Stepwise pH-Gradient Elution for the Preparative Separation of Natural Anthraquinones by Multiple Liquid-Liquid Partition

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Liquid-Liquid Distribution, pH-Gradient, Anthraquinone

Preparative-scale separation of substituted anthraquinones by multiple liquid-liquid partition was studied using isopropylmethyl ketone (IMK)/aqueous phosphate buffer (aq.) as the solvent system and the Hietala apparatus with 100 partition units as the partition equipment. The lower (aq.) phase was chosen as mobile, while the upper (IMK) phase remained stationary. Hence, the principle of stepwise pH-gradient elution could be utilized to separate the components in two complex mixtures of hydroxyanthraquinones and hydroxyanthraquinone carboxylic acids, isolated from the fungus *Dermocybe sanguinea*. In spite of the nonlinearity of the partition isotherms for these anthraquinones, implying deviations from the Nernst partition law, remarkable separations were achieved for the components in each mixture. Every anthraquinone carboxylic acid showed markedly irregular partition behavior, appearing in the effluent as two more or less resolved concentration zones. Such splitting was attributed to the formation of relatively stable sandwich-dimers, which were in a slow equilibrium with the monomers in the more nonpolar organic phase. At lower pH-values, only the polar monomers were distributed into the mobile aqueous phase and moved forward, whereas the nonpolar sandwich-dimers remained almost entirely in the stationary organic phase and lagged behind. When the pH of the mobile aqueous phase was raised high enough, the firmly linked dimers were monomerized and emerged from the apparatus as a second concentration profile. Intermolecular hydrogen bonding and π-π interaction between the π-systems of two anthraquinone molecules in a parallel orientation were considered responsible for the nonlinear and markedly irregular partition behavior of the natural anthraquinones studied. The nonlinearity of the partition behavior of the hydroxyanthraquinones lacking the carboxyl group, appeared merely as excessive broadening of the experimental concentration profile, which impaired the resolution between the components only insignificantly. Thus, *e.g.* the main components, dermocybin and emodin, could both be obtained from Separation 1 in a purity of at least 99%.

Introduction

In our recent articles (Hynninen *et al.*, 2000; Räisänen *et al.*, 2000), we described the application of new 1D and 2D TLC techniques using silica plates to separate variously substituted anthraquinones isolated from the fungus *Dermocybe sanguinea*. These analytical-scale separations were quite successful and led to the discovery of five new, earlier in *D. sanguinea* unidentified anthraquinone compounds. However, when similar anthraquinone mixtures were attempted to separate on a preparative scale by column chromatography (CC) on silica gel, insurmountable difficulties were encountered. Part of the anthraquinone sample was strongly adsorbed to the top of the silica layer and the extensive tailing and overlapping of the concentration zones hindered resolution seriously. Apparently, these difficulties were caused by the very high tendency of the natural anthraquinones to form self- and mixed aggregates, due to the presence of carbonyl, hydroxyl and carboxyl groups, capable of forming intermolecular hydrogen bonds. To avoid such difficulties, pure multiple liquid-liquid partition (MLLP) methods offer better possibilities, while they allow one to adjust the pH of the aqueous phase so high

Abbreviations: CC, column chromatography; CCD, countercurrent distribution; 1D, one-dimensional; 2D, two-dimensional; GC-MS, gas chromatography-mass spectrometry, Gluc*p*, glucopyranoside; IMK, isopropylmethyl ketone; MLLP, multiple liquid-liquid partition; MSD, Martin-Synge distribution; TLC, thin-layer chromatography.

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that the anthraquinone molecules undergo deprotonation, producing anionic forms, which repel one another.

When planning the liquid-liquid partition system, we chose IMK as the organic upper phase and phosphate buffer (aq.) with variable pH as the lower phase. IMK was selected as the organic phase because of its limited miscibility with water and because it has a carbonyl group, which was expected to compete with the carbonyl groups of the anthraquinones for hydrogen bond formation and, in this way, to hinder the intermolecular aggregation. Using the IMK/aq. as phase system, our preliminary determinations of the partition coefficients of the anthraquinones in Fractions 1 and 2, isolated from *D. sanguinea* (Hynninen *et al.*, 2000), indicated that the hydroxyanthraquinone carboxylic acids were distributed noticeably into the aqueous phase, when the pH was around 7.5, whereas the hydroxyanthraquinones without carboxyl groups, required for that a pH value in the range 9–12. In their dianionic form, the anthraquinones would be expected to exist predominantly in the aqueous phase, whereas in their neutral form, the molecules would occur significantly also in the organic phase, where their aggregation is possible at higher concentration levels. The equilibrium reactions expected to occur in the organic and aqueous phases of the IMK/aq. system are shown in Fig. 1.

