1. Introduction

Polyphenolic components from *Genista* species have been well characterised because of their potential as antioxidants and as therapeutic leads;¹ ² however, the identification of dyer’s greenweed (*Genista tinctoria* L.) in historical textiles has been the subject of only limited studies.³ ⁴ Although historically weld (*Reseda luteola* L.) was probably the most widely used European yellow dye plant, it is reported that other dye plants, including dyer’s greenweed (*Genista tinctoria* L.) and sawwort (*Serratula tinctoria* L.), were used as substitutes.⁵ The flavones luteolin and apigenin, the isoflavone genistein, and the glycosides of these are known dye components of *Genista* species.⁶ But genistein, and its glycosides, are also the main dye components found in other varieties of broom; notwithstanding, the dye source for historical textiles is usually ascribed to dyer’s greenweed.⁷ ⁸ Whilst genistein is the principle component on which this attribution is made,⁹ our studies of the dye extracts of historical samples show the presence of additional dye components that could enhance this identification. This paper presents a comprehensive study of the structure of these additional dye components using UPLC-PDA‡ and ESI-MS/MS techniques. To achieve this, an analytical method was developed for a range of flavonoid and isoflavonoid dyes, allowing a more efficient separation of several isomeric dye components from textile samples. This method was then used to determine the relative amount of dyestuffs present in raw materials, modern yarns and historical yarns; examining differences between the plant extract and the dye components adsorbed onto textiles, and relating variations in component ratios to dyeing processes such as over-dyeing, and to the effects of photo-degradation. Data from a selection of historical yarns sampled from mid-sixteenth century English tapestries from the Burrell Collection in Glasgow, UK and the Bodleian Library in Oxford, UK were then placed in context using the results of this study.

2. Experimental

2.1 Materials and chemicals

2.1.1 Plant and textiles. Dyer’s broom (*Genista tinctoria* L.) from Fibrecrafts (George Weil & Sons Ltd.) was used to prepare the reference material. Alum mordanted wool (YW2) and silk (YS3a) yarns were dyed, and over-dyed (YS3b–d), as part of the Monitoring of Damage to Historic Tapestries project (FP5, EC contract number EVK4-CT-2001-00048).⁹

2.1.2 Flavone and isoflavone standards. Luteolin (3',4',5,7-tetrahydroxyflavone), genistein (4',5,7-trihydroxyisoflavone), apigenin (4',5,7-trihydroxyflavone), prunetin (4',5-dihydroxy-7-methoxyisoflavone), and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) standards were purchased from Sigma-Aldrich; glycitein (4',7-dihydroxy-6-methoxyisoflavone) standard

‡ As commonly adopted by the field, the abbreviation UPLC has been used for the technique of ultra-high pressure liquid chromatography (UHPLC).
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was purchased from Cayman Chemical Company; and chrys-
eriol (4',5,7-trihydroxy-3'-methoxyflavone) and diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) standards were purchased from Extrasynthese. All commercial standards were >98% purity.

An authentic sample of isorutin (4',7-dihydroxy-5'-methoxyisoflavone) was prepared from genistein by selective acetylation (Ac₂O, py) to give 7,4'-diacetoxy-5-hydroxyisoflavone,[18] methylation of the remaining free hydroxyl [(MeO)₂SO₂K₂CO₃, acetone] under high dilution conditions[19] to give 7,4'-diacetoxy-5-methoxyisoflavone, followed by hydrolysis (NaHCO₃ aq., MeOH : THF)[18] of the acete groups which allowed isolation of isorutin in >95% purity after chromato-
graphy (see ESI 1).[22]

2.1.3 Solutions of standards. The UPLC® system was cali-
ibrated using stock solutions of flavonoid and isoflavonoid standards: (1) a solution containing luteolin and genistein (1.00 ± 0.01 mg of each standard) in H₂O : MeOH [25 mL, 1 : 1 (v/v); equivalent to 40 µg mL⁻¹]; (2) a solution containing apigenin, chrysoeriol (0.20 ± 0.01 mg of each standard) in H₂O : MeOH [10 mL, 1 : 1 (v/v); equivalent to 20 µg mL⁻¹]; (3) a solution of diosmetin (1.00 ± 0.01 mg) in H₂O : MeOH [25 mL, 1 : 1 (v/v); equivalent to 40 µg mL⁻¹]. Diluted solutions were then prepared with components at concentrations of 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.02 and 0.01 µg mL⁻¹, by dilution with H₂O : MeOH [1 : 1 (v/v)] using calibrated micro-pipettes.

