Simultaneous Analysis of the Coloring Compounds in Indigo, Phellodendron bark, and Madder Dye Using HPLC-DAD-MS

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Abstract

Indigotin, indirubin, berberine, palmatine, alizarin, and purpurin are major pigments of indigo plant, Phellodendron bark, and madder. The six pigments were examined using the HPLC-DAD-MS instrument for the purpose of the simultaneous detection of the pigments in a single sample run. The HPLC-DAD-MS method examined the individual pigment solutions in DMSO, a solution containing 6 pigments, and the DMSO extract of the silk dyed with a dye solution of 5 pigments excluding indirubin. The retention times of the HPLC chromatograms, λ max of the uv-vis absorption bands in the DAD analyses, and the molecular ions detected for the compound peaks in the MSD analyses were consistent throughout the analyses of individual pigment solutions, mixed pigment solutions, and dye extracted from silk dyeing. The developed instrumental method of the simultaneous detection of six pigments can identify dye in an exhumed textile if the textile is dyed using any one (or multiple) pigments of indigo, Phellodendron bark, or madder plant.

Key words: Indigo, Phellodendron bark, Madder, Dye identification, HPLC-DAD-MS

I. Introduction

Textiles excavated from ancient burials are important historical materials which can provide documentary evidence of the past culture and technology. However, the original color of these textiles are often completely lost due to the serious change of color and staining which occur within the burial environment. Considering that color was an important visual means of expressing social identity, the exhumed textiles without the preservation of their original colors provide only limited information regarding the past clothing culture. To fully utilize the exhumed textiles as the documentary evidence of the past, it is necessary to investigate the nature of the original color of the badly faded textiles. This can be done through the identification of dye which were used to color these textile pieces.

Different research efforts were carried out to identify dye in museum textiles using instrumental methods such as thin layer chromatography (Schweppe, 1989) and gas chromatography (Ahn & Obendorf, 2004; Casas-Catalán & Doménech-Carbó, 2005; Colombini et al., 2007; Grosjean et al., 1987). TOF-SIMS and MALDI-TOF methods were recommended as the non-destructive methods which identify the molecular ion of the pigment by ion sputtering the sample surface (Lee et al., 2008; Soltzberg et al., 2007). Among the many instrumental methods, the high performance liquid chromatography (HPLC), equipped with a diode array detector (DAD) or (and) a mass selective detector (MSD), has been by far the most often used me-

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ethod for the identification of dye in ancient textiles. The ability of the HPLC to separate and quantify the pure compounds from a solvent mixture using either the isocratic or the gradient elution system (Wikipedia, 2013b), the ability of the DAD to detect and identify the compounds by their absorbances in the ultra violet and visible wavelength regions, the ability of the MSD to detect and identify the molecular ions of the compounds using the electrospray ionization or the atmospheric pressure chemical ionization modes (Wikipedia, 2013a), all add power to the ability of HPLC-DAD-MS to identify dye in unknown ancient samples.

To list a few recent research which used the HPLC method, Zhang et al. (2007) identified that the yellow dyes of A.D. 1050-1200 pre-Columbian textiles of the northern Peru were flavonol and luteolin glycoside types. Valianou et al. (2009) identified that the dyes used in the 13-16th century textiles of Mamluk Dynasty of Egypt were madder, cochineal, curcumin. Petrovicu et al. (2010) examined that the dyes of red silk yarns of the 17th century Bucharest, Romania were alizarin, purpurin, and carminic acid using the reversed phase liquid chromatography. Mantzouris et al. (2011) identified alizarin, carminic acid, or fustic from the 16-20th century textiles from Greece. Ahn et al. (2012) identified berberine and palmatine from the textiles of the 17th century Chosun Dynasty of Korea. Petrovicu et al. (2012) identified indigotin from the 19th century textiles from Kilim and alizarin, purpurin from the 16th century red silk thread from historic collections of Romania.

