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An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE)☆

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ABSTRACT

The simultaneous quantification of madecassoside, asiaticoside, madecassic acid and asiatic acid in *Centella asiatica* by HPLC-UV is proposed. Asiaticoside was used as reference for the quantification of herosides and asiatic acid for aglycones. The evaluation of the extraction efficiency of the four molecules led to use Soxhlet extraction for 8 h. The method was validated and was found to be accurate in the concentration range of 1.0–3.0 mg/ml for asiaticoside and 0.5–2.0 mg/ml for asiatic acid with CV <3% for all investigated compounds. LOD and LOQ were, respectively, 0.0113 and 1.0 mg/ml for asiaticoside and 0.0023 and 0.5 mg/ml for asiatic acid. This method was shown to be convenient for routine analysis of samples of *C. asiatica*.

1. Introduction

Centella asiatica (APIACEAE) is an ethnomedicinal herbaceous species, originated from India which grows spontaneously in subtropical regions: China, Malaysia, Australia, America, South Africa and Madagascar. In Madagascar, the plant is largely used by the local population and is the second medicinal species exported [1]. *C. asiatica* is claimed to have a number of medicinal properties and is used in Ayurvedic medicine for the treatment of leprosy, skin tuberculosis, wound healing, stomach aches, arthritis, varicose veins, high blood pressure and as a memory enhancer [2]. Recently, several studies demonstrated that extracts of the plant possess antioxidant activity [3,4], have antiproliferative effects in tumor cells [5], improve venous wall alterations in chronic venous hypertension and protect the venous endothelium [6]. Asiaticoside, one of its active molecules, is reported to cause changes in gene expression and to induce type I collagen synthesis in human fibroblasts [7–9]. Madecassoside was reported to have an anti-rheumatoid effect and wound healing properties [10,11].

Triterpenoids of *C. asiatica* (Fig. 1) are components of medicinal drugs and are much used in cosmetic preparations for skin care [12,13]. Collected during all year long, considerable differences of the triterpenoid contents were observed according to geographic regions, phenotype and genotype [14,15], so the assessment of a validated analytical method is necessary. This will also help local population to determine the best cultivating and harvesting conditions.

Most studies led on *C. asiatica* report the quantification of herosides [16,17], acids [18] or acids and heterosides but with insufficiently validated methods [14,15,19–21]. Several reported methods were based on HPLC differing only in the mobile phase composition or in the detection system. Xingyi et al. [16] proposed a method only dedicated for the quantification of madecassoside and asiaticoside using acetonitrile/water (29/17, v/v) in isocratic mode. Quantification of only madecassoside, asiaticoside and its isomer has been reported by Zhang et al. [17] using ELSD detector. An addition of β-cyclodextrin in the mobile phase was used for the quantification of the sole madecassic acid [18]. The existing methods for the simultaneous quantification of madecassoside, asiaticoside, madecassic acid and asiatic acid used either acetonitrile/water [19], acetonitrile/water with TFA 0.1% [20] or acetonitrile/water each containing 0.05% of H_3PO_4 as mobile phase [21], together with detection at 205 nm. Most methods did not give good resolution or were not suitable for LC–MS and all were insufficiently validated [19–21]. The monography of the European Pharmacopeia [22] quantifies total triterpenoids (Fig. 2a) but our attempts to reproduce the separa-

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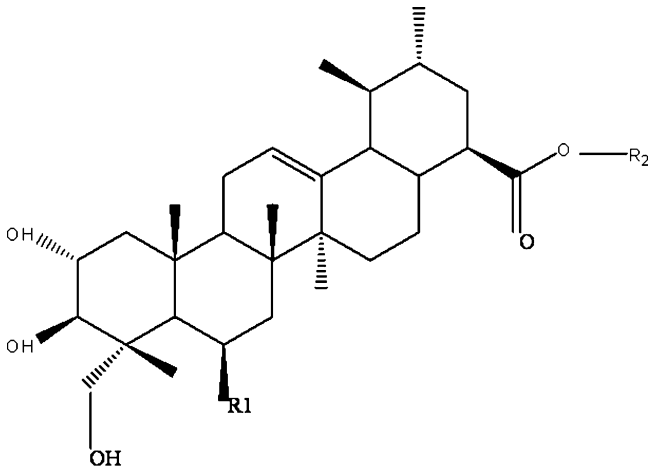

madecassoside	**R_1= OH**	**R_2 = Glu-Glu-Rha**
asiaticoside	**R_1= H**	**R_2 = Glu-Glu-Rha**
madecassic acid	**R_1= OH**	**R_2 = H**
asiatic acid	**R_1= H**	**R_2 = H**

Fig. 1. Structure of triterpenes of *Centella asiatica* (Glu: glucose, Rha: rhamnose).

tion did not allow us to quantify precisely these compounds. Only the peak corresponding to madecassoside was clearly identified, asiaticoside and the two aglycones eluted during the washing part of the gradient and were not distinguished (Fig. 2b). This was also observed by the European Pharmacopeia expert group in which the monography is under revision.

The main objective of this work is thus to improve the existing methods based on HPLC-UV and validate it for the simultaneous quantitation of madecassoside, asiaticoside and their aglycones, madecassic and asiatic acids in *C. asiatica*.

2. Experimental

2.1. Chemicals and plant material

Asiaticoside (99.2%, HPLC), madecassoside (97.94%, HPLC), asiatic acid (99%, HPLC) and madecassic acid (95%, HPLC) were purchased from Extrasynthese (Genay, France). Acetonitrile and methanol HPLC grade were from Prolabo, VWR (Leuven, Belgium).

Fresh leaves of *C. asiatica* (L.) Urban were collected in December 2007 and January 2008 at the East and High Plateau regions of Madagascar. Leaves were separated from stems, dried at 40 °C, powdered and sifted with a sieve of 355 μm meshes [22]. The powdered leaves were stored at ambient temperature in obscurity and in a dry area.

One gram dried powdered leaves [22] were extracted by Soxhlet for 8 h with 100 ml of methanol. The extracts were evaporated to dryness under reduced pressure. The dried crude extract was dissolved in 10 ml of methanol, filtered through a 0.45 filter (Whatman, New Jersey, USA).

2.2. Apparatus

The HPLC Waters 2690 separation module (Waters, Milford, MA, USA) used consisted of a pump, an autoinjector, a UV spectrophotometric detector Kromaton (Angers, France), all controlled by Borwin software (Borwin, Rostock, Germany).

For the determination of mass spectra, a LCQ Advantage Thermo Finnigan (Waltham, MA, USA) was used piloted by X-Calibur software.

Table 1
Gradient conditions for HPLC.

Time (Min)	Pump A, water (%)	Pump B, acetonitrile (%)
0	80	20
15	65	35
30	35	65
35	20	80
40	20	80
45	80	20
55	80	20

Chromatographic separation was performed with a reversed phase RP-18 LiChroCART® column (250 mm × 4 mm I.D.; particle size: 5 μm). Mobile phase was a gradient of acetonitrile/water (Table 1), a flow rate of 1 ml/min and detection at 206 nm.

2.3. Standards solutions

Stock solutions of asiaticoside and asiatic acid were prepared in methanol at 5.0 and 2.5 mg/ml, respectively, and stored at 0 °C. Dilution was done for each experiment. Three concentrations (m = 3) of asiaticoside (0.5, 2.5 and 5.0 mg/ml) and of asiatic acid (0.25, 1.0 and 2.5 mg/ml) were used. Each concentration was analyzed two times (n = 2) for 3 days (k = 3).

