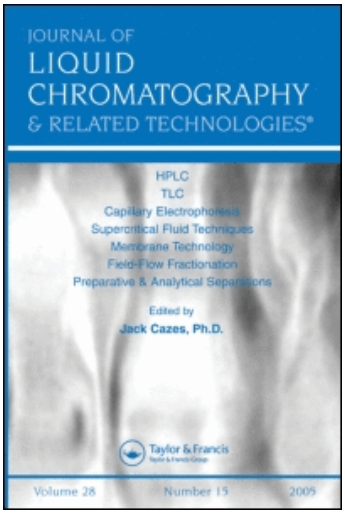

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A. C. J. Hermans-Lokkerbol[a]; R. Van Der Heijden[a]; R. Verpoorte[a]
[a] Division of Pharmacognosy Center for Bio-Pharmaceutical Sciences Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

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SOLVENT SYSTEM SELECTION FOR SEPARATION OF ANTHRAQUINONES BY MEANS OF CENTRIFUGAL PARTITION CHROMATOGRAPHY; APPLICATION TO AN EXTRACT OF A *RUBIA TINCTORUM* HAIRY ROOT CULTURE

A. C. J. HERMANS-LOKKERBOL*, R. VAN DER HEIJDEN, AND R. VERPOORTE
Division of Pharmacognosy
Center for Bio-Pharmaceutical Sciences
Gorlaeus Laboratories
Leiden University, P. O. Box 9502
2300 RA Leiden, The Netherlands

ABSTRACT

Two two-phase systems were selected for the separation of anthraquinones by means of centrifugal partition chromatography, using alizarin, purpurin and quinizarin as test substances. These systems were applied to the separation of anthraquinones, extracted from a *Rubia tinctorum* hairy root culture. Some of the anthraquinones present were identified: alizarin, alizarin 1-methyl ether, lucidin and nordamnacanthal.

*To whom correspondence should be addressed

INTRODUCTION

For the isolation of natural products liquid-liquid partition chromatography has since long been valued; the advantages are extensively described in literature [1 and references cited therein]. One of the older techniques, using liquid-liquid partition, is droplet counter-current chromatography (DCCC), introduced in 1970 [2]. A stationary phase is immobilized in vertically placed columns, which are interconnected by capillaries. For the transport of the mobile phase through these capillaries the driving force of a pump is needed. Gravity is however the driving force for the movement of the mobile phase through the stationary phase and for the immobilization of the stationary phase in the columns. In centrifugal partition chromatography (CPC) a centrifugal force retains the stationary phase; the separation columns are horizontal channels engraved in polychlorotrifluoroethylene plates, and the longitudinal axe of the channels is parallel to the centrifugal force. This technique, first described in 1982 by Murayama *et al.* [3], has been studied in detail by Berthod *et al.*[4-9]. One of the interesting options of CPC is mode reversion: after a certain time of elution the mode in which the CPC-apparatus is run is changed; this is done by reversing the direction of the flow and interchanging mobile phase and stationary phase. As a consequence of this, compounds, which were strongly retained in the first mode, are eluted shortly after the mode reversion. In this way extreme long chromatographic runs can be avoided.

An extensive inventory of physical-chemical parameters, concerning liquid-liquid separation techniques is given by Miething and Rauwald [10]; data are given of polarity, interfacial tension (γ), viscosity (η), density (ρ) and composition of each of the phases for 2-phase systems, containing chloroform, MeOH and water in several ratios. Important physical parameters for a good CPC-performance are γ, η, and difference in density of the two phases ($\Delta\rho$). The pressure in the CPC-apparatus is limited to 60 bar. The pressure is proportional to $\Delta\rho$ and to the square of the angular speed (ω). So a low $\Delta\rho$ results in a low backpressure and thus allows a higher rotation speed of the centrifuge, which improves the efficiency [5].

Low viscosity enables fast mass transfer of the solutes within the phases, which improves the efficiency. A low value of γ enables fast mass transfer of the solutes between the two phases and a high separation capacity, but can also be the cause of flooding. In that case the two phases form an emulsion within the channels and small

drops of stationary phase are eluted together with the mobile phase. This results not only in a noisy detector signal but also in a considerable loss of efficiency, capacity and reproducibility of the chromatographic system because of the loss of stationary phase. The phenomenon can appear in both descending and ascending mode, but sometimes it occurs in only one of both. Diminishing the flow rate and increasing the rotation speed of the centrifuge can stop the flooding. It should be noted that an increase of rotation speed is restricted by the backpressure of the system ($P \sim \omega^2$). Temporary flooding can occur during the elution of a high concentration of a solute, which lowers the interfacial tension below the critical value for a good performance. Foucault and Nakanishii had the same experience during a CPC-separation of proteins [11]. It also happened during the elution of the test solutes in one of our systems.

