Dyes and Pigments 154 (2018) 290–295

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Degradation of lucidin: New insights into the fate of this natural pigment present in Dyer's madder (*Rubia tinctorum* L.) during the extraction of textile artefacts

Lauren Ford[a,b], Christopher M. Rayner[b], Richard S. Blackburn[a,*]

[a] School of Design, University of Leeds, Leeds LS2 9JT, UK
[b] School of Chemistry, University of Leeds, Leeds LS2 9JT, UK

ARTICLE INFO

Keywords:
Madder
Rubia tinctorum
Lucidin
Dyes
Textiles
Extraction
Reactivity
HPLC analysis

ABSTRACT

The complex mixtures of colorants present in different madder species can provide significant information about which plant species or technique was used to dye the fibres of historical textile artefacts, hence, when extracting and analysing colorants from textile artefacts as much of this information as possible should be preserved. Historical textiles are most commonly extracted with 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*), but this solvent system hydrolyses dye glycosides and may also induce chemical reactions. One of the primary components in Dyers' madder (*Rubia tinctorum* L.) is lucidin primeveroside, but it is rarely seen in artefacts, nor is the corresponding aglycon lucidin. It has been demonstrated that the hydrochloric acid method causes hydrolysis of anthraquinone glycosides to their aglycon counterpart. Herein it is demonstrated that lucidin is not stable in such acidic conditions and degrades rapidly to xanthopurpurin. This is confirmed by HPLC, LC-MS and ^{1}H NMR, which also provide evidence of the mechanism of degradation being a retro-aldol process.

1. Introduction

Natural colorants are complex mixtures of many different molecules and plant dyes are often a mixture of aglycons of the parent colorant moiety and their glycosidic counterparts. The ratio of the abundance of these molecules can provide significant information about which plant species was used to dye the fibres or the technique used for the dyeing process. In the context of historical textiles, this information is of paramount importance for conservation and restoration purposes, as well as the generation of information on the ethnographic origins of the artefacts.

Colorants obtained from the roots of Dyers' madder (*Rubia tinctorum* L.), are grouped collectively in the Colour Index as C. I. Natural Red 8, and have been used as a red dyestuff for centuries. Over 35 anthraquinonoid compounds have been reported to be extractable from madder roots [1], however, many of these compounds are artefacts of inherent reactivity during analytical extraction methods and are suspected as not being not present *in planta*; for example, anthraquinones that contain a 2-methoxymethyl- or a 2-ethoxyethyl group are formed during extraction with hot methanol or ethanol, respectively [1,2]. When extracting and analysing colorants from textile artefacts as much information should be preserved as possible in order to gain better insight on how they were dyed and the plant species from which the dye originated, hence, it is important to limit the damage to the colorant molecule in the extraction process. However, extraction of artefacts is not straightforward as the dyes are strongly bound to the substrate *via* a mordant metal (typically Al^{3+}); the most common literature extraction procedure uses a 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*) mixture [3–8], as the strong acid enables displacement of the dye molecules from their mordant metal complex [9]. However, such conditions may also induce a chemical reaction, hence, it is vital that fundamental understanding of the reactivity of such natural dyes is developed alongside the analysis of the components within the mixture. If the conditions of extraction and analysis of these dyed textiles changes the ratios of the compounds present, or modifies their structure, then valuable information on that artefact will be lost or potentially misinterpreted.

Only relatively recently has there been significant evidence confirming the primary anthraquinone components in *Rubia tinctorum* roots as the glycosides ruberythric acid (**1**) and lucidin primeveroside (**3**) [10–14]; the majority of literature has pointed to alizarin (**2**) as the major anthraquinone present, and whilst it does occur in the plant, it is in much lower concentrations than its glycoside [11,12,14]. We have previously suggested [15] that acidic conditions used in extraction and

* Corresponding author.
E-mail address: r.s.blackburn@leeds.ac.uk (R.S. Blackburn).

https://doi.org/10.1016/j.dyepig.2018.03.023
Received 10 January 2018; Received in revised form 14 February 2018; Accepted 13 March 2018
Available online 14 March 2018

