Flavor Improvement of Soybean Preparations by Genetic Removal of Lipoxygenase-2[1]

C.S. Davies[a], S.S. Nielsen[b] and N.C. Nielsen[c]
[a]Department of Agronomy, [b]Department of Food Science and [c]USDA/ARS and Department of Agronomy, Purdue University, W. Lafayette, Indiana

Lipoxygenase-mediated oxidation of fatty acids has long been implicated in the production of off-flavors in soybean products. This assumption was tested by use of preparations from soybean lines nearly isogenic to the cultivar Century that lack the lipoxygenase isozyme or isozyme combinations L_1, L_2, L_3, $L_1 + L_3$, or $L_2 + L_3$. Near-isogenic lines were used to ensure that any effects observed were due to elimination of lipoxygenase isozymes, and not to other unrecognized genetic differences between lines. Full-fat soy flour and unblanched soymilk preparations from the lines were evaluated by a six-member taste panel for eight flavor and/or aroma attributes common to soybeans. By comparison to near-isogenic controls, removal of the L_2 isozyme from soymilk preparations produced significantly lower scores for beany, rancid and oily flavor and aroma attributes, as well as higher scores for dairy and cereal flavor and aroma attributes. Similar trends were noted for soy flour flavor attributes. Thiobarbituric acid (TBA) numbers, a measure of lipid oxidation, were lower in homogenized soy flour suspensions from lines lacking L_2. Removal of the L_1 and L_3 isozymes did not result in improved flavor scores or lower TBA numbers. Total oil content and the fatty acid profile of the near isolines did not vary appreciably. The results indicated that genetic removal of the L_2 isozyme may reduce off-flavors in soy products.

Soybean oil contains between 7 and 9% linolenic acid (18:3) and about 55% linoleic acid (18:2). Oxidation of these fatty acids during processing and storage of soybeans poses a significant problem to the food industry. Breakdown products from fatty acids have been associated with grassy-beany and rancid flavors (1,2). These oxidation products are considered responsible, at least in part, for reduced consumer acceptability of soy products. The flavor problems can occur in protein as well as oil products because the undesirable compounds are reactive and can bind covalently to proteins during processing (3,4).

Considerable indirect evidence has implicated seed lipoxygenase as a major factor contributing to the oxidation of lipids during processing, thereby lowering product quality (3,5). This enzyme catalyzes the hydroperoxidation of *cis-cis* pentadienes and is present in relatively large quantities (about 2% of the protein) in soybean seeds (6). The hydroperoxides formed by lipoxygenase activity and their breakdown products are known to exhibit off-flavors (3,7). Furthermore, lipoxygenase inactivation by heat during processing has been correlated with the improvement of sensory evaluation scores (8). Heat treatments are widely used to inactivate lipoxygenase, but they are expensive, can produce undesirable alterations of the proteins and themselves can add to the flavor profile of soy products.

Three lipoxygenase isozymes, denoted L_1, L_2 and L_3, are present in soybean seeds (6). They differ from one another in reaction products, pH optima, substrate specificity and mobility in SDS gels (6,9,10). Each of the isozymes is encoded by a single gene, denoted Lx_1, Lx_2 and Lx_3, respectively. Null-alleles (designated lx_1, lx_2 and lx_3) for each of the three lipoxygenase gene loci have been documented (9,11-13). Their identification has, for the first time, permitted direct testing of the effect of each of the lipoxygenase isozymes on the generation of off-flavors. Here we report the results of sensory evaluation of soy flour and soymilk samples as well as TBA (thiobarbituric acid) analysis to measure lipid oxidation of soy flour samples prepared from these lines. This was done by comparing preparations made from seeds of near-isolines that genetically lacked one or two of the isozymes, to preparations from a near-isogenic control that contained the isozymes. Both the sensory and chemical data implicate the L_2 isozyme as a principal contributor to off-flavors and provide evidence that genetic elimination of this isozyme could be used to reduce the flavor problems.

