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Short communication

Mass spectrometric identification of new minor indigoids in shellfish purple dye from *Hexaplex trunculus*

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ABSTRACT

Analysis of natural dyes in historical objects is important for both conservation purposes and to determine the origin and culture that produced it. Identification of a particular dye is usually made based on the presence of its main components, while consideration of minor components is important for differentiating between dyes originating from closely related species. Tyrian purple is one of the oldest dyes known to man and derives from different species of marine molluscs. In all of these species, indigotin, indirubin and their brominated analogues are the main colouring compounds. Here, we describe the identification of minor indigoids found in extracts of the pigment obtained from one of the Tyrian purple species, *Hexaplex trunculus*. Identification of these compounds was made based on isotopic patterns and accurate mass measurements of protonated molecular ions and their high collision energy fragments obtained in LC–MS/MS experiments. The unknown compounds appeared to be analogues of indirubin and its mono- and dibrominated derivatives with one CO group in the indirubin backbone substituted by a CNH group. Identification of these compounds facilitates the detection of dyestuffs from *H. trunculus* in historical objects and increases our knowledge about the dye biosynthesis and technology of Tyrian purple production.

1. Introduction

Tyrian purple (Royal purple, shellfish purple or Purple of the Ancients) is a natural dye discovered at least 3500 years ago, which can be obtained from the hypobranchial glands of marine molluscs, such as Mediterranean shellfishes *Hexaplex trunculus* L., *Bolinus brandaris* L. and *Stramonita haemastoma*, along with more than 20 other species distributed around the world [1–3]. The dyestuff was used in ancient times for textile dyeing, ceramic colouring and painting pigment preparation. Tyrian purple has characteristic dark blue to violet shades due to its main colouring components: indigotin, indirubin and their brominated derivatives [1,4]. Characterisation of different molluscs species used to produce Tyrian purple, by extraction of the main colouring components with specific solvents and their subsequent analysis using reversed-phase HPLC systems coupled to UV–vis and/or MS detection or MS alone, has been described previously [1,5–11].

Identification of minor components in extracts from historical purple dyes is important for several reasons. Firstly, the presence of specific components and their ratios can often be used to differentiate between pigments from closely related species [1,6,12,13] or even different genders of molluscs used for dyeing [14]. This may be crucial information for determining the origin of the object. Additionally, identification of minor components can be helpful in elucidating dye synthesis pathways, either at the precursor level in animals or in the dye production process. In the latter case, the presence of minor components may provide additional clues about the technological skills of the culture that produced the dye.

The aim of our study was to identify four unknown indigoids detected previously in extract from *H. trunculus* by LC–UV–vis spectroscopy [15]. These compounds were proposed to have *cis*-indirubin structures on the basis of comparative spectral and retention data [9,16]. In this work we adopted an LC–MS/MS approach to identify the unknown indigoids using both the isotopic patterns and accurate mass measurements of their protonated molecular ions, as well as comparison of their high collision dissociation (HCD) fragmentation patterns with those obtained for known indigoids present in the same extract. This