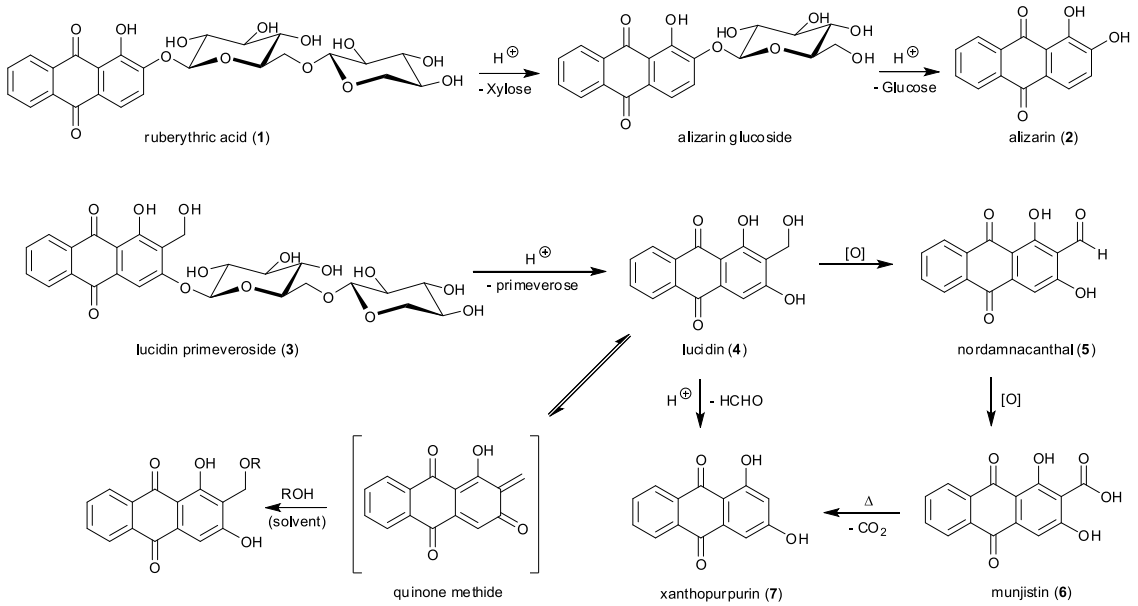


Fig. 1. Possible inter-relationships between anthraquinone compounds found in *Rubia tinctorum* based on chemical or biochemical interconversion.

analysis of dyes in previous studies may have led to observations that alizarin was the primary component, it being the product of ruberythric acid hydrolysis (**1** → **2**). However, despite high concentrations of lucidin primeveroside (**3**) in *Rubia tinctorum* roots [11,12,14], the aglycon lucidin (**4**) is rarely detected (and then only in low and trace concentrations *in planta* and in textile artefacts [10–12,16]) even when acidic conditions are used that would promote hydrolysis (**3** → **4**); it is suspected that the reactive nature of lucidin means that it is readily converted to other compounds. As Fig. 1 shows, lucidin (**4**) can be oxidised to nordamnacanthal (**5**), and studies have suggested this is catalysed by endogenous oxidase enzymes in the plant [17,18]. It is possible that nordamnacanthal (**5**) can form munjistin by the action of endogenous oxidase; subsequently xanthopurpurin (**7**) may be formed through decarboxylation of munjistin (**6**). It has also been proposed [19] that another enzymatic reaction can occur that converts lucidin into the quinone methide (Fig. 1). It is thought this intermediate may be able to be formed by acidic conditions, but the actual intermediate is too difficult to isolate due to it being a very strong electrophile and addition at the double bond by any nucleophile is highly likely [12].

However, these enzymes are probably denatured in the dyeing process and hence this mode of degradation probably is not responsible for these compounds in historic artefacts. Mouri & Laursen [12] recently confirmed that, unless *R. tinctorum* roots were "warmed in water" for prolonged periods (hence, providing enzymatic incubation conditions), significant concentrations of anthraquinone glycosides were present in the dyebath and on dyed wool fibre. They demonstrated that steaming madder roots or boiling them in water for 30 s was sufficient to deactivate the hydrolytic enzymes. An initial extraction process by boiling the madder root was typically performed in the Japanese *Kusaki-zome* dyeing method and typical European madder dyeing processes historically involved heating the dyebath to 75–80 °C, which would most probably also denature the endogenous enzymes present [20].

