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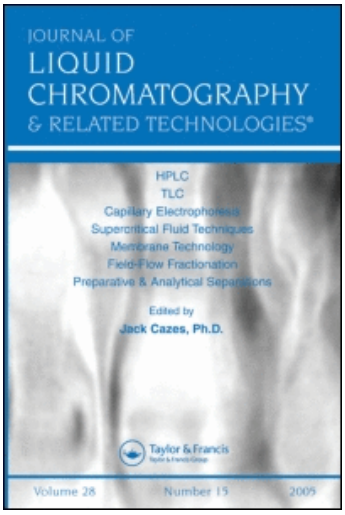

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Abstract: Ion-Exchange centrifugal partition chromatography using benzalkonium chloride as a strong exchanger (SIXCPC) was successfully used to purify rosmarinic acid from a crude extract that was produced by callus culture. The purification process was carried out on a gram scale using the ternary biphasic system $CHCl_3$: *n*-BuOH : water 4.5:1:4.5 v/v/v in the ascending mode (mobile aqueous phase and stationary organic phase). Two particular points are discussed: the influence of benzalkonium chloride on ternary solvent system stability and the advantage of injecting the analytes as sodium salts rather than molecular acids.

Keywords: Rosmarinic acid, Purification, Ion exchange, Centrifugal partition chromatography

INTRODUCTION

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Fig. 1).

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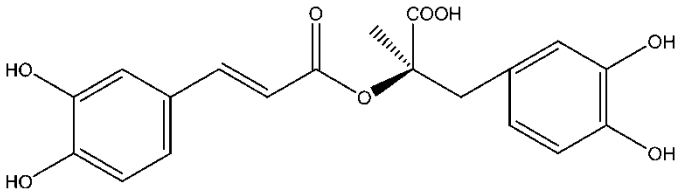

Figure 1. Structure of rosmarinic acid.

Its presence as an accumulated defence compound has mainly been reported in the Boraginaceae and Lamiaceae families. Several interesting biological activities were found for this compound, such as antiviral, antibacterial, anti-inflammatory and antioxidant.[1] Interest in rosmarinic acid has grown as it has been recognized as a good health promoting substance in medicinal plants, herbs and spices. For this reason, the biotechnological production of rosmarinic acid from plant cell cultures has been undertaken by many groups. Its purification from culture medium is not straightforward; we propose an original and efficient method to reach this goal. The purification of organic acids, such as isomers of hydroxy-cinnamic acid has previously been developed in our group, using Ion-Exchange Centrifugal Partition Chromatography (IXCPC).[2] For this purpose, benzalkonium chloride was selected as a *strong* anion-exchanger and iodide as the displacer. The resulting technique will be referred to as SIXCPC. The same methodology was applied to the one-step purification of rosmarinic acid from the crude ethanolic extract of *Lavandula vera* cell suspension. High purity rosmarinic acid was obtained in high yield (3.4% of dry extract). The protocol should be amenable to preparative purification of rosmarinic acid.

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EXPERIMENTAL

Reagents

Benzalkonium chloride was a commercial mixture containing isomers,with an alkyl tail from C_8 to C_{10} in proportion less than 5%; C_{12}: 50 to 60%; C_{14}: 30 to 40% and C_{16} to C_{18} less than 5%. It was provided by Acros Organics (Noisy le Grand, France). Sodium iodide was also purchased from Acros Organics. Chloroform, *n*-butanol and sodium hydroxide came from Carlo Erba (Rodano, Italy). Water was purified by de-ionization and inverse osmosis.

CPC Apparatus

The separations were performed on a FCPC Kromaton Technologies apparatus (Fig. 2) (Angers, France). This machine is a hydrostatic CCC

Figure 2. Kromaton Technologies FCPC® 200 mL rotor and partition disk. Note the connecting ducts centred on the bottom and the top of each cell. The upper and lower cell walls are made of inter-disk Teflon® gaskets.

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chromatograph with a rotor made of 20 circular partition disks (1320 partition cells, column capacity: 200 mL). The rotation speed can be adjusted from 200 to 2000 rpm, producing a centrifugal force field in the partition cell of about 120 *g* at 1000 rpm and 470 *g* at 2000 rpm.

