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# *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.



## 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

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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 (18.2 M Ω) 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 × 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 × 1.0 mm i.d. and 234 mL × 3.2 mm i.d. on one bobbin; 95 mL × 2.0 mm i.d. and 98 mL × 2.0 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 × 0.8 mm i.d.; 80 mL × 1.6 mm i.d.; 230 mL × 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 centre 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:

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

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