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Polyphenols in countercurrent chromatography. An example of large scale separation[1]

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Abstract. Polyphenols are sometimes difficult to separate in classical liquid chromatography. Countercurrent chromatography uses a biphasic liquid system to separate the components of a mixture. A centrifugal field allows to use a liquid stationary phase in an open tube. The phase density difference and the centrifugal field are the only parameters allowing the equilibrium between the two liquid phases. The big advantage of the technique in preparative separation is the dual-mode capability of CCC. The role of the phases can be switched during a run. The mobile phase becomes stationary and *vice versa*. Then no injected material can be left in the machine. The large scale separations of a flavonoid mixture and two tannin samples are described: choice of the biphasic liquid system, analytical study, scaling-up, injection protocol, two step separation in case of dual-mode use. It was possible to inject 26 g of a tannin sample in one run.

Keywords. Countercurrent chromatography – polyphenol – preparative chromatography – flavonoid tannin.

Introduction

Polar compounds are commonly found in vegetal extracts. Saponins, cardenolides, iridoids, alkaloids, antibiotics, tannins, flavonoids, quercetrins xanthones, lignans and other polyphenols or sugar or glycoside derivatives are examples of polar compounds very commonly found in vegetal extracts and used as active principles for their pharmacological properties [1]. They are often difficult to separate by HPLC and countercurrent chromatography can be a very useful tool to purify them [2-3].

Countercurrent chromatography (CCC) is the separation technique that uses a liquid stationary phase. The mobile phase is also a liquid phase, *i.e.*, a biphasic liquid system is used. It is important to realize that in most CCC separations there are absolutely no countercurrent flowing liquids. The name of the technique was coined by Yoishiro Ito, its inventor [4]. Publishing more than 250 articles using it, he made the CCC acronym so related to the liquid-liquid centrifugal partition chromatography technique that it is now accepted worldwide [5].

The advantages of the use of a liquid stationary phase are:

- a simple retention mechanism (liquid-liquid partition);
- no irreversible solute adsorption;
- high loading capability;
- large range of polarity;
- little solute modification, degradation or denaturation;
- original selectivity;
- few pH limitations;
- low cost.

The main problem in CCC is to retain the liquid stationary phase when the liquid mobile phase is pushed through it. Centrifugal forces are used in all modern CCC apparatuses [2, 3, 5, 6]. The liquid stationary phase is retained in channels by a constant centrifugal field in hydrostatic (HS) apparatuses [7]. It is retained in coils of Teflon tubes by a variable field in hydrodynamic (HD) apparatuses [6]. Another problem is the efficiency reduced to a maximum of one plate per channel for HS apparatuses and two to three plates per coil turn for HD machines. The efficiency of CCC machines ranges from 100 plates to 2000 plates depending on the geometrical characteristic of the machine but also on the biphasic liquid system used and on the operating conditions.

In this work, the separation of polyphenols of vegetal origin is described and the scaling-up of a tannin separation is presented.

Experimental

CCC Apparatuses

Three different HD CCC apparatuses were used. They are described in table I. The analytical machine was constructed by the late SFCC company (Société Française de Chromato Colonne) (Éragny, France) that discontinued its CCC machine production in 1992. The two Kromaton, models 2 and 3, preparative machines were built by SEAB (64, rue Pasteur, 94807 Villejuif, France).

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Table I. Instrumental characteristics of the hydrodynamic CCC machines.

Model	*characteristics*	*volume mL*	*ratio β*	*tube length m*	*tube diameter mm*	*number of turns*
SFCC 2000	3 spools rotating vertically	52	0.56	29	1.6	133
		156	0.56	87	1.6	400
Kromaton 2	2 spools rotating horizontally	94	0.67	19	2.5	30
		1070	0.67	218	2.5	345
Kromaton 3	2 spools rotating horizontally	190	0.80	94	1.6	125
		396	0.80	197	1.6	260
		981	0.80	200	2.5	260
		1972	0.80	402	2.5	520

The SFCC machine can work with one or three spools. The spools of the Kromaton machines are coiled with different tubes. The β ratio is r/R, the ratio of the spool diameter over the rotation diameter (distance between the rotor axis and the spool axis). All three machines have a temperature regulation system.

