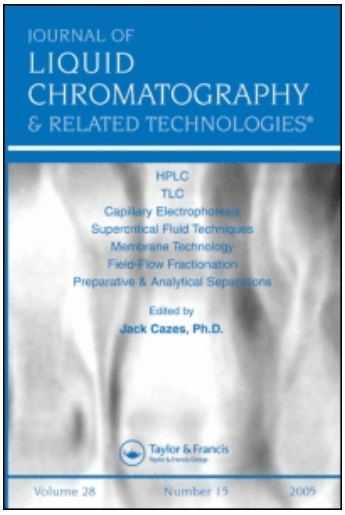

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HPLC ANALYSIS OF ANTHRAQUINONE DERIVATIVES IN MADDER ROOT (*Rubia tinctorum*) AND ITS CELL CULTURES

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ABSTRACT

A sensitive and reproducible RP-HPLC method was developed for the characterization of madder root and its cell cultures extracts and for the determination of anthraquinone derivatives as glycosides (ruberythric acid, lucidin primveroside) and aglycones (alizarin, lucidin, purpurin) in them.

The fingerprint chromatographic patterns of the extracts were obtained on C_{18} silica column, using gradient with acetonitrile-water-acidic buffer eluent system. For quantitative measurements, the anthraquinone derivatives were separated by isocratic elution in the same type of mobile phase. The chief components were identified and quantitatively determined.

The natural plant and its cell suspension cultures were compared to each other. The preparative fractionation of the extracts was achieved by gel chromatography, HPLC and selective extractions with solvent series, solid-phase extraction techniques and SFE.

INTRODUCTION

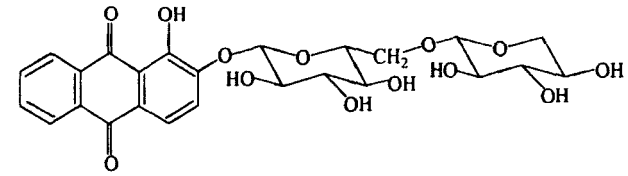
The natural plant derived food colors tend to be frequently used, and the need is growing for heat- and light-resistant natural substances. Madder pigments seem to satisfy these requirements.

The natural dyes obtained from rubiaceous plants have been highly esteemed, since ancient times in the East and West, because of the excellent dyeing qualities and light fastness. In recent years, however, rubiaceous plants have found little use in dyeing since the advent of chemical dyes. Some compounds of pharmacological interest, were found in them with hopeful applications. So, their plant cell cultures are a potential source for production phytochemicals of high economic value.

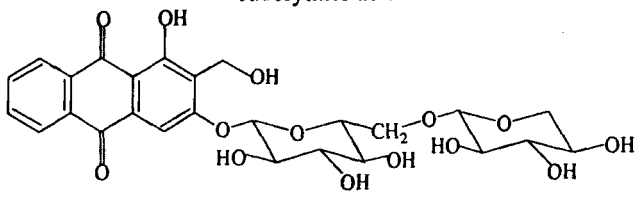
In Rubia species, many biologically active compounds, mostly secondary plant products, metabolites, are found, for example: anthraquinones, hydroxyanthraquinones, their derivatives and glycosides, naphthohydroquinones, naphthoquinones, their derivatives and glycosides, naphthoquinone dimers, as well as peptides. The formulas of our measured components are given in Fig. 1.

Rubia tinctorum (madder root), the source of natural dye, produces anthraquinone pigments and secondary metabolites in the roots and, also, in the cultured cells, one of them being alizarin[4]. The herbal drugs, consisting of crude Rubia extracts, have some compounds of pharmacological interest with the activity of dissolving bladder and kidney stones (especially Ca-oxalate and Ca-phosphate) in the urinary tract. This effect has been clinically tested and some medicines are produced: Cystenal® by SPOFA (Praha, Czechoslovakia), Rubia Teep® tablets from Madaus (Köln, FRG). All parts of Rubia tinctorum contain pigment complexes of different quantity and rather different composition, but the underground organs (rhizome and roots) have the highest pigment content.[22]

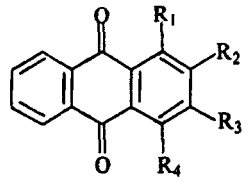
It has been shown, that Rubia tinctorum produces lucidin, in addition to alizarin, and that these hydroxyanthraquinones are present as glycosides which decompose in rat to the genotoxic hydroxyanthraquinones lucidin and



ruberythric acid



lucidin primveroside



	R_1	R_2	R_3	R_4
anthraquinone	H	H	H	H
2-hydroxy-anthraquinone	H	OH	H	H
anthraquinone-1-methylether	OCH_3	H	H	H
1-hydroxy-2-methyl-anthraquinone	OH	CH_3	H	H
alizarin	OH	OH	H	H
alizarin-2-methylether	OH	OCH_3	H	H
anthragallol	OH	OH	OH	H
lucidin	OH	CH_2OH	OH	H
purpurin	OH	OH	H	OH

Figure 1. Formulas of the measured compounds.

