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Analysis of Indigo-type compounds in natural dyes by negative ion atmospheric pressure photoionization mass spectrometry

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ABSTRACT

Atmospheric Pressure Photoionization (APPI) in Mass Spectrometry (MS) has been utilized for a number of indigo-related compounds and was found to exhibit an excellent response. All structures were ionized in negative ion mode yielding almost exclusively deprotonated molecules. Their product ion mass spectra were also recorded and showed characteristic losses mainly of small neutrals such as CO, HBr and $CONH_2$. APPI-MS was applied further to the analysis of indigo dyestuffs of historical importance. HPLC with single ion monitoring (SIM) was employed for the separation and detection of the compounds. A simple HPLC gradient that separated the components in less than 10 min was developed. MS/MS spectra of the colouring components were also recorded and compared to that of the reference substances. The composition of Tyrian purple originating from *Murex trunculus* (*Hexaplex trunculus*), was by far the most complex, whereas some of the structures were also detected in *Purpura haemastoma* (*Stramonita haemastoma*) and *Plicopurpura pansa* (*Plicopurpura patula* subs. *pansa*). Further, a number of synthetic indigo dyes, produced at different times of the 19thcentury by different manufacturers, were analyzed; similar spectra were obtained suggesting that these were highly pure. Overall, the developed analytical procedure was very efficient offering high sensitivity and selectivity. APPI was proved suitable for ionizing the species under investigation producing clear mass spectra with characteristic fragment ions and may be used successfully in the future for the characterization of similar structures in historical art objects.

1. Introduction

Tyrian purple, also known as Royal purple, Purple of the Ancients and shellfish purple is one of the oldest and most infamous dyes, used since the ancient times. Its cost was inevitably extremely high, being over a hundred times greater than that of garments dyed with kermes and indigo, a mixture of which gave an inferior shade of red–blue [1]. The colour of the dye is derived exclusively from marine shellfish of the Muricidae and Thaididae families. The precursors giving the colour are contained in the hypobranchial glands of the molluscs. In ancient times, these were extracted by salting them and then by heating them in water, after which the garments were immersed in order to 'drink' the dye [2]. In more recent times, the hypobranchial glands were carefully extracted and their content was spread directly on to cloth. Exposure of the garments to air and light was essential for the colour to be developed [3]. The first record of dyeing with Tyrian purple was found in the 16th century BC in Crete [4]. Phoenicians were the forerunners of the art of dyeing with purple and the cities of Tyre and Sidon were established as leaders of the purple dyeing industry [5].

The impressive "iodine like" colour that Hermione was producing was highly prized in the East for over a 1000 of years and, therefore, was established as rival to the red colour of Tyrian purple. The colour difference was attributed to the different species of molluscs processed in the two areas. *Purpura haemastoma* that were processed in Tyre produced a red colour known as the famous Tyrian purple. In Hermione, molluscs of the *Murex trunculus* species were in abundance; these gave a much more intense "iodine like"

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colour compared to Tyrian purple, also known as “porphyra”. The first attempt to investigate the identity of the purple was reported in the late 19th century but was unsuccessful due to limited amount of the dye [6]. Some years later, Friedländer identified 6,6′-dibromoindigo (DBI) as the major colouring component. Since then, a number of analytical techniques have been used to confirm the presence of DBI in various species of molluscs [7].

Indigo may be obtained from species of many different plants found in several parts of the world. These include species of the *Indigofera* genus, *Isatis tinctoria*, or woad and *Polygonum tinctorium*. For dyeing, indigo and its respective isomers needs to be reduced to the respective leuco-form that bonds with the fibers; subsequent oxidation into its original insoluble form allows for the colour to be developed.

In terms of analysis, various classical procedures have been used in the past for the identification of the colouring components of natural dyestuffs, with microchemical analysis, thin-layer chromatography (TLC) and HPLC being amongst the most popular, especially for the analysis of historically important samples [4]. Thin-layer chromatography (TLC) was the method of choice for many years, but was gradually replaced or used in addition to HPLC, since the former was limited to qualitative analysis. HPLC with UV–Vis detection offered higher sensitivity but also lacked specificity since the peaks detected corresponded to the UV absorbance of the compounds. With the advent of API sources, mass spectrometry was coupled successfully to HPLC and since then it has been used for the analysis of indigoid compounds in a number of samples including natural sources [8–13] molluscan species [14], art objects [15–18] and Coptic textiles [19]. The analysis of *M. trunculus* by HPLC–APCI-MS revealed seven indigoid structures with 6-bromoindirubin and 6′-bromoindirubin being unresolved, however, in LC terms [14]. Indigo photo-oxidation products in various solutions have also been reported [20].