Other hardware

Only the column is special in CCC. The peripherical materials, pumps, valves, detectors and recorders or integrators are classical LC hardware. A Shimadzu LC-10AS pump was used for flow rates up to 9.9 mL/min. A Shimadzu LC8A prep-pump was used when higher flow rates were desired. A Shimadzu SPD-6A UV detector and a Cunow DDL 21 evaporative light scattering detector were used connected to a Shimadzu C-R5A and a C-R6A integrator, respectively. Touzard et Matignon (Dukert, Courtabœuf, France) was the supplier. Thin layer chromatography was done with Merck 10×10 plates code 60-F254.

Solvents

Ethyl acetate, butanol and butanone (methyl ethyl ketone or MEK), methyl isobutyl ketone (MIBK) were from SDS (Peypin, France). Methanol and acetic acid were obtained from Fluka (Sigma-Aldrich, St Quentin Fallavier, France). They were used as received. The CCC relevant physico chemical properties are listed in table II. Water was deionized and distilled.

Samples

The Quercetrin extract was obtained from L. Light & Co. Ltd., Colinbrode, England. The Hamamelis extract was furnished by C.A. Erdelmeier of Willmar Schwabe GmbH, Karlsruhe, Germany. The tannin mixture was the product number 48812, Tannic acid, of Fluka.

Protocol

Since a centrifugal field is used to hold the liquid stationary phase in the CCC machine, a CCC "column" exists as long as the machine rotor is rotating. Unless otherwise indicated, we used the following protocol to prepare a CCC "column": first the CCC machine is filled at a high flow rate with the liquid phase chosen to be the stationary phase. 200 min (more than 3 hours) were needed to fill the 2L machine at 10 mL/min. Then the rotor is started at the desired speed and the mobile phase is pushed in the machine in the correct way: from head to tail (top to bottom) if the mobile phase is the denser liquid or from tail to head if it is the lighter phase [3, 6, 8]. The mobile phase equilibrates with the stationary phase turns after turns displacing part of the later. Only the stationary phase is seen exiting the machine. It is collected in a graduated cylinder. When the mobile phase exits out of the machine, the CCC column is ready. The collected stationary phase volume corresponds to V_M, the volume of the mobile phase inside the machine that is the dead volume. The stationary phase volume retained by the machine is $V_S = V_T - V_M$. These volumes are used to calculate P, the liquid-liquid partition coefficient of an injected solute using its retention volume, V_R:

$$V_R = V_M + PV_S = V_T + (P - 1)V_S \quad (1)$$

If the partition coefficient is known, then Eq. (1) allows to predict accurately the retention volume of the solute using the phase volumes inside the CCC "column." Often the retention ability of a CCC machine is rated by the phase retention factor, Sf:

$$Sf = V_S/V_T \quad (2)$$

The Sf factor depends on the CCC machine and also on the biphasic liquid system used and the operating conditions [2, 6].

Table II. Physico-chemical properties of the solvents used.

Solvent	*density* g/cm^3	*viscosity* *cP*	*Solubility* % w/w 20 °C		*Polarity*		
			solvent in water	*water in solvent*	δ	*Snyder*	*Reichardt*
1-butanol	0.810	2.95	7.8	20.1	27.2	3.9	60.2
butanone	0.805	0.43	24	10	19.2	4.7	32.7
ethyl acetate	0.901	0.45	8.7	3.3	18.2	4.4	22.8
methanol	0.791	0.55	4	4	29.3	5.1	76.2
water	0.998	1.0	4	4	48.6	10.2	100

δ is the Hildebrand parameter. Butanone is also called methyl ethyl ketone (MEK).

