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Short communication

Preparative separation of glycoalkaloids α-solanine and α-chaconine by centrifugal partition chromatography

Jacques Attoumbré [a], David Lesur [b], Philippe Giordanengo [c,d], Sylvie Baltora-Rosset [e,*]

[a] S.I.P.R.E – Comité Nord, rue des champs Potez, 62217 Achicourt, France
[b] Laboratoire des Glucides FR3517, UPJV, 33 rue St Leu, 80039 Amiens cedex, France
[c] Université de Picardie Jules Verne, 33 rue Saint Leu, 80039 Amiens cedex 1, France
[d] Institut Sophia Agrobiotech, CNRS 7254 – INRA 1355, Université de Nice Sophia Antipolis, 400 route des Chappes, BP167, 06903 Sophia Antipolis, France
[e] EDYSAN EA 4698, Université de Picardie Jules Verne, Faculté de Pharmacie, 1 rue des Louvels, 80037 Amiens cedex, France

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ABSTRACT

The main glycoalkaloids of a commercial potato cultivar, α-chaconine and α-solanine, were extracted from sprouts of *Solanum tuberosum* cv. Pompadour by a mixture of $MeOH/H_2O/CH_3COOH$ (400/100/50, v/v/v). In these conditions, 2.8 ± 0.62 g of crude extract were obtained from 50 g of fresh sprouts and the total glycoalkaloid content was determined by analytical HPLC at 216.5 mg/100 g. α-Chaconine and α-solanine were separated in a preparative scale using centrifugal partition chromatography (CPC). In a solvent system composed of a mixture of ethyl acetate/butanol/water (15/35/50, v/v/v), α-chaconine (54 mg) and α-solanine (15 mg) were successfully isolated from the crude extract in one step of purification. The purity of isolated compounds was determined to be higher than 92% by HPLC analysis.

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1. Introduction

Steroidal glycoalkaloids constitute an important class of biologically active compounds in potato (Solanaceae) plant family which is a very valuable crop that provides high-quality nutrition to billions of people around the world. These secondary metabolites are involved in plant resistance to pests and predators and have been shown to be toxic to a wide range of organisms from fungi to humans [1,2]. Glycoalkaloids (GAs) are usually distributed in all plant organs with a main localization in sprouts, flowers and skin [3]. Their structures and concentrations largely depend on potato lines and environmental factors and it has been shown that GAs can accumulate to high levels in greened, stored, damaged, and irradiated tubers for example [4].

α-Chaconine and α-solanine are the major potato glycoalkaloids (PGAs) in commercial cultivars and are intensively studied because of their greatest contribution to the total GA content and their bioactivity. The potential human poisoning effect of PGAs has led to the implementation of safety regulations limiting their content in edible tubers to 20 mg/100 g fresh weight involving the development of numerous reliable analytical methods [3]. Moreover, biological activity of PGAs is not limited to their toxicity and they have been reported to possess pharmacological properties [2]. To study and validate their potential health-promoting effects in humans, effective extraction procedures and preparative separation methods are needed. Various analytical methods have been reported for the determination of GAs. The simplest methods, such as gravimetric and colorimetric ones, lack the required specificity and suffer from contamination by other potato components. Other techniques such as immunoassays rely on the specificity of antibodies to get better sensitivity and to eliminate tedious sample preparation process but are not suitable to recover isolated products [5]. The main methods used to detect, quantify and isolate GAs are chromatographic techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC) or liquid chromatography electrospray ionization-mass spectrometry (LC–ESI-MS) [6–9]. Because of their complex chemical structures (hydrophobic 27-carbon skeleton of steroidal alkamine attached to a hydrophilic trisaccharide), serious technical difficulties are associated with PGAs quantitative analysis and isolation. GC analysis requires chemical derivatization. As GAs show no suitable chromophore for HPLC–UV methods, their detection has to be made at around 200–210 nm where many compounds absorb light leading to tedious sample preparations to overcome background noise. Solid-phase extraction (SPE) currently used to purify GAs from plant matrixes before their analysis by HPLC often leads to extremely variable results for recoveries [7,8]. Moreover, even after SPE clean-up, the analysis of GAs extracts by HPLC requires a strict

* Corresponding author. Tel.: +33 03 2282 7770; fax: +33 03 2282 7469.
E-mail address: sylvie.baltora-rosset@u-picardie.fr (S. Baltora-Rosset).

