Analysis of Furanoid Esters in Soybean Oil and the Effect of Variety and Environment on Furanoid Ester Content[1]

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ABSTRACT: An improved method was developed to analyze the major furanoid esters in soybean oil. The method is based on urea fractionation of the methyl esters, silver ion chromatography, and gas chromatography of the furanoid concentrate. Activation of the soybean lipoxygenase decreased the amount of furanoid ester recovered from the oil, but the degumming of crude soybean oil and the choice of solvent used to extract soybean lipids caused no change in furanoid ester content. Fifty-six soybean varieties, representing a wide range in maturity group and geographical origin, were grown in Puerto Rico and used to determine the range of furanoid ester contents. Furanoid ester II ranged from 0.033–0.29 mg/g, and ester III ranged from 0.058–0.27 mg/g. The two major furanoid esters were positively correlated with each other and with maturity group. Growth environment as well as variety caused significant differences in furanoid content.

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KEY WORDS: Analysis, effect of growth environment, effect of variety, furanoid fatty acids.

Fatty acids containing a furan ring, or furanoid fatty acids, were first discovered by Morris *et al.* (1) in the seed oil of *Exocarpus cupressiformis*. More than 30 furanoid acids have been reported in a variety of animal and plant sources.

The physiological role of these acids remains a matter of speculation. They may be involved in animal reproductive rhythms (2) and embryo development (3). Batna and Spitellar (4) suggest that they may act as antioxidants in plants. Furanoid acids inhibit lysyl oxidase and may be useful in the treatment of diseases that entail excess collagen and elastin deposition (5). Their biosynthetic pathway is not clear.

Besides the seed oil of *E. cupressiformis*, furanoid esters have been found in algae (*Chlorophyta* sp.), mushroom (*Agaricus bisporus*), and yeast (*Saccharomyces cerevisiae*); they are also found in the roots and shoots of grasses (*Poaceae* sp.), clover (*Trifolium pratense*), dandelion (*Taraxacum officinale*), chive (*Allium sativum*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), cabbage (*Brassica oleracea*), orange (*Citrus sinensis osbeck*), lemon (*Citrus limon*), strawberries (*Fragaria* sp.), and birch leaves (*Betula pendula*) (6) as well as the latex of *Hevea brasiliensis* (7). Furanoid esters occur in greater amounts in the green parts of plants than in the stems, roots, and seeds.

Three furanoid acids were identified in soybean oil by Guth and Grosch (8) and are shown in Figure 1. Guth and Grosch (8) reported that the three furanoid esters totaled 250–390 mg/kg in soybean oil, 70–120 mg/kg in wheat germ oil, 13–36 mg/kg in rapeseed oil, and 17–24 mg/kg in corn oil. Furanoid esters were absent in olive and sunflower oils. Analyses of the soybean cultivar Century and five soybean genotypes that lacked one or two of the three lipoxygenase isoenzymes revealed that their seed oils contained 190–225 mg/kg of furanoid ester II and 91–132 of ester III (9). The concentrations of furanoid esters were not correlated with lipoxygenase activities.

The oxidation of furanoid fatty acids has been studied by Boyer *et al.* (10), Zabolotsky *et al.* (11), and Batna and Spitellar (4,12). These studies indicated that the furan ring in furanoid acids could be co-oxidized by linoleate hydroperoxide with the ring opening to form a conjugated dioxoene. The dioxoene formed from dialkylfuranoids was more stable that those of tri- and tetraalkylfuranoids, which underwent further reaction. Tetraalkyl-substituted furanoid acids were also capable of scavenging free radicals generated during oxidation reactions and suppressing the oxidation of linoleic acid (12,13). The trialkyl furanoids were about one-half as effective as the tetraalkyl varieties in retarding oxidation, and the dialkyl showed no significant activity. Guth and Grosch (8) reported that furanoid acids II and III of soybean oil could be easily photooxidized to produce a strong flavor compound, 3-methylnonane-2,4-dione; they believed it to be important in the flavor of photooxidized soybean oil.

To isolate and measure furanoid esters in lipids, acyl groups are generally converted to methyl esters and concentrated by some combination of the following techniques: catalytic hydrogenation of the unsaturated esters other than the furanoid esters, formation of urea inclusion compounds (2,14,15), and silver ion chromatography (2,7). Final quantitation usually has been accomplished by gas chromatography (GC). Guth and Grosch (8) added the deuterated furanoid ester methyl 10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoate-8,9-d2 as an internal standard before concentrating the furanoid esters and determining the furanoid content by chemical ionization

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GC–mass spectrometry (MS).

