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Carnosol purification. Scaling-up Centrifugal Partition Chromatography separations

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Abstract

This paper illustrates the use of a recently proposed protocol allowing predicting the scale-up on hydrostatic columns of countercurrent chromatography (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 the 35-mL CPC was transferred as a 1.9 g load on the 254-mL medium size CPC and 9 g load on the 812-mL CPC. The maximum productivity of 3.1 mg of carnosol per hour obtained on the small 35-mL rotor was transferred on the 254-mL CPC giving 8.3 mg/h and, on the larger 812-mL rotor 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 few simple experiments.

Keywords

Centrifugal partition chromatography; countercurrent chromatography; scale-up; rosemary; carnosol; throughput.

INTRODUCTION

Countercurrent chromatography (CCC) is the preparative purification technique that uses two liquid phases to perform compound separation [1-3]. The challenge of CCC columns is to retain the liquid stationary phase without any solid support. Centrifugal fields are used. Two types of CCC columns reached commercial development: the hydrodynamic CCC columns and the hydrostatic CCC design called Centrifugal Partition Chromatographs (CPC). Both types of CCC columns are available from laboratory scale to industrial scale. They are however based on very different concepts. The hydrodynamic CCC columns are based on coils

of open tubes rotating with a planetary motion needing two axes of rotation [1-3]. The technical conception of hydrostatic CCC columns or CPC instruments rely on rotors made of twin-cell chambers that rotate around a single axis. All CCC columns, small or large, hydrostatic or hydrodynamic, can work with the same given biphasic liquid system for a particular separation.

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 [4-6]. For hydrostatic CCC instruments, many examples tend to prove that large-scale CPC instruments perform better than small-volume CPCs [7, 8]. Therefore the exact amount of crude may be significantly higher than predicted by the simple linear value of the column volumes. How can the purification

capability of a large volume CCC column be fully exploited? Such studies were scarcely reported in the literature [7, 8]. 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 maintaining the desired purification level [9]. This method is used in this work to optimize the purification of carnosol from crude rosemary solvent extracts by CPC.

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 [10-18]. This pharmacologic activity relies on three main active ingredients: carnosol, carnosic acid and rosmarinic acid. While the two acids are easily isolated via ion-exchange [19], carnosol is quite difficult to isolate from rosemary herb, because of its high instability towards heat, light, solvents [20-24] and its low content in plants. Due to its therapeutic potential, providing carnosol high purity standards has become now 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.

There is a work using hydrodynamic CCC to isolate carnosol from Rosemary leaves [25]. Injecting 715 mg of methanol extract of rosemary leaves in a 325 mL hydrodynamic PC Inc CCC instrument with the hexane-ethyl acetate-methanol-water (70/30/14/8 v/v) biphasic system and the upper organic mobile phase at 1.5 mL/min, Fisher was able to obtain 66 mg of 95% pure carnosol in 5 hours [25]. There was no attempt to increase the yield or the productivity. No carnosol purification by CPC were found. This paper presents a CPC carnosol separation on a small 25-mL CPC column. It will then illustrate the rapid CPC method development that was performed on rosemary extracts to isolate significant amounts of pure carnosol using a mid-scale 200-mL rotor and going almost industrial scale with a 1-L rotor. It demonstrates that the methodology developed using standard compounds [9] can be applied to real samples. The method allows for simple and reliable

predictable scale-up on hydrostatic CPC instruments.

EXPERIMENTAL SECTION

Chemicals

A Rosemary leave extract was purchased from Cooper industries, Melun, France (extract #1 042 000). 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).

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 concentration.

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.

HPLC Analysis

The HPLC analysis of rosemary extracts has been adapted from a published method [26]. An Alliance Waters 2690 instrument was used equipped with a 5 µm Zorbax SB-Aq 3 x 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.

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 were placed in test tubes and 400 mg of rosemary extract were added in at least two test tubes. 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.

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 liter-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 to 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 denser and 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 rotor volume (i.e. 20 mL/min to fill the 35-mL rotor in about 3 min, 40 mL/min to fill the 254-mL rotor in less than 10 min and 80 mL/min filling the 812-mL rotor in 15 min). Then the rotation speed was increased so as to reach the desired centrifugal field. The mobile phase, the upper organic phase, was then introduced in the appropriate ascending direction (also called tail-to-head [1-3]), 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 injecting and measuring the retention volume of an un-retained compound (new coccine red) or using the collected volume of displaced lower stationary phase. Injections were performed through the loop, with the sample dissolved either in the mobile (organic upper) phase or in the stationary (aqueous lower) phase. 15 mL fractions were automatically collected.

The commercial rosemary dry 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 CPC loop.

RESULTS AND DISCUSSION

CPC method development

The HPLC analysis of the commercial dry extract revealed that the sample was quite complex with a low carnosol content estimated at 1.6 mg carnosol per gram of rosemary leaves extract (0.16% w/w). The good solvents for carnosol are methanol, ethanol, methyl-*ter*-butyl ether (MTBE) or dimethyl sulfoxide [27, 28]. Methanol was banned from the tested solvent systems because it was found to enhance the degradation of carnosol into carnosic acid [20, 23, 29]. Therefore it was decided to construct a

solvent system based on ethanol. A panel of 24 solvent systems were tested using shake-flask method. A solvent system made of heptane/MTBE/ethanol/water was selected. Both heptane and water are not prone to dissolve carnosol, but they are required to generate the biphasic system. Moreover, water dissolves most of the rosemary unwanted compounds, therefore increasing the selectivity between carnosol and other compounds that partitioned between the two phases.