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pH control to observe efficient detection and separation and the composition of the mobile phase is also an important factor to ensure good solubility of GAs. The need to use buffers to set a suitable pH for the HPLC technique limits its use for the development of easy preparative procedures since a final extraction step is necessary to remove salts and to recover the isolated product.

Only few protocols for GAs preparative isolation are described in the literature. α-Chaconine and α-solanine were isolated at a preparative scale by Soulé et al. by MPLC. 86.7 mg and 66.5 mg were, respectively, obtained from 1 kg of potato peel [10]. The authors mentioned the simplicity but the low yield obtained in the MPLC separation. Moreover, the purity of solanine (85%) is not sufficient for further use. Recently, Paul et al. developed an efficient LC–MS protocol for preparative scale isolation and quantification of two steroidal alkaloids solamargine and solasonine from *Solanum xanthocarpum* [11]. However, in addition to the equipment which is not readily available, this method requires the use of formic acid in the mobile phase. This may involve additional processing of the purified compounds according to their future use because GAs can be hydrolyzed in the presence of acid when they are evaporated to dryness [7].

Countercurrent chromatography (CCC) is a chromatographic technique which benefits from some advantages when compared with LC techniques: (i) no non-specific adsorption to a solid support, (ii) higher selectivity, (iii) higher sample loading capacity, (iv) reduction of solvent quantity and (v) shorter separation time. Fukuhara et al. described an efficient semi-preparative scale isolation of GAs from *Solanum incanum* by the sequential use of rotation locular countercurrent chromatography and droplet countercurrent chromatography (RLCC and DCC) [12]. 170 mg of pure compounds were obtained from 550 g of fresh ripe fruits of *S. incanum*. A new GA, arudonine, was also isolated by CCC by bioassay-guided fractionation of a root bark extract of *Solanum arundo* [13]. A pH-zone-refining CPC was successfully implemented for the preparative isolation of two GAs, solamargine and solasonine [14]. Only solamargine was recovered in one step by CCC while a further step using MPLC was necessary to yield pure solasonine. In addition when using this technique, removal of acid or base from the separated products may be inconvenient [15].

The aim of this study was to develop an efficient method for the isolation and purification of α-chaconine and α-solanine from fresh sprouts of *Solanum tuberosum* (cv. Pompadour) without sample clean-up and in the shortest time. The objective was fully achieved by the use of CPC which led to the successful separation of the two GAs of high purity used for further chemical modulations and biological studies.

2. Experimental

2.1. Chemical reagents

α-Chaconine and α-solanine used as reference standards were purchased from Extrasynthese (France). All organic solvents were analytical grade and purchased from Prolabo (France).

2.2. Apparatus

The CPC instrument used in this study is a SPOT CPC 100 Light (Armen Instrument) fitted with a rotor of 10 circular partition disks (1000 partition cells: 0.1 ml per cell; total column capacity of 100 ml). Rotation speed can be chosen from 0 to 4000 rpm. The effluent was monitored by a Lash 06 DAD detector (ECOM, Prague) equipped with a preparative flow cell operating at 202 and 210 nm and collected by a LS 5600 (Armen) fraction collector.

The HPLC used was a Shimadzu HPLC System including a LC-20AT pump and a SPD-M20A diode-array detector.

LC–MS spectra were performed on a Waters 2695 Alliance coupled with a quadrupole mass spectrometer ZQ (Water-Micromass, Manchester, UK) equipped with an electrospray ion source (ESI-MS). LC–ESI–MS were recorded in the positive and negative ion mode. The capillary voltage was ±3.5 kV and a cone voltage range from ±20 to ±60 V was used. Data acquisition and processing were performed with MassLynx V4.0 software.

