PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **16**, 342–348 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002.pca.855

A validated method for the quantification of pimarane and trachylobane diterpenes in the leaves of *Croton zambesicus* by capillary gas chromatography

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A sensitive and accurate method, combining Soxhlet extraction, solid-phase extraction and capillary gas chromatography, is described for the quantitative determination of four new diterpenes (*ent*-trachyloban-3β-ol, *ent*-18-hydroxy-trachyloban-3-one, *ent*-trachyloban-3-one and isopimara-7,15-dien-3β-ol) from the leaves of *Croton zambesicus*. This is the first method describing the quantification of trachylobane diterpenes in a crude extract. It has been fully validated in order to be able to compare the diterpene composition in other samples of *C. zambesicus*, which is an important source of trachylobanes. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: Capillary gas chromatography; diterpenes; trachylobane; pimarane; *Croton zambesicus*; Euphorbiaceae.

INTRODUCTION

Croton zambesicus Muell. Arg. (Euphorbiaceae) (Syn. *C. amabilis* Muell. Arg., *C. gratissimus* Burch.) is a Guineo-Congolese species that is widespread in Tropical Africa (Adjanohoun *et al.*, 1989) and grows as a shrub or small tree reaching 10 m in height. A decoction of the leaf is used in Benin as an anti-hypertensive or anti-microbial (for urinary infections) agent, and also to treat fever associated with malaria (Watt and Breyer-Brandwikj, 1962; Adjanohoun *et al.*, 1989). *Croton* species are well known for their diterpenoid content, and various types of this class of compound, including phorbol esters, clerodane, labdane, kaurane, trachylobane and pimarane, have been isolated from the genus. Few phytochemical studies have been made on *Croton zambesicus* and only clerodane, labdane and trachylobane diterpenes have been isolated from the stem bark of this plant (Ngadjui *et al.*, 2002). Recently, however, we have isolated new cytotoxic pimarane and trachylobane diterpenes from a dichloromethane extract of the leaves of *C. zambesicus* (Block *et al.*, 2002, 2004).

Trachylobanes are poorly studied diterpenes that are characterised by a pentacyclic carbon skeleton with a tricyclo 3,2,1,0 octane system for the rings C, D and E. Since their first identification, they have been isolated from a wide range of families and genera of plants, but mainly from the Asteraceae (*Helianthus*), Labiateae (*Sideritis*), Annonaceae (*Xylopia*) and Euphorbiaceae (*Croton*) (Fraga, 1994). Since, to the best of our knowledge, no quantitative method for the determination of this type of diterpene has been reported in the literature, we developed a capillary gas chromatography technique in order to determine the amounts of pimarane and trachylobane diterpenes in the leaves of *Croton zambesicus*. The method involved Soxhlet extraction followed by solid-phase extraction (SPE), which was optimised in order to eliminate impurities from the crude extract prior to GC determination of diterpenes. The sample preparation protocol allowed the separation and quantification of three trachylobane [*ent*-trachyloban-3β-ol (**1**), *ent*-18-hydroxy-trachyloban-3-one (**2**), *ent*-trachyloban-3-one (**3**)] and one pimarane [isopimara-7,15-dien-3β-ol (**4**)] diterpenes. The method was fully validated in order to be applied to the quantitative determination of diterpenes in different samples of *C. zambesicus*.

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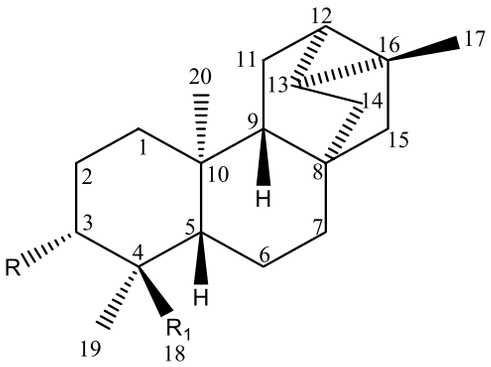
Contract/grant sponsor: Fonds Spécial de Recherche, Université Catholique de Louvain.
Contract/grant sponsor: Belgian National Fund for Scientific Research; Contract/grant number: 9.4553.04.
Contract/grant sponsor: European Commission; Contract/grant number: HPRN-CT-1999-00054.