1-hydroxy-anthraquinone.[2,3,5,6,13,27,28,30,33] Alizarin, produced in cell cultures, can be used as an indicator for the production of the anthraquinone metabolites in cultured Rubia cells.

Many methods have been reported for the separation of the naturally occuring free anthraquinone aglycones. These have been based on paper chromatography,[8,23] thin-layer chromatography,[15,17] centrifugal partition chromatography,[7] counter-current chromatography,[35] low-pressure column chromatography,[21,27] and high performance liquid chromatography (HPLC).[11,14,16,18-20,25,29,31,32]

The adsorption of anthraquinone pigments on Amberlite XAD-2 resin was described previously, but the method was not suitable for routine use with cell and tissue culture extracts.[29] Column purification, however, was practical and C_{18} cartridges proved to be effective in the HPLC determination of anthraquinone in pulping liquors.[11]

Recently, some HPLC systems have been elaborated for analysis of madder extracts. Odake et al.[12] applied Nucleosil-508 (4.6×250 mm) column and methanol with 0.1% phosphoric acid was the solvent.

According to Westendorf et al.[28] a Merck- and a Spectra Physics HPLC-system were applied for analysis of Rubia extracts with acetonitrile gradient in 0.1% acetic acid.

Quercia[14] offered for HPLC determination of some anthraquinone glycosides: Permaphase ODS or Micropack Si 10 (250×2 mm) columns with various mixtures of methanol/water (for RP-HPLC) or of methanol/n-pentane (for NP-HPLC).

MATERIALS

Chemicals were obtained from the following sources: alizarin from Merck (Darmstadt, FRG), ruberythric acid (depur, referred in this paper as glycoside mixture) from Carl Roth GmbH & Co. (Karlsruhe, FRG) and Rubia Teep from Madaus (Köln, FRG). Lucidin was synthesized according to the method described by Murti et al.[10]

The other derivatives were synthesized in our lab.[1,26] A pure, authentic, ruberythric acid sample was synthesized by Cs. Weber[26] in our lab, according to a modified method of Zemplén and Bognár.[34] The plant cell cultures were prepared and treated in the laboratory of Department of Plant Anatomy, Eötvös University.

EXPERIMENTAL

Sample Preparation

Dried and powdered material (10 mg), obtained from R. tinctorum cell culture, was suspended in 2.5 mL of 80% (v/v) ethanol,[15] sonicated for 5 min (Branson 2200 sonicator) and soaked at 80°C for 0-10 h. The extract was separated by centrifugation, the residue was mixed with 1.5 mL of 80% (v/v) ethanol, incubated for 4 h at 80°C and centrifuged again. The combined supernatants were evaporated to dryness and the residue was dissolved in 1 mL of 80% (v/v) ethanol and used as the crude extract in further HPLC studies.

Purification of the Crude Extracts

The crude extract (1 mL) was diluted tenfold with water and passed dropwise through preactivated SPE cartridges (Bond Elut LRC C_8 1 cc; Analytichem, Harbor City, CA, USA), which were then washed with 2 mL of water, followed by 1 mL of methanol-water (30:70, v/v). After drying the cartridges with air, the fraction containing the anthraquinones was eluted from the tube with 1 mL of methanol-water (80:20, v/v).

HPLC of the Extracts

a.) Chromatography was performed using a Beckman 342 HPLC system, equipped with a Beckman 114 M solvent-delivery module, a Beckman 420 controller, a Beckman 165 variable-wavelength detector and an Altex 210 loop injector (20 μL loop volume). The ratiograms (254/280 nm) were recorded with a BBC Goertz Metrawatt SE-120 two-channel recorder (BBC) and chromatograms (254 nm, quantitation signal), with a Merck-Hitachi-2000 chromato-integrator. The ratio threshold was set at 2%. The components were separated on an ODS Hypersil (5 μm particle size) reverse phase column (125×4 mm I.D.) (Shandon Southern Products, Runcorn, UK). The isocratic elution of components was accomplished using methanol-5% acetic acid (pH 3.0) (70/30) at a flow-rate of 1.0 mL/min. Peaks were identified by comparing their retention times, ratiogram plots and on-line detection of the UV spectra with those of standards

b.) Separations were performed on a Knauer system consisting of two pumps Model 64 with analytical pumphead, a gradient programmer Model 50B, an injection valve with 20 μL sample loop and an UV spectral photometer