We used CPC for the analysis of the anthraquinone-content of a *Rubia tinctorum* hairy root culture [12]. Many plants of the family Rubiaceae are known to contain substantial amounts of anthraquinones [13]. Naturally occurring anthraquinones are used as dyes. Madder, *Rubia tinctorum* L., is well known for this purpose; the main product is alizarin. Also cell and tissue cultures of plants in the family Rubiaceae can produce large amounts of anthraquinones; in some cases the percentages even exceed those of the parent plant [14].

For the analysis and separation of the anthraquinone derivatives by means of the CPC-apparatus a 2-phase system had to be selected. The choice of such a system was done by using alizarin (1,2-dihydroxy-9,10-anthraquinone), purpurin (1,2,4-trihydroxy-9,10-anthraquinone) and quinizarin (1,4-dihydroxy-9,10-anthraquinone) as model compounds. For the separation of the anthraquinones several 2-phase systems are described in literature, but none of them for CPC. The system hexane - EtOAc - EtOH - water 5:2:4:1 has been described by Inoue *et al.* [15] for anthraquinone separation by DCCC. Zhang *et al.* [16] used a high speed counter-current chromatograph and the system n-hexane - EtOAc - MeOH - water 9:1:5:5 for separation of anthraquinones. Both systems were tested for CPC-performance as well as several other systems containing hexane, water, MeCN or MeOH and an organic modifier (THF, EtOAc or $CHCl_3$). Selection of the $CHCl_3$ - MeOH- water systems used, was guided by data of the physical-chemical parameters obtained from Miething and Rauwald [10].

In plants anthraquinones can occur as such or in the form of glycosides. The samples which were analyzed consisted of the aglycones, the glycosides together with the aglycones, or the aglycones obtained after hydrolysis of the glycosides.

The identification of all anthraquinones present in the *R.tinctorum* hairy root extract was not the aim of this study; nevertheless some of these compounds were identified.

MATERIALS AND METHODS

Apparatus

A modular Sanki (Kyoto, Japan) Centrifugal Partition Chromatograph (type LLN) was used for all experiments. It consists of a power supply, model SPL, a centrifuge model NMF, a loop sample injector plus flow director model FCU–V, equipped with a 3.4 ml loop, and a triple head constant flow pump, model LBP–V. The centrifuge is equipped with 12 cartridges; in the experiments described here only 6 were used, which contain in total 2400 channels with a total volume of 125 ml. To a UVIS 200 detector (Linear Instruments Corporation, Reno, NV, USA) a Panasonic Pen-recorder model VP–6722A was connected. Fractions were collected by means of a LKB (Bromma, Sweden) 17000 minirac fractioncollector.

Chemicals

Alizarin was obtained from Sigma (St. Louis, MO, USA) and purpurin from Aldrich (Milwaukee, Wis, USA). Quinizarin was a contamination in the purpurin. Chloroform, toluene, MeCN, MeOH, EtOAc and EtOH were distilled before use. Acetic acid, formic acid and THF were p.a. quality (Baker, Deventer, The Netherlands) and hexane (mixed isomers) was Baker grade. Whenever n-hexane (Baker 'Analyzed') was used this is indicated in the text.

For the TLC-screening of the CPC-fractions 20 x 20 cm 0.25 mm silicagel 60 F_{254} glass plates (Merck, Darmstadt, Germany) were used. For preparative TLC home made plates, silicagel 60 PF_{254} (Merck) 0.5 mm layer were used; they were washed respectively with MeOH-25% NH_4OH 100:1, with MeOH and finally with chloroform before use.

Solvent systems

CPC–solvent systems were prepared by mixing the solvents thoroughly at room temperature for 1 h; the two phases were separated before use, leaving some ml of the lower layer in the upper layer and vice versa, guaranteeing saturation.
System C1 was composed of hexane – MeOH – water 100:99:1. In descending mode, with a centrifugal speed of 1200 rpm and a flow rate of 4.6 ml/min, the pressure was 38 bar and V_s, the volume of the stationary phase in the cartridges, was 75 ml. In ascending mode (1200 rpm; 4.5 ml/min) the pressure was 36 bar and V_s 74 ml.

System C2, as used for the analysis of the *R. tinctorum* hairy root extract, consisted of n-hexane – EtOAc – MeOH – water 9:1:5:5. In ascending mode, with centrifugal speed of 700 rpm and flow rate 4.6 ml/min, the pressure was 45 bar and V_s 83.5 ml. After reversion of mode,at a flow rate of 4.6 ml/min and 1000 rpm the pressure was 40 bar. The volume of the new stationary phase in the cartridges was circa 45 ml.