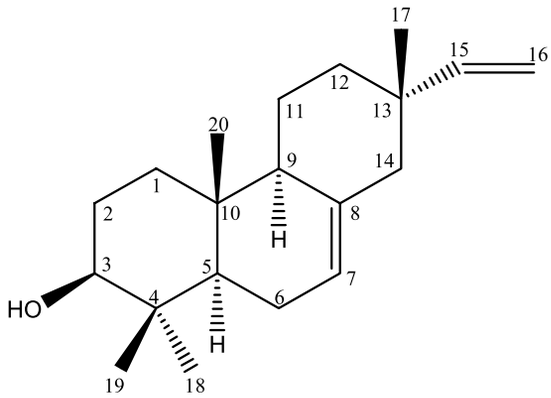
EXPERIMENTAL

Chemicals and reagents. Dichloromethane, hexane, methanol and ethyl acetate (HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Heneicosane (C_{21}) from Fluka (Buchs, Switzerland) was used as internal standard at a concentration of 0.1 mg/mL in dichloromethane. Reference diterpenes were previously isolated and identified from *C. zambesicus* in our laboratory (Block *et al.*, 2002, 2004).

Plant material. Leaves of *C. zambesicus* were collected on 6 February 2003 in Agon in the Atlantic Department in Benin (20 km north of Cotonou) and dried

Received 4 March 2004
Revised 30 September 2004
Accepted 10 October 2004





		R	R_1
1	*ent*-Trachyloban-3β-ol	OH	CH_3
2	*ent*-18-Hydroxy-trachyloban-3-one	=O	CH_2OH
3	*ent*-Trachyloban-3-one	=O	CH_3

4 Isopimara-7,15-dien-3β-ol

immediately in the dark at 25°C. Plant material was identified by the botanist Professor V. Adjakidje (Université d'Abomey-Calavi, Benin) and a voucher specimen deposited in the herbarium of the National Botanical Garden of Belgium in Meise (reference BR S.P. 848.108).

Extraction of the plant material. Powdered dried leaves (10 g) were extracted with dichloromethane (400 mL) in a Soxhlet apparatus for 8 h and the resulting extract was dried under reduced pressure at 30°C. This procedure was performed in triplicate in order to determine the extraction rate. The optimum extraction time was determined by evaluating the amount of diterpene extracted after different times of Soxhlet extraction. After each time period (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 h) solvent from the Soxhlet was replaced by fresh solvent. The Soxhlet extracts were evaporated under reduced pressure at 30°C, dissolved in equal volumes (200 mL) of internal standard solution [0.1 mg/mL of heneicosane (C_{21}) in dichloromethane], and introduced into the GC system. The recovery rate of the Soxhlet extraction was determined as follows. One sample (10 g) of leaves (S1) and another of leaves (10 g) spiked with 1 mg of diterpene 3 (S2) were submitted to an 8 h Soxhlet extraction with 400 mL dichloromethane. These solutions were evaporated to dryness and dissolved in 10 mL internal standard solution. A third solution (S3) was prepared by dissolving 1 mg of 3 in 10 mL internal standard solution. Aliquots (1 µL) of S1, S2 and S3 were injected into the GC in triplicate. The efficiency of the Soxhlet extraction process was evaluated using **3** as test substance and was calculated using the formula:

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

where *S*1 is the response of **3** in the plant extract, *S*2 is the response of **3** in the plant spiked with 1 mg of **3**, and *S*3 is the response of the same amount of **3** alone in the internal standard solution.