Experiments performed in this study have been aimed at an investigation of the response of the APPIion source [21] for ionizing indigo-related structures, since these are coloured dye compounds and a photoionization technique may have advantages over ESI and APCI [22–25]. Indigo-related standards were used as reference samples. APPI was found to exhibit an excellent response and, therefore, was applied further to investigations regarding (i) the composition of Tyrian purple originating from molluscs collected from the Hermione coastlinein Greece (*Murex trunculus*, *P. haemastoma*) and Mexico (*Plicopurpura pansa*) and (ii) the analysis of indigo dyes, part of the collection exhibited in the Manchester Museum of Science and Industry (Manchester, UK). Additional analytical techniques were also employed for the analysis of the samples, including UV/Vis, colour and reflection measurements.

2. Materials and methods

2.1. Chemicals and reagents

N,*N* − dimethylformamide (99.9%, DMF) was purchased from Sigma–Aldrich (Dorset, UK). Acetonitrile (>99.5%, BDH Laboratory Supplies), water and toluene (98%) were HPLC grade (Aldrich, Dorset, UK). Synthetic indigo (IND, 22,929-6) was purchased from Sigma–Aldrich (Dorset, UK). 6-bromoindigo (MBI, 19393) and 6,6′-dibromoindigo (DBI, 13,681) were purchased from Molecular Diversity Preservation International (MDPI, Basel, Switzerland).

2.2. MS and HPLC–MS analysis

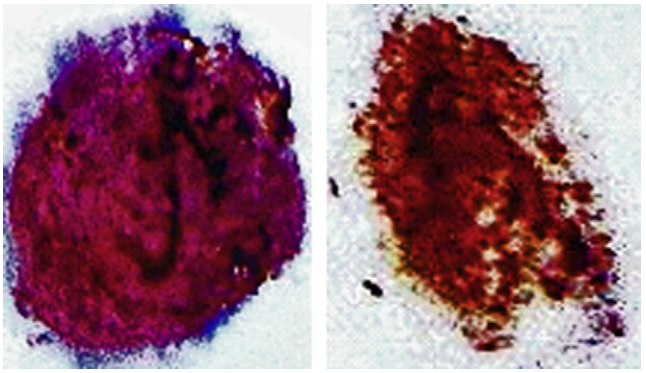
All separations were carried out using an Agilent 1100 (Agilent Technologies) HPLC system. The column was a Phenomenex Luna C18 (2), 150 × 4.6 mm, 3 μm. The mobile phase consisted of H_2O/Acetonitrileand the flow rate was 0.9 mL min^{-1}. Samples were injected and separated at ambient temperature. The DAD signals monitored were 290, 300, 320, 340, 613 nm, chosen according to the UV/Vis absorbance of the compounds. A linear gradient of 65–100% acetonitrile in 10 min was employed and baseline separation of the indigo-related compounds was achieved. The HPLC was interfaced to an API 365 mass spectrometer through an APPI ion source (Applied Biosystems/MDS Sciex). Liquid nitrogen (Cryospeed, Lancaster) blow-off was used as the nebulizer, curtain, collision and lamp gas. A binary pump (Perkin Elmer Series 200 LC) was employed to deliver the HPLC-grade dopant toluene at a flow rate of 0.1 mL min^{-1}. For infusion, the samples were delivered with a Harvard Apparatus syringe pump at a flow rate of 0.1 mL min^{-1}. In this instance, the scan rate of the first quadrupole was set at 1 scan sec^{-1} and the recorded spectra were constructed of 30 scans. For the selected ion monitoring mode, the scan rate of the quadrupole was set at 5 scans s^{-1}. Source parameters were optimized to provide best *S*/*N* for the ions under investigation. These were declustering potential −34 V, focusing potential −102 V, entrance potential −4.2 V, ion spray voltage −1350 V, source temperature 475 °C and nitrogen nebulizer and curtain gas, 9 and 10 L min^{-1} respectively.

