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Application of a new optimization strategy for the separation of tertiary alkaloids extracted from *Strychnos usambarensis* leaves☆

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ABSTRACT

The HPLC separation of six alkaloids extracted from *Strychnos usambarensis* leaves has been developed and optimized by means of a powerful methodology for modelling chromatographic responses, based on three steps, i.e. design of experiments (DoE), independent component analysis (ICA) and design space (DS). This study was the first application of a new optimization strategy to a complex natural matrix. The compounds separated are the isomers isostrychnopentamine and strychnopentamine, 10-hydroxyusambarine and 11-hydroxyusambarine, also strychnophylline and strychnofoline. Three LC parameters have been optimized using a multifactorial design comprising 29 experiments that includes 2 center point replicates. The parameters were the percentage of organic modifiers used at the beginning of a gradient profile which consisted in different proportions of methanol (MeOH) and acetonitrile (MeCN), the gradient time to reach 70% of organic modifiers starting from the initial percentage and the percentage of MeCN found in the mobile phase. Subsequent to the experimental design application, predictive multilinear models were developed and used in order to provide optimal analytical conditions. The optimum assay conditions were: methanol/acetonitrile-sodium pentane sulfonate (pH 2.2; 7.5 mM) (33.4:66.6, v/v) at a mobile phase flow rate of 1 ml/min during a 40.6 min gradient time. The initial organic phase contained 3.7% MeCN and 96.3% MeOH. The method showed good agreement between the experimental data and predictive value throughout the studied parameters space. Improvement of the analysis time and optimized separation for the compounds of interest was possible due to the original and powerful tools applied. Finally, this study permitted the acquisition of isomers profiles allowing the identification of the optimal collecting period of *S. usambarensis*.

1. Introduction

Malaria still remains the major parasitic infection, affecting hundreds of countries around the world, especially tropical regions in Africa, Asia and Latin America. The impact of the disease in terms of morbidity and mortality, as well as the ever increasing emergence of resistance to currently used treatments enhances the importance of developing new drugs. The plant world represents a huge, varied, promising and a countless source of new potential therapeutic substances.

1.1. Alkaloids from Strychnos usambarensis leaves

Our study concerns *S. usambarensis* Gilg (Loganiaceae). This small tree growing widely in Eastern Africa, particularly in Rwanda, is used in traditional pharmacopoeias. It is well known not only for its use as arrow poison due to quaternary curarizing alkaloids contained in the roots [1,2] but also for its tertiary alkaloids isolated from leaves because some of them possess antiplasmodial [3] and cytotoxic properties [4–7]. These activities could be related to their original structure: they are all asymmetrical monoterpenoid bisindolic usambarane-type alkaloids. The major compounds are isostrychnopentamine (Fig. 1A) and its epimer strychnopentamine (Fig. 1B), 10-hydroxyusambarine (Fig. 1C) and its position isomer 11-hydroxyusambrine (Fig. 1D), strychnophylline (Fig. 1E),

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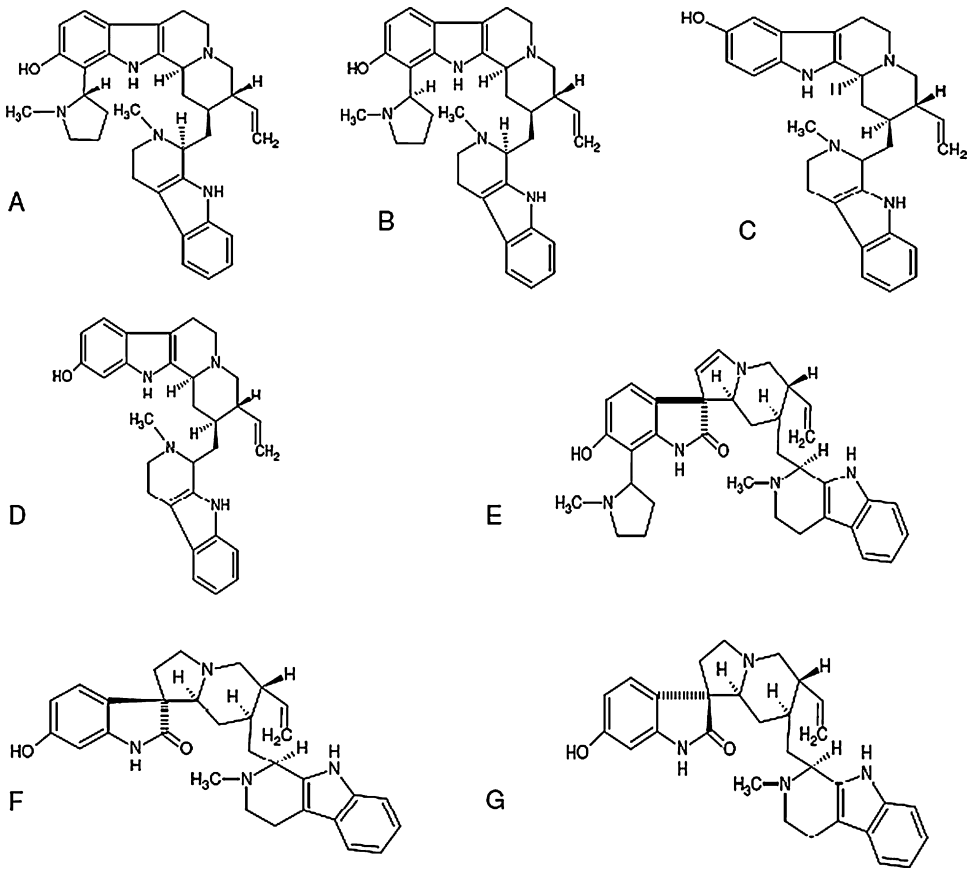