Clean-up of the extract. The dichloromethane extract (50 mg) was dissolved in 1 mL dichloromethane and applied to an SPE cartridge filled with 1 g silica gel (Bakerbond spe Si; J. T. Baker). The SPE cartridge was dried for 2 h under vacuum at room temperature and then eluted with 5 mL hexane (F1), followed by 8 mL hexane:ethyl acetate (85:15) (F2) and finally with 10 mL methanol (F3); eluate F2 contained purified diterpenes. This operation was performed in triplicate on the same dichloromethane extract, and fractions F2 were evaporated to dryness under a stream of nitrogen at room temperature. All F2 fractions were dissolved in 5 mL internal standard solution before injection into the GC. In order to determine the absolute recovery of the SPE purification, the SPE clean-up was performed in triplicate as previously described. The 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 (Chapuzet *et al.*, 1997; Hubert *et al.*, 1999, 2003). Both solutions were injected into the GC system. For each diterpene, the area ratio in both solutions was measured and the corresponding recovery was calculated from the following formula:

$$\frac{\text{Area ratio of diterpene in F2}}{\text{Area ratio of diterpene in dichloromethane extract}} \times 100$$

GC-FID analysis. GC analyses were performed on a FOCUS GC (ThermoFinnigan, Rodano, Italy) equipped with a J&W Scientific (Folsom, CA, USA) DB-XLB column (15 m × 0.25 mm i.d.; 0.25 µm film thickness). Samples were introduced using splitless injection (1 µL injection volume; 250°C inlet temperature; 10 mL/min split flow; 0.02 min splitless time). The column oven temperature was programmed as follows: starting temperature 50°C, held for 1 min and then increased at 10°C/min to 200°C (held for 3 min), then increased at 3°C/min to 215°C, and finally at 10°C/min to 300°C and held for 5 min. Helium was used as carrier gas at a flow of 1.8 mL/min, and the temperature of the detector (FID) was set at 300°C. Data were recorded and processed using ChromCard software (ThermoFinnigan).

GC-MS analysis. In order to confirm the specificity and selectivity of the GC method, GC-EIMS analyses were performed using a ThermoQuest (Rodano, Italy)

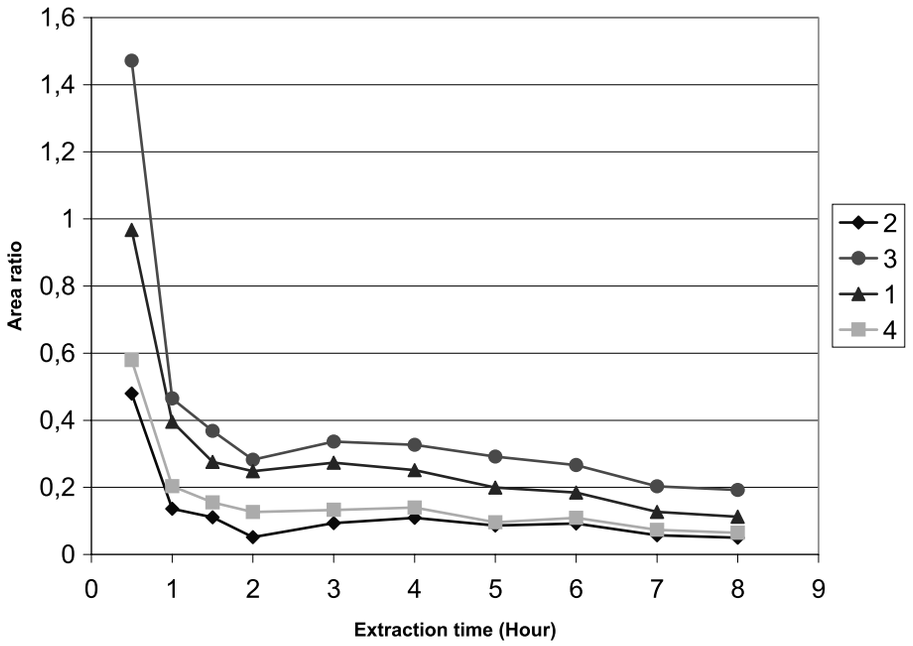


Figure 1. Diterpene responses in the dichloromethane extract of *C. zambesicus* after different times of Soxhlet extraction. **1**, *ent*-Trachyloban-3β-ol; **2**, *ent*-18-hydroxy-trachyloban-3-one; **3**, *ent*-trachyloban-3-one; and **4**, isopimara-7,15-dien-3β-ol.

