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Short Communication

Isolation and determination of alizarin in cell cultures of *Rubia tinctorum* and emodin in *Dermocybe sanguinea* using solid-phase extraction and high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method was developed for the determination of the non-glycosidic anthraquinones alizarin (1,2-dihydroxy-9,10-anthracenedione), emodin (1,3,8-trihydroxy-6-methyl-9,10-anthracenedione) and anthraquinone (9,10-anthracenedione). The anthraquinones were separated by isocratic elution on a 125 × 4.6 mm I.D. column containing ODS Hypersi15 reversed-phase material using methanol-5% acetic acid (pH 3.0) (70:30) as the mobile phase. Free alizarin was determined in plant cell suspension cultures of *Rubia tinctorum* and free emodin in mushrooms *(Dermocybe sanguinea).* The effective extraction of anthraquinones from plant cells was achieved with 80% (v/v) ethanol after incubation for 10 h at 80°C. Prepurification and concentration of anthraquinones in the plant cell and mushroom extracts were effected by a solid-phase technique using C_8 cartridges.

INTRODUCTION

Rubia tinctorum, the source of a natural dye, produces anthraquinone pigments in the roots and also in the cultured cells, one of them being alizarin [1]. The herbal drugs consisting of crude ***Rubia*** extracts have the activity of dissolving bladder and kidney stones. It has been shown that ***R. 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 1-hydroxyanthraquinone [2]. Alizarin, produced in cell cultures, can be used as an indicator for the production of the anthraquinone metabolites in cultured ***Rubia*** cells.

Dermocybe sanguinea, a wild mushroom also used as a natural dye, contains several anthraquinone pigments, the most important being emodin [3]. It was shown previously that ***D. sanguinea*** extracts are genotoxic, which is only partially ex-

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plained by emodin [4]. Further research on other anthraquinones and genotoxic compounds of ***D. sanguinea,*** as mushrooms or cultured cells, needs more sophisticated HPLC methods for isolation, identification and determination.

Many methods have been reported for the separation of the naturally occurring free anthraquinone aglycones. These have been based on paper chromatography [5], thin-layer chromatography (see, for example, refs. 6 and 7), low-pressure column chromatography [8] and high-performance liquid chromatography (HPLC) (see, for example, refs. 4 and 9-l 5). 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 [14]. Column purification, however, was practical and C_{18} cartridges proved to be effective in the HPLC determination of anthraquinone in pulping. liquors [12].

In this paper we describe an isocratic HPLC method for the extraction and purification of alizarin from ***R. tinctorum*** plant cells and emodin from ***D. sanguinea*** mushroom cells. 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 and emodin.

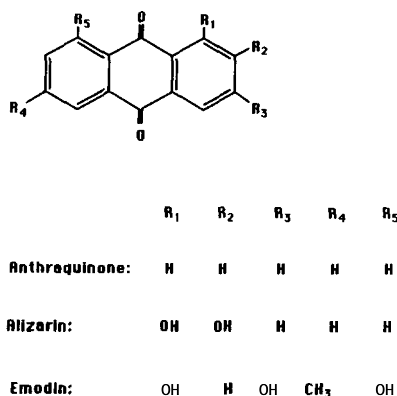
EXPERIMENTAL

Chemicals

Alizarin (1,2-dihydroxy-9, IO-anthracenedione) and anthraquinone (9, IO-anthracenedione) were purchased from Reanal (Budapest, Hungary), emodin (1,3,8-trihydroxy-6-methyl-9,10-antracenedione) from Aldrich Chemie (Steinheim, Germany), ethanol from ALKO (Helsinki, Finland), methanol of HPLC grade from Rathburn (Walkerburn, UK) and acetic acid of analytical-reagent grade from Merck (Darmstadt, Germany). Water was purified with Millipore Milli-Q UF Plus equipment. The structures of the anthraquinones studied are shown in Fig. 1.

Plant and mushroom material

Rubia tinctorum L. cell suspension cultures, grown in a basic Murashige and Skoog medium containing indoleacetic acid (IAA) (1 .O mg/l), naphthaleneacetic acid (NAA) (0.1 mg/l) and kinetin (0.2 mg/l) (cultivated by Dr. J. Kretovics, ELTE University, Budapest, Hungary), were used for the analysis. Specimens of ***Dermocybe sanguinea*** (identified by H. Heikkilä, Department of Natural History, Museum of Kuopio, Kuopio, Finland) were collected from the Kuopio area in Eastern Finland. The mushrooms were extracted as fresh or dried samples or they were frozen within 8 h of collection and stored at − 20°C until extraction.