2.3. Preparation of crude extract

50 g of fresh sprouts of *S. tuberosum* cv. Pompadour were ground and extracted by 550 ml of $CH_3OH/H_2O/CH_3COOH$ (400/100/50, v/v/v) under agitation for 12 h at room temperature. After filtration, the mixture was concentrated under reduced pressure to obtain a syrup consistence. This residue was dissolved in 75 ml of CH_3OH and 75 ml of NH_4OH (commercial solution at 30%) was added under cooling. The precipitate was recovered from the mixture by centrifugation at 5000 rpm for 30 min. 2.8 g of residue were obtained, re-dissolved in CH_3OH and stored at 4 °C for subsequent analysis and separation. The operation was repeated 3 times.

2.4. HPLC and LC–MS analyses

HPLC analyses of the crude extract and of the CPC peak fractions were conducted on a 250 mm × 4.6 mm, 5-μm, Prevail reverse-phase C18 column (Grace) using a linear binary gradient of H_2O–H_2KPO_4 0.1 M (solvent A) and CH_3CN (solvent B) with a flow rate of 1 ml/min as follows: 20–40% B (0–15 min), 40–80% B (15–30 min), 80% B (30–35 min), 80–20% B (35–40 min). The HPLC eluate was monitored at 202 nm. 20 μl were used for injection which was repeated 3 times.

LC–MS analyses of the samples were conducted on a 250 mm × 4.6 mm, 5-μm, Prevail reverse-phase C18 column (Grace) using a linear binary gradient of H_2O (solvent A) and CH_3CN (solvent B) both containing 0.1% (v/v) formic acid, with a flow rate of 1 ml/min as follows: 20–40% B (0–15 min), 40–80% B (15–30 min), 80% B (30–45 min), 80–20% B (35–40 min). The HPLC eluate was monitored at 202 nm.

The calibration curves were prepared using six different concentrations of the two glycoalkaloids in CH_3OH. 20 μl of each solutions ranging from 1 to 0.03125 mg/ml (2-fold serial dilutions) were injected in triplicate in the column. Calibration graphs were plotted based on linear regression analysis of the peak area vs. concentration, the curves showed good linearity (r^2 = 0.983 for chaconine and 0.988 for solanine).

2.5. CPC separation

2.5.1. Selection of the two-phase solvent system

The solvent system was selected according to the distribution coefficient K_C of α-solanine and α-chaconine. The K_C value was determined by HPLC analysis. Suitable amount of crude extract was dissolved in the tested solvent system and vortexed for 30 s. After separation and evaporation under reduced pressure, the residue of each layer was dissolved in 500 μl of methanol for HPLC analysis of its GA content. The K_C was calculated according to the ratio: concentration in the stationary phase/concentration in the mobile phase [16].

2.5.2. Collection and analysis of fractions

The solvent system used for separation was ethyl acetate/butanol/water in the ratio 15/35/50 (v/v/v). Biphasic system was prepared just before use by thoroughly mixing volumes of solvent in the above ratio. After the equilibration was

