

# Identification of anthraquinone markers for distinguishing *Rubia* species in madder-dyed textiles by HPLC

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**Abstract** The anthraquinone components of the roots of various species of madder (*Rubia* spp.) have been used for millennia as red colorants in textiles, carpets and other objects. Although many species of *Rubia* are known, only a few of them have been used widely for dyeing. Furthermore, though nearly 70 anthraquinones have been found in *Rubia* species, only a few of these occur consistently at relatively high levels. Knowledge of the plant dyestuffs is helpful for establishing the location of production, production method and/or history of the dyed object. Using plant material and dyed textile fibers obtained from a number of sources, and HPLC with photodiode array and mass spectrometric detection for analysis, we have been able to identify marker anthraquinones that permit differentiation of the more common species of madder used for dyeing in Eurasia. Textiles dyed with all of the species examined contain varying amounts of purpurin, but only those dyed with *Rubia akane* contain large amounts of 6-hydroxyrubiadin (1,3,6-trihydroxy-2-methylanthraquinone) or its glycosides. Textiles dyed with *R. tinctorum* contain primarily alizarin, whereas those dyed with *R. cordifolia* and *R. peregrina* contain mostly purpurin, munjistin and pseudopurpurin, but little or no alizarin or 6-hydroxyrubiadin. The latter two species cannot reliably be distinguished from each other, however. The plants themselves often contain glycoside precursors not usually seen in the dyed materials.

**Keywords** HPLC · Mass spectrometry · Madder · *Rubia* species · Dye analysis

## Introduction

The roots of various species of *Rubia* (madder) and other members of the family, Rubiaceae, have been used for millennia as sources of red dyes for textiles and other materials, especially yarns for making carpets. All of these root-derived dye molecules are anthraquinones, whose general stability to light and to washing, in addition to the widespread availability of the plants themselves, have resulted in their having been used throughout the world [1]. Like most plants, the various species of *Rubia* tend to have been localized to different parts of the world—at least until modern times—so identification of species used is often helpful in identifying the source of production of a dyed object or providing information about its history and technology of dyeing.

Although there have been several reviews [1–4] describing the anthraquinone components of species of *Rubia*, little quantitative information is available, except for *R. tinctorum* [5, 6]. Thus it is difficult to know from these reviews whether a specific component represents a major amount or only a trace, in a particular species. A systematic attempt to distinguish between certain species of *Rubia* in dyed textiles was made about 25 years ago by Wouters [7], using HPLC with photodiode array detection. In those studies, dyed textile fibers were extracted using strong acid, so information about glycosides was unavailable.

In the present study, we have expanded the number of commonly used species analyzed to include *R. akane*, which was commonly used in the Far East, and have added on-line mass spectrometric detection, which greatly aids characterization of minor anthraquinone components and of others for which no standards are available. In addition we have used mild extraction methods that preserve glycosidic

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linkages, permitting detection of some previously unreported anthraquinone dye components.

## Experimental

### Plant materials

Plant specimens were obtained from colleagues, all of whom are plant taxonomists, botanists or experienced dyers, in France (*R. tinctorum* L. and *R. peregrina* L.); Japan (*R. akane* Nakai = *R. argyi* (H. Lév. et Vaniot) H. Hara ex Lauener; *R. cordifolia* L. var. *pratensis* Maxim.); Myanmar (*R. angustissima* Wall. ex G. Don), Nepal and Bhutan (*R. cordifolia* L., *R. sikkimensis* Kurz); and China (*R. yunnanensis* Diels, *R. schumannensis* Pridge and unidentified *Rubia* species from Sichuan and Tibet). Other specimens of *R. cordifolia* and *R. tinctorum* were purchased from Tanaka-nao (Tokyo) and Kremer Pigments (Germany). It is assumed that most of these specimens were dried at ambient temperature. However in some cases, the fresh, dye-containing roots were heat-treated. Sometimes textile samples dyed with these plants were also provided.

### Inactivation of endogenous enzymes

Endogenous enzymes that promote deglycosylation or other reactions in plant roots were inactivated by heating root specimens, either by steaming or placing them in near-boiling water for about 30 s, within a short time after being dug up and washed. Subsequently these specimens were allowed to dry completely at ambient temperature.

### Extraction of plant material

Samples of plant specimens were extracted by heating about 10 mg of the ground or chopped, dry plant with 1.0 mL of methanol/water (1:1) at 65 °C for 1 h. The supernatant liquid was removed by pipet and was centrifuged at 12,000 rpm for about 5 min, after which the clear supernatant was subjected to analysis as described in section “[Analysis of dye components](#)”. This solution was diluted with methanol/water (1:1) if necessary.

In some cases, the plant extracts were heated in 2 M HCl at 95 °C for 60 min to hydrolyze *O*-glycosides or to promote decarboxylation.

### Dyed textiles

Specimens of Nara period (8th C. Japan) silk textiles and Indian textiles, as well as threads from textiles dyed during the 20th century by R. Uemura, were provided by the Museum of Fine Arts, Boston. Other specimens were obtained from the

Victoria and Albert Museum, London, the Zanjan (Iran) Anthropology Museum and the National Museum of Mongolia. In addition, many specimens of wool textiles and carpets from China, Tibet and other parts of the Middle East and Central Asia were analyzed.