(or RI detector) with analytical flow cell (Knauer GmbH, Bad Homburg, FRG). Column effluents were monitored at 254 or 280 nm. The columns were: 250×4 mm Superpac PEP S C_2/C_{18} RP 5 μm (Pharmacia LKB, Uppsala, Sweden); 250×4 mm BST-Nucleosil C_{18} RP, 5 μm (BST, Budapest, Hungary); 300×4 mm Polygosil 60-10NH_2 (Macherey Nagel, Düren, Germany); 250×4 mm MOS-Hypersil C_8 RP 6 μm (Shandon Southern Products, Runcorn, UK); 250×4 mm BST Rutin 10 C_{18} Pep 5 μm (BST, Budapest, Hungary); 4.6×250 mm Beckman Ultrasphere ODS 5 μm (USA) (Fig. 2). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Budapest, Hungary). The chromatograph was operated isocratically and gradient with flow rates between 0.8 and 2 mL/min. Peaks were identified with standard addition (co-injection) using more eluent systems isocratically and with gradient elution, too.

Eluent systems were:

Isocratic:

acetonitrile - 0.05 M phosphate buffer, pH 4.5 55:45 (v/v) for alizarin;
acetonitrile - 0.05 M acetate buffer, pH 4.5 43:57 (v/v) for lucidin measurements.
acetonitrile - water 82.5:17.5 (v/v) for carbohydrate-determinations
acetonitrile - 0.02 M acetate buffer, pH 4
43:57 (v/v) for hydroxyanthraquinones
22:78 (v/v) for anthraquinone glycosides

Gradient:

1. Eluent A:
acetonitrile - 0.05 M phosphate buffer, pH 4.5 - methoxy-ethanol 15:80:5 (v/v/v)
Eluent B:
acetonitrile - 0.05 M phosphate buffer, pH 4.5 - methoxy-ethanol 80:15:5 (v/v/v)
Gradient: linear, 20→90% B in A over 40 min;

2. Eluent A:
acetonitrile - 0.02 M acetate buffer, pH 4 15:85 (v/v)
Eluent B:
acetonitrile - 0.02 M acetate buffer, pH 4 85:15 (v/v)
Gradient: linear, 10%B→90%B or 20%B→90% B in A over 40 min.

For preparative separations ammonium acetate buffer was used in place of phosphate one. Samples were prepared with methanol extraction. KNAUER system was applied for preparative HPLC with preparative pumpheads, flow cell and injector. The column was 120×16 mm KNAUER Lichroprep RP18 15-25 μm (Knauer GmbH, Bad Homburg, FRG).

