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## ARTICLE

# Historical textile dyeing with *Genista tinctoria* L.: A comprehensive study by UPLC-MS/MS analysis

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Polyphenolic components from *Genista* species have been well characterised because of their potential as anti-oxidants and as therapeutic leads; however, the identification of dyer's greenweed (*Genista tinctoria* L.) in historical textiles has been the subject of only limited studies. This paper presents a comprehensive UPLC-PDA MS/MS study of reference and historical yarns dyed with this species. Several so far unreported dye components that could assist with the identification of this dye source, were characterised by MS/MS. Furthermore, the effect of photo-degradation and textile preparation techniques (such as over-dyeing) on the dye fingerprint was investigated and the results correlated with those obtained from historical samples from the Burrell and Bodleian collections, UK.

## 1. Introduction

Polyphenolic components from *Genista* species have been well characterised because of their potential as anti-oxidants and as therapeutic leads;<sup>1,2</sup> however, the identification of dyer's greenweed (*Genista tinctoria* L.) in historical textiles has been the subject of only limited studies.<sup>3,4</sup> 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.<sup>5</sup> The flavones luteolin and apigenin, the isoflavone genistein, and the glycosides of these are known dye components of *Genista* species.<sup>6</sup> But genistein, and its glycosides, are also the main dye component found in other varieties of broom; notwithstanding, the dye source for historical textiles is usually ascribed to dyer's greenweed.<sup>7,8</sup> Whilst genistein is the principle component on which this attribution is made,<sup>4</sup> 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<sup>§</sup> 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 (YSb-d), as part of the Monitoring of Damage to Historic Tapestries project (FP5, EC contract number EVK4-CT-2001-00048).<sup>9</sup>

**2.2.2 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 was purchased from Cayman Chemical Company; and chrysoeriol (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 isopruneitin (4',7-dihydroxy-5-methoxyisoflavone) was prepared from genistein by selective acetylation (Ac<sub>2</sub>O, py) to give 7,4'-diacetoxy-5-hydroxyisoflavone,<sup>10</sup> methylation of the remaining free hydroxyl [(MeO)<sub>2</sub>SO<sub>2</sub> K<sub>2</sub>CO<sub>3</sub>, acetone] under high dilution conditions<sup>11</sup> to give 7,4'-diacetoxy-5-methoxyisoflavone, followed by hydrolysis (NaHCO<sub>3</sub> aq., MeOH:THF)<sup>10</sup> of the acetate groups which allowed isolation of isopruneitin in >95% purity after chromatography (see SI 1).<sup>12</sup>

**2.2.3 Solutions of standards.** The UPLC® system was calibrated using stock solutions of flavonoid and isoflavonoid standards: 1. a solution containing luteolin and genistein (1.00 ± 0.01 mg of each standard) in H<sub>2</sub>O:MeOH [25 mL, 1:1 (v/v); equivalent to 40 µg mL<sup>-1</sup>]; 2. a solution containing apigenin, chrysoeriol (0.20 ± 0.01 mg of each standard) in H<sub>2</sub>O:MeOH [10 mL, 1:1 (v/v); equivalent to 20 µg mL<sup>-1</sup>]; 3. a solution of

1 diosmetin ( $1.00 \pm 0.01$  mg) in  $\text{H}_2\text{O}:\text{MeOH}$  [25 mL, 1:1 (v/v);  
2 equivalent to  $40 \mu\text{g mL}^{-1}$ ]. Diluted solutions were then prepared  
3 with components at concentrations of 20, 10, 5, 1, 0.5, 0.1,  
4 0.05, 0.02 and  $0.01 \mu\text{g mL}^{-1}$ , by dilution with  $\text{H}_2\text{O}:\text{MeOH}$  [1:1  
5 (v/v)] using calibrated micro-pipettes.

## 7 2.2 UPLC-PDA and ESI MS Systems.

8 **2.2.1 UPLC-PDA.** The UPLC chromatographic method  
9 was developed using a Waters Acquity UPLC<sup>®</sup> system with  
10 sample detection using a Waters PDA detector (250 to 500 nm).  
11 Data were collected by Waters Empower 2 software and  
12 processed with Origin 8.5 (OriginLab, Northampton, MA,  
13 USA). Sample extracts were automatically injected *via* a  
14 Rheodyne injector with a  $10 \mu\text{L}$  sample loop. The bandwidth  
15 (resolution) was  $1.2 \text{ nm}$  with a sampling rate of  $5 \text{ points s}^{-1}$ . The  
16 method used a PST BEH C18 reverse phase column,  $1.7 \mu\text{m}$   
17 particle size,  $150 \times 2.1 \text{ mm}$  (length  $\times$  i.d.), set-up with inline  
18 filter. The total run time was 37.33 min at a flow rate of  $250 \mu\text{L}$   
19  $\text{min}^{-1}$  and the column was maintained at  $55 \pm 1 \text{ }^\circ\text{C}$ . A binary  
20 solvent system was used; A = 0.02 % aqueous  $\text{HCOOH}$  (pH 3),  
21 B = MeOH. The elution program was isocratic for 3.33 min  
22 (77A: 23B) then a linear gradient from 3.33 min to 29.33 min  
23 (10A: 90B) before recovery of the initial conditions over 1 min  
24 and equilibration over 7 min.