In this study, an efficient method was developed to analyze the furanoid esters in soybean oil, the range of variation of the two major furanoid esters in 56 soybean varieties was measured, and observations were made on the effect of growth environment on the furanoid content.

MATERIALS AND METHODS

Materials. Soybean oil was obtained from a local grocery store. Cod liver oil was purchased from ICN Biochemicals, Inc., Cleveland, OH.

Soybean plant introductions, representing a wide range in maturity and geographical origin, were provided by Randall L. Nelson, USDA, Agricultural Research Service, Urbana, IL. Forty seeds of each variety were planted in Puerto Rico in May 1995 and harvested in September 1995. Six soybean varieties with extreme furanoid acid contents, PI 398672, PI 398955, FA4, PI 165929, PI 171444, and PI 323276, were regrown in Puerto Rico in January 1996 and harvested in April 1996. Environmental effects on furanoid ester content were determined in four additional soybean varieties from the Iowa State University Agronomy Department that were grown in Washington, Ames, and Waterloo, IA in 1994.

Preparation of cartridges. Silver nitrate cartridges were prepared by adding 2 mL of a 20.4% silver nitrate solution to a 900-mg Maxi-Clean™ silica cartridge (Alltech Associates, Deerfield, IL) by using a syringe. The cartridges were dried for 6 h with dry air at 200 mL/min. Silver ion thin-layer plates were prepared by spreading a slurry of 37 g of silica gel in 77 mL water that contained 3.7 g silver nitrate to give three 20 × 20 cm 0.4 mm plates.

Preparation of standards. To prepare furanoid fatty acid standards from cod liver oil, 100 mL oil was converted to methyl esters by using methanolic sodium methoxide. The furanoid esters were concentrated by urea fractionation using 40 g esters, 360 g urea, and 400 mL methanol. The mixture was refluxed for 3 min and stored at 4°C for 12 h. The urea complex was removed by filtration, and the filtrate was diluted with 150 mL of 1% aqueous hydrochloric acid. Yield of furanoid concentrate was 4 g. A silver-ion TLC plate, streaked with 200 µL of the esters, was developed in 3:7 diethyl ether/hexane (vol/vol). Bands were visualized by spraying with 0.2% 2,7-dichlorofluorescein in ethanol and viewing under ultraviolet radiation. The band migrating between saturates and monoenes was collected and extracted with diethyl ether. The ether was removed under a nitrogen stream, and the furanoid ester concentrate was dissolved in hexane.

Concentration of the furanoid esters. During the analysis of furanoid fatty acids in soybeans, oxidation was minimized by using amber vials and performing each step in dim lighting and removing dissolved oxygen from solvent by boiling them briefly before using. For the analysis, 10 g soybeans were ground in a Wiley Mill (Arthur H. Thomas Co. Scientific Apparatus, Philadelphia, PA), fitted with a 20-mesh delivery unit. The resulting powder was soaked in 25 mL 2:1 chloroform/methanol (vol/vol) overnight, and the oil was removed by filtration. The filtrate was mixed with 3 mL water, and the chloroform layer was collected and evaporated under a stream of nitrogen. One gram of the residual oil was dissolved in 1 mL hexane, 7 mL 1 M sodium methoxide was added, and the mixture was held at 50°C for 15 min with occasional shaking. After adding 5 mL water, the fatty acid methyl esters (FAME) were extracted with hexane. The FAME were used for the fatty acid profile and evaporated under a nitrogen stream to remove the hexane. The FAME were subjected to urea fractionation by using 0.5 g FAME, 5 g urea, and 5 mL methanol. The mixture was refluxed for 3 min and stored at 4°C for 12 h. The urea complex was removed by filtration and washed with 6 mL of urea-saturated methanol. The filtrate and washings were acidified with 5 mL boiled water and 4 drops 1 M hydrochloric acid. The FAME in the filtrate were extracted four times with 1.5 mL hexane. The FAME solution from the urea filtrate extract was reduced to 100 µL and applied to a silver ion cartridge; 150 µL hexane was used to make the transfer. The cartridge was eluted with 7% diethyl ether in hexane. The first 1 mL of eluate was discarded, and the next 3.5 mL contained the furanoid concentrate. The next 1.5 mL of eluate was routinely collected and examined but typically only contained about 4% of the total furanoid ester. To the fractions containing the furanoid concentrate, 200 µL of squalene standard solution (0.15 mg/mL) was added, and the resulting solution was reduced to about 200 µL under a stream of nitrogen and analyzed by GC.

