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Separation of protoberberine quaternary alkaloids from a crude extract of *Enantia chlorantha* by centrifugal partition chromatography

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Abstract

High-performance centrifugal partition chromatography (HPCPC) has been successfully applied to the separation of four protoberberine quaternary alkaloids, namely palmatine, jatrorrhizine, columbamine and pseudocolumbamine, from a methanolic extract (M_1, 1.47 g) of *Enantia chlorantha* Oliver stem bark. For their isolation, two successive biphasic solvent systems composed of dichloromethane–methanol–water (48:16:36, v/v) were selected. The aqueous-rich phase was the stationary phase and the organic-rich phase was the mobile phase. The first system, containing potassium perchlorate, allowed to isolate 600 mg of palmatine, and to obtain 146 mg of a mixture (M_2) containing only jatrorrhizine, columbamine and pseudocolumbamine. The second biphasic system, prepared with water alkalinized with sodium hydroxide, was employed to isolate the M_2 components. This system applied to the purification of 70 mg of M_2 allowed to obtain 16 mg of jatrorrhizine and 13 mg of columbamine. To obtain pseudocolumbamine (16 mg), the elution mode was reversed, the aqueous-rich phase becoming the mobile phase, and the organic-rich phase becoming the stationary one. Analytical reversed-phase high-performance liquid chromatography, NMR, high-resolution mass spectrometry and UV spectrometry were used to verify the identity and the purity of the isolated compounds.

Keywords: *Enantia chlorantha*; Centrifugal partition chromatography; Alkaloids; Palmatine; Jatrorrhizine; Columbamine; Pseudocolumbamine

1. Introduction

Malaria is caused by infection with protozoal parasites of the genus *Plasmodium* transmitted by the bites of infected female mosquitoes of the genus *Anopheles*. Approximately 40% of the world population live in areas with the risk of malaria. Each year, 300–500 million people suffer from acute malaria, and 0.5–2.5 million die from this disease.

In African countries, the use of medicinal plants is commonplace for the treatment of malaria symptoms such as fever. Among these plants, *Enantia chlorantha* Oliver (Annonaceae) is used. The purification of stem bark extracts of *E. chlorantha* afforded mainly four quaternary alkaloids of protoberberine type: palmatine, jatrorrhizine, columbamine and pseudocolumbamine [1,2]. Two minor aporphine alkaloids (7-hydroxydehydronuciferine and 7-hydroxydehydronornuciferine) were also isolated [3].

Various biological activities of stem bark extracts or protoberberine alkaloids of *E. chlorantha* have been described [4–7] such as antiplasmodial effects (in vitro and in vivo), hepatoprotection, anticandidal and antibacterial activities (in vitro). Among these activities, antiparasitical and antibacterial interesting properties were linked to protoberberine alkaloids and conduced some authors to synthesize derivatives of these alkaloids to study their structure–activity relationships [8–13].

Different techniques have been used to analyze and/or fractionate protoberberine alkaloids from plant extracts. Most of the procedures used for the fractionation of these alkaloids included preliminary liquid–liquid separation of the tertiary alkaloids, followed by precipitation of the

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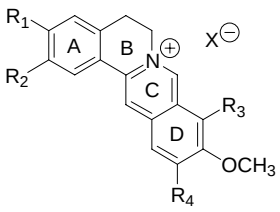
quaternary alkaloids with Mayer's Reagent (potassium iodomercurate). After the replacement of the iodomercurate anions by chloride anions with an anion exchanger resin, the protoberberine alkaloids were separated over a silica column, either directly [14] or after reduction by $NaBH_4$, and analyzed by gas chromatography and NMR [1,2]. Circular thin layer chromatography [15], optimum performance laminar chromatography (OPLC) [16], capillary electrophoresis [17–19] and HPLC [20–25] were employed for the analysis of protoberberine alkaloids in different plants, but the separation of these alkaloids was often insufficient. Analytical and preparative separation of palmatine, berberine, epiberberine and coptisine (protoberberine alkaloids) from *Coptis chinensis* Franch, by high-speed counter-current chromatography (HSCCC) was carried out with a chloroform-methanol-0.2 M HCl (4:1.5:2, v/v) biphasic system [26].

As neither satisfactory analytical analysis nor preparative separation of *E. chlorantha* protoberberine alkaloids had been performed, we decided to optimize firstly the analytical HPLC separation of palmatine, jatrorrhizine, columbamine and pseudocolumbamine and then, their isolation with the simplest and shortest method, so as to continue the prospecting of structure–activity relationships.

The isolation of those very polar compounds was the challenge of this study since their adsorption on silica is high and their chemical structures are very closely related (Fig. 1): jatrorrhizine, columbamine and pseudocolumbamine are position isomers; palmatine differs from them only by a methoxy group that replaces one hydroxy group on the A ring of the molecule. To perform such a preparative chromatographic separation, high-performance centrifugal partition chromatography (HPCPC) seemed to be an appropriated technique [27–29]. HPCPC, like HSCCC, is a non-solid support preparative liquid–liquid chromatographic method, belonging to counter-current chromatographic techniques (CCC) based on partition of solutes between two immiscible liquid phases. The solutes are separated according to their distribution constants (K_C) expressed as the ratio of their concentration in the stationary phase to their concentration in the mobile phase. CCC avoids problems related to interactions of the injected material with a solid support, such as irreversible adsorption or degradation. Moreover, there is generally no problem due to saturation of the stationary phase, allowing CCC to be efficient in preparative separations.

All modern CCC apparatuses use a centrifugal field to maintain in the "column" one liquid phase, playing the role of the stationary phase, the other liquid phase being pumped through it and thus playing the role of the mobile phase. Two types of CCC apparatuses, hydrodynamic and hydrostatic machines, are commercially available. The hydrodynamic CCC machines use a variable-gravity field produced by a two-axis gyration mechanism and a rotary seal-free arrangement for the column (spools containing coiled PTFE tubes). Due to the planetary motion of the apparatus spools, the centrifugal field changes in intensity and direction. When the centrifugal field is high, phase decantation occurs. When the centrifugal field direction reverses, the separated liquid phases commingle in an emulsion-like state. So alternating decantation and mixing zones appear in the spool. These apparatuses, mainly developed by Ito and co-workers, are referred to as counter-current chromatographs. The hydrostatic CCC machines use a constant-gravity field produced by a single-axis rotation mechanism and two rotary seal joints for inlet and outlet of the mobile phase. The column itself consists of a series of discrete partition cells engraved in the rotor and connected by ducts in cascade. The mobile phase is pumped from cell to cell and flows through the stationary phase in the centrifugal direction when it is the more dense phase (this operating mode is called the descending mode) or in the centripetal direction when it is the less dense one (the ascending mode). Mass transfer occurs in each cell while the two phases are in contact. Hydrostatic CCC apparatuses, mainly developed by Nunogaki (Sanki Engineering company, Japan) are usually named CPC apparatuses.

