Chemical Analysis and Elucidation of Anthraquinone and Flavonoid Type Compounds with Applications to Historical Artefacts and Sustainability

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The candidate confirms that the work submitted is her own, except where work has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors of this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Section 3.3 contains work from a jointly authored publication. In this work, the NMR experiments and synthesis of the reference material is directly attributed to the author of this thesis. The HPLC analysis and purification of the compounds is attributed to Jose Rodriguez, as referenced in the thesis text.

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Abstract

This thesis describes the effects of different solvents have on the extraction profile of natural dyes from dye plants: madder, weld, golden rod, and chamomile. HPLC has been used to build a fingerprint of each dye plant profile and thus used to compare to the profiles of back extraction from textiles for natural dye identification in historical artefacts. The use of solid phase extraction is compared to extraction methods with no purification step which is favourable for anthraquinone dyes but results in a major loss of glycosidic compounds when using yellow dyestuffs. Supported by ^{1}H and ^{13}C NMR data, the conclusive X-ray crystal structure of the natural dye ruberythric acid is presented which has never been achieved prior to this research. In a collaboration with food science two of the main components of chamomile are fully characterised by 1D and 2D, ^{1}H and ^{13}C NMR. These compounds are usually referred to as ferulic acid derivatives in the literature but their actual structure is reported herein.

The thesis also discusses the relative dye uptake of anthraquinone compounds onto wool textiles which were measured by HPLC. Sorption isotherms for the main anthraquinones in madder; ruberythric acid, pseudopurpurin and alizarin are compared for more in-depth understanding on the method of adsorption of these compounds. Herein the glycosidic compounds in madder are shown to have a higher adsorption capacity than the aglycons. Ruberythric acid is shown to follow a Tempkin isotherm with the highest degree of correlation but both alizarin and ruberythric acid show good fitting with the Freundlich isotherm also. Pseudopurpurin was shown to follow a Freundlich isotherm with the highest degree of linearity but did also show some fitting to the Langmuir isotherm. The isotherms allow data to be collected on the energy of adsorption and draw conclusions on the effect the functional groups have on the dyeing capability which is studied herein for the first time on individual anthraquinone components.

Studies were carried out on the acid-sensitive colorants present in madder which are degraded in the textile back extraction process. Anthraquinone aglycons alizarin and purpurin are usually identified in analysis following harsh back extraction methods, such as those using solvent mixtures with concentrated hydrochloric acid at high temperatures. Herein, a softer novel 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*.

A study into the breakdown compounds of the aglycon; lucidin under acidic conditions used for traditional back extractions was also undertaken. Here it is observed that lucidin is converted into xanthopurpurin in a retro aldol like mechanism. This report discusses some of the issues raised by using these harsh back extraction methods and the problems faced in using them to analyse historic artefacts.

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1 Introduction

Colours today are essential to everyday life and are commonly used to attract attention and give a desired message. A wide range of colours and hues have been developed and are now readily available as chemists have strived to develop many synthetic compounds which can be used to impart colour. However, synthetics as an alternative to natural colorants can be damaging to human health and/or hazardous to the environment. This is putting increasing pressure on companies using these chemicals to assess their environmental impacts. Therefore, with health legislation for cosmetics, food and pharmaceuticals constantly changing researchers are looking back to natural alternatives to use in products. Research is ongoing in the fields of natural dyes and actives for sustainable purposes, and they can be used as attractive marketing tools in cosmetic and pharmaceutical industries.[1]

Before the advent of synthetic alternatives colour came from plant, animal or mineral sources. Colorants are either a dye or a pigment. A dye refers to a soluble compound that can impart colour by attaching onto, or being held within the object being coloured. Pigments are insoluble in most solvents and are applied to the surface of an object.[2] It is not really understood how or why the first dyeing happened, but it is thought to have probably been accidental.[3] The art of dyeing has evolved through time, with Chinese Chronology dating 3000 BC mentioning dye houses.[4] Later on civilizations began to strive to understand how mordants and salts improve the dyeing process. Surviving artefacts and manuscripts on dyeing can give information on culture and heritage of the region they were found.[5] These methods were not always recorded in writing and often passed down from teacher to student, hence some methods of dyeing have been lost. Scientific analysis of these artefacts can help us to fill in the unknown gaps of how these shades and colours were created throughout our history.

Colour, although not as obtainable as it is today, played a big part of history. It was often reserved for the wealthy and powerful and became a sign of status within society. There has always been a romantic attachment to the colour red, perhaps due to its likeness to the colour of blood which had a very strong meaning in ancient times. Red Ochre was present in civilisation at around 30,000 BC and was used to cover the cranium and body of human corpses.[4] During the Neolithic period human kind began to gather seeds and evolve from hunter gatherers to farmers, it is thought that during this period vegetable dyes such as madder became available to use and a number of colours began to be produced from plants.[6] Dioscorides wrote about the usefulness of madder dyeing and the dyestuff was known to the dyers of Egyptian, Greek and Roman eras.[5]

There is a wide variety of textiles that have been preserved by different natural phenomenon such as peat bogs of Scotland[7] or very dry conditions in burial sites in China, Xinjiang[8]. The preservation of these textiles in burial sites can allow for the easy identification of the tannic and organic matter but sometimes can cause discoloration on a molecular level of the colorant compounds present.[9] In some cases the original colour is difficult to decipher just by observation but chemical analysis can give information of what the original colour would have been. In other cases fibres have been well cared for and kept in optimum conditions to minimise the degradation of fibre or colorant, often the case with museum owned artefacts. An example of this, is the identification of silk fibres from the Ottoman period which were analysed by chromatographic methodology to reveal their dye stuff make up by comparison with aglycon references of common dyes.[10] These samples were provided by the Topkapi Palace Museum, Istanbul and the colour was visible to the human eye before analysis. Historians and conservationists strive to protect these insights to the past. To do this the dye components and fibre properties must be understood. Exciting collaborations between chemistry and history help to develop more information on these artefacts and uncover cultural heritage mysteries.

1.1 History of Madder

Plants belonging to the *Rubiaceae* family give a red dye upon extraction of the roots (see Figure 1.1 showing *Rubia tinctorum* a cultivated plant from the *Rubiaceae* family). The majority of the *Rubiaceae* family grows wild. There are many different strains present in nature throughout the world. Until modern eras the species of madder used depended on the geographical location and which species was available in that area. The establishment of trading routes, particularly after 1600's between Europe and the east complicate identification of madder root in artefacts using just its geographical location.[4] Therefore to fully identify the root used, compositional analysis of the dyestuff in question would need to be carried out.

Rubia tinctorum is by far the most widely used species in Europe for dyeing and was cultivated, hence it is often given the name 'dyer's madder'.[11,12] The name *Rubia tinctorum* is derived from the Latin meaning 'dyer's red' and again shows the prominence of this plant in the dyeing industry. Archaeological finds in Britain have shown madder to be dyed onto wool dated from the ninth to the eleventh centuries AD, these finds have been mainly around the geographical areas of Coppergate, York and London (late Anglo-Saxon eras).[4] *Rubia tinctorum* has been identified on a number of different coloured textiles, Coptic textiles dating from the seventh to the ninth centuries AD varying from yellow to orange to red were shown to have all been dyed with *Rubia tinctorum* by chromatographic methods.[13]

Rubia cordifolia, also known as munjeet, was prominent in the exuberant Indian cotton industry until the 1750's and was another major species of dye plant belonging to the *Rubia* family.[12] The roots of *Rubia cordifolia* are native to the South, South East and East Asia.[11] The roots of this plant have been identified on samples of historical textiles from China, where the colour red holds strong cultural values and was used to display social ranking.[14]

Another species of madder dye plant used in East Asia is *Rubia akane* which was used as a red dye plant across much of Japan.[11] This particular dye plant has until recently been relatively easy to identify due to the presence of the unusual dye compound 6-hydroxyrubiadin (more information is given on the structure of the anthraquinones found in these roots in the following chapters).[11] However recent studies by Han *et al.* have shown that *Rubia cordifolia* grown in China contains 6-hydroxyrubiadin and its glycosylated derivatives in its chemical composition.[14] This finding shows how important it is to fully identify all of the compounds in the dye plant and ensure that all marker pigments are fully considered before a conclusion is made.

Rubia peregrina, also known as wild madder is very difficult to distinguish chemically from *Rubia cordifolia* due to both having the high presence of pseudopurpurin in their extracts.[11] However when trying to make conclusions on which dye plant was used in historical textiles sometimes it is necessary to take into account both geographical locations of where the textile was thought to be made or found and the chemical marker compositions. An example of this is the identification of *Rubia peregrina* samples from funeral figurines found in Macedonia, Greece dated from the Hellenistic period third to the second century BC. The high levels of pseudopurpurin found in the extracts could lead to conclusions that this particular artefact was dyed with either *Rubia peregina* or *Rubia cordifolia*. However due to the fact that *Rubia peregrina* was most common in the Mediterranean area, other than *Rubia tinctorum* which shows a different composition, the dye compound in the textile was concluded to be *Rubia peregina*.[15]

Other more minor species of madder dyes are *Rubia sikkimensis* which were used to produce the ‘reds of Naga’ famous in the north eastern provinces of India.[4]

Another dye plant of the *Rubiaceae* family worth mentioning is the Galium species, also known as Bedstraw. The name of the dye plant comes from the Greek word *gala* meaning milk and gives coral and rose dye colours with alum mordants.[4] Native to temperate regions of Europe, North Africa and Asia this dye plant was also used as a

red dye and produces similar compounds to those in the *Rubia* species however its use was not as wide spread as *Rubia spp.* varieties.[12]

Figure 1.1. Roots and aerial parts of a Rubia tinctorum plant.[16]

Madder was a very important dye and was widely used across many artefacts from textiles to paintings. The British Museum has reported madder to be the colorant source for many objects in their collection from Greek, Roman, Parthian and Egyptian eras.[17] Its common use is often attributed to its ease to grow and cultivate, which means the supply was plentiful and was far less expensive than insect red dyes.[4] Madder also probably owes some of its success to the number of shades which can be created from the plant material; not only on its own but in combination with other dyes. For example, mixed with indigo it produces a purple which was known as the best process for imitating the expensive and very exclusive shellfish purple.[12] Different shades of madder can be produced based on the age of the plant, the processing of the roots and the concentration at which it was applied. The type of mordant used and the method of applying the mordant can also drastically change the appearance of the textile colour

when dyed with madder.[12] This is due to the chromophore in the anthraquinone complex. Conjugation of double bonds around the ring allows for delocalisation around the whole molecule and creates a colour. However upon coordination to a metal the energy level of the delocalised system is modified and a colour change is observed.[18] Methods of mordanting are explained in further detail in section 1.7. The colorants extracted from the madder plant are grouped in the Colour Index as C.I. Natural Red 8.

The presence of madder throughout history is made apparent by the number of ancient languages which have a word for the plant or dye material. Words for madder appear in Ancient oriental languages, Biblical period languages, and Old European languages.[4] The first known textile dyed with madder was the belt of Tutankhamun which dates back to 1350 BC.

Madder was widely used to dye wool, but was also a huge dyestuff for cotton and silk textiles. When used to dye cotton it is usually referred to as Turkey red. Creating the Turkey red colour was a laborious process which could take up to a month.[19] When dyeing with madder, the fibres must first be treated in order to increase the fastness of the dyestuff. For cotton this process involves a long succession of treatments such as boiling in oil, excrement and wood ash and being left to ferment. This process was often repeated many times and allowed to ferment in the sun hence its original links to warmer climates such as Turkey and India.[4] Although this dyeing technology was created and developed in the East, it was adopted in Europe around the middle of the eighteenth century.[20] Yarn dyed with the Turkey red technology was manufactured in Glasgow by 1785 and this is thought to be the first instance of a dye house able to create the Turkey red colour in Great Britain.[20] This textile production was also set up in Manchester at the end of the eighteenth century.[21]

The pre-treatment for proteinaceous fibres such as wool and silk is a lot simpler. The fibre is usually pre-treated with the desired mordant, dried and then can be added into the dyebath.[12]

In 1826 *Rubia tinctorum* was found to contain two components, alizarin and the more rapidly fading purpurin by Pierre-Jean Robiquet.[22] The synthesis of alizarin was a race against time with only one day between two patents of the same synthesis being filed in 1868. The first by German chemists Karl Graebe and Karl Lieberman working for BASF in 1868 and the second, a day later, by English chemist William Henry Perkin.[23] The production of synthetic madder was far cheaper and less time consuming than the lengthy process of growing and extracting colorants from natural madder. This ultimately lead to the collapse of madder farming and helped to open up the world of synthetic organic chemistry as we know it today. However research carried out by the national museums Scotland has suggested that the decline of 'natural' madder was actually rather slow and it was not until 1961 that the United Turkey Red Company (which produced dyed fabric using natural madder dyes) ceased trade.[21]

Nowadays most dyes are made synthetically, however, production can be harmful to the environment and some synthetic dyes can be toxic to human health.[24] If the industry were to move back to using natural dyes on industrial scales the process would rely on the ability to produce a dye extract rich in the desired colorants or use a hybrid of natural and synthetic dyes.[25,26] Efforts to incorporate natural dyes back into industrial processes have been reviewed by Dr. David Hill at the University of Bristol.[27] Optimisation of dye bath conditions and full understanding of mordant dye interactions could lower the amount of dyestuff needed for successful dyeing. Multiple optimisation methods have been tested such as the pH of the extraction dye bath, optimum temperature for extraction, material to liquor ratio and the particle size of dye plant upon extraction.[28,29] There have also been some efforts to change the characteristics of the dye molecules themselves by chemical modification in order to change the polarity of the dyes for use with man-made fibres.[30]

Throughout history madder has not only been used to dye textiles, but has also been mentioned for its medicinal properties by Pliny, Hippocrates and Dioscorides.[4] The

root was said to be boiled in wine or water, some honey or sugar is added for sweetening and the solution is consumed.[4] The roots of the plant have been reported to have antimicrobial, antifungal, hypotensive, antiviral[31,32] and antimalarial properties[26,33]. Madder root was also a common herbal medicine for the treatment of kidney stones due to the compounds present in the extracts being able to coordinate to calcium which causes the kidney stones.[34] However, lucidin, a major component of *Rubia tinctorum* is known to have genotoxic properties.[34–36] Also the metabolite of alizarin; 1-hydroxyanthraquinone is thought to have mutagenic activity.[37] These properties of the compounds in madder has resulted in a ban from the food or medicinal market in the EU.[38] Therefore producing an extract which could easily be purified and does not contain these compounds would be advantageous to industry.

1.2 History of Yellow textile colorants

Yellow dye plants have been used on their own and with other dyes to produce a wide range of colours.[12] Chemical analysis of yellow dye bearing plants have shown that it is the flavonoid components of the plant which give the yellow colour. The main flavone luteolin is a dye component in many species of dye plant from different botanical families.[1,39] The yellow dyes suffer most dramatically from fading, and the loss of colour is often observed.[40] The flavones such as luteolin are said to be the most stable yellow colorants to photo degradation and is why their presence in favoured dye plants is so common.[41] The flavones have been shown to be much more stable to UV light than the flavonols which are another class of compounds extracted from yellow dye plants.[42] This is contrasting from the anthraquinones mentioned in section 1.4 and the blue dyes from indigo which are very stable to light. Often shades which were once green in colour fade to the underlying blue of the indigo dye underneath.[12]

Differing from the red dyes, many plants produce the compounds responsible for the yellow colour and hence there was not only one species that predominated.[19,40] The

use of potentially hundreds of different yellow dye plants throughout history makes analysis of yellow dyed textiles very interesting. Often the glycosides hold the key to dye stuff identification in yellow dyed threads. Marker pigments from certain species can be used to identify a plant which has been used to dye a material. The combination of certain compounds and their abundance in the textile help to make a 'fingerprint' of each plant that could be detected on the textile.[39]

Figure 1.2. Weld dye plants before harvest.[43]

One of the preferred medieval yellow dye plants in Europe was weld (*Reseda luteola* L.) shown in Figure 1.2 also sometimes called dyer's mignonette.[12] Dye analysis of textiles obtained from the monastery of Simonos Petra were shown to contain weld as their major dye stuff.[44] Research by Zhang and Laursen highlights the importance of very careful analysis of yellow dyes in their studies on 3000 year old textile fragments from the Taklamakan Desert region of Xinjiang, China. Luteolin-7-O-glucoside was shown to be the major dye compound in these dye extracts, usually indicative of weld (*Reseda luteola*), but identification of other minor compounds lead to the correct identification that in fact neither of these textiles actually contained dye from weld.[2] Weld was also shown to be a major yellow dye plant in the analysis of a selection of yellow

and green yarns from 15th- 17th century tapestries analysed as part of the Monitoring of Damage to Historical Tapestries (MODHT) project.[42,45] Weld contains luteolin and its glycoside derivatives[1] as well as other minor flavones such as apigenin and its associated glycosidic derivatives, but in much smaller quantities upon comparison to luteolin and derivatives. It is a very hardy plant which can tolerate poor or even chalky soils and can grow up to 130 cm in height. The species is easily distinguished by the shape of the petals of the flowering part of the plant, the upper petal is clawed with 4-8 lobed limbs; the lower petals can be clawed or clawless.[12] The whole plant is used to produce the dye and historically the dye bath was produced by extracting with water. The importance of weld as a dye plant is observed through the references to it in account books of clothiers from medieval Europe.[12] As mentioned previously, many other dye plants can be used to create a yellow colour from a similar palette of colorants but throughout this thesis a select few have been chosen for thorough investigation. Although there are many dye plants used for producing yellow dyes throughout history this thesis will focus on dye plants which contain the dye compound luteolin. More detailed discussion of the reasons for the choice of dye plants introduced in this section is given in chapter 3.

Chamomile has been used throughout history as a food supplement and for its medicinal purposes. It is well documented that flavonoids have very good antioxidant and anti-inflammatory properties.[46] It is due to the properties of these compounds and the abundance of them in chamomile plants that make it a popular plant extract.[47,48] Dyer's Chamomile (*Anthemis tinctoria*) has also been used as a yellow dye, referred to as dyers chamomile.[12,45] Chamomile was not used as extensively as weld for dyeing, but the enormous amounts of chamomile used for tea across the world could make it a good waste stream from which to obtain some interesting compounds for sustainable applications (Figure 1.3).

Figure 1.3. Chamomile plants before harvest.[49]

Golden Rod, *Solidago virgaure* is another yellow dye plant, again the main components present in the plant are from the flavonoid family of compounds. However, golden rod is also reported to contain a main component quercetin (C.I. Natural Yellow 10).[50] The plant has short yellow petals which contain the colorant for dye extraction (Figure 1.4). Some recent studies have researched into using golden rod to extract natural dyes in scales to rival that of synthetic dyes.[50,51] Golden rod has a high abundance of natural polyphenols which make the extracts active against oxidants.[52]

Figure 1.4. Golden rod flowers.[53]

The yellow dyes, like dyer's madder can be used to dye silk, wool, cotton and linen by using an alum mordant. The importance of these colorants can be observed in the account books of clothiers, the dyers' tariffs of the wool guilds and the first treatises on dyeing.[12] Weld was a major source of the yellow dyes, but also often blended with indigo to produce green colours. The quantities of dyestuffs needed to dye with yellow dye plants are much more than the quantities needed for madder, often requiring 100% or more on mass of fibre of the dye plant.[12]

1.3 Cultivation, Harvesting and Processing of Dye Plants

Madder production was a successful industry up until the 19th century when synthetic alternatives took over the market. The ideal harvest time is three years after planting which gives the plants peak yield of dyestuff.[54] The rhizomes of the plant are used for the dyeing of materials and contain the majority of colorant components. Studies have shown the leaves and stems do not contain any of the dyestuffs at all.[35]

The stems of the cultivated *Rubia tinctorum* are very delicate and hence usually lie along the floor once a certain height is reached as the stems are unable to support the weight of the plant. The plant has very small yellow flowers which bloom in July and

their fruit are small black berries which ripen in September. Mature plants' rhizomes are covered in a dark rind with a red colour underneath, the central part of the rhizome is white and does not hold much of the colorant content of the plant.[26]

Weld dye plants are simple looking and the stems can grow up to 130 cm tall, with small yellow flowers grouped along the stem. Weld is able to grow in sandy and rocky regions and can live through poor soil conditions.[12] In some areas it can still be found to be growing wild. The whole of the plant is usually used for dyeing but studies have shown that the colour is more concentrated to the leaves and root.[12]

The dye plants are collected whole and dried in drying sheds in clusters making effort to lose as little material as possible. However, research has shown that this method decreases the overall yield of colorants, drying at 40°C is preferential but this also causes a decrease in the glycosidic compounds and an increase in aglycons, probably by hydrolysis of the sugars. Therefore the best method of storage if the glycosidic compounds are desired is to deep-freeze the plants until extraction can be carried out.[12] Freezing the dye plants could however have some implications if the dye plant is to be used in experiments designed to mimic those of historical recipes. Historically it would not have been possible to freeze dye plant samples and dried plants would be used in dye houses. The freezing and thawing of the plants could have some effect on the chromatographic profile of the dye plant and hence if the aim is to simulate a historical recipe this method of storage should be avoided.[12] However it is important to understand what effects storage conditions have on dye plants before the colorants are even extracted from the plant.

Chamomile plants produce daisy shaped flowers which contain most of its colouring properties. Often it is only the flowers which are harvested for use as a dye for the food industry. Chamomile has high yields of the flavonoids responsible for the health benefits associated with the plant when compared to other flavonoid containing herbs.[47]

The dyeing ability of chamomile gives a much warmer yellow when compared to weld which gives a lemon yellow shade with an alum mordant.[55]

Golden rod is a dye plant very similar to weld, it is very hardy and produces small yellow flowers which are responsible for most of its colour.[55] These flowers must be harvested when young, as older plants contain less colour. Golden rod has also been shown to have some potential for use in cosmetic applications as it contains high yields of natural antioxidants and polyphenolic content.[52]

1.4 Compounds in Madder

The alglycon compounds found in madder are shown in Figure 1.5. They are all hydroxy anthraquinones differing in the number and position of hydroxyl groups, with other functional groups, such as carboxylic acids and aldehydes occasionally present. Methyl or ethyl ether compounds are often observed when ethanol or methanol is used as the extraction solvent so are believed to be artefacts of the extraction process rather than occurring naturally in the plant.[34,56] The dyestuffs are usually found in the plant in their glycosidic form (Figure 1.6) and can be hydrolysed enzymatically or under harsh conditions such as strongly acidic solutions.[25] The anthraquinone compounds present in madder plants are thought to be linked to the plants defence. They are stored in the plant material as glycosides and upon crushing release hydrolysis enzymes to hydrolyse the glycosidic bond.[35]

Madder compounds containing carboxylic acid functional groups easily undergo decarboxylation and hence are often not observed in analysis of madder plant colorants.[57,58] The decarboxylation of the anthraquinones in madder is easy to occur due to the electron donating groups of the hydroxyl groups in the ring and the keto tendency of these groups stabilising the intermediate upon the elimination of carbon dioxide through decarboxylation. Even heat in the drying process is sufficient to cause decarboxylation of the carboxylic anthraquinones.[17] Fresh madder is shown to yield a

higher content of the carboxylic containing pseudopurpurin than dried madder.[17] The degradation of compounds present in madder species are explained and investigated further in chapters 4 and 5.

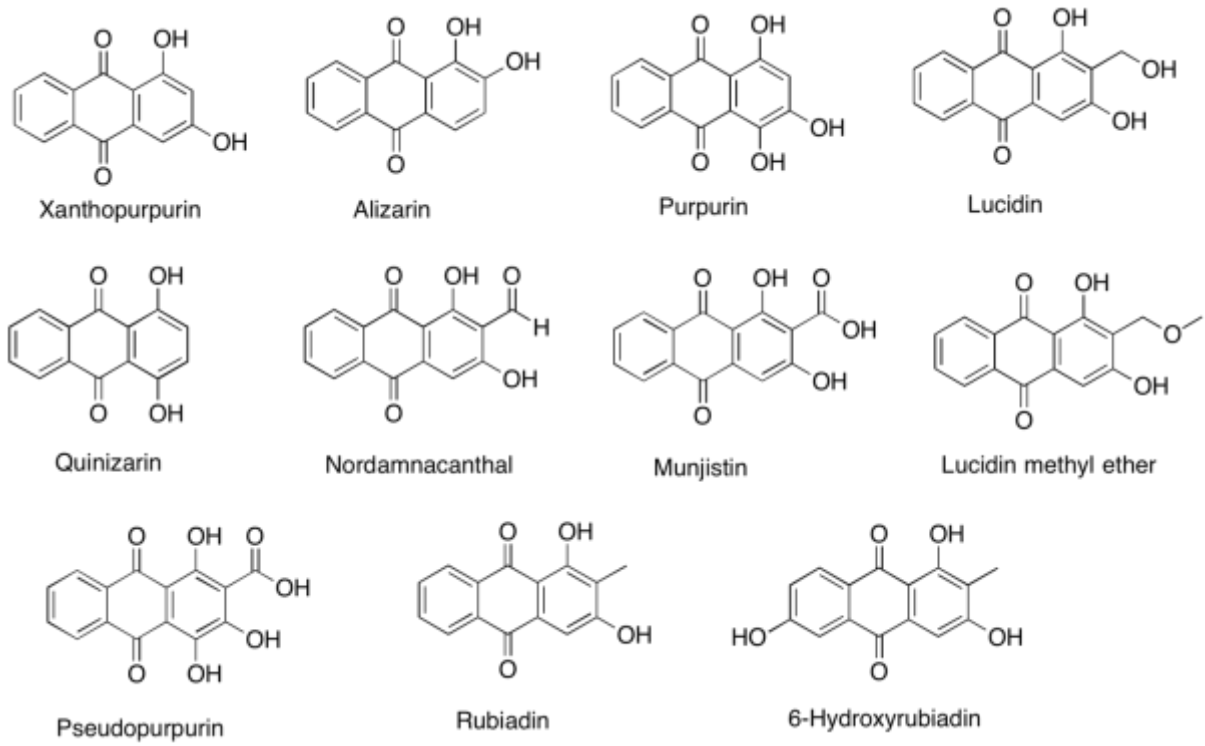


Figure 1.5. Aglycon compounds present in madder root extracts.

The relative abundance of each compound in the dye liquor or extract can be used to identify which species of madder is being dyed with when analysing textiles. This is highly important to the field of cultural heritage and can give some indications on preservation strategies. As previously mentioned species of madder common to the East were *Rubia cordifolia* and *Rubia akane*; and madder species common to the west were *Rubia tinctorum* and *Rubia peregina*.[59] However as touched on previously the geographical location of artefacts and the species of madder which is local to that area is not enough to fully identify the dye on a textile. For example fibres from an ancient graveyard at Yingpan, Xinjang were shown to contain high concentrations of alizarin and purpurin indicating the use of *Rubia tinctorum* which was not a common dye plant in this region.[60] The textiles found in this burial site are of high importance as they give

examples of trade routes between the East and the West at the time of the Han (206 BC- 220 AD) and Tang (618- 907 AD) dynasties. The textiles natural preservation allows historians to not only identify the dyestuff as *Rubia tinctorum*, regarded as a western dye, but also displayed both western and eastern motifs in the design of the textile which shows the designers knowledge of both eastern and western trends.[60]

However the composition of components in the plant make up are also important when analysing an extract to be used for industrial purposes where knowing the optimum time of harvest is crucial.[61] Derksen et al. have developed robust methods to study the optimum time of harvest of madder roots by measuring both the relative abundance of dye compounds in the roots and the total biomass weight. These studies compared plants harvested after two and three years and show an increase in the total biomass weight and the concentration of alizarin after three years. However other colorant species such as purpurin and pseudopurpurin do not show any increase in concentration when harvested at three years compared to two years.[62]

Some compounds are also marker pigments for certain species, for instance the presence of munjistin has been previously used as a marker pigment in the identification of *Rubia cordifolia*.[11] This then undergoes a decarboxylation reaction similar to that of pseudopurpurin to give xanthopurpurin and hence the presence of these compounds in textile analysis is used to identify the madder species used in the original dyeing. Marker compounds present in textiles are not always a robust way of analysing the dyestuff in a textile and often there are many factors to consider when identifying dyes in artefacts of historic importance. This is shown by the studies in Glasgow mentioned previously which identify 6-hydroxyrubiadin (usually a marker for *Rubia akane*) in back extracts of textiles dyed with *Rubia cordifolia* from China.[14]

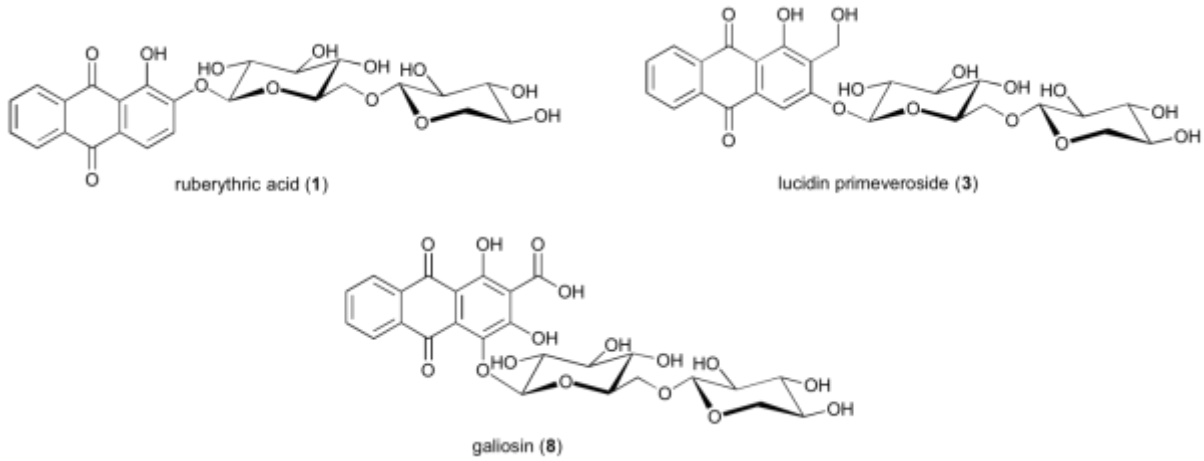
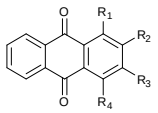


Figure 1.6. Glycoside derivatives of the anthraquinones detected in madder root extracts.

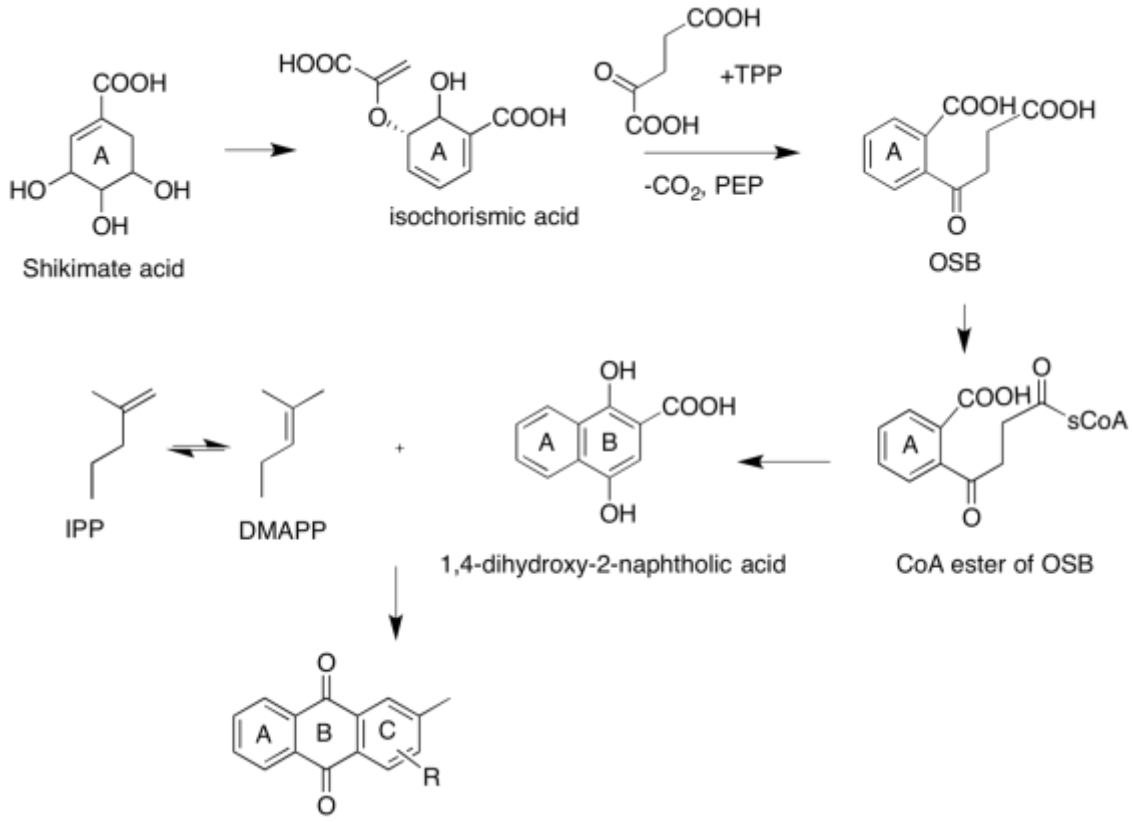
The abundance of the glycoside compounds in the dye plant can also give some information on the historical textile. Which is essential in understanding trade routes and dyeing recipes which were used. This is important when analysing textiles for eras which do not have any dyeing manuscripts available and the dye technique is not known. For instance, in some recipes madder root was treated with acid before dyeing to help release the colorants from the plants, this dye was named ‘garancine’ and does not contain any glycosides.[59] In analysis of textiles these compounds must be preserved so that more information on the method of dyeing and also the origin of the dye plant can be identified in historical artefacts. The glycosidic and carboxylic derivatives in the madder plant are very sensitive to acidic solvents and hence are easily hydrolysed, therefore the method of extraction from textiles must be carefully chosen to ensure that the chemical composition of the mixture is not changed upon extraction for analysis. A full overview of the anthraquinones found in madder roots in the literature is given in Table 1.1.

Table 1.1. Compounds present in madder root extracts found in the literature.

Number	Common name					Mass (Da)	References
		R_1	R_2	R_3	R_4		
1	ruberythric acid	OH	OGlc+Xyl	H	H	534	11,33,56,63–6542
2	alizarin	OH	OH	H	H	240	11,33,56,59,63–6542
3	lucidin primeveroside	OH	CH_2OH	OGlc+Xyl	H	564	11,33,56,63–65
4	lucidin	OH	CH_2OH	OH	H	270	33,64,66
5	nordamnacanthal	OH	CHO	OH	H	268	11,33,67
6	munjistin	OH	COOH	OH	H	284	11,33
7	xanthopurpurin	OH	H	OH	H	240	11,33,68
8	galiosin	OH	COOH	OH	OGlc+Xyl	594	11,33,59
9	pseudopurpurin glucoside	OH	COOH	OH	OGlc	462	11,33
10	pseudopurpurin	OH	COOH	OH	OH	300	11,33,63,68
11	purpurin	OH	H	OH	OH	256	11,33,59,65
12	rubiadin primeveroside	OH	CH_3	OGlc+Xyl	H	548	64
13	rubiadin	OH	CH_3	OH	H	254	11,64
14	1-hydroxy-2-methyl-AQ	OH	CH_3	H	H	238	68
15	2-hydroxy-AQ	H	OH	H	H	224	68
16	2-(hydroxy methyl)-AQ	H	CH_2OH	H	H	238	11
17	anthragallol	OH	OH	OH	H	256	68

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

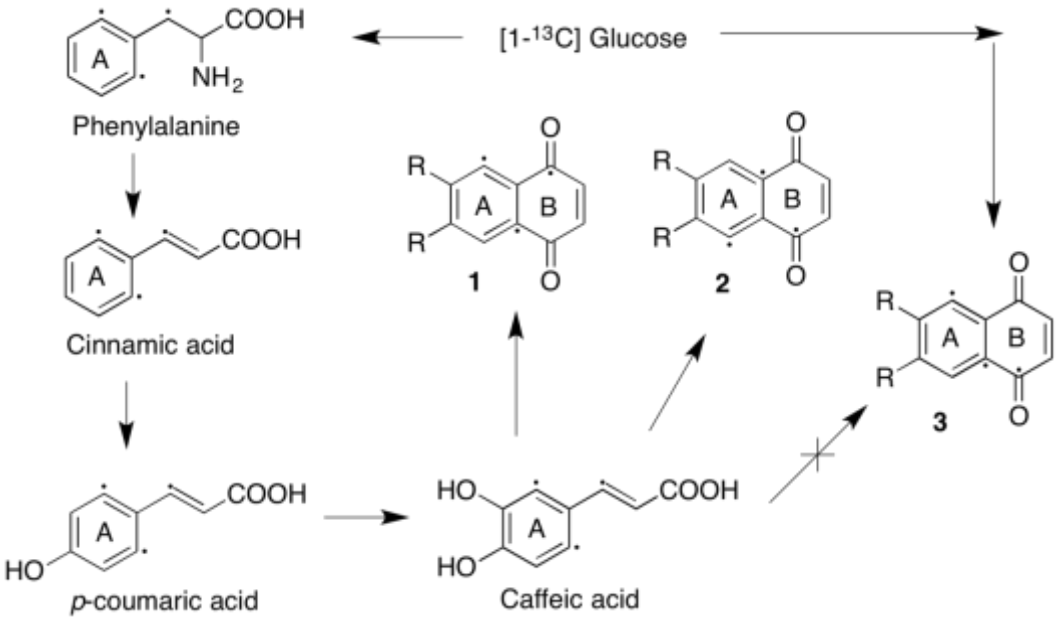
To understand the presence of certain compounds in madder plants the biosynthesis of these plant secondary metabolites must be considered. There are two main pathways that produce anthraquinones in plants; the polyketide or the chorismate/o-succinylbenzoic acid pathway. Anthraquinones from *Rubiaceae* species have been shown to follow the chorismate/o-succinylbenzoic acid pathway shown in Scheme 1.1 for the biosynthesis of ring A and B.[69]



Scheme 1.1. Chorismate/o-succinylbenzoic acid biosynthetic pathway of anthraquinones.[69]

[1-^{13}C] glucose was incorporated into lucidin primeveroside produced by *Rubia tinctorum* cells. The pattern of labelled carbon atoms could not be attributed to either the polyketide pathway or the phenylpropanoid pathways see Scheme 1.2. This is strong evidence that rings A and B are derived from the chorismate/o-succinylbenzoic acid and ring C is suggested to form from isopentenyl phosphate (IPP) via the terpenoid

pathway.[70] This suggested biosynthetic pathway results in an anthraquinone which is only substituted on ring C and can then be further oxidised and glycosylated enzymatically.



Scheme 1.2. Phenylpropanoid biosynthesis of anthraquinones. Diagram shows the expected carbon labelling patterns from this synthetic pathway (patterns 1 & 2). Compound 3 is the labelling pattern followed by the anthraquinones from Rubia tinctorum. Black dots represent the labelled carbons.[69]

From Scheme 1.1 the product formed is methylated in the 2 position of ring C. The ortho/para directing capabilities of the methyl group and the presence of the carbonyl β to the 1-position of the C ring could help the oxidation of the 1-position. Most of the anthraquinones in higher plants are oxidised in position 1 (Figure 1.5). When trying to understand the abundance of these compounds in plants and the presence of the anthraquinone backbone it is very important to understand how they are made in the plant. Many of these compounds present in the different plants differ only by an OH group or the position of the OH groups which makes the sensitivity of the instruments when analysing the compound of high importance. This work done on *Rubia tinctorum* cells explains why anthraquinones which follow this biosynthetic pathway only have

substitution on ring C. Which is what has been observed throughout the literature in the marker compounds which identify *Rubia tinctorum*.[11]

1.5 Compounds in Yellow dye plants

The yellow dye plants contain many polyphenols, the majority of these polyphenolics that give the yellow colour are flavones (Figure 1.7). The main two flavone compounds identified in these plants are apigenin, luteolin and their glycosidic derivatives. Due to the fact that there are only two main compounds used to identify yellow dye plants and they are present in many different species it becomes more important to preserve the glycosidic components in these extracts.[39,71] Many yellow dye plants show different abundances of certain glycosides and hence the ratios of these compounds can be used to identify which yellow dye plant was used to originally dye the textile. Therefore 'soft' extraction methods are favoured for the extraction of yellow dyes so as to preserve the presence of the glycosidic materials for plant identification.[39,71] These dye plants were chosen for study due to them all containing the aglycons luteolin and apigenin and their glycosidic derivatives. As mentioned in section 1.2 golden rod (*Solidago virgaure*) also contains quercetin however for this study it is the only the yellow flavones which are of interest to the study in order to set up robust chromatographic methods for measuring

the presence of this class of compounds. Quercetin is a flavanol and hence has not been included for comparison in Figure 1.7.

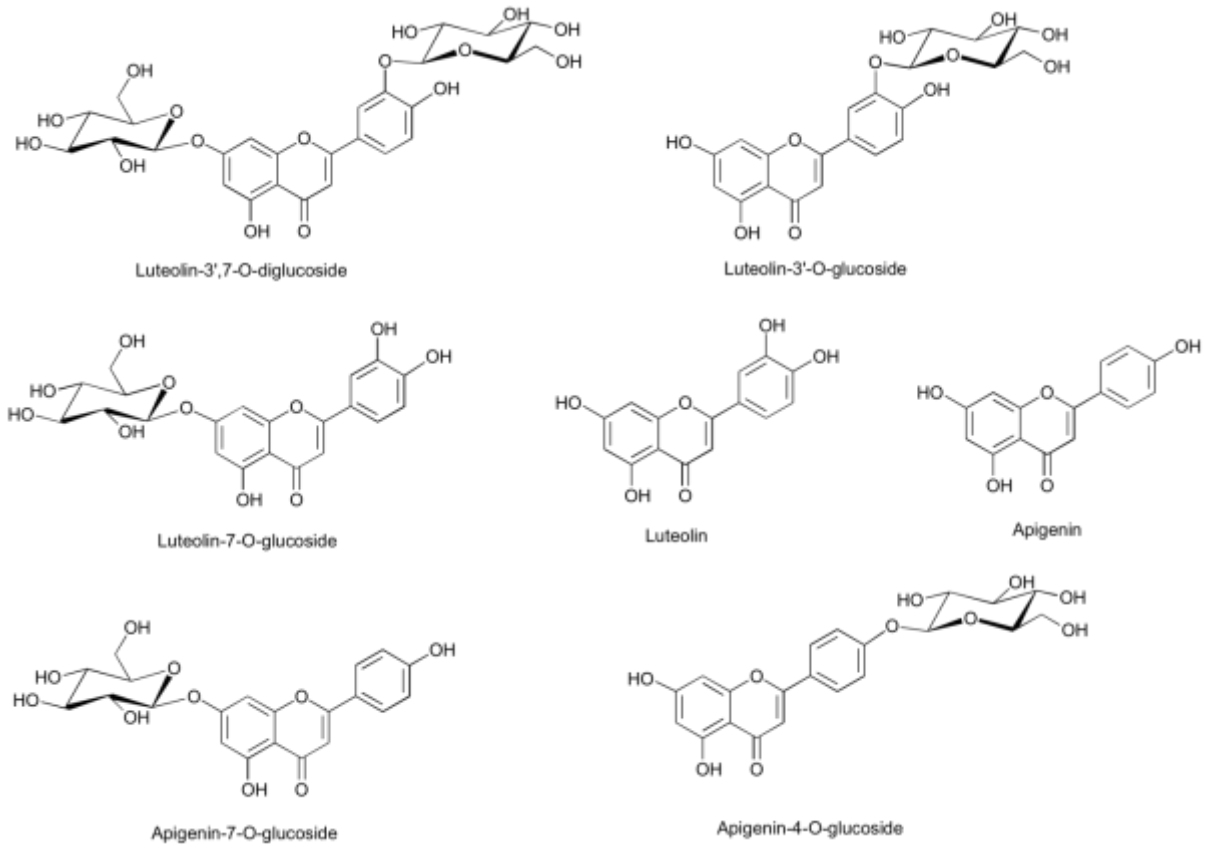


Figure 1.7. Flavone compounds found in Chamomile, *Solidago virgaure* and Weld which contribute to the yellow colour of these dye plants.

Again, it is interesting to understand the biosynthesis of these compounds and their role within the plant. Flavones are synthesised through the phenylpropanoid pathway which involves the conversion of phenylalanine to caffeic acid.[72] The caffeic acid is then converted into 4-coumaroyl CoA and enzymatically transformed by chalcone synthase to produce a chalcone, from which all flavonoids derive. The flavonoid is then modified by a series of enzymes, such as isomerases, reductases and hydroxylases.[72] The chalcone then undergoes a ring closure to a flavanone via a Michael type addition.[73] This process is catalysed by chalcone isomerase and is stereoselective but it can also occur unselectively at room temperature.[74] Transferases can then modify the plant in the final stages of biosynthesis in order to add sugar groups which help the solubility of these

compounds in aqueous medium.[72] The process of biological synthesis of these compounds explains why these plants also commonly contain other compounds such as cinnamic acid which are not useful as dye compounds but may have antioxidant or other favourable properties. In understanding the biological synthesis of the compounds of interest it can also help scientists to understand how these compounds may degrade as often the retro pathway is feasible. Also the reason for these intermediate compounds being present in the extraction can be deciphered if their role in the synthesis of secondary metabolites can be understood. Some compounds may not have got to the end of their biosynthetic process when the extraction was carried out and hence it is important to understand what these intermediates are and whether they will have any effect in the chromatographic profiling of the dye plant. This research aims to produce some robust methods to distinguish between the flavones present and display the importance the glycosides play in distinguishing between these species.

1.6 Extraction Isolation and Purification

Plant dyes are extracted with solvents to release the chemicals required for dyeing. The solvent used can have an effect on which compounds are released from the plant. The polarity of the solvent is key to extracting the compounds needed systematically. Plant dyes usually consist of glycosidic components and hydrolysed aglycon materials. The plants usually synthesise glycosidic components, probably to provide some water solubility to the compounds. Hence when the compounds are in their glycosidic form they are more polar and are soluble in more polar solvents such as alcohols or water. If the extraction is first done with an organic solvent such as ethyl acetate then smaller, less polar compounds will be extracted into the solution. However, if more polar solvents are used such as ethanol, methanol or water then more polar compounds would be expected to be extracted into the solution.

If it is necessary to obtain only some of the compounds from the plant, different extraction solvents may be applied in succession as shown in Figure 1.8. These extractions are modern extraction methods and solvents and could be utilised to extract different anthraquinones into different solvents.[66] Optimising the extraction of certain dye compounds could be useful in creating extracts which contain partially purified extracts. This could help to create quality extracts used in sustainable applications.[75]

However, in regards to historical textiles, the recipes used to recreate the original dyebaths must be kept as close to the original procedures as possible. Most extractions of plants would have been done in water throughout history due to the inaccessibility of organic solvents and the prices of solvents such as ethanol. This is confirmed by manuscripts, where available, in ancient documents but is an educated guess for other textiles.[12] Ancient documents describing dyeing with madder date as far back as the New Empire of the Egyptian era (1500 BC) which describe fast reds from aluminium mordanting and dyeing with madder. Pliny recorded that Egyptian alum has the best properties in reference to dyeing with mordants.[5] The *Papyrus Anastasi* suggests madder was used in ancient Egypt when discussing working conditions and describes observations of the workers as ‘his hands are red from madder like those of a man covered in blood’.[5] Later documents also show the use of a ‘madder broth’ throughout the medieval period.[76] Therefore any experiments done to recreate the historic methods would be done in a water bath using aqueous extraction.

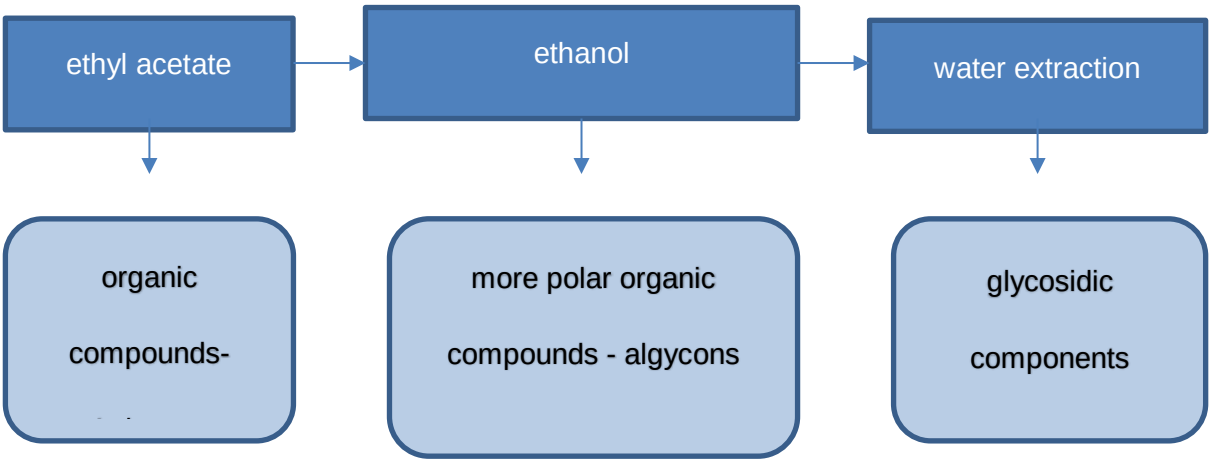


Figure 1.8. Solvent polarity and the compounds from plants most likely to be extracted.

1.7 Dyeing with Mordant Dyes

The colorant components which are extracted from madder and yellow dye plants are mordant dyes. The term mordant means to 'bite' in French and it is used to help bind the dye to the fibre. The mordant is a polyvalent metal which forms a coordination complex with the amino acids on the wool and the hydroxyl groups on the colorant compounds. The mordant is usually an inorganic salt which dissociates in solution to become a metal ion, however, organic mordants can sometimes be used such as tannins.

The most commonly used metal mordant is alum or potassium aluminium sulfate ($Al_2(SO_4)_3{\cdot}K_2SO_4{\cdot}24H_2O$) also abbreviated to potash alum.[18] Historically this was probably extracted from the minerals kalinite, alunite and leucite, however, today it is commercially available to use. Other salts used as mordants in textile dyeing were iron sulfate ($FeSO_4{\cdot}7H_2O$) and tin chloride ($SnCl_2$);[77,78] these metal ions act as Lewis acids in solution meaning they can accept electrons. This allows the metal ion to form complexes with the water and release a proton into the solution and lower the overall pH.[18] Historically cream of tartar (potassium hydrogen tartrate) was used in the mordanting process to soften the wool. Originally this salt was deposited and collected from wine casks during wine production (Figure 1.9). However, today we use a purified form which

is commercially available. The most common historical recipe used 15% omf (on mass of fibre) alum and 6% omf cream of tartar for mordanting processes.[12]

Figure 1.9. Crystals of potassium tartrate formed on the inside of a wine cork.

Dyeing with a mordant is relatively simple when used in the wool dyeing procedure. The mordant dissolves in water and the desired loading of mordant is done by placing the wool fibre into an aqueous solution of the chosen mordant and moderate heating applied. The amino acids in the fibre are susceptible to complexation with the mordant. The amino acids glutamic and aspartic acid are particularly susceptible as due to their acidic nature; they have a low pK_a of 4 which makes them easy to deprotonate in solutions with a pH higher than 4, which means they are deprotonated in neutral solutions.[18] The negative deprotonated acid can then complex with the positive metal cation which can then coordinate to the dye when added to the dyebath.

When the dye is deprotonated and coordinated, a colour change is observed due to a change in energy of the delocalised system of the chromophore. When light from the electromagnetic spectrum is absorbed by a colorant compound, certain wavelengths are reflected and the surface being observed appears coloured. The presence of π

electrons in the delocalised system allows transition from the π-π* antibonding energy levels. This transition only occurs when light of a certain wavelength is absorbed; the absorbance of this light corresponds to a colour in the visible spectrum. All the other wavelengths of light will then be reflected hence showing as a colour rather than white light and hence can be observed by the human eye.[79] For example anthraquinones display a red colour and would absorb the blue/violet end of the spectrum with wavelengths of around 420-450 nm and reflect the red end of the visible spectrum (Figure 1.10).

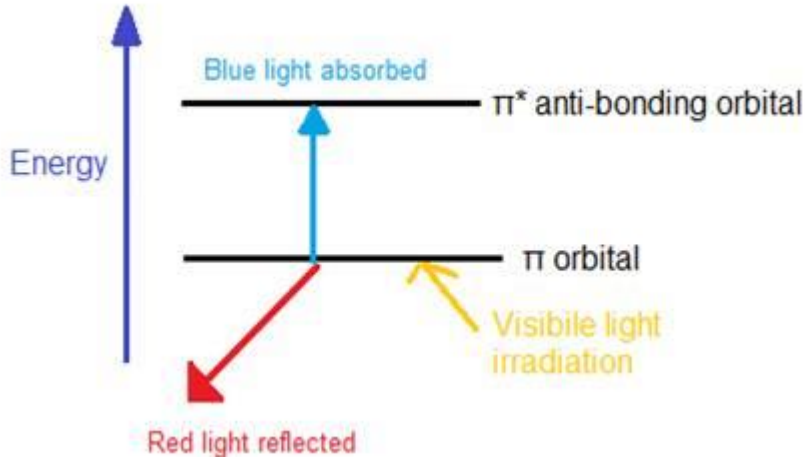


Figure 1.10. Representation of the absorption of certain wavelengths of light on a chromophore and reflection of the other wavelengths causing the object to appear coloured.

The chromophore moiety is therefore what controls the colour of the dye, however other chemical groups can be introduced to the chromophore structure which can change the size of the energy gap between the π and π* orbitals and therefore modify the colour slightly. These functional groups are called auxochromes which include: hydroxyl groups (-OH), carboxyl groups (-COOH), amino groups and sulphonic groups (-SO_3H). These have the ability to change the size of the energy gap due to the electronegativity of the groups.[79]

The type of mordant used and the metal incorporated into the complex also changes the appearance of the colour significantly. This is because upon complexation

the metal becomes incorporated into the delocalised system of the chromophore and lowers the overall energy gap of the electronic transition because metal ions have low lying energy levels. Different metals have different energy levels and therefore the type of metal mordant used can change the colour of the dyed fabric quite considerably due to the wavelength of light being absorbed varying.[18]

Protonated phenol ligands are not generally good chelators in coordination chemistry due to their relatively high pK_a values of around 9-10. However, in the presence of certain metal cations such as Fe^{2+}, Cu^{2+} or Al^{3+} the proton is more easily displaced and thus metal chelation can occur (Scheme 1.3).[80] This is due to the strong electronegativity of the oxygen atoms and their interaction with positive metal cation centres which allows exchange between labile hydrogens and metal cations. This is not the case for aliphatic alcohols in which case the oxygen anion created is not stabilised by the mesomeric effect typical of phenols.[80] The mesomeric effect describes the effect of stabilisation through resonance of delocalised systems. The presence of more than one hydroxyl group on a ring also helps to lower the pK_a of the phenol groups as they activate the ring from incorporation of the lone pair of electrons from the oxygen making the ring more electrophilic.

Scheme 1.3. Metal chelation of a phenol.

Bidentate ligands (two functional groups coordinating to the same metal) can bind to metal centres much more easily that monodentate ligands (only one available site for coordination to a metal), hence, catechol which is two phenol groups adjacent to one another can coordinate to Fe^{3+} at pH 7 but phenol does not. There are many catechol groups in the flavonoids extracted from yellow dye plants and the anthraquinones

extracted from madder plants, hence, chelation to the mordant metals on the wool is favourable (Figure 1.11). It is important to understand how mordanting works and how these dyes coordinate to the metal for developing back extractions to analyse dyes on textiles. If the mordant-metal bonds can be broken without causing degradation to the dye then a successful back extraction can be achieved. It is also important to have as much understanding of the mordant as possible for optimising dye conditions when using natural dyes industrially for sustainable applications.[27]

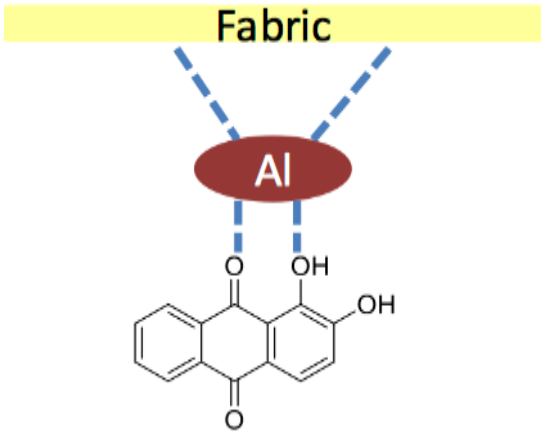


Figure 1.11. Mordant metal binding to the fabric and the dye substrate.

The anthraquinone dyes extracted from madder have two structural properties which make them good mordant dyes: (i) they must have a hydroxyl group adjacent to one of the carbonyl groups in either C-1 or C-4; (ii) hydrogen bonding must be weakened by an electron donating substituent at C-2.[81] Anthraquinones which only have one free hydroxyl group are not important to the dyeing as they do not complex easily with the mordant and therefore do not have affinity to stay on the fibre.[81]

Dyeing with madder has four main steps in the process to the final dyed textile: (i) pre-washing/scouring; (ii) pre-mordanting; (iii) dyeing; (iv) washing. Most literature uses the pre-mordanting method,[82–84] however, different shades and hues have been reported by applying an additional mordant after dyeing (before washing) or in the dyebath directly.[18] Post-mordanting can also be done, in which case the fabric is first

dyed and then treated with a metal mordant after dyeing to form a complex on the fibre. It is also possible to add a mordant into the dyebath itself, this is usually referred to as meta-mordanting.

The actual structure of the metal complex to the anthraquinones is still not fully understood as there are many ways in which the complex could occur. Kiel and Heertjes suggested that complexation occurred through the carbonyl and the adjacent hydroxyl group (Figure 1.12).[85] However, there can also be coordination through the catechol moiety where there is a hydroxyl group in the 2 position on the ring. In actuality, there is probably coordination on both sites of the compound.

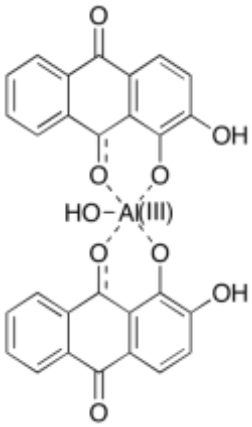


Figure 1.12. Metal complex of alizarin to aluminium.[85]

The conditions of coordination can also be affected by the pH and solvent. For example, the OH in the β position next to the carbonyl has a much higher pK_a than the OH in the γ position due to the intramolecular hydrogen bond between the lone pair of electrons on the carbonyl and the β hydroxyl in position 1. This means a much higher pH is needed in order to deprotonate this hydrogen. This type of coordination is the same as that of the flavones which could bind through the phenol group adjacent to the carbonyl or the catechol moiety as shown in Figure 1.13. Currently there is no crystal structure data on the metal coordination of these dye complexes which allow conclusive elucidation on the site of coordination of the polyphenol and the metal.

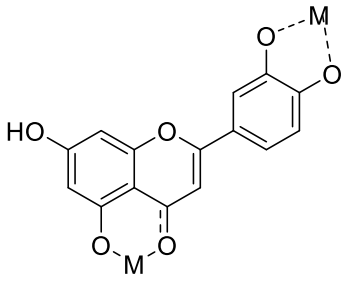


Figure 1.13. Possible metal coordination sites of luteolin.

The ability of these compounds to bind to metals is not only a property that is useful to dyeing but also a process that contributes to the health benefits of these compounds. It has been suggested that the flavonoids not only possess radical scavenging antioxidant chemistry but also the ability of these compounds to bind to iron can suppress Fenton chemistry-mediated damage.[86,87] Radical scavenging capabilities of the flavonoids has also been shown to improve when complexed to some metals.[87] It is important to understand these qualities of the dye compounds when working with the plant extracts. Metal complexes can be used to help purify material without the need for using columns which could be useful in sustainable large scale preparation of these compounds.[88] It is also good to know the radical scavenging activity of complexes as this property gives the compound good anti-oxidative qualities which could be attractive to consumers in extracts of these plants.