MLLP may be defined as a process consisting of a great number of successive liquid-liquid partitions. The different forms of pure MLLP methods (liquid-liquid partition chromatography not included) have been classified on the basis of the nature of phase transfer, which can be stepwise or continuous, and on the basis of the nature of sample feeding, which can be batchwise or continuous (Hynninen, 1976a). Of the pure MLLP methods, the Countercurrent Distribution, CCD, developed by the Craigs (Craig and Craig, 1956; Hynninen, 1976a) and the Martin-Synge Distribution, MSD, (Hynninen, 1976b, 1976c) can be theoretically mastered. In each case, it is possible to calculate the theoretical distribution profiles for the solutes and the optimal operating conditions for their separation. The advantages of using the principle of stepwise pH-gradient elution were realized quite early when separating dicarboxylic porphyrins (Ellfolk *et al.*, 1969; Hynninen and Ellfolk, 1973) and Mg-free chlorophyll derivatives (Hynninen, 1973). In this paper, we will describe the application of the stepwise pH-gradient elution to separate substituted anthraquinones on a preparative scale by means of the Hietala apparatus (Hietala, 1960). The concentration profiles of the solutes in the Hietala apparatus generally obey the theory of Martin and Synge (Martin and Synge, 1941; Hynninen, 1976b).

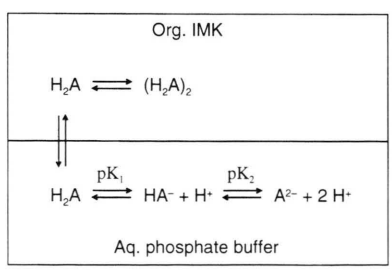


Fig. 1. Equilibrium reactions of a substituted anthraquinone in the organic isopropylmethyl ketone (IMK) phase and the aqueous buffer phase. The vertical arrows denote the mass transfer through the interphase.

Experimental

Chemicals and fungal anthraquinones

All chemicals were of analytical-reagent grade, if not mentioned differently. Isopropylmethyl ketone was from Fluka AG (Buchs, Switzerland) and HPLC-grade ethyl acetate from Lab-Scan (Dublin, Ireland). Ethanol was of Aa- or AaS-grade and purchased from Primalko (Rajamäki, Finland). Toluene, acetic acid 99.8%, formic acid 100%, sodium hydroxide, sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate were purchased from E. Merck (Darmstadt, Germany). TLC aluminum sheets precoated with silica gel 60 without fluorescent indicator were purchased from E. Merck. Distilled water was used. The fungal anthraquinones, Fractions 1 and 2, were the same as described earlier (Hynninen *et al.*, 2000; Räisänen *et al.*, 2000).

Equipment

For MLLP, an apparatus designed by Hietala (Hietala, 1960) and manufactured by Karppinen Oy (Helsinki, Finland) was used. There were 100 partition units in the apparatus, each having a vol-

ume of 13.5 ml. Teflon tubing was used to connect the tubes to each other in series and to convey liquids to and from the glass-tube train. A micropump giving a flow rate of 5 ml/min and an automatic fraction collector (LKB) were used as ancillary equipment. The absorbance at the selected wavelength was measured from each fraction using a Perkin-Elmer 55B spectrophotometer.

The UV/Vis absorption spectra of the purified anthraquinone compounds were recorded on a Varian Cary 5 E UV-Vis-NIR spectrophotometer (Varian Optical Spectroscopy Instruments, Mulgrave, Victoria, Australia) using Starna Ltd. quartz-glass cuvettes, type 21, pathlength 10 mm (Starna Ltd., Romford, Great-Britain).

