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Mild extraction methods using aqueous glucose solution for the analysis of natural dyes in textile artefacts dyed with Dyer's madder (Rubia tinctorum L.)

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Abstract

Madder (Rubia tinctorum L.) has been widely used as a red dye throughout history. Acid-sensitive colorants present in madder, such as glycosides (lucidin primeveroside, ruberythric acid, galiosin) and sensitive aglycons (lucidin), are degraded in the textile back extraction process; in previous literature these sensitive molecules are either absent or present in only low concentrations due to the use of acid in typical textile back extraction processes. Anthraquinone aglycons alizarin and purpurin are usually identified in analysis following harsh back extraction methods, such those using solvent mixtures with concentrated hydrochloric acid at high temperatures. Use of softer extraction techniques potentially allows for dye components present in madder to be extracted without degradation, which can potentially provide more information about the original dye profile, which varies significantly between madder varieties, species and dyeing technique. Herein, a softer extraction method involving aqueous glucose solution was developed and compared to other back extraction techniques on wool dyed with root extract from different varieties of Rubia tinctorum. Efficiencies of the extraction methods were analysed by HPLC coupled with diode array detection. Acidic literature methods were evaluated and they generally caused hydrolysis and degradation of the dye components, with alizarin, lucidin, and purpurin being the main compounds extracted. In contrast, extraction in aqueous glucose solution provides a highly effective method for extraction of madder dyed wool and is shown to efficiently extract lucidin primeveroside and ruberythric acid without causing hydrolysis and also extract aglycons that are present due to hydrolysis during processing of the plant material. Glucose solution is a favourable extraction medium due to its ability to form extensive hydrogen bonding with glycosides present in madder, and displace them from the fibre. This new glucose method offers an efficient process that preserves these sensitive molecules and is a step-change in analysis of madder dyed textiles as it can provide further information about historical dye preparation and dyeing processes that current methods cannot. The method also efficiently extracts glycosides in artificially aged samples, making it applicable for museum textile artefacts.

Keywords

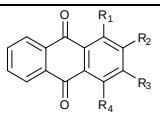
Madder; Rubia tinctorum; dyes; textiles; extraction; reactivity; HPLC analysis.

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 nature of the glycosides and 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 dye 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. Dioscorides wrote about madder's usefulness in dyeing red and the dyestuff was well known to the dyers of Egypt, Greek and Roman eras [3]; however, only in the 16th Century did dyeing recipes start to be readily available in literature [4]. Over 35 anthraquinonoid compounds have been reported to be extractable from madder roots [5], 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 [5,6].

Table 1. Anthraquinone derivatives observed in the roots of Rubia tinctorum L.

Number	Common name					Mass (Da)	References
		R_1	**R_2**	**R_3**	**R_4**		
1	ruberythric acid	OH	OGlc+Xyl	H	H	534	[5,7-11]
2	alizarin	OH	OH	H	H	240	[5,7-11]
3	lucidin primeveroside	OH	CH_2OH	OGlc+Xyl	H	564	[5,7-11]
4	lucidin	OH	CH_2OH	OH	H	270	[7,8,12]
5	nordamnacanthal	OH	CHO	OH	H	268	[7,9,13]
6	munjistin	OH	COOH	OH	H	284	[7,9]
7	xanthopurpurin	OH	H	OH	H	240	[7,9,14]
8	galiosin	OH	COOH	OH	OGlc+Xyl	594	[7-9]
9	pseudopurpurin glucoside	OH	COOH	OH	OGlc	462	[7,9]
10	pseudopurpurin	OH	COOH	OH	OH	300	[5,7,9,14]
11	purpurin	OH	H	OH	OH	256	[7,9,11]
12	rubiadin primeveroside	OH	CH_3	OGlc+Xyl	H	548	[8]
13	rubiadin	OH	CH_3	OH	H	254	[8,9]
14	1-hydroxy-2-methyl-AQ	OH	CH_3	H	H	238	[14,15]
15	2-hydroxy-AQ	H	OH	H	H	224	[14]
16	2-(hydroxy methyl)-AQ	H	CH_2OH	H	H	238	[9]
17	anthragallol	OH	OH	OH	H	256	[14]

Glc, glucose; Xyl, xylose; OGlc+Xyl, O-primeveroside, O-(6-O-β-D-xylopyranosyl-D-glucosyl); AQ, anthraquinone.

Table 1 summarises the compounds that have been reported as being extracted from Rubia tinctorum roots (excluding products of reaction with the extraction solvent); however, many of these compounds may not be present, or

present in low concentrations, in planta and may actually be a result of reaction during harvesting, drying, or processing of the madder root before it is used for dyeing. 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. HPLC-DAD has become an important tool in analysing samples of historical interest due to the complex mixtures of colorants involved when dyeing with natural dyes [16,17]. 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 [18-23], as the strong acid enables displacement of the dye molecules from their mordant metal complex [24]. However, such conditions may also induce chemical reaction, and hence it is vital that a fundamental understanding of the reactivity of such natural dyes is developed alongside the analysis of the components within the mixture.

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**) [5,7-11]; 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 [8,9,11]. Anthraquinone glycosides can be hydrolysed to their aglycon counterparts (Figure 1); this may occur in the roots of the plant catalysed by endogenous enzymes, but could also be during chemical processing, especially under acidic conditions. Ruberythric acid was first isolated from madder (unspecified Asian species) by Rochleder in 1851 [25]; Schunck later demonstrated that the glycoside could be hydrolysed to alizarin in planta catalysed by one of the first enzymes ever studied, which he isolated and called "erythrozyme" [26]. Hill & Richter [27] demonstrated that boiling madder roots in water destroyed (denatured) the hydrolytic enzymes and the glycosides could be readily extracted. Rubiadin primeveroside (**12**) occurs in very low concentrations in Rubia tinctorum roots (<2%) [8], and is most likely the origin of trace amounts of rubiadin (**13**) detected in Rubia tinctorum samples [8,9], as a result of hydrolysis (**12**→**13**). Galiosin (**8**) and pseudopurpurin glucoside (**9**) occur in low concentrations in Rubia tinctorum roots [7-9], and are both most probably the origin of low amounts of pseudopurpurin (**10**) in Rubia tinctorum samples [5,7,9,14], also as a result of hydrolysis (**8**→**9**→**10**).

Under the strongly acidic conditions used in many literature methods [28], it is suggested herein that acidic conditions used in extraction and analysis of dyes in previous studies may have led to observations that alizarin was the primary component [18-23], it being the product of ruberythric acid hydrolysis (**1**→**2**), most likely via the glucoside, although isolation of the glucoside has not been reported. Even when 'milder' acids, such as 0.5 M citric acid [29] and 2 M trifluoroacetic acid (TFA) [30], have been used in the extraction of artefacts, alizarin has been the main compound detected. More recently, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) have been employed to remove dyes from artefacts [29,31], but neither solvent has been demonstrated as able to remove mordanted dyes nor have they shown an ability to remove anthraquinone glycosides intact; it is also likely that DMSO can oxidise lucidin to nordamnacanthal through a Kornblum/Swern type oxidation [32], hence DMSO is not an appropriate solvent for use in madder extraction.