25 **2.2.2 UPLC-ESI MS.** UPLC-ESI MS analysis was  
26 performed using a Waters Acquity UPLC<sup>®</sup> system coupled with  
27 a Waters Synapt G2 (Waters Corporation, Manchester, UK)  
28 equipped with an electrospray ionization source, using the  
29 gradient described above. MS and tandem MS (MS/MS)  
30 analysis were conducted in negative ionization mode. Source  
31 conditions included a capillary voltage of 2.5 kV, a sample  
32 cone voltage of 35 V, a desolvation temperature of  $300 \text{ }^\circ\text{C}$  and  
33 a source temperature of  $120 \text{ }^\circ\text{C}$ . Argon was used as a collision  
34 gas. Precursor ions were selected on the basis of  $m/z$  and  
35 retention time. For MS/MS the collision energy was optimised  
36 to establish characteristic fragmentation patterns.  
37 Representative data using a collision energy of 25eV to  
38 illustrate fragmentation patterns is presented. Data was  
39 collected using Masslynx 4.1 (Waters Corporation, Milford,  
40 MA, USA) and processed with Origin 8.5 (OriginLab,  
41 Northampton, MA, USA).

## 42 2.3 Plant and Yarn Extractions.

43 **2.3.1 Plant Extraction.** 1.5 g of dried leaves of dyer's  
44 greenweed were extracted in 100 mL ( $\text{MeOH}:\text{H}_2\text{O}$  1:1, v/v) in  
45 an ultrasonic bath for 2 hours at  $40^\circ\text{C}$ . A fraction of the extract  
46 was centrifuged for 10 min at 10,000 rpm and filtered using a  
47 PTFE Phenomenex syringe filter ( $0.2 \mu\text{m}$ , 4 mm) for UPLC-  
48 PDA analysis. A second fraction of the extract was subjected to  
49 the hydrolysis procedure described below for yarn analysis and  
50 again analysed by UPLC-PDA.

51 **2.3.2 Yarn Extraction.** The yarns (0.1-0.5 mg) were  
52 extracted with 37%  $\text{HCl}:\text{H}_2\text{O}:\text{MeOH}$  [ $200 \mu\text{L}$ , 2:1:1 (v/v/v)], at  
53  $100 \text{ }^\circ\text{C}$  for 10 min.<sup>13</sup> After ambient cooling to room  
54 temperature, the extract was centrifuged for 10 min at 10,000  
55 rpm and then filtered directly into Waters UPLC vials<sup>®</sup>  
56 (residual volume of  $9 \mu\text{L}$ ) using a PTFE Phenomenex syringe  
57 filter ( $0.2 \mu\text{m}$ , 4 mm). The extract was then cooled with liquid  
58 nitrogen and dried under vacuum using a freeze drier system.  
59 The dry residue was then reconstituted with  $\text{H}_2\text{O}:\text{MeOH}$  [ $40$   
60  $\mu\text{L}$ , 1:1 (v/v)] - allowing a single injection of  $10 \mu\text{L}$ .

## 61 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 illuminance  
and UV levels using an Elsec IRLOG 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.<sup>14</sup>

## 62 3. Results and Discussion

### 63 3.1 UPLC and MS study of flavone/isoflavone standards

64 **3.1.1 UPLC Method Development.** The UPLC method was  
65 evaluated based on several performance characteristics: the  
66 repeatability, the specificity, the limit of detection / quantification  
67 and the linearity.<sup>15</sup> The method was developed to provide an  
68 improved separation and quantification of 5 dye standards: the  
69 flavones luteolin (1) and apigenin (3), the isoflavone genistein (2),  
70 and the two *O*-methylated flavones chrysoeriol (4) and diosmetin  
71 (5). For all the dye standards the average variation on 12  
72 measurements ranged between 0.04 - 0.06 %, and the method  
73 allowed a significantly greater separation of several dye components  
74 compared to that achieved in a recent UPLC study.<sup>16</sup> The method  
75 allowed a complete separation of the flavone luteolin (1) and the  
76 isoflavone genistein (3) with an  $R_s$  factor of 3.13; while the  
77 separation of the flavone apigenin (3) and the *O*-methylated flavone  
78 chrysoeriol (4) was increased to a value of 2.88, compared to 0.78  
79 obtained by conventional HPLC.<sup>17</sup> Finally, the method also allowed  
80 the separation of the *O*-methylated regio-isomers chrysoeriol (4) and  
81 diosmetin (5) with an  $R_s$  factor of 1.32 (Figure 1). The limits of  
82 detection (LoD) and limits of quantification (LoQ) were calculated  
83 based on the average value of the baseline noise  $H_{\text{noise}}$  of several  
84 solvent blanks, considering all data points. The baseline of the UV  
85 detector at 254 nm averaged  $(9 \pm 1) \times 10^{-4}$  AU, resulting in detection  
86 limits ranging from 0.5 ng for genistein to 1.9 ng for apigenin for an  
87 injection volume of  $5 \mu\text{L}$  (Table 1). These values equalled or  
88 improved upon the values published for luteolin (1), genistein (2)  
89 and apigenin (3) in other UPLC/UHPLC studies.<sup>16,18</sup>

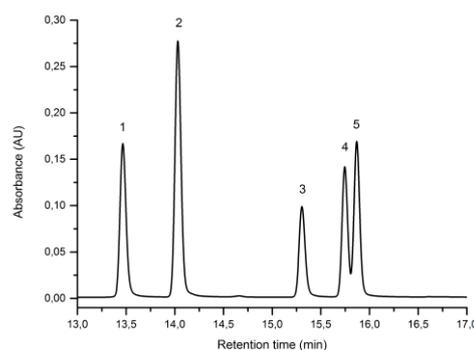
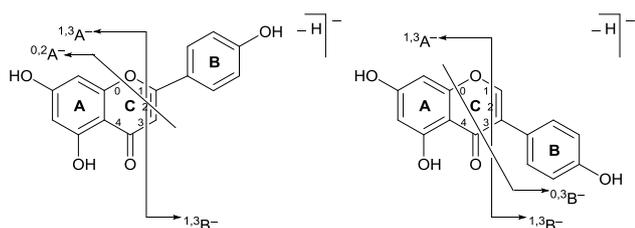


Figure 1: UPLC Method using PST BEH C18,  $1.7 \mu\text{m}$ ,  $2.1 \times 150 \text{ mm}$  column, showing the separation of luteolin (1), genistein (2), apigenin (3), chrysoeriol (4) and diosmetin (5). Standard solution at  $10 \mu\text{g mL}^{-1}$  recorded at 254 nm ( $5 \mu\text{L}$  injection volume).

