57]. This is because very small, aged and degraded historical/archaeological samples are usually available and thus improved HPLC signals are desirable. Based on the data provided in Figs. 3 and 4, DMSO was selected for further studies. In particular, treatment temperature and time were optimised as described next.

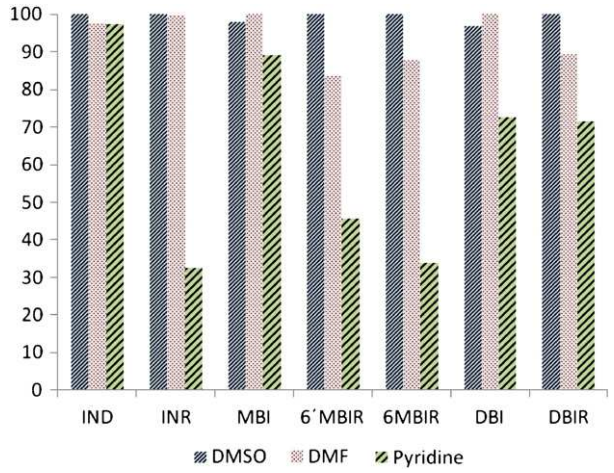

Fig. 3. Integrated HPLC peak areas (288 nm) measured for compounds extracted from Carthaginian purple and normalised to the highest value (taken as 100%) that was recorded for one of the three tested solvents. Samples were treated at 80 °C for 15 min.

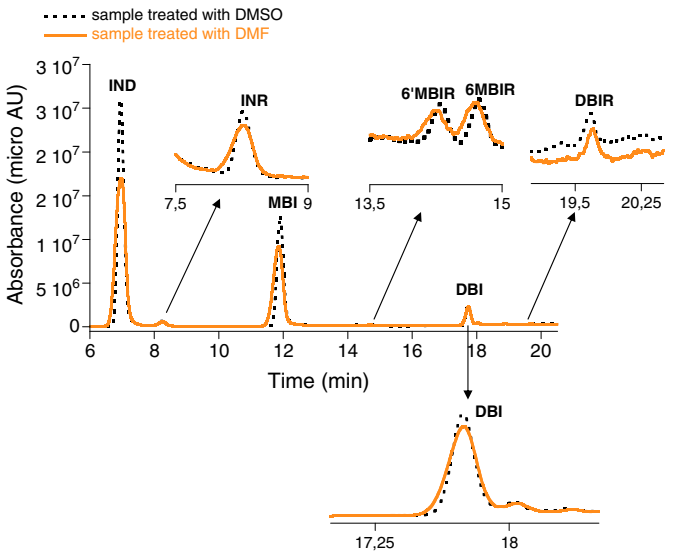

Fig. 4. HPLC chromatograms collected at 288 nm for Carthaginian molluscs treated with DMSO and DMF at 80 °C for 15 min.

The effect of temperature on the efficiency of DMSO to solubilise Tyrian purple is revealed in Fig. 5. A similar qualitative behaviour is recorded for IND, INR, 6′MBIR, 6MBIR and DBIR: the solubilised quantity of each compound increases when the treatment temperature is raised from 40 to 60 and then to 80 °C. Further increase of the temperature to 100 °C results in a slight decrease of the HPLC peak area. The reduction of the peak area is pronounced when the temperature is further increased to 120 °C. Degradation mechanisms developed at elevated temperatures (100, 120 °C) might be responsible for these results. In summary, treatment of the sample at 80 °C resulted in the best yields for IND, INR, 6′MBIR, 6MBIR and DBIR. The two brominated indigotins appear to be more stable to elevated temperatures, as the HPLC peak areas of MBI and DBI did not reduce at 100 but only at 120 °C. For MBI absolutely no difference was recorded between 80 and 100 °C. For DBI, treatment at 100 °C resulted in a better yield compared to 80 °C. Overall, according to the data provided in Fig. 5 the best yields were recorded when the DMSO bath of the Carthaginian sample was heated at 80 °C to solubilise the purple material.

