Development and Validation of a High Performance Liquid Chromatographic Method for Quantitative Determination of Aporphine Alkaloids from Different Samples of *Cassytha filiformis*

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Original Paper

Abstract

A sensitive and accurate procedure based on an alkaloid extraction coupled to an HPLC-UV-MS determination has been developed for the separation and quantification of the major aporphines in *Cassytha filiformis*. The extraction step and the liquid chromatography conditions were optimized in order to improve the selectivity of the method. The HPLC mobile phase consisted of a mixture of water containing 10 mM ammonium acetate adjusted to pH 3 with acetic acid-acetonitrile (90 : 10, v/v) (A) and acetonitrile (B) used in a gradient mode (0 to 40 %). The stationary phase was an RP-select B (5 μm) column. The method was completely validated using cassythine, one of the major aporphines in our samples, as reference standard and successfully applied to the determination of these pharmacologically interesting aporphines in seven different batches of *C. filiformis*. The detection and quantitation limits of cassythine were found to be 13 and 20 μg/mL, respectively. The results showed variations in the total alkaloid content in samples from 0.11 to 0.43 %.

Key words
Aporphine alkaloids · *Cassytha filiformis* · Lauraceae · Validation · Extraction · Quantification · HPLC

Introduction

Cassytha filiformis is a sprawling parasitic herb which is widely distributed along the seashores throughout the tropics. Several aporphinoid alkaloids have been isolated from samples originating from Brazil [1], Australia, New Guinea [2], Taiwan [3] and Benin [4], but compositions are quite variable depending on the origins. Wu et al. demonstrated that a methanolic extract of a Taiwan plant sample exhibited significant vasorelaxing activity as well as an inhibitory effect on platelet aggregation induced by several aggregating agents. A bio-guided fractionation of this extract allowed them to isolate cathafiline, cathaformine, predicentrine, ocoteine, actinodaphnine and *N*-methylactinodaphnine having antiplatelet actions. The last two compounds also showed vasorelaxing properties while ocoteine was shown to be a selective α_1-adrenoceptor antagonist in isolated rat thoracic aorta [5]. In African traditional medicine the practitioners use the whole plant mostly as aqueous extracts to fight several infections, parasites or to treat cancers [6]. Investigations of a sample of *C. filiformis* from Benin showed that the alkaloid fraction and isolated aporphines (neolitsine, cassythine, dicentrine and actinodaphnine) possessed cytotoxic properties against cancer and non-cancer cell lines *in vitro* [4]. More recently, we evaluated the antitrypanosomal activity of this alkaloid fraction and isolated aporphines and showed that their activity could be at least partially related to an interaction with DNA and inhibition of to-

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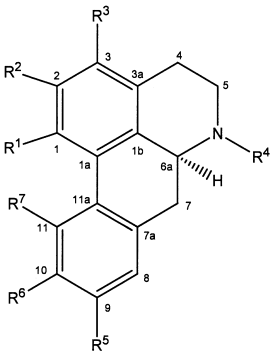
poisomerases [7]. Data on alkaloids from *C. filiformis* showed that compositions were quite variable depending on the origin of the samples. The composition could also vary according to the host plant, as it is a parasitic plant, and the seasons of the year. That is the reason why we decided to quantify aporphines in several samples. Few studies described the separation and the quantification of aporphines by HPLC. Most of them deal with the quantification of boldine and other aporphines in *Poemus boldus*, a medicinal plant described in several Pharmacopoeias [8], [9], [10], [11]. As far as we know the only other publications were made by Sun et al. who studied the possibility of the separation by HPLC of eight aporphines found in many Lauraceous species and quantified those in *Neolitsea sericea* var. *aurata* [12] and Tseng et al. who analyzed a mixture of nine aporphine alkaloids by HPLC-NMR [13]. These methods, applied to the tested samples, did not allow a good separation of the major aporphines or an ESI detection. Therefore, a new HPLC method coupled to UV detection had to be developed and validated for the separation and quantification of these pharmacologically interesting aporphines in *C. filiformis*. In order to confirm the structure of these compounds and demonstrate the specificity of the present HPLC-UV method, the mobile phase was selected to be compatible with ESI-MS detection which has not been used in routine analysis. The method was fully validated by considering different parameters such as selectivity, response function, trueness, precision, accuracy and linearity. The method was successfully used to quantify aporphines in different batches of *C. filiformis* and will be used in a long-term follow-up study.



Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1 Boldine	$O\text{-}CH_3$	OH	H	CH_3	OH	$O\text{-}CH_3$	H
2 Actinodaphnine	$-O\text{-}CH_2\text{-}O-$		H	H	OH	$O\text{-}CH_3$	H
3 Cassythine	$-O\text{-}CH_2\text{-}O-$		$O\text{-}CH_3$	H	OH	$O\text{-}CH_3$	H
4 Dicentrine	$-O\text{-}CH_2\text{-}O-$		H	CH_3	$O\text{-}CH_3$	$O\text{-}CH_3$	H
5 Norneolitsine	$-O\text{-}CH_2\text{-}O-$		H	H	$-O\text{-}CH_2\text{-}O-$		H
6 Neolitsine	$-O\text{-}CH_2\text{-}O-$		H	CH_3	$-O\text{-}CH_2\text{-}O-$		H
7 Cassythidine	$-O\text{-}CH_2\text{-}O-$		$O\text{-}CH_3$	H	$-O\text{-}CH_2\text{-}O-$		H

Fig. **1** Structures of boldine (**1**) and the identified aporphines **2** – **7** of *Cassytha filiformis*.

Table **1** Various batches of *Cassytha filiformis* collected on different hosts and seasons (dry or rainy) of the year 2002 – 2003. Ekpe and Seme are geographically close and have the same type of soil

Batch of C. filiformis	*Place of collection*	*Season of collection*	*Host plant of Cassytha filiformis*
1	Ekpe	Short rainy season	*Acacia auriculiformis* (Mimosaceae)
2	Ekpe	Long dry season	*Acacia auriculiformis* (Mimosaceae)
3	Seme	Long rainy season	*Anacardium occidentale* (Anacardiaceae)
4	Seme	Long rainy season	*Mangifera indica* (Anacardiaceae)
5	Seme	Long dry season	*Mangifera indica* (Anacardiaceae)
6	Seme	Long rainy season	*Azadirachta indica* (Meliaceae)
7	Seme	Long dry season	*Hyptis suaveolens* (Lamiaceae)

Materials and Methods

Chemicals and reagents

Boldine (**1**), purity 99%, (Fig. **1**) was purchased from Federa (Brussels, Belgium). Alkaloids **2** – **6** (Fig. **1**) were isolated from *C. filiformis* as previously described [4], [14] while the isolation and structural determination of cassythidine (**7**) followed the same purification procedure [4]. The structure of cassythidine (**7**) was established by combining MS and NMR spectra analysis and comparing with literature data [2]. All solvents and reagents were of analytical grade. Water was purified by a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Acetonitrile, methanol and diethyl ether were obtained from Carlo Erba (Milan, Italy), glacial acetic acid, ammonia solution (25%) and dichloromethane from J. T. Baker (Phillipsburg, NJ, USA). Nylaflo® membrane filters (0.45 μm) for the mobile phase were supplied by Pall Corporation (New York, USA) and PVDF membranes (0.45 μm) for the preparation of samples before HPLC injection were from Whatman (Germany). Ammonium acetate and anhydrous sodium sulfate came from Merck (Darmstadt, Germany).

Plant material

Aerial parts of *C. filiformis* L. (Lauraceae) were collected at Seme or Ekpe, Oueme, Benin and identified by Prof. V. Adjakidje. Plant materials were taken from various host plants and at different seasons of the years 2002 and 2003 (dry or rainy seasons) (Table **1**). Batch 1 was used for all the steps of the validation protocol. A voucher of *C. filiformis* has been deposited at the Herbarium of the Belgian National Botanical Garden, at Meise (BR-S.P. 848 105). The collected plants were air-dried and kept at room temperature until analysis. Just before extraction, the dried plant materials were ground and passed through a sieve (Retsch® 355 μm) to obtain homogenous powders.