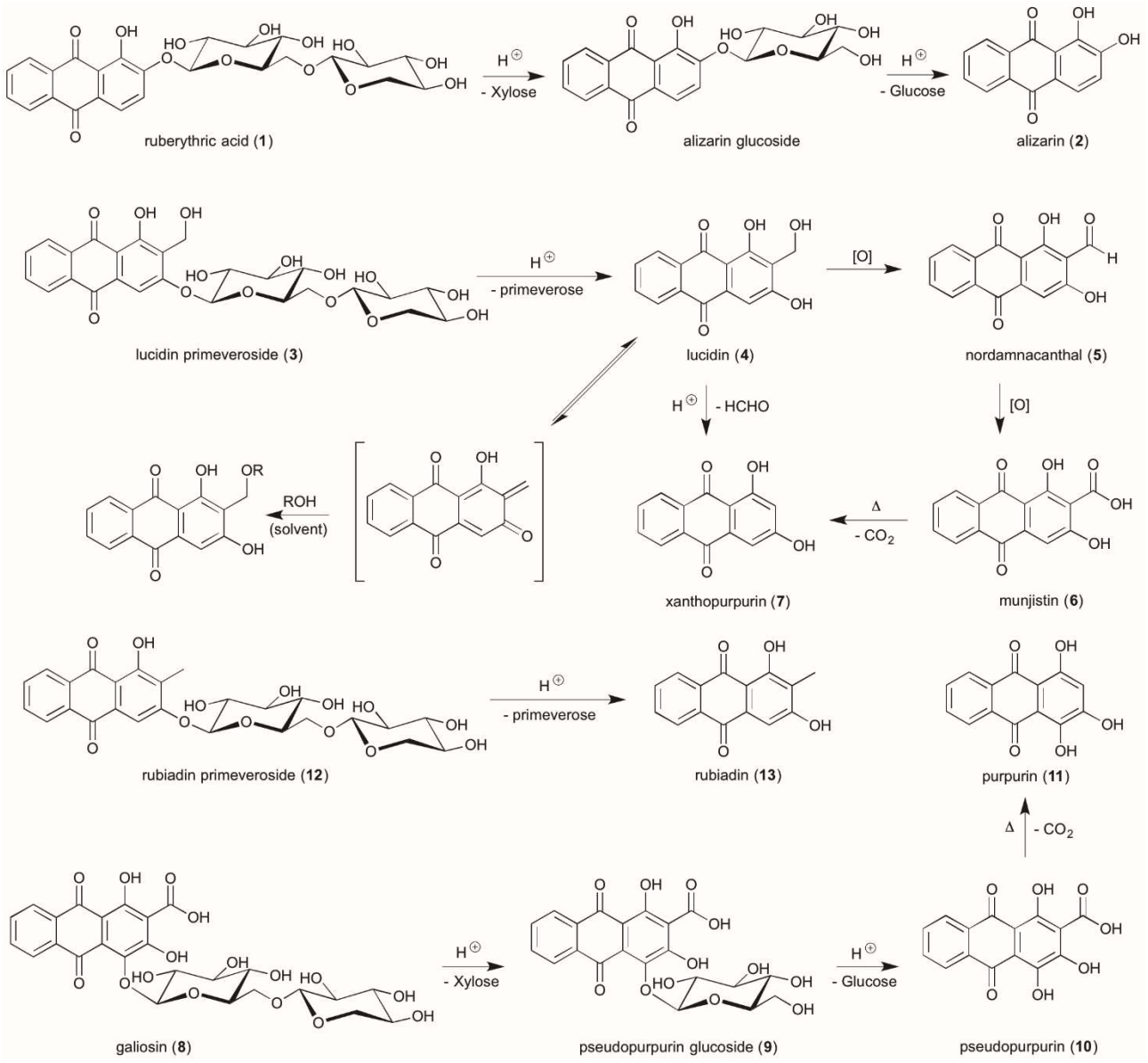


Figure 1. Possible inter-relationships between anthraquinone compounds found in Rubia tinctorum based on chemical or biochemical interconversion.

The stability of different anthraquinone glycosides varies. Hill & Richter [27] demonstrated that galiosin (**8**) is very unstable and is readily hydrolysed to pseudopurpurin (**10**), which is as a result of glycoside substitution at the α-hydroxy in the 1-position; ruberythric acid (**1**) and rubiadin primeveroside (**12**) have glycoside substitution at the β-hydroxy in the 2-position, making them significantly more stable to hydrolysis; the presence of a carboxylic acid moiety within galiosin at the 2-position may also catalyse hydrolysis. After alizarin, the major compound identified as being present in Rubia tinctorum in literature is purpurin (**11**) [18-23]; however, it is suggested that purpurin is formed through decarboxylation of pseudopurpurin during the drying of the roots [33].

Interestingly, despite high concentrations of lucidin primeveroside (**3**) in Rubia tinctorum roots [8,9,11], the aglycon lucidin (**4**) is rarely detected (and then only in low and trace concentrations in planta and in textile artefacts [7-9,12]) 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 Figure 1 shows, lucidin (**4**) can be oxidised to nordamnacanthal (**5**), and studies have suggested this is catalysed by endogenous oxidase enzymes in the plant [5,34,35]. Despite munjistin (**6**) being observed in Rubia tinctorum roots [7,9] and several other Rubia species [9,27,36,37], a glycoside, munjistin glucoside, has only ever been detected in Japanese madder (Rubia akane Nakai) [9], suggesting that munjistin is formed in planta through some other mechanism. It is possible that nordamnacanthal (**5**) can be further oxidised to form munjistin; in contrast, Hill & Richter [27] proposed that munjistin (**6**) was formed by natural reduction of pseudopurpurin (**10**), although this seems less likely. Xanthopurpurin (**7**) is formed through decarboxylation of munjistin (**6**); xanthopurpurin may also be formed directly from lucidin (**4**) through an acid (or base)-catalysed loss of formaldehyde through a retro-aldol type process (Figure 1).

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. This should be considered alongside known or anticipated reactivity of the molecules within the dye mixture. Mild extraction methods may also provide information about the dyeing process, for example, in 1860, Fabre patented [38] a process to produce "garancine", which involved intentionally breaking down the glycosides in madder by treatment with sulfuric acid before dyeing to obtain more free anthraquinones, which was believed to increase the efficiency of extraction of the dye components from the madder root.

This paper compares four different textile extraction techniques for identification of colorants present in different varieties of Rubia tinctorum: HCl/methanol/water [18]; aqueous citric acid solution [29]; aqueous TFA solution [29,30]; and a novel extraction method using an aqueous glucose solution.

2. Materials and methods

2.1. Materials and solvents

Natural cream wool, heavy weight with plain even weave was purchased from Whaley's, Bradford. Three madder root types were purchased from George Weil & Sons: 'Iranian madder', grown in Shiraz, Iran received as ground and dried material; 'Turkish madder', sourced from a wholesaler received as dried roots; and 'English madder', grown by Dr. David Hill at Bristol University received as dried roots. Alizarin and purpurin standards were purchased from Sigma-Aldrich. A mixture of the glycosides lucidin primeveroside and ruberythric acid was purchased from Apin Chemicals. All solvents used were of HPLC grade and purchased from Sigma-Aldrich. HPLC grade water was obtained by distillation on site.