**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,<sup>3,19-21</sup> and have allowed the characterisation of several fragmentation mechanisms using secondary and tertiary mass spectrometry ion fragmentation (MS<sup>2</sup> and MS<sup>3</sup>).<sup>3,20</sup> A formal notation which has been adopted for these fragmentation mechanisms is indicated in Figure 2; <sup>22,23</sup> it is based upon glycoside nomenclature with letters to indicate the ring carrying the charge and superscript numbers to indicate bond scission. Studies of isoflavonoids using negative ESI and MS<sup>n</sup> have identified detailed mechanisms for the loss of small neutral molecules including CO, CO<sub>2</sub> and ketene (C<sub>2</sub>H<sub>2</sub>O),<sup>21</sup> 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<sub>3</sub>]<sup>•-</sup> or [M - H - glycoside]<sup>•-</sup> ions.<sup>21</sup>

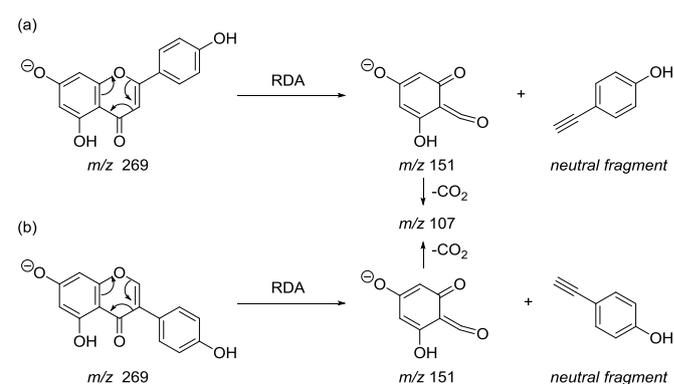


**Figure 2:** Formal notation used for the retrocyclization fragmentation of the [M - H]<sup>-</sup> ions of (a) flavonoids and (b) isoflavonoids under negative ion electrospray ionisation conditions.

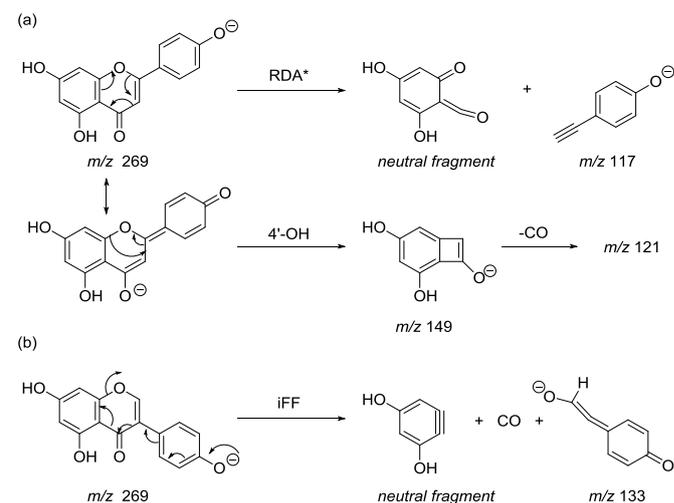
Alongside the standards used for optimisation of the UPLC conditions, several *O*-methylated isoflavones related to genistein (**2**) [isoprunitin (**6**), glycitein (**7**), prunetin (**8**) and biochanin A (**9**)], which were putative candidates for the minor components found in historical extracts, 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 hydrogen bond between the 5-OH and the carbonyl of the C-ring (*cf.* genistein vs isoprunitin, Table 1), in these cases shorter retention times were observed due to reduced binding to the stationary phase by the C-ring carbonyl.

Prior to sample analysis, ESI conditions were optimised using a reference standard solution containing luteolin, genistein, apigenin, chrysoeriol and diosmetin at 10 µg/mL 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]<sup>-</sup>, 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, <sup>1,3</sup>A<sup>-</sup>, Scheme 1); this is more pronounced in the case of flavonoids and is frequently accompanied by the loss of CO<sub>2</sub>.<sup>20</sup> However, when deprotonated on the B-ring, flavonoids show breakdown by the equivalent retro-Diels-Alder mechanism (RDA\*, <sup>1,3</sup>B<sup>-</sup>, Scheme 2), whereas there is little evidence for this in the isoflavonoids. Instead, an alternative 4'-OH isoflavonoid-specific fragmentation pathway predominates (iFF, <sup>0,3</sup>B<sup>-</sup>, Scheme 2). This is in direct contrast to our previously reported 4'-OH flavonoid-specific pathway (4'-OH, <sup>0,2</sup>A<sup>-</sup>, Scheme 2).<sup>20</sup> As expected, upon methylation both flavonoids and isoflavonoids have as a principal fragment the [M - H - CH<sub>3</sub>]<sup>•-</sup> ion, which typically occurs as the base peak and is accompanied by neutral losses of CO and CO<sub>2</sub>; retrocyclization accounts for only a very small proportion of the fragmentation in these cases.