GC conditions. For GC, approximately 1 µL of sample was injected with a split ratio of 1:5 into a Hewlett-Packard 5890A GC (Hewlett-Packard Co., Avondale, PA), fitted with a flame- ionization detector and a 30 m × 0.32 mm SPB-1 column with a 0.25-µm film thickness. The injector temperature was 270°C, detector temperature was 280°C, and column temperature was held at 150°C for 1 min, programmed to 270°C at 40°C/min, and held for 10 min. Carrier gas was helium at 1.4 mL/min, supplemented with a 20 mL/min bypass; hydrogen at 30 mL/min; and air at 380 mL/min. Flame-ionization correction factors, calculated with the equation of Ackman and Sipos (16), were applied to the furanoid esters; the correction factor for squalene was determined empirically.

To analyze the major soybean FAME, the GC was equipped with a 15 m × 0.25 mm SP-2330 column with a 0.20-µm film thickness, operated isothermally at 190°C. Injector temperature was 230°C, and detector temperature was 230°C.

GC–MS electron impact analyses were performed with a Hewlett-Packard 5890A gas chromatograph and an HP 5970 mass selective detector (Hewlett-Packard Co., Avondale, PA) at 70 eV. GC–MS chemical ionization analyses were performed with a Finnigan TSQ700 instrument (Finnigan, Bremen, Germany) with isobutane as the reagent gas, a DB 1701 column, and a mass range from 125–650.

Evaluations. To test the effect of extraction method on furanoid ester content, soybeans were ground as before in a Wiley Mill and extracted by either hexane or 2:1 chloro-

form/methanol (vol/vol). Some of the oil extracted with chloroform/methanol was freed of solvent and degummed with 3% water for 1 h and centrifuged. Furanoid ester content was analyzed in duplicate.

To test the effect of lipoxygenase activity on furanoid ester content, 100 g Kenwood soybeans were ground in a Wiley Mill as before, and the resulting powder was sprayed with various amounts of water to activate lipoxygenases and stored in the dark. After 48 h, oil was extracted with 1:1 methylene chloride/methanol (vol/vol) for 20 min, and the extract was tested for lipoxygenase activity by the method of Hammond *et al.* (17). A mixture of 35:65 soybean powder and water (wt/vol) was incubated in the dark for 36 h, extracted with methylene chloride/methanol and tested for furanoid fatty esters as described previously. Furanoid ester content was analyzed in duplicate. Statistical analyses were carried out by standard methods.

RESULTS AND DISCUSSION

Mass spectra of the furanoid methyl esters of soybean oil gave results in agreement with Guth and Grosch (8). The three furanoid esters shown in Figure 1 were found. The amount of ester I was an order of magnitude smaller than that of the other esters.

Because there was no commercial source of furanoid esters, a furanoid ester concentrate from cod liver oil was used to verify the analytical procedure. Of the six furanoid esters of cod liver oil, III and IV were used to verify the analytical method.

Furanoid esters were assumed to have a hydrogen flame detector response in the GC that could be calculated by the equation of Ackman and Sipos (16). The authors verified that methyl esters of saturated fatty acids C_{16}–C_{24} gave GC response factors in accord with the equation. The correction factor for squalene, calculated by the equation, did not agree with the observed value, so the observed value was used. The concentrations of furanoid methyl esters I, III, and IV in the concentrate from cod liver oil were 0.016, 0.023, and 0.049%, respectively, after using these correction factors.

Preliminary tests showed that urea fractionation of soybean FAME removed the saturates but left the furanoid esters contaminated with major amounts of unsaturated FAME. Further purification of the urea filtrate with silver ion TLC plates was effective but time consuming, and furanoid esters were easily oxidized on the surface of the silica gel (18). Separation on silica gel cartridges preloaded with silver nitrate was optimized by using the furanoid concentrate from cod liver oil added to the FAME in the urea filtrate from soybean oil. By using 7% diethyl ether in hexane as an eluant, the authors were able to separate the furanoid esters from the unsaturates in about 1 min. Recoveries of the furanoid esters III and IV were 92–100%. The recovery of ester III was corrected for the amount present in the soybean oil FAME.