Choice of the liquid system

How to define solvent polarity

Solvent polarity is roughly related to the interactions between the solvent molecules. If there are no interaction or only Van der Waals interactions between the molecules of a pure solvent, this solvent is considered as apolar, *e.g.*, alkanes, silicon oils, perfluorinated solvents. If there are interactions between the solvent molecules, then the solvent is said to be polar. Different polarity degrees are possible. Weak interactions (dipoles-induced dipoles) will correspond to weakly polar. Strong interactions are found in very polar solvents (hydrogen bonds, ionic interactions in room temperature ionic liquids). At the moment, there are different polarity scales such as the Hildebrand scale based on the solubility parameter, δ, defined as the work necessary to separate two solvent molecules. In chromatography, the Snyder polarity scale, based on the eluting power of the solvent in thin layer chromatography (TLC), is commonly used. In organic synthesis, the Reichardt scale is most often used. It is related on the transition energy for the solvatochromic absorption band of a pyridinium -N-phenoxyde betaine dye. Table II lists the polarity value in the three scales for the solvent used. In the Hildebrand and Reichardt scales, the order is the same: water is the most polar solvent, then methanol, butanol, butanone and ethyl acetate is the less polar. In the Snyder scale, butanol is considered as the less polar solvent. Its high viscosity reduces its eluting power in TLC. Such differences between the different polarity scales are very common [9].

Two or three solvent systems?

The two solvent biphasic liquid system is the most simple and convenient to use in CCC. It is simple because the mixing of the two solvents will produce the two liquid phases that are one solvent saturated by the other. The butanone-water system forms two liquid phases particularly useful in CCC because of their high polarity. The aqueous denser phase contains 76 % w/w water and 24 % w/w butanone (28 % v/v). The upper organic phase contains 10 % w/w water and 90 % w/w butanone (92 % v/v). In CCC, the phase that is used as the mobile phase is depleted more rapidly than the other phase. There is no problem to prepare more mobile phase. The mutual saturation of the butanone-water system is fast.

Often it is not possible to find a biphasic system able to perform the desired separation with only two solvents, then three solvents are used: a good solvent for the sample, a second solvent as good for the sample as possible and making a second liquid phase with the first solvent and a third solvent that partitions between the two phases. Ternary phase diagrams are used [7, 10]. The third solvent allows a fine tuning of the partition of the sample components between the two phases. Given the number of possible combinations of three solvents (and more), it can be difficult to find the best biphasic liquid system. General methods to find rapidly the liquid system to perform a good CCC separation were exposed in the literature [11-13]. Figure 1 shows the ternary mass diagram of the water (good solvent)-ethyl acetate (second phase solvent)-butanol system that was used for the separation of tannin samples. Point A locates the composition of the prepared mixture. Points B and C locate the composition of the upper organic and lower aqueous phase obtained when the A mixture is well equilibrated. The BC line is the tie-line corresponding to the A biphasic mixture. Table III lists the corresponding w/w and v/v compositions.

Separation of a flavonoid sample

The sample called “Quercitrin extract” was fractionated using the simple butanone-water biphasic system. A rapid analysis using the small volume coil of the Kromaton 2 machine showed in 20 min at 2 mL/min (organic mobile phase) that the fractionation of the sample was possible. However, the small coil did not have a sufficient efficiency to resolve fully the sample: only two peaks were obtained.

The large volume coil of the Kromaton 2 machine was then filled with the butanone saturated aqueous stationary phase in more than two hours at 8 mL/min. Next the rotor was started at 400 RPM and the organic mobile phase was

Table III. Composition of the A, B and C points of Figure 1.

Figure 1	*butanol*		*ethyl acetate*		*water**		*density*
composition	*% w/w*	*% v/v*	*% w/w*	*% v/v*	*% w/w*	*% v/v*	*g/cm³*
A global mixture	3.0	3.5	44.3	46.5	52.7	50	B and C phases
B upper phase	4.9	5.5	90.6	90.5	4.5	4	0.90
C lower phase	1.2	1.5	8.5	9.3	90.3	89.2	0.99

* in most experiments, water was actually a 1 % acetic acid solution (pH 2.8)

Table IV. Chromatographic parameters corresponding to Figure 2.

compound	*tr* *min*	*Vr* *mL*	*N* *plates*	*k*	$P_{butanone/water}$	$P_{water/butanone}$
kaempferol	67	201	800	0.95	10	0.10
quercetin	86	258	700	1.52	6.2	0.16
kaempferol rhamnoglucoside	100	300	640	1.90	4.9	0.20
quercitrin	129	387	(600)*	2.75	3.4	0.29

* estimated value (peak off scale)
k: retention factor calculated as $P_{water/butanone} * V_S/V_M$
Experimental CCC conditions described in Figure 2 caption

pumped into the rotating machine at 3 mL/min in the tail-to-head direction. The stationary phase retention factor was 90.4 % (V_M = 102 mL, V_S = 968 mL, V_T = 1070 mL). The flavonoid sample was separated in four components with 650 mL and in 4 hours. Figure 2 shows the CCC chromatogram of a 0.33 g injection. The compounds were identified using mass spectrometry as described in a recent article [14].

