Comparative Studies of Immobilized Lipase- and Acid-catalyzed Fatty Acid Methyl Ester Synthesis for Seed Lipid Analysis

Chang-Soo Kim, Si-Kyung Lee, and Young Soo Keum*

Department of Bioresources and Food Science, College of Life and Environmental Sciences, Konkuk University, Seoul 05029, Korea

Received August 7, 2015
Revised December 30, 2015
Accepted March 27, 2016
Published online June 30, 2016

*Corresponding Author
Tel/Fax: +82-2-450-3758
E-mail: rational@konkuk.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract Fatty acids are one of the most important nutrients in food. Acid- or base-catalyzed transesterification methods are commonly used for the analysis of fatty acids. However, several drawbacks were reported for these methods, including the isomerization and degradation of fatty acids. Lipase-catalyzed reactions are usually undertaken at mild conditions, preventing such problems. In this study, commercial resin-bound lipase from *Candida antartica* was tested for possible application in fatty acid methyl ester analysis. Experimental parameters, including temperature, reaction time, and re-cycling were evaluated. The optimized condition was (5–10 mg lipid, 0.5 mL of MeOH, and 50 mg Novozyme 435 in 2 mL toluene, 80°C for 1 h). In optimized condition, the lipase-catalyzed methods yielded similar results with the chemical method. In overall, lipase-catalyzed transesterification can be a useful alternative to acid-catalyzed methods for fatty acid analysis.

Keywords: lipase, fatty acid methyl ester, analysis, *Candida antartica*

Introduction

Fatty acids (FA) and their metabolites (e.g., triacylglycerol, TAG) are some of the most important nutrients in vegetable oils. Most seed oils consist predominantly of TAGs, which are esters of FAs with glycerol (1). In addition to common FAs, some seed oils contain unusual FAs. For example, ricinoleic acid is the most abundant FA in castor oil, while the edible mushroom *Cantharellus cibarius* contains a high amount of acetylenic FA (2). It was found that minor constituents of seed oils can cause detrimental health effects such as stercularic acid and malvalic acid in cotton seed oil (3). Consequently, reliable analytical methods for FAs are extremely important. Because of analytical difficulties with intact TAGs, the lipids in analytical samples are transformed into several derivatives. FA contents in food are usually analyzed in the form of fatty acid methyl esters (FAMES), which can be prepared by acid- or alkali-catalyzed transesterification (4–6). However, these methods are known to have several drawbacks. For example, conjugated linoleic acids (CLAs) can be decomposed (7). It is well-known that some unusual FAs can be degraded into a mixture of non-natural derivatives during common analytical procedures. Cyclopropene-containing FAs, toxic constituents of cotton seed oil can be polymerized (8). In overall, it is necessary to develop more reliable transformation methods for accurate measurement of FA constituents in food or in biological samples. Industrial applications of enzymes are commonly found in the food industry, pharmaceuticals,

cosmetics, and household products (9,10). Several depolymerizing enzymes (e.g., amylase, cellulase, pectinase, etc.) are used for the production of simple sugars or for the removal of polymers, while lipases are currently one of the most important elements in the production of biodiesel (10). Among numerous enzymes, thermostable lipases and xylanases are widely used in industrial and research purposes (11). Some microbial lipases have additional interesting features. For example, lipase B from *Candida antartica* is compatible with many organic solvents (e.g., hydrocarbons, alcohol, and toluene) and is thermo-stable (12). These properties make microbial lipase ideal tools for biodiesel production. Lipases from animal resources (e.g., porcine pancreatic lipase) can be used to produce modified TAGs from other lipids (13). Several applications of lipases have been reported in relation to structural analysis of lipids. For example, lipases from *Mucor miehei* and *Rhizopus oryzae* were used to analyze the positional composition of FAs on TAGs (14,15). And many lipase-catalyzed methods for FAME analysis were reported (13–16). However, its application on unusual lipids was limited.

The objective of this study is to develop enzymatic methods for the determination of FA contents in seed lipids with resin-bound lipase. FAMES from various seed oils were prepared with the lipase. The reaction conditions, including temperature, substrate structures and reaction time were optimized and the seed lipids with unusual FAs were analyzed using the improved method.