1.8 Analysis of Compounds in Dye Plants

As explained in chapters 4 and 5, the dye composition and relative abundance of each compound in the mixture is used to identify the plant it came from.[10,46] This makes analysis of these compounds by HPLC the most effective way of identification as it allows separation of the components in these complex mixtures. HPLC-DAD is a useful analytical tool which separates compounds for analysis according to their polarity. The column used can be either normal or reversed phase. Normal phase elution has a polar

stationary phase meaning that the more polar compounds (compounds with many polar functional groups such as –OH or -COOH) will have more interaction with the column and less polar compounds (those with high aromaticity or long alkyl chains) will have less interaction with the column. The opposite is true for reversed phase. The interaction with the column allows compounds in complex mixtures to be separated as compounds interacting with the stationary phase will elute slower than compounds which favour the mobile phase. The reversed phase chromatogram is more common in natural extract analysis.[10,62,89,90] Not only is this method a useful tool for separating compounds but also quantitative analysis of each component in the mixture can be obtained from the peak area using this method. The compounds extracted from plants are relatively polar, especially compounds containing sugars and hence reversed phase HPLC is often employed for analysis, usually an octadecyl carbon chain (C18) column is used, comparisons have also been made between UHPLC and HPLC.[91,92] Using a reversed phase HPLC column increases the hydrophobic properties of the stationary phase. This results in polar compounds eluting faster as they will have the least interaction with the column and the less polar compounds will have more interaction with the stationary phase and be slower to elute (Figure 1.14). Due to these interactions between the column and substrate, resolution between the glycosidic compounds and the aglycons in dye plants is usually good. However, separation of similar compounds in the dye mixture is not trivial.

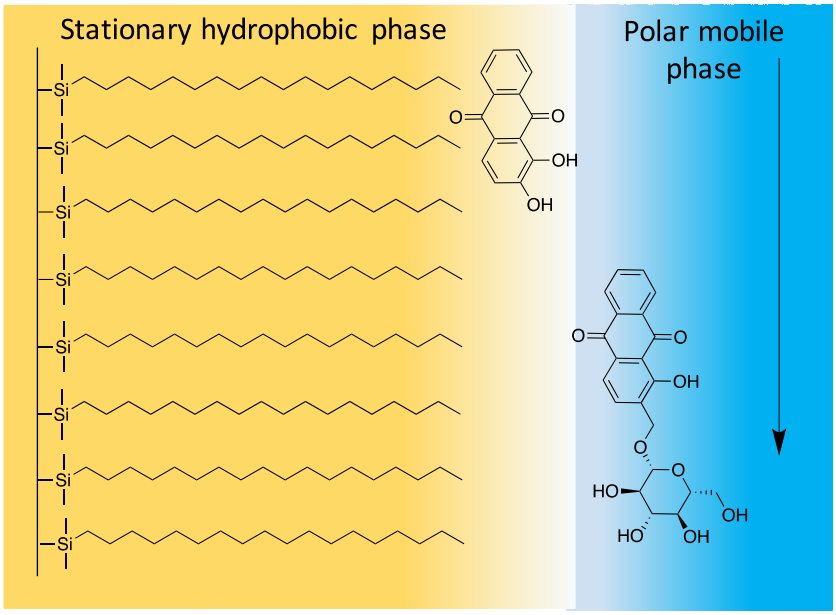
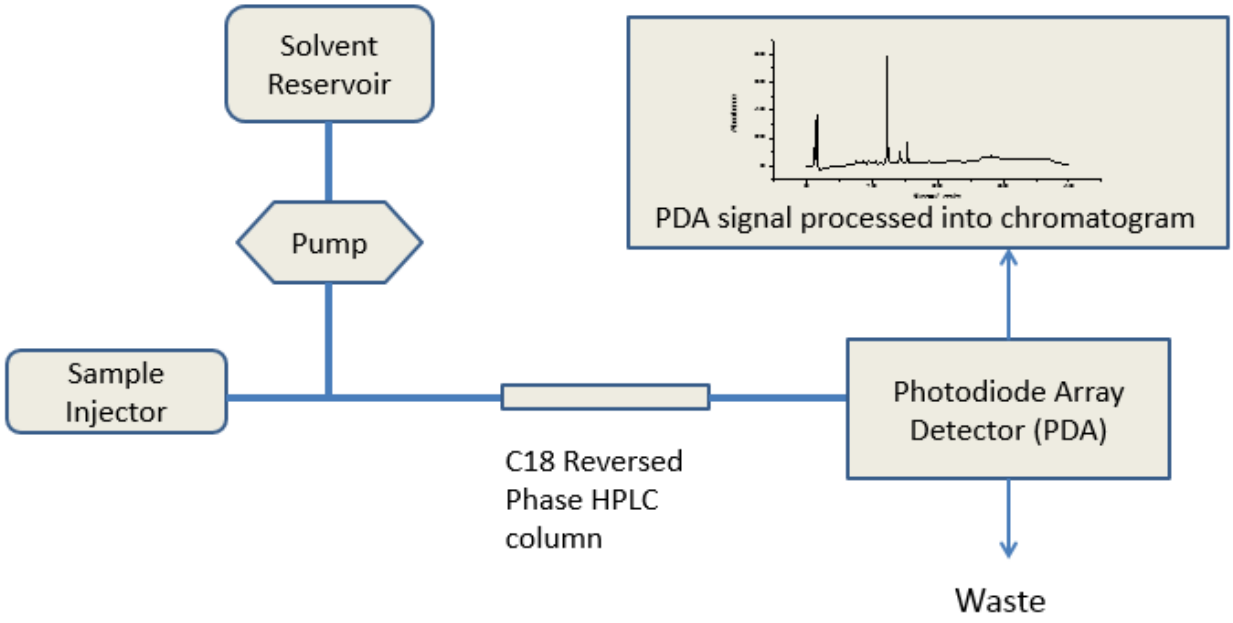


Figure 1.14. Representation of reversed phase HPLC, C18 column and a polar mobile phase showing the more favoured interaction of the polar glycosidic compounds with the less polar aglycon.

The presence of the chromophore makes dyes ideal for analysis by high performance liquid chromatography with a diode array detector (HPLC DAD).[93,2] A basic schematic of the layout of a HPLC system is displayed in Scheme 1.4.



Scheme 1.4. Basic schematic of a HPLC system fitted with a C18 column and a photodiode array detector.

The two factors used to identify compounds by HPLC DAD are the retention time in the chromatogram as they are separated out on the column as well as the characteristic UV/vis spectrum given by the use of a DAD detector. Quite often the retention time of a compound is not enough to confirm the identity of that peak in the spectrum and scientists rely on the detector to give more information about the peak in question. When a diode array detector (DAD) is used the electronic spectral characteristics of the compound being detected can also be observed. This gives more information about the compound being identified and therefore gives more reliable results. The different dye compounds usually have a unique absorbance maxima pattern which can be used to identify the dye compound in question. The absorbance maxima are different for some isomers which have the same mass and therefore some compounds can be distinguished by these methods where they could not be distinguished by other means (*e.g*. Figure 1.15).

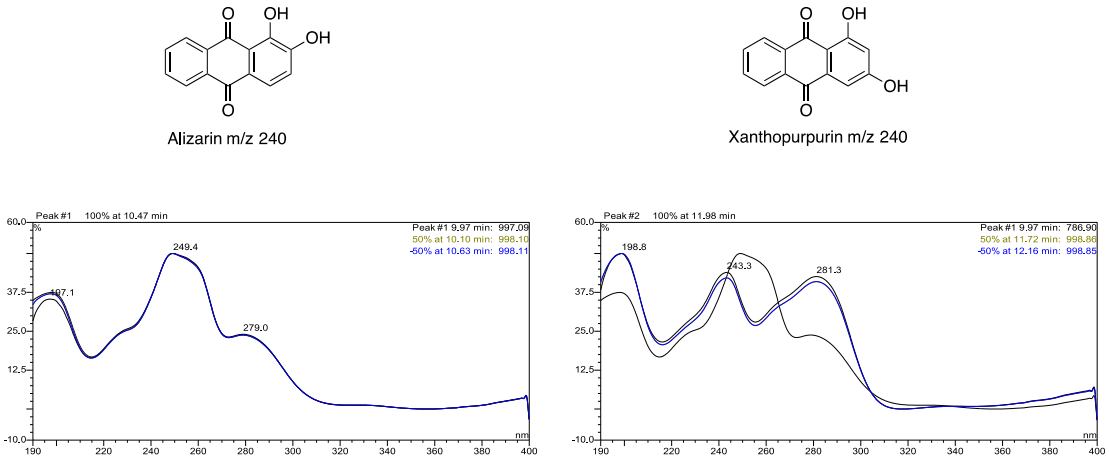


Figure 1.15. Chemical structures, masses and UV/vis spectral data of the dye compounds in madder plants; alizarin and xanthopurpurin.

Another method of detection is mass spectrometry (MS), this gives the mass of the compound giving the liquid chromatography peak. It is a reliable tool as the mass and fragmentation pattern often gives an indication of the compound in question.[2,14,94,95] Whist it is often useful to know the mass of each peak in the chromatogram it is difficult to distinguish between isomers of the same mass without knowing their retention times or having authentic samples. Fragmentation can sometimes give some indication of the structure of the compound creating the peak, for example, the loss of carbon dioxide or the loss of a sugar. However, often MS/MS experiments are required to reveal structural detail by mass spectrometry.[42]

Dye plants usually contain many different derivatives of the same family of compounds, and hence isomers often arise which have different retention times but the same mass and hence cannot be identified using only the molecular mass of the compound, for example see Figure 1.15. The presence of isomers of the same mass and identification by mass spectroscopy can also sometimes lead to the misinterpretation of some compounds in dye plants. Ferulic acid derivatives in chamomile extracts are widely cited as different compounds throughout the literature

and is a good example of how using only the mass for identification can be misleading.[46,96] These structures are discussed in further detail in Section 3.3.

Gas chromatography (GC) has been used as a method of detection for historical dyes on artefacts. However, many dye plants produce large polar compounds and they must be derivatised in order to be analysed by GC-MS.[33] Trimethylsilylation (TMS) derivatisation is to be preferred, this has been compared to tert-butyldimethylsilyl (TBDMS) and MTBSTFA reagent which gave low responses or multiple peaks for alizarin.[33] Therefore this method is a lengthy process and methods where the extraction bath can be analysed directly are often preferred. Due to the need for an extra derivatisation which is another step in the analysis which could cause discrepancies in the results GC was not considered as an analysis technique in this research.

Another separation technique considered for use in historical artefact identification is capillary electrophoresis. This technique can achieve good separations when the compounds for identification are negatively charged, hence using a buffer system or in slightly alkaline conditions.[97] Under these conditions a photochemical degradation of purpurin was observed and resulted in fading of the solution. This can be resolved by lowering the pH of the solution which then results in a compromise of the separation of the peaks.[97] Capillary electrophoresis however, has been used to separate some of the flavonoids in chamomile and linden with very short run times.[98] Due to the effects of the conditions for electrophoresis on purpurin and alizarin mentioned above and the need for alkaline conditions, this method of analysis was also deemed not suitable for this research. It would be advantageous if the analysis technique used could distinguish between yellow and red dyes when they are both dyed onto the same yarn. The need for alkaline conditions and the effect of this on the anthraquinones from madder mean that this method, whilst providing information on some dye extracts is not ideal for routine analysis.

NMR spectroscopy is a routine analysis used in organic chemistry for giving structural information on unknown compounds. Elements with odd mass numbers such as ^{1}H or ^{13}C have magnetic moments and hence give NMR signals. The chemical shift of the signal can be used to determine its molecular environment and the coupling constants can give further information on neighbouring molecular environments. From these parameters, a bigger picture can be built of the structure of the compound in question. Whilst this technique is used occasionally in analysis of natural extracts, usually coupled with HPLC it is more often employed to identify isolated compounds.[99,100]

Some methods can be used which do not require sampling of the textile at all, these techniques are often referred to as non-destructive techniques. Fourier-transform infra-red spectroscopy (FT-IR) is often used for this, hand held machines are also available to study large textiles or artefacts that are difficult to move such as cave paintings or large displays. FT-IR techniques can give information on the character of the molecular vibrations and be used to identify the presence of certain functional groups. It is often used as a complimentary technique to Raman spectroscopy, this in itself has a number of limitations such as its low sensitivity in the absence of resonance enhancement.[101] Using these techniques it is difficult to distinguish between plants of similar colorant components as there is no separation of the colorants and the results are not quantitative. However, these techniques are useful to use alongside destructive techniques which require sampling as they can give information on the organic binders, resins, varnishes or finishes of artefacts.[102,103]

1.9 Back Extraction of Historical Textiles

The method of analysis is an important consideration when identifying textiles from historic artefacts. The method of sampling is also another huge parameter which can hinder results if not carefully selected. In order to analyse dye compounds from textiles by HPLC or other chromatographic methods they must first be solubilised.

The first methods to do this were developed by Wouters et al. and involved using very strong acid of the ratios 37 % hydrochloric acid (HCl): water: methanol (2:1:1, v/v/v).[104] This strongly acidic solution allows for H^+ coordination into the metal mordants in the textile and releases the dye compound. Unfortunately, the extreme acidity of the solution also results in the breakdown of some of the compounds in the extract and a resultant loss of information see chapter 5. This technique is still used in the cultural heritage field as it is efficient in removing the anthraquinone aglycons from the wool,[10,13,105,106] however, it is more dis-favoured when analysing yellow dyes.[39,107] This is because the yellow dye extracts contain many glycosidic compounds which are easily degraded under these conditions.[39,71]

Other methods have tried to minimise the breakdown of compounds when using Wouter's method by employing the use of weaker acids. The aim of this is that the H^+ ions will still interact with the mordant metals on the textile but will allow the solubilisation of the dye compounds without degradation. Other acids used to extract textiles are; hydrofluoric acid (HF)[57], citric acid,[108] trifluoroacetic acid (TFA),[108] oxalic acid[8,84,108] and formic acid[109]. The majority of these methods still degrade glycosides into aglycons which is especially detrimental for the identification of yellow dyestuffs. Reviews have shown the extent of degradation by comparing different methods against each other but each method which involves acid and heat causes some degree of degradation to glycosidic compounds in textile analysis.[107]

Another method that has been used to extract mordant dyes from wool is the use of a good aluminium chelator such as ethylenediaminetetraacetic acid (EDTA).[77,110] EDTA contains four carboxylic acid moieties in its structure which makes it a good metal chelator due to the presence of electronegative oxygen in its structure. EDTA is a good chelator due to the chelation effect, the increased affinity to the metal due to the presence of more than one site for possible chelation. Again, it is thought that this metal complexing agent will complex to the mordant metals and allow the release of the dye

compounds into a neutral solution to give the least degradation to the dye compounds. This procedure is commonly used with solvents more able to dissolve the aglycon materials such as a mixture of acetonitrile and methanol, or dimethylformamide (DMF).[77,110] These techniques have shown advantageous results when compared to very harsh methods such as that of using hydrochloric acid in yellow dyestuffs.[77]

Other examples of 'soft' extractions have looked at using organic solvents for better solubility such as DMSO in reaction mediums, occasionally used with acid to identify dyes from historical artefacts.[14] The use of DMSO activated by acid, especially when combined with primary alcohols such as those present in lucidin can cause unwanted side effects such as the Swern oxidation which can make it difficult for the identification of lucidin under these conditions.[111]

The research in this field is uniform in the idea that a 'soft' extraction that is efficient at extracting both glycosides and aglycons from textile materials would be ideal for analysis by HPLC. Also, due to the common use of different dye plants used on the same textile in order to get a wider palette of colours, it is desirable for the back-extraction techniques to efficiently extract different families of compounds.

From the literature, methods have been developed which are robust in detecting aglycon compounds when analysing dye plants. These aglycon compounds are very important for the analysis of dyed textiles and can lead to dyestuff identification. However, there is an overall loss of information when acidic methods are used due to the degradation of some of the sensitive compounds in plant extracts. Therefore, development of a less detrimental back extraction technique would be advantageous in order to gain as much information as possible on the original dyeing of historical textiles whilst still providing the detail needed for robust identification. It could also be possible to use a number of back extraction techniques which result in more compounds being identified, for example the use of a soft extraction to identify glycosides and then the use of an acidic extraction.

1.10 Aims of Research

The main aims of this research were to further establish work done by Robert Henderson *et al* at the University of Leeds of novel 'soft' back extractions using glucose. These novel extraction techniques were applied to small sample sizes herein which simulate the sample sizes used by museums. The main aim of this work is to establish a method which is able to cause the least degradation to any of the compounds in madder and hence can retain as much information for cultural and heritage interests as possible. These novel extractions were compared to other back extraction techniques currently being used in the cultural heritage sector with the aim to see how well it performed in comparison. The aim was to get full chromatographic data of all of the back extractions for three different types of madder to compare the results.

In order to better understand how this extraction method works the project aims were to fully elucidate compounds present in the complex matrix presented by plant extracts and provide robust methods of analysis for elucidation of these compounds and to minimise the presence of unknown peaks in chromatograms.

This project aims to highlight the importance of full structural characterisation for compounds in complex mixtures when using HPLC as an analysis tool. The aim of the project was to decipher as much information as possible about the chemical compositions of the plants chosen to study in the thesis. The aim was to have chemical compositions which would look similar when hydrolysed and therefore display the importance of preserving compounds in the same form *in planta* for dyestuff analysis; for example, as the glycosides.

The project aims to synthesise compounds which are not available commercially in order to conclusively identify all peaks in the chromatograms. The synthesis of compounds which are not commercially available was also done to understand their

behaviour in solutions and in the conditions of back extractions, for example, strongly acidic conditions.

This project also aims to investigate efficient and cost effective methods to purify natural anthraquinone and flavonoid dye compounds. This was done through extraction in different solvents and using SPE resins to investigate if an extract which is easier to work with and contained less unwanted materials such as sugars and tannins was viable.

Another project aim was to understand as much as possible how compounds in the dye baths interact with mordanted wool when being adsorbed onto the fibre. This project strives to understand which compounds are adsorbed most easily onto the wool and the mechanisms of adsorption of individual compounds in the extracts to see what effect different functional groups have on the dyeing capability of wool.

2 Extractions of Madder

Colorants need to be extracted from the roots of the madder plant in order to be used as a dyestuff. A number of different solvents can be used for this extraction giving a slightly different chromatographic profile depending on the polarity of the chosen solvent. The differing chemical profiles can then be used as a fingerprint in order to identify dyestuffs in artefacts. Therefore, it is important to understand fully the chromatographic profiles of the dyestuffs being studied and the effects of the solvents on extraction before embarking on any analysis of artefacts.

This chapter of work was done to develop robust reference analytical data which can be used for comparison when these extracts are dyed onto wool. The main aim of this study was to highlight the chemical differences between the dye plants chosen to study. In displaying the differences between the different plant compositions, their back extractions and dyeing capabilities can be studied in later chapters. Although the work done in this chapter highlights differences in the dye compositions from *Rubia tinctorum,* only one sample from each region is tested herein. Therefore, this study aims to show that the chemical compositions from the chromatographic fingerprints can be used to distinguish between these particular plants and that the methods developed do not modify the chemical composition. However, this does not mean that every plant from that region would have the same fingerprint. In order to show a trend in regional chemical composition changes, more samples from each region would be needed for study, and the cultivation, harvesting and processing before extraction of each dye plant would need to be the same in order to ensure the variables are minimised.

Extractions using water, ethanol and ethyl acetate were done to analyse the chemical content of the dye extracts. HPLC was used as the method of analysis as it is an effective method of measuring the chemical composition of the complex matrix of compounds obtained when looking at plant extracts. HPLC is effective because of its

ability to separate the compounds in the dye extract, this is advantageous as it is possible to see the number of compounds present and also the relative ratios of compounds abundant in the mixture. HPLC is also useful as it is possible to take aliquots from liquid extracts which can be directly analysed. One limitation of this technique is that good separation sometimes requires long run times. This however can be overcome by optimisation of the solvent system and the type and length of column used.[1,58,91] The use of HPLC is also advantageous due to the fact that different detectors can be used. Herein HPLC coupled with mass spectrum and diode array detection was utilised. This allowed identification by both the UV/vis spectra given by that particular compound and the mass of the compound. Using both techniques is advantageous because some of the compounds have the same mass and hence can only be distinguished by their retention time and spectral data. Whereas other compounds can have very similar spectral data but are of diffing mass and hence can be easily distinguished from one another. Negative ionisation was chosen because literature states that it is more robust at detecting the anthraquinone aglycons being studied. Whilst the glycosides can be detected in both positive and negative ionisation, the aglycons were only ionised in the negative ionisation.[63]

Three types of *Rubia tinctorum* were chosen for comparison: English Madder, Turkish Madder and Iranian Madder (Figure 2.1). English madder was grown in Bristol and was dried in air in the dark. Turkish and Iranian madder samples were supplied by a leading dye master in Shiraz, Iran, where the madder is sourced locally for use in carpets and weaving; these samples are more likely to have been dried in the traditional way first by steaming the roots and then drying in the sunlight.[4] However all dye plants were bought from the commercial source George Weil and hence there could be potential for discrepancies in products supplied. Although HPLC analysis throughout this study for each dye plant source supplied was consistent and hence the chance of being supplied a different product is minimised.

Figure 2.1. Dried madder samples as obtained from supplier from left to right Iranian madder, English madder and Turkish madder.

Historically water would have been used as the dye extraction solvent and the dyeing would take place straight after the filtration to remove the plant residues; extraction of madder would have taken place at varying temperatures for textile dyeing. However, this is problematic for analysis, as it can be difficult to remove water as a solvent after extraction due to the high temperatures required to evaporate. Hence, the dye extracts are usually diluted and analysed in water by liquid chromatography methods. Unfortunately, this is not acceptable if other methods of analysis are going to be used. For example attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy, as samples have to be in a solid state, or for nuclear magnetic resonance (NMR) spectroscopy; specific solvents (such as deuterated solvents) need to be used and water cannot be present in the analysis. This can be overcome when analysing compounds by FTIR by using different preparation techniques such as preparing potassium bromide disks but cannot be overcome for NMR. However, preparing solutions for IR using these alternative methods can still be problematic due to diminished signals because of the presence of solvent in much higher amounts than the

compound in question. Water can be removed by freeze drying samples, and although this is time-consuming, analysis of each method is only required once, and water *in situ* can be used for dyeing under usual conditions. Extractions can also be carried out in other solvents such as ethanol, methanol or aqueous mixtures of these solvents for sustainable applications. It is interesting to compare historic water-based methods with alternative solvents to determine if there is a more efficient way of extracting the dye compounds from the plant roots. The use of different solvents for extraction of the colourants from the plants is not novel but does give a better understanding of how the colourants of interest for this thesis behave under different polarity conditions.[34]

For analytical and research purposes after extraction, dyebaths can be further treated in different ways depending on the desired product. Solid phase extraction (SPE) using Amberlite XAD 7HD can be used to remove free sugars and other undesired compounds from the solution in order to concentrate and purify the colorant materials, and comparison of the compounds present before and after SPE was carried out. This work was to establish if the use of a resin is selective to some of the anthraquinones or whether all of the anthraquinones can be collected and concentrated by SPE. As SPE is used as a method to remove free sugars and non-phenolic compounds, this could provide a dye extract that is richer in the desired compounds and does not contain any compounds which could inhibit fixation or compete with the dye onto the wool mordant sites. The application of solid phase extraction has been previously studied by Derksen *et al*. but only for the separation of alizarin from other organic compounds in aqueous extracts.[81] Whilst SPE is a method commonly employed for concentrating organic compounds and separating desired materials herein is the first known example of purification of the coloured anthraquinones from the roots using an amberlite resin. However studies using SPE resins have previously been carried out on cell cultures from *Rubia tinctorum*.[112]

Water, ethanol and ethyl acetate were used as extraction solvents due to their different polarities to test the effect of this parameter on the compounds extracted. Sequential extractions were done to identify the effect of the gradual increase of solvent polarity on the compounds obtained (Figure 2.2) for order of polarity of the three solvents. This involved an initial extraction at reflux temperature in ethyl acetate and then filtration of the solids, which were then collected and further extracted with ethanol, filtered and collected for subsequent extraction with water. The resulting filtrates were dried under vacuum in a rotary evaporator >40 °C, or in the case of water, by freeze-drying. The samples were analysed by taking aliquots straight from the dyebath, diluting with methanol to provide a 1:1 methanol: water solution, and performing high performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC-MS). In each case the results were compared.

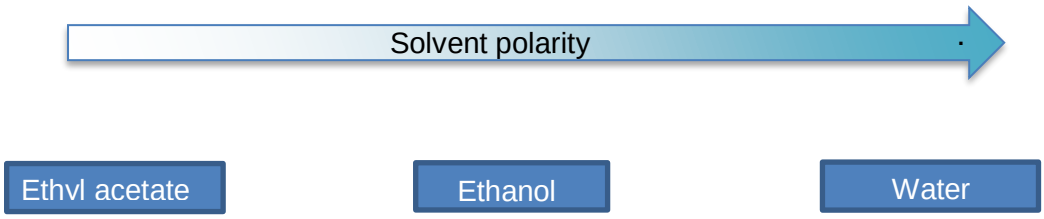


Figure 2.2. Polarity of the three different solvents chosen for the study of extraction from madder plants.

2.1 Extraction of different madder varieties

Iranian, Turkish and English madder were extracted as described in experimental section 6.3. Extraction was carried out in these three different solvents to measure the effect of polarity on the compounds extracted. These three solvents, or solvents with similar polarities have been previously used to extract from the roots of *Rubia tinctorum* and this is not a novel application but gives a good basis for understanding the effect of solvent on the specific samples available for this study.[113,114] Also it is important to build

a solid background of understanding of these specific dye plants and their chromatographic profiles. Observing which solvent the compounds are most easily extracted in gives an indication of their solubility and which solvents would be most favourable when back extracting from textiles. Table 2.1 summarises the compounds identified when extracted with ethyl acetate, ethanol and water. Total yields (mass of extract after extraction and solvent evaporation) of all of the extractions are summarised in Table 2.2.In all madder types, water yields the highest amount by mass of dye extract. English madder has the smallest mass of material extracted when water and ethanol are used as the solvent, but the highest with ethyl acetate. This is possibly as a result of a lower level of free sugars in the material displayed later in NMR studies of plant extracts (Figure 2.14) and higher abundance of less polar aglycons see chromatograms (Figure 2.5). It is noted that pseudopurpurin is not observed in the extracts summarised in Table 2.1. Although pseudopurpurin is reported to be present in fresh samples of *Rubia tinctorum* it is known to decarboxylate under storage or drying conditions.[115] These extractions were carried out on dried roots and hence it is probable that any pseudopurpurin present in the original fresh roots was decarboxylated and hence not observed herein.

Table 2.1. Compounds identified in Iranian, Turkish, and English madder when extracted with ethyl acetate, ethanol and water. + indicates compound present.

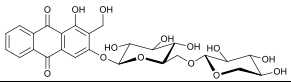
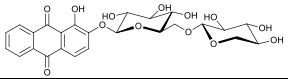
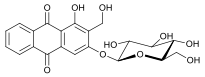
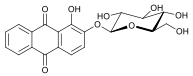
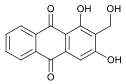
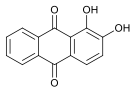
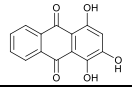
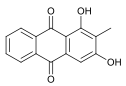
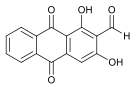
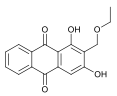
Identified Compound	Structure	Mass LC-MS $[M-H]^-$	λ_{max} (nm)	Iranian			Turkish			English		
				Ethyl Ac	Ethanol	Water	Ethyl Ac	Ethanol	Water	Ethyl Ac	Ethanol	Water
Lucidin primeveroside (3)		563	200, 246, 285	+	+	+	+	+	+		+	+
Ruberythric acid (1)		533	224, 256	+	+	+	+	+	+		+	+
Lucidin glucoside (3)'		n.d.	200, 246, 285	+								
Alizarin glucoside (1)'		n.d.	224, 256	+								
Lucidin (4)		269	200, 247, 288	+	+		+	+		+		+
Alizarin (2)		239	198, 249, 279	+	+	+	+	+	+	+	+	+
Purpurin (11)		n.d.	210, 255, 294	+								
Rubiadin (13)		n.d.	247, 285	+	+					+	+	+
Nordamnacanthal (5)		267	258, 285	+		+				+		+
Lucidin ethyl ether (4')		297	n.d.		+			+				

Table 2.2. Yields of Iranian, Turkish and English madder with different extraction solvents.

Madder type	Yield from extraction solvent (g)		
	Ethyl acetate	Ethanol	Water
Iranian	0.09	0.60	3.70
Turkish	0.09	0.56	3.20
English	0.19	0.12	2.90

2.1.1 Extraction of Iranian madder

The HPLC chromatograms of extracts from Iranian madder root after extraction with ethyl acetate, ethanol, and water are shown comparatively in Figure 2.3, the samples were analysed in triplicate by HPLC. Extraction with ethyl acetate (Figure 2.3a) demonstrates that the main peak observed is alizarin (2), which is expected as alizarin is not very polar, hence the use of a non-polar solvent should extract the non-polar compounds from the dye plant. The glycosides lucidin primeveroside (3) and ruberythric acid (1), which are present in high amounts in the plants, are also observed. Unfortunately, the complete resolution was not achieved between the glycosidic compounds. Optimisation of the method on the HPLC system was attempted by changing the gradient parameters of the mobile phase and the temperature of the oven but resolution of the glycosides was unfortunately never obtained. This could have been due to the age of the column being used for these studies. The only C18 column available for use on the HPLC was over three years old and although it had been well maintained there could have been some damage to the long carbon chains of the stationary phase causing unresolved peaks. However, this was a qualitative study to distinguish which compounds are present in the plants chosen and therefore although it was not ideal, resolution of the peaks in the chromatograph was not vital for this study.

It is unexpected that extraction of these large polar sugars is possible with ethyl acetate, but the high concentrations of these compounds present in the plant probably led to some extraction. The aglycon lucidin (4) was also observed along with its oxidised derivative nordamnacanthal (5). Peaks corresponding to alizarin monoglucoside and lucidin monoglucoside are also detected; it is unclear whether these peaks are due to degradation of ruberythric acid (1) and lucidin primeveroside (3), respectively, in the extraction process or if these compounds are present *in planta*. Analysis by LC-MS showed peaks with a molecular weight matching lucidin primeveroside (*m*/*z* = 563) and ruberythric acid (*m*/*z* = 533), as well as the molecular weights of lucidin (*m*/*z* = 269), alizarin (*m*/*z* = 239) and nordamnacanthal (*m*/*z* = 267). The data for all mass spectra discussed is displayed in Table 2.3.

The main compositional change between these three solvent systems is the higher abundance of the aglycons in the ethyl acetate solvent. This is expected as explained above due to the less polar nature of the aglycons. It is shown in the chromatograms that some of the aglycons can only be extracted using ethyl acetate. For example, purpurin is only observed when extracted with ethyl acetate in the research conducted herein and hence the use of this solvent as an initial extraction could be diagnostic in identifying these compounds in plant extracts.

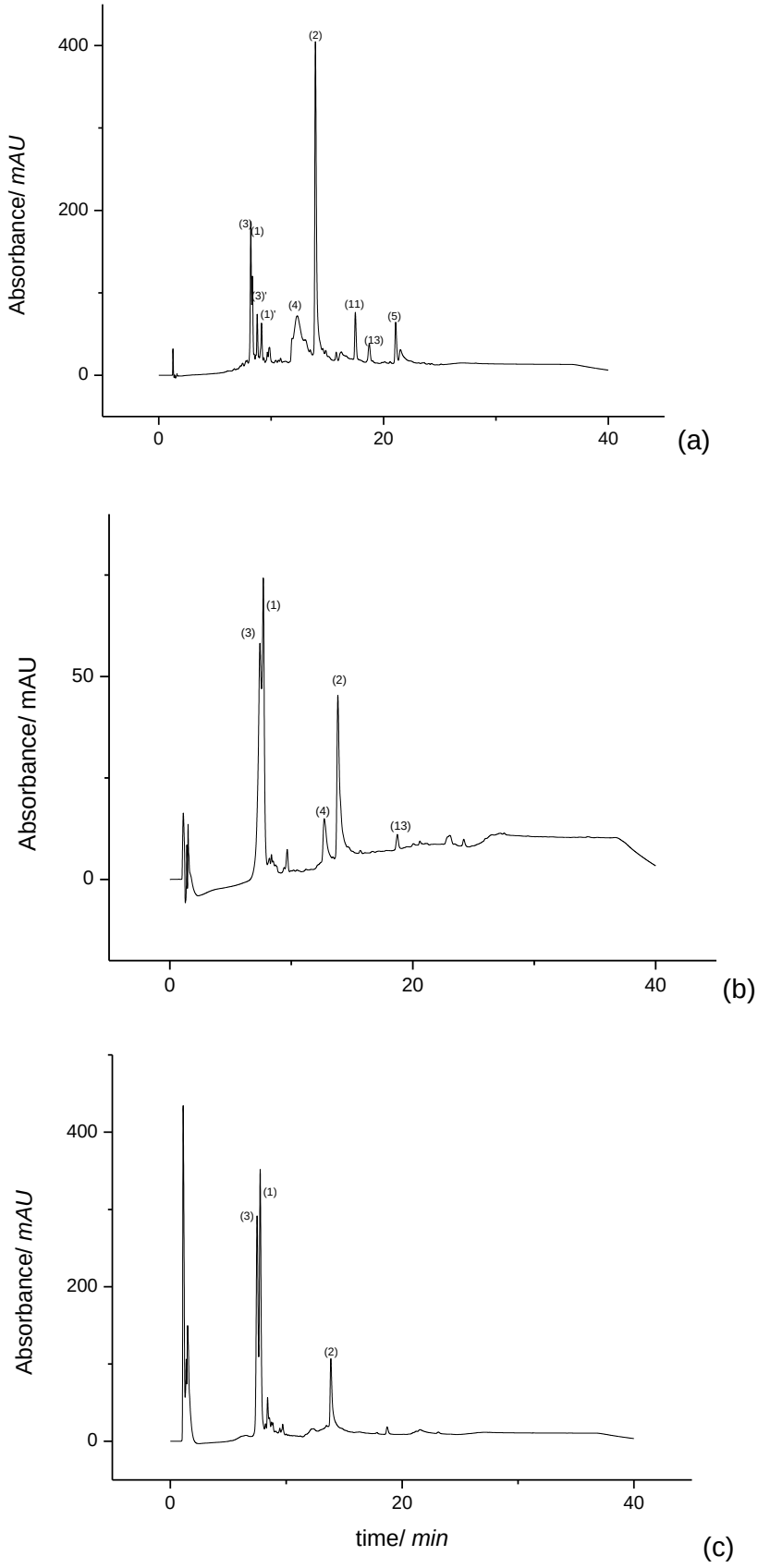
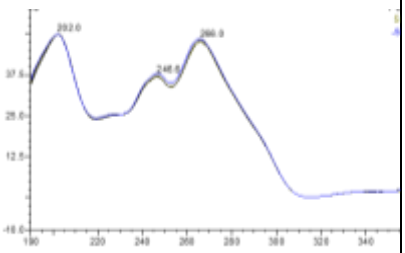
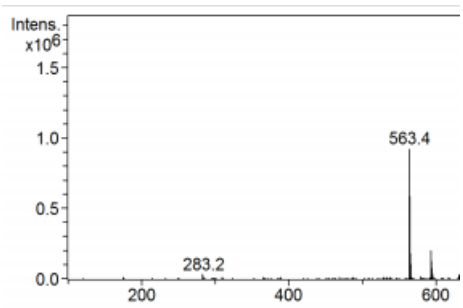
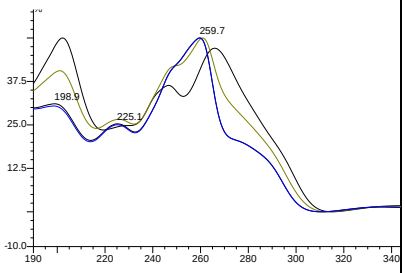
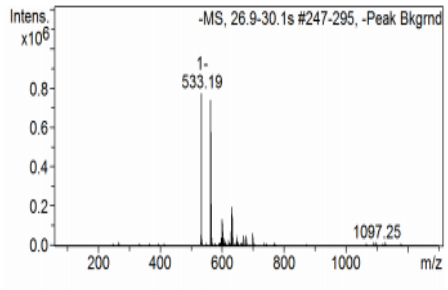
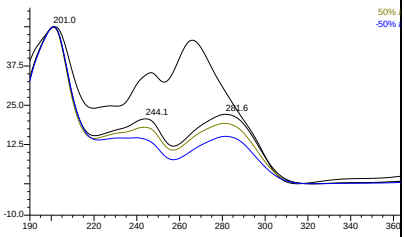
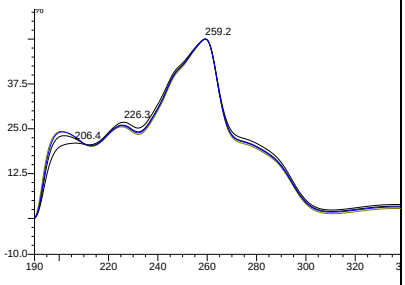
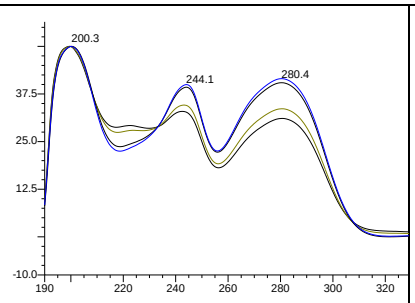
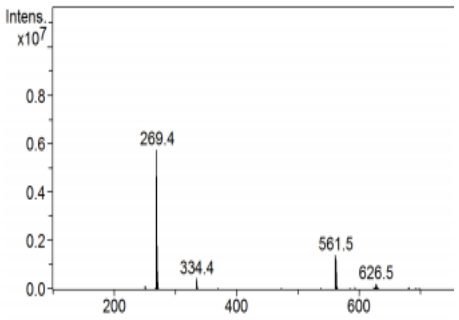
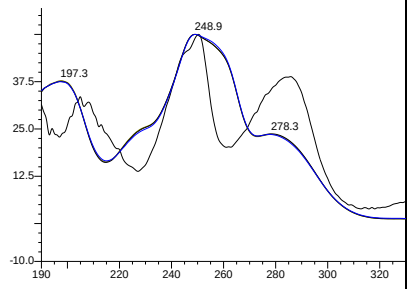
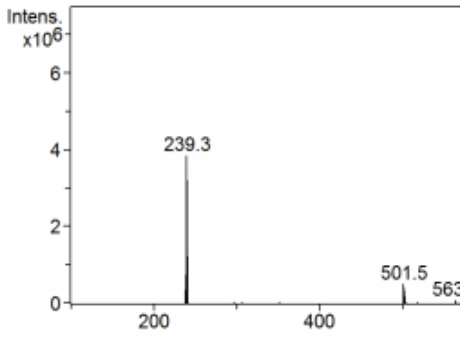
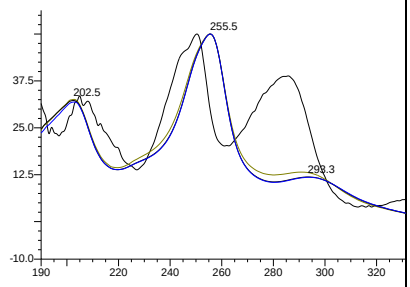
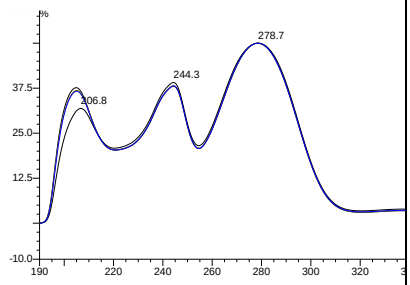
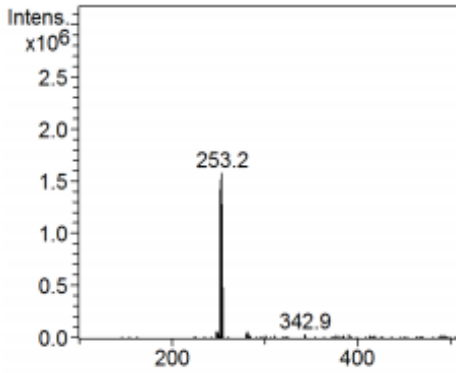
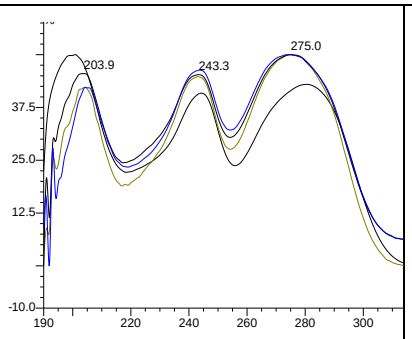
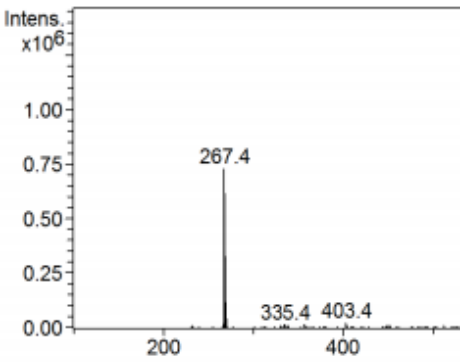
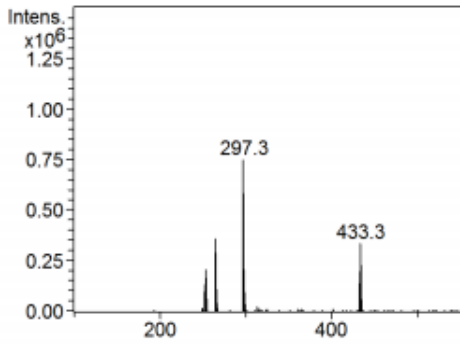


Figure 2.3. HPLC chromatogram of extracts from Iranian madder root after extraction with: (a) ethyl acetate; (b) ethanol; (c) water.

Table 2.3 UV/vis and mass spectrum data of all compounds identified in the HPLC chromatograms of madder varieties using different solvents.

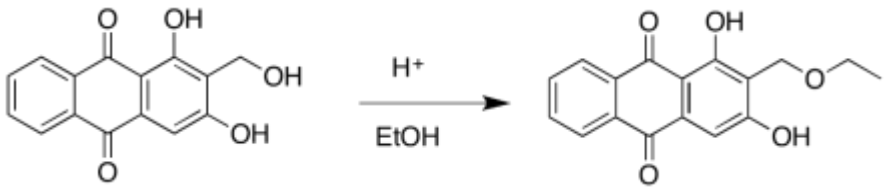
Compound Identified	UV/vis trace from HPLC	Mass Spectrum
Lucidin Primeveroside		
Ruberythric acid		
Lucidin Glucoside		-
Alizarin Glucoside		-

Lucidin		
Alizarin		
Purpurin		-
Rubiadin		

Nordamnacanthal		
Lucidin Ethyl Ether	-	

Extraction with ethanol (Figure 2.3b) demonstrates that the main peak observed is the combined peak of the glycosides lucidin primeveroside (3) and ruberythric acid (1). Ethanol is a more polar solvent than ethyl acetate so it would be expected to extract the more polar compounds more favourably. The peaks corresponding to the aglycons lucidin (4) and alizarin (2), and a very small peak assigned to rubiadin (13), were also observed. LC-MS showed the main peaks with a molecular weight corresponding to be the glycosides of lucidin primeveroside (*m*/*z* = 563) and ruberythric acid (*m*/*z* = 533). There were also corresponding masses for lucidin (*m*/*z* = 269), alizarin (*m*/*z* = 239) and a peak giving a mass of 297 in the negative ionisation mode which corresponds to the ethyl ether derivative of lucidin (4'). This is not a compound present *in planta*, but an artefact of lucidin reacting with the ethanol at the temperature of the extraction (Scheme 2.1).[56] These artefacts formed in the extraction solution are undesirable in an extraction procedure as they modify the ratio of coloured compounds in the mixture and can

therefore lead to false identification of dyestuff. All of the spectral data for the peaks observed in these reactions were matched to data shown in Table 2.3.



Scheme 2.1. Formation of the lucidin ethyl ether artefact in acidic conditions when ethanol is used as a solvent.

From Table 2.1 it is interesting to note that some compounds can only be detected by certain methods. This is probably due to the very small amount of these compounds present in the dyebath and also due to the complexity of the mixture to be analysed. Lucidin ethyl ether is tentatively assigned by the correct mass, but could not be identified on the HPLC chromatogram as there is insufficient data to assign it. There are two small peaks in the chromatogram which have not been assigned, one of these could be due to lucidin ethyl ether which elutes after 20 minutes. However, the authentic standard was not available commercially and hence identification of this compound on the HPLC could not be confirmed therefore its identification was based solely on the mass and literature findings.[25,62,116]

Extraction with water (Figure 2.3c) demonstrates that the main peak observed is the combined peak of the glycosides lucidin primeveroside (3) and ruberythric acid (1), which are extracted in high yields. Again, there is some extraction of the aglycon alizarin (2). LC-MS data shows extraction of water yields the compounds: ruberythric acid (*m*/*z* = 533), lucidin primeveroside (*m*/*z* = 563), alizarin (*m*/*z* = 239) and nordamnacanthal (5) (*m*/*z* = 267).

In summary, it is observed that the main compounds in Iranian madder root are the glycosidic compounds ruberythric acid (1) and lucidin primeveroside (3) for water and ethanol extraction. These two extraction techniques also give a higher yield by mass

of the crude material extracted and dried which would suggest more anthraquinone content. The aglycons alizarin (2) and lucidin (4) are also present but in smaller concentrations and are more easily extracted when ethyl acetate is used as the extraction medium as displayed in the chromatographic profiles however the ethyl acetate extraction does give a lower overall yield of dried extract. This shows that following a recipe of dyeing from historical times where water would have been used as the extraction solvent, the dyer would have resulted in an extract rich in the glycosides if using Iranian madder treated under the same conditions. Understanding the dyebath composition is important when analysing historical textiles as it will give further information on the possible reactions and transformations of each compound in the dyebath when extracting them back from the textile.

2.1.2 Extraction of Turkish madder

The same extraction process was carried out with Turkish madder in order to evaluate if the origin of the plant (where it was grown) had an effect on the composition of the colorants present *in planta*. The Turkish madder obtained from George Weil & Sons Ltd. went through a very similar processing procedure to that of Iranian madder, hence we would expect to see a very similar extraction profile of these dyestuffs. All peaks in the chromatograms were matched to the UV/vis and mass spectra data shown in Table 2.3.

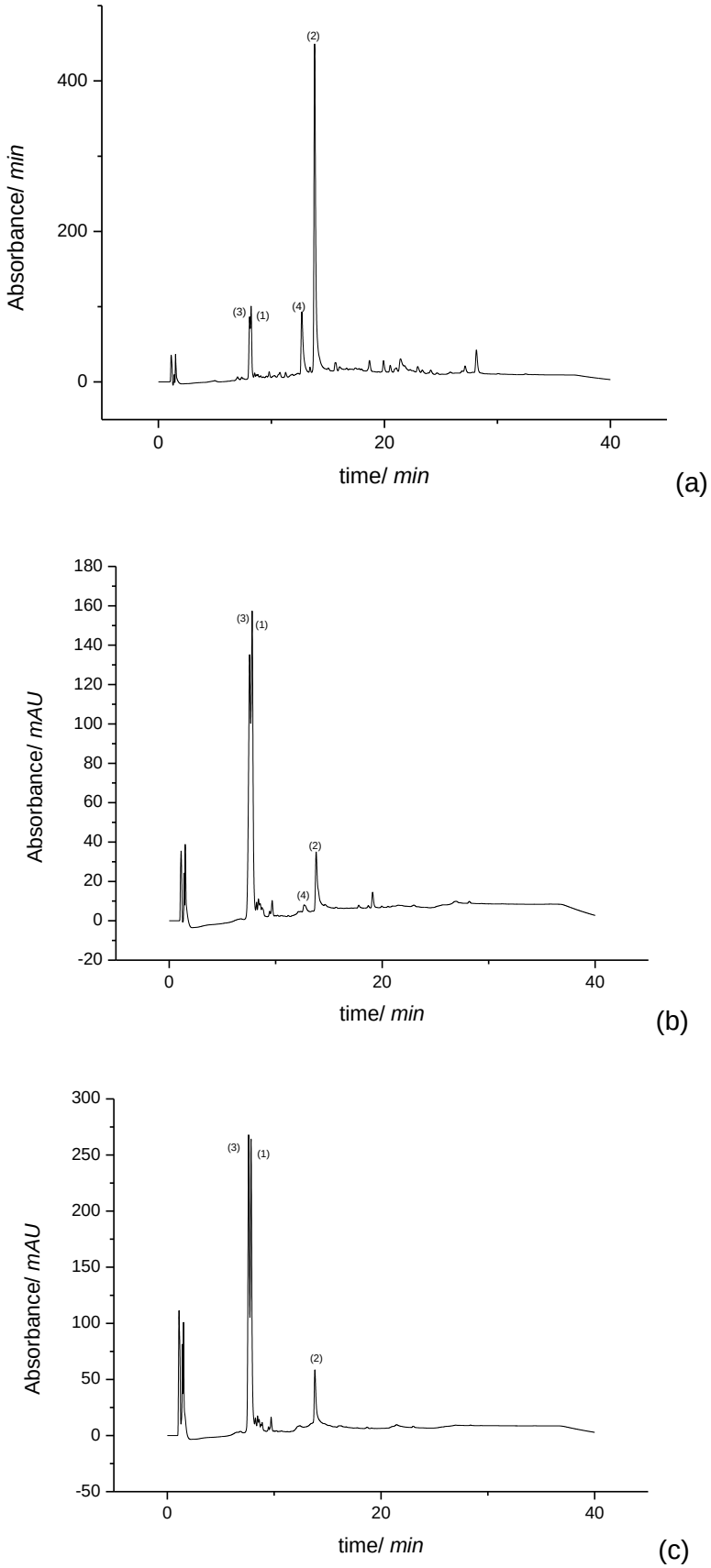


Figure 2.4. HPLC chromatogram of extracts from Turkish madder root after extraction with: (a) ethyl acetate; (b) ethanol; (c) water.

Ethyl acetate extraction of Turkish madder (Figure 2.4a) was very similar to that of Iranian madder; the main compound in the extract was alizarin (2) some lucidin (4) was also extracted, but not in as high concentrations as that of alizarin. There was again some extraction of the glycosides lucidin primeveroside (3) and ruberythric acid (1), but this was probably due to them being present in high amounts in the plant. LC-MS results showed the mass for the glycosides lucidin primeveroside (*m*/*z* = 563) and ruberythric acid (1) (*m*/*z* = 533) as well as those for lucidin (4) (*m*/*z* = 269) and alizarin (2) (*m*/*z* = 239). This confirms the results observed by HPLC. This result was to be expected as alizarin (2) has a high affinity for the ethyl acetate due to it not being very polar, it has a very limited solubility in water, hence, a larger peak would be expected in the extraction with ethyl acetate.

Ethanol extraction of Turkish madder (Figure 2.4b) yielded mainly the glycosidic compounds lucidin primeveroside (3) and ruberythric acid (1). There is some alizarin extracted and detected by HPLC, but it is not a major compound present. The compounds observed by LC-MS were lucidin primeveroside (*m*/*z* = 563), ruberythric acid (*m*/*z* = 533), lucidin (4) (*m*/*z* = 269), alizarin (2) (*m*/*z* = 239) and lucidin ethyl ether (4') (*m*/*z* = 297), which is an artefact of the ethanol extraction process described in section 2.1.

Water extraction of Turkish madder (Figure 2.4c) again yielded the glycosides ruberythric acid and lucidin primeveroside and the aglycon alizarin. The glycosides lucidin primeveroside (*m*/*z* = 563) and ruberythric acid (*m*/*z* = 533) were detected by mass upon LC-MS analysis. Alizarin was not detected by LC-MS in this sample, possibly due to the high concentration of glycosides in the extract. It is expected that Turkish madder would behave in the same way as Iranian madder as they are both grown in similar climates and are thought to have been through a similar drying process. The peaks in the chromatograms were assigned based on matching to the UV/vis and mass spectra data shown in Table 2.3.

2.1.3 Extraction of English madder

Extractions were carried out as described for Iranian and Turkish madder. HPLC analysis of the ethyl acetate extraction of English madder (Figure 2.5a) shows two major compounds in the chromatogram corresponding to alizarin (2) and rubiadin (13). This is probably due to these two compounds having the fewest polar functional groups on the anthraquinone backbone, hence favouring solubility in a non-polar solvent. There are fewer glycosides present in this extraction when compared to the same extraction with Turkish and Iranian madder, but this could be due to there being smaller amounts of the glycosides present in English madder. This difference could be due to the way the dyestuff was dried; English madder bought from George Weil & Son Ltd. was grown by Dr. David Hill at Bristol University and most likely dried in the dark in an outbuilding, due to the colder climates of the UK. It is likely in this case that the drying conditions herein do not denature the endogenous enzymes in the madder root and hence the aglycons could be enzymatically formed from hydrolysis of the glycosides. LC-MS data in the negative ionization mode shows four peaks corresponding to lucidin (m/z = 269), alizarin (m/z = 239), rubiadin (m/z = 253) and nordamnacanthal (m/z = 267). This extraction method is therefore suitable if an extract rich in the aglycons alizarin and rubiadin is desired. There are small amounts of lucidin present in the dyebath.

HPLC analysis of the ethanol extraction of English madder Figure 2.5b shows the appearance of the main compound alizarin. There is also again the appearance of the peak corresponding to rubiadin, however, in the ethanolic extraction there is also the appearance of two more peaks corresponding to the glycosides: lucidin primeveroside and ruberythric acid. English madder extracted with ethanol did not ionise any of the compounds in negative ionisation mode. This could have been due to the lower concentration of the English madder samples in the analysis of ethanol extractions due to solubility issues.

Figure 2.5c showed the main peak in the extract as alizarin (2). There is also a broad peak corresponding to lucidin (4) in the chromatogram that was not present in either of the above chromatograms. The peaks corresponding to the glycosides are also present when water is used as an extraction method of English madder. The ratio of glycosides to aglycons differs in English madder to those of Turkish or Iranian origin. In general, the water extract of English madder is much more complex than the other samples, this could be due to the way the plants are treated when drying and hence more of the aglycon compounds are observed, alternatively these differences could be *in planta*. Either way, these differences in the extracts of the plant provide a good platform on which to study the dyeing mechanisms and to test back extractions of complex mixtures with different known compositions. LC-MS data shows masses of lucidin primeveroside (m/z = 563), ruberythric acid (m/z = 533), alizarin (m/z = 239), and nordamnacanthal (m/z = 267). Again, all peaks in the chromatogram were matched to the characteristic spectra displayed in Table 2.3.

It would be interesting to further study more samples of madder, specifically grown in the UK to gain further understanding if these differences were *in planta* or due to other variables such as drying or storage conditions.

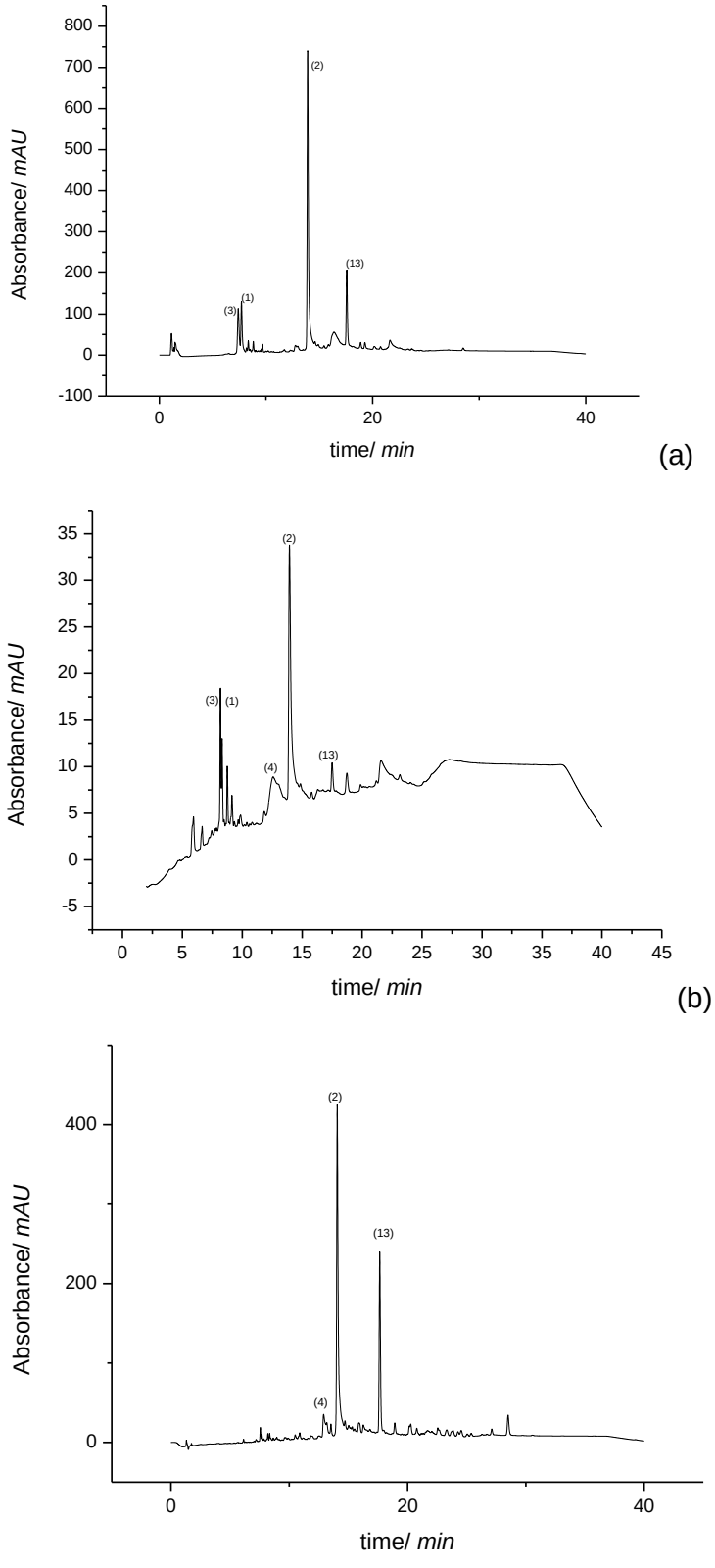


Figure 2.5. HPLC chromatogram of extracts from English madder root after extraction with: (a) ethyl acetate; (b) ethanol; (c) water.

It was noted that in all three types of madder extracted, the ethanol chromatogram was of a much lower concentration and therefore had a higher signal to noise ratio. This was due to the way the samples were prepared for HPLC. As explained in experimental section 6.3 the samples were all evaporated to dryness after extraction in order to measure their crude yields. They were then re-dissolved in a 1:1 mixture of methanol: water for HPLC analysis. It was of upmost importance to ensure that all of the components of the mixture were fully re-dissolved and hence all of the compounds important to this study would be detected by HPLC. The ethanol extracts proved more difficult to re-dissolve in all cases and hence needed more solvent for full solubilisation and therefore are displayed in the chromatograms at a lower concentration.

2.2 Aqueous extractions of madder followed by Solid Phase Extraction (SPE)

Solid phase extraction of the plant extracts was carried out to concentrate and purify the colorant compounds in the extraction. The aim of this study was to try to purify some of the compounds from the extract in an efficient and sustainable way. The use of SPE was chosen as the solvents required for this partial purification are water and ethanol which are both 'green' solvents.[117] Thus using this method would be a sustainable option for the partial purification of these extracts. Water extraction of all three types of madder were compared by HPLC and LC-MS to the samples that had undergone SPE using Amberlite XAD 7HD resin. Amberlite XAD 7HD was chosen for study due to its availability in the lab. The resin is a nonpolar resin which can be used for adsorption of organic materials from aqueous solution. It has a pore size large enough for the compounds of interest to this study to be adsorbed and therefore was considered a good option for the use of SPE in partial purification of anthraquinones from aqueous extracts. SPE allows concentration of the dye compounds within the solution and in this case removes many of the free sugars which are very polar and hence will not be adsorbed to the resin and

other unwanted non-phenolic compounds from the dye solution shown in Figure 2.6. Polyphenolic compounds in the madder extract have high affinity for the resin and adhere to the resin when washed with water. Non-phenolic compounds in the extract have more affinity for the water and stay in solution and are separated from the mixture. Subsequently, the resin can be washed with ethanol to remove bound polyphenols and allow them to be concentrated into the ethanolic solution, which can then be evaporated to dryness and the compounds isolated can be analysed.