### Extraction of dyed textiles or fibers

Dyed textile or yarn specimens were extracted using a “soft” procedure, namely, by heating approximately 0.1–1 mg of fibers in 200 µL of a solution of pyridine/water/1.0 M oxalic acid in water (95:95:10) at 100 °C for 15 min, as described in detail in [8].

### Analysis of dye components

Extracts of plant material or of dyed silk or wool were analyzed by HPLC with photodiode array and mass spectrometric detection using an Agilent 1100 high performance liquid chromatography system consisting of an automatic injector, a gradient pump, a Hewlett-Packard series 1100 photodiode array detector, and an Agilent series 1100 VL on-line atmospheric pressure ionization electrospray ionization mass spectrometer, essentially as described earlier [9]. Operation of the system and data analysis were done using ChemStation software, and detection was generally done in the negative ion [M-H]<sup>-</sup> mode. Separation of dye components was made, in the majority of cases, on a Vydac C18 reversed phase column (2.1 µm dia. × 250 µm long; 5-µm particle size). Columns were eluted with acetonitrile-water gradients containing 0.1 % formic acid in both solvents. In the course of this work, which includes HPLC runs spanning several years, a number of gradients and columns were used, but because the types of stationary and mobile phases were unchanged, the elution times of peaks were always in the same order.

## Results and discussion

Much of our recent work has been aimed at trying to identify the specific biological sources of dyes used to color textiles and other materials in the past. The present study was initiated because analysis of some ancient textiles from Japan and Mongolia produced ambiguous results, in some cases detection of anthraquinone dyes that seemed never to have been described in textiles before. Subsequently we obtained species of madder from numerous sources for comparative studies. In some cases we were able to obtain plant material that had been heat-treated immediately after collection to inactivate endogenous glycosidases that decompose labile precursors.

It should be noted that many of the anthraquinones described in [2] are found in nearly all of the *Rubia*

species. However in our experience most of them occur in minor and often variable amounts, so the following discussion is primarily concerned with the major, distinguishing components.

### *Rubia akane*

The most compositionally distinctive of the *Rubia* species we examined was *R. akane* (now known as *R. argyi*), which was used for centuries as a red dyestuff in Japan. This species is unusual because of the presence primarily of derivatives of 6-hydroxyrubiadin (1,3,6-trihydroxy-2-methyl-anthraquinone; compound **5** in Table 1 and Scheme S1). Although these have been reported as occurring in many of species of *Rubia* (see [2]), they are dominant in *R. akane* and barely detectable in the other common species examined in this study. HPLC analysis of extracts of roots of *R. akane* that have been dried at ambient temperature shows the presence of large amounts of compounds with molecular

masses of 578, 620 and 662 Da, which correspond, respectively, to rhamnosylglucosyl-6-hydroxyrubiadin (**4**), and its monoacetates (**2** and **3**) and diacetate (**1**) (Fig. 1b). Because we did not characterize these compounds ourselves, we do not know which isomers are present, but structural analysis has been done by others [2]. Treatment of these extracts with HCl results in disappearance of the peaks of masses 578, 620 and 662 Da and the appearance of a large peak with  $M=270$  Da, which corresponds to the mass of 6-hydroxyrubiadin (**5**) (Fig. 1d), confirming that the higher mass peaks were glycosides, which are labile to acid (Scheme S2). On the other hand, treatment of the same extract with alkali (KOH) resulted in the disappearance of peaks at 620 and 662 Da, and an increase in the peak at 578 Da (Fig. 1f), which is consistent with the higher mass peaks being esters (acetates) (Scheme S2).

Analysis of *R. akane* roots that had been boiled or steamed shortly after the plants were dug up revealed the presence of two early eluting peaks with masses of 594 Da

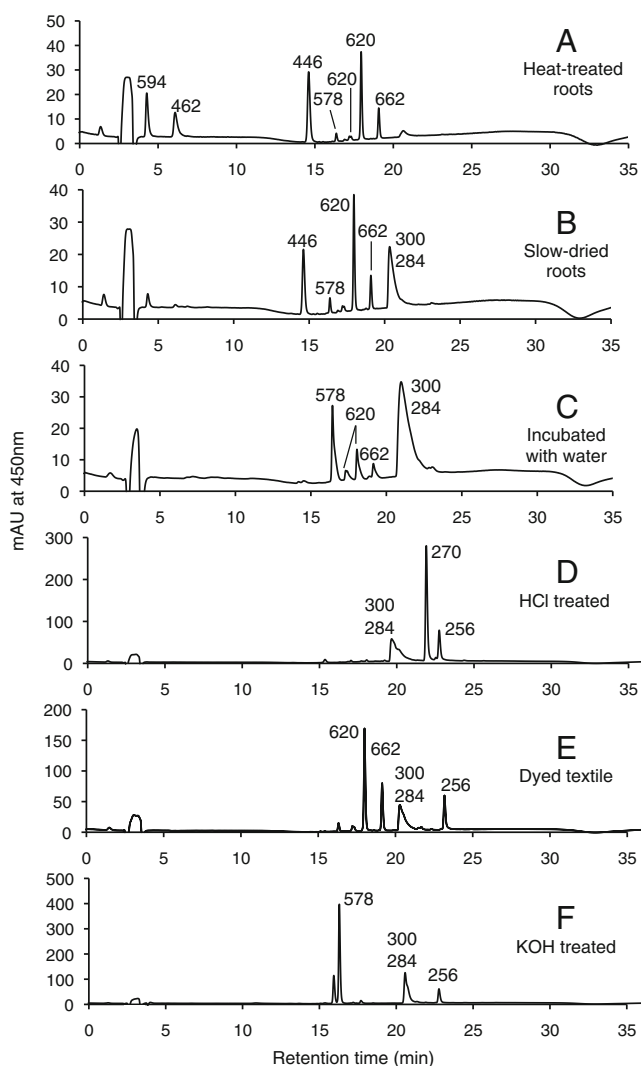
**Table 1** List of compounds found in extracts of various species of *Rubia* or *Rubia*-dyed textiles

