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Validation of a method for the determination of sterols and triterpenes in the aerial part of *Justicia anselliana* (Nees) T. Anders by capillary gas chromatography

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ABSTRACT

An accurate and sensitive method, combining soxhlet extraction, solid phase-extraction and capillary gas chromatography is described for the quantitative determination of one triterpene (lupeol) and three sterols (stigmasterol, campesterol and β-sitosterol) and the detection of another triterpene (α-amyrin) from the aerial part of *Justicia anselliana*. This is the first method allowing the quantification of sterols and triterpenes in this plant. It has been fully validated in order to be able to compare the sterol and triterpene composition of different samples of *J. anselliana* and therefore help to explain the allelopathic activity due to these compounds. This method showed that the aerial part of *J. anselliana* contained (292 ± 2) mg/kg of lupeol, (206 ± 1) mg/kg of stigmasterol, (266 ± 2) mg/kg of campesterol and (184 ± 9) mg/kg of β-sitosterol.

1. Introduction

From the family Acanthaceae, *Justicia anselliana* (Nees) T. Anders is an afrotropical plant that is found in Mali (white stream level), Guinea (Agoué zone), Liberia (Cape Palmas), Ghana (Koug-Akuse), Nigeria (South and North), Togo (Toblékolé) and Benin (Zang-nanado) [1]. It is an aquatic plant which requires a lot of water and which is one of the 43 Acanthaceae species that constitutes the flora of Benin [2]. Leaves and roots are remedies for heart diseases and root decoction is used against the testicles inflammation [3].

In the Ouémé valley (south Benin) where *J. anselliana* is a weed of waste places and occupies about 12–18% of the uncultivated soils [4], it was identified as very dangerous for cowpea *Vigna unguiculata* (L.) Walp. According to the empirical observations described by farmers, this grass forms a very intimate association with cowpea and at an advanced stage of its development; it leads to the discoloration of cowpea leaves and puts an end to the cowpea development. Allelopathy is defined as the effect of one plant (or microorganisms) on the growth of another plant through the release of chemical compounds into the environment [5,6]. Previous works on the allelopathic effect of *J. anselliana* showed that alcoholic extracts of its aerial parts produced more significant effects on growth parameters such as seedlings, elongation and weight of the cowpea small plant, than extracts of the root [7]. Recently, we have isolated from the alcoholic extract of *J. anselliana*'s aerial parts, sterols and triterpenes which showed allelopathic effect on cowpea [8].

As the knowledge of the quantity of sterols and triterpenes in the crude extract of the plant can help to explain their activity, we decided to quantify these compounds in *J. anselliana*. Several methods in the literature report quantitative assay for the determination of these sterols and triterpenes [9–14], but none of the methods tested was suitable to our plant extracts as sterols and triterpenes peaks were not well separated from other constituents.

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Furthermore, most of these methods used derivatization of the compounds before GC analysis and/or were not fully validated. We report the development and validation of a new capillary gas chromatography technique for determination of sterols and triterpenes in the aerial part of *J. anselliana* by GC-FID without derivatization. This was possible by the development of low bleeding high temperature resisting columns such as the DB-XLB used here.

2. Experimental

2.1. Chemical and reagents

Dichloromethane (DCM), hexane, methanol and ethyl acetate of HPLC grade were obtained from Fisher Scientific (Tournai, Belgium). Triacontane (C_{30}) was obtained from Fluka AG (Buchs SG, Switzerland). The reference sterols and triterpenes were obtained from Extrasynthèse (Genay, France).

2.2. Plant material

Aerial parts of *J. anselliana* were collected in Ouémé valley (South Benin), in January 2005. Voucher specimen (nr: AA6295/UNB) were deposited at the National Herbarium of the University of Abomey - Calavi (Republic of Benin).

2.3. Internal standard

Triacontane (C_{30}) was used as internal standard at a concentration of 0.1 mg/ml in DCM. The responses taken into account were therefore the ratio of the area of the analyte peak over the area of the C_{30} peak.

2.4. Extraction of the plant material

Dried and powdered aerial parts (10 g) were extracted with DCM (400 ml) in a soxhlet apparatus for 8 h. The extract was dried under reduced pressure at 30 °C. This procedure was performed during 3 days ($k = 3$) on three different samples ($n = 3$) of the same batch of aerial parts in order to determine the extraction rate. The time required to obtain the highest extraction rate of sterols and triterpenes was selected by monitoring the evolution of sterol and triterpene responses at different times of soxhlet extraction. At each time tested (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 h), solvent from the soxhlet was replaced by fresh solvent. Solutions of soxhlet extracts were evaporated under reduced pressure at 30 °C, dissolved in equal volume (10 ml) of internal standard solution [0.1 mg/ml of triacontane (C_{30}) in DCM] and introduced into the GC system.

