Microchim Acta (2008) 162: 371–380
DOI 10.1007/s00604-007-0866-0
Printed in The Netherlands

Microchimica Acta

Original Paper

Towards the identification of characteristic minor components from textiles dyed with weld (*Reseda luteola* L.) and those dyed with Mexican cochineal (*Dactylopius coccus* Costa)

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Received 28 February 2007; Accepted 11 September 2007; Published online 10 December 2007

Abstract. The identity of a minor flavonoid component observed in extracts of textile samples dyed with weld (*Reseda luteola* L.) is confirmed as chrysoeriol (3′-*O*-methylluteolin) by HPLC-PDA analysis. HPLC-PDA, HPLC-MS and NMR techniques have been used to show that the unknown dcII component found in extracts of textiles dyed with Mexican cochineal (*Dactylopius coccus* Costa) is most probably the 7-*C*-glycoside of flavokermesic acid. In addition, the unknown dcIV and dcVII components have been shown to be isomeric with carminic acid, most probably differing only in the stereochemistry of the sugar moiety.

Keywords: Natural dyes; flavonoids; anthraquinones; historical textiles

Dyes derived from biological sources were the main source of textile colouring material until the mid to late 19th century. The accurate identification of the original dye source used to colour textiles can be important, both from the point of view of the historian and the conservator. When combined with documentary evidence on dyeing practices, patents or trade routes, information regarding how, when or where a textile was dyed can sometimes be elucidated [1]. For the conservator, the dye and dyeing methods used on the textile can influence the strength and ageing properties of the yarns and therefore provide information regarding the present condition of the object [2].

Evidence for the use of particular biological sources in the dyeing of historical textile yarns can often be obtained by examining the acid hydrolysed extract of a small sample from the object [3, 4]. Although the major colouring component is often the same in many natural dye sources, minor constituents can be characteristic of the particular species used. These specific 'marker components' can then be compared with those from freshly dyed reference material, thus indicating the use of particular biological sources. Such analysis is most effectively achieved using high performance liquid chromatography with photo diode array detection (HPLC-PDA), enabling the retention time (R_t) and UV-Vis spectral information of each component in the extracts to be compared against an 'in-house' library of standards. In addition, mass spectrometry (MS) is often used for further investigative work, particularly using electrospray ionisation (ESI) with an ion trap analyser which allows collisionally induced dissociation (CID) MS^n experiments.

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A major reason for chemically identifying all of the dye components is the potential difficulties caused by photo-degradation reactions, which can alter the nature and relative amounts of the dye components found in the acid hydrolysed extracts of aged yarns [5, 6]. These changes can make the unequivocal determination of the biological dye source from an aged textile somewhat problematic. The chemical characterisation of these dye 'marker components' is therefore desirable, both to rationalise their use as species markers and to help explain the analytical results obtained from historical samples [7].

Experimental

Materials

Weld and Mexican cochineal were supplied by Verfmolen De Kat, Netherlands (www.verfmolendekat.net). The authentic flavonoids (chrysoeriol **3** and diosmetin **4**) were obtained from Apin Chemicals Ltd, U.K. (www.apinchemicals.com) and carminic acid **5** was purchased from Sigma-Aldrich, U.K. (www.sigmaaldrich.com).

Instrumentation

All mordantings and dyeings were prepared as part of the Monitoring of Damage to Historical Tapestries project [8]. The dyeing was performed in a large-scale dyeing facility and followed medieval methods and recipes as closely as possible.

For high performance liquid chromatography, (HPLC), a Waters 600 gradient pump and a Waters 2996 PDA detector were controlled by Waters Empower software, which also collected and manipulated the data. Solvents were sparged using a vacuum in-line degasser and chromatographed peaks were monitored at 254, 275 and 430 nm although the PDA detector measured all spectral information between 250 and 750 nm. The bandwidth (resolution) was 2.4 nm, and response time was 1 sec.

Mass analysis was performed using a Thermoquest Finningan LCQ^{TM} mass spectrometer, operating in negative ion mode and controlled by LCQ Navigator^{TM} software, tuned to the deprotonated molecular ion peak of carminic acid **5**. Sample solutions in methanol or deuteriated methanol (MeOD) were directly injected into the source *via* a syringe pump delivering 8 μL min^{-1}. Peaks were selected for CID with a band-width of 1.0 atomic mass units (amu) and the relative collision energy required for the MS^n experiments was usually around 20–25%. Data were collected and processed by the LCQ Navigator^{TM} software.

A Finnigan MAT Spectra^{TM} system was used as the HPLC unit for LC-MS, composed of an AS3000 autosampler, a P4000 pump and V2000 single wavelength, tuneable UV-visible detector. This was connected in series to the Thermoquest Finningan LCQ^{TM} mass spectrometer described above, operating in negative ion mode. The sample solution (20 μL) was injected *via* the autosampler, using the same column and gradient system as the HPLC PDA analysis, although a post-column splitter reduced the flow into the mass spectrometer to *ca.* 0.30 mL min^{-1}.

NMR spectra were recorded at 600 MHz using a Bruker AVA600 instrument.

Dye extraction

The sample preparation for the extraction of dye components is based on the commonly used hydrolysis procedure with hydrochloric acid [9]. The dyed yarn (typically 0.1–0.5 mg) was placed in a 2 mL glass test tube, to which was added a 2:1:1 (v/v/v) mixture of 37% hydrochloric acid:methanol:water (400 μL). The tube was then placed in a water bath at 100 °C and heated for precisely 10 min. After rapid cooling under cold water, the extract was filtered using a 5 μm Analytichem polypropylene frit under positive pressure. The test tube was rinsed with methanol (200 μL) and the combined filtrates dried by rotary vacuum evaporation over a water bath at 40 °C. The dry residue was then reconstituted with methanol (25 μL) and water (25 μL).