Compound		Substituent <sup>a</sup>				Mass (Da)	Where found <sup>b</sup>
Number	Common name	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>6</sub>		
<b>1</b>		CH <sub>3</sub>	OGlc+Rha+2Ac	H	OH	662	Hi, Lo, T
<b>2</b>		CH <sub>3</sub>	OGlc+Rha+Ac	H	OH	620	Hi, Lo, T
<b>3<sup>c</sup></b>		CH <sub>3</sub>	OGlc+Rha+Ac	H	OH	620	Hi, Lo, T
<b>4</b>		CH <sub>3</sub>	OGlc+Rha	H	OH	578	Hi, Lo, T
<b>5</b>	6-Hydroxyrubiadin	CH <sub>3</sub>	OH	H	OH	270	T+HCl
<b>6</b>	Galiosin	COOH	OH	OGlc+Xyl	H	594	Hi
<b>7</b>	Pseudopurpurin+Glc	COOH	OH	OGlc	H	462	Hi
<b>8</b>	Munjistin+Glc	COOH	OGlc	H	H	446	Hi, Lo
<b>9</b>	Munjistin	COOH	OH	H	H	284	B, T
<b>10</b>	Pseudopurpurin	COOH	OH	OH	H	300	B, T
<b>11</b>	Xanthopurpurin	H	OH	H	H	240	B, T, T+HCl
<b>12</b>	Purpurin	H	OH	OH	H	256	B, T, T+HCl
<b>13</b>	Ruberythric acid	OGlc+Xyl	H	H	H	534	Lo
<b>14</b>	Alizarin	OH	H	H	H	240	B, T, T+HCl
<b>15</b>	Lucidin primeveroside	CH <sub>2</sub> OH	OGlc+Xyl	H	H	564	Lo
<b>16</b>	Lucidin	CH <sub>2</sub> OH	OH	H	H	270	P+HCl
<b>17</b>	Rubadin	CH <sub>3</sub>	OH	H	H	254	Lo, T
<b>18</b>	Nordamnacanthol	CHO	OH	H	H	268	Lo

<sup>a</sup> Refers to anthraquinone substituents in structure above: Glc, glucose; Rha, rhamnose; Xyl, xylose; Ac, Acetyl

<sup>b</sup> Abbreviations: Hi, in extract of fresh plant heated to High temperature and then dried; Lo, in extract of plant dried only at ambient temperature; T, in extract of dyed textile; T+HCl, in dyed-textile extract heated with HCl; B, in dye bath of lab-dyed textile; P+HCl, in plant extract heated with HCl

<sup>c</sup> Isomer of **2**; generally seen only in very low amount



**Fig. 1** HPLC profiles for *Rubia akane*. Elution was monitored at 450 nm. The numbers over the peaks are the molecular masses of the principal or otherwise significant components, which are identified in Table 1 (also Schemes S1–S5). See text for details

and 462 Da (Fig. 1a). Mass spectral fragmentation data show the presence of an ion with  $M=300$  Da, which is indicative of pseudopurpurin (**10**), so we suggest that these are, respectively, the xylosylglucosyl (primeverosyl) (**6**) and glucosyl (**7**) derivatives of pseudopurpurin, probably linked at the 4-hydroxyl group. Most likely, compound **6** is the same as the unstable galiosin (4-*O*-primeverosylpseudopurpurin) found by Hill and Richter in 1936 in fresh *R. tinctorum* [10]. Only traces of the 594-Da and 462-Da peaks are seen (Fig. 1b) in roots that have been dried at ambient temperature, suggesting that the roots contain endogenous enzymes that rapidly deglycosylate the pseudopurpurin glycosides [10]. Note also the increase in size of the peak for pseudopurpurin ( $M=300$ )+munjistin ( $M=284$ ), which do not separate in our HPLC system, in going from Fig. 1a to b.

