Identification of α-Parinaric Acid in the Seed Oil of *Sebastiana brasiliensis* Sprengel (Euphorbiaceae)[1]

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ABSTRACT: Besides some usual fatty acids, the seed oil of *Sabastiana brasiliensis* (Euphorbiaceae) contains up to 39% (estimated by ultraviolet spectroscopy) of α-parinaric acid (*cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid). The fatty acids were analyzed by gas chromatography and gas chromatography/mass spectrometry as their methyl esters. The structure of α-parinaric acid was proven by a combination of chemical and spectroscopic methods, conducted with the crude oil, the methyl ester mixture, and the isolated fatty acid methyl ester. Complete assignment of the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) shifts of α-parinaric acid was carried out by two-dimensional NMR experiments.
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KEY WORDS: Euphorbiaceae, NMR spectroscopy, α-parinaric acid, *Sebastiana brasiliensis.*

Sebastiana brasiliensis (Euphorbiaceae) is a shrub or tree 3–5 m in height, which grows in Brazil, Uruguay, Argentina, Paraguay, and Bolivia (1). The plant yields globose capsular fruits of about 1–1.5 cm that contain three oily seeds. The Brazilian popular names of *"leiteiro"* (milk producer) or *"pau-leiteiro"* (milk wood) point to its high latex content. In the present study, as a part of our current research project about seed oils from plants of Brazil, the fatty acid (FA) composition and the nuclear magnetic resonance (NMR) spectroscopic data of the seed oil and its isolated C_{18} conjugated tetraenoic acid were investigated. This is the first chemical report about the plant *S. brasiliensis.*

MATERIALS AND METHODS

The fruits from *S. brasiliensis* were collected in 1994 and 1995 in different locations of the State Rio Grande do Sul, Brazil [National Park of Turvo, Derrubadas (SBT), in the region of Caçapava do Sul (SBC), and Santana da Boa Vista (SBV)]. For extraction of the oil, the air-dried seeds were repeatedly crushed and homogenized with a pestle in a mortar under petroleum ether (40–60°C). After filtration, the solvent was removed by vacuum distillation at 30°C, and the residue was flushed with nitrogen and stored in petroleum ether solution at −18°C until use. The yield was 25.8% (SBT), 23.4% (SBC), and 26.3% (SBV) of a pale-yellow oil. To minimize autoxidation, all solvents were bubbled with nitrogen before use and contained 0.001% butylated hydroxytoluene.

Transesterification to the fatty acid methyl esters (FAME) was carried out with 0.5 N sodium methoxide in anhydrous methanol at room temperature under nitrogen as described by Christie (2). For preparation of the maleic adducts, a part of the FAME mixture was refluxed under N_2 for 2 h with toluene containing maleic anhydride (3). Preparative separation of the FAME fraction was performed with 0.6-mm preparative silica layers with petroleum ether/diethyl ether (80:20, vol/vol) as solvent system. For the isolation of α-parinaric acid ME from the total FAME, 0.6-mm preparative silica layers were used and developed twice with ether/diethyl ether (94:6, vol/vol) (4). The conjugated FAME was detected under ultraviolet (UV) (254 nm), and the usual FAME were visualized under UV (340 nm) after spraying with 2′,7′-dichlorofluorescein. The thin-layer chromatography (TLC) bands of interest were scraped off, eluted with diethyl ether and analyzed by UV, infrared (IR), and NMR spectroscopy. Oxidative splitting of α-parinaric acid ME by permanganate-periodate was carried out by the method of Rudloff (5). The oxidation products were analyzed after methylation in 2% H_2SO_4/MeOH solution by gas chromatography (GC).

A Hewlett-Packard (Palo Alto, CA) 5890 GC Series II GC, a flame-ionization detector (FID; 230°C), and a split/splitless injector (1:50, 230°C) with glass insert and H_2 as carrier gas (head pressure 150 kPa) were used to analyze the FAME. Separation of the compounds was achieved with an HP-20M (Hewlett Packard) capillary column (50 m × 0.2 mm × 0.2 μm), at an oven temperature of 195°C. Peak integration was performed with an HP3392A integrator (Hewlett-Packard). For estimation of the equivalent chainlength (ECL) values, the retention times were measured from the time of elution of the solvent, considered as unretained solute.