2.3. Sample preparation

Indigoid structures are characterized by their low solubility to common organic solvents.[14,15] In our experiments, all standards were dissolved in warm *N*,*N*-dimethylformamide (DMF) at a concentration of 1 mg L^{-1}.

M. trunculus and *Purpura haemastoma*molluscs were freshly acquired by local divers off Hermione coastline (Greece). The glands of the mollusc were directly brushed on cotton and the colour of the dye was developed in response to air (Picture 1). Samples were shipped to Manchester via the National Archaeological Museum (Athens, Greece). Pieces of the fabric were weighed and soaked in DMF and the dye was extracted at 110 °C for 2 min. The solutions were further diluted to a final concentration of approximately 1 mg L^{-1}, as measured by a UV/Vis spectrophotometer (Perkin Elmer, Lambda 40). The same procedure was applied for extracting the dye from wool samples dyed with *P. pansa* originating from Mexico.

The indigo dyes obtained from the Manchester Museum of Science and Industry (Manchester, UK) were in their original form and similarly to the standards were dissolved in warm DMF. The solutions were placed in the ultrasonic bath for approximately 15 min and were further diluted to a final concentration of 1 mg L^{-1}prior to MS and HPLC–APPI-MS analysis.



Picture 1. *Murex trunculus* (left) and *Purpura haemastoma* (right) dyes brushed on cotton.

2.4. Colour characterization by reflectance measurements

The colourimetric properties of the *M. trunculus* and *P. haemastoma* samples were characterized in the *CIE L*a*b** colour space. The instrument settings (Spectroeye™ portable spectrophotometer, GretagMacbeth) chosen were: gas filled tungsten light source, D50 daylight illuminant, 2° observer angle. In addition, values by means of *CIE L*C*h** coordinates were obtained. The reflectance (*%R*) was obtained between 380 and 720 nm at 10 nm intervals. The curve of each dye was the average of five individual measurements at different points of the stains.

3. Results & discussion

3.1. Analysis of standards by APPI

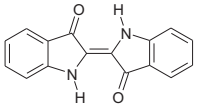
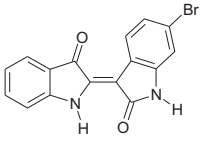
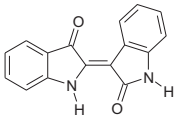
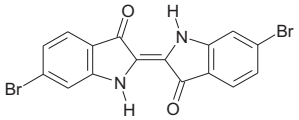
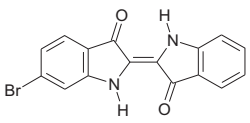
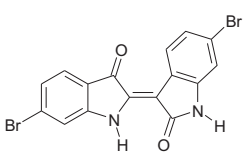
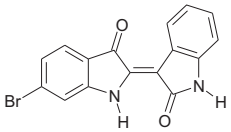
For the optimization of the ion source parameters, indigo, 6-bromoindigo and 6,6′-dibromoindigo were infused in the APPI ion source at a concentration of 1 mg L^{-1}. The structures are depicted in Table 1 along with their chemical formula and respective monoisotopic masses. The MS analysis was undertaken in negative ion mode, due to the high electron affinity of the Br atoms present. Low DP was chosen so as to allow minimum fragmentation of the ions under investigation. The APPI-MS spectra of the standards are shown in Fig. 1. For indigo (Fig. 1A), the base peak corresponded to the $[M - H]^-$ ions detected at *m/z* 261. Loss presumably of a CO from the deprotonated molecule ion and further loss of $-NH_2$ yielded peaks at *m/z* 233 and *m/z* 217, respectively. The loss of a CO group is characteristic of the indigo structure and has been observed under positive EI conditions from the molecular ion [26,27] as well as from protonated molecules formed in CI, ESI and APCI [11,13,16,19]. Loss of the $-NH_2$ group was detected also with these techniques but was undetected under EI conditions. Using API sources, the authors also reported the elimination of both CO groups from the protonated molecules yielding ions at a mass difference of −56 Da. Such a loss was not observed in APPI under the mild ion source conditions utilized. The same fragmentation pattern was observed in tandem MS experiments of the *m/z* 261 ion where indigo fragmented similarly and yielded all the above-mentioned ions (data not shown). The APPI spectra of 6-bromoindigo and 6,6′-dibromoindigo are shown in Fig. 1B and C respectively. MBI yielded $[M - H]^-$ ions as the base peak at *m/z* 339/341 with the characteristic isotopic pattern (1:1) of the bromine atom. Fragment ions were produced at lower masses and were attributed to the losses of $-CONH_2$ (*m/z* 295/297) and HBr (*m/z* 259) from the deprotonated molecule. Tandem MS experiments for the *m/z* 259 ion produced fragment ions at *m/z* 231 (−28 Da), *m/z* 215 (−44 Da) and *m/z* 156 (−103 Da), characteristic losses of the indigo molecule (data not shown). Fragmentation of the central double carbon bond and further elimination of a CO neutralproduced the doublet at *m/z* 196/198. These ions were also observed in the analysis of 5-bromoisatin (monoisotopic mass 224.94 Da), an isomer of 6-bromoisatin that is an intermediate to the generation pathway of purple originating from molluscs [7] (data not shown here). In DBI MS analysis, the base peak corresponded to the triplet deprotonated molecules detected at *m/z* 417/419/421 ($[M - H]^-$). The characteristic pattern of 1:2:1 clearly indicates the presence of two bromines in the structure. Similar fragmentation to that of MBI was observed with ions arising from the loss of $-CONH_2$ (*m/z* 373/375/377) and HBr (*m/z* 337/339).

