Measurement of α-Linolenic Acid in the Development of Edible Oil Flax

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A thiobarbituric acid-gas liquid chromatograph combination procedure is described for rapid screening of individual or half-seeds of flax for α-linolenic acid (ALA) in the development of an edible oil flax. The thiobarbituric acid test may be used to screen as many as 1000 seeds in a single day. However, the test needs to be complemented by quantitative determination of fatty acids by gas chromatography. The latter technique has been used to analyze half-seed of flax. The combination has worked extremely well for the isolation of low ALA mutants of flax.

Flax, the sixth largest oilseed crop in the world, is grown for industrial (linseed) oil, primarily because of its α-linolenic acid (ALA; 18:3 ω-3) content. Of the many species in the genus *Linum*, the only cultivated species (*L. usitatissimum*) contains high concentrations of ALA, which may range from 46–71% (1). A generally similar range in ALA, (45.5–64.2%) was reported more recently by Green and Marshall (2) in a diverse collection of 214 accessions of the same species. The cool northern climes of the Prairies, where most of the Canadian flax is grown, delay maturity of the crop and provide a longer period for oil and fatty acid synthesis. Canadian grown flax rarely contains less then 40% oil, 15% linoleic acid (LA; 18:2 ω-6) and 55% ALA.

Recently, interest has developed in the nutritional value of ω-3 fatty acids, particularly in fish oil, which contains eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3). These long-chain fatty acids reduce synthesis in the human body of prostaglandin (PGE2) and thromboxane (TXA2), both of which constrict smooth muscle and coronary arteries and thus may contribute to atherosclerosis and thrombosis (3). Dietary ALA from vegetable oils such as soybean, canola and flax is converted by chain elongation and desaturation to EPA and DHA. An ingestion of 30 ml per day of linseed oil by human subjects led to increases of 328 and 182% of EPA and DHA, respectively, in serum phospholipids (4). A long-term study of one year duration also showed an increase in EPA on ingestion of vegetable oils rich in ALA and LA, although no changes were noticed in plasma total cholesterol (5). However, in a more recent study (6), milled flaxseed added directly to meals of healthy individuals for four weeks reduced serum cholesterol by 8.5% and increased ALA and EPA in red cell phosphatidylcholine by 100%.

Traditional uses of linseed oil in paints, varnishes and linoleum have been steadily declining due to availability of water-based, synthetic substitutes. This has destabilized flax production on the Canadian Prairies. Conversion of linseed oil to edible oil with ALA reduced to levels present in soybean and canola oils (ca. 10%) could promote the production of flax in Saskatchewan, where it is not possible to grow most edible oilseed crops due to a short growing season. Green and Marshall (2) and Green (7) have reported flax mutants, produced by chemical mutagenesis and recombination of the mutant genes, that contain less than 2% ALA. This decrease in ALA was accompanied by an increase in LA from 14 to 51%, due to inhibition of two desaturase enzymes involved in the biosynthesis of ALA (8). Thus mutation breeding, in conjuction with traditional methods of hybridization and selection, offers the possibility of producing flax cultivars with various fatty acid compositions.

Development of edible oil flax cultivars requires screening for ALA on a large number of mutants or segregating flax lines. This paper describes rapid determination of ALA on whole or half-seed by the thiobarbituric acid test and gas liquid chromatography.

MATERIALS

Flax samples. Samples of flax (*Linum usitatissimum*), grown in 1987 and 1988, were taken from field experiments grown annually at the Kernen Crop Research Farm, University of Saskatchewn, Saskatoon. The air-dried, clean seed (moisture content 7–8%) was used as is in all experiments.

Filter paper sheets, Whatman No. 1 (46 × 57 cm) were cut in quarters to 11.5 × 14 cm size. Self-adhesive Matte film, Transtext, size 8 1/2 × 11 cm, was obtained from a local supplier (Engineering Technical Supplies Ltd., Saskatoon, Canada).