When the exhumed textiles have some evidence of original color remaining in the piece, the identification of dye can be efficiently carried out since the standard dye can be selected for comparison based on the color. However, because almost all exhumed textiles look tan in color (Choo et al., 2002), any natural dye which were used in the past can become the potential candidate. Therefore, it is necessary to examine the possibility of a number dyes simultaneously.

In this paper, the HPLC-DAD-MS instrument was used to examine the six major pigments which belong to indigo plant, _Phellodendron_ bark, and madder plant for the purpose of detecting the six pigments simultaneously in a single sample run. First, six dye solutions of individual pigment were examined, and second a dye solution containing all six pigments was examined using the HPLC-DAD-MS method to detect the six pigment compounds from the samples. The investigation of mixed dye solution was intended to simulate the possibility of composite dyeing of two or more dyes in one fabric, which was often practiced in the past to produce a mid-hue. It was also intended to simulate the possible staining of dye from adjacent clothing artifact within the coffin burial. The developed HPLC-DAD-MS method was finally applied to examine the dye extracted from the silk sample, which was dyed in this study with the mixed dye solution, to verify that the method can be successfully used in the detection of dye in the textiles as well.

Indigo, _Phellodendron_ bark and madder were selected based on the fact that they were the most representative dyestuffs of the past which color the fabrics in blue, yellow, and red color types, and also because their major pigments with high purity can be purchased from the reliable global vendors. The six pigments under investigation were indigotin and indirubin of indigo plant, berberine and palmatine of _Phellodendron_ bark, and alizarin and purpurin of madder. The chemical characteristics of the six pigments are shown in <Table 1>. The method developed in this research can be applied in the future identification of dye in the exhumed textiles which have been dyed with indigo, _Phellodendron_ bark, and madder.

**II. Experimental**

1. **Materials**

Indigotin (95%), indirubin, alizarin, berberine chloride form (berberine in the following) (97%), palmatine chloride hydrate (palmatine in the following) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purpurin (85%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO, HPLC grade), acetonitrile (HPLC grade), HPLC water (HPLC grade), and sodium hydrosulfite were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic acid (88%, ACS grade) was purchased...
from Macron Chemicals (Phillipsburg, NJ, USA). Aluminium potassium sulfate was purchased from Shinyo Pure Chemicals (Osaka, Japan). HPLC sample was filtered using a glass fiber enhanced 0.45µm syringe filter by Alltech Associates (Deerfield, IL, USA). Silk used for dyeing was the Standard Adjacent Fabrics for Colorfastness (KS K0905) purchased from KATRI (Seoul, Korea). Water used for dyeing was purified using Milli-Q Integral System by EMD Millipore (Darmstadt, Germany).

2. Methods

1) Preparation of Pigment Solution

The pigment solutions for the HPLC analysis were prepared in DMSO in two different modes- separate solution of six individual pigments and a mixed solution of six pigments. For individual pigment solution, each of the six pigments were dissolved using 80mL DMSO in separate beakers. The concentrations were 0.05g/L for indigotin, 0.1g/L for indirubin, and 0.2g/L for berberine, palmatine, alizarin, and purpurin. Lower concentrations of indigotin and indirubin were used due to the low solubility of indigotin and the amount of indirubin available for the analysis. For the mixed pigment solution, all six pigments were dissolved together in a single beaker using 300mL DMSO. The concentrations were 0.016g/L for indigotin and indirubin, 0.033g/L for berberine, palmatine, alizarin, and purpurin. Each sample was filtered using the glass fiber enhanced 0.45µm syringe filters for the HPLC-DAD-MS analysis.