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

	Soxhlet extraction	SPE clean-up	Total
Diterpenes[a]	Recovery ± SD (%) (n = 9)	Recovery ± SD (%) (n = 9)	Total recovery ± SD (%) (n = 9)
1	97.0 ± 2.7	100 ± 0.9	97.0 ± 3.6
2		51.4 ± 1.2	49.9 ± 3.9
3		96.9 ± 2.4	94.0 ± 5.1
4		96.6 ± 3.1	93.7 ± 5.8

[a] **1**, *ent*-Trachyloban-3β-ol; **2**, *ent*-18-hydroxy-trachyloban-3-one; **3**, *ent*-trachyloban-3-one; and **4**, isopimara-7,15-dien-3β-ol.

Trace GC 2000 instrument connected to a Trace MS detector. The capillary column and chromatographic protocols were as indicated above, and the operating conditions of the electron impact detector were 250°C source temperature and 310°C GC interface temperature. Data were recorded and processed using Xcalibur 1.1 software (ThermoQuest). The MS of peaks in the SPE fraction (F2) and in the dichloromethane extract were analysed and compared with reference compounds. Diterpenes were identified by comparison of their retention times and MS with the corresponding pure diterpenes previously isolated from *C. zambesicus* (Block *et al.*, 2002, 2004).

Data analysis. Validation data were recorded and processed using the e.noval internet validation package (version alpha 1.0 software; Arlenda, Liège, Belgium).

RESULTS AND DISCUSSION

Extraction and SPE clean-up

The first step in developing a method for the quantitative determination of pimarane and trachylobane diterpenes in the leaves of *Croton zambesicus* was to select the most appropriate Soxhlet extraction time, thus the response of each diterpene in the dichloromethane extract after different times of extraction was evaluated. As shown in Fig. 1, the bulk of the diterpenes had already been extracted after 0.5 h; after this time, the extracted amount decreased rather quickly up to 2 h, and then slowly to reach a steady-state after 7 h. After 8 h, the extraction was almost complete and no significant difference between 7 and 8 h could be observed. Consequently, an extraction time of 8 h in the Soxhlet apparatus was finally selected in order to guarantee a good reproducibility of the process. Figure 1 also shows that the other diterpenes present exhibited almost the same behaviour as **3**, suggesting that the extraction rate of **3** could be applied to these as well. As presented in Table 1, the mean extraction rate for diterpene **3** was in the order of 97.0 ± 2.7% [n (number of replicates) = 9].

The second step of the method involved the SPE purification of the dichloromethane extract in order to avoid interference from high-boiling-point compounds that could damage the GC column. As shown in the chromatograms (Fig. 2) of the dichloromethane extract and SPE F2 eluate, the fraction F2 contained the purified diterpenes. Very good recoveries (>95%) were