On the one hand this paper describes the optimization of HPLC analysis of *E. chlorantha* main protoberberine alkaloids, for their K_C determination and the purity control of the isolated compounds. On the other hand the selection of solvent systems for HPCPC separations, based on solubility and partition coefficient studies, in relation with pH and the nature of the anions present, is described as well as the rapid and efficient isolation of palmatine, jatrorrhizine, columbamine and pseudocolumbamine from a methanolic extract of *E. chlorantha*.



Protoberberine alkaloids	R_1	R_2	R_3	R_4
Palmatine	OCH_3	OCH_3	OCH_3	H
Jatrorrhizine	OH	OCH_3	OCH_3	H
Columbamine	OCH_3	OH	OCH_3	H
Pseudocolumbamine	OCH_3	OH	H	OCH_3

Fig. 1. Structure of the main alkaloids of *E. chlorantha*.

2. Experimental

2.1. HPCPC apparatus

The experiments were performed using a LLB high-performance centrifugal partition chromatograph (Sanki Eng., Kyoto, Japan). The total column volume is 220 ml. A four-port switching valve incorporated in the HPCPC apparatus allows to operate in either the descending or the ascending mode. This HPCPC system was equipped with a quaternary solvent delivery pump Waters model 600 (Waters, Milford, MA, USA). The effluent was continuously monitored at 270 nm with a Waters 486 UV detector—the main line (90% of the flow) going to a fraction collector ISCO type Retriever 500 (ISCO, Lincoln, NB, USA)—the other line (10% of the flow) going to an evaporative light scattering detection (ELSD) system Sedere Type Sedex 45 (Sedere, Vitry-sur-Seine, France). The nebulisation gas was compressed air at 2.2 bar. The temperature of the nebuliser was set at 25 °C. The samples were manually injected through a Rheodyne valve (Rheodyne, Cotati, CA, USA) with a 15 ml sample loop.

2.2. HPLC apparatus

The HPLC apparatus used was a Waters LC system, consisting of a 600 model pump, an in-line degasser, a 717 plus autosampler and a 996 photodiode array detector. Instrument monitoring and data acquisition were performed using Empower software (Waters). The mobile phase was composed of two solvents: A (water with 0.075%, v/v, $HClO_4$) and B (methanol with 0.075%, v/v, $HClO_4$). Each sample was dissolved in 800 μl of methanol and 200 μl of A and filtered through a 0.45 μm Millex-HV filter (SLHVR04NL, Millipore). The injected volume was comprised between 5 and 50 μl, depending on the concentration of the sample. Analyses were performed with a Nova-Pak C_{18} column from Waters (150 mm × 3.9 mm i.d., 5 μm particle size). The linear gradient was 20–100% solvent B in 30 min and 10 min with 100% solvent B. The flow rate was set at 1 ml min^{-1}. The UV absorption spectrum of each signal was recorded between 210 and 400 nm and the chromatograms were extracted at 270 nm. The analyses were performed at 30 °C.

2.3. Reagents and material

All organic solvents (methanol, ethyl acetate, dichloromethane and butan-1-ol) and chemical reagents (perchloric acid, formic acid, acetic acid, triethylamine, sodium dodecylsulfate, diethylamine, sodium hydroxide, hydrochloric acid, ammonium acetate and sodium acetate) used for the extraction, the HPLC analysis and the preparation of the two-phase solvent systems were commercial analytical grade and were purchased from VWR International (Fontenay-sous-Bois, France). Analytical grade potassium perchlorate was purchased from Merck (Darmstadt, Germany). Distilled water was employed.

For HPLC analysis HPLC-grade methanol, purchased from Carlo Erba (Val de Rueil, France), and water (Milli-Q water system, Millipore, Bedford, MA, USA) were used.

The stem barks of *E. chlorantha* were collected in Estuaire region in Gabon, and identified by Dr. H. Bourobou-Bourobou of the National Herbarium of Gabon, where voucher specimens are deposited. The barks were scraped and pulverized.

2.4. Preparation of methanolic extract M_1

A 430 g batch of *E. chlorantha* powdered bark was first degreased with 3000 ml of hexane for 10 h, and then extracted with 3000 ml of methanol for 50 h in a Soxhlet apparatus. The methanolic extract was evaporated to dryness at 40 °C under reduced pressure to give M_1 (32.3 g, 7.5% yield).

2.5. Measurement of the distribution constants K_C and control of the collected HPCPC fractions

Shake flask experiments were performed to determine the distribution constants K_C of the solutes between the two phases of the tested systems [27]. The two-phase solvent systems were composed of methanol, water and either ethyl acetate, butan-1-ol or dichloromethane. Each system was prepared at room temperature by stirring the solvents, at different volume ratios, before separating them into upper and lower phase. We evaluated both the influence of the pH and the influence of the nature of various anions introduced in the aqueous phase. Either $HClO_4$, $KClO_4$, NaOAc, NH_4OAc, HCl, NaOH or Et_2NH were added to the water at different concentrations. The same two-phase solvent system with distilled water was taken as a reference. To measure the distribution constants, an aliquot (10 mg) of M_1 was dissolved in 10 ml of the aqueous-rich phase and the solution was gently shaken with an equal volume of the organic-rich phase. The two phases were then separated and the solute concentrations in each phase were quantified by HPLC. For each solute, the distribution constant K_C, which is defined as the solute affinity for the aqueous-rich stationary phase, was calculated as follows:

$$K_C = \frac{[C]_{\text{aqueous-rich phase}}}{[C]_{\text{organic-rich phase}}} = \frac{A_{\text{aqueous-rich phase}}}{A_{\text{organic-rich phase}}} \quad (1)$$

where $[C]_{\text{aqueous-richphase}}$ and $[C]_{\text{organic-richphase}}$ are the solute concentrations in the aqueous- and the organic-rich phases, respectively; $A_{\text{aqueous-rich phase}}$ and $A_{\text{organic-rich phase}}$ are the HPLC solute peak areas measured for the aqueous- and the organic-rich phases, respectively. For each solute, the theoretical retention volume V_R was calculated as follows:

$$V_R = V_M + K_C V_S \quad (2)$$

where V_M and V_S are the mobile phase and the stationary phase volumes inside the HPCPC "column", respectively.

After HPCPC separations, identical fractions were pooled and carefully evaporated to dryness. For structural determination and purity control, the combined fractions showing one peak by HPLC were analyzed by ^{1}H NMR and ^{13}C NMR on a Bruker AC-200P (200 MHz) or a Bruker AM-400 (400 MHz) spectrometers and by high-resolution mass spectrometry on a Waters-Micromass LCT. Infrared spectra were recorded on a Bruker Vector 22 apparatus and UV-Vis spectra were recorded on a Shimadzu UV160 spectrophotometer.