2.2 General Procedures and Instrumentation

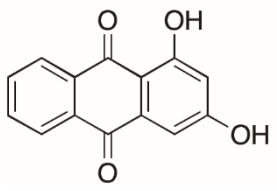
Nuclear magnetic resonance (NMR) spectra recorded for H^1 NMR at 300.13 MHz and 500.21 MHz and ^{13}C at 75.45 MHz on a Bruker DPX300 and DRX500 spectrometer. Chemical shifts are given in parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm) for proton resonances. The proton coupling constants are corrected and given in Hz and expressed 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 x 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 minutes 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 dye mixtures

2.3.1 Xanthopurpurin

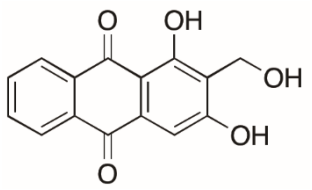
This method was based on that of Murti et al. [39]. Anhydrous aluminium chloride (4.8 g, 40 mmol) and sodium chloride (1.2 g, 20 mmol) were heated to 150 °C until molten. To this, a mixture of phthalic anhydride (1.84 g, 8 mmol) and resorcinol (0.80 g, 8 mmol) was added slowly. The temperature was then slowly increased to 165 °C and maintained for 4 hours. The reaction mixture was then cooled to 0 °C and 2M aqueous hydrochloric acid solution was added and stirred for 15 minutes. The reaction mixture was then heated to reflux for 30 minutes, 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. Product was collected as a yellow/orange amorphous solid (28 mg, 1.2% yield).



^{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, H4), 8.18 (dd, J= 7.5, 1.7 Hz, 1H, H1), 7.95 (app td, J= 1.7, 7.6 Hz, 1H, H3), 7.92 (app td, J= 1.7, 7.6 Hz, 1H, H2), 7.15 (d, J= 2.3 Hz, 1H, H5), 6.62 (d, J= 2.3 Hz, 1H, H6). ^{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 2. IR (ATR), ν (cm^{-1}): 3360, 1633, 1598, 1451, 1258. Melting point: 261-264 °C. λ_{max} (log ε) in MeOH: 412 nm (4.15).

2.3.2. Lucidin

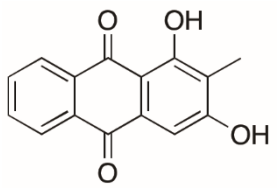
This method was based on that of Murti et al. [39]. Xanthopurpurin (20 mg, 0.08 mmol) was dissolved in 5% aqueous sodium hydroxide solution (0.5 ml). Aqueous formaldehyde 37% (30 µl, 0.4 mmol, 5 equivalents) was then added and stirred at room temperature for 3 hours and the reaction was monitored by 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 to give lucidin in a yellow amorphous solid (21 mg, 87.5% yield).



^{1}H NMR (500 MHz, DMSO): δ 11.33 (s, 1H, OH), 8.22 (dd, J= 7.5, 1.5 Hz, 1H, H4), 8.15 (dd, J= 7.0, 1.5 Hz, 1H, H1), 7.77 (app td, J= 1.6, 7.2 Hz, 1H, H3), 7.74 (app td, J= 1.6, 7.2 Hz, 1H, H2), 7.26 (s, 1H, H5), 4.83 (broad s, 1H, OH), 4.55 (s, 2H, H6). ^{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 2. IR (ATR), ν (cm^{-1}): 3400, 1634, 1558, 1365, 1338. Melting point: 301-305 °C. λ_{max} (log ε) in MeOH: 410 nm (3.66).

2.3.3. Rubiadin

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 2-methyl resorcinol (0.99 g, 8 mmol) was added slowly. The temperature was then slowly increased to 165 °C and maintained for 4 hours. The reaction mixture was then cooled to 0 °C and 2M aqueous hydrochloric acid solution was added and stirred for 15 minutes. The reaction mixture was then heated to reflux for 30 minutes, after which it was cooled to room temperature and extracted with ethyl acetate (4 × 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 to give yellow plates (0.41 g). Yellow powder was then recrystallised in ethanol (0.39 g, 19% yield).



^{1}H NMR (501 MHz, MeOD) δ 8.19 (dd, J = 7.5, 1.3 Hz, 1H, H4), 8.10 (dd, J = 7.5, 1.3 Hz, 1H, H1), 7.75 (app td, J = 1.7, 7.3 Hz, 1H, H3), 7.72 (app td, J = 1.7, 7.3 Hz, 1H, H2) 7.17 (s, 1H, H5), 2.06 (s, 3H, H6). ^{13}C NMR (126 MHz, MeOD) δ 188.05, 183.63, 164.21, 164.18, 135.53, 135.59, 134.92, 134.75, 133.57, 132.44, 128.02, 127.74, 119.28, 108.43, 8.51. HRMS: m/z (ESI-) calculated for $C_{15}H_{10}O_4$ [M-H]$^-$: 253.0579; found [M-H]$^-$:253.0534. HPLC retention time and mass data of negative ion can be found in Table 2. IR (ATR), ν (cm^{-1}): 3387, 2917, 2356, 1698, 1577, 1292 cm^{-1}. Melting point: 286-291 °C (from EtOH). λ_{max} (log ε) in MeOH: 410 nm (4.46).

2.4. Dyeing procedure

Wool samples (5 g) were first scoured using HPLC grade water containing 1 g dm^{-3} Sandozin NIN (non-ionic detergent) in a Roaches Pyrotec S Rotodyer dyeing machine, using a liquor-fibre ratio of 200:1, at 50 °C for 2 hours. Samples were then washed in deionised water and air-dried. Scoured wool samples were then mordanted in an aqueous solution of 77% on mass of fibre (omf) potassium aluminium sulfate and 15% omf potassium hydrogen tartrate in a Roaches Pyrotec S Rotodyer dyeing machine, using a liquor-fibre ratio of 200:1, at 50 °C for 2 hours. Wool samples were then rinsed with deionised water and immersed directly in the dyebath. Dyebaths were previously prepared by heating dried and ground madder root (0.62 g, 31% omf) in water (100 ml) in a Roaches Pyrotec S Rotodyer dyeing machine, at 90 °C for 3 hours; the red solution was then filtered to remove any solids and pre-treated wool was immersed in the solution and temperature maintained at 90 °C for 3 hours with rotation. After dyeing, wool samples were then rinsed and left to dry in air and stored away from light.

2.5. Extraction methods

2.5.1. HCl extraction

This method was based on one used by Wouters [18], which has been used similarly by many other authors. A solution of 37% HCl: methanol: water (2:1:1, v/v/v) was prepared and 0.5 ml of this solvent was used to extract each wool sample (2 mg) at 90°C for 15 mins. The solution was then filtered and evaporated to dryness at reduced pressure using a Buchi rotary evaporator at 20 mbar below 40°C and then re-dissolved in methanol: water (1:1, v/v). Samples were then subjected to HPLC-DAD analysis. Each type of madder was extracted and the experiment was repeated twice for each type of madder dyed wool.

2.5.2. Citric acid extraction

This method was based on that of Valianou et al. [29]. An aqueous solution of 0.5 M citric acid was prepared 0.5 ml of this solvent was used to extract each wool sample (2 mg) at 90°C for 15 mins. The solution was then filtered and evaporated to dryness at reduced pressure using a Buchi rotary evaporator at 20 mbar below 40°C and re-dissolved in methanol: water (1:1, v/v). The samples were then subjected to HPLC-DAD analysis. Each type of madder was extracted and the experiment was repeated twice for each type of madder dyed wool.

2.5.3. Trifluoroacetic acid (TFA) extraction

This method was based on methods by Valianou et al. [29] and Mantzouris et al. [30]. An aqueous solution of 2 M TFA was prepared and 0.5 ml of this solvent was used to extract each wool sample (2 mg) at 90°C for 15 mins. The solution was then filtered and evaporated to dryness at reduced pressure using a Buchi rotary evaporator at 20 mbar below 40°C and re-dissolved in methanol: water (1:1, v/v). The samples were then subjected to HPLC-DAD analysis. Each type of madder was extracted and the experiment was repeated twice for each type of madder dyed wool.