A mixture of IND, MBI and DBI standards were prepared at a ratio of 1:1:1 at a final concentration of 1 mg L^{-1} and were then injected into the HPLC. The DAD was connected online and the signal at 600 nm was monitored. The MS was operated in the selected ion monitoring (SIM) mode for the $[M - H]^-$ ions at *m/z* 261 (IND), *m/z* 339 (MBI) and *m/z* 419 (DBI). The resulting chromatograms are depicted in Fig. 2.The MS was significantly more sensitive than the DAD in which extensive ramping of the baseline occurred and the signal was very weak. Limits of detection (LODs) were calculated at1 pg for the $[M - H]^-$ ions in SIM.

3.2. Analysis of Tyrian purple samples

Two pieces of dyed cotton were obtained; one was stained with the dye produced by *M. trunculus* and the other by *Purpura haemastoma* molluscs collected on the Hermione coast in Greece. The

Table 1
Structures of the proposed constituents of Tyrian purple.

Name	Structure	Name	Structure
Indigo (IND) $C_{16}H_{10}O_2N_2$ 262.07 amu		6′-bromoindirubin (MBR′) $C_{16}H_9O_2N_2Br$	
Indirubin (INR) $C_{16}H_{10}O_2N_2$		6,6′-dibromoindigo (DBI) $C_{16}H_8O_2N_2Br_2$ 417.90 amu	
6-bromoindigo (MBI) $C_{16}H_9O_2N_2Br$		6,6′-dibromoindirubin (DBR) $C_{16}H_8O_2N_2Br_2$	
6-bromoindirubin (MBR) $C_{16}H_9O_2N_2Br$ 339.98 amu			