Extraction of the alkaloids

Fifty grams of dried and powdered samples of *C. filiformis* were macerated four times with 250 mL of methanol acidified with 1% of acetic acid at 50 °C in a refluxing water bath for 1 h. Between each maceration the residue was filtered and washed with 50 mL of the same solvent. The extracts were combined and concentrated under reduced pressure. The residue was dissolved in 400 mL of an aqueous solution acidified by acetic acid

(1%) then filtered. The filtrate was washed three times with 150 mL of ether. The aqueous acid layer was basified (pH 9.5) with NH_4OH 25% and extracted three times with 200 mL of dichloromethane. The dichloromethane layers were combined and dried over anhydrous Na_2SO_4 and evaporated to dryness. 50 milligrams of the residue obtained were dissolved in 50 mL of methanol. After filtration (0.45 μm membrane), 20 μL of the solution of alkaloid extract (1 mg/mL) were injected into the HPLC system. This standardized protocol was applied to each sample of *C. filiformis*. For LC-MS experiments, the solution of alkaloid extract (1 mg/mL) was diluted with methanol to obtain solutions of about 500 μg/mL before membrane filtration.

Chromatographic conditions and instrumentation

A LiChrospher 60, RP-select B column, (250×4 mm, 5 μm particle size) equipped with a guard column (4×4 mm) packed with the same sorbent was used throughout all chromatographic experiments (Merck). Prior to use, solvents were filtered over a 0.45 μm membrane filter and sonicated for 10 min in a Retsch URG Transsonic. The mobile phase consisted of (A) water containing 10 mM ammonium acetate adjusted to pH 3 with acetic acid-acetonitrile (90 : 10, v/v) and (B) acetonitrile. A gradient elution program was applied as follows: 0 – 2 min linear increase from 0 to 5% B; 2 – 5 min hold on 5% B; 5 – 31 min linear increase to 10% B; 31 – 40 min hold on 10% B; 40 – 45 min linear increase to 15% B; 45 – 74 min linear increase to 20% B; 74 – 79 min linear increase to 40% B in order to wash the column; 79 – 81 min linear decrease to 0% B. Prior to the next injection, the column was re-equilibrated with the same phase for 9 min. The flow rate was kept constant at 0.7 mL/min. Peaks were detected at 307 nm. The HPLC-UV separation was performed using a LaChrom HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an L-7200 autosampler and an L-7400 UV detector. A Kontron 440 diode array detector was also used during the analytical development phase. The chromatographic data were recorded and processed by the LaChrom D-7000 HPLC System Manager (version 3) software. The HPLC-electrospray ionization (ESI)-MS system consisted of Thermo Separation Products (TSP, San Jose, CA): a P1000XR pump, an AS 3000 autosampler and a UV 6000LP DAD detector. The chromatographic data were processed by Excalibur software version 1.2. Mass spectra were acquired using an ion trap LCQ mass spectrometer (Finnigan, San Jose, CA) equipped with an electrospray ionization source in the positive mode. The operating parameters were as follows: the spray needle voltage was set at 5.1 kV, capillary voltage was 18 V and capillary temperature was 250 °C. Sheath gas flow rate was set at 45 PSI and auxiliary gas flow rate at 2 PSI. For all the MS-MS experiments, collision induced dissociation (CID) was recorded at a relative collision energy of 33%.

The validation data were processed by e.noval® version 1.0. software (Arlenda, Belgium) and the statistical data of the dosage with SPSS 10.1.4. for Windows software (SPSS Inc, Chicago, IL, USA).

