Formation of Volatile Carbonyl Compounds and Cooxidation of β-Carotene by Lipoxygenase from Wheat, Potato, Flax, and Beans

Werner Grosch,* Gudrun Laskawy, and Franziska Weber

Partially purified lipoxygenases from wheat, potato, bean seed, and flax seed were differentiated with respect to the formation of volatile carbonyl compounds (during the oxidation of linolenic acid), the specific activity of carotene bleaching (in the presence of linoleic acid), and the peroxidation specificity. The enzyme from beans forms significantly more carbonyl compounds than the wheat, potato, and flax lipoxygenases. 2,6-Nonadienal (potato) and 2-hexenal (bean) were identified as the predominant flavor components. The enzyme from bean and also that from potato bleach β-carotene with a higher specific activity than the lipoxygenases from wheat and flax which are poor carotene "oxidases". The lipoxygenases from wheat, flax, and potato oxidize linolenic acid mainly to one hydroperoxide (either the 9- or the 13-isomer). Only the bean lipoxygenase peroxidizes this substrate to 9- and 13-hydroperoxides in a ratio of 1:1. The relationship between the peroxidation specificity of the lipoxygenases and their cooxidation potential is discussed.

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is widely distributed in the plant kingdom. In the presence of molecular oxygen the enzyme catalyzes the oxidation of polyunsaturated fatty acids, which contain a 1,4-*cis*,*cis*-pentadiene system. The involvement of this enzyme in the formation of aroma substances from unsaturated fatty acids and in the bleaching of carotenoids is important for the quality of many plant foodstuffs.

Studies on the lipoxygenases of soybeans and peas have shown two types of the enzyme. Lipoxygenases with an optimum at pH 6.5, which can react not only with the free acids but also with the methyl esters, attack the terminal double bonds of the unsaturated system (Roza and Francke, 1973). They oxidize linoleic acid to 9- and 13- and α-linolenic acid to 9- and 16-hydroperoxides in an approximate relationship of 1:1 (Christopher et al., 1972; Leu, 1974; Roza and Francke, 1973). In addition to the hydroperoxidation reaction these enzymes form a great range of volatile carbonyl compounds in sensorily significant amounts (Grosch et al., 1974; Grosch and Laskawy, 1975). Also, they can cooxidize with high velocity carotenoids (Arens et al., 1973; Weber et al., 1974). In contrast to pH 6.5 lipoxygenase from soybeans and peas, the lipoxygenase with an optimum pH at 9–10 shows a higher peroxidation specificity. It oxidizes linoleic acid predominantly to the 13-hydroperoxide (Hamberg and Samuelsson, 1967), forms only traces of volatile carbonyl compounds (Grosch and Laskawy, 1975), and is a poor carotenoid "oxidase" (Weber et al., 1974).

The question arises, from the differences of the soybean and pea lipoxygenase with low and high peroxidation specificity in formation of volatile carbonyl compounds and carotenoid bleaching velocity, as to whether this relationship is valid for lipoxygenases from other plants. This article deals with the investigations with semipurified lipoxygenases from potatoes, wheat, flax seeds, and beans.

EXPERIMENTAL SECTION

Materials and Reagents. Potatoes (*Solanum tuberosum* L. var. Princess), wheat seeds (*Triticum aestivum* L. var. Kolibri), bean seeds (*Phaseolus coccineus* L. var. Weisse Riesen), and flax seeds (*Linum usitatissimum*) were used. Linolenic acid (>99%, Nu Chek Prep), Tween 80 (Schuchardt), and piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Pipes buffer, Sigma) were also used. The other chemicals were of analytical grade.

Deutsche Forschungsanstalt für Lebensmittelchemie, 8000 München 40, West Germany.

Enzyme Assays. Lipoxygenase was assayed at 23 °C at pH 6.5 using a modification of the substrate described by Surrey (1964). The assay mixture contained 1 mM linoleic acid, 0.3 μl/ml of Tween 80, and 82 mM sodium phosphate. In 2 ml of reaction mixture one unit caused a ΔA_{234}^{1cm} of 1.0 between 30 and 60 s. To compare the lipoxygenase activity with the carotene bleaching velocity the photometric assay procedures described earlier (Weber et al., 1974) were used.

Peroxidase and catalase activities were determined according to procedures in Bergmeyer (1970).

Hydroperoxide isomerase was determined according to Zimmerman and Vick (1970b).

