Schinus terebinthifolius countercurrent chromatography (Part II): Intra-apparatus scale-up and inter-apparatus method transfer

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Abstract

Countercurrent chromatography (CCC) is being widely used across the world for purification of various materials, especially in natural product research. The predictability of CCC scale-up has been successfully demonstrated using specially designed instruments of the same manufacturer. The reality is that the most of CCC users do not have access to such instruments and do not have enough experience to transfer methods from one CCC column to another. This unique study of three international teams is based on innovative approach to simplify the scale-up between different CCC machines using fractionation of *Schinus terebinthifolius* berries dichloromethane extract as a case study. The optimized separation methodology, recently developed by the authors (*Part I*), was repeatedly performed on CCC columns of different design available at most research laboratories across the world. Hexane – ethyl acetate – methanol – water (6:1:6:1, v/v/v/v) was used as solvent system with masticadienonic and 3β-masticadienolic acids as target compounds to monitor stationary phase retention and calculate peak resolution. It has been demonstrated that volumetric, linear and length scale-up transfer factors based on column characteristics can be directly applied to different i.d., volume and length columns independently on instrument make in an intra-apparatus scale-up and inter-apparatus method transfer.

Key-words: *Schinus terebinthifolius*, intra-apparatus scale-up, inter-apparatus method transfer, countercurrent chromatography, high performance countercurrent chromatography, high speed countercurrent chromatography

1. Introduction

Countercurrent chromatography is a liquid-liquid partition chromatography, in which the liquid stationary phase is retained in the apparatus using centrifugal force instead of a solid support [1]. Separation is based on the partition of compounds between the two immiscible liquid phases [2].

The use of a liquid stationary phase leads to many advantages over the conventional techniques, for example, 100% sample recovery as no solid support is used [1], high loading capacity due to the larger amount of stationary phase in the column [3], easy and predictable scale-up from the analytical to preparative scale [4].

Because of its feasibility and development of more robust equipment, increasing attention has been given to CCC scale-up over the past few years [5-8]. However, the reality for those trying to work in this field is the difficulty in matching apparatus and columns from different manufacturers, especially when transfer methodology from one country, instrument and scale to another. In the literature, there is only one example of direct transfer, gluraphanin separation, which was done by trial and error [9].

Differences in instrument design (columns geometry and their arrangement on a rotor) directly affect important parameters in CCC: stationary phase retention, mixing/settling and, as consequence, peak resolution. Stationary phase retention is a measure of hydrodynamic equilibrium of a solvent system in a column, while resolution is a measure of efficiency of the mixing and settling process [10]. The direct transfer of operating conditions between instruments of different manufacturers or even between different models of the same manufacturer will not give the same results. In these case, scale-up theory cannot be directly applied, making method transfer highly complex and time consuming.

Almost all available CCC equipment on the market and in research labs contains more than one column, often with different i.d. (tubing internal diameter), volume and length [3]. Therefore, the aim of this work was to look how the scale-up approach can be simplified to make it easier for any researchers to use their current CCC equipment for scale-up separations. Hence, two new terms have been introduced to make classification more clear. The first is an intra-apparatus scale-up to describe scale-up between different columns mounted in the same instrument. In this case, the scale-up calculations can be easily applied, since most of design parameters are maintained. The second one is inter-apparatus scale-up to describe scale-up between instruments of different makes. This is the most common situation for both academia and industry.

2. Experimental

2.1 General

Organic solvents used for the preparation of crude extracts and CCC separations were HPLC grade, purchased from Tedia Brazil (Rio de Janeiro, Brazil) or Sigma (Deisenhofen, Germany). All aqueous solutions were prepared with dionised water (18.2MΩ) purified by Milli-Q water system (Merck Millipore, USA).

2.2 Equipment

Analytical, semi-preparative and/or preparative CCC separations were performed on four different instruments representing three column arrangements currently available within the CCC community. All columns are made of fluorinated polymers (**Table 1**):

- Spectrum DE centrifuge (Dynamic Extractions, Tredegar, UK) equipped with two counterbalancing bobbins containing two perfluoroalkoxy polymer (PFA) multi-layer columns each (22 mL; 0.8 mm i.d. and 125.5 mL; 1.6 mm i.d.). The rotation speed is adjustable from 200 to 1600 rpm.
- Pharma Tech CCC 1000 (Pharma-Tech Research Corp., Baltimore, MD, USA) equipped with three bobbins containing one polytetrafluoroethylene (PTFE) multi-layer column each (about 285 mL × 2.6 mm i.d. each with total volume of 850 mL connected in series or 15 ml x 0.8 mm i.d. each with total volume of 45 mL connected again in series). The rotation speed is adjustable from 0 to 1200 rpm.
- Quattro HT-Prep countercurrent chromatograph (AECS, Bridgend, UK) equipped with two counterbalancing bobbins containing two PTFE multi-layer columns each (26 mL x1.0 mm i.d. and 234 mL x3.2 mm i.d. on one bobbin; 95 ml x2.0 mm i.d. and 98 ml x2.0 mm i.d. on another bobbin). The 95 and 98 mL columns connected in series gave 193 mL column used for the separations. The rotation speed is adjustable from 0 to 865 rpm.
- Multilayer Coil Separator - Extractor countercurrent chromatograph (P.C. Inc., Potomac, Maryland, USA) equipped with three PTFE multi-layer columns (15 mL x 0.8 mm i.d.; 80 mL x 1.6 mm i.d.; 230 mL x 1.6 mm i.d.) mounted on a single bobbin and counterbalanced with a counterweight. The rotation speed is adjustable from 0 to 1200 rpm.

All CCC systems were connected to a constant flow pump and a fraction collector. Only Spectrum DE and Quattro HT-Prep had in-built temperature control and it was set at 30°C.

