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Occurrence and Formation of Bitter-Tasting Trihydroxy Fatty Acids in Soybeans

Christiane Moll, Ursula Biermann, and Werner Grosch*

A quantitative method for the determination of the mixture of 9,12,13-trihydroxyoctadeca-10-enoic acid and 9,10,13-trihydroxyoctadeca-11-enoic acid (Tri-OH) in legumes is reported. Storage of a soybean flour at 22 °C increased the Tri-OH content from 0.03 to 0.05% in 3 months. Components of soybeans, glutathione, and horse radish peroxidase (HRP) were tested for their ability to form Tri-OH from linoleic acid hydroperoxides. Most effective was a protein fraction from soybeans containing lipoxygenase and peroxidase activities, followed by HRP and by proteins. In the latter case thiol groups are involved in the Tri-OH formation.

A bitter taste whose intensity increases during maturation contributes to the flavor of soybeans (Rackis et al., 1972). In stored soybeans Sessa et al. (1974, 1976) localized the bitter taste to one lysolecithin and two lecithin fractions, which together represented at least 0.08% of the defatted flour. The authors found that autoxidized soy lecithins are extremely bitter with thresholds in the range of 0.006%. Further experiments suggested that not the phosphocholine moiety of the lecithin molecule but a bound oxidized fatty acid is responsible for the bitter taste.

Enzymatic oxidation of linoleic acid by use of a protein preparation of soybeans with lipoxygenase and peroxidase activities generates fatty acids which after emulsification with a sugar ester taste bitter (Baur et al., 1977). The main bitter substance was identified as a mixture of 9,12,13-trihydroxyoctadec-*trans*-10-enoic acid and 9,10,13-trihydroxyoctadec-*trans*-11-enoic acid (Tri-OH refer to both isomers). The Tri-OH exhibits a bitter threshold of 0.6–0.9

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μmol/mL that increases threefold when the double bond is hydrogenated (Baur and Grosch, 1977). The occurrence of Tri-OH in bitter-tasting soybean flakes was established by Sessa et al. (1977). Several authors have assumed that the linoleic acid hydroperoxides (LOOH) which arise from the lipoxygenase catalysis are the precursors of the Tri-OH. Results of Gardner et al. (1974) support this idea because Tri-OH was identified among the numerous products which were obtained after the decomposition of LOOH by Fe(III)–cysteine. A mechanism to explain the formation of the Tri-OH under these conditions was proposed by Gardner (1975).

In the present paper the quantitative determination of Tri-OH in stored soybeans is reported. Besides this some components of soybeans are tested for their ability to form the Tri-OH.

EXPERIMENTAL SECTION

Materials and Reagents. Soybeans (Harburger Ölwerke, Brinckmann and Mergell); ground soybeans (200 g) were stored in a desiccator over a saturated solution of ammonium nitrate (63% relative humidity) at 22 °C for 3 months; linoleic acid (Sigma, grade III), Tween 80 (Schuchardt), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes buffer, Sigma), glutathione (GSH, Boehringer), horse radish peroxidase (Boehringer, grade I), bovine serum albumin (Merck), Sephadex G-200 (Pharmacia), silica gel 60 (0.063–0.2 mm, Merck) were also used; 9,10,12,13-tetrahydroxystearic acid was prepared by oxidation of linoleic acid with $KMnO_4$ according to Kass and Radlove (1942) and purified as described earlier (Baur and Grosch, 1977); the other chemicals were of analytical grade.

Gel Chromatography of Soybean Proteins. The following steps were performed at 0–4 °C. Ground soybeans (20 g; defatted with light petroleum) were stirred for 45 min with 200 mL of 0.1 M sodium phosphate buffer (pH 6.8). The slurry obtained was filtered through two layers of cheesecloth and centrifuged at 10000*g* for 30 min. From the supernatant 45 mL containing 600 mg of protein was applied to a Sephadex G 200 column (2.5 × 100 cm) which was equilibrated with the buffer and ascendingly eluted with 0.1 M sodium phosphate buffer (pH 6.8). Fractions of 9 mL/tube were collected and assayed for UV absorption and lipoxygenase and peroxidase activity. The effluents 118–234 mL (fraction 1), 235–324 mL (fraction 2), 325–396 mL (fraction 3), and 397–522 mL (fraction 4) each were combined, concentrated to 20 mL by ultrafiltration (system "Amicon", membrane PM 10), and used for incubation experiments with LOOH substrate.