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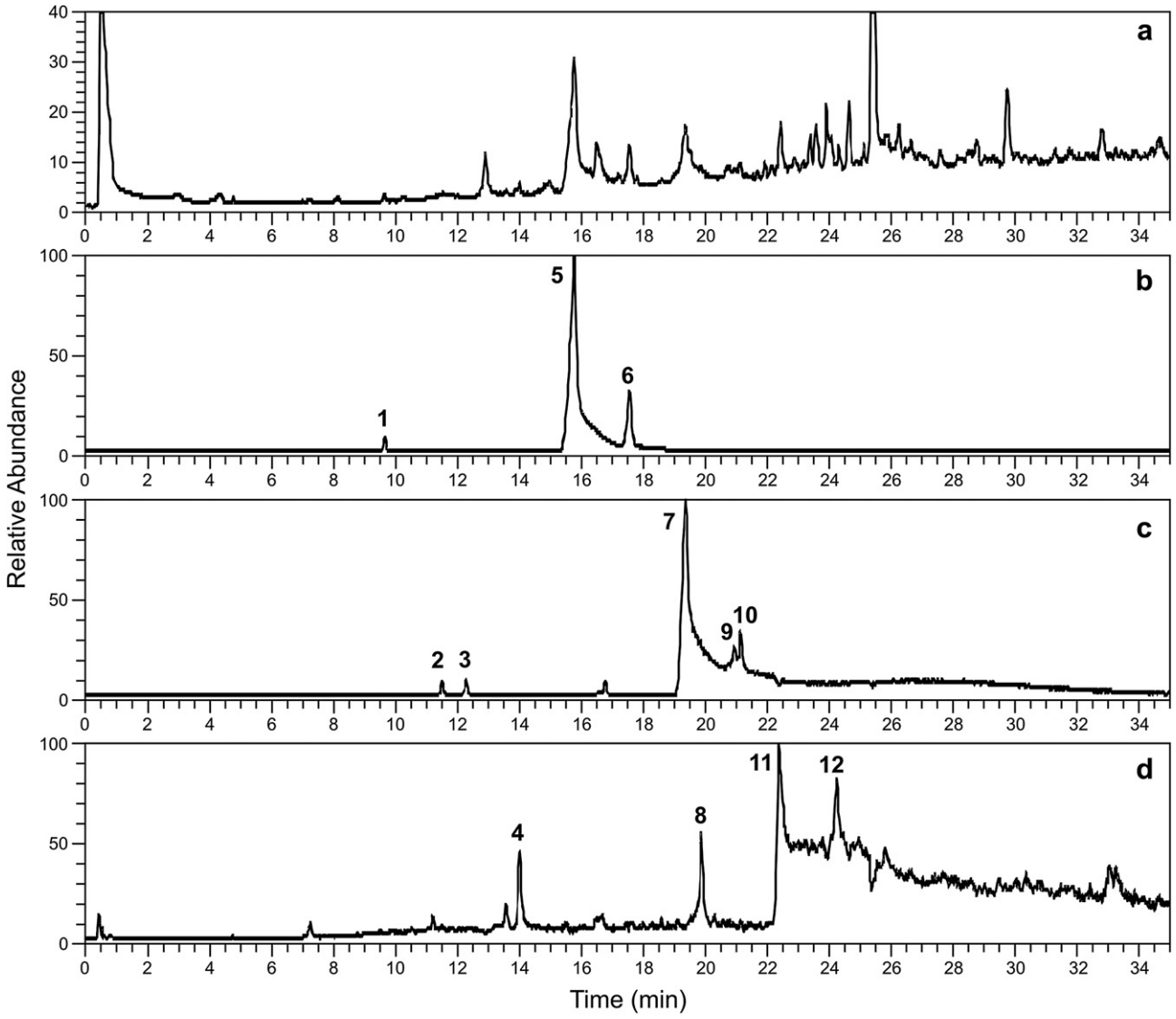

Fig. 1. TIC of the extract from *Hexaplex trunculus* dye (a) and chromatograms for selected mass ranges (b – *m*/*z*: 261.5–263.5, c – *m*/*z*: 340–341 and d – *m*/*z*: 417–423) showing previously identified compounds: 5 – indigotin, 6 – indirubin, 7 – 6-bromoindigotin, 9 – 6-bromoindirubin, 10 – 6′-bromoindirubin, 8 – 6,6′-dibromo-*iso*-indigotin, 11 – 6,6′-dibromoindigotin, 12 – 6,6′-dibromoindirubin, and four not identified indigoid derivatives: 1 – compound A, 2 – compound B, 3 – compound C, 4 – compound D.

approach confirmed the presence of an indirubin skeleton for the newly characterised compounds. Further, rather than *cis*-indirubins as suggested by previous studies [15,16], our results demonstrate they are *trans*-structures, containing an imine group in place of the ketone group on the pyrrole moiety.

2. Materials and methods

2.1. Chemicals and standards

Acetonitrile (HPLC grade) was purchased from Fischer Scientific (Loughborough, UK), formic acid (puriss p.a., for mass spectroscopy – 98%) was purchased from Sigma–Aldrich (Steinheim, Germany), dimethyl sulfoxide (DMSO) (reagent ACS) was purchased from Acros Organics (Geel, Belgium), methanol (HPLC grade) was obtained from J.T. Baker (Deventer, Holland); all aqueous solutions were prepared using deionised MilliQ water. Indigotin (Ind), 6-bromoindigotin (6-BrInd), 6,6′-dibromoindigotin (6,6′-2BrInd), 6,6′-dibromo-*iso*-indigotin (6,6′-2Br-*iso*-Ind), indirubin (Inr) and 6′-bromoindirubin (6′-BrInr) standards were provided by Dr. Christopher J. Cooksey (Watford, United Kingdom). Identification of 6-bromoindirubin (6-BrInr) was carried out by comparison with previously obtained chromatographic data [15].

2.2. Sample of purple pigment and dye extraction

The *H. trunculus* purple sample was supplied by Inge Bösken-Kanold (Lacoste, France). The pigment had been obtained by precipitation of the dye on a talc substrate using local molluscs from the Mediterranean French coast. No further information about the preparation procedure for this sample is known. To extract the dye, 16.6 mg of sample was placed in an Eppendorf tube and extracted with 1 mL DMSO for 25 min in an ultrasonic bath, then filtered using a centrifuge filter Ultrafree-MC, Durapore PVDF, 0.22 μm pore size from Millipore (Bedford, MA, USA) at 14000 rpm for 15 min at room temperature. The obtained filtrate was submitted for LC–MS/MS analysis.