MATERIALS AND METHODS

Lipoxygenase lines. The soybean lines evaluated in this study are near-isogenic for the lipoxygenase null-alleles lx_1, lx_2, lx_3, $lx_1 + lx_3$ and $lx_2 + lx_3$. They lack the L_1, L_2, L_3, $L_1 + L_3$ or $L_2 + L_3$ isozymes, respectively. The lines were developed by backcrossing the alleles into the cultivar Century (14). Soy flour was prepared from the following experimental lines: L_1-5 (L_1less phenotype, lx_1lx_1 genotype, backcross generation 5), L_2-3, L_3-5, L_1L_3-2-2 and L_2L_3-2-4. Soymilk was prepared from the following lines: L_1-4, L_2-2, L_3-4 and L_1L_3-2-2.

The lipoxygenase lines were grown along with the cultivar Century at one location at the Purdue Agronomy Farm, West Lafayette, Indiana, during the summer of 1985. The seed lots for $L_1$5, L_2-3, L_3-5, L_2L_3-2-4 and Century-1 were obtained from bulked seeds of 3-6 plants. The seed lots for L_1L_3-2-2, L_1-4, L_2-2, L_3-4 and Century-2 were a bulk of three to six 3-ft rows for each of the five lines. The Century seeds were used as a control for sensory analysis and TBA tests. The soymilk and soy flour anchor samples were prepared from a 1984 harvest of Century.

The plants and seeds from the evaluated lines were similar in size, shape and color to those of Century from the same location. Fatty acid compositions were determined in duplicate for a 10-g sample of seed meal from each of the isolines as reported earlier (15).

Soy flour and soymilk preparation. Soy flour was prepared by grinding 15 g of seeds in an Osterizer blender at "liquify" speed for one min, and the flour was sieved through a 20-mesh screen to remove seed coat fragments.

[1]Cooperative research between USDA/ARS and the Purdue Agricultural Experiment Station. This is Paper Journal No. 10858 from the Purdue Agricultural Experiment Station.

*To whom correspondence should be addressed at the Department of Agronomy, Purdue University, W. Lafayette, IN 47906.

Unblanched soymilk was prepared by soaking 50 g of rinsed seeds overnight at 4 C in 150 ml of triple distilled, deionized water. The seeds were rinsed three times with fresh water, drained and ground in 125 ml water for two min at high speed in a Waring blender. The homogenate was poured into a beaker, and 25 ml of water was used to rinse the blender jar. The rinse was added to the homogenate, and the resulting mixture was allowed to stand one hr at room temperature. The mixture was filtered through three layers of cheesecloth to obtain the soymilk. Samples were prepared fresh for each day of testing. Because there was an insufficient quantity of seeds available when these experiments were done, soymilk samples were not prepared for L_2L_3-2-4.

Sensory evaluation. Six subjects from the Kansas State University Sensory Analysis Center evaluated the flavor and aroma intensity of the soymilk and soy flour preparations. The subjects, each with at least one year of professional experience in flavor profiling, were trained to identify eight attributes common to soy products using the standards listed in Table 1. Before testing began, the subjects jointly agreed on a value for each of the standards and on a value for preparations from a control sample of Century seeds for each attribute. This sample of Century seeds was from a different seed lot than the experimental samples, and served as the anchor for use by the subjects during the test periods. Anchor and standard intensities are listed in Table 1 with a description of the standard for each attribute. The technique of attribute scaling, similar to that of Kalbrener et al. (7), was used to quantify the flavor or aroma intensity of the preparations. With this technique, the subjects rated the samples on an integer scale of 1-10. This scale was subdivided into the following categories: 1, bland; 2-4, weak; 5-6, moderate; 7-8, strong; 9-10, very strong. Both scores and samples from the anchor were available as references for each subject during the test periods.

The samples were coded and presented in a random order. A minimum of 10 min was required between samples to allow the subjects' taste senses to return to a neutral state. Crackers, distilled water, apples and peanut butter were used to help cleanse the mouth of both flavor and aftertaste.

