Analysis of Triacylglycerols Containing Cyclopropene Fatty Acids in *Sterculia foetida* (Linn.) Seed Lipids†

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The distribution of cyclopropene acyl moieties in triacylglycerols of *Sterculia foetida* (Linn.) seed lipids was determined after conversion into oxo derivatives which are stable under GLC conditions and readily separated. The triacylglycerols after derivatization were analyzed by spectroscopic and chromatographic techniques. It was found that the triacylglycerols of *S. foetida* seed lipids are composed of four types of molecular species I–IV in the ratio 6:41:33:20, respectively. The minor molecular species I contains common long-chain acyl moieties without a cyclopropene ring. The molecular species II, III, and IV were shown to have one, two, and three cyclopropene acyl moieties, respectively. Pancreatic lipase hydrolysis revealed that the oleoyl and linoleoyl moieties are preferentially esterified at the *sn*-2 position of glycerol, while the palmitoyl moieties are mostly located at the *sn*-1,3 positions. Similarly, the sterculoyl moieties show a preference for the *sn*-2 position and the malvaloyl moieties for the *sn*-1,3 positions.

INTRODUCTION

It is well-known that the cyclopropene fatty acids (CPFA) such as sterculic (9,10-methylene-9-octadecenoic) acid and malvalic (8,9-methylene-8-heptadecenoic) acid show carcinogenic (Sinnhuber et al., 1976) and cocarcinogenic (Lee et al., 1971) activities. Sterculic acid is more carcinogenic than malvalic acid (Pawlowski et al., 1985). The unusual physiological properties (Roehm et al., 1971) shown by such lipids are due to the presence of a cyclopropene ring in the fatty acid chain.

sterculic acid, $n = 7$
malvalic acid, $n = 6$

Much attention has been paid to the quantitation of CPFA and the evaluation of their biological activities, but less interest (Schuch et al., 1986; Christie, 1970) has been shown in the analysis of triacylglycerols containing cyclopropene acyl moieties, despite their occurrence in some edible fats (Berry, 1982). It was of interest to analyze such triacylglycerols for the positional distributions of the sterculoyl and malvaloyl moieties. The difficulties encountered in the analysis and separation of molecular species of triacylglycerols containing CPFA moieties are attributed to the strained and reactive cyclopropene ring. Direct gas–liquid chromatography of intact triacylglycerols containing CPFA moieties is not feasible because these substances undergo rapid thermal polymerization at high temperatures. The cyclopropene groups also react readily with silver nitrate to produce different derivatives depending on the organic solvent used in these reactions (Kircher, 1965; Johnson et al., 1967; Raju and Reiser, 1966). Consequently, separation of these triacylglycerols according to the number of CPFA moieties cannot be accomplished by argentation TLC. There is some evidence that the CPFA moieties partially inhibit the activity of lipase, which raises problems in the determination of their positional distribution by lipolysis (Litchfield, 1972). Attempts to resolve these triacylglycerols by GLC after the CPFA moieties were converted into more stable cyclopropane (Litchfield et al., 1967) and mercapto derivatives (Schneider et al., 1968) were not very successful.

A number of methods have been reported (Conway et al., 1985) for the analysis of fatty acid methyl esters containing CPFA moieties. These include the use of a well-conditioned glass column packed with nonpolar stationary phase at low temperature (150 °C) (Fisher and Schuller, 1981; Bianchini et al., 1981) and hydrogenation of cyclopropene moieties to cyclopropane moieties using heterogeneous (Cornelius et al., 1965; Hammonds and Shone, 1966) and homogeneous (Bland et al., 1984) catalysts followed by GLC analysis. These methods could not be used for the successful analysis of triacylglycerols into their molecular species possessing CPFA moieties.

Keeping in view the above difficulties, we believed the best way to analyze the CPFA-containing triacylglycerols would be to modify the cyclopropene group to an oxygenated derivative under very mild conditions so that they can be easily separated and analyzed by conventional chromatographic techniques.