The authors also verified that the furanoid esters III and IV of cod liver oil were recovered when added to 0.5 g of soybean FAME and subjected to urea fractionation, silver ion cartridge separation, and GC. The recovery on 10 samples averaged 96.8 ± 3.13%. When the FAME in the urea complex were examined, no furanoid fatty acids could be detected when the FAME were fractionated by silver ion cartridges or when the urea complex were recrystallized and the filtrate FAME was examined. Experience showed that a typical standard deviation for the analytical method was ± 0.005 mg/g FAME.

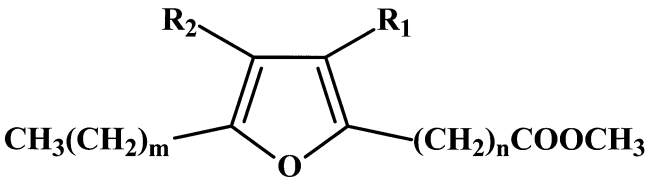

FIG. 1. The general structure of furanoid fatty acids. Following the designation of Guth and Grosch (8) for furanoid acids found in soybean oil for I, $n = 8$, $m = 4$, R_1 is CH_3, and R_2 is H; furanoid acid II, $n = 8$, $m = 4$, and both R_1 and R_2 are CH_3; furanoid acid III, $n = 10$, $m = 4$, and both R_1 and R_2 are CH_3; furanoid acid IV (found in cod liver oil), $n = 10$, $m = 2$, and both R_1 and R_2 are CH_3.

Effect of isolation conditions on furanoid ester recovery. No significant differences ($P < 0.05$) in furanoid ester content could be observed when soybean oil was extracted with hexane or chloroform/methanol, and degumming the oil extracted with chloroform/methanol to remove the phospholipid had no effect on furanoid ester content. Addition of water to ground soybean seed (cv. Kenwood) activated its lipoxygenases. Maximum lipoxygenase activity occurred when 65 wt% water was added to the ground seeds. Oil was extracted from the samples immediately after adding water and after

TABLE 1
Variations in Furanoid Methyl Ester Content (mg/g FAME) of Selected Soybean Varieties Having Low and High Values

	Furanoid Ester Content (mg/g FAME)			
Soybean Varieties	II	III	II + III	Maturity Group
A19	0.033	0.260	0.293	I
PI 567374	0.054	0.092	0.146	IV
PI 339865A	0.055	0.134	0.189	IV
PI 423770	0.068	0.075	0.143	IV
FA4	0.070	0.092	0.162	—
PI 171444	0.226	0.272	0.498	VI
PI 323276	0.227	0.252	0.479	VII
PI 471903	0.232	0.154	0.386	VI
PI 165929	0.262	0.201	0.463	VII
PI 283327	0.290	0.122	0.412	VI
PI 253658 B	0.104	0.082	0.186	I
FA2	0.104	0.084	0.188	—
PI 105579	0.090	0.088	0.178	IV
PI 398672	0.076	0.088	0.164	III
PI 398884	0.146	0.221	0.367	IV
PI 445683	0.210	0.255	0.465	VII

storage for 2 d. After 2 d of storage, the percentage of furanoid II was reduced 25%, whereas that of III was reduced 34%. Thus, furanoid esters are not synthesized by activating lipoxygenase. These results agree with those of Guth *et al.* (9) who found that the furanoid ester content from soybean varieties that lack various lipoxygenases was normal.

Survey of soybean varieties. Fifty-six soybean varieties grown in Puerto Rico were harvested in September 1995 to examine genotypic differences in furanoid ester content. These varieties represented a wide range of maturity and geographic origin. Results from 16 of those lines are shown in Table 1. Variation in furanoid ester II and III content among all 56 lines is given in Figures 2 and 3. Results were not reported for furanoid ester I (<10% of total furanoids in all genotypes). Furanoid II ranged from 0.03–0.29 mg/g FAME, and furanoid III ranged from 0.06–0.27 mg/g. The combined total ranged from 0.16–0.49 mg/g. Among these genotypes, it appeared that soybeans in maturity groups VI and VII exhibited significantly greater levels of furanoid esters II plus III.

Effect of growth condition on furanoid ester content. The furanoid ester content of soybean oil was affected by both variety and growth conditions when four soybean varieties were grown in Washington, Ames, and Waterloo, IA, in 1994. The cv. Kenwood had a typical fatty acid profile, whereas YB29ZA had a low linolenate content (2.8%), YA7343Z006 had a low saturated fatty acids content (6.4%), and WA91.283091 had a high palmitate content (24.2%). The furanoid ester contents are shown in Table 2. The three locations exhibited small but significant differences in furanoid content. Soybeans from Waterloo had significantly higher levels of furanoid II and III than did those from Ames and Washington, and there was no significant difference for II and III between soybeans from Ames and Washington.