Protein Determination. During chromatography protein was measured by the absorbance at 280 or 230 nm. For more accurate measurements the biuret method, as described by Beisenherz et al. (1959), was used.

Lipoxygenase Isolation. All steps were performed at 0–4 °C.

Potatoes. Potatoes were peeled and cut into pieces. The pieces (250 g) were ground with 450 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 30 mM ascorbic acid in a Waring Blender. The slurry obtained was filtered through two layers of cheesecloth and centrifuged at 13000*g* for 20 min. The supernatant was precipitated with 40% ammonium sulfate saturation for 60 min, the precipitate was discarded, and the supernatant was precipitated with 60% ammonium sulfate saturation. The precipitate was collected and dissolved in 50 ml of 10 mM sodium phosphate buffer (pH 7.0). After centrifugation the supernatant was applied to a Sephadex G 50 column (3 × 25 cm) which was equilibrated and eluted with the 10 mM sodium phosphate buffer (pH 7.0). The eluent (30 ml) in the range of void volume (void volume being determined by chromatography of Blue Dextran 2000) was collected. This solution (25 ml) containing 242 mg of protein was applied to a DEAE-cellulose column (2.5 × 32 cm) which was equilibrated with the above 10 mM phosphate buffer. Elution was performed with the same buffer and a linear salt gradient of 0.0 M NaCl (1000 ml)–0.8 M NaCl (1000 ml). Fractions of 9.5 ml/tube were collected and assayed for uv absorption and lipoxygenase activity. The fractions 45–63 containing lipoxygenase were combined for the oxidation experiments.

Wheat. Ground seeds (80 g) (defatted with light petroleum) were stirred for 60 min with 800 ml of 10 mM sodium phosphate buffer (pH 7.5). As described under the section on potatoes a protein fraction was precipitated with ammonium sulfate in the saturation range 30–60%. From

the residue dissolved in 50 ml of 10 mM sodium phosphate buffer (pH 7.5) the lipoxygenase containing protein fraction was separated by gel and DEAE-cellulose chromatography as described above. Only the pH values of the buffer and the salt gradient were changed. Both steps were performed with 10 mM sodium phosphate buffer (pH 7.5). Protein (295 mg) was applied to the DEAE-cellulose column which was eluted with the same buffer and a linear salt gradient of 0.0 M NaCl (800 ml)–0.4 M NaCl (800 ml). The fractions 30–51 (9.5 ml/tube) containing lipoxygenase were combined for the oxidation experiments.

Flax Seed. Ground seeds (20 g) (defatted with acetone) were extracted with 200 ml of 0.1 M sodium phosphate buffer (pH 7.4) and heat treated for 10 min at 55 °C to destroy hydroperoxide isomerase activity as described by Zimmerman and Vick (1970b). The supernatant (60 ml) of the heat treatment was precipitated with 50% ammonium sulfate saturation.

The precipitate was dissolved in 20 ml of 50 mM sodium phosphate buffer (pH 6.6) and clear centrifuged. The supernatant (6 ml) containing 150 mg of protein was applied to a Sephadex G 200 column (2.5 × 90 cm) which was equilibrated and upward eluted with the same buffer. Fractions 22–30 (9.5 ml/tube) containing lipoxygenase were combined for the oxidation experiments.

Beans. Ground seeds (10 g) (defatted with light petroleum) were stirred for 60 min with 100 ml of 10 mM sodium phosphate buffer (pH 6.8) and then filtered through one layer of cheesecloth and centrifuged with 15000*g* for 30 min. The supernatant (50 ml) was chromatographed on a Sephadex G 50 column as described under the section on potatoes. Only the pH of the phosphate buffer was changed to 6.8. Protein (206 mg) was applied to the DEAE-cellulose column. The elution conditions are given in Figure 2. The fractions 21–30 and 38–45 (9.5 ml/tube) containing the lipoxygenases B-1 and B-2 were combined for the oxidation experiments.

Oxidation Experiments. The preparation of the substrates and the incubation procedures were carried out as described in a previous paper (Grosch and Laskawy, 1975). The reaction system (400 ml) contains 100 mg of linolenic acid, 5 ml of a 0.001% solution of Tween 80, and the following buffer substances: a mixture of 15 mM sodium citrate and 1 mM sodium phosphate for the experiment at pH 5.8 with the lipoxygenase from wheat; a mixture of 10 mM sodium acetate and 2 mM sodium phosphate for the experiment at pH 5.5 with the lipoxygenase from potato; and 2.5 mM Pipes buffer for the experiments at pH 6.5 with lipoxygenases from flax and beans. After addition of the enzyme preparation the reaction mixture was stirred for 20 min at 10 °C.