2.6. HPCPC separation procedures

2.6.1. Isolation of palmatine

To isolate palmatine we selected a two-phase solvent system composed of dichloromethane, methanol and water. The mobile phase (dichloromethane-rich phase) and the stationary phase (aqueous-rich phase) were prepared by mixing dichloromethane, methanol and water in a 48:16:36 (v/v) ratio. The two phases were then separated and degassed in an ultrasonic bath.

The HPCPC column, stocked with methanol, was firstly emptied with N_2 (I quality, Air Liquide, Paris La Défense, France) at 2 bars and then filled with the aqueous-rich stationary phase at 10 ml min^{-1} in the ascending mode. The apparatus was then rotated at 700 rpm while the organic-rich mobile phase, which is heavier than the stationary one, was pumped through the column in the descending mode at 9 ml min^{-1}. A clear mobile phase eluting at the tail outlet of the column indicated that the hydrostatic equilibrium was established in the column (73% retention stationary phase).

One hundred to two hundred and fifty milligrams of M_1 were mixed with 15 ml of stationary phase containing 10^{-4} to 2.5×10^{-4} mol $KClO_4$, corresponding to a 0.5 molar ratio between the perchlorate anions and the main protoberberine alkaloids. The resulting suspension was injected with the Rheodyne injection valve without stopping the flow. The pressure drop was 17–21 bar. One collected fraction (5–15 ml) out of four was analyzed by HPLC. When necessary to identify the fractions containing pure compounds, more fractions were analyzed.

After all the components of interest (i.e. palmatine, jatrorrhizine, columbamine and pseudocolumbamine) were eluted (800 ml) the solutes that were still retained in the stationary phase of the HPCPC column were eluted by switching the system in "dual mode" [29]: the organic-rich phase became the stationary phase while the aqueous-rich mobile phase was pumped at 9 ml min^{-1} in the ascending mode.

At the end of each run, the rotation was stopped and the HPCPC column was emptied with N_2 at 2 bars in the descending mode. Methanol was then pumped through the column at 10 ml min^{-1} in the ascending mode to wash it.

A total of 1.47 g of M_1 was injected. Fractions containing palmatine were pooled and yielded 600 mg (40.8% of M_1, >95% purity). Fractions containing only jatrorrhizine, columbamine and pseudocolumbamine were pooled and evaporated to give a mixture M_2 (146 mg, 9.9% of M_1) that was further separated by HPCPC with another solvent system. Fractions containing the most polar components of M_1 were pooled and evaporated to give a mixture M_3 (350 mg, 23.8% of M_1).

2.6.2. Isolation of jatrorrhizine, columbamine and pseudocolumbamine

To separate jatrorrhizine, columbamine and pseudocolumbamine, we selected the same two-phase solvent system as for palmatine isolation but water was replaced by an aqueous solution containing NaOH (5.7×10^{-3} mol l^{-1}). The separation conditions were as described above except for the flow rate that was set at 3 ml min^{-1}. The sample suspension was prepared with 70 mg of mixture M_2 and 15 ml of stationary phase.

Jatrorrhizine and columbamine were eluted within 1100 ml of mobile phase (organic-rich phase). Pseudocolumbamine was eluted after switching the system in "dual mode", within 200 ml of mobile phase (aqueous-rich phase). To recover the acidic phenol functions of these compounds, the fractions were neutralized by 0.01 M hydrochloric acid in methanol and desalted by solid phase extraction on C_{18} cartridges (Sep-Pak, Waters).

Fractions containing jatrorrhizine (16 mg, 22.9% of M_2, >95% purity), columbamine (13 mg, 18.6% of M_2, >95% purity) and pseudocolumbamine (16 mg, 22.9% of M_2, >95% purity) were pooled and evaporated. The recovery of each compound from M_2 was between 60 and 70%. Low recovery can be explained by some sublimation of these compounds during the elimination of the residual water under reduced pressure at 45 °C.

3. Results and discussion

3.1. M_1 components analysis by HPLC

As previously described [1,2], the stem bark of *E. chlorantha* mainly contains quaternary alkaloids (protoberberine type) that are highly polar compounds (Fig. 1). Their reversed-phase HPLC analysis is difficult due to their possible strong interactions with the residual silanols of the stationary phase. To overcome these difficulties, it is preferable either to use a column with few free silanols (type B alkyl-silica columns) or to modify the mobile phase by adding triethylamine or by acidifying it.

In the literature, protoberberine quaternary alkaloid separations were described using columns that have very low "silanol activity" (e.g. Cosmosil columns [30], Nacalai Tesque) and an acidic mobile phase [20,21]. Other separations were described with a mobile phase containing ion-pairing reagents such as sodium dodecylsulfate or octanesulfonate [22–25].

To carry out the analytical separation of protoberberine alkaloids we tested three columns: a type A column (Nova-Pak C_{18}) and two type B columns—Inertsil ODS-3 (150 mm ×

4 mm i.d., 5 μm particle size, GL Sciences) and Symmetry Shield C_{18} (150 mm × 4.6 mm i.d., 3.5 μm particle size, Waters).

With the Nova-Pak C_{18} column and a methanol–water linear gradient (20–100% methanol), strong electrostatic interactions were observed between the quaternary ammoniums and the ionized free silanols of the stationary phase, which induced total retention of these compounds. This was not the case with the Symmetry Shield C_{18} or the Inertsil ODS-3 columns, but with these columns insufficient resolution was obtained even after the attempts to improve separation by mobile phase modification (with acid–base or ion-pairing reagent addition).

The most satisfactory separations were obtained with the Nova-Pak C_{18} column and a modified mobile phase. We studied the influence of both the nature of the organic modifier (methanol or acetonitrile) and addition of acid–base or ion-pair reagents. So we compared the separation of methanolic extract M_1 with binary mobile phases: solvent A (water containing different acid–base and/or ion-pairing reagents) and solvent B (methanol containing the same reagents as solvent A). Reagents employed were either acetic acid, formic acid, triethylamine, sodium dodecylsulfate or perchloric acid. The following elution gradient was always used: 20–100% solvent B in 30 and 10 min with 100% solvent B. Fig. 2A–E represent the expanded chromatograms showing the separation of the four main components of M_1 (palmatine, jatrorrhizine, columbamine and pseudocolumbamine) obtained with different mobile phases. When acetic acid was employed at 8.3×10^{-3} mol l^{-1}, in both aqueous and methanolic mobile phases, all the protoberberine alkaloids were irreversibly retained in the column. This is probably due to a still high level of ionized silanols on the stationary phase because acetic acid is a weak acid and the pH of the aqueous phase is therefore too high (pH 3.4). When the pH of the mobile phase was lowered by adding more acetic acid (0.17 mol l^{-1}, pH of the aqueous phase 2.6) or by adding formic acid (8.3×10^{-3} mol l^{-1}, pH 2.9) the retention of these compounds was lowered significantly but led to unsatisfactory chromatograms (Fig. 2A and B), because broad peaks and still too high retention times were obtained. If triethylamine was added to the acidic mobile phases (Fig. 2C), the interactions with the free silanols were lowered leading to less broad peaks and to less retention,