Lipoxygenase Isoenzymes L-1 and L-2. The isoenzymes were isolated and partially purified by precipitation with ammonium sulfate, gel filtration, and DEAE-cellulose chromatography as described previously (Grosch et al., 1977).

Enzyme Assays. Lipoxygenase was assayed as described in a previous paper (Grosch et al., 1977). Peroxidase activity was determined according to the procedure in Bergmeyer (1970).

Protein Determination. During chromatography protein was measured by the absorbance at 280 nm. The protein contents of the preparations used for the incubation experiments were determined by the biuret method (Beisenherz et al., 1953).

Linoleic Acid Substrates (pH 9.0 or 6.8). Linoleic acid (1.7 mmol) was dissolved in 20 mL of water containing 0.32 μL of Tween 80 by addition of 5 mL of 1 N NaOH. The solution was adjusted to pH 9 or 6.8 with HCl and made up to 200 mL with 0.1 M sodium borate buffer (pH 9.0) or 0.1 M sodium phosphate buffer pH 6.8.

Linoleic Acid Hydroperoxide (LOOH). Linoleic acid substrate (200 mL, pH 9.0) was oxidized with 4 mg of soybean lipoxygenase at 3 °C for 120 min. The isolation, purification by TLC, and determination of the concentration was carried out as described by Streckert and Stan (1975) and analysis of LOOH as described by Grosch et al. (1976).

LOOH Substrate. Tween 80 (0.3 μL) was added to a solution of 1.7 mmol LOOH in methanol. Methanol was removed in vacuo and the LOOH/Tween 80 mixture dissolved in 20 mL of highly dilute NaOH (pH 9). The solution was made up to 200 mL with 0.1 M sodium phosphate buffer (pH 6.8) and the pH was corrected to 6.8 with dilute HCl.

Hemoprotein Determination. The procedure of Hartree (1955) was followed.

Taste Analysis. Fresh or stored ground soybeans (5 g) were suspended in 20 mL of tap water. The taste of the suspension was evaluated by four members of the institute.

Tri-OH Content of Stored Soybean Flour. Ground soybeans (3 g) were extracted with 100 mL of $CHCl_3$–methanol (1:1, v/v). After filtration 0.6 mg of 9,10,12,13-tetrahydroxystearic acid dissolved in 0.6 mL of methanol was added to the extract as an internal standard. The solvent was removed in vacuo and the residue dissolved in 100 mL of diethyl ether/methanol (9:1, v/v). The free acids were methylated with diazomethane as described by Schlenk and Gellerman (1960).

For transesterification of the extracted lipids the solvent was evaporated in vacuo and the residue taken up in a mixture of 10 mL of diethyl ether and 24 mL of light petroleum, and 4 min after addition of 6 mL of 0.5 M sodium methylate in methanol the reaction system was diluted with 40 mL of diethyl ether, washed with 3 × 50 mL water, and dried over sodium sulfate.

After concentration to 2 mL, the organic phase was applied to a column (30 × 1.8 cm) with silica gel 60 in cyclohexane. The column was eluted stepwise with 150 mL of cyclohexane/diethyl ether (8:2, v/v) and 300 mL of diethyl ether/methanol (1:1, v/v). The last named effluent was collected and concentrated.

Quantitative Analysis of Tri-OH. The methyl esters of fatty acids were chromatographed on silica gel plates (0.5 mm) for a distance of 12 cm with the upper phase of the mixture isooctane/ethyl acetate/water (1:1:2, v/v/v). The compounds were located by spraying a small strip on the side of the plate with 5% phosphomolybdic acid in 96% ethanol and by heating the strips with a hair dryer. The silica gel band which contained the methyl esters of the Tri-OH and the 9,10,12,13-tetrahydroxystearic acid (internal standard) were scraped off and extracted with 50 mL of warm methanol. After removal of the methanol (in vacuo) the methyl esters of the hydroxy fatty acids were dissolved in 1 mL of pyridine and silylated according to Graveland (1973a). The analysis followed by gas chromatography (Graveland, 1973a). Conditions: 1 m × 1.2 mm column of 3% silicone JXR on Gas-Chrom Q (100–120 mesh), oven temperature, 210 °C; injector and detector (FID) temperature, 250 °C; helium as carrier gas (15 mL/min). The quantities of the Tri-OH were determined on the basis of the internal standard. For identification of the methylated and silylated Tri-OH in the gas chromatogram, the JXR column was coupled via Biemann–Watson separator with a Varian CH 7 mass spectrometer; temperature of the ion source 200 °C, energy 70 eV.