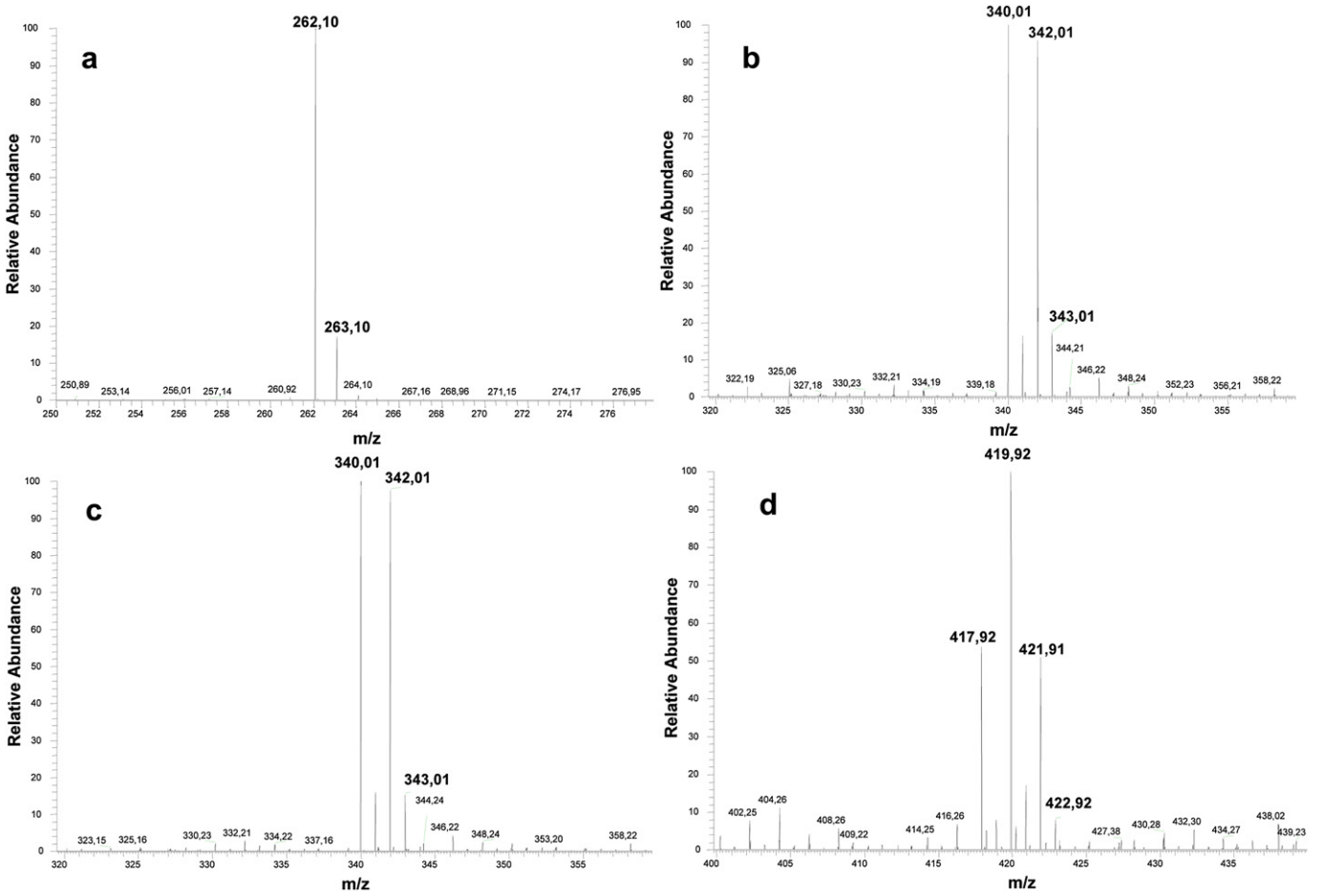

Fig. 2. Mass spectra of unknown compounds A–D obtained in ESI positive ion mode: a) unknown compound A, b) unknown compound B, c) unknown compound C, d) unknown compound D.

2.3. Liquid chromatography mass spectrometry

All LC–MS/MS analyses were carried out using an LTQ Orbitrap XL system with a HESI source coupled to an Accela LC system comprising an HPLC with a built-in degasser and autosampler units (Thermo Scientific, San Jose, CA, USA). 5 μL of the sample was injected on a Hypersil Gold C18 column (50 × 2.1 mm i.d., 1.9 μm; Palo Alto, CA, USA) maintained at 40 °C. A constant flow rate of 0.3 mL/min was used with the following gradient: 5% B isocratic for 1 min, then up to 95% B for 40 min followed by isocratic 95% B for 5 min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The column was re-equilibrated for 5 min before the next injection.