We have previously suggested [15] that xanthopurpurin may also be formed directly from lucidin (**4**) through an acid (or base)-catalysed loss of formaldehyde through a retro-aldol type process (Fig. 1), but there is no literature to support this proposal. The absence of lucidin in the analysis of artefacts dyed with madder is rarely acknowledged, or it is stated that lucidin is degraded into unknown products [9]. Lucidin is the only commonly reported anthraquinone detected in the roots of *Rubia tinctorum* to contain a primary alcohol, which could make its degradation unique. As described in our previous research [15], use of the 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*) solvent system causes hydrolysis of anthraquinone glycosides present in madder root, with the result that only aglycons are detected in back extraction experiments.

Herein it is suggested that when such acidic methods of extraction are used to solvate the dye compounds, degradation of the aglycon lucidin may also occur. The purpose of the research described is to study the degradation of lucidin under the conditions of extraction involved with the common 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*) solvent method.

2. Materials and methods

2.1. Materials and solvents

All chemicals were purchased from Sigma-Aldrich. All solvents used were of HPLC grade and also purchased from Sigma-Aldrich. HPLC grade water was obtained by distillation on site.

2.2. General procedures and instrumentation

Nuclear magnetic resonance (NMR) spectra recorded for ^{1}H NMR at 300.13 MHz and 500.21 MHz and ^{13}C at 75.45 MHz on a Bruker *DPX300* and *DRX500* spectrometer. Chemical shifts are given in parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm) for proton resonances. The proton coupling constants are corrected and given in Hz and expressed, *e.g.* as multiplicities, singlet (s), broad singlet (bs), doublet (d), double doublet (dd), triplet (t) and quartet (q). High resolution electrospray (ESI+) mass spectrometry was performed on a Bruker MaXis Impact spectrometer, *m/z* values are reported in Daltons to four decimal places. Liquid Chromatography with Mass Spectrometry (LC-MS) was carried out for analysis synthetic references. LC analyses were carried out at room temperature on a Phenomenex Hyperclone C_{18} column, 5 µm particle size, 250 × 4.6 mm I.D. column equipped with a pre-column. Chromatography was carried out using two solvents: (A) water and 0.1% formic acid solution and (B) acetonitrile and 0.1% formic acid solution. A linear gradient programme was applied: of 0–3 min 0–100% increase of solvent B. The flow rate during the experiment was 1.0 ml min^{-1}. Injections were made by a Basic Marathon autosampler equipped with a 20 µl loop. The method

was carried out on an Agilent 1200 LC using a Bruker HCT Ultra Ion Trap for the MS detection and a Diode Array Detector. The ESI (electrospray ionisation) parameters in the negative ion mode were as follows: spray voltage 4000 V (applied to the spray tip needle), dry gas 10 $dm^3 min^{-1}$, dry temperature 365 °C, capillary 60 nA, nebulizer 65 psi, nebulising gas N_2. UV/visible spectrophotometry was carried out using a Jasco V-530 UV/visible/NIR spectrophotometer at 2 nm intervals. Spectral properties and wavelength of maximum absorbance (λ_{max}) were evaluated. Infrared spectra were recorded on a Bruker Alpha Platinum ATR. Samples were analysed in the solid phase and absorption maxima (ν_{max}) are given in wave numbers (cm^{-1}) to the nearest whole wavenumber.

2.3. Synthesis of references for chemical components of madder root

2.3.1. Xanthopurpurin

This method was based on that of Murti et al. [21]. Anhydrous aluminium chloride (4.8 g, 40 mmol) and sodium chloride (1.2 g, 20 mmol) were heated to 150 °C until molten. To this, a mixture of phthalic anhydride (1.84 g, 8 mmol) and resorcinol (0.80 g, 8 mmol) was added slowly. The temperature was then slowly increased to 165 °C and maintained for 4 h. The reaction mixture was then cooled to 0 °C and 2 M aqueous hydrochloric acid solution was added and stirred for 15 min. The reaction mixture was then heated to reflux for 30 min, after which it was cooled to room temperature and extracted with ethyl acetate (3 × 30 ml). The ethyl acetate extracts were then washed successively with saturated sodium bicarbonate solution (30 ml), dried with magnesium sulphate and evaporated to dryness.