The extract solution was diluted with methanol (1:5, v/v) for the preparation of the validation standards and spiked with three known concentrations of a stock mixture of asiaticoside and asiatic acid. Each validation standard was analysed three times (n = 3) for 3 days (k = 3).

2.4. Evaluation of the extraction

The extraction kinetic was established by evaluating the peak area (HPLC analysis) of each compound after 4, 6, 8 and 10 h (n = 3). The most appropriate Soxhlet extraction time was determined using these data.

2.5. Validation of the method

All the reference compounds are commercially available but asiaticoside and asiatic acid were selected to achieve the validation of the method and to quantify madecassoside and madecassic acid, respectively, because we observed identical response factors for the two osides and the two aglycones in HPLC-UV (Table 2). Consequently, choosing only two references reduces the cost of the analysis.

As *C. asiatica* is a biological matrix, relatively large acceptance limits are prescribed [23]. Validation of the method was done for 3 days by testing the following criteria: response function, linearity, trueness, precision (repeatability and intermediate precision), accuracy, limits of detection (LOD) and quantification (LOQ), and quantification range.

Statistical analyses of data were done using the e-noval V2.0 (Arlenda-Liège) software.

3. Results and discussions

3.1. Optimization of the extraction

Several extraction modes were proposed such as maceration, sonication or Soxhlet. Soxhlet extraction was selected as it is easier to control. As illustrated in Fig. 3, no significant difference was observed after 8 and 10 h of extraction. Consequently, an extraction time of 8 h was used for all experiments. This result corroborates the European Pharmacopeia findings indicating that the problem observed with the European Pharmacopeia method is due

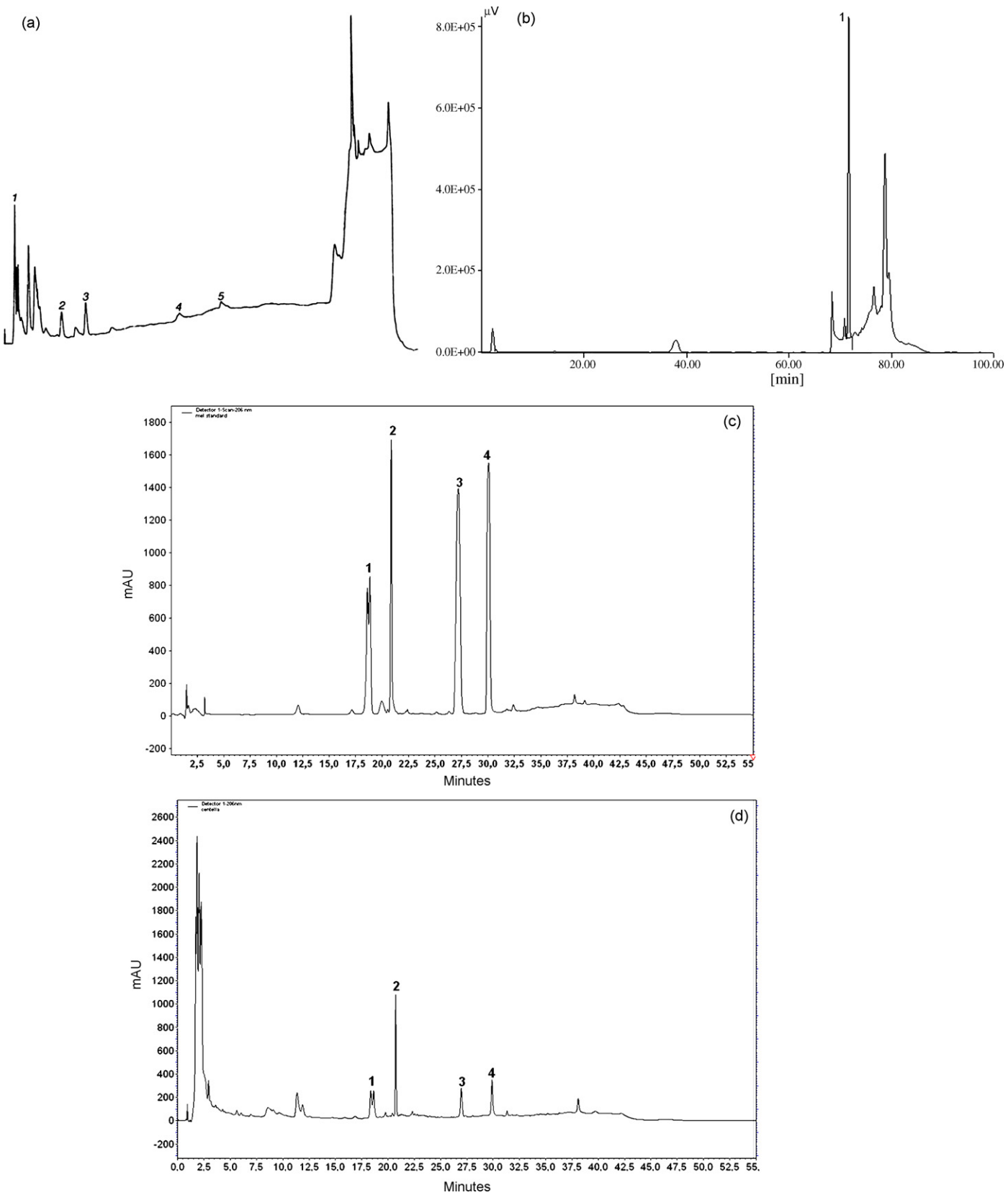

Fig. 2. (a) Chromatogram of a crude extract of *Centella asiatica* [22] (1: solvent, 2: madecassoside (TR = 5.8), 3: asiaticoside (TR = 8.1), 4: madecassic acid (TR = 17.6), 5: asiatic acid (TR = 21.7)); (b) chromatogram of a crude extract of *Centella asiatica* obtained in our lab with European Pharmacopoeia method (1: madecassoside); (c) chromatogram of reference standards with the developed method (1: madecassoside and its isomer (asiaticoside B), 2: asiaticoside, 3: madecassic acid, 4: asiatic acid); (d) chromatogram of a crude extract of *Centella asiatica* with the developed method.

Table 2
Peaks area ratio of the different compounds investigated with asiaticoside.

	Compounds			
	Madecassoside	Asiaticoside	Madecassic acid	Asiatic acid
Reference standards purity	97.94%	99.2%	95%	99%
Peak area ratio[a] ($n=2$)	1 ± 0.5[b]	1.0 ± 0.2[b]	2.1 ± 1.3[b]	2.1 ± 0.3[b]

[a] Area ratio for each compound was calculated using the response of asiaticoside (major compound) as reference.
[b] RSD (%).

to the chromatographic conditions and not an extraction failure [22].

3.2. Method validation

Selectivity and peak purity were analysed by the comparison of retention times and mass spectra with reference compounds. Mass spectra were analysed at three levels (beginning, middle and end) of each peak investigated and found to be comparable (Figs. 2c–d and 4a–d). Comparison of the chromatogram of an extract of *C. asiatica* from European Pharmacopoeia method (Fig. 2a) and the chromatogram obtained with our method (Fig. 2d) shows its good resolution and the interest of this developed method for the quantification of aglycones. We also observed that madecassoside and its isomer (asiaticoside B also called terminoloside) were slightly separated, but as both are considered to be active and are usually not separated; we added both areas and considered both peaks as one madecassoside peak to allow comparison with other results. Calibration standards of asiaticoside and asiatic acid were prepared without matrix ($m=3$, $n=2$). Different regression models were tested such as: weighted ($1/X$) quadratic regression, weighted ($1/X^2$) quadratic regression, quadratic regression, weighted ($1/X$) linear regression, weighted ($1/X^2$) linear regression, linear regression after logarithm transformation, linear regression after square root transformation, weighted ($1/X$) linear regression, linear regression. Accuracy profiles were plotted to determine the most suitable regression model [24]. Fig. 5a and b shows the accuracy profiles obtained with the quadratic regression as response function for both standards. It was selected as the most adequate one as the 95% β-expectation tolerance intervals were totally included inside the ±20% acceptance limits for each concentration level of the validation standards for both analytes.

