RED, REDDER, MADDER

Analysis and isolation of anthraquinones from madder roots (*Rubia tinctorum)*

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Proefschrift
ter verkrijging van de graad van doctor
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Nationale driekleur

Van oorsprong was onze Prinsenvlag oranje-wit-blauw. Het oranje was een combinatie van meekraprood en curcumageel. Dit geel was echter niet licht-echt. Hoewel dus een vlag met een oranje band was voorgeschreven, gaf de praktijk, door het verbleken van het geel, een rode te zien.

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NN08201,3051

De door Abma uitgevoerde milieu levenscyclusanalyse van synthetische alizarine versus natuurlijke alizarine is vanwege de geringe beschikbaarheid aan informatie niet juist.

- *A.-J. Abma. De groene kant van rood. Milieugerichte levenscyclusanalyse van rode textielkleurstoffen: alizarine uit meekrap en synthetische kleurstoffen, Rapport C86, Chemiewinkel Rijksuniversiteit Groningen, 1998, 82p.*
- *H. W. J. M. Sengers, J. G. Meeusen. Review 'De groene kant van rood'-milieugerichte levenscyslusanalyse van rode textielkleurstoffen: alizarine uit meekrap en synthetische kleurstoffen. vertrouwelijk rapport, LEI, Den Haag, 2001, 32p.*
- *Ch. E. Dutilh. Milieuoverwegingen bij biologische produktie van alizarine. vertrouwelijk rapport, Amsterdam, 2001, 4p.*

In tegenstelling tot wat de auteurs beweren heeft de hydrolyse van ruberythrinezuur tot alizarine niet onder invloed van het enzym β-glucosidase plaats gevonden.

- *T. Masawaki, M. Taya, S. Tone. Selective solvent extraction of ruberythric acid from madder roots, and subsequent hydrolysis with β-glucosidase. Journal of Fermentation and Bioengineering, 1996, 81, 567-569.*
- *Dit proefschrift.*

Het gebruik van het woord buffer voor een oplossing met alleen een zuur of een base, die wordt toegevoegd ter bevordering van de ionisatie tijdens HPLC-MS, geeft bij de lezer verwarring over de aard van de toegevoegde oplossing.

- *M. M. Nindi, B. V. Kgarebe, J.-L. Wolfender, B. M. Abegaz. Electrospray liquid chromatography-mass spectrometry of the leaf extract of Rhamnus prinoides. Phytochemical Analysis, 1999, 10, 69-75.*

Het verbieden van planten, die voorheen werden gebruikt als kruidenpreparaten, op basis van toxiciteit of mutageniciteit van een bepaalde gevonden component is niet terecht.

- *Staatsblad van het Koninkrijk der Nederlanden. 56 Besluit van 19 januari 2001, houdende vaststelling van het warenwetbesluit kruidenpreparaten. 2001, 1-12*
- *J. Westendorf, H. Marquardt, B. Poginsky, M. Dominiak, J. Schmidt, Genotoxicity of naturally occurring hydroxyanthraquinones. Mutation Res., 1990, 240, 1-12.*
- *E. Madaus withdraw Rubia Teep® tablets from market in 1981. 1996, personal mail communication.*
- *Dit proefschrift.*

Het geven van een letterlijk Nederlandse vertaling van een Engelstalige samenvatting in een proefschrift is volkomen nutteloos.

- *Een groot deel van de proefschriften geschreven door promovendi met de Nederlandse notionaliteit.*

Het weer wordt niet mede mogelijk gemaakt door Miss Etam.

- *RTL 4, 2 september 2001*

De toename van de bewegingsruimte voor dieren in een dierentuin is de afgelopen decennia omgekeerd evenredig toegenomen in vergelijking met de ruimteverandering voor dieren in de bioindustrie.

Stellingen behorend bij het proefschrift: Red, Redder, Madder. Analysis and isolation of anthraquinones from madder roots (*Rubia tinctorum*).

Wageningen, 4 september 2001 G.C.H. Derksen

Voor Opa Steuten

Verfrecept

Neem in de buitenlucht gedroogde meekrapwortelen en maal deze fijn in een koffiemolen. Neem 60 dl handwarm water en voeg al roerend met een garde 3 eetlepels (20 g) van de gemalen meekrap toe. Roer dit geheel 30 minuten en zorg ervoor dat de oplossing handwarm blijft. Na 30 minuten kan het geheel worden verwarmd tot koken. Laat het geheel één uur voorzichtig koken, met de deksel op de pan. Giet daarna de oplossing zo heet mogelijk door een pantysok en vang de vloeistof op. Neem vervolgens een theelepel (5g) aluin en los dit op in 50 dl handwarm water. Voeg 20 gram wol toe en verwarm geheel 30 minuten tot net niet koken, laat daarna afkoelen. Voeg de wol toe aan de meekrapvloeistof (vul aan tot 60 dl met water) en verwarm het geheel 1 uur tot net niet koken (90°C). Na afkoelen kan de wol eruit worden gehaald en drie keer goed gespoeld met koud water en gedroogd.

Contents

Chapter 1

General introduction*

1.1 History, taxonomy and cultivation

1.1.1 Name, botany and occurrence of Rubia tinctorum

The Roman writer Plinius already used the name Rubia for the madder plant, because of the red colour of the roots. Tinctorum is derived from the Latin word for dyeing[1]. The genus Rubia belongs to the tribe Rubieae. The genera Galium and Asperula belong also to the Rubieae. The tribe is part of the subfamily Rubioideae. Other genera within the Rubioideae are Coffea, Cephaelis and Morinda. As well as the subfamily Cinchonoideae, the subfamily Rubioideae belongs to the Rubiaceae. The Rubiaceae are part of the order of the Gentianales (see figure 1.1)[2].

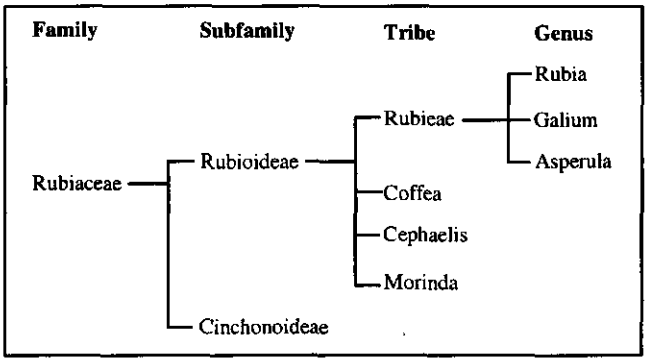

***Figure 1.1** Botanical position of the genus Rubia tinctorum within the Rubiaceae.*

* This chapter is based on the review:
Goverdina C.H. Derksen, Teris A. van Beek, *Rubia tinctorum* L. Atta-ur-Rahman (ed.), In *Studies in Natural Product Chemistry*. Elsevier, Amsterdam. Vol. 26, in press.

The stalks of the madder plant are so weak that they often lie along the ground, preventing the plant from rising to its maximum height of 60-100 cm. With little spines at the edges and midrib the plant can cling to other plants. The flower-shoots spring from the joints in pairs, the loose spikes of yellow, starry flowers blooming only in the second or third year, in June. The thick fleshy stalks that compose the perennial are about 0.6 cm thick. From the rhizome roots run under the surface of the soil for some distance, sending up shoots (figure 1.2). The root is covered with a small blackish bark, the husk. Beneath this layer the root consists of a red coloured layer, the intermediate layer. The innermost layer of the root consists of large vascular bundle cells, the pith. These cells are surrounded by a small concentration of red dye. The highest concentration of red dye is found in the intermediate layer, less in the pith and almost nothing in the husk (figure 1.3)[3-6]. It is not clear how the red dye is stored in the intermediate layer. It is probably stored in compartments outside the cells, which are possibly divided by a pectin layer[6].

Rubia tinctorum is native in Southern and Southeast Europe, in the Mediterranean area, Asia Minor and in the Caucasus. Nowadays the plant also grows in China and Japan, up to the Malaysian Archipelago, in the Western part of North America, in Mexico and South America. In earlier days madder was cultivated in Central and Western Europe. Nowadays most of the plants are found in the wild[1].

1.1.2 History

Madder roots have been used for dyeing textiles in many parts of the world since ancient times. One of the eldest known textiles dyed with madder is a belt found in Tutankhamun's grave and dates back to 1350 BC. During the times of the Pharaohs in Egypt neither alum nor another mordant was used during dyeing. In the surroundings of the Dead Sea a wool pouch dyed with madder from 135 AD was found. In China some archaeological grave treasures revealed the use of madder for dyeing fabrics. The ancient Greeks used madder for dyeing and the main red dye in the Roman Empire was obtained from madder. Around the time of the birth of Christ the quality of the dyed product improved by using a mordant. The dyeing knowledge got lost due to the migration of many nations after the decline of the Roman Empire. Only in the Byzantine Empire and in the East the dyeing technique was still practised. During the unrest between 600 and 900 AD a lot of dyers roamed off to Italy. From Italy they wandered to France, the Rhineland and as far as England. During the Middle Ages Charlemagne stimulated the cultivation of madder. He inserted the plant on the list 'Capitulare de villis'. On this list all the plants occurred which were allowed to be cultivated on land owned by the state (France, Belgium, Holland and Italy). The Flemish were very experienced

***Figure 1.2** Rubia tinctorum L. (Madder).*

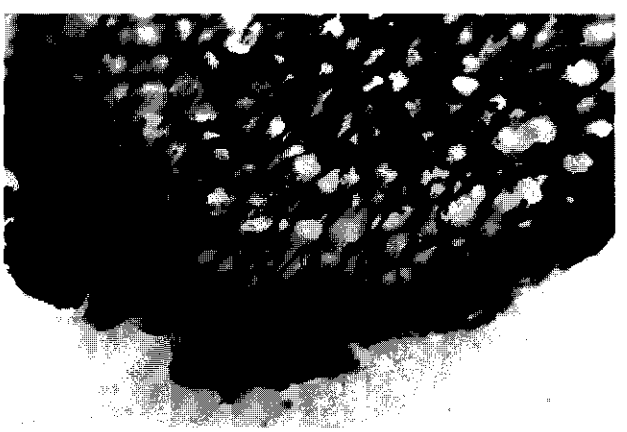

***Figure 1.3** Microscopic picture of a cross section of madder root[6]. The highest concentration of red dye is found in the intermediate layer, less in the pith and almost nothing in the husk.*

in the dyeing of textiles. They used just three plants as dye sources, *Reseda luteola* ('Wau', yellow), *Rubia tinctorum* (red) and *Isatis tinctoria* ('Waid', blue). With these three plants they could create almost any colour. The textiles that were dyed during the Middle Ages were mainly derived from animal sources, like wool. The dyeing of plant based textiles was not developed during the Middle Ages. Dyeing of cotton and other plant based materials was still the secret of the East. During the Middle Ages the technique of dyeing was mainly based on oral tradition and only a few written manuscripts have been found. From the seventeenth century more books and articles on dyeing were published. In 1678 the Dutch East India Company (VOC) introduced the cotton printing trade in Europe. In the 17th and 18th century the Turkish red dye technique was introduced in Europe. In 1747 Greek/Turkish dyers established in France (Rouen) a Turkish ruled red textile industry. Here the madder culture was the most famous of the 18th century. In the 19th century the madder cultivation also developed in the Alsace. From 1600-1900 there was a heavy trade in madder dyes throughout Europe. In 1868 Graebe and Liebermann discovered how to prepare alizarin synthetically. At the end of the 19th century the madder culture rapidly declined, due to the cheaper production of synthetic alizarin[1]. About one century later madder is again cultivated on a commercial scale in The Netherlands in the province Groningen.

1.1.3 Cultivation, harvesting and processing of madder

There is evidence that madder was already cultivated in The Netherlands in the 12th century. Especially in the deltas of the Dutch province Zeeland, the cultivation of madder was highly developed and was long considered as the finest madder in Europe[7-9].

The cultivation of madder started at the end of April or beginning of May. The young madder shoots were planted in well-manured soil. New plants were kept free of weeds and the soil around the roots was kept loose. In the autumn, after the foliage had died the plant was covered with five to ten cm of soil. In this way the roots were protected against frost. In the following year the roots produced shoots. In the third year the weight of the roots increased 70%. At the end of the third year in September and October the roots were harvested. The harvest was a heavy and time-consuming job. The farmer needed a lot of experience to dig out the entire root intact. The remaining soil was removed by shaking. The roots were left in the open air for drying during a couple of days[7-9].

The classical preparation of the roots was divided in two main processes: drying and stamping. After four to five days the madder was placed inside the madder drying house. The roots that had to be dried were first laid on the bars of the lowest floor. After a couple of days

the roots were moved higher and higher in the drying house. An oven at the ground level heated the drying house. After drying the roots were trashed to loosen the earth and the husk of the roots. If the husk was not removed the product was sold as 'onberoofde' (unrobbed). The trash that was left after the threshing was called the 'Mullen'. This product had the lowest dyeing quality. The (robbed) roots were placed in a stamp trough fitted with iron plates where they were ground to powder[9]. The stamped product was sieved through coarser and finer sieves, which gave products of different quality. The toughest part of the root remained and was stamped again and sieved. This process was repeated a couple of times. In this way first the outside of the root was collected and finally the innerparts of the root. Von Wiesner wrote that the part of the root that was sieved first possessed the highest dyeing power. In contrast to Von Wiesner, De Kanter wrote that the inner part, i.e. the last part sieved had the highest dyeing quality[5,10].

In former days, two main qualities were distinguished called 'Gemene' and 'Krap'. 'Krap' had a higher dyeing quality than 'Gemene'. The madder product 'Two and One' consisted of two parts 'Krap' and one part 'Gemene'. The madder product 'One and One' consisted of one part 'Gemene' and one part 'Krap'[7-10]. According to Van Dijk-van der Peijl the outer part of the 'robbed' root was called 'Gemene' and the inner part 'Krap'[7-9]. According to microscopic analysis of some cross-sections of madder root the concentration of red colour is not the highest in the centre of the root. The highest concentration of red dye was found in the layer between the husk and pith of the root (figure 1.3) [3,5,6]. In the 18th century, France became an important competitor in madder cultivation, due to improved production methods. An innovation was the treatment of dried and ground roots with sulphuric acid. The woody roots were destroyed and charred, while alizarin itself remained unaffected. The residue after drying and powdering was called 'Garancin'. 'Garancin' possessed a colouring capacity, which was four to six times higher than that of the original madder. 'Garancin' was favoured over the madder products from Zeeland and was a commercial success[3,5,7-9]. Another madder product was 'Madder Flower'. The ground madder was macerated in a solution of diluted sulphuric acid, washed, pressed, dried and ground. In this way sugars were removed and glycosides hydrolysed possibly through partial fermentation (some yeast was added). An enzyme which occurs naturally in madder (syn. Erythrozym) may have played a role in the hydrolysis as well. 'Madder Flower' had almost twice the dyeing capacity of the original madder. When 'Madder Flower' was further extracted with methanol and precipitated with water the product was called 'Azale'[3,5].

1.2 Synthesis and biosynthesis

1.2.1 Synthesis of alizarin

In 1868 Graebe and Liebermann showed that alizarin could be reduced to anthracene by the then novel procedure of zinc distillation. At that time the exact structure of alizarin was still unknown. They suggested that the structure of alizarin was 1,2-dihydroxyanthraquinone[11]. In 1868 they verified this hypothesis by a total synthesis of alizarin. In the knowledge, that two of the halogen atoms in chloranil could be replaced by hydroxyl groups on treatment with alkali, they prepared a dibromoanthraquinone. They assumed that it would behave in the same way and indeed on fusion with NaOH it yielded a compound in all respects identical with the natural alizarin obtained from madder[12]. This was the first total synthesis of a natural pigment (figure 1.4).

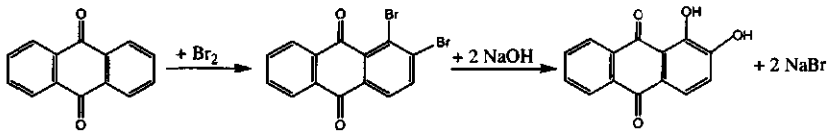

***Figure 1.4** Conversion of alizarin by treatment of anthraquinone with two bromine atoms and on treatment with alkali introduced by Graebe and Liebermann [13].*

Owing to the cost of bromine this route was however not economically viable. Almost at the same moment Graebe, Liebermann and Caro in Germany and Perkin in England found a similar, alternative reaction pathway for the synthesis of alizarin. Graebe, Liebermann and Caro patented this reaction. They introduced the sulphonation of anthraquinone to 2-anthraquinone sulphonic acid and the subsequent conversion to alizarin (figure 1.5)[12].

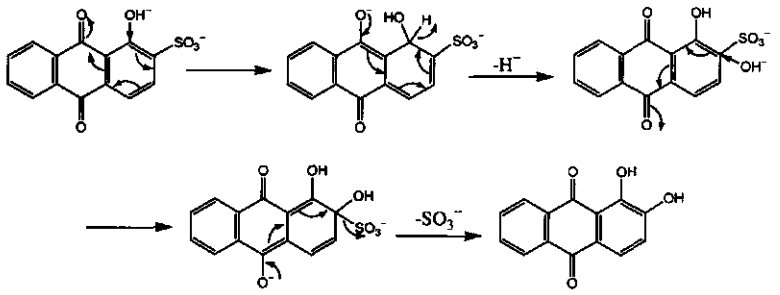

***Figure 1.5** Synthesis of alizarin introduced by Graebe, Liebermann and Caro[13].*

Alizarin plants were built in Germany and England[14-16]. The procedure developed by Koch (B.A.S.F.) is based on the sulphonation of anthraquinone with oleum followed by alkali fusion under addition of an oxidising agent to prevent reduction of the quinone (figure 1.6)[17].

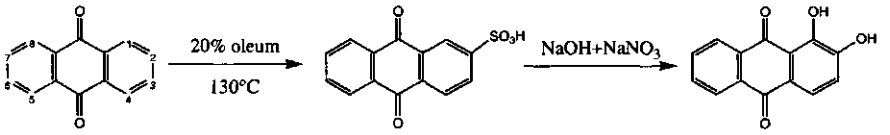

***Figure 1.6** Synthesis of alizarin based on sulphonation with oleum and alkali fusion[17].*

For the synthesis of highly pure alizarin, one has to start with pure 2-anthraquinone sulphonic acid during the alkali smelt. This can be obtained by choosing the reaction conditions and oleum concentration in such a way that only 40% anthraquinone will be sulphonated. Of the then formed anthraquinone sulphonic acids about 85% is the desired isomer. The remaining sulphonic acids consist of traces 1-anthraquinone sulphonic acid and about 15% 2,6-and 2,7-disulphonic acids. This result can be achieved with 20% oleum, a reaction temperature of 130°C, a reaction time of 4 hours and a weight ratio SO_3 to anthraquinone of 1:1. This method gives the highest possible yield of 2-sulphonic acid. After the reaction, the solution is diluted with 80% sulphuric acid, until the remaining oleum is converted into 90% sulphuric acid. Under these conditions the unreacted anthraquinone precipitates after which it is filtered, washed until neutral and dried[12,17-19]. In the next step the filtrate is diluted with 40% sulphuric acid and NaCl is added till the sodium salt of 2-anthraquinone sulphonic acid precipitates. The other sulphonic acids remain in solution. The salt is converted with excess base and sodium nitrate into the sodium salt of alizarin in an autoclave at a temperature of 180°C. This reaction takes 7 hours with a yield of approximately 95%[17]. After dilution with water, the alizarin is separated from the reaction solution by acidifying and filtration. Finally the residue is washed till it is free from acids and then mixed with 20% water to form a paste. As such it is used for dyeing. The product was sold as Alizarinred BB 20% (B.A.S.F.) or ditto B_2 20% (ICI)[17]. These companies do not sell alizarin anymore. Alizarin is still produced and sold by the companies Buckeye Chemical & Colour Co., USA (Fl 75,-/kg), Transpek, India and Mitsui Toatsu Dyes, Japan.

1.2.2 Biosynthesis

Leistner in Germany and Burnett in Great-Britain have performed much work on the biosynthesis of anthraquinones[20-26]. Two different pathways for the biosynthesis of

anthraquinones in plants exist: either the polyketide pathway or the shikimate pathway[22]. Anthraquinones synthesised according to the former pathway are substituted in both ring A and C. Examples are emodin and chrysophanol. These polyketide-derived anthraquinones are found in Rhamnaceae, Polygonaceae and Leguminosae[20,22,26,27]. Anthraquinones formed by the latter pathway (figure 1.7) are characterised by a substitution in one ring only. These anthraquinones like alizarin are found in Rubiaceae (e.g. *Rubia tinctorum*)[13,20-22,25,28,29].

Ring A and one of the carbonyl groups of the anthraquinones are derived from shikimic acid[24]. The other carbonyl group and the remainder of ring B are derived from α-ketogluturate that originates in turn from glutamate[21]. The acetyl coenzyme ester of *o*-succinylbenzoic acid is cyclized to 1,4-dihydroxy-2-naphtoic acid[28]. Symmetrical 1,4-naphtoquinone is not an intermediate in the biosynthesis as has been shown in labelling studies[21-23,25,29,30]. The intermediate 1,4-dihydroxy-3-prenyl-2-naphtoic acid is formed by incorporation of dimethylallyl pyrophosphate at the unsubstituted carbon of the quinone ring[31]. Finally cyclisation of the prenyl with the C-1 carbon will lead to ring C of the anthraquinone. If the isopentenyl unit cyclizes on the OH group at C-10 this will lead to a pyran, for example mollugin[22,23,25,29,30].

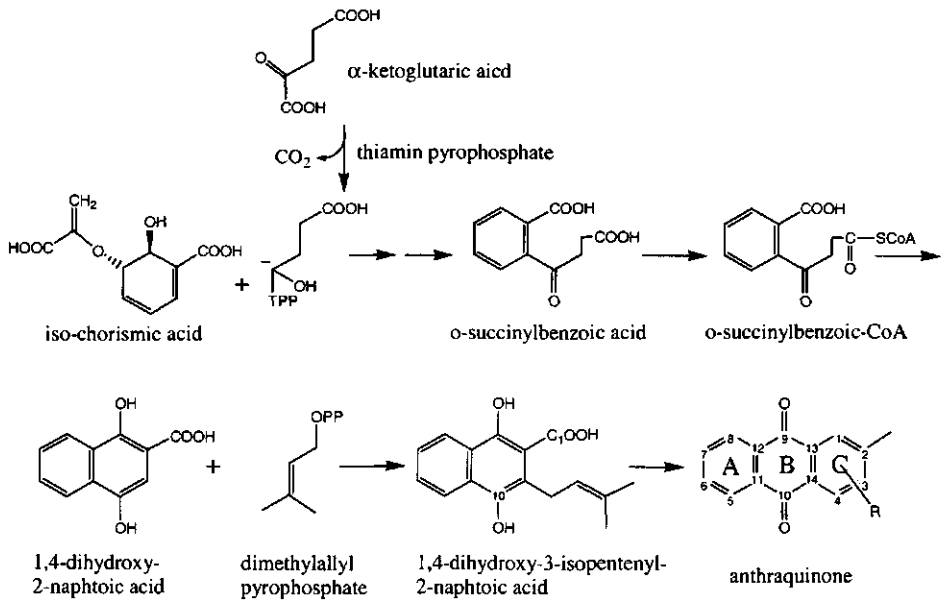

***Figure 1.7** Biosynthesis of anthraquinones by the shikimate pathway[28].*

The biosynthesis of these anthraquinones parallels those of the menoquinones in bacteria and naphtoquinones of plants for example juglone, vitamin K and lawsone. These compounds are also derived from shikimic (or chorismic) and α-ketoglutaric acids via *o*-succinylbenzoic acid[21,22]. 1,4-Dihydroxy-2-naphtoic acid is the branching point in the biosynthesis of menoquinones, naphtoquinones and anthraquinones[28].

Recently it was shown by Eichinger *et al.* in an elegant biosynthetic study that mevalonate was *not* the precursor of the C-ring of Rubia anthraquinones[32]. Until a few years ago it was thought that isopentenyl pyrophosphate was always derived from mevalonate. However it has since been shown that two different pathways exist: (1) the mevalonate pathway, and (2) the deoxyxylulose pathway (operational in plastids). In the latter pathway pyruvate and glyceraldehyde 3-phosphate - via 1-deoxy-D-xylulose 5-phosphate - are the precursors of terpenoids. Cell cultures of *Rubia tinctorum* were fed with [1-^{13}C]- or [U-$^{13}C_6$]-glucose. Amino acids were obtained by hydrolysis of biomass, and their ^{13}C labelling patterns were used to reconstruct the labelling patterns of acetyl CoA, pyruvate, phosphoenol pyruvate, erythrose 4-phosphate, and α-ketoglutarate by retrobiosynthetic analysis. The labelling patterns were used to predict the labelling patterns of lucidin primeveroside via three different hypothetical pathways (polyketide, mevalonate and deoxyxylulose). The observed labelling pattern was in excellent agreement with the pattern predicted on the basis of the precursors *o*-succinylbenzoate and dimethylallyl pyrophosphate derived via the deoxyxylulose pathway[32].

Anthraquinones in *Rubia tinctorum* are mostly present as glycosides. Glycosylated secondary products differ from the free aglycones in two properties: they show increased water solubility and decreased chemical reactivity. Because of the better water solubility the glycosides are stored in the plant vacuole and the glycosides are less reactive toward cell compounds. This is probably the reason why glycosylated compounds, rather than free aglycones are accumulated[33]. Research on the biosynthesis of anthraquinones has been mostly focused on the aglycone. Little research has been carried out on the modification reactions on the aglycone part and on the glycosylation and storage of anthraquinone glycosides in *Rubia tinctorum*. In Morinda the anthraquinone glycosides are stored in the vacuole. The enzymatic mechanism for hydrolysis of glycosides and glycosylation of anthraquinones is present or rapidly induced in cells that do not produce these compounds themselves[34].

1.3 Extraction, isolation and purification of anthraquinones in *Rubia tinctorum*

1.3.1 Extraction

Many articles deal with the extraction of anthraquinones from the plant, root or cell cultures of *Rubia tinctorum*. The isolation procedure depends on whether the free aglycones or the glycosides are desired. The anthraquinones can be isolated by sequential extraction with solvents of increasing polarity[35-39]. The different extract solutions can be further purified by a liquid-liquid partitioning step. As a first extraction step a non-polar solvent can be used such as ether, benzene, chloroform, dichloromethane or ethyl acetate[35,40-45]. The anthraquinone glycosides, however, should be extracted by using water, ethanol, methanol or water-ethanol mixtures[35,41,45-52]. The extraction can be performed at different temperatures. During the extraction of anthraquinones from plant material with hot methanol or ethanol artefacts can be formed. These anthraquinones show the presence of 2-methoxymethyl or 2-ethoxymethyl groups respectively e.g. lucidin was partly converted to the corresponding ω-ethyl ether. This reaction is highly temperature-dependent[53]. Thus for the extraction of anthraquinones the use of hot methanol or ethanol should be avoided[28]. Also basic solutions are used for the extraction of anthraquinones or the fractionation of apolar plant extracts, e.g. sodium carbonate, sodium bicarbonate or sodium hydroxide[36,37,45,49,54]. Most of the anthraquinones in madder are phenols. In general phenols dissolve well in basic solutions[45]. Masawaki *et al.* (1996) used this property for the simultaneous extraction of both aglycones and glycosides. When an aqueous KOH solution (50 mmol/m^3) was used as extraction solvent, both ruberythric acid and lucidin primeveroside were extracted. When chloroform was used as extraction solvent, the anthraquinone primeverosides were not extracted into chloroform and only a small amount of alizarin and lucidin was extracted. Selective extraction from dried pulverised madder roots of the anthraquinones alizarin and lucidin into chloroform and their primeverosides into the aqueous phase was achieved using a chloroform-water two-phase extraction with pH adjustment to pH=5[55].

In many cases the glycosides in the extract are subsequently hydrolysed in aqueous (2-5%) H_2SO_4 or HCl solution at 80-100°C[47,48,51,56]. In this process the main glycosides of madder, ruberythric acid and lucidin primeveroside, are converted to their aglycones alizarin and lucidin with the simultaneous release of glucose and xylose. During the hydrolysis most of the aglycones precipitate[45]. Direct acidic hydrolysis of madder root extract gives a black precipitate. An aqueous extract of madder root also contains asperuloside, an iridoid glycoside. On warming with dilute acids i.e. the conditions of the hydrolysis asperuloside gives first a green colour and then a tarry black precipitate[47,57]. Partial hydrolysis of

anthraquinone glycosides can be achieved with an aqueous solution of NaOH[58]. Another possibility is the hydrolysis of the glycosides with hydrolases. Masawaki *et al.* claimed that ruberythric acid, in a two phase chloroform-water solution, could be selectively and completely converted to alizarin within 6 hours by enzymatic hydrolysis with ß-glucosidase at 50°C at pH=5. In contrast only 60% of ruberythric acid was converted to alizarin in a one-phase aqueous solution after 6 hours. This could be explained by the fact that alizarin exerts an inhibitory effect on the enzymatic hydrolysis of ruberythric acid in the solution. In the two-phase extraction the enzymatic hydrolysis of ruberythric acid could proceed effectively because the inhibitory effect of alizarin was reduced by the extraction of alizarin into the $CHCl_3$ layer[55]. In this experiment lucidin primeveroside was not enzymatically hydrolysed by β-glucosidase[55].

The anthraquinones can be further purified by precipitation or by chromatography. Precipitation can be achieved with reagents as lead acetate[59]. Precipitation was very common in older days. Nowadays components are mainly purified by chromatography.

1.3.2. Column chromatography

Crude extracts of anthraquinones can be further fractionated by column chromatography. Many column materials have been used for the purification of anthraquinones of *Rubia tinctorum*. Eluents used for normal phase column chromatography usually consist of a series of solvents of increasing polarity (table 1.1).

***Table 1.1** Column material and eluents used for the separation of anthraquinones by low-pressure column chromatography.*

Product name	Material	Solvent	Reference
Silica gel	SiO_2	methanol	35
		acetone	36
		chloroform	36
		petroleum ether 40-60	36
		benzene	36
		chloroform-methanol	38
		hexane-ethyl acetate	38
		hexane	43
		chloroform-methanol	60
Sephadex G-25	crosslinked dextran	0.5% aq. sodium carbonate	49
Sephadex LH20	crosslinked hydroxy-propylated dextran	methanol	35
		methanol	51
		chloroform-methanol	43
Amberlite XAD-2	non-ionic polymeric absorbent	water-methanol	58
Dowex 50 (H^+)	strongly acidic cation-exchange resin	methanol	51

Pre-purification and concentration of anthraquinones occurring in plant extracts can be effected by solid-phase extraction (SPE) using C8 cartridges. A crude ethanol extract was diluted tenfold with water and passed drop-wise through pre-activated SPE cartridges. These cartridges were then washed with water followed by methanol-water (30-70). After drying the cartridge with air the anthraquinone fraction was eluted from the column with methanol-water (80:20)[46,51].

1.3.3 Paper chromatography and thin layer chromatography

Paper chromatography has been frequently used in the past. Shibata *et al.* (1950)[61] applied paper chromatography for the separation and identification of anthraquinone pigments. Petrol ether (bp. 45-70°C) saturated with 97% methyl alcohol was used as solvent. After development the spots were revealed with magnesium acetate[61]. n-Butanol-acetic acid-water (4:1:5) was used as eluent for the separation of both the glycosides and aglycones[62].

Nowadays thin layer chromatography (TLC) has replaced paper chromatography. TLC is now frequently used for the planar separation of anthraquinones, the most frequently used adsorbent being silica[28]. Almost every research group has developed their own solvent system in table 1.2 some examples of eluents are given for the separation of anthraquinones in *Rubia tinctorum* extracts. Preparative TLC has been applied for the purification of anthraquinones from *Rubia tinctorum*[63].

Detection of anthraquinones on TLC plates is very simple due to their yellow-orange colours. Some anthraquinones show a colour when observed in UV_{254} light and most of them show colours when observed in UV_{366} light. A change in colour is also observed when hydroxyanthraquinones are sprayed with a solution of KOH or NaOH in methanol (5% w/v). This detection method for hydroxyanthraquinones is called the Bornträger reaction. The colour changes from yellow-orange to red-purple due to the ionisation of the OH group(s). The mesomerism is enlarged and results in a shift of the absorption maximum to higher wavelength[45,66]. Another identification test is the exposure of the spots to NH_3 vapour; a colour change of the anthraquinone spots is observed[28,44]. As already mentioned spots can also be revealed by spraying with 0.5% magnesium acetate in methanol and heating at 90°C for five minutes. Hydroxyanthraquinones react with this reagent if they have at least one hydroxyl group in the 1-position. Depending on the position of the hydroxyl groups in the anthraquinone nucleus different coloured spots were produced with this reagent. Compounds, which contain two hydroxyl groups in the 1,2-position, give a violet colour, those with two in the 1,3-position an orange-red or pink colour and those with two in the 1,4-position give a purple colour[61].

Table 1.2 Eluents used for the separation of anthraquinones by silica gel TLC.

Eluent	Reference
n-butanol saturated with 6M ammonia-methanol 4:1	47
ethyl formate-formic acid-toluene 7:2:5	47
ethyl formate-formic acid-toluene 4:1:5	47
toluene-pyridine-acetic acid 10:1:1	47
benzene-ethyl acetate 1:1	47
n-butanol-ethanol-water 4:1:5	47
n-butanol-pyridine-water-benzene 5:3:3:1	47
n-butanol-pyridine-water 6:4:3	47
methanol-10 vol-% aq. acetic acid 6:4	56
methanol-10 vol-% aq. acetic acid 7:3	56
methanol-10 vol-% aq. acetic acid 8:2	56
chloroform-hexane-ethyl acetate-acetic acid 40:40:15:5	56
toluene-acetic acid 9:1	56
n-butanol-ethanol-ammonia 6:2:3	49
benzene-ethyl formate-formic acid 40:24:1	49
chloroform-ethyl acetate 6:1	49
ethyl acetate-methyl ethyl ketone-formic acid-water 5:2:0.2:1	49
toluene-methanol 9:1	64
chloroform-methanol-25% ammonia 85:14:1	64
benzene-ethanol 8:2	51
benzene-ethyl acetate-methanol 40:30:5	44,65
chloroform-benzene-ethyl acetate-acetic acid 40:40:15:5	44
methanol-10 vol-% aq. acetic acid 8:2	41
chloroform-benzene-ethyl acetate-ethanol 8:8:3:1	43
benzene-acetone 9:1	43
chloroform-methanol 9:1	38,43
hexane-ethyl acetate 9:1	38
hexane-ethyl acetate 8:2	38
chloroform-methanol 8:2	38
chloroform-methanol 7:3	38

1.3.4 Gas liquid chromatography (GLC)

For GLC studies of hydroxyanthraquinones more volatile derivatives have to be prepared, because the anthraquinones as such are not sufficiently volatile. Several derivatives, namely methyl ethers, trimethylsilyl (TMS) ethers and trifluoroacyl derivatives have been separated on SE-30, OV-17, and UC-W98 phases[67,68]. All anthraquinone derivatives other than reductively silylated ones gave excessive tailing when chromatographed[67]. Reductive silylation was therefore considered the method of choice for derivatization of hydroxyanthraquinones[67]. The influence of the number and position of substituents in the

anthraquinone nucleus on GLC behaviour is clear. An increase in retention time was observed with an increase in the number of hydroxyl groups. The GC elution order largely follows the molecular weights. Within the group of dihydroxyanthraquinones, 1,4-dihydroxyanthraquinones had shorter retention times than 1,2- or 1,3-dihydroxyanthraquinones. Hydroxyanthraquinones have longer retention times than the corresponding methylanthraquinones. Retention time was also decreased when a hydroxyl group was replaced by a methoxyl group[67-69].

1.3.5 High pressure liquid chromatography

In spite of the excellent separation of the anthraquinones by GLC, one preferably uses high pressure liquid chromatography (HPLC) for the separation of these compounds. For an HPLC separation the anthraquinones do not have to be silylated, which saves time and prevents possible losses during the derivatisation. Several screening methods for anthraquinones in

***Table 1.3** HPLC systems used for the separation of anthraquinones of Rubia tinctorum.*

HPLC column	Eluent	Compound	Reference
KONTRON RP18	0.1% aq. acetic acid-acetonitrile gradient	gly + agly	40
RP-C-18 Nova pak	methanol-10% aq. acetic acid isocratic	agly	56
Shin-Pack CLC-ODS	methanol-5% aq. acetic acid 7:3 isocratic	agly	35
Shin-Pack CLC-ODS	methanol-5% aq. acetic acid 6:4 isocratic	agly	35
Hypersil 5 RP	isocratic elution methanol-5% aq. acetic acid 7:3	agly	46
TSK ODS 120 T	methanol–10% aq. acetic acid 3:7 gradient	gly	48
TSK ODS 120 T	methanol-10% aq. acetic acid 3:2 gradient	agly	48
Armsfer-C8	acetonitrile- 4% aq. acetic acid gradient	alizarin	50
Nucleosil-508	methanol-0.1% H_3PO_4	gly + agly	51
ODS Hypersil RP	isocratic methanol- 5% aq. acetic acid 7:3	gly + agly	51
Superpac PEP S C_2/C_{18} RP	acetonitrile-0.02 M ammonium acetate buffer pH 4 15:85 gradient	gly + agly	51
RP C_{18} LiChroCART	methanol-10% aq. acetic acid isocratic	agly	41
Alltima end-capped C_{18}	water-acetonitrile gradient	gly + agly	70

Rubia tinctorum, based on HPLC have been described in the literature[40,41,46,51,56]. C_{18} reversed-phase materials are mostly used with an occassional mentioning of silica gel.

In general, the HPLC methods focused on the aglycones or even only on alizarin. In most cases the madder extract is first hydrolysed and the total quantity of alizarin is determined after HPLC separation[41,56]. Three HPLC methods for the simultaneous analysis of both glycosides and aglycones were described[40,51,70].

In all the articles the anthraquinones of *Rubia tinctorum* were (quantitatively) measured through determination of the ultraviolet (UV) absorbance at 254 nm[40,41,46,51,56,70,71], 280 nm[48,50,51] or at visible wavelengths more specific for anthraquinones as 430 nm[72], 480 nm[41] or 500 nm[41]. The eluents used for the HPLC separation of the glycosides generally consisted of mixtures of water and acetonitrile or water and methanol[28]. Most research groups added some acid to suppress tailing of the anthraquinone peaks[70]. In table 1.3 the HPLC systems used for the separation of anthraquinones of *Rubia tinctorum* are described.

1.3.6 Liquid-liquid chromatography techniques

Liquid-liquid partition chromatography has been applied successfully for the separation of anthraquinone glycosides and aglycones[29,63,73,74]. A liquid-liquid separation technique called droplet counter current chromatography (DCCC) has been developed in 1970 by Tanimura *et al.*[75]. The liquid separation is based on the partitioning of the different compounds in the sample between many tiny mobile phase droplets, which move through the stationary phase, and the stationary phase. DCCC was used for the separation of anthraquinone glycosides or aglycones[73,74]. The solvent system chloroform-methanol-water 5:5:3 was used for the separation of the two main anthraquinone glycosides of *Rubia tinctorum*[70].

During a DCCC experiment gravity is the driving force for the movement of the mobile phase through the stationary phase. In the newer technique of centrifugal partition chromatography (CPC) centrifugal forces drive the process. Separation by CPC is much faster than by DCCC. Hermans-Lokkerbol *et al.* optimised the solvent system for the separation of both the anthraquinones aglycones and glycosides in a *Rubia tinctorum* extract with CPC. The solvent systems n-hexane-ethyl acetate-methanol-water 9:1:5:5 and chloroform-methanol-water-acetic acid 5:6:4:0.05 were used and offer different selectivity. The first system was used for the separation of anthraquinone aglycones and the second system for the separation of aglycones and glycosides[63].

1.3.7 Capillary electrophoresis

Recently the separation of 10 anthraquinone aglycones and two glycosides from Rheum by capillary electrophoresis was described[76] and compared with HPLC separation. From the investigated anthraquinones also alizarin and purpurin occur in madder. Because all of the anthraquinones can be charged by means of complexation with a borate buffer, capillary zone electrophoresis (CZE) with a 90 cm x 75 µm fused silica capillary was used. The detection window was located at 80 cm. The voltage was 23 kV and the temperature 20°C. The injection was pressure controlled (1.2 sec at 200 mbar). The total run time was 39 minutes compared to 63 minutes for the HPLC separation.

Best resolution was obtained at a 30 mM borate concentration, a pH value of 10.56 and with acetonitrile as organic modifier. The detection limit was about ten times higher than the corresponding HPLC detection limit[76]. CZE has some merit for the fast analysis of anthraquinones although further research is necessary to see if it can be successfully employed for the quantitation of madder extractives.

