# Preparation of Malvalic and Sterculic Acid Methyl Esters from *Bombax munguba* and *Sterculia foetida* Seed Oils

**E. Fehling, S. Schönwiese, E. Klein, K.D. Mukherjee, and N. Weber***

Institut für Biochemie und Technologie der Fette, Bundesanstalt für Getreide-, Kartoffel- und Fettforschung, D-48147 Münster, Germany

**ABSTRACT:** A method has been developed for the preparation of highly pure malvalic (*cis*-8,9-methyleneheptadec-8-enoic) and sterculic (*cis*-9,10-methyleneoctadec-9-enoic) acid methyl esters starting from *Bombax munguba* and *Sterculia foetida* seed oils. The methyl esters of these oils were prepared by sodium methylate-catalyzed transmethylation followed by cooling (6°C) the hexane solution of crude methyl esters and separation of insoluble fatty acid methyl esters by centrifugation in the case of *B. munguba* and by column chromatography in the case of *S. foetida*. Subsequently, the saturated straight-chain fatty acid methyl esters were almost quantitatively removed by urea adduct formation. Finally, methyl malvalate and methyl sterculate were separated from the remaining unsaturated fatty acid methyl esters, in particular methyl oleate and methyl linoleate, by preparative high-performance liquid chromatography on $C_{18}$ reversed-phase using acetonitrile isocratically. Methyl malvalate and methyl sterculate were obtained with purities of 95–97 and 95–98%, respectively.
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**KEY WORDS:** *Bombax munguba* oil, cyclopropene fatty acids, malvalic acid methyl ester, preparative HPLC, *Sterculia foetida* oil, sterculic acid methyl ester, urea fractionation.

Cyclopropene fatty acids are widely distributed in the seed oils of many plant families of the order Malvales (1,2). Small proportions of cyclopropene fatty acids, e.g., malvalic (*cis*-8,9-methyleneheptadec-8-enoic) and sterculic (*cis*-9,10-methyleneoctadec-9-enoic) acids, which are present in crude cottonseed oil, are of special interest owing to their harmful biological effects when fed to animals (3–7).

The presence of cyclopropene fatty acids in seed oils can be roughly checked by the Halphen test (8). Identification by gas chromatography (GC) and high-performance liquid chromatography (HPLC) analysis (9,10) is difficult, however, because no cyclopropene fatty acid standards are commercially available. This paper presents a simple method for the preparation of both methyl malvalate and methyl sterculate of high purity, which may be useful for GC and HPLC comparison as well as biochemical and enzymatic studies. The starting materials were seeds of *Bombax munguba* (11) and *Sterculia foetida* (1,2), which are known to be good sources of cyclopropene fatty acids.

*To whom correspondence should be addressed at the Institut für Biochemie und Technologie der Fette, Bundesanstalt für Getreide-, Kartoffel- und Fettforschung, Piusallee 68, D-48147 Münster, Germany.
E-mail: ibtfett@uni-muenster.de

## EXPERIMENTAL PROCEDURES

*Materials.* Distilled solvents were used throughout. HPLC-grade acetonitrile was purchased from Rathburn Chemicals (Walkerburn, Scotland). Silica gel, urea, and sodium methylate were products of E. Merck (Darmstadt, Germany). *Bombax munguba* Mart. seeds were collected at University of Paraiba, João Pessoa, Brazil (11); *S. foetida* L. seeds were a gift from Professor Helmut K. Mangold, Münster, Germany.

*Preparation of fatty acid methyl esters (FAME).* Seeds of *B. munguba* (10 g) were ground in an electric coffee grinder and the meal extracted in a Soxhlet extractor for 6 h with 200 mL hexane. The hexane was evaporated and the oil transmethylated with sodium methylate in methanol (11). After addition of water and 2 N hydrochloric acid the acidic reaction mixture was extracted repeatedly with diethyl ether. Ether extract (5.1 g of crude FAME) was evaporated to dryness, dissolved in hexane (1 g/3 mL) and cooled to 6°C for 3 d in a refrigerator. Insoluble material was removed from the hexane solution by centrifugation and the supernatant was used for urea fractionation.

After removal of the seed coats, *S. foetida* seeds were ground and extracted as described above, yielding 5.1 g oil/10 g seeds. Triacylglycerols were separated from polar nontriglyceride compounds of the oil by flash chromatography using Silica Gel 60 (glass column 20 × 2 cm) in hexane using hexane/diethyl ether (95:5, vol/vol) and hexane/diethyl ether (9:1, vol/vol), 75 mL each, as the eluents. The triacylglycerol fraction (2.7 g) was transmethylated and the resulting crude methyl esters were purified by flash chromatography as described above with 75 mL hexane/diethyl ether (95:5, vol/vol). Purified FAME were used for urea adduct formation.