The recovery rate of soxhlet extraction was determined as follows. First, one sample (10 g) of aerial parts (S1) and another sample of 10 g of aerial parts spiked with 1 mg of lupeol or stigmasterol (S2L or S2S) were submitted to an 8 h soxhlet extraction with 400 ml of DCM. These solutions were evaporated to dryness and dissolved in 10 ml internal standard solution. Finally, 1 mg of lupeol or stigmasterol was directly dissolved in 10 ml internal standard solution to prepare solutions (S3L or S3S). 1 μl aliquots of S1, S2L, S2S, S3L and S3S were injected into the GC. The efficiency of the soxhlet extraction process was evaluated using lupeol and stigmasterol as test substances for triterpenes and sterols, respectively. The recovery rate was calculated using the following formula:

$$\frac{S2 - S1}{S3} \times 100.$$

2.5. Clean up of extract

50 mg of the dichloromethane extract dissolved in 1 ml DCM were applied onto a SPE cartridge filled with 1 g silica gel (Bakerbond spe Si, 1 g, J.T. Baker, Phillipsburg, NJ, USA). The SPE cartridge was dried for 2 h under vacuum at room temperature and then eluted with 5 ml of hexane (F1), followed by 8 ml of hexane–ethyl acetate (80–20) (F2) and finally with 10 ml of methanol (F3); eluate F2 contained the purified triterpenes and sterols. The fractions F2 were evaporated under a nitrogen flux to dryness at room temperature. All F2 fractions were dissolved in 5 ml internal standard solution before injection into the chromatographic system. In order to determine the absolute recoveries of the SPE purification, the SPE clean-up was performed on the three different DCM extracts obtained from three different samples ($n = 3$) of the same batch of plant material and during three different days ($k = 3$) as previously described. The nine F2 eluates were dissolved in 5 ml internal standard solution and, at the same time, 50 mg dichloromethane extract were dissolved directly in 5 ml internal standard solution [15–19]. Both solutions were injected into the GC system. For each sterol and triterpene, the area ratio in both solutions was measured and the corresponding recovery was calculated using the following formula:

$$\frac{\text{Area ratio of sterol or triterpene in F2}}{\text{Area ratio of sterol or triterpene in dichloromethane extract}} \times 100.$$

2.6. Total recovery

The total recovery of all sterols and triterpene of interest was determined as follows. 10 g of aerial parts (S1) and 10 g of aerial parts spiked separately with 1 mg of each sterol or triterpene ($S2_x$) were submitted to an 8 h soxhlet extraction with 400 ml of dichloromethane. These solutions were evaporated to dryness and submitted to SPE clean-up by applying 50 mg as previously described, to give F2(S1) and $F2_x(S2_x)$ dissolved in 10 ml of internal standard solution. Finally, 1 mg of each sterol or triterpene was directly dissolved in 10 ml of internal standard solution to prepare solution ($S3_x$). 1 μl of F2(S1), of each $F2_x$ ($S2_x$) and $S3_x$ were injected in triplicate. The total recovery was calculated using a total of nine samples obtained from three different samples of the same batch of aerial parts of *J. anselliana* and during three different days according to the following formula:

$$\frac{F2(S2) - F2_x(S1)}{S3_x} \times 100$$

where $F2_x$ ($S1_x$) is the response of each sterol or triterpene of interest in the F2 SPE cleaned fraction of plant extract, $F2_x$ ($S2_x$) is the response of each sterol or triterpene of interest respectively in the $F2_x$ SPE cleaned fraction of each plant extract spiked with each corresponding sterol or triterpene and $S3_x$ is the response of the same amount of corresponding sterol or triterpene alone in the internal standard solution.

2.7. GC-FID analysis

GC analysis were performed on a FOCUS GC (ThermoFinigan, Rodano, Italy) equipped with a 15 m × 0.25 mm i.d.; 0.25 μm film thickness DB-XLB column (J&W Scientific Column from Agilent Technologies, Folsom, CA, USA). Samples were introduced using splitless injection (injected volume: 1 μl, inlet temperature: 300 °C, split flow: 10 ml/min, splitless time: 0.80 min). Oven temperature was programmed as followed: temperature starts at 200 °C, increases of 10 °C/min to 320 °C (held for 25 min). Helium was used as carrier gas at a constant flow rate of 1.2 ml/min. Temperature of FID detector at 320 °C. Data were recorded and processed by ChromCard software (ThermoFinnigan, Rodano, Italy).

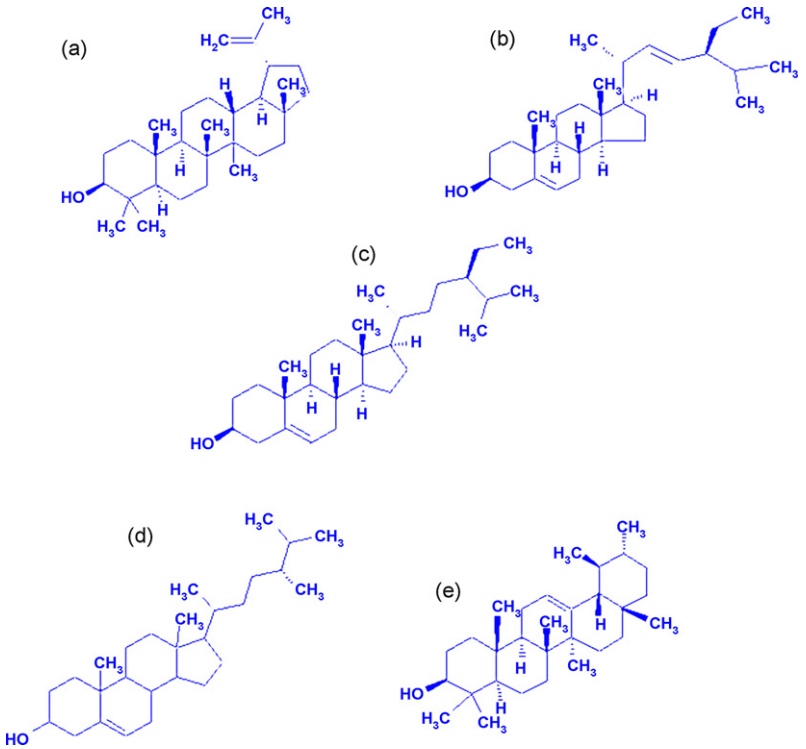

Fig. 1. Structure of quantified sterols (stigmasterol, campesterol, β-sitosterol) and triterpenes (lupeol, α-amyrine). A, lupeol; B, stigmasterol; C, β-sitosterol; D, campesterol; E, α-amyrin.