2.2 UPLC-PDA and ESI MS systems

2.2.1 UPLC-PDA. The UPLC chromatographic method was developed using a Waters Acuity UPLC® system with sample detection using a Waters PDA detector (250 to 500 nm). Data were collected by Waters Empower 2 software and processed with Origin 8.5 (OriginLab, Northampton, MA, USA). Sample extracts were automatically injected via a Rheodyne injector with a 10 µL sample loop. The bandwidth (resolution) was 1.2 nm with a sampling rate of 5 points per s. The method used a PST BEH C18 reverse phase column, 1.7 µm particle size, 150 × 2.1 mm (length × i.d.), set-up with inline filter. The total run time was 37.33 min at a flow rate of 250 µL min⁻¹ and the column was maintained at 55 ± 1 °C. A binary solvent system was used; A = 0.02% aqueous HCOOH (pH 3), B = MeOH. The elution program was isocratic for 3.33 min (77A : 23B) then a linear gradient from 3.33 min to 29.33 min (10A : 90B) before recovery of the initial conditions over 1 min and equilibration over 7 min.

2.2.2 UPLC-ESI MS. UPLC-ESI MS analysis was performed using a Waters Acuity UPLC® system coupled with a Waters Synapt G2 (Waters Corporation, Manchester, UK) equipped with an electrospray ionization source, using the gradient described above. MS and tandem MS (MS/MS) analyses were conducted in negative ionization mode. Source conditions included a capillarvoltage of 2.5 kV, a sample cone voltage of 35 V, a des-
olvation temperature of 300 °C and a source temperature of 120 °C. Argon was used as a collision gas. Precursor ions were selected on the basis of m/z and retention time. For MS/MS the collision energy was optimised to establish characteristic fragmentation patterns. Representative data using a collision energy of 25 eV to illustrate fragmentation patterns is pre-
sented. Data was collected using Masslynx 4.1 (Waters Corpor-
ation, Milford, MA, USA) and processed with Origin 8.5 (OriginLab, Northampton, MA, USA).

2.3 Plant and yarn extractions

2.3.1 Plant extraction. 1.5 g of dried leaves of dyer’s 
greenweed were extracted in 100 mL (MeOH : H₂O 1 : 1, v/v) in 
a 18916 bath for 2 hours at 40 °C. A fraction of the extract 
was centrifuged for 10 min at 10 000 rpm and filtered using a 
PTFE Phenomenex syringe filter (0.2 µm, 4 mm) for UPLC-PDA 
analysis. A second fraction of the extract was subjected to the 
hydrolysis procedure described below for yarn analysis and 
again analysed by UPLC-PDA.

2.3.2 Yarn extraction. The yarns (0.1–0.5 mg) were extrac-
ted with 37% HCl : H₂O : MeOH [200 µL, 2 : 1 : 1 (v/v/v)], at 100 
°C for 10 min.[13] After ambient cooling to room temperature, the 
extract was centrifuged for 10 min at 10 000 rpm and then 
filtred directly into Waters UPLC vials® (residual volume of 9 
µL) using a PTFE Phenomenex syringe filter (0.2 µm, 4 mm). The 
extract was then cooled with liquid nitrogen and dried under 
vacuum using a freeze drier system. The dry residue was then 
reconstituted with H₂O : MeOH [40 µL, 1 : 1 (v/v)] – allowing a 
single injection of 10 µL.

2.4 Accelerated light-ageing system

Accelerated light-ageing experiments were performed at 
National Museums Scotland using a Complete Lighting Systems 
light box (St Albans, UK), working at ambient temperature and 
relative humidity (RH). These levels were monitored hourly, 
together with the intensity of illumiance and UV levels using an 
Elsec IROLG environmental Logger and the data periodically 
downloaded to computer. Wool yarns dyed with weld (Reseda 
luteola L.) and others with the single dye component genistein 
were removed over a period of 4571 hours and 1 mg of yarn 
was subsequently hydrolysed following the procedure described 
above and investigated by HPLC analysis following the method 
used by Peggie et al. in order to obtain the degradation profiles 
of luteolin and genistein components.[14]