Trueness [25,26] is expressed in relative bias (%) at each concentration level of the validation standards. Relative bias was less than 3% (Table 3) for asiaticoside and 10% for acid asiatic showing the excellent trueness of the method.

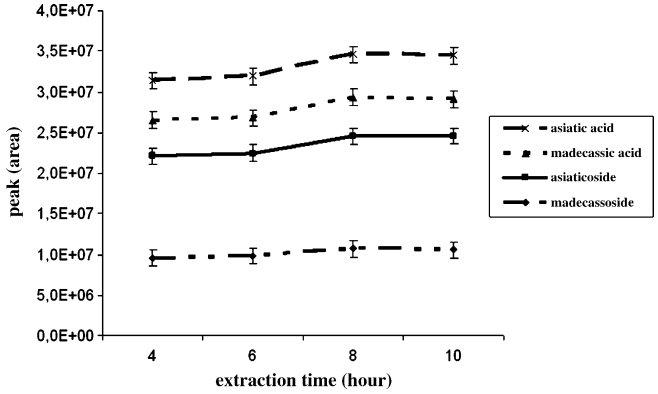

Fig. 3. Madecassoside, asiaticoside, madecassic and asiatic acids responses after different times of Soxhlet extraction of *Centella asiatica* ($n=3$, RSD <1%).

Precision was evaluated in terms of standard deviation (SD, mg/ml) and relative standard deviation (RSD %) values for repeatability and intermediate precision [27,28]. As seen in Table 3, RSD (%) for repeatability and intermediate precision did not exceed 4%.

Accuracy allows to evaluate total error, the sum of systematic and random errors of the tests results [24–28]. For asiaticoside and asiatic acid, as illustrated in Fig. 5a and b, their respective accuracy profiles show that the relative upper and lower 95% β-expectation tolerance limits are totally included inside the acceptance limits set at ±20%. The method can thus be considered as accurate between

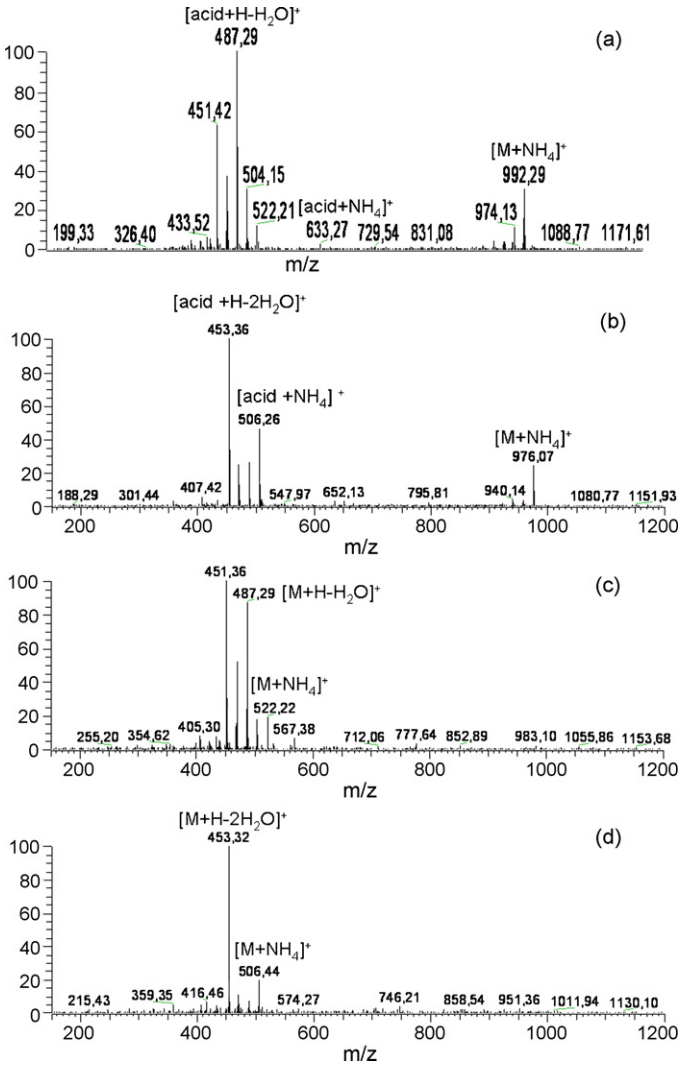

Fig. 4. Positive ion mode mass spectra obtained for peaks of the methanol extract of *Centella asiatica*. (a) madecassoside $[M+NH_4]^+=992$; (b) asiaticoside $[M+NH_4]^+=976$; (c) madecassic acid $[M+NH_4]^+=522$; (d) asiatic acid $[M+NH_4]^+=506$.

1.0 and 3.0 mg/ml for asiaticoside and between 0.5 and 2.0 mg/ml for asiatic acid.

For asiaticoside and asiatic acid, LOD (the smallest quantity of the analyte that can be detectable in the sample, but not quantifiable) were 0.0113 and 0.0023 mg/ml, respectively. These results were estimated using the mean intercept of the calibration model and the residual variance of the regression. The LOQ (the smallest quantity quantifiable in the sample) were determined with the accuracy profiles as they are the smallest concentration levels where the 95% β-expectation tolerance limits remain inside the ±20% acceptance limits [24–26]. In other words, they are the smallest concentration levels with a maximum total error of 20%. As shown in Fig. 5a and b, the LOQ are the smallest concentration levels of the validation standards, i.e. 1.0 and 0.5 mg/ml for asiaticoside and asiatic acid, respectively.

Uncertainty of the measurement characterises the dispersion of the values that could reasonably be attributed to the measurer [29,30]. It was evaluated using expanded uncertainty where true value can be observed with a confidence level at 95%. Table 4 shows that relative expanded uncertainties were less than 10% for asiaticoside and asiatic acid which means that the unknown true value is located at a maximum of ±10% around the measured result.