1.4 Secondary metabolites occurring in *Rubia tinctorum*

The most important components of *Rubia tinctorum* are the anthraquinones. The anthraquinones found in *Rubia tinctorum* differ in the nature of the substituents and the substitution pattern. Due to the biosynthetic route of anthraquinones (*vide supra*) in Rubiaceae these substituents are only found on carbon atoms 1, 2, 3 and 4. A hydroxyl group is frequently encountered as substituent. In literature a total of 36 different anthraquinones were isolated and identified from madder root (appendix). The basic anthraquinone structure is depicted in figure 1.8.

Alizarin is the most well known anthraquinone from madder. In 1826 alizarin was first isolated from *Rubia tinctorum* by Colin and Robiquet[77]. After the first isolation of alizarin many other anthraquinones were isolated from *Rubia tinctorum* for example purpurin, munjistin, rubiadin, pseudopurpurin, nordamnacanthal, lucidin, xanthopurpurin and anthragallol. Ruberythric acid was first isolated in a crystalline form by Rochleder in 1851[78]. Ruberythric acid consists of the aglycone alizarin and a disaccharide. Many years later the sugar moiety was identified as primeverose, a disaccharide of xylose and glucose[79]. Lucidin is another anthraquinone in madder that is mainly present as its glycoside form. The sugar moiety is also primeverose. In the literature of the 19th century and early 20th century the occurrence of other glycosides was reported: rubiadin glucoside[5,78,80] galiosin (pseudopurpurin 1-ß-primeveroside), rubiadin 3-ß-primeveroside, rubiadin glucoside, rubianin

(a C-glucoside)[1,3,5,81], rubiadin 3-ß-D-glucoside, galiosin (pseudopurpurin β-primeveroside)[82]. However in more recent literature these findings have not been confirmed. In recent literature only ruberythric acid and lucidin primeveroside have been reported. Recently three new glycosides were found in *Rubia tinctorum*: lucidin glucoside, 2-hydroxymethylanthraquinone 3-glucoside and 3,8-dihydroxy-2-hydroxymethylanthraquinone[38]. Because the proposed biosynthetic route for anthraquinones in *Rubia tinctorum* does not allow substitutions in both anthraquinone rings, the report of the latter compound needs to be confirmed.

Some anthraquinones isolated from *Rubia tinctorum* are believed to be artefacts for example the anthraquinones which show the presence of a 2-methoxymethyl or 2-ethoxymethyl group. These anthraquinones have been formed during extraction with boiling methanol or ethanol[28,53,83]. According to Schweppe, the anthraquinones purpurin (1,2,4-trihydroxyanthraquinone) and purpuroxanthin (1,3-dihydroxyanthaquinone) are formed from respectively pseudopurpurin (3-carboxy-1,2,4-trihydroxyanthraquinone) and munjistin (2-carboxy-1,3-dihydroxyanthraquinone) during drying of the roots[1]. Some anthraquinones were only isolated once from *Rubia tinctorum*. It is thus doubtful whether these anthraquinones are really present in madder, especially if there are few spectral data available as for example with quinizarin-2-carboxylic acid (appendix).

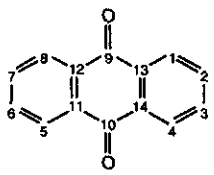

Figure 1.8 The basic anthraquinone structure and the numbering of the carbon atoms chosen in this research.

1.5 Dyeing with madder

1.5.1 Compounds responsible for the dyeing effect

The roots of *Rubia tinctorum* have been used for dyeing textiles in many parts of the world since ancient times. Madder was widely cultivated in Western Europe for the dye industry until the beginning of the twentieth century. *Rubia tinctorum* contains useful anthraquinone mordant dyes. Anthraquinone derivatives are good mordant dyes if they satisfy the following conditions:

1. The anthraquinone has a hydroxyl group at the C-1 (or C-4) position next to one of the carbonyl groups.
2. The hydrogen bonding must be weakened by a substituent at C-3 with an -I or -M effect, or a substituent at C-2 with a +I or +M effect[17].

The anthraquinones in madder with only one free phenolic group are of no dyeing importance[1].

At the end of the nineteenth century alizarin could be produced synthetically. As the colouring capacity of alizarin was very similar to that of dried madder roots, the cultivation of madder quickly came to an end and only synthetic alizarin was used for dyeing textile[1].

1.5.2 Theory and practice of dyeing with madder

A lot of different formulas for dyeing with madder have been described in the literature. The recipes can be divided in two main classes, according to the origin of the material to be dyed, the number of process steps and the necessary chemicals.

1. The Alizarin red procedure, for dyeing animal derived fibres such as wool
2. The Turkish red procedure, for dyeing plant derived fibres such as cotton[1]

In a lot of modern handbooks on dyeing, the difference between these procedures is not taken into account[9]. But actually it is as *complicated* to dye vegetable fibres like cotton, flax or hemp with madder derived dyestuff, as it is *uncomplicated* to dye an animal fibre with a madder dyestuff. In contrast with cotton, wool fibre can be dyed red that is hearty and has a good chroma with a much easier dyeing recipe using only alum as mordant. For madder dyeing of a plant-derived fibre like cotton yarns a much more complicated recipe has to be used with a large number of dyeing steps and mordant components[1,9,17]. This must be due to the different composition of both fibres. Cotton (a vegetable fibre) consists of 94% cellulose[1], which is built of ß-glucopyranosyl residues joined by 1→ 4 linkages. Wool (an animal fibre) consists of the protein α-keratin[84].

In the Alizarin red procedure the main steps are:

1. Pre-treatment
2. Mordanting
3. Dyeing
4. Washing

Between the mordanting and the dyeing step the wool does not have to be dried.[1].

In the Turkish red procedure the following steps can be distinguished:

1. Pre-treatment; The yarn or cloth is cleaned from fatty and waxy components.
2. Oiling; The fibre is repeatedly treated with oil (Tournant oil or Turkish red oil).
3. Treatment with tannic acid; Fixing agent for the alum mordant.
4. Treatment with mordant; The mordant is necessary as fixative for the dye.
5. Fixation of the mordant; The fixation is mostly achieved by drying of the textile at a low temperature. Kiel (1961) showed that the formation of $Al(OH)_3$ out of aluminium acetate mordant and sulphonate mordant is essential for the fixation. He showed that the fixation was maximal if the pH lies between 4 and 9 during the fixation.
6. Dyeing; The stained cloth is dyed with madder. A colour complex is formed between the dye, the mordant and the calcium.
7. Washing; The excess dye and other used compounds are removed from the cloth[17].

Other compounds can be used in the dyeing process to change the dyeing result. Most of these compounds give a qualitatively different product. For example the use of the alternative mordant $FeSO_4 . 7H_2O$ results in a darker and blacker colour. Copper gives a warmer and deeper colour. If the textile is placed in a tin bath after drying, the colour turns more yellow. It is assumed that the Sn(II) ions displace some of the calcium ions. This process is named "aviveren" in Dutch[17].

In the past a lot of authors have proposed different structures for the coloured complex that will be formed during the dyeing process. It was already known before 1868 that besides dye and mordant also calcium is essential for the complex. The pKa values of the alizarin hydroxyl groups at position 1 and 2 are respectively 12.0 and 8.2 making the 2-hydroxyl group the more acidic one. During the formation of the dye complex a calcium ion reacts with the 2-hydroxyl while an aluminium ion forms a complex between the 1-hydroxyl and the carbonyl group. The ratio between both alizarin and aluminium and between alizarin and calcium is 2:1[17].

Based on IR spectra of alizarin-metal ion complexes Kiel suggested that the complex must have an Al^{3+} chelated in the ß-ketol (C-1 or C-4) position of the alizarin molecule. Thirty-five years later Soubayrol *et al.* proved with ^{27}Al NMR in the solid state that the complex is a binuclear co-ordination complex (figure 1.9) of the structure proposed by Kiel[85].

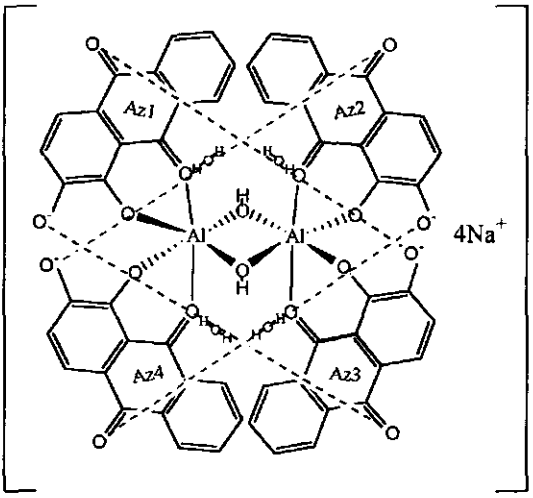

Figure 1.9 *Structure for the dye complex suggested by Soubayrol et al.*[85].

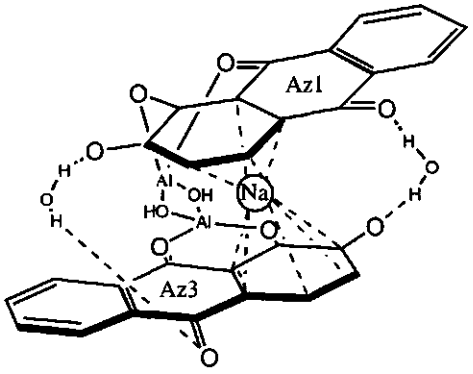

Figure 1.10 *Three-dimensional structure of one half of the binuclear dye complex suggested by Soubayrol et al.*[85].

The binuclear species consists of a tetra anion skeleton formed around two Al^{3+} ions linked by two hydroxyl bridges[85]. Depending on the nature of the cation and the drying conditions, the complex is surrounded by a variable number of water molecules, $[Al_2(\mu\text{-}OH)_2Na_2(C_{14}H_6O_4)_4(H_2O)_4](H_2O)_2(Na)_2$ and $[Al_2(\mu\text{-}OH)_2Ca_2(C_{14}H_6O_4)_4(H_2O)_4](H_2O)_2$

respectively. The four-alizarinate ligands are probably bound with their eight free oxygen atoms to the four H_2O molecules as shown in figure 1.9.

Alizarin molecules Az1 and Az3 partially overlap each other with two water molecules in front of the complex. Similarly molecules Az2 and Az4 overlap each other with the two remaining water molecules at the rear of the complex. The alizarin entities, which overlap each other with two benzene rings form a sandwich structure inside of which two Na^+ or two Ca^{2+} cations are probably confined (figure 1.10). The ionic radii of Na^+ and Ca^{2+} fit nicely into a benzene hexagon of 2.4 Å diameter, which therefore supports the formation of $(C_6H_6)_2Na$ sandwich subunits. Deformation of the four O-Al bonds in the core is probably responsible for the decrease in the apparent co-ordination number of Al in the closed complex, (a lot of strain for including the bulk of the ligands) leading to an atypical chemical shift for ^{27}Al near 24 ppm. If instead K^+ or Ba^{++} are used as ions the complexes $[Al_2(\mu\text{-}OH)_2(C_{14}H_6O_4)_4(H_2O)](H_2O)_5(K)_4$ and $[Al_2(\mu\text{-}OH)_2(C_{14}H_6O_4)_4(H_2O)_3](H_2O)_3(Ba)_2$ are formed. These are called binuclear open structure complexes. The bulkier K^+ or Ba^{++} cations are not able to enter the alizarin sandwich structure and rather than having Al-O-Al bridges that are too strained, the structures remain open and the water molecules are no longer necessary. In this case, the co-ordination number six gives standard ^{27}Al chemical shifts near zero and probably standard O-Al bond lengths near 1.86 Å as in$[(Al_2)\mu\text{-}OH)_2]^{4+}$ core complexes[85]. In the case of Ba^{2+}, the stable complex is a trihydrate, with probably two alizarinates connected by two water molecules, the other two ligands remaining free[85].

Inspection of molecular models showed that the two water molecules of each dibenzene sandwich may be replaced by a cellobiose entity representing a unit of the cellulose fibre. This possibility provides a possible explanation for the fixation of this dye on cotton fabric[85].

1.6 Non-dyeing uses and biological activity of madder

1.6.1 Non-dyeing uses

Extracts of madder root (*Rubia tinctorum*) contain some compounds of pharmacological interest. Crude extracts have been used for the treatment of bladder and kidney stones, especially those consisting of calcium oxalate and calcium phosphate in the urinary tract. *In vitro* experiments showed that ruberythric acid prevented the formation of calcium phosphate and calcium oxalate[86]. Extracts of madder roots have been used as ingredients of phytopharma-ceuticals[28,40,44,46,47,50,51,53,56,86,87]. Rubia Teep® tablets from Madaus (Köln, Germany) are an example of a madder phytopharmaceutical. With regard to the effect against

stones no clinical trials exist[88]. Thus in view of the potential toxicity a negative advice about the medicinal use of madder was released by the German Commission E. Madaus withdrew Rubia Teep® from the market in 1990 due to possible mutagenic properties of lucidin (*vide infra*).

Alizarin can be used to stain calcium deposits in soft tissues. Dermato-pathologists use it to detect dermal calcium in disorders such as pseudoxanthoma elasticum and calcinosis cutis[89]. It harmlessly stains also living tissues. Bones of animals that ate madder turned pink or red. The active compound responsible for the staining is supposed to be pseudopurpurin[89].

Alizarin is also used diagnostically as a marker for the study of bone growth. Alizarin and radioactive calcium are deposited similarly in growing bones, including those of the skull. Radioactive calcium and alizarin are deposited similarly in growing bones. However radioactive calcium is toxic for both the investigator and the patient, a disadvantage which does not arise with the use of alizarin[13,90].

Another application of madder extract is its use as food colourant. Natural food colourants are used rather than synthetic ones, because of a consumer preference for natural products. It is widely believed that natural colourants are generally safer than synthetic ones. Madder root extract has been used as a food colourant in confections, boiled fish and soft drinks in Japan due to their colours with distinctive heat and light resistant properties. Madder root anthraquinones turn purple after reacting with proteins in foods[51,56,91,92].

1.6.2 Mutagenic and carcinogenic activity of Rubia tinctorum extracts

Because of the application of *Rubia tinctorum*, extracts in phytopharmaceuticals and as food colourants studies on the safety of these products have been carried out. Brown and Dietrich (1979) were the first to report lucidin to be mutagenic[65]. Later different authors confirmed that this anthraquinone shows mutagenicity in several strains of *Salmonella typhimurium*[40,49,65,71,93-96]. The mutagenicity of lucidin was also tested in a battery of genotoxicity assays: mutagenicity in bacterial cells[49] and mammalian cells[49,53,65,71,97], induction of DNA repair in primary rat hepatocytes[53] and *in vivo* transformation of C3H/M2-mouse fibroblasts[53,65,91,98]. Lucidin induced unscheduled DNA synthesis in primary rat hepatocytes and transformed C3H/M2 mouse fibroblasts[40,65,71,97]. The genotoxic effect of lucidin is a matter of some concern in products containing Rubia extracts. For example, Rubia Teep® tablets contain small amounts of lucidin and lucidin primeveroside[51] and products with madder root extract are used as a food colourant. The glycoside lucidin primeveroside (a compound of madder root) is metabolised in rats to the genotoxic lucidin[40,71,94,97] and rubiadin[99,100].

In a thorough study Kawasaki *et al.* (1992) elaborated that the mutagenicity of madder is not exclusively due to lucidin. Dried roots of madder were extracted with different solvents and these extracts were fractionated by chromatography. Twenty compounds were isolated from the roots of *Rubia tinctorum* and these compounds were tested for their mutagenicity in *Salmonella typhimurium* strain TA 100 and/or TA98. 1-Hydroxy-2-methylanthraquinone, lucidin-ω-methylether, rubiadin, xanthopurpurin, 7-hydroxy-2-methyl-anthraquinone, lucidin, lucidin-ω-ethylether, lucidin primeveroside and the non-anthraquinone compound mollugin showed mutagenicity[35]. The non-anthraquinone compound mollugin is a direct mutagen[35].

Kawasaki *et al.* further studied the mutagenicity of 25 anthraquinones to determine the structure-mutagenicity relationship. Lucidin and the alkoxy derivatives (lucidin-ω-methylether and lucidin-ω-ethylether) as such showed mutagenicity. Rubiadin that can be regarded as lucidin reduced at the hydroxymethyl group, showed mutagenicity only after metabolic activation. They concluded that 1,3-dihydroxyanthraquinones possessing a methyl or hydroxymethylgroup on carbon-2 are mutagenic. An oxygenated state of the benzylic carbon-2 is essential for direct mutagenicity[35].

Lucidin primeveroside exhibited mutagenic potential in *Salmonella typhimurium* TA100 without S9 (post mitochondrial supernatant fraction) mix but not with the addition of this preparation[101]. When the glycoside was treated with hesperidinase during the preincubation period, it became more active. In that case it was active both in the presence and absence of the S9 metabolic activation mix. This pointed in the direction of lucidin as the actual direct and indirect mutagen. When the preincubation period was prolonged, higher mutagenicity was found confirming the hypothesis about lucidin as the responsible compound[101]. The data confirmed earlier findings about the mutagenicity of lucidin.

These mutagenic studies showed that lucidin can be metabolised to a reactive compound which forms covalent adducts with DNA and possibly other macromolecules. It was reported that lucidin forms ethers and esters upon heating with alcohol or acids. This supports the reactive character of lucidin[40,83,97]. Lucidin ethylether and methylether also showed mutagenicity[35,71]. Kawasaki *et al.* (1994) proved that lucidin forms an adduct with the nucleic acids adenine and guanine under physiological conditions. The reactivity of adenine is higher than that of guanine. No adducts of pyrimidine were detected[102]. These adducts were identified as products from a reaction at the benzylic position of lucidin with a nitrogen atom of the base. This indicated the formation of an exomethylenic compound as an electrophilic intermediate[102]. Poginsky *et al.* (1991) also suggested the formation of an electrophilic intermediate that could react with DNA[40] (figure 1.11). Compounds like nordamnacanthal (2-formyl-1,3-dihydroxyanthraquinone) and munjistin (2-carboxy-1,3-dihydroxyanthraquinone) do not possess an oxymethylene group on carbon 2 and are not

mutagenic since an easy dehydration to an exocyclic methylene group is not possible in these compounds. Substitution of the 1,3 dihydroxy group drastically decreases the mutagenicity because the exomethylenic electrophile derived from the substituted compound, like lucidin primeveroside cannot exist as a stable tautomer.

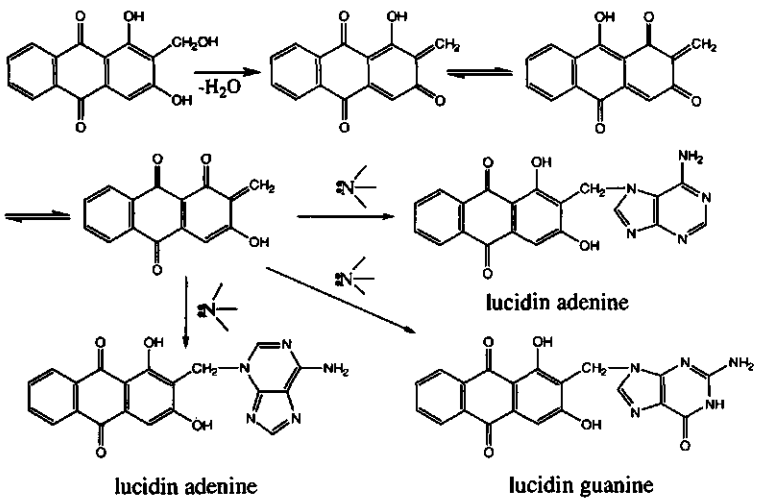

Figure 1.11 *Structures of lucidin-base adducts and the proposed reaction for the formation of the lucidin adducts*[40,102].

To elucidate the possible carcinogenicity of madder roots, three groups of rats received either a normal diet or a diet supplemented with 1% or 10% madder for more than two years[103]. After this period all surviving animals were sacrificed and their organs studied. Weight gain and morbidity were not different among the three groups. Non-neoplastic lesions related to the treatment were evident in the liver and kidneys of both sexes. Moreover, dose-dependent increases in benign and malignant tumour formation were observed in the liver and kidneys of treated animals. ^{32}P-Post-labelling analysis showed an increase in the overall level of DNA adducts observed in the liver, kidney and colon of rats treated with 10% madder root in the diet. HPLC analysis of ^{32}P-labelled DNA adducts revealed a peak co-migrating with an adduct obtained after *in vitro* treatment of deoxyguanosine-3'-phosphate with lucidin. These observations implied that the long-term medicinal use of madder by humans is associated with the risk of formation of malignant tumours[103].

1.6.3 Biological activity of Rubia extracts

In two assays antioxidant activity was established for alizarin[104]. The antibacterial and antifungal activity of aqueous (containing glycosides) and ether (containing aglycones) extracts of madder was tested. The aqueous extract showed antibacterial activity against *Sh. largei-sachsii*. The ether extract was active against *Sh. largei-sachsii, Staphylococcus aureus* and *Streptococcus haemolyticus*. Madder powder and the total aglycones showed fungal activity against *Candida albicans, Geotrichum candidum, Geotrichum louberi, Rhodotorula rubra, Rhinoclaviella* sp. and *Saccharomyces cervisiae*[105]. The pure anthraquinones nordamnacanthal and alizarin showed antifugal activity[106]. Of 45 tested pure compounds purpurin was the most effective one against flagellates[107].

1.7 Motivation for and outline of this thesis

The roots of *Rubia tinctorum* (madder) are the source of a natural dye (alizarin) and they have been used to dye textiles in many parts of the world since ancient times. At the end of the 19th century the use of madder for dyeing rapidly declined due to the invention of synthetic alizarin and its large-scale production[1,13,108]. Nowadays there is a renewed interest in dyes from madder root, due to different reasons.

1. Increasing costs of the production of synthetic alizarin. The formed polluting side products have to be removed from the final product which takes nowadays already more than 50% of the production costs and this will increase due to the growing environmental restrictions.
2. Environmental concerns. During the synthesis of alizarin a lot of polluting side products are formed (1000 mass-%), like carbonised tar, polluted sulphuric acid and base, large amount of salts and breakdown products of anthraquinones.
3. Renewed interest in natural dyes.
4. The need for potential alternative crops to create a greater variety in agricultural products.

An important element in the revitalisation of madder is the preparation of a dye formulation, which can compete in quality and price with synthetic alizarin.

For the development of an economically feasible industrial process for the large-scale production of a dye preparation from madder root, research has been performed on madder root. Good methods must be developed for the isolation, purification and analysis of alizarin and other anthraquinones. In chapter 1 of this thesis, an overview is given about the available literature information on *Rubia tinctorum*. A HPLC procedure has been developed for the separation of anthraquinones in a madder root extract. On-line identification of the

anthraquinones in madder root is important for the fast analysis of the main anthraquinones. The development of such a procedure is reported in chapter 2. After identification of the most important anthraquinones, different approaches were investigated to increase the extraction yield of alizarin from madder root (chapter 3). Beside alizarin also the amount of lucidin present or formed has to be taken into account. Because of the mutagenity of lucidin, this compound is not desirable in a dye preparation. The formation of lucidin has to be avoided or the lucidin present has to be eliminated. This is described in chapter 3. Different madder cultivars were screened for their content of the most important anthraquinones (chapter 4). For this screening a fast and reliable quantification method had to be developed, which is also described in chapter 4. Finally alizarin must be isolated. The isolation procedure must achieve an extraction yield of ≈90%, be cheap, not labour intensive, not use toxic solvents and have few process steps. An overview of different approaches, which were used for the isolation of alizarin from the rest of the plant material and a discussion of their usefulness, is given in chapter 5.

Chapter 2

High-pressure liquid chromatography coupled on-line with UV, diode-array or mass spectrometric detection for the separation and identification of anthraquinones in roots of *Rubia tinctorum* L.*

2.1 Introduction

Beside the main anthraquinone, alizarin **8**, 36 other anthraquinones have been reported from *Rubia tinctorum* [1,13,28,108]. The anthraquinones found in *Rubia tinctorum* differ in the nature of the substituents and the substitution pattern (Appendix A). Several screening methods for anthraquinones in *Rubia tinctorum* extracts, based on reversed-phase high pressure liquid chromatography (RP-HPLC), have been described in the literature[40,41,46,51,56,70]. In general, HPLC analysis is based on separation and detection of the aglycones. The madder extract is hydrolysed and the total quantity of alizarin **8** is determined[41,56]. A few RP-HPLC methods

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*A part of this chapter is submitted as:
Goverdina C.H. Derksen, Harm A.G. Niederländer, Teris A. van Beek, High-performance liquid chromatography-mass spectrometry of anthraquinones from *Rubia tinctorum* L., *J. Chromatograpr. A*, submitted.

describe the simultaneous analysis of both glycosides and aglycones[40,51,70]. In all cases the anthraquinones are detected with ultra violet-visible light (UV-VIS) spectroscopy after HPLC separation.

For nearly a decade coupling of an HPLC to a mass spectrometer (MS) is an important method for rapid identification of compounds in plant extracts. There are different types of interfaces available for coupling HPLC to MS in order to accomplish nebulization and vaporization of liquid, ionisation of sample, removal of excess solvent, and transfer of the ions into the mass analyser[109]. These include electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) which are used by different authors for the ionisation of phenols and their glycosides[110-120]. Literature is available on high performance liquid chromatography-mass spectrometry (HPLC-MS) of phenolic naphtoquinones and hydroxyfluorenones[113], xanthones[118,119], flavonoids[111,112,116,117], naphthalenes[117], gallotannins and ellagitannins[116], naphtodianthrones[111] and their glycosides[111,114,117-120]. In contrast to phenols like flavonoids, only few publications are available on the HPLC-MS detection of anthraquinones. Nindi *et al.* described an HPLC-MS analysis for the components of a leaf extract of *Rhamnus prinoides*. They applied ESI with both positive (PI) and negative ionisation (NI), utilizing four different additives. They succeeded to ionise two out of four anthraquinones present in the leaf extract[117]. Mueller *et al.* made use of an APCI interface for the NI of anthraquinones in whole plant extracts. Separation of the components was accomplished with an HPLC-RP-column with a gradient system with the eluents ammonium acetate (10 mM) in water and acetonitrile (40 to 72% ACN over 16 min). They identified the anthraquinone aglycones emodin, chrysophanol and physcion[115].

Due to the large number of different anthraquinones that have been isolated and identified from *Rubia tinctorum* by different authors, a method for unambiguous on-line identification of anthraquinones in plant extracts is essential. In this study an HPLC method was developed for the separation, identification and quantitation of the anthraquinones in a madder root extract.

2.2 Experimental

2.2.1 Chemicals

Acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from LAB-SCAN Analytical Sciences (Dublin, Ireland). Ultra pure water was obtained from a combined Seradest LFM 20 Serapur Pro 90 C apparatus (Seral, Ransbach, Germany). All HPLC solvents were degassed by vacuum filtration over a 0.45 µm membrane filter (Type RC,

Schleicher & Schuell) prior to use. Ammonium formate, ammonium acetate, triethylamine, formic acid, and EDTA disodium salt dihydrate were obtained from Acros (Geel, Belgium), ammonia and acetic acid from Merck (Darmstadt, Germany). Reference compounds were obtained as described in chapter 4.

2.2.2 Extraction and sample preparation

Dried and powdered three year old root material of *Rubia tinctorum* (2.5 g) was refluxed with 100 ml water-ethanol (1:1)(v/v). After 3 hours the suspension was filtered under reduced pressure on a Büchner and a sample of 500 µl of the filtrate was taken. The remainder of the filtrate was evaporated to dryness under reduced pressure. The resulting extract was suspended in 100 ml 2% H_2SO_4 (v/v) solution and refluxed for 48 hours. At t = 0, ½, 1, 2, 4, 12, 24, 48 hours a sample of 500 µl was taken. All samples (500 µl) were diluted with 2000 µl water-methanol (1:1)(v/v). The diluted samples were filtered over a 0.45 µm membrane filter and analysed by HPLC.

2.2.3 HPLC-conditions

HPLC separation was carried out at room temperature on an Alltima end-capped C_{18} column, 100 Å pore size, 5 µm particle size, L 250 mm, ID 4.6 mm column equipped with a pre-column. Prior to use solvents were filtered over a 0.45 µm, ø 50 mm membrane filter (Type RC, Schleicher & Schuell) and sonicated for 15 min in a Retsch Transsonic 570 (Emergo, Landsmeer, The Netherlands). Chromatography was carried out using two solvents: A-ammonium formate/formic acid buffer (0.2 M, pH=3) + EDTA (30 mg $litre^{-1}$) and B-acetonitrile. A linear gradient programme was applied: 0-6 min 27%B; 6-20 min linear increase to 60%B; 20-23 min hold on 60%B; 23-25 min linear increase to 70%B; 25-35 min hold on 70%B, 35-40 min linear decrease to 27%B. The flow-rate during the experiment was 1.0 ml min^{-1}. Peaks were detected at 254 nm.

2.2.4 HPLC-DAD instrumentation

Analysis was performed with an HPLC system consisting of a 450G ternary low-pressure gradient HPLC pump and a 340S diode array detector. Injections were made by a Basic Marathon autosampler equipped with a 20 µL loop. The system was connected to a Pentium computer with CM PCS1 Chromeleon system control (Separations, H.I-Ambacht, The Netherlands).

2.2.5 *HPLC-MS instrumentation*

The HPLC system consisted of TSP SpectraSYSTEM including a SN4000 controller, an HPLC quaternary pump P4000, an autosampler AS3000 and a UV2000 detector. After UV detection the eluate was introduced into the MS system. A Finnigan LCQ ion trap mass spectrometer was equipped with a Finnigan electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) interface. Data were processed by Finnigan Xcalibur software system on a Gateway computer (ThermoQuest, Breda, The Netherlands).

2.2.6 *Continuous flow analysis*

For tuning, standard solutions of alizarin (1.3 mM alizarin in methanol) and ruberythric acid (1.3 mM ruberythric acid in methanol) were used. These solutions were separately introduced into the interface by continuous flow analysis using a syringe (Hamilton, Nevada) at a flow-rate of 3 μl min^{-1}. Averaged spectra were recorded over a period of 3 min: the scan range was 50-2000 *m/z* at a scan rate of 0.50 s. The syringe and outlet of the HPLC apparatus were coupled to the MS interface by a T-piece. During continuous flow analysis of alizarin or ruberythric acid, the eluent and flow-rate that will be used during on-line HPLC-MS measurements was simultaneously added with the HPLC pump. This eluent consisted of 73% solvent A and 27% solvent B. Adding the HPLC eluent mimics the eluent/sample composition, which enters the MS-interface during on-line HPLC-MS analysis. Both PI and NI were used. When the ESI interface was used, various additives were introduced (by continuous flow from a second parallel attached syringe at 3 μl min^{-1}) into the anthraquinone flow to promote the formation of ions. The additives were in order of increasing acidity: 6.25 mM triethylamine, 5% NH_3 in water, 25 mM ammonium acetate, acetic acid (2 ml litre^{-1}), trifluoroacetic acid (0.5 ml litre^{-1}).

2.2.7 *HPLC-ESI-MS analysis*

Because the sensitivity of ESI decreases rapidly at higher flow rates, the flow-rate was kept at 0.2 ml min^{-1} and HPLC separation was obtained with a smaller Alltima end-capped C-18 column (L 250 mm, ID 2.1 mm, 100 Å pore size, 5 µm particle size). After HPLC separation, 5% NH_3 in water was added at a flow-rate of 3 μl min^{-1} by a syringe which was connected by a T-piece.

The ESI parameters in negative ion experiments were as follows: spray voltage 4.5 kV (applied to the spray tip needle), sheath gas (N_2) 79 arbitrary units (arb.), auxiliary gas (N_2) 55 arb. units, heated capillary temperature 200°C and capillary voltage –12.00 V.

2.2.8 HPLC-APCI-MS analyses

The conditions for the APCI interface were as follows: vaporizer temperature 450°C, sheath gas 80 arb. units, auxiliary gas 49 arb., discharge current 10 μA, heated capillary temperature 150 °C. The mass spectrometer was operated in the NI mode. Nitrogen was used as the nebulizing gas. The flow-rate was kept at 1.0 ml min^{-1} when extracts were analysed by liquid chromatography. No additive solution was added post-column.

2.2.9 MS-MS experiments

During MS^2 experiments helium was used as collision gas. Only a single parent ion was kept in resonance (isolation width 1-3 *m/z*), all other ions were ejected from the trap without mass analysis. The ion was then agitated and allowed to fragment by collision-induced dissociation (CID). The collision energy was adjusted experimentally to give >90% yield of fragmentation by varying the relative collision energy from 10 to 40%. During MS^n measurements ($n \geq 3$) this procedure was repeated for one of the daughter ions.

2.3 Results and discussion

2.3.1 Continuous flow analysis of pure standards

The initial continuous flow analysis studies were aimed at determining the conditions under which it is possible to ionise anthraquinone aglycones and anthraquinone glycosides present in madder roots. The two interfaces ESI and APCI were compared. In order to determine the optimum ionisation conditions for the anthraquinones, alizarin **8** (an anthraquinone aglycone) and ruberythric acid **2** (an anthraquinone glycoside) were used as test compounds (table 2.1). Alizarin **8** (1.3 mM alizarin in methanol) and ruberythric acid **2** (1.3 mM ruberythric acid in methanol) were separately introduced into the ESI interface and the APCI interface. During introduction of alizarin or ruberythric acid, the eluent that will be used for on-line HPLC-MS measurements was added with an HPLC pump.

With the ESI probe, continuous flow analysis of alizarin **8** without further addition of additive did not give rise to any detectable ionisation in the PI and NI mode. To aid the formation of ions various additives (see experimental) were used. The highest signal intensity for alizarin with the ESI interface was obtained in the NI mode with a triethylamine additive solution. Triethylamine is a base but a major drawback of this additive in MS applications is that it interferes with the ionisation of other samples and molecules over a long period of

time. Therefore, ammonia instead of triethylamine was chosen as additive solution for the analysis of alizarin **8**. The results of the negative ionisation of alizarin **8** with the ESI probe are given in figure 2.1a. The molecular ion of alizarin **8** $[M-H]^-$ with an *m/z* of 239.3 is the base peak of the spectrum and the MS was tuned for maximum response on this peak. In figure 2.1b the MS^2 spectrum for fragmentation of the molecular ion at *m/z* 239.3 is depicted. The MS^2 signal of *m/z* 239.3 gives a base peak $[M-H-C=O]^-$ at *m/z* 211.2.

Alizarin **8** was also introduced into the APCI interface (in the NI mode) by continuous flow analysis, adding only the eluent that will be used for HPLC-MS measurements. The results of this experiment are depicted in figure 2.1c and the MS^2 spectrum for fragmentation of the molecular ion at m/z 239.2 in figure 2.1d. It turned out that for the ionisation of the compounds with the APCI probe no additive, other than the buffer present in the HPLC eluent, was required. The fact that no additional additive solution had to be added post-column, made the analysis of alizarin **8** with the APCI interface easier than with the ESI interface. Both ESI and APCI interfaces were tested for the ionisation of alizarin **8** in PI mode (with or without addition of various additives) but no ionisation of the aglycone occurred.

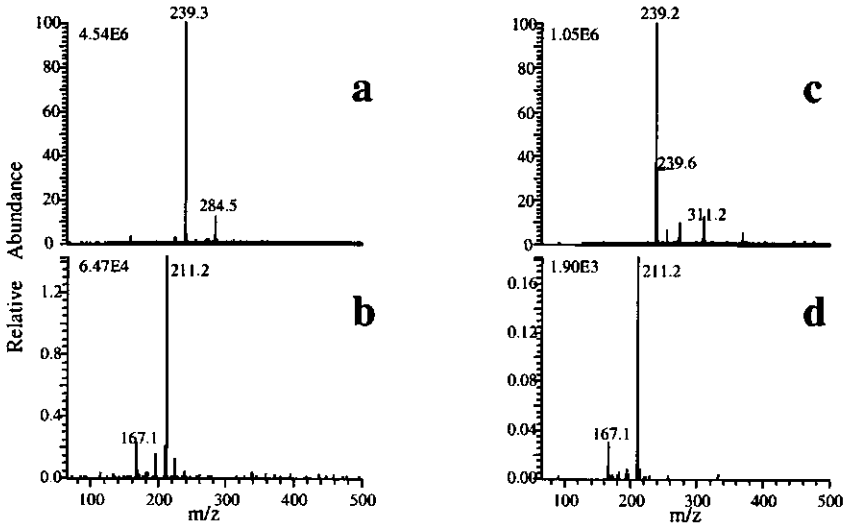


Figure 2.1. *Figure 2.1a is parent ion spectrum of alizarin 8. Figure 2.1b is the CID daughter ion spectrum of the fragment at m/z 239 of figure 2.1a. Both spectra were obtained with ESI in NI mode and as additive ammonia was used. Figure 2.1c is parent ion spectrum of alizarin. Figure 2.1d is the CID daughter ion spectrum of fragment m/z 239 of figure 2.1c. Both spectra were obtained with APCI in NI mode.*

The same experiments were carried out with a solution of ruberythric acid **2**. Using the ESI interface the highest signal intensity was obtained in the PI mode with ammonium acetate as additive or with ammonium formate used as buffer present in the HPLC eluent. The result of the positive ionisation of ruberythric acid **2** is given in figure 2.2a. The main peak is the ion $[M+NH_4]^+$ at *m/z* 552.0 but also some other abundant ions, such as $[2M+NH_4]^+$ at *m/z* 1085.9, $[3M+NH_4]^+$ at *m/z* 1619.6 and [M–primeverose+H]$^+$ at *m/z* 241.2, were detected. Additional spectra were obtained by applying a series of voltage offsets (20 and 40V) to investigate the CID of the major ions of $[M+NH_4]^+$, $[2M+NH_4]^+$ and $[3M+NH_4]^+$ which resulted in the formation of *m/z* 241.3 and *m/z* 552.0.

The result of continuous flow analysis of ruberythric acid **2** with ammonia as additive in the NI mode is depicted in figure 2.2b. The most abundant ion was [M–H]$^-$ at *m/z* 533.1 but also the ions [M–primeverose–H]$^-$ at *m/z* 239.3 and [2M–H]$^-$ at *m/z* 1066.9 were detected. CID of the major ion at *m/z* 533.1 gave a fragment at *m/z* 239.2. The signal intensity of the observed peaks (with ammonia as additive) was lower in the NI mode than in the PI mode (with ammonium acetate as additive). Nevertheless later experiments were performed in the NI mode, given the fact that the anthraquinone aglycones can only be ionised in the NI mode. The ionisation of the anthraquinone glycosides in the NI mode was sufficiently strong to be applicable during on-line MS experiments. Ionisation of ruberythric acid, in both negative and positive ionisation mode, gave a pattern of M, 2M and 3M molecular ions, which does not disappear by increasing the temperature of the heated capillary up to 300°C. This ionisation pattern is characteristic for the anthraquinone glycosides and is not seen for the anthraquinone aglycones. This is due to the sugar part of these glycosides.

The result of the continuous flow analysis of ruberythric acid **2** with the APCI interface in the NI mode is depicted in figure 2.2c. No additive, other than the additive present in the added HPLC eluent, was needed. The main ion that was formed was [M–primeverose–H]$^-$ at *m/z* 239.4 and a minor ion was [M–H]$^-$ at *m/z* 533.2.

Continuous flow experiments of a ruberythric acid **2** solution with the ESI interface gave [M–H]$^-$ as most abundant ion, whereas with the APCI interface the most abundant ion was [M–primeverose–H]$^-$. These continuous flow results confirm that ESI is clearly a "soft ionisation" technique, as compared to APCI.

None of the MS or MS^n spectra (applying ESI or APCI with any additive in NI or PI mode) showed an *m/z* signal corresponding to an ion of ruberythric acid **2** minus xylose. Xylose is the terminal sugar of the sugar primeverose. It can be concluded that these ionisation techniques are not suitable for determining the sugar sequence of ruberythric acid.