3. Results and discussion

3.1 UPLC and MS study of flavone/iso flavon e standards

3.1.1 UPLC method development. The UPLC method was 
evaluated based on several performance characteristics: the 
repeatability, the specificity, the limit of detection/quantifica-
tion and the linearity.[15] The method was developed to provide 
an improved separation and quantification of 5 dye standards: 
the flavones luteolin (1) and apigenin (3), the isoflavone genis-
tein (2), and the two O-methylated flavones chrysoeriol (4) and 
diosmetin (5). For all the dye standards the average variation on 
12 measurements ranged between 0.04–0.06%, and the method 
allowed a significantly greater separation of several dye 
components compared to that achieved in a recent UPLC 
study.[16] The method allowed a complete separation of the
flavone luteolin (1) and the isoflavone genistein (3) with an $R_s$ factor of 3.13; while the separation of the flavone apigenin (3) and the O-methylated flavone chrysoeriol (4) was increased to a value of 2.88, compared to 0.78 obtained by conventional HPLC.\textsuperscript{17} Finally, the method also allowed the separation of the O-methylated regio-isomers chrysoeriol (4) and diosmetin (5) with an $R_s$ factor of 1.32 (Fig. 1). The limits of detection (LoD) and limits of quantification (LoQ) were calculated based on the average value of the baseline noise $H_{\text{noise}}$ of several solvent blanks, considering all data points. The baseline of the UV detector at 254 nm averaged $(9 \pm 1) \times 10^{-4}$ AU, resulting in detection limits ranging from 0.5 ng for genistein to 1.9 ng for apigenin for an injection volume of 5 μL (Table 1). These values equalled or improved upon the values published for luteolin (cf. genistein vs. prunetin, Table 1), except for where methylation disrupted the chelating motif formed by the 5-OH and the carbonyl of the C-ring (cf. genistein vs. isoprunetin, Table 1), in these cases shorter retention times were observed due to reduced binding to the stationary phase.

Prior to sample analysis, ESI conditions were optimised using a reference standard solution containing luteolin, genistein, apigenin, chrysoeriol and diosmetin at 10 μg mL$^{-1}$ amongst others to maximise signal and minimise in-source fragmentation. Standard solutions were then used to characterise fragmentation patterns in the MS/MS experiments. For the MS/MS studies the deprotonated standards, [M – H]$^-$, were subjected to fragmentation at collision energies ranging from 10–40 eV; with 25 eV determined to be optimal as it resulted in significant fragmentation across all species. The results of MS/MS fragmentation at this collision energy are presented in Table 1, with fragment ion intensities reported as a percentage of the base peak intensity (% BPI). Direct comparison of MS/MS fragmentation of deprotonated flavonoids and isoflavonoids under these conditions has allowed us to identify the following trends. When deprotonated on the A-ring, both flavonoids and isoflavonoids show cleavage due to a retro-Diels–Alder mechanism (RDA, $^{1,1\text{A}}$, Scheme 1); this is more pronounced in the case of flavonoids and is frequently accompanied by the loss of CO$_2$.\textsuperscript{20} However, when deprotonated on the B-ring, flavonoids show breakdown by the equivalent retro-Diels–Alder mechanism (RDA*, $^{0,2\text{B}}$, Scheme 2), whereas there is little evidence for this in the isoflavonoids. Instead, an alternative 4’-OH isoflavonoid-specific fragmentation pathway predominates (iFF, $^{0,2\text{A}}$, Scheme 2).\textsuperscript{20} As expected, upon methylation both flavonoids and isoflavonoids have as a principal fragment the [M – H – CH$_3$]$^-$ ion, which typically occurs as the base peak and is accompanied by neutral losses of CO and CO$_2$ retrocyclization accounts for only a very small proportion of the fragmentation in these cases.

