HPLC analysis of the natural scale insect, madder and indigoid dyes

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A linear gradient elution method has been applied to the HPLC analysis of plant and scale insect red anthraquinonoid mordant dyes and molluscan blue and red-purple indigoid vat dyes. The method enables the use of the same elution program for the determination of different chemical classes of dyes. In addition, it significantly shortens the retention times of natural anthraquinonoid dyes over those previously published. For the first time a new dye, probably dibromoindirubin, has been detected in the Murex trunculus sea snail. The dye families investigated include the ones most often found on ancient textiles and shards from dyeing vessels in Israel and other regions.

INTRODUCTION

Analysing the colorants applied to ancient textiles can help in understanding the processes associated with one of the oldest of chemical technologies: textile dyeing. Such an investigation of the culture of ancient peoples combines history, archaeology, botany, entomology, marine zoology and forensic chemistry. The dye analysis usually involves extracting the colorant from a small thread that has been removed from an archaeological textile fragment. The excavated textile pieces are themselves often small, so that only a minute quantity of the artefact can be sacrificed for the analytical
investigation. This results in an extracted dye sample that could weigh only a few nanograms, a quantity which requires the use of highly sensitive instrumentation.

The applicability of the high-performance liquid chromatography (HPLC) method to the analysis of ancient textile dyes was successfully demonstrated by Wouters [1]. This method is generally more efficient than the other analytical and instrumental techniques that have been used for the analysis of mixtures of natural dyes. The latter include mass spectrometry [2], thin-layer chromatography [3], ultra-violet and visible light spectrophotometry [4,5], and infra-red spectrometry [6].

The chemical structures of the natural dyes investigated in the present study are presented in Figure 1. The plant sources of indigo are typically Indigofera tinctoria L (indigo) and Isatis tinctoria L (woad) [7]. The brominated indigoids are only obtainable from marine molluscs. Along the Mediterranean coast, the most common whelk species from which the ‘true purple’ dye (6,6’-dibromoindigo) can be produced are Murex (Trunculariopsis) trunculus, Murex brandaris and Thais (Purpurea) haemastoma [8]. The 6-monobromoindigo dye, indigotin, and possibly some indirubin may also be formed from the M trunculus species [2,9].

<table>
<thead>
<tr>
<th>Substance</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
<th>R₇</th>
<th>R₈</th>
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<tbody>
<tr>
<td>Madder</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Purpurin</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<tr>
<td>Insect</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carminic acid</td>
<td>OH (c)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>COOH</td>
<td>CH₃</td>
<td></td>
</tr>
<tr>
<td>Flavokermesic acid</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>COOH</td>
<td>CH₃</td>
<td></td>
</tr>
<tr>
<td>Kermesic acid</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>COOH</td>
<td>CH₃</td>
<td></td>
</tr>
<tr>
<td>Laccaic acid A</td>
<td>OH (a)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>COOH</td>
<td>COOH</td>
<td></td>
</tr>
<tr>
<td>Laccaic acid B</td>
<td>OH (b)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>COOH</td>
<td>COOH</td>
<td></td>
</tr>
</tbody>
</table>

![Chemical structures](image)

**Figure 1** Chemical structures of anthraquinonoid, indigoid and indirubinoid dyes (corrected structures)
The hydroxy- and the carboxyhydroxy-anthraquinonoid colorants may be derived from plants such as the historically important Rubia tinctorum L. (madder), which contains alizarin and purpurin among other dye components [1,3]. Another source of carboxyhydroxy-anthraquinonoid dyes are scale insects (Coccoidea). The major dyes from each cocid species include [10]:
(a) Kermesic and flavokermesic acids from Kermes vermilio (oak kermes)
(b) Carminic acid from Porphyrophora hamelii Brandt (Armenian cochineal), Porphyrophora polonica Planchon (Polish cochineal) and Dactylopius coccus Costa (American cochineal)
(c) Laccaic acids A and B from Kerria lacca Kerr (Indian lac).

Previously an HPLC gradient elution method for the analysis of indigoid dyes, first described in a Chinese article, has been outlined in this Journal [9]. In the present study the indigoid elution method was extended to different chemical classes of dyes: the hydroxy- and the carboxyhydroxy-anthraquinonoids from plant and insect sources. The indigoids and anthraquinonoids are the colorants most commonly found on ancient textiles and pottery fragments of dyeing vats in Israel and other regions [6,11,12].

EXPERIMENTAL

Dye extraction procedures
Prior to HPLC analysis, an extracted dye solution was prepared using analytical-grade solvents. The dyes were extracted from the fibres of about 1 cm of dyed thread according to the procedure appropriate to each chemical class of dye. A red dyeing from a mordant dye from a plant or insect source was treated for a few minutes with equal portions of 3 mol/l hydrochloric acid and methanol at 100°C. The dye solution was then evaporated to remove the acid and then redissolved in about 0.25 ml methanol. For a blue plant dyeing or a purple mollusc dyeing, the yarn was treated for about 1 min with near-boiling NN-dimethylformamide (DMF) to strip the vat dye. Each extracted dye solution was subsequently filtered through a 0.45 μm PTFE or nylon syringe filter.

Reference dye solutions
The standard dyes used as references were commercial synthetic dyes, the natural raw dyestuff or obtained from natural wool and cotton dyeings.

The commercially available dyestuffs included: carminic acid (Fluka), alizarin (Merck), purpurin (Aldrich) and indigotin (BASF). The first three dyes were dissolved in methanol, while indigotin was extracted in DMF. A methanolic solution of kermesic and flavokermesic acids was obtained from a Kermes vermilio insect from Algeria (Ashill Colour Studio, Shefford, UK). An additional standard solution was also obtained by extraction from a wool dyeing produced from this species. Laccaic acids A and B were extracted from a red dyeing produced from the lac insect.