The effect of treatment time on the efficiency of DMSO to solubilise Tyrian purple is revealed in Fig. 6, which shows that treatment for 1 min results in poor yields. Longer treatment times are necessary to increase the solubilised quantities of the purple components. Treatment time of 15 min is a reasonable selection according to the results of Fig. 6.

3.2. Analysis of *H. trunculus* samples

Using the sample treatment conditions selected according to the above (DMSO, 15 min and 80 °C) four *H. trunculus* samples described in paragraph 2.3 as T1, T2, T3 and T4, were analysed. The relative compositions of the four samples, measured as HPLC peak areas at 288 nm, are shown in Table 4. HPLC data collected in other studies on Muricidae snails, the provenance (region/country) of the molluscs and the solvent used to extract and solubilise the purple dye in each study are included in the table. To the best of our knowledge, Table 4 includes all the relative compositions (HPLC peak areas) of Tyrian purple molluscs that have been reported in the open literature. Relative compositions of samples T8 and B3 (molluscs from the Saronikos sea, Greece) were previously collected by our group [14] but are reported herein for the first time.

It is important to note that the data of Table 4 were measured using 288 nm as monitoring wavelength except for samples T11 (298 nm was used [16]), T12 and H4. The relative compositions of T12 and H4 samples were measured at 283 nm using unpublished HPLC chromatograms that were kindly provided by Dr. M. Papanastasiou (personal communication, 2011).

The four samples investigated here (T1, T2, T3 and T4) are indigotin-rich (IND > 34%) and monobromoindigotin rich (MBI > 31%) *H. trunculus* snail pigments. Relatively small percentages for the other purple components are reported in Table 4.

A comparison of T3 (Tunisian-red, prepared in sunlight) and T4 (Tunisian-blue, prepared in dark) shows that the product T3 contains more brominated indigotins (63.7%) than T4 (43.8%). This difference probably reflects the composition of the four precursors reported for *H. trunculus* [58]. Of the four precursors (1–4), two (1,3) are unsubstituted in the 2-position and consequently will react to produce pigment in the dark, and two (3,4) are brominated. The relative amounts were reported to be 45%, 5%, 30% and 20%. Consequently, in

Table 3
Relative (%) integrated HPLC (288 nm) peak areas measured for the components of Carthaginian purple (T1), after treatment with three solvents.

Solvent	IND	INR	MBI	6′MBIR	6MBIR	DBI	DBIR
DMSO	62.6	1.2	31.9	0.2	0.3	3.7	0.1
DMF	61.6	1.2	32.8	0.2	0.2	3.9	0.1
Pyridine	65.3	0.4	31.0	0.1	0.1	3.0	0.1

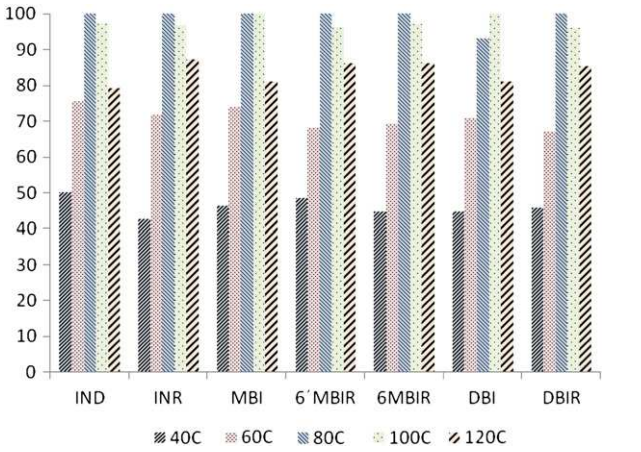

Fig. 5. Integrated HPLC peak areas (288 nm) measured for compounds extracted from Carthaginian purple and normalised to the highest value (taken as 100%) that was recorded for one of the five tested temperatures. Samples were treated with DMSO for 15 min.