3.1.2 UPLC-MS study. Previous studies have shown that mass spectrometric analysis of flavonoid and isoflavonoid dyes may be achieved effectively using electrospray ionisation (ESI) mass spectrometry in negative mode,\textsuperscript{2,19–22} and have allowed the characterisation of several fragmentation mechanisms using secondary and tertiary mass spectrometry ion fragmentation (MS$^2$ and MS$^3$).\textsuperscript{2,19–22} A formal notation which has been adopted for these fragmentation mechanisms is indicated in Fig. 2;\textsuperscript{2,22} it is based upon glycoside nomenclature with letters to indicate bond scission. Studies of isoflavonoids using negative ESI and MS$^0$ have identified detailed mechanisms for the loss of small neutral molecules including CO, CO$_2$ and ketene (C$_2$H$_2$O)$_2$,\textsuperscript{21} processes which are common across these classes. In addition, for methylated, or glycosylated, flavonoids and isoflavonoids the primary fragmentation mechanism has been shown to be radical cleavage resulting in the detection of [M – H – CH$_3$]$^-$ or [M – H – glycoside]$^-$ ions.\textsuperscript{23}

Alongside the standards used for optimisation of the UPLC conditions, several O-methylated isoflavones related to genistein (2) [isopruniten (6), glycitein (7), prunetin (8) and biochanin A (9)], which were putative candidates for the minor components found in historical extracts, were selected for study by UPLC-MS. With this larger group of compounds some general trends in elution times could be deduced. Not surprisingly, compounds with an increased hydroxyl substitution pattern were shown to elute at shorter retention times under the UPLC conditions employed (cf. luteolin vs. apigenin, Table 1). Methylation generally gave rise to longer retention times (cf. genistein vs. prunetin, Table 1), except for where methylation disrupted the chelating motif formed by the 5-OH and the carbonyl of the C-ring (cf. genistein vs. isoprunetin, Table 1), in these cases shorter retention times were observed due to reduced binding to the stationary phase.

Prior to sample analysis, ESI conditions were optimised using a reference standard solution containing luteolin, genistein, apigenin, chrysoeriol and diosmetin at 10 μg mL$^{-1}$ amongst others to maximise signal and minimise in-source fragmentation. Standard solutions were then used to characterise fragmentation patterns in the MS/MS experiments. For the MS/MS studies the deprotonated standards, [M – H]$^-$, were subjected to fragmentation at collision energies ranging from 10–40 eV; with 25 eV determined to be optimal as it resulted in significant fragmentation across all species. The results of MS/MS fragmentation at this collision energy are presented in Table 1, with fragment ion intensities reported as a percentage of the base peak intensity (% BPI). Direct comparison of MS/MS fragmentation of deprotonated flavonoids and isoflavonoids under these conditions has allowed us to identify the following trends. When deprotonated on the A-ring, both flavonoids and isoflavonoids show cleavage due to a retro-Diels–Alder mechanism (RDA, $^{1,1\text{A}}$, Scheme 1); this is more pronounced in the case of flavonoids and is frequently accompanied by the loss of CO$_2$.\textsuperscript{20} However, when deprotonated on the B-ring, flavonoids show breakdown by the equivalent retro-Diels–Alder mechanism (RDA*, $^{0,2\text{B}}$, Scheme 2), whereas there is little evidence for this in the isoflavonoids. Instead, an alternative 4’-OH isoflavonoid-specific fragmentation pathway predominates (iFF, $^{0,2\text{A}}$, Scheme 2).\textsuperscript{20} As expected, upon methylation both flavonoids and isoflavonoids have as a principal fragment the [M – H – CH$_3$]$^-$ ion, which typically occurs as the base peak and is accompanied by neutral losses of CO and CO$_2$ retrocyclization accounts for only a very small proportion of the fragmentation in these cases.
Table 1  Flavonoid and isoflavonoid standards used to calibrate the UPLC-PDA and ESI-MS systems