Xanthopurpurin (**7**) was collected as a yellow/orange amorphous solid, 28 mg, 1.2% yield, m.p. 261–264 °C. IR (ATR), ν (cm^{-1}): 3360, 1633, 1598, 1451, 1258. λ_{max} (log ε) in MeOH: 412 nm (4.15). ^{1}H NMR (500 MHz, DMSO): δ 12.76 (s, 1H, OH), 11.32 (s, 1H, OH), 8.23 (dd, J = 7.5, 1.7 Hz, 1H), 8.18 (dd, J = 7.5, 1.7 Hz, 1H), 7.95 (app td, J = 1.7, 7.6 Hz, 1H), 7.92 (app td, J = 1.7, 7.6 Hz, 1H), 7.15 (d, J = 2.3 Hz, 1H), 6.62 (d, J = 2.3 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) δ 158.2, 157.8, 157.4, 157.0, 134.0, 133.8, 126.6, 126.2, 118.9, 116.0, 113.2, 110.4, 108.1, 107.5. HRMS: *m/z* (ESI-) calculated for $C_{14}H_8O_4$ $[M-H]^-$:239.0423; found $[M-H]^-$:239.0354. HPLC retention time and mass data of negative ion can be found in Table 1.

2.3.2. Lucidin

In order to gain a better understanding of the effect that 37% hydrochloric acid has on lucidin during extraction, a pure sample of lucidin was synthesised to observe any changes in its structure under the back extraction conditions. This method was based on that of Murti et al. [21]. Xanthopurpurin (20 mg, 0.08 mmol) was dissolved in 5% aqueous sodium hydroxide solution (0.5 ml). Aqueous formaldehyde 37% (30 µl, 0.4 mmol, 5 equivalents) was then added and stirred at room temperature for 3 h and the reaction was monitored by LC-MS. Once completion was observed the solution was precipitated with 10% aqueous hydrochloric acid solution (~1 ml) until a yellow precipitate was observed. The yellow precipitate was then extracted with ethyl acetate (3 × 1 ml), dried with magnesium sulphate and then evaporated to dryness. This was then separated on a short flash silica column with 70% ethyl acetate, 30% hexane.

Lucidin (**4**) was collected as a yellow amorphous solid, 21 mg, 87.5% yield, m.p. 301–305 °C. IR (ATR), ν (cm^{-1}): 3400, 1634, 1558, 1365, 1338. λ_{max} (log ε) in MeOH: 410 nm (3.66). ^{1}H NMR (500 MHz, DMSO): δ 11.33 (s, 1H, OH), 8.22 (dd, J = 7.5, 1.5 Hz, 1H), 8.15 (dd, J = 7.0, 1.5 Hz, 1H), 7.77 (app td, J = 1.6, 7.2 Hz, 1H), 7.74 (app td, J = 1.6, 7.2 Hz, 1H), 7.26 (s, 1H), 4.83 (broad s, 1H, OH), 4.55 (s, 2H, CH_2). ^{13}C NMR (126 MHz, MeOD) δ 159.9, 159.6, 159.4, 159.1, 158.7, 158.4, 119.4, 117.1, 114.8, 112.6, 54.7, 54.5, 54.3, 54.2, 54.0. HRMS: *m/z* (ESI-) calculated for $C_{15}H_{10}O_5$ $[M-H]^-$:269.0528; found $[M-H]^-$:269.0464. HPLC retention time and mass data of negative ion can be found in Table 1.

2.4. Chemical degradation of lucidin

2.4.1. With methanol

Pure lucidin (2 mg) synthesised and purified as described above was dissolved in 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*) (0.5 ml) and heated to 100 °C for 15 min. After this time an aliquot of the reaction mixture was taken for LC-MS and HPLC analysis. The remaining reaction mixture was evaporated to dryness and re-dissolved in deuterated acetone for NMR analysis. Deuterated DMSO was also evaluated as a solvent for NMR analysis, but deuterated acetone provided better solubility. Deuterated methanol could not be used as it would interfere with the results forming the methyl ether adduct.