Soybean cultivar YA7343Z006 had a significantly higher level of furanoid ester II than Kenwood and YB29ZA, while WA91.283091 had a significantly lower level of II than Kenwood and YB29ZA. Soybean WA91.283091 had a significantly higher level of furanoid ester III than Kenwood and

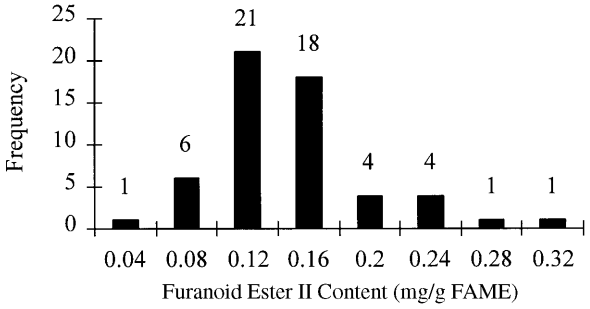

FIG. 2. The distribution frequency of soybeans with various percentages of furanoid methyl ester II in their oils.

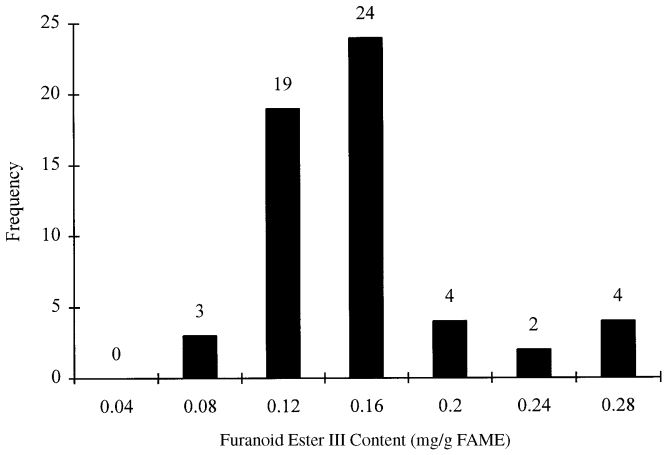

FIG. 3. The distribution frequency of soybeans with various percentages of furanoid methyl ester III in their oils.

TABLE 2
Effect of Environment on Furanoid Methyl Esters II and III in Four Soybean Varieties Grown at Three Iowa Locations

		Locations			
	Varieties	Washington	Ames	Waterloo	Means[a]
Furanoic ester II (mg/g FAME)	Kenwood	0.116	0.127	0.140	0.128^{c}
	YB29ZA	0.125	0.125	0.142	0.131^{c}
	YA7343Z006	0.139	0.144	0.145	0.143^{b}
	WA91.283091	0.029	0.030	0.035	0.031^{d}
	Means[a]	0.102^{y}	0.107^{y}	0.115^{x}	
Furanoid ester III (mg/g FAME)	Kenwood	0.108	0.113	0.119	0.113^{c}
	YB29ZA	0.104	0.102	0.110	0.106^{d}
	YA7343Z006	0.110	0.115	0.112	$0.112^{c,d}$
	WA91.283091	0.140	0.140	0.156	0.145^{b}
	Means[a]	0.116^{y}	0.118^{y}	0.124^{x}	

[a]Means for each furanoid ester with the same *b,c* or *d* superscripts do not differ significantly for varieties at the 5% level. Means for each furanoid ester with the same *x,y* superscripts do not differ significantly for locations at the 5% level. Values are the average of duplicate analyses.

YA7343Z00, and YB29ZA had a significantly lower level of III than Kenwood and YA7343Z006. In addition, it was noted that time of planting affected furanoid content of the 56 lines grown in Puerto Rico. Seed harvested in Fall 1995 (Table 1) generally contained lower levels of furanoid methyl ester II plus III than the same varieties harvested in Spring 1996. As an example, furanoid ester levels in PI 398672 increased from 0.17 mg/g FAME (Fall) to 0.27 mg/g FAME (Spring). A similar response was found in genotypes with inherently greater furanoid content. PI 323276 contained 0.48 mg/g FAME (Fall) and 0.65 mg/g FAME (Spring). Therefore, these environmental effects did not change the ranking of furanoid content among soybean genotypes.

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