2.5.4. Glucose extraction

An aqueous solution of 0.4% D-(+)-glucose in HPLC grade water was prepared and 0.5 ml of this solvent was used to extract each wool sample (2 mg) at 90 °C for 15 mins, which corresponded to a 1:1 mass ratio of glucose to wool sample.

The solution was then filtered and evaporated to dryness at reduced pressure using a Buchi rotary evaporator at 20 mbar below 40°C and re-dissolved in methanol: water (1:1, v/v). The samples were then subjected to HPLC-DAD analysis. Each type of madder was extracted and the experiment was repeated twice for each type of madder dyed wool.

2.6. High Performance Liquid Chromatography with Photodiode Array Detection (HPLC-DAD)

HPLC conditions were carried out at 30 °C with a Nova-Pak C18, 5 μm particle size, 15 x 3.9 cm I.D. column equipped with pre-column on a Dionex Ultimate 3000 series UHPLC binary pump system with online degasser and photodiode array detection (DAD). Conditions were solvent A: water (HPLC grade) in 0.1% formic acid; solvent B: acetonitrile in 0.1% TFA. Linear gradient programme applied of 0-6 minutes 27% B; 6-20 minutes linear increase to 60% B; 20-23 minutes hold at 60% B; 23-25 minutes linear increase to 70% B; 25-35 minutes hold at 70% B; 35-40 minutes linear decrease to 27% B. Method followed as described in the literature [5]. The flow rate was 1.0 ml min^{-1} and peaks were detected at 254 nm. Detection range was 190-400 nm, scan speed set to 0.002 mins and the data collection rate was 5 Hz. Quantification of dye components was conducted at 254 nm relative to standards, in accordance with literature methods [18,29,30].

2.7. Artificial ageing of dyed wool

To implement the photodegradation of dyed wool samples by light irradiation, bespoke equipment was constructed and a method applied based on the work of Colombini et al. [40]. The apparatus consisted of a wooden box of dimensions 1 m (width) x 1 m (length) x 0.44 m (height). Within the box both the irradiation element and the sample area was contained within a circular steel frame of 84 cm diameter and 38.5 cm height. Wool samples dyed with Turkish madder were mounted on to cardboard cut-outs and stapled in place then they were mounted at varying distances from the centre on a spherical section of mesh to enable uniform irradiation and energy delivery. Illumination was provided by a xenon arc bulb, wherein the bulb was an XBO 450 w/4 xenon short arc lamp, producing 450 watts with a luminous flux of 13000 lm. The light source element was located 24 cm from the base. Owing to a 310° range of irradiation, and the height of the box, the maximum area available for sample mounting was ca. 227 cm x 46 cm, hence the number of samples irradiated was dependent on the specific test procedure or requirement of processing. The delivery of energy to the dyed wool sample was controlled by the duration of exposure, in every instance the samples were exposed to 400 hours of light and the distance from the bulb was 30 cm. The box was fitted with a fan to expel ozone and facilitate air exchange. Testing was conducted in ambient conditions where the temperature and humidity were maintained at 25 °C and 65% RH, respectively.

3. Results and discussion

Comparative HPLC analysis of the original dyebaths of the three different Rubia tinctorum varieties was carried out and assigned by comparison to analytical standards (Table 2). From Figure 2 it can be seen that “Iranian madder” (Figure 2A) and “Turkish madder” (Figure 2B) have similar colorant profiles: the primary components are the glycosides lucidin primeveroside (**3**) and ruberythric acid (**1**), with significant concentrations of the aglycons alizarin (**2**) and purpurin (**11**). However, the profile of “English madder” (Figure 2C) is considerably different; the main components present are lucidin

(**4**) and alizarin (**2**); English madder only displays small amounts of the glycosides lucidin primeveroside (**3**) and ruberythric acid (**1**), which may have been caused through either enzymatic or chemical hydrolysis during drying/processing of the roots. All chromatograms also contain a small peak which has been assigned to nordamnacanthal (**5**) as it matches the mass observed by LC-MS.

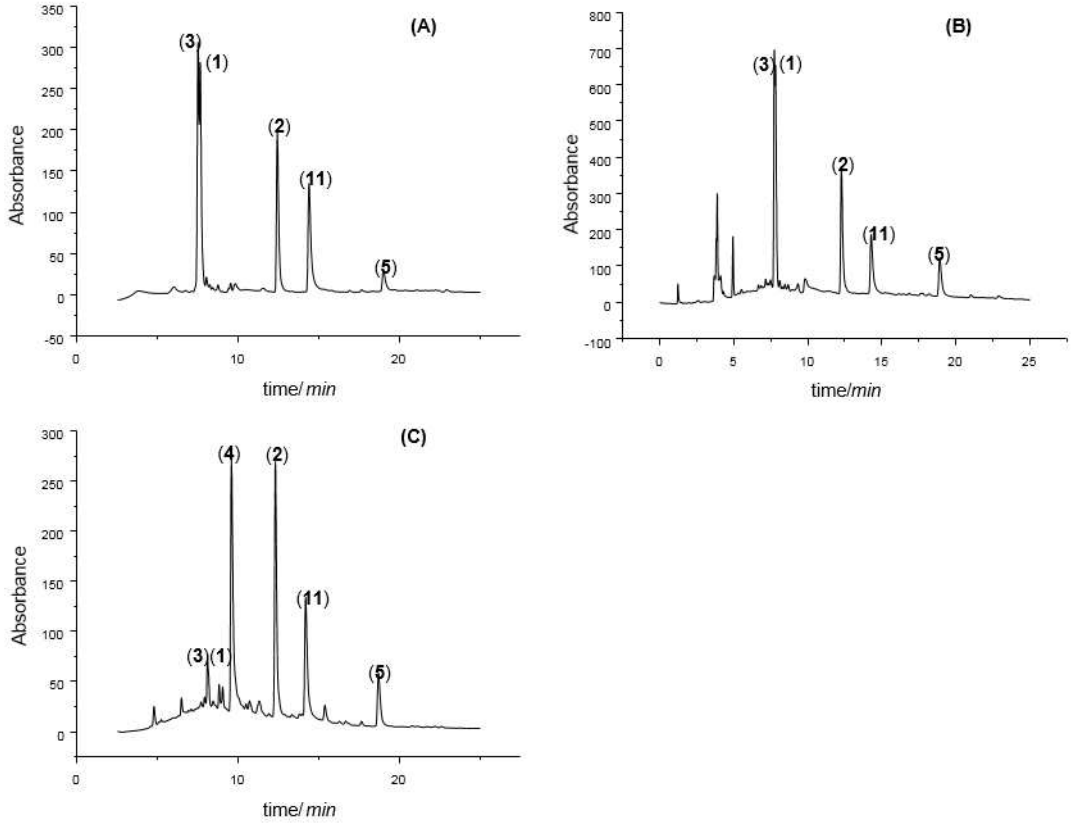


Figure 2. HPLC chromatograms of dyebaths prepared by extracting roots of different Rubia tinctorum varieties at 90 °C for 3 hours (no wool added): (A) Iranian madder; (B) Turkish madder; (C) English madder.