2.4.2. Without methanol

Pure lucidin (2 mg) synthesised and purified as described above was dissolved in 37% hydrochloric acid: water (1:1, *v/v*) (0.5 ml) and heated to 100 °C for 15 min. After this time an aliquot was taken for LC-MS and HPLC analysis. The remaining reaction mixture was evaporated to dryness and re-dissolved in deuterated acetone for NMR analysis.

3. Results and discussion

The HPLC chromatogram of lucidin after heating in 37% hydrochloric acid: water (1:1, *v/v*) (Fig. 2) showed a decrease in the lucidin peak (4) and a peak appearance at retention time 11.57 min (7); this new peak has the same UV/vis data and retention time as that of the synthesised xanthopurpurin standard (Table 1). The LC-MS also shows two peaks with the same molecular weights as lucidin (*m/z* = 269) and xanthopurpurin (*m/z* = 239). These results suggest that under aqueous acidic conditions that mimic those used in textile back extractions [20], lucidin is partially degraded to xanthopurpurin.

It is hypothesised that in this case the reaction probably proceeds through a retro-aldol type mechanism (Scheme 1). The ability of this reaction to occur is unique to aromatic systems containing multiple hydroxyl groups as hydroxyl groups on an aromatic ring are electron donating, and usually in equilibrium with a low concentration of the keto tautomer. The keto tendency of the hydroxyl groups in positions one and three on the lucidin aromatic ring in acidic conditions drive the reverse aldol condition. The electron donating ability of the other hydroxyl group in the ring also provides stabilisation to the ketone tautomer. The loss of formaldehyde also provides an entropic driving force for this potentially reversible reaction.

The HPLC chromatogram of the madder extract prepared using 37%

Table 1
Compounds identified by HPLC-DAD and LC-MS in hydrochloric acid degradation of lucidin.

Solvent	Anthraquinone derivative assigned to HPLC peak	Retention time (min)	UV λ_{max} values for compound identification (nm)	Molecular ion, *m/z*, from LC-MS $[M-H]^-$
37% hydrochloric acid: water (1:1, *v/v*)	lucidin (4)	9.9	244.6, 280.9	269.0
	xanthopurpurin (7)	11.6	243.5, 280.7	239.0
37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*)	lucidin (4)	9.9	244.5, 280.2	269.4
	xanthopurpurin (7)	11.6	243.5, 280.7	239.4
	lucidin methyl ether (8)	12.3	244.5, 281.3	283.4

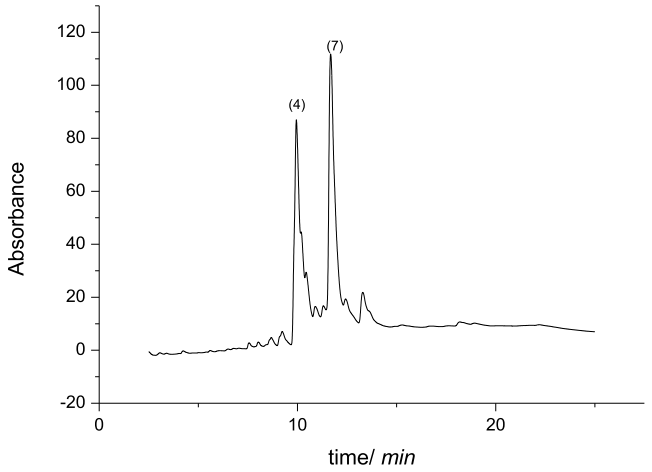


Fig. 2. HPLC chromatogram of lucidin after heating in 37% hydrochloric acid: water (1:1, *v/v*).