Fig. 1. Chemical structures of (A) isostrychnopentamine, (B) strychnopentamine, (C) 10-hydroxyusambarine, (D) 11-hydroxyusambarine, (E) strychnophylline, (F) strychnofoline and (G) isostrychnofoline.

strychnofoline (Fig. 1F) and its epimer isostrychnofoline (Fig. 1G). From a therapeutically point of view, isostrychnopentamine (ISP), and to a lesser extent, its isomer strychnopentamine (SP) seem to be the most promising substances [8]. ISP proved to be active *in vitro* and *in vivo* against *Plasmodium falciparum* with under μM concentrations [9]. In the recent years, ISP also showed a potential anti-tumor activity against apoptosis-resistant cancer cells [10,11].

1.2. Analytical and biostatistical methods

To our knowledge, this work is one of the first studies dedicated to the chromatographic separation of these tertiary alkaloids extracted from *S. usambarensis* leaves, representing also the first application of a new optimization strategy to a complex natural matrix. Considering the complexity of the plant material, the difficulty lies in finding a method that could separate and identify all the structurally similar compounds (epimers and isomers). Two LC procedures have been described in the 1990s about alkaloids separation of *Strychnos* species. The first one exposed the isolation of monomeric indole alkaloids in *Strychnos nux-vomica* and *Strychnos ignatii* seeds [12], and the second one, the separation of bisindolic alkaloids in *S. usambarensis* roots [13]. Both were assessed by reversed-phase chromatography using acetonitrile (MeCN), an ion-pairing reagent (sodium acetate and sodium salt of heptanesulfonic acid, respectively) and phosphate buffer (pH ~3.00).

The goals of the present study were to develop and optimize HPLC conditions for the separation of six target alkaloids from *S. usambarensis* leaves that are strychnopentamine, isostrychnopentamine, 10-hydroxyusambarine, 11-hydroxyusambarine, strychnophylline and strychnofoline, respectively. To achieve this, three methodologies were combined. Firstly, design of experiments (DoE) was implemented to gather experimental data in order to achieve statistical modelling. The major advantage of using design of experiments to develop this method is that it allows all potential factors to be evaluated concurrently, systematically and quickly [14–16]. Secondly, a design space was built over the design of experiment domain to simultaneously optimize the chromatographic method separation and assess the robustness of its future use [17–19]. Finally, independent component analysis methodology was used to facilitate peaks detection and identification even for co-eluted peaks [20]. Another aim was to reduce analysis cost using methanol (MeOH) as organic modifier rather than MeCN because at the time of the study, the market presented itself with a paucity regarding the last one.

Ion-pairing chromatography was used in this study as it is an efficient strategy to control the retention of protonised bases by reversed phase liquid chromatography (RP-LC) [21–24]. Additionally this new method showed to be precise and suitable to qualitatively identify different samples of *S. usambarensis* leaves collected over a period of three years in various seasons.

2. Materials and methods

2.1. Chemicals

Orthophosphoric acid 85% (pro analysis), sodium carbonate, dichloromethane, methanol and acetonitrile of HPLC-grade were obtained from Merck (Darmstadt, Germany). The 1-pentanesulfonic acid (sodium salt monohydrate 99%) was purchased from Acros Organics (Geel, Belgium). The ultra pure water

was obtained with a Milli-Q system (Millipore Plus 185, Billerica, MA, USA).

2.2. Plant material

All five *S. usambarensis* leaves batches were collected by two of the authors (M.F. and L.A.) in Akagera National Park, Rwanda at different periods of time (June 2007, November 2007, August 2008 and 2009, February 2010). They were quickly air-dried in Rwanda before storage in Belgium at a temperature of 15 °C. A voucher specimen of the plant was deposited in the herbarium of the National Botanical Garden of Belgium at Meise.

2.3. Extraction of the tertiary alkaloids from S. usambarensis leaves

For each batch of leaves, powdered dried material was macerated in sodium carbonate and then percolated with distilled ethyl acetate until total extraction of the alkaloids. The extract was acidified by acetic acid 1% and washed by distilled dichloromethane to remove chlorophyll and pigments. The resulting acidic solution was basified to pH 8.0 using sodium carbonate and repeatedly extracted with distilled dichloromethane. The organic solvent was dried over sodium sulfate and concentrated to yield the alkaloid extract.