Standard stock solutions

Stock solutions of boldine (S1) and cassythine (S2) were prepared independently by dissolving the appropriate amount of each compound in methanol in order to obtain a final concentration of 52 and 1000 μg/mL, respectively. The S1 solution was only used to evaluate the efficiency of the extraction step. The S2 solution was first used as calibration standard solution to estimate the amount of cassythine in the alkaloid extract before starting the pre-validation step. Furthermore, three different concentrations of cassythine were added to a solution of 1 mg/mL of the alkaloid extract. The added concentrations of cassythine were 20, 165 and 510 μg/mL, respectively. These latter solutions were used to confirm the amount of cassythine in the alkaloid extract by means of the standard addition method. The same spiked solutions were then used to select the response function during the pre-validation step. Finally, the S2 solution of cassythine was also used as calibration standard solution in the validation step as well as during the batch analysis of plant materials.

Peak identification

Alkaloids were identified by comparison of their retention times, UV and mass spectra with the corresponding pure compounds previously isolated from *C. filiformis* [4], [14]. Identification of compound A (Fig. **2**) is not complete as we did not manage to obtain enough pure compound for refined NMR structure determination. Nevertheless, MS data showed that it may be nor-boldine or one of its isomers [14].

Results and Discussion

Method development

Different procedures were tested to obtain an optimal and well-suited time extraction of the alkaloids from the plant material: the Soxhlet method; single, double, triple and quadruple macerations with or without heating. Due to its poor reproducibility, the time-consuming percolation process was discarded. The best results were obtained with the quadruple maceration at 50 °C as proposed by Kartal et al. for the extraction of other types of alkaloids [15]. In addition to these preliminary extraction experiments [4], the liquid-liquid extraction step was standardized, a pH value of 9.5 and a 200-mL volume of dichloromethane in triplicate were selected.

The chromatographic separation was performed on a LiChrospher 60 RP-select B column by using an acidic mobile phase, these conditions being adapted to the analysis of this type of ba-

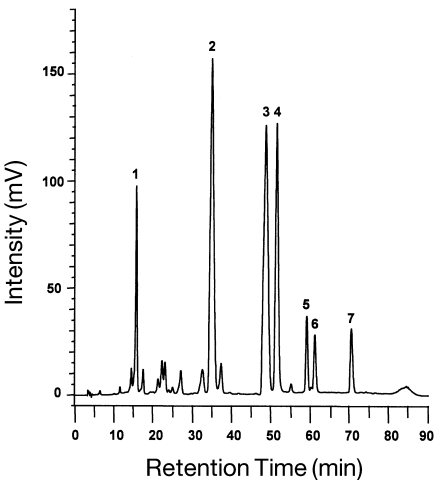


Fig. **2** Typical chromatogram of the total alkaloid extract (1 mg/mL) obtained by using the extraction step on the batch 5 of *Cassytha filiformis* coupled to the LC method. Key to peak identity: 1, compound A; 2, actinodaphnine; 3, cassythine; 4, dicentrine; 5, norneolitsine; 6, neolitsine; 7, cassythidine.

sic compounds [12]. On the basis of the DAD spectra analysis, the detection wavelength was selected at 307 nm corresponding to the most specific detection wavelength for the aporphines of *C. filiformis*. The ammonium acetate buffered solution and its concentration in the HPLC mobile phase were chosen to be compatible with ESI-MS detection. A flow rate of 0.7 mL/min was also selected to be well-suited for HPLC-MS and HPLC-UV analysis. However, due to the complexity of the alkaloid extract, a gradient program had to be developed and a long analysis time was needed to achieve a good separation of the different alkaloids of the extract (Fig. **2**). Under these conditions, the resolution values between each aporphine peak-pairs were higher than 1.5 and no significant interferences were observed at the time of retention of the different alkaloids of interest.

Method validation

The overall validation strategy involves four steps: evaluation of the extraction efficiency, determination of cassythine content in one batch of alkaloid extract, a pre-validation phase and the validation phase. As no alkaloid identified in the tested samples of *C. filiformis* [4] was commercially available, cassythine, purity 97%, one of the major aporphines from our batch of *C. filiformis* [4], was selected as reference standard to express the results. However, it was checked that the UV response factors of the other aporphine alkaloids were very close.