<table>
<thead>
<tr>
<th>Component</th>
<th>Structure</th>
<th>PDA $R_t$ (min)</th>
<th>LoD (LoQ)</th>
<th>$\lambda_{\text{max}}$ MeOH (nm)</th>
<th>MS/MS $R_t$ (min)</th>
<th>$[M - H]^{-}$</th>
<th>MS/MS [CE: 25 eV] $m/z$ (% BPI) fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FLAVONES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luteolin (1)</td>
<td><img src="image" alt="Luteolin Structure" /></td>
<td>13.47</td>
<td>0.9 (2.6)</td>
<td>252</td>
<td>291 (sh)</td>
<td>349</td>
<td>13.56</td>
</tr>
<tr>
<td>Apigenin (3)</td>
<td><img src="image" alt="Apigenin Structure" /></td>
<td>15.26</td>
<td>1.9 (5.7)</td>
<td>267</td>
<td>300 (sh)</td>
<td>338</td>
<td>15.58</td>
</tr>
<tr>
<td>Chrysoeriol (4)</td>
<td><img src="image" alt="Chrysoeriol Structure" /></td>
<td>15.69</td>
<td>1.2 (3.4)</td>
<td>250</td>
<td>268</td>
<td>290 (sh)</td>
<td>349</td>
</tr>
<tr>
<td>Diosmetin (5)</td>
<td><img src="image" alt="Diosmetin Structure" /></td>
<td>15.87</td>
<td>1.2 (3.7)</td>
<td>250</td>
<td>268</td>
<td>290 (sh)</td>
<td>348</td>
</tr>
<tr>
<td><strong>ISOFLAVONES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein (2)</td>
<td><img src="image" alt="Genistein Structure" /></td>
<td>14.03</td>
<td>0.5 (1.6)</td>
<td>260</td>
<td>332 (sh)</td>
<td>14.16</td>
<td>269</td>
</tr>
<tr>
<td>Isoprunetin (6)</td>
<td><img src="image" alt="Isoprunetin Structure" /></td>
<td>12.17</td>
<td>—</td>
<td>255</td>
<td>332 (sh)</td>
<td>12.16</td>
<td>283</td>
</tr>
<tr>
<td>Glycitein (7)</td>
<td><img src="image" alt="Glycitein Structure" /></td>
<td>12.91</td>
<td>—</td>
<td>235 (sh)</td>
<td>257</td>
<td>320</td>
<td>12.98</td>
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<tr>
<td>Prunetin (8)</td>
<td><img src="image" alt="Prunetin Structure" /></td>
<td>18.93</td>
<td>—</td>
<td>260</td>
<td>332 (sh)</td>
<td>19.04</td>
<td>283</td>
</tr>
</tbody>
</table>
3.2 Dye components characterised in *Genista tinctoria* L.

3.2.1 UPLC-PDA study. In common with other recent studies, UPLC-PDA investigation of the extract of dyer’s greenweed leaves reveals a complex mixture of flavonoid glycosides and minor amounts of their aglycone equivalents; after acid hydrolysis the same extract exhibits a similar composition to the acid hydrolysed extracts from wool (YW2) and silk (YS3a) reference yarns (Fig. 3). Both fibre extracts present a very similar dye component profile; when analysed at 254 nm the main dye components luteolin (1), genistein (2), and apigenin (3) appear to be of quite similar intensity on each substrate (wool vs. silk; see ESI 4†). In addition, UPLC analysis shows that both luteolin methyl-ethers chrysoeriol (4) and diosmetin (5) are present at low levels in the acid hydrolysed extracts (Fig. 3B). Chrysoeriol (4) was found to average 1.3 and 1.2% (wool and silk, respectively), while diosmetin (5) was found to average 2.4 and 2.3% (wool and silk, respectively) of the total relative amount of the flavonoids present in the extracts (see ESI 4†). The presence of the methylated regioisomers chrysoeriol and diosmetin in the acid hydrolysed extracts of yarn dyed with *Genista tinctoria* L. has not been reported previously; but is not surprising given that chrysoeriol has been reported to occur in some *Genista* species. Four unknown components were also characterised in the acid hydrolysed extracts of both silk and wool yarns; some of these are thought to be aglycones as they were also found in the extract of dyer’s greenweed leaves before acid hydrolysis (Fig. 3).

Thus, these components might be useful markers for the identification of *Genista tinctoria* L. and were named Gt1, Gt2, Gt3, and Gt4 (Rt = 10.06 min, Gt2 (Rt = 11.67 min), Gt3 (Rt = 12.17 min) and Gt4 (Rt = 14.61 min) respectively; they all exhibit a maximum absorption between 255 and 261 nm, which indicates an isoflavonoid structure. Of these, Gt3 is often present at levels which are detectable in historical textile samples; hence we have further investigated the structure of these components by MS/MS, and examined the effect of the dyeing process on their uptake onto wool and silk fibres.

3.2.2 UPLC-MS study. In an attempt to identify the Gt components unambiguously, the extract from the reference yarn YW2 was studied further by UPLC-ESI-MS (Fig. 3, lower). Although detection at 254 nm indicates the presence of a clear peak for Gt1, the ESI-MS BPI profile clearly shows the presence of several overlapping species with a very similar retention time, thus it was not possible to obtain further information about Gt1.