2.8. GC–MS analysis

In order to confirm the specificity and the selectivity of GC method, GC-EIMS analysis were performed on a TRACE GC 2000 series (ThermoQuest, Rodano, Italy), equipped with an autosampler AS2000 (ThermoQuest). The GC system was interfaced to a Trace MS mass spectrometer (ThermoQuest) operating in the electron-impact mode. The same capillary column (DB-XLB; column length 15 m × 0.25 mm with a 0.25 μm film thickness) was used with the same condition concerning injection, helium flow rate and oven temperature program. The GC interface temperature was set at 320 °C. The electron energy was 70 eV and the ion source was at 250 °C. Data were recorded and processed with Xcalibur 1.1 sofware (ThermoQuest). Mass spectra of peaks in the SPE fraction (F2) and in the dichloromethane extract were analysed and compared to reference compounds.

2.9. Peak identification

Sterols and triterpenes were identified by comparison of their retention times and mass spectra with the reference compounds.

2.10. Response factor

The response factors of all sterols and triterpenes of interest (Fig. 1) were evaluated and compared by injecting equal concentration of each sterol or triterpene (0.2 mg/ml in the internal standard solution).

2.11. Standard addition method

Four different concentrations of lupeol and stigmasterol (5, 25, 100, and 500 μg/ml; $m = 4$) were added to a 2 mg/ml solution of F2 eluate. These spiked eluates as well as the eluate itself (F2) were used to determine the amount of lupeol and stigmasterol in F2 by means of standard addition method but also to test a possible effect of the matrix in the quantitative determination of sterol and triterpenes. All these solutions were injected in triplicate ($n = 3$).

2.12. External calibration curve

An external calibration curve was constructed by injecting in triplicate ($n = 3$) four different concentrations (5, 25, 100, and 500 μg/ml in DCM + C_{30}) of lupeol and stigmasterol ($m = 4$). This curve was first used to estimate the amount of lupeol and stigmasterol in the final extract. Secondly, in the prevalidation and validation phases, this operation was repeated during 3 different days ($k = 3$) in order to determine the intra and interday precision, trueness and accuracy of the present method. Finally, this calibration curve was used in routine analysis.

2.13. Data analysis

Validation results and accuracy profiles were obtained using the Internet based software e.noval v1.1a (Arlenda, Liège, Belgium).

2.14. Bioassays with cowpea seeds

The allelopathic activities [20] of different compounds (at 200 ppm concentration) were tested on cowpea (*Vigna unguiculata* (L.) Walp) seeds [8].

3. Results and discussion

3.1. Soxhlet extraction and SPE clean-up

10 g of dried and powdered *J. anselliana* aerial part yielded 346.4 ± 1.1 mg ($n \times k = 3 \times 3 = 9$) crude dichloromethane extract and

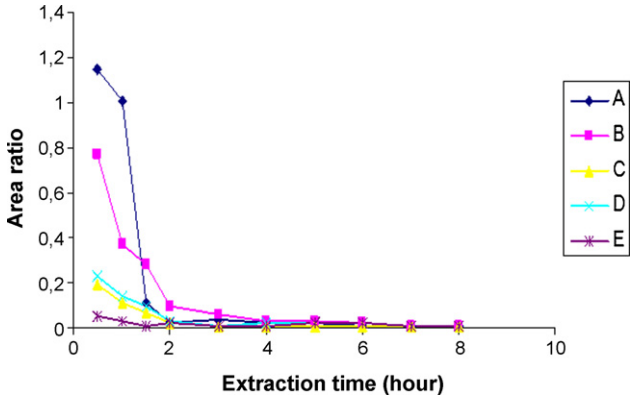

Fig. 2. Sterols and triterpenes responses in the dichloromethane versus extraction time. A, lupeol; B, stigmasterol; C, β-sitosterol; D, campesterol; E, α-amyrin.

50 mg of dichloromethane extract submitted to the SPE clean-up gave 12.7 ± 0.6 mg ($n \times k = 3 \times 3 = 9$) of SPE fraction F2.

The first step in developing a method for the quantitative determination of sterols and triterpenes in the aerial part of *J. anselliana* was to select the appropriate soxhlet extraction time, thus the response of each sterol and triterpene in the dichloromethane extract was evaluated after different extraction times. As illustrated in Fig. 2, a high amount of sterols and triterpenes was extracted already after 1/2 h. Afterwards, the extracted amount decreased rather quickly up to 2 h and reached a steady-state after 6 h. After 8 h, the extraction was almost complete and no significant difference between 7 and 8 h could be observed. An extraction time of 8 h in the soxhlet apparatus was finally selected in order to guarantee a good reproducibility for the process. As presented in Table 1, the mean extraction rates for lupeol and stigmasterol were respectively 93.8 ± 2.2% ($n \times k = 3 \times 3 = 9$) and 96.5 ± 2.0% ($n \times k = 3 \times 3 = 9$).