Selection of operation conditions

In order to obtain accurate values for the partition coefficient, the phase ratio, $\alpha = v_m/v_s$, where v_m is the volume of the mobile phase and v_s the volume of the stationary phase in a partition unit, must be accurately measured. For this purpose all the tubes to be used in the separation process were first filled with the mobile phase only and the volume of solvent needed was measured. The partition tubes were then turned to a position determined by the desired phase ratio. Keeping the tubes in this position, stationary phase was pumped in until the mobile phase no longer emerged from the train. The volume of the displaced mobile phase was measured considering volumes of the inlet and outlet tubings (32 ml). The volume of the mobile phase in a partition unit, v_m, was determined to be 3.68 and 3.70 ml in Separations 1 and 2, respectively. The volume of the stationary phase in a partition unit, v_s, was calculated by subtracting the volume of the mobile phase from the total volume of the partition unit (13.5 ml); v_s was 9.82 ml in Separation 1 and 9.80 ml in Separation 2. The phase ratio, $\alpha = v_m/v_s$, was 0.375 and 0.378, respectively. In the separations, a shaking frequency of 36 cycles/min, an amplitude of ± 30°, and a flow rate of 2–5 ml/min were used.

Fractionation by multiple liquid-liquid partition

In a separatory funnel, a 300 mg amount of Fraction 1 (Separation 1) or Fraction 2 (Separation 2) was dissolved in 88.0 ml of IMK and 33.5 ml of the aqueous phosphate buffer, whose pH was adjusted to 6.0 in Separation 1 and to 4.5 in Separation 2. The first nine tubes of the train were emptied of solvents and the upper and lower phases of the feeding anthraquinone mixture were divided equally between the tubes. The loaded tubes were closed with Teflon stoppers and the fractionation was started. The effluent was collected in fractions of 6 to 17 ml. The absorbance of each fraction was measured at the wavelength of 400, 445 or 500 nm. The total volume of effluent emerged from the apparatus was 6250 ml in Separation 1 and 8810 ml in Separation 2. The experimental and theoretical distribution profiles in Separations 1 and 2 are shown in Figs. 2 and 3, respectively. On the basis of the distribution profiles, the fractions in the same concentration zone were combined. After combining the fractions, the aqueous buffer solution was neutralized and the anthraquinone pigments were extracted into IMK in a separatory funnel. The IMK phase was collected and evaporated to dryness.

Results and Discussion

MLLP Separation 1

Fig. 2 presents the separation of Fraction 1 by MLLP. Excellent resolution was obtained for the components endocrocin (Ec), dermocybin (D), emodin (E) and physcion (P), and only the concentration zones denoted with Dr_I and Dr_{II}, both representing dermorubin, remained partially overlaid. The partition coefficients k of the anthraquinone compounds in Separation 1 are compiled in Table II and their structures are shown in Table I. The compounds were identified by 1D and 2D TLC methods, electronic (UV/Vis) absorption spectra, and GC-MS (Hynninen *et al.*, 2000; Räisänen *et al.*, 2000; and references therein).

An interesting feature in the concentration profiles of the separation is that dermorubin was eluted in two partially resolved concentration zones, Dr_I and Dr_{II}. This result can be interpreted in terms of the equilibrium reactions occurring in the organic and aqueous phases (Fig. 1). In the beginning of the separation, the pH of the aqueous phase was adjusted to 6.0. Under those conditions, part of dermorubin existed in the organic phase as monomers, which were in a very slow equilibrium with the firmly-linked sandwich dimers of the compound (Hynninen *et al.*, 2000). The monomers

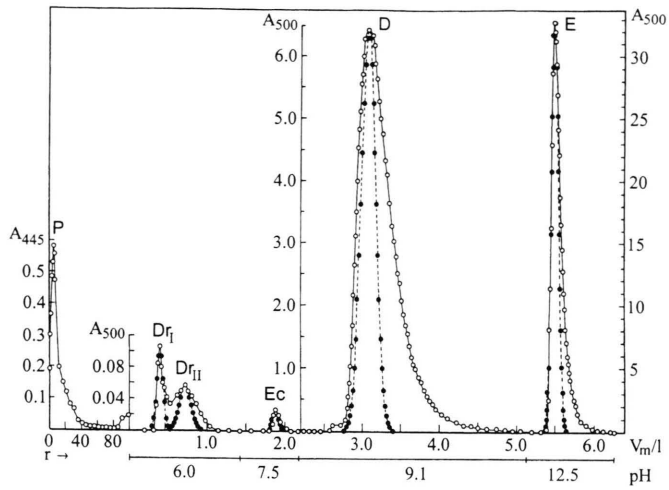
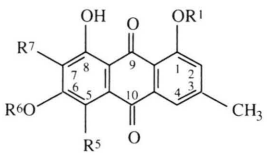
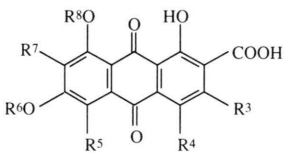