2.3 Preparation of crude extract, two-phase solvent system and sample solution

Schinus terebinthifolius berries dichloromethane extract, solvent system and sample preparation methodology was taken from a previously published work by the authors [11]. However, in this research the original solvent system was modified by replacing Heptane with Hexane in Alkane-Ethyl acetate-Methanol-Water 6:1:6:1 (v/v/v/v) as this change does not affect solvent system properties [12].

2.4 G-level, Column Cross Sectional Area and Column Length calculations

Not all CCC instrument manufacturers provide data required for the calculation of fluctuating *g*- level, especially for multilayer columns. Therefore, in this work *g*-level calculation was done in a traditional way, at the point of column (bobbin) centre, (**Table 1**) using the following formula:

$$g\text{-}level = \frac{R\ \omega^2}{9.81}$$

where R is a rotor radius, distance between the central axis of device and the center of a bobbin around which column is wound; measured in meters; ω is the rotational speed of a column in radians/s and 9.81 is the earth's gravity acceleration at sea level measured in m/s^2.

Calculation of Cross Sectional Area (A) and Length (L) for each column was done using the following formulas:

$$\mathrm{A} = \frac{\pi\ d^2}{4} \qquad \mathrm{L} = \frac{V}{\mathrm{A}}$$

where d is internal diameter in millimeters and V is the column volume in milliliters.

2.5 Extra-column volume measurement

The extra column volume (V_{ext}) was determined (**Table 1**) as follows: the CCC set up (column, flying leads, tubing connecting column with pump and fraction collector) was entirely filled with mobile phase (MP). Then, stationary phase (SP) was pumped in and the displaced MP volume was measured using a cylinder. The column volume (V_c) given by the manufacturer was then subtracted from total system volume (V_{sys}) :

$$V_{ext} = V_{sys} - V_c$$

Each measurement was made until obtaining three equal values.

2.6 Analytical Separation Procedure

Three experimental procedures were carried out using each apparatus:

(1) Injection after reaching hydrodynamic equilibrium. The column was entirely filled with the SP, set rotating at required speed and MP was pumped into the column. After the MP front emerged indicating that hydrodynamic equilibrium has been established, the sample solution was injected through the injection valve. For each instrument hydrodynamic equilibrium was established at rotational speed 10% lower than maximum recommended by the instrument's manufacturer. Prior the injection the rotation was increased to the recommended maximum. Elution of 0.8 Vc occurred before extrusion.

(2) Injection with a mobile phase front (without equilibration): the column was entirely filled with the SP and set rotating at maximum speed. Sample injection was done after MP has passed the injection valve [13] to create a buffer zone between the SP and the sample solution. Again, equilibrium was established at a rotational speed 10% lower than the maximum recommended and, prior to the injection, the rotation was increased to the recommended maximum. Elution of 1.6 Vc was allowed before extrusion of the column content was performed.

(3) Same procedure as (1) but elution of 1.6 Vc was permitted before extrusion took place. The elution was based on Vc in procedures (1) and (3) due to the elution of the first target compound with Kd < 1.

2.7 Stationary phase retention calculation

For separations with injection before equilibration (procedure 2), the stationary phase retention (S_f) was calculated using the formula below as a ratio of SP volume to total column volume. The amount of SP eluted from the column during equilibrating is equal to the MP volume displacing it. Quite often sample injection causes additional stripping of stationary phase due to differences in the physico-chemical properties (density, viscosity, etc) between solvent system and a sample solution. Therefore, after sample injection this SP volume was corrected by measuring the SP stripping volume in the collected fractions and the final corrected stationary phase retention (S_f*) was obtained.

$$S_f = (V_c - V_m) \times 100 / V_c$$

$$S_f^* = [V_c - (V_m + V_{str})] \times 100 / V_c$$

For separations with injection without equilibration, only the corrected stationary phase retention (S_f*) was calculated by measuring both the amount of stationary phase that displaced from the column and stripping volume in the collected fractions.

2.8 Resolution calculation

Resolution (Rs) was calculated using the following formula, which makes the assumption that all peaks are symmetrical:

$$Rs = \frac{2\,(V2 - V1)}{W2 + W1}$$

Where V is the peak volume and W is the width volume of two consecutive compounds (**Table 2**). This calculation was based on TLC analysis (for detailed information, see S1 in [11] and Apendix II in [19]). Dried CCC fractions were solubilized in 0.5 mL (analytical runs); 1.0 mL (semi-preparative runs) and 2.0 mL (preparative runs) of dichloromethane-methanol 1:1 (v/v). The same volume of each CCC fraction was carefully spotted on TLC plates.

Two target compounds, 3β-masticadienonic and masticadienolic acids, were not eluting consecutively in the CCC run. Therefore, there were two resolution values calculated. The Rs1 was resolution between 3β-masticadienonic acid and impurity, and Rs2, resolution between impurity and masticadienolic acid (**Table 2**).

2.9 Scale-up factor calculation from analytical to preparative separation

In this work, traditional and non-traditional methodologies to scale-up were combined (**Tables 3 and 4**).

- linear scale-up factor (SUF) was applied to columns with different length and i.d.
- volumetric SUF was applied to columns with similar length but different i.d.
- length transfer factor (TF) was applied to columns with different length but same i.d.

Calculations can be visualized as follows:

$$Linear\ SUF = \frac{A2}{A1} \qquad Volumetric\ SUF = \frac{V2}{V1} \qquad Length\ TF = \frac{L2}{L1}$$

Where A is a cross-sectional area, V is the volume and L is the length of a column.