The second step of the method involved the SPE purification of dichloromethane extract in order to avoid interference from high-boiling-point compounds that could damage the GC column. Fig. 3 shows the chromatogram of the SPE eluate (F2) that contained the purified sterols and triterpenes. As can be seen in Table 1, good SPE clean-up recoveries (>80%) were obtained for the compounds investigated, even for α-amyrin whose resolution is incomplete.

3.2. Method validation

The validation involved three main steps: (I) determination of the content of stigmasterol (sterol) and lupeol (triterpene) in the plant material; (II) a pre-validation phase; and (III) a validation phase or formal validation step.

Table 1
Recovery of the extraction and clean-up steps.

Compounds	Soxhlet extraction Recovery ± S.D. (%) ($n \times k = 3 \times 3$)	SPE clean-up Recovery ± S.D. (%) ($n \times k = 3 \times 3$)	Total Total recovery ± S.D. (%) ($n \times k = 3 \times 3$)
Triterpenes			
Lupeol	93.8 ± 2.2	95.2 ± 1.0	90.3 ± 3.7
α-Amyrin		81.5 ± 4.2	80.0 ± 3.2
Sterols			
Stigmasterol	96.5 ± 2.0	93.9 ± 2.6	91.6 ± 3.4
Campesterol		85.6 ± 1.6	82.1 ± 3.2
β-Sitosterol		84.2 ± 5.2	81.8 ± 3.5

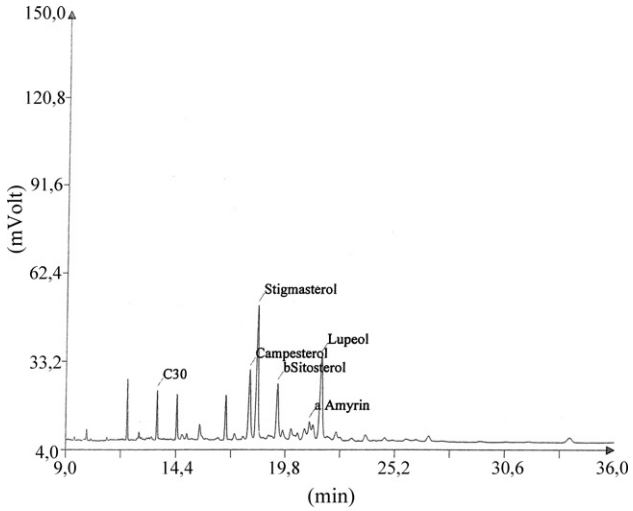

Fig. 3. Typical GC-FID chromatogram of the SPE (F2) eluate of *Justicia anselliana*. (1) Internal standard; (2) campesterol; (3) stigmasterol; (4) β-sitosterol; (5) α-amyrin; (6) lupeol (for chromatographic protocol see Section 2).

For the quantification of the sterols and triterpenes, equal concentrations of each compound (0.2 mg/ml) were injected into the GC system and their respective FID detector response factors determined. As presented in Table 2, no significant differences of response factors were observed between the sterols (0.55, 0.56 and 0.52) and between the triterpenes (0.97 and 0.96) but the response factors of the sterols were very different compared to the triterpenes. Consequently, stigmasterol could be used as reference for the quantitative determination of the other sterols and lupeol for other triterpenes.

Two different quantitative approaches were used to determine the amount of lupeol and stigmasterol in the dichloromethane extract. In the first technique, an external standard calibration curve using known amounts of lupeol and stigmasterol at four different concentration levels ranging from 5 to 500 μg/ml was constructed ($m = 4$). The following regression equations were found by plotting the peak area ratio (Y) versus the analyte concentration (X) in μg/ml: $Y = 0.0011X - 0.0005$ with $r^2 = 0.9999$ for lupeol and $Y = 0.0005X + 0.0071$ with $r^2 = 0.9998$ for stigmasterol. Using these equations, concentrations of 76.03 ± 0.83 μg/ml for lupeol and 77.80 ± 3.24 μg/ml for stigmasterol were found in the injected plant extract material studied. In order to confirm these results, the standard addition method was then used [21] in which four concentration levels (5, 25, 100, and 500 μg/ml) of lupeol and stigmasterol were added to the dichloromethane extract to give up the following regression equations: $Y = 0.0011X + 0.0815$ ($r^2 = 0.9999$) for lupeol and $Y = 0.0005X + 0.0309$ ($r^2 = 0.9995$) for stigmasterol. With these equations, concentrations of 76.30 ± 1.10 μg/ml for lupeol and 76.20 ± 2.90 μg/ml for stigmasterol were found in the injected plant extract. The comparison of the concentrations calculated by these two different quantitative approaches for lupeol (76.03 ± 0.83 μg/ml and 76.30 ± 1.10 μg/ml) and stigmasterol (77.80 ± 3.24 μg/ml and 76.20 ± 2.9 μg/ml) shows no significant difference from each other ($p < 0.05$). In addition, the slopes of the

Table 2
Comparison of response factors of the different compounds investigated.