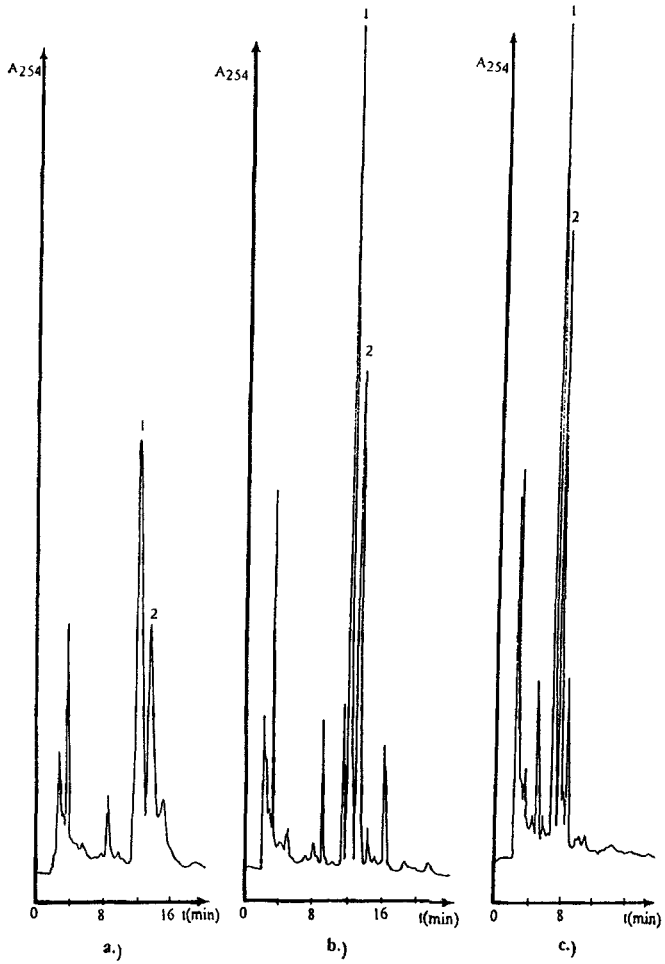


Figure 2. Chromatographic pattern of aqueous extract of Rubia tinctorum cell suspension culture (RF 413) on three different columns. Columns: a.) BST Rutin 10 C_{18} Pep (250×4.6 mm) b.) Beckman Ultrasphere ODS 5μ (250×4 mm) c.) MOS Hypersil C_8 RP 5 μm (250×4 mm). The other conditions were the same. Eluent: acetonitrile: 0.02M ammonium acetate buffer pH 4 22:78 (v/v). Injection volume: 20μL. Detection: 254 nm. Flow rate: 1 mL/min. Peaks: 1. lucidin primveroside, 2. ruberythric acid.

c.) Pharmacia LKB-HPLC system was used sometimes with Superpac PEP S C_2/C_{18} RP 5 μm, 250×4 mm (Pharmacia LKB, Uppsala, Sweden) column in the eluent systems above.

Table 1

TLC R_F Values of Rubia Components

Compounds	R_F Values		
	S1	S2	S3
anthraquinone	0.95	0.80	0.84
2-hydroxy-anthraquinone	0.84	0.75	0.87
anthraquinone-1-methylether	0.91	0.82	0.80
alizarin	0.82	---	0.77
alizarin-2-methylether	0.87	0.87	0.81
anthragallol	0.65	---	---
lucidin	0.89	0.80	0.66
purpurin	0.78	---	---

S1: -eluent: benzene:ethanol (80:20); Kieselgel 60 (Merck)
S2: -eluent: benzene:ethanol (80:20; Kieselgel 60 (Merck) desact.
S3: -eluent: benzene:ethyl acetate-methanol (40:30:5);[36,37] Kieselgel G (Merck)

PREPARATIVE PURIFICATION

5 g of lyophilized, fermented, washed bio-mass was treated with methanol in Soxhlet-extractor. The extract was evaporated in vacuo to dryness. The extraction-step was repeated with dichlorometane. The material was 0.16 g after removal of the dichlorometane and the residue (1.61 g) was extracted with water. The water solutions were lyophilized (or evaporated). 0.30 g of crude product was dissolved in 10 mL of water and applied into a column (42×2 cm) of Dowex 50 (H^+ form). After washing (H_2O) the column was eluted with methanol. 20 mg substance was obtained. It was fractionized further by GPC on Sephadex LH-20 column (65×2 cm) with methanol (Fig. 3). Four chief fractions with different colour bands were collected and evaporated in vacuo to dryness, respectively. The eluates were analyzed by HPLC.

RESULTS AND DISCUSSION

The analysis has been started with TLC studies of Rubia extracts (Table 1). The efficiency of TLC separations were not enough for our studies with Rubia extracts because of great number of components.

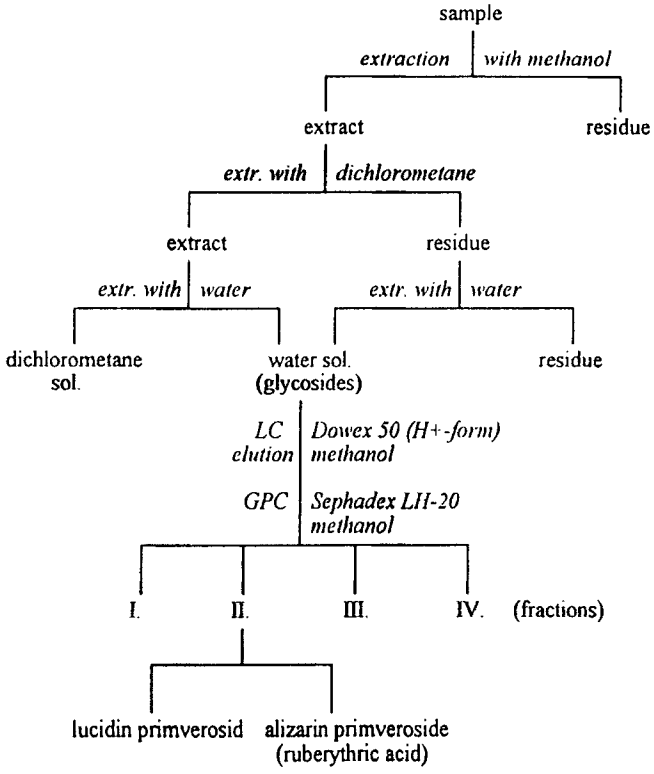


Figure 3. Flow chart. Extractions and column liquid chromatography for isolation of anthraquinones and their glycosides.