Table **2** Validation results

Validation Criterion		
Extraction efficiency (n^a = 3)		
Boldine (**1**)	Samples (n^a = 3)	Recovery (%; mean ± SD^b)
without matrix	1	100.2 ± 4.7
	2	104.2 ± 5.8
	3	99.1 ± 0.4
	Mean ± SD^b(N^c = 9)	101.2 ± 4.4
within matrix	1	82.1 ± 2.2
	2	71.9 ± 1.6
	3	71.0 ± 3.6
	Mean ± SD^b(N^c = 9)	75.0 ± 5.8
Trueness (k^d = 3; n^a = 3)		Cassythine (**3**)
Absolute bias : μg/ml (relative bias: %)		
20.0 μg/ml		1.25 (6.2)
166.7 μg/ml		0.19 (0.1)
509.3 μg/ml		25.62 (5.0)
Precision (k^d = 3; n^a = 3)		
Repeatability (RSD^e%)		
20.0 μg/ml		2.2
166.7 μg/ml		1.3
509.3 μg/ml		0.8
Intermediate precision (RSD^e%)		
20.0 μg/ml		3.9
166.7 μg/ml		2.6
509.3 μg/ml		0.8
Accuracy (k^d = 3; n^a = 3)		
β-expectation confidence limit (μg/ml)		
20.0 μg/ml		19.2 – 23.3
166.7 μg/ml		155.4 – 178.3
509.3 μg/ml		526.8 – 543.1

[a] Number of replicates (n).
[b] Standard deviation (SD).
[c] Total number of experiments (N).
[d] Number of series (day) (k).
[e] Standard deviation (RSD).

Determination of the extraction efficiency: The efficiency of the standardized extraction method [16] was evaluated by using boldine (1) which is a commercialized aporphine absent from the plant but structurally related to the aporphines of *C. filiformis* (see Fig. **1**). As shown in Table **2**, the recovery rate was first evaluated by means of samples without matrix. The mean recovery was about 101% showing that the extraction of boldine alone is complete. Then, the same amount (13 mg) of boldine was added to three 50 g samples of *C. filiformis* and extracted by the standardized procedure. The mean recovery was about 75% (Table **2**). It can be deduced that the plant material influenced the level of recovery of the extraction. However, as the relative standard deviation values for the mean recoveries (within and without matrix) were very close, this process was shown to be sufficiently reproducible.

Determination of cassythine content in alkaloid extract and pre-validation phase: In order to determine the amount of cassythine in the alkaloid extract (batch 1) two different quantitative methods were used. In the first technique, a calibration curve with one concentration level of about 1000 μg/mL of pure cassythine dissolved in methanol was used (n = 3) [17]. The following regression equation was found: y = 62430 x. A cassythine concentration in batch 1 of 156.3 ± 6.0 μg/mL was found. In order to confirm this result the standard addition method was then used [18], three different concentration levels of cassythine of about 20, 160 and 510 μg/ml were added to a methanolic solution of 1 mg/mL of the alkaloid extract of *C. filiformis* batch 1 (n = 3, k = 3). The following regression equation was found: y = 65763 x + 10228124 with r^2 = 0.9994. This equation was solved for x and a cassythine concentration in sample batch 1 of 155.3 ± 4.3 μg/mL was found. The concentrations calculated by these two different quantitative methods are not significantly different. These preliminary experiments seem to demonstrate that no matrix effect can be allotted to the alkaloid extract and that a higher concentration level (1000 μg/mL of cassythine in methanol) could be used for the routine analysis of the different batches of the plant materials. Nevertheless, these assumptions had been checked during the pre-validation phase [17]. The experiments achieved during this step also allow us to select the appropriate model for the calibration curve in the validation step. For that purpose, the novel approach based on two-sided 95% β-expectation tolerance intervals for total measurement error – including both bias and precision – of the calibration standards has been used [17]. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e., 15% according to the Food and Drug Administration (FDA) document [19]. As illustrated in Fig. **3**, the most suitable accuracy profile was the quadratic regression model. However, except for the accuracy profile corresponding to the linear regression model, all the other profiles were within the acceptance limits (cf., Fig. **3A, C, D** and **E**). Consequently a calibration curve built from the highest concentration level (1000 μg/mL) of cassythine in methanol was selected for the validation step. Considering the time spent for the sample preparation and the analysis of the extracts, this choice was of particular interest. Indeed, this approach not only allowed us to reduce the number of calibration standards and their analysis time but also to avoid the preparation of the calibration standards in the matrix. In addition,