The molluscan indigoid dyes were obtained from several sources. The 6,6’-dibromoindigotin dye was extracted from a cotton sample dyed with the extract from Purpura patula pansa Gould sea snail, according to the hot DMF indigoid dye extraction procedure described above. The related 6-monobromoindigotin dye was generated from the above-mentioned purple dyeing according to the following two procedures, each of which caused the partial debromination of the dibrominated species.

Procedure A
A sample of the original purple dyed cotton was immersed in a reducing solution of 5% sodium hydroxide and 5% sodium dithionite for about 20 min at 60°C under normal room lighting conditions. The dyeing was subsequently removed from the leuco solution and allowed to undergo oxidation in the air to the final purple shade. This new dyeing was then extracted by means of hot DMF.

Procedure B
Monobromoindigotin was generated by irradiating the original blue DMF-extracted solution overnight at 288 nm in the closed UV/VIS spectrophotometer cell compartment.

A solution of the brominated indigoid dyes was also produced from the dissected hypobranchial glands of M. trunculus species caught off the Israeli Mediterranean coast. About a hundred M. trunculus snails were collected (14 May 1993) in water about 1 m deep at Achivz Beach, Israel. The glands were all dissected at the beach and the final purple colour was developed under the sun. A DMF solution of indirubin was also obtained from the synthetic dye at University College, London.

Chromatographic system
A Varian Vista 5500 LC instrument was used for the chromatographic measurements, with a Merck 150 x 4 mm Lichrosorb 15537 RP-18 standard column (7 μm), 10 μl sample loop and a 1.0 ml/min flow rate. Data processing was on a PC using LabCalc software (Galactic Industries, USA). The linear gradient elution scheme is indicated in

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Methanol (%)</th>
<th>Water (%)</th>
<th>Phosphoric acid (%)</th>
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<tbody>
<tr>
<td>0–1</td>
<td>30</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>1–3</td>
<td>30–60</td>
<td>60–30</td>
<td>10</td>
</tr>
<tr>
<td>3–15</td>
<td>60–90</td>
<td>30–0</td>
<td>10</td>
</tr>
<tr>
<td>≥15</td>
<td>90</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1 Linear gradient elution method for HPLC analysis of mordant and indigoid dyes
Table 1 [9]. The optimum detector wavelengths used were:
(a) For madder dyes 430, 275 and 255 nm
(b) For scale insect dyes 480, 430 and 275 nm
(c) For indigos 600 and 288 nm
(d) For indirubinoids 550 and 288 nm.

RESULTS AND DISCUSSION

All 12 dyes investigated were dissolved in a methanol/DMF solution, and the resulting chromatogram of this solution is depicted in Figure 2. The retention times relative to alizarin, R, are quoted in Table 2. With all the indigoids and anthraquinonoids investigated the absolute retention time of the final eluting dye was under 18 min. For many of the dyes, the relative retention times are reproducible to within about 0.1% or better.

Besides dibromoindigotin, monobromoindigotin and indigotin, the other indigoid dye generated from the gland of the M trunculus species was most probably dibromoindirubin; no indirubin was detected. Its absorbance greatly increased at a detector wavelength set at 550 nm over that at 600 nm, and its R value was higher than that of dibromoindigotin. Since the brominated indigotin species, both monobromoindigotin and dibromoindigotin, absorb in the visible region at about 600 nm, close to indigotin, it is likely that a brominated indirubin would also absorb at a $\lambda_{\text{max}}$ close to that for indirubin (about 550 nm).

In addition, the R value of indirubin is somewhat higher than that of its isomer indigotin, so that the R value of dibromoindirubin should also show a similar relationship to that of its isomer dibromoindigotin. In fact, the R value ratio between indirubin and indigotin (1.15) is identical to the new dye/dibromoindigotin ratio. This spectral and chromatographic evidence indicates that the new dye found was probably dibromoindirubin. This is the first time that evidence for the presence of the dibromoindirubin dye in the glandular extract of a M trunculus species has been found. On the basis of chromatographic data, Wouters has conjectured that a 'brominated indirubin' was present in M brandaris and T haemastoma, but not in M trunculus [13]. It is most probable that vat dyesings with an extract that contains dibromoindirubin will also yield monobromoindirubin and eventually also indirubin. The latter dyes are formed by the debromination of dibromoindirubin caused by the exposure of the reduced dye solution to light.

Figure 2 and Table 2 show that both plant and insect anthraquinonoids, as well as plant and molluscan indigoids, may be efficiently separated and detected via the HPLC method using the same elution program. Previously published HPLC determinations of these dyes relied on different elution schemes for the anthraquinonoids and for the indigoids [1,9,10,14]. In addition, the chromatographic system and the elution conditions used in this study significantly shorten the retention times of the anthraquinonoids, compared with those of other systems that have been used [1,10]. For example, the R value of the hydroxyanthraquinone purpurin is reduced from about 38 min to less than 12 min [1]. It has yet to be determined whether this method produces a similar separation of the various minor dye components from a given dyestuff as the ones previously published [1,10].

CONCLUSIONS

The HPLC gradient elution method, along with a suitable chromatographic system, is universally effective in the relatively fast separation and identification of major red, blue, and purple natural dyes used on ancient textiles. For the first time evidence for the existence of the dye...
dibromoindirubin in a Murex trunculus extract has been presented. The colorants investigated are those most often found on ancient textile dyeings and archaeological fragments from dyeing vats in Israel and other regions.

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REFERENCES