The LTQ Orbitrap XL was calibrated in the positive ion mode on the day of analysis using a mixture of caffeine, MRFA peptide and Ultramark 1600. The instrument settings were subsequently tuned with indigotin solution in methanol following a standard optimization procedure for all lens voltages and settings of the HESI

Table 1
Accurate mass measurements of $[M + H]^+$ ions for known and unknown indigoid compounds in extract from dye sample obtained with *Hexaplex trunculus*; in all cases mass of the lowest mass isotope in the MS spectrum is presented.

Number	Compound	t_R [min]	Elemental composition of $[M + H]^+$ ion	Calculated mass of $[M + H]^+$ ion	Measured mass of $[M + H]^+$ ion	Error of the mass measurement [ppm]
1	Compound A	9.7	$C_{16}H_{12}ON_3$	262.0980	262.0981	0.16
2	Compound B	11.5	$C_{16}H_{11}ON_3Br$	340.0085	340.0087	0.45
3	Compound C	12.3	$C_{16}H_{11}ON_3Br$	340.0085	340.0085	−0.11
4	Compound D	14.0	$C_{16}H_{10}ON_3Br_2$	417.9191	417.9191	0.17
5	Indigotin	15.8	$C_{16}H_{11}O_2N_2$	263.0821	263.0819	−0.43
6	Indirubin	17.6	$C_{16}H_{11}O_2N_2$	263.0821	263.0821	0.25
7	6-Monobromindigotin	19.4	$C_{16}H_{10}O_2N_2Br$	340.9926	340.9926	0.13
8	6,6′-Dibromo-*iso*-indigotin	19.9	$C_{16}H_9O_2N_2Br_2$	418.9031	418.9031	−0.06
9	6-Monobromoindirubin	21.0	$C_{16}H_{10}O_2N_2Br$	340.9926	340.9927	0.34
10	6′-Monobromoindirubin	21.2	$C_{16}H_{10}O_2N_2Br$	340.9926	340.9927	0.25
11	6,6′-Dibromoindigotin	22.4	$C_{16}H_9O_2N_2Br_2$	418.9031	418.9030	−0.30
12	6,6′-Dibromoindirubin	24.3	$C_{16}H_9O_2N_2Br_2$	418.9031	418.9030	−0.13