The analysis of the intact roots and the cell suspension culture was developed, and then carried out in our laboratory by HPLC. After optimisation alizarin, lucidin, purpurin, anthraquinones, their derivatives, and ruberythric acid were detected in the intact roots as well as in the cell suspension culture (Table 2). The pharmacological products (as Cystenal®) show similar chromatographic pattern, as previous ones.

We have developed a gradient HPLC method for „finger-print" chromatographic analysis of different Rubia extracts and cultures. This system is efficient for measurement of both anthraquinone aglycones and its glycosides, too (Fig. 4). At first, isocratic methods were applied for the extraction and purification of alizarin from Rubia tinctorum plant cells.

Table 2

(HPLC) Chromatographic Data of Rubia Components

Compound	Gradient System, t_R (retention time) min	Isocratic System, t_R min	k' (t_0=2.1 min)	eluent I:II (v/v)
anthraquinone	28.2	26.0	11.38	43:57
2-hydroxy-anthraquinone	20.1	9.8	3.67	43:57
1-hydroxy-2-methyl-anthraquinone	37.1	23.4	10.14	57:43
alizarin	20.4	10.5	4.00	43:57
alizarin-2-methylether	26.9	24.0	10.38	43:57
anthragallol	16.7	6.4	2.05	43:57
lucidin	18.1	7.7	2.67	43:57
purpurin	25.6	18.2	7.67	43:57
ruberythric acid	8.1	14.1	5.70	22:78
lucidin primveroside	7.7	13.1	5.24	22:78

Eluent: I: acetonitrile II: 0.02 M ammonium acetate buffer
Column: Beckman Ultrasphere ODS 5μm (250×4 mm)
Flow rate: 1 mL/min

For clean up of the samples, Soxhlet-extraction and solid phase extraction were applied. Isolation and subsequent purification using solid-phase extraction (SPE) with C_8 cartridges, following HPLC analysis and UV detection, provided a fast, sensitive and easy method for the determination of free alizarin.

Our results show that the pigments of Rubia tinctorum could be produced in large scale plant cell culture for industrial purposes. According to the analytical results, it is possible to optimize the production toward alizarin or ruberythric acid.

Acetic acid or acidic buffer (phosphate, acetate pH 4-5) in the mobile phase, affected the peak shape of the anthraquinones, the strongest effect being with alizarin. The washing and elution process with SPE removed most of the impurities having short or long retention times, lowering the detection limit in the HPLC analysis. It is possible, that some of the impurities removed by SPE and unknown components in the purified eluate, are other anthraquinones (free

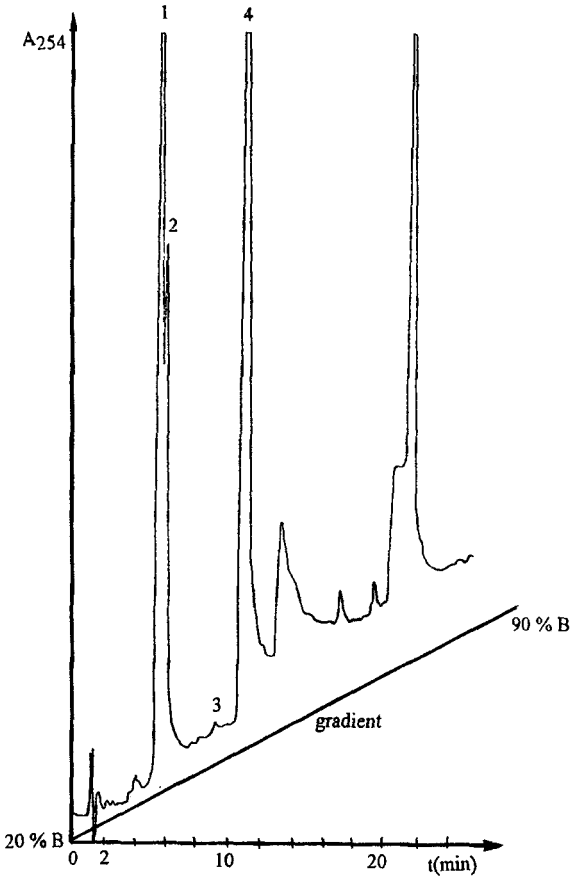


Figure 4. Chromatographic pattern of Rubia tinctorum root extract (with methanol). Column: Superpac PEP S C_2/C_{18} RP 5μm (250×4 mm). Eluent: A: acetonitrile : 0.02 M ammonium acetate buffer pH 4 15:85, B: acetonitrile : 0.02M ammonium acetate buffer pH 4 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. lucidin primveroside, 2. ruberythric acid, 3. lucidin, 4. alizarin.