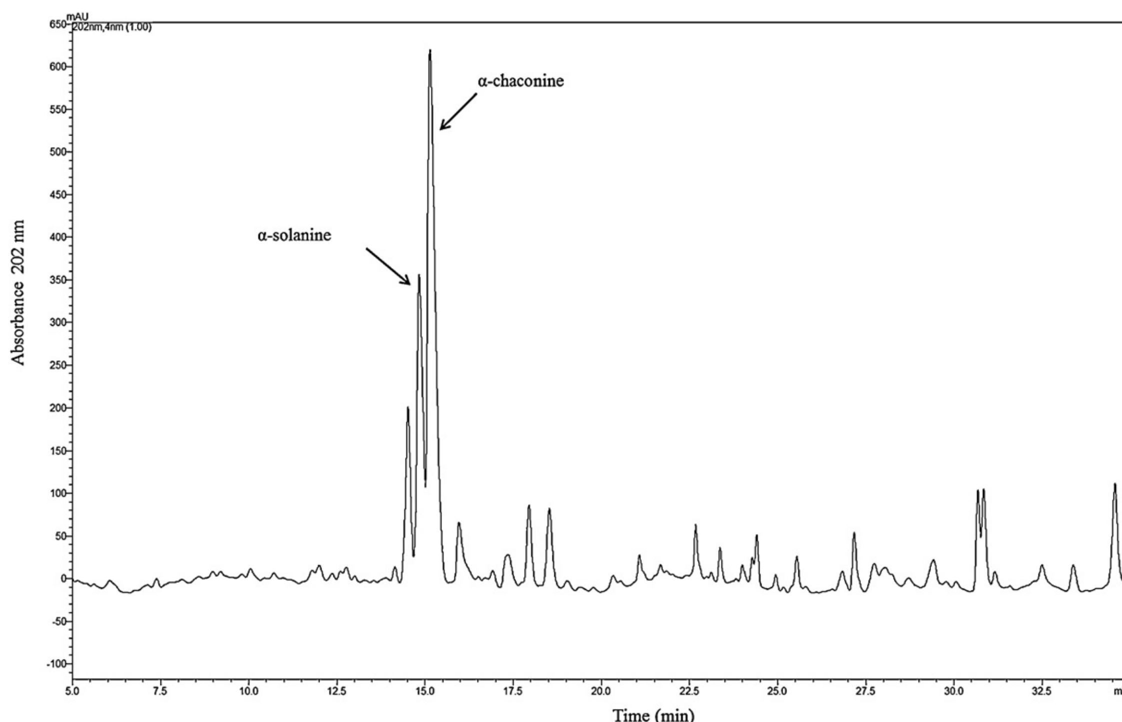

Fig. 1. Chromatogram of the crude extract of *S. tuberosum* (cv. Pompadour) sprouts by HPLC.

established, the stationary phase (lower phase in the ascending mode) was pumped into the column at a flow-rate of 30 ml/min while the apparatus was run at 500 rpm according to the equilibration mode of the apparatus. After injection of the sample (0.8 g of crude extract in 10 ml of the stationary phase) the mobile phase was perfused at 2500 rpm at a flow-rate of 8 ml/min under 26–28 b during the runs. The eluent was monitored at 202 nm and fractions of 15 ml were collected and analyzed by HPLC. Fractions collected before the fraction number 8 contained no GAs.

The volume of the stationary phase displaced by the mobile phase was measured and used to determine V_M. The stationary phase volume (V_S) was calculated according to the relation $V_C = V_M + V_S$ since the column volume (V_C) is known. The stationary phase retention was expressed as V_S/V_C. The general chromatographic retention equation which directly links retention volume of a solute (V_R) with its partition coefficient was used: $V_R = V_M + K_C \cdot V_S$ to estimate the K_C observed during the separation. The selectivity (separation factor) and the number of theoretical plates were calculated as follows: $\alpha = K_{C(\text{solanine})}/K_{C(\text{chaconine})}$ and $N = (4\,V_R/W)^2$ (W: peak width at the baseline) [16].

3. Results and discussion

The 2.8 ± 0.62 g of crude extract produced from the treatment of 50 g of fresh sprouts were analyzed by HPLC (Fig. 1). Two main peaks with retention times (Rt) of 14.96 min and 15.18 min were detected and were consistent to those of authentic samples of α-solanine and α-chaconine, respectively. From the calibration curves, the amounts of α-chaconine and α-solanine that could be recovered from the crude extract were estimated to74.13 ± 11.62 mg and to 34.13 ± 10.75 mg, respectively. Considering that these two compounds mainly contribute to the total GA content of commercial cultivar, we estimated that the average content of GAs alkaloids in sprouts of *S. tuberosum* cv. Pompadour was 216.5 mg/100 g FW. These results are consistent with the literature data (200–700 mg/100 g FW in sprouts depending on potato species [3]) and also confirmed that α-chaconine contributed more largely than α-solanine (68% vs. 32%) to the total potato GA content [2].

