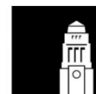
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Comparative sorption isotherms for colorants present in Dyers' madder (Rubia tinctorum L.) provide new insights into historical dyeing

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

[a]School of Design, University of Leeds, Leeds LS2 9JT, United Kingdom; [b]School of Chemistry, University of Leeds, Leeds LS2 9JT, United Kingdom; *Tel: +44 (0)113 343 3757; E-mail: r.s.blackburn@leeds.ac.uk

Abstract

Dyers madder (Rubia tinctorum L.) has been famously used throughout history as a source of red dye. The sorption onto mordanted wool of the major colorant components of R. tinctorum, alizarin and the glycosides ruberythric acid and lucidin primeveroside, is studied herein. Sorption of a purified 1:1 mixture of ruberythric acid:lucidin primeveroside most closely followed a Temkin isotherm (R^2 0.925), whereas alizarin followed a Freundlich isotherm (R^2 0.940). These results were compared to HPLC chromatograms of English, Turkish and Iranian varieties of R. tinctorum before and after dyeing, where it was observed that the glycosides are shown to have the highest uptake onto wool. The higher sorption energy of the purified 1:1 ruberythric acid:lucidin primeveroside mixture (–11.4 kJ mol^{-1}) compared to alizarin (–5.8 kJ mol^{-1}) is in agreement with the HPLC results, indicating that the ruberythric acid/lucidin primeveroside mixture has a substantially higher affinity for wool compared to alizarin. Not only do the glycosides show higher affinity for the wool, but greater interactions between adsorbed species suggest a more extensive aggregation of dye on the surface of the wool. These observations are in contrast to much literature and bring into question previous conclusions that alizarin was the main dyeing species throughout history.

Keywords

Madder; Rubia tinctorum; alizarin; HPLC analysis; adsorption.

1. Introduction

Colorants obtained from the roots of Dyers' madder (Rubia tinctorum L.) have been used for centuries as a red dyestuff; they are grouped collectively in the Colour Index as C. I. Natural Red 8. In 1826, Robiquet & Colin reported that R. tinctorum root contained two colorants, alizarin (**2**) and the more rapidly fading purpurin (**11**) [1,2]. The majority of 19th and 20th century literature similarly concluded that the major colorant present in extracts from R. tinctorum was alizarin. However, plant biosynthesis typically results in formation of both glycosides (compounds with sugar moieties attached) as well as aglycons (compounds without sugar moieties). Glycosides and aglycons typically have very different chemical and physical properties, for example, glycoside derivatives have substantially increased water solubility over their aglycon counterparts. The 2-O-linked disaccharide of alizarin, ruberythric acid, was actually first isolated from an unspecified species of madder by Rochleder in 1851 [3], but it is only relatively recently that significant evidence has confirmed the primary anthraquinone components in R. tinctorum roots in planta are the glycosides ruberythric acid (**1**) and lucidin primeveroside (**3**) [4-8]. Whilst the aglycon alizarin does occur in planta, it is in much lower concentrations than its glycoside; total glycosides present in R. tinctorum roots are in at least a five-fold concentration over total aglycons, with individual glycoside concentrations significantly higher than their aglycon counterparts [6,8]. Table 1 summarises the compounds that have been reported as being extracted from R. tinctorum roots; 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, and the levels of the anthraquinonoid compounds present may vary with age [9]. Other compounds may be formed as part of the analysis process, and it is know that anthraquinones that contain a 2-methoxymethyl- or a 2-ethoxymethyl group are formed during solvent extraction with hot methanol or ethanol, respectively [4,10].

Anthraquinone O-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 processing, especially under acidic conditions. Schunck demonstrated that ruberythric acid (**1**) could be hydrolysed to alizarin (**2**) in planta, catalysed by one of the first enzymes ever studied, which he isolated and called "erythrozyme" [11]; this is most likely via the glucoside, although isolation of the glucoside has not been reported. Hill & Richter [12] demonstrated that boiling madder roots in water destroyed (denatured) the hydrolytic enzymes and the glycosides could be readily extracted. They also found that the stability of different anthraquinone glycosides varies and 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.