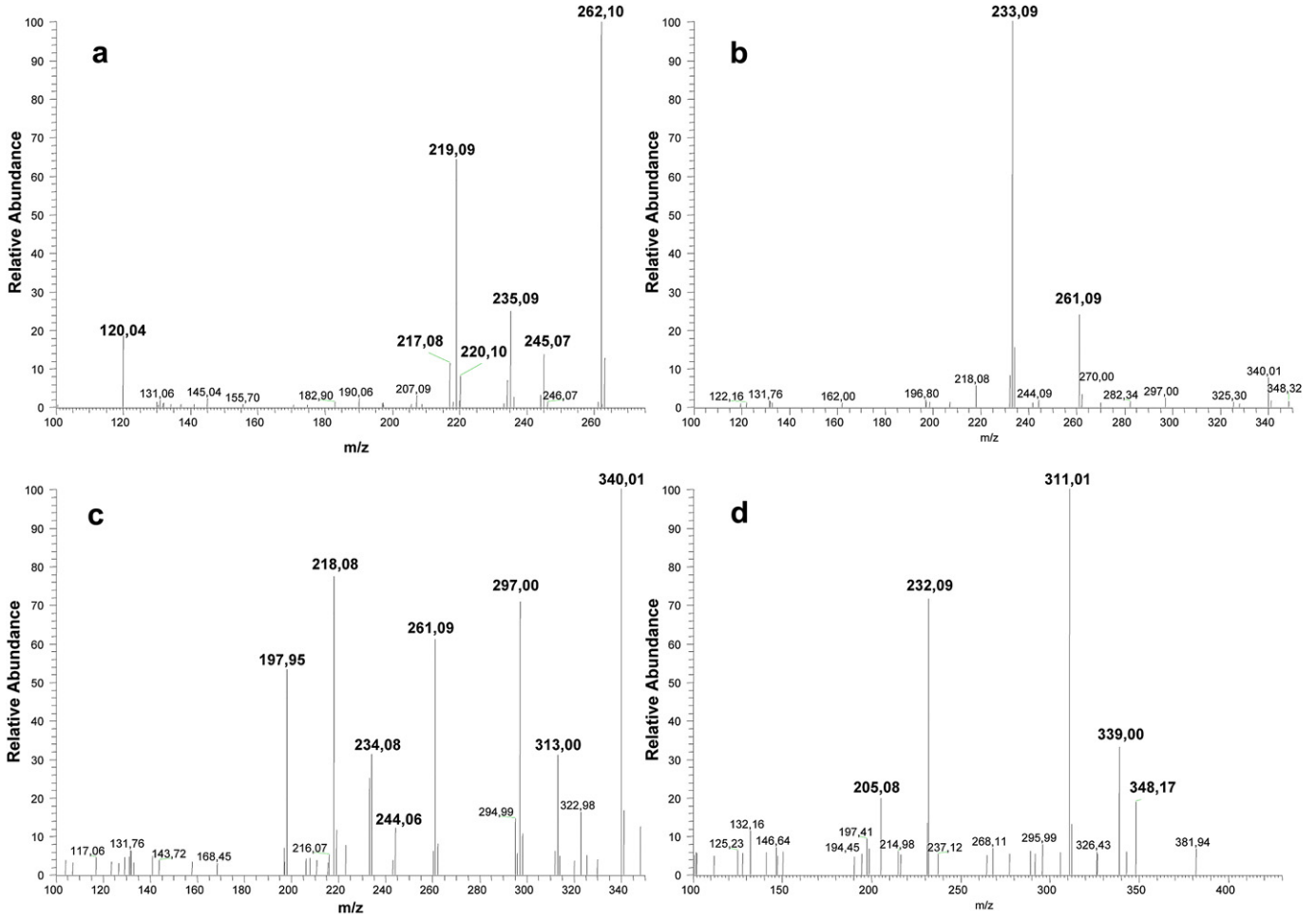

Fig. 3. MS/MS spectra acquired in HCD cell for normalised collision energy equal 55: a) unknown compound A, b) unknown compound B, c) unknown compound C, d) unknown compound D.

source. The HPLC eluent was sprayed into the mass spectrometer at 4.5 kV, with nebulizer and sheath gases set to 52 and 36 arbitrary units, respectively. The heated capillary temperature was 310 °C and the heater temperature was 300 °C. The capillary voltage and tube lenses were set to 41 V and 110 V, respectively. Data were acquired using a scan range *m*/*z* 150–1500 in positive ion mode. A scan event cycle comprised a full scan mass spectrum at a resolving power of 30.000 (at *m*/*z* 400) and three corresponding data-dependent MS/MS events acquired at a resolving power of 7500. Data-dependent HCD fragmentation was triggered by the most abundant ion from the parent mass list, with the value of the minimal signal set to 500 and employing normalised collision energies of 15, 35 and 55; the most informative was the 55 collision energy (more fragments were observed) and spectra obtained for this energy were used for structure elucidation. Data acquisition and evaluation was performed with Xcalibur software version 2.0.7 (Thermo Scientific, San Jose, CA, USA). Possible fragmentation pathways of the compounds of interest were evaluated with Mass Frontier™ 5.0 software (Thermo Scientific, San Jose, CA, USA).

3. Results and discussion

In Fig. 1, a total ion chromatogram (TIC) and chromatograms for selected mass ranges of the extract from the *H. trunculus* sample are presented, showing it contains previously identified indigotin, indirubin and their derivatives (6-bromoindigotin, 6- and 6′-bromoindirubins as well as 6,6′-dibromoindigotin, 6,6′-dibromo-*iso*-indigotin and 6,6′-dibromoindirubin). In addition, several compounds that were not identified based on UV–vis spectral characteristics of indigoids but were proposed to have indirubin-like structures were also detected (compounds A–D) [15]. Peak tailing was observed for the major compounds present in the sample (indigotin, 6-bromoindigotin and 6,6′-dibromoindigotin), which is a typical feature when purple is subjected to reversed-phase chromatography. The effect is particularly pronounced when highly concentrated extracts are analysed to monitor minor components present in the dye and may be avoided by heating the column [15].

3.1. Mass spectra

In Fig. 2, mass spectra of unknown compounds A–D are presented. For all the indigoid standards used, only $[M+H]^+$ ions were observed; hence, we assumed that the mass spectra of the unknown compounds also corresponded to $[M+H]^+$ ions. This was supported by the fact that no other ions arising from loss of some functional groups (ex. CO_2 or H_2O) from the molecular ion in the HESI source were observed. Compound A had one unit lower mass than indigotin and indirubin. Compounds B and C had one unit lower masses than monobrominated indigoids and exhibited the same isotopic pattern, which is characteristic of molecules with one bromine atom present in the structure (ratio of M and M+2