A first test of separation of the two GAs by preparative HPLC was performed but the results were not satisfactory. Only 0.35% of α-chaconine (95% purity) was obtained after two sequential steps of HPLC separation and subsequent extraction with chloroform to remove salts, while the amount of α-solanine was too low and was not quantified. Given this poor result that was not really surprising in the light of the literature, we decided to implement a separation by CPC.

Separation of natural products using CPC is based on the partition behaviors of target compounds between immiscible solvents used as a mobile and stationary phases. Key points in performing CPC separation are a good solubility of the sample in the solvent system as well as the determination of a suitable two-phase system. As shown in Table 1, the mixture of ethyl acetate/butanol/water (15/35/50, v/v/v) led to the best results with the compounds of interest almost equally distributed between the two phases (K_C value around 1).

Fig. 2 shows a typical CPC chromatogram obtained during the separation of 0.8 g of crude alkaloid extract. Each fraction collected during the separation process was analyzed by HPLC (Fig. 2(A)–(C)). It should be noted that the elution order of the GAs was reversed during the two methods used in this study: α-chaconine was first eluted by CPC and presented a higher retention time by HPLC demonstrating that α-chaconine is more polar than α-solanine. For

Table 1
The K_C (distribution coefficient) values of α-chaconine and α-solanine in different solvent systems.

Solvent system composition Ethyl acetate/butanol/water	K_C value α-Chaconine	K_C value α-Solanine
10/40/50	0.37	0.59
15/35/50	0.77	1.25
20/30/50	1.25	2.00