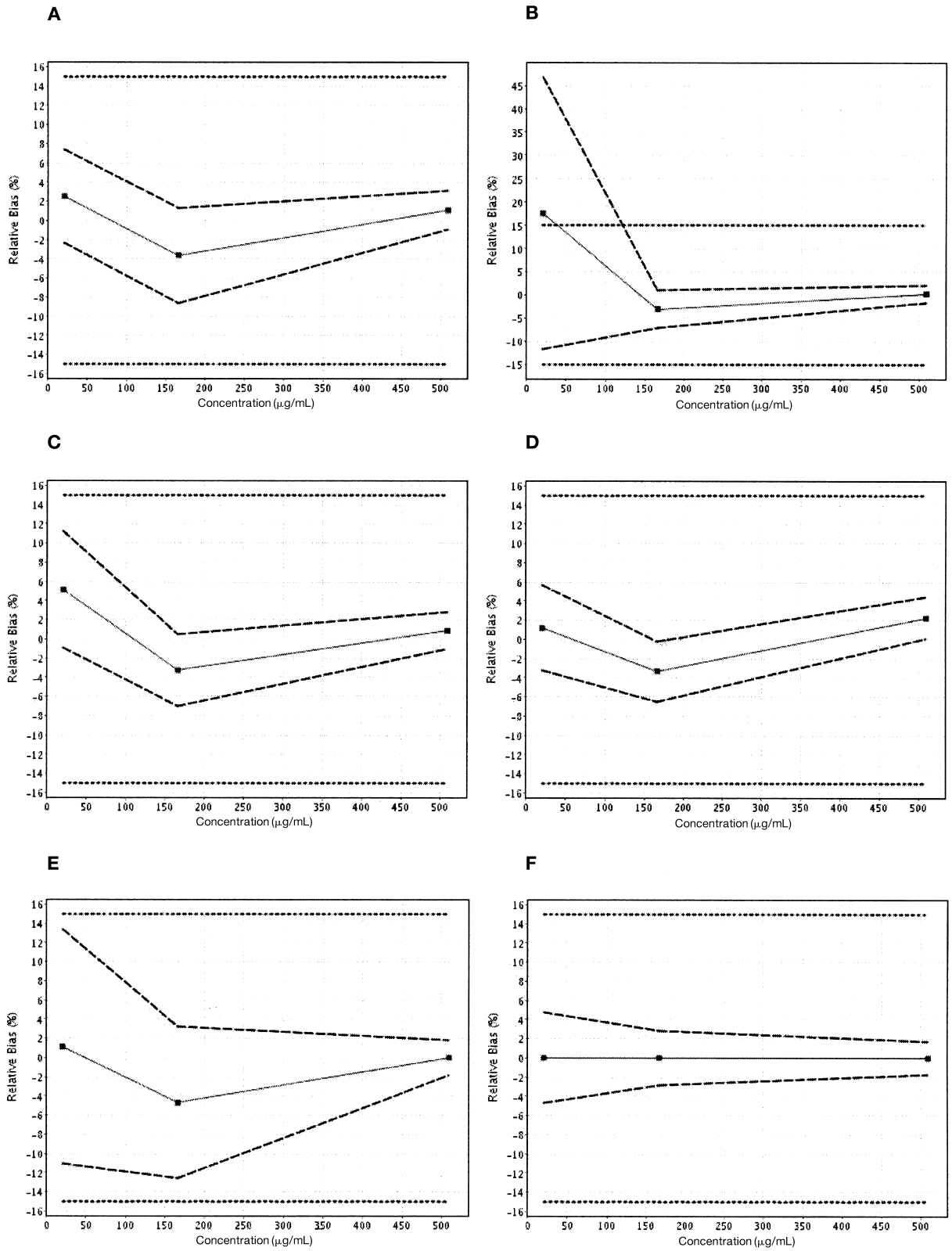


Fig. **3** Accuracy profiles of cassythine (concentration in µg/mL) using (A) a weighted linear regression model with a weight equal to 1/X, (B) a linear regression model, (C) a linear regression model after square root transformation, (D) a linear regression model after logarithm transformation, (E) a linear regression through 0 fitted with only the highest concentration level, (F) a quadratic regression.

these pre-validation experiments confirmed those performed during the determination of cassythine in a batch of alkaloid extract.

Validation phase: The stability [19] of the analyte was first checked. No significant degradation of the stock solution of cassythine and the solution of the alkaloid extract stored for 30 days at 4 °C was observed.

The selectivity [16], [17] of the method was studied by analyzing several individual batches of *C. filiformis*. A typical chromato-

gram of alkaloid extract is presented in Fig. **2**. Peaks were identified by comparison of their retention time (R_T) with the corresponding pure compounds previously isolated from *C. filiformis* and HPLC-MS analysis at different levels of the peaks [14]. The HPLC-MS data confirmed that no interferences were observed at the time of retention of the different alkaloids.

The trueness, precision and accuracy [17] results are presented in Table **2**. Compared to the regulatory requirements fixed [19], the bias did not exceed the values of 15 %, the RSD values were relatively low – less than 4 % for the lowest concentration level of the range – and the proposed method was accurate, since the different confidence limits of the bias did not exceed the acceptance limits irrespective of the concentration level.

The method linearity [16], [17] was demonstrated by the following regression equation: $y = 1.054\ x - 3.545$ with $r^2 = 0.9995$.

