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Carnosol purification. Scaling-up centrifugal partition chromatography separations

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ABSTRACT

This paper illustrates the application of a recently proposed protocol allowing the scale-up prediction on hydrostatic countercurrent chromatography columns (centrifugal partition chromatographs or CPC). A commercial extract of rosemary (*Rosmarinus officinalis* L.) was used as the starting material containing 0.48% of carnosol, an active pharmaceutical ingredient with great potential. After a rapid method development on a small-scale 35-mL CPC instrument that allowed for the determination of the solvent system and maximum sample concentration and volume, the purification was transferred on two larger instruments using the "free space between peaks" method. The method takes into account the technical limitations of the larger instruments, such as pressure and/or maximum centrifugal field, and allows, by simply running an analytical-sized injection on the large scale rotor, to give an accurate prediction of the maximum sample load and best throughput. The 0.27 g of rosemary extract maximum load on a 35-mL CPC was transferred as a 1.9 g load on a 254-mL medium size CPC and 9 g load on a 812-mL CPC. The maximum process efficiency of 3.1 mg of carnosol per hour obtained on the small 35-mL column was transferred on the 254-mL CPC giving 8.3 mg/h and, on the larger 812-mL column 49.4 mg of carnosol could be obtained per hour. If the scaling-up in CPC instruments is not directly homothetic, it can be highly predictable through a few simple experiments.

1. Introduction

The use of countercurrent chromatography (CCC) for natural product research and active ingredient production has increased in the past decades [1,2]. Once the delicate selection of the biphasic liquid system constituting both the stationary and the mobile phases in CCC has been performed, it is of primary concern to be able to scale-up the separation in order to produce a sufficient amount of the molecule of interest. For that purpose, two types of CCC columns have reached commercial development: the hydrodynamic CCC columns and the hydrostatic CCC design called centrifugal partition chromatography (CPC). Both types of CCC columns are available from laboratory scale to industrial scale. The hydrodynamic CCC columns are based on coils of open tubes rotating with a planetary motion needing two axes of rotation. The technical conception of hydrostatic CCC columns or CPC instruments rely on rotors made of twin-cell chambers that rotate around a single axis. These differences strongly impact the scale-up strategy.

In hydrodynamic CCC systems, the scale-up was demonstrated to be linear simply considering the ratio of the columns volumes and/or the ratio of the tube sections [3–5]. For hydrostatic CCC instruments, many examples tend to prove that large-scale CPC instruments perform better than small-volume CPCs [6,7]. Therefore the exact amount of crude may be significantly higher that predicted by the simple linear value of the column volumes. The question remains: how can the purification capability of a large volume CPC column be fully exploited? Such studies are infrequently found in the literature [6,7]. We recently proposed a protocol called the "free-space between peak method", which we applied on the separation of a maximum amount of selected GUESS compounds. We showed that it is possible, starting with a single analytical injection on a large scale instrument, to predict the exact maximum quantity of sample that can be injected to maintain the desired purification level [8]. This method is used in this work to optimize the purification of carnosol from crude rosemary solvent extracts by CPC.

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Rosemary (*Rosmarinus officinalis* L.) is a well known aromatic and medicinal herb commonly used in the Mediterranean diet. Recent studies have shown the many benefits it could provide for treatment of cancers and heart diseases [9–17]. This pharmacologic activity mainly relies on three main active ingredients: carnosol, carnosic acid and rosmarinic acid. While the two acids are easily isolated via ion-exchange [18], carnosol is quite difficult to isolate from rosemary herb, because of its high instability towards heat, light, solvents [19–23] and its low abundance in plants. Due to its therapeutic potential, providing high purity carnosol standards has now become of major importance for the pharmaceutical industry, not only to pursue research and toxicological studies, but also to provide a reliable standard for quality control of new drugs.

Carnosol have already been isolated from Rosemary leaves by CCC [24]. 715 mg of a Rosemary leaves methanol extract was injected in a 325 mL instrument with a hexane-ethyl acetate-methanol-water (70/30/14/8 v/v) biphasic system and an upper organic mobile phase at 1.5 mL/min. Fisher was able to obtain 66 mg of 95% pure carnosol in 5 h [24]. There was no attempt to increase the yield or the productivity. In CPC instruments, no carnosol purification were found. This paper presents a CPC method development for carnosol separation on a small 25-mL CPC rotor. It will then illustrate the rapid CPC transfer that was performed on rosemary extracts to isolate significant amounts of purified carnosol using a mid-scale 200-mL rotor and going industrial scale with a 1-L rotor. It demonstrates that the methodology developed using standard compounds [8] can be applied to a real sample. The method allows for simple, reliable and predictable scale-up on hydrostatic CPC instruments.