Materials and Methods

Materials Soybean oil, castor oil (*Ricinus communis*), and jojoba oil (*Simmondsia chinensis*) were obtained from a local market. The following seeds were purchased from commercial providers: *Firmiana simplex*, *Helichrysum* sp., *Hydnocarpus kurzii*, *Litchi chinensis*, *Momordica cochinchinensis*, *Osteospermum* sp., *Pinellia ternata*, and *Raphanus sativus*. Lipase B from *Candida antarctica*, immobilized on acrylic resin (Novozyme 435) was kindly provided by Daejong Co. (Seoul, Korea). Solvents and other reagents were obtained from Sigma-Aldrich Korea, Alfa Aesar Korea, and Samchun Co. (Seoul, Korea).

Extraction of triacylglycerols from seeds Seeds were pulverized with a high speed blender (Warring, Stamford, CT, USA). Powdered samples (1 g) were extracted for 6 h with *n*-hexane (50 mL) using a Soxhlet extractor. After removal of the solvent under reduced pressure, the residue was dissolved in toluene (20 mL) and used for transesterification experiments as described below.

Chemical methods for fatty acid methyl esters Two different acid-catalyzed methods were tested: methanolic HCl (method A) and BF₃-methanol (method B). For the chemical transesterification experiments, soybean oil was used. Methanolic HCl was prepared from concentrated hydrochloric acid (20 mL) and methanol (80 mL). Aliquots of oils (5–10 mg) were mixed with toluene (2 mL) and methanolic HCl (1 mL) or BF₃-methanol (1 mL). The mixture was heated to 80°C for 1 h. After cooling, water (5 mL) and hexane (5 mL) were added and the mixture was briefly shaken. The organic layer was collected and washed with water (5 mL) then passed through a short column of anhydrous sodium sulfate. The eluates were concentrated to dryness. The residue was dissolved in *n*-hexane (2 mL) and used for gas chromatography-mass spectrometry (GC-MS). To test the reaction efficiency, the method B were applied on selected seed TAGs, containing unusual fatty acids and the results were compared with those from enzymatic method.

Enzymatic transesterification For the optimization study, commercial soybean oil was used. Novozym 435 (1, 5, 10, 50, and 100 mg) was added to an oil solution (5–10 mg oil in 2 mL of toluene). MeOH (0.5 mL) was added and mixed thoroughly. The mixture was heated to room temperature, 40, 60, and 80°C for defined periods (30 min, 1, 2, or 4 h). After cooling, aliquots of the solution were collected and analyzed with GC-MS. The reactivity of unusual FAs was tested with seed-extracted triacylglycerols under the above optimized conditions. To determine the stability of catalytic activity at elevated temperatures (60 and 80°C), the enzyme bead (50 mg), previously used for the same reaction with soybean oil, was washed with hexane (3 mL×3) and dried under vacuum for 1 h. The dried Novozym 435 bead was repeatedly used for the above-mentioned transesterification reaction. The stability was expressed as the ratio of FAME obtained from

soybean oil (1 mg) in each run compared to the amount in the initial reaction. All experiments, including chemical and enzymatic reactions were performed in triplicates.

Statistical analysis Standard deviations and averages of experimental data were calculated with Statistica (StatSoft Inc., Tulsa, OK, USA).

Gas chromatography-mass spectrometry (GC-MS) Quantitative analysis of the reaction was performed with GC-MS. The GC-MS conditions were as follows: Shimadzu GC-2010 SE GC-MS; column: Rts-5MS (Restek Ltd., Bellefonte, PA, USA, 30 m, 0.25 mm i.d., 0.25 mm thickness). Column temperature: 70°C (10 min), increased at a rate of 3°C/min to 290°C (20 min); injector temperature, 250°C; interface temperature, 275°C. Mass spectra were obtained in electron impact mode in full scan (*m/z* range 70–700). Percent composition of marker fatty acids was calculated from peak area of marker fatty acid, divided by the peak area of all fatty acid, then multiplied by 100.

Results and Discussion

Chemical transesterification Soybean oil was transformed into FAMES using chemical methods. In general, the BF₃-methanol method (method B) was superior to the methanolic HCl method (method A) (Table 1). It is well-known that method A usually takes a long reaction time (16). Appreciable amounts of free FAs and monoacylglycerols were found in samples produced with method A. Complete conversion of acylglycerol by this protocol was achieved after 4 h while the reaction with method B was completed within 1 h. BF₃-methanol (method B) is one of the most common acidic catalysts for FAME analysis (17). The method can be applied to various lipids, including triacylglycerols, phospholipids, and sphingolipids. In addition, short reaction time is another important advantages of this method over method A (17). However, the reagent is harmful and several artifacts have been reported (18). In this study, the analytical results from method B were used for the comparison of enzymatic reactions.