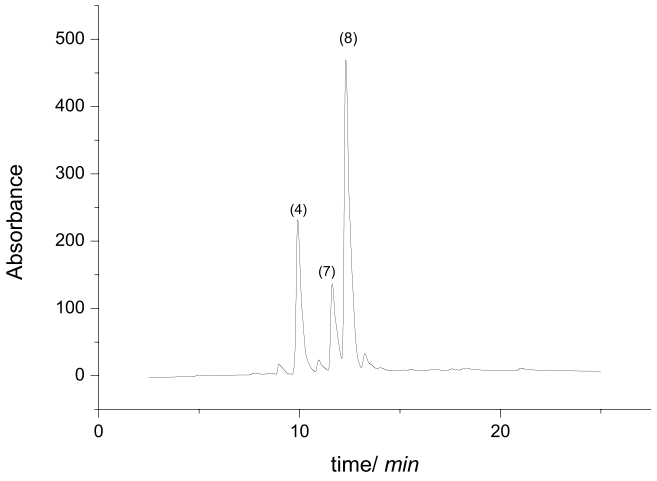
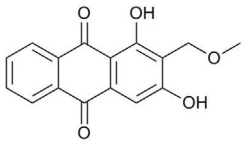


Fig. 3. HPLC chromatogram of lucidin after heating in 37% hydrochloric acid: methanol: water (2:1:1, *v/v/v*).

hydrochloric acid: methanol: water (2:1:1, *v/v/v*) (Fig. 3) showed a decrease in the lucidin peak (4) and formation of the xanthopurpurin peak (7). However, in this reaction there was also the formation of a third peak (Table 1) which has the largest peak height and peak area in the chromatogram. LC-MS also showed three peaks in the chromatogram; lucidin (m/z = 269), xanthopurpurin (m/z = 239) and the third peak which gives a mass of m/z = 283, which corresponds to the methyl ether of lucidin (**8**) [13]. It is well documented that ether products can be formed when using alcohol in the solvent system when extracting dye compounds and in extraction from textile artefacts [1,2,13]. The methyl ether product is expected to be much more stable than the methyl hydroxyl present in lucidin; the primary alcohol in lucidin can potentially be displaced by nucleophilic attack of alcoholic solvents such as ethanol or methanol. Under dry or acidic conditions this primary alcohol is most likely removed as water and formation of the quinone methide can take place. The quinone methide is very susceptible to nucleophilic addition [12] and this process is reversible – addition of water will reform lucidin whereas attack by methanol will form the lucidin methyl ether. It is important to note that whilst lucidin can undergo the retro-aldol process, this is not feasible for the methyl ether, and reformation of the quinone methide would be the main reaction pathway available. LC-MS also showed evidence of the formation of a dimeric species (**9**) with the mass of m/z = 491.



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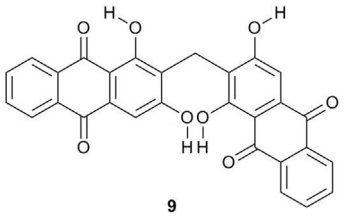

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It is noted herein that the UV/vis spectra of each peak was very similar for these compounds. For this reason, mass spectra data and ^{1}H NMR were used to fully assign the peaks and observe the reaction as it proceeds. The NMR experiments are not trivial to analyse as lucidin has limited solubility in most solvents or is only soluble at low concentrations. In addition to this the NMR signal corresponding to xanthopurpurin can appear diminished due to the proton between the two

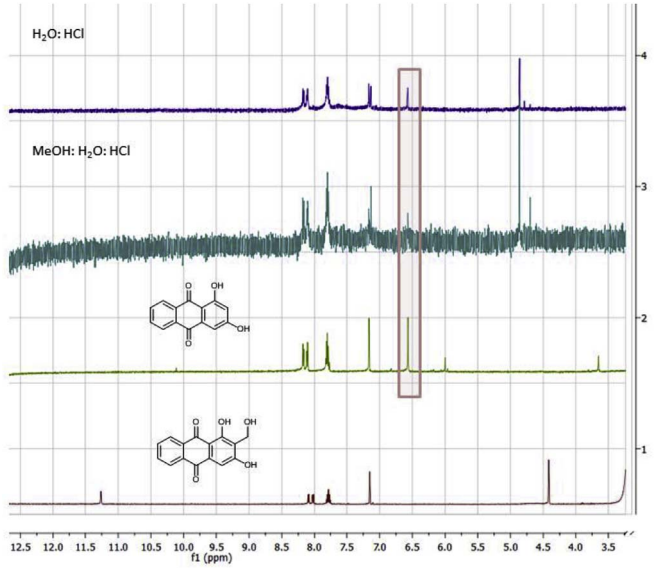
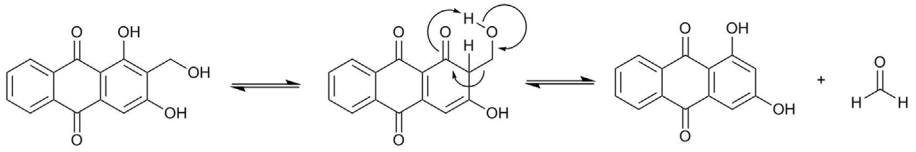