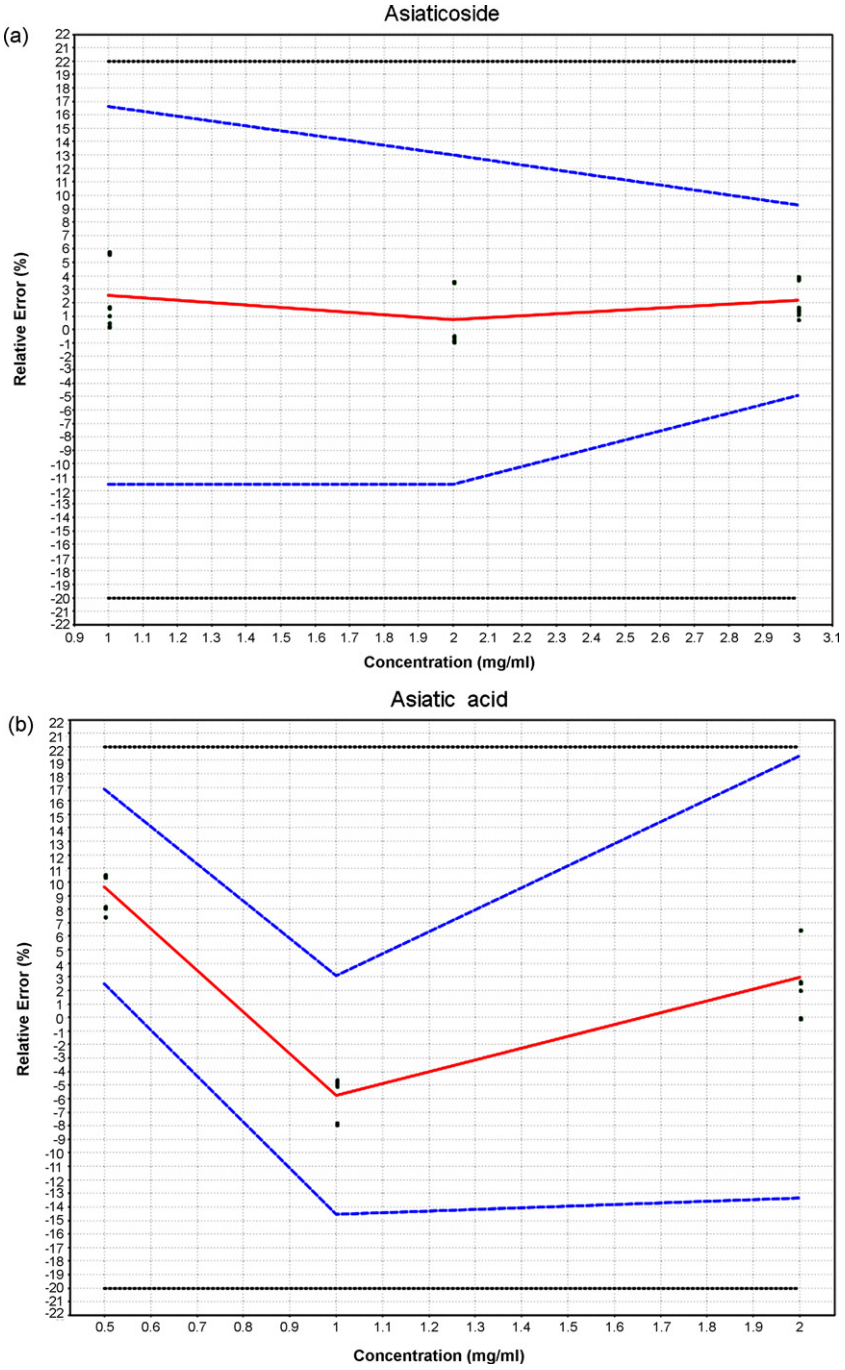

Fig. 5. Accuracy profiles of asiaticoside (a) and asiatic acid (b) obtained with quadratic regression. The plain line is the relative bias, dashed lines are the β-expectation tolerance limit (β = 95%) and dotted lines represent the acceptance limit (±20%). The dots represent the relative back-calculated concentrations of the validation standards and are plotted according to their targeted concentration.

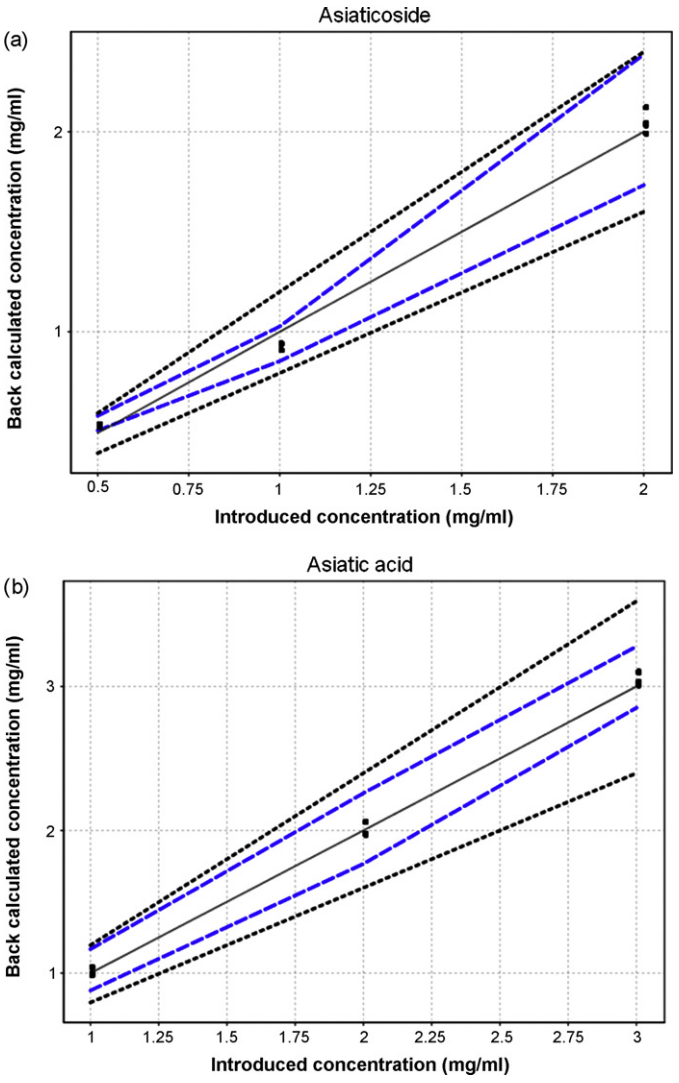

Fig. 6. Linear profiles of asiaticoside (a) and asiatic acid (b). The continuous line is identity line ($y = x$), the dotted lines are the upper and lower acceptance limits in absolute values and the dashed lines are the upper and lower β-expectation tolerance limits ($\beta = 95\%$).

The linearity demonstrated the relationship between introduced and calculated concentration [25,26,28] using β-expectation tolerance interval approach. The concentrations of the validation standards were back-calculated in order to determine, by concentration level, the mean relative bias as well as the upper and lower β-expectation tolerance intervals. The acceptance limits were set at ±20%. In order to demonstrate method linearity, a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equations obtained for asiaticoside and asiatic acid with their coefficient of determination are presented in Table 3.

The slopes values obtained for the two standards were, respectively 1.02, 1.023. Fig. 6a and b demonstrate the linearity of the results.

3.3. Application to samples of Centella asiatica

Three samples of *C. asiatica* were collected in the East and High Plateau regions of Madagascar. Extracts were analyzed and the results obtained by our method and the data described in the literature [14,15] are given in Table 5. Our results are in the same range than those obtained in the previous works. The heterosides are more abundant than aglycones and asiaticoside is often the major compound. Nevertheless, differences occur between samples and those analyzed here seemed less rich than the others, but we have to point out that all these other methods were not validated so the reliability of these results needs caution. This proposed method gives low RSD (%) values and is reliable. These results stress the importance of a good quantification method to determine the best culture and harvesting conditions.

A validated method for the quantification of asiaticoside, madecassoside, asiatic and madecassic acids in drug samples of *C. asiatica* was developed. This method allows the simultaneous quantification of madecassoside, asiaticoside, madecassic acid and asiatic acid. Most of other validated methods only quantified some of these active molecules. Furthermore, the method described in European Pharmacopeia does not give good signal to noise ratio for the accurate quantification of aglycones and was not reproducible in our laboratory. The method developed and validated was then successfully applied to quantify these four compounds in different samples of *C. asiatica* collected in Madagascar.