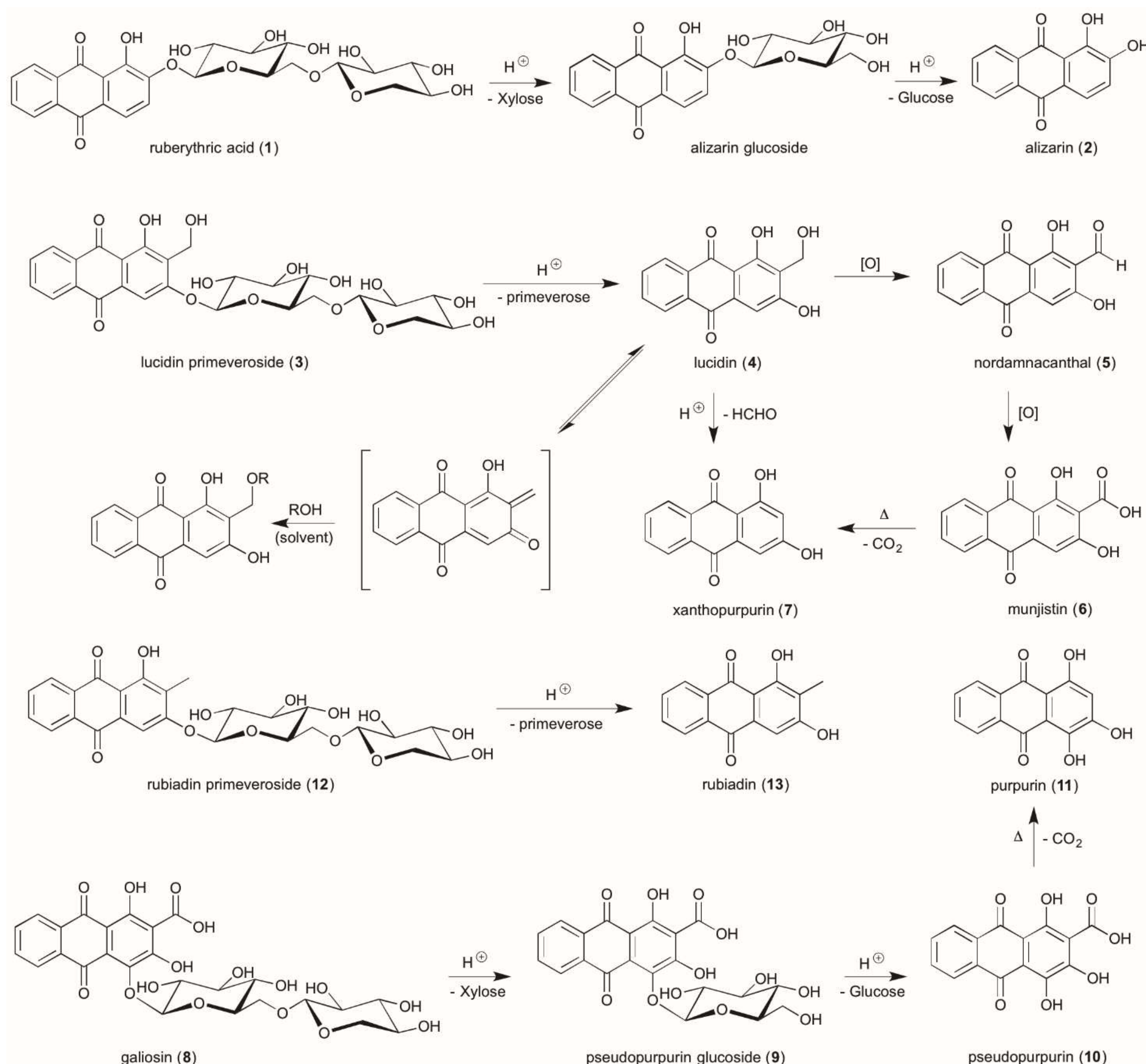


Figure 1. Possible inter-relationships between anthraquinone compounds found in R. tinctorum based on chemical or biochemical interconversion.

Mouri & Laursen [13] 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 seconds 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 [14] and typical European madder dyeing processes historically involved heating the dyebath to 75-80 °C [15], which would most probably also denature the endogenous enzymes, ensuring any glycosides present in the root were also present in the dyebath.

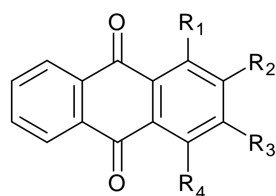
Table 1. Compounds detected in R. tinctorum L. in literature. ++ indicates major compound in planta, + significant amount, tr. very low or trace amount.

Compound	Mass (Da)	References
ruberythric acid (alizarin primeveroside) (**1**)	534	++ [5-8,12,13,16-18]
alizarin (**2**)	240	+ [4-8,13,16-19]
lucidin primeveroside (**3**)	564	++ [4-8,16,18]
lucidin (**4**)	270	tr. [5,6,16,18,20]
nordamnacanthal (**5**)	268	+/tr. [5,13,16,18,21]
munjistin (**6**)	284	+ [5,13,18,19]
xanthopurpurin (**7**)	240	+/tr. [5,13,16-18,22]
galiosin (pseudopurpurin primeveroside) (**8**)	594	+ [5,12]
pseudopurpurin glucoside (**9**)	462	+ [5,13]
pseudopurpurin (**10**)	300	+ [5,12,13,17-19,22]
purpurin (**11**)	256	+/tr. [5,8,12,13,16,18,19]
rubiadin primeveroside (**12**)	548	+ [6,12]
rubiadin (**13**)	254	+/tr. [13,16,18,19]
1-hydroxyanthraquinone (**14**)	224	+ [17]
1-hydroxy-2-methyl-AQ (**15**)	238	+ [18,22,23]
anthragallol (**16**)	256	+ [22]
2-hydroxyanthraquinone (**17**)	224	+ [22]
2-(hydroxy methyl)-anthraquinone (**18**)	238	+ [22]
rubianine (**19**)	402	+ [24,25]

O-primeveroside, O-(6-O-β-D-xylopyranosyl-D-glucopyranosyl).

Interestingly, despite high concentrations of lucidin primeveroside (**3**) in R. tinctorum roots [6,8,13], the aglycon lucidin (**4**) is rarely detected (and then only in low and trace concentrations in planta [5,6,13,20]) 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 [26,27]. Munjistin (**6**) is observed in R. tinctorum [5,13], but a glycoside (munjistin-3-O-glucoside) has only ever been detected in R. cordifolia and R. akane [13], suggesting that munjistin is formed in planta (especially in other species) through a different mechanism. It is possible that nordamnacanthal (**5**) can be further oxidised to form munjistin; in contrast, Hill & Richter [12] 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**) [28]; 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). Purpurin (**11**) is identified in R. tinctorum [5,8,12,13,16,18,19], however, it is suggested that it is formed through decarboxylation of pseudopurpurin (**10**) during drying of the roots [28]. It is also likely that the pseudopurpurin glycosides galiosin (**8**) and pseudopurpurin glucoside (**9**) occur in planta – their presence has been detected in low concentrations in R. tinctorum [5,12,13], and are most probably the origin of pseudopurpurin (**10**) as a result of hydrolysis (**8**$\rightarrow$**9**$\rightarrow$**10**) [4-6,13]. Rubiadin primeveroside (**12**) occurs in very low concentrations (<2%) [6,12] and is most likely the origin of trace amounts of rubiadin (**13**) detected, as a result of hydrolysis (**12**$\rightarrow$**13**). Several other simple aglycon anthraquinone derivatives (**14-18**) have been observed in low concentrations in R. tinctorum. Despite all other anthraquinone glycosides found in R. tinctorum being the O-glycoside, Schunck claimed to have isolated the C-glycosyl anthraquinone "rubianine" from the roots of R. tinctorum in 1893 [24], the structure of which was confirmed by ^{1}H and ^{13}C NMR spectroscopy almost 100 years later [25]; rubianine (**19**) is essentially the 2-C-glucoside of xanthopurpurin.