Weld

Mordanting and dyeing of wool with weld

Alum (22.5 g) was dissolved in boiling water (75 L) and scoured wool hanks (1.5 kg) were immersed and boiled for 2 h before being wrung out. Chopped weld (dried whole plant; 750 g) and potassium carbonate (75 g) were added to boiling water (75 L) and the wet, mordanted wool was immersed and boiled for 1 h before being removed, wrung and rinsed with both cold and hot water.

HPLC-PDA analysis of weld extracts

The chromatographic method for the analysis of weld used a Phenomenex Sphereclone ODS2, 5 μm particle size, 150 × 4.6 mm (length × internal diameter), reverse phase column, with a guard column containing the same stationary phase. This was enclosed in a heat-controlled chamber and maintained at 25 ± 1 °C. Sample extracts were injected *via* a Rheodyne injector with a 20 μL sample loop. The total run time was 35 min at a flow rate of 1.2 mL min^{-1}. A tertiary solvent system was used; A = 20% (v/v) MeOH (aq.), B = MeOH, C = 5% (v/v) ortho-phosphoric acid (aq.). The elution programme was isocratic for 3 min (67A:23B:10C) then a linear gradient from 3 to 29 min (0A:90B:10C) before initial conditions recovery over 1 min and equilibration over 5 min.

The luteolin methyl ether spiking experiments with chrysoeriol **3** and diosmetin **4** were based on the dye extraction protocol outlined above. However, the dry residues from the extracted weld-dyed wool yarns were reconstituted with 1:1 (v/v) MeOH:H_2O solutions of chrysoeriol **3** (50 μL, *ca.* 10 μg mL^{-1}) or diosmetin **4** (50 μL, *ca.* 10 μg mL^{-1}) respectively.

Cochineal

Mordanting and dyeing of wool with cochineal

Alum (106 g) was dissolved in boiling water (50 L) together with D-tartaric acid (69 g), sodium chloride (30.4 g) and sandalwood (30.4 g). Scoured wool hanks (1.3 kg) were immersed and boiled for 2 h, before the heat was turned off and the bath allowed to cool for 24 h. The hanks were removed, but not wrung. Gum arabic (53 g), alum (1.3 g), turmeric (8.7 g) and sodium chloride (5.4 g) were all dissolved in boiling water (50 L), before ground cochineal (36.2 g) was added and approximately half of the pre-mordanted wool (670 g) was immersed in the bath and left to boil for 1 h. The wool was then rinsed in cold water.

HPLC-PDA analysis of cochineal extracts

The general instrumentation and chromatographic conditions for the analysis of cochineal extracts were the same as those described above for weld, with the exception that a different solvent system was employed to allow direct comparison with the LC-MS data. Thus, the tertiary solvent system was; A = H_2O, B = MeOH, C = 5% (v/v) formic acid (aq.). The elution programme was isocratic for 2 min (67A:23B:10C) then a linear gradient from 2 min to 30 min (0A:90B:10C) before initial conditions recovery over 2 min and equilibration over 8 min.

Semi-preparative extraction of colouring components from dried cochineal

Ground cochineal (0.21 g) was added to a solvent mixture of 37% hydrochloric acid:methanol:water [2:1:1 (v/v/v), 20 mL]. The mixture was placed in a water bath at 100 °C and heated for precisely 10 min. After rapid cooling under cold water, the extract was filtered using a 5 μm Analytichem polypropylene frit under positive pressure. The solution was dried by rotary vacuum evaporation over a water bath at 40 °C and the dry residue reconstituted with methanol (3 mL) and water (3 mL). The reconstituted solution was then chromatographed on a semi-preparative scale using the following conditions. A Phenomenex Luna C18(2) 10 μm particle size, 60 × 21.20 mm, reverse phase column was used at ambient temperature. Sample extracts were injected *via* a Rheodyne injector with a 200 μL sample loop. The total run time was 35 min at a flow rate of 8 mL min^{-1}. A tertiary solvent system was used; A = 20% (v/v) MeOH (aq.), B = MeOH, C = 5% (v/v) formic acid (aq.). The elution programme was isocratic for 3 min (67A: 23B: 10C) then a linear gradient from 3 min to 29 min (0A: 90B: 10C) before initial conditions recovery over 1 min and equilibration over 5 min. Three separate fractions, corresponding approximately to dcIV, dcVII and flavokermesic acid **7** were collected. The respective fractions from nine injections were then combined and evaporated to dryness (Fig. 6).

NMR analysis of the cochineal components

1-D proton spectra, acquired in D_2O solution, were obtained from a carminic acid **5** reference and from fraction 2 respectively (Table 4). Assignments were aided by information from correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and total correlation spectroscopy (TOCSY) experiments.

Results and discussion

Weld

The main constituents of the yellow dye source weld (*Reseda luteola* L.) are well documented [10–12]; luteolin **1** (Fig. 1) is the major flavonoid component observed in the plant extracts, present as the parent compound and as its 7-glucoside, 3′-glucoside and 3′,7-diglucoside. The presence of these luteolin-*O*-glucosides on dyed textile yarns has been confirmed and their usefulness as characteristic marker species demonstrated when a suitable 'soft' extraction procedure is chosen [13]. However, when acid hydrolysis conditions are used to extract the colouring components from dyed yarn, any flavonoid-*O*-glycosides present are converted into their parent aglycones. Luteolin **1** is not a specific marker for weld, therefore the identification of other minor components is required for species identification.