Fig. 4. Stacked ^{1}H NMR of lucidin breakdown experiments. From top to bottom: H_2O: HCl; MeOH: H_2O: HCl; xanthopurpurin standard; lucidin standard.

hydroxyl groups being labile for exchange with deuterium providing further evidence for the presence of the keto-equilibrium form.

The ^{1}H NMR spectra shown in Fig. 4 shows an increase in the proton signals highlighted in the coloured box; this proton corresponds to the aromatic signal between the two hydroxyl groups in xanthopurpurin [22]. The appearance of this signal shows that lucidin has degraded to xanthopurpurin most likely through the retro-aldol type reaction proposed. There is also evidence for the formation of formaldehyde (as indicated in Scheme 1). Under the reaction conditions, where water (or methanol) is present, formaldehyde would not be expected to be observed as it would exist primarily as the hydrate ($HOCH_2OH$). This equilibrium, and resulting ^{1}H NMR signals are quite sensitive to concentration, temperature and pH, but it is known that $HOC\underline{H}_2OH$ has a chemical shift of *ca.* 4.6 ppm and the oligomer $HOC\underline{H}_2OC\underline{H}_2OH$ slightly



Scheme 1. Mechanism of the proposed retro-aldol type mechanism of lucidin in acidic aqueous conditions.

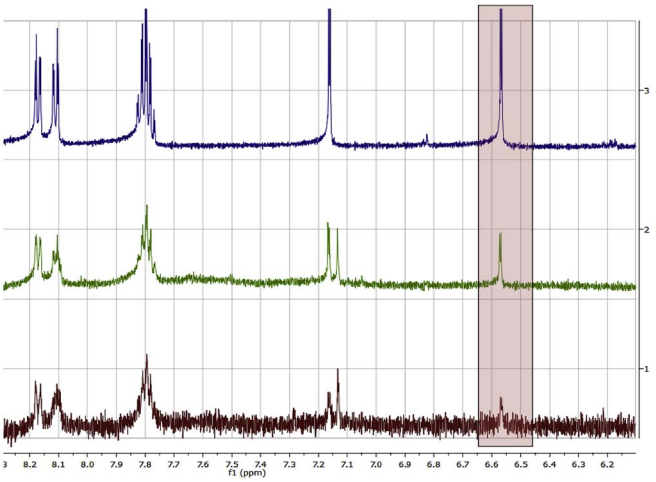


Fig. 5. Stacked ^{1}H NMR spectra showing meta-coupling of the aromatic signals: xanthopurpurin standard (blue; top); H_2O: HCl (green; middle); MeOH: H_2O: HCl (red; bottom). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher around 4.7 ppm [23]. There are definitely appropriate (if small) signals in this region of the spectrum consistent with this explanation however unambiguous assignment has not been possible so far. It is feasible that liberated formaldehyde may re-add to xanthopurpurin at a different position (*e.g.* C4 rather than C2) to form an isomeric product, although the presence of the adjacent carbonyl makes this centre much less nucleophilic, and so is unlikely. Other reactions, such as *O*-alkylation on phenolic groups, are feasible, but there is no evidence to suggest this could occur at anything more than a small equilibrium concentration.