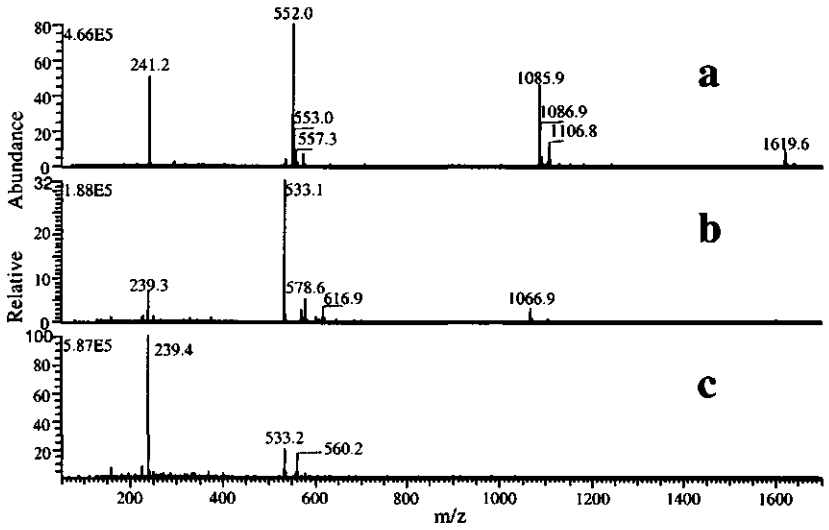


Figure 2.2. *Figure 2.2a, 2.2b and 2.2c are ruberythric acid* **2** *parent ion spectra. Figure 2.2a was obtained with ESI in PI mode and as additive ammonium acetate was used. Figure 2.2b was obtained with ESI in NI mode and as additive ammonia was used. Figure 2.2c was obtained with APCI in NI mode.*

2.3.2 LC-MS analysis of a reference sample

For the development of an HPLC separation of the anthraquinone glycosides and aglycones in *Rubia tinctorum* extracts, a reference sample containing the most reported anthraquinones in literature was prepared. This reference sample consisted of the glycosides: lucidin primeveroside **1** and ruberythric acid **2** and the aglycones: lucidin **7**, alizarin **8**, purpurin **10** and quinizarin **11**. Figure 2.3 and figure 2.4 show the HPLC trace of this reference sample on respectively a reversed phase column with an ID of 4.6 mm and a flow of 1.0 ml min^{-1} and a column with an ID of 2.1 mm with a corresponding flow of 0.2 ml min^{-1}.

After HPLC separation the individual analytes were introduced on-line via an ESI interface to the mass spectrometer. Ammonia was added post-column which is necessary for the negative ionisation of both the glycosides and aglycones, as was established in the previous section based on continuous flow analysis. The ammonia was added post-column to avoid interference with the separation of the components. The UV-254 nm and total ion current (TIC) traces are depicted in figure 2.3. All the components in the standard mixture were well detectable at 254 nm. Ionisation of all the anthraquinones was not equally easy. Base peak spectra of these components did not show an improved signal to noise ratio as

compared to the related TIC spectra. The glycosides and lucidin were easily ionised, whereas the alizarin and purpurin peaks were less detectable and the peak for quinizarin could not be distinguished from the noise at all. Both glycosides were well detectable in the NI mode with ammonia as additive. Thus it was not necessary to perform an additional analysis in the PI mode.

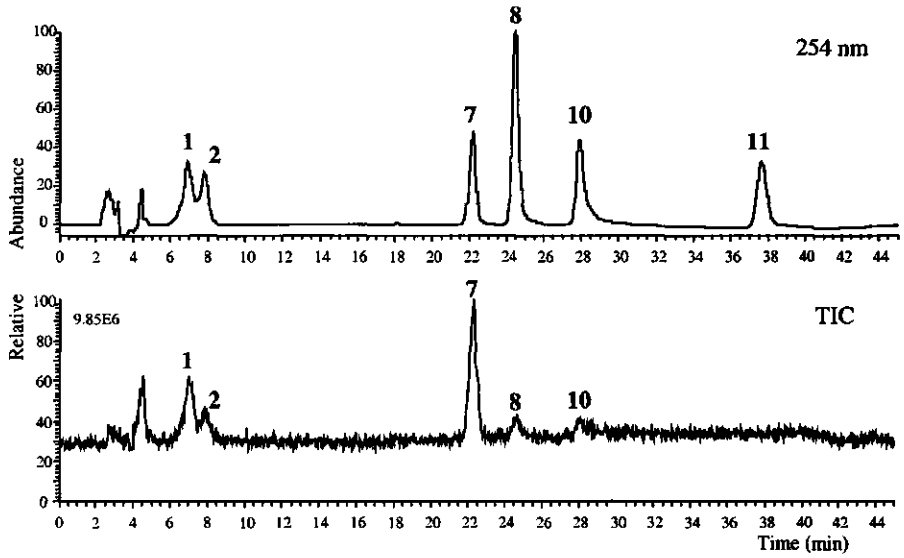


Figure 2.3. *UV (254 nm) and TIC traces of anthraquinone reference sample obtained with ESI interface in NI mode, lucidin primeveroside* ***1****, ruberythric acid* ***2****, lucidin* ***7****, alizarin* ***8****, purpurin* ***10****, quinizarin* ***11****.*

The same standard mixture was also sampled with the APCI interface. An advantage of this interface is that no post-column addition of any additive solution is necessary (i.e. the HPLC additive suffices). Another advantage of APCI over ESI is its compatibility with a higher eluent flow-rate. The UV-254 nm and TIC traces are depicted in figure 2.4. All the components in the standard mixture are clearly observed by UV detection at 254 nm. The TIC trace of the standard anthraquinone solution analysed in the APCI mode (figure 2.4) is quite similar to the TIC trace in the ESI mode (figure 2.3). It can therefore be concluded that the ionisation of the different anthraquinones in the APCI mode is comparable with ionisation in the ESI mode.

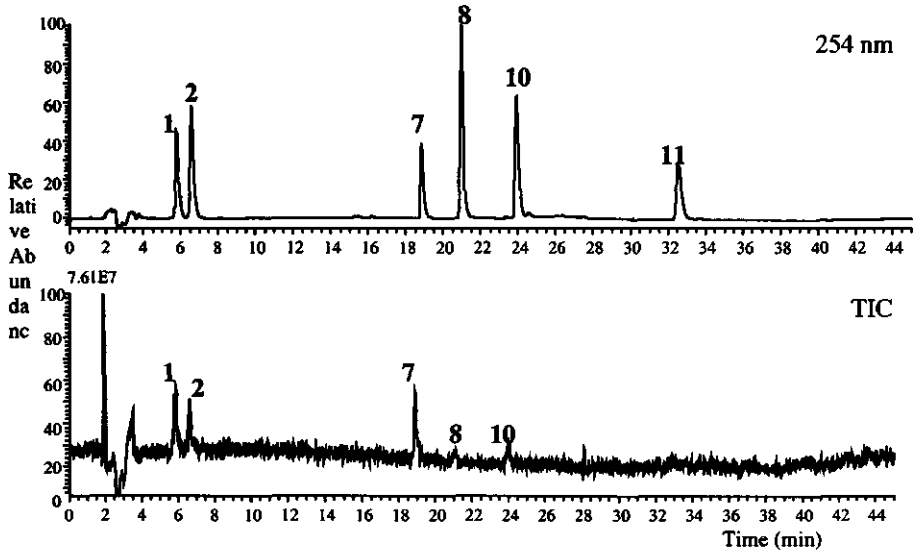


Figure 2.4. *UV (254 nm) and TIC traces of anthraquinone reference sample obtained with the APCI interface in NI mode, lucidin primeveroside 1, ruberythric acid 2, lucidin 7, alizarin 8, purpurin 10, quinizarin 11.*

The fact that not all the anthraquinones can be equally well ionised must be due to differences in substitution pattern. To investigate the influence of different substitution patterns on the ionisation, eight pure anthraquinones were separately injected and analysed (using ESI with ammonia additive in NI mode). All the analysed anthraquinones gave a clearly visible peak in the UV chromatogram at 254 nm, this in contrast to the TIC. Purpurin **10**, lucidin **7**, xanthopurpurin **9**, 2,6-dihydroxyanthraquinone **16** and alizarin **8** were easily ionised. 2-Hydroxymethylanthraquinone **13**, anthraquinone **14**, 1,8-dihydroxyanthraquinone **15**, and quinizarin **11** could not be detected at all. Summarising, anthraquinones with a hydroxy group γ to the carbonyl (position 2, 3, 6 and/or 7) could be ionised. Anthraquinones with only (a) hydroxy group(s) β to the keto function of the molecule (position 1, 4, 5 and/or 8) could not be ionised. These results agree with the results of Nindi *et al.*, who reported that the anthraquinone emodin (1,6,8-trihydroxy-3-methylanthraquinone) was detectable due to the hydroxy group at position 6, but the anthraquinones chrysophanol (1,8-dihydroxy-3-methylanthraquinone) and physcion (1,8-dihydroxy-3-methyl-6-methoxyanthraquinone) in an extract from *Rhamnus prinoides* were not detectable[117]. A hydroxy group β to the carbonyl has a much higher pKa value than a hydroxy group γ to the carbonyl group due to an internal hydrogen bond between the β-OH and the carbonyl group. In case of alizarin these

pKa's are respectively 12 and 8.2. In this research a 0.1 M acidic buffer solution (HPLC flow) was mixed post-column with a 5% ammonia solution, which resulted in an overall pH of 9. Under these conditions a hydroxy group at the 2-position is deprotonated, which is confirmed by the colour change of the solution (yellow→purple). The solution has insufficient basicity to abstract a proton, the common route in negative ionisation, from an anthraquinone with only hydroxy group(s) β to the keto group[113]. Furthermore, 2-hydroxymethylanthraquinone **13** cannot be ionised because of the high pKa value of the hydroxymethyl. This hydroxy group is not a phenolic hydroxy group and the corresponding anion is not stabilised by mesomerism. So ionisation of lucidin **7** is due to the hydroxy group at R_3 and not due to the hydroxymethyl group at R_2.

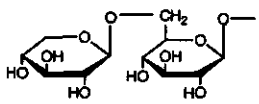
2.3.3 LC-MS analysis of an ethanol-water extract of madder roots

An ethanol-water (1:1) extract of *Rubia tinctorum* was analysed with the above-developed HPLC-ESI-MS method. The UV-254 chromatogram and mass spectra of the numbered peaks are depicted in figure 2.5. The glycosides lucidin primeveroside **1** and ruberythric acid **2** are the major anthraquinone components in the extract. Lucidin primeveroside **1** showed ions at $[M-H]^-$ *m/z* 563.0, $[2M-H]^-$ *m/z* 1126.9, $[3M-H]^-$ *m/z* 1691.7, $[M-primeverose]^-$ *m/z* 269.2, and $[M+HCOO^-]^-$ *m/z* 608.7. Ruberythric acid **2** showed ions at $[M-H]^-$ *m/z* 533.1, $[2M-H]^-$ *m/z* 1066.8, $[3M-H]^-$ *m/z* 1600.7, $[M-primeverose]^-$ *m/z* 239.3, and $[M+HCOO^-]^-$ *m/z* 578.4.

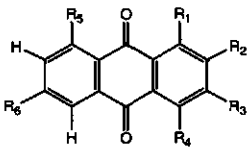
Two unknown anthraquinone peaks appeared at t=15.6 (peak **5**) and t=16.0 (peak **6**) min. The extract was also analysed with high-pressure liquid chromatography diode-array detection (HPLC-DAD). For peak **5** absorption maxima were found at 259 and 494 nm and for peak **6** at 248, 288 and 420 nm. In figure 2.5 the mass spectra of both peaks are depicted. Peak (**5**) at t=15.6 min had a base peak at *m/z* 299.1 while peak (**6**) at t=16.0 min had a base peak at *m/z* 283.1. Assuming that both anthraquinones ionise like the anthraquinones of the standard solution, these peaks would correspond with the $[M-H]^-$ ions. In that case the corresponding molecular masses of both peaks were respectively 300 and 284. MS^2 spectra of these base peaks showed a fragment at *m/z* 255.3 for the parent ion at *m/z* 299.1 and a fragment at *m/z* 239.3 for the parent ion at *m/z* 283.1. This agreed with decarboxylation of the parent ion (i.e. $[M-H-CO_2]^-$). The values found with MS, MS^2 and DAD were compared with values found in literature[13,121]. From this comparison, the two unknown peaks were most probably pseudopurpurin **5** (2-carboxy-1,3,4-trihydroxyanthraquinone, MW=300, Rt=15.6 min) and munjistin **6** (2-carboxy-1,3-dihydroxyanthraquinone, MW=284, Rt=16.0 min) (table 2.1). To confirm this assignment, the ethanol-water (1:1)(v/v) extract was refluxed in sulphuric acid and the solution was analysed with HPLC-MS. If the two peaks were really

pseudopurpurin **5** and munjistin **6** these components should give purpurin **10** and xanthopurpurin **9** after decarboxylation with sulphuric acid. Indeed, after acidic hydrolysis the two peaks for pseudopurpurin **5** and munjistin **6** had disappeared and two new peaks had appeared in the chromatogram. The elution times and masses of these two peaks corresponded with those of commercially available purpurin **10** (MW=256) and synthesised xanthopurpurin **9** (MW=240).

***Table 2.1** Anthraquinone structures, the numbers in the table correspond with the numbers in the text and in the figures and vice versa.*



primeverose



anthraquinone aglycone moiety

No:	Name	R_1	R_2	R_3	R_4	R_5	R_6	MW
1	Lucidin primeveroside	OH	CH_2OH	primeverose	H	H	H	564
2	Ruberythric acid	OH	primeverose	H	H	H	H	534
3	Lucidin glucoside	OH	CH_2OH	glucose	H	H	H	432
4	Alizarin glucoside	OH	glucose	H	H	H	H	402
5	Pseudopurpurin	OH	COOH	OH	OH	H	H	300
6	Munjistin	OH	COOH	OH	H	H	H	284
7	Lucidin	OH	CH_2OH	OH	H	H	H	270
8	Alizarin	OH	OH	H	H	H	H	240
9	Xanthopurpurin	OH	H	OH	H	H	H	240
10	Purpurin	OH	H	OH	OH	H	H	256
11	Quinizarin	OH	H	H	OH	H	H	240
13	2-Hydroxymethyl anthraquinone	H	CH_2OH	H	H	H	H	238
14	Anthraquinone	H	H	H	H	H	H	208
15	1,8-Dihydroxy anthraquinone	OH	H	H	H	OH	H	240
16	2,6-Dihydroxy anthraquinone	H	OH	H	H	H	OH	240

The UV-254 nm chromatogram of the ethanol-water extract (figure 2.5) shows a large peak, not numbered, at *t*=4.5 min. This peak was separated by means of HPLC with an eluent with a higher water content in a number of minor peaks (not shown) and analysed with DAD. Only one of these minor peaks turned out to be an anthraquinone, which was not further analysed.

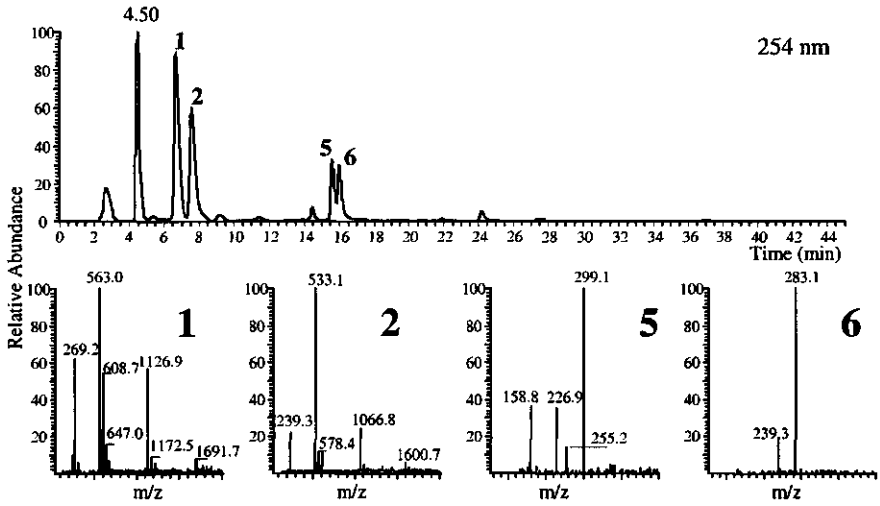


Figure 2.5. *UV (254 nm) trace of a crude extract of Rubia tinctorum roots and corresponding parent ion mass spectra of the peaks, obtained with an ESI interface in NI mode, lucidin primeveroside 1, ruberythric acid 2, pseudopurpurin 5, munjistin 6.*

During the first hour of the refluxing of the extract with sulphuric acid two peaks appeared at Rt=10.4 min (peak **3**) and Rt=13.1 min (peak **4**). In figure 2.6 the chromatogram and corresponding MS and UV-VIS spectra are depicted. The UV-VIS spectra of both unknown peaks were identical to the UV-VIS spectra of respectively lucidin primeveroside **1** (maxima at 247, 266 and 406 nm, match factor 999.25) and ruberythric acid **2** (maxima at 231, 260 and 416 nm, match factor 999.15). CID of the ions at *m/z* 431.0, 476.7 and 862.8 of the compound eluting at Rt=10.4 min (**3**) gave a fragment ion at *m/z* 269.1. CID of the ion at *m/z* 401.0 of the compound eluting at Rt=13.1 min (**4**) gave a fragment ion at *m/z* 239.1. From these spectra it was concluded that the two peaks were the mono glucosides of lucidin **7** and alizarin **8**, respectively lucidin glucoside **3** (Rt=10.4 min) and alizarin glucoside **4** (Rt=13.1 min). Lucidin glucoside **3** gave the ions $[M-H]^-$ at *m/z* 431.0, $[2M-H]^-$ at *m/z*

(Rt=13.1 min). Lucidin glucoside **3** gave the ions $[M-H]^-$ at *m/z* 431.0, $[2M-H]^-$ at *m/z* 862.8, $[M-glucoside]^-$ at *m/z* 269.1, $[M+HCOOH-H]^-$ at *m/z* 476.7 and $[M-glucose-H]^-$ at m/z 269.1. Alizarin glucoside **4** gave the ions $[M-H]^-$ at *m/z* 401.1 and $[M-glucose-H]^-$ at m/z 239.1.

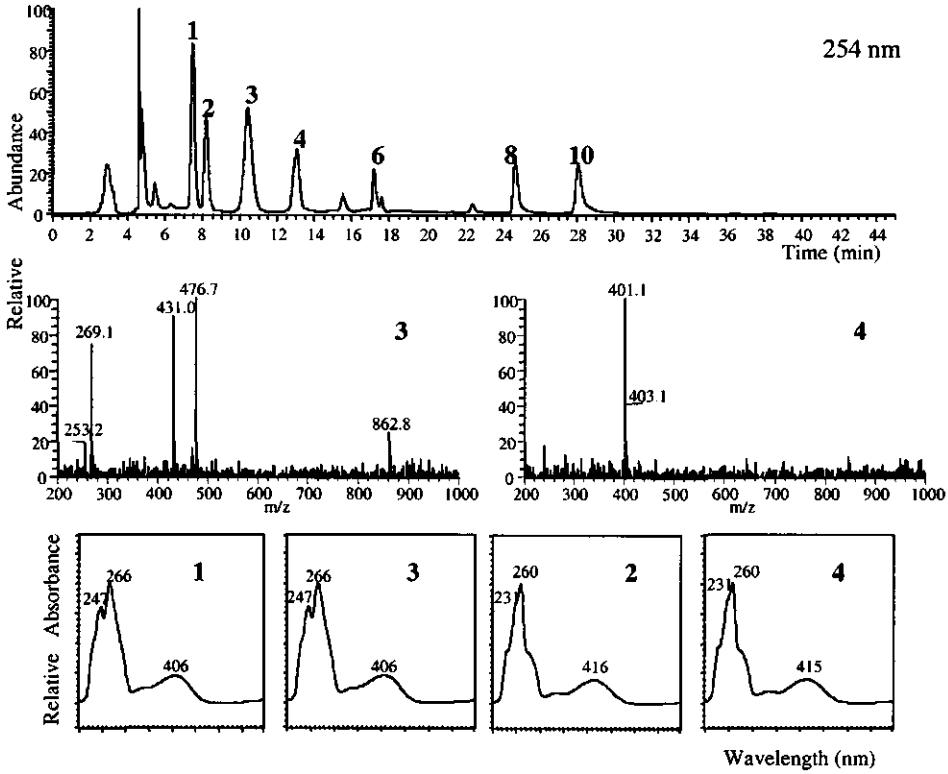


Figure 2.6 *UV (254 nm) trace of an acidic hydrolysate of an aqueous alcoholic extract of Rubia tinctorum roots (after 30 min). The parent ion mass spectra obtained with ESI interface in the NI mode and the UV-VIS spectra of the peaks 3 and 4 are depicted, lucidin primeveroside **1**, ruberythric acid **2**, lucidin glucoside **3**, alizarin glucoside **4**, munjistin **6**, alizarin **8**, purpurin **10**.*

2.3.4 *RP-HPLC separation of anthraquinones*

In an earlier article[70] a method was developed for the separation of anthraquinones glycosides and aglycones in madder root. The separation was performed with a water-acetonitrile gradient. However on-line mass spectrometric detection pointed out the presence of carboxylic anthraquinones (pseudopurpurin **5** and munjistin **6**) in a madder root extract. These carboxylic acids are partially deprotonated at the pH of a water-acetonitrile mixture. If the solution is not acidic enough these compounds will eluete at the dead time. At an acidic pH these compounds are protonated. The best separation of the carboxylic anthraquinones was obtained with a formiate buffer of pH=4. Finally a buffer of pH=3 was chosen because at this pH also the compounds xanthopurpurin **9** and purpurin **10** were separated. For adequate comparison of the peaks in an HPLC analysis of a madder root extract buffering of the eluent is necessary.

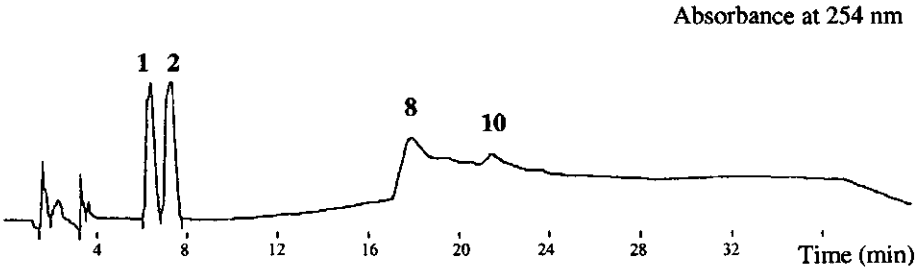


***Figure 2.7** An HPLC (UV 254 nm) trace obtained of an anthraquinone reference sample if no EDTA was added to solution A of the eluent. The reference sample consisted of lucidin primeveroside **1**, ruberythric acid **2**, alizarin **8** and purpurin **10**.*

According to the literature low metal contamination of the column can give chelate formation of compounds to geminal silanols. The metal contamination probably arises from the stainless-steel instruments and frits when the column is eluted or stored with neat acetonitrile or methanol[122-125]. Alizarin is used for dyeing of textile. During the dyeing process alizarin forms a complex with metal ions like aluminium, calcium and iron. During the formation of the dye complex a calcium ion reacts with the 2-hydroxyl while an aluminium ion forms a complex between the 1-hydroxyl and the carbonyl group. The ratio between both alizarin and aluminium and between alizarin and calcium is 2:1 (§1.5.2)[17,85].

A complex formation could probably also be established between geminal silanol groups, metal ions and alizarin in RP-HPLC chromatography. To remove the metal EDTA was added to the eluent. The complex formation was successfully avoided. If no EDTA was added a chromatogram as depicted in figure 2.7 was obtained. Notwithstanding the peak doubling the glycosides could still be separated and identified. However the aglycones elueted as unidentifiable broad peaks.

2.4 Conclusion

An HPLC method was developed for the simultaneous separation of anthraquinone glycosides, anthraquinone carboxylic acids and anthraquinone aglycones in extracts of madder root. On-line UV (254 nm), diode-array and mass spectrometric detectors were used for the identification of the main anthraquinones in madder root. Two useful HPLC-MS methods were developed for the identification of anthraquinones. One uses an APCI interface and the other uses an ESI interface. Both methods are based on negative ionisation of the anthraquinone glycosides and aglycones. Positive ionisation did not give rise to ionisation of the aglycones. However the glycosides can also be analysed in positive mode. APCI is easier to apply than ESI, because no base has to be added post-column to induce ionisation of the anthraquinones and a higher flow-rate of the eluent entering the interface is allowed. A disadvantage of APCI, however, is that the ionisation of the compounds is not as soft as with ESI. Especially with the ionisation of less stable compounds like the glycosides this is a disadvantage, because it leads to more pronounced in-source fragmentation making the parent ion more difficult to observe. Co-eluting salt components can suppress or enhance the analyte signal during the ionisation process[126]. In this research on-line UV detection is much more sensitive for analysis of anthraquinones than on-line mass detection. Consequently, HPLC-UV is the method of choice for quantitative analysis of anthraquinones.

The HPLC-MS method used in this research could not be used for the ionisation of anthraquinones with only hydroxy group(s) β to the keto function. For ionisation of these anthraquinones in the ESI mode an additive has to be added post-column, which increases the pH of the solution above 10. Under the experimental conditions the pH of the eluent after post-column addition of ammonia was 9.0. With a four times diluted buffer concentration the pH of the solution was 10.3 after addition of ammonia, still too low for ionisation of β-hydroxyls. To preserve the separation of the different anthraquinones it is not possible to change the buffer for water or to develop an eluent system at higher pH. So the developed HPLC-MS method is not suitable for the identification of all the anthraquinones. According to the literature (appendix A) most identified anthraquinones in madder root contain a hydroxy group at the 2 or 3 position γ to the keto function, which can be ionised by the post-column

addition of ammonia. However mass spectrometry is not the only method available for detection. Anthraquinones that can not be detected with ms can still be recognised as a possible anthraquinone by UV-430 nm or DAD. All the pH values were measured before the addition of acetonitrile

HPLC-DAD and HPLC-MS are helpful for the identification of unknown anthraquinones in extracts. Time consuming isolation of anthraquinones becomes unnecessary. This is proven in this study with the identification of the anthraquinones: pseudopurpurin **5**, munjistin **6**, lucidin glucoside **3** and alizarin glucoside **4**. It can be concluded that the main anthraquinones in madder root are the glycosides lucidin primeveroside **1** and ruberythric acid **2** and the carboxylic anthraquinones pseudopurpurin **5** and munjistin **6**. Beside these also a small amount of the aglycones alizarin and purpurin was detected.

Chapter 3

Hydrolysis of anthraquinone glycosides from *Rubia tinctorum* L.*

3.1 Introduction

Nowadays the use and production of natural dyes becomes more popular due to the growing awareness for the environment. Increasing efforts are being made to revive madder cultivation and to increase the utilisation of a madder derived dye in the textile branch. For the formation of a commercially useful dye preparation from madder the glycoside ruberythric acid should be hydrolysed to the aglycone alizarin **8**, which is the main dye component. Beside ruberythric acid **2** another glycoside, lucidin primeveroside **1**, with the same sugar moiety as ruberythric acid **2** (primeverose) is present in madder root. A major drawback of the hydrolysis of madder root is the conversion of lucidin primeveroside **1** to the mutagenic aglycone lucidin **7**. Brown and Dietrich (1979) were the first to report that lucidin **7** is mutagenic[65]. Later different authors confirmed that this anthraquinone shows mutagenicity[40,49, 53,65,71,91,93-98].

In this chapter different procedures for the hydrolysis of ruberythric acid **2** from madder root into the useful dye component alizarin **2** are compared. Additionally the effect of these procedures on the formation of other anthraquinone aglycones, in particular lucidin **7** is studied.

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* A part of this chapter is submitted as:
Goverdina C.H. Derksen, Martijn Naayer, Teris A. van Beek, Anthony Capelle, Ingrid K. Haaksman, Henk A. van Doren and Æde de Groot, Production of anthraquinone dyes from madder root. Part I: The chemical and enzymatic hydrolysis of the anthraquinone glycosides, *Phytochem. Anal.*, submitted.

3.2 Experimental

3.2.1 Chemicals

The following enzyme preparations were used in this research: β-glucosidase (SIGMA), arabinofuranosidase A and B (DSM), fermizyme HA 1000 (DSM), multifect XL (Genecor), multifect CL (Genecor), multifect cellulase GC (R) (Genecor), xylanase (FFI), roxazym G2 (Hoffmann LaRoche), pectinex 100L (NOVO), pectinex AR (NOVO), pectinex BE 3L (NOVO), pectinex ultra SP-L (NOVO), pentopan 500BG (NOVO), bio-feed plus CT (NOVO). "Acetone powder" of tea leaves was a gift of Prof. Sakata, Faculty of Agriculture at Shizuoka Univeristy in Japan. Fresh tea leaves were collected at the greenhouses of Unifarm in Wageningen. Madder root was a gift of Rubia B.V. (Zuidbroek, The Netherlands). Other chemicals and reference compounds were obtained as described in chapter 2 and chapter 4, respectively.

3.2.2 HPLC analysis

The HPLC conditions were the same as described in § *2.2.3*. The HPLC-apparatus consisted of a Gilson 305 piston pump, Gilson 306 piston pump, Gilson 811C dynamic mixer and a Gilson 805 manometric module (Goffin-Meyvis, Bergen op Zoom, The Netherlands). The equipment was connected with a Gilson 234 auto injector (Goffin-Meyvis, Bergen op Zoom, The Netherlands) and a Shimadzu Corporation Chromatopac C-R3A integrator ('s-Hertogenbosch, the Netherlands). Detection was performed on a Gilson 116 UV detector (Goffin-Meyvis, Bergen op Zoom, The Netherlands). HPLC-DAD and HPLC-MS experiments were carried out as described in chapter 2.

3.2.3 Sample treatment

During the different experiments described in this article, samples of 500 μl were taken. Samples were removed with a pipette with a wide orifice to make sure that both dissolved compounds and precipitated compounds (anthraquinones) were taken from the suspension. All samples (500 μl) were diluted with 2000 μl water-THF-formic acid (1:1:0.005) v/v/v to dissolve both polar and apolar compounds, like anthraquinone glycosides and aglycones. The diluted samples were filtered over a 0.45 μm, ø 25 mm membrane filter (Type RC, Schleicher & Schuell) and analysed by HPLC.

3.2.4 Plant material, madder root (MR)

The roots were dried at 45°C in an oven with forced ventilation during 1 week. The dried roots were powdered in a Retsch Grindomix GM200 for 1 min at 7000 rpm, for 1 min at 8500 rpm and for 1 min at 10,000 rpm (Emergo, Landsmeer, The Netherlands).

3.2.5 Water-ethanol extract (WEE)

Dried and powdered madder root (MR) (2.5 g) was refluxed in 100 mL water-ethanol (1:1) (v/v). After 3 hours the suspension was filtered under reduced pressure on a Büchner funnel with a filterpaper (Schleicher & Schuell) after which a sample of 500 µl of the filtrate was taken. The remainder of the filtrate was lyophilised with a Christ Alpha 1-2 freeze dryer (Salm en Kipp bv, Breukelen, The Netherlands). In this article this extract is named water-ethanol extract (WEE)

3.2.6 Treatment with acid or base

Madder root (MR) (2.5 g) or water-ethanol extract (WEE) was suspended in a solution of 100 mL 1 M hydrochloric acid, 1 M sulphuric acid or 1 M sodium hydroxide, the suspension was heated up to 80°C or 100°C and left at this temperature for 48 hours. At t = 0, ½, 1, 2, 4, 12, 24, 48 hours a sample of 500 µl was taken.

3.2.7 The nature and kinetics of the acidic hydrolysis reaction

Madder root (MR, 2.5 g) or water-ethanol extract (WEE) was suspended in a solution of 100 mL 1 M sulphuric acid, 15 mg lucidin was added and the suspension was refluxed for 24 hours. A sample (500 µl) was taken.

Pure lucidin primeveroside (15 mg) was suspended in 100 mL of 1 M sulphuric acid and refluxed for 48 hours. At t = 0, ½, 1, 2, 4, 12, 24, 48 hours a sample of 500 µl was taken.

A water-ethanol extract (WEE) was suspended in a solution of 100 mL 1 M sulphuric acid, the suspension was refluxed for 24 hours, filtered under reduced pressure on a Büchner funnel and a sample of 500 µl of the filtrate was taken. The filtrate was refluxed another 24 hours and filtered under reduced pressure on a Büchner funnel and a sample of 500 µl of the filtrate was taken. Again the filtrate was refluxed 24 hours and filtered under reduced pressure on a Büchner funnel after which a sample of 500 µl of the filtrate was taken. Finally 15 mg of pure lucidin was added to the last filtrate and the suspension was refluxed for 24 hours. After refluxing a final sample (500 µl) was taken.

3.2.8 Treatment with enzymes

A suspension of madder root (MR, 2.5 g) or water-ethanol extract (WEE) in 100 mL 0.02 M sodium acetate buffer pH 4.5 was treated with different enzyme solutions and stirred at 45°C for 48 hours.

- The extract (WEE) was incubated with 1 mL β-glucosidase (500 mg/ 10 mL buffer solution).
- The suspensions (WEE and MR) were incubated with 1 mL pectinex BE 3L or 1 mL arabinofuranosidase B (500 mg/10 mL buffer solution).
- The suspensions (WEE and MR) were incubated with "acetone powder" from tea leaves.
- The suspensions (WEE and MR) were incubated with "acetone powder" from madder root.
- Nothing was added (blank).

At t = 0, ½, 1, 2, 4, 12, 24, 48 hours a sample of 500 μl was taken.

3.2.9 Selection of xylosidase and glucosidase

The following enzymes were tested: arabinofuranosidase A, multifect XL, xylanase, multifect CL, multifect cellulase GC (R), pectinex 100L, pectinex AR, pectinex BE 3L, pectinex ultra SP-L, roxazym G2 (liquid enzymes) fermizyme HA 1000, arabinofuranosidase B, pentopan 500BG, bio-feed plus CT (powdery enzymes). Liquid enzyme (0.5 mL) was diluted to 2 mL with buffer solution, and 100 mg of powdery enzyme was diluted in 2 mL of buffer solution and centrifuged during 10 min at 10.000 rpm/min and added to a solution of β-D-*p*-nitrophenol xyloside and –glucoside. The enzymes were analysed for their β-xylosidase and β-glucosidase activity with the aid of a robot-system (Zymark). With the aid of an auto-analyser (Skaler) the colour reagent was added and the activity was measured as a function of the released *p*-nitrophenol. These experiments were performed by TNO, Zeist, the Netherlands[127]. The enzymes with the highest activity from this experiment were diluted 500 times and tested.

3.2.10 Preparation of "acetone powder" from tea leaves or madder root

Fresh madder root or fresh tea leaves were finely chopped, crushed in dry-ice acetone with an Ultra-Turrax T-50 (Rowa Techniek B.V., Leiderdorp, The Netherlands) and then filtered on a Büchner. The residue was washed with chilled acetone (-20°C) until the filtrate became colourless. The residue was spread on filter paper in a fume hood and occasionally turned over to evaporate the acetone. The residual powder "acetone powder" was kept frozen[128].

3.2.11 Testing of the nature of the hydrolysis reactions by endogenous enzymes

Madder root (2.5 g) was added to and stirred in solutions (100 mL) of respectively:
– 100 mL 0.02 M sodium acetate buffer pH 4.5 at 100°C.
– A solution of ethanol : water (1:1) at 45°C.
– A solution of 1 M NaOH at 45 °C
After 48 hours a sample of 500 μl was taken.

Madder powder (2.5 g, MR) was stirred in 100 mL buffer (0.02 M sodium acetate pH 4.5) for 12 hours under a nitrogen atmosphere in a closed reaction vessel at 45°C. After 12 hour a sample (500 μl) was taken and the vessel was opened and nitrogen was replaced by air. The solution was stirred for 12 hours at 45°C. At t = 0, ½, 1, 2, 4, 12 hours a sample (500 μl) was taken.

Madder powder (2.5 g, MR) was stirred in 100 mL buffer (0.02 M sodium acetate buffer pH 4.5) for 12 hours under a nitrogen atmosphere in a closed reaction vessel at 45°C. After 12 hours the suspension was refluxed for 2 hours. After 2 hours a sample (500 μl) was taken, the vessel was opened and nitrogen was replaced by air. The solution was stirred for one week at 45°C. At t = 0, ½, 1, 2, 4, 12, 24, 48 hours and one week a sample (500 μl) was taken.

3.2.12 Effects on the stability of endogenous madder enzymes

The endogenous enzymatic activity was tested under the following conditions:
– Influence of different temperatures. Solution of madder root (2.5 g) was stirred in 100 mL 0.02 M sodium acetate buffer pH 4.5 at the temperatures 4°C, 20°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C and under reflux.
– Influence of pH. A solution of madder root (2.5 g) was stirred in ultrapure water at pH 2, 4, 6, 8, 10 and 12. The buffer solutions were:
  50.7 mL 0.1 M glycine/0.1 M NaCl + 49.3 mL 0.1 M HCl buffer (pH=2),
  62.0 mL 0.1 M citric acid + 38 mL 0.2 M $NaHPO_4 \cdot 2H_2O$ (pH=4),
  37.4 mL 0.1 M citric acid + 62.6 mL 0.2 M $NaHPO_4 \cdot 2H_2O$ (pH=6),
  27.9 mL 0.2 M TRIS + 72.1 mL 0.1 M HCl (pH=8),
  62.5 mL 0.1 M glycine/0.1 M NaCl + 37.5 mL 0.1 M NaOH buffer (pH=10),
  46 mL 0.1 M glycine/0.1 M NaCl + 54 mL 0.1 M NaOH buffer (pH=12).
– Instead of an acetate buffer (0.02 M sodium acetate buffer pH 4.5) madder root (2.5 g) was stirred in ultrapure water (100 ml) and tap water 100 ml).
In all these experiments a sample of 500 μl was taken at t = 0, ½, 1, 2, 4, 12, 24 and 48 hours.

3.3 Results and discussion

The main anthraquinones in a water-ethanol (1:1) extract of madder root are the compounds lucidin primeveroside **1**, ruberythric acid **2**, pseudopurpurin **5** and munjistin **6** (figure 3.1, figure 3.2). The roots contain only minor amounts of the desired component, alizarin **8**. To increase the amount of alizarin **8** the glycoside ruberythric acid **2** has to be converted to alizarin **8** by hydrolysis of the disaccharide, primeverose (6-*O*-β-D-xylopyranosyl-β-D-glucose). Lucidin primeveroside **1** contains the same disaccharide, primeverose as ruberythric acid **2**. During hydrolysis of ruberythric acid **2** to alizarin **6**, lucidin **7** can be formed out of lucidin primeveroside **1**. Lucidin **7** is, however, a known mutagenic agent[40,49,53,65,71,91,93-98] and thus not desirable.

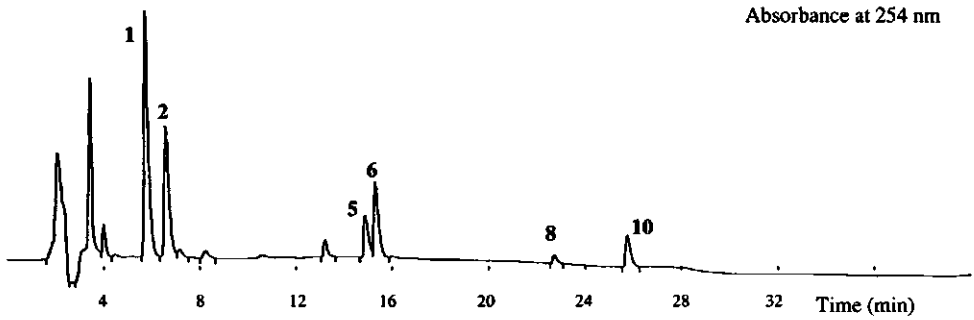


***Figure 3.1** HPLC-trace of a water-ethanol 1:1 extract of madder root, lucidin primeveroside 1, ruberythric acid 2, pseudopurpurin 5, munjistin 6, alizarin 8, purpurin 10.*

3.3.1 Formation of alizarin with strong acid or base

Extract (WEE) and madder root (MR) were treated with 100 mL of a 1 M solution of sulphuric acid or hydrochloric acid at 80°C or 100°C to hydrolyse ruberythric acid into alizarin and xylose and glucose. During the hydrolysis samples were taken at regular intervals and after dilution analysed by HPLC to follow the conversion in time. During heating the solution coloured green and black precipitates were formed due to asperuloside, a non-anthraquinone compound present in *Rubia tinctorum*. Like all iridoid glucosides, asperuloside is unstable in acidic solutions[38,47,129].