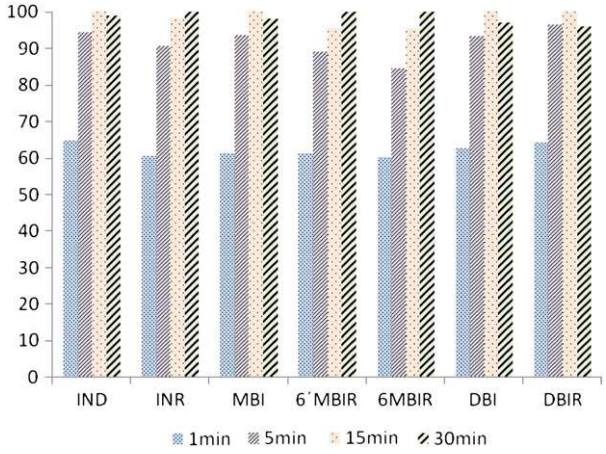

Fig. 6. Integrated HPLC peak areas (288 nm) measured for compounds extracted from Carthaginian purple and normalised to the highest value (taken as 100%) that was recorded for one of the four tested treatment times. Samples were treated with DMSO at 80 °C.

the dark the product would contain 46% (T4 43.8%) of brominated indigotins, and in the light, where all precursors produce pigment, 63% (T3 63.7%) of brominated indigotins. Although these results may be indicative of the different preparation conditions, the reported differences on the relative compositions of the two samples T3 and T4 are within deviations occurring between other *H. trunculus* snails included in Table 4.

3.3. Analysis of historical and archaeological samples

Two historical (E1 and E2) and five archaeological (Ak1, Ak2, Ak3, Ra and Tri) samples were included in the study, as described in paragraph 2.5. As no chemical evidence for the use of Tyrian purple in the two historical samples was available, it was decided first to use XRF prior to HPLC. Because bromine (Br) is contained in several components of Tyrian purple (Fig. 1), non-invasive X-ray techniques can be used to provide evidence about the presence of the purple dye [18]. It is reported that the Kα and Kβ lines of bromine (Br) were recorded in the XRF spectra of both E1 and E2 samples, thus revealing the use of Tyrian purple by the dyers of the Byzantine epitaphios. The presence of Tyrian purple in the five archaeological samples had been previously shown [19,20,23,24] and therefore the application of the XRF method on these samples was not necessary.

The archaeological and historical samples were treated with hot DMSO (80 °C for 15 min) and subjected to HPLC. The chromatograms collected for samples Ak1 and E2 are shown in Fig. 7, as examples. Relative (%) integrated HPLC (288 nm) peak areas are summarized in Table 5 for all historical and archaeological samples. Previously collected, but unpublished, data at 288 nm for Ak1, after treatment with hot DMF, are included in the table [19]. The following conclusions can be reached from the results of Table 5:

i. Monobromoindirubins were detected in very small amounts in the DMSO extract of Ak1. These compounds were not detected in the DMF extract of the same sample, investigated previously [19]. This is probably because DMSO results in better extraction yields for the monobromoindirubins, compared to DMF (Fig. 3). Likewise, we report that monobromoindirubins were not detected in the other archaeological samples (Ak2, Ak3, Tri and Ra) when these were previously treated with DMF [23,24]. According to Table 5, except for Ak2, monobromoindirubins were detected in the archaeological samples after treatment with DMSO. Consequently, the improved DMSO treatment method, suggested in the present report, is more informative regarding the compositions of the purple pigments, which were used in the Late Bronze Age samples of the Aegean islands.

Table 4
Relative (%) integrated HPLC peak areas of Tyrian purple components, investigated in the present (samples T1, T2, T3 and T4) and previous studies, as noted. Data were measured using 288 nm as monitoring wavelength except for samples T11 (298 nm was used), T12 and H4 (283 nm was used), as noted.