System C3, as used for the analysis of the *R. tinctorum* hairy root extract, consisted of chloroform - MeOH - water - acetic acid 5:6:4:0.05. In ascending mode (centrifugal speed 700 rpm, flow rate 3.1 ml/min) the pressure was 51 bar and V_s 61 ml. After reversion of mode, with the same flow and centrifugal speed, the pressure was 50 bar.

TLC solvent systems used, with hR_F-values of alizarin (A), purpurin (P)and quinizarin (Q):

	A	P	Q
S1: $CHCl_3$ - MeOH 9:1	58	26	81
S2: $CHCl_3$ - MeOH 3:1			
S3: $CHCl_3$ - MeOH 1:9	70 (t)[1]	68(t)	81 (t)
S4: $CHCl_3$ - MeOH - 25% NH_4OH 85:14:1	24 (t)	02	82
S5: hexane - EtOAc 1:1	27	06	50 (t)
S6: hexane - EtOAc 1:2		21	85
S7: *toluene* - EtOAc - HCOOH 75:24:1	49	40	69
S8: toluene - MeOH 9:1	36 (t)	15 (t)	75

[1] (t) = tailing

All hR_F-values given are a result of development in a saturated chamber. Nevertheless there were distinct differences between hR_F-values of the same compound, determined on several occasions. Though the hR_F-values of the testcompounds were determined on several days, for each solvent system they were assessed on the same plate. The hR_F-values, as given for alizarin 1-methyl ether,

lucidin and nordamnacanthal were determined on the same day and -for each solvent system- on the same plate.

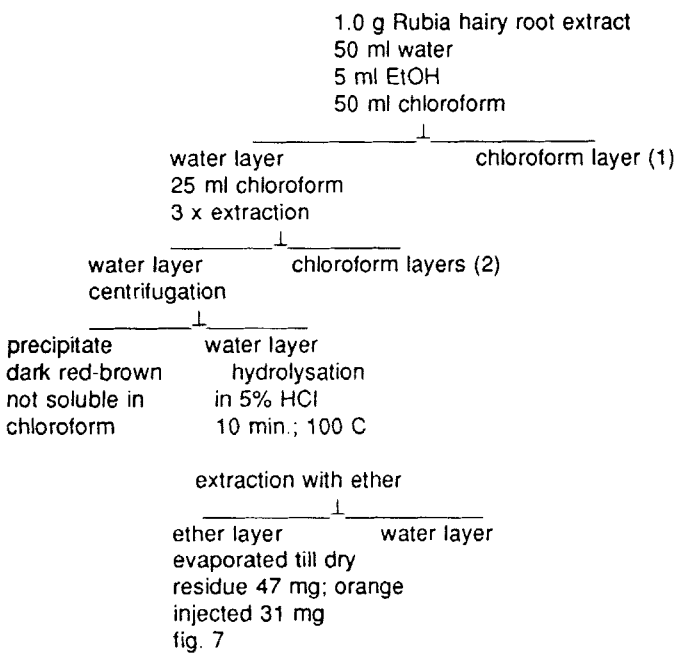
Preparation of *R. tinctorum* hairy root extract

Hairy root cultures were initiated and subcultured as described in [12]. At the end of the growth cycle biomass and medium were separated. Biomass was stored at -18°C. 111 g of biomass was extracted twice with EtOH 96% (overnight, continuous stirring at 4°C). After filtration, the extract was concentrated under reduced pressure. Yield 2.4 g.

Sample preparation

The injected sample for system C2 (see experiment fig. 5) was prepared by suspending 200 mg of the *R. tinctorum* hairy root extract in 4 ml water and extracting twice with 4 ml chloroform. The collected chloroform extracts were evaporated until dryness and taken up in 4 ml lower + 2 ml upper phase. An aliquot of 3.4 ml (consisting of both layers) was injected.

The samples for the experiments of figures 6 and 7 (also CPC solvent system C2) were prepared according to the following scheme.



The combined chloroform layers (1 and 2) were concentrated to dryness; brown-yellow residue; 61 mg. Of this circa 25 mg was injected (see fig. 6)

The injection sample for system C3 (see experiment fig. 8) was prepared by taking up 15 mg of the *R. tinctorum* hairy root extract in 1 ml stationary (lower) and 2.5 ml mobile (upper) phase; of this 3.4 ml was injected.

For the experiment of figure 9 part of the collected fractions 8-11 of fig 5, corresponding with the 49-77 ml eluate-fraction, were concentrated to dryness, taken up in 1.5 ml of the lower phase and injected in system C3. Injected amount circa 0.7 mg.