2.10 Analyses of obtained CCC fractions

Each CCC fraction was analyzed by TLC (Merck Art. 05554, Darmstadt, Germany) developed with chloroform-ethyl acetate 3:1 (v/v). Plates were sprayed with universal reagent (3% vanillin solution in methanol with 10% H_2SO_4) followed by heating at 105˚C. Results were compared to previous TLC analysis [11] to identify the target compounds.

3. Results and discussion

3.1 Pre-separation procedures

Before separations, the extra-column volume (V_{ext}) of each CCC set up was measured, which is the system volume (V_{sys}) minus the column volume (V_C) and it includes the inlet and outlet flow lines and all delivery tubes. A large V_{ext} leads to increase in the retention volume of a target compound, delaying its elution out of the column, and causing errors in calculations of solute partition and peak resolution. This delay is equal to the V_{ext} plus a volume that depends on the phase (MP or SP) used for sample injection [1;5;16].

The effect is negligible for columns of a large volume but becomes significant for small analytical columns and/or large sample volumes. The measured volumes are shown in **Table 1** and vary from 1.1 to 53.3% of the Vc. These values were taken in consideration for stationary phase retention calculation of analytical separations only, where the retention volumes were corrected based on Vext [11]. The sample injection was kept at 5% Vc because it was seen that a sample volume up to 5% Vc has very little impact on resolution [13].

It is worth noting that *g*-level values have been used by CCC equipment manufacturers to differentiate high-speed (HSCCC) from high-performance (HPCCC) equipment and widely exploited as a branding tool. HSCCC machines are rotated at speeds that create 20 to 80 times the earth's gravity acceleration, while HPCCC instruments are designed to provide higher *g*-levels, typically 240 x *g* [14]. This difference affects separation time and sample loading: high-performance equipment can maintain satisfactory stationary phase retention and, consequently, peak resolution, at higher flow-rates. The *g*-level values corresponding to the rotational speed used for each instrument in this study are as follows: 243.2 *g* for Spectrum DE rotating at 1600 rpm; 89.4 *g* for Pharma Tech CCC rotating at 1000 rpm; 78.5 *g* for Quattro HT-Prep rotating at 860 rpm and 82.7 *g* for P.C. Inc. rotating at 860 rpm (**Table 1**).

Calculations of tubing (column) length and cross sectional area of each CCC column (**Table 1**) were required to determine which scale-up approach is appropriate.

3.2 Analytical Separations

Firstly, all three experimental procedures (see *Analytical Separation procedure, section 2*) were carried out at analytical scale in each apparatus. All analytical columns have 0.8 mm i.d. apart from Quattro HT-Prep, which has 1.0 mm. The optimized conditions of sample loading - (sample concentration (100 mg/mL) and sample volume (5% Vc) - were established in previous work [11]. Elution flow-rate was set at 0.5 mL/min for high-speed and 0.75 mL/min

for high-performance equipment in order to maintain similar stationary phase retention. Results were analyzed in terms of Sf, Sf* and Rs (**Tables 1** and **2**).

Each equipment provided similar Sf and Sf* (**Table 1**), confirming that the chosen solvent system is stable in the presence of the 100 mg/mL concentration sample solution and that the 5% Vc injection volume did not cause perturbations in the hydrodynamic equilibrium under the selected operating conditions. A difference was observed in the stationary phase retention between experimental procedures 1 and 2 and slightly higher values of Sf were obtained when sample solution was injected after column equilibration. This is interesting because it is considered to be quite a common practice to inject with a solvent front (without equilibrating) to make experiment shorter.

It can be seen from **Table 2** that for procedure (1) Rs1 is higher than Rs2 for all machines and separation was not achieved in P.C. Inc. For this particular separation, analytical Spectrum DE (43.8 m, 243.2 *g*, Rs1 1.47, Rs2 1.16) with the longest column at the highest *g*, gave as good results as analytical Quattro HT-Prep (33.1 m, 78.5*g*, Rs1 1.55, Rs2 1.00) with *g*-field 3 times lower but with a column having a slightly wider internal diameter. This set of experiments clearly demonstrates the importance of the column length as the shortest column of P.C. Inc. (23.9 m) gave the worst results.

Changing separation procedure to (2) by removing an equilibration step and injecting the sample with the solvent front, led to decrease in Rs1 value while Rs2 value increases in all equipment with Spectrum DE providing the best separation (**Table 2**). The reason that Rs2>Rs1 for most of the instruments in procedure 2 is that the elution time is longer (collection of one Vc) giving more time for the separation and the reason Rs1>Rs2 in procedure 1 is that the separation is shorter (collection of one Vs) and the second target, masticadienolic acid (Kd > 1) was retained in the column until extrusion. This experiment emphasizes that the solvent system should be equilibrated first to be hydrodynamically stable at the moment of injection to achieve the best results. The injection is interfering with stationary phase retention, mixing/settling and peak resolution.

The difference in results between procedure (1) and (3) is that Rs1 value was maintained in Spectrum DE and improved in Pharma Tech CCC, P.C. Inc. and Quattro HT-Prep while Rs2 improved in all machines (**Table 2**), which was expected. The longer elution step gives more time for compounds to separate [1].

3.3 Scale-up from analytical to semi-preparative and preparative scales

The best-resolved analytical separations obtained with procedure (3): injection after column equilibration and elution of two Vs before extrusion, was chosen to be scaled up between columns inside the same equipment, and was called intra-apparatus scale-up (**Table 4**). According to traditional scale-up theory, which is based on increases of both cross-sectional area and column length [5], there are two different ways to scale-up depending on the column characteristics: linear scale-up, based on column cross sectional area, should be applied to different length and different i.d. columns [5;17-18] while volumetric scale-up, based on column volume, should be applied to same length but different i.d. columns [5-6]. For columns having different length but same i.d., a length transfer factor, was applied. Traditionally, when *n* of such columns are connected in series and the flow rate kept the same, peak resolution would increase as a factor of $\sqrt{n}$ but also the separation time [15]. In this work, a non-traditional approach to scale-up via connecting identical i.d. columns in series includes increase of the flow-rate to maintain the separation time.

