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Effect of *Digitalis lanata* matrix composition on the lanatoside C partition coefficient and its consequence on rotation locular counter-current chromatography efficiency

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Abstract

Lanatoside C standard samples had their partition coefficients (*K*) determined in dichloromethane–methanol–water (4:4:3), (5:6:4) and (7:13:8), by the shake flask method and the obtained values were similar. On the other hand, *K* for lanatoside C accomplished by calculation from rotation locular counter-current chromatography (RLCC) traces of *Digitalis lanata* pre-purified extracts, as well as by the shake flask method, in the same solvent systems, showed a significant variation. Such variation was attributed to differences in the pre-purification procedures for *D. lanata* extract, which resulted in starting materials for RLCC with very distinct cardenolide profiles, reflecting, thus, in the *K* values for lanatoside C and, consequently, in the lanatoside C purity at the end of the chromatographic process. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Digitalis lanata*; Partition coefficients; Matrix effects; Counter-current chromatography; Lanatosides; Cardenolides

1. Introduction

Chromatography is usually described in terms of the capacity factor, k', rather than of the partition coefficient, *K*. The stationary phase in conventional systems is retained by adsorption or chemical bonding to a solid supporting matrix. Solute adsorption to the matrix surface often contributes to its retention and hence, k' is determined by a mixed mechanism and is not always directly predictable from the partition coefficient. On the other hand, in counter-current chromatography (CC) the solid supporting matrix is absent and separation is therefore entirely dependent on the more fundamental partition coefficient, *K* [1]. Hence, *K* can be predicted from counter- current chromatograms, without the interference of adsorption processes, which can not be discarded in the determination of *K* by high-performance liquid chromatography (HPLC) methods. The following equation can be used to calculate *K* from a counter-current chromatogram [1]:

$$K = \frac{V_R - V_m}{V_m} \cdot \frac{(1 - S_F)}{S_F} \qquad (1)$$

where V_m represents the volume of the mobile phase, V_R the volume of retention of a solute, and S_F is assumed to be the volume ratio between stationary and mobile phases remaining in the columns.

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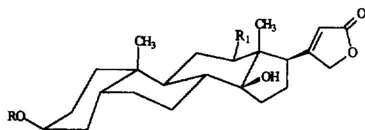

Cardenolide	*R	R_1
Desacetyllanatoside C	Glc-β1–4-Dox-β1–4-Dox-β1–4-Dox-β1–	OH
Lanatoside C	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	OH
Digoxin	Dox-β1–4-Dox-β1–4-Dox-β1–	OH
Lanatoside A	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	H
Digitoxin	Dox-β1–4-Dox-β1–4-Dox-β1–	H

*Glc, glucose; Dox, digitoxose; α-AcDox, α-acetyldigitoxose

Fig. 1. Chemical structures of cardenolides.

Rotation locular counter-current chromatography (RLCC) is an all liquid chromatographic method developed by Ito and Bowman [2,3]. The use of RLCC in the isolation of several classes of natural products was reported [4,5]. RLCC shows a low chromatographic resolution and consequently it has rarely been employed as a single method for the isolation of pure compounds. In a previous paper, we reported the isolation of lanatoside C (Fig. 1) from *Digitalis lanata* extracts [6]. In that work we investigated different pre-purification procedures for *D. lanata* extracts, before injection into the RLCC system. We observed that the composition of the pre-purified *D. lanata* extracts seemed to have an influence over the chromatographic efficiency, in other words, that the matrix composition could alter the K values for lanatoside C, reflecting in its final purity, at the end of the chromatographic process [6].

The scope of the present work is to measure the influence of the matrix composition in the partition coefficient of a single component and its effect on the efficiency of a counter-current chromatographic process. To perform this study we evaluated the variation of the K values for lanatoside C, in several *D. lanata* extracts, of different compositions.

2. Experimental

2.1. Preparation of plant material

The 12-month-old leaves of *D. lanata* were collected in an experimental cultivar at Itatiaia, Brazil. The dense rosette leaves were dried at 40°C for 48 h (1000 *g*) and then percolated with 70% MeOH (3×3 l) at room temperature, for 72 h. The extract was evaporated to dryness under reduced pressure, at 50°C, to give a dark green residue (400 g). Portions of this crude hydromethanolic extract were submitted to different methods for pre-purification, before injection into the RLCC system.