CPC- experiments

All experiments were performed using 6 cartridges.

For the experiments with system C1 the flow rate was 4.6 ml/min and centrifugal speed 1200 rpm. Experiments with system C2 were performed with flow rate 4.6 ml/min and centrifugal speed 700 rpm. The first 70 ml after the injection peak were collected in 3.5 ml fractions; after that the fraction size was 7 ml. After reversion of mode the flow rate was not changed; the centrifuge was set at 1000 rpm, resulting in a backpressure of 40 bar; 5 ml-fractions were collected. For the experiments with system C3 the conditions were: flow rate 3.1 ml/min, centrifugal speed 700 rpm, fraction-size 6 ml; after reversion of mode: 3 ml/min, 700 rpm and fraction-size 3 ml.

Fractions were screened on TLC (system S4, occasionally also S5); the results of the screening are given in the figures.

Identification of the anthraquinones

The numbers as appointed to compounds **1-10** in the experiments correspond with their R_F-sequence in TLC-system S4 (see fig. 4); similarity of these compounds, as eluted in the different experiments, was ascertained by means of co-TLC, using at least 3 solvent systems.

Alizarin (1,2-dihydroxy-9,10-anthraquinone), compound **8** was identified by NMR, MS, co-TLC with the authentic sample in S3, S4, S5, S7 and by the retention volume in the CPC-experiments.

Alizarin 1-methyl ether, compound **4** was identified by UV, MS and co-TLC with the reference-compound in S4, S5 and S7; hR_F-values respectively 32, 29 and 35.

Lucidin (1,3-dihydroxy-2-hydroxymethyl-9,10-anthraquinone), compound **7** was identified by UV, MS and co-TLC with the reference-compound in S4, S5 and S7; hR_F-values respectively 11, 9 and 26.

Nordamnacanthal (1,3-dihydroxy-2-carboxy-9,10-anthraquinone), compound **6** was identified by co-TLC with the reference-compound in S4, S5 and S7; hR_F-values respectively 25, 29 and 67. Nordamnacanthal proved to be the major product in *R. tinctorum* hairy root cultures [12]; identification by means of MS and NMR.

RESULTS AND DISCUSSION

For the separation of anthraquinones, several solvent systems were tested for their physical suitability for CPC and to study their selectivity for the separation of the test compounds alizarin, purpurin and quinizarin.

Inoue *et al.* [15] described the system hexane - EtOAc - EtOH - water 5:2:4:1 for the separation of anthraquinones by means of DCCC. By using this relatively non-polar system in CPC, quinizarin could be separated from alizarin and purpurin (not shown in figures). The latter two were poorly separated and eluted in tailing peaks. After the run a substantial amount of the anthraquinones still proved to be retained in the stationary phase. Moreover during the elution of alizarin and purpurin flooding occurred. This solvent system did not allow a good CPC-performance.

A non-polar system which did allow a good CPC performance (physically) was hexane - MeOH - water 100:99:1 (system C1); this system was used with high efficiency for 1-naphtol, DDT (2,2-p-chlorophenyl-1,1,1-trichloroethane) and DDD (1,1-p-chlorophenyl-2,2-dichloroethane) [5]. However, in descending mode alizarin and purpurin were hardly retained and eluted shortly after the solvent front as one peak (fig. 1a); the front part of the peak contained relatively more of the alizarin, indicating some resolution. This indicates that their partition coefficients are small (about 0.2). Quinizarin was eluted with baseline separation, having a partition coefficient of about 0.6 in this system. By starting in the ascending mode, the separation of alizarin and purperin could be improved by varying the elution time before mode reversion (fig. 1b-

