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Short communication

High-performance liquid chromatographic method for the analysis of anthraquinone glycosides and aglycones in madder root (*Rubia tinctorum* L.)

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Abstract

A HPLC method has been developed for the simultaneous characterisation of anthraquinone glycosides and aglycones in extracts of *Rubia tinctorum* L. The anthraquinones are separated on an end-capped C_{18}-RP column with a water–acetonitrile gradient as eluent and measured with UV detection at 250 nm. With this method the glycosides lucidin primeveroside and ruberythric acid and the aglycones lucidin, alizarin, purpurin, quinizarin and 2,6-dihydroxyanthraquinone can be analysed. Lucidin which is not commercially available was synthesised starting from resorcinol and phthalic anhydride. The glycosides ruberythric acid and lucidin primeveroside are commercially available as a mixture and were separated by droplet counter-current chromatography in ascending flow with chloroform–methanol–water as eluents prior to their use as standards.

Keywords: *Rubia tinctorum* L.; Anthraquinones; Lucidin primeveroside; Ruberythric acid; Lucidin; Alizarin; Purpurin; Quinizarin

1. Introduction

The roots of *Rubia tinctorum* L. (madder) are the source of a natural dye. The dye components are anthraquinones and the most important one is alizarin (**5**). Ruberythric acid (**2**) is the glycoside of alizarin (**5**) with the sugar moiety primverose (6-*O*-β-D-xylopyranosyl-β-D-glucose). Other anthraquinone aglycones in madder are, purpurin (**6**), lucidin (**4**) and quinizarin (**7**) [1]. The most common anthraquinone glycosides are ruberythric acid (**2**) and lucidin primeveroside (**1**) [2].

Several screening methods of anthraquinones in *Rubia tinctorum* L., based on reversed-phase high-performance liquid chromatography (RP-HPLC), have been described in the literature [2–6]. In general, HPLC analysis are based on the aglycones. The madder extract is hydrolysed and the total quantity of alizarin (**5**) is determined [4,6]. The only two HPLC methods described in the literature for the simultaneous analysis of both glycosides and aglycones fail to give baseline separation of the glycosides, ruberythric acid (**2**) and lucidin primeveroside (**1**) [2,5].

In this paper we describe an HPLC method for the quantitative detection of the most common anthra-

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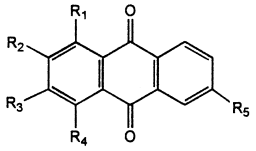
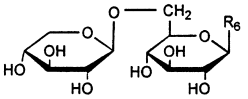
Table 1
Structures of the anthraquinones



anthraquinone aglycone moiety



primeveroside
R_6 = -O-aglycone

	Name	R_1	R_2	R_3	R_4	R_5
1	Lucidin primeveroside	OH	CH_2OH	*O*-Primeveroside	H	H
2	Ruberythric acid	OH	*O*-Primeveroside	H	H	H
3	2,6-Dihydroxyanthraquinone	H	OH	H	H	OH
4	Lucidin	OH	CH_2OH	OH	H	H
5	Alizarin	OH	OH	H	H	H
6	Purpurin	OH	OH	H	OH	H
7	Quinizarin	OH	H	H	OH	H
8	Purpuroxanthin	OH	H	OH	H	H

quinone glycosides and aglycones of *Rubia tinctorum* L., ruberythric acid (**2**), lucidin primeveroside (**1**), lucidin (**4**), alizarin (**5**), purpurin (**6**) and quinizarin (**7**) (Table 1).

2. Experimental

2.1. Chemicals

Crude ''ruberythric acid'' was obtained from Carl Roth (Karlsruhe, Germany). Acetonitrile (HPLC grade) and chloroform 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). All HPLC solvents were degassed prior to use by vacuum filtration over a 0.45-μm membrane filter (Type RC, Schleicher and Schuell).

2.2. Reference compounds

Alizarin (**5**) (1,2-dihydroxy-9,10-anthracenedione), purpurin (**6**) (1,2,4-trihydroxy-9,10-anthracenedione), quinizarin (**7**) (1,4-dihydroxy-9,10-anthracenedione) and 2,6-dihydroxyanthraquinone (**3**) (2,6-dihydroxy-9,10-anthracenedione) were purchased from Acros (Geel, Belgium).