	Region	Solvent	Components								Reference	Note
			IND	INR	MBI	6'MBIR	6MBIR	DBI	DBIR	Other		
Hexaplex trunculus L.												
T1	Carthage, Tunisia	DMSO	62.6	1.2	31.9	0.2	0.3	3.7	0.1			Carthaginian sample (paragraph 2.3)
T2	Croatia	DMSO	48.1	4.3	36.3	2.0	1.4	6.8	1.1			Croatian sample (paragraph 2.3)
T3	Tunisia	DMSO	34.9	0.4	49.4	0.3	0.3	14.3	0.4			Tunisian-red sample (paragraph 2.3)
T4	Tunisia	DMSO	53.5	1.5	39.0	0.3	0.7	4.8	0.2			Tunisian-blue sample (paragraph 2.3)
T5	Tarragona, Spain	Pyridine	56.0	0.0	37.0			7.0	0.0		8	Sample was stained cotton (not vat)
T6	Tarragona, Spain	Pyridine	53.0	14.0	33.0			0.0	0.0		8	Sample was dyed wool (vat)
T7	Akhziv, Israel	DMF	4.05	0.0	17.79			60.0	18.16		11,30	Data were collected at 600 nm in [11] and converted to 288 nm in [30]
T8	Saronikos, Greece	DMF	30.2	7.0	26.2	5.9	8.2	13.6	8.9		14	Data were collected in [14] and reported, for the first time, herein
T9	Akhziv, Israel	DMSO	0.35	0.0	7.36	0.0	0.73	67.89	23.68	0.0	30	
T10	Spain	DMSO	38.91	3.19	39.49	1.14	1.84	4.06	9.90	1.46	30	
T11	France	DMSO	5.97	2.17	34.81	4.24	2.57	37.53	8.48	4.23	16	Data were collected at 298 nm
T12	Hermione, Greece	DMF	21.9	5.3	25.6	1.2	4.0	15.6	2.5	24.0	(*)	Data were collected at 283 nm
Bolinus brandaris L.												
B1	Tarragona, Spain	Pyridine	0.0	0.0	0.0			85.0	15.0		8	Sample was stained cotton (not vat)
B2	Tarragona, Spain	Pyridine	0.0	0.0	6.0			81.0	13.0		8	Sample was dyed wool (vat)
B3	Saronikos, Greece	DMF	trace	0.0	1.6	0.0	0.0	97.2	1.2		14	Data were collected in [14] and reported, for the first time, herein
B4	Fiumicino, Italy	DMSO	0.0	0.0	0.85	0.0	0.0	59.27	2.35	37.53	30	
Stramonita haemastoma												
H1	Tarragona, Spain	Pyridine	0.0	0.0	3.0			91.0	6.0		8	Sample was stained cotton (not vat)
H2	Tarragona, Spain	Pyridine	trace	0.0	3.0			91.0	6.0		8	Sample was dyed wool (vat)
H3	Israel	DMSO	0.0	0.0	0.66	0.0	0.0	65.44	10.46	23.45	30	
H4	Hermione, Greece	DMF	30.3	0.0	3.8	0.0	0.0	31.0	1.9	32.9	(*)	Data were collected at 283 nm

(*) Data were calculated using chromatograms which were kindly provided by Dr. M. Papanastasiou (personal communication, 2011).