The solvents were pumped using a Dionex P580HPG 4-ways binary high-pressure gradient pump (Sunnyvale, CA, USA). The samples were introduced into the CPC column via a low pressure injection valve (Upchurch, CIL Cluzeau, Sainte-Foy-La-Grande, France) equipped with a 21 mL sample loop. The effluent was monitored with a Dionex UVD 170S detector equipped with a preparative flow cell (6 μL internal volume, path length of 2 mm). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The experiments were conducted at room temperature (22 ± 1°C).

Plant Cell Culture

The *Lavandula vera* callus culture (marked as *L. vera* MM) was obtained from the stem explants of the oleaginous Bulgarian variety "Druzba"[3] and was

maintained in Linsmayer-Skoog agar medium[4] supplemented with 30 g · L^{-1} sucrose and 0.2 mg · L^{-1} 2,4-dichlorphenoxy acetic acid at 26°C for more than 10 years.

L. vera MM cell suspension was cultivated for 12 days in a 3L (2.2 L working volume) laboratory bioreactor (New Brunswick, BioFlo 110) containing LS modified nutrient medium[5] at culture conditions as follow: agitation −100 rpm, temperature −26°C and concentration of dO_2−30% of air saturation. For inoculation, 20% (v/v) cell suspension *L. vera* MM was grown on a shaker for 7 days. After the cultivation was complete the cell biomass was separated by filtration and was used for further extraction of rosmarinic acid.

Rosmarinic Acid Extraction

The rosmarinic acid was extracted from cell biomass with 50% (v/v) ethanol/water (three times during 20 minutes) at 70°C. The extract was evaporated to dryness, the dry residue was dissolved in a small volume of 70% (v/v) ethanol and was then stored for 24 hours at 10°C. The precipitate was separated by filtration and the filtrate was lyophilized using an ALPHA 1–2 lyophilizer (Martin Christ GmbH, Germany). The obtained dry material was used for purification experiments.

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Preparation of Solvent Systems

Biphasic systems were prepared by mixing $CHCl_3$, *n*-BuOH, and water (4.5:1:4.5 v/v/v) in a separatory funnel, shaking them vigorously and allowing them to settle until the phases became limpid. The aqueous phase was adjusted to ∼pH 7 by adding drops of a 1 M NaOH solution. After phase separation, 21 g of benzalkonium chloride was added in 250 mL of the organic stationary phase. This corresponds to a concentration of 230 m*M* considering an average molecular weight of 360 g · mol^{-1} for the mixture of benzalkonium chloride isomers. This organic phase was then equilibrated with a small quantity of aqueous phase. The mobile phase was prepared by dissolving NaI in the aqueous phase (3.8 g · L^{-1}, 25 m*M*).

Preparation of Sample Solutions

Five grams of the crude ethanolic extract was dissolved in 20 mL of fresh aqueous phase (without NaI). The pH was exactly 7 without any adjusting. This aqueous solution was equilibrated with the benzalkonium-containing organic phase to restore the saturation of the aqueous phase. In order to allow the sample to transfer to the stationary organic phase as

benzalkonium-rosmarinate ion-pairs in the first cells of the CPC column, 80 mL of the NaI-free mobile phase were pumped after sample injection for 40 min.

CPC Experimental Conditions

Before the experiment, water was pumped in the rotating column, followed by the injection of two column volumes of the organic stationary phase in ascending mode, at 20 mL/min and at 500 rpm. The sample solution was injected at 2 mL/min and 1300 rpm. Then 80 mL of pure mobile phase (without NaI) were pumped in at 2 mL/min. The outgoing phase corresponding to this step did not contain any acid. The displacement mobile phase (NaI containing) was then pumped at 2 mL/min, and the fractions collected every 5 min. The outgoing aqueous phase was monitored at 311 nm by the online UV detector. Stationary phase retention was 70% ($V_S = 140$ mL) at the beginning of the run and decreased to 40% ($V_S = 80$ mL) at the end of the experiment. The back pressure was approximately 60 bars at the beginning of the experiment decreasing to about 35 bars at the end of the separation.