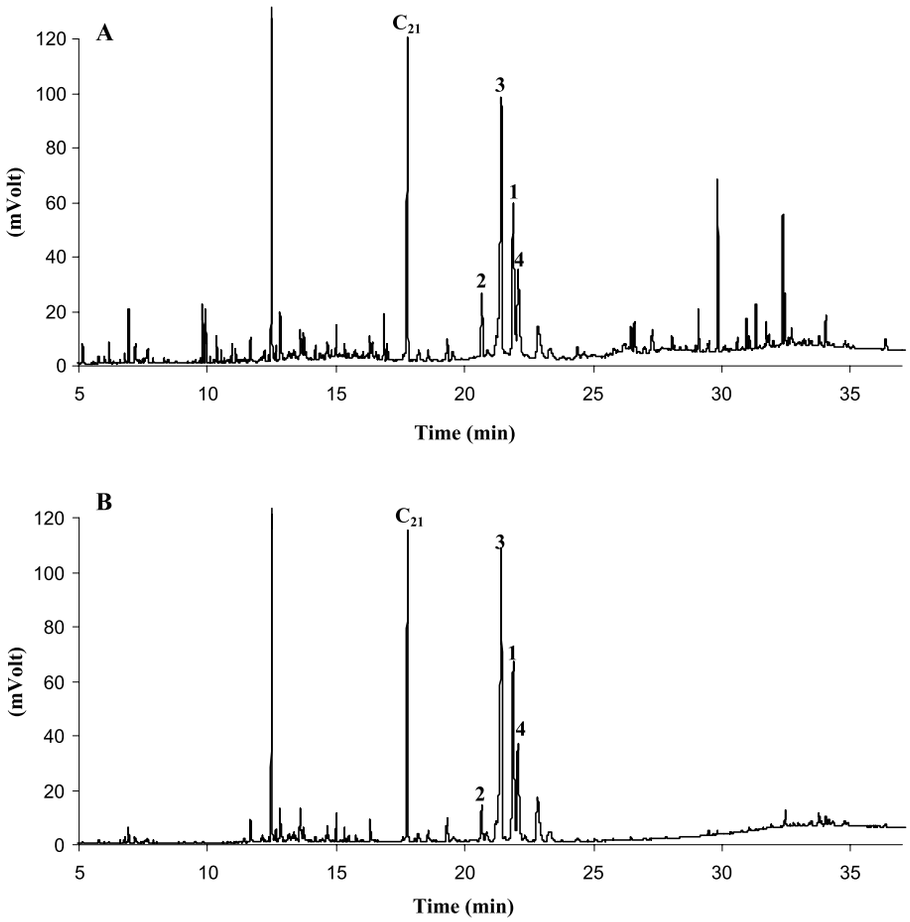


Figure 2. Typical GC-FID chromatograms of (A) the dichloromethane extract of *C. zambesicus* and (B) the SPE eluate F2. Key to peak identities: **1**, *ent*-trachyloban-3β-ol; **2**, *ent*-18-hydroxy-trachyloban-3-one; **3**, *ent*-trachyloban-3-one; **4**, isopimara-7,15-dien-3β-ol; $\mathbf{C_{21}}$, internal standard. (For chromatographic protocol see the Experimental section.)

obtained for most diterpenes (Table 1) except for **2**, which is the more polar diterpene.

Validation of the method

The validation involved three main steps: (i) determination of the content of diterpene **3** in the plant material; (ii) a pre-validation phase; and (iii) a validation phase or formal validation step. As none of the diterpenes present in *C. zambesicus* were commercially available, **3** was selected as the reference substance because the isolated amount of **3** was the highest of all diterpenes. For the quantification of the other diterpenes, equal concentrations of each diterpene (0.1 mg/mL) were injected into the GC system and their respective FID detector response factors determined. As presented in Table 2, no significant differences between the response factors of the diterpenes under investigation were observed and thus **3** could be further used as a calibration standard for the quantitative determination of the other diterpenes.

Two different quantitative methods were employed to determine the amount of **3** in the dichloromethane extract. In the first technique, an external standard calibration curve, using known amounts of **3** at three different concentration levels ranging from 5 to 500 µg/mL, was constructed (n = 3). The regression equation (y = 0.0076x – 0.0099; r^2= 0.9991) was determined by plotting the peak area ratio (y) vs the analyte concentration (x) in µg/mL, and using this equation, the concentration of **3** in the injected plant extract studied was found to be 99.4 ± 3.2 µg/mL. In order to confirm this result, the standard addition method was used (Miller and Miller, 2000) in which three concentration levels (5, 100 and 500 µg/mL) of purified **3** were added to the dichloromethane extract yielding the regression equation y = 0.0076x + 0.7612 (r^2 = 0.9999). This equation was solved for x, and a concentration of **3** of 100.4 ± 1.1 µg/mL was found in the injected plant extract. The concentrations determined by these two different quantitative methods were very close and not significantly different from each other (t_{cal} 1.38; t_{table} 2.16; α = 0.05). In addition, no significant difference between the slopes of the two

Table 2. Comparison of response factor of diterpenes 1–4 investigated

	Diterpenes[a]			
	1	**2**	**3**	**4**
Area Ratio (n = 3)	0.74	0.76	0.75	0.74
SD	0.01	0.04	0.02	0.01

[a] **1**, *ent*-trachyloban-3β-ol; **2**, *ent*-18-hydroxy-trachyloban-3-one; **3**, *ent*-trachyloban-3-one; and **4**, isopimara-7,15-dien-3β-ol.