2.2. RLCC separations

Separations were performed on an RLCC-A apparatus (Tokyo Rikakikai, Tokyo, Japan), equipped with UV detector (Knauer). The wavelength employed was 225 nm. The biphasic solvent system dichloromethane–methanol–water was used in three different compositions, respectively, (5:6:4); (7:13:8) and (4:4:3). Solvents of commercial grade were employed. Biphasic solvent systems were prepared in a 2-l separatory funnel, before use, and allowed to equilibrate for 2 h to separate the phases. The solvent system upper layer was employed as mobile phase and rotation was performed at 80 rpm, in all the experiments.

2.3. Experiment RLCC-1

A portion of the crude hydromethanolic extract (50 g) was dissolved in 30% MeOH (1500 ml). Pigments were removed by shaking with polyvinylpolypyrrolidone (PVPP) (150 g), following adsorption of the filtrate on Amberlite XAD-7 (150 g). The resin was washed with water (3×900 ml) and 30% MeOH (3×900 ml). Cardenolides were recovered by elution with 70% MeOH (3×900 ml). This extract was concentrated in a rotatory evaporator under reduced pressure, at 40°C, to ca. 300 ml and partitioned with $CHCl_3$–iso-PrOH (3:2, v/v) (3×90 ml). The organic layer was concentrated in a rotatory evaporator under reduced pressure, at 40°C and the obtained residue (1.7 g), dissolved in dimethylsulfoxide (2 ml), was submitted to flash chromatography in a reversed-phase silica column (LichroPrep RP-8, 40–63 μm Merck; pressure 1.2 bar) (460×25 mm I.D.). Elution was performed with a MeOH–water gradient: fractions 1–3 (60:40; 770 ml); fractions 4–7 (75:25; 700 ml); fractions 20–23 (80:20; 220 ml). Fraction 1 (228 mg) was dissolved

in 4 ml of the stationary and mobile phases (1:1; v/v) and injected into the RLCC system, using dichloromethane–methanol–water (4:4:3) as solvent system. The RLCC flow-rate was 0.7 ml/min and the lanatoside C retention volume (V_R) was 590 ml.

2.4. Experiment RLCC-2

A portion of the crude hydromethanolic extract (64 g) was dissolved in 70% MeOH (600 ml) and partitioned with light petroleum (2×300 ml). The hydromethanolic layer was concentrated in a rotatory evaporator under reduced pressure, at 50°C, to ca. 300 ml and partitioned with $CHCl_3$–iso-PrOH (3:2, v/v) (3×300 ml). The residue from the $CHCl_3$–iso-PrOH layer (3.2 g) was chromatographed in a silica gel column (silica gel 60, 70–230 mesh, Merck; 310×25 mm I.D.) using a gradient of EtOAc–EtOH as follows: fractions 1–7 (98:2; 2110 ml); fractions 8–11 (96:4; 5310 ml). Fractions 10 and 11 were combined and concentrated in a rotatory evaporator under reduced pressure, at 40°C, to give a residue (343 mg), which was dissolved in 4 ml of the stationary and mobile phase (1:1, v/v) for injection into the RLCC system, using dichloromethane–methanol–water (5:6:4) as solvent system. The RLCC flow-rate was 0.6 ml/min and the lanatoside C retention volume (V_R) was 570 ml.

2.5. Experiment RLCC-3

Fifty g of the crude hydromethanolic extract were submitted to the procedures described in Section 2.3. The single difference is that elution was performed with the following MeOH–water gradient: fractions 1–18 (50:50; 1200 ml); fraction 19 (60:40; 50 ml); fractions 20–23 (80:20; 220 ml). Combined fractions 15–18 (231 mg) were dissolved in 4 ml of the stationary and mobile phases (1:1; v/v) and injected into the RLCC system, using dichloromethane–methanol–water (5:6:4) as solvent system. The RLCC flow-rate was 0.7 ml/min and the lanatoside C retention volume (V_R) was 537 ml.

2.6. Experiment RLCC-4

Fifty g of the crude hydromethanolic extract were submitted to the procedures described in Section 2.3. The single difference is that elution was performed isocratically with MeOH–water (70:30): fractions 1–6 (1200 ml). Combined fractions 1–2 (510 mg) were dissolved in 4 ml of the stationary and mobile phases (1:1; v/v) and injected into the RLCC system, using dichloromethane–methanol–water (7:13:8) as solvent system. The RLCC flow-rate was 0.5 ml/min and the lanatoside C retention volume (V_R) was 490 ml.