Soy flour samples were evaluated only for flavor, while soymilk samples were evaluated for both flavor and aroma. For soy flour samples, each subject received approximately three-fourths teaspoon of flour in a 30-ml glass beaker covered with a watch glass. To allow for full development of flavor and feeling in the mouth, about three small tastes (one-eighth teaspoon) of sample were taken before its characteristics were rated. For soymilk samples, each subject received approximately 15 ml of milk at room temperature in a 30-ml glass beaker covered with a watch glass. The aroma attributes were rated first by a series of short sniffs; this was completed in three min. Each subject took three sips before beginning to rate the flavor attributes of the soymilk. The subjects then sipped as much milk as needed to complete the response form. They could then check the control if they desired and change their rating as appropriate.

Soy flour samples were rated for flavor in one experiment, and soymilk samples were rated for aroma and flavor in a second experiment conducted one week later. Both experiments consisted of three test periods conducted on the mornings of three consecutive days (1 test period per day). Each sample was rated once by each subject during a test period.

Mean values for scores from the six subjects were obtained for each combination of attribute, day and

TABLE 1

Standards and Intensity Values Assigned to Standards and Anchors Used for Evaluation of Flavor and Aroma Attributes

			Anchor intensity		
			Soymilk[a]		Soyflour
Attribute	Standard	Standard intensity	A	F	F
Dairy/milky	Carnation nonfat dry milk		4	5	—
	Liquid	7	—	—	—
	Powder	8	—	—	—
Cereal	Quaker quick grits	6	4	3	4
Beany	Lima bean extract[b]	9	5	6	8
Rancid	Aged Wesson oil	9	8	8	3
Oily	Soybean oil	5	7	7	4
Chalky	2% Calcium carbonate	5	—	4	3
Bitter	0.03% Caffeine	5	—	4	3
Astringent	0.1% Tannic acid	5	—	3	2

[a]A, aroma, F, flavor.

[b]100 g dry lima beans (Brown's Best Baby Lima Beans) were pulverized in a blender for one minute; the resulting flour was then mixed with 500 ml distilled deionized water and held for 1 hr; this mixture was then strained through four layers of previously washed cheesecloth and the strained liquid was used as the standard.

lipoxygenase line. These values were analyzed statistically by analysis of variance using a randomized complete block design for each flavor or aroma attribute. Each lipoxygenase line was considered a treatment, and day was considered a treatment, and day was considered a blocking factor. Statistical significance was based on an F-protected LSD test.

TBA analysis. The 2-thiobarbituric acid (TBA) assay was used as a measure of lipid oxidation in soy flour prepared from seeds of Century and lipoxygenase lines $L_1$5, L_2-3, L_3-5 and L_1L_3-2-2. The distillation method of Tarladgis et al. (16) as modified by Rhee and Watts (17) was used. A suspension of 4 g flour in 50 ml deionized, distilled water was homogenized with a polytron for up to 10 min. To inactivate lipoxygenase immediately after mixing, the sample was acidified to pH 1.1 to 1.2 by addition of hydrochloric acid (acid/water, 1:2, v/v) and 150 μl of a 15% solution of butylated hydroxy toluene (BHT) in ethyl alcohol. The mixture was quantitatively transferred to a 500-ml flask, and the volume was adjusted to 100 ml with water. Boiling chips and 0.5 ml of antifoam A concentrate (Sigma) were added. The mixture was distilled until exactly 50 ml of distillate was collected (13 to 14 min). A 3-ml aliquot of the distillate was combined with 3 ml of 0.02 M TBA in water, boiled for 35 min and its absorbance determined at 530 nm. "TBA numbers" were calculated by converting the absorbance reading to mg malonaldehyde per 1000 g of sample, according to the procedure of Tarladgis et al. (16). Soy flour samples homogenized for different periods were distilled in duplicate, and then triplicate aliquots of distillate were reacted with the TBA reagent.

RESULTS AND DISCUSSION

A summary of scores obtained from sensory evaluation of the soymilk and soy flour preparations of the lipoxygenase lines is shown graphically in Figure 1. Pertinent statistical data that relate to these data are shown in Table 2. The score for each attribute was recorded at the time it was most intense. Subjects reported that samples with high scores for the beany, bitter, astringent and rancid attributes had less intense "aftertastes" that linger after swallowing. The aftertaste, however, was not scored.

The most striking observation from sensory evaluation of the preparations was the large difference between scores of the L_2-less line and those of the other lines in the soymilk samples. For example, when the soymilk was evaluated for aroma, all significant differences could be attributed to the L_2-less line. For both aroma and flavor evaluations, the differences were greatest for the rancid attribute.