Following the rules, scale-up and transfer factors (**Tables 3 and 4**) were used to increase elution and extrusion flow-rates, fraction size and sample volume proportionally . Sample concentration was maintained at 100 mg/mL in all runs. Rotation, and consequently the *g*-force, was kept constant in each equipment during scale-up [5].

3.3.1 Spectrum-DE

Linear scale-up was applied to scale-up from Spectrum DE analytical 22 mL to semi-preparative 125.5 mL column, as they differ in length and i.d., by a factor of 4.0 (**Table 3 and 5**). Results showed that resolution increased from 1.47 and 1.45 to 1.86 and 1.65 for Rs1 and Rs2 (**Table 2**) respectively, which can be explained by both the greater stationary phase retention (due to larger volume and longer length) and the smaller extra column volume proportion [5].

Considering that the Spectrum DE is a high-performance equipment, two other experiments were tested in semi-preparative scale to try to double sample throughput. The goal was to evaluate increased amount of sample using two different procedures, always keeping separation time the same. The first experiment consisted of doubling the flow-rate from 3.0 to 6.0 mL/min, thus halving the running time by keeping fraction volume. The second experiment was keeping parameter the same but increasing the sample volume to 10%Vc.

Although Sf dropped and stationary phase carry over was observed with the increase of flow-rate in the first experiment (**Table 1**), the resolution values did not show significant decrease

(**Table 2**). It seems that the effect of doubling the sample volume had more impact on the quality of the separation, as the peak broadening caused resolution values to drop below 1.5, especially for Rs1. This suggests that it could be more advantageous to keep same sample amount and increase flow rate than to increase the injected sample by doubling the sample volume in one separation procedure to achieve the same throughput.

3.3.2 Pharma Tech CCC1000

This equipment has three identical columns that can be used connected in series or as single column set-up, in latter case less sample would be injected. The linear scale-up calculation was applied to transfer method from analytical (15 mL, 1 column) to semi-preparative (285 mL, 1 column) scale by a factor of 10.6, as columns differ in both length and i.d. (**Table 3**). Results presented similar Sf (**Table 1**) and resolution values (**Table 2**) demonstrating coherence for predicted parameters.

Furthermore, the column length approach was used to scale-up from the 285 mL (1 column) to the 855 mL (3 columns connected in series), by a factor of 3.0, as the columns differ only in length while maintaining the i.d. (**Table 3**). These three columns are connected to each other by a flying lead on the rotor, inside the equipment, which means that it is under centrifugal force while running. The operating conditions predicted using this scale-up approach caused maximum pressure limit for this equipment (100 psi). In this experiment, the effect of the lower Sf (**Table 1**) seemed to be overcome by the increase in column length and the lost of stationary phase did not compromise the quality of the separation. The slightly better resolution values observed (**Table 2**) could be related to the smaller proportional extra-column volume and higher flow rates, in comparison to the first scale-up experiment. Therefore the lower percentage Vext was achieved as the columns were connected in series internally [5]. Also, the increase in three times column length, and consequently increase in the number of mixing/settling steps, contributed to the improved results (**Table 5**). Additionally, there is an optimal relationship between tubing i.d. and flow rate, especially for the case of columns with larger i.d. [4], which could justify the better results when using the flow rate of 15 mL/min.

Interestingly, if methodology transfer from 285 mL (1 column) to 855 mL (3 columns) would have been done with no flow-rate increasing, resolution would increase $\sqrt{3}$ (= 1.73) in a three times longer run [15]. Using the scale-up length factor, while keeping running time the same, Rs1 and Rs2 increased by 1.03 and 1.22 times, respectively.

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335 *3.3.3 Quattro HT-Prep CCC*

336 Quattro HT-Prep design is well suited for volumetric intra-apparatus scale-up as 4 columns of
337 26, 95, 98 and 224 mL volume are about 30 m long each (**Table 1**). Since this instrument has
338 two similar semi-preparative columns (95 and 98 mL; 2.0 i.d.), it has also the possibility to
339 increase a column volume by connecting them in series. The columns can be connected in the
340 external part of the equipment, out of the rotor, which means that part of the flying leads is
341 under *g*-field while running and part is not. Therefore, as a consequence the extra column
342 volume is bigger than if columns would be connected inside.
343 The volumetric approach was applied to transfer method from analytical 26 mL to semi-
344 preparative 98 mL by a factor of 3.8 and from semi-preparative 98 mL to preparative 224 mL
345 by a factor of 2.2 (**Table 3**). The length factor was calculated to scale-up from the 98 mL (1
346 column) to the 193 mL (2 columns) (**Table 3**). The two-fold increased length of the 193 mL
347 column should be able to compensate the effects of lower Sf caused by the twice higher flow
348 rate when using columns with the same i.d. [5]. Alternatively, a linear scale-up factor of 2.2
349 could be calculated to transfer parameters from 193 to 224 mL column (**Table 3**).
350 Higher Sf values were achieved (**Table 1**), in comparison to the analytical experiments, when
351 the method was transferred from the 98 to 224 mL column. As expected, connecting two
352 identical columns in series with total volume of 193 mL and ramping up the flow-rate,
353 resulted in a drop of the stationary phase retention values. The latter were also comparable to
354 the ones observed in analytical scale (**Table 2 and 5**), with slight improvement of Rs2 at
355 preparative scale, probably due to the better Sf. The increase in volume by either doubling
356 column length or increasing the column cross sectional area seem to have similar positive
357 effect on the quality of the separation process, specially for compounds eluting after Kd=1.
358 Although Rs1 dropped, values obtained were still equal or above 1.5, indicating a base line
359 separation.
360

361 *3.3.4 Multilayer Coil Separator P.C. Inc.*

362 Linear scale-up factor, based on column cross sectional area, was applied to increase
363 parameter values from the analytical 12 mL to the semi-preparative 80 mL column by 4.0. To
364 scale-up from semi-preparative 80 mL to the preparative 270 mL column, a length factor of
365 3.4, based on column length, was used (**Table 3**). In both cases resolution between the target
366 compounds improved as column volume increased (**Table 2**). This is due to the fact that
367 columns are longer and have larger i.d. than analytical one [1].