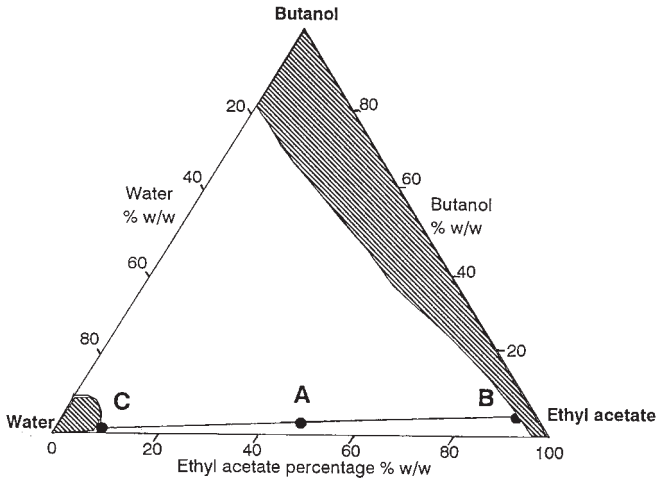

Fig. 1. The ternary mass diagram of the system water-ethyl acetate-butanol at 20 °C. Hatched areas: monophasic compositions (not usable in CCC). Composition A was prepared to perform the tannin separation. It gives Compositions B and C whose formulas are listed in Table III.

Table IV lists the chromatographic parameters that can be established from the CCC chromatogram. The retention volumes give the butanone/water partition coefficients of the compounds. These physico chemical parameters are precious data in hydrophobic studies, in quantitative structure-activity relationships (QSAR) and quantitative structure-retention relationships (QSRR) in pharmacological or chromatographic studies, respectively. CCC is a very powerful tool to determine accurately the liquid-liquid partition coefficients of solutes [8, 9, 15].

Table IV and Figure 2 also show that CCC compensates easily its low efficiency by a high selectivity power. Kaempferol and quercetin as well as the rhamnoglucoside derivative and quercitrin differ by only an oxygen atom in the R1 position (Fig. 2). This oxygen atom produces a 60 % and 45% increase of the $P_{water/butanone}$ coefficient, respectively (Table IV). Since the *k* retention factor is directly proportional to the P coefficient, the same change is observed on the values. This produced a selectivity factor, α, of 1.6 between kaempferol and quercetin and 1.5 between the glucoside derivatives ($\alpha = k_2/k_1$). Such selectivity factors are high enough to produce an almost baseline resolution of the compound with an efficiency of only about 600 plates.

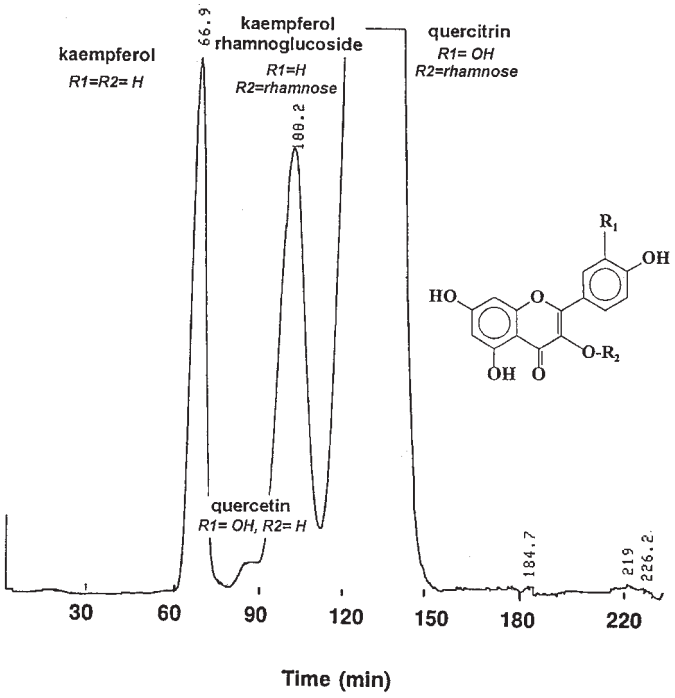

Fig. 2. CCC chromatogram of the quercetin sample. Mobile phase: butanone 3 mL/min, tail to head. Stationary phase: aqueous. V_T = 1070 mL, V_M = 102 mL, V_S = 968 mL, 400 rpm. Injection: volume 9 mL of a 37 g/L solution, mass 330 mg. Detection: UV 330 nm.