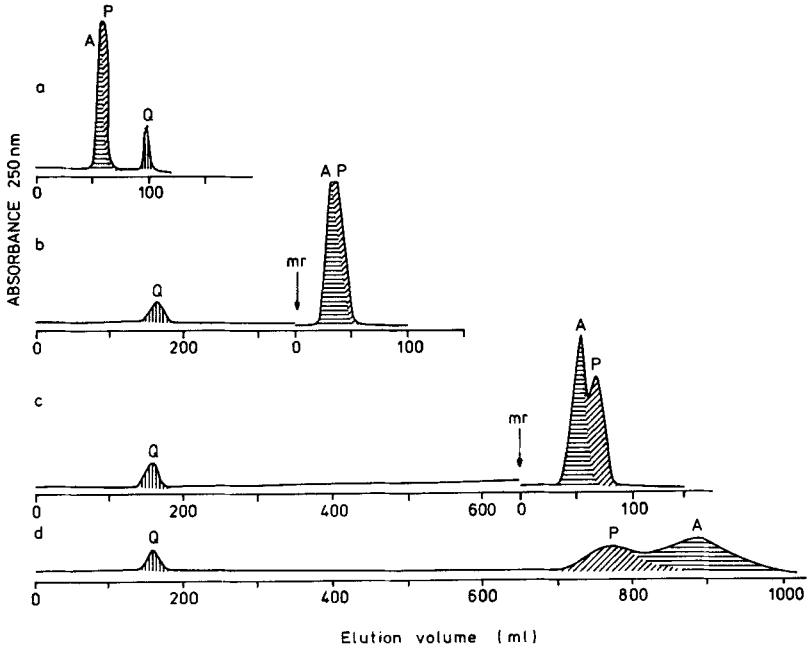


Figure 1: Optimization of CPC-separation of alizarin (A), purpurin (P) and quinizarin (Q) using the mode reversion option. Solvent system C1 (hexane - MeOH - water 100:99:1) flow rate 4.6 ml/min, centrifugal speed 1200 rpm.

1a: descending mode
1b: ascending mode and mode reversion after 350 ml
1c: ascending mode and mode reversion after 650 ml
1d: ascending mode

d). In figure 1b, the initial mode was ascending, so quinizarin was eluted first and alizarin and purpurin were strongly retained. At the moment of mode reversion, indicated by the arrow, alizarin and purpurin were localized in the third of the six cartridges. After mode reversion they were hardly retained. In figure 1c alizarin and purpurin were at the moment of mode reversion almost at the end of their way through the CPC-apparatus. In figure 1d there was no mode reversion. Resolution between alizarin and purpurin was about the same in 1c and 1d but in 1d the run time was longer and the elution volume was larger.

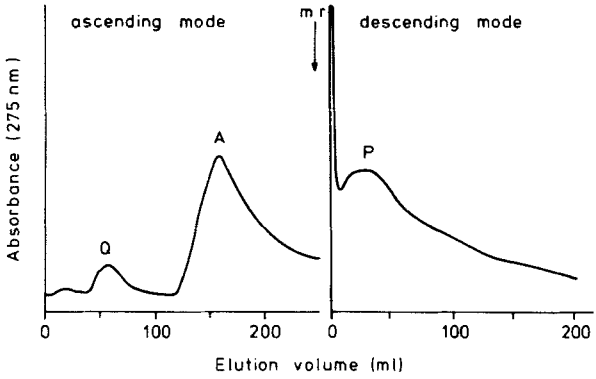


Figure 2: Analysis of the test mixture. A = alizarin; P = purpurin; Q = quinizarin. Separation by means of solvent system C2 (n-hexane - EtOAc - MeOH - water 9:1:5:5); flow rate 4.6 ml/min; centrifugal speed 700 rpm, after mode reversion 1000 rpm. mr = mode reversion.

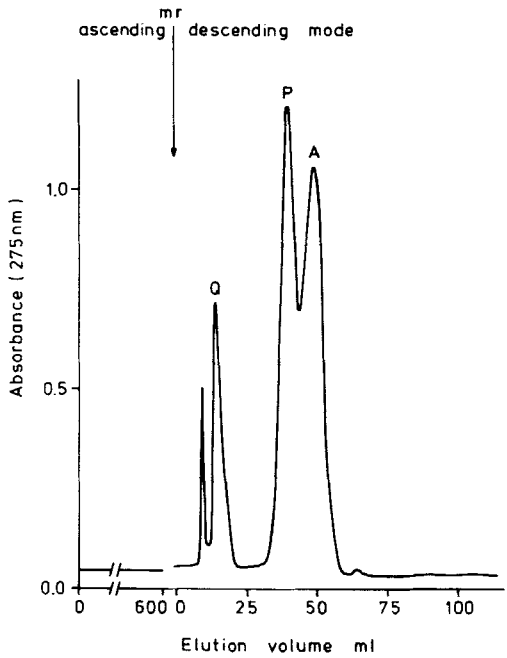


Figure 3: Analysis of the test mixture. A = alizarin; P = purpurin; Q = quinizarin. Separation by means of solvent system C3 (chloroform - MeOH - water - AcOH 5:6:4:0.05); flow rate 3.1 ml/min; centrifugal speed 700 rpm in both modes. mr = mode reversion.

