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# Isolation of indole alkaloids from *Catharanthus roseus* by centrifugal partition chromatography in the pH-zone refining mode

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**Abstract**

Centrifugal partition chromatography (CPC) in the pH-zone refining mode allowed a preparative and efficient isolation of vindoline, vindolinine, catharanthine and vincaleukoblastine from a crude mixture of *Catharanthus roseus* alkaloids. The separation protocol was tested with a synthetic mixture of vindoline, catharanthine and vincaleukoblastine. The fraction content was analyzed and the results compared with theoretical chromatograms obtained by numerical simulation. The increase in injected sample mass results in an improvement of the purity of the isolated compounds. This observation, confirmed by theory, is of prime importance for the development of preparative pH-zone refining CPC as a preparative separation method. 

*Keywords:* Counter-current chromatography; *Catharanthus roseus*; Centrifugal partition chromatography; pH-Zone-refining counter-current chromatography; Alkaloids; Indole alkaloids

## 1. Introduction

*Catharanthus roseus* G. Don (Apocynaceae) is a plant that produces indole alkaloids used in anticancer chemotherapy. The preparation and the purification of these compounds have been intensively studied [1]. Aerial parts of the plant contain between 0.2 and 1% of a mixture of about 90 different alkaloids. The most abundant ones are the monomers like catharanthine and vindoline, bearing the indole and the indoline chromophore respectively (Fig. 1). The dimers resulting from the coupling of two such compounds present interesting pharmaceutical activities. Anticancer drugs like leurocristine and vincaleukoblastine (or vinblastine or VLB) belong to this category (Fig. 1). Industrial-scale production of these molecules proceeds in two different ways. The first one follows the classical extraction and isolation scheme. This approach is hampered by the complexity of crude extracts and by the low proportion of valuable dimeric compounds. The other way resorts to the hemisynthesis or the biomimetic coupling reaction that uses monomers as starting material. Both methods require the development of an efficient, selective, and preparative chromatographic technique able to isolate dimers and monomers from complex extraction mixtures. This article shows that this goal is achieved by means of Centrifugal Partition Chromatography (CPC) when used in the pH-zone refining mode [2].

CPC is a liquid–liquid partition chromatographic method generally referred to as counter-current

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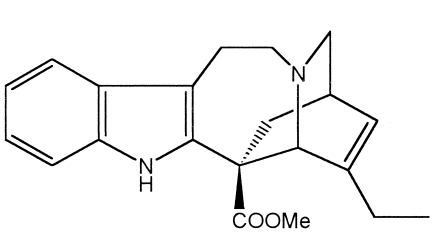


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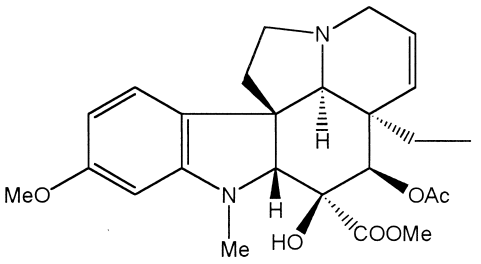


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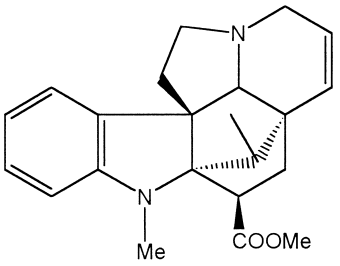
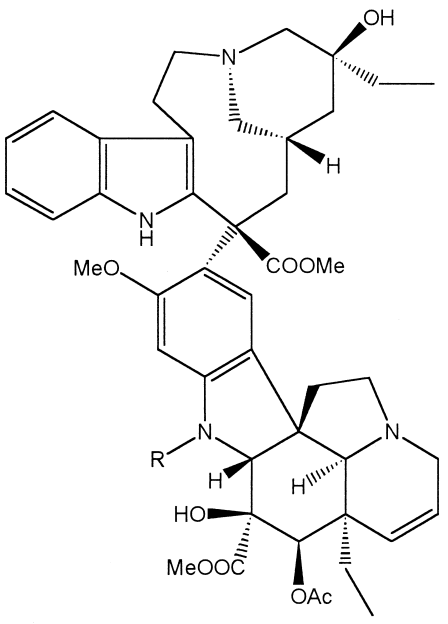



vindolinine




R=CHO : vincristine
R=Me : vincaleukoblastine

Fig. 1. Structures of interesting alkaloids found in the aerial parts of *Catharanthus roseus*.

chromatography (CCC) [3], even though there is, strictly speaking, no counter-current between the liquid phases. One is mobile and the other one is kept stationary inside of the column by a constant centrifugal field. The column itself is a rotor built as a disk stack in which partition cells are engraved. The CPC technique has the ability to separate fragile compounds because there is no irreversible adsorption or chemical reaction of the injected material with a solid support [4]. Moreover, there is generally no problem due to saturation of the solid-state phase, allowing CPC to be efficient in preparative separations [5]. A high selectivity is obtained by a careful choice of the biphasic solvent system, thus allowing the separation of compounds with very similar structures [6].