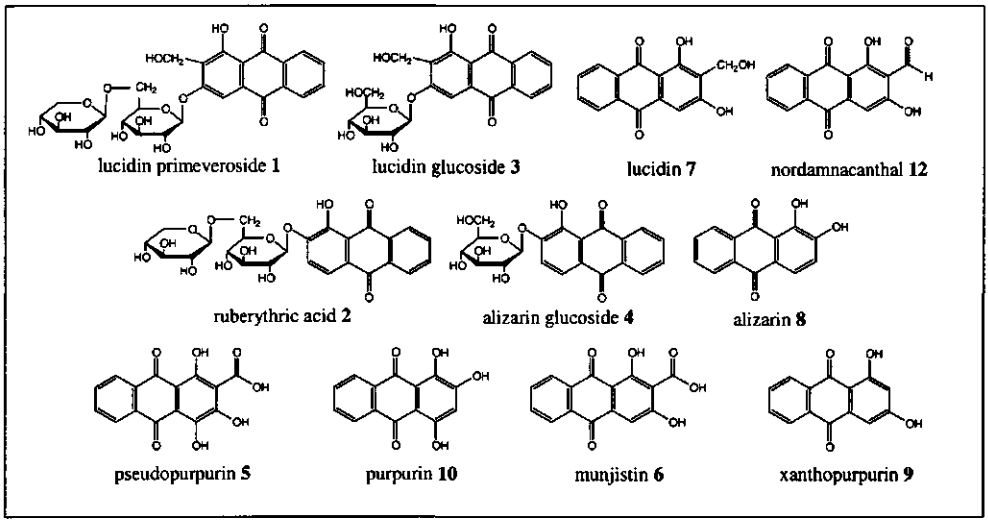


***Figure 3.2** Structure and name of anthraquinones occurring in this chapter. The numbers correspond with the numbers in the text, tables and figures.*

In figure 3.3 the time course of the acidic hydrolysis of *R. tinctorum* (1 M sulphuric acid, 100°C) is shown by HPLC traces at t=0, t=1 and t=24 hours, respectively (figure 3.3a, 3.3b, 3.3c). The anthraquinone peaks were identified with HPLC-DAD and HPLC-MS as described in chapter 2. First the terminal xylose of the disaccharide primeverose is released, which resulted in the formation of the monoglucosides: lucidin glucoside **3** and alizarin glucoside **4** from lucidin primeveroside **1** and ruberythric acid **2**, respectively (figure 3.3b). The carboxylic acid pseudopurpurin **5** is decarboxylated to purpurin **10**. After 1 hour also some lucidin **7** and alizarin **8** was formed from lucidin glucoside **3** and alizarin glucoside **4**, respectively. After 24 hours (figure 3.3c) the solution consisted mainly of the anthraquinones alizarin **8**, xanthopurpurin **9** and purpurin **10**. Ruberythric acid **2** was completely hydrolysed to alizarin **8**. Pseudopurpurin **5** and munjistin **6** were decarboxylated to yield purpurin **10** and xanthopurpurin **9**, respectively.

During the time-course of the experiment lucidin primeveroside **1** is hydrolysed to lucidin glucoside **3** and some lucidin **7** is also formed (figure 3.3b) but finally no mutagenic lucidin **7** is recovered in the solution (figure 3.3c). No other peaks were found in the chromatogram, which could explain the disappearance or conversion of lucidin **7** or its conversion to another anthraquinone. The compounds eluting at t=4 min were not anthraquinones according to HPLC-DAD.

Some experiments were performed to find out why no lucidin **7** is recovered after 24 hours of refluxing madder root (MR) or madder root extract (WEE) in a strongly acidic solution. Pure lucidin primeveroside **1** was refluxed in a solution of 1 M sulphuric acid. In this experiment lucidin primeveroside **1** is completely hydrolysed to lucidin **7**.

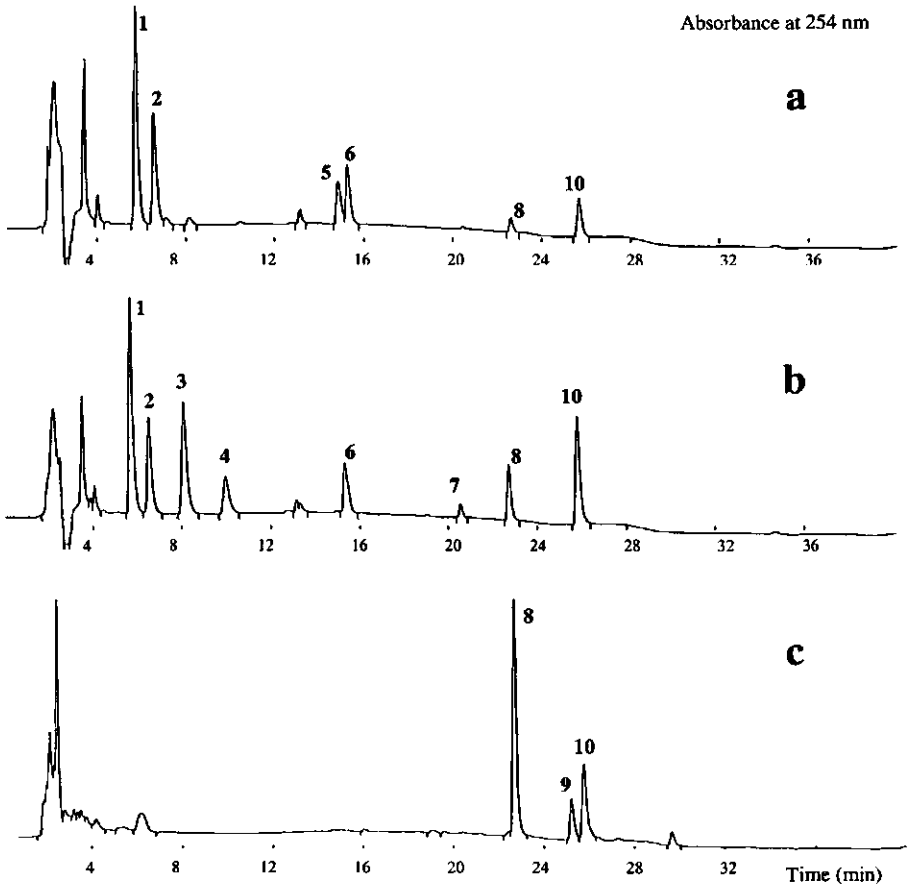


***Figure 3.3** HPLC-trace of the acidic treatment of madder root at different times. Figure 3.3a start of the experiment, in figure 3.3b after 1 hour of refluxing, figure 3.3c after 24 hours of refluxing. lucidin primeveroside **1**, ruberythric acid **2**, lucidin glucoside **3**, alizarin glucoside **4** pseudopurpurin **5**, munjistin **6**, lucidin **7**, alizarin **8**, xanthopurpurin **9**, purpurin **10**.*

The fact that refluxing an acidic solution of madder root resulted in the formation of lucidin glucoside **3** and some lucidin **7** after 1 hour (figure 3.3b), shows that probably lucidin **7** reacted with plant dissoluble compounds and not the glycoside lucidin primeveroside **1**. To prove this pure lucidin was added to a refluxing solution of 1 M sulphuric acid of madder root extract (WEE). After 24 hours of refluxing none of the added lucidin **7** was traceable in the solution. Probably lucidin **7** binds covalently with (a compound of) the plant material. The fact that none of the added lucidin **7** was recovered from the acidic water-ethanol extract (WEE) suggests that lucidin **7** forms a precipitate with a plant compound which is soluble in hot water-ethanol. In a last experiment an extract (WEE) was refluxed twice during 24 hours in an acidic solution and in both cases the solution was filtered to remove the (black) precipitation (insoluble asperuloside), which was formed during the refluxing. To the last filtrate, pure lucidin **7** was added and again the solution was refluxed for 24 hours. After refluxing lucidin **7** could be detected in the solution. But when the solution was refluxed for another 24 hours no lucidin was recovered and again a black preticipate was formed.

An ethanol-water extract (WEE) and madder root (MR) were also suspended in a solution of 1 M sodium hydroxide or potassium hydroxide and heated at 80°C or 100°C. Samples at different times were taken. The HPLC trace depicted in figure 3.4 (1 M NaOH, 100°C) was obtained after 24 hours. Ruberythric acid **2** was converted to alizarin **8**. Beside alizarin **8** a broad range of minor (un)identified anthraquinones was formed.

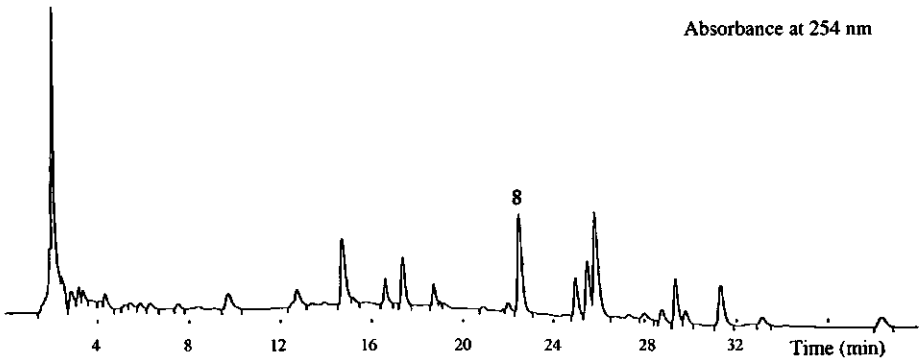


***Figure 3.4** HPLC-trace of madder root treated with 5% NaOH solution, alizarin 8.*

3.3.2 Formation of alizarin with hydrolases

Beside hydrolysis of the glycosides by acid or base also the hydrolysis by enzymes was investigated. According to Masawaki *et al.* (1996) ruberythric acid **2** can be hydrolysed to alizarin **8** by the enzyme β-glucosidase isolated from almonds[55]. These findings could not be reproduced. β-Glucosidase is an exo-enzym, which means that it will hydrolyse a terminal β-glucose. The sugar moiety of ruberythric acid **2**, primeverose (= 6-O-β-D-xylopyranosyl-β-D-glucose) has a terminal xylose and cannot be hydrolysed by β-glucosidase.

For the enzymatic hydrolysis of ruberythric acid **2** a β-xylosidase and a β-glucosidase are required. Several commercial enzyme preparations, which most likely have β-glucosidase and β-xylosidase activity, were added to a solution with β-D-*p*-nitrophenol xyloside or glucoside. The hydrolytic activity of these commercial enzyme preparations was determined by measuring the amount of *p*-nitrophenol released. Two enzyme preparations with the highest enzymatic activity were selected. These were pectinex BE 3L (liquid) with a β-xylosidase activity of 0.09 units/mg and β-glucosidase activity of 0.13 units/mg and arabinofuranosidase (powder) with activities of 1.75 units/mg and 2.22 units/mg.

The hydrolytic activity of pectinex BE 3L and/or arabinofuranosidase on the madder glycosides was tested by adding these enzyme preparations to a buffered solution of madder extract (WEE) and stirring the solution at 45°C. During the hydrolysis samples of the solutions were taken at regular intervals and analysed by HPLC after dilution. Due to the β-xylosidase activity of the commercial enzyme preparations the glycosides lucidin primeveroside **1** and ruberythric acid **2** were initially hydrolysed to lucidin glucoside **3** and alizarin glucoside **4**, respectively. After 5 hours the glycosides were fully hydrolysed to the corresponding aglycones lucidin **7** and alizarin **8**. The carboxylic acids, pseudopurpurin **5** and munjistin **6** were not converted (figure 3.5).

Some Japanese researchers[128,130] found a glycosidase in tea leaves, which is responsible for the aroma formation in tea. They found that this enzyme is a β-primeverosidase responsible for the formation of primeverose from glycosides of geraniol and linalool. They unambiguously proved that this enzyme is indeed a β-primeverosidase and not a combination of a β-xylosidase and a β-glucosidase[130]. Because the sugar moiety of the two main glycosides in madder root is also a primeveroside, it was tried if an extract of tea leaves could be used for the hydrolysis of ruberythric acid **2** in madder root. "Acetone powder" prepared from tea leaves was added to a buffered solution of madder root extract (WEE) and stirred for 48 hours at 45°C. After 48 hours half of the amount of lucidin primeveroside **1** and ruberythric acid **2** in WEE was hydrolysed to lucidin **7** and alizarin **8**. During the time course

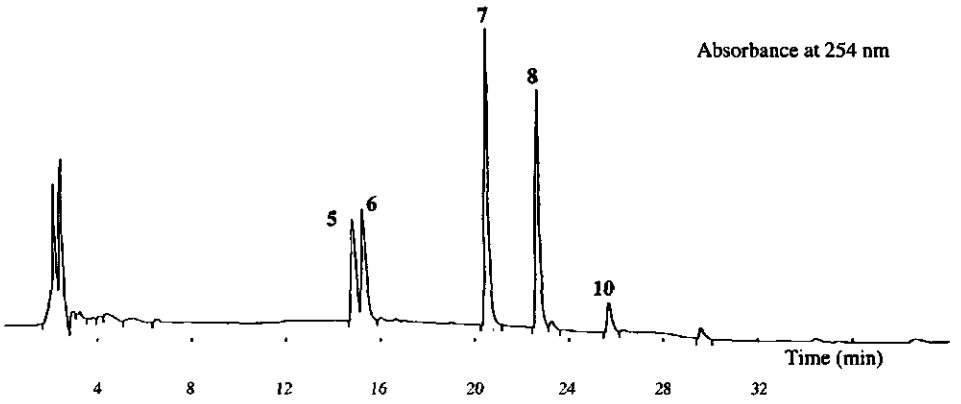


***Figure 3.5** HPLC-trace of the hydrolysis of madder root with the commercial enzyme preparation arabinofuranosidase, pseudopurpurin **5**, munjistin **6**, lucidin **7**, alizarin **8**, purpurin **10**.*

of the experiment no lucidin glucoside **3** or alizarin glucoside **4** was detected, which confirms the presence of a β-primeverosidase. Acetone powder of tea leaves could be used for the release of alizarin **8** in spite of the fact that alizarin **8** is chemically totally different from the monoterpenes linalool and geraniol which are the aglycones in tea glycosides.

3.3.3 Hydrolysis by endogenous enzymes

Fresh madder roots were extracted with acetone in the same way as described for the tea leaves to determine if they contain primeverosidase activity similar to tea leaves[128]. This "acetone powder" of madder root was incubated with madder root extract (WEE) dissolved in 0.02 M acetate buffer pH=4.5 at 45°C for 48 hours. Due to the fact that madder root extract (WEE) is prepared with ethanol at high temperatures (refluxing) it does not have any endogenous enzyme activity anymore. After 1 hour all the ruberythric acid **2** was converted to alizarin **8**. Lucidin primeveroside **1** had disappeared but no lucidin **7** was formed. At t=34.4 min an unknown peak had appeared.

When a suspension of madder root (MR) was stirred under identical conditions as above without the addition of "acetone powder", the same HPLC-trace was obtained, depicted in figure 3.6. This proves that it is not necessary to use fresh madder root for the hydrolysis of madder root by its endogenous enzymes. In spite of the fact that madder root (MR), which

was used in all the described experiments, was dried at 45°C, it still contained endogenous enzymes that could be activated.

The unknown peak at t=34.4 min was analysed with HPLC-DAD and HPLC-ESI-MS as described in chapter 2. Absorption maxima were found at 259, 296 and 412 nm and its mass spectrum showed a base peak at *m/z* 267.3. MS^2 spectra of the base peak showed a fragment at *m/z* 239.3. The unknown peak was identified as nordamnacanthal **12** (1,3-dihydroxy-2-formylanthraquinone, MW=268)[35,42,64,83,108,131]. Instead of the expected conversion of lucidin primeveroside to lucidin, the corresponding anthraquinone aldehyde nordamnacanthal was formed during the stirring of madder root.

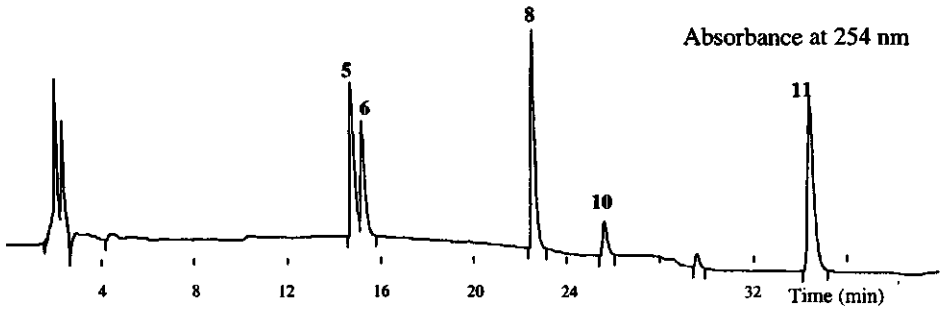


***Figure 3.6** HPLC-trace of madder root stirred in buffer solution at 45°C, pseudopurpurin 5, munjistin 6, alizarin 8, purpurin 10, nordamnacanthal 12.*

In order to demonstrate that the hydrolysis is an enzymatic reaction, the following control experiments were performed. A suspension of madder root (MR) was added to a buffer solution at 100°C, causing denaturation of the enzymes. After 15 min of refluxing the madder suspension was cooled to 45°C and stirred for one week but the glycosides were not hydrolysed. If madder root was stirred in a solution of water-ethanol (1:1) v/v at 45°C no hydrolysis occurred either. The endogenous enzymes were denaturated by the ethanol.

In order to get a better idea about the reaction mechanism of the conversion of lucidin primeveroside **1** to nordamnacanthal **12** instead of the expected lucidin **7** some additional experiments were performed. Madder root was stirred in buffer for 12 hours under a nitrogen atmosphere in a closed reaction vessel. After 12 hours the vessel was opened, a sample was taken, and nitrogen was replaced by air. Stirring was continued for 12 hours and the time-course of the conversion was followed. In the first 12 hours (under nitrogen) the glycosides were hydrolysed and the anthraquinones alizarin **8** and lucidin **7** were formed. After allowing

air into the solution lucidin **7** was converted to nordamnacanthal **12** within 6 hours. This proved that the conversion of lucidin primeveroside **1** to lucidin **7** to nordamnacanthal **12** takes place in two steps; first the glycoside is hydrolysed by a hydrolase and secondly lucidin **7** is converted to nordamnacanthal **12**. For the conversion of lucidin **7** to nordamnacanthal **12** oxygen is obligatory. Under a nitrogen atmosphere no nordamnacanthal **12** was formed but only lucidin **7**.

In the above-described experiment (first only nitrogen, later air) it took 6 hours before all the formed lucidin **7** was converted to nordamnacanthal **12**. When the madder root (MR) suspension was exposed to the air immediately lucidin primeveroside **1** was converted into nordamnacanthal **12** within one hour and during this time course no lucidin **7** was detected at all. It is assumed that the difference in reaction speed is caused by the fact that lucidin **7** is oxidised very rapidly and has no chance to precipitate. However, when the reaction is performed under nitrogen lucidin **7** is able to establish the dissolution equilibrium. Lucidin **7** is very poorly soluble in water and after opening of the vessel the enzyme oxidation will go much slower.

To establish that the conversion of lucidin **7** into nordamnacanthal **7** is enzymatic, the previous experiment was repeated, but before opening the reaction vessel the reaction mixture was heated to 100°C to inactivate any enzymes that might be present. After one week of stirring the suspension in the air it was found that no lucidin **7** had been converted into nordamnacanthal **12**. It can be concluded that the presence of only oxygen is not sufficient for the formation of nordamnacanthal **12**. An enzyme (an oxidase) catalyses the formation.

3.3.4 Characteristics of the conversion reaction by endogenous enzymes

The influences of pH and temperature on the endogenous enzyme activity in madder root were investigated.

Because the conversion of the glycosides is enzymatic it is important to keep the reaction temperature below a value that would lead to denaturation of the enzymes. The reaction was tested at different temperatures and the time-course of the experiments (ultrapure water) was followed. In figure 3.7 the results are depicted for sampling at t=30 min and at 4 hours, respectively. It was found that the conversion is optimal when the temperature lies between 35 and 65°C at a reaction time of about 30 min. At longer reaction times, for example 4 hours, the temperature maybe lower, down to 0°C. At temperatures above 65°C, the enzymes are slowly denaturated, although there will still be some conversion.

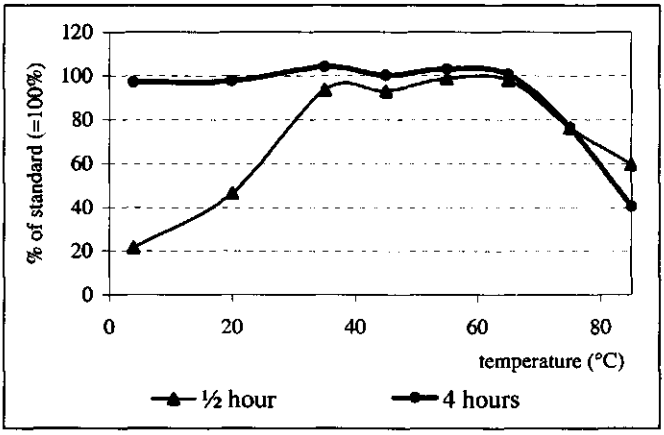


***Figure 3.7** Influence of temperature on the formation of alizarin in madder root by endogenous enzymes after ½ hour and 4 hours.*

The effect of the pH on the conversion of the glycosides at 45°C is depicted in figure 3.8. The figure shows the percentage of conversion for two different reaction times (30 min and 4 hours). If the suspension is stirred for four hours, the pH optimum will lie between 6 and 8. At higher and lower pH's the percentage conversion lowers rapidly. If the solution is stirred for 30 min the conversion will only be complete at a pH of around 6.

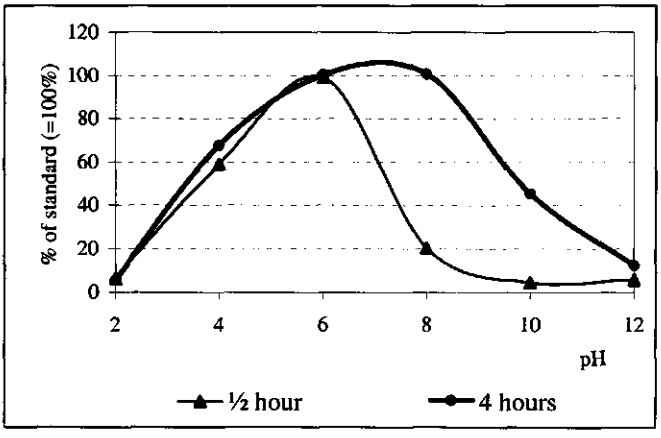


***Figure 3.8** Influence of pH on the formation of alizarin in madder root by endogenous enzymes after ½ hour and 4 hours.*

A buffer system is not necessary for the conversion of the glycosides. Demineralized water, ultrapure water and even tap water can be used instead of a 0.02 M acetate solution pH=4.5. The conversion of the glycosides in a suspension of madder root in ultrapure water (pH=6) at 45°C is completed in less than 45 min. If a suspension of madder root is stirred in ultrapure water (pH=6) at room temperature the reaction is completed within 90 min.

3.4 Conclusion

For the production of an alizarin dye preparation from madder root, the glycoside ruberythric acid should be hydrolysed. If madder root (MR) is treated with strong acid, strong base, or endogenous enzymes ruberythric acid **2** is successfully hydrolysed into alizarin **8** without the formation of the mutagenic anthraquinone lucidin **7**. Refluxing madder root in basic solutions gives a suspension with a large number of minor unidentified anthraquinones. Therefore this method is less suitable for use in dye preparation, especially because it is unknown whether some of these anthraquinones are mutagenic. The anthraquinones that are formed by treatment of madder root with strong acid are alizarin **8**, purpurin **10** and xanthopurpurin **9**. Conversion of the glycosides by endogenous enzymes present in madder root gives alizarin **8** and nordamnacanthal **12**, which are not mutagenic[35].

Production of an alizarin **8** dye preparation from madder root has to compete in quality and price with synthetic alizarin **8**. During the treatment of madder root (MR) with acid the solution has to be refluxed for at least 12 hours to convert the glycosides. In case of endogenous enzymes the suspension only needs oxygen to avoid the formation of lucidin, but this can easily be achieved by stirring the suspension in water. When devising a commercial dye extraction method, use of these endogenous enzymes could be favourable.

Until recently extracts from *Rubia tinctorum* found widespread use in the treatment of urinary tract infections and for the dissolution of bladder stones. Detection of mutagenic lucidin **7**[35,65,71] led to the withdrawal of phytopharmaceuticals containing *Rubia tinctorum* extracts from the market without further investigation of their content of mutagenic anthraquinones like lucidin **7**. In this research it was proven that with a simple treatment of madder root with strong acid or endogenous enzymes, the presence or formation of lucidin **7** in madder root extracts can be avoided.

For the further development of a commercial dye preparation from madder root some more research has to be performed on the isolation and extraction of alizarin from the plant material and the other anthraquinones present in madder root.

Chapter 4

Two extraction methods for the quantitative determination of alizarin and other anthraquinones in *Rubia tinctorum* L. and the screening of different cultivars

4.1 Introduction

One important element in the revitalisation of natural alizarin **8** as commercial product is that farmers should be able to provide plentiful amounts of cheap, high-quality, vegetable materials. This must be achieved by the selection of a *Rubia tinctorum* cultivar with the best agronomic characteristics like plant density, root density, root thickness, resistance and amount of ruberythric acid **2** and alizarin **8**.

For the determination of the amount of anthraquinones in madder roots a good screening method is necessary. For the quantification of anthraquinones in cell suspension cultures most researchers used the method developed by Zenk *et al.* 1975[31,34,50,64,83,132-135]. With this method the absorption of an 80% ethanol extract of the cell suspension culture is measured at 434 nm the absorption maximum of alizarin **8**. A modification of this method consisting of adding one drop of 5% KOH and measuring at λ=520 nm, is also used for quantification purposes[64,87,136]. This method is very fast. A disadvantage is that no distinction can be made between the different anthraquinones present in extracts. To obtain information about the quantity of individual anthraquinones an on-line separation like GC, HPLC or CE with detection will be necessary. A fine GLC method has been described in which the hydroxyanthraquinones are reductively silylated[67,68,137]. Most published methods for analysis of anthraquinones make use of HPLC. One preferably uses a method based on liquid chromatography, like HPLC because then the anthraquinones do not have to be

silylated, which saves time and prevents possible losses during the derivatisation. The HPLC methods described in literature quantify only one or two anthraquinones in madder root extracts, for example the quantitative analysis of alizarin[46,138]. The determination of free and bound alizarin **8** (ruberythric acid **2**) is determined by an alcoholic and alcoholic-acidic extract of the plant[41,56]. A quantification method is described mainly based on the compound lucidin **7**[139]. Kriszan *et al.* described the quantification of both the glycosides lucidin primeveroside **1** and ruberythric acid **2** (no base-line separation) and the corresponding aglycones[51]. Recently a CE method for the separation and quantitative analysis of a mixture of commercially available anthraquinones was published. The mixture contained only two madder root related anthraquinones (alizarin **8** and purpurin **10**)[76].

In this study the development of a rapid, simple and reliable method for the isolation and quantification of the most abundant anthraquinones in *Rubia tinctorum* is described. Several *Rubia tinctorum* cultivars were screened.

4.2 Experimental

4.2.1 Reference compounds

Crude "ruberythric acid" was obtained from Carl Roth GmbH & Co (Karlsruhe, Germany). Alizarin **8** (1,2-dihydroxyanthraquinone) and purpurin **10** (1,2,4-trihydroxyanthraquinone) were purchased from ACROS (Geel, Belgium). Lucidin primeveroside **1**, ruberythric acid **2**, lucidin, xanthopurpurin **9**, pseudopurpurin **5**, munjistin **6** and nordamnacanthal **12** were not commercially available.

Lucidin primeveroside **1** and ruberythric acid **2** were separated and purified by droplet counter-current chromatography (DCCC). The DCCC apparatus was from Tokyo Rikakikai Co, LTD, an EYELA model type DCCC-A. The model consisted of 300 tubes with an ID of 2 mm. The connecting Teflon tubing had an ID of 0.5 mm. The DCCC was used in the ascending mode. The eluent system used, was $CHCl_3$-MeOH-H_2O (5:5:3) (v/v/v). Crude "ruberythric acid **2**" (500 mg) was dissolved in 7 ml of the ascending fluid. During the elution the flow was 0.48 ml/min. The total separation time was 15½ hour. Fractions of 9 ml were collected and analysed with HPLC. The fractions with pure lucidin primeveroside **1** (0.026 g) and ruberythric acid **2** (0.029 g) crystallised and were obtained by filtration[70].

Lucidin and xanthopurpurin **9** were synthesised in two steps according to the method of Murti *et al.*[47]. Column chromatography over silica was used to purify xanthopurpurin **9**, which is the product of the first step. The column was eluted with petroleum ether

(40/60)-ethyl acetate-formic acid (75:25:1) (v/v/v). Part of the xanthopurpurin **9** was converted into lucidin in the second reaction step[47,70].

Pseudopurpurin **5**, munjistin **6** and nordamnacanthal **12** were isolated from *Rubia tinctorum*. Dried madder root (50 g) was stirred in ultrapure water (2000 ml) at room temperature for two hours. The aqueous layer was extracted twice with ethyl acetate (500 ml) and the inmiscible layers were separated with a separatory funnel. The ethyl acetate layers were combined and contained the desired nordamnacanthal **12**. The extract was dried under reduced pressure. The aqueous layer was filtrated and the filtrate contained the carboxyl anthraquinones: pseudopurpurin **5** and munjistin **6**.

The dried ethyl acetate extract (1.2 g) was dissolved in THF-water (1:1) and added to a C_{18} column (15 g). The column was eluted with THF-water (1:1) (v/v) (before use the eluent was bubbled with N_2) and 18 coloured fractions were collected. The last coloured fraction (no: 18) was obtained by eluting the column with 100% THF. The fractions were analysed with HPLC. The fractions with nordamnacanthal **12** were placed in the refrigerator and after one night most of the nordamnacanthal **12** had precipitated. The nordamnacanthal **12** was obtained by filtering the solution. The remaining solutions and filtrates with nordamnacanthal **12** were pooled. *t*-Butylmethylether (10%) was added which caused phase separation. The organic layer was dried and pooled with the precipitated nordamnacanthal **12**. Nordamnacanthal **12** (0.057 g) was stored in the freezer under N_2.

The filtrate of the aqueous layer (1800 ml) was added to a Sephadex L20 column and eluted with ultrapure water. Three different coloured fractions were obtained: brown, red and yellow. The fractions were analysed with HPLC. Fractions were freeze-dried with a Christ Alpha 1-2 freeze dryer (Salm en Kipp, Breukelen, The Netherlands) and gave respectively pseudopurpurin **5** (0.087 g, red fraction) and munjistin **6** (0.032 g, yellow fraction).

4.2.2 Purity reference compounds

The purity of all the reference compounds was checked by means of UV/VIS λ_{max} (HPLC-DAD, buffer-acetonitrile), MS (HPLC-ESI-NI), qualitative 400 MHz ^{1}H-NMR, 100 MHz ^{13}C-NMR and quantitative 400 MHz ^{1}H-NMR spectroscopy. Maleic acid was used as standard for the quantitative determination[140]. DMSO-d_6 (Acros) was used as solvent.

Lucidin primeveroside **1**: UV/VIS: λ_{max} 204, 247, 266, 406 nm. MS: 563.0 (M–H, 100), 1126.9, 1691.7, 269.2, 608.7. ^{1}H-NMR: δ: 13.04 (1 OH, s, OH-aglycone), 8.21 (2H, m, H-5,8), 7.94 (2H, m, H-6,7), 7.48 (1H, s, H-4), 5.14 (1 H, d, J = 7.2 Hz, gluc1-H), 4.63 (2 H, dd, J = 33.4 J = 11.3 Hz, CH_2OH), 4.16 (1 H, d, J = 7.4 Hz, xyl1-H), 3.97 (1H, d, J =9.8 Hz, gluc6B-H), 3.70 (m, sugar-H), 3.37 (m, sugar-H), 3.06 (m, sugar-H). ^{13}C-NMR: δ: 162.90

(C-1), 124.53 (C-2), 162.75 (C-3), 107.26 (C-4), 127.81 (C-5), 135.74 (C-6), 135.60 (C-7), 127.40 (C-8), 187.99 (C-9), 182.34 (C-10), 133.81 (C-11), 133.66 (C-12), 112.27 (C-13), 134.65 (C-14), 51.82 (CH_2OH), 101.69 (C-gluc1), 74.14 (C-gluc2), 76.59 (C-gluc3), 70.03 (C-gluc4), 77.28 (C-gluc5), 68.89 (C-gluc6), 104.95 (C-xyl1), 74.14 (C-xyl2), 76.73 (C-xyl3), 70.36 (C-xyl4), 66.50 (C-xyl5).

Ruberythric acid **2**: UV/VIS: λ_{max} 200, 231, 260, 416 nm. MS: 553.1 (M–H, 100), 1066.8, 1600.7, 239.3, 578.4. ^{1}H-NMR: δ: 12.64 (1 OH, s, OH-aglycone), 8.23 (2H, m, H-5,8), 7.96 (2H, m, H-6,7), 7.75 (1H, d, J = 8.5 Hz, H-3), 7.64 (1H, d, J = 8.6 Hz, H-4), 5.45 (1 OH, bs, gluc2-OH), 5.23 (1 OH, d, J = 4.8 Hz, gluc3-OH), 5.21 (1 OH, d, J = 2.8 Hz, gluc4-OH), 5.08 (1H, d, J = 7.4 Hz, gluc1-H), 5.02 (1 OH, d, J = 2.8 Hz, xyl2-OH), 4.96 (1-OH, d, J =4.7 Hz, xyl3-OH), 4.93 (1-OH, d, J = 5.0 Hz, xyl4-OH), 4.16 (1H, d, J = 7.4 Hz, xyl1-H), 3.99 (1H, d, J = 10.5 Hz, gluc6B-H), 3.67 (1H, m, xyl5-H), 3.60 (1H, m, gluc5-H), 3.55 (1H, m, gluc6A-H), 3.39 (1H, m, gluc2-H), 3.31 (1H, m, gluc3-H), 3.27 (1H, m, xyl4-H), 3.19 (1H, m, gluc4-H), 3.06 (1H, m, xylH-3), 2.98 (1H, m, xylH-2), 2.96 (1H, m, xylH-5). ^{13}C-NMR: δ: 152.62 (C-1), 152.08 (C-2), 121.67 (C-3), 121.22 (C-4), 127.67 (C-5), 136.04 (C-6), 135.20 (C-7), 127.47 (C-8), 188.20 (C-9), 180.64 (C-10), 132.89 (C-11), 134.27 (C-12), 100.76 (C-13), 125.69 (C-14), 99.69 (C-gluc1), 73.88 (C-gluc2), 77.34 (C-gluc3), 70.41 (C-gluc4), 76.96 (C-gluc5), 69.17 (C-gluc6), 104.86 (C-xyl1), 74.28 (C-xyl2), 77.46 (C-xyl3), 70.47 (C-xyl4), 66.45 (C-xyl5).

Xanthopurpurin **9**: UV/VIS: λ_{max} 225, 282, 415 nm. MS: 239.3 (M–H, 100). ^{1}H-NMR: δ: 12.62 (1 OH, s), 8.04 (2H, m, H-5,8), 7.80 (2H, m, H-6,7), 7.01 (1H, d, J = 2.3 Hz, H-4), 6.48 (1H, d, J = 7.6 Hz, H-2).

Lucidin **7**: UV/VIS: λ_{max} 203, 246, 281, 413 nm. MS: 269.2 (M–H, 100). ^{1}H-NMR: δ: 13.21 (1H, s, OH-1), 11.36 (1H, s, OH-2), 8.19 (2H, m, H-5,8), 7.92 (2H, m, H-6,7), 7.26 (1H, s, H-4), 4.55 (2H, s, -CH_2-).

Pseudopurpurin **5**: UV/VIS: λ_{max} 259, 494 nm. MS: 299.1 (M–H, 100), 255.2. ^{1}H-NMR: δ: 17.02 (1H, s, COOH), 15.85 (H, s, OH), 13.61 (H, s, OH), 8.23 (2H, m, H-5,8), 7.88 (2H, m, H-6,7). ^{13}C-NMR: δ: 126.95 (C-5), 135.53 (C-6), 134.00 (C-7), 126.75 (C-8), 136.17 (C-11), 132.50 (C-12).

Munjistin **6**: UV/VIS: λ_{max} 248, 288, 420 nm. MS: 283.1 (M–H, 100), 239.3. ^{1}H-NMR: δ: 16.45 (1H, s, COOH), 16.14 (1H, s, OH), 8.04 (2H, m, H-5,8), 7.77 (2H, m, H-6,7), 6.86 (1H, s, H-4). ^{13}C-NMR: δ: 167.44 (C-1), 111.77 (C-2), 168.75 (C-3), 106.40 (C-4), 126.88 (C-5), 135.41 (C-6), 133.56 (C-7), 127.29 (C-8), 184.08 (C-9), 179.86 (C-10), 136.03 (C-11), 132.99 (C-12), 108.54 (C-13), 137.68 (C-14), 175.40 (COOH).

Nordamnacanthal **12**: UV/VIS: λ_{max} 216, 260, 296, 424 nm. MS: 267.2 (M–H, 100). ^{1}H-NMR: δ: 10.39 (1H, s, CHO), 8.21 (2H, m, H-5,8), 7.95 (2H, m, H-6,7), 7.15 (1H, s, H-4).

^{13}C-NMR: δ: 166.53 (C-1), 109.08 (C-2), 167.21 (C-3), 113.05 (C-4), 127.00 (C-5), 136.01 (C-6), 134.88 (C-7), 126.49 (C-8), 186.19 (C-9), 181.10 (C-10), 132.79 (C-11), 132.71 (C-12), 107.93 (C-13), 138.30 (C-14), 191.42 (CHO).

4.2.3 Plant material

Roots from several *Rubia tinctorum* cultivars and one *Rubia cordifolia* species were collected on 12 November 1999 in Zuidbroek, Groningen, The Netherlands. Two and three year old roots from the same cultivar were collected in Borkel en Schaft, Brabant, The Netherlands. Herbarium accession numbers are available on request from the department of Taxonomy of Wageningen University, The Netherlands. The roots were processed as described in § *3.2.4*.

4.2.4 Solubility of alizarin and anthraquinone glycosides

The solubility of alizarin **8** in different solvents was tested. The absorption spectrum (200-600 nm) of alizarin **8** in methanol was determined. Alizarin **8** has an absorption maximum at 438 nm. A calibration curve of alizarin **8** in methanol at 438 nm was established with an ordinate minimum of 0 and ordinate maximum of 0.57 Absorption Units (AU). Different solvents: toluene, acetone, chloroform, ultrapure water, methanol, THF, *n*-propanol, petroleum ether, *t*-butyl-methylether, ethanol-water 1-1, 2 M trifluoracetic acid, ethyl acetate, 2 M sulphuric acid, *i*-propanol, diethylether, dichloromethane, ethanol, acetonitrile and 0.5 M NaOH were saturated with alizarin **8**. The solutions were sonicated for 15 min in a Retsch Transsonic 570 (Emergo, Landsmeer, The Netherlands). After sonication the solution was filtered over a 0.45 μm membrane filter. The filtrate was diluted until the absorption of the filtrate at 438 nm was between 0 and 0.57 AU. The alizarin **8** concentration was based on the calibration curve of alizarin **8** in methanol at 438 nm. An exception is the absorption maximum of alizarin **8** in an alkaline solution. In case of the basic solution, the filtrate was diluted and after dilution it was acidified with hydrochloric acid. UV/VIS spectra were obtained with a Perkin Elmer UV/VIS spectrometer Lambda 18 (Boston, USA).

For the determination of the solubility of the main glycoside ruberythric acid **2** this compound was needed in pure form. Ruberythric acid **2** is not commercially available in pure form. Purification of this compound is labour intensive and has a low yield (§ *4.2.3*). To prevent the use of a lot of pure ruberythric acid **2** for solubility experiments, instead of the pure compound a mixture of polar anthraquinones extracted from madder root was used. Madder root is extracted with ethanol-water (1:1) as described in § *4.2.7*. The filtrate was dried under reduced pressure. The dried filtrate was dissolved in methanol filtered and the absorption spectrum (200-600 nm) of the solution was determined. The solution had an

absorption maximum at 408 nm. HPLC-DAD examination of the solution revealed that absorption at 408 nm was due exclusively to anthraquinones in the solution. The main anthraquinones in an ethanol-water extract of madder root are the glycosides and the carboxylic acids. A calibration curve at four concentrations of the dried filtrate in methanol at 408 nm was established with an ordinate minimum from 0 and ordinate maximum of 0.93 AU. Different solvents: ethanol, acetone, chloroform, methanol, THF, methanol-water (1:1), ethanol-water (1:1), ethyl acetate, water and 0.5 M NaOH were saturated with dried madder root extract. The solutions were sonicated for 15 min, filtered and diluted until the absorption of the filtrate at 408 nm was between 0 and 0.93 AU. The anthraquinone concentration was based on the calibration curve of the dried filtrate in methanol at 408 nm. Except the alkaline solution, which was acidified after dilution. UV/VIS spectra were obtained.

4.2.5 Extraction efficiency and sample size

Dried and powdered root material (2.5 g) was refluxed with 100 ml water-ethanol (1:1) (v/v). After 2 hours the suspension was filtered under reduced pressure. A sample of 100 µl of the filtrate was taken and diluted with 900 µl water-methanol-formic acid (1:1:0.005) (v/v/v). The amount of the filtrate was determined in ml's. The residue was refluxed with 100 ml water-ethanol (1:1) (v/v). After 2 hours the suspension was filtered under reduced pressure. A sample of 500 µl of the filtrate was taken. The residue was refluxed again with 100 ml water-ethanol (1:1) (v/v). After 2 hours the suspension was filtered under reduced pressure. A sample of 500 µl of the filtrate was taken. The residue was refluxed again with 100 ml THF. After 2 hours the suspension was filtered under reduced pressure. A sample of 500 µl of the filtrate was taken All samples of 500 µl were diluted with 500 water-methanol-formic acid (1:1:0.005) (v/v/v). The amount of filtrate was determined in ml's (decant in measure cylinder).