Incubation Experiments. From the LOOH or linoleic acid substrate (pH 6.8), 10 mL was mixed with the solutions of proteins or other compounds as listed in Table

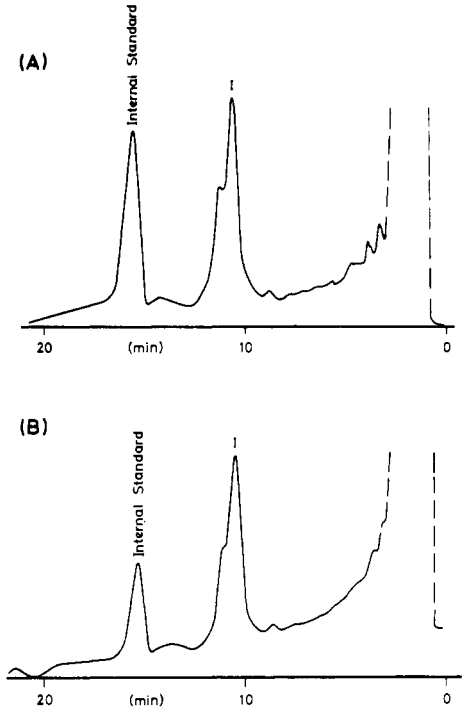

Figure 1. Gas chromatographic analysis of the silylated and methylated Tri-OH isolated from ground soybeans before (A) and after storage (B).

II. The reaction system was made up to 20 mL with 0.1 M sodium phosphate buffer (pH 6.8). After an incubation of 2 h at 22 °C under stirring in the presence of air, 2 mg of 9,10,12,13-tetrahydroxystearic acid dissolved in 2 mL of methanol was added. The solution was adjusted to pH 4 with dilute HCl and thrice extracted with 70 mL of diethyl ether. The combined extracts were washed thrice with 50 mL of water, dried over sodium sulfate, and concentrated to 3 mL. After methylation with diazomethane and concentration, the quantitative analysis of the Tri-OH was done as given above.

RESULTS

Tri-OH in Soybeans. An analytical method was developed for determination of Tri-OH in soybeans. The main steps are conversion of the free and bound fatty acids to methyl esters, separation of the polar from the nonpolar fatty esters by column chromatography, isolation of Tri-OH and the internal standard by TLC, and quantitative analysis of the silylated esters by GLC. As internal standard 9,10,12,13-tetrahydroxystearic acid was chosen because it is absent in stored and unstored soybeans.

Peak I of the gas chromatograms (Figure 1) which result from the analysis of ground soybeans before and after storage was identified as Tri-OH by comparison of the mass spectra with that of a reference substance.

The lipids of soybeans consist of about 50% linoleic and 10% linolenic acid (Cowan et al., 1973). For this reason it is possible that the Tri-OH peak contains 9,12,13-trihydroxyoctadeca-10,15-dienoic acid, which according to Graveland (1973b) can result from the enzymatic oxidation of linolenic acid. Graveland (1973b) has shown that in the mass spectra of such a trihydroxyoctadienoic acid the ion m/e 171 is a key fragment and the base peak. In contrast to the mass spectrum of the pure Tri-OH (Figure 2A) in the mass spectrum of the Tri-OH (Figure 2A), the ion m/e 173 is the base peak and the ion m/e 171 is absent. In the mass spectrum of the trihydroxy acids isolated from soybeans (Figure 2B) the ion 171 occurs besides the ion 173. For this reason it is very likely that the soybeans