Table 3
Validation results in crude extract of *Centella asiatica*.

Validation criteria	Asiaticoside		Asiatic acid	
Response function	Quadratic regression Calibration range (3 points) 0.5–5 mg/ml		Quadratic regression Calibration range (3 points) 0.25–2.5 mg/ml	
Trueness	Concentration (mg/ml)	Relative bias (%)	Concentration (mg/ml)	Relative bias (%)
	1	2.6	0.5l	9.7
	2	0.7	1	−5.8
	3	2.2	2	3
Precision	Repeatability (SD mg/ml)	Intermediate precision (RSD %)	Repeatability (SD mg/ml)	Intermediate precision (RSD %)
	0.2313	2.9	0.2421	1.5
	0.0987	2.5	0.1427	1.8
	0.252	1.5	0.1997	3.3
Accuracy	β-Expectation lower and upper tolerance limits of the relative error (%)		β-Expectation lower and upper tolerance limits of the relative error (%)	
	−11.5, 16.6		2.5, 16.9	
	−11.6, 13.0		−14.6, 3.1	
	−5.0, 9.3		−13.4, 19.3	
Linearity				
Slope	1.02		1.023	
Intercept	−0.005007		−0.01017	
r^2	0.998		0.9912	

Table 4
Estimates of the measurement uncertainties related to asiaticoside and asiatic acid, at each concentration level investigated in validation using quadratic regression model.

Asiaticoside	Concentration level (mg/ml)	Mean introduced concentration (mg/ml)	Uncertainty of the bias (mg/ml)	Uncertainty (mg/ml)	Expanded Uncertainty (mg/ml)	Relative expanded uncertainty (%)
	1.0	1.000	0.01644	0.03294	0.06588	6.6
	2.0	2.000	0.02860	0.05723	0.1145	5.7
	3.0	3.000	0.02554	0.05146	0.1029	3.4
Asiatic acid	Concentration level (mg/ml)	Mean introduced concentration (mg/ml)	Uncertainty of the bias (mg/ml)	Uncertainty (mg/ml)	Expanded uncertainty (mg/ml)	Relative expanded uncertainty (%)
	0.5	0.5000	0.004286	0.008628	0.01726	3.4
	1.0	1.000	0.01031	0.02065	0.04131	4.1
	2.0	2.000	0.03809	0.07625	0.1525	7.6

Table 5
Comparison of the content of triterpenes in *Centella asiatica* samples analysed by our method ($n = 3$) in % of the dry plant with results given in previous works [14,15].

Sample	Madecassoside	Asiaticoside	Madecassic acid	Asiatic acid
Sample 1	1.7 ± 0.04	2.0 ± 0.02	0.95 ± 0.039	0.98 ± 0.03
Sample 2	1.27 ± 0.013	1.63 ± 0.04	<LOD	<LOD
Sample 3	1.64 ± 0.02	1.75 ± 0.02	0.72 ± 0.02	0.72 ± 0.08
CA-1 [14]	4.76 ± 1.342	5.23 ± 0.025	1.97 ± 0.007	1.89 ± 0.08
CA-2 [14]	4.28 ± 0.124	4.52 ± 0.138	1.88 ± 0.07	1.79 ± 0.102
CA-3 [14]	5.89 ± 0.15	6.42 ± 0.48	0.23 ± 0.00	0.16 ± 0.07
CA-4 [14]	3.23 ± 0.06	3.37 ± 0.26	0.37 ± 0.09	0.44 ± 0.17
CA-5 [14]	4.74 ± 0.23	4.58 ± 0.63	0.23 ± 0.08	0.25 ± 0.10
CA-6 [14]	2.38 ± 0.18	2.67 ± 0.27	0.42 ± 0.29	0.36 ± 0.28
CA-7 [14]	2.76 ± 0.29	3.09 ± 0.24	0.12 ± 0.05	0.09 ± 0.07
Leaves type-1 [15]	3.62 ± 0.29	3.68 ± 0.04	0.11 ± 0.01	0.05 ± 0.02
Leaves type-2 [15]	2.76 ± 0.15	3.13 ± 0.14	0.59 ± 0.12	0.64 ± 0.14

Mean ± RSD %. CA: *Centella asiatica* [14].

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References

[1] G. Péchard, M. Antona, S. Aubert, D. Babin, Bois et Forêts des Tropiques 284 (2005) 2.
[2] P. Boiteau, A.R. Ratsimamanga, Thérapie 1 (1956) 11.
[3] G. Jayashree, G.K. Muraleedhara, S. Sudarslal, V.B. Jacob, Fitoterapia 74 (2003) 5.
[4] A. Gnanapragasam, K. Kuma Ebenezar, V. Sathish, P. Govindaraju, T. Devaki, Life Sci. 76 (2004) 5.
[5] M. Yoshida, M. Fuchigami, T. Nagao, H. Okabe, K. Matsunaga, J. Takata, et al., Biol. Pharm. Bull. 28 (2005) 1.
[6] L. Incadela, M.R. Cesarone, M. Cacchio, M.T. De Sanctis, C. Santavenere, M.G. D'Auro, M. Bucci, G. Belcaro, Angiology 52 (Suppl. 2) (2001) S9.
[7] C.D. Coldren, P. Hashim, J.M. Ali, S.K. Oh, A.J. Sinskey, C. Rha, Planta Med. 69 (2003) 8.
[8] L. Lu, K. Ying, S. Wei, Y. Liu, H. Lin, Y. Mao, Br. J. Dermatol. 151 (2004) 3.
[9] J. Lee, E. Jung, Y. Kim, J. Park, S. Hong, J. Kim, C. Hyun, S.Y. Kim, D. Park, Planta Med. 72 (2006) 4.
[10] M. Liu, Y. Dai, X. Yao, Y. Li, Y. Xia, Z. Gong, Int. Immunopharmacol. 8 (2008) 11.
[11] M. Lui, Y. Dai, Y. Li, F. Huang, Z. Gong, Q. Meng, Planta Med. 74 (2008) 8.
[12] E.K. Park, S.H. Park, K.R. Patent No. 007051377. Rep. Kor. Kongkae Taeho Kongbo (2007).
[13] D.H. Park, J.S. Lee, G.S. Jung, K.R. Patent No. 2007111785 Rep Kor. Kongkae Taeho Kongbo (2007).
[14] D. Randriamampionona, B. Diallo, F. Rakotonirina, C. Rabemanantsoa, K. Cheuk, A.M. Corbisier, J. Mahillon, S. Ratsimamanga, M. El Jaziri, Fitoterapia 78 (2007) 7.
[15] T.J. Jacinda, R. Meyer, I.A. Dubery, Plant Cell. Tissue Organ Culture 94 (2008) 1.
[16] L. Xingyi, F. Lijuan, Zhongguo Yaoshi (Wuhan, China) 11 (2008) 11.
[17] F.L. Zhang, Y.J. Wei, J. Zhu, Z.N. Gong, Biomed. Chromatogr. 22 (2008) 2.
[18] P. Jian, K. Guiqing, Y. Chuanxun, Z. Beibei, J. Risheng, Y. Yuan, J. Chromatogr. 25 (2007) 3.
[19] B.T. Schaneberg, J.R. Mikell, I.A. Khan, Pharmazie 58 (2003) 6.
[20] P.K. Inamdar, R.D. Yeole, A.B. Ghogare, N. de Souza, J. chromatogr. A 742 (1996) 1.
[21] B. Günther, H. Wagner, Phytomedecine 3 (1996) 59.
[22] European Pharmacopoeia, 6th ed., 2008 (http://www.edqm.eu).
[23] Analytical Methods committee, Analyst 120 (1995) 2303.
[24] E. Rozet, C. Hubert, A. Ceccato, W. Dewé, E. Ziemons, F. Moonen, K. Michail, R. Wintersteiger, B. Streel, B. Boulanger, Ph. Hubert, J. Chromatogr. A 1158 (2007) 126.
[25] E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, R. Oprean, S. Rudaz, B. Boulanger, Ph. Hubert, J. Chromatogr. A 1158 (2007) 111.
[26] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G.M. Luzard, C. Nivet, L. valet, J. Pharm. Biomed. Anal. 36 (2004) 579.
[27] Guidance for industry: Bioanalytical Method Validation, US Department of Health and Human Services, US Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Rockville, May 2001.
[28] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G.M. Luzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[29] Eurachem/Citac Guide Quantifying the uncertainty in analytical measurement, 2nd ed., 2000.
[30] CBEA-4/16, EA Guidelines on the Expression of Uncertainty in Quantitative Testing, 2004, http://www.european-accreditation.org.