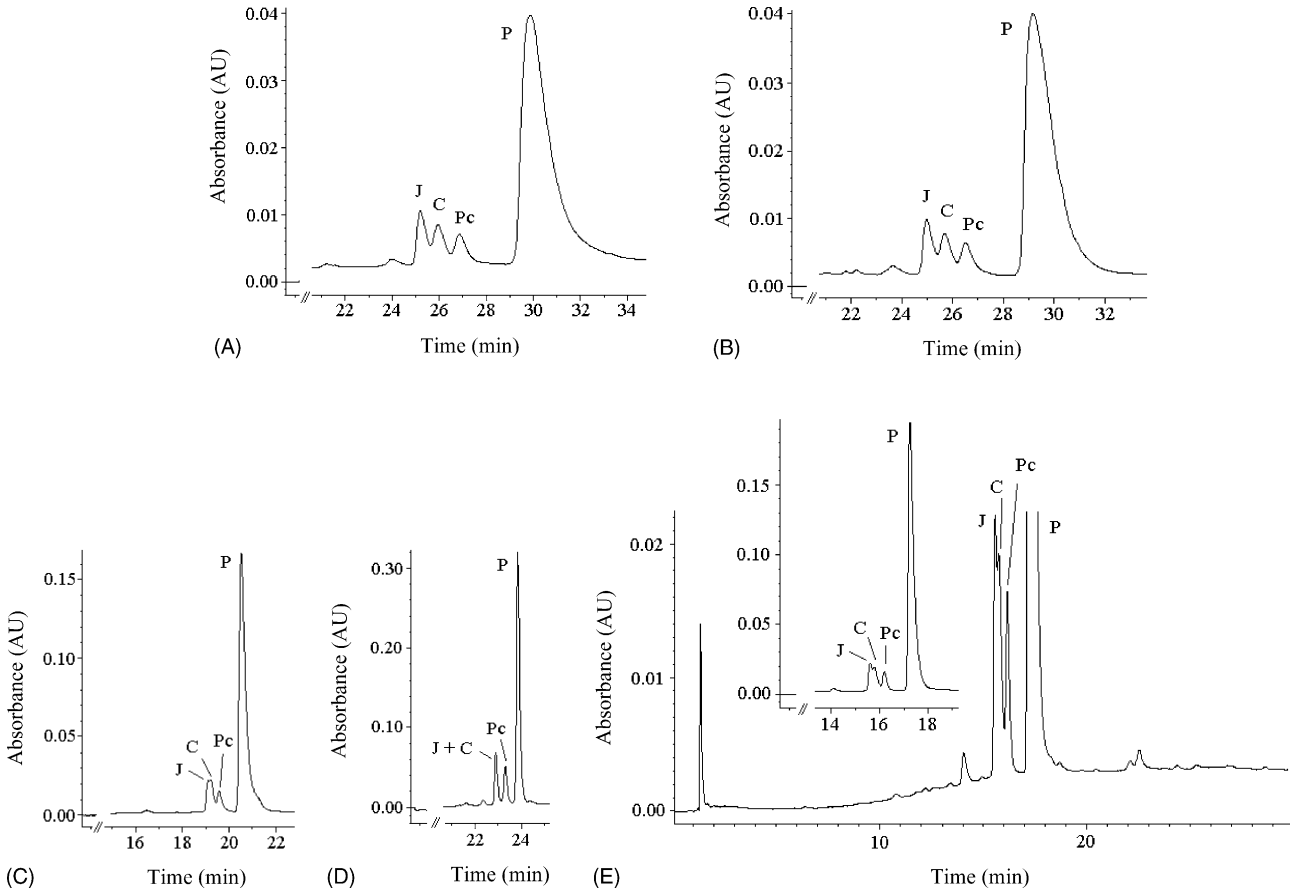


Fig. 2. Enlargements of M_1 chromatograms, showing the separation of the four main protoberberine alkaloids, obtained with different reagents added to both water and methanol mobile phases: (A) formic acid (8.3×10^{-3} M, pH* 2.9); (B) acetic acid (0.17 M, pH* 2.6); (C) acetic acid and triethylamine (0.17 and 7.2×10^{-4} M, respectively, pH* 2.7); (D) sodium dodecylsulfate (8.3×10^{-3} M); (E) perchloric acid (8.3×10^{-3} M, pH* 2.0). HPLC conditions: column, Nova-Pak C_{18} (150 mm × 3.9 mm i.d., 5 μm particle size); elution flow rate, 1 ml min^{-1}; elution gradient, 20–100% methanol in 30 min and 10 min at 100% methanol; UV absorbance detection at 270 nm. (*) pH of the aqueous phase.

but with insufficient resolution between the peaks of jatrorrhizine and columbamine. Another solution to prevent the electrostatic interactions with the residual silanols of the stationary phase was to add ion-pairing reagents to the mobile phase. Sodium dodecylsulfate added to both aqueous and methanolic phases at 8.3×10^{-3} mol l^{-1} led to narrow peaks (Fig. 2D) however jatrorrhizine and columbamine were not separated in the column. The most satisfying chromatographic profile (Fig. 2E) was obtained when perchloric acid was added to both aqueous and methanolic phases at a concentration of 8.3×10^{-3} mol l^{-1} (pH of the aqueous phase 2.0). In this case perchloric acid acts both as an acid and an ion-pairing reagent. As it is easier and cheaper to use it than sodium dodecylsulfate in chromatographic gradients, we chose to add this reagent to the mobile phase, even if the separation between jatrorrhizine and columbamine is not sufficient. As a matter of fact, the resolution between jatrorrhizine and columbamine was considered to be acceptable to follow HPCPC separations. When we needed more accuracy, formic acid was added to both aqueous and methanolic phases (8.3×10^{-3} mol l^{-1}) instead of perchloric acid.

Quantification of palmatine, jatrorrhizine, columbamine and pseudocolumbamine in mixtures M_1 and M_2 was realized by HPLC. We found that palmatine, jatrorrhizine, columbamine and pseudocolumbamine contents in M_1 were 51, 5, 6 and 7% (w/w), respectively. The global content of these four components being around 69% (w/w) of M_1. Therefore we could consider that 1 mg of M_1 contains around 2×10^{-6} mol expressed as protoberberine alkaloids. Jatrorrhizine, columbamine and pseudocolumbamine contents in M_2 were 37, 27 and 33% (w/w), respectively.