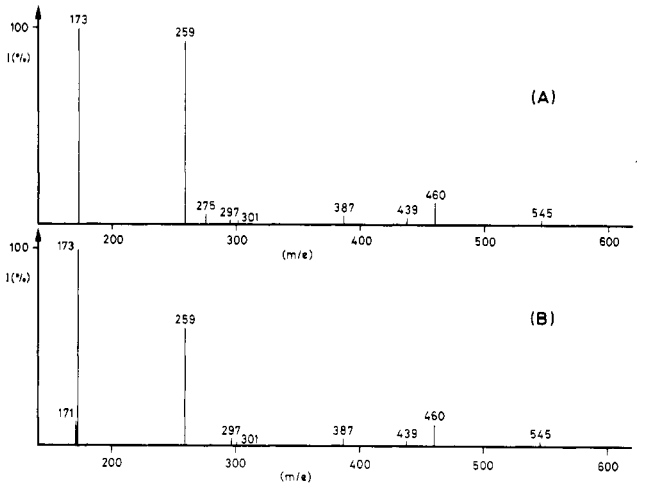

Figure 2. Mass spectra of silylated and methylated Tri-OH (A) and peak I (B) from the gas chromatogram presented in Figure 1B.

Table I. Tri-OH in Soybean Flour

sample of soybean flour	μg of Tri-OH/g of flour
1. after milling	320
2. stored (3 months at −80 °C)	360
3. stored (3 months at 22 °C, 63% relative humidity)	520

contain a trihydroxy octadecadienoic acid besides the Tri-OH.

The results of the quantitative determination of Tri-OH are listed in Table I. At the beginning of the storage experiments the soybeans contained 0.03% Tri-OH. Storage of the soybean flour at 22 °C but not at −80 °C leads to an increase of the Tri-OH to 0.05%.

Storage of the soy flour at 22 °C led to a change in its taste. After 3 months the predominant note was still the green-beany one; however, a slight increase in the bitter part of the taste was noted.

Formation of Tri-OH. A part of linoleic acid is converted to Tri-OH during an aerobic incubation with high amounts of the purified lipoxygenase isoenzymes L-1 and L-2 (experiment 1 and 2 in Table II). This observation agrees with the earlier results (Arens and Grosch, 1974), according to which increased concentrations of a purified lipoxygenase from peas favored the formation of the Tri-OH.

The LOOH preparation used in the incubation experiments was determined to be an isomeric mixture of 13-hydroperoxyoctadeca-9,11-dienoic (75%) and 9-hydroperoxyoctadeca-10,12-dienoic (25%) acids.

Experiment 3 (Table II) indicates that incubation of the LOOH alone yields 1.8 μmol of Tri-OH. This amount is nearly doubled by addition of a soybean extract which contained all water-soluble components (experiment 4).

The Tri-OH increases further when the protein fractions which were obtained by gel chromatography of the soybean extract (Figure 3) are present in the reaction system (experiments 5–8). Differences in the ability of the fractions to produce the Tri-OH are noticed when the protein concentration is raised to 80 mg/20 mL. Under these conditions fraction 2 which, as shown in Figure 3, contains the lipoxygenase and peroxidase converts about 25% of the LOOH to Tri-OH. In contrast the amount of Tri-OH is only slightly increased in the experiments with high concentrations of the fractions 1 and 3.

The lipoxygenases L-1 and L-2 also produce Tri-OH during incubation with LOOH (experiments 9 and 10). In

Table II. Formation of Tri-OH in 20 mL of 0.1 M Sodium Phosphate Buffer (pH 6.8)

no.	preparation or compound added	mg of protein	substrate (85 μmol)	Tri-OH formed during incubation of 2 h at 22 °C, μmol
1	L-1	30	18:2[a]	5.6
2	L-2	30	18:2[a]	6.7
3	without		LOOH	1.8
4	extract[b]	30	LOOH	3.1
5a	fraction 1 (Figure 3)	30	LOOH	4.7
5b	fraction 1	80	LOOH	6.5
6a	fraction 2 (Figure 3)	30	LOOH	4.5
6b	fraction 2	80	LOOH	22.8
7a	fraction 3 (Figure 3)	30	LOOH	4.7
7b	fraction 3	80	LOOH	6.8
8	fraction 4 (Figure 3)	25	LOOH	5.2
9	L-1	30	LOOH	10.4
10	L-2	30	LOOH	5.2
11	BSA[c]	80	LOOH	5.4
12	BSA-SH[c]	80	LOOH	7.2
13	GSH (85 μmol)		LOOH	8.5
14	peroxidase	1	LOOH	6.1
15	$FeSO_4$ (0.36 μmol)[d]		LOOH	2.3