Lucidin primeveroside (**1**) and ruberythric acid (**2**) are not commercially available in pure form and were separated and purified by droplet counter-current chromatography (DCCC). The DCCC apparatus was from Tokyo Rikakikai, an EYELA Model type DCCC-A. The model consisted of 300 tubes with an I.D. of 2 mm, the connecting PTFE tubing had an I.D. of 0.5 mm. The DCCC was used in the ascending mode. The eluent system used, was $CHCl_3$–MeOH–water (5:5:3). Crude ''ruberythric acid'' (500 mg) was dissolved in 7 ml of the ascending fluid. During the elution the flow was 0.48 ml/min. Fractions of 9 ml were collected. The total separation time was 15 h 30 min.

Lucidin (**4**) is not commercially available and was synthesised in two steps according to the method of Murti et al. [7]. Column chromatography over silica was used to purify purpuroxanthin, which is the product of the first step. The column was eluted with petroleum ether (40:60)–ethyl acetate–formic acid (75:25:1). Purpuroxanthin was then converted to lucidin [7].

The compounds **1**, **2** and **4** were identified by 400 MHz ^{1}H-NMR and ^{13}C-NMR [8,9].

Their purity was determined by means of quantita-

tive 400 MHz ^{1}H-NMR. Maleic acid was used as internal standard and DMSO-d_6 was used as solvent [10].

2.3. Sample preparation for HPLC

Dried and powdered three-year-old root material of *Rubia tinctorum* (2.5 g) was refluxed with 100 ml water–ethanol (75:25). After 6 h 200 μl of the extract was filtered over a 0.45-μm membrane filter (Type RC, Schleicher and Schuell). One hundred μl of the filtered extract was diluted with 900 μl water–methanol (1:1) and analyzed by HPLC.

2.4. HPLC

The HPLC apparatus consisted of a Waters 600E multisolvent delivery system, equipped with a Waters 994 programmable photodiode array detector. Data were processed using Waters 991 PDA software. Analysis was carried out at room temperature on an Alltima end-capped C_{18}, 100 Å pore size, 5 μm particle size, 250 mm×3.2 mm I.D. column. Chromatography was carried out using two solvents: (A) water; (B) acetonitrile in a linear gradient programme (Table 2). The flow-rate of the mobile phase was 1.0 ml/min. Injections were made by a Gilson 231 Sample Injector equipped with a 10-μl loop. Peaks were detected over the 200–600 nm range of the absorption spectrum and all chromatograms were plotted at 250 nm. The t_R values for lucidin primeveroside (**1**), ruberythric acid (**2**), 2,6-dihydroxyanthraquinone (**8**), lucidin (**4**), alizarin (**5**), purpurin (**6**) and quinizarin (**7**) were respectively, 8.1; 9.1; 19.1; 22.2; 23.8; 25.8 and 34.0 min (Fig. 2).

Table 2
Gradient table for HPLC analysis

Time (min)	Solvent A (%)[a]	Solvent B (%)[b]
Initial	73	27
6	73	27
20	30	70
35	30	70
40	73	27
45	73	27

[a] Solvent A=double-distilled water.
[b] Solvent B=acetonitrile.

3. Results and discussion

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 are necessary to record linear calibration curves. By means of HPLC analysis it was shown that commercially available ''ruberythric acid'' contained two anthraquinone glycosides [2,5]. By 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% other unidentified constituents.

Different authors have isolated ruberythric acid (**2**) and lucidin primeveroside (**1**) from Rubiaceae plant material by one or more extraction steps followed by one or more column chromatography (Sephadex LH-20, Dowex 50, Amberlite XAD-2) [2,5,7,9,11]. 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 could cause irreversible adsorption on the solid stationary phase during the chromatographic procedure [12]. We tried unsuccessfully to separate and isolate the glycosides by medium-pressure liquid chromatography (MPLC), with borate impregnated silica gel [13]. Hermans-Lokkerbol et al. [14] used CPC with a solvent mixture of $CHCl_3$–MeOH–water–acetic acid (5:6:4:0.5) to separate glycosides of *Rubia tinctorum*. Inoue et al. [15] used DCCC with a solvent mixture of $CHCl_3$–MeOH–water (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 ruberythric acid (**2**) and lucidin primeveroside (**1**) by DCCC (Fig. 1). Only one mixed fraction was collected. In one DCCC run 26 mg lucidin primeveroside and 29 mg ruberythric acid were purified from 500 mg crude ''ruberythric acid''. The separation of the glycosides was achieved far more readily with DCCC than by conventional chromatography [2,5,7,9,11].