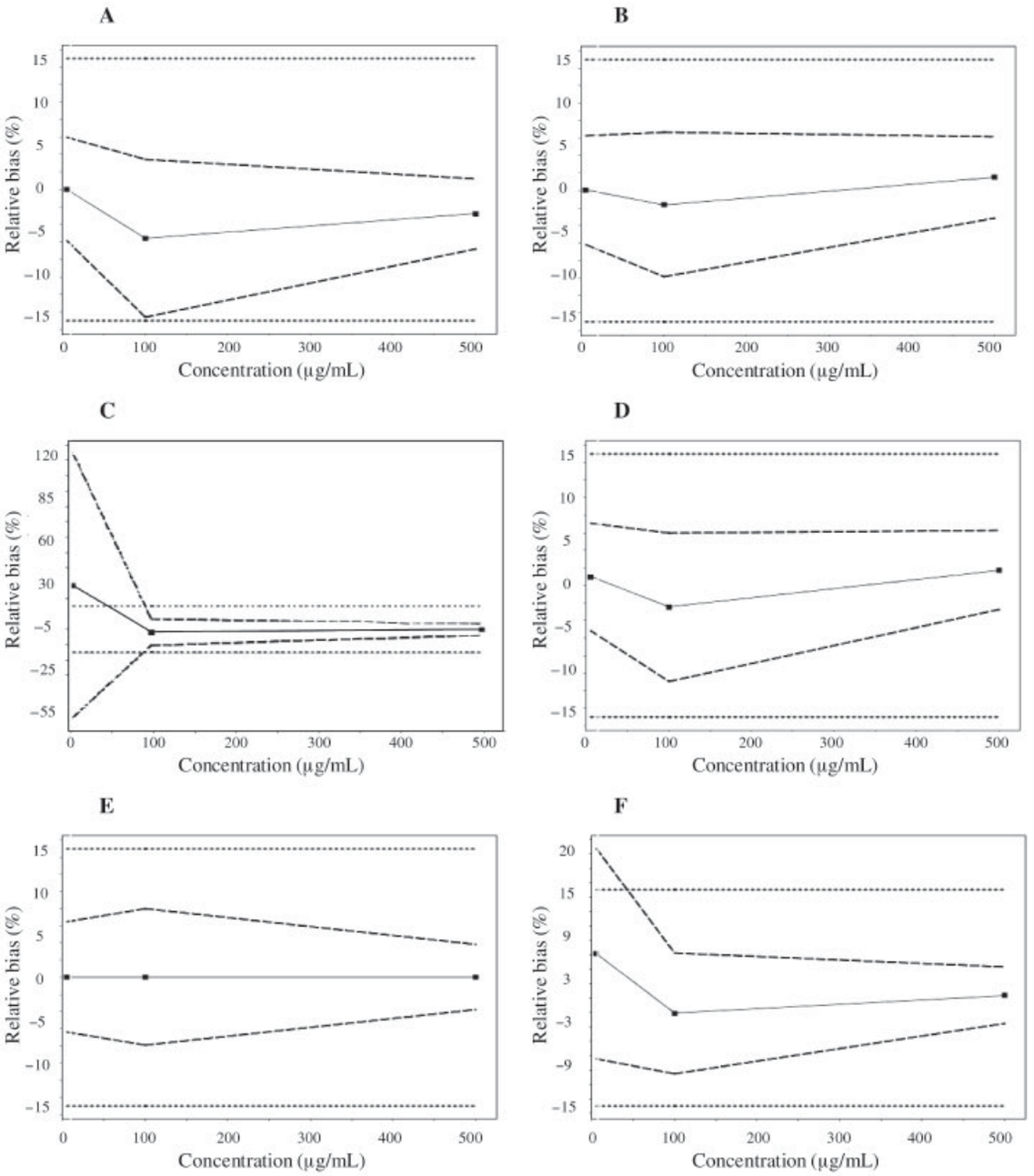


Figure 3. Accuracy profiles of the concentration (µg/mL) of *ent*-trachyloban-3-one (**3**) using (A) linear regression through 0 fitted with the highest concentration level only, (B) weighted linear regression model with a weight equal to $1/X^2$, (C) linear regression model, (D) linear regression model after logarithm transformation, (E) quadratic regression, and (F) linear regression model after square root transformation.

calibration curves was observed (t_{cal} 1.89; t_{table} 2.03; $\alpha = 0.05$). Thus, these preliminary experiments demonstrate that an external calibration curve of **3** 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 (Chapuzet *et al.*, 1997; Hubert *et al.*, 1999, 2003), 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 [k (number of analyses per day) = 3] were constructed in the range 5–500 µg/mL [m (number of concentration levels) = 3]. Each concentration was injected three times during 3 days. The accuracy profiles obtained by applying different regression models are presented in Fig. 3, and comparison of these led to the conclusion that the quadratic regression model [Fig. 3(E)] was the most suitable. However, except for the accuracy profiles corresponding to the linear regression model and the linear regression model after square root transformation, all other profiles were also within the acceptance limits [cf. Fig. 3(A), (B), (D) and (E)]. Regarding these latter accuracy profiles, the well-known weighted linear regression model was chosen for regression analysis.

During the validation step, several criteria were evaluated, such as stability of diterpenes, selectivity of the method, response function, trueness, precision, accuracy, linearity and limits of detection and quantitation. For the investigation of the stability of diterpenes, stock