### Table 1 (Contd.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Structure</th>
<th>PDA Rt (min)</th>
<th>LoD (ng)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; MeOH (nm)</th>
<th>MS/MS Rt (min)</th>
<th>[M – H]&lt;sup&gt;–&lt;/sup&gt; m/z</th>
<th>MS/MS (CE: 25 eV) m/z (% BPI) fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochanin A</td>
<td><img src="biochanin_a.png" alt="Image" /></td>
<td>19.30</td>
<td>—</td>
<td>260</td>
<td>14.95</td>
<td>283 (9.5)</td>
<td>[M – H]&lt;sup&gt;–&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>332 (sh)</td>
<td>19.45</td>
<td>283 (9.5)</td>
<td>[M – H – CH&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;–&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>268 (100)</td>
<td>19.45</td>
<td>268 (100)</td>
<td>[M – H – CH&lt;sub&gt;3&lt;/sub&gt; – CO]&lt;sup&gt;–&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>240 (5.3)</td>
<td>19.45</td>
<td>240 (5.3)</td>
<td>[M – H – 2H – CH&lt;sub&gt;3&lt;/sub&gt; – CO]&lt;sup&gt;–&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>239 (7.3)</td>
<td>19.45</td>
<td>239 (7.3)</td>
<td>[M – 2H – CH&lt;sub&gt;3&lt;/sub&gt; – CO]&lt;sup&gt;–&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>211 (3.5)</td>
<td>19.45</td>
<td>211 (3.5)</td>
<td>[M – 2H – CH&lt;sub&gt;3&lt;/sub&gt; – 2CO]&lt;sup&gt;–&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The ions annotated with RDA correspond to retro-Diels–Alder fragments (1,3A<sup>-</sup>) and 4’-OH iso- 
  flavonoid-specific fragmentation (iFF, 0,3B<sup>-</sup>). The ions annotated with RDA* correspond to retro-Diels–Alder 
  fragments where ionisation is in the B-ring (1,3B<sup>-</sup>). The ions annotated with iFF correspond to the iso- 
  flavonoid-specific fragmentation (iFF, 0,3B<sup>-</sup>).
However, the components Gt3-4 were readily isolated by ESI-MS analysis, allowing further study by MS/MS (ESI 3†).

**Fragmentation of Gt2.** The minor component Gt2 exhibits a maximum absorption at 260 nm which indicates an isoflavonoid structure, with [M – H]⁻ at m/z 285 that corresponds to the isoflavone genistein with an additional OH. Several isomeric isoflavone structures corresponding to this level of hydroxyl substitution have been reported in the literature of which 5,7,3',4'-tetrahydroxyisoflavone (orobol); 6,7,3',4'-tetrahydroxyisoflavone; 2,5,7,4'-tetrahydroxyisoflavone and 7,8,3',4'-tetrahydroxyisoflavone exhibit a maximum absorption between 258 and 262 nm. Detailed MS/MS studies showed a fragmentation pattern similar to genistein (Table 2); and the decrease in retention time under UPLC conditions relative to biochanin A would support additional hydroxylation in the B-ring; in addition, an increased retention time of 2.93 min relative to orobol (Table 2) fits well with the equivalent flavonoid pairing of luteolin and diosmetin where methylation of the 4'-OH leads to an increased retention time of 0.37 min (Table 1). However, alternative structures for Gt4 include 3'-O-methylorobol, the isoflavonoid equivalent of chrysoeriol (4), or 7-O-methylorobol possibly formed by alkylation of a 7-O-glycosidic species under the acidic extraction conditions, as recently reported in a study of the constituents of sawwort (Serratula tinctoria L.). However, it is clear that it is not the 5-O-methylated derivative as this, by analogy with isoprunetin, would be expected to elute much earlier in the UPLC chromatogram.