The raw scores for the dairy and beany flavor attributes in full-fat soy flour samples varied more than those for soymilk samples. This is reflected in the larger LSD values obtained for these attributes after statistical treatment of scores generated by the panel (Table 2). These data indicated that panel members had more difficulty classifying differences in flavor intensity for the attributes in soy flour than in soymilk.

The mean scores for the dairy, cereal and beany attributes showed the same trends for soy flour as the

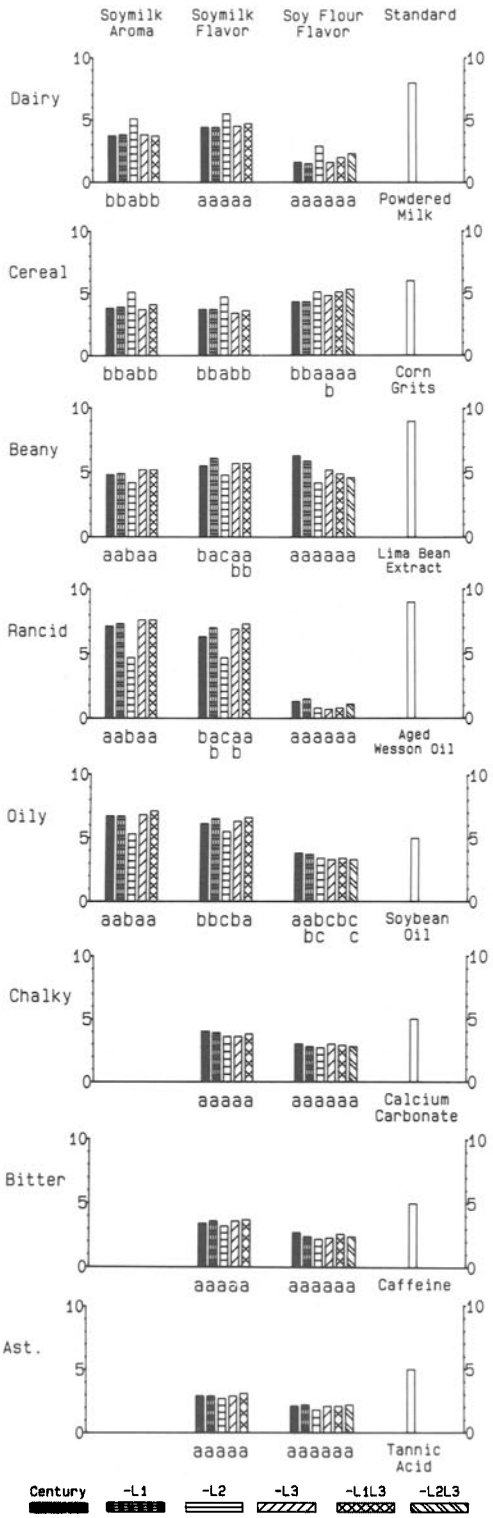

FIG. 1. Taste panel scores (y axis) of soymilk and soy flour preprations from seeds lacking one or two of the lipoxygenase isozymes. Preparations of Century seeds that contain all three isozymes were included as a control. The scores of 110 were subdivided into categories: 1, bland; 24, weak; 56, moderate; 78, strong; and 910, very strong intensity. Each bar represents the mean of 18 evaluations (six judges averaged over three days). Bars with the same letter are not statistically different at the 5 level of significance. LSD and P values for each cluster of bars are listed in Table 2. Intensity values for the standards used to train the subjects are included for reference.

differences detected in the soymilk evaluations (Fig. 1). For example, the average scores for cereal flavor in full-fat soybean flour from seeds that lacked the L_2 isozyme were higher than scores for flour from Century seeds that had the isozyme. Lower scores for beany flavor were obtained for flour from the L_2-less isoline compared to Century controls. The differences in cereal scores for soy flour from the Century control vs the L_2- and L_2L_3-less lines were significant at the 95% level (Table 2 and Fig. 1), although distinction between flavors of flour from the L_2-less genotype and the other null alleles was not clear as for soymilk samples. LSD analysis indicated that the differences in beany attribute between flour from Century and the L_2-less lines were significantly different at the 95% level. However, since the P value was significant only to the 93% level (Table 2), this difference was not indicated as significant in Figure 1. No statistical significance was associated with the dairy scores for soy flour samples, probably because of the large standard deviations associated with these data. Scores for the rancid attribute were substantially lower in soy flour samples compared to soymilk; they were apparently not as important a factor in their flavor profile.