Before and after the incubation 0.5 ml of the reaction system was pipetted into 3 ml of methanol. After dilution to 5 ml with water the extinction at 234 nm was measured. The diene formation was determined by using a molar extinction coefficient at 234 nm of 25000 l. mol^{-1} cm^{-1} (Johnston et al., 1961). Immediately after incubation the pH of the reaction system was changed to pH 8 with 1 N NaOH. This was followed by the addition of 10 ml of methanol and 15 ml of barium chloride solution (3 g of $BaCl_2 \cdot 2H_2O$ dissolved in 10 ml of water). The precipitate formed was filtered off at 4 °C (refrigerator) and the filtrate acidified with 5 ml of cooled 85% H_3PO_4. 2,4-Dinitrophenylhydrazine (50 mg) dissolved in 15 ml of cooled 85% H_3PO_4 was added. The isolation and identification of the 2,4-dinitrophenylhydrazones (DNP) were carried out as described in an earlier paper (Grosch et al., 1974).

Table I. Amounts of Monocarbonyl Compounds Formed from Linolenic Acid

Lipoxygenase preparation	mg of protein[a]	Substrate converted to dienes, %	Monocarbonyl compd, μmol
Bean B-2 (pH 6.5)	2.9	55	3.2
Wheat (pH 5.8)	11.8	43	1.3
Flax (pH 6.5)	22	72	1.3
Potato (pH 5.5)	12.8	46	1.5

[a] In the reaction mixture of 400 ml.

Determination of the Hydroperoxide Isomers. The preparation of the reaction system and the incubation procedure are described under the section on oxidation experiments. After the incubation the reaction mixture was acidified (pH 3.0) with diluted HCl and extracted 3 times with 300 ml of diethyl ether. The diethyl ether solution was washed twice with 100 ml of water, dried over Na_2SO_4, concentrated, and applied to silica gel HF_{254} plates. After developing twice with the solvent system *n*-heptane–diethyl ether–acetic acid (50:50:1, v/v/v) the hydroperoxides (LOOH) were located on the plates by ultraviolet absorption, scraped off, and eluted with diethyl ether. Ethanol (20 ml) was added and the diethyl ether removed under vacuum. Then the LOOH were reduced with $NaBH_4$ to hydroxy acids according to the method of Zimmerman and Vick (1970a) and methylated with diazomethane as described by Schlenk and Gellerman (1960). For purification the unsaturated hydroxy methyl esters were chromatographed on silica gel HF_{254} by developing with the solvent system isooctane–diethyl ether (2:3, v/v). After locating the esters on the plates by ultraviolet absorption the isomers were scaped off together and eluted with diethyl ether. The solution was evaporated to dryness under N_2 and taken up in 5 ml of absolute methanol. The esters were hydrogenated with palladium on Norite as catalyst. The mixture of the hydroxy methyl stearates was purified on thin layers of silica gel HF_{254} using isooctane–diethyl ether (2:3, v/v) as the developing solvent. The ester mixture was eluted with diethyl ether and analyzed for the ratio of 9- and 13-isomers by a mass spectrometer provided with a direct insertion probe. The inlet temperature was 47 °C, the source temperature 200 °C, and the electron potential 70 eV. Isomer ratios were determined from the relative intensities of mass peaks 158 and 187 for the 9-isomer and mass peaks 214 and 243 for the 13-isomer.

RESULTS

Preparation of the Lipoxygenase. Chromatography of the ammonium sulfate preparations gave for potato and flax seeds one peak with lipoxygenase activity. The eluent which contained the enzyme was combined and tested for the formation of volatile carbonyl compounds from linolenic acid and for the cooxidation of β-carotene. The wheat proteins were separated into three peaks with lipoxygenase activity (Figure 1). The eluents, 285–485 ml, containing the first two peaks were combined for further experiments. The two bean protein fractions with lipid peroxidation activity (B-1 and B-2 in Figure 2) were separately investigated. The enzyme B-1 is, for reasons as yet unclear, only active when highly diluted. It was therefore only possible to measure the velocity of the β-carotene cooxidation. All the lipoxygenase preparations were free from peroxidase and catalase activity. The hydroperoxide isomerase had been removed from the flax