Temperature effects on enzymatic transformation The enzymatic conversion rate was found to be highly dependent on temperature (Table 1). A limited amount of triacylglycerol was converted to FAMES at room temperature and at 40°C with Novozym 435. According to the GC-MS analysis, the major components at these conditions were free FAs (e.g., oleic acid, and palmitic acid) and monoacylglycerol (glycerol ester of oleic and palmitic acids). However, triacylglycerol from soybean oil was rapidly converted to FAMES at 60 and 80°C. Further optimization studies were performed at both temperatures.

Effects of enzyme amounts and reaction time To optimize the amount of the catalysts, different quantities of Novozym 435 were

Table 1. Percent composition of fatty acid in soybean oil, determined with HCl-catalyzed, BF₃-methanol, and enzymatic methods

Fatty acid	HCl-catalyzed	BF ₃ -MeOH	Novozym 435 ³⁾			
			Room temp.	40°C	60°C	80°C
12:0	ND ¹⁾	ND	ND	ND	ND	ND
14:0	ND	0.1±0.05	ND	ND	ND	0.1±0.05
16:0	10.4±0.5	12.3±1.1	2.9±1.2	8.5±1.1	12.3±0.3	12.3±0.3
16:1	ND	ND	ND	ND	0.1±0.05	ND
18:0	8.0±1.2	5.5±1.3	0.3±0.1	4.8±0.4	5.7±0.9	5.7±0.4
18:1	28.4±0.9	23.8±1.3	32.1±3.9	25.7±2.7	23.9±1.0	23.9±1.4
18:2	47.3±2.1	53.0±1.8	60.5±4.3	57.6±3.5	52.5±0.9	52.5±0.8
18:3	5.9±0.7	5.2±1.0	4.2±1.0	3.3±0.9	5.4±1.1	5.4±1.0
Conversion (%) ²⁾	28.5±3.5	100±1.8	18.2±4.7	65.3±6.3	100±2.1	100±3.1

¹⁾ND, Not detected²⁾Relative conversion (%) of triacylglycerol to FAME, compared to BF₃-methanol method.³⁾For the experiment, oil (1 mg) was reacted with methanol (0.5 mL) or Novozym 435 (100 mg) in toluene (2 mL) at the specified temperatures for 1 h. **Fig. 1.** Effects of the amount of Novozym 435 (A) and reaction time (B) on the conversion of soybean oil to FAMES.

treated at 60 and 80°C for 1 h, with the amounts of the other reagents fixed as described in the experimental section. According to GC-MS, complete conversion was achieved with 50 and 100 mg of Novozym 435. Smaller amounts of enzyme (10 mg) yielded less transformation (Fig. 1). In general, higher reaction rates were observed at 80°C. In consideration of the above results, the optimum conditions were selected as 50 mg enzyme and 80°C for the reaction. The reaction time was optimized at these conditions. The concentration of FAMES increased rapidly within 60 min and there were no noticeable changes observed at 90 and 120 min (Fig. 1). In overall, the optimized conditions for Novozym 435-catalyzed FAME synthesis were summarized as 5-10 mg lipid, 0.5 mL MeOH, and 50 mg enzyme in 2 mL of toluene, 80°C for 1 h (method C).

Fig. 2. Effects of repeated use of Novozym 435 (50 mg) on the conversion of soybean oil to FAMES at 60°C (blank bar) and 80°C (black bar), 1 h.

Stability of catalytic activity during repeated use Enzymes are generally considered expensive catalysts for synthetic applications. Other obstacles to chemical synthesis are catalytic stability at high temperatures and in organic solvents. However, a number of thermostable enzymes are frequently used in industry (19). In addition to its stability at elevated temperatures, Novozym 435 is well-known to be compatible with many different organic solvents (12,20). In this study, the catalytic stability during repeated use was tested at two different temperatures. FAME conversion during 1 h of reaction was evaluated with re-cycled enzyme from the previous reaction. The residual activities were 80 and 100% at 80 and 60°C after 10 cycles (Fig. 2 and 3). The results indicated that Novozym 435 is a promising catalyst for FAME production.