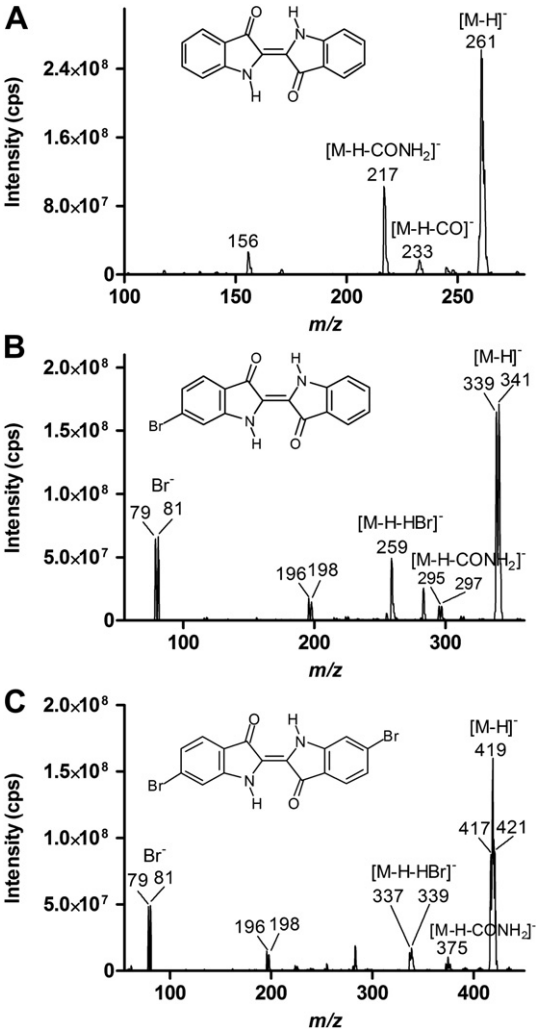


Fig. 1. APPI-MS spectra of IND (A), MBI (B) and DBI (C) standards in DMF.

glands were extracted and their content was directly brushed on the wool; the colour was developed on exposure to air. For the analysis of the stains and before these were extracted into solution, non-destructive techniques were utilized. These included the measurement of the colourimetric properties of each of the stains according to the *CIE L*a*b** and *CIE L*C*h** coordinate systems using a reflectance spectrophotometer, the reflection of the dyes on the visible range and elemental analysis by SEM. The dyes were then transferred to solution and their UV/Vis absorbance spectra were recorded followed by MS and HPLC–APPI-MS analysis.

3.2.1. Colour characterization

The colour characterization of the stains was obtained by reflectance using a colourimeter (SpectroEye™, Gretag Macbeth), calibrated to a white standard surface ($L^* = 94.81$, $a^* = 2.28$, $b^* = -8.47$ & $L^* = 93.03$, $C^* = 8.93$, $h^* = 275.61$). Random readings were taken from the dyed areas since the stains were non-uniform and darker and lighter areas were formed. The reference was subtracted from the sample readings (average of three measurements on each spot) and their average value is given in Table 2 along with their standard deviation. The high value of the standard deviation is attributed to the in-homogeneity of the stains, since the dyes were found to exhibit similar lightness (L^* axis) with values obtained in the range of −34 to −42. In the red–green region both species exhibited positive values towards red with *P. haemastoma* showing slightly higher values than *M. trunculus*. Differences were obtained in the blue–yellow axis with *M. trunculus* exhibiting negative values (towards blue), whereas *P. haemastoma* positive values (towards yellow). The visible reflectance spectra of the two dyes were also recorded. Both dyes gave identical spectra with maxima at 430 nm and 520 nm (data not shown). The latter is indicative of the presence of DBI on the wool. *M. trunculus* showed an additional shoulder at about 650 nm, an area where the absorption of indigo would be expected; this peak has been identified before on dyed wool with *M. trunculus* [28].

After the dyes were transferred in DMF, their UV/Vis absorbance (Perkin Elmer, Lambda 40) was recorded. The visible absorbance of indigoid compounds has been studied extensively and was shown to vary with solvent, concentration, temperature and chemical substituents of the indigo ring [7]. Bromination of the indigo ring was shown to have a small effect on λ_{max} and indigoid compounds generally show a strong absorption maximum in the region of 605 nm. Both of the dyes exhibited strong absorption in the regions of 300 and 610 nm. The results were identical with Tyrian purple in DMF originating from the Mexican snail *P. pansa* [29].