Table II. Results of the Analysis of the Monocarbonyl Compounds (as DNP) from Linolenic Acid

Compd no.	Mobility in relation to alcanal-DNP,[a] carbon no.	λ_{max} in $CHCl_3$, nm	Mol wt[b]	Results of identification	mol %
		Oxidation with the Lipoxygenase from Potatoes			
1a	2	355		Acetaldehyde	2
2a	3	358		Propanal	11
3a	3-4	375		2-Pentenal	3
4a	4-5	375	278	2-Hexenal	15
5a	6	375	318	2,6-Nonadienal	46
6a	3-4	390		2,4-Heptadienal	8
7a	7-8	395		3,5-Octadien-2-one	9
8a	3-4	410		2,4,6-Nonatrienal	6
		Oxidation with the Lipoxygenase from Beans (B-2)			
1b	3	358		Propanal	21
2b	4-5	375	278	2-Hexenal	53
3b	3-4	390	290	2,4-Heptadienal	7
4b	7-8	395	304	3,5-Octadien-2-one	4
5b	3-4	410	316	2,4,6-Nonatrienal	4

[a] Mobility on thin-layer chromatography (TLC); Kieselgur/Carbowax-cyclohexane. [b] Determined by mass spectrometry of the DNP.

Table III. Cooxidation of β-Carotene

Lipoxygenase preparation in the reaction system	Protein, μg/ml	Diene formation[a] (15-30 s), μM	β-Carotene[b] decrease (15-30 s), μM	β-Carotene decrease (μM)/diene formation (μM)
Bean B-1 (pH 6.5)	1.6	1.0	0.28	0.28
Bean B-2 (pH 6.5)	1.15	3.2	0.54	0.17
Wheat (pH 5.5)	2.7	2.75	0.16	0.06
Flax (pH 6.5)	10.5	0.6	0.04	0.07
Potato (pH 5.5)	1.3	1.0	0.15	0.15

[a] The substrate contains 1 mM linoleic acid, 0.3 μl/ml of Tween 80, and 50 mM sodium phosphate buffer (pH 6.5) (flax and bean) or 0.1 M sodium acetate buffer (pH 5.5) (wheat and potato). [b] The substrate contains the substances shown under footnote *a* and also 9.34 μM β-carotene.

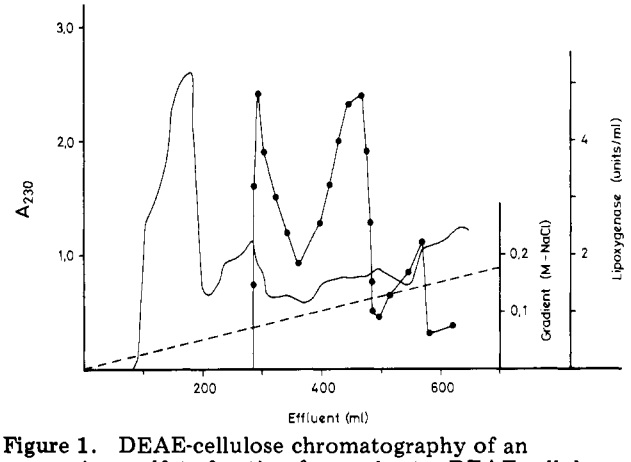

Figure 1. DEAE-cellulose chromatography of an ammonium sulfate fraction from wheat. DEAE-cellulose column (2.5 × 30 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.5); 295 mg of protein dissolved in the same buffer was applied. Elution (32 ml/h) was performed with a linear NaCl gradient (– – –) in the buffer: (——) protein; (•-•) lipoxygenase activity at pH 6.5.

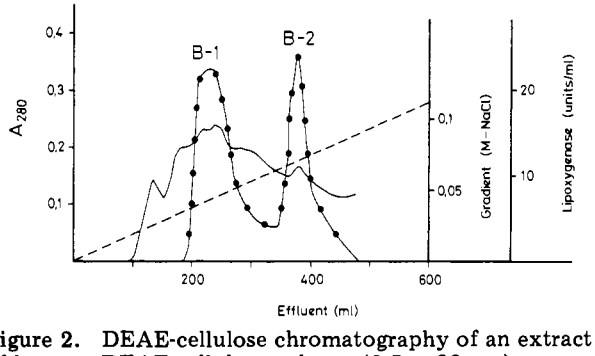

Figure 2. DEAE-cellulose chromatography of an extract of beans. DEAE-cellulose column (2.5 × 30 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8); 206 mg of protein dissolved in the same buffer was applied. Elution (32 ml/h) was performed with a linear NaCl gradient (– – –) in the buffer: (——) protein; (•-•) lipoxygenase activity at pH 6.5.

seed preparation through heat denaturation.