Structures of lipids on enzymatic activity Enzyme-catalyzed reactions frequently occur in a structure-selective manner. For example, several lipases were successfully applied for the chiral resolution of racemic drugs (21). However, stereo-selectivity can be a serious drawback when the catalysts are used for structurally diverse

Table 2. Marker fatty acids for lipids and catalytic activity of Novozym 435

Seeds	Marker fatty acids	Relative conversion rate (%) ¹⁾	Marker fatty acid FAME (%) ²⁾		Statistical difference of FAMES ³⁾	
			B	C	Common	Marker
<i>Firmiana simplex</i>	Sterculic acid	108±7	2±1	13±2	N	Y
<i>Helichrysum</i> sp.	Crepenynic acid	105±6	2±1	10±2	N	Y
<i>Hydnocarpus kurzii</i>	Hydnocarpic acid	100±2	36±5	35±4	N	N
<i>Litchi chinensis</i>	Dihydrosterculic acid	110±9	3±1	11±2	N	Y
<i>Momordica cichinchinensis</i>	Eleostearic acids (α - and β -)	102±4	53±7	59±2	N	N
<i>Osteospermum</i> sp.	α -Dimorphecolic acid	98±4	21±9	20±6	N	N
<i>Pinellia ternata</i>	13-Phenyltridecanoic acid	100±5	2±1	3±1	N	N
<i>Raphanus sativus</i>	C20-C22 fatty acids	100±11	39±1	41±1	N	Y
<i>Ricinus communis</i>	Ricinoleic acid	101±8	83±6	85±2	N	N
<i>Simmondsia chinensis</i>	Fatty wax, No or trace TAG	89±18	ND ⁴⁾	ND	ND	ND

¹⁾Percent of conversion of TAG or lipids to total FAMES by Novozym 435, compared to BF₃-MeOH method.

²⁾Percent of marker fatty acid FAME to total FAMES, measured by BF₃-MeOH (B) and Novozym 435 method (C).

³⁾Respective N and Y for statistically not different and different between methods, $p < 0.05$. Common FAMES include hexadecenoic acid, palmitic acid, linolenic acid, linoleic acid, stearic acid, and eicosanoic acid.

⁴⁾ND, Not determined

Fig. 3. Percent composition of individual FAMES to total FAMES of initial enzymatic reaction during repeated use (1, 4, and 10 times) at optimized conditions with reaction temperature at 60 (A) and 80°C (B).

substrates. Common structural features of FAs in foods and other biological samples are (a) straight-chained, (b) 10-22 carbons, and (c) 0-6 *cis*-double bonds. However, numerous microorganisms and plants can produce lipids with unusual FAs. For example, cyclopropane-FAs (e.g., dihydrosterculic acid and lactobacillic acid) are useful biomarkers for lactic acid-producing bacteria and are used for the production of dairy products (22). In this study, lipids with unusual FAs were prepared from various seeds or vegetable oils and the catalytic efficiency of Novozym 435 was tested. The selected seeds and oils are known to contain unusual FAs, including FAs with aromatic or alicyclic rings (e.g., cyclopropane, cyclopentene, and phenyl), triple bonds, conjugated polyenes, and hydroxyl groups (23-31). Major lipids in jojoba oil are fatty wax (esters of common FAs with long-chain fatty alcohol), rather than TAG (32). The marker FAs for each lipid are summarized in Table 2. Seed lipids from *H. kurzii* and *P. ternata* contain cyclopentene- and phenyl-terminated FAs, respectively (26,29). The concentrations of these FAs produced with Novozym 435 were similar to those produced using the acid-catalyzed method (Table 2) and the result well-coincided

with the literature (26,29). Hydroxylated FAs are usually minor constituents of plant lipids. However, some plant seeds, including *Osteospermum* spp., castor bean (*Ricinus communis*), and *Trewia* spp. contain high amounts of hydroxy FAs (28,31,33). In addition to TAGs, some of these plants can produce estolides, which are polyesters of hydroxy FAs and TAGs (33). The contents of hydroxy FAs in *Osteospermum* seed and castor oil were close to the literature values (28,33), indicating that the enzymatic method used in this study is applicable to hydroxy FA-containing TAGs. Although polyunsaturated FAs (PUFAs) are some of the most common FAs in plant lipids, conjugated PUFAs are found in a limited number of plants. Eleostearic acids (α - and β -acid) are the most abundant FAs in *Momordica* seed TAGs (27). Radish seed (*Raphanus sativus*) is used in Chinese medicine. Recent study indicates that *Brassica* seeds contain unusually high amounts of eicosenoic acids and docosenoic acids. According to this study, the concentrations of both acids were 39-41% of total FAs, which coincides with the literature (30). In general, the results from BF₃-MeOH (method B) and Novozym 435 (method C) were closely correlated. However, the concentrations of marker acids in some