14: R_1 = OH; R_2 = R_3 = R_4 = H.
15: R_1 = OH; R_2 = CH_3; R_3 = R_4 = H.
16: R_1 = R_2 = R_3 = OH; R_4 = H.
17: R_1 = H; R_2 = OH; R_3 = R_4 = H.
18: R_1 = H; R_2 = CH_2OH; R_3 = R_4 = H.
19: R_1 = OH; R_2 = C-glucose; R_3 = OH; R_4 = H.

Although many compounds are observed in planta, there is few studies dedicated to understanding which dyes in R. tinctorum are actually adsorbed onto mordanted wool during dyeing processes and there has been no known study on the sorption of individual dye compounds in the extracts [29]. Due to the vastly differing functional groups on the dyes, it would be expected that the dyes would perform differently when applied to wool. Most literature that has focused on analysis of historical textiles has concluded that alizarin is the effective dyeing agent when using madder, but

other authors more recently argue [6,13,16,29] that this is a function of acidic extraction techniques that degrade dye glycosides, such those using solvent mixtures with concentrated hydrochloric acid at high temperatures. In the analysis of museum textile artefacts and in an effort to understand our dyeing history, it is important to gain fundamental knowledge about the sorption properties of the dye component in madder.

Herein, a study of the dyeing properties of the main individual dye compounds in R. tinctorum root is carried out on mordanted wool; the aim is to establish how the chemistry of these different compounds affects their interaction with the wool or the mordant site. Two different species were compared: (i) a purified 1:1 mixture of ruberythric acid:lucidin primeveroside; and (ii) alizarin.

2. Theory

Sorption isotherms are used to identify and quantify the affinity of a sorbent for a substrate. Herein, the affinity of different anthraquinones, which contain different functional groups, for mordanted wool is considered.

2.1. Langmuir isotherm

The Langmuir isotherm [30,31] describes sorption onto specific homogeneous sites within an adsorbent. Langmuir's model of adsorption depends on the assumption that intermolecular forces decrease rapidly with distance and consequently predicts the existence of monolayer coverage of the adsorbate (dye) at the outer surface of the adsorbent (mordanted wool). It is then assumed that once a sorbate molecule occupies a site, no further adsorption can take place at that site. Moreover, the Langmuir equation is based on the assumption of a structurally homogeneous adsorbent where all sorption sites are identical and energetically equivalent and there is no interaction between molecules adsorbed on neighbouring sites. Theoretically, the sorbent has a finite capacity for the sorbate. Therefore, a saturation value is reached beyond which no further sorption can take place. The saturated or monolayer (as $C_t \rightarrow \infty$) capacity can be represented by the expression represented in equation 1:

$$q_e = \frac{K_L C_e}{1 + a_L C_e} \quad (1)$$

where q_e is the equilibrium concentration of sorbate on the sorbent (solid-phase) (mg g^{-1}), C_e is the equilibrium sorbate concentration in solution (mg dm^{-3}), K_L (dm^3 g^{-1}) and a_L (dm^3 mg^{-1}) are Langmuir constants. The constants K_L and a_L are evaluated through linearization of equation 1 (equation 2).

$$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e \quad (2)$$

Therefore, a plot of C_e/q_e versus C_e should yield a straight line of intercept value $1/K_L$ and slope a_L/K_L if the isotherm obtained through experiment observes the Langmuir expression. The theoretical monolayer capacity is q_0 and is numerically equal to K_L/a_L. However, the linearity of equation 2 is only respected at low solution concentrations, where the model follows Henry's law: as C_e becomes lower, a_LC_e is much less than unity and $q_e = K_LC_e$.

2.2. Freundlich isotherm

The Freundlich isotherm [32] suggests that sorption energy exponentially decreases on completion of the sorptional centres of an adsorbent and describes heterogeneous systems, which are characterized by the heterogeneity factor $1/n_F$. When n = 1/n, the Freundlich equation reduces to Henry's law. Hence, the empirical equation (equation 3) can be written:

$$q_e = K_F {C_e}^{1/n_F} \quad (3)$$

where q_e is the equilibrium concentration of sorbate on the sorbent (solid-phase) (mg g^{-1}), C_e is the equilibrium sorbate concentration in solution (mg dm^{-3}), K_F is the Freundlich constant (dm^3 g^{-1}), and $1/n_F$ is the heterogeneity factor. The capacity constant K_F and the affinity constant n_F are empirical constants dependent on several environmental factors. A linear form of the Freundlich isotherm can be obtained by taking logarithms of equation 3 (equation 4).

$$\ln q_e = \ln K_F + \frac{1}{n_F} \ln C_e \quad (4)$$

Therefore, a plot of $\ln q_e$ versus $\ln C_e$ should yield a straight line of intercept value $\ln K_F$ and slope $1/n_F$ if the isotherm obtained experimentally observes the Freundlich expression; if $n > 1$, then the adsorption is favourable. The Freundlich isotherm is another form of the Langmuir approach for adsorption on an "amorphous" surface where the amount of adsorbed material is the summation of adsorption on all sites. The Freundlich isotherm is derived by assuming an exponential decay energy distribution function inserted into the Langmuir equation. It describes reversible adsorption and is not restricted to the formation of the monolayer.