Recently, a method was developed (Schuch et al., 1986) for the analysis of triacylglycerols containing CPFA moieties; it involves the conversion of the cyclopropene group into stable α,β-unsaturated keto derivatives. This method was based on the experiments conducted by Kircher (1965). We have adapted a similar methodology to analyze the triacylglycerols of *Sterculia foetida* seed lipids containing 72% cyclopropene fatty acids.

MATERIALS AND METHODS

Materials. The matured seeds of *S. foetida* (Hindi; Jangli badam) were collected from the trees grown at the campus of the Indian Institute of Chemical Technology (Hyderabad, India). Analytical grade reagents and adsorbent (silica gel G) for thin-layer chromatography (TLC) were from E. Merck Ltd. (Bom-

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Table I. Composition (% m/m) of Acyl Moieties[a] of Molecular Species of Triacylglycerols in *S. foetida* Seeds Obtained by Derivatization with $AgNO_3$/Acetonitrile/Acetone

fraction	relative proportion, %	14:0	16:0	16:1	18:0	18:1	18:2	18:3	19:CE	18:CE	others (unidentified)
I	6	tr[c]	24.8	tr	9.5	23.8	41.8				
II	41	tr	32.2	tr	1.5	15.0	10.7	0.9	28.2[b]	9.3[b]	2.1
III	33	tr	17.1		0.7	5.1	2.3	2.9	53.5[b]	14.4[b]	4.0
IV	20								65.0[b]	34.0[b]	0.9

[a] Acyl moieties are designated by number of carbon atoms:number of double bonds. 19:CE, sterculoyl; 18:CE, malvaloyl. [b] Calculated as the sum of keto and methoxy keto derivatives of 19:CE and 18:CE, respectively. [c] Trace.

bay, India). Distilled solvents were used throughout. Porcine pancreatic lipase was purchased from Sigma Chemical Co. (St. Louis, MO).

^1H NMR spectra were recorded on a Varian (Palo Alto, CA) A 60 spectrometer in $CDCl_3$, and chemical shifts were determined in parts per million downfield from tetramethylsilane used as internal standard. Total methyl esters of groundnut, linn, and coconut seed oils were used as reference standards. Methyl esters of total lipids from *S. foetida* seed were used as reference standards for methyl sterculate and methyl malvalate. Methyl tridecanoate served as internal standard.

Lipid Extraction and Derivatization. The dried seeds were finely powdered and extracted with light petroleum ether (40–60 °C) in a Soxhlet apparatus on a water bath for 1–2 h. Total lipids and lipid fractions were converted to methyl esters by transmethylation using benzene/methanol/sulfuric acid (10:85:4 v/v/v) (Ahmad et al., 1986). Methyl esters were purified by silica gel (60–120 mesh) column chromatography.

Cyclopropene groups present in the acyl moieties of triacylglycerols and methyl esters were converted to α,β-unsaturated keto derivatives (Schuch et al., 1986; Kircher, 1965). Triacylglycerols (up to 20 mg) were shaken with 1 mL of 0.1 M $AgNO_3$ in acetonitrile/acetone (1:1 v/v) for 24 h at room temperature under a nitrogen atmosphere. The reaction mixture was then diluted by adding excess water. Lipids were extracted with hexane, and the hexane extract was washed with 5% (v/v) HCl followed by 5% (w/v) sodium bicarbonate. The solvent was removed under nitrogen at 50 °C.

Analysis of Fatty Acids. Methyl esters were analyzed by gas–liquid chromatography (GLC) using a 10% Silar 5 CP column (1.8 m × 2 mm) in a Tracor 540 instrument equipped with a flame ionization detector (Tracor Instruments, Austin, TX). Nitrogen (50 mL/min) was used as carrier gas, and the column temperature was programmed from 150 to 230 °C at 4 °C/min. Peaks were identified by comparing the retention times with those of authentic reference standards, and the peak areas were calculated by triangulation. Figures reported are area percent of the individual peaks.