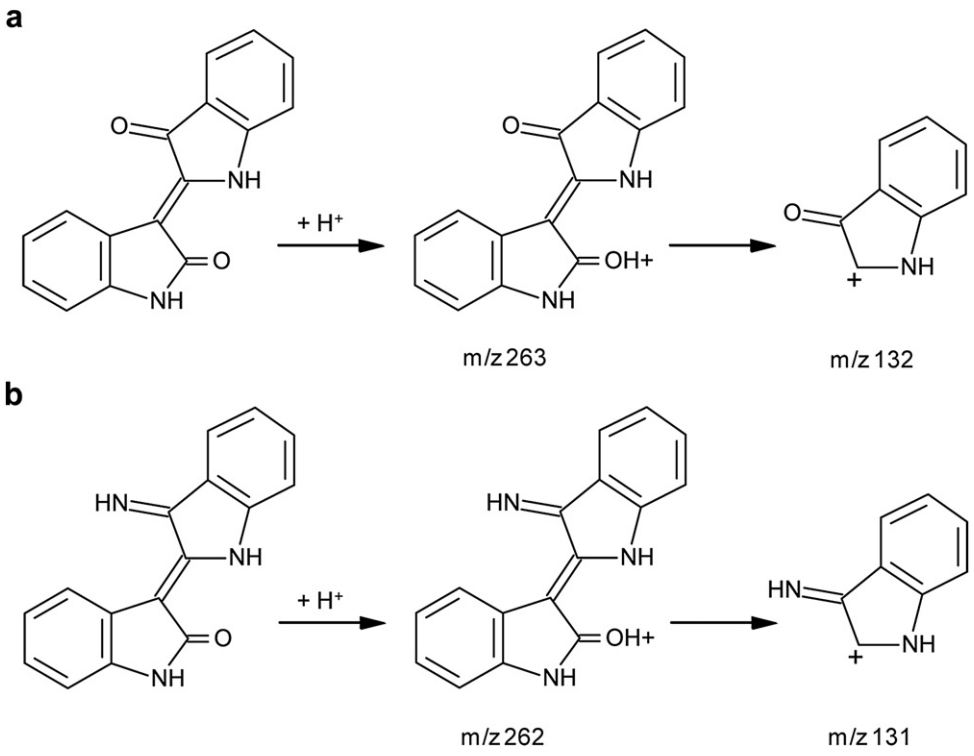

Fig. 4. Structures and fragmentation pathway proposed by Mass Frontier™ for observed fragment of indirubin (a) and compound A (b) in HCD of protonated molecular ions.

isotopes equal to 100:94 [17]). Compound D exhibited one unit lower mass than dibrominated indigoids and an isotopic pattern that was characteristic of dibrominated compounds (ratio of M to M+2 to M+4 equal to 51:100:47 [17]). This suggests that the unknown compounds are closely analogous to their respective brominated and non-brominated indigoids.

Accurate mass measurements (with a very good mass accuracy below 0.5 ppm) for the $[M + H]^+$ ions of all the discussed compounds are presented in Table 1. The one unit mass differences observed for the unknown compounds compared to their respective analogues may be attributed to a several structures. However, considering both the previously published UV–vis spectra [15] and the fact that *H. trunculus* is known for its ability to synthesise indigoid precursors, a structure possessing the indirubin backbone seems likely. Analysis of the possible elemental compositions based on the accurate mass measurements with carbon, hydrogen, oxygen and nitrogen atoms included in the search criteria resulted in the lowest mass error for compounds with elemental composition $C_{16}H_{12}ON_3$ for compound A, its monobrominated derivatives for isomeric compounds B, C and dibrominated derivative for compound D. For compound A, we propose the structure shown in Fig. 4b, resulting from substitution of a CO group in the indirubin molecule with a CNH group. Based on the isotope pattern, we also suggest that compounds B and C are monobrominated derivatives, and D is a dibrominated derivative of compound A. Further confirmation of the proposed structures was obtained by performing a comparative analysis of the MS/MS spectra of both known and unknown indigoids present in the purple dye extract.

3.2. MS/MS spectra

Initially, the HCD MS/MS spectrum of compound A was compared with that obtained for indigotin and indirubin (Fig. 3a, Table 2). The high similarity of these spectra suggests that all the

Table 2
Accurate mass measurements of MS/MS ions for HCD fragmentation of indirubin and unknown compound A.

Mass fragment	Elemental composition of the fragment	Transition	Calculated mass of the fragment	Measured mass of the fragment	Error of the mass measurement (ppm)
Indirubin (fragmented ion $[M + H]^+$: $m/z = 263$)					
245	$C_{16}H_9N_2O$	$[M - H_2O + H]^+$	245.0715	245.0714	−0.56
235	$C_{15}H_{11}N_2O$	$[M - CO + H]^+$	235.0871	235.0869	−1.14
219	$C_{15}H_9NO$	$[M - CO_2 + H]^+$	219.0922	219.0919	−1.66
132	C_8H_6NO	[a]	132.0449	132.0443	−4.61
Compound A (fragmented ion $[M + H]^+$: $m/z = 262$)					
245	$C_{16}H_9ON_2$	$[M - NH_3 + H]^+$	245.0715	245.0710	−1.83
244	$C_{16}H_{10}N_3$	$[M - H_2O + H]^+$	244.0875	244.0867	−3.16
235	$C_{15}H_{11}ON_2$	$[M - CNH + H]^+$	235.0871	235.0866	−2.25
234	$C_{15}H_{12}N_3$	$[M - CO + H]^+$	234.1031	234.1025	−2.70
219	$C_{15}H_{11}N_2$	$[M - CONH + H]^+$	219.0922	219.0916	−2.98
131	$C_8H_7N_2$	[a]	131.0609	131.0605	−3.53

[a] See Fig. 3.

