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Development of an HPLC method for the determination of tetranortriterpenoids in *Carapa guianensis* seed oil by experimental design

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ABSTRACT

Carapa guianensis crabwood, popularly known in Brazil as "andiroba", is a tree that grows in South and Central America and is used by the local population mainly for its anti-inflammatory and insect anti-feeding activities. Scientific studies on this plant have led to the development of an insect-repellent candle and the investigations of the anti-inflammatory properties of its more important biomarkers—tetranortriterpenoids. These compounds, together with glycerides and fatty acids, are present in the seed oil, the most important commercial product from this plant. The growing scientific and commercial interest in "andiroba oil" has urged the development of adequate analytical methods for assessing its quality. Central composite experimental design is a useful statistical method for the development and optimization of HPLC methods, and has been used for a variety of samples. The aim of this work is to develop a HPLC method for the determination of tetranortriterpenoids in "andiroba" oil, by means of central composite experimental design, as well as to prevalidate this method.

1. Introduction

Carapa guianensis Aubl, known as "andiroba", is a tall tree that grows at South and Central America, mainly in the Amazon river basin [1]. The species has been described as a good source of wood and is thus a good option for reforesting [2–5]. Nevertheless, *C. guianensis* is mostly recognized for its uses in traditional medicine. In 1993, Hammer and colleagues interviewed the caboclo community of Marajó island (Northeast of Brazil) and found that *C. guianensis* cortex and the oil extracted from its seeds were currently used as insect repellent, wound healing, treatment of arthritis, throat inflammation, diarrhea, diabetes, ear infection and even uterine cancer [6]. Andiroba oil is the main product extracted from the large seeds of *C. guianensis,* whose main constituents are glycerides, fatty acids, with tetranortriterpenoids (TNTP) as a minor group [7]. The presence of TNTP in *C. guianensis* is documented since 1960s [8–10] and recent papers have reported the anti-feeding properties of the bagasse, the byproduct of the andiroba seed oil extraction, leading to the development of an insect repellent candle [7]. Chemical structures of TNTP isolated from *C. guianensis* are shown in Fig. 1. Anti-inflammatory properties of the oil and isolated TNTP have also been investigated with encouraging results, indicating that the latter may also be responsible for this therapeutic property as well [11–13]. Despite the high therapeutic potential of this plant, there are no analytical methods available for the quantitative determination of TNTP in *C. guianensis*, in order to correlate the observed activities with the amount of TNTP in the extracts. On the other hand, rising commercial interest in this species has made andiroba oil into one of the most important products produced in the Brazilian Amazon [14] being sold either as raw material or as a component of phytomedicine or cosmetic products. It is evident that there is a need for analytical methods to assay andiroba oil and its content of TNTP, in order to guarantee the quality of products derived from it.

Central composite design (CCD) is a statistical method that has been applied to optimize HPLC experimental conditions, such as the resolution and time of analysis for Sudan dyes separation [15] and in the optimization of the resolution of acidic mixtures containing ferulic, syringic and *p*-cumaric acids, etc., on a monolithic stationary phase [16]. Wsól and Fell applied CCD to optimize the analysis of oracin, a new cytostatic drug, and its enantiomers [17]. De Beer et al. applied CCD to optimize the separation of cough-syrup compounds, comparing this method to factorial designs and concluded

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that, despite being more laborious, CCD was the more reliable [18]. CCD provides valuable information about the experimental conditions, such as the significance of each factor individually and of interactions between factors studied for the experimental output [19]. Moreover, it gives response surface graphs that can be adjusted to the desirable response, known as desirability, which describes the experimental response for factor value variation, hence indicating the combination of factor values for the optimum response. This calculation is accomplished by a polynomial regression according to the equation below

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3$$

where Y is the experimental response and a_x the coefficients of the factors and interactions and X_x stands for each factor. This equation describes the experimental response and includes a term that accounts for each factor and interaction between factors [19].

The objective of the present work is to develop an HPLC analytical method for the determinations of tetranortriterpenoids in *C. guianensis* oil by means of a central composite experimental design.

2. Experimental

2.1. Solvents

NANOpure™ Diamond System (Barnstead, Dubuque, USA) treated water and acetonitrile and methanol HPLC-grade from Vetec (Duque de Caxias, Brazil) were used in the development of the HPLC method. Chloroform and acetonitrile P.A. grade from Vetec (Duque de Caxias, Brazil) were used in the solid phase extraction.