Ito introduced the pH-zone-refining mode in CPC as a variant of displacement chromatography [7]. It is devoted to the purification of compounds whose electric charge depends on pH value. For example, a

mixture of free bases is injected in the organic stationary phase along with a base stronger than all the compounds to separate. This stronger base is called the retainer base. The bases are moved along the column by pumping through an acidic aqueous solution of the displacer. Pure products are then isolated in the effluent as salts. The column is used in the descending mode, assuming that the density of the aqueous mobile phase is greater than the density of the organic stationary phase. In the ascending mode, base salts and an acidic retainer introduced in the aqueous stationary phase can be displaced by a base mixed to the mobile organic phase. Separation of organic acids or of their salts proceeds in a similar way.

A characteristic of displacement chromatography is the liberation of the products from the column by contiguous blocks arranged according their p$K_a$ values and also partition coefficients. In a block, the concentration of the different species (and so the pH) are constant and fixed by the acid–base and repartition equilibrium constants. The overlap between blocks is minimum and does not vary with the amount of injected sample.

The present study first shows simulated and then real separations of a mixture of two monomers and a dimer from *C. roseus*. The tuned protocol is then applied to the separation of a crude alkaloid extract. The preparative aspect of CPC in pH-zone refining mode is evaluated by increasing the mass of injected sample. The agreement between numerical simulations and experimental chromatograms is discussed.

## 2. Experimental

### 2.1. CPC apparatus

The separations were performed using a HPCPC Sanki Series 1000 column (Tokyo, Japan). The column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 250 ml. The column is connected to the injector and the detector through two high-pressure rotary seals. A four-port valve, included with the CPC, allows it to be operated in either the ascending or the descending mode. The HPCPC was connected to a Techlab (Erkerode, Germany) TIP50 gradient pump. Detection was performed with an UV/Vis detector ISCO type $V^4$ (Lincoln, NB, USA), set at 254 nm and a micro flow pH electrode (Broadley James, Irvine, CA, USA) connected to a pH meter type PHM240 (Radiometer, Copenhagen, Denmark). Fractions were collected with a collector model Superfrac manufactured by Pharmacia (Uppsala, Sweden). Sample injections were carried out by a Rheodyne valve type 7125 (Altech Associates, Deerfield, IL, USA), through a 10-, 15- or 20-ml loop.

### 2.2. Reagents

Methyl *tert.*-butyl ether (MtBE, puro), acetonitrile ($CH_3CN$, puro) chloroform ($CHCl_3$, puro), methanol (MeOH, puro) and hydrochloric acid (HCl 37%) were purchased from Carlo Erba, (Rodano, Italy); triethylamine (TEA) from Aldrich (Sigma–Aldrich, Steinheim, Germany). The pure alkaloids (vindoline, catharanthine, vindolinine and vincaleukoblastine) came from our laboratory. The tartaric solution of a crude alkaloid extract from *Catharanthus roseus* was a kind gift from Omnichem (Louvain la Neuve, Belgium).

### 2.3. Preparation of solvent phases and sample solutions

MtBE, $CH_3CN$ and water were thoroughly equilibrated in suitable proportions (4:1:5, v/v) and the two phases separated. The upper organic phase was made basic with TEA at the concentrations of 8 m*M* when it was used as the mobile phase (ascending mode) and 10 m*M* when it was used as the stationary phase (descending mode). The lower aqueous phase was acidified by HCl used as a retainer at a concentration of 10 m*M* (ascending mode) or used as a displacer at 8 m*M* (descending mode).

In the case of the separation of a synthetic mixture of three pure alkaloids; vindoline, catharanthine and vincaleukoblastine were dissolved in 10 ml of stationary phase in their salt or basic form following which phase was chosen as stationary.

In the case of the purification of the crude alkaloid extract, the aqueous solution of tartrate alkaloids was lyophilized and dissolved in the mobile phase to be injected.

### 2.4. Separation procedure

The column was first filled with the stationary phase. Then, the rotation was brought to 800 rpm, the sample was injected and finally the mobile phase was pumped into the column in ascending or descending mode at a flow-rate of 3 ml/min, resulting in 35–40 bar backpressure and 75–85% of stationary phase retention. The beginning and the end of the separation were checked by UV absorbance measurement at 254 nm and the evolution of the pH was continuously monitored using an on-flow pH electrode connected to a pH meter.

### 2.5. Characterization of the alkaloids

All fractions were checked by TLC on Whatman K6F plates (Whatman, Maidstone, UK) developed with $CHCl_3$–MeOH (97:3). The fraction of interest and all isolated compounds were analyzed by $^1H$ and $^{13}C$-NMR spectroscopy in $C^2HCl_3$. Spectra were recorded at 300 MHz on a a.c. 300 Bruker spectrometer (Wissembourg, France).