Table 3
Accurate mass measurements of MS/MS ions for HCD fragmentation of 6-bromoindirubin and unknowns: compound B and compound C.

Mass fragment	Elemental composition of the fragment	Transition	Calculated mass of the fragment	Measured mass of the fragment	Error of the mass measurement (ppm)
6-Bromoindirubin (fragmented ion $[M + H]^+$: $m/z = 341$)					
323	$C_{16}H_8N_2OBr$	$[M - H_2O + H]^+$	322.9820	322.9826	1.86
313	$C_{15}H_{10}N_2OBr$	$[M - CO + H]^+$	312.9976	312.9972	−1.37
297	$C_{15}H_{10}N_2Br$	$[M - CO_2 + H]^+$	297.0027	297.0024	−0.99
262	$C_{16}H_{10}N_2O_2$	$[M - Br + H]^+$	262.0742	262.0738	−1.67
261	$C_{16}H_9N_2O_2$	$[M - HBr + H]^+$	261.0664	261.0655	−3.38
234	$C_{15}H_{10}N_2O$	$[262\text{-}CO]^+$	234.0793	234.0790	−1.21
218	$C_{15}H_{10}N_2$	$[262\text{-}CO_2]^+$	218.0844	218.0840	−1.92
132	C_8H_6NO	[a]	132.0449	132.0444	−4.38
Compound B (fragmented ion $[M + H]^+$: $m/z = 340$)					
313	$C_{15}H_{10}ON_2Br$	$[M - CNH + H]^+$	312.9976	312.9969	−2.33
297	$C_{15}H_{10}BrN_2$	$[M - CONH + H]^+$	297.0027	297.0032	1.47
261	$C_{16}H_{11}ON_3$	$[M - Br + H]^+$	261.0902	261.0895	−2.92
233	$C_{15}H_{11}N_3$	$[261\text{-}CO]^+$	233.0953	233.0947	−2.39
218	$C_{15}H_{10}N_2$	$[261\text{-}CONH]^+$	218.0844	218.0840	−1.78
Compound C (fragmented ion $[M + H]^+$: $m/z = 340$)					
323	$C_{16}H_8BrON_2$	$[M - NH_3 + H]^+$	322.9820	322.9817	−0.96
313	$C_{15}H_{10}ON_2Br$	$[M - CNH + H]^+$	312.9976	312.9964	−3.89
297	$C_{15}H_{10}BrN_2$	$[M - CONH + H]^+$	297.0027	297.0022	−1.70
261	$C_{16}H_{11}ON_3$	$[M - Br + H]^+$	261.0902	261.0893	−3.42
260	$C_{16}H_{10}ON_3$	$[M - HBr + H]^+$	260.0824	260.0810	−5.26
234	$C_{15}H_{10}ON_2$	$[261\text{-}CNH]^+$	234.0793	234.0786	−2.88
233	$C_{15}H_{11}N_3$	$[261\text{-}CO]^+$	233.0953	233.0946	−2.90
218	$C_{15}H_{10}N_2$	$[261\text{-}CONH]^+$	218.0844	218.0839	−2.47
131	$C_8H_7N_2$	[a]	131.0609	131.0599	−7.65

[a] See Fig. 3.

compounds belong to the same structural class. The proposed elemental compositions of the main HCD MS/MS fragments based on the accurate mass measurements are presented in Table 2. For indigotin and indirubin, the observed fragments resulted from loss of H_2O, CO and CO_2 (but not $CONH_2$ as formerly postulated [9]) molecules from one or two carboxyl groups of the indigoid backbone. For compound A, loss of H_2O and CO molecules associated with the CO group was also observed, as well as analogous losses of CNH and NH_3 molecules from the hypothetical CNH group and a CONH molecule from the CO and CNH groups combined. These additional fragmentations for compound A, which closely resemble the HCD pattern expected for both indirubin and indigotin with one carbonyl group substituted by a CNH group, provide further evidence for the proposed structure of this compound. Additionally, the presence of ions at m/z 132 for the known indigoids and m/z 131 for compound A suggests they have analogous structures with CO/CNH group differences, as indicated by analysis of the fragmentation pathway in Mass Frontier™ (Fig. 4). When the ratios of MS/MS fragments for compound A with the relevant ones obtained for indigotin and indirubin are compared, it seems likely that compound A is an indirubin analogue with one carbonyl group replaced by a ketimine group. The indirubin-like structure for these compounds was also proposed previously based on analysis of their UV–vis spectra [15]. Two isomers are theoretically possible for compound A because there are two CO groups that can be substituted in the indirubin molecule (Fig. 4a). We propose that the structure shown in Fig. 4b is the most probable based on the high similarity of the UV–vis and MS/MS spectra of the unknown compounds with their indirubin analogues. To confirm the exact structure, further investigations, employing other techniques, e.g. NMR, and significantly larger samples of raw material than used here, are required.