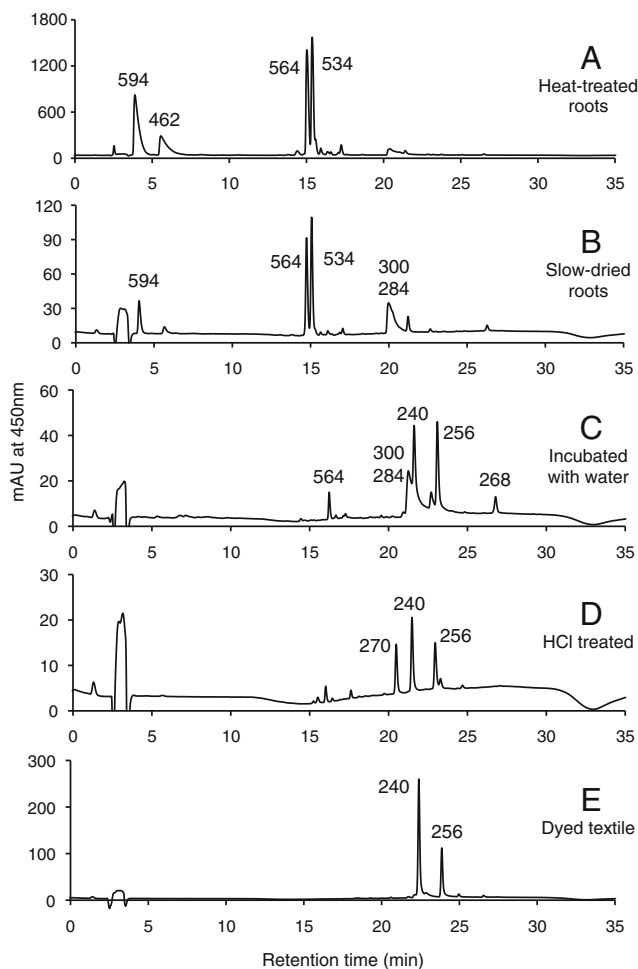
Also present in heat-treated roots (Fig. 1a) or in roots dried at ambient temperature (Fig. 1b) is a compound having a mass of 446 Da, which disappears (Fig. 1c) when the roots are incubated with water at a moderate (50 °C) temperature. We attribute this peak to the glucoside of munjistin (**9**), which presumably is converted by endogenous enzymes to munjistin, as seen with the increase of the pseudopurpurin +munjistin ( $M=300+284$ ) peak in Fig. 1c. In addition, warming of the roots with water also causes an increase in the 578-Da peak and decrease of the 620-Da and 662-Da peaks, which could be caused either by endogenous deacetylases or to simple aqueous hydrolysis of the acetates.

Finally, analysis of the extract of a contemporary dyed silk textile (Fig. 1e) shows the presence of the glycoside acetates (**1–3**) at 662 and 620 Da, pseudopurpurin +munjistin (**10 + 9**) and purpurin (**12**), the latter being identified by its mass (256 Da) and distinctive UV/Vis spectrum (cf. [5]). Purpurin presumably arises, in this instance at least, during the dyeing process, since we have observed (data not shown) that pseudopurpurin is fairly easily decarboxylated to purpurin. Derksen et al. have also shown, in *R. tinctorum*, that the pseudopurpurin decarboxylates much more rapidly than munjistin does [11].

#### *Rubia tinctorum*

*Rubia tinctorum* is the most common species of madder in the West and has been cultivated since ancient times as a source of a stable, red dye [3]. The analysis and identification of anthraquinone components of *R. tinctorum* have been described in several reports by Derksen and coworkers [cf. 6, 11, 12]. The only systematic report of the use of HPLC to distinguish between *Rubia tinctorum* and other species of *Rubia* was that of Wouters in 1985 [7].

Extracts of textiles dyed with *R. tinctorum* are characterized by the presence of a large alizarin (**14**) peak and a generally smaller (or sometimes practically non-existent) purpurin (**12**) peak, as seen in Fig. 2e. However, it is well known [5, 11] that the roots of *R. tinctorum* contain an alizarin precursor, ruberythric acid (alizarin primeveroside, **13**) as well as the primeveroside of lucidin (**15**), which appear here as peaks with respective masses of 534 Da and 564 Da, as seen in Fig. 2a and b (see also Scheme S3). These peaks generally are converted to alizarin (**14**) and lucidin (**16**), respectively, by endogenous enzymes before or during the dyeing process [11] (see Fig. 2c), though they occasionally appear in dyed textiles (Table S1), apparently depending on how the roots were processed. What has never been adequately explained is where purpurin comes from, except by decarboxylation of pseudopurpurin (see Fig. 2b). However, as can be seen in Fig. 2a, the heat-treated *R. tinctorum* roots appear to contain the same pseudopurpurin precursors, **6** (galiosin) and **7**, found in *R. akane*



**Fig. 2** HPLC profiles for *Rubia tinctorum*. Monitoring, labeling and identification of peaks as in Fig. 1. In profile C, the peak for pseudopurpurin+munjistin (300 Da+284 Da) has shifted to a later elution time. See text for discussion of this phenomenon

