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Evaluation of a methylation procedure to determine cyclopropenoids fatty acids from *Sterculia striata* St. Hil. Et Nauds seed oil

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Abstract

Cyclopropenoids fatty acids (CPFA) from *Sterculia striata* seed oil were characterized by gas chromatography/mass spectrometry (GC/MS) and quantified by gas chromatography–flame ionization detector (GC–FID) after derivation to fatty acid methyl esters using a cold base-catalyzed procedure. ^{1}H nuclear magnetic resonance (NMR) analysis were done in oil and fatty acid methyl esters derivatives to quantify CPFA and verify artifacts formation during the base-catalyzed reaction. Similar quantities of CPFA were found in *S. striata* and *Sterculia foetida* seed oils before and after a base-catalyzed methylation by NMR analysis, with no artifact formation. These results were compatible with those obtained by GC–FID analysis. Transmethylation with KOH in methanol was an appropriated method to prepare cyclopropenoids fatty acids methyl esters and quantify them by GC and NMR analysis.

Keywords: Cyclopropenoids fatty acids (CPFA); *Sterculia striata*; Methylation

1. Introduction

Several studies on seeds of Sterculaceae, Malvaceae, Filiceae and Bombacaceae family describe the occurrence of cyclopropenoid fatty acid derivatives. The most commonly ones found are sterculic (9,10-methylene-9-octadecenoic) and malvalic (8,9-methylene-8-heptadecenoic) acids [1,2].

Studies with the oil from seeds of *S. foetida*, *S. tom*entosa and *S. tragacanta* (Sterculaceae) have reported high content of cyclopropenoids fatty acids (CPFA) [3,4]. Compounds containing cyclopropenoid ring are associated with several biological properties, such as: insecticide, antifungal, antibiotic, antiviral, hormonal, carcinogenic or antitumoral activities and enzyme inhibitor [5,6]. The effects of CPFA in animals have been the subject of several investigations, including co-carcinogenic and carcinogenic activities [7–9]. Sterculic acid is an inhibitor of Δ^9-desaturase which converts stearic acid into oleic acid and is potentially noxious to man, since it can alter the cellular membranes permeability and inhibit the cellular reproduction [10].

The "chichá" nut, or *Sterculia striata* seed, also known in Brazil as "amendoim-da-mata" and "castanha de macaco" [11], is consumed raw by the fauna and cooked or toasted by man [12]. Some nutritional features of "chichá" nuts were evaluated by Oliveira et al. [13], in which several macronutrients such as lipids (28.6%), protein (22.5%) and carbohydrates (45.8%), were quantified. We have reported previously some physical and chemical characteristics of *S. striata* seed oil and verified the occurrence of CPFA [14].

CPFA are labile compounds and there are several analytical problems to determine these compounds using gas chromatography. The cyclopropenoid groups are destroyed by heating and acid media during extraction and esterification process [9]. CPFA can also be decomposed thermally in the injector or in polar columns during the GC analysis. The main methods used for the analysis of CPFA are hydrogen bromide titration, nuclear magnetic resonance (NMR), selective derivations followed by gas chromatographic

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analysis of the products or direct GC analysis of fatty acid methyl esters formed [15–20]. GC analysis have the advantage to permit the determination of all fatty acids composition.

Base-catalysed esterification of cyclpropenoid fatty acids is necessary to prepare stable fatty acid methyl esters to GC analysis [18]. Spitzer used a base-catalysed esterification with sodium methoxide (0.5 mol L^{-1}) at room temperature to prepare cyclopropenoids fatty acid methyl esters and some derivatives for structure elucidation [19,20].

Therefore, as the CPFA are unstable under many experimental conditions applied to methylation and gas chromatographic analysis, the objective of this work was evaluate a simple and fast cold base-catalyzed procedure to prepare CPFA methyl esters from *S. striata* seed oil. Additionally, NMR analysis were carried out parallel with gas chromatography to evaluate artifact formation and quantify cyclopropenoid groups.

2. Experimental

2.1. Samples, standards and reagents

The fruits of *S. striata* were collected on September 2000, at Universidade Federal do Piauí Campus, Teresina-PI. *S. foetida* seed oil from Paraíba state, Brazil was used as reference since its fatty acids composition is well known [1,2].

Mixture of fatty acid methyl esters standards from C_4 to C_{24} with certificate of composition were purchased from Supelco Park (Bellefonte, PA, USA). Heneicosanoic acid methyl ester ($C_{21:0}$) standard with 99% of purity, used as internal standard in GC–FID analysis, $CDCl_3$ and tetramethylsilane were purchased from Sigma Chemical (Saint Louis, USA). Methanol and *n*-hexane (HPLC grade) were obtained from EM Science (USA). All other solvents and chemicals were of reagent grade.

2.2. Oil extraction

Seeds (500 g) were ground, homogenized and oil was extracted with cold ethyl ether. About 10 g of the sample were dissolved in 50 mL of ethyl ether and extracted during 12 h in contact with the solvent. The organic extract was filtered and dried with anhydrous sodium sulfate. The ethyl ether was removed under vacuum. The oil obtained was esterified to determine fatty acids composition by gas chromatography.

2.3. Methylation procedure

Fatty acid methyl esters were prepared following in general lines IUPAC methodology without heating [21]. A 100 mg sample of raw seed oil was accurately weighed into a 20 mL centrifuge tube and dissolved in 5 mL of the internal standard solution of a methyl ester fatty acid $C_{21:0}$ in *n*-hexane (3 mg mL^{-1}). A methanolic KOH solution (2 mol L^{-1}) was added (0.2 mL). The tube has been sealed and mixed vigorously for 30 seconds in a vortex shaker. Saturated NaCl solution (2.0 mL) was added and the organic phase was separated. An aliquot (1.0–2.0 μL) of the hexane solution was submitted to GC analysis.

2.4. Gas–liquid chromatography

The fatty acid methyl esters were analyzed in a Shimadzu gas chromatograph, model GC-17A with a flame ionization detector (FID). The components were separated in a fused silica capillary column, SP 2340 (Supelco Park, Bellefonte, PA, USA) (60m with internal diameter of 0.25 cm and film thickness of 0.20 μm). The following chromatographic conditions were observed: column temperature programmed, 60 °C (2 min), 15 °C min^{-1} to 135 °C (1 min), rate of 3 °C min^{-1} to 215 °C (10 min); injector temperature: 230 °C; detector temperature: 240 °C; carrier gas: hydrogen; gas linear speed: 20 cm s^{-1}; split: 1:50. Fatty acid methyl esters were identified by co-injection of standards and quantified using internal standard (heneicosanoic acid methyl ester ($C_{21:0}$) – Sigma Chemical – USA – 99% of purity). The correction factors for flame ionization detector were determined by a derived chromatogram from the analysis of the reference mixture of known methyl esters (FAME C_4–C_{24}) under operation conditions identical with those used for the sample. FID correction factors were calculated in relation to palmitic acid methyl ester ($C_{16:0}$) following AOCS method [22]. The correction FID factors used for malvalic and sterculic fatty acids were the same as closer fatty acids methyl esters eluted in chromatogram ($C_{18:1}$ and $C_{18:2}$). The internal standard ($C_{21:0}$) was used to calculate the concentration of cyclopropenoids fatty acids and the other main fatty acids (Table 1). The calculation was done considering the mass and area relation between fatty acids and internal standard.

2.5. Gas–liquid chromatography–mass spectrometry

The not identified fatty acid methyl esters were analyzed in a gas chromatograph with mass detector (GC 17A GC/MS–QP 5000-Shimadzu) managed by software (Class-5000-Shimadzu).

The samples were submitted to the previously mentioned esterification process, and the chromatographic conditions were the same for those used for GC–FID analysis. The analysis with mass detector were carried out on the following conditions: scan mode; interface temperature of 240 °C; carrier gas helium; flow 1.0 mL min^{-1}; acquisition time from 9 to 45 min and solvent cut in 8 min; mass range from 40 to 350, electron energy of 70 eV; multiplier voltage of 1.3 kV; analyzer quadrupole. The compounds were identified through comparison with mass spectra published for methyl malvalate and methyl sterculate [23] and of the NIST 62 and NIST 12 libraries.

Table 1
Fatty acids composition from *Sterculia striata* and *Sterculia foetida* seed oils (fatty acid methyl ester $C_{21:0}$ as internal standard)

Peak	Fatty acid	*Sterculia foetida* (g/100 g of oil) $N = 3$		*Sterculia striata* (g/100 g of oil) $N = 3$	
		Mean ± S.D.	R.S.D.%	Mean ± S.D.	R.S.D.%
1	$C_{16:0}$	15 ± 1	6.7	20.8 ± 0.8	3.8
2	$C_{16:1}$	0.13 ± 0.02	15.4	2.06 ± 0.08	3.9
3	$C_{18:0}$	1.66 ± 0.08	4.8	2.80 ± 0.09	3.2
4	Malvalic acid[a]	5.4 ± 0.5	9.3	3.9 ± 0.2	5.1
5	$C_{18:1}$ *cis* w9 + w7	5.6 ± 0.6	10.7	30 ± 1	3.3
6	Sterculic acid[a]	54 ± 2	3.7	11.2 ± 0.6	5.4
7	$C_{18:2}$ *cis*/*cis*	7.7 ± 0.4	5.2	9.6 ± 0.3	3.1
8	$C_{18:3}$	0.20 ± 0.02	10.0	0.18 ± 0.02	11.1
9	$C_{20:0}$	0.15 ± 0.02	13.3	0.56 ± 0.03	5.4

S.D. – standard deviation, R.S.D.% – relative standard deviation percentage.
[a] Identification by comparison with published mass spectra [23].

2.6. ^1H NMR analysis

Samples of oil extracted by cold process and methyl esters, prepared without heating following the IUPAC methodology [21] were analyzed by ^1H NMR [3,4] in a 500 MHz Bruker DRX 500 spectrometer. The samples were prepared in a tube of 5 mm internal diameter using $CDCl_3$ as solvent and tetramethylsilane as internal standard. The ^1H NMR spectra of the oil and the methyl esters show a singlet at δ 0.77 regarding to the two hydrogens of the cyclopropenoid ring and a triplet at δ 0.88 due to terminal methyl hydrogen groups of all fatty acids or the methyl esters which compose the triacylglycerols. The percentage of CPFA was calculated by dividing the area of the singlet at δ 0.77 (hydrogens of the cyclopropenoid ring) by the area of the triplet at δ 0.88 (terminal methyl hydrogen groups of all fatty acids) and multiplying by 150. The samples were analyzed in triplicate.

3. Results and discussion

Since CPFA are unstable in many experimental conditions for gas chromatographic analysis, some precautions were taken to minimize these problems. The lipid extraction was carried out by a cold process and fatty acid methyl esters were obtained by a cold base-catalysed process. The fatty acids composition determined from *S. striata* and *S. foetida* oils are showed in Table 1. GC–FID fatty acid profile of *S. foetida* (A) and *S. striata* (B) seeds are shown in Fig. 1. Fig. 2 shows a total ion chromatogram of fatty acid methyl esters of *S. striata* oil. GC/MS analysis indicated the presence of compounds bearing cyclopropenic groups (peaks 4 and 6). These compounds were eluted with the same retention times for *S. foetida* and *S. striata*. It is well known that in *S. foetida* oil, there is more than 50% of CPFA, including sterculic (9,10-methylene-9-octadecenoic) and malvalic (8,9-methylene-8-heptadecenoic) acids [1,2,24]. The mass spectra of the compounds 4 and 6 were compatible with the methyl malvalate and methyl sterculate ones, respectively. (Figs. 3 and 4) [23].

The mean percentage of CPFA in the *S. striata* seed oil by GC analysis with FID detector was 15.1 ± 0.8.

^1H NMR analysis showed no artifact formation when cold base-catalyzed process was used. ^1H NMR spectra of the methyl esters obtained in basic catalyzed process showed no signals for triacylglycerols (δ 4.1–4.3) indicating that the esterification was quantitative. These spectra showed an additional signal at δ 3.66, characteristic for methyl esters hydrogens (–C(O)OCH_3). Thus peaks at δ 3.66 (OCH_3) and at δ 0.88 (terminal CH_3 groups) showed the same area indicating a complete methylation. The percentages of CPFA obtained by ^1H NMR analysis directly in oil extracted in a

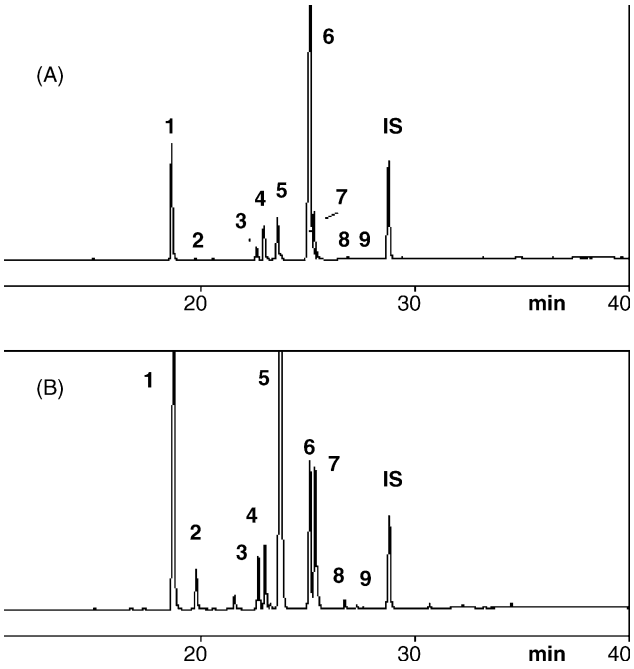


Fig. 1. GC–FID chromatogram of the fatty acid methyl esters from *S. foetida* (A) and *S. striata* (B) seed oils: (1) palmitic acid $C_{16:0}$; (2) palmitoleic acid ($C_{16:1}$ *cis* 9); (3) stearic acid ($C_{18:0}$); (4) not identified by FID*; (5) octadecenoic acid ($C_{18:1}$ *cis* w9 +w7); (6) not identified by FID*; (7) linoleic acid ($C_{18:2}$ *cis*/*cis* 9, 12); (8) araquidic acid $C_{20:0}$); (9) linolenic acid ($C_{18:3}$ *cis*/*cis*/*cis* 9, 12, 15); IS – Internal standard ($C_{21:0}$). * The possible structures were evaluated by GC/MS analysis and compared with NIST 62 and NIST 12 mass spectra libraries and mass spectra published for methyl malvalate and methyl sterculate [23].

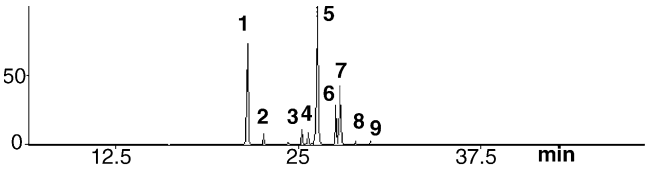


Fig. 2. Total ion chromatogram of fatty acid methyl esters of *S. striata* seed oil. The possible structures were evaluated by comparison with NIST 62 and NIST 12 mass spectra libraries and mass spectra published for methyl malvalate and methyl sterculate [23]. (1) Palmitic acid $C_{16:0}$; (2) palmitoleic acid ($C_{16:1}$ *cis* 9); (3) stearic acid ($C_{18:0}$); (4) malvalic acid; (5) oleic + vacenic acids ($C_{18:1}$ *cis* 9 + *cis* 11); (6) sterculic acid; (7) linoleic acid ($C_{18:2}$ *cis*/*cis* 9, 12); (8) araquidic acid ($C_{20:0}$); (9) linolenic acid ($C_{18:3}$ *cis*/*cis*/*cis* 9, 12, 15).

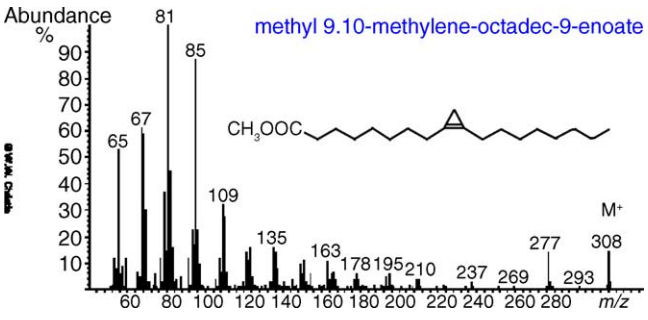


Fig. 3. Mass spectrum of sterculic acid methyl ester [23].

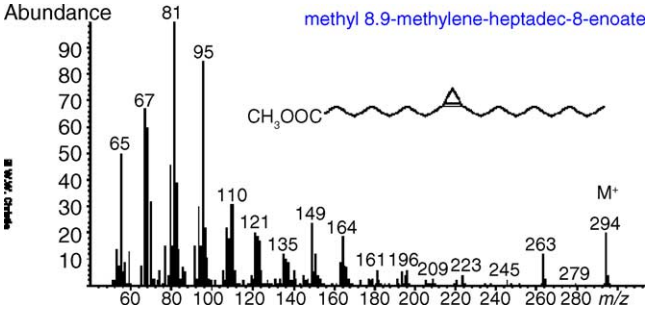


Fig. 4. Mass spectrum of malvalic acid methyl ester [23].

cold process and in its correspondent fatty acid methyl esters obtained under base-catalyzed process in *S. foetida* and *S. striata* are shown in Table 2. The mean contents of CPFA in *S. striata* oil and in fatty acid methyl esters were similar, 15.5 ± 0.2 and 15.3 ± 0.3%, respectively, showing no decomposition of CPFA under basic catalysis. The percentage of CPFA in *S. foetida* and *S. striata* determined by ^{1}H NMR and by GC–FID analysis were comparable (Tables 1 and 2). Bianchini et al. [16] have shown that for kapok seed oil there was a good agreement of the results of CPFA contents with NMR, HBr titration and GC methods. GC analysis was carried out with a previous derivation of CPFA by argentation of methyl esters and directly, through a base-catalyzed procedure of transmethylation using sodium, anhydrous methanol and heating by 20 min. Kapok seed oil contains about 10% of CPFA, which is similar to the concentration found in *S. striata* seed oil.

Cottonseed oil has low quantities of CPFA (<2%), which are eliminated in the refining process. However, the concentration of CPFA found in *S. striata* compromise oil for use in food due to health implications caused by ingestion of cyclopropenoid acids.

The mean percentage of CPFA in *S. foetida* determined by GC analysis was 59 ± 3% (Table 1) and through NMR analysis varied from 56.4 ± 0.1% (raw oil) to 57.2 ± 0.4% (fatty acid methyl esters) (Table 2). These amounts were in agreement with the literature, since the percentage of CPFA in *S. foetida* oil was previously determined as 55.6 ± 1.9% by NMR, 57.8 ± 2.1% by Halphen reaction and 62.6 ± 0.7 using catalyzed hydrogenation reaction and GC analysis [15]. Pawlowski et al. [3] found for *S. foetida* oil 49.2% of CPFA determined by NMR, and 51–55% by Halphen reaction using methyl sterculate as standard.

The results in the present paper showed that cyclopropenoids fatty acid methyl esters formation and GC analysis, in *S. striata* seed oil, had good repeatability with acceptable values of the relative standard deviation percentage (R.S.D.%), i.e. about 5%, for triplicates (Table 1). Recommended temperature for chromatographic column, Carbowax 20 M, in CPFA analysis is below 190 °C [16]. In this experiment, was used a capillary column with poly(biscyanopropyl siloxane) as stationary phase and programmed column temperature, starting at low temperature (60 °C) and reaching temperatures higher than 190 °C at a slow rate. These conditions would probably have contributed to reduce decomposition of cyclopropenoids rings.

A cold base-catalyzed method, similar to the one used in this paper has been applied to prepare fatty acid methyl esters in fat milk and margarine, rich in volatile short-chain fatty acids [25,26]. Berdeaux et al. [27] has tested five methylation procedures, including base-catalyzed method using KOH in methanol to quantify major oxidation short-chain glycerol-bound compounds. This procedure has showed

Table 2
CPFA content of *Sterculia striata* and *Sterculia foetida* seed oils[a]

	Crude oil $N = 3$			Fatty acid methyl esters obtained by base-catalyzed method $N = 3$		
	A	*B*	*B*/*A* × 150 total CPFA (%)	*A*	*B*	*B*/*A* × 150 total CPFA (%)
Sterculia striata	1.54 ± 0.7	0.16 ± 0.07	15.5 ± 0.2	2.1 ± 0.6	0.22 ± 0.06	15.3 ± 0.3
Sterculia foetida	3.7 ± 1.0	1.4 ± 0.4	56.4 ± 0.1	5.4 ± 1.0	2.0 ± 0.4	57.2 ± 0.4

A = CH_3 terminal signal area; *B* = Cyclopropene CH_2 signal area.
[a] Determinated from the ^{1}H NMR high field region (δ 0.5–1.5).

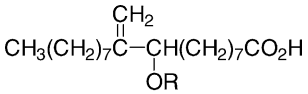
good values of repeatability and recovery with no artifacts formation.

Sterculic acid polymerizes at a significant rate at room temperature and it is stable as methyl ester. The polymerization reaction involves opening of the cyclopropene ring. The free carboxylic group induces the opening of the ring with the formation of allyl derivatives of type 1–6, as main products [25].

S. foetida oil was also submitted to an acid transmethylation procedure, using methanol in 2% of sulfuric acid and heating during 45 min [28], to verify artifact formation through ^{1}H NMR analysis.

In the ^{1}H NMR spectrum of methyl esters, obtained in acid media, we observed two wide singlets at δ 5.95 and 5.68, characteristic of *gem*-dissubstituted double bound. This data confirmed the opening of the cyclopropenoids fatty acid rings and suggested the formation of derived of the types 1 and 2. The possibility of formation of types 5 and 6 was eliminated, because signals of hydrogen in conjugated diene were not observed.

The results in the present study indicate that the procedure applied, which include transmethylation with KOH in methanol was simple, fast and appropriated to prepare cyclopropenoids fatty acids methyl ester from *S. striata* seed oil and quantify by GC analysis.



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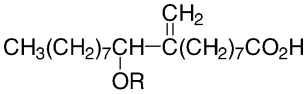

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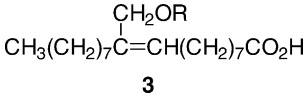

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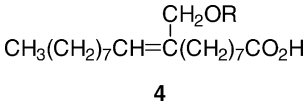

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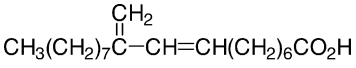

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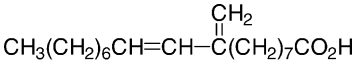

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