### 2.6. Simulated separations

Software was developed in order to simulate pH-zone-refining separations. The column is artificially divided into sections that mimic the theoretical plates. Each section contains a fraction of the mobile and stationary phases. The chromatographic development is decomposed into the repetition of two elementary processes: the equilibration of all the chemical species present in each section and the pumping of the mobile phase. The latter stage requires the introduction of fresh mobile phase into the first section of the column, the simultaneous migration of all the mobile phases of each section toward the next one, except for the last section whose content is analyzed to determine pH, concentration and purity profiles. The most demanding part of the calculation is the equation resolution imposed by the partition and acid–base equilibria, constrained by charge and mass balance relationships. The model proposed by Ito [8] is generalized to the separation of acids or bases, whatever the number of ionization sites of each compound [9].

## 3. Results and discussion

The separation of alkaloids by means of pH-zone-refining CPC concerns, to date, only mixtures with a limited number of components [10]. The complexity of the crude alkaloid extract from *C. roseus* merited some preliminary studies. An artificial mixture of two monomers, vindoline and catharanthine, and of the dimer vincaleukoblastine was first considered in computer simulations. Calculation requires the knowledge of the thermodynamic constants of the partition and acid–base equilibria (Table 1). The former are determined by standard UV methods [11,12] while the latter were found in literature data [13]. The monomers are potential dibases, while the dimer is a potential tetrabase. However, the low basicity of nitrogen atoms conjugated to the aromatic systems leads to the consideration that only the unconjugated tertiary amino sites are basic. The $pK_a$ values reported in Table 1 concern these sites. The volumes and species concentration required as parameters for the simulation are selected according to the real separation conditions. In Fig. 2A, compound amounts and pH are drawn as a function of the number of collected fractions. The volume of each fraction is equal to the volume of the mobile phase within a single column section: 0.25 ml. Purity profiles are drawn in Fig. 2B. The purity is defined as the molar fraction of the currently leaving compound in the effluent. The current compound is the most concentrated in the effluent. The result of the simulation shows that a separation of the three molecules of interest can be achieved with high resolution. The steady-state concentration of each compound should reflect the number of protonation sites. The dibase should be two times less concentrated than monobases because all the substances are

Table 1
Partition coefficients and $pK_a$ values of vindoline, catharanthine and vinblastine

| | $K_p=[AlcN]_{org}/[AlcN]_{aq}$ | $pK_a$ value in 66% DMF |
|---|---|---|
| Vindoline ($\lambda$, nm) | 37.4 (316[a]) | 5.5 |
| Catharanthine ($\lambda$, nm) | 66.5 (284.6[a]) | 6.8 |
| Vinblastine ($\lambda$, nm) | 17.0 (260[a]) | 7.4 |

[a] $\lambda$ used for partition coefficients calculation using the UV method [12].