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Application of design of experiments and design space methodology for the HPLC-UV separation optimization of aporphine alkaloids from leaves of *Spirospermum penduliflorum* Thouars

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ABSTRACT

Spirospermum penduliflorum Thouars (Menispermaceae) is an endemic species of Madagascar traditionally used as vasorelaxant. Recently, two aporphine alkaloids known to possess antihypertensive activity (dicentrine and neolitsine) were isolated and identified from the leaves of this plant. In the present study, a HPLC-UV method allowing the separation of all alkaloids and the quantification of dicentrine in the alkaloidic extract of leaves was developed using design of experiments and design space methodology. Three common chromatographic parameters (i.e. the mobile phase pH, the initial proportion of methanol and the gradient slope) were selected to construct a full factorial design of 36 experimental conditions. The times at the beginning, the apex (i.e. the retention time) and the end of each peak were recorded and modelled by multiple linear equations. The corresponding residuals were normally distributed which confirmed that the models can be used for the prediction of the retention times and to optimize the separation. The optimal separation was predicted at pH 3, with a gradient starting at 32% of methanol and a gradient slope of 0.42%/min. Good agreement was obtained between predicted and experimental chromatograms. The method was also validated using total error concept. Using the accuracy profile approach, validation results gave a LOD and LOQ for dicentrine of 3 μg/ml and 10 μg/ml, respectively. A relative standard deviation for intermediate precision lower than 10% was obtained. This method was found to provide accurate results in the concentration range of 10–75 μg/ml of dicentrine and is suitable for routine analysis.

1. Introduction

Nowadays, the traditional medicine still holds an important place to cure diseases in developing countries. It is mainly due to the non accessibility of modern medicine by the local population. The safety, effectiveness and quality of finished herbal medicinal products depend on the quality of their source materials, which can include hundreds of natural constituents, and how elements are handled through production processes.

Spirospermum penduliflorum Thouars (Menispermaceae) was chosen among a list of medicinal plants used by Malagasy population because it is an endemic species of Madagascar. This plant is rich in alkaloids. The decoction of all parts is traditionally used as anticholinergic and vasorelaxant [1] and the decoction of leaves is also used for the treatment of malaria and as a chloroquine adjuvant. The dried leaves are also smoked for pulmonary tuberculosis treatment. The decoction of roots was taken as cholagogue, tonic and for hepatic disorders [2]. Different studies on root extracts of *S. penduliflorum* allowed the isolation of some active molecules: columbine (clerodane-type diterpenoid), palmitine (protoberberine-type quaternary alkaloid) and limacine (bisbenzylisoquinoline alkaloid) [3]. Recently, Raoelison et al. found a vasorelaxant activity on isolated rat aorta using a methanol/dichloromethane leaves extract (under publication). Two aporphine alkaloids were then isolated by bioguided fractionation: dicentrine (Fig. 1) and neolitsine both known to possess antihypertensive activities. In their experimental model, dicentrine gave an EC50 value of 0.15 ± 0.04 μg/ml on rat aorta relaxation.

In the previous studies, Teng et al. also demonstrated that dicentrine is a strong vascular α-1 adrenoceptor antagonist

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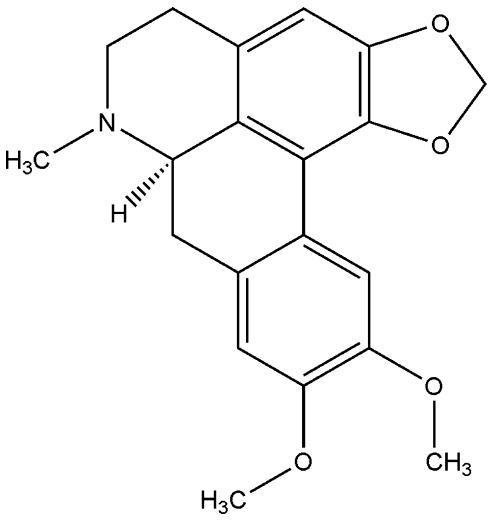

Fig. 1. Chemical structure of dicentrine.

having antiplatelet [4–6], and antiarrhythmic activities [7]. It confirms the traditional use of this plant as an antihypertensive. Lai et al. [8] and Tsai et al. [9] proposed two HPLC methods for the quantification of dicentrine in rat plasma and urine. The first method was not validated [8]. The second method [9] was fully validated but the separation of some peaks was not complete. Besides, the present matrix was more complex than plasma and urine. Therefore, a HPLC method coupled with UV and MS detection was developed to separate and quantify these molecules and to control the quality and effectiveness of this plant. The optimization of chromatographic methods for plant extracts is often intricate and can be time consuming. In fact, it is a thorny problem to separate components because of the number of compounds, the similarities between the chromatographic behaviours of some of them while some others have widely distinct physico-chemical properties (e.g. polarities, pK_a, Log P). Usually, optimization of a chromatographic method is achieved by changing parameters one by one which is commonly called "one factor/variable at a time" methodology. On the contrary, design of experiments (DoE) methodology investigates the factors effects and the interactions between them by modifying multiple factors at a time. Combined with design space (DS) [10–15], it leads to a powerful methodology (DoE-DS) allowing the identification of optimal conditions for the achievement of robust analytical methods. In the present study, DoE-DS methodology was used to develop a method for the separation of aporphin alkaloids contained in leaves extract of *S. penduliflorum*. The method was then fully validated using the total error concept and the accuracy profile approach for the quantification of dicentrine in these leaves extracts [16,17].

2. Materials and methods

2.1. Chemicals and reagents

Dicentrine reference standard was purchased from Sequoia Research Products Ltd. (Pangbourne, Ukraine). The reagents were purchased as follows: methanol HPLC grade, ethoxyethane from VWR (Leuven, Belgium), dichloromethane from Sigma Aldrich (St. Louis, MO, USA), ammonia solution (25%) from J.T. Baker (Deventer, Netherlands), acetic acid from Fisher scientific (Erembodegem, Belgium), ammonium formate and anhydrous sodium sulphate from Merck (Darmstadt, Germany).