Fig. 2. Separation of the anthraquinones in Fraction 1 by multiple liquid-liquid partition (MLLP) using IMK/0.1 M aqueous phosphate buffer with varied pH as solvent system. The sample was fed into tubes r = 0, ..., 9. Number of tubes used in the fractionation = N = 100. Volume of the mobile phase (buffer) in a partition unit = v_m = 3.68 ml; volume of the stationary phase (IMK) in a partition unit = v_s = 9.82 ml. Total volume of effluent emerged from the apparatus = V_m = 6250 ml; flow rate = 2–5 ml/min. Filled circles (●) denote the theoretical values and open circles (○) the experimental values, the latter obtained by measuring the absorbances at selected wavelengths of the effluent fractions. The absorbances of the IMK phases in the tubes were recorded at 445 nm after the addition of 0.2 ml acetic acid into each tube. For the compound abbreviations, see Table I and the text. The operational partition coefficients of the components are collected in Table II. A_{445} = absorbance at wavelength 445 nm, V_m/l = volume of effluent emerged from the apparatus in litres, r = tube number.

Table I. Structures and abbreviations of the anthraquinone compounds from the fungus *Dermocybe sanguinea*. Gluc*p* = glucopyranoside.





Compound	R^1	R^5	R^6	R^7
Emodin (**E**)	H	H	H	H
7-Chloroemodin (**Cl-E**)	H	H	H	Cl
5,7-Dichloroemodin (**Cl_2-E**)	H	Cl	H	Cl
Emodin-1-β-D-Gluc*p*	Gluc*p*	H	H	H
Physcion (**P**)	H	H	CH_3	H
Dermocybin (**D**)	H	OH	CH_3	OH
Dermocybin-1-β-D-Gluc*p*	Gluc*p*	OH	CH_3	OH

Compound	R^3	R^4	R^5	R^6	R^7	R^8
Endocrocin (**Ec**)	CH_3	H	H	H	H	H
5,7-Dichloroendocrocin	CH_3	H	Cl	H	Cl	H
Dermolutein (**Dl**)	CH_3	H	H	H	H	CH_3
Dermorubin (**Dr**)	CH_3	OH	H	H	H	CH_3
5-Chlorodermorubin (**Cl-Dr**)	CH_3	OH	Cl	H	H	CH_3
Austrocorticone (**A**)	$COCH_3$	H	H	CH_3	H	CH_3
4-Hydroxyaustrocorticone	$COCH_3$	OH	H	CH_3	H	CH_3

were rapidly distributed for a greater part into the aqueous phase due to the presence of one isolated HO-group and one COOH-group, both of which are capable of intermolecular hydrogen-bonding (the intramolecular hydrogen bonds formed by the phenolic HO-groups with the proximal quinonyl oxygen(s) may be assumed to be strong and remain intact). At pH 6.0 of the aqueous phase, the carboxyl-group presumably existed entirely in its deprotonated form, COO^-. The negative charge of the carboxylate anion enabled solvation and hindered intermolecular association (aggregation), thus increasing considerably the solubility of the carboxylic acid in the aqueous phase. While spend-

ing more time in the mobile aqueous phase, the monomers migrated faster forward in the tube train and emerged as Dr_I, whereas the relatively stable sandwich dimers, being externally essentially non polar, lingered longer in the stationary IMK phase and lagged behind. Due to the migration of the monomeric dermorubin in the tube train, its concentrations in the phases of the tubes left behind, diminished and, hence, more monomers were withdrawn from the dimer – monomer equilibrium in the organic phase. After a sufficiently low concentration level was reached, the rest of the firmly-linked sandwich dimers were disrupted and the monomers produced were transferred into the mobile aqueous phase, where aggregation was less likely. Thus, that part of dermorubin, which, in the beginning of the fractionation, existed as sandwich dimers in the stationary IMK-phase, finally emerged from the tube train as a second concentration zone, Dr_{II}.

After all dermorubin had emerged from the tube train, the pH of the aqueous phase was raised to 7.5, which led to the elution of the component Ec, identified as the anthraquinone carboxylic acid endocrocin. Due to its very low concentrations, there were no signs of anomalous partition behavior for Ec. On raising the pH of the aqueous phase to 9.10, component D was eluted from the apparatus and was characterized as dermocybin. The eastern side of the experimental concentration profile of D shows clear excessive broadening, which can be interpreted to arise also from aggregation phenomena in the organic IMK-phase. For dermocybin, however, the dimers or aggregates are dissociated into monomers much faster than in the case of anthraquinone carboxylic acids, because the molecules of the D dimer are held together by weaker forces. These weaker forces are likely to result in the formation of relatively unstable dimers with open conformations and not in the formation of such sandwich dimers as predictable for the anthraquinone carboxylic acids, *e.g.* dermorubin (Hynninen *et al.*, 2000).