2.2. Reference

7-Oxo-7-deacetoxy-gedunin used as reference in prevalidation was isolated in-house and characterized by GC–MS, ^{1}H NMR and ^{13}C NMR, its purity was determined by thermal analysis and proved to be 94.7% (data not shown).

2.3. Carapa guianensis oil sample

Andiroba oil was purchased from Brasmazon (Pará, Brazil).

2.4. Solid phase extraction

The SPE method was performed in a 5-ml glass syringe 12 mm in diameter and 73 mm in height (Jinan, China). About 500 mg of silica gel 60 (mesh 0.063–0.200 mm; Merck, Darmstadt, Germany) were conditioned between two wool disks, by using 10 ml of chloroform under pump pressure. The solvent was collected and the column recharged, avoiding its drying out. A sample of 1 ml of approximately 25 mg/ml solution of andiroba oil in chloroform was applied to the top and let flow slowly down the column. The next step was the elution of the glycerides and fatty acids by applying 40 ml of a mixture of chloroform—*n*-hexane 95:5 in sequential portions of 5 ml each. The tetranortriterpenoids were then eluted with three successive portions of 5 ml of a 95:5 chloroform–acetonitile mixture. Finally, for the elution of minor components, 10 ml of acetonitrile were applied.

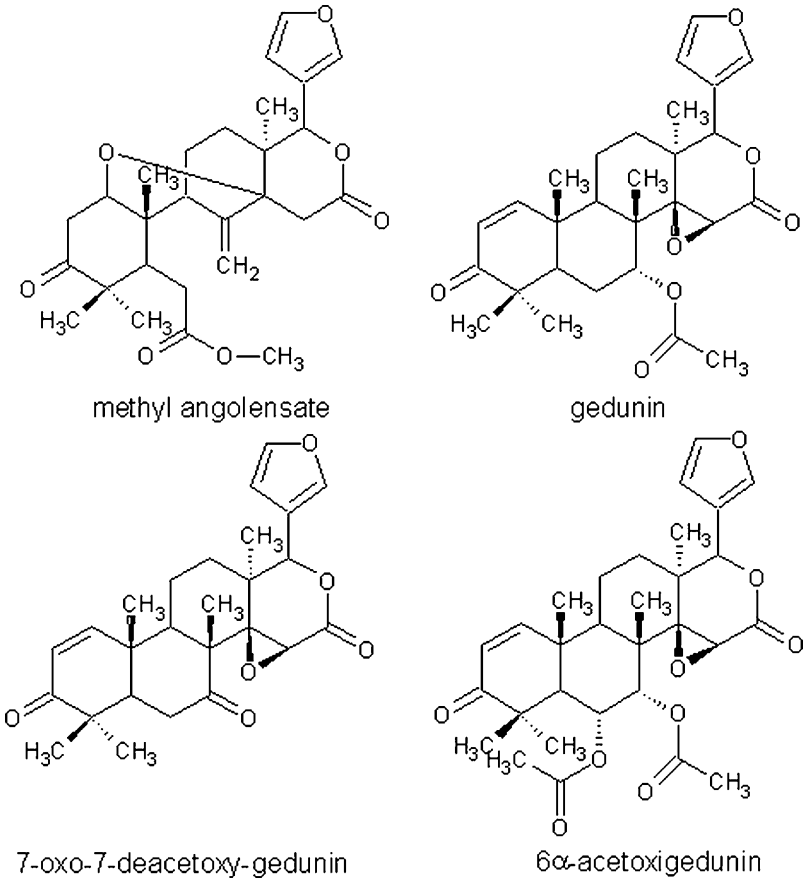

Fig. 1. Tetranortriterpenoids found in *C. guianensis* seed oil.

2.5. High performance liquid chromatography

The HPLC method was developed on a Shimadzu Prominence™ instrument (Kyoto, Japan) with the following modules: Communications Bus Module CBM-20A, Liquid Chromatograph Pump LC-20AT equipped with a gradient valve, Diode Array Detector SPD-M20A and Auto Sampler SIL-20A. The column used was a Hibar™ Lichrospher 100 RP-18 (250 mm × 4 mm, 5 μm particle size, Merck, Darmstadt, Germany). The mobile phase was filtered through a 0.45-μm filter and degassed before use. The wavelength used for acquisition was 210 nm. All samples were filtered with 0.45 μm filter before analysis.