2.3. Temkin isotherm

Unlike the Langmuir equation, the Temkin isotherm [33] takes into account the interactions between adsorbed species and adsorbates to be adsorbed and is based on the assumption that the free energy of sorption is a function of the surface coverage. When more sorbates are adsorbed, the chance for

the incoming sorbates in getting adsorbed is correspondingly reduced; that is, adsorption takes place on a non-uniform surface. The Temkin isotherm takes the following form (equation 5):

$$q_e = \frac{RT}{b_T}\ln(K_T C_e) \quad (5)$$

where K_T is the equilibrium binding constant corresponding to the maximum binding energy, b_T is the Temkin isotherm constant. Equation 5 can be linearized as equation 6:

$$q_e = B_T \ln K_T + B_T \ln C_e \quad (6)$$

where B_T = RT/b_T. The Temkin isotherm contains a factor that explicitly takes into the account adsorbing species-adsorbent interactions. This isotherm assumes that (i) the heat of adsorption of all the molecules in the layer decreases linearly with coverage due to adsorbent-adsorbate interactions, and that (ii) the adsorption is characterized by a uniform distribution of binding energies, up to some maximum binding energy. A plot of q_e versus lnC_e enables the determination of the isotherm constants B_T and K_T from the slope and the intercept, respectively. B_T is the Temkin isotherm constant, K_T is the equilibrium binding constant (dm^3 g^{-1}) corresponding to the maximum binding energy, and b_T is the Temkin constant related to the heat of adsorption (J mg^{-1}).

2.4. Adsorption energy

Thermodynamic data such as adsorption energy (ΔG) can be obtained from K_L, K_F and K_T (equation 7), where K is constant in terms of dm^3 mol^{-1}, T is temperature, and R is the gas constant.

$$-\Delta G = RT \ln K \quad (7)$$

3. Materials and methods

3.1. Materials

Alizarin was obtained from Sigma-Aldrich and used as received for both dyeing and as an analytical standard as it was determined by HPLC to be 99%+ pure. A sample of "ruberythric acid" was purchased from Apin Chemicals (Abingdon, UK), but HPLC analysis showed it to be a mixture of ruberythric acid and lucidin primeveroside. Natural cream wool, heavy weight with plain even weave was purchased from Whaley's (Bradford, UK). Three R. tinctorum root types were purchased from George Weil & Sons (Guildford, UK): Iranian madder, grown in Shiraz, Iran was received as ground and dried material; Turkish madder, sourced from a wholesaler was received as dried roots; and English madder, grown by Dr. David Hill at Bristol University, was received as dried roots. All

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

3.2. Purification of "ruberythric acid" by solid-phase extraction (SPE)

NMR analysis of the ruberythric acid/lucidin primeveroside mixture showed the presence of free sugars in the sample, which were removed by the following SPE procedure. Amberlite XAD-7HD resin was soaked in deionised water for 60 minutes at room temperature to remove any salts and activate the resin; the quantity of resin used was a 1:1 ratio by mass of the original material. The Amberlite resin was then packed into a column and washed again with water. An aqueous solution of the ruberythric acid/lucidin primeveroside mixture (0.2 g in 10 ml) was added to the column, and then subsequently washed with water (5 x 100 ml) to remove free sugars. The column was subsequently eluted with ethanol (2 x 100 ml), the ethanol extracts collected, and then evaporated under reduced pressure on a rotary evaporator and the yield of each sample recorded. The resulting solid was re-dissolved in methanol:water (1:1, v/v) and analysed by LC-MS and HPLC.

The purified ruberythric acid/lucidin primeveroside mixture (0.1 g) was ground up in a pestle and mortar and placed under a high vacuum for 2 hours to remove any residual moisture. A ^{1}H NMR spectrum of this was then recorded in DMSO-d_6. ^{1}H NMR, δH (500 MHz, $(CD_3)_2SO + D_2O$); 8.30 − 8.14 (m, 2H), 8.00 – 7.92 (m, 2H), 7.74 (d, 1H, J = 8.5 Hz), 7.63 (d, 1H, J = 8.5 Hz), 7.48 (s, 1H), 3.0 – 5.5 (m, vb). Analysis of the ^{1}H NMR spectrum revealed a 1:1 molar ratio mixture of ruberythric acid lucidin primeveroside.

This purified 1:1 ruberythric acid:lucidin primeveroside mixture was used as the effective dyeing agent in isotherm studies and as the analytical standard for the "glycosides". It is acknowledged that use of this mixture of glycosides is not ideal, but it was not possible to obtain sufficient quantities of pure ruberythric acid.

3.3. Dyeing process

Wool samples (5 g) were initially 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 subsequently mordanted in an aqueous solution of 15% on mass of fibre (omf) potassium aluminium sulfate and 6% 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 into the dyebath. Dyebaths were prepared by heating dried and ground madder root (0.62 g, 12% omf) in water (100 ml) at 90 °C for 3 hours; the resulting 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.

3.4. Sorption isotherms

Calibration curves for alizarin and the purified 1:1 ruberythric acid:lucidin primeveroside mixture were calculated. A range of known concentrations were prepared in 1:1 (v/v) methanol:water and their absorbance at λ_{max} measured using UV/Vis spectroscopy. Linear regression of plots of absorbance vs. concentration was performed and the gradient of the straight line calculated; in each case linear regression had correlation of $R^2 > 0.95$.

For isotherm experiments, a stock aqueous solution was made for each of alizarin and the purified 1:1 ruberythric acid:lucidin primeveroside mixture. These stock solutions were then diluted using deionised water to give dyebaths of five different known concentrations. Mordanted wool (0.5 g) was immersed in the prepared dyebaths using a liquor-fibre ratio of 500:1; this was a particularly long liquor ratio by modern standards, but typical of the liquor ratio that would have been observed in historical dyebaths in relation to the actual individual dye components. Dyebaths were heated in a Roaches Pyrotec S Rotodyer dyeing machine at 90 °C for 2 hours, which was determined with much experience to be sufficient for each dye to achieve equilibrium (this was established through repetitions of the experiment up to 8 hours, and for efficiency in subsequent work 2 hours was employed).