Gas chromatography-mass spectrometry (GC/MS) analysis was performed with the NERMAG AUTOMASS (Paris,

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France), operating at an ionization energy of 70 eV, a source temperature of 225°C and an interface temperature of 220°C. Separation of the FAME derivatives was carried out with a DB 23 column (J&W Scientific, Folsum, CA) with a temperature program (160–230°C; 2°C/min). Helium was used as carrier gas (0.5 bar).

IR spectra of the oil and the FAME were obtained with a Shimadzu FTIR-8101 (Tokyo, Japan) in a liquid film. UV spectra of the oil and the FAME were recorded from 400 to 200 nm in cyclohexane solution with a Shimadzu UV-2201 spectrophotometer. The UV estimation of the content of α-parinaric acid in the oil was carried out by an established method (6).

The ^{1}H NMR, ^{1}H ^{1}H two-dimensional shift correlation (COSY), ^{13}C NMR, distortionless enhanced polarization transfer (DEPT), and ^{1}H ^{13}C 2D heteronuclear shift correlation (HETCOR) experiments were performed with the crude oil on a BRUKER AMX500 (Karlsruhe, Germany) spectrometer. The ^{1}H NMR and ^{13}C NMR spectra of α-parinaric acid ME were run in deuteriochloroform solution on a Varian VLX-200 (Palo Alto, CA) with trimethylsilyl as internal standard.

RESULTS AND DISCUSSION

GC and GC/MS analyses of the FAME of the seed oil samples of *S. brasiliensis* showed the presence of the usual FA with a substantial amount of α-linolenic acid (Table 1), identified by co-injection of standard compounds and by comparison of the mass spectra with the spectra of standards. Furthermore, a GC peak with retention characteristics (ECL 23.22) that are not usually found in seed oils eluted after the common FAME on the Carbowax GC column. The mass spectrum of this compound, subsequently identified as α-parinaric acid, showed a molecular ion at *m/z* 290 (19%), corresponding to an empirical formula $C_{19}H_{30}O_2$. This suggested a C_{18} chain with four double bonds, and the high ECL-value indicated here that the compound must have another configuration of the olefinic system as the usual methylene-interrupted conjugated *cis*-FA. High ECL values on capillary columns coated with medium polar stationary phase material have been observed for FA with conjugated double bonds (7,8). The ions *m/z* 275 (M – CH_3, 0.2%), *m/z* 261 (M – CH_3CH_2, 1.1%), and *m/z* 259 (M-OCH_3, 1.9%) were present in the high-mass range. In the low-mass range, the highly unsaturated hydrocarbon fragments [*m/z* 55 (30%), *m/z* 67 (23%), *m/z* 79 (51%), *m/z* 91 (100%), *m/z* 105 (63%), *m/z* 119 (30%), *m/z* 133 (22%), *m/z* 147 (15%), *m/z* 161 (4%), and *m/z* 175 (2%)] were found to be more intensive than the typical oxygen-containing methyl ester fragments [*m/z* 74 (7%) and *m/z* 87 (7%)]. The base peak at *m/z* 91, which may be due to tropyllium ions, formed by cyclization and rearrangements, is typical for highly unsaturated FAME (9). In contrast to the mass spectrum of the ME of the conjugated ixoric acid (*cis,cis,cis,trans*-8,10,12,14-octadecatetraenoic acid) (10), no ions that could enable the location of the double bonds were present. Although the peak at *m/z* 147 from the ion series $C_nH_{2n-7}^+$ could be explained by an allylic cleavage [$CH_3CH_2(CH{=}CH)_4CH_2^+$], the diagnostic value for determination of the double-bond positions was considered low because other more intensive signals of the same $C_nH_{2n-7}^+$ series, such as *m/z* 119 ($C_9H_{11}^+$) and 105 ($C_8H_9^+$), also appeared with abundance. The latter ions can be explained only by bond migration and subsequent cleavage, a well-known phenomenon that prevents the localization of double bonds in most of the unsaturated FAME (11).