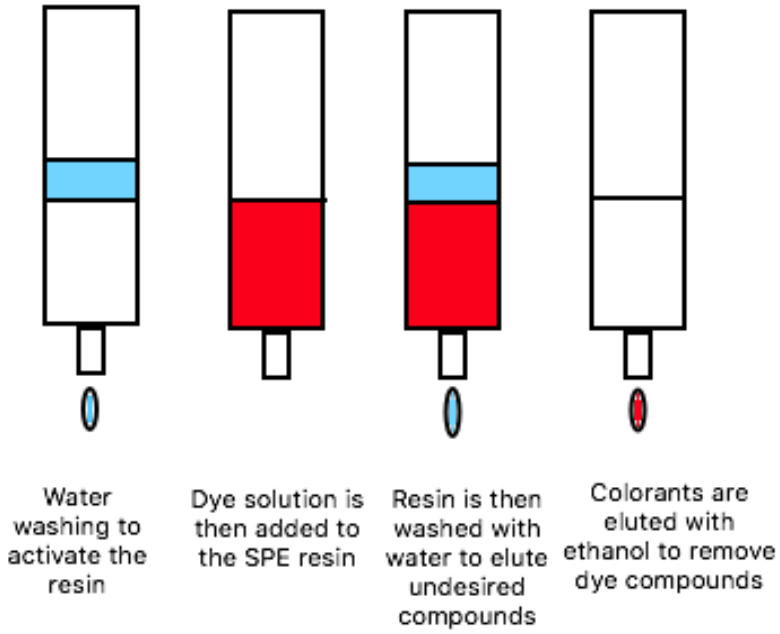


Figure 2.6. Typical SPE process of madder extracts to purify the phenolic compounds in the matrix.

2.2.1 Extraction of Iranian madder with SPE

Water extraction of Iranian madder was carried out as described in experimental section 6.3. The extract was then analysed by HPLC and LC-MS. Water extraction of Iranian madder yielded the glycosidic compounds as major peaks in the extract; there is also a small peak corresponding to alizarin, but it is not a major compound in the extract (Figure 2.7a). LC-MS data shows extraction with water yields the compounds: ruberythric acid (m/z = 533), lucidin primeveroside (m/z = 563), alizarin (m/z = 239) and nordamnacanthal

(*m*/*z* = 267). In the UV/vis trace only the glycosides lucidin primeveroside and ruberythric acid are observed.

After SPE (Figure 2.7b) some of the glycosidic compounds appear to have been separated from the extract. One of the most significant effects is the consistency of the extracts before and after SPE. Before SPE the extracts are very hard to handle as the resultant solid is hygroscopic; however, after SPE the residual solid is a free flowing powder that is easy to handle. The powder obtained after SPE is much easier to weigh out and can be stored in sample vials without the need for nitrogen and sealing. Whereas colorant extracts from freeze drying water samples are problematic as their hygroscopic nature means that they absorb water and hence become difficult to accurately weigh out due to their increased water content giving a higher mass. This could affect the accuracy of the liquor ratios and hence the repeatability of experiments using these extracts. This problem is enhanced also by the texture of the extracts after exposure to the air they are very sticky and hard to accurately weigh out. This change in consistency is probably due to the presence of free sugars in the extract before SPE.

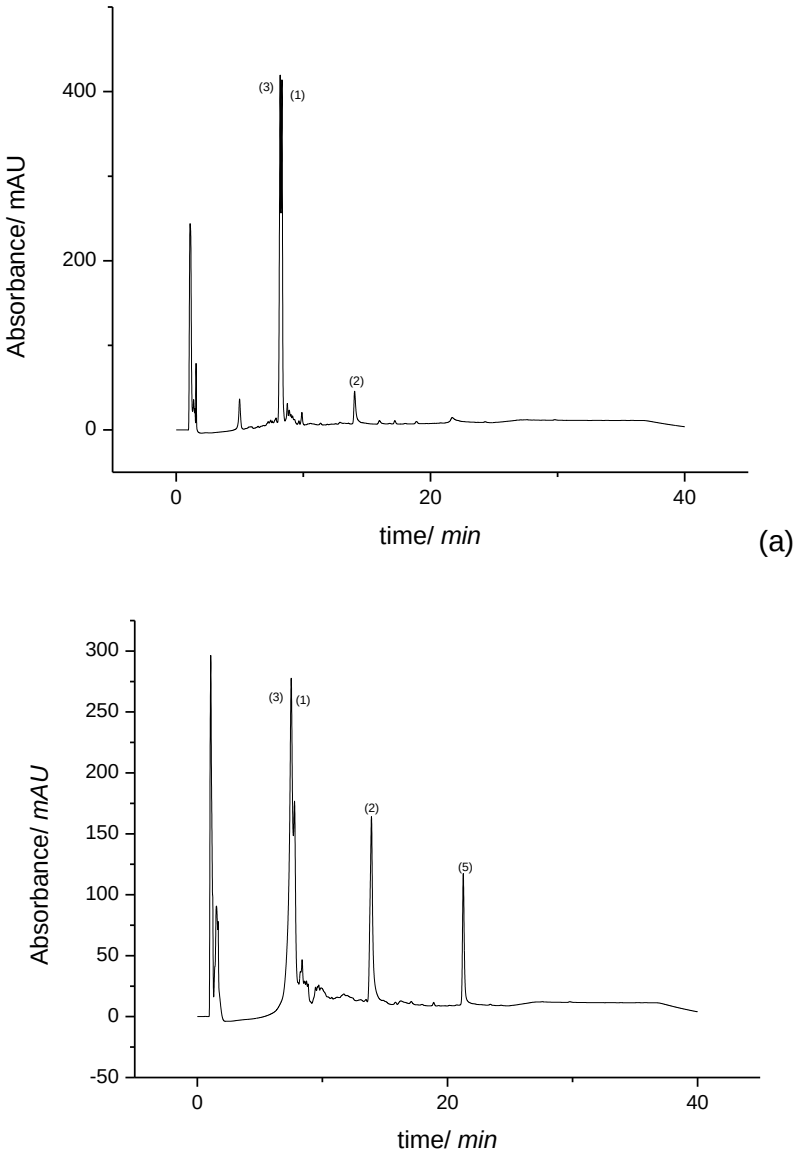
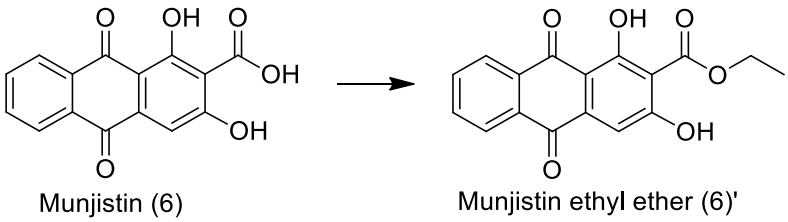


Figure 2.7. Water extraction of Iranian madder (a), followed by SPE (b).

LC-MS chromatograms after SPE show mass peaks for lucidin primeveroside (*m*/*z* = 563), ruberythric acid (*m*/*z* = 533), alizarin (*m*/*z* = 239), rubiadin (*m*/*z* = 253), purpurin (*m*/*z* = 255) and nordamnacanthal (*m*/*z* = 267). Interestingly after SPE there is also a peak present at *m*/*z* = 311 (Figure 2.8), this mass corresponds to munjistin ethyl ether.[33] Munjistin has not been observed in any of the previous samples, however, by concentrating the dye compounds using SPE it may be that minor compounds are more easily observed by chromatography methods of analysis. The peaks identified in these

extracts by HPLC were assigned based on their characteristic data matching those displayed in Table 2.3.

The ethyl ester product of munjistin is also probably an artefact of the ethanolic extraction to remove the dyes from the resin. The carboxylic acid part of the compound can easily be converted to the ethyl ester in a simple addition of the alcohol (Scheme 2.2). This reaction could occur naturally but these ethyl ether compounds are only observed when ethanolic solvents have been used in the extraction or SPE process. This suggests they are artefacts of the solvent addition to the carboxylic moiety on the anthraquinone rather than occurring naturally in the plant.[56]



Scheme 2.2. Formation of munjistin ethyl ester from munjistin.

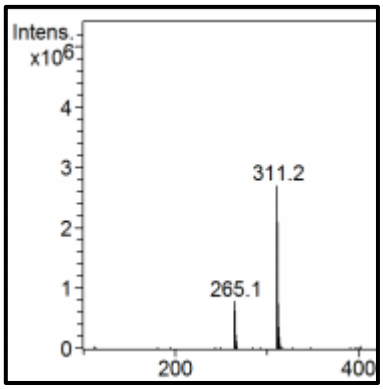


Figure 2.8. Mass spectrum data of munjistin ethyl ester

The overlaid ^{1}H-NMR of the extracts before and after SPE show that the majority of the sugar peaks which arise between 3.0-5.5 ppm, highlighted in the pink box, are removed by the SPE process (Figure 2.9). This demonstrates that the free sugars of the extract have been successfully removed by SPE, resulting in more desired compounds

in the final extract. Before SPE the ratio of aromatic peaks between 6-8.5 ppm and the sugar groups between 2.5-5 ppm is 1:6, whereas after SPE the ratio falls to 1:2. There are still some sugar peaks left in the ^{1}H NMR spectrum after SPE, but these are probably mostly due to the glycosidic peaks of lucidin primeveroside and ruberythric acid. This also explains the 1:2 ratio of sugar peaks to aromatic peaks, these compounds contain two sugars for every aromatic compound.

Although the peaks in this region are diagnostic for the peaks present in sugars a reference of the sugar D-glucose is displayed in Figure 2.10 to display the sugar peaks present in this region. The anomeric peak is slightly higher usually between 4.5-6 ppm due to its position between two electronegative oxygens causing more deshielding and hence displays a peak at a higher ppm.[118] This reference also displays the OH groups in the region of 4-5 ppm due to the use of pure reference material and using d_6 DMSO as the deuterated solvent. DMSO is aprotic and hence there can be no proton exchange and therefore OH signals will be observed under conditions completely deficient of water. If any water is present in the sample there would be some proton exchange with deuterium and hence these signals appear diminished or cannot be seen at all which is why these peaks are not seen in the extract but can be seen in the standard, as the standard probably contains some residual water.[119]

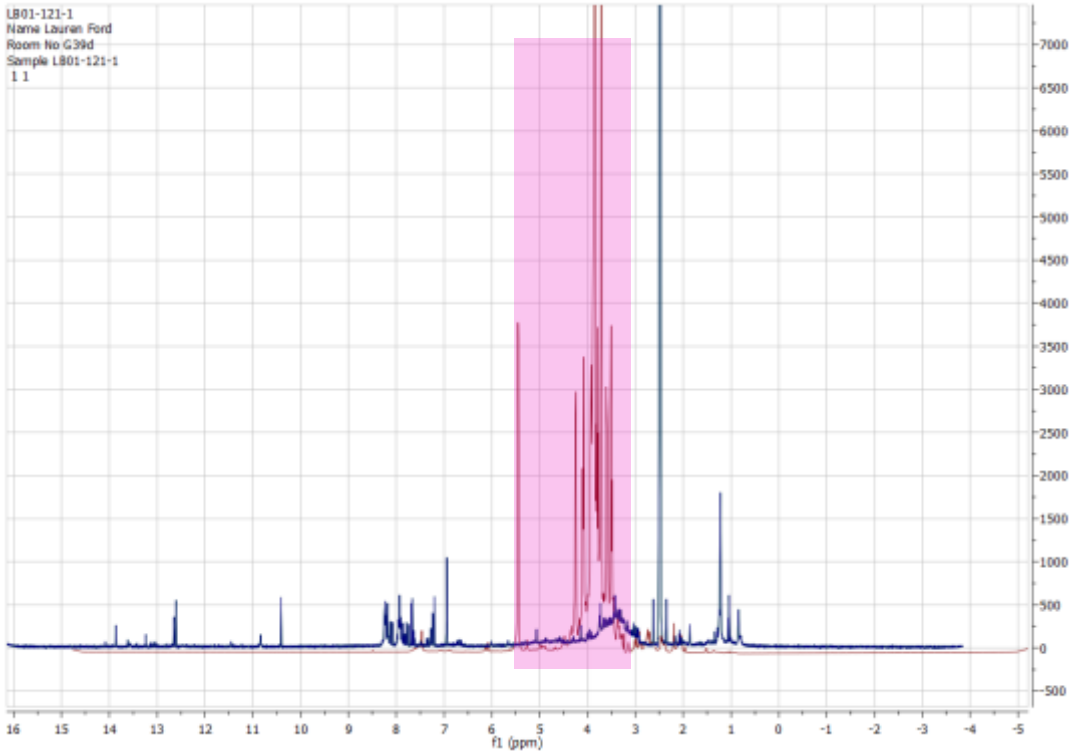


Figure 2.9. NMR spectra before (red) and after (blue) SPE of Iranian madder extract.

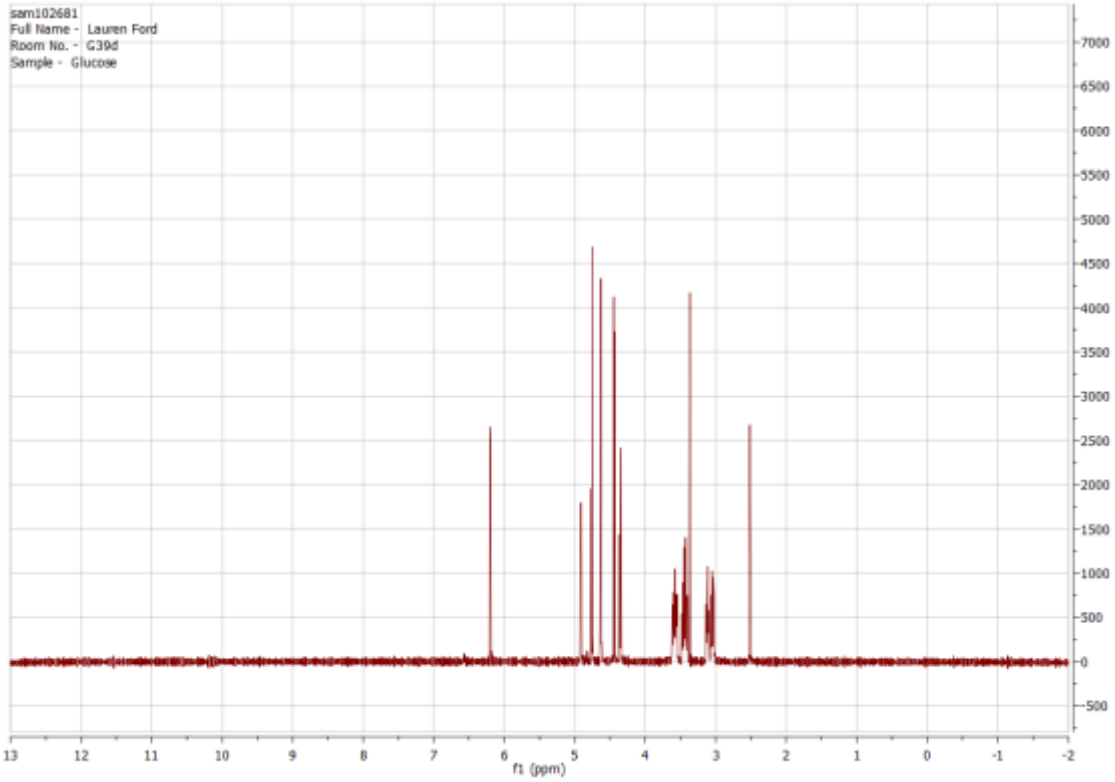
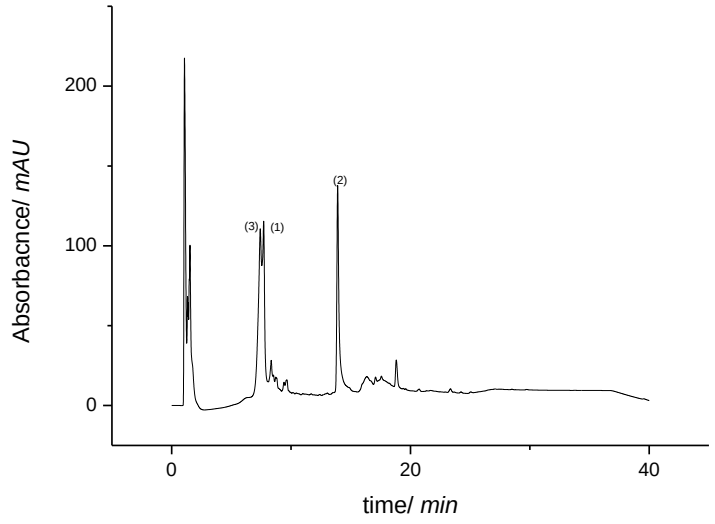


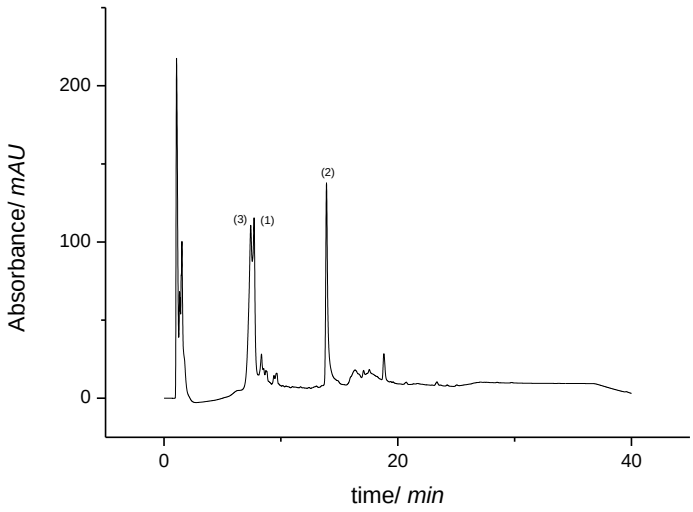
Figure 2.10 Reference NMR of D-glucose showing sugar peaks in the region of 3-5 ppm

2.2.2 Extraction of Turkish madder with SPE

Water extraction of Turkish madder was carried out as described in experimental section 6.3. The solution obtained after extraction was analysed by HPLC and LC-MS. The two main peaks in the water extract of Turkish madder (Figure 2.11a) are the glycosides lucidin primeveroside (3) and ruberythric acid (1), and the aglycon alizarin (2). There is also a small peak corresponding to nordamnacanthal (5). This is also observed in the LC-MS chromatogram, the masses observed for the water extract of Turkish madder are lucidin primeveroside (*m*/*z* = 533), ruberythric acid (*m*/*z* = 533), alizarin (*m*/*z* = 239), rubiadin (*m*/*z* = 253) and nordamnacanthal (*m*/*z* = 267).



(a)



(b)

Figure 2.11. HPLC chromatogram showing Turkish madder extracted with water (a) and after SPE (b).

The composition of Turkish madder extract after SPE (Figure 2.11b) is very similar to that before SPE when analysed by HPLC. The glycoside compounds and alizarin are the main peaks in both chromatograms. The peaks assigned in the HPLC chromatograms herein were done so by matching to the characteristic data given in Table 2.3. Again, before the SPE treatment of Turkish madder the sample was very hygroscopic and sticky whereas afterwards it was a free flowing powder that was easy to handle, probably due to the removal of free sugars. The ^{1}H-NMR spectra before and after SPE are shown in Figure 2.12, where it is observed that before SPE the major peaks in the spectrum correspond mainly to free sugars (highlighted in the pink box in the range of 2.5-5.5 ppm), whereas after SPE there is a reduction in the integration of the sugar peaks in comparison to the aromatic region between 6-8.5 ppm. The ratio of the integration of the range 2.5-4.5 ppm to 6-8.5 ppm before SPE was 10:1, whereas after SPE in ratio changed to 5:2. This suggests the successful removal of free sugars in the extraction and concentration of the useful dye compounds without any changes to the compositions.

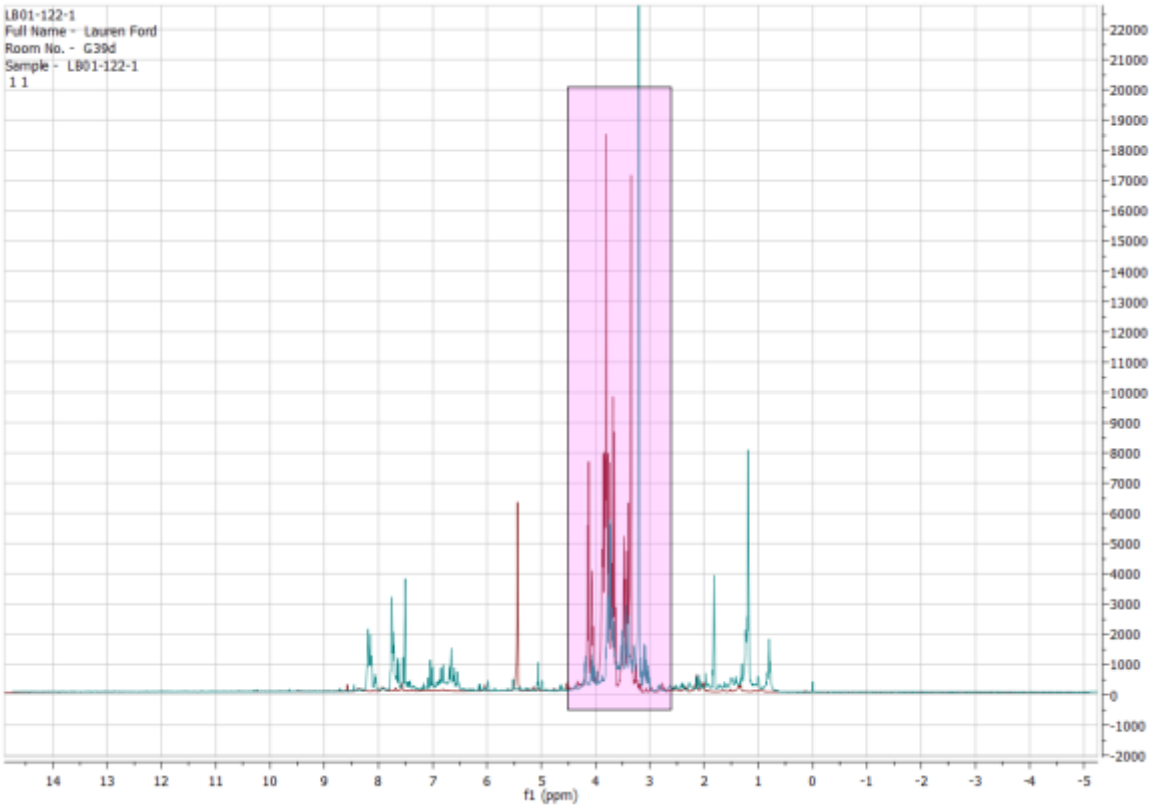


Figure 2.12. NMR showing Turkish madder before (red) and after (blue) SPE.

2.2.3 Extraction of English madder with SPE

English madder was extracted in water as described in experimental section 6.3. The chromatogram before SPE (Figure 2.13a) showed the main peak present was alizarin; the glycosides lucidin primeveroside (3) and ruberythric acid (1) are also present, as well as aglycons lucidin (4), purpurin (11), rubiadin (13) and nordamnacanthal (5). By LC-MS the compounds observed before SPE are: lucidin primeveroside (*m*/*z* = 563), alizarin (*m*/*z* = 239), rubiadin (*m*/*z* = 253), purpurin (*m*/*z* = 255) and nordamnacanthal (*m*/*z* = 267).

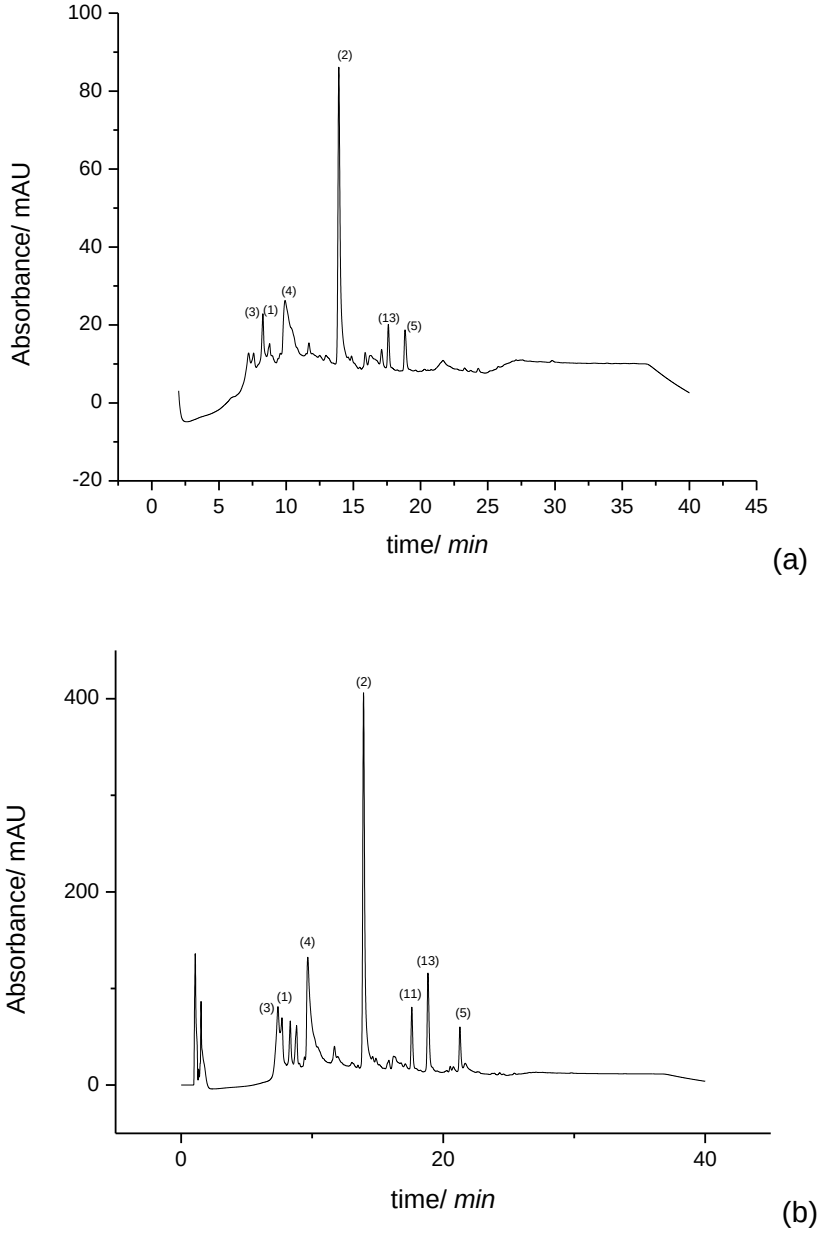


Figure 2.13. Chromatogram of English madder extracted with water (a) and after SPE (b).

The chromatogram after SPE (Figure 2.13b) is very similar to before SPE in terms of the main peaks present in the extracts. There are some differences in the chromatogram such as the presence of purpurin (11), which is probably due to a lower general concentration of the colourant compounds in the extract and hence all of the anthraquinone compounds are easier to detect as there is not one peak swamping the chromatogram. In the LC-MS there are also some differences, the compounds detected

are: lucidin primeveroside (*m*/*z* = 563), alizarin (*m*/*z* = 239), rubiadin (*m*/*z* = 253), purpurin (*m*/*z* = 255), munjistin ethyl ether (*m*/*z* = 311) (Figure 2.8) and nordamnacanthal (*m*/*z* = 267). The presence of munjistin ethyl ester is probably an artefact that arises due to the extraction from the resin with ethanol as described in Scheme 2.2. There is also another peak in the LC-MS chromatogram which elutes just after alizarin which also has a mass of 239 in negative ionisation mode. This compound could be due to xanthopurpurin, this is explained in more detail in chapter 5 which is a degradation compound from lucidin and munjistin. In the mass spectrum of this peak there is also a very small peak of 311 which is again assigned to mujistin ethyl ester artefacts from the SPE process (Figure 2.8).

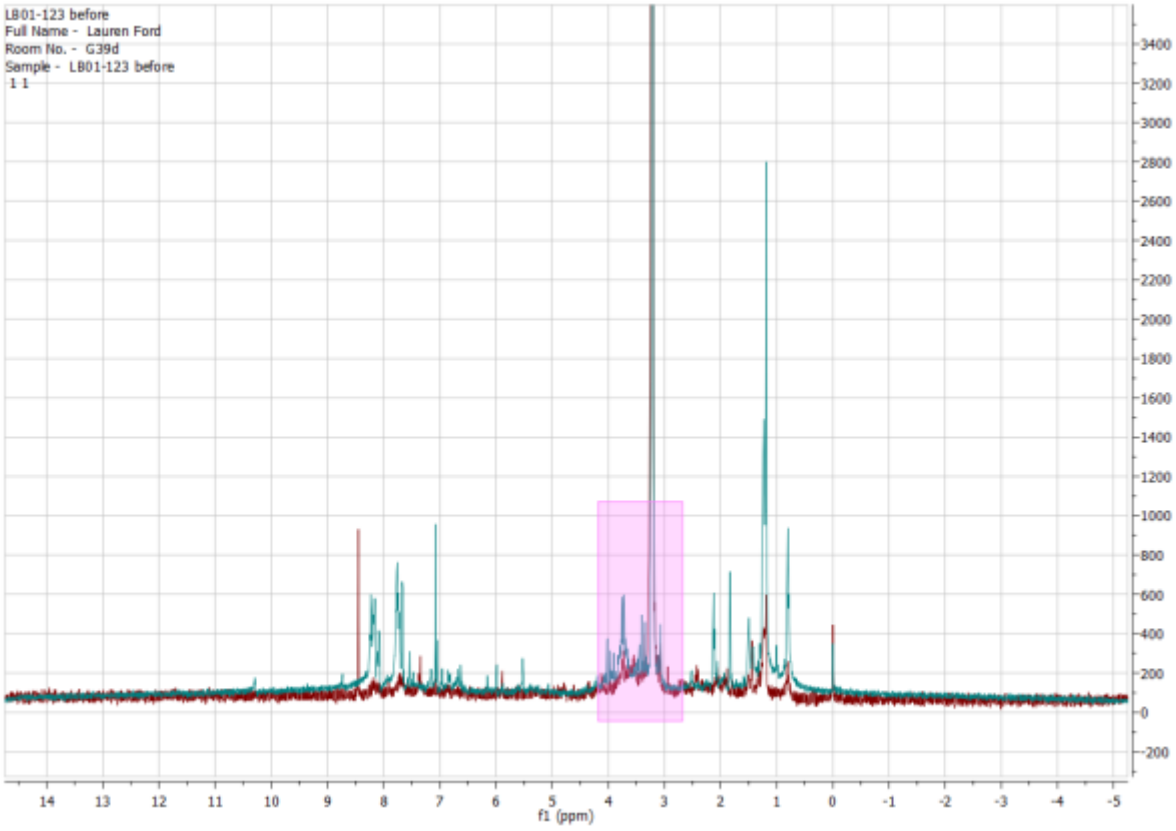


Figure 2.14. Superimposed NMR spectra of English madder extracts before (red) and after (blue) SPE.

The ^{1}H-NMR spectra of English madder before and after SPE (Figure 2.14) are quite different to that of Iranian and Turkish madder because there are less free sugars

present in the original water extract. The spectra after SPE is not significantly different to that of the sample before SPE. This is apparent in the freeze-dried samples as they are also a lot less hygroscopic and sticky than the Iranian and Turkish madder samples. This is interesting because if the drying process is the reason for the change in the ratio of the anthraquinones in the plant extracts due to hydrolysis of the sugars, it would be expected that more free sugars would be observed in the extracts. However, this is not the case and hence it could be possible that the differences in the English madder ratios are *in planta*.

In all of the SPE extracts the free sugars were successfully removed by SPE and the successful removal is displayed by NMR. NMR is useful for showing the free sugar content as the diagnostic peaks are localised to between 3.0 – 5.5 ppm and hence their relative ratio to the aromatic peaks displayed in the spectrum can show whether their removal is successful or not.

In all cases the powder obtained was much easier to use and store after SPE due to it being less hygroscopic. This could be useful when using these dyes as a sustainable alternative to synthetic dyes as the extracts could be supplied directly to dye houses. However herein only one SPE resin was tested for its purifying ability, in future work different SPE resins could be tested on these plant extracts to see the effects different resins had on the final compound composition. It would be interesting to see if a SPE resin could be used to purify a class of compounds from the extract; for example, to separate the glycosides from the aglycons.

2.3 Crystal structure elucidation of ruberythric acid

It is observed in the extraction research described in this chapter thus far that the glycosidic compounds ruberythric acid and lucidin primeveroside are major compounds in the extraction of madder root, and the main components of aqueous and ethanolic extractions of Iranian and Turkish madder. This suggests that historical artefacts

analysed using these methods of extraction could have these compounds present as their major colorant component. This is assuming the conditions they were stored in do not change their chemical composition. It could be possible that through time the chemical composition of the dyed textile could change due to degradation of the colorant compounds on the wool. Studies have shown that the anthraquinones in madder roots undergo greater degradation in anoxic conditions when compared to degradation in oxygen rich conditions however this study focused on the degradation of alizarin.[120] Henderson *et al.* displayed the presence of the glycosidic compounds in back extraction results after artificial ageing suggesting that these compounds could be expected in historical artefacts even after ageing.[121] However each historical artefact is different and the conditions of storage of historical artefacts varies massively which could cause some degradation of the glycosides under real ageing conditions. Despite this it is still important to know if these compounds are still present in the artefact to gain as much information on the dye as possible.

This study looks in detail at the glycosidic compound ruberythric acid and its non-covalent interactions in solution and crystal structure. This is important to understand when thinking about the dyeing mechanisms of these major components of *Rubia tinctorum.*

Isolation of these glycosidic compounds was achieved using ethanol as the extraction solvent. The ethanol extracts were an orange solution and upon solvent evaporation left an orange powder which became darker and sticky if left open to the air overnight. The orange powder obtained was soluble in water, methanol and ethanol. HPLC analysis has shown that the largest peaks were attributable to the glycoside components, suggesting that they are much more abundant *in planta* than the aglycon derivatives. Hence, if there is no further treatment to the dyebath then the main colorant components are the glycosides and not the aglycons; although these compounds are well cited there is limited information on the exact structure of these compounds. For

many studies analysing natural dyes, it is only alizarin which is studied or identified in extracts for the presence of *Rubia tinctorum*.[44,60,122] More recently sensitive techniques have allowed for a more in depth analysis of the colorant compounds in *Rubia tinctorum* by HPLC-DAD and MS.[2,44,57,62,77] These compounds are being recognised as being important references for the analysis of the use of *Rubia tinctorum* in textiles and therefore it is important to know their conclusive structure in order to understand their interactions. Herein the madder root extract was recrystallised repeatedly to successfully obtain a good quality crystal for x-ray crystallography.

Dried extracts of the madder roots were dissolved in methanol and after 5 days a red amorphous solid was found in the bottom of the flask. The liquid was decanted off and the red solid was dried under reduced pressure. Deuterated DMSO-d_6 was then added into the flask and left for a further 4 days, after which a large yellow crystal was obtained. The crystal was confirmed by ^{1}H and ^{13}C NMR data (Figure 2.15) to be ruberythric acid and the x-ray crystal structure was obtained for the first ever full crystal structure elucidation of this compound. This product is the second known example of a glycoside containing anthraquinone crystal structure, after the recent crystal structural elucidation of lucidin primeveroside by Henderson *et al.*[56] The structure of ruberythric acid is a derivative of alizarin and contains a primeveroside moiety consisting of a glucose molecule and a terminal xylose attached through the β-phenolic oxygen of the anthraquinone. Both sugar moieties are linked at the anomeric centre with β stereochemistry which is identified by the high coupling constants (J = 7.5 Hz) of both sugar anomeric protons. This large coupling constant is due to the *trans*-coplanar relationship with the proton on the adjacent carbon.

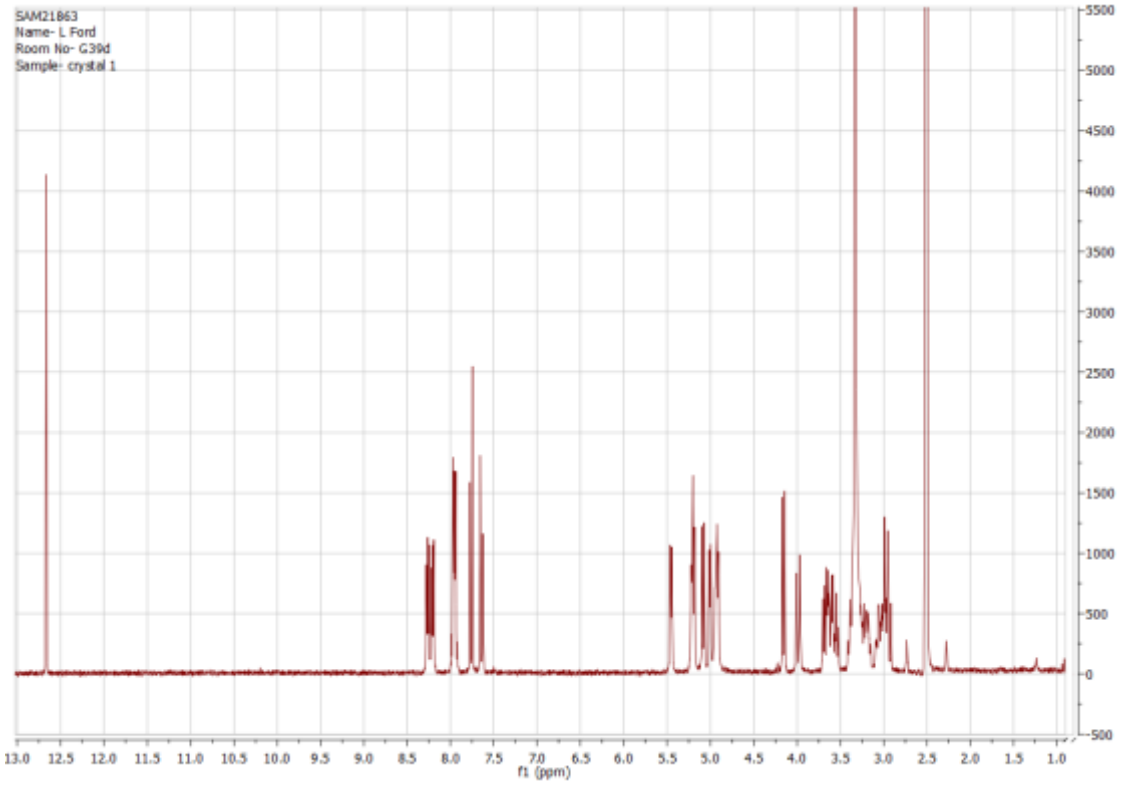


Figure 2.15. ^{1}H NMR spectra of ruberythric acid.

The isolation and absolute configuration of the structures of the glycosidic components in *Rubia tinctorum* has helped to understand the compounds and how they bind to the textile. It can be observed from Figure 2.16 that there is an intramolecular hydrogen bond between the carbonyl of the anthraquinone and the β-hydroxyl group. Literature studies suggest that this is the binding site to the mordant and hence its dyeing binding site.[85] However, there are other areas of the compound rich in hydroxyl groups which could also have some interaction with other dye compounds on the wool or metal ions themselves; the sugar moieties. These results are in accordance to previously hypothesised findings that suggest madder adsorption does not follow ideal monolayer adsorption when the glycosides are present.[121] Madder extracts have previously been shown to follow a Langmuir isotherm which is limited to monolayer adsorption however this study was looking primarily at the aglycon compounds mixture.[29] However this crystal structure gives conclusive physical evidence of these hydrogen bonding

interactions which could be causing the multilayer adsorption of sugar containing anthraquinone mixtures.

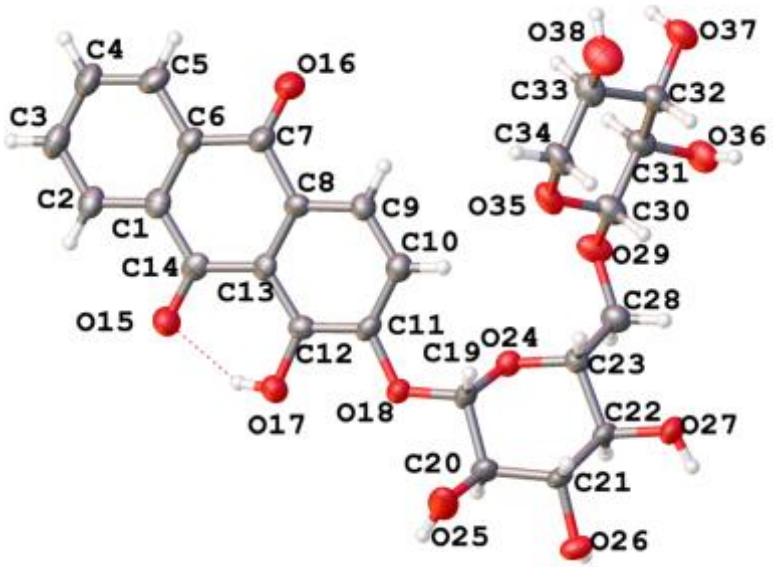


Figure 2.16. Structure of the glycosidic compound ruberythric acid extracted from *Rubia tinctorum* showing the intramolecular hydrogen bond between the carbonyl and the adjacent hydroxyl of the anthraquinone backbone.

It is probable that both the sugars and the hydroxyls on the anthraquinone backbone take part in the dyeing of the wool. In the crystal packing the hydrogen bonding of the molecule comes from the sugar moieties solely, the anthraquinone backbone does not take part in any of the bonding (Figure 2.17). However, this at least in part, could be due to the packing within the crystal lattice. The crystal packing shows that the major intermolecular interactions are due to the hydrogen bonding between the sugars. Each ruberythric acid molecule forms hydrogen bonds to another two hydrogen bonding molecules. The terminal xylose forms two hydrogen bonds with the glucose of another ruberythric acid compound. The elucidation of this crystal structure has allowed for observation of these interactions for the first time.

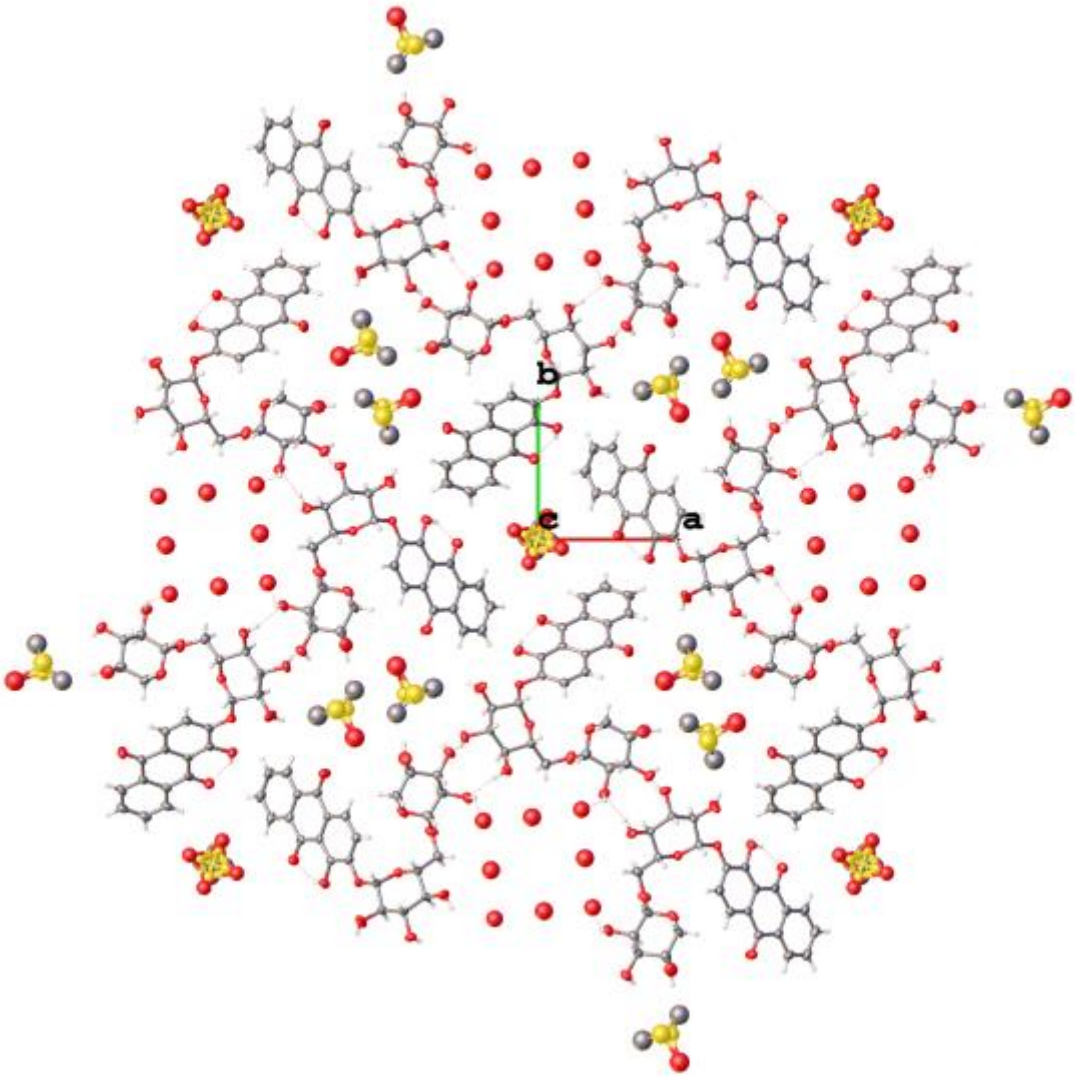


Figure 2.17. Showing the crystal packing hydrogen bonding interactions of ruberythric acid.

The anthraquinone backbone does take part in some π-stacking shown in of the layers of the crystal shown in Figure 2.18. The π-stacking and hydrogen bonding activities of ruberythric acid could mean that there is more than one layer of dyestuff deposited onto the wool in the dyeing process which has been previously studied in isotherms of madder extracts.[29] This crystal structure elucidation provides some evidence of the type of interaction which could help to form multilayers of dye aggregation when a glycosidic compounds are present in the dyebath. The hydrogen bonding of the sugar groups within the ruberythric acid moiety suggests that there could be formation of multilayers through hydrogen bonding to form aggregates on the surface

of the wool. Understanding these interactions will help to form a back extraction technique that can interrupt these aggregates and release the dye compounds into solution.

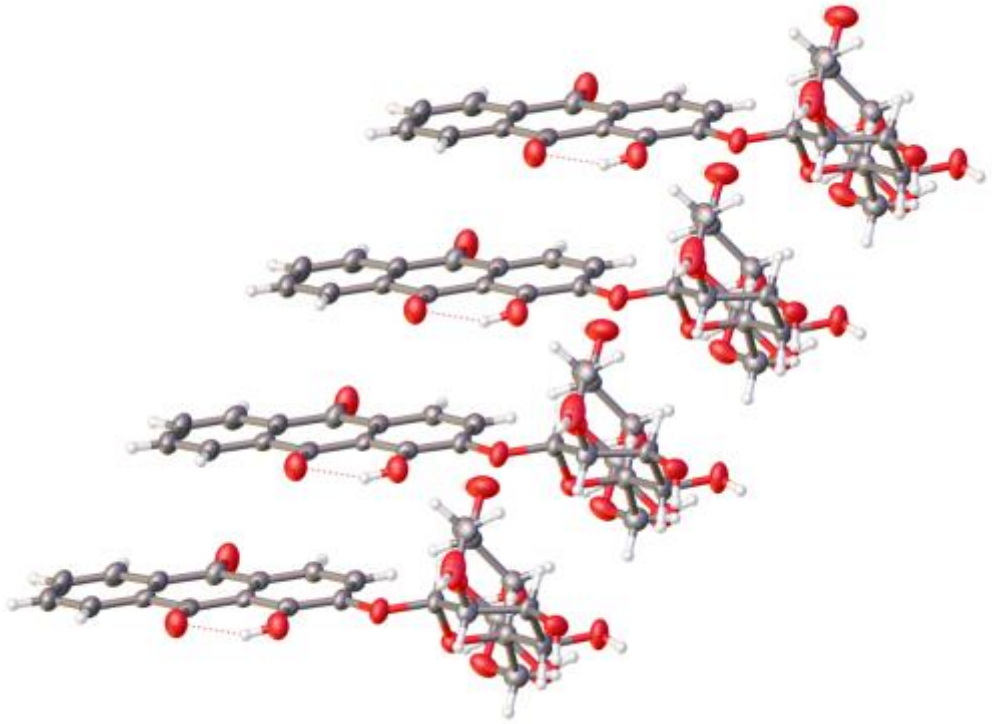


Figure 2.18. π-stacking interactions of ruberythric acid.

2.4 Conclusions

There are distinct differences in the compositions of the three types of madder shown herein. The Turkish and Iranian madder plants have high concentrations of the glycosides lucidin primeveroside and ruberythric acid, whereas the English madder does not contain any glycosides and the main components in its dye composition are lucidin and alizarin. It is unclear whether these differences are due to processing of the dye plants (such as the way they were dried) and therefore give different profiles on analysis or if these differences are *in planta*. This could be clarified with further work to gain more samples from the regions in question and further study into the trends of the chromatographic profile of *Rubia tinctorum* from different regions. Seeing these

compositions is important in the next step of this research as these differences must be observed when the plant dyestuffs are dyed onto and extracted back off wool in an analysis of the dye compounds.

It is also clear that the polarity of the solvent has a huge effect on the compounds extracted out of the dyestuff; the more polar the solvent system for extraction the more polar compounds are extracted. Less polar compounds, alizarin and rubiadin, are extracted in all of the solvents tested; lucidin is extracted in smaller quantities when ethyl acetate was used as the solvent system, which suggests that it is more polar than alizarin and rubiadin. Lucidin is a mutagenic compound in madder roots,[34,35] hence extraction of colourants from madder without extraction of lucidin could be useful in the field of food colourants or cosmetics.

SPE shows some interesting results by LC-MS. Presence of the carboxylic acid containing compounds in madder munjistin and pseudopurpurin have been widely documented,[11,107,110] however, herein they have not been identified by either HPLC or LC-MS in any of the solvents used for extraction. In the LC-MS chromatogram of the solid phase extracted madder samples there is a peak of m/z = $[MH]^-$ = 311 corresponding to the peak of the munjistin ethyl ester. The ethyl ester product of munjistin is probably more stable than the munjistin as the stable leaving group of CO_2 is no longer available once the ethyl ester is formed. This is only observed in the mass spectrum and is not noticeable in the UV/vis trace which suggests that these compounds are only detectable by mass. The ethyl ester product of munjistin is probably formed in the ethanol extraction of these compounds from the SPE resin. The munjistin is still only present in very small concentrations however, it is only observed after purification by SPE and not in the water or ethanol extractions which suggests munjistin has a high affinity to the SPE resin (as the parent compounds) and then is in high enough concentrations to be detected in the dyebath after this treatment. Therefore, this treatment of the dye extracts

could be used to help identify trace amounts of the carboxyl containing compounds in historical textiles.

The SPE process also concentrates the glycosides lucidin primeveroside and ruberythric acid. NMR spectra revealed the free sugars extracted into the solution are successfully removed from the samples by SPE. The more polar compounds are concentrated in the final solution using the SPE method, this could therefore be a good method to gain high quantities of these compounds for analysis methods and dyeing using these natural materials. The SPE also produces a dye extract which is less hydroscopic and easier to handle which could be preferred when using natural dyes in much larger scales. If there was to be a resurgence of the use of natural dyes this could be an easy way of making a dye that is easy to store and use.

Novel methods for the detection of sugar removal after SPE processes were used which utilise NMR spectroscopy. Using NMR, the successful removal of the free sugar compounds was observed. This was the first known study to test the effectiveness of amberlite XAD 7HD with the anthraquinone compounds from *Rubia tinctorum* dye plant extracts. The first ever crystal structure of the anthraquinone glycoside; ruberythric acid was obtained and fully characterised. This confirms some of the non-covalent interactions between dye compounds in the sugar containing anthraquinones and allows for full understanding of the structure of this compound.

3 Extraction of yellow dyes

The dyestuffs used to create yellow textiles historically were much more diverse than those used for red dyes. Often dyers used whatever was available to them, this makes identifying historic yellow dyes from artefacts much more difficult. However, in Europe, weld was often the most favoured yellow dye plant.[107] When analysing textiles dyed with yellow dyes, not only are there more plants to distinguish from one another, but the components in the dyestuffs themselves are not as robust to chemical change as some of the other dyes such as; anthraquinones from madder or indigo from woad. Flavones are known to degrade into hydroxybenzoic acids through light and oxygen activation.[40,120]

Dye components in yellow dye plants belong to the flavonoid class of compounds; the majority of the yellow colorants found are glycosylated, which aids the solubility of these compounds in water for the dyeing process. The glycosylation of these compounds means that removal of these dyes from textiles in the analysis process is not a trivial feat and very ‘soft’ methods must be employed for the solvation for analysis so as not to destroy or degrade the glycosylation present.[39,71,123] Degradation compounds of yellow dyes have been studied in some detail, but these compounds have very small molecular sizes and the detection can be very limited. Therefore, it is important to understand which compounds are present in the dye extraction baths before the dyeing takes place in order to try to understand what may still be dyed onto the textile.

The dye plants were chosen for this study based on them all having some abundance of luteolin and apigenin and the corresponding glycosides.[12] This study was done to compare these natural dye plant extracts in ethanol and water to create chromatographic profiling of the chemical composition of each plant extracts. These

plants were chosen due to their differing profiles from extraction when the glycosidic compounds are present. Due to the main flavone aglycons being the same in all of the extracts it was considered that upon acid hydrolysis these plant dyes would show back extracts that would be fairly similar in composition. Therefore, this study aimed to create chromatographic profiles of each plant extract which could be used for comparison for back extracts in future work. Unfortunately, due to the time scales of this project, the extraction studies were all that could be carried out in this report but these results could be utilised in future work studying the dyeing capability and back extraction of these compounds from textiles.

Samples herein were extracted in ethanol and water. These were also compared to samples which were subjected to solid phase extraction (SPE) and all samples were analysed by HPLC and LC-MS. As described in section 2.2, the SPE step removes free sugars and non-phenolic compounds from the extract so that the desired compounds can be purified to some extent. The dye plants studied were: weld (*Reseda luteola*), chamomile (*Matricaria chamomilla*) both aerial parts and flowers only and golden rod (*Solidago canadensis*). All of the dye plants used in these studies were supplied by George Weil. Ltd and hence were from a commercial supplier. As discussed with the madder varieties it is possible when buying from a commercial supplier that discrepancies between products supplied could occur, but chromatographic profiles of all of the dye compounds supplied throughout the course of this work displayed consistent chromatographic profiles. The aerial parts and flowers only were studied in this thesis due to their availability from the supplier. It was considered interesting to see if there was a noticeable different from plants as a whole compared to just the flowers.

Standards of luteolin and apigenin were obtained from LKT laboratories. Other compounds were identified from the literature and corresponding UV/vis data or by mass. These standards gave a good basis in order to identify the aglycons in the sample. Their characteristic UV/vis traces then allowed for identification of these dye compounds in

other samples. The UV/vis traces of apigenin and luteolin are shown in Figure 3.1. These also fit well with corresponding UV/vis data found in the literature of apigenin and luteolin.[90] Although the UV/vis traces of luteolin and apigenin are very similar there are distinct differences between them, the large broad absorbance peaks at 337 nm for apigenin and 348 nm for luteolin. The smaller absorbance peak for luteolin is at 253 nm and has a distinctive shoulder whereas the apigenin UV/vis shows a narrow absorbance at 267 nm with no shouldering. These distinctive UV/vis traces can be observed when these peaks are identified in the HPLC chromatogram and is used to confirm the peak identity along with its retention time. An overview of the colorants identified in the yellow dye plants chosen for analysis can be seen in Table 3.5.

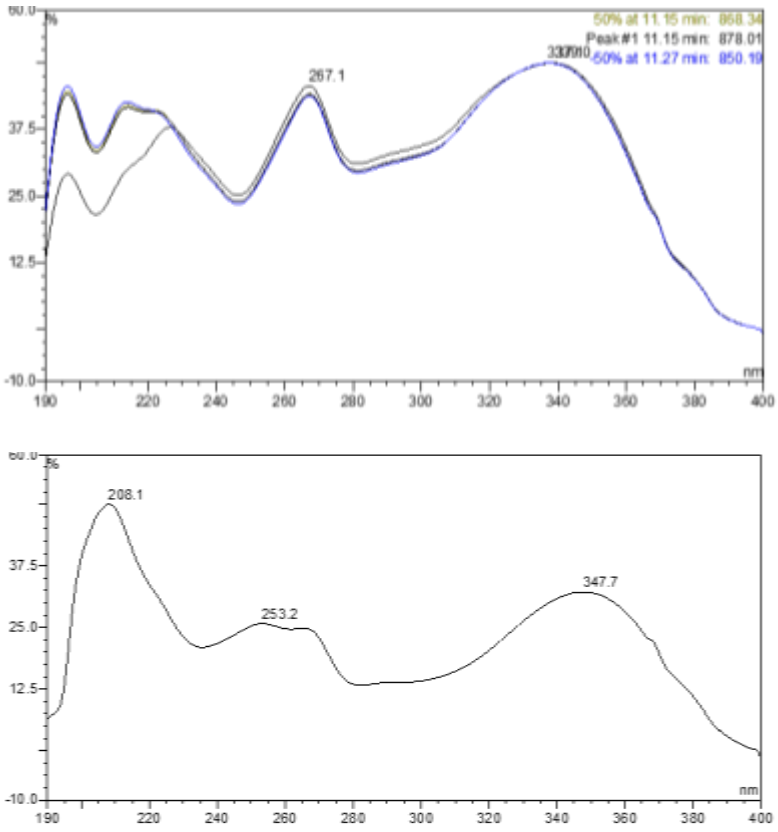


Figure 3.1. UV/vis characteristic traces of apigenin (top) and luteolin (bottom) for HPLC identification.

3.1 Ethanol extraction of yellow dye plants

Each dye plant was extracted with ethanol as described in section 6.3. Ethanol was chosen as a solvent due to its polarity and hence should be suitable to extract polar glycosidic compounds but also some of the aglycon compounds. It was also considered due to the 'green' nature of the solvent and its ease to remove after extraction.[117] Ethanol can be removed by solvent evaporation after extraction on a rotary evaporator. In comparison to water which has to be freeze dried this is much more favourable. After extraction, each dye plant liquor was filtered and an aliquot was taken for HPLC and LC-MS analysis. The compounds in the yellow dye plants were identified where possible based on authentic samples or by mass and UV/vis trace matches with compounds in the literature. However, the UV/vis traces in literature vary from paper to paper so characterisation based on matching UV/vis data alone was avoided.[90,107] One problem with identifying peaks based on UV/vis data alone is that the solvent can have an effect on the UV/vis maxima especially at lower wavelengths.[124] Therefore different solvent gradients and the solvent choice in HPLC elution can have an effect on the UV/vis trace observed. However, these problems can be overcome by using authentic samples to measure the UV/vis data on the gradient programme being used and then using the UV/vis data from these sample peaks to identify these compounds in the mixtures from the plants.