Different ratios of heptane/MTBE/ethanol/water were tested. The ascending (or tail-to-head) mode with the upper organic phase was preferred to provide larger carnosol retention in the ethanol-rich lower aqueous stationary phase and recover carnosol in an easy-to-evaporate organic phase. The CPC method development was carried out on the small scale 35-mL rotor in order to gain time, solvents and sample [30].

Increasing the amount of ethanol while keeping the amount of MTBE constant, leads to a better affinity of carnosol and other impurities for the lower phase. As the ratios ethanol/MTBE moved from 2:1 to 4:1, the carnosol partition coefficient K shifted from 1.5 to 4.4 (Fig. 1A-C). The adjacent impurity was also more retained with its K shifting from 1.2 to 2.6. However, a significant selectivity increase was observed from $\alpha = 1.27$ ($1.5/1.2$) to $\alpha = 1.69$ ($4.4/2.6$) giving a baseline resolution between the two peaks with the richer ethanol composition 4:1:4:1 v/v. The 3:1:2:1 system could not fractionate all the target constituents of the crude sample (Fig. 1B) whereas the 4:1:4:1 system fractionated four peaks with good resolution, with the carnosol peak being baseline separated from other compounds (Fig. 1C). Therefore, the heptane/MTBE/ethanol/water 4:1:4:1 v/v system was selected for all further separations.

Fractions between 18 and 27 min were collected for carnosol analysis as illustrated by Fig. 2B-D. It is clear that Fraction 23 (apex of the CPC carnosol peak in Fig. 1C), is pure carnosol. All impurities have been removed when compared to the crude extract analysis (Fig. 2A) allowing obtaining a pure carnosol peak in less than 30 min on the small 35-mL rotor. In order to produce significant amounts of carnosol usable as a chemical standard, the sample loading on the small 35-mL column must be maximized, then a scale-up study should be made to transfer the purification to larger instruments so that

adequate and optimized carnosol productivity can be reached.

Scale-up methodology

The free-space between peaks method

The first step in the scaling-up methodology that we proposed in a recent paper [9] is the quick determination on a small-scale rotor of the free-space available between the compound of interest and its closest impurity. In our case, carnosol partitions in the selected solvent system heptane/MTBE/ethanol/water 4:1:4:1 v/v with a K coefficient of 4.2. It means that carnosol is mostly (80%) located in the aqueous lower phase. So, the lower phase of such system was used to selectively solubilize carnosol from the commercialized dry extract. The commercial rosemary dry extract was dissolved in the 4:1:4:1 lower phase at a concentration of 500 mg/mL. A significant portion of the extract did not dissolve in this phase. The mixture was centrifuged and the solid material was filtered out and discarded. Evaporating the filtrate, it was determined that the maximal amount of crude extract that could be dissolved in the saturated lower phase was 180 mg/mL containing 870 μg of carnosol or 0.48% w/w of the extracted crude material as determined by HPLC (Fig. 2A). This extraction step enriched the rosemary commercial sample in carnosol by about three times. In all further parts of this work, the extracted and filtrated lower phase solution containing 180 mg/mL crude rosemary will be used as the mother liquor containing the carnosol to be purified.

Using this sample, an analytical injection was performed on the 35-mL lab-scale rotor injecting 90 mg (0.5 mL of the 180 mg/mL solution). The resulting chromatogram is shown by Fig. 3A. The free-space volume, ΔV , was calculated as [9]:

$$\Delta V = \Delta V_r - 2(\sigma_A + \sigma_B) \quad (1)$$

ΔV_r is the difference between the retention volumes of the less retained solute A, an unwanted impurity, and the most retained solute B, carnosol, in mL; ΔV is expressed in mL, σ is the volume standard deviation of each peak. Fig. 3A illustrates the determination of a free-space ΔV of 18.7 mL between carnosol and its closest

impurity for the small-scale CPC of rosemary extract.

The next step of the "free space between peaks" method is to increase the sample injected mass keeping on the same small-scale rotor as much as possible, i.e. up to get touching bands on the compound of interest. Fig. 3B shows the chromatograms obtained increasing the injected volume of 180 mg/mL aqueous solution of the rosemary extract. Since the 180 mg/mL solution is the maximum possible concentration for the extract purified, the loading study can only be done increasing the injection volume. A very limited loss of stationary phase was observed since the sample is injected in the stationary phase. Fig. 3B shows the chromatogram evolution during the loading study. Above 1.5 mL (270 mg) injected, the early eluting impurity peak starts to overlap with the carnosol peak. The maximal column load $V_{\text{max.inj}}$ is 1.5 mL in our experimental conditions. This maximum 270 mg load on the small 35-mL rotor contains 1.3 mg of pure carnosol (0.48%). The mass per ΔV proportionality factor of the method is 14.4 mg/mL (270 mg over 18.7 mL). The "free space between peaks" method states that the maximum load on a larger CPC column is related by this proportionality factor to the ΔV between peaks obtained with an analytical injection done on the larger column with the same liquid system [9].