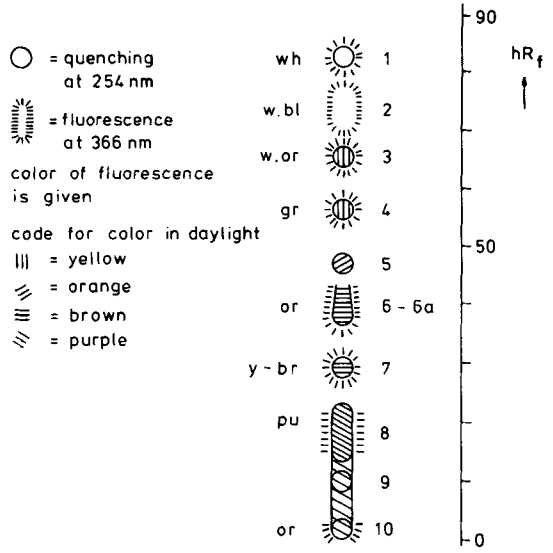


Figure 4: Thin-layer chromatogram of the sample as injected for figure 5. Solvent system S4 (see under Methods). Abbreviations for colours of fluorescence: w = weak; wh = white; bl = blue; or = orange; gr = green; y =yellow; br = brown; pu = purple;. hR_F value of 1 = 84; of 10 = 0.
Corresponding numbers in all figures refer to corresponding compounds.

Replacing in this system, methanol by acetonitrile (i.e. hexane - MeCN - water 100:99:1) resulted in similar separations. Additional organic modifiers were tested, EtOAc, chloroform and THF, each added respectively to the system C1 to give the ratio 100:99:1:0.5. All were tested in both ascending and descending mode, but no improvement in resolution between alizarin and purpurin could be detected. Also the increase of water content in the lower phase by using a system with ratio 100:95:5 did not result in higher resolution.

Zhang *et al.* [16] used the system n-hexane - EtOAc - MeOH- water 9:1:5:5 (system C2) in a high speed counter-current chromatograph. This system with a relative high polarity (water and MeOH content each 25%) met the physical requirements for a good CPC-performance. In ascending mode the elution sequence

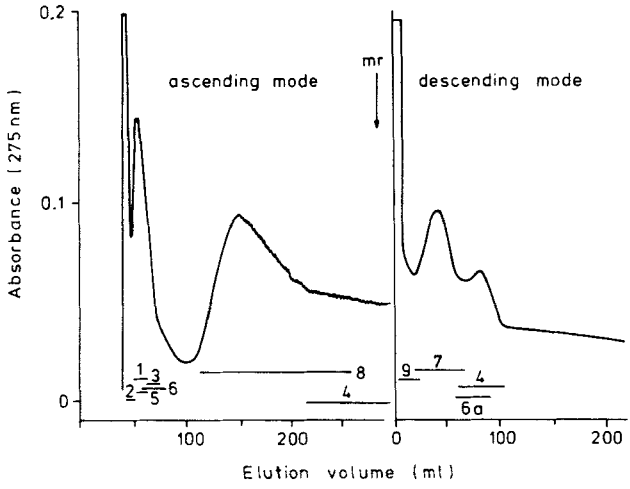


Figure 5

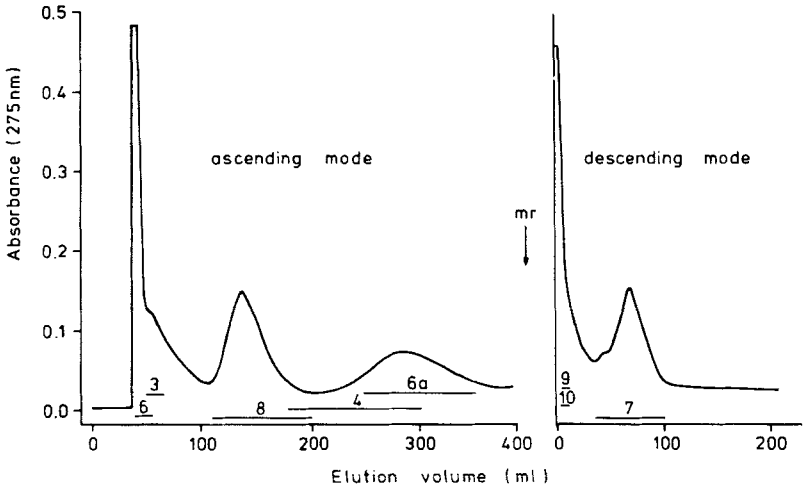


Figure 6

Figures 5-7: Analysis of several anthraquinone containing samples, prepared from *Rubia tinctorum* hairy root extract. CPC-solvent system and conditions as described under figure 2. More detailed information about sample preparation is given under Methods. mr = mode reversion.
Corresponding numbers in all figures refer to corresponding compounds.

5 and **6**: Anthraquinone-aglycones, present as such in the hairy root extract. Injected amount for figure 6 is 25 mg.

7: Anthraquinone-aglycones obtained after hydrolysis of the glycosides. Amount injected 31 mg.