3.2.2. APPI-MS & HPLC–APPI-SIM analysis

The first mass spectrometric studies on the composition of *M. trunculus* were reported early on the 1990s, when EI high resolution MS was utilized to investigate the composition of the dye produced by the molluscs.[9] The authors identified indigo and 6,6′-dibromoindigo as major constituents of the dye; the presence of 6-bromoindigo was also addressed but questions were raised by the same group since mono-debromination of DBI during volatilization was observed yielding MBI.[8] MBI was detected in *Murex* extracts, along with that of indirubin a year later by HPLC–UV/Vis [30] and verified by EI MS at almost the same period.[10] Data were presented by the group of Karapanagiotis who analyzed the composition of *M. trunculus* and identified all the above components along with the isomers of MBI (MBR and MBR′) by HPLC–DAD.[14]These analyses verified the observations of Baker

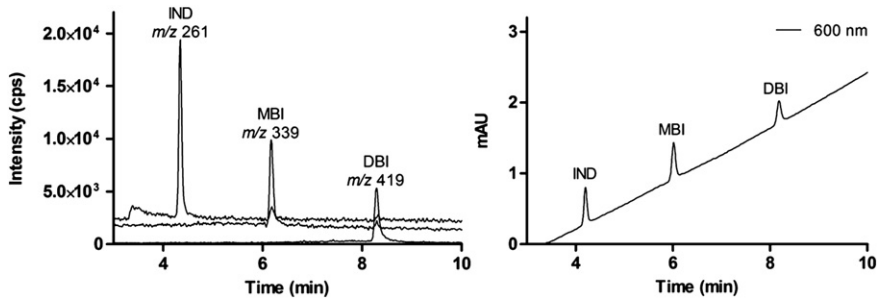


Fig. 2. HPLC–DAD-SIM of a mixture of IND, MBI, DBI standards in DMF.

Table 2
*CIE L*a*b** and *CIE L*C*h** color space measurements of the two dyes.

	L^*	a^*	b^*	L^*	C^*	h^*
Murex trunculus	−38.9	14.4	−1.9	−34.5	11.2	47.6
	±5.5	±3.6	±2.2	±4.5	±2.8	±7.7
Purpura haemastoma	−38.0	16.5	7.8	−35.4	11.0	72.5
	±7.7	±4.6	±6.1	±7.3	±4.1	±5.1

who by investigating the chemistry of various shellfish concluded that, by far, the most complex gland composition is that shown by *M. trunculus* [31]. *P. haemastoma* species have been analyzed to a much lesser extent; in 1992 Wouters reported DBI as a major colour constituent of the dye and provided spectral data (UV absorbance) of a second indigoid compound proposed to be DBR [32]. The latter was identified by Karapanagiotis' group [14].

The mass spectra of the dyes originating from *M. trunculus* and *P. haemastoma* stains are depicted in Fig. 3.The MS analysis of *M. trunculus* (Fig. 3A) showed deprotonated molecules for IND, MBI and DBI at *m*/*z* 261, *m*/*z* 339 and *m*/*z* 417, with their characteristic fragments detected at lower masses. MS/MS experiments for all indigoid structures were also undertaken and were shown to be consistent with the product ion mass spectra of the standards (data not shown). HPLC was essential for the separation of the indigoid isomers, indirubins. The ions chosen to be monitored over time were the *m*/*z* 261 (IND, INR), *m*/*z* 339 (MBI, MBR, MBR′) and *m*/*z* 419 (DBI, DBR) and are presented in Fig. 4. The DAD was also connected online but was operated in the spectral mode where the UV/Vis spectra of all the eluted components were recorded in the range of 270–650 nm. The UV/Vis spectra of the bromoindirubins are also featured (inset) since at the time of the analysis the presence of MBR and MBR′ had not been reported in the literature, whereas DBR had been proposed but not verified.[33] The MBR and MBR′ exhibited maxima at 300, 366 and 540 nm and were in very good agreement with the spectra of synthesized bromoindirubins in *N*,*N*-dimethylformamide; the DBR maxima were slightly higher due to the second bromine atom contained in the molecule [34].

