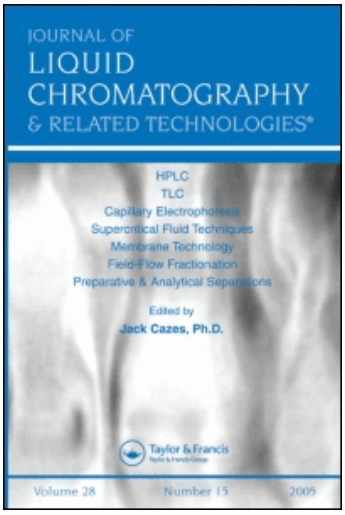

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LARGE SCALE CENTRIFUGAL PARTITION CHROMATOGRAPHY IN PURIFICATION OF POLYPHENOLS FROM *OROBANCHE RAPUM*

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ABSTRACT

Centrifugal partition chromatography linked with evaporative light scattering detection has been investigated to achieve the large scale-up isolation of oraposide which is a phenylpropanoid glycoside having a radical-scavenging activity. A two-phase solvent system has been studied to improve the yield of pure product and to decrease the cost of purification. A comparison with preparative HPLC has been made.

INTRODUCTION

Previous work[1] has shown interest in Centrifugal Partition Chromatography (CPC) for the isolation of phenylpropanoid glycosides such as oraposide and verbascoside from *Orobanche rapum* extract. In fact, the high content of these two compounds gives the plant a radical-scavenging activity which is interesting for the cosmetic and pharmaceutical industries. Oraposide has been patented by Parfums Christian Dior;[2] however, the quantity of crude extract purified by the

binary system used (ethylacetate/acetonitrile/water; 26.2/25.8/48) is too small (less than 300 mg). Because of the high toxicity and cost of acetonitrile, a new system has been developed, in order to increase the quantity of pure oraposide which is unavailable commercially for biological assays and as standards for titration.

Since the bleeding of the liquid stationary phase involves a high noise baseline in UV detection,[3] evaporative light scattering detection (ELSD) was set to monitor the compound elution. ELSD also permits the use of highly UV-absorbing solvents in the binary system.

EXPERIMENTAL

CPC Apparatus

Analyses were performed using a Sanki Model LLB-M (Sanki Engineering, Kyoto, Japan). A TSP Pump Model P 100 (Thermo Separation Products, Les Ulis, France) was used to deliver liquid phases at a flow-rate of 4 mL/min. Detection of solutes was carried out by using ELSD Model Sedex 45 (Sedere, Alfortville, France) set in derivation with a split 1/10 of the mobile phase. This mode of detection enabled a highly UV absorbing solvent such as acetone and ethylacetate to be used.

HPLC Apparatus

Analysis of CPC fractions was performed using a Merck pump Model (Merck, Darmstadt, Germany) at a flow-rate of 1.5 mL/min. The column used was a Lichrospher RP 18, 5 μm (125 x 4 mm I.D.) from Merck. Gradient elution was carried out with solvent A (water with 0.1% of trifluoroacetic acid) and solvent B (acetonitrile with 0.1% of trifluoroacetic acid) from 90% A to 75% A during 20 min. The retention times were respectively 13.20 min and 15.07 min for verbascoside and oraposide. Detection was performed by UV detection at 330 nm. The quantitative determination was described in previous work.[1]

Equipment

Since our previous work, the extraction method has been gradually modified in order to enhance, fivefold, the solubility of the crude extract in the injection solvent with a similar content of oraposide and verbascoside.

RESULTS AND DISCUSSION

Determination of a New Solvent System

Systems with ethyl acetate/acetonitrile/water enable each polyphenol[1] to be easily purified. However, the time of oraposide purification is too long and

the injected amount too small (300 mg of extract in 3mL of aqueous liquid phase). Another system has, therefore, been investigated. The aim was to modify the system slightly to decrease retention, to maintain a good selectivity, and to increase the solubility of compounds for scale-up purification of oraposide.

The partition coefficient K_i of a solute *i* between two immiscible liquid phases *w* and *o*, respectively aqueous and organic, is defined by the following equation:[3]

$$RT \ln K_i = V_i (\delta_w - \delta_o) (\delta_w + \delta_o - 2\delta_i) \quad (1)$$

where V_i is the molar volume of the solute *i* and δ is the solubility parameter defined by Hildebrand.[5] Here K_i represents the ratio $C_{i(s)} / C_{i(m)}$ where $C_{i(s)}$ is the concentration in the stationary phase and $C_{i(m)}$ the concentration in the mobile phase.