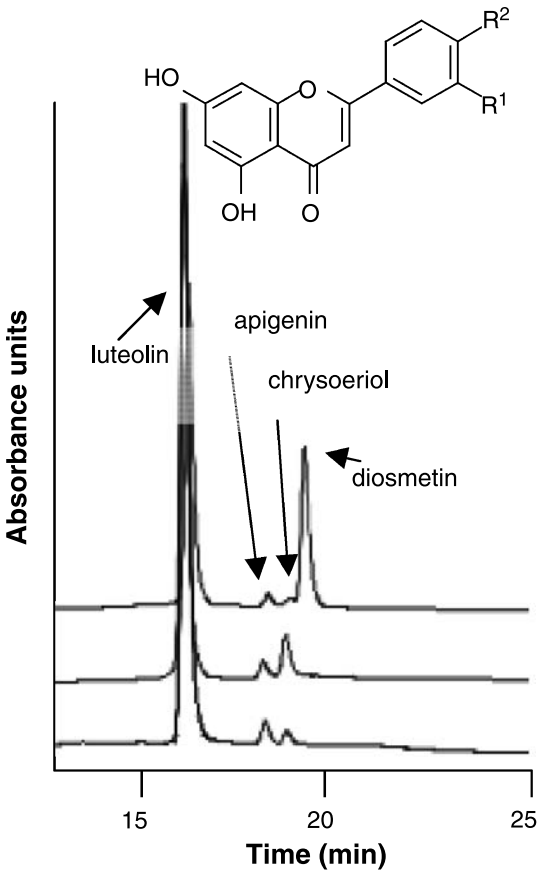

Fig. 1. The main flavonoid constituents in the acid hydrolysed extract from a weld (*Reseda luteola* L.) dyed yarn (**1**–**3**), together with an additional luteolin methyl-ether (**4**). Luteolin (**1**) $R^1 = R^2 = OH$; Apigenin (**2**) $R^1 = H$, $R^2 = OH$; Chrysoeriol (**3**) $R^1 = OMe$, $R^2 = OH$; Diosmetin (**4**) $R^1 = OH$, $R^2 = OMe$. HPLC chromatograms all monitored at 254 nm. Top trace; Weld extract spiked with diosmetin (**4**). Middle trace; Weld extract spiked with chrysoeriol (**3**). Bottom trace; Weld extract

In the acid hydrolysed extracts of weld-dyed textiles, luteolin **1** is observed together with relatively minor amounts of apigenin **2** and a component identified as a luteolin methyl ether by HPLC-PDA and mass spectrometric analysis (*cf*. Fig. 1, bottom trace) [14]. Previous research demonstrated that the methoxy group was present on the B-ring [6]; thus the minor component must be either chrysoeriol (3′-methoxy-5,7,4′-trihydroxyflavone) **3** or diosmetin (4′-meth-

oxy-5,7,3′-trihydroxyflavone) **4**. Both of these isomers are known natural products and have been reported as minor constituents in extracts from feverfew (*Tanacetum parthenium* L.) [15]. The identification of this minor component is important as it is a key marker for weld, distinguishing the acid hydrolysed extracts from those obtained from aged sawwort (*Serratula tinctoria* L.) dyed yarn, which otherwise may show a very similar dye profile [7, 16]. Its characterisation may also help in the elucidation of minor components found in the acid hydrolysed extracts from textiles dyed with other species, such as dyers greenweed (*Genista tinctoria* L.), where the minor ether components have yet to be identified [6].

Solutions of authentic samples of both **3** and **4** were chromatographed under identical conditions to that of the weld reference. A comparison of retention time and UV-Vis spectra suggested that the luteolin methyl ether present in the acid hydrolysed extracts of weld dyed yarn was chrysoeriol **3**. This was confirmed by spiking experiments, where a solution of either chrysoeriol **3** or diosmetin **4** was used to reconstitute the dry residue from the acid hydrolysis of a weld dyed yarn and compared with an un-spiked weld extract (Fig. 1).

The weld extract (Fig. 1, bottom trace) contains three peaks, luteolin **1**, apigenin **2** and a luteolin methyl ether **3** or **4**. In the weld extract reconstituted with a spiked chrysoeriol **3** solution (Fig. 1, middle trace), no new peaks are visible, however, the area of the peak corresponding to the luteolin methyl ether increased. Furthermore, the spectral purity of this peak remained constant. In contrast, an additional peak, with a retention time corresponding to the diosmetin **4** isomer, is present in the diosmetin **4** spiked solution (Fig. 1, top trace). Thus, the luteolin methyl ether isomer in the acid hydrolysed extracts from weld dyed yarn is chrysoeriol **3**.

The identification of chrysoeriol **3** completes the characterisation of the components observed in the acid hydrolysed extracts from weld-dyed yarn. Its presence in the extracts, together with apigenin and luteolin, allows yarn dyed with weld to be confidently distinguished from yarn dyed with alternative biological species [7, 14–16].

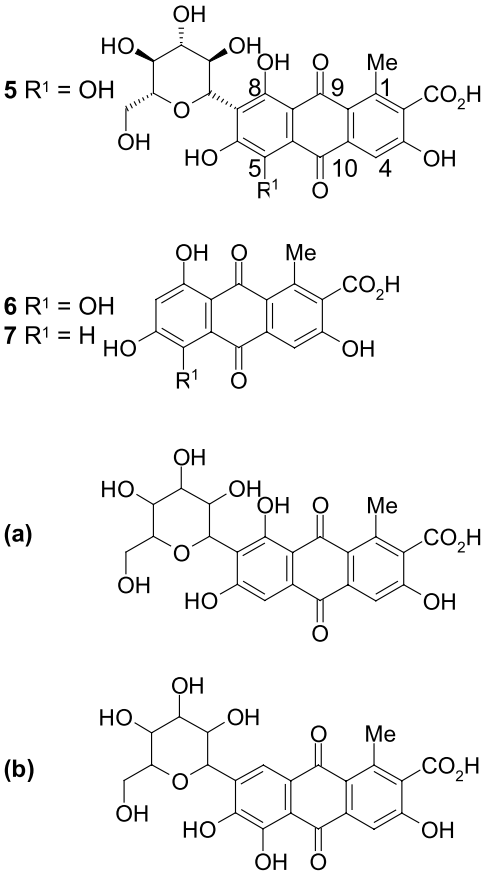

Fig. 2. The structures of the characteristic constituents **5**–**7** observed in the acid hydrolysed extracts of Mexican cochineal (*Dactylopius coccus* Costa) dyed yarns. Carminic acid (**5**) $R^1 = OH$; Kermesic acid (**6**) $R^1 = OH$; Flavokermesic acid (**7**) $R^1 = H$. In addition, the proposed structure (a) for the unknown component dcII, based on flavokermesic acid (**7**), together with (b) an alternative dcII structure. The stereochemistry of the sugar moiety is currently unknown

Mexican cochineal

Mexican cochineal (*Dactylopius coccus* Costa) is one of the coccid dye sources, a term which encompasses all the scale insect dyes obtained from the females of different species [17]. These insects contain a variety of red anthraquinone colouring components (Fig. 2) which can include *C*-glycosides such as carminic acid **5**. These are not hydrolysed during the acid extraction procedure, in contrast to the *O*-glycosides encountered during analysis of the yellow flavonoid sources.