2.5.1. Preliminary method

This method employed the equipment described above with mobile phase composition of acetonitrile–water–methanol 56:44 (v/v), 1.0 ml/min flow and injection volume of 10 μl.

2.5.2. Optimized method

This method employed the equipment described above with mobile phase composition of acetonitrile–water–methanol 35:35:30 (v/v/v), with flow of 0.9 ml/min and injection volume of 20 μl.

2.6. Experimental design

Two optimization experiments of central composite experimental design were performed. The factors under study included flow, volume of injected sample and composition of mobile phase expressed as the amount of acetonitrile. Tables 1 and 2 show the runs of both experiments respectively. Designs were generated and analyzed in the Statistica software version 5.5.

2.7. Prevalidation

The final method was prevalidated based on the complete method described by Grdinić and Vuković [20]. Six concentrations with four replicates of 7-oxo-7-deacetoxy-gedunin were analyzed, the areas were computed and linearity, precision, LOD and LOQ were calculated.

2.8. Tetranortriterpenoids determination

The amount of each tetranortriterpenoid was determined by means of the calibration curve calculated in the prevalidation assay, the working range was from 1.0 to 50.0 μg/ml. All tetranortriterpenoids were quantified as a function of 7-oxo-7-deacetoxy-gedunin.

Table 1
First central composite experimental design

Run	Mobile phase composition[a] (%)	Flow (ml/min)	Volume of injected sample (μl)
1	51.0	0.5	5.0
2	51.0	0.5	15.0
3	51.0	1.5	5.0
4	51.0	1.5	15.0
5	61.0	0.5	5.0
6	61.0	0.5	15.0
7	61.0	1.5	5.0
8	61.0	1.5	15.0
9	49.2	1.0	10.0
10	62.8	1.0	10.0
11	56.0	0.4	10.0
12	56.0	1.6	10.0
13	56.0	1.0	3.2
14	56.0	1.0	16.8
15	56.0	1.0	10.0
16	56.0	1.0	10.0
17	56.0	1.0	10.0

[a] Amount of acetonitrile in mobile phase.

Table 2
Second central composite experimental design

Run	Mobile phase composition[a] (%)	Flow (ml/min)	Volume of injected sample (μl)
1	25.0	0.5	10.0
2	25.0	0.5	30.0
3	25.0	1.5	10.0
4	25.0	1.5	30.0
5	45.0	0.5	10.0
6	45.0	0.5	30.0
7	45.0	1.5	10.0
8	45.0	1.5	30.0
9	22.1	1.0	20.0
10	47.9	1.0	20.0
11	35.0	0.4	20.0
12	35.0	1.6	20.0
13	35.0	1.0	7.1
14	35.0	1.0	32.9
15	35.0	1.0	20.0
16	35.0	1.0	20.0

[a] Amount of acetonitrile in mobile phase.

3. Results and discussion

3.1. Method development

A preliminary method was based on the method used by Pereira [7] to monitor extracts of *C. guianensis*. The region of TNTP elution was established by analyzing a selected TNTP enriched extract (data not shown). The composition of the mobile phase in the middle of this region was calculated by interpolation to be acetonitrile–water 56:44 (v/v). As a next step, this mobile phase composition was assayed experimentally in the isocratic mode, keeping the flow at 1.0 ml/min. The oil sample used was treated by solid phase extraction to yield a concentrated sample of tetranortriterpenoids. Tetranortriterpenoids present in the mixture were identified by injection of isolated samples. They were 7-oxo-7-deacetoxy-gedunin, methyl angolensate, 6α-acetoxygedunin and gedunin.