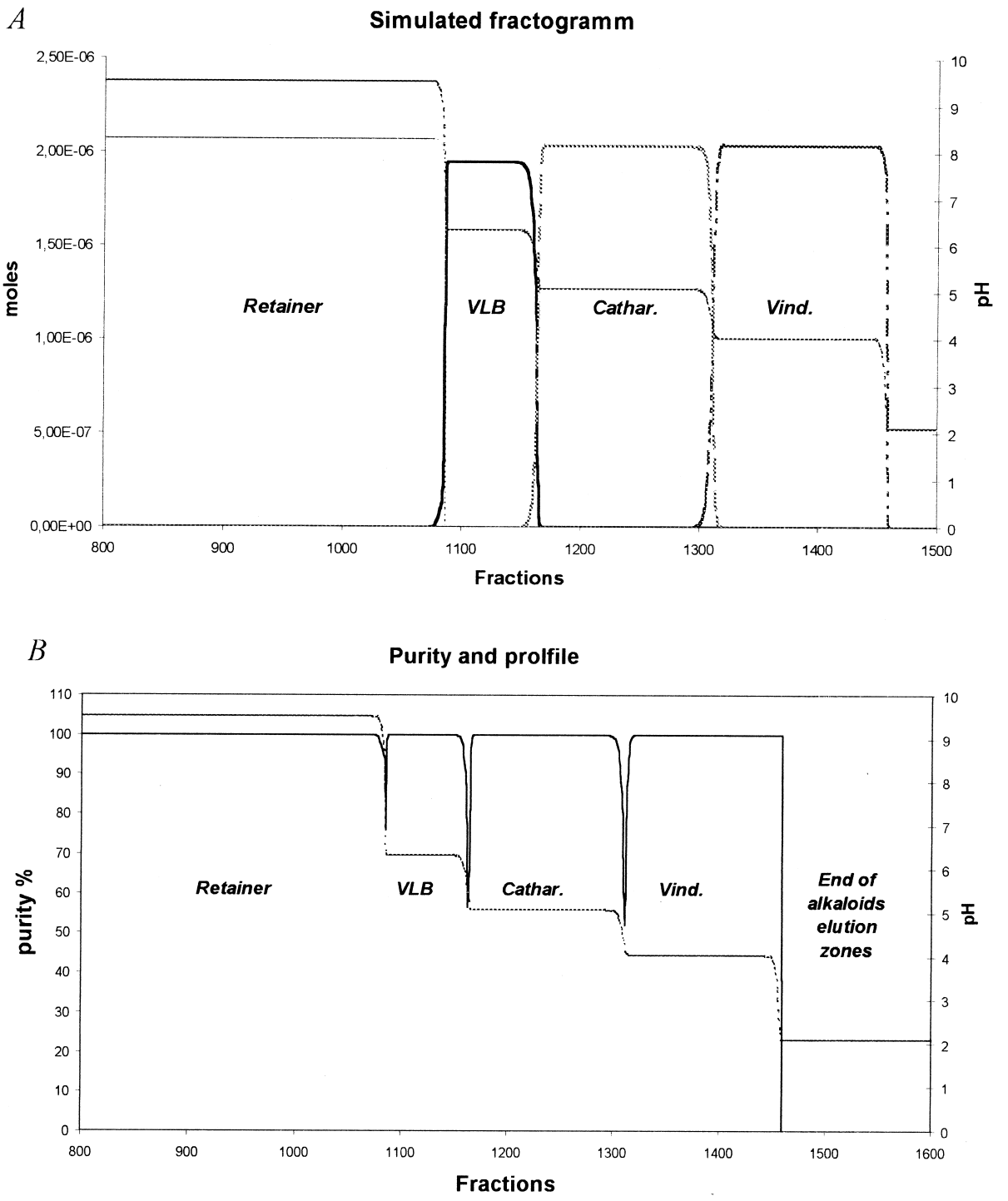



Fig. 2. (A) Simulated fractogram of a mixture of vindoline, catharanthine and vincaleukoblastine plus retaining base (TEA) in the organic stationary phase. The sample was in the first theoretical plate. [TEA]=10 m*M*, [HCl]=8 m*M*, [vindoline]=200 m*M*, [catharanthine]=200 m*M*, [VLB]=100 m*M* (concentrations in the first theoretical plate), number of theoretical plates=150, volume of stationary phase=1.5 ml, volume of aqueous mobile phase=0.25 ml. (B) Purity and pH profile obtained for the same simulated separation.

isolated as salts of a displacer whose concentration is constant. The calculated concentration of 0.5 is experimentally determined as being 0.85. This type of discrepancy was already observed in real separations involving the mixture of a diacid and monoacids [14]. In the present case, the dibase is actually eluted as a mixture of the mono and the diprotonated forms. Computer simulations show that the fully diprotonated species is eluted only when both p*K*a values are identical. The monobasic behavior is enhanced by the increase of the difference between the p$K_a$ values.

After these theoretical results, the separation of a purposely prepared mixture of vindoline, catharanthine and vinblastine was performed using the biphasic solvent system methyl *tert.*-butyl ether–acetonitrile–water 4:1:5 and classical retainer and displacer (hydrochloric acid and triethylamine) previously used by Ito (Table 2). The ascending and the descending modes were tested and led to approximately the same resolution. UV detection at 254 nm was completed with on-line pH monitoring to observe pH plates and thus the transitions between the different zones of elution. Fig. 3 presents the two chromatograms and Table 3 the results. Only a few fractions containing mixed products were collected, and yields or recoverability are high (>80%).

The graph in Fig. 4 shows a global similarity between concentrations obtained by computer simulation (calculated from data in Fig. 2) and from the true separation. Nevertheless, it appears that the calculation overestimates concentrations of vindoline and vincaleukoblastine. This may be due to the weak dibasic character of vindoline and to the weak

Table 2
Experimental conditions for the separation of vindoline, catharanthine and vincaleukoblastine

| | Ascending mode | Descending mode |
|---|---|---|
| Apparatus | HPCPC Sanki Series 1000 | |
| Solvent system | MtBE–$CH_3CN$–water | |
| Stationary phase (retainer) | Aqueous (HCl 10 m*M*) | Organic (TEA 10 m*M*) |
| Mobile phase (displacer) | Aqueous (HCl 8 m*M*) | Organic (TEA 8 m*M*) |
| Rotation (rpm) | 800 | |
| Flow-rate (ml/min) | 3 | |
| Back pressure (bar) | 39 | 37 |
| Detection | UV (254 nm) and online pH detection | |
| Fraction time (min) | 1 | 1 |