Interestingly, the Sf was maintained after volumetric increase of flow rate in the preparative column, even though it had same i.d. as the semi-preparative one (**Table 1 and 5**). Also, no stationary phase carry over was observed, even after the increase in sample volume, showing that this equipment is able to keep the system's hydrodynamic equilibrium. Additionally, the higher flow rate seemed to improve the mixing steps as peaks were sharper in preparative scale. Moreover, the lower relative Vext and over 3 times increase in column length will contributed to the positive effect on peak resolutions, which were higher in the preparative than in the semi-preparative run.

3.4 Inter-apparatus method transfer

The overall results discussed above showed that it is feasible to adapt the method developed in one CCC machine to another, even when they have different column volumes and design. However, some essential parameter adjustment must be taken into account to assure matching conditions.

For instance, applying linear scale-up from the analytical Spectrum DE 22 mL column (0.8 mm i.d.) to semi-preparative Pharma Tech CCC 285 mL column (2.6 mm i.d.), Quattro HT-Prep 98 mL column (3.1 mm i.d.) or Coil Separator P.C. Inc. 80 mL column (1.6 mm i.d.), would give a factor of 10.6, 6.2 and 4.0, respectively, (**Table 3**) leading to flow rate and sample volume values similar to the ones calculated from the analytical columns of the same equipment.

Different situation would be observed, however, if this transfer would be done from Spectrum DE 22 mL analytical column to preparative Pharma Tech CCC 855 mL, Quattro HT-Prep 193 mL and Coil Separator P.C. Inc. 270 mL columns because linear scale-up would lead to the same values obtained for semi-preparative columns described above (**Table 3**), not matching the parameters that could be used in reality. This happens because the linear approach, based on cross sectional area and the same stationary phase retention, only considers the column i.d. and does not take into account the total column volume and/ or column length directly. In this case, the sample loading could be calculated on the basis of percentage of Vc. The flow rate should be optimized according to Sf and maximal pressure values to improve scale-up results.

Other variables that will influence the quality of a separation in columns with similar volume are the column length and geometry. Longer columns will have a higher number of theoretical plates, in other words, higher efficiency [1]. Different aspects of column design can be comprised under 'geometry', such as helical angle (that will also influence in the

number of turns of the coiled column), β-value (that will determine the efficiency of the mass transfer rates and hydrodynamic behavior of different kinds of solvent systems) and if the columns are single or connected in series (due to possibility of diffusion in the Vext). Unfortunately not all these parameters were available for the instruments used in this work.

It is also important to consider the type of CCC used: high performance machines are specially designed to run at higher rotational speeds and flow rates. The higher number of mixing steps generated by the larger number of rotating cycles will lead to sharper peaks and better chromatographic resolution in a shorter separation time. One suggestion when transferring a method developed in a HPCCC to a HSCCC device could be to reduce the sample loading proportionally to the rotational speed (*g*-force level) in order to obtain similar peak resolutions. Additionally, when transferring the method between HSCCC equipment, it is possible to maintain the same *g*-level via adjusting rotational speed in order to achieve same Sf for a given flow rate.

Using methodology described, the experiments performed in this study showed that, in general, all equipment was able to deliver equally efficient fractionation of the target compounds from the *S. terebinthifolius* dichloromethane extract.

4. Conclusion

Intra- and inter-apparatus scale-up is feasible. The approaches described in this work will help different users to save time, solvent and sample by applying the proposed strategies to method transfer.

Based on column characteristics and independently on a CCC instrument make, volumetric and linear scale-up theory can be directly applied to same length but different i.d. columns and different length and i.d. columns, respectively. A novel approach to scale-up by increasing length in columns with same i.d. has been successfully demonstrated. Flow-rate, fraction size and sample volume must be increased according to the calculated factor while sample concentration and *g*-force should be kept constant. However, the more careful consideration should be taken when methodology is being transferred between HPCCC and HSCCC instruments.

There is a lack of published inter-compatibility studies on CCC instruments, but method transfer and reproducibility of the CCC technology is viable when using scale-up theory based on column characteristics. Collating data of such examples will make these scale-up approaches even more robust and easy to use.

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References

[1] W.D. Conway. Counter-Current Chromatography: Apparatus, Theory and Applications, VCH Publishers Inc., NY, 1990.

[2] A. Marston, K. Hostettmann. Developments in the application of counter-current chromatography to plant analysis, J. Chromatogr. A 1112 (2006) 181-194.

[3] A. Berthod. Counter-current Chromatography: From the Milligram to the Kilogram, In Advances In Chromatography, Taylor & Francis Inc., NY, 2009.

[4] I.A. Sutherland, L. Brown, A.S. Graham, G.G. Guillon, D. Hawes, L. Janaway, R. Whiteside, P. Wood. Industrial scale-up of countercurrent chromatography: predictive scale-up, J. Chromatogr. Sci. 39 (2001) 21-28.

[5] P. Wood, S. Ignatova, L. Janaway, D. Keay, D. Hawes, I. Garrard, I. Sutherland, Counter-current chromatography separation scaled up from an analytical column to a production column, J. Chromatogr. A 1151 (2007) 25–30.