2.4. HPLC alkaloids solutions

For each sample, approximately 10 mg of the alkaloid extract was precisely weighed and dissolved in 1 ml of mobile phase, specifically a mixture of MeOH/MeCN (96.3:3.7, v/v) and aqueous solution of pentane sulfonate (7.5 mM) (33.4:66.6, v/v) adjusted to pH 2.2 with orthophosphoric acid (0.5%). These solutions were then filtered through a 0.45 μm HVLP filtration membrane into vials for injection in the HPLC system.

2.5. Instruments and conditions

The HPLC system used was a Waters 2695 separation module coupled to a Waters 996 Photodiode array detector. The chromatographic separation was performed on a LiChrospher® 60 RP-select B column (250 mm × 4 mm i.d.; particle size 5 μm) from Merck. The UV detector wavelength was set at 273 nm for the entire study. Temperatures of the column and of the samples were set at 25 °C and 10 °C, respectively. Injections of 5 μl of the alkaloids extracts were accomplished. pH measurements were performed with a SevenEasy Mettler-Toledo pH meter (Schwerzenbach, Switzerland) on aqueous eluent component before the addition of the organic modifier.

In the optimized procedure, the mobile phase used for the separation was a 33.4:66.6 (v/v) mixture of an organic phase and an aqueous solution of sodium pentane sulfonate (7.5 mM) adjusted with orthophosphoric acid (0.5%) to pH 2.2. The organic phase, containing 3.7% MeCN and 96.3% MeOH was delivered at a flow rate of 1.0 ml/min during a 40.6 min gradient time using gradient profile.

2.6. Design of experiments, independent component analysis and design space

To define the DoE only the factors that have a significant effect over the separation had to be selected as well as establishing their minimal and maximal limits. The DoE to be selected had to allow the modelling of response surfaces of individual analytes from a complex mixture applying a limited number of experiments in order to find the optimal analytical conditions [25]. Among the classes of experimental designs that are appropriate for obtaining data that will permit the estimation of the coefficients, of all main effects and interactions are the full factorial designs. A full factorial design (3^3) was selected for this study given the complexity of the mixture and the lack of a priori data on the compounds chromatographic behaviour. It was also employed in order to enable a better understanding of the chromatographic separation and to allow estimation of interactions and quadratic effects. The three factors selected as well as their three levels are given in Table 1. They are:

Table 1
Factors and levels selected for the full factorial design. $\%OM_{initial}$: initial percentage of organic modifiers mixture of the mobile phase; T_G: gradient time; %MeCN: percentage of acetonitrile added into the organic modifiers mixture of the mobile phase.

Facteurs	Niveaux		
$OM_{initial}$ (%)	25	35	45
T_G (min)	25	35	45
MeCN (%)	2.5	7.5	12.5

- The percentage of organic modifiers mixture used at the beginning of the gradient profile consisting of different proportions of MeOH and MeCN.
- The gradient time to reach 70% of organic modifier starting from the initial percentage.
- The percentage of MeCN found in the mobile phase.

This full factorial design leads to 29 experiments including 2 additional independent repetitions at the center of the experimental domain (leading therefore to a total of 3 center points).

The chromatograms obtained from the realisation of the 3^3 full factorial design, allowed peaks to be detected and indexed at their beginnings (t_B), apexes (t_A) and ends (t_E) which where then transformed in retention factors in order to predict the chromatographic behaviour of the compounds following the methodology proposed by Lebrun et al. [17,26]. In order to model the logarithm of the retention factors, multiple linear equations have been used which enable the computation and optimization of the separation factor S [17–20]. The optimization criterion considered in this study is the separation factor S, firstly because it is a natural criterion and secondly, its value and its associated error are easily computable [17,27,28]. The separation, S, represents the difference between the retention time at the beginning (t_B) of the second peak and the retention time at the end (t_E) of the first peak of the critical pair. Independent component analysis (ICA) is a blind source separation method that could be used to automate the way the peaks are indexed and identified [29,30].

Design space can be defined as the robust zone where the quality of the method separation is kept in spite of small environmental variations and this region for method robustness can also be envisioned as the space within the experimental domain (also known as the knowledge space) where the critical separation S is at least 0 min. The design space is defined by the set of all factors where the predicted probability that the separation S is higher than 0 min must be higher than a certain quality level (π), given the uncertainty of the estimation of model parameters θ [17–20]. In other words, the design space defines a region of experimental conditions where $S \geq 0$ min is obtained at least π times out of one hundred (e.g. 80 times out of 100).

2.7. Software

JMP v 8.0.2 is the statistical software that provided the technologies and algorithms for building and deploying the DoE method applied in this study.