In order to modify the solvent system, acetonitrile has been replaced by acetone as these two solvents are in the same group (group VI characterizing the solvents with dipole interactions) according to Snyder.[6] The polarity of acetone is lower than that of acetonitrile : 5.1 and 5.8 respectively in the Snyder scale; 9.77 and 11.75, respectively in the solubility parameter scale,[7] the difference being principally due to the larger dipole interactions with acetonitrile.

Thus, various mixtures of ethylacetate/acetone/water (mixtures IIa to IId, see Table 1) have been considered instead of the ethylacetate/acetonitrile/water mixtures (mixtures Ia to Id used in the previous work[1]). The polarities of aqueous and organic liquid phases are defined by δ_w and δ_o respectively and the values were calculated from the δ values of the pure solvents as follows:

$$\delta_\omega = \Phi_1\delta_1 + \Phi_2\delta_2 + \Phi_3\delta_3 \quad (2)$$

where δ_1, δ_2 and δ_3 represent the parameter of each solvent in the mixture and Φ their volume fraction.

As shown in Table 1, the values $\delta + \delta_o$ obtained with acetonitrile mixtures (I systems) are larger than the values with acetone mixtures (II systems) for a similar value of δ_w-δ_o. Thus, for a given value δ_w-δ_o, ln Ki will be smaller with acetone mixture.

Moreover, for a given system (I or II), as δ_w+δ_o varies very weakly, if $\delta_w + \delta_o < 2\ \delta i$, the increase of δ_w-δ_o involves a decrease of ln Ki which is < 0. If $\delta_w + \delta_o \approx 2\ \delta i$, the effect of a δ_w-δ_o increase will be very small and ln Ki will be quasi constant.

Table 1

Composition of the Two Two-Phases Systems, and Corresponding Calculated Values of δ_w and δ_0

	Aqueous Mobile Phase				Organic Stationary Phase					
System	**EtOAc**	**ACN**	**Water**	δ_w	**EtOAc**	**ACN**	**Water**	δ_0	$\delta_w + \delta_0$	$\delta_w - \delta_0$
I a	5.7	7.3	87	21.8	81.5	12.5	6	10.3	32.1	11.5
I b	5.8	14.2	80	21.0	64.9	24	11.1	11.3	32.3	9.7
I c	6.4	20.6	73	20.2	49.9	32.3	17.8	12.5	32.7	7.7
I d	8.1	27.5	64.4	19.1	37.85	36.25	25.9	13.8	32.9	5.3
	Aqueous Mobile Phase				**Organic Stationary Phase**					
System	**EtOAc**	**Acetone**	**Water**	δ_w	**EtOAc**	**Acetone**	**Water**	δ_0	$\delta_w + \delta_0$	$\delta_w - \delta_0$
II a	8.7	3.9	87.4	21.7	90.8	5.5	3.7	9.7	31.4	12.0
II b	9.2	18.2	72.6	19.7	71.7	22.4	5.9	10.1	29.8	9.6
II c	12.7	23.6	63.7	18.4	62.8	29.1	8.1	10.5	28.9	7.9
II d	14.3	30.9	54.8	17.2	51.8	36.4	11.8	11.0	28.2	6.2

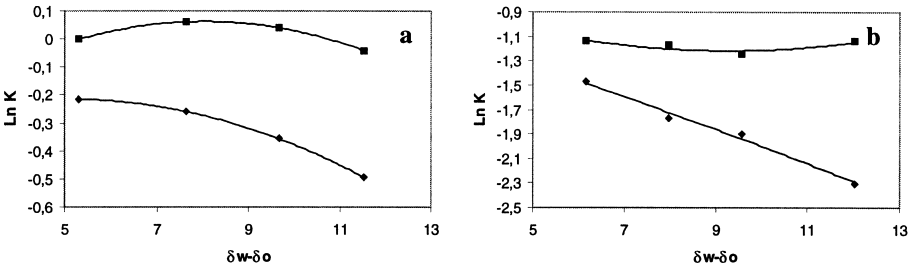

Figure 1. Influence of δ_ω-δ_o value on Ln Ki. (a) System EtOAc/ACN/Water. (b) EtOAc/Acetone/Water

Experimental results shown in Figure 1 illustrate this: elution determined by K_i value will be faster with acetone mixtures than with acetonitrile mixtures. The comparison of K_i from Figure 1 confirms the reversed phase liquid chromatographic behavior where verbascoside, which is more hydrophilic, is eluted before oraposide and illustrates the larger hydrophobicity of oraposide observed in Micellar Electrokinetic Chromatography.[8] Moreover, the variation of K_i as a function of $\delta_w - \delta_o$ shows that $\delta_i \approx (\delta_w + \delta_o)/2$ for oraposide and $\delta_i > (\delta_w + \delta_o)/2$ for verbascoside. Thus the selecti-vity increases as the polarity difference $\delta_w - \delta_o$ between the two phases increases.