2.7. Experiment RLCC-std

A mixture of cardenolide standards, composed of desacetyllanatoside C (3.7 mg), lanatoside C (6.9 mg), digoxin (6.5 mg), lanatoside A (3.6 mg) and digitoxin (2.7 mg), was dissolved in 4 ml of the stationary and mobile phases (1:1; v/v) and injected into the RLCC system, using dichloromethane–methanol–water (5:6:4) as solvent system. The RLCC flow-rate was 0.7 ml/min. Retention volumes: desacetyllanatoside C, 450 ml; lanatoside C, 700 ml; digoxin, 1000 ml. Lanatoside A and digitoxin remained in the RLCC columns, after elution with 1430 ml of mobile phase.

2.8. Calculation of the partition coefficient from RLCC chromatograms

Calculations were performed using Eq. (1). The retention volume (V_R) of the cardenolide was determined directly in the chromatogram. The mobile phase volume remaining in the RLCC columns was estimated by emptying the columns content and measuring the volume in a cylinder. It was assumed to be the average value of 400 ml for all the calculations.

2.9. Determination of the partition coefficient by the shake flask method

A standard sample of the cardenolide was accurately weighted (1.00 mg) and dissolved in MeOH LiChroSolv grade (1.00 ml). An aliquot of this solution was taken (200 μl) and transferred to a 2-ml Eppendorf flask. This aliquot was concentrated until residue in a heating block, under air flow. The obtained residue was dissolved in equal volumes of the solvent system lower and upper phases (1.00 ml). The Eppendorf flask was then vigorously shaken for 3 min, in a vortex shaker. After that, the flask was

centrifuged by 12 000 rpm, for 10 min, to allow the complete separation of phases. Aliquots of each one of the phases were taken (500 μl) and then concentrated until residue. Following, these residues were dissolved in MeOH LiChroSolv grade (300 μl) that was finally injected into the HPLC system. K was determined from the peaks areas ratio between the organic and aqueous layers. In each case, K was determined in duplicate. The peak areas used for the determination of K were the average of three injections. For the determination of K for lanatoside C in the pre-purified *Digitalis lanata* extracts, a similar procedure was adopted and a sample of 10 mg was employed, in each case.

2.10. HPLC analysis

Analyses were carried out in a Hewlett-Packard 1090 apparatus serie II with diode-array detection (Walbronn, Germany). An ODS C_{18} column (100× 2.1 mm I.D.) was employed (Hewlett-Packard, Grom, Germany) with temperature of 40°C, flow-rate of 0.2 ml/min and wavelength of 220 nm. A gradient elution of water (A) and 84% CH_3CN (B) was employed: 0–5 min 85% A, 15% B; 5–10 min 80% A, 20% B; 10–12 min 80% A, 20% B; 12–25 min 73% A, 27% B; 25–35 min 55% A, 45% B; 35–50 min 40% A, 60% B; 50–52 min 5% A, 95% B. The composition of the starting material for RLCC and the purity of the isolated cardenolides were estimated by normalization of the peak areas, without considering differences between extinction coefficients of the cardenolides or the existent impurities.

2.11. Chemicals

Acetonitrile of chromatographic grade LiChroSolv was obtained from Merck (Darmstadt, Germany). Water was purified using the Milli-Q^{50} purification system (Millipore, Eschborn, Germany). Lanatoside A and lanatoside C were obtained from Roth (Karlsruhe, Germany); desacetyllanatoside C, digoxin and digitoxin were obtained from Boehringer Mannheim (Mannheim, Germany).

3. Results and discussion

The focal point of a counter-current chromatogram is the centrality of $K=1$. If the extra-column dead volumes are neglected, solutes with $K=1$ will be eluted when one column volume of mobile phase has passed through the column [7]. Based on this assumption, we selected dichloromethane–methanol–water (5:6:4), (7:13:8) and (4:4:3) as solvent systems and determined K values for lanatoside C standard samples by the shake flask method. The obtained K values did not show a significant variation in the three solvent systems (Table 1) and theoretically, all of them could be employed as solvent system for RLCC, for the purpose of isolating lanatoside C.