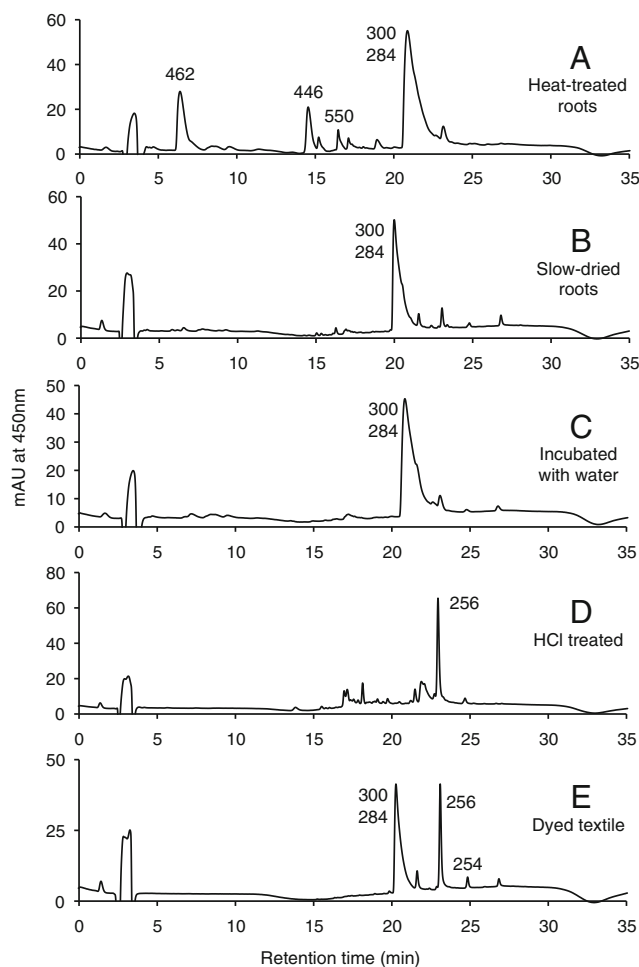
(Scheme S1). As in the case of *R. akane*, these compounds are greatly diminished (cf. Fig. 2b) or absent in roots that have been dried at ambient temperature, suggesting the presence of endogenous enzymes as proposed earlier [10].

Lucidin is unusual in that it never (in our experience) appears in dyed textiles and also that it is reputed to be mutagenic—or to be converted to mutagenic compounds. Normally lucidin is not observed except in plant material as its glycoside (15) since endogenous enzymes convert it to other substances (see [5, 11]). However if lucidin primeveroside-containing extracts (Fig. 2b) are treated with HCl, which hydrolyzes glycosides and inactivates enzymes, lucidin is seen as a peak showing a mass of 270 Da eluting ahead of alizarin (Fig. 2d). What happens to lucidin in the dye bath is uncertain. Derkesen et al. [11] have presented evidence that lucidin is oxidized to nordamnacanthol (18) in the presence of oxygen by endogenous enzymes (Scheme S4). On the other hand Ishii et al. [13] have demonstrated that lucidin can be converted enzymatically to a quinone

methide, a species known to be a powerful alkylating agent for nucleophiles, including DNA (Scheme S4).

### *Rubia cordifolia*

*Rubia cordifolia* is common in South, Southeast and East Asia. The primary colorants in roots dried at ambient temperature are pseudopurpurin (10) and munjistin (9) which elute as a single broad, tailing peak in our HPLC system (Fig. 3a). The reason for the tailing, and occasional changes in shape and elution time, is likely related to the use of dilute formic acid in the HPLC mobile phase. Formic acid is favorable for producing both positive and negative ions in the mass spectrometer, but is not acidic enough to completely suppress ionization of munjistin and pseudopurpurin carboxyl groups. Thus these partially ionized molecules can interact with polar groups on the stationary phase of HPLC column, depending on the degree of end-capping of silanol



**Fig. 3** HPLC profiles for *Rubia cordifolia*. Monitoring, labeling and identification of peaks as in Fig. 1. Note the appearance of a small peak for rubiadin ( $M=254$  Da), which elutes at 24–25 min, in both *R. cordifolia* and *R. peregrina*. The small peak at about 22 min (e.g., in Fig. 3e) has not been identified, but it is not alizarin, based on its elution time

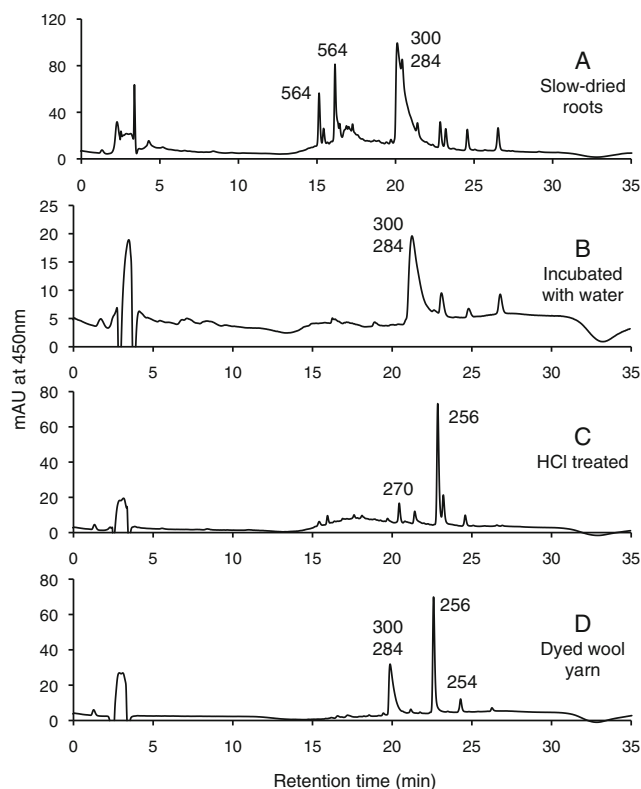


groups. Wouters [7] reported the same phenomenon. Nevertheless, pseudopurpurin and munjistin can readily be differentiated by ion extraction mass spectrometry.