Reagents. These were petroleum ether (boiling range, 35–60°C), concentrated sulfuric acid and methanol (both ACS grade), and thiobarbituric acid (product no. 30408; 4,6-dihydroxy-2-thiopyrimidine), 99% purity, obtained from British Drug House, Pool, England, or from Sigma Chemical Company, St. Louis, MO (lot. no. 39F-0530).

METHODS

Thiobarbituric acid test (TBA). Single seeds were used for this test. The self-adhesive Matte film was marked in a grid pattern to accommodate 100 seeds in a 10 × 10 square. The seeds were placed on the film, to which they attached firmly and were immobilized. The Matte film sheet was then covered with two sheets of filter paper (taped at the corners), and the seed was crushed by gently going over it back and forth with a marble rolling pin. The crushed seed was left overnight to allow oil spots to move to the top sheet. The lower sheet contained the crushed seed and was discarded. The upper sheet containing the oil spots was placed for 30 min under a high intensity UV lamp (Canlab model G-54) and the lamp height was adjusted exactly to 20

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cm. After the UV exposure, the sheet was dipped in freshly prepared 2% TBA solution (9), blotted to soak excess of the TBA solution and heated at 110°C for 20 min, sandwiched between two tightly clamped 1.0 mm thick glass plates padded with two filter paper sheets. The developed sheet was examined visually to distinguish between deep red (high ALA) and lighter red or orange (low ALA) spots.

Linolenic acid determination by gas-liquid chromatography (GLC). Whole or half-seed were used for linolenic acid determination by GLC. In the latter case, seed was cut with a sharp scalpel in the ratio of 60:40; the larger section contained the embryo and a portion of the cotyledons, while the smaller was made up entirely of cotyledons. The cotyledon section was held with forceps and squeezed into 1.0 ml of methanol containing 2% sulfuric acid (10), which was held in a 3.5 ml screw cap vial (Pierce Chemical Co., Rockford, IL). The vial was heated at 100°C for 1 hr, cooled to room temperature, and then 1.0 ml of petroleum ether was added. After shaking, the ether layer was quantitatively transferred with a Pasteur pipette to a 2.0 ml vial which was then capped, sealed with a crimper and transferred to an automatic sampler. The GLC was operated under the following conditions: 6 ft. glass column packed with Supelco GP 3% SP 2310/2% SP-2300 on 100/120 Chromosorb W (Supelco, Bellefonte, PA); injection and detector temperatures, 250°C; oven temperature, 190°C; carrier gas (nitrogen) flow rate adjusted to complete the run in about 10 min; and a Hewlett-Packard 3385 A automation system calculated each fatty acid as percent of the total fatty acids.

RESULTS AND DISCUSSION

The TBA test. Figure 1 shows sections of sequential development of red spots on the interaction of ALA with TBA. Unequal spots on the right were due to highly variable oil, and hence ALA content, of individual flax seeds. However, the color intensity of spots was not influenced by oil content due to sensitivity of the TBA test.

The mechanism of the red color complex formed from oxidation products of ALA (9,12,15-octadecatrienoic acid) and TBA is shown in Figure 2. This mechanism had been investigated in a number of studies on autoxidation of polyunsaturated fatty acids (11-13). Three separate steps in the mechanism have been recognized. The first is the formation, on autoxidation, of four isomeric peroxide radicals by removal of hydrogen from carbon 11 or 14 of ALA. Oxidation is facilitated by heat, light, ultraviolet radiation and metal ions (13). Two of the unsaturated peroxides (β, ν) undergo cyclization to form a five-membered ring peroxide which is the non-volatile precursor for formation of malonaldehyde (11). There is some disagreement on the origin of malonaldehyde. Pryor *et al.* (12) reported that the non-volatile precursor is at least partly a bicyclic endoperoxide (2,3-dioxanoborane), which decomposes to form malonaldehyde. Nevertheless, malonaldehyde is the key intermediate for TBA reaction, and was the major component identified from oxidized and UV-irradiated unsaturated fatty acid esters and squalene (14). The cyclic peroxide giving rise to malonaldehyde appears only on the oxidation of methylene-interrupted polyunsaturated fatty acids with three or more double bonds (11). Therefore, the TBA test is specific for linseed oil where ALA (18:3) is the major fatty acid. In the final step, malonaldehyde condenses with two moles of TBA to yield the highly colored complex, which absorbs at 535 nm.