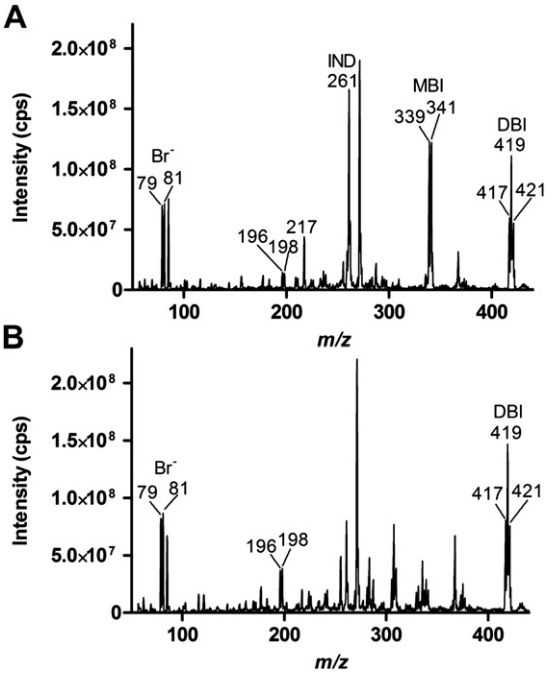


Fig. 3. APPI-MS analysis of the dyes obtained from *Murex trunculus* (A) and *Purpura haemastoma* (B).

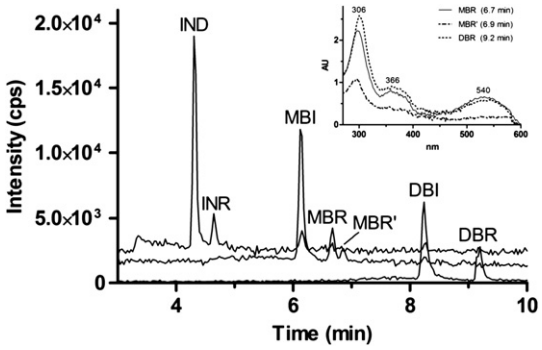


Fig. 4. HPLC–SIM-MS of the dye originating from *Murex trunculus* molluscs; the UV/Vis spectra of MBR, MBR′ and DBR are also depicted (inset).

The mass spectrometric analysis of *P. haemastoma* showed the presence of DBR mainly as well as that of MBI at a very low abundance (Fig. 3B). Other ions were also detected in the range of *m*/*z* 260–380; none of them could be identified as fragments of DBI and MS/MS studies were inconclusive for their structural identification. The corresponding chromatogram of the sample is shown in Fig. 5. DBI and DBR produced major peaks whereas MBI and its respective indirubins were of very low abundance. Those analogs are reported herein for the first time to be present in *P. haemastoma* species.

Plicopurpura pansa is another source of Tyrian purple. The chemistry involved in obtaining the dye is rather different to that of other Muricidae. In the Muricidae molluscs, the precursors are indoxyls, brominated and non-brominated that lead to a range of compounds including IND, MBI and DBI and to their respective isomers. *P. pansa* however contain brominated indoxyls that lead solely to the formation of brominated products.[29] Another major difference is that the hypobranchial gland of the Muricidae species needs to be extracted in order to obtain the dye and, therefore, the animals are killed. In contrast, *P. pansa* may be 'milked' to obtain the dye by pressing slightly the valve that closes its mouth without harming the animals. A few drops of the colouring fluid are obtained thus and the colour is developed. The animals are then returned to the sea and repetitive milking may take place since their hypobranchial gland is very active [35].

The MS analysis of *P. pansa* composition (data not shown) showed DBI as the major peak and was consistent with studies showing DBI as the main colourant of the dye [29,36]. The identity of the ion was confirmed further by MS/MS. At a higher mass, another bromine-containing ion was detected at *m*/*z* 435 indicating probably an oxidation product of DBI (+16 Da); its product ion spectrum yielded ions at *m*/*z* 391 (−44 Da presumably corresponding to CO_2) and bromide ions. MBI (*m*/*z* 339) was present at

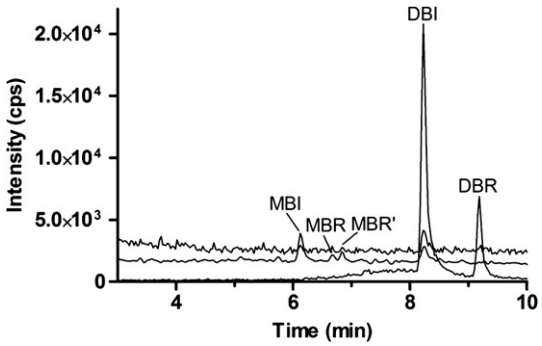


Fig. 5. HPLC–SIM-MS of the dye originating from *Purpura haemastoma* molluscs.

low levels. At the low mass end of the spectrum, ions were detected at m/z 224/226 corresponding to the $[M - H]^-$ of 6-bromoisatin. 6-bromoisatin is an intermediate in the formation pathway of 6,6′-dibromoindirubin [36]. Its identity was verified by MS/MS and produced characteristic fragment ions at m/z 196, m/z 146, m/z 117 and m/z 79, also observed in the tandem MS analysis of the corresponding standard. The presence of 6-bromoisatin, in combination with the absence of 6,6′-dibromoindirubin indicated that probably the time allowed for the colour formation reaction to be completed was insufficient.