2) Silk Dyeing

Separate dyebath was prepared for berberine, palmatine, alizarin, and purpurin by mixing 0.025g of
each pigment in 370mL of purified water in separate beakers. This corresponded to 1% o.w.f. dye concentration and 1:30 liquor ratio. Approximately 40cm × 20cm size (2.45g) silk fabric sample was mordanted with 0.49g of aluminium potassium sulfate in 370mL water for 1 hour at 60°C. The mordanted silk was thoroughly washed using running tap water and rinsed with purified water. The mordanted silk sample was dyed first using berberine dyebath for 1 hour at 60°C. After thorough rinsing, the silk was mordanted again using a fresh mordanting solution of 0.49g aluminium potassium sulfate in 370mL water. The mordanted silk was then dyed using the palmatine dyebath. Following this process, the silk was dyed with alizarin and then purpurin. The silk was premordanted before each dyeing process using a fresh mordanting solution. The silk dyed consecutively with berberine, palmatine, alizarin, and purpurin was cut into approximately 1.7g size for the indigotin dyeing. A 255mL indigotin dyebath was made with 0.017g dye and about three folds of sodium hydrosulfite in purified water. The dyebath was adjusted to pH 11 using the NaOH and dyeing was conducted for 30 minutes at room temperature. The silk was then thoroughly rinsed with running cold water and air dried so that the leuco form of indigotin could freely oxidize to the insoluble indigotin within the silk.

3) Color Measurement
The color of dyed silk was measured using the JS-555 spectrocolorimeter (Color Techno System, Japan) under D65 illuminant and 10° standard observer.

4) Extraction of Dye from the Dyed Silk Sample
Approximately 0.5cm × 0.5cm size dyed silk specimen was place in a small beaker with 0.4mL of mixed solution of HCl/methanol/water (2:1:1 v/v/v). The beaker was placed in 110°C oven for 10 minutes, cooled, and then placed above NaOH pellets inside a vacuum desiccator until the liquid completely evaporated. An aliquot of 1.5mL of DMSO was added to the completely dried out beaker for the final extraction process. By this process, the silk sample turned white and the DMSO extract showed the color of dye. The DMSO extract was filtered using the glass fiber enhanced 0.45µm syringe filters into an HPLC vial and the sample was analyzed using the HPLC-DAD-MS instrument.

5) HPLC-DAD-MS Analysis
An Agilent 1200 series binary HPLC-DAD-MS system (Foster City, CA) equipped with a diode-array detector (DAD) and a mass selective detector (MSD) consisting of a single quadrupole mass analyzer was used for sample analysis. The mass detector was operated in both Selective Ion Monitoring (SIM) mode and Scan Mode using the atmospheric pressure chemical ionization (APCI) source in the positive ionization mode. LC separation was achieved by Restek Ultra C-18 column (length 250mm × I.D. 4.6mm, particle size 5µm). The gradient elution applied in the analysis using solvent A (acetonitrile) and solvent B (1% formic acid in water) was: 0-5.7 min, 90-20% B; 5.7-10 min, 20-61% B, 10-15 min, 61% B, 10-15 min, 61% B. The flow rate was 1.0mL/ min, and the injection volume was 20µL. Detection wavelength for the DAD was set for 255, 265, 288, 542, 606nm with the spectrum detection range 190-700nm. The column temperature of the MSD was 25°C and the ionization source was operated with drying gas (N2) flow 11.0L/m, drying gas temperature 300°C, vaporizer temperature 250°C, nebulizer pressure 50 psi, capillary voltage of 5kV in positive ion mode, chargeing voltage 1.3kV, fragmentor voltage 95V, and mass range m/z 82-380.

III. Results and Discussion
1. Analysis of Individual Pigment Solutions
The results of HPLC-DAD-MS analyses on the individual pigment solutions are illustrated in <Fig. 1>. Each pigment which was dissolved in DMSO eluted in a clean single peak in their HPLC chromatograms. The retention times for the peak of each pigment compound were 9.9-10.5 min for indigotin, 10.0-12.0 min for indirubin, 6.2-6.7 min for berberine, 6.2-6.6 min for palmatine, 8.8-9.2 min for alizarin, and 9.9-10.4 min for purpurin. The intensities of the peaks of indigotin and indirubin were lower than berberine, palmatine, and alizarin due to the lower concentration used in indigotin and indirubin pigment solutions. The
low peak intensity of purpurin must be attributable to the relatively low purity of the purpurin purchased (85%).