Compounds	Triterpenes		Sterols		
	Lupeol	α-Amyrin	Stigmasterol	Campesterol	β-Sitosterol
Area ratio ($n = 3$)	0.97	0.96	0.55	0.56	0.52
S.D.	0.01	0.01	0.02	0.01	0.01

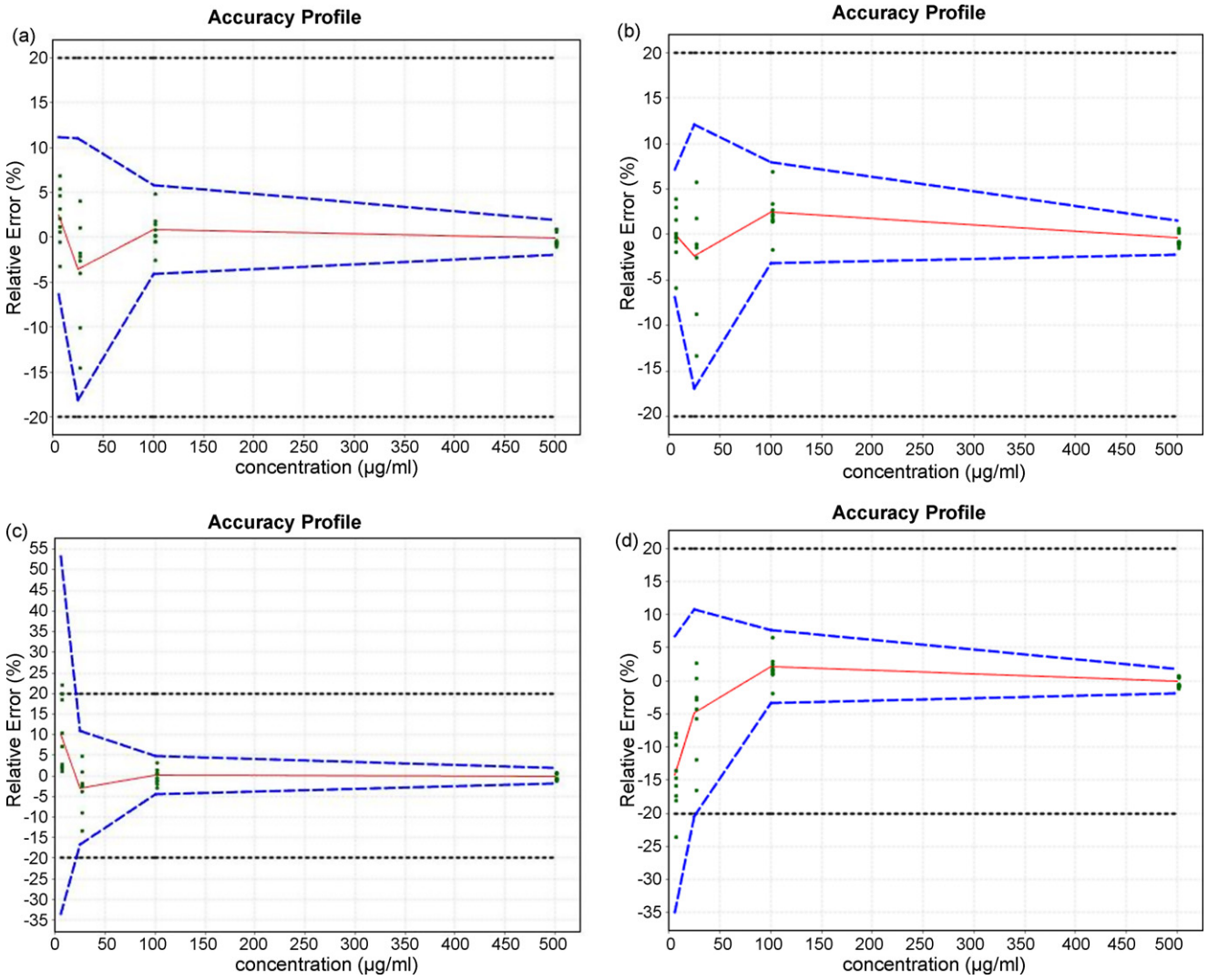

Fig. 4. Accuracy profiles of the concentration (µg/ml) of lupeol using (A) weighted "1/*X*" quadratic regression, (B) weighted "1/*X*" linear regression, (C) quadratic regression, and (D) linear regression. Relative bias (—); acceptance limits (■■■■■■); beta expectation tolerance limits (– – – –); relative back-calculated concentrations (■).

two calibration curves were equal. These preliminary experiments demonstrated that an external calibration curve of lupeol or stigmasterol could be used for the routine analysis and that no matrix effect could be allotted to the soxhlet extract (slope equality).