MS/MS spectra of the unknown brominated indigoids are presented in Fig. 3a and b. Like the non-brominated compounds, the spectra of compounds B and C and their identified counterparts are clearly similar. A summary of the accurate mass measurements of the HCD MS/MS fragments for these compounds is presented in Table 3. Similar losses of fragments to the unbrominated analogues were observed, i.e. loss of H_2O, CO_2 and CO molecules, as well as loss of a bromine atom and HBr group from the known indigoids, while for the unknown compounds there were additional losses of CONH, CNH and NH_3 groups, confirming the proposed structures. Once

Table 4
Accurate mass measurements of MS/MS ions for HCD fragmentation of 6,6′-dbromoindirubin and unknown compound D.

Mass fragment	Elemental composition of the fragment	Transition	Calculated mass of the fragment	Measured mass of the fragment	Error of the mass measurement (ppm)
6,6′-Dibromoindirubin (fragmented ion $[M + H]^+$: $m/z = 419$)					
340	$C_{16}H_9O_2N_2Br$	$[M - Br + H]^+$	339.9847	339.9839	−2.41
312	$C_{15}H_9ON_2Br$	$[M - Br - CO + H]^+$	311.9898	311.9889	−2.99
233	$C_{15}H_9ON_2$	$[M - 2Br - CO + H]^+$	233.0715	233.0716	0.39
205	$C_{14}H_9N_2$	$[M - 2Br - 2CO + H]^+$	205.0766	205.0760	−2.79
Compound D (fragmented ion $[M + H]^+$: $m/z = 418$)					
339	$C_{16}H_{10}ON_3Br$	$[M - Br + H]^+$	339.0007	339.0011	1.02
311	$C_{15}H_{10}N_3Br$	$[M - Br - CO + H]^+$	311.0058	311.0052	−2.08
232	$C_{15}H_{10}N_3$	$[M - 2Br - CO + H]^+$	232.0875	232.0867	−3.20
205	$C_{14}H_9N_2$	$[M - 2Br - CO - CNH + H]^+$	205.0766	205.0763	−1.14

again, ions at *m*/*z* 132 and *m*/*z* 131 were observed, in agreement with the fragmentation pathway presented in Fig. 4. Comparison of the relative ratios of MS/MS fragments indicated that compound B is a 6′-brominated indirubin analogue with one carbonyl group replaced by a ketimine group, whereas compound C is an equivalent 6-brominated indirubin analogue. The "inversion" of elution order of these 6- and 6′- isomers compared to monobrominated indirubines is consistent with their UV–vis spectra [15].

According to the MS/MS spectra, a close analogy between known dibrominated indigoids and compound D was also evident (Fig. 3d, Table 4), although in this case fewer fragment ions were detected in comparison with the other compounds. The observed main fragments of the known compounds corresponded to loss of one bromine atom, one bromine atom and a CO group, two bromine atoms and a CO group or two bromine atoms and two CO groups. Additional losses of two bromine atoms, CO and CNH groups were observed for compound D. Comparison of the relative ratios of MS/MS fragments suggested that compound D is a 6,6′-dibromoindirubin analogue with one CO group replaced by a CNH group.

4. Conclusions

In this paper, we presented the results of LC–MS/MS identification of minor indigoids found in extracts from *H. trunculus* samples. The unknown compounds displayed UV–vis spectra that were characteristic of indigoids and had molecular masses one unit lower than known mollusc colouring components, suggesting they were analogues of indirubin and its mono- and dibrominated derivatives, with one CO group substituted by a CNH group, namely indirubin-3′-monoimines. Our results demonstrate the advantages of using advanced mass spectrometric techniques for the precise identification of dye structures, especially for compounds for which accurate structure determination is difficult based on chromatographic and UV–vis spectral data.

Identification of these compounds provides a basis for explaining their presence in the *H. trunculus* pigment. However, the origin of these compounds requires further elucidation, i.e. to determine whether they result from biochemical processes taking place in the animal or the dye production process, since we do not currently have sufficient information about the pigment preparation process. We have previously detected these compounds in other purple artefacts and aim to investigate whether biochemical pathways are responsible for the synthesis of these compounds in future work. Information from these studies will increase our knowledge of one of the oldest known natural dyes and will thus facilitate the characterisation of *H. trunculus* purple in historical objects. On the other hand, if the compounds are found to originate from the dye preparation procedure, their presence may help to reveal the technological processes used to produce Tyrian purple dye.

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