Although the main colouring components from different species have been characterised [9, 18–20], the structures of some important minor components remain unknown. In some cases, the relative amounts of minor components observed in the acid hydrolysed extracts can allow different coccid dyestuffs to be distinguished from one another. For example, the relative amount of one such unknown component (labelled dcII), can allow yarn dyed with Mexican

(*Dactylopius coccus* Costa), Polish (*Porphyrophora polonica* Linnaeus) or Armenian (*Porphyrophora hameli* Brandt) cochineal to be distinguished [19]. This can be of considerable importance in establishing the provenance of a sample, since the introduction of Mexican cochineal to Europe can be attributed to Spanish imports from the 16th century onwards [17].

The HPLC-PDA chromatogram (monitored at 430 nm) obtained from the acid hydrolysed extract of a Mexican cochineal dyed wool sample is shown in Fig. 3. The components were identified as structurally related anthraquinones by matching their retention times and UV-visible spectra to either an in-house library of standard compounds or with previously published data [18].

The main colouring component was identified as carminic acid **5**, with a minor amount of flavokermesic acid **7** also present [21]. A trace of kermesic acid **6**, confirmed by HPLC-MS, was also observed. Three significant unknown components were observed in the chromatogram which, in order of elution, have been labelled dc (*Dactylopius coccus*) II, dcIV and dcVII, by Wouters and Verhecken [18]. The following experiments were conducted in an attempt to characterise these components.

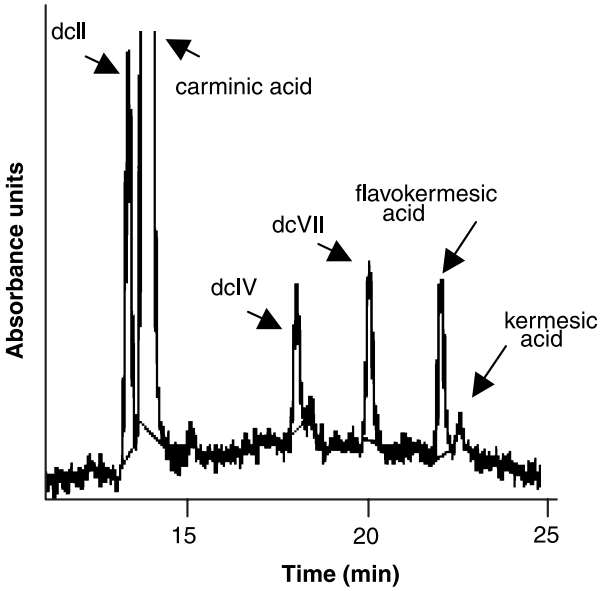

Fig. 3. HPLC-PDA chromatogram (monitored at 430 nm) of a Mexican cochineal dyed wool extract, rescaled to show the minor components

The UV-Vis spectra of the eluting components are shown in Fig. 4. By inspection, the dcII component ($\lambda_{max} = 285.7$ and 435.0) appears to be related to flavokermesic acid **7** ($\lambda_{max} = 285.7$ and 432.6), while dcIV