3.1.1 Weld

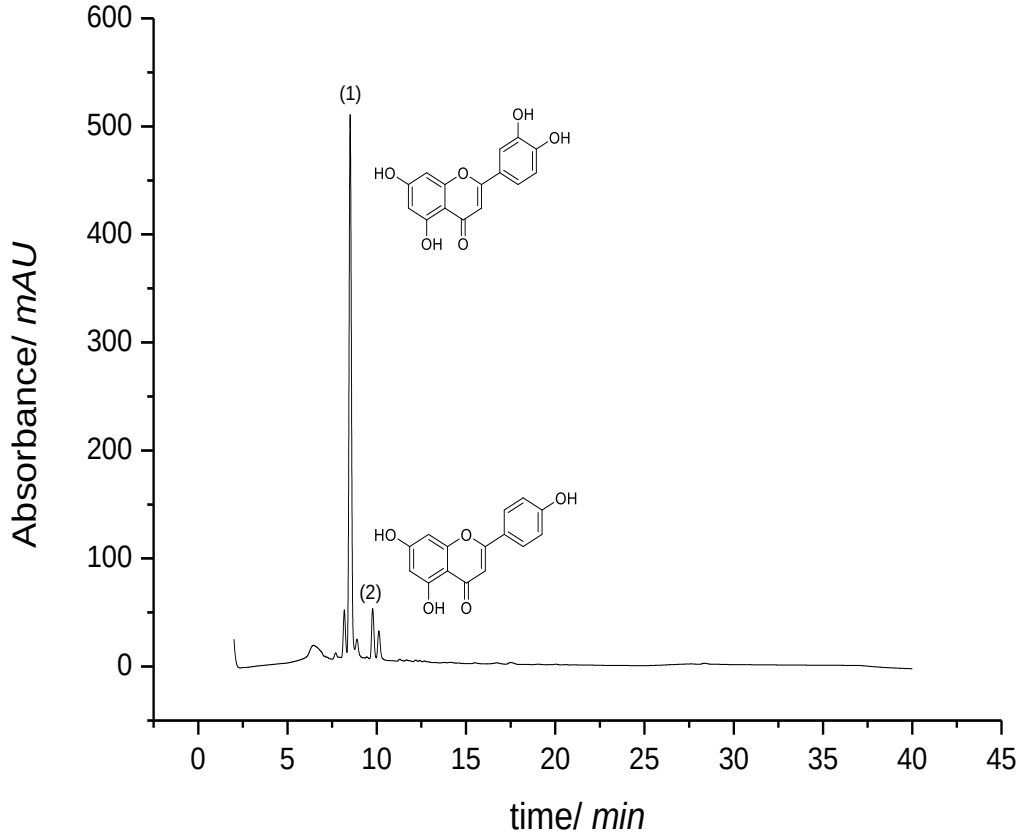


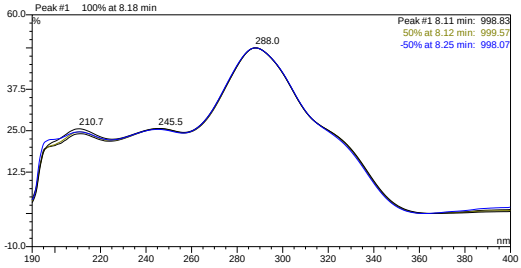
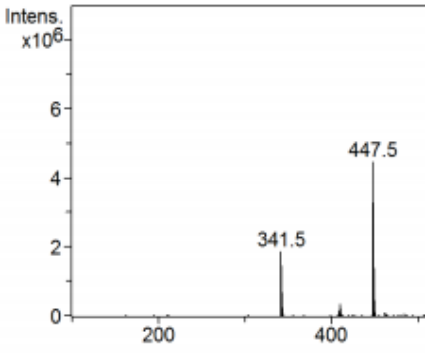
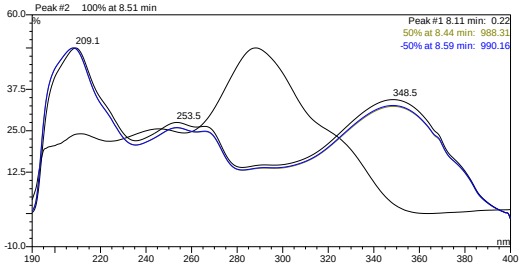
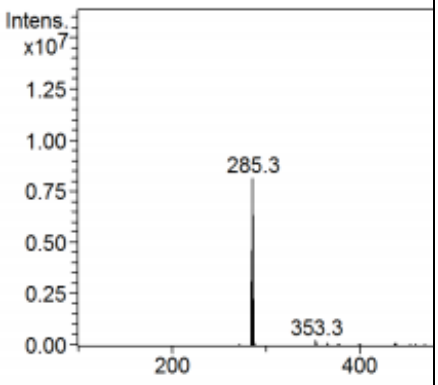
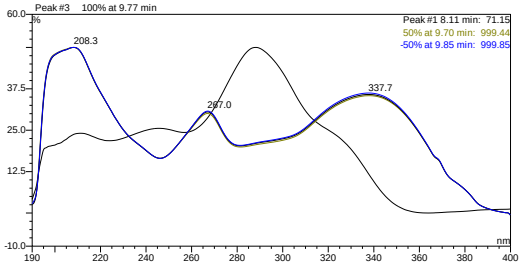
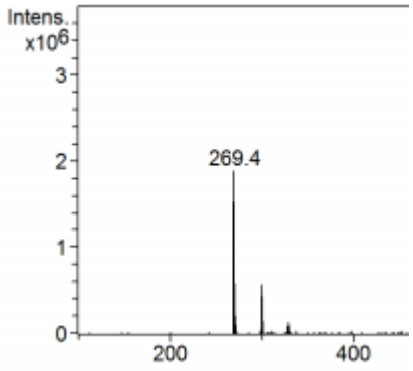
Figure 3.2. Ethanol extraction of weld HPLC chromatogram (1) luteolin (2) apigenin identified with reference to authentic samples.

The main compound present in the chromatogram of ethanol extraction of weld is luteolin (1), which has been previously described as one of the main components in weld;[12] apigenin (2) is also present of in the chromatogram identified from the authentic samples, but in much lower concentrations than luteolin (Figure 3.2). LC-MS of the extract shows peaks corresponding to luteolin (1) m/z= 285, apigenin (2) m/z= 269, and a glycoside of luteolin with a mass of m/z= 447. It is unclear from this mass the position of the glycoside moiety, but luteolin-7-*O*-glycoside is heavily cited in the literature as one of the main compounds in weld;[48,125,126] luteolin-4-*O*-glucoside has also been reported to have been observed in extracts but is less commonly present in samples.[89] This

compound is not identified by HPLC-DAD, although could be attributed to the small broad peak at around 6 minutes, but mass in LC-MS identifies a luteolin glycoside, which in this case is used to identify this glycoside tentatively by its mass spectrum. All spectra are summarised in Table 3.1. These results for the liquid extraction of weld align with previous research from the University of Edinburgh where luteolin and apigenin were identified as the main compounds from back extracted wool. In this study chrysoeriol, which is a methylated isomer of luteolin was also observed.[42,45] However this work was done using the common back extraction method 37% hydrochloric acid: methanol: water (2:1:1), the use of strong acid and methanol could have resulted in a methanol adduct. This could explain why this compound is not observed herein as although ethanol is used as the extraction solvent the solvent is not acidic and hence ether formation is less likely to occur.

One observation to note is that the sugars observed herein differ from those seen in *Rubia tinctorum* from previous chapters. These two dye plants are from completely different botanical families, as shown in Chapter one the anthraquinones follow a different biosynthesis pathway to the flavonoids. Therefore, the addition of sugars to these secondary metabolites is followed by different biosynthetic pathways.

Table 3.1. The UV/vis spectral data and mass spectral data of compounds observed in ethanol extracts of weld.

Compound Identified	UV/vis data	Mass Spectrum data
Luteolin Glucoside		
Luteolin		
Apigenin		

It is interesting to note that the ratio of luteolin aglycon to luteolin glycosides is much higher in favour of the aglycon when extracting weld with ethanol, particularly because

much of the literature states that the glycosidic forms are more common in flavonoid-bearing plants.[71] Therefore, if an extract rich in luteolin was required this could be a simple and effective method of acquiring it. It is unclear if the presence of high amounts of the aglycon occurs *in planta* or if it is caused by the drying process, as previously seen in madder. The drying process can have a large effect on the yield of plant glycosides; freeze drying has been shown to be the best drying method to keep these compounds intact.[12] Whilst the variability which is created by the various drying conditions causes problems with the reproducibility of the dye plant extracts; herein this study aimed to observe the chemical composition in these plant extracts. The aim was then to be able to maintain a chemical profile as similar as possible to the original extract in back extractions from wool for future work.

3.1.2 Golden Rod

The chromatogram of golden rod extracted with ethanol is much richer in glycosidic components (Figure 3.3) when compared to weld, the glycosidic compounds are more polar due to the sugar groups with many hydroxyls present and, hence, are eluted earlier in the chromatogram. This chromatogram is much more complex when compared to weld (Figure **3.2**) with many compounds present in the sample; the aglycons luteolin (1) and apigenin (2) are highlighted and can be observed in the extraction but they are not the main components of the extraction liquor. LC-MS of the golden rod extract gives masses of m/z 609, m/z 447, m/z 431, m/z 285 and m/z 269. The corresponding compounds of these masses are summarised in Table 3.5, UV/vis and mass spectra are displayed in Table 3.2.

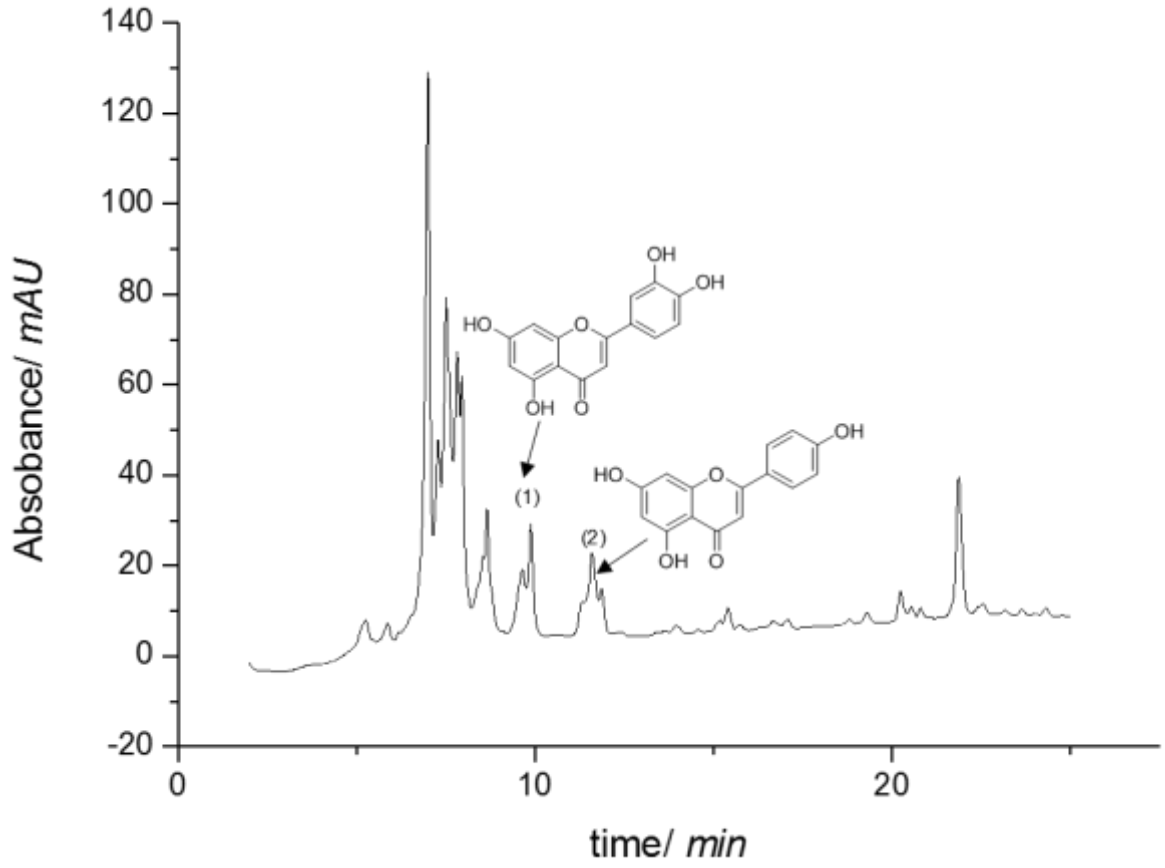
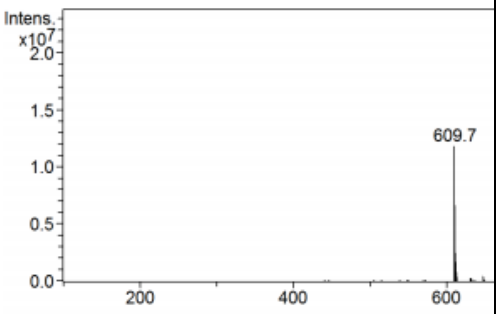
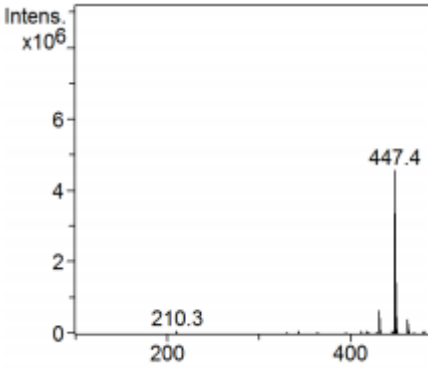
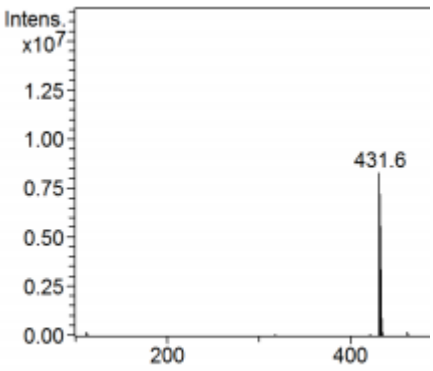
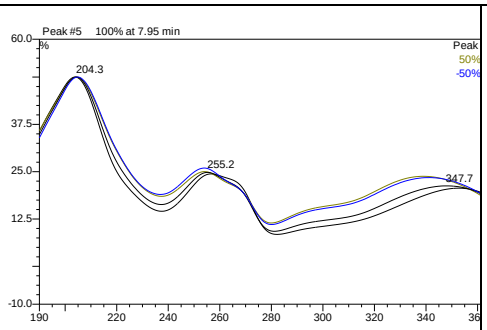
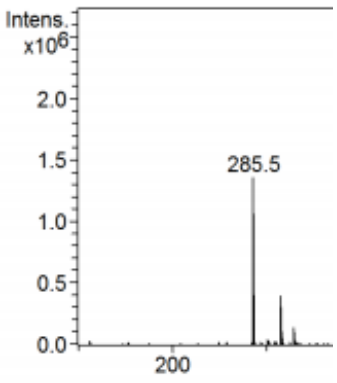
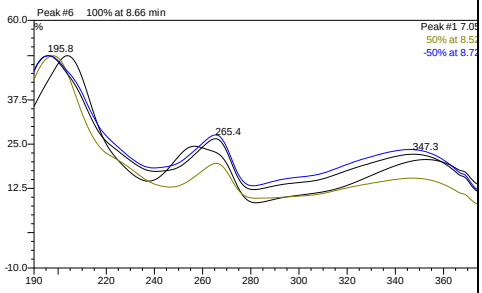
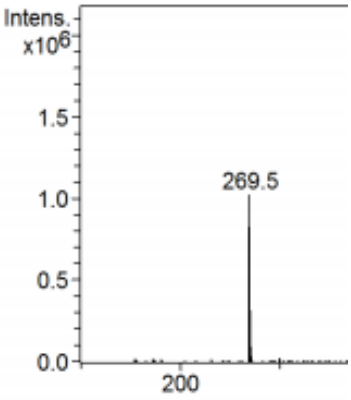


Figure 3.3. Chromatogram of ethanolic extracts of golden rod displaying luteolin (1) and apigenin (2) identified with reference to authentic samples.

Whilst a method optimisation was attempted for the yellow dye compounds by changing the gradient of the mobile phase and the temperature of the column compartment full separation was never achieved. As previously discussed this could be due to the age of the column being used for these studies. The glycosidic components in the extract can only be identified by mass, as the samples were not available commercially for comparison to authentic samples and the scope of the project did not provide enough time to purify and isolate each compound for full elucidation. DAD detection is not very reliable for the glycosidic components as many literature sources contradict λ_{max} values of the detected compounds,[90,107] which makes identification of yellow glycosidic dyes very difficult.

Table 3.2. The UV/vis spectral data and mass spectral data of compounds observed in ethanol extracts of golden rod.

Compound Identified	UV/vis data	Mass Spectra data
Luteolin-3,7-O-glucoside	Unresolved	
Luteolin-O-glucoside	Unresolved	
Apigenin-O-glucoside	Unresolved	

Luteolin		
Apigenin		

3.1.3 Chamomile flowers

Chamomile flowers were extracted as described in section 6.3 at 90 °C in ethanol. An aliquot of the extract was taken and analysed by HPLC and LC-MS. This chromatogram again consists of mainly the glycosidic derivatives of the flavones in the plant and the chromatogram appears very complex. The glycosidic compounds are eluted between 5-10 minutes, they are not well resolved, but the ratio of glycosides to aglycons can be observed.

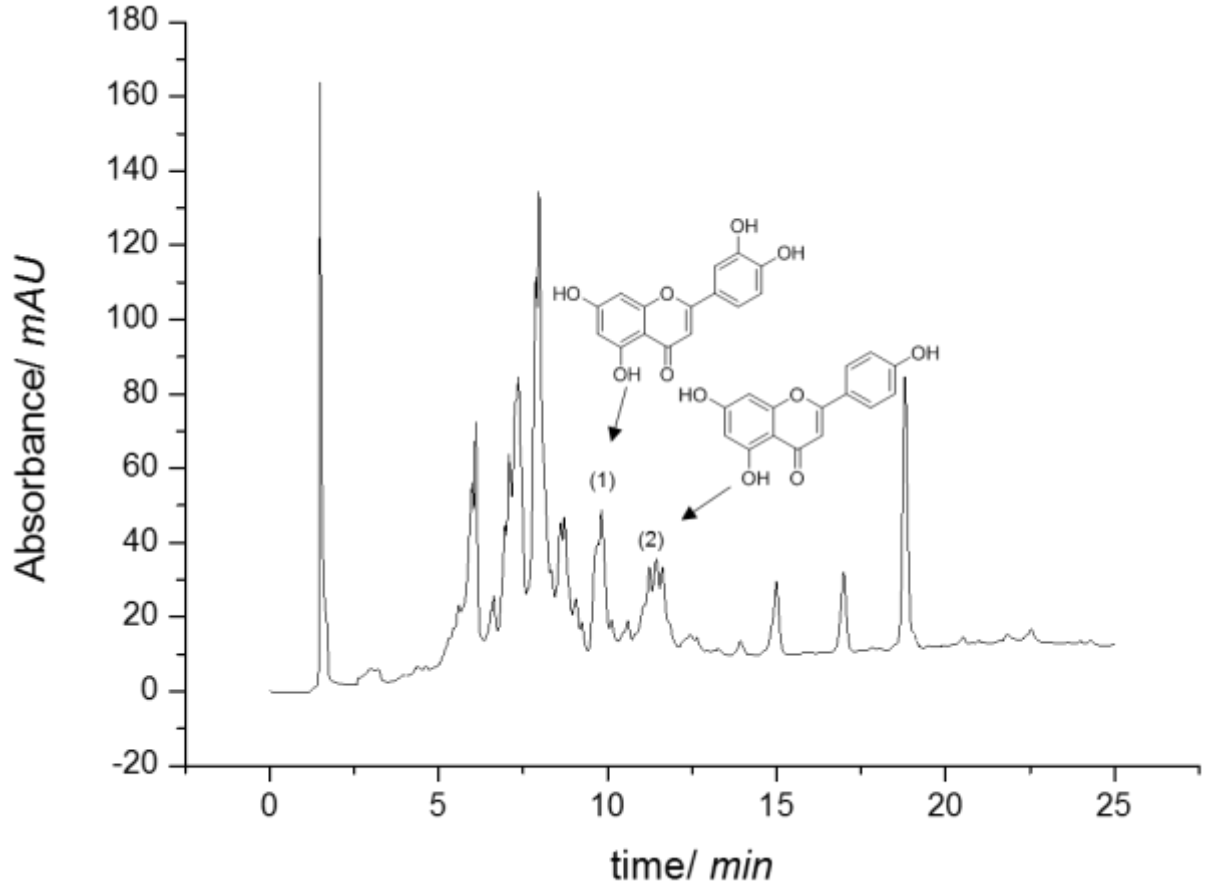


Figure 3.4. HPLC chromatogram of crude ethanolic extracts of chamomile flowers displaying luteolin (1) and apigenin (2) compounds detected with reference to authentic samples.

The aglycons identified by authentic samples are labelled luteolin (1) and apigenin (2) in Figure 3.4. The LC-MS of the sample showed peaks with masses m/z 609, m/z 447, m/z 431, m/z 285 and m/z 269. These peaks were assigned to the flavones in the sample however, there were two other peaks in the spectrum which were not well resolved with masses of m/z 711 and a fragment of the mass spectrum at m/z 355 (**Figure 3.5**). These are assigned to two cinnamic acid derivatives in the sample, which are fully elucidated and explained further in case study section 3.3. Cinnamic acids are precursors to the biosynthesis of flavonoids (section 1.5), and herein give distinctive peaks in the chromatogram and hence the structure of them was considered important when analysing this plant matrix.

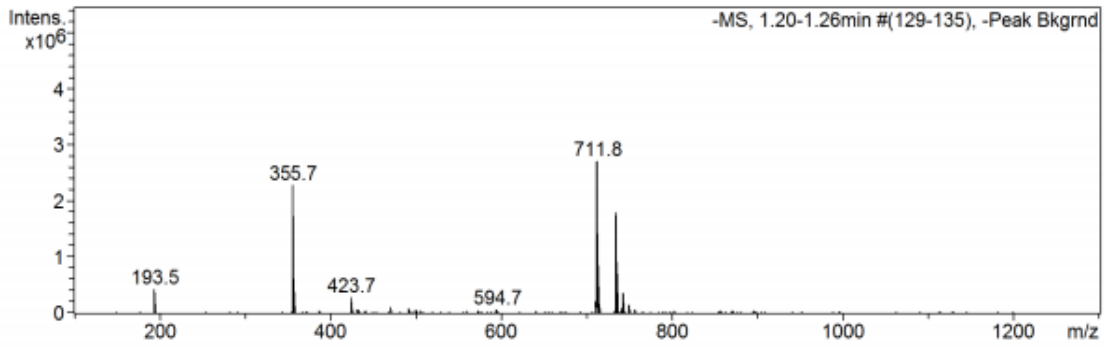
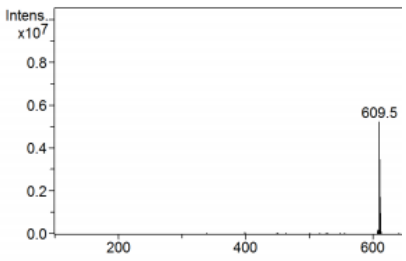
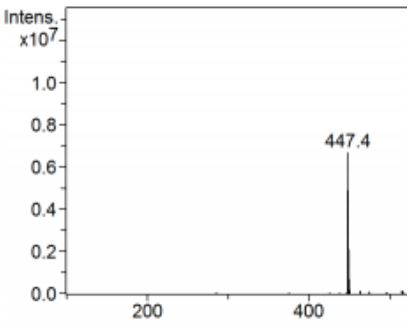
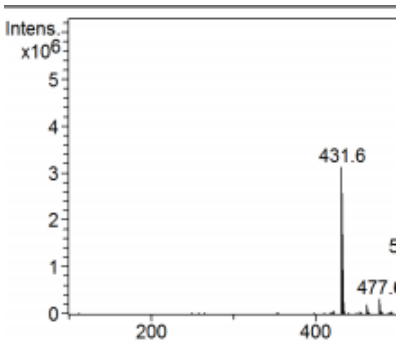
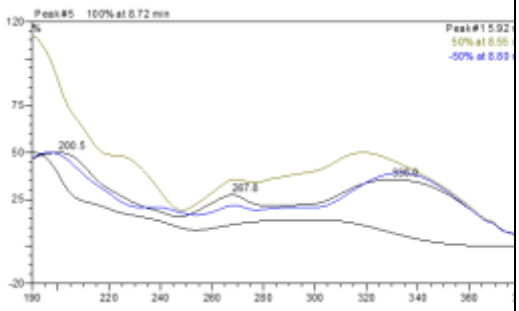
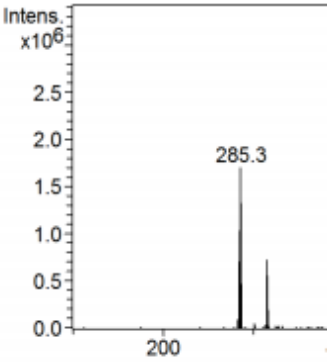
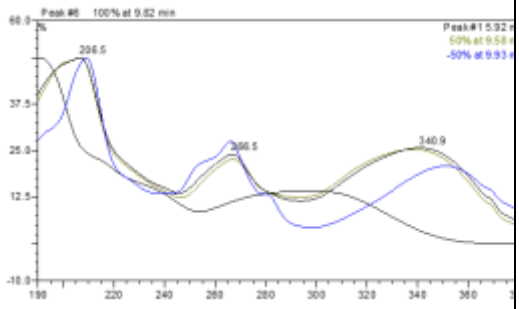
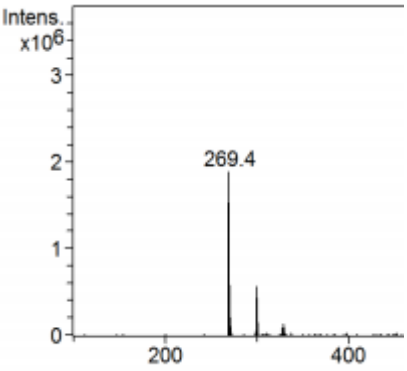


Figure 3.5. Mass spectra of the cinnamic acid derivatives in ethanol extracts of chamomile flowers

The flavone compounds identified in chamomile flowers are summarised in Table 3.5, the mass spectra and UV/vis data are displayed in Table 3.3.

Table 3.3. Mass spectra and UV/vis data of flavone compounds identified in ethanol extracts of chamomile flowers.

Compound Identified	UV/vis data	Mass Spectral data
Luteolin-3,7-O-glucoside	Unresolved	
Luteolin-O-glucoside	Unresolved	

Apigenin-O-glucoside	Unresolved	
Luteolin		
Apigenin		

3.1.4 Aerial parts of chamomile

The aerial parts of the chamomile plant were extracted as described in experimental section 6.3 at 90°C in ethanol. An aliquot of the extract was taken and analysed by HPLC and LC-MS. The chromatogram of the ethanol extract of the aerial parts of the chamomile plant is shown in Figure 3.6. The aglycons luteolin (1) and apigenin (2) are indicated in the chromatogram, again there are many glycosidic components in this

sample extract. As expected it is very similar in profile to that of chamomile flowers extracted with ethanol.

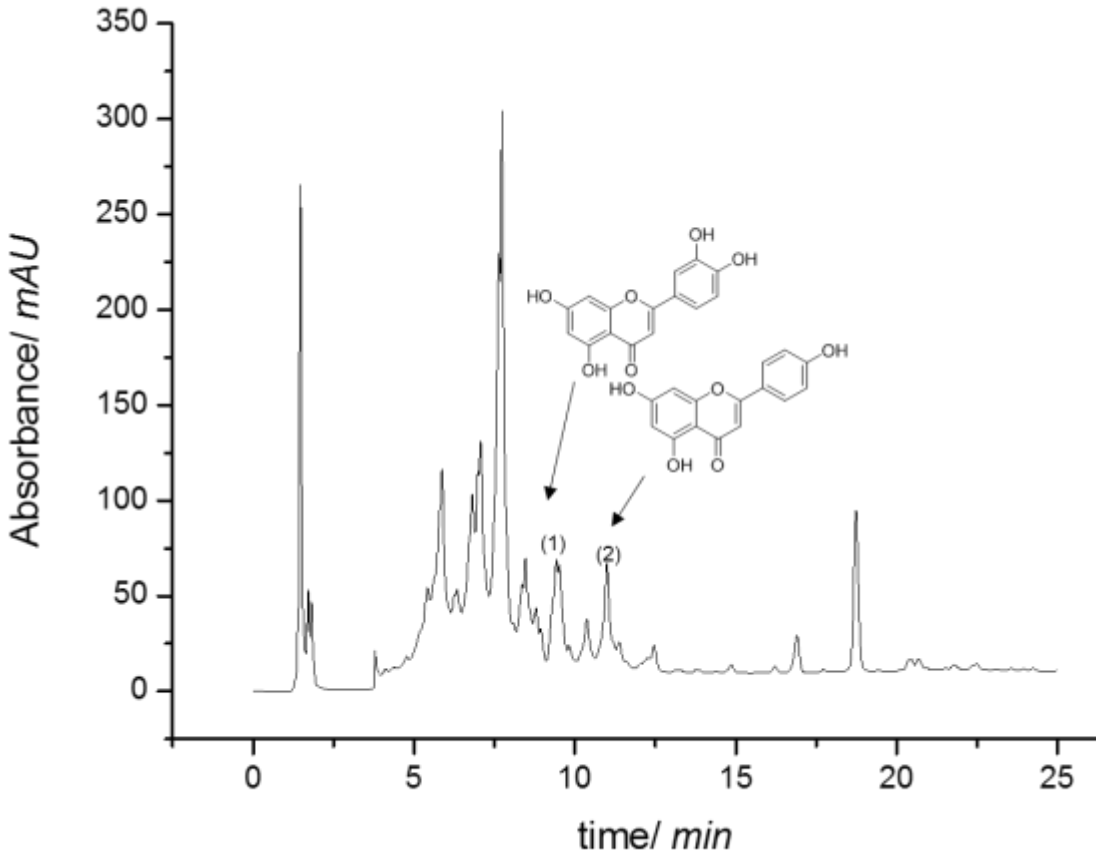


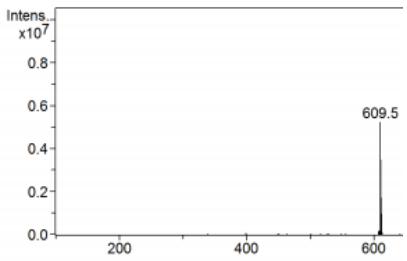
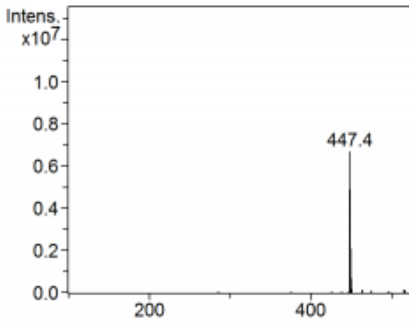
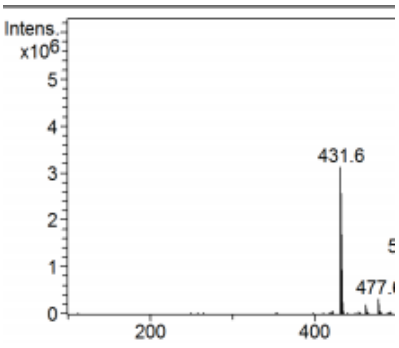
Figure 3.6. HPLC chromatogram of ethanol extracted aerial chamomile plants displaying luteolin (1) and apigenin (2) identified from authentic samples.

The masses of several glycosidic compounds were identified in the extracts by LC-MS. The masses identified corresponded to lutoelin-3',7-*O*-glucoside m/z= 609, luteolin-*O*-glucoside m/z 447, apigenin-*O*-glucoside m/z 431, luteolin m/z 285 and apigenin m/z 269. The flavones are all very close in elution time on the LC-MS but there was a peak giving a mass of m/z 711 and a fragment at m/z 355 in the mass spectrum. This has been assigned to a hydroxycinnamic acid or a derivative of one of the hydroxycinnamic acids. This class of compounds are fully elucidated and explained in further detail in section 3.3.

It is interesting to note that in the chromatogram of the aerial parts of the chamomile plant there is no peak at 15 minutes in the sample. This peak is present in the chamomile flowers in quite significant amounts. However, it does not match any standards tested herein, the UV/vis trace associated with the sample is indicative of a hydroxycinnamic acid with a λ_{max} of 316.[127]

The flavone compounds present in the extract are summarised in Table 3.5, UV/vis and mass spectral data are given in Table 3.4.

Table 3.4. UV/vis and mass spectra of compounds identified from ethanol extracts of chamomile plants.

Compound Identified	UV/vis data	Mass Spectra
Luteolin-3,7-O-glucoside	Not resolved	
Luteolin-O-glucoside	Not resolved	
Apigenin-O-glucoside	Not resolved	

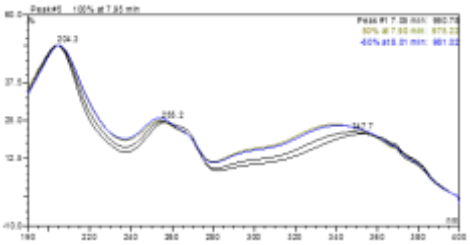
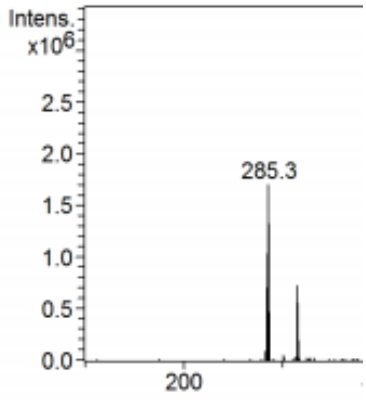
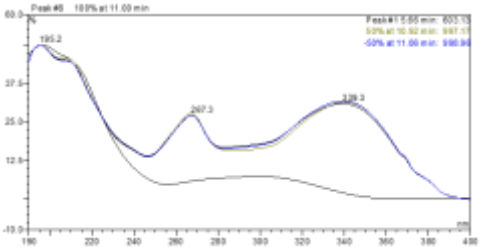
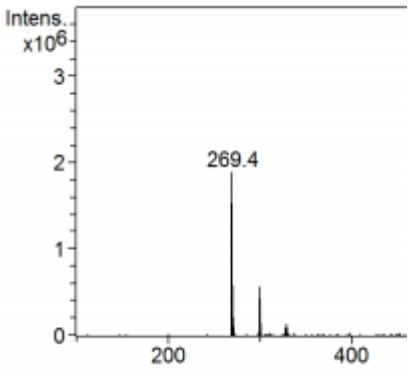
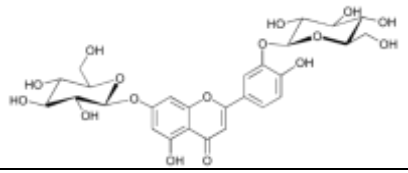
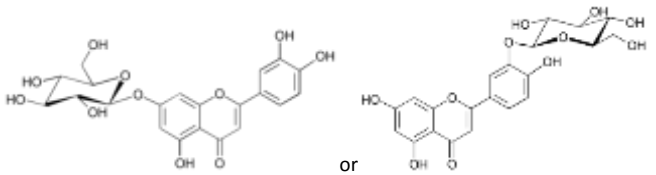
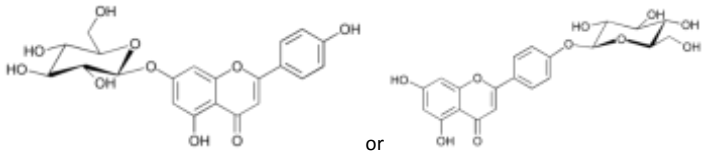
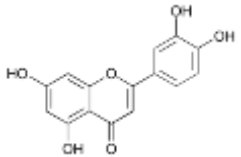
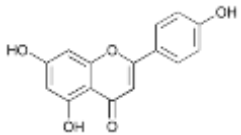
Luteolin		
Apigenin		

Table 3.5. Summary of compounds identified in ethanolic extracts of weld, golden rod, chamomile flowers and chamomile plant.

Flavonoid derivative assigned to HPLC peak	Structure	Molecular ion (LC-MS [M-H]$^-$)	UV λ_{max} (nm)	Weld	Golden rod	Chamomile flowers	Chamomile plant
Luteolin-3',7-*O*-glucoside		609	Not detected		+	+	+
Luteolin-*O*-glucoside	 or	447	Not detected	+	+	+	+
Apigenin-*O*-glucoside	 or	431	Not detected		+	+	+
Luteolin (1)		285	209, 253, 348	+	+	+	+
Apigenin (2)		269	208, 267, 337	+	+	+	+

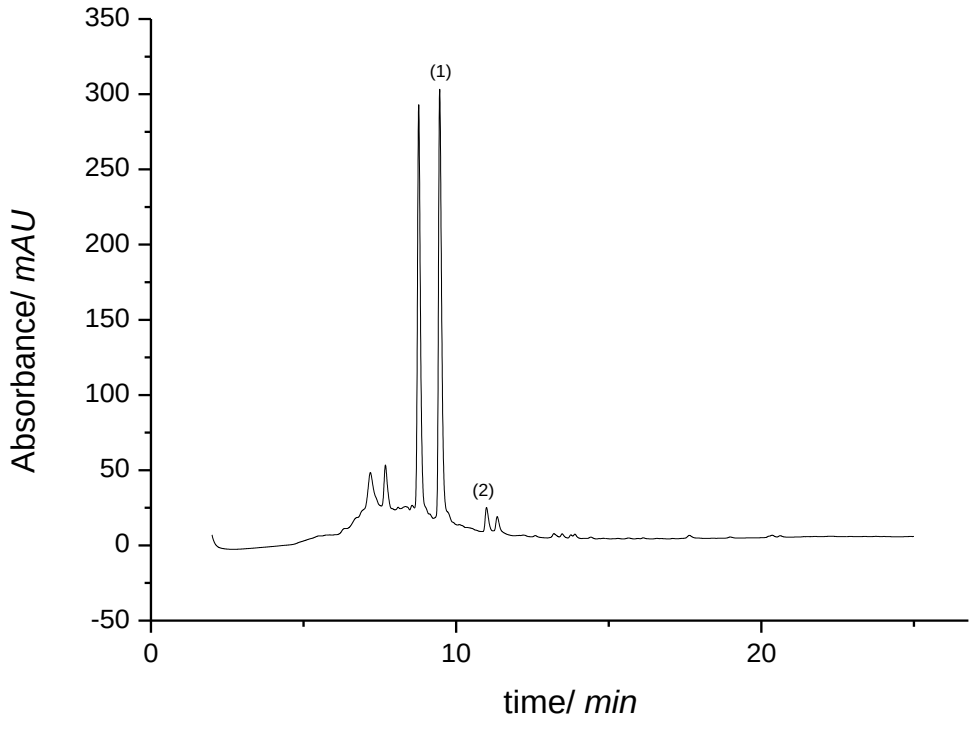
3.2 Water extraction of Yellow dye plants followed by SPE

As described in section 2.2, SPE is a technique which can be used to remove free sugars and unwanted polyphenol compounds from the dye plant extract. Herein SPE was conducted using Amberlite XAD 7HD resin to determine if the extract could be purified using this method. In all SPE experiments, the resulting sample obtained was a yellow/brown amorphous solid all of similar consistency. This differs from the freeze-dried water samples which were hygroscopic and had a sticky consistency, similar to water extraction experiments using madder, which suggests that there are many free sugars in the solutions which are removed in the SPE process. It was also observed that after washing the resin with water, the resultant wash water was a coloured solution which suggests some of the dye compounds are washed out of the extract in this step. However, this colour could also be due to compounds present in the plant other than flavonoids such as tannins.

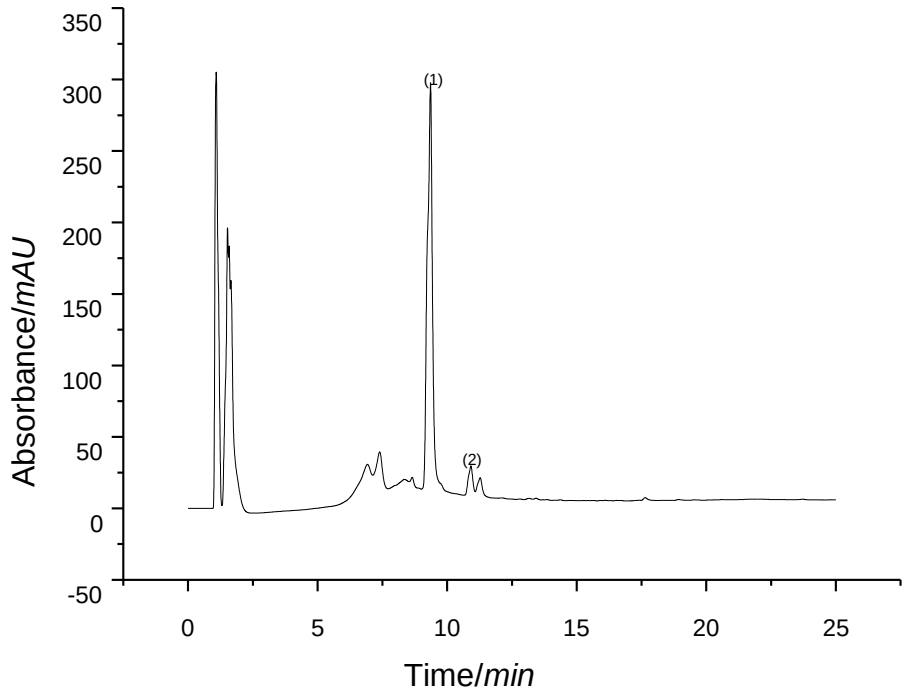
3.2.1 Weld

Weld was extracted with water as described in experimental section 6.3 at 90 °C. Aliquots were taken from the extraction solvent and analysed by HPLC and LC-MS. From the chromatogram of water extract of weld shown in Figure 3.7a, two main peaks are observed, which are assigned to luteolin-*O*-glucoside, which was assigned by the mass and matching UV/vis data from the literature[107] and luteolin, which was assigned based on matching UV/vis data to that of the authentic sample. This differs from the chromatogram where ethanol is used as the extraction solvent (Figure 3.2); in that case there was only one main peak observed in the chromatogram corresponding to luteolin. This suggests that water is a better solvent for extracting glycosidic compounds in the

weld dye plant. The peaks in the chromatogram profiles are identified based on the spectra matching those displayed in Table 3.1.



(a)



(b)

Figure 3.7. Chromatogram showing water extraction of weld (A) and chromatogram showing after SPE extraction of the water extract.

As water is more likely to be the solvent in historical recipes, it is expected that glycosidic components would be observed in a textile dyed with weld, however, it is difficult to know if other glycosides are present due to the drying procedure carried out on the raw plant material before being extracted. The LC-MS of the water extracted sample showed masses of m/z 447, m/z 285, there was no distinguishable peak for apigenin in the LC-MS of this sample, but apigenin was detected in small amounts from the HPLC of the dye plant.

The chromatogram after SPE of the water extracts is significantly different to that of the water extracts shown in Figure 3.7b. The peak corresponding to luteolin-*O*-glucoside is not present after SPE whereas there was a peak for this compound in the water extracts. This suggests that it could have degraded on the resin or the glycosidic form of luteolin does not have strong attraction to the SPE resin and therefore is eluted out of the sample in the water wash with the sugars and the other non-phenolic compounds. The latter is more likely in this case because water and ethanol are the only solvents used in this reaction and hence degradation using these solvents would not be expected. Due to the nonpolar nature of the resin used in the experiments herein the glycosides could be too polar to adsorb efficiently to the resin. However previously it was shown that anthraquinones with a primeveroside (disaccharide of glucose and xylose) adhered efficiently to this resin. It could be that the spatial configuration of the glycoside of luteolin does not allow successful adsorption to this resin. Further experiments of this resin on other dye plants which have an abundance of different glycosidic chemical components to show the ability of this resin to adsorb other glycosidic compounds and are discussed further in the next sections.

This study suggests that if a sample rich in glycosides is required, an SPE using Amberlite XAD-7HD is not a suitable resin for flavonoid preparation and other resins

must be considered. A number of resins could be tested to try and achieve a more efficient purification that does not remove the glycosidic compound luteolin-*O*-glucoside.

3.2.2 Golden rod

The chromatogram of golden rod extracted with water is shown in Figure 3.8a. Again, there is a peak tentatively assigned to luteolin-*O*-glucoside, which is present in abundance after extraction with water. After SPE (Figure 3.8b) the peak corresponding to the luteolin-*O*-glucoside is decreased, which is in agreement with the results shown previously. LC-MS analysis of the water extracts of golden rod showed peaks with the corresponding masses: lutoelin-3',7-*O*-glucoside m/z= 609; luteolin-*O*-glucoside m/z 447; luteolin m/z 285; and apigenin m/z 269. The peak for apigenin glycoside is no longer present in the mass spectrum or the HPLC of this extract, which suggested that this compound is lost in the SPE resin wash process and hence must have low affinity for the resin used in these experiments. The peaks in the chromatogram profiles are identified based on the spectra matching those displayed in Table 3.2.

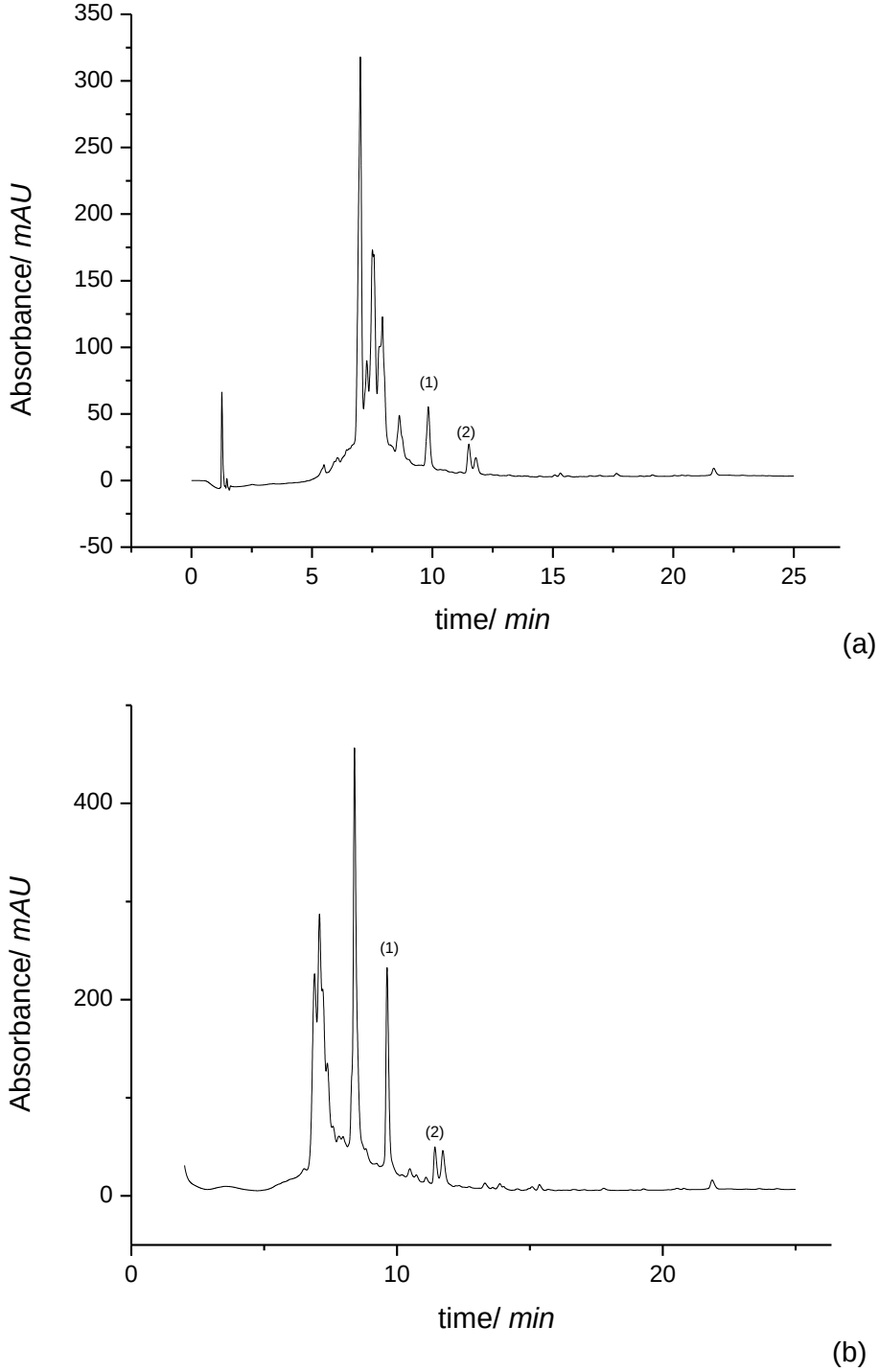


Figure 3.8. Golden rod water extraction before SPE (a) and after SPE (b).

Figure 3.9. Extracts of golden rod with water as a solvent (left) and ethanol as solvent (right).

Figure 3.9 shows the colour difference between golden rod extracted from ethanol, compared to that extracted with water; the colour difference is due to more chlorophyll extracted with ethanol. This difference in the colour of the plant extracts is the same across all of the yellow dye plants tested, which suggests that the flavonoids have a much higher affinity for the water compared to ethanol. This could also be one of the reasons SPE with this particular resin is not as efficient for flavonoid glycosides due to the decreased solubility of the compounds in ethanol.

From Figure 3.8b it is observed that although there is a decrease in the peak assigned to luteolin-7-*O*-glucoside, other glycosidic compounds are still present in the mixture, however, many of the peaks shown in the chromatograms do show decreased peak ratios when compared to luteolin. LC-MS of the extract after SPE displayed peaks with the corresponding masses: lutoelin-3',7-*O*-glucoside m/z= 609; luteolin-*O*-glucoside m/z 447; luteolin m/z 285; and apigenin m/z 269. The mass of apigenin-*O*-glucoside was no longer detected in the extract and hence probably had a weak interaction with the resin and hence was washed out in the water washes.

3.2.3 Chamomile flowers

Chamomile flowers were extracted with water at 90 °C as described in section 6.3. Figure 3.10 shows the chromatogram of the chamomile flowers extracted with water. The aglycons of luteolin (1) and apigenin (2) are labelled. There are many polar compounds present in the chromatogram which elute earlier than the aglycons due to glycosidic derivatives of the flavonoids present in the sample. LC-MS analysis showed peaks corresponding to lutoelin-3',7-*O*-glucoside m/z= 609, luteolin-*O*-glucoside m/z 447, apigenin-*O*-glucoside m/z 431, luteolin m/z 285 and apigenin m/z 269. The peaks in the chromatogram profiles are identified based on the spectra matching those displayed in Table 3.3.

The chromatogram after SPE shown in Figure 3.10b, displays considerably less glycosidic compounds present after SPE of the extract. The peaks corresponding to the aglycons luteolin and apigenin however, are well retained and the peak areas of these compounds do not change considerably before and after SPE. A large decrease in the peak area of luteolin-*O*-glucoside is observed after SPE which is in accordance of all of the above results after SPE. The LC-MS analysis of the extract after SPE gave the peaks for the corresponding masses lutoelin-3',7-*O*-glucoside m/z= 609, luteolin-*O*-glucoside m/z 447, luteolin m/z 285 and apigenin m/z 269. Again apigenin-*O*-glucoside was lost in the SPE process suggesting it has poor affinity to the SPE resin chosen for this study. The peaks in the chromatogram profiles are identified based on the spectra matching those displayed in Table 3.4.

The preliminary experiments done on the yellow dye plants herein do show some differences in chromatographic profiles before and after SPE. There are also differences between the dye plant profiles which are easily observed. These differences could be used to identify dyestuffs of historic importance. Table 3.5 shows the glycosides are shown to be a major contributor to the colourant compounds in the plant and hence when analysing yellow dyes a soft extraction procedure must be used as all of the dye plants

show the presence of luteolin and apigenin aglycons and therefore are not useful for dyestuff identification. The aglycons therefore can only be used as markers in the identification that the textile was dyed with a yellow dye bearing plant.

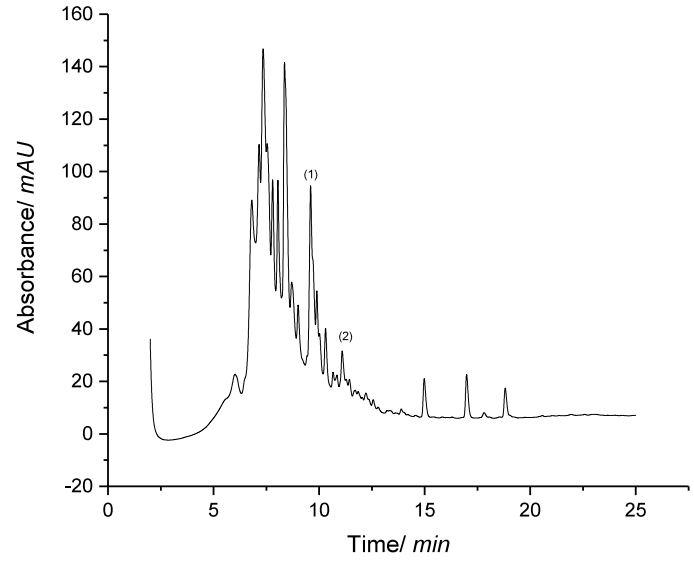


Figure 3.10. HPLC chromatograms of chamomile flowers extracted with water (a) and after SPE (b).

Table 3.6 shows the yields of the dye plants, weld is shown to produce the highest yield of dried extract when extracted with ethanol (9 %) and golden rod shows the highest yield of dried extract after the SPE process (18 %). These yields are of the crude extracts

and hence the content of the desired flavone compounds in the extracts are not considered. However, the SPE process does concentrate some of the desired compounds in the extracts and hence the yield after the SPE process gives a better indication of colorant content. Considering this, it could be assumed that golden rod produces a higher yield of the colourant compounds according to the results herein. In general water is shown to produce higher yields as an extraction solvent, possibly due to the presence of glycosides in the plant providing a more favourable solubilisation into the more polar water solvent.

Table 3.6. Yields of the total dried mass of the extracts of each dye plant with water and ethanol solvents and SPE.

Dye plant	Yield with ethanol extraction (%, w/w)	Yield with water extraction and SPE (%, w/w)
Weld	9%	14%
Golden rod	6%	18%
Chamomile flowers	4%	11%

3.3 Characterisation of cinnamic acids in German chamomile

Herein a case study is provided of a method development to provide in depth structural and spatial characterisation of two previously disputed compounds in chamomile extracts. Due to the research done on the extractions of chamomile and previous identification of methoxycinnamic acids in the extracts a collaboration was set up with Professor Gary Williamson's research group in the School of Food Science and Nutrition within the University of Leeds. The group is researching the actives in chamomile for use in modulating carbohydrate digestion and sugar absorption, which is achieved by inhibiting the α-glycosidase activity which has been shown to be a promising method for reducing the progression of type 2 diabetes.[128]

An extract of German chamomile was used for the study with the aim to identify and isolate the active components which show inhibition to α-glycosidase in the plant. The first active compound in the plant was found to be apigenin-7-*O*-glucoside which was identified by a known standard. Two other products had been isolated from German chamomile extracts by semi-preparative HPLC and were found to be active. The identity of these compounds is widely disputed throughout the literature. The study into these compounds provided a more in-depth knowledge of NMR and the importance of utilising multiple NMR techniques in the full elucidation of complex structures found in natural products. Herein it is shown that 1D NMR is not sufficient to provide the information needed for the proof of which isomer this structure displays, this can only be achieved by 2D NMR experiments.

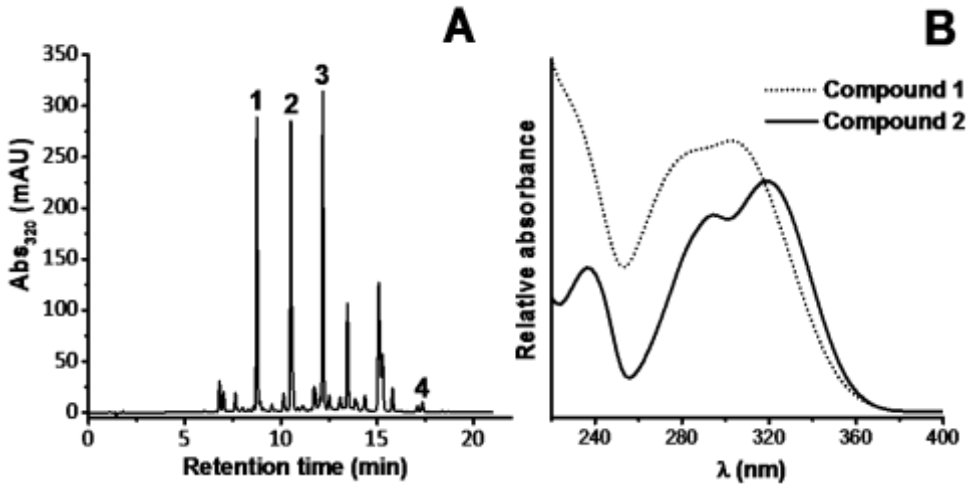


Figure 3.11. A: HPLC-DAD chromatogram of GC extract recorded at 320 nm. (1) (Z)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid (2) (E)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid (3) Apigenin-7-O-glucoside (4) Apigenin. B: UV/vis data for the two identified compounds. Provided by Jose Rodriguez.

HPLC analysis of the extract and sample preparation was done by Jose Rodriguez of the Williamson group in the School of Food Science as described in section 6.10, to obtain the chemical profile of the plant extract (Figure 3.11). Apigenin 7-*O*-glucoside was found to be the main peak in the chromatogram. There was also the presence of two other unknown peaks in the extract, these showed as two distinct peaks

both giving $[M-H]^-$ masses of m/z 355 and 711 as the main constituents (Figure 3.5). These have previously been reported as glycosidic derivatives of ferulic acid.[46] Another group suggested that these peaks were corresponding to a ferulic acid hexoside dimer.[129] The data of these compounds from the literature match those found in the chamomile plants tested herein. However, due to the apparent uncertainty in the literature, the true identity of these compounds was calculated by using nuclear spectroscopic methods.

The samples were prepared by using semi preparative HPLC, the separated fractions were collected and tested for purity using LC-MS. The semi preparative HPLC method had to be completed a few times and purified fractions combined in order to get enough sample for analysis. The samples were then freeze dried and stored at -20°C until ready for analysis.

The chemical characterisation was carried out by ^{1}H and ^{13}C NMR; 1D and 2D experiments were utilised to identify the compounds present in the sample. The fractions were dissolved in d_6 DMSO and submitted for ^{1}H and ^{13}C analysis. It was originally thought that the presence of the other double bond peaks in the spectra could have been due to the presence of an impurity of chlorogenic acid in the extract. However, when the NMR spectra was overlayed with the NMR spectra of a standard of chlorogenic acid it became apparent that these peaks were not due to the presence of chlorogenic acid (Figure 3.12).

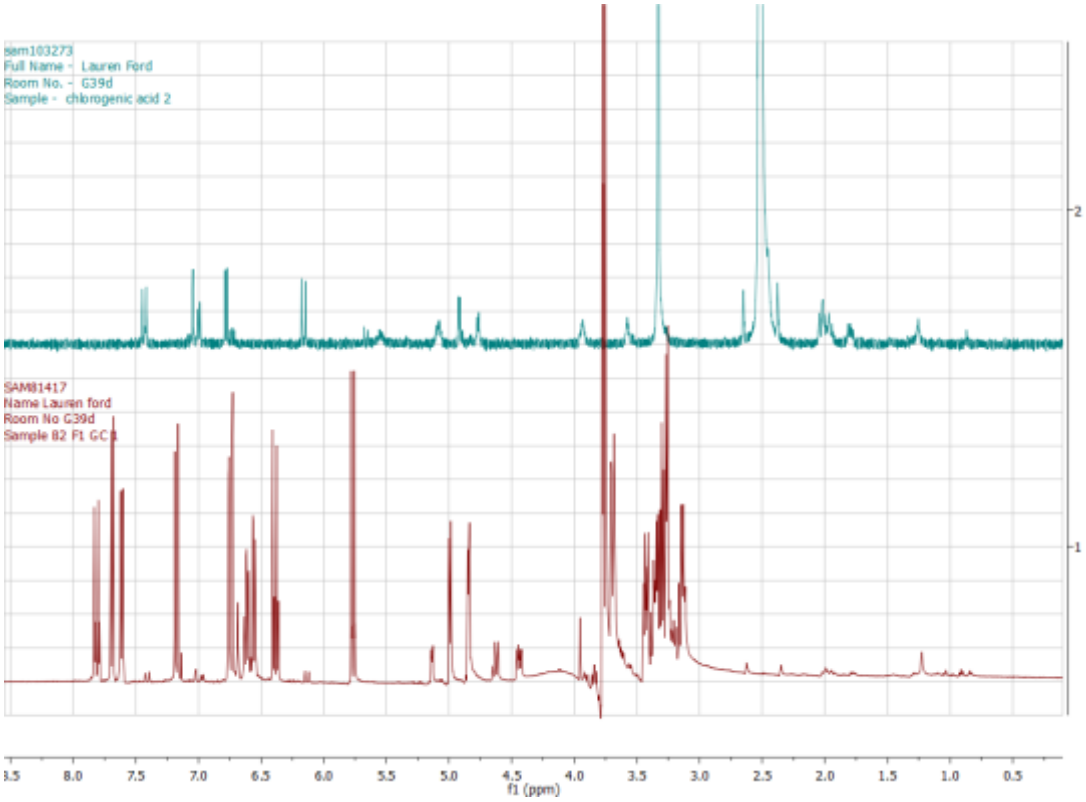


Figure 3.12. Overlayed spectra of chlorogenic acid (green) with the mixture of methoxycinnamic acid isomers (red).

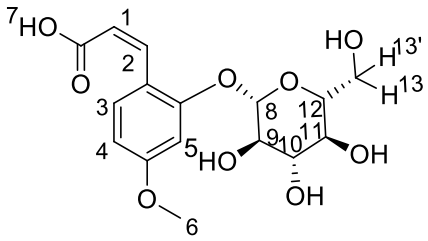


Figure 3.13. Structure of (Z)-2-D-Glucopyranosyloxy-4-methoxycinnamic acid.