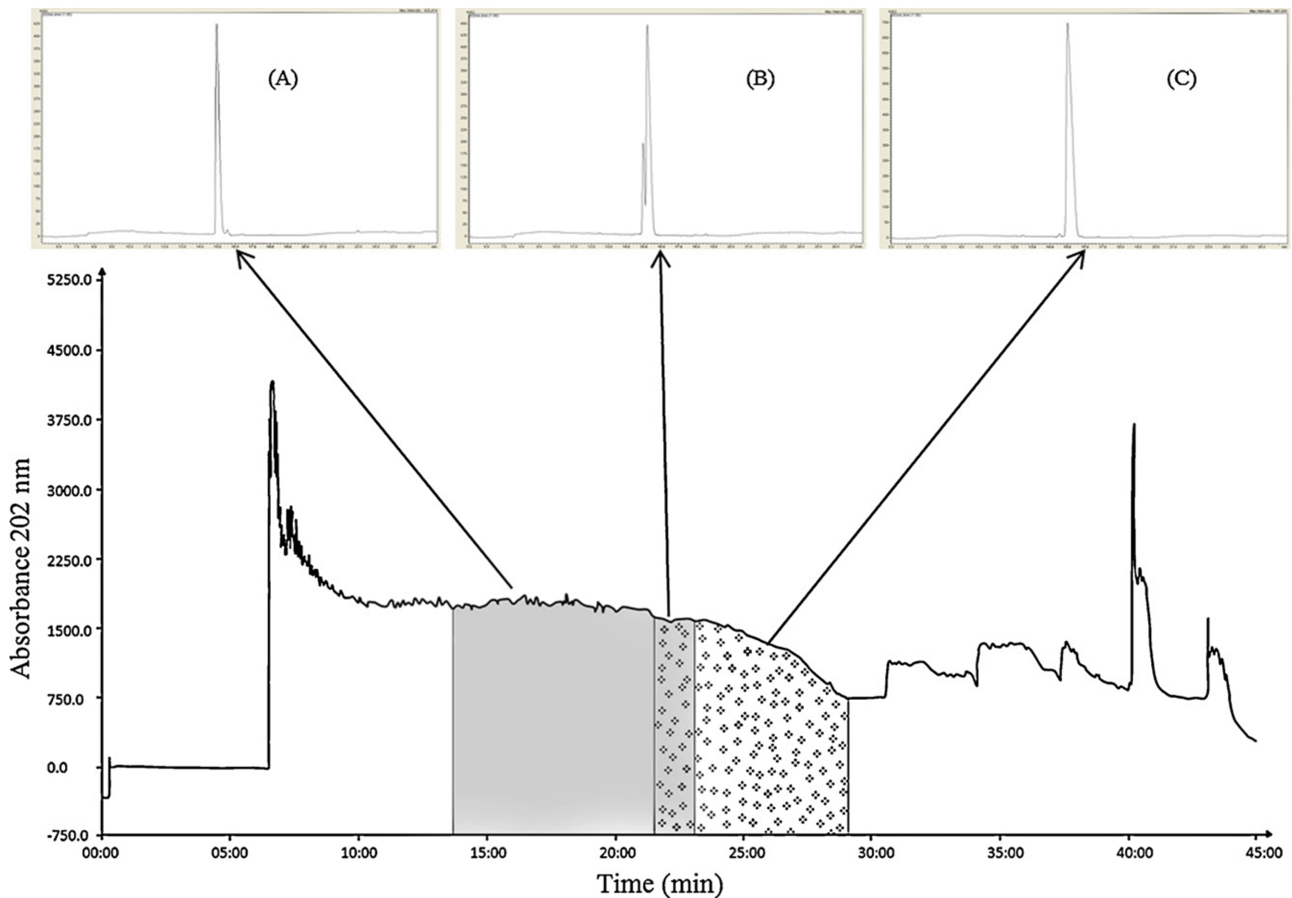

Fig. 2. CPC chromatogram and HPLC control [(A) α-chaconine, (B) α-solanine and α-chaconine, (C) α-solanine] for the separation of crude extract from *S. tuberosum* (cv. Pompadour) sprouts.

each fraction, analysis of HPLC calibration curves allowed to determine the amounts of each of the two glycoalkaloids (Fig. 3) as well as their purity (Table 2). Table 2 also presents for all the fractions, the yield of each compound calculated on the mass of the crude extract as well as the value of the cumulative yield throughout the purification process.

From these results, it appears that α-chaconine was easily isolated under the conditions used with a global yield of 1.92%. Moreover, four of the five fractions collected showed very high purities. On the other hand, the isolation of α-solanine was less effective, the amount recovered was less important (yield 0.53%) and only three fractions had a purity greater than 90%. To support this difference observed for the two compounds, the efficiency of the purification process can be calculated on the basis of the values obtained by analytical HPLC on the crude extract. 54 mg of α-chaconine (corresponding to F8 to F13 excepted F10) were obtained while an amount of 74 mg was expected resulting in an

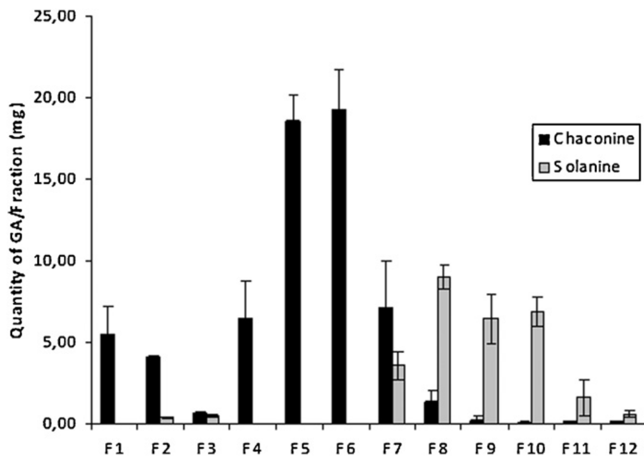

Fig. 3. α-Chaconine and α-solanine contents of the 12 CPC fractions as determined by HPLC analysis. The values represent the means ± S.D. of three independent experiments.

Table 2
α-Chaconine and α-solanine amounts isolated from the fractionation of the crude extract (2.8 g) of *Solanum tuberosum* (cv. Pompadour) sprouts by CPC.