[6] I. Sutherland, P. Hewitson, S. Ignatova. Scale-up of counter-current chromatography: Demonstration of predictable isocratic and quasi-continuous operating modes from the test tube to pilot/process scale, J. Chromatogr. A 1216 (2009) 8787-8792.

[7] I. Sutherland, S. Ignatova, P. Hewitson, L. Janaway, P. Wood, N. Edwards, G. Harris, H. Guzlek, D. Keay, K. Freebairn, D. Johns, N. Douillet, C. Thickitt, E. Vilminot, B. Mathews. Scalable Technology for the Extraction of Pharmaceutics (STEP): The transition from academic knowhow to industrial reality, J. Chromatogr. A 1218 (2011) 6114-6121.

[8] I. Sutherland, C. Thickitt, N. Douillet, K. Freebairn, D. Johns, C. Mountain, P. Wood, N. Edwards, D. Rooke, G. Harris, D. Keay, B. Mathews, R. Brown, I. Garrard, P. Hewitson, S. Ignatova. Scalable Technology for the Extraction of Pharmaceutics: Outcomes from a 3-year collaborative industry/academia research programme, J. Chromatogr. A 1282, (2013) 84-94.

[9] D. Fisher, I.J. Garrard, R. van den Heuvel, I.A. Sutherland, F.E. Chou, J.W. Fahey. Technology Transfer and Scale Up of a Potential Cancer-Preventive Plant Dynamic Extraction of Glucoraphanin, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1913-1922.

[10] I.A. Sutherland, A.J. Booth, L. Brown, B. Kemp, H. Kidwell, D. Games, A.S. Graham,G.G. Guillon, D. Hawes, M. Hayes, L. Janaway, G.J. Lye, P. Massey, C. Preston, P.Shering, T. Shoulder, C. Strawson, P. Wood, Industrial scale-up of countercur-rent chromatography, J. Liq. Chromatogr. Relat. Technol. 24 (2001) 1533–1553.

[11] M.N. Vieira, F.N. Costa, G.G. Leitão, I. Garrard, P. Hewitson, S. Ignatova, P. Winterhalter, G. Jerz. Schinus terebinthifolius scale-up countercurrent chromatography (Part I): High performance countercurrent chromatography fractionation of triterpene acids with off-line detection using atmospheric pressure chemical ionization mass spectrometry, J. Chromatogr. A 1389 (2015) 39-48.

[12] A. Berthod, M. Hassoun, M.J. Ruiz-Angel. Alkane effect in the Arizona liquid systems used in countercurrent chromatography, Anal. Bioanal. Chem. 383 (2005) 327–340.

[13] W.D. Conway, L.R. Chadwick, H.H.S. Fong, N.R. Farnsworth, G.F. Pauli. Extra-Column Volume in CCC, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1799-1818.

[14] H. Guzlek, P.L. Wood, L. Janaway. Performance comparison using the GUESS mixture to evaluate counter-current chromatography instruments, J. Chromatogr. A 1216 (2009) 4181-4186.

[15] Q.-Z. Du, C.-Q. Ke, Y. Ito. Separation of epigallocatechin gallate and gallocatechin gallate using multiple instruments connected in series, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 203-208.

[16] Philip L. Wood, David Hawes, Lee Janaway & Ian A. Sutherland. Determination of J-type centrifuge extra-coil volume using stationary phase retentions at differing flow rates, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 1417-1430.

[17] I.A. Sutherland, G. Audo, E. Bourton, F. Couillard, D. Fisher, I. Garrard, P. Hewitson, O. Intes. Rapid linear scale-up of a protein separation by centrifugal partition chromatography, J. Chromatogr. A 1190 (2008) 57

[18] Y. Yuan, B. Wang, L. Chen, H. Luo, D. Fisher, I.A. Sutherland, Y. Wei. How to realize the linear scale-up process for rapid purification using high-performance counter-current chromatography, J. Chromatogr. A 1194 (2008) 192

[19] MN Vieira. Studies on the Use of Countercurrent Chromatography for the Preparative Isolation and Identification of Natural Compounds Active against Neglected Tropical Diseases, Thesis, 2015.

Table 1. CCC equipments used and experimental conditions

	Vc (mL)	I.D. (mm) / Cross Sectional Area (mm^2)	Length (m)	Extra-coil volume (mL / %)	ω (rpm)	*g* level (x *g*)	Elution / Extrusion Flow-rate (mL/min)	Fraction size (mL)	Sf (%)	Sf* (%)	Sample volume at 100 mg/mL (mL)
Spectrum DE	22.0	0.8 / 0.5	43.8	4.5 / 20.5	1600	243.2	0.75 / 1.5	0.75	84 (1) 78 (2) 87 (3)	84 (1) 78 (2) 87 (3)	1.1
	125.5	1.6 / 2.0	62.5	7.0 / 5.6			3.0 / 6.0	3.0	89 (3)	89 (3)	4.4
							6.0 / 12.0	6.0	84 (3)	80 (3)	8.8
							3.0 / 6.0	6.0	86 (3)	84 (3)	13.0
Pharma Tech CCC 1000	15.0	0.8 / 0.5	29.9	8.0 / 53.3	1000	89.4	0.5 / 1.0	0.5	87 (1) 86 (2) 87 (3)	87 (1) 86 (2) 87 (3)	0.75
	285.0	2.6 / 5.3	53.7	8.0 / 2.8			5.0 / 10.0	5.0	86 (3)	84 (3)	8.0
	855.0	2.6 / 5.3	161.1	8.0 / 0.9			15.0 / 30.0	15.0	80 (3)	68 (3)	24.0
Quattro HT-Prep CCC	26.0	1.0 / 0.79	33.1	1.5 / 5.8	865	78.5	0.5 / 1.0	0.5	81 (1) 79 (2) 80 (3)	80 (1) 78 (2) 79 (3)	1.3
	98.0	2.0 / 3.1	31.2	3.0 / 3.1			2.0 / 4.0	2.0	89 (3)	89 (3)	5.0
	193.0	2.0 / 3.1	61.5	5.0 / 2.6			4.0 / 8.0	4.0	79 (3)	78 (3)	10.0
	224.0	3.2 / 8.0	27.9	3.0 / 1.3			4.5 / 9.0	4.5	92 (3)	92 (3)	11.0
Coil Separator P.C. Inc.	12.0	0.8 / 0.50	23.9	4.0 / 33.3	860	82.7	0.5 / 1.0	0.5	67 (1) 65 (2) 67 (3)	67 (1) 65 (2) 67 (3)	0.6
	80.0	1.6 / 2.0	39.8	4.0 / 5.0			2.0 / 4.0	2.0	90 (3)	90 (3)	2.4
	270.0	1.6 / 2.0	134.4	4.0 / 1.5			6.0 / 12.0	6.0	90 (3)	90 (3)	8.0