(*Z*)-2-D-Glucopyranosyloxy-4-methoxycinnamic acid (Figure 3.13) present as a mixture of E and Z in a 1:3 ratio (Figure 3.14), E peaks eliminated and Z peaks picked at: ^{1}H NMR (501 MHz, DMSO-d_6) δ 7.70 (d, *J*= 8.5 Hz, 1H, H3), 7.19 (d, *J* = 12.5 Hz, 1H, *cis*-alkene, H2), 6.74 (d, *J* = 2.5 Hz, 1H, H5), 6.56 (dd, *J* = 8.5, 2.5 Hz, 1H, H4), 5.78 (d, *J* = 12.5 Hz, 1H, *cis*-alkene, H1), 4.85 (d, *J* = 7.5 Hz, 1H, anomeric proton, H8), 3.77 (s, 3H, H6), 3.72 (broad d, *J*= 11.7 Hz, 1H, H13), 3.47 (broad dd, *J*= 11.7, 6.0 Hz, 1H, H13'), 3.38 (ddd, *J*= 10.0, 6.2, 1.6 Hz, 1H, H12), 3.34-3.29 (m, 2H, H9 & H10)*, 3.16

(apparent t, *J*= 8.8 Hz, 1H, H11). ^{13}C NMR (126 MHz, DMSO-d6) δ 167.7, 161.0, 160.7, 156.7, 136.32, 131.1, 117.53, 116.73, 101.11, 100.5, 77.24, 76.86, 73.27, 69.81, 60.72, 55.33. *Overlayed by strong signal from water.

ESI-MS: *m/z* 355.1035 $[M-H]^-$.

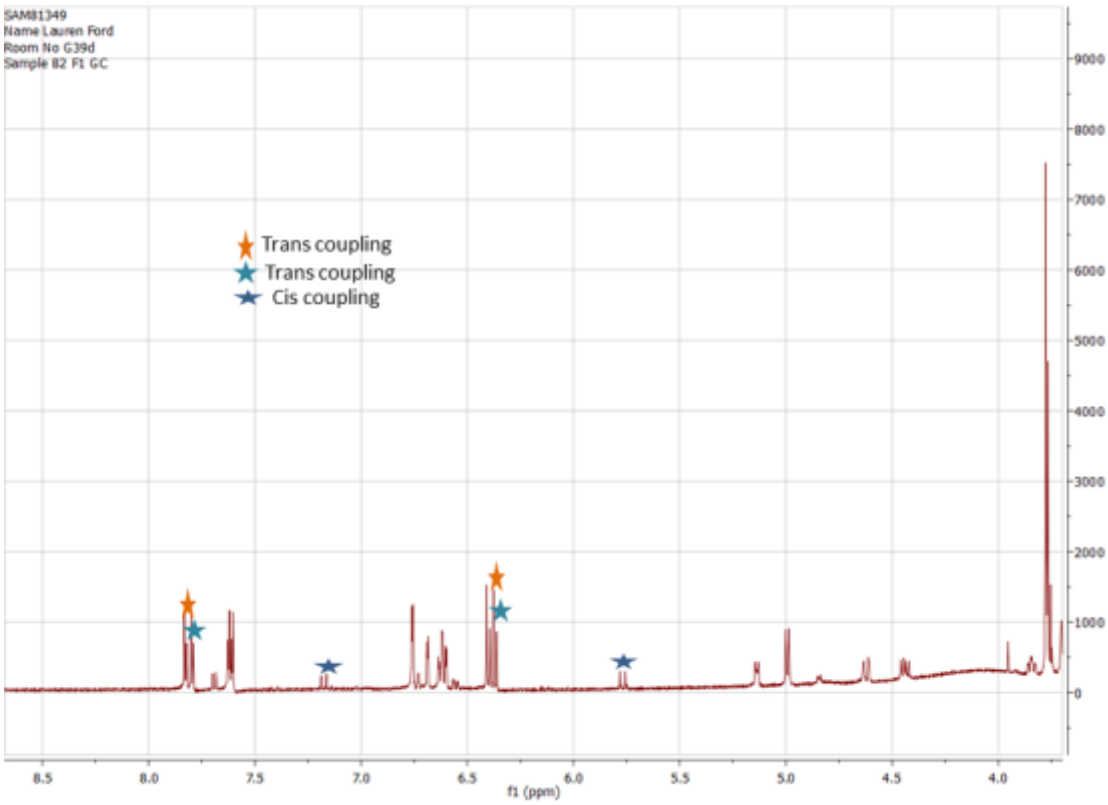


Figure 3.14. ^{1}H NMR of (*Z*)-2-D-Glucopyranosyloxy-4-methoxycinnamic acid present as a mixture of E and Z in a 1:3 ratio. Trans and cis coupled peaks highlighted.

The *trans* conformation of the alkene is more thermodynamically stable and hence it is thought that through time and with the presence of UV light isomerisation of the isomers occurs.[130] Therefore, a pure sample of (*Z*)-2-D-Glucopyranosyloxy-4-methoxycinnamic acid was never obtained by NMR due to isomerisation before the sample ran on the NMR.

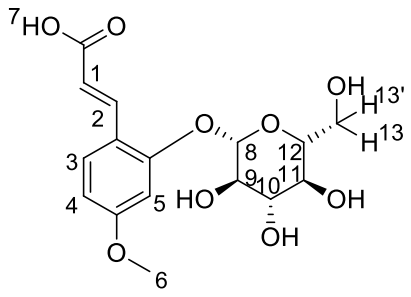


Figure 3.15. Structure of (E)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid.

Analysis of pure (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid (Figure 3.15): ^{1}H NMR (501 MHz, DMSO-d_6) δ 12.00 (s, 1H, H7), 7.82 (d, *J* = 16.2 Hz, 1H, *trans*-alkene, H2), 7.62 (d, *J* = 8.7 Hz, 1H, H3), 6.76 (d, *J* = 2.4 Hz, 1H, H5), 6.62 (dd, *J* = 8.6, 2.4 Hz, 1H, H4), 6.39 (d, *J* = 16.2 Hz, 1H, *trans*-alkene, H1), 5.16 (broad s, 1H (OH)), 4.98 (d, *J* =7.5 Hz, 1H, anomeric proton, H8), 4.89 (broad s, 2H (OH)), 4.58 (broad s, 1H, (OH)), 3.78 (s, 3H, H6), 3.72 (broad d, *J*= 11.7 Hz, 1H, H13), 3.47 (broad dd, *J*= 11.7, 6.0 Hz, 1H, H13'), 3.38 (ddd, *J*= 10.0, 6.2, 1.6 Hz, 1H, H12), 3.34-3.29 (m, 2H, H9 & H10)*, 3.16 (apparent t, *J*= 8.8 Hz, 1H, H11). ^{13}C NMR (126 MHz, DMSO-d6) δ 168.08, 162.08, 157.07, 138.76, 129.32, 116.73, 116.05, 108.03, 101.11, 100.02, 77.24, 76.86, 73.27, 69.81, 60.72, 55.33. *Overlayed by strong signal from water. ESI-MS: *m/z* 355.1035 $[M-H]^-$ Spectra shown in experimental section 6.10.

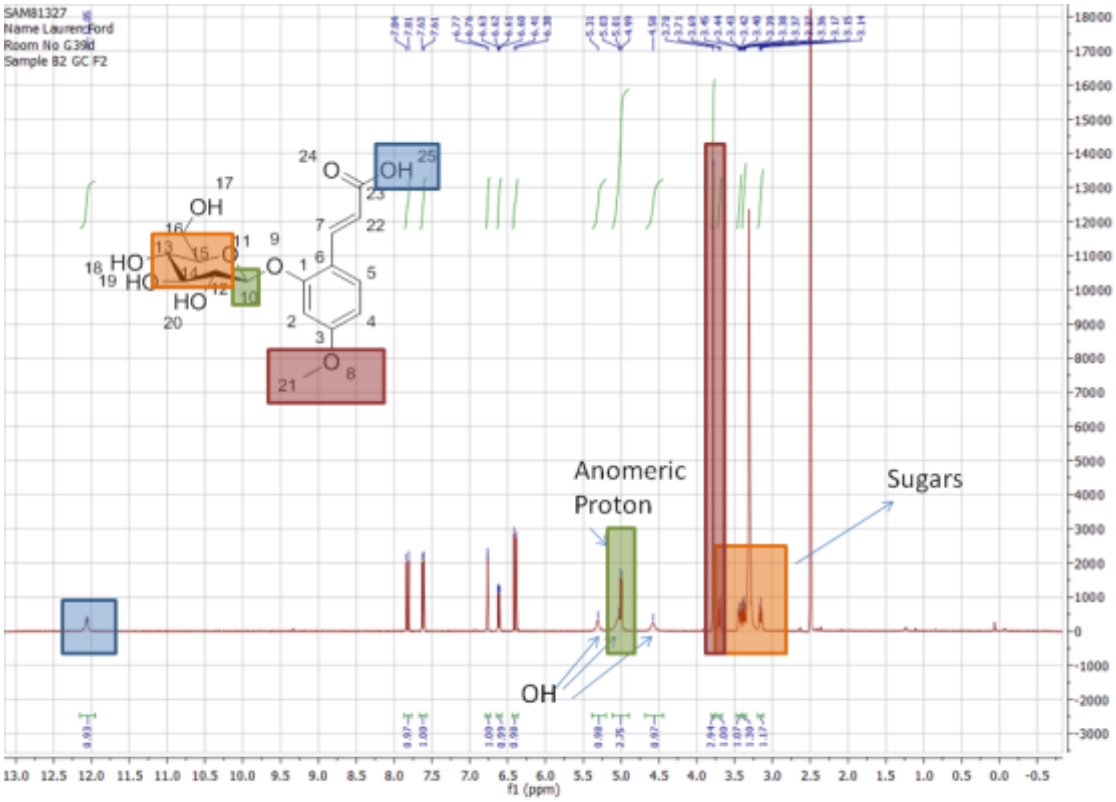


Figure 3.16. ^{1}H NMR (DMSO d_6) characterisation of pure (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid.

A pure sample of the *trans* isomer (Figure 3.15) was obtained by the semi-preparative HPLC method described in section 6.1 and analysed by NMR as shown in Figure 3.16. This solution also isomerised when left exposed to UV light to the *cis* isomer. Therefore, both of these samples were able to change conformation in daylight, which made analysis of these compounds much more difficult as the integrals were not constant. The NMR spectra was solved by identifying the compound in the pure fraction analysed straight after preparative HPLC and taking these peaks from the other spectra.

The trans and cis isomers were fully elucidated and therefore the peaks corresponding to these structures in the HPLC chromatographs could be conclusively identified. It was important to observe that a change in conformation of the alkene in these experiments resulted in the elution of two separate peaks. This study also gives more insight into the important impacts the external conditions have when studying

complex mixtures from plants. Herein it is shown that when the solution containing the extract is exposed to UV light a change in the abundance of the *cis* and *trans* isomers occurs. However, these solutions were of the purified extracts and the isomers may behave differently in the complex mixture of the full plant extract. Reversible and conformational changes due to external conditions such as UV light is however an important aspect to identify and consider when trying to analyse plant extracts.

However, this only tells half of the story, ^{1}H and ^{13}C NMR on their own are not very useful for identifying the full structure of these complex compounds for conclusive identification. They give the characteristic peaks of this kind of system but do not conclusively identify where each functional group is on the aromatic ring. The hydrogen showing as a doublet with *ortho* only coupling of 8.7 Hz indicates that there is a hydrogen on the position next to it on the aromatic ring and there is nothing in the *meta* positions of this hydrogen. The hydrogen showing as a doublet with the coupling constant of 2.4 Hz indicates that there are no hydrogens directly next to it in the ring and but there is another hydrogen *meta* to it and hence a small coupling is observed. The double doublet in the spectra couples to both of these hydrogens and therefore it must be *ortho* to one and *meta* to the other. The coupling constants at 4.85 and 4.98 of the anomeric protons for the E and Z isomers of the glycosidic cinnamic acids respectively both have coupling constants of 7.5 Hz. This coupling of the anomeric proton is indicative of a β-glycoside present in both of the compounds. The coupling shown by this aromatic system is displayed in Figure 3.17.

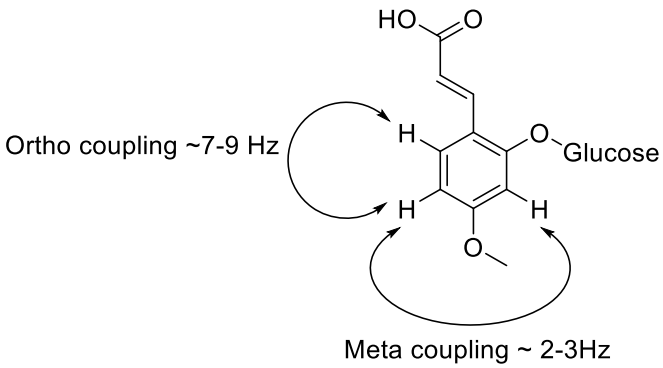


Figure 3.17. Ortho and meta coupling of aromatic systems.

The *cis* and *trans* conformations of the compounds can also be calculated from the coupling constants of H1 and H2 of each isomer. The large coupling constant of 16 Hz signifies a *trans* isomerisation and the smaller coupling of 12 Hz around the double bond indicates the *cis* conformer present in the compound.

This gives an indication of the pattern of substitution around the ring, a *cis* and *trans* double bond present, and a β-glycoside linkage, but does not give any insights to which groups are on each position of the ring. Figure 3.18 shows the structure of ferulic acid which would also follow this pattern of substitution. Therefore, the two dimensional ^{1}H NMR was consulted in order to determine the positions of the functional groups on the structures presented.

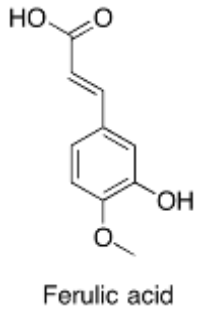


Figure 3.18. Structure of *trans* ferulic acid.

The NOSEY (nuclear Overhauser effect spectroscopy) can be used to show the hydrogens close in space to one another. Hence the hydrogens enhanced in this

spectrum must be in close proximity but not coupled to one another and therefore the placement of the functional groups around the ring can be calculated. This technique was employed as the only way to elucidate the correct isomeric form was to observe the spatial correlations between the hydrogens close in space. The relevant NOSEY enhancements and the percentage of enhancement are given in Figure 3.19 and the NOSEY spectra is displayed in Figure 3.20.

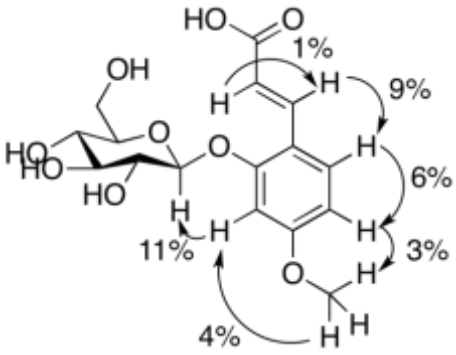


Figure 3.19. Cinnamic acid structure displaying the relevant NOSEY enhancements and the percentage of enhancement of each interacting hydrogen.

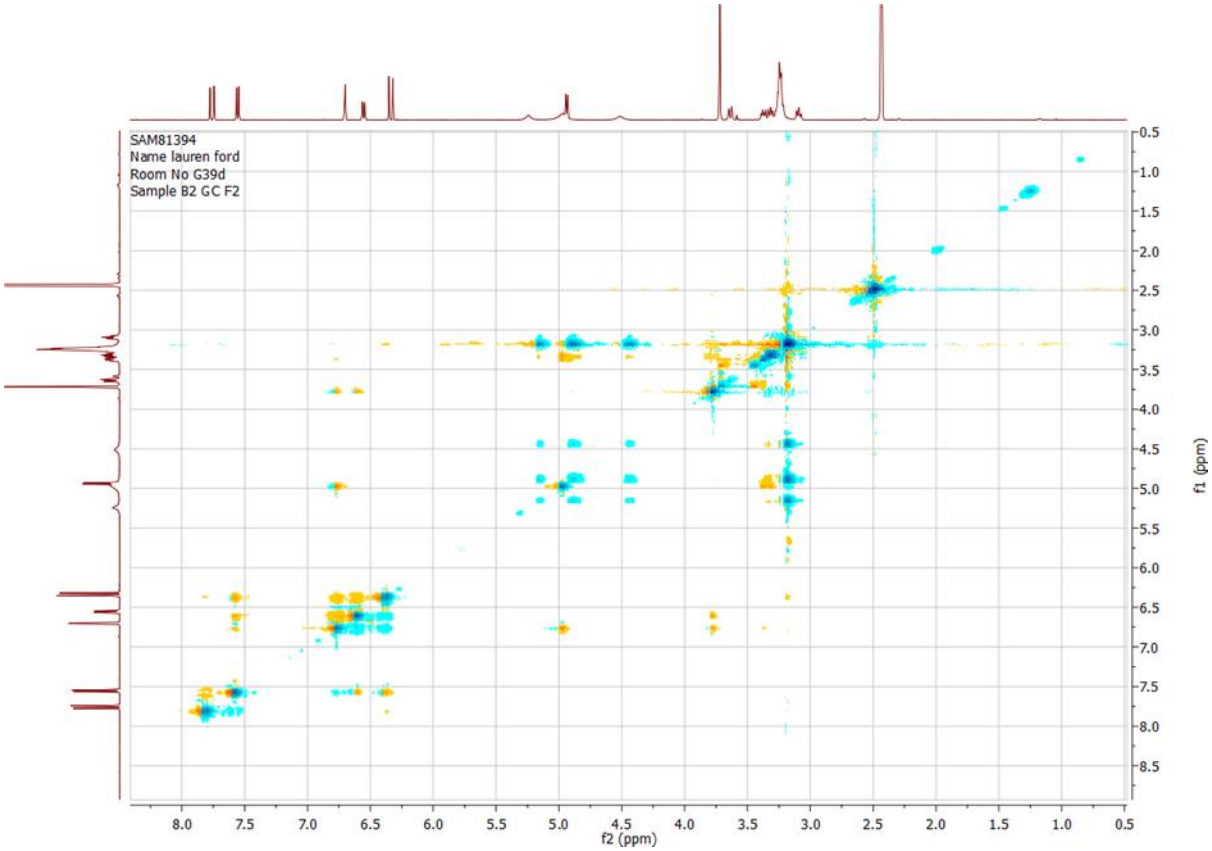


Figure 3.20. NOSEY enhancement spectra of the isolated (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid.

From these spectra, the proposed structure of the compounds is fairly certain but further experiments were done to solidify these findings and ensure the correct structures were identified. The aglycon (Figure 3.21) was prepared as described in experimental section 6.10 and displayed in Scheme 3.1. Due to the nature of the functional groups present in this compound the aglycon can easily form a lactone by an intramolecular ring closing mechanism which results in reformation of the starting material.

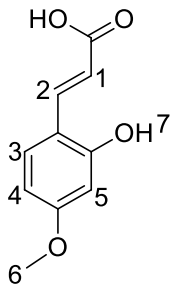
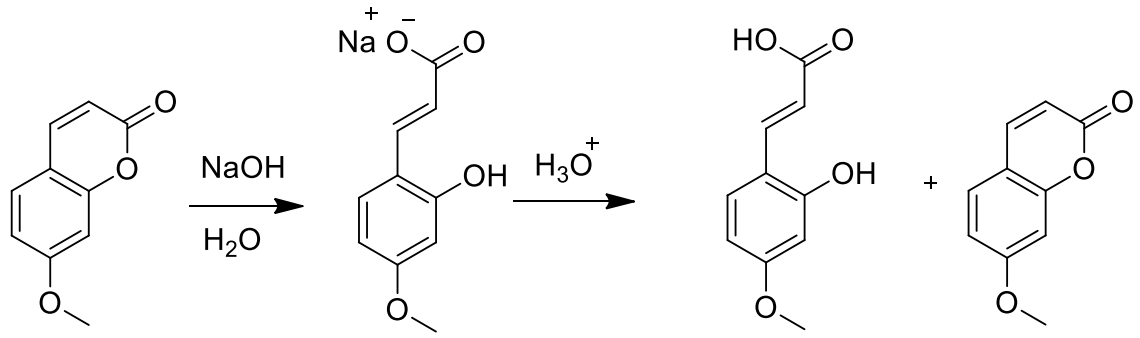


Figure 3.21. Aglycon of methoxycinnamic acid found in German chamomile.

The synthesis was approached by opening the coumarin lactone which is available to buy commercially and is inexpensive. This was hydrolysed using basic conditions as shown in Scheme 3.1. After 4 hours stirring at room temperature in the basic solution aliquots were taken and showed full conversion into the *trans* carboxylate by NMR in D_2O. The solution was then acidified with 1 M aqueous HCl, extracted and purified as described in experimental section 6.10. The low yield of this reaction was due to the ring closure to reform the coumarin in acidic conditions.



Scheme 3.1. Scheme showing the method of preparation of the (E)-2-hydroxy-4-methoxycinnamic acid aglycon from 7-methoxy coumarin.

To try to overcome this ring closure step the reaction was done in the dark to try to prevent isomerisation to the *cis* isomer. However, the coumarin was still the main product after the work up so it is thought that the acid could aid isomerisation into the *cis* isomer which is then able to close the ring in an intramolecular mechanism. However small quantities were purified of the (E)-2-hydroxy-4-methoxycinnamic acid which were then analysed by NMR and HPLC. This synthesis of the algycon was carried out in order to further ensure the correct isomer had been identified by NMR. The synthesised aglycon was then given to Jose to match with hydrolysed extracts from the plant (Figure 3.22). From the HPLC-DAD (Figure 3.22 A) the peak for ferulic acid does not match that of the hydrolysed compound whereas the peak of the synthesised compound does correlate to the hydrolysed peak. This experiment gives further proof of the correct structure presented herein. The synthesis of the aglycon and all NMR studies were done herein and the HPLC associated with this case study was done in Food Science by Jose Rodriguez.

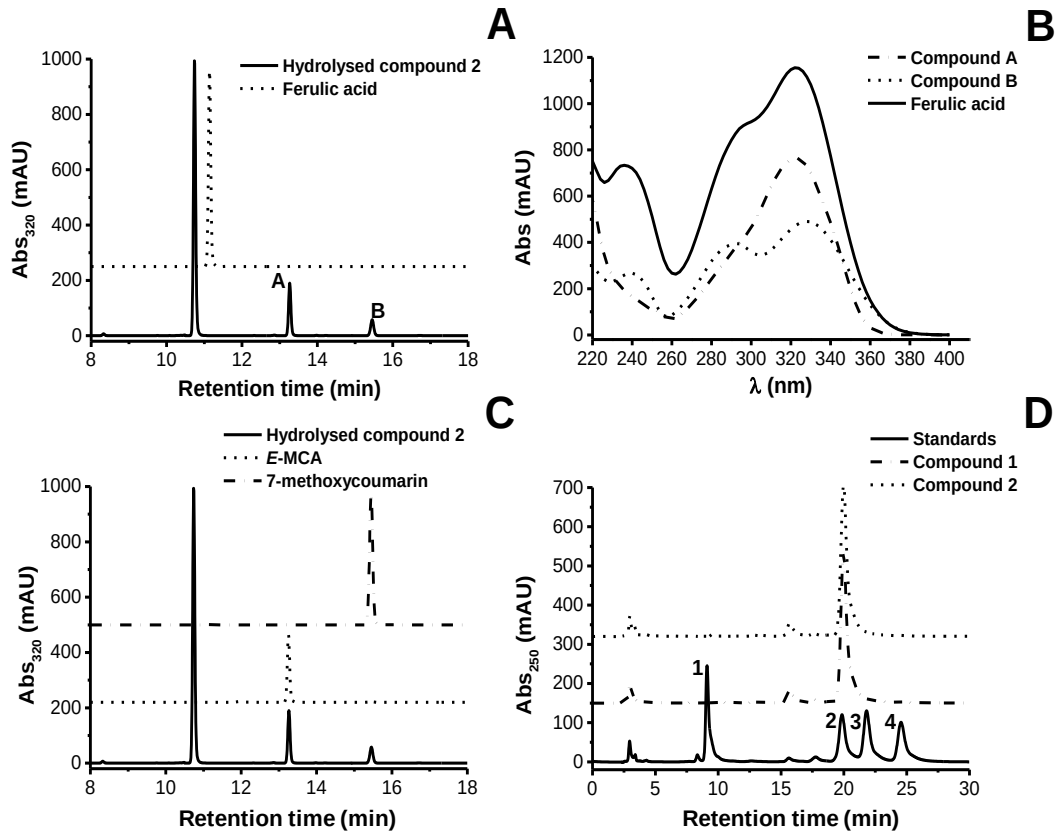


Figure 3.22. Analysis of compound 2 after acid hydrolysis. (A) HPLC-DAD chromatogram of the hydrolysed fraction and comparison of the resulted compounds with ferulic acid standard. (B) UV/Vis spectra of ferulic acid standard and compounds resulted from acid hydrolysis. (C) Comparison of the resulted compounds after acid hydrolysis with standards of (*E*)-MCA and 7-methoxycoumarin. (D) HPLC-DAD chromatograms of PMP derivatives of standard monosaccharides: 1) D-mannose; 2) D-glucose; 3) D-galactose; 4) D-Xylose.

3.4 Conclusions

The SPE resin used herein does not adsorb some of the glycosidic dye compounds in the extract very effectively and hence they are lost from the solution during this process. Further work could look at the efficiency of different resins on the glycosidic compounds in these dye plants to try to establish a better SPE process. A less polar SPE resin could be chosen and assessed to see its efficiency of adsorbing the glycosidic compounds for better purification. This would be advantageous as the dye compounds from the solution could be separated from other compounds present which could hinder the dyeing capabilities of the plant extract. These compounds are also not only used for

dyeing but have some medicinal and health benefits too and hence an extract rich in these compounds could be of high value to the food and cosmetics industries.[47,98]

Further work also needs to be done to fully identify all of the compounds in the chromatograms. The HPLC programme could be amended in order to achieve better separation of the glycosidic compounds in the dye plants on the chromatogram. Also a study needs to be done which characterises all of the compounds present by chemical methods such as NMR or X-ray crystal structure in order to definitively identify the correct λ_{max} of each compound for conclusive identification.

This work fully identifies two compounds in the extracts of German chamomile which contribute to the inhibition of pancreatic α-amylase. The UV-Vis spectra and the diagnostic MS fragments displayed by these polyphenols logically suggest that these compounds are (Z) and (E)-ferulic acid glycosides. It is for this reason many reports have mistaken these compounds for the ferulic acid derivatives. However the correct structures are fully elucidated herein by 1D and 2D ^{1}H and ^{13}C NMR. This ensures that the correct structure can be identified from natural extracts using the data provided. For full understanding of the plant extract it was considered important to gain as much information about these compounds as possible and observe how they behave in solution due to their peaks being present in the extraction from the plants. A better understanding of the importance of in depth NMR studies in the structure and characterisation of compounds to confirm peaks in HPLC chromatograms was also gained from this chapter of work. This case study has exemplified how even if mass spectral data and UV/vis spectral data are obtained the complete structural information of the compound is still difficult to conclusively identify using only these techniques.

4 Dyeing with natural dyes

In the previous chapters the extractions of the yellow dye plants and madder are considered. Once these compounds have been extracted they can be applied to a fabric substrate. This chapter examines the dyeing mechanisms involved with these complex mixtures of dye compounds onto wool substrates. As described in section 1.7, dyeing wool requires the use of a mordant in order to fix the dyestuff onto the fibre, theoretically providing a binding site for sorption. Historically this was alum (potassium aluminium sulfate),[3,4,12] and hence this was used herein; cream of tartar (potassium hydrogen tartrate) was also used historically to help soften the wool and allow uptake of the mordant onto the wool. Cream of tartar is a weak acid and hence will release H^+ upon dissolving in water. The decreased pH of the solution helps assist the mordanting procedure and softens the wool due to the presence of the slightly acidic solution. Other 'dye assistants' could be used such as oxalic acid, but cream of tartar is often favoured with alum mordanting.[19]

Wool was first scoured with a non-ionic detergent and then pre-mordanted using potassium aluminium sulfate and potassium hydrogen tartrate, as described in experimental section 6.5.1. The dyebath was prepared using the method described in experimental section 6.5, using 30% omf of the chosen dye plant matter. The pre-treated wool was then immersed in the dyebath and heated for 1 hour at 90 °C.

This study was done to simulate textiles from historic artefacts with the aim to observe which compounds were adsorbed onto mordanted wool. The extracts of madder chosen for study were the water extracts of the roots of madder, the previous chapters have given robust chromatographic profiling of these plants extracts in order to identify all peaks in the experiments. Water extractions were chosen for study due to the use of water in historical recipes for dyeing.[12] This study was done to better understand which compounds in these extracts are actually adsorbed onto the wool during these

processes and in what abundance. Isotherm studies were applied to the individual compounds in the dye plants, although the adsorption properties of madder roots as a complex mixture have been studied previously[29] the individual dye components and the effect of different functional group on the adsorption properties have not been studied and hence are not yet fully understood.

4.1 HPLC and UV/vis dyeing studies with Iranian, Turkish and English madder

Aliquots were taken from the dyebaths before and after dyeing for analysis by HPLC and UV/vis using a standalone UV/vis spectrometer; samples were diluted by a factor of 4. The full experimental procedure is detailed in experimental section 6.9.

From the visible spectrum of Iranian madder (Figure 4.1a), it can be seen that there is reduced absorbance across the whole spectrum after dyeing which suggests most of the compounds in the dyestuff were adsorbed onto the fibre. The area under the curve was integrated using the origin software to show the percentage decrease across the spectrum. Iranian madder was shown to decrease in absorbance by 82 % when the integrations of the curves were subtracted from one another and the percentage calculated. The peak at 430 nm corresponding to the maximum wavelength absorbance of alizarin decreases rapidly, suggesting uptake onto the fibre which was expected due to the common presence of alizarin in back extractions from textiles dyed with *Rubia tinctorum* roots.[57,60,90,106,122] There is also a shoulder at 530 nm which completely disappears after dyeing; the visible spectra of purpurin shows a similar shoulder and hence this could be assigned to uptake of purpurin; this small shoulder is also present in commercial samples of ruberythric acid (Figure 4.2). It was originally thought that the sample of ruberythric acid may have contained some purpurin but HPLC analysis confirmed the sample contained only lucidin primeveroside and ruberythric acid and

hence this shoulder must also be due to the absorbance profile of either ruberythric acid or lucidin primeveroside.

The visible spectrum of Turkish madder before and after dyeing (Figure 4.1b) also shows the decrease of absorbance across the whole spectrum after dyeing, suggesting again that most of the colorant compounds in the dyebath have been adsorbed onto the wool. The adsorbance decrease of Turkish madder was shown to be 67 % from the integration calculations from the curves. The results from this are very similar to the results shown for Iranian madder, which is to be expected as chromatograms showing the extractions of these two types of madder are very similar in terms of composition.

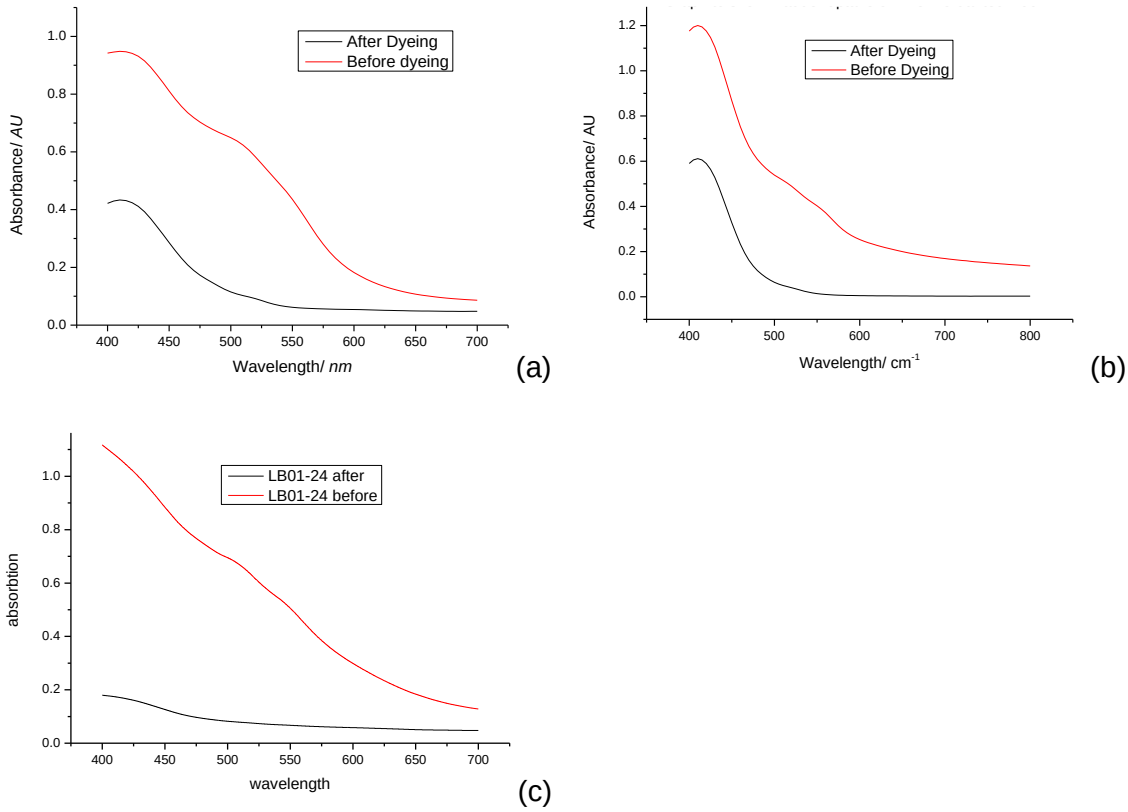


Figure 4.1. Visible spectra of the dyebaths, before, and after dyeing with (a) Iranian, (b) Turkish, and (c) English madder.

The visible spectrum of English madder before and after dyeing (Figure 4.1c) shows a big decrease in absorbance across the whole spectrum accounting to a 94 % decrease in absorbance calculated from the areas under the curve. This shows that most of the dye components present in English madder have been adsorbed onto the wool. English madder shows the highest decrease in absorbance of all three of the dye plants tested herein. This is possibly due to the nature of the compounds present in this extract having high adherence to the mordanted wool. However, in all cases there is a large decrease in absorbance across the whole spectrum suggesting good uptake of colorants onto the fibre.

The compounds present in the root which do not contain a chromophore could not be detected using this method. However, this study aims to understand the dyeing capability of the anthraquinones from the roots of *Rubia tinctorum* and hence the compounds of interest can be detected by UV/vis. A study into the compounds which do not contain a chromophore could be interesting to understand if there is any degradation of compounds in the dye process this is discussed further in section 7.1.

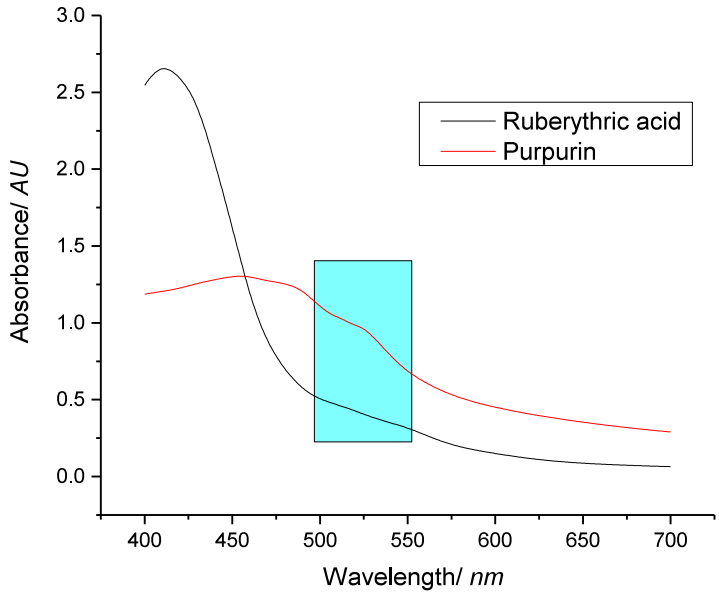


Figure 4.2. UV/visible spectrum of purpurin and ruberythric acid with the shoulder at 530 nm highlighted.

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; if the peak area decreased then it indicated that that compound was adsorbed onto the fibre. The peak areas were obtained from the software from the HPLC system and the changes in peak area could be calculated by subtraction of the peak area after dyeing from the peak area before dyeing. The glycosides lucidin primeveroside and ruberythric acid are not fully resolved, as the commercial standard labelled “ruberythric acid” was found upon analysis to be a mixture of the two compounds, hence changes in the peak area of these two compounds were combined to give one value for the “glycosides”. Whilst it is not ideal to have unresolved peaks, for this study the main aim was to see what effect the functional groups of the anthraquinone backbone had on the dyeing capability. Due to the key functional group on lucidin primeveroside being the same; a primeveroside sugar moiety, their dyeing capabilities were studied as one peak. The peak area can be measured and therefore the changes in the peak area indicate the effect of the sugar groups on the dyeing capabilities.

This study demonstrates which compounds are adsorbed by the fibre and provides more comprehensive data on which compounds should be present in a back extraction of a textile dyed with these dyes. A summary of the compounds found in the roots of the three madder varieties and their uptake onto wool can be found in Table 4.1.

HPLC analysis of the compounds present in a dyebath of Iranian madder before and after dyeing (Figure 4.3a) reveals that the concentration, as indicated by the change in peak area (Table 4.1), of all dye compounds in the dyebath solution decreased upon the addition of mordanted wool; some are not present at all in the dyebath after dyeing. This suggests that all dye compounds expected to be adsorbed in the dyeing procedure are adsorbed onto the wool under these conditions, and confirms that all of the

compounds present in the water extracts of Iranian madder should be present in an efficient back extraction technique.

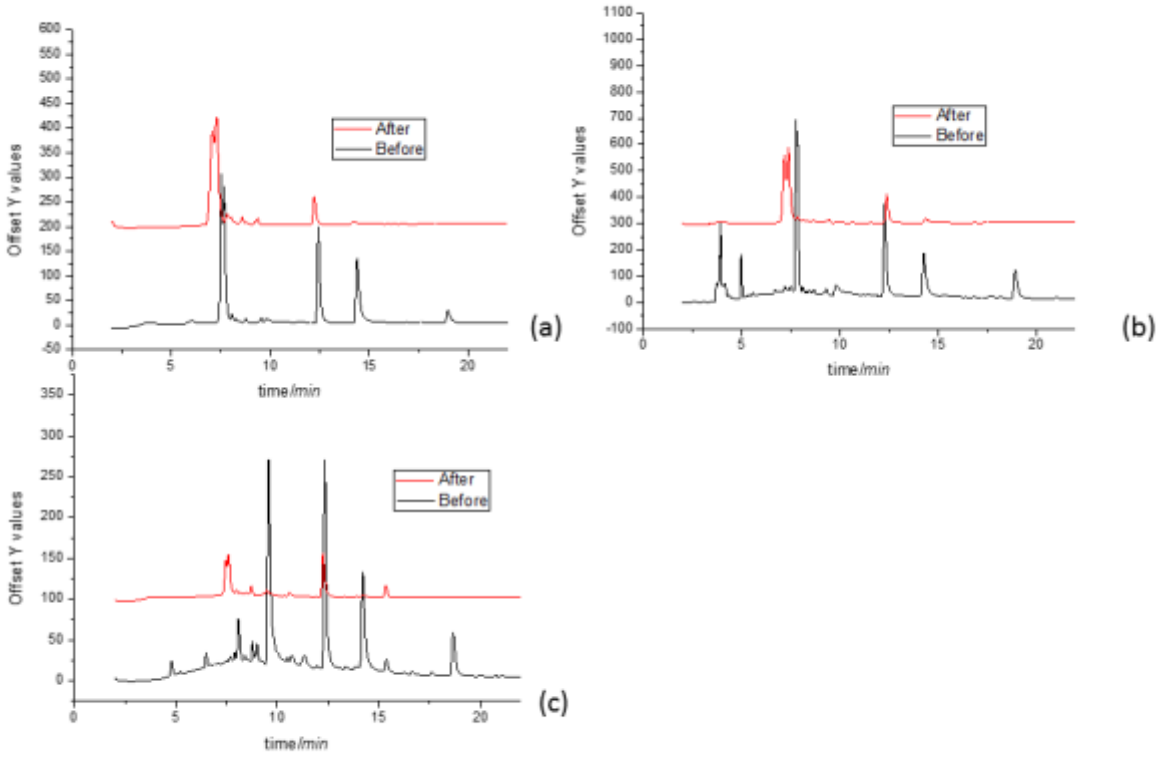
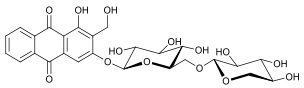
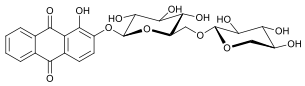
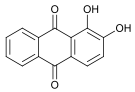
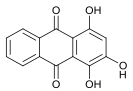
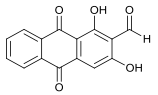
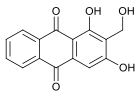
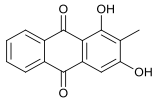


Figure 4.3. HPLC of water extracted (a) Iranian, (b) Turkish, and (c) English madder before (black) and after (red) dyeing on mordanted wool measured at 254 nm.

Table 4.1. Comparison of compounds present in different madder dyebaths: % of total peak area 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 to HPLC peak	Structure	UV/vis λ_{max} values measured at 254 nm for compound identification (nm)	Iranian		Turkish		English	
			% of total peak area before dyeing	% change of peak area after dyeing	% of total peak area before dyeing	% change of peak area after dyeing	% of total peak area before dyeing	% change of peak area after dyeing
Lucidin primeveroside (3)		200, 246, 285	52.5	18.2	37.9	16.4	5.8	7.1
Ruberythric acid (1)		224, 256						
Alizarin (2)		198, 249, 279	23.0	57.7	20.4	69.0	28.6	76.9
Purpurin (11)		210, 255, 294	20.4	81.0	12.2	85.0	16.9	100.0
Nordamnacanthal (5)		214, 259, 297	4.1	100.0	8.4	100.0	8.6	100.0
Lucidin (4)		200, 247, 288	n.d	n.d	n.d	n.d	35.8	100.0
Rubiadin (13)		225, 248, 281	n.d	n.d	n.d	n.d	2.0	11.0

HPLC chromatograms of Turkish madder before and after dyeing (Figure 4.3b) are very similar to those of Iranian madder; each peak area decreased after dyeing, which shows that every major component of the dye adsorbed onto the fibre. 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). It is assumed that the nordamnacanthal is being adsorbed onto the wool rather than undergoing a chemical change and hence showing a diminished peak. Due to the absence of reducing agents in the dye bath it would be highly unlikely that the nordamnacanthal in this dyebath would be reduced back to lucidin. The other reaction it could undergo would be oxidation as the dyeing process is done in an open vessel and hence interaction with oxygen is possible but this would result in munjistin which would display a peak in the HPLC chromatogram but is not observed herein.

HPLC chromatograms of English madder before and after dyeing (Figure 4.3c) show a very different composition to that of Turkish and Iranian madder. As described in chapter 2, English madder has much lower concentrations of glycosides and consists mainly of the aglycons lucidin, alizarin and purpurin. The HPLC of the English madder extracts after being dyed with mordanted wool still show a peak of the glycosidic compounds. However, the peak area is reduced suggesting there has been some adsorption. After dyeing the peaks of the glycosides and alizarin are more similar in size, however as explained further below this is due to the higher response factor of alizarin when using the DAD detector.

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 and is given by the equation:

Response factor = Peak Area / Concentration

The response factor is therefore essentially a ratio between the concentration of a peak and the response that the chosen detector gives for that peak in the form of the peak area. Therefore, if a standard of a known concentration was used in the HPLC profile, the peak area obtained from that known concentration can be used to calculate the response factor. 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 glycosides and alizarin, which is representative of each madder variety as these are the most significant peaks in the chromatograms and both of these compounds are commercially available. Response factors of the compounds found in madder root are summarised in Table 4.2; R^2 values provide a representation how reliable the data is from the calibration plot based on linear regression; clearly high correlation is observed as both R^2 values are >0.99 all calibration plots are shown in experimental section 6.9. It is observed that the response factor for alizarin is much greater than for the glycosides, which demonstrates that a much smaller mass of alizarin is needed to give a large peak on the HPLC chromatogram. The consequence of this is that due to the largely differing response factors the peak area ratio using UV/vis as a detector at this wavelength does not give an even representation of the concentration of these compounds. This could result in there being limitations on the limits of detection when analysing the glycosides in historical artefacts due to their lower response factor. However, this study displays the importance of considering the response factor and expressing the HPLC chromatogram peaks in concentrations rather than peak area ratios. This is also an important factor to consider if it is necessary for molar quantification of all compounds in a plant extract if plant extracts were to be used in industry. Another way to do this would be by isolation of all compounds and calculate the yields of each compound by weight but if commercial standards are available this method allows for fast quantification of the calibrated compounds in a plant extract.

From the change of concentration of the dyestuffs before and after dyeing, it can be seen that for both Iranian and Turkish madder there is a 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 lucidin primeveroside and ruberythric acid, and not alizarin as suggested in much literature that focuses on analysis of textile artefacts.[44,58,122] It is unclear why the Iranian madder allows for more adsorption of the glycosides when compared to Turkish madder. It could be due to compounds such as enzymes or tannins present in the complex dye matrix which could not be detected by HPLC or UV/vis which could be either helping or hindering the dyeing process.

Whilst it is clear that the glycosides are major compounds in these dye plants, the presence of the glycosides as the main dye compounds adsorbed on to the wool will still depend on the dye bath conditions before dyeing. If acid is used in the extraction of the colorants from the root the glycosides will not be present due to hydrolysis.[25] It is due to this hydrolysis that alizarin is often referred to as the main compound in the roots of *Rubia tinctorum* due to its identification in textiles dyed with madder. It is still routine for the analysis of historical textiles to extract with strong acid and therefore the main compounds observed are the aglycons.[44,106,108] However the work herein shows that the adsorption of glycosides is a major factor to the colorant of a dyed textile when they are present in the dye bath.

Table 4.2. 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 (mmol g^{-1})		
			Iranian	Turkish	English
Ruberythric acid and lucidin primeveroside (glycosides)	0.996	174	0.0146	0.0188	0.0065
Alizarin	0.994	2280	0.0034	0.0074	0.0050

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). Although some lucidin was synthesised for research in this project the quantities obtained through synthesis were very small. All of the synthesised compound was used in the study of the breakdown of lucidin (chapter 5) and hence there was not enough spare to perform calibration curves. Despite the fact English madder contains low concentrations of glycosides in comparison with Turkish and Iranian madder, there are still a higher number of moles of glycosides adsorbed with respect to alizarin.

From this study, the absolute change in concentration of the two major classes of compounds in madder, the glycosides (lucidin primeveroside and ruberythric acid) 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 *Rubia* spp.[11,26,61,62] This study also shows that in analysing dyed textiles, one may expect to observe greater molar concentrations of glycosides in back extraction in comparison with alizarin. However, it is important to consider that the glycosides are more sensitive to external conditions in the dye bath

such as; the presence of acid. The hydrolysis of these compounds in some dyeing recipes and methods to extract from textiles for analysis also contribute to alizarin being referred to as the main dye compound in *Rubia spp* due to the fact that they are not observed under these conditions and it is only the aglycons such as alizarin that can be detected.

4.2 Sorption isotherm studies

Isotherm studies were carried out for three of the main compounds found in madder, each of which had different functional groups; the aim was to establish how the chemistry of these compounds affects their interaction with the wool. The three different components used were: pseudopurpurin, which has a carboxylic acid moiety; a mixture of ruberythric acid and lucidin primeveroside, each of which have glycoside moieties; and alizarin, which has the basic hydroxyl anthraquinone backbone. Pseudopurpurin was donated from the British Museum and analysed by ^{1}H NMR before use see experimental section 6.9. Although pseudopurpurin (**Figure 4.4**) was not detected in the HPLC analysis of the three *Rubia tinctorum* varieties used herein, it is easily decarboxylated under the drying conditions and hence is still useful to analyse. Pseudopurpurin is highly cited as being present in fresh madder samples. Due to the three electron donating hydroxyl groups around the aromatic ring containing the carboxylic acid the decarboxylation of pseudopurpurin is very easy.[25,33,81] It also is thermodynamically more stable due to entropic favourability of two products over one. Therefore, pseudopurpurin can degrade through loss of carbon dioxide to purpurin. However, the carboxylic functional group is still important to understand in terms of dye compounds extracted from these plants and hence the dyeing properties of pseudopurpurin was considered and the adsorption isotherm plotted herein. Pseudopurpurin is also a good representative of other carboxylate containing anthraquinones present in *Rubia cordifolia* (Indian madder) such as munjistin, which is

a major dye used historically and, hence, worthy of comparison. The glycoside mixture was purchased from Apin chemicals as ‘ruberythric acid’, but HPLC analysis showed it to be a mixture of ruberythric acid and lucidin primeveroside. NMR analysis also showed that the mixture obtained from Apin chemicals contained many peaks in the region of 3-4 ppm due to free sugars in the sample; these were removed by SPE and the purified glycosides were used in the present study. Alizarin was used as obtained from Sigma Aldrich.

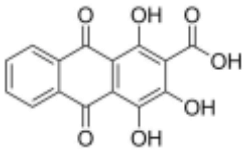


Figure 4.4. Pseudopurpurin structure

Adsorption isotherms are used to identify and quantify the affinity of a sorbent to a substrate. In this case this is the affinity of the anthraquinones containing different functional groups to the mordanted wool. A range in concentrations must be used when dyeing in order to measure the affinity of the dyestuffs at all concentrations. By plotting the solid-phase concentration of dye uptake against the dye still in the solution, the liquid-phase concentration gives a graphical representation of the dye equilibrium in question and by application of different isotherm models thermodynamic data can be obtained. It is important for this research to gain as much understanding as possible about how these compounds are being adsorbed onto the wool. Understanding their binding properties enables a better understanding of how these compounds are interacting with the wool and therefore how to back extract them with the least degradation possible. Whilst studies have previously been done to assess the adsorption properties of a madder extract as a whole, this is the first known adsorption isotherm study into the separated components of *Rubia tinctorum*.[131]

The Langmuir isotherm[132,133] 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 (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 and hence even if the concentration is increased there will be no more adsorption once all monolayer sites are filled. The saturated or monolayer capacity can be represented by the expression represented in equation 4.1:

$$q_e = \frac{K_L C_e}{1 + a_L C_e} \tag{4.1}$$

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 linearisation of equation 4.1 (equation 4.2).

$$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e \tag{4.2}$$

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 4.2 is only respected at low solution concentrations, where the model follows Henry's law: as C_e becomes lower, $a_L C_e$ is much less than unity and $q_e = K_L C_e$.

The Freundlich isotherm[134,135] suggests that sorption energy exponentially decreases on completion of the sorptional centres of an adsorbent and describes heterogeneous systems, which are characterised by the heterogeneity factor $1/n_F$. When

n (the exponential factor) = 1/n_F, the Freundlich equation reduces to Henry's law. Hence, the empirical equation (equation 4.3) can be written:

$$q_e = K_F C_e^{\,1/n_F} \tag{4.3}$$

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 4.3 (equation 4.4).

$$\ln q_e = \ln K_F + \frac{1}{n_F} \ln C_e \tag{4.4}$$

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. 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.

Unlike the Langmuir equation, the Temkin isotherm[136] 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 to get adsorbed is correspondingly reduced; that is, adsorption takes place on a non-uniform surface. The Temkin isotherm takes the following form (equation 4.5):

$$q_e = \frac{RT}{b_T} \ln(K_T C_e) \tag{4.5}$$

where K_T is the equilibrium binding constant corresponding to the maximum binding energy, b_T is the Temkin isotherm constant, T is the temperature (K), and R is the ideal gas constant (8.3145 J mol^{-1} K^{-1}). The equation can be linearised as (equation 4.6):

$$q_e = B_1 \ln K_T + B_1 \ln C_e \quad (4.6)$$

Where $B_1 = RT/b_T$.

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 characterised 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_1 and K_T from the slope and the intercept, respectively. K_T is the equilibrium binding constant (dm^3 mol^{-1}) corresponding to the maximum binding energy and constant B_1 is related to the heat of adsorption.

Thermodynamic data such as adsorption energy can be obtained from K_L, K_F and K_T (equation 4.7), where K is constant in terms of dm^3 mol^{-1}.

$$-\Delta G = RT \ln K \quad (4.7)$$

To perform the isotherm experiments a stock solution was made of the chosen dyestuff and then the solution was diluted to give five dyebaths of different known concentrations; absorbance of the dyebaths was then measured before and after dyeing. Using Beer-Lambert law the concentration still left in solution after dyeing can be calculated from its absorbance and therefore the concentration of the dye adsorbed onto the wool can be calculated by taking this value away from the concentration in the original dyebath. Once all of the concentrations have been obtained then a plot of q_e against C_e can be obtained.

4.2.1 Sorption Isotherm of pseudopurpurin

Figure 4.5 shows a plot of q_e *vs*. C_e for pseudopurpurin at five different concentrations. Linearisation of the isotherm was attempted by application of all three models and it was found that the Freundlich model (lnq_e *vs*. lnC_e) gave the most reliable fit, with an R^2 value of 0.95 (Figure 4.5 (b)). The R^2 value displays how well the data points fit into a straight line, the closer the R^2 value is to one the better the fit.

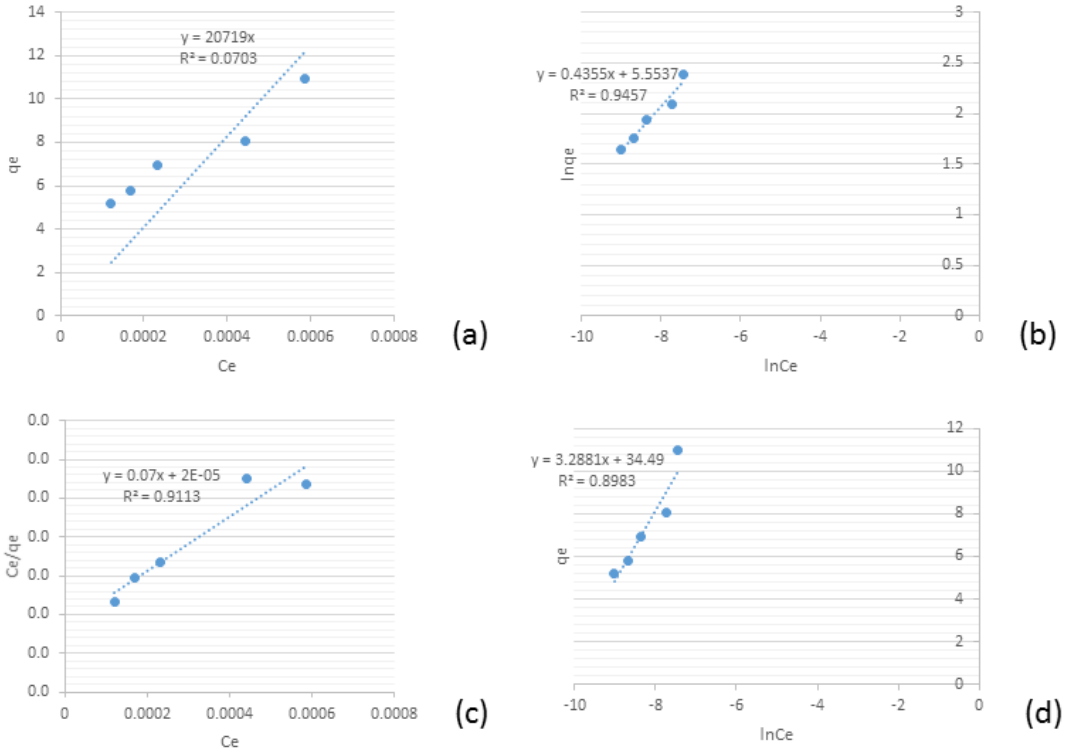


Figure 4.5. Plot of q_e vs. C_e for pseudopurpurin at different concentrations and linearization by application of the isotherms (a) Nernst (b) Freundlich (c) Langmuir and (d) tempkin models.

Pseudopurpurin shows a good fit to the Freunlich isotherm. This suggests that pseudopurpurin adsorption could be reversible and not restricted to monolayer formation only. This could be due to the presence of the carboxylic acid moiety on the dyestuff, the Freundlich equation is used to display a heterogeneous system of different adsorption energies dyeing due to a non-uniform surface. Due to the presence of charged

carboxylate moieties on the molecule, it would be expected to display a different energy of adsorption based on the environment of the site to which it was adsorbing. This could be a mordant metal site or a charged site on an aggregation of other dye compounds. The Langmuir equation does still show a good fit for the adsorption of pseudopurpurin dye compounds. However, the increased linearity shown with the Freundlich consideration of amorphous coverage suggests that this is a better fit for the adsorption mechanism of pseudopurpurin.

4.2.2 Sorption isotherm of ruberythric acid

Figure 4.6 shows a plot of q_e *vs*. C_e for ruberythric acid at five different concentrations. Linearisation of the isotherm was attempted by application of all three models and it was found that the Temkin model (q_e *vs*. lnC_e) gave the most reliable fit, with an R^2 value of 0.93. However, a good fitting with the Freundlich model (lnq_e *vs.* lnC_e) was also observed for the adsorption of ruberythric acid onto mordanted wool.

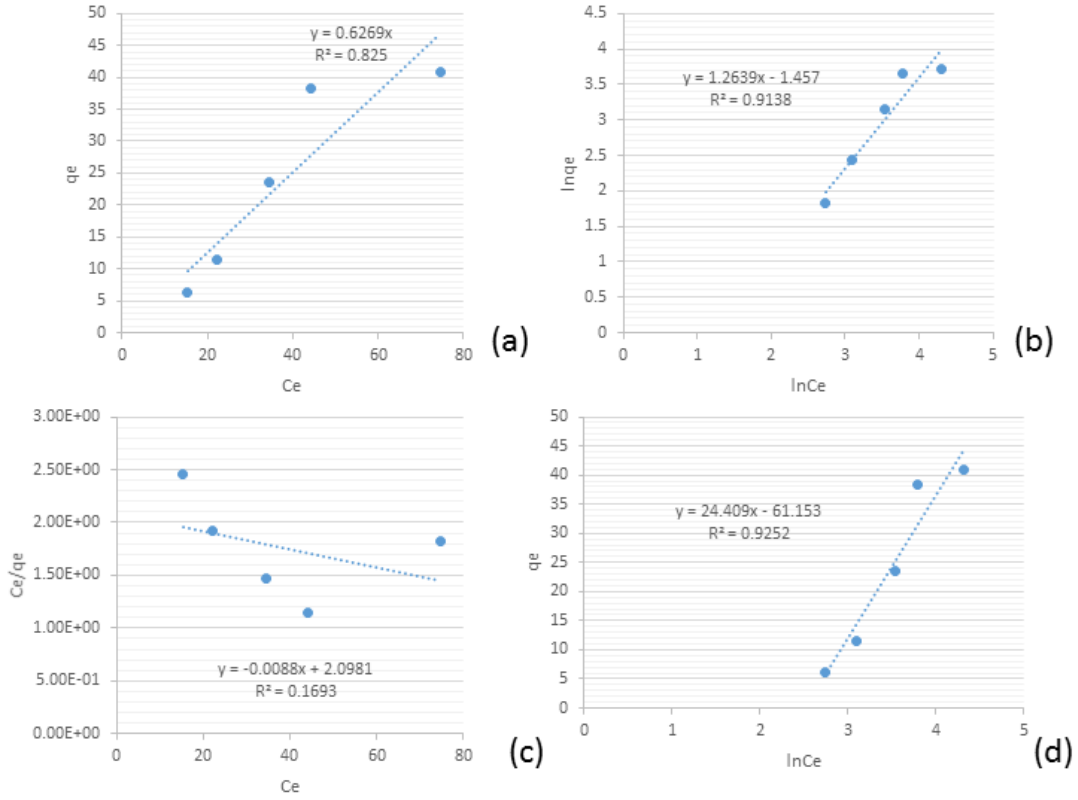


Figure 4.6. Plot of q_e vs. C_e for ruberythric acid at different concentrations and linearisation by application of the (a) Nernst (b) Freundlich (c) Langmuir (d) Temkin model.

Here the effect of the glycoside is considered in the dyeing capabilities. It is observed that in this case the Langmuir isotherm is not followed at all which suggests that the adsorption mechanism followed by dyes which contain a glycoside derivative is not monolayer adsorption. This suggests aggregation of these types of dye compounds in multilayers possibly due to hydrogen bonding between the sugars as shown in Chapter 2.

4.2.3 Sorption Isotherm of Alizarin

Alizarin was used as a dyestuff in this study because it is heavily cited in literature as one of the major components in madder root.[12,56,62,137] It is also a good example of a simple hydroxyl anthraquinone representative of many similar isomers present in the plant. Figure 4.7 shows a plot of q_e *vs*. C_e for alizarin at five different concentrations.