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Fraction Analysis

Quantification was performed on a customized Dionex Summit HPLC system, equipped with a P580 pump, an ASI-100 automated injector, a STH column oven and a UVD340S diode array detector and a C_{18} Uptisphere 5HDO-25QS (250 × 4.6 mm i.d., 5 μm particle size) column (Interchrom, Montluçon, France). The mobile phases were acetonitrile/water mixtures acidified by TFA 200 μL/L. The acetonitrile/water gradient was set as follows: the starting composition contained only 10% v/v acetonitrile. It was raised to 30% v/v acetonitrile in 20 min, then to pure acetonitrile (100% v/v) in 5 min and maintained for another 5 min. Eventually a decrease to 90% acetonitrile was made in 5 min to finish the analysis for 5 min at 90% acetonitrile. The flow was 1 mL/min. The temperature of the column oven was set at 25°C. All the chromatographic data management was ensured by the Chromeleon software 6.0.1 version (Dionex, USA). Evaporation under vacuum and centrifugation was performed in a Jouan RC10.22 centrifugal evaporator (Jouan Inc, Wincester, VA, USA).

Pseudo Ternary Diagram[6]

A solubility isotherm (or a binodal curve) of benzalkonium chloride in the $CHCl_3$: *n*-BuOH : water 4.5:1:4.5 v/v/v was built. Pre-defined ratios (w/w) of stationary phase were successively added to 150 mg of benzalkonium chloride until the appearance of the conjugated phase. The coordinates of

these points are plotted to design the ternary diagram, presented in its orthogonal form.

RESULTS AND DISCUSSION

SIXCPC

Basically, strong ion-exchange CPC can be performed by dissolving a lipophilic quaternary ammonium salt in the organic stationary phase. The analytes in their carboxylate form (water-soluble ion-pairs) are extracted in the stationary phase as lipophilic benzalkonium ion-pairs. Then a displacer-containing aqueous mobile phase is pumped through the stationary phase, and performs a "re-extraction" of the analytes in the mobile phase as hydrophilic species.[2,7,8]

The chromatographic process involved here (SIXCPC) is a particular case of development by displacement, and therefore implies that analytes progress in the CPC column as an isotachic train. In each cell, the analyte which shows the greatest affinity for the benzalkonium cation (*i.e.* the strong anion exchanger) in the organic stationary phase excludes competitively those with lower affinity. Thus, it acts as a displacer by forcing them to solubilize in the aqueous mobile phase and to progress in the column. The shock layer is the overlapping region between two separated anions, each of them showing a steep concentration drop or rise at the column outlet. The sharp front of the analyte train is formed by competition with the chloride anion (*i.e.*, the carrier) on the exchanger whereas the end of the train is maintained as a shock layer by iodide anion as displacer. Once the analytes are separated by mutual exclusion, they progress in the CPC column as neighboring segments (Fig. 3).

In this development mode, separation only depends on the association constant ratios of the different anions with the benzalkonium cation. Graphical representations of simulated separations have been drawn from numerical modelling (Fig. 4). The latter uses a numerical model of the chromatographic process that was previously described to predict column content and effluent composition at each time. This simulation confirms the

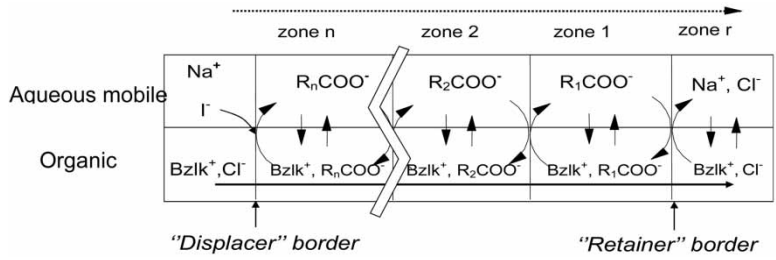

Figure 3. Isotachic train in the displacement mode.

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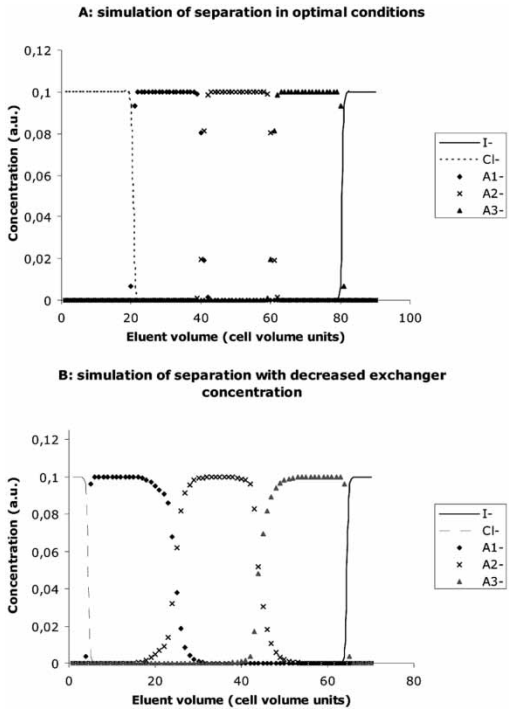