Table 3. Validation results for the developed method

		Validation criterion[a]		
Response function ($k = 3$, $m = 3$, $n = 3$) weighting factor: $1/X^2$	Range (μg/mL)	5–500	5–500	5–500
	Slope	7.50×10^{-3}	7.45×10^{-3}	7.47×10^{-3}
	Intercept	9.77×10^{-4}	1.93×10^{-3}	2.06×10^{-3}
	r^2	0.9989	0.9990	0.9997
	Concentration	*Absolute bias (μg/mL)*	*Relative bias (%)*	
Trueness ($k = 3$, $n = 3$)	5 μg/mL	0.08	1.60	
	100 μg/mL	−0.26	−0.26	
	500 μg/mL	8.25	1.65	
	Concentration	*Repeatability (RSD %)*	*Intermediate precision (RSD %)*	
Precision ($k = 3$, $n = 3$)	5 μg/mL	4.62	4.62	
	100 μg/mL	1.63	1.63	
	500 μg/mL	0.25	0.41	
	Concentration	*β-Expectation tolerance limit (μg/mL)*		
Accuracy ($k = 3$, $n = 3$)	5 μg/mL	4.63–5.54		
	100 μg/mL	96.54–103.0		
	500 μg/mL	503.1–513.4		
	Range (μg/mL)	5–500		
Linearity ($k = 3$, $m = 3$, $n = 3$)	Slope	1.018		
	Intercept	−0.919		
	r^2	0.9999		
Limit of detection	1.1 μg/mL			
Limit of quanatitation	5.0 μg/mL			

[a] k = number of analysis per day; m = number of concentration levels; n = number of replicates.

solutions in dichloromethane were stored for 30 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 diterpenes could be observed.

The selectivity of the analytical method was investigated in order to assure that the method could be used to quantify **3** and the other diterpenes in the presence of other constituents in the SPE eluate (F2) and in the dichloromethane extract. No endogenous sources of interferences were observed at the retention times of the analytes (Fig. 2).

As previously mentioned, for the determination of the response function, a weighted linear regression with three concentration levels (5, 100 and 500 μg/mL) was employed (Table 3). The determination coefficient (r^2) obtained for the regression line of **3** demonstrated the excellent relationship between peak area ratio and concentration.