Table 3
Separations results for the ascending and descending modes

| Fractions | Volume (ml) | Alkaloid(s) | Mass (mg) | mMol | Yield (%/injected products) | Concentration (m*M*) |
|---|---|---|---|---|---|---|
| *Ascending mode* | | | | | | |
| 62–70 | 27 | Vind. | 96 | 0.21 | 95 | 7.77 |
| 71 | 3 | Vind+Cathar. | 8 | | | |
| 72–79 | 24 | Cathar. | 67 | 0.20 | 83.3 | 8.33 |
| 80 | 3 | Cathar.+VLB | 6 | | | |
| 81–86 | 18 | VLB | 89 | 0.11 | 84.6 | 6.11 |
| *Descending mode* | | | | | | |
| 58–63 | 18 | VLB | 96 | 0.11 | 84.6 | 5.96 |
| 64 | 3 | VLB.+Cathar. | 7 | | | |
| 65–72 | 24 | Cathar. | 74.5 | 0.20 | 80 | 8.33 |
| 73 | 3 | Cathar.+Vind. | 9 | | | |
| 73–81 | 27 | Vind. | 98.5 | 0.20 | 91 | 7.4 |

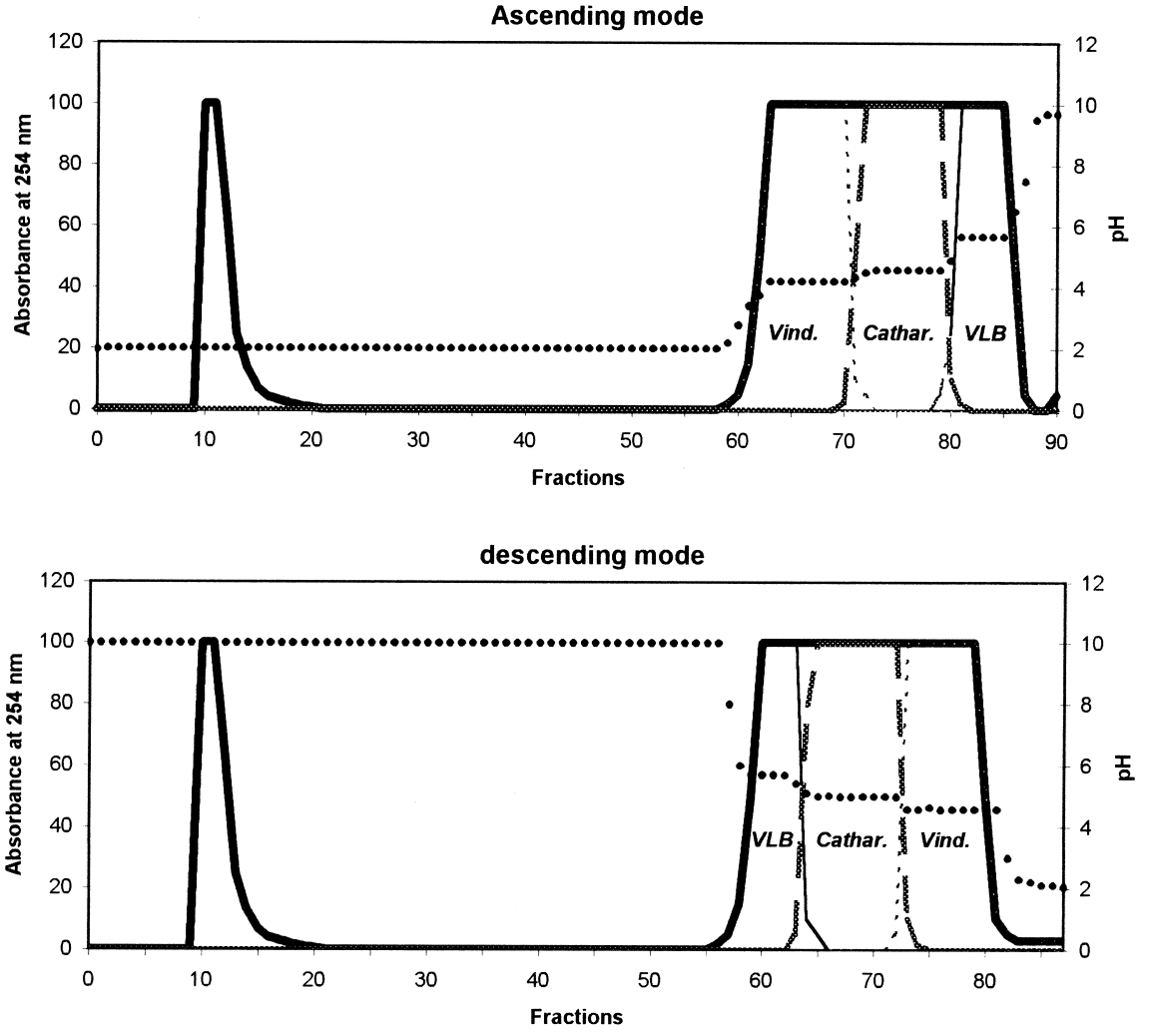



Fig. 3. UV chromatogram and pH profile for the separation of vindoline, catharanthine and vincaleukoblastine. Sample: ascending mode: 110 mg (0.22 mmol) of vindoline chlorhydrate, 90 mg (0.24 mmol) of catharanthine chlorhydrate and 118 mg (0.13 mmol) of vincaleukoblastine sulfate in 10 ml of aqueous stationary phase; descending mode: 100 mg (0.22 mmol) of vindoline, 84 mg (0.25 mmol) of catharanthine and 105 mg (0.13 mmol) of vincaleukoblastine in 10 ml of organic stationary phase. For other experimental conditions, see Table 2.

tribasic character of vincaleukoblastine. The extra basic site would be located on the indoline nucleus, considering that indole itself is not basic enough to be significantly protonated. This hypothesis is confirmed by the good agreement observed between real and computed steady-state concentration for catharanthine, a compound that bears two nitrogen atoms, a basic one, and another one which is part of an indole nucleus.