Scaling-up to semi-preparative 254-ml rotor

The attempted first transfer was performed on a semi-preparative rotor with a 200-mL column (exact volume 254 mL). This type of CPC rotor is nowadays the most widespread rotor in laboratories, as it is usually employed both for method development and small production. The analytical injection volume should not pass 1% of the column volume. A 2-mL injection volume was selected corresponding to 360 mg of crude rosemary extract. The much higher weight of the 254-mL and its larger number of cells generating more pressure did not allow spinning it at 2200 rpm. The maximum rotor rotation was only 1800 rpm. High flow rates give quick separations but large volumes of stationary phase are flushed out of the column (low Sf killing resolution) [1-3]. Low flow rates allow for high Sf and good resolution but the experiments are long and the productivity low [31]. Several flow rates were tested between 5 and 50 mL/min to select the 20 mL/min flow rate as being high enough to give

acceptable separation duration and low enough to maintain enough liquid stationary phase. The separation of 2 mL (360 mg) of crude sample at 20 mL/min and 1800 rpm allowed measuring a ΔV volume of 131 mL.

The "free space between peaks" method give a $\Delta V_1 / \Delta V_2$ ratio of $131/18.7 = 7$ that can be multiplied by the 270 mg maximum load on the 35-mL rotor to predict a 1.9 g load on the 254-mL rotor. Fig. 4A presents the separation obtained following the method. The carnosol peak is baseline separated from the leading impurity with touching bands as expected. The 1.9 g injection allows obtaining 9.12 mg of pure carnosol in 50 min (productivity 10.9 mg/h) using one liter of upper organic phase.

As previously observed [9], the productivity could be improved at the cost of a slightly lower purity. Increasing the flow rate is interesting since it will produce purified material more quickly. Since there will be stationary phase losses, the resolution will decrease which can be compensated by lowering the mass load. The analytical 35-mL rotor had a maximum productivity at 40 mL/min (2200 rpm). Making a 2-mL analytical injection on the 254-mL rotor spinning at 1800 rpm and with a 40 mL/min flow rate produced a ΔV of 62 mL only. This tells that it will be possible to inject $62/18.7 = 3.3$ times the maximum mass loaded on the 35-mL rotor that is 0.9 g or 5 mL of 180 mg/mL crude rosemary extract. Fig. 4B shows the baseline separation of the 0.9 g injected mass done in 20 min. This allowed obtaining 4.32 mg of pure carnosol in 20 min (productivity 13 mg/h) using 0.8 liter of organic mobile phase.

Linear scale-up

Direct linear scale-up was used with hydrodynamic CCC columns [4, 32, 33]. The ratio of the two column volumes is the transfer factor. The optimized separation on a small analytical CCC column is directly transferred on a large scale CCC column by using the same biphasic liquid system and a flow rate increased by the column volume ratio. The injected volume is also multiplied by the column volume ratio. Trying a direct linear scale-up in our case gives a column volume ratio of 7.3 ($254 \text{ mL}/35 \text{ mL} = 7.3$). So the optimized analytical separation with a 5 mL/min flow rate and 1.5 ml (270 mg) injected (Fig. 3B) should be linearly transferred onto the 254-mL rotor with a flow rate of 37

mL/min injecting 11 mL (2 g) of rosemary extract. Fig. 4C shows the chromatogram obtained in these conditions with a poor separation of carnosol (arrow). It must be noted that the 2200 rpm rotor rotation of the small 35-mL column could not be maintained as it should have been; the experimental pressure would pass the maximum permitted pressure on the 254-mL column. As opposite to reported studies [7, 9], in this case, the linear transfer towards the larger column cannot provide identical or better separation, due to pressure limitation on the higher scale rotor. When scaling-up a CPC separation, we strongly suggest users to set first the rotation speed as high as permitted by pressure limitations; then select the best flow rate to achieve the purpose.

Scale up on larger 1-L rotor

In order to prove that the "free space between peaks" method was applicable to industrial level, the carnosol purification was achieved on a 1-liter rotor (exact volume 812 mL). On this kind of rotor, method development and loading studies must be optimized regarding to the amount of solvent and hours spent for that purpose.

Two analytical injections at maximum rotation speeds, 1800 rpm, were performed to measure the free-space ΔV . The first one was performed at 50 mL/min, which is a relatively low flow rate for the 814-mL rotor since 17 min are needed for one column volume. However this 50 mL/min flow rate should provide the best resolution. The second analytical injection was performed at 100 mL/min. The free-space ΔV obtained at 50 mL/min was 623 mL allowing to calculate the predicted maximum load on the 814-mL rotor as $623/18.7 \times 0.27 = 9$ g. The ΔV obtained at 100 mL/min was lower, only 436 mL, with a maximum load predicted as $436/18.7 \times 0.27 = 6.3$ g only. However the throughput at 100 mL/min should be higher than that at 50 mL/min.

The predicted quantities of sample were injected at their respective flow rates and the experimental separations are illustrated in Fig. 5. It can be easily observed that baseline resolution of the carnosol peak with touching bands was obtained in both cases. 43 mg of carnosol were obtained in 60 min in the maximum load configuration (Fig. 5A) giving a productivity of 43 mg/h. In one hour, three liters of mobile phase were used. With the 100 mL/min configuration

(Fig. 5B), 30 mg of carnosol were obtained in 30 min that is a productivity of 60 mg/h.