The limit of quantitation (LOQ) was fixed to 20 μg/mL, i.e., the smallest concentration level quantitatively determined with a well defined accuracy [17] (Table **2**) and the limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression [18]. By applying this computation method, the LOD was equal to 13 μg/mL.

Finally, due to the unknown amount of alkaloid expected in the plant materials, the influence of the dilution procedure, which is intended to be used in routine for samples with a concentration higher than the upper limit of the range, has to be checked [17]. Two dilution factors (2 and 5 for concentration levels of 1000 and 2500 μg/mL, respectively) were investigated and no significant effect was observed. The mean values (recovery ± SD) for dilution factors 2 and 5 were 101.6 ± 2.1 and 102.0 ± 3.8 %, respectively.

Application to samples of *C. filiformis*

Alkaloids were identified by comparison of their retention times, UV and mass spectra before quantitation. According to the validation step, a linear calibration curve passing through zero built from one concentration of pure cassythine (1000 μg/mL) was used for calibration. 1 mg/mL solutions of alkaloid extracts of each sample of *C. filiformis* obtained by the standardized protocol were injected three times and analyzed by HPLC-UV. In each sample, the seven major separated aporphines were quantified and expressed as cassythine. The amount (μg aporphines expressed in cassythine by g of dried plant of *C. filiformis*) of quantifiable aporphines in *C. filiformis* was calculated with the following equation:

$$Aporphine\ (\mu g/g) = \left[\frac{C\ x\ Q}{W}\right] x\ \frac{1}{f}$$

where Q is the amount (mg) of the total alkaloid extract obtained by the standardized extraction; W is the weight (g) of the dried plant *C. filiformis* used for the start of standardized extraction; f is level of extraction recovery; C (μg/ml) is the aporphine concentration calculated from the equation of the calibration curve of pure cassythine. The results of the quantification (mean of three injections) for the seven samples are given in Fig **4**.