An aliquot of the dyebaths was taken before and after dyeing and diluted with an equal volume of methanol to give a 1:1 (v/v) methanol:water solution. Absorbance of these dyebath aliquots was measured at λ_{max} using UV/Vis spectroscopy, and dye concentrations subsequently calculated from calibration plots. From this data, dye adsorbed by the fibre (q_e) and dye remaining in solution (C_e) at equilibrium was calculated. Using this data, an isotherm plot of q_e vs. C_e was constructed for each of alizarin and the purified 1:1 ruberythric acid:lucidin primeveroside mixture.

3.5. UV/visible spectrophotometry

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. Aliquots were taken from the dyebaths for analysis by UV/vis; samples were diluted by a factor of 2 for the visible analysis to allow for a maximum absorbance between 0 and 1. Alizarin had λ_{max} at 430 nm and the purified 1:1 ruberythric acid:lucidin primeveroside mixture had λ_{max} at 520 nm.

3.6. High performance liquid chromatography with photodiode array detection (HPLC-DAD)

Aliquots were taken from the dyebaths for analysis by HPLC-DAD. Analysis experiments 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). Mobile phase employed was 0.1% w/v aqueous formic acid (A) and a 0.1% w/v solution of TFA in acetonitrile (B). Programme applied: isocratic hold period of 0–6 min 27% B (73% A); 6–20 min linear increase to 60% B; 20–23 min hold at 60% B; 23–25 min linear increase to 70% B; 25–35 min hold at 70% B; 35–40 min linear decrease to 27% B. 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 min 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.

4. Results and discussion

4.1. HPLC dyeing studies of Iranian, Turkish and English madder

To fully elucidate the compounds being adsorbed onto the wool, the dyebaths were analysed by HPLC-DAD before and after dyeing to ascertain the level of adsorption of the dyestuff onto wool. Changes in the resultant peak area on the HPLC chromatogram of each dye compound were calculated; a decrease in peak area indicated that the compound adsorbed onto the fibre. The glycosides lucidin primeveroside and ruberythric acid in the mixture were not fully resolved, as the commercial standard labelled “ruberythric acid” was found on analysis to be a mixture of these two compounds, hence, changes in the peak area of these two compounds were combined to give one value for the “glycosides”. A summary of the compounds found in the roots of the three R. tinctorum varieties and their uptake onto wool can be found in Table 2.

HPLC analysis of the compounds present in a dyebath of Iranian madder before and after dyeing (Figure 2a) reveals that the concentration of all dye compounds in the dyebath decreased upon the addition of mordanted wool; some are not present at all in the dyebath after dyeing. This suggests that all dye compounds present in the dyebath are adsorbed onto the wool to some degree under these conditions. HPLC chromatograms of Turkish madder before and after dyeing (Figure 2b) are very similar to those of Iranian madder; each peak area decreased after dyeing, which shows that every major component of the dye is adsorbed onto the fibre to some degree. For both Iranian and Turkish madder, nordamnacanthal was completely adsorbed onto the fibre and is not detected at all after dyeing, although it was only present as a small component of the whole dye compound mixture for each variety (relative peak area 4.1% and 8.4%, respectively). HPLC chromatograms of English madder before and after dyeing (Figure 2c) show a very different composition to that of Turkish and Iranian madder. English madder has much lower concentrations of glycosides and consists mainly of the aglycons lucidin, alizarin and purpurin, most probably as a result of drying processes that have caused in situ hydrolysis of the glycosides.

Table 2. Comparison of compounds present in dyebaths different R. tinctorum varieties: % of total peak area (TPA) before dyeing represents contribution to 100% of area of all peaks observed in HPLC; % change of peak area after dyeing represents reduction in individual peak area on dyeing.

Anthraquinone derivative assigned (HPLC peak number)	UV λ_{max} values (nm)	Iranian		Turkish		English	
		% of TPA before	% change of TPA after	% of TPA before	% change of TPA after	% of TPA before	% change of TPA after
Ruberythric acid (1)	224, 256	52.5	18.2	37.9	16.4	5.8	7.1
Lucidin primeveroside (3)	200, 246, 285						
Alizarin (2)	198, 249, 279	23.0	57.7	20.4	69.0	28.6	76.9
Lucidin (4)	200, 247, 288	n.d	n.d	n.d	n.d	35.8	100.0
Nordamnacanthal (5)	214, 259, 297	4.1	100.0	8.4	100.0	8.6	100.0
Purpurin (11)	210, 255, 294	20.4	81.0	12.2	85.0	16.9	100.0
Rubiadin (13)	225, 248, 281	n.d	n.d	n.d	n.d	2.0	11.0

HPLC chromatogram peak areas are not directly proportional to the concentration of the compounds in the dyebath, as each compound has a different response factor when detected by the DAD; the response factor can be calculated from a calibration plot of the peak area against a known concentration. Unfortunately only the response factors of the glycosides and alizarin could be obtained due to the fact that the majority of compounds in R. tinctorum are not commercially available; calibrations were plotted for the glycoside mixture and alizarin, which is representative of each R. tinctorum variety as these are the most significant peaks in the chromatograms and both of these compounds are commercially available. Response factors for the purified 1:1 ruberythric acid:lucidin primeveroside mixture (glycosides) and alizarin are summarised in Table 3. It is acknowledged that the two glycosides will have individual response factors, but herein it has been assumed that they are the exactly the same for the purposes of calculations. These assumptions had to be made for these experiments due to it not being possible to obtain pure ruberythric acid in sufficient quantities. Likely inequalities in response factors of ruberythric acid and lucidin primeveroside means that variation of the ratio of each would influence calculated figures, however, the same purified same was used throughout for all experiments and as the analytical standard, minimising associated variances.