Amounts of Volatile Monocarbonyl Compounds. In these experiments 340 units of each lipoxygenase preparation were incubated with the linolenic emulsion. In this way varying amounts of the substrate were oxidized to compounds containing a diene system (Table I). One reason is probably the differing stabilities of the lipoxygenase during the 20-min incubation. Monocarbonyl compounds appeared in all reaction systems (Table I). In relation to the amounts of diene compounds (mainly hydroperoxides) produced, only very small amounts of volatile carbonyl compounds were formed by lipoxygenases from wheat, flax, and potatoes. In this respect they behave in the same way as the soy lipoxygenase L-1 (Grosch and Laskawy, 1975). The bean enzyme B-2 clearly forms more carbonyl compounds (3.2 μmol) than arise in the other three experiments.

Table IV. Specificity of Linolenic Acid Peroxidation

Lipoxygenase preparation	% hydroperoxide group in	
	Position 9	Position 13
Wheat	85	15
Potato	98	2
Bean B-2	51	49

Monocarbonyl Compounds from Linolenic Acid. From four experiments with the potato enzyme, 6 μmol of monocarbonyl compounds was isolated as DNP; 5.2 μmol gave eight compounds which could be identifed as described in Table II. Only five of these carbonyl compounds are formed by the lipoxygenase B-2 from beans. About 14 μmol was taken for analysis, of which 12.5 μmol was identified as shown in Table II. According to the results of the quantitative analysis the potato enzyme

Table V. Reactivity of Different Lipoxygenases

No.	Lipoxygenase preparation	pH	Peroxidation specificity[a]	Volatile carbonyl[a] compd, μmol	Carotene bleaching, β-carotene decrease (μM)/diene formation (μM)
1	Potato	5.5	9-LOOH	1.5	0.15
2	Wheat	5.8	9-LOOH	1.3	0.06
3	Flax	6.5	13-LOOH[c]	1.3	0.07
4	L-1 soybean	8.5	13-LOOH[d]	1.5	0.06
5	B-2 bean	6.5	13-:9-LOOH (1:1)	3.2	0.17
6	L-2 soybean	6.5	16-:9-LOOH (1:1)[b]	6.9	0.55
7	L-3 soybean	6.5		7.1	0.43

[a] Linolenic acid as substrate. [b] Results of Roza and Francke (1973). [c] Results of Zimmerman and Vick (1970a). [d] Results of Hamberg and Samuelsson (1967).

forms primarily 2,6-nonadienal, whereas for the lipoxygenase B-2 from beans 2-hexenal predominates.

Cooxidation of β-Carotene. To characterize the cooxidation potential of the lipoxygenase preparations the velocity of the carotene bleaching was related to the velocity with which linoleic acid was oxidized to dienes (Table III). Of the two bean lipoxygenases the preparation B-1, which is only active at high dilution, has the highest cooxidation potential. It is comparable to that of the soy and pea lipoxygenase with a neutral pH optimum (Arens et al., 1973; Weber et al., 1974) and is twice as high as that of the lipoxygenase from potato and the second lipoxygenase from beans (B-2). In contrast to these the enzymes from wheat and flax are very weak cooxidizing lipoxygenases. In this they resemble the alkaline soy lipoxygenase L-1 (Weber et al., 1974). When the linoleic acid was replaced by the same concentration of a mixture of 13- and 9-LOOH (75:25) in the test with the wheat lipoxygenase, the velocity of carotene bleaching sank still further.

Specificity of Linolenic Acid Peroxidation. This specificity of the preparations from wheat, potato, and beans (B-2) was determined. In agreement with the results of Galliard and Phillips (1971) and Graveland (1970) the lipoxygenases from potato and wheat oxidize predominantly C-9 of the substrate (Table IV). In contrast to this B-2 oxidizes the linolenic acid to the 9- and 13-isomers in the ratio 1:1. Here B-2 differs from the neutral soy lipoxygenase which according to Roza and Francke (1973) forms the 9- and 16-LOOH from this fatty acid.