For both soymilk and soy flour samples, no differences between the lines were identified for the chalky, bitter or astringent attributes. Additional statistically significant differences occurred between lines other than those that involved the L_2-less phenotype, but the differences were small and inconsistent.

TBA numbers of homogenized flour suspensions from the L_2-less line were significantly lower ($\alpha = 0.05$) after two min of homogenization than those of Century, as well as those of the L_1-less, L_3-less and L_1L_3-less lines (Fig. 2). This difference increased slowly after 4, 7 and 10 min of homogenization. As expected, TBA numbers from all lines, including the L_2-less line, increased with increasing times of homogenization.

Based on comparison of non-isogenic lines with and without L_1, Hildebrand and Kito (18) concluded that mutants lacking this isozyme had less capacity to generate TBA-reactive oxidation products. This result conflicts with the one shown in Figure 2, where L_1-less allele does not cause a detectable reduction in the rate of oxidation compared to the near-isogenic control. The bases for this inconsistency is unclear, although several obvious differences exist between the two experiments. In addition to using non-isogenic lines where varietial differences could exist, Hildebrand and Kito used a direct extraction method for TBA analysis rather than the distillation method used in this study. The advantages of the distillation method have been noted in the literature (19). In our experience, the distillation procedure was required to obtain precise and reproducible results. Inconsistent values were obtained using the direct extraction method. The absorbence values obtained for malonaldehyde-like products when soybean samples were reacted with TBA in the direct

TABLE 2

P and LSD Values of Taste Panel Scores for Eight Sensory Attributes[a]

Attribute	Soymilk aroma	Soymilk flavor	Soy Flour flavor
Dairy			
P>F	0.0001[b]	0.0653	0.2771
LSD	0.22	0.79	1.40
Cereal			
P>F	0.0293[c]	0.0048[b]	0.0277[c]
LSD	0.83	0.57	0.63
Beany			
P>F	0.0306[c]	0.0036[b]	0.0681
LSD	0.60	0.48	1.45
Rancid			
P>F	0.0003[b]	0.0011[b]	0.1883
LSD	0.87	0.90	0.75
Oily			
P>F	0.0004[b]	0.0037[b]	0.0110[c]
LSD	0.52	0.46	0.27
Chalky			
P>F	—	0.1743	0.8705
LSD	—	0.39	0.61
Bitter			
P>F	—	0.3279	0.8643
LSD	—	0.51	0.83
Astringent			
P>F	—	0.1875	0.2669
LSD	—	0.32	0.40

[a]Data illustrated in Figure 1.
[b]Significant at α=0.01%.
[c]Significant at α=0.05%.