On the basis of the pre-validation protocol proposed by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) Commission [15–18], the experiments carried out during step (II) permitted analysis of the response function and selection of the appropriate model for the calibration curve for the validation step. For this purpose, three external calibration curves were constructed in the range 5–500 µg/ml [m (number of concentration levels) = 4]. Each concentration level was independently prepared three times (n = 3) and the external calibration curves were prepared during 3 days (k = 3). Different regression models were tested and their quality was assessed by means of the accuracy profiles as shown in Figs. 4 and 5 for lupeol and stigmasterol, respectively. The tested regression models were the simple linear, the weighted linear, the quadratic and the weighted quadratic. As can be seen from the accuracy profiles in Fig. 4, only two regression models allowed to accurately quantify lupeol in the whole range studied: the weighted (1/X) quadratic and the weighted (1/X) linear. This last model was selected as it diminished the bias observed at the lowest concentrations and as it is the simplest and easiest model to use. For stigmasterol, none of the tested models allowed to quantify accurately over the whole range studied as shown in Fig. 5. The chosen model was therefore the one which gave the lowest limit of quantification, with the smallest bias, i.e. the weighted (1/X) quadratic model.

During the validation step, several criteria were evaluated, such as sterols and triterpenes stability, selectivity of the method, response function, trueness, precision, accuracy, linearity and limit of detection and quantification [22–24]. For the sterols and triterpenes stability investigation, stock solutions in dichloromethane were stored for 31 days at 4 °C and injected into the GC–MS in order to follow the response of each compound and to detect apparition of new peaks. No significant degradation of sterols and triterpenes were observed.

In order to assure that the method could be used to quantify lupeol and stigmasterol with the other sterols and triterpenes in the presence of the other constituents present in the SPE elute (F2) and in the dichloromethane extract, the selectivity of the analytical method was investigated. No endogenous sources of interferences were observed at the retention times of the analytes in the F2(SPE) extract (Fig. 3).

As previously mentioned, for the determination of the response function, a 1/X weighted linear regression (for lupeol) and 1/X

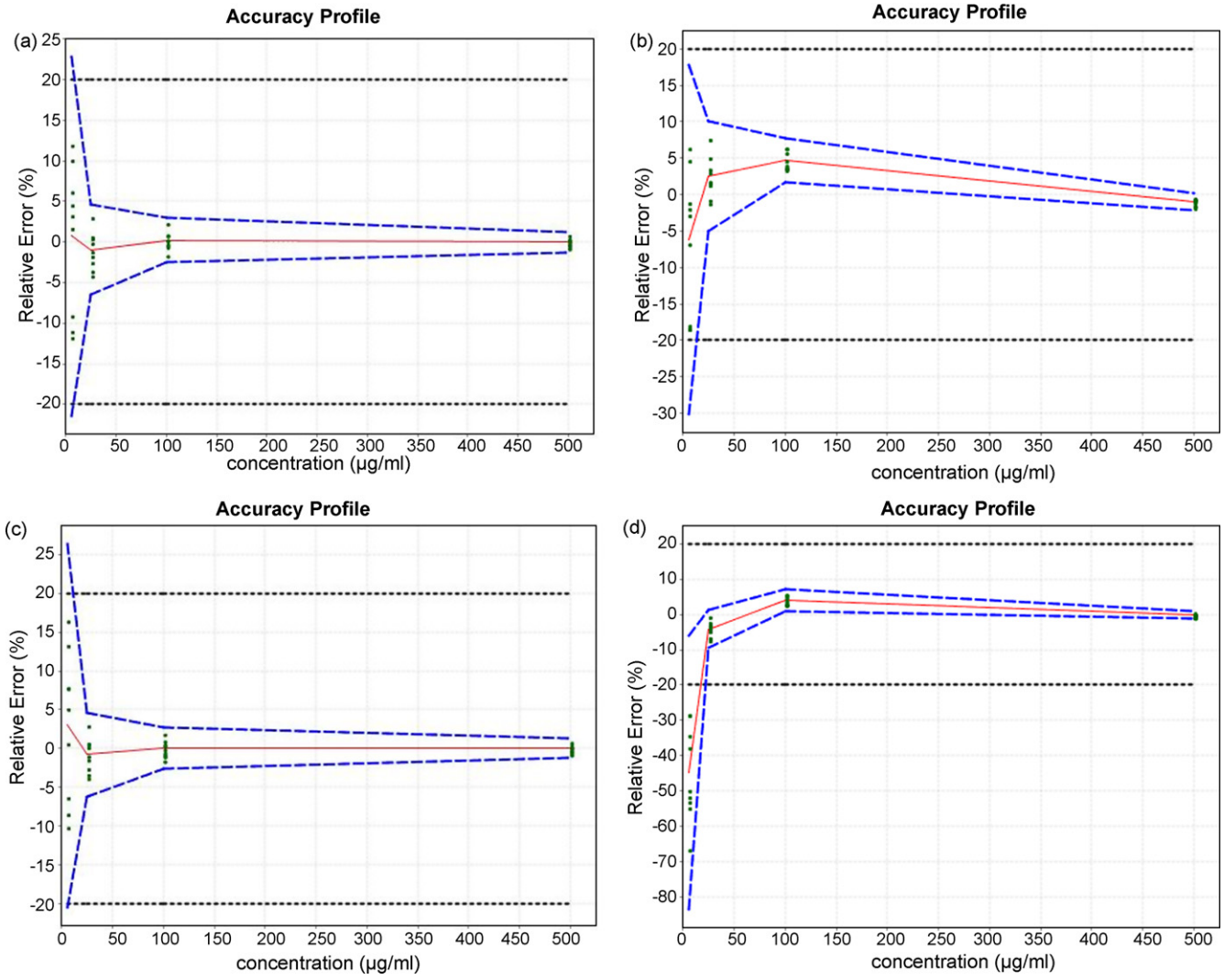

Fig. 5. Accuracy profiles of the concentration (μg/ml) of stigmasterol using (A) weighted "1/*X*" quadratic regression, (B) weighted "1/*X*" linear regression, (C) quadratic regression, and (D) linear regression. Relative bias (—); acceptance limits (■■■■■■); beta expectation tolerance limits (– – – –); relative back-calculated concentrations (■).