	R1	R2	R3	R4	R5
Anthraquinone:	H	H	H	H	H
Alizarin:	OH	OH	H	H	H
Emodin:	OH	H	OH	CH3	OH

Fig. 1. Structures of alizarin, anthraquinone and emodin.

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 [16], 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.

D. sanguinea was extracted with 94% (v/v) ethanol for 48 h at room temperature, the filtered extract was concentrated with a rotary evaporator and the residue was acidified [4]. The precipitate which was formed after acidification was used, in addition to the ethanol extract, as the crude extract of ***D. sanguinea*** for this study.

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 three anthraquinones was eluted from the tube with 1 ml of methanol-water (80:20,v/v).

HPLC of the extracts

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 D-2000 chromate-integrator. The ratio threshold was set at 2%. The components were separated on an ODS Hypersil (5-μm particle size) reversed-phase column (125 mm × 4.0 mm I.D.) (Bischoff Chromatography, Leonberg, Germany). 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.

RESULTS AND DISCUSSION

The results from the extraction 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 (Fig. 2). The typical free alizarin content was 2 mg/g in dry *R. tinctorum* ma-

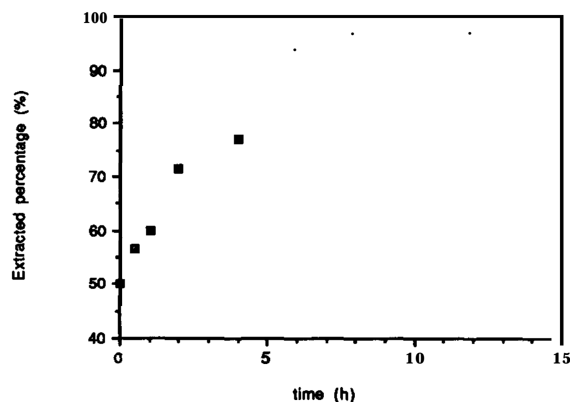


Fig. 2. Effect of incubation time on the extraction of alizarin from ***Rubia tinctorum*** L. cells at 80°C with ethanol-water (80:20, v/v).

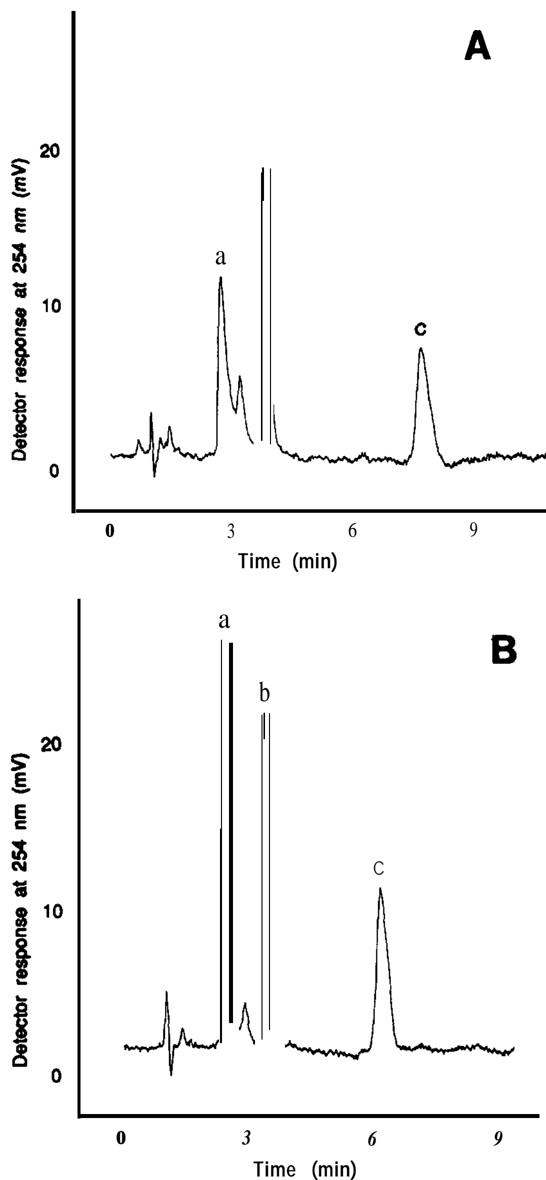


Fig. 3. Isocratic HPLC separation of a standard solution containing 3.3 μg/ml each of (a) alizarin, (b) anthraquinone and (c) emodin on a Hypersil ODS column. The mobile phase was (A) methanol-water (70:30) (pH 7.0) or(B) methanol-5% acetic acid (70:30) pH 3.0.

terial, with a range of 0.4–4 mg/g depending on the culture. *D. sanguinea* contained emodin at about 1.4 mg/g dry mass and 0.3 mg/g fresh mass, with the content depending on the location and age of the mushroom.