Comparing performances

The transfer methodology applied on a preparative scale rotor required only two analytical injections to accurately predict the production rates. It is undoubtedly less time- and solvent-consuming than any sample load study on the production scale rotor [31].

During the various scale-up presented, we highlighted the fact that, typical of CPC instruments and not hydrodynamic ones, at maximum rotation speed for a given rotor, a low flow rate provides adequate operating conditions for maximal load, useful for batch production while a higher flow rate may provide a larger throughput, suitable for routine larger production.

Table 1 compiles the various operating conditions and results, in regards with quantities, solvents consumption and experiment duration including the required equilibration times between experiments to be as close as possible to real-world practice. It clearly shows that scale-up in CPC is not linear but can easily be predicted in any operating conditions, using a simple analytical injection. For example, the preparative 812 mL rotor is 23 times larger than the laboratory 35-mL rotor. In a batch use, it can produce 45 mg per cycle or 32 times more than the 35-mL rotor (1.4 mg per cycle, Table 1). On a routine process, it exhibits a throughput of almost 50 mg of carnosol purified per hour compared to 3.1 mg per hour for the small rotor. That is 15.9 times higher. While not linear, scale-up in CPC is definitely and accurately predictable. It is interesting to finish this study noting the small change in carnosol productivity per solvent used: the 35-mL rotor produces 9 mg per liter of organic mobile phase when the 812-mL rotor produces 14.3 mg/L. The intermediate 254-mL rotor had the worst volume productivity of 5.6 mg/L.

CONCLUSION

The purification of carnosol from *Rosmarianus officinalis* is an example of predictable scale-up process by CPC. The

recently proposed method was first investigated on a laboratory scale 35-ml rotor and then predictably scaled-up to a mid-scale 254-mL rotor and further to a preparative scale 812-mL rotor. While linear scale up in CPC is not practical, this study clearly shows that a separation that is rapidly developed at laboratory scale, using minimum amounts of time and solvents, can be transferred at any larger scale in a predictable manner. The "free space between peaks" methodology in elution mode provides a simple way to predict maximum load on any CPC rotor and any operating conditions. While maximum rotation speed is highly recommended, the flow rate needs to be adjusted to the user goal on the production instrument, using few analytical injections. The purification of carnosol at industrial scale level was performed successfully demonstrating the predictive potential of the "free space between peaks" method in scaling-up CPC separation.

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Table 1 Compilation of scale-up conditions and results in the carnosol purification from a rosemary extract.

	Laboratory scale		Semi-prep scale		Preparative scale	
	25ml rotor		200ml rotor		1000 ml rotor	
Exact volume	35 ml		254 ml		812 ml	
Objective	Maximum load	Throughput	Maximum load	Throughput	Maximum load	Throughput
Criteria	ΔV_{max}	$\Delta V/t_{max}$	ΔV_{max}	$\Delta V/t_{max}$	ΔV_{max}	$\Delta V/t_{max}$
Flow rate (mL/min)	5	10	20	40	50	100
Rotation speed	2200 rpm		1800 rpm		1800 rpm	
Centrifugal field	545 g		365 g		365 g	
Stationary phase retention, Sf	54 %	46 %	66 %	46 %	68 %	49 %
Pressure (bars)	48	46	54	47	53	54
Injection volume	1.5 mL	1 mL	10.5 mL	5 mL	50 mL	35 mL
Duration of a cycle*	39 min	17 min	71 min	33 min	83 min	38 min
	0.65 h	0.28 h	1.18 h	0.55 h	1.38 h	0.63 h
Rosemary extract injected weight (g)	0.27	0.18	1.90	0.90	9.04	6.32
Throughput crude g/h	0.42	0.64	1.61	1.64	6.53	10.0
Throughput carnosol/cycle	1.4 mg	0.9 mg	9.5 mg	4.5 mg	45.0 mg	31.5 mg
Production carnosol mg/h	2.1	3.1	7.9	8.3	32.3	49.4
carnosol per volume mg/L	10.8	9	10.3	5.6	17.6	14.3
Solvent consumption/cycle	130 mL	100 mL	0.92 L	0.80 L	2.55 L	2.20 L
Solvent consumption/h	190 mL/h	350 mL/h	770 mL/h	1.47 L/h	1.83 L/h	3.45 L/h

*A full cycle comprises equilibrium (1 column volume), elution and extrusion (1 column volume). Flow rate remains the same for all three operations.