The partition coefficient determined for a single standard sample is constant in a selected solvent system, at a fixed temperature, a law that was first recognized by Nerst in 1891 [8]. However, this simple law may not hold true in all the experiments because of molecular interactions between the solute

Table 1
Comparison of lanatoside C (LC) partition coefficients (K) accomplished by different procedures

Dichloromethane–methanol–water solvent system							
Composition	Pre-treated *D. lanata* extracts	LC in the starting material (%)	Obtained LC (%)	Calculated K^a	Experimental K^b	Experimental K^c	Variation between K^b and K^c (%)
4:4:3	RLCC-1	21	56	0.635	0.723	0.943	30
5:6:4	RLCC-2	22	91	0.568	0.717	0.510	29
5:6:4	RLCC-3	60	96	0.458	0.717	0.638	11
7:13:8	RLCC-4	13	57	0.300	0.705	0.612	13

[a] Values calculated from RLCC chromatograms, using Eq. (1), for pre-treated *Digitalis lanata* extracts (see Section 2).
[b] Values determined experimentally for standard samples, using the shake flask method (see Section 2.9).
[c] Values determined experimentally for pre-treated *Digitalis lanata* extracts, using the shake flask method (see Section 2.9).

and the solvent molecules and the effects of concentration of the solute molecules [8]. Thus, it is expected that a complex matrix, such as a vegetal extract, may have influence on the *K* value of a component. Such possible interference can be expected in *Digitalis* extracts, where one can find over 70 cardenolides with a large range of polarity [9]. In order to evaluate such effect on *K* for lanatoside C, partition coefficients were calculated by Eq. (1), from RLCC chromatograms performed with dichloromethane–methanol–water (4:4:3), (7:13:8) and (5:6:4) as solvent systems. The accomplished data were compared with those obtained by the shake flask method for standard samples (Table 1). The calculated values presented a considerable variation that can be attributed to the peculiarities in the pre-purification methods for the extracts, before injection into the RLCC system. The employed pre-purification procedures (Fig. 2) resulted in starting materials for RLCC with very distinct cardenolide profiles, as attested by their HPLC chromatograms (Fig. 3).

The pre-purification performed in experiment RLCC-1 included adsorption in PVPP and XAD-7 resins, followed by reversed-phase chromatography (Fig. 2), which led to a starting material for RLCC consisting of cardenolides with a large range of polarity (Fig. 3) and a lanatoside C content of 21%. Silica gel chromatography was the pre-treatment employed in experiment RLCC-2 (Fig. 2), which also resulted in a starting material comprising cardenolides of diverse polarity (Fig. 3), with a similar lanatoside C content (22%). Pre-purification procedures performed in experiments RLCC-3 and RLCC-4 were similar to that employed in experiment RLCC-1, but different eluents were employed for the reversed-phase chromatography (Fig. 2). Combined fractions eluted with methanol–water (50:50) from the reversed-phase column were employed as starting material for RLCC-3, which was composed of medium polar cardenolides (Fig. 3), with a high lanatoside C content (60%). In experiment RLCC-4 (Fig. 2), elution from the reversed-phase column was performed with methanol–water (70:30). The starting material accomplished with this eluent was composed of several cardenolides of varied polarity (Fig. 3), lanatoside C comprising 13% of it.

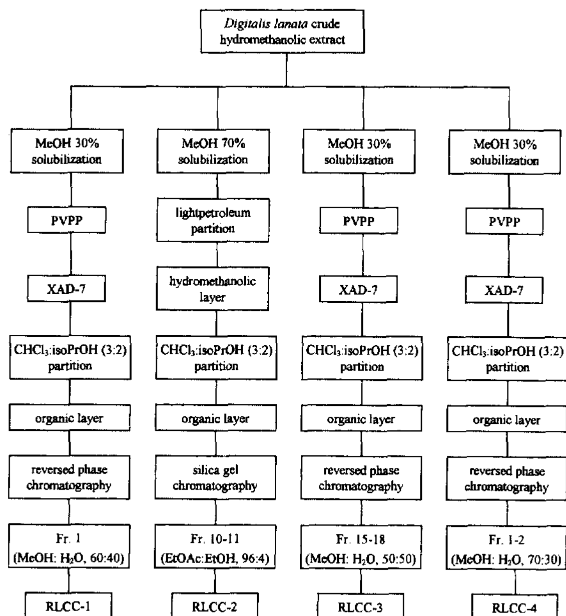

Fig. 2. Sequences of pre-purification for *Digitalis lanata* hydromethanolic extract. See Sections 2.3–2.5 for detailed description.

The differences in the cardenolide profiles of the starting material for RLCC reflected considerably on *K* for lanatoside C and resulted in the isolation of this cardenolide with distinct purity (Table 1). Therefore, the type of pre-treatment applied to the extract plays an important role when isolating substances by RLCC, since it determines the composition and concentration of the matrix which, in turn, will affect *K* and consequently the efficiency of the chromatographic process.