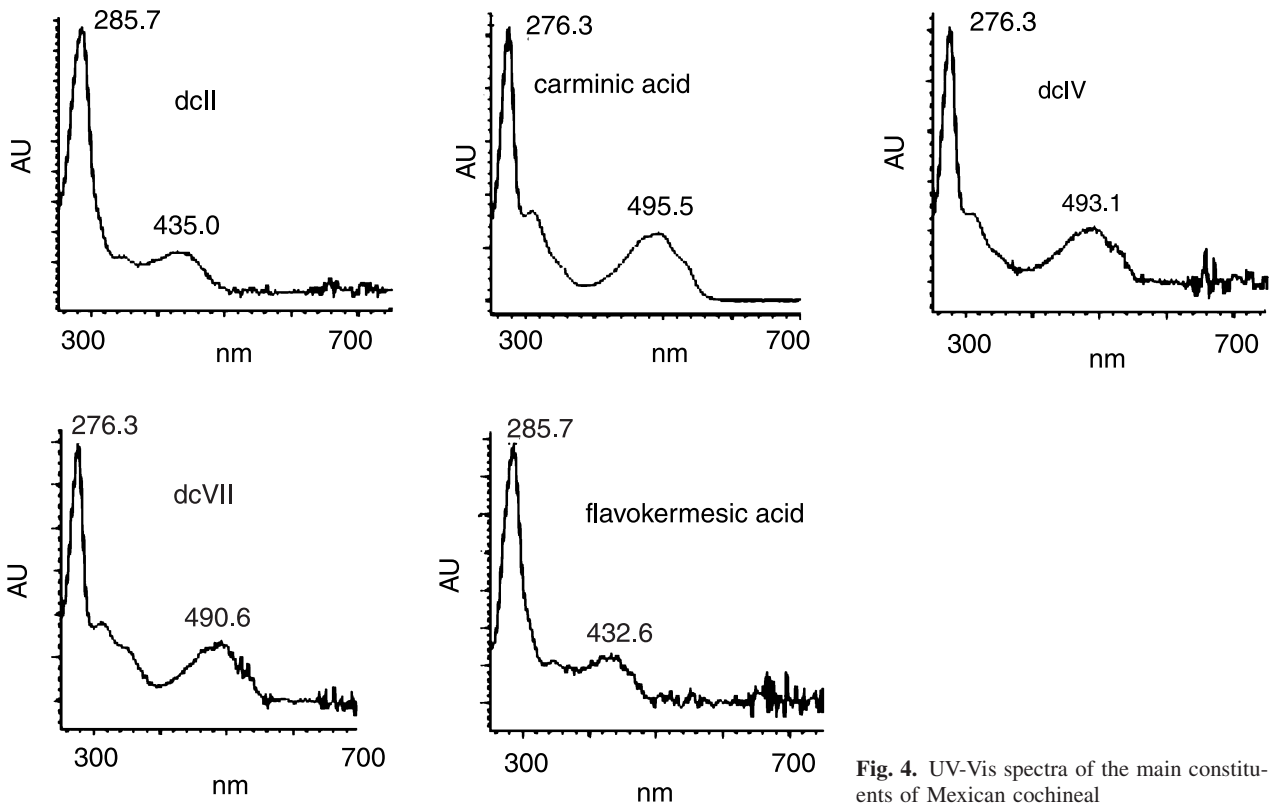

Fig. 4. UV-Vis spectra of the main constituents of Mexican cochineal

($\lambda_{max} = 276.3$ and 493.1) and dcVII ($\lambda_{max} = 276.3$ and 490.6) have UV-Vis spectra similar to that of carminic acid **5** ($\lambda_{max} = 276.3$ and 495.5). Further characterisation of these components requires a knowledge of the mass spectrometry of anthraquinones, but only a few publications are available [22, 23].

A directly injected methanolic solution of raw cochineal gave a good mass spectrum under negative ion electrospray conditions. Spectra were also recorded using deuteriated methanol (MeOD) as a solvent. Under these conditions, molecular ion clusters were observed corresponding to sequential replacement by deuterium of the hydrogen atom in each of the OH groups (with the exception of the ionised OH of the carboxylic acid moiety). In each case, the ion corresponding to the most fully deuteriated species was chosen for CID experiments. Thus fragmentation pathways under CID conditions could be investigated.

The base peak in the raw cochineal extract was, as expected, the deprotonated molecular ion ($m/z = 491$) of carminic acid **5**. The results of the MS^2 and MS^3 (MeOH/D) experiments on carminic acid **5** are summarised in Table 1. Significantly, both the $m/z = 357$ and $m/z = 327$ ions were observed from MS^2 analysis of $m/z = 491$ and from the MS^3 analysis on the resulting base peak at $m/z = 447$.

These data show that the initial fragmentation of carminic acid **5** involves decarboxylation of the carboxylic acid moiety (to give $m/z = 447$), followed by the loss of a neutral fragment of 90 Da (containing 2 exchangeable protons) to give $m/z = 357$ or loss of a 120 Da fragment (containing 3 exchangeable protons) to give $m/z = 327$. The two latter fragments originate from breakdown of the sugar residue. Identical losses have also been reported in recent studies of flavonoid C-glycoside fragmentation under negative ion conditions [24–26], and the observed product ions, formed by cross-ring cleavage, are characteristic for a hexose. Hence, with the exception of the initial decarboxylation process, which is not diagnostic in this series of anthraquinones, the major breakdown pathways involve the hexose moiety.

HPLC-MS investigations of the acid hydrolysed extracts from dyed textile yarns confirmed the presence of carminic acid **5** ($m/z = 491$, $[M\text{-}H]^-$). Further analysis revealed a component eluting at the dcII position ($m/z = 475$, $[M\text{-}H]^-$), with a molecular ion 16 Da less than carminic acid **5** (Fig. 5). The MS^2 and MS^3 (MeOH/D) experiments on the $m/z = 475$ component, isolated from the directly injected solution of raw cochineal, showed that it had similar breakdown peaks to carminic acid (Table 2). For example, in both cases the initial MS^2 base peak is due to the decarboxylation of the carboxylic acid moiety, followed in the MS^3 experiment by loss of a 90 Da or 120 Da

Table 1. Collision induced dissociation (CID) of carminic acid **5** ($m/z = 491$) and MS^3 fragmentation of [carminic acid-H-44]$^-$ ($m/z = 447$) under negative ion conditions

m/z: MS^2 491 (relative abundance)	Neutral fragment lost (possible species)	Number of exchangeable protons (from deuteriated studies)	
		Observed fragment	Neutral fragment
447 (100%)	44 (CO_2)	8	0
357 (5%)	134	6	2
327 (3%)	164	5	3
m/z: MS^3 491, 447			
429 (28%)	18 (H_2O)	6	2
357 (100%)	90 ($C_3H_6O_3$)	6	2
327 (52%)	120 ($C_4H_8O_4$)	5	3

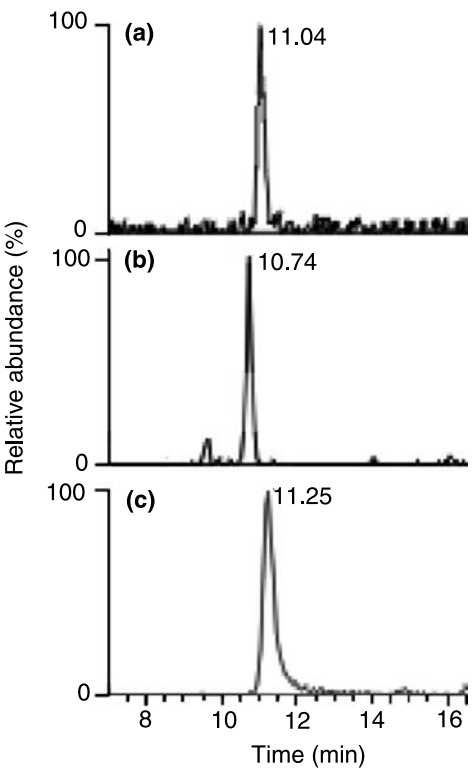

Fig. 5. HPLC-MS of acid hydrolysed extracts from Mexican cochineal dyed yarn. (a) The UV-Vis signal (monitored at 430 nm) (b) Negative ion MS^2 chromatogram showing the presence of ions at $m/z = 475$ corresponding to the dcII position and (c) Negative ion MS^2 chromatogram showing the presence of ions at $m/z = 491$ at the carminic acid **5** position