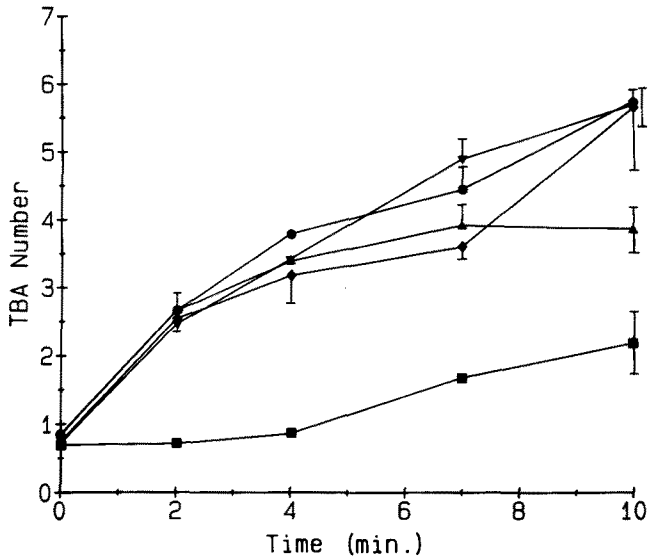

FIG. 2. Effects of homogenization time on TBA number (malonaldehyde equivalent) for soy flour preparations from Century (•) and lipoxygenase lines L_1-5 (L_1-less) (▲), L_2-3 (L_2-less) (■), L_3-5 (L_3-less) (▼) and L_1L_3-2-2 (L_1L_3-less) (◆). The soy flour/water suspensions were homogenized for 0, 2, 4, 7 or 10 min before addition of acid and butylated hydroxytoluene. Values at each time point are the averages of duplicate distillations and triplicate distillate samples reacted with the TBA reagent. Verticle bars at time points reflect standard deviations. Standard deviations greater than 0.1 for the duplicate distillation samples are indicated by vertical lines on one or both sides of the data points. Data points with no vertical lines have a standard deviation of less than 0.1.

extraction method were considerably greater at 450 nm than at 530 nm. The latter is the absorption maximum of the complex formed between TBA and the malonaldehyde derived from 1,1,3,3-tetraethoxypropane standards. By contrast, distilled soy flour samples had an absorption maximum at 530 nm and a very low absorption at 450 nm.

The sensory data and TBA results we have reported indicate that both off-flavors and lipid oxidation were lower in sample preparations from the L_2-less line. We attribute these differences specifically to the L_2 isozyme, since the experiment was designed such that other genetic and environmentally induced differences among lines that could have affected lipid oxidation were minimized. Genetic influences were reduced by use of the near-isolines, and the lines were grown in the same location to control environmental variability.

The performance of the L_2-less line in the sensory evaluations could have reflected large differences among lines in either total oil content or levels of polyunsaturated fatty acids. Although small differences were observed (Table 3), they were considered unlikely to be responsible for the large changes in sensory perception observed among lines.

Differences among the three isozymes in pH optima and reaction products probably accounted for some of the variations between isolines that were observed. L_1 has a pH optimum of 9.0-9.5 (6) and is therefore probably not active in the preparations evaluated. However, L_2 and L_3 have pH optima between 6.0 and 7.0 (6) and were probably active. During lipoxygenase-catalyzed oxidation, the *cis,cis* 1,4-pentadiene structures in linoleic and linolenic acid yield either 13-hydroperoxy or 9-hydroperoxy fatty acids. These isomers produce different breakdown products. Hexanal (from 18:2) and hexenal (from 18:3) are produced from the 13-hydroperoxy isomer, whereas 9-carbon aldehydes are considered to arise from the 9-hydroperoxide (20,21). Both hexenal and hexanal have a grassy-beany flavor and substantially lower flavor thresholds than the corresponding 9-carbon compounds (22). If the isozymes vary in the ratio of 13- to 9-hydroperoxy isomers produced, the level of hexenal or hexanal in the product could be altered significantly and result in the different flavor perceptions observed in these experiments.

The higher scores for the dairy and cereal attributes in the preparations from the L_2-less line could be due to the phenomena of masking and counteraction (23,24). These two terms are used in connection with the control of objectionable odorants. Each component of a mixture displays some ability to mask the other component, and the perceived intensity of chemicals in a mixture is lower than the sum of the intensities of the unmixed components. Thus, when the beany, oily and rancid attributes were reduced in intensity by removal of breakdown products from the L_2 isozyme, the subjects may have perceived an increase in the cereal and dairy attributes, even if no absolute intensity differences were present.

The results we described have clear practical implications for the soybean industry. The data indicate that genetic elimination of the L_2 isozyme may reduce some of the off-flavors associated with soy products and thereby increase their consumer acceptability. However, only after these or similar genetic lines have been evaluated in a number of commercially feasible systems will a clear understanding of the value of removing the L_2 isozyme be reached.