In order to confirm the differences between *K* values accomplished for standard samples (K^b) and the values obtained for complex matrixes (K^c), we determined *K* for lanatoside C for pre-treated *Digitalis lanata* extracts, using the shake flask method (Table 1). Again, *K* values varied significantly according to the matrix composition. The greatest variation of *K* in these experiments, in a percentile basis, related to differences in values obtained for standard samples, was 30% and 29%, respectively, for RLCC-1 and RLCC-2 experiments (Table 1). It is worthwhile to note that the maximal variations in *K* values were manifested in those pre-purified extracts with various components at high concentrations (Fig. 3).

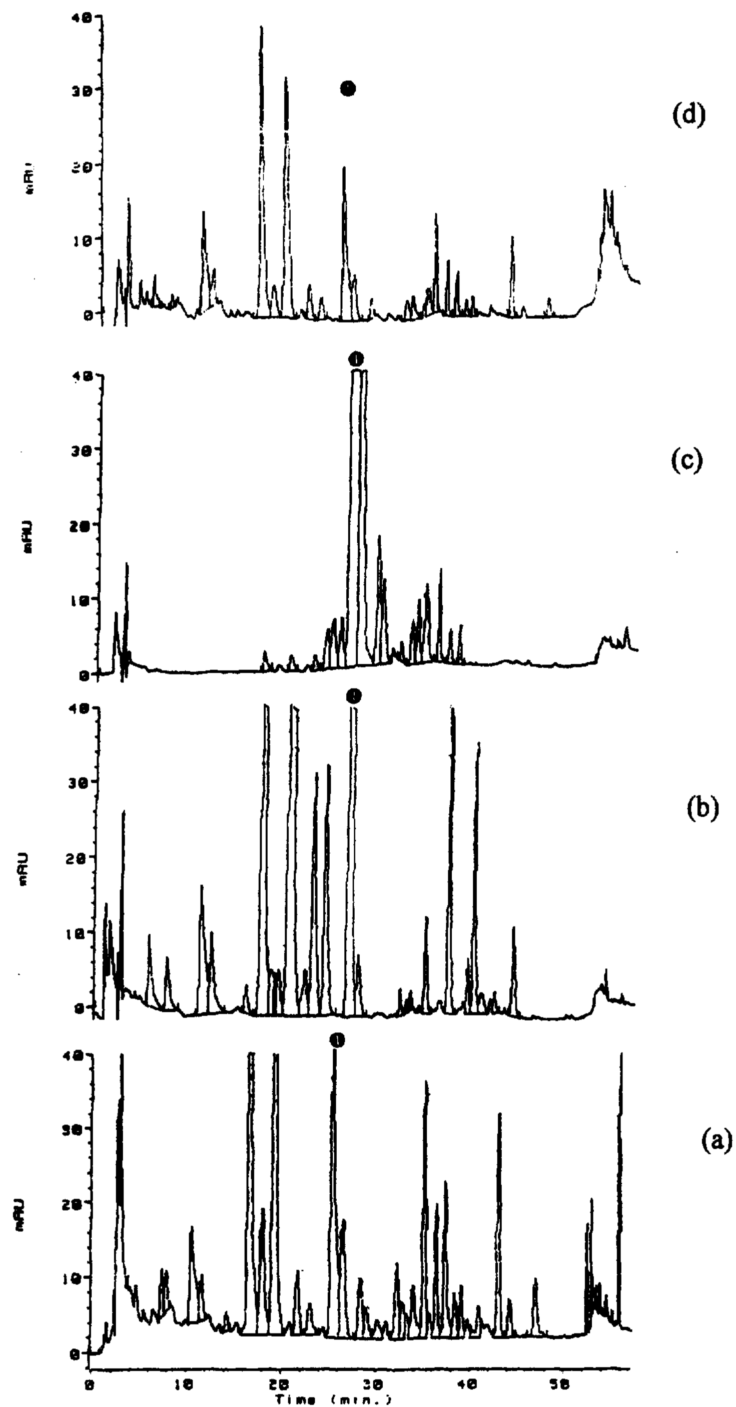

Fig. 3. HPLC chromatograms of starting materials for RLCC, accomplished by different pre-purification methods. (a) Experiment RLCC-1, (b) experiment RLCC-2, (c) experiment RLCC-3 and (d) experiment RLCC-4. Chromatographic conditions: see Section 2. (1) Lanatoside C.