As in the case of *R. akane* (Fig. 1a) and *R. tinctorum* (Fig. 2a), we detected apparent glycosides for pseudopurpurin and munjistin, in this case the glucosides of pseudopurpurin (7, 462 Da) and of munjistin (8, 446 Da), as seen in Fig. 3a. These peaks are not seen in roots dried at ambient temperature (Fig. 3b). Warming the dried roots with water produced no major change in the profile (compare Fig. 3c with b). However, analysis of silk dyed with *R. cordifolia* showed a large purpurin (12) peak with  $M=256$  Da (Fig. 3e). There are at least two possible explanations for the large purpurin peak. The first is that it arises from decarboxylation of pseudopurpurin during the dyeing process, and the second is that the textile fibers have a strong preference for the more hydrophobic purpurin molecule. We have noted [8] that small, hydrophobic (as seen by their late elution times on reversed phase HPLC columns) molecules seem to be selectively absorbed by textile fibers. In fact, we have noticed in the present study (data not shown) that the purpurin peak is significantly larger, relative to the pseudopurpurin/munjistin peak, for the first batch of wool yarn dyed in a dye bath than for the second batch of yarn dyed in the same bath, indicating that the less polar purpurin is selectively adsorbed during the first dyeing. Cardon [14] has also discussed how selective adsorption of dyes can result in different shades from one dye bath to another. Heating of plant extracts (e.g., those seen in Fig. 3b or c) with HCl results in decarboxylation of both munjistin and pseudopurpurin to form purpurin (12) and xanthopurpurin (11,  $M=240$  Da), which co-elute in our system, but are easily detectable by ion extraction mass spectrometry (Fig. 3d).

#### *Rubia peregrina* (wild madder)

Comparison of Figs. 3e and 4d shows that it is difficult, if not impossible, to tell whether a textile fiber was dyed with *R. peregrina* or with *R. cordifolia*. However, it is very easy to distinguish the plants, themselves. As seen in Fig. 4a, *R. peregrina* contains a peak at about 15 min that appears to be lucidin primeveroside (15,  $M=564$  Da), as well as an isomer of this compound, whose structure we have not been able to determine. Confirming the assignment of the 564-Da peak as 15 is the presence of a peak with a mass ( $M=270$  Da) and an elution time consistent with lucidin (16) in the HCl-treated extract (Fig. 4c), as also seen for *R. tinctorum* (Fig. 2d). Although we were unable to obtain heat-treated plant material for this study, Cuoco et al. [15] have reported finding a large amount of galiosin (6) in *R. peregrina*, as well as lucidin primeveroside (15). Note also that Fig. 4a shows a number of small, unlabeled peaks. These are



**Fig. 4** HPLC profiles for *Rubia peregrina*. Monitoring, labeling and identification of peaks as in Fig. 1. Note the appearance of a small peak for rubiadin ( $M=254$  Da), which elutes at 24–25 min, in both *R. cordifolia* and *R. peregrina*

anthraquinones, judging from their UV/Visible spectra, but vary in size from sample to sample, even within the same species, and therefore are unreliable as markers.

#### Minor constituents in *Rubia* species

As can be seen in the review of Singh et al. [2], most species of *Rubia* contain many of the same anthraquinones, though relative amounts are not given. In the course of the present study, we have also observed the presence of many minor peaks (cf. Fig. 4a), whose size and even presence vary from specimen to specimen. In some cases we can guess at their identity, but in others, we cannot. Some examples are  $M=550$  Da (purpurin primeveroside?) in *R. cordifolia* (Fig. 3a),  $M=548$  (rubiadin primeveroside?) in *R. peregrina*,  $M=254$  (rubiadin, which elutes after purpurin) in several species, and  $M=284$  Da (phycion?), which elutes after rubiadin). There are many possible causes for this variation, including environmental effects (soil, climate, age, time of harvesting, etc.), how the plant was processed after harvesting, and variation in the dyeing recipe, all of which are unknown in historical textiles. In any event these minor constituents cannot be used reliably for identifying particular species of *Rubia*.

### Other species of *Rubia*

Besides the four species discussed above, there are many other species of *Rubia*, several of which are listed in the review article by Singh et al. [2], but we have found no reports so far that any of them were used in large amounts for dyeing, except, perhaps, for *R. sikkimensis*, (Naga madder) [1]. We have obtained small specimens of *R. sikkimensis* (East Nepal), *R. yunnanensis* (Yunnan, China), *R. angustissima* (Myanmar), *R. schumanniana* and *R. chinensis* (Sichuan, China), some unidentified species from Sichuan and Tibet, and *R. cordifolia* var. *pratensis* (Japan) (see Table S1). Many of these contain significant amounts of 6-hydroxyrubiadin (**5**) glycosides, indicating that they are relatives of *R. akane*, though others seem to be more similar to *R. cordifolia*. In any event, in most of these cases, we have only a single, small specimen of each species—not enough to generalize from.