Green and Marshall (9) used the TBA test to isolate two reduced ALA mutants from flax treated with ethylmethanesulfonate. The mutants contained about one-half the ALA (19.1-23.4%) present in the parent cultivar. Using the TBA test, we obtained 53 plants with reduced ALA from 2430 mutation 1 plants (15). The TBA test thus lends itself to preliminary screening for ALA in individual seeds of flax. In our laboratory, 600-800, and sometimes up to 1000 seeds have been screened daily with the TBA test. The test requires an experienced technician with a visual ability to distinguish low and high ALA spots. Appropriate

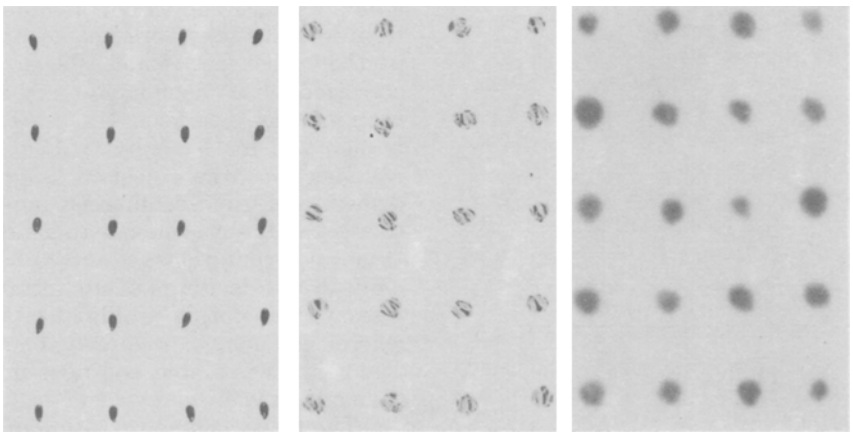

FIG. 1. Development of thiobarbituric acid test for preliminary screening of α-linolenic acid in single seeds of flax. Left, flaxseed attached to Matte film; center, crushed seeds; right, red color spots.