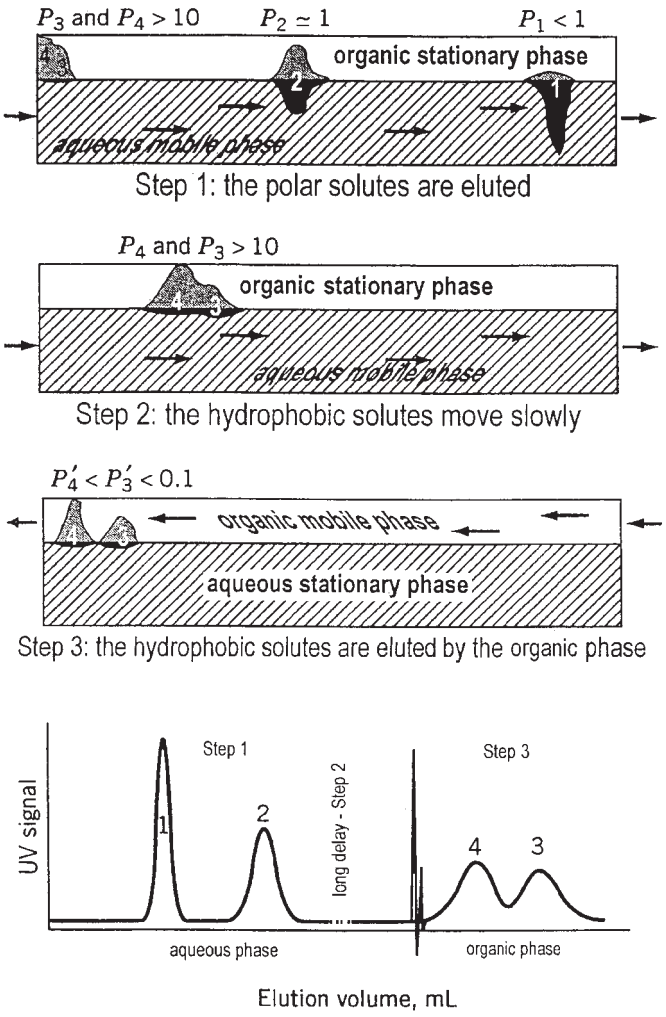

Fig. 3. The dual-mode use of a CCC machine. Steps 1 and 2 are done with the aqueous phase in the head to tail direction. Step 3 is done with the organic light phase in the tail to head direction. Bottom: the corresponding chromatogram.

Mass separation of tannins

Tannins are polar polyphenols of vegetal origins. They are difficult to separate because in a vegetal extract numerous isomers with a very similar molecular basis coexist [19, 20]. The use of a liquid-liquid system to fractionate tannin samples produces original selectivities and preparative capabilities that were already used [3, 6-7, 16, 19, 20]. We present here the separation of the tannin sample sold by Fluka with the name "Tannic acid" product 48812 valid only for the batch #365176/1 (Feb. 12, 1997). We bought a second 250 g pot (batch #395301/1 (May 5, 1998)) that gave different chromatograms. The commercial tannin mixture was used to select the suitable liquid system and investigate the loading capabilities of our hydrodynamic CCC machines.

Optimization using the low volume machine

The SFCC 2000 machine was used to search for the optimal liquid system. Using and adapting the Margraff approach [12], it was found that the composition A that separates in organic phase B and aqueous phase C (Fig. 1) of the water-butanol-ethyl acetate system was able to partition correctly the tannin sample. Table III lists the exact compositions of the phases.