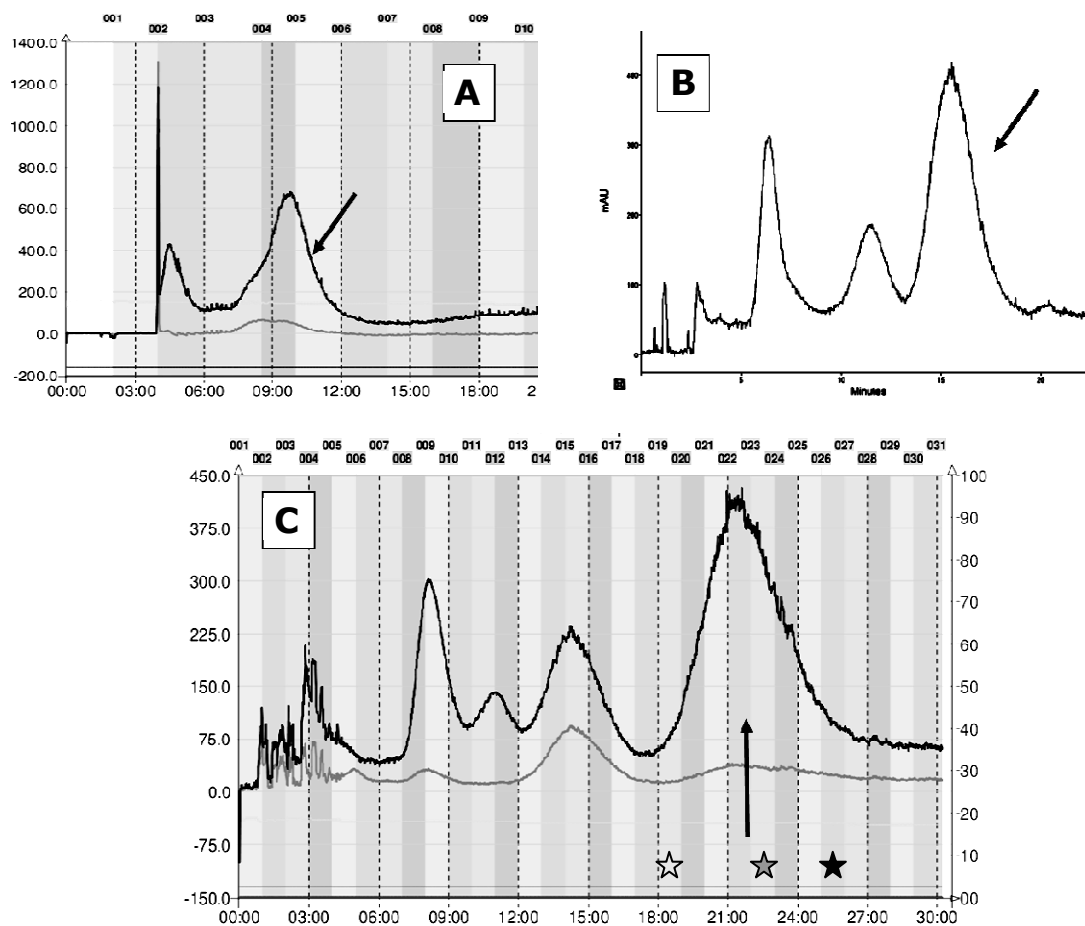


Figure 1. CPC chromatograms of the rosemary extract (1 mL@180 mg/mL) obtained with the 35-mL rotor and different liquid system compositions of the heptane/MTBE/ethanol/water system. **A**-3:1:2:1 v/v (Sf = 63%, P = 56 bar, K = 1.5) , **B**-3:1:3:1 v/v (Sf = 61%, P = 47 bar, K = 3.0) , **C**-4:1:4:1 v/v (Sf = 62%, P = 46 bar, K = 4.4). Rotor rotation: 2200 rpm, upper phase mobile phase flow rate: 5 mL/min in the ascending direction, detection UV 210 nm. The arrows point at the carnosol peaks. The stars indicate the three fractions analyzed in Figure 2 B, C and D.