2. Experimental section

2.1. Chemicals

A rosemary leaves extract was purchased from Cooper industries (Melun, France). According to the manufacturer, the solid extract was made of a 30% ethanol cold infusion, followed by low-pressure solvent evaporation at 45 °C to preserve the active compounds. The ratio plant/extract is 5/1 (w/w). Solvents were supplied by Sigma-Aldrich (Isle d'Abeau, France) and the analytical standards carnosol, carnosic acid and rosmarinic acid were obtained from Phytolab (Vestenbergsgreuth, Germany).

2.2. Sample preparation

The analytical standards were prepared in methanol, with carnosol and rosmarinic acid at 250 μg/mL and carnosic acid at 170 μg/mL concentrations.

The commercial rosemary extract was dissolved in the lower phase of the selected biphasic system and sonicated for 10 min. The mixture was then centrifuged at 5000 rpm for 3 min to remove any remaining solid material and the supernatant was collected and directly injected in the CPC instrument.

2.3. HPLC analysis

The HPLC analysis of rosemary extracts has been adapted from a published method [25]. An Alliance Waters 2690 instrument was used equipped with a 5 μm Zorbax SB-Aq 3 × 150 mm column and a DAD detection system set up at 214 nm. A gradient elution was performed with the solvents A: water with 0.1% acetic acid and B: acetonitrile. The gradient program was: 90% A + 10% B for 0.5 min, next increasing B from 10% to 24% in 23 min, and next increasing B from 24% to 100% pure B in 38 min. The flow rate was 0.4 mL/min with an injection volume of 20 μL. One analysis cycle lasted 62 min.

2.4. Selection of the two-phase solvent system

Different two-phase solvent systems were tested. The appropriate system was selected based on the partition coefficient, *K*, ratio of the compound concentration in the stationary phase over the concentration in the mobile phase. The *K* values of the main compounds found in the crude sample should be different enough so that compounds can be separated in a reasonable amount of time. The *K* values were determined as follows: the selected two-phase solvent system was prepared and let for full equilibration in a separation funnel at room temperature. 2 mL of upper and lower phases was placed in test tubes and 400 mg of rosemary extract was added. Each test tube was shaken for 1 min and then sonicated for 10 min for optimal solubilization. The two phases were separated and centrifuged at 5000 rpm. The supernatant was collected and dissolved in a 1/10 ratio with the same upper or lower phase, then injected in the HPLC system. A blank analysis of each upper/lower phase was also carried out to check on possible solvent peaks. The *K* value was calculated as the peak area of the compound in the lower phase divided by the peak area of the compound in the upper phase at the same retention time in the HPLC chromatogram.

2.5. Centrifugal partition chromatography

The CPC system was a FCPC A from Kromaton Rousselet Robatel (Annonay, France). This unit can be mounted with different rotors easily interchanged. A 25 mL-rotor was used for method development; a 200 mL- rotor and a 1 L-rotor were operated for scale-up studies. The exact volumes of the three rotors mounted in the FCPC A system were experimentally measured as 35 mL, 254 mL and 812 mL, respectively. The FCPC A chamber has a water circulation system that allows for cooling down to 25 °C using a Julabo cryostat (Colmar, France). An integrated chromatographic device, model Spot Prep II by Armen Instrument (Saint-Avé, France), was equipped with a quaternary pump (1–250 mL/min), a stainless steel injection loop (10 or 50 mL) and a dual wavelength detection system (set at 210 and 254 nm). The Spot Prep II also includes a fraction collector and a data treatment and control unit using the Armen Glider Prep software. The UV signal was also externally treated using the chromatographic AZUR software (Datalys, Grenoble, France).

The liquid stationary phase was the lower aqueous phase of the selected biphasic systems. It was loaded in the selected rotor rotating at 600 rpm, with a high flow rate depending on the column volume (i.e. 20 mL/min to fill the 35-mL column in about 3 min, 40 mL/min to fill the 254-mL column in less than 10 min and 80 mL/min filling the 812-mL column in 15 min). Then the rotation speed was increased to reach the desired centrifugal field. The mobile phase, the upper organic phase, was then introduced in the appropriate ascending direction, at the desired flow rate. The displaced aqueous stationary phase volume was collected at the column exit until equilibrium was reached as noted by a stabilization of the increasing driving pressure associated with a growing layer of upper mobile phase seen toping the lower phase in the collection vessel. The stationary phase retention was either measured by injecting and measuring the retention volume of an un-retained compound or using the collected volume of displaced lower stationary phase. Injections were performed through the loop, with the sample dissolved in the stationary (aqueous lower) phase. 15 mL fractions were automatically collected.

The commercial rosemary extract was dissolved in the aqueous lower phase of the selected solvent system at a concentration of 500 mg/mL. The insoluble matter was precipitated at the bottom of a test tube using centrifugation, while the supernatant was directly injected in the 10 or 50 mL sample loop.