The preliminary work shows that the desired separation is workable and that the conclusions that may be drawn from the results of computer simulations make sense. In the second part of this work, the purification of a crude extract of *C. roseus* alkaloids (as salts of tartaric acid) was performed using the previously described operating conditions. Four separations were achieved with an increasing mass of injected sample, in order to investigate the prepara-

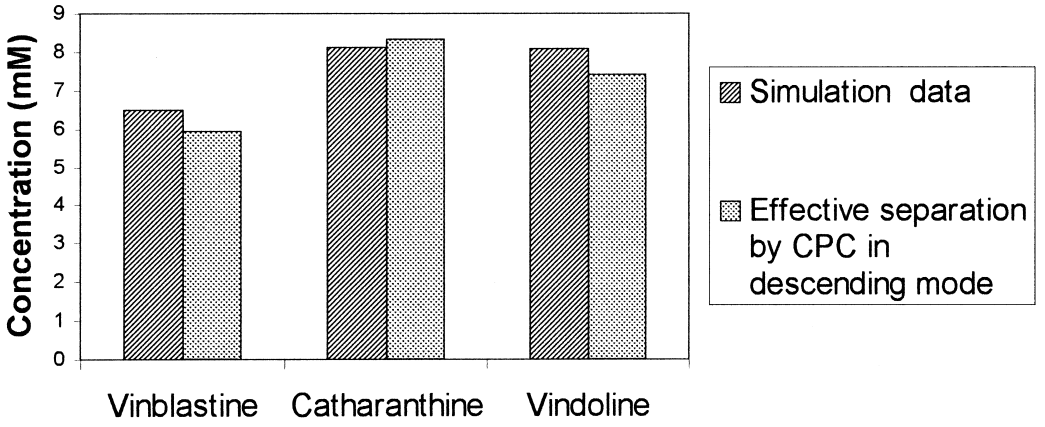



Fig. 4. Comparison of the different alkaloid concentrations in the effluent between the simulation and the real separation in the ascending mode.

Table 4
Experimental conditions for four separations (800 mg, 1.6 g, 2.4 g and 7 g) of tartrate alkaloids from *Catharanthus roseus*

| | Separation 1 | Separation 2 | Separation 3 | Separation 4 |
|---|---|---|---|---|
| Sample (injection volume) | 800 mg (10 ml) | 1.6 g (15 ml) | 2.4 g (20 ml) | 7 g (20 ml) |
| Apparatus | HPCPC SANKI Series 1000 | | | |
| Elution mode | Ascending | | | |
| Biphasic solvents system | MtBE–$CH_3CN$–water (4:1:5) | | | |
| Stationary phase (retainer) | Aqueous (HCl 10 m*M*) | | | |
| Mobile phase (displacer) | Organic (TEA 8 m*M*) | | | |
| Rotation (rpm) | 800 | | | |
| Flow-rate (ml/min) | 3 | | | |
| Back pressure | 37–40 | | | |
| Detection | UV 254 nm/on line pH monitoring | | | |
| Fraction time (min) | 2 | | | |

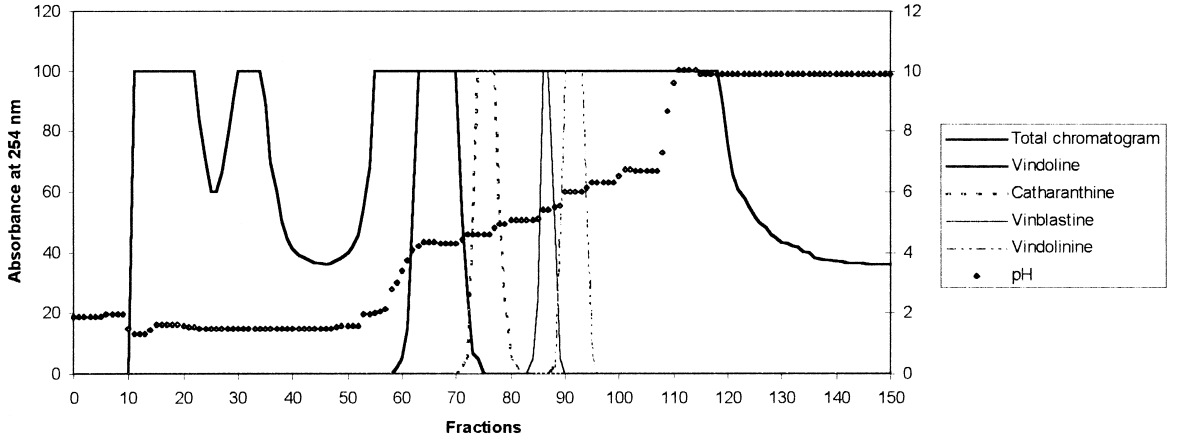



Fig. 5. UV chromatogram and pH profile for the separation with 2.4 g of tartrate alkaloids from *Catharanthus roseus*. For experimental conditions, see Table 4.