It was not possible to separate the sample directly. The dual-mode was used. Figure 3 explains graphically this unique way to operate a CCC machine using the liquid nature of the stationary phase [7, 17]. Step 1 is the normal way to use a chromatograph with a mobile, say aqueous, and a stationary, say organic, phase. The aqueous denser phase moves in the head to tail way. In Step 2, it is shown that solutes 3 and 4 would need a huge mobile phase volume to elute. They are hydrophobic with a high affinity for the organic apolar phase. The answer to elute them is to switch the phase role: the stationary phase becomes the mobile phase and *vice versa*. Step 3 shows that solutes 3 and 4 are easily eluted by the organic mobile phase in the opposite tail to head way. Furthermore, the separation initiated in Step 2 is improved in Step 3 since 4 moves faster than 3 with the organic mobile phase. The advantages of the dual mode use

were evidenced in the partition coefficient determination [8, 15] and general uses of CCC [7, 18].

The tannin sample was fractionated in 12 peaks. 7 peaks eluted in the head to tail way with an aqueous mobile phase of composition C (Fig. 1). 5 more peaks eluted in the reversed tail to head way with the composition B organic mobile phase (not shown by Figure 4, see Table V). The paramount advantage of the dual mode use of CCC is that nothing can be left in the machine if the organic phase volume, V_{org}, pushed in the reversed tail to head way is higher than:

$$V_{org} \geq V_{aq}\, V_S/(V_{aq} - V_M) \qquad (3)$$

in which the subscript aq, S, and M refer to the volume of aqueous phase pushed in the head to tail direction after the sample injection in Steps 1 and 2 (Fig. 3), the volume of organic phase that was retained and stationary in Steps 1 and 2, and the volume of aqueous phase that was mobile in Steps 1 and 2, respectively. The V_S volume of organic phase in the machine should remain the same during Step 3. Usually it does [7, 9, 15, 17, 18, 20].

Tannins are very sensitive to small pH changes, they oxidize readily in basic media. To stabilize the aqueous phase pH at a 2.8 value, 1 % v/v acetic acid was added to water before preparing the biphasic system. Table V lists the retention values and partition coefficients corresponding to the peaks obtained. The partition coefficients listed correspond to the affinity of the compounds for the organic phase ($P_{org/aq}$). The affinity for the aqueous phase would be measured by the inverse: $P_{aq/org} = 1/P_{org/aq}$. The peak elution order from 1 to 7 (Fig. 4) corresponds to the increasing $P_{org/aq}$ order. The P values are obtained by Eq. 1. The mode inversion is done after a volume V_{aq} of aqueous phase is pumped in the head to tail direction. Then, the mobile phase becomes the organic phase pumped in the tail to head direction (Fig. 3). The peaks elute in decreasing $P_{aq/org}$ order (Table V). The P value is calculated as $P = V_{aq}/V_R$ [15, 17]. The volume of organic phase needed to elute all the hydrophobic tannins out of the CCC machine is 95 mL (Eq. 3). When 100 mL of organic phase are pumped in the tail to head direction, it is certain that no injected compound remains in the machine. This experiment shows that CCC was able to separate peaks corresponding to compounds with P values ranging from 0.08 (hydrophilic) to 67 (less polar). However tannins are so complex that the separated peaks did not correspond to a single component. A thin layer chromatography plate was done for every peak. All of them, except maybe peak #8 showed more than one spot after elution with an AcOEt-MIBK-Acetic acid (50-50-1 v-v-v) mobile phase and revelation with a $FeCl_3$ pulverization (blue spots). Evaporating the collected fraction, it was found that Peak #8 contained more than 80% of the injected mass.

Scaling-up the separation

The tannin separation was scaled-up injecting larger amounts on the same machine. A 30 g/L solution was prepared by dissolving the exactly weighted amount of tannins in the adequate aqueous phase volume. A 300 g/L solution was prepared by dissolving 30 g of the tannin sample in a mixture of 5 mL of aqueous and 5 mL of organic phase. The resulting solution was monophasic. Figure 4 shows the chromatogram obtained when 30 mg (1 mL of the 30 g/L solution, Fig. 4-A) and 750 mg (2.5 mL of the 300 g/L solution, Fig. 4-B) were injected in the 150 mL machine. The loading capability of the CCC machine are demonstrated. Almost no broadening can be seen on Peaks #6 and #7 between the 30 mg and 750 mg injections. Peaks 1, 4 and 5 broaden somewhat, but they correspond to a twenty-time higher amount of tannins. The retention volumes are not affected by the concentration increase.