Five samples of 2.5 g madder root were stirred in 100 ml water for 1 hour at 45°C. After 2 hours 25, 50, 100, 200 or 400 ml THF-water-formic acid (1:1:0.005) (v/v/v) was added and stirred. After 1 hour and after 64 hours samples of 1000 µl were taken. The experiment was performed in duplicate. Two series of four different suspensions of madder root were stirred in water for 1 hour at 45°C: 2.5 g in 100 ml, 1.25 g in 50 ml, 0.63 g in 25 ml and 0.25 g in 10 ml. After 1 hour respectively 400 ml, 200 ml, 100 ml and 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) was added to the different suspensions and stirred. After 1 hour stirring, samples of 1000µl were taken. Six portions of 0.25 g madder root were stirred in 10 ml of water of 45°C. After 1 hour 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) was added to all the solutions. Two were refluxed 1 hour, two were stirred at 45°C and two were

stirred at room temperature for one hour. After 1 hour samples of 1000 µl were taken. All samples were were diluted with 1000 µl THF-water-HCl (1:1:0.05) (v/v/v).

4.2.6 Extraction and sample preparation for glycoside determination (direct method)

Dried and powdered root material (2.5 g) was refluxed with 100 ml water-ethanol (1:1) (v/v). After 2 hours the suspension was filtered under reduced pressure. A sample of 100 µl of the filtrate was taken and diluted with 900 µl water-methanol-formic acid (1:1:0.005) ((v/v/v). The amount of the filtrate was determined in ml's (decant in measuring cylinder). The residue was refluxed with 50 ml water-ethanol (1:1) (v/v). After 2 hours the suspension was filtered under reduced pressure. A sample of 500 µl of the filtrate was taken and diluted with 500 µl of water-methanol-formic acid (1:1:0.005) (v/v/v). The amount of the filtrate was determined in ml's.

4.2.7 Extraction and sample preparation for aglycone determination (indirect method)

Dried and powdered madder (0.25 g) was stirred in 10 ml of ultrapure water for 1 hour at 45°C (or 2 hours at 25 °C). After stirring, 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) was added and stirred for another 30 min at room temperature. A sample of 1000 µl was taken and diluted with 1000 µl of THF-water-formic acid (1:1:0.005) (v/v/v).

4.2.8 Liquefaction

Madder root (0.25 g) was suspended in 10 ml water or 10 ml of 0.02 M sodium acetate buffer (pH 4.5) after which 200 µl of the enzyme preparations Viscozyme L and Celluclast 1,5 L was added (triplicate). The commercial enzyme preparation Viscozyme L is a pectinase with also some cellulolytic and hemicellulolytic activity (NOVO Nordisk) and the preparation Celluclast 1,5 L has mainly cellulase activity (NOVO Nordisk). One half of the samples was stirred for 2 hours while the other half of the samples was stirred for 80 hours at 45°C. After stirring 40 ml of THF-water-formic acid (1:1:0.005) (v/v/v) was added. The diluted samples were stirred for 30 min at 45°C. 1000 µl of the sample was diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v).

4.2.9 HPLC analysis

All the diluted samples were filtered over a 0.45 μm, ø 25 mm membrane filter (Type RC, Schleicher & Schuell) and analysed by the HPLC method described in chapter 3. HPLC-DAD and HPLC-MS experiments were carried out as described in chapter 2.

4.2.10 Recovery

Madder root (7.5 g) was refluxed in 100 ml ethanol-water (1:1) (v/v). After 2 hours the suspension was filtered. Ethanol-water (1:1) (v/v) was added to the filtrate until the final volume was 300 ml. The filtrate was divided in three portions of 100 ml and transferred to three round bottom flasks. From every flask a sample of 100 μl was taken and diluted with 900 μl methanol-water-formic acid (1:1:0.005) (v/v/v). The sample was filtered (0.45 μm, ø 25 mm membrane filter, Type RC, Schleicher & Schuell) and analysed by HPLC. The filtrates were refluxed, filtered and the residue was refluxed and filtered again as described in § *4.2.6*. Samples were taken from the filtrate and analysed and the recovery was determined.

Madder root (1 g) was stirred in 40 ml water at 45°C. After 1 hour 200 ml of water (1:1) was added. The solution was heated and filtered. The filtrate was cooled and three portions of 50.00 ml were taken from the filtrate and suspended in three round bottom flasks. From every solution a sample (500 μl) was taken and diluted with 500 μl THF-water-formic acid (1:1:0.005) (v/v/v). The samples were stirred (30 min), filtered and analysed by HPLC. The three madder root water filtrates were lyophilised. The dried samples were suspended in 10 ml of water and treated as described in § *4.2.7*. Samples were taken, analysed on HPLC and the recovery was determined.

4.3 Results and Discussion

4.3.1 Purification and identification of standards

For a quantitative HPLC analysis of the anthraquinone content in *Rubia tinctorum* extracts, sufficient amounts of the various anthraquinones present (figure 4.1) are necessary to construct linear calibration curves. By means of HPLC analysis it was shown that commercially available "ruberythric acid" contained two anthraquinone glycosides[34,40,51,70]. By means of quantitative HPLC and NMR it was proven that commercial "ruberythric acid" consisted of 12.5 % lucidin primeveroside **1**, 7.7 % ruberythric acid **2** and ≈ 80% sucrose and other unidentified constituents[34].

Different authors have isolated lucidin primeveroside **1** and ruberythric acid **2** from *Rubia tinctorum* plant material by one or more extraction steps followed by one or more column chromatography steps (Sephadex LH-20, Dowex 50, Amberlite XAD-2)[35,38,40,47,51,55,58,70]. Purification of these glycosides by column chromatography is time consuming and the yield is low because of the poor separation of the glycosides. Furthermore, the acidic hydroxyl groups in polyphenols can cause irreversible adsorption to the solid stationary phase (silica) during the chromatographic separation[74]. Attempts to separate and isolate the glycosides by medium pressure liquid chromatography (MPLC), with borate impregnated silica gel[141] were unsuccessful. Hermans-Lokkerbol *et al*[63] used CPC with a solvent mixture of $CHCl_3$: MeOH : H_2O : acetic acid (5:6:4:0.5) to separate glycosides of *Rubia tinctorum*. Inoue *et al.*[73] used DCCC with a solvent mixture of $CHCl_3$: MeOH : H_2O (5:5:3) to separate the anthraquinone glycosides of *Morinda citrifolia* . The same solvent conditions were used successfully in this research to separate the glycosides lucidin primeveroside **1** and ruberythric acid **2** by DCCC.

Lucidin **7** is not a major aglycone in *Rubia tinctorum*, however it can be formed by hydrolysis of lucidin primeveroside **1** the major glycoside. Lucidin **7** is a mutagenic compound[40,49,53,65,71,91,93-98] and not commercially available; some effort was made to get lucidin **7** pure. Lucidin **7** was successfully synthesised by the method of Murti *et al.*[47].

A couple of research groups isolated nordamnacanthal **12** from *Rubia tinctorum* by column chromatography or CPC[35,43,63,64]. In this research nordamnacanthal **12** was isolated from *Rubia tinctorum*. First nordamnacanthal **12** was formed by stirring madder root in water (chapter 3) and after formation the compound was extracted with ethyl acetate and purified by C_{18} column chromatography. As eluent THF-water (1:1) was used. During the purification of nordamnacanthal **12** with C_{18} column chromatography, the compound is partially oxidised to munjistin **6**. This can be partially inhibited by using an eluent, which is saturated with N_2 before use. The formed munjistin **6** can be easily separated from nordamnacanthal **12** by adding 10% *t*-butyl methyl ether to the THF-water fraction. THF and water will separate with the nordamnacanthal **12** in the THF- *t*-butyl methyl ether layer and munjistin **6** in the aqueous layer. Nordamnacanthal **12** is stable in a solution of THF.

Munjistin **6** and pseudopurpurin **5** were purified by column chromatography[25,36,62,142]. In this research munjistin **6** and pseudopurpurin **5** were isolated from an aqueous *Rubia tinctorum* extract and by additional Sephadex LH20 column chromatography. First the brown polar compounds eluted from the column followed by the anthraquinone carboxylic acids, which could be distinguished by a change in colour. First pseudopurpurin **5** was eluted as a red band and secondly munjistin **6** as a yellow band.

The purities of the anthraquinone glycosides and aglycones used for the calibration curves are reported in table 4.1. Commercial purpurin **10** was contaminated with quinizarin **11**[63].

4.3.2 HPLC method

The HPLC method described in chapter 2 was used for determining the anthraquinone glycosides and aglycones in one run. For the most important anthraquinones occurring in madder root linear calibration curves, based on the peak area, were obtained. In table 4.1 the purity, maximum anthraquinone concentration used for the calibration curve, calibration curve equation, correlation coefficient and molar extinction coefficients of the different anthraquinones are reported. Detection was carried out by UV at 254 nm.

Table 4.1 *Purity, maximum anthraquinone concentration used for the calibration curve, calibration curve equation, correlation coefficient (r^2) and molar extinction coefficients (ε) at 254 nm and at 434 nm.*

compound	**purity %**	**min-max mg/L**	**calibration curve x= mg/L y= area**	**r^2**	**ε 254 nm L/mol cm**	**ε 434 nm L/mol cm**
lucidin primeveroside **1**	92	0- 46.2	y= 5835x + 539	1.0000	$2.19 \cdot 10^4$	$4.52 \cdot 10^3$
ruberythric acid **2**	86	0- 43.9	y= 7694x + 727	0.9999	$3.33 \cdot 10^4$	$5.78 \cdot 10^3$
pseudopurpurin **5**	54	0- 53.8	y= 9814x – 7343	0.9999	$2.89 \cdot 10^4$	$3.87 \cdot 10^3$
munjistin **6**	52	0- 51.9	y= 20820x – 915	1.0000	$3.51 \cdot 10^4$	$6.59 \cdot 10^3$
lucidin **7**	74	0- 65.5	y= 13028x – 2681.7	1.0000	$0.80 \cdot 10^4$	$4.73 \cdot 10^3$
alizarin **8**	96	0-127.1	y= 15094x + 19666	1.0000	$2.82 \cdot 10^4$	$6.03 \cdot 10^3$
purpurin **10**	53	0- 54.2	y= 14427x + 1261.2	0.9997	$4.64 \cdot 10^4$	$7.65 \cdot 10^3$
nordamnacanthal **12**	53	0- 53.1	y= 9369x - 12734	0.9976	$3.00 \cdot 10^4$	$5.60 \cdot 10^3$

4.3.3 Sample treatment

The fresh roots were dried at the air for one week and ground in a mixer at three different increasing speed revolutions. With this grinding procedure the endogenous enzymes were not denaturated. The water content of the fresh roots varied from 62-69%. Comparison of the chromatograms obtained from fresh roots and from the same roots after drying did not show any degradation or changes as a result of the drying step. The roots could be stored for at least four years at room temperature without significant deterioration of the anthraquinones or endogenous enzymes.

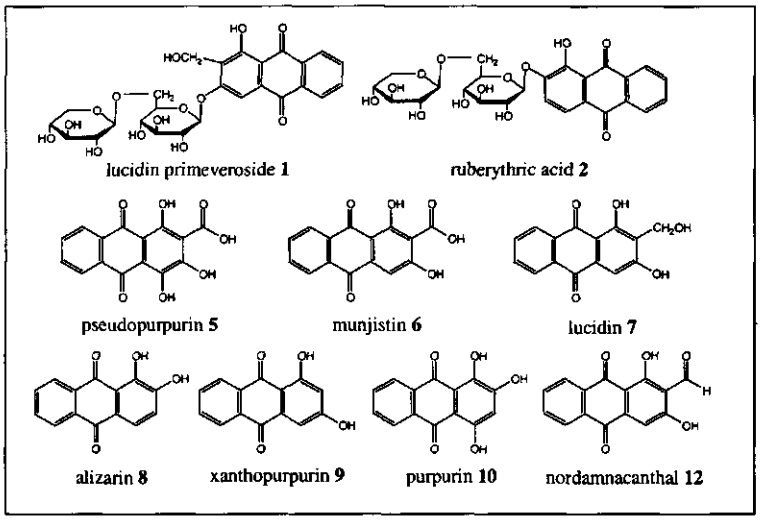


Figure 4.1 *Structures and names of anthraquinones described in this chapter. The numbers correspond with the anthraquinones and numbers in the text, tables and figures.*

4.3.4 Quantification of glycosides in madder root

As already mentioned the main anthraquinones in madder root are the glycosides: ruberythric acid **2** and lucidin primeveroside **1** and the anthraquinone carboxylic acids: munjistin **6** and pseudopurpurin **5** (figure 3.1). For the large-scale screening of madder root a selective and quantitative extraction step of these anthraquinones in madder root has to be developed.

Several solvents were tested for their ability to dissolve these compounds. The dried glycoside extract dissolves best in solvents like water (25 absorption unit (AU)), methanol (31 AU), 0.5 M NaOH (42 AU), methanol-water 1:1 (42 AU), ethanol-water 1:1 (44 AU). According to these results an extraction method was developed for the isolation of anthraquinone glycosides with ethanol-water (1:1).

Extraction of madder root with ethanol-water was performed at different temperatures: reflux, 80°C, 45°C and room temperature. It turned out that refluxing was the best because at high temperatures filtration of the extract was much faster and a higher yield of anthraquinones was obtained. At lower temperatures the filter paper got clogged almost immediately and the filtration could take more than one hour while anthraquinones could precipitate and stay in the residue. Grinding of madder root in more coarse particles did not solve this problem and with increasing particle size the standard deviation increased also.

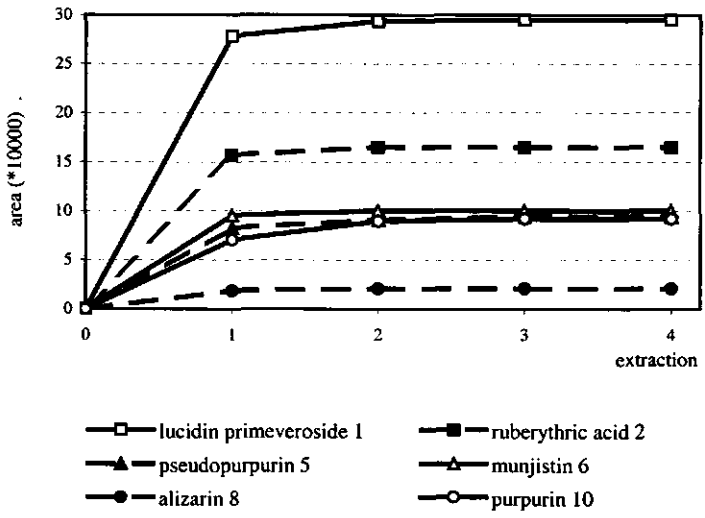


***Figure 4.2** Extraction curves of lucidin primeveroside **1**, ruberythric acid **2**, pseudopurpurin **5**, munjistin **6**, alizarin **8** and purpurin **10** with refluxing solution of ethanol-water (1:1).*

The extraction curve of lucidin primeveroside **1** and ruberythric acid **2** was determined by repeating the extraction of the madder root four times see figure 4.2. During the first more than 95% of the total extracted anthraquinone glycosides (97% of ruberythric acid **2**) was already extracted and after two extractions more than 99% of the total extracted anthraquinone glycosides was extracted. As final control after three extractions the remaining residue was extracted with boiling THF but no significant amount of anthraquinones was obtained.

The amount of sample and solvent that are necessary for the above described extraction procedure are quite large. The same was tried with half of the amount of sample (1.25 g) and solvent (50 ml). It turned out that the reproducibility decreased.

According to these extraction efficiency experiments a direct extraction method was developed based on two extraction steps of 2.5 g madder root in 100 and 50 ml ethanol-water respectively. The method is described in § *4.2.6*. The amount of glycosides and other anthraquinones that was determined in 1 g of madder root with this method is depicted in table 4.2. The experiment was done in triplicate and the RSD is depicted in table 4.2. From one of the three filtrates of the first extraction step five samples (100 μl) were taken and diluted with ethanol-water (900 μl) and analysed by HPLC. The RSD of these five samples

was calculated to determine the reproducibility of taking a sample from the same suspension, table 4.2.

Finally recovery experiments were performed. In an ethanol-water extract of madder root the amount of anthraquinones was determined. The extract was divided in three portions and these were dried separately. After drying the samples were treated with boiling ethanol-water as described in § 4.2.6. The amount of recovered anthraquinones was determined. The recovery was 93.8 %, 95.4 %, 94.8 %, 95.4 %, 96.4 % and 107.8 % for lucidin primeveroside **1**, ruberythric acid **2**, pseudopurpurin **5**, munjistin **6**, alizarin **8** and purpurin **10**, respectively. The high recovery of purpurin **10** could be caused by the fact that pseudopurpurin **5** is very easily decarboxylated. The other values are all around 95%. After refluxing the solution in ethanol-water, it is immediately filtrated to avoid precipitation of the anthraquinones. Nevertheless before all the solution has passed through the filter it has cooled down a bit. So the residue (rest of root material) may contain some anthraquinones that were precipitated during the filtration, which could be a reason for the lower recovery of these anthraquinones.

4.3.5 Quantification of aglycones in madder root suspension

In chapter 3 it was already described that the glycosides in madder root are easily converted into their aglycones by endogenous enzymes. This characteristic was used in developing a quantification method. During stirring of madder root in water ruberythric acid **2** is hydrolysed to alizarin **8** and lucidin primeveroside **1** is converted to nordamnacanthal **12** by the endogenous enzymes. Alizarin **8** and nordamnacanthal **12** will precipitate because these components do not dissolve in water. For an accurate quantification method based on this method the anthraquinones should be dissolved again before a sample can be taken. If the aglycones are not dissolved a sample of the solution will not be representative. The solubility of the main aglycone, alizarin **8** was determined in chloroform (1.9 g/l), ethyl acetate (2.8 g/l), acetone (3.7 g/l), ethanol (3.9 g/l) and 0.5 M NaOH (5.6 g/l) and THF (25.7 g/l). According to these results an extraction method was developed with water and THF.

Madder root (2.5 g) was stirred in water (100 ml) for one hour at 45°C. After one hour 400 ml of THF-water-formic acid (1:1:0.005) (v/v/v) was added and stirred at room temperature to dissolve the anthraquinones. Higher temperatures (45°C and reflux) were tried but did not yield a higher amount of anthraquinones. The amount of anthraquinones was determined by HPLC.

The main goal was to develop an accurate method using a smaller sample and less solvent than with the ethanol-water method. First the amount of THF-water–formic acid necessary to dissolve the anthraquinones after enzymatic conversion was investigated. The results are depicted in figure 4.3. The volume of solvent could be reduced from 400 to 200 ml

THF-water-formic acid if 2.5 g of madder root was used. The recovery and the RSD of these extractions were comparable. At lower amounts of solvent the amount of extracted anthraquinone decreased and the RSD increased. The results depicted in table 4.2 were obtained after 1 hour. When the suspensions were stirred longer (24 hours) the same results were obtained.

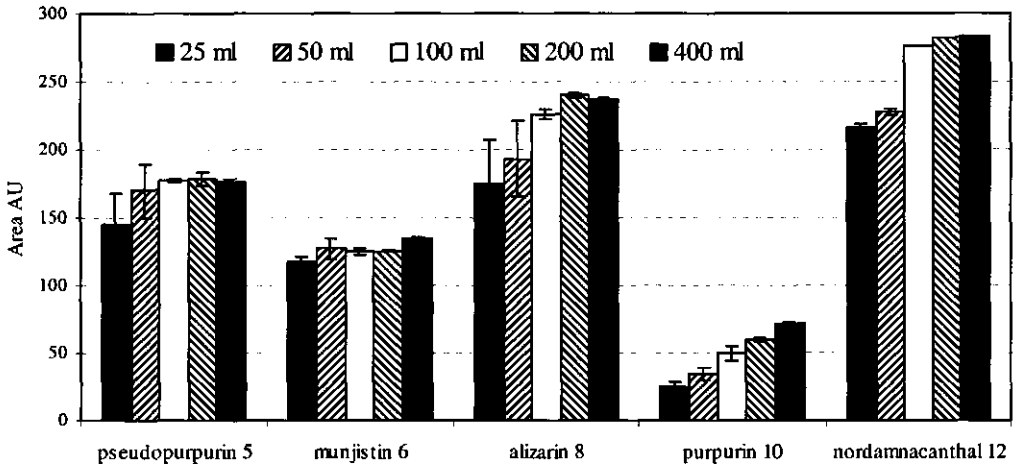


***Figure 4.3** Extraction efficiency of anthraquinones by adding different amounts of THF-water-formic acid (1:1:0.005) (v/v/v) after stirring 2.5 g madder root in 100 ml of water for 1 hour at 45°C.*

Secondly the scale of the experiment was varied but the ratio between madder root, water and added THF-water-formic acid was kept constant (1 g : 160 ml). The results are depicted in figure 4.4.

Adding 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) after stirring 0.25 g ground madder root in 10 ml ultra-pure water was a good extraction method for the quantitative determination of the anthraquinones with an acceptable standard deviation. With this indirect method the amount of sample and the amount of solvent could be decreased ten times relative to the direct method. The amount of anthraquinones and the relative standard deviation (triplicate) is depicted in table 4.2.

In an acidified solution at room temperature or 45°C pseudopurpurin **5** will be decarboxylated to purpurin **10** (see chapter 2). In the method described above an acidified THF-water solution was added to the madder root suspension, which gave decarboxylation of pseudopurpurin **5**. It is possible that all the purpurin **10** that is detected in the solution is

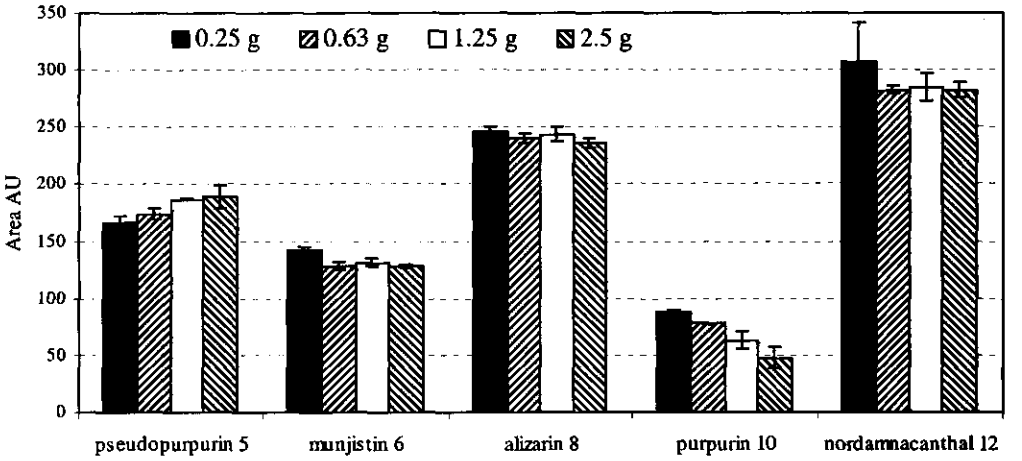


Figure 4.4 Extraction efficiency of anthraquinones by varying the scale of the experiment: 0.25 g madder root + 10 ml water + 40 ml THF-water-formic acid or 0.63+25+100 or 1.25+50+200 or 2.5+100+500, respectively.

formed out of pseudopurpurin **5**. If no acid was added to the suspension no purpurin **10** could be detected. However if no acid is added to the madder suspension only half of the amount of the formed nordamnacanthal **12** will dissolve in the THF-water solution. For a quantitative determination of the amount of nordamnacanthal **12** acid is obligatory. The native amount of pseudopurpurin **5** available in madder root can be calculated by multiplying the amount of purpurin **10** with 300/256 (the Mw of pseudopurpurin **5** divided by the Mw of purpurin **10**). It is not clear whether purpurin **10** is present in the native plant or only pseudopurpurin **5**. In all the analyses of madder root extracts performed for this thesis purpurin **10** was found. Because purpurin **10** is formed out of pseudopurpurin **5** very easily this can already happen during the harvesting and/or drying of the roots, which is also mentioned in the literature[1].

The recovery experiments were performed for a madder root water extract with known amounts of the different anthraquinones. The recovery was 104%, 115%, 108%, 106% and 110% for pseudopurpurin **5**, munjistin **6**, alizarin **8**, purpurin **10** and nordamnancathal **12**, respectively. The recovery values are on average 5-10% higher than the 100% expected. During the determination the suspension is stirred at 45 °C. In spite of closing the flask with a rubber stop maybe some water or THF evaporated due to the higher temperature.

The extraction method with ethanol-water did not give the same amounts of pseudopurpurin **5** and munjistin **6** as the THF-water method (table 4.2). To check if the extraction solvent caused this, madder root was also extracted with THF-water without prior

conversion of the glycosides. In this case the amount of pseudopurpurin **5** and munjistin **6** increased but still less than found after conversion. This could point at a pseudopurpurin glycoside and a munjistin glycoside but no proof for this could be found with HPLC-DAD or HPLC-MS.

Table 4.2 *The concentration of anthraquinones determined with a direct ethanol-water extraction and the concentration determined with the indirect method consisting of first an incubation with the endogenous enzymes and secondly extraction with THF-water. The corresponding RSD of five samples from five extracts and the RSD of five samples from the same extract.*

compound	**direct method mg/g root**	**RSD**	**RSD 1 extract**	**indirect method mg/g root**	**RSD**	**RSD 1 extract**
lucidin primeveroside **1**	24.58	3.5	1.2	0	-	-
ruberythric acid **2**	10.11	3.3	1.2	0	-	-
pseudopurpurin **5**	3.72	5.6	1.7	8.64	4.4	1.3
munjistin **6**	2.30	6.1	1.0	3.69	4.5	2.3
alizarin **8**	0.54	7.6	0.7	6.33	2.6	1.9
purpurin **10**	2.08	12.5	3.9	1.06	8.6	8.2
nordamnacanthal **12**	0.65	20.0	-	12.53	5.9	1.8

4.3.6 Liquefaction

Although unlikely it is still possible that not all anthraquinones are released by these extraction methods and perhaps some stay bound to the rest of the plant material. First the polysaccharide composition of the madder root was determined, by acidic hydrolysis and sugar analysis with a high pressure anion exchange chromatography (HPAEC). This determination was carried out by TNO, Zeist, the Netherlands[143,144]. About 30% of the plant consist of polysaccharides, with cellulose as main component but also some hemicellulose and pectin is present. To release anthraquinones bound to cell material, madder root was treated with commercial liquefaction enzymes. Madder root was treated with two enzyme preparations: Viscozyme L and Celluclast 1,5 L. Viscozyme L is a pectinase with also some hemicellulolutic and cellulolytic activity. Celluclast 1,5 L is a cellulase. The amount of anthraquinones that was determined in these samples was the same as found without liquefaction enzymes. Use of the liquefaction enzyme preparations Viscozym L and Celluclast 1,5 L does not release more anthraquinones from madder root than extraction with organic solvents. This indicates that the anthraquinones in madder root are not stored inside plant cells or connected to polysaccharides or cellulose or other plant cell material.

4.3.7 Screening of Rubia tinctorum cultivars

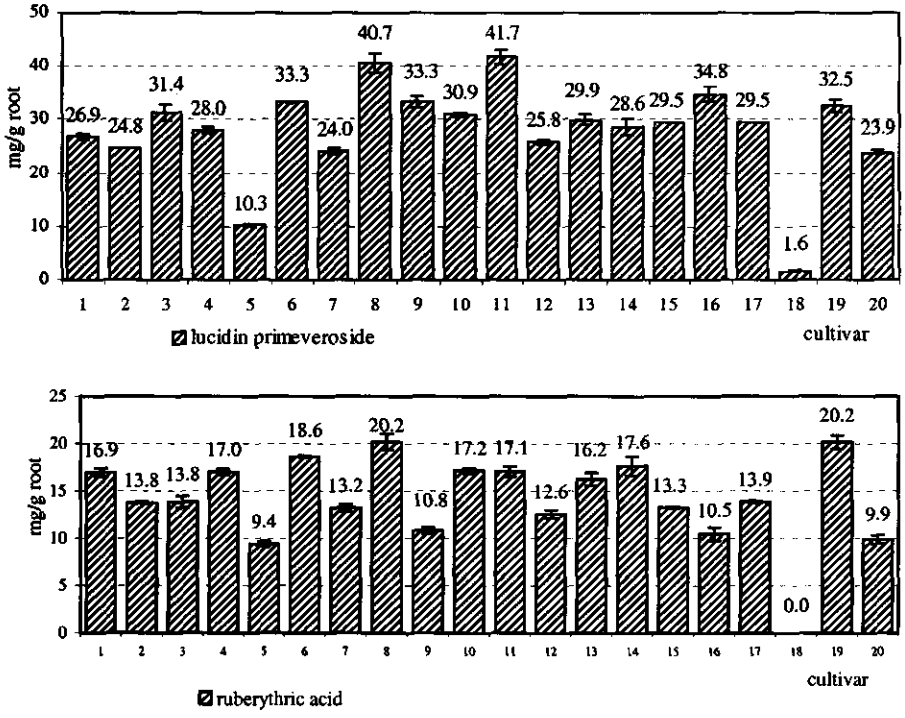
Different cultivars of *Rubia tinctorum* cultivated in Noordbroek, Groningen, The Netherlands were collected. These cultivars were screened for their content of lucidin primeveroside **1** and ruberythric acid **2** with the direct ethanol-water method described in § *4.2.6.* The concentration of pseudopurpurin **5**, munjistin **6**, alizarin **8**, purpurin **10** and nordamnacanthal **12** was determined with the indirect THF-water method (conversion with endogenous enzymes) described in § *4.2.7.* The results are depicted in figures 4.5 and 4.6. According to the manufacturer all the roots tested were *Rubia tinctorum* cultivars with exception of batch no 18, which should be *Rubia cordifolia.* Batch 18 was analysed but no ruberythric acid **1** and/or alizarin **8** was detected in the sample, only a small amount of lucidin primeveroside **1** was detected. According to the literature *Rubia cordifolia* should contain alizarin **8**[1,28]. So according to these results the roots could either not belong to *Rubia cordifolia* or *Rubia tinctorum.*

The original concentration of lucidin primeveroside **1** and ruberythric acid **2** can be calculated from the concentration of nordamnacanthal **12** and alizarin **8** as determined by the indirect method. On average the concentration of alizarin **8** that was determined by recalculation was 18% higher than the concentration of ruberythric acid as determined with the direct ethanol-water extraction. For nordamnacanthal **12** the THF-water method gave a 7% higher result than expected from the direct ethanol-water method. This is a deviation that cannot be ignored. Especially for cultivar 16 the difference is relatively large. The results of the recovery experiments in § *4.3.4* and § *4.3.5* show that the glycosides determination gave in general 5% lower values expected while the aglycones determination gave values that were 5-10% too high. These recovery differences can explain the different values found in the screening experiments.

The concentrations that were found for lucidin primeveroside **1**, ruberythric acid **2**, alizarin **8** and nordamnacanthal **12** are depicted in figure 4.5. The highest concentration of lucidin primeveroside **1** was found in the cultivars 11, 8 and 16. The lowest concentration was found for cultivar 5. The highest concentration of nordamnacanthal **12** is determined for 11, 8, 16. The lowest concentration is determined for 20. The highest concentration of ruberythric acid **2** with the direct method and the indirect method was found for cultivars 8 and 19. But also cultivars 6 and 14 have a high concentration of ruberythric acid **2** and alizarin **8**. The cultivars 5, 20, 9 and 16 have the lowest concentration of ruberythric acid **2**. The lowest concentration of alizarin **8** was found in cultivars 20, 9 and 16.

Cultivar 5 has a much higher concentration of alizarin **8** (10.0 mg/g) determined with the indirect method than expected from the concentration of ruberythric acid **2** (9.4 mg/g) found with the direct method. With the indirect method a concentration of alizarin **8** should be

found that is half the amount of the concentration of ruberythric acid **2** determined with the direct method. Due to the enzymatic conversion of ruberythric acid, with a molecular weight of 534, is converted to alizarin **8,** which has a molecular weight of 240. However in figure 4.5 it is not depicted that during the direct ethanol-water extraction (1:1) (v/v) beside 10.9 mg/g ruberythric acid **2** also 3.2 mg/g alizarin **8** was extracted. The same was found for lucidin primeveroside **1** and nordamnacanthal **12** in the same cultivar 5. So before the enzymatic reaction was applied on these madder roots the concentration of alizarin **8** and nordamnacanthal **12** was already relatively high. In all the other cultivars the concentration of alizarin **8** found with the direct extraction with ethanol-water extraction varied between 0 and 0.6 mg/g. This explains why cultivar 5 has the lowest concentration of ruberythric acid **2** and lucidin primeveroside **1** but not the lowest concentration of alizarin **8** and nordamnacanthal **12.**



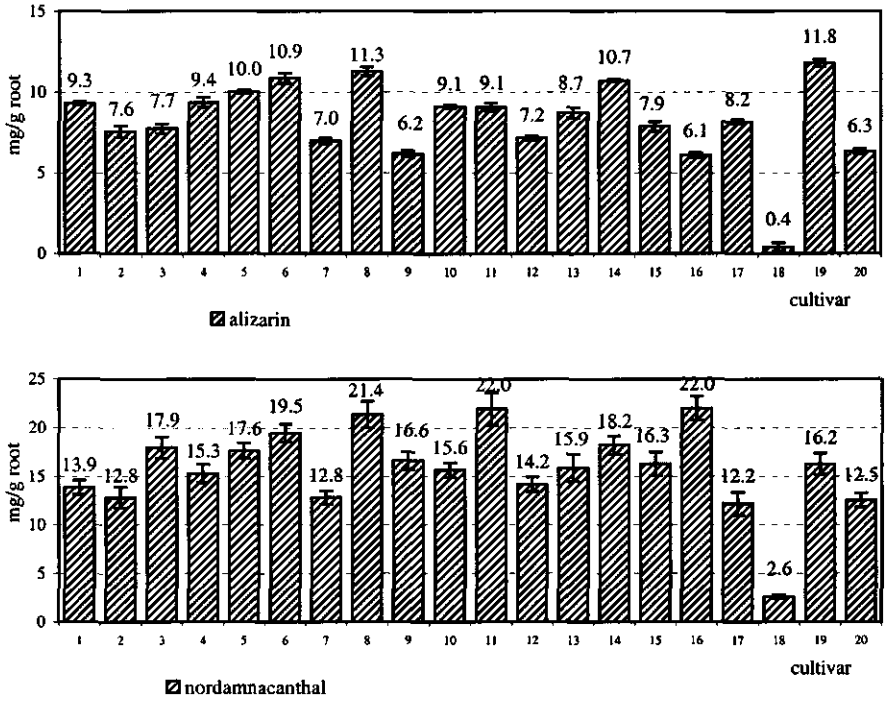


Figure 4.5 *The concentration of lucidin primeveroside* ***1*** *and ruberythric acid* ***2*** *in mg/g root determined with the direct ethanol-water extraction method of different cultivars of Rubia tinctorum. The concentration of nordamnacanthal* ***12*** *and alizarin* ***8*** *in mg/g determined with the indirect hydrolysis THF-water extraction method of different cultivars of Rubia tinctorum.*

The concentration of pseudopurpurin **5**, mujistin **6** and purpurin **10** found with the indirect THF-water extraction method is depicted in figure 4.6. The highest amount of pseudopurpurin **5** + purpurin **10** in madder root is found in cultivars 16 and 8. The lowest amount is found for cultivars 11, 12 and 20. For munjistin **6** the highest level in madder root is found for sample 17 and 19 the lowest for 7 and 14. Because pseudopurpurin **5** is easily converted to purpurin **10** during the analysis (§ *4.3.5*) the amount of pseudopurpurin **5** and purpurin **10** is depicted in one graph.

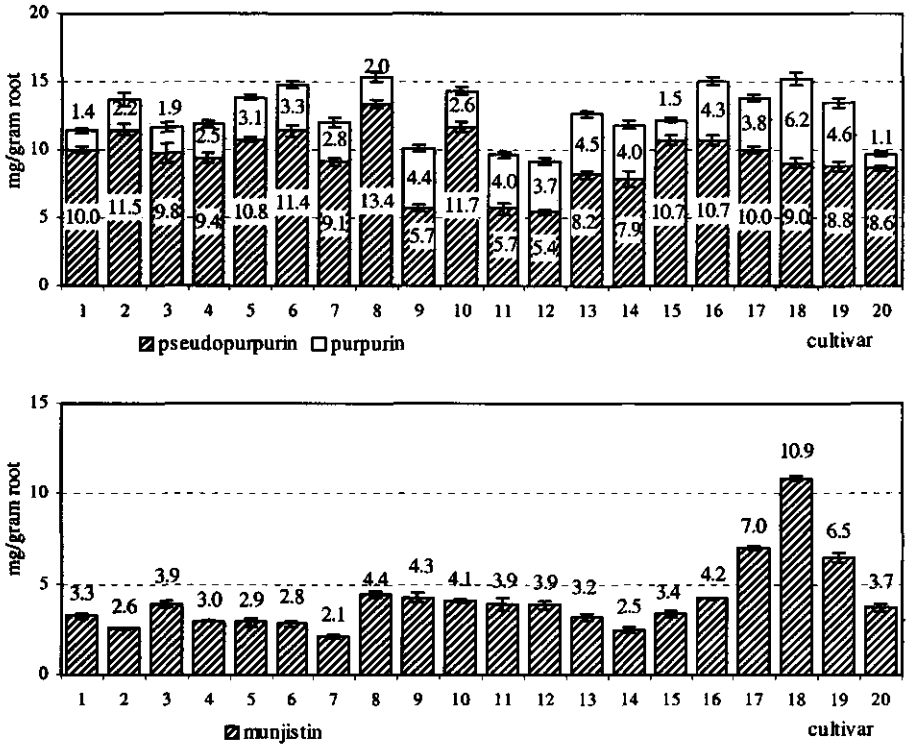


***Figure 4.6** The concentration pseudopurpurin **5**, munjistin **6** and purpurin **10** found in different cultivars of Rubia tinctorum after conversion of the glycosides and extraction with THF-water.*

4.3.8 Influence of harvest year on anthraquinone content

In earlier days madder root was harvested in the autumn of the third year (chapter 1). For a more economically feasible production it would be preferable if the harvesting could already take place in the second year. In this case the expense of one more year of cultivation must be compared to the difference between the amounts of alizarin **8** that can be isolated after two and three years. The concentration of anthraquinones was determined with the indirect quantification method described in § *4.2.7*. The results for two and three year old madder roots are depicted in table 4.3. If madder root will be cultivated three years instead of two years, the concentration of alizarin **8** in the roots is a factor 1.3 higher. Beside the content of alizarin **8** in the root also the total weight of madder root harvested per hectare is important. After two years 2000 kg dry roots to a hectare are harvested after three years this increased with 10-15%.

Table 4.3 Amount of anthraquinones (mg/g root) in two and three year old madder root, and ratio three/two year.

compound	two year	three year	ratio
pseudopurpurin **5**	7.7	7.4	1.0
munjistin **6**	5.8	6.2	1.1
alizarin **8**	6.7	8.7	1.3
purpurin **10**	3.7	3.5	0.9
nordamnacanthal **12**	11.3	13.4	1.2

4.4 Conclusion

Two methods were developed for the quantitative analysis of anthraquinones in madder root. Refluxing madder root twice in boiling ethanol-water (1:1) (v/v) was suitable for the extraction of anthraquinone glycosides. Lucidin primeveroside **1** and ruberythric acid **2** were quantitatively determined with HPLC. With the other method lucidin primeveroside **1** and ruberythric acid were first converted to the aglycones nordamnacanthal **12** and alizarin **8**. After conversion the aglycones were extracted with THF-water-formic acid (1:1:0.005) (v/v/v).

Compared to the direct ethanol-water extraction method for the quantitative determination of the glycosides, the indirect method needs less material and is simpler to perform. In the direct method the suspension must be refluxed, filtrated and the remaining residue has to be refluxed again, which is labour intensive. In the indirect method the temperature is much lower and the suspension just needs to be stirred. The method was performed at 45 °C but can also be performed at room temperature in case no oven is available. Then the suspension must be stirred for two hours instead of one hour. However one has to realise that the compounds determined with the THF-water method are actually artefacts of the anthraquinones originally present in madder root. Alizarin **8** is an artefact of ruberythric acid **2**, nordamnacanthal **12** an artefact of lucidin primeveroside **1**, and purpurin **10** an artefact of pseudopurpurin **5**.