Comparison of contents of the seven samples of *C. filiformis*

The comparison was first done individually for each aporphine. In other words the contents of a single compound are compared for all the samples injected. To allow this comparison a statistical ANOVA test was applied to the data. P values < 0.05 indicated a significant difference between samples. All batches were found to be significantly different from each other except samples 3 and 6 for actinodaphnine and samples 4 and 6 for dicentrine. The total amount of quantifiable aporphines was also found to be significantly different from each other. Even if the same standardized extraction was applied to all the batches of plant, the yields of total quantifiable aporphines could vary from 0.11 to 0.43 % of dried plant. As can be seen from Fig. **4**, the species seems to have an effect on the total amount of quantifiable aporphines as exemplified by samples 1 and 2 having a high yield even if the season of collection changed. The same fact can be observed for samples 4 and 5, this time with a lower yield. The family may also have an effect as all samples from one family (Anacardiaceae) (samples 3, 4, 5) have a low content. The host plant and the period of the collection had both an effect on the quantity of aporphines and their relative proportions but a clear relationship according to the general effect of the season or the host plant cannot be deduced. Samples collected on the same host

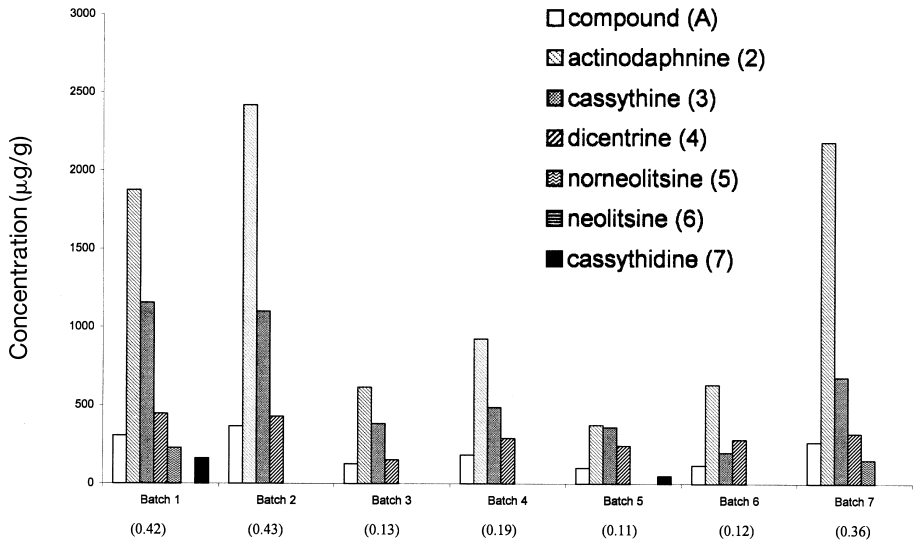


Fig. **4** Different concentrations (μg aporphines expressed as cassythine by g of dried plant of *C. filiformis*) of quantifiable aporphines in the seven batches analyzed of *C. filiformis*. Values in parenthesis are the total alkaloid content in each batch expressed in percentage (%)

plant but at diverse seasons of the year were all different. The same facts were observed for samples collected at the same period but on different host plants (variations are more important). Nevertheless the four major aporphines with actinodaphnine as the dominant one, are the same in all samples (Fig. **4**), but in variable proportions. According to the literature this does not seem to be the case with samples from other continents for which the major aporphines are different [1], [2], [3]. This may be due to an additive effect of the soil. We plan now to apply the validated procedure to other samples of *C. filiformis* from different countries to obtain further information on the role of these factors on the aporphine contents.

In conclusion, a selective and accurate procedure based on an alkaloid extraction coupled to an HPLC-UV-MS system has been developed for the determination of the major aporphines in *C. filiformis*. The extraction step and the chromatographic conditions were optimized in order to improve the selectivity of the procedure which was fully validated using cassythine as reference standard, one of the major aporphines. Finally, the procedure developed was validated and successfully applied to the determination and quantification of these aporphines in seven different batches of *C. filiformis*. Due to the frequent use of this plant in traditional medicine, this method may be used to complete this preliminary study by a long-term follow-up study.

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