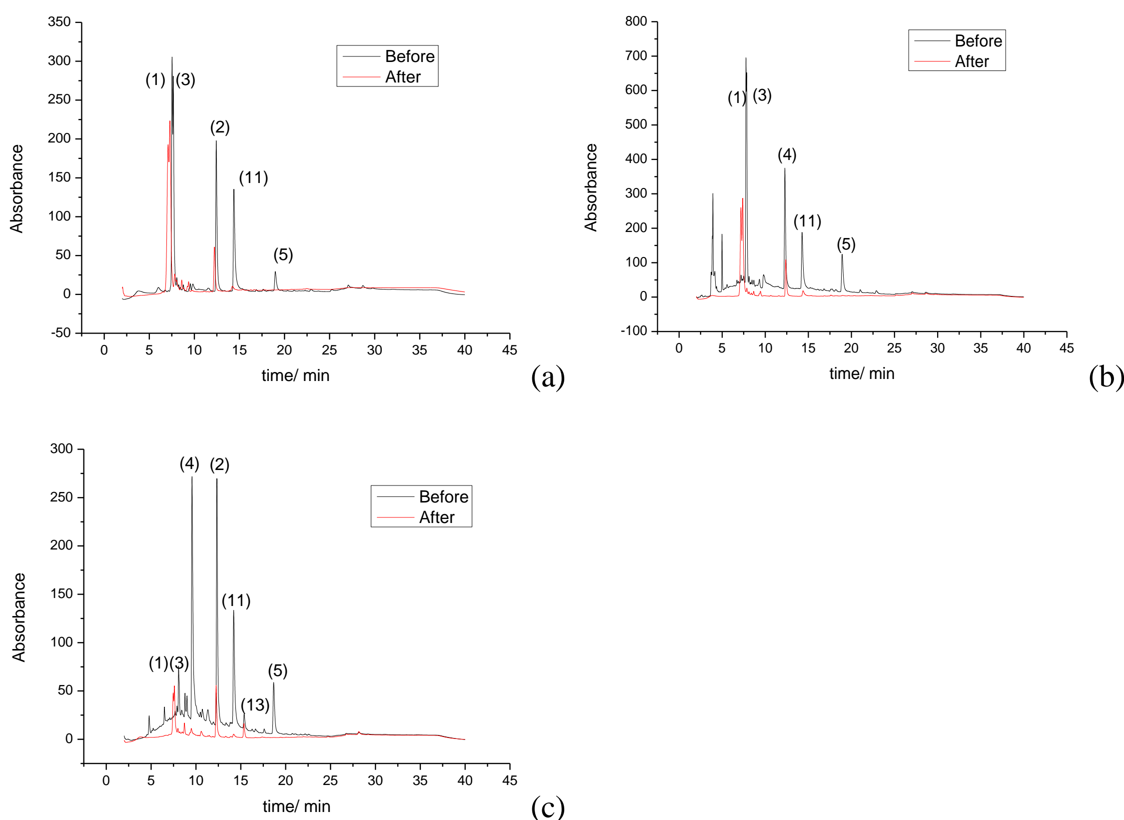


Figure 2. HPLC of (a) Iranian, (b) Turkish, and (c) English madder before (black) and after (red) dyeing on mordanted wool.

R^2 values from the calibration plot linear regression clearly show high correlation as both R^2 values are >0.99. It is observed that the response factor for alizarin is much greater than for the glycosides, which is consistent with the higher ratio of mass of the conjugated system to total mass of alizarin compared to the corresponding ratio for the glycosides. However, it is particularly interesting to note that the difference in response factors between the glycoside mixtures and the aglycon is an order of magnitude, which is much larger than would be expected based just on molecular mass. This suggests that glycosylation is a significant influence on absorption intensity, which is worthy of further investigation. From the change of concentration of the dyestuffs, it can be seen that for both Iranian and Turkish madder there is significantly greater adsorption of glycosides onto wool in comparison with alizarin. In the case of Iranian madder, 4.3 times the number of moles of glycosides are adsorbed with respect to alizarin, and in the case of Turkish madder, 2.5 times the number of moles of glycosides are adsorbed with respect to alizarin, which suggests that the main dye compounds adsorbed onto the wool are the glycosides (purified 1:1 ruberythric acid:lucidin primeveroside mixture), and not alizarin as suggested in most literature. In the case of English madder, the major peak of lucidin is completely adsorbed onto the wool (the response factor for lucidin could not be calculated due to the unavailability of pure lucidin to perform a calibration curve, hence the molar quantities of lucidin adsorbed onto the wool could not be reported). Despite the fact that English madder contains low concentrations of glycosides, in comparison with Turkish and Iranian madder, it is still observed that a higher number of moles of glycosides are adsorbed (6.5 μmol g^{-1}) in comparison with the number of moles of alizarin adsorbed (5.0 μmol g^{-1}).

Table 3. Comparison of the data fitting of the calibration curves (R^2) of the glycosides and alizarin and their consequential response factors.

Dye compound	**R^2 value**	**Response factor**	**Dye uptake onto wool fibre (μmol g^{-1})**		
			Iranian	**Turkish**	**English**
1:1 ruberythric acid:lucidin primeveroside mixture	0.996	174	14.6	18.8	6.5
Alizarin	0.994	2280	3.4	7.4	5.0

From this study, the absolute change in concentration of the two major classes of compounds in R. tinctorum, the glycosides and alizarin, can be calculated. It was shown that the glycosides are the main contributor to the dyeing components of both Iranian and Turkish madder, and the molar adsorption of these two compounds is much greater than the aglycon, alizarin, which is often referred to as the main dye component of R. tinctorum.