Ideally, an effective extraction method should reveal differences between madder types grown in different locations, i.e. Iran, Turkey and England; it is currently unclear whether the differences between these plants are due to different treatments upon drying/processing or if the origins of the differences in the ratios of the colorants are in planta. Nonetheless, it is interesting to determine if these subtle differences are observed in extraction of dyed wool samples. Textile back extractions were carried out on wool dyed with the three different Rubia tinctorum varieties and compared to the original dyebaths. It is worth noting that at these higher concentrations some of the peaks retention times are shifted slightly higher. This has been seen observed in dilutions creating the calibration curves of the dye, but each peak can be identified by the distinctive UV trace of each compound, as shown in Table 2.

Table 2. Compounds identified by HPLC-DAD and LC-MS analysis of madder roots.

Anthraquinone derivative assigned to HPLC peak	Retention time (min)	UV λ_{max} values for compound identification (nm)	Molecular ion, m/z, from LC-MS $[M-H]^-$
lucidin primeveroside (**3**)	7.5	246, 285	563
ruberythric acid (**1**)	7.5	224, 259	557
lucidin (**4**)	9.5	247, 288	269
alizarin (**2**)	11.9	249, 279	239
xanthopurpurin (**7**)	12.3	243, 280	239
purpurin (**11**)	13.5	255, 294	255
rubiadin (**13**)	15.2	248, 275	253
nordamnacanthal (**5**)	17.2	259, 297	267

3.1. HCl: methanol: water extraction

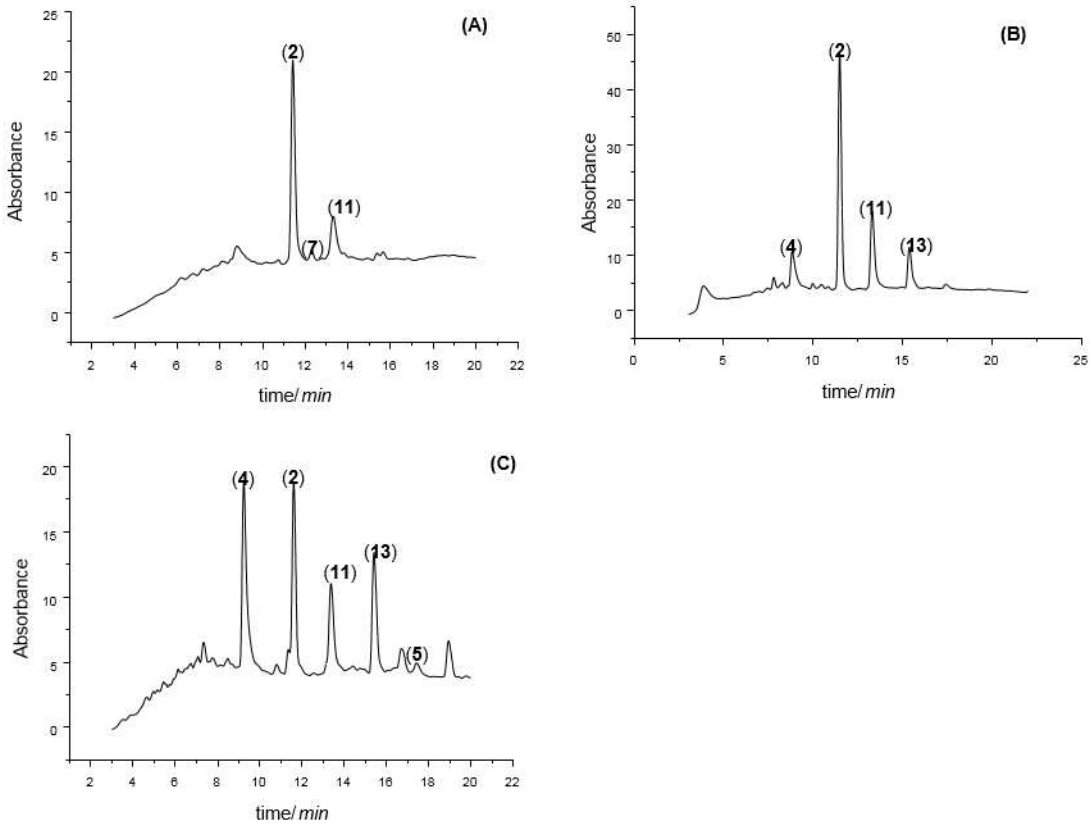


Figure 3. HPLC chromatograms of wool dyed with extracts of roots of different Rubia tinctorum varieties, extracted with 37% HCl: methanol: water (2:1:1, v/v/v): (A) Iranian madder; (B) Turkish madder; (C) English madder.

37% HCl: methanol: water (2:1:1, v/v/v) is used throughout the literature for extraction of natural colorants from textile substrates and artefacts. From the HPLC chromatograms (Figure 3), the main peaks present following HCl extraction of

wool samples dyed with the three types of madder are alizarin (**2**) and purpurin (**11**) for all varieties. In the extraction of Turkish madder (Figure 3B) and English madder (Figure 3C), rubiadin (**13**) is present after back extraction of the textile samples, but absent in the original dyebath; this could be a breakdown product of the extraction procedure as documented in literature [28,29], notably a possible hydrolysis product following breakdown of rubiadin primeveroside (**12**), which is present in very low quantities and may be masked by other anthraquinone glycoside peaks in the HPLC-DAD analysis of the original dyebaths.

No glycosides are present in the Iranian madder (Figure 3A) or Turkish madder (Figure 3B) extractions, which is not representative of the original dyebaths (Figures 2A and 2B) where the glycosides ruberythric acid (**1**) and lucidin primeveroside (**3**) are observed in significant concentrations; this highlights the need for a milder extraction technique as it is important to preserve the glycosides in order to gain the most detailed information on the compounds used in the original dyeing. Lucidin (**4**) is present in extraction of Turkish madder (Figure 3B) when it is not observed in the corresponding original dyebath (Figure 2B), which is most likely as a result of hydrolysis of lucidin primeveroside to lucidin during the acidic back extraction process, demonstrating that this HCl: methanol: water technique is limited in terms of its ability to extract a true representative sample of dye components from the textile sample.

3.2. Citric acid extraction

The theory behind the citric acid technique is that the weaker acid provides an extraction process with a less detrimental effect to acid-sensitive compounds present in the madder [29]; it is also possible that citric acid is able to chelate to the mordant metal (Al^{3+}), which may aid extraction by disrupting the dye-metal complex. Despite the milder acid, there is still evidence of detrimental effects, as observed in by HPLC (Figure 4): the glycosides lucidin primeveroside (**3**) and ruberythric acid (**1**) are observed in very low concentrations in Iranian madder, significantly lower than in the original dyebath, and not observed at all in Turkish or English madder; the main peaks observed in all three samples are the aglycons alizarin (**2**) and purpurin (**11**). The possible degradation product rubiadin (**13**) is present, but only in the case of English madder (Figure 4C), wherein a peak assigned to lucidin (**4**) is also present, but in lower concentrations compared to the original dyebath. Nordamnacanthal (**5**), which is found in all original dyebaths, is present in the case of both Iranian and Turkish madder and in similar peak ratios to the original dyebaths. Nordamnacanthal (**5**) is not present in English madder extracted with citric acid (Figure 4C) when it is present in the original dyebath. This could be the effect of many other aglycons competing for sites on the mordanted wool in the dyeing procedure and hence may not be as easily adsorbed onto the wool as its more polar derivative lucidin.