The statistical analysis of the data was performed by means of an in-house computer program. The coding used was based on R

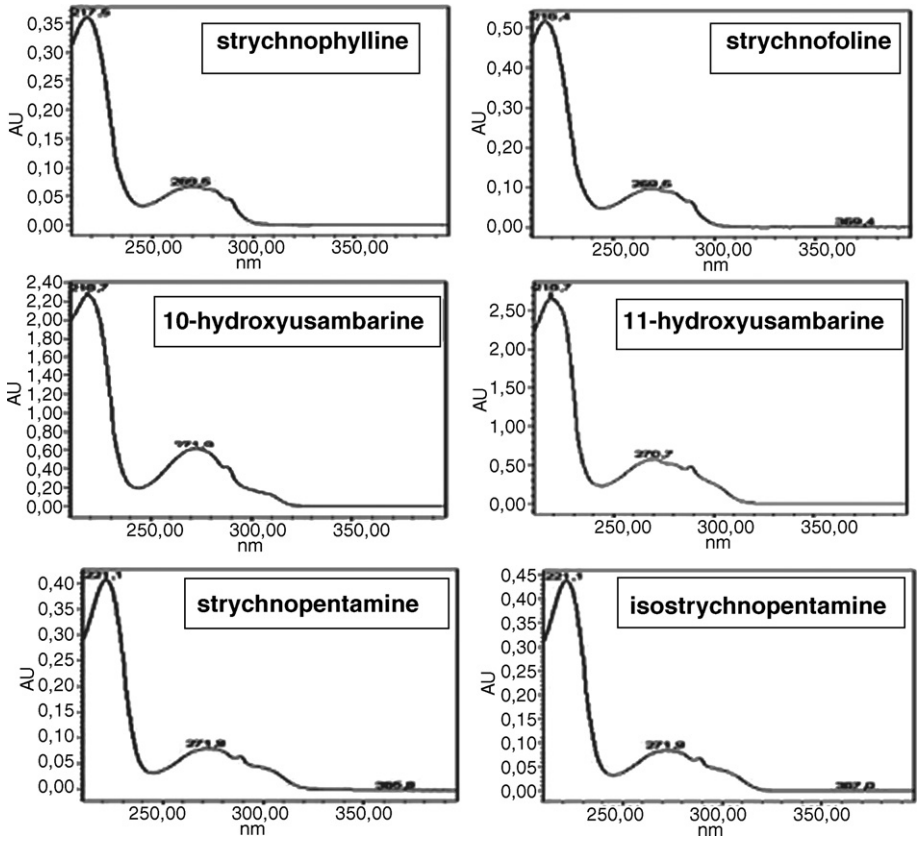

Fig. 2. UV spectra of studied alkaloids.

2.12 statistical language [31] for Windows which is open access and available at http://www.r-project.org.

3. Results and discussion

3.1. Experimental design

The full factorial design employed to optimize the separation, also assisted the development of a better understanding of the interaction of the selected chromatographic factors on separation quality. The three HPLC factors chosen to be optimized were selected based on their known strong influence on selectivity, empirical preliminary experiments and certain instrumental limitations. For instance, given the fact that work on *S. usambarensis* was a joint study, there was need for a column available in both laboratories that could additionally authorize a preparative liquid chromatography transfer, which in this case was represented by LiChrospher® 60 RP-select B. Also the experiments were conducted at a temperature of 25 °C and a pH 2.2 which assured compounds stability. Instead of heptane sulfonate [12], sodium pentane sulfonate was chosen as the ion-pairing agent because of its short alkyl chain that forms an ion-pair complex which is less hydrophobic resulting to a smaller retention time. From preliminary experiments, a small percentage of MeCN was added to the mobile phase because its presence improved the shape of peaks, thus helped reduce the costs of analysis, especially due to the actual shortage and high price of MeCN, in a potential preparative context.

Hence, the three parameters selected for the optimization process were gradient time, percentage of organic modifier and percentage of MeCN in the mobile phase composition. Table 1 shows the established ranges over which the chromatographic factors were to be varied, as follows: percentage of initial organic modifiers mixture (25–45%), gradient time (25–45 min) and percentage of MeCN in the mobile phase (2.5–12.5%).

The randomized sequence, in which the 29 experiments (including the 2 additional independent repetitions at the center of the experimental domain) were performed, helps minimize the effect of environmental variables that can introduce a bias on the measurements. Replicates of the central point, that were carried out using freshly prepared buffer, offer the possibility of calculating the independent estimate of the experimental error so that lack-of-fit and the statistical significance of the factors effects could be tested. Additionally, these 3 independent repetitions allowed for an overall test of quadratic effects.

3.2. Optimization analysis – ICA

Given the similarity of the UV-spectra for all 6 compounds of interest shown in Fig. 2, their almost identical chromatographic behaviour as well as the great number of potential endogenous compounds interfering with the 6 peaks of interest, the identification becomes very delicate. At this stage ICA is usually an interesting tool because it allows a numerical separation for the co-eluted peaks and refines the estimation of the peak retention time at the beginning, apex and end. However in this case, the contribution of ICA is not very significant mostly because of the similarity of UV-spectra of the compounds of interest. Nonetheless, ICA was useful for the unknown endogenous compounds.