3.2. Choice of the two-phase solvent system for HPCPC separations

As palmatine represents more than half of M_1, it was better to first separate it from the other protoberberine alkaloids and then to separate the other alkaloids.

The first step to optimize the chromatographic conditions of the HPCPC, was to find solvents in which M_1 was the most soluble and then to select the biphasic solvent systems. Various solvents covering a wide range of polarity, including water, methanol, butan-1-ol, acetic acid, ethyl acetate, dichloromethane and heptane were tested. Water, methanol, butan-1-ol, ethyl acetate and dichloromethane were selected as best solvents for M_1. The dichloromethane–methanol–water, ethyl acetate–methanol–water and butan-1-ol-methanol–water biphasic systems were studied in first intention.

The distribution constants (K_C) of the protoberberine alkaloids between the two phases of these biphasic systems were quantified with the shake flask method coupled with quantitative HPLC analysis. The K_C values determined by HPLC and obtained for the protoberberine alkaloids of M_1 with the three solvent systems are compiled in Table 1.

With the biphasic solvent system containing butan-1-ol, all K_C values were close to 1, and the attempts to improve K_C differences by modifying pH or by adding anions were unsuccessful.

With the biphasic solvent systems containing either dichloromethane or ethyl acetate, jatrorrhizine, columbamine and pseudocolumbamine were more soluble in the aqueous-rich phase ($K_C > 20$). Considering palmatine, its K_C value was also above 20 in the system containing ethyl acetate but with the dichloromethane–methanol–water system palmatine became more soluble in the dichloromethane-rich phase ($K_C = 6.3$). To find the best conditions for the separation of the main protoberberine alkaloids, we studied both the influence of pH and the nature of various anions present in the aqueous phase on K_C values.

3.3. Influence of the pH of the aqueous phase on K_C values

To study the influence of pH, dichloromethane–methanol–water (48:16:36, v/v) and ethyl acetate–methanol–water (50:10:40, v/v) two-phase solvent systems were prepared by replacing water by aqueous solutions containing either hydrochloric acid, sodium hydroxide or diethylamine. Among strong acids, hydrochloric acid was chosen to minimize complexation or ion-pairing between its anions and the protoberberine quaternary ammoniums.

To determine K_C values of M_1 protoberberine alkaloids, shake flask experiments were performed with 10 mg of M_1 (containing about 2×10^{-5} mol of the main protoberberine alkaloids) and 10 ml of each phase of the biphasic solvent system. The amount of acid or base, added to the aqueous phase, corresponded to either 10^{-5}, 4×10^{-5} or 10^{-4} mol. K_C values calculated when acid or base were added to the

Table 1
Distribution constants[a] (K_C) of the M_1 main protoberberine alkaloids in various biphasic solvent systems

Protoberberine alkaloids	CH_2Cl_2–MeOH–water (48:16:36, v/v)	AcOEt–MeOH–water (50:10:40, v/v)	BuOH–MeOH–water (46:10:44, v/v)
Jatrorrhizine	45.8	46.5	0.8
Columbamine	23.5	20.1	0.9
Pseudocolumbamine	42.8	53.2	1.0
Palmatine	6.3	59.4	0.9

[a] K_C values determined with shake flask method coupled with HPLC and obtained for jatrorrhizine, columbamine, pseudocolumbamine and palmatine with dichloromethane–methanol–water, ethyl acetate–methanol–water and butan-1-ol-methanol–water two-phase solvent systems. K_C values were calculated as the solute affinity for the aqueous-rich stationary phase (Eq. (1)).

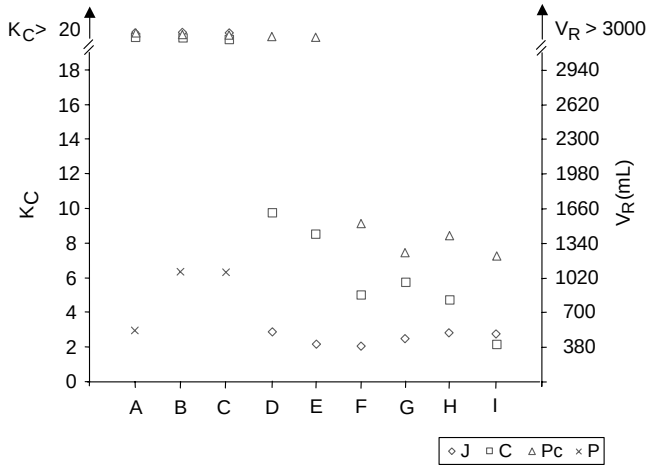


Fig. 3. Distribution constants[a] (K_C) and theoretical retention volumes[b] (V_R) of palmatine[c] (×), jatrorrhizine (◇), columbamine (□) and pseudocolumbamine (△) obtained with the dichloromethane–methanol–water two-phase solvent system prepared with water containing[d] (A) 1.4×10^{-2} M hydrochloric acid, pH 1.8; (B) 1.4×10^{-3} M hydrochloric acid, pH 2.8; (D) 1.4×10^{-3} M diethylamine, pH 10.8; (E) 1.4×10^{-3} M sodium hydroxide, pH 11.1; (F) 5.7×10^{-3} M diethylamine, pH 11.3; (G) 1.4×10^{-2} M diethylamine, pH 11.5; (H) 5.7×10^{-3} M sodium hydroxide, pH 11.8; (I) 1.4×10^{-2} mol sodium hydroxide, pH 12.2. Distilled water (C) was used as a reference.

[a] K_C of solutes were measured with shake flask method coupled with HPLC quantitative analysis, and K_C values were calculated as the solute affinity for the aqueous-rich stationary phase (Eq. (1)).

[b] Theoretical retention volumes were deduced from K_C values (Eq. (2)).

[c] K_C of palmatine when pH is above 10 are not represented because of the degradation of this alkaloid in alkaline methanolic medium.

[d] Concentrations and pH are given for the aqueous phase used to prepare the biphasic solvent system.

aqueous phase (different pH of the aqueous phase) were compared with those obtained when the biphasic system was prepared with distilled water.

In all cases, the best two-phase solvent system was the dichloromethane–methanol–water system, as observed with neutral water. Fig. 3 shows K_C (left scale), quantified by HPLC and obtained with the dichloromethane–methanol–water biphasic systems, as a function of pH of the aqueous phase. Theoretical retention volumes as a function of pH are also presented (right scale). When K_C values are above 20 we consider that the compounds are mainly present in the aqueous-rich phase and that the HPCPC retention volumes are too high to perform an HPCPC experiment. As described in the literature [31] palmatine degradation was observed in alkaline methanolic medium, so K_C values could neither be calculated nor be presented on Fig. 3 with these conditions.