Linearisation of the isotherm was attempted by application of all three models and it was found that the Freundlich model (lnq_e *vs*. lnC_e) gave the most reliable fit, with an R^2 value of 0.82.

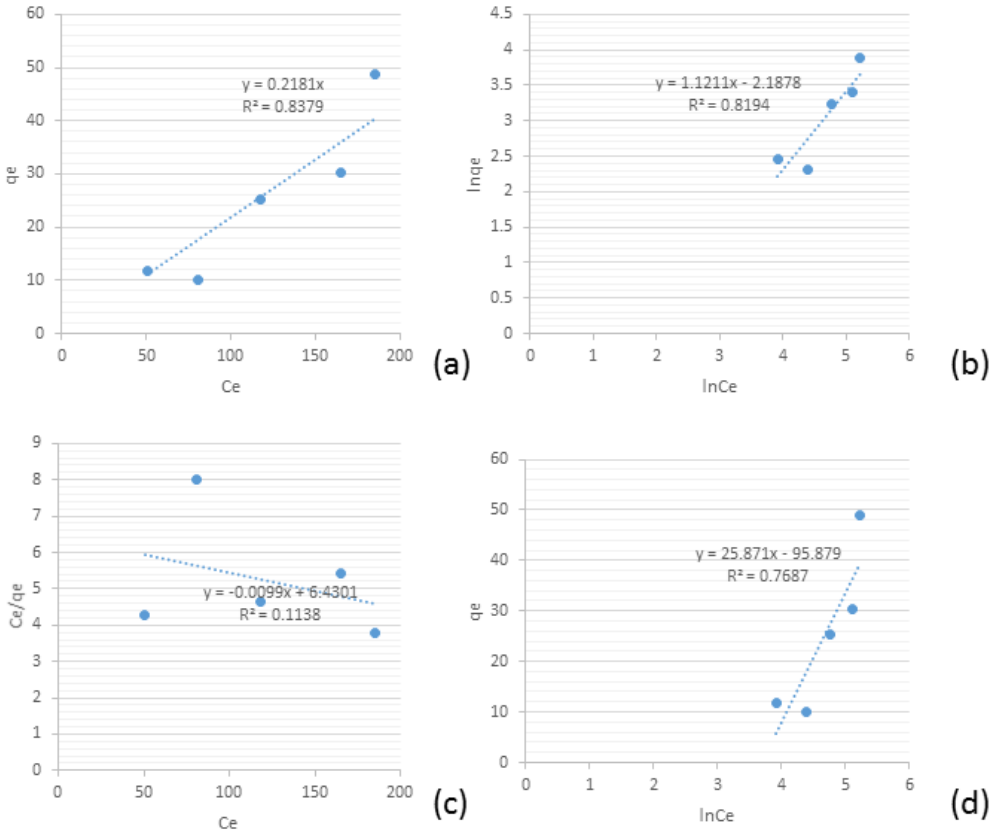


Figure 4.7. Plot of q_e vs. C_e for alizarin at different concentrations and linearisation by application of the (a) Nernst (b) Freundlich (c) Langmuir (d) Temkin model.

The adsorption mechanisms of alizarin were much more similar to those displayed by ruberythric acid than pseudopurpurin in that there is reasonable linearity shown when fitted with Freundlich and Tempkin isotherms but no linearity shown at all to the Langmuir isotherm. This is logical as ruberythric acid is the glycosidic derivative of alizarin, however, in this case the alizarin dye adsorption follows Fruendlich more closely than the Tempkin model.

4.2.4 Isotherm comparison

A summary of the data obtained from these dyeing studies can be found in Table 4.3, which shows that the functional moieties on hydroxyanthraquinones present in madder dye plants have a large effect on sorption properties. Although the plot of q_e vs. C_e for the Nernst isotherm fitting was done, none of the adsorption trends could be described as fitting a Nernst isotherm because none of the plotted graphs go through the origin. All three of the anthraquinones chosen to study in this chapter show some correlation to a Temkin isotherm (fitting $R^2 > 0.7$ for all three chosen compounds), and it is observed that ruberythric acid has greater sorption energy (–11.4 kJ mol^{-1}) in comparison with alizarin (–5.4 kJ mol^{-1}) when fitted to the Tempkin isotherm, suggesting that the 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. Pseudopurpurin shows the highest sorption energy (-14.4 kJ mol^{-1}) suggesting that the carboxylic acid aids dye adsorption.

Unlike the Langmuir equation, the Temkin isotherm also takes into account interactions between adsorbed species and adsorbates to be adsorbed; constant B_1 is related to the heat of adsorption and takes into the account adsorbing species-adsorbent interactions, and it is observed that interactions between ruberythric acid molecules (24.4 J mol^{-1}) are similar to the corresponding interactions between alizarin molecules (25.9 J mol^{-1}) suggesting that their interactions are similar. This could be due to the π-π stacking of the flat anthraquinone backbone as observed in section 2.3. It is proposed that due to the higher sorption energy of ruberythric acid, in comparison with alizarin, these greater adsorbent-adsorbate and adsorbate-adsorbate interactions are results of hydrogen bonding interactions from the glycoside moiety which is also shown by the crystal structure elucidation of ruberythric acid. Although the highest sorption energy is observed by pseudopurpurin when following a Tempkin isotherm it also displays the lowest B_1 constant suggesting less interaction between the pseudopurpurin molecules. This could be due to the presence of the carboxylic acid being deprotonated to a

carboxylate and therefore there could be some like charge repulsion creating a weaker interaction between these compounds on the surface of the fibre.

All three dye compounds also show good correlation with the Freundlich isotherm in which more than one layer of sorption is suggested. This result adds to the findings of the crystal structure of ruberythric acid discussed in section 2.3 which shows extensive hydrogen bonding of the sugar moiety suggesting that a multiple layer adsorption would take place through aggregation of the ruberythric acid molecules.

Pseudopurpurin most closely follows a Freundlich isotherm, which suggests that sorption is not restricted to the formation of the monolayer, unlike the Langmuir isotherm. The multilayer sorption observed is similar to ruberythric acid and alizarin sorption, suggesting that the hydroxyanthraquinone backbone follows this sorption process; it is interesting that pseudopurpurin has the greatest sorption energy (–19.0 kJ mol^{-1}) of all three dye compounds, and it is suggested that this is as a result of the anionic carboxylate moiety which is actively involved in ionic interactions with cationic functions in the mordanted fibre. Although pseudopurpurin does not most closely follow a Langmuir isotherm, it is the only one of the three madder components evaluated that shows some significant correlation to that isotherm (R^2 = 0.911). Alizarin and ruberythric acid do not show any correlation to the Langmuir isotherm. Langmuir's model of adsorption describes site-specific adsorbent-adsorbate interactions, hence, supporting the theory that the carboxylate moiety is significantly involved in sorption. The fact that ruberythric acid and alizarin do not correlate to the Langmuir isotherm at all suggests that they are capable of forming interactions other than the site specific ones between the mordant and dye for example hydrogen bonding of the sugars and π-π stacking of the anthraquinone backbone. Understanding the pattern of adsorption of these compounds onto mordanted wool can help to develop the most effective and efficient back extraction techniques for analysis of these dyes on textiles.

Table 4.3. Freundlich, Langmuir and Temkin isotherm data for dyeings with pseudopurpurin, ruberythric acid and alizarin.

Dye	M_w (g mol^{-1})	*Freundlich*			
		R^2	K_F (dm^3 mol^{-1})	n_F	–ΔG (kJ mol^{-1})
Alizarin	240.21	0.819	26.94	0.9	9.9
Pseudopurpurin	300.22	0.946	538.8	2.3	19.0
Ruberythric acid	534.47	0.914	124.5	0.8	14.6

Dye	M_w (g mol^{-1})	*Langmuir*			
		R^2	K_L (dm^3 mol^{-1})	q_0 (mg g^{-1})	–ΔG (kJ mol^{-1})
Alizarin	240.21	0.113	--	--	--
Pseudopurpurin	300.22	0.911	193.0	5.9	15.9
Ruberythric acid	534.47	0.169	--	--	--

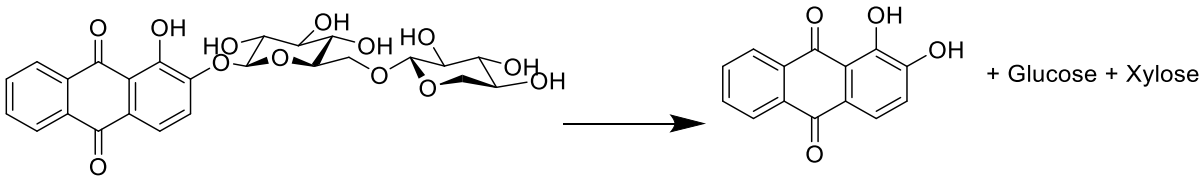
Dye	M_w (g mol^{-1})	*Temkin*			
		R^2	K_T (dm^3 mol^{-1})	B_1 (J mol^{-1})	–ΔG (kJ mol^{-1})
Alizarin	240.21	0.769	5.9	25.9	5.4
Pseudopurpurin	300.22	0.898	119.7	3.3	14.4
Ruberythric acid	534.47	0.925	43.6	24.4	11.4

5 Back Extractions of Wool Dyed with Rubia *tinctorum* Extracts and Analysis of the Effect of These Conditions on the Compounds in Madder

In order to identify madder dyes by HPLC analysis on historical textiles and artefacts, extremely small samples of yarns and fibres of a few milligrams are selected from the discrete areas of the coloured parts of a textile. The need for sampling is not ideal as it is a destructive technique for the artefact and once the sample is taken it cannot be replaced. However, HPLC analysis provides superior results over other non-destructive methods due to the separation that can be achieved of the compounds in the dye mixture and allows detection of very small amounts of components. The separation of these compounds allows identification of the ratios of compounds present in the dyestuff. As presented in the previous chapters the ratio of compounds in the madder root provide a 'fingerprint' of that dyestuff for the unambiguous identification of the type of madder used to dye that fibre or fragment. In order to achieve the most effective identification of the dyestuff the presence of the dye components must not be compromised in the solvation technique used for liquid chromatography analysis methods.

Back extractions reported in the literature were originally based on strongly acidic conditions using 2:1:1 mixture of 37 % hydrochloric acid: methanol: water (*v*/*v*/*v*) and heating to high temperatures.[10,13,122] However, under these conditions the extraction methods used cause hydrolysis of the glycosides and usually results in alizarin and sometimes purpurin being the only compounds observed in the extract when analysing madder dyed textiles using *Rubia tinctorum*.[25] The sugars in the glycosidic compounds

are easily cleaved from the dye in acidic conditions due to protonation of the glycosidic bond which is then broken into the free sugar and the chromophore (see Scheme 5.1).



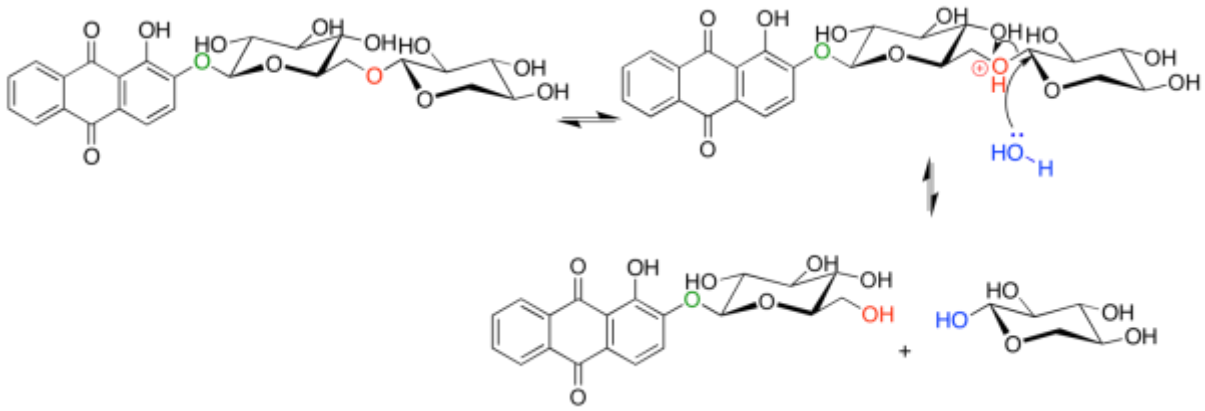
Scheme 5.1. Hydrolytic cleavage of sugars from ruberythric acid.[25]

This degradation changes the chemical and physical properties of the dye compound and results in it being eluted at a different time in the chromatogram and usually shows a different UV/vis signal. The change in retention time is due to the loss of the sugar moieties and hence the decrease in polarity. Sugars are very polar due to the presence of many hydroxyl groups in the structure of a sugar ring. The terminal xylose is degraded first as the hemiacetal bond of the glucose-xylose bond (shown in red in Scheme 5.2) is much easier to break than that of the glucose-anthraquinone glycosidic linkage (shown in green in Scheme 5.2). This is because the protonation of the glucose-xylose linker is much easier to hydrolyse as the oxygen is much more electronegative. The lone pair of the oxygen glycosidic linker next to the anthraquinone is incorporated into the conjugated system of the anthraquinone and hence protonation and degradation is a lot more difficult due to this increased stabilisation. This mechanism is proposed based on common hemiacetal and carbohydrate chemistry under acidic conditions.[130]

It has been observed herein that the glycosides are one of the major compound classes in the extracts of *Rubia tinctorum* and if these compounds are broken down in the back extraction procedure there is an overall loss of information on the dyestuff. Whilst the observation of these compounds is not essential to the generic dye identification, which can be distinguished by certain marker aglycon anthraquinones[11], there is still lots to be gained by observing these compounds in textiles of cultural interest.

The presence of these compounds in the textile back extraction can give information on the origin of the dye plants, possible trade routes and the techniques used to make the textile.[57,77] As described in Section 1.9, a common dye extraction method in France was to extract the roots of the plant in aqueous acidic media, therefore in these extracts the glycosides would not be present.[12] Another preparation method of the madder roots involved steaming of the roots in specialised pits for 10-12 hours.[4] This resulted in denaturisation of the endogenous enzymes and hence inhibit the hydrolysis reaction caused by the presence of these enzymes. Therefore, if the glycosides are present in a historic textile it could indicate this method of dye plant preparation. Whilst it is unclear whether the degradation of the colour is hindered by the glycoside derivatisation, studies have shown that the glycosidic compounds are still detected after artificial ageing.[121,138] There have been some studies into the degradation of alizarin, purpurin and madder root extracts on mordanted and unmordanted wool. The madder root in this study was shown to be very rich in purpurin by HPLC and did not contain the glycosidic compounds in significant amounts and therefore the madder root extract behaved very similarly to that of pure purpurin.[139] Alizarin was also shown to display more degradation in this study as measured by the larger decrease in peak area when compared to purpurin. This is surprising as due to the extra hydroxyl group present in the purpurin structure, the electron donating nature of these hydroxyls and the keto tendency of phenols in an aromatic ring making purpurin more chemically reactive it would be expected that purpurin would degrade more rapidly than alizarin.[140]

More recently the importance of observing the glycosides in the artefacts in question has pushed researchers to develop ‘softer’ extraction techniques.[57,77,110] The aim of these extraction methods is to extract the dyestuff from the textile in sufficient amounts for the detection of the coloured compounds but to cause the minimum degradation or chemical change to these compounds in the process. Herein an evaluation of the commonly used extraction methods was carried out on the three madder types evaluated in the previous chapters.



Scheme 5.2. Hydrolysis of the terminal xylose of ruberythric acid under acid conditions.

The aim was to develop a dye extraction method which could identify whether or not the textile being extracted had been dyed with madder root extract containing glycosides. Previous chapters have shown that the glycosides are present in some bought samples of *Rubia tinctorum* and that they are adsorbed onto the wool fibres in the dyeing process. Herein a back extraction process which distinguishes between the differing chromatographic profiles of extracts observed in previous chapters is desired.

Three types of madder: Iranian, Turkish and English were dyed on the wool samples as described in experimental section 6.5. Small samples of 2 mg were taken of all of the dyed wool samples to keep conditions as similar as possible to what would be done in a museum environment. The textiles were analysed by HPLC and the compounds were identified by the use of authentic standards of alizarin, purpurin, and ruberythric acid/lucidin primeveroside bought mixture. Rubiadin, xanthopurpurin and lucidin were chemically synthesised and fully elucidated before being used for a standard for peak identification see experimental section 6.7 for the synthesis conditions. If the standard could not be synthesised or was not available commercially, mass spectrometry is used to identify the compound this was only the case for nordamnacanthal.

Table 5.1. Anthraquinones detected in *Rubia tinctorum* extracts and back extractions.

Anthraquinone derivative assigned to HPLC peak	Retention time (min)	λ_{max} (nm)	Mass Spectrometry $[M-H]^-$
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

5.1 HCl back extraction

The HCl back extraction was developed by Wouters *et al.* and was the first method developed to observe the dyestuff used in historical textiles by HPLC analysis.[141] This method employs the use of 2:1:1, 37 % hydrochloric acid (HCl): methanol: water (*v*/*v*/*v*). It has since been adopted to analyse many artefacts[10,122], but usually only aglycons are observed due to degradation of the sugar containing compounds. This method of analysis is still routinely used to identify the dyestuffs in textiles. The method is efficient for identification of the aglycon materials and gives high limits of detection for aglycon compounds.[108] Research done in the CHARISMA EU funded project involved identification of dyestuffs from the same reference materials across different labs and comparison of the results was done. This study was to identify if the results were consistent across multi-laboratory where different scales etc. were being used.[142] This work details guidance to minimise differences in results due to location of the extraction.

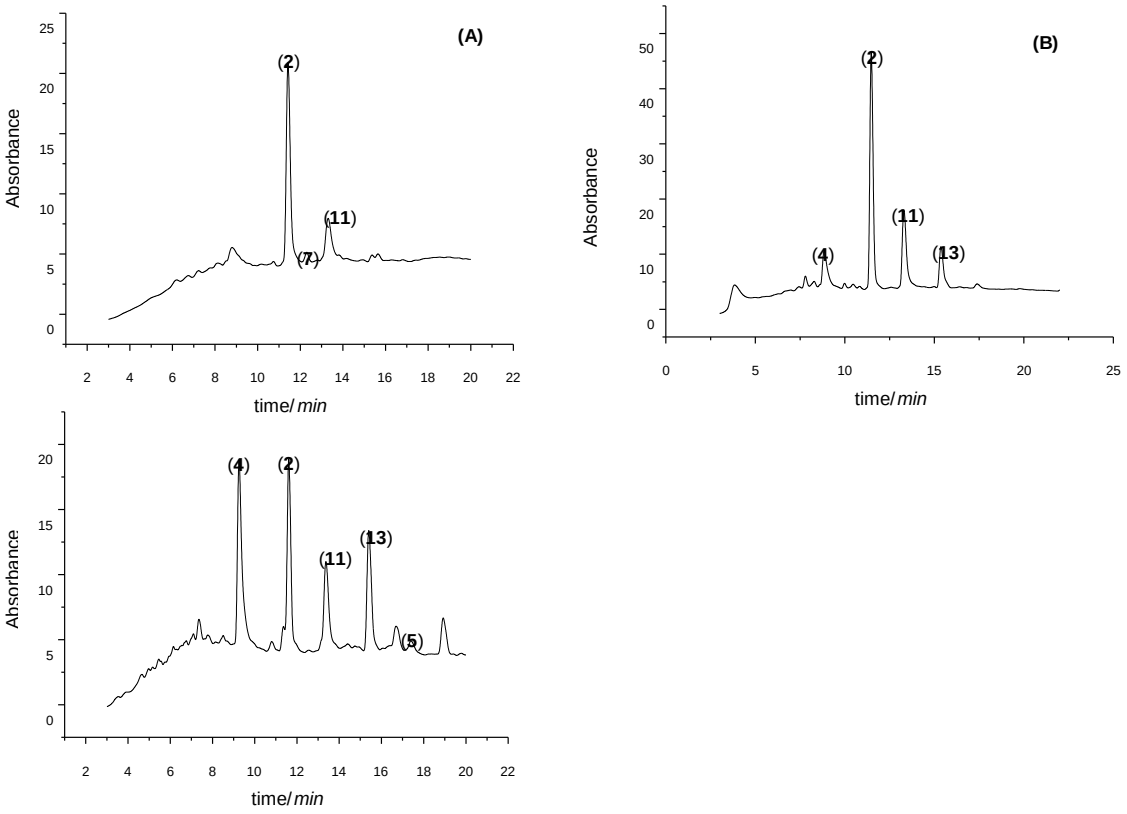


Figure 5.1. HPLC chromatograms of wool dyed with extracts of roots of different R. tinctorum varieties, extracted with 37% HCl: methanol: water (2:1:1, v/v/v): (A) Iranian madder; (B) Turkish madder; (C) English madder.[138] Peaks present in the chromatograms can be identified by their number (4) lucidin (2) alizarin (11) purpurin (13) rubiadin (5) nordamnacanthal.

From the HPLC chromatograms (Figure 5.1) the main compounds present in the back extractions of all samples are alizarin (2) and purpurin (11). In the back extraction of Turkish and English madder using this method rubiadin (13) is observed. This is not present in large amounts in the original dye bath and hence could be a degradation product of another compound present in the extracted dye as described in the literature.[77,108] There is no presence of the glycosides ruberythric acid (1) or lucidin primeveroside (3) in this back extraction which is not representative of the original dyestuff as these are the main peaks present in the original dyebaths of Iranian and Turkish madder. The HCl extraction as displayed in this study can be used to identify anthraquinone aglycon compounds such as alizarin etc. Therefore, it can be used to

indicate a madder type dye is present, through detection of these marker pigments, but the chemical compositional detail needed to be more confident in further conclusions about the making or origin of the dyed textile is lost. This is why improved more mild methods have been developed for analysis of textiles when more information on the chemical composition is required.

5.2 Citric acid back extraction

Citric acid was developed as a back extraction method for two reasons. Firstly, it is a weaker acid than hydrochloric acid and hence the acidity of the solution may be able to remove the dye from the wool but have a less detrimental effect on the acid sensitive compounds in the dyestuff.[108] Secondly it is also possible that citric acid could coordinate to the mordant metal (Al^{3+}), which could help the dye extraction by disrupting the dye-mordant complex and thus releasing the dye into the solution. Citric acid has been shown biologically to form complexes with Al^{3+} in order to detoxify aluminium in soil and therefore it is suggested that it could complex to the mordant metals on the wool.[143]

Despite being a weaker acid there is still evidence of detrimental effects observed in the HPLC chromatograms (Figure 5.2). The glycosides lucidin primeveroside (3) and ruberythric acid (1) are present in very small amounts in Iranian madder, much lower than in the original dyebaths. They are also not present at all in the back extractions of Turkish and English madder which is not representative of the original dyestuff. The main peaks observed in all three of the samples are alizarin (2) and purpurin (11). The possible degradation product rubiadin (13) is present, but only in the case of English madder and in small amounts in Iranian madder. A peak assigned to lucidin (4) is also present in English madder which is expected due to its presence in the original dye baths, but here it is present in a lower relative peak area compared to the original dyebath. Lucidin is also detected in small amounts in Iranian madder when back extracted with citric acid, which is not present in the original dye bath and therefore probably a product of

hydrolysis in the acidic conditions. 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 however it is not observed in English madder. The absence of nordamnacanthal in English madder is surprising as nordamnacanthal is an oxidation product of lucidin which is present in high amounts in English madder.[33] The absence of the glycosides in these chromatograms for Iranian and Turkish madder deem this an unsuccessful back extraction.

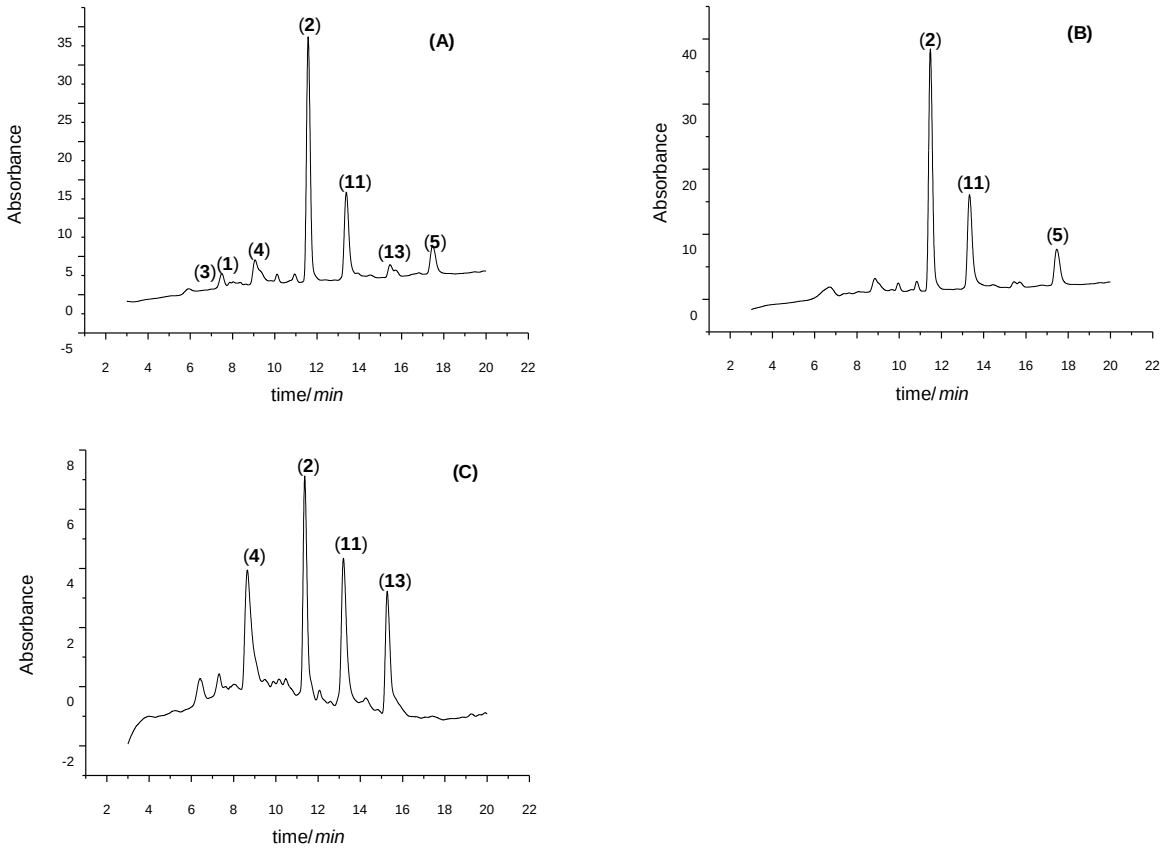


Figure 5.2. HPLC chromatograms of wool dyed with extracts of roots of different R. tinctorum varieties, extracted with 0.5 M citric acid: (A) Iranian madder; (B) Turkish madder; (C) English madder. Compounds in the chromatograms identified by their number (1) ruberythric acid (3) lucidin primeveroside (4) lucidin (2) alizarin (11) purpurin (13) rubiadin (5) nordamnacanthal.

5.3 TFA acid back extraction

This back extraction procedure utilises again a mildly acidic system in the hope that the compounds could be extracted but not degraded.[44,107,109] Again, the main compounds

observed are the aglycons alizarin (2) and purpurin (11) from the HPLC chromatograms shown in Figure 5.3. There are no glycosidic compounds detected in any of the samples when 2 M TFA is used as an extraction medium, this is probably due to the acid sensitivity of these compounds in solution. A broad peak is present in the Iranian madder extraction that has a similar retention time as the glycosides, but the UV/vis data does not correspond, hence this could not be assigned to lucidin primeveroside (3) or ruberythric acid (1). It is notable that the HPLC chromatograms of the Turkish and Iranian madder samples also contain Lucidin in the extraction analysis; however, lucidin is not observed as a product in the original dye baths of Turkish madder and hence is probably present due to the hydrolysis of lucidin primeveroside. Lucidin is observed to some extent in all of the back extractions using TFA in the extraction solvent.

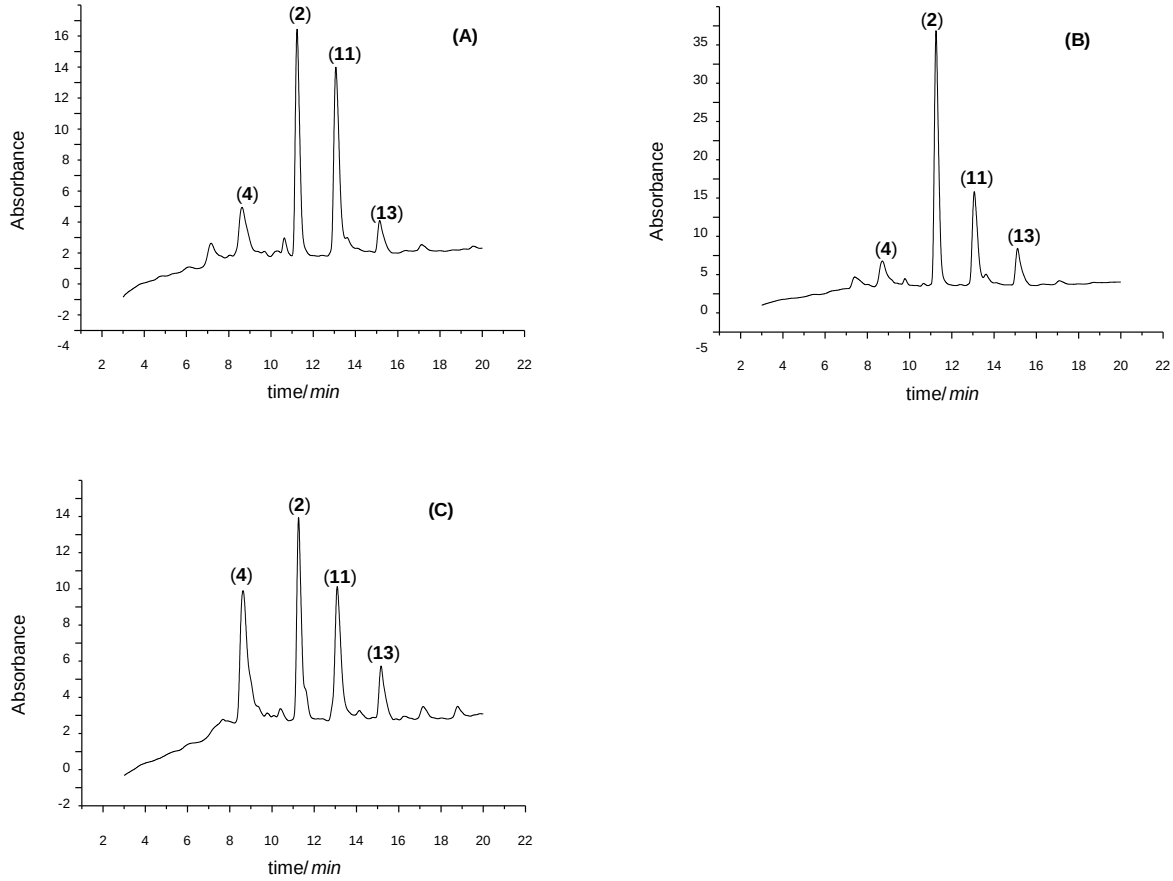


Figure 5.3. HPLC chromatograms of wool dyed with extracts of roots of different R. tinctorum varieties, extracted with 2 M TFA: (A) Iranian madder; (B) Turkish madder; (C) English madder.

Again, the lack of the glycosidic compounds in these extraction methods suggests hydrolysis into the aglycons lucidin and alizarin. This is therefore resultant of a loss of information on the original artefact and consequently deemed an unsuccessful ‘soft’ extraction but successful in identifying the aglycons in the madder root dyed textile artefacts.

5.4 Glucose back extraction

An aqueous glucose solution (0.4 %, *w*/*v*) was used to extract all three types of madder. Although glucose is used in this method to extract from the dyed textiles, it cannot be added on to the compounds to form a glycoside. The addition of a glucose to alizarin is a complex synthesis and cannot be done in water.[121] The compounds observed herein are also primeveroside derivatives rather than only glucoside and hence if there was an addition of glucose a different retention time would be expected and/or a different UV/vis trace. It is observed in the HPLC chromatograms (Figure 5.4) of the back extractions using glucose solution that the glycosides are present in the Iranian and Turkish madder samples. This is the first time that lucidin primeveroside (3) and ruberythric acid (1) have been present in back extractions from madder dyed wool samples in this research. Accordingly, it would seem that of the different extraction solvents tested herein, the glucose method is the only one that allows extraction of dyed wool samples and also preserves the dye compounds in the same molecular form as observed in the original dyebath. Importantly, it would seem that the glucose extraction method does not cause significant hydrolysis of the glycosidic components lucidin primeveroside (3) and ruberythric acid (1). It is interesting to note that the chromatogram present in Figure 5.4 (b) is more similar in composition to the ethyl acetate extracts observed in Figure 2.4 (a) rather than the water extractions observed in Figure 2.4 (c). It is unclear why there are a higher presence of other anthraquinones in this back extraction and it could only be

assigned to some degradation in the dyeing procedure. None the less the glycosidic compounds can still be observed and detected in this back extraction.

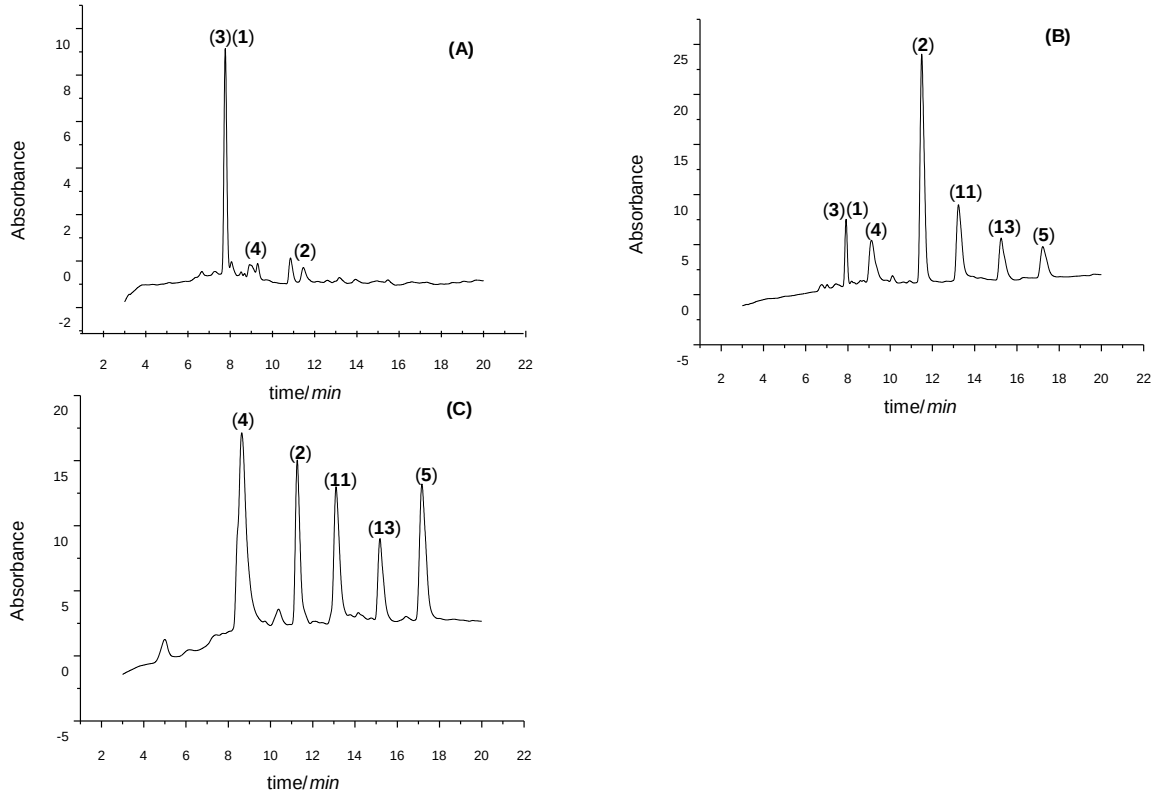


Figure 5.4. HPLC chromatograms of wool dyed with extracts of roots of different R. tinctorum varieties, extracted with 0.4% aqueous glucose solution: (A) Iranian madder; (B) Turkish madder; (C) English madder.

In the glucose extraction method lucidin (4) is observed as a main peak for the extracted English madder samples, which corresponds to the HPLC analysis of the original dyebath. Lucidin is also observed in low concentrations in the Turkish madder samples, in agreement with observations from the TFA extraction. It is unclear why the Turkish and Iranian madder back extractions look so different whereas their original dye baths were very similar. These analyses were repeated hence anomalous results were minimised but is something which could be analysed in more detail to determine whether the difference is in the dyeing capability of the plant extract or in the back extraction capability. The differences could be due to compounds present in the complex plant matrix which do not have a chromophore and hence are not observed in the back

extractions, but could be causing some degradation on the wool or in the dyeing, or back extraction processes.

Both Turkish madder and English madder yield alizarin (2) and purpurin (11) under these extraction conditions, however, the aglycons are only observed in trace quantities in the Iranian madder samples. HPLC analysis of the dyebaths before and after dyeing reveals that the most significant decrease in peak size is that of the alizarin peak. However, due to alizarin having a large response factor on the HPLC detector this corresponds to a much smaller decrease in the molar concentration than that of the glycosides. Therefore, in extraction and analysis of the wool samples, it would be expected that the glycosides peak would be the largest observed, but the aglycons alizarin and purpurin should still be present. Glucose solution appears to be a favourable medium for extraction of the dyestuffs in madder for many reasons:

1) Glucose can competitively bind to the sugar moieties in the anthraquinone glycosides as it is capable of multiple hydrogen bonding interactions. Therefore, it is able to displace the glycosylated dye compound and release it into solution.
2) The neutral pH of the solution allows the glycosidic dyes to remain intact.

It is unlikely that there is only one dye compound per mordant metal across the whole fibre surface as shown by the dyeing isotherms displayed in chapter 4. It is more probable that there will be a presence of multilayers of dye compounds held through hydrogen bonding and π-π stacking interactions that could be disrupted by the interaction of glucose with the aggregates. The crystal packing of ruberythric acid and lucidin primeveroside[56] show that all of the hydrogen bonding interactions in the packing are from the sugar moieties of the compounds. This supports the argument that glucose would be able to disrupt these aggregates by forming competitive hydrogen bonding interactions and thus releasing dye compounds into solution.

Glucose has many -OH groups in its structure which are able to form complexes with aluminium (Al^{3+}) which could also allow for dye release into solution. Competitive

binding to the aluminium mordant from glucose would allow the dyes to be released into a neutral solution and hence degradation of the compounds is avoided. This is observed in the case of English madder, there is some back extraction of the algycons from the dyed wool, this cannot be due to disruption of aggregation of glycosides as the glycosides were only present in small amounts before dyeing and hence the glucose must be interacting with the mordant metals themselves.

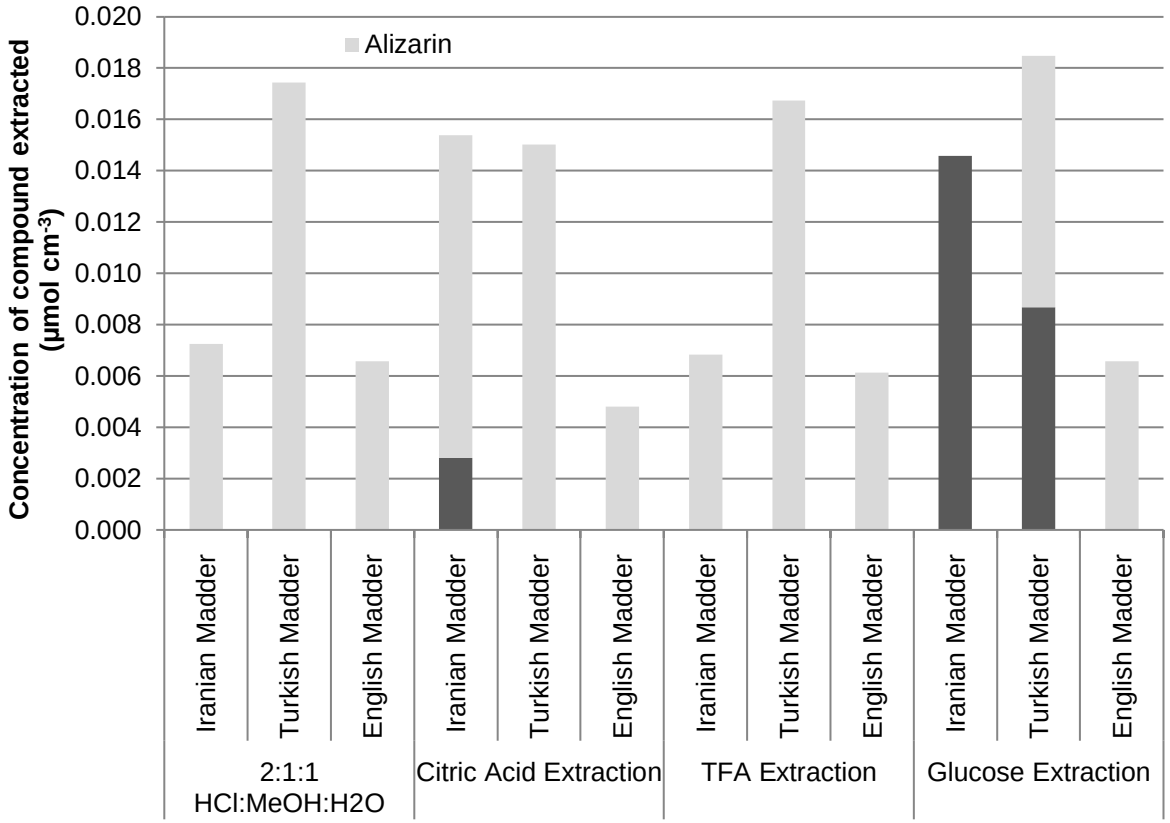


Figure 5.5. Concentration of anthraquinone glycosides (primarily ruberythric acid and lucidin primeveroside) extracted from wool samples in comparison with concentration of alizarin (main anthraquinone aglycon) extracted.

Quantitative comparison of the glycosidic compounds ruberythric acid (1) and lucidin primeveroside (3) to the main alglycon present in back extractions; alizarin (2) can be seen in Figure 5.5. This highlights the efficiency of glucose as a back extraction method in its ability to remove the glycosidic anthraquinones without 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, confirming no

anthraquinone glycosides were present in the starting dyebath as observed in chapters 2 & 4. 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 chapter 4. 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. It is also proposed that this extraction method could be used in combination with other back extraction techniques which can extract the aglycon materials under harsher conditions to ensure all compounds in the textile being studied are detected. This method does not cause any visible degradation to the wool fibre, so could be used in combination with the 37 % HCl: water: methanol method for full identification of all compounds. The glucose method could be carried out first as a detection of the glycoisdic compounds if they are present and then a second step involving harsher conditions for identification of algycons which were not extracted in this 'soft' method.

5.5 Conclusions of Back Extractions

Extraction of dye compounds in aqueous glucose solution provides an effective sample preparation technique, which minimises hydrolysis of the glycosidic components of the dye plant. It is also highly efficient on a 2 mg scale, which makes it applicable to museum textile artefacts which would require small sample sizes. The short preparation time of the extraction is also ideal for museums as the short extraction times could result in less degradation but also gives a reliable detection of the glycosidic compounds. Studies on

museum objects are tested using the methods that will give the highest quality and quantity of components for conclusive identification which is shown herein for detection using glucose. HPLC-DAD analysis allowed conclusive identification of each peak based on retention times of each peak and confirmed by UV/vis data obtained. Glucose solution is a favourable extraction medium as it is able to form many hydrogen bonding interactions with the dye compounds, and hence disrupts mordant metal complexes and dye aggregates which help to solubilise the dye compounds for removal from the fibre. The neutral pH of the extraction method ensures that acid sensitive compounds such as the glycosides (ruberythric acid, lucidin primeveroside and galiosin) and sensitive aglycons (lucidin) remain intact in the extraction procedure. This is important as these compounds give a lot of information on the original dyestuff or dyeing method, which is of upmost importance to conservators or historians when analysing these historic artefacts. 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 provides an efficient ‘soft’ extraction which allows solubility of the sensitive compounds in madder with minimal degradation. One suggested area for improvement of the glucose method is in the separation of the glycosides in liquid chromatography 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. Another question posed by this research is the presence of lucidin in acidic back extractions. It is noted that the glycosides are probably degraded by the acid and hence only the glycosides are observed but in most cases it is alizarin which is present in the back extraction and lucidin is not always seen. This poses the question of what happens to lucidin under acidic conditions.

When strong acid is used in the extraction procedure to back extract English madder a small shoulder is observed on the alizarin peak. This could be due to the presence of xanthopurpurin which is not observed in the samples herein. Xanthopurpurin

is an isomer of alizarin and hence will have a similar polarity and due to the age of this column may not be fully resolved. However, this could not be confirmed due to poor separation. There is more discussion about the presence of xanthopurpurin in strong acidic back extraction conditions in Section 5.6.

LCMS of the back extractions performed herein would have been useful for obtaining more data for compound identification of the textile's dye composition. Unfortunately, this method of analysis was not available when this study was performed. However, although it would have been useful to have more data on the peaks and be able to display their mass spectrum, it was not needed for peak identification due to all compounds being identified by synthesised or bought standards. Other than nordamnacanthal which was identified at a later date by oxidation of lucidin to nordamnacanthal and identified by its mass (Figure 5.6). This peak was then identified on these HPLC chromatograms by the retention time and UV/vis trace (Chapter 2, Table 2.3).

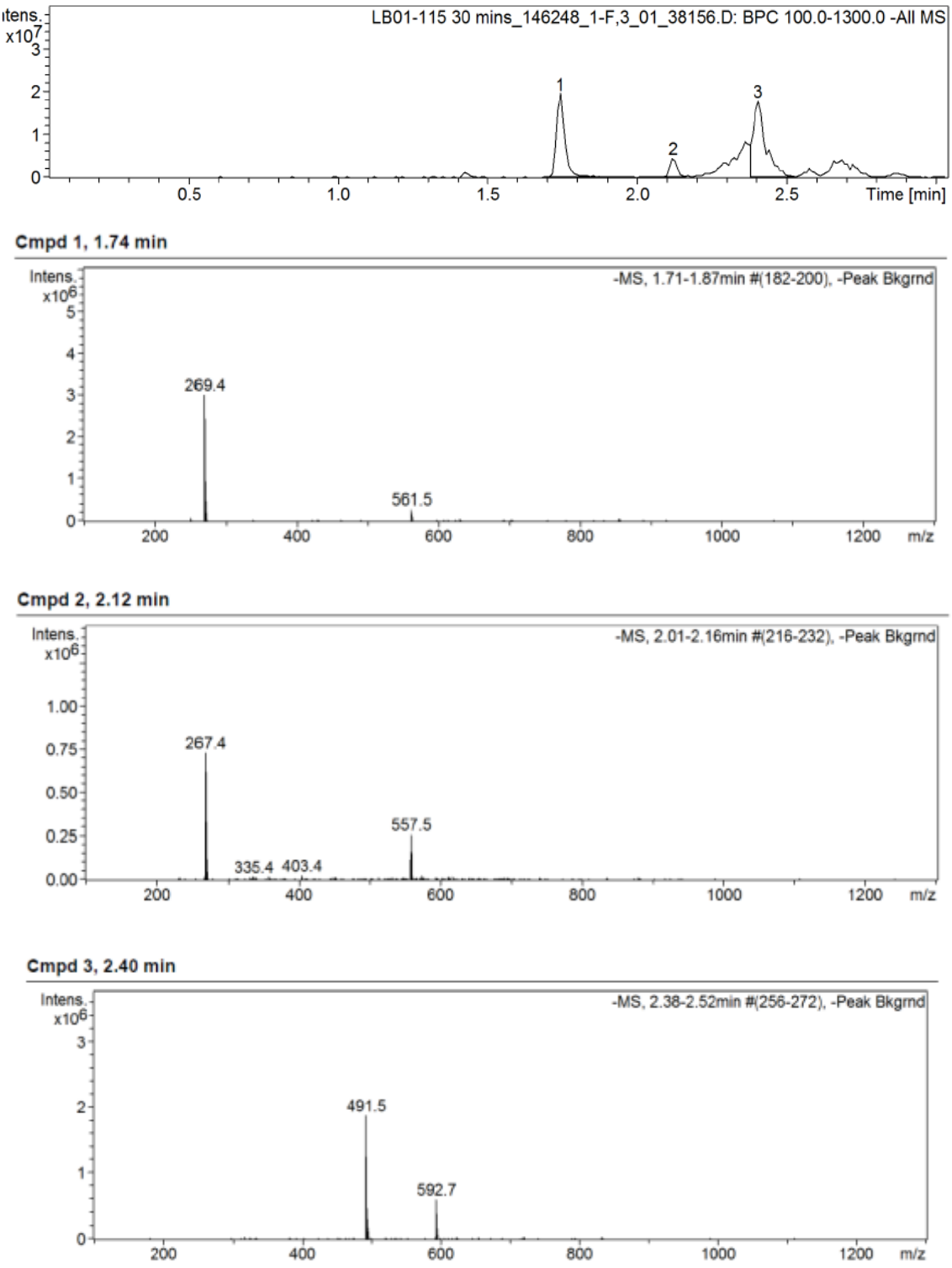


Figure 5.6. From top to bottom LCMS chromatogram of lucidin oxidised by DMSO and HCl (1:1), mass spectrum of lucidin, mass spectrum of nordamnacanthal and mass spectrum of a proposed dimer of lucidin under the mass spectrum or strong acid conditions.

5.6 Synthesis and Breakdown of Lucidin Containing Derivatives and Consequential Reactions in Acidic Back Extraction Conditions

As described in the previous chapters the acidic hydrolysis of some of the compounds in the madder root dyes lead to the detection of only aglycons in back extraction experiments. The literature suggests that from the identification of these sensitive compounds it is not only possible to identify that madder was used to dye the textile in question,[11] but also distinguish the species of madder root, information about trade routes and the processing methods used to create the dye.[57,77,108,138] Herein it is suggested that when acidic methods of extraction are used to solvate the dye compounds; degradation of aglycons such as lucidin could also occur.

Lucidin is a natural product that can be extracted from the roots of madder (*Rubia spp.*).[34,35,66] It is a major component of the madder root extract in its glycosylated form, lucidin primeveroside, which can be hydrolysed by enzymes or acid to lucidin.[25,56] However, many studies reported in the literature only observe alizarin and purpurin in the back extractions from artefacts that have been dyed with *Rubia tinctorum*.[10,33,122,144,145] This is misleading as extracts rich in the glycosides lucidin primeveroside and ruberythric acid would be expected to hydrolyse to lucidin and alizarin upon acid extraction. As shown in chapter 4 alizarin has a very large response factor when measured on the HPLC and hence even if there is only small amounts present it creates a larger peak area for the same molar concentration when compared to the glycosides and hence is easier to detect when using a DAD detector.

If upon analysis of these textiles the conditions used to extract the dyes change the ratios of the mixtures or modify the actual compounds themselves then this valuable information on that dyestuff will be lost as the chemical composition will be changed. The absence of lucidin in artefact analysis dyed with madder is rarely acknowledged or stated

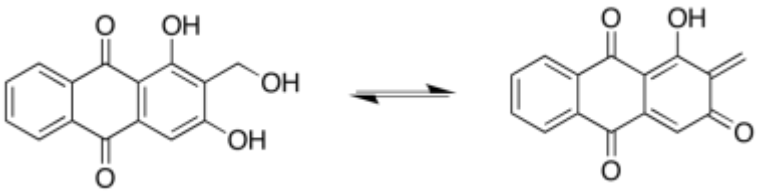
as being broken into unknown degradation products.[57] Lucidin contains a primary alcohol and is the only commonly reported anthraquinone detected in the roots of *Rubia tinctorum* to contain a primary alcohol which could make its degradation unique.[63]

The breakdown of lucidin under acidic conditions was studied in order to understand why these compounds are not observed in the back extractions of madder dyed artefacts. The fact that lucidin is not observed in acidic analysis of historic artefacts could be due to one of two reasons:

1) First the dye could have degraded naturally on the artefact through time and hence is not present in the dyed artefact to detect.
2) Secondly the use of very strong acid in back extractions is not only detrimental to the glycosidic compounds but also to some of the more sensitive aglycons such as lucidin. If this is the case the breakdown products need to be identified to ensure that these products are not being confused with other compounds in the extract.

Degradation of the artefacts naturally could be through exposure to UV light in museum displays,[139,146] oxidation upon exposure to oxygen in the air,[139] microorganism degradation[146] and tensile strength degradation of the actual fibres.[42]

Studies reported in the literature have identified that endogenous enzymes in the madder plant cause oxidation of lucidin to nordamnacanthal.[25,62] There is also another enzymatic reaction that can occur that converts lucidin into the quinone methide (Scheme 5.3).[2]

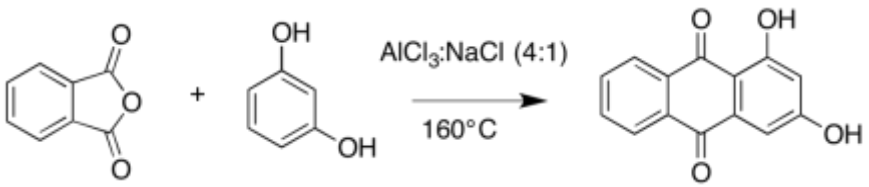


Scheme 5.3. Elimination reaction of lucidin to form quinone methide.

However, these enzymes are probably denatured in the dyeing process and hence this mode of degradation probably is not responsible for these compounds in historic artefacts. It is thought this intermediate may be able to be formed by acidic conditions but the actual intermediate is too difficult to isolate due to it being a very strong electrophile. In this quinine methide intermediate the aromaticity of the compound is broken and hence there will be an addition at the double bond by any nucleophile.[11] In order to gain better understanding of the effect that acid is having on these solutions, a pure sample of lucidin was synthesised to observe any changes in its structure under the back extraction conditions. Lucidin was synthesised in a two-step process as described in experimental section 6.7.2. This first step involves the synthesis of the precursor xanthopurpurin via a Friedel-Crafts acylation mechanism as described in section 6.7.1 see Scheme 5.4. This reaction involved an aluminium melt at high temperatures which was the most effective method, other methods tested which were not successful are shown in Table 5.2. Achieved yield was 28 mg (1.2 %)

Table 5.2. Summary of methods tested to try to improve yield of xanthopurpurin.

Methods tested	**Reference of method**
One pot synthesis using alum as catalyst and water as reaction solvent- phthalic anhydride did not dissolve in water	[147]
The use of aluminium melt with methanesulfonic acid as a catalyst	[148]
Order of addition of phthalic anhydride and resorcinol	-
Scale of the reaction/size of aluminium melt compared to reagents	-



Scheme 5.4. First step of the synthesis of Lucidin; synthesis of Xanthopurpurin.

The successful synthesis of xanthopurpurin was characterised by ^{1}H NMR (Figure 5.7) and LCMS (Figure 5.8).

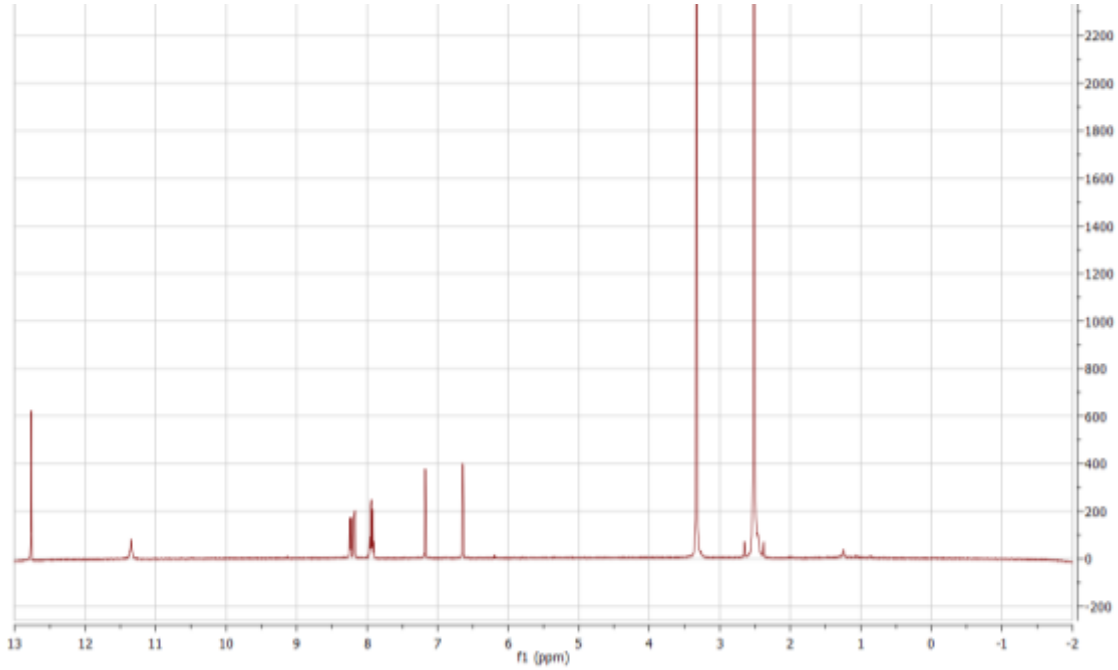


Figure 5.7. ^{1}H NMR spectra of xanthopurpurin

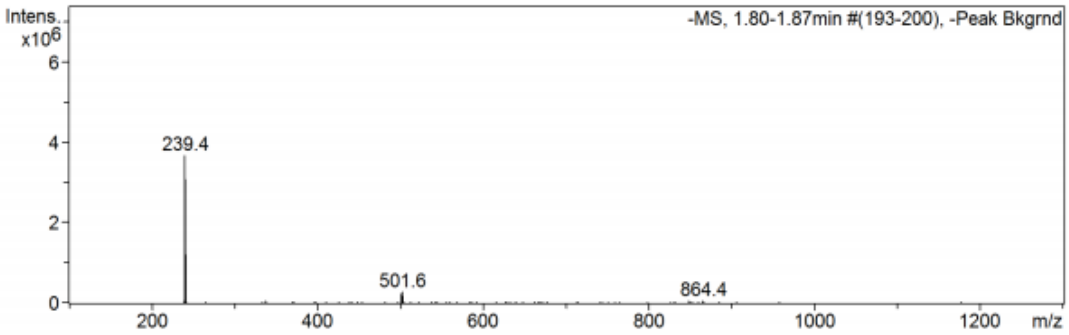
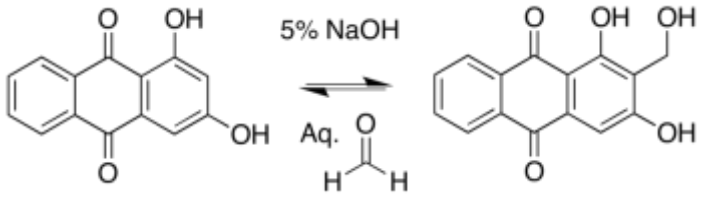


Figure 5.8. LCMS of xanthopurpurin

The second step is an aldol type mechanism under basic conditions using a formaldehyde solution (Scheme 5.5), as explained in experimental section 6.7.2. Aliquots were taken to monitor reaction procedure by LC-MS, if left overnight formation of a dimeric species occurred with the mass of m/z= 491.



Scheme 5.5. Second synthesis step of lucidin, aldol type mechanism.