Figure 4. Simulated separations of three analytes A_1^-, A_2^-, A_3^-, with Cl^- as retainer and I^- as displacer. For numerical data, see Fig. 3 in Ref. 2. Simulation (A) shows an ideal separation with steep evolution of analyte content in the shock layers. If column capacity is lowered by decreasing the exchanger concentration (B), the shock layers become wider.

displacement mode with the analytes flowing out as contiguous blocks at constant concentration equal to that of the displacer.

Influence of Benzalkonium Concentration on the Biphasic System Stability

In ion-exchange CPC, the capacity of the column is related to the preparative ability of the technique. It is closely related to the concentration of the ion-exchanger (benzalkonium chloride in this work) in the organic stationary phase. However, the dissolution of a large amount of benzalkonium chloride can dramatically disturb the phase composition of the initial biphasic system ($CHCl_3$:*n*-BuOH:water 4.5:1:4.5, v/v/v). A monophasic system may even be obtained. In order to evaluate the influence of benzalkonium chloride on the initial biphasic system, the strategy developed in our recent study of mass overload in CPC[6] was applied. It requires the

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construction of the "mobile phase/stationary phase/benzalkonium chloride" pseudo ternary phase diagram. The position of the binodal curve maximum indicates if the biphasic system is "robust" towards the addition of a large quantity of benzalkonium chloride or not, and can be used for exchanger maximum mass determination.

Figure 5 presents the resulting pseudo ternary diagram and shows that the system "mobile phase/stationary phase/benzalkonium chloride" is rather robust. The exchanger concentration used in this work (84 g/L of organic stationary phase) necessitates only a small addition of aqueous phase to restore the saturation.

The Injection Step

Theoretically, two protocols can be used to inject the analytes in the column. Indeed, the lipophilic ion-pairs can be generated before or during the injection. The latter appears to be more efficient for two reasons:

a) the preparation of the benzalkonium ion-pairs before the CPC run implies an additional time-consuming step,

b) the generation of benzalkonium ion-pairs during the injection step (see Experimental) induces a pre-fractionation of the sample in the head of

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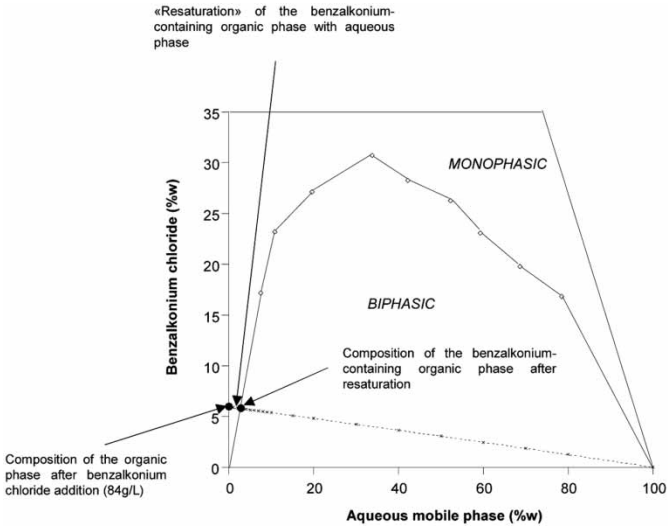

Figure 5. Pseudo ternary diagram of the system benzalkonium chloride-aqueous mobile phase-organic stationary phase (in%, w/w) and representation of the re-saturation step of the organic phase after benzalkonium addition.