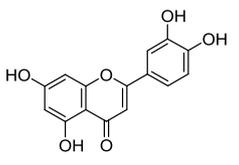
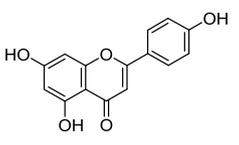
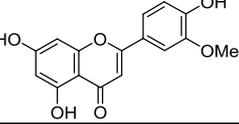
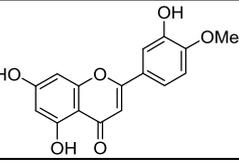
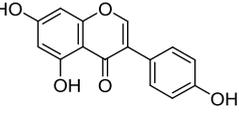
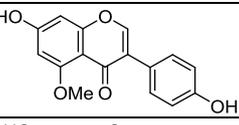
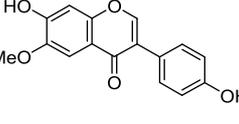
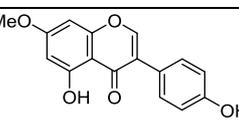
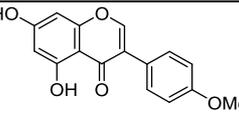


**Scheme 1:** Retro-Diels-Alder (RDA, <sup>1,3</sup>A<sup>-</sup>) MS/MS fragmentation mechanisms for [M - H]<sup>-</sup> with ionisation at the 7-OH, illustrated for: (a) the flavonoid apigenin; and (b) the isoflavonoid genistein.



**Scheme 2:** MS/MS fragmentation pathways for [M - H]<sup>-</sup> with ionisation at the 4'-OH, illustrated for: (a) the flavonoid apigenin, with retro-Diels-Alder\* (RDA\*, <sup>1,3</sup>B<sup>-</sup>) and 4'-OH flavonoid-specific (4'-OH, <sup>0,2</sup>A<sup>-</sup>) mechanisms; and (b) the isoflavonoid genistein, with the 4'-OH isoflavonoid-specific fragmentation (iFF, <sup>0,3</sup>B<sup>-</sup>) mechanism.

**Table 1:** Isoflavonoid and flavonoid standards used to calibrate the UPLC-PDA and ESI-MS systems.

Component	Structure	PDA R <sub>t</sub> (min)	LoD (LoQ) (ng)	λ <sub>max</sub> MeOH (nm)	MS/MS R <sub>t</sub> (min)	[M - H] <sup>-</sup> m/z	MS/MS (CE: 25 eV) m/z (% BPI) fragment
<b>FLAVONES</b>							
Luteolin (1)		13.47	0.9 (2.6)	252 291 (sh) 349	13.56	285	285 (100) [M - H] <sup>-</sup> 217 (12.5) [M - H - C <sub>3</sub> O <sub>2</sub> ] <sup>-</sup> 199 (17.8) 175 (23.1) [M - H - C <sub>3</sub> O <sub>2</sub> - C <sub>2</sub> H <sub>2</sub> O] <sup>-</sup> 151 (45.2) RDA 149 (20.2) (4'-OH) 133 (63.5) RDA* 121 (6.6) (4'-OH)-CO 107 (12.1) RDA-CO <sub>2</sub>
Apigenin (3)		15.26	1.9 (5.7)	267 300 (sh) 338	15.58	269	269 (100) [M - H] <sup>-</sup> 225 (28.5) [M - H - CO <sub>2</sub> ] <sup>-</sup> 201 (12.9) [M - H - C <sub>3</sub> O <sub>2</sub> ] <sup>-</sup> 183 (9.5) 159 (12.6) [M - H - C <sub>3</sub> O <sub>2</sub> - C <sub>2</sub> H <sub>2</sub> O] <sup>-</sup> 151 (58.7) RDA 149 (54.9) (4'-OH) 121 (11.3) (4'-OH)-CO 117 (79.1) RDA* 107 (17.4) RDA-CO <sub>2</sub>
Chrysoeriol (4)		15.69	1.2 (3.4)	250 268 290 (sh) 349	15.80	299	299 (4.4) [M - H] <sup>-</sup> 284 (100) [M - H - CH <sub>3</sub> ] <sup>•</sup> 256 (30.1) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 151 (1.3) RDA
Diosmetin (5)		15.87	1.2 (3.7)	250 268 290 (sh) 348	15.93	299	299 (1.3) [M - H] <sup>-</sup> 284 (100) [M - H - CH <sub>3</sub> ] <sup>•</sup> 256 (4.7) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 151 (1.0) RDA
<b>ISOFLAVONES</b>							
Genistein (2)		14.03	0.5 (1.6)	260 332 (sh)	14.16	269	269 (100) [M - H] <sup>-</sup> 241 (2.0) [M - H - CO] <sup>-</sup> 224 (6.4) [M - 2H - CO <sub>2</sub> ] <sup>-</sup> 213 (3.9) [M - H - 2CO] <sup>-</sup> 201 (8.2) [M - H - C <sub>3</sub> O <sub>2</sub> ] <sup>-</sup> 197 (6.8) [M - H - CO - CO <sub>2</sub> ] <sup>-</sup> 159 (13.8) [M - H - C <sub>3</sub> O <sub>2</sub> - C <sub>2</sub> H <sub>2</sub> O] <sup>-</sup> 133 (20.4) iFF 107 (9.1) RDA-CO <sub>2</sub>
Isoprunitin (6)		12.17	--	255 332 (sh)	12.16	283	283 (30.7) [M - H] <sup>-</sup> 268 (63.0) [M - H - CH <sub>3</sub> ] <sup>•</sup> 240 (100) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 196 (6.7) [M - H - CH <sub>3</sub> - CO - CO <sub>2</sub> ] <sup>•</sup>
Glycitein (7)		12.91	--	235 (sh) 257 320	12.98	283	283 (2.6) [M - H] <sup>-</sup> 268 (100) [M - H - CH <sub>3</sub> ] <sup>•</sup> 240 (58.2) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 196 (4.3) [M - H - CH <sub>3</sub> - CO - CO <sub>2</sub> ] <sup>•</sup>
Prunetin (8)		18.93	--	260 332 (sh)	19.04	283	283 (6.4) [M - H] <sup>-</sup> 268 (100) [M - H - CH <sub>3</sub> ] <sup>•</sup> 240 (7.7) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 196 (1.2) [M - H - CH <sub>3</sub> - CO - CO <sub>2</sub> ] <sup>•</sup>
Biochanin A (9)		19.30	--	260 332 (sh)	19.45	283	283 (9.5) [M - H] <sup>-</sup> 268 (100) [M - H - CH <sub>3</sub> ] <sup>•</sup> 240 (5.3) [M - H - CH <sub>3</sub> - CO] <sup>•</sup> 239 (7.3) [M - 2H - CH <sub>3</sub> - CO] <sup>-</sup> 211 (3.5) [M - 2H - CH <sub>3</sub> - 2CO] <sup>-</sup>