Preparative Scale

The system chosen for the purification of oraposide is system IIa because of its better selectivity. The injected quantity is about 2 grams of extract dissolved in 10 mL of mobile phase. The retention of the stationary phase was > 70% in the descending mode. As this retention is high and as the polar impurities are eluted in void volume before the first peak of verbascoside, the pollution of organic stationary phase was weak at each run and three successive purifications could be achieved, with a final retention of stationary phase of approximately 65%.

By maintaining the rotation at high speed, the loop valve is again loaded, then the mobile phase flow-rate is increased at the desired flow-rate. Between each injection, 5 to 10% of stationary phase are replaced in descending mode. The reproducibility of the three purifications is shown in Figure 2.

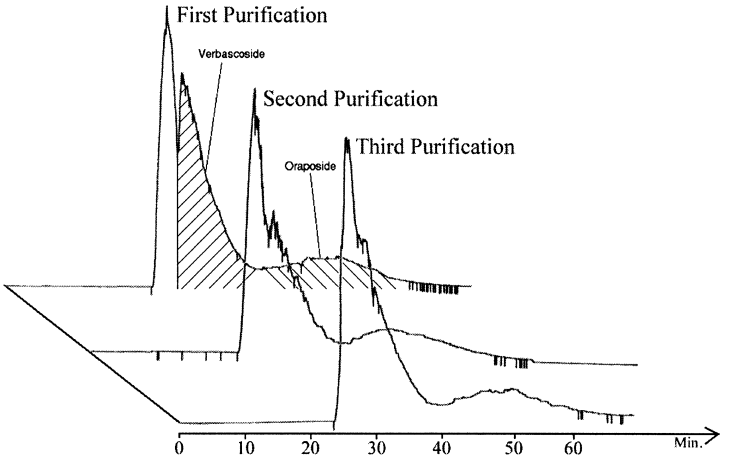

Figure 2. Three successive purifications with the same stationary phase. System EtOAC/Acetone/Water. Descending mode (aqueous mobile phase), Flow rate = 4mL/min., Rotational Speed : 1100 rpm. ELSD detection.

Finally, 6 gram of extract is purified and the yield of oraposide is approximately 450 mg with a 100% purity. Verbascoside is also purified (yield 1200 mg, 80% purity).

The optimization of all the parameters (solvent system, crude extract, injection conditions) has allowed purification of 6 gram of extract instead of

Table 2

Comparison Between CPC and Preparative Scale HPLC for a 2 grams Purification of Raw *Orobanche Rapum* Extract

	CPC	HPLC
Injected amount	2 gr	2 gr
Injected volume	10 mL	30 mL
Stationary phase nature	EtOAc/Me_2CO/H_2O (90.9/5.4/3.7, v/v/v)	C18 bonded silica
Stationary phase quantity	240 mL	240 gr
Mobile phase nature	EtOAc/Me_2CO/H_2O (8.7/3.9/87.4, v/v/v)	H_2O/EtOH + TFA (80/20, v/v) + 0.1%
Mobile phase quantity	220 mL	1500 mL
Mobile phase flow-rate	4 mL/min	100 mL/min
Analysis time	55 min	15 min

300 mg, i.e. 20 fold more at a lower cost because acetone is approximately half the price of acetonitrile.

Table 2 compares the cost of purification of 2 grams of extract between CPC and HPLC preparative. CPC is more attractive than HPLC preparative because it uses only 220 mL of mobile phase compared to 1500 mL for HPLC. Moreover CPC does not require solid support. In fact, the price of the C18 packing used in HPLC is about $1500 per kilogram, while in CPC the stationary phase is only a hydro-organic phase. Moreover, the apparatus used in CPC consists of an analytic HPLC pump, whereas a more expensive pump is required for preparative HPLC. This scale-up potential of CPC versus HPLC with a standard machine is in agreement with the work of Sutherland.[9]

CONCLUSION

An optimized two-phase CPC system has enabled the isolation of a large amount of phenylpropanoid glycosides having a patented radical-scavenging activity. The solvents chosen are less expensive and less toxic than those used previously and a high scale-up is achieved in three successive runs with a standard LC apparatus in a shorter time.

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