3.3 Effect of textile preparation and ageing on the dye fingerprint

3.3.1 Over-dyeing process. The composition of dyestuffs extracted from historical textile samples is known to vary due to the effects of ageing, but it is also thought to be dependent on workshop practices such as over-dyeing (where a yarn is treated with successive dyebaths in order to achieve the desired colour or hue). Silk reference samples prepared by over-dyeing with successive dyer’s greenweed dye baths (YS3a-d, one to four baths respectively) were examined by UPLC (Fig. 5). While the
silk reference YS3a shows the highest level of Gt3 and genistein (2) compared to luteolin (1), this is gradually reduced as the textile is over-dyed (YS3b–d). A number of explanations for this have been suggested including that luteolin (1) binds the mordant more efficiently than the isoflavone genistein (2), so that the latter is displaced with each additional dyeing, or that genistein (2), degrades preferentially under the dyebath conditions, or other reasons. The degradation of genistein (2), and Gt3, in the dyebath would be supported by several studies of soy products which showed that isoflavonoid compounds, especially genistein (2), degraded easily at elevated temperatures. However, in the context of dyeing a gradual equilibration to give the most stable dye/mordant/proteinaceous fibre complex is a more attractive explanation for this variation. Particularly as a displacement mechanism might also be expected to apply to Gt3, which should not bind as strongly due to the modified chelating motif provided by the 5-O-methylation, and in support of this the ratio of Gt3 to luteolin is the most noticeably reduced.

<table>
<thead>
<tr>
<th>Unknown component</th>
<th>PDA Rt (min)</th>
<th>λmax MeOH (nm)</th>
<th>MS/MS Rt (min)</th>
<th>[M – H]– m/z</th>
<th>MS/MS (CE: 25 eV) m/z (% BPI) fragment</th>
<th>Proposed structure</th>
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<td>Gt1</td>
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<td>11.96</td>
<td>285</td>
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<td>257 (13.2) [M – 2H – CO2]–</td>
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<tr>
<td></td>
<td></td>
<td>290 (sh)</td>
<td></td>
<td></td>
<td>240 (6.4) [M – H – 2CO]–</td>
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<td></td>
<td>229 (12.3) [M – H – C3O2]–</td>
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<td>217 (12.7) [M – H – C5O3]–</td>
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<td>198 (9.8) iFF</td>
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<td>Gt2</td>
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<td>260</td>
<td>11.96</td>
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<td>290 (sh)</td>
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<td>240 (100) [M – H – CH3 – CO]–</td>
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<td>330 (sh)</td>
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<td>211 (3.4) [M – 2H – CH3 – 2CO]–</td>
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<td>196 (14.8) 184 (10.1)</td>
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<td>255 (6.0) [M – 2H – CH3 – CO]–</td>
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<td>227 (5.5) [M – 2H – CH3 – 2CO]–</td>
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<td>14.89</td>
<td>299</td>
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<td>261</td>
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<td>284 (100) [M – H – CH3]–</td>
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<td></td>
<td>295 (sh)</td>
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<td>256 (7.3) [M – H – CH3 – CO]–</td>
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<tr>
<td></td>
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<td>340 (sh)</td>
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<td>255 (6.0) [M – 2H – CH3 – CO]–</td>
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<td></td>
<td></td>
<td>227 (5.5) [M – 2H – CH3 – 2CO]–</td>
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<td></td>
<td></td>
<td></td>
<td>200 (6.4) iFF</td>
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</table>

Table 2: Investigation of Gt compounds

* The ions annotated with iFF correspond to the isoflavonoid-specific fragmentation (0,3B–).
through subsequent over-dyeing processes (YS3a–d). The presence of both colourless components, genistein (2) and Gt3, in high quantities on wool and silk yarns after a single dyebath would clearly affect the colour achieved and might explain why dyer’s greenweed was judged to be of lower quality as a dyestuff than weld due to the need for multiple dyeing processes to obtain the desired yellow colour.

3.3.2 Light ageing. An investigation into the relative degradation rates of luteolin (1) and genistein (2) was carried out using reference wool yarn YW1 dyed with weld (Reseda luteola L.) and an alum mordanted wool yarn ‘dyed’ with pure genistein that were aged under accelerated conditions. The results were then compared with the degradation of luteolin in weld and have been plotted in Fig. 6, where the area of each component, per mg of yarn (monitored at 254 nm) has been expressed as a percentage of the area obtained in the un-aged sample extract. The degradation rate of both components can be fitted to a double exponential model, predicted by previous work. This is best explained, at least to a first approximation, by the two different degradation rates associated with the dye on the surface of the yarn and the dye in the bulk of the yarn. After 4000 h of exposure in the light box, the amount of luteolin observed in the extract (per mg of yarn) had fallen to ca. 10% of that observed in the original, un-aged, extract. In contrast, the amount of genistein observed in the extract after 4000 h of ageing had only fallen to ca. 35% of that observed in the original, un-aged, extract; thus confirming the observation that genistein (on an alum mordanted wool substrate) has a relatively slow photo-degradation rate compared to luteolin. These results suggest that although the relative amount of genistein initially present on the fibre is dependent on the dyeing process, its presence in the acid hydrolysed extract should act as a “marker” in historical samples.