### Correlation of HPLC elution times with anthraquinone structural characteristics

The polarities of anthraquinone components listed in Table 1 show an interesting correlation not only with their HPLC elution times, as seen in Table 2, but also their appearance in plant material and dyed material. Generally only the group 4, and sometimes group 2 or 3, anthraquinones are seen in dyed textiles, in cases where the madder has been fully processed before being used for dyeing. The group 1 compounds are seen only in madder that has been heat-treated to inactivate glycosidases, indicating that fresh roots contain certain very active glycosidases. However, as has been shown by Derksen et al. [11], and the present work (see Fig. 2b), even roots that have been dried at ambient temperature contain glycosidase activity.

### Distinguishing between *Rubia* species based on HPLC analysis

In the analysis of textile specimens, one has the choice of extraction using one of the “soft” protocols, e.g. [8, 9], which preserve glycosidic linkages, or the traditional “hard” method [7], which involves heating with 2 M–3 M HCl, with resulting cleavage of glycosidic linkages and partial or complete decarboxylation of some compounds, especially pseudopurpurin. Depending on one’s objectives, either method can be used to

distinguish between the three most widely used types of madder, namely *R. akane*, *R. tinctorum* and *R. cordifolia* (summarized in Table S2). *R. akane* is rather unique in that the primary aglycone is 6-hydroxyrubiadin (**5**), which is the major product seen after heating with HCl, whereas the glycosides, particularly those with molecular masses of 578 Da and 620 Da are seen with using “soft” extraction. Essentially none of these compounds is seen in the other species of madder. *R. tinctorum* is also distinctive because only this species contains large amounts of alizarin, which predominates regardless of which extraction method is used.

*R. cordifolia* and *R. peregrina* can be distinguished from the aforementioned two species because they contain neither alizarin nor 6-hydroxyrubiadin in significant amounts, but they cannot be distinguished from each other. It has been suggested [7, 16] that the presence of rubiadin (**17**) in dyed textiles is indicative of *R. peregrina*, but we have found comparable amounts of rubiadin, which elutes about two minutes later than purpurin, in both *R. cordifolia* and *R. peregrina* (see Figs. 3e and 4d), and also in *R. tinctorum*. Furthermore Cuoco et al. [15] did not find significant amounts of rubiadin in their specimen of *R. peregrina*, but did find it in some of their *R. tinctorum* specimens. Singh et al. [2] report rubiadin being present in four species of *Rubia*. Finally, since rubiadin is one of the more hydrophobic anthraquinones, it could be expected to occur in abnormally high and variable amounts in dyed textiles depending on the dyeing protocol used.

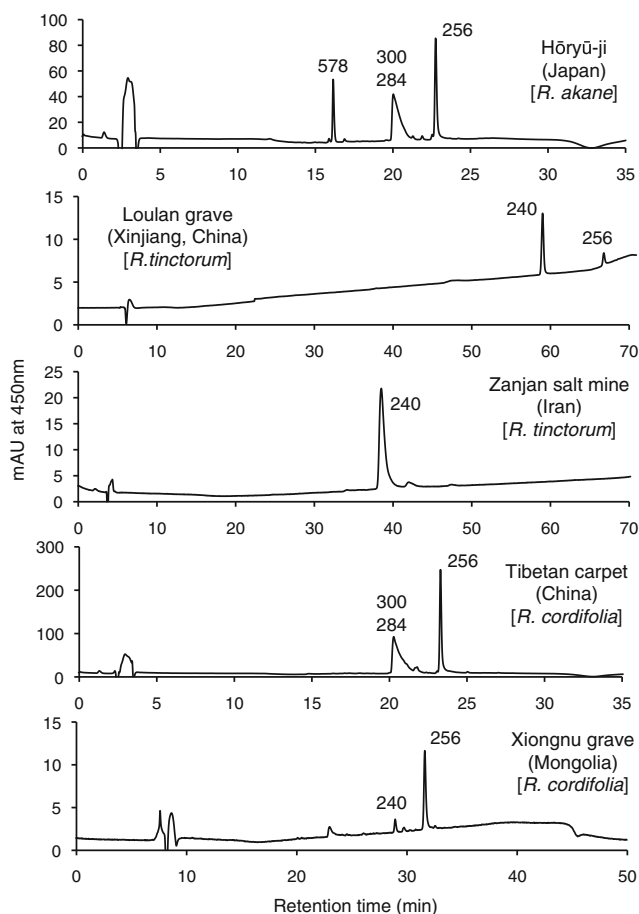
Several years ago the existence of “Tibetan madder” was suggested [17], based on the presence of purpurin and the absence of alizarin seen in HCl extracts of wool fibers from Tibetan carpets. According to our own results and those of Wouters [7], these criteria would also be met by *R. cordifolia* and *R. peregrina*, but *R. cordifolia* was ruled out because of a report [18], based on thin-layer chromatograms, that *R. cordifolia* contains alizarin. Our own results, and Wouters’ [7], contradict the presence of alizarin, so we propose that “Tibetan” madder is probably *R. cordifolia*. This suggestion is strengthened by historical records of imports of *R. cordifolia* from Bhutan into Tibet [19].