The acidification of the aqueous-rich phase by hydrochloric acid had a small effect on K_C or V_R. To observe a significant change of K_C (two-fold decrease) pH must be sufficiently acidic (pH 1.8). As protoberberine alkaloids are quaternary ammoniums, the addition of acid would not induce any modification of their acid–base equilibrium, so this effect may be due to slight interactions between the chloride anions and the protoberberine quaternary ammoniums (salting out effect).

Increasing pH above 10.5, by adding either sodium hydroxide or diethylamine, induced a significant decrease of K_C values of jatrorrhizine, columbamine, and pseudocolumbamine, as compared with distilled water, which is correlated with the pH increase. Their increasing solubility in the dichloromethane-rich phase is probably due to their phenolic group ionization, as demonstrated by the color change of their solutions from yellow to red-brown, leading to zwitterionic compounds with a global electric charge equal to zero. The best conditions to separate jatrorrhizine, columbamine, and pseudocolumbamine by HPCPC would be those obtained with the aqueous-rich phase containing either diethylamine (pH 11.3–11.5, Fig. 3F–G) or sodium hydroxide (pH 11.8, Fig. 3H).

As previously explained, alkaline methanolic medium would not be appropriated for purification of samples containing palmatine, so the first step would be to separate palmatine from the other protoberberine alkaloids, with an appropriate biphasic solvent system, and then to separate jatrorrhizine, columbamine and pseudocolumbamine with the alkaline biphasic solvent system.

3.4. Influence of the nature of various anions introduced in the aqueous phase on K_C values

It has been noticed, in HPLC analyses, that ion-pairing can occur between protoberberine quaternary ammoniums and perchlorate ions. These ion-pairing interactions could increase the solubility of these compounds in the organic-rich phase. Consequently interactions between protoberberine quaternary ammoniums and different anions were studied regarding both the influence of the nature of the anions (perchlorate, chloride and acetate) and their concentration (anion to protoberberine ratio). Ion-pairing reagents such as sodium dodecylsulfate were not employed as they would be difficult to eliminate and are expensive reagents.

As previously, to determine the K_C values of protoberberine alkaloids, shake flask experiments were performed with 10 mg of M_1 (containing about 2×10^{-5} mol of the main protoberberine alkaloids) and 10 ml of each phase of the biphasic solvent system. The biphasic solvent systems studied were dichloromethane–methanol–water (48:16:36, v/v) and ethyl acetate–methanol–water (50:10:40, v/v). The amount of each anions added to the aqueous phase corresponded to either 10^{-4}, 10^{-5} or 10^{-6} mol.

The most interesting results (satisfactory differences between protoberberine alkaloids K_C values and K_C values under 20) were obtained when the molar ratio between the anions and the main protoberberine alkaloids was 0.5, corresponding to an addition of 10^{-5} mol of anions. So we will focus our discussion only on the results obtained with this molar ratio.

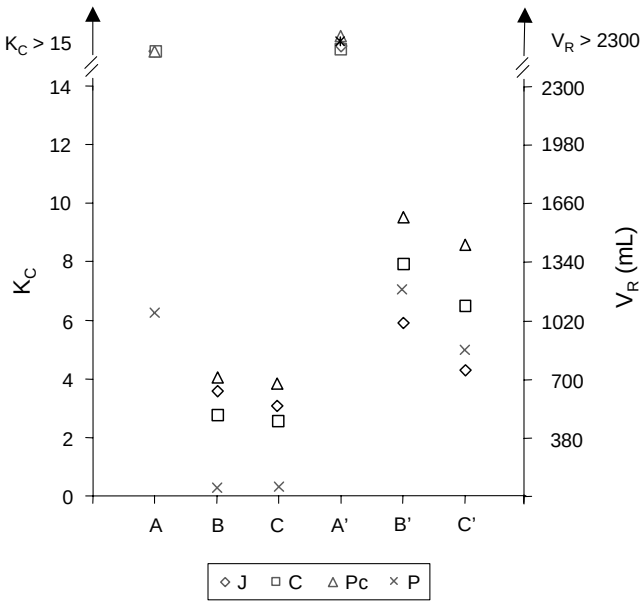


Fig. 4. Distribution constants[a] (K_C) and theoretical retention volumes[b] (V_R) of palmatine (×), jatrorrhizine (◇), columbamine (□) and pseudocolumbamine (△) obtained with the dichloromethane–methanol–water (A–C) and the ethyl acetate–methanol–water (A′–C′) two-phase solvent systems prepared with water containing[c] (B and B′) 1.4×10^{-3} M perchloric acid, pH 2.8; (C and C′) 1.4×10^{-3} M potassium perchlorate, pH 6.2. Distilled water (A and A′) was used as a reference.

[a] K_C of solutes were measured with shake flask method coupled with HPLC quantitative analysis, and K_C values were calculated as the solute affinity for the aqueous-rich stationary phase (Eq. (1)).

[b] Theoretical retention volumes were deduced from K_C values (Eq. (2)).

[c] The molar ratio between the added anions and the main protoberberine alkaloids was equal to 0.5. Concentrations and pH are given for the aqueous phase used to prepare the biphasic solvent system.

In both biphasic solvent systems, addition of chloride and acetate anions did not induce any modifications of K_C as compared to distilled water, so these anions could not be used to separate the protoberberine alkaloids by HPCPC.

On the contrary, perchlorate anions (Fig. 4B, B′, C and C′) added to water led to a significant decrease of K_C for every protoberberine alkaloid, in both biphasic solvent systems, as compared to distilled water (Fig. 4A and A′). This was correlated with the increase of perchlorate concentration. The K_C decrease is the result of an increasing solubility of these compounds in the dichloromethane- or in the ethyl acetate-rich phases, which is probably due to ion-pair formation.

As observed in previous experiments, the solubility of the protoberberine alkaloids is higher in the dichloromethane-rich phase than in the ethyl acetate-rich phase. Moreover, in the dichloromethane–methanol–water biphasic system containing perchlorate ions, K_C values for palmatine differed more from those of the other protoberberine alkaloids than in the ethyl acetate–methanol–water biphasic system. Consequently palmatine could be separated from the other alkaloids by using a dichloromethane–methanol–water biphasic system containing perchlorate anions in a 0.5 molar ratio between perchlorate anions and the main protoberberine alkaloids.