Analysis of Triacylglycerols. Triacylglycerols were isolated from the total lipids by silica gel (60–120 mesh) column chromatography using petroleum ether/diethyl ether (95:5 v/v) as eluting solvent. Cyclopropene groups in the acyl moieties of triacylglycerols were converted to α,β-unsaturated keto derivatives as described above. These derivatized triacylglycerols were fractionated into four fractions (I–IV; R_f values: I, 0.88; II, 0.64; III, 0.47; IV, 0.3) by TLC using petroleum ether/diethyl ether (70:30 v/v) as developing solvent. The bands were detected under UV light and extracted from silica gel with diethyl ether. Aliquots of each molecular species of triacylglycerols were transmethylated and the resulting methyl esters analyzed by GLC. Similarly, methyl esters from total lipids of *S. foetida* were treated with $AgNO_3$/acetonitrile/acetone and used as reference standards. Relative proportions of molecular species were determined using methyl tridecanoate as internal standard.

Lipase hydrolysis of the four classes of triacylglycerols was essentially the same as that described by Luddy et al. (1964) except that we used gum arabic (acacia) in place of sodium cholate as emulsifier. The resulting 2-acylglycerols were isolated by boric acid TLC (Thomas et al., 1965) using petroleum ether/diethyl ether/acetic acid (70:30:2 v/v/v) as developing solvent. Components were viewed under UV light after the plates were sprayed with 2′,7′-dichlorofluorescein. The lipids were extracted from silica gel as described by Christie (1973). The 2-acylglycerols were analyzed by GLC for acyl composition after transmethylation.

Table II. Acyl Composition (% m/m) of *S. foetida* Seed Lipids after Derivatization

	present work			
		total lipids		reported in the literature (Bohannon and Kleiman, 1978)[d]
acyl moieties[a]	TG[c]	*c*	*d*	
14:0	tr			
16:0	19.1	12.5	12.3	14.7
16:1	tr	tr	tr	tr
18:0	0.2	0.2	0.3	1.4
18:1	7.9	4.0	4.0	4.9
18:2	2.3	1.9	2.1	4.5
18:3	1.2	0.3	0.3	
20:0	1.2	3.3	3.4	1.8
20:1				0.2
19:CE	53.2[b]	58.8[b]	59.0[e]	65.1[e]
18:CE	13.1[b]	19.0[b]	18.6[e]	6.3[e]
others (unidentified)	1.8			

[a] See Table I. [b] Calculated as the sum of keto and methoxy keto derivatives of 19:CE and 18:CE, respectively. [c] Treated with $AgNO_3$/acetonitrile/acetone. [d] Treated with $AgNO_3$/methanol. [e] Calculated as the sum of the methyl ether and keto derivatives of 19:CE and 18:CE, respectively.

RESULTS AND DISCUSSION

The acyl composition of each of the triacylglycerol fractions isolated by preparative TLC was determined quantitatively by GLC (Table I) after acid-catalyzed transmethylation, and their relative proportions were determined using methyl tridecanoate as internal standard. During transmethylation, it was observed that the α,β-unsaturated keto derivative was partially converted to methoxy keto derivative as observed by GLC. The methyl esters of α,β-unsaturated ketones and methoxy ketones gave distinct peaks in GLC. The sum of the area of these peaks was considered as the percentage of CPFA moieties. The methoxy keto derivative was characterized by ^1H NMR spectral analysis. It showed NMR signals at δ 3.6 (3 H, singlet, $COOCH_3$), 3.4 (2 H, doublet, $CHCH_2OCH_3$), 3.25 (3 H, singlet, OCH_3), 2.16–2.6 (5 H, multiplet, methine and methylene protons α to carbonyl groups), 1.3 (chain methylene protons), and 0.9 (terminal methyl protons). We assume that the attack of the lone pair of electrons present in methanol took place on a carbocation generated by enol formation in the acidic medium (a Michael-type addition). In basic medium too, the partial conversion of an α,β-unsaturated ketone into methoxy ketone has been reported (Schuch et al., 1986).