### Analysis of *Rubia* in ancient textiles

Figure 5 shows HPLC profiles of extracts of five textiles of historical or archaeological interest. All were extracted

**Table 2** Correlation between retention times and chemical structures of anthraquinone dye derivatives

	Retention time	Functionality (besides OH)	Compound
Group 1	<15 min	Carboxyl+glycosyl	<b>6, 7, 8</b>
Group 2	15–20 min	Glycosyl only	<b>1–4, 13, 15</b>
Group 3	20–22 min	Carboxyl only	<b>9, 10</b>
Group 4	>22 min	No glycosyl or carboxyl	<b>5, 11, 12, 14, 16–18</b>



**Fig. 5** HPLC profiles of extracts of textiles of historical interest. Note that different HPLC columns and gradients were used, making direct comparisons of elution times impossible

using mild protocols [8, 9] that leave glycosidic linkages intact. The Nara specimen is approximately 1300 years old and comes from a fragment of a textile from Hōryū-ji, the oldest temple extant in Japan. The presence of a large peak at  $M=578$  Da (in addition to large peaks for pseudopurpurin, munjistin and purpurin) indicates the presence of the glycoside **4** (Table 1, Scheme S1), which is a marker for *R. akane*. The absence of a large acetate peak at  $M=620$  Da, seen in textiles dyed more recently with *R. akane* (Fig. 1e), probably means that the acetyl derivative(s) of **4** hydrolyzed, in the presence of traces of water, over a period of 1300 years.

Two specimens shown in Fig. 5 appeared to have been dyed with *R. tinctorum*. One specimen came from a grave in Loulan, located at the eastern end of the Tarim Basin in Xinjiang, China, and is estimated to be about 3000 years old. The second came from fabrics found in a salt mine in Zanjan, Iran, and is estimated to be around 2000 years old. Both of these specimens contain only alizarin and purpurin, suggesting that they were dyed with *R. tinctorum*. We have analyzed many other ancient red-dyed specimens from the Tarim Basin region and all of them show profiles typical of

*R. tinctorum* (cf. Table S1). In this regard, analytical data for a large number of other textiles from the Tarim Basin have been published [20]. In most of those that were apparently dyed with madder, the most abundant anthraquinone is alizarin, indicating that they were dyed with *R. tinctorum*, rather than *R. cordifolia*.

Although *Rubia tinctorum* is known to have been used for centuries in Europe and the Middle East [1], the use of this plant seems to have extended east in Central Asia as far as eastern Xinjiang Province in China for 3000 years or more. In one respect, this is not particularly surprising since there is abundant evidence that the earliest settlers in that part of the world came from Central Asia [21]. However, it is not known whether this species of *Rubia* was introduced by the early settlers or whether it was endemic to the region.

The remaining two profiles in Fig. 5 were produced by extracts from a Tibetan “Wangden” carpet, which is probably no older than about 250 years, and from a silk textile from a Xiongnu tomb in Mongolia, dating back approximately 1800–2000 years. The dyestuff in both of these cases could be either *R. cordifolia* or *R. peregrina*, based on the prominence of purpurin and pseudopurpurin/munjistin, and the absence or very low abundance of alizarin. In both cases, we suspect they were dyed with extracts of *R. cordifolia*. In the case of the carpet, it is known that *R. cordifolia* was imported from Bhutan into Tibet [19]. The Xiongnu textile was probably produced in China. In the first place *R. cordifolia* was cultivated for dyeing in China at least 3000 years ago [22] and, in the second, there is a long history of silk being used for barter and tribute by the Chinese in dealings with the Xiongnu and other neighboring peoples [23]; if the silk was imported from China, it seems likely that it was dyed in China, too. Nevertheless, although *R. akane* and *R. tinctorum* can be ruled out for these specimens, one cannot absolutely discount *R. peregrina*.

#### Other genera of Rubiaceae

The present study deals only with the most common species of *Rubia* and a few others that we were able to obtain, but there are many other sources of anthraquinone dyes, in particular many species of *Relbunium*, widely used in ancient times in South America, *Oldenlandia umbellata* (= *Hedyotis umbellata*, chay root), a source of alizarin once widely cultivated in India, many species of *Galium*, found in temperate regions worldwide, and *Morinda* used in south Asia [1, 3, 24, 25]. In most, if not all, cases the dye is extracted from the roots of these plants. There could be some uncertainty in deciding between the genera (*Rubia*) that we studied and others. For example, *Relbunium* species are reported [26], like *Rubia cordifolia* and *Rubia peregrina*, to contain primarily purpurin. However, *Relbunium* species seem to be limited to South America [26], so one has to



consider the likely age and origin of the textile, as well as the dye analysis before coming to a conclusion about a particular object.

## Conclusions

The major finding from this work is that it is relatively easy to distinguish *R. akane*, *R. tinctorum* and *R. cordifolia*/*R. peregrina* from each other, as they contain 6-hydroxyrubiadin, alizarin and purpurin (only), respectively, as unique markers. We were not able to find reliable markers to distinguish *R. peregrina* from *R. cordifolia*, however. Rubiadin is not a reliable marker for *R. peregrina* as it occurs in varying amounts in textiles dyed with a number of species of madder.

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