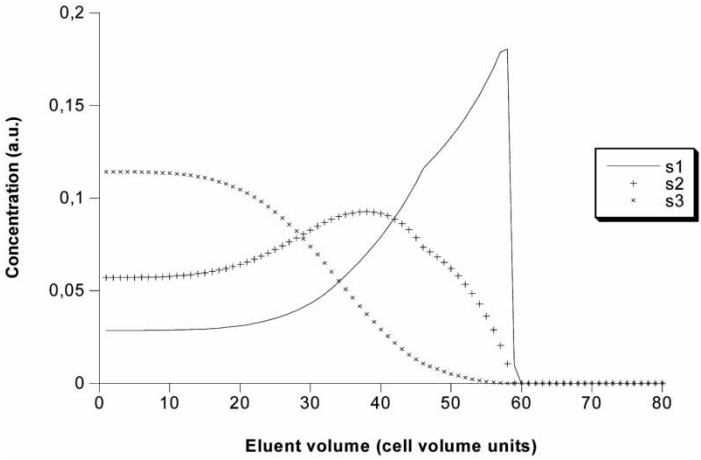

Figure 6. Simultated injection of three analytes A_1^-, A_2^-, A_3^-, with Cl^- as retainer and I^- as displacer. In this simulation $K_{I^-/Cl^-} = 10^6$, $K_{I^-/A1^-} = 50$, $K_{I^-/A2^-} = 100$, and $K_{I^-/A2^-} = 200$. The injection volume is equal to five cell volumes. Each analyte is injected at a concentration of 0.8 (arbitrary units). The ratio $v = V_{stat}/V_{mob}$ of the phase volume in each cell is equal to 1, the displacer and retainer concentration is fixed to 0.05 and 0.2, respectively.

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the CPC column. This phenomenon is due to the different association constants of the different analytes with the exchanger and can be predicted by numerical modelling (Fig. 6).

Rosmarinic Acid Purification

Figure 7 shows the CPC chromatogram, recorded at 275 nm, obtained after the injection of 5 g of the crude ethanolic extract (dissolved in 19 mL of NaI-free aqueous stationary phase and 1 mL of the organic mobile phase). Fractions 83 to 86 were found to be identical. They were pooled together, evaporated to dryness, and then analysed by RP-HPLC. They contained 335 mg of rosmarinic acid (HPLC purity: ~90%). The volume of the pooled fractions corresponds to 40 mL that leads to a concentration of 23 m*M* for rosmarinic acid. It is consistent with the concentration fixed at 25 m*M* for the displacer (NaI). The poor quantity of isolated rosmarinic acid could be explained by the low rosmarinic content in the injected crude extract, the latter containing mainly sugars used as nutrition in the culture media.

CONCLUSION

Ion-exchange CPC using a strong exchanger (SIXCPC) was successfully used to purify rosmarinic acid from a crude extract that was produced by callus

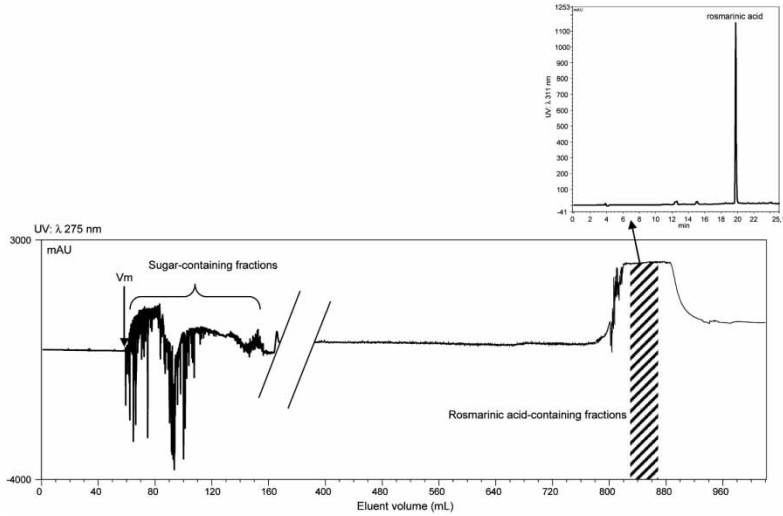

Figure 7. Chromatogram of rosmarinic acid purification using SIXCPC on a FCPC Kromaton 200 mL column. Biphasic solvent system: $CHCl_3$:*n*-BuOH:water 4.5:1:4.5 v/v/v, benzalkonium chloride in the organic stationary phase: 233 m*M*, sodium iodide in aqueous mobile phase: 25 m*M*. Ascending mode, rotation speed: 1300 rpm, back pressure: 58–61 bars, retention: 70%–40%.

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culture. The purification process was carried out on a gram scale. Its scaling up to a production-compatible process is feasible, because care was taken to ensure a high column capacity. The same approach will be used in a near future for the purification of natural and synthetic anionic organic molecules.

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