Fraction	Purity[a] (%)	Yield[b,c] (%)	Cumulated yield (%)
F8	100.0	0.20[b]	–
F9	91.9	0.15[b]	0.35
F10	58.8	–	–
F11	100.0	0.23[b]	0.58
F12	100.0	0.66[b]	1.24
F13	100.0	0.68[b]	1.92[b]
F14	66.3	–	–
F15	87.0	–	–
F16	95.8	0.23[c]	–
F17	98.4	0.24[c]	0.47
F18	93.0	0.06[c]	0.53[c]
F19	78.3	–	–

[a] Purity based on HPLC results.
[b] Yields of α-chaconine based on the weight of the crude extract for the fractions of purity above 90%.
[c] α-Solanine based on the weight of the crude extract for the fractions of purity above 90%.

Table 3
Experimental results obtained in CPC separation estimated on the basis of HPLC results of Fig. 3 (see Section 2).

	α-Chaconine	α-Solanine
V_R (HPLC) (ml)	240	285
Peak widths W (ml)	90	75
K_C calculated preliminary in vials	0.77	1.25
K_C observed during the run	3.54	4.36

isolation efficiency of 73%. On the other hand, 15 mg of α-solanine were isolated on an expected quantity of 34 mg giving an efficiency of only 44%. We saw before in the introduction that studies to isolate GAs from plant material are often unique studies and it is therefore difficult to have comparison points, however our results are quite satisfactory within the context of the isolation of natural products. Indeed, this result may be related to the work of Soulé et al. [10] who have isolated only about 86 mg α-chaconine from 1 kg of potato peel.

Under the conditions of CPC purification used, we never observed usual chromatographic peaks but chromatograms similar to those obtained during the implementation of pH-zone-refining CPC [14]. To evaluate the parameters of the GAs purification process, we estimated that the HPLC contents shown in Fig. 3 had a shape that could be considered in a first approximation as a Gaussian peak. From this hypothesis, we were able to evaluate the retention volumes as well as the peak width at the baseline for each of the two GAs. Based on these results, some parameters which allow to get a first overview of the performance of the process were calculated (Table 3).

For efficient CPC separation, the K_C of the target compounds should lie in the range $0.5 < K_C < 2.0$. Moreover, the separation factor α between any two components should be greater than 1.5 [17]. In our case, the α value calculated from the K_C preliminary extraction data set is 1.62, whereas the α value obtained by the estimated values of Table 3 is 1.23.

Given the values of the parameters Sf = 0.55 and $\alpha = 1.62$ a much higher resolution of our peaks was expected [16]. Several explanations can be proposed to explain the shape of the chromatogram. Firstly, the column loading is an important factor. In our separation conditions, we introduced 800 mg of sample on a column of 100 ml, i.e. a loading value of 8 mg/ml which is high compared to the average loading of 2.2 mg/ml observed in the literature and could contribute to produce peak broadening and overlapping [18]. This peak broadening as well as the differences between the α values could also be explained by the particular nature of the compounds that we try to separate. According to Pauli et al. [18], highly concentrated or problematic samples can cause disruptions to the solvent system equilibrium inside a running CC instrument and result in trouble during the separation. The amphiphilic complex nature of our sample can cause a significant stationary phase loss during the run and/or can disturb the mass transfer. In support of this explanation in relation to the chemical structure of the studied compounds, Berthod et al. recently showed a discrepancy between the measured and calculated separation factors during the separation of benzoic acid that could be explained by the specific nature of the compound [19].

4. Conclusion

In this study, CPC was successfully implemented for the isolation of polar and close Rt steroidal alkaloids α-chaconine and α-solanine whereas their separation was almost impossible by semi-preparative HPLC. Despite an unusual form of chromatogram and disruptions related to the nature of their structures, we obtained the alkaloids of interest with good yields and high purity (higher than 93%). These results demonstrate the high effectiveness of this methodology for providing significant amounts of these bioactive glycoalkaloids for further chemical modulations and biological studies, we were thus able to increase the yield from 0.35% to 1.92% for α-chaconine using CPC rather than HPLC. The conditions developed in this study allow to isolate large amounts of complex compounds within a few hours, without any preliminary cleanup or concentrations steps and avoid the problem of poor detection of alkaloids in UV. In addition, a scaling can easily be considered which is quite crucial in our case to achieve our future research objectives.

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