Once all the chromatograms were obtained from the operational chromatographic conditions, the peaks were manually detected and indexed at the beginning, apex and end of each retention time. The logarithm of the retention factor was modelled employing multiple linear equations in order to enable the computation, the

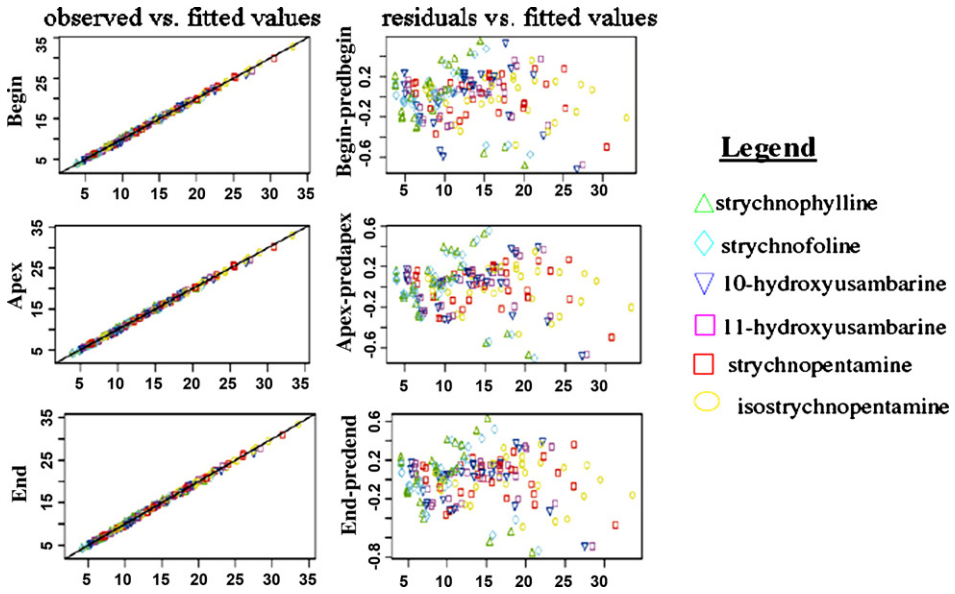

Fig. 3. Actual original responses of retention times (begin, apex and end) versus predicted one (predapex). On the right are the residuals.

optimization of the separation and to create multivariate responses surface model [20]. The importance of checking the fit quality of a model is stressed here because of its strong correlation with the poor accuracy of the prediction of the retention times. In order to asses the quality of the modelling, the observed values and the predicted ones for the beginning, apex and end were compared. A good correlation between these values was observed meaning that the data is well fitted (all $R^2 > 0.998$). To illustrate this, Fig. 3 presents the scatter plots of the observed and predicted retention times of peaks.

The residuals which represent the differences between the predicted output from the model and the measured output from the data set are in a range of [−0.8; 0.6]. And also a normal distribution of residuals was noted as observed in Fig. 3 which shows that the model chosen provided a good explanation of the data.

The optimization criterion considered in this study was the separation factor S which is defined as the difference between the beginning of a peak and the end of the precedent peak. The propagation of the predictive error through the criterion was analyzed to offer confidence for the optima as proposed by Lebrun et al. [17]. In order to asses the robustness, the probability to attain a separation superior or equal to 0 was calculated by means of Monte Carlo simulations and the response surfaces obtained were then replaced by probability surfaces in order to find a region of $S \geq 0$ [18].

3.3. Design space computation

Considering the aforementioned conditions, the design space corresponds to the region where this probability is superior to a preset quality level (π), in this case $\pi = 30\%$. The expected probability to have well-separated peaks at the optimal condition is $\pi = 45\%$ as illustrated in Fig. 4. This design space also corresponds to a robustness zone in accordance with ICH Q8 [32] guidelines. Working within this robust zone defined in the experimental domain is convenient because it is not considered as a modification in the analytical method.

Despite the relatively poor design space probability (30%), a good agreement between the predicted chromatogram and the experimental chromatogram was observed. Indeed, a comparison between the predicted and the real processed chromatogram at the optimum conditions is illustrated in Fig. 5. The chromatogram recorded at the optimum conditions is obtained by employing a 33.4:66.6 (v/v) mixture of organic phase and an aqueous solution of sodium pentane sulfonate (7.5 mM) adjusted with orthophosphoric acid (0.5%) to pH 2.2. The initial organic phase was a mixture (3.7% MeCN and 96.3%) delivered at a flow rate of 1.0 ml/min during a 40.6 min gradient time using a gradient profile. The chromatogram obtained at the optimum conditions illustrated in Fig. 6A was also compared to the initial chromatogram shown in Fig. 6B obtained based on a previous publication [12] and recorded before the application of experimental design and