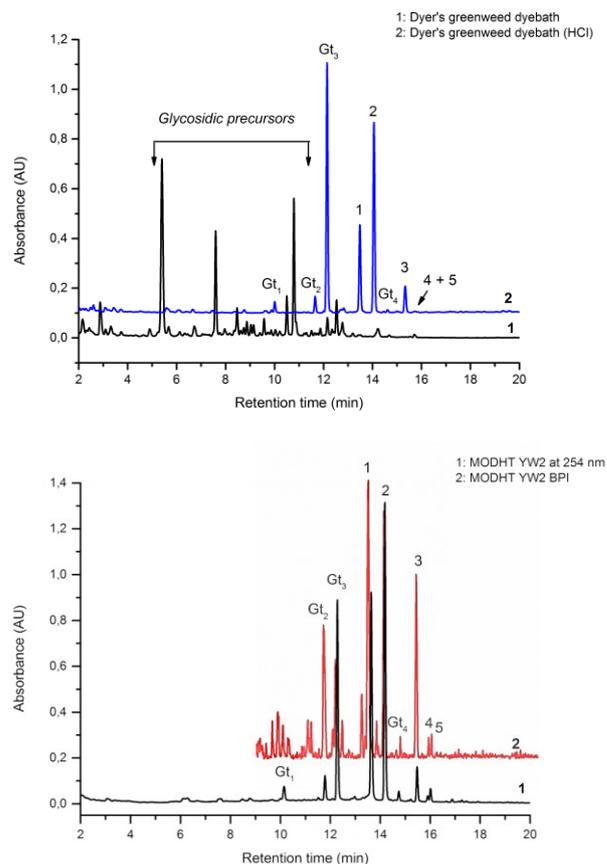
The ions annotated with RDA correspond to retro-Diels-Alder fragments (<sup>1,3</sup>A<sup>-</sup>). The ions annotated with RDA\* correspond to retro-Diels-Alder fragments where ionisation is in the B-ring (<sup>1,3</sup>B<sup>-</sup>). The ions annotated with iFF correspond to the isoflavonoid-specific fragmentation (<sup>0,3</sup>B<sup>-</sup>).

### 3.2 Dye components characterised in *Genista tinctoria* L.

**3.2.1 UPLC-PDA Study.** In common with other recent studies,<sup>24</sup> 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 (Figure 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 SI 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 (Figure 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 SI 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.<sup>25</sup> 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 (Figure 3). Thus, these components might be useful markers for the identification of *Genista tinctoria* L. and were named Gt<sub>1</sub> (R<sub>t</sub> = 10.06 min), Gt<sub>2</sub> (R<sub>t</sub> = 11.67 min), Gt<sub>3</sub> (R<sub>t</sub> = 12.17 min) and Gt<sub>4</sub> (R<sub>t</sub> = 14.61 min) respectively; they all exhibit a maximum absorption between 255 and 261 nm, which indicates an isoflavonoid structure. Of these, Gt<sub>3</sub> 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 (Figure 3, Lower). Although detection at 254 nm indicates the presence of a clear peak for Gt<sub>1</sub>, 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 Gt<sub>1</sub>. However, the components Gt<sub>2-4</sub> were readily isolated by ESI-MS analysis, allowing further study by MS/MS (SI 3).

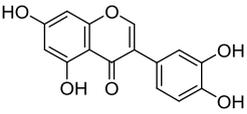
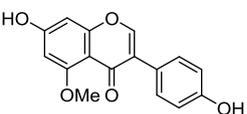
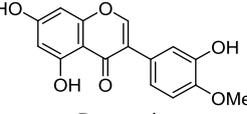
**Fragmentation of Gt<sub>2</sub>.** The minor component Gt<sub>2</sub> exhibits a maximum absorption at 260 nm which indicates an isoflavonoid structure, with [M - H]<sup>-</sup> at m/z 285 that corresponds to the isoflavone genistein with an additional OH. Several isomeric isoflavone structures corresponding 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



**Figure 3:** (A - Upper): UPLC chromatogram of a solution of dyer's greenweed extract and its acid hydrolysed equivalent, both monitored at 254 nm. (B - Lower): UPLC chromatogram of the acid hydrolysed reference MODHT YW2 monitored at 254 nm and MS BPI trace. Indicated on Figures 3A and 3B are the flavones luteolin (**1**) and apigenin (**3**), the isoflavone genistein (**2**), together with the four unidentified components Gt<sub>1</sub>, Gt<sub>2</sub>, Gt<sub>3</sub> and Gt<sub>4</sub> and the two luteolin methyl-ethers chrysoeriol (**4**) and diosmetin (**5**).

maximum absorption between 258 and 262 nm.<sup>26</sup> Detailed MS/MS studies showed a fragmentation pattern similar to genistein (Table 2); and the decrease in retention time under UPLC-MS conditions of 2.2 min relative to genistein compares favourably with the flavonoid pairing of luteolin and apigenin where an additional hydroxyl group in the B-ring decreases the retention time by 2.02 min (Table 1). These data, combined with the UV-vis spectra, suggest that Gt<sub>2</sub> corresponds to 5,7,3',4'-tetrahydroxyisoflavone (orobol), a hydroxylated isoflavone that has been identified in several *Genista* species growing in Italy and also in *Genista tinctoria*.<sup>27,28</sup>