3.2. Central composite design

In order to optimize the TNTP's chromatographic resolution a central composite experimental design was generated with the following parameters: mobile phase composition, flow and volume of the injected sample. Mobile phase composition was expressed as the amount of acetonitrile and water was the complimentary volume. Table 1 shows each run of this experiment. The results of this first experiment using binary mobile phase revealed that gedunin was co-eluted with another unknown substance, indicating that the desired separation would require further optimization. A ternary mobile phase composition was then assayed and a preliminary composition was established as acetonitrile–water–methanol 35:35:30 v/v/v. A second central composite design was then generated using the same factors. It is not possible to vary the amounts of all three solvents in such a design when analyzing then as just one factor (mobile phase composition), water was then chosen to be kept constant at 35% in the mobile phase composition since modifications of its amount led to poorer separations. Therefore only the amounts of acetonitrile and methanol were varied complimentary to each other, hereby expressed as the amount of the first. Each run is displayed in Table 2. The experimental outputs were

Table 3
Experimental output

Experiment	Res 1	Res 2
1	0.462	1.905
2	0.000	1.325
3	0.000	1.421
4	0.000	1.194
5	3.139	1.168
6	2.041	0.526
7	2.252	0.825
8	1.590	0.218
9	0.000	1.530
10	2.453	0.672
11	1.755	1.415
12	1.361	1.037
13	1.747	1.393
14	1.089	0.953
15	1.511	1.240
16	1.526	1.210

considered as the resolution between methyl angolensate and its adjacent peak (Res 1) and the gedunin and its adjacent peak (Res 2) as well, for they showed the poorer separation. The results are shown in Table 3.

For Res 1 all factors studied were significant by linear regression; by quadratic regression only flow was non significant. This means that all factors affect this experimental output. Interactions were significant but since their effect estimate were smaller than the factor's own effect estimates, this was probably due to the high significance obtained for the individual factors. For Res 2 all three factors were significant by linear regression and only mobile phase composition was significant by quadratic regression. No interaction was significant for this output. Results are shown in Fig. 2. In order to establish the optimum condition response surface graphs were generated by fixing the optimum resolution of both peaks to the value of 1.5 (desirability 1.0) and setting the factors to optimum value. The greater significance of the mobile phase composition

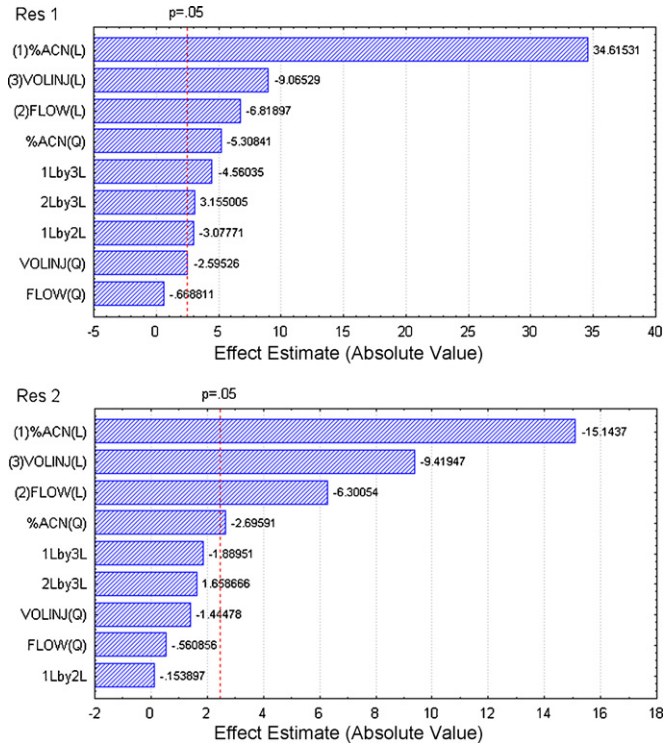

Fig. 2. Pareto graphs for Res 1 and Res 2.

was also shown as a wavelike shape of the surface on graphs that relates this factor to the other two (Fig. 3) and the optimum value was determined as 34,995%. Since mobile phase composition was the most significant factor for both outputs, it was the first factor to be fixed at 35% of acetonitrile in the mobile phase, since it is the optimum value and comprises the wider range of both flow and injection volume (Fig. 3). Hence, the optimized mobile phase composition was acetonitrile–water–methanol 35:35:30 (v/v/v). Flow, whose optimum value was 1.0 ml/min, was the next factor fixed. Since the optimum region for the mobile phase composition established was large, five runs were carried out with the following flows: 0.8; 0.9; 1.0; 1.1; 1.2 ml/min. The best results were comprised between 1.0 and 0.9 ml/min in accordance to the model, the lesser was chosen since it requires a smaller amount of solvent. The optimum value for the volume of injected sample was 7 μl, but when fixing this factor it is necessary to take into account the low absorbance of TNTP and the prevalidation to be done further, for both reasons it is interesting to fix this factor at the larger possible value. For instance, the volume of injected sample was fixed at 20 μl since the larger volume for this flow is still in the optimum zone (Fig. 3). A chromatogram of andiroba oil prepared sample using the optimized method is shown in Fig. 4.