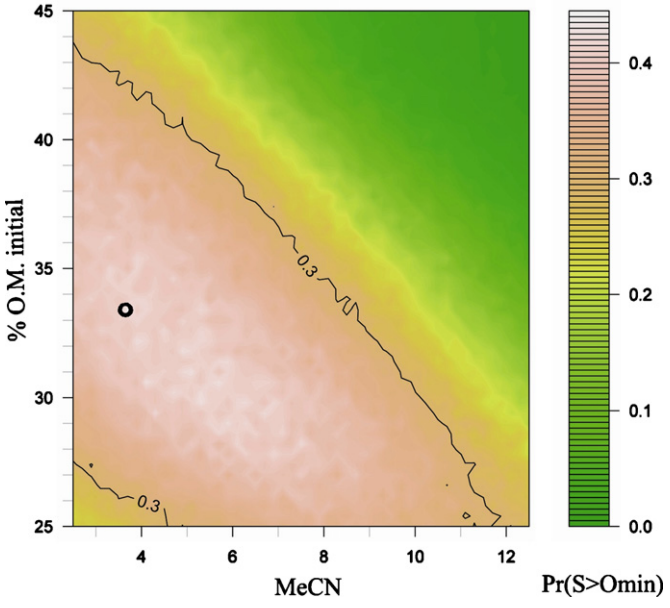

Fig. 4. Representation of the design space of the method on the experimental domain. Probability surface for the separation criterion $S > 0$ Pr($S > 0$ min) with the design space ($\pi = 0.3$ or 30%) included inside the black lines where the expected probability to have well-separated peaks at the optimal condition is $\pi = 45\%$. The black circle represents the optimal condition: gradient time of 40.6 min, proportion of MeCN in the organic modifiers ($\%OM_{initial}$) mixture of 3.7% and proportion of the organic modifiers mixture in the mobile phase of 33.4%.

Table 2
Variation of the extracts concentration (expressed as percentage) of the 6 compounds of interest during a 3 years period. June 2007 content was arbitrarily chosen as reference.

Period	Percentage					
	Strychnofoline	Strychnophylline	10-Hydroxyusambarine	11-Hydroxyusambarine	Strychnopentamine	Isostrychnopentamine
June 2007	100	100	100	100	100	100
November 2007	202.6	136.3	86.5	223.3	23.3	78.5
August 2008	103.7	101.7	235.6	2337.7	188.2	121.8
August 2009	166.1	105.9	241.4	2121.2	225.2	120.5
February 2010	389.1	274.7	283.7	1086.8	103.8	42.1

design space approach. The chromatogram illustrated in Fig. 6B was assessed by reversed-phase chromatography using acetonitrile (MeCN), an ion-pairing reagent (sodium salt of heptanesulfonic acid) (1 g in 420 ml) and fitted at pH 3.2 with phosphoric acid (0.5%). Firstly, improvement of separation for the isomers of interest (strychnopentamine and isostrychnopentamine), of the analysis time and of the peak shape quality was noted. Secondly, an increase in the separation is also noticed regarding the other two pairs of isomers that are 10-hydroxyusambrine–11-hydroxyusambrine (R_S = 1.5) and strychnophylline–strychnofoline (R_S = 4.3).

3.4. Qualitative comparative phytochemical characterization

The optimized HPLC-UV method was then used to compare the potential differences of its phytochemical constituents over the collection periods of plant samples. As shown in Table 2 where the lot of June 2007 was arbitrarily chosen as percentage reference, *S. usambarensis* displayed differences in the concentration of its phytochemical constituents according to the period it was collected. Chromatograms showed important differences between the analyzed samples with an emphasis on isostrychnopentamine and strychnopentamine as illustrated in Fig. 7A–E.

The *S. usambarensis* samples collected in August and in February showed the highest content of hydroxylated derivatives: 10- and 11-hydroxyusambarine. Comparing the various periods under study, the highest concentration in isostrychnopentamine was obtained for the month of August, which is also equal to the concentration of its isomer, strychnopentamine SP. However, for the month of November, there was a marked difference in concentration between ISP and SP, as well as between 10-hydroxyusambarine and its 11-hydroxy isomer. Consequently, it is reasonable to