**Table 2:** Investigation of Gt compounds.

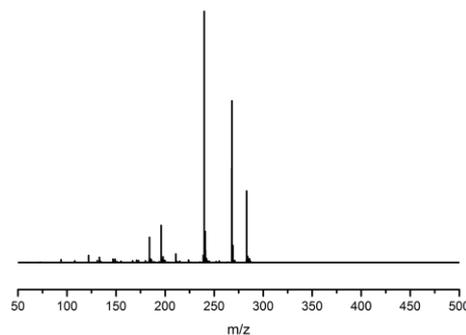
Unknown component	PDA R <sub>t</sub> (min)	λ <sub>max</sub> , MeOH (nm)	MS/MS R <sub>t</sub> (min)	[M - H] <sup>-</sup> m/z	MS/MS (CE: 25 eV) m/z (% BPI) fragment	Proposed Structure
Gt <sub>1</sub>	10.06	218 (sh) 255 290 (sh)	--	--	--	Not identified
Gt <sub>2</sub>	11.67	260 290 (sh) 330 (sh)	11.96	285	285 (100) [M - H] <sup>-</sup> 268 (8.5) 257 (13.2) [M - H - CO] <sup>-</sup> 240 (6.4) [M - 2H - CO <sub>2</sub> ] <sup>-</sup> 229 (12.3) [M - H - 2CO] <sup>-</sup> 217 (12.7) [M - H - C <sub>3</sub> O <sub>2</sub> ] <sup>-</sup> 198 (9.8) 149 (14.3) iFF	 Orobol
Gt <sub>3</sub>	12.17	255 330 (sh)	12.38	283	283 (28.5) [M - H] <sup>-</sup> 268 (64.8) [M - H - CH <sub>3</sub> ] <sup>+*</sup> 240 (100) [M - H - CH <sub>3</sub> - CO] <sup>+*</sup> 211 (3.4) [M - 2H - CH <sub>3</sub> - 2CO] <sup>-</sup> 196 (14.8) [M - H - CH <sub>3</sub> - CO - CO <sub>2</sub> ] <sup>+*</sup> 184 (10.1)	 Isopruneitin
Gt <sub>4</sub>	14.61	219 (sh) 261 295 (sh) 340 (sh)	14.89	299	299 (12.8) [M - H] <sup>-</sup> 284 (100) [M - H - CH <sub>3</sub> ] <sup>+*</sup> 256 (7.3) [M - H - CH <sub>3</sub> - CO] <sup>+*</sup> 255 (6.0) [M - 2H - CH <sub>3</sub> - CO] <sup>-</sup> 227 (5.5) [M - 2H - CH <sub>3</sub> - 2CO] <sup>-</sup> 200 (6.4)	 Pratensein

The ions annotated with RDA correspond to retro-Diels-Alder fragments (<sup>1,3</sup>A<sup>-</sup>). The ions annotated with RDA\* correspond to retro-Diels-Alder fragments where ionisation is in the B-ring (<sup>0,3</sup>B<sup>-</sup>). The ions annotated with iFF correspond to the isoflavonoid-specific fragmentation (<sup>0,3</sup>B<sup>-</sup>).

**Fragmentation of Gt<sub>3</sub>.** The minor component Gt<sub>3</sub> exhibits a maximum absorption at 254 nm, with [M - H]<sup>-</sup> at m/z of 283 that corresponds to an *O*- or *C*-methylated isoflavonoid related to genistein. Detailed MS/MS studies (Figure 4) showed the presence of a [M - H - CH<sub>3</sub>]<sup>+\*</sup> fragment ion at m/z 268, characteristic of an *O*-methylated isoflavone. In addition, the base peak at CE 25 eV was found to be at m/z 240 corresponding to the fragment ion [M - H - CH<sub>3</sub> - CO]<sup>+\*</sup>. The close match of the MS<sup>2</sup> fragmentation pattern to that of the authentic standard, combined with the correlation of retention time and the UV-vis data allowed Gt<sub>3</sub> to be unambiguously identified as 5-*O*-methyl genistein or isopruneitin (6). This was confirmed by a spiking experiment under the UPLC conditions in which authentic isopruneitin was added to the extract from reference yarn YW2 (SI 4). Although it has not previously been identified in historical textile extracts, the presence of high levels of isopruneitin and its associated glycoside in the *Genista tinctoria* L. textile extract is not surprising, as it is one of the main components reported in *Genista* species,<sup>29</sup> and has been previously characterised in several *Genista* species and also *Genista tinctoria* L. plant extracts.<sup>28,30</sup>

**Fragmentation of Gt<sub>4</sub>.** Finally, the minor component Gt<sub>4</sub> eluted closely to genistein (2) in the UPLC chromatogram at 14.61 min and exhibited a maximum absorption at 261 nm and [M - H]<sup>-</sup> at m/z 299. Further MS/MS study showed a fragmentation pattern similar to biochanin A (8) with an extra hydroxyl group; the base peak was found to be a [M - H - CH<sub>3</sub>]<sup>+\*</sup> fragment ion at m/z 284, characteristic of an *O*-methylated isoflavone. This could correspond to pratensein (3'-hydroxybiochanin A), which is the isoflavonoid equivalent of diosmetin (5).<sup>31</sup> Again a significant decrease in retention time under UPLC-MS 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)