weighted quadratic regression (for stigmasterol) with four concentration levels (5, 25, 100, and 500 μg/ml) were employed. The determination coefficient (r^2) obtained for the regression line of lupeol and stigmasterol demonstrated the excellent relationship between peak area ratio and concentration as shown in Tables 3 and 4. The validation was performed on three different days ($k = 3$) and using validation standards prepared at four concentration levels ($m = 4$) ranging from 5 to 500 μg/ml in the plant matrix. Each validation standards was independently prepared in triplicate each day of the validation.

As can be seen from Tables 3 and 4, trueness was expressed in terms of absolute bias (in μg/ml) or relative bias (%) and was assessed by means of the validation standard in the plant matrix at four concentration levels ranging from 5 to 500 μg/ml ($k = 3$, $n = 3$). The mean values were very close to the theoretical concentrations, illustrating the good trueness of the method.

For each concentration level of the validation standard, the variances of repeatability and of intermediate precision, as well as the corresponding relative standard deviation (R.S.D.), were computed from the estimated concentration. As can be seen in Table 3, the R.S.D. values were relatively low, less than 5% except at the concentration level of 25 μg/ml for lupeol (R.S.D. = 5.9%) and at the lowest concentration of the range (5 μg/ml) for stigmasterol (R.S.D. = 9.1% - Table 4). It should be noted that the variability was mainly due to interday rather than intraday variation, illustrating the good precision of the developed method.

The accuracy of the method was also evaluated: Tables 3 and 4 show the upper and lower β-expectation tolerance limits [17–18,22–24] expressed in μg/ml and presented as a function of the introduced concentrations. As can be seen from these results, the proposed method was accurate, since the different tolerance limits did not exceed the acceptance limits (15%) for each concentration level of lupeol. For stigmasterol, the method was accurate for all concentration level tested except the lowest one (5 μg/ml). Therefore, the range in which this method will give accurate measurements was reduced from 8.148 to 500 μg/ml.

In order to demonstrate the linearity of the results, a regression line was fitted on the estimated or back-calculated concentrations of all the series ($N = 36$) as a function of the introduced concentrations by applying a line regression model based on the least squares method. The following regression equation was found: $Y = 0.7227 + 0.9955X$ (with $r^2 = 0.9998$) for lupeol and $Y = 1.626 \times 10^{-3} + 1.000X$ (with $r^2 = 1.0000$) for stigmasterol,

Table 3
Validation results for the developed method for lupeol.

Criterion of validation[a]					
Response function ($k=3$, $m=4$, $n=3$)	Range (μg/ml)	5–500	5–500	5–500	
Weighting factor: 1/X	Slope	1.12×10^{-3}	1.13×10^{-3}	1.12×10^{-3}	
	Intercept	-2.59×10^{-3}	-2.56×10^{-3}	-2.30×10^{-3}	
	r^2	0.9999	0.9997	0.9999	
Trueness ($k=3$, $n=3$)	Mean introduced concentration (μg/ml)	Mean back-calculated concentration (μg/ml)	Absolute bias (μg/ml)	Relative bias (%)	
	5.0	5.0	5.4×10^{-03}	0.1	
	25.0	24.4	−0.6	−2.4	
	100.0	102.4	2.5	2.4	
	500.0	498.1	−1.9	−0.4	
Precision ($k=3$, $n=3$)	Concentration (μg/ml)	Repeatability (R.S.D.%)	Intermediate precision (R.S.D.%)		
	5	2.8	2.9		
	25	5.9	5.9		
	100	2.2	2.2		
	500	0.8	0.8		
Accuracy ($k=3$, $n=3$)	Concentration (μg/ml)	β-Expectation tolerance limit (μg/ml)			
	5	4.65–5.36			
	25	20.76–28.04			
	100	96.90–107.9			
	500	488.7–507.5			
Linearity ($k=3$, $m=4$, $n=3$)	Range (μg/ml)	5–500			
	Slope	0.9955			
	Intercept	0.7227			
	r^2	0.9998			
Limit of detection (μg/ml)		2.932			
Limit of quantitation (μg/ml)		5.00			

[a] k = number of days of analysis; m = number of concentration levels; n = number of independent replicates.

Table 4
Validation results for the developed method for stigmasterol.