2.2. Plant material

Fresh leaves of *S. penduliflorum* were collected in December 2009 in the Eastern Region of Madagascar. A voucher specimen (AML 13) was deposited at the herbarium of the "Institut Malgache de Recherches Appliquées" (IMRA). Leaves were dried at ambient temperature and reduced to powder. In order to have a homogenous powder, the dried powdered leaves were passed through a sieve of 710 μm meshes.

2.3. Apparatus

Chromatographic separations were performed with a reversed-phase C18 LiChroCART® column (250 mm × 4 mm i.d.; particle size: 5 μm) (Merck, Darmstadt, Germany), a HPLC Waters 2690 separation module (Waters, Milford, MA, USA) coupled to a UV spectrophotometric detector Kromaton (Angers, France); all controlled by Borwin software (Borwin, Rostock, Germany). The column was maintained at 30 °C. For the peaks identification, a LCQ Advantage Thermo Finnigan (Waltham, MA, USA) mass spectrometer with ESI ion source was used, piloted by X-Calibur software.

Method validation experiments were performed using a Merck Hitachi HPLC system consisting of a pump L6200, an automatic injector AS 2000 A, and a UV detector L400; all piloted by the Borwin software.

3. Experimental

3.1. Plant extraction

One gram of powdered leaves was macerated in a refluxing water bath at 50 °C for 1 h with 50 ml of methanol–acetic acid 99% (99:1, v/v). The residue was washed with 10 ml of the same solvent and filtered through a filter paper (No 5, Whatman). These operations were repeated 4 times in order to have an extraction of 4 h.

The extracts were combined and evaporated to dryness under reduced pressure. Dried crude extract was diluted in 50 ml of water acidified with 1% of acetic acid, filtered and washed twice with 30 ml of ethoxyethane. Liquid-liquid extraction was performed four times with 50 ml of dichloromethane. Organic phases were dried over anhydrous sodium acetate and evaporated to dryness. The dried crude extract was diluted to 40 ml of methanol, filtered through a 0.45 μm filter (Whatman). Finally, 20 μl of this solution were injected into the HPLC system.

3.2. Experimental design

Preliminary assays were performed to identify the critical factors (i.e. the factors having the highest effect on the responses) to be further used during optimization procedure. These preliminary tests also allowed for the establishment of the ranges of the factors. Different columns were therefore tested to change chromatographic selectivity: a LiChroCART® RP-18e column (250 mm × 4 mm i.d.; particle size: 5 μm), a LiChroCART® RP-select B column (250 mm × 4 mm i.d.; particle size: 5 μm) and

Table 1
Chromatographic parameters studied for the method robust optimization. pci: methanol proportion at the beginning of the gradient.

Parameter	Levels			
pH	2.5	4.5	7	
Gradient slope (%/min)	0.4	0.6	1	
pci (%)	20	30	40	50

Table 2
Gradient conditions tested for the robust optimization of the HPLC method.

pci (%)	Gradient slope (%/min)					
	0.4		0.6		1	
	Time (min)	MeOH (%)	Time (min)	MeOH (%)	Time (min)	MeOH (%)
20	0	20	0	20	0	20
	100	60	66.6	60	40	60
	110	60	77	60	50	60
	111	20	78	20	51	20
30	0	30	0	30	0	30
	75	60	50	60	30	60
	85	60	60	60	40	60
	86	30	61	30	41	30
40	0	40	0	40	0	40
	50	60	33.3	60	20	60
	60	60	43	60	30	60
	61	40	44	40	31	40
50	0	50	0	50	0	50
	25	60	16.6	60	10	60
	35	60	26	60	20	60
	36	50	44	50	21	50

a Aluspher® C8 column (250 mm × 4 mm i.d.; particle size: 3 μm) were tested. pH and gradient slopes were also varied.

The LiChroCART RP-select B column was found to be the most adequate for the chromatographic separation offering the best selectivity among the tested columns.

The method optimization involved a full factorial design set up with 3 chromatographic factors: pH and gradient slope both at 3 levels and methanol proportion at the beginning of the gradient (pci) was studied with 4 levels (see Table 1). Table 2 summarizes the gradient conditions which ensue from the DOE. All the peaks are eluated at 60% of methanol, then 12 different gradient times are defined using pci and gradient slope and use for 3 pH levels. Finally a total of 43 analyses were performed including the 36 conditions defined by the DoE. The conditions at pH 2.5, pci = 30%, slope = 1%/min; pH 4.5, pci = 30%, slope = 0.6% and pH 4.5, pci = 50%, slope = 0.4%/min were carried out in duplicate and 4 independent repetitions were performed at pH 4.5, pci = 40%, slope = 0.6%/min. These independent repetitions were carried out to estimate the responses variability over the whole experimental domain: at some extreme points (i.e. pH 2.5, pci = 30%, slope = 1%/min and pH 4.5, pci = 50%, slope = 0.4%/min) and at the two points which are the closest to the DoE geometrical center (i.e. pH 4.5, pci = 30%, slope = 0.6% and pH 4.5, pci = 40%, slope = 0.6%/min).

3.3. Design of experiments and design space methodology

DoE provides a structured methodology for the determination of effects and the interactions between factors affecting a process and the output response of that process [18]. The most common objective of chromatographic methods development is to determine the operating conditions which allow obtaining an optimal separation of all compounds in the shortest analysis time. Factors which have the highest effects on the response (and therefore on the separation) must be selected. Usually, the chromatographic resolution (R_S) between two adjacent peaks is modelled to assess separation. Nevertheless, R_S cannot be selected as the response of multiple linear models due to discontinuities when selectivity changes and peaks interbred [10,19]. Hence, the beginnings, apex and ends (t_B, t_R, t_E, respectively) of each peak were measured and the selected responses were the logarithm of the retention factor ($\log(k_{tR})$ with $k_{tR} = (t_R - t_0)/t_0$ and t_0: the column dead time) and the logarithm of the half-widths ($\log(w_l)$ and $\log(w_r)$ with $w_l = k_{tR} - k_{tB}$ and $w_r = k_{tE} - k_{tR}$). These responses were modeled by multiple linear equations (see Eq. (1)).

$$\log(k_{tRi}) = \beta_0 + \beta_1 \cdot \mathrm{pH} + \beta_2 \cdot \mathrm{pH}^2 + \beta_3 \cdot \mathrm{pci} + \beta_4 \cdot \mathrm{pci}^2 + \beta_5 \cdot \mathrm{slope} + \beta_6 \cdot \mathrm{slope}^2 + \beta_7 \cdot \mathrm{pH} \cdot \mathrm{pci} + \beta_8 \cdot \mathrm{pH} \cdot \mathrm{slope} + \beta_9 \cdot \mathrm{pci} \cdot \mathrm{slope} + \beta_{10} \cdot \mathrm{pH} \cdot \mathrm{pci} \cdot \mathrm{slope} + \varepsilon \quad (1)$$

where $\log(k_{tRi})$ represents the logarithm of the retention times (t_R) of the ith peak. Logarithms of peaks half-width were modelled by the same equations. The β_1 to β_{10} are the parameters of the mathematic model, pH, pci and slope are the factors of the design of experiments and ε is representative of the residual error assumed normally distributed. Furthermore, a new separation criterion, S, was used to ease the predictive error propagation from the modelled responses to this criterion [10–12,19]. S is defined as the difference between t_B (the beginning) of the second peak and t_E (the end) of the first peak of the critical pair (i.e. the two most proximate peaks). The error affecting the modelled responses was estimated and propagated to S using Monte Carlo simulations and allowed to compute the DS. The DS corresponds to a region of the experimental domain where the probability to attain $S > 0$ (i.e. baseline-resolved peaks) is higher than a selected quality level [10–12]. The quality level and the design space shapes are also representative of the robustness of a method [20–23]. This is expressed in Eq. (2).

$$DS = \{x_0 \in \chi | E_\theta[P(S > \lambda)|\theta] \geq \pi\} \quad (2)$$

where x_0 is a point in the experimental domain, χ. λ is the threshold on the separation criterion S and π is the quality level. P and E correspond to the estimators of probability and mathematical expectation, respectively.