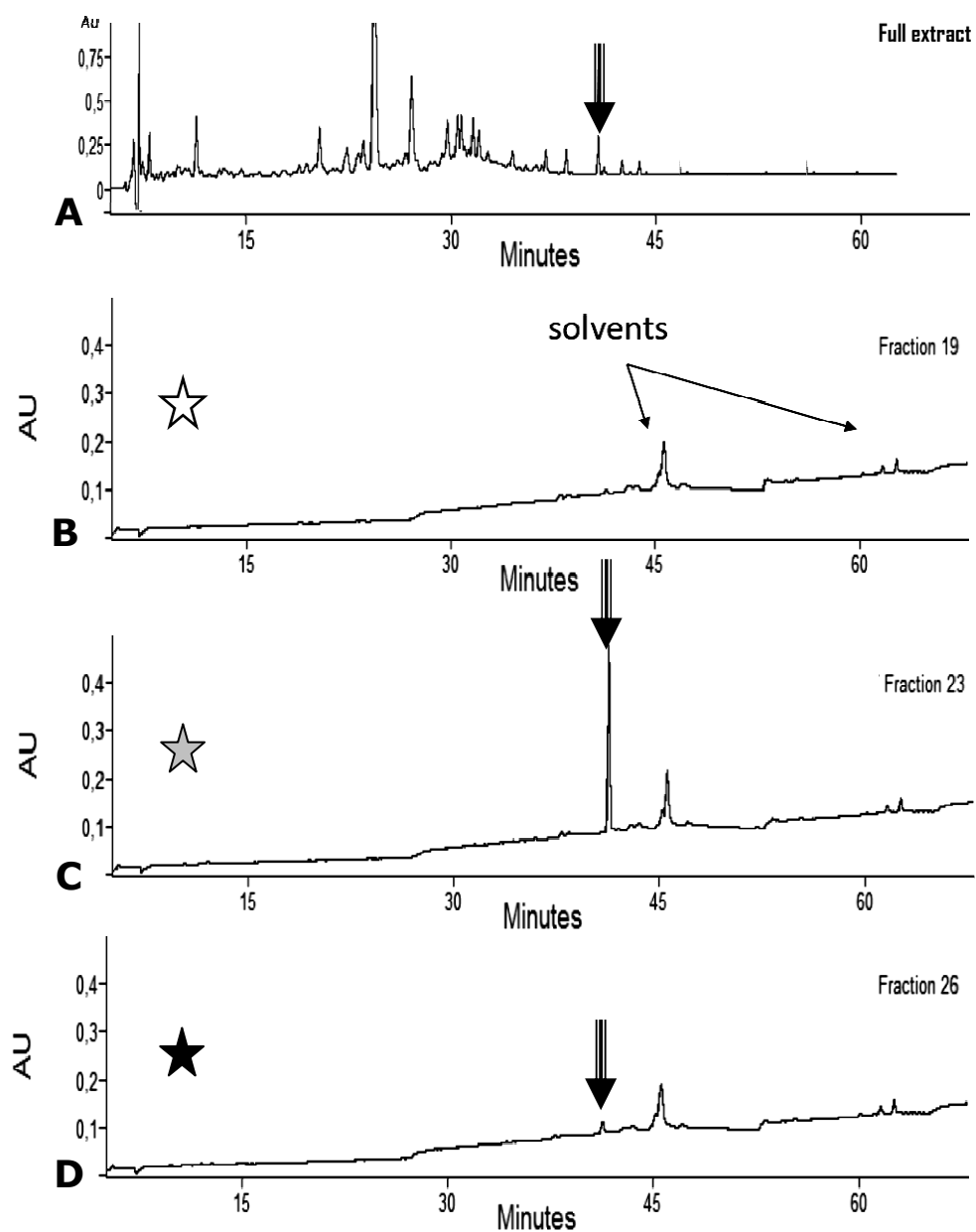


Figure 2. HPLC analyses of **A**-rosemary extract solubilised in methanol/water 90/10, **B**-Fraction 19, **C**-Fraction 23, and **D**-Fraction 26, in upper phase of the system heptane/MTBE/ethanol/water 4:1:4:1 v/v (stars in Figure 1C). Column 5 μ m Zorbax SB-Aq 3x150 mm, gradient elution with acetonitrile-water 0.4 mL/min flow rate, 20 μ L injected, UV detection 254 nm. The vertical down-arrows point the carnosol peaks.