To obtain more structural information about the unknown compound, the crude oil and the FAME mixture were exam-

TABLE 1
The Fatty Acid Composition of the Seed Oil of *Sebastiana brasiliensis* (Euphorbiaceae)

Fatty acid	Peak area % sample SBT[a]	Peak area % sample SBC[b]	Peak area % sample SBV[c]	ECL-value
12:0	<0.1	<0.1	<0.1	
14:0	<0.1	<0.1	<0.1	
16:0	7.3	9.7	8.8	
16:1(*c*9)	<0.1	<0.1	<0.1	16.26
18:0	4.4	5.6	5.1	
18:1(*c*9)	8.4	8.9	8.7	18.20
18:1(*c*11)	0.3	0.4	0.7	18.28
18:2(*c*9,*c*12)	12.0	10.8	10.3	18.61
18:3(*c*9,*c*12,*c*15)	34.0	42.3	30.5	19.20
20:0	0.3	0.1	0.2	
20:1(*c*11)	0.5	0.3	0.5	20.15
18:4(*c*9,*t*11,*t*13,*c*15)	32.5	21.4	35.1	23.22
18:4(*c*9,*t*11,*t*13,*c*15) (estimated by UV)	36.1	25.4	39.3	

[a]Sample collected in the National Park of Turvo, Derrubadas.
[b]Sample collected in the region of Caçapava do Sul.
[c]Sample collected in the region of Santana da Boa Vista. ECL, equivalent chainlength; UV, ultraviolet; *c, cis; t, trans.*

ined further. The UV spectrum of the oil showed maxima at 321, 306, 293, and 282 nm, indicating the presence of a compound with four conjugated double bonds with *cis,trans,trans,cis*-configuration (12). In agreement with these UV data, the IR bands at 994 cm^{-1} (s) and 952 cm^{-1} (m), which are characteristic for the conjugated *trans-trans* linkage (12), could be observed. Because the identified usual FAME GC peaks in the mixture do not show this type of UV and IR bands, it was concluded that the compound represented by the unknown GC peak must be responsible for these characteristic absorptions. The presence of adjacent *trans-trans* alkene groups in the unusual FAME was indicated moreover by the fact that only the GC peak of this compound decreased strongly after reaction of the FAME mixture with maleic anhydride, proving that the unknown FA must have formed a maleic anhydride adduct (12).

Further confirmation of the configuration of the conjugated double bonds was achieved by analysis of the ^{1}H and ^{13}C NMR spectroscopic data of the oil. Besides the signals of the glycerides of saturated FA and oleic, linoleic, and linolenic acids, which could be attributed by comparison with standard compounds and data in the literature (13–15), some additional characteristic signals appeared in the NMR spectra. Assignment of each peak was made in conjunction with ^{1}H NMR, COSY, ^{13}C NMR, and HETCOR spectroscopic experiments.

In addition to the usual $-CH_3$ signals at δ 0.85, the ^{1}H NMR spectrum revealed the terminal methyl group triplet, deriving from α-linolenic acid, at δ 0.97 (13), which was slightly overlapped with a further triplet at δ 1.0. The latter could be assigned to the terminal methyl group of the conjugated FA by HETCOR and COSY experiments. The complete linear array of the conjugated FA chain of the hydrocarbon backbone was given by the COSY spectrum of the seed oil. Starting from the terminal methyl triplet at δ 1.0, coupling was observed with the allylic methylene at δ 2.2, which in turn was coupled to the signal at δ 5.45 in the olefinic region, thus giving evidence for an ω3 arrangement in the conjugated FA. The allylic proton multiplet at about δ 2.2 was clearly separated from the allylic proton multiplet of the usual unsaturated FA at δ 2.05 (13). The allylic nature of these protons was proven by both their chemical shift and their correlation to olefinic protons (δ 5.3-5.4). The latter signals coupled also with the multiplet at about δ 6.0, which was further correlated to the signal at about δ 6.5. Further coupling was seen between the multiplet at δ 6.5 and δ 6.25 (Fig. 1). Because the chemical shifts of the symmetric olefinic protons of the *cis-trans-trans-cis* system are close together, the eight protons at these double bonds appeared as four pairs of multiplets, centered at δ 5.44, δ 6.0, δ 6.25, and δ 6.48, respectively. More detailed analysis of the spectra of the COSY and HETCOR experiments allowed the assignment of the center of each proton signal as shown in Tables 2 and 3. The analysis of the coupling constants of the multiplets, centered at δ 6.3 and δ 6.5, which were attributed to the four *trans*-protons of the conjugated system (H-11 to H-14), was possible by computer iteration. Using the computer program RACCOON for spectra