or glycosidic), as they are known to be synthesized in R. tinctorum.[4] Stepwise development of the SPE cartridges seems to be useful for the purification of other anthraquinones, but if the cartridge size or sample type is changed then the SPE process should be optimized again.

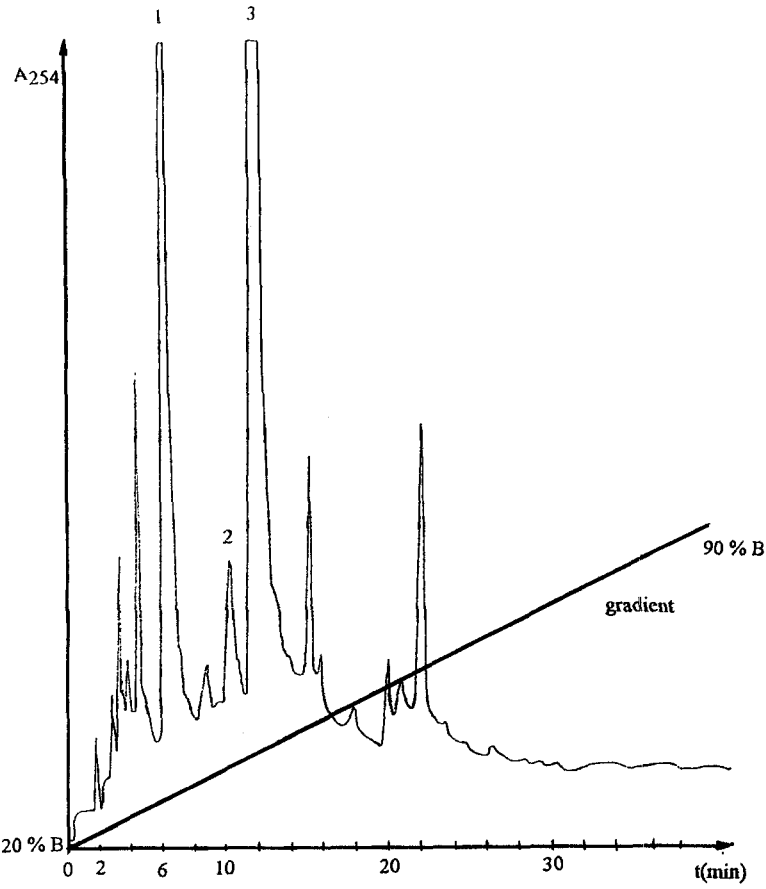


Figure 5. Chromatographic pattern of extract (with methanol) of Rubia tinctorum cell suspension culture (25/2). Column: Superpac PEP S C_2/C_{18} RP 5 μm (250×4 mm). Eluent: A: acetonitrile: 0.02 M ammonium acetate buffer pH 4 15:85, B: acetonitrile: 0.02 M ammonium acetate buffer pH 4, 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. ruberythric acid, 2. lucidin, 3. alizarin.

This isocratic method is useful for studies with alizarin and other anthraquinones produced by R. tinctorum and, should also, facilitate the further isolation, identification and determination of their glycosides, too. Non-glycosidic forms of anthraquinone derivatives can be used as marker molecules in further studies of anthraquinone biosynthesis in cultured R. tinctorum, e.g.,