*Urea adduct formation.* Urea (15 g), moistened with methanol (0.3 mL/g urea) was added in small portions to 1 g of the above *B. munguba* or *S. foetida* FAME fraction dissolved in 40 mL hexane; after each addition, the mixture was vigorously shaken (12). After standing overnight, the mixture

was centrifuged and the supernatant removed. The pellet was washed twice with hexane. In the case of *B. munguba,* the combined hexane extracts were washed with water, dried with anhydrous sodium sulfate, concentrated, and the unsaponifiable components were removed from FAME by flash chromatography (glass column 2 × 20 cm) on Silica Gel 60 (Merck) in hexane using 75 mL hexane/diethyl ether (9:1, vol/vol) as the eluent. FAME (100 mg) were eluted with the latter solvent mixture. After evaporation of solvents, this fraction was dissolved in acetonitrile and used for the isolation of malvalic and sterculic acid methyl ester by preparative reversed phase (RP)–HPLC.

*Sterculia foetida* FAME of hexane supernatant of urea fractionation were washed with water, dried over anhydrous sodium sulfate, and used directly for the isolation of both cyclopropenoid FAME by preparative RP–HPLC.

*Preparative RP–HPLC.* The HPLC system consisted of a Gilson pump 303 (Villier le Bel, France) equipped with a pump head 25SC and a manometric module 803C; a Gilson HM Holochrome UV/Vis detector (set to a wavelength of 210 nm) and a Gilson model 131 refractive index (RI) detector (thermostated to 30°C), which were used in series; and a Kontron Instruments data system 450-MT2 (Milan, Italy). FAME were separated isocratically with acetonitrile on a Merck Hibar RP-18 column (250 × 25 mm i.d. and precolumn 30 × 25 mm i.d., packed with LiChrospher 100, 10 µm). The flow rate was set at 17.5 mL/min. Injections (up to 90 mg methyl esters) were carried out with a Rheodyne 7125 sample injector (Cotati, CA) equipped with a 1 mL sample loop. Fractions were collected at intervals of 0.3 min with a Frac-100 fraction collector (Pharmacia Biotech, Uppsala, Sweden). The detector signals and fraction changes were recorded by a Pharmacia chart recorder equipped with an event marker.

*GC and GC–mass spectrometry (MS) analyses.* GC was carried out in a Hewlett-Packard (Waldbronn, Germany) HP-5890 Series II gas chromatograph fitted with a flame-ionization detector. FAME were chromatographed on a 0.20 µm DB-23 fused-silica capillary column (J&W Scientific, Folsom, CA), 40 m × 0.18 mm i.d., and on a 0.52 µm HP-1 fused-silica capillary column (Hewlett-Packard), 25 m × 0.32 mm i.d**.** The methyl esters were separated on the DB-23 column, using hydrogen as the carrier gas, at 160°C for the first 2 min, followed by linear programming from 160 to 178°C at 1°C/min and from 178 to 240°C at 8°C/min. The final temperature was kept constant for 2 min. FAME separation on the HP-1 column was conducted using nitrogen as the carrier gas beginning at 180°C for 2 min, followed by linear programming from 180 to 220°C at 2°C/min and from 220 to 240°C at 15°C/min. With both columns the split ratio was 1:10 and the injector and flame-ionization detector temperatures were 270°C. Peaks in gas chromatograms were assigned by comparison of their retention times with those of known standards and mixtures of methyl esters that had been analyzed earlier (11). Peak areas and percentages were calculated using

**TABLE 1**
**Fatty Acid Composition of Fractions Obtained During Various Steps of Enrichment of Methyl Malvalate and Methyl Sterculate Starting from Fatty Acid Methyl Esters of Total Seed Lipids of *Bombax munguba* and *Sterculia foetida***