As can be seen from Table 3, trueness was expressed in terms of absolute bias (in μg/mL) or relative bias (%) and was assessed by means of validation standard in the matrix at three 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 time-dependent intermediate precision, as well as the corresponding relative standard deviation (RSD), were computed from the estimated concentrations. As can be seen from Table 3, the RSD values were relatively low, less than 5% for the lowest concentration of the range (5 μg/mL). It should be noted that the variance was mainly due to inter-day rather than intra-day variation, illustrating the good precision of the developed method.

The accuracy of the method was also evaluated: Table 3 shows the upper and lower β-expectation tolerance limits (Hubert *et al.*, 2003) 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 limits of the bias did not exceed the acceptance limits (15%) for each concentration level.

In order to demonstrate the linearity of the method, a regression line was fitted to the estimated or back-calculated concentrations of all the series ($N = 27$) as a function of the introduced concentrations by applying a linear regression model based on the least squares method. The following regression equation was found: $y = -0.9188 + 1.0018x$, where y = back-calculated concentration (μg/mL) and x = introduced concentration (μg/mL): the coefficient of determination (r^2) was 0.999.

Finally, the limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression (Miller and Miller, 2000). By applying this method, the LOD of the developed method was found to be 1.1 μg/mL. As the accuracy profile was within the acceptance limits, the LOQ was fixed at 5 μg/mL, i.e. the smallest concentration level investigated. Indeed, trueness, precision and accuracy were all demonstrated at this concentration level (Table 3).

Quantification of diterpenes

For quantification of diterpenes in plant samples, the same external calibration curve (5, 100 and 500 μg/mL) as mentioned above was used, and aliquots (1 μL) of the SPE F2 eluate were analysed by GC-FID in triplicate ($n = 3$). As no significant differences between the response factors of the different diterpenes were observed,

Table 4. Amount of diterpenes 1–4 in the leaves of *C. zambesicus*

Diterpene	Amount ± SD (mg diterpene/g dried leaves)
1 *ent*-Trachyloban-3β-ol	0.858 ± 0.004
2 *ent*-18-Hydroxy-trachyloban-3-one	0.248 ± 0.023
3 *ent*-Trachyloban-3-one	1.285 ± 0.008
4 Isopimara-7,15-dien-3β-ol	0.474 ± 0.002

the amount of each diterpene in the leaves was calculated using the following formula:

$$\text{Diterpene (mg/g dry leaves)} = \frac{C \times 5 \times 13.06}{10\,000 \times R}$$

where C is the concentration of diterpenes in µg/mL calculated from the equation of the weighted linear regression model, R is the total recovery of corresponding diterpene (see Table 2) 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 13.06 is required because only 50 mg from the 653 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 are presented in Table 4.

The present method has been developed and fully validated for the quantitative determination of four major biologically active new diterpenes previously isolated from a dichloromethane extract of the leaves of *Croton zambesicus*. Unfortunately, because of lack of reference compounds, not all the diterpenes present in this extract could be identified and quantified; nevertheless, our study is the first report concerning the quantification of trachylobane diterpenes. Quantification by GC-FID was preferred to HPLC-UV because of the poor UV absorption of trachylobanes and the poor separation of diterpenes 1 and 4. The results show that, together with β-caryophyllene, β-sitosterol and α-amyrin (identified by GC-MS and by comparison with reference compounds), the trachylobanes are the main compounds in the dichloromethane extract. The leaves of *C. zambesicus* seem to be an important source of trachylobane diterpenes, and it will be of interest to compare, using this validated method, the amount of these compounds in other samples of *C. zambesicus* collected at different periods and from different locations.

Acknowledgements

The authors wish to thank J. P. Van Heleputte and M. C. Crutzen-Fayt for their skilful technical assistance. The work was funded by grants from the Fonds Spécial de Recherche of the Université Catholique de Louvain and from the Belgian National Fund for Scientific Research (fund number 9.4553.04). D. Brkic was under contract in the frame of a research training network founded by the European Commission (HPRN-CT-1999-00054).

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