4.2. Sorption isotherms of the purified 1:1 ruberythric acid:lucidin primeveroside mixture and alizarin

Sorption of the purified 1:1 ruberythric acid:lucidin primeveroside mixture was studied to provide an understanding of how anthraquinone glycosides interact with and adsorb onto the wool fibre. Figure 3 shows an isotherm plot of q_e vs. C_e for the purified 1:1 ruberythric acid:lucidin primeveroside mixture; linearization of the isotherm was attempted by application of all three models and it was found that the Temkin model (q_e vs. $\ln C_e$) gave the most reliable fit ($R^2 = 0.93$), although significant fit to the Freundlich isotherm was also observed ($R^2 = 0.91$). Figure 4 shows an isotherm plot of q_e vs. C_e for alizarin; linearization of the isotherm was attempted by application of all three models and it was found that the Freundlich model ($\ln q_e$ vs. $\ln C_e$) gave the most reliable fit, with an R^2 value of 0.94, although significant fit to the Temkin isotherm was also observed ($R^2 = 0.88$). There was no observed association with the Langmuir isotherm for either alizarin or purified 1:1 ruberythric acid:lucidin primeveroside mixture ($R^2 < 0.2$).

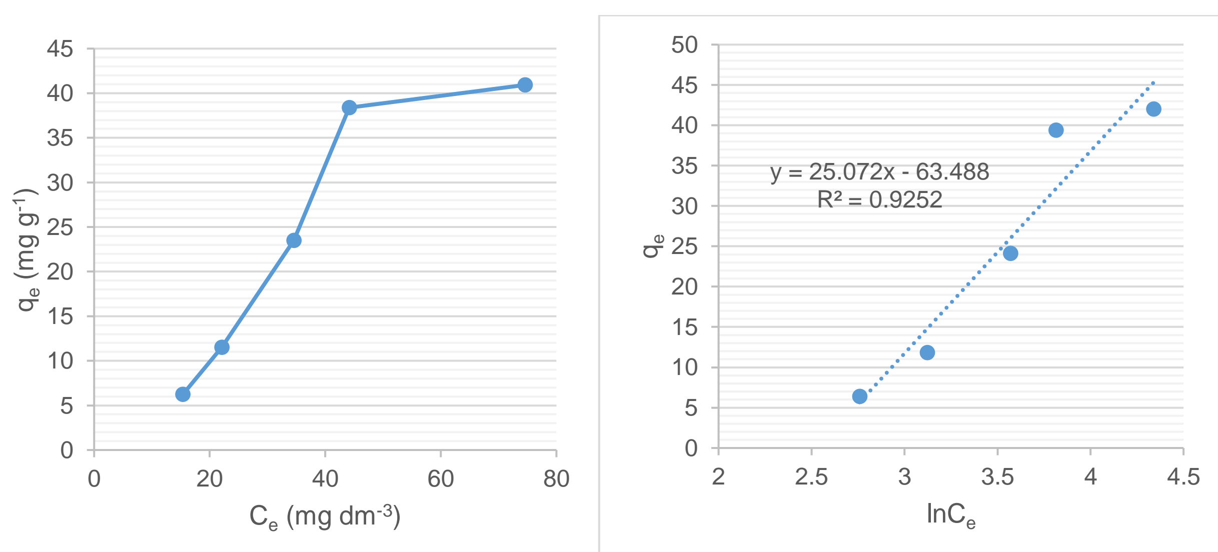


Figure 3. Plot of q_e vs. C_e for the purified 1:1 ruberythric acid:lucidin primeveroside mixture at different concentrations and linearization of the isotherm by application of the Temkin model (q_e vs. $\ln C_e$).

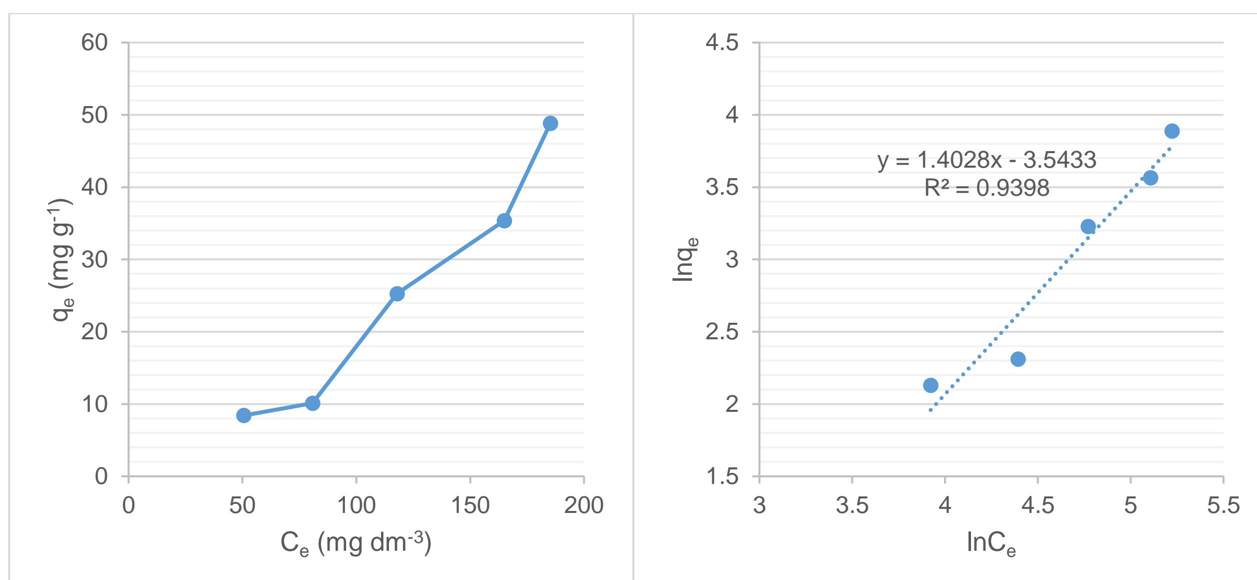


Figure 4. Plot of q_e vs. C_e for alizarin at different concentrations and linearization of the isotherm by application of the Freundlich model ($\ln q_e$ vs. $\ln C_e$).