3.3. Prevalidation

Validation of an analytical method is a complex and a time consuming process and, for this reason, some authors have described the need for a prevalidation step at the end of the development process, thus assuring that methods that enter validation process are not going to fail [21,22]. Grdinić and Vuković [20] suggested two methods of prevalidation: (i) full prevalidation with 24 measurements (four replicates at six concentrations) and (ii) exploratory prevalidation, with 8 measurements (two replicates at four concentrations). In the present study the full model was adopted, which employs a calibration curve, and permits calculation of the validation parameters. Selectivity was determined by peak spectral purity and the central composite experimental designs. The variety of different analysis conditions in each of these experiments allowed a search for peaks undergoing coelution which, in fact, was not observed in any method. Linearity was determined by multiple regression analysis. The determination coefficient (r^2) was estimated to be 0.99978 and the working range, corrected by reference purity, was established between 0.95 and 47.35 μg/ml. Multiple regression analysis also estimated no significant difference between the intercept and zero. This result admits determinations from a unique calibration level, once working in the fixed range above. Precision was measured at repeatability level by calculation of relative standard deviations at all concentrations and proved to be satisfactory. The results are displayed in Table 4. Limits of quantitation and detection were calculated using the standard error of the intercept calculated by the multiple regression analysis. The LOD calculated was 0.3 μg/ml and the LOQ was 0.93 μg/ml—being the lower boundary of the calibration curve.

Table 4
Method repeatability

Calibration level[a] (μg/ml)	RSD (%)
0.9	2.4
9.5	0.3
18.9	1.0
28.4	0.8
37.9	0.7
47.4	1.0

[a] Corrected by reference purity.