(1) Procedure (1); (2) Procedure (2) and (3) Procedure (3); according to *Analytical Separation Procedure* in *Experimental*
Parameters scaled up according to scale-up and transfer factors calculated on **Table 3** are shown in gray.
I.D. means tubing internal diameter

Table 2. Resolution calculation for the target compounds (calculated according to *Resolution calculation* in *Experimental*)

	Vc (mL)	Solvent Front (F)	1st target 3β-Masticadienolic acid		Impurity		2nd target Masticadienonic acid		Resolution[1]	
			width / peak (Fr)	width / peak (mL)	width / peak (Fr)	width / peak (mL)	width / peak (Fr)	width / peak (mL)	Rs1	Rs2
Spectrum DE	22.0	(1) 18	34-45 / 40	12.75 / 25.5*	50-58 / 54	10.5 / 36.0*	59-70 / 65	12.75 / 44.25*	1.47	1.16
		(2) 20	34-46 / 42	13.5 / 30.0*	50-57 / 54	9.75 / 36.0*	62-74 / 69	16.5 / 47.25*	1.26	1.42
		(3) 18	33-45 / 40	13.5 / 25.5*	50-57 / 54	9.75 / 36.0*	60-75 / 70	15.75 / 48.0*	1.47	1.45
	125.5	(3) 9	26-33 / 29	21.0 / 87.0	38-45 / 42	21.0 / 126.0	49-61 / 56	36.0 / 168.0	1.86	1.47
		(3) 13	27-34 / 30	42.0 / 180.0	38-44 / 41	36.0 / 246.0	47-59 / 54	72.0 / 324.0	1.69	1.44
		(3) 11	27-38 / 33	33.0 / 99.0	42-50 / 46	24.0 / 138.0	52-72 / 66	60.0 / 198.0	1.37	1.43
Pharma Tech CCC 1000	15.0	(1) 18	25-31 / 28	3.0 / 6.0*	32-40 / 37	4.0 / 10.5*	41-50 / 46	4.5 / 15.0*	1.29	1.06
		(2) 19	26-34 / 31	4.0 / 7.5*	34-39 / 38	2.5 / 11.0*	42-53 / 48	5.5 / 17.0*	1.08	1.25
		(3) 20	27-33 / 28	6.0 / 6.0*	33-37 / 35	2.0 / 9.5*	39-51 / 46	6.0 / 15.0*	1.40	1.38
	285.0	(3) 11	27-37 / 31	50.0 / 155.0	44-55 / 49	55.0 / 245.0	57-83 / 75	130.0 / 375.0	1.71	1.41
	855.0	(3) 20	41-49 / 44	120.0 / 660.0	54-63 / 59	135.0 / 885.0	72-85 / 78	195.0 / 1170.0	1.76	1.72
Quattro HT-Prep CCC	26.0	(1) 15	32-44 / 38	6.0 / 17.5*	50-60 / 55	5.0 / 26.0*	60-70 / 65	5.0 / 31.0*	1.55	1.00
		(2) 17	34-45 / 39	5.5 / 18.0*	45-57 / 53	6.0 / 25.0*	60-72 / 67	6.0 / 32.0*	1.22	1.17
		(3) 15	34-44 / 40	5.0 / 18.5*	51-60 / 56	4.5 / 26.5*	65-80 / 74	7.5 / 35.5*	1.60	1.50
	98.0	(3) 9	33-43 / 40	20.0 / 80.0	51-59 / 55	16.0 / 110.0	70-84 / 73	28.0 / 146.0	1.67	1.64
	193.0	(3) 11	37-45 / 40	32.0 / 160.0	50-60 / 54	40.0 / 216.0	76-88 / 77	48.0 / 308.0	1.55	2.09
	224.0	(3) 9	33-44 / 39	49.5 / 175.5	50-59 / 54	40.5 / 243.0	71-85 / 79	63.0 / 355.5	1.50	2.17
Coil Separator P.C. Inc.	12.0	(1) 7	16-24 / 20	4.0 / 6.0*	23-36 / 29	6.5 / 10.5*	31-37 / 34	3.0 / 13.0*	0.62	0.52
		(2) 9	23-28 / 25	2.5 / 8.5*	25-31 / 28	3.0 / 10.0*	29-36 / 33	3.5 / 12.5*	0.50	0.77
		(3) 7	16-24 / 20	4.0 / 6.0*	25-33 / 28	4.0 / 10.0*	35-45 / 39	5.0 / 15.5*	1.00	1.22
	80.0	(3) 11	27-39 / 33	24.0 / 66.0	41-47 / 44	12.0 / 88.0	51-77 / 61	52.0 / 122.0	1.22	1.06
	270.0	(3) 11	30-41 / 36	66.0 / 216.0	46-55 / 49	54.0 / 294.0	61-77 / 71	96.0 / 426.0	1.30	1.76