The 300 g/L solution had an elevated viscosity. Injection loops with volumes higher than 2.5 mL could be made but a pressure increase due to the sample viscosity limited their use (1/16" tubing, 0.5 mm i.d.). The Kromaton CCC machine can use 1.6 mm and 2.5 mm i.d. tubing in its injection port. A 66 mL loop was made allowing to inject 20 g when the 300 g/L solution was prepared. The 2L spool configuration was equilibrated with the Table III liquid system. The organic phase retention ratio was only 26% at 420 rpm. The 66 mL injection of the viscous tannin solution produced an important stationary phase leak. The injected plug forms a piston pushing the biphasic liquid system out of the machine and no separation occurs. After some unsuccessful injections, the protocol was modified as follows: 17 min were needed to inject the sample at 4 mL/min, then 120 mL of aqueous mobile phase were further introduced in the machine at 4 mL/min for 30 more minutes, next the flow rate was stopped, the mode valve was switched in the opposite way (tail to head) and the flow rate was resumed for

Table V. Chromatographic parameters of the tannin separation presented in Figure 4.

peak	*tr* *min*	V_R *mL*	*P*	*calculation*
1	40.0	80.1	0.08	
2	0.81	258	0.22	$P = (V_R - V_M)/V_S$
3	0.805	111.3	0.48	V_M = 74 mL
4	74.4	148.7	0.96	Vs = 78 mL
5	89.6	179.2	1.35	V_T = 152 mL
6	0.998	289.0	2.76	Sf = 52%
7	193.6	387.2	4.02	
Mode inversion after 215 min (not shown in Figure 4)				
8	3.21	6.42	67.0	
9	5.48	10.96	39.2	$P = V_{aq} / V_R$
10	9.31	18.62	23.1	V_{aq} = 430 mL
11	23.8	47.6	9.0	
12	37.8	75.6	5.7	

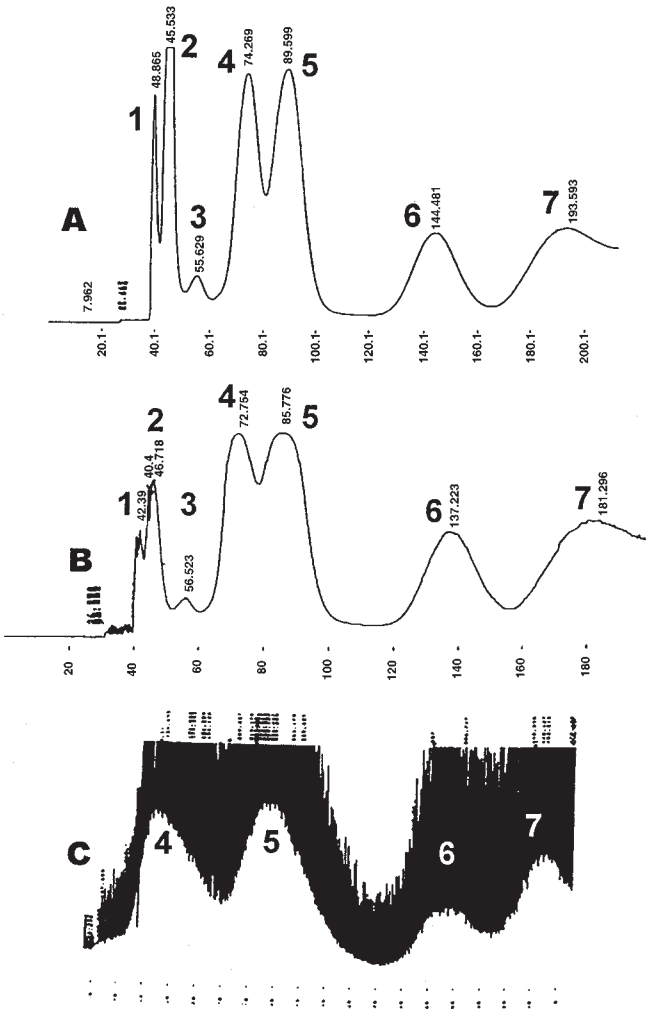

Fig. 4. Separation of a vegetal tannin sample. A- separation of 30 mg (1 mL of 30 g/L solution) by the SFCC machine (V_T = 152 mL). B- Scaling-up with the same machine. Separation of 0.75 g injected in 2.5 mL of a 300 g/L solution. 800 rpm, aqueous mobile phase in the head to tail direction, 2 mL/min, Sf = 56 %. C- Large scale separation (20 g in 66 mL of a 300 g/L solution) with the Kromaton 3 2L-preparative machine. 420 rpm, 4 mL/min, Sf = 26 %. 280 nm UV detection. Liquid system: see Table III.