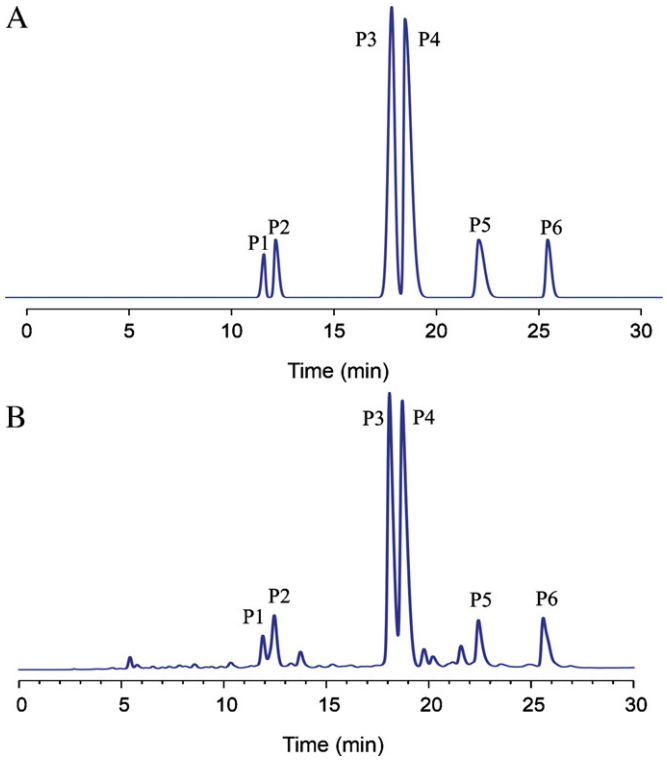

Fig. 5. Comparison between the predicted chromatogram (A) and the observed chromatogram (B) at the optimal condition obtained by employing a 33.4:66.6 (v/v) mixture of organic phase and an aqueous solution of sodium pentane sulfonate (7.5 mM) adjusted with orthophosphoric acid (0.5%) to pH 2.2; the initial organic phase was a mixture (3.7% MeCN and 96.3%) delivered at a flow rate of 1.0 ml/min during a 40.6 min gradient time using a gradient profile. P1: strychnophylline, P2: strychnofoline, P3: 10-hydroxyusambarine, P4: 11-hydroxyusambarine, P5: strychnopentamine and P6: isostrychnopentamine.

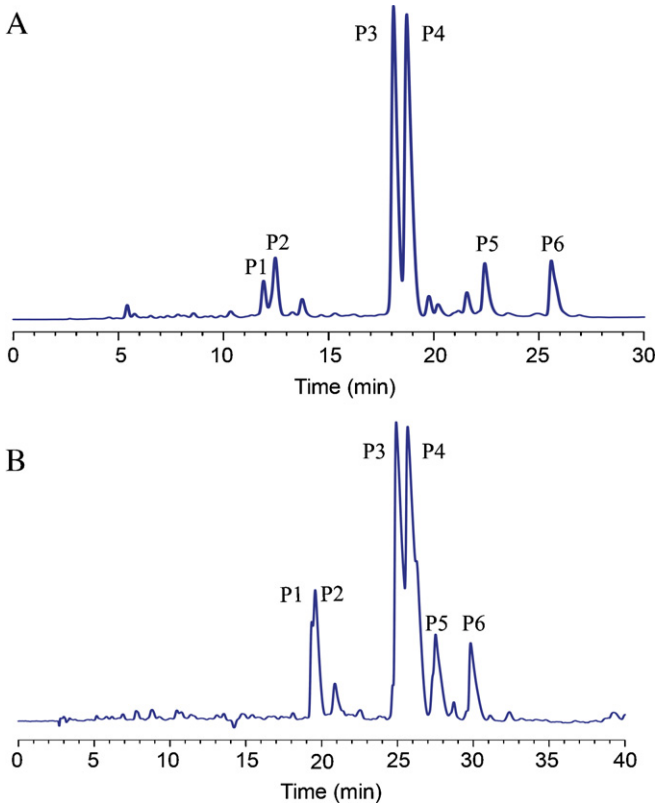

Fig. 6. Comparison between the observed chromatogram at the optimal condition obtained by employing a 33.4:66.6 (v/v) mixture of organic phase and an aqueous solution of sodium pentane sulfonate (7.5 mM) adjusted with orthophosphoric acid (0.5%) to pH 2.2; the initial organic phase was a mixture (3.7% MeCN and 96.3%) delivered at a flow rate of 1.0 ml/min during a 40.6 min gradient time using a gradient profile (A) and the one at the initial conditions based on acetonitrile (MeCN), aqueous solution of sodium salt of heptanesulfonic acid (1 g in 420 ml) and fitted at pH 3.2 with phosphoric acid (0.5%), delivered at 1.0 ml/min (B). P1: strychnophylline, P2: strychnofoline, P3: 10-hydroxyusambarine, P4: 11-hydroxyusambarine, P5: strychnopentamine and P6: isostrychnopentamine.