There was an overlap between the retention times of indigotin and purpurin, and also between berberine and palmatine. However, it was possible to distinguish indigotin and purpurin since the two pigment compounds exhibited different $\lambda_{\text{max}}$ at the visible range of DAD uv-vis absorption bands, owing to the difference in their color. The $\lambda_{\text{max}}$ of indigotin was 607 nm which was close to the $\lambda_{\text{max}}$ reported in the literature using DMSO as the solvent (Oh & Ahn, 2013), exhibiting the blue color. The $\lambda_{\text{max}}$ of purpurin was 480 nm which was close to the $\lambda_{\text{max}}$ reported by the manufacturer (Cayman Chemical, 2012), exhibiting the dark brown color (Sigma-Aldrich, 2012). Indigotin and purpurin
were also distinguishable by their protonated molecular ion \([\text{M+H}]^+\) detected by the mass selective detector (MSD), which were m/z 263 and m/z 257 respectively. The protonated molecular ion \([\text{M+H}]^+\) is the typical form of major molecular ion observed in the positive ionization mode of APCI MSD analysis (Ren et al., 2007).

The \(\lambda_{\text{max}}\) of alizarin, 428nm, was similar to berberine and palmatine despite the fact that madder colors the fabrics in reddish orange color. This was due to the fact that the solvent used for this analysis was DMSO. During the experiment, it was found that alizarin gave a yellowish hue in DMSO while it gave a more reddish orange hue in water. The result also explains that although alizarin is the major coloring compound of madder plant, the color of madder dyed fabric is the result of the concerted effect of alizarin, purpurin, and other coloring compounds present in madder plant.

Berberine and palmatine were not distinguishable by the uv-vis absorption bands (visible \(\lambda_{\text{max}}\) was 426nm for berberine and 427nm for palmatine) since the two pigment compounds showed almost identical spectra owing to the fact that they were both quaternary protoberberine alkaloids (Zhang et al., 2009) and that they had the same yellow color. The two compounds were distinguishable through their mass spectra since the molecular ions of berberine and palmatine were detected as m/z 338 and m/z 354 respectively. These molecular ions of berberine and palmatine corresponded to \([\text{M+H}]^+\) of berberine chloride and palmatine chloride reported in the literature which used the APCI positive mode in the MSD analysis (Ren et al., 2007).

2. Analysis of the Mixed Pigment Solution

The six pigment compounds were successfully detected from the mixed pigment solution (Fig. 2). The retention times were 9.8-10.2 min for indigotin, 10.7-11.4 min for indirubin, 6.2-6.9 min for berberine, 6.4-6.9 min for palmatine, 8.8-9.0 min for alizarin, and 9.8-10.2 min for purpurin, which fell within the retention times detected in individual pigment solutions. The \(\lambda_{\text{max}}\) of the uv-vis absorption spectra were indigotin 607nm, indirubin 542nm, berberine 427nm, palmatine 428nm, alizarin 429nm, and purpurin 481nm, which were either identical or only 1nm shifted from those of the individual pigment solutions. The mass spectrum of each pigment peak gave the same molecular ion \([\text{M+H}]^+\) for indigotin, indirubin, alizarin and purpurin and \([\text{M+H}_2]^+\) for berberine and palmatine as those of the individual pigment solutions.

The peak intensity of the HPLC chromatogram and the uv-vis absorption bands, however, were much lower than those of the individual pigment solutions. This was because the concentration of each pigment used in the mixed pigment solution was lower than the concentration used in the individual pigment solutions. The concentration of indigotin in the mixed pigment solution was about 1/3 of the individual pigment solution. And the concentrations of indirubin, berberine, palmatine, alizarin, and purpurin were about 1/6 of the concentrations of the individual pigment solutions. The differences in the peak intensities roughly corresponded to the differences between the concentrations of the pigments in the mixed pigment solution and the individual pigment solution.