| | Fatty acid composition[a] (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Step/fraction | 16:0 | 18:0 | Δ9-18:1 | 18:2 | 18:1CE[b] | 19:1CE[b] | Others | CE/SAT[c] |
| *Bombax munguba* | | | | | | | | |
| Starting material | 58.3 | 3.8 | 5.3 | 5.6 | 0.5 | 18.4 | 8.1 | 0.30 |
| 1. Cooling | | | | | | | | |
| Pellet | 88.0 | 3.8 | 1.3 | 1.3 | Trace | 4.7 | 0.9 | 0.05 |
| Supernatant A | 48.2 | 3.5 | 7.7 | 8.0 | 0.9 | 29.0 | 2.7 | 0.58 |
| 2. Urea adduct formation | | | | | | | | |
| Pellet | 56.6 | 3.9 | 8.7 | 7.2 | Trace | 19.5 | 4.1 | 0.32 |
| Supernatant B | 0.4 | 0.7 | 2.1 | 12.2 | 1.8 | 77.6 | 5.2 | 72.2 |
| 3. Preparative RP–HPLC | | | | | | | | |
| 18:1CE | n.d.[d] | n.d. | n.d. | 2.6 | 97.4 | n.d. | Trace | — |
| 19:1CE | n.d. | n.d. | 0.6 | n.d. | n.d. | 98.5 | 0.9 | — |
| *Sterculia foetida* | | | | | | | | |
| Starting material[e] | 27.0 | 3.7 | 5.6 | 6.3 | 6.7 | 45.0 | 5.7 | 1.7 |
| 1. Column chromatography | 24.4 | 3.6 | 5.4 | 5.3 | 6.9 | 47.0 | 7.4 | 1.9 |
| 2. Urea adduct formation | | | | | | | | |
| Pellet | 29.8 | 4.0 | 7.4 | 5.5 | 6.2 | 40.3 | 6.8 | 1.4 |
| Supernatant | 0.5 | 1.2 | 1.7 | 5.4 | 8.7 | 73.1 | 9.4 | 48.1 |
| 3. Preparative RP–HPLC | | | | | | | | |
| 18:1CE | n.d. | n.d. | n.d. | 2.9 | 95.6 | n.d. | 1.5 | — |
| 19:1CE | n.d. | n.d. | 0.6 | n.d. | n.d. | 95.3 | 4.1 | — |

[a]Fatty acids are designated by number of carbon atoms:number of double bonds. Δ indicates the position of a double bond.
[b]18:1CE = 18:1 cyclopropenoic fatty acid, i.e., malvalic acid; 19:1CE = 19:1 cyclopropenoic fatty acid, i.e., sterculic acid.
[c]Ratio total cyclopropenoic acids/saturated fatty acids.
[d]n.d. = not detected; trace, <0.2%.
[e]Triacylglycerols after separation of polar nontriglyceride compounds by flash chromatography. RP–HPLC, reversed-phase–high-performance liquid chromatography.

Hewlett-Packard GC ChemStation software. GC–MS was performed as described earlier (13).

## RESULTS AND DISCUSSION

Seed oils of the plant families of Bombacaceae and Malvaceae, e.g., cotton and kapok seed oil, contain toxic cyclopropenoic fatty acids and cannot be used for the preparation of food and feed without technical processing for their removal (14). Pure cyclopropenoic fatty acids, such as malvalic and sterculic acids, which are of interest for lipid analysis as well as biochemical studies and chemical reactions, are not commercially available. Fractionation by urea adduct formation is known to yield concentrates of cyclopropenoic fatty acids together with unsaturated fatty acids (15). Separation of cyclopropenoic fatty acids by spinning band distillation requires relatively large proportions of starting material (16). $AgNO_3$-chromatography is not suitable for the separation of cyclopropenoic fatty acids because of ring opening and other derivatization reactions catalyzed by silver ions (17,18). Methods used for the preparative isolation of cyclopropenoic fatty acids in good purity include repeated urea fractionations and crystallizations (9,19,20) as well as countercurrent distribution (19). We report here a simple method involving preparative RP–HPLC for the preparation of highly pure cyclopropenoic fatty acids, e.g., malvalic and sterculic acids, in appropriate quantities that could be of interest for chemical, biochemical, and analytical purposes.

Table 1 shows the different steps of enrichment of methyl malvalate and methyl sterculate starting from FAME of total seed lipids of *B. munguba* and *St. foetida*. Fatty acid composition of the samples at the various fractionation steps was determined by GC. It should be noted that methyl malvalate was not separated from methyl stearate on the polar DB-23 column, whereas this critical pair of FAME was well resolved on the unpolar HP-1 column.

Crude FAME from *B. munguba* oil contained high proportions of methyl palmitate, which were reduced by precipitation from hexane solution at low temperature. It is obvious from Table 1 that the ratio of cyclopropenoid fatty acids/saturated fatty acids (CE/SAT) was almost doubled in supernatant A of *B. munguba* FAME after cooling (CE/SAT = 0.58) as compared to the starting material (0.30). The proportion of saturated fatty acids, in particular palmitic acid (16:0), was dramatically reduced in the supernatant B after urea adduct formation (CE/SAT = 72.2). Simultaneously, the proportion of unsaturated fatty acids, especially linoleic acid, increased with each fractionation step. Separation of cyclopropenoic acids from other unsaturated fatty acids was, however, not possible by $AgNO_3$-chromatography for reasons stated above.