10 min. This was designed to dissolve the plug of injected phase and to force the sample to partition between the two liquid phases. After that, the flow rate was stopped again, the machine was let to equilibrate for 5 min, the mode valve was switched back in the correct position (head to tail) and the flow rate was resumed at 4 mL/min. Figure 4-C shows part of the obtained chromatogram. The retention volume of Peak #7 was 3.3 L (almost 14 hours retention time).

The important noise observed on the chromatogram 4-C is due to a significant stationary phase leak. Microdroplets of stationary phase pass through the detector producing the numerous spikes making the noise. The organic stationary phase loss was measured to be 20 mL/h. The Sf phase retention ratio dropped from an initial 26 % value (V_S = 515 mL) to Sf = 11 % (V_S ~210 mL) at the end of the head to tail phase of the preparative tannin separation. The injection protocol and the separation done with a continuously decreasing stationary phase volume modified somewhat the chromatogram aspect. It is still possible to note that the peak looks gaussian with minimal distortion (Fig. 4-C). This was not true during the tail to head phase of the separation. A huge and broad peak was obtained, but it was not possible to locate the 5 peaks obtained with the analytical CCC machine. This is likely due to the stationary phase volume reduction. The stationary phase loss could easily be reduced or even stopped by increasing the centrifugal field (rotation speed). The apparatus we used was a prototype limited to a 450 rpm maximum rotation speed. This rotation speed may be not enough to create a centrifugal field song enough to retain correctly polar biphasic liquid systems. The rotor mass is in the 30 kg range when loaded with the liquids. The overall rotation diameter (rotor and spool) is in the half meter range. It is obviously difficult to make a well-balanced rotor of this size and mass. The small vibrations generated by the minute out-of-balance may became dramatic and dangerous when the rotation speed is increased. This mechanical problem is under investigation and it should be resolved since a 600 to 800 rpm speed (2 to 4 times higher centrifugal field) seems desirable.

The protocol established with the commercial tannin sample was applied to a real sample, a Hamamelis extract, proposed by Schwabe (Germany). This extract had been purified to refine the glucoside hamameli-tannin. It contained a large majority of the desired tannin along with non desired tannins. The analytical separation of the Hamamelis tannin sample with the Table III biphasic liquid system showed 4 peaks: 3 impurities (25 % w/w) and a major peak containing about 3/4 of the injected mass. The UV spectra of the collected fractions were too similar to allow the characterization of the hamameli-tannin. We suppose that the major tannin was the desired one. The preparative separation with the 2L Kromaton machine was done in two steps: 11 hours (2.6 L) in the head to tail direction with the aqueous mobile phase and 6 hours (1.4 L) in the opposite tail to head direction with the organic mobile phase. The major peak (more than 70 % of the injected mass) was the first to elute with a 390 min retention time or 1560 mL retention volume. If this peak was Hamameli-tannin, it was completely eluted in 420 min (1.7 L). If the other tannins are not wanted, it is possible to stop the CCC machine after 420 min, to empty it rapidly using a compressed gas and to start a new purification of 25 g. With these conditions, the throughput of the production can be estimated to 20 g in 8 hours or 2.5 g/h.

Conclusion

The preparative capability of CCC is one more time demonstrated. Large volume CCC machines that are appearing on the market, are able to separate large mass of sample in one

run giving throughput comparable with classical prep-LC. The original selectivity obtained with the biphasic liquid system chosen is associated with the paramount advantage of the guaranteed total recovery of the injected mass. Other chromatographic modes not presented in this work, such as displacement chromatography or pH zone refining [19], are able to separate even higher mass of sample in one run. As the reliability of the CCC machines increases (and the noise they generate decreases !), they will be more and more used as powerful tools to purify large mass of compounds. CCC machines are already parts of production processes in the pharmaceutical industry.

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