3.5. HPCPC separations

The study of the influence of the composition of the two-phase solvent systems on K_C, such as the nature of the organic solvent (dichloromethane, ethyl acetate, butan-1-ol), the pH and the nature of the added anions in the aqueous phase, led us to conclude that two successive biphasic solvent systems could be employed to separate the M_1 main protoberberine alkaloids: first a dichloromethane–methanol–water (48:16:36, v/v) system containing perchlorate anions (0.5 molar ratio between perchlorate anions and the main protoberberine alkaloids) to isolate palmatine, and then a dichloromethane–methanol–water (48:16:36, v/v) system with water containing either diethylamine (5.7×10^{-3} to 1.4×10^{-2} M, pH 11.3–11.5) or sodium hydroxide (5.7×10^{-3} M, pH 11.8) to isolate jatrorrhizine, columbamine and pseudocolumbamine.

In both systems, the addition of either perchlorate ions, diethylamine or sodium hydroxide, led to an increasing solubility of the protoberberine alkaloids in the organic-rich phase, allowing us to choose this phase as the mobile phase for the HPCPC experiments.

As alkaline pH is not recommended for palmatine isolation [31], palmatine would be firstly isolated by HPCPC with the biphasic system containing perchlorate ions.

3.5.1. HPCPC isolation of palmatine with perchlorate anions

As K_C (or V_R) variations are correlated with the perchlorate anion concentrations we needed to introduce the exact amount of perchlorate anions in the injected sample.

So as to determine what perchlorate source was better for the separation of palmatine, two types of HPCPC were carried out: the first one using $KClO_4$ and the other one using $HClO_4$. Similar separations were obtained with both experiments. However, we preferred the use of $KClO_4$ to avoid acidification of the sample by $HClO_4$.

Moreover, as the number of theoretical plates (N) can vary with the flow rate [27], we studied its effect on the protoberberine alkaloid separation. Two HPCPC experiments were then carried out with 3 or 9 ml min^{-1} flow rates. In both cases N was found between 200 and 300, so only palmatine could be correctly separated from the other compounds (the theoretical resolution between palmatine and columbamine was above 4.5 whereas the theoretical resolutions between columbamine, jatrorrhizine and pseudocolumbamine were always below 1). Consequently, to decrease the duration of experiments, it was decided to carry out the isolation of palmatine at 9 ml min^{-1} and to collect the other protoberberine alkaloids together for further HPCPC separation. However, in order to compare their theoretical retention volumes with those obtained experimentally, we first analyzed

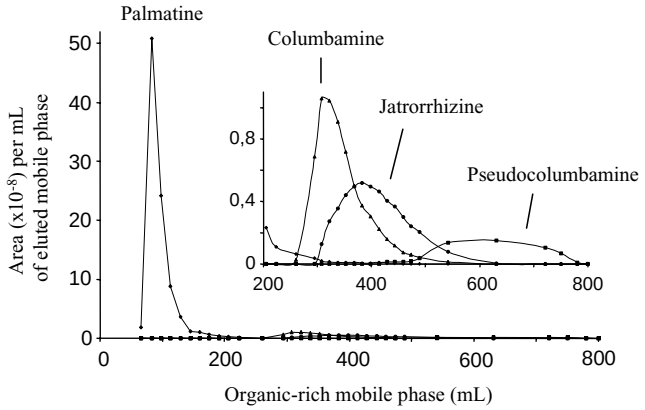


Fig. 5. Elution profile of palmatine, jatrorrhizine, columbamine and pseudocolumbamine from mixture M_1 in an HPCPC run. The biphasic solvent system was composed of dichloromethane–methanol–water. The injected sample contained potassium perchlorate (molar ratio between perchlorate anions and protoberberine alkaloids equal to 0.5). The flow rate was 9 ml min^{-1} at 700 rpm and fractions were collected every 2–5 min. The content of the fractions was quantified by peak area measurement at 270 nm by HPLC (same conditions as for Fig. 2).

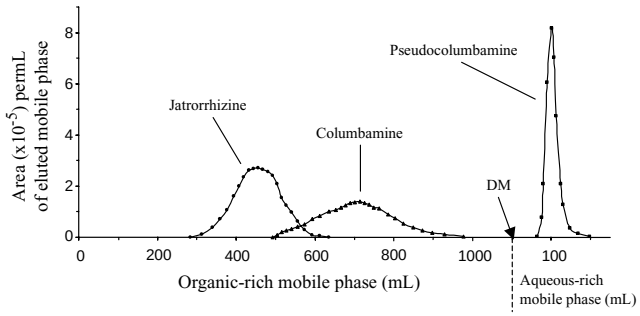


Fig. 6. Elution profile of jatrorrhizine, columbamine and pseudocolumbamine from mixture M_2 in an HPCPC run. The biphasic solvent system was composed of dichloromethane–methanol–water containing sodium hydroxide (pH 11.8). The flow rate was 3 ml min^{-1} at 700 rpm and fractions were collected every 2–5 min. The content of one fraction out of two was quantified by peak area measurement at 270 nm by HPLC (same conditions as for Fig. 2). At the point marked DM, the system was switched in the "dual mode".

all the fractions by HPLC. The retention volumes of the protoberberine alkaloids determined by the reconstruction of the chromatogram were close to their theoretical values (Fig. 5). Fractions containing palmatine were pooled. As can be observed in Fig. 5, about 50% of pseudocolumbamine could theoretically be recovered with this system. However we chose to pool all the fractions containing jatrorrhizine, columbamine and pseudocolumbamine to obtain the mixture M_2 that was further fractionated by another HPCPC experiment.

The compounds too polar to be eluted in "normal mode" were eluted in "dual mode", pooled and evaporated to give a mixture M_3.

3.5.2. Isolation of jatrorrhizine, columbamine and pseudocolumbamine by HPCPC with either diethylamine or sodium hydroxide

To determine whether diethylamine or sodium hydroxide was better for the separation of jatrorrhizine, columbamine and pseudocolumbamine from mixture M_2, two types of HPCPC were carried out: the first one with the dichloromethane–methanol–water biphasic system prepared with water containing diethylamine (10^{-2} M, pH 11.4) and the other one with the dichloromethane–methanol–water biphasic system prepared with water containing sodium hydroxide (5.7×10^{-3} M, pH 11.8). As the resolution was higher at 3 ml min^{-1} than at 9 ml min^{-1}, the experiments were carried out with this flow rate. In both cases, the separations were similar. However, we preferred the use of sodium hydroxide rather than diethylamine. As a matter of fact, diethylamine and diethylammonium are both soluble in organic- and aqueous-rich phases, and were difficult to eliminate from the samples, even after solid phase extraction on C_{18} cartridges. On the contrary, sodium hydroxide is not soluble in the organic-rich phase, therefore jatrorrhizine and columbamine were not contaminated by it. As pseudocolumbamine was eluted with the aqueous-rich phase, fractions containing this compound were contaminated by an excess of sodium hydroxide, which was eliminated by solid phase extraction desalting on C_{18} cartridges, after its transformation into NaCl by HCl addition.