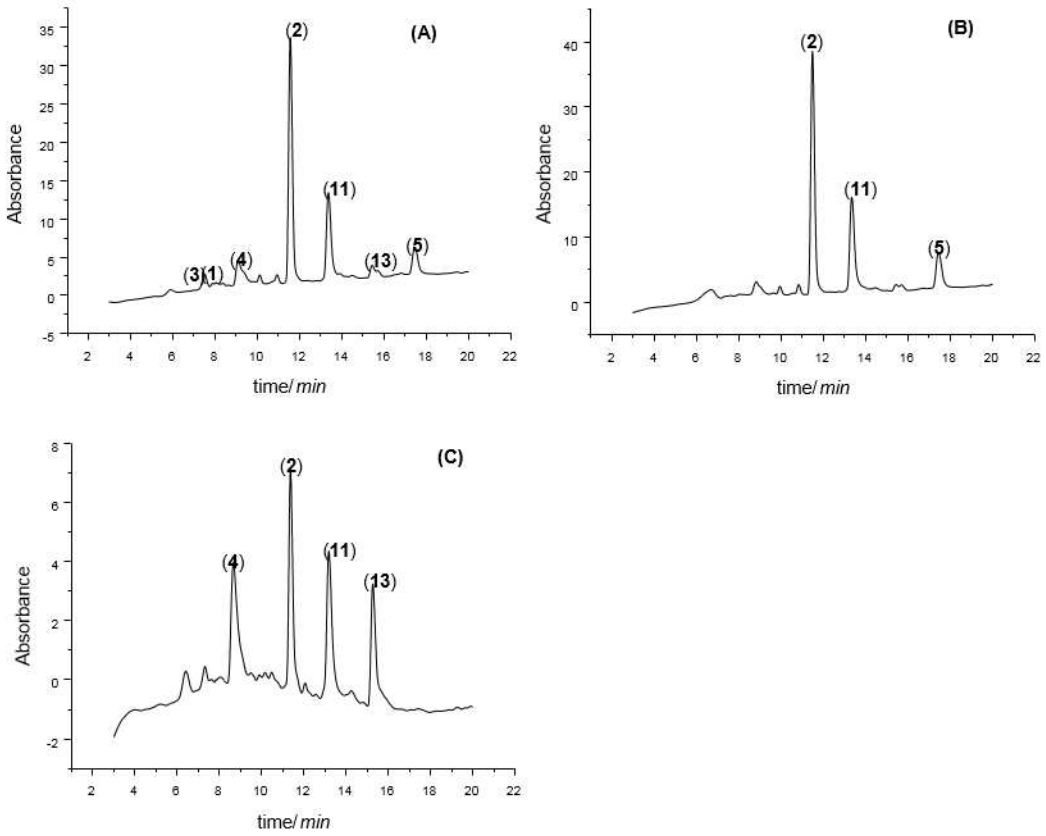
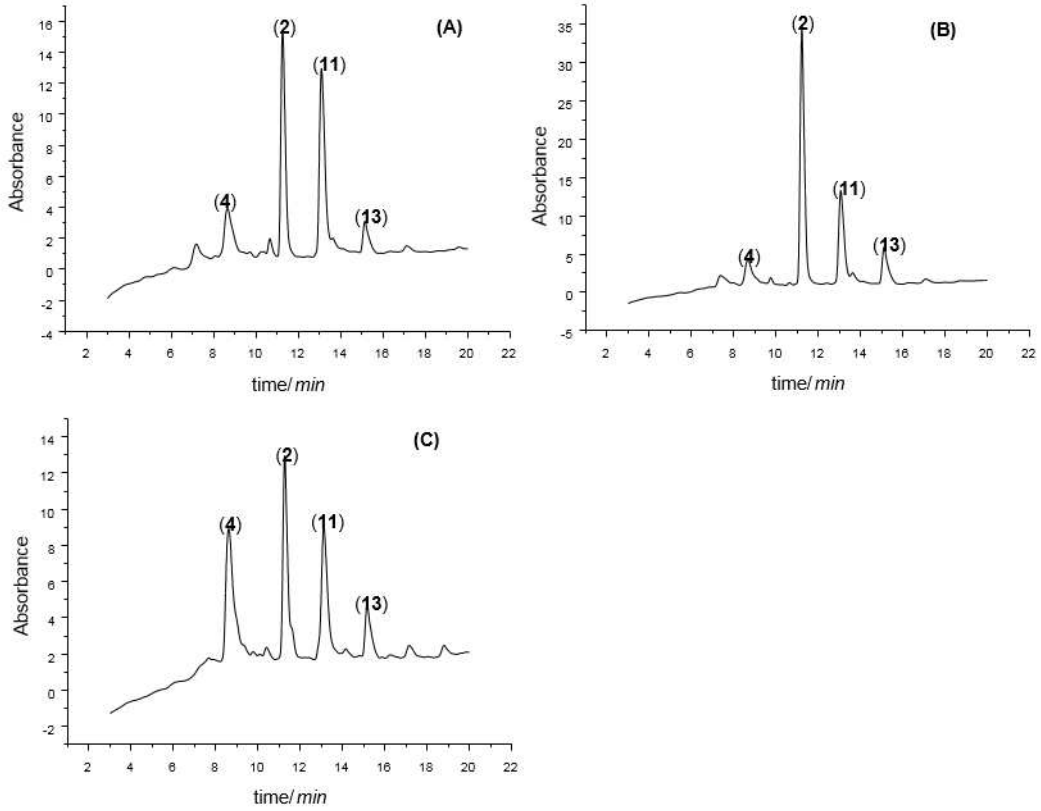


Figure 4. HPLC chromatograms of wool dyed with extracts of roots of different Rubia tinctorum varieties, extracted with 0.5 M citric acid: (A) Iranian madder; (B) Turkish madder; (C) English madder.

3.3. Trifluoroacetic acid (TFA) extraction

As can be observed in Figure 5, the peaks present are the aglycons alizarin (**2**) and purpurin (**11**) in all cases; no glycosides are present in the samples, which is probably due to them being very sensitive to acid hydrolysis under these conditions. A broad peak is present in the Iranian madder extraction (Figure 5A) that has a similar retention time as the glycosides, but the UV data did not correspond, hence this could not be assigned to lucidin primeveroside (**3**) or ruberythric acid (**1**). This extraction procedure is very efficient for the extraction of English madder (Figure 5C) as it the closest to resembling the original dyebath; the main peak in English madder samples is that of lucidin (**4**) as seen in the original dyebath. It is notable that the HPLC chromatograms of the Turkish madder samples (Figure 5B) also contain lucidin in the extraction analysis; however, lucidin is not observed as a product in the original dyebaths of Turkish madder (Figure 2B) and hence is probably present due to the hydrolysis of lucidin primeveroside. Rubiadin (**13**) is present after back extraction of all the samples using the TFA method; as discussed previously, this is likely the hydrolysis product rubiadin primeveroside (**12**), which is present in very low quantities and may be masked by other anthraquinone glycoside peaks in the HPLC-DAD analysis of the original dyebaths. It is also notable that nordamnacanthal (**5**) is not present in any of the samples extracted with TFA when it is present in the original dyebath, which may be as a result of the TFA extraction process not efficiently extracting nordamnacanthal or as a result of degradation of the compound during back extraction.



352

353 **Figure 5.** HPLC chromatograms of wool dyed with extracts of roots of different Rubia tinctorum varieties, extracted
354 with 2 M TFA: (A) Iranian madder; (B) Turkish madder; (C) English madder.