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), anthraquinone (156-20 000 ng/ml) and emodin (312-10 000 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 six identical plant cell culture samples) and the recovery from the SPE step was estimated to be more than 99% (n = 3) for alizarin; however the recovery of emodin was about 78%. When methanol-5% acetic acid (90: 10, v/v) was used for elution, the recovery was increased to 95%, including emodin. This was more effective than elution with 100% methanol (data not shown).

Acetic acid in the mobile phase affected the peak shape of the anthraquinones, the strongest effect being with alizarin (Fig. 3). The use of acetic acid also shortened the retention time of emodin, but only a slight effect was seen with the other compounds (Fig. 3). In the standard alizarin a small peak due to some degradation product, an isomer or impurity, was seen, the relative amount of which seemed to decrease when acetic acid was used (Fig. 3).

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 (Figs. 4 and 5). It is possible that some of the impurities removed by SPE and unknown components in the purified eluate are other anthraquinones (free or glycosidic), as they are known to be synthesized in both *R. tinctorum* and *D. sanguinea* [1,3]. A higher concentration of methanol (60%, v/v) in water was needed to remove the impurities with short retention times found in the

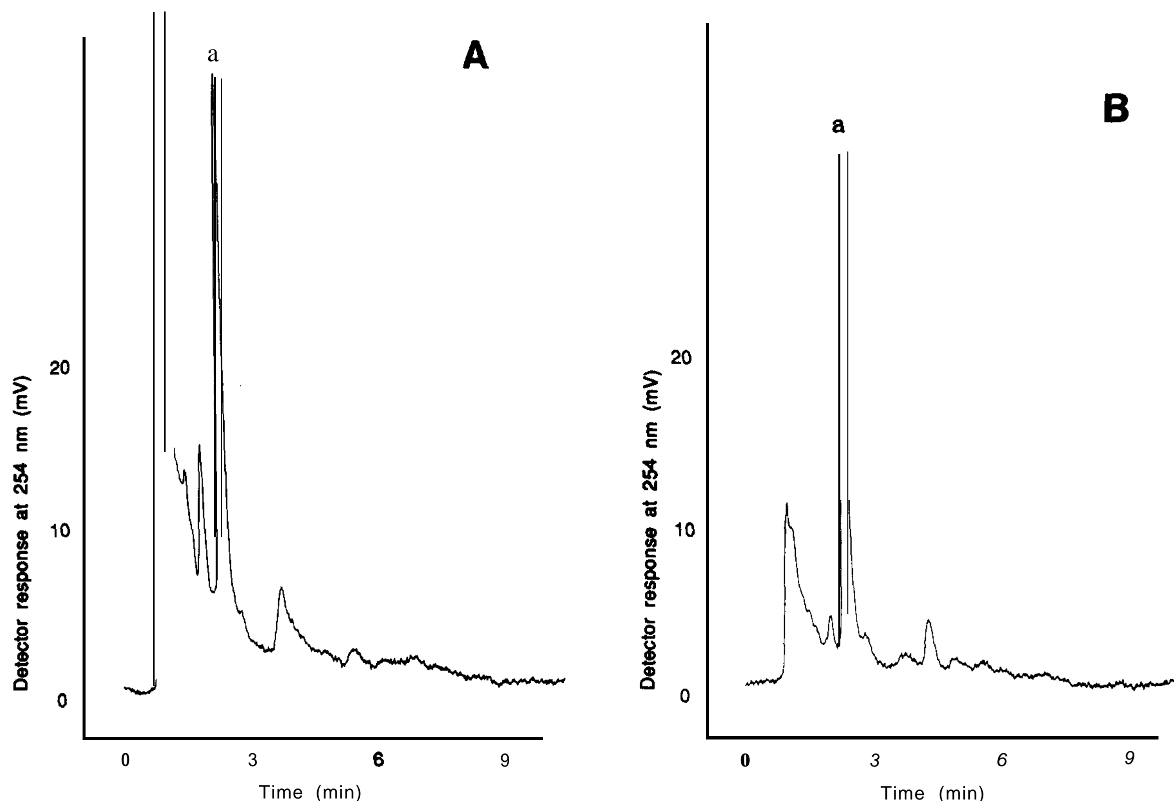


Fig. 4. HPLC of (a) alizarin (A) in a crude extract of *R. tinctorum* L. cell suspension culture and (B) after C_8 SPE purification. Alizarin concentration, 4.0 μg/ml.