The differences in K values do not seem to arise from the use of distinct solvent system compositions for the RLCC separations, since K for lanatoside C standard samples, determined by the shake flask method, were similar in all the three tested solvent systems (Table 1). A remarkable illustration of how the composition of the starting material affects K is accomplished by experiments RLCC-2 and RLCC-3. In both experiments, dichloromethane–methanol–water (5:6:4) was employed as solvent system for RLCC but the partition coefficients for lanatoside C were very distinct (Table 1). The starting material for experiment RLCC-2 had a lanatoside C content of 22% and K value was 0.510. On the other hand, experiment RLCC-3 had a starting material with a 60% lanatoside C content and K value was 0.638. Such astonishing difference in K values for lanatoside C, representing 25% of variation, was not observed for K determined for a standard sample, where the maximal variation was 2.5%, between experiments RLCC-1 ($K=0.723$) and RLCC-4 ($K=0.705$). Thus, the observed differences are due to the composition of the starting material, which, in turn, is a consequence of the pre-treatment procedures.

These results have demonstrated that the composition of a complex matrix, like a plant extract, modify considerably the K value of a component, thus affecting counter-current efficiency. In order to evaluate if a less complex matrix has also an influence on K, we prepared a mixture of cardenolide standard samples and determined their partition coefficients by calculation from RLCC chromatograms. Hence, a mixture of standard cardenolides, including desacetyllanatoside C, lanatoside C, digoxin, lanatoside A and digitoxin (Fig. 1) were injected into the RLCC system, employing dichloromethane–methanol–water (5:6:4) as solvent system and the K values were calculated from the chromatogram, by means of Eq. (1). Each standard cardenolide sample has also had its K determined, individually, by the shake flask method. The values of K obtained by calculation from the RLCC chromatogram were considerably different from the experimental K (Table 2), in a way similar to that observed in the preceding experiments with *D. lanata* extracts (Table 1).

Table 2
Comparison between the partition coefficients (K) of cardenolide standard samples experimentally determined by the shake flask method and those calculated from a RLCC chromatogram

Cardenolide	Dichloromethane–methanol–water (5:6:4)	
	Experimental K^a	Calculated K^b
Desacetyllanatoside C	0.114	0.167
Lanatoside C	0.717	1.002
Digoxin	1.863	2.004
Lanatoside A	4.553	ne
Digitoxin	19.680	ne

[a] Values determined experimentally for standard samples, using the shake flask method (see Section 2.9).
[b] Values calculated from RLCC chromatograms, using Eq. (1) (see Section 2).
ne = Not eluted from RLCC columns under the experimental conditions.

In conclusion, the different pre-treatment procedures for a *Digitalis lanata* hydromethanolic extract, before injection into the RLCC system, resulted in starting materials with very distinct cardenolide profiles. Such differences affected the partition coefficients of lanatoside C and reflected, subsequently, in RLCC efficiency. Generally speaking, these results have demonstrated that the selection of pre-treatment procedures for a plant extract to be fractionated by counter-current chromatography is crucial, since it will affect the partition coefficient of a component, and, consequently, the efficiency of the whole chromatographic process.

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References

[1] W.D. Conway, in: N.B. Mandava, Y. Ito (Eds.), Counter-Current Chromatography: Theory and Practice, Marcel Dekker, New York, 1988, Ch. 4, pp. 443–461.
[2] Y. Ito, R. Bowman, J. Chromatogr. Sci. 8 (1970) 315.
[3] Y. Ito, R. Bowman, Anal. Chem. 43 (1971) 69A.
[4] J.K. Snyder, K. Nakanishi, K. Hostettmann, M. Hostettman, J. Liq. Chromatogr. 7 (1984) 243.

[5] I. Kubo, G.T. Marshall, F.J. Hanke, in: N.B. Mandava, Y. Ito (Eds.), Counter-Current Chromatography: Theory and Practice, Marcel Dekker, New York, 1988, Ch. 6, pp. 493–507.
[6] F.C. Braga, W. Kreis, A.B. de Oliveira, J. Chromatogr. A 756 (1996) 287.
[7] W.D. Conway, J. Chromatogr. A 538 (1991) 27.
[8] B.N. Mandava, J.M. Ruth, in: N.B. Mandava, Y. Ito (Eds.), Counter-Current Chromatography: Theory and Practice, Marcel Dekker, New York, 1988, Ch. 2, pp. 27–77.
[9] M. Wichtl, W. Bühl, G. Huesmann, Dsch. Apoth. Ztg. 127 (1987) 2391.