Lucidin (**4**) is not a major aglycone in *Rubia tinctorum* but it is the hydrolysis product of lucidin primeveroside (**1**). Because lucidin (**4**) is considered

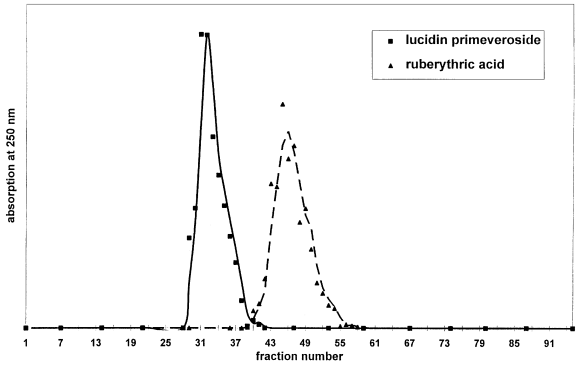


Fig. 1. Reconstructed chromatogram of DCCC separation.

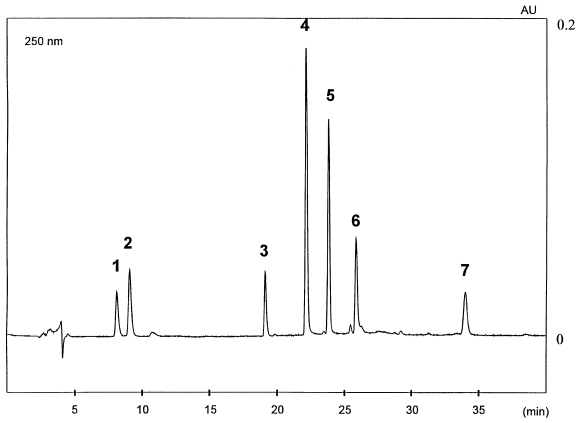


Fig. 2. HPLC trace of anthraquinone standards at 250 nm.

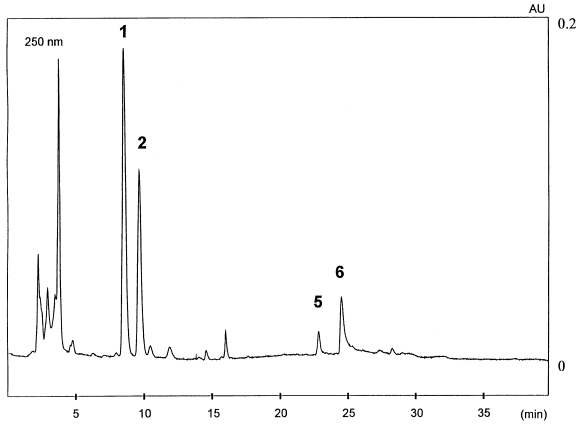


Fig. 3. HPLC trace of a crude extract of *Rubia tinctorum*.

to be a mutagenic compound [5,8,11] and not commercially available some effort was made to get lucidin pure. Lucidin (**4**) was successfully synthesised by the method of Murti et al. [7].

The purities of the antraquinone glycosides and aglycones used for the calibration curves are reported in Table 3. Commercial purpurin (**6**) was contaminated with quinizarin (**7**) [14].

3.2. HPLC method

A HPLC method was developed for the characterisation of anthraquinone glycosides and aglycones in madder root extracts in one run (Fig. 2). Fig. 3 shows an HPLC run of an extract of *Rubia tinctorum*. Linear calibration graphs, based on the peak area with good correlation (Table 3) were obtained for ruberythric acid (**2**), lucidin primeveroside (**1**), lucidin (**4**), alizarin (**5**), purpurin (**6**) and quinizarin (**7**). The highest sensitivity was obtained by monitoring at 250 nm, the wavelength where an anthraquinone shows its maximum absorbance. The detection limits are reported in Table 3. The advantage of this HPLC procedure over previously published procedures is the baseline separation and quantification of all the glycosides and aglycones in one HPLC

Table 3
Purity, correlation coefficient and detection limits of anthraquinones **1**–**8**

No.	Name	Purity (%)	Correlation coefficient linear calibration graphs	Limit of detection (ng)
1	Lucidin primeveroside	81	1.0000	3.9
2	Ruberythric acid	89	0.9998	2.0
3	2,6-Dihydroxyanthraquinone	56	1.0000	1.5
4	Lucidin	74	1.0000	1.9
5	Alizarin	95	0.9999	3.4
6	Purpurin	55	0.9997	17.1
7	Quinizarin	93	0.9999	1.5

run. Furthermore, very sharp peaks are obtained and no acid had to be added to the water to suppress tailing of the anthraquinone peaks.

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