Table 2. Collision induced dissociation (CID) of the unknown dcII component ($m/z = 475$) and MS^3 fragmentation of $[dcII\text{-}H\text{-}44]^-$ ($m/z = 431$) under negative ion conditions

m/z: MS^2 475 (relative abundance)	Neutral fragment lost (possible species)	Number of exchangeable protons (from deuteriated studies)	
		Observed fragment	Neutral fragment
431 (100%)	44 (CO_2)	7	0
413 (5%)	62	5	2 (possibly [(base peak) – 18]$^-$)
385 (4%)	90	5	2
355 (2%)	120	–	–
341 (26%)	134	5	2
311 (7%)	164	4	3
m/z: MS^3 475, 431			
413 (7%)	18 (H_2O)	6	1
371 (3%)	60	–	–
353 (5%)	78	–	–
341 (100%)	90 ($C_3H_6O_3$)	5	2
311 (27%)	120 ($C_4H_8O_4$)	4	3
269 (23%)	162 ($C_6H_{10}O_5$)	4	3
268 (3%)	163	–	–

fragment, indicating the presence of a hexose sugar moiety. The deuteriated experiments confirm that the deprotonated molecular ion of dcII ($m/z = 475$) contains one less exchangeable proton than carminic acid **5** ($m/z = 491$), while the corresponding fragments observed in the MS^2 experiments also contain one less exchangeable proton; dcII therefore has only 3 phenolic groups on the anthraquinone core (compared with 4 on the carminic acid **5** core).

The position of this ''missing'' substituent on the anthraquinone core of dcII can be ascertained by analysis of UV-Vis spectra. Several systematic investigations of the electronic spectra of substituted anthraquinones have been undertaken [27–29], and it has been shown that the longest wavelength $\pi \rightarrow \pi^*$ absorption band must be due to an intramolecular electron transfer transition from the substituent to the anthraquinone nucleus [30]. Furthermore, the spectra of 1-substituted anthraquinones are shifted to longer wavelengths compared with their 2-substituted analogues by *ca.* 50–60 nm due to the presence of intramolecular hydrogen-bonding between the substituent and the adjacent carbonyl group of the quinone. By comparing the λ_{max} values of the intramolecular electron transfer band in carminic acid **5** with the equivalent absorption band in dcII (Table 3) it is apparent that a phenolic group adjacent to the quinone must be absent from the dcII component.

Table 3. λ_{max}/nm in substituted anthraquinones

	λ_{max} (intramolecular electron transfer band)/nm	Difference/nm
Carminic acid	496	63
dcII	433	
1-substituted anthraquinone (OH) [30]	406	
2-substituted anthraquinone (OH) [30]	354	52

Although two possible structures for dcII remain (the –OH group from position 5 or 8 could be absent in dcII compared to carminic acid **5**), the structure is most likely to be that shown in Fig. 2(a), by comparison with flavokermesic acid **7**, a known constituent of cochineal, rather than the unknown isomeric compound shown in Fig. 2(b). This hypothesis is corroborated by examining the biosynthetic route to carminic acid **5**, which proceeds *via* a polyketide to flavokermesic acid **7** and then kermesic acid **6**, before a C-glucoside is added to form the final product [31]. It is highly feasible that the C-glucoside may also be added to the flavokermesic acid precursor, producing the proposed dcII structure. The alternative dcII structure would require a completely different biosynthetic route.

Neither mass spectrometry or UV-Vis spectroscopy can provide detailed information regarding the stereochemistry of the glycosidic linkage or of the sugar itself. However, the precedent of other major sugars in this series would indicate that it is a glucose moiety and we suggest that dcII is a 7-*C*-glucoside of flavokermesic acid **7**.

To investigate the remaining unknown constituents of *Dactylopius coccus* Costa. (dcIV and dcVII), a hydrolysed solution of the raw cochineal used for the dyeing of the model tapestries was utilised. In an attempt to separate the minor components from carminic acid **5**, the solution was chromatographed on a semi-preparative scale. Three separate fractions, corresponding approximately to dcIV, dcVII and flavokermesic acid **7**, were collected. The respective fractions from nine injections were then combined and evaporated to dryness. Re-injection of the reconstituted fractions onto the analytical column confirmed the enrichment of the minor components and the removal of carminic acid **5** (and dcII) (Fig. 6).

These fractions were analysed using HPLC-MS. The unknown dcIV and dcVII peaks (present in both

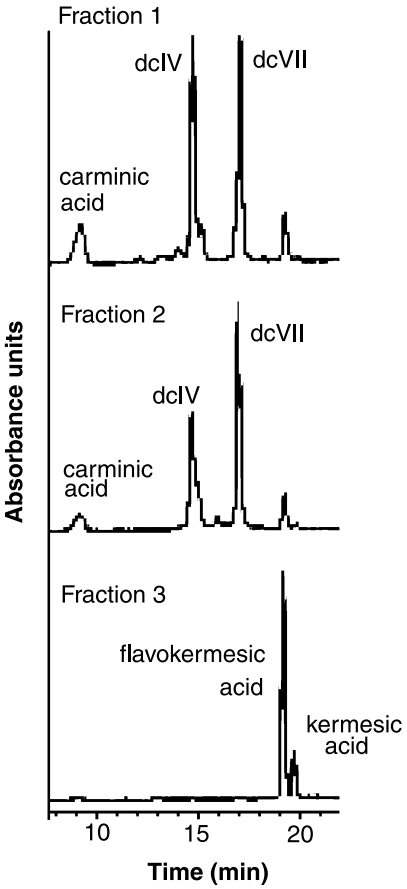

Fig. 6. HPLC-PDA chromatograms (monitored at 430 nm) of the three fractions (Fractions 1 – 3) from the semipreparative column. The dry residue of each was diluted with methanol before analysis

fraction 1 and fraction 2) were found to be isomeric with carminic acid **5**, with all components producing a deprotonated molecular ion of $m/z = 491$. Furthermore, no significant differences were observed in the MS^n fragmentation patterns of the two unknown components (dcIV and dcVII) when compared with the fragmentation of carminic acid **5** (Fig. 7). The HPLC MS^3 analysis of fraction 3 confirmed the presence of both flavokermesic acid **7** and kermesic acid **6**. However, further (MS^3) analysis provided little additional structural information.