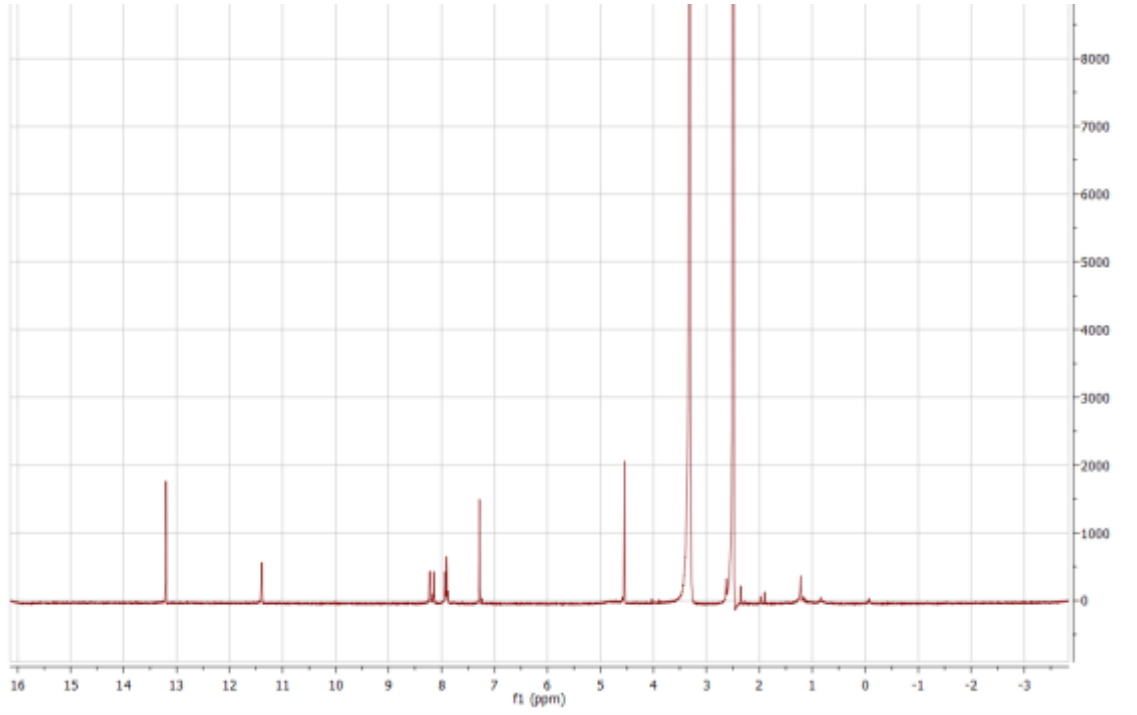


Figure 5.9. ^{1}H NMR spectra of lucidin in deuterated DMSO d_6.

Figure 5.10. High resolution mass spectrum of lucidin

Lucidin was purified and fully characterised by ^{1}H NMR (Figure 5.9), the LC-MS (Figure 5.10) indicated one compound. The pure lucidin was then dissolved under two conditions, to simulate back extraction conditions of historic artefacts. The compounds were dissolved in two different conditions: with and without the presence of methanol in the acidic medium used for the back extraction of artefacts, heated to 100°C and stirred for 15 minutes as described in experimental section 6.7. They were then evaporated to dryness and analysed by ^{1}H NMR and HPLC-DAD.

5.7 HCl: water breakdown of lucidin

The HPLC chromatogram for the water extraction without the presence of methanol (water: HCl, 1:1, *v*/*v*) carried out as described in experimental section 6.8.2, showed that there was a decrease in the lucidin peak and a peak appearance at retention time 11.57 min. This new peak has the same UV/vis data and retention time as that of the xanthopurpurin data shown in Table 5.3. The LC-MS also show two peaks that have the molecular weight of, lucidin (m/z= 269) and xanthopurpurin (m/z= 239). These results

suggest that under acidic conditions in water which mimic those used in textile back extractions, lucidin is partially degraded to xanthopurpurin (Figure 5.11).

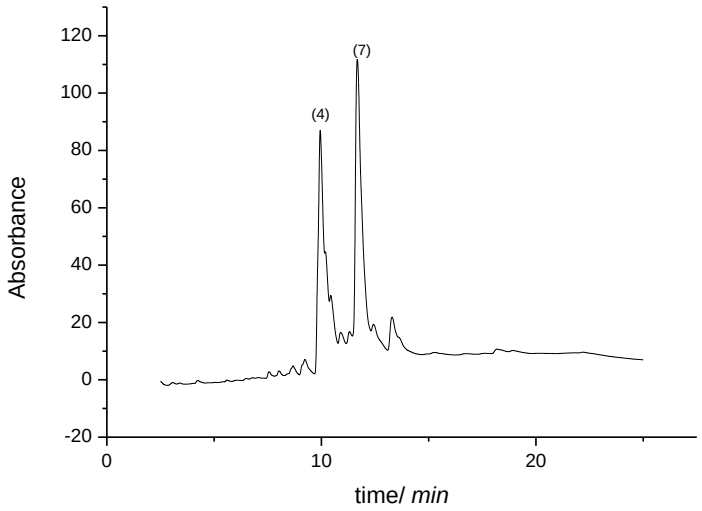
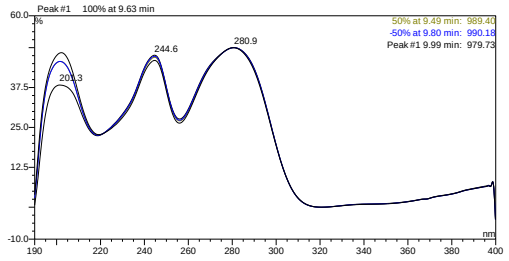
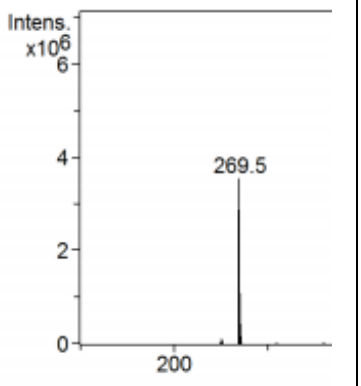
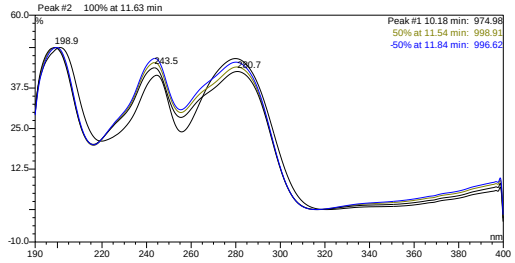
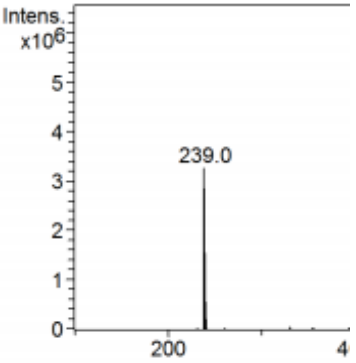
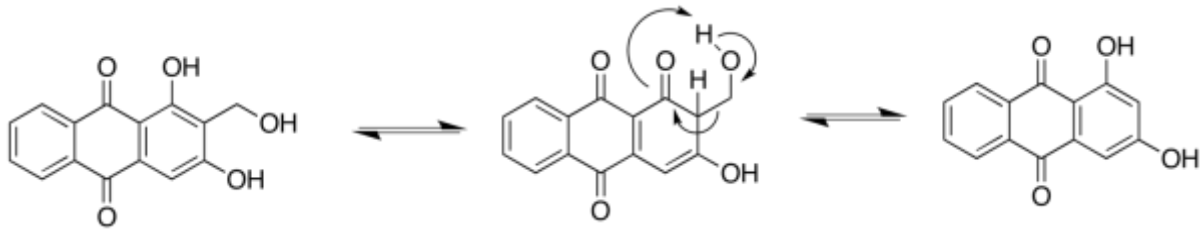


Figure 5.11. Chromatogram of lucidin after heating in water: hydrochloric acid (1:1, v/v). (4) lucidin (7) xanthopurpurin.

Table 5.3. Experimental data of the peaks shown in the chromatogram of lucidin heated in water and HCl.

Compound Identified	UV/vis data	Mass spectrum
Lucidin (4)		
Xanthopurpurin (7)		

It is hypothesised that in this case the reaction probably proceeds through a retro-aldol type mechanism (Scheme 5.6). The ability for this reaction to occur is unique to aromatic systems containing multiple hydroxyl groups. Hydroxyl groups on an aromatic ring are electron donating. The keto tendency of the hydroxyl groups in positions one and three on the lucidin aromatic ring in acidic conditions drive the reverse aldol condition. The electron donating ability of the other hydroxyl group in the ring also provide some stabilisation to the ketone transition state.



Scheme 5.6. Mechanism of the proposed retro aldol type mechanism of lucidin in acidic aqueous conditions.

The results of our experiments suggest that lucidin is not stable in acidic conditions and degrades rapidly to xanthopurpurin when heated in acidic water conditions used for extraction of historical textiles. NMR studies showing this degradation are displayed in Figure 5.14. Whilst labelled ^{14}C NMR experiments could have helped elucidate the exact mechanism, the difficulty in synthesising lucidin meant that it was not possible to synthesise the radiolabelled lucidin. Many recent literature sources have started to use mass spectrometry coupled with HPLC as a technique to provide additional structural data when analysing historic artefacts.[2,77,109,149,150] Xanthopurpurin and alizarin elute very closely on a C18 column and hence could co-elute as one peak in many systems. The two compounds also have the same mass as they are isomeric forms of one another and hence if their UV/vis spectra are not analysed in detail they could be mistaken for the same compound and hence only alizarin would be detected in the sample.

5.8 Methanol: Water :HCl breakdown of lucidin

The solvent system used in the literature for back extraction of dyes from textile artefacts composed of a 1:1:2 v/v/v ratio of methanol: water: HCl, was used in the second experiment. The reaction was run as described in experimental section 6.8.2.

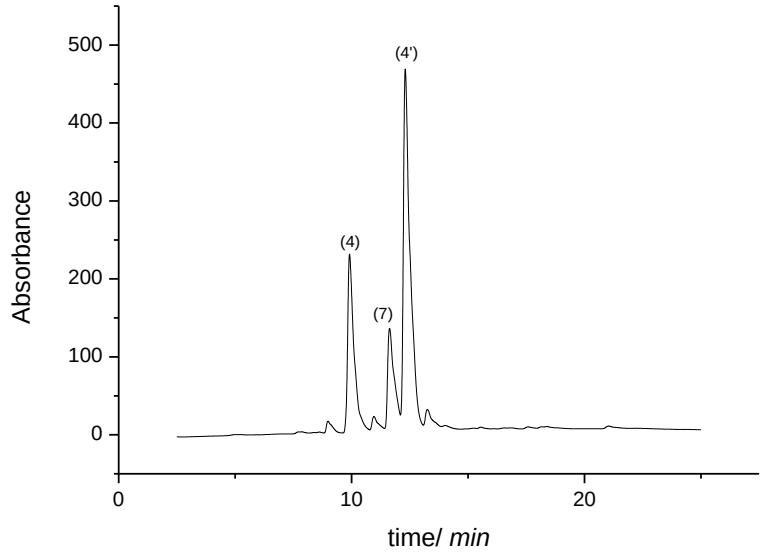
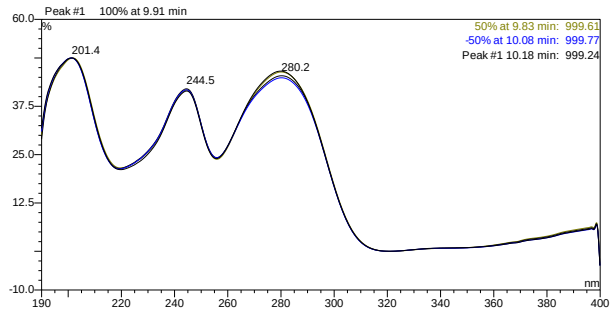
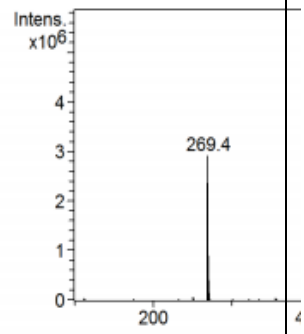
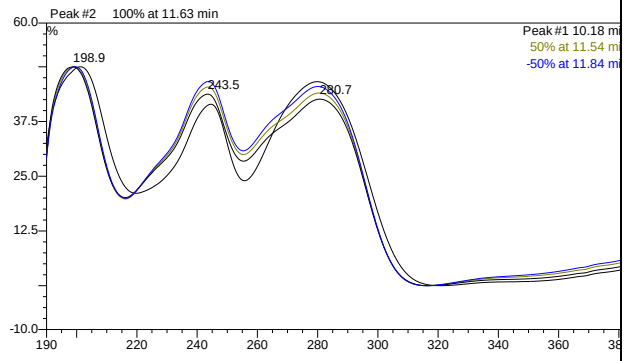
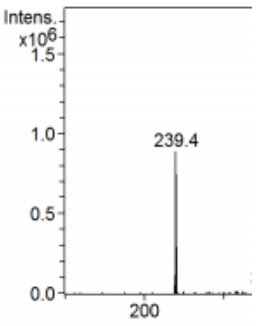
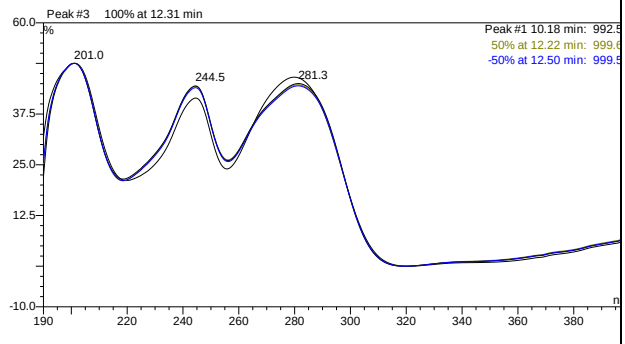
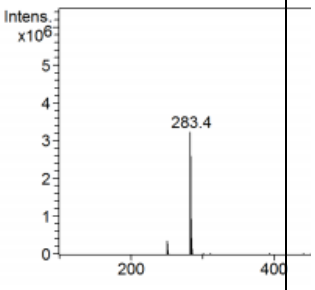


Figure 5.12. HPLC chromatogram of lucidin breakdown with methanol: water: HCl (1:1:2, v/v/v). (4) Lucidin (7) xanthopurpurin (4') methyl lucidin.

From the chromatogram of the breakdown (Figure 5.12) there is again a decrease in the lucidin peak and formation of the xanthopurpurin peak. However, in this reaction there was also the formation of a third peak which has the largest peak height and peak area in the chromatogram. LC-MS also showed three peaks in the chromatogram; lucidin (m/z= 269), xanthopurpurin (m/z= 239) and the third peak which gives a mass of m/z= 283. This mass corresponds to the methyl ether of lucidin. The experimental data of this chromatogram is shown in (Table 5.4). It is well documented that ether products can be formed when using alcohol in the solvent system when extracting dye compounds from the plants as described in chapter 2. It appears that this is also the case when back extracting from historic textiles. The methyl ether product is expected to be much more stable than the methyl hydroxyl which is present in the lucidin compound. The primary alcohol in the lucidin compound is easily displaced by nucleophilic attack of alcoholic solvents such as ethanol or methanol. Under dry or acidic conditions this primary alcohol can also be removed as water and formation of the quinone methide can take place (Scheme 5.3). The quinone methide is very susceptible

to nucleophilic attack[11] due to the broken aromaticity and hence this process is reversible and attack by water will reform lucidin whereas attack by methanol will form the methyl lucidin (Figure 5.13).

Table 5.4. Experimental data of the peaks shown in the chromatogram of lucidin breakdown in HCl: water: methanol solvent system.

Identified Compound	UV/vis data	Mass Spectrum
Lucidin (4)		
Xanthopurpurin (7)		
Lucidin methyl ether (4')		

It is noted herein that the UV/vis spectra of each peak was very similar for these compounds. For this reason, mass spectra data and ^{1}H NMR were used to fully assign

the peaks and observe the reaction though changes in the ^{1}H NMR spectra (Figure 5.15). The NMR experiments are not trivial to analyse as lucidin has limited solubility in most solvents or is only soluble at low concentrations. In addition to this the NMR signal corresponding to xanthopurpurin can appear diminished due to the proton between the two hydroxyl groups being labile for exchange with deuterium due to electron donation from the hydroxyl groups. The NMR spectra shown in Figure 5.14 show an increase in the proton signals highlighted in the coloured box. This proton corresponds to the aromatic signal between the two hydroxyl groups in xanthopurpurin.[151] The appearance of this signal shows that the lucidin has degraded to xanthopurpurin most likely through the retro aldol type reaction proposed above. Figure 5.15 shows the aromatic NMR spectra expanded in the aromatic region which displays the appearance of meta coupling (2-3 Hz). This meta coupling signals that there is a hydrogen in the meta position of the ring. This coupling further confirms the degradation to xanthopurpurin which displays this meta coupling.

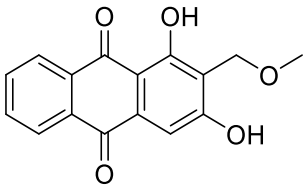


Figure 5.13. Structure of the proposed methyl ether product.

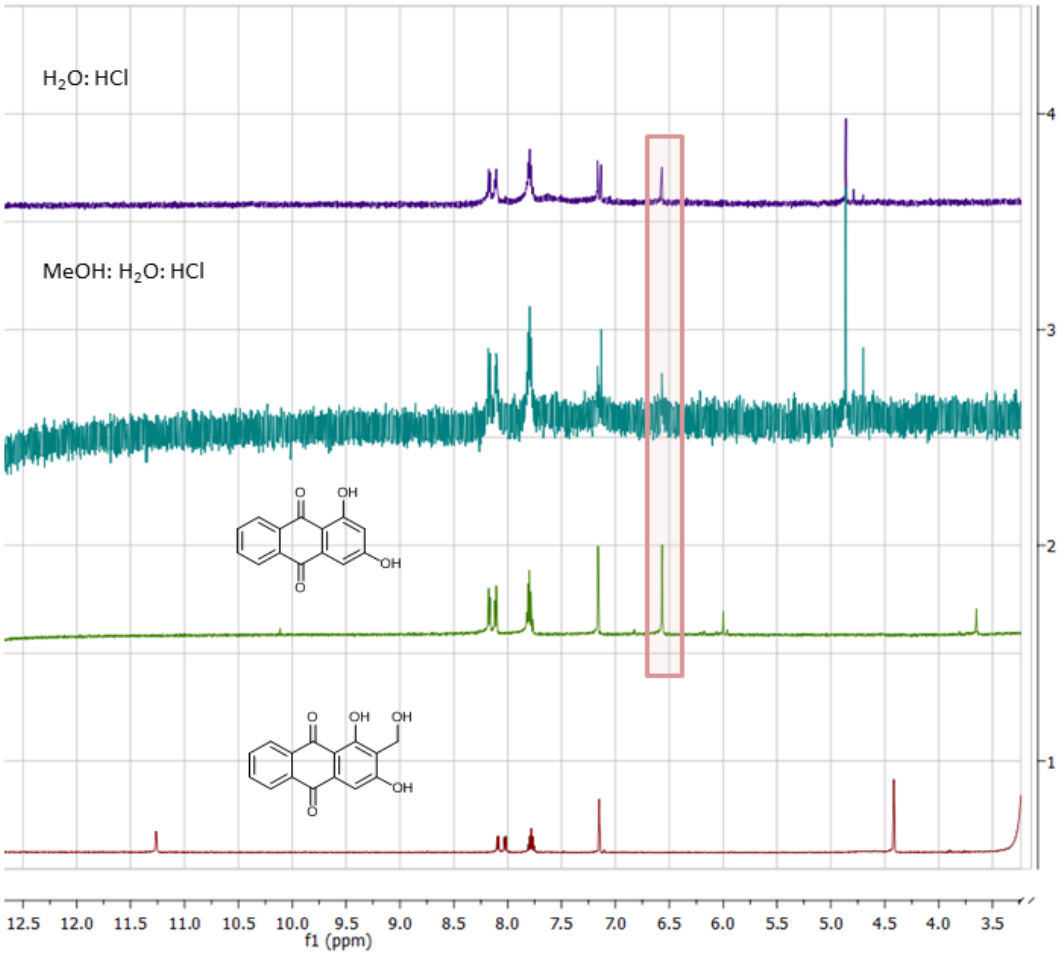


Figure 5.14. Stacked ^{1}H NMR of lucidin breakdown experiments. From top to bottom H_2O: HCl, MeOH: H_2O: HCl, xanthopurpurin standard, lucidin standard.

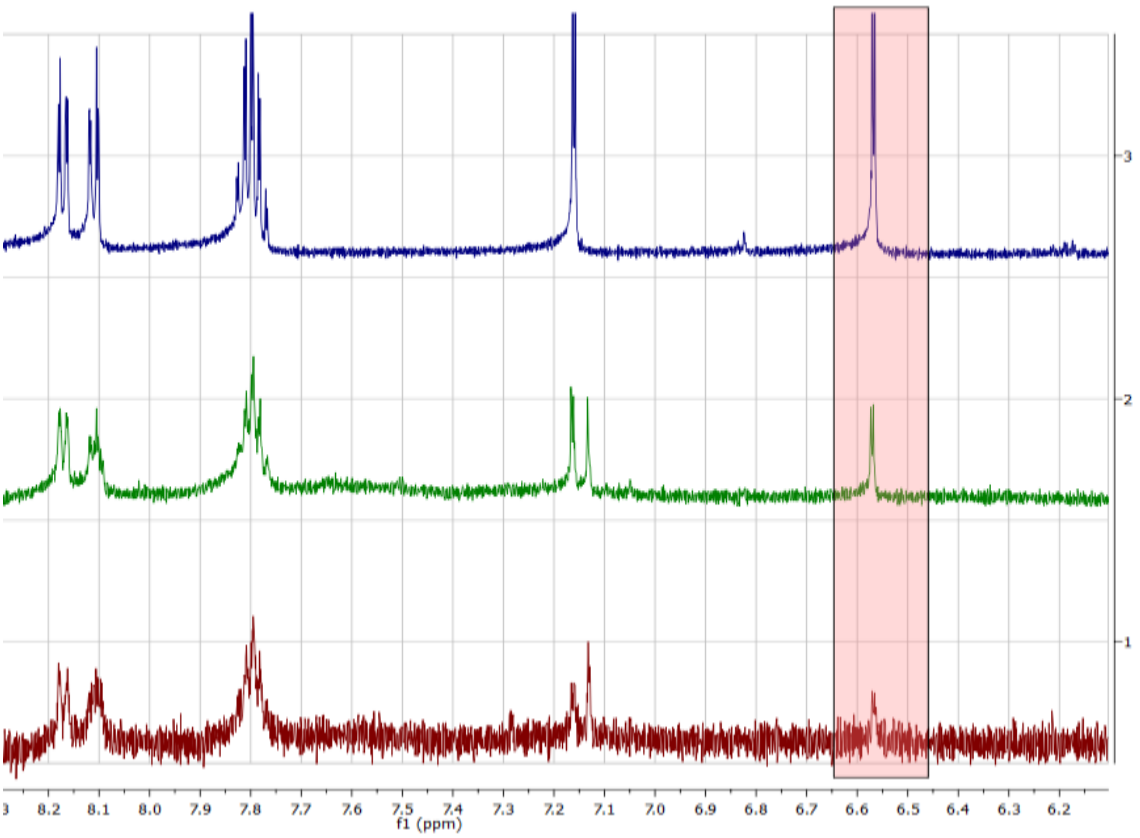


Figure 5.15. Stacked ^{1}H NMR spectra showing meta-coupling of the aromatic signals. From top to bottom; xanthopurpurin standard (blue), H_2O: HCl (green), MeOH: H_2O: HCl (red).

Upon closer inspection, the meta-coupling constant between the two protons on the xanthopurpurin can be observed in the degradation studies. This further confirms the degradation of lucidin into xanthopurpurin see Figure 5.15. A singlet corresponding to lucidin present in the breakdown experiments is also observed, indicating that the breakdown of lucidin is not complete and there is still some left in the reaction medium. This result is confirmed in the HPLC chromatograms shown in Figure 5.11 and Figure 5.12, where lucidin is still present in both of these experiments but in smaller concentrations.

This reaction was also tested in deuterated solvents deuterium oxide and deuterated methanol. This was to try to monitor the progress of the reaction by NMR however, this resulted in the deuterated reaction product of the lucidin methyl ether as observed by the mass m/z= 286. This causes problems for analysis by NMR experiments

due to the methanol adduct being the deuterated product and hence is not displayed in the NMR spectrum.[118] However the mass spectrum shows the lucidin methyl ether ion m/z= 283 + 3 for the deuterated methanol adduct due to deuterium showing an M + 1 mass (Figure 5.16). This is another result showing the formation of the lucidin methyl ether.

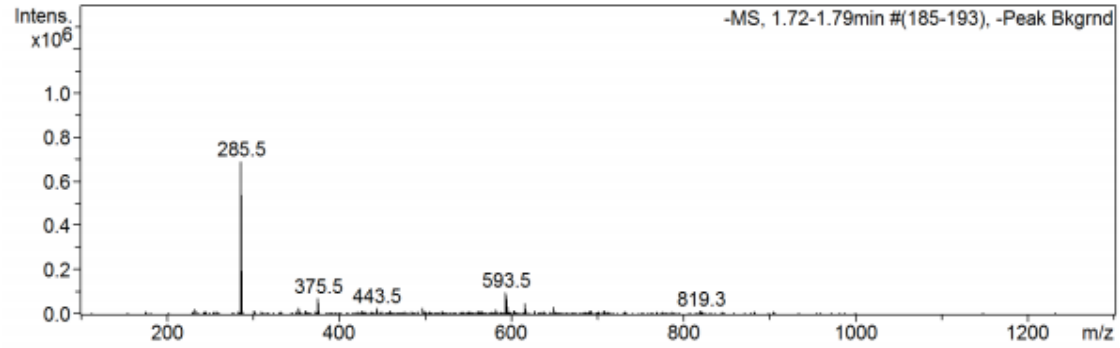


Figure 5.16. Mass spectrum of deuterated methyl ether product of lucidin.

The breakdown of lucidin into xanthopurpurin is important for the field of conservation scientists and conservators. Previously the presence of xanthopurpurin has been used as an indicator that the sample probably contained munjistin, which is a carboxylic acid derivative of xanthopurpurin.[11] Munjistin can be easily decarboxylated to form xanthopurpurin,[25] this reactivity is again due to the keto tendency of the electron donating hydroxyl groups on the ring. The carbonyl can leave as carbon dioxide to form xanthopurpurin. Munjistin is a marker pigment which can be used to prove the presence of *R. cordifolia* in dyed textiles. Therefore, by using acid in the extraction process information is lost on the dyed material as munjistin is decarboxylated to xanthopurpurin which is present in acid extractions of both *R. tinctorum* and *R. cordifolia*.

Herein it is shown that xanthopurpurin is not solely formed from munjistin but can also be formed by degradation of lucidin in the harsh acidic conditions that are used in the extraction conditions. Therefore, when identifying madder species based on ‘marker’ pigments, the method of extraction used in the experimental should be questioned before

concluding on the species originally used for dyeing. The possible degradation pathways observed herein and from the literature are summarised in Figure 5.17.

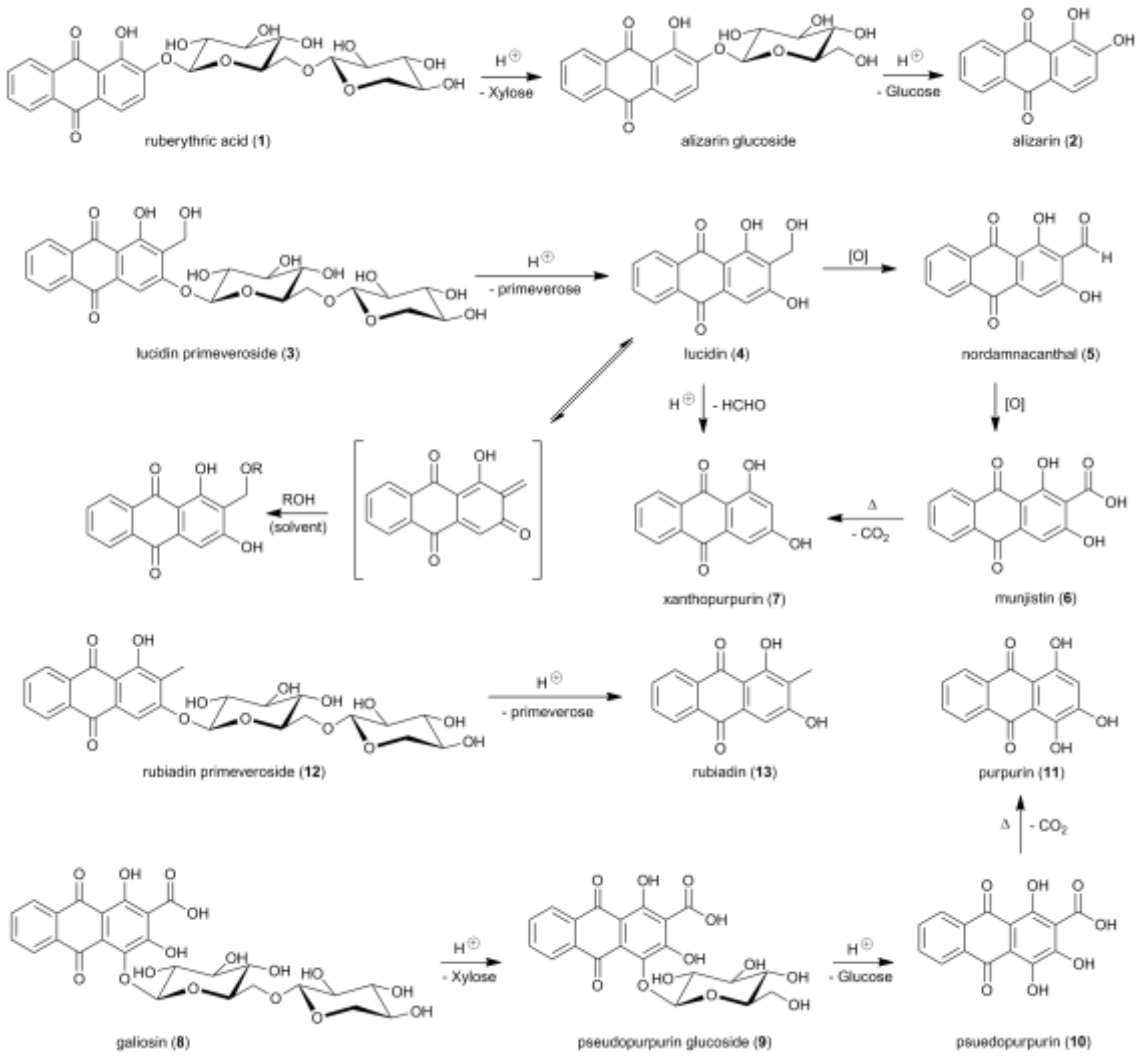


Figure 5.17. Compounds present in madder root and their proposed degradation products.

5.9 Comparison of DMSO/oxalic acid extraction to novel glucose method

DMSO/oxalic acid is a recent mild extraction method currently being used to analyse historical textiles of cultural interest.[14] This method was not originally used for comparison between the analytical methods due to it being a slightly different technique that does not use aqueous solvents and the possible oxidation of primary alcohols in the solution by a Swern oxidation (Figure 5.6). However, for completeness this study was compared to the novel back extraction method using glucose proposed in this body of work.

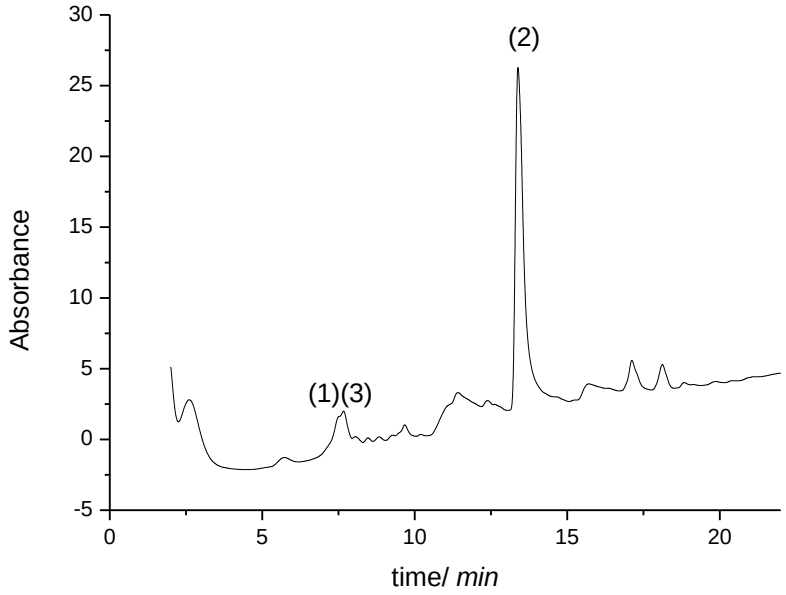


Figure 5.18. DMSO/oxalic acid extraction of Turkish madder samples. (1) Ruberythric acid (3) lucidin primeveroside (2) alizarin.

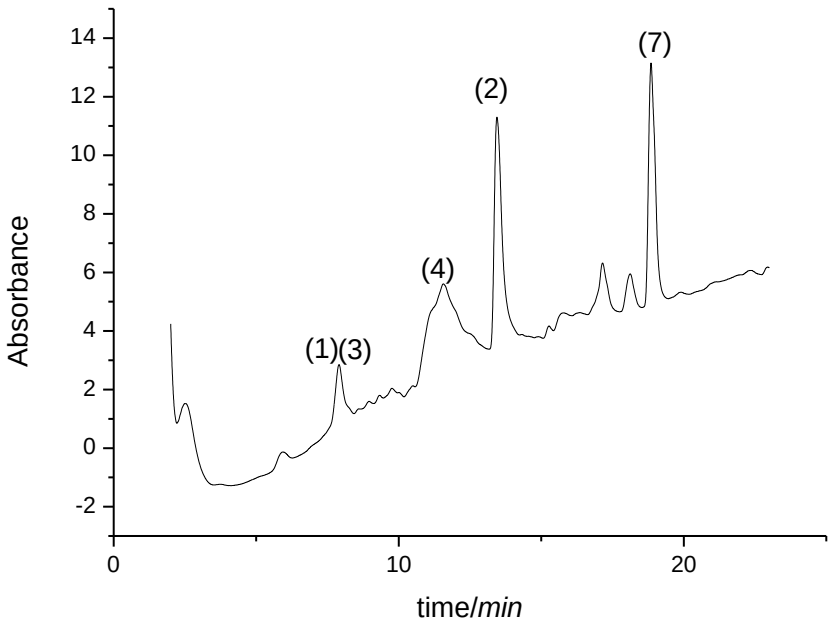


Figure 5.19. DMSO/oxalic acid extraction of English madder dyed wool. (1) Ruberythric acid (3) lucidin primeveroside (4) lucidin (2) alizarin (7) nordamnacanthal.

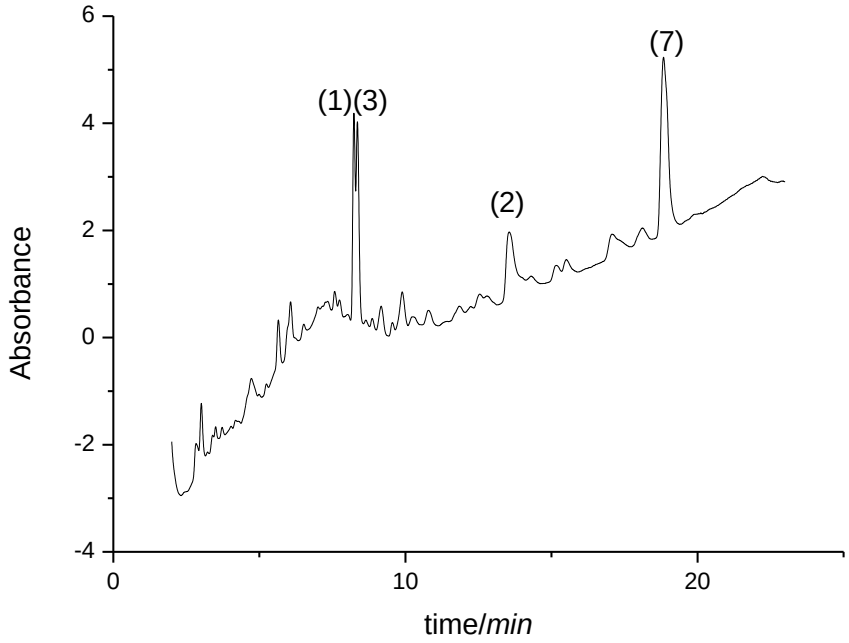


Figure 5.20. Glucose extraction of Turkish madder dyed wool. (1) Ruberythric acid (3) lucidin primeveroside (2) alizarin (7) nordamnacanthal.

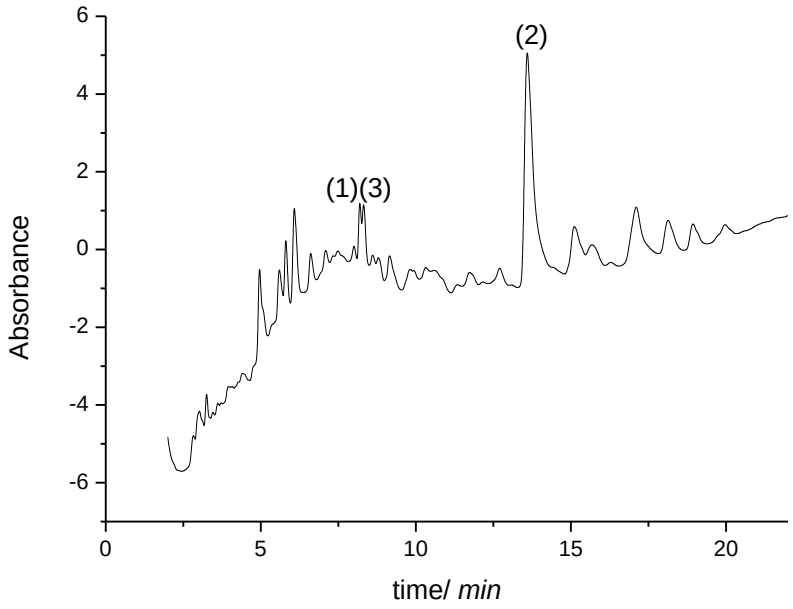


Figure 5.21. Glucose extraction of English madder dyed wool. (1) Ruberythric acid (3) lucidin primeveroside (2) alizarin.

The back-extraction chromatograms displayed above were extracted from wool dyed for the original back extraction experiments and hence the wool dyed samples were at least 3 years old when this extraction was carried out. It is for this reason that the extraction using these techniques was not performed on Iranian madder dyed samples as unfortunately there was no Iranian madder dyed samples left to analyse. However, due to the similarities between Iranian and Turkish madder shown throughout this thesis an extraction using Turkish madder was deemed to be sufficient. The age of the samples means there could have been some degradation to some of the compounds: such as lucidin to nordamnacanthal in the dyestuff due to oxidation as the wool samples were stored in an environment open to oxygen and light.[25]

The DMSO/oxalic acid extraction shows a good identification of the agycon components of the dyed material (Figure 5.18). The glycosides are also observed when this method of extraction is used on Turkish madder dyed wool. However, the relative ratio of glycosides to the aglycon alizarin is much smaller than what was observed in the original

dye baths for Turkish madder. This could either be due to poor extraction of the glycosides from the wool or from degradation in the extraction conditions. When this is compared to the glucose method for extraction of Turkish madder (Figure 5.20) a much higher relative ratio of the glycosidic compounds are observed when compared to the alizarin peak. This is probably due to the sugar in the solution disrupting the aggregation shown by the crystal structure as described in chapters 4 and 5. When the DMSO/oxalic acid back extraction is applied to English madder dyed samples a much more favourable back extraction is observed (Figure 5.19) when compared to the glucose extraction method (Figure 5.21). This is probably due to the extracted compounds being mostly the aglycon materials which show limited solubility in water. Therefore, the DMSO method is better at solvating these materials and hence is better at detecting compounds from mainly aglycon extracts. The DMSO method also provides a better signal to noise ratio in the chromatograms when extracting these dyed wool samples. It is unclear why the lucidin peak in these extractions is so broad. Due to these extractions being done at a much later date the column on the HPLC system had been changed and hence these conditions were not optimised on this new column and the same elution programme was used as that for the old column. Therefore, it is not clear whether this is how lucidin behaves on this column or the broadening is due to unresolved peaks in the chromatogram.

From these results, it is shown herein that the glucose method proposed is more efficient at extracting glycosidic compounds from the dyed wool material but the DMSO/oxalic acid method provided superior results in detecting aglycon compounds from the textiles. It is therefore suggested that the glucose method could be used to extract from dyed artefacts as an initial extraction procedure; to see if there are any glycosides present in the textile being extracted. Then follow this analysis with an extraction technique which provides superior analysis of alglycon materials such as the DMSO/oxalic acid method or HCl: methanol: water method to provide a full detailed overview of all dye compounds adsorbed in the wool.

6 Experimental

6.1 General methods

Nuclear magnetic resonance (NMR) spectra were recorded for ^{1}H at 300 and 500 MHz and ^{13}C at 75 and 125 MHz on a Bruker *DPX300* or *DRX500* spectrometer. Chemical shifts are reported in parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm) for proton resonances. The proton coupling constants are reported as corrected values and are given in Hz (multiplicities, s singlet, bs broad singlet, d doublet, t triplet, q quartet and apparent multiplicities are described as app). To aid characterisation 2-D correlation spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOSEY), Heteronuclear Single-Quantum Correlation spectroscopy (HSQC) and Heteronuclear Multiple-Bond Correlation spectroscopy (HMBC) pulse sequences were utilised. COSY is the most commonly used 2D NMR experiment used to identify spins which are coupled to one another in a homonuclear through bond correlation. NOSEY is used to detect nuclei which are spatially close to one another. HMBC is used to detect heteronuclear correlations over long ranges of a few bond. HSQC detects correlations between nuclei of two different types separated by one bond.[118]

UV/Vis analysis was carried out using a *Jasco V630* UV-Vis spectrophotometer.

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.[56,58] The flow rate was 1.0 ml min^{-1} and peaks were detected at 254 nm.

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.

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 NI (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. Negative ionisation mode was found to be more sensitive for the compounds in madder root and hence all analysis was done in the negative ionisation mode.[150]

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.

Solvents were removed under reduced pressure using a Buchi rotary evaporator at 20 mbar, followed by further drying under high vacuum at 0.5 mmHg.

A suitable single crystal was selected for X-ray analysis and immersed in an inert oil. Measurements were carried out at 120K on an Agilent SuperNova diffractometer equipped with an Atlas CCD detector and connected to an Oxford Cryostream low temperature device using mirror monochromated Cu Kα radiation (λ = 1.54184 Å) from

a Microfocus Nova X-ray source. The structure was solved by direct methods using SHELXS and refined by a full matrix least squares technique based on F2 using SHELXL97.

To measure the melting points 2-3mm of sample was placed in the bottom of the capillary tube and the tube places in the heating block. The 'melting point' was measured as the range from the appearance of the first liquid droplet until complete melting of the crystals. The measurements were carried out on a Mettler Toledo melting point system.

6.2 Materials

Natural cream wool, heavy weight with plain even weave, natural cream colour, width 150 cm (60”), approx 430 gsm, 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. Pseudopurpurin standard was supplied by Dr. David Hill and Vincent Daniels from the British Museum. Alizarin and purpurin standards were purchased from Sigma-Aldrich as were all other synthesis chemicals. A mixture of the glycosides lucidin primeveroside and ruberythric acid was purchased from Apin Chemicals. Aluminium potassium sulfate dodecahydrate and potassium tartrate were purchased from sigma Aldrich. All solvents used were of HPLC grade and purchased from Sigma-Aldrich. HPLC grade water was obtained by distillation on site.

6.3 Extraction procedures

6.3.1 Ethyl acetate extraction

Madder root (10 g) of all three varieties was extracted with ethyl acetate (300 ml) at reflux temperature for 60 minutes. After extraction, the solution was filtered and evaporated to

dryness under reduced pressure on a rotary evaporator. The orange solid was then re-dissolved in methanol: water (1:1, *v*/*v*) for analysis by LC-MS and HPLC. Yield: Iranian madder 0.09 g, Turkish madder 0.09 g, English madder 0.19 g.

6.3.2 Ethanol extraction of madder previously extracted with ethyl acetate

Madder root filtrate (10 g) from section 6.3.1 was extracted with ethanol (300 ml) at reflux temperature for 60 minutes. After extraction, the solution was filtered and the solvent evaporated under reduced pressure on a rotary evaporator. The resultant solid was then re-dissolved in methanol: water (1:1, *v*/*v*) for analysis by LC-MS and HPLC. Yield: Iranian madder 0.60 g, Turkish madder 0.56 g, English madder 0.12 g.

6.3.3 Water extraction of madder previously extracted with solvents

Madder root filtrate (10 g) from section 6.3.2 was extracted with deionised water (300 ml) at 90 °C for 60 minutes. After extraction, the solution was filtered and the solvent removed by lyophilisation after freezing in liquid nitrogen. The solid was then re-dissolved in methanol: water (1:1, *v*/*v*) for analysis by LC-MS and HPLC. Yield: Iranian madder 3.7 g, Turkish madder 3.2 g, English madder 2.9 g.

6.3.4 Water extraction of natural dyes

Dye plants (10 g) were extracted with water (300 ml) at 90°C for 60 minutes. The solution was then filtered and solvent was removed by lyophilisation and yield recorded (Table 6.1). The solid was then re-dissolved in methanol: water (1:1, *v*/*v*) and analysed by HPLC and LC-MS. NMR experiments were run in deuterated methanol d_4 with the addition of small amounts of DMSO d_6 if the solvation was not achieved. The resultant solid was very hygroscopic and sticky.

Table 6.1. Table showing the yields of dyes from the water extraction of three types of madder chosen to study after freeze drying.

Dye Plant	Yield (g)
Iranian madder	4.1 g
Turkish madder	3.3 g
English madder	3.3 g

6.3.5 Ethanol extraction

Madder root of each variety (10 g) was extracted with ethanol (300 ml) at reflux temperature for 60 minutes. After extraction, the solution was filtered and the solvent evaporated under reduced pressure on a rotary evaporator, for each sample the yield was measured (Table 6.2). The resultant solid was then re-dissolved in methanol: water (1:1, *v*/*v*) for analysis by LC-MS and HPLC. NMR experiments were run in deuterated methanol d_4 adding DMSO d_6 to aid solution if needed.

Table 6.2. Table to show the extraction yields of the different dye plants when extracted with ethanol.

Dye Plant	Yield (g)
Iranian madder	0.83 g
Turkish madder	0.65 g
English madder	0.24 g
Weld	0.9 g
Golden rod	0.6 g
Chamomile flowers	0.4 g
Chamomile plant	0.5 g

6.3.6 Solid Phase Extraction (SPE)

Water extracted dye plant liquor was prepared as described in section 6.3.4 for all varieties of dye plants. Amberlite XAD-7HD 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 plant material. The Amberlite resin was then applied to the column, and washed with water. The aqueous madder extract was then applied to the resin and washed with water (5 x 100 ml) to remove free sugars. The resin was then washed with ethanol (2 x 100 ml) and the ethanol extracts collected. The ethanol solution was then evaporated under reduced pressure on a rotary evaporator and the yield of each sample recorded. The resulting solid was then re-dissolved in methanol: water (1:1, *v*/*v*) and analysed by LC-MS and HPLC. NMR experiments were run in deuterated methanol d_4 with addition of DMSO d_6 to aid solution if needed.

Table 6.3. Table to show the extraction yields of dye plants after SPE.

Dye plant	Yield (g)
Iranian madder	1.7 g
Turkish madder	1.6 g
English madder	1.4 g
Weld	1.4 g
Golden rod	1.8 g
Chamomile flowers	1.1 g

6.4 Recrystallization of ruberythric acid and crystal structure determination

Madder root ethanolic extract from section 6.3.5 (1.5 g) was re-dissolved in methanol (150 ml) and sonicated to ensure full solvation; any remaining solid was removed by filtration. The solution was then left for 5 days after which an amorphous solid was observed in the bottom of the flask. The liquid was then decanted off and the remaining solid was dried on the high vacuum system. Deuterated DMSO (~3 ml) was then added to the flask and left open to the air for a further 4 days to give a large yellow crystalline needle. Deuterated solvent was used due to the small amounts of compounds available and the need for NMR analysis throughout the process. An X-ray crystal structure of the compound was recorded, and a segment of the crystal was analysed by ^{1}H and ^{13}C NMR spectroscopy in DMSO-d_6.

^{1}H NMR (500 MHz): δ 8.27 (d, 1H, J=9.2 Hz, H^{2}), δ 8.22 (d, 1H, J=9.2 Hz, H^{5}), δ 7.97 (app t, 2H, J= 8.9 Hz, H^{3} and H^{4}), δ 7.77 (d, 1H, J=8.5 Hz, H^{9}), δ 7.65 (d, 1H, J=8.5 Hz, H^{10}) aromatic peaks, δ 5.47 (d, 1H, J=5.3 Hz, H^{25}), δ 5.22 (d, 1H, J=5.0 Hz, H^{27}), δ 5.20 (d, 1H, J=5.5 Hz, H^{26}), δ 5.09 (d, 1H, J=7.5 Hz, anomeric glucose H^{19}), δ 5.02 (d, 1H, J=4.8 Hz, H^{36}), δ 4.95 (d, 1H, J=4.7 Hz, H^{38}), δ 4.92 (d, 1H, J=5 Hz, H^{37}), δ 4.17 (d, 1H,

J=7.5 Hz, anomeric xylose H^{30}). Other sugar hydrogens are seen as multiplets between δ 3.9-2.9.

^{13}C NMR d_6 DMSO (500 MHz): δ 188.47, 180.92, 151.74, 151.12, 135.17, 134.31, 133.39, 132.96, 126.79, 126.58, 125.87, 120.79, 120.42, 116.23, 103.96, 99.85, 76.58, 76.44, 76.07, 73.40, 72.99, 69.57, 68.26, 65.55, 40.05, 38.94. HRMS: m/z (ESI+) calculated for $C_{25}H_{26}O_{13}$ $[M+ Na]^+$:557.1373; found $[M+ Na]^+$: 557.1275.

IR spectrum shows characteristic peaks at 3255 cm^{-1} (OH stretching), 1667 cm^{-1} and 1632 cm^{-1} (ketones).

Ruberythric acid crystallised as orange needles in a tetragonal cell and was solved in the *I*4 space group, with one molecule and disordered solvent (modelled as one and a half molecules of water and one and a quarter molecules of DMSO d_6) in the asymmetric unit. There was significant disordered solvent in the asymmetric unit. This has been modelled as one and a half molecules of water and one and a quarter molecules of DMSO d_6. One molecule of DMSO d_6 has been modelled with sulfur disordered across two positions in a 50:50 ratio. The quarter molecule of DMSO d_6 is disordered across a four-fold rotational axis. The correct composition of this DMSO d_6 has been modelled, but molecular structure has not been refined. Disordered solvent was refined isotopically. No hydrogen atoms were added to the disordered solvent during refinement, but the correct number of atoms has been added to the molecular formula in the CIF.

6.5 Dyeing methods

6.5.1 Dyeing with mordant

Wool samples 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 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.[12] 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, 12 % omf) in water liquor- fibre ratio 500:1 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.

6.5.2 Dyeing without mordant

Wool samples 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. 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, 12 % omf) in water, liquor-fibre ratio 500:1 in a *Roaches Pyrotec S Rotodyer* dyeing machine, 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.

6.6 Back extraction methods

6.6.1 HCl extraction

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.[104] 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.

6.6.2 Citric acid extraction

An aqueous solution of 0.5 M citric acid was prepared and 0.5 ml of this solvent was used to extract each wool sample (2 mg) at 90°C for 15 mins.[108] 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.

6.6.3 TFA extraction

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.[44,108] 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.

6.6.4 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. 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.

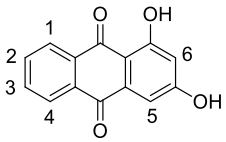
6.6.5 DMSO/oxalic acid extraction

Two step extraction method using DMSO and Oxalic acid was used for extraction of the wool samples (2 mg). The first step involved immersion of the wool with DMSO (0.1 ml) and heated to 80°C for 10 minutes. The solvent was then removed from the wool vial and retained. The wool was then immersed in a mixture of oxalic acid: acetone: water: methanol (1:30:40:30, *v/v/v/v*) (0.1 ml) and heated to 80°C for further 15 minutes. The two aliquots were then combined and analysed by HPLC-DAD. Each type of madder was extracted and the experiment was repeated twice for each type of madder dyed wool.

6.7 Synthesis of standards

6.7.1 Xanthopurpurin

This method was based on that of Murti *et al*.[152] 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). (Figure 5.9)

^{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 (Figure 6.4).

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 can be found in Chapter 2 (Figure 6.1) UV/vis from chromatogram (Figure 6.2).

IR (ATR), v/cm^{-1}: 3360, 1633, 1598, 1451, 1258 cm^{-1} (Figure 6.3). Melting point: 261-264°C.

λ_{max} (log ε) in MeOH: 412 (4.15) nm.

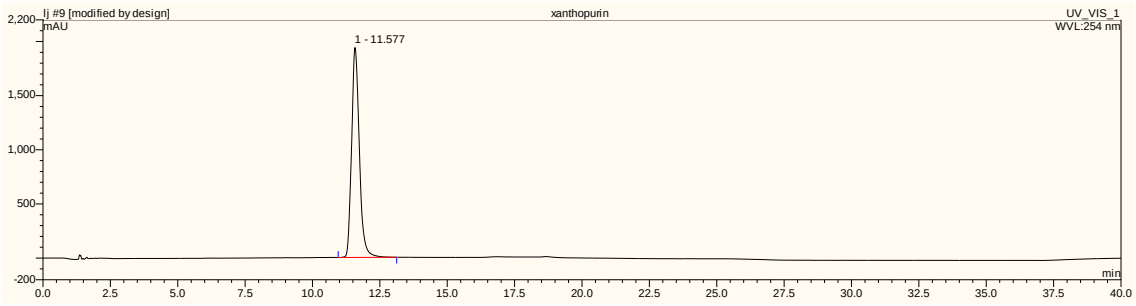


Figure 6.1. HPLC chromatogram of xanthopurpurin.

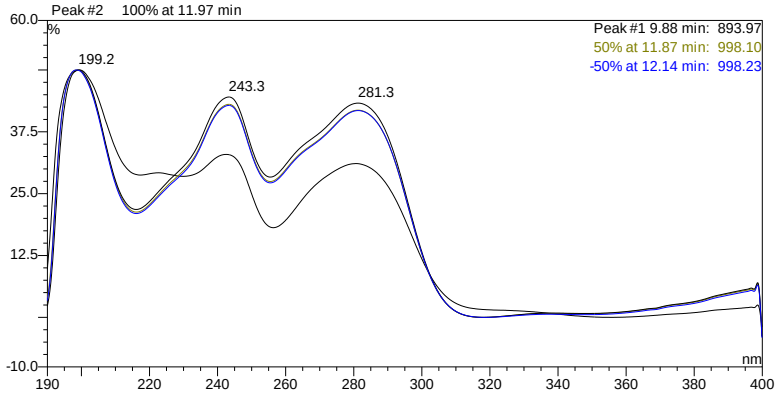


Figure 6.2. UV/vis spectrum of xanthopurpurin.

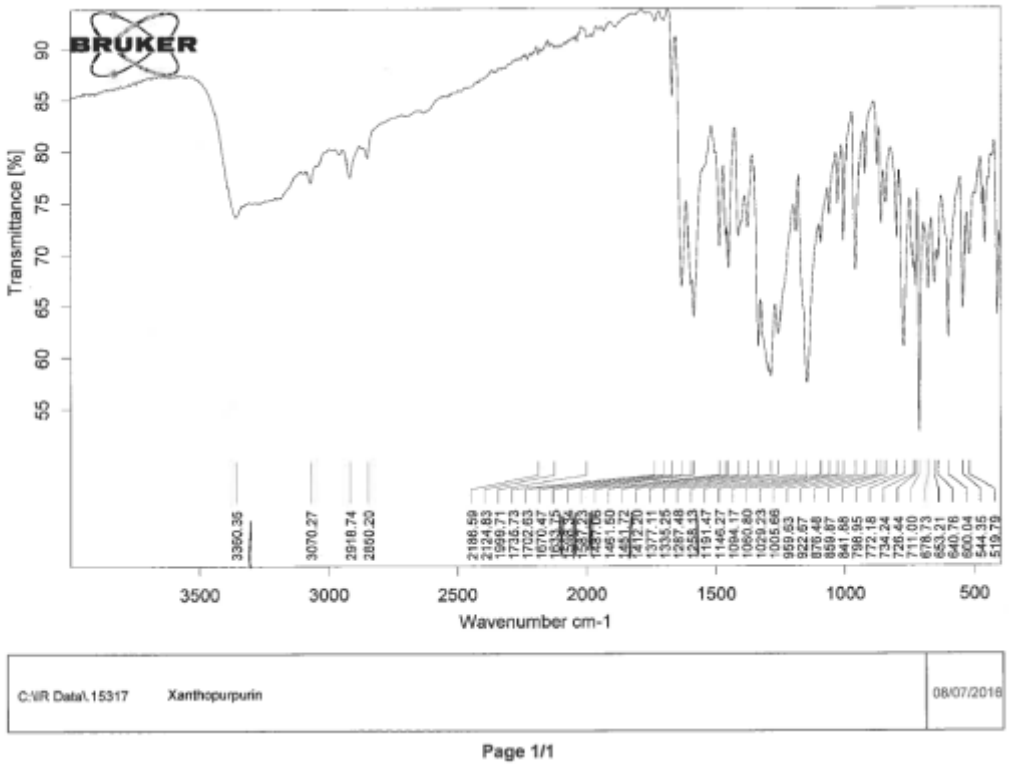


Figure 6.3. IR spectrum of Xanthopurpurin.

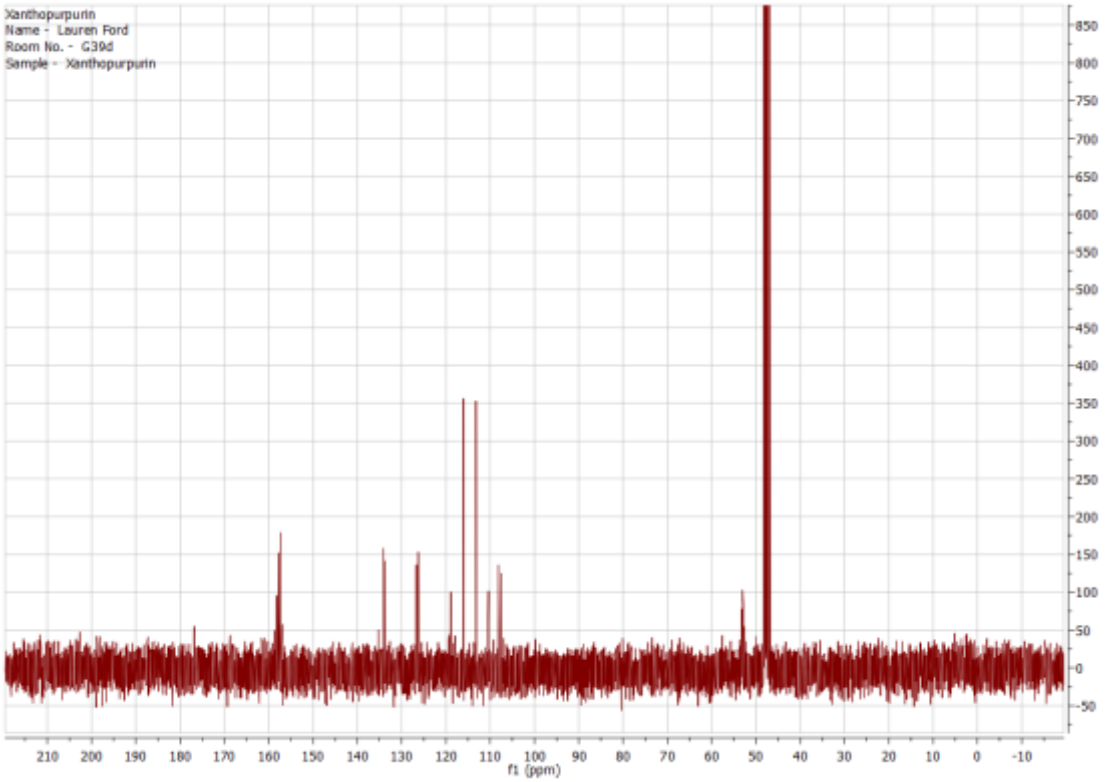
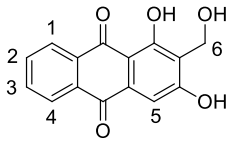


Figure 6.4. ^{13}C NMR spectrum of xanthopurpurin.

6.7.2 Lucidin

This method was based on that of Murti *et al*.[152] 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 equiv.) 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). (Figure **5.9**)

^{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 (Figure 6.8).

HRMS: m/z (ESI-) calculated for $C_{15}H_{10}O_5$ $[M-H]^-$:269.0528; found $[M-H]^-$:269.0464. (Figure 5.10)

HPLC (Figure 6.5) retention time and mass data can be found in chapter 2. UV/vis spectrum for peak in the chromatogram is shown in Figure 6.6.

IR (ATR), v/cm^{-1}: 3400, 1634, 1558, 1365, 1338 cm^{-1} (Figure 6.7). Melting point: 301-305°C.