3.4. Chromatographic conditions

The mobile phase consisted of methanol and ammonium formate buffer (20 mM). Experiments were carried out in gradient mode (see Table 2 for gradients). The pH of the aqueous solution was adjusted to 2.5, 4.5 or 7 with concentrated formic acid or ammonia. The column was thermostated at 30 °C, flow rate was 1 ml/min and the detection was performed at a wavelength of 307 nm.

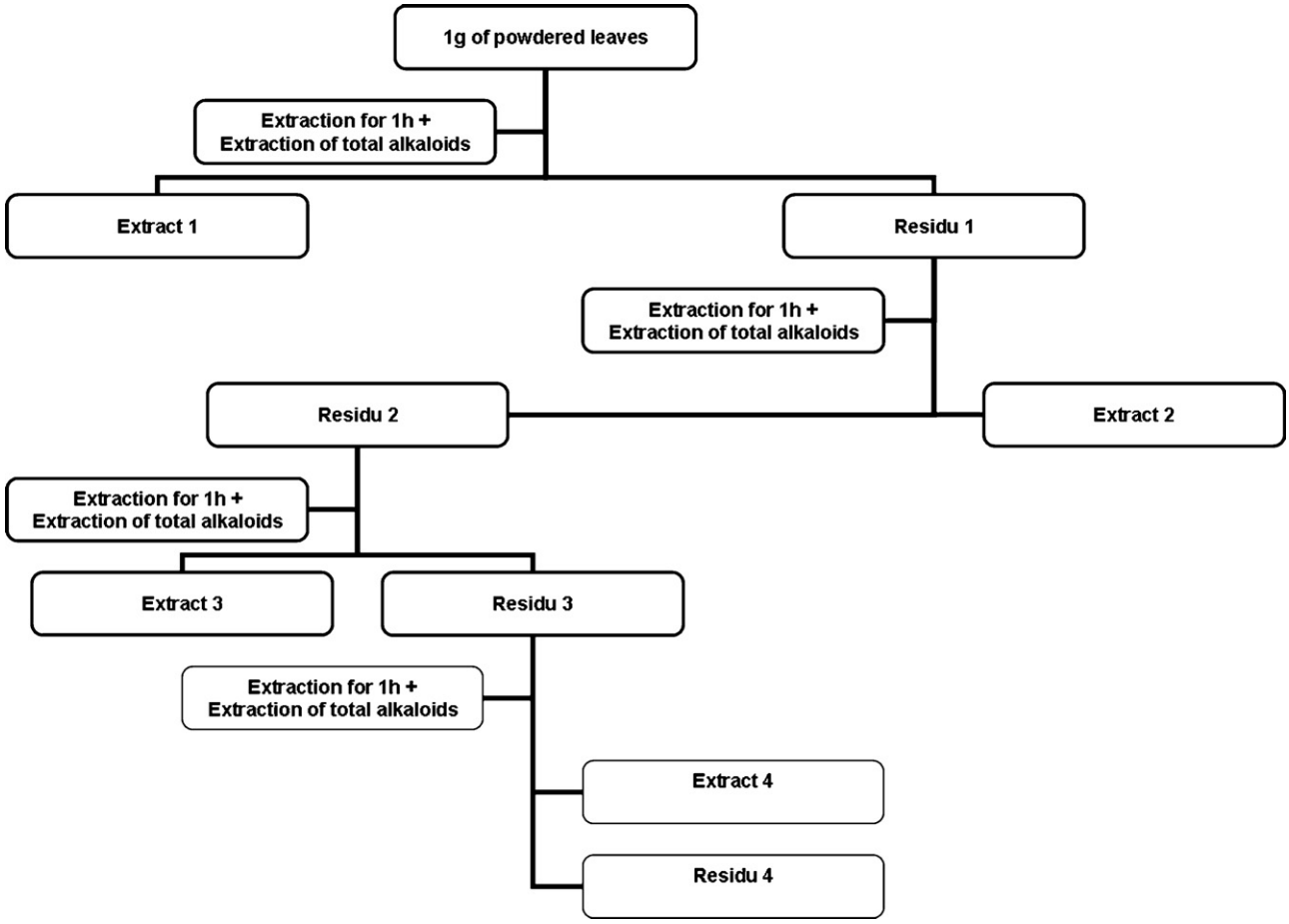

Fig. 2. Extraction procedure performed to assess extraction efficiency.

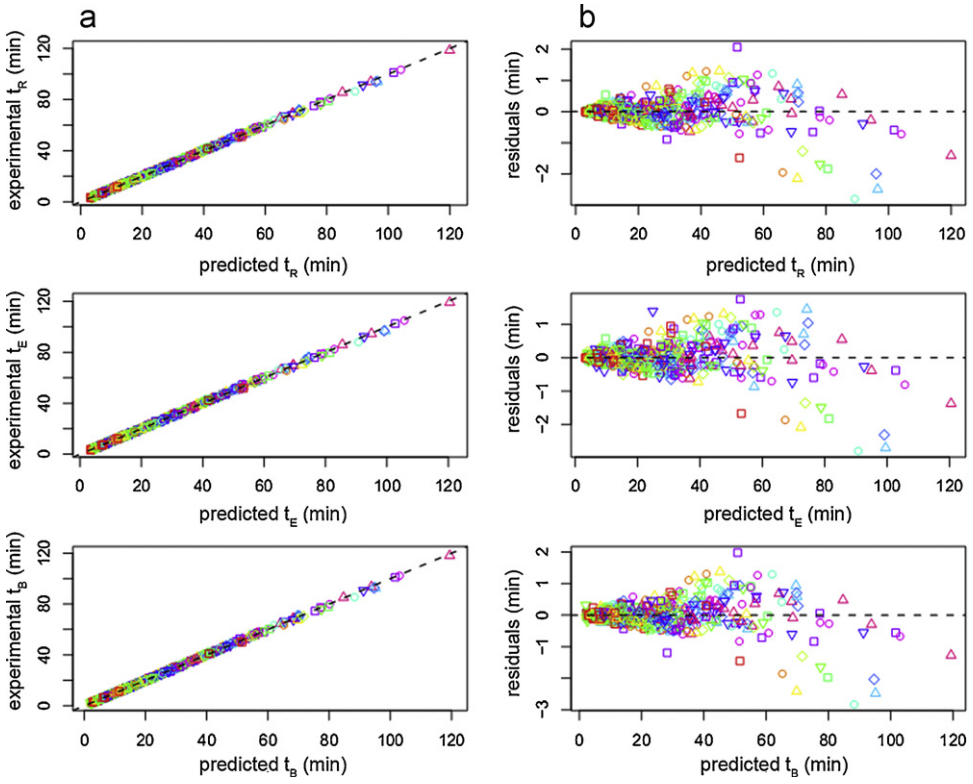

Fig. 3. (a) Correlation between predicted and experimental responses of total alkaloids of *Spirospermum penduliflorum*. (b) Corresponding residual distributions.