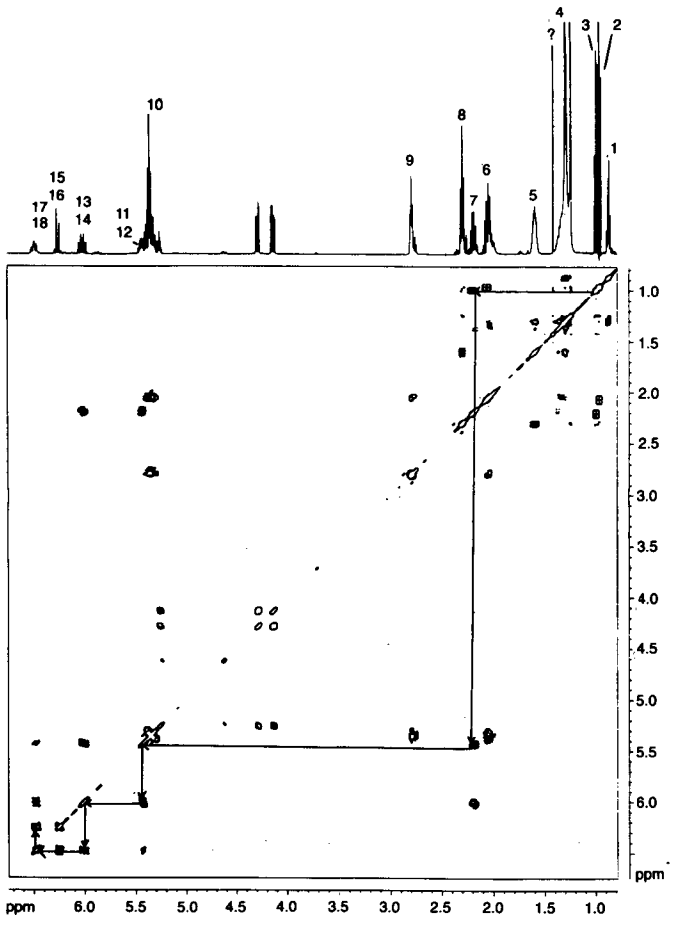

FIG. 1. ^{1}H ^{1}H two-dimensional shift correlation spectrum of the seed oil of *Sebastiana brasiliensis* (Euphorbiaceae), which shows key correlations between coupled protons of α-parinaric acid (500 MHz). The signal numbers correspond with the signal numbers in Table 2.

simulation (P.F. Schatz, University of Wisconsin, Madison, WI), we could approximate the real NMR spectrum for these olefinic protons by using $J_{11,12}$ and $J_{13,14}$ = 14 Hz and $J_{12,13}$ = 11.5 Hz. The high value for the coupling constant $J_{11,12}$ and $J_{13,14}$ was expected for the protons of the *trans-trans* structure. The symmetry of the COSY pattern in the double-bond range was identical to that published for *cis,cis,trans,trans,cis*-5,8,10,12,14-eicosapentaenoic acid (17), also containing a *cis-trans-trans-cis*-system. The chemical shift differences between the symmetric proton signals of the latter compound were more significant than for α-parinaric acid, which can be explained by a polarization effect of the *cis*-olefinic bond between C_5 and C_6 on the conjugated double-bond system in the $C_{20:5}$ FA.

In the ethylenic region of the ^{13}C NMR spectrum, eight signals, attributable to eight olefinic carbons (DEPT experiment), could be observed (Table 3). Because the other signals in this region were clearly assigned to the usual FA, it was concluded that these peaks derive from the olefinic carbons of the conjugated FA. By a HETCOR experiment, the carbon shifts of the corresponding allylic methylene groups were

TABLE 2
^{1}H Nuclear Magnetic Resonance (NMR) Data (in ppm relative to trimethylsilyl) of the Seed Oil of *Sebastiana brasiliensis* and Their Assignments[a]