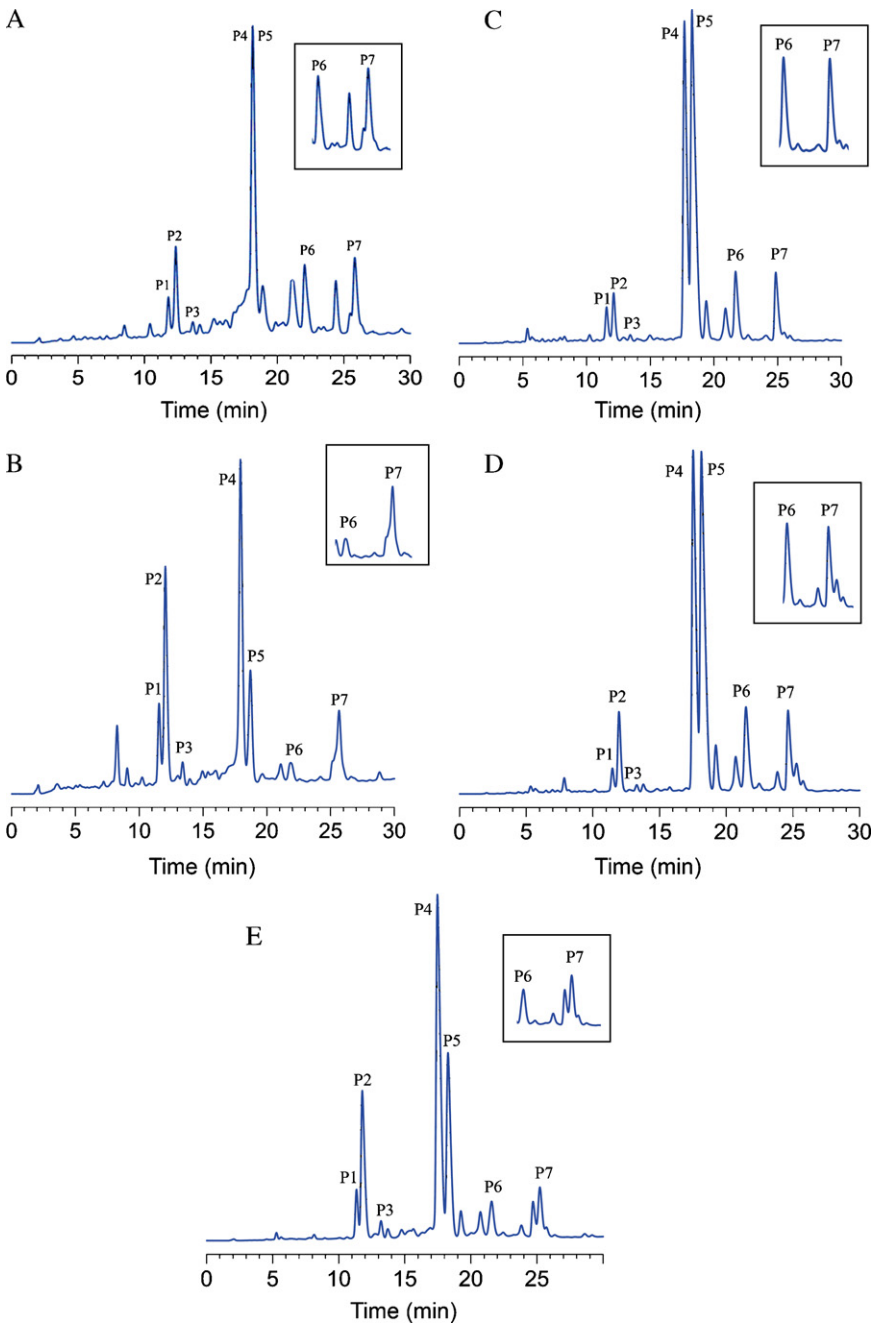

Fig. 7. (A) Chromatogram obtained for the period of June 2007 (P1: isostrychnofoline, P2: strychnofoline, P3: strychnophylline, P4: 10-hydroxyusambarine, P5: 11-hydroxyusambarine, P6: isostrychnopentamine and P7: strychnopentamine). (B) Chromatogram obtained for the period of November 2007 (P1: isostrychnofoline, P2: strychnofoline, P3: strychnophylline, P4: 10-hydroxyusambarine, P5: 11-hydroxyusambarine, P6: isostrychnopentamine and P7: strychnopentamine). (C) Chromatogram obtained for the period of August 2008 (P1: isostrychnofoline, P2: strychnofoline, P3: strychnophylline, P4: 10-hydroxyusambarine, P5: 11-hydroxyusambarine, P6: isostrychnopentamine and P7: strychnopentamine). (D) Chromatogram obtained for the period of August 2009 (P1: isostrychnofoline, P2: strychnofoline, P3: strychnophylline, P4: 10-hydroxyusambarine, P5: 11-hydroxyusambarine, P6: isostrychnopentamine and P7: strychnopentamine). (7) Chromatogram obtained for the period of February 2010 (P1: isostrychnofoline, P2: strychnofoline, P3: strychnophylline, P4: 10-hydroxyusambarine, P5: 11-hydroxyusambarine, P6: isostrychnopentamine and P7: strychnopentamine).

conclude that during the dry season the specie is a much richer source of alkaloids of interest than in the rainy season.

4. Conclusions

A HPLC method developed using a new optimization strategy was used for the separation of tertiary alkaloids extracted from *S. usambarensis* leaves. The methodology involves three steps: design of experiments (DoE), independent component analysis (ICA) and design space (DS) which allowed finding the robust analytical conditions where the separation meet the desired requirements. This was the first time that this strategy was applied to a complex matrix.

The partial replacement of acetonitrile by methanol was successful, allowing reducing costs and maintaining a high quality of results in respect with the ICH Q8 guidelines and the design space approach.

An optimal separation was achieved especially for strychnopentamine, isostrychnopentamine, 10-hydroxyusambarine and 11-hydroxyusambarine. A good separation was also obtained for

another pair of isomers that is represented by strychnophylline and strychnofoline, succeeding so to acquire for the first time, to our knowledge, a LC-UV profile for the compounds of interest.

In addition, the method resulting from the strategy of simultaneous multifactorial optimization reduced overall assay development time and provided information regarding separation and sensitivity due to the detection of new compounds in the analyzed mixture of plant origin, compounds whose separation and identification are still delicate.

This study has also allowed the identification of the highest concentration period for alkaloids which present the most interesting pharmacological activity.

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