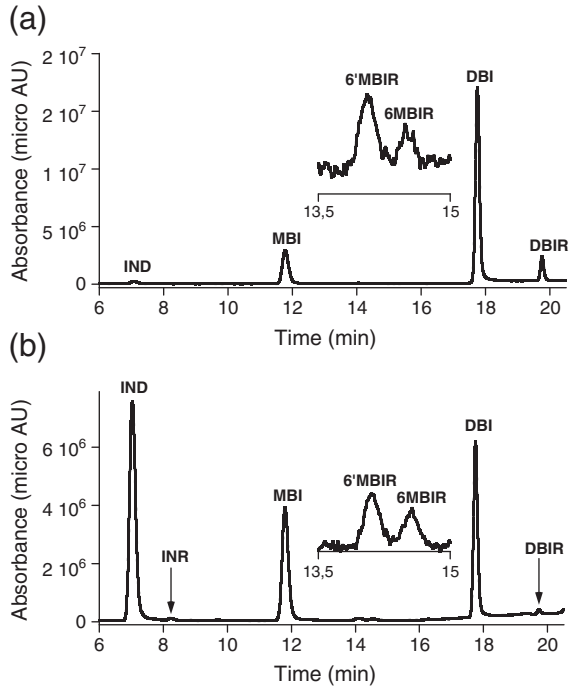

Fig. 7. HPLC chromatograms at 288 nm collected for samples: (a) Ak1, a purple pigment found in Akrotiri, 17th c. BC and (b) E2, removed from a Byzantine epitaphios, 14th c. AC.

ii. The relative compositions reported for the DMSO and DMF extracts of Ak1 do not have major differences, considering that the monobromoindirubins detected only in the DMSO extract were found in very small amounts. This is in agreement with the results of Table 3, which showed that the relative composition of the purple dye is not affected by the solvent (DMSO, DMF or pyridine) selected for sample treatment.

iii. The results reported in Table 5 for the five archaeological (Ak1, Ak2, Ak3, Tri and Ra) samples of the Late Bronze Age, are qualitatively similar, as they all correspond to low amounts of IND (indigotin-poor samples), high, moderate and small amounts of DBI, MBI and DBIR, respectively. INR was not detected and monobromoindirubins were detected in very small amounts. These observations may suggest that similar production methods and/or raw sources may have been applied and used to treat the five samples, excavated from three different Aegean sites [24].

iv. The results reported for the two historical (E1 and E2) samples, removed from the same Byzantine object, are similar as they both correspond to high amounts of IND (indigotin-rich samples) and moderate amounts of MBI and DBI. The other components were detected in very small amounts.

Table 5
Relative (%) integrated HPLC (288 nm) peak areas measured for the archaeological (Ak1, Ak2, Ak3, Tri, Ra) and historical (E1, E2) samples.

Sample	Solvent treatment	IND	INR	MBI	6′MBIR	6MBIR	DBI	DBIR
Ak1	DMF[a]	0.7	0.0	25.9	0.0	0.0	66.3	7.1
	DMSO	1.1	0.0	20.0	0.3	0.1	71.5	7.0
Ak2	DMSO	1.7	0.0	18.8	0.0	0.0	77.6	1.9
Ak3		3.2	0.0	26.9	0.4	0.2	65.5	3.8
Tri		2.5	0.0	24.2	0.5	0.1	65.9	6.9
Ra		1.6	0.0	15.5	tr	tr	77.3	5.6
E1		48.3	tr	25.7	0.4	0.3	24.8	0.5
E2		47.7	0.4	25.2	0.5	0.2	25.4	0.6

[a] Data were collected in [19] and reported, for the first time, herein.

v. Conclusions (iii) and (iv) reveal the differences in the relative compositions of the archaeological and historical samples; major differences are observed in the relative percentages of IND, DBI and DBIR. On the other hand, MBI is contained in similar relative amounts in both archaeological and historical samples, ranging roughly from 15% to 27%. Significant amounts of MBI (>7%) have been recorded only on *H. trunculus* samples according to the data of Table 4 and a previously published report [30]. This may be considered as a first indication regarding the potential use of *H. trunculus* molluscs in the historical and archaeological samples. However, a more systematic investigation must include the total relative compositions of the samples i.e. all the major purple components. For this reason, a PCA analysis is carried out next.