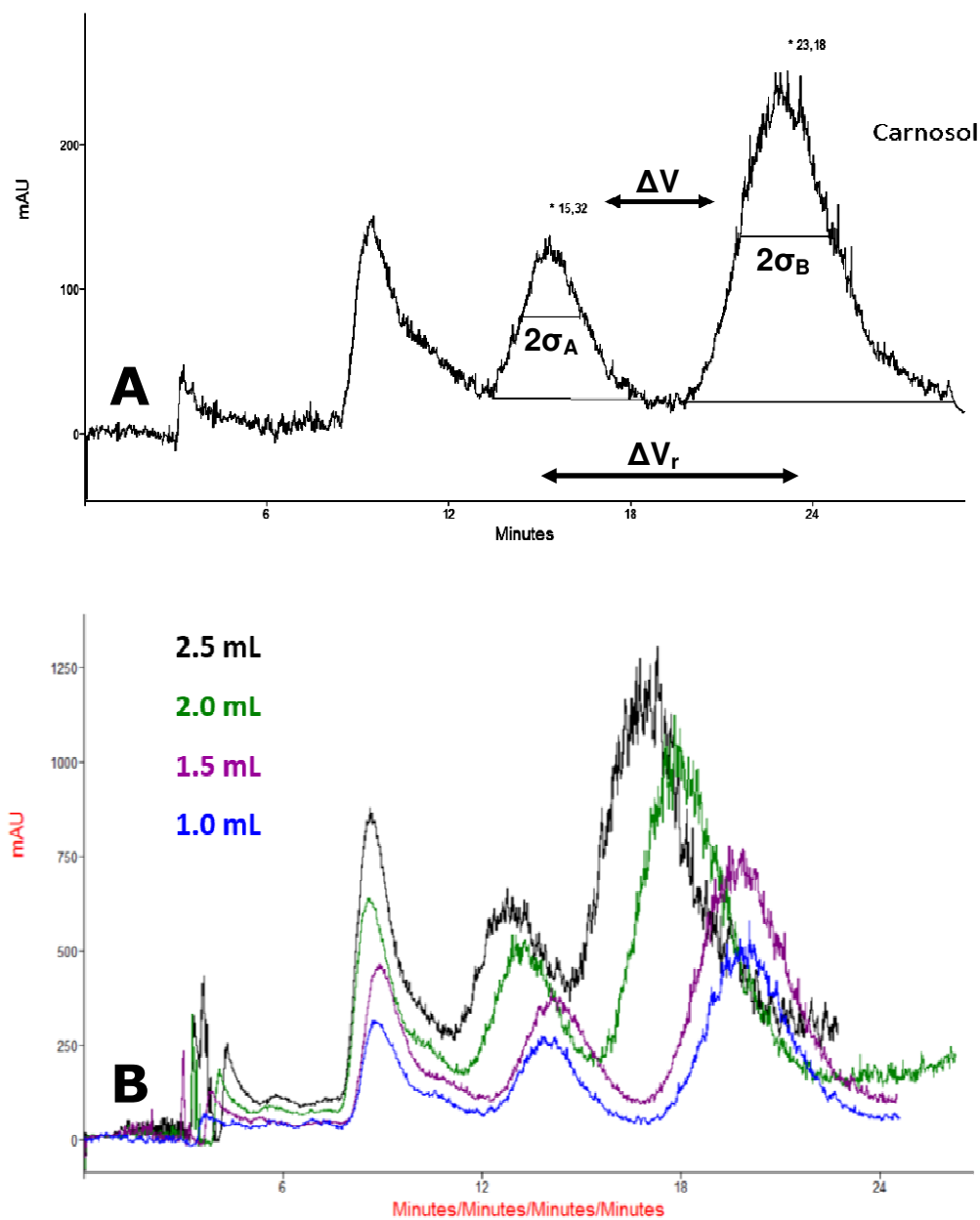


Figure 3. Analytical injections of rosemary extract on a 35 mL-rotor. Solvent system heptane/MTBE/ethanol/water 4:1:4:1 v/v, rotor rotation 2200 rpm, organic upper phase flow rate in the ascending mode = 5 mL/min, K carnosol = 4.4, UV detection 210 nm. **A**-injection of 90 mg (0.5 mL of 180 mg/mL solution in the aqueous phase); S_f = 59%, P = 48 bar; **B**- overlay of loading injections 180 mg (1 mL) S_f = 54%, 270 mg (1.5 mL) S_f = 54%, 360 mg (2 mL) S_f = 50% and 450 mg (2.5 mL) S_f = 46%.