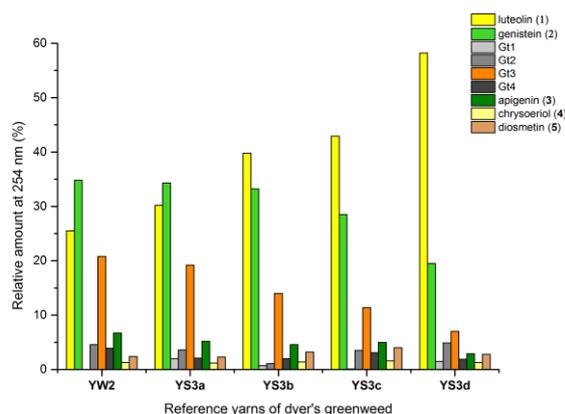
fits well with the equivalent flavonoid pairing of luteolin and diosmetin where methylation of the 4'-OH leads to an increased retention time of 2.37 min (Table 1). However, alternative structures for Gt<sub>4</sub> include 3'-*O*-methylorobol, the isoflavonoid equivalent of chrysoeriol (4), or 7-*O*-methylorobol possibly formed by alkylation of a 7-*O*-glycosidic species<sup>32</sup> under the acidic extraction conditions, as recently reported in a study of the constituents of sawwort (*Serratula tinctoria* L.).<sup>33</sup> However, it is clear that it is not the 5-*O*-methylated derivative as this, by analogy with isopruneitin, would be expected to elute much earlier in the UPLC chromatogram.



**Figure 4:** MS/MS fragmentation of the [M - H]<sup>-</sup> ion (m/z 283) of unknown component Gt<sub>3</sub> under negative ion electrospray ionisation conditions (CE: 25 eV).

### 3.3 Effect of Textile Preparation and Ageing on the Dye Fingerprint

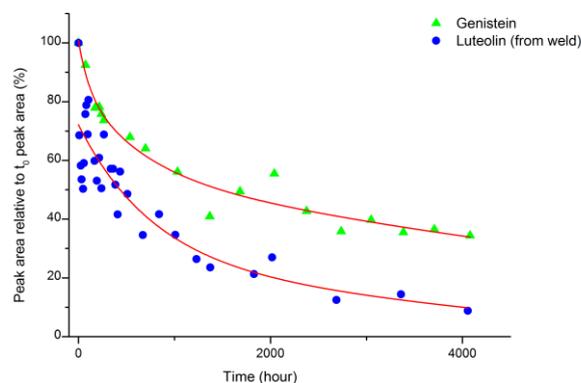
**3.3.1 Over-dyeing process.** The composition of dyestuffs extracted from historical textile samples is known to vary due to the effects of ageing, but it is also thought to be dependent on workshop practices such as over-dyeing (where a yarn is treated with successive dyebaths in order to achieve the desired colour or hue).<sup>4,8</sup> 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 (Figure 5). While the silk reference YS3a shows the highest level of Gt<sub>3</sub> 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),<sup>4</sup> so that the latter is displaced with each additional dyeing, or that genistein (2), degrades preferentially under the dyebath conditions,<sup>4</sup> or other reasons. The degradation of genistein (2), and Gt<sub>3</sub>, 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.<sup>34,35</sup> 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 Gt<sub>3</sub>, 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 Gt<sub>3</sub> to luteolin is the most noticeably reduced through subsequent overdyeing processes (YS3a-d). The presence of both colourless components, genistein (2) and Gt<sub>3</sub>, 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 than that of weld due to the need for multiple dyeing processes to obtain the desired yellow colour.<sup>8</sup>



**Figure 5:** Relative amounts of the flavonoid dyes present in the acid-hydrolysed extracts of wool (YW2) and silk (YS3a) MODHT reference samples and a series of over-dyed (YS3b-d) samples; monitored at 254 nm.

**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

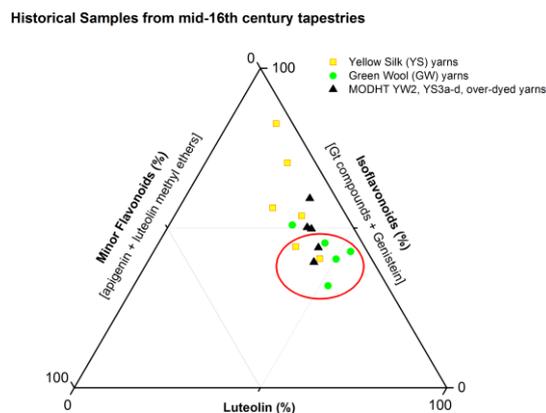
weld and have been plotted in Fig. 6, where the area of each component, per mg of yarn (monitored at 254 nm) has been expressed as a percentage of the area obtained in the un-aged sample extract. The degradation rate of both components can be fitted to a double exponential model, predicted by previous work.<sup>3</sup> 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.<sup>4</sup>



**Figure 6:** The photo-degradation rates of genistein (2) and luteolin (1).

**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 (SI 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 Gt<sub>3</sub> and Gt<sub>4</sub>, 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 Gt<sub>3</sub> 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 (Figure 7) highlights the differences in composition that result from these differences in yarn preparation and allows the dye profile of the historical samples (SI, 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 photodegradation studies presented above, the scattering of the composition might also reflect variation in the levels of photo-degradation of the samples.



**Figure 7:** Ternary representation of the relative amounts of dye components characterised in the acid hydrolysed extracts of historical silk and wool yarns and over-dyed silk reference yarns (MODHT YW2 and YS3a-d), monitored at 254 nm. Dyestuffs are grouped as follows: i. luteolin (1); ii. isoflavonoids [Gt<sub>3</sub> and genistein (2) and \* Gt<sub>1</sub>, Gt<sub>2</sub> and Gt<sub>4</sub> for reference samples]; and iii. minor flavone components [apigenin (3), chrysoeriol (4), diosmetin (5)]. The red circle indicates the historical samples that it is thought were subjected to an over-dyeing process.

#### 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 Gt<sub>1-4</sub> 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 Gt<sub>2</sub> as orobol, Gt<sub>3</sub> as isopruneitin and Gt<sub>4</sub> as pratensein (or a related *O*-methylated isoflavone). We believe that these, in particular Gt<sub>3</sub>, might aid with the identification of dyer's greenweed as a plant dye source on historical textiles. This robust method was also used to study the effects of over-dyeing and photodegradation 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.