λ_{max} (log ε) in MeOH: 410 (3.66) nm.

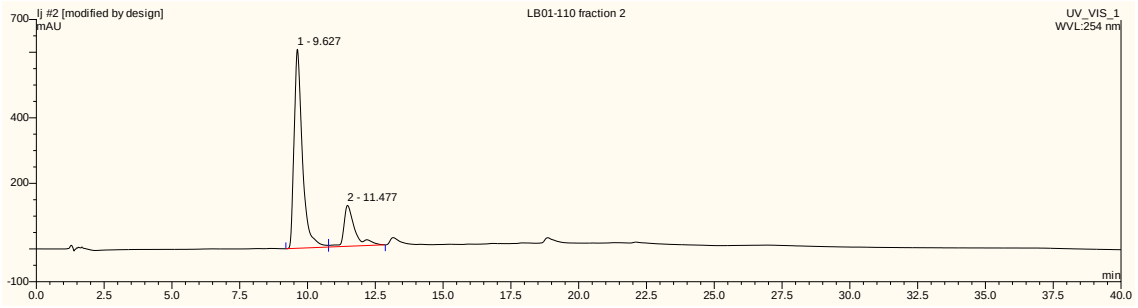


Figure 6.5. HPLC chromatogram shows lucidin with some degradation into xanthopurpurin.

The degradation shown in the HPLC chromatogram of lucidin is thought to be due to the slightly acidic elution of the column conditions. NMR or HRMS did not show any presence of xanthopurpurin in the purified synthetic compound.

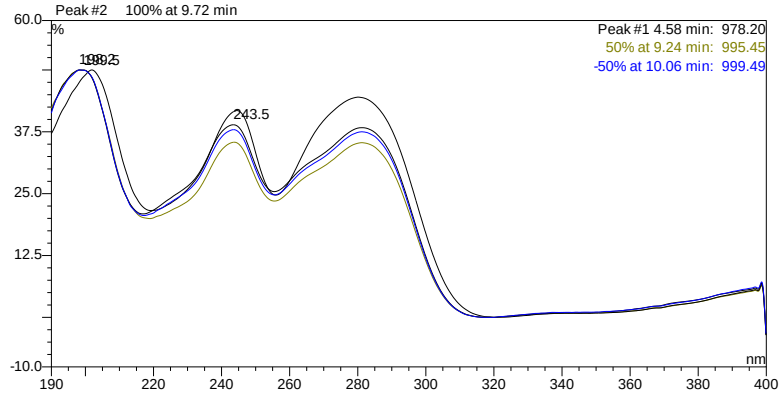


Figure 6.6. UV/vis spectrum of lucidin.

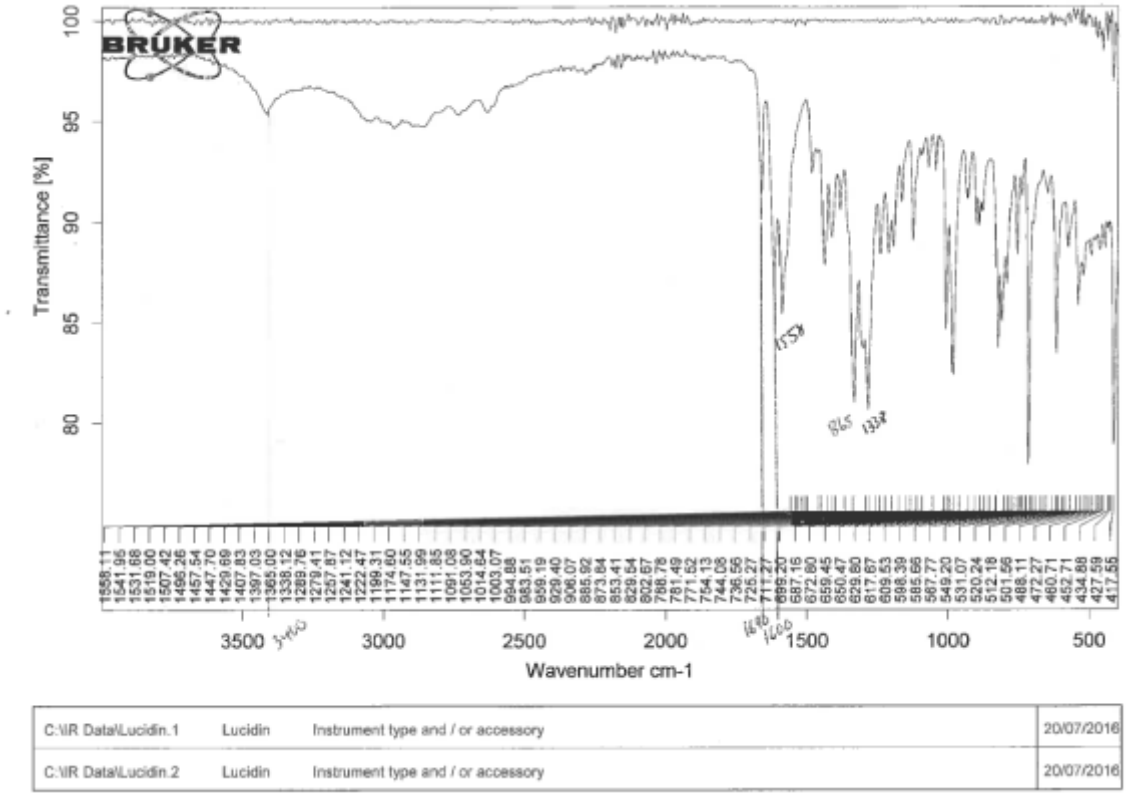


Figure 6.7. IR spectrum of lucidin.

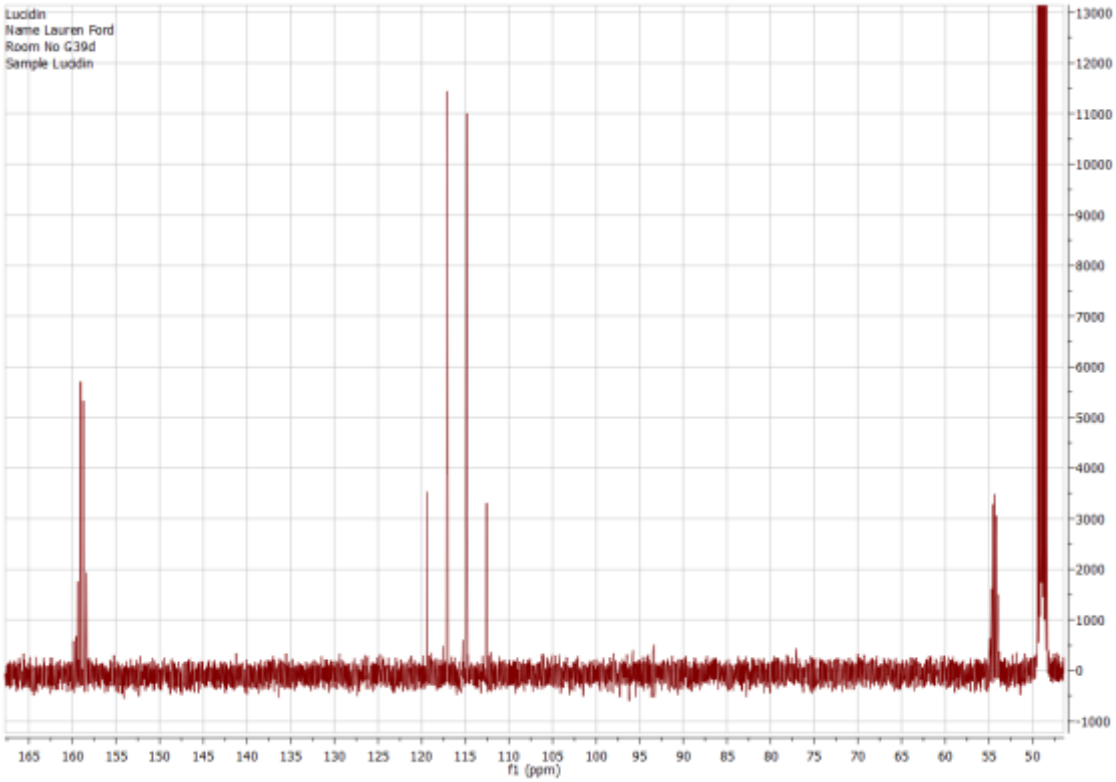


Figure 6.8. ^{13}C NMR of lucidin.

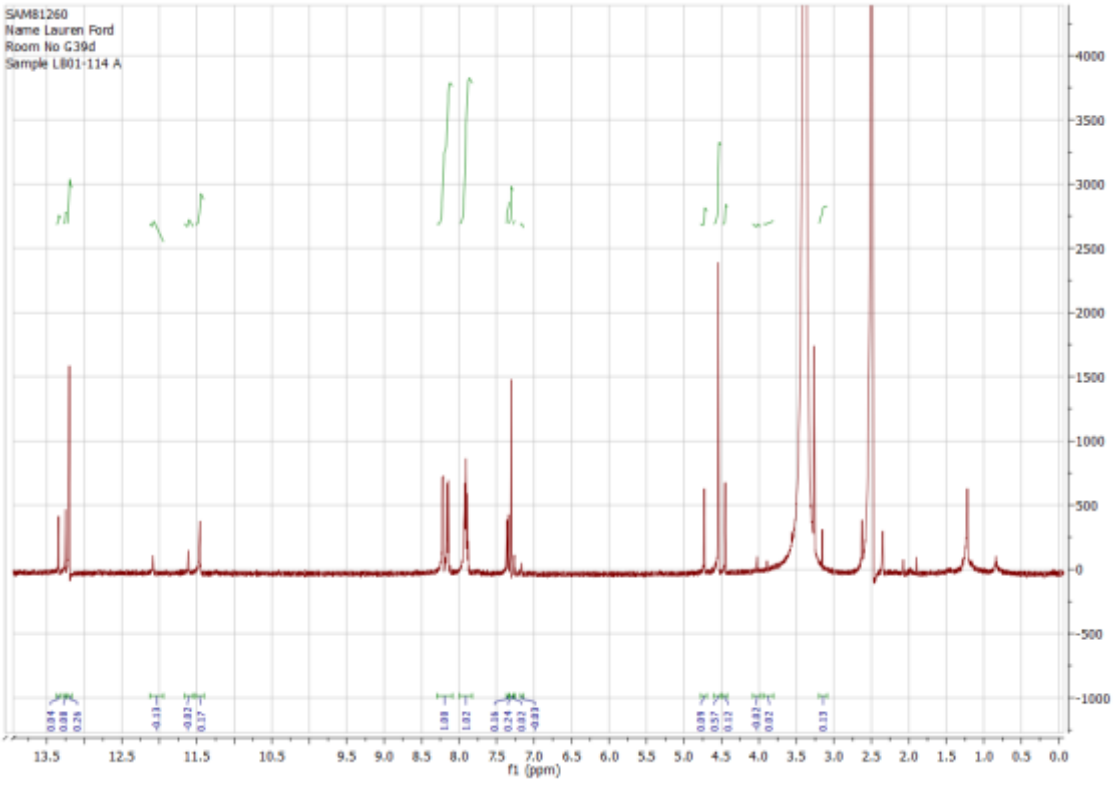
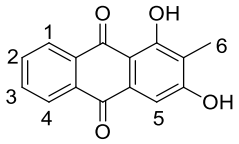


Figure 6.9. NMR of reaction of lucidin in methanol with acid.

6.7.3 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).[116]



^{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) (Figure 6.13).

^{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 (Figure 6.14).

HRMS: m/z (ESI-) calculated for $C_{15}H_{10}O_4$ $[M-H]^-$: 253.0579; found $[M-H]^-$:253.0534 (Figure 6.11).

HPLC retention time and mass data can be found in chapter 2 (Figure 6.10) UV/vis spectrum of peak in the HPLC chromatogram displayed in Figure 6.12.

IR (ATR), v/cm^{-1}: 3387, 2917, 2356, 1698, 1577, 1292 cm^{-1} (Figure 6.15). Melting point: 286-291°C (from EtOH).

λ_{max} (log ε) in MeOH: 410 (4.46) nm.

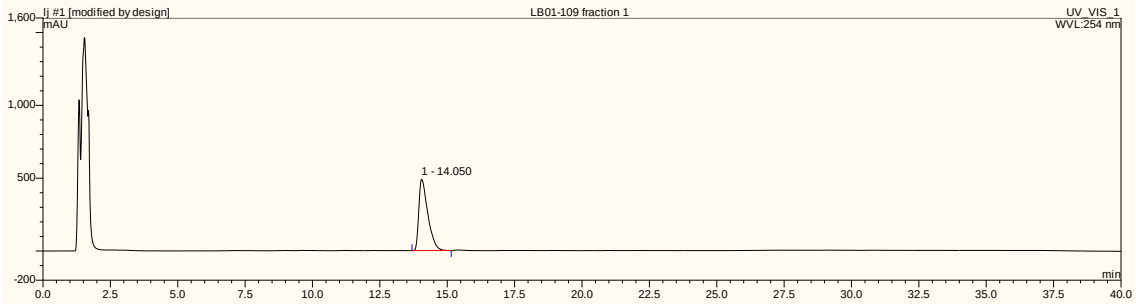


Figure 6.10. HPLC chromatogram of synthesised rubiadin.

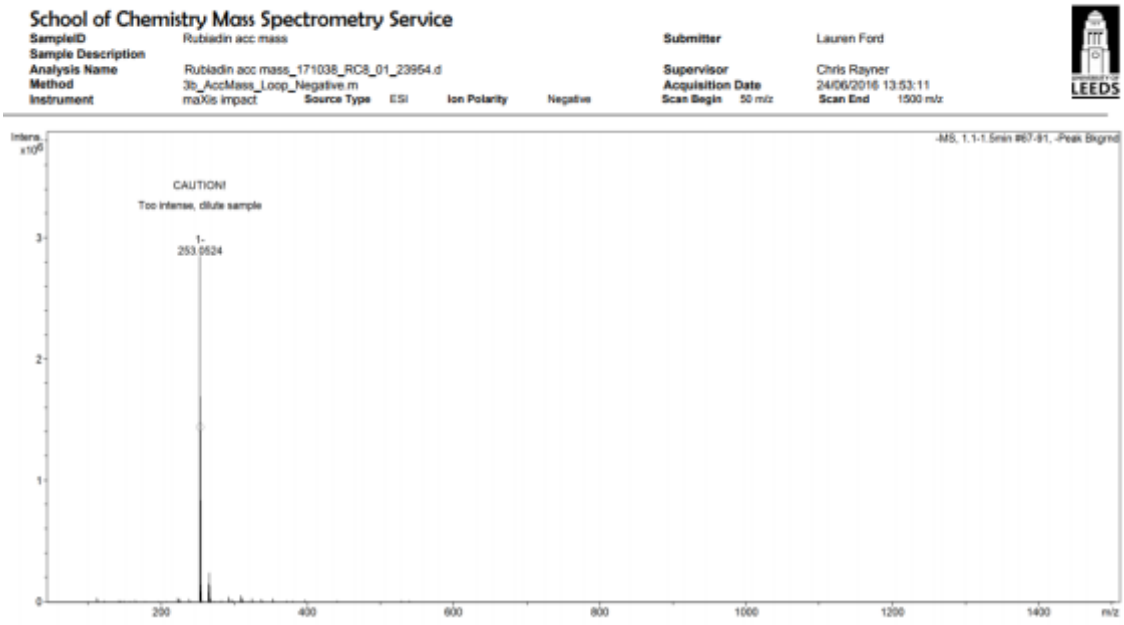


Figure 6.11. High resolution mass spectrum of rubiadin.

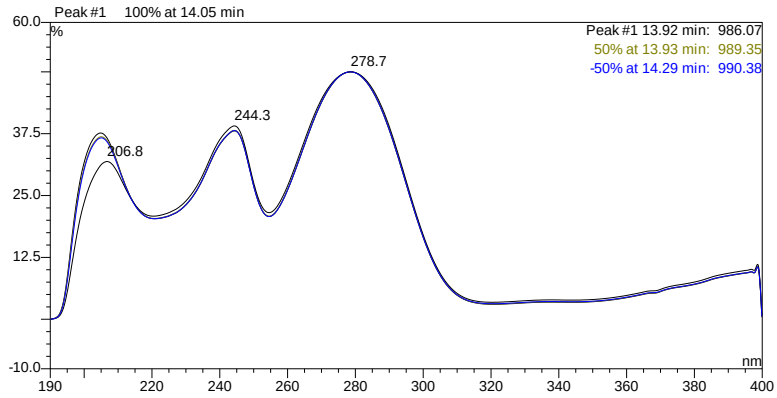


Figure 6.12. UV/vis spectrum of rubiadin.

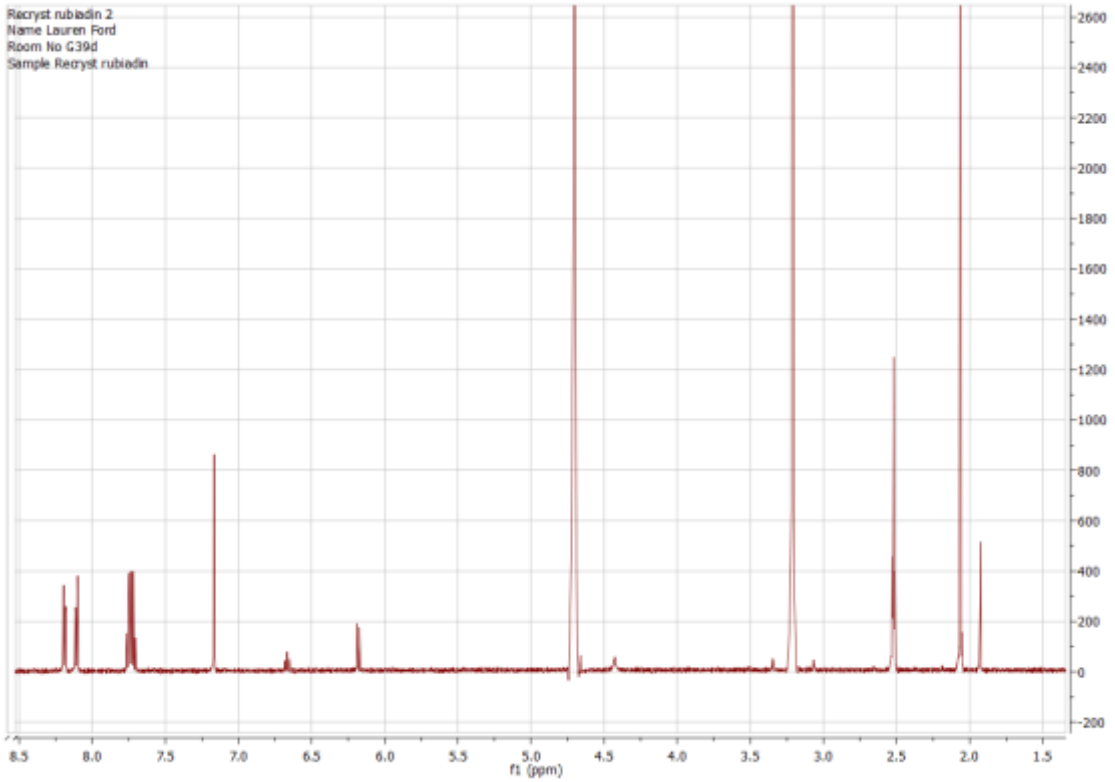


Figure 6.13. ^{1}H NMR spectra of rubiadin.

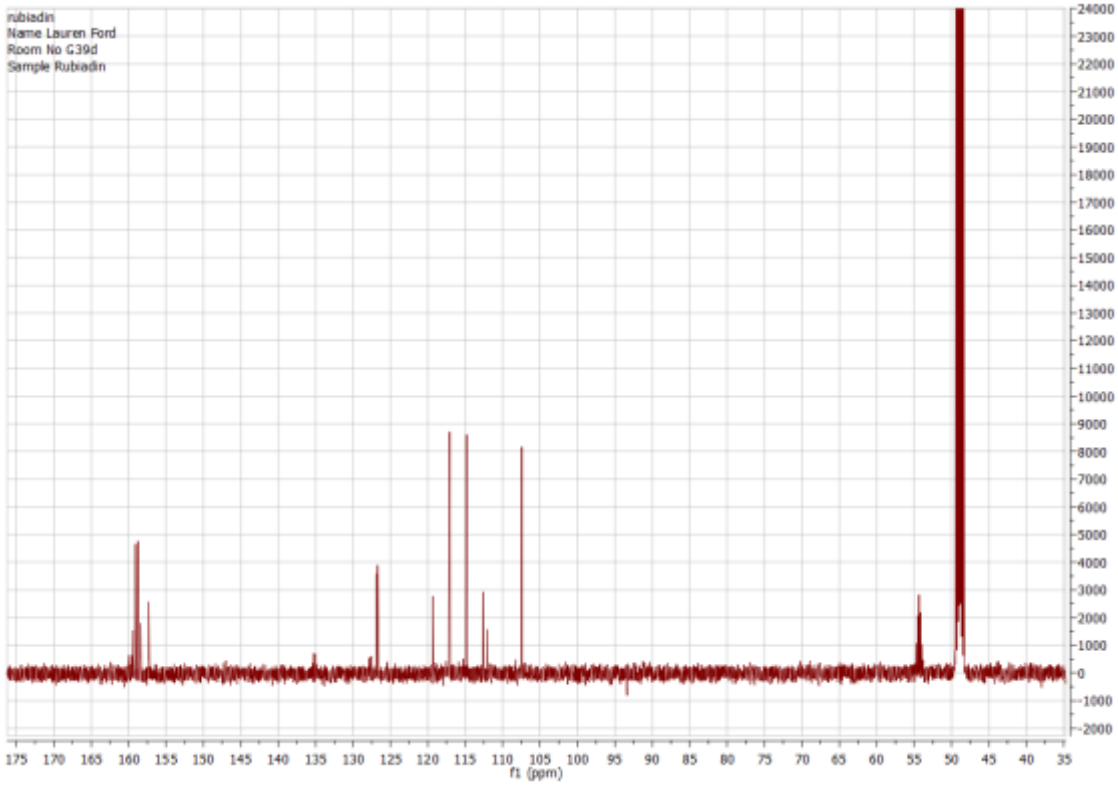


Figure 6.14.^{13}C NMR spectra of rubiadin.

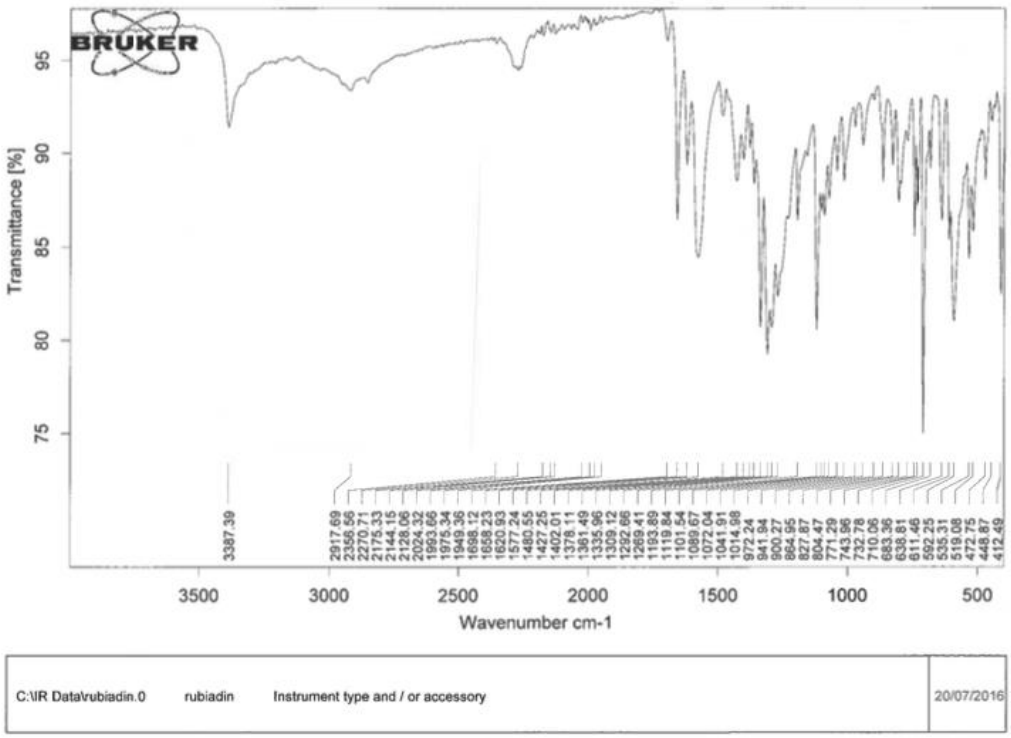


Figure 6.15. IR spectrum of synthesised rubiadin.

6.7.4 NMR of Pseudopurpurin

The ^{1}H NMR of pseudopurpurin donated by the British museum was obtained to test the purity of the sample for consequential use in dye isotherm studies (Figure 6.16). The NMR shows a spectrum corresponding to that of pseudopurpurin and this standard was therefore used hereafter as a standard of pseudopurpurin and to study the dyeing mechanisms of pseudopurpurin in isotherm experiments.

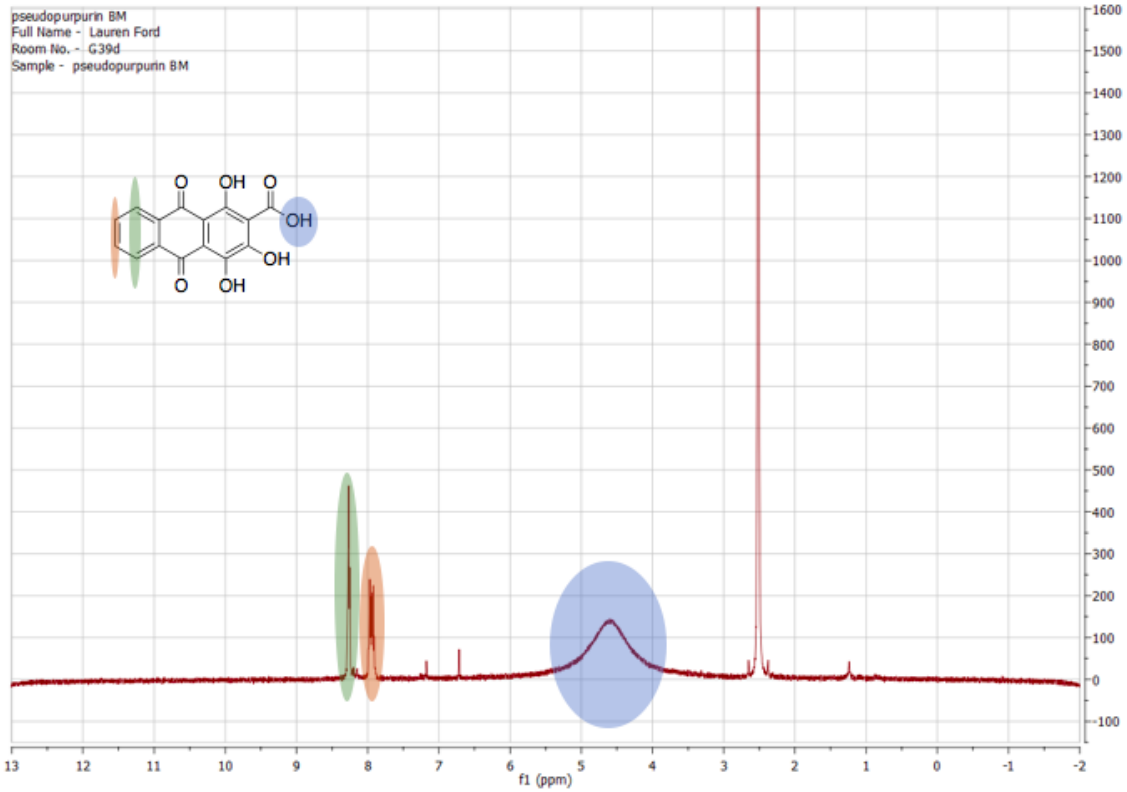


Figure 6.16. ^{1}H NMR of pseudopurpurin from the British museum in d_6 deuterated DMSO. Hydrogen peaks detected by NMR are highlighted.

6.8 Degradation experiments of lucidin

6.8.1 With methanol

Pure lucidin (2 mg) from experimental section 6.7.2 was dissolved in 1:1:2, v/v/v, methanol: water: HCl (0.5 ml) and heated to 100°C for 15 minutes. After this an aliquot was taken for LC-MS and HPLC analysis. The remaining reaction mixture was then evaporated to dryness and re-dissolved in deuterated acetone for NMR analysis. Deuterated DMSO was also tested as a solvent for NMR analysis but acetone provided better solubility. Deuterated methanol could not be used as it would interfere with the results forming the methyl ether adduct.

6.8.2 Without methanol

Pure lucidin (2 mg) from experimental section 6.7.2 was dissolved in 1:1, v/v, water: HCl (0.5 ml) and heated to 100°C for 15 minutes. After this an aliquot was taken for LC-MS and HPLC analysis. The remaining reaction mixture was then evaporated to dryness and re-dissolved in deuterated acetone for NMR analysis. Deuterated DMSO was also tested as a solvent for NMR analysis but acetone provided better solubility. Deuterated methanol could not be used as it would interfere with the results.

6.9 Dyeing isotherms

Calibration curves of each dye compound were plotted using the λ_{max} absorbance value of 5 different known concentrations. In all cases a good fit was found achieving an r^2 value > 0.95. The gradient of each calibration was then used to calculate the concentration of unknown dye bath concentrations after dyeing in order to measure uptake onto the wool. Each piece of wool was dyed for 30 minutes, this was based on a previous study dyeing mordanted wool with Iranian madder (Figure 6.17). Although this study was done on Iranian madder and not the individual dye compounds being studied herein it was assumed that the kinetics would be similar. The compounds used in this isotherm study were limited, only small quantities of pseudopurpurin donated by the British museum were available. Further work could establish that 30 minutes is efficient to establish dyeing equilibrium for the individual compounds.

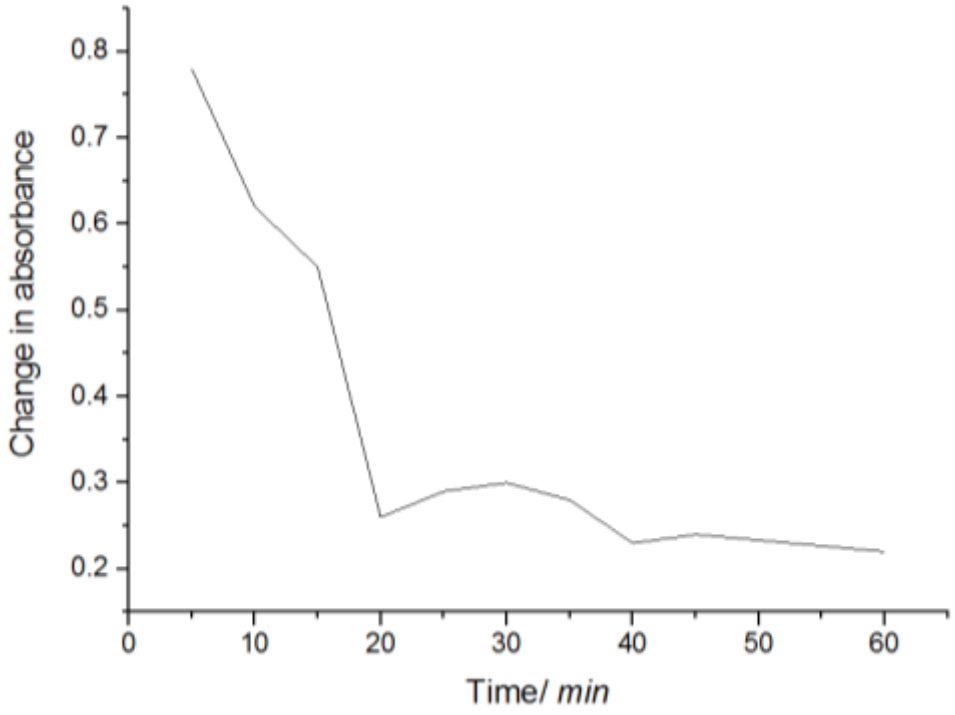


Figure 6.17. Kinetic study of dyeing Iranian madder onto mordanted wool. Absorbance values were compared to the initial dye bath and the change in absorbance recorded by subtracting the absorbance of the aliquot from the absorbance of the initial dyebath absorbance. All samples were diluted by a factor of 4 and absorbance was measured at 510 nm.

6.9.1 Pseudopurpurin Isotherm

Pseudopurpurin (0.1 g, 0.3 mmol) was dissolved in a 1:1 methanol: water solution (v/v, 200 ml). This stock solution was then diluted to 5 %, 10 %, 20 %, 30 % and 40 % using deionised water, in each case making the dye solution up to 100ml. These different dilutions were then used to dye mordanted wool, the wool was pre-mordanted using the procedure from section 6.6.6. The mordanted wool (1 g) was immersed in each of the dye baths and heated with stirring to 90°C for 30 minutes. An aliquot of the dye bath was taken before and after dyeing and the visible absorbance was measured. The λ_{max} of the compound dyeing onto the wool was used to calculate the decrease in concentration before and after dyeing, for pseudopurpurin the absorbance values were taken at 530 nm. Using the data obtained from the absorbance of the dye bath solutions each isotherm was plotted and the fitting compared to indicate the most probable mechanism

of adsorption. Calibration curve used for the fitting of the isotherm is displayed in Figure 6.18.

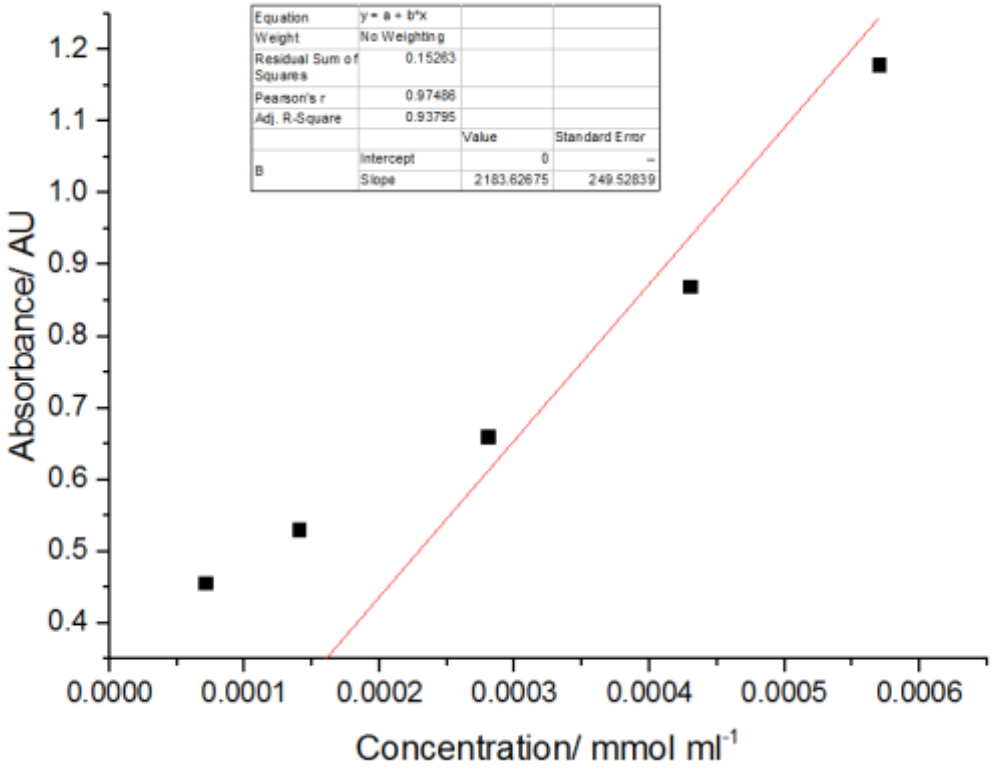


Figure 6.18. Calibration curve of pseudopurpurin at five concentrations measured at 530 nm displaying an R^2 value of 0.97.

6.9.2 Alizarin Isotherm

Alizarin was dissolved in water liquor fibre ratio 500:1. Different concentrations were prepared: 1, 2, 3, 4, & 5% omf. These different concentrations were then used to dye mordanted wool, the wool was pre-mordanted using the procedure from section 6.6.6. The mordanted wool was immersed in each of the dye baths and heated with stirring to 90°C for 30 minutes. An aliquot of the dye bath was taken before and after dyeing and the visible absorbance was measured. The λ_{max} of the compound dyeing onto the wool was used to calculate the drop in concentration before and after dyeing, for alizarin the absorbance values were taken at 430 nm. Using the data obtained from the absorbance of the dye bath solutions each isotherm was plotted and the fitting compared to indicate

the most probable mechanism of adsorption. Calibration curve used for the fitting of the isotherm is displayed in Figure 6.19.

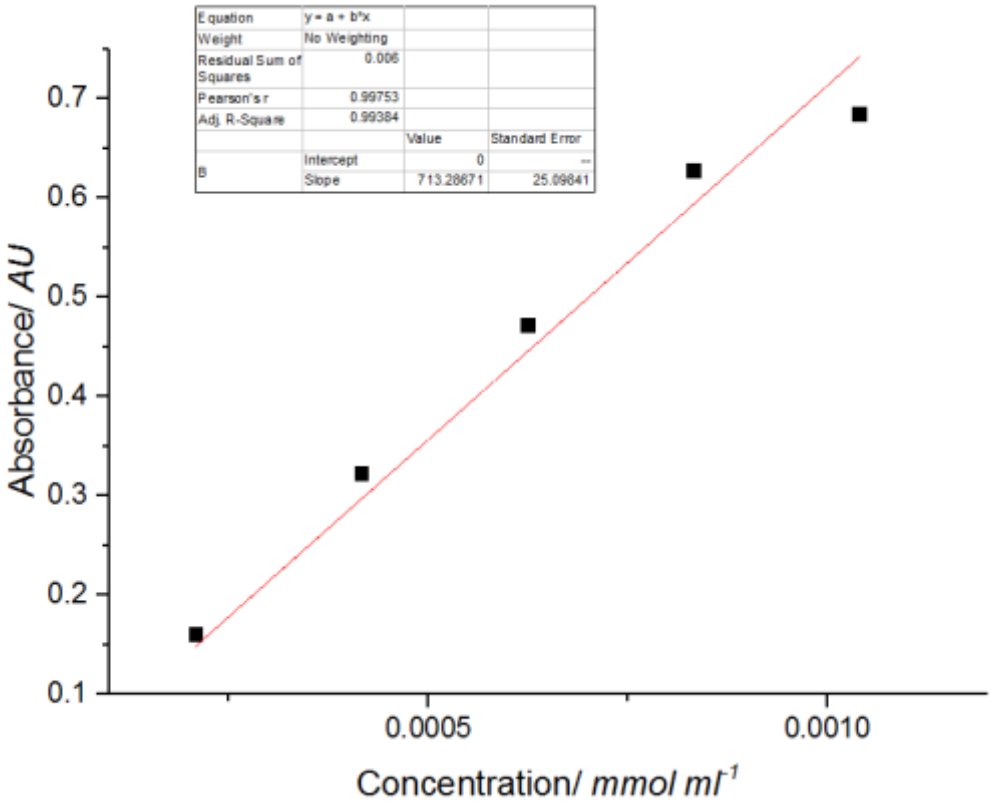


Figure 6.19. Calibration curve of alizarin at five concentrations measured at 430 nm showing an R^2 value of 0.99.

6.9.3 Ruberythric Acid Isotherm

The remaining standard bought from Apin chemicals was purified by SPE using the same procedure as in section 6.3.6, yield= 0.04 g. Ruberythric acid was dissolved in water liquor fibre ratio 500:1. Different concentrations were prepared: 1, 2, 3, 4, & 5% omf. These different concentrations were then used to dye mordanted wool. The wool was pre-mordanted using the procedure from section 6.6.6. In each dye bath the mordanted wool was immersed in the dye bath and heated with stirring to 90°C for 30 minutes. An aliquot of the dye bath was taken before and after dyeing and the visible absorbance was measured. The λ_{max} of the compound dyeing onto the wool was used to calculate the drop in concentration before and after dyeing, for the ruberythric acid: lucidin primeveroside mixture the absorbance values were taken at 520 nm. Using the data

obtained from the absorbance of the dye bath solutions each isotherm was plotted and the fitting compared to indicate the most probable mechanism of adsorption. Calibration curve used for the fitting of the isotherm is displayed in Figure 6.20.

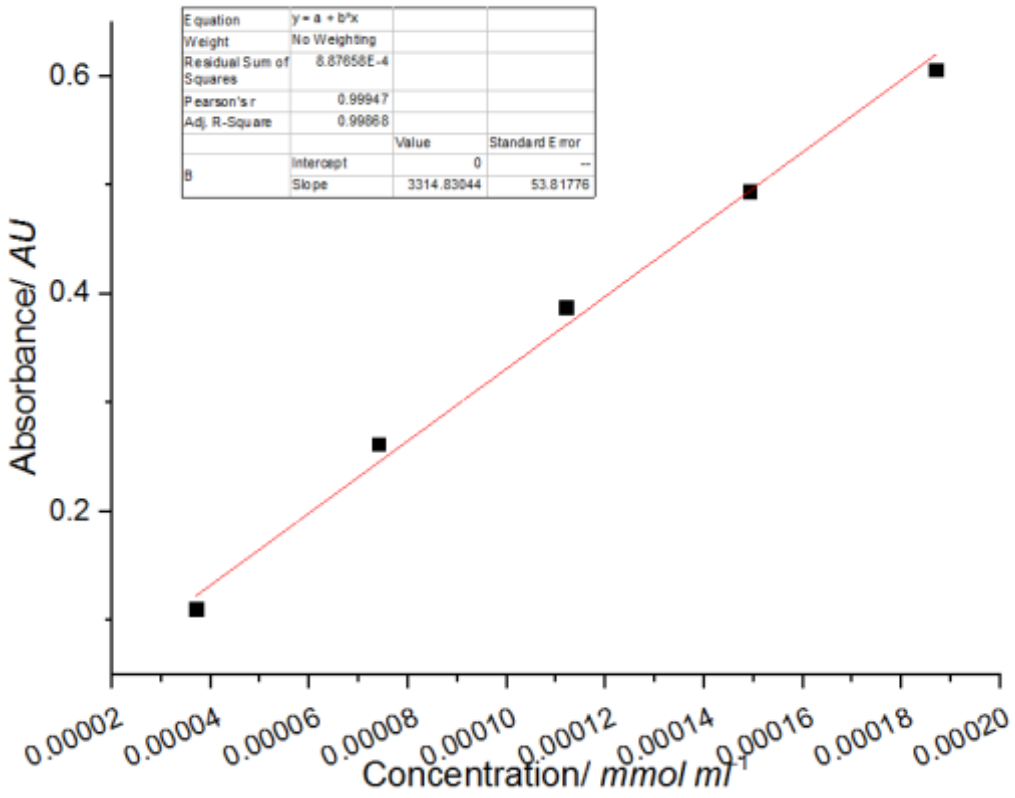


Figure 6.20. Calibration curve of the glycosides measured at 520 nm and of five concentrations displaying a R^2 value of 0.99.

6.10 Characterisation of cinnamic acids from chamomile

Preparative HPLC was carried out by Jose Villa Rodriguez in the department of Food science as follows:

6.10.1 Detection and quantification of major polyphenols in German chamomile

Initial polyphenol profiling of GCE was performed on an HPLC-DAD system (1200 series; Agilent Technologies, Berkshire, UK) equipped with a Kinetex C18 analytical column (150 x 2.1 mm I.D., 2.6 µm; Phenomenex, Cheshire, UK) maintained at 35 °C. GC suspension was prepared as previously described at 1 mg cm^{-3} and 10 µL was injected

and separated using a 41 min gradient of premixed 5 % acetonitrile in water (5:95, v/v) (A) and premixed 5% water in acetonitrile (5:95, v/v) (B), both modified with 0.1 % formic acid at 0.25 cm^3 min^{-1}. The gradient utilized started at 0 % solvent B and increased to 10 % (5 min), 25 % (10 min), 35 % (20 min), 50 % (25 min), and held at a plateau up to 30 min. The gradient was increased 100 % at 30.5 min and returned to 0 % solvent B over 5.5 min before initial starting conditions were resumed for a 6 min column re-equilibration. Online detection was carried out at 320 nm and used for quantification and presentation. The detection and quantitative analysis of the targeted compounds were conducted by comparison with those of authentic standards and using a 5-point linear calibration curve, respectively. The limit of detection and quantification were calculated for the individual polyphenols.

6.10.2 Semi-preparative isolation of methoxycinnamic acid derivatives

Two methoxycinnamic acid derivatives were isolated using an ÄKTA Purifier System (GE Healthcare, Fairfield, CT, USA) controlled by a PC running GE Unicorn software (5.11) equipped with an Gemini C6 phenyl column (250 x 10 mm I.D., 5 µm; Phenomenex, Cheshire, UK), UV detector UV-900, pH/conductivity detector pH/C-900, fraction collector Frac-950, gradient mixer, and pump P-900. GC suspension (150 mg cm^{-3}) was loaded manually (1 cm^3) using a syringe through a sample loop of 1 cm^3 and eluted using water containing 0.1% TFA (solvent A) and methanol (Solvent B) at a flow rate of 2.3 cm^3 min^{-1} as follows: 0-12.8 min linear gradient to 24 % B; 12.8-25.6 min isocratic at 24 % B; 25.6-111 min linear gradient to 100 % B; 111-123.8 min isocratic at 100 %B, 123.8-125 min linear gradient to 5 % B: 125-150.6 min isocratic at 5 %B. The elution was followed at 320 nm and fractions containing the separated compounds (Figure 6.21) were collected and analysed for purity by LC-MS (Figure 6.21). Multiple semi-preparative separations were done and the fractions for each peak combined, freeze-dried, and stored at -20 °C.

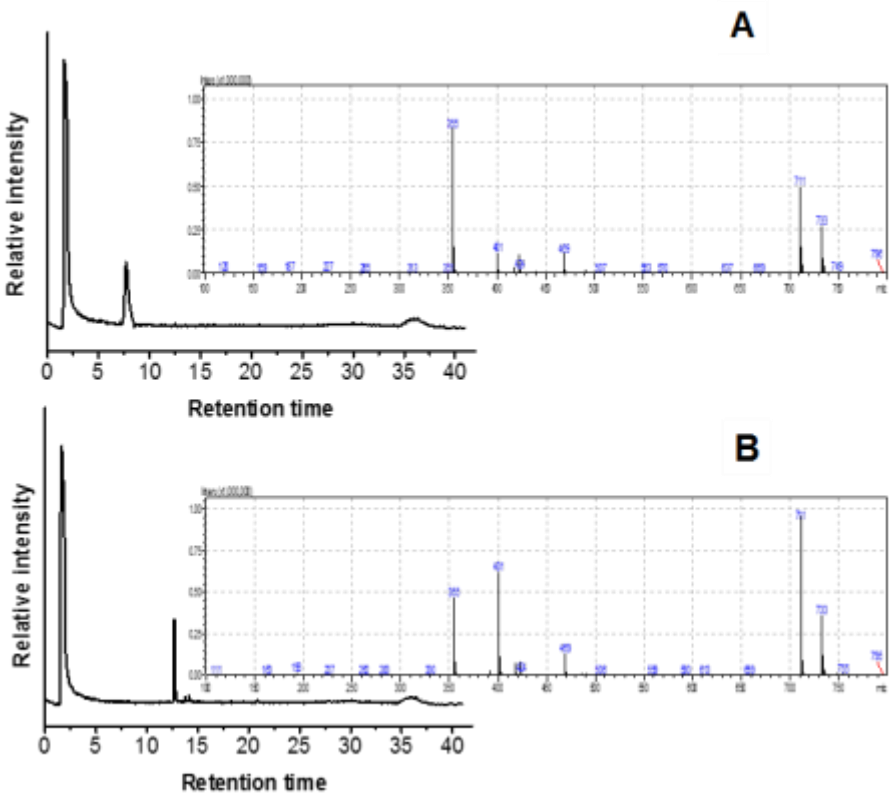
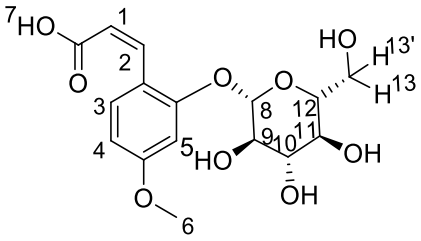


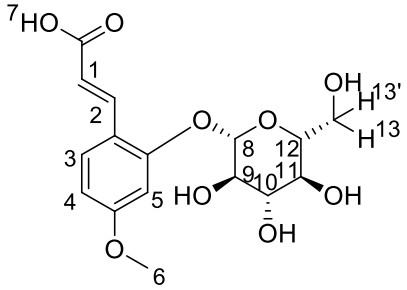
Figure 6.21. ESI-MS data obtained for the A: (*Z*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid and B: (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid

Purified samples were then analysed by ^{1}H and ^{13}C NMR.



(*Z*)-2-D-Glucopyranosyloxy-4-methoxycinnamic acid present as a mixture of E and Z in a 1:3 ratio, E peaks eliminated and Z peaks picked at: ^{1}H NMR (501 MHz, DMSO-d6) δ 7.70 (d, *J*= 8.5 Hz, 1H, H3), 7.19 (d, *J* = 12.5 Hz, 1H, *cis*-alkene, H2), 6.74 (d, *J* = 2.5 Hz, 1H, H5), 6.56 (dd, *J* = 8.5, 2.5 Hz, 1H, H4), 5.78 (d, *J* = 12.5 Hz, 1H, *cis*-alkene, H1), 4.85 (d, *J* = 7.5 Hz, 1H, anomeric proton, H8), 3.77 (s, 3H, H6), 3.72 (broad d, *J*= 11.7 Hz, 1H, H13), 3.47 (broad dd, *J*= 11.7, 6.0 Hz, 1H, H13'), 3.38 (ddd, *J*= 10.0, 6.2, 1.6 Hz, 1H, H12), 3.34-3.29 (m, 2H, H9 & H10)*, 3.16 (apparent t, *J*= 8.8 Hz, 1H, H11). ^{13}C

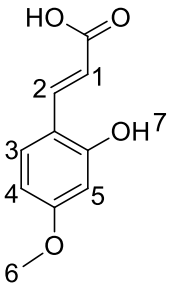
NMR (126 MHz, DMSO-d6) δ 167.7, 161.0, 160.7, 156.7, 136.32, 131.1, 117.53, 116.73, 101.11, 100.5, 77.24, 76.86, 73.27, 69.81, 60.72, 55.33. *Overlayed by strong signal from water. ESI-MS: *m/z* 355.1035 $[M-H]^-$.



Analysis of pure (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic acid: ^{1}H NMR (501 MHz, DMSO-d6) δ 12.00 (s, 1H, H7), 7.82 (d, *J* = 16.2 Hz, 1H, *trans*-alkene, H2), 7.62 (d, *J* = 8.7 Hz, 1H, H3), 6.76 (d, *J* = 2.4 Hz, 1H, H5), 6.62 (dd, *J* = 8.6, 2.4 Hz, 1H, H4), 6.39 (d, *J* = 16.2 Hz, 1H, *trans*-alkene, H1), 5.16 (broad s, 1H (OH)), 4.98 (d, *J* =7.5 Hz, 1H, anomeric proton, H8), 4.89 (broad s, 2H (OH)), 4.58 (broad s, 1H, (OH)), 3.78 (s, 3H, H6), 3.72 (broad d, *J*= 11.7 Hz, 1H, H13), 3.47 (broad dd, *J*= 11.7, 6.0 Hz, 1H, H13'), 3.38 (ddd, *J*= 10.0, 6.2, 1.6 Hz, 1H, H12), 3.34-3.29 (m, 2H, H9 & H10)*, 3.16 (apparent t, *J*= 8.8 Hz, 1H, H11). ^{13}C NMR (126 MHz, DMSO-d6) δ 168.08, 162.08, 157.07, 138.76, 129.32, 116.73, 116.05, 108.03, 101.11, 100.02, 77.24, 76.86, 73.27, 69.81, 60.72, 55.33. *Overlayed by strong signal from water. ESI-MS: *m/z* 355.1035 $[M-H]^-$. Key NOESY enhancements shown in Figure 3.19. The hydrolysed standard of the *(E)-2-hydroxy-4-methoxycinnamic acid* were prepared as follows for further identification with hydrolysis products of the natural product extracted from the plant.

Synthesis of (E)-2-hydroxy-4-methoxycinnamic acid 7-Methoxy coumarin (0.44 g, 2.5 mmol) was dissolved in an aqueous solution of 0.5 M NaOH (20 cm^3) and stirred for 4 hours at room temperature. Aliquots were taken and showed full conversion to the *trans* carboxylate by NMR in D_2O. The solution was then acidified with 1 M aqueous HCl and extracted with dichloromethane (3 × 10 cm^3). The solvent was then evaporated and eluted with ethyl acetate on a flash silica column. This yielded the (*E*)-2-hydroxy-4-

methoxycinnamic acid (*E*-MCA) (32 mg, 0.165 mmol, 6.6 % yield). The low yield was due to the ring closing to reform the coumarin in acidic conditions.



^{1}H NMR (500 MHz, Acetone) δ 9.25 – 9.21 (broad s, 1H, H7), 7.95 (d, *J* = 16.1 Hz, 1H, H2), 7.54 (d, *J* = 8.5 Hz, 1H, H3), 6.40 (d, *J*= 2.2 Hz, 1H, H5), 6.37 (dd, J= 2.2, 8.5 Hz, 1H, H4) 6.33 (d, J= 16.1 Hz, 1H, H1), 3.66 (s, 3H, H6). The synthesised compound was analysed by HPLC-DAD as described in section 2.3. ESI-MS: *m/z* 193.06 $[M-H]^-$

7 Conclusions and Future Work

This thesis provides full chromatographic profiling of compounds in dye plants which provide red and yellow colorants upon extraction from the plant materials. *Rubia tinctorum* grown in three different regions was studied for its chemical composition when extracted with solvents of differing polarity; ethyl acetate, ethanol and water. The chemical compositions of the compounds identified in these solvents were compared to the chemical composition after SPE using an amberlite resin. SPE provided an extract with a similar chemical composition as a water extract but a much easier to handle powder was obtained due to the hygroscopic properties of freeze dried water extracts. The free sugars were successfully removed from the extract as observed by ^{1}H NMR. The crystal structure was obtained of ruberythric acid which allowed for the full elucidation of this compound for the first time; the full ^{1}H NMR and ^{13}C NMR data were also obtained for this compound. The crystal structure herein was shown to display extensive hydrogen bonding in the sugar moieties and an intramolecular hydrogen bond between the carbonyl and the adjacent hydroxyl.

This thesis also studied the yellow dyes for their flavonoid chemical composition. The HPLC profiles of *Solidago virgaure* (golden rod), *Anthemis tinctoria* (dyer's chamomile) and *Reseda luteola* (weld) were obtained as references of their chromatographic profile. Again, the SPE of the dye plants were compared to solvent extraction methods using water and ethanol. Amberlite XAD 7HD was used as the SPE resin and a loss of glycosidic compounds was observed in some cases. Due to the efforts to characterise compounds in these extracts herein a collaborative study with Food Science was set up and lead to the full elucidation by 1D and 2D ^{1}H NMR of two previously unknown peaks in the chromatograms of chamomile. These peaks were present in both dyers chamomile

(*Anthemis tinctoria*) and German chamomile (*Matricaria chamomilla*). These two compounds are fully characterised herein and all NMR data displayed.

The dyeing characteristics of the compounds from madder root were studied herein. The extracts of Iranian, Turkish and English madder were compared by HPLC before and after dyeing to observe the chemical compositional changes upon adsorption to wool. The change in the relative peak areas was recorded; the peak area for all compounds decreased after dyeing showing adsorption onto the wool. The response factors of compounds available commercially were recorded and therefore the molar concentration relative to the peak area could be calculated. This displayed that although a smaller change in peak area for the glycosides was observed a larger molar concentration was adsorbed onto the wool when compared to the change in peak area of alizarin and the relative molar concentration adsorbed. Herein the adsorption isotherms were plotted of the separated components of *Rubia tintorum* to observe the effect different functional groups have on the dyeing capabilities. The compounds studied were ruberythric acid (glycosidic), pseudopurpurin (carboxylic) and alizarin (hydroxyl). The isotherms suggested that multilayers of dye adsorption occurred onto mordanted wool. All three compounds tested displayed correlation with the Freundlich isotherm fitting however a slightly better fitting was achieved for ruberythric acid when fitted to the Tempkin isotherm.

A novel back extraction was developed for the analysis of dyestuff in historical textiles by HPLC utilising a glucose solution. This glucose solution provided superior results for the detection of glycosides when compared to strong and weak acidic back extractions. However, for the analysis of aglycons detection is poorer than other methods such as DMSO/oxalic acid or HCl/ methanol/ water. The glucose method was also compared to an extraction technique currently being used in cultural heritage sectors using DMSO/ oxalic acid.[14] Whist the glucose method provided better results in detecting the glycosidic compounds in the dye extracts, the DMSO/ oxalic acid method was better for detecting

aglycon compounds and had a higher signal to noise ratio. The compounds which cannot usually be referenced due to their unavailability commercially were synthesised herein. By synthesising these compounds, a full study into the breakdown products which occur when lucidin is placed in strong acidic conditions could be undertaken. Herein it is shown that lucidin is broken down into xanthopurpurin through a proposed retro aldol reaction, this degradation pathway is studied by HPLC and ^{1}H NMR.

7.1 Future Work

The subject topics studied in this PhD project has led to new unanswered questions. Herein the samples of *Rubia tinctorum* grown in different regions provide a different chemical composition depending on where they are grown. It would be interesting to study more samples from each of these regions to gain more robust evidence of these chemical changes being due locational growing conditions. For this study, the samples would need to be grown in different locations but undergo the same drying and storage conditions. The samples would also all have to be harvested at the same time to ensure that the roots are of the same age, the time of harvested must also be consistent. Whilst it is understood that it is difficult to obtain samples which meet all of this criterion it would be an interesting study none the less. The effects of the different drying conditions on the chemical compositions would also be very interesting. Dyeing studies between fresh madder and dried madder could also be very insightful. HPLC could be utilised to see if there is a different chemical composition between the fresh and dried material. Also the absolute effects different drying conditions have on the chemical composition of the extracted dye plants could be done. For example, freeze drying of madder could be compared to heated drying.

The use of solid phase extraction was tested to see if an extract richer in the colorant compounds could be achieved using this technique. Herein only one SPE resin was tested for the partial purification of these compounds. Future work could analyse the

effect of different SPE resins to see if there is any effect of the different resins on the retention of the colorant compounds in the plant extracts or if a different resin could separate one class of compounds, for example; the glycosides.

A new column could be used to better separate the glycosidic compounds on the chromatogram. Whilst method development was carried out during the course of this PhD, a longer column could be tested for future work to see if they can be separated by changing this parameter. This could also help to separate the glycosides extracted from the yellow dye plants. Separation and full characterisation of all of the chemical components of the yellow dye plants or comparison with reference samples which have been full identified by NMR would provide more conclusive data on the chemical composition of these plant extracts. Synthesis of the compounds not commercially available could be carried out to confirm the presence of these compounds in a chromatogram.

The dyeing studies herein have shown that there is a decrease in all peaks in the chromatogram after dyeing, and therefore they are assumed to be being adsorbed onto the wool surface. However more detailed studies could be done to ensure that the decrease in the peak area is not due to degradation of the compounds. For example, the dye solution could be exposed to the conditions used for dyeing; in solution, at the same temperature and HPLC used to detect any changes in the peak area.

One other main topic of study which was not achieved during the course of this PhD is the effect of glycosylation on the degradation of these compounds. It would be interesting to do a conclusive study to see if the glycosylation of ruberythric acid provided less degradation upon artificial ageing when compared to the aglycon alizarin.

Herein it is shown that the glucose extraction is favourable for extracting the glycosidic compounds for analysis to see if they are present in the dyed textile. This back extraction method now needs to be applied to different species of *Rubia spp*. It will be interesting to see if this method is still suitable when there are different glycosylated

species present. This will be especially interesting when the glycosylated species are alkylated on the sugar moiety as this could affect the packing of the dye on the textile and the ability of the sugars to disrupt this packing.

It would be important to consider the effects conditioning of the wool would have on the samples tested. Herein there was no conditioning of the wool carried out which means that in more humid environments the wool could seem heavier due to the water content in the wool sample. It is important to eliminate this variable to ensure the dye liquor ratios are correct. This could make a big difference in the samples used in museum artefacts where very small sample sizes are used. Future work could look at the effects conditioning of the wool has on these studies as this is not something which is routinely done in artefact analysis.

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