*Sterculia foetida* oil was characterized by high proportions of polar nontriglyceride compounds which were separated by flash chromatography. After transmethylation of the triacylglycerols, followed by separation of unsaponifiable compounds by a second flash chromatography, FAME were ready for urea fractionation. Table 1 shows that the composition of FAME derived from *S. foetida* oil was hardly changed by chromatographic separation of unsaponifiable compounds (CE/SAT = 1.7 vs. CE/SAT = 1.9), whereas the proportion of saturated fatty acids, in particular palmitic acid (16:0), was greatly reduced in the supernatant after urea fractionation (CE/SAT = 48.1).

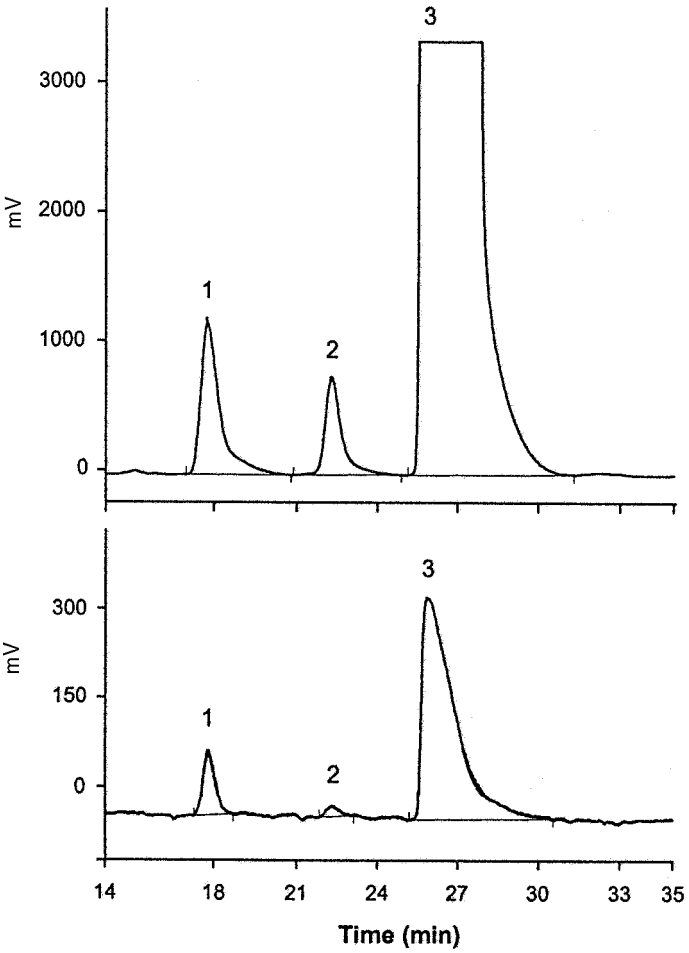


**FIG. 1.** Preparative reversed-phase–high-performance liquid chromatography of the supernatant of urea adducts of fatty acid methyl esters of *Bombax munguba* seed oil, enriched with malvalic (18:1CE) and sterculic (19:1CE) acid methyl esters. The various fractions collected at intervals of 0.3 min were combined according to their fatty acid methyl ester composition as determined by gas chromatography (upper trace: ultraviolet detection, lower trace: refractive index detection; 1, methyl linoleate; 2, methyl malvalate; 3, methyl sterculate + methyl oleate).

Finally, preparative HPLC on $C_{18}$ reversed-phase with ultraviolet and RI detection was used to separate methyl oleate and methyl linoleate from malvalic and sterculic acid methyl esters (Fig. 1). Methyl linoleate was removed by RP-HPLC owing to a higher polarity than methyl malvalate and methyl sterculate, whereas methyl oleate overlapped with methyl sterculate during elution from the $C_{18}$ column. As a consequence, the first fractions of the methyl sterculate peak, which contained a mixture of oleic and sterculic acid methyl esters, were collected separately. Similarly, the last fractions of the

methyl sterculate peak, containing small proportions of palmitic acid methyl ester, were separated from the middle fraction, which contained sterculic acid methyl ester (*m/z* 308, $[M]^+$) in a concentration of 95–98%. Malvalic acid methyl ester (*m/z* 294, $[M]^+$) was isolated in a concentration of 95–97%, as was confirmed by GC and GC–MS analysis (Table 1). Each injection (90 mg methyl esters) in preparative HPLC provided around 1 mg methyl malvalate and 55–60 mg methyl sterculate from *B. munguba,* and 3–5 mg methyl malvalate and 40–45 mg methyl sterculate from *S. foetida*.

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