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#### Notes and references

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<sup>§</sup> As commonly adopted by the field, the abbreviation UPLC has been used for the technique of ultra-high pressure liquid chromatography (UHPLC).

Electronic Supplementary Information (ESI) available: Preparation of an authentic sample of isopruneitin; (ESI<sup>+</sup>) MS<sup>2</sup> fragmentation of flavonoid and isoflavonoid standards at 25 eV; UPLC-PDA and (ESI<sup>+</sup>) MS<sup>2</sup> fragmentation of Gt components at 25 eV; UPLC-PDA investigation of references and historical yarns. See DOI: 10.1039/b000000x/

- 1 N. C. Veitch, *Nat. Prod. Rep.* 2013, **30**, 988-1027.
- 2 V. Sharma and K. G. Ramawat, In *Natural Products*; K. G. Ramawat and J. M. Mérillon, Ed.; Springer-Verlag Berlin Heidelberg: 2013, p 1849-1865.
- 3 E. S. B. Ferreira, University of Edinburgh, PhD Thesis, 2002.
- 4 D. A. Pegg, University of Edinburgh, PhD Thesis, 2006.
- 5 D. Cardon, *Dyes Hist. Archaeol.* 1995, **13**, 59-73.
- 6 E. S. B. Ferreira, H. McNab A. N., Hulme and A. Quye, *Chem. Soc. Rev.* 2004, **33**, 329-336.
- 7 D. Cardon, *Le monde des teintures naturelles*; Belin Paris, 2003
- 8 J. H. Hofenk de Graaf, *The colourful past*; Archetype Publications, 2005.
- 9 A.-M. Hacke and A. Quye, Appendix 2 in *Wrought in gold and silk: Preserving the Art of Historic Tapestries*, A. Quye, K. Hallet and C. Herrero Carretero Eds, NMS Enterprises Ltd. – Publishing, Edinburgh, UK.
- 10 N. Al-Maharik and N. P. Botting, *Tetrahedron*, 2003, **59**, 4177-4181.
- 11 B. J. Compton, L. Larsen and R. T. Weavers, *Tetrahedron*, 2011, **67**, 718-726.

## Journal Name

- 12 S. A. Adensanya, M. J. O'Neill and M. F. Roberts, *Phytochemistry*, 1985, **24**, 2699-2702.
- 13 J. Wouters and A. Verhecken, *Stud. Conserv.*, 1989, **34**, 189-200.
- 14 D. A. Peggie, A. N. Hulme, H. McNab and A. Quye, *Microchim. Acta*, 2008, **162**, 371-380.
- 15 C. Hartmann, J. Smeyers-Verbeke, D. L. Massart and R. D. McDowall, *J. Pharm. Biomed. Anal.*, 1998, **17**, 193-218.
- 16 A. Serrano, M. van Bommel and J. Hallett, *J. Chromatogr. A*, 2013, **29**, 102-111.
- 17 L. G. Troalen, The University of Edinburgh, PhD thesis, 2013.
- 18 Villela, A., van der Klift, E.J.C., Mattheussens, E.S.G.M., Derksen, G.C.H., Zuilhof, H., van Beek, T.A. *J. Chromatogr. A*, 2011, **1218**, 8544-8550.
- 19 A. N. Hulme, H. McNab, D. A. Peggie and A. Quye, *Phytochem.* 2005, **66**, 2766-2770.
- 20 H. McNab, E. S. B. Ferreira, A. N. Hulme and A. Quye, *Int. J. Mass Spectrom.*, 2009, **284**, 57-65.
- 21 K. Ablajan, *J. Mass. Spectrom.*, 2011, **46**, 77-84.
- 22 R. E. March, M. Xiu-Sheng, C. D. Metcalfe, M. Stobiecki and L. Marczak, *Int. J. Mass Spectrom.*, 2004, **232**, 171-183.
- 23 J. Kang, L. A. Hick and W. E. Price, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 857-868.
- 24 M. Łuczkiwicz, D. Głód, T. Bączek and A. Buciński, *Chromatographia*, 2004, **60**, 179-185.
- 25 F. Tosum, C. Akyuz Kizilay and A. U. Tosun, *Chem. Nat. Compd.*, 2009, **45**, 83-84.
- 26 S. E. Kulling, D. M. Honig, T. J. Simat and M. Metzler, *J. Agric. Food Chem.*, 2000, **48**, 4963-4972.
- 27 C. Noccioli, L. Luciardi, S. Barsellini, C. Favro, A. Bertoli, A. Bader, M. C. Loi and L. Pistelli, *Chem. Nat. Compd.*, 2012, **48**, 672-673.
- 28 D. Rigano, V. Cardile, C. Formisano, M. T. Maldini, S. Piacente, J. Bevilacqua, A. Russo and F. Senatore, *Chem-Biol. Interact.*, 2009, **180**, 211-219.
- 29 J. B. Harborne, *Phytochemistry*, 1969, **8**, 1449-1456.
- 30 O. Boumaza, R. Mekkiou, R. Seghiri, D. Sarri, S. Benayache, V. P. Garcia, J. Bermejo and F. Benayache, *Chem. Nat. Compd.*, 2006, **42**, 730-731.
- 31 M. Chen, L. Liu and X. Chen, *Can. J. Chem.*, 2013, **11**, 1147-1154.
- 32 I. I. Ozimina and V. A. Bandyukova, *Khim. Prir. Soedin.*, 1985, 507-510.
- 33 K. Lech, K. Witkoś and M. Jarosz, *Anal Bioanal. Chem.*, 2014, **406**, 3703-3708.
- 34 Y. Ungar, O. F. Osundahunsi and E. Shimoni, *J. Agric. Food Chem.*, 2003, **51**, 4394-4399.
- 35 B. Eisen, Y. Ungar and E. Shimoni, *J. Agric. Food Chem.*, 2003, **51**, 2212-2215.