The above results of the mixed pigment solution indicated that the six pigments can be simultaneously detected by a single sample run using the HPLC-DAD-MS method developed in this study. The result also implies that each pigment compound can be detected even when the exhumed textiles were dyed using any two or multiple of indigo, \textit{Phellodendron} bark, and madder plant, and also when the staining of dye from adjacent clothing artifact occurred within the coffin burial.

3. Analysis of Dye Extracted from the Silk Sample

<Table 2> shows the color data of the silk sample dyed consecutively with berberine, palmatine, alizarin, purpurin, and then indigotin, premordanted with aluminium potassium sulfate before each dyeing procedure. After dyeing with 5 pigments was completed, the dye was extracted from the silk using the mixed solution of HCl/methanol/water (2:1:1 v/v/v). The extract of the silk sample was analyzed using the same HPLC-DAD-MS method as the liquid dyes and the results are shown in <Fig. 3>.

The 5 pigment compounds were all successfully detec-
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Ted from the DMSO extract of the silk sample dyeing. The retention times of the extract were 9.9-10.0 min for indigotin, 6.2-6.7 min for berberine, 6.4-6.6 min for palmatine, 8.9-9.0 min for alizarin, and 10.0-10.3 min for purpurin. The retention times of the silk extract matched the retention times of the pigment compounds detected in the individual pigment solutions and in the mixed pigment solutions. The \( \lambda_{\text{max}} \) of the uv-vis absorption spectra were indigotin 606nm, berberine 427nm, palmatine 427nm, alizarin 428nm, and purpurin 481nm, which also matched the \( \lambda_{\text{max}} \) of the pigment compounds in the individual and the mixed solutions. The mass spectrum of each pigment peak gave the same molecular ion \([M+H]^+\) or \([M+H_2]^+\) as those of the individual pigment solutions. The results indicate that the HPLC-DAD-MS method developed in this study, toge-

### Table 2. Color data of the silk sample consecutively dyed with 5 pigments

<table>
<thead>
<tr>
<th>K/S (( \lambda_{\text{max}} ): 520nm)</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>H</th>
<th>V</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.18</td>
<td>35.04</td>
<td>28.3</td>
<td>2.69</td>
<td>1.09 R</td>
<td>3.41</td>
<td>5.34</td>
</tr>
</tbody>
</table>

![Fig. 2. HPLC-DAD-MS results of mixed pigment solution.](image)

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ther with the present method of extracting the dye from fiber, can be used to detect and identify indigotin (and highly likely indirubin as well), berberine, palmatine, alizarin, and purpurin from the textile sample.

IV. Conclusions

In this paper, the HPLC-DAD-MS instrument was used to examine the six major pigments of indigo plant, *Phellodendron* bark, and madder in DMSO solvent for the purpose of detecting the six pigments simultaneously in a single sample run. When the individual pigment solutions were examined, indigotin, indirubin, berberine, palmatine, alizarin, and purpurin eluted at 9.9-10.5 min, 10.0-12.0 min, 6.2-6.7 min, 6.2-6.6 min, 8.8-9.2 min, and 9.9-10.4 min, respectively. Indigotin and purpurin whose HPLC peaks overlapped were distinguishable through their uv-vis absorption bands of the DAD analysis and their molecular ions detected by the MSD analysis. Berberine and

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Fig. 3. HPLC-DAD-MS results of the dye extracted from silk dyed with the mixed dye solution of 5 pigments in water.
palmatine were distinguishable through their MSD molecular ions. The HPLC-DAD-MS results of mixed pigment solution and the extraction from the silk dyeing were consistent with those of the individual pigment solutions. The results of this study indicated that the six pigments can be simultaneously detected by a single sample run using the HPLC-DAD-MS method developed in this study. The developed instrumental method of simultaneous detection of six pigments can be used to identify dye in exhumed textiles if the textile has been dyed in the past using any one or multiple of indigo, Phellodendron bark, and madder plant.

References


acterization of tertiary and quaternary alkaloids from *co-