Criterion of validation[a]					
Response function ($k=3$, $m=4$, $n=3$)	Range (μg/ml)	5–500	5–500	5–500	
Weighting factor: 1/X	Slope	5.10×10^{-4}	4.98×10^{-4}	4.97×10^{-4}	
	Intercept	5.52×10^{-3}	5.99×10^{-3}	6.04×10^{-3}	
	Quadratic term	-8.68×10^{-8}	-6.32×10^{-8}	-6.01×10^{-8}	
	r^2	0.9999	0.9999	1.0000	
Trueness ($k=3$, $n=3$)	Mean introduced concentration (μg/ml)	Mean back-calculated concentration (μg/ml)	Absolute bias (μg/ml)	Relative bias (%)	
	5.0	5.1	3.6×10^{-02}	0.7	
	25.0	24.8	−0.2	−0.9	
	100.0	100.2	0.2	0.2	
	500.0	500.0	-4.0×10^{-02}	−7.9	
Precision ($k=3$, $n=3$)	Concentration (μg/ml)	Repeatability (R.S.D.%)	Intermediate precision (R.S.D.%)		
	5	9.1	9.2		
	25	2.3	2.3		
	100	1.1	1.2		
	500	0.5	0.5		
Accuracy ($k=3$, $n=3$)	Concentration (μg/ml)	β-Expectation tolerance limit (μg/ml)			
	5	4.93–6.14			
	25	23.37–26.15			
	100	97.52–103.0			
	500	493.8–506.2			
Linearity ($k=3$, $m=4$, $n=3$)	Range (μg/ml)	5–500			
	Slope	1.0000			
	Intercept	1.626×10^{-03}			
	r^2	1.0000			
Limit of detection (μg/ml)		0.4537			
Limit of quantitation (μg/ml)		8.148			

[a] k = number of days of analysis; m = number of concentration levels; n = number of independent replicates.

where Y = back-calculated concentration (expressed in μg/ml) and X = introduced concentration (expressed in μg/ml).

The limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression [20]. By applying this method, the LOD of the developed method was found to be 2.932 μg/ml for lupeol and 0.4537 μg/ml for stigmasterol. As the accuracy profile was within the acceptance limits in the whole range of concentration tested for lupeol, the limit of quantitation (LOQ) was fixed at 5 μg/ml (i.e. the smallest concentration level investigated; Table 3) while it was determined as the smallest concentration within the acceptance limits for stigmasterol (8.148 μg/ml; Table 4).

3.3. Quantification of sterols and triterpenes

The external calibration curve (5, 25, 100, and 500 μg/ml) as mentioned above was used for quantification of sterols and triterpenes. The 1 μl aliquots of the SPE F2 eluate were analysed by GC-FID in triplicate (n = 3). No significant differences between the response factors of the different triterpenes were observed. However, the response factors of the sterol was different from those of triterpenes, the amount of each sterol and triterpene in the aerial part of *J. anselliana* was therefore calculated using the following formula:

$$\text{Sterol or triterpene(mg/g dry aerial part)} = \frac{C \times 5 \times 6.93}{10000 \times (R/100)}$$

where C is the concentration of triterpenes in μg/ml calculated from the equation of the $1/X$ weighted linear regression model or the concentration of sterols in μg/ml calculated from the equation of the $1/X$ weighted quadratic regression model, R is the total recovery (in percent) of corresponding sterol or triterpene (see Table 2), 100 is a factor necessary because R is in percentage and 5 is a dilution factor due to the dissolution of the whole SPE fraction F2 in 5 ml dichloromethane. Whilst C was calculated in μg/ml, the dilution factor 6.93 is required because only 50 mg from the 346.35 mg of dichloromethane extract were deposited on the SPE column, and 10,000 is the conversion factor of μg/10 g into mg/g. The results of quantification of the sterol and triterpenes in aerial parts of *J. anselliana* are presented in Table 5.

With this quantitative method, 1 g of *J. anselliana* contained 292.44 μg of lupeol, 266.37 μg of campesterol, 206.06 μg of stigmasterol and 184.25 μg of β-sitosterol. The comparison of these results with the allelopathic activities of these sterols at a concentration of 200 μg/ml each (Fig. 6, which confirmed the previous results [8]) showed that only lupeol, stigmasterol and campesterol can account, at least in part, for the allelopathic effects on cowpea (*Vigna unguiculata*) germination as α-amyrin (for which only detection was possible using the developed method) is not present in sufficient amount and β-sitosterol is much less effective.

Table 5
Amount of sterols (stigmasterol, campesterol, β-sitosterol) and triterpene (lupeol) in the aerial part of *J. anselliana*.

Compounds	Amount ± S.D. (mg sterol or triterpene/kg dried aerial part)
Triterpene	
Lupeol	292 ± 2
Sterol	
Stigmasterol	206 ± 1
Campesterol	266 ± 2
β-Sitosterol	184 ± 9

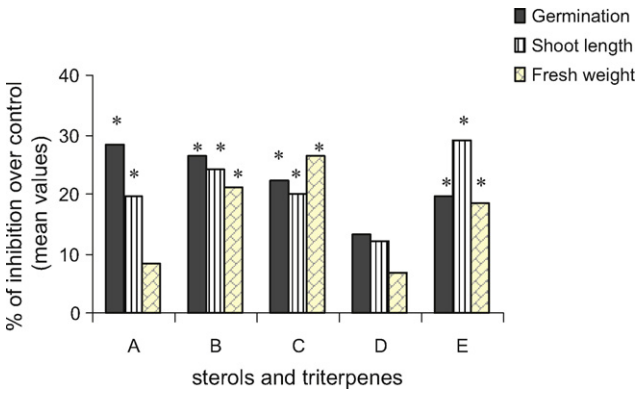

Fig. 6. Effects at 200 μg/ml of sterols and triterpenes quantified or identified in Justicia anselliana on cowpea (*Vigna unguiculata*). n = 6, *: p-value < 0.05. A: lupeol, B: stigmasterol, C: campesterol, D: β-sitosterol and E: α-amyrin.

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