Table 4. Freundlich and Temkin isotherm data for dyeings with the purified 1:1 ruberythric acid:lucidin primeveroside mixture and alizarin.

Dye	**M_w (g mol^{-1})**	**Freundlich**			
		R^2	**K_F (dm^3 mol^{-1})**	**n_F**	**–ΔG (kJ mol^{-1})**
Alizarin	240	0.940	6.9	0.7	5.8
1:1 ruberythric acid:lucidin primeveroside mixture	549*	0.914	127.0	0.8	14.6
Dye	**M_w (g mol^{-1})**	**Temkin**			
		R^2	**K_T (dm^3 mol^{-1})**	**b_T (J mg^{-1})**	**–ΔG (kJ mol^{-1})**
Alizarin	240	0.876	5.2	100.6	5.0
1:1 ruberythric acid:lucidin primeveroside mixture	549*	0.925	43.6	120.4	11.4

*Average M_w of ruberythric acid (534) and lucidin primeveroside (564) in a 1:1 mixture.

A summary of the data obtained from these dyeing studies can be found in Table 4, which shows that the functional moieties on hydroxyanthraquinone have a large effect on sorption

properties. It is observed that the purified 1:1 ruberythric acid:lucidin primeveroside mixture has substantially greater sorption energy (–11.4 kJ mol^{-1}) in comparison with alizarin (–5.8 kJ mol^{-1}), suggesting that glycosides are more likely to interact with the adsorbent (pre-mordanted wool fibre) than aglycons, although both will be likely to adsorb to some degree. Unlike the Langmuir equation, the Temkin isotherm also takes into account interactions between adsorbed species and adsorbates to be adsorbed; constant b_T is related to the heat of adsorption and takes into the account adsorbing species-adsorbent interactions, and it is observed that interactions between glycoside molecules (120.4 J mg^{-1}) are higher than corresponding interactions between alizarin molecules (100.6 J mg^{-1}). It is proposed that the greater interactions and sorption energy of the glycosides, in comparison with alizarin, are as a result of greater adsorbent-adsorbate and adsorbate-adsorbate interactions due to greater hydrogen bonding interactions resultant from the glycoside moiety; clearly alizarin also forms multilayers, if not to the same extent. Dyes capable of multiple hydrogen bonding interactions would be expected to bind particularly strongly as each hydrogen bond would reinforce each other in a chelate-type effect. This is particularly the case when cooperative hydrogen bonding (where an alcohol acts as both a hydrogen bond donor and acceptor) can occur across numerous sites on the dye and substrate. In addition, such multiple binding and dye adsorption would displace water molecules, especially those adjacent to the relatively hydrophobic core of the dye, leading to significant entropy driven binding.

Previous X-ray crystallography research by the authors [8] and Henderson et al. [7] on crystal packing of ruberythric acid and lucidin primeveroside, respectively, demonstrated that the main intermolecular interactions between separate glycoside molecules are due to hydrogen bonding between the sugar moieties. Each ruberythric acid molecule forms hydrogen bonds to another two ruberythric acid molecules, which are through the terminal xylose, which forms two hydrogen bonds with the glucose of the primeveroside of another ruberythric acid molecule [8]; similar observations were made for lucidin primeveroside [7]. Although care should be taken relating X-ray crystal structures to real substrate binding, the multiple hydrogen bonding and cooperativity observed strongly suggest this plays a very important role. There is also evidence of π-π stacking between the anthraquinone rings of the glycosides.

What is notable about the crystal structures of ruberythric acid and lucidin primeveroside is that they both display significant interaction between the sugars in the lattice; however, the anthraquinone aglycon moiety does not provide any intermolecular hydrogen bonding interactions within the lattice, showing only the well cited intramolecular hydrogen bonding. These observations lead to questions of how these dye molecules interact with textile fibre substrates as hydrogen

bonding interactions are cited as the main attractive force operating between natural anthraquinonoid dye molecules and textile polymer fibres.

The importance of glycosides in binding interactions has previously been overlooked, and this insight is not only interesting in terms of fundamental sorption properties, but also aids the development of methods and analytical techniques for extraction of the dyes from artefacts. The authors have previously demonstrated that glucose solution is highly effective at back extracting both anthraquinone glycosides and aglycons from wool dyeings [16]; the work herein confirms the formation of multilayer aggregated forms of adsorbed anthraquinones from R. tinctorum and that glucose is able to displace this hydrogen-bonded network and effectively release the dye compounds from fibre without the use of acid.

5. Conclusions

From this study, the absolute change in concentration of the two major classes of compounds in R. tinctorum, glycosides (lucidin primeveroside and ruberythric acid) and alizarin, can be calculated from HPLC chromatograms. It was shown that the glycosides are the main contributor to the dyeing components of both Iranian and Turkish madder, and the molar adsorption of these two compounds is much greater than the aglycon, alizarin, which is often referred to as the main dye component of R. tinctorum. Comparative work using sorption isotherms for the purified 1:1 ruberythric acid:lucidin primeveroside mixture and alizarin demonstrates that chemical differences in these compounds results in different adsorption mechanisms onto mordanted wool. The higher sorption energy of the glycosides (–11.4 kJ mol^{-1}) compared to alizarin (–5.8 kJ mol^{-1}) is in agreement with the HPLC results, indicating that the purified 1:1 ruberythric acid:lucidin primeveroside mixture has higher affinity for wool compared to alizarin. Not only do the glycosides show a higher affinity for the wool, but the interactions between the adsorbed species and adsorbates also show a higher level of interaction and hence suggest a level of aggregation of dye on the surface of the wool. This is the first known sorption study of the isolated compounds from R. tinctorum root and provides insights into understanding uptake of the different components in Dyers' madder onto wool fibre during dyeing. These observations are in contrast to much literature and bring into question conclusions that alizarin was the main dyeing species throughout history.

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