3.3.3 Historical tapestry samples. These studies have allowed us to contextualise the analytical data acquired from a small selection of historical yarns (yellow silk and green wool) sampled from mid-sixteenth century English tapestries from the Burrell Collection in Glasgow, UK and the Bodleian Library in Oxford, UK (ESI 4†). The dye profiles from these yarns are characterised by the presence of the flavones luteolin (1) and apigenin (3), the isoflavone genistein (2), the O-methylated flavone chrysoeriol (4) and the isoflavonoid compounds Gt3 and Gt4, along with indigotin in the green yarns. Interestingly, diosmetin (5) was only identified in two historical samples, highlighting the need for further investigation into the light fastness of both O-methylated flavone isomers. The presence of both the isoflavone genistein and the methylated isoflavonoid compound Gt4 in most of the historical yarns investigated would suggest that they exhibit similar photo-degradation rates. It was observed that the relative amounts of isoflavonoid dyes were very variable in historical yarns, which would suggest that these variations could be related to the textile preparation, as was observed in the reference yarns subjected to over-dyeing processes. A ternary representation of the relative amounts of the dyestuff components (Fig. 7) highlights the differences in composition that result from these differences in yarn preparation and allows the dye profile of the historical samples (ESI, Tables 2 and 3†) to be compared with those of over-dyed silk references. From these observations, it does appear that most of the yellow yarns were not over-dyed (with an isoflavonoid content of >50%), while in contrast several of the green yarns show a profile which more closely matches that of the over-dyed silk samples, i.e. with a higher ratio of luteolin : genistein. However, in light of the photo-degradation studies presented above, the scattering of the composition might also reflect variation in the levels of photo-degradation of the samples.

4. Conclusions

This UPLC study provides a greatly improved method for the identification of a range of flavonoid and isoflavonoid dyestuffs from historical textile samples, including the regio-isomeric compounds chrysoeriol and diosmetin. This method has allowed the unambiguous identification of additional dye
components which occur in the plant extract of *Genista tinctoria* L. and also in the acid hydrolysed extracts of reference and historical yarns dyed with this species. Four unknowns were also identified in the extracts, designated Gt₁₋₄ on the basis of their relative retention times, and further study of these by UPLC-MS/MS allows us to suggest a structural identification of Gt₁ as orobol, Gt₂ as isoprenutin and Gt₄ as pratensein (or a related O-methylated isoflavone). We believe that these, in particular Gt₃, might aid with the identification of dye’s greenweed as a plant dye source on historical textiles. This robust method was also used to study the effects of over-dyeing and photo-degradation on the relative compositions of dyestuff components on wool and silk substrates, allowing us to contextualise a selection of historical yarns sampled from mid-sixteenth century English tapestries. The application of UPLC and MS techniques to the study of historical textile samples opens up exciting possibilities for heritage applications, as objects from which sampling was previously not possible may now be examined due to the greatly reduced requirements for sample size and subsequent limit of detection obtained, enabling the identification of very minor components in heavily degraded samples. Given the clear benefits which this study demonstrates and the wealth of information which these methods provide, we anticipate that those working in the field of heritage science will rapidly adopt UPLC as the method of choice for natural product dyestuff analysis.

**Acknowledgements**

We thank Jane Rowlands and Patricia Collins (Burrell Collection, Glasgow, UK) and David Howell (Bodleian Library, Oxford, UK) for allowing sampling of the tapestries; Andrew Simpson from Waters UK for technical support on both HPLC and UPLC systems; Lorraine Gibson, Strathclyde University, Glasgow UK, for advice during method development; Logan Mackay, University of Edinburgh for assistance with UPLC-MS. Financial assistance was provided through the Science and Heritage programme (studentship to LGT, Grant ref. CDA08/411), the EC (contract number EVK4-CT-2001-00048, Monitoring of Damage to Historic Tapestries project), Glasgow Museums and National Museums Scotland. Tapestry detail in the graphical abstract reproduced courtesy of Glasgow Museums Collection.

**Notes and references**