If the indirect method is selected the HPLC analysis time can be reduced. With the present HPLC method the first anthraquinone (pseudopurpurin **5**) elutes only at t=15 min. So the gradient system could start at a higher percentage of acetonitrile because no glycosides are present. A disadvantage is that no glycosides can be determined if the enzymatic conversion was not complete.

The concentration of alizarin **8** varied between 6.1 and 11.8 mg/g root for the different cultivars screened in this research. This difference has to be taken into account when a cultivar is selected for the commercial production of alizarin **8**. Beside the content of

alizarin **8** in the root also agronomic characteristics like plant density, root density, root thickness, resistance against abiotic and biotic influences and cost/benefit of two or three year old roots are very important. These factors were not investigated in this research.

Chapter 5

Investigation of different procedures to isolate alizarin from *Rubia tinctorum* L. roots

5.1 Introduction

As already described in chapter 3 somewhere during dye preparation from madder root, ruberythric acid has to be hydrolysed to alizarin **8**. Ruberythric acid **2** can easily be converted to alizarin **8** by stirring ground madder root in water. Under these circumstances the endogenous enzymes will hydrolyse the glycoside ruberythric acid **2** into alizarin **8** and the monosaccharides. This method is much easier and cheaper than hydrolysis by acids, especially if it has to be used in industry on a commercial scale. Beside alizarin **8** also the carboxyanthraquinones pseudopurpurin **5** and munjistin **6** and the aldehyde nordamnacanthal **12** are present in the mixture. Possibly this procedure could be used in the production of an alizarin **8** preparation from madder root. However first a good separation procedure has to be developed for the water insoluble alizarin **8** and the rest of the plant material.

In this chapter research is described about the different routes to isolate alizarin **8** from madder root and to select a method for a large-scale dye preparation. The methods were compared with respect to ease of operation, yield and economic feasibility.

5.2 Experimental

5.2.1 Chemicals

Ammonium formate, formic acid, ethanol, EDTA and NaOH were obtained from Acros (Geel, Belgium). Surfactants Brij 35, 58, 76, 78, 92, 98 and Tween 20, 40, 80 and 85 were obtained from Acros (Geel, Belgium). SPE materials C_{18}, CH, C_8, CBA, CN(EC), ENV^+ and SCX were obtained from International Sorbent Technology (Hengoed, United Kingdom);

OASIS and Bond elut were from Waters Corporation (Milford, Mass, USA); PBA was obtained from Analytichem international (Zug, Switzerland). Resin materials Amberlite IR 120 (H), paricle size 0.30-1.18 mm (14-52 mesh), Amberlite IRA-400 (CI) particle size 0.30-1.18 mm (14-42 mesh), Dowex 50W-X8 (H) particle size 0.075-0.15 mm (100-200 mesh) were obtained from BDH Chemicals Ltd (Poole, United Kingdom). Amberlite XAD-4 was obtained from SERVA, Dowex Optipure was obtained from Sigma-Aldrich, Extrelute was obtained from Merck and Chelex 100 Resin from Biorad (Hercules, USA). Plant material was processed as described in § *3.2.4*

5.2.2 HPLC analysis

All the samples were filtered over a 0.45 µm, ø 25 mm membrane filter (Type RC, Schleicher & Schuell) and analysed by the HPLC method described in chapter 3. HPLC-DAD and HPLC-MS experiments were carried out as described in chapter 2.

5.2.3 Reference sample

Dried and powdered madder (0.25 g) was stirred in 10 ml ultrapure water for 1 hour at 45°C. After stirring 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) was added and stirred for 30 min at 45°C. A sample of 1000 µl was taken and 1000 µl of THF-water-formic acid (1:1:0.005) (v/v/v) was added. The diluted samples were filtered over a 0.45 µm ø 0.25 mm membrane filter and analysed by HPLC. This procedure was taken as a reference in all experiments, corresponding to the maximum amount of anthraquinones from madder root.

5.2.4 Particle size and amount of water

A series of experiments was carried out to determine the influence of the particle size of madder and the influence of the amount of water on the conversion of glycosides to aglycones. To determine the minimal amount of water necessary for the conversion, madder root (0.25 g) was stirred in different amounts of water: 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml. The madder root was further treated according the procedure described for the reference sample.

Dried madder root was divided in 5 different batches. All batches were ground in a hammer mill with a sieve of 6 mm. Batch 2, 3, 4 and 5 were further ground in a hammer mill with a 1.5 mm sieve. Batch 3 was further ground in a cutting mill with a 1.5 mm sieve. Batch 4 and 5 were further ground in a hammer mill with a sieve of 1.5 mm. Finally batch 5 was ground in a cutting mill with a sieve of 0.75 mm. The different treatments are depicted in

table 5.1. After grinding and sieving part of the batch was used for the determination of the anthraquinone concentration according to the method described in §5.2.5 (triplicate). Contrary to the reference sample the suspensions of batch 1, 2, 3, 4 and 5 were stirred for 1 day and batch 1 and 5 also for 10 days.

Table 5.1 Grinding sizes for madder root.

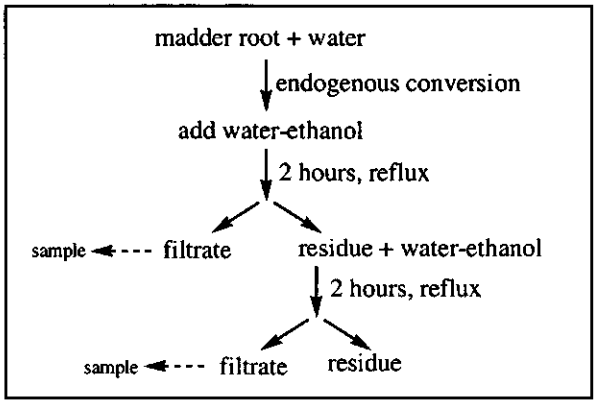
batch	KS 6mm	KS 3mm	KS 1.5mm	RE 1.5mm	RE 0.75mm
1	X				
2	X	X			
3	X	X		X	
4	X	X	X		
5	X	X	X		X

KS = Hammer mill with 6, 3 or 1.5 mm sieve
RE = Cutting mill with 1.5 or 0.75 mm sieve

5.2.5 *Extraction with water or water–ethanol after enzymatic formation of aglycones*

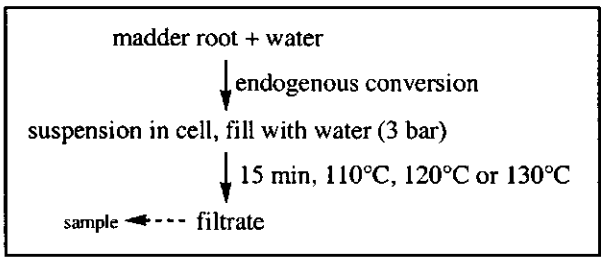
Madder root (2.5 g) was stirred in 100 ml ultrapure water for 1 hour at 45°C. After 1 hour 100 ml of water-ethanol (1:1) (v/v) was added and the suspension was refluxed for 2 hours. After refluxing the suspension was filtered immediately (as hot as possible) over a Büchner funnel. The amount of filtrate was determined and a sample of 100 µl was taken, which was diluted with 900µl THF-water-formic acid (1:1:0.005) (v/v/v). The residue was suspended in 100 ml water–ethanol (1:1) and refluxed for 2 hours and filtered. The amount of filtrate was determined and a sample of 1000 µl was taken and diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v). Again the residue was suspended in 100 ml water–ethanol (1:1) (v/v) and the reflux experiment was repeated twice. In total the 2.5 g of madder root was extracted 4 times with water-ethanol (1:1) (v/v). The complete extraction procedure was repeated 5 times with different ratios of water-ethanol. Water-ethanol 2:1, 4:1, 9:1 and plain water were used. The extraction scheme is depicted in scheme 5.1.

With stainless steel HPLC tubing an HPLC pump was connected in-line with a 10 ml and 5 ml high-pressure cell. These stainless high-pressure cells were normally used in a supercritical fluid extraction device. In the second cell (5ml) a 0.45 µm filter was placed at the entrance and exit of the cell to avoid root material from entering the tubing. The last cell was connected with 2 m stainless steel HPLC tubing of ø 0.25 mm. The tubing and cells were placed in an oven. The end of the tubing stuck just out of the oven into a tube with 2 ml of cold water. Then water was pumped through the system at a flow of 1 ml/min. The overall pressure in the system was approximately 4 bar.



Scheme 5.1 *Extraction of madder root with water or water-ethanol after enzymatic formation of aglycones.*

The 5 ml cell was filled with madder root suspension. Ultrapure water was pumped through the system at a flow of 1.0 ml/min. When the system was filled with water the tubing that stuck outside the oven was closed. Within 15 min the oven was heated up to 110°C, 120°C or 130°C. The over pressure (3 bar), which is higher than the atmospheric pressure (1 bar) prevents the water from boiling. After reaching the set temperature, the system remained at this temperature for 30 min. After 30 min the tubing was opened and the water was pumped with 1.0 ml/min out of the system into the cold water in the test tube. When the red colour of the water that came out the tubing, had faded away the flow was stopped. The amount of filtrate was determined and a sample (1000 μl) of the filtrate was taken and according to the colour diluted with 1000 μl or 2000 μl of THF-water-formic acid (1:1:0.005) (v/v/v) and analysed on the HPLC. The procedure is depicted in scheme 5.2 and figure 5.1.



Scheme 5.2 *Extraction scheme of madder root with water above 100°C and under pressure after enzymatic formation of aglycones.*

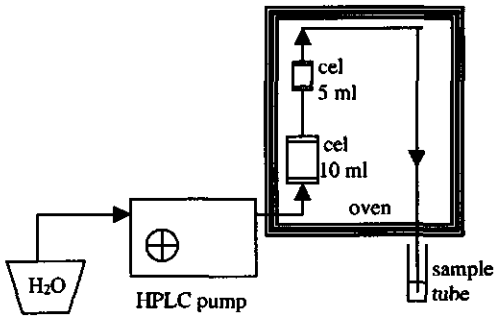


Figure 5.1 Extraction procedure of madder root with water above 100°C and under pressure after enzymatic formation of aglycones

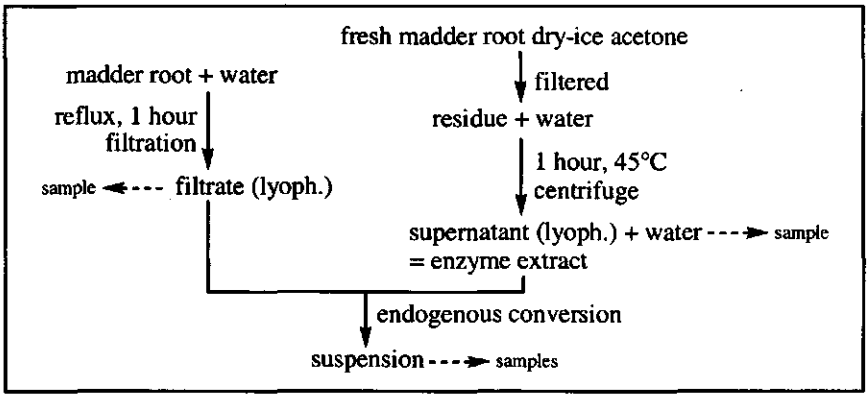
5.2.6 *Extraction of glycosides and conversion into aglycones*

Dried and powdered madder root (2.5 g) was added to boiling ultrapure water (100 ml). The suspension was refluxed for 1 hour before immediate filtration over a Büchner funnel. An aqueous extract containing glycosides was obtained. A sample of the filtrate (500 µl) was taken.

Fresh madder root was finely chopped and crushed in dry-ice acetone by an Ultra-Turrax T-50 (Rowa Techniek B.V., Leiderdorp, The Netherlands) and then filtered on a Büchner. The residue was washed with chilled acetone (-20°C) until the filtrate became colourless. The residue was spread on filter paper in a hood and occasionally turned over to evaporate the acetone. The residual "acetone powder" was kept frozen[128]. "Acetone powder" (2.5 g) was stirred for 1 hour at 45 °C in ultrapure water (200 ml). The suspension was centrifuged at 3600 rpm for 20 min in a Mistral 1000 from MSE (U.K). The supernatant was lyophilised in a Christ Alpha 1-2 freeze dryer (Salm en Kipp, Breukelen, The Netherlands). A freeze-dried enzyme extract was obtained.

The freeze-dried enzyme extract was dissolved in 125 ml ultrapure water. A sample (1000 µl) was taken. Glycoside extracts were treated with 50 ml and 5 ml of the enzyme extract solution. The solutions were stirred at 45 °C and at t=0, 1, 2, 3, 4, 8 and 24 hours a sample was taken (500 µl). The complete procedure is also depicted in scheme 5.3.

All samples, 500 µl in this experiment, were diluted with 2000 µl THF-water-formic acid (1:1:0.005) (v/v/v) stirred and filtrated over a 0.45 µm ø 25 mm membrane filter. 1000 µl of the filtrate was diluted in 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v). An exception was the sample of enzyme extract solution (1000 µl) which was diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v).

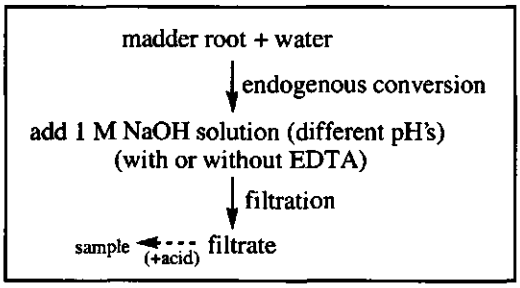


***Scheme 5.3** Extraction of madder root with boiling water followed by enzymatic conversion of the glycosides by an enzyme extract isolated from madder root.*

5.2.7 Extraction with an alkaline solution

Madder root (0.25 g) was stirred in 10 ml water at 45°C. After 1 hour a solution of 1 M NaOH was added. The pH of the solution was adjusted to 10 or 13, which gave a colour change from yellow to purple. Water was added to 50 ml and the suspension was stirred for 30 min at room temperature. After stirring ± 2000 µl of the suspension was filtrated on a membrane filter (scheme 5.4). A sample was taken (1000 µl).

A solution of pure alizarin 8 (0.0792 g) in THF (100 ml) was made. The solution was divided in 10 parts of 10 ml and the separate solutions were dried with a rotary evaporator. The dried samples were suspended in 10 ml ultrapure water or tap water and stirred for 1 hour at 45°C. After 1 hour 1 M NaOH solution (corresponding to the sample in ultrapure or tap water) was added until the solution had a pH of 10 or 13. Ultrapure water or tap water was



***Scheme 5.4** Extraction of madder root with an alkaline solution after enzymatic conversion.*

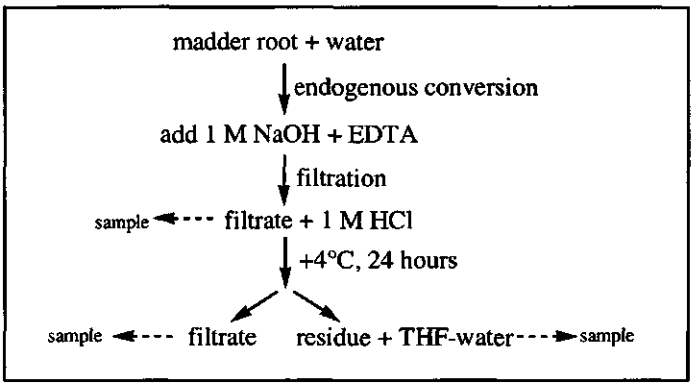
added to 50 ml. The solution was stirred for 30 min and filtered over a membrane filter (scheme 5.4).

Four series of madder root (0.25 g) were stirred in 10 ml ultrapure or in 10 ml tap water at 45°C for 1 hour. To the madder root suspensions in tap water, 1 M NaOH solution in tap water was added until the suspension had a pH of 10 or 13. Experiment was performed in duplicate. To the madder root suspensions in ultrapure water 1 M NaOH solution in ultrapure water was added until the suspension had a pH of 10 or 13 (duplicate). The solution changed from yellow to purple when the pH was increased. To the suspensions ultrapure water or tap water was added to 50 ml. The solution was stirred for 30 min and filtrated over a membrane filter (scheme 5.4). A sample was taken (1000 μl).

Madder root (0.25 g) was stirred in different concentrations of EDTA solution in ultrapure water (10 ml) at 45°C. Concentrations of 1, 0.8, 0.6, 0.5 and 0.1% were used (triplicate). After 60 min 20 ml of 1 M NaOH in ultrapure water was added and 20 ml of ultrapure water. The solution (2000 μl) was filtrated over a membrane filter (scheme 5.4). A sample was taken (1000 μl).

Madder root (0.25 g) was stirred in a solution of 0.6 % EDTA in ultrapure water (10 ml) at 45°C. After 1 hour 20 ml, 2000 μl or 1000 μl 1 M NaOH in ultrapure water was added. Ultrapure water was added to 50 ml. The pH of the solution was determined and the solution was filtrated (2000 μl) over a membrane filter (scheme 5.4). A sample was taken (1000 μl).

Madder root (0.25 g) was stirred in a solution of 0.6 % EDTA in ultrapure water (10 ml) at 45°C. After 1 hour 20 ml 1 M NaOH and 20 ml ultrapure water was added. The pH of the solution was measured and the solution was filtered over a paper filter. A sample was



Scheme 5.5 Extraction of madder root with an alkaline solution after enzymatic conversion. After extraction the solution was acidified to precipitate the anthraquinones.

taken (1000 μl). The rest of the filtrate was acidified with a 1 M HCl solution, which resulted in a colour change (from purple to orange/yellow). The solution was stored in the refrigerator for 24 hours. A second filtration (over a membrane filter) was performed and a sample of the filtrate was taken (1000 μl). The residue was dissolved in 50 ml THF-water and a sample was taken (1000 μl) (scheme 5.5). All samples (1000 μl) were diluted with 1000 μl of THF-water-formic acid (1:1:0.005) (v/v/v). that resulted in a colour change. The samples were analysed by HPLC.

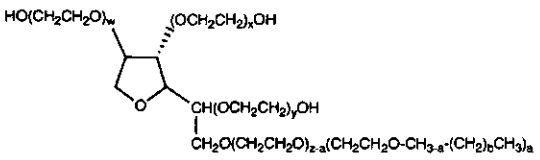
5.2.8 Extraction of madder root with surfactants

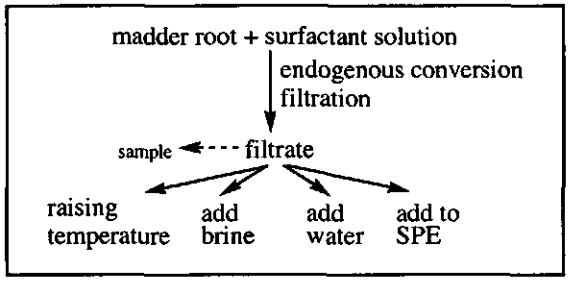
Madder root (0.25 g) was dissolved in 50 ml of 1, 0.75 or 0.5% surfactant solution. The following surfactants were used Brij 35, 58, 76, 78, 92, 98 and Tween 20, 40, 80, 85 (table 5.2). The solution was stirred for 1 hour at 45°C and was filtrated. A sample (500 μl) was taken from the solution and diluted with (500 μl) THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. In order to isolate the aglycones and separate the surfactant, the filtrate solution was treated in different ways (scheme 5.6).

- Water up to 300 ml was added and in between the additions, the surfactant solutions were kept in the fridge for precipitation.
- Raising the temperature up to 75°C.
- Addition of brine.
- Solid phase extraction see § *5.2.10.*

***Table 5.2** Surfactants.*

name	compound	formula
Brij 35	polyoxyethylene (23) lauryl ether	$C_{12}H_{25}(OCH_2CH_2)_{23}OH$
Brij 58	polyoxyethylene (20) cetyl ether	$C_{16}H_{33}(OCH_2CH_2)_{20}OH$
Brij 76	polyoxyethylene (10) stearyl ether	$C_{18}H_{37}(OCH_2CH_2)_{10}OH$
Brij 78	polyoxyethylene (20) stearyl ether	$C_{18}H_{37}(OCH_2CH_2)_{20}OH$
Brij 92	polyoxyethylene (2) oleyl ether	$C_{18}H_{35}(OCH_2CH_2)_2OH$
Brij 98	polyoxyethylene (20) oleyl ether	$C_{18}H_{35}(OCH_2CH_2)_{20}OH$
Tween 20	polyoxyethylene (20) sorbitan monolaurate	w+x+y+z = 20, b=9, a=1
Tween 40	polyoxyethylene (20) sorbitan monopalmitate	w+x+y+z = 20, b=14, a=1
Tween 80	polyoxyethylene (20) sorbitan monooleate	w+x+y+z = 20, b=16, a=1
Tween 85	polyoxyethylene (20) sorbitan trioleate	w+x+y+z = 20, b=16, a=3





***Scheme 5.6** Extraction of madder root with surfactant after enzymatic conversion.*

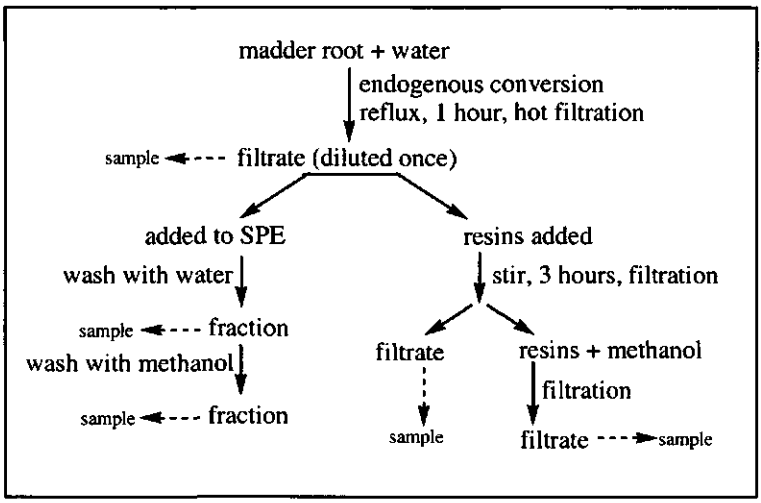
5.2.9 Elution of anthraquinones from solid phase materials

Madder root (2.5 g) was stirred in water (100 ml) for 2 hours at 45°C. The suspension was refluxed for 1 hour and filtered as hot as possible over a Büchner funnel. The filtrate was diluted once with ultrapure water. A sample of the filtrate (500µl) was diluted with 2000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. The rest of the filtrate was added to different solid phase materials like commercially available resins and commercially available ready-made solid phase extraction cartridges (SPE).

Filtrate (9 ml) was added to 1.5 g of resin material. The suspension was stirred for 3 hours at room temperature. After stirring the suspension was filtrated over a Büchner funnel and 1000 µl of the filtrate was diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. The resins (residue) were stirred for 2 hours in 9 ml methanol and filtrated (scheme 5.7). The filtrate was analysed by HPLC without further dilution. The following commercial resins were tested: Amberlite IRA-400 (Cl) (strongly basic anion exchanger, quarternary ammonium, polystrene), Amberlite IR-120 (H) (strongly acidic cation exchanger, cross linked polystyrene), Dowex 50W8 (H) (strongly acidic cation exchanger, cross linked polystyrene), Amberlite 200 Na^+-form (strongly acidic cation exchanger), Amberlite XAD-4 (non-ionic, phenol recovery) and Chelex 100 Resin (weak cation chelating).

The diluted filtrate was also passed through activated SPE cartridges. The columns were activated by eluting the column with 4 ml methanol and 4 ml of ultrapure water. After activation 4 ml of filtrate was passed through the column. The column was washed *n* times with 4 ml of ultrapure water until all colour had faded away. Finally *n* times 4 ml methanol were added to elute absorbed components (scheme 5.7). All coloured samples were analysed. The aqueous samples (1000 µl) were diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. Methanol samples were not diluted but analysed directly by

HPLC. The following commercially available SPE materials were tested: C_{18}, CH, C_8, (non polar, trifunctional silane), CN(EC) (non-polar, polar for aqueous matrix) PBA (covalent), CBA (weak cation exchanger), SCX (strong cation exchange) and ENV$^+$ (non-polar, hyper cross-linked styrene divinyl benzene), OASIS (non polar).



Scheme 5.7 *Fractionation of madder root suspension with solid phase materials or resins.*

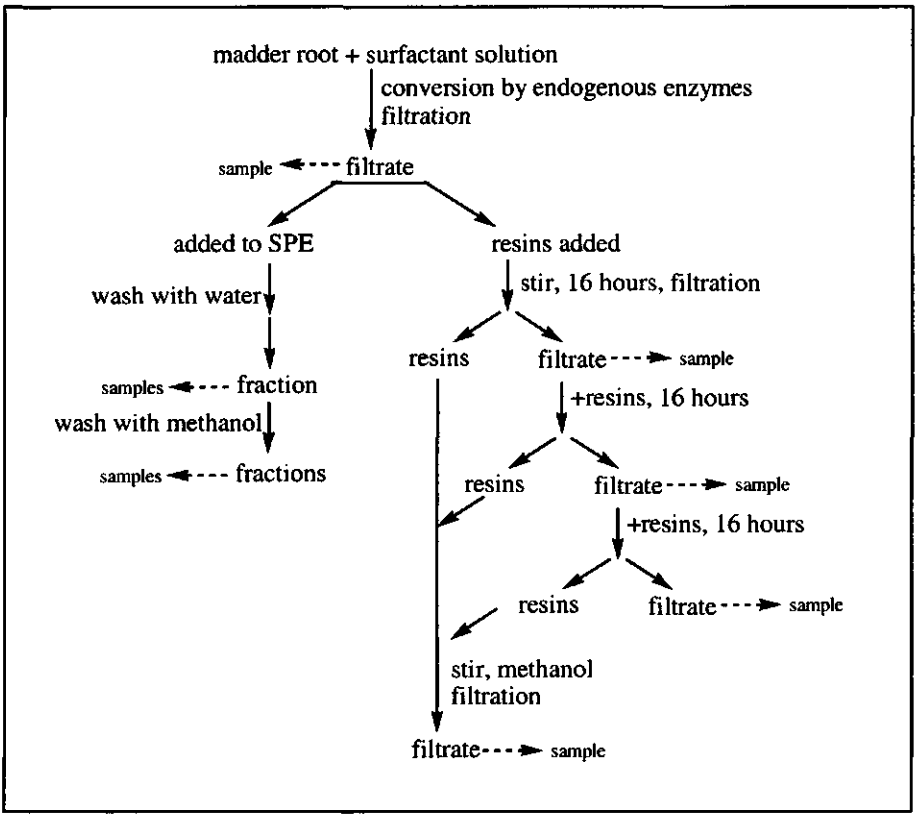
5.2.10 Extraction of madder root with surfactants and fractionation of the extract with solid phase materials

Madder root (0.5 g) was stirred in 100 ml of 0.75% Brij 78 solution for 2 hours at 45°C. After stirring the solution was filtrated. A sample 1000 μl was taken from the solution and diluted once with (1000 μl) THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. The rest of the filtrate was added to commercially available solid phase cartridges or resin material: Dowex 50W8(H) resins, Amberlite IRA-120 (Cl) resins and SPE cartridges with C_{18} sorbent.

The filtrate (15 ml) was added to 2 g of Dowex 50W8 or Amberlite IRA-120 resin material. The suspension was stirred 16 hours at room temperature. After stirring the suspension was filtrated and a sample (1000 μl) was taken. The rest of the filtrate was again added to 2 g of resins, stirred overnight, filtrated, a sample (1000 μl) was taken and the remaining filtrate was again treated with 2 g resins. The 3 (residues) resins were individually stirred overnight in 15 ml of methanol and filtrated. The extraction scheme is depicted in scheme 5.8. The filtrate was analysed by HPLC without dilution. The other (aqueous) samples

(1000 µl) were diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC.

The filtrate obtained after stirring madder root in surfactant solution and filtration, was also passed through activated C_{18} SPE cartridges. Applying 4 ml methanol and 4 ml of ultrapure water preactivated the column. Then 4 ml of the surfactant filtrate was passed through the column. The column was washed *n* times with 4 ml of ultrapure water until no foaming was observed anymore. The extraction scheme is depicted in scheme 5.8. Samples were taken (1000 µl). Finally *n* times 4 ml of methanol were added to elute absorbed components. Samples (1000 µl) were taken. All coloured samples were analysed. Methanol samples were analysed directly by HPLC. All the other samples (1000 µl) were diluted with 1000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC.



***Scheme 5.8** Extraction of madder root with surfactants after enzymatic conversion. After filtration the extract was further treated with solid phase material or resins.*

5.3 Results and discussion

5.3.1 Effect of the amount of water and the grinding size of madder root on the formation of alizarin

In chapter four a quantitative determination of aglycones was developed. In this method 0.25 g of madder root was stirred in 10 ml of water. In this research the minimum amount of water was determined that was obligatory for the conversion of the glycosides. A reduction of the amount of water is not applicable for an accurate quantitative determination, but can be very useful for a large-scale preparation of a dye product. The amount of water was decreased in steps from 10 ml to 0.5 ml. It turned out that 2.0 ml of water was still enough to convert ruberythric acid **2** and lucidin primeveroside **1** into alizarin **8** and nordamnacanthal **12**. If the amount of water was reduced further beside alizarin **8** and nordamnacanthal **12** also lucidin **7** was formed. The limiting factor of the conversion reaction is the supply of oxygen to the suspension. Due to the small amount of water the suspension is very thick and stirring is almost impossible. However oxygen is compulsory for the formation of the non-mutagenic nordamnacanthal **12** from the mutagenic lucidin **7**. It was tried to supply more oxygen by blowing air through the suspension or by making use of water enriched with oxygen (water was bubbled through with air) but this was not successfully. Because there was little water in the suspension the water was evaporated by the air that was bubbled through the suspension before the conversion was completed.

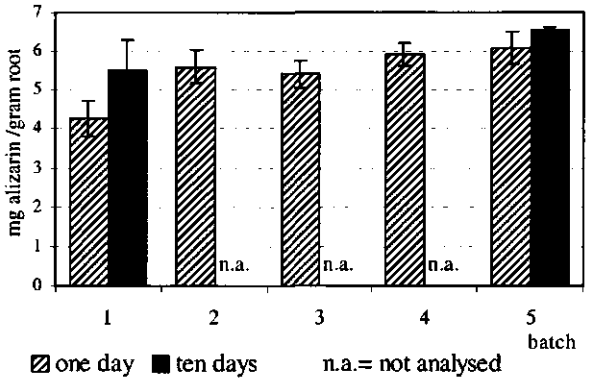


***Figure** 5.2 Influence on the particle size of ground madder root on the extraction of alizarin 8 (mg alizarin 8 / g root). Particle size decreases from batch 1 to 5 (see table 5.1). Experiments were performed on batch 2, 3 and 4 for 1 day and for batch 1 and 5 for 1 and 10 days.*

The particle size of madder root is important for the extraction speed (figure 5.2). As the particle size decreases, the extraction of anthraquinones improves. After 1 day 30% less alizarin **8** was extracted for batch 1 (largest particle size) compared to batch 5 (smallest particle size) (see figure 5.2). According to the HPLC trace batch 1 still contained glycosides. It can be concluded that for suspensions of batch 1, the conversion of glycosides into aglycones was not complete. The reaction time was increased. Madder roots of batch 1 and 5 were stirred for 10 days and compared with each other. Now no glycosides were detected anymore in batch 1. The extraction of anthraquinones from batch 1 was increased and the difference with batch 5 was 16% (figure 5.2). However a long extraction time is not preferable for an industrial method.

5.3.2 Extraction with water or water–ethanol after enzymatic formation of aglycones

Alizarin **8** that is formed after the hydrolysis of ruberythric acid **2** is not soluble in water, which hampers for the separation of alizarin **8** from the rest of the plant material. If alizarin **8** could be dissolved it would be possible to separate the dye component from the rest of the insoluble plant material by filtration. In this research the solubility of the anthraquinones especially of alizarin **8** in different ethanol-water mixtures was investigated. First the glycosides were converted to the aglycones by stirring madder root in water. After conversion the suspension was refluxed in ethanol-water for 2 hours and filtrated. This extraction was repeated 3 times for the residue. The extraction scheme is depicted in scheme 5.1. The amount of alizarin **8** in the filtrates was compared to a reference sample and depicted in figure 5.3. With a mixture of ethanol-water (1:1) (v/v) all the anthraquinones could be extracted in two extraction steps. More than 95% of the total amount of alizarin **8** was already extracted in the first step. The amount of extracted alizarin **8** decreased with an increasing % of water in the extraction solution. The other anthraquinones showed the same extraction behaviour as alizarin **8**.

According to the literature the extraction of anthraquinones from plant material with hot methanol or ethanol should be avoided because artefacts can be formed. The presence of 2-methoxymethyl or 2-ethoxymethyl groups respectively caused by the partial conversion of lucidin to the corresponding ω-ether is an example of such artefacts. This reaction is highly temperature-dependent[28,53]. In this research artefacts were found if the madder root suspension was extracted with refluxing 100% ethanol. At Rt = 29.9 min an unknown anthraquinone compound was detected. HPLC-MS analysis gave as main ion $[M\text{-}H]^-$ at *m/z* 297.2 and an ion $[M\text{-}OC_2H_5]^-$ at *m/z* 253.4. According to these results the unknown compound might be lucidin ω-ethylether (1,3-dihydroxy-2-ethoxymethylanthraquinone).

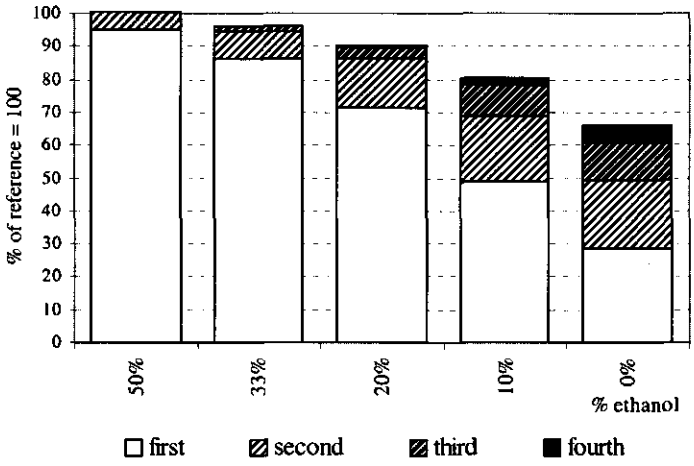


Figure 5.3 Percentage of alizarin 8 extracted from madder root suspensions using water-ethanol with different % of ethanol. Every suspension was extracted four times.

After extraction the solution has to be filtered as hot as possible otherwise the filter will clog very rapidly and alizarin **8** will precipitate and remain in the residue. If only boiling water is used as extraction solvent 60% of the alizarin **8** that is present will be extracted in four extraction steps. The solubility of alizarin **8** increases with increasing temperatures. To increase the extraction yield of alizarin **8** in pure water the extraction was performed at temperatures higher than 100°C. For this an stainless steel flow cell was placed in an oven. An HPLC pumped water through the cell, which was filled with madder root suspension. To prevent the water from boiling the cell was connected with tubing with a length of 2 m and 0.25 mm i.d., which caused a pressure of 3 bar in the cell (see also figure 5.1, scheme 5.2). In this system madder root suspension was extracted at 110°C, 120°C and 130°C. The results of these extractions were compared with the cumulative amount of alizarin **8** extracted in the reference sample (=100%). The results are depicted in figure 5.4. For comparison in this figure also the amount of alizarin **8** extracted with refluxing water after 1 and after 4 extractions is depicted. At a temperature of 130°C 71% of the total amount of alizarin **8** the madder root suspension was extracted. An advantage of this extraction procedure relative to the extraction with boiling water is that less extraction solvent has to be used. The ratio between solvent (ml) and suspension (mg) was around 1:2. Furthermore no ethanol was used and more alizarin **8** was extracted than with refluxing water. The extract was kept in the refrigerator but almost no alizarin **8** and/or other anthraquinones precipitated from the water.

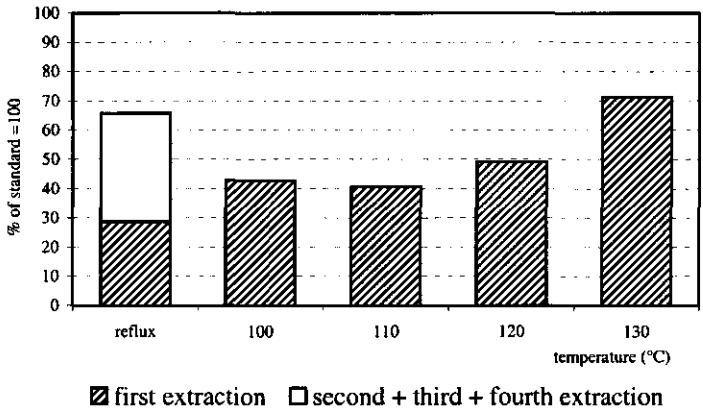


Figure 5.4 *Percentage of alizarin* **8** *extracted from madder root suspension. Madder root suspension was extracted under 3 atm. pressure at 100°C, 110°C, 120°C and 130°C. The yields are compared with the amount of alizarin* **8** *extracted with water under reflux conditions after one extraction and after four extractions.*

5.3.3 Extraction of glycosides and conversion into aglycones

In § *5.3.2* it was reported that alizarin **8** could be isolated for 100% from a madder root suspension by extraction with refluxing water-ethanol (1:1) (v/v). If only water was used as extraction solvent not all the alizarin **8** could be extracted. A maximum of 71% alizarin **8** was retrieved. In this experiment it was investigated if the amount of isolated alizarin **8** could be increased by an initial extraction of ruberythric acid **2** with water followed by a hydrolysis to alizarin **8** afterwards.

For the extraction of the glycosides it is important that no conversion of the glycosides takes place during the extraction, because extraction of aglycones proceeds poorly. To avoid this the, madder root was added while the water was already boiling. The extraction recoveries of the anthraquinones with water-ethanol (1:1) (v/v) (v/v) or water were compared and depicted in figure 5.5. Extraction of madder root with boiling water gave 93 % of the amount of ruberythric acid obtained with boiling ethanol-water (1:1) (v/v). The glycoside water extract was divided in 12 equal volumes and these were freeze-dried.

After extraction of the glycosides with boiling water, freeze-dried enzyme extract (§ *5.2.8*) was added to the solution. After stirring for 2 hours at 45°C the glycosides were

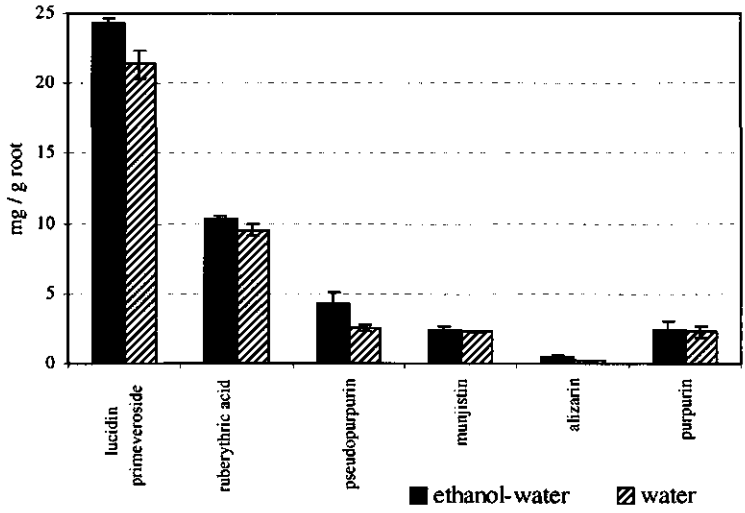


Figure 5.5 Amount of anthraquinones (mg/g root) obtained after extraction of madder root with boiling ethanol-water (1:1) (v/v) or boiling water.

converted to alizarin **8** and nordamnacanthal **12**. The glycosides obtained from 2.5 g madder root were converted by an enzyme extract (5 ml) obtained from 0.15 g madder root. The total amount of alizarin **8** that was obtained was 82 % compared to the amount of alizarin **8** in a madder root reference sample.

5.3.4 Extraction with an alkaline solution

Anthraquinones in madder root are phenols. In general phenols dissolve well in basic solutions[45]. In this research it was investigated if this property could be used for the extraction of alizarin **8** from madder root suspension. Alizarin **8** has hydroxyl groups at the 1 and 2 position with pKa values of respectively 12.0 and 8.2. After stirring of madder root in water 1 M NaOH solution was added until the suspension had a pH of 13. At this pH all the hydroxyl groups of alizarin **8** are ionised, which was also indicated by a colour change of the suspension from yellow to purple due to the increased resonance structures of the formed ion. After the addition of NaOH the suspension was filtered. The amount of alizarin **8** that was found in the filtrate was only 43% of the total amount of alizarin **8** found in a reference sample of madder root suspension (figure 5.6). The rest of the alizarin **8** was found in the residue.