3.3. Analysis of indigo dyes

Seven indigo dyes were kindly provided by the Manchester Museum of Science & Industry (MSIM); these were part of the dyes collection exhibited in MSIM. The dyes were synthesized by different manufacturers at different periods in the 20th century, though precise information was not available. The aim of this work was to investigate their purity.

Dyes were in the form of powders or minerals.Minute amounts were weighed and dissolved in DMF to a final concentration of 1 mg L^{-1}. The UV/Vis absorbance of all dyes gave identical UV/Vis spectra (613 nm maximum) and were in accordance with UV/Vis spectra reported in the literature [7,30] as well as with the indigo standard used in this study. For the MS analysis, a high declustering potential of −49.90 V was utilized in the APPI ion source to allow for in-source fragmentation of the dyes; the focusing potential was optimized accordingly and was −142.3 V. All mass spectra were identical, with fragment ions detected at a higher abundance compared to the spectrum of indigo (Fig. 1A) that was recorded under milder declustering potential (−34 V). The base peak corresponded to the m/z 217 produced by the elimination of the $-CONH_2$ group from the $[M - H]^-$ ion. The deprotonated molecules of indigo were detected at m/z 261 and m/z 260 corresponding to $[M - H]^-$ and $[M - H_2]^-$ ions. Losses of a CO neutral from those yielded fragment ions at m/z 233 and m/z 232, respectively [13,26,27]. The m/z 205 peak was assigned as the fragment ion produced from the elimination of both CO groups from the $[M - H]^-$ deprotonated molecule, detected also in MS studies of indigo with ESI and APCI [16,19]. A high abundance peak was detected at m/z 156 but could not be assigned; MS/MS of this ion failed to produce fragment ions at lower masses, even at higher energies (50 eV), indicating a very stable structure. Tandem MS experiments were conducted for the $[M - H]^-$ and $[M - H_2]^-$ for all indigo dyes to verify the origin of the previously mentioned ions; the same fragmentation pattern was observed for all dyes yielding the characteristic losses as discussed above. To investigate the purity of the dyes further, the samples were analyzed by HPLC–APPI-MS. No detectable impurities were found. Of interest, however, was the brownish colour of the solutions produced, when they were left exposed to the laboratory daylight conditions for three days. Different shades of brown were formed in this period, indicating different kinetics of degradation. Preliminary results showed the presence of is at in as a degradation product, but this is to be investigated further under controlled photo-degradation conditions.

4. Conclusions

The applicability of the APPI ion source was tested for a number of indigo-related compounds and exhibited an excellent response. All structures were ionized in negative ion mode yielding almost exclusively deprotonated molecules. Their product ion mass spectra were also recorded and showed characteristic losses mainly of neutrals such as CO and HBr. APPI-MS was applied further to the analysis of indigo dyestuffs of historical importance.

M. trunculus was found to exhibit by far the most complex composition, producing an intense 'iodine-like' colour. Analysis of *P. haemastoma* showed a simpler composition constituting mainly of DBI and its respective indirubin. The high sensitivity of the ion source also revealed the presence of minute amounts of MBI and its analogs in the species. DBI was also found to be the main constituent of the colour produced by *P. pansa*. Deviations obtained in the chemical analysis of the molluscs provided at different periods of time suggested that the final composition and, therefore, the colour of the dyes are affected by a number of parameters (i.e., the time of the year, the environmental and physiological condition of the animals, the oxygen availability, the age, gender and prey of the species) and therefore cannot be used as a robust criterion for distinguishing the various species of molluscs. Other methodologies that will take into consideration these parameters need to be developed, as mass spectrometry is limited to the identification of the constituents of individual dye samples.

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