The most abundant component E was eluted from the apparatus at pH 12.5 and was identified as emodin. The slight excessive broadening of its concentration profile may also be attributed to the formation of weakly-linked aggregates in the organic phase, comparable to those of dermocybin. The component P, which remained largely in the feeding tubes of the apparatus, was identified as physcion. Its very low partition coefficient, k = 0.008, can be ascribed to the missing of isolated hydroxy-groups, prone to intermolecular hydrogen-bonding and deprotonation. Apparently, the two phenolic hydroxyls at positions 1 and 8 (Table I) of physcion form so strong hydrogen bonds with the proximal carbonyl oxygen that the molecule remains externally nonpolar, being partitioned entirely into the organic phase, even at pH 12.5 of the aqueous phase.

It should be noticed while inspecting Fig. 2 that the absorbance scales for the two main components, emodin and dermocybin, are enormously higher than those for the other components. Thus, the amounts of endocrocin, dermorubin and physcion in Fraction 1 were very small. The separation yielded 164 mg (55%) of pure emodin, obtained from the fractions in the range V_m = 5426–6249 ml, and 87 mg (29%) of pure dermocybin, obtained from the fractions in the range V_m = 2803–4352 ml. On the basis of the UV/Vis spectrophotometric and GC-MS measurements, the purity grades of the emodin and dermocybin preparations were 99% (Räisänen *et al.*, 2000).

MLLP Separation 2

Fig. 3 presents the separation of Fraction 2, which was a complex mixture, consisting mainly of anthraquinone carboxylic acids. According to our previous study (Räisänen *et al.*, 2000), Fraction 2 contained fourteen different anthraquinone compounds. Inspection of the separation results in Fig. 3 reveals right away very irregular partition behaviour for several of the components, which were identified utilizing the results from the 2D TLC analyses, as well as those from the UV/Vis and mass spectral measurements (Räisänen *et al.*, 2000; and references therein). The values of the operational partition coefficients k of the compounds are collected in Table II.

The separation was started by adjusting the pH of the mobile aqueous phase to 4.5, which led to the appearance of the components Dr_I, Cl-Dr_I and A in the effluent fractions between V_m = 0.0–1.0 l. Though components Dr_I and Cl-Dr_I, which were identified as dermorubin and 5-chlorodermorubin (Table I), emerged from the tube train as a sharp main peak, a long tail can be seen to follow the

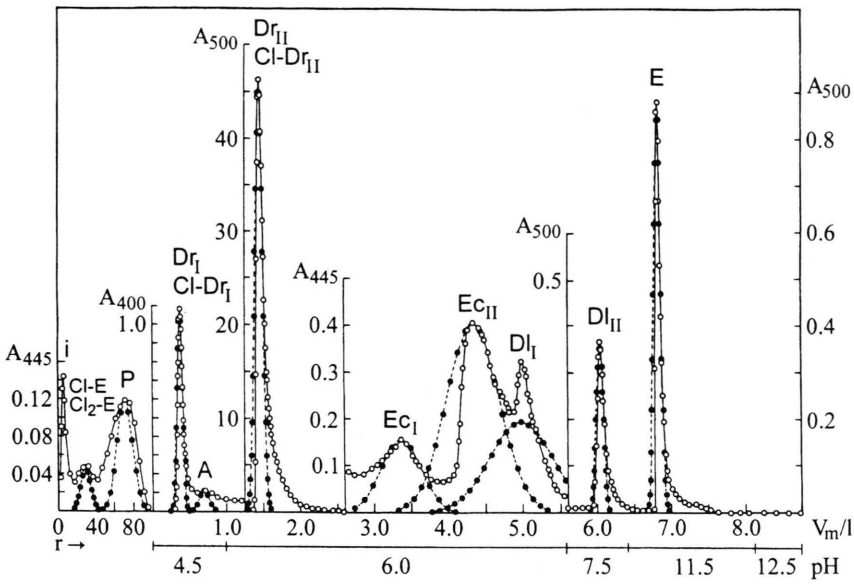


Fig. 3. Separation of the anthraquinones in Fraction 2 by MLLP using IMK/0.1 M aqueous phosphate buffer with varied pH as solvent system. The sample was fed into tubes r = 0, ..., 9. N = 100. v_m = 3.70 ml; v_s = 9.80 ml. V_m = 8810 ml; flow rate = 2–5 ml/min. The symbols (●) and (○) are as in the Fig. 2 legend. For the compound abbreviations see Table I and the text. The operational partition coefficients of the components are collected in Table II.