[a] 18:2 linoleic acid. [b] Ground soybeans (defatted with light petroleum) were extracted as described in the Experimental Section under "Gel Chromatography of Soybeans Proteins". [c] Bovine serum albumin (BSA) was incubated with (for experiment 11) and without (for experiment 12) GSH according to Libenson and Jena (1963). The BSA and BSA-SH were separated from the reagents by chromatography on a Sephadex G-50 column (100 × 2.5 cm). [d] Phosphate buffer was replaced by Pipes buffer (pH 6.8). Before (15 min) and after (10 min) the start of the reaction the system was saturated with nitrogen to prevent rapid oxidation of the ferrous state. After 10 min the reaction was continued under aerobic conditions.

contrast to L-2, which is not more effective than the other soybean proteins, the isoenzyme L-1 transforms significantly more LOOH into Tri-OH.

The difference in the amount of Tri-OH formed in experiments 11 and 12 indicates that the thiol groups of a protein increase breakdown of the LOOH to Tri-OH. This conclusion is also supported by the increase of Tri-OH in consequence of the additions of GSH to the LOOH solution (experiment 13).

Fraction 2 (80 mg) contained 1 mg of hemoprotein calculated on the basis of horse radish peroxidase. In experiment 14 this amount of peroxidase was added to the reaction system. It was found that the conversion of the LOOH to Tri-OH is in the same range as in the experiments with the soy proteins.

In the last trial the amount of ferrous ions which occurs in 30 mg (0.3 μmol) of lipoxygenase was added to the LOOH. No significant effect was observed (experiment 15) since the difference from the control experiment (experiment 3) is only a marginal increase of 0.4 μmol of Tri-OH.

DISCUSSION

Storage of ground soybean flour at room temperature leads to an increase in the content of Tri-OH possibly as consequence of an enzymatic peroxidation. What effect this has upon the development of a bitter taste in soybeans is, however, still open question. To answer this the distribution of Tri-OH in the lipid classes must be determined. In all probability the intensity of the bitter taste depends upon the extent to which water-soluble phosphatides with a Tri-OH as acyl residue are formed during storage since one may assume that the threshold of bitter taste of Tri-OH joined to a phospholipid is lower than that of free Tri-OH or of a Tri-OH esterified to a triglyceride.

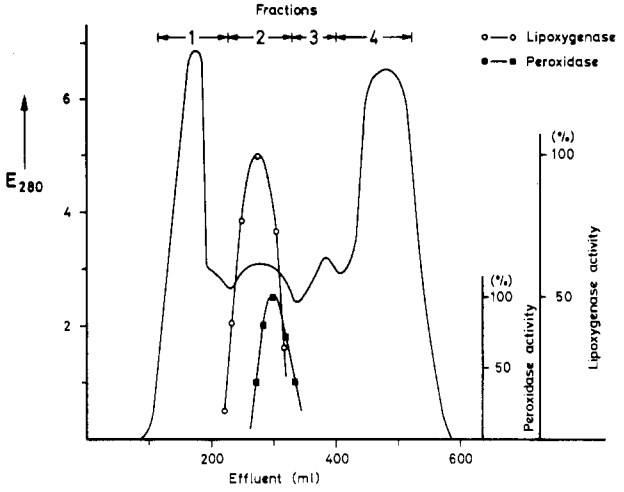

Figure 3. Gel chromatography of an extract from ground soybeans on Sephadex G-200 (the details are described under Experimental Section).

After incubation of linoleic acid with a defatted soybean flour or with a high concentration of soybean proteins, no LOOH are detectable (Baur et al., 1977). The LOOH are only intermediates of the lipoxygenase catalysis which are modified by subsequent reactions to a range of products.

The results of the model experiments reported here indicate that the breakdown of the LOOH to Tri-OH is furthered by all the investigated protein fractions of the soybeans and, however, by bovine serum albumin. It seems that thiol groups are involved in the Tri-OH formation.

The experiment in which the horse radish peroxidase was incubated with LOOH establishes that hemoproteins are more active than proteins or ferrous ions in producing Tri-OH.