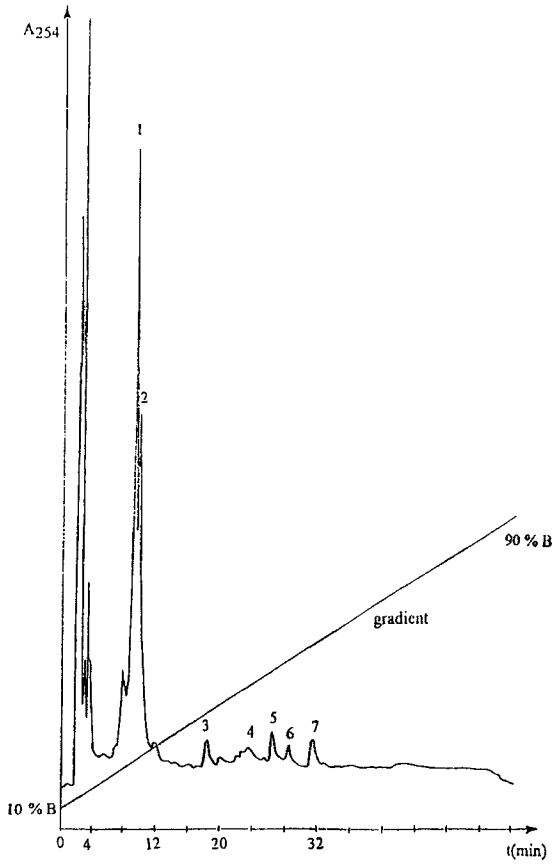


Figure 6. Chromatographic pattern of extract (with methanol) of Rubia tinctorum cell suspension culture (RF 413). Column: MOS-Hypersil C_8 RP 6μm (250×4 mm). Eluent: A: acetonitrile: 0.02 M ammonium acetate buffer pH 4, 15:85, B: acetonitrile: 0.02 M ammonium acetate buffer pH 4 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.0 mL/min. Peaks: 1. lucidin primveroside, 2. ruberythric acid, 3.-7. hyroxy anthraquinones.

alizarin, produced in cell cultures, can be used as an indicator for the production of the anthraquinone metabolites in cultured Rubia cells. For chromatographic identification of the peaks the standard additions method was used at different chromatographic conditions.

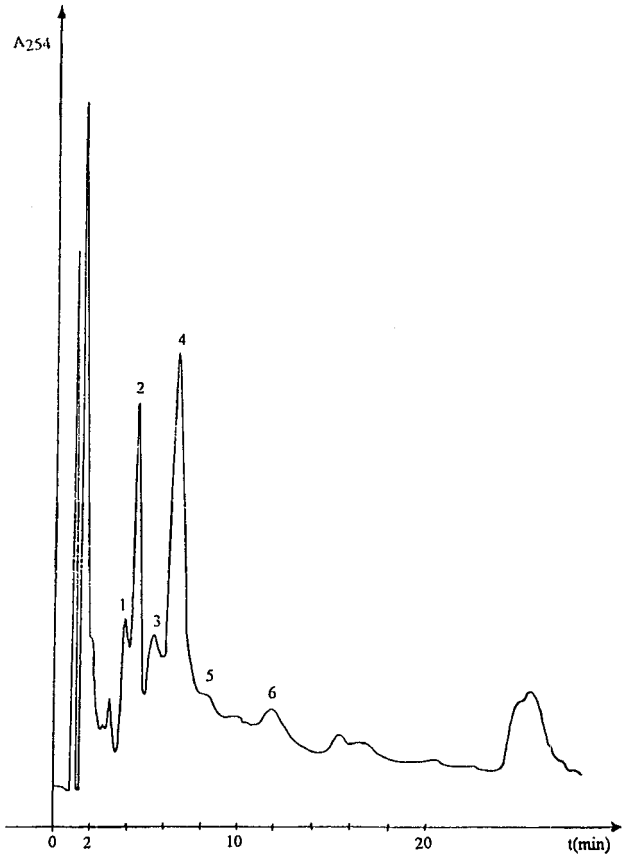


Figure 7. Chromatographic pattern of supercritical fluid extract of Rubia tinctorum cell suspension culture (84). Column: Superpac PEP S C_2/C_{18} RP 5 μm (250×4 mm). Eluent: acetonitrile:0.02 M ammonium acetate buffer pH 4, 43:57 (v/v). Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. lucidin, 2. anthragallol, 3. alizarin, 4. 2-hydroxy-anthraquinone, 5. alizarin-2-methylether, 6. anthraquinone.

The results from the extraction of alizarin with ethanol at 80°C at different times, indicate that incubation for 10 h was needed to achieve the maximum recovery for cultured plant cell suspension material. The typical free alizarin content was 2 mg/g in dry Rubia tinctorum material, with a range of 0.4-4 mg/g depending on the culture.

Linear calibration graphs (based on the peak heights in mm) with good correlation ($r^2 > 0.999$), were obtained for alizarin (range 78-10 000 ng/mL) and anthraquinone (156-20 000 ng/mL), the first value of the range showing the detection limit at a signal-to-noise ratio of 3.

The precision of the whole assay was 1.5% (from identical plant cell culture samples) and the recovery from the SPE step was estimated to be more than 99% (n=3) for alizarin.