To determine whether the conversion of CPFA moieties into α,β-unsaturated ketones (Kircher, 1965) is quantitative or not, the acyl composition of $AgNO_3$/methanol-treated triacylglycerols was compared with that of $AgNO_3$/acetone/acetonitrile-treated triacylglycerols. The results (Table II) are in good agreement with the acyl composition of *S. foetida* seed lipids reported earlier (Bohannon and Kleiman, 1978). Treatment of triolein with $AgNO_3$/ac-

Table III. Positional Distribution[a] of Acyl Moieties (% m/m) in Molecular Species of Triacylglycerols of *S. foetida* Seed Obtained by Derivatization with $AgNO_3$/Acetonitrile/Acetone

acyl moieties[b]	molecular species according to number of keto groups							
	I(0)[c]		II(1)[c]		III(2)[c]		IV(3)[c]	
	sn-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3
14:0	0.2		0.2		0.1			
16:0	3.7	35.2	11.5	42.5	6.0	22.4		
16:1	0.2		1.4					
18:0		14.0		2.3		1.1		
18:1	30.9	20.3	35.6	4.5	5.1	5.1		
18:2	64.5	30.5	35.5		2.4	2.2		
18:3	0.2		0.7	0.9		4.3		
19:CE			13.1[d]	35.6[d]	80.3[d]	40.1[d]	87.8[d]	54.0[d]
18:CE			1.3[d]	13.0[d]	4.0[d]	19.6[d]	12.2[d]	46.0[d]
others (unidentified)	0.3		0.6	2.0	1.5	5.2		

[a] Composition of acyl moieties at the *sn*-2 position was determined by lipolysis with pancreatic lipase; composition of the acyl moieties at the *sn*-1,3 positions was calculated from the composition of acyl moieties at the *sn*-1,2,3 positions (Table I) and the *sn*-2 position. [b] Acyl moieties are designated by number of carbon atoms:number of double bonds. 19:CE, sterculoyl; 18:CE, malvaloyl. [c] Numbers in parentheses indicate number of keto groups. [d] Calculated as the sum of keto and methoxy keto derivatives of 19:CE and 18:CE, respectively.

etonitrile/acetone caused no hydrolysis of acyl linkages, suggesting that the reaction is specific for CPFA moieties but not for acyl linkages and other double bonds present in the fatty acid chain.

It is evident from the acyl composition of each fraction (Table I) that the triacylglycerols of *S. foetida* seed lipids contain four types of molecular species (fractions I–IV) differing in the number of cyclopropene acyl moieties per molecule. Fractions I (6% of the total TG), II (41% of the total TG), III (33% of the total TG), and IV (20% of the total TG) contain none, one, two, and three CPFA moieties per molecule since they contained no keto, ~33% keto, ~66% keto, and 100% keto derivatives, respectively.

Positional distribution of acyl moieties in the four classes of triacylglycerols was determined by pancreatic lipase hydrolysis. We have used gum arabic (acacia) instead of sodium cholate as emulsifier and stabilizer of emulsion in all lipolytic reactions because it gives better results (Jensen, 1983). Lipolyzed products of each triacylglycerol fraction were separated by TLC. The 2-acylglycerols containing α,β-unsaturated keto acyl moieties and the common long-chain acyl moieties had the same R_f values and remained at the bottom of the plates.

The positional distribution of acyl moieties in the molecular species of triacylglycerols of *S. foetida* seeds is given in Table III. The sterculoyl moieties are preferentially located at the *sn*-2 position of the triacylglycerol molecules. In triacylglycerols of *Bombax munguba* (Schuch et al., 1986) and *Bombacopsis glabra* (Christie, 1970) seed lipids also, most of the sterculoyl moieties are present at the *sn*-2 position.

It is clear from the data obtained after lipase hydrolysis of each fraction that in fractions I and II the oleoyl and linoleoyl moieties are preferentially located at the *sn*-2 position, while in fraction III these two acids are equally distributed between the *sn*-2 and *sn*-1,3 positions. In the first three fractions, palmitoyl moieties are more concentrated at the *sn*-1,3 positions. Triacylglycerols containing one CPFA (fraction II) have abundant sterculoyl and malvaloyl moieties at the *sn*-1,3 positions. In fractions III and IV, the sterculoyl and malvaloyl moieties are enriched at the *sn*-2 and *sn*-1,3 positions, respectively.

ABBREVIATIONS USED

18:CE, malvaloyl moiety; 19:CE, sterculoyl moiety; CPFA, cyclopropene fatty acid; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; TG, triacylglycerols; TLC, thin-layer chromatography.

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