The good separations obtained with this biphasic solvent system allowed us to obtain jatrorrhizine, columbamine and pseudocolumbamine. Fig. 6 shows the reconstituted chromatogram of the HPCPC carried out with sodium hydroxide. Measured retention volumes, determined by the reconstruction of the chromatograms were close to the calculated ones for both systems.

4. Conclusion

Protoberberine alkaloids such as palmatine, jatrorrhizine, columbamine and pseudocolumbamine, which are very polar compounds and which have similar chemical structures, have been isolated in two steps by HPCPC from a methanolic extract of *E. chlorantha*. They were obtained with high purity (>95%) in sufficient amount for in vitro biological evaluation. As higher amounts of those alkaloids can be obtained, in vivo biological evaluation could be performed.

These separations involved either ion-pairing between the quaternary ammoniums and perchlorate anions, or the ionization of the phenolic compounds by addition of sodium hydroxide or diethylamine. This suggests that those methods could be used for the separation of other quaternary alkaloids as well, rather than using classical preparative normal or reversed-phase HPLC. Indeed if we had chosen preparative RP-HPLC, because of the poor solubility of these compounds in methanol–water systems, it would be necessary to carry out many injections. For example, to isolate palmatine from 200 mg of M_1, it would have been necessary to

carry out about five injections. The overall injections would require about 10 liters of mobile phase whereas HPCPC solvent consumption was only 3 liters. Moreover, the column cost would be very expensive. Even if the solubility of these compounds in dichloromethane–methanol mixtures is higher, we could not choose preparative normal phase HPLC because of the potential adsorptions of these quaternary ammoniums on the column. The drawbacks linked to the use of a solid stationary phase were overcome with the use of HPCPC, which features a high loadability and a unique selectivity due to the infinite number of possible biphasic liquid systems offering a wide polarity range. Moreover, irreversible solute adsorption on stationary phase does not occur in HPCPC experiments since the stationary phase is a liquid. Furthermore the components retained in the stationary phase can easily be recovered either by reversing the stationary and mobile phases during the run (dual mode), or by extruding the stationary phase (after stopping the column rotation and mobile phase flow) while collecting fractions.

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References

[1] M. Hamonnière, M. Leboeuf, A. Cavé, R.R. Paris, Plantes Méd. Phytothér. 9 (1975) 296.
[2] L. Jalander, R. Sjöholm, P. Virtanen, Collect. Czech. Commun. 55 (1990) 2095.
[3] P. Wafo, B. Nyasse, C. Fontaine, B.L. Sondengam, Fitoterapia 70 (1999) 157.
[4] E.O. Agbaje, A.O. Onabanjo, Ann. Trop. Med. Parasit. 85 (1991) 585.
[5] J.O. Moody, P.J. Hylands, D.H. Bray, Pharm. Pharmacol. Lett. 2 (1995) 80.
[6] P. Virtanen, V. Lassila, K.-O. Söderström, Pathobiology 61 (1993) 51.
[7] C.W. Wright, S.J. Marshall, P.F. Russell, M.M. Anderson, J.D. Phillipson, G.C. Kirby, D.C. Warhurst, P.L. Schiff, J. Nat. Prod. 63 (2000) 1638.
[8] D.L.C. McCall, J. Alexander, J. Barber, R.G. Jaouhari, A. Satoskar, R.D. Waigh, Bioorg. Med. Chem. Lett. 4 (1994) 1663.
[9] B. Nyasse, E. Nkwengoua, B. Sondengam, C. Denier, M. Willson, Pharmazie 57 (2002) 358.
[10] J.L. Vennerstrom, D.L. Klayman, J. Med. Chem. 31 (1988) 1084.
[11] K. Iwasa, M. Kamigauchi, M. Ueki, M. Taniguchi, Eur. J. Med. Chem. 31 (1996) 469.
[12] K. Iwasa, H.-S. Kim, Y. Wataya, D.-U. Lee, Eur. J. Med. Chem. 33 (1998) 65.
[13] K. Iwasa, Y. Nishiyama, M. Ichimaru, M. Moriyasu, H.-S. Kim, Y. Wataya, T. Yamori, T. Takashi, D.-U. Lee, Eur. J. Med. Chem. 34 (1999) 1077.
[14] S.K. Chattopadhyay, A.B. Ray, D.J. Slatkin, P.L. Schiff, Phytochemistry 22 (1983) 2607.
[15] A. Baerheim-Svendsen, A.M. van Kempen-Verleun, R. Verpoorte, J. Chromatogr. 291 (1984) 389.
[16] J. Pothier, N. Galand, P. Tivollier, C. Viel, J. Planar Chromatogr. 6 (1993) 220.
[17] J.D. Henion, A.V. Mordehai, J. Cai, Anal. Chem. 66 (1994) 2103.
[18] M. Unger, D. Stöckigt, D. Belder, J. Stöckigt, J. Chromatogr. A 767 (1997) 263.
[19] Y.-M. Liu, S.-J. Sheu, J. Chromatogr. 623 (1992) 196.
[20] S.-J. Lin, H.-H. Tseng, K.-C. Wen, T.-T. Suen, J. Chromatogr. A 730 (1996) 17.
[21] W.-C. Chuang, D.-S. Young, L.K. Liu, S.-J. Sheu, J. Chromatogr. A 755 (1996) 19.
[22] A. Bonora, B. Tosi, G. Dall'Olio, A. Bruni, Phytochemistry 29 (1990) 2389.
[23] T. Misaki, K. Sagara, M. Ojima, S. Kakizawa, T. Oshima, H. Yoshizawa, Chem. Pharm. Bull. 30 (1982) 354.
[24] H.S. Lee, Y.E. Eom, D.O. Eom, J. Pharm. Biomed. Anal. 21 (1999) 59.
[25] N. Fabre, C. Claparols, S. Richelme, M.-L. Angelin, I. Fourasté, C. Moulis, J. Chromatogr. A 904 (2000) 35.
[26] F. Yang, T. Zhang, R. Zhang, Y. Ito, J. Chromatogr. A 829 (1998) 137.
[27] A.P. Foucault, Chromatographic Science Series, vol. 68, Marcel Dekker, New York, 1995.
[28] A.P. Foucault, L. Chevolot, J. Chromatogr. A 808 (1998) 3.
[29] A. Berthod, Comprehensive Analytical Chemistry Series, vol. 38, Elsevier, Amsterdam, 2002.
[30] J.J. Gilroy, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 1000 (2003) 757.
[31] R. Marek, P. Seckarova, D. Hulova, J. Marek, J. Dostal, V. Sklenar, J. Nat. Prod. 66 (2003) 481.