Fig. 4. Representative GC-MS total ion chromatograms of FAMES from *Firmiana simplex* seed TAGs by $\text{BF}_3\text{-MeOH}$ (A) and optimized enzymatic method (B). FAs for FAMES abbreviation: 16:0, palmitic acid; 16:1, hexadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; M, malvalic acid; S, stercularic acid; DS, dihydrostercularic acid.

lipids (*Firmiana*, *Litchi*, and *Helichrysum*) were significantly different, depending on the method (Table 2). The results from enzymatic method were more closely related to the literature values than method B. For example, cyclopropene-/cyclopropane-containing FAs (stercularic acid and dihydrostercularic acid) were successfully derivatized to the corresponding FAMES by optimized enzymatic method (method C) (Fig. 4). It has to be mentioned that base-catalyzed methods (e.g., sodium methoxide) are much more commonly used to prepare methyl esters of cyclopropenoic/acetylenic FA (25,34,35). Although the enzymatic method (method C) was either equally efficient or superior to method B, lipase-catalyzed transesterification was not a promising methods for fatty wax (jojoba oil) (Table 2). The conversion of total lipids to FAMES by enzymatic method was approximately 89% of method B.

In overall, Novozym 435 is an extremely useful catalyst for FAME preparation from TAGs. Its advantages over the chemical method are a) short analysis time since no additional procedures (e.g., solvent extraction, dehydration, and evaporation etc.) are required, b) repeated use, and c) strong activity with diverse FAs.

Acknowledgments This work is supported by Konkuk Univeristy fund, 2013-A019-0030.

Disclosure The authors declare no conflict of interest.

References

- Spitzer V. Screening analysis of unknown seed oils. *Lipids* 101: 2-19 (1999)
- Hong SS, Lee JH, Jeong W, Kim N, Jin HZ, Hwang BY, Lee HJ, Lee SJ, Jang DS, Lee D. Acetylenic acid analogues from the edible mushroom *Cantharellus cibarius* and their effects on the gene expression of peroxisome proliferator-activated receptor-gamma target genes. *Bioorg. Med. Chem. Lett.* 22: 2347-2349 (2012)
- Ames BN. Dietary carcinogens and anticarcinogens. oxygen radicals and degenerative diseases. *Science* 221: 1256-1264 (1983)
- Ruiz-Rodriguez A, Reglero G, Ibanez E. Recent trends in the advanced analysis of bioactive fatty acids. *J. Pharmaceut. Biomed.* 51: 305-326 (2010)
- Ruiz-Jiménez J, Priego-Capote F, Luque de Castro MD. Identification and quantification of *trans* fatty acids in bakery products by gas chromatography-mass spectrometry after dynamic ultrasound-assisted extraction. *J. Chromatogr. A* 1045: 203-210 (2007)
- Kim BH, Lumor SE, Akoh CC. Trans-free margarines prepared with canola oil/palm stearin/palm kernel oil-based structured lipids. *J. Agr. Food Chem.* 56: 8195-8205 (2008)
- Golay P, Dionisi F, Hug B, Giuffrida F, Destailats F. Direct quantification of fatty acids in dairy powders with special emphasis on trans fatty acid content. *Food Chem.* 101: 1115-1120 (2006)
- Rinehart KL, Goldberg SI, Tarimu CL, Culbertson TP. Cyclopropene rearrangement in the polymerization of stercularic acid. *J. Am. Chem. Soc.* 83: 225-231 (1961)
- Akoh CC, Chang SW, Lee GC, Shaw JF. Biocatalysis for the production of industrial products and functional foods from rice and other agricultural produce. *J. Agr. Food Chem.* 56: 10445-10451 (2008)
- Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. *Enzyme Microb. Tech.* 39: 235-251 (2006)
- Zamost BL, Nielsen HK, Starnes RL. Thermostable enzymes for industrial applications. *J. Ind. Microbiol.* 8: 71-82 (1991)
- Anderson EM, Larsson KM, Kirk O. One biocatalyst-many applications: The use of *Candida antarctica* B-lipase in organic synthesis. *Biocatal. Biotransfor.* 16: 181-204 (1998)
- Sahin N, Akoh CC, Karaali A. Lipase-catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes. *J. Agr. Food Chem.* 53: 5779-5783 (2005)
- Dourtoglou T, Stefanou E, Lalas S, Dourtoglou V, Poulos C. Quick regiospecific analysis of fatty acids in triacylglycerols with GC using 1,3-specific lipase in butanol. *Analyst* 126: 1032-1036 (2001)
- Foglia TA, Konkerton EJ, Sonnet PR. Regioselective analysis of triacylglycerols by lipase hydrolysis. *J. Am. Oil Chem. Soc.* 72: 1275-1279 (1995)
- Ichihara K, Fukubayashi Y. Preparation of fatty acid methyl esters for gas-liquid chromatography. *J. Lipid Res.* 51: 635-640 (2010)
- Ackman RG. Remarks on official methods employing boron trifluoride in the preparation of methyl esters of the fatty acids of fish oils. *J. Am. Oil Chem. Soc.* 75: 541-545 (1998)
- Fulk WK, Shorb MS. Production of an artifact during methanolysis of lipids by boron trifluoride-methanol. *J. Lipid Res.* 11: 276-277 (1970)
- Haki GD, Rakshit SK. Developments in industrially important thermostable enzymes: A review. *Bioresource Technol.* 89: 17-34 (2003)
- Lerin L, Ceni G, Richetti A, Kubiak G, Oliveira JV, Toniazio G, Treichel H, Oestreicher EG, Oliveira D. Successive cycles of utilization of Novozym 435 in three different reaction systems. *Braz. J. Chem. Eng.* 28: 181-188 (2011)
- Ghanem A, Aboul-Enein HY. Application of lipases in kinetic resolution of racemates. *Chirality* 17: 1-15 (2005)
- Caligiani A, Marseglia A, Palla G. An overview on the presence of cyclopropane fatty acids in milk and dairy products. *J. Agr. Food Chem.* 62: 7828-7832 (2014)
- Shimadate T, Iino T, Hosoyama Y. Cyclopropenoid fatty acids from the seed oil