Table II. Operational partition coefficients of the anthraquinones in the MLLP Separations 1 and 2. The operational partition coefficient k is defined as c_m/c_s, where c_m and c_s are the anthraquinone concentrations in the mobile and the stationary phases, respectively, in a partition unit.

Compound	Separation 1		Separation 2	
	k	pH	k	pH
Dermorubin, Dr_I	27.2	6.0	111.5	4.5
5-Chlorodermorubin, Cl-Dr_I			111.5	4.5
Austrocorticone, A			2.91	4.5
Dermorubin, Dr_{II}	2.73	6.0	5.24	6.0
5-Chlorodermorubin, Cl-Dr_{II}			5.24	6.0
Endocrocin, Ec, Ec_I	6.67	7.5	0.374	6.0
Endocrocin, Ec_{II}			0.291	6.0
Dermolutein, D_{II}			0.239	6.0
Dermolutein, Dl_{II}			9.11	7.5
Dermocybin, D	1.58	9.10		
Emodin, E	15.8	12.5	9.59	11.5
Physcion, P	0.008	12.5	0.081	11.5
7-Chloroemodin, Cl-E			0.033	11.5
5,7-Dichloroemodin, Cl_2-E			0.033	11.5

main peak. In the long tail, there was hidden a small amount of component A, which was identified as austrocorticone (Table I).

After the pH of the aqueous phase was adjusted to 6.0, a very intensive concentration peak, denoted as Dr_{II} and Cl-Dr_{II}, appeared in the effluent at $V_m \approx 1.2$ l. Now the main part of dermorubin and 5-chlorodermorubin emerged from the apparatus, the splitting of the concentration profile resembling that observed for dermorubin in Separation 1. Noteworthy is that the concentration level before the intensive peak of Dr_{II} and Cl-Dr_{II} never reaches zero and that the rear side of this peak shows clear excessive broadening. The concentration profile does not reach the zero level, because of the continuous slow monomer – dimer equilibrium, which allows small amounts of monomer to distribute into the aqueous phase and emerge from the tube train. Very small amounts of 4-hydroxyaustrocorticone and 5,7-dichloroendocrocin (Table I) were eluted together with Dr_{II} and Cl-Dr_{II}.

The experimental concentration profiles of the effluent in the range V_m = 2.6–6.5 l exhibit even more pronounced deviations from the theoretical profiles. Careful analysis of the components in this effluent range revealed the presence of endocrocin in two incompletely resolved concentration zones, Ec_I and Ec_{II}. Also dermolutein was split into two concentration zones, Dl_I, and Dl_{II}. The Dl_I part overlaps with Ec_{II} seriously, making its contribu-

tion to the total absorption difficult to analyze (the theoretical distribution curve of Dl_I may have lower intensities). The Dl_{II} part emerged from the apparatus quite soon after raising the pH of the aqueous phase to 7.5.

The concentration zone E appeared in the effluent after raising the pH of the aqueous phase to 11.5. The 2D TLC analysis indicated emodin as the main compound, but there were also traces of emodin- and dermocybin-1-β-D-glucopyranosides present in this zone. The components, which still remained in the tube train of the apparatus, were identified as physcion (P), 7-chloroemodin (Cl-E) and 5,7-dichloroemodin (Cl_2-E) (Table I). The material that remained in the feeding tubes contained unidentified impurities (i).

An interesting conclusion from the foregoing results is that the anthraquinone carboxylic acids dermorubin, 5-chlorodermorubin, endocrocin and dermolutein all regularly exhibited anomalous partition behaviour, which can be interpreted in terms of the equilibrium reactions in the phases (Fig. 1), in a fashion similar to that described for dermorubin in Separation 1. Apparently, all these anthraquinone carboxylic acids can form, in the organic phase, externally relatively nonpolar, firmly-linked sandwich dimers (Hynninen *et al.*, 2000), which are in a slow equilibrium with the monomers.

Acknowledgements

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