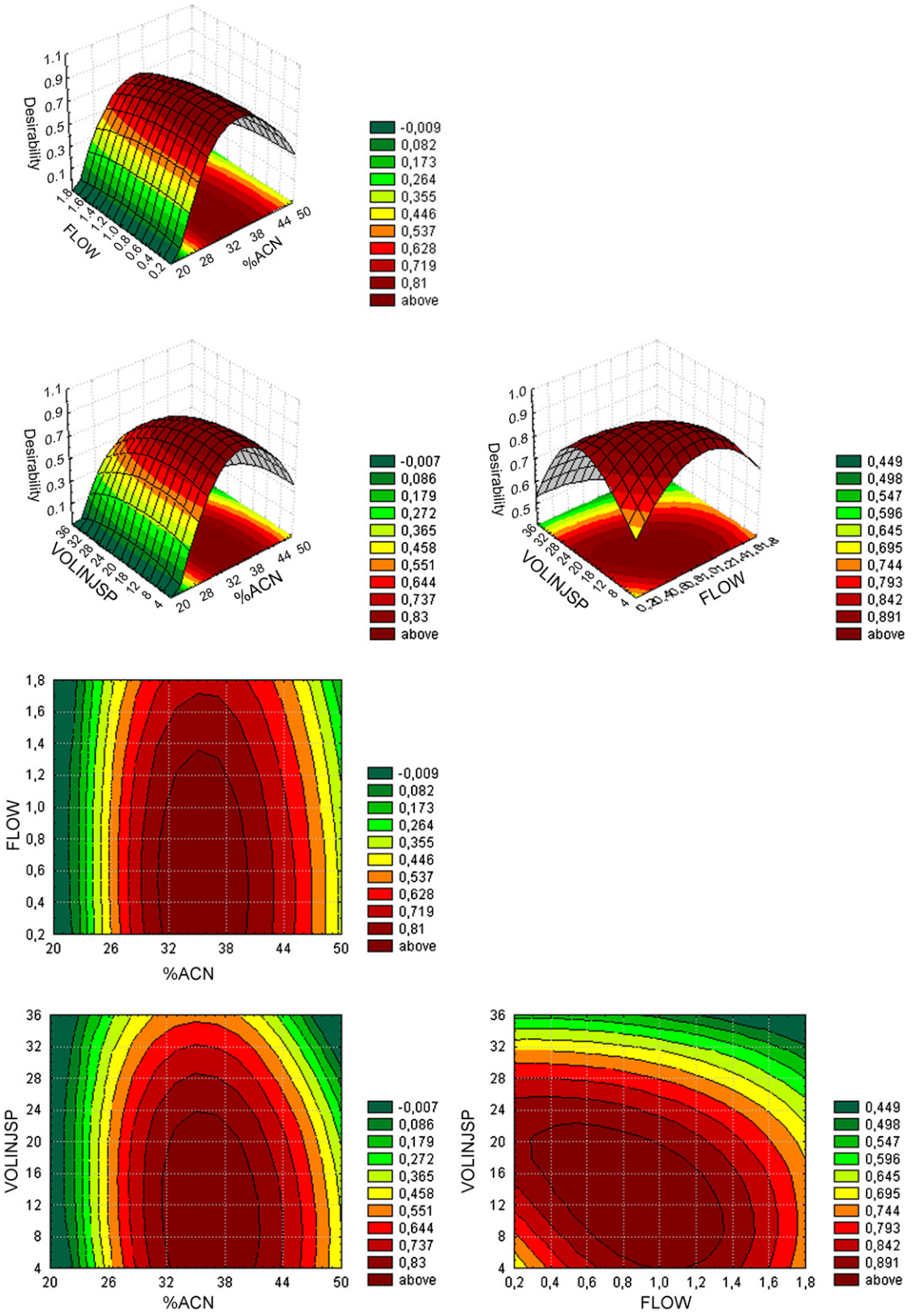

Fig. 3. Response surface graphs describing optimal conditions for Res 1 and Res 2 (desarability 1.0 = resolution 1.5).

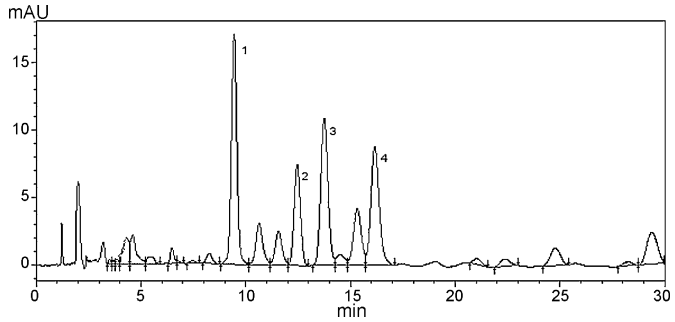

Fig. 4. Chromatogram of tetranortriterpenoids at optimal conditions. (1) 7-Oxo-7-deacetoxy-gedunin, (2) methyl angolensate, (3) 6α-acetoxygedunin, (4) gedunin.

Table 5
Amount of tetranortriterpenoids in andiroba oil

Tetranortriterpenoid	Amount (mg/g)
7-Oxo-7-deacetoxy-gedunin	2.48
Methyl angolensate	1.15
6α-Acetoxygedunin	1.82
Gedunin	1.62
Total	7.07

3.4. Tetranortriterpenoids determination

TNTP content was calculated from the prevalidation analytical curve and these results are shown in Table 5. As the only available reference was 7-oxo-7-deacetoxy-gedunin, the other tetranortriterpenoids were quantified using the same calibration reference. This is a straightforward procedure, given that they are structurally similar and the differences in their molecular weight are not significant for this purpose. The results show the amount of this substances in *C. guianensis* seed oil ranging from 2.48 mg/g to as low as 1.15 mg/g.

4. Conclusion

An analytical method for the determination of tetranortriterpenoids was developed and optimized by central composite experimental design. This experimental design allowed the assaying of the selected factors simultaneously, including interactions between factors, by means of a rational approach in order to reach the optimum conditions. In addition, experimental design aided the validation procedure on selectivity tests, in this case by detection of a compound coeluting with gedunin in a binary mobile phase system. These results demonstrated the value of CCD for HPLC method development and prevalidation. Four tetranortriterpenoids detected in this sample of andiroba oil were determined by this method and prevalidation indicates that the method may be validated.

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