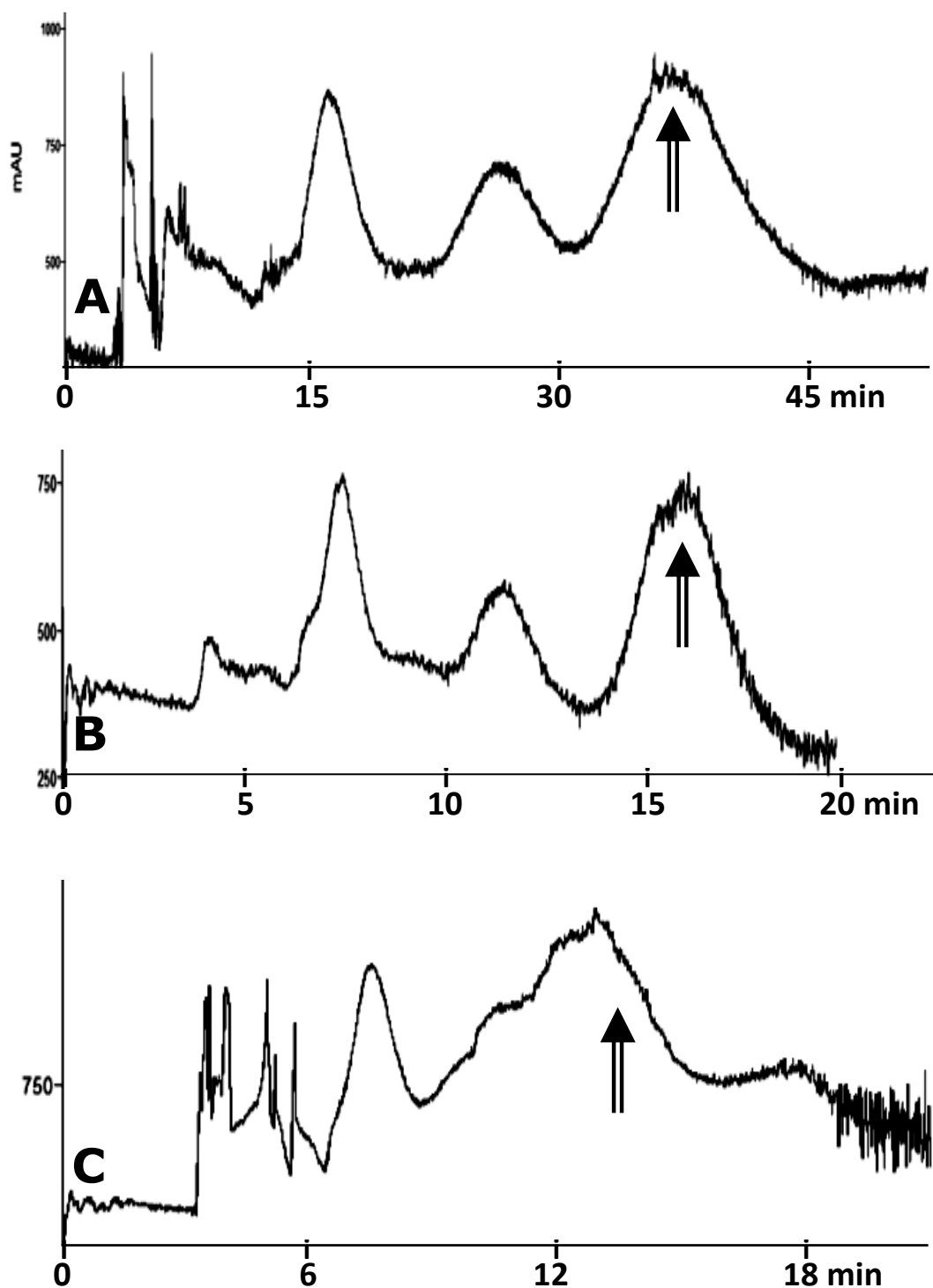


Figure 4. Semi-preparative chromatograms of rosemary extract on a 254-mL CPC column. Solvent system heptane/MTBE/ethanol/water 4:1:4:1 v/v, rotor rotation 1800 rpm, organic upper mobile phase in the ascending mode, $K_{\text{carnosol}} = 4.4$ (arrows), UV detection 210 nm. **A**-maximal load, flow rate: 20 mL/min, injection of 1.9 g (10.5 mL of 180 mg/mL solution in the aqueous phase); $S_f = 66\%$, $P = 54$ bar; **B**-maximum throughput, flow rate: 40 mL/min, injection of 0.9 g (5 mL); $S_f = 46\%$, $P = 47$ bar; **C**- linear scale-up, flow rate: 37 mL/min, injection of 2 g (11 mL) $S_f = 46\%$, $P = 47$ bar.

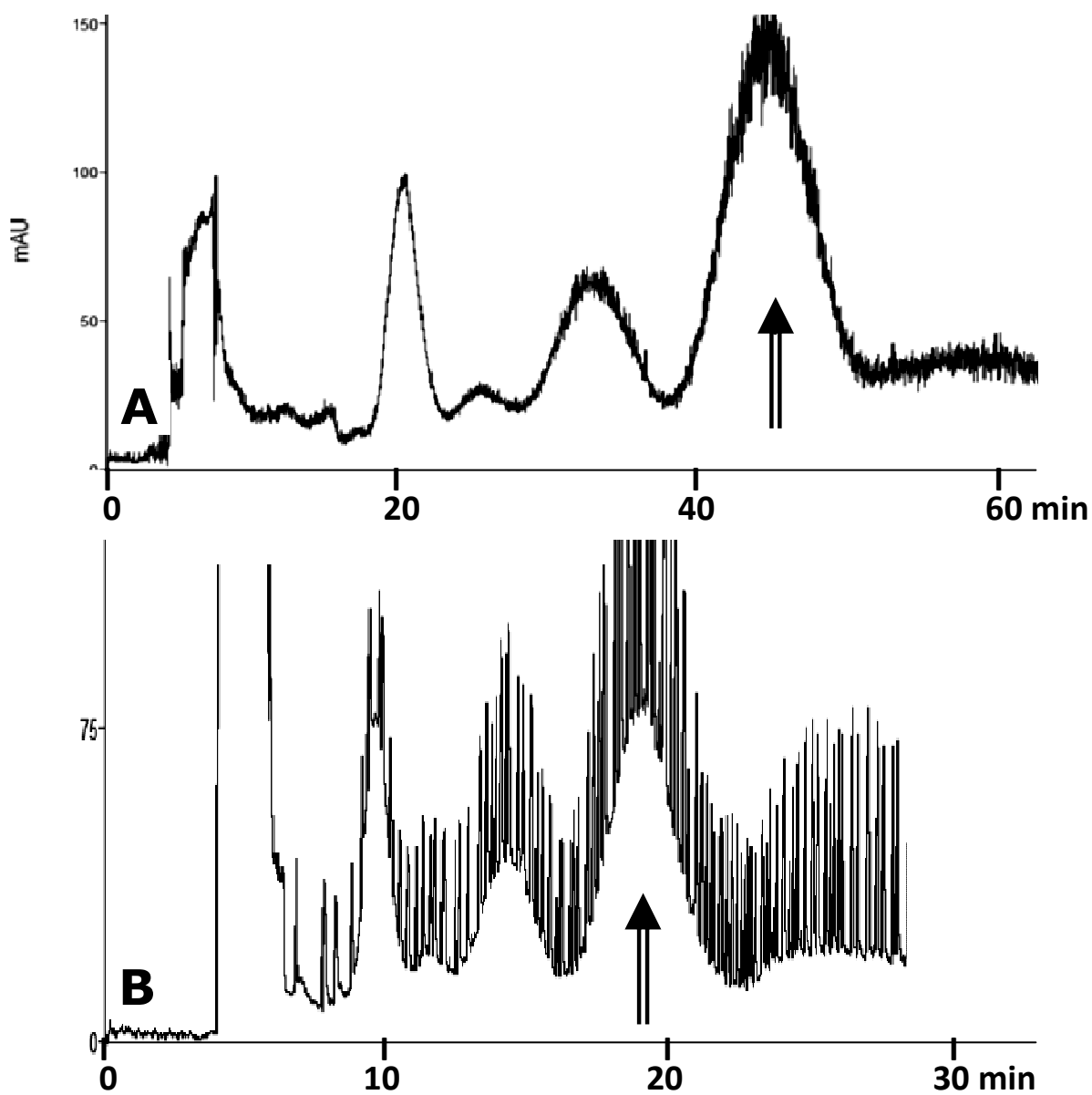


Figure 5 Preparative injections of rosemary extract in a 812-mL rotor. Solvent system heptane/MTBE/ethanol/water 4/1/4/1 v/v, ascending mode. **A-** Maximal loading performed at 50 mL/min, 1800 rpm., $V_{inj} = 50$ mL (9.0 g), $Sf = 68\%$, $P = 53$ bar. **B-** Maximal throughput performed at 100 mL/min, 1800 rpm., $V_{inj} = 35$ mL (6.3 g), $Sf = 49\%$, $P = 54$ bar. The arrows points at carnosol.