*Volume corrected according to Vext; (Fr) means fraction number; (1) Procedure (1); (2) Procedure (2) and (3) Procedure (3); according to *Analytical Separation Procedure* in *Experimental*

Table 3. Scale-up and transfer factors calculation for intra- and inter-apparatus method transference

		Spectrum DE		Pharma Tech CCC 1000			Quattro HT-Prep CCC				Coil Separator P.C. Inc.		
		V 22.0 i.d. 0.8 L 43.8	V 125.5 i.d. 1.6 L 62.5	V 15.0 i.d. 0.8 L 29.9	V 285.0 i.d. 2.6 L 53.7	V 855.0 i.d. 2.6 L 161.1	V 26.0 i.d. 1.0 L 33.1	V 98.0 i.d. 2.0 L 31.2	V 193.0 i.d. 2.0 L 61.5	V 224.0 i.d. 3.2 L 27.9	V 12.0 i.d. 0.8 L 23.9	V 80.0 i.d. 1.6 L 39.8	V 270.0 i.d. 1.6 L 134.4
Spectrum DE	V 22.0 i.d. 0.8 L 43.8		Linear A2/A1 4.0	Length L2/L1 0.68	Linear A2/A1 10.6	Linear A2/A1 10.6	Linear A2/A1 1.6	Linear A2/A1 6.3	Linear A2/A1 6.3	Linear A2/A1 16.0	Length L2/L1 0.6	Linear A2/A1 4.0	Linear A2/A1 4.0
Pharma Tech CCC 1000	V 15.0 i.d. 0.8 L 29.9	Length L2/L1 1.5	Linear A2/A1 4.0		Linear A2/A1 10.6	Linear A2/A1 10.6	Volumetric V2/V1 1.7	Volumetric V2/V1 6.5	Linear A2/A1 6.3	Volumetric V2/V1 14.9	Length L2/L1 0.8	Linear A2/A1 4.0	Linear A2/A1 4.0
Quattro HT-Prep CCC	V26.0 i.d. 1.0 L 33.1	Linear A2/A1 0.64	Linear A2/A1 2.6	Volumetric V2/V1 0.58	Linear A2/A1 6.8	Linear A2/A1 6.8		Volumetric V2 / V1 3.8	Linear A2/A1 4.0	Volumetric V2 / V1 8.6	Volumetric V2/V1 0.5	Volumetric V2/V1 3.1	Linear A2/A1 2.6
Coil Separator P.C. Inc.	V 12.0 i.d. 0.8 L 23.9	Length L2/L1 1.8	Linear A2/A1 4.0	Length L2/L1 1.3	Linear A2/A1 10.6	Linear A2/A1 10.6	Linear A2/A1 1.6	Volumetric V2 / V1 8.6	Linear A2/A1 6.3	Volumetric V2 / V1 18.7		Linear A2/A1 4.0	Linear A2/A1 4.0

V in mL; i.d. in mm, L in m. In grey: intra-apparatus scale-up. In white: inter-apparatus scale-up.

Table 4. Overview of scale-up details

	Vc1 → Vc2 (mL)	Scale-up method applied to increase flow-rate, sample volume and fraction size	Change in Rs1 / Rs2 (%)
Spectrum DE	22.0 → 125.5	Linear A2/A1 (2.0/0.5=4)	+26.5 / +1.38
Pharma Tech CCC 1000	15.0 → 285.0	Linear A2/A1 (5.3/0.5=10.6)	+22.1 / +2.17
	285.0 → 855.0	Length L2/L1 (161.1/53.7=3)	+2.92 / + 22.0
Quattro HT-Prep CCC	26.0 → 98.0	Volume V2/V1 (98/26=3.8)	+4.38 / +9.33
	98.0 → 193.0	Length L2/L1 (61.5/27.9=2)	-7.19 / +27.4
	98.0 → 224.0	Volume V2/V1 (224/98=2.2)	-3.23 / +3.83
Coil Separator P.C. Inc.	12.0 → 80.0	Linear A2/A1 (2.0/0.5=4)	+22.0 / -13.1
	80.0 → 270.0	Length L2/L1 (134.4/39.8=3.3)	+6.56 / +66.0

Table 5. Overview of experimental details and results of scale-up

	Vc (mL)	Fractions in Elution + Extrusion[(3)]	Experiment time (min)	Sample injected (mg)	Sample recovery (%)	Solvent consumption (mL)	Productivity (mg/min) (mg/mL)
Spectrum DE	22.0	47 + 29	76	110	99.2	57	1.4 1.9
	125.5	67 + 42	109	440	98.6	327	4.0 1.3
Pharma Tech CCC 1000	15.0	48 + 30	78	75	99.0	39	0.96 1.9
	285.0	91 + 57	148	800	99.5	740	5.4 1.1
	855.0	91 + 57	148	2400	99.1	2220	16.2 1.1
Quattro HT-Prep CCC	26.0	84 + 52	136	130	98.2	68	0.96 1.9
	98.0	78 + 49	127	500	99.5	254	3.9 2.0
	193.0	77 + 48	125	1000	99.4	500	8.0 2.0
	224.0	80 + 50	130	1100	99.5	585	8.5 1.9
Coil Separator P.C. Inc.	12.0	38 + 24	62	60	98.2	31	0.97 1.9
	80.0	64 + 40	104	240	98.9	208	2.3 1.2
	270.0	72 + 45	117	800	98.8	702	6.8 1.1

[(3)] Procedure (3); according to *Analytical Separation Procedure* in *Experimental*