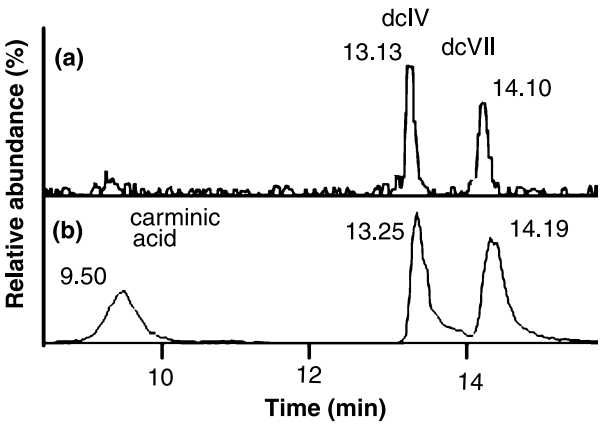

Fig. 7. HPLC-MS analysis of fraction 1. (a) The UV-Vis signal (monitored at 430 nm), (b) Negative ion MS^3 chromatogram showing that carminic acid, dcIV and dcVII have the same molecular ion at $m/z = 491$ and breakdown peak at $m/z = 447$

The UV-Vis and mass spectral data therefore indicate that dcIV and dcVII must differ from carminic acid **5** only in the nature of the sugar moiety. This difference is likely to be stereochemical, since mass spectrometry does not usually provide information regarding the α/β geometry of the glycosidic linkage, nor can it usually distinguish diastereomeric sugar units.

In an attempt to gain stereochemical information regarding the dcIV and dcVII components, NMR techniques were employed. The ^1H NMR spectrum of fraction 2 revealed a highly complex spectrum, but three signals were clearly resolved in the range (δ_H 5–6) (Table 4). These are most likely to be due to the anomeric protons from dcIV, dcVII and carminic acid

Table 4. ^1H NMR chemical shifts (δ/ppm) and coupling constants (J/Hz) for the β-D-glucopyranose sugar component of carminic acid **5** and the unknown 'sugar 1' and 'sugar 2' components, performed in D_2O on a Bruker AVA600 spectrometer

H assignment	^1H Chemical shift (δ/ppm)	Coupling constant (J/Hz)
Carminic acid reference		
1 (anomeric)	5.02	d, 9.9
2	4.30	m
3	3.51	m
4	3.49	m
5	3.45	m
6(1)	3.90	d,d, 2.4 and 12.2
6(2)	3.76	d,d, 5.6 and 12.2
Sugar 1 in fraction 2		
1 (anomeric)	5.78	d, 3.3
2	4.58	m
3	4.43	m
4	4.46	d,d, 3.5 and 8.4
5	4.09	m
6(1)	3.85	d,d, 3.1 and 11.4
6(2)	3.76	d,d, 5.4 and 11.4
Sugar 2 in fraction 2		
1 (anomeric)	5.42	d, 2.9
2	4.28	m
3	4.32	m
4	4.06	d,d, 2.5 and 7.7
5	4.10	m
6(1)	3.80	d,d, 3.6 and 11.7
6(2)	3.68	d,d, 5.7 and 11.7

5 (indeed, the anomeric signal from carminic acid **5** was easily identified by comparison with the NMR spectrum of an authentic sample).

It is clear from the data in Table 4, that the two unknown sugars have very different anomeric coupling constants ($^3J = 3.3$ and 2.9 Hz) from that seen in carminic acid **5** ($^3J = 9.9$ Hz). This suggests that they are either α-sugars (such as α-glucose), or that the hydroxyl group adjacent to the anomeric position is of mannose type, since the dihedral angles between the C-1 and C-2 protons are close to 180° in the β-anomers of glucose and galactose, but around 60° for the β-anomer of mannose and the α-anomers of glucose, galactose and mannose. According to the Karplus equation, the coupling constants (J) for the former should be around 10 Hz while those of the latter should be much less, at around 3 Hz. Conclusive assignment to dcIV and VII will require a complete analysis of the sugar spin systems obtained from pure samples.

Conclusion

The luteolin methyl ether marker component in the acid hydrolysed extracts from weld dyed yarn is confirmed as chrysoeriol **3** (3′-methoxy-5,7,4′-trihydroxyflavone).

The series of analytical studies on Mexican cochineal have provided a more complete structural characterisation of the minor components found in the acid hydrolysed extracts of dyed yarn. It is likely that dcII is a glycoside – presumably a glucoside – of flavokermesic acid **7**; the dcIV and dcVII components are isomers of carminic acid **5**, differing only in the stereochemistry of the sugar moiety.

Acknowledgements. This work was funded by the EC, contract number EVK4-CT-2001-00048 (Monitoring of Damage to Historic Tapestries project). We are most grateful to Mr. A.T. Taylor (MS) and Mr. J. Bella (NMR) for expert technical assistance and to Dr. A.-M. Hacke (The University of Manchester) for the preparation of dyed reference yarns.