Fig. 5 shows the ^{1}H NMR spectra expanded in the aromatic region which displays the appearance of meta coupling (2–3 Hz), which indicates that there is a hydrogen in the *meta* position of the ring, further confirming the degradation to xanthopurpurin (which displays this meta coupling). Fig. A1 shows the ^{1}H NMR spectra of lucidin methyl ether and displays integration between the singlet (H4, 7.30 ppm) and the methyl group corresponding to the methyl ether (OCH_3, 3.27 ppm). Upon closer inspection, the meta-coupling constant between the two protons on the xanthopurpurin can be observed in the degradation studies, which further confirms the degradation of lucidin into xanthopurpurin. A singlet corresponding to lucidin present in the breakdown experiments is also observed, indicating that the breakdown of lucidin is not complete and some remains in the reaction medium. This result is confirmed in the HPLC chromatograms (Figs. 2 and 3) where lucidin is still present in both of these experiments, but in lower concentrations.

This reaction was also examined in deuterium oxide and deuterated methanol, however, this resulted in the deuterated reaction product of the lucidin methyl ether (m/z = 286), seen in Fig. 6, which caused problems in analysis by NMR due to the methanol adduct being the deuterated product and, hence, not visible in the ^{1}H NMR spectrum [24]. However, the mass spectrum shows the lucidin methyl ether ion (m/z = 283 + 3) for the deuterated methanol adduct, which confirms formation of the lucidin methyl ether product.

Degradation of lucidin into xanthopurpurin is important for the field of conservation scientists and conservators. Previously the presence of xanthopurpurin has been used as an indicator that the sample probably contained munjistin, which is a carboxylic acid derivative of xanthopurpurin [12]. Munjistin can be easily decarboxylated to form xanthopurpurin [18], this reactivity is again due to the keto tendency of the hydroxyl groups on the ring. Munjistin is a marker pigment which can be used to prove the presence of *R. cordifolia* in dyed textiles [20], therefore, by employing an acid extraction process information relevant to the analysis of the dyed materials is potentially lost as munjistin is decarboxylated to xanthopurpurin and lucidin is degraded to xanthopurpurin, causing problems in determining whether the original artefact was dyed with *R. tinctorum* or *R. cordifolia*. This is further complicated by the fact that xanthopurpurin and alizarin elute very closely on a C18 column and hence could co-elute as one peak in many systems. The two compounds also have the same mass, hence, if their UV/vis spectra are not analysed in detail they could be mistaken for the same compound and hence alizarin could be wrongly assigned in the sample.

4. Conclusions

It has been demonstrated herein that lucidin is not stable in acidic conditions and degrades rapidly to xanthopurpurin when heated in aqueous acid conditions that are typically used for extraction of historical textiles dyed with madder. This is confirmed by HPLC, LC-MS and ^{1}H NMR, which have also provided support for the proposed mechanism of degradation being a retro-aldol process. Different madder varieties and species and different origins have different chromatographic profiles *in planta*, hence, the most effective artefact extraction technique would be the one that preserves the colorants in the dyeings in the form as applied. As most existing methods cause some form of acid-catalysed degradation to colorant moieties, it is vital to future development of analytical techniques to examine historical textiles, that milder and effective extraction techniques are developed to enable better-informed identification of the original dyestuff and to provide more information about the botanic, geographic and ethnographic origins of the dyes.

Acknowledgments

Funding: The authors would like to thank The Clothworkers' Foundation for the provision of financial support to LF to allow her to conduct her PhD studies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2018.03.023.

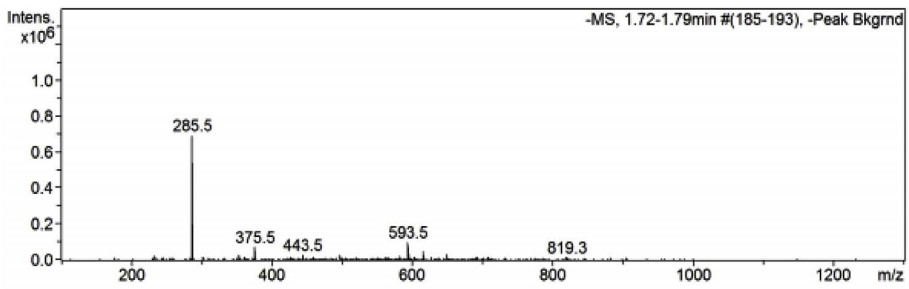


Fig. 6. Mass spectrum of deuterated methyl ether product of lucidin.

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