- of *Firmania planatifolia*. Prog. Nat. Sci. 11: 33-39 (1976)
24. Gaydou EM, Ralaimanarivo A, Bianchini JP. Cyclopropanoic fatty acids of litchi (*Litchi chinensis*) seed oil. A reinvestigation. J. Agr. Food Chem. 41: 886-890 (1993)
 25. Powell RG, Smith CR, Wolff IA. *Helichrysum* seed oil. I. separation and characterization of individual acids. J. Am. Oil Chem. Soc. 42: 165-169 (1965)
 26. Zhang JY, Wang HY, Yu QT, Yu XJ, Liu BN, Huang ZH. The structures of cyclopentenyl fatty acids in the seed oils of Flacourtiaceae species by GC-MS of their 4,4-dimethyloxazoline derivatives. J. Am. Oil Chem. Soc. 66: 242-246 (1989)
 27. Chang MK, Conkerton EJ, Chapital DC, Wan PJ, Vadhw OP, Spiers JM. Chinese melon (*Momordica charantia* L.) seed: Composition and potential use. J. Am. Oil Chem. Soc. 73: 263-265 (1996)
 28. Hopkins CY, Chisholm MJ. Occurrence in seed oils of some fatty acids with conjugated unsaturation. Can. J. Chemistry 43: 3160-3164 (1965)
 29. Meija J, Soukup VG. Phenyl-terminated fatty acids in seeds of various aroids. Phytochemistry 65: 2229-2237 (2004)
 30. Barthelet VJ. (*n*-7) and (*n*-9) *cis*-monounsaturated fatty acid contents of 12 *Brassica* species. Phytochemistry 69: 411-417 (2008)
 31. Sreenivasan B, Kamath NR, Kane JG. Studies on castor oil. I. fatty acid composition of castor oil. J. Am. Oil Chem. Soc. 33: 61-66 (1956)
 32. Miwa TK. Jojoba oil wax esters and derived fatty acids and alcohols: Gas chromatographic analyses. J. Am. Oil Chem. Soc. 48: 259-264 (1971)
 33. Madrigal RV, Smithe CR. Estolide triglycerides of *Trewia nudiflora*. Lipids 17: 650-655 (1982)
 34. Schneider EL, Loke SP, Hopkins DT. Gas-liquid chromatographic analysis of cyclopropenoid fatty acids. J. Am. Oil Chem. Soc. 45: 585-590 (1968)
 35. Fehling E, Schonwiese S, Klein E, Mukherjee KD, Weber N. Preparation of malvalic and sterculic acid methyl esters from *Bombax munguba* and *Sterculia foetida* seed oils. J. Am. Oil Chem. Soc. 75: 1757-1760 (1998)