References

1. Quye A, Cheape H, Burnett J, Ferreira E S B, Hulme A N, McNab H (2003) An historical and analytical study of red, pink, green and yellow colours in quality 18th- and early 19th-century scottish tartans. Dyes Hist Archaeol 19: 1
2. Hallet K, Howell D (2005) Size exclusion chromatography of silk – inferring the tensile strength and assessing the condition of historic tapestries. Preprints to the ICOM Committee for Conservation 14th Triennial meeting in The Hague, Vol. 2, p 911
3. Petroviciu I, Wouters J (2002) Analysis of natural dyes from Romanian 19th- and 20th-century ethnographical textiles by DAD-HPLC. Dyes Hist Archaeol 18: 57
4. Trojanowicz M, Orska-Gawrys J, Surowiec I, Szostek B, Urbaniak-Walczak K, Kehl J, Wrobel M (2004) Chromatographic investigation of dyes extracted from coptic textiles from the national museum in Warsaw. Stud Conserv 49: 115
5. Ferreira E S B, Quye A, McNab H, Hulme A N (2002) Photooxidation products of quercetin and morin as markers for the characterisation of natural flavonoid yellow dyes in ancient textiles. Dyes Hist Archaeol 18: 63
6. Ferreira E S B (2002) New approaches towards the identification of yellow dyes in ancient textiles. PhD Thesis, The University of Edinburgh
7. Peggie D A (2006) The development and application of analytical methods for the identification of dyes on historical textiles. PhD Thesis, The University of Edinburgh
8. Hacke A-M (2006) Investigation into the nature and ageing of tapestry materials. PhD Thesis, The University of Manchester
9. Wouters J (1985) High performance liquid chromatography of anthraquinones: analysis of plant and insect extracts and dyed textiles, Stud Conserv 30: 119
10. Kaiser R (1993) Quantitative analyses of flavonoids in yellow dye plant species weld (*Reseda luteola* L.) and sawwort (*Serratula tinctoria* L.). Angew Bot 67: 128
11. Yuldashev M P, Batirov E Kh, Malikov V M, Yulasheva N P (1996) Flavonoids of *Psoralea drupaceae* and *Reseda luteola*. Chem Nat Compd 32: 923
12. Cristea D, Bareau I, Vilarem G (2003) Identification and quantitative HPLC analysis of the main flavonoids present in weld (*Reseda luteola* L.). Dyes Pigments 57: 267
13. Zhang X, Laursen R A (2005) Development of mild extraction methods for the analysis of natural dyes in textiles of historical interest using LC-Diode Array Detector-MS. Anal Chem 77: 2022
14. Ferreira E S B, Quye A, Hulme A N, McNab H (2003) LC-Ion Trap MS and PDA-HPLC – Complementary techniques in the analysis of flavonoid dyes in historical textiles: the case study of an 18th century herald's tabard. Dyes Hist Archaeol 19: 13
15. Williams C A, Harborne J B, Geiger H, Hoult J R S (1999) The flavonoids of *Tanacetum parthenium* and *T. vulgare* and their anti-inflammatory properties. Phytochemistry 51: 417
16. Hulme A N, McNab H, Peggie D A, Quye A (accepted) The chemical characterisation of aged and unaged fibre samples dyed with sawwort (*Serratula Tinctoria*) using PDA HPLC and HPLC ESI MS. Dyes His Archaeol 22 (in press)
17. Hofenk de Graaff J H (2004) The colourful past, 1st edn, Abegg-Stiftung and Archetype Publications Ltd, p 147
18. Wouters J, Verhecken A (1989) The scale insect dyes (Homoptera: Coccoidea). Species recognition by HPLC and Diode-Array analysis of the dyestuffs. Annals Soc Ent Fr (N.S.) 25, 4: 393
19. Wouters J, Verhecken A (1989) The Coccid insect dyes: HPLC and computerized diode array analysis of dyed yarns. Stud Conserv 34: 189
20. González M, Méndez J, Carnero A, Lobo M G, Afonso A (2002) Optimizing conditions for the extraction of pigments in cochineals (*Dactylopius coccus* Costa) using response surface methodology. J Agr Food Chem 50: 6969
21. Wouters J, Verhecken A (1987) The chemical nature of flavokermesic acid. Tetrahedron Lett 28, 11: 1199
22. Derksen G C H, Niederländer H A G, van Beek T A (2002) Analysis of anthraquinones in *Rubia tinctorium* L. by Liquid Chromatography coupled with Diode-Array UV and mass spectrometric detection. J Chromatogr A 978: 119

23. Mosi A A, Reimer K J, Eigendorf G K (1997) Analysis of polyaromatic quinones in a complex environmental matrix using gas chromatography ion trap tandem mass spectrometry. Talanta 44: 985
24. Cuyckens F, Claeys M (2004) Mass Spectrometry in the structural analysis of flavonoids. J Mass Spectrom 39: 1
25. March R E, Miao X, Metcalfe C D, Stobiecki M, Marczak L (2004) A fragmentation study of an isoflavone glycoside, genistein-7-O-glucoside, using electrospray quadrupole time-of-flight mass spectrometry at high mass resolution. Int J Mass Spectrom 232: 171
26. Stobiecki M (2000) Application of mass spectrometry for identification and structural studies of flavonoid glycosides. Phytochemistry 54: 237
27. Sharma A, Kumar P, Zaidi Z H (1998) Effect of hydroxy substituents on the electronic absorption spectrum of 9,10-anthraquinone. Asian J Phys 7, 1: 162
28. Fain V Y, Zaitsev B E, Ryabov M A (2003) A quantum-chemical study of prototropic tautomerism in 1-Hydroxy-9,10-anthraquinones. Russ J Gen Chem 73, 4: 621
29. Labhart H von (1957) Zur quantitativen beschreibung des einflusses von substituenten auf das absorptionsspektrum ebener molekeln. Anwendung auf anthrachinon. Helv Chim Acta 40, 5: 1410
30. Yoshida Z, Takabayashi F (1968) Electronic spectra of mono-substituted anthraquinones and solvent effects. Tetrahedron 24: 913
31. Morgan E D (2004) Biosynthesis in insects, 1st edn. The Royal Society of Chemistry, p 134