3.4. Principal component analysis (PCA)

A covariance based PCA (Minitab 15) analysis was carried out to investigate if it is possible to achieve the distinction of the three Mediterranean molluscan species, using the data of Table 4, and to identify the biological purple sources of the archaeological and historical samples, using the data of Table 5. Prior to the PCA analysis, some of the data of Table 4 were treated according to the following. (i) As mentioned previously the percentages reported in Table 4 for samples T12 and H4 were measured using unpublished chromatograms, which were collected by Papanastasiou at 283 nm. We converted the original 283 nm to 288 nm values using the relative absorbance of each purple component at the two wavelengths. This approach has been adopted previously by Koren [30]. We followed the same procedure to treat the data of sample T11, as these were originally reported at 298 nm [16]. (ii) Monobromoindirubins were usually not reported in old investigations and therefore the available compositional data for 6′MBIR and 6MBIR are extremely rare. Furthermore, whenever these compounds were reported they were found in very small amounts, as it is evidenced by the data of Table 4. For these reasons, we decided to exclude 6′MBIR and 6MBIR from our considerations for the PCA analysis. The percentage relative compositions were re-calculated considering only five compounds: IND, INR, MBI, DBI and DBIR.

The relative compositions obtained according to the above for the purple molluscs and historical/archaeological samples using the data of Tables 4 and 5 respectively, are summarized in Table 6. The values for the reference, molluscan samples included in Table 6 were subjected to a covariance based PCA analysis. The first two components which explain cumulatively the 98.1% of the structure information of the original four dimensional space (IND, MBI, DBI, DBIR) were used. Then the values for the historical/archaeological samples of Table 6 were included in the PCA plot, shown in Fig. 8. The discussion next focuses initially on the reference, molluscan samples and then it is extended to the historical and archaeological samples.

Fig. 8 suggests that it is impossible to separate *B. brandaris* and *S. haemastoma* species using the data currently available in the literature. The PCA scores obtained for the *brandaris* and for most of the *haemastoma* samples, except for H4, are very similar. The PCA score for the H4 sample is different from the scores obtained for the other three *haemastoma* (H1, H2 and H3) samples. This is because the molluscs from Hermione (H4) are surprisingly rich in IND and poor in DBI. On the contrary, IND is not present (H1, H3) or it is contained as a trace (H2) in the other three *haemastoma* samples, which furthermore contain high amounts of DBI.

Fig. 8 shows that the separation of *H. trunculus* from *brandaris* and *haemastoma* species is not easy, especially when the discussion focuses on T7, T9, and maybe T11, *trunculus* samples. Samples T1, T2, T3, T4, T5, T6, T8, T10 and T12, correspond to negative first principal components (PC1), which results in the separation of these *trunculus* samples from the *brandaris* and *haemastoma* species. According to Fig. 8, the second principal component (PC2) does not contribute

Table 6
Relative (%) integrated HPLC (288 nm) peak areas of molluscs and historical/archaeological samples used for the PCA analysis.

Sample	IND	INR	MBI	DBI	DBIR
Hexaplex trunculus L.					
T1	62.9	1.2	32.1	3.7	0.1
T2	49.8	4.4	37.6	7.1	1.1
T3	35.1	0.4	49.7	14.4	0.4
T4	54.0	1.5	39.4	4.9	0.2
T5	56.0	0.0	37.0	7.0	0.0
T6	53.0	14.0	33.0	0.0	0.0
T7	4.05	0.0	17.79	60.00	18.16
T8	35.2	8.1	30.5	15.8	10.4
T9	0.35	0.00	7.41	68.39	23.85
T10	40.72	3.34	41.33	4.25	10.36
T11[a]	10.3	2.9	44.4	36.8	5.6
T12[b]	27.8	7.5	37.4	23.2	4.1
Bolinus brandaris L.					
B1	0.0	0.0	0.0	85.0	15.0
B2	0.0	0.0	6.0	81.0	13.0
B3	0.0	0.0	1.6	97.2	1.2
B4	0.00	0.00	1.36	94.88	3.76
Stramonita haemastoma					
H1	0.0	0.0	3.0	91.0	6.0
H2	0.0	0.0	3.0	91.0	6.0
H3	0.00	0.00	0.86	85.48	13.66
H4[b]	41.2	0.0	6.0	49.4	3.4
Historical and archaeological samples					
Ak1	1.1	0.0	20.1	71.8	7.0
Ak2	1.7	0.0	18.8	77.6	1.9
Ak3	3.2	0.0	27.1	65.9	3.8
Tri	2.5	0.0	24.4	66.2	6.9
Ra	1.6	0.0	15.5	77.3	5.6
E1	48.6	0.0	25.9	25.0	0.5
E2	48.0	0.4	25.4	25.6	0.6