| Fractions | Alkaloids | Quantities (mg) | Yields / alkaloid extracts (%) |
|---|---|---|---|
| | **SEPARATION 1** | | |
| [57:58] | vindoline | 35 | 4.3 |
| [59:62] | Impure catharanthine | 30 | — |
| [65] | Impure vinblastine | 23 | — |
| [67] | vindolinine | 19 | 2.3 |
| | **SEPARATION 2** | | |
| [66:71] | vindoline | 110 | 6.87 |
| [72:75] | catharanthine | 60 | 3.7 |
| [78] | vinblastine | 21 | 1.3 |
| [82] | vindolinine | 45 | 2.8 |
| | **SEPARATION 3** | | |
| [63:72] | vindoline | 189 | 7.8 |
| [73:80] | catharanthine | 120 | 5 |
| [86:87] | vinblastine | 41 | 1.7 |
| [92:95] | vindolinine | 76 | 3.1 |
| | **SEPARATION 4** | | |
| [64:94] | vindoline | 577 | 8.2 |
| [96:123] | catharanthine | 423 | 6.0 |
| [141:147] | vinblastine | 146 | 2.0 |
| [162:95] | vindolinine | 231 | 3.3 |

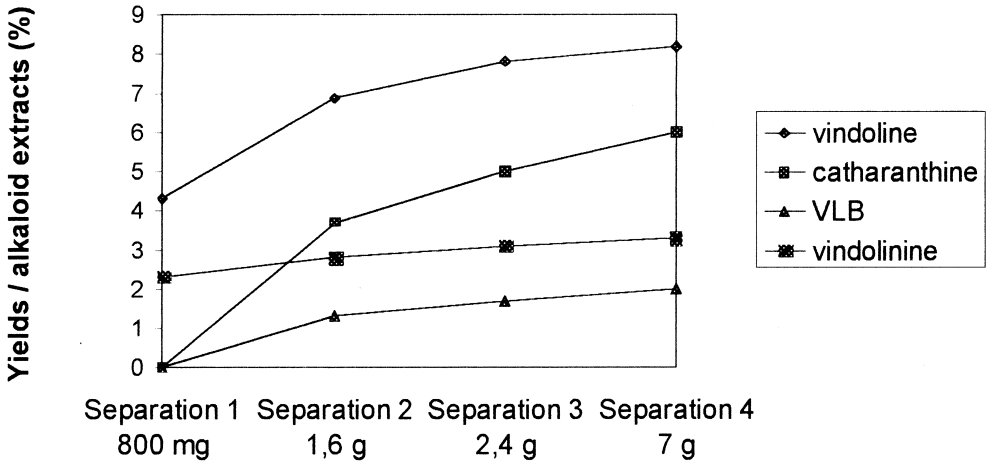



Fig. 6. Results and yields of pure products for the four separations of tartrate alkaloids from *Catharanthus roseus*.

tive aspects of pH-zone refining. Experimental details are listed in Table 4. The ascending mode was selected, because the removal of an organic mobile phase is easy and the alkaloids are recovered as free bases.