The reason for the fivefold increase in Tri-OH formation when a high concentration of fraction 2 was incubated with LOOH (experiment 6b Table II) is not clear. It is possible that some type of inhibition is involved until lipoxygenase, peroxidase, and other proteins which promote the formation of Tri-OH are in excess.

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Partial Purification of a Lipoxygenase from Apples

In-Sook Kim and Werner Grosch*

A membrane-bound lipoxygenase (EC 1.13.11.12) was partially purified from apples by differential centrifugation and gel chromatography. The enzyme had a pH optimum at 6.0 and converted linoleic acid predominantly into the 13-hydroperoxyoctadeca-9,11-dienoic acid. Reversible inhibition was obtained with ethylenediaminetetraacetic acid disodium salt and cyanide. Hemoproteins were not involved in the lipid peroxidation activity.

According to a proposal of Tressel et al. (1970) a lipoxygenase-catalyzed formation of hydroperoxides and a subsequent cleavage by an "aldehyde lyase" in apples is the pathway involved in the breakdown of unsaturated fatty acids to volatile carbonyl compounds like hexanal and 2-*trans*-hexenal. In agreement with the first postulated reaction, we recently found (Grosch et al., 1977) that a homogenate of apples is highly specific in peroxidizing linoleic acid to the 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH). In this paper we present further evidence for the occurrence of a lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) in apples.

EXPERIMENTAL SECTION

Materials and Reagents. The following materials were used: apples ("Golden Delicious" from a local market), linoleic acid, and linolenic acid (99%, Nu Chek Prep), dithioerythritol (Sigma), Tween 20 (Schuchardt), ethylenediaminetetraacetic acid disodium salt (EDTA; Merck), Sepharose CL 2 B (Pharmacia), and Triton X-100 (Serva). The other chemicals were of analytical grade.

Methods. *Determination of Lipoxygenase Activity.* The linoleate peroxidation was assayed at 25 °C by two different techniques. Method I: The oxygen consumption was determined polarographically with a Clark oxygen electrode according to Delieu and Walker (1972). The apparatus was purchased from Bachofer, Reutlingen (Germany). Unless otherwise stated incubation mixtures contained 0.64 mM linoleic acid, 0.2 μL/mL of Tween 20 and 2 mL of 0.1 M sodium acetate buffer (pH 6.0). The mixture was stirred continuously, and after attaining equilibrium at 25 °C, enzyme solution was injected. Enzyme activities were calculated from initial rates of O_2 uptake, assuming an initial dissolved oxygen concentration of 0.25 μmol/mL. One unit of lipoxygenase catalyzed the uptake of 1 μmol of O_2/min. Method II: From the substrate described unter method I, 3 mL was pipetted into a 1-cm silica glass cuvette. After addition of the enzyme solution, the change in absorbance at 234 nm was measured.

Protein Determination. During chromatography protein was measured by the absorbance at 280 nm. For more accurate measurements the method of Lowry et al. (1951) with bovine serum albumin as standard was used.

Detection of Hematin. Using the method of Hartree (1955), the Sepharose fraction (1.2 mg of protein) was treated with 3 mL of a reagent containing 0.1 N NaOH, pyridine, and sodium dithionite. The visible spectrum was recorded between 400 and 650 nm.

Determination of the Hydroperoxide Isomers. *Fatty Acid Emulsion.* Ten milligrams of linoleic acid was (with the addition of 8 μL of Tween 20 and some drops of 1 N NaOH) dissolved in 10 mL of H_2O. The solution was diluted to 50 mL with 0.1 N sodium acetate buffer (pH 6.0). The pH of the emulsion was corrected to 6.0 with dilute HCl.

Incubation. The combined fractions from the Sepharose column with lipoxygenase activity (7 units) were added to the fatty acid emulsion after degassing with O_2 (5 min). The reaction mixture was stirred for 20 min at 25 °C. Immediately after incubation the reaction mixture was acidified (pH 4.0) with diluted HCl and extracted twice with 50 mL of diethyl ether. The diethyl ether solution was washed twice with 30 mL of water, dried over Na_2SO_4, and concentrated to 27 mL. After addition of 3 mL of methanol, the fatty acids were methylated with diazomethane as described by Schlenk and Gellerman (1960). For purification the methyl esters were chromatographed on silica gel HF_{254} by developing with isooctane/diethyl

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