TABLE 3

Total Oil Content and Fatty Acid Profile of Seeds from the Soybean Isolines

Line[a]	Oil %	Fatty acid %				
		16:0	18:0	18:1	18:2	18:3
L1-5	21.1	11.5	3.2	21.3	56.4	7.6
L1-4	19.7	11.5	3.2	20.8	56.7	7.6
L2-3	19.7	12.3	3.2	20.5	56.7	7.2
L2-2	19.6	12.0	3.2	21.6	55.9	7.3
L3-5	19.0	12.1	3.2	17.5	58.5	8.8
L3-4	19.3	12.0	3.2	18.0	57.9	8.7
L1L3-2-2	18.8	11.3	3.2	20.1	57.4	8.0
Century-1	20.6	11.5	3.3	21.2	56.2	7.8
Century-2	20.5	11.7	3.3	19.1	58.0	7.9

Values are the average of duplicate determinations on a 5-g sample of seed meal from each line.

[a]Lipoxygenase phenotype and genotype, backcross generation and conditions of growth for each line are given in Materials and Methods.

ACKNOWLEDGMENTS

The work was supported in part by grants from the American Soybean Association Research Foundation. B. Smith supervised the taste panel, B. Weber performed the TBA assays, J.F. Cavins performed the fatty acid analysis and Z. Vickers provided suggestions on sensory evaluation.

REFERENCES

1. Dutton, H.J., C.R. Lancaster, C.D. Evans and J.C. Cowan, *J. Am. Oil Chem. Soc. 28*:115 (1951).
2. Hammond, E.G., in *World Soybean Res. Conf. III Proc.*, edited by R. Shibles, Westview Press, Boulder and London, 1985, p. 251.
3. Rackis, J.J., D.J. Sessa and D.H. Honig, *J. Am. Oil Chem. Soc. 56*:262 (1979).
4. Gardner, H.W., *J. Agric. Food Chem. 27*:220 (1979).
5. Wolf, W.J., *J. Agr. Food Chem. 23*:136 (1975).
6. Axelrod, B., T.M. Cheesebrough and S. Laakso, *Methods Enzymol. 71*:441 (1981).
7. Kalbrener, J.E., K. Warner and A.C. Eldridge, *Cereal Chem. 51*:406 (1974).
8. Mustakas, G.C., W.J. Albrecht, J.E. McGhee, L.T. Black, G.N. Bookwalter and E.L. Griffin Jr., *J. Am. Oil Chem. Soc. 46*:623 (1969).
9. Kitamura, K., C.S. Davies, N. Kaizuma and N.C. Nielsen, *Crop Sci. 23*:924 (1983).
10. Matoba, T., H. Hidaka, K. Kitamura, N. Kaizuma and M. Kito, *J. Agric Food Chem. 33*:856 (1985).
11. Hildebrand, D.F., and T. Hymowitz, *Crop Sci. 22*:851 (1982).
12. Davies, C.S., and N.C. Nielsen, *Crop Sci. 26*:460 (1986).
13. Kitamura, K., T. Kumagai and A. Kikuchi, *Japan J. Breed. 35*:413 (1985).
14. Davies, C.S., and N.C. Nielsen, *Crop Sci. 27*:370-371.
15. Wilcox, J.R., J.F. Cavins and N.C. Nielsen, *J. Am. Oil Chem. Soc. 61*:97 (1984).
16. Tarladgis, B.G., B.M. Watts, M.T. Younathan and L. Dugan Jr., *J. Am. Oil Chem. Soc. 37*:44 (1960).

17. Rhee, K.S., and B.M. Watts, *J. Food Sci. 31*:664 (1966).
18. Hildebrand, D.F., and M. Kito, *J. Agric. Food Chem. 32*:815 (1984).
19. Kakuda, Y., D.W. Stanley and F.R. van de Voort, *J. Am. Oil Chem. Soc. 58*:773 (1981).
20. Sessa, D.J., *J. Agric. Food Chem. 27*:234 (1979).
21. Grosch, W., and D. Schwenke, *Lebensm.-Wiss. Technol. 2*:109 (1967).
22. Dixon, M.D., and E.G. Hammond, *J. Am. Oil Chem. Soc. 61*:1452 (1984).
23. Cain, W.S., and M. Drexler, *Ann. N.Y. Acad. Sci. 237*:427 (1974).
24. Cain, W.S., *Chem. Senses & Flavor 1*:339 (1975).

[Received September 12, 1986]