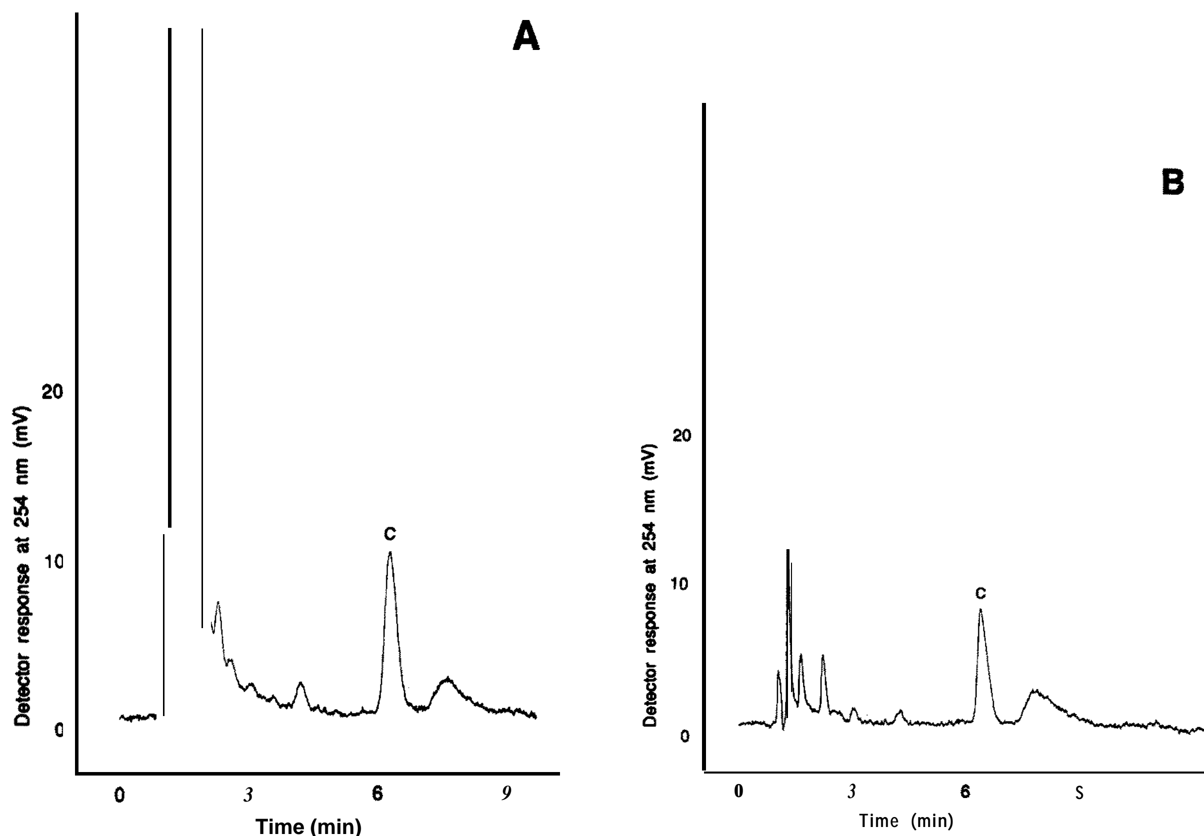


Fig. 5. HPLC of (c) emodin, (A) in a crude extract of *D. sanguinea* and (B) after C_8 SPE purification. Emodin concentration, 3.0 μg/ml.

ethanol extracts of dried mushrooms. Stepwise development of the SPE cartridges seems to be useful for the purification of other anthraquinones in mushrooms, but if the cartridge size or sample type is changed then the SPE process should be optimized again.

This isocratic HPLC technique 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 emodin and other anthraquinone components of cultured ***D. sanguinea*** mushrooms. Further studies are needed to identify the glycosidic and other anthraquinones of ***Dermocybe*** species. The combination of SPE, HPLC assay and hydrolysis of the glycosides makes it possible to separate the two forms of anthraquinones present in plant or mushroom material.

Non-glycosidic forms of alizarin and emodin can be used as marker molecules in further studies of anthraquinone biosynthesis in cultured ***R. tinctorum*** and ***D. sanguinea,*** respectively.

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