355

356 **3.4. Glucose extraction**

357 An aqueous glucose solution (0.4% w/v) was used to extract wool samples dyed with the three madder types. It was
358 observed from HPLC chromatograms (Figure 6) of the extraction of the wool samples that the glycosides lucidin
359 primeveroside (**3**) and ruberythric acid (**1**) are present in the extraction from Iranian madder (Figure 6A) and Turkish
360 madder (Figure 6B), which has not been observed in significant concentrations for any of the other extraction methods
361 evaluated in this research. Accordingly, it would seem that the glucose method is the only one that allows preservation of
362 the glycosidic components lucidin primeveroside (**3**) and ruberythric acid (**1**) adsorbed onto the fibre and also enables
363 their extraction. As can be seen from extraction of Turkish madder (Figure 6B) and English madder (Figure 6C), the
364 glucose method is also able to extract aglycons as both samples yield alizarin (**2**) and purpurin (**11**), however the aglycons
365 are only observed in trace quantities in the Iranian madder samples (Figure 6A); HPLC analysis of the dyebaths before
366 and after dyeing reveals that the most significant decrease in peak size was that of the two glycosides as a result of their
367 sorption onto the wool fibre, and only a small decrease in the peaks of alizarin and purpurin was also observed, so in the
368 case of the Iranian madder it may be that there was limited adsorption of the aglycons onto the fibre during dyeing, hence
369 why there was little removed during glucose extraction. In the glucose extraction method lucidin (**4**) is observed as a main
370 peak for the extracted English madder samples (Figure 6C), which corresponds to the HPLC analysis of the original
371 corresponding dyebath (Figure 2C), the glucose method does not extract any glycosides from the English madder as there
372 was only trace concentration of the glycosides present in the corresponding original dyebath (Figure 2C), most likely as

a result of hydrolysis during processing of the madder prior to receipt. Lucidin is also observed in low concentrations in the Turkish madder samples, in agreement with observations from the TFA extraction.

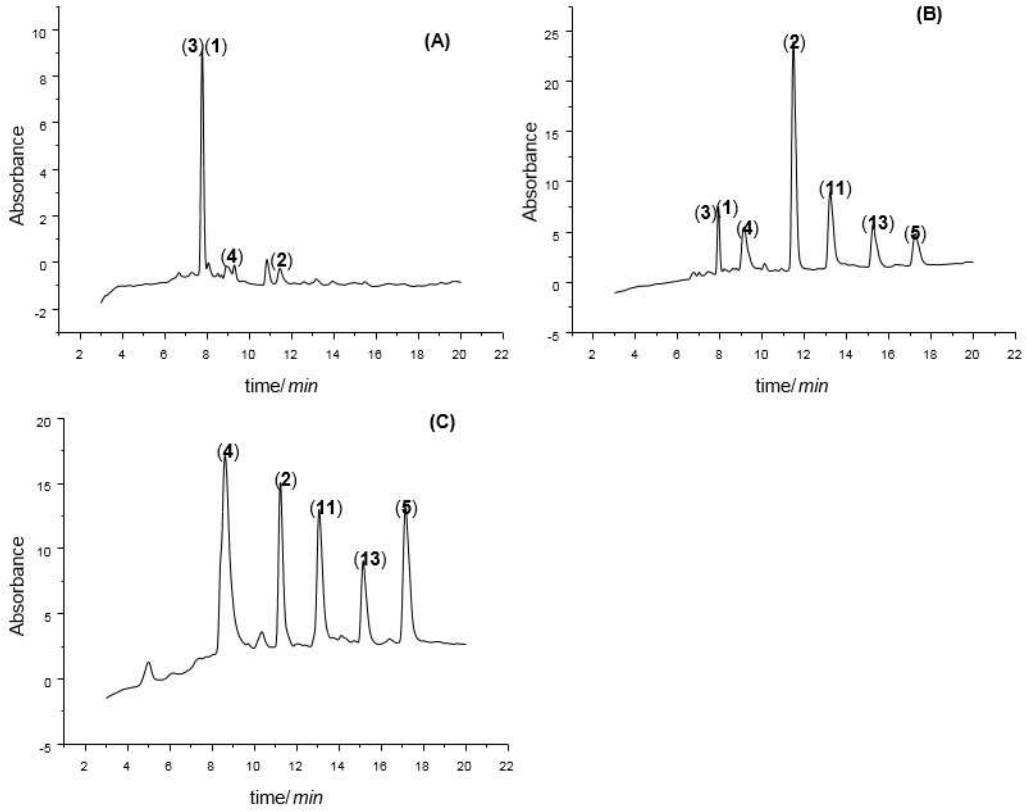


Figure 6. HPLC chromatograms of wool dyed with extracts of roots of different Rubia tinctorum varieties, extracted with 0.4% aqueous glucose solution: (A) Iranian madder; (B) Turkish madder; (C) English madder.

An aqueous solution of 0.4% glucose and using 0.5 ml of this solvent to extract a 2 mg wool sample corresponded to a 1:1 mass ratio of glucose to wool sample; it was found through varying the concentration of the glucose that this was sufficient to enable efficient extraction and increasing glucose concentration above a 1:1 ratio did not improve the extraction efficiency. When no glucose was present, the water alone was not able to extract any dye components from the sample in significant concentrations. Temperatures below 90 °C did not extract as high concentrations of both the glycoside and aglycon dye components, but glycosides were still detected. Longer extraction times were also applied, but 15 minutes was found to be sufficient for the samples tested and extending the time did not afford significant advantages. Other sugars (xylose, galactose, fructose, and sucrose) were also evaluated to see the efficiency of the extraction technique, and it was observed that although other sugars were able to extract glycosides efficiently, the relative concentration of alizarin extracted was not as high as for glucose extractions; it was thought that this may be related to the nature of the primary hydroxyl group in glucose and its ability to bind with and disrupt the Al-dye mordant complex. Full details of these optimisation and alternative sugar experiments are available [41].

It is suggested that the glucose solution provides a favourable extraction medium for several reasons. Glucose can competitively bind to sugar moieties in the anthraquinone glycosides as it is capable of multiple hydrogen bonding

interactions, thus displacing glycosylated dye into aqueous solution. It is unlikely that there is one dye interaction per mordant metal atom and the presence of multiple layers of dye (through H-bonding and π-π interactions between dye molecules) means that there is propensity for aggregates to be disrupted and the dye displaced from fibre. Crystal structures of the glycosidic components lucidin primeveroside (**3**) [10] and ruberythric acid (**1**) [11] display significant hydrogen bonding between sugar moieties, and the anthraquinone backbones do not hydrogen bond at all, which supports the argument that glucose can disrupt dye aggregates by forming extensive hydrogen bonding interactions with the sugar moieties in the dye molecules. It is also evident that the glucose extraction method is able to overcome the interactive forces between the mordant metal (Al^{3+}) and the dye as the glucose solution is still an effective extraction medium in the case of English madder where it would appear that there are no anthraquinone glycosides in the initial dye extracts. It is possible that glucose can also competitively bind to the mordant and disrupt dye-metal interactions. It should be noted that some pale residual colour remained on each sample after back extraction, but this was observed in all cases and not particular to any one method. This is most probably due to low concentrations of particularly strongly bound residual components, which could be potentially analysed through alternative techniques using fibre digestion.