Signal	δ	m	J (Hz)	Assignments[b]
1	0.88	*t*	7.5	CH_3– (S, M, L)
2	0.968	*t*	7.51	CH_3– (Ln)
3	1.0	*t*	7.5	CH_3– (P)
4	1.28	*m*	—	–$(CH_2)_n$– (all FA)
5	1.58	*m*	—	CH_2–CH_2–COOR (all FA)
6	2.05	*m*	—	–CH=CH–CH_2– (M, L, Ln)
7	2.2	*m*	—	–CH=CH–CH_2– (P; H-8/H-17)
8	2.3/2.308	*t*	7.6	–CH_2–COOR (all FA)
9	2.76/2.80	*t*	6.5	–CH=CH–CH_2–CH=CH– (L, Ln)
10	5.35	*m*	—	–CH=CH– (M, L, Ln)
11	5.43	*m*	—	H-9 (P)
12	5.45	*m*	—	H-16 (P)
13	6.0	*tt*	10.8; 1.0	H-10 (P)
14	6.03	*tt*	10.9; 1.0	H-15 (P)
15	6.25	*m*	14; 11.5[c]	H-13 (P)
16	6.258	*m*	14; 11.5[c]	H-12 (P)
17	6.478	*m*	14; 10.8[c]	H-11 (P)
18	6.488	*m*	14; 10.8[c]	H-14 (P)

[a]Assignments are based on ^{1}H ^{1}H-homonuclear and ^{1}H ^{13}C two-dimensional heteronuclear shift correlation experiments and on comparison with standard compounds and literature data (Refs. 13,17). The chemical shifts of α-parinaric acid were confirmed by ^{1}H NMR analysis of the isolated α-parinaric acid methyl ester. Signals of the glycerol backbone are omitted.
[b]Abbreviations: S, saturated; M, monoenes; L, linoleate; Ln, linolenate; P, α-parinate.
[c]Values used for the computer simulation of the ^{1}H NMR spectrum of these protons.

identified at δ 27.97 and δ 21.3. The first of these signals can be attributed to a methylene group adjacent to a *cis*-double bond (14). The latter signal appeared in the same range as the signal for the ω2 carbon from linolenic acid (14), suggesting that the second allylic methylene group of the conjugated FA must also be in α-position to a *cis*-double bond in ω2 position of the FA chain. The allylic chemical shift changes of the conjugated FA were +0.69 ppm in comparison with the signal due to the same carbons of the nonconjugated linolenic FA (see Table 3). In accordance with these observations, the lack of ^{13}C-signals in the range at δ 32.5, which derive from carbons in allylic position to a *trans*-double bond (14,16), proved the absence of a –CH_2–CH=CH(*trans*)-structure, and confirmed the UV data that the configuration of the conjugated double-bond system must be *cis-trans-trans-cis*. These ^{13}C NMR data were in good agreement with the published ^{13}C NMR data of the oil of *Impatiens balsamina* (Balsaminaceae), known to contain α-parinaric acid (16). In contrast to the work of Tulloch (16), who measured the ^{13}C NMR spectrum at 25 MHz, the signals of α-parinaric acid in the double-bond range were well resolved when operating at 125 MHz (Fig. 2).

All of these data demonstrate that the conjugated FA from the oil of *S. brasiliensis* is identical with *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid. To confirm the NMR assignments and the location of the double bonds, the conjugated FA was isolated by preparative TLC and further examined. Due to the system of conjugated double bonds, the compound was easily detectable on the layer under UV_{254}. It was well separated from the nonconjugated acids under the experimental conditions. The latter fraction was detected under UV after spraying the plates with 2′,7′-dichlorofluorescein. UV and IR spectra of the conjugated FAME were nearly identical with the data obtained from the crude seed oil of *S. brasiliensis* and with data in the literature data concerning α-parinaric acid ME (12), whereas the nonconjugated FAME fraction did not show any absorption in the described regions. The ^{1}H and ^{13}C NMR spectra of the isolated compound could

TABLE 3
Chemical Shifts of the ^{13}C Atoms (in ppm relative to trimethylsilyl) of the Seed Oil of *Sebastiana brasiliensis* and Their Assignments[a]