DISCUSSION

Under consideration are both the results arrived at here and those of earlier works (Grosch and Laskawy, 1975; Weber et al., 1974) compiled in Table V. When referring to their reactivity in foodstuffs we can differentiate between two types of lipoxygenases. The first group consists of lipoxygenases 1–4 in Table V which oxidize both linoleic and linolenic acids very specifically. The 9- (potato and wheat) or the 13-hydroperoxide (flax, L-1 from soy) is formed. We suggest that the peroxyl radicals formed from the active centers convert so quickly into hydroperoxides that they can neither break down nor react with other substrate or polyene molecules. The formation of volatile monocarbonyl compounds and the cooxidation of β-carotene thus take place very slowly. Aroma substances only arise directly to a very small extent from the catalysis of these lipoxygenases. However, precursors are formed with the hydroperoxides which, either through heating or through reaction with other components of the foodstuffs such as traces of heavy metals or ascorbic acid (Grosch, 1976), can break down into volatile carbonyl compounds.

The soy lipoxygenases L-2 and L-3 (6 and 7 in Table V) are considerably more reactive than the first group of lipoxygenases. Their peroxidation specificity is lowered in that they oxidize the linolenic acid to two products. A part of the peroxyl radicals initially formed can apparently react further since they do not immediately appear as hydroperoxides. At the surface of the enzyme, or in its neighborhood, reactions are started which are well known from the autoxidation process of olefins. This results in a cooxidation of the substrate and of compounds whose H-atoms are activated through double bonds for an H abstraction.

The bean lipoxygenase B-2 (5 in Table V) stands between the two groups of lipoxygenases. The enzyme forms in equal amounts two hydroperoxides from linolenic acid (9- and 13-LOOH). Unlike the two soy enzymes L-2 and L-3, however, it does not oxidize the terminal C-atoms of the system of three double bonds. In contrast to the lipoxygenases 1–4 (Table V), B-2 forms volatile monocarbonyl compounds in greater amounts although it does not reach the quantities which arise from the catalysis of the soy enzymes L-2 and L-3.

ACKNOWLEDGMENT

The authors express their thanks to V. Mundy for her help in translating the manuscript. The mass spectrometric analysis by K.-H. Fischer and G. Szonyi is gratefully acknowledged.

LITERATURE CITED

Arens, D., Seilmeier, W., Weber, F., Kloos, G., Grosch, W., *Biochim. Biophys. Acta* **327**, 295 (1973).
Beisenherz, G. H., Boltze, J., Bücher, Th. Czok, R., Garbade, K. H., Meyer-Arendt, E., Pfleiderer, G., *Z. Naturforsch. B* **8**, 555 (1953).
Bergmeyer, H. U., Methoden der Enzymatischen Analyse, 2nd ed, Verlag Chemie, Weinheim/Bergstr., Germany, 1970.
Christopher, J., Pistorius, E. K., Axelrod, B., *Biochim. Biophys. Acta* **284**, 54 (1972).
Galliard, T., Phillips, D. R., *Biochem. J.* **124**, 431 (1971).
Graveland, A., *J. Am. Oil Chem. Soc.* **47**, 352 (1970).
Grosch, W., *Z.-Lebensm.-Unters.-Forsch.*, in press (1976).
Grosch, W., Laskawy, G., *J. Agric. Food Chem.* **23**, 791 (1975).
Grosch, W., Laskawy, G., Fischer, K. H., *Lebensm.-Wiss. Technol.* **7**, 335 (1974).
Hamberg, M., Samuelsson, B., *J. Biol. Chem.* **242**, 5329 (1967).
Johnston, A. E., Zilch, K. T., Selke, E., Dutton, H. J., *J. Am. Oil Chem. Soc.* **38**, 367 (1961).
Leu, K., *Lebensm. Wiss. Technol.* **7**, 82 (1974).
Roza, M., Francke, A., *Biochim. Biophys. Acta* **316**, 76 (1973).
Schlenk, H., Gellerman, J. L., *Anal. Chem.* **32**, 1412 (1960).
Surrey, K., *Plant Physiol.* **39**, 65 (1964).
Weber, F., Laskawy, G., Grosch, W., *Z. Lebensm.-Unters.-Forsch.* **155**, 142 (1974).
Zimmerman, D. C., Vick, B. A., *Lipids* **5**, 392 (1970a).
Zimmerman, D. C., Vick, B. A., *Plant Physiol.* **46**, 445 (1970b).

Received for review October 17, 1975. Accepted January 16, 1976. We are grateful to the Deutsche Forschungsgemeinschaft Bonn-Bad Godesberg for supporting this work.