The experiment was repeated but now the madder root was suspended in tap water or ultrapure water. After conversion to the suspensions in tap water a 1 M NaOH solution in tap water was added and to the suspensions in ultrapure water 1 M NaOH solution in ultrapure water was added. The pH of the suspensions were adjusted to pH =10 or pH = 13. Tap water or ultrapure water was added to 50 ml. All the nine suspensions (including a reference sample) were filtrated. In all the samples in which tap water was used the alizarin **8** was retained in the residue and nothing was found in the filtrate. When instead of tap water, ultrapure water was used and the pH was increased to 13, alizarin **8** (96%) was recovered in the filtrate. At pH 10, 60% of the alizarin **8** was recovered in the filtrate (figure 5.6). The same results were obtained when the experiment was repeated with other alkaline solutions like KOH or NH_4OH (no pH=13). In contrast with ultra-pure water, tap water contains ions like calcium. Alizarin **8** forms a complex with such ions, which is also a important for the dyeing properties of the molecule[17,85,108]. When alizarin **8** is dissolved in an alkaline solution the alizarin **8** molecules ionise and are able to form a complex with ions present in tap water. This is also indicated by the fact that a purple complex precipitated from an alkaline suspension of pure alizarin **8** in tap water after 4 hours room temperature.

Based on the previous results with pure alizarin **8** a series of madder root suspensions were diluted with an alkaline solution in tap water or ultrapure water until the pH was 10 or 13. The results are also depicted in figure 5.6. Compared to a reference sample the amount of alizarin **8** retained in the extraction with alkaline solution (ultrapure water, pH 13) was still much lower. Due to the fact that madder root contains ions like calcium it will be impossible to avoid complex formation of alizarin **8** by using just ultrapure water.

To overcome this problem of complex formation between alizarin **8** and metal ions EDTA was added. EDTA forms complexes with nearly all polyvalent metal ions and also with many monovalent cations. Instead of 10 ml of ultrapure water, different solutions of EDTA in ultrapure water were added to madder root. After stirring for 1 hour at 45°C an alkaline solution was added and the pH was adjusted to 13. The suspension was filtered and the amount of alizarin **8** in the filtrate was determined. The recovery of alizarin **8** according to a reference sample was for a 1, 0.8, 0.6, 0.5 and 0.1% EDTA solution respectively 96, 93, 96, 90 and 54%. On the basis of these results further experiments with alkaline solutions were done with a solution of 0.6% EDTA in ultrapure water. Looking at the other anthraquinones it was found that in a solution of 0.6% EDTA the recovery of nordamnacanthal **12** was only 6.5%. The other anthraquinones showed the same extraction efficiency as alizarin **8**.

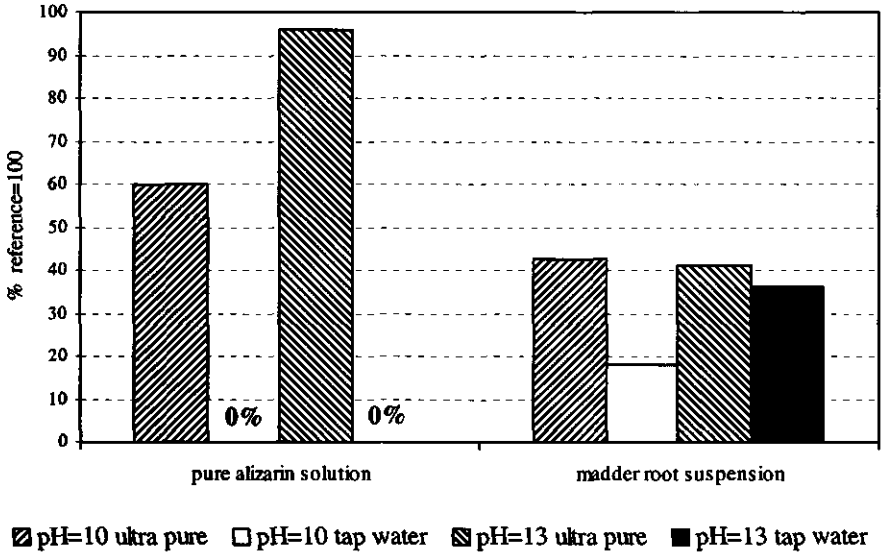


***Figure 5.6** Percentage of alizarin 8 recovered from pure alizarin 8 solution or madder root suspension using ultrapure water at pH=10 or pH=13 and tap water at the same pH.*

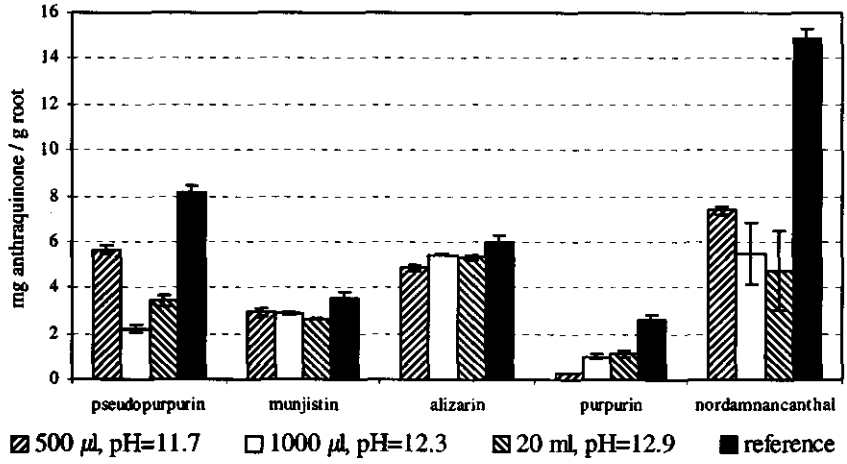


***Figure 5.7** Amount of anthraquinones (mg/g root) obtained from 0.6% aqueous EDTA solution after extraction with NaOH at different pH values.*

The amount of NaOH and the corresponding pH of the solution necessary for the solubilisation of alizarin **8** were investigated. The results are depicted in figure 5.17. Madder root was stirred with an aqueous solution of 0.6% EDTA at 45°C. After conversion of the glycosides 500 µl, 1000 µl or 20 ml 1 M NaOH solution was added. Ultrapure water was added up to 50 ml. The different suspensions had a pH of 11.7, 12.3 or 12.9 respectively. Adding 1000 µl gave the same extraction yield of alizarin **8** as 20 ml 1 M NaOH. The extraction method with 1000 µl 1 M NaOH gave an extraction yield of 91% compared with the reference sample. The recovery of the other anthraquinones was lower than for alizarin **8** especially for nordamnacanthal **12**. Maybe these compounds were converted to other anthraquinones however no other unknown peaks were observed in the HPLC trace. Probably these anthraquinones are less soluble at this pH level.

The insoluble plant material was easily removed by filtering the alkaline madder root suspension. HCl (1 M) was added to the filtrate till a colour change from purple to orange occurred. The filtrate was stored in a refrigerator to allow the precipitation of the anthraquinones. In a second filtration step, the anthraquinones were retained on the filter, see also scheme 5.5.

5.3.5 Extraction with surfactants

Water-soluble surfactants were used in these experiments to increase the solubility of alizarin **8** in an aqueous madder root suspension. Madder root was stirred in 50 ml 1% surfactant solution at 45°C for 60 min. After stirring, the suspension was filtrated and the amount of anthraquinones in the filtrate was determined with HPLC. The surfactants Brij 92, 98, 76, 78, 58, 35 and Tween 40, 80, 85, 20 (table 5.2) were tested and compared with the reference sample. The results are depicted in figure 5.8. Compared to the standard sample it was found that the surfactants Brij 58, 76, 78 and 98 gave the best results. The recovery of alizarin **8** for these surfactants was ≥ 96%. These surfactants were also tested at lower concentrations of 0.75 and 0.5%. When a solution of less than 0.75% surfactant was used the amount of dissolved alizarin **8** decreased rapidly. All other anthraquinones available in madder root suspension showed a similar behaviour as alizarin **8** with the exception of nordamnacanthal **12**. According to the reference sample the amount of nordamnacanthal **12** varied between 28% and 35%.

The anthraquinones could be separated from the rest of the insoluble plant material by filtration of the suspension. After filtration the surfactant had to be separated from alizarin **8**. The addition of water as an attempt to separate the surfactant from the anthraquinones was unsuccessfully. The introduction of an electrolyte, sodium chloride, and the manipulation of

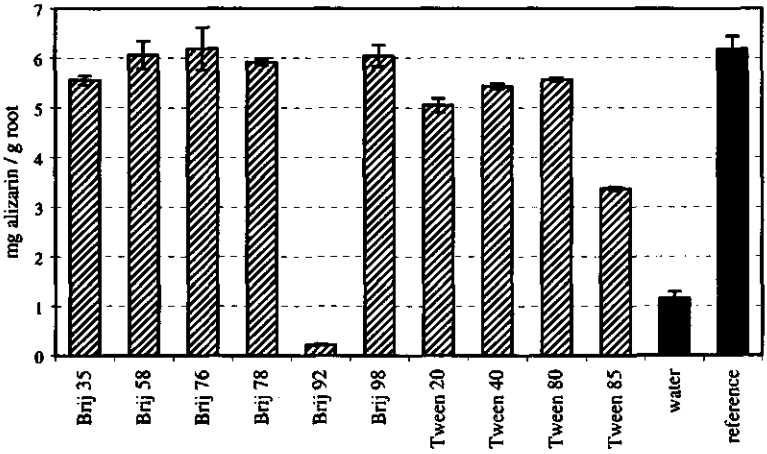


Figure 5.8 *Amount of alizarin* **8** *extracted from madder root suspension with different kinds of surfactant solutions compared with the amount found by THF-water extraction after prior enzymatic hydrolysis of the glycosides (reference sample).*

temperature (raising or decreasing), did not affect the properties of this solution. For separation of alizarin **8** and surfactant different SPE materials and other chromatographic materials were tested. These experiments are described in § *5.3.7.*

5.3.6 Purification of anthraquinones with solid phase materials

Solid phase materials have been widely used for the extraction and concentration of organics at low levels from aqueous systems. A lot of different solid phase materials are available as commercial product. Different materials were tested on their ability to separate alizarin **8** from other compounds and other anthraquinones in madder root. For these experiments, the filtrate of an aqueous extract of madder root suspension was taken as reference. The filtrate was added to commercially available solid phase materials and the amount and composition of the anthraquinones after eluting was determined by HPLC. Due to the poor solubility of the aglycones in water the aglycones will precipitate easily in an aqueous solution of madder. So before every experiment the solution had to be filtered and the concentration of the different anthraquinones in the filtrate had to be determined.

From the resins that were tried, the best performance was shown by Dowex 50W-X8(H^+) and Amberlite resin IRA-120(H^+). These are both strongly acidic cationic exchangers based on polystyrene. It is thought that the most important interactions are of an apolar nature and that the ionic part is less relevant. On the other hand the recovery obtained from using an anionic resin, also based on polystyrene, amberlite IRA-400(Cl^-), was very low. All the alizarin **8** was absorbed by the material and could not be eluted with methanol. Chelex 100 Resin did not interact with alizarin **8** and most of the analyte was not retained (figure 5.9).

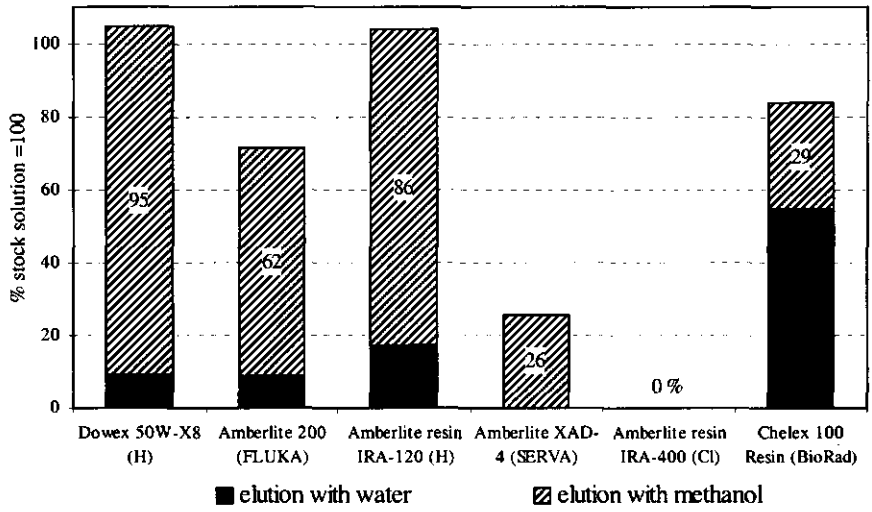


***Figure 5.9** Percentage of alizarin 8 retained from a stock solution that was added to different resin materials.*

From the SPE cartridges C_{18}, C_8, and CH gave a maximum retention of all alizarin **8**. CN(EC) showed also a strong interaction but not all the material was recovered by elution with methanol. When ready to use solid phase cartridges with materials like PBA, CBA, OASIS, SCX and ENV^+ were used part of the alizarin **8** was found in the breakthrough, the other part was retained. Not all the alizarin **8** retained to the material was eluted by methanol; a fraction was retained by the sorbent. The extraction of alizarin **8** using these sorbents would take more steps, which is not practical (figure 5.10).

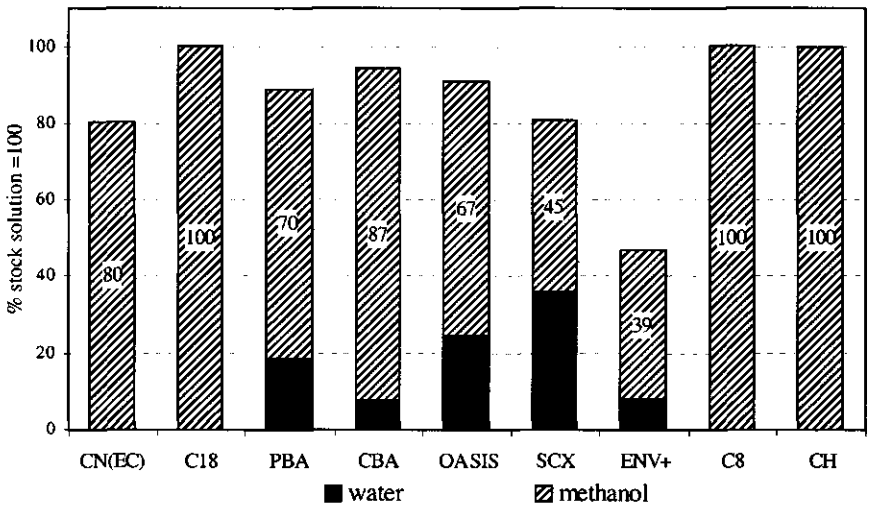


Figure 5.10 *Percentage of alizarin* ***8*** *retained from a stock solution that was added to different solid phase materials.*

5.3.7 Extraction and isolation of alizarin with surfactants and solid phase materials

As already described in § *5.3.5* a 0.75 % solution of Brij 58, 76, 78 or 98 can be used for the extraction of alizarin **8** and other anthraquinones from a madder root suspension. After filtration the filtrate consists of anthraquinones, water-soluble compounds from madder root and surfactant. In this experiment it was tested if solid phase materials could be used to isolate and concentrate alizarin **8** from the filtrate of a madder root suspension treated with a 0.75% Brij 78 solution. Dowex 50W(H) and Amberlite IRA-120(H) resins and C_{18} SPE cartridges were used.

After enzymatic conversion the madder root suspension was extracted with surfactant and further isolated by adding Dowex 50W(H) or Amberlite IRA-120(H). After extraction the filtrate was stirred with resin material twice. The procedure is depicted in scheme 5.8. From both resins Dowex showed the highest recovery. A recovery of 87% of the alizarin **8** present in madder root was obtained. For Amberlite the recovery of alizarin **8** was 74%. Another advantage of Dowex compared to Amberlite was that in the first extraction of the filtrate a bigger amount of alizarin **8** was retained, 72% compared to 48% for Amberlite.

A major disadvantage of both methods is the ratio resin-filtrate (4 g-15 ml) that had to be used for extraction. The amount of resins needed is so high that stirring becomes almost impossible. Furthermore the chromatogs of the methanol fractions show other components absorbed, especially with Amberlite. Several compounds, which elute in the beginning of the chromatograms (polar compounds), were detected in these chromatograms (figure 5.11).

When SPE cartridges with C_{18} material were used a high recovery of 96% was obtained. C_{18} also showed selectivity for alizarin **8** when compared to other important anthraquinones such as pseudopurpurin **5**, munjistin **6**, purpurin **10** and nordamnacanthal **12**. For example purpurin **10** is eluted mainly with the first elution solvent, water in contrast to alizarin **8** which elutes only during the second step with methanol. A methanolic extract of mainly alizarin **8**, pseudopurpurin **5** and munjistin **6** was obtained (figure 5.12). Besides the material gave a more pure extract with respect to interferences (other not studied more polar compounds). Most of the surfactant available in the extract was removed by SPE on C_{18}. When washing the column with water, after application of the sample, in the first two or three fractions, most of the surfactant was removed. Due to this fact it is important to work with a vacuum only slightly beneath 1 atm as otherwise the foaming produced by the surfactant will interfere with the elution.

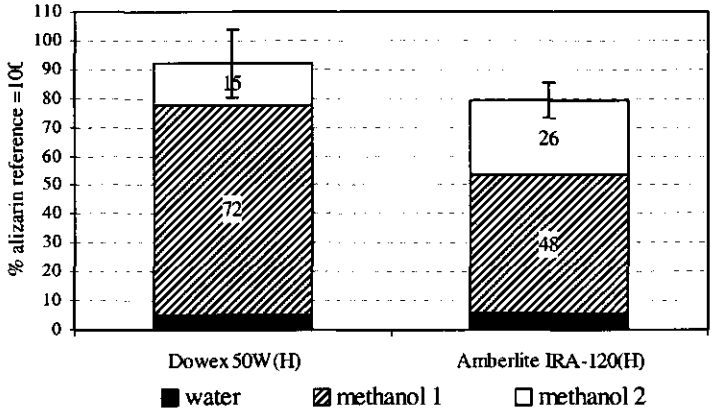


***Figure 5.11** Percentage of alizarin 8 retained if the surfactant Brij 78 was used for the extraction of the anthraquinones from a madder root suspension and further treatment of the filtrate with Dowex 50W (H) and Amberlite IRA-120 (H).*

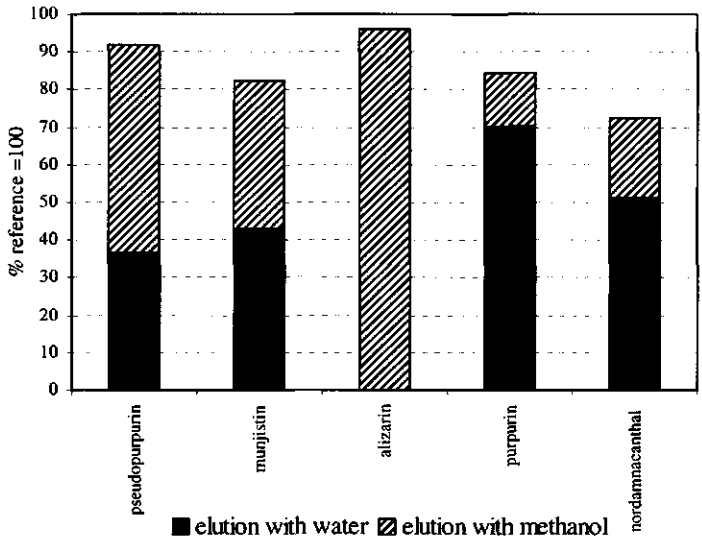


Figure 5.12 Percentage of alizarin 8 after extraction of an enzymatic converted madder root suspension with surfactant Brij 78 and further treatment of the filtrate with C_{18} solid phase material.

5.4 Conclusion

In this research the endogenous enzymes, already available in madder root, were used for the hydrolysis of the glycosides to increase the amount of free alizarin **8**. This method for hydrolysis of ruberythric acid is by far the easiest. Madder root only has to be stirred in water at 45°C (chapter 3) and within 1 hour all the ruberythric acid is hydrolysed. However after conversion an extraction procedure has to be used to separate alizarin **8**, which is not soluble in water from the rest of the plant material.

Only extraction with refluxing ethanol-water (1:1) (v/v) gave a 100% recovery of alizarin **8** from the rest of the insoluble plant material. With this method a lot of solvent has to be used and a high temperature is needed, which makes it less attractive. Extraction of madder root with boiling water gave a low recovery of 66% alizarin **8** and a lot of water is necessary (four extraction steps). Less solvent was needed if water was used under pressure at 130°C. In this case the recovery increased to 71%. Much energy is needed to reach such a high temperature. Furthermore the anthraquinones did not precipitate after cooling the solution, which makes further purification difficult. The extraction with ethanol-water (1:1) (v/v) is worthwhile scaling up. Reusing the extraction solvent will reduce the costs of this extraction.

To make use of the ability of alizarin **8** to ionise at higher pH, madder root suspension (prior enzymatic conversion) was dissolved in an alkaline solution. Unfortunately this gave a low recovery of alizarin **8** due to the formation of insoluble alizarin **8** complexes with polyvalent ions present in madder root. If madder root was stirred in a solution of EDTA, complex formation of alizarin **8** with metal ions was avoided and a recovery of 91% alizarin **8** was obtained. The advantage of this method is that alizarin **8** is separated from the insoluble plant material in the first step (solvation with base) and separation of alizarin **8** from other soluble plant constituents could be achieved by adding acid in a second step. The anion of alizarin **8** will be protonated and alizarin **8** will precipitate. A disadvantage is the use of a large amount of EDTA and the formation of a large amount of NaCl during the procedure. EDTA is quite expensive due to the high percentage needed compared to the amount of alizarin **8** isolated (EDTA mass-% : alizarin **8** mass-% 194:1). Relative large amount of salt (waste) is formed compared to the amount of isolated alizarin **8** (NaCl mass-% : alizarin **8** mass-% 37:1). Due to the large amount of waste formed scale experiments are less attractive.

Extraction of alizarin **8** with a non-ionic surfactant from the Brij series with a polyoxyethylene part >10 enabled a 96% recovery of alizarin **8**. After extraction alizarin **8** was further concentrated and purified from the water-soluble compounds of the plant and from some of the other anthraquinones with solid phase C_{18} material. Probably this method is rather expensive due to the expensive materials like surfactant and C_{18} material, which have to be used. On the other hand these materials can be reused. Scaling up experiments have to be performed.

In contrast to the other methods in one method ruberythric acid was not first hydrolysed. In this method first the anthraquinones were isolated from the insoluble plant material with boiling water. After isolation the glycosides were converted by adding a crude extract of fresh madder root that contained endogenous enzyme activity. This gave a recovery of 82% alizarin **8**. An advantage of this method is that the water soluble precursor of alizarin **8** (ruberythric acid **2**) is isolated which circumvents the difficult separation step of precipitated free alizarin **8** and insoluble plant material. A disadvantage is the use of a lot of extraction solvent at a high temperature. Furthermore additional madder root is needed for the preparation of enzyme extract. These roots cannot be used for alizarin **8** isolation. Probably the amount of extra fresh madder root can be decreased if more research is done on the purification of the enzymes and possibly by the immobilisation of the enzymes.

A larger scale experiment has to be performed. In this research a number of different methods were investigated for their capacity to isolate alizarin **8** and to separate it from the rest of the madder root plant material. Most attention was paid to the recovery of alizarin **8**. Not much attention was given to the total amount of extract obtained from madder root or the separation of alizarin **8** from the other anthraquinones. Nevertheless on the basis of these

experiments insight was obtained which methods are suitable and which are not suitable for further scale up investigations. In this research all the experiments were performed on a small scale which varied between 2.5 and 0.25 g of madder root. A selection based on practical applicability and alizarin **8** yield showed three extraction routes, which are interesting for further scale up investigations. In further scaling up experiments more attention must be paid to parameters like amount of extract obtained, further purification of alizarin **8** from other extracted compounds, in particularly anthraquinones, costs and industrial applicability of the procedure.

Chapter 6

Preliminary scale-up experiments for the isolation of alizarin from madder root

6.1 Introduction

Different possibilities were described (see chapter 5) for the isolation of alizarin **8** from madder root. The following aims were formulated for an industrially applicable isolation procedure:

1. as few as possible extraction steps
2. large amounts of organic solvents should be avoided
3. low energy requirements
4. the method should be technically feasible on an industrial scale
5. the procedure should not be labour intensive
6. during the process few waste products should be formed

Based on the results obtained in chapter 5 and on the requirements mentioned above, a selection was made and three isolation methods were selected as promising. The advantages and disadvantages of these methods were compared and are summarised in table 6.1. In method 1 and 3 the glycosides in madder root are first converted by endogenous enzymes (chapter 3) and extracted afterwards. In method 2 this sequence conversion is reversed.

Till now between 0.25 and 2.5 g madder root was used for all experiments. In this chapter the amount of madder root was increased to 100-250 g. Beside the amount of alizarin **8** isolated, also other parameters such as the amount of dry extract and the amount of other anthraquinones were calculated.

Table 6.1 Three promising methods for isolation of alizarin 8 from madder root and the advantages and disadvantages of each method.

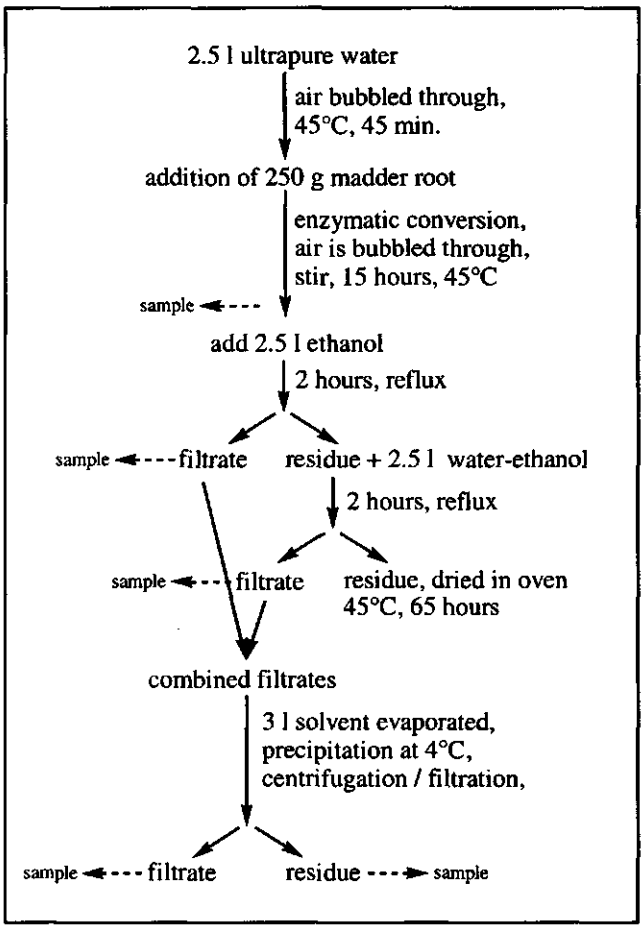
no:	method	advantages	disadvantages
1	conversion by endogenous enzymes, extraction with boiling ethanol-water, filtration	easy to apply, 100 % extraction of alizarin **8**	large amount of (organic) solvent, high energy (refluxing)
2	first extraction of glycosides, conversion by adding an enzyme extract prepared from fresh madder roots	use of water, easy to apply	high energy requirement (refluxing)
3	conversion by endogenous enzymes in aqueous surfactant solution, filtration, isolation with SPE C_{18}	use of water, no high temperatures	technically more difficult, more expensive materials

6.2 Experimental

6.2.1 Extraction with water–ethanol 1:1 after enzymatic formation of aglycones

A flask with 2.5 l of ultrapure water was placed in an oven. Air was bubbled through the water at 45°C. After 45 min 250 g of madder root was added. Air was bubbled through the suspension while it was stirred at 45°C. After 15 hours a sample of 10 ml (suspension) was taken. This sample was diluted with 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) and stirred for 30 min at room temperature. After stirring a sample of 500 µl was taken.

The remaining suspension was added to 2.5 l of ethanol in a round bottom flask of 10 l. The suspension was refluxed for 2 hours. After refluxing the suspension was filtered immediately (as hot as possible) over a Büchner funnel with a filterpaper (Schleicher & Schuell). A sample (500 µl) was taken from the filtrate (3.8 l). The remaining residue was added to 2.5 l ethanol-water 1:1 in a round bottom flask (5 l). The suspension was refluxed for 2 hours. After refluxing the suspension was filtered as hot as possible over a Büchner funnel with a filterpaper. A sample (500 µl) was taken from the filtrate (2.3 l). The residue was dried in an oven at 45°C for 60 hours. Both filtrates were combined and from the suspension 3 l solvent was evaporated under reduced pressure. The remaining suspension was stored at 4°C. After two days the chilled suspension was centrifuged at 6000 rpm in a Heraeus Varifuge F. The supernatant was filtered over a Büchner funnel with a filterpaper (Schleicher & Schuell). The pellet of the centrifugation and the residue of the filtration were combined and dried. Finally 14.7 g of solid material was obtained. A sample (106.8 mg) was taken and diluted in 100 ml THF-water-formic acid (1:1:1:0.005) (v/v/v), filtered and analysed by HPLC. The extraction is depicted in scheme 6.1.



Scheme 6.1 *Extraction of madder root with water-ethanol after enzymatic formation of aglycones.*

6.2.2 Extraction of glycosides and conversion into aglycones

Ultrapure water (10 l) was heated to 80°C. Dried and powdered madder root (250 g) was added. The suspension was refluxed for 2 hours before immediate filtration over a Büchner funnel with a filterpaper (Schleicher & Schuell). An aqueous extract (8.6 l) containing glycosides was obtained. The aqueous glycoside extract was placed in an oven and air was bubbled through. The suspension was cooled to 45°C. A sample (500 μl) of the extract was taken.

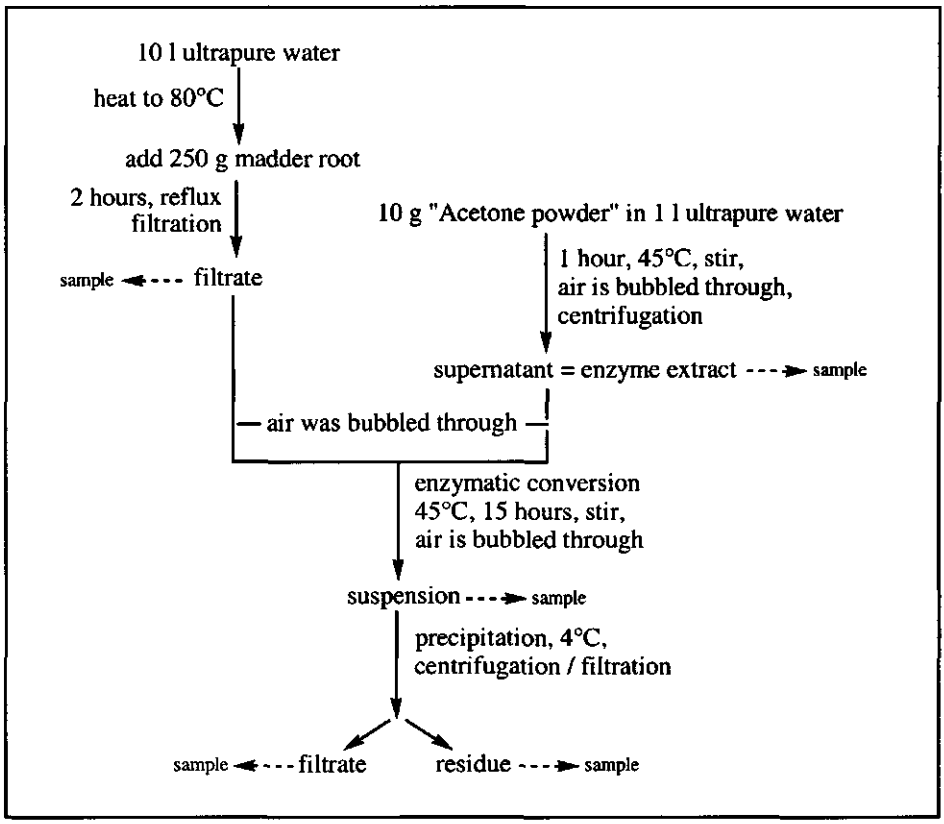
Air was bubbled through 1 l ultrapure water at 45°C. After 45 min 10 g of "Acetone powder" was added and the suspension was stirred for 1 hour at 45°C. "Acetone powder" was prepared as described in § *5.2.6.* The suspension was centrifuged at 3600 rpm during 20 min. A sample (500 µl) of the supernatant was taken.

The remaining supernatant (0.86 l) was added to the remaining of the aqueous glycoside extract (7.5 l). Air was bubbled through the suspension while stirring at 45°C. After 15 hours a sample (500 µl) was taken and the remaining suspension cooled to 4°C. After 15 hours the suspension (precipitation) was centrifuged at 6000 rpm. The supernatant was filtered over a Büchner funnel with a filterpaper. The pellet of the centrifugation and the residue of the filtration were combined and dried. A dried extract of 3.2 g was obtained. From the dried residue 51 mg was dissolved in 25 ml THF-water-formic acid (1:1:0.005) (v/v/v) and a sample (500 µl) was taken. The complete procedure is depicted in scheme 6.2.

6.2.3 Extraction of madder root with surfactant and fractionation of the extract with solid phase material

Air was bubbled through 6 l ultrapure water and stirred at 45°C. After 1.5 hours 1 l of 0.75% Brij 78 solution was added. Finally madder root 100 g was added. Air was bubbled through the suspension while it was stirred at 45°C. After 15 hours a sample of 10 ml (suspension) was taken. This sample was diluted with 40 ml THF-water-formic acid (1:1:0.005) (v/v/v) and stirred for 30 min at room temperature. After stirring a sample of 500 µl was taken. The remaining suspension was filtered over a Büchner funnel with a filterpaper (Schleicher & Schuell). The residue was dried in an oven at 45°C for 65 hours.

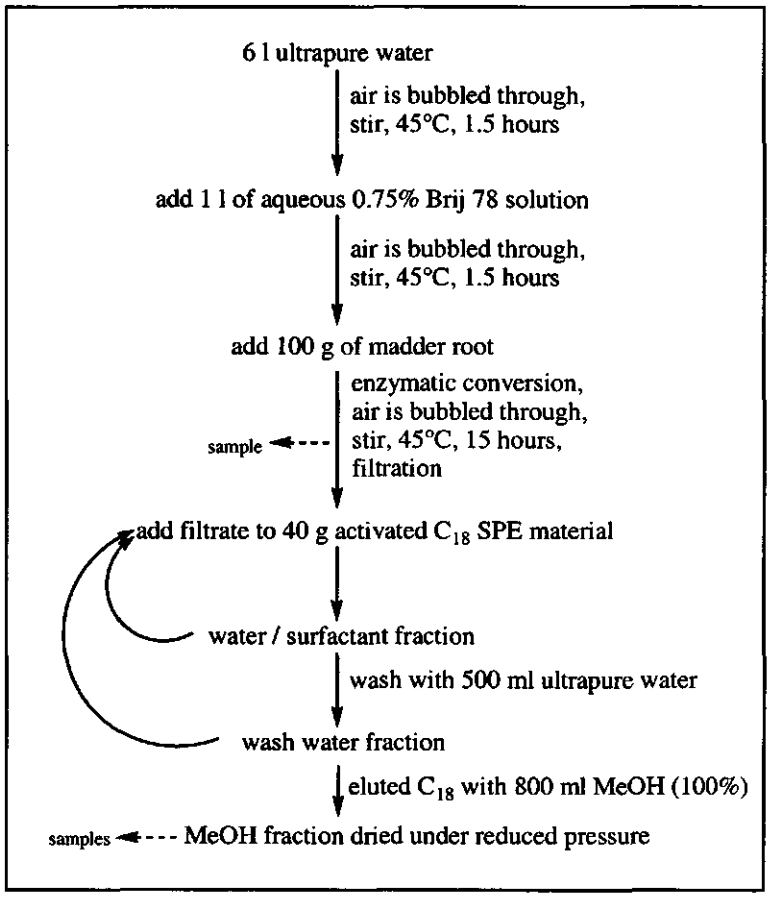
Instead of an SPE cartridge, a glass filter (pore size 40) was filled with 40 g of the same C_{18} material as used in § *5.2.10.* The C_{18} material was activated by eluting the column with 100 ml methanol and 150 ml 2% methanol in water. After activation the filtrate (7 l) obtained after stirring madder root in surfactant solution and filtration, was passed through the 40 g activated C_{18} material. After obtaining the filtrate the C_{18} material was washed with 500 ml ultrapure water. Finally the C_{18} column was washed with 8 times 100 ml MeOH (100%). With exception of the methanol fractions (8 times 100 ml), all the other fractions (7 l filtrate and water fraction of the wash step) were applied again on the C_{18} column and the procedure was repeated. The MeOH (100%) filtrates were dried under reduced pressure and respectively 9.0 and 8.1 g solid material were obtained. The solid materials were analysed on HPLC, 47.6 mg of extract 1 and 94.0 mg of extract 2 were diluted in 50 and 100 ml THF-water-formic acid (1:1:0.005) (v/v/v), respectively.



***Scheme 6.2** Extraction of madder root with boiling water followed by enzymatic conversion of the glycosides by an enzyme extract isolated from madder root.*

6.2.4 HPLC analysis

All samples of 500 µl obtained in this experiment, were diluted with 2000 µl THF-water-formic acid (1:1:0.005) (v/v/v) and stirred. The suspensions were filtered over a 0.45 µm, ø 25 mm membrane filter (Type RC, Schleicher & Schuell) and analysed by the HPLC method described in chapter 3.



***Scheme 6.3** Extraction of madder root with surfactants after enzymatic conversion. After filtration the extract was purified on C_{18} material.*

6.3 Results and discussion

6.3.1 Enzymatic conversion

As already mentioned in chapter 3 oxygen is obligatory for the enzymatic conversion of lucidin primeveroside **1** to nordamnacanthal **12**. In chapter 3 and 4 2.5 or 0.25 g madder root were stirred in respectively 100 or 10 ml ultrapure water. Stirring the solution was enough to provide the oxygen needed. In chapter 5 it was described that for 0.25 g madder root a

minimum of 2.0 ml of ultrapure water is needed to prevent the formation of lucidin **7**. When there is less than 2 ml ultrapure water not enough oxygen can be supplied by stirring. In this chapter the amount of madder root was increased to 250 g. The first procedure that was followed was the same as in § *5.2.6*. First 2.5 l ultrapure water was added to 250 g madder root and the suspension was stirred at room temperature. However in this case both lucidin **7** and nordamnacanthal **12** were formed. If the same experiment was repeated at 45°C this also resulted in the formation of the unwanted lucidin **7**. If the suspension was stirred longer (in total 5 days) the amount of lucidin **7** was kept decreasing and the amount of nordamnacanthal **12** increasing but lucidin **7** did not disappear completely.

Just stirring is not enough for a complete conversion of the glycosides ruberythric acid **2** and lucidin primeveroside **1** to alizarin **8** and nordamnacanthal **12** in a madder root sample of 250 g in 10 l. To obtain complete conversion air was bubbled through the ultrapure water at 45°C before the root material was added. After addition of the madder root, air was bubbled through the suspension while it was stirred at 45 °C during the complete reaction time. As already mentioned in chapter 3 these results stress that oxygen is the limiting factor for the oxidation reaction. If this reaction has to be carried out at still larger scale (e.g. 250 kg) the problem of supplying enough oxygen could become a problem once more. The circumstances have to be chosen so that a maximum amount of oxygen can be supplied to the reaction. Probably this can be accomplished by providing fine bubbles of pure oxygen (or air) and choosing a reaction vessel in which the suspension has a large contact area with the air.

Another parameter that becomes more important in scaling-up experiments is the temperature. In § *3.3.4* it was described that the optimum temperature lies between 35°C and 65°C is. At smaller scale (0.25-2.5 g) the reaction could be performed at room temperature (chapter 3). However if the sample is scaled-up to 250 g it turned out that the reaction could better be performed at the optimum temperature to prevent the formation of lucidin **7** due to a too low reaction temperature.

6.3.2 Extraction with water–ethanol 1:1 after enzymatic formation of aglycones

The results of the extraction procedure with ethanol-water (scheme 6.1) are depicted in figure 6.1 and table 6.2. After extractions with ethanol-water (1:1), evaporation, precipitation and filtration a dry extract of 14.7 g was obtained from 250 g madder root. The main anthraquinones in the extract are pseudopurpurin **5**, munjistin **6**, alizarin **8**, purpurin **10** and nordamnacanthal **12**. From the average amount of 6.3 mg alizarin **8** available in 1 g of madder root 4.9 mg was extracted with this scale-up method, which corresponds to 78 % of the total amount of alizarin **8** present in madder root. From the 14.7 g dry extract obtained 5.5 g or 35 % are anthraquinones.