[a] Data were collected at 298 nm [16] and converted to 288 nm herein.
[b] Data were collected at 283 nm (Papanastasiou) and converted to 288 nm herein.

significantly in the separation of *trunculus* from the other two Mediterranean species. Consequently, our discussion is focused on PC1. It is important to note that the negative sign of PC1 for samples T1, T2, T3, T4, T5, T6, T8, T10 and T12 is originated mainly because of the contributions coming from the relative amounts of IND and DBI; the contributions of the other components to the score of PC1 are not that significant. These *trunculus* samples are rich in IND and poor in DBI. This is not true for samples T7, T9 and T11 which contain small amounts of IND (<11% in Table 6) and relatively high amounts of DBI (>36% in Table 6) corresponding thus to a positive PC1, which brings them close to the, IND-poor and DBI-rich, *brandaris* and *haemastoma* samples. The separation of the Akhziv *trunculus* samples T7 and T9 from *brandaris* and *haemastoma* species becomes further problematic if we consider that these are microsamples from the same pigment, which were analysed with different HPLC instruments and conditions [11,30]. The variation in the PCA scores of T7 and T9, raised because of the different experimental procedures, is comparable with the difference in the PCA scores between the Akhziv *trunculus* samples and the *brandaris*/*haemastoma* samples (Fig. 8).

The two historical samples (E1 and E2) correspond to negative PC1, approaching thus most of the *trunculus* samples. However, overall, the relative compositions of E1 and E2 are not very different from the composition of *haemastoma* sample H4, and this is depicted in the plot of Fig. 8.

The PCA scores obtained for the archaeological samples (Ak1, Ak2, Ak3, Tri and Ra) were comparable, because their relative compositions were similar. Therefore, the data points of these samples are placed close in the PCA plot of Fig. 8. Interestingly, the Akhziv *trunculus* samples, T7 and T9, are included in the same regime with the archaeological samples. Consequently, in a first attempt to comment on the possible biological source of the purple pigments used in the samples of the Late Bronze Age, one could argue that *H. trunculus* could be the molluscan raw source. It is noteworthy that Z. Koren reached the same conclusion for the purple source of the Ak1 sample, following a totally different approach that considered the relative amounts of only MBI and DBI [30]. Koren's calculations were based on relative compositional data which were obtained by our group using DMF for sample treatment. However, considering the discussion provided previously for the reference molluscan samples we have to suggest that more data is necessary to draw a clear conclusion regarding the origin of the purple pigments used in the 17th c. BC samples of the Aegean. This includes the (i) analysis of more molluscs – especially for the *brandaris* and *haemastoma* species where the data is very limited – and (ii) investigation of the effects of several variables (e.g. age and sex of the snails, pigment/dye preparation conditions etc.) on the composition of Tyrian purple.

4. Conclusions

The conclusions of the study are summarized as follows:

- Among DMSO, DMF and pyridine, which have been suggested in the past to treat and solubilise Tyrian purple samples [8,11,14,16,30,49,50], pyridine gave clearly the poorest results considering the extracted and solubilised relative quantities of IND, INR, 6′MBIR, 6MBIR, MBI, DBI and DBIR, measured as HPLC peak areas. The efficiencies of DMSO and DMF were almost comparable, but DMSO resulted in better yields for the brominated indirubins. Furthermore, better signals (i.e. higher HPLC peaks) were recorded in the chromatogram collected after treating the Tyrian purple sample with DMSO. These conclusions were reached after treating the purple samples in solvent baths heated at 80 °C for 15 min.
- Although the solvent affects the HPLC peak areas and heights recorded in the chromatogram, it does not have any major effect on the relative composition of the purple dye. Comparable relative compositions were recorded for Tyrian purple samples treated with DMSO, DMF and pyridine.
- Systematic studies using DMSO for the solubilisation of Tyrian purple showed that the best treatment temperature and time correspond to 80 °C and 15 min, respectively.
- Principal component analysis (PCA) was applied, to investigate if it is possible to achieve the distinction of the three Mediterranean molluscan species (*H. trunculus* L., *B. brandaris* L. and *S. haemastoma*), using all the HPLC quantitative results which could be found (Table 4), including data collected in the present study with the analyses of four *H. trunculus* L. samples from Tunisia and Croatia. The results suggest that it is impossible to separate *B. brandaris* and *S. haemastoma* species (Fig. 8). A slight separation of *H. trunculus* samples from the other two species is recorded in the PCA plot. However, the PCA scores of the *trunculus* samples exhibit a large variation and for this reason the interpretation of the results must be carefully considered.
- The improved method (sample treatment with DMSO at 80 °C for 15 min) was used to extract and solubilise Tyrian purple contained in two historical and five archaeological samples, which were extracted from a Byzantine epitaphios and found in islands of the Aegean Sea, respectively. The presence of true purple in the textile historical samples was first revealed with XRF. The presence of Tyrian purple in the archaeological samples was known from previous studies [19,20,23,24]. The identified compounds and relative (%) integrated HPLC peak areas are summarized in Table 5.
- The improved treatment method resulted in the identification of monobromoindirubins in the DMSO extracts of the archaeological samples, which were not detected in the previous studies where DMF was used for sample treatment [19,20,23,24].
- The relative compositions of the historical and archaeological samples were included in the PCA plot, which provided indications that

Variable	Coefficients	
	PC1	PC2
IND	–0.483	–0.718
MBI	–0.348	0.627
DBI	0.800	–0.183
DBIR	0.075	0.241

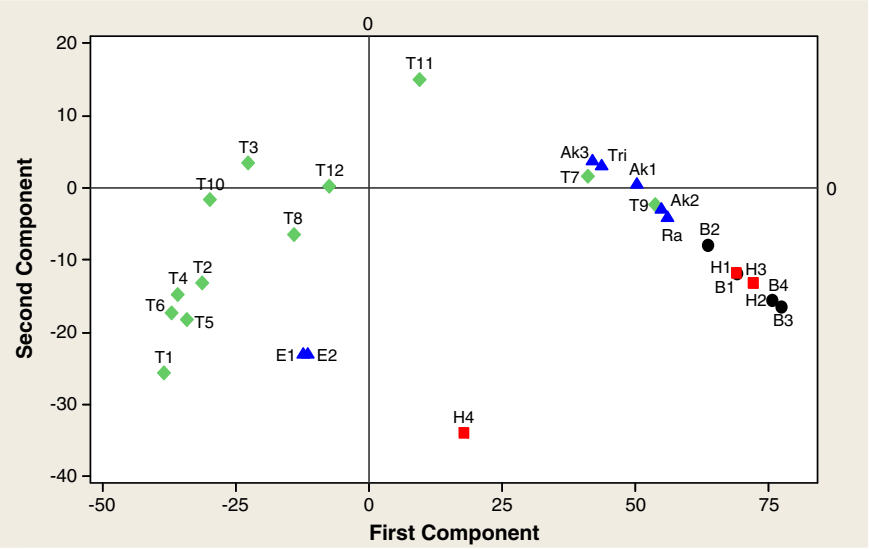

Fig. 8. PCA scores for reference samples of *Hexaplex trunculus* L. ("T" samples, presented by diamonds), *Bolinus brandaris* L. ("B" samples, presented by dots) and *Stramonita haemastoma*, ("H" samples, presented by squares) and historical and archaeological samples (presented by triangles) using the values of Table 6. The coefficients used to obtain the scores for the two components (PC1 and PC2) are shown in the table.

the samples may have been treated with *H. trunculus* molluscs, although this result should be further examined as the distinction of the three Mediterranean mollusc species is not clear, as described above.

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