Our optimized HPLC measurements were applied for

- qualitative characterization („finger print") of extracts (Fig. 4-7),
- identification of components in extracts (Fig. 4-7),
- quantitative determinations of chief components (as alizarin, lucidin, ruberythric acid etc.) (Table 3),
- comparison of natural plant and cell cultures (Fig. 4-6),
- indication of anthraquinones biosynthesis via their measurements,
- structure determination of components in extracts,
- semipreparative and preparative fractionation, separation and purification of components,
- control of pharmaceutical products containing extracts,
- checking the purity of authentic standard samples,
- characterization of commercial ruberythric acid,
- qualitative comparison of different fractionation methods (as Soxhlet, LC, SFE etc.) (Fig. 4-7) and
- purity control of chemically synthesized anthraquinone products.

Preparative HPLC was performed on RP C_{18} preparative columns; methanol or acetonitrile, ammonium acetate buffer (pH 4)/water gradient system. Fractions were collected, checked by anal. HPLC, evaporated or lyophilized, then crystallized (e.g. from hot water to yield ruberythric acid). The structure of isolated compound was identified by UV, IR, NMR and MS data.

Table 3

Anthraquinone Constituents (content %)

	Alizarin	Lucidin	Ruberythric Acid	Lucidin Primveroside
madder root	0.15 - 0.17	$0.2x10^{-3}$ -$9.0x10^{-3}$	4 -5	2 - 3
cell culture homogenates	0.03 - 0.19	$0.9x10^{-3}$ -$9.6x10^{-3}$	3.8 - 5	0.08 - 0.1

After preparative GPC fractionation (on Sephadex LH-20 column) we have found, that the first fraction contains more compounds, second fraction has two, very similar ones (very well crystallizable yellow needles) (Fig. 3), the chief part of third fraction was found to be alizarin, the fourth fraction contains purpurin .

For structure determination the fraction II. was hydrolyzed with dilute (5%) hydrochloric acid: glucose and xylose (HPLC identification: Polygosil 60-10NH_2 column; eluent: acetonitrile-water 82.5:17.5 (v/v); flow rate: 1.5 mL/min; det.: RI) were obtained in addition to alizarin and lucidin. The MS, IR, UV data were in good agreement with those of ruberythric acid.

Suzuki and coworkers[24] isolated from their homogenate on very similar manner lucidin-ethylether, pseudopurpurin, alizarin, purpurin and ruberythric acid. Data of this ruberythric acid was identical with those of authentic ruberythric acid isolated from roots by the method of Hill and Richter[27] based on lead complex precipitation.

In order to elucidate the biogenesis of anthraquinones in madder, Burnett and Thompson[4] isolated nineteen anthraquinones, all substituted in one benzenoid ring only, from mature plants of R. tinctorum by extraction with solvent-series of different polarity.

Their method was applied for fractionation of cell culture extract, too. The comparison of fractions could be checked by HPLC.

In order to replace traditional solvent extraction method such as Soxhlet one, nowadays there is a better way: supercritical fluid extraction using carbon dioxide. Madder root and cell culture homogenates were treated in a preliminary experiment with CO_2 according to SFE. SFE experiments were performed by B. Simándy, Technical University, Budapest. The extracts were studied by HPLC. It was found, that the chromatographic patterns have

changed: carbon dioxide guaranted a substantial extraction (yield 6.7 %) for apolar compounds, as aglycones (2-hydroxy-anthraquinone, anthragallol etc) (Fig. 7).

We have found, that the authentic ruberythric acid (depur, produced from Carl Roth GmbH & Co. (Karlsruhe, FRG) contains two substances. Westendorf found, similarly, that it contained, in addition to alizarin primveroside, substantial amounts of lucidin primveroside too.

The anthraquinones were identified qualitatively and quantitatively on the basis of cochromatography with authentic compounds and comparison of their hydrolysis products.

Rubia Teep® was also analysed by HPLC and the glycosides ruberythric acid and lucidin primeveroside, as well as the free aglycons alizarin and lucidin, were observed. Small amount of lucidin was detected (present in free form and as the glycoside derivative) in each tablet of Rubia Teep®. Its HPLC pattern demonstrates two peeks: one is ruberythric acid, the second was identified as lucidinprimveroside.

These two compounds were found in our root extracts and cultured cells too. Their purity were characterized by HPLC, TLC and ^{1}H-NMR spectroscopy.

Our results demonstrated very well the sensitivity and efficiency of HPLC methods for chemical characterization of madder root and its cell cultures.

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