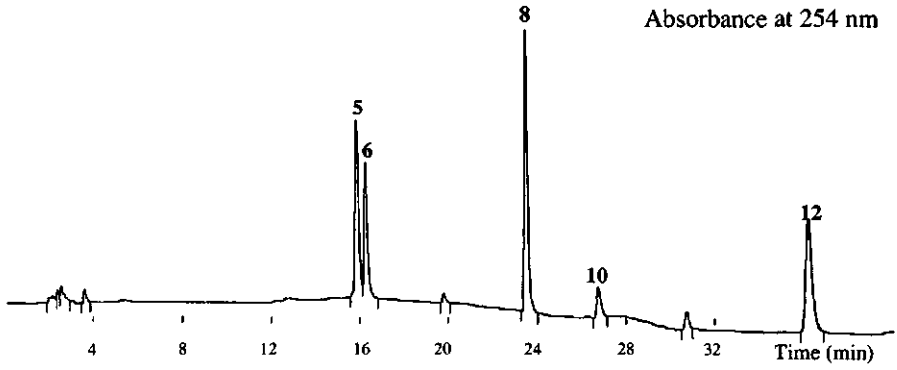


Figure 6.1 *HPLC-trace of sample of dry extract obtained with ethanol-water (1:1) (v/v) extraction after enzymatic conversion as described in scheme 6.1. For peak assignments, see table 6.2.*

Table 6.2 *Yield of ethanol-water extraction, amount (mg) of anthraquinone in 1 g extract, total amount (g) of anthraquinone extracted from 250 g of madder root and amount (mg) of anthraquinone extracted from 1 g of root material.*

compound	mg /g extract	total amount (g)	mg /g root
pseudopurpurin **5**	107.3	1.6	6.3
munjistin **6**	33.2	0.5	2.0
alizarin **8**	83.2	1.2	4.9
purpurin **10**	15.2	0.2	0.9
nordamnacanthal **12**	134.8	2.0	7.9

6.3.3 Extraction of glycosides and conversion into aglycones

The results of the extraction procedure with water followed by conversion with an enzyme extract ("acetone powder") (scheme 6.2) are depicted in figure 6.2 and table 6.3. After extraction with water, conversion with an "acetone powder" enzyme extract, precipitation, centrifugation and filtration a dry extract of 3.2 g was obtained from 250 g madder root. The conversion reaction with the isolated enzyme extract ("acetone powder") was successful.

The main anthraquinones detected in the extract were pseudopurpurin **5**, munjistin **6**, alizarin **8**, purpurin **10**, nordamnacanthal **12** and an unknown not further identified anthraquinone (t=30.7 min) (see figure 6.3). From the average amount of 6.3 mg alizarin **8** present in 1 g of madder root only 1.2 mg was extracted with this scale-up method, which corresponds to 19 % of the total amount of alizarin **8**. From the 3.2 g dry extract obtained 1.2 g or 38 % were anthraquinones. Most of the anthraquinones in madder root stayed in the solution and did not precipitate at 4°C during the night in the refrigerator. In the scale-up experiment with refluxing ethanol-water (§ *6.3.2*) more alizarin **8** was obtained with precipitation at 4°C. In the ethanol-water experiment after evaporation 3 l solvent (mainly water) was left before the precipitation step. In the experiment with "acetone powder" a larger amount water (8.4 l) was present before the precipitation. Maybe the larger amount of water prevented the precipitation of alizarin **8**. Another difference in this experiment ("Acetone powder") was that madder root was only extracted with (refluxing) water, i.e. no other solvent such as ethanol was used. It is possible that madder root contains a kind of natural surfactant which is also extracted with refluxing water and which keeps the alizarin **8** in solution and prevents its precipitation.

The isolation of alizarin **8** from the water suspension obtained after the conversion has to be improved. First it must be investigated if the amount of water used for the refluxing or conversion can be reduced to get a solution from which the anthraquinones precipitate more easily. Another possibility is to reduce the amount of solvent after the conversion reaction likewise the ethanol-water refluxing experiment. Extraction of the anthraquinones with an organic solvent after conversion is not an option. This procedure ("Acetone powder") was developed to avoid the use of an organic solvent such as ethanol. If an additional step with ethanol has to be added, than the procedure described in § *6.2.1* could better be followed, giving probably the same result. Maybe further purification with C_{18} chromatography after conversion is an option.

Table 6.3 *Yield of extraction procedure with "Acetone powder", amount (mg) of anthraquinone in 1 g extract, total amount (g) of anthraquinone extracted from 250 g of madder root and amount (mg) of anthraquinone extracted from 1 g of root material.*

compound	mg /g extract	total amount (g)	mg /g root
pseudopurpurin **5**	25.0	0.1	0.3
munjistin **6**	19.0	0.1	0.2
alizarin **8**	94.1	0.3	1.2
purpurin **10**	28.5	0.1	0.4
nordamnacanthal **12**	200.2	0.6	2.6

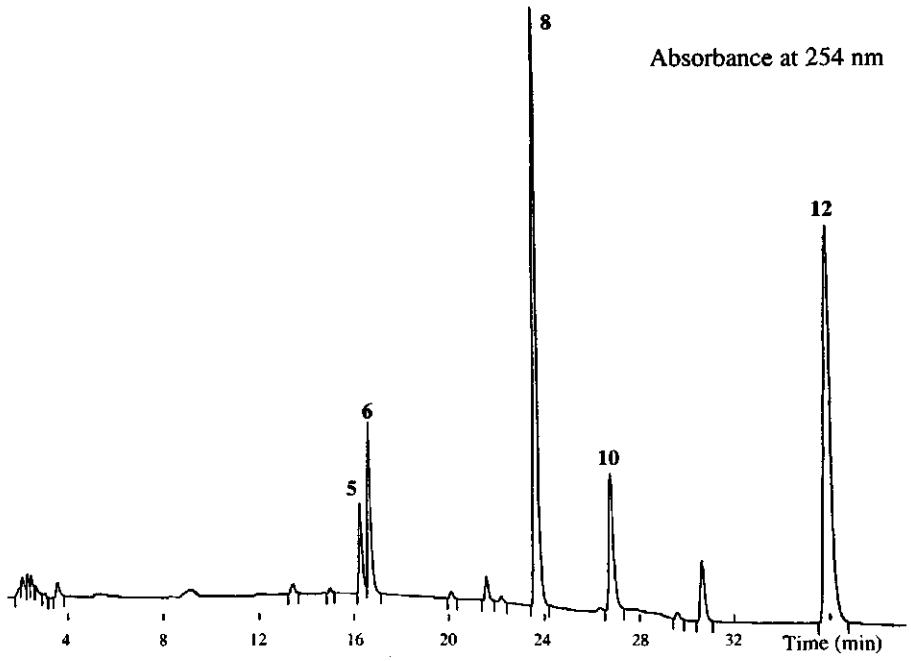


Figure 6.2 *HPLC-trace of sample of dry extract obtained with extraction with "Acetone powder" described in scheme 6.2. For peak assignments, see table 6.3.*

6.3.4 Extraction of madder root with surfactant and further purification with C_{18} material

The results of the extraction procedure with surfactant and filtration over C_{18} material (scheme 6.3) are depicted in figure 6.3 and table 6.4. After extraction with surfactant, twice chromatography over C_{18} material and evaporation of the solvent a dry extract of 17.1 g was obtained from 100 g madder root material. The main anthraquinone in the extract is alizarin **8** but also pseudopurpurin **5**, munjistin **6**, purpurin **10**, nordamnacanthal **12** and an unknown not further identified anthraquinone (t=30.7 min) were detected (see figure 6.3). HPLC-MS analysis of the unknown anthraquinone (t=30.7 min) gave as main ion $[M-H]^-$ at *m/z* 253.2 and CID of the ion gave a fragment ion at *m/z* 225.1. According to these results the unknown anthraquinone might be rubiadin (1,3-dihydroxy-2-methylanthraquinone). From the average amount of 6.3 mg alizarin **8** available in 1 g of madder root 6.2 mg was extracted with this scale-up method, which corresponds with 98 % of the total amount of alizarin **8** available in madder root. After the first chromatography step over C_{18} (the first methanol fraction) 82 %

of the total amount of alizarin **8** isolated was obtained. From the 17.1 g dry extract obtained only 1.9 g or 11 % are anthraquinones.

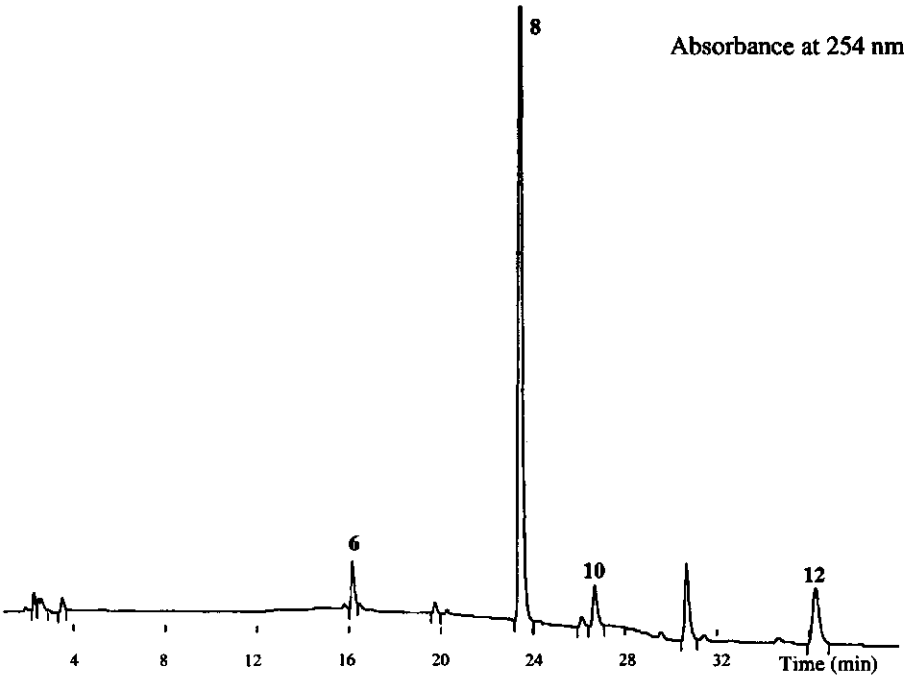


***Figure 6.3** HPLC-trace of sample of dry extract obtained with extraction method (scheme 6.3) with surfactant and purification over C18 material. For peak assignments, see table 6.4.*

***Table 6.4** Yield of extraction method with surfactant and C_{18} material, amount (mg) of anthraquinone in 1 g extract, total amount (g) of anthraquinone extracted from 250 g of madder root and amount (mg) of anthraquinone extracted from 1 g of root material.*

compound	mg /g extract	total amount (g)	mg /g root
pseudopurpurin **5**	2.0	0.1	0.2
munjistin **6**	6.9	0.1	0.6
alizarin **8**	70.4	1.2	6.2
purpurin **10**	5.8	0.2	0.5
nordamnacanthal **12**	18.2	0.3	1.6

6.4 Conclusion

The experiments described in this chapter were the first try outs for scaling-up the isolation of alizarin **8** from madder root. From the tested extraction procedures the second method with the "acetone powder" gave the lowest yield of extracted alizarin **8**. The first and second method with the ethanol-extraction and "acetone powder" gave the extracts with the highest percentage of total anthraquinones. In the third method almost all the alizarin **8** available in madder root was isolated but the percentage of anthraquinones in the extract was very low. Before further scaling-up experiments are performed the conditions of these experiments have to be optimised. All three methods still have opportunity to become an industrially feasible method. For the second approach ("acetone powder") a solution has to be found for the isolation of alizarin **8** from the aqueous suspension. Furthermore the purification of alizarin **8** in method one (ethanol-water reflux) and method two ("acetone powder") from the other anthraquinones has to be investigated. Probably C_{18} chromatography could be incorporated in the isolation process of scheme 6.1 and 6.2 to increase the amount of alizarin **8** with regard to the other anthraquinones. For the third method (surfactant) the percentage anthraquinones in the extract has to be increased.

In this thesis not much attention was paid to the dyeing ability of extracts from madder root. The dyeing capacity of the extracts obtained with the three different scale-up procedures should be investigated. The composition of the anthraquinones and other unknown compounds in the dry extract could influence the dyeing. Different anthraquinones have different dyeing abilities (see chapter 1). Probably components as nordamnacanthal **12** or the glycosides are poorer dyes than pseudopurpurin **5** and alizarin **8**. To avoid trial and error research more research should be carried out to elucidate the complex formation between textile and dye compound.

Another point of research is the production costs of the three different processes. If these three methods are reproduced and optimised for the amount of materials and solvents, cost calculations have to be performed for the determination of the price of the product. In these cost calculations parameters as price of solvents and other materials, energy needed and cost of labour have to be considered. When the best method is selected it has to be scaled up again to for instance 250 kg. Furthermore plant breeding might increase the initial amount of ruberythric acid.

Chapter 7

General discussion

The first aim of this research was the development of a reliable method for the identification and quantification of the main anthraquinones in madder root and various extracts. For this a suitable HPLC-UV method was investigated (chapter 2). This method was extended with an HPLC-MS and an HPLC-DAD method for the on-line identification of anthraquinones in madder root. For the construction of calibration curves for the quantitative analysis, several anthraquinones, which were not commercially available, were isolated. The major anthraquinones present in madder root were investigated. The main anthraquinones obtained in these extracts were lucidin primeveroside **1**, ruberythric acid **2**, pseudopurpurin **5**, munjistin **6**, alizarin **8**, purpurin **10** and nordamnacanthal **12**. In the literature also other minor anthraquinones have been described. In this thesis no attention was paid to the minor anthraquinones, which can be obtained after multi-step isolation.

In madder root alizarin **8** is mainly present as its glycoside ruberythric acid **2**. For the development of a natural alizarin **8** dye from madder root ruberythric acid **2** needs to be converted to free alizarin **8**. Hydrolysis of ruberythric acid **2** is not difficult and could be achieved by treatment of madder root with a refluxing strongly acidic or basic solution, with an enzyme mixture of xylosidase and glucosidase or with endogenous enzymes. Of these methods the conversion of ruberythric acid **2** with endogenous enzymes is most promising for the preparation of a commercial dye product. Compared to the other methods this method fulfils three conditions for an economically feasible production method:

1. low energy requirement
2. use of simple chemicals as water and oxygen
3. no mutagenic lucidin is formed.

The endogenous enzymes were also successfully applied in the development of a fast and reliable method for the large scale screening of anthraquinones in different madder root cultivars. Madder root was suspended in water and the endogenous enzymes converted the glycosides to the aglycones. The aglycones were extracted with THF-water and analysed.

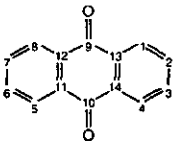
In chapter 5 different approaches were described for the isolation of alizarin **8** from madder root. The following aims were formulated for an industrially applicable isolation procedure:

1. as few as possible extraction steps
2. large amounts of organic solvents should be avoided
3. low energy requirements
4. the method should be technically feasible on an industrial scale
5. the procedure should not be labour intensive
6. an extraction yield of ≈90%

Based on the results obtained in chapter 5 and on the requirements mentioned above, a first selection was made and three isolation methods were selected for preliminary scale-up experiments. These three procedures were scaled-up to 100-250 g. In all three methods the endogenous enzymes were used for the conversion of the glycosides. It turned out that supplying sufficient oxygen to the suspension is critical because otherwise lucidin **7** is formed. All three methods need further optimisation but in principle all three have potential to be used on an industrial scale. For further scale-up experiments parameters like the ratio of alizarin **8** to dry extract, further purification of alizarin **8** from the other anthraquinones and the dyeing quality should be taken into account. At this stage cost calculations have to be made for the determination of the price of the product.

Appendix

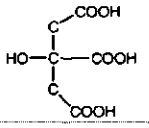
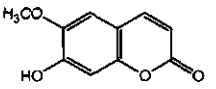
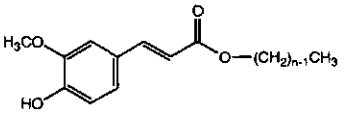
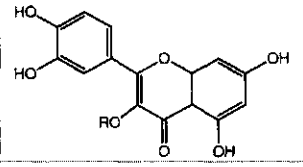
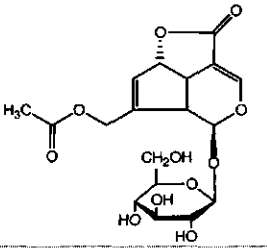
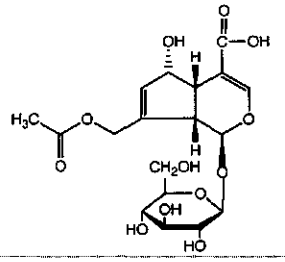
List of anthraquinones and other secondary metabolites from *Rubia tinctorum* reported in literature

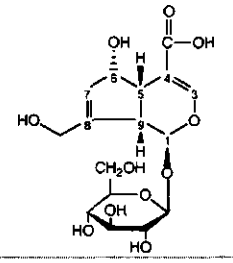
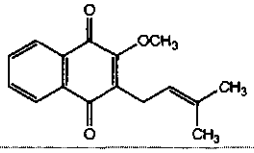
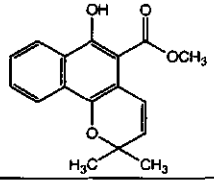


parent anthraquinone structure

Name		**isolation**
Alizarin	1,2-Dihydroxyanthraquinone	1-18
Alizarin dimethylether	1,2-Dimethoxyanthraquinone	3
Alizarin 1-methylether	2-Hydroxy-1-methoxyanthraquinone	3,9
Alizarin 2-methylether	1-Hydroxy-2-methoxyanthraquinone	1,3,8
Anthragallol	1,2,3-Trihydroxyanthraquinone	3,8
Anthragallol 2,3-dimethylether	1-Hydroxy-2, 3-methoxy-anthraquinone	3
Anthragallol 3-methylether	1,2-Dihydroxy-3-methoxy-anthraquinone	3
Christofin	1,4-Dihydroxy-2-ethoxymethyl-anthraquinone	13
2-Hydroxyanthraquinone		3,8
1-Hydroxy-2-hydroxymethylanthraquinone		19,20
1-Hydroxy-2-methylanthraquinone		1,3,5

2-Hydroxymethylanthraquinone-3-O-ß-D-glucoside		12
2-Hydroxymethyl-8-hydroxyanthraquinone-3-O-ß-D-glucoside		12
2-Hydroxymethylquinizarin	1,4-Dihydroxy-2-hydroxymethyl-anthraquinone	20,21
7-Hydroxytectoquinone	2-Hydroxy-7-methylanthraquinone	1,22
Lucidin	1,3-Dihydroxy-2-hydroxymethyl-anthraquinone	1,3,5,7-10,23
Lucidin ω–ethylether	1,3-Dihydroxy-2-ethoxymethyl-anthraquinone	1,12
Lucidin glucoside	1-Hydroxy-2-hydroxymethyl-anthraquinone-3-O-ß-D-glucoside	12
Lucidin ω-methylether	1,3-Dihydroxy-2-methoxymethyl-anthraquinone	1
Lucidin primeveroside	1-Hydroxy-2-hydroxymethyl-anthraquinone-3-O-ß-D-primeveroside	1,5,8,10,12
2-Methoxyanthraquinone		3
1-Methoxy-2-methylanthraquinone		3
Munjistin	2-Carboxy-1,3-dihydroxy-anthraquinone	3,18,24
Munjistinmethylester	2-Carboxymethyl-1,3-dihydroxy-anthraquinone	1,25
Nordamnacanthal	1,3-Dihydroxy-2-formylanthraquinone	1,9,25,26
Pseudopurpurin	2-Carboxy-1,3,4-trihydroxy-anthraquinone	3,5,14,18
Purpurin	1,2,4-Trihydroxyanthraquinone	3,5,7,13,14,18,20
Quinizarin	1,4-Dihydroxyanthraquinone	13,20,21
Quinizarin-2-carboxylic acid	2-Carboxy-1,4-dihydroxyanthraquinone	20
Ruberythric acid	1-Hydroxyanthraquinone-2-O-ß-D-primeveroside	1,5,8,10,27
Rubiadin	1,3-Dihydroxy-2-methylanthraquinone	1,3,14,18
Rubianin	1-Hydroxyanthraquinone-2-C-ß-D-glucoside	28
Tectoquinone	2-Methylanthraquinone	1,25
Xanthopurpurin	1,3-Dihydroxyanthraquinone	1,3,5,18
Xanthopurpurin dimethylether	1,3-Dimethoxyanthraquinone	3
Xanthopurpurin 3-methylether	1-Hydroxy-3-methoxyanthraquinone	3

Non-anthraquinone compounds		
Carboxylic acids	Citric acid 	29,30
Coumarins	Scopoletin 	1
Cinnamic acid derivatives 	Hexadecyl ferulate n=16	1
	Octadecyl ferulate n=18	1
	Eicosyl ferulate n=20	1
Flavonoids 	Hyperoside R=ß-D-glucose	31
	Rutin R=6-O-ß-D-rhamnose-ß-D-glucose	31
Iridoids	Asperuloside 	12,32
	Asperulosidic acid 	32

	Monotropein 	32
Naphtoquinones	Lapachol methylether 	1
Miscellaneous	Mollugin 	1

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Summary

The roots of *Rubia tinctorum* L. (madder) are the source of a natural dye. The dye components are anthraquinones with alizarin being the main dye component. Alizarin as such is present in madder root in only small quantities, most of the alizarin is present as its glycoside ruberythric acid. The sugar in this disaccharide is primeverose. Madder roots have been used to dye textiles in many parts of the world since ancient times. From 1600-1900 there was a heavy trade in madder throughout Europe. Madder root was an important export product for Holland. In 1868 Graebe and Liebermann discovered how to prepare alizarin synthetically. At the end of the 19th century the madder culture rapidly declined due to the cheaper production of synthetic alizarin. Production of synthetic alizarin gives polluting side products. Nowadays the use and production of natural dyes becomes more popular due to the growing awareness for the environment and the need for alternative crops. An important element in the revitalisation of madder as an industrial crop is that the dye preparation from madder should be able to compete in quality and price with synthetic alizarin. Due to this renewed interest this research was initiated with this thesis as result.

In the literature a total of 36 different anthraquinones have been reported from madder root. Both anthraquinone glycosides and aglycones have been described. For the simultaneous identification of the anthraquinone glycosides and aglycones in extracts of madder root a high-pressure liquid chromatography method (HPLC) was developed. The anthraquinones were separated on an end-capped C_{18}-RP column with a water-acetonitrile gradient as eluent and measured with ultra violet (UV) detection at 250 nm. For the identification of anthraquinones on-line a mass spectrometer (MS) and a diode-array detector were used. For on-line MS detection both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) could be used. Anthraquinone glycosides could be ionised in both the positive (PI) and negative ionisation (NI) mode. For anthraquinone aglycones ionisation was only obtained in the negative ionisation (NI) mode. However the efficiency of ionisation of hydroxyanthraquinone aglycones depended much on the pKa value of the compound. With ESI a modifier like ammonia had to be added post column to the eluent to ionise the compounds, which was not necessary with the APCI interface. UV detection still proved to be the method of choice for the quantitative analysis of anthraquinones, as UV detection is

sensitive, simple and cheap. HPLC-DAD and HPLC-MS provide useful additional information for the identification of anthraquinones in plant extracts.

The main anthraquinones in an ethanol-water extract of madder root are the glycosides lucidin primeveroside and ruberythric acid and the anthraquinones pseudopurpurin and munjistin, which contain a carboxylic acid moiety. Beside these compounds also small amounts of the aglycones alizarin and purpurin could be detected and sometimes also lucidin was present.

For the production of a commercially useful dye preparation from madder, the glycoside ruberythric acid should be hydrolysed to the aglycone alizarin, which is the main dye component. An intrinsic problem of the hydrolysis of ruberythric acid in madder root is the simultaneous conversion of lucidin primeveroside to the unwanted mutagenic aglycone lucidin. Madder root was treated with strong acid, strong base or enzymes to convert ruberythric acid into alizarin. The anthraquinone composition of the suspensions was analysed with HPLC-UV, HPLC-DAD and HPLC-MS.

Refluxing madder root under acidic conditions during 1 hour gave lucidin glucoside and ruberythric glucoside, which are respectively the first hydrolysis products of lucidin primeveroside and ruberythric acid. Refluxing for one night gave as products: alizarin, xanthopurpurin and purpurin. The glycoside ruberythric acid was hydrolysed to alizarin. The carboxylic acids pseudopurpurin and munjistin were decarboxylated to purpurin and xanthopurpurin. Lucidin primeveroside disappeared but no lucidin could be detected in the extract. It was not investigated what happened to lucidin under these conditions. Refluxing madder root under basic conditions gave a madder root suspension with a lot of minor unidentified anthraquinones.

Stirring of dried madder root in water at room temperature for 90 min gave a suspension with pseudopurpurin, munjistin, alizarin and nordamnacanthal. Nordamnacanthal originates from lucidin primeveroside, which is hydrolysed to lucidin and subsequently oxidised to the corresponding aldehyde nordamnacanthal by an endogenous hydrolase and oxidase respectively. Nordamnacanthal is not mutagenic. During this conversion oxygen is obligatory and can be added by stirring the suspension. This stirring is an easy method for simultaneously hydrolysing ruberythric acid and to getting rid of the mutagenic lucidin.

For the quantitative determination of the main anthraquinones two different extraction methods were developed and compared. In the first method madder root (2.5 g) was extracted twice with boiling ethanol-water (1:1) (100 and 50 ml) and analysed with HPLC. In the second method the anthraquinone glycosides were first converted to the aglycones by the endogenous enzymes. After conversion the aglycones were extracted with THF-water-formic acid (1:1:0.005) (v/v/v) and analysed by HPLC. With the ethanol-water extraction the anthraquinone glycosides lucidin primeveroside and ruberythric acid could be determined.

With the THF-water-formic acid extraction the amount of pseudopurpurin, munjistin, alizarin and nordamnacanthal could be determined. This method is easier to apply than the method with ethanol-water. However one has to keep in mind that the compounds determined are actually artefacts of the anthraquinones really present in madder root.

For the quantitative determination of the main anthraquinones by HPLC-UV calibration curves were constructed. Lucidin primeveroside and ruberythric acid were purified by droplet counter current chromatography (DCCC). Pseudopurpurin and munjistin were purified by Sephadex LH-20 column chromatography. Nordamnacanthal was purified by C_{18} column chromatography.

Different madder root cultivars were screened for their anthraquinone composition and amount of the main anthraquinones. The concentration of alizarin varied from 6.1 to 11.8 mg/g root. If madder root was cultivated for three instead of two years the amount of alizarin increased from 6.7 mg/g to 8.7 mg/g.

A number of different methods were compared for their capacity to isolate alizarin from the rest of the plant material. To make a first selection attention was mainly paid to the yield of alizarin. Except for one method, madder root was first stirred in water. As already discussed, stirring in water is the easiest way to hydrolyse ruberythric acid. After stirring for one hour at 45°C the suspension was treated in different ways to extract alizarin from the rest of the plant material.

Extraction with refluxing ethanol-water (1:1) gave a 100% recovery of alizarin from the rest of the insoluble plant material. If instead of ethanol-water (1:1) (v/v) boiling water was used an extraction recovery of 66% was obtained after four extraction steps. If the water was heated to 130°C under pressure, less water had to be used and the recovery of alizarin was 75%. The extraction of alizarin with an alkaline solution is impossible due to the formation of insoluble alizarin polyvalent metal ion complexes. If madder root was stirred in a solution of EDTA instead of ultra pure water, complex formation of alizarin was avoided and a recovery of 91% was obtained. Extraction of alizarin with a non-ionic surfactant from the Brij series enabled a 96% recovery of alizarin. After separation the surfactant has to be removed, which can be achieved by using a solid phase extraction (SPE) step with C_{18} material.

Another route that was investigated was the formation of alizarin by endogenous enzymes after isolation of ruberythric acid. Madder root was extracted with boiling water and after filtration the glycosides were converted by an enzyme extract of fresh madder root. Alizarin was obtained in a recovery of 82%. This last method circumvents the precipitation of free alizarin and its troublesome separation from insoluble plant parts.

Three routes were selected as most promising for an industrial application. These three methods were tested at a larger scale. The first method consisted of the following steps:

conversion of madder root (250 g) by endogenous enzymes, extraction of madder root with refluxing ethanol-water, hot filtration, evaporation to half of the original volume and precipitation at 4°C. An extract of 14.7 g was obtained which consisted for 35 % of anthraquinones. Of the total amount of alizarin available in the starting material 78% was extracted. The second method consisted of the following steps: extraction of madder root (250 g) with refluxing water, hot filtration, conversion of the glycosides in the filtrate by a madder root enzyme extract and precipitation at 4°C. An extract of 3.2 g was obtained of which 38 % were anthraquinones. Of the total amount of alizarin available in the starting material 19% was extracted. The third method consisted of the following steps: extraction of madder root with an aqueous surfactant solution, twice C_{18} chromatography for extracting alizarin, elution of alizarin with methanol and evaporation. An extract of 17.1 g was obtained of which 11 % were anthraquinones. Of the total amount of alizarin available in the starting material 98% was extracted.

For the development of an economically feasible route these three methods have to be further optimised. After optimisation the three routes have to be compared in terms of amount of extract obtained, alizarin content, dyeing capacity, costs and industrial applicability of the procedure.

Meekrap, terug van weggeweest

Korte inhoud van dit proefschrift

Inleiding (hoofdstuk 1)

Meekrap is een plant waaruit een rode kleurstof voor textielverf kan worden gewonnen. Van 1600 tot 1900 was er een levendige handel in de gedroogde en gemalen wortelen van dit gewas. Vooral de Zeeuwse meekrap gaf hoge kwaliteit en was internationaal befaamd. De rode kleurstof in de meekrap heet alizarine. In 1868 ontdekten de scheikundigen Graebe en Liebermann een methode om alizarine chemisch te produceren. Deze alizarine was veel goedkoper, waardoor de meekrapteelt aan het eind van de 19de eeuw was verdwenen.

Bij de chemische productie van alizarine komen veel milieuvervuilende nevenproducten vrij. Door de toenemende milieueisen is de prijs van alizarine verdubbeld. Om meekrap opnieuw te introduceren als kleurstofbron, moeten de 19deeeuwse teelt-, oogst- en verwerkingsmethoden worden vertaald naar moderne technieken. Vanaf september 1996 heb ik onderzoek gedaan naar de ontwikkeling en verbetering van de verwerkingsmethoden van meekrapwortelen. De resultaten heb ik beschreven in dit proefschrift.

Identificatie van kleurstoffen in meekrapwortels (hoofdstuk 2 en 4)

Na een uitvoerig literatuuronderzoek bleek, dat naast alizarine, er nog 36 andere kleurstoffen in meekrap zitten. Eerst heb ik een methode ontwikkeld om te allen tijde te kunnen bepalen welke kleurstoffen aanwezig zijn in een meekrapmengsel. Hiervoor worden de kleurstoffen eerst gescheiden en daarna één voor één bekeken en benoemd. Het scheiden gebeurt met een HPLC-kolom op basis van het verschil in oplosbaarheid van de kleurstoffen. Hierdoor komen de stoffen één voor één van de kolom af. Daarna stromen de stoffen langs een lichtmeter. Als een stof langs de lichtmeter, UV-lamp, stroomt dooft het licht even. Van bekende stoffen zoals alizarine weet ik op welk tijdstip ze langs de UV-lamp komen. Geeft de licht meter op dat tijdstip een signaal dan weet ik dus dat er alizarine in het mengsel zat. Voor onbekende stoffen gebruik ik een MS- meter. Een MS-meter is eigelijk een zeer nauwkeurige balans. Deze meter weegt de stof die langs komt. Elke stof heeft een eigen gewicht waaraan deze herkenbaar is.

Deze HPLC methode heb ik later dermate uitgebreid dat ik naast de identiteit van een stof ook kan zeggen hoeveel van deze stof aanwezig is. Hiermee heb ik van een groot aantal verschillende meekrapplanten bepaald welke kleurstoffen erin zitten en hoeveel. Zo heb ik gevonden dat driejarig wortelmateriaal 30% meer kleurstof bevat als tweejarig materiaal terwijl het droog gewicht maar met 10% toeneemt.

Vrijmaken van meer rode kleurstof (hoofdstuk 3)

Uit proeven met de HPLC methode bleek dat alizarine niet de belangrijkste kleurstof in meekrap is. Dit is ruberythrinezuur. Ruberythrinezuur kan worden afgebroken tot alizarine. De volgende stap was het vinden van een geschikte manier om dit ruberythrinezuur af te breken tot alizarine. Dit heb ik op drie verschillende manieren geprobeerd.

Bij de eerste manier werd meekrap lange tijd gekookt in water met zwavelzuur. In de eerste helft van de 19de eeuw behandelden de Fransen meekrap ook al met zwavelzuur. Zij verkregen hierdoor een product dat een tweemaal betere verfkracht had dan de andere meekrapkleurproducten die tot dan toe te koop waren. Ik concludeerde inderdaad dat tijdens het koken van meekrap in een zwavelzuur oplossing, het ruberythrinezuur wordt afgebroken tot alizarine. Nadelen van dit proces zijn het gebruik van zwavelzuur en het energieverbruik om het mengsel langere tijd op 100°C te houden. Om deze redenen ging ik op zoek naar een meer milieu en energetisch vriendelijke manier om ruberythrinezuur af te breken.

Bij de tweede manier heb ik meekrap behandeld met enzymen. Enzymen zijn natuurlijk en kunnen worden gebruikt in water. In eerste instantie heb ik gebruik gemaakt van in de handel verkrijgbare enzymen. Door meekrap samen met de enzymen in water te roeren bij 45°C, werd alle ruberythrinezuur afgebroken tot alizarine. Een nadeel was dat er ook lucidine werd gevormd. Lucidine is een mutagene stof en kan dus kanker veroorzaken. Deze stof is absoluut ongewenst in het eindproduct.

Bij de derde manier werd meekrap gemalen, gedroogd (<60°C) en geroerd in water bij 45°C. Het bleek dat tijdens het roeren ruberythrinezuur werd afgebroken door een enzym dat van nature al aanwezig is in meekrap. Dit enzym komt vrij doordat de meekrap is gemalen. Een tweede enzym, dat ook van nature voorkomt in meekrap, zorgde ervoor dat er géén lucidine werd gevormd. Dit tweede enzym heeft echter zuurstof nodig om zijn werk goed te doen. Deze zuurstof kan eenvoudig worden toegediend door het mengsel te doorborrelen met lucht (roeren). Deze laatste manier om alizarine te vormen, is goedkoop en er is weinig energie voor nodig.

Zuiveren van het kleurstofproduct (hoofdstuk 5 en 6)

Na de omzetting blijft er een mengsel van alizarine, andere kleurstoffen, plantenresten en water over. Nu moet nog een manier worden gevonden om alizarine te zuiveren van de rest.

Aangezien alizarine niet oplosbaar is in water kan het niet gescheiden worden door filtreren, zoals bijvoorbeeld koffie wordt gezet. Ik heb een aantal manieren bedacht om alizarine te zuiveren. Al deze manieren heb ik uitgeprobeerd en bepaald hoe hoog de opbrengst alizarine was.

Uiteindelijk heb ik drie methoden geselecteerd om alizarine te zuiveren uit bewerkte meekrap. Deze drie voldoen het beste aan de volgende criteria:

- hoge opbrengst aan alizarine uit de bewerkte meekrap
- weinig gebruik van chemische stoffen
- lage energie gebruik
- uitvoerbaar op industriële schaal
- arbeidsextensief
- weinig nevenproducten

Deze drie methoden zijn uitgeprobeerd op grotere schaal. In plaats van 0.25 of 2.5 gram meekrap is nu 250 gram meekrap gebruikt.

Bij de eerste methode werd gedroogde en gemalen meekrap geroerd in water waardoor alizarine werd gevormd. Daarna werd alcohol toegevoegd en werd het mengsel aan de kook gebracht. Door de hoge temperatuur en de alcohol lost alizarine op. Na 2 uur koken werd het mengsel gefiltreerd en de vloeistof voor de helft verdampt. De overgebleven vloeistof werd in de koelkast geplaatst. Door de lage temperatuur lost alizarine slecht op en zal dus neerslaan. Na één nacht werd het mengsel wederom gefiltreerd en de vaste stof die op het filter bleef liggen, bestaat voor 35% uit kleurstoffen. Van de totale hoeveelheid alizarine die maximaal uit de plant kan worden gehaald (100%) werd met deze methode 78% verkregen.

Bij de tweede methode werd de gedroogde en gemalen meekrap 2 uur in water gekookt en daarna gefiltreerd. Het ruberythrinezuur zit dan in de vloeistof. Na afkoelen van de vloeistof werd een enzymmengsel toegevoegd. Het enzymmengsel had ik van tevoren uit verse meekrap gehaald. Deze enzymen zorgen ervoor dat ruberythrinezuur wordt afgebroken tot alizarine en dat er geen lucidine wordt gevormd. Na het roeren werd de oplossing in de koelkast gezet voor tenminste één nacht en hierna gefiltreerd. De vaste stof die achter blijft op het filter, is het kleurstofpreparaat en bestaat voor 38% uit kleurstoffen. Dit kleurstofproduct bevat 19% van de alizarine totaal aanwezig in meekrap.

Bij de derde methode werd meerkap geroerd in water met een beetje zeep. Tijdens het roeren, wordt ruberythrinezuur afgebroken. Door de zeep blijft het alizarine opgelost. Ook na filtreren zit de alizarine nog in de vloeistof. Zo is het alizarine gescheiden van de vaste delen van het plantenmateriaal. Hierna wordt de vloeistof met een materiaal gemengd dat kleurstoffen absorbeert en andere stoffen doorlaat. Daarna worden de kleurstoffen uit het absorptiemateriaal gehaald met een beetje alcohol. De alcohol verdampt en het

kleurstofpreparaat blijft over en bestaat voor 11% uit kleurstoffen. Dit kleurstofproduct bevat 98% van de alizarine totaal aanwezig in meekrap

Van een geslaagd onderzoek tot industriële schaal

Alle drie de methodes zijn redelijk tot goed geschikt voor een industriële toepassing. Nu moeten deze drie methodes worden geoptimaliseerd en gecombineerd tot één betere methode. Daarna kan deze methode worden opgeschaald tot bijvoorbeeld 250 kg en weer verder geoptimaliseerd worden.

Met de resultaten van dit vierjarig onderzoek kan een industrieel proces worden opgezet waarmee meekrap wordt verwerkt tot een commercieel kleurstofproduct. Hiermee is de honderd jaar geleden verdwenen meekrapcultuur klaar voor een gemoderniseerde terugkeer.

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G.C.H. Derksen, T.A. van Beek, Æ. De Groot, A. Capelle.
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Curriculum vitae

Op 19 december 1970 werd Dorien (Goverdina Christina Helena) Derksen geboren te Steenbergen, Noord Brabant. In 1987 behaalde zij haar MAVO diploma aan de Petrus Canisius te Steenbergen. De schooltijd werd vervolgd aan het Mollerlyceum te Bergen op Zoom waar zij in 1989 en 1991 respectievelijk haar HAVO en Atheneum diploma behaalde. In 1991 begon zij aan de studie levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit Wageningen. Tijdens deze studie werd een vijfmaands afstudeervak gedaan in de levensmiddelenchemie, specialisatie eiwitten bij de vakgroep levensmiddelenchemie en Microbiologie onder begeleiding van dr. H. Gruppen, J. de Groot en prof A. Voragen. Vervolgens werd in het kader van een Erasmus project een afstudeervak gedaan in de organsiche chemie, specialisatie synthese bij de vakgroep Organic Chemistry aan de University of Cardiff, Wales, onder begeleiding van dr. S.P. Bew, prof. D.W. Knight (Cardiff) en dr. M. Franssen en prof Ae. de Groot Wageningen Universiteit (voormalig Landbouwuniversiteit Wageningen). De studie werd afgerond met een vijfmaands stage bij het Nederlands Instituut voor Zuivelonderzoek (NIZO) in Ede onder begeleiding van dr. S. Visser en dr. K.G. de Kruif met als specialisatie melkeiwitten. Zij behaalde het diploma levensmiddelentechnolgie in september 1996. In ditzelfde jaar werd begonnen met een promotie onderzoek bij de afdeling Fytochemie aan de leerstoelgroep Bio-organische Chemie aan de Wageningen Universiteit. Van september 1996 tot november 2000 onder begeleiding van dr. T.A. van Beek, prof. Ae. de Groot en prof A. Capelle. De resultaten van het van september 1996 tot november 2000 uitgevoerde onderzoek staan beschreven in dit proefschrift. Sinds maart 2001 is zij werkzaam als onderzoeker bij Numico-research in Wageningen.

The cover shows a painting titled "Het keuren van de meekrap" (inspection of the madder). The painting is exhibited in the "Zeeuws museum" in Middelburg, The Netherlands.

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