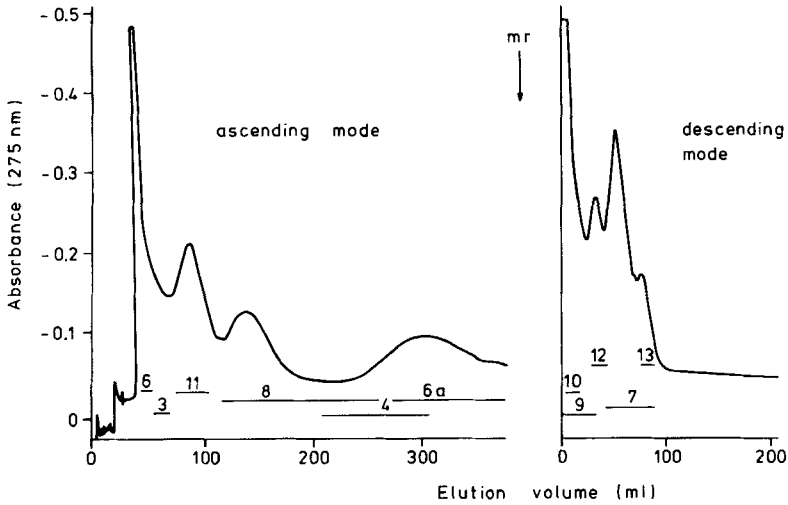


Figure 7

was as in the systems described above: first quinizarin, then alizarin and purpurin (fig. 2). This system allowed good separation of the three anthraquinones.

In a system composed of chloroform, MeOH and water the hydrophobicity can be adjusted by variations in the ratio MeOH/water. The selectivity of such a system is different compared to system C2 because of the differences in solvent parameters. The system 50:57:30 (for chloroform, MeOH and water respectively) has a low difference in density of the two phases [10], resulting in a low backpressure, allowing a higher rotation speed which improves efficiency. But also the interfacial tension is low [10] and this indeed results in a large degree of flooding in both modes. The interfacial tension in the system with ratio 5:6:4 is almost twice as high (1.5 v 0.8 dyne/cm, but also the difference in density between the two layers is much larger (0.3860 v. 0.3109 g/cm^3;) [10]. This system proved to give good results for the separation of the test substances when the CPC-apparatus was run in ascending mode, followed by reversed mode after 450 ml (equal to 7.2 times the volume of the stationary phase). Shortly after the reversion the anthraquinones were eluted in the order quinizarin, alizarin, purpurin with baseline separation but some tailing for purpurin. Addition of acetic acid to this system

to give the ratio 5:6:4:0.05 suppressed the tailing of purpurin and also changed the elution sequence to quinizarin, purpurin, alizarin (fig. 3).

By CPC the dihydroxy-anthraquinones alizarin and quinizarin and the trihydroxy-anthraquinone purpurin can be well separated. By changing the pH, selectivity can be influenced. Some of the systems studied were selected for the separation of plant tissue extracts containing anthraquinones.

Applications to anthraquinone extracts of a *Rubia tinctorum* hairy root culture.

In the extract of the *R. tinctorum* hairy root culture anthraquinones are present in free and glycosylated form. The aglycones were indexed by numbers, based on their elution in a TLC-system (Fig. 4). After isolation, by CPC and subsequent preparative TLC, the following anthraquinones were identified as indicated in Materials and Methods:

compound **4**: alizarin-1-methylether,

compound **6**: nordamnacanthal,

compound **7**: lucidin,

compound **8**: alizarin.

Based on their fluorescence behaviour (fig. 4) compounds **1** and **2** were regarded as being non-anthraquinonal, the other compounds were isolated in too small amounts to allow identification.

The system n-hexane-EtOAc-MeOH-water 9:1:5:5 (system C2) was used for the analysis of anthraquinone aglucones. Chloroform soluble anthraquinones were chromatographed in the ascending mode, followed by mode reversion after 300 ml (fig. 5) or 400 ml (fig. 6 + 7). Alizarin-1-methylether (**4**), eluted after about 270 ml; so at the moment of mode reversion at 300 ml (fig. 5), part of **4** was still present in the last cartridge and this part was eluted at the end of the run in descending mode. In figures 6 and 7, where the mode was reversed after 400 ml, all of **4** was eluted in the ascending mode. A similar effect can be seen for **6a**.

In the preparation of the sample for fig. 7 the water soluble anthraquinone-glycosides were first separated from the aglycones and subsequently hydrolysed to obtain the anthraquinone-part of these glycosides. Compounds **11-13** , present in this sample, could not be detected in the samples of figures 5 and 6; from this we conclude that these anthraquinones are present only in glycosidic form.