A quantitative comparison of extraction methods (Figure 7) further exemplifies the efficiency of the glucose extraction method in its ability to remove glycosidic anthraquinone components without causing hydrolysis of the sugar moieties. A further important observation was that the concentration of alizarin in English madder in each extraction method, including glucose, was equal (within experimental error), confirming no anthraquinone glycosides were present in the starting dyebath as observed in Figure 2C. Furthermore, although alizarin is extracted from Turkish madder-dyed samples by the glucose method, it is at a much lower concentration than for other extraction methods, confirming that both alizarin and anthraquinone glycosides were present in the starting dyebath as observed in Figure 2B. This further demonstrates the advantages of the glucose method; dye components may be hydrolysed fully or partially before adsorption onto the fibre (e.g. during drying or processing of madder roots, or in the original dyebath), and this new method provides differentiation of this from hydrolysis in back extraction. This is a step-change in analysis of madder dyed textiles as it can provide further information about historical dye preparation and dyeing processes that current methods cannot.

The intention of the work described herein was to develop a technique that could be applied to museum objects and historical textile artefacts; however, further validation is desirable before using such valuable substrates. An alternative approach was to artificially age freshly dyed samples using UV photodegradation, based on the work of Colombini et al. [40]. Samples dyed with an alternative source of Turkish madder were used for this work as they had been demonstrated to have the greatest concentration of glycosides present in the fresh dyeings when analysed with the novel glucose method (Figure 6A). Dyeings were aged in a xenon light chamber for 400 hours and fading of the samples was observed, although significant colour remained. Aged dyeings and fresh dyeings were subsequently extracted using the HCl, TFA and glucose methods previously described herein and analysed as before. Considering the relative ratios of lucidin primeveroside, ruberythric acid and alizarin, it was observed that for the aged samples extracted with the HCl and TFA methods, only alizarin was observed and the glycosides not detected, which compared exactly to samples before ageing. For samples extracted using the glucose method, the ratio of lucidin primeveroside:ruberythric acid:alizarin extracted before ageing was 0.8:0.9:1.0, and the same ratio after ageing was 0.8:0.8:1.0, so only minimal change was observed. It is thought that the packing of the dyes on the fibre surface should not change considerably after ageing if these dyes are present in the original textile; moreover, the preservation of the glycosides after artificial ageing in the same ratio as alizarin, suggests that glycosides are able to form complexes with the mordant metal, which is known to improve light fastness [42], and demonstrates that alizarin glycosides are able to survive ageing. This work on aged

samples demonstrates that this novel glucose method can be applied to historical textiles, and work is ongoing in this area to exemplify this on actual artefacts.

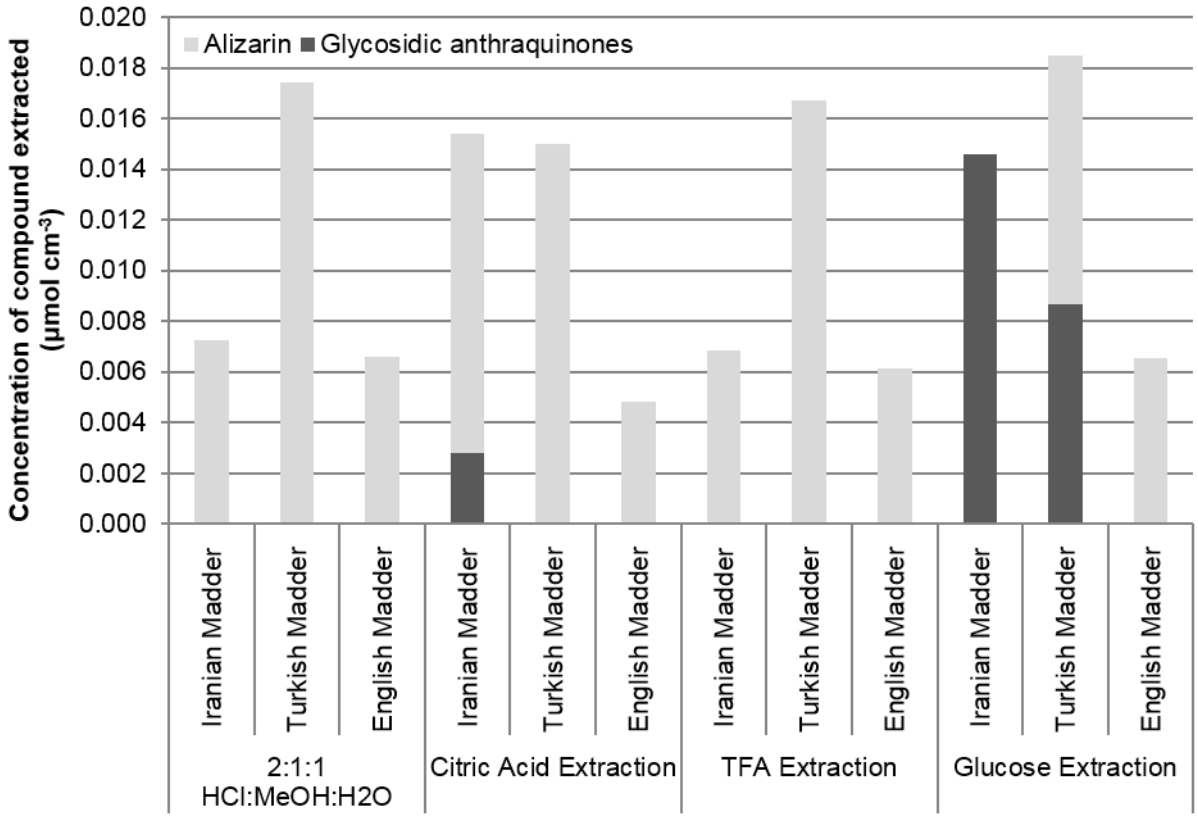


Figure 7. Concentration of anthraquinone glycosides (primarily ruberythric acid and lucidin primeveroside) extracted from wool samples in comparison with concentration of alizarin (main anthraquinone aglycon) extracted.

4. Conclusions

Extraction in aqueous glucose solution provides a highly effective method for extraction of madder dyed wool on a 2 mg scale, which would be applicable for museum textile artefacts. HPLC-DAD analysis allowed conclusive identification of each peak based on UV data and retention times obtained. Glucose solution is a favourable extraction medium due to its ability to form extensive hydrogen bonding with dye molecules present in madder, particularly glycosides, to solubilise the molecules for expeditious removal from fibre. It is extremely important that acid-sensitive molecules present in madder, such as glycosides (lucidin primeveroside, ruberythric acid, galiosin) and sensitive aglycons (lucidin), are preserved in the textile extraction process to enable better-informed identification of the original dyestuff and dyeing process. Acid-sensitive anthraquinonoid colorants are either not present at all or only in small amounts in the previously studied textile back extractions, particularly those including acid in the method, due to their sensitivity. This new glucose method offers an efficient process that preserves these sensitive molecules and is a step-change in analysis of madder dyed textiles as it can provide further information about historical dye preparation and dyeing processes that current methods cannot. The method also efficiently extracts glycosides in artificially aged samples, making it applicable for

museum textile artefacts. One suggested area for improvement of the glucose method is in the separation of the glycosides in LC through the use of alternative methods, which would be especially useful to be able to separately quantify the different glycosides present, especially those present in lower concentrations.

This technique is used to detect glycosides contained in the textile if they are present and does not have any detrimental effect on these compounds and hence give more information on the historical textile being examined. This detection method is a very gentle method which could be used as a first step to detect these acid sensitive compounds followed by a stronger acidic back extraction to remove any aglycons still left on the textile.

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