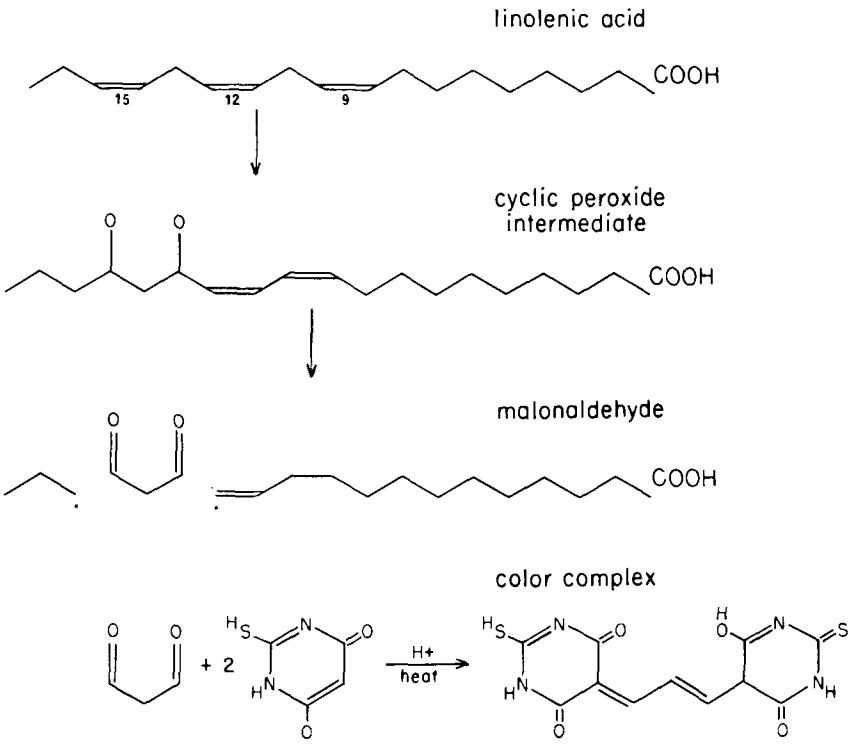

FIG. 2. Mechanism involved (simplified from references 11 and 12) in thiobarbituric acid test for rapid screening of α-linolenic acid in single seeds of flax.

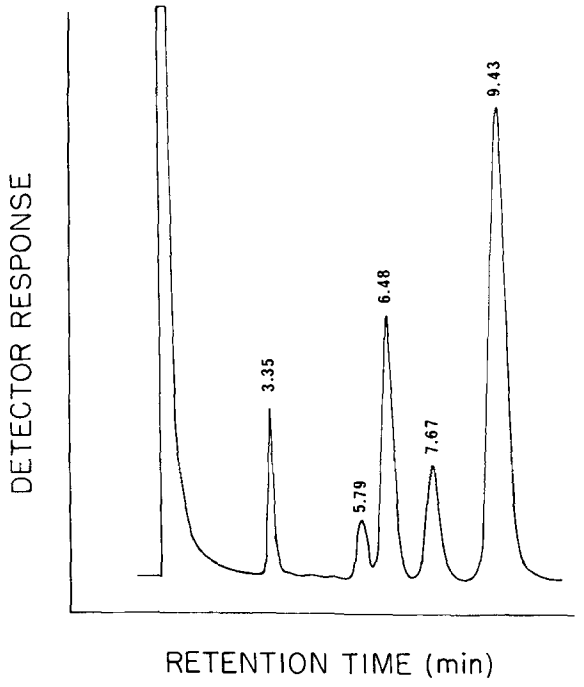

FIG. 3. Gas-liquid chromatograph elution profile of fatty acid methyl esters prepared from half-seed of flax. The five peaks, excluding the solvent peak, represent 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids, respectively.

standards containing different concentrations of ALA may be prepared by mixing freshly extracted linseed oil with sunflower, corn or other oil not containing ALA. However, the test is not fool-proof and may miss desirable spots. For that reason, it needs to be complemented by quantitative analysis.

Gas liquid chromatography. In flax, like some other oilseeds, the fatty acid composition is determined by genotype of the developing embryo. Therefore, half-seed analysis of fatty acids allows faster development of cultivars than analysis of a bulk seed sample from a single plant (16). In our laboratory, individual seeds, from lots partially screened by the TBA test and suspected to contain reduced levels of ALA, are surface sterilized with alcohol and sodium hypochlorite and sectioned as described earlier. The cotyledon portion is analyzed for ALA by GLC (see Methods). The embryo section, where desired, is grown on agar in petri dishes, and the seedling is subsequently transferred to a pot in the greenhouse to generate a new flax plant. Under our conditions, about 70 half-seeds are analyzed daily in two loadings of an automatic sampler. Figure 3 shows the elution profile of fatty acid methyl esters, where each analysis requires about 10 min to complete. The column is stable and does not need packing more than once a year.

The fatty acid composition of individual flax seeds is highly variable, and the inter-seed coefficient of variability (CV) for 18:1, 18:2 and 18:3 fatty acids ran from 5-23% (17). In our experience, at least 25 seeds corre-

sponding to about 100 mg of ground seed are needed to reduce CV to less than 5% and to obtain a representative analysis. For an average analysis of this kind, seed may be ground in a Krups coffee grinder, and ground seed is then hydrolyzed with 5 ml of the methanol-sulfuric acid mixture for 3 hr at 80°C for analysis of the fatty acid methyl esters.

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