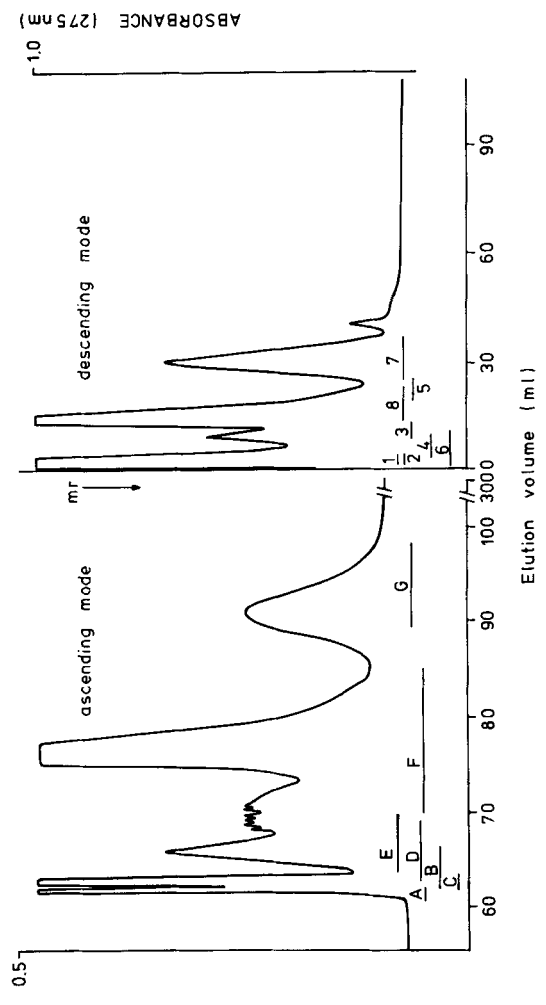


Figure 8: Analysis of an anthraquinone extract of *R. tinctorum* hairy roots. The sample contains both glycosides and aglycones. CPC-solvent system and conditions as described under figure 3. Amount injected is 15 mg. For sample preparation see Methods. mr = mode reversion.
Corresponding numbers in all figures refer to corresponding compounds.

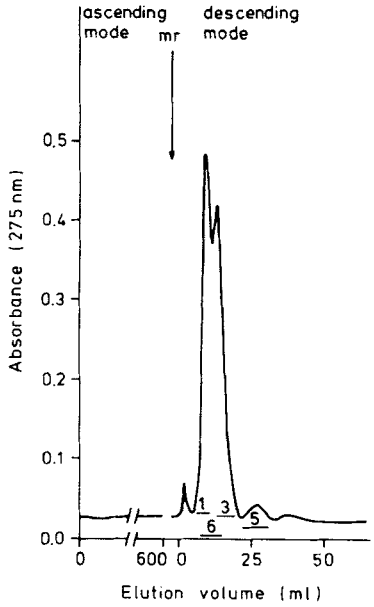


Figure 9: Analysis of the combined fractions 8-11 (corresponding with eluate fraction 50-64 ml) of figure 5. CPC-solvent system and conditions as described under figure 3. The amount injected is circa 0.7 mg. For sample preparation see Methods. mr = mode reversion.
Corresponding numbers in all figures refer to corresponding compounds.

Analysis of an extract containing glycosides and aglycones.

The acetic acid containing system (C3) was also used for the analysis of a *R. tinctorum* hairy root extract, containing both anthraquinone-glycosides and aglycones (fig. 8).

The glycosides, marked A-G, eluted in the ascending mode (which in this case means that the polar phase is the mobile phase). The glycosides were not identified. The aglycones eluted after mode reversion, the elution sequence being different from the sequence in system C2.

The difference in selectivity between the two systems C2 and C3 was used for a further purification of compounds **1**, **3**, **5** and nordamnacanthal **6**. The combined fractions 8-11 (corresponding with eluate fraction 50 - 64 ml) of the C2 separation (fig. 5) were used for a separation in system C3, the results of which are shown in figure 9.

CONCLUSIONS

CPC is a useful, fast and mild technique for the separation of anthraquinones, which can be performed on analytical and preparative scale. A major advantage is that both glycosides and aglycones can be separated in one run. The two systems n-hexane - EtOAc - MeOH- water 9:1:5:5 v/v/v/v and chloroform - MeOH - water - acetic acid 5:6:4:0.05 v/v/v/v offer different selectivity.

ABBREVIATIONS

DCCC: droplet counter-current chromatography; CPC: centrifugal partition chromatography; TLC: thin-layer chromatography; Me: methyl; Et: ethyl; Ac: acetyl; THF: tetrahydrofuran.

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