In a first experiment the injected sample mass was

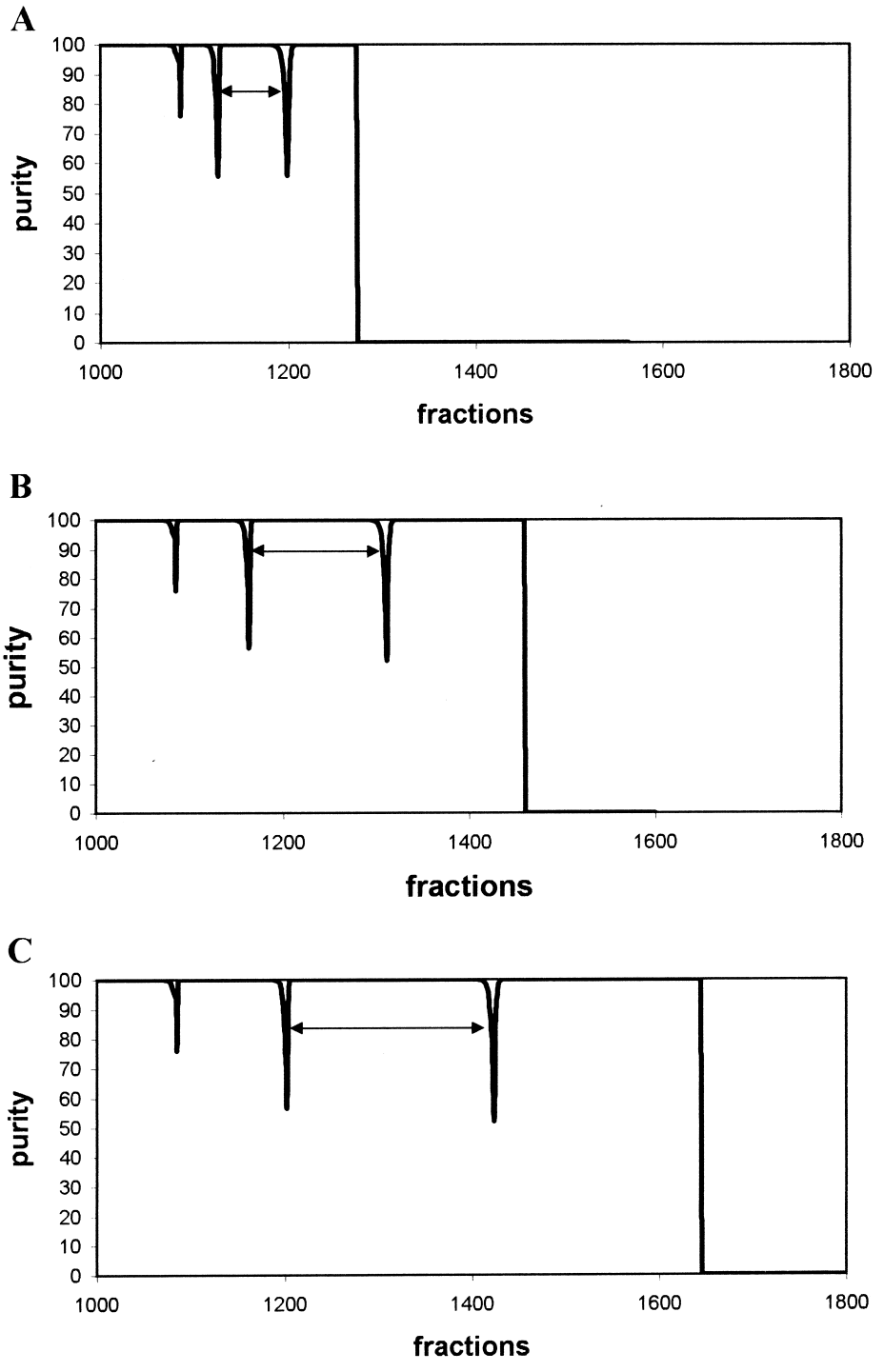



Fig. 7. Simulated purity profile obtained for three increasing quantities of vindoline, catharanthine and vincaleukoblastine. A: [vindoline]= 100 m*M*, [catharanthine]=100 m*M*, [VLB]=50 mM; B: [vindoline]=200 m*M*, [catharanthine]=200 m*M*, [VLB]=100 mM; [vindoline]= 300 m*M*, [catharanthine]=300 m*M*, [VLB]=150 m*M*. Other parameters are identical to Fig. 2.

0.8 g. Only two pure products were isolated: catharanthine and vindoline, the major monomer alkaloids produced by *C. roseus*. Upon increase of the injected mass (1.6 g, 2.4 g, and 7.0 g), four compounds were isolated: three monomers (vindoline, catharanthine, and vindolinine) and one dimer (vincaleukoblastine). They were identified by co-tlc and comparison of their $^{1}H$ and $^{13}C$ NMR data with those of reference compounds. The chromatogram and the elution profiles for one of these separations is presented in Fig. 5.

Yield evolution in separated compounds is reported in Fig. 6. It shows that the yields increase with the amount of injected sample. This is a surprising situation in chromatography, in which mass overloading has a positive effect. Even the increase in injected volume, required for the solubilization of the sample, has no noticeable impact on the effluent volume during transitions between the steady-state periods. Some insight in the origin of the improvement brought by mass overloading was gained by computer simulations. Typical separation conditions are used but with varying injected sample masses. As the concentration of the effluent is driven by the concentration of the displacer, an increase of the sample mass causes a lengthening of the elution time for each component of the mixture, without affecting the transition zones. Therefore, the period of time during which pure compounds are eluted are longer, relative to the overall separation time. This result is clearly visible in the graphs in Fig. 7, in full agreement with experimental data. The purity enhancement may also be understood by considering that each compound in the column behaves as a retainer for the compound just behind. An increase of sample mass thus allows a better organization of the repartition of the molecules in the column. From a pragmatic point of view, the observed phenomenon has highly interesting applications for the design of preparative and pilot-scale separations.

## 4. Conclusion

This work presents a successful application of pH-zone-refining CPC to the preparative purification of *C. roseus* alkaloids. A computer program designed for the simulation of the chromatographic process correctly interprets the experimental chromatograms. It predicts the positive effect of mass overloading on the purity of the separated products. This effect is experimentally observed, and is of prime importance for the development of pH-zone-refining CPC as a preparative purification method.

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