Peak	^{13}C-Shift	Assignments[b]	Peak	^{13}C-Shift	Assignments
1	14.16/14.21	CH_3– (S, M, L)	18	127.83/127.84	C-10 (Ln; 1, 3/2-glyceride)
2	14.34	C-18 (Ln)	19	127.96	C-12 (L)
3	14.37	C-18 (P)	20	128.14	C-11 (P), C-10 (L)
4	20.63	C-17 (Ln)	21	128.21	C-14 (P)
5	21.33	C-17 (P)	22	128.24	C-10 (P)
6	22.67/22.78	CH_3–CH_2–CH_2– (S, M, L)	23	128.30	C-12 (Ln)
7	24.91/24.95	C-3 (S, M, L, Ln, P)	24	128.36	C-13 (Ln)
8	25.61	C-11 (L)	25	128.89	C-15 (P)
9	25.70	–CH=CH–CH_2–CH=CH– (Ln)	26	129.76/129.78	C-9 (O; 2/1,3-glyceride)
10	27.28	–CH=CH–CH_2–CH_2– (M, L, Ln)	27	130.04/130.09	C-10 (O), C-9 (L)
11	27.97	C-8 (P)	28	130.26	C-13 (L)
12	29-30	–$(CH_2)_n$– (all FA)	29	130.28	C-9 (Ln)
13	31.61	C-16 (L)	30	132.01	C-16 (Ln)
14	31.99	CH_3–CH_2–CH_2– (M)	31	132.83	C-9 (P)
15	32.01	CH_3–CH_2–CH_2– (S)	32	132.88	C-12 (P)
16	34.09/34.12/34.26	C-2 (S, M, L, Ln, P)	33	132.91	C-13 (P)
17	127.19	C-15 (Ln)	34	134.52	C-16 (P)

[a]Assignments are based on distortionless enhanced polarization transfer, ^{1}H-^{13}C-two-dimensional heteronuclear shift correlation experiments and on comparison with standard compounds and data in the literature (Refs. 13–15). The chemical shifts of α-parinaric acid were confirmed by ^{13}C NMR analysis of the isolated α-parinaric acid methyl ester. Signals of glycerol and carboxyl carbons are omitted.
[b]Abbreviations as in Table 2; O, oleate; FA, fatty acid.

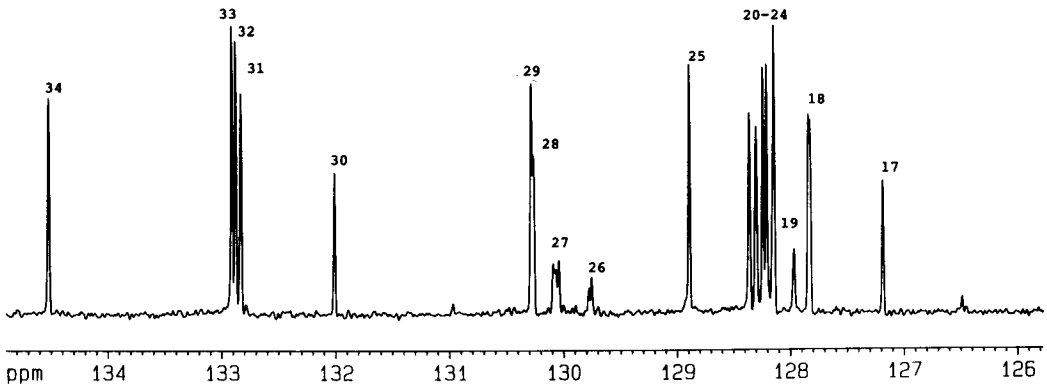

FIG. 2. 125 MHz ^{13}C nuclear magnetic resonance olefinic spectrum of the seed oil of *Sebastiana brasiliensis* (Euphorbiaceae). The chemical shifts of the numbered signals are given in Table 3.

confirm all NMR signal assignments for α-parinaric acid made on the crude oil (Tables 2 and 3). Location of the double-bond system was proven moreover by the oxidative splitting of the isolated α-parinaric acid ME. GC analysis of the oxidation products showed that azelaic acid ME was the only resulting dibasic acid; thus, the conjugated double-bond system must begin at C_9 of the FA chain.

Chemotaxonomical considerations. The plant family Euphorbiaceae is known to contain a great variety of unusual C_{18} FA derivatives, e.g., α-eleostearic, α-kamlolenic, ricinoleic, and vernolic acid (18,19). Up to now, α-parinaric acid was found only in the oils from some species of the plant families Balsaminaceae and Chrysobalanaceae (20), and the seed oil of *S. brasiliensis* is the first example in the plant family Euphorbiaceae that contains this FA. α-Parinaric acid was not detectable in other species of *Sebastiana